STUDYING THE EXTRACTION OF MACRO AND MICRONUTRIENT ELEMENTS IN DRIED STEVIA PLANT LEAVES AND STEMS USING PRESSURISED HOT WATER EXTRACTION

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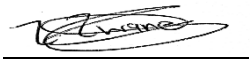
DECLARATION

I declare that this dissertation is my work, unaided work. It is being submitted for the Degree of Master of Science at the University of Witwatersrand, Johannesburg. It has not been submitted before for any university degree or examination.



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ABSTRACT

Stevia rebaudiana is economically significant because it is utilised as a natural sweetener. This study extracted the macronutrients and micronutrient elements from *Stevia rebaudiana* using the pressurised hot water extraction (PHWE) technique. The PHWE system consisted of deionised water heated at 81-100ºC and transported through the extraction cell inside an oven. The cell was filled with mixed amounts of dried *Stevia rebaudiana* powder (1.5 g) and diatomaceous earth. As part of the optimisation, the extraction was done at 50, 100, and 150 ºC at a constant flow rate of 1 mL/min. Microwave digestion was used to compare the quantity of extracted metals to total metal analysis, followed by ICP-OES analysis, in contrast to macronutrients. Calculating the extraction half-lives revealed that the extraction followed first-order reaction kinetics. This may be due to the metals being strongly linked to the ligand. The order of amounts extracted of the micronutrient elements was Fe > Zn > Al> Ni > Co > Cr with Fe being highest in amount extracted and Cr least. The extraction temperature influenced the order of extraction mentioned above. An increase influenced macronutrient elements in extraction time, and the extraction order was K > Ca > Mg. When the metals removed under optimal conditions were compared to the total metals in *Stevia rebaudiana* biomass for both leaves and stems, it was discovered that PHWE could extract more macronutrient elements than micronutrient elements. The average recovery for macronutrient elements was 1249 mg/kg ± 3, and the average recovery for micronutrient elements was 11.802 mg/kg ± 0.3. Micronutrients extracted at 50,100 and 150 ºC resulted in 2-200 mg/kg concentrations and macronutrients 100-15000 mg/kg. This suggests that *Stevia rebaudiana* extracts for both stem and leaves from PHWE can easily be used for value additions such as dietary supplements in other foodstuffs and beverages.

DEDICATION

I dedicate this dissertation to my late mother, Duduzile Cynthia Zwane.

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I want to thank my supervisors, Prof. Luke Chimuka and Dr Ida Risenga at the University of the Witwatersrand, for their assistance and guidance throughout my MSc. The Post Graduate students in the Environmental Analytical Chemistry research group and APES have contributed immensely to my personal and professional growth. Importantly, I appreciate my close friends and family's encouragement and contribution.

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List of Abbreviation

Al	Aluminium
As	Arsenic
A.S.E.	Accelerated solvent extraction
B.A.C.	Bioactive compounds
Cd	Cadmium
Ca	Calcium
CO_2	Carbon dioxide
Cr	Chromium
Co	Cobalt
Cu	Copper
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ICP-MS	Inductively Coupled plasma mass spectrometry
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
Fe	Iron
Mg	Magnesium
Mn	Manganese
M.A.E.	Microwave assisted extraction
Ni	Nickel
Pb	Lead

PHWE	Pressurised hot water extraction
P.L.E.	Pressurised Liquid Extraction
R.O.S.	Reactive oxygen species
Se	Selenium
Ag	Silver
S.F.E.	Supercritical fluid extraction
UHPLC	Ultra-high-performance liquid chromatography
U.S.A	United states of America
WHO	World Health Organization
Zn	Zinc

CHAPTER 1

1.0 Introduction

Natural herbs have been studied extensively for their therapeutic benefits, including *Stevia rebaudiana*. Food chain members may be at risk through metal contamination of food supplies (Hajar et al.,2014). High concentration of metals in food is a primary concern to environmentalists and health practitioners; they pose a threat to human natural health and environmental threat. Intracellular accumulations of metals result in the generation of oxygen radicals, e.g., reactive oxygen species (R.O.S.) (Manyi-Loh et al., 2018). Exposure to arsenic (As), mercury (Hg), etc., are linked to different health effects and diseases (Manyi-Loh et al., 2018). *Stevia rebaudiana* is a small bushy shrub known to be native to `Paraguay (Jarma-Orozco et al., 2012). Approximately 10% of its leaves contain steviosides, which are intensely sweet compounds that are said to be 150 to 300 times sweeter than commercial sugar (Samsudin and Aziz, 2013). The dry extracts from *Stevia rebaudiana* leaves contain flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycinnamic acids, oligosaccharides, free sugars, amino acids, lipids, and trace elements which have significant health benefits (Muanda et al., 2011).

Similar to basil, *Stevia rebaudiana* grows best in hot or warm climates. Plants grown in warm climates will grow 24 inches tall and wide, but cooler summers will experience less growth than hot summers. Plants need light to photosynthesise, but excessive light can harm them (Ceunen and Geuns, 2013; Osman et al., 2013). *Stevia rebaudiana* grows well in full sun, with at least 15 hours of light per day (Jarma-Orozco et al., 2012).

It contributes significantly to human welfare, is adaptable to various climatic conditions, and has a high sweet content (Pleasant, 2013). Perennial plants have unique regeneration capacity after frost damage and are easy to propagate by seeds, stem cuttings, and root division (Sing and Kaul, 2005). Due to its extensive root system and fragile stems, *Stevia rebaudiana* produces small oval leaves (Kamal-Alahmed, 2018). It has green leaves which grow in pairs. It has flower heads that are white sprouts and are soft and eventually become hard at maturity (Yadav et al., 2011). The seeds (figure 2.0) are small brownish-black in colour (achenes) and are scattered with the aid of pappus bristles such as that of dandelions (Yadav et al., 2011).

Stevia rebaudiana, in its natural habitat, thrives in sunny conditions, but several studies are ongoing to determine optimal conditions for accumulating steviol glycosides (Kumar et al., 2013). Among the eight terpene glycosides found in the leaves of this species are stevioside,

rebaudioside (A, B, C, D, and E), and dulcoside A and C (Salazar et al., 2018; Martins et al., 2016; Yadav et al., 2011). Stevioside and rebaudioside A are responsible for the sweetness of the leaves. Among the other glycosides, bitter stevioside has the most unpleasant taste. High levels of it limit *Stevia rebaudiana* consumption (Kovačević et al., 2018). *Stevia rebaudiana* molasses (industrial waste of stevia) has a good potential for the extraction of some steviol glycoside, a source of carbohydrates with zero calories (López-Carbón et al ,2019).

Three glucose molecules are attached to aglycones (the steviol moiety) to form stevioside. Using liquid chromatography, U.V., and mass spectrometry, sweet stevia glycosides are derived from steviol aglycones (Cacciola et al., 2011). Stevia glycosides have valuable biological properties that could lead to health benefits. Regular consumption of these compounds reduces sugar, radionuclides, and cholesterol in the blood, improves cellular regeneration and blood coagulation, suppresses neoplastic growth, and strengthens blood vessels (Konoshima and Takasaki, 2002).

Researchers have demonstrated that steviol glycoside has anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumour, antidiarrheal, diuretic, and immunomodulatory effects (Boonkaewwan et al., 2006). Studies have shown that stevioside is not carcinogenic, mutagenic, or teratogenic (Kamal-Alahmed, 2018). There are many uses for *Stevia rebaudiana*, including cancer treatment (Yasukawa et al., 2002), diabetes treatment (Lailerd et al., 2004), obesity treatment, cavity treatment, hypertension treatment (Dyrskog et al., 2005), fatigue treatment, depression treatment, and yeast infection treatment.

Due to the delicacy and medicinal properties of the leaves, the species has gained both scientific and commercial interest (Brandle and Telmer, 2007). Brazilians and Japanese use it as a non-caloric sweetener. Researchers studied whether *Stevia rebaudiana* is a better sweetener than commercially available xylitol (Mogra and Dashora, 2009). Pressurised hot water extraction (PHWE), used in sample preparation to remove organic pollutants and metal nutrients from food items for food safety analysis, is appropriate since stevia in foods and beverages requires safety monitoring. PHWE was first developed primarily to extract organic chemicals from soil and plant samples, but as time went on, its eco-friendliness helped it gain popularity (Teo et al., 2010). In addition to regulating essential variables like temperature, pressure, flow rate, and additives, it is a speedy procedure used in various extraction methods (Mohan et al., 2014).

As a result of higher temperatures and pressure, the material used in PHWE extraction should not corrode, and chrome-containing alloys as a material are recommended (Mohan et al., 2014).

Since the PHWE extraction method requires less energy, it is typically considered eco-friendly (Koubaa et al., 2015). Applying temperatures between 100 and 374°C and increased pressures keeps the water in a liquid condition and may be used again in the extraction process without evaporating. Previous research has utilised this extraction method for the environmentally friendly recovery of bioactive chemicals and steviol glycosides (Kovaevi et al., 2018).

Over the last few decades, the amount of heavy metal pollution in the environment has significantly grown (Tchounwou et al., 2014). In urban or rural settings, anthropogenic activities like mining discharge metals into the environment through the soil (Aremu et al., 2010). Due to the weathering of rocks and soils, metals find their way into aquatic systems. Ozone depletion is also a result of volcanic eruptions and human activities that use, refine, and mine metals (Aremu et al., 2010). Because the *Stevia rebaudiana* plant is susceptible to contamination during its growth, development, and processing, a substantial study must examine how metals accumulate in Stevia (Sharma et al., 2006). During growth, soils and water can directly contaminate the *Stevia rebaudiana* plant with heavy metals (Hajar et al., 2014). Metals can be categorised as either necessary or not. Copper (Cu), manganese (Mn), selenium (Se), and zinc (Zn) are micronutrients that are crucial for the activity of enzymes as well as for the survival of living things (Yannick et al., 2018).

Micronutrients are essential nutrients living organisms require in varying amounts (Islam et al., 2015). Micronutrients have varying daily intake limits based on the type of nutrient (Cho-Ruk et al., 2006). The human body cannot produce vitamins or minerals, so micronutrients must be obtained from food (Mooradian et al.,2017). That is why they are also known as essential nutrients. Vitamins are organic compounds from plants (Mooradian et al.,2017). Plants that lack these micronutrient elements struggle to grow and exhibit deficiency symptoms (Islam et al., 2015), and higher-level chlorosis may occur (Havlin, 2002). The heavy metals in stevia herb and their toxicity are poorly documented (Sharma et al., 2006).

The leaves and stems of *Stevia rebaudiana* found elements such as Al, Cd, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ag, Co, Ca, Mn and Ni in research conducted by "Hajar et al. (2014). The above study differs through the extraction process and the type of instrument used. PHWE was used for the primary extraction process in this study, an effective green process. ICP-OES was also used as the instrument of analysis. This work aimed at studying the composition of macro and micronutrient elements in dried stevia plant leaves and stems using pressurised hot water extraction. This study investigates the extraction of macro and micronutrient elements from

dried stevia leaves and stems using PHWE. This method involves using water at high temperatures and pressures to extract the desired compounds, resulting in a more efficient extraction process than traditional methods. PHWE also has the advantage of being environmentally friendly, as it uses only water as a solvent and can be easily scaled up for industrial applications.

The findings of this study could have significant implications for the development of new products containing stevia and for the nutritional value of these products. By identifying the optimal conditions for PHWE, it may be possible to increase the yield of macro and micronutrient elements from stevia, making it a more valuable and sustainable source of these essential nutrients. This study has the potential to contribute to the growing body of research on the extraction of bioactive compounds from plant materials using green technologies.

CHAPTER 2

2 Literature review

2.0 Background

Moises Santiago Bertoni categorised and characterised *Stevia rebaudiana* botanically in 1899 (Kamal-Alamad, 2018). Known initially as *Eupatorium rebaudianum,* it was renamed *S. rebaudiana* Bertoni in 1905. *(Kamal-Alamad, 2018). Stevia rebaudiana* is a plant native to Paraguay (South America) with natural sweetener molecules (Jarma-Orozco et al., 2020) known as steviol glycosides.

Stevia rebaudiana is a sweet herb containing natural non-caloric sweeteners (Cardello et al., 1999). Its versatility and tremendous impact on human lives give it immense worth (Pleasant, 2013). It is a perennial plant with a remarkable ability to regenerate after frost damage, and it may be easily propagated from seed, stem cuttings, or root division (Sing and Kaul, 2005). *Stevia* has a deep taproot and brittle stems that bear tiny, elliptical leaves (Shock, 1982). *Stevia rebaudiana* has white, tender-at-first, but subsequently firm flower buds (Shock, 1982). The root system is superficial with few lateral roots; the seeds are small brownish-black in colour (achenes) and are distributed with the aid of pappus bristles such as dandelions, as shown in figure 2.1.

Macro and micronutrient elements present in *Stevia rebaudiana* have health benefits. Thus, its consumption rapidly increases with time. Macronutrients are essential nutrients needed in more

significant amounts for vital functions, while the micronutrients elements are needed in smaller amounts in the human body.

The function of tissues is maintained by micronutrients, which play a vital role in metabolism. It is, therefore, essential to consume good supplements, but excessive supplementation may be harmful (Shenkin, 2006).

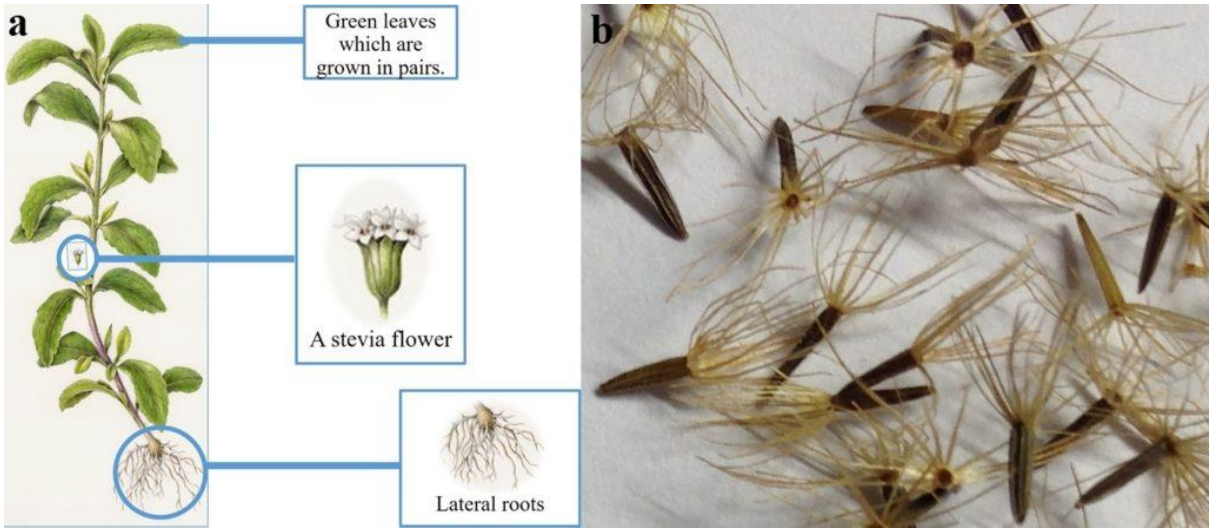


Figure 2.1 Morphological structure of Stevia rebaudiana (a) and seeds (b)

Source: Goettemoeller and Ching (1999).

Prior research compared the effects of three dried *Stevia rebaudiana* leaf extracts made with water (A extract), 96% ethanol (E extract), and a 4:1 combination of glycol and water. *Stevia rebaudiana* leaves' micronutrients and macronutrients were investigated in the study (Beben et al., 2015). According to the research, *stevia* extracts using various solvents contain a sizable quantity of physiologically active phytochemicals (Beben et al., 2015). However, this research extracts macro and micronutrients from *Stevia rebaudiana* leaves and stems using the PHWE technique and water as the solvent.

2.1 *Stevia rebaudiana* uses economic importance

Due to its significance as a source of non-caloric sweeteners and steviol glycosides with the potential in the food sector to reduce sugar consumption, *Stevia rebaudiana* Bertoni leaves are gaining more and more attention (Wölwer-Rieck, 2012). According to studies, consuming certain species may improve carbohydrate metabolism by lowering blood sugar levels in diabetic and non-diabetic consumers (Lemus-Mondaca et al., 2012). Consuming *Stevia*

rebaudiana may lower heart rate since it benefits managing hypertension and hyperglycemia (Jay et al., 2020). Additionally, it can be taken as a herbal supplement to help cure illnesses including cancer, diabetes, obesity, cavities, and hypertension (Yasukana et al., 2002; Lailerd et al., 2004). *Stevia rebaudiana* enhances urinal function in addition to having hypoglycemic, hypotensive, vasodilating (Dyrskog et al., 2005), taste-improving, sweetening, anti-fungal, anti-viral, anti-inflammatory, and anti-bacterial effects (Parson, 2001). It is trustworthy for treating diabetes since it is non-toxic and does not impact blood sugar levels (Alan, 2002).

It has been proven in various studies and documents that *stevia* has natural constituents that are beneficial to human health (Jay et al., 2020). Table 2.1 highlights the uses of *stevia* in different countries (Taylor, 2005). *Stevia rebaudiana* has also been found to have antimicrobial activity (de Cock et al., 2016). The bacterial diseases can be reduced by adding *stevia* in mouthwash and toothpaste, resulting in decreased gum and tooth decay (de Cock et al., 2016). Mild *stevia* leaf tea induces relief for an upset stomach. A wet *Stevia* leaf bag provides a cooling effect on the eyes like a cucumber. The leaves tighten the skin and can be an anti-ageing agent (Kamal-Alahmed, 2018).

Table 2.1 Medicinal uses of stevia in different countries

Source: Taylor (2005)

Country	Medicinal uses
Brazil	It is typically used for cavities, depression, diabetes, fatigue, heart support, hypertension, hyperglycemia, infections, obesity, sweet cravings, tonic, urinary insufficiency, and wounds.
Paraguay	Diabetes
South America	Diabetes, hypertension, infections, obesity
United States	Candida, diabetes, hypertension, hyperglycemia, infections and as a vasodilator.

