

Development of various green extraction techniques and water extraction online particle formation for *Moringa oleifera*



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Declaration

I declare that this research is my own, unassisted work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Yannick Belo Nuapia

This 9th day of May 2019 in Johannesburg

List of publications

1. **Statistical comparison of two modelling on pressurized hot water extraction of vitamin C and phenolic compounds from *Moringa oleifera* leaves.** Yannick Nuapia, Ewa Cukrowska, Hlanganani Tutu, Luke Chimuka. Published in South Africa Journal of Botany (2018), doi.org/10.1016/j.sajb.201809.001 in press.
2. **Chemometric approach on the behaviour of macro and micro-nutrient elements in the *Moringa oleifera* grown in Southern Africa when extracted by pressurized hot water extraction technique.** Yannick Nuapia, Phatsimo G Matshediso, Ewa Cukrowska, Hlanganani Tutu, Luke Chimuka. Submitted to Food Chemistry Journal. JFC-D-18-00936.
3. **Pressurized hot water extraction with on-line particle formation of *Moringa oleifera* leaves.** Yannick Nuapia, Said Al-Hamimi, Larissa Cunico, Margareta Sandahl, Hlanganani Tutu, Luke Chimuka, Ewa Cukrowska, Charlotta Turner. Manuscript in preparation.
4. **Ultra-high-pressure supercritical fluid extraction and chromatography of oil from *Moringa Oleifera* and *Perigrina* seeds.** Yannick Nuapia Belo, Said Al-Hamimi, Luke Chimuka, Charlotta Turner. Accepted for publication in Analytical and Bioanalytical Chemistry. Manuscript ID: ABC-00424-2019.R1.

Contribution of the authors

The principal author was involved in conceptualizing the idea, did experiments, analysed data and wrote all manuscripts and published papers presented in this thesis. Co-authors helped with analysis of results, revised the drafted manuscripts and made suggestions for improvement.

Abstract

Moringa oleifera is an excellent source of bioactive compounds which exhibit several nutritional and medicinal properties. Different parts of the plant are used as source material of these compounds. This thesis provides data on: (i) pressurized hot water extraction of vitamin C, polyphenols and other nutrients from *Moringa oleifera* leaves and, (ii) ultra-high supercritical fluid extraction and characterization of oil compounds from *Moringa oleifera* and *peregrina* seeds. In addition, data on water extraction with on-line particle formation developed for drying aqueous extract is also given. The details of the current work are described as follows:

The first part of the work involved the optimization of pressurized hot water extraction for producing *Moringa oleifera* extract rich in vitamin C and polyphenolic compounds mainly kaempferol and quercetin. This work has been published in South Africa Journal of Botany and it is presented in this thesis as **Paper 1**. Response surface methodology (RSM) and artificial neural network (ANN) were used to understand the influence of main parameters such as temperature, time and flow rate in the extraction process. The optimal conditions for an efficient pressurized hot water extraction were found to be 91°C for temperature, 60 min for extraction time and 0.3 ml min⁻¹ for flow rate. The predicted concentrations of kaempferol, quercetin and vitamin C was found to be 3.92 ± 0.30 (with a recovery of 94.23%), 4.74 ± 0.32 (with recovery of 93.55%) and 0.357 ± 0.19 (with a recovery of 85.02%) g per 100 g of dry powder, respectively.

Also, macro and micro-nutrients were extracted using pressurized hot water extraction from *Moringa oleifera* leaves. This work has been submitted to Food Chemistry Journal and it is presented in this thesis as **Paper 2**. Response surface design of the experiment was applied to investigate the influence of two independent factors which are temperature (50 - 200°C) and time (5 – 60 min) on the behavior pattern of macro and micro-nutrients from the leaves of *Moringa oleifera*. The obtained results showed that the macro-elements such as Ca, K, Mg, and Na, were less influenced by the extraction temperature while, the micro-nutrients like Al, Co, Cr, Cu, Fe, Ni and Zn depended on the extraction temperature.

The recovery of macro-nutrients ranged from 89 ± 2 to $99\pm 1\%$ while that of micro-nutrients ranged from 23 ± 2 to $37\pm 4\%$. Therefore, at optimum conditions, more macro-nutrients than micro-nutrients were extracted. This study showed that pressurized hot water extraction was successfully used to extract metal species from *Moringa oleifera* leaves just like organic compounds.

Pressurized hot water hot extraction was used for the recovery of polyphenol compounds, vitamin C, macro and micro-nutrients from the *Moringa oleifera* leaves (**Paper 1 and 2**). The technique uses water as a green solvent and has emerged as a sustainable alternative for the extraction of bioactive compounds from the plant. The main disadvantage of using water as a solvent, compared to organic solvents, is the difficulties to concentrate the obtained extracts. The presence of water might be detrimental to the stability of the extract and increases the cost of transportation of a final commercial product since the amount of bioactive compounds per mass of water extract is low. Drying the water extracts involves extra process steps to obtain a commercial product. Thus, in **Paper 3**, pressurized hot water extraction of antioxidants and other essential chemicals from *Moringa oleifera* leaves combined with one-step on-line drying technique, based on the use of supercritical carbon dioxide, was optimized. This extraction process is also called WEPO (water extraction with on-line particle formation). A full factorial design was applied to investigate the influence of three qualitative such as CO₂ pressure, CO₂ flow rate and nitrogen pressure and five quantitative factors including nitrogen position, use of vacuum, length of the tubing, the internal diameter of the nozzle and additional nitrogen on the particle size and distribution (nm), water content (%) and the amount (mg) of powder collected. These factors were first screened, and qualitative factors were found to be the most important parameters for the optimization of the WEPO technique. The obtained optimum conditions for fine and dried particles were found to be 90.95 bars CO₂ pressure, 16 ml min⁻¹ flow rate of CO₂ and 26 bars nitrogen pressure. With these conditions, the amount of powder collected, water content and the average size of particles were 150 ± 10 mg, $3.58 \pm 0.56\%$ and 384.33 ± 21.36 nm, respectively. Results were compared with those from extracts obtained by continuous flow PHWE followed by a freeze and spray drying techniques. The results showed that the WEPO process gave fast results with

well-defined particles and uniform particle sizes and exhibiting high water solubility as compared with other techniques.

Ultra-high-pressure supercritical fluid extraction method was optimized and applied to the extract, and characterized oil from two *Moringa* species namely, *Moringa oleifera* and *peregrina* seeds (**Paper 4**). A full factorial design was used to investigate the influence of pressure and temperature (40 to 80 MPa and 40 to 70°C, respectively), on the amount of oil extracted from crushed seeds. The results revealed that pressure has significant positive influence on the amount of oil extracted. The optimum conditions were found at 80 MPa and 57°C. The extracted amounts obtained at optimum conditions were 396 ± 23 and 529 ± 26 mg per sample mass, for *Moringa oleifera* and *peregina* seed oil, respectively. The extraction kinetics study revealed a mainly solubility-controlled extraction of oil; and 28 g of CO₂ was required to extract 400 mg of oil per gram of seeds using the developed method. Addition of ethanol into the extraction vessel prior to the extraction increased the polar fraction in the oil extracted. The developed method increased the extracted oil by two folds compared to a sonication method. The obtained oil consisted mainly of glycerolipids, sterol esters and phospholipids. Phospholipids, campesterol and stigmasterol esters were found higher in *Moringa oleifera* while cholesterol esters were more abundant in *Moringa peregrina*.

Dedications

This thesis is dedicated to my wife and children Zoe Belo Kena Nuapia and Immaculate Belo Kena Nuapia for being there for me during my doctoral research studies. Special feeling of gratitude to my loving parents, Henry Belo Nuapia and Marceline Iyodja whose words of encouragement and push for tenacity still ring in my ears.

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List of abbreviations

DG	:	Diacylglycerol
FFAs	:	free fatty acids
GXL	:	Gas expanded liquid
LPC	:	Lysophosphatidylcholine
MAE	:	Microwave-assisted extraction
MG	:	Monoacylglycerol
MUFAs	:	Monounsaturated fatty acid
PC	:	Phosphatidylcholine
PE	:	Phosphatidylcholine,
PI	:	Phosphatidylinositol,
PG	:	Phosphatidylglycerol
PHWE	:	Pressurized hot water extraction
PUFAs	:	Polyunsaturated fatty acid
SCF	:	Supercritical fluid
SC-CO ₂	:	Supercritical carbon dioxide
SE	:	Soxhlet extraction
SER	:	Sterol ester
SFE	:	Supercritical fluid extraction
TG	:	Triacylglycerol
UAE	:	Ultrasound-assisted extraction
WHO	:	World Health Organization

CHAPTER 1

1 Introduction

The use of plants as the potential source of natural compounds is growing. Several research studies have reported that 50% of all drugs in clinical use worldwide are from natural products and their derivatives (Acosta et al., 2016; Raut and Wairkar, 2018; Romero-gonz et al., 2016; Wai et al., 2018). In many cultures, plant-based extracts play a vital role in human health care system. Such plants are called nutraceuticals. They are used as nutritional or medicinal supplements (Acosta et al., 2016).

The term nutraceutical is derived from the words 'nutrition' and 'pharmaceutical' (Wai et al., 2018). It is food of which natural derivative products may provide medicinal and nutritional benefits (Wai et al., 2018). A plant extract is recognized as nutraceutical after there is sufficient clinical data to demonstrate such benefit in human or animal health (Rossi et al., 2018; Gentile et al., 2019).

Nutraceutical products occur at the crossroads of the food, cosmetics, and pharmaceutical ingredients. They range from dietary supplements, isolated nutrients from herbal products, processed foods and beverages (Mokhtari et al., 2017). Chemically, nutraceutical products could contain phenolic compounds, isoprenoid, carbohydrate, vitamins, fatty acid and structural lipids, amino acids, probiotics, prebiotics and minerals (Bhatt et al., 2017). Some of the above chemicals play an important role in human and animal homeostasis and in maintaining the optimal immune response (López-gutiérrez et al., 2015). The deficiency or excessive intakes of these compounds can have negative impacts on human health.

Worldwide, nutraceuticals have been accepted as supplementary therapy to the conventional drugs (Sharma et al., 2019). The healthcare industry has seen the growing demand for nutraceutical products due to their anti-carcinogenicity,

antioxidative, antimutagenicity and antiaging properties (Gentile et al., 2019; Lotha et al., 2018; Mokhtari et al., 2017; Mollica et al., 2018; Spazzafumo et al., 2016). Some studies on the benefits of nutraceutical products have clearly shown an insightful relationship between optimal health and diet. Particularly with respect to reducing the risk posed by degenerative diseases such as diabetes, cancer, osteoporosis, stroke and heart disease (Mollica et al., 2018).

World Health Organization (WHO) reported that over 65% of the worldwide population used plants as sources of conventional medicines for their primary health care (WHO, 2005). Due to the benefits that the nutraceutical products provide, their development has arisen as an important tool, which can improve the mental and physical well-being of the consumer. Increased cost of health care and the desire of the population to improve their quality of life as well as life expectancy led pharmaceutical and food industries to develop several extraction and drying techniques which can improve the isolation and conservation of bioactive compounds from plant material, as sources of nutraceutical ingredients (Lotha et al., 2018).

One of the challenges when extracting phytochemicals (nutraceutical candidates) such as phenolic compounds and vitamin C is to reduce thermal degradation throughout the recovery process and, at the same time, allow the extraction to go to completeness. Conventionally, mass transfer and speed of extraction are enhanced by increasing the temperature as it is the case for pressurized hot water extraction (PHWE), microwave-assisted extraction and solvent assisted extraction. However, this certainly leads to thermal degradation of sensitive compounds like polyphenols and vitamin C. An alternative is to optimise the technique such as pressurized hot water extraction for a complete extraction and no degradation of target compounds (**Paper 1 and 2**). PHWE is advantageous in that it uses water as solvent which is readily available, cheap and not toxic. However, water is not a good medium for the long-term stability of bioactive compounds, so, a way of removing it from the extract is needed. For large applications of such extracts in food and cosmetic industries, storage space of large volumes can be a problem and some applications

like lotion formulations are not water based so the extract must rather be in powder form. In this thesis, *Moringa oleifera* extract from the PHWE was able to be dried by converting it into particle form, i.e., a powder made of micron-sized particles. The extract, in its particle form, had the potential for value addition expansion in as far as its application in the food and cosmetic industries is concerned. One way to reduce the amount of water in the extract is to spray-dry it. This technique is rather costly and time-consuming and may also lead to degradation of the bioactive compounds due to heat, light and oxygen (Plaza and Turner 2015). In this thesis, reducing the amount of water in the *Moringa oleifera* extract was achieved by water extraction followed by an online particle formation (WEPO), based on the use of supercritical carbon dioxide (Sievers et al., 2001, Rodriguez-Meizoso et al., 2012; Andersson et al., 2012). In this novel method, the PHWE extract is mixed continuously with supercritical carbon dioxide in a small-volume at T-junction, resulting in a spray of small-sized water droplets that are rapidly dried by a stream of hot nitrogen. The results of this work are presented in **Paper 3**.

On the other hand, the development of a high-diffusivity fluid such as a supercritical fluid (SCF) seems to be more important for the extraction of non-polar compounds such as lipid from oil. Supercritical carbon dioxide (SC-CO₂) above 31°C and 7.4 MPa gives fast mass transfer (Costa et al., 2012). Sc-CO₂ is a well-known solvent for its low viscosity and fast diffusion properties. Solubility of an analyte in Sc-CO₂ is generally suggested to be controlled by two parameters, the vapor pressure of the analyte and its interactions with the Sc-CO₂. These two parameters are influenced by temperature and pressure (Andersson et al., 2012).

Therefore, temperature and pressure are principal factors that can control the extraction process in supercritical fluid extraction (SFE). Optimisation of these two factors is important in SFE to obtain fast, efficient and selective extraction method. Temperature in SFE has contradictory effects depending on the operational range and type of analytes. For instance, temperature can increase mass transfer properties and enhance vapor pressure of the analytes, which leads to increase in recovery. Moreover, high temperature decreases the density of the ScCO₂ and also changes

the solubility parameters and subsequently, may lower the solvation power. Increasing the pressure naturally leads to higher density. For example, increasing the pressure at 50°C from 20 MPa to 80 MPa increases the density from 0.785 to 1.043 g ml⁻¹. The increase in the density is associated with an increase in solubility parameters. Using ultrahigh pressure Sc-CO₂ extraction might enhance the extractability, alter the selectivity and improve the extraction rate. Ultrahigh pressure Sc-CO₂ was used for defatting food ingredients like almond and cocoa powder (Ghafoor et al., 2010).

Pressure between 60 and 85 MPa showed the best results to extract the maximum amount of oil from coriander seeds (Basa'ar et al., 2017). Sc-CO₂ extraction technique has been applied to extract oil from Moringa seeds (Barakat et al. 2016; Boulaadjoul et al., 2018). The influence of extraction pressure and temperature on the oil yield and quality was assessed in the range of 20 and 50 MPa and between 40 and 100°C, respectively. In most studies pressure was a significant factor impacting the extracted amount. In these studies, the composition of the oil was evaluated through analysis of fatty acids by GC with FID or MS after hydrolysis and derivatization (Ee et al., 2008). Such an investigation does not provide complete information as to whether the fatty acids originally present are as free fatty acids or are attached to glycerolipids, phospholipids, or glycolipids. For this reason, in the present thesis, extraction method of oil from Moringa seeds based on ultrahigh pressure supercritical fluid extraction (UHPSFE) was developed and optimised and ultrahigh performance supercritical fluid chromatography coupled to high resolution mass spectrometry (UHPSFC/QTOF-MS) was used for a comprehensive lipid profiling analysis of the obtained oil. This work has been submitted at the Journal of Agricultural and Food Chemistry and presented as **Paper 4**.

CHAPTER 2

2 Literature review

2.1 Review of bioactive compounds from *Moringa oleifera* leaves and their health benefits

The research on bioactive compounds is essential for the control of many human sicknesses (Ahmed et al., 2016). Medicinal plants with antioxidant, anti-inflammatory and anti-microbial activities are a source of novel phytochemicals (Oroian and Escriche, 2015). *Moringa oleifera* a genus species of moringaceae family, is generally distributed in the subtropical and tropical region of Africa, the Middle East, and Asia (Gupta et al., 2017). In South Africa, *Moringa oleifera* plant is mostly grown in the provinces of Limpopo, North West, some parts of Gauteng and KwanZulu-Natal. It is used as a medicinal plant, vegetable and nutraceutical food that contains essential nutritional and herbal chemicals with various therapeutic and pharmacological activities (Cuellar-Núñez et al., 2018).

The screening of the bioactive compounds from *Moringa oleifera* leaves has been reported in several studies (Cuellar-Núñez et al., 2018a; Gopalakrishnan et al., 2016; Saucedo-Pompa et al., 2018). They have reported that the leaves are an excellent source of compounds such as carotenoids, essential amino, flavonoids, phenolic acids, polyphenols, and vitamins. *Moringa oleifera* has traditionally been applied in the treatment of diabetes, hypertension, malaria, parasitic diseases and skin diseases (Falowo et al., 2018; Saucedo-Pompa et al., 2018). It has been proved that it exhibits pharmacological activities such as antioxidant (Guillén-Román et al., 2018), anti-inflammatory, anti-hyperglycemic, anti-hyperlipidemic, and anti-cancer (Cuellar-Núñez et al., 2018a; Singh et al., 2009). This section reviewed, the *Moringa oleifera* bioactive compounds (mainly phenolic compounds and fatty acids) and its nutritional and medicinal applications.

2.1.1 Phytochemical compounds

Traditionally, the general health benefit of a diet has been associated with the presence of vitamins, lipid, carbohydrates, proteins, and minerals (McCarthy and O’Gara, 2015). However, several studies have demonstrated that the health benefit of nutraceutical foods is mostly related to the contribution of non-nutrient compounds especially bioactive compounds (Gupta et al., 2017; McCarthy and O’Gara, 2015; Nouhi et al., 2018). A bioactive compound can be defined as any natural substance found at low concentration in plants and food-derived (vegetables, nuts oils, grains and fruits) that exhibit several physiological activities which contribute to the proper functioning of the human organism (Cuellar-Núñez et al., 2018). Various classifications have been proposed relating to their botanical categorisation such as clinical function, pathways, and biochemical classes (Ohta et al., 2017). The compounds like fatty acids, organic acid, and phytosterols are primary metabolites and perform an essential metabolic function for plants. The secondary metabolites (phytochemicals) do not participate in the central plant metabolic reactions (He et al., 2018a). However, they play a vital role in the living organism or human body system.

Phytochemicals are mostly classified according to their chemicals structure such as inorganic (minerals) and organic (alkaloids, phenolic compounds, unsaturated fatty acid, phytosterols, oligosaccharides, polysaccharides, organosulfur compounds, and nitrogen-contain compounds). The classification of bioactive compounds is shown in Figure 2.1.

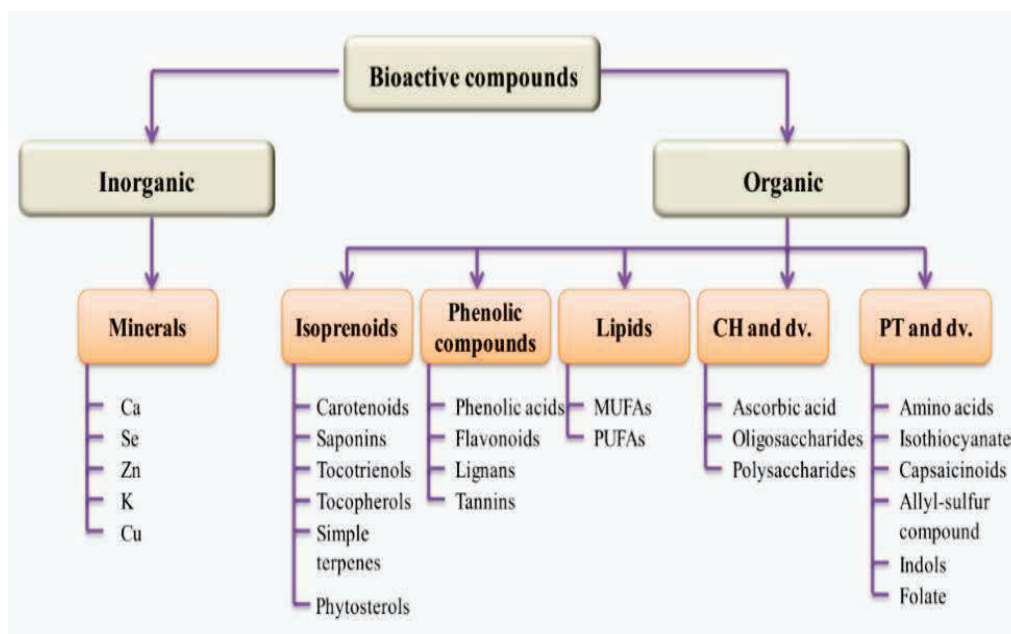


Figure 2.1. Classification of bioactive compounds (Nouhi et al., 2018)

2.1.1.1 Polyphenol compounds

Polyphenol compounds such as phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids are natural substance extracted from the plants (Hamany Djande et al., 2018a). They are biogenetically produced by the malonic and shikimic pathways as presented in Figure 2.2. They possess in their structure one or several aromatic rings which are linked with hydroxide groups. Hence, over 8000 phenolic compounds with various chemical compositions have been reported from Moringa plants (Castillo-López et al., 2017; Lin et al., 2018; Nouman et al., 2016). They are usually combined with other different molecules such as proteins, lipids, carboxylic acids, and carbohydrates (Prasanth et al., 2011).

Amongst the phenolic compounds, the flavonoids are the polyphenols with fifteen carbons and two aromatic rings linked by a three-carbon bridge. Other flavonoids are phenolic acid compounds such as stilbenes and gallic acid (Hamany Djande et al., 2018a). The stilbenes are the most commonly distributed phenolic compounds in all the plant kingdom (Vergara-Jimenez et al., 2017).

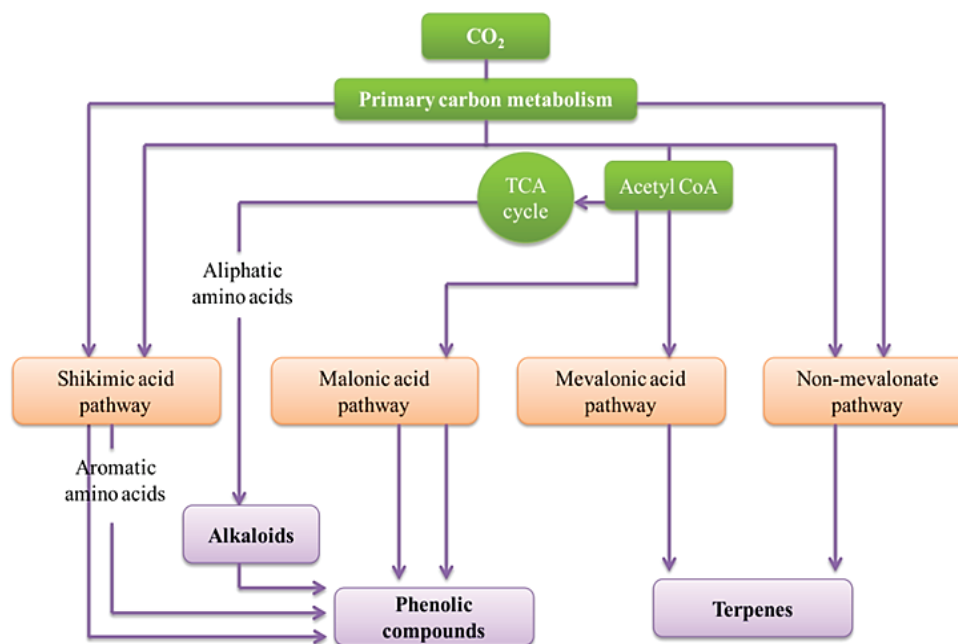


Figure 2.2. Malonic and shikimic pathways for the synthesis of polyphenol compounds (Prasanth et al., 2011)

More than 500 flavonoid compounds have been reported from various plants (Maldini et al., 2014; Rajanandh and Kavitha, 2010; Rodríguez-Pérez et al., 2015). They exhibit many pharmacology activities such as antifungal, antibiotic, antioxidant and antiviral. Epidemiological studies have shown that the ingestion of flavonoids protects the body against several chronic diseases related to oxidative stress, cancer and cardiovascular illness (Lin et al., 2018). *Moringa oleifera* leaves have been reported to supply a high amount of flavonoid compounds such as kaempferol and quercetin (Coppin et al., 2013). Several studies have reported a total concentrations of kaempferol and quercetin ranging from 3,41 to 11 g per 100 g and 1,23 to 14,34 g per 100 g, respectively (Matshediso et al., 2015; Prasanth et al., 2011; Vats and Gupta, 2017). They are linked with sugars such as rhamnose, glucose, and rutinose, which are usually found as monosaccharides, disaccharides, or oligosaccharides via C- or O-glycosylation in *Moringa oleifera* (Makita et al., 2017). They are also conjugated with the glycoside group such as galactosyl moiety, rhamnosyl, xylosyl, apiosyl, acetyl, malonyl, succinoyl. Coppin et al.

(2013) have found six quercetin, and kaempferol derivatives from the *Moringa oleifera* leave samples from Ghana and Zambia.

Makita et al. (2016) also identified the same number of quercetin, and kaempferol derivatives from *Moringa oleifera* leaves from South Africa and Namibia. They have found other compounds such as multiflora B, isorhamnetin and apigenin (known as vitexin). These compounds have gained particular attention as beneficial and health agents because of their antioxidant activities (Makita et al., 2017b; Maldini et al., 2014; Rodríguez-Pérez et al., 2016). Specifically, vitexin and its derivatives have been shown to exhibit anti-Alzheimer, anti-diabetic and anti-inflammatory activities (Ramabulana et al., 2016). Flavonoids have been demonstrated to be a powerful antioxidant than many vitamins (Vats and Gupta, 2017). For all these reasons, *Moringa oleifera* leaves can be used as food ingredients and nutraceutical food.

2.1.1.2 Fatty acids

Fatty acids are carboxylic acids with a hydrocarbon chain of an even number of carbon atoms ranging from 16 - 26. They contain 0 to 6 carbon-carbon double bonds that mainly possess cis-geometry, however, some fatty acids with trans-geometry are also reported in the literature (Crompton and Dunstan, 2018; Outram and Zhang, 2018; Rashid et al., 2008). Fatty acids have different classes such as triacylglycerols, waxes and fats (Flores et al., 2018; Afolabi et al., 2018).

Fatty acids with a single bond among adjacent carbon atoms are called saturated and those with at least one double bond in their structure are defined as unsaturated (Stroescu et al., 2013). Within these groups, those with one double bond are defined as monounsaturated fatty acids (MUFAs) and while those with two or more double bonds are generally known as polyunsaturated fatty acids (PUFAs). The PUFAs can be classified in two groups such as short and long chain fatty acids (Porto et al., 2016). The nomenclature of the fatty acids is also based on the total chain length and the position of the bonds (Nkukwana et al., 2014). The double bonds of the unsaturated fatty acids are numbered from the methyl group defining the metabolic

family, represented as n-x or ω -x, where the x represents the position of the first double bond from the methyl end (Vorster et al., 2018).

Due to their structural variety, fatty acids are used for various purposes ranging from food, cosmetic, to producing detergents, soaps, coatings, lubricants and among other products. Among them, 20-25 fatty acids are generally distributed in nature. They have a length of 10 to 22 carbons in their chain (Boulaadjoul et al., 2018). They are found in large amounts in animal fats and plant oils (Wang et al., 2017). The oils from vegetables and seeds represent approximately 97% of total oil production in the world (Outram and Zhang, 2018). They are a source of myristic, lauric, stearic, palmitic, linoleic, oleic, erucic and α -linoleic fatty acids. This thesis also explored the extraction of lipid compounds extracted from seeds of *Moringa oleifera* and *peregrine* plants.

2.1.2 Nutritional properties

Each part of the *Moringa oleifera* plant has essential nutrients. Its leaves are a rich source of minerals such as calcium, copper, iron, magnesium, potassium, and zinc (Castillo-López et al., 2017). It is also a source of vitamins like vitamin A, vitamin B, nicotinic acid, pyridoxine, folic acid, and vitamins D, E and C (Nouhi et al., 2018). The leaves have a low calorific value, therefore, it is used in the diet of obese people (Lin et al., 2018). The pods are an excellent source of fibre. It is applied for treating digestive problems and colon cancer (Cuellar-Núñez et al., 2018). It was reported from literature that immature pods contain about 46.78%, 20.66%, and 30% of fibre, protein and amino acids, respectively (Gupta et al., 2017). Its high amount of minerals makes the tree essential for development and growth for young children (Cuellar-Núñez et al., 2018). For instance, the calcium is considered as one of the essential mineral for the bone and teeth (Oyeyinka and Oyeyinka, 2018).

As compared with other products, for example, 1 kg of milk can provide 300 to 400 mg of calcium, a similar amount of Moringa leaves can provide 1,000 mg of Ca,

and Moringa dry powder can offer more than 4,000 mg of Ca (Omotoso et al., 2018).

Also, Moringa powder can substitute iron tablets, in the treatment of anemia (Lin et al., 2018). For example, beef contains only 2 mg of iron per g of meat, while Moringa leaf powder contains 28 mg of iron per g of dry leaves (Falowo et al., 2018). It was reported that *Moringa oleifera* leaves contain a higher level of iron than spinach (Saucedo-Pompa et al., 2018). Zinc intake is vital for the synthesis of RNA and DNA, and sperm growth (Andrade et al., 2011). The leaves of *Moringa oleifera* have an approximate level of 25.5-31.03 mg zinc per kg. This amount corresponds to the daily requirement of zinc in the diet (Falowo et al., 2018). The seeds of *Moringa oleifera* plant are an excellent source of polyunsaturated fatty acids (PUFAs) such as linoleic acid, linolenic acid and oleic acid (Barakat and Ghazal, 2016). The PUFAs have an essential role in the metabolism of cholesterol. Several studies have shown that moringa seed oil contains 76% of PUFAs, making it ideal for substituting olive oil in the market (Andrade et al., 2011).

2.1.3 Medicinal properties

Moringa oleifera has exhibited several medicinal activities which explain the use of the plant in many diseases (Cuellar-Núñez et al., 2018). It has been applied in traditional medicine in India and several African countries (Olurisha et al., 2016). The presence of bioactive compounds with several physiological and pharmacology activities makes it an excellent medicinal agent (Cohen-Zinder et al., 2017). In this section, the effect of moringa against several ailments is reviewed.

2.1.3.1 *Moringa oleifera* as an anti-diabetic agent

Several clinical essays of *Moringa oleifera* extract have successfully shown a positive response in patients suffering from diabetes type 1 and 2 (Paula et al., 2017; Rajanandh et al., 2012). Type 1 is characterized by a non-production of insulin by the patient. The insulin is a hormone that sustains the blood sugar level at the required average value. Type 2 diabetes also called insulin resistance diseases (Olurisha et al., 2016), is a type of diabetes caused by the non-sensibility of beta-

cells for recognizing the sugar levels, hence reducing the production of insulin (Elwan et al., 2018). Several studies have shown that *Moringa oleifera* can act as an anti-diabetic agent (Chin et al., 2018; Elwan et al., 2018; Karthivashan et al., 2015b; Paula et al., 2017; Vijay and Kumar, 2012). A study done in rats demonstrated that the aqueous extract of *Moringa oleifera* could stop the streptococcin induced type 1 diabetes and reactivate the sense of the beta cell to recognize the sugar level for type 2 diabetes (Yassa and Tohamy, 2014).

In a similar study, the patient with diabetes was fed with the *Moringa oleifera* dry powder (Paula et al., 2017). Results found that the sugar level dropped faster when eaten the Moringa powder. Same study reported that the antioxidant concentrations increase in rats serum when treated with 500 mg of moringa seed powder per kg body-weight. These experiments prove that the antioxidant compounds present in Moringa powder can reduce the reactive oxygen species. Therefore, it can activate the sensitivity of beta cells to the level of sugar and increase the production of insulin.

The polyphenolic compounds such as kaempferol and quercetin due to their structures are able to exhibit antioxidant activities. Thus, they are able to scavenge reactive oxygen species (Coppin et al., 2013). Such compounds present in Moringa reduce the oxidant activities of the reactive oxygen species released from mitochondria, thereby protecting the beta cells and in turn keeping hyperglycemia under control (Vijay and Kumar, 2012). Diabetes leads to several complications like nephropathy, and retinopathy. The use of *Moringa oleifera* as a nutraceutical might help to prevent diabetes problems in humans.

2.1.3.2 *Moringa oleifera* as an anticancer agent

Cancer is one of the common diseases (de Andrade Luz et al., 2017). Around 14.1 million cancer cases are prevalent in the world (Coz-Bolaños et al., 2018). Many factors such as lack of exercise, radiation exposure, unbalanced diet, and smoking can lead to the development of cancer (Zhou et al., 2018). The proposed cancer treatment such as chemotherapy, radiation, and surgery are not affordable for all

classes of people in the community, and also, they have several side effects (Metwally et al., 2017).

Antioxidant compounds can also be considered as one of the alternative ways to reduce cancer. The *Moringa* plant contains natural antioxidant compounds that can be used as anticancer agents (Ezhilarasi et al., 2016). It is a safe and reliable nutraceutical product (Coz-Bolaños et al., 2018). Studies have reported that *Moringa* liquid extract inhibits the development of the cancer cell (Cuellar-Núñez et al., 2018b). Some studies have shown that the aqueous extract of *Moringa oleifera* may be used as anti-nonproliferative cancer compounds (Jaja-Chimedza et al., 2018).

