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DANIEL BARBOSA ALCÂNTARA

DEVELOPMENT OF TWO DIFFERENT LIQUID MOBILE PHASE SEPARATION SYSTEMS (AF4-ICP/MS AND UPLC-ESI/QDA) FOR SELENOMETHIONINE ANALYSES IN YEAST AND CASHEW NUT (*Anacardium Occidentale*) SAMPLES

FORTALEZA

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I dedicate this work to my mother Mrs. Ana Cristina, to my beloved wife Jamylle Venancio and all the people that cheered for its conclusion.

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It is our choices that show what we truly are, far more than our abilities. J.K. Rowling.

RESUMO

O selênio (Se) é um micronutriente essencial para mamíferos, onde desempenha importantes funções bioquímicas. Sua principal fonte em alimentos vem do Se-aminoácido conhecido como selenometionina (SeMet), que é uma espécie de Se não volátil e solúvel em soluções aquosas. Este trabalho descreve o desenvolvimento de dois diferentes sistemas de separação que empregam fase móvel líquida, o fracionamento assimétrico por campo e fluxo de fluxo cruzado (sigla em inglês '*AF4')* e a cromatografia líquida de ultra-performance (sigla em inglês '*UPLC*'), para análise de SeMet em amostras de fermento e castanhas de caju, respectivamente. Desse modo, diferentes técnicas de preparação de amostra foram avaliadas quanto a compatibilidade com o sistema AF4 acoplado a espectrometria de massa com plasma indutivo (sigla em inglês '*ICP-MS*'), onde a extração alcalina forneceu as melhores condições de recuperação, uma vez que minimizou a hidrólise das ligações peptídicas das Se-proteínas, o que favoreceu a análise por AF4. O método desenvolvido (AF4-ICP/MS com extração alcalina) foi validado e aplicado em suplementos comerciais de femento enriquecido com Se, onde forneceu resultados em concordância com aqueles obtidos por métodos já bem reconhecidos. Para o sistema UPLC com detecção utilizando um analisador de massas de quadrupolo único acoplado ao detector de fotodiodos (sigla em inglês '*QDa*') e ionização por eletrospray (sigla em inglês '*ESI*'), um peparo de amostra por microextração líquido-líquido assistida por ultrassom (sigla em inglês '*UALLME*') foi otimizado através de um planejamento experimental para análise SeMet em amostras de castanha de caju, onde as melhores recuperações foram obtidas ao se trabalhar com solvente de extração em valores extremos de pH, além de ser obervado uma forte interação entre as variáveis 'massa de amostra' e 'pH do solvente de extração' na concentração de SeMet obtida. A genisteína apresentou-se como um padrão interno alternativo à Se-metil selenocisteína em corrigir o sinal analítico da SeMet; além da matriz da castanha de caju interferir positivamente na quantificação do analito pelo sistema cromatográfico. Ainda, o método UALLME-UPLC-ESI/QDa foi validado e aplicado em amostras reais de castanha de caju. Os resultados sugerem o uso eficiente do AF4 como um método alternativo às técnicas tradicionais de separação em muitas aplicações; além de destacar a necessidade de se expandir as pesquisas a respeito da SeMet em castanhas de caju provenientes do estado do Ceará (Brasil) devido à importância desta oleaginosa para a economia local.

Palavras-chave: SeMet; extração alcalina-AF4-ICP/MS; UALLME-UPLC-ESI/QDa; fermento; castanha de caju.

ABSTRACT

Selenium (Se) is an essential micronutrient for mammals and it plays important biochemical functions in the organism. Its principal source in foods comes from the Selenomethionine (SeMet) compound, which is a non-volatile Se species soluble in aqueous solutions. This way, this work described the method development of two different liquid mobile phase separation systems (asymmetrical flow field flow fractionation – AF4 and ultra-performance liquid chromatography – UPLC) to analyze SeMet in yeast and cashew nut samples, respectively. Thus, several popular sample preparation techniques were evaluated for their suitability to determine SeMet in selenized yeast by AF4 coupled to inductively plasma mass spectrometry (ICP-MS), where alkaline extraction provided the best recovery/determination conditions since it minimized hydrolysis of the protein peptide bonds optimally required for AF4 separation. From the different AF4 membranes evaluated, the 5 and 10 kDa regenerated cellulose (RC) provided similar results, while the 500 kDa RC resulted in a significant analyte loss. The developed alkaline-AF4-ICP/MS method was validated and applied in real commercial Se yeast supplements providing results in accordance with already recognized methods. For the UPLC system (detection using a single quadrupole mass analyzer with photo diode array detector – QDa and electrospray ionization - ESI) an ultrasound assisted liquid-liquid microextraction (UALLME) was optimized through an experimental design to analyze SeMet in cashew nut sample. Results showed best recoveries when working with extracting solvent in extreme values of pH. Also it was detected strong interaction between the variables ‘sample mass’ and ‘extracting solvent pH’ in the analyte response (SeMet concentraition). Genistein presented as a suitable SeMet internal standard when was compared its efficiency with the SeMet similar compound (Selenium Methyl Selenocystin – SeMeSeC). The matrix matched calibration strategy was used to minimize the influence of coextractives in the analyte analyses by the UPLC-ESI/QDa. The UALLME-UPLC-ESI/QDa method was validated and applied in real cashew nut samples. Outcomes suggest the efficient use of the AF4 as an alternative method to traditional separation techniques for many applications; besides to highlight the importance to expand the researches about SeMet in cashew nuts from Ceará state (Brazil) due to the importance of this oilseed to the local economy.

Keywords: SeMet; alkaline-AF4-ICP/MS; UALLME-UPLC-ESI/QDa; yeast; cashew nut.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAS	Atomic Absorption Spectrometry
AF4	Asymmetrical Flow Field Flow Fractionation
AFS	Atomic Fluorescence Spectrometry
APCI	Atmospheric Pressure Chemical Ionization
apoEr2	Apolipoprotein E Receptor 2
C18	Octadecylsilane
CE	Capillary Electrophoresis
CNS	Central Nervous System
CPE	Cloud Point Extraction
CRM	Certified Reference Material
CT	Centrifugation Time
D	Diffusivity
DIO	Iodothyronine Deidinases
DIW	Deionized Water
DLLME	Dispersive Liquid-Liquid Micro Extraction
DRC	Dynamic Reaction Cell
EFFF	Electrical FFF
ESI	Electrospray Ionization Source
FA	Formic Acid
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFF	Field Flow Fractionation
FIAS	Flow Injection Analysis System
FlFFF	Flow Field Flow Fractionation
GC	Gas Chromatography
GPx	Glutathione Peroxidases
HG	Hydride Generation
HPLC	High-Performance Liquid Chromatography
ICP-MS	Inductively Plasma Mass Spectrometry

ICP-OES	Inductively Plasma Optical Emission Spectrometry
IS	Internal Standard
ISe	Inorganic Se
k_{ow}	Octanol-Water Partition Coefficient
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Coupled to Tandem Mass Spectrometer
LLME	Liquid-Liquid Micro Extraction
LOD	Limit of Detection
MALS	Multi-Angle Light Scattering
ME	Matrix Effect
MeSeC	Methylselenocysteine
MetSeMet	Methyl-Selenomethionine
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSA	Methanesulfonic Acid
n.d.	Not Detected
NRC	National Research Council of Canada
QDa	Single Quadrupole Mass Analyzer with a Photodiode Detector Array
qTOF	Quadrupole in Series with the Time of Flight
R	Correlation Coefficient
RC	Regenerated Cellulose
RDA	Recommended Dietary Allowance
RMS	Root Mean Square Radius
RNAA	Radiochemical Neutron Activation Analysis
RSD	Relative Standard Deviation
RT	Retention Time
SDS	Sodium Dodecyl Sulfate
Se	Selenium
SeCys	Selenocystein
SeFFF	Sedimentation FFF

SeHCy	Selenohomocystine
SELM-1	Selenium Enriched Yeast
SeMeSeC	Selenium Methyl Selenocystin
SeMet	Selenomethionine
SeP	Selenoproteins
SePP	Selenoprotein P
SeY	Se-Enriched Yeast
Si-OH	Silanol Groups
SIR	Selected Ion Recording
SRM	Selected Reaction Monitoring
T3	Triiodothyronine
T4	Thyroxine
ThFFF	Thermal FFF
TrxR	Thioredoxin Reductases
UALLME	Ultrasound Assisted LLME
UB	Ultrasonic Bath
UP	Ultrasonic Probe
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
UV/Vis	Ultraviolet-Visible
υ	Velocity

CONTENTS

1 INTRODUCTION

Selenium (Se) is recognized as an essential micronutrient for most organisms where its principal exposure route is through the diet (Zhang, Huang *et al.*, 2020). The most bioavailable species of this element in foods are the organic species (selenoproteins - SeP), obtained through conversion of inorganic Se (ISe) present in the soil by plants (Adadi, Barakova *et al.*, 2019). Therefore, the content of Se available to human diet are influenced by the levels of ISe presented in the soil, and in certain geographic areas, where its amount is low, the Se consumption by the population is often below than the one considered adequate for diseases prevention. According to Kieliszek (2020), the Se deficiency may contribute to the incidence of cardiovascular diseases, such as Keshan disease, besides being associated with the occurrence of several types of cancers, especially in the thyroid tissues (Kieliszek, 2019). And for Qazi et al (2019), Se plays an important role in male sexual maturation once it is associated with the sperm capsule formation and with the sperm flagellum generation that is involved with its mobility (Qazi, Angel *et al.*, 2019).

After Clark et al (1996) demonstrate the potential of selenium-enriched yeast supplement in cancers prevention, the selenium supplementation has gotten very popular in the last years (Clark, Combs *et al.*, 1996). Although the selenomethionine (SeMet), selenocystein (SeCys), selenium methyl selenocystein (SeMeSeC) and others selenium amino acids are the main monomeric units that make up the SeP (Vicente-Zurdo, Romero-Sánchez *et al.*, 2020), Se has been supplemented mainly as SeMet once it is the major selenium specie found naturally in foods (rice, beans, nuts, cashews, etc) (Pyrzynska e Sentkowska, 2020). Also, Se from the SeMet is the most easily absorbed by the human body (Kubachka, Hanley *et al.*, 2017).

Thus, due to the crescent interest in Se analyses, significant efforts have been made in the field of development of analytical methods for Se species speciation and total Se determination in complex matrices in recent years. Liquid chromatography (LC) coupled to mass spectrometry (MS) or high-performance liquid chromatography (HPLC) coupled to inductively plasma mass spectrometry (ICP-MS) (Gao, Luo *et al.*, 2017; Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018; Arias-Borrego, Callejón-Leblic *et al.*, 2020; Gawor, Ruszczynska *et al.*, 2020) are currently the most commonly used detection systems for Se species analyses; while ICP-MS (Juranović Cindrić, Zeiner *et al.*, 2018; İçelli, Öz *et al.*, 2020; Kilic e Soylak, 2020) and

inductively plasma optical emission spectrometry (ICP-OES) (Esteki, Vander Heyden *et al.*, 2017; Lima, Leonardo W, Stonehouse, Gavin C *et al.*, 2019; Karasakal, 2020) are the main systems used to total Se determination.

However, for the analysis of trace elements in complex matrices like foods, an efficient extraction method is an essential requirement once coextractives (e.g. lipids, proteins, sugars, etc) may interfere at the instrumental analyses (Alcântara, Fernandes *et al.*, 2019). The preliminary preparation of the sample aims to extract, isolate and concentrate the target element at appropriate levels for quantification, in addition to remove possible interfering compounds from sample matrix and oftentimes chemically convert the analyte to a detectable form (Bouvarel, Delaunay *et al.*, 2020).

Therefore, numerous preparation techniques have been developed for the extraction of different species of Se in biological samples. Classical enzymatic hydrolysis with proteases, for example, has been used as a Se extraction technique in several food samples for many years, but according to Bierla et al (2012), inconsistent recoveries can be obtained as a result of the batch variability of proteases and the need of rigorous control of the conditions for optimal operation (Bierla, Szpunar *et al.*, 2012). Methanesulfonic acid (MSA) solubilization with reflux step has previously been used to efficiently extract SeMet from yeast with higher efficiency than the enzymatic extraction (Wrobel, Kannamkumarath *et al.*, 2003; Yang, Lu, Sturgeon, Ralph E *et al.*, 2004; Bierla, Szpunar *et al.*, 2012), but the loss of about 2 % of SeMet by decomposition can be an inconvenience of this method.

Emerging technologies that minimize the drawbacks of the conventional enzymatic hydrolysis with use of different energies supplies (microwave, ultrasound, etc) to enhance protein disruption have been widely developed in the literature, decreasing the time and amount of enzymes needed for Se species extraction (Zhou, Wang *et al.*, 2017; Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018; Franck, Perreault *et al.*, 2019). Also, López-García et al (2013) reported the use of the liquid-liquid microextraction (LLME) technique to concentrate several Se species present in edible oils, and, in contrast the common way this method is used (analyte extracted from the aqueous phase to an organic phase), the authors highlighted it was efficient in extract analytes from oil samples into a slightly acidic aqueous medium (López-García, Vicente-Martínez *et al.*, 2013). The LLME is a simple and low cost technique with shorter time, low solvent and amount of sample consumptions (John, Kuhn *et al.*, 2017).

Thus, several strategies can be used to efficiently extract (enzymatic, acid, alkaline, aqueous solutions, etc) and quantify (HPLC-ICP/MS, LC-MS/MS, etc) SeMet in foods. At this work distinct methodologies were developed for SeMet determination. The asymmetrical flow field flow fractionation (AF4) coupled to ICP-MS was employed for analyses in yeast where the compatibility of different extraction methods with the mechanism of separation occurring in the AF4 apparatus were evaluated. Also, the LC coupled to a single quadruple MS technique was used to analyze SeMet in cashew nut samples with an optimized LLME assisted by ultrasound sonication (UALLME) as sample preparation method.

As several researches have reported that oilseeds products are considered the best source of bioavailable Se, a bibliographic survey about analyses of different Se species in a lot of kind of nuts was conducted for the literature review of this thesis. Information about the main Se sources, biochemistry and trends in analytical chemistry are presented and systematized through bar charts.

2 LITERATURE REVIEW

2.1 Selenium Sources and Biochemistry

In nature Se occurs in both inorganic (e.g. selenite, selenate, selenide) and organic (e.g. SeMet, SeMeSeC and SeCys) forms. High quantities of this element are provided by foods like meat, seafood, eggs, cereals, yeast, garlic, broccoli, nuts and turnip cabbage; being available as different compounds depending on its source (Adadi, Barakova *et al.*, 2019). For example, in water, fish and cabbage, it is present principally as selenate, while in garlic, onions and broccoli the organic form SeMeSeC is the most abundant; whereas SeMet is the main Se containing molecule in cereals, plant foods and yeast; SeCys in animal foods and meats; and recently was discovered selenoneine in chicken, tuna and mackerel (Rayman, 2012).

The bioavailability of Se in foods is variable and depends of its chemical form. According to Drutel et al (2013) Se in its organic form, mainly from the selenoamino acid SeMet, is more easily bio accessible by mammals and constitute 50 – 80 % of the total Se present in plants and grains (Drutel, Archambeaud *et al.*, 2013). Cereals and oilseeds products are considered the best sources of Se (bioavailability more than 80 % against 20 – 50 % for seafood, for example). Kumar and Priyadarsini et al (2014) highlight Brazil nuts as the richest source of bioavailable selenium, despite this type of nut be neither of easy availability and nor commonly consumed (Santhosh Kumar e Priyadarsini, 2014). Actually, the bibliographic survey presented at Figure 1a, shows that researches focus mainly on Brazil nuts, and it was the oilseed with the largest variety of selenium form studied. Lima et al (2019), for example, analyzed the variation of Se concentration in two Brazil nut batches grown in Brazil, and found values ranging from 28 to 49 mg kg^{-1} as total Se (Lima, Leonardo W., Stonehouse, Gavin C. *et al.*, 2019). According to the authors, selenium presented in Brazil nut was mainly identified in organic form. Also, in studies performed by Moreda-Piñeiro et al (2018) in Brazil nut matrix harvest in Bolivia, SeMet and SeCys were detected in all analyzed samples (Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018).

However, quantities of Se containing molecule in foods may vary widely according to the inorganic Se content of the soil where they grew up. Most European countries, for example, have low levels of Se in the soil, which explains the mild to moderate deficiency of this mineral in the diet of the population from that region. Dietary Se deficiency have also been

reported in regions of China (Dinh, Cui *et al.*, 2018) and Russia (Skalny, Burtseva *et al.*, 2019), besides that, Se deficiencies causing *myxoedematous cretinism* are observed in large parts of Central Asia (Stuss, Michalska-Kasiczak *et al.*, 2017). These observations make the researches about the Se content in nuts from different countries of great importance. Figure 1b shows that studies are concentrated mainly in nuts from Brazil and Turkey, where Brazil is the country with the largest number of samples with a considered high concentration of Total Se (between 1 – 100 mg kg^{-1}). In analyses performed by da Silva, Mataveli and Arruda (2013), for example, a value of 54.8 mg kg^{-1} for total Se was quantified in Brazil nuts from Brazil, where, according to them, only SeMet was found as bioaccessible Se compound after gastrointestinal digestion and corresponded to 74 % of the total Se (Da Silva, Mataveli *et al.*, 2013). Lopes et al (2016), found values for total Se that reached 1.68 mg kg^{-1} in Brazilian babassu coconut (Lopes, G. S., Silva, F. L. F. *et al.*, 2016).

Selenium has been identified to be integral part of more than 20 distinct selenoprotein (SeP), including the selenoprotein P (SePP) (a Se stock in the body), and enzymes like glutathione peroxidases (GPx), thioredoxin reductases (TrxR) and iodothyronine deidinases (DIO) (Kuršvietienė, Mongirdienė *et al.*, 2020). In humans, its plasma concentration is directly related to dietetic Se absorbed and shall vary in a range of 60 and 120 µg L^{-1} (Zhang, Li *et al.*, 2019). Once absorbed this essential micronutrient incorporates the GPx protein, which is transported into the liver to be converted in SePP that is distributed to various organs like brain, kidney, heart, spleen, muscles and gonads (Santhosh Kumar e Priyadarsini, 2014; Donadio, Rogero *et al.*, 2018). Thus, deficiency of SeP in the organism is related to several immune responses and diseases such as cancer, Keshan disease, Alzheimer, virus infections, male infertility, neurological problems and abnormalities responses of thyroid hormone. Therefore, is very common the practice of selenium supplementation in regions where its deficiency is detected, and, after Food and Drug Administration (FDA) approval, SeMet is the principle compound used as Se source in supplements (normally Se-enriched yeast – SeY) once it has been identified as the major component of grains (Drutel, Archambeaud *et al.*, 2013; Watanabe, De Lima *et al.*, 2020).

However, the effects of Se on humans can be either beneficial or detrimental (if ingested at high amounts) (Vinceti, Mandrioli *et al.*, 2014; Vinceti, Filippini *et al.*, 2018). Daily dose recommendations vary from country to country, but doses should not exceed 400 µg dia^{-1}

for safe administration. Neurotoxic effects induced by Se compound include: increase of the levels of dopamine in central nervous system (CNS) (Rasekh, Davis *et al.*, 1997; Babür, Tan *et al.*, 2019), degeneration of cholinergic neurons (Estevez, Mueller *et al.*, 2012; Naderi, Salahinejad *et al.*, 2018), inhibition of glutamate uptake (Souza, Stangherlin *et al.*, 2010; Naderi, Salahinejad *et al.*, 2018), increase of thiobarbituric acid reactive substances and lipid peroxidation (Mudron e Rehage, 2018; Jóźwiak e Politycka, 2019), among others. Also, studies have found toxic neurological manifestation in mice due both form of Se (inorganic and organic), but selenite has shown to be much more toxic than SeMet. Actually, inorganic Se induced apoptosis in cultured mouse cortical neurons even at low levels of concentrations (Xiao, Qiao *et al.*, 2006; Chen, Sun *et al.*, 2019), moreover, for Drutel, Archambeaud and Caron (2013) SeMet is nonspecifically incorporated into proteins, which make it safer and devoid of direct toxicity for consumption even at high doses, that is another reason for supplementation occur mainly through SeMet (Drutel, Archambeaud *et al.*, 2013). So, due to these sets of characteristics favorable to SeMet, this Se compound has been the principle object of study by the scientific community in most nut matrices as is observed in Figure 1c. Several works have highlighted SeMet as the major Se component in diverse nuts, like in Brazil nuts (Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018), peanut (Gao, Luo *et al.*, 2017), monkeypot nut (Németh e Dernovics, 2015), walnut (Tadayon e Mehrandoost, 2015), etc.

However, another oilseed very popular worldwide but that has not received much attention from the scientific community is the cashew nut. This nut is produced by more than 30 countries where Vietnam (276,263 tons), India (83,093 tons), Netherland (35,655 tons), United Arab Emirates (18,990 tons) and Brazil (15,588 tons) are the five largest producers of the nut in shell and together they account for more than 80 % of the world production (Fao, 2020). In 2016, Brazil exported about 3.1 % of the total volume in the world corresponding to revenue of about 129,588,000 US$ (Ibge, 2020). Only the Northeast region accounts for 99.7 % of the Brazilian exports where the state of Ceará stands out with 61.9 % of the cashew acreage in this region which corresponds to 61.6 % when considering the whole country (Brainer e Vidal, 2018). In 2017 the production of the Ceará of this oilseed was about 81 mil tons (Adece, 2020). These data show the importance of studying the SeMet content in cashew nuts from the northeast region of Brazil due to its high economic importance generating job and income.

It is known Se follows different metabolic routes when ingested, and it depend on its chemical state (strongly related to the ingested source) which determine its absorption rate. The majority of Se is absorbed in duodenum (mainly from selenite by passive diffusion), followed by jejunum and ileum (mainly from selenate by cotransport with sodium ions) and is transported across the intestinal brush border actively or passively (Whanger, 1976; Ha, Alfulaij *et al.*, 2019). Inorganic Se is absorbed by simple process of diffusion, however much of the Se released from inorganic sources may re-combine with other components making insoluble complexes which is excreted reducing its absorption (Whanger, 1976; Ha, Alfulaij *et al.*, 2019).

The organic Se, on the other hand, is actively absorbed via amino acid transport mechanism (Combs e Combs, 1986; Chen, Sun *et al.*, 2019). The SeMet, for example, has the same mechanism of absorption with the methionine. Initially, all the dietary SeMet is incorporated into the protein and can be metabolized to Se-adenosyl methionine and futher to Se-adenosyl homocysteine, in which is converted to SeCys by the enzymatic activity of the cystathionine β-synthase and cystathionine δ-lyase (Mahima 2012; Ha, Alfulaij *et al.*, 2019). The SeCys can be incorporated into proteins or degraded by SeC-lyase enzyme releasing the elemental Se (Mahima 2012; Ha, Alfulaij *et al.*, 2019). Researchers have proved that organic Se have 120 – 200 % more bioavailability in comparison to sodium selenite in cattle (Liao, Brown *et al.*, 2011), pig (Mahan e Parrett, 1996; Zhang, Zhao *et al.*, 2020) and guinea pig (Mahima 2012); still, if the diet is rich in low molecular weight proteins like vitamins (mainly A, C and E), the Se bioavailability can get increased (Pieczyńska e Grajeta, 2015; Gong e Xiao, 2018).

Then, as the balance of Se in the body is critical to prevent the organism from several kind of disorders, below are summarized some impacts on human health due to deficits of this micronutrient.

2.1.1 Fertility: Se plays a significant role in reproductive system functions. Several studies have reported correlations between its intake and disorders of procreation processes. In men, Se is essential for sperm mobility through two SeP: the SePP, synthesized in the liver, is transported to the testis where is absorbed by the Apolipoprotein E Receptor 2 (apoEr2); and the GPx, found in the mitochondria, is the midpiece sheath of the sperm tail (Santhosh Kumar e Priyadarsini, 2014; Qazi, Angel et al., 2019). Quantification of Se in spermatozoa has demonstrated about 0.8 and 0.2 fg of Se in midpiece and head, respectively (Kehr, Malinouski et al., 2009). According to

Pieczyńska and Grajeta (2015), SePP deficiency in a group of selected mice has induced numerous disorders of the structure of the spermatozoa tail (Pieczyńska e Grajeta, 2015). It is known the seminal fluid also presents high concentration of SePP which is important to protect the sperm during the storage, genital tract passage and final journey (Ahsan, Kamran *et al.*, 2014; Domosławska, Zdunczyk *et al.*, 2018). So, enough Se supplies for SePs is crucial to maintain a good semen characteristics for quality and fertility.

For women, is observed a significant decrease of Se concentration in the blood during the second and third trimester of pregnancy, probably due to an increase demand for oxygen in the mother's body and to a developing fetus (Pieczyńska e Grajeta, 2015; Grieger, Grzeskowiak *et al.*, 2019). According to Pieczyńska and Grajeta (2015) deficiency of Se in pregnant women may lead to dysfunction in the nervous system of the fetus (Pieczyńska e Grajeta, 2015).

2.1.2 Brain disorders: SePP transports Se to the brain and increases the probability of the apoER2 receptor in facilitate the SePP uptake at the blood-brain barrier (Burk e Hill, 2009). Studies have demonstrated that interruption of selenium supply to the brain impair the SePP-apoER2 pathway causing neurodegeneration, and can cause irreversible changes in the neuronal cells leading to cognitive impairment, seizures, Parkinson, Alzheimer, etc (Valentine, Abel *et al.*, 2008; Vicente-Zurdo, Romero-Sánchez *et al.*, 2020).

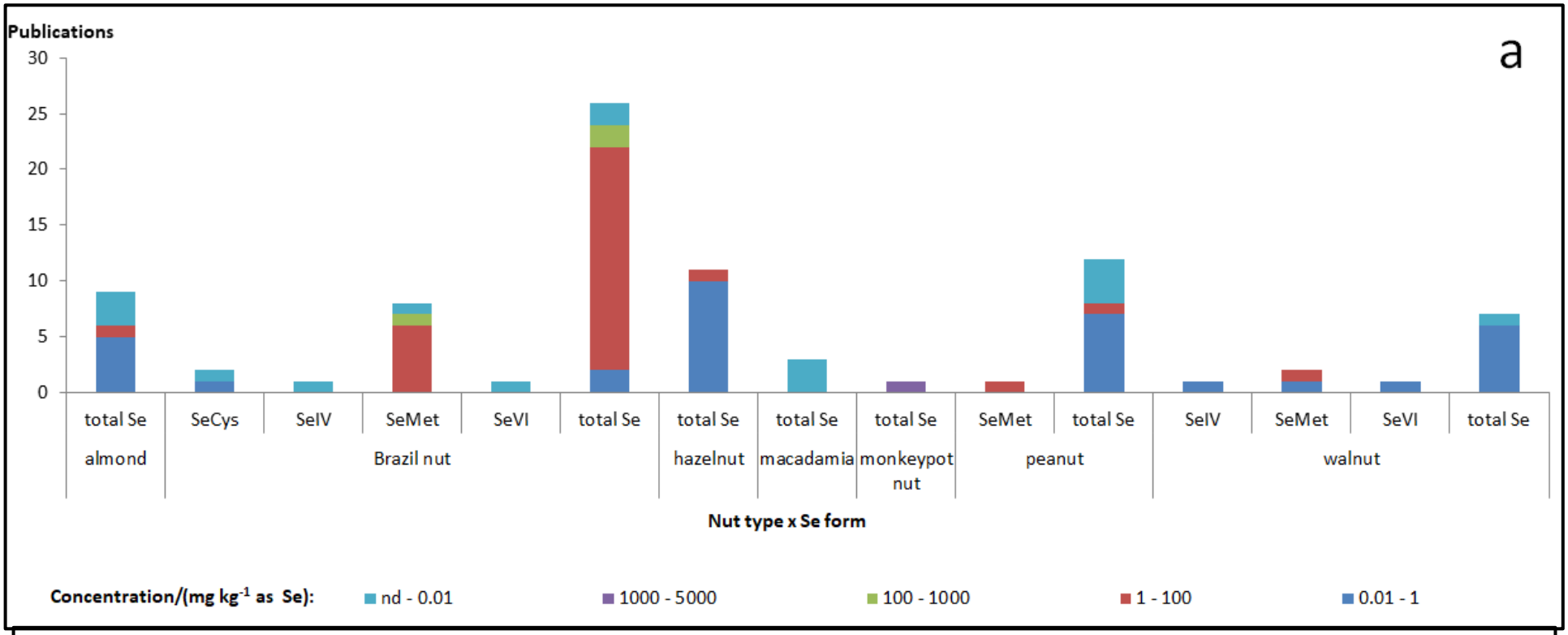
2.1.3 Cardiovascular disorders: Low level of Se in plasma is associated with the increased risk of cardiovascular diseases. One example is the Keshan disease, a potentially fatal form of cardiomyopathy that was first reported on China and occurs due to the Se deficiency combined with infection by coxsackie B virus (Holmgren, 2009). Studies have suggested that supplementation with Se could reduce the risks associated with cardiovascular diseases once Se prevents the oxidative modification of lipids, platelet aggregation and inflammations (Cominetti, De Bortoli *et al.*, 2012; Zhang, X., Li, X. *et al.*, 2018; Méplan e Hughes, 2020).

2.1.4 Thyroid dysfunction: Thyroid is one of the organs with the highest selenium content. This micronutrient plays an important role, through the GPx protein, in protect the thyroid cells from hydrogen peroxide generated in the cells to be used by the thyroid peroxidades in the synthesis of

the actives thyroid hormones triiodothyronine (T3) and thyroxine (T4) (metabolism-regulating hormones responsible for the development of several important functions in organism systems, like in the CNS) (Schomburg e Köhrle, 2008; Stuss, Michalska-Kasiczak *et al.*, 2017). Women at the reproductive age with autoimmune thyroiditis are prone to develop hypothyroidism that affect the fertility, course of pregnancy and the development of the child, besides to get thyroid dysfunction after delivery (Drutel, Archambeaud *et al.*, 2013; Ibrahim, Kerkadi *et al.*, 2019). Also, during pregnancy, the CNS of the fetus takes place in the first and second trimester and is mainly determined by the transport of the mother's thyroid hormones T3 and T4 through the placenta (Pieczyńska e Grajeta, 2015). Studies performed by Negro et al (2007) demonstrated the benefits of SeMet supplementation in the management of autoimmune thyroid disorders in pregnant woman, decreasing the thyroid inflammatory activity, post-partum thyroid disease and permanent hypothyroidism, as well as, being effective against Hashimoto disorder (Negro, Greco *et al.*, 2007).