Because of the rise in people with diabetes and health-conscious consumers, *Stevia rebaudiana* has emerged as a promising and renewable raw ingredient in the food industry (Savita et al.,2004). The demand for *stevia* leaves, a natural sugar substitute with no caloric impact, has steadily risen in foreign markets (Marketline,2014). Growing *stevia* requires relatively little

water, which is a significant perk at a time when fresh water is becoming increasingly rare. It requires around 5 per cent as much water as sugar cane (Marketline, 2014). Farmers' interest in growing stevia has increased in recent years. This is because the crop can only be propagated by expensive means, such as stem cutting or tissue culture; the payoff can occur over 2-3 years after planting (Singh et al., 2005).

The value of the *Stevia rebaudiana* market was estimated to be worth $410m in 2016, and considering the current rate of growth, the market has risen steadily (Marketline, 2014). Although *Stevia rebaudiana* is a plant native to South America, its growth in many areas of the world is universal; for centuries, its gained popularity due to its sweet taste (Shah, 2019). Products with *Stevia rebaudiana* are a healthy alternative to sugary drinks. Toxicological studies based on stevioside suggested no mutagenic effect or toxicity. Steviol, an aglycones glycoside that is thermostatic, pH-stable, and non-fermented, is included in this stevioside (Geuns 2003), making it simple to employ in cooked food and beverages without sacrificing quality (Abdullateef and Osman 2011).

Steviol glycosides in *Stevia rebaudiana* are considered of great medicinal and nutraceutical importance worldwide (Gutiérrez et al.,2020). However, Rebaudioside A is the essential glucoside with higher economic value and preferable organoleptic and physicochemical characteristics (Hearn and Subedi, 2009; Palmer et al., 2013). These steviol glycoside compounds in extracts or purified form have economic importance in the food and pharmaceutical industries (Álvarez-Robles et al., 2016; Angelina et al., 2016; Marcinek and Krejpcio, 2017). Steviol glycosides and steviol belong to diterpene and possess a structure similar to that of steroids (Figure 2.2).

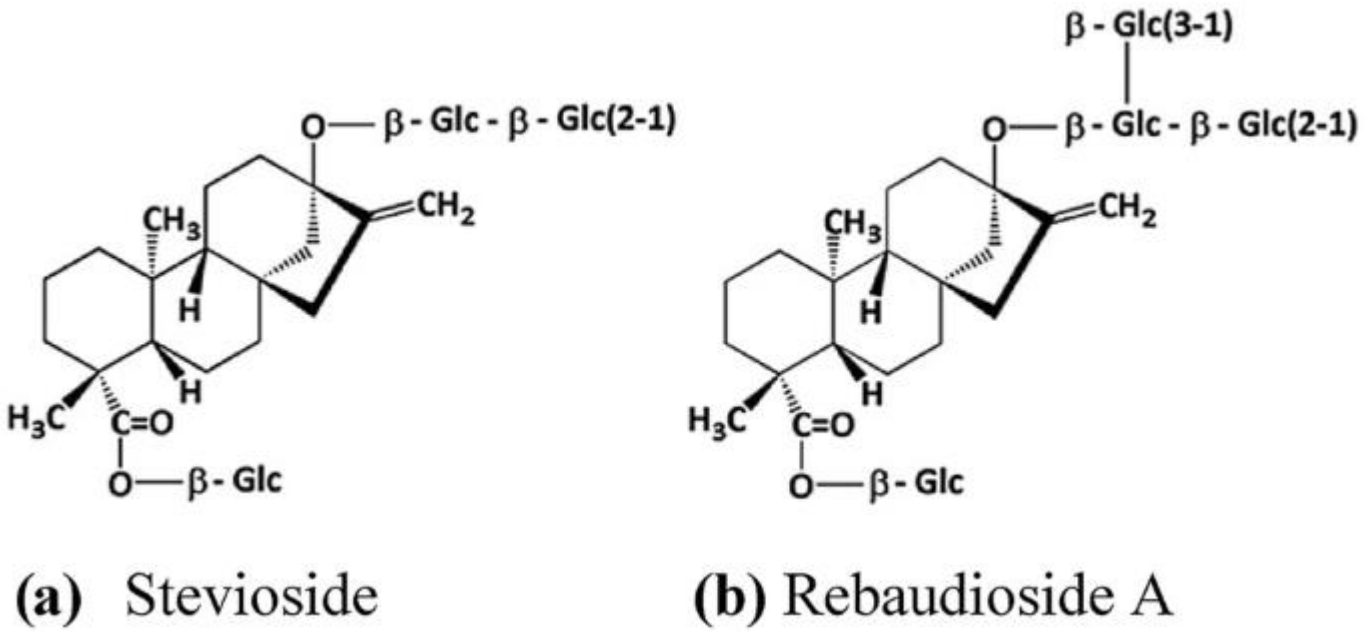


Figure 2.2 Chemical structure of stevioside (a), and rebaudioside A(b)

Source: Panagiotou et al., (2018)

2.1.1 Nutrients and chemical composition of *Stevia rebaudiana*

Stevia rebaudiana contains protein, calcium, phosphorous, and other nutrients (Snehal and Madhukar, 2012). Studies indicate that extracts from dried leaves contain 10% amino acids, 18% proteins, 33% carbohydrates, and 39% reducing sugars, while in the fresh leaves extract, the levels are 25%, 19%, 31%, and 25%, respectively (Snehal and Madhukar, 2012). Sterols, triterpenes, flavonoids, tannins, and essential oil with rich proportions of aromatics, aldehyde, monoterpenes, and sesquiterpenes have been reported (Siddique et al., 2016).

The nutrient distribution of *stevia* leaves is presented in Table 2:2.

Anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumour, antidiarrheal, diuretic, and immunomodulatory qualities are a few valuable features in *Stevia rebaudiana* extracts (Ferrazzano et al., 2016).

Table 2. 2 Nutrient distribution of stevia leaves depending on the applied drying method

Source: Gasmalla et al., (2014)

Component	%	sun-dried	microwave-dried
Protein	10.73	7.46	4.45
Fats	6.13	4.39	4.18
Ash	12.06	8.06	4.65
Carbohydrates	63.10	69.85	73.99
Dietary Fibre	5.03	5.76	4.35
Reducing sugars	4.5	4.8	5.30

Flavonoids, alkaloids, chlorophylls, oligosaccharides, free sugars, amino acids, lipids, and trace elements are all found in the stevia leaf extract that has been dried (Komissarenko et al., 1994). The biochemical composition of *stevia* has been compared to different medicinal plants, as seen in Table 2.3.

Low-calorie, high-potency sweeteners made from *Stevia rebaudiana* leaves are available in several forms. Based on these findings, *stevia* seems likely to be employed in food and non-food goods, including cosmetics and medicines, due to its many beneficial characteristics. According to data in Table 2.3, compared to the plants listed, *stevia* much outperforms them. Carbs, proteins, crude fibre, fat, and ash are more concentrated.

Table 2. 3 Stevia rebaudiana functional food in comparison to different aromatic and medicinal plants (mg/ 100 g) of dried weight

Source: Khiraoui et al., (2017)

	Stevia	Rooibos tea	Olive leaves	Oregano	Stinging nettle
Carbohydrates	51.50-56.72	25	27.5	1.6	ND
Proteins	11.75-16.23	15	5.4	0.2	6.5
Crude Fibre	17.43-19.13	ND	7	0.8	5.3
Fat	3.86-5.78	2-3	6.5	0.18	1.6
Ash	7.37-11.28	5	3.6	ND	5.6

2.1.3 Heavy metals composition in *Stevia rebaudiana*

It is easy to contaminate *Stevia rebaudiana* plants during their growth, development, and processing. Extensive research is required to explore *stevia*'s types of heavy metals (Hajar et al., 2014). "Heavy metals" typically refer to metals with a specific density greater than 5 g/cm3. (2014) Jaishankar et al. are a significant source of metals discharged into the environment by various human activities, such as pollution (Aremu et al., 2010). Plant development may be inhibited by metal absorption in excessively contaminated soil. However, certain plant species have collected significant levels of heavy metals without indicating stress, which might be dangerous for people and animals (Oliver, 2007).

The extraction reported by "Hajar et al. 2014" presented a variety of elements such as: As Co, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ca, Mn and Ni. The stevia plant, including its leaves, stems, and flowers, has shown remarkable resistance to heavy metals at concentrations much below the maximum allowable for plants. This means it has been used in traditional medicine and the food industry. Table 2:5 shows some reported presence of metals in *stevia.*

Table 2. 4 The reported presence of some metals in Stevia rebaudiana (leaves, stems, and flowers) by using ICP-MS

Source: Hajar et al., (2014)

Heavy metals	Leaves (mg/kg^{-1})	Stems (mg/kg^{-1})
Arsenic (As)	0.05316	0.438
Cadmium (Cd)	0.3515	0.361
Chromium (Cr)	5.0103	3.87
Copper (Cu)	6.9411	8.11
Iron (Fe)	623	472
Magnesium (Mg)	189.56	1760
Lead (Pb)	0.5233	0.513
Selenium (Se)	0.00492	0.04536

Zinc (Zn)	44.4235	27.9
Aluminium (Al)	58.887	108.84
Silver (Ag)	0.0676	0.0317
Cobalt (Co)	0.0842	0.0611
Calcium (Ca)	259.44	558
Manganese (Mn)	6.0358	6.336
Nickel (Ni)	1.6737	1.53

Micronutrients like chromium, manganese, iron, cobalt, copper, zinc, selenium, molybdenum, and iodine are found in *Stevia rebaudiana*, along with the macronutrients like sodium, magnesium, phosphorus, sulphur, chlorine, potassium, and calcium (Szefer and Nriagu, 2007; Adotey et al., 2009). As a result, *stevia* is a substance that protects, regulates, and maintains the body's metabolism because of its high mineral content. According to research by Choudhary and Bandyopadhyay (1999), *stevia* leaves have high nutritional value.

Iron, zinc, and manganese help the immune system but also protect against illnesses caused by free radicals (Jimoh and Oladiji, 2005). Haemoglobin production is iron-dependent. Again, *stevia* leaves' increased iron content aids the body in keeping its haemoglobin levels stable. Anaemia, caused by a lack of iron in the body, can be treated using sugary concoctions made from *stevia* leaves. Anaemia is one of the numerous nutritional problems plaguing people in third-world nations. Concentrations of heavy metals in *Stevia rebaudiana* leaves were found to be within the typical range in prior research (Abou-Arab et al., 2010). The ramifications of the results (Abou-Arab et al., 2010) may be considered.

2.2 Modern Green Extraction Techniques

2.1.2 Natural and artificial sweeteners

Natural decomposable sweeteners have fewer side effects and symptoms, such as headaches, depression, and seizures. (Kroye, 2010). Sugar alcohols make up of natural sweeteners, including sorbitol, xylitol, lactitol, mannitol, erythritol, trehalose, and maltitol. Rare sugars found in nature have lately gained popularity as a new type of sweetener (Mooradian et al., 2017). They lead to increased use of natural sweeteners (Mooradian et al., 2017). Naturally

occurring sugars are preferable to artificial sugars due to their non-caloric status (Khiraoui et al., 2017).

In addition to its use as a natural sweetener, *stevia* possesses antimicrobial properties that work in two ways to prevent the spread of bacteria and other pathogens. For one, it may help reduce the frequency of colds and cases of flu, and for another, it has inspired a variety of new types of mouthwash and toothpaste (Bhutia and Sharangi, 2016).

Potential health risks associated with sugar substitutes include glucose intolerance and ineffective weight loss (Pang et al., 2021). According to animal studies, artificial sweeteners may be addictive to animals (Li et al., 2018). According to research, people who use artificial sweeteners habitually tend to gain weight (Pang et al., 2021). Table 2:4 compares non-natural sweeteners commonly used in the U.S.A. (Mooradian et al., 2017). Wastewater treatment plants collect all artificial sweeteners used by companies and homes, which may not be limited to the food sector (Kroye, 2010).

In the current water treatment system, molecules like sucralose and aspartame cannot be removed, adversely affecting ecosystems (Li et al., 2018). Decreases in the brain's antioxidant system might harm people (Abhilash et al., 2012). To combat the degradation of natural sweeteners in the environment, it is possible to use artificial alternatives.

Table 2. 5 Comparison of high-intensity non-natural sweeteners available in the U.S.

Source: Mooradian et al., (2017).

Table 2. 4 compares the intensity of sweetness of non natural sweeteners that are available in the united states to the sweetness of sucrose. The above indicates that the non natural sweetners may be harmful as they are too sweet compared to natural sweetners such as sucrose.

Sweetener	Regulatory status	sweetness compared to sucrose
Ace-k	Approved as a sweetener and flavour enhancer in foods (except in meat and poultry)	200x
Advantame	Approved as a sweetener and flavour enhancer in foods (except in meat and poultry)	20,000x
Aspartame	Approved as a sweetener and flavour enhancer in foods generally	200x
Neotame	Approved as a sweetener and flavour enhancer in foods (except in meat and poultry)	7000-13,000x
Saccharin	Approved as a sweetener only in certain special dietary foods	200-700x
Sucralose	Approved as a sweetener in foods generally	600x

The significance of naturally sweetened protein drinks with low carbohydrate contents for consumers has also been shown by prior research (Gerdes, 2012; Jacobson, 2015; Oltman et al., 2015). As a result of the aforementioned, protein drinks can now be sweetened with non-nutritive sweeteners in place of sugar to keep their sweetness. Plants are the source of natural non-nutritive sweeteners, often sweet glycosides (Kim and Kinghorn, 2002).

Regardless of the study, *Stevia rebaudiana* is now considered a sweetener, including the above nutrients (Siddique et al., 2016). *Stevia rebaudiana* is known to be at least 50 - 100 times sweeter than sucrose (Siddique et al., 2016). Synthetic sweeteners, like saccharin, have been linked to a possible increase in cancer risk. However, stevia has many components (beyond the steviosides and rebaudioside). As reported by Gasmella et al. (2014), steviol glycosides, one of the most critical components of *Stevia rebaudiana*, are responsible for the plant's sweetening properties and hence aid in the creation of low-calorie meals. The most significant rebaudioside is rebaudioside A, which may be found in the leaves of *Stevia rebaudiana* at a concentration of 2-4% dry matter. In contrast to steviosides, it has the most extended shelf life of all

glycosides and no harsh aftertaste (Marcinek and Krepjcio, 2017). Their chemical makeup might vary depending on how the stevia leaves are prepared (Marcinek and Krepjcio, 2017).

2.2.1 Supercritical fluid extraction (S.F.E.*)*

"Supercritical fluid extraction" (S.F.E.) refers to an extraction method in which supercritical fluids achieve the desired component separation. CO_2 is commonly used as a vaporisation liquid because of its speed and convenience (Marcus,2019). By adjusting the conditions of heat, pressure, and co-solvent, the parameters of this method may be readily adjusted. Selective extraction of valuable components from natural materials, or elimination of undesirable ones, is what supercritical fluid extraction is all about (Marcus, 2019). There are two main applications for supercritical fluid extraction: (1) eliminating undesired chemicals, including organic pollutants, toxins, and pesticides, and (2) extracting essential bioactive components like flavours, colourants, and other biomolecules (Pereira & Meireles, 2010). The S.F.E. is a suitable method for extracting both low- and high-polar chemical molecules (Manjare and Dhingra, 2019). Instead of the S.F.E. method, liquid extraction is required to remove metal ions (Manjare and Dhingra, 2019).

Rarely are *stevia* leaves treated with S.F.E. By creating a two-step procedure that reduces CO2-soluble substances such as alcohols, aliphatic hydrocarbons, sesquiterpenes, and many others, followed by S.F.E. in a polar co-solvent, the bitter components of *stevia* leaves may be partially eliminated (Pasquel et al., 2000). Higher glycoside yields were found when water was utilised as a co-solvent in S.F.E. in plant species by Pasquel et al. (Yoda et al., 2003).

Since it offers an alternative to established procedures like organic solvent extraction and steam distillation, extracting essential oil components using S.F.E. has drawn considerable attention, particularly in the food, pharmaceutical, and cosmetic sectors (Pourmortazavi and Hajimirsadeghi, 2007). Traditional organic solvent extraction has been used extensively to create natural product extracts, but it has drawbacks such as limited selectivity, high energy costs, and the potential loss of volatile chemicals during the solvent removal process. Supercritical fluids are therefore preferred for extracting flavours from natural materials (Malaman et al., 2011).

As shown in figure 2.3, S.F.E. can be considered eco-friendly and has many benefits over traditional liquid solvent extractions, such as speed, selectivity, cleanliness, minimal solvent volumes needed, and the ability to control the extract's chemical profile via selective precipitation of compound classes (Capuzzi et al., 2013).

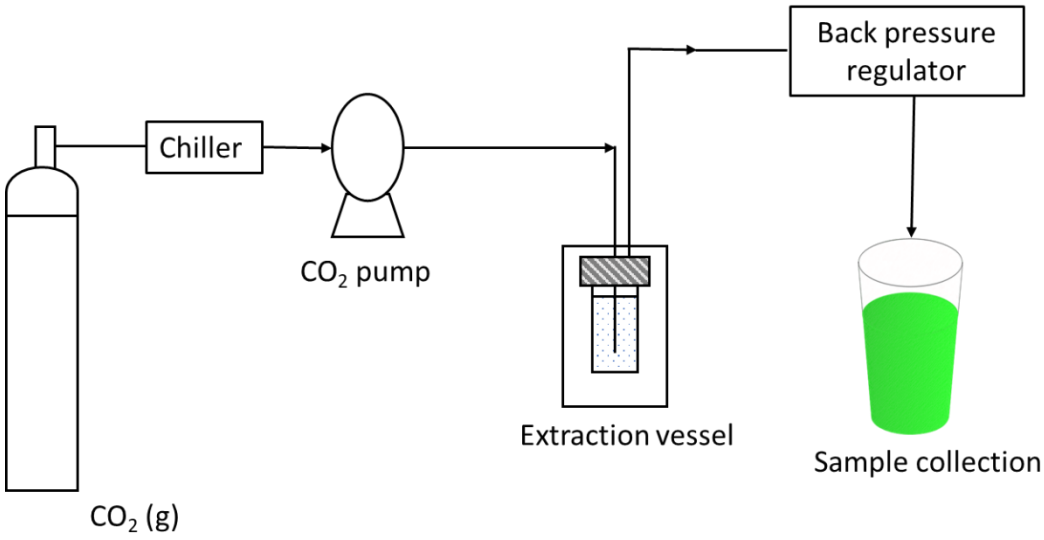


Figure 2.3 Supercritical fluid extraction

Source: Wei et al., (2005).