Moreover, many studies have shown that the anti-proliferative activity of cancer can be caused by the inability to produce a reactive oxygen species in damaged cells (Zhou et al., 2018). Reactive oxygen species generated in the cells lead to apoptosis (Ezhilarasi et al., 2016). This is further proved by the up-regulation of caspase 3 and caspase 9, which are part of the apoptotic pathway (Coz-Bolaños et al., 2018). Furthermore, the reactive oxygen species produced by *Moringa* antioxidant compounds is specific and targets only cancer cells making it an ideal anticancer preventive agent (Elwan et al., 2018). It has also been reported that the extracts increased the expression of glutathione-S-transferase, which inhibits the expression of oxidant agents (Karthivashan et al., 2015). Therefore, the *Moringa oleifera* plant has a high content of antioxidant compounds that can be used for prevention of cancer (Cuellar-Núñez et al., 2018).

2.1.3.3 Effects of *Moringa* on obesity

Obesity is an excessive or abnormal body fat. It is considered as a significant public health problem in the world. It was reported by WHO that 40% of women and 39% of men are overweight (Metwally et al., 2017). Also, more than 340 million children and adolescents are obese with a distribution of 19% for boys and 18% for girls (Saeed et al., 2016).

Moreover, factors such as lack of physical exercise and consumption of fast food with a high cholesterol content, resulting in the accumulation of high energy intake are usually associated with a risk of obesity (Khan et al., 2014). Besides, in the living organism, excess energy is conserved in the form of lipids. When the body produces more and more energy without using it, this is converted into lipid and is deposited in several organs (Metwally et al., 2017).

These physiological disorders can induce several chronic diseases such as ischemic heart, atherosclerosis, osteoarthritis, hypertension and diabetes (Esakkimuthu et al., 2016; Jagadeesha et al., 2018; Jaja-Chimedza et al., 2018). An in vivo investigation with rats has shown that ethanol extract from *Moringa oleifera* leaves can be used for prevention of obesity. It was reported that the clinical response of *Moringa oleifera* leaves extract was similar to the anti-hypercholesterolemic drug such as simvastatin (de Andrade Luz et al., 2017; Metwally et al., 2017). Also, the crude methanol extract of *Moringa oleifera* leaves were assessed for anti-obesity activity and hypocholesterolemic in fat rats. The authors of this study observed a significant decrease of cholesterol concentrations in liver, serum and kidney (Jagadeesha et al., 2018; Khan et al., 2014). Moreover, similar results were obtained with mice fed with *Moringa oleifera* leaves (Karthivashan et al., 2015b).

2.1.3.4 Other ailments

Moringa oleifera has shown activity in preventing other diseases (de Andrade Luz et al., 2017; Cuellar-Núñez et al., 2018). It has been reported that the plant can be used as an efficient neuroprotector agent (Rajanandh et al., 2012). Cerebral ischemia is due to the obstruction of blood movement inside the brain (Singh et al., 2009). It leads to reperfusion and lipid peroxidation, which in turn results in the production of reactive oxygen species. *Moringa oleifera*, as an antioxidant agent, can significantly reduce the formation of reactive oxygen species (Olurishe et al., 2016). Therefore, it also has a function protecting the brain (Vijay and Kumar, 2012).

Moringa products can be used to prevent madness, as it has been shown to be a promoter of spatial memory (Omotoso et al., 2018). The leaf extracts can reduce

the acetylcholine esterase activity, thereby improving cholinergic function (Saucedo-Pompa et al., 2018). Khan et al. (2014) have reported that the use of *Moringa oleifera* leaves can increase the synthesis of the proteins, reduce the amount of creatinine and urea in the blood. Also, it can prevent renal dysfunction. In addition, a dosage of 350 mg and 500 mg of Moringa leaves powder daily could reduce the acidity in gastric ulcer and therefore can be used as an antiulcer agent (Choudhary et al., 2013) .

In traditional medicine, Moringa has been prescribed for patients with AIDS (Jaja-Chimedza et al., 2018). According to traditional knowledge, Moringa boosts the immune system of a HIV positive person (Vijay and Kumar, 2012). However, this affirmation is still under investigation by several researchers in the world. The research studies in rats revealed that the hydro-alcoholic extract of Moringa leaves decreased the amount of TNF-alpha, IL-1 and rheumatoid factor (He et al., 2018). This is evidence that Moringa is a powerful medicine for osteoarthritis (Choudhary et al., 2013).

Microbial diseases are widespread, and there is a need for antimicrobial agents, *Moringa oleifera* has been proven to be an excellent antimicrobial agent (Vijay and Kumar, 2012). A study by Mahdi et al. (2017) has shown that the extracts of *Moringa oleifera* can act against bacteria such as *Staphylococcus aureus*, *Vibrio cholera*, and *Bacillus subtilis*.

2.2 Overview of the extraction techniques of bioactive compounds

Nowadays, the use of plants as a source of nutraceutical ingredients in the pharmaceutical, food and cosmeceutical industries is increasing (Wang and Weller, 2006). It is essential to apply different extraction techniques for maximum recovery of beneficial compounds from the plants. The extraction of analytes of interest from the matrix sample is one of the crucial stages of all analytical procedures (Sood and Gupta, 2015; Pintać et al., 2018). It plays a significant role in the potency of the extraction. Thus, it is important to have an extraction technique that is capable of

extracting all the essential compounds in short time without degrading the quality of the final product.

Natural products from plants are usually a mixture of large a range of phytochemicals with various chemical structures and polarities (Piana et al., 2018). The solubility of natural compounds is governed by the polarity of the solvent used, chemical nature of the mixture, and its probable interaction with other components in the matrix composition (Leyva-Jiménez et al., 2018; Moreira et al., 2018). For instance, the polyphenolic compounds are usually combined with other compounds such as protein and carbohydrates (Jurinjak Tušek et al., 2018).

The complexity of the plant matrix makes it challenging to develop an appropriate extraction process for the recovery of all type of bioactive compounds from the plant (Piana et al., 2018). Sometimes, an additional stage can be essential to eliminate the unwanted compounds which could interact with the needed components and cause much errors in the final results (Kou et al., 2018a). Several extraction techniques are applied to recover phytochemicals from plants (Giacometti et al., 2018b). They are namely traditional extraction techniques such as Soxhlet. The modern ones include pressurized hot water extraction (PHWE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) (Wang and Weller, 2006). The modern extraction techniques are preferred because take less time use, less organic solvents and give high recovery. They use high pressure and temperature to speed up the extraction. Various parameters influence the extraction process such as solvent to sample ratio, extraction time, chemical composition, temperature, physical characteristics of the matrix and solvent used (Moreira et al., 2018). These parameters influence the extraction in various ways, depending on the technique applied. Different extraction techniques are discussed in the following section.

2.2.1. Traditional extraction technique

Traditional extraction techniques have been continually used for the recovery of phytochemicals from plants (Pintać et al., 2018). The technique is based on the mass transfer capacity of the compound of interest from solid or liquid sample to a suitable solvent (Sánchez-Camargo et al., 2017). The amount of the compound extracted depends on the solubility capacity among the target analyte and other components of the sample matrix in the solvent used for recovery (Giordano et al., 2017).

Several conventional extraction techniques such as solid-liquid (SLE), solid-phase extraction (SPE), liquid-liquid (LLE), Soxhlet extraction (SE), maceration and hydro-distillation are applied to extract phytochemicals from plants (Caldas et al., 2018). The solvent used is chosen in relation to the polarity of the target compounds (Pintać et al., 2018). In this respect, nonpolar solvent like chloroform, hexane, petroleum ether, ethyl acetate, dichloromethane, and their different mixtures are employed to extract nonpolar compounds such as fatty acid (Bodoira et al., 2017a).

Polar solvents like acetone, methanol, ethanol, and their aqueous mixture are employed to recover polar and semi-polar compounds such as polyphenols, saponins, and others (Caldas et al., 2018). As comparing SPE to LLE and, it is important to highlight that the SPE does not need a large amount of organic solvent. Therefore, it is possible to automate the online systems (Pereira et al., 2017). Moreover, in SPE, various range of cartridges with different packing size are employed (Bajer et al., 2018). In this situation, the different sorbents for example, polymeric materials, C₁₈, functionalized sorbents, carbon or a combination of those cited above are generally used for extracting polyphenolic compounds. Nevertheless, silica-based cartridges have been generally used for this purpose (Alghamdi et al., 2018). Conventionally, recovery of the fatty acids is done by maceration, hydro-distillation or Soxhlet.

Soxhlet extraction (SE) and maceration in various proportions of water-ethanol mixtures have been employed for the recovery of flavonoids and phenolic compounds from *Moringa oleifera* leaves (Al-Owaisi et al., 2014; Castro-López et al., 2017). Also, the combinations of water-methanol have been used for the extraction of polyphenolic compounds from different parts of the *Moringa oleifera* plant by SE (Prabakaran et al., 2018). However, some studies reported that the maceration extraction technique with methanol as solvent might recover tannins more efficiently than SE performs (Castro-López et al., 2017). Whereas, the latter is more active on extraction of polyphenol compounds (Haas et al., 2018).

Amongst these extraction techniques, SE is the most frequently applied for extracting fats and triglycerides from food matrices and seeds (Pereira et al., 2017). Also, maceration with ethanol was used for removing unsaturated fatty acids from *Moringa oleifera* seed oils (Prabakaran et al., 2018). While, SE extraction technique with methanol: chloroform has been reported to give the highest oil yield from *Moringa oleifera* seeds (Tsaknis and Lalas, 2002), it is important to highlight the mechanical extraction methods are also often used to extract oils from seed (Anwar et al., 2006).

Despite the fact that traditional extraction techniques are useful for recovering different types of bioactive compounds, they are generally solvent and time consuming and they are associated with risk of toxicity for environmental and human well-being (Porto et al., 2013). Therefore, the development of modern extraction techniques like PHWE, UAE, MAE, and SFE are emerging as an alternative. These techniques present a significant advantage over traditional methods. These advantages are: less degradation, elimination of additional sample clean up, reduction in organic solvent consumption, concentration steps before chromatographic analysis, selectivity, improvement in kinetics and extraction efficiency and ease of automation (Masci et al., 2016).

2.2.2. Ultrasound-assisted extraction technique (UAE)

The technique has been largely employed during the last two decades for removing phytochemical compounds by pharmaceutical, food and cosmetic companies (Prakash Maran et al., 2017). Ultrasound is defined as a mechanical wave which requires an elastic medium for propagation (Dal Prá et al., 2017). It is based on variation between the chemical and physical properties of the sample matrix after their interactions with ultrasound waves (Tomšik et al., 2016).

The cavitation effect eases the extraction process and enhancing the mass transfer between sample and solvent by disrupting the plant-cell walls. Moreover, the parameters such as frequencies, temperature, solvents used, and ultrasound power affect the quality of the UAE (Albuquerque et al., 2018). Several researchers have reported that increasing temperatures increase the yield of extraction by enhancing the diffusion rates and solubility of the target analytes in the appropriate solvent (Giacometti et al., 2018).

Nevertheless, it should be noted that some compounds can be degraded by increasing the temperature. Also, some impurities from the sample can be solubilized at this condition (Goltz et al., 2018). Several research studies have demonstrated that ultrasound frequency does not a significant influence in the yield of the extraction (Kou et al., 2018). In addition, the increase of ultrasound power results in the collapse and promotion of many cavitation bubbles (Irakli et al., 2018). UAE technique has been mainly employed to extract polyphenolic compounds from different parts of the plants. Goltz et al. (2018) reported that the extraction efficiency of different phenolic acids was significantly higher as compared with the maceration extraction technique. Poodi et al. (2017) have reported the used of UAE methods to recover anthocyanins and flavanones from various plant samples with promising results.

Several authors have reported that UAE seems to be more effective than traditional extraction techniques, with giving similar extraction efficiency for total phenolic

and flavonoid compounds (Bindes et al., 2018). Besides, the UAE technique has also been applied for extracting phytochemicals like kaempferol and quercetin from *Moringa oleifera* leaves (Wu et al., 2018). It was demonstrated that the UAE was more efficient than SE (Tomšik et al., 2016), and it has been employed for the extraction of triglycerides from *Moringa oleifera* seed (Giacometti et al., 2018). Moreover, the technique is less time consuming than Soxhlet for the recovery of fatty acids and triacylglycerols from *Moringa oleifera* seeds (Garba et al., 2015). Another research study done by Irakli et al. (2018) showed that UAE was the best option for the removal of fatty acids, like palmitic acid and linoleic acids, from seeds as compared with traditional extraction technique such as SE.

In summary, UAE is much faster and low cost than the traditional extraction techniques. Nevertheless, the efficiency of the technique depends on the nature of the target analytes. Besides, it should be kept in mind that the distribution of the ultrasonic wave in the vessel is not uniform. It is limited to the vicinity of the ultrasound probe, making its application on a big scale a difficult task (Mehmood et al., 2019).

2.2.3 Supercritical fluid extraction

A supercritical fluid is extremely compressed gas that has a density close to that of liquid (Hatami et al., 2019). Supercritical fluid extraction (SFE) is a technique which uses a supercritical fluid solvent such as carbon dioxide. Currently, the technique is commercially often used by the food and pharmaceutical industries to extract compounds on a large scale (Costa et al., 2012). It is a fast, clean and efficient technique, for the extraction of various phytochemicals from the plant. It has an advantage that it can perform the extraction of bioactive compounds or other target analytes without the production of toxic residues. Also, it allows the extraction of high purity bioactive compounds (Basa'ar et al., 2017). Because of their relatively high diffusivity and low viscosity, supercritical fluids have better transport properties than other organic solvents. They can easily diffuse into the solid materials, thereby, generating a faster extraction rate (Hall et al., 2018). The

main characteristic of the technique is the possibility to change the density of the fluid by variation of the temperature and pressure (Tsitsagi et al., 2018).

Moreover, several supercritical fluids can be used, for the recovery of non-polar phytochemicals. Carbon dioxide (CO_2) is most regularly employed for removal of the target compounds. It presents many advantages such as it is environmentally friendly, non-flammable, inert, readily available, noncorrosive, cheap, odourless and tasteless (Garca - Pérez et al., 2017). The density of the supercritical fluid CO_2 is like that of the liquid whereas its viscosity is strongly close to the gases, at its critical temperature and pressure of 31.1°C and 7.38 MPa , respectively (Tsitsagi et al., 2018). The quantity of the energy used is generally insignificant as compared with water. It is easily evaporated from the extract because of its modest depressurization (Hatami et al., 2012). The recycling of SCCO_2 is recognized as an environmentally friendly process. Changes to the temperature and pressure close to the critical point (Figure 2.3) can affect the properties of the SCCO_2 such as viscosity, density and dielectric.

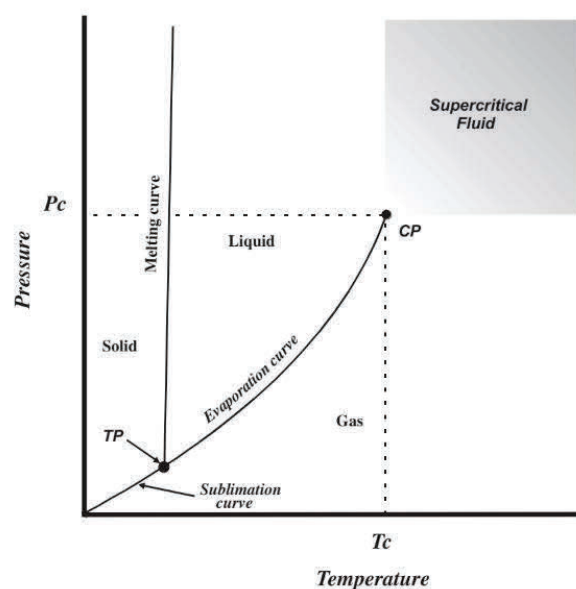


Figure 2.3. Phase diagram of pure substance. TP: triple point; CP: critical point; P_c : critical pressure; and T_c : critical temperature. These parameters are specific to each substance (Torres-ossandón et al., 2018).

One of the major disadvantages of SCCO₂ is that its integration in the analytical process needs sophisticated equipment, which makes its use extra difficult (Todd and Baroutian, 2017). However, the SCCO₂ is employed as the solvent in several fields such as the discovery of new compounds from the natural source, food and textile industry, coating in furniture production and automobile, and polymer manufacturing. Also, the SCCO₂ is used in the recovery of many non-polar compounds including essential oils and lipids (Campone et al., 2018). The SFE technique involves mainly two different steps which is the extraction of the target compounds by the supercritical CO₂ from the sample and separation of the substance from the supercritical fluid after the expansion. The overflow description of the equipment is shown in Figure 2.4.

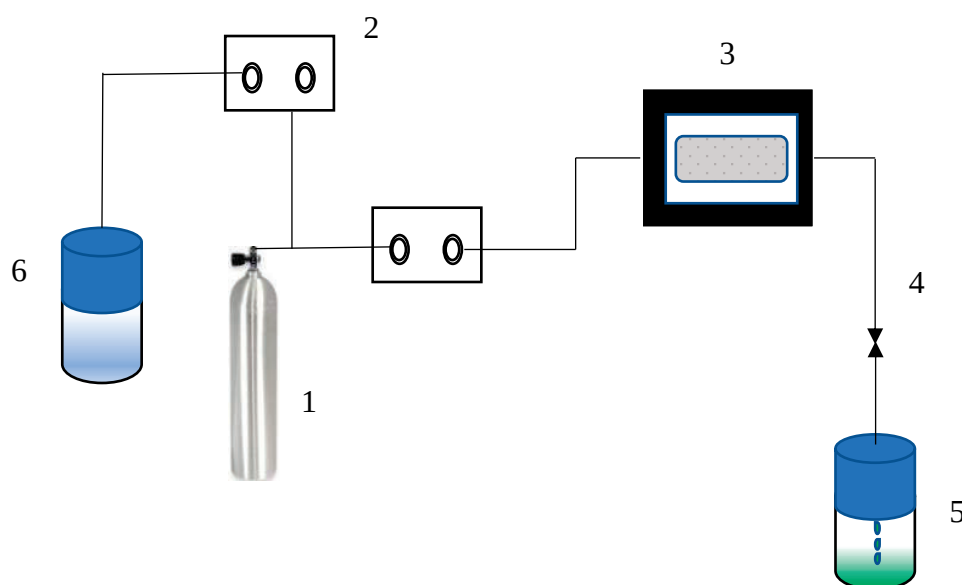


Figure 2.4. Schematic representation of supercritical fluid extraction system. it consists of a container for the solvent used for the extraction process, carbon dioxide (CO₂) in this case (1), a pump that permits the chosen CO₂ flow (2), an oven containing the extraction vessel (3), a back pressure regulator which help the pressurization of the system (BPR) (4), a trapping container (5) and optionally a modifier pump (6) (Todd and Baroutian, 2017).

The important factors to optimize in SFE are pressure, temperature, extraction time and modifier or co-solvent percentage. For example, Campone et al. (2018) investigated the influence of temperatures from 32 to 86°C in the extraction of lycopene from tomato. Results revealed that the rate of lycopene recovery increased at higher temperatures. Another study done by Villanueva-Bermejo et al. (2018) has reported that a temperature of 70°C was the optimal condition for efficient recovery of total phenolic content (TPC) from *Eucalyptus globulus* bark.

Furthermore, the solvating power of the solvent are strongly influenced by the pressure, and consequently the extraction. It is important to highlight that increasing the pressure, results in an increase in the solvation power but decreases the extraction selectivity (Duval et al., 2016). Many research studies using SFE have found out that pressure from 150 – 300 bars and extraction temperature from 30 - 60°C, are mostly used for the recovery of various phytochemicals from diverse plant materials (Liu et al., 2018). In a study by Hatami et al. (2019), CO₂ pressures above 200 bars have been used to recover triglycerides from seeds. The CO₂ has a low polarity which makes it more suitable for the recovery of nonpolar phytochemicals from plant samples. For instance, it has been employed for extraction of oils, unsaturated fatty acids and tocopherol-enriched oils from various plants (Hall et al., 2018).

However, when the compounds of interest are more polar, a co-solvent such ethanol is added in smaller amount together with CO₂ to enhance the polarity of the carbon dioxide supercritical fluid solvent. This helps to increase the extraction efficiency of most polar compounds. Casas et al. (2008) improved the extraction of various polar polyphenols from rosemary leaves by adding 6.6% of ethanol as a modifier to the SFE solvent. The similar process was also used by Ghafoor et al. (2010) for the recovery of polyphenolic from grape. Besides, as observed in other extraction methods (Giacometti et al., 2018), the extraction time has an important factor in the process. In the extraction of phenolic compounds 120 min was found to be optimum time (Ferrentino et al., 2018). The best way to establish an efficient recovery is to perform an extraction rate until all compounds are extracted from the sample (Lima et al., 2018; Santos-Zea et al., 2018). For example, to achieve a complete extraction

of oil from chia seeds, Villanueva-Bermejo et al. (2018) used an extraction time of 160 min.

Currently, it has been shown that instead of adding a co-solvent to scCO₂, compressed liquid CO₂ can be added to an organic solvent, thereby creating a so-called gas-expanded liquid (GXL). In terms of physical properties, a GXL has similar density compared to the organic solvent without CO₂ added, and viscosity is somewhere between a conventional liquid and an SCF (Pimentel-Moral et al., 2018). GXLs are interesting options to SCFs, since more diverse physicochemical properties can be obtained from a variety of different green organic solvents, than for scCO₂ with or without a cosolvent added. This has been demonstrated by Grijó et al. (2018) for methanol, ethanol, and acetone among other solvents, giving a range of obtainable dielectric properties (Todd and Baroutian, 2017). Applications of GXLs as solvents have mainly been demonstrated in particle formation, polymer processing and in homogeneous and heterogeneous catalysis (see an excellent review article by Jessop and Subramaniam (Brandão et al., 2017) but only in a few cases as extraction solvent (Hall et al., 2018). In comparison with conventional extraction (CE) Casas et al. (2007) concluded that SFE was more selective than the steam-distillation method. Nevertheless, Bodoira et al. (2017) reported that SE extraction was the most effective, allowing 58.93% of sesame oil vs. 26.47% obtained by SFE (Yildiz et al., 2010).

2.2.4. Pressurized hot water extraction

Pressurized hot water extraction (PHWE) is also called pressurized liquid extraction (PLE) when other solvents with exception of water are employed. The technique involves the extraction of target analytes at high temperature and pressure (Náthia-Neves et al., 2017). The fundamental principles of PHWE approach are described as below: water is a highly polar solvent with a high dielectric constant (ϵ) at room temperature and atmospheric pressure due to the presence of the extensive hydrogen-bonded structure.

Hence, traditionally water is not considered as a suitable extraction fluid for non-polar or organic compounds at room temperature. When the temperature of the water increases, there is a steady decrease in its viscosity, permittivity, and surface tension but an increase in its diffusivity properties (Náthia-Neves et al., 2017). With enough pressure to keep water in the liquid phase at high temperature, the initial value of the dielectric constant of 80ϵ at 25°C decreases to 27ϵ at 250°C and 50 bars, which falls between those of methanol ($\epsilon = 33$) and ethanol ($\epsilon = 24$) at 25°C (Castro-Puyana et al., 2017; Karásek et al., 2018; Piao et al., 2014; Plaza and Turner, 2015; Saini and Keum, 2016). The most exciting benefit of PHWE is that the solvents is under its supercritical or liquid state during the extraction process, which, allows, the extraction of a wide range of bioactive compounds (Machado et al., 2015, Bursa et al., 2018). The description of the apparatus used to perform PHWE is displayed in Figure 2.5.

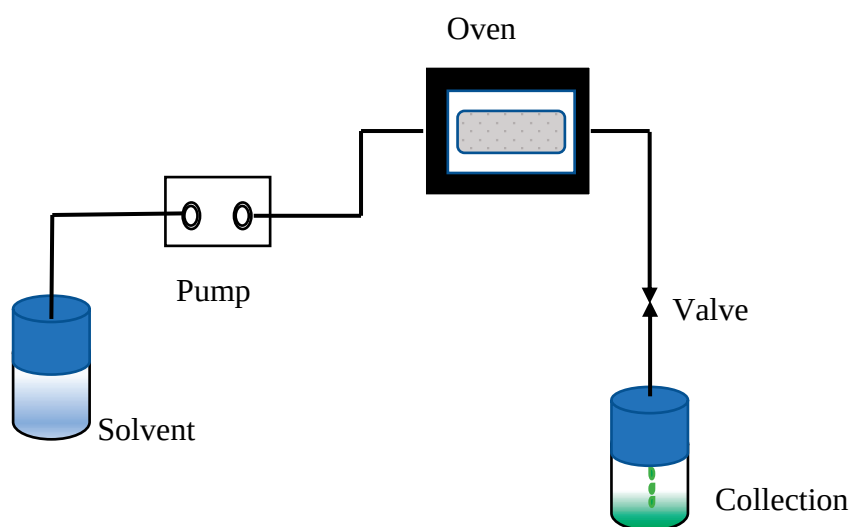


Figure 2.5. Schematic diagram of pressurized hot water extraction system.

For an efficient extraction recovery, the temperature, the flow rate and extraction time are essential to be considered and need optimisation. At high temperature, the viscosity of the solvent drops, therefore eases its diffusion through the matrix and improves the extraction process (Leyva-Jiménez et al., 2018). Matshediso et al. (2012) assessed the influence of various temperatures, ranging from $50 - 200^{\circ}\text{C}$ in pressurized hot water extraction of total phenolic compounds (TPC), myricetin,

quercetin, and kaempferol from *Moringa oleifera* leaves. Optimum temperature for an efficient extraction of polyphenolic compounds was found at 100°C. At the temperature above 150°C, the bioactive compounds including kaempferol, quercetin and other polyphenolic compounds started to degrade. A recent study published by Osmar et al. (2018) revealed that TPC was significantly recovered from certain plants at a temperature above 192.4°C as compared with conventional extraction (CE) at 80-90°C and UAE at 25°C (del Pilar Sánchez-Camargo et al., 2017). Therefore, temperatures from 100 - 200°C demonstrated to be more suitable for the recovery of polyphenolic compounds using the PHWE technique (Okiyama et al., 2018).

On the other hand, pressure has been highlighted to not be an essential factor affecting PHWE extraction. A number of researchers have failed to determine any significant influence of pressure into the extraction process (Otero et al., 2019). Concerning the extraction time, its impact is strictly connected to the operation mode, whether dynamic or static (Náthia-Neves et al., 2017).

Overall, PHWE performs an efficient extraction of phytochemicals than traditional extraction techniques. Several researchers have concluded that PHWE requires a less amount of total solvent used, easy manipulation of sample and solvents and shorter extraction time in comparison with SE and UAE (Karásek et al., 2018; Prado et al., 2016; Vazquez-Roig and Picó, 2015). Besides, PHWE has often been used for the extraction of phenolic compounds (Bursa et al., 2018). But, nowadays, it is used for a wide range of applications such extraction of vitamins, proteins, sugars, lipids and removal of organic contaminants in foodstuffs and environmental samples (Boyaci et al., 2015; Domínguez-Rodríguez et al., 2017; Millar et al., 2016). Compared with SFE, it shows a better extractible capacity with polar and semi polar compounds. Also, it is able to provide higher extraction efficiency. SFE could be more selective (Leyva-Jiménez et al., 2018).

2.2.5. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is one the modern sample preparation techniques increasingly being used in the field of phytochemical extraction (Fu et al., 2018). The technique uses microwave frequencies that consists of magnetic and electric fields, which move perpendicular to each other with a frequency from 0.3 to 300 GHz. They are measured as an electromagnetic wave that can diffuse through certain samples matrix and interact with the compounds and, therefore, produce heat (Liu et al., 2017). The association of these mass gradients and heat working in the identical direction accelerates the process and enhances the extraction yield (Liu et al., 2018). Just like other modern extraction technique, the extraction time, temperature and solvent are the main parameters to be optimized (Ferreira et al., 2018).

It is crucial to notice that water and ethanol combined together enhance the heating capacity of the solvent mixture, which results in the degradation of the target analytes if the extraction is running for a long period of time (Chan et al., 2015). However, Backes et al. (2018) reported that the extraction efficiency of polyphenolics from tea leaves did not improve with a shorter extraction time from 4 to 8 minutes. Besides, they have also reported that the recovery of phenolic compounds through MAE was far higher than that obtained by maceration, water or thermal reflux (Munir et al., 2018). Also, MAE optimized at 46% ethanol was the best condition for extracting total phenolic as compared with UAE and traditional solvent extraction (Chan et al., 2015). Xie et al. (2014) reported that the chemical composition of oils extracted with MAE and SE were typically similar, however the relative contents of the same fatty acids showed some differences. MAE provided an advantage in that it recovered higher amounts of fatty acids as compared with other techniques (Yuan et al., 2018). The main benefit of MAE is its ability to extract a large range of analytes.

2.3 Analytical tools used for identifying and quantifying bioactive compounds

Analytical techniques are essential for the development of nutraceuticals (Tohma et al., 2016). They provide information about the chemical and physical stability of the target analytes, easing the choice and design of the dosage form, and allowing their quantification in the sample matrices (Singh et al., 2013).

They are divided into two main groups including separation techniques, which are employed to isolate the compounds from a mixture; and spectrometric methods, which offer accurate knowledge on the chemical structural characteristics of each analyte (Downey and Rochfort, 2008). Various techniques have been applied to identify and quantify polar and non-polar compounds from sample matrix such as plant materials (Obboh et al., 2015; Yang et al., 2016). Liquid chromatography (HPLC) and capillary electrophoresis are mostly used to separate semi polar and polar compounds, while the gas chromatography (GC) is employed mostly to analyse volatile compounds (Rassem et al., 2018). Due to the interferences between compounds inside the sample matrices, other techniques such as mass spectrometry and nuclear magnetic resonance are used in combination with above techniques to improve the identification, purification and quantification of the target compounds (Hameed et al., 2016; Obboh et al., 2015).

2.3.1 Chromatographic techniques

Isolation of target analytes is an essential stage in the determination of unknown compounds from diverse matrices (Motilva et al., 2013). Hence, chromatography consists of various separation methods based on the different distribution properties between the mobile and stationary phase (Rodriguez-aller et al., 2013).

In chromatography, the analytes are transported by the mobile phase to a stationary phase (Downey and Rochfort, 2008). The compounds in the sample naturally exhibit differential partitioning or interactions with the two phases (Yang et al., 2013). This partitioning mostly depend the chemistry of the compounds. Therefore, each compound has a specific equilibrium constant with a different elution rate. Consequently, compounds which have a strong interaction with the stationary phase

will remain for a long period on the column and have longer retention times than compounds with less or no affinity with the stationary phase (Tohma et al., 2016). Then, these analytical techniques are often applied to characterise the bioactive compounds such as polyphenolic compounds, fatty acid and others from plant extract. The following section will explain in detail each applied techniques.

2.3.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC), also called liquid chromatography is the most commonly applied separation method for complex matrices such as plant materials (Huang et al., 2004). Its instrumentation usually constitutes the following parts including a pump, an injector, a column, a detector, and a data acquisition system (Figure 2.6). In the HPLC, the mobile phase consists of a single solvent or a mixture of solvents in which the pH is regularly modified with, acids, bases or buffer solutions depending on the analyte to be isolated (Motilva et al., 2013). The mobile phase moves through the column with the help of the pump.

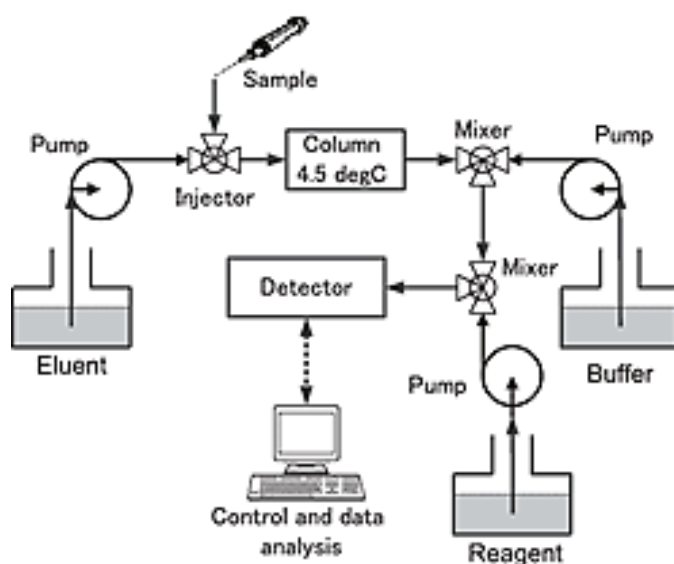


Figure 2.6. Schematic diagram of HPLC system

The stationary phase is packed in the column and its physical and chemical properties are important to predict further separation (Rodríguez-Pérez et al., 2015).

Currently, the reverse phase chromatography is the most widely used HPLC mode for the isolation of polar mixture (Tohma et al., 2016; Yannick et al, 2018).

The selection of a mobile phase depends on the analytes of interest, the column employed and the coupled detection system, whereas the choice of a suitable column is directly linked to the nature of the analytes to be isolated (Rodriguez-aller et al., 2013). Once, all the compounds are eluted out of the column, they are detected in the detector, produce an analytic signal, which is connected to a chromatogram. The retention time and intensity of the signal depends on the amount of the target compound. Hence, each compound is represented as a single peak with specific peak area and retention time (RT).

The column is the essential compartment of HPLC system. For polyphenolic compounds, C₁₈ is the most commonly used column. It is packed with a settlement of octadecylsilyl groups. The size of the column can range from 10 to 30 cm long, the internal diameter from 3.9 to 4.6 mm and a particle size of 1.8 to 10 µm (Alarcón-flores et al., 2016). The use of C₁₈ columns frequently involves the application of binary solvent systems comprising of acidic aqueous solutions and organic solvents including acetonitrile, ethanol or methanol as mobile phases (Iitta, 2003). The isolation of several polar polyphenolic compounds is strongly dependent on the acidity and basic condition of the mobile phase (Tohma et al., 2016). The presence of acids in the aqueous mobile phase can lead to the ionization of these compounds, so enhancing their separation in the column (Alarcón-flores et al., 2016).