2.1.5 Cancer: The exact mechanism of the chemoprotective effect of Se on cancer prevention is unknown, but epidemiological studies have indicated that exist an inverse correlation between the Se intake and the risk of several cancers occurrence (gastrointestinal, lung, skin, prostate, thyroid, etc) in humans and animals (Kuršvietienė, Mongirdienė *et al.*, 2020). Studies have reported the protective effect of the supplementation with selenium-enriched yeast on total cancer incidence (Clark, Combs *et al.*, 1996; Karunasinghe, Ng *et al.*, 2020). Hurst et al (2012), for example, suggested that Se was effective in slow the progression of prostate cancer (Hurst, Hooper *et al.*, 2012); and according to Ibiebele et al (2013) the Se supplementation was associated with about 60 % reduction in gastrointestinal cancers (Ibiebele, Hughes *et al.*, 2013). Also, for Brigelius-Flohé and Maiorino (2013), the GPx is the most important SeP antioxidant in the colon and provides defense against colon cancer (Brigelius-Flohé e Maiorino, 2013). Despite it all, more studies are needed to complete understand the role of Se on cancer prevention and also to recognize which form is more effective in which kind of cancer.

Figure 1 – Inventory about Se species analyses in different nuts in terms of quantity of publication: (a) type of nut x Se form x concentration; (b) total Se concentration x sample from; (c) Se form x nut; (d) Se form x extraction method; (e) Se form x detection method; (f) detection method x LOD; (g) detection method x Se form x concentration.



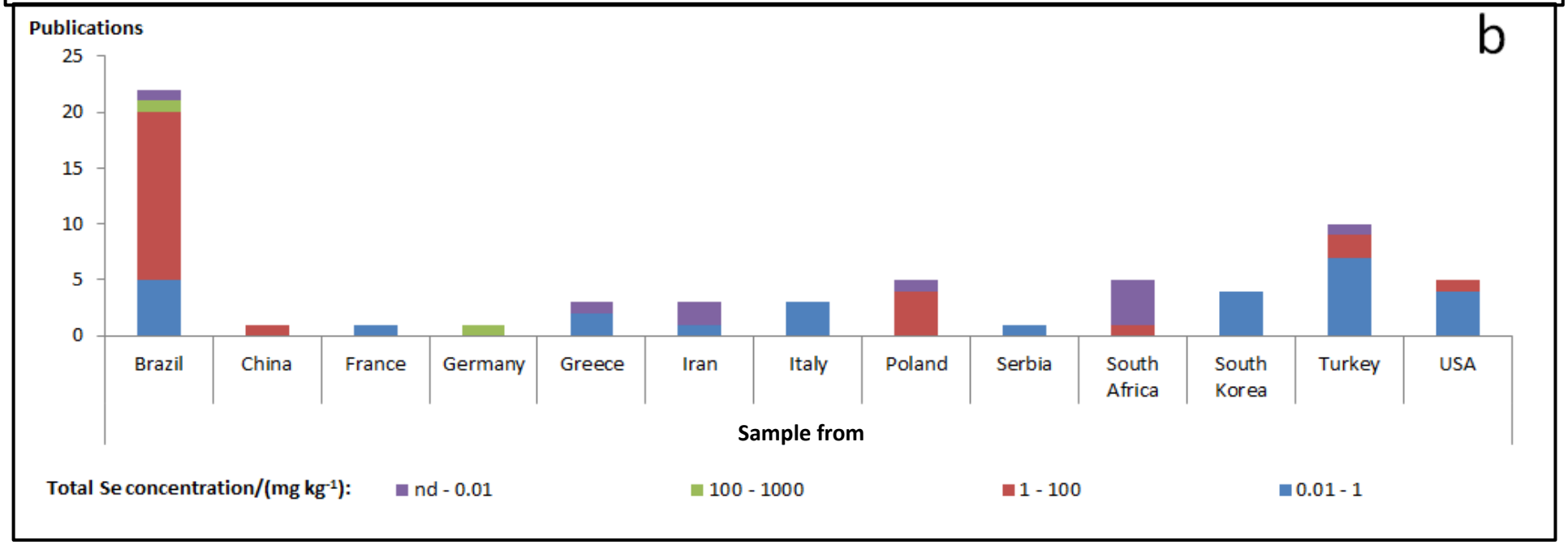
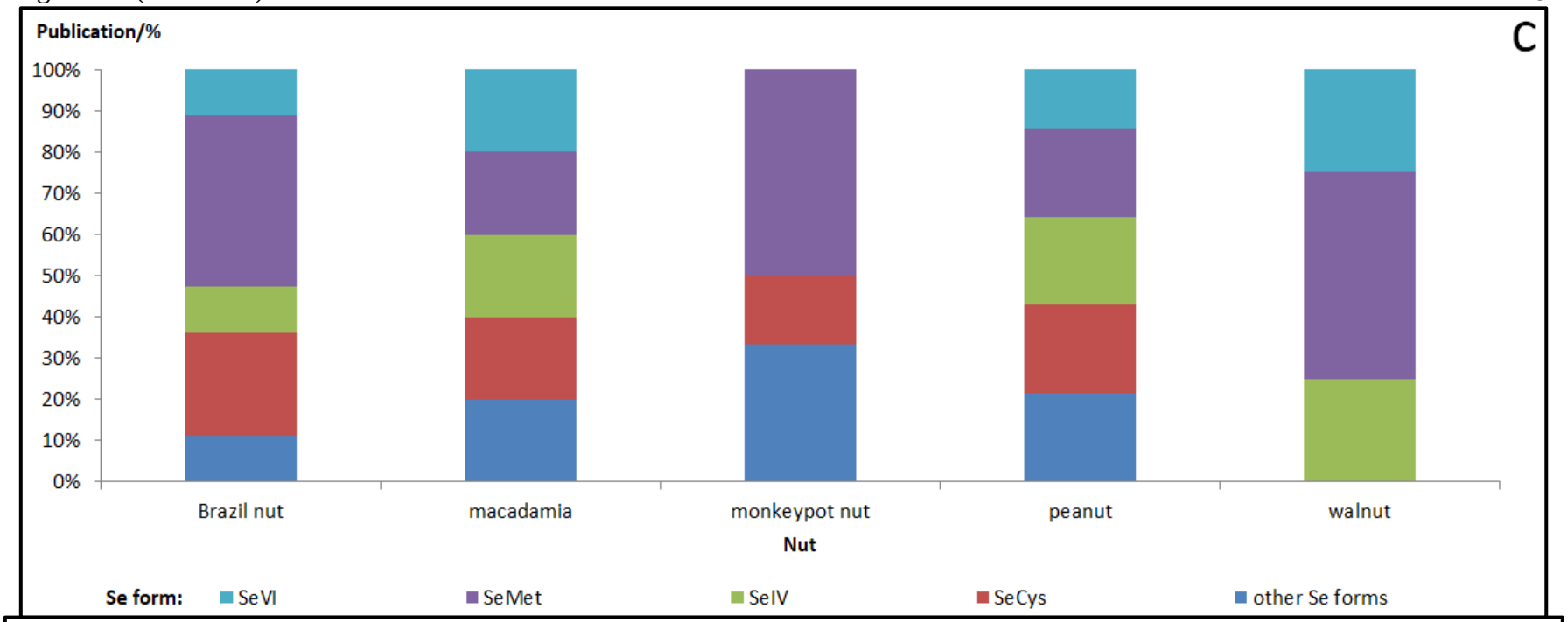


Figure 1 – (*continued*)



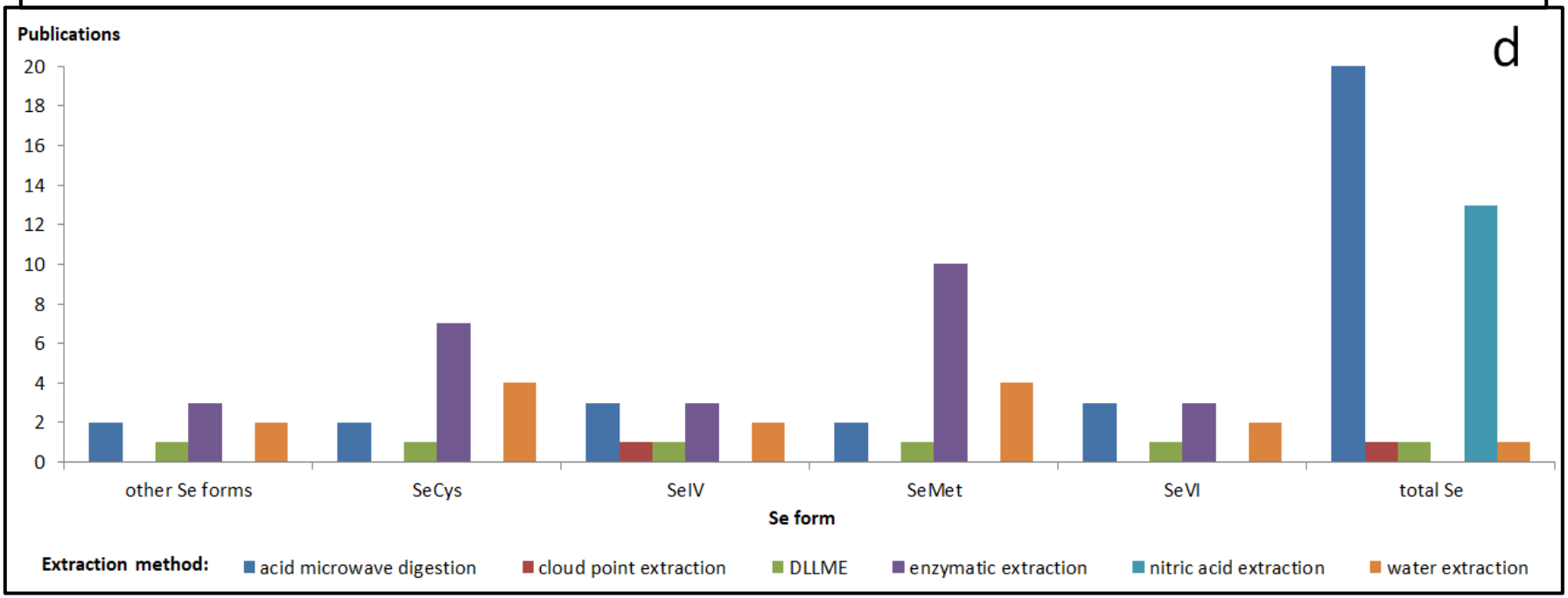
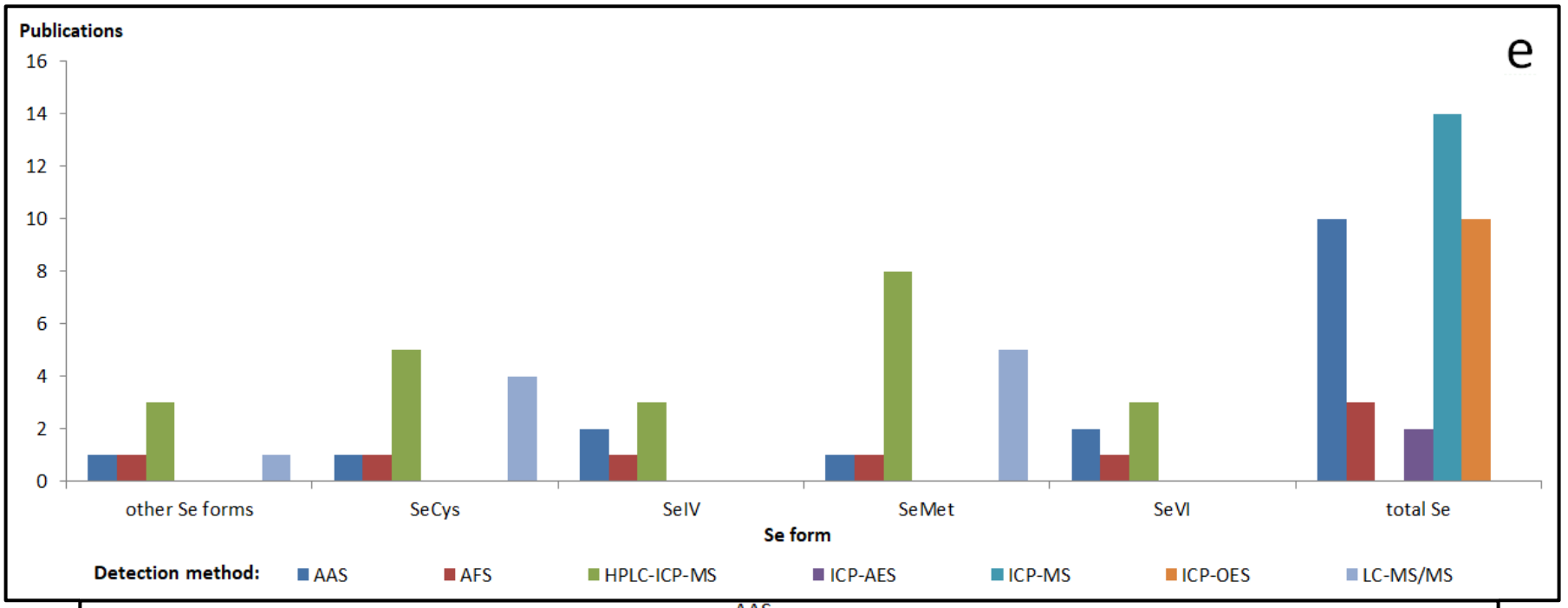


Figure 1 – (*continued*)



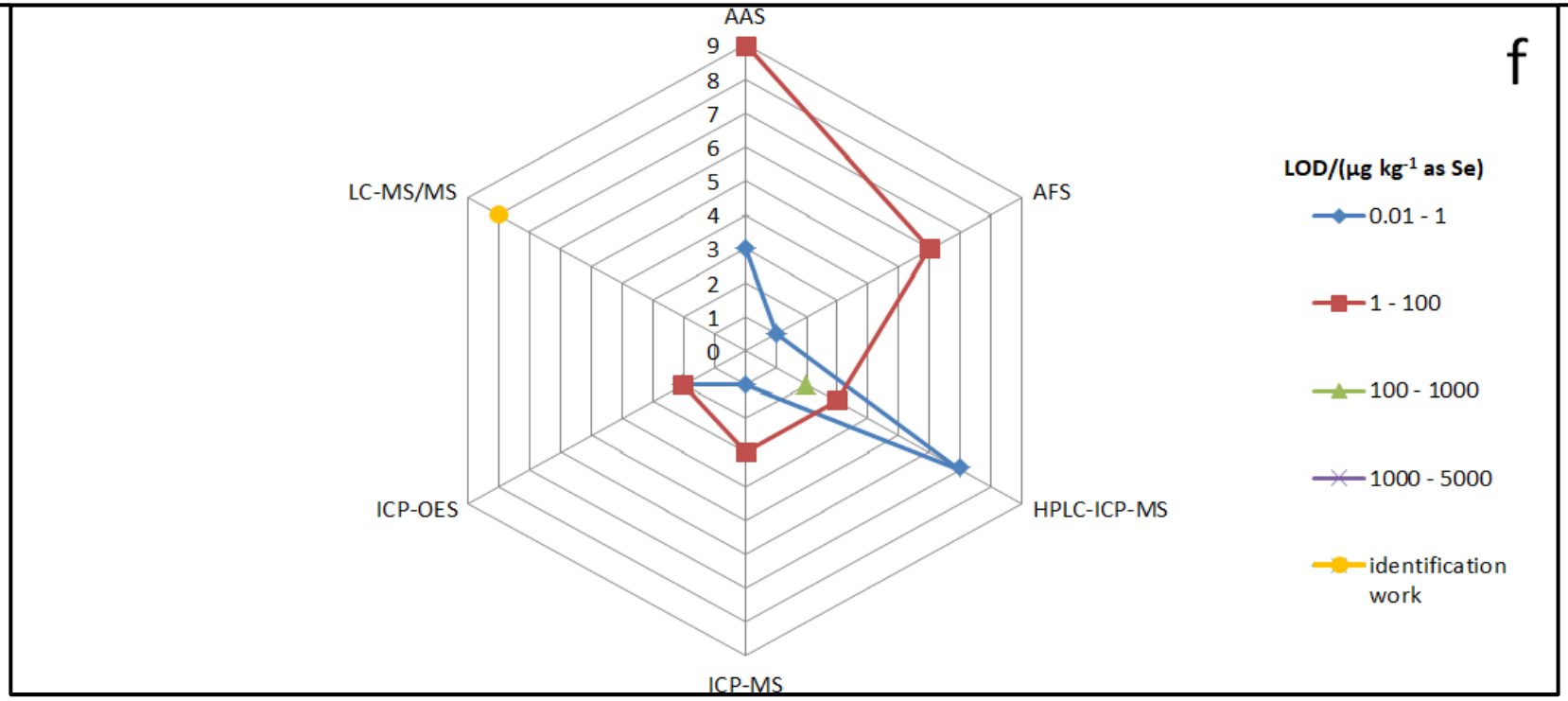
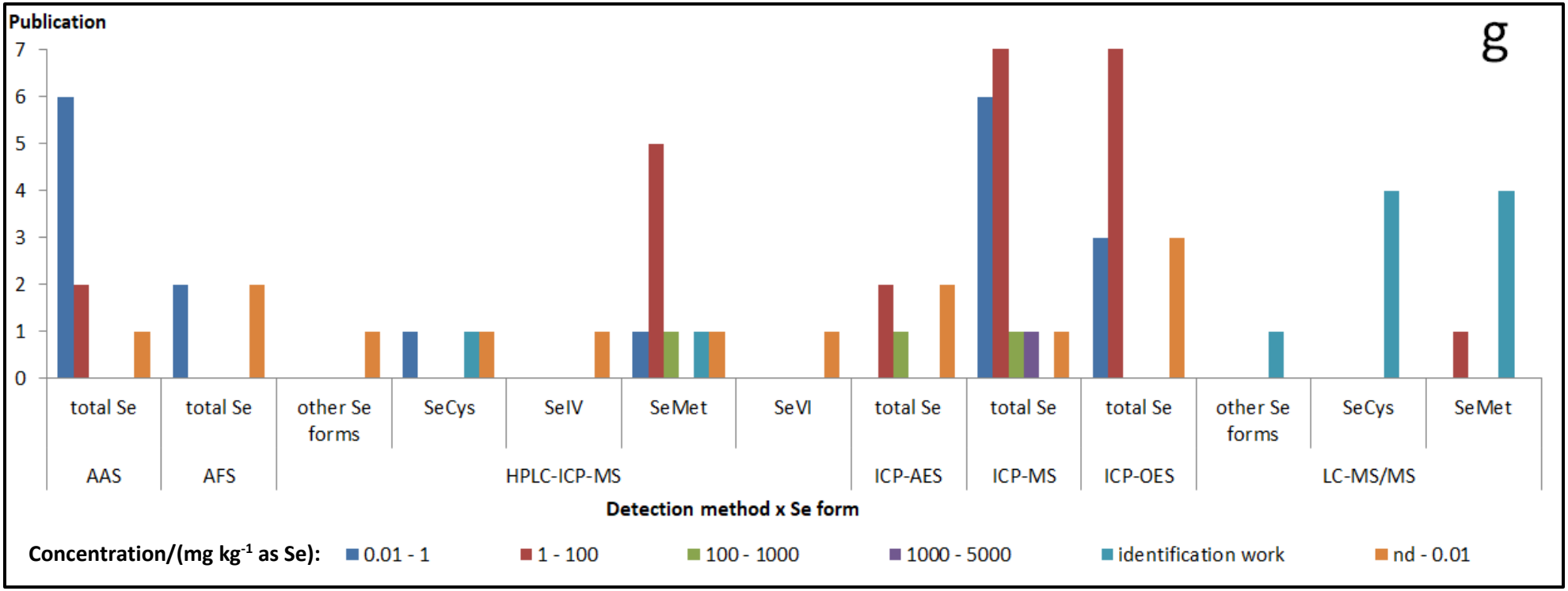


Figure 1 – (*continued*)



Source: author, 2020.

Se: Selenium; SeMet: Selenomethionine; SeCys: Selenocystein; nd: not detected; USA: United Stat of America; DLLME: Dispersive Liquid-Liquid Micro Extraction; LOD: Limit of Detection; AAS: Atomic Absorption Spectroscopy; AFS: atomic fluorescence spectrometry ; HPLC-ICP-MS: high-performance liquid chromatography coupled to inductively plasma mass spectrometry; ICP-AES: inductively coupled plasma atomic emission spectrometer; ICP-MS: inductively plasma mass spectrometry; ICP-OES: inductively plasma optical emission spectroscopy ; LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry.

2.2 Analyses of Total Se and Se Containing Molecules in Nuts

Despite its importance to economic value and health issues, up to now there is not an official approved method for determination of Se in foods. However, before any chemical analysis in a given matrix, some questions should be raised, which correspond the steps of the analytical process. These steps include decisions about the sampling size, the appropriate sample preparation method and, among other factors, which quantification and detection system is sensible to the target compound. These questions can be asked through the knowledge of the physical and chemical properties of the analyte, the sample matrix composition and the mechanism of detection of the instrumental system for quantification.

This topic presents relevant trends regarding the main sample preparation methods and detection systems used for Se species and total Se analyses in different nut matrices, and aim to clarify researchers in future researches related to this theme.

2.2.1 Sample preparation methods for Se and Se containing molecules extraction and applications for nut analyses

Previous preparation of the sample is an essential requirement in food analyses once these matrices normally presents high complexity, and compounds like lipids, sugars, pigments and other substances with high molar masses are possible coextractives that may interfere the instrumental analysis (Alcântara, Fernandes *et al.*, 2019). Therefore, the sample preparation aim to isolate and concentrate the target element at appropriate levels, besides to remove interferences from the sample matrix, improving the performance of the analytical method in terms of selectivity, sensibility, precision and accuracy (Maciel, De Toffoli *et al.*, 2019). Also, an efficient sample pre-treatment preserves the detection system from inconveniences ensuring a longer instrument life (Cabrera, Caldas *et al.*, 2016).

Usually this is the most time-consuming and laborious step of the analytical process, which there are great possibilities of errors mainly through losses of analyte or sample contamination (Xia, Yang *et al.*, 2019). So, ideally these methods should be easy to manipulate and fast, with as few steps as possible, besides being of low cost, rugged, effective and meet the requirements of green chemistry (Alcântara, Paz *et al.*, 2018).

To choose a suitable pre-treatment procedure the characteristics of the analyte, the sample nature and the analytical technique employed for determination must be taken into account. The physical and chemical properties (e.g. charge, polarity, volatility, pKa, etc) are fundamental parameters for the analyte, while for the sample matrix, its physical state, sample size, content of organic matter (e.g. fat, pigments, proteins, etc) are some of the important variables (Jardim, 2010).

For Se species extraction from foods, all those requirements for sample preparation are also necessary and over the years numerous methods have been developed to this aim. Messaoudi et al (2020), for example, used the liquid-liquid extraction employing high oxidative reagents (H_2SO_4, HNO_3, H_2O_2 and HCl) as sample digestion solvents to determine total Se in pennyroyal (*Mentha pulegium L*) by Radiochemical Neutron Activation Analysis (RNAA) (Messaoudi, Begaa *et al.*, 2020). According to the authors, the Se concentration obtained varied from 0.043 to 0.054 mg kg^{-1} and kept close to the minimal Food and Agriculture Organization (FAO) recommendations. Hirtz and Gunther (2020), on the other hand, determined the total Se content in selenized yeast reference material SELM-1 using microwave digestion (3:1 HNO_3:H_2O_2) and ICP-MS detection obtaining values in accordance with the certified range (2059 ± 0.064 µg kg^{-1}). Additionally, they also evaluated the efficiency of an ultrasonication-assisted extraction method employing TRIS-buffer as solubilization solvent for Se species speciation, and reported an extraction yield near 100 % for SeMet and methylselenocysteine (MeSeC) from supplements. The authors attributed the good performance of this sample preparation to its least invasive feature, preserving the chemical identity of the monomeric Se units (Hirtz e Günther, 2020). And Liang et al (2018), optimized an alkaline treatment to extract SeP from Se-enriched rice, where the most significant factor affecting the extraction was the NaOH concentration (optimal value of 0.14 mol L^1) (Liang, Lan *et al.*, 2018).

Figure 1d presents a bibliographic survey about the main methods employed to extract Se species from nuts in the last years. It is observed that the preferred procedure for organic Se (SeMet and SeCys) determination is normally based on enzymatic hydrolysis extraction. For speciation analyses, methods must be capable of quantitatively extract each Se containing molecules without altering the nature of the individual species, and enzyme extraction with use of proteases and lipases as assisted enzymes are the most efficient approach to quantitative extract Se compounds from biological samples with no degradation of the

selenoamino acids (Zhao, Zheng *et al.*, 2011; Gawor, Ruszczynska *et al.*, 2020). Studies have reported that the protease K, for example, is efficient to cleave the peptide bonds of the amine group of the methionine, while the carboxyl group remains intact preserving the identity of the compound (Wrobel, Kannamkumarath *et al.*, 2003). Vonderheide et al (2002), examined various sample preparation approaches with the goal of Se preservation after speciation in Brazil nuts samples, and the treatment with the proteinase K was the most efficient (Vonderheide, Wrobel *et al.*, 2002). For Moreda-Piñeiro et al (2018), methods employing proteases have been the most widely used to release protein-bound compound in several foods as these enzymes mimics the physiological conditions of the human intestine (Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018).

However, due to the long period required to disrupt the cell membranes by enzymes, the dependency of an accurate sample/enzyme ratio for good extraction, the possibility of incomplete release of the species after treatment and costs associated with the proteases are some limitations of the enzymatic hydrolysis and the reason to this method going out of use in recent years as a single preparation method. In studies performed by Egressey-Molnar et al (2011), a time of 24 hours over shaken was needed to complete extract SeMet from monkeypot nut using protease XIV (Egressy-Molnár, Vass *et al.*, 2011). And Vonderheide et al (2002) reported that SeMet quantification in Brazil nuts could be underestimated due the incomplete enzymatic hydrolysis of the proteins by the proteinase K (Vonderheide, Wrobel *et al.*, 2002). Also Yang et al (2004) related that for maximum recovery of SeMet from SELM-1 certified reference material (CRM), a significant high amount of costly protease XIV was needed (400 mg), making this procedure relatively expensive for routine analysis (Yang, Lu, Sturgeon, Ralph E *et al.*, 2004).

Thus, novel and emerging technologies that minimize the drawbacks of enzymatic hydrolysis have been widely developed. The most promising technologies include the use of microwave, ultrasound and high hydrostatic pressurized energies (Franck, Perreault *et al.*, 2019). These techniques used in combination with enzymatic hydrolysis enhance the disruption of cell membranes by application of energies before or at the same time of the enzymes acting chemically, decreasing the time and amount of proteases needed for an appropriate extraction (Zhou, Wang *et al.*, 2017). Moreda-Piñeiro et al (2018) used the pressurized-assisted enzymatic hydrolysis and the microwave-assisted enzymatic hydrolysis with protease XIV to release Se species from Brazil nuts and reported that the hydrolyses can be completed in 7 and 12 minutes respectively, which is a considerably shortened time when compared with the classic enzymatic

extraction (Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018). Also Peachey et al (2008) suggested the use of microwave energy to assist the enzymatic treatment as a newly method to efficiently extract SeMet from selenized yeast samples with a dramatic reduction of extraction time in comparison with the conventional enzymatic methodologies (Peachey, Mccarthy *et al.*, 2008).

An alternative method to enzymatic extraction to release SeMet from nut matrices was developed by Wrobel et al (2003) with the use of 4 mol L^{-1} methanesulfonic acid (MSA) at reflux (125 °C) for 8 h for protein hydrolysis (Wrobel, Kannamkumarath *et al.*, 2003). According to the authors, better cleavage of SeMet was observed in this procedure compared to the conventional enzymatic hydrolysis and significantly higher amount of SeMet was found in yeast and nuts when this acid treatment was employed. Yang et al (2004) compared the efficiency of SeMet extraction in a yeast CRM by fourteen different methods including acid (with MSA or HCl), alkaline (with tetramethylammonium hydroxide) and enzymatic (with protease K, XIV, VIII or pronase + lipase) digestions and the MSA refluxed was found to be the most efficient (Yang, Lu, Sturgeon, Ralph E *et al.*, 2004). Yet, according to them, among the enzymatic hydrolysis, the one that used 20 mg pronase and 10 mg lipase got the highest extraction efficiency, but recoveries remained nearly 50 % lower than the MSA extraction.

The drawback of the Wrobel et al (2003) method using MSA is the loss of about 2 % of SeMet by decomposition (Wrobel, Kannamkumarath *et al.*, 2003), furthermore it was developed exclusively to SeMet analyses, and therefore its efficiency for other Se species extraction is unknown. Thus the enzymatic treatment in combination with different energies supplies (microwave, ultrasound, etc) has remained as the most used strategy for several Se species extraction, including SeMet, in recent years.

The extraction yield of most proteins strongly depends of the media pH once amino acids are amphoteric species that can act as an acid or a base depending on the pH value. If values close to the isoelectric pH are used the zwitterion forms of most of these molecules predominate and their solubility is impaired (Phongthai, Lim *et al.*, 2016). In studies performed by Xiong et al (2016), the highest extracted protein yield was obtained at extreme values of pH (< 2.5 and > 11.5), while significant lower extractions were observed in intermediates pH (between 3.5 and 8) (Xiong, Gao *et al.*, 2016). However, the survey presented in Figure 1d shows that extraction using only water remained as the second most used technique to extract SeMet, SeCys and Se VI. This can be explained by the fact that lots of the works that employed

water as extraction solvent aimed to perform only an identification study of the Se species (Dernovics, Giusti *et al.*, 2007; Bierla, Szpunar *et al.*, 2012; Da Silva, Mataveli *et al.*, 2013; Németh, Reyes *et al.*, 2013; Bakirdere, Volkan *et al.*, 2015; Both, Shao *et al.*, 2018). This kind of pretreatment is simple, of low cost and environmentally friendly and is convenient to be used when only qualitative results are requested. Da Silva et al (2013), for example, relate that SeMet and SeCys were the main species identified after water treatment in Brazil nuts samples (Da Silva, Mataveli *et al.*, 2013). And Németh et al (2013) report identification of selenohomocystine (SeHCy), SeMet and methyl-selenomethionine (MetSeMet) in monkeypot nut samples after water extraction (Németh, Reyes *et al.*, 2013).