2.2.2 Pressurised liquid extraction (P.L.E.)

P.L.E., or A.S.E., is an automated technique for extracting organic compounds from solid or semi-solid samples using organic solvents at elevated temperatures and pressures. Its advantages include using small amounts of reagents, high efficiencies, high percentage recoveries, and good reproducibility (Wang et al., 2020). P.L.E. has been widely used to extract drug residues in various matrices. It is also effective for extracting veterinary drug residues in animal tissues and pesticide residues in vegetables and fruits (Tanaka et al., 2007).

Organophosphate esters, typically present in the soil, have been extracted using P.L.E. (Tanaka et al., 2007). Many studies have shown that OPEs can bioaccumulate because of their toxicity (Luo et al., 2018). As shown in Figure 2.4 (Luo et al., 2018), the extract is purified as it exits the cell and runs through the purification material. The extraction yield of steviol glycosides from *stevia* leaves was shown to be strongly affected by temperature, static time, and several cycles; however, there were no interactions between these independent factors, as shown by response surface analysis. Thus, the conventional A.S.E. approach was rapid, efficient, and repeatable and may be utilised for screening S. rebaudiana genotypes. It is quicker, greener, more convenient, and uses less energy than traditional and alternative extraction techniques (Jentzer et al., 2015).

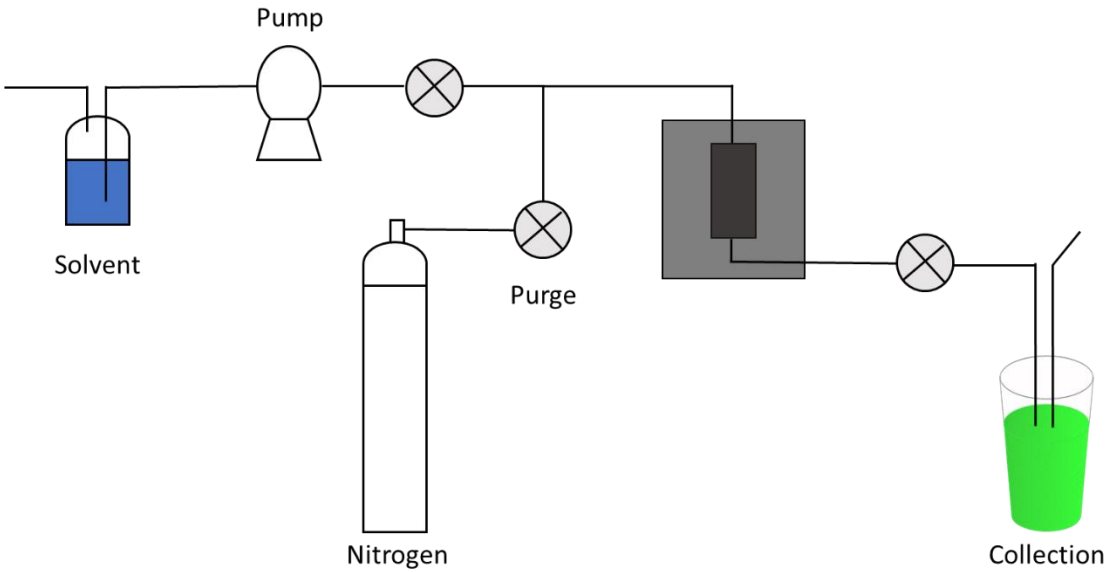


Figure 2.4 Pressurised liquid extraction (Soria et al.,2012)

The A.S.E. approach can improve sample throughput in *stevia* germplasm assessment screening processes. The response surface approach investigates the effects of A.S.E.'s many variables on extraction yield, temperature, static duration, and total cycles (R.S.M.). By taking the steps mentioned above, we may reduce the number of trials while simultaneously predicting, via a mathematical model, the impact of the relevant elements on the response of interest (Bharathi et al., 2011).

2.2.3 Microwave-assisted extraction (M.A.E.*)*

In the 1990s, membrane-assisted extraction (M.A.E.) technology was widely utilised to remove organic residues from solid materials. During M.A.E., solvents are heated in a microwave to separate analytes from the rest of the sample. High temperatures and a suitable solvent are crucial to the success of this method (Lopez-Avila,2003). A diagram of the M.A.E. plan is presented in Figure 2.5. A molecule's dielectric constant determines how well microwaves may enter; a more significant dielectric constant results in more excellent microwave absorption. Sample extraction with M.A.E. can be done in an open or closed container. The choice depends heavily on the solvent's value; if it is high, a closed vessel will be needed, and the solvent will be heated much past its boiling point.

Extraction of sample components indicated by higher will occur in the surrounding cold solvent if the solvent has a low value and the open-vessel mode is used (Lehotay and Schenck, 2003). Simply put, the faster the system achieves the extraction temperature, the greater the dielectric constant and the more energy the molecules absorb. While M.A.E. offers a higher sample

throughput than Soxhlet and ultrasound-assisted extraction, it is limited to thermally stable chemicals due to the elevated temperature required for extraction (de la Guardi and Armenta,2011).

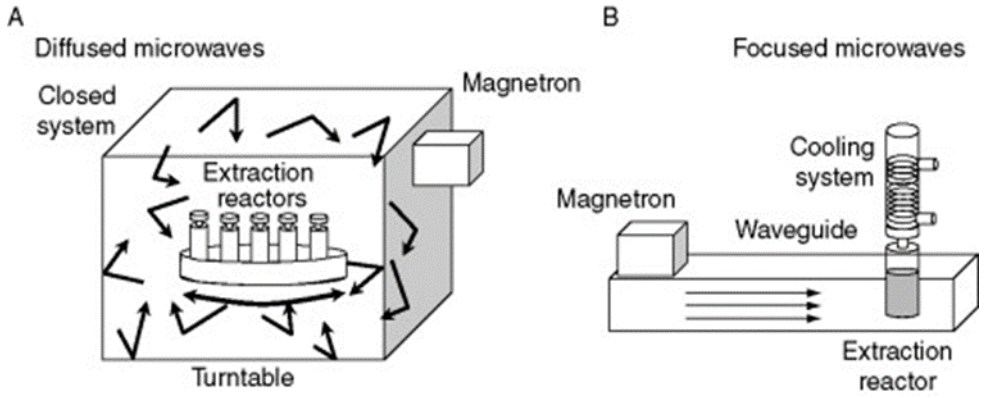


Figure 2.5 Scheme of Microwave Assisted Extraction

Source: Lopez-Avila (2003).

Instead of using organic solvents, acids extract metals from solid solids using the same technique. The extraction process is then called microwave digestion. M.A.E. allows for extracting active compounds from medicinal plants and herbal supplements such as *Stevia rebaudiana*. It is advantageous because it uses microwaves to disrupt cell membranes and releases intracellular lipids into the organic solvent (Kumar et al., 2021). Natural components such as flavonoids, glycosides, polysaccharides, terpenoids, and essence have also been extracted using the method mentioned above (Fu and Feng, 2003).

2.2.4 Pressurised hot water extraction (PHWE)

PHWE is a practical green extraction technique using pressurised water at a high temperature and regulated pressure. Temperature, extraction time, flow rates, and the addition of modifiers are the primary factors to consider such as in static PHWE, convection is accomplished using a stirrer in order to speed up mass transfer (Teo et al., 2010). When compared to the standard industrial extraction procedures, it is recognised to be a comparatively quick process. The main advantage of the above process is the reduced solvent consumption and extraction time (Ameer et al., 2017). PHWE has proven to be a better-suited method to isolate components from plants. It has numerous advantages compared to the previously mentioned extraction approaches (Plaza et al., 2013). The solvent is kept in a liquid state due to the elevated temperatures and pressures, enhancing the extraction by better mass transfer (figure 2.6). PHWE provides

advantages over conventional extraction methods, such as being “greener”, faster and more efficient.

The recovery of Bioactive compounds (B.A.C.) is conducted with a "5-Stages Universal Recovery Process": (i) macroscopic pretreatment, (ii) macro and micro-molecular separation, (iii) extraction, (iv) purification, and (v) product formation (Galanakis, 2012). Extraction is essential due to the retrieval of target components and bioresources; therefore, PHWE is also helpful in recovering nutritional components in plants and maybe a suitable technique for scale-up to industrial applications (Deng et al., 2015).

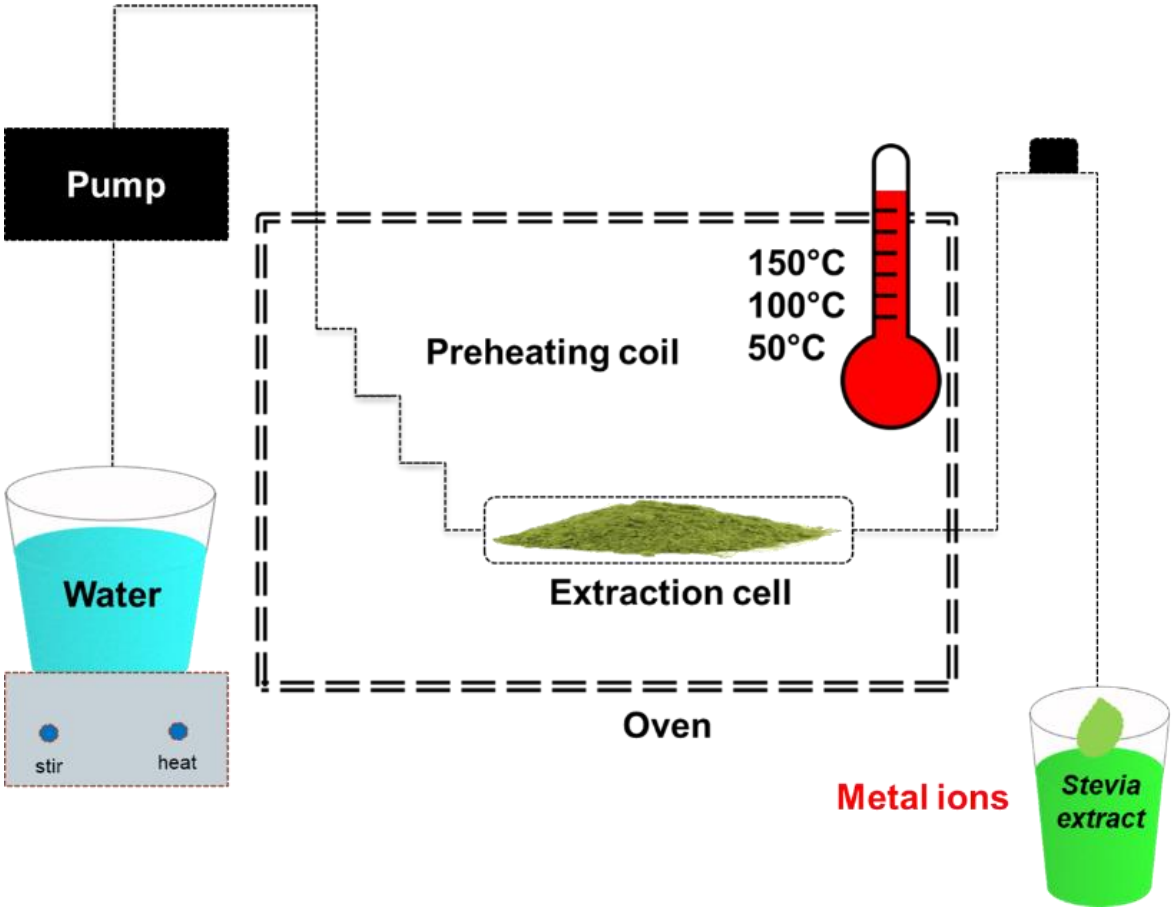


Figure 2.6 Schematic diagram of PHWE

Source: Smith (2002)

At the high temperatures and pressures required for PHWE, water takes on the properties of an organic solvent, becoming less polar and exhibiting a dielectric constant similar to that of ethanol and methanol (Smith, 2002). Due to harmful solvent residues, several extraction methods have fallen out of favour. PHWE has been used to extract phenolic chemicals from plants in the food and pharmaceutical sectors using PHWE (Fernadez-Perez et al., 2001).

Optimising the temperature, flow rate, and extraction time is crucial for a successful extraction recovery. The solvent loses some of its viscosity by increasing the temperature, allowing it to diffuse more easily into the matrix and facilitate extraction (Leyva-Jiménez et al., 2018). Because water is a solvent, items made using PHWE pose no threat to human health.

For food safety analysis and environmental monitoring, PHWE is also employed in sample preparation to remove organic pollutants from soils/sediments and food (Teo et al.,2010). Using this method, one may acquire *Stevia rebaudiana* extract quickly and flexibly. Steviol glycosides, followed by total phenolics, were the primary factors in classifying stevia extracts from Bertoni leaves (Gawe-Bben et al., 2015).

2.3 Chemical analysis in *Stevia Rebaudiana*

2.3.1 Liquid chromatography with mass spectrometry

Liquid chromatography is partition chromatography that uses analysis of non-volatile or thermally labile compounds that cannot be analysed by gas chromatography (Synder, 1992). It depends on the solutes' interaction between two immiscible liquid phases prepared by two or more kinds of solvents (Tong, 2020). In liquid chromatography, the mobile phase is a liquid; the stationary phase is a fine powder. This can be alumina or silica, referred to as the normal phase, or alumina or silica capped with octadecylsilane (ODS), known as the reverse phase. Reverse phase columns are usually non-polar and consist of a polar mobile phase made from water and water-soluble solvents such as methanol and acetonitrile (Provini and Galassi, 1993). The above technique has gained popularity for the quality control of plants and the characterisation of secondary metabolites in plants such as *Stevia rebaudiana* (Thirumal and Laavu, 2017).

The technique involves physically separating a sample with liquid chromatography and identifying charged components (ions) under a magnetic field, as seen in Figure 2.7 (Parasuraman et al., 2015). The Liquid chromatograph does sample separation, and the separated species are then sprayed into the mass spectrometer for ionisation and detection (Parasuraman et al., 2015). In a mass analyser and detector, ions are sorted by mass-to-charge ratio and counted (Stromberg and Mukundan, 2020). The detector may amplify the signal from each ion, giving rise to a mass spectrum. The mass of individual particles and molecules and inferences about their chemical structures may all be gleaned from a sample's mass spectrum (Korfmache,2005; Lim and Lord, 2002). Liquid chromatography combined with the mass spec

is a valuable technique for analysing complicated botanical extracts and correctly identifying chemical components in medicinal plants (Boligon and Athayde, 2014).

For polar lipids such as phospholipids, ultra-high-performance liquid chromatography (UHPLC) further enhances the separation power of high-performance liquid chromatography (HPLC) (Stromberg and Mukundan, 2020). Panja and Mukhopadhyay (2019) use HPLC to identify stevia's steviosides and rebaudioside.

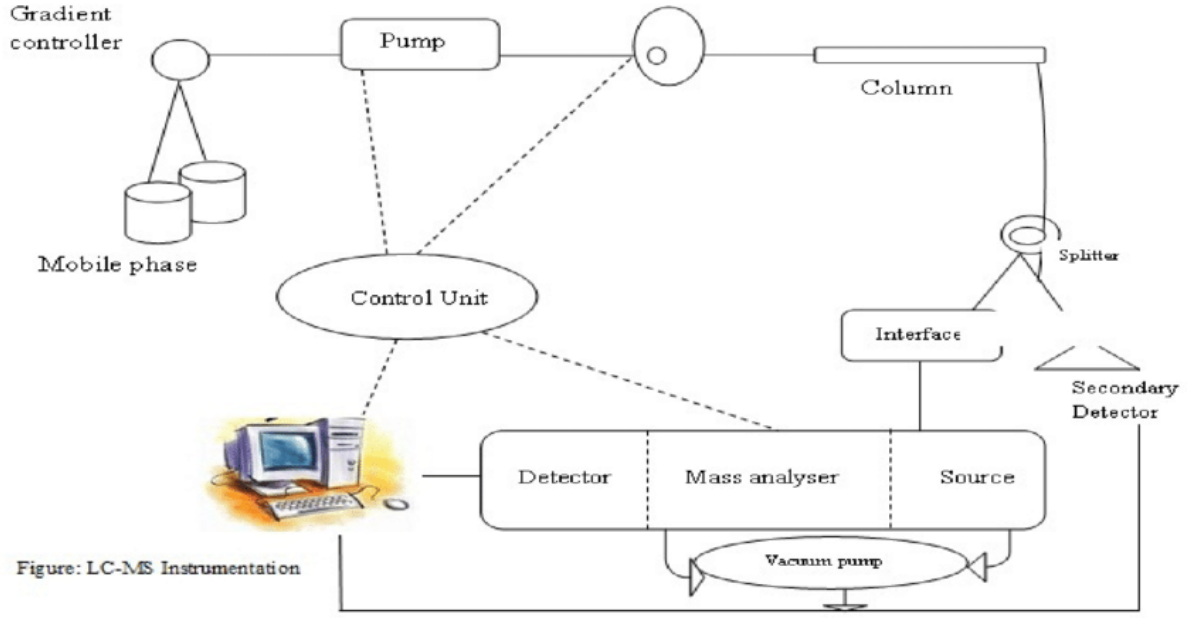


Figure 2.7 Image showing LC-MS instrumentation

Source: Parasuraman et al., (2015)

2.3.2 Gas chromatography with mass spectrometry

Analysis of organic substances and gases uses the gas chromatography (G.C.) method (Hou et al.,2006). High-resolution gas chromatography is necessary for examining environmental materials due to the difficulty separating some substances (Provini and Galassi., 1993). In order to analyse combinations of organic substances, the aforementioned methodologies are merged into a single technique. As seen in Figure 2.8, gas chromatography can separate the constituents of a mixture, whereas mass spectrometry can describe each constituent separately (Hou et al.,2006).