Furthermore, flavonoids are semi polar compounds, and are easily isolated by the organic solvent (Rodriguez-aller et al., 2013). Therefore, acetonitrile and acidified aqueous solution have been shown to be better combination solvents to separate these compounds (Downey and Rochfort, 2008). A number of researchers have reported that the mixture of methanol and acidified water as mobile phase is an excellent selection for the separation of polyphenolic compounds from various plant materials (Mendiola et al., 2008; Muñoz et al., 2018; Yang et al., 2013). The timing of the analysis is another factor which has significant influences for

separation of natural compounds such as polyphenolic (Waterman et al., 2014). The best setting time for analysis has been shown between 10 and 150 minutes at a flow rate range from 0.2 and 1.5 mL min⁻¹ (Motilva et al., 2013). As can be seen above, HPLC offers excellent versatility that is not found in any other chromatographic system because of its ability to easily separate a wide variety of chemical mixtures from plants. After separation, the next step involves detecting components as they elute of the column.

2.3.3 HPLC - UV-Vis detector

HPLC technique using UV-Vis as detector, is widely applied to characterize many compounds (Hameed et al., 2016). The technique has good excellent resistance to changes in flow, temperature, high precision and linearity (Muñoz et al., 2018). In addition, UV-Vis detector is more robust than many other detection systems. Several molecules can absorb UV-Vis radiation; therefore, the detector is considered universal (Ee et al., 2008). There are three kinds of UV-Vis detectors available in the market: diode array detectors (DADs), fixed wavelength and variable wavelength detectors.

The first detector it is mostly use for the identification of phytochemicals. The DAD detector can provide a full spectrum of UV-Vis absorption of compounds that rapidly pass through a measurement cell. The use of DAD associated with HPLC allows the assessment of unknown compounds from plant materials. For instance, HPLC-DAD was applied to identify and quantify 16 different anthocyanidins extracted from grape juice (Ee et al., 2008). While, this detection system can be suitable for solving analytical problems. However, it presents the disadvantage of low selectivity and does not provide chemical structural information, so that it is not able to precisely identify the compounds without using commercial standards.

2.3.4 HPLC coupled to mass spectrometry

Nowadays, mass spectrometry (MS) is playing an important role in several stages of characterization and identification of phytochemical in plant (Tohma et al., 2016). The technique is excellent in speed and sensitivity. Hence, it has become a standard tool in pharmaceutical, food and cosmetic industries (Sneha et al., 2017). The MS is based on the formation of anions or cations that can be magnetically or electrically fragmented according to their mass-to-charge ratio (m/z). Its analysis can provide valuable information such as structure and purity of the analytes (Standing and Vestal, 2015). While various mass spectrometers are available, they all contain an interface such as an ionization source, ion separation system according to their m/z (mass analyser) and a detector coupled with a signal processor (Figure 2.7).

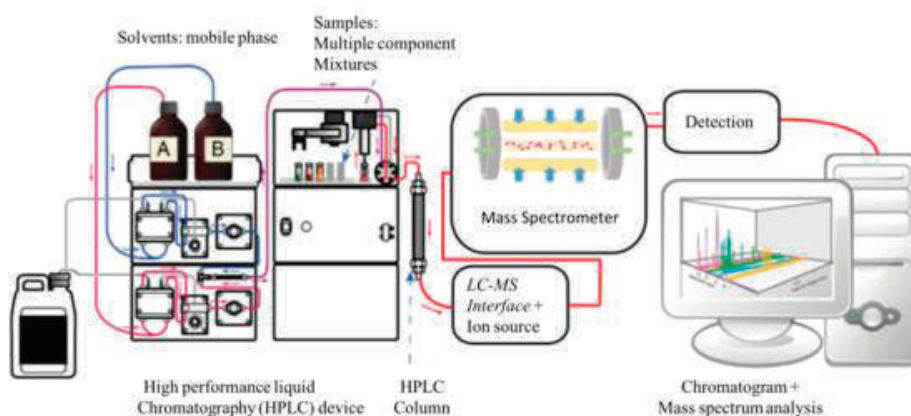


Figure 2.1. Schematic diagram of a HPLC coupled to mass spectrometry

In addition, HPLC coupled with mass spectrometry (MS) needs the use of an interface, in which the analytes from the column are evaporated and ionized (Rascón et al., 2018). Therefore, converting the ionic species sample in the gas phase before isolation. For instance, the electrospray ionization (ESI) is generally the mostly used interface for identification of polyphenol compounds. It is largely useful for the analysis of high molecular weight, polar, and labile compounds (Yanini et al., 2018).

The ions are generated at atmospheric pressure by passing a solution sample over a small capillary with internal diameter $< 250\ \mu\text{m}$. It has a potential variance from a counter-electrode at voltages between ± 500 and $\pm 4500\ \text{V}$ (Figure 2.8).

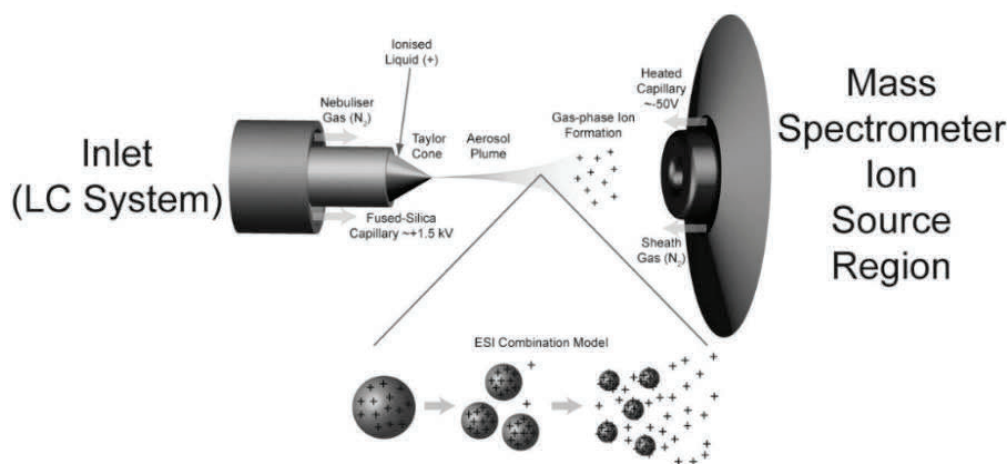


Figure 2.8. Schematic diagram of an electrospray ionization source (Yanini et al., 2018)

Once the gas phase ions have been formed, the next stage is to isolate each ion as a function of its m/z before detection. Presently, four main mass analysers such as quadrupole flight time (QTOF), quadrupole (Q), ion trap (IT) and time of flight (TOF) are used. They differ in price, size, ability to perform tandem mass spectrometry (MS/MS) experiments, resolution and range mass (Standing and Vestal, 2015). The current thesis assesses the application of a quadrupole time-of-flight mass spectrometer (QTOF). In the time-of-flight (TOF) quadrupole analyser, ions produced are extracted and accelerated at high speed by an electric field in an analyser. Hence, They are separated according to their m/z ratios. The ions reached a velocity which is inversely proportional to its value m/z . Thus, the ions with high m/z value take longer to pass through the analyser, while smaller ions reach the detector faster (Sneha et al., 2017).

The higher sensitivity of the QTOF, it is due to funnels in the ion transmission zone, which avoid ion loss during transmission. It contains a quadrupole and collision cell

before the flight time tube. A quadrupole (Q) contains four parallel cylindrical bars, allowing the choice of ions according to their fragmentation in a collision, cell helped by a collision gas (generally nitrogen). The quadrupole acts as a sort of mass filter, the fragmented ions being separated in the TOF base on their m/z ratio in the same manner as before (Yahia et al., 2017). These matching results are the best association of specific performance characteristics, like accurate mass measurement, and ability to perform fragmentation (Jackson et al., 2018). QTOF provides the capacity to assess masses from 50 to 20000 m/z with high resolution and accuracy of 3 ppm for internal calibration and 5 ppm with external calibration for precursor fragments and ions. QTOF is a powerful tool that may be applied for bioavailability and pharmacokinetic studies, and metabolomics. In addition, QTOF has been widely used for the initial characterization of phytochemical from various plant matrices (Tamires et al., 2019).

Therefore, the combined technique of HPLC and mass spectrometry is often applied for the identification and quantification of polyphenolic compounds from *Moringa oleifera* leaves. For instance, Makita et al. (2016) have used a mass spectrometry technique for the identification of 14 flavonoids from the leaves of *Moringa oleifera* harvested in both South Africa and Namibia. Meanwhile, Coppin et al. (2013) have identified 12 flavonoids from *Moringa oleifera* leaves collected from sub-Saharan Africa.

2.3.5 Supercritical fluid chromatography

Supercritical chromatography (SFC) is a separation technique that bonds the gap between liquid and gas chromatography (Arnáiz et al., 2012). The technique can be used for a broad spectrum of components, ranging from volatile compounds to macromolecules (Poe et al., 2012). The mild temperatures required for the isolation, combined with the ease of separation of components, makes SFC ideal for the identification and quantification of diverse compounds from a complex sample matrix (Yi et al., 2012). These are the advantages of the SFC as compared to other methods of chromatography. However, the analysis of polar and very volatile

molecules remains the domain of liquid and gas chromatography, respectively. The schematic diagram of the SFC equipment is shown in Figure 2.9.

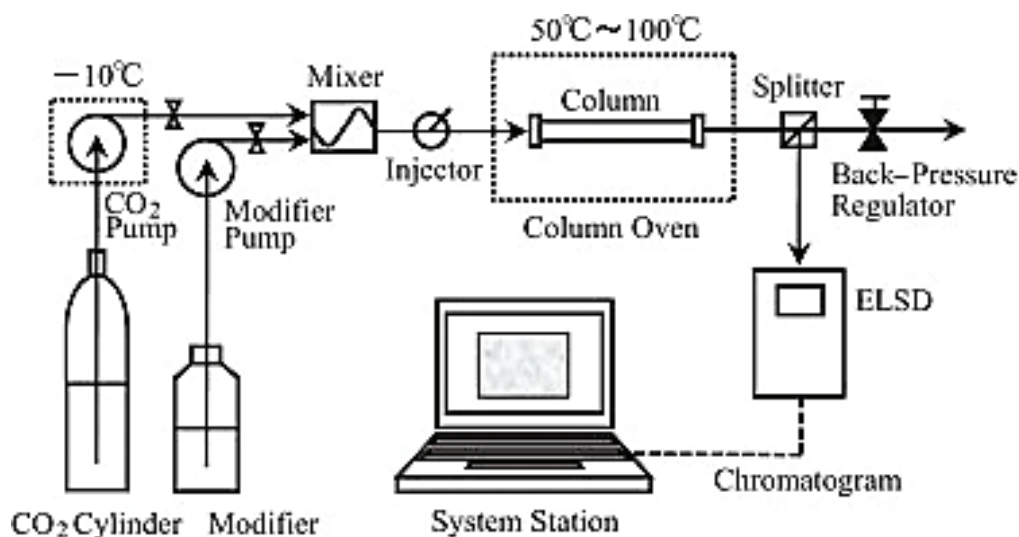


Figure 2.9. Schematic diagram of a supercritical chromatography system

The improved resolution gained from using SFC is directly related to the tunability of the eluent density and system temperature (Kaczmarek et al., 2012). SFC provides a unique and efficient tool for both analytical and preparative separation techniques. The most common mobile phase used in SFC is carbon dioxide (CO_2). It has been applied for the separation of many drugs and metabolites (Rajendran, 2012).

The use of a co-solvent is common because of the low solubility of polar compounds in dense CO_2 (Saito, 2013; Rahmat et al., 2009). The co-solvent provides an extra degree of tunability, which can further reduce running time and improve resolution (Storbeck et al., 2018; Bamba et al., 2012). In the last decade, many considerable research and analytical approaches of SFC has been accomplished. Several research and review papers have been published in the literature (Huang et al., 2018, Planeta et al., 2012, Poe et al., 2012, Abrahamsson et al., 2012).

2.4 Drying techniques for plant extracts

Drying is the removal of moisture or water from an aqueous product by evaporation under controlled condition (Acosta-Esquivarosa et al., 2009). It is the oldest technique used to preserve food by human. The drying process inhibits microbial and enzyme activities and guarantees the expected shelf-life of the product. Also, it helps the storage and easy handling of the final product (Mohtar et al., 2014). In the manufacturing world, it is an essential technique in agricultural, chemical, biotechnology, ceramics, polymer, pulp and paper, pharmaceutical and mineral industries (Adeyemi, SB, Ogundele KO, 2014). Conventionally, the sun drying technique was employed to remove moisture from food and agricultural products (Vuthijumnok et al., 2013). It is the most widely used drying methods worldwide because it is easy, convenient and cheap.

Although sun drying needs slight expertise or capital, there are several difficulties in using this technique for dehydration of food products. A number of research studies have reported that food dried through sun drying have an undesirable change in their quality (Papillo et al., 2018, Ogundele, 2014; Vuthijumnok et al., 2013). The technique is strongly weather dependent, and there is loss of product aromas and vitamins due to direct sunlight and long periods of drying. Also, lack of sufficient control during the drying process, contamination of the product with dust and soil, large space requirements and non-uniformity of dried products are disadvantages of the sun drying technique (Mohtar et al., 2014, Abu Bakar Sajak et al., 2016). These difficulties can be overcome using novel drying techniques such as freeze and spray drying, and particle formation. In this section, the above the techniques cited, are reviewed.

2.4.1 Spray drying

Spray drying has become the most important method for removing water in a slurry or liquid including coffee, egg powders and milk. It is a straight forward procedure, in which liquid extracts are transformed directly into powder (Miller et al., 2018).

It is widely applied in the food industry for the manufacture of agglomerates and food powders. It is the most economic drying approach because of its ease of handling expenses. It is composed of the following steps: (a) pumping liquid to the atomization device; (b) atomizing the liquid by using a nozzle such as the rotary disc atomizer, pressure nozzle, pneumatic nozzle, and ultrasonic nozzle; (c) drying of generated droplets in hot drying media; and (d) separating the dried particles from exhaust air (Papillo et al., 2018). The parameters such as drying medium mass flow rate, nozzle parameters, air-droplet contact systems, drying medium temperature, drying media (nitrogen, air, etc.), feed mass flow rate, powder and air discharge systems and chamber shape strongly affect the process of spray drying (Sablan et al., 2018).

Cardoso et al. (2017) have reported that the recovery of the spray drying is about 4 to 5 times more than the freeze drying due to its low electricity used and faster drying process. Another author has reported that spray drying performs 4 times more economically than vacuum drying and 8 times more than freeze drying (Oyarzún-ampuero et al., 2016). Furthermore, spray drying shows an advantage of relatively smaller drying contact time from 5 to 100 secs (Jotha et al., 2016; Papillo et al., 2018). The powder generated by spray drying is extremely stable due to the low water activities and moisture content (Tom et al., 2018). Under these conditions, the powder obtained are relatively resistant to oxidative and microbiological degradation, and enzymatic activities (Ballesteros et al., 2017). The powder generated from spray drying have a low particle size which leads to a product with higher bioavailability (Rojas et al., 2017). Therefore, the technique is mostly used to produce plant extract powder in pharmaceutical and food industries (Papillo et al., 2018). Although, all the advantages of spray drying technique, it operates at high drying temperature such as 150 – 220°C “inlet” air temperature and 50 - 80°C outlet air temperature (Francisco et al., 2018), which can cause degradation of thermolabile compounds such as vitamin C, anthocyanins and polyphenolic (Lan et al., 2018). In addition, spray drying have a low thermal efficacy due to hot air circulating in huge volumes into the drying chamber.

2.4.2 Freeze drying

The freeze drying is employed for heat sensitive compounds such as pharmaceuticals and certain biological substances and other foodstuffs, which cannot be heated even to reasonable temperatures in a normal drying process (Zhang et al., 2018). In this technique, the moisture is vaporized by sublimation from the frozen foodstuffs being dehydrated in a vacuum chamber. It has many benefits over hot-air drying method like loss of aroma and taste (Rafaella et al., 2018). Freeze drying is composed of three steps such as the freezing, primary drying, and secondary drying stage (Cardoso et al., 2017).

The freeze dryers have several design parameters including cooling rate, freezing temperature and time at freezing stage, target product temperature, chamber pressure, and dryer capacity at primary drying stage, and heating rate and chamber pressure at secondary drying stages. Several research papers have demonstrated the use of freeze drying in removing of water from aqueous extract without causing any degradation (Zhang et al., 2018, Rafaella et al., 2018, Cardoso et al., 2017).

2.4.3 Particle formation with supercritical fluid

The use of dense gases in the particle formation process have gained interest in the last decades. It is a promising and an alternative technique to overcome the limitation of the traditional drying methods (Sreedhar et al., 2017). The most applied technique such as spray drying, and recrystallization used solvent evaporation, face several disadvantages such as high temperature, shear force, presence of unwanted compounds and electrostatic charge (Temelli and Science, 2018). Dense gas approach can be the solution to these problems by using the properties of the vicinity of the critical point (Nuchuchua et al., 2017). These gases exhibit the properties such as gas-like transport, liquid-like densities and the compressibility which lets the solvent to be adjusted with minor variation of temperature and pressure (Nuchuchua et al., 2017).

Generally, carbon dioxide is the most dense gas used in the particle formation process and it is easily evaporated at room temperature. Carbon dioxide (CO₂) has relatively low toxicity and is a non-flammable fluid. It expands easily at room temperature and separates from the final product (Cai et al., 2008). Many green chemistry principles are in fact satisfied, specifically in what concerns pollution prevention, lower toxicity and the use of several available resources (Sie et al., 2003). Nevertheless, as reported by Padrela et al. (2018), it is important to know that the use of CO₂ can generate a product with great characteristics offering a performance rather than just an environmental benefit, which makes the technology an effective alternative to establish a good industrial process.

Therefore, several scientific works have been published and the suitability of supercritical CO₂ has been shown both for the composites and precipitation of pure compounds, presenting an improvement of the technology mostly in relation with the production of smaller particle size with better distribution, as well as good morphology control (Tabernero et al., 2012). Several techniques such as Rapid Expansion of Supercritical Solutions (RESS), Particles from Gas-Saturated Solutions (PGSS), Gas Anti-solvent (GAS), Aerosol Solvent Extraction System (ASES), Supercritical-Assisted Atomization (SAA) and Carbon Dioxide Assisted Nebulization with a Bubble Dryer (CAN-BD) have been applied to remove water from the aqueous extract. This thesis has focused on the development of a drying process with similar principles to the CAN-BD technique.

The CAN-BD was invented by Sievers et al. (2000). The technique uses supercritical carbon dioxide fluid as an aid in the aerosolization of solutions. The use of CO₂ leads to the generation of microbubbles and small droplets that dry faster than aerosol formed with conventional methods (Nuchuchua et al., 2017). The temperatures required to dry the nebulized solutions are lower than those applied in the spray-drying technique. This makes CAN-BD more appropriate for the processing of thermally labile or sensible compounds. The CAN-BD process generates particles less than 3 times in diameter than spray drying (Cho et al., 2015). The mechanism process is explained as follows: the supercritical CO₂ is mixed intimately in a low-volume T-shaped fitting with an aqueous or solution containing

a dissolved solute. The mixture is expanded to atmospheric pressure to generate an aerosol, which is dried by preheated nitrogen in the drying chamber to form a fine powder. This principle was adopted to develop a new drying process called water extraction with online particle formation (WEPO). The technique is an association of pressurized hot water extraction with CAN-BD. It was applied first by Anderson et al. (2012) in the extraction and direct drying of the onion extract. Therefore, in this thesis, WEPO was used to dry the aqueous pressurized hot water extract of *Moringa oleifera* leaves.

CHAPTER 3

3 Research objectives

3.1 General objectives

Plants are an excellent source of essential compounds which exhibit several nutritional and herbal activities. Also, these compounds can be used as ingredients or additives in food, pharmaceutical and cosmetic industries. Hence, studies on the potential use of plant-based ingredients as nutritional and herbal supplements are on the increase. These nutraceuticals are aimed at improving the quality of human life. The overall main of this thesis was to apply environmentally friendly and non-toxic solvents in the extraction of the bioactive compounds from *Moringa oleifera* plant and develop a fast and cheaper drying technique in order to keep the bioactive compounds stable in the extract for a long period of use.

3.2 Specific objectives

The following specific objectives were established:

- ✓ To optimize pressurized hot water extraction for thermolabile compounds from *Moringa oleifera* leaves using two statistical modelling approaches (Paper 1).
- ✓ To assess the influence of various parameters of pressurized hot water extraction for macro and micro-nutrients from *Moringa oleifera* leaves using response surface methodology approach (paper 2).
- ✓ To develop a water extraction method with on-line particle formation of *Moringa oleifera* aqueous extract (paper 3)
- ✓ To develop an ultra-supercritical fluid extraction method of oil from *Moringa oleifera* and *peregrina* seeds (paper 4)

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CHAPTER 4:

Publication and manuscripts

This section presents the published article and manuscripts that must be considered for examination of the thesis. The referencing in each manuscript is formatted according to requirements of the journal in which they were published or submitted. The design including topics, subtopics and referencing styles are therefore different for all four documents. However, the published article has been formatted to suit the requirement of this thesis submission. The Figures and Tables have been inserted in appropriate positions close to where they are mentioned in the body text.

4.1 Paper 1

This paper entitled “Statistical comparison of two modelling methods on pressurized hot water extraction of vitamin C and phenolic compounds from *Moringa oleifera* leaves” has been published in South Africa Journal of Botany. The article compared the efficacy of response surface methodology (RSM) and artificial neural network (ANN) on the optimisation of pressurized hot water extraction of thermolabile compounds.

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Statistical comparison of two modelling methods on pressurized hot water extraction of vitamin C and phenolic compounds from *Moringa oleifera* leaves

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Abstract

Moringa oleifera is an excellent source of bioactive compounds which exhibit nutritional and medicinal properties. Pressurized hot water extraction is known as a green, environmentally, faster and efficient extraction technique. However, it is facing a challenge with the degradation of thermolabile compounds at higher temperature. This research was performed to investigate the influence of extraction temperature, time and flow rate of pressurized hot water extraction in the recovery of vitamin C and phenolic compounds from *Moringa oleifera* leaves. Response surface methodology (RSM) and artificial neural network (ANN) were used as estimates and predictive models with maximum responses. The optimal pressurized hot water extraction conditions were identified at: 91°C for temperature, 60 min for extraction time and 0.3 ml min⁻¹ for flow rate, giving a predicted concentration of 3.92±0.30, 4.74±0.32 and 0.357±0.19 g per 100 g of dry powder for kaempferol, quercetin and vitamin C, respectively. Evaluation of the two models through the statistical error parameters showed that ANN model gave a good predictive and estimation capabilities than RSM. Therefore, ANN can be recommended for use the optimization method for the pressure hot water extraction.

Keywords: ANN; *Moringa oleifera* Lam; phenolic compounds; Pressurized hot water extraction; RSM; Vitamin C.

1. Introduction

The use of plants as sources of bioactive compounds has received more attention in the food and pharmaceuticals industries. *Moringa oleifera* Lam, has been reported as an excellent source of phytochemicals such as phenolic compounds which exhibit several activities as compared to other tropical plants (Rodríguez-Pérez et al., 2015). The recovery of essential compounds from *Moringa oleifera* has received the interest of many investigators because of the nutritional and medicinal benefits it can provide. For instance, the liquid extract of *Moringa oleifera* is used for antitumor, anti-inflammatory and anti-microbe treatment in traditional and modern medicine (Doria et al., 2016; Martínez-González et al., 2017; Wright et al., 2017). The nutritional and medicinal values of the plant have led researchers to use several extraction techniques to enhance the recovery yield of the bioactive compounds from *Moringa oleifera* leaves (Wang et al., 2017; Xi and Yan, 2017).

Pressurized hot water extraction (PHWE) has been demonstrated to be an excellent technique for the extraction of phytochemicals from plants (Plaza and Turner, 2015). The technique is based on the use of high pressure and temperature to keep the water in the liquid state during the entire extraction process (Matshediso et al., 2015). PHWE has been receiving more interest due to the benefits it can offer compared to other conventional and non-conventional extraction methods (Hohnová et al., 2017). Several examples of the applicability of PHWE for the recovery of phenolic compounds from plants have been reported in the literature (Hohnová et al., 2017; Masike et al., 2017; Matshediso et al., 2015). Temperature has been reported to be the principal parameter which affects the selectivity and recovery of phenolic compounds by PHWE (Khoza et al., 2016).

Some authors have also reported maximum temperatures above 200°C proposed for the PHWE to achieve a complete extraction of the phenolic and flavonoid compounds from the plants (Rodríguez-Pérez et al., 2015). Nevertheless, the recovery of thermolabile compounds such as vitamin C is low in the final extract when it is extracted at high temperatures (Kaleem et al., 2016). For instance,

Moringa oleifera leaves are high in vitamin C (Ahmed et al., 2016). It is a thermolabile compound which is easily degraded at high temperatures (Merichel et al., 2007). There is a need to design an extraction technique which allows higher extraction of bioactive compounds with less or no degradation of the thermolabile ones. To obtain a sufficient design, it is important to employ statistical techniques for optimization.

Response surface methodology (RSM) is a statistical and mathematical modelling approach used to assess various regression analysis of quantitative data obtained from experiments to explain simultaneous multivariate equations (Ferrerres et al., 2017). It is able to model several factors (input) to responses (output), evaluate the interactions between factors and find the optimum condition of the experiment. Response surface methodology has been widely used in modelling and optimization of many extraction techniques for active compounds from plants (Derrien et al., 2017; Vivek et al., 2016). It is mostly used to design experiments with more than two variables because it helps to decrease the number of experimental runs in a full factorial design, and also reduces the running cost and time significantly (Belwal et al., 2016; Wong et al., 2017).

Artificial neural network (ANN) forms part of the machine learning techniques that fall under the broad category of artificial intelligence. They are inspired by the simulation of the human brain. ANN looks like the biological brain in two aspects, namely: training the system to the needed process for the network to get knowledge from its environment and storing acquired information by the synaptic weights or interneuron connection strength (Sabour and Amiri, 2017). These properties give ANN an excellent ability and flexibility in data fitting, prediction and modelling of non-linear relationships (Huang et al., 2017). Compared to RSM, ANN is a promising powerful and higher modelling statistical tool, due to its ability to learn from observations and to conclude through generalization, and predictive modelling of complex non-linear processes (Ameer et al., 2017). In the literature, ANN has been applied for optimization of several processes (Ameer et al., 2017; Chamoli, 2015; Pilkington et al., 2014; Prakash Maran et al., 2013).

To our knowledge, there are no studies through literature which compares two optimization techniques such as RSM and ANN approaches for PHWE of bioactive compounds from *Moringa oleifera* leaves. Additionally, there are no studies investigating the behaviour of vitamin C when it is coextracted with phenolic compounds by pressurized hot water extraction from *Moringa oleifera*.

2. Materials and methods

2.1. Chemicals and Reagents

All the chemicals were of analytical grade. Hydrochloric acid; diatomaceous earth; orthophosphoric acid and meta-phosphoric acid reagent were purchased from Sigma-Aldrich (Johannesburg, South Africa). The organic solvents such as methanol, acetic acid and acetonitrile were HPLC grade and purchased from Sigma-Aldrich. The ultrapure water used was obtained from a Milli-Q system (Millipore, Meck, Johannesburg, SA). Quercetin; kaempferol and vitamin C standards were also purchased from Sigma-Aldrich (Johannesburg, South Africa).

2.2. Sample preparation

Matured *M. oleifera* leaves were collected from Limpopo Province, South Africa in January 2017. The leaves were oven-dried at 48°C for 48 hours. The dry leaves were crushed with a pestle and mortar. The powder was put in the zip plastic bag and stored in a refrigerator at 4°C.

2.3. Experimental design for RSM

Statistical approaches to design experiments are important in method development because they involve investigating the inter-relationships between parameters that affect the efficiency of the method. In the current study, a central composite design of experiment was applied to investigate the interactive effect of temperature, time and flow rate on the recovery of bioactive compounds from *Moringa oleifera* leaves

using the PHWE technique. Design Expert 11 (Stat Ease Inc., Minneapolis, USA) was used as the statistical software.

The independent factors and their respective levels have been selected in agreement with the preliminary experiments carried out in our laboratory by Matshediso et al. (2015). In accordance with CCD, twenty experimental runs were performed to assess the recovery procedure. Each run represented a single combination of factors levels. For each experimental run, the amount of kaempferol, quercetin and vitamin C were determined as presented in Table 1. The data from CCD were analyzed by response surface regression for fitting the second-order polynomial model whose equation is described elsewhere (Prakash Maran et., 2013).

The experimental data were evaluated using multiple regression analysis. The F-test was used to evaluate the significance of the coefficients of regression. The modelling was performed with a quadratic model such as linear, squared, and interaction terms. The values of R^2 , adjusted R^2 , and prediction error sum of squares (PRESS) were applied to check the model adequacies. For each response, analysis of variance (ANOVA) was used to determine the significant terms in the model, and the results were presented in Table 2. The response surfaces were generated through the regression coefficients.

Table 4.1.1. Experimental and predicted data with response of independent factors

Run	Factors			Kaempferol (g/100g)			Quercetin (g/100g)			Vitamin C (g/100g)		
	X ₁ (°C)	X ₂ (min)	X ₃ (ml min ⁻¹)	Experimental Data	RSM Predicted	ANN Predicted	Experimental Data	RSM Predicted	ANN Predicted	Experimental Data	RSM Predicted	ANN Predicted
1	50.00	5.00	0.30	1.05	1.32	1.09	1.04	1.29	1.06	0.183	0.149	0.186
2	125.00	32.50	1.15	2.32	2.57	2.40	2.89	2.65	2.92	0.129	0.184	0.172
3	50.00	5.00	2.00	0.16	0.36	0.26	0.51	0.3	0.51	0.107	0.101	0.108
4	50.00	32.50	1.15	1.53	1.25	1.53	1.68	1.26	1.78	0.225	0.202	0.228
5	125.00	5.00	1.15	2.04	2.28	2.03	2.11	2.38	2.11	0.17	0.141	1.700
6	200.00	5.00	0.3	0.81	0.62	0.71	0.73	0.92	0.73	0.038	0.051	0.380
7	200.00	32.50	1.15	0.41	0.69	0.50	0.66	0.75	0.66	0.039	0.025	0.390
8	200.00	5.00	2.00	0.12	0.06	0.12	0.17	0.1	0.17	0.012	0.010	0.120
9	50.00	60.00	2.00	1.04	1.1	1.07	1.26	1.32	1.26	0.244	0.221	0.244
10	125.00	32.50	1.15	2.30	2.47	2.33	2.88	2.65	2.92	0.159	0.184	0.172
11	125.00	32.50	1.15	2.44	2.47	2.30	2.91	2.65	2.92	0.169	0.184	0.172
12	125.00	32.50	0.30	3.31	2.79	3.32	3.40	3.2	3.61	0.268	0.215	0.268
13	125.00	32.50	1.15	2.52	2.47	2.40	2.91	2.65	2.92	0.201	0.184	0.172
14	50.00	60.00	0.30	2.36	2.54	2.36	2.71	2.92	2.71	0.303	0.317	0.303
15	125.00	32.50	2.00	1.79	1.91	1.79	2.22	2.34	2.22	0.156	0.163	0.156
16	125.00	60.00	1.15	3.13	2.89	3.12	3.49	3.11	3.50	0.243	0.214	0.243
17	200.00	60.00	2.00	0.22	0.19	0.22	0.42	0.32	0.42	0.029	0.035	0.029
18	125.00	32.50	1.15	2.30	2.57	2.40	2.94	2.65	2.92	0.163	0.184	0.172
19	125.00	32.50	1.15	2.50	2.47	2.40	3.12	2.65	2.92	0.209	0.184	0.172
20	200.00	60.00	0.30	1.44	1.79	1.44	1.65	1.44	1.65	0.058	0.065	0.058

Table 4.1.2: ANOVA for response surface modelling showing linear, quadratic and interaction relations of each variable and coefficient for model prediction

Source	DF	Kaempferol				Quercetin				Vitamin C			
		CE	SS	F-value	p-value	CE	SS	F-value	p-value	CE	SS	F-value	p-value
Model	9	2.48	18.56	39.45	0.0001	2.83	21.99	42.54	0.0001	1.84	12.85	15.51	0.0001
A-Temperature	1	-0.3152	0.9937	19.01	0.0014	-0.3561	1.27	22.08	0.0008	-0.8875	7.88	85.61	0.0001
B-Time	1	0.4011	1.61	30.78	0.0002	0.4953	2.45	42.71	0.0001	0.3663	1.34	14.59	0.0034
C-Flow rate	1	-0.564	3.18	60.85	0.0001	-0.5162	2.66	46.39	0.0001	-0.3021	0.9127	9.92	0.0003
AB	1	-0.1835	0.2693	5.15	0.0466	-0.1562	0.1953	3.4	0.095	-0.2754	0.6068	6.6	0.028
AC	1	0.0392	0.0123	0.2348	0.6384	0.0262	0.0055	0.096	0.7631	0.0995	0.0793	0.8622	0.375
BC	1	-0.1217	0.1184	2.27	0.1632	-0.1979	0.3134	5.45	0.0417	0.0195	0.0031	0.0333	0.8588
A ²	1	-1.59	6.92	132.36	0.0001	-1.71	8.04	140	0.0001	-0.7078	1.38	14.98	0.0031
B ²	1	0.03	0.0025	0.0472	0.8324	-0.0818	0.0184	0.3204	0.5839	0.0363	0.0036	0.0394	0.8466
C ²	1	-0.0084	0.0002	0.0037	0.9528	0.0382	0.004	0.0698	0.797	0.0929	0.0238	0.2584	0.6222
PRESS		2.79	2.79			2.52				3.32			
R ²		0.9502				0.9579				0.9185			
Adj R ²		0.9280				0.9316				0.8730			
Pred R ²		0.8536				0.8887				0.7585			
Lack of Fit	5		0.3517	2.06	0.2239		0.2701	0.8872	0.5506		0.4901	1.14	0.4446
Pure Error	5		0.171				0.3044				0.43		

2.4. Artificial neural network modelling

ANN can be used as a polynomial regression modelling tool. It has the capability to model complex non-linear relationships between the input factors and the output responses. In this study, multilayer perceptron (MLP) with a back-propagation feed-forward ANN model for mapping layers of input factors and output response variables were used and trained for predictive modelling. The extraction temperature (X_1), time (X_2) and flow rate (X_3) were the inputs while the amount of kaempferol (Y_1), quercetin (Y_2) and vitamin C (Y_3) were outputs to the model. The general neural network architecture is shown in Figure 4.1.1.