Several other methods have been reported in the literature with the aim to extract Se species from nuts, like cloud point extraction (CPE) (Depoi e Pozebon, 2012; Tadayon e Mehrandoost, 2015), dispersive liquid-liquid micro extraction (DLLME) (López-García, Vicente-Martínez *et al.*, 2013) and alkaline extraction (Chunhieng, Pétritis *et al.*, 2004; Kannamkumarath, Wrobel *et al.*, 2005; Alcântara, Nascimento *et al.*, 2020). According to John et al (2017) the LLME is a simple and low cost technique with low time, solvent and amount of sample consumptions (John, Kuhn *et al.*, 2017). For Xiong et al (2016) the alkaline treatment presents good performance in extract water-soluble amino acids (Xiong, Gao *et al.*, 2016); and the use of sodium dodecyl sulfate (SDS) buffered alkaline digestion has been reported in the literature as an alternative method to this aim due to its non-destructive character capable to preserve the chemical identity of the Se species (Kannamkumarath, Wrobel *et al.*, 2005; Alcântara, Nascimento *et al.*, 2020).

Microwave digestion (normally with nitric acid as oxidation solvent) followed by nitric acid solubilization, were the most used extraction methods when the goal was the total Se analysis in nuts (Figure 1d). Total Se is the sum of Se atoms from all Se species, which means a complete degradation of all Se proteins is necessary to release the Se element, thus a method with high power of digestion is needed, and wet decompositions are quite convenient to this purpose. These methods use acids with oxidizing potential with heating for complete mineralization of the organic matter. The wide use of nitric acid as solubilization solvent can be justified by its considerable power of digestion at high temperatures and its capacity to dissolve several elements including Se (Astolfi, Protano *et al.*, 2020). Also the risk of explosion when HNO_3 is in contact with organic matter is low, besides a reagent of high purity, very convenient

to elemental trace analyses, is easily obtained by sub boiling distillation (Harris, 2015c). In combination with HNO_3 several works have used few amounts of hydrogen peroxide (H_2O_2) as an auxiliary oxidizing agent (Esteki, Vander Heyden *et al.*, 2017; Gao, Luo *et al.*, 2017; Juranović Cindrić, Zeiner *et al.*, 2018; İçelli, Öz *et al.*, 2020; Karasakal, 2020).

In a hermetically sealed ambient resistant to high pressure, faster reactions can be obtained once a sufficiently higher temperature is reached. Proteins and amino acids are completely digested at these conditions, with use of HNO_3, in a considerable low time. Microwave furnaces meet this technology with safety (which justify the greatest utilization of this technique to total Se determination in nuts, Figure 1d), and the sample temperature rise by direct absorption of the electromagnetic radiation is an advantage over the conventional conductive heating methods (Krug, 2008; Wathudura, Peiris *et al.*, 2020). Works have reported that significant higher amounts of trace elements can be obtained in samples treated with microwave digestion than with open vessel acid digestion by heating (Kilic e Soylak, 2020). According to Tarantino et al (2017) the use of microwave digestion offers advantages of minimal risk of sample contamination, feasibility for treatment of micro-samples and capability for treatment of several samples at a time with small volumes of high purity solvents that are costly to be obtained (Tarantino, Barbosa *et al.*, 2017).

Karasakal (2020) compared the efficiency of extraction of different amounts of HNO_3/H_2O_2 (2+1) as oxidation solvents for total Se determination in almond, walnut and coconut oils using microwave digestion and ICP-OES detection, and observed that concentrations generally decreased when amounts of HNO_3/H_2O_2 mixtures increased (Karasakal, 2020). According to the authors, the total Se concentration varied in the range of 2.97 to 3.80 mg kg^{-1} in walnut, of 2.52 to 3.97 mg kg^{-1} in almond and of 2.49 to 4.51 mg kg^{-1} in coconut oils. Içelli et al (2020) reported values for total Se concentration between 0.014 and 0.016 mg kg^{-1} in hazelnuts from Turkey (İçelli, Öz *et al.*, 2020); while Gao et al (2017) quantified a maximum total Se concentration of 17.01 mg kg^{-1} in Chinese peanut samples (Gao, Luo *et al.*, 2017).

The downside of microwave digestion technique is the reduced sample masses used for digestions to prevent explosion due to the pressurized atmosphere, which can impair the sensitivity of the method, but the use of highly sensitive and selective detection methods overcome this limitation. The next topic aims to outline the major detection and quantification

systems used for Se species and total Se determinations in nut samples, where their main advantages and limitations are clarified.

2.2.2 Main detection and quantification systems used for Se and Se containing molecule analyses in nuts.

Before any data treatment, the choice of a suitable quantification method is the last step of the analytical process and it is very important to determine the success in the Se species analyses in foods. Thus, the physical-chemical properties of the target compound, the complexity of the sample matrix and the detection mechanism of the instrumental system are some of the variables that must be take into account to get an accurate and reliable result.

ICP-MS and ICP-OES were commonly used for the total Se determination (Figure 1e). In ICP, the ionization of the sample occurs in the last step of the inductively coupled plasma of argon shortly afterward the occurrence of desolvation, vaporization and atomization (Beauchemin, 2017). The high temperature involved in this process enable a wide atomization spectrum and a wide calibration range (Phan-Thien, Wright *et al.*, 2012; Wilschefski e Baxter, 2019).

When coupled to MS, ICP can be a powerful tool for the determination of total Se in several foods. Using the dynamic reaction cell (DRC), interferences from the sample matrix (e.g. coeluting compounds) and the ones associated with the system (e.g. isobaric interferences like $^{40}Ar^{+}_{2}$ for ^{80}Se) are minimized increasing the selectivity and sensitivity of the method (Németh, Reyes *et al.*, 2013; Leblanc, Kawamoto *et al.*, 2019; Pyrzynska e Sentkowska, 2019). Lopes et al. (2016), for example, monitored the ^{82}Se by ICP-MS in Brazil nuts and babassu coconut using the DRC and obtained an improvement of about three times in the LOD value (about 7.00 µg kg^{-1}) (Lopes, G. S., Silva, F. L. *et al.*, 2016).

However, the OES detector can be a more affordable possibility in the coupling with ICP, getting sufficiently good data quality for many investigations. LOD values in the range of 1 to 100 µg kg^{-1} (Figure 1f) have been obtained by both techniques (ICP-MS and ICP-OES) (Tošić, Mitić *et al.*, 2015).

When hyphenated with a separation system, the ICP can provide valuable information about the quantity of the various Se species since the detection is associated to the

retention time of each compound previously separated (Pyrzynska e Sentkowska, 2019). HPLC is a very interesting separation technique which brings the possibility of identification, purification and quantification of several compounds at a time, especially the least volatile ones (Silva e Collins, 2011; Prathap, Dey *et al.*, 2013; Pyrzynska e Sentkowska, 2019; Sentkowska, 2019). In this review was noted that the non-volatile Se species, like SeMet and SeCys, were mainly quantified when the HPLC separation system was linked to the ICP-MS detection (Figure 1e) (Gao, Luo *et al.*, 2017; Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018).

Compared with the use of the ICP by itself, the utilization of HPLC-ICP-MS yielded a greater sensitivity where was observed trends to obtain LODs in the range of 0.01 to 1 μg kg^{-1} as Se for the coupled system against 1 to 100 μg kg^{-1} as Se for the ICP-MS system (Figure 1f). However, is important to note that while the HPLC-ICP-MS apparatus had its use mainly aiming the quantification of the different Se species in nuts, the ICP-MS or ICP-OES were very convenient for the determination of total Se where there is no need for a previous separation of the species, which cause an increase of the analyses time.

Liquid chromatography coupled to tandem mass spectrometer (LC-MS/MS) systems were also quite employed for organic Se analyses (e.g. SeMet and SeCys) as show Figure 1e. However, they were used mainly for identification purpose (Figure 1g and Figure 1f). These techniques presents the possibility of operating in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) modes that provides additional data of structural information of molecules by the utilization of their mass spectrums, so they are a powerful tool to identify and to confirm the presence of a particular compound in the matrix (Alcântara, Paz *et al.*, 2018; Oiram Filho, Alcântra *et al.*, 2018; Alcântara, Fernandes *et al.*, 2019). A lot of works used the mass analyzer single quadrupole in series with the time of flight (qTOF) once this connection presents a high resolution capacity, where while the quadrupole works as a mass filter, the time of flight is able to differentiate masses in the order of four decimal places. As expected, SeMet and SeCys were identified in several monitoring works in the most varied of nut matrices (Figure 1g) (Chunhieng, Pétritis et al., 2004; Dumont, De Pauw et al., 2006; Dernovics, Giusti et al., 2007; Németh, Reyes et al., 2013; Németh e Dernovics, 2015).

Several other methods have been reported in the literature to determine Se species in nut matrices, such as atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS). These techniques can become a powerful strategy for the determination of

Se IV, arising from the reaction between Se VI and $NaBH_4$ in acid medium, when associated with Hydride Generation (HG) (Takase, Pereira *et al.*, 2002; Zam, Alshahneh *et al.*, 2019). Among the advantages of these systems is the possibility to reduce interferences caused by the matrix since the analyte is detached from it as the hydride is generated improving the selectivity and sensibility (De Lima, Do Lago *et al.*, 2013; Zam, Alshahneh *et al.*, 2019).

Thus, different strategies have been described in the literature to analyze the different Se species in foods, where several possibilities regarding the sample preparation and detection systems were developed by the scientific community, and the time of analyses, the target Se compound, the type of nut, the robustness and affordability are some of the factors involved for the choice of a suitable analytical method.

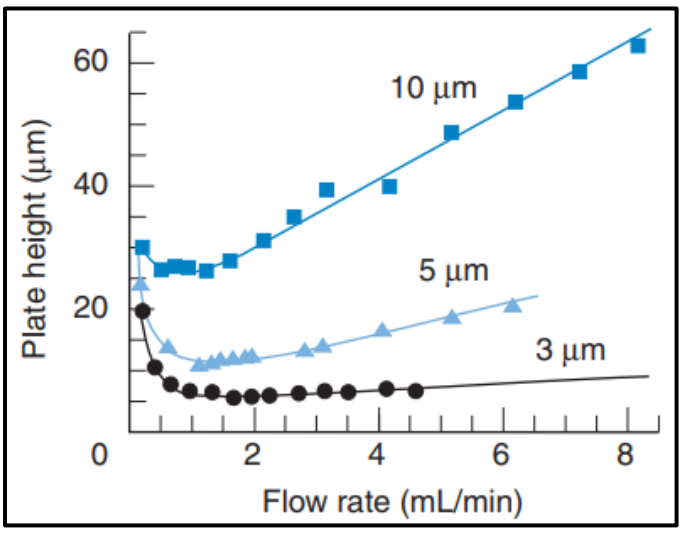
2.3 Considerations about Separation Techniques Employing Liquid Mobile Phase

2.3.1 Liquid chromatography (LC) coupled to mass spectrometer (MS) and applications for SeMet analyses in foods

LC is a separation technique for soluble substances and it uses high pressure to force a liquid mobile phase, whose function is to transport the injected sample towards the detector, pass through a packed column containing very fine particles that are usually involved by a chemically bonded liquid film constituting the stationary phase (Harris, 2015a). The differentiated interactions of different compounds between the mobile and stationary phases separate them.

The efficiency of separation in a packed column increases as the size of the stationary phase particles decreases, where a significant decreasing on size permits to improve the peaks resolution or to maintain the same resolution while decreasing the run time (Douglas Skoog, 2014). The better resolution provided by small particles is a result of the uniform flow of mobile phase through the column that reduce the 'multiple path' term from the van Deemter equation (Harris, 2015a); also in a more packaged column, the distance in which the solute must diffuse in the mobile and stationary phases is in the order of the particle size, decreasing the 'finite equilibration time' term in the van Deemter equation as well (Douglas Skoog, 2014; Harris, 2015a). Figure 2 presents the van Deemter plots for different diameter particles, where the smaller the particle size the lower the plate height, and less sensitive to changes in flow rates the plate height is.

Figure 2 – Plate height as a function of flow rate for stationary phase particle diameters of 10, 5, and 3 µm.



Source: Harris, 2015a.

HPLC, that is a high pressure liquid chromatography technique (maximum pressure in the range of 600 bar), normally presents particle sizes between 3 and 5 µm (Harris, 2015a; Maciel, De Toffoli *et al.*, 2020). However well designed systems resistant to very high pressures (in the range of 1200 bar) that use columns with particle diameters lower than 2 µm already exists in the market and are a registered trademark of waters Corporation called as ultra-performance liquid chromatography (UPLC) (Gumustas, Kurbanoglu *et al.*, 2013; Harris, 2015a). Thus, different of the conventional HPLC, its special version UPLC presents the advantage to get the separation of several compounds in a very low time with less solvent consumption, obtaining a more effective data in terms of accuracy, selectivity and sensitivity (Kurbanoglu, Karsavurdan *et al.*, 2019).

When coupled to MS techniques, the sensitivity and selectivity can be further increased due the possibility to work in the selected ion recording (SIR) acquisition mode (Alcântara, Paz *et al.*, 2018; Barbosa, Martins *et al.*, 2018; Alcântara, Fernandes *et al.*, 2019; Fernandes, Alcântara *et al.*, 2019). The ACQUITY® QDa® detector presents this technology and once uses a single quadruple analyzer it is an affordable possibility, presenting a mass accuracy of about ± 0.2 Da (Cooper, 2013; Jagadabi, Nagendra Kumar *et al.*, 2018).

The connection between LC and MS techniques is possible through the electrospray ionization source (ESI) interface. ESI ejects preexisting ions from the solution into the gas phase and, as the mass spectra is normally simple due a little fragmentation of the analyte, it is well suited for the study of charged macromolecules such as proteins or its amino acids once these species can get a net negative or positive charge depending on the medium pH (Harris, 2015b). Several works have reported the use of ESI for proteins disintegration (Németh, Reyes *et al.*, 2013; Németh e Dernovics, 2015; Du, Lu *et al.*, 2020; Xue, He *et al.*, 2020; Yi, Ren *et al.*, 2020), and its application to SeMet analyses is very appropriated once this Se amino acid can change its net charge depending on the medium used to extract it. In studies performed by Alcântara et al (2020), acid treatments yielded positively charged SeMet species, while in alkaline treatments negatively charged species were formed (Alcântara, Nascimento *et al.*, 2020).

Table 1 summarizes papers from the literature that evaluated SeMet in different matrices regarding the concentration level achieved, quantification technique used and the figure of merits obtained, where due its low vapor pressure and, therefore, the need of derivatization in gas chromatography (GC) analyses, SeMet has been determined mainly by LC techniques

coupled to ESI tandem mass spectrometry (MS/MS) or ICP/MS. Zhang, Zhang and Zhang (2018) used the UPLC-ESI-MS/MS apparatus for selenium speciation of the blood plasma in rats after intragastric administration of Se-yeast (Zhang, Zhang *et al.*, 2018).

In this context, one of the objectives of this work was to develop a method to analyze SeMet in cashew nuts by UPLC-ESI/QDa system employing an ultrasound assisted LLME (UALLME) sample preparation. Since the variation in the analytical signal is a common inconvenience, even for robust detection systems such as many chromatography apparatus, SeMeSeC and genistein were tested as SeMet internal standards (IS), and their efficiency to correct its analytical signal were compared.

Table 1 – Survey regarding the type of sample analyzed, quantification technique used, concentration of SeMet found and figures of merit.

Reference	Sample	Instrumentation	SeMet Conc./(mg kg^{-1})	LOD/(µg kg^{-1})	Accurace/%	Linear range/(µg L^{-1})
Alcântara et al, 2020	Yeast (CRM-SELM-1)	AF4-ICP/MS	25 to 3320	0.49	105	-
Lima et al, 2019	Brazil nut	HPLC-ICP/MS	29 to 49	-	-	-
Gao et al, 2018	Rice	HPLC-ICP/MS	0.0239 and 0.0366	0.03	93.7 to 109	0.2 to 50
Zhang, Zhang and Zhang, 2018	Plasma rats	UPLC-MS/MS	-	-	79.4 to 95.4	2 to 200
Kubachka et al, 2017	Capsule and tablets suplements	HPLC-ICP/MS	n.d. to 1631.8	0.6	75%	5 to 10
Jagtap et al, 2016	Fish tissues	HPLC-ICP/MS	1.6 to 5.4	-	52 to 95%	0 to 100
Bodnar and Konieczka, 2016	Sprout samples (brocoli, cabage)	HPLC-ICP/MS	2.03 to 10.5	-	-	20 to 60
Fang et al, 2015	Rice and wheat	HPLC-ICP/MS	0.027 to 0.046	1.1	80.5 to 98.4%	2.5 to 400
Torres et al, 2014	Olive oil	UPLC-MS/MS	n.d.	-	84 to 94 %	-
Silva, Mataveli and Arruda, 2013	Brazil nuts and plankton CRM	HPLC-ICP/MS	Only qualitative analysis	0.4	91 % from plankton CRM	-
Hsieh and Jiang, 2013	Wheat and food suplements	HPLC-ICP/MS	0.94 to 1.94	0.04 to 0.07	104	0.10 to 10
Zhaoa et al, 2011	Rice	CE-ICP/MS	0.136 to 0.143	0.5	95 to 102%	10 to 400
Rebane, Herodes and Leito, 2011	Onion	HPLC-MS/MS	0.011 to 0.021	1	-	3.2 to 490
Vacchinaa et al, 2010	Yeast (CRM-SELM-1)	HPLC-ICP/MS	-	2.48	103 %	5 to 100
Fang et al, 2009	Se-enriched rice	HPLC-ICP/MS	0.085	0.77	74%	2.5 to 124
Bierla et al, 2008	Edible animal tissues	HPLC-ICP/MS	0.14 to 0.40	-	59.5 to 108 %	-
Su et al, 2008	Garlic and rabbit serum	HPLC-UV/Vis/nano-TiO2-chemil.	0.019 to 0.181	12	88 to 96 %	0.05 to 12.4
Gosetti et al, 2007	Commercial supplements	HPLC-MS/MS	107.6	3.5	-	11 to 100
McSheehy et al, 2005	Yeast (CRM-SELM-1)	LC/ESI-MS	-	0.95	108 %	-
Yang et al, 2004	Yeast (CRM-SELM-1)	GC/MS	-	0.9	107 %	-
Vonderheide et al, 2002	Brazil nuts	HPLC-ICP/MS	n.d. to 11.0	-	92 to 105 %	5 to 250

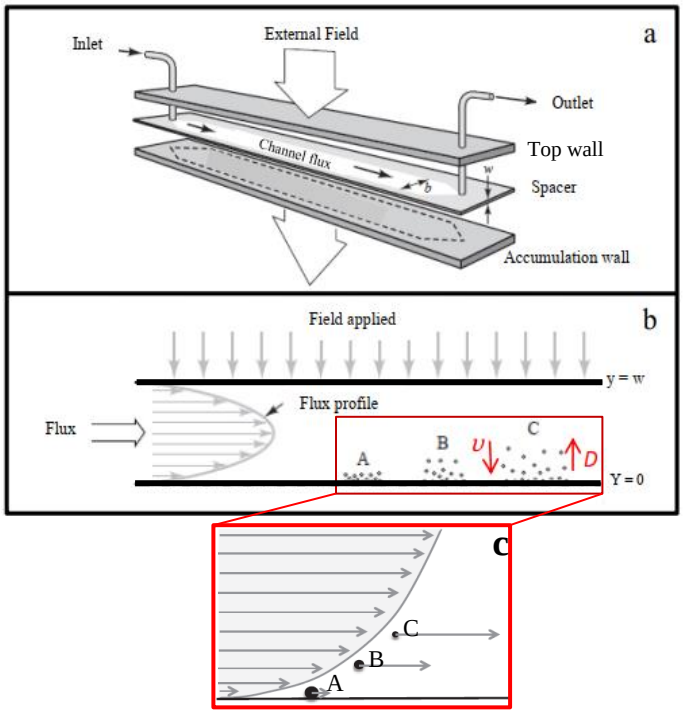
Note: AF4: Asymmetric Flow Field Flow Fractionation; CE: Capillary Electrophoresis; CRM: Certified Reference Material; ESI: Electrospray; GC: Gas Chromatography; HPLC: High-Performance Liquid Chromatography; ICP/MS: Inductively Plasma Mass Spectrometry; LC: Liquid Chromatograph; LOD: Detection Limit; MS: Mass Spectrometry; n.d.: Not Detected; SELM-1: Selenium Enriched Yeast Reference Material; SeMet: Selenomethionine; UPLC: Ultra-Performance Liquid Chromatography; UV/Vis: Ultraviolet-Visible; (-): not available.

2.3.2 Field Flow Fractionation (FFF)

Field Flow Fractionation (FFF) is one of the most versatile families of separation techniques known and is suitable for separation of macromolecule, colloids and particulates in a very broad molar mass range (Malik e Pasch, 2016). Its concept was first described by Giddings in 1966, but only recently its applications and advantages over other separation techniques have been demonstrated.

The separations in FFF occur inside a channel with the length ranging from 25 to 100 cm and width from 1 to 3 cm. Figure 3a demonstrate a typical FFF channel. Basically it is composed of two blocks (top wall and accumulation wall) and a spacer (thickness from 50 to 500 μm) (Douglas Skoog, 2014). The sample is injected in the channel inlet and an external field is applied perpendicular to the face channel (Figure 3a).

Figure 3 – (a) Scheme of a general FFF channel; (b) Principle of separation inside a channel; (c) Zoom of the region close to the accumulation wall.



Source: Skoog et al (2013) modified by the author.

The sample components interacts with the field which causing their migration toward the accumulation wall in a velocity (υ) determined by the intensity of the interaction (Zhang, X.,

Li, Y. *et al.*, 2018). As the diffusivity (*D*) of the particle acts against the field force, the sample components achieve a concentration distribution (steady state) close to the accumulation wall when υ and *D* get the equilibrium (Schimpf, Caldwell *et al.*, 2000; Zhang, X., Li, Y. *et al.*, 2018). Figure 3b illustrates this distribution for three different substances (A, B and C). The separation is obtained once different components have different υ and *D* values, where the higher the υ and the lower the *D*, the particle gets closer the bottom wall.

Only after the stationary profile is achieved, the flux of mobile phase inside the channel is started (Douglas Skoog, 2014). This flux must be laminar with a parabolic profile, where the highest linear velocity is at the center of the channel (Figure 3c). Thus, components farther from the bottom wall get a higher velocity than those closer the wall, and so, elute first (Figure 3c).

The classification of the FFF depends of the physical nature of the field. The most known are the electrical, thermal and sedimentation FFF. The choice of a specific technique is a result of the physical chemical characteristic of the target compound. In electrical FFF (EFFF), used for charged species, an electric field is applied perpendicular to the flux direction of mobile phase (Petersen, Shiri *et al.*, 2018). Species with higher charge are directed more efficiently to the accumulation wall. So, lower charge species elute first (Schimpf, Caldwell *et al.*, 2000).

In thermal FFF (ThFFF), a thermal field is employed through a temperature gradient along the channel thickness in order to induce the diffusion movement that depends of the molecule thermal coefficient (Greyling e Pasch, 2019). When the channel is placed inside a centrifuge, where a centrifugal field is applied, the sedimentation FFF (SeFFF) is obtained (Ivaneev, Ermolin *et al.*, 2020). The SeFFF is used for separation of high molecular weight molecules, where the components with bigger size and density get closer the accumulation wall and have a higher retention time value (Giddings, 1995; Ivaneev, Ermolin *et al.*, 2020).

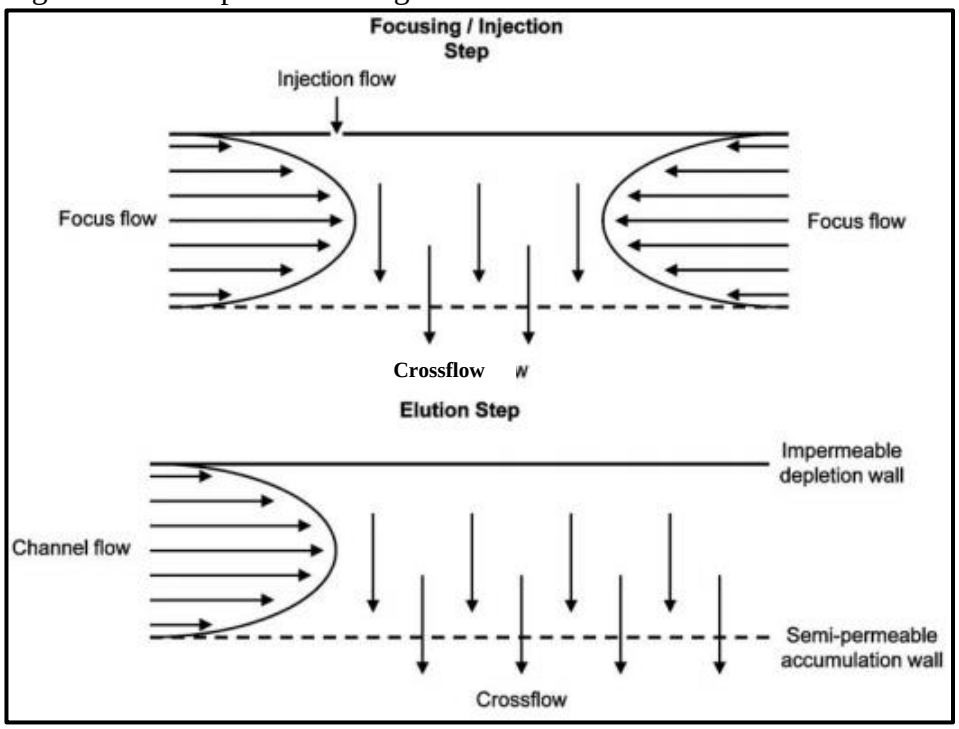
Another sub-class, the flow field flow fractionation (FlFFF), is considered the most versatile of the FFF techniques once the external field is replaced by a secondary flow (named crossflow) of mobile phase that is transverse to the main flux (Zhang, X., Li, Y. *et al.*, 2018). Inside the FlFFF channel there is a porous membrane placed on the accumulation wall which is directly related to the amount of analyte that reaches the detector (Marioli e Kok, 2019; Alcântara, Nascimento *et al.*, 2020). If the analyte gets a high velocity of migration, due a strong interaction to the crossflow, and if its size was lower than the membrane pore size, it can be lost

by crossing the membrane, going to the crossflow waste (Schimpf, Caldwell *et al.*, 2000; Marioli e Kok, 2019; Alcântara, Nascimento *et al.*, 2020). In some applications of the FlFFF an asymmetric channel can be used to favor the crossflow, in this case the FlFFF is normally named as asymmetrical flow field flow fractionation (AF4).

2.3.2.1 Generalities of Flow Field Flow Fractionation (FlFFF) and coupling with atomic spectrometry techniques

The FlFFF separation is a result of the size and mass properties of the particles. Basically it depends of three modes (injection, focus and elution) that directly influence the intensity and retention time of the analyte (Cao, Pollastrini *et al.*, 2009; Marioli e Kok, 2019). Essentially, in the injection mode, the sample is introduced to the channel and focused in a thin band towards the membrane with the aid of two opposing streams of the carrier flow (crossflow) (Figure 4, Focusing/Injection step). After the equilibrium between the crossflow and the analyte brownian diffusion is achieved, the elution mode starts where the carrier flow goes through the channel in a parabolic laminar flow profile (Figure 4, Elution step). As smaller particles are positioned higher in the channel, where the flow is faster, they elute before the large ones.

Figure 4 – Principle of working of FlFFF.



Source: Cao, Pollastrini and Jiang (2009).

FlFFF technique holds a great promise for the analysis and characterization of protein aggregates and particles once it enables size based separation of molecules in a wide range of molecular mass (10^3 to 10^{10} g mol^{-1}) (Wimuktiwan, Shiowatana *et al.*, 2015). Its attractiveness is the protein speciation with matrix-free separation mechanism. According to Tügel et al (2015), in traditional separation techniques, the high force of interaction between the analyte and the column stationary phase can cause structural disruption and denaturation of the protein chain, and analytical methods involving the speciation under mild conditions to avoid degradation of the original form of the analyte is increasingly required (Tügel, Runyon *et al.*, 2015). Thus, the FlFFF has been used as an alternative method to chromatographic in some applications since it allows an interaction between the analyte and the field strong enough to occurs separation, but not too strong to cause degradation. For Pornwilar and Siripinyanond (2014) FlFFF consists of a gentle separation method capable of keeping the biological activity of the analyte (Pornwilard e Siripinyanond, 2014).

The FlFFF has been used to characterize cells (Zhang e Lyden, 2019), proteins (Loiseleux, Rolland-Sabaté *et al.*, 2018), viruses (Eskelin, Poranen *et al.*, 2019), polymers (Zielke, Fuentes *et al.*, 2018), etc. Ratanathanawongs and Lee (2006), reported its use to monitor the distribution of lipoprotein particles used as biomarkers for coronary artery disease (Ratanathanawongs Williams e Lee, 2006). The authors mentioned that this method presented more adequate application than size exclusion chromatography (SEC) in the separation of high molecular weight proteins in wheat.

However, as already commented, the use of a sensible detection method, that allows quantification of multiple analytes, is essential for speciation analyses in complex matrices. For Pornwilar and Siripinyanond (2014), due the high selectivity and sensitivity, the coupling of FlFFF with atomic spectrometry techniques, such as ICP-OES and ICP-MS, is recommended when elementary speciation based on size is needed (Pornwilard e Siripinyanond, 2014). Recently, the FlFFF technique coupled to ICP/MS system has been successfully used for separation and determination of several macromolecules. Wimuktiwan, Shiowatana and Siripinyanond (2015), for example, investigated the association between bovine serum proteins (albumin and glubulin) with silver nanoparticles by FIFFF-ICP/MS, showing an alternative method to this purpose (Wimuktiwan, Shiowatana *et al.*, 2015). Kim, Lim and Moon (2016) coupled the FlFFF to an ICP/MS system for size separation and detection of plasma

metalloproteins from lung cancer patients (Kim, Lim *et al.*, 2016). According to the authors, the gentle separation mechanism of the technique minimizes the rupture of the metal-protein complex, concluding that relative amounts of seven (Mn, Ni, Cu, Zn, Zr, I and Ba) metalloproteins studied in diseased patients were altered when compared to healthy patients.

These results show the potential of the FlFFF-ICP/MS technique as a powerful screening method for substances associated with proteins such as selenoproteins, opening a large number of analytical opportunities for its use in protein and amino acids analyses in biological samples. Thus, this study also aimed to develop a methodology for SeMet analysis in selenized yeast certified reference material (CRM SELM-1) using the FlFFF with asymmetric channel (AF4) coupled to an ICP/MS system, where different sample preparations (water, acid and alkaline extractions) were examined and their compatibility with the separation mechanism of the AF4 apparatus were compared.

3 OBJECTIVES

3.1 General

Method development and validation of the techniques AF4-ICP/MS and UPLC-ESI/QDa to analyze SeMet in yeast and cashew nut (*Anacardium Occidentale*) samples, respectively.