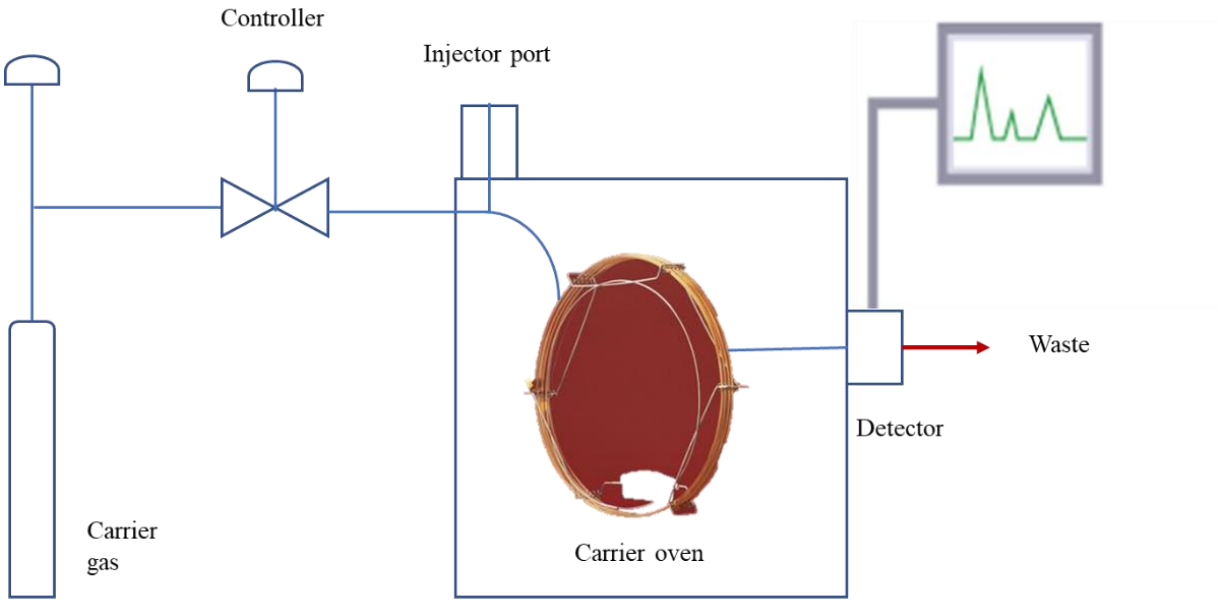


Figure 2.8 Gas-chromatography with mass spectrometry

Hou et al. (2006)

This dual approach is beneficial for forensic and environmental studies and studies involving large numbers of organic chemicals (Medeiros, 2018). Complete descriptions of the analytical procedures used to detect nerve agents and sulphur mustard traces in these samples have been published in previous studies (Black et al., 1994). Full scan mass spectra were used to confirm positive results where available; when concentrations were low, further chemical ionisation GC-MS-SIM, higher resolution GC-MS-SIM, and GC-tandem MS/MS with M.R.M. were used (Black et al., 1994).

Gas chromatography is a technique for separating substances that uses a carrier gas (such as helium) in a fused silica capillary column. The eluate collected in the fused silica capillary column is carried to the mass spectrometer utilising the carrier gas. An MS ionises molecules and separates them by mass and electric charge. The ion signal is mapped against the mass-to-charge ratio in a mass spectrum. The mass, chemical composition, and elemental or isotopic fingerprint of a sample may all be calculated from its spectra. High molecular compounds are thermally destroyed at high temperatures (500-1400 °C) in an inert atmosphere using the analytical pyrolysis technique connected to GC/MS (Kusch, 2017).

Using GC-MS, qualitative analysis of several T. vulgaris organic crude extracts revealed a wide range of high and low-molecular-weight chemicals. Most GC-MS-identified chemicals recovered from the crude extracts have essential biological roles (Hashmi et al., 2013). Gas chromatography-mass spectrometry (GC-MS) was employed in a prior investigation to identify

the phytocomponents in an ethanolic leaf extract of *Stevia rebaudiana* (Ramya et al.,2021). A total of 40 different compounds were identified in a GC-MS chromatogram. Oleic acid, Phytol, and Isosteviol were the secondary metabolites found (Ramya et al.,2021).

2.3.3 Inductively Coupled plasma optical emission (ICP-OES)

Atomic spectroscopy uses Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) to analyse materials by breaking them down into a cloud of hot gases that include free atoms and the components of interest (Hou et al., 2006). When the sample is heated to extremely high temperatures, its atoms get excited and ionised. Excited atoms or ions can return to their ground states through thermal or radiative energy transfers (Hill et al., 2007). The concentration of the components of interest can be calculated from the intensity of the light emitted at various wavelengths (Hou et al., 2006). Various food materials can be quantitatively determined using ICP-OES regularly (Hill et al.,2007).

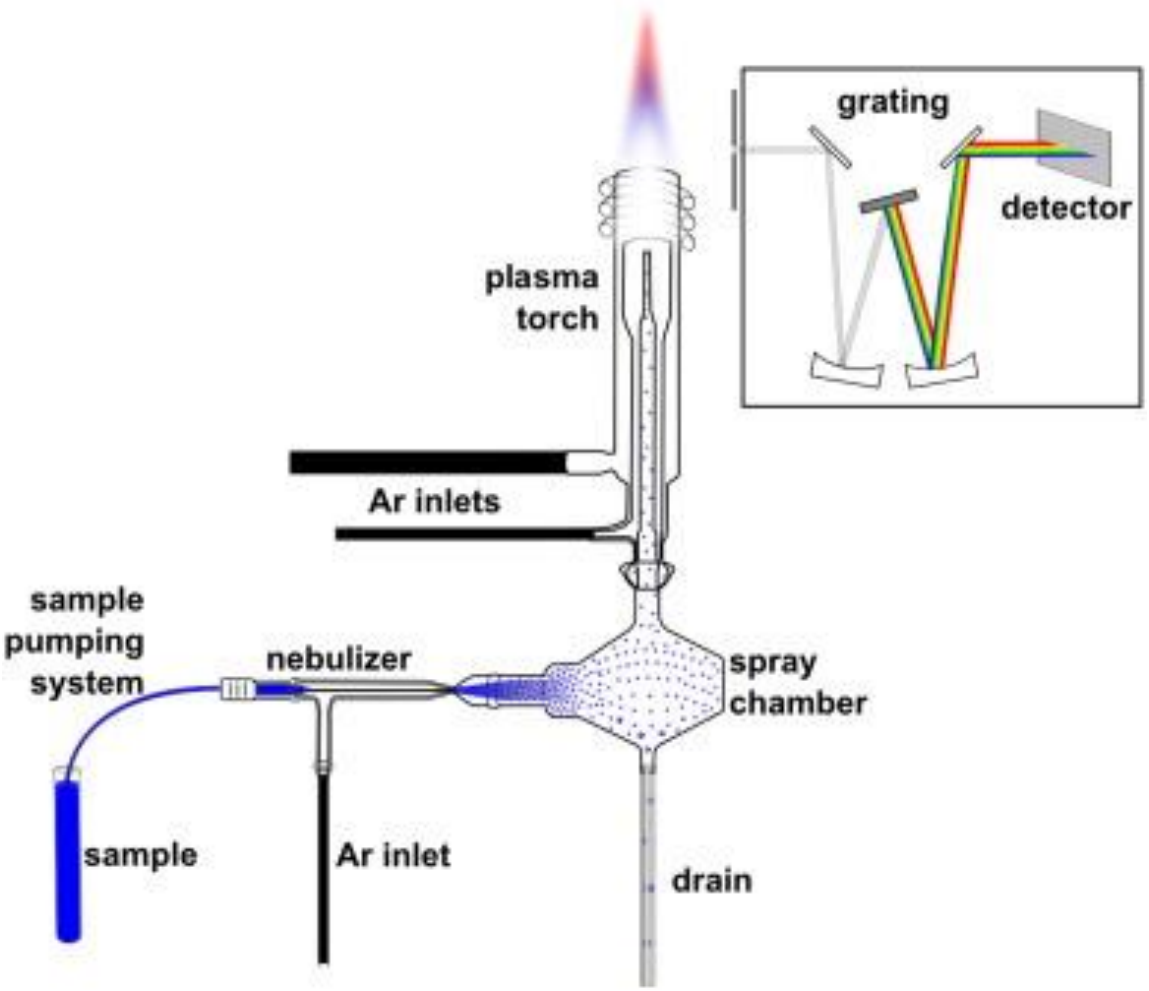


Figure 2.9 Schematic diagram of ICP-OES

Source: Hou et al., (2006)

Using the instrumental analytical technique ICP-OES, elemental traces may be quantified in liquid solutions down to 10 ng/L for some specific elements (Hill et al., 2007). This type of atomic emission spectroscopy involves the atomisation and excitation of an inductively linked plasma torch. Plasmas, typically argon-based, operate at far lower temperatures than flames and graphite furnaces. Excitation (atomisation) is far more effective (Harvey, 2000; Harris, 2010). As a result, the signals produced have better detection limits, more accuracy, and a more comprehensive linear response range (Harvey, 2000; Harris, 2010).

The most effective method for analysing trace elements in complex matrices, such as cement pore solutions and solid samples, is inductively coupled plasma-optical emission spectrometry (ICP-OES). Acids must extract solid samples to release metals in solution before analysing with ICP-OES. Microwave digestion is often the preferred extraction technique before ICP-OES analysis. ICP-OES can simultaneously measure the concentration of up to 70 elements, considering the matrix interferences (Hou et al., 2006). ICP-OES Metals were available in varying concentrations in the herb plant samples such as *Stevia rebaudiana* and Moringa (Alhogbi, 2018). The trace element determination of herbal plants using acid digestion was observed through ICP-OES (Alhogbi, 2018).

2.3.4 Inductively Coupled plasma mass spectrometry (ICP-MS)

ICP-MS is a robust and susceptible technology for detecting certain elements that identify important and unidentified chemicals. The plasma's high temperature and high ion density make it the perfect atomiser and element ioniser for all materials and matrices introduced by several specialised devices (Ammann,2007). ICP-MS is primarily utilised in various disciplines, including geochemistry, environmental and life sciences, industries (food, chemical, semiconductor, nuclear), forensic science, and archaeology, to identify metals in solid and liquid samples (Amman,2007). The method has continually advanced since the first commercially accessible instrument was introduced in 1983 (Amman,2007). Several manufacturers make dependable and durable equipment for detecting multiple element isotopes with shallow detection limits (ppt) and high spectral resolution (10 000). (Montaser, 1998; Nelms, 2005).

ICP-MS can quantitatively measure the elements to determine the overall quantity of a given element. Compared to other ionisation techniques, such as flame ionisation, plasma ionisation has the benefits of taking place in a chemically inert environment, which prevents oxide formation, and plasma ionisation is more thorough (Nelms, 2005). It is employed in the

analysis of samples of matter to ascertain their elemental composition, the structure of their inorganic, organic, and biological molecules, the qualitative and quantitative composition of their complex mixtures, the composition and structure of their solid surfaces, and the isotopic ratios of their atoms (Skoog, 2007). Figure 2.4.4 shows a schematic representation of ICP-MS.

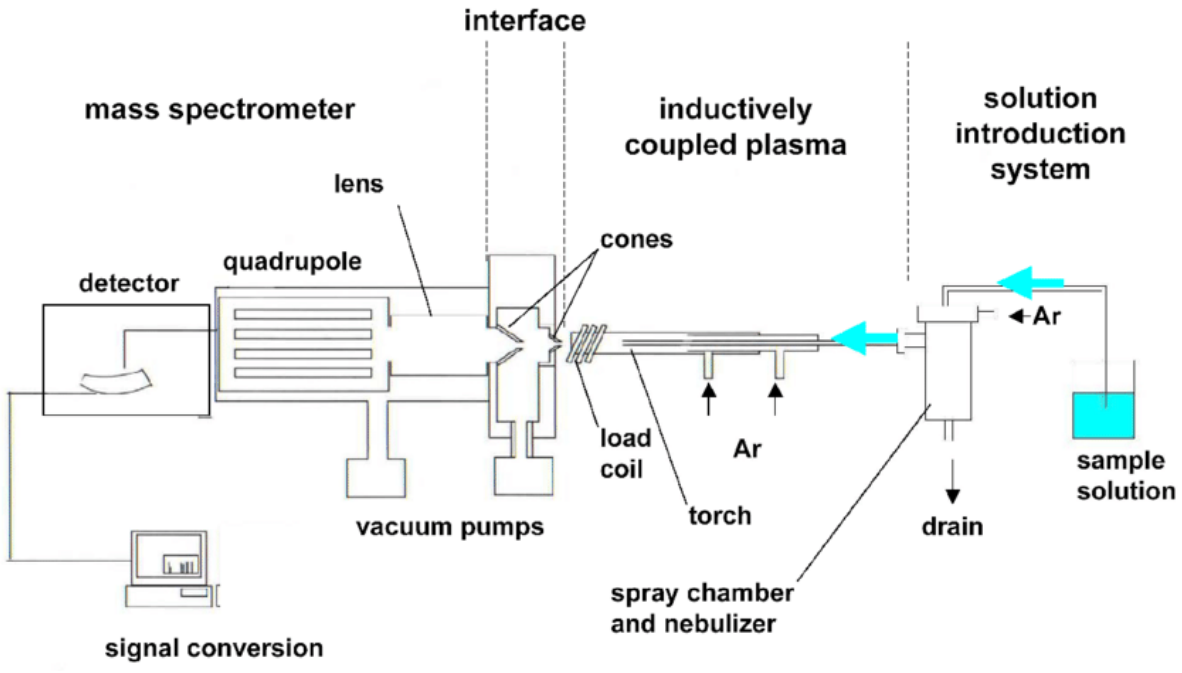


Figure 2.10 Inductively Coupled plasma mass spectrometry (Gilstrap, 2009).

Basic chemical information, such as total single-element concentrations or elemental and isotope ratios, can be obtained by I.C.P. - M.S. alone. In this context, the method has proven to be an excellent analytical tool because of its high sensitivity, high throughput, and low total cost of ownership. When measuring multiple parameters in a single sample and thousands of samples yearly, the per-parameter cost of an experimental life cycle is comparable to that of a single sample vial. Despite a somewhat more excellent material and operational cost (Ar consumption), ICP-MS is financially beneficial (Ammann, 2007). When matrix interferences are eliminated, and the technique is reliable, ICP-MS may regularly identify major and trace elements in plant samples (Masson et al., 2010).

For their 2014 study, Hajar et al. utilised an ICP-MS to analyse their samples (ICP-MS). *Stevia rebaudiana* has been shown to accumulate heavy metals while extracting its leaves, stems, and flowers (Hajar et al., 2014). ICP-MS can identify ultra-trace elements in plant material because of how efficiently it processes ions during the ion extraction process, transports ions through the mass spectrometer, and detects them (Wilschefski and Baxter 2019). In the past, fifteen heavy elements, including lead, were measured in *Stevia rebaudiana* leaves, stems, and flowers

using the inductively coupled plasma mass spectrometer Elan 9000: As, Cd, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ag, Co, Ca, Mn and Ni (Wilschefski and Baxter 2019).

CHAPTER 3

General and specific objectives

3.1 General objectives

The main focus of this project was to study the presence and behaviour of macro and micronutrient elements in dried *stevia* leaves using pressurised hot water extraction techniques at various extraction temperatures.

3.2 Specific objectives

I. To investigate how PHWE temperature and extraction time influence the extractability of macro and micronutrient elements in the dried powder *stevia* plant.
II. To compare the recovery of macro and micronutrient elements extracted by PHWE at optimum conditions to the total metals using microwave digestion.
III. To evaluate whether the extract from *stevia* plant parts by PHWE can be used as a possible food supplement for macro and micronutrient elements.

3.3 Hypothesis

Macronutrient elements are not tightly bound to stevia plant leaves in organic compounds compared to micronutrient elements. Thus, macronutrient elements will be extracted at low PHWE temperature compared to micronutrient elements.

The hypothesis that macronutrient elements are not firmly bound to stevia plant leaves in organic compounds compared to micronutrient elements suggests that the extraction of these elements will vary depending on the temperature used in pressurized hot water extraction (PHWE). According to this hypothesis, macronutrient elements will be extracted at a lower temperature than micronutrient elements. This hypothesis has significant implications for the study of the extraction of macro and micronutrient elements in dried stevia plant leaves and stems, as it implies that the extraction efficiency of these two types of elements will vary significantly based on the extraction conditions used. Further investigation is needed to determine the validity of this hypothesis and its potential impact on the extraction of macronutrient and micronutrient elements in stevia plant leaves.

3.4 Justification

The cultivation and use of poisonous *stevia* plants is relatively simple. Since the properties of the metals (macro and micro) present in plant biomass might change depending on the soil where the plants are grown, further study is required (Hajar et al., 2014). *Stevia* is a natural sweetener becoming increasingly popular as a sugar substitute due to its low glycemic index and zero-calorie content. However, *stevia* leaves and stems are also a rich source of various macro and micronutrients, such as iron, calcium, and magnesium, which have various health benefits. The extraction of these nutrients from the plant material is necessary to determine *stevia*'s potential as a source of these essential elements. Pressurised hot water extraction (PHWE) is a novel and efficient method for extracting various plant compounds, including nutrients. Therefore, a study exploring the extraction of macro and micronutrient elements in dried *stevia* plant leaves and stems using PHWE is justified. The findings of such a study would contribute to a better understanding of the nutritional value of *stevia* and could potentially be used in developing new functional foods and dietary supplements.

3.5 Novelty

The study exploring the extraction of macro and micronutrient elements in dried *stevia* plant leaves and stems using pressurised hot water extraction represents a novel contribution to plant extraction. Previous studies have primarily focused on extracting steviol glycosides, the sweet-tasting compounds in *stevia* plants, using various extraction methods. However, this study goes beyond previous research by investigating the extraction of a broader range of compounds, specifically the macro and micronutrient elements essential for human health. Moreover, pressurized hot water extraction as a technique is noteworthy, as it is a relatively green and efficient method that has received limited attention in *stevia* extraction. Therefore, this study represents a valuable addition to the literature, providing new insights into the nutritional value of *stevia* plants and the potential use of pressurized hot water extraction as an extraction technique.