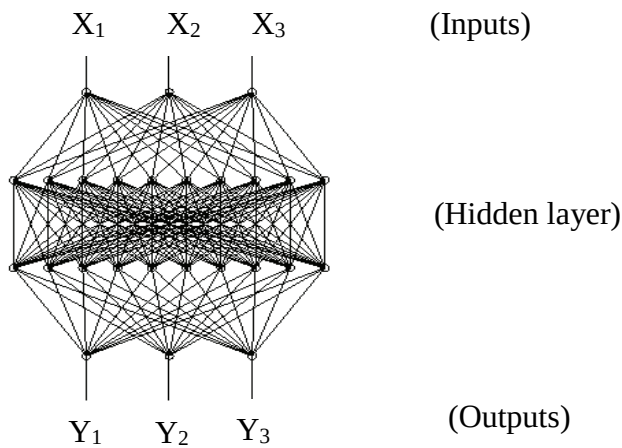


Figure 4.1.1. Artificial neural network architecture

The number of neurons in the input layer is fixed by the number of inputs and in the output layer by the number of outputs (Ameer et al., 2017). The topology of the neural network developed was designated as 3-2h- 3 (three input neurons, two hidden layers and three output neurons). The neural network topologies were designed, trained, validated, and tested with the experimental data and hidden layers were varied from 2 to 10. To build the artificial neural network, 70% (14 runs) of the dataset employed to develop the RSM modelling were applied for network training, 15% (3 runs) for validation and the remaining 15% (3 runs) for network testing. The training process was run by a trial and error search method to get a minimum of mean square error (MSE).

In the current investigation, the numbers of input and output neurons were already determined by CDD. Thus, the selection was limited to the determination of the appropriate number of neurons in the hidden layer. Neural network tools (MATLAB 2018a, Mathworks Johannesburg, South Africa) and Design expert 11 (Stat Ease Inc., Minneapolis, USA) were used for artificial neural network modelling.

Artificial neural network is a potential simulation and optimization modelling tool with a powerful prediction and estimation capabilities. The best numbers of neurons in the hidden layer of the neural network were assessed by changing the number of neurons in the hidden layer and also for various combinations of ANN-specific parameters like learning rate, momentum coefficient, maximum numbers of training cycle and initialization. The adequate capacity of the model was determined by choosing the weights, resulting in the least set RMSE between experimental and their corresponding predicted values. Each layer's initial weight and bias value was designed to determine the structure of the trained network.

The training was terminated after 1000 epochs or iterations of the network. The predicted data of ANN model are presented along with the RSM predicted and experimental values in Table 4.1.1. Network training was carried out on different feed-forward neural networks of many topologies. They were trained and then tested by determining the number of neurons in the hidden layer. The ANN topology with the lowest RMSE and highest R^2 values was selected as the optimum condition with better reliability and precision. Based on this criterion, the optimum ANN topologies for an efficient extraction of kaempferol, quercetin and vitamin C by pressurized hot water extraction was 3:10:3 which expressed numbers of neurons in the input, hidden layers, and output layers, respectively.

2.5. Comparison of RSM and ANN models

The absolute average deviation (AAD), root mean square error (RMSE), mean absolute error (MAE), standard error of prediction (SEP), model predictive error (MPE), and chi-square statistic (χ^2) and correlation coefficients (R^2) were used to

assess the fitting and predictive efficiency between experimental and predicted data of the models. The equations used to calculate these parameters are presented in Table 4.1.3. The model abilities were also evaluated by plotting the predicted values of RSM and ANN against the corresponding experimental values.

Table 4.1.3: Equations of error functions

Error	Equation
Absolute average deviation	$\text{ADD (\%)} = \left[\frac{\sum_{i=1}^n (Y_{iexp} - Y_{ical} / Y_{iexp})}{n} \right]$ (2)
Root mean square error	$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^n (Y_{i,e} - Y_{i,p})^2}{n}}$ (3)
Mean absolute error	$\text{MAE} = \frac{1}{n} \sum_{i=1}^n Y_{i,e} - Y_{i,p} $ (4)
Standard error of prediction (%)	$\text{SEP (\%)} = \frac{\text{RMSE}}{Y_{i,e}} \times 100$ (5)
Model predictive error (%)	$\text{MPE (\%)} = \frac{100}{n} \sum_{i=1}^n \left \frac{Y_{i,e} - Y_{i,p}}{Y_{i,p}} \right $ (6)
Chi square (χ^2)	$\chi^2 = \sum_{i=1}^n \frac{(Y_{i,p} - Y_{i,e})^2}{Y_{i,p}}$ (7)
Correlation R^2	$R^2 = \frac{\sum_{i=1}^n (Y_{i,p} - Y_{i,e})^2}{\sum_{i=1}^n (Y_{i,p} - Y_e)^2}$ (8)

2.6. Pressurized hot water extraction procedure

A green extraction platform was designed using a laboratory-built pressure hot water extraction system and equipped with an old gas chromatography (GC) oven using programmed heating of the sample in the cell, a HPLC pump and a hot plate (Figure 4.1.2). This was in accordance with the method described by Matshediso et al. (2015) with some modifications. For each run, a mass of 5 g of *Moringa oleifera* powder and 5 g of diatomaceous earth were both mixed and filled into the PHWE extraction cell. Before each extraction, the cell loaded with the powder was pre-heated at a specific extraction temperature for 10 min.

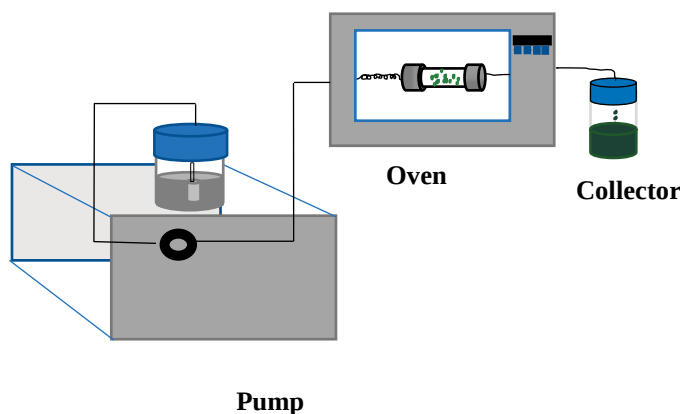


Figure 4.1.2. Pressurized hot water extraction system

The dynamic extractions were performed under different extraction condition as specified by the CDD matrix (Table 1). During the extraction process, the collected vessels were covered with an aluminium foil and put in a cooling box with ice to avoid any degradation of the bioactive compounds. After each extraction cycle, the liquid extracts were transferred to the 50 mL centrifuge tubes. The tubes were closed tightly and stored at -18°C , prior to further analysis.

2.7. Conventional sonication extraction method

In order to study the efficiency of the PHWE technique, the total amount of target bioactive compounds in *Moringa oleifera* leaf powder was first determined using sonication extraction methods (reference). For total quercetin, kaempferol and total phenolic, a mass of 2 g of *Moringa oleifera* leaf powder was mixed with 20 mL of 80% methanol in a 50 mL conical flask covered with the aluminium foil on the top and sonicated for 4 h in darkness. After filtration, 10 mL of the extract was mixed with 20 mL of 1% (v/v) HCl in 80% methanol in a 50 mL round-bottomed flask to hydrolyze the flavanols. The flask was fitted with a reflux condenser. The mixture was refluxed at 90°C for 2 h in the water bath system to obtain aglycons of flavanols. The extract was cooled to room temperature and then sonicated for 5 min to remove air and filtered through a $0.45\ \mu\text{m}$ filter paper (Millipore, Bedford, MA, USA). Finally, the extract was filtered and taken for analysis using UHPLC-DAD

-qTOF-MS (Dionex ultimate 3000, Thermofisher, South Africa, Johannesburg). The extraction was repeated several times until the analytes could not be detected. The amount of each fraction was combined to estimate the total amount of quercetin and kaempferol in the powder.

2.8. UHPLC-DAD -qTOF-MS analysis of bioactive compounds

An elution gradient of 0.1% orthophosphoric acid in water and acetonitrile were applied as elution solvent at a flow rate of 1.0 mL min⁻¹ as follows: 90% (water) for 5 min and decreased for 45% for the next 15 min. The initial conditions were further set at 90% (water) in the last 5 min. About 10 µL of the extract was injected into the UHPLC-DAD -qTOF-MS. The column temperature was kept at 50°C. The wavelength of the detector scan range was set between 200 and 500 nm. Compounds were identified in accordance with the retention time and absorbance spectra of pure standards. The quantification of the compounds was performed using a calibration curve.

3. Results and discussion

3.1. Response surface modelling

The CDD was performed in order to determine the optimum PHWE condition for an efficient extraction of kaempferol, quercetin and vitamin C from *Moringa oleifera* leaves. The results are shown in Table 1. The regression models were obtained by fitting the linear, interactive and quadratic models to the experimental data. The adequacy of the models was assessed by model summary statistics and the results are given in Table 3. The model significance was evaluated by probability values (P-values), while the evidence of goodness of fit was assessed by R² values for prediction and experimental values. It can be observed from Table 3 that a higher Fisher's F-test values and lower P-values indicate the relative significance of each term. Most of the terms in the designed quadratic model were found to be statistically significant (p<0.05). The suitability of the model was assessed through the lack of fit. As indicated by the R² values in Table 3, the

statistical significance of designed regression models were fairly high. For all the terms, lack of fit values are non-significant ($p > 0.05$) which implied that the quadratic model was valid, reliable and precise. The model fitting revealed that the independent factors (temperature, time and flow rate) significantly affected the responses content of kaempferol, quercetin and vitamin C. The multiple linear regression (MLR) equations were used to draw contour plots and 3D response surfaces which elucidated the interaction effects of independent factors (Figure 3).

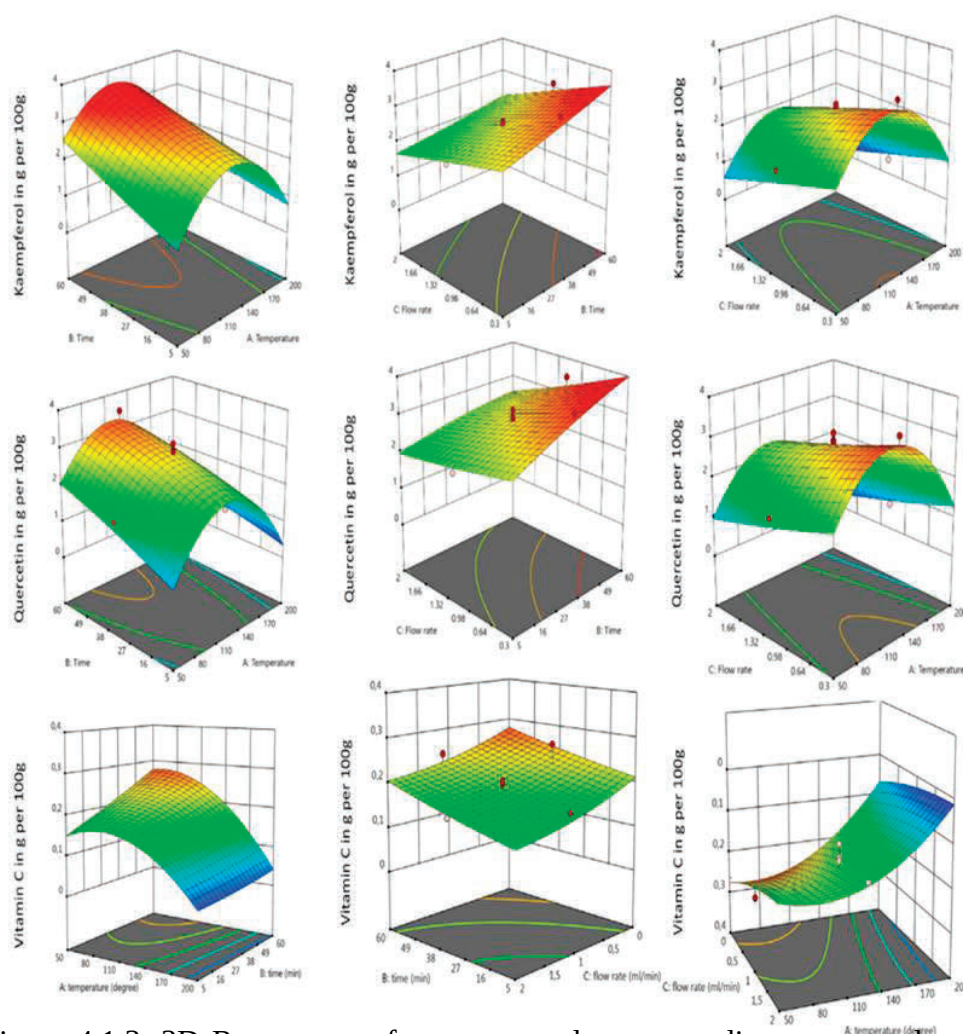


Figure 4.1.3. 3D Response surface curve and corresponding contour plot showing effect of independent factors of PHWE on maximum extraction of bioactive compounds from *Moringa oleifera*, all concentrations are expressed in g per 100 g.

The 3D plots explain the influence of different interaction effects of independent variables on the responses. Data fitting with respect to the RSM model was assessed by plotting predicted data against observed values, as shown in Figure 4.1.3. Also, ANN model-predicted values were plotted against experimental values of all three response variables (Figure 4.1.3).

On the plot, we can observe that all points are located closer to the predicted **straight line** (Figure 4.1.3 A-F), indicating that the ANN model has a potential predicted capability for the experimental data for all three response variables (Y1, Y2, Y3) with a reliable precision and validity. Data fitting demonstrated that all data points were well concentrated around the selected unity-slope line for all three response variables (kaempferol, quercetin, vitamin C). The second-order polynomial equation was fitted with the experimental results obtained from the CDD. The final equation obtained in terms of coded factors is given by the following equations:

$$\begin{aligned} \text{Kaempferol} = & 2.48188 - 0.315222 X_1 + 0.40111 X_2 - 0.564 X_3 - 0.183472 X_1 X_2 + \\ & 0.0391672 X_1 X_3 - 0.121667 X_2 X_3 - 1.58615 X_1^2 + 0.029958 X_2^2 + - \\ & 0.008372 X_3^2 \end{aligned} \quad (9)$$

$$\begin{aligned} \text{Quercetin} = & 2.82821 + -0.356111 X_1 + 0.495334 X_2 - 0.516224 X_3 - 0.156249 \\ & X_1 X_2 + 0.02625 X_1 X_3 - 0.197917 X_2 X_3 - 1.71014 X_1^2 - 0.0818099 \\ & X_2^2 + 0.0381901 X_3^2 \end{aligned} \quad (10)$$

$$\begin{aligned} \text{Vitamin C} = & 1.84091 + -0.8875 X_1 + 0.366334 X_2 - 0.302111 X_3 - 0.275416 X_1 X_2 \\ & + 0.0995836 X_1 X_3 + 0.0195841 X_2 X_3 - 0.707847 X_1^2 + 0.036318 \\ & X_2^2 + 0.092983 X_3^2 \end{aligned} \quad (11)$$

The results revealed that the equations showed the real relationship between the independent factors and the responses. The F-values and P-values of 39.45 and 0.0001; 42.54 and 0.0001; 15.51 and 0.0001, for the amount of kaempferol, quercetin and vitamin C, respectively, which implied that the models were significant. The values of R^2 reported in Table 4.1.2 suggested that 90% of the data were compatible. Further discussion of Figure 4.1.4 will be provided later where RSM and ANN predictions are compared.

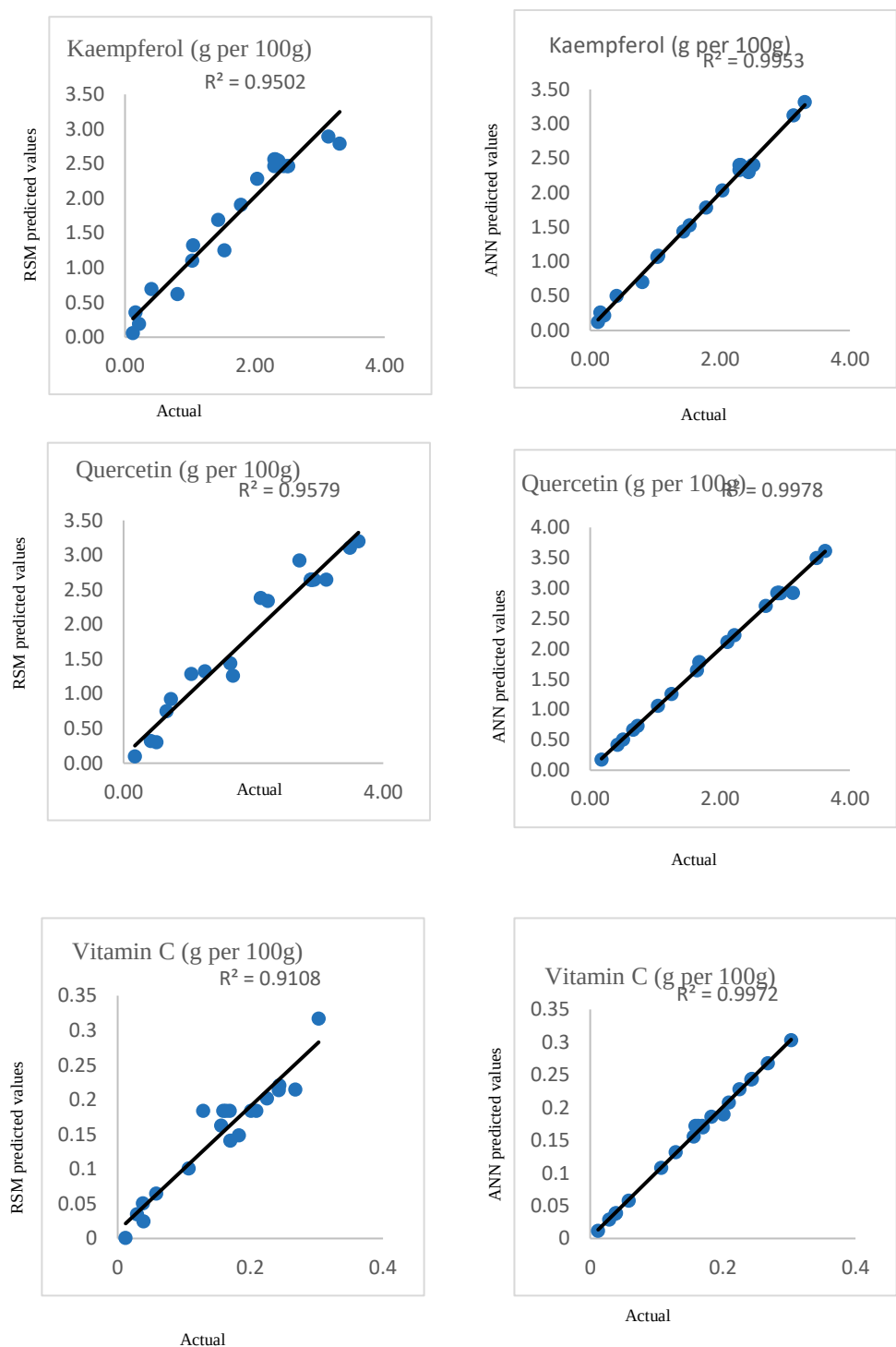


Figure 4.1.4 Comparison of experimental and predicted data given by RSM and ANN

3.2. Effect of process factor on the maximum extraction of bioactive compounds from *Moringa oleifera* leaves

3.2.1. Kaempferol and Quercetin determination

The amount of kaempferol and quercetin extracted under different PHWE extraction conditions are presented in Table 4.1.1. The highest concentration of 3.32 and 3.40 g per 100 g for kaempferol and quercetin, respectively were obtained in the experimental run 12 under the following extraction conditions: 125°C, 32.5 min and 0.3 ml min⁻¹ for temperature, time and flow rate, respectively. The increase in kaempferol and quercetin amount extracted with high extraction temperature and time, and low flow rate can be attributed to an increase in the contact time and interaction of extraction solvent with the powdered sample matrix, resulting in improved diffusion rate of bioactive compounds from the powder of *Moringa oleifera* leaves. Also at high temperature water becomes less polar and favours the solubility of the medium polarity compounds (Plaza and Turner, 2015). Some authors have reported that the temperature is the main factor that affects the extraction efficiency and selectivity in PHWE (Khoza et al., 2016; Plaza and Turner, 2015). As expected, the maximum temperature tested (200°C) was proposed as the optimum condition in PHWE in terms of extraction yield (Khoza et al., 2016). However, for the extraction of thermolabile compounds, high temperatures may cause their degradation in the extracts as reported by Matshediso *et al.* (2015). Merichel et al (2013) have also reported that the yield of the pressurized hot water extraction of phenolic compounds was dramatically increased by the rise of temperature. They have found that at a temperature above 130°C, the new compounds are forming by Maillards and caramelization reactions. They have set the optimum condition to extract the maximum amount of phenolic compounds with minimal formation brown color at 120°C and 3 min. Rodriguez-Perez *et al.* (2016) have comprised an optimum condition of 125°C and 60 min for efficiency extraction of phenolic compounds from *Moringa oleifera* leaves.

3.2.2. Vitamin C

The amount of vitamin C extracted at different PHWE conditions are presented in Table 1. The interactive effect of temperature, time and flow rate on the extraction of vitamin C from *M. oleifera* by PHWE technique is presented as a response surface in Figure 4.1.3. The highest amount extracted (3.03 g per 100 g) was found in the experimental run 14 (Table 4.1.1). Vitamin C is one of the most thermolabile compounds. Higher temperature does not favour vitamin C. It was reported through the literature that at a temperature above 95°C, the amount of vitamin C in the juice or plants started to decrease dramatically (Herbig and Renard, 2017; Kaleem et al., 2016). The results found in our investigation were in agreement with those in literature.

3.3. Comparison between RSM and ANN models

Both, ANN and RSM modelling approaches were compared with their predictive performance and estimation capabilities. The statistical parameters such as AAD (%), RMSE, MAE, SEP (%), MPE (%), and chi-square (χ^2), have been calculated by Eq. (2–8, presented in Table 4.1.3) to compare the models. The obtained results of the statistical analysis and comparison between RSM and ANN models are shown in Table 4.1.4.

Table 4.1.4. Comparison between RSM and ANN modeling

	Kampferol (g per 100 g)		Quercetin (g per 100 g)		Vitamin C (g per 100 g)	
	RSM	ANN	RSM	ANN	RSM	ANN
AAD	1.01	0.0019	2.12	0.21	1.084	0.09
RMS						
E	0.59	0.01	1.24	0.12	0.34	0.0027
MAE	0.34	0.006	0.72	0.07	0.33	0.03
SEP	1.74	0.029	3.12	0.3	1.09	0.009
MPE	2.91	0.057	5.72	0.52	3.36	0.2989
	0.02					
χ^2	9	0.00018	0.123	0.0011	0.034	0.0003

The results showed that RSM models have larger values of the statistical parameters than ANN models, indicating that the ANN model has higher modelling ability than the RSM models for extraction of bioactive compounds from *Moringa oleifera* by PHWE. Linear regression analysis was carried out between the response (amount of kaempferol, quercetin and vitamin C extracted) values predicted by ANN and RSM models with their corresponding experimental value. It is shown in Figure 4.1.4. From these plots, The ANN model predictions are much closer to the line of perfect prediction than the RSM models.

Also, the ANN model showed higher R^2 values as compared to R^2 values of RSM, which proved the superiority of the ANN model in terms of predictive and estimation capabilities. Thus, the ANN model shows a significantly higher generalization capacity than the RSM models. This higher predictive accuracy of the ANN can be attributed to its universal ability to approximate the nonlinearity of the system, whereas the RSM is restricted to a second-order polynomial (reference). The generation of ANN models requires a large number of iterative calculations, whereas it is only a single step calculation for a RSM. The ANN model may require a high computational time to create a design and is more costly than a RSM. ANN is also useful as it is flexible toward the addition of new experimental data for model generation.

A further advantage of ANN is that its training process can be optimized using different strategies such as genetic algorithms, Bayesian statistics, swarm optimization techniques, step-wise regression coupled with ANN modelling, and support vector machine (Hafizi et al., 2013; Huang et al., 2017; Orhan et al., 2012).

3.4. Simultaneous extraction of bioactive compounds

With a focus on simultaneous extraction of bioactive compounds, composite desirability functions were used to identify the universal optimum conditions. The response surface showed that the optimum conditions to extract quercetin by PHWE technique from *Moringa oleifera* were the temperature of 118°C, time of 57 min

and flow rate of 0.3 ml min⁻¹ (desirability of 0.962). For kaempferol, the optimum condition was 117.90°C, 53 min and 0.3 ml min⁻¹ (desirability of 0.946). The predicted amount of quercetin and kaempferol at these conditions were 4.01 and 3.52 g per 100 g, respectively. The optimum condition to extract a maximum amount of vitamin C was at a temperature of 82°C, extraction time of 60 min and flow rate of 0.3 ml min⁻¹ (desirability of 0.906). The predicted amount was 0.31 g per 100g. Vitamin C is the least stable among the investigated analytes and as such it has the lowest optimum extraction temperature. Therefore, the universal conditions were investigated in these ranges. This was done by setting the software to consider a simultaneous maximum extraction of kaempferol, quercetin and vitamin C. The predicted optimum condition which can extract a maximum amount of kaempferol, quercetin and vitamin C were obtained when temperature, extraction time and flow rate were set at 91°C, 60 min and 0.3 ml min⁻¹, respectively. The amounts of bioactive compounds expected to be extracted under these conditions using the two predicted models are shown in Table 5. The universal extraction conditions were applied to the *Moringa oleifera* powder to determine the exact extracted amounts. It was observed that the ANN predicted values for the extraction of all the bioactive compounds were within the standard deviation of the experimental values compared to RSM predicted values.

3.5. Efficiency of the PHWE on bioactive compounds

The amount of the quercetin, kaempferol and vitamin C extracted using the PHWE technique was compared with the total amounts obtained using exhaustive solid-liquid extraction techniques. The results are presented as percentage recoveries in Table 5. The results show that the percentage recoveries of the PHWE ranged from 86 – 94%. These results prove that PHWE is an efficient technique which can be used in the extraction of bioactive compounds from *Moringa oleifera* leaves. The percent recovery found in the current study is similar with the one reported by Vilku et al. (2007) in the pressurized hot water extraction of phenolic compounds from apple.

Table 4.1.5 Predicted and experimental extraction values of each compound under universal conditions

Compounds	RSM Predicted	ANN Predicted	Experimental
Quercetin (g per 100 g)	3.47	4.09	3.92±0.30
Kaempferol (g per 100 g)	3.91	4.75	4.74±0.32
Vitamin C (g per 100 g)	0.31	0.365	0.357±0.19

4. Conclusion

The current study was carried out to investigate the influence of temperature, time and flow rate on the extraction of bioactive compounds from *Moringa oleifera* leaves. RSM and ANN were performed as optimization techniques. The evaluation of different error statistical parameters such as AAD, RMSE, MAE, MPE, χ^2 and R^2 have shown that ANN is an excellent statistical tool in terms of prediction and estimation capabilities compared to the RSM model. The optimization of PHWE process by RSM and ANN suggested that maximum values of kaempferol, quercetin and vitamin C were 4.09, 4.75 and 3.65 g per 100 g respectively under optimum conditions of 91°C, 60 min and 0.30 ml min⁻¹, while the RSM is the most commonly used statistical tool for optimization. ANN has shown to be a lot better alternative to RSM with better predictive and estimation capacities for an efficient optimization of pressurized hot water extraction of kaempferol, quercetin and vitamin C from *Moringa oleifera* leaves. To conclude, the optimization of PHWE using the two techniques provide an effective guideline for a maximum extraction of bioactive compounds from *Moringa oleifera* leaves.

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Compliance with Ethical Standards

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Conflict of Interest: All authors declare that they have no conflict of interest.

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4.2 Paper 2

This paper entitled “Chemometric approach on the behaviour of macro- and micro-nutrient elements in the *Moringa oleifera* grown in Southern Africa extracted by pressurised hot water extraction technique” has been submitted to Food chemistry Journal. The article reports the application of pressurized hot water extraction of metal species from *Moringa oleifera* leaves.

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Chemometric approach on the behaviour of macro- and micro-nutrient elements in the *Moringa oleifera* grown in Southern Africa extracted by pressurised hot water extraction technique

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Abstract

Moringa oleifera leaves are widely used in traditional medicine as a food supplement for its high antioxidant and vitamin content. Pressurised hot water extraction (PHWE) technique as a green approach was used for the recovery of the macro and micro-elements from dry leaves powder of *Moringa oleifera*. In this study, response surface methodology was applied to assess the influence of temperature (50 - 200°C) and time (5 – 60 min) on the behaviour pattern of macro and micro nutrients from the leaves of *Moringa oleifera* when extracted by pressurised hot water extraction technique. The elements such as Ca, K and Mg (macro-elements), and Al, Co, Cr, Cu, Fe, Ni and Zn (micro-elements) from the leaves were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). The behaviour of extracted elements was compared in terms of recovery to the total concentration in the leaves. Extraction of the metals at different temperatures and times revealed their different behaviour. The removal of macro-nutrients from the *Moringa oleifera* leaves were less influenced by the temperature but highly influence by the extraction time. However, the amounts of micro-elements extracted were found to be very depended on the extraction temperature. Hence, the recovery for macro-nutrients ranged from 89±2 to 99±1% and while for micro-nutrients it ranged from 23±2 to 37±4%. From the results, it

can be observed that more macro-nutrients are extracted than microelements and the pressurized hot water extraction can be efficiently applied for the extraction of the nontoxic metals from plant materials.

Keywords: macro- and micro-nutrients, *Moringa oleifera*, *response surface*, pressurised hot water extraction.

1. Introduction

Moringa oleifera, which is one of the 13 known *Moringa* species, is coming to the forefront as a result of scientific evidence that it is an important source of essential nutrients and has medicinal properties (Anwar et al., 2007; Hazra et al., 2012; Williams, 2013; Nadeem et al., 2015). *Moringa oleifera* leaves are known as a source of antioxidants as well as macro and micro-elements (Nadeem et al., 2015). These nutrients play a vital role in human physiology (He et al., 2011). For instance, the macro-nutrient such as Ca, K and Mg are essential for living organisms. They are involved in several metabolism mechanisms. The human body need 300 to 3000 mg per day of macro-nutrients (Williams, 2013). Micro-nutrients participate in several enzymatic reactions as coenzymes (Williams, 2013). They are needed in trace amounts while at high concentrations, they can be toxic to humans and animals (MacBurney et al., 2004). In order to evaluate the nutritional content of the *Moringa oleifera* leaves, target compounds have to be extracted. Thus, various nutrients both elements and organic compounds have been extracted from *Moringa oleifera* leaves using different extraction techniques (MacBurney et al., 2004; Igbal and Bbanger, 2006; Pakade et al 2013; Williams, 2013).

Conventional extraction techniques used for extraction of compounds from herbal plants are distillation and solvent extraction (He et al., 2011). These are now becoming less popular because they are often result in toxic solvent residues. Thus, a move now is towards modern sample extraction techniques especially green ones such as supercritical fluid extraction (Turner et al., 2002) and pressurised hot water extraction (PHWE) (Kroholm et al., 2007; Roudsari et al., 2009; Mustafa and Turner, 2011). In pressurised hot water extraction, as water is heated at high

temperature and pressure, it behaves as an organic solvent and hence becomes less polar with a dielectric constant that compares to that of ethanol and methanol (Smith, 2012). The use of subcritical water as a solvent for the extraction of essential compounds especially in plant materials has an added advantage in that such extracts can be used in value addition in various food products without minimal solvent health risk. This eliminates another step often followed that produces non-toxic extracts when using solvent evaporation (Carr et al., 2011). The application of pressurized hot water extraction of phenolic compounds has been reported (Fernandez-Perez et al., 2001).

The extraction mechanism of PHWE involves the control of some essential parameters such as pressure, flow rate and temperature (Mustafa and Turner, 2011). The results from these studies reported that 120°C is the optimum temperature for an efficient recovery of the phenolic compounds without degradation and beyond this temperature, other compounds form and exhibit different structures and chemical properties compared to the target compounds (Mustafa and Turner, 2011). On the other hand, very little studies have been done on metal extraction from plant material using PHWE (Priego-Lopez et al, 2002; Fernandez-Perez et al., 2001). No such studies have been reported on the Moringa plant. This is very important for use of Moringa extract in food value addition as metals are also often needed in the final extract. Therefore, the goal of this work was to develop and optimise a PHWE method that maximizes the macro-nutrients extraction yield and at the same time minimizes the recovery of micro-nutrients from *Moringa oleifera* leaves.