3.2 Specific

- ✓ Optimize the experimental conditions of the AF4 separation, including the use of membranes with different pore sizes (5 kDa, 10 kDa and 500 kDa);
- ✓ Evaluate the efficiency of different procedures to extract SeMet from yeast SELM-1 sample and their compatibilities with the AF4-ICP/MS system;
- ✓ Evaluate the suitability of genistein as a potential SeMet IS for the UPLC-ESI/QDa system;
- ✓ Optimize the experimental conditions of the UALLME sample preparation through an experimental design to analyze SeMet in cashew nuts by UPLC-ESI/QDa;
- ✓ Validate both methodologies and apply them in real samples.

4 MATERIALS AND METHODS

The experiments carried out in this work were conducted through a partnership between the research groups: Laboratório Multiusuário de Química de Produtos Naturais (LMQPN) from Brazilian Agricultural Research Corporation (Embrapa), Chemical Metrology from National Research Council of Canada (NRC) and Laboratório de Análise de Traços (LAT) from Federal University of Ceará (UFC).

4.1 Chemical, Reagents and Solutions

All chemicals used were of analytical reagent grade or higher quality. High purity deionized water (DIW) was produced by reverse osmosis of tap water followed by deionization (Barnstead/Thermolyne, Dubuque, IA, USA) to yield an 18 MΩ-cm resistivity. Methanesulfonic acid (99.0 % v v^{-1}), nitric acid (90.0 % v v^{-1}), sodium dodecyl sulfate (SDS) (99.0 % w w^{-1}) and 2-mercaptoethanol (14.3 mol L^{-1}) were purchased from Merck (Canada). Formic acid (FA) (95.0 % v v^{-1}) and Hydrochloric acid (HCl) (37.0 % v v^{-1}) were obtained from Merck (Canada and Brazil). Methanol (99.9 % v v^{-1}) and acetonitrile (99.9 % v v^{-1}) were purchased from Merck (Brazil). Glycerol (99.9% v v^{-1}) and Tris Base were obtained from Fisher Scientific (Canada). Sodium hydroxide (NaOH) (97.0 % w w^{-1}) was purchased from EMD Serono (Canada) and from Dinâmica (Brazil). n-hexane (97.0 % v v^{-1}) was purchased from Vetec (Brazil) and acetone (99.5 % v v^{-1}) from Neon (Brazil).

SeMet (99.6 % w w^{-1}) and SeMeSeC (98.0 % w w^{-1}) powder standards were purchased from Fisher Scientific. The genistein (98.0 % w w^{-1}) powder standard was obtained from Merck (Brazil), while the CRM for selenium enriched yeast (SELM-1) was provided by the National Research Council of Canada (NRC, Canada).

For the experiments analyzed by AF4-ICP/MS, a stock solution of SeMet (1000 mg L^{-1}) in 0.5 % nitric acid was prepared and stored under refrigeration until use. The working solutions (5, 10, 25 and 50 µg L^{-1}) were prepared daily with dilutions in a solution of 0.05 % (w v^{-1}) SDS. The SDS buffer was prepared according to Kushnirov (2000) (Kushnirov, 2000) and consisted of 0.06 mol L^{-1} tris-HCl, 5 % (v v^{-1}) glycerol, 2 % (w v^{-1}) SDS, 4 % (v v^{-1}) 2-mercaptoethanol and 0.0025 % (v v^{-1}) bromophenol blue. The stock tris-HCl 15 mol L^{-1} (pH 6.8)

was prepared dissolving the base tris in DIW and adjusting the pH to 6.8 with concentrated HCl (Kushnirov, 2000; Hou, Ding *et al.*, 2017). All plastic and glass labware were cleaned by immersion in 5 % (v v^{-1}) HNO_3 for at least 24 hours and thoroughly rinsed with DIW before use.

For the experiments analyzed by UPLC-ESI/QDa were obtained the stock solutions of SeMet (10 and 1000 mg L^{-1}), SeMeSeC (10 and 1000 mg L^{-1}) and genistein (10 and 100 mg L^{-1}) in a 10 % (v v^{-1}) water solution of methanol. From them were prepared the SeMet work solutions of 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, 3.0, 4.0 and 6.0 mg L^{-1} in the solvent (dilutions using ultrapure water) and in the matrix (dilutions using the extracts of the cashew nut from the sample C), where SeMeSeC (1.00 mg L^{-1}, for external standard calibration) and genistein (0.2 mg L^{-1}, for external standard and matrix matched calibrations) were used as IS to get the 'area SeMet/area IS x SeMet concentration' analytical curves. Standards were stored at 4 °C until use and all plastic and glass labware were cleaned by immersion in acetone solution (10 % v v^{-1}) for at least 24 hours.

The extracting solvents for UALLME sample preparation optimization were prepared as follow: HCl 0.1 mol L^{-1} (pH 1.00) by diluting an amount of the commercial reagent in ultrapure water, the NaOH 0.1 mol L^{-1} (pH 13) by dissolving an amount of the measured mass of the commercial reagent in ultrapure water, and for the pH close to 7.00 ultrapurified water was used. The NaOH 0.01 mol L^{-1} solution was prepared by further diluting the NaOH 0.1 mol L^{-1} solution.

The mobile phase used for the AF4 system consisted of a 0.05 % (w v^{-1}) solution of SDS that was previously passed through a 0.45 µm Millipore membrane filter. And for UPLC-ESI/QDa apparatus, water and acetonitrile (both with 0.1 % formic acid) were used as mobile phases previously filtered in a 0.22 µm Millipore membrane.

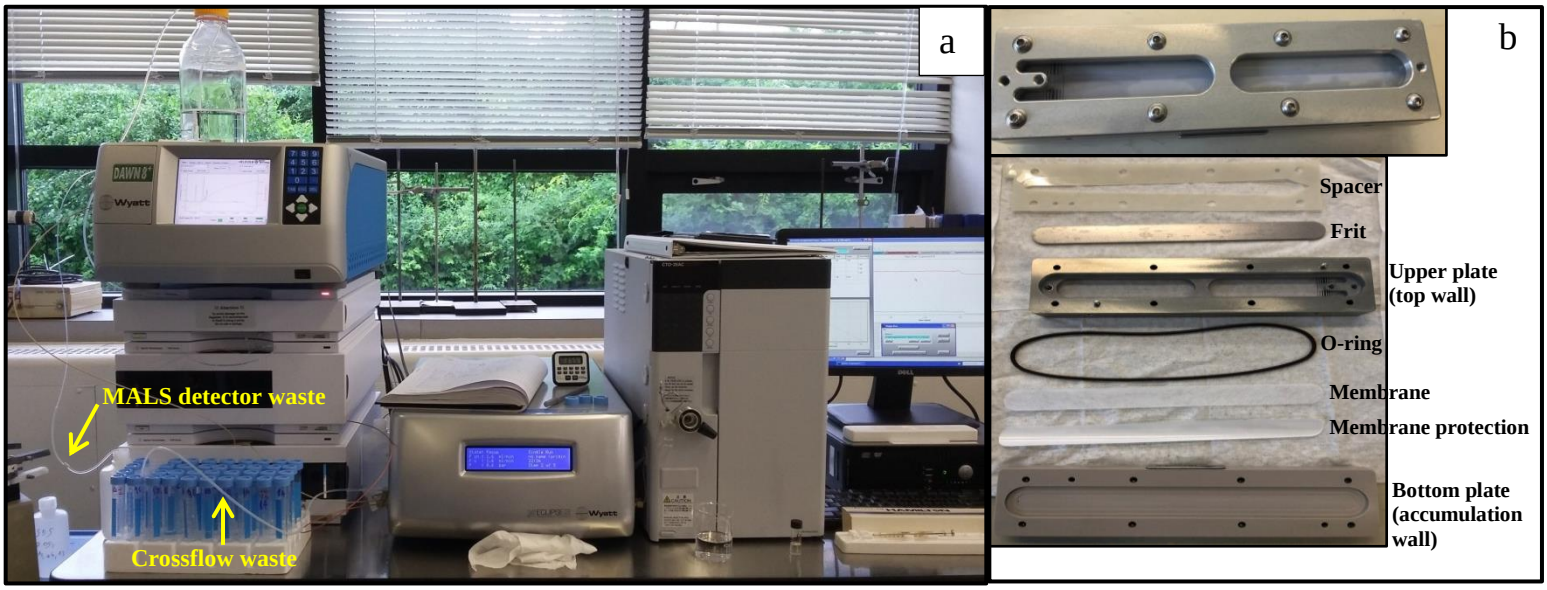
4.2 Instrumentation

An Eclipse 2 FFF separation system (Wyatt Technology, Germany) equipped with a series 1100 isocratic pump (Agilent Technologies model G1310A, Netherlands), series 1100 degasser (Agilent model G1379A), series 1100 ultraviolet (UV) detector (Agilent model G1314A) and Multi-Angle Light Scattering (MALS) detector (Wyatt Technologies model DAWN HELEOS II 8+, Germany) with manual injection based on a 50 µL sample loop was

used (Figure 5a). Separations were achieved using a 350 µm spacer in an asymmetrical channel flow (Long LC: 246 mm) (Figure 5b). Several regenerated cellulose (RC) membranes (Wyatt Technology, Germany) with different pore sizes (5, 10 or 500 kDa) were tested.

The AF4 system was coupled with an ELAN DRC II inductively coupled plasma mass spectrometer (PerkinElmer Sciex, Shelton, CT) equipped with a Meinhard concentric nebulizer coupled to a cyclonic spray chamber (Glass Expansion Inc., Pocasset MA) (Figure 6). The ICP/MS was operated in standard mode; instrument operating parameters included: RF power of 1100 W; plasma Ar gas, auxiliary Ar gas and nebulizer gas flows of 15 L min^{-1}, 1.20 L min^{-1} and 1.14 L min^{-1}, respectively; *m/z* per reading cycle: ^{78}Se; dwell time: 10 ms; sweeps per reading: 4; number of replicates: 1. Quantification was performed using peak area.

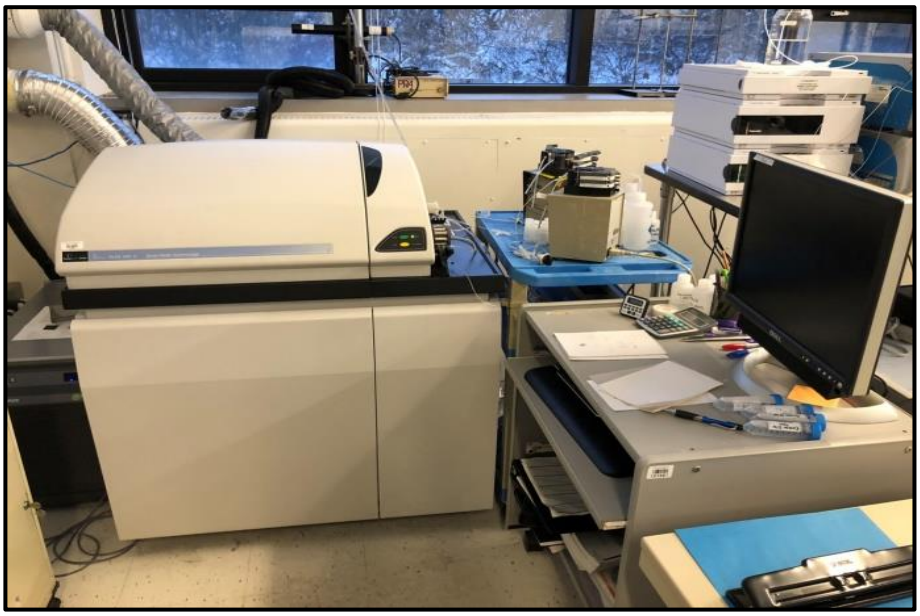
Figure 5 – (a) AF4 apparatus used in the study; (b) AF4 channel and its parts.



Source: author, 2020

An in-house assembled flow injection analysis system (FIAS) was used to generate the calibration curves for Se (using SeMet standard). The FIAS was inserted between the outlet of the AF4 system and the ICP/MS nebulizer through a T-connector. Figure 7a presents a scheme of the FIAS-AF4-ICP/MS connection and Figure 7b describes the FIAS system used. When the valve is in position 1, the 50 µL loop is filled with the sample while the mobile phase is directed to the ICP/MS; switching the valve to position 2 permits the mobile phase to flush the sample loop to the ICP/MS.

Figure 6 – ICP/MS system used in the study.



Source: author, 2020

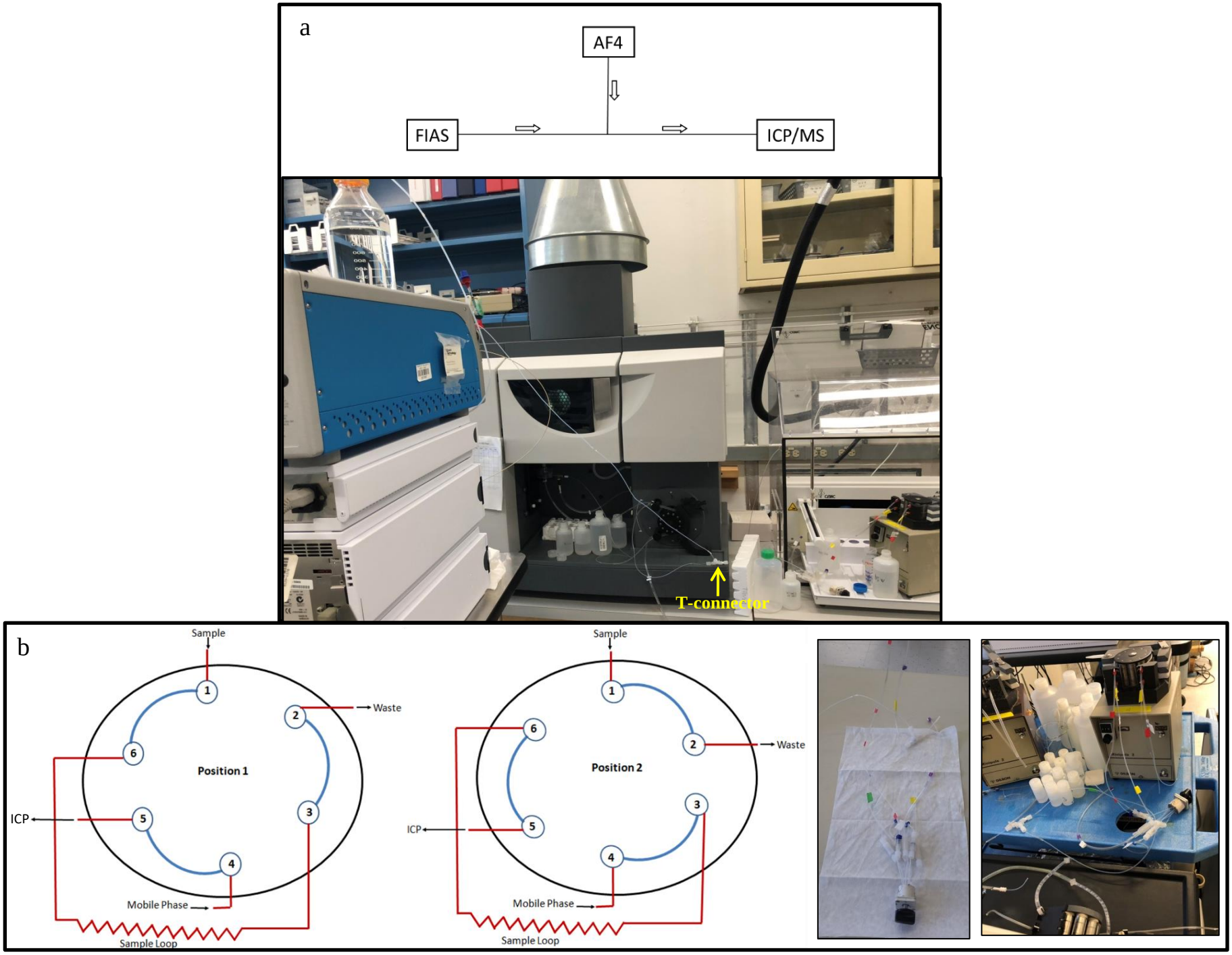
A hot plate (Digiprep Jr block heater, SCP Science, Canada), ultrasonic bath (UB) (Branson model 3510, USA), ultrasonic probe (UP) (Branson sonifier model 450, USA) and model AB150 pH meter (Fisher Scientific , USA) were used during various sample treatments tested for the AF4-ICP/MS system. Also the Open Chrom® 1.2.0 Alder software was used to integrate the AF4-ICP/MS peak area and Astra 5 software of the Wyatt technologies company was used for size measurements.

An ACQUITY® UPLC coupled to a single quadrupole mass analyzer in series with a photodiode detector array (QDa) from Waters Corporation (Milford, MA, USA) were also used as separation and quantification systems (Figure 8). Chromatographic runs were performed with a Waters ACQUITY® UPLC BEH octadecylsilane (C18) column (150 mm x 2.1 mm, 1.7 µm) at a fixed temperature of 40 °C and mobile phase consisting of water (A) and acetonitrile (B), both with 0.1 % formic acid. The flow rate was kept at 0.3 mL min^{-1}, with an injection volume of 5 µL (through an autosampler) and the gradient ranging as follow: 98.0 % A for two minutes, then varying from 98.0 % to 0.0 % A by 0.5 min which stayed for 2 min, and then returning to 98% A by 0.5 min where remained for 3 min, getting a total time of 8 min that includes the column conditioning in each running (Lira, Dionísio *et al.*, 2020).

ESI worked at positive mode with a fixed temperature of 120 °C, desolvation temperature of 350 °C, nitrogen desolvation gas flow of 350 L h^{-1} and capillary and cone voltages of 3 kV and 15 V respectively. All analyses were performed with the MS working at SIR mode, monitoring the *m/z* ratios of the protonated molecules $[M+H]^+$ which are 198.00, 183.98 and 271.06 for SeMet, SeMeSeC and genisteine respectively. The instrument was controlled by the Masslynxfi 4.1 software program (Waters Corporation, Milford, USA).

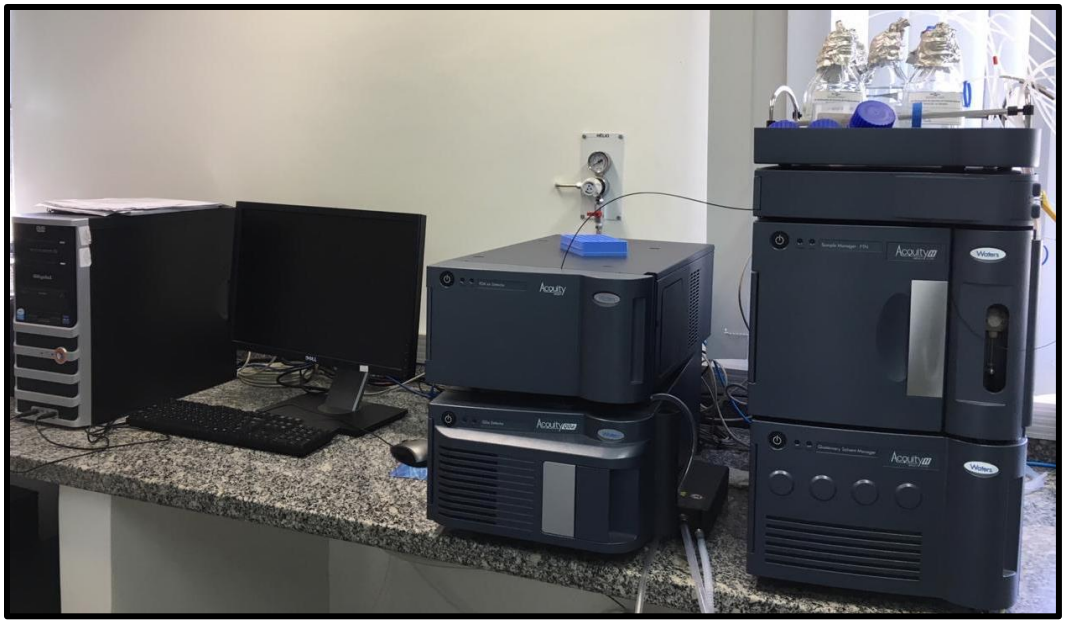
For the UALLME sample preparation an analytical balance (FA-2104N, Bioprecisa, Brazil), UB (1440D, Odontobras, Brazil), vortex (K45-2820, Kasvi basic, Brazil) and centrifuge (Eppendorf Centrifuge 5430, Merck, Brazil) were used.

Figure 7 – (a) Connection between FIAS, AF4 and ICP/MS; (b) Operation of the FIAS system at each position. Arrows indicate the flux direction.



Source: author, 2020

Figure 8 – UPLC-ESI/QDa system used in the study.



Source: author, 2020

4.3 Optimization of the AF4 separation conditions

AF4 separation was optimized in order to obtain a more intense peak with lesser retention time according to Table 2. The mobile phase used for the AF4 system consisted of a 0.05 % (w v^{-1}) solution of SDS that was previously filtered with a 0.45 µm milipore membrane. Optimization was performed using three separate injections of 50 µL of SELM-1 extracts (MSA, filtered and without reflux) and using the MALS detector.

Table 2 – Conditions for optimization of the AF4 separation method.

Condition	Steps	Time/min	Mode	X start/(mL min^{-1})	X end/(mL min^{-1})	FF/(mL min^{-1})
1	1	3.00	Elution	1.00	1.00	-
	2	1.00	Focus	-	-	1.5
	3	2.00	Focus + Injection	-	-	1.5
	4	0.70	Focus	-	-	1.5
	5	20.00	Elution	1.00	0.00	-
2	1	3.00	Elution	1.00	1.00	-
	2	1.00	Focus	-	-	1.5
	3	2.00	Focus + Injection	-	-	1.5
	4	0.40	Focus	-	-	1.5
	5	20.00	Elution	1.00	0.00	-
3	1	3.00	Elution	1.00	1.00	-
	2	1.00	Focus	-	-	2.0
	3	2.00	Focus + Injection	-	-	2.0
	4	0.70	Focus	-	-	2.0
	5	20.00	Elution	1.00	0.00	-
4	1	3.00	Elution	1.00	1.00	-
	2	1.00	Focus	-	-	1.0
	3	2.00	Focus + Injection	-	-	1.0
	4	0.70	Focus	-	-	1.0
	5	20.00	Elution	1.00	0.00	-
5	1	3.00	Elution	1.00	1.00	-
	2	1.00	Focus	-	-	2.0
	3	2.00	Focus + Injection	-	-	2.0
	4	0.40	Focus	-	-	2.0
	5	20.00	Elution	1.00	0.00	-

Note: (-) not available for the step; X start = Flow of the field when the step begins; X end = Flow of the field when the step finished; FF = Focus Flows.

4.4 Sample Preparations Evaluated for the AF4 Suitability Study

Several sample preparation strategies were examined for the SeMet analyses in AF4, including acid extraction using methanesulfonic acid (with and without a reflux step), room temperature acid extraction using formic acid, alkaline extraction using SDS buffer and water extraction (manual shaking and with use of ultrasound assisted device). These are detailed below:

4.4.1 Acid extraction with methanesulfonic acid (MSA) (without reflux): This procedure was similar to that described by Mester et al (2006) (Mester, Z., Willie, S. *et al.*, 2006). Typically, a nominal 0.25 g of sample was accurately weighed into a vessel and 6 mL concentrated MSA and 16.75 mL DIW were added (resulting in a MSA concentration of 4 mol L^{-1}). Samples were vigorously shaken by hand for at least 30 seconds.

4.4.2 Acid extraction with methanesulfonic acid (MSA) (with reflux): The same procedure described above was followed but the sample was further submitted to reflux on a hot plate (125 °C) for 16 h with glass beads added to serve as anti-bumping granules (Wrobel, Kannamkumarath *et al.*, 2003).

4.4.3 Acid extraction using formic acid: This procedure was similar to that described by Lopes et al (2016) (Lopes, G. S., Silva, F. L. F. *et al.*, 2016). An accurately weighed nominal sample mass of 0.25 g was manually extracted by shaking the sample with 10 mL of concentrated formic acid and 15 mL of DIW for at least 30 seconds.

4.4.4 Alkaline extraction using SDS buffer: This procedure is described in Kushnirov (2000) (Kushnirov, 2000). A nominal 0.25 g sample was accurately weighed into a vessel and mixed with 10 mL of DIW and 10 mL of a 0.2 mol L^{-1} solution of NaOH, incubated for 5 min at 100^{o}C followed by addition of 5 mL of SDS buffer. Samples were vigorously shaken by hand for 30 seconds.

4.4.5 Water extraction: A nominal 0.25 g of accurately weighed sample was placed in a vessel and 7 ml of DIW was added. The mixture was vigorously shaken by hand for at least 30 seconds.

4.4.6 Water extraction in an ultrasonic bath (UB): Sample treatment was identical to the above but the mixture was placed in an ultrasonic bath for 30 min at 25 °C.

4.4.7 Water extraction using an ultrasonic probe (UP): A nominal 0.25 g of accurately weighed sample was placed in a vessel and 7 ml of DIW added. A 6 mm diameter Ti US probe tip was immersed to a depth of at least 1 cm into the mixture (ensuring that the tip was centered in the tube) and 60 W ultrasonic power applied for 30 min at room temperature.

All samples were analyzed with or without further filtration through a 0.22 µm millipore membrane syringe filter (Merck, Canada). Samples were stored at 4 ^{o}C and then further diluted with DIW prior to analyses.

4.5 Study of the Genistein Suitability as a Potential SeMet IS for the UPLC-ESI/QDa System

The evaluation of the suitability of genistein as a candidate SeMet internal standard (IS), when the UPLC-ESI/QDa is employed as quantification system, was performed plotting the calibration curves of SeMet in the presence of SeMeSeC (1.0 mg L^{-1}) and genistein (0.2 mg L^{-1}). Experiment was realized using ultra-purified water as solvent. The angular coefficient (AC) of both curves were compared using equation 1.

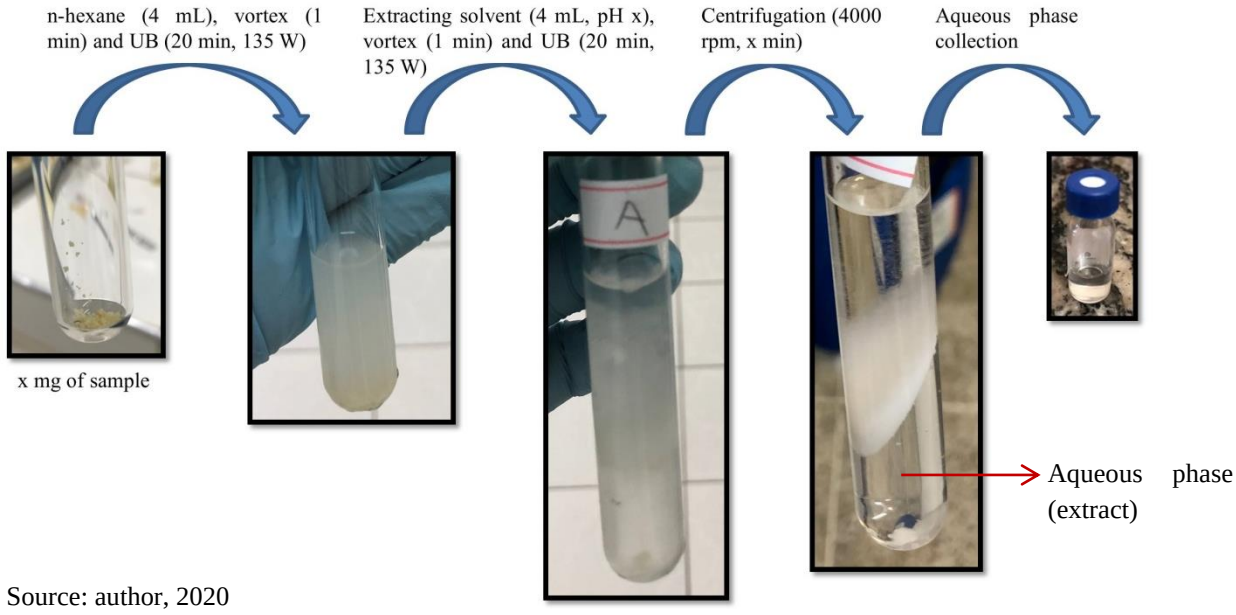
$$error/\% = \frac{(higher\ AC - lower\ AC)}{(higher\ AC)}\ x\ 100 \qquad (1)$$

The $'error/\%'$ corresponds to the percentage error associated with the use of the non-similar compound as SeMet IS, where, the lower its value the more appropriate the use of genistein to correct the variations in the instrumental signal of the analyte.

4.6 Ultrasound-Assisted Liquid-Liquid Microextraction (UALLME) and Experimental Design Optimization

The optimization of the UALLME method was performed using a three factors and two levels experimental design with central point. The Statistica software was used to generate the experimental design model and to process the data. Table 3 summarizes the experiments, illustrated by Figure 9, where an amount of sample mass (varying from 10 to 90 mg according the statistical planning) was placed in a test tube in which 4 mL of n-hexane was added. The tube content was shaken by vortex during 1 min and taken to the UB for 20 min with fixed power of 135 W at room temperature. After that, 4 mL of aqueous extracting solvent (with pH varying of 1 to 13, according the statistical planning) was added and the solution was shaken again by vortex for 1 min and kept over ultrasonic bath treatment (135 W at room temperature) for 20 min. Afterwards, the sample tube was placed for centrifugation (the time was defined according the statistical planning and varied from 5 to 35 min) at 4000 rpm in order to separate the phases. The aqueous phase (extract) was collected and stored in an amber bottle (previously cleaned with neutral detergent and acetone) and stored at 4 °C refrigeration until chromatographic analysis. The experiments 1 to 8 were performed in triplicate, and the central point (experiment 9) was accomplished in six replicates.

Figure 9 – Illustration of the steps of the sample preparation process. Note: ultrasonic bath (UB).



Source: author, 2020

Table 3 – Experimental design for UALLME optimization.

Factors	Levels		
	-	0	+
Sample mass/mg	10	50	90
Centrifugation time/min	5	20	35
Extraction solvent pH	1	7	13

Experiments	Sample mass/mg	Centrifugation time/min	Extraction solvent pH
1	10	5	1
2	90	5	1
3	10	35	1
4	90	35	1
5	10	5	13
6	90	5	13
7	10	35	13
8	90	35	13
9	50	20	7

Note: UALLME: ultrasound-assisted liquid-liquid microextraction.