CHAPTER 4

Materials and methods

4.1 Materials and chemicals

The analytical grade reagents utilised in this investigation, such as nitric acid (HNO_3), hydrochloric acid (HCl), and hydrogen peroxide (H_2O_2), were acquired from Sigma-Aldrich (Johannesburg, South Africa). Sigma-Aldrich provided the diatomaceous earth (SiO_2) utilised as the disperser in the PHWE extraction cell (Johannesburg, South Africa). Deionised water was supplied via the Milli-Q-RO4 water system (Millipore, Bedford, MA, U.S.A.), which was employed as a solvent.

Nitric acid (HNO_3), hydrochloric acid (HCl), and hydrogen peroxide (H_2O_2) are commonly used in studies exploring the extraction of macro and micronutrient elements in plant materials. These chemicals are essential for breaking down the complex organic compounds in plant tissues and releasing the bound minerals. Pressurised hot water extraction is an efficient technique for extracting nutrients from plant samples, but it can leave some minerals trapped in the residual matrix. Adding nitric acid and hydrochloric acid in the extraction process can help dissolve the mineral residues and increase the recovery of nutrients. Hydrogen peroxide is often used as an oxidising agent to convert the metallic ions to their highest oxidation state, making them more soluble in the solvent. Including these chemicals in the study exploring the extraction of macro and micronutrient elements in dried *stevia* plant leaves and stems using pressurised hot water extraction is justified as it can provide more accurate and comprehensive information about the nutrient content and bioavailability of the plant material.

4.2 Equipment

Analytical balance purchased from Ohaus (Johannesburg, South Africa) weighed the solid reagents and *stevia*-dried powder. Anton Paar multiwave 3000 microwave digester (Anton Paar, Switzerland) was used to extract the metals from *stevia* powder biomass. In order to analyse and quantify metals in the samples, Spectro, Genesis ICP-OES (Kleve, Germany) was used to filter samples and run 0,25 mm filtrate (Manicaa, Midrand, South Africa) for 20 minutes.

The Anton Paar Multiwave 3000 Microwave Digester and Spectro Genesis ICP-OES (Kleve, Germany) are essential tools in the study exploring the extraction of macro and micronutrient elements in dried *stevia* plant leaves and stem using pressurised hot water extraction. The Multiwave 3000 Microwave Digester enables fast and efficient digestion of samples, allowing

for complete extraction of nutrients from the plant material. On the other hand, the Spectro Genesis ICP-OES provides accurate and precise measurements of the extracted elements, allowing for a thorough analysis of the nutrient content in the sample. Without these tools, it would not be easy to obtain reliable data on the nutrient content of the *stevia* plant material, which is crucial for understanding its potential as a source of valuable nutrients. Therefore, using these tools is justified and necessary for the success of this study.

4.3 Growing the *stevia* plants in the greenhouse

Stevia rebaudiana seedlings were purchased from a nursery in Centurion area, Pretoria, South Africa nursery. The plants were purchased as tiny pot plants, and a transfer was done into small black plastic bags so the plants could be given enough room to grow and for the shoots to start flowering in a greenhouse at the University of the Witwatersrand School of Animal, Plant and Environmental Science (A.P.E.&S). Plants were watered twice weekly, and the soil was toppled if necessary. Temperatures in the greenhouse ranged between 26 to 29 degrees Celsius. The plants were grown for about 90 days

Growing the stevia plants in the greenhouse at temperatures between 26 to 29 degrees Celsius is justified in the study exploring the extraction of macro and micronutrient elements in dried *stevia* plant leaves and stems using pressurised hot water extraction. This temperature range is optimal for the growth of *stevia* plants, as they are native to warmer regions and thrive in warm, humid conditions. Additionally, higher temperatures can increase the concentration of steviol glycosides, which are the compounds responsible for the plant's sweetness. Pressurised hot water extraction is a commonly used method for extracting bioactive compounds from plant materials and is effective for extracting nutrients from stevia. Therefore, growing the stevia plants in a controlled greenhouse environment at the optimal temperature range will provide the highest quality and quantity of plant material for the extraction process, resulting in more accurate and representative data regarding the macro and micronutrient content of the dried stevia plant leaves and stems.

4.4 Harvesting of Stevia Plants

Stevia branches were cut at least four to five inches of the stem, and the leaf and stem branches were then rinsed with deionised water immediately after harvest and left to air dry in a sterile environment in the shade for 10-14 days. The stevia was then further air dried to ensure moisture content and complete dryness of the sample material. The stevia was crushed into a

fine powder using a blender and stored in aluminium foil to prevent exposure to sunlight until extractions using PHWE.

The process of cutting the stevia branches at least four to five inches of the stem and rinsing the leaf and stem branches with deionized water immediately after harvest, and then leaving them to air dry in a sterile environment in the shade for 10-14 days is critical for the study exploring the extraction of macro and micronutrient elements in dried stevia plant leaves and stems using pressurised hot water extraction. This process ensures that the stevia branches are free from extraneous materials and contaminants that may interfere with the extraction process. The air-drying process in a sterile environment in the shade helps preserve the integrity and quality of the plant material, ensuring that the macro and micronutrient elements are not degraded or altered. This critical process is essential for obtaining accurate and reliable results in extracting macro and micronutrient elements in dried stevia plant leaves and stems using hot water extraction.

In the study exploring the extraction of macro and micronutrient elements in dried stevia plant leaves and stems using pressurised hot water extraction (PHWE), the decision to crush the stevia into a fine powder using a blender and store it in aluminium foil to prevent exposure to sunlight was a critical step. The fine powder increases the surface area of the plant material, allowing for better contact between the stevia and the hot water during the extraction process. Furthermore, storing the powder in aluminium foil protects it from exposure to sunlight, which can degrade some of the micronutrients in the stevia plant. By taking these precautions, the study was able to accurately extract and analyze the macro and micronutrient elements present in the stevia plant, which is essential for understanding its potential uses in the food and beverage industry.

4.5 Extraction of heavy metals using PHWE from dried stevia leaves

The PHWE system consisted of an extraction cell house in a G.C. oven for controlling the extraction temperature. Deionised water was used as the extraction solvent and was pumped by a highly pressurised liquid chromatography pump. The deionised water was heated at 80-100ºC and pumped through the system during extraction. For each run, 1.5 g of *Stevia rebaudiana* and 1.5 g of diatomaceous earth were mixed and filled into the Pressurised hot water extraction cell. Before each extraction, the extraction solvent was allowed to run through the system for at least 60 min. This ensured that all the void volumes in the extraction flow system, including the cell, were filled with solvent. Afterwards, the system was kept idle for

10 minutes to warm the cell to the predetermined extraction temperature. The extractions were carried out in three copies at a 1 mL/min flow rate. 20 mL of the extract were collected following PHWE extraction at 50, 100, and 150°C with a continuous flow rate. The extraction period was adjusted after changing the extraction temperature by up to 100 minutes. After each extraction cycle, the liquid extracts were put into the 50 mL centrifuge tubes. Before the further investigation, the tubes were firmly capped and kept at -18°C.

4.6 Microwave digestion of the plants

A mass of 1.50 ± 0.005 g of homogenised dried stevia and 1.5 ± 0.005 g. The Multiwave 3000 microwave digester was used to process diatomaceous earth (Sigma Aldrich, Johannesburg, South Africa) that had been weighed using an analytical balance and then placed into acid-washed digestion tubes (PTFE-TFM liners) containing 6 ml HCl, 2 mL HNO_3, a drop or two of H_2O_2, and 6 mL stevia sample. After being capped and put onto rotor segments, the digestive tubes were placed into the microwave digestion apparatus. The samples received 20 minutes of radiation exposure. The solutions were transferred to 50 mL conical tubes after being cooled in the original tubes. Deionised water was added for further testing.

Microwave digestion is a widely accepted sample preparation technique in the elemental analysis due to its numerous advantages. Microwave digestion offers fast and efficient decomposition of plant materials, allowing for the release of all analytes from the matrix. Additionally, it significantly reduces the time required for digestion, and it requires a smaller sample size. These benefits result in increased accuracy and precision, reduced reagent consumption, and decreased laboratory contamination. Therefore, using microwave digestion to prepare the plant samples in this study will ensure reliable and reproducible results in extracting macro and micronutrient elements.

4.7 Analysis of the samples using ICP-OES

Inductively coupled plasma optical emission spectroscopy was used to evaluate the liquid extract of the pressured hot water extraction and microwave-assisted digestion for metal content (ICP-OES). Metals were analysed using the plant's standard procedure, AOAC Official Method 2015.01. (US EPA, 2015).

The decision to analyse metals using the plant's standard procedure, AOAC Official Method 2015.01, in a study exploring the extraction of macro and micronutrient elements in dried stevia plant leaves and stems using pressurised hot water extraction is justified for several reasons. Firstly, AOAC's Official Methods are widely recognized and accepted as the gold standard for

analytical procedures in the food industry. These methods are rigorously tested and validated, providing highly accurate and reliable results. Secondly, the AOAC Official Method 2015.01 is specifically designed to determine minerals in plant materials, including metals such as calcium, iron, zinc, and magnesium. Therefore, it is highly appropriate for analysing the metallic elements in dried stevia plant leaves and stems. Finally, using a standardized method such as AOAC Official Method 2015.01 ensures that the results obtained are comparable and can be easily compared to other studies conducted using the same method, facilitating the interpretation of the results and enhancing the reliability and validity of the study.

CHAPTER 5

Results and Discussion

5.1 Studying the influence of PHWE parameters on metal behaviour in stevia-dried biomass powder

Influence of extraction temperature

5.1.1 Micronutrients elements behaviour with PHWE temperature

Fig. 5.1 shows the influence of extraction temperature for micronutrient elements. Most micronutrient elements were not influenced by extraction temperature as concentrations at 50°C were similar to other temperatures. This indicates that these elements are not tightly bound to organic chemicals found in stevia leaves, like phenolic compounds. However, elements such as Co, Cr, and Ni have less amounts extracted at 150°C than Al, Fe, and Zn, showing similar concentrations at various extraction temperatures. Nuapia et al. (2020) discovered that an increase in the extraction temperature increased Moringa's amounts of extractable micronutrients. Fig 5.1 shows the opposite trend, possibly due to morphological differences between stevia and Moringa.

The finding that most micronutrient elements were not influenced by extraction temperature in the pressurised hot water extraction of dried stevia plant leaves and the stem is an actual result with implications for both research and industry. The study demonstrated that extraction at 50°C was just as effective in extracting these micronutrients as at higher temperatures, indicating that temperature may not be a critical factor in optimizing extraction efficiency for these elements. This has important practical implications for the development of efficient and cost-effective extraction methods for micronutrients in various plant materials. Additionally, this finding raises questions about the extraction mechanisms for these micronutrients and suggests further research to understand the underlying processes and identify the key factors influencing extraction.

In figure 5.1, Fe has the highest concentration extracted with 12.37 mg/kg at 150°C, whereas Co has the lowest with 2.08 mg/kg at the same temperature. Because it contributes to protein synthesis and enzyme activation, Fe is required in higher amounts than other micronutrients (McDowell et al.,1986). The results show that temperature increase affected the extraction of some micronutrients by either increasing or decreasing their concentration. When the temperature was increased, the concentration of Cr gradually decreased, possibly indicating that some micronutrient elements have a higher selectivity for extraction. Studies have found

that good selectivity comes from combining different transition metals with well-designed ligands (Chang et al.,2022).

The high concentration of Fe in the stevia plant indicates that it can serve as a potential source of iron, a vital nutrient necessary for various physiological processes in the human body. On the other hand, the low concentration of Co in the stevia plant leaves and stems may suggest that it is not a reliable micronutrient source. These findings provide crucial insights into the nutrient composition of the stevia plant, which can be valuable for food and nutrition researchers and manufacturers. Additionally, these results demonstrate the effectiveness of pressurised hot water extraction in isolating and quantifying macro and micronutrient elements in plants.

The finding that the concentration of Cr gradually decreased with an increase in temperature is an essential result of the study on extracting macro and micronutrient elements in dried stevia plant leaves and stems using pressurized hot water extraction. This result suggests that temperature significantly affects the extraction of Cr from the stevia plant. It also raises questions about the potential loss of other essential nutrients in stevia leaves and stems under high-temperature extraction conditions. These findings emphasize the importance of carefully controlling the temperature during the extraction process to optimize the yield of desirable nutrients while minimizing the loss of other valuable components. Further research is needed to explore the optimal temperature conditions for extracting various macro and micronutrients in stevia plant leaves and stems.

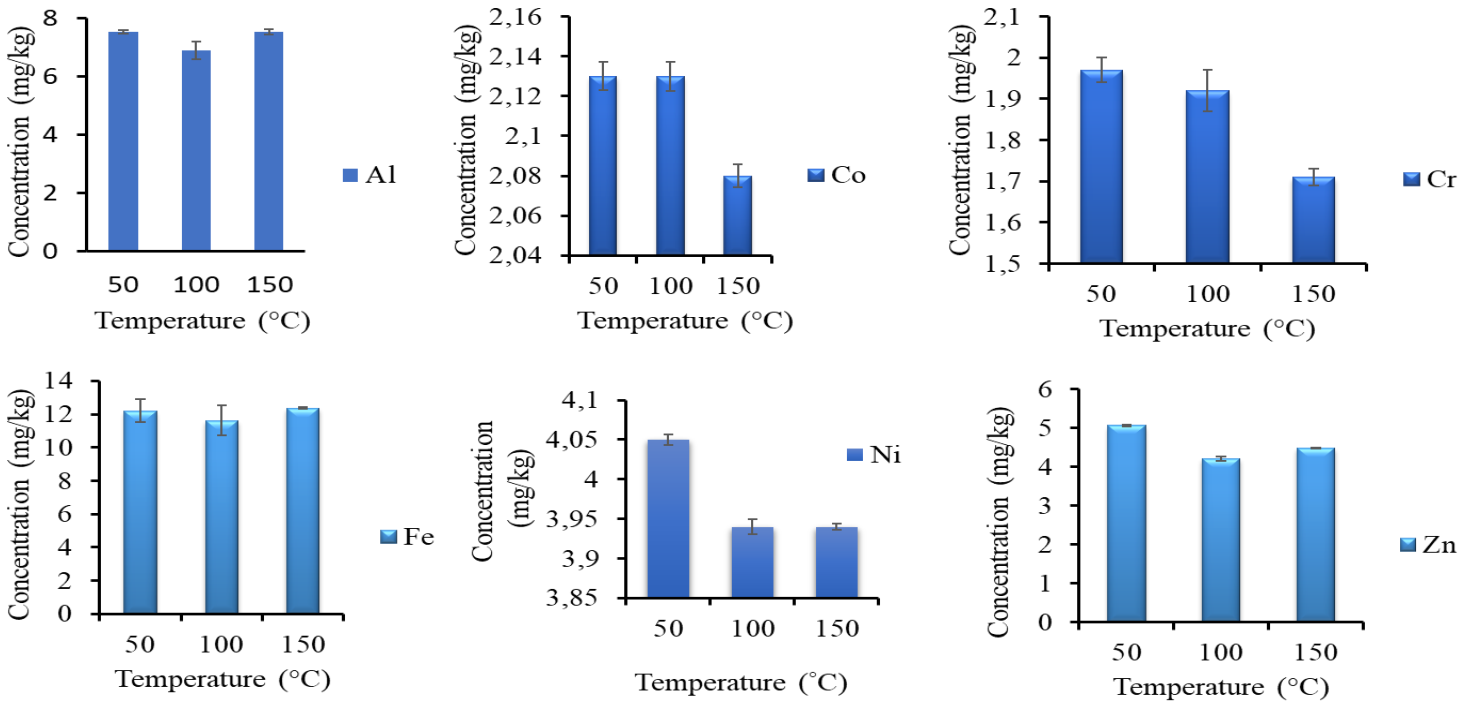


Figure 5.1 Comparison of extraction temperature of some micronutrient's elements in stevia at a constant extraction time of 20 minutes

5.1.2 Macronutrients elements behaviour with PHWE temperature

The macronutrients extracted from stevia at different temperatures presented in figure 5.2 extracted were influenced by temperature. Only Ca showed an increase in amounts extracted with increased temperature. Essentially K and Mg were not much influenced by temperature increase. This indicates that Ca is more tightly bound to organic chemicals in stevia leaves. A study by Kroyer (2010) in the liquid extraction of metals from stevia found that higher temperatures around 120°C favoured extraction of macronutrients; applications made with stevioside resulted in good stability observed at elevated temperatures up to 120°C hourly. Nuapia et al. (2020) found that the extraction of macronutrient elements from Moringa oleifera leaves was enhanced by increasing the extraction time more than by increasing the extraction temperature. The extraction of metals and dissolution of the product are both facilitated by high temperatures (Chang et al.,2022).

The finding that only Ca showed increased amounts extracted with increased temperature is significant in several ways. First, it suggests that increasing the temperature can optimise the extraction of Ca from dried stevia plant leaves and stems using pressurised hot water extraction. This could be useful for producing Ca-enriched extracts that could be used to develop functional foods and nutraceuticals. Second, the finding highlights that different macro and micronutrient elements in dried stevia plant leaves and stems may have different responses to changes in temperature during extraction. This underscores the need for a more nuanced approach to extracting these elements, where the specific characteristics of each element are taken into account.

The finding that K and Mg were not much influenced by temperature suggests that other factors may affect the extraction of these elements. Future research could explore these factors and develop new methods for extracting K and Mg from dried stevia plant leaves and stems. Additionally, the finding underscores the importance of exploring extracting macro and micronutrient elements from other plant species using pressurised hot water extraction. This could lead to developing new and improved methods for extracting these elements, which could have significant implications for the production of functional foods and nutraceuticals.