2. Materials and methods

2.1. Chemicals and reagents

All reagents used were of analytical grade. Diatomaceous earth was purchased from Sigma Aldrich (Johannesburg, South Africa). Metals elements used as standards were purchased from Spectro South African branch (Johannesburg, South Africa). Ultra-pure water from Milli-Q, Millipore (MA, USA) was used in all the experiments.

2.2. Plant material

Leaf samples of *Moringa oleifera* were collected from a moringa farm in Hammanskraal, Pretoria (South Africa). The samples were rinsed with distilled water immediately after harvest and left to air dry in a sterile environment in the shade. The leaves were pounded with a pestle and mortar and sieved with a 25 μm sieve and stored in an opaque container for later use.

2.3. Design of experiment

The effects of temperature (50-200°C) and time (5 – 60 min) on the extraction of trace metals were evaluated using the response surface methodology. Full factorial design comprised of 12 experiments with 3 center point replicates which was created in MODDE Pro (Sartorius Stedim Biotech, Malmö, Sweden). Influence of the variables was evaluated by calculating the recovery of Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Na, Ni, Pb and Zn. During the optimization process, the flow rate was kept constant at 0.5 ml min⁻¹ according to our previous work (Yannick et al., 2018). Multiple linear regression was used to calculate the fitting model and response surface. The adequacy of the models was evaluated by the R² and Q² values (where R² shows the model fit and Q² shows an estimate of the future prediction precision). Further, predicted vs. observed plot were done and coefficient plots obtained. The optimum processing conditions were obtained by using graphical and numerical analysis based on the criteria of the desirability function and the response surface plots.

2.4. Pressurised hot water extraction procedure

The pressurized hot water extraction system that was used has been previously reported (Yannick et al., 2018). It consists of an extraction cell housed in a GC oven for controlling the extraction temperature. The liquid through the system was pumped by an HPLC pump. Deionized water heated at 80°C was pumped through the system during extraction. For each run, a mass of 5 g of *Moringa oleifera*

powder and 5 g of diatomaceous earth (as dispersing agents) were both mixed and filled into the PHWE extraction cell. Before each extraction, the cell loaded with the powder was pre-heated at a specific extraction temperature for 10 min. Dynamic extractions were performed under different extraction condition as specified by the CDD matrix (Table 1). After each extraction cycle, the liquid extracts were transferred to the 50 mL centrifuge tubes. The tubes were closed tightly and stored at -18°C, prior to further analysis.

2.5. Metal analysis

The total metal content was analysed using inductive coupled plasma optical emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro, Germany). The standard method for metals from plants was applied (AOAC Official Method 2015.01 (US EPA,2015)).

2.6. Quality assurance

Appropriate quality assurance procedures and precautions were carried out to ensure reliability of the results. Deionised water was used throughout the study. Glassware was properly cleaned, and the reagents were of analytical grade. Reagent blank determinations were used to correct the instrument readings. Standards were prepared for each metal from their stock solution for calibration of the instrument. Precision and accuracy of analysis were checked through repeated analysis.

Table 4.2. 1. Experimental conditions and the corresponding extracted metals (mg kg⁻¹) from PHWE

Run Order	Temperature	Time	Al	Ca	Co	Cr	Cu	Fe	K	Mg	Ni	Zn
12	50.00	5.00	29.91	19808.52	5.50	3.02	21.25	71.37	39726.52	14094.50	2.51	21.25
5	125.00	5.00	80.43	20690.13	20.73	10.89	80.01	260.76	41859.12	14178.12	9.46	80.09
3	200.00	5.00	164.20	20734.60	35.06	19.26	135.47	452.62	40886.32	14246.32	16.01	135.47
4	50.00	32.50	30.99	22996.45	7.52	4.13	29.07	97.13	52760.61	16838.51	3.44	29.07
6	125.00	32.50	82.73	23011.52	23.51	12.91	90.82	303.44	52026.43	16920.22	10.74	90.82
1	200.00	32.50	185.84	23675.46	38.92	21.38	150.37	502.41	52615.22	16998.43	17.77	150.37
2	50.00	60.00	38.02	29044.67	17.15	9.42	66.28	221.45	56497.13	22521.11	7.83	66.28
8	125.00	60.00	84.00	30144.73	26.56	14.59	102.63	342.89	56673.21	22165.22	12.13	102.63
9	200.00	60.00	198.47	30682.14	43.59	23.94	168.41	562.69	56716.32	22560.41	19.91	168.41
10	125.00	32.50	86.86	24162.23	24.68	13.56	95.36	318.62	54127.72	17766.22	11.27	95.36
7	125.00	32.50	87.69	24392.12	24.92	13.69	96.27	321.65	45548.32	16935.42	11.38	96.27
11	125.00	32.50	83.55	23241.60	23.74	13.04	91.73	306.48	50446.74	17089.44	10.84	91.73

3. Results and discussion

3.1. Design of experiment optimization approach

Temperature and time were chosen for the investigation of the behaviour of macro- and micro-nutrient elements in the *Moringa oleifera* extracted by pressurised hot water extraction technique. The model was created and calculated by fitting using partial least squares. The fitted model showed a total explained variance of 89 – 99 % ($R^2 = 0.89 - 0.94$) and a cross-validated predictability of 90 - 96 % ($Q^2 = 0.85$), where R^2 shows the model fit and Q^2 shows an estimation of the future prediction and precision. The linearity of the predicted versus observed values plot (Figure 4.2.1) highlighted the validity of the model and its capability to predict the best condition of the extraction within the range of the design.

The coefficients plot (Figure 4.1.2) reveals that the extraction of macro-nutrients depended on the extraction time while the recovery of the micro-nutrients was more influenced by the temperature.

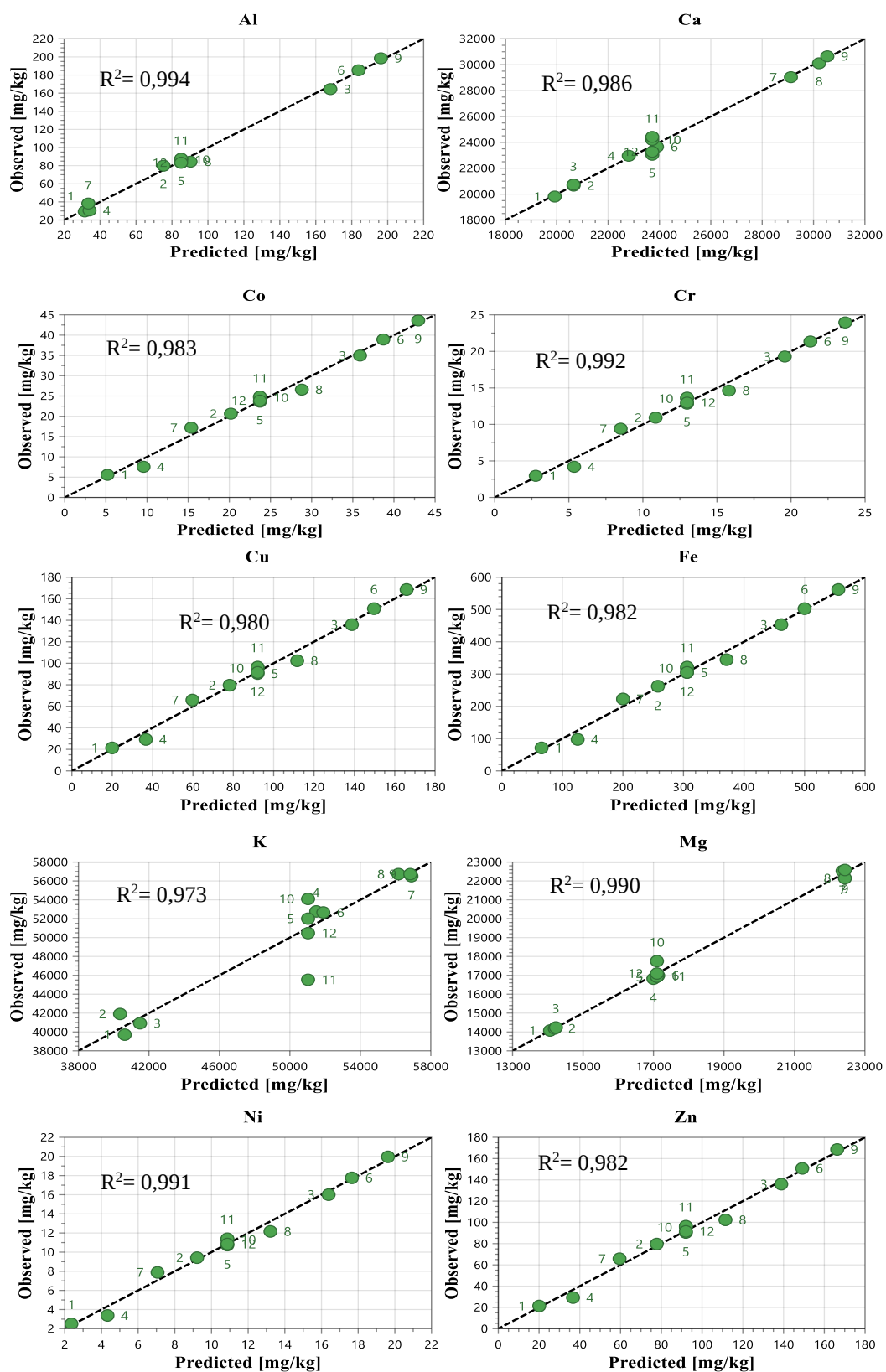


Figure 4.2.1. Predicted versus observed values plot of the designed model

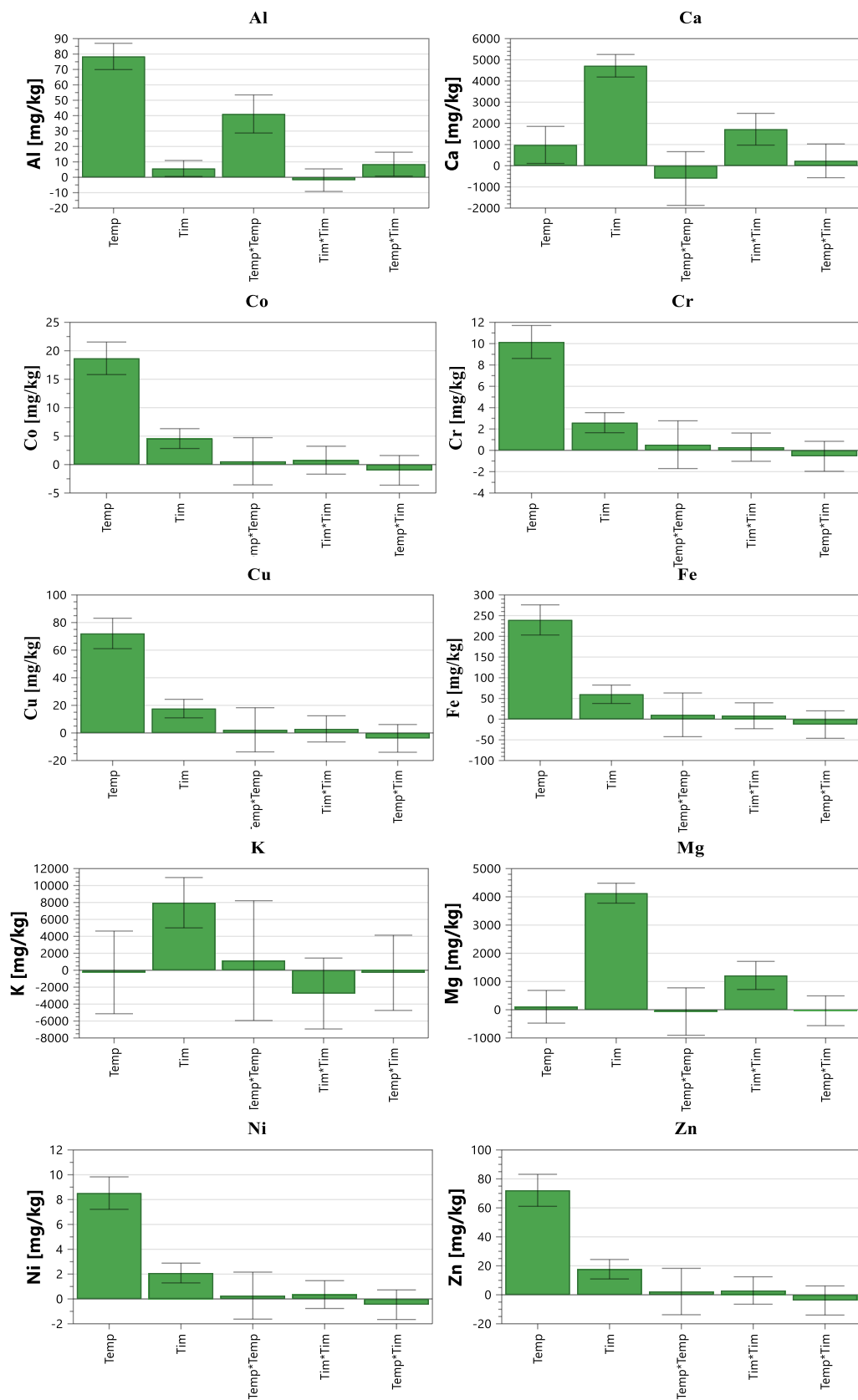


Figure 4.2. 2. Coefficient of plot of the interaction of different factors

3.2. Effect of the selected parameters on the extraction of metals

The 3 D contour plots presented in Figure 3 showed the amount of macronutrients extracted were more dependent on extraction time and not on the temperature. The results suggest that the macro-element in the *Moringa oleifera* leaves are not tightly bound to organic ligands like carbohydrate in the plant leaves. This means even at low temperature macro-nutrient can be extracted at high amount by just increasing the extraction time. This is very important for value additions of the extract in the food industry, extraction temperature need not to compromise some organic compounds like polyphenolic compounds and vitamin C. The behaviour of micronutrient elements during PHWE are also shown in the 3 D contour plot (Figure 4.2.3). The results indicate that micronutrients generally were extracted at high temperature. This observation is direct opposite to the extraction of macronutrients of which more were extracted at any temperature. This might be due to differences in the chemistry of binding of macronutrients and micronutrient elements in the *Moringa oleifera* leaves (Brunetti et al., 2013; Kanayama et al., 2013). This suggest that micro-nutrients are tightly bound to the ligands in the plant cells.

3.3. Predicted conditions for a maximum extraction of healthy metals from *Moringa oleifera* leaves

The results of different predicted conditions for a maximum extraction of healthy metals from the leaves are presented in Table 2, this consists of five runs set from 80 to 100°C, since a long extraction time shown to be in favor for a maximum extraction of the nontoxic metals. Therefore, the time was kept constant at 60 min. The results show that all the predicted values were within the standard deviation of the experimental value. It emphasis that the partial lest squares model can be used to predict the extraction condition of the metal from the pressurized hot water extraction. After analysis of the results, it was observed that after 90°C, the amount of toxic metals started to increase.

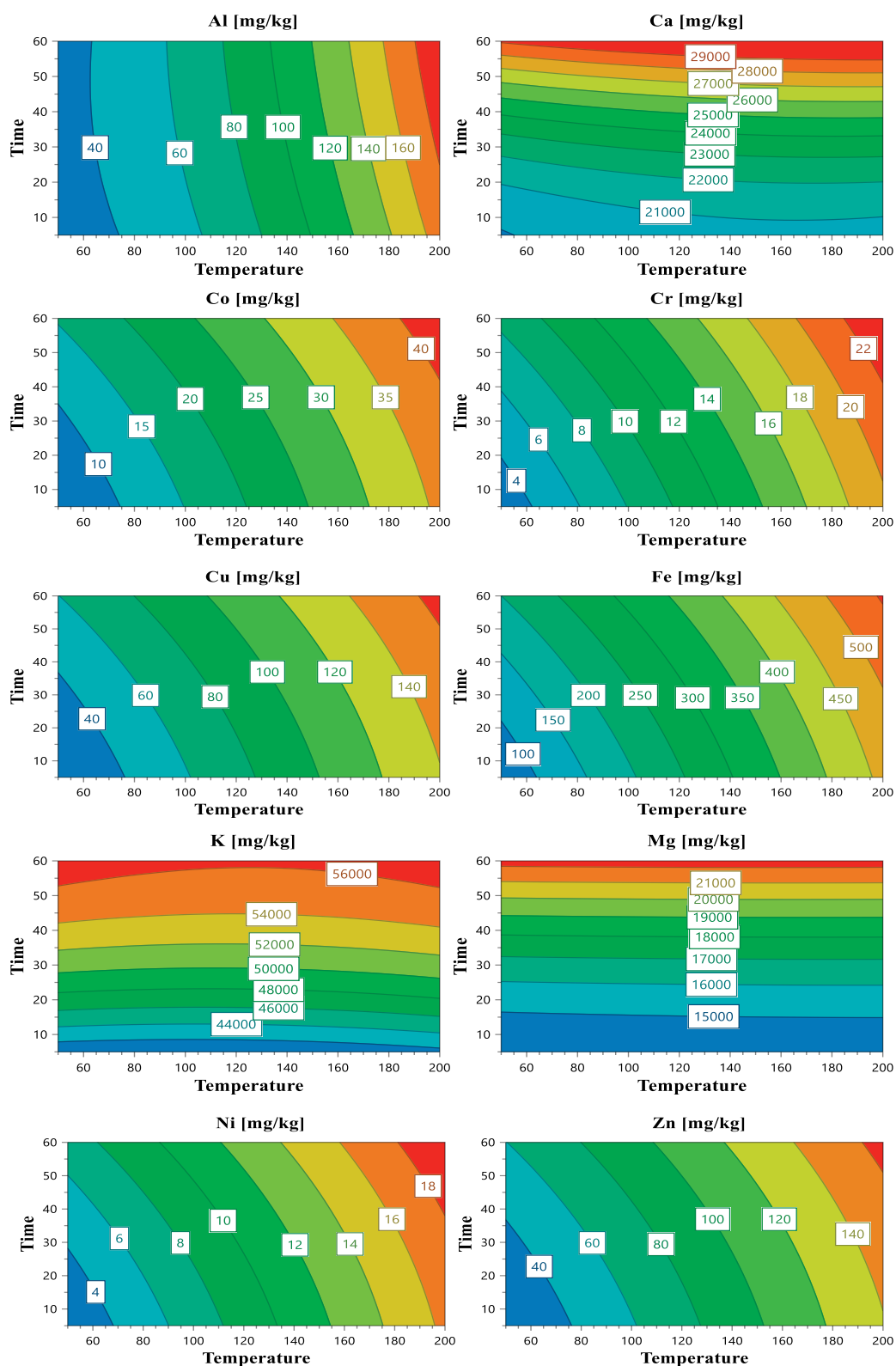


Figure 4.2.3. Contour plot of the impact of temperature and time on the amount of metal species extracted

In addition, this temperature is suitable for the extraction of antioxidant compounds as reported in our previous work on the pressurized hot water extraction of kaempferol, quercetin and vitamin C from *Moringa oleifera* leaves (Yannick et al., 2018). Therefore, it was concluded that the best conditions of PHWE are at 90°C and 60 min for extraction temperature and time, respectively.

3.4. Recovery of metals extracted by pressurized hot water extraction from *Moringa oleifera* leaves

The results of total metal concentration in the leaves were compared from those extracted by PHWE (Table 3). Table 3 shows that moringa leaves are rich in macro and micro elements. Macro elements were almost 100% extracted with recovery ranging from 88.84 ± 2.09 – $98.49 \pm 1.34\%$. For the micro elements the recovery ranged from 22.53 ± 2.31 – $38.46 \pm 1.89\%$ except for iron with recovery of $71.22 \pm 2.12\%$. These differences in the amount extracted between macro and micro elements reflected, how tightly, they are bound to organic ligands.

Table 4.2.2. Predicted vs observed value of metals extracted (N=3)

Factor	Al (mg kg ⁻¹)		Ca (mg kg ⁻¹)		Co (mg kg ⁻¹)		Cr (mg kg ⁻¹)		Cu (mg kg ⁻¹)		Fe (mg kg ⁻¹)		K (mg kg ⁻¹)		Mg (mg kg ⁻¹)		Ni (mg kg ⁻¹)		Zn (mg kg ⁻¹)	
Temp	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs
80.00	50.48	48.25 ±4.60	30197.00	30396.00 ±40.15	20.70	21.00 ±8.96	11.34	10.00 ±6.49	80.07	78.56 ±6.89	267.02	280.00 ±30.25	56440.00	56650.00 ±34.96	22408.00	22689.00 ±38.92	9.46	11.56 ±5.69	80.07	86.96 ±7.56
85.00	54.08	50.18 ±5.80	30216.00	31156.00 ±25.62	21.60	22.36 ±5.21	11.82	12.46 ±8.75	83.52	86.16 ±5.69	278.42	290.00 ±46.56	56388.00	56459.00 ±50.68	22413.00	22895.00 ±57.83	9.87	13.56 ±7.56	83.52	91.56 ±9.56
90.00	57.89	53.78 ±5.96	30236.00	31235.00 ±32.55	22.50	24.26 ±9.56	12.31	15.23 ±6.89	86.98	90.26 ±8.45	289.87	300.00 ±45.89	56342.00	56560.00 ±45.62	22418.00	22906.00 ±68.92	10.28	15.56 ±6.59	86.9	95.56 ±8.79
95.00	61.90	59.86 ±8.92	30256.00	31465.00 ±21.55	23.40	28.16 ±9.16	12.70	19.26 ±8.45	90.45	95.12 ±7.56	301.38	325.00 ±38.95	56400.00	56800.00 ±56.93	22422.00	23045.00 ±80.63	12.69	18.56 ±9.56	90.46	100.23 ±11.23
100.00	66.16	64.56 ±8.08	30265.00	32012.00 ±30.55	24.30	30.49 ±9.98	13.28	20.25 ±9.16	93.93	98.23 ±8.16	312.94	345.00 ±49.86	56566.00	56987.00 ±65.93	22526.00	23156.00 ±15.00	15.10	20.69 ±7.96	93.94	109.56 ±16.56
80.00	50.48	48.25 ±4.60	30197.00	30396.00 ±40.15	20.70	21.00 ±8.96	11.34	10.00 ±6.49	80.07	78.56 ±6.89	267.02	280.00 ±30.25	56440.00	56650.00 ±34.96	22408.00	22689.00 ±38.92	9.46	11.56 ±5.69	80.07	86.96 ±7.56

Pred: Predicted; Obs: observe. To all the experiments, the extraction time was constant at 60 min

Table 4.2.3. Recovery of metals extracted by pressurized hot water extraction (N=3)

	PHWE (mg kg ⁻¹)	Microwave digestion (mg kg ⁻¹)	Recovery (%)
Al	54±11	146±13	37±4
Ca	31235±256	32001±214	98±3
Co	24±10	77±11	32±1
Cr	15±7	68±9	23±2
Cu	90±9	235±5	39±2
Fe	214±24	300±46	71±2
K	56560±286	57426±346	99±1
Mg	22906±689	25783±329	89±2
Ni	16±7	46±4	34±2
Zn	96±9	286±5	34±2

As compared with the PHWE, the total concentrations of macronutrients are slightly higher. This is more pronounced for Ca, K and Mg which are much higher in *Moringa oleifera* leaves. For micronutrients, the total concentrations were very high in the extract. Again, differences in total metal content can be attributed to the binding chemistry of the metals in the organic matter (Brunetti et al., 2013; Kanayama et al., 2013). The results indicate that macronutrients are extracted more in *Moringa oleifera* leaves. Thus, complexes with soft metals and soft bases are much more stable. This may suggest that *Moringa oleifera* has high amounts of such ligands.

Overall, the amount of metals extracted using PHWE were lower than the amount recovered using the microwave assisted extraction though actual difference depends on metal type and plant species. This is consistent with literature because MAE is much more efficient. In MAE, heating occurs in a targeted and selective manner with practically no heat being lost to the environment as the heating occurs in a closed system. One other reason microwave extraction is efficient is because there

is a synergistic combination of the heat and mass transfer working in the same direction as opposed to PHWE where the mass transfer occurs from outside to the inside of the substrate.

4. Conclusions

Pressurized hot water is a promising green extraction technique for obtaining essential compounds from *Moringa oleifera* leave. The technique has demonstrated to be able to extract metals like organic compounds. Response surface design was applied to investigate the behaviour of two dependant factors such as temperature and time on the recovery of metals by the pressurized hot water extraction. The results revealed that the extraction of micro-elements depended on the temperature. The optimum conditions were found to be at 90°C and 60 min for temperature and extraction time, respectively. Under this condition macro-elements are more extracted than micro-elements. This selectivity of PHWE lead to produces an extract with a high amount of healthy metals and less amount of those needed in trace elements. This is important in order to produce a nontoxic extract for the food industry.

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4.3 Paper 3

This paper entitled “Pressurised hot water extraction with on-line particle formation of *Moringa oleifera* leaves” is under preparation for submission to Journal. In this article, water extraction with on-line particle formation, based on the use of supercritical fluid, was optimised for drying Moringa aqueous extract. The manuscript is in preparation for submission

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Pressurised hot water extraction with particle formation on-line of *Moringa oleifera* leaves

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Abstract

An on-line process for pressurized hot water extraction of antioxidants from *Moringa oleifera* leaves as well as drying of the extract in one step by particle formation based on the use of supercritical carbon dioxide has been optimized. This process has been called water extraction with on-line particle formation (WEPO). Design of experiment was used to investigate the influence of 3 quantitative factors including CO₂ pressure (60 – 80 bar), CO₂ flow rate (8 -10 ml min⁻¹) and nitrogen pressure (10 -20 bar) as well as 5 qualitative factors (nitrogen position, vacuum, length of the tubing, internal diameter of the nozzle and additional nitrogen). The factors were first screened using Factorial Factor Res IV and optimized by the Box Behnken methodology. The amount of powder collected, water content, size and morphology of particles and polyphenolic content were studied to evaluate the efficiency of the WEPO process. The optimum conditions to obtain a fine and dried particle were set as 90.95 bar CO₂ pressure, 16 ml min⁻¹ flow rate of CO₂ and 26 bar nitrogen pressure. With these conditions, the amount of powder collected, water content and average size of particle were 150±10 mg, 3.58±0.56% and 384.33±21.36 nm, respectively. These results were compared with the results from extracts obtained by continuous flow PHWE followed by freeze and spray drying techniques. The results showed that WEPO is a suitable and promising process to obtain, in only one step, fine and dried particles.

Keywords: *Moringa oleifera*, online particle formation, pressurized hot water extraction

1 Introduction

Nowadays, the demand for natural compounds from plants is increasing due to their use as ingredients in food, cosmetics and pharmaceutical industries (Hamany Djande et al., 2018a, Chin et al., 2018)). Natural compounds also called phytochemicals exhibit several nutritional and medicinal properties such as: antioxidant, antimicrobial, anti-cancer and neuroprotector. *Moringa oleifera* is one the plants in the vegetal kingdom which is considered as an excellent sources of bioactive compounds (Rajanandh et al., 2012). It is originally from India and widely distributed in tropical and subtropical regions (Ohta et al., 2017). Due to its health benefits, all parts of the plant are used for diverse purposes (Zhong et al., 2018). The leaves are most applied as vegetables, tea and food supplement (Makris, 2018). Several studies have reported that *Moringa oleifera* leaves are a rich source of polyphenolic, vitamins, proteins and minerals (Gupta et al., 2017). A number of clinical studies have demonstrated the pharmacology activities such as anti-inflammatory, anti-bacteria, anti-cancer and immunosuppressant activities of *Moringa oleifera* extract (Cohen-Zinder et al., 2017).

The qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of a proper extraction method. In the isolation process of bioactive compounds from plants, conventional extraction techniques such as Soxhlet, maceration and sonication are generally used (Hamany Djande et al., 2018b). These techniques are time consuming and use an important volume of organic solvent which are toxic. As an alternative to these problems, pressurized hot water extraction (PHWE) has been introduced as a green extraction technique (Nuapia et al., 2018). It uses a supercritical water as a solvent for the recovery of the bioactive compounds from plants. At high pressure and temperature water has the solubility properties of ethanol and methanol which leads to the extraction of polar and semi polar compounds (Ongkowijoyo et al., 2018). This extraction

technique was applied in the recovery of several bioactive compounds from plants, also, it was reported to be more efficient for the recovery of phenolic compounds (Rodríguez-meizoso et al., 2012). PHWE has been applied widely to extract phenolic compounds from moringa leaves (Matshediso et al., 2015, Nuapia et al., 2018).

Among all these advantages, PHWE extract contains a large amount of water, this might be harmful to the stability of the bioactive compounds due to the increase in micro-organism and enzymatic activities. Also, it is difficult to transport and store a liquid extract (Acosta-Esquivarosa et al., 2009). These are the most significant limitations of PHWE application on an industrial scale. To overcome this, drying the liquid extract is a better option for a suitable commercial product. Freeze, spray and hot air drying has been often applied to remove water from the extract (Mohtar et al., 2014). However, these techniques presented several disadvantages such as time consuming and exposed the extract to risk of degradation of the bioactive compounds. The use of supercritical fluid is seen to be a promising alternative drying process. Supercritical fluid carbon dioxide (SC-CO₂) has been applied as an anti-solvent to precipitate compounds from organic solutions. SC-CO₂ and organic solvents are miscible above a certain temperature and pressure, while the compound is insoluble in the mixture and it precipitates and can be dried by a hot nitrogen (Sievers et al., 2007; Anderson et al., 2012).

In the case of aqueous solution, these processes are not suitable for drying due to the low solubility of water in SC-CO₂. Instead, SC-CO₂ can be used as a dispersion medium as in the so-called CAN-BD process (Carbon Dioxide Assisted Nebulisation by a Bubble Dryer). Therefore, SC-CO₂ and water form an emulsion that is fragmented down in fine water droplets by the expansion of CO₂ after depressurisation. The fine droplets formed are dried by hot nitrogen (Rodríguez-meizoso et al., 2012). Recently, CAN-BD has been employed to obtain powders of DNA and RNA, antibiotics, antiviral (Sievers et al., 2007), water-soluble drugs (Nuchuchua et al., 2017), vaccines (Tabernero et al., 2012) and proteins (Padrela et al., 2018) from aqueous solution. Therefore, there is few research studies, where,

CAN-BD is being used to dry a complex matrix such as plants extract. Water extraction with online particle formation was for the first time reported by Rodríguez-Meizoso et al. (2012) and Andersson et al. (2012). It is an on-line process for pressurised hot water extraction (PHWE) of antioxidants from plants as well as drying in one step by particle formation based on the use of supercritical carbon dioxide (SCCO₂) the process was called WEPO[®] (water extraction with on-line particle formation).

The technique is faster and produced fine particles with a good bioavailability and less exposing of the compounds to air contact. As reported by Anderson et al (2012), the amount of the powder collected with WEPO was far lower than the one with freeze and spray drying. Thus, the current work assessed the effect of different parameters of WEPO such as temperature, pressure of CO₂, flow rate of CO₂ and nitrogen pressure on increasing the amount of powder collected and decreasing the size of the particle obtained. Response surface methodology was used as the statistical tool for the optimization of the process.

2. Materials and methods

2.1 Materials

Matured *Moringa oleifera* leaves were collected from Limpopo Province, South Africa in December 2017 and shipped to Lund University, Sweden for experiment works. The leaves were put in the plastic bags and stored in a freezer at -20°C. To avoid variation from the raw material, the same batch was used throughout the whole project. Ultrapure CO₂ was bought from Air Products (Amsterdam, Netherlands), Nitrogen 4.5 grade was provided by Strandmoller (Klampenborg, Denmark). Acetonitrile and methanol use for UHPLC analysis were of HPLC grade and purchased from Sigma (Lund, Sweden). Formic acid (98–100% purity) was obtained from EMSURE (Darmstadt, Germany). Gallic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin-4'-O-β-D-glucose, quercetin 3,4'-diglucoside, quercetin and kaempferol were purchase from

Polyphenols Laboratories S.A. (Sandnes, Norway). Ethanol (95% purity) was used for cleaning of the equipment.

2.2. Equipment description

The structure of the built system designed to carry out the water extraction with particle formation on-line (WEPO) is shown on Figure 4.3.1. The apparatus designed in this work combines two processes: the continuous flow PHWE of *Moringa oleifera* leaves sample and the continuous production of an aerosol from the liquid extract by mixing the water extract with SC-CO₂. The formed aerosol is dehydrating on -the-fly by a hot N₂ flow. Thus, extract and precipitation take place in the same system within a shorter time.

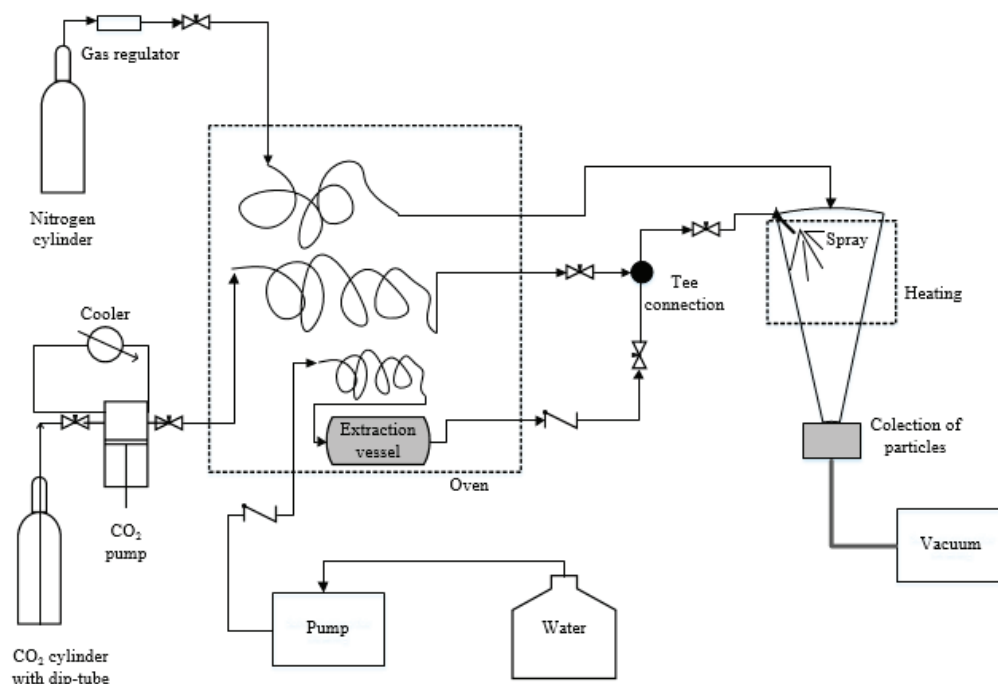


Figure 4.3.1. Scheme of the WEPO equipment.