SELM-1 CRM was used as sample for the optimization of the extraction procedure. Once optimized, the method was validated using the cashew nut matrix, and then applied in commercial samples of three different brands (A, B and C) to determine their SeMet contents. Nuts were obtained as natural raw material without shell and, before pre-treatment, cashew nuts (5 seeds per brand) were crushed and homogenized using an industrial blender, and then stored

in a small plastic packaging with seal. Also, before any injection in the chromatography system, solutions were filtered using a 0.22 µm PTFE filter.

4.7 Estimation of the Cashew Nut Matrix Effect in SeMet analysis by UPLC-ESI/QDa

The intensity of the matrix effect (ME) caused by the cashew nut extract was estimated using Equation 2 by comparison of the angular coefficients obtained through external standard calibration (dilutions in ultrapure water) and matrix-matched calibration (dilutions using the sample extracts obtained by the optimized UALLME method). Genistein (0.2 mg L^{-1}) was used as internal standard (IS) in both curves.

$$ME/\% = \frac{(MAC - SAC)100}{SAC} \quad (2)$$

ME/% is the calculated matrix effect in percentage and *MAC* and *SAC* are the angular coefficients of the curves prepared in the matrix and in the solvent respectively, do not considering the influence of the sample matrix in the analyte instrumental response if the result remains within the range of -20 to 20 % (Alcântara, Paz *et al.*, 2018; Barbosa, Martins *et al.*, 2018; Oiram Filho, Alcântra *et al.*, 2018; Fernandes, Alcântara *et al.*, 2019).

5 RESULTS AND DISCUSSION

5.1 AF4-ICP/MS Method Development for SeMet Analysis in Yeast

5.1.1 Optimization of the AF4 method

The AF4 separation is achieved in two steps, encompassing analyte focusing and elution. Optimizations of both are important as they directly influence the intensity and retention time of the analyte. During the focusing step, the sample is concentrated into a thin band close to the membrane with the aid of two opposing streams of the carrier liquid (i.e, crossflow configuration). A steady-state is achieved reflecting the influence of the forces attributed to the crossflow and brownian diffusion. Once the elution step begins, the carrier liquid passes through the channel within in a parabolic laminar flow profile, separating sample components based on their size-to-mass ratios. Due to brownian diffusion, smaller particles are positioned higher in the channel, where the carrier flow is faster, and consequently they elute first.

Figure 10a presents fractograms obtained under various conditions (presented at Table 2). A sharper elution peak is obtained when, during step 4, the focus time is decreased from 0.70 to 0.40 min (condition 1 to 2). This shorter exposure time, under the influence of the crossflow field, permitted the analyte to concentrate further away from the semipermeable membrane surface, incurring smaller losses due to adsorption and/or permeation (M-M e Siripinyanond, 2014). A shorter retention time could also be observed for condition 2 (9.2 versus 6.95 min for conditions 1 and 2, respectively).

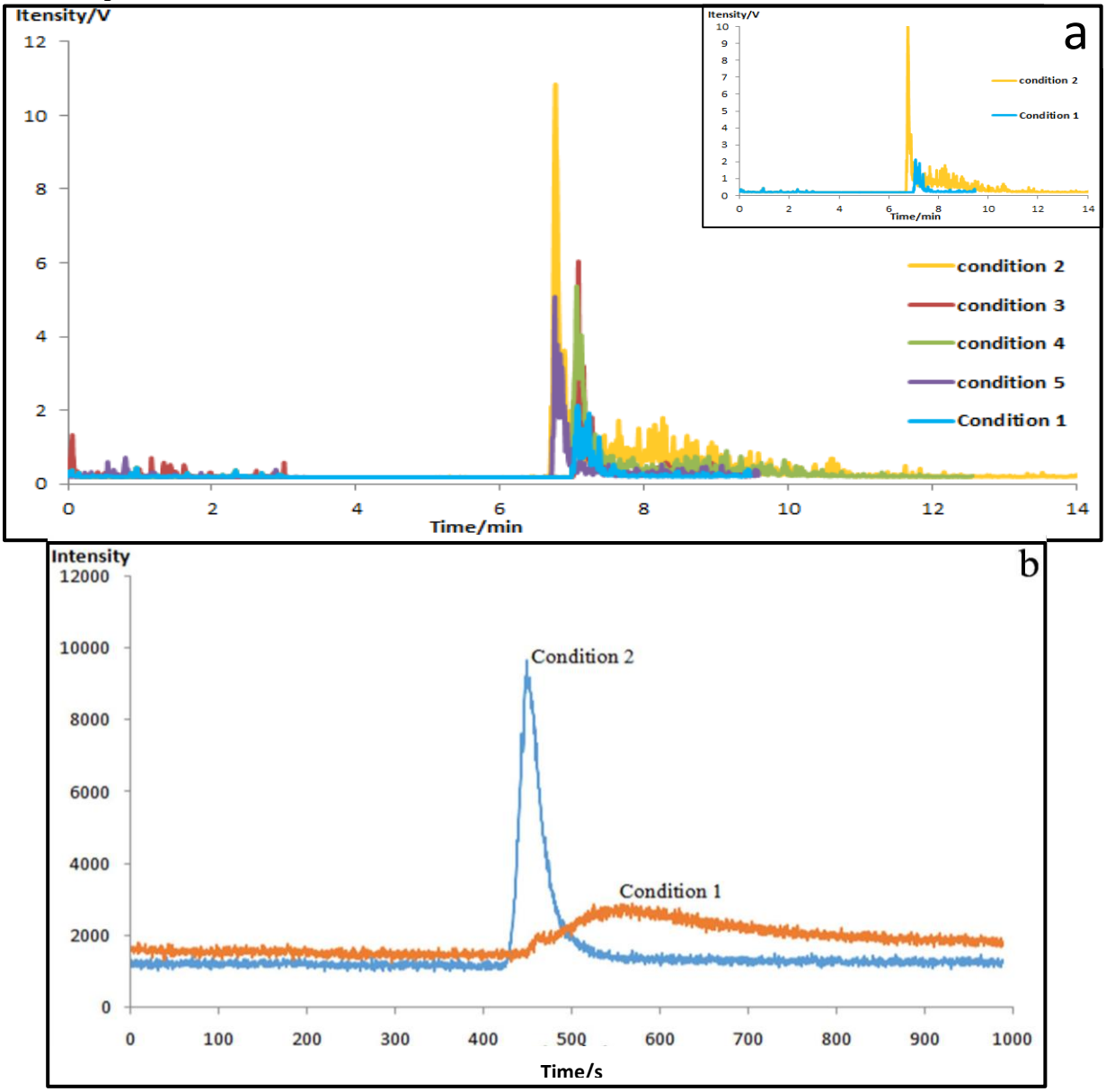
When the AF4 system was coupled to the ICP-MS, a more symmetrical transient peak profile, with smaller base width was obtained using condition 2 when compared to condition 1. This is evident in Figure 10b. According to Meisterjahn et al (2016), one of the main reasons for peak broadening is the different velocities of individual components, resulting from the parabolic crossflow profile created within the channel (Meisterjahn, Wagner *et al.*, 2016). Thus, decreasing the focus time to 0.4 min was enough to achieve a steady state, but not too long to permit diffusions which would cause the peak broadening.

For the optimization of the channel flow rate, when the focus flow rate was increased from 1.5 to 2.0 mL min^{-1} (in steps 2 to 4, conditions 2 to 5), peak intensity decreased drastically.

According to Lee et al (2016), increasing the intensity of the external field will cause the signal intensity to decrease because of enhanced interaction of the analyte with the membrane due to their closer proximity (Lee, Lee *et al.*, 2016). Thus, for further studies, condition 2 was adopted.

It should be mentioned that the analysis time when using AF4 was significantly lower than typical values reported in the literature for determination of SeMet using HPLC-ICP-MS (i.e., 20 min) (Wrobel, Kannamkumarath *et al.*, 2003).

Figure 10 – Fractograms obtained under various AF4 conditions. Sample: SELM-1 extract (MSA, filtered and without reflux). Membrane used: 10 kDa RC; (a) AF4 coupled to MALS. (b) AF4 coupled to ICP-MS.



Source: author, 2020

5.1.2 Influence of extraction methods and AF4 membrane size

Accurate quantitation of any species requires methods that exhibit high extraction efficiency, and are capable of preserving the identity of the species of interest (Alcântara, Fernandes *et al.*, 2019).

It is well known that SeMet is the major form of Se found in foods supplements such as selenized yeast by incorporation into the yeast protein (Mcsheehy, S., Yang, L. *et al.*, 2005; Kubachka, Hanley *et al.*, 2017) and it is the main form of Se present in NRC CRM SELM-1 as confirmed by GC-MS and LC-MS analyses of this material (Mester, Z., Willie, S. *et al.*, 2006) .

Several sample preparation procedures were evaluated for extraction of SeMet from SELM-1, including neutral (water, with or without assistance from an ultrasonic bath/probe), acidic (using MSA or formic acid) and alkaline (using SDS buffer) extractions. Performance for each procedure is based on results of 10 replicate injections. Three AF4 membranes (regenerated cellulose (RC)) of different pore sizes (5, 10 and 500 kDa) were also tested. Note that these were the only RC membrane pore sizes available from the AF4 manufacturer at the time these studies were performed.

Results presented in Table 4 show that extractions using only water were the least effective, especially evident when no ultrasonic treatments (probe or bath) were used. Protein solubilization usually takes place at extreme values of pH (< 2 and > 12), thus the use of only water to extract SeMet was ineffective since the solution pH is near neutral and isoelectric points of most proteins are achieved, greatly decreasing their solubility (Xiong, Gao *et al.*, 2016). Although the use of ultrasonic energy enables rapid cell membrane disruption (Peachey, Mccarthy *et al.*, 2008), it was not effective for quantitative recovery of SeMet from SELM-1.

On the other hand, acidic extraction using MSA (without reflux) was more efficient than extraction with formic acid; however, neither treatment achieved an efficiency above 40 %. According to Xiong et al (2016), protein extraction yield is strongly associated with the solubility of the target protein (Xiong, Gao *et al.*, 2016). These authors verified that acidic extractions were more efficient for proteins which are more easily dissolved in saline solutions.

Table 4 – SeMet concentration and its relative standard deviation (RSD/%, n = 10) obtained for SELM-1 using different sample extraction preparation strategies and different AF4 membranes.

	SeMet Concentration, mg kg^{-1} (RSD, %)		
Sample preparation	**RC Membrane, 5 KDa**	**RC Membrane, 10 KDa**	**RC Membrane, 500 KDa**
FA, not filtered	255 (16)	238 (11)	97.5 (7)
FA, filtered	356 (17)	336 (1)	-
MSA, without reflux and not filtered	1187 (3)	838 (13)	175 (11)
MSA, without reflux and filtered	639 (2)	406 (4)	-
MSA, refluxed and not filtered	77 (4)	67 (13)	-
MSA, refluxed and filtered	39 (4)	32 (22)	-
SDS buffer, not filtered	3282 (5)	3166 (99)	327 (6)
SDS buffer, filtered	3320 (9)	2962 (4)	235 (10)
Water, not filtered	-	70 (7)	-
Water, filtered	-	25 (19)	-
Water, with ultrasonic bath and not filtered	-	144 (13)	-
Water, with ultrasonic bath and filtered	-	124 (14)	-
Water, with ultrasonic probe and not filtered	-	134 (12)	-
Water, with ultrasonic probe and filtered	-	100 (8.1)	-

Note: SeMet concentration in SELM-1 CRM is 3190 ± 290 mg kg^{-1}; (-) Not performed; RC: regenerated cellulose; FA: formic acid, MSA: methanesulfonic acid; SDS: sodium dodecyl sulfate.

Although it was earlier reported that use of MSA with a reflux step permitted quantitative extraction of SeMet from yeast (based on subsequent determinations by GC-MS, GC-ICP-MS or HPLC-ICP-MS techniques) (Yang, Lu, Sturgeon, Ralph E. *et al.*, 2004; Bierla, Szpunar *et al.*, 2012), the same conclusion could not be supported by this study when using AF4 coupled to ICP-MS for SeMet analysis. When using MSA extraction combined with a reflux step, poor recoveries were obtained. It should be noted that the lowest pore size membrane used in this study had a molecular weight cut-off of 5 kDa, suggesting that the poor recovery may be a result of the complete hydrolysis of the enriched yeast protein to its constituent Se amino acid SeMet and its permeation through the membrane.

It was noted that for all sample preparation procedures used, when different membranes were examined, higher recoveries for SeMet were obtained when employed the membrane with the smallest pore size (5 kDa), decreasing drastically with the use of the one with 500 kDa. It was also observed that for both 5 and 10 kDa membranes, good agreement with the certified value for SELM-1 could be obtained when the SDS buffer extraction procedure was adopted. This sample preparation procedure was the only one which achieved complete recovery (based on 5 and 10 kDa membranes), showing that the size of the SeMet containing species is an important property that needs to be taken into consideration when using AF4 separation technique.

A further important parameter is the influence of the surface charge of the SeMet containing species generated by the extraction protocol. Recovery is directly influenced by the interaction of the resultant extracted species with the AF4 membrane material (which is negatively charged). These parameters (size and charge) are further discussed later.

No significant difference was observed when samples were filtered or not filtered prior to AF4 separation. When using unfiltered samples, larger particles were still present during the AF4 separation and those could be accumulated in the membrane decreasing the amount of analyte available for the quantification. On the other hand, filtered samples are more convenient to use as they prevent the instrument lines/valves from clogging due to larger particles, thereby increasing lifetime and requiring less maintenance.

This study shows that when using AF4 for quantitation of SeP, a milder extraction procedure, such as the alkaline SDS buffer, which will lead to incomplete hydrolysis of the peptides and proteins, is desired. In contrast to HPLC and GC separation techniques, wherein

complete hydrolysis of yeast protein into its constituent amino acids is necessary for quantitative recovery of SeMet (Mcsheehy, S., Yang, L. *et al.*, 2005), a partial extraction procedure is more appropriate for AF4 as the size of the resultant analyte species significantly affects the recovery.

5.1.3 Fractionation analysis in AF4 system

Theoretically, the membranes used with AF4 are semi-permeable, preventing the sample components (considering their size is larger than the molecular cut-off of the membrane used) from passing through, but allowing the solvent to easily exit the channel. In practice, especially for small sized analytes, there is a fraction of the analyte that may cross the semi-permeable membrane, resulting in a loss of the detected species. According to Meisterjahn et al (2016), the membrane should prevent particle losses by permeation through the accumulation wall either due to steric hindrance by the pore size or by promoting sufficient electrostatic repulsion (Meisterjahn, Wagner *et al.*, 2016). Consequently, properties such as size and molecular surface charge may influence the ability of the analyte to pass into/through the membrane. Since different sample extraction methods may alter the analyte properties (i.e., size and charge), the analytical signal will be strongly dependent of the extraction protocol employed.

In order to verify the amount of analyte that effectively reaches the detector, fractions of the crossflow waste (i.e, solution which passed through the membrane) were separately collected during the injection, focus and elution steps. The fraction that reaches the detector, corresponding to the elution peak, was also collected. Figure 5a shows how this experiment was performed, using a 10 kDa RC membrane. The collected fractions were analyzed by ICP-MS using standard solution nebulization. Selenium was not present in the fractions collected during the focus and elution steps for any of the various extraction strategies studied. A very small fraction of Se (less than 3%) was found in the injection step for the simple water extraction, but when a probe or ultrasonic bath was added to this treatment, the amount of Se increased substantially (to about 50% of total Se).

When using MSA extraction (with reflux), a large amount of analyte crossed the 10 kDa RC membrane during the injection step (about 25 % of total Se). This may account for the very low values recovery with this methodology, showing that the use of a reflux step is not

advisable when the AF4 system is used as it increase the degradation of the yeast protein and enhances releases of the simpler SeMet amino acid.

For extractions using formic acid (both filtered and unfiltered), no significant amount of Se was detected in the injection step (less than 1 %) even though the amount present in the detector fraction was only about 10% of the certified value. This was likely due to the effect of solubilization previously discussed and the properties of the membrane surface discussed in the topic *5.1.3.1*.

The fractions corresponding to the collected elution peak (reaches the detector) were also further analyzed by GC-MS in order to confirm the analyte identity. The peak and corresponding mass spectrum profile confirmed that it was SeMet.

5.1.3.1 Influence of molecular charge in AF4 analyses

The surface charge of SeMet containing species is influenced by the pH of the medium (Xiong, Gao *et al.*, 2016). For example, acid treatment will yield a positively charged species (Xiong, Gao *et al.*, 2016) while for an alkaline treatment, such as SDS buffer, a negatively charged species will be formed (Xiong, Gao *et al.*, 2016). On the other hand, for pH values close to neutral, such as occurs with a water extraction, a neutral species is more likely obtained (Phongthai, Lim *et al.*, 2016).

Ulrich et al (2012) evaluated the influence of the pH on the zeta potential of regenerated cellulose membranes and observed a negative feature over a wide range of pH (2 to 11) (Ulrich, Losert *et al.*, 2012). Since the surface of the regenerated cellulose has a net negative charge, the different charged states of the SeMet containing species created with each extraction protocol will interact differently with the RC membrane. Electrostatic repulsion between the negatively charged SeMet containing species generated under alkaline treatment and the membrane is thus expected, and may be one of the reasons that a large amount of analyte does not cross the membrane for samples treated with SDS buffer, contributing to the highest recoveries observed (Table 4). Strong electrostatic repulsion between the analyte and the membrane material increases colloidal stability and recovery, beside favoring earlier elution (Meisterjahn, Wagner *et al.*, 2016).

On the other hand, when analyte and membrane materials are oppositely charged, analyte deposition onto the accumulation wall may increase (Bendixen, Losert *et al.*, 2014). The positively charged SeMet containing species obtained when using acidic extractions may result in strong interactions with the membrane, where it is retained and consequently not detected in either the crossflow waste or detector fractions. This could explain the lower recoveries obtained using the formic acid extraction.

As previously noted, extractions using only water were the least effective (Table 4), since neutral molecules would be obtained, facilitating the analyte containing species crossing through the membrane, as a weakly (or absent) electrostatic interaction is present. In this case, permeability would be governed mainly by molecular size.

Interactions between analyte and membrane have been previously reported, especially for engineered nanoparticles. For instance, Hagendorfer et al (2012) verified strong interaction between silver nanoparticles and a polyvinylidendifluoride AF4 membrane (Hagendorfer, Kaegi *et al.*, 2012). Incomplete recoveries during AF4 separation were observed due to gold nanoparticles adhering to the RC and polyethersulfone membranes (Schmidt, Loeschner *et al.*, 2011). Studies have also shown that recoveries were dependent on the nanoparticle type and membrane material used (Ulrich, Losert *et al.*, 2012). To the authors' knowledge, no information is currently available in the literature concerning analyte-membrane interactions involving different charged SeMet containing species.

5.1.3.2 Influence of species size in AF4 analyses

In real samples, analytes are usually not found in isolation and often form aggregates with many matrix components, consequently increasing their size (Alcântara, Paz *et al.*, 2018; Alcântara, Fernandes *et al.*, 2019). Nevertheless, the use of different types of extraction procedures may also modify the size of the resultant analyte.

Sizes, estimated by MALS as root mean square radius (RMS), were obtained for the SeMet containing species arising from the different extraction procedures used. A Berry first-order fit method was employed for the calculation of the RMS (Table 5). RMS values varied from 482 to 138 nm for the different extraction strategies, with the largest obtained for extractions using SDS buffer (356 ± 6 and 482 ±11 nm for filtered and non-filtered solutions,

respectively), confirming that this milder extraction led to incomplete hydrolysis of the yeast protein containing SeMet, as earlier suggested. Thus, the largest RMS observed combined with the electrostatic repulsions between the SeMet containing species and the AF4 membrane, contributed to the quantitative recovery of the SeMet in CRM SELM-1 when using SDS extraction.

Table 5 – SeMet molecular size from different sample preparation in 10 kDa RC membrane estimated by Berry model first-order fit using MALS detector and Astra 5 software.

Sample preparation	**RMS/nm ± RSD**
FA, not filtered	163 ± 5
FA, filtered	160 ± 5
MSA, no refluxed and no filtered	155 ± 4
MSA, no refluxed and filtered	209 ± 16
MSA, refluxed and no filtered	140 ± 9
MSA, refluxed and filtered	138 ± 16
SDS buffer, not filtered	482 ± 11
SDS buffer, filtered	356 ± 6
Water, not filtered	164 ± 7
Water, filtered	ND
Water, with ultrasonic bath and not filtered	146 ± 4
Water, with ultrasonic bath and filtered	ND
Water, with ultrasonic probe and not filtered	149 ± 11
Water, with ultrasonic probe and filtered	ND

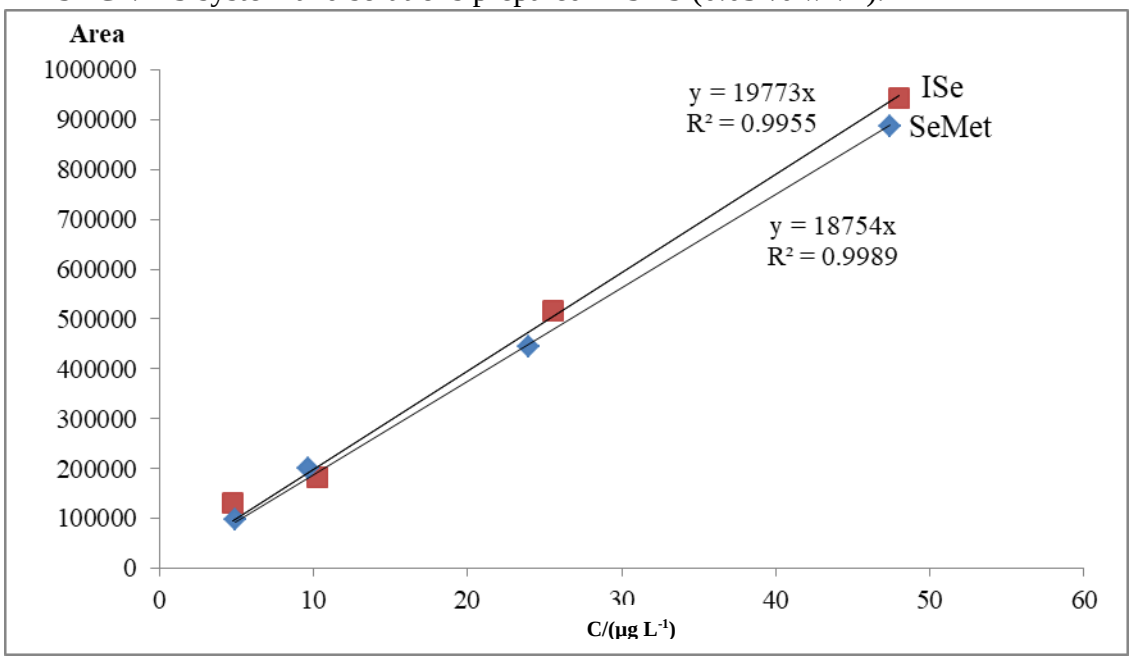
Note: ND: not detected by MALS; RMS: root-mean-square radius; FA: formic acid; MSA: methane sulfonic acid; SDS: sodium dodecyl sulfate; RSD: relative standard deviation.

As previous discussed, when a reflux step was combined with the MSA extraction, more than 20 % of the SeMet crossed the membrane. According to Wrobel et al (2003), refluxing the sample for a long period, as used in this study, promotes cleavage of inter- and intra-molecular bonds of the protein containing SeMet and may also eliminate the analyte solvation shell (Wrobel, Kannamkumarath *et al.*, 2003). Similar effects were evident here, since refluxed samples showed the lowest size values for the SeMet containing species, between 138 and 140 nm for filtered and non-filtered samples (Table 5), facilitating its passage (and loss) through the semi-permeable membrane.

When SeMet or inorganic Se standards were injected into the AF4 system, the majority (about 98%) crossed the membrane, mostly during the injection step. The same behavior was observed when samples were spiked with SeMet or inorganic Se standards. Attempts to perform standard addition calibrations before the separation with the AF4 were not successful. Thus, calibration was undertaken using a flow injection system (FIAS) connected to the ICP-MS system. The FIAS system mimics the transient peak profile obtained when using AF4. Evaluation of FIAS data was also based on peak area response.

Although it is known that the major selenoamino acid present in selenized yeast is SeMet, both inorganic Se and SeMet standards were tested for calibration purposes (using 4-point calibration) and no significant difference was detected between their angular coefficients for FIAS-ICP/MS system (Figure 11). Se was monitored with the ICP-MS at *m/z* 78 in both cases.

Figure 11 – Comparison of angular coefficients of two Se species. Curves obtained by FIAS-ICP/MS system and solutions prepared in SDS (0.05 % w v^{-1}).



Source: author, 2020
Se: selenium; ISe: inorganic selenium; SeMet: selenomethionine; FIAS: flow injection analysis system.

5.1.4 Method validation and application in commercial yeast supplements of Se

A LOD of 0.49 µg L^{-1} (for SeMet as Se) was obtained using the proposed methodology, based on a 3*s* criterion where *s* is the estimate of the standard deviation of at least 10 repetitive measurements of the blank. Blanks included all reagents and manipulations and were run in parallel with samples. Compared to other methods reported in the literature, including LC-ICP-MS and GC-ICP-MS, an improvement of about 2-fold in the LOD was achieved (Yang, Mester *et al.*, 2004; Giráldez, Ruiz-Azcona *et al.*, 2015). Correlation coefficients (R) higher than 0.9955 were obtained for calibration functions. Instrumental precision, expressed as relative standard deviation (RSD/%) for ten replicate injections of samples submitted to the alkaline SDS page buffer extraction, was lower than 5.0 %.

Sample preparation using alkaline extraction was the only method that generated satisfactory accuracy when using AF4 coupled to ICP-MS, achieving SeMet concentrations in agreement with the certified value (3190 ± 290 mg kg^{-1}). This was possible due the favorable size and charge properties of the SeMet-containing species obtained when using alkaline extraction. The intra-assay precision was about 3.8 % based on independent analyses, using the SDS buffer protocol, performed on three different days (Alcântara, Paz *et al.*, 2018; Barbosa, Martins *et al.*, 2018; Oiram Filho, Alcântra *et al.*, 2018).

The accuracy of the proposed method was further evaluated by determination of SeMet in several commercial selenium supplements. Results, obtained using SDS buffer extraction, were compared with those using HPLC-ICP-MS based on MSA reflux (Leblanc, Kawamoto *et al.*, 2019) and are presented in Table 6. It is evident that good agreement with both methods has been established for these materials.

These results show that alkaline extraction combined with AF4-ICP/MS detection is an effective method for determination of SeMet in selenized yeats and can be efficiently applied to real samples having a similar matrix.

The rapid analysis and lower solvent consumption can be highlighted as the main advantages of the developed method over the traditional one where samples need to be refluxed for several hours. A further advantage of the proposed methodology is related to the mode of

separation. The AF4 channel provides a longer lifetime than chromatographic columns, as it can be periodically cleaned (including the membrane). This is not the case for chromatographic columns, which can lose their ability to separate compounds over time due to loss of active sites on the stationary phase or even due to loss of the stationary phase itself. So, the cost associated to maintain the separation technique working in good performance can be lower for the AF4 system. Also, AF4 is considered a greener technique as smaller amounts of solvent, or no organic solvent, is used when compared to chromatographic techniques. AF4 coupled to ICP-MS has the potential to increase its use in the field of separation techniques making studies of method development of great importance.

Table 6 – Comparison of different methodologies for SeMet analyses in different yeast samples.

Sample	Form of Se as presented on label	Labeled Se[a] (μg Se/tablet)	Average tablet mass/g	SeMet (μg Se/tablet) ± SD	
				FFF-ICP-MS	HPLC-ICP-MS[b]
1	Selenium (yeast)	200	0.346	147 ± 30	135.1 ± 26.9
2	SelenoExcell® selenium (as high selenium yeast)	200	0.504	142.5 ± 24	131.2± 20.2

Note: [a]total selenium is the sum of Se methionine and inorganic selenium; [b]data from LeBlanc et al (2019) (sample 1 refers to S3, sample 2 refers to S6); SD: standard deviation.

5.1.5 Final considerations about the developed alkaline-AF4-ICP/MS method

This study showed that when coupled to an ICP-MS, AF4 can be used for speciation purposes as an alternative method to traditional chromatographic techniques such as LC. AF4 allows sufficient interaction between the analyte and the field to enable species separations but does not lead to protein degradation as common observed in traditional chromatographic techniques. One of the main requirement for using AF4 for speciation purposes is that incomplete hydrolysis of all peptides and proteins occurs in order to avoid permeation of the analyte through the AF4 membrane. This could be achieved with the use of mild extraction conditions such as alkaline extraction with SDS buffer as exemplified in this study for the determination of SeMet in selenized yeast. If the extraction method promotes cleavage of inter- and intra-molecular bonds of the protein, the size of the resultant analyte may be too small, increasing the chances of the

analyte containing species crossing the AF4 membrane, as was evident when using classical extraction methods such as MSA with a reflux step.

In order to achieve complete recoveries, the possible interactions between analyte and membrane should be taken into consideration, and it can be strongly influenced by the pH medium used to extract the target compound.

The optimized AF4 separation conditions combined with an alkaline extraction (SDS buffer) provided the best accuracy for the determination of SeMet in selenized yeast due to the high efficiency of extraction in addition to appropriate size and charge properties required for the AF4 separation. This methodology presented suitable precisions (instrument and intra-assay), linearity and LOD and can be efficiently applied to real samples of yeast to provide a robust, fast and low cost methodology for quantitation of SeMet.

5.2 UALLME-UPLC-ESI/QDa Method Development for SeMet Analysis in Nuts

5.2.1 Study of the use of genistein as a candidate SeMet IS for the UPLC-ESI/QDa system

Since low amounts of sample are usually injected into the chromatographic systems, particularly when using ultra-pressure techniques, and due variations in the electric current when analyses are performed in different days, the lack of reproducibility can be a frequent inconvenience which impairs the accuracy of the analytical results. This drawback is corrected by the use of a suitable internal standard (IS) that must have chemical similarities with the analyte, but should not be present in the sample matrix.

Therefore, SeMeSeC could be an appropriate IS for SeMet due to their chemical similarities. Zhang, Zhang and Zhang (2018) used this compound as IS for SeMet analysis by UPLC-MS/MS in rats plasma (Zhang, Zhang *et al.*, 2018). However, several works have reported its presence in many nut matrices. Moreda-Piñeiro et al (2018), for example, found SeMeSeC in a concentration that reached 0.81 mg kg^{-1} in Brazil nut samples by HPLC-ICP/MS (Moreda-Piñeiro, Sánchez-Piñero *et al.*, 2018); and Németh et al (2013) identified several Se species in monkeypot nut by LC-QToF/MS, where SeMeSeC was one of them (Németh, Reyes *et al.*, 2013). So, the suitability of genistein (a non-similar SeMet compound) as a candidate SeMet IS was evaluated through comparison of the calibration curves (see equation 1) obtained when using the similar (SeMeSeC) and the non-similar SeMet compounds as ISs, where the advantage of using the genistein, is the significant lower possibility of this compound be present in cashew nut samples compared to SeMeSeC, guaranteeing more accuracy quantifications.