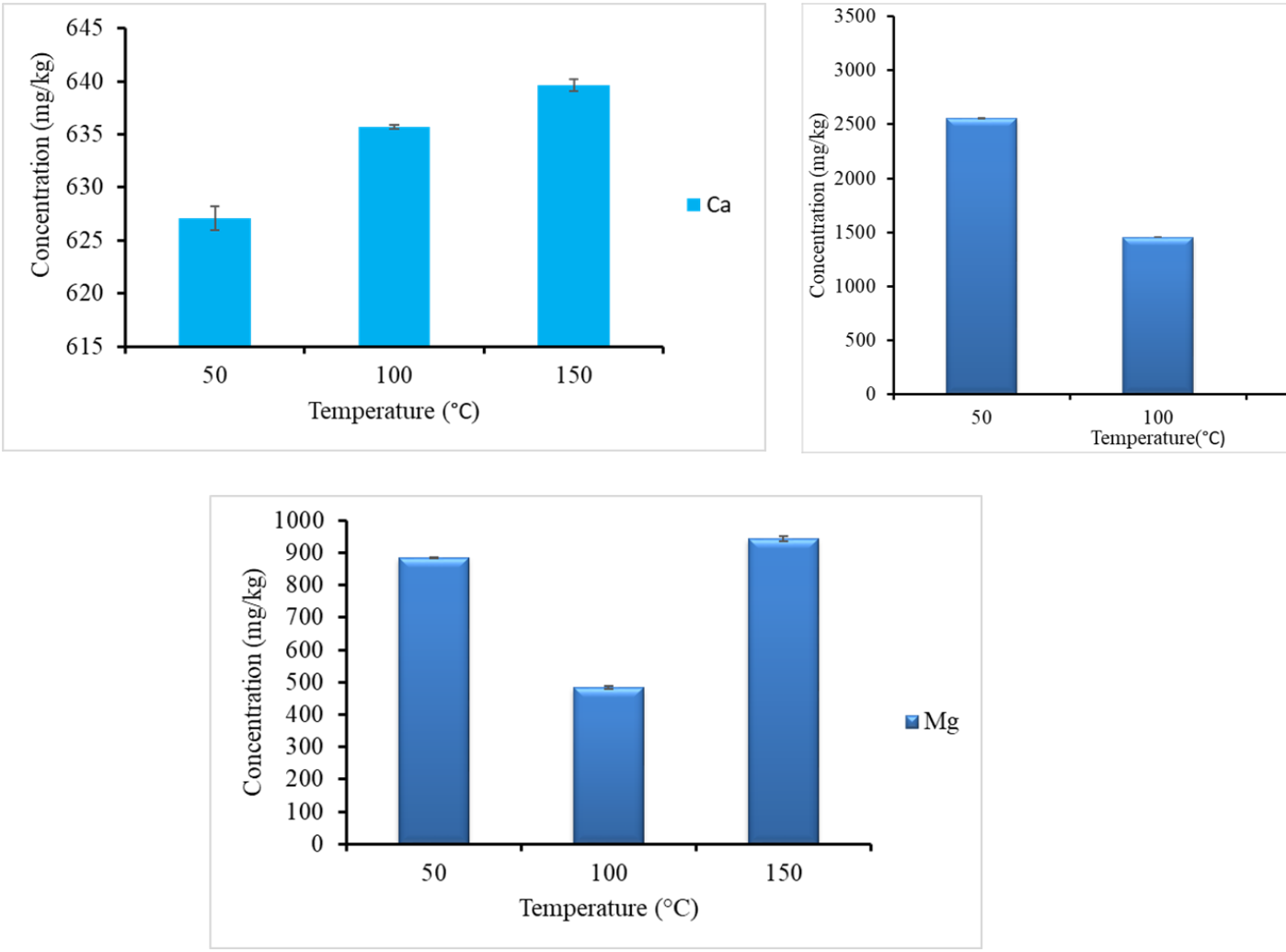


Figure 5.2 Comparison of the influence of extraction temperature on macronutrients in PHWE of stevia at 20 minutes.

5.2 Influence of extraction temperature and time

5.2.1 Micronutrients elements at various extraction times and 50, 100, and 150ºC

Results for varying the extraction time and temperature for micronutrient elements are shown in Figures 5.3, 5.4, and 5.4. The results show that the amount extracted increased with extraction time in all cases. This means that releasing these metals from leaves of organic compounds into the extraction solvent is time-dependent. According to a study by Ghoreishi et al. (2008), the extraction yield rises as its extraction period rises from 10 to 90 minutes. However, increasing the extraction time from 90 to 180 minutes had no additional impact. The extraction temperature impacts the overall amount removed for several metals. A higher temperature is known to boost extraction yields and speed up the process as the extraction rate is raised. But in this study many micronutrients were not influenced by extraction temperature

as the total amounts extracted at 50, 100, and 150^{o}C were almost similar except for iron, nickel, and cobalt. Nickel and cobalt amounts extracted at 150°C were much higher, meaning these are slightly more tightly bound to chemical compounds in stevia leaves. The micronutrients could be tightly bound to the ligand (Brunetti et al., 2013; Kanyama et al., 2013). Temperature increases, for instance, can boost extraction yields and hasten the process by increasing the rate of extraction, which can be helpful tools for analysts looking to optimise and expedite the extraction procedure (Chang et al.,2022).

The study found that the number of extracted elements increased with extraction time in all cases. This finding is significant because it suggests a longer extraction time is required to obtain a higher yield of macro and micronutrient elements from Stevia plant leaves and stems. This finding is consistent with previous studies showing that plant material extraction yield increases with longer extraction times.

The study also found that releasing these metals from leaves of organic compounds into the extraction solvent is time-dependent. This finding is significant because it suggests that the mechanism by which these elements are extracted from the plant material depends on when the material is in contact with the extraction solvent. This finding is consistent with previous studies that have shown that plant material's extraction mechanism depends on factors such as temperature, pressure, and solvent composition. This study's pressurised hot water extraction technique effectively and efficiently extracts macro and micronutrient elements from Stevia plant leaves and stems. This technique is advantageous over traditional extraction techniques because it reduces the time required for extraction and requires less solvent. Pressurised hot water also eliminates the need for organic solvents, which are harmful to the environment and pose health risks to the operators.

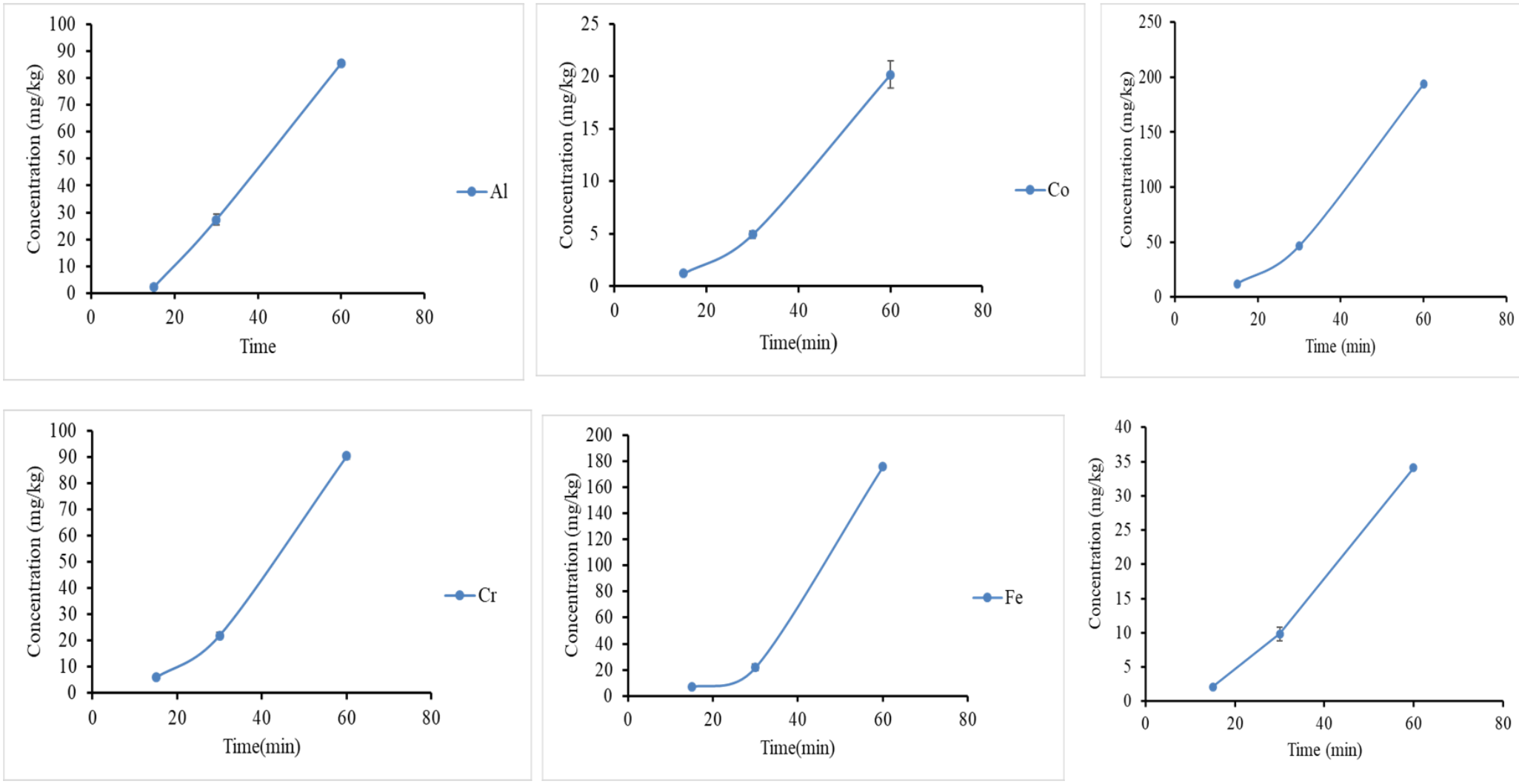


Figure 5.3 Micronutrients extracted at 50ºC with variation in extraction time.

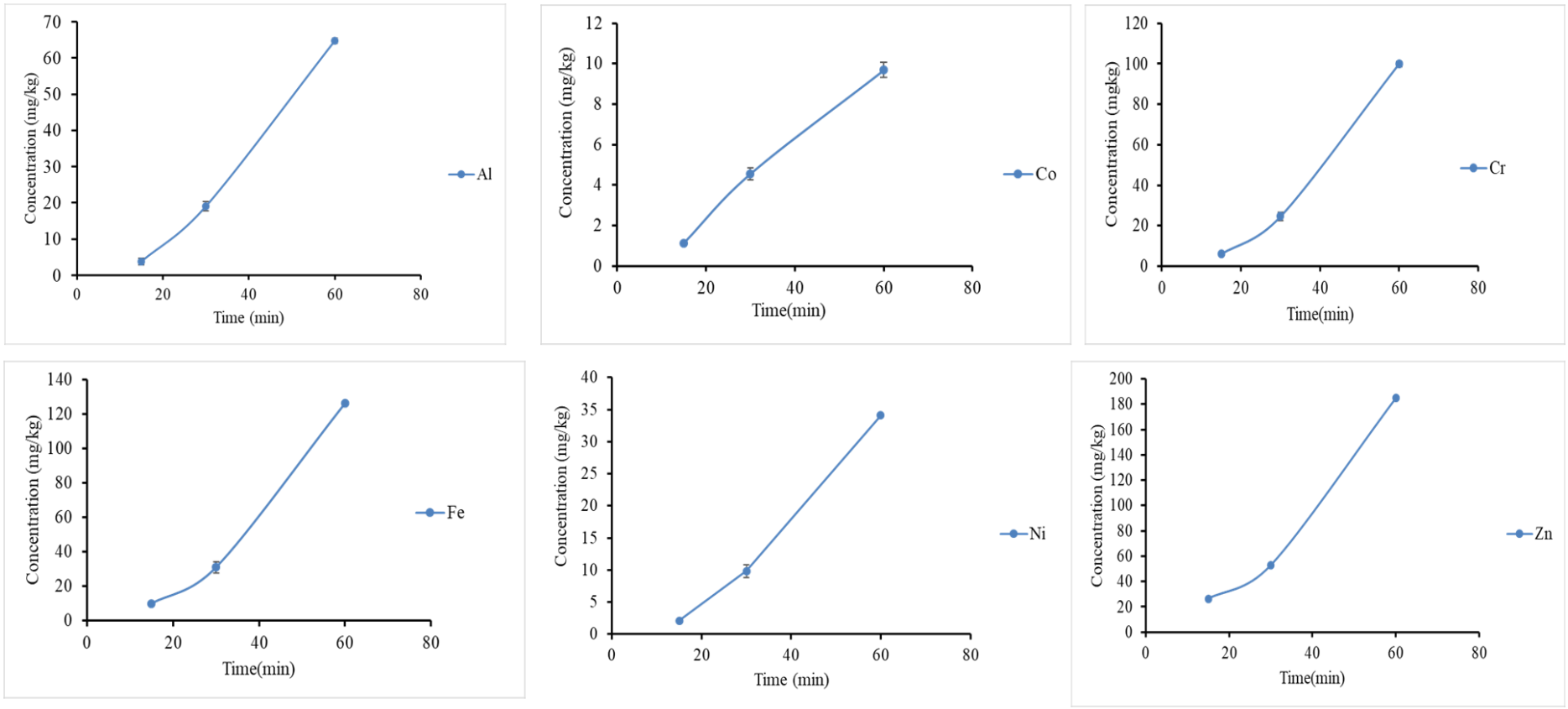


Figure 5.4 Micronutrients extracted at 100ºC with variation in extraction time.

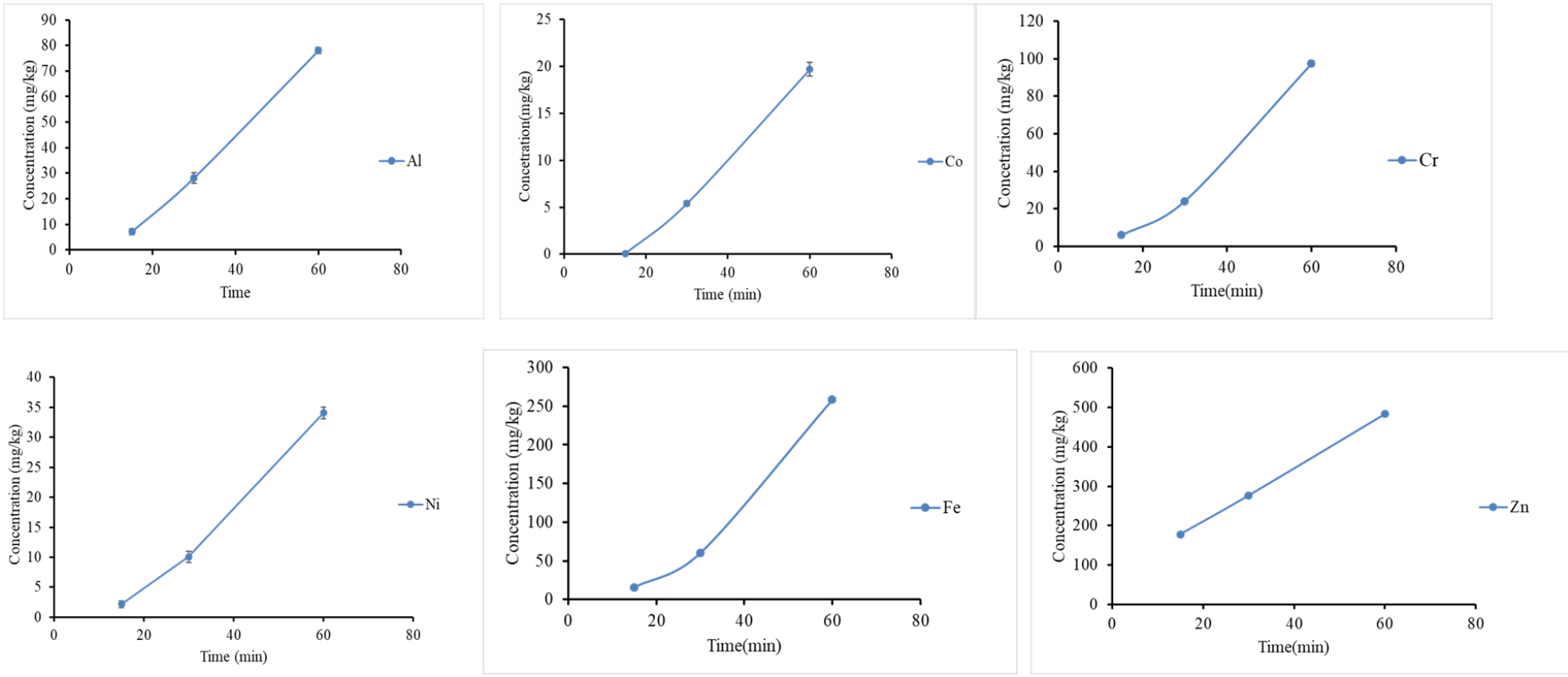


Figure 5.5 Micronutrients extracted at 150ºC with variation in extraction time.

5.2.2 Macronutrients element's behaviour in PHWE at various times and temperatures

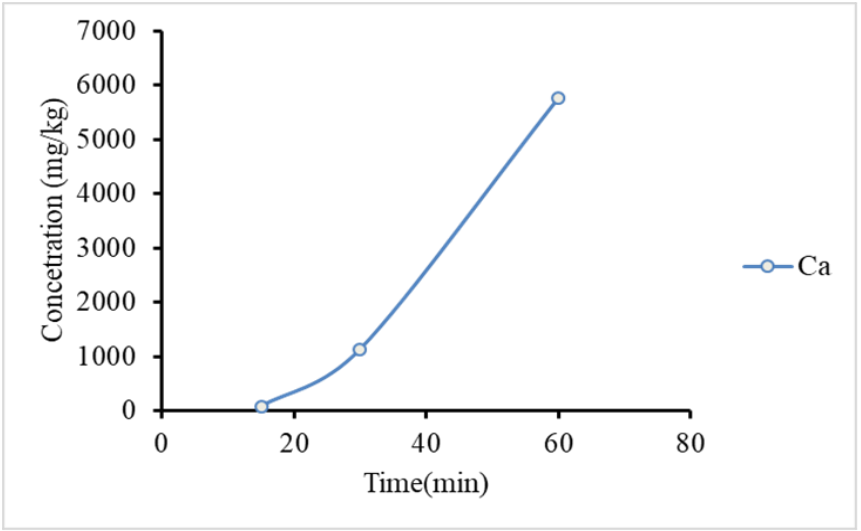
In Figures 5.6, 5.7, and 5.8, the macronutrients favour a longer extraction time, while the graphs indicate linearity. The macronutrients were favourable towards an increase in extraction time. At 50 and 100ºC $p > 0.05$ (Table A5 and A6 in appendix), there was statistically no significant difference in the behaviour of the macronutrients at the specific temperatures. Contrary to the above at 150ºC $p < 0.05$ (Table A8), statistically, there is a significant difference between the macronutrients. Mg, K, and Ca show linearity in their graphs; at 100 ºC (figure 5.7), the graphs have a steep slope indicating higher extraction of the macronutrients. Extending the extraction process may remove large quantities of macronutrient components (Nuapia et al.,2020). Plant cells' biochemical processes depend on magnesium. Mg^{2+} is abundant because it is essential for controlling mitochondria operations, such as their volume, ion composition, and ATP generation (Pasternak et al.,2010). Other crucial plant processes that need calcium include enhancing other nutrients' transportation inside the plant and their uptake by roots (Pasternak et al.,2010).