The WEPO system comprises of a HPLC pump (515 HPLC pump, Waters, Milford, MA, USA) delivering water at a constant flow rate through 1/16 in. stainless steel tubing to the extraction cell (10 ml) located in an oven (5890A, Hewlett Packard, nowadays Agilent Technologies, Santa Clara, CA, USA). The temperature used

throughout all the experiments was 120°C. From the cell, the extract was led to a tee fitting where it was mixed with SCCO₂ to create an emulsion that is immediately exited into atmospheric pressure through a nozzle. The obtained aerosol was sprayed into a 5 L glass chamber (custom made) and dried with hot dry N₂ (120°C). A needle valve was placed before the nozzle to help restrict the pressure during the process. The CO₂ was pumped by an Isco syringe pump (Model 260D, Teledyne Isco, Thousand Oaks, CA). The particles were collected from a filter in the bottom of the chamber with the help of the vacuum.

2.3 Experimental design and statistical assessment

The response surface methodology was applied to establish the experimental protocol for the water online particle formation from PHWE extract of *Moringa oleifera* leaves. It was used to fix the best combination of the different parameters for optimum amount of particle collected with smaller size and good distribution. Response surface was employed to screen the influence of eight parameters sprayed in two groups (qualitative and quantitative factors), on the amount, size and distribution of the particle collected. The parameters are presented in Table 1. Factorial factor Res IV was employed as a screening model. The important parameters were optimized using Box Behnken. The screening and optimization design comprised of 19 and 15 runs, respectively, with 3 replicates were created in MODDE 10.1 (Sartorius Stedim Biotech, Malmö, Sweden).

Table 4.3.1. Screening parameters

Parameters	Abbr.	Type	Settings
CO ₂ Pressure	PrCO ₂	Quantitative	60 to 80
FlowCO ₂	FCO ₂	Quantitative	5 to 10
Nitrogen Pressure	PrNit	Quantitative	10 to 20
Nitrogen Position	NP	Qualitative	top. bottom
Nozzle internal diameter	IDNoz	Qualitative	0.25 cm. 0.5 cm
Vacuum	Vac	Qualitative	without. with
Length	Len	Qualitative	45mm. 60 mm
Extract Nitrogen	Ext	Qualitative	with. without

2.4. Experimental procedure

Pressurized hot water extraction conditions were performed in accordance with the process describe by Andersson et al. (2012). The WEPO procedure was first performed under different conditions as described by the screening methodology (Table 2). For each run, a mass of 2 g of *Moringa oleifera* powder was filled into the PHWE extraction cell. The process started with 5 min of static extraction by filling the cell with water at the desired temperature (120°C) and other parameters were set according to the design experiment build, with valve closed (see Figure 4.3.1). The CO₂ was pumped through the system at the desired pressure and temperature, with the flow rate controlled by the nozzle. The flow of hot N₂ was directed in accordance with the condition prescribe experimental design. The extraction continued in a constant flow mode by opening valve V₁ and setting the water flow rate at the chosen constant value of 0.3 ml min⁻¹.

Table 4.3.2. Experimental with response of independent parameters

Run Order	CO ₂ Pressure (bar)	Flow CO ₂ (bar)	Nitrogen Pressure (bar)	Nitrogen Position	Nozzle internal diameter (cm)	Vaccum	Length (mm)	Extract Nitrogen	Amount of powder (mg)	Water content (%)	Particle size (um)
4	60	5	10	top	0.25	without	45	with	25.56	32.78	1086
15	80	5	10	top	0.25	with	60	without	35.96	30.85	959
2	60	10	10	top	0.5	without	60	without	43.15	27.78	1258
10	80	10	10	top	0.5	with	45	with	37.52	25.56	998
16	60	5	20	top	0.5	with	60	with	58.45	15.07	888
19	80	5	20	top	0.5	without	45	without	43.89	17.09	908
17	60	10	20	top	0.25	with	45	without	46.89	20.19	878
1	80	10	20	top	0.25	without	60	with	69.23	14.89	789
18	60	5	10	bottom	0.5	with	45	without	102.45	11.78	748
6	80	5	10	bottom	0.5	without	60	with	164.56	9.06	639
9	60	10	10	bottom	0.25	with	60	with	168.2	8.99	501
12	80	10	10	bottom	0.25	without	45	without	105.56	12.03	457
11	60	5	20	bottom	0.25	without	60	without	163.52	8.09	438
14	80	5	20	bottom	0.25	with	45	with	129.23	5.48	409
7	60	10	20	bottom	0.5	without	45	with	130.69	5.55	398
13	80	10	20	bottom	0.5	with	60	without	165.26	7.89	391
3	70	7.5	15	top	0.25	without	45	with	25.58	16.48	875
5	70	7.5	15	top	0.25	without	45	with	36.89	18.49	905
8	70	7.5	15	top	0.25	without	45	with	35.45	17.58	789

The water extract meets the SCCO₂ in a low dead-volume tee fitting, forming an emulsion that goes through a restrictor and reaches the expansion-drying chamber. In this chamber, the pressure and temperature are below the critical point of the CO₂. So, the volume of the emulsion is rapidly expanded because the SCCO₂ becomes a gas. The aerosol formed in the chamber is dried by the hot N₂ current. After the desired extraction time, valve V₁ is closed and the water flow is stopped while SCCO₂ and hot N₂ flows are continued for a few more minutes. By closing valve V₁, the entrance of water droplets in the chamber due to a possible residual pressure in the extract line is avoided. Particles are trapped in the bottom of chamber outlets with the help of the vacuum. Once, the important parameters were determined, the optimization process was started in accordance with the protocol displayed in Table 4.2.3.

Table 4.3.3. Experimental design for an optimization

Run Order	CO ₂ pression (Bar)	Flow rate CO ₂ (ml min ⁻¹)	Nitrogen (Bar)	Particle size (nm)	Amount of powder (g)
2	60	8	20	495	133,56
12	100	8	20	381	133,05
14	60	16	20	426	132,48
7	100	16	20	321	131,15
1	60	12	10	569	100,45
9	100	12	10	371	100,98
10	60	12	30	516	142,09
3	100	12	30	465	143,08
15	80	8	10	489	101,65
13	80	16	10	429	100,89
11	80	8	30	529	141,26
8	80	16	30	512	169,89
5	80	12	20	309	131,21
4	80	12	20	302	119,49
6	80	12	20	356	131,56

2.5 UHPLC-DAD analysis

Ultimate UHPLC with a DAD that used 200, 280, 350, 370 and 520 nm wavelength, flow rate at 0.5 mL min⁻¹, injection volume at 10 µL, temperature at 50 °C, back pressure at 110 bars, column was a Waters Xselect C18 150 x 3 mm, 3.5 µm particle size. The percentage of the composition of the mobile phase gradient used in

running the samples in the HPLC where A is water with 1% formic acid and B is acetonitrile with 1% formic acid. The gradient composition are describe as follow: 0 min 95 (A) and 5 (B) %, 2 min 87 (A) and 13 (B) %, 8 min 86 (A) and 14 (B), 20 min 50 (A) and 50 (B) %, 23 min 95 (A) and 5 (B) % and 25 min 95 (A) and 5 (B) %. The polyphenolic compounds were identified and quantified based on their retention times and peak areas, respectively using standards.

2.6. Water content

The water content of the WEPO particles, spray and freeze-dried extracts were evaluated by gravimetric analysis using an analytical balance.

2.7. Scanning electron microscopy (SEM)

The particles from WEPO, freeze and spray dried extracts were observed in a JSM 6700F scanning electron microscope (JEOL. Tokyo. Japan) using a 10-kV beam. The samples were first coated with a thin gold layer by a SCD 004 sputter coater (Oerlikon Balzers. Balzers. Liechtenstein). The size and distribution of the particles were assessed by using image J software (NIH, Image Processing and Analysis, United State).

3. Results and discussion

3.1. Screening

Response surface methodology (RSM) is a statistical and mathematical and statistical tool which can be applied to evaluate and optimize multivariable systems by finding the best relationship between the response and the set of independent variables. In this study eight factors, as displayed in Table 1, were screened and optimized for maximum amount of powder collected with smaller particle size. The screening model was created and calculated by fitting using multiple linear regression. The fitted model showed a cross-validated predictability of 65% ($Q^2 =$

0.65) and total explained variance of 95% ($R^2=0.95$) and, where Q^2 shows an estimation of the future prediction and R^2 shows the model fit. Fig. 2 shows the linearity of the predicted versus observed values plot highlighted the validity of the model and its ability to predict the most important parameters to be optimized.

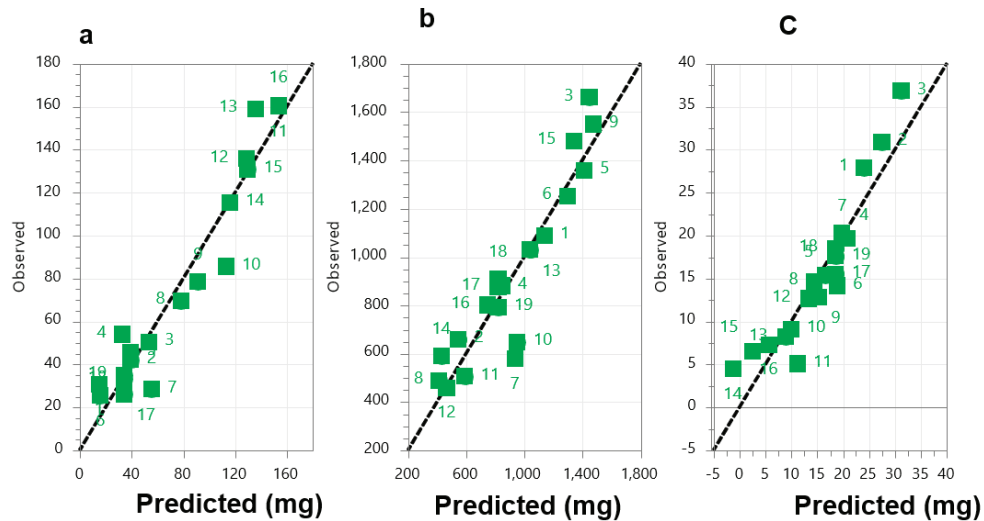


Figure 4.3.2. Predicted versus observed: Amount of powder (a), Particle size and distribution (b), water content (C).

The coefficients plot displayed in Figure 3a reveals that nitrogen pressure, nitrogen position in the vessel and the length of the nozzle have significant influence on the amount of the powder collected with p -value of 0.001. The dehydration of the particle forming are faster with increasing hot nitrogen pressure. This emphasis that the particles are more dried, and not stick to the wall of the vessel. They are directly pulled down by the vacuum in the bottom of the vessel and collected. Moreover, the position of the nitrogen nozzle allows the particle to precipitate on the top or bottom of the vessel. According to the coefficient plot in Figure 4.3.3b, when the nitrogen nozzle was place in the bottom of the vessel, it was observed that the particles were forming near to the vacuum and collector. Therefore, they were easily collected.

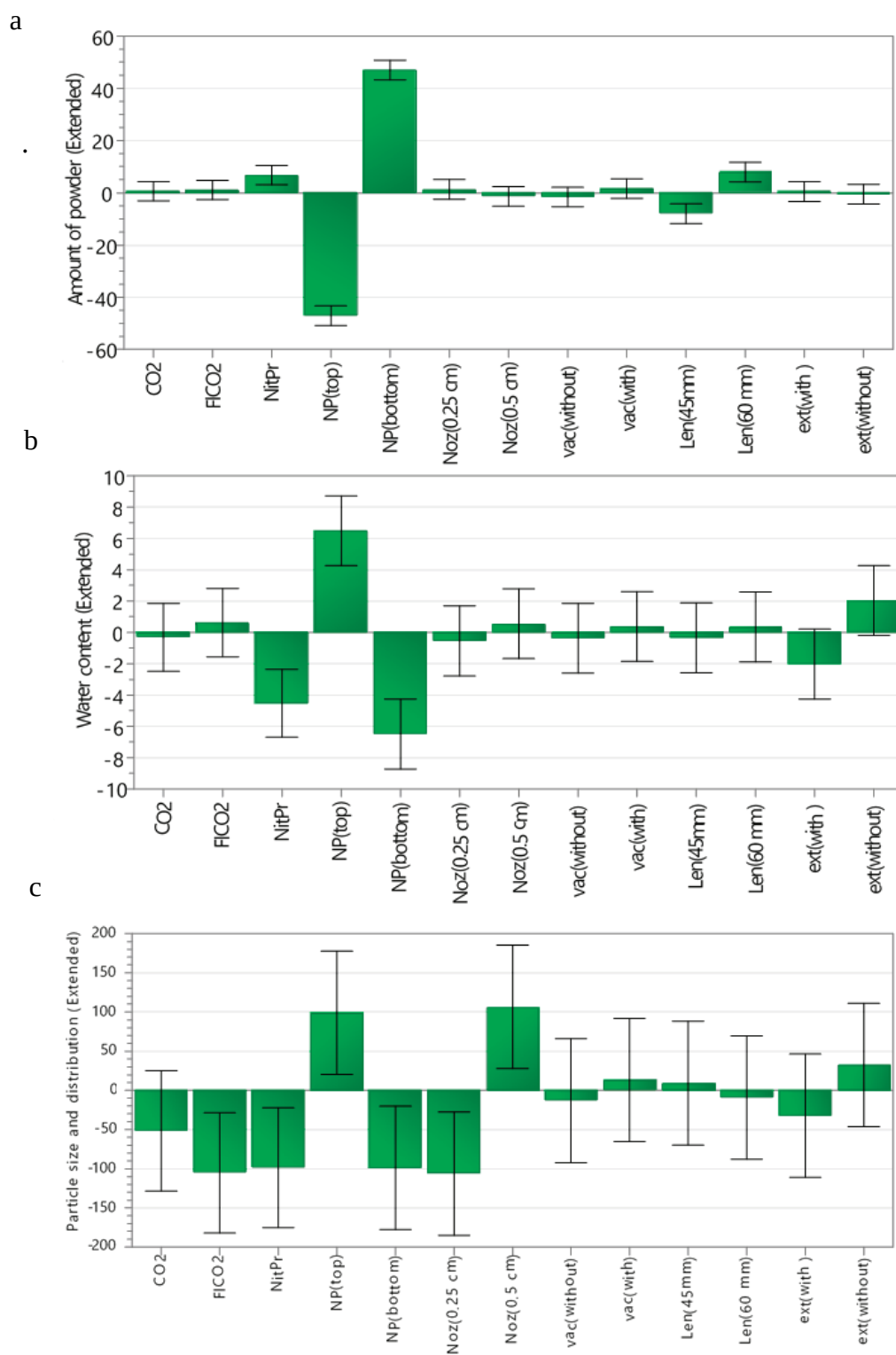


Figure 4.3.3. Coefficient plot: Amount of powder (a), Water content (b), Particle size and distribution (c).

Furthermore, Increasing the nitrogen pressure significantly decreases the amount of the water in the particle. CO₂ pressure, flow rate of CO₂, nitrogen pressure, position of the nitrogen and the size of the nozzle have an influence on the particle size (Figure 4.3.3c). Then, increasing the pressure and flow rate of CO₂ decreases the size of the particle formed

At this condition, the particle did not have enough time to agglomerate in the restriction nozzle, then they are directly flushed out with a high CO₂ pressure and only fine particles were precipitated rather than big one. These observations are displayed in Figure 4.3.4 a and b from the SEM.

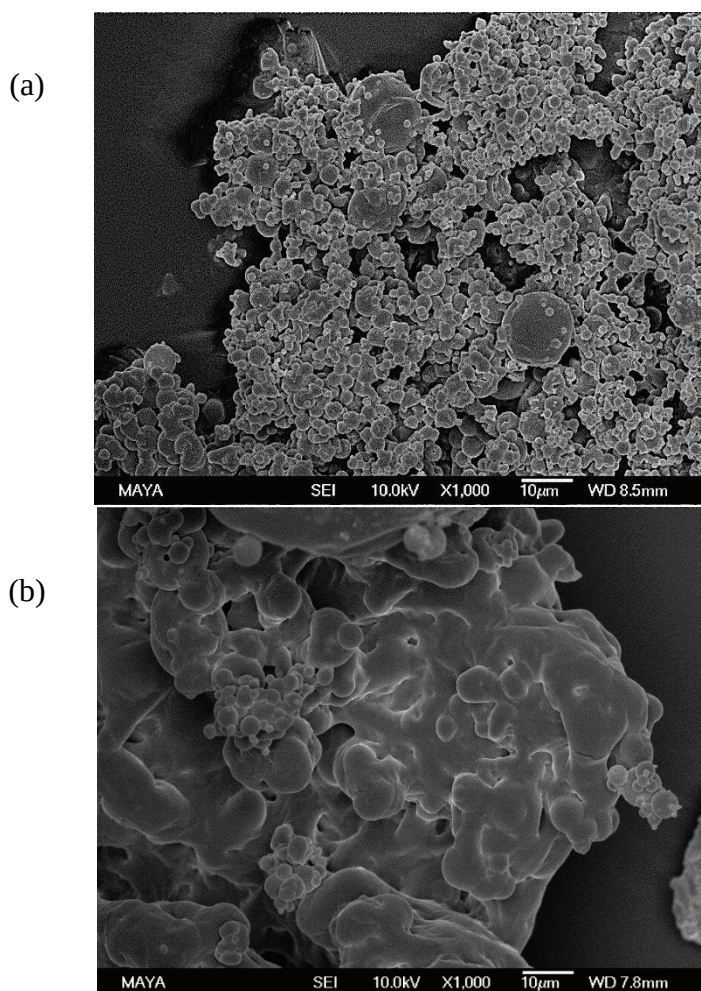


Figure 4.3.4 (a) SEM image at CO₂ Pressure: 100 bars, Flow rate CO₂: 16 ml min⁻¹, Nitrogen pressure: 20 bars, (b) SEM image at CO₂ pressure: 60 bars, Flow CO₂: 8ml min⁻¹, Nitrogen pressure: 20 bars.

In addition, with hot nitrogen as the drying agent, at high nitrogen pressure up to 30 bars, the particles have low water content, however, at this condition the particles are breaking apart, as seen in Figure 4.3.5. In summary, parameters such as CO₂ pressure and flow rate, and nitrogen pressure were considered as the important parameters which show to have a significant influence on the amount collected, water content and particle size. Thus, increasing these parameters could lead to collection of high amounts of powder with low water content and small particle size.

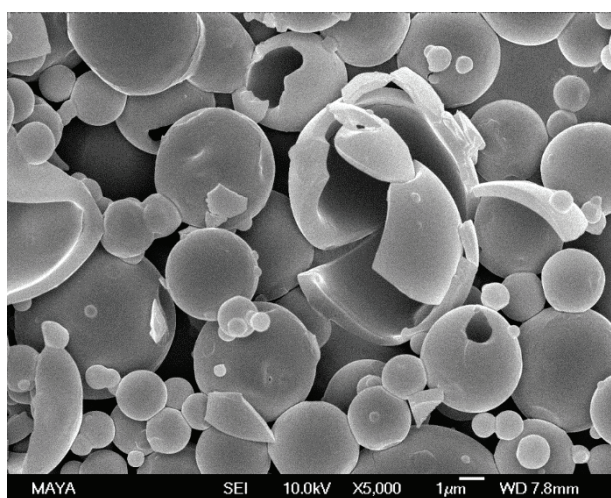


Figure 4.3.5. SEM image at high nitrogen pressure

Other parameters such as nitrogen position, vacuum, length of the tubing, ID of the nozzle and the applying of the extra nitrogen are kept fixed at the conditions which are favored for the optimisation.

3.2. Optimization of water extraction with on-line particle formation

The effects of CO₂ pressure and flow rate, and nitrogen pressure on WEPO were optimized using a response surface methodology. Box Behnken design comprised of 15 runs with 3 centre point replicates was created in MODDE 10.1 (Sartorius Stedim Biotech. Malmö. Sweden) to investigate the influence CO₂ pressure (60 – 100 bar) and flow rate (8 -16 ml min⁻¹), and nitrogen pressure (10 -30 bar) on the WEPO. During the optimization process, nitrogen position was placed in the bottom

of the vessel, the length of the tubing ID of the nozzle used were 60 and 0.25 mm, respectively.

The extra nitrogen pressure of 30 bars was employed at the end of each run. Multiple linear regression (MLR) was used to estimate response surface and the fitting model. The adequacy of the models was assessed by the R^2 and Q^2 values (where R^2 shows the model fit and Q^2 shows an estimate of the future prediction precision). The optimum processing conditions were obtained by using graphical and numerical analysis based on the criteria of the desirability function and the response surface plots. Increasing the CO₂ pressure and flow rate have increased the amount of particles collected. On the other hand, nitrogen pressure showed insignificant impact (p-value 0.100), although it has positive impact on removing water from the particles. The response surface is represented by contour plots in Figure 4.3.6 shows the direct and interaction impact of CO₂ pressure and flow rate on the amount of powder collected and the size of particle.

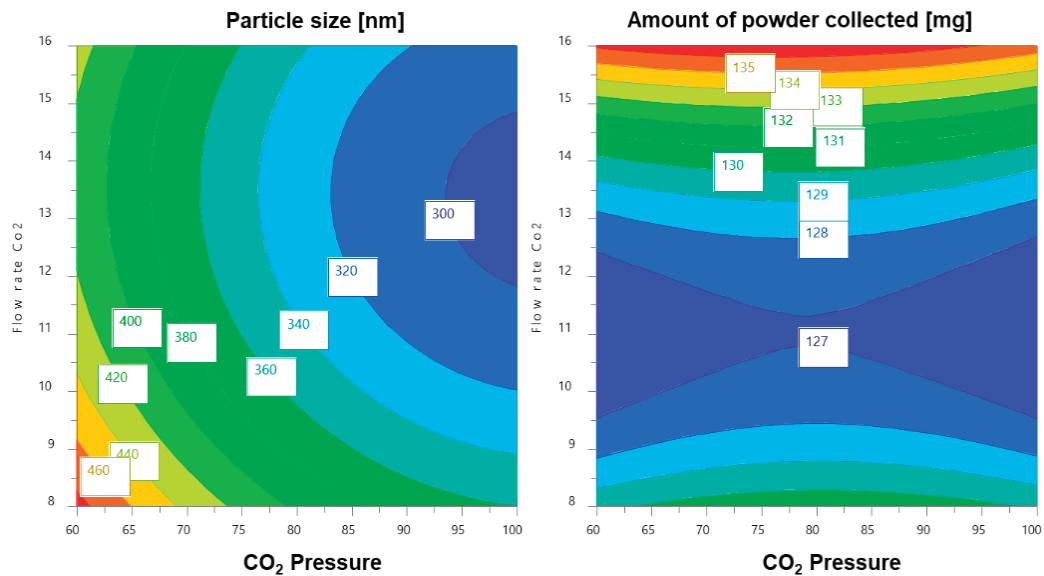


Figure 4.3.6. Contour plot of the interaction of the selected parameters on the particle size and amount of powder collected.

Increasing the CO₂ pressure up to 100 bars and flow rate up to 16 ml min⁻¹ raised amount of powder collected from 127 mg to about 135 mg. Also, increasing CO₂ pressure and flow rate produce fine particles with a uniform distribution. The desirability function was used to find the setting of parameters where the maximum yield can be obtained. The best condition was found at 90.95 and 26 bars, 16 ml min⁻¹, for CO₂ pressure, nitrogen pressure and CO₂ flow rate, respectively. The predicted value of the amount of powder collected and particle size at the best conditions were 153 nm and 391 mg. To validate the model, the WEPO process was repeated at the best condition and found 384.33 ± 21.36 nm for particle size and 150 ± 10 mg for powder collected which fits very well to the predicted value.

As compared with the previous work done on particle formation of plant extract, this result is a significant improvement in the water extraction with on-line particle formation, as compared with the first work done by Rodríguez-meizoso et al., 2010 and Anderson et al. (2012). They collected a mass from 5 to 10 mg, while with the new strategy, the mass of powder was 13,5 times more. However, similar results were obtained for the particle size.

3.3. Amount of powder collected and particle size

The amount of the powder obtained from the WEPO was compared with other drying techniques such as freeze and spray drying. The results of the three techniques are summarised in Table 4.3.3. The maximum amount collected in mg was 2,38 and 1,33 times lower than the results for freeze and spray drying. These differences were due to that lost during the collecting process of the particles in the WEPO process. However, WEPO shows the production of particles with small size as compare with other drying techniques.

3.4 Polyphenolic content in particles

PHWE extract, freeze, spray and WEPO particles were analysed by UHPLC–DAD to identify the polyphenolic compounds and to determine their content. The polyphenolic compounds present in the extracts were gallic acid, catechin,

chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin-4'-O- β -diglucoside, quercetin-3,4'-diglucoside, quercetin and kaempferol. The concentration of these compounds in the reconstituted extracts were also compared with the fresh *Moringa oleifera* leave extract (PHWE extract). The concentration of the polyphenolic compounds present in the samples is shown in Figure 4.3.7. The obtain results concluded that the level of polyphenol compounds in different extract from the different drying process are comparable, meaning that the WEPO process produces extracts with a similar composition as the freeze and spray drying process.

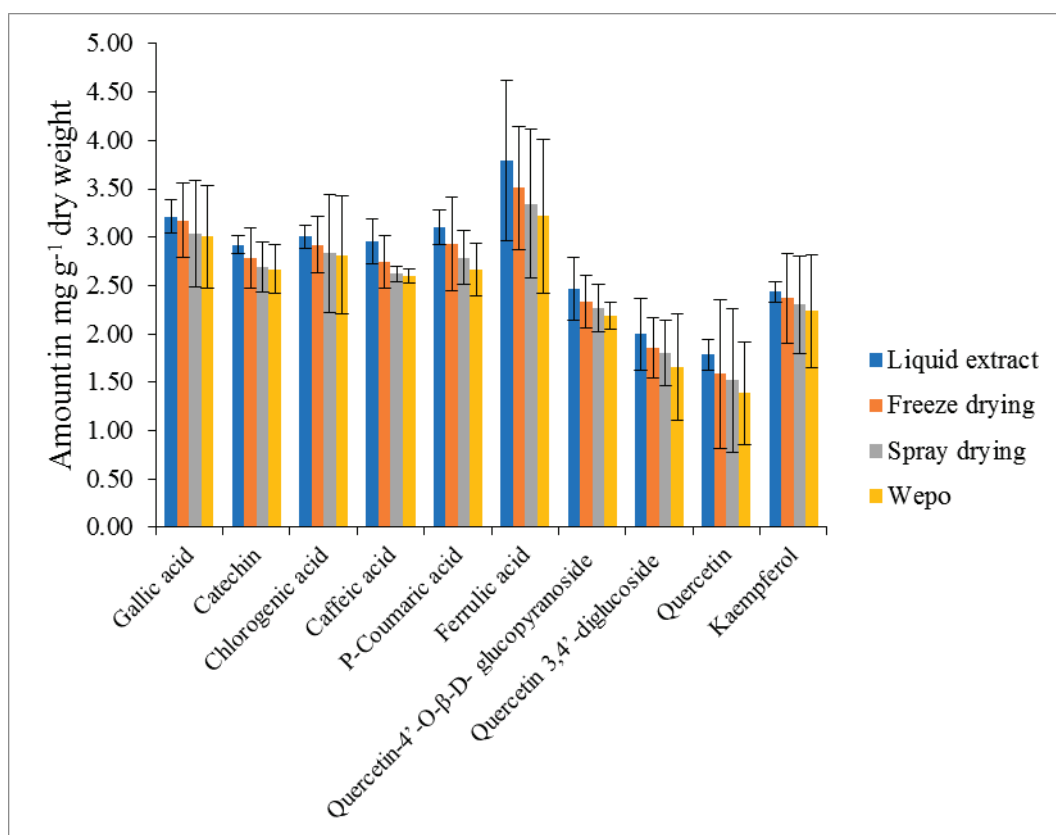


Figure 4.3.7. Polyphenolic content in different reconstituted extract (N=3)

Furthermore, the concentration of different flavonoids is also comparable to the fresh extract, directly determined by UHPLC–DAD after the extraction. One drawback, however is that the WEPO process shows a lower total yield of polyphenolic due to the loss of the particles, as discussed above.

3.5 Scanning electron microscopy analysis of the particles

The analysis of the particles obtained from WEPO, freeze and spray drying are presented in Fig. 8 and 9. The outcome results show that the spray drying and WEPO process produces spherical particles of the *Moringa oleifera* leave extract (Figure 4.3.8). The size of the particles from WEPO ranged from 362.97 to 405.69 nm while of the particle from spray drying ranged from 134 to 212 um.

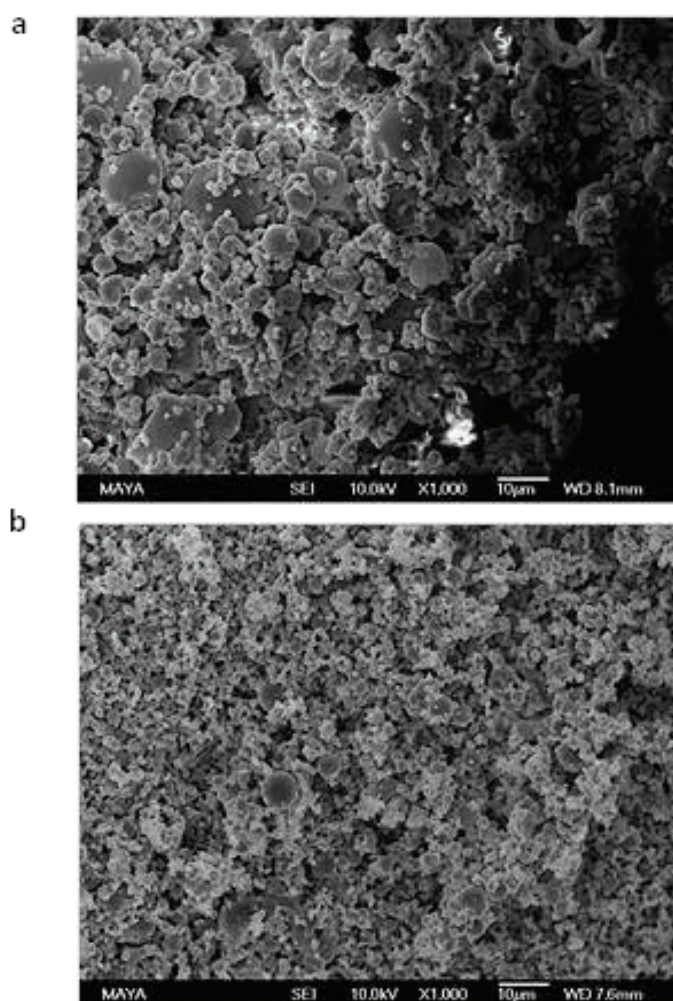


Figure 4.3. 8. SEM image of spray drying (a) and WEPO (b)

Moreover, the freeze-dried extract did not produce particles, but rather a continuous fibrous network (Figure 4.3.9). The morphology of the WEPO particles could be the reason why it was possible to solubilise them in aqueous solution by manually shaking the sample vial, while the spray and freeze-dried extracts need long periods of shaking for complete dissolution in water. This difference shows a good solubilisation rate and perhaps bio-accessibility of the active compounds present in the WEPO particles, as compared to those obtained by spray and freeze drying.

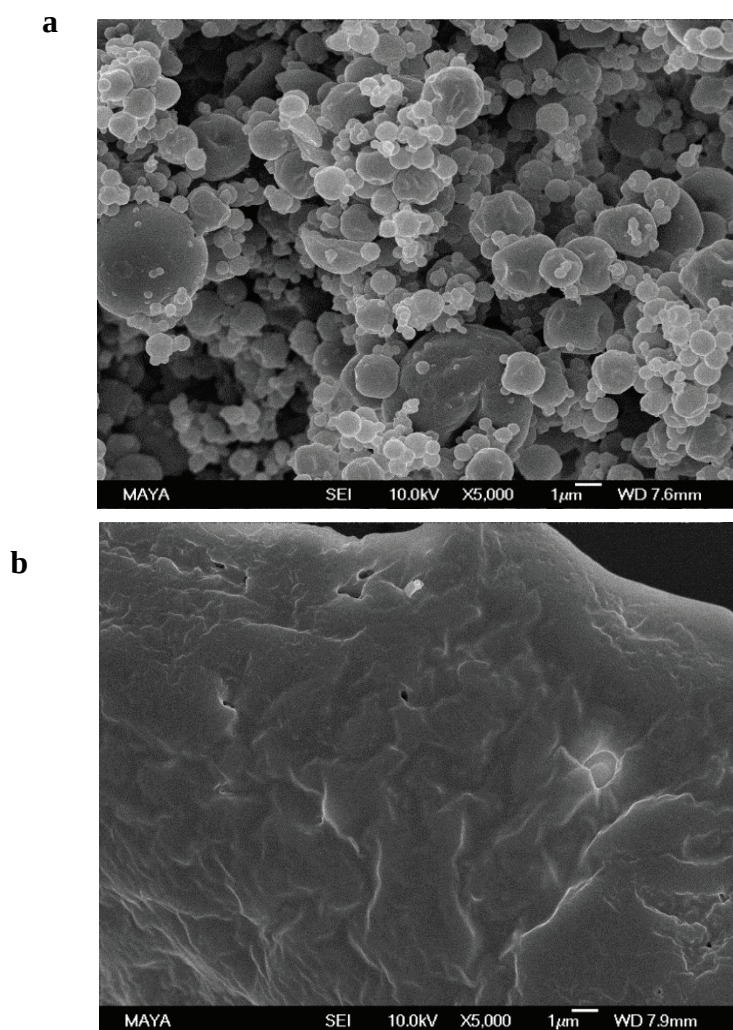


Figure 4.3.9. SEM image of WEPO (a) and Freeze-drying (b)

4 Conclusions

WEPO is as a suitable and promising process to obtain, in only one step, fine and dried particles. Also, it is an important drying process which avoid the agglomeration of particles. The temperature, pressure, CO₂, nitrogen, ID and length of nozzle, nitrogen in the vessel was optimized. SEM images has confirmed the good distribution and size of WEPO particles. As expected, particle ID decreases with pressure of CO₂ and the smaller ID of nozzle. Adding extra nitrogen after the end of the process may fragment the particles.