Table 7 presents the percentage error associated to the use of genistein to correct the SeMet instrumental signals, calculated according to equation 1, where, the low value, about 2.00 %, indicates whatever the curve used to quantify the SeMet in real samples, the results will be similar, despite no chemical similarity between genistein and SeMet. Thus, for future experiments, genistein was used as IS for SeMet analyses in the UPLC-ESI/QDa system.

Genistein has been used as IS mainly for flavonoid compounds in liquid and gas chromatography systems, such as for analyses of icaritin by GC-MS (Shen, Wong *et al.*, 2007) and genkwanin and puerarin by HPLC-MS/MS (Song, Zhang *et al.*, 2013; Sun, Xue *et al.*, 2015).

The authors of the present work have not found in the literature studies about the genistein suitability as SeMet IS for LC quantifications yet.

Table 7 – Comparison of genistein with SeMeSeC as potential internal standard for SeMet analysis in UPLC-ESI/QDa system.

Internal Standard	Angular Coefficient	Linear Coefficient	R	Error/%
SeMeSeC	1.7066	0.3869	0.9973	1.959
Genistein	1.7407	-0.0048	0.9993	

Note: Data of the curves prepared in ultrapure water were used; SeMet: selenomethionine; SeMeSeC: Se-methylselenocystin; R: correlation coefficient; error/%: percentage error associated to the angular coefficients.

5.2.2 UALLME experimental design optimization

In complex matrices such as foods, the previous preparation of the sample is an essential requirement aiming to remove interferents from the sample matrix, as well as to isolate and concentrate the analyte at apropriated levels for the instrumental analysis (Alcântara, Fernandes *et al.*, 2019). However, most of these methods employ large amount of solvents that can increase the limit of detection and also they present several steps that are usually time-consuming. The LLME techniques have been reported in the literature as alternative sample preparation methods that allow the isolation of the analyte in a significantly short time with low solvent consumption (López-García, Vicente-Martínez *et al.*, 2013; John, Kuhn *et al.*, 2017).

Therefore, in this work a LLME method was developed, using ultrasound energy (method named as UALLME), throug an experimental design of three factors in two levels with central point as well described in section 4.6. After introduction of each reagent (n-hexane and extracting solvent) an ultrasonic bath step was employed to increase the solubilization of compounds in each phase. n-Hexane was used to remove the oil soluble fraction of the sample,

while was expected that the analyte be dispersed in the aqueous extraction solvent. In studies peformed by Alcântara et al (2020), ultrasonic energies (using probe or bath) increased the yield of Se species extracted in water preparations (Alcântara, Nascimento *et al.*, 2020).

Table 3 (section 4.6) sumarizes the experiments performed using the SELM-1 CRM, where the SeMet concentration in mg kg^{-1} was used as the response. Figure 12 presents the results after introduction of the data in the software statistica, where is observed through the Pareto chart (Figure 12a) that none variable by itself (sample mass, centrifugation time or pH) remained as a significant parameter to influence the response. As the interactions between the centrifugation time (CT) with the other variables (sample mass or pH) also remained as a non significant parameters, the time which the sample is kept in centrifugation does not influence the sample preparation procedure. This step is used to separate the organic and the aqueous phase after the introduction of the extracting solvent, and the results indicate that a low CT is enough to get the phases separated. So, the time of 5 minutes was used in the experiments.

The interaction 'sample mass x pH' negatively influenced the analyte extraction (Figure 12a), indicating the SeMet concentration recovery increases when working with small amounts of sample at low pH. However, the response surface chart 'pH x sample mass (keeping the CT = 5 min)' presented at Figure 12b shows that the amount of SeMet recovered presents maximum values when working in extreme pHs. These results confirm previous experiments performed by Xiong et al (2016), that reported the highest extracted protein yield was obtained at solutions with pH < 2 and pH > 12 (Xiong, Gao *et al.*, 2016). According to the authors the isoelectric point of most proteins is achieved at pHs near neutral which greatly decreases their solubility in solutions presenting this range of pH. For the Se amino acid SeMet, the isoelectric point occurs in the pH range of about 4 to 8 (see Figure 13).

Figure 12b also shows that the alkaline treatment is more advantageous than the acid solubilization once high efficiency of extraction was obtained even when using low amounts of sample. In studies performed by Xiong et al (2016), solutions with pH > 10.5 extracted more water-soluble proteins (Xiong, Gao *et al.*, 2016), such as those containing SeMet (also confirmed by the SeMet octanol-water partition coefficient – $k_{ow} = 4.99 \times 10^{-04}$, indicating its greater affinity for water than for organic solvents). Nevertheless, if it is necessary to work with acid solutions as extraction solvent, a significantly good efficiency of extraction for SeMet in yeast can be achieved when working with large amounts of sample (>70 mg) (Figure 12b).

Figure 12 – Results of the UALLME experimental design using the SELM-1 CRM: (a) Pareto chart, and reponses surfaces for (b) pH x sample mass (for CT = 5 min), (c) sample mass x CT (for pH = 1.00), (d) sample mass x CT (for pH = 13.00), (e) pH x CT (for sample mass = 80 mg) and (f) pH x CT (for sample mass = 20 mg).

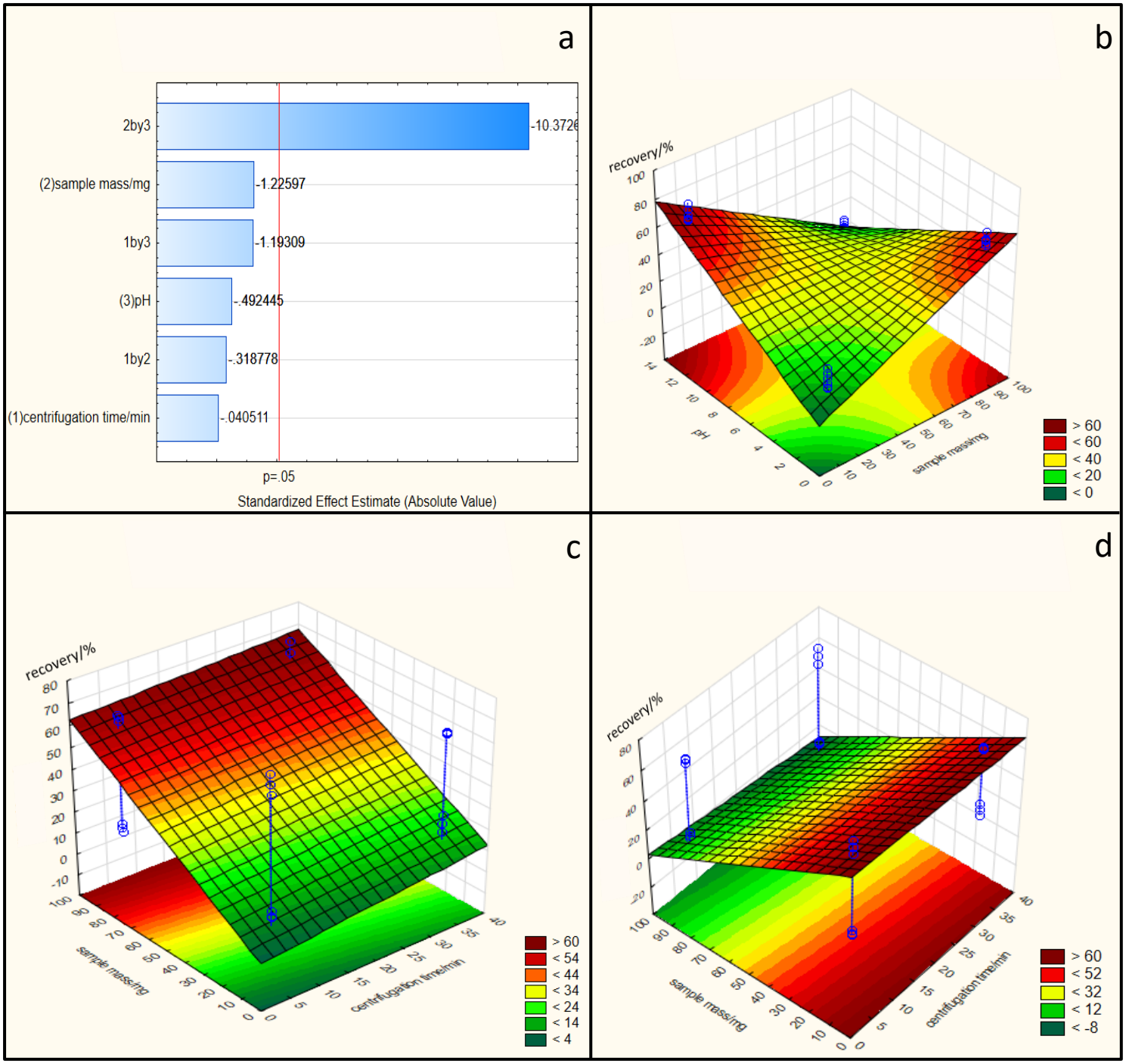
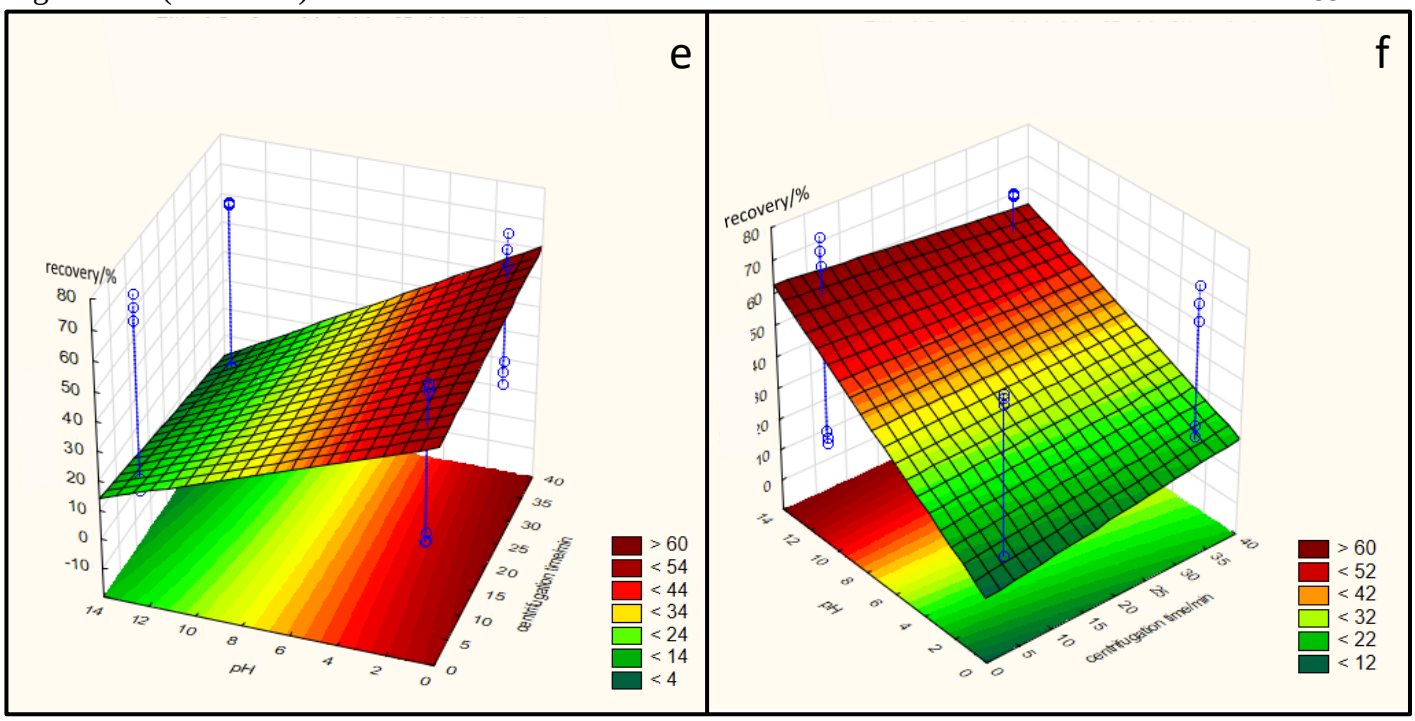


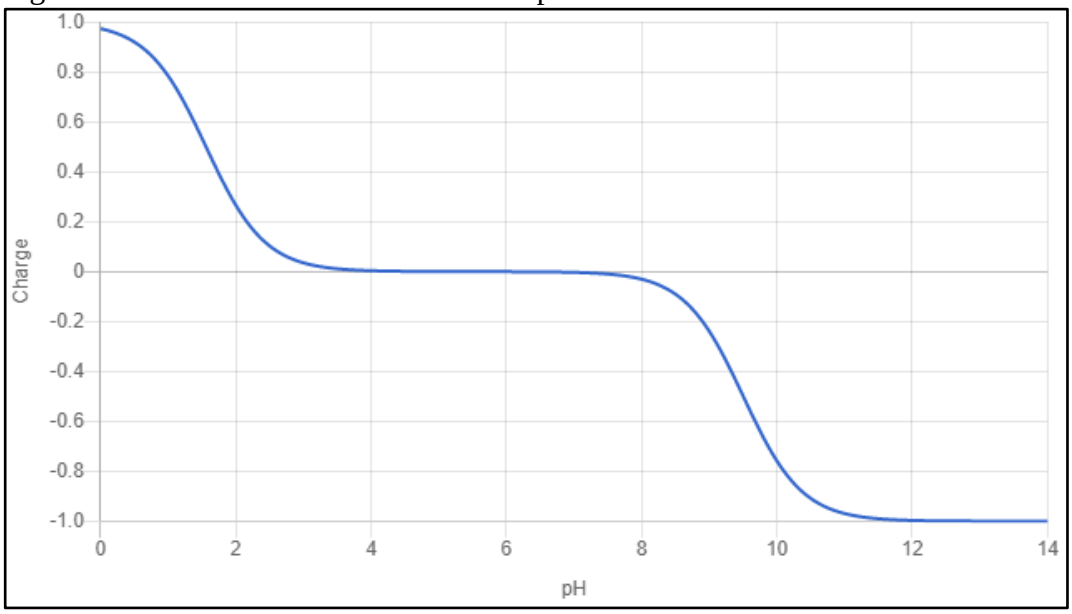
Figure 12 – (*continued*)



Source: Author, 2020.

Recovery/% corresponds the percentage of SeMet recovered from the SELM-1 CRM; UALLME: ultrasound-assisted liquid-liquid microextraction, CRM: certified reference material, CT: centrifugation time. From Figure 12a: (1) effect of the sample mass, (2) effect of the centrifugation time, (3) effect of the pH, (1 by 2) interaction between sample mass and centrifugation time, (1 by 3) interaction between sample mass and pH, (2 by 3) interaction between centrifugation time and pH.

Figure 13 – Selenomethionine isoelectric point chart.

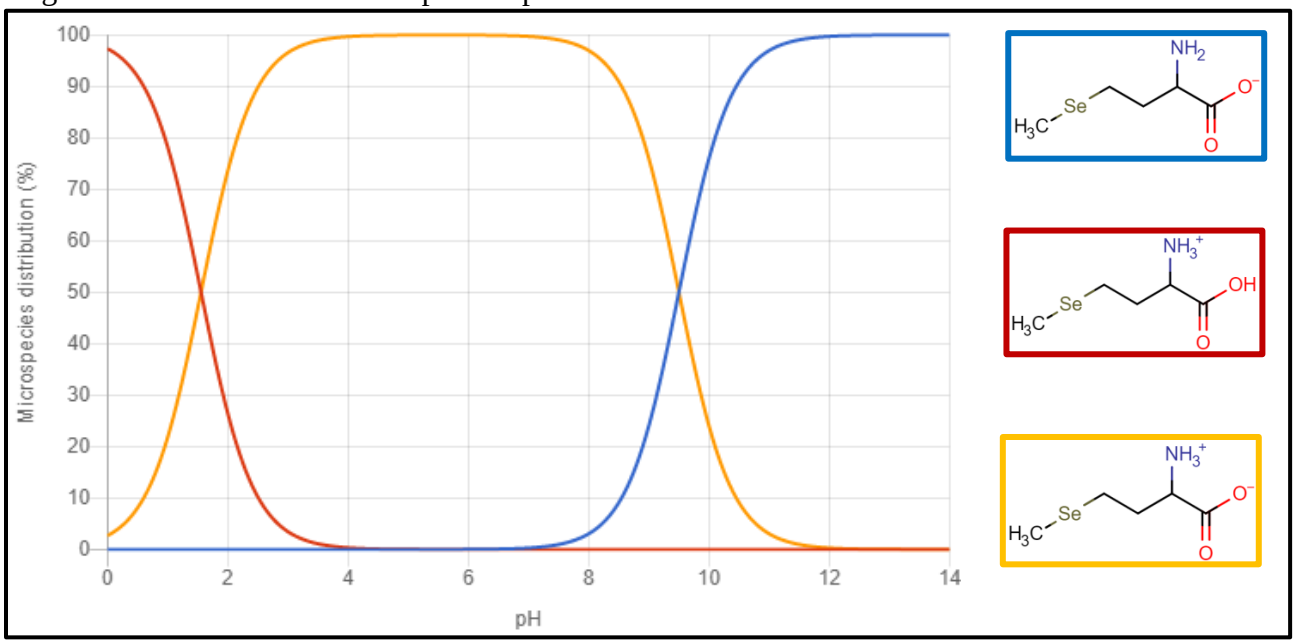


Source: Chemicalize, 2020. https://chemicalize.com/app

As the sample mass was not a limiting factor, in the present work it was decided to use a 0.1 mol L^{-1} HCl ($pH = 1.00$) solution as extracting solvent. At this pH, about 80 % of the

SeMet is in its positive ionic form (see the SeMet species speciation chart, Figure 14) becoming more soluble in aqueous solutions than its neutral specie, probably increasing its extraction rate. Also, the pH of the final extract was ajusted to about 2.50, before instrument injections, with diluted NaOH solution (0.01 mol L^{-1}), to preserve the column stationary phase (optimal operation: 2 < pH < 12) and to get a solution more compatible with the mobile phase (0.1 % formic acid solution, $pH \cong 2.66$). At these conditions (low pHs) the positive ionization of the analyte in the ESI source is further improved, increasing the sensitivity of the instrumental analysis. Therefore, considering the limited amount of sample available to perform analyses and the acid pH of the final extract, the sample mass was fixed in about 80 mg for further experiments.

Figure 14 – Selenomethionine species speciation chart.



Source: Chemicalize, 2020. https://chemicalize.com/app

Through the response surface charts 'sample mass x centrifugation time', is possible to note when the pH is fixed in 1 and 13 (Figures 12c and 12d respectively), the working range of the sample mass should be high and low, respectively, to get the highest SeMet recovery, as already discussed. This fact is also observed in the Figures 12e and 12f (response surface charts 'pH x centrifugation time') when the mass is fixed in 80 mg and 20 mg respectively, where, for the larger measured mass, an extraction solvent at low pH must be used for the highest response,

while, for the smaller mass, a high pH extraction solvent is desired. All those Figures confirm the non significance of the CT in the response, since, if the variable on the 'x' axis is kept constante ('sample mass' for the Figures 12c and 12d or 'pH' for the Figures 12e and 12f), whatever the value of the CT, the result of the SeMet extraction will be similar.

The UALLME optimal condition, considering a final acid medium, was found using a CT = 5 min, a measured sample mass of 80 mg and the 0.1 mol L^{-1} HCl as extracting solvent. This LLME procedure meets the requirements of the recommended sample preparation techniques once it is a simple method (being easily transferred between labs), consuming low amounts of reagents (only 4 mL of n-hexane and extracting solvent) and presents a considerable low time since the longest step is 20 min (ultrasonic bath performed twice per extraction with a total time of 40 min); it is still a candidate extraction procedure for routine analyses as multiple samples can be processed in a single batch. López-García et al (2013) also used a LLME procedure to extract Se species from edible oils using an acidic aqueous medium as solubilization solvent, and they reported the importance of the ultrasonic energy to favor the dispersion of the ionic solvent into the oil phase (López-García, Vicente-Martínez *et al.*, 2013).

Unlike of an univariate optimization, that is usually employed, the use of an experimental design for method development was quite convenient, as it allowed a multivariate optimization which the influence of the interactions between variables on the analyte response was checked. In the case of this work, the extraction solvent pH is closely linked to the sample mass on the SeMet recovery from the CRM.

5.2.3 Matrix effect evaluation and method validation

A validation process was performed in order to ensure the development of a reliable method and an accurate interpretable information about the sample. This analytical stage shall guarantee the method meets the requirements of the analytical application of high metrological quality results (Ribani, Bottoli *et al.*, 2004; Barbosa, Martins *et al.*, 2018). It should be performed whenever an existing method is modified or when an entirely new method is developed (Ribani, Bottoli *et al.*, 2004). This way the applicability of the developed UALLME-UPLC-ESI/QDa method for SeMet analysis in cashew nuts was assessed through the linearity, selectivity, sensitivity, precision, accuracy and linear range studies.

As the sample nature can influence the performance of the analytical method causing overestimation or underestimation of the analyte signal, a matrix effect (ME) study was performed. Despite the mechanisms governing these effects are not completely understood in LC, is believed it is especially related to the MS technique, occuring mainly at the ionization source (Stahnke and Alder, 2015). According to Villagrasa et al (2007), coeluting residual matrix components can affect the ionization efficiency of the target analyte leading to erroneous results (Villagrasa, Guillamón *et al.*, 2007), so this is an important parameter that should become an integral part of the quantitative LC-ESI/MS method development and validation. Also, studies have recognized that the physicochemical properties of the analyte such as, polarity, octanol-water partition coefficient (K_{ow}), molecular weight, etc, are closely related to the intensity of the ME too (Alcântara, Paz *et al.*, 2018).

5.2.3.1 Cashew nut matrix effect in SeMet analysis by LC-ESI/MS

The matrix effect was evalluated through comparison of the angular coefficients of calibration curves prepared by dilutions in ultrapure water (external standard calibration) and in the sample extracts (matrix-matched calibration), both in the presence of 0.2 mg L^{-1} genistein, using the equation 2 (see section 4.7). Figure 15 shows the analytical curves, where a positive matrix effect of 46.9 % was found, demonstrating the cashew nut matrix influence the SeMet analysis by an overestimation of the analytical signal.

Studies have reported that in LC-ESI/MS the ME occurs mainly in the ESI source once analytes are less efficiently ionized if matrix molecules with higher proton affinity are present (Kebarle and Verkerk, 2009; Stahnke and Alder, 2015). However, different of the observed in this work, that mechanism is related to suppression of the analytical signal where a negative ME should be detected. So, is believed the positive value obtained in here could be related principally to the LC system, especially inside the column.

In the stationary phase support from LC columns there are about 8 µmol of silanol groups (Si-OH) / m^2; when working in reverse phase mode they are reacted with a chemically bonded non polar stationary phase (normally octadecylsilane like the one used at this work) (Maldaner, Collins *et al.*, 2010). However, the efficiency of this process depends on the column manufacturer and at most only 4 µmol of Si-OH / m^2 are derivatized (Harris, 2015a). The free

silanol group generates its dissociated form ($Si\text{-}O^-$) in a wide range of pH, that strongly retains protonated bases (e.g. RNH_3^+) (Harris, 2015a). SeMet becomes this form at low pH (Figure 14), which probably favored its $Si\text{-}O^-$ adsorption when its water solutions were injected (e.g. external standard calibration); but when SeMet was injected in the presence of the sample matrix (e.g. matrix-matched calibration), a competitive process may have occurred between the analyte and the several matrix components to adsorve on the column's active sites, favoring the SeMet avaiability to the mobile phase, probably incrasing its analytical signal and justifying its response overestimation.

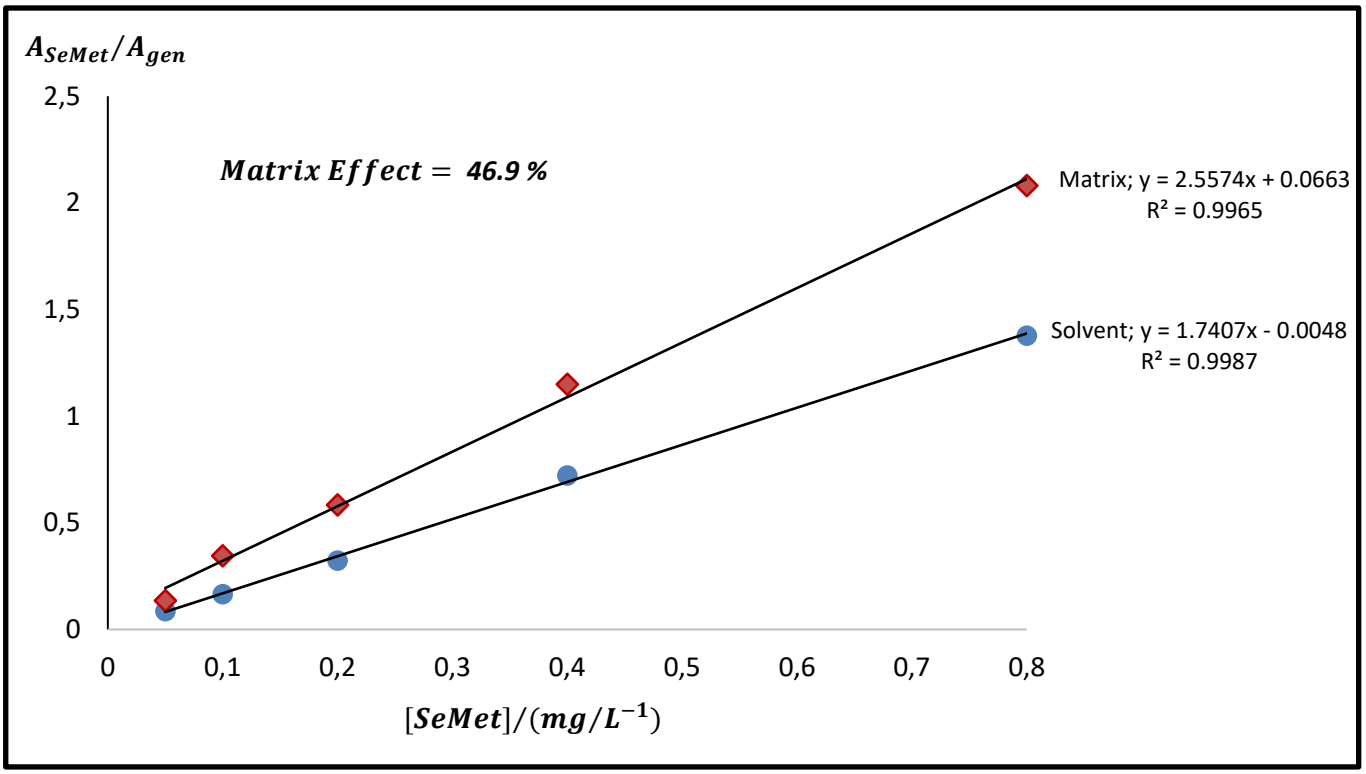
Nevertheless, Pinho et al (2009) do not rule out the contribution of adsorption mechanisms occurring inside the MS system, that can further increase the positive ME due competition between the analyte and sample coextrectives in the deposition on metallic surfaces from the MS compartments (e.g. source, analyzer, etc) (Pinho, Neves *et al.*, 2009).

Among several substances, the phospholipids were identified as the main reason for the occurrence of ME in biological samples in the LC-ESI/MS detection (Silvester and Smith, 2012; Stahnke and Alder, 2015). For plants and its parts (e.g. fruits, seeds, etc), the analyte quantification is significantly influenced by the high content of their essencial oils, where coextractives like lipds, pigments and other components can still remain solubilized in the extract, even after the sample purification (Pinho, Neves *et al.*, 2009).

Studies have reported the interference of sample matrices in analyses of several compounds by LC-ESI/MS, like pesticides quantification in cashews (De Oliveira Silva, De Castro *et al.*, 2014), benzoxazinoid derivatives in plant materials (Villagrasa, Guillamón *et al.*, 2007) and acylcarnitines in human urine (Abe, Suzuki *et al.*, 2017). Silva et al (2019) reported positive MEs for most analytes when employed the LC-ESI/MS system in pesticide analyses from cabbage (Silva, De Menezes *et al.*, 2019). According to the authors, a comparison of the efficiency of the ESI and the atmospheric pressure chemical ionization (APCI) souce showed the ME was more intense when the APCI was used.

Thus, considering these circumstances associated to the influence of cashew nut coextractives in SeMet analyses by UPLC-ESI/QDa (ME out of the range -20 – 20), the matrix-matched calibration must be used for the quantification of the analyte in real samples.

Figure 15 – Superposition of solvent and matrix SeMet calibrations.



Source: Author, 2020.
A_{SeMet} / A_{gen}: ratio between the chromatographic peak areas of selenomethionine and genistein, [SeMet]: selenomethionine concentration.

5.2.3.2 UALLME-UPLC-ESI/QDa method validation for SeMet analyses in cashew nuts

Table 8 summarizes the figures of merit for the developed UALLME-UPLC-ESI/QDa method. Limits of detection (LODs) and quantification (LOQs) were estimated by successive dilutions, where the working solutions were analyzed starting from the most diluted one, until the value of the sinal to noise ratio (S/R) gets close to 3.3 and 10.0, respectively. The results remained as 10 and 50 µg L^{-1} for LOD and LOQ, respectively, for both curves (in the solvent and in the matrix). Lower LODs have been reported in the literature when using the LC tandem mass spectrometry (LC-MS/MS) (Mcsheehy, Shona, Yang, Lu *et al.*, 2005) or the ICP-MS/MS coupled with LC (Gao, Luo *et al.*, 2017) to analyze SeMet. However, the use of the QDa analyzer working in SIR mode demonstrated to be an alternative strategy once it got a significant high sensitivity even using only one mass filtrer (single quadrupole MS), that is a more affordable possibility. The use of an ultra pressure separation system also contributed to this

satisfactory result, since the analyte dilution factor inside the UPLC column is lower than in HPLC column where dimensions are larger and a higher mobile phase flow rate is used (Harris, 2015a).