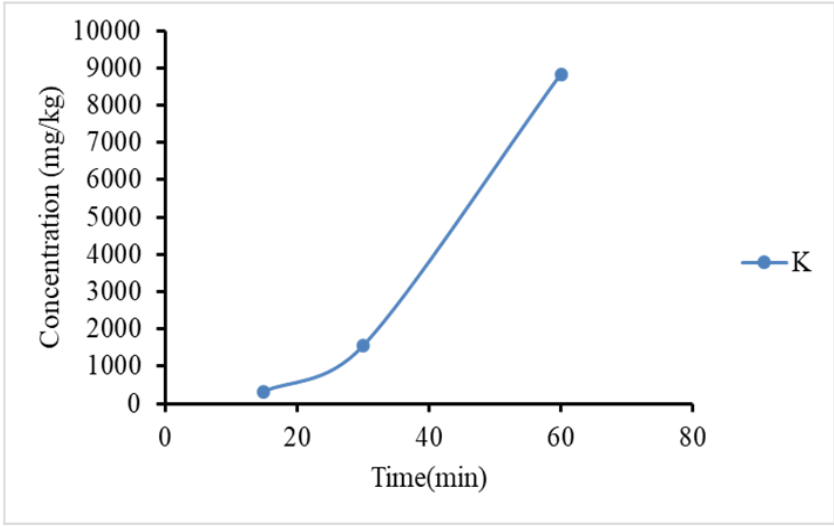
Understanding what macronutrients are and their importance in the extraction process is essential. Macronutrients are nutrients that living organisms, including humans, require in relatively large amounts. These include carbohydrates, proteins, and fats, which are all critical for the proper functioning of the body. In the extraction process, macronutrients play a crucial role in determining the quality and quantity of the extracted elements. The study found that macronutrients favour a longer extraction time. This is an important finding as it suggests that the extraction process must be carefully controlled to ensure the optimum extraction time. This is particularly important when considering the extraction of micronutrient elements, which may be more sensitive to changes in extraction time.

Furthermore, the study found that there was statistically no significant difference in the behaviour of the macronutrients at specific temperatures of 50 and 100ºC. This finding suggests that the extraction process may be more robust at these temperatures and less sensitive to changes in extraction time. However, at 150ºC, there was a statistically significant difference between the macronutrients, indicating that the extraction process is more sensitive at this temperature. The linearity observed in the graphs is also an exciting finding. Linearity suggests that the extraction process is consistent and predictable, which is essential when considering the reproducibility of the extraction process. The linearity observed in the graphs may result from the controlled conditions under which the extraction process was carried out.

The macronutrients Nitrogen, Phosphorus, Potassium, Sulphur, Calcium, and Magnesium is often categorised as minerals that plants need in relatively high quantities (El-Ramady et al.,2014). Macronutrients are components of organic macromolecules, such as proteins and nucleic acids, and also serve as osmotically active substances like potassium (Shehata and El-Ramady, 2012; Niklaus,2007). According to studies, organic acids occur during plant cell metabolism, and calcium, along with magnesium and potassium, aids in neutralising these acids, which is why they are found in high plant concentrations (Pasternak et al.,2010).

Figure 5.6 shows calcium, potassium and magnesium concentrations obtained at 50°C at different extractions times such as 15, 30 and 60 minutes. While Figure 5.7 gives us the concentrations for the same macronutrients at 100°C. Figure 5.8 the macronutrients are extracted at 150°C at various extraction times.





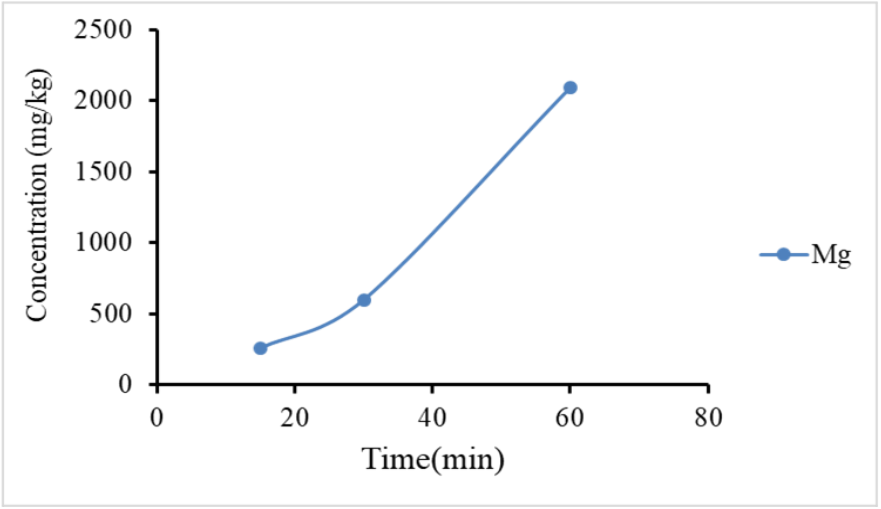


Figure 5.6 Macronutrients extracted at 50ºC at various extraction times

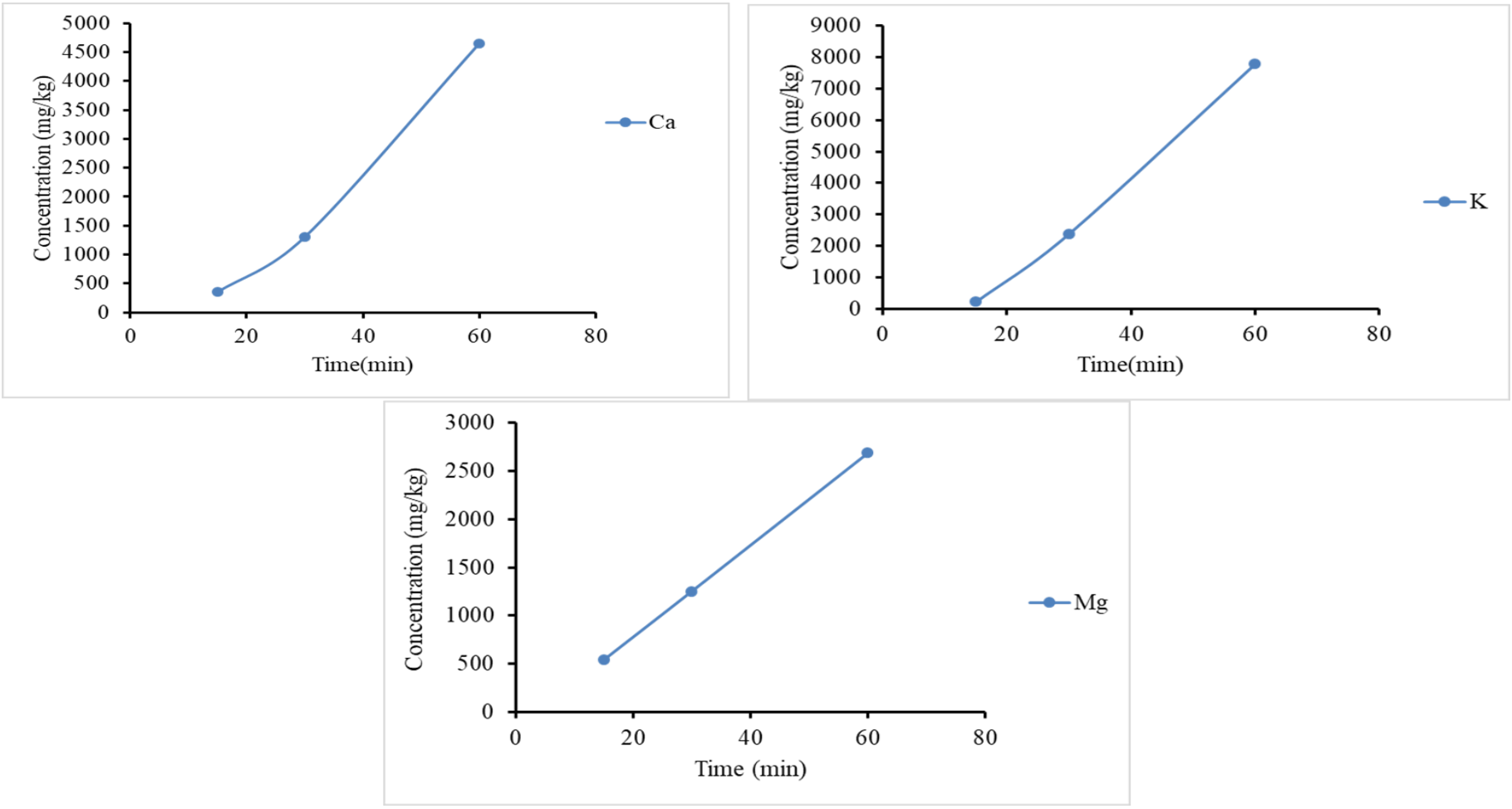


Figure 5.7 Macronutrients extracted at 100ºC at various extraction times

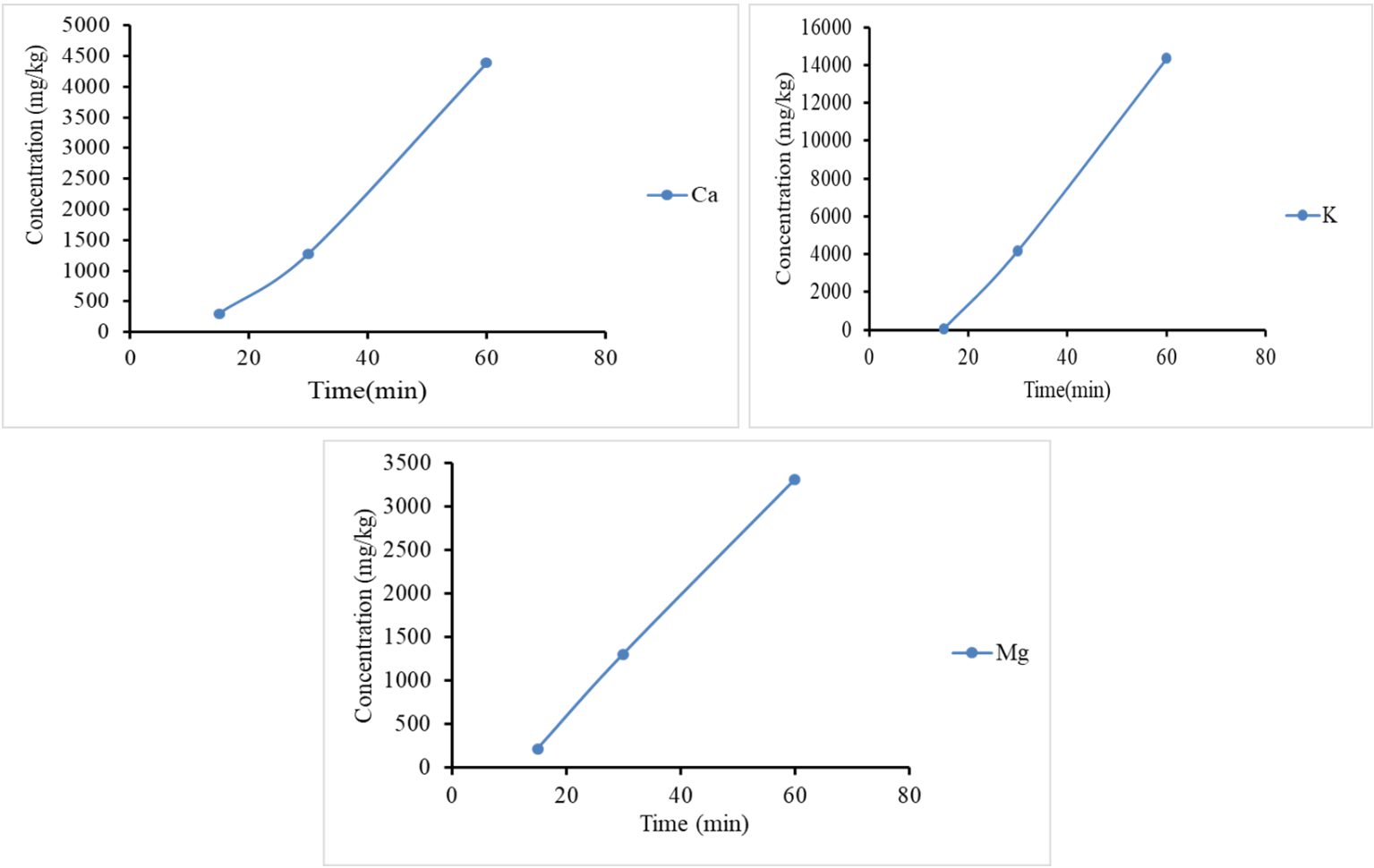


Figure 5.8 Macronutrients extracted at 150ºC at various extraction times

5.3 Comparison of total metal analysis to PHWE

Macro-nutrient components had a recovery rate of 82% to 91%, whereas micro-nutrients had a recovery rate of 25% to 49%. Copper and iron, two essential micronutrients, were recovered at 74% and 82%, respectively. The findings that the macro-nutrient components had a much higher recovery rate than the micro-nutrients in the dried stevia plant leaves and stem are significant in understanding the potential nutritional benefits of using stevia as a sweetener. While the plant is primarily known for its sweetening properties, it also contains many essential nutrients, including vitamins and minerals. The fact that the recovery rate for macro-nutrients was so much higher than that for micro-nutrients suggests that using stevia as a source of these nutrients may not be as efficient as previously thought. This information may be particularly relevant for individuals seeking to incorporate stevia into their diet to increase their nutrient intake.

The fact that copper and iron, two essential micronutrients, were recovered at a higher rate than other micro-nutrients is also noteworthy. Copper and iron are essential for various bodily functions, including forming red blood cells and maintaining a healthy immune system. The relatively high recovery rate for these two nutrients may make stevia a handy source of these nutrients for individuals who have specific nutritional needs or deficiencies. However, it is also worth noting that the recovery rate for these nutrients was still not as high as that for some of the macro-nutrients, which suggests that using stevia as a primary source of copper and iron may not be as efficient as other dietary sources. The findings support the viability of using PHWE stevia extracts in food production. Using PHWE, Nuapia et al. (2020) determined that the recovery of macro-nutrient components in Moringa varied from 88 to 98%, while the recovery of micro-nutrients ranged from 21 to 46%. The above results correlate with the results found in this study, as seen in table 5.1. Table 5.1 compares the total metal obtained in stevia leaves to that extracted by PHWE at 100^{o}C.

Table 5.1 Comparison of total metals in stevia leaves compared to that extracted by PHWE.

Metals	***PHWE (mg kg^{-1})***	***Microwave Digestion (mg kg^{-1})***	***Recovery (%)***
Al	7.52 ±0.05	14.672 ± 0.65	51 ± 4.43
Ca	639.63 ± 0.56	700 ±2.53	91.4 ± 0.96
Co	9.68 ±0.37	19.68 ± 0.75	49.19 ±0.65
Cr	1.97± 0.03	7.66 ±0.02	25.70 ±0.02
Cu	29.60 ± 0.03	39.67 ± 0.04	74.61 ± 0.06
Fe	12.37 ±0.04	15.00 ± 0.01	82.47 ± 0.15
K	2338.36 ± 2.81	2577.66 ±7.60	90.72 ± 2.40
Mg	771.29± 4.62	936 ± 1.05	82 ± 1.40
Ni	16.43 ± 1.32	38 ± 0.08	43.25 ± 0.05
Zn	5 .05 ± 0.03	9±0.013	55.5 ± 0.016

5.4 Comparison of the extraction mass transfer using first-order reaction kinetics

The natural logarithm of a metal extracted concentration versus time resulted in a linear graph (Figure 5.9 and 5.10). This demonstrates that the extraction of metals from *Stevia rebaudiana* leaves followed the first-order reaction kinetics. Using the first-order reaction kinetics, half-lives were calculated at 100^{o}C. The results are shown in Figure 5.9. Results indicate that between 3 and 6 minutes, half of the metals in the leaves were extracted by PHWE. Thus, to almost quantitatively extract the metals at 100^{o}C, one needs about 12minutes. Almost all metals are half extracted by 3 mins, indicating similar attachment strength to chemical ligands found in stevia leaves. However, an exception is an aluminium, with a half-life of nearly 6 mins. This suggests that aluminium is more tightly bound to chemical compounds in stevia leaves. In similar studies using PHWE, anthocyanin extraction from red onions followed a first-order reaction model (Westerberg et al., 2010). In a study by Heravi et al. (2015), sage medicinal plants' kinetic and thermodynamic parameters were examined. Rate constants were calculated under experimental conditions and found to be pseudo-first order similar to stevia. Table5.4 shows the rate constants calculated from the plots in figure 5.9. and figure 5.10. For a first-order reaction, a plot of the natural logarithm of the concentration of a reactant versus time is

a straight line with a slope of −*k*. The slope of the line in figure 5.9 was used to calculate the rate constant (k),

slope= log $[C]_a$ – Log $[C]_b$ / T_a -T_b, [C] is concentration and T is time.