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4.4 Paper 4

This paper entitled “Ultra-high-pressure supercritical fluid extraction and chromatography of oil from *Moringa Oleifera* and *Moringa Peregrina* seeds” has been submitted to Agriculture and Food Chemistry Journal. The article reports an ultra-high-pressure supercritical fluid extraction technique that was developed, optimised and applied to extract oil from Moringa seeds. Accepted manuscript for publication in Analytical and Bioanalytical Chemistry. ID: ABC-00424-2019.R1

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Ultrahigh pressure supercritical fluid extraction and chromatography of oil from *Moringa Oleifera* and *Moringa Peregrina* seeds

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Abstract

An ultrahigh pressure supercritical fluid extraction method was optimized and applied to extract oil from two moringa species namely, *Moringa oleifera* and *Moringa peregrina* seeds. A full factorial design was used to investigate the direct and interaction influence of pressure and temperature in the range of 40 to 80 MPa and 40 to 70°C, respectively, on the extracted amount of oil from crushed seeds. The results revealed that pressure has positively significant influence on increasing the extracted amount in a short time. The best condition was found at 80 MPa and 57°C. The extracted amounts obtained at the best condition using neat CO₂ as solvent were 396 ± 23 and 529 ± 26 mg oil per gram of seeds for *Moringa oleifera* and *Moringa peregrina*, respectively. The extraction kinetics study revealed a mainly solubility-controlled extraction of oil; and 28 g of CO₂ was required to extract 400 mg of oil per gram of seeds of *Moringa oleifera* using the developed method. Addition of ethanol to the sample prior to the extraction, increased the polar extracted fraction in the oil as well as the total yield. The developed method

increased the extracted oil by two folds compared to a reference method based on solvent sonication. The obtained oil consisted mainly of glycerolipids, sterol esters and phospholipids. Phospholipids, campesterol and stigmasterol ester concentrations were found to be higher in *Moringa oleifera* while cholesterol ester was more abundant in *Moringa peregrina*.

Keywords: *Ultrahigh pressure supercritical fluid extraction, Moringa oleifera, Moringa peregrina, lipid profiling, sonication, phospholipids*

Introduction

The Moringa tree belongs to the family of the Moringaceae. It is widely distributed from tropical and sub-tropical countries. Moringa family includes 13 species of which *Moringa oleifera* is the most widely cultivated species in Africa and India. It is also known as the ‘drumstick’ or ‘horseradish’ tree. *Moringa peregrina* grows naturally covering a wider range from the Dead Sea area sporadically along the Red Sea coasts to northern Somalia and around the Arabian Peninsula to the mouth of the Persian Gulf [1]. Due to the medicinal and nutritional values of Moringa every part of the tree including leaves, stem, flower and seed has been utilized. As a source of various therapeutic bioactive compounds, it is applied in medicinal and pharmacological products. The leaves are an excellent source of flavonoids, phenolics, saponins, proteins and vitamins [2]. The seed can contain up to 34 to 60% of oil called “ben oil”. It is rich in behenic, oleic, stearic and palmitic acids. Moringa seed contain up to 65% of oleic acid than palm, soybean and sunflower oil [1]. The quality of moringa seeds oil is similar to olive as reported in literature [3, 4]. The oil is an excellent source of unsaturated fatty acids, and vitamins A and E. In addition, the Moringa seed oil has antiseptic, antimicrobial and anti-inflammatory activities [3]. These exceptional nutritional and medicinal values of the oil lead to their use in the cosmetic, food and pharmaceutical industries. The quality of the *Moringa oleifera* oil has received the interest of many investigators to develop a novel technology for an efficient extraction of edible oil.

The oil is mostly extracted by the physical method such as hydraulic and screw press. These traditional techniques were produced low oil yield and were time consuming. To solve the problem, several industries use organic solvent extraction to increase the oil recovery. Solvents such as petroleum ether, n-hexane, chloroform and methanol are applied for the extraction of oil. Solvent extraction techniques have several disadvantages to the oil quality (due to solvent residues) and to the environment as those solvents are energy-demanding and toxic. The conventional techniques of oil extraction should be substituted with nontoxic, efficient and environmentally suitable approaches.

Extraction using supercritical carbon dioxide (ScCO_2) as a solvent offers several benefits for pharmaceutical and food industries. Low toxicity, non-flammable, high diffusivity, gas like viscosity and liquid like density are the main advantages of the ScCO_2 [5]. In addition, its near-ambient critical temperature makes it suitable for the extraction and processing of thermolabile high value-added products without degradation [6]. Moreover, ScCO_2 solvent can be removed from the final products by simply releasing the pressure, leaving almost no solvent residues [7]. ScCO_2 is a well-known solvent for its low viscosity and fast diffusion properties. Solubility of a compound in ScCO_2 is generally suggested to be controlled by two parameters – the vapor pressure of the compound and its interactions with the ScCO_2 [8]. These two parameters in turn are influenced by temperature and pressure. Therefore, temperature and pressure are principal factors that can control the extraction process in supercritical fluid extraction (SFE).

Optimization of the pressure and temperature is important in SFE to obtain fast, efficient and selective extraction methods. Temperature in SFE has contradictory effects depending on the operational range and type of analytes. For example, temperature can enhance mass transfer properties and increase vapor pressure of the analytes, which leads to an increase in recovery. On the other hand, high temperature decreases the density of the ScCO_2 and also changes the solubility

parameters and subsequently, may lower the solvation power [8]. Increasing the pressure naturally leads to higher density [9, 10]. For instance, increasing the pressure at 50 °C from 20 MPa to 80 MPa increases the density from 0.785 to 1.043 g/ml. The increase in the density is associated with an increase in solubility parameters. King et al. estimated the change in hydrogen bonding parameter (δ_h) of ScCO₂ when the pressure increased from 10 to 90 MPa to 5.57 to 9.12 [10]. Using ultrahigh pressure ScCO₂ extraction might enhance the extractability, alter the selectivity and improve the extraction rate. Ultrahigh pressure ScCO₂ was used in defatting food ingredients like almond and cocoa powder [11]. Pressure between 60 and 85 MPa showed the best results to extract the maximum amount of oil from coriander seeds [11]. Recently, extraction under 130 MPa was achieved to obtain apple seed oil [12]. The high pressure increased extracted oil amount from 10 to 190 mg/g dry apple seeds compared to 30 and 130 MPa.

ScCO₂ extraction technique has been applied widely to extract oil from moringa seeds [13–15]. The impact of extraction pressure and temperature on the oil yield and quality was investigated in the range of 20 and 50 MPa and between 40 and 100 °C, respectively. In most studies pressure was a significant factor impacting the extracted amount. In these studies, the composition of the oil was evaluated through analysis of fatty acids by GC with FID or MS after hydrolysis and derivatization. Such analysis does not provide full information whether the fatty acids are originally present as free fatty acids or attached to glycerolipids, phospholipids, or glycolipids. To the best of our knowledge, there are no studies implementing ultrahigh pressure (> 50 MPa) ScCO₂ for the extraction of oil from *Moringa* species seeds. Moreover, there are no comprehensive lipid profiling studies of the oil extracted from *Moringa* seeds. Hence, this study aims to develop an extraction method of oil from *Moringa* seeds based on ultrahigh pressure supercritical fluid extraction (UHPSFE) using a design of experiment (DoE) approach. Furthermore, we aim to conduct a comprehensive lipid profiling analysis of the obtained oil using ultrahigh performance supercritical fluid chromatography coupled to high resolution mass spectrometry (UHPSFC/QTOF-MS).

Experimental

Chemicals and Material

Ultrapure CO₂ (99.999%) was provided by Air Products (Amsterdam, Netherlands). LC-MS grade methanol was purchased from Scharlau (Barcelona, Spain). Heptane (99.5%) was purchased from Alfa Aesar (Karlsruhe, Germany). Ethanol 99.7% and HPLC grade chloroform were from VWR (VWR Chemicals, France). MS-grade ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using a Milli-Q purification system (Millipore, Billerica, MA).

Plant samples

Two species of moringa were subjected for extraction and analysis; *Moringa oleifera* from South Africa and *Moringa peregrina* from Oman. Prior to processing, the samples were dried at 50°C for 8 h and the water content was determined gravimetrically and found 3.5 – 4.6% weight. The dried samples were crashed to a powder and kept in a sealed container until extraction.

Extraction with UHPSFE

A home-built extraction system was assembled consisting of an ISCO 65 D syringe-pump (Teledyne Isco, Thousand Oaks, CA), a GC oven where the extraction vessel was placed, and a needle valve connected to a restrictor to control the flow rate. The extractions were started by weighing and loading 1 g of seed powder into a 5 ml stainless-steel extraction vessel and mixed with 4 g of 3 mm glass beads. The extraction vessel was fitted with metallic filters (0.45 µm pore size) at the inlet and the outlet. The extractions were carried out in continuous flow rates ranging between 2.2 – 2.8 ml min⁻¹ controlled manually by a needle valve. The extracts were collected in pre-weighted glass tubes to record the extract weight. The system

was flushed with the CO₂/n-heptane fluid mixture after each run for cleaning. The extracted oil was weighted and kept in a freezer. A 30 mg mass of the oil was re-dissolved in 1 ml of a mixture n-heptane:isopropanol (1:1, v:v) for chromatographic analysis.

Design of experiment

The effects of pressure (P) and temperature (T) on the extraction yield of oil were evaluated using a response surface methodology. Full factorial design comprised of 11 experiments with 3 centre point replicates was created in MODDE 10.1 (Sartorius Stedim Biotech, Malmö, Sweden) to investigate the influence of temperature (40–70°C) and pressure (40–80 MPa) on the extraction amount of oil. During the optimization process, the flow rate was kept in the range of 2.2 – 2.8 ml min⁻¹ while the total solvent extraction volume was fixed to 40 ml per extraction. The volume was fixed due to the low capacity of the Isco pump which is 65 ml. Multiple linear regression (MLR) was used to calculate the fitting model and response surface. The adequacy of the models was evaluated by the R² and Q² values (where R² shows the model fit and Q² shows an estimate of the future prediction precision), predicted vs. observed plot and coefficient plots. The optimum processing conditions were obtained by using graphical and numerical analysis based on the criteria of the desirability function and the response surface plots.

Extraction with sonication

Ultrasonic solvent extractions were performed according to the procedure describe by Xhaxhiu & Wenclawiak with some modification [16]. Briefly, 2 g of crushed seed were dispersed into a 100 ml conical flask filled with 25 ml pure *n*-heptane. The samples were placed in a sonication water bath for 20 minutes at 60°C. The extract was collected, and the residue was subjected to extraction with fresh solvent. This step was repeated twice. The extracts were combined and filtered. The solvent

was removed using rotary evaporator and the extracted amounts were measured gravimetrically. 30 mg mass of the oil was re-dissolved in 1 ml of a mixture n-heptane:isopropanol (1:1, v:v) for chromatographic analysis.

Lipid profiling by UHPSFC

Samples were analysed by ultrahigh performance supercritical fluid chromatography (UHPSFC) according to our previous method [17]. Aliquot of 2 μ l of reconstituted oil was injected in Acquity UPC² (Waters, Milford, MA) equipped with an Acquity Diol UPC² column (100 mm $\times$ 3 mm, 1.7 μ m, Waters). The flow rate and column temperature were set at 1.5 mL min⁻¹ and 48 °C, respectively. Active back pressure regulator (ABPR) was set at 131 bars to control the pressure at the outlet of the column. Methanol containing 20 mM of ammonium formate was used as a modifier, and the gradient of the modifier added to the CO₂ was as follow: 0 min, 1%; 3 min, 4%; 4 min, 19%; 8 min, 45%; 11 min, 45%; 12 min, 1%; 14 min, 1%. The injector needle was washed with methanol after each injection.

The Acquity UPC² was connected to a Xevo 2G QTOF-MS (Waters). Two T-pieces (Waters) were used for backpressure control and infusion of methanol (0.2 mL min⁻¹) as a makeup liquid. The MS was operated in positive and negative ESI mode with a scan range of m/z 80–1200. In positive ESI mode, the capillary voltage was 3.2 kV, the sampling cone voltage 34 V, the source temperature 120 °C, the drying gas temperature 420 °C, the cone gas flow 40 L h⁻¹ and the drying gas flow 690 L h⁻¹. In negative ESI mode, the capillary voltage was 2.6 kV, the sampling cone voltage 42 V, the source temperature 120°C, the drying gas temperature 390°C, the cone gas flow 40 L h⁻¹ and the drying gas flow 740 L h⁻¹. A collision energy ramp between 15 and 55 eV was used for MSE, targeted MS/MS and data dependent MS/MS in both positive and negative ESI. Data was acquired using MassLynx v 4.1 (Waters Technology).

Data analysis

Raw data (m/z) generated by UHPSFC were processed using the open source software package MZmine 2.28 (<http://mzmine.sourceforge.net/>) [18]. Data were generated by targeted peak detection with a m/z tolerance of 0.5 mDa or 5 ppm and a retention time tolerance of 0.2 min, respectively. Data were visualized by principal component analysis (PCA) calculated in SIMCA-P+ 12.0.1 (Sartorius Stedim Biotech, Malmö, Sweden). Data were compared and analyzed statistically using ANOVA with t-test and p -value < 0.05 is considered significant.

Results and discussion

Optimization of the extraction process

Pressure and temperature were selected for optimization because they are the main factors that influence the solubility of oil in ScCO₂ solvent by tuning the density of the fluid and subsequently, its solvation power. The extracted amounts of oil obtained from *Moringa oleifera* at different experiments of the design are presented in Table 1. The model was created and calculated by fitting using multiple linear regression. The fitted model showed a total explained variance of 94% ($R^2=0.94$) and a cross-validated predictability of 85% ($Q^2= 0.85$), where R^2 shows the model fit and Q^2 shows an estimation of the future prediction and precision (Fig.S1A).

Table 4.4.1. Experimental conditions and the corresponding extracted oil amount from *Moringa oleifera* seeds

Exp	Run	Pressure	Temperature	Extracted amount
No	Order	(MPa)	(°C)	of oil (mg)
1	9	40	40	238
2	3	40	70	270
3	7	80	40	330
4	1	80	70	359
5	5	60	40	299
6	6	60	70	317
7	11	40	55	296
8	10	80	55	396
9	2	60	55	338
10	4	60	55	320
11	8	60	55	310

The linearity of the predicted versus observed values plot (Figure S1B) underlined the validity of the model and its capability to predict the best condition of the extraction within the range of the design. The coefficients plot (Figure 4.4.1A) reveals that pressure has a significant influence on the extracted amount of the oil with a p -value of 0.001. Increasing the extraction pressure increased the extracted amount of oil. Whereas, temperature has contrast impact on the extracted amount of oil but overall its influence was minor.

The response surface represented by a counter plot in Figure 4.4.1B shows the direct and the interaction impact of pressure and temperature on the extracted amount. Increasing the pressure from 40 to 80 MPa at 55°C raised the yield from 285 mg to about 395 mg. This is probably due to the increment in density of the fluid from 0.907 to 1.03 g ml⁻¹.

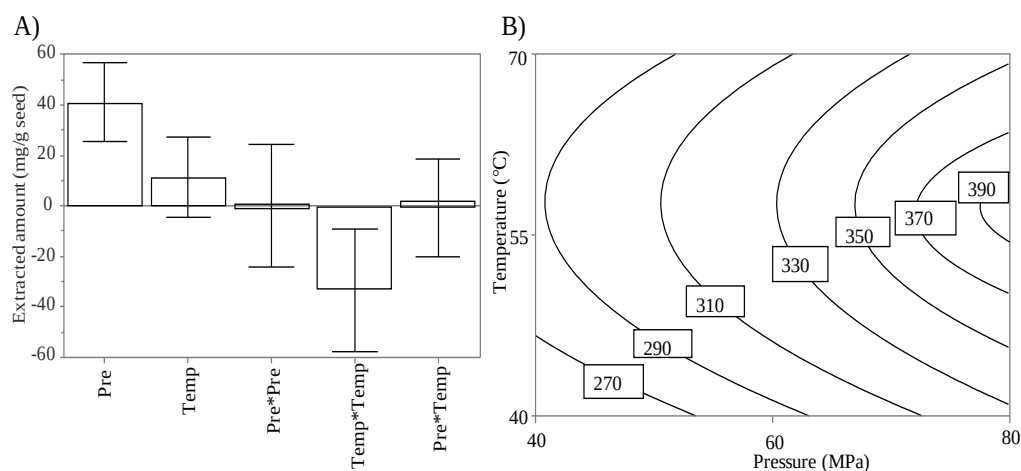


Figure 4.4.1. The plots obtained from the DoE model. (A) The coefficient plot of the examined variables and their influence on the extracted amount of oil from *Moringa oleifera* seeds. (B) The contour plot showing the extracted amount of oil (mg) at different settings of temperature and pressure.

At a high density, the space between CO₂ molecules diminishes and that enhances the interaction between oil and CO₂, leading to a greater oil solubility in CO₂ [19, 20]. In addition, a higher pressure is leading to greater rupturing of cell walls and more solvent penetration and enhanced mass transfer [21]. Nguyen et al. found that the solubility of *Moringa oleifera* oil increased by 3.4 times as the pressure increased from 20 to 30 MPa at a constant temperature [22]. Rai et al. also found pressure to be the most significant parameters influencing oil yield from *Moringa oleifera* seeds using SFE [15].

Increasing the temperature from 40 to about 55°C also increased the yield. This can be explained by the enhancement of mass transfer due to a decrease in solvent viscosity. Temperature influences positively through increasing the vapor pressure of the analytes, which might increase the extracted amount [8]. However, a further increase in temperature has contradictory effects on the extracted amount, which might be explained by the of reduction in solvent's density. It is clear that the optimal condition is found at the highest value of pressure (80 MPa) and around 55°C. For a systematic optimization, a desirability function was used to find the setting of pressure and temperature where the maximum extracted amount can be

found. The best condition was found at 57°C and 80 MPa. The predicted value of the yield at the best condition was 389 mg. To validate the model, extraction was repeated at the best condition and was found to be 396 ± 23 mg (n=3) which fits well to the predicted value.

The effect of volume of the extraction solvent was examined for two extraction cycles (the pump was refilled between them) each cycle with 40 ml at the optimal condition. The results showed that an additional 40 ml extraction cycle did not increase the extracted amount, which indicates that the extraction is completed in the first cycle. Therefore, an extraction kinetics study was performed by collecting fractions each 10 ml of used CO₂. Figure 4.4.2A shows extraction curves obtained at two different pressures, 40 and 80 MPa, at a fixed temperature of 57°C and an average flow rate of 2.2 ml min⁻¹. The volume of CO₂ (ml) is converted to mass (g) by calculating the density of CO₂ at 5°C (chiller temperature) and pressures of 40 and 80 MPa using online software (www.peacesoftware.de). At 80 MPa the extracted amount is about 400 mg g⁻¹ seeds compared to 278 mg g⁻¹ seeds at 40 MPa. The extraction kinetics curve in Figure 4.4.2A reveals that more than 50% of the extractable oil is extracted after flushing the sample with 10 g of CO₂ at 80 MPa.

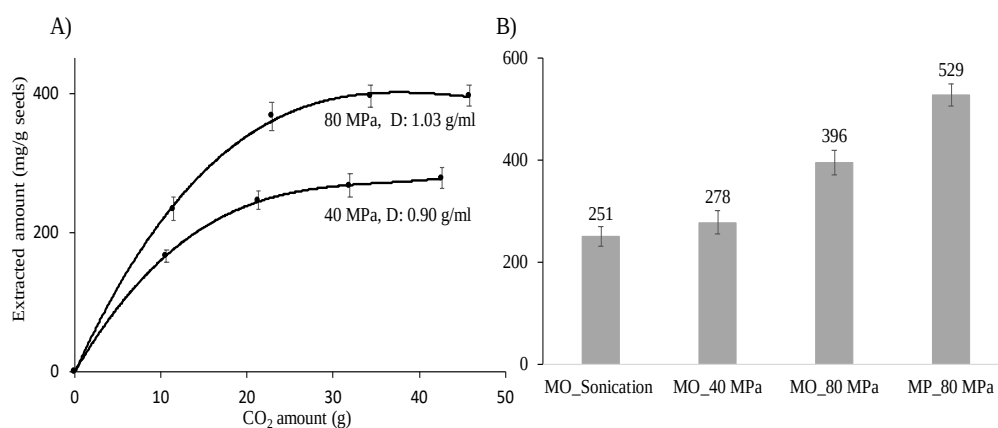


Figure 4.4. 2. (A) Extracted oil amount (mg) vs. solvent amount (g) from *Moringa oleifera* (MO) using neat ScCO₂ at 40 and 80 MPa and temperature of 57 °C. (B) Extracted oil amount from MO seeds using SLE with sonication, SFE at 40 and 80 MPa, and from *Moringa peregrina*.

In addition, the extraction process is completed after using about 28 g of CO₂ at 80 MPa as indicated by plateau curve afterward. In comparison, flushing the sample with 40 g of CO₂ at 40 MPa is not adequate to complete the extraction of the oil as shown in Figure 4.4.2A in the lower curve. The curve of 40 MPa indicates that the extracted amount increases as more solvent is flushed. The flow rate was not optimized in this study since it was manually controlled which inherently introduces errors. The optimal extraction condition was to use 40 ml of ScCO₂ at 80 MPa and 57°C.

The optimised UHPSFE method was compared with a conventional method based on sonication treatment. To make the comparison fair, the solvent/sample ratio (40:1 g: g) was kept equivalent to the UHPSFE method. The obtained extracted amount from the sonication treatment was 251 ± 22 mg oil g⁻¹ seed. This amount represents about 63% of the amount obtained by SFE at 80 MPa. Beside using organic solvent, the conventional method takes 1 hour for extraction and about 20 min for filtration and solvent evaporation. Whereas, when the SFE method was run at a flow rate of 2 ml min⁻¹, it takes about 20 min to complete the extraction without a need for clean-up procedure. The developed SFE method at the optimal conditions was used to extract oil from *Moringa peregrina* (MP) seeds for comparison with *Moringa oleifera* (MO). At the optimal conditions SFE, the oil amount extracted from MP seeds was 529 ± 26 mg. Fig. 2B. Summarizes the obtained amount of oil at different pressure conditions, using different extraction techniques and different *Moringa* species. Nguyen et al. obtained 378 mg oil g⁻¹ *Moringa oleifera* seeds using an SFE method at 29 MPa, 7 hours extraction time and in presence of 10% (v:v) ethanol [22]. This indicates that including modifier (organic solvent) might increase the extracted amount of the oil. Ideally, modifier is added as a co-solvent with ScCO₂ throughout the extraction process.

Due to the limitation of the availability of ultrahigh pressure liquid pump in our laboratory which can maintain the pressure up to 80 MPa or above, the impact of presence of modifier was studied by adding (1 ml) ethanol into the extraction vessel

before the extraction. Addition of ethanol combined with 5 min static extraction followed by dynamic extraction with 40 ml neat ScCO₂ at 80 MPa and flow rate around 2 ml min⁻¹. The extracted amount with modifier was 461 ± 26 mg and 583 ± 31 mg per gram of *Moringa oleifera* and *Moringa peregrina*, respectively. The limitation of ScCO₂ is that its polarity is often too low to obtain efficient extractions, because the analytes lack sufficient solubility, or the solvent has a poor ability to cause desorption of the analytes from active matrix sites. The presence of the modifier increases the solubility since the solvent is capable of binding with the analytes with different interactions including dipole-dipole and hydrogen bonding [23, 24]. Also, the modifier enhances desorption of the analytes present in micro/nanopores in the matrix to the Nernst diffusion layer. Furthermore, it has been found that the presence of the modifier in the ScCO₂ plant matrix samples causes swelling of the matrix which facilitates the solvent penetration and enhances mass transfer of the analytes [25]. Rai et al. reported that addition of 5 and 10% ethanol to ScCO₂ (v:v) at 30 MPa increased the extracted amount of oil from *Moringa oleifera* seeds by 1.3 and 1.9 times, respectively [15].

Extraction selectivity

Screening the MS data obtained from both positive and negative ESI modes for pooled oils obtained at different conditions from *Moringa oleifera* seeds, showed that several lipid classes could be identified based on retention time, exact mass *m/z* and MS/MS fragmentation patterns [17, 26]. These classes include sterol ester (SER), triacylglycerol (TG), diacylglycerol (DG), monoacylglycerol (MG), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylcholine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and free fatty acids (FFAs). Typical chromatograms obtained from *Moringa perigrina* are shown in Fig. 3.

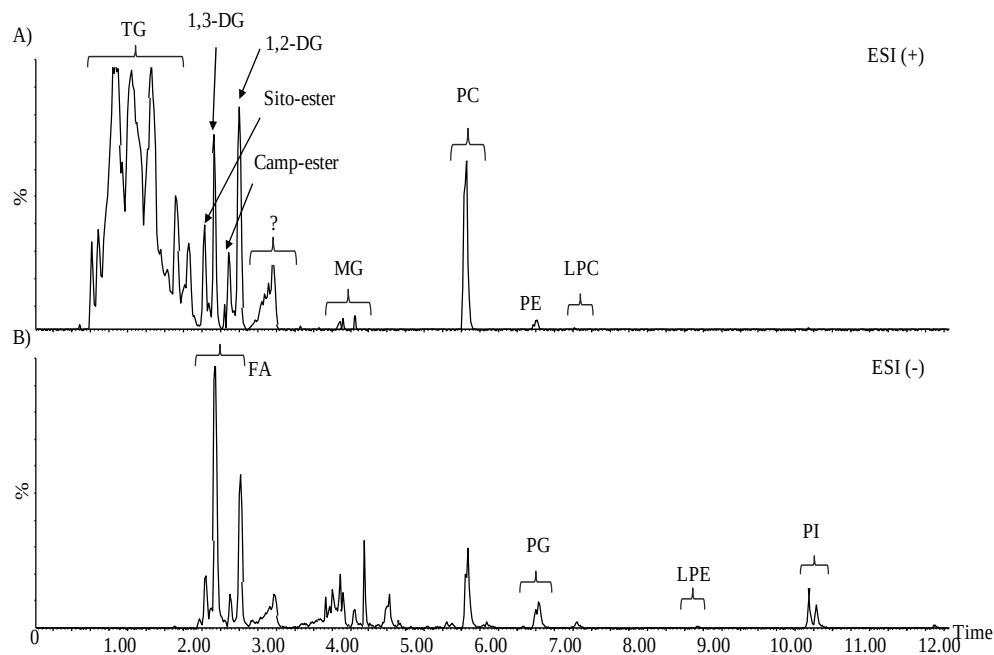


Figure 4.4.3. Example of chromatograms obtained from the oil extracted from *Moringa peregrina* using the developed extraction method. (A) in positive ESI and (B) in negative ESI.

There is one lipid class detected at relatively high abundance eluted between 3.0 and 3.5 which was not included in the discussion of the oil composition due to uncertainty in the identification. This class of lipids has masses in positive mode between m/z 892.7 and 1046.9, and they have main fragments in MS/MS at m/z 603.5, 631.5 and 659.5. This class of lipids is not well ionized in negative ESI mode. This class also has a feature of fatty acids as can be recognized by differences in m/z by two Daltons. In addition, the MS/MS spectra in negative mode reveals the presence of fatty acids at masses m/z 281.2, 255.2, 283.4 and 295.2. In general, this class of lipid is detected uniquely in MP and might belong to glycolipids but this is not fully confirmed. Targeted peak picking for more than 300 lipid species (excluding this class) was followed to study the lipid composition and relative quantities of these species in the oil obtained at different extraction conditions (40 and 80 MPa), different extraction techniques (SFE and sonication) and two moringa species (*oleifera* and *peregrina*). The resulted data were visualized by a PCA model (described variation, $R^2=0.91$; predictive ability, $Q^2=0.77$) as shown in Figure 4.4.4.

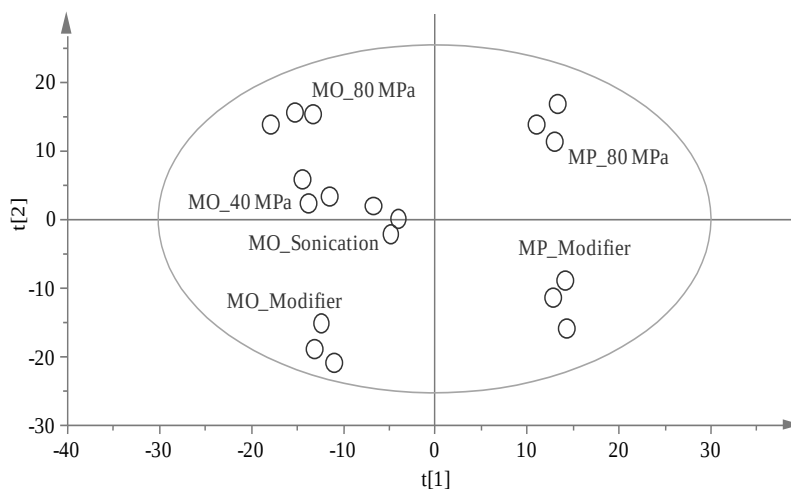


Figure 4.4.4. PCA score plot of MS data of oil samples from two moringa species obtained under different extraction techniques and conditions.

The score plot shows a clear clustering between extraction methods and moringa species. The first component separates the data according to the origin of the moringa species while the second component separates extraction method either as neat ScCO₂ or in presence of the modifier. The PCA model reveals that extraction selectivity varies with different pressure and in presence of the modifier. To understand the influence of these conditions on the selectivity, separated PCA models were calculated and fitted (supporting information).

Effect of extraction pressure on the oil composition

As mentioned above, the effect of pressure on enhancing the extracted amount is clear. It was suggested that high pressure increased the dipole moment of ScCO₂ and its electron donation and accepting capacity increased that enables it to react with polar head groups [27]. The PCA model (Fig. S2) and Table S1 present the data for oil extracted under 40 and 80 MPa at fixed temperature (57°C). The score plot of the PCA shows the clustering of the seed samples based on the extraction pressure conditions. The loading plot indicates that most of the lipid species are extracted in high amounts under high pressure. The main detected components in

the oil are TGs, DGs and SEs. The clustering of the data discloses that at high pressure, lipids with longer fatty acid chains and a higher degree of unsaturation are extracted more in comparison with lower pressure. Interestingly, the impact of high pressure in tuning the polarity of the ScCO₂ is obvious in the extraction of species belonging to polar classes like PC, PC, PE, LPC and LPE. Some polar lipid species are only found in 80 MPa extracts but at relatively low intensities (Table S1). So, high density of ScCO₂ is more selective toward neutral lipids (nonpolar) such as sterols and glycerolipids. However, detection of some of the phospholipid species using neat ScCO₂ is an indication of the presence of phospholipids in significant amounts in moringa seeds. In order to investigate the polar lipids, oil extracted at 80 MPa in the presence of ethanol was analysed and compared to neat ScCO₂.

Effect of modifier

The most significant aspect of addition of modifier is that it increases the solubility range of the solvent to include more polar lipids. Although 1 ml of the modifier was added to the sample after being placed in the extraction vessel, it was adequate to increase the extracted amount of the oil significantly compared to neat ScCO₂. The analysis showed that the modifier enhanced extraction of polar lipids to include phosphatidylinositol (PI) and phosphatidylserine (PS) (Table S2). This could be explained by the increase of dipole–dipole and hydrogen bond interactions between ethanol and the oil components with polar function groups [28]. Table 4.4. 2. shows phospholipid species detected significantly higher under addition of ethanol as a modifier.

Table 4.4. 2. Polar lipid species detected higher in the oil from *Moringa oleifera* extracted under 80 MPa with modifier compared to neat ScCO₂.

Lipid class	Class species
PC	32:0, 32:1, 34:0, 34:1, 34:2, 35:1, 36:0, 36:1, 36:2, 36:3, 38:0, 38:1, 38:2, 38:5, 40:1
PE	32:2, 34:0, 34:1, 34:2, 36:1, 36:2, 36:4, 38:4, 38:5, 42:6
LPC	16:0, 18:0, 18:1, 20:4
LPE	16:0, 18:1
PI	32:1, 34:1, 34:2, 36:2, 40:6, 42:6
PS	36:2, 38:1, 40:3, 42:1

Another remark from addition of modifier is that, lipids form TGs, DGs and FAs with higher degree of unsaturation and longer carbon chains are extracted significantly more under the addition of ethanol. This finding is in agreement with our finding in separation of lipids using SFC [17], where lipids with higher degree of saturation and longer carbon chain elute late with higher percentage of organic co-solvent.