Three types of precisions were evaluated and they were expressed as relative standard deviation (RSD/%). The instrumental precision based at ten injections of the SeMet standard (1 mg L^{-1}) in the instrumental system was 1.2 %; and the intraday precisions, obtained through external standard calibration, performed in three different days, were 3.7 and 5.7 % when using the SeMeSeC and genistein as IS respectively. Once all experiments were carried out in at least three independent replicates, was possible to obtain the intra-assay precision which remained less than 17 %.

The correlation coefficients (R) for all calibration functions (in the solvent and in the matrix, obtained by five levels of concentrations starting from the LOQ) were higher than 0.99, and the linear ranges were found to be 0.05 – 2.00 mg L^{-1} for external standard calibration and 0.05 – 4.00 mg L^{-1} for matrix-matched calibration; while the selectivity of the analytical method was guaranteed through the use of the selected ion recording (SIR) acquisition mode, monitoring the *m/z* ratio corresponding to the protonated molecule $[M + H]^+$ for the target analyte (198.00 for the protonated SeMet molecule).

The accuracy was estimated through the recovery tests spiking the cashew nut matrix at three levels of SeMet concentrations (0.4, 2.0 and 4.0 mg L^{-1}) right after the mass measurements of the sample C (calculation performed according to equation 3; C_f: analyte concentration in the intentionally contaminated sample, C_i: analyte concentration in the non-intentionally contaminated sample and C_a: added concentration of the analyte); and through the estimation of the SeMet concentration in the SELM-1 CRM. These experiments were realized performing three independent replicates of sample preparation. Despite the satisfactory result when using the spiking tests (recovery of SeMet between 78.5 and 120.6 %), the concentration of SeMet obtained from the SELM-1 remained in the range of 60 and 70 % of the certificated value (3190 mg kg^{-1}).

$$recovery/\% = \frac{C_f - C_i}{C_a} x\ 100 \qquad (3)$$

It is known, the NRC CRM SELM-1, SeMet is incorporated into the yeast protein to mimics it shape present in real food samples, whereas, when the SeMet is added in the cashew nut sample using a standard solution (like in recovery tests) the analyte is in its free form that is more easily recovered by the sample preparation process. For Ribani et al (2004) the limitation of the recovery procedure is that the spiked analyte normally is not in the same form as the one in the sample (Ribani, Bottoli *et al.*, 2004).

Thus, the acid UALLME developed method combined with the UPLC-ESI/QDa detection demonstrated as an efficient method for SeMet determination in cashew nut matrices, although its accuracy could be further improved, for example, with the use of a digestion step in the sample preparation method to try release the SeMet compound from the protein chain.

Table 8 – Results of the figures of merit and analyses in commercial cashew nut samples.

Sensitivity	**Solvent**	**LOD/(mg L^{-1})**	0.01
		LOQ/(mg L^{-1})	0.05
	Matrix	**LOD/(mg L^{-1})**	0.01
		LOQ/(mg L^{-1})	0.05
Precision	**Instrumental**	1.2[a]	
	Intra-Assay	**From the UALLME Optimization Experiments /(RSD/%)**	0.7 – 16.7[b]
		From the Recovery Tests/(RSD/%)	2.0 – 6.7[b]
	Intra-Day	**Solvent Curve using SeMeSeC as IS/(RSD/%)**	3.7
		Solvent Curve using Genistein as IS/(RSD/%)	5.7
Accuracy	**Mean Recovery[c]/% ± SD**	**0.4[d]**	78.5 ± 5.3
		2.0[d]	113.0 ± 2.2
		4.0[d]	120.6 ± 3.6
	[SeMet]/mg kg^{-1} recovered from the CRM[e]	1914 – 2233[b]	
SeMet Linear Range	**External Standard Calibration/mg L^{-1}**	0.05 – 2.00	
	Matrix-Matched Calibration/mg L^{-1}	0.05 – 4.00	
Real Samples Quantification		**Brand A/(mg kg^{-1})**	< LOQ
		Brand B/(mg kg^{-1})	< LOQ
		Brand C/(mg kg^{-1})	< LOQ

Note: [a]value in terms of RSD/%; [b]range considering several experiments; [c]through recovery tests; [d]fortification levels in 'mg L^{-1}' considering a final extract volume of 4 mL; [e][SeMet] in SELM-1 CRM is 3190 mg kg^{-1} ± 290 mg kg^{-1}. LOD: detection limit, LOQ: quantification limit, UALLME: Ultrasound-Assisted Liquid-Liquid Microextraction, RSD: relative standard deviation, SeMeSeC: Se-methylselenocystin, IS: internal standard, SD: standard deviation.

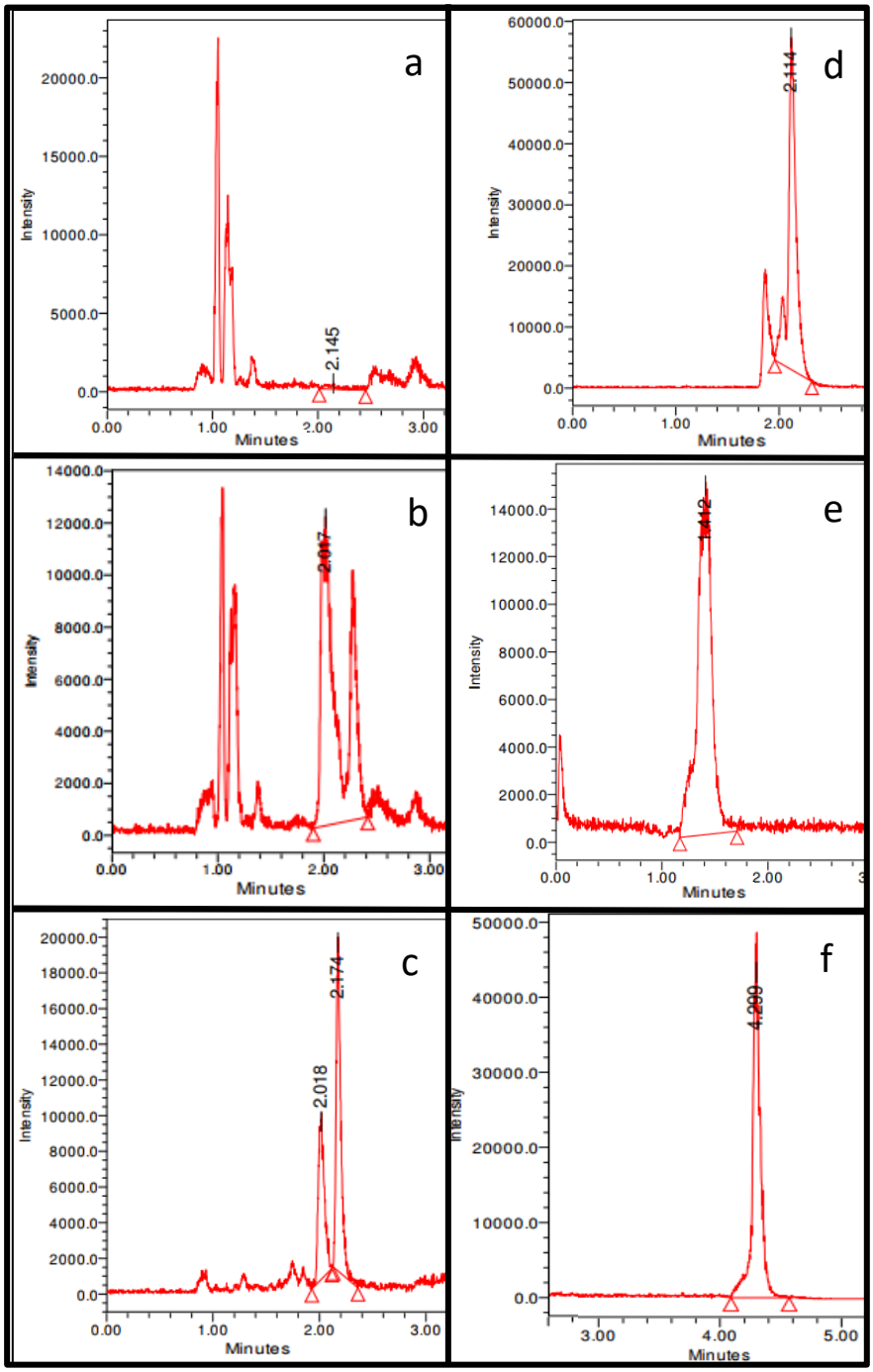
5.2.4 Analysis of commercial cashew nut samples

The developed and validated UALLME-UPLC-ESI/QDa method was applied for the determination of SeMet in commercial cashew nut samples from three different brands named as A, B and C. Figure 16 shows chromatograms for the extract of the sample C without analyte spike, the sample C extract spiked with 1.0 mg L^{-1} SeMet standard and the extract for the selenized yeast CRM SELM-1, besides to presents the chromatograms of the SeMet (0.8 mg L^{-1}), SeMeSeC (1.0 mg L^{-1}) and genistein (0.2 mg $L^{-}1$) standards in the solvent for comparison purposes. The other samples (A and B) obtained a chromatographic profile very similar to that presented for sample C (Figure 16a), the only difference is that they got more intense peaks in the retention time (RT) of about 1.00 min.

Comparing the chromatograms, and knowing the SeMet is the major form of Se present in SELM-1 CRM (Mester, Zoltán, Willie, Scott *et al.*, 2006), is convenient to consider the peaks at about RT = 2.1 min from 'Figures 16b and 16c' correspond to the target compound. The appearance of two peaks representing SeMet can be justified by an analyte degradation during sample pre-treatment or during instrumental analysis, principally due interaction mechanisms occuring inside the column. According to Pornwilard and Siripinyanond (2014), the chromatographic techniques have shown to cause structural disruption of protein chains due strong interactions of them with the column stationary phase (Pornwilard and Siripinyanond, 2014). But, another theory, which may be a more accurate one, can be associated with the pH adjustment of the sample extract to about 2.50 before instrumental injection. At this pH the SeMet is present in the solution at two different species, the protonated one (positive ionic form) and the neutral molecule (not dissociated) (Figure 14), which probably are represented by both peaks around 2.1 min (Figures 16b and 16c).

It is important to consider the quantification analyses were performed using the SIR mode by monitoring the fragment of mass corresponding to the SeMet protonated molecule (*m/z* = 198.00), which significantly increases the sensitivity and selectivity of the instrumental analysis. But, even so, no peaks appeared in the retantion time of the SeMet (about 2.1 min) in the sample chromatograms (Figure 16a for sample C that was similar for samples A and B), indicating a probable absence of analyte in the analyzed samples or its presence at a concentration level below the LOQ (Table 8).

Figure 16 – UPLC-ESI/MS chromatograms in SIR mode. (a) Pure extract of the sample C (*m/z* = 198.00), (b) sample C extract spiked with SeMet at 1.0 mg L^{-1} (*m/z* = 198.00), (c) SELM-1 CRM extract diluted 10 times before injection (*m/z* = 198.00), (d) SeMet standard in the solvent at 0.8 mg L^{-1} (*m/z* = 198.00), (e) SeMeSeC standard in the solvent at 1.0 mg L^{-1} (*m/z* = 183.98), (f) genistein standard in the solvent at 0.2 mg L^{-1} (*m/z* = 271.06).



Source: Author, 2020.

Nevertheless, this result may be more strongly related with the sample preparation process than to the detection system. As already discussed at section 5.2.3.2, the level of SeMet recovered from the CRM was a little lower than the expected value, probably due the non effective release of all SeMet molecules traped in the protein chains by the sample pre treatment, possibly causing a false negative result for SeMet. Peaks appearing at 1.00 min in the sample chromatograms (Figure 16a), for example, may be related to SeMet containing proteins that also presents the fragment of mass of 198.00.

However, this fact does not diminish the relevance of the developed UALLME sample preparation method, where its conditions (pH, CT and sample mass) have already been optimized, and presents advantages like the highlighted in the topic 5.2.2. So, in order to further improve the developed sample preparation process, a digestion step will be added to try to get the SeMet concentration recovered from the SELM-1 CRM in agreement with the certified value, thus, the method will be still more accurate to be applied in real samples. Explanation about how this improvement will be performed is presented in the topic "Perspective".

As far as the authors known, this is the first attempt to quantify SeMet in cashew nuts sold at market in Ceará state (from Brazil), and studies indicate the need for researches to be expanded due the great importance of this oilseed to the local economy, where only the state of Ceará accounts for 61.6 % of the cashew acreage for the whole country (Brainer and Vidal, 2018).

5.2.5 Final considerations about the developed UALLME-UPLC-ESI/QDa method

A simple and rapid ultrasound-assisted liquid-liquid microextraction (UALLME) method was efficiently optimized for the parameters pH, centrifugation time and measured sample mass to analyze SeMet in cashew nuts. Considering the instrumental analysis by UPLC-ESI/QDa, the genistein proved to be an alternative internal standard for SeMet, despite does not present chemical similarity with it.

The developed UALLME in combination with UPLC-ESI/QDa using quantification at SIR mode presented satisfactory sensitivity, linearity, selectivity, instrumental, intra-assay, intraday precisions and accuracy in terms of recovery tests. Also, cashew nut matrix positively influenced in the analyte quantification.

Accuracy by the use of a CRM material and analyses in commercial samples demonstrated the need of performing a little improvement in the developed extraction method, but which does not diminish its relevance and advantages for SeMet analyses in real cashew nut samples.

6 CONCLUSIONS

Chromatography is a separation technique which presents a wide field of application providing high quality data in terms selectivity and accuracy in a lot of areas of the scientific research. But new technologies have been developed in the market and the AF4 stands out due to advantages related to its mechanism of separation which is considered of low cost, but also presenting results of good quality.

Thus, this work presented the theory of the different mechanisms that govern the analytical separations of these two systems, highlighting their advantages and disadvantages, besides demonstrating their applicability in similar areas of the knowledge. This way, the UPLC and AF4 methods were well developed and validated to analyze SeMet in different matrices (cashew nut and yeast, respectively), where the choice of a given separation system should take into account the goal of the analytical research, the mechanism of separation of the different species, the analyte properties, the sample preparation used before instrumental analysis, the long and short term costs associated with each system and, still, which detection system is going to be used in the coupling.

7 PERSPECTIVE

Currently, with the development of new technologies for genetic editing, is possible to access the DNA of individuals (animal, plants, etc) to add, remove or replace genes in order to obtain products with a specific characteristic (Funari, Castro-Gamboa *et al.*, 2013). However, is necessary to know which substances or metabolites must be manipulated to obtain the desired property, such metabolites are called biomarkers. Before any genetic editing, a metabolomics study must be performed in order to identify the possible biomarkers responsible for the expected changes (Funari, Castro-Gamboa *et al.*, 2013).

Alves (2016), for example, through the metabolomics analyses to identify biomarkers responsible for the defense mechanism of the early dwarf cashew tree leaf against anthracnose, identified about nine resistant and three susceptible biomarkers when evaluating healthy and diseased plants (Alves, 2016). Such results may accelerate the selection of viable genotypes, saving time and resource, to obtain an anthracnose resistant product.

Once suitable levels of Se in the human body are associated with prevention of several illnesses, a metabolomics study with the aim to identify possible SeMet biomarkers can be very interesting to assist the genetic engineering in produce products (such as nuts) with adequate levels of SeMet containing protein. This product, of natural origin, could be administered instead selenium-based supplements in diseases prevention.

Thus, the perspective of this study is to apply the developed UALLME-UPLC/ESI-QDa method to analyze SeMet in several cashew nuts samples present in the active germplasm bank (BAG) of the Embrapa company, and thus, to obtain a database about the SeMet concentration in these clones. This way a metabolomics study, in cashews which present the highest and the lowest levels of the analyte, can be conducted in order to identify the biomarkers associated with the presence of SeMet in the samples.

To guarantee the success in this research the UALLME method will be further improved with the introduction of a digestion process in the sample preparation. This step, added before the centrifugation, will be optimized using the factorial planning, as the one used in this work, where the time and temperature of digestion can be the factors varying on two levels (with central point) and the recovered SeMet concentration from the SELM-1 CRM can be the response. Table 9 shows an example of how this experiment can be performed. The objective of

this experiment is to get the accuracy of the developed method further improved and, thus, being even more appropriated to be used as a screening method for SeMet analysis in cashew nuts.

Table 9 – Experimental design for the digestion step that will be introduced in the UALLME method.

Factors	**Levels**		
	-	**0**	**+**
Time of digestion/min	5	20	35
Temperature of digestion/°C	50	100	150

Experiments	**Time of digestion/min**	**Temperature of digestion/°C**
1	35	150
2	5	50
3	35	50
4	5	150
9	20	100

REFERENCES

ABE, K. H.; SUZUKI, *et al.* Matrix effect–corrected liquid chromatography/tandem mass-spectrometric method for determining acylcarnitines in human urine. **Clinica Chimica Acta**, Japan, v. 468, p. 187-194, 2017.

ADADI, P.; BARAKOVA, N. V. *et al.* Designing selenium functional foods and beverages: A review. **Food Research International**, Russia, v. 120, p. 708-725, 2019.

ADECE, A. D. D. D. E. D. C.-. Frutas do Ceará. https://www.adece.ce.gov.br/, Ceará June.06.2020. 2020.

AHSAN, U.; KAMRAN, Z. *et al.* Role of selenium in male reproduction—A review. **Animal Reproduction Science**, Pakistan, v. 146, n. 1, p. 55-62, 2014.

ALCÂNTARA, D. B., FERNANDES, T. S. *et al.* Diagnostic detection systems and QuEChERS methods for multiclass pesticide analyses in different types of fruits: An overview from the last decade. **Food chemistry**, Brazil, 2019.

ALCÂNTARA, D. B.; NASCIMENTO, R. F. *et al.* Evaluation of different strategies for determination of selenomethionine (SeMet) in selenized yeast by asymmetrical flow field flow fractionation coupled to inductively coupled plasma mass spectrometry (AF4-ICP-MS). **Analytical Methods**, Brazil, v. 12, p. 3351-3360, 2020.

ALCÂNTARA, D. B.; PAZ, M. S. *et al.* Organophosphorus Pesticide in Sapodilla (Manilkara zapota) Fruit. **Journal of the Brazilian Chemical Society**, Brazil, v. 29, n. 10, p. 2180-2188, 2018.

ALVES, P. D. C. C. Estudos metabolômicos associados a resistência de folhas de cajueiro (Anacardium Occidentale L.) à antracnose. Brazil, 2016.

ARIAS-BORREGO, A.; CALLEJÓN-LEBLIC, B. *et al.* A novel HPLC column switching method coupled to ICP-MS/QTOF for the first determination of selenoprotein P (SELENOP) in human breast milk. **Food Chemistry**, Spain, v. 321, n. 15, 2020.

ASTOLFI, M. L.; PROTANO, C. *et al.* A new rapid treatment of human hair for elemental determination by inductively coupled mass spectrometry. **Analytical Methods**, Italy, v. 12, n. 14, p. 1906-1918, 2020.

BABÜR, E.; TAN, B. *et al.* Deficiency but Not Supplementation of Selenium Impairs the Hippocampal Long-Term Potentiation and Hippocampus-Dependent Learning. **Biological trace element research**, [S.I.], v. 192, n. 2, p. 252-262, 2019.

BAKIRDERE, S.; VOLKAN, M. *et al.* Speciation of selenium in supplements by high performance liquid chromatography-inductively coupled plasma-mass spectrometry. **Analytical Letters**, [S. I.], v. 48, n. 9, p. 1511-1523, 2015.

BARBOSA, P. G. A.; MARTINS, F. I. C. C. *et al.* Statistical analysis for quality adjustment of the analytical curve for determination of pesticide multiresidue in pineapple samples. **Food analytical methods**, Brazil, v. 11, n. 2, p. 466-478, 2018.

BEAUCHEMIN, D. Inductively Coupled Plasma Mass Spectrometry Methods. **Encyclopedia of Spectroscopy and Spectrometry (Third Edition)**. Oxford: Academic Press, p.236-245, 2017.

BENDIXEN, N.; LOSERTS, S. *et al.* Membrane–particle interactions in an asymmetric flow field flow fractionation channel studied with titanium dioxide nanoparticles. **Journal of Chromatography A**, Switzerland, v. 1334, p. 92-100, 2014.

BIERLA, K.; SZPUNAR, J. *et al.* Comprehensive speciation of selenium in selenium-rich yeast. **TrAC Trends in Analytical Chemistry**, France, v. 41, p. 122-132, 2012.

BOTH, E. B.; SHAO, S. *et al.* Selenolanthionine is the major water-soluble selenium compound in the selenium tolerant plant Cardamine violifolia. **Biochimica et Biophysica Acta (BBA)-General Subjects**, China, v. 1862, n. 11, p. 2354-2362, 2018.

BOUVAREL, T.; DELAUNAY, N. *et al.* Selective extraction of cocaine from biological samples with a miniaturized monolithic molecularly imprinted polymer and on-line analysis in nano-liquid chromatography. **Analytica Chimica Acta**, France, v. 1096, p. 89-99, 2020.

BRAINER, M. D. C.; VIDAL, M. D. F. Cajucultura nordestina em recuperação. **Informe ETENE**, Brazil, v. 1, n. 5, 2018.

BRIGELIUS-FLOHÉ, R; MAIORINO, M. Glutathione peroxidases. **Biochimica et Biophysica Acta (BBA) - General Subjects**, Germany, v. 1830, n. 5, p. 3289-3303, 2013.

BURK, R. F.; HILL, K. E. Selenoprotein P—Expression, functions, and roles in mammals. **Biochimica et Biophysica Acta (BBA) - General Subjects**, USA, v. 1790, n. 11, p. 1441-1447, 2009.

CABRERA, L. D. C.; CALDAS, S. S. *et al.* Evaluation of alternative sorbents for dispersive solid-phase extraction clean-up in the QuEChERS method for the determination of pesticide residues in rice by liquid chromatography with tandem mass spectrometry. **Journal of separation science**, Brazil, v. 39, n. 10, p. 1945-1954, 2016.

CAO, S.; POLLASTRINI, J. *et al.* Separation and characterization of protein aggregates and particles by field flow fractionation. **Current pharmaceutical biotechnology**, USA, v. 10, n. 4, p. 382-390, 2009.

CHEN, D.; Sun, H. *et al.* Selenium bio-absorption and antioxidant capacity in mice treated by selenium modified rice germ polysaccharide. **Journal of Functional Foods**, China, v. 61, 2019.

CHUNHIENG, T.; PÉTRITIS, K. *et al.* Study of selenium distribution in the protein fractions of the Brazil nut, Bertholletia excelsa. **Journal of Agricultural and Food Chemistry**, France, v. 52, n. 13, p. 4318-4322, 2004.

CLARK, L. C.; COMBS, G. F. *et al.* Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin: a randomized controlled trial. **Jama**, USA, v. 276, n. 24, p. 1957-1963, 1996.

COMBS, G. F. e S. B. Combs. 5 - ABSORPTION, EXCRETION, AND METABOLISM OF SELENIUM. In: G. F. Combs e S. B. Combs (Ed.). The Role of Selenium in Nutrition: **Academic Press**, 1986. 5 - ABSORPTION, EXCRETION, AND METABOLISM OF SELENIUM, p.179-204

COMINETTI, C.; DE BORTOLI, M. C. *et al.* Brazilian nut consumption improves selenium status and glutathione peroxidase activity and reduces atherogenic risk in obese women. **Nutrition Research**, Brazil, v. 32, n. 6, p. 403-407, 2012.

COOPER, J. Improving Sensitivity and Selectivity for Primary Aromatic Amines Analysis with ACQUITY QDa Detector. **Consumer Products Testing**, USA, 2013.

DA SILVA, E. G.; MATAVELI, R. L. V. *et al.* Speciation analysis of selenium in plankton, Brazil nut and human urine samples by HPLC–ICP-MS. **Talanta**, Brazil, v. 110, p. 53-57, 2013.

DE LIMA, G. C.; DO LAGO, A. C. *et al.* Determination of selenium using atomically imprinted polymer (AIP) and hydride generation atomic absorption spectrometry. **Analytica chimica acta**, Brazil, v. 768, p. 35-40, 2013.

DE OLIVEIRA SILVA; DE CASTRO, R. R. C. *et al.* Liquid chromatography–electrospray ionization–tandem mass spectrometry method for determination of twenty multi-class pesticide residues in cashew. **LWT-Food Science and Technology**, Brazil, v. 59, n. 1, p. 21-25, 2014.

DEPOI, F. D. S.; POZEBON, D. The use of cloud point extraction and hydride generation for improving the Sb and Se limits of detection in ICP OES. **Journal of the Brazilian Chemical Society**, Brazil, v. 23, n. 12, p. 2211-2221, 2012.

DERNOVICS, M.; GIUSTI, P. *et al.* ICP-MS-assisted nanoHPLC-electrospray Q/time-of-flight MS/MS selenopeptide mapping in Brazil nuts. **Journal of Analytical Atomic Spectrometry**, France, v. 22, n. 1, p. 41-50, 2007.

DINH, Q. T.; CUI, Z. *et al.* Selenium distribution in the Chinese environment and its relationship with human health: a review. **Environment international**, China, v. 112, p. 294-309, 2018.

DOMOSŁAWSKA, A.; ZDUNCZYK, S. *et al.* Selenium and vitamin E supplementation enhances the antioxidant status of spermatozoa and improves semen quality in male dogs with lowered fertility. **Andrologia**, Poland, v. 50, n. 6, 2018.

DONADIO, J. L.; ROGERO, M. M. *et al.* SEPP1 polymorphisms modulate serum glucose and lipid response to Brazil nut supplementation. **European journal of nutrition**, Brazil, v. 57, n. 5, p. 1873-1882, 2018.

SKOOG, D. W.; HOLLER, J.; CROUCH, S. Fundamentos de química analítica: Cengage Learning. 1088 p., USA, 2014.

DRUTEL, A.; ARCHAMBEAUD, F. *et al.* Selenium and the thyroid gland: more good news for clinicians. **Clinical Endocrinology**, France, v. 78, n. 2, p. 155-164, 2013.

DU, L.; LU, W. *et al.* Detection of milk powder in liquid whole milk using hydrolyzed peptide and intact protein mass spectral fingerprints coupled with data fusion technologies. **Food Science & Nutrition**, [S.I.], v. 8, n. 3, p. 1471-1479, 2020.

DUMONT, E.; DE PAUW, L. *et al.* Speciation of Se in Bertholletia excelsa (Brazil nut): a hard nut to crack? **Food Chemistry**, USA, v. 95, n. 4, p. 684-692, 2006.

EGRESSY-MOLNÁR, O.; VASS, A. *et al.* Effect of sample preparation methods on the D, L-enantiomer ratio of extracted selenomethionine. **Analytical and bioanalytical chemistry**, Hungary, v. 401, n. 1, p. 373-380, 2011.

ESKELIN, K.; PORANEN, M. M. *et al.* Asymmetrical Flow Field-Flow Fractionation on Virus and Virus-Like Particle Applications. **Microorganisms**, Finland, v. 7, n. 11, 2019.

ESTEKI, M.; VANDER HEYDEN, Y. *et al.* Qualitative and quantitative analysis of peanut adulteration in almond powder samples using multi-elemental fingerprinting combined with multivariate data analysis methods. **Food Control**, Belgium, v. 82, p. 31-41, 2017.

ESTEVEZ, A. O.; MUELLER, C. L. *et al.* Selenium induces cholinergic motor neuron degeneration in Caenorhabditis elegans. **NeuroToxicology**, USA, v. 33, n. 5, p. 1021-1032, 2012.

FAO, F. a. a. O. O. T. U. N.-. Production. http://www.fao.org/faostat/en/#data/QC 2020.

FERNANDES, T. S. M.; ALCÂNTARA, D. A. *et al.* Matrix effect evaluation and method validation of organophosphorus pesticide residues in bell peppers (Capsicum annuum L.) by GC–MS determination. **International Journal of Environmental Analytical Chemistry**, Brazil, p.1-16, 2019.

FRANCK, M.; PERREAULT, V. *et al.* High hydrostatic pressure-assisted enzymatic hydrolysis improved protein digestion of flaxseed protein isolate and generation of peptides with antioxidant activity. **Food research international**, Canada, v. 115, p. 467-473, 2019.

FUNARI, C. S.; CASTRO-GAMBOA, I. *et al.* Metabolômica, uma abordagem otimizada para exploração da biodiversidade brasileira: estado da arte, perspectivas e desafios. **Química Nova**, Brazil, p.1605-1609, 2013.

GAO, L.; LUO, D. *et al.* Se in Se-enriched peanut, and losses during peanut protein preparation. **International Journal of Food Science & Technology**, [S.I.], v. 52, n. 3, p. 843-850, 2017.

GAWOR, A.; RUSZCZYNSKA, A. *et al.* Determination of Selenium Species in Muscle, Heart, and Liver Tissues of Lambs Using Mass Spectrometry Methods. **Animals**, Poland, v. 10, n. 5, 2020.

GIDDINGS, J. C. Measuring colloidal and macromolecular properties by FFF. **Analytical chemistry**, [S.I.], v. 67, n. 19, p. 592A-598A, 1995.

GIRÁLDEZ, I.; RUIZ-AZCONA, P. *et al.* Speciation of selenite and selenoamino acids in biota samples by dual stir bar sorptive extraction-single desorption-capillary gas chromatography/mass spectrometry. **Microchemical Journal**, Spain, v. 122, p.197-204, 2015.

GONG, J.; XIAO, M. Effect of Organic Selenium Supplementation on Selenium Status, Oxidative Stress, and Antioxidant Status in Selenium-Adequate Dairy Cows During the Periparturient. **Period**. **Biological Trace Element Research**, China, v. 186, n. 2, p. 430-440, 2018.

GREYLING, G.; PASCH, H. Thermal Field-Flow Fractionation of Polymers: **Springer**, South Africa, v. 1, 2019

GRIEGER, J. A.; GRZESKOWIAK, L. E. *et al.* Maternal selenium, copper and zinc concentrations in early pregnancy, and the association with fertility. **Nutrients**, Australia, v. 11, n. 7, 2019.

GUMUSTAS, M.; KURBANOGLU, S. *et al.* UPLC versus HPLC on drug analysis: advantageous, applications and their validation parameters. **Chromatographia**, Turkey, v. 76, n. 21, p. 1365-1427, 2013.

HA, H. Y.; Alfulaij, N. *et al.* From selenium absorption to selenoprotein degradation. **Biological Trace Element Research**, USA, v. 192, n. 1, p. 26-37, 2019.

HAGENDORFER, H.; KAEGI, R. *et al.* Characterization of Silver Nanoparticle Products Using Asymmetric Flow Field Flow Fractionation with a Multidetector Approach – a Comparison to Transmission Electron Microscopy and Batch Dynamic Light Scattering. **Analytical Chemistry**, Switzerland, v. 84, n. 6, p. 2678-2685, 2012.