Table 5.2 Table showing the rate constants calculated using the slope of graphs (Fig 5.9)

Metals	Rate constant (k)
Ni	$6.1 \times 10^{-1}\ s^{-1}$
Fe	$4.8 \times 10^{-1} s^{-1}$
Co	$6.1 \times 10^{-1} s^{-1}$
Al	$3.2 \times 10^{-1} s^{-1}$
Zn	$6.2 \times 10^{-1} s^{-1}$
Cr	$6.1 \times 10^{-1} s^{-1}$
Ca	$6.1 \times 10^{-1} s^{-1}$
K	$6.1 \times 10^{-1} s^{-1}$
Mg	$6.2 \times 10^{-1} s^{-1}$

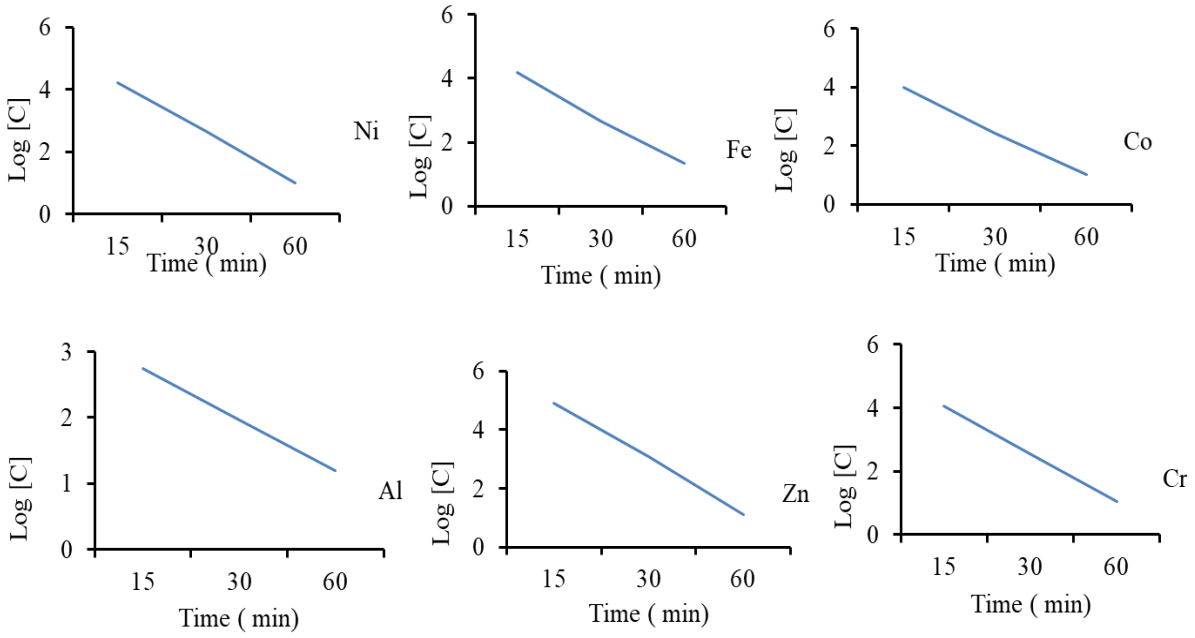


Figure 5.9 First-order reaction kinetics observed for micronutrient elements extracted by PHWE

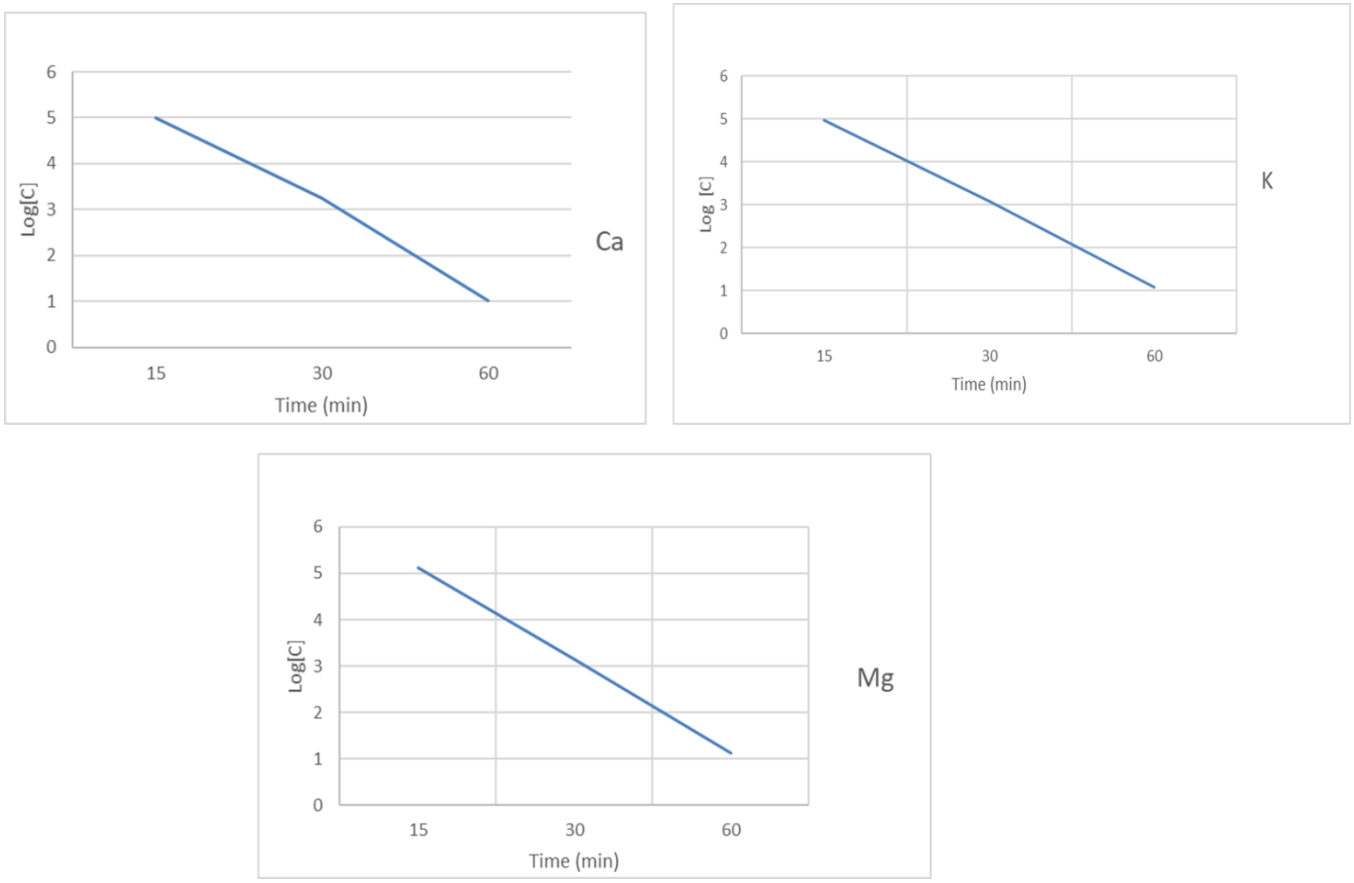


Figure 5. 10 First-order reaction kinetics observed for macronutrient elements extracted by PHWE

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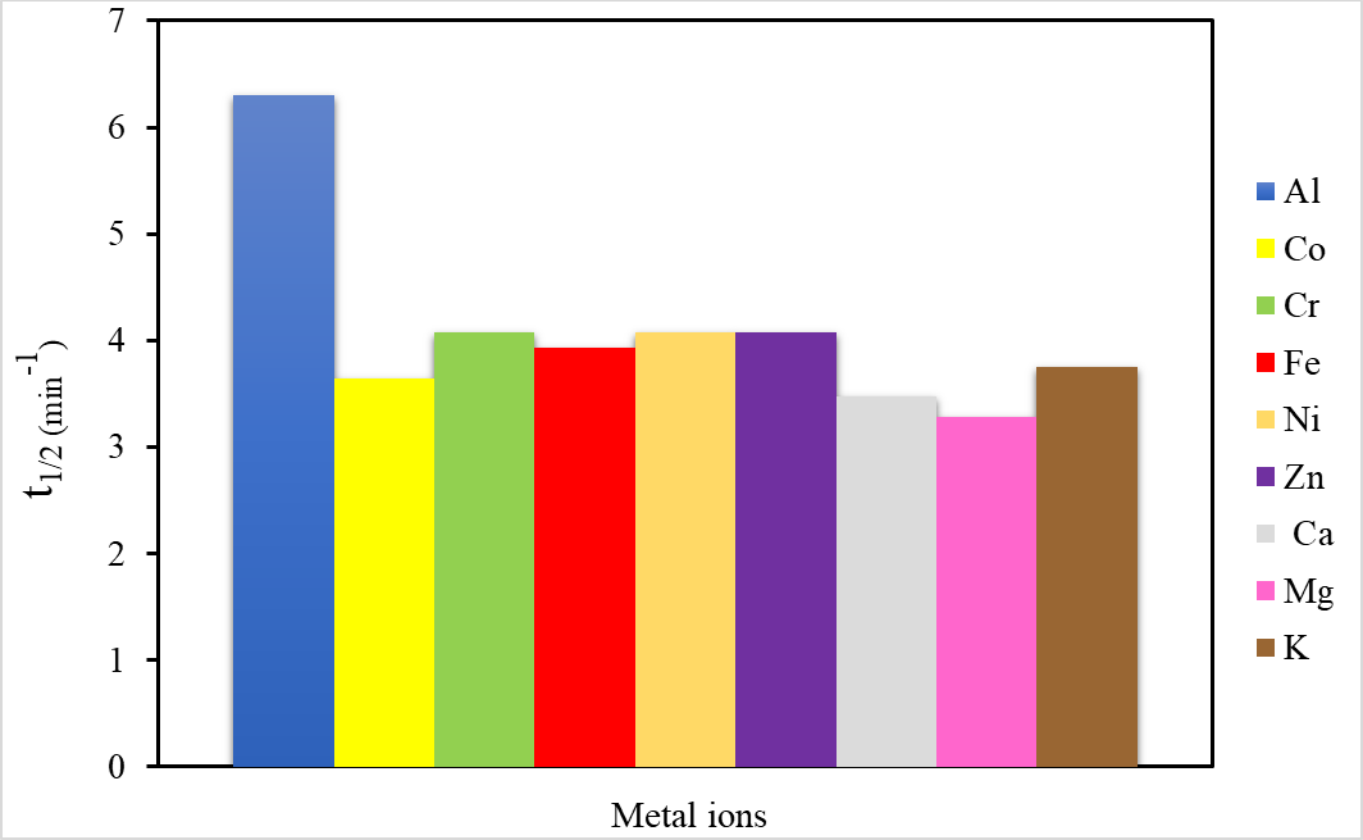

Figure 5. 11 Half-lives graphically demonstrated for the first-order reaction of macro and micronutrients.

CHAPTER 6

Conclusion and Recommendations

6.1 Conclusion

In conclusion, the findings of this study suggest that an increase in PHWE temperature and extraction time leads to an increase in the extractability of both macro and micronutrients in dried stevia plants. This suggests that PHWE can be an effective and efficient method for extracting nutrients from plant materials. The results also demonstrate that the order of extraction of micronutrients in dried stevia plants is Fe>Zn>Al>Ni>Co>Cr. This knowledge can help identify the essential micronutrients in stevia plants and optimise extraction conditions to target those nutrients specifically. However, it is essential to note that the results of this study were obtained using dried stevia plant materials, and further research is needed to investigate the effects of PHWE on the extraction of nutrients in fresh plant materials. Additionally, while the order of extraction of micronutrients provides valuable insight, it is essential to understand

the overall composition and nutritional value of the extracted materials. Therefore, future studies should aim to comprehensively evaluate the nutritional composition and bioavailability of the extracted nutrients and their potential applications in the food and agriculture industries. An increase influenced macronutrients in extraction time, and the extraction order was K>Ca>Mg. The total metal recovery of macro-nutrient elements ranged from 88 to 98%, while in micro-nutrients, it ranged from 21 to 46% using microwave digestion, proving to be higher than that of PHWE at optimum conditions. PHWE can quantitatively extract macro and micronutrients, and most nutrients are safe; therefore, *Stevia rebaudiana* could be used as a food supplement.

6.2 Recommendations

The study investigated the efficiency of PHWE for extracting macro and micronutrient elements from stevia plant leaves and stems. The researchers found that PHWE is an efficient method for extracting nutrients, particularly the macroelements potassium (K), calcium (Ca), and magnesium (Mg), from stevia leaves and stems. The study also showed that extraction efficiency is influenced by various factors such as temperature, pressure, and extraction time.

Based on the results of this study, several recommendations can be made regarding extracting macro and micronutrient elements from dried stevia plant leaves and stems using PHWE. First, it is recommended to use high pressure and temperature for efficient extraction of nutrients.

Second, it is recommended to use finely ground stevia plant material to increase the surface area available for extraction. This will improve the contact between the plant material and the extraction solvent, increasing nutrient extraction efficiency.

Third, it is essential to consider the environmental impact of the extraction process. The use of pressurised hot water can consume a significant amount of energy, which can contribute to greenhouse gas emissions. Therefore, it is recommended to use renewable energy sources, such as solar or wind energy, to power the extraction process.

Fourth, it is essential to consider the economic viability of the extraction process. The equipment used for PHWE can be high, impacting the extracted nutrients' overall cost. Therefore, it is recommended to consider the cost-benefit analysis of the extraction process, including the value of the extracted nutrients and the cost of the equipment.

As such, extracting macro and micronutrient elements from dried stevia plant leaves and stems using PHWE is an efficient method for nutrient extraction. The recommendations outlined above can help improve the extraction process's efficiency, sustainability, and economic viability. By following these recommendations, researchers and industries can effectively extract nutrients from stevia plant material for use in food, pharmaceutical, and other industries.

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APPENDIX

Table A 1 Micro nutrients at 20 mins at 50°C ,100°C and 150°C

F-statistic value = 532.53152

P-value = 0

Data summary

GroupsN	Mean	Std.Dev.	Std Error
Group 1(Al)	3	7.2167	0.3213 0.1855
Group 2(Co)	3	2.113	0.0289 0.0167
Group 3(Cr)	3	1.8667	0.138 0.0797
Group 4(Fe)	3	12.07	0.3894 0.2248
Group 5 (Ni)	3	3.9767	0.0635 0.0367
Group 6 (Zn)	3	4.5833	0.4343 0.2508

ANOVA summary

Source	Degrees of freedom	Sum of squares	Mean squares	F stat	P-value
Between groups	5	221.1417	44.2283	567.7652	0
Within groups	12	0.9348	0.0779		
Total	17	222.0765			

Table A 2 Micro nutrients at 50°C

F-statistic value = 8.23848, P-value = 0.0014

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Al)	3	38.35	42.67	24.6355	
Group 2(Co)	3	8.39	10.1315	5.8494	
Group 3(Cr)	3	39.31	44.8254	25.88	
Group 4(Fe)	3	33.1	38.3416	22.1365	
Group 5(Ni)	3	15.44	16.6213	9.5963	
Group 6(Zn)	3	312.39	156.0294	90.0836	
ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	5	206128.159	41225.6318	8.2385	0.0014
Within Groups	12	60048.4245	5004.0354		

Table A 3 Micro nutrients at 100°C

F-statistic value = 2.31385

P-value = 0.10856

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Al)	3	29.24	31.7246	18.3162	
Group 2(Co)	3	8.76	10.0464	5.8003	
Group 3(Cr)	3	43.52	49.6421	28.6609	
Group 4(Fe)	3	3465.28	3902.7826	2253.2726	
Group 5(Ni)	3	16.43	17.854	10.308	
Group 6(Zn)	3	88.01	84.8532	48.99	
ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	5	29391243.8982	5878248.7796	2.3138	0.1086
Within Groups	12	30485605.1444	2540467.0954		
Total:	17	59876849.0426			

Table A 4 Micro nutrients at 150°C

F-statistic value = 2.91713

P-value = 0.05971

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1	3	37.77	36.4011	21.0162	
Group 2	3	8.46	9.8661	5.6962	
Group 3	3	42.57	48.3832	27.9341	
Group 4	3	111.42	129.0579	74.5116	
Group 5	3	1613.81	1572.0736	907.6371	
Group 6	3	83.98	96.7502	55.8587	
ANOVA Summary					
Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Stat	P-
	DF	SS	MS		Value
Between Groups	5	6080259.3869	1216051.8774	2.9171	0.0597
Within Groups	12	5002390.5212	416865.8768		
Total:	17	11082649.9081			

Table A 5 Macro nutrients at 20 mins at 50°C, 100° C and 150°C

F-statistic value = 1.17714

P-value = 0.37045

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Ca)	3	634.1367	6.406	3.6985	
Group 2(K)	3	1571.13	1376.6885	794.8315	
Group 3(Mg)	3	771.2867	250.277	144.4975	
ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	2	1536515.8713	768257.9356	1.1771	0.3704
Within Groups	6	3915901.6792	652650.2799		
Total:	8	5452417.5505			

Table A 6 Macronutrients at 50°C for 15,30 and 60 mins

F-statistic value = 0.0805

P-value = 0.92364

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Ca)	3	2323.1267	3026.8966	1747.5796	
Group 2(K)	3	1888.5567	2110.8423	1218.6954	
Group 3(Mg)	3	2779.12	2924.8481	1688.6618	
ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	2	1189883.9659	594941.9829	0.0805	0.9236
Within Groups	6	44344989.3013	7390831.5502		
Total:	8	45534873.2671			

Table A 7 Macro nutrients at 100°C for 15,30 and 60 mins

F-statistic value = 0.42594

P-value = 0.67147

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Ca)	3	2103.4	2258.7901	1304.1131	
Group 2(K)	3	3465.28	3902.7826	2253.2726	
Group 3(Mg)	3	1493.53	1094.9754	632.1843	
ANOVA Summary					
Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Stat	P-Value
	DF	SS	MS		
Between Groups	2	6114456.6138	3057228.3069	0.4259	0.6715
Within Groups	6	43065631.7307	7177605.2884		
Total:	8	49180088.3445			

Table A 8 Macro nutrients at 150°C for 15,30 and 60 mins

F-statistic value = 9.91254

P-value = 0.01254

Data Summary					
Groups	N	Mean	Std. Dev.	Std. Error	
Group 1(Ca)	3	1990.55	2137.0199	1233.809	
Group 2(K)	3	10412.14	4779.9594	2759.7109	
Group 3(Mg)	3	83.98	96.7502	55.8587	
ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	2	181229076.2786	90614538.1393	9.9125	0.0125
Within Groups	6	54848453.0397	9141408.8399		
Total:	8	236077529.3183			