UHPSFE vs. Sonication

Sonication treatment is considered as an efficient way to enhance the recovery of bioactive compounds from a plant matrix [29]. As mentioned earlier in the extraction optimization section, sonication assisted extraction gives lower recovery than UHPSFE. A PCA plot (Fig. S3) was calculated for the data obtained from UHPSFE using neat ScCO₂ and sonication extracts illustrates differences in the selectivity. The data reveals that there are some unique lipid species extracted either under sonication or UHPSFE. In general, polar lipids that belong to phospholipids are extracted in higher amount with sonication compared to UHPSFE. Several species like PC 42:2, LPC 22:1, FA 22:4, PE 38:2, PI 32:2 and MG 22:6 are extracted uniquely under sonication. This can be attributed to the effect of organic solvent to cause swelling of the particles and impact of sonication to destruct the

cell wall and therefore better contact and interactions of solvent with the particles. Other species of lipid are extracted at different ratios between UHPSFE and sonication without noticeable trends. The results also revealed that UHPSFE is more selective toward campesterol and stigmasterol esters than sonication. Studies reported that there were no differences in the fatty acids composition of oil obtained by SFE or sonication [30, 31]. Thus, UHPSFC using neat ScCO₂ might increase the extracted amount of the oil but has minor influence on the composition of the oil and fatty acid profiling compared to sonication assisted extraction.

Oil composition from *Moringa oleifera* and *Moringa peregrina*

As a final comparison in this study, we compared the oil composition extracted from *Moringa oleifera* and *peregrina* using the developed UHPSFE method with modifier (Table S3). Figures. S4 and S5 illustrates the chromatograms for the oil from *Moringa oleifera* and *peregrina* in both (+/-) ESI-QTOF, respectively. Although *Moringa oleifera* has lower content of oil than *Moringa peregrina*, its composition is rich in phospholipids. For example, many species of LPC, PC and LPE are detected uniquely in *Moringa oleifera*. In addition, most of campesterol and stigmasterol esters are significantly higher in *Moringa oleifera* compared to *Moringa peregrina*. However, the cholesterol ester is more abundant in *Moringa peregrina*. Fahad et al., reported previously that cholesterol is not detected in *Moringa oleifera* while stigmasterol is significantly higher than in *Moringa peregrina* [32]. On other hand, lipids with higher degree of unsaturation are more pronounced in *Moringa peregrina* than *Moringa oleifera*. Fig. 5 shows detected masses in *Moringa peregrina* and *Moringa oleifera* in ESI (-) between minute 2 and 3 where free fatty acids elution is expected. *Moringa Peregrina* is a rich source of very long chain fatty acids (C>22) such as lignoceric acid (C24), montanic acid (C28) and melissic acid (C30).

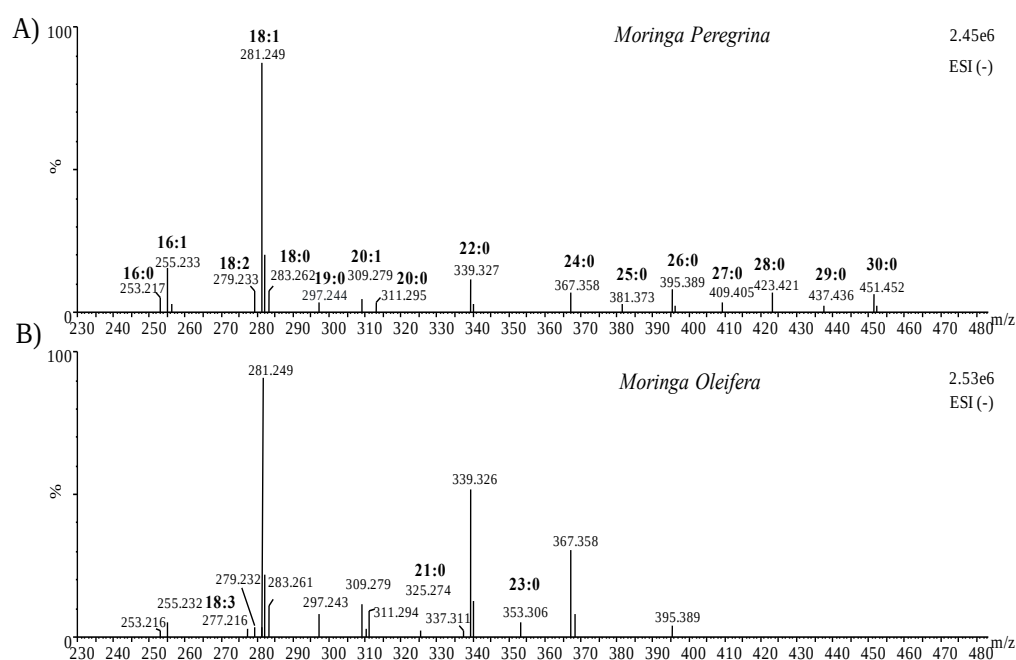


Figure 4.4.5. Extracted masses eluted between minute 2 and 3 in UHPSFC/QTOF in (-) ESI from oil samples for *Moringa peregrina* (A) and *Moringa oleifera* (B).

For short chain fatty acids both species have similar profiling although some species detected only in *Moringa oleifera* like heneicosylic acid (C21). From these results, we can observe the diversity of the lipid composition amount in the same plant species.

Conclusions

An extraction method based on UHPSFE was developed to obtain oil from moringa seeds. Response surface methodology was used to optimise the operation conditions of pressure and temperature. Pressure was found to be the most important factor influencing the oil extractability. Extraction at 80 MPa and 57°C was the optimal condition to achieve the highest extracted amount in the investigated range. Extraction at 80 MPa increased the extracted amount to 396 mg g⁻¹ seeds compared to 278 mg g⁻¹ seeds at 40 MPa. The presence of the modifier (ethanol) increased the extracted amount and also increased the abundance of polar lipids mainly phospholipids in the extracted oil. The results showed that sonication extraction is

not as efficient to extract the oil from the seeds as the novel UHPSFE method. UHPSFC analysis revealed that moringa oil is composed of TGs, DGs, MGs, campesterol and stigmasterol ester, FAs, PCs, LPCs, PE, LPE, PI and PS. *Moringa peregrina* has a higher content of oil than *Moringa oleifera* seeds. Phospholipids are more abundance in *Moringa oleifera* while *Moringa peregrina* has uniquely very long chain fatty acids.

Acknowledgment

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Supporting information 1

Ultra-high-pressure supercritical fluid extraction and chromatography of oil from *Moringa Oleifera* and *Moringa Perigrina* seeds

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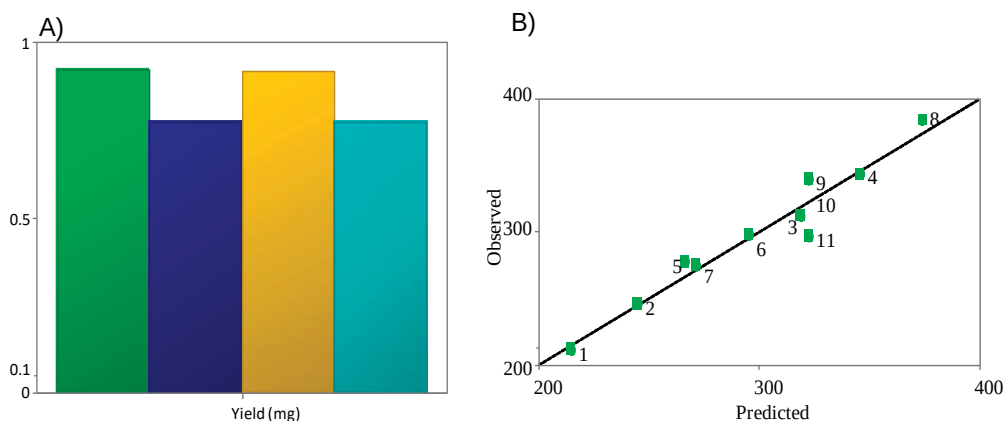


Fig S 1. (A) DOE model fitting shows the R^2 (94%) and Q^2 (85%) values with high value of validity and repeatability. (B) The plot shows the linear relationship between predicted values and observed data from the experiments.

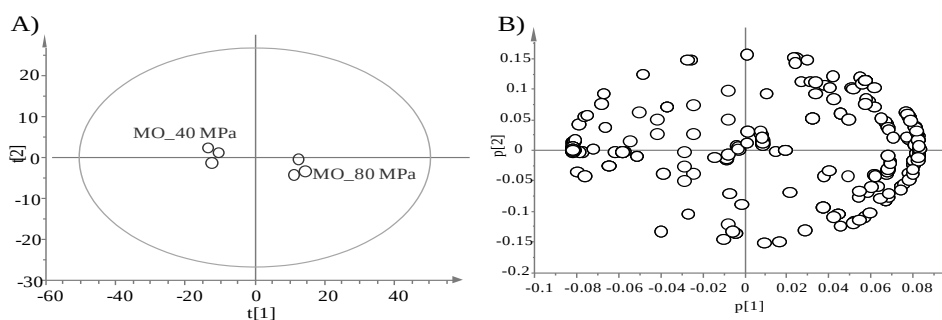


Fig S 2. PCA model shows the discrimination in the MS data obtained SFE at 40 and 80 MPa in both modes (+ and -) from *Moringa Oleifera* (MO), (A) the score plot and (B) the loading plot. Data are clustering in the loading plot toward the side of 80 MPa

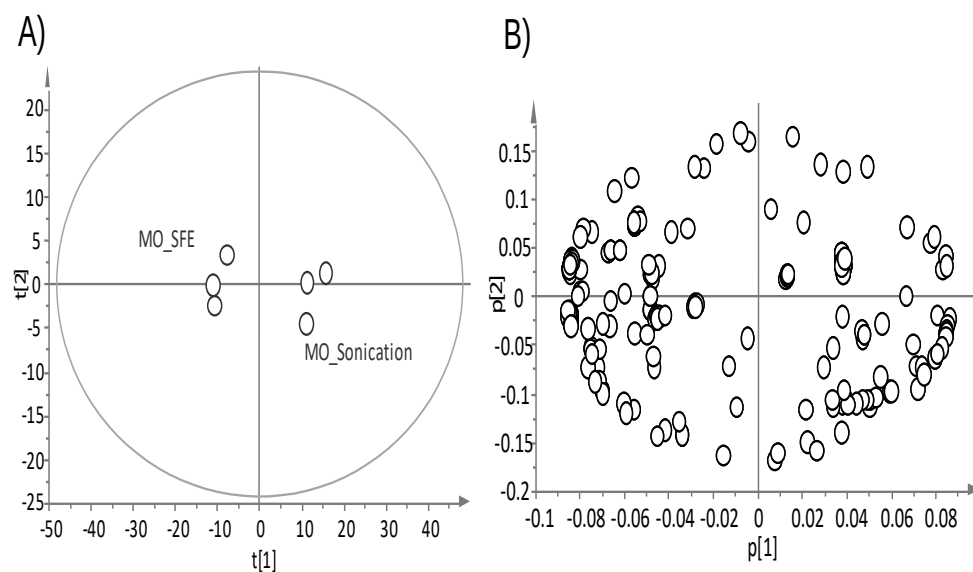


Fig S 3. PCA model shows the discrimination in the MS data obtained from *Moringa Oleifera* at the optimal condition of UHPSFE and conventional SLE method (sonication). (A) is the score plot and (B) is the loading plot

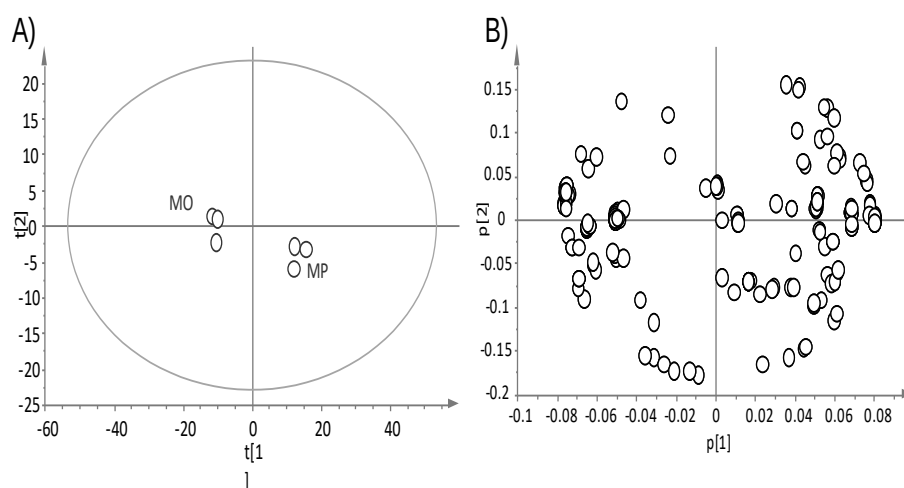


Fig S 4. Figure S4. PCA model shows the discrimination in the MS data obtained at the optimal condition of UHPSFE from both *Moringa Oleifera* (MO) and *Moringa Peregrina* (MP), (A) the score plot and (B) the loading plot.

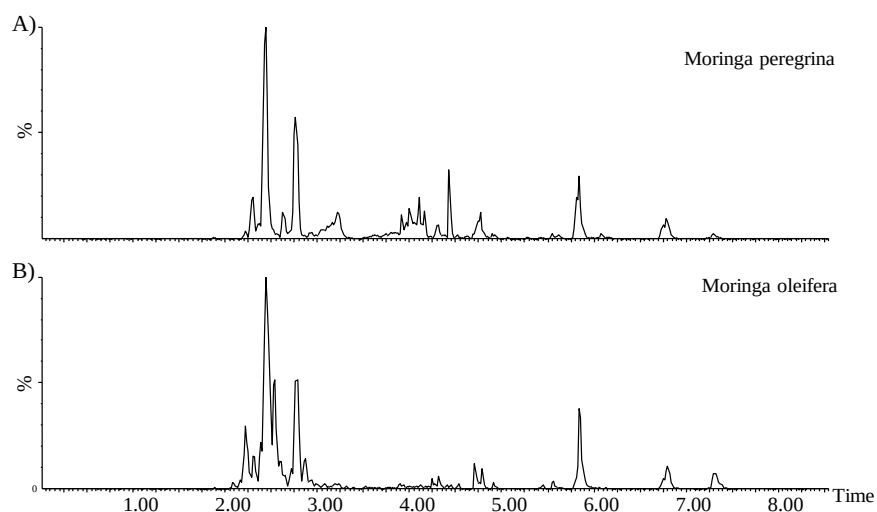


Fig S 2. Base peak intensity (BPI) chromatograms in negative ESI for *moringa peregrina* (A) and *moringa oleifera* (B)

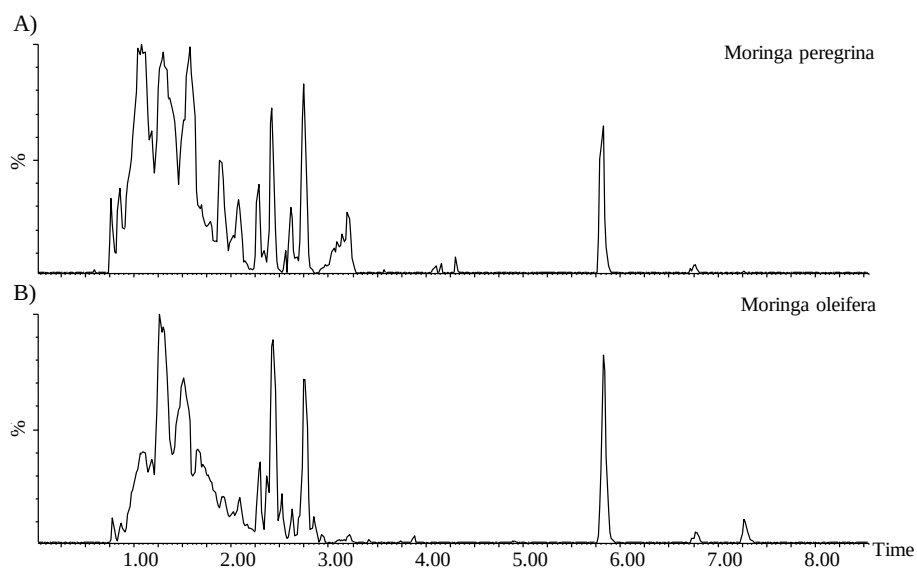


Fig S 6. Base peak intensity (BPI) chromatograms in positive ESI for *moringa peregrina* (A) and *moringa oleifera* (B)

Supporting information 2

Ultra-high-pressure supercritical fluid extraction and chromatography of oil from *Moringa Oleifera* and *Moringa Perigrina* seeds

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Table 1a: Relative peak area of lipids detected in oil extracted from *Moringa Oleifera* seeds at 40 and 80 MPa.

Lipid species were detected higher at 80 MPa		
Identification	80 MPa	40 MPa
TG 54:7	1±0.47	0.06±0.01
20:0-Campesterol	1±0.12	0.22±0.03
16:0-Campesterol	1±0.25	0.25±0.03
TG 42:0	1±0.26	0.27±0.03
1,2 DG 36:6	1±0.1	0.31±0.04
TG 55:5	1±0.25	0.35±0.04
TG 50:4	1±0.07	0.45±0.05
TG42:2	1±0.3	0.46±0.05
TG 44:5	1±0.26	0.47±0.06
TG 60:3	1±0.03	0.52±0.06
TG 58:2	1±0.06	0.52±0.06
TG 55:4	1±0.07	0.52±0.06
TG 49:3	1±0.14	0.52±0.06
18:3-Campesterol	1±0.1	0.55±0.06
TG 58:3	1±0.03	0.56±0.07
TG 53:3	1±0.07	0.56±0.07
TG 52:4	1±0.05	0.57±0.07
TG 49:1	1±0.06	0.57±0.07
TG 44:2	1±0.22	0.57±0.07
TG 48:3	1±0.07	0.58±0.07
TG 42:1	1±0.25	0.58±0.07
TG 46:5	1±0.23	0.59±0.07
TG 60:2	1±0.04	0.6±0.07
TG 51:3	1±0.05	0.6±0.07
TG 49:2	1±0.07	0.6±0.07
TG 53:4	1±0.08	0.6±0.07
TG 49:0	1±0.12	0.61±0.07
TG 56:5	1±0.09	0.62±0.07
TG 56:4	1±0.07	0.63±0.07
TG 51:2	1±0.08	0.63±0.07
TG 46:1	1±0.21	0.63±0.07
TG 53:5	1±0.04	0.64±0.07
TG 60:3	1±0.16	0.64±0.07
1,2 DG 35:4	1±0.02	0.65±0.08
TG 54:6	1±0.08	0.66±0.08
TG 46:2	1±0.15	0.66±0.08

Lipid species were detected higher at 80 MPa		
Identification	80 MPa	40 MPa
TG 50:0	1±0.09	0.67±0.08
TG 54:5	1±0.1	0.67±0.08
TG 50:1	1±0.1	0.67±0.08
TG 48:0	1±0.1	0.67±0.08
TG 56:0	1±0.14	0.67±0.08
TG 53:6	1±0.06	0.68±0.08
TG 48:1	1±0.05	0.69±0.08
TG 51:3	1±0.08	0.69±0.08
TG 55:2	1±0.1	0.69±0.08
TG 53:2	1±0.03	0.7±0.08
TG 54:3	1±0.04	0.7±0.08
TG 51:5	1±0.08	0.7±0.08
TG 60:5	1±0.26	0.71±0.2
1,3 DG 34:4	1±0.02	0.71±0.2
1,3 DG 35:5	1±0.11	0.72±0.25
TG 50:3	1±0.07	0.72±0.08
1,2 DG 35:6	1±0.07	0.72±0.08
TG 54:2	1±0.05	0.74±0.09
TG 58:4	1±0.11	0.74±0.09
TG 55:6	1±0.02	0.75±0.09
TG 52:3	1±0.07	0.75±0.09
TG 56:1	1±0.06	0.75±0.09
TG 52:0	1±0.12	0.75±0.09
TG 56:3	1±0.12	0.76±0.09
TG 54:4	1±0.08	0.78±0.09
TG 54:1	1±0.1	0.78±0.09
1,2 DG 35:3	1±0.02	0.79±0.09

Lipid species were detected higher at 40 MPa		
Identification	neat CO ₂	Modifier
1,3 DG 38:1	1±0.03	1.24±0.14
1,3 DG 34:3	1±0.14	1.45±0.17
MG 20:4	1±0.04	1.46±0.17
1,2 DG 35:0	1±0.23	1.47±0.17
TG 55:3	1±0.07	1.69±0.2
1,2 DG 32:3	1±0.1	1.7±0.2
TG 60:5	1±0.26	1.7±0.2
1,3 DG 34:4	1±0.02	1.71±0.2
1,2 DG 38:4	1±0.08	1.71±0.2
1,3 DG 32:3	1±0.1	1.71±0.2
MG 18:0	1±0.19	1.79±0.21
1,3 DG 35:5	1±0.11	2.17±0.25
MG 18:1	1±0.1,2	2.28±0.27
MG 16:0	1±0.87	3.32±0.39

Lipid species were detected uniquely at 80 MPa	
PC 34:1	
PC 32:2	
LPC 16:0	
PC 36:1	
PE 38:2	
PE 36:2	
PE 34:1	

Table S2: Relative peak area of lipid species detected in the oil obtained from *Moringa oleifera* seeds using neat CO₂ and CO₂ with modifier (ethanol) at 80 MPa.

Lipid species were detected higher at 80 MPa with modifier (ethanol)		
Identification	Modifier	neat CO ₂
20:2-Stigmasterol	1±0.03	0.01±0
FA 16:1	1±0.33	0.01±0.01
22:2-Sitosterol	1±0.03	0.01±0.01
20:3-Campesterol	1±0.1	0.01±0.02
1,3 DG 36:6	1±0.02	0.02±0
TG 50:4	1±0.03	0.02±0
1,3DG 32:2	1±0.03	0.02±0
20:0-Campesterol	1±0.03	0.02±0
TG 58:5	1±0.03	0.02±0
FA 18:3	1±0.15	0.02±0.02
TG 50:5	1±0.36	0.02±0.04
18:3-Sitosterol	1±0.04	0.03±0
TG 53:3	1±0.12	0.03±0
20:2-Sitosterol	1±0.06	0.03±0
20:1-Stigmasterol	1±0.04	0.03±0
MG 18:2	1±0.05	0.04±0.03
FA 20:1	1±0.23	0.04±0.04
16:1-Campesterol	1±0	0.05±0.02
1,3 DG 36:7	1±0.02	0.05±0.06
FA 22:4	1±0.31	0.05±0.09
18:2-Stigmasterol	1±0.06	0.06±0
18:1-Stigmasterol	1±0.05	0.06±0.01
22:1-Stigmasterol	1±0.18	0.07±0.03
16:2-Campesterol	1±0.01	0.08±0
22:0-Sitosterol	1±0.08	0.08±0.01
16:2-Sitosterol	1±0.02	0.08±0.01
22:1-Sitosterol	1±0.04	0.08±0.01
20:1-Campesterol	1±0.04	0.08±0.01
TG 60:4	1±0.12	0.08±0.02
16:3-Campesterol	1±0.04	0.09±0.01
1,3 DG 36:3	1±0.02	0.09±0.01
TG 60:3	1±0.32	0.09±0.01
22:2-Campesterol	1±0.04	0.1±0.01
18:2-Sitosterol	1±0.01	0.1±0.01
16:2-Stigmasterol	1±0.01	0.1±0.02
1,3 DG 38:3	1±0.01	0.1±0.02
FA 18:0	1±0.18	0.11±0.09

Lipid species were detected higher at 80 MPa with modifier (ethanol)		
Identification	Modifier	neat CO2
20:2-Campesterol	1±0.03	0.1,2±0.04
FA 20:0	1±0.14	0.1,2±0.15
TG 60:2	1±0.06	0.1,3±0
1,2 DG 38:3	1±0.04	0.14±0.03
1,2 DG 35:0	1±0.08	0.16±0.04
TG 44:3	1±0.49	0.17±0.08
20:3-Sitosterol	1±0.06	0.19±0.01
18:3-Campesterol	1±0.11	0.21±0.02
16:1-Sitosterol	1±0.03	0.22±0.01
1,3 DG 36:5	1±0.05	0.23±0.02
16:3-Sitosterol	1±0.04	0.23±0.06
20:0-Sitosterol	1±0.03	0.24±0.03
1,3 DG 34:4	1±0.07	0.25±0
20:1-Sitosterol	1±0.05	0.25±0.03
22:0-Campesterol	1±0.11	0.25±0.08
16:0-Sitosterol	1±0.02	0.27±0.02
1,2 DG 32:3	1±0.01	0.29±0.03
1,3 DG 32:3	1±0.01	0.29±0.03
16:3-Stigmasterol	1±0.04	0.33±0.03
1,3 DG 34:3	1±0.01	0.34±0.05
1,3 DG 38:4	1±0.02	0.39±0.03
1,3 DG 35:5	1±0.04	0.4±0.05
16:0-Campesterol	1±0.09	0.41±0.1
18:2-Campesterol	1±0.06	0.44±0.03
18:3-Stigmasterol	1±0.11	0.45±0.08
1,3 DG 32:0	1±0.07	0.45±0.29
1,2 DG 38:0	1±0	0.51±0.05
1,3 DG 35:6	1±0.03	0.57±0.04
1,2 DG 38:1	1±0	0.75±0.03
1,2 DG 32:0	1±0.07	0.75±0.1,2

Lipid species were detected uniquely at 80 MPa with modifier (ethanol)		
22:2-Stigmasterol	20:3-Campesterol	PI 36:5
22:3-Sitosterol	LPC 22:1	LPC 20:3
PC 34:0	PC 35:3	LPC 20:2
PC 42:2	PC 37:3	18:0-Campesterol
MG 22:6	18:1-Campesterol	LPC 16:1
PC 35:1	PC 36:1	LPC 18:0
PC 36:1	FA 18:2	LPC 18:1
PE 36:2	22:3-lesterol	LPC 16:2
PC 34:1	PC 35:4	LPC 18:3
PC 32:2	22:3-Campesterol	PC 34:5
PE 38:2	PC 37:2	PE 34:0
PE 34:1	LPC 18:2	FA 22:3
LPC 19:0	PE 38:2	PE 32:1
PE 36:2	FA 20:2	LPE 17:0
FA 22:1	LPC 19:1	PC 35:5
LPC 20:5	PI 36:0	PC 40:2

Table S3: Relative peak area of lipid species detected in the oil obtained from *Moringa Oleifera* (MO) and *Moringa Peregrina* (MP) seeds using neat CO₂ with modifier (ethanol) at 80 MPa.

Species detected higher in MP		
Identification	M.P	MO
16:0-Campesterol	1±0.34	0.02±0.02
22:1-Stigmasterol	1±0.04	0.04±0
22:2-Sitosterol	1±0.04	0.04±0
22:0-Campesterol	1±0.58	0.04±0.02
20:1-Stigmasterol	1±0.03	0.05±0
20:2-Sitosterol	1±0.03	0.05±0
MG 20:4	1±0	0.05±0.01
20:0-Sitosterol	1±0.08	0.06±0
18:3-Stigmasterol	1±0.03	0.06±0
MG 18:1	1±0.04	0.06±0.01
22:0-Stigmasterol	1±0.18	0.06±0.01
22:1-Sitosterol	1±0.18	0.06±0.01
1,3 DG 38:4	1±0.02	0.07±0
16:3-Stigmasterol	1±0.03	0.07±0
18:0-Stigmasterol	1±0.05	0.08±0
18:1-Sitosterol	1±0.05	0.08±0
20:0-Stigmasterol	1±0.04	0.08±0.01
20:1-Sitosterol	1±0.04	0.08±0.01
18:0-Sitosterol	1±0.03	0.09±0
LPC 20:4	1±0.02	0.1±0.01
1,3 DG 38:0	1±0.04	0.12±0.01
1,2 DG 38:0	1±0.04	0.12±0.01
16:0-Sitosterol	1±0	0.13±0
LPE 18:1	1±0.01	0.14±0.01
1,2 DG 36:4	1±0.05	0.14±0.01
PC 40:1	1±0.11	0.14±0.14
LPC 18:1	1±0.01	0.16±0
LPC 20:1	1±0.04	0.17±0
16:0-Stigmasterol	1±0	0.18±0.01
16:1-Sitosterol	1±0	0.18±0.01
1,3 DG 38:1	1±0	0.19±0.01
1,2 DG 38:1	1±0	0.19±0.01
PE 34:1	1±0.46	0.21±0.02
1,2 DG 34:3	1±0.01	0.21±0.05
1,2 DG 38:4	1±0.14	0.22±0.02
20:2-Stigmasterol	1±0.06	0.22±0.06

Species detected higher in MP		
Identification	M.P	MO
20:2-Stigmasterol	1±0.06	0.22±0.06
20:3-Sitosterol	1±0.06	0.22±0.06
20:1-Campesterol	1±0.06	0.26±0
LPC 18:0	1±0.1	0.26±0.01
PC 36:4	1±0.03	0.26±0.02
PC 42:1	1±0.06	0.26±0.26
18:3-Sitosterol	1±0.11	0.27±0
18:2-Stigmasterol	1±0.11	0.27±0
18:3-Campesterol	1±0.16	0.27±0.01
1,2 DG 34:4	1±0.06	0.28±0
1,3 DG 34:4	1±0.06	0.29±0
22:2-Campesterol	1±0.29	0.34±0.01
LPC 16:1	1±0.05	0.35±0.27
PC 40:4	1±0.18	0.35±0.35
PC 38:2	1±0.02	0.36±0.01
TG 60:4	1±0.02	0.37±0.02
18:0-Campesterol	1±0.02	0.37±0.04
18:2-Campesterol	1±0.01	0.37±0.18
TG 54:5	1±0.06	0.39±0.02
16:2-Sitosterol	1±0.01	0.39±0.1
TG 58:4	1±0.02	0.47±0
TG 60:3	1±0.03	0.47±0.01
TG 60:2	1±0.01	0.48±0.02
TG 52:5	1±0.11	0.48±0.03
TG 60:6	1±0.18	0.48±0.05
1,3 DG 35:5	1±0.04	0.49±0.01
1,3 DG 36:0	1±0.02	0.49±0.02
20:2-Campesterol	1±0.03	0.49±0.04
PE 36:2	1±0.09	0.49±0.09
PE 34:2	1±0.25	0.5±0.1
TG 60:5	1±0.08	0.5±0.11
PC 36:3	1±0.01	0.51±0.02
PC 33:2	1±0.01	0.52±0.04
PE 36:1	1±0.04	0.52±0.11
18:2-Sitosterol	1±0.06	0.54±0.12

Species detected higher in MP		
Identification	MP	MO
18:1-Stigmasterol	1±0.06	0.54±0.12
20:3-Stigmasterol	1±0.11	0.55±0.02
1,2 DG 32:3	1±0.01	0.56±0.01
1,2 DG 35:4	1±0.01	0.56±0.02
LPE 16:0	1±0.07	0.56±0.06
PC 35:5	1±0.02	0.58±0.06
TG 54:0	1±0.04	0.59±0.1,3
PC 37:1	1±0.01	0.6±0.02
TG 58:3	1±0	0.6±0.02
PC 36:1	1±0.01	0.6±0.03
PC 36:2	1±0.03	0.6±0.04
16:2-Campesterol	1±0.02	0.62±0.01
PC 36:0	1±0.01	0.64±0.02
1,3 DG 36:1	1±0.03	0.67±0.01
TG 54:1	1±0.05	0.68±0.01
PC 35:4	1±0.02	0.68±0.02
PC 34:3	1±0.02	0.71±0.02
PC 37:2	1±0.02	0.72±0.07
PC 38:0	1±0.02	0.73±0.02
PC 38:3	1±0.05	0.74±0.1
PC 35:1	1±0	0.75±0.03
16:2-Stigmasterol	1±0.04	0.77±0.02
16:3-Sitosterol	1±0.04	0.77±0.02
1,3 DG 38:3	1±0.04	0.77±0.02
TG 55:6	1±0.08	0.79±0.08
PE 38:5	1±0.1	0.81±0.03
TG 54:2	1±0.08	0.81±0.06
TG 52:0	1±0.03	0.83±0.02
TG 56:3	1±0.05	0.85±0.03
TG 53:3	1±0.06	0.86±0
1,3 DG 35:2	1±0.04	0.86±0.01
TG 54:4	1±0.04	0.89±0.04
1,2 DG 38:3	1±0.02	0.91±0.02
TG 56:2	1±0.03	0.91±0.02
16:1-Stigmasterol	1±0.01	0.39±0.1
1,3 DG 38:2	1±0.01	0.39±0.1
TG 58:5	1±0.02	0.43±0.03
PC 38:1	1±0.02	0.44±0.01
TG 58:2	1±0.04	0.45±0.01
1,2 DG 38:2	1±0.01	0.45±0.11
PE 32:0	1±0.1	0.45±0.22
TG 56:5	1±0.06	0.46±0.11

Species detected higher in MO		
Identification	MP	MO
CE 20:1	1±0.2	2.27±0.14
TG 46:0	1±0.04	2.15±0.06
TG 50:2	1±0.04	2.22±0.07
PC 32:2	1±0.05	2.26±0.08
PE 38:1	1±0.38	2.26±0.18
TG 50:0	1±0.03	2.39±0.1
TG 50:1	1±0.03	2.42±0.11
PC 33:1	1±0.08	2.51±0.2
PC 32:0	1±0	2.52±0.16
TG 48:2	1±0.03	2.59±0.07
TG 53:1	1±0.01	2.75±0.07
CE16:0	1±0.11	2.76±0.04
TG42:1	1±0.1,2	2.82±0
TG 49:0	1±0.15	2.85±0.12
TG42:2	1±0.05	2.88±0.07
1,3 DG	1±0.18	2±0.49
CE 18:3	1±0.14	3.03±0.04
TG 50:3	1±0.04	3.11±0.05
TG 49:1	