HARRIS, D. C. High-Performance Liquid Chromatography. In: (Ed.). Quantitative Chemical Analysis. New York: W.H.Freeman & Co Ltd, 2015a. High-Performance Liquid Chromatography, p.928

_____. Mass Spectrometry. In: (Ed.). Quantitative Chemical Analysis. New York: W.H.Freeman & Co Ltd, 2015b. Mass Spectrometry, p.928

_____. Sample Preparation. In: (Ed.). Quantitative Chemical Analysis. New York: W.H.Freeman & Co Ltd, 2015c. Sample Preparation, p.928

HIRTZ, A; GÜNTHER, K. Determination of Total Selenium and Extractability of Selenium in Commercially-Available Dietary Supplements by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). **Analytical Letters**, [S.I.], p. 1-16, 2020.

HOLMGREN, J. L. A. Selenoproteins. **The journal of biological chemistry**, Sweden, v. 284, 2009.

HOU, F.; DING,W. *et al.* Alkali solution extraction of rice residue protein isolates: Influence of alkali concentration on protein functional, structural properties and lysinoalanine formation. **Food Chem**, China, v. 218, p. 207-215, 2017.

HURST, R.; Hooper, L. *et al.* Selenium and prostate cancer: systematic review and meta-analysis. **The American Journal of Clinical Nutrition**, United Kingdom, v. 96, n. 1, p. 111-122, 2012.

IBGE, I. B. D. G. E. E.-. Levantamento Sistemático da Produção Agrícola - LSPA. https://www.ibge.gov.br/pt/inicio.html, June.06.2020. 2020.

IBIEBELE, T. I.; HUGHES, M. C. *et al.* Dietary antioxidants and risk of Barrett's esophagus and adenocarcinoma of the esophagus in an Australian population. **International Journal of Cancer**, Australia, v. 133, n. 1, p. 214-224, 2013.

IBRAHIM, S. A.; KERKADI, A. *et al.* Selenium and health: an update on the situation in the Middle East and North Africa. **Nutrients**, Qatar, v. 11, n. 7, 2019.

İÇELLI, O.; ÖZ, E. *et al.* Analysis of Conventionally and Magnetic-Field Dried Fruit and Nuts for Mycotoxins by High-Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS) and Trace Elements by Inductively Coupled Plasma–Mass Spectrometry (ICP-MS). **Analytical Letters**, Turkey, v. 53, n. 5, p.7 35-745, 2020.

IVANEEV, A. I.; ERMOLIN, M. S. *et al.* Sedimentation Field-flow Fractionation in Thin Channels and Rotating Coiled Columns: From Analytical to Preparative Scale Separations. **Separation & Purification Reviews**, Russia, p. 1-17, 2020.

JAGADABI, V.; NAGENDRA KUMAR, P. V. *et al.* A Stability-Indicating UPLC Method for the Determination of Potential Impurities and Its Mass by a New QDa Mass Detector in Daclatasvir Drug Used to Treat Hepatitis C Infection. **Journal of Chromatographic Science**, [S.I.], v. 57, n. 1, p. 44-53, 2018.

JARDIM, I. Extração em fase sólida: fundamentos teóricos e novas estratégias para preparação de fases sólidas. **Scientia Chromatographica**, Brazil, v. 2, n. 1, p. 13-25, 2010.

JOHN, J. J.; KUHN, S. *et al.* Ultrasound assisted liquid–liquid extraction with a novel interval-contact reactor. **Chemical Engineering and Processing: Process Intensification**, Bélgica, v. 113, p. 35-41, 2017.

JÓŹWIAK, W; POLITYCKA, B. Effect of selenium on alleviating oxidative stress caused by a water deficit in cucumber roots. **Plants**, [S.I.], v. 8, n. 7, 2019.

JURANOVIĆ CINDRIĆ, I.; ZEINER, M. *et al.* Mineral composition of elements in walnuts and walnut oils. **International Journal of Environmental Research and Public Health**, Sweden, v. 15, n. 12, 2018.

KANNAMKUMARATH, S. S.; WROBEL, K. *et al.* Studying the distribution pattern of selenium in nut proteins with information obtained from SEC-UV-ICP-MS and CE-ICP-MS. **Talanta**, USA, v. 66, n. 1, p. 153-159, 2005.

KARASAKAL, A. Determination of Trace and Major Elements in Vegan Milk and Oils by ICP-OES After Microwave Digestion. **Biological Trace Element Research**, Turkey, p. 1-11, 2020.

KARUNASINGHE, N.; NG, L., *et al.* Selenium supplementation and prostate health in a New Zealand cohort. **Nutrients**, New Zealand, v. 12, n. 1, 2020.

KEBARLE, P.; VERKERK, U. H. Electrospray: from ions in solution to ions in the gas phase, what we know now. **Mass spectrometry reviews**, Canada, v. 28, n. 6, p. 898-917, 2009.

KEHR, S.; MALINOUSKI, M. *et al.* X-Ray Fluorescence Microscopy Reveals the Role of Selenium in Spermatogenesis. **Journal of Molecular Biology**, USA, v. 389, n. 5, p.808-818, 2009.

KIELISZEK, M. Selenium–fascinating microelement, properties and sources in food. **Molecules**, Poland, v. 24, n. 7, 2019.

KILIC, S.; SOYLAK, M. Determination of trace element contaminants in herbal teas using ICP-MS by different sample preparation method. **Journal of Food Science and Technology**, Turkey, v. 57, n. 3, p. 927-933, 2020.

KIM, J. Y.; LIM, H. B. *et al.* Online miniaturized asymmetrical flow field-flow fractionation and inductively coupled plasma mass spectrometry for metalloprotein analysis of plasma from patients with lung cancer. **Analytical chemistry**, Korea, v. 88, n. 20, p. 10198-10205, 2016.

KRUG, F. J. Decomposições assistidas por radiação microondas. In: F. J. E. Krug (Ed.). Métodos de preparo de amostras: fundamentos sobre preparo de amostras orgânicas e inorgânicas para análise elementar. Piracicaba, 2008. Decomposições assistidas por radiação microondas, p.340

KUBACHKA, K. M.; HANLEY, T. *et al.* Evaluation of selenium in dietary supplements using elemental speciation. **Food Chemistry**, USA, v. 218, p. 313-320, 2017.

KURBANOGLU, S.; KARSAVURDAN, O. *et al.* Recent Advances on Drug Analyses Using Ultra Performance Liquid Chromatographic Techniques and their Application to the Biological Samples. **Current Analytical Chemistry**, Turkey, v. 15, n. 3, p. 277-293, 2019.

KURŠVIETIENĖ, L.; MONGIRDIENĖ, A. *et al.* Selenium anticancer properties and impact on cellular redox status. **Antioxidants**, Lithuania, v. 9, n. 1, 2020.

KUSHNIROV, V. V. Rapid and reliable protein extraction from yeast. **Yeast**, Russia, v. 16, n. 9, p.857-60, 2000.

LEBLANC, K. L.; KAWAMOTO, M. S. *et al.* Quantitation of Selenomethionine in Multivitamins and Selenium Supplements by High Performance Liquid Chromatography Inductively-Coupled Plasma Mass Spectrometry. **Food Analytical Methods**, Canada, v. 12, n. 6, p. 1316-1326, 2019.

LEE, W.C.; LEE, B. T. *et al.* Optimisation, evaluation and application of asymmetrical flow field-flow fractionation with single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) to characterise silver nanoparticles in environmental media. **Microchemical Journal**, Korea, v. 129, p. 219-230, 2016.

LIANG, P.; LAN, X. *et al.* Study on Extraction Method of Selenium-enriched Rice. **Agricultural Biotechnology**, China, v. 7, n. 3, p. 165-169, 2018.

LIAO, S. F.; BROWN, K. R. *et al.* Dietary Supplementation of Selenium in Inorganic and Organic Forms Differentially and Commonly Alters Blood and Liver Selenium Concentrations and Liver Gene Expression Profiles of Growing Beef Heifers. **Biological Trace Element Research**, USA, v. 140, n. 2, p. 151-169, 2011.

LIMA, L. W.; STONEHOUSE, G. C. *et al.* Selenium accumulation, speciation and localization in Brazil nuts (Bertholletia excelsa HBK). **Plants**, USA, v. 8, n. 8, p. 289, 2019.

LIRA, S. M.; DIONÍSIO, A. P. *et al.* Metabolic profile of pitaya (Hylocereus polyrhizus (F.A.C. Weber) Britton & Rose) by UPLC-QTOF-MSE and assessment of its toxicity and anxiolytic-like effect in adult zebrafish. **Food Research International**, Brazil, v. 127, 2020.

LOISELEUX, T.; ROLLAND-SABATÉ, A. *et al.* Determination of hydro-colloidal characteristics of milk protein aggregates using asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering and differential refractometer (AF4-MALLS-DRi). **Food Hydrocolloids**, Fance, v. 74, p. 197-206, 2018.

LOPES, G. S.; SILVA, F. L. *et al.* An evaluation of the use of formic acid for extraction of trace elements from Brazil nut and babassu coconut and its suitability for multi-element determination by ICP-MS. **Journal of the Brazilian Chemical Society**, Brazil, v. 27, n. 7, p. 1229-1235, 2016.

LÓPEZ-GARCÍA, I.; VICENTE-MARTÍNEZ, Y. *et al.* Nonchromatographic speciation of selenium in edible oils using dispersive liquid–liquid microextraction and electrothermal atomic absorption spectrometry. **Journal of agricultural and food chemistry**, [S.I.], v. 61, n. 39, p .9356-9361, 2013.

M-M, P.; SIRIPINYANOND, A. Field-flow fractionation with inductively coupled plasma mass spectrometry: past, present, and future. **Journal of Analytical Atomic Spectrometry**, Thailand, v. 29, n. 10, p. 1739-1752, 2014.

MACIEL, E. V. S.; DE TOFFOLI, A. L. *et al.* New materials in sample preparation: Recent advances and future trends. **TrAC Trends in Analytical Chemistry**, Brazil, v. 119, p. 115633, 2019.

MACIEL, E. V. S.; DE TOFFOLI, A. L. *et al.* Miniaturized liquid chromatography focusing on analytical columns and mass spectrometry: A review. **Analytica Chimica Acta**, Brazil, v. 1103, p. 11-31, 2020.

MAHAN, D. C; PARRETT, N. A. Evaluating the efficacy of selenium-enriched yeast and sodium selenite on tissue selenium retention and serum glutathione peroxidase activity in grower and finisher swine. **Journal of Animal Science**, USA, v. 74, n. 12, p. 2967-2974, 1996.

MAHIMA, A. K. V.; KUMAR, A. *et al*. Inorganic Versus Organic Selenium Supplementation: A Review. **Pakistan Journal of Biological Sciences**, India, v. 15, n. 9, p. 418-425, 2012.

MALDANER, L.; COLLINS, C. H. *et al.* Fases estacionárias modernas para cromatografia líquida de alta eficiência em fase reversa. **Química Nova**, Brazil, v. 33, n. 7, p. 1559-1568, 2010.

MALIK, M. I.; PASCH, H. Field-flow fractionation: new and exciting perspectives in polymer analysis. **Progress in Polymer Science**, Pakistan, v. 63, p. 42-85, 2016.

MARIOLI, M.; KOK, W. Y. Recovery, overloading, and protein interactions in asymmetrical flow field-flow fractionation. **Analytical and bioanalytical chemistry**, Netherlands, v. 411, n. 11, p. 2327-2338, 2019.

MCSHEEHY, S.; YANG, L. *et al.* Determination of methionine and selenomethionine in selenium-enriched yeast by species-specific isotope dilution with liquid chromatography-mass spectrometry and inductively coupled plasma mass spectrometry detection. **Anal Chem**, Canada, v. 77, n. 1, p. 344-349, 2005.

MEISTERJAHN, B.; WAGNER, S. *et al.* Silver and gold nanoparticle separation using asymmetrical flow-field flow fractionation: Influence of run conditions and of particle and membrane charges. **Journal of Chromatography A**, Austria, v. 1440, p. 150-159, 2016.

MÉPLAN, C.; HUGHES, D. J. The Role of Selenium in Health and Disease: Emerging and Recurring Trends: **Multidisciplinary Digital Publishing Institute**, Ireland, 2020.

MESSAOUDI, M.; BEGAA, S. *et al.* Radiochemical separation by liquid-liquid extraction for the determination of selenium in Mentha pulegium L.: Toxicity monitoring and health study. **Applied Radiation and Isotopes**, Algeria, v. 159, 2020.

MESTER, Z.; WILLIE, S. *et al.* Certification of a new selenized yeast reference material (SELM-1) for methionine, selenomethinone and total selenium content and its use in an intercomparison

exercise for quantifying these analytes. **Analytical and Bioanalytical Chemistry**, Canada, v. 385, n. 1, p. 168-180, 2006.

MOREDA-PIÑEIRO, J.; SÁNCHEZ-PIÑERO, J. *et al.* Selenium species determination in foods harvested in Seleniferous soils by HPLC-ICP-MS after enzymatic hydrolysis assisted by pressurization and microwave energy. **Food Research International**, Spain, v. 111, p. 621-630, 2018.

MUDRON, P.; REHAGE, J. Effects of vitamin E and selenium supplementation on blood lipid peroxidation and cortisol concentration in dairy cows undergoing omentopexy. **Journal of animal physiology and animal nutrition**, Germany, v. 102, n. 4, p. 837-842, 2018.

NADERI, M.; SALAHINEJAD, A. *et al.* Dopaminergic dysregulation and impaired associative learning behavior in zebrafish during chronic dietary exposure to selenium. **Environmental Pollution**, Canada, v. 237, p. 174-185, 2018.

NEGRO, R.; GRECO, G. *et al.* The Influence of Selenium Supplementation on Postpartum Thyroid Status in Pregnant Women with Thyroid Peroxidase Autoantibodies. **The Journal of Clinical Endocrinology & Metabolism**, Italy, v. 92, n. 4, p. 1263-1268, 2007.

NÉMETH, A.; DERNOVICS, M. Effective selenium detoxification in the seed proteins of a hyperaccumulator plant: the analysis of selenium-containing proteins of monkeypot nut (Lecythis minor) seeds. **JBIC Journal of Biological Inorganic Chemistry**, Hungary, v. 20, n. 1, p. 23-33, 2015.

NÉMETH, A.; REYES, J. F. G. *et al.* The relationship of selenium tolerance and speciation in Lecythidaceae species. **Metallomics**, Hungary, v. 5, n. 12, p. 1663-1673, 2013.

OIRAM FILHO, F.; ALCÂNTRA, D. B. *et al.* Development and validation of a reversed phase HPLC method for determination of anacardic acids in cashew (Anacardium occidentale) nut shell liquid. **Journal of chromatographic science**, Brazil, v. 56, n. 4, p. 300-306, 2018.

PEACHEY, E.; MCCARTHY, M. *et al.* Acceleration of enzymatic hydrolysis of protein-bound selenium by focused microwave energy. **Journal of Analytical Atomic Spectrometry**, United Kingdom, v. 23, n. 4, p. 487-492, 2008.

PETERSEN, K. E.; SHIRI, F. *et al.* Exosome isolation: Cyclical electrical field flow fractionation in low-ionic-strength fluids. **Analytical chemistry**, USA, v. 90, n. 21, p. 12783-12790, 2018.

PHAN-THIEN, K. Y.; WRIGHT, G. C. *et al.* Inductively coupled plasma-mass spectrometry (ICP-MS) and-optical emission spectroscopy (ICP–OES) for determination of essential minerals in closed acid digestates of peanuts (Arachis hypogaea L.). **Food chemistry**, [S.I.], v. 134, n. 1, p. 453-460, 2012.

PHONGTHAI, S.; LIM, S. T. *et al.* Optimization of microwave-assisted extraction of rice bran protein and its hydrolysates properties. **Journal of cereal science**, Korea, v. 70, p. 146-154, 2016.

PIECZYŃSKA, J.; GRAJETA, H. The role of selenium in human conception and pregnancy. **Journal of Trace Elements in Medicine and Biology**, Poland, v. 29, p. 31-38, 2015.

PINHO, G. P.; NEVES, A. A. *et al.* Efeito de matriz na quantificação de agrotóxicos por cromatografia gasosa. **Química Nova**, Bazil, v. 32, n. 4, p. 987-995, 2009.

PORNWILARD, M. M.; SIRIPINYANOND, A. Field-flow fractionation with inductively coupled plasma mass spectrometry: past, present, and future. **Journal of Analytical Atomic Spectrometry**, Thailand, v. 29, n. 10, p. 1739-1752, 2014.

PRATHAP, B.; DEY, A. *et al.* A review-importance of RP-HPLC in analytical method development. **International journal of novel trends in pharmaceutical sciences**, [S.I.], v. 3, n. 1, p. 15-23, 2013.

PYRZYNSKA, K.; SENTKOWSKA, A. Liquid chromatographic analysis of selenium species in plant materials. **TrAC Trends in Analytical Chemistry**, Poland, v. 111, p. 128-138, 2019.

_____. Selenium in plant foods: speciation analysis, bioavailability, and factors affecting composition. **Critical Reviews in Food Science and Nutrition**, p.1-13, 2020.

QAZI, I. H.; ANGEL, C. *et al.* Role of selenium and selenoproteins in male reproductive function: a review of past and present evidences. **Antioxidants**, China, v. 8, n. 8, 2019.

RASEKH, H. R.; DAVIS, M. D. *et al.* The effect of selenium on the central dopaminergic system: A microdialysis study. **Life Sciences**, USA, v. 61, n. 11, p. 1029-1035, 1997.

RATANATHANAWONGS WILLIAMS, S. K.; LEE, D. Field-flow fractionation of proteins, polysaccharides, synthetic polymers, and supramolecular assemblies. **Journal of separation science**, USA, v. 29, n. 12, p. 1720-1732, 2006.

RAYMAN, M. P. Selenium and human health. **The Lancet**, United Kingdom, v. 379, n. 9822, p. 1256-1268, 2012.

RIBANI, M.; BOTTOLI, C. B. G. *et al.* Validação em métodos cromatográficos e eletroforéticos. **Química nova**, Brazil, v. 27, n. 5, p. 771-780, 2004.

SANTHOSH KUMAR, B.; PRIYADARSINI K. I. Selenium nutrition: How important is it? **Biomedicine & Preventive Nutrition**, India, v. 4, n. 2, p. 333-341, 2014.

SCHIMPF, M. E.; CALDWELL, K. *et al.* Field-flow fractionation handbook: John Wiley & Sons. [S.I.], 2000

SCHMIDT, B.; LOESCHNER, K. *et al.* Quantitative Characterization of Gold Nanoparticles by Field-Flow Fractionation Coupled Online with Light Scattering Detection and Inductively Coupled Plasma Mass Spectrometry. **Analytical Chemistry**, Denmark, v. 83, n. 7, p. 2461-2468, 2011.

SCHOMBURG, L.; KÖHRLE, J. On the importance of selenium and iodine metabolism for thyroid hormone biosynthesis and human health. **Molecular Nutrition & Food Research**, Germany, v. 52, n. 11, p. 1235-1246, 2008.

SENTKOWSKA, A. Chromatographic Analysis of Selenium Species. In: (Ed.). **Selenium in the Environment and Human Health**: IntechOpen, 2019. Chromatographic Analysis of Selenium Species

SHEN, P.; WONG, S. P. *et al.* Sensitive and rapid method to quantify icaritin and desmethylicaritin in human serum using gas chromatography–mass spectrometry. **Journal of Chromatography B**, Republic of Singapore, v. 857, n. 1, p. 47-52, 2007.

SILVA, C. G. D.; COLLINS, C. H. Aplicações de cromatografia líquida de alta eficiência para o estudo de poluentes orgânicos emergentes. **Química Nova**, Brazil, v. 34, n. 4, p. 665-676, 2011.

SILVA, R. D. O.; DE MENEZES, M. G. *et al.* Efficiency of ESI and APCI ionization sources in LC-MS/MS systems for analysis of 22 pesticide residues in food matrix. **Food chemistry**, Bazil, v. 297, 2019.

SILVESTER, S.; SMITH, L. Profiling phospholipid elution in reversed-phase LC–MS/MS bioanalytical methods in order to avoid matrix effects. **Bioanalysis**, United Kingdom, v. 4, n. 8, p. 879-895, 2012.

SKALNY, A. V.; BURTSEVA, T. I. *et al.* Geographic variation of environmental, food, and human hair selenium content in an industrial region of Russia. **Environmental research**, Russia, v. 171, p. 293-301, 2019.

SONG, Y.; ZHANG, S. *et al.* Determination of genkwanin in rat plasma by liquid chromatography-tandem mass spectrometry: application to a bioavailability study. **Journal of pharmaceutical and biomedical analysis**, China, v. 84, p. 129-134, 2013.

SOUZA, A. C. G.; STANGHERLIN, E. C. *et al.* Diphenyl diselenide and diphenyl ditelluride: neurotoxic effect in brain of young rats, in vitro. **Molecular and Cellular Biochemistry**, Brazil, v. 340, n. 1, p. 179-185, 2010.

STAHNKE, H.; ALDER, L. Matrix effects in liquid chromatography-electrospray ionization-mass spectrometry. In: (Ed.). **Mass spectrometry for the analysis of pesticide residues and their metabolites**: Wiley Online Library, 2015. Matrix effects in liquid chromatography-electrospray ionization-mass spectrometry, p.161-186

STUSS, M.; MICHALSKA-KASICZAK, M. *et al.* The role of selenium in thyroid gland pathophysiology. **Endokrynologia Polska**, Poland, v. 68, n. 4, p. 440-465, 2017.

SUN, D.; XUE, A. *et al.* Simultaneous determination of acetylpuerarin and puerarin in rat plasma by liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study

following intravenous and oral administration. **Journal of Chromatography B**, China, v. 995, p. 24-30, 2015.

TADAYON, F.; MEHRANDOOST, S. Determination and speciation of Se (IV), Se (VI), and selenomethionine in iranian walnut samples by spectrophotometry after cloud point extraction. **Journal of Analytical Chemistry**, Iran, v. 70, n. 11, p. 1336-1341, 2015.

TAKASE, I.; PEREIRA, B. H. *et al.* A geração química de vapor em espectrometria atômica. **Química Nova**, Brazil v. 25, n. 6B, p. 1132-1144, 2002.

TARANTINO, T. B.; BARBOSA, I. S. *et al.* Microwave-assisted digestion using diluted nitric acid for multi-element determination in rice by ICP OES and ICP-MS. **Food analytical methods**, Brazil, v. 10, n. 4, p. 1007-1015, 2017.

TOŠIĆ, S. B.; MITIĆ S. S. *et al.* Elemental composition of edible nuts: fast optimization and validation procedure of an ICP-OES method. **Journal of the Science of Food and Agriculture**, Serbia, v. 95, n. 11, p. 2271-2278, 2015.

TÜGEL, I.; RUNYON, J. R. *et al.* Analysis of polysaccharide and proteinaceous macromolecules in beer using asymmetrical flow field-flow fractionation. **Journal of the Institute of Brewing**, Sweden, v. 121, n. 1, p. 44-48, 2015.

ULRICH, A.; LOSERT S. *et al.* Critical aspects of sample handling for direct nanoparticle analysis and analytical challenges using asymmetric field flow fractionation in a multi-detector approach. **Journal of Analytical Atomic Spectrometry**, Switzerland, v. 27, n. 7, p. 1120-1130, 2012.

VALENTINE, W. M.; ABEL, T. W. *et al.* Neurodegeneration in Mice Resulting From Loss of Functional Selenoprotein P or Its Receptor Apolipoprotein E Receptor 2. **Journal of Neuropathology & Experimental Neurology**, USA, v. 67, n. 1, p. 68-77, 2008.

VICENTE-ZURDO, D.; ROMERO-SÁNCHEZ, I. *et al.* Ability of selenium species to inhibit metal-induced Aβ aggregation involved in the development of Alzheimer's disease. **Analytical and Bioanalytical Chemistry**, Spain, p. 1-13, 2020.

VILLAGRASA, M.; GUILLAMÓN, M. *et al.* Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. **Journal of Chromatography A**, Spain, v. 1157, n. 1-2, p. 108-114, 2007.

VINCETI, M.; FILIPPINI, T. *et al.* Environmental selenium and human health: an update. **Current environmental health reports**, USA, v. 5, n. 4, p. 464-485, 2018.

VINCETI, M.; MANDRIOLI, J. *et al.* Selenium neurotoxicity in humans: Bridging laboratory and epidemiologic studies. **Toxicology Letters**, Italy, v. 230, n. 2, p. 295-303, 2014.

VONDERHEIDE, A. P.; WROBEL, K. *et al.* Characterization of selenium species in Brazil nuts by HPLC− ICP-MS and ES-MS. **Journal of Agricultural and Food Chemistry**, Ohio, v. 50, n. 20, p. 5722-5728, 2002.

WATANABE, L. M.; DE LIMA, L. F. *et al.* Association between creatine kinase activity, oxidative stress and selenoproteins mRNA expression changes after Brazil nut consumption of patients using statins. **Clinical Nutrition**. Brazil, 2020.

WATHUDURA, P. D.; PEIRIS, C. *et al.* Microwave and open vessel digestion methods for biochar. **Chemosphere**, Sri Lanka, v. 239, 2020.

WHANGER, P. D.; PEDERSEN, N. D. *et al*. Absorption of selenium and selenomehionine from digestive tract segmentes in rats. **Proc. Soc. Exp. Biol. Med.**, [S.I.], v. 153, p. 295-297, 1976.

WILSCHEFSKI, S. C.; BAXTER, M. R. Inductively coupled plasma mass spectrometry: introduction to analytical aspects. **The Clinical Biochemist Reviews**, Australia, v. 40, n. 3, 2019.

WIMUKTIWAN, P.; SHIOWATANA, J. *et al.* Investigation of silver nanoparticles and plasma protein association using flow field-flow fractionation coupled with inductively coupled plasma mass spectrometry (FlFFF-ICP-MS). **Journal of Analytical Atomic Spectrometry**, Thailand, v. 30, n. 1, p. 245-253, 2015.

WROBEL, K.; KANNAMKUMARATH, S. S. *et al.* Hydrolysis of proteins with methanesulfonic acid for improved HPLC-ICP-MS determination of seleno-methionine in yeast and nuts. **Analytical and Bioanalytical Chemistry**, [S.I.], v. 375, n. 1, p. 133-138, 2003.

XIA, L.; YANG, J. *et al.* Recent progress in fast sample preparation techniques. **Analytical Chemistry**, China, v. 92, n. 1, p. 34-48, 2019.

XIAO, R.; QIAO, J. T. *et al.* Sodium selenite induces apoptosis in cultured cortical neurons with special concomitant changes in expression of the apoptosis-related genes. **NeuroToxicology**, China, v. 27, n. 4, p. 478-484, 2006.

XIONG, G.; GAO, X. *et al.* Comparative study of extraction efficiency and composition of protein recovered from chicken liver by acid–alkaline treatment. **Process Biochemistry**, China, v. 51, n. 10, p. 1629-1635, 2016.

XUE, C.; HE, Z. *et al.* Effects of amides from pungent spices on the free and protein-bound heterocyclic amine profiles of roast beef patties by UPLC–MS/MS and multivariate statistical analysis. **Food Research International**, China, 2020.

YANG, L.; MESTER, Z. *et al.* Determination of Methionine and Selenomethionine in Yeast by Species-Specific Isotope Dilution GC/MS. **Analytical Chemistry**, Canada, v. 76, n. 17, p. 5149-5156, 2004.

YANG, L.; STURGEON, R. E. *et al.* Comparison of extraction methods for quantitation of methionine and selenomethionine in yeast by species specific isotope dilution gas

chromatography–mass spectrometry. **Journal of chromatography A**, Canada, v. 1055, n. 1-2, p. 177-184, 2004.

YI, Y.; REN, G. *et al.* Simultaneous determination of deuterated vortioxetine and its major metabolite in human plasma by UPLC-MS/MS and application to a pharmacokinetic study in healthy volunteers. **Journal of Chromatography B**, China, v. 1138, 2020.

ZAM, W.; ALSHAHNEH, M. *et al.* Methods of Spectroscopy for Selenium Determination: A Review. **Research Journal of Pharmacy and Technology**, Syrian Arab Republic, v. 12, n. 12, p. 6149-6152, 2019.

ZHANG, C.; HUANG, Y. *et al.* Selenium sources differ in their potential to alleviate the cadmium-induced testicular dysfunction. **Environmental Pollution**, China, v. 267, 2020.

ZHANG, H.; LYDEN D. Asymmetric-flow field-flow fractionation technology for exomere and small extracellular vesicle separation and characterization. **Nature Protocols**, USA, v. 14, n. 4, p. 1027-1053, 2019.

ZHANG, K.; ZHAO, Q. *et al.* Effect of different selenium sources on growth performance, tissue selenium content, meat quality, and selenoprotein gene expression in finishing pigs. **Biological trace element research**, China, v. 196, n. 2, p. 463-471, 2020.

ZHANG, S. Q.; ZHANG, B. H. *et al.* Quantification of selenomethionine in plasma using UPLC–MS/MS after the oral administration of selenium-enriched yeast to rats. **Food chemistry**, China, v. 241, p. 1-6, 2018.

ZHANG, X.; LI, X. *et al.* Selenium and cardiovascular disease: Epidemiological evidence of a possible U-shaped relationship. In: (Ed.). **Selenium**: Springer, 2018. Selenium and cardiovascular disease: Epidemiological evidence of a possible U-shaped relationship, p.303-316

ZHANG, X.; LI, Y. *et al.* Field-flow fractionation: A gentle separation and characterization technique in biomedicine. **TrAC Trends in Analytical Chemistry**, Republic of Korea, v. 108, p. 231-238, 2018.

ZHANG, Y.; LI, H. *et al.* Plasma selenium levels and risk of new-onset diabetes in hypertensive adults. **Journal of Trace Elements in Medicine and Biology**, China, v. 56, p. 6-12, 2019.

ZHAO, Y.; ZHENG, J. *et al.* Speciation analysis of selenium in rice samples by using capillary electrophoresis-inductively coupled plasma mass spectrometry. **Talanta**, China, v. 84, n. 3, p. 983-988, 2011.

ZHOU, Q.; WANG, H. *et al.* Effects of ultrasound irradiation on enzymatic hydrolysis of protein and application for the determination of tetracyclines in complex matrices. **Drug testing and analysis**, China, v. 9, n. 10, p. 1586-1593, 2017.

ZIELKE, C.; FUENTES, C. *et al.* Co-elution phenomena in polymer mixtures studied by asymmetric flow field-flow fractionation. **Journal of Chromatography A**, Sweden, v. 1532, p. 251-256, 2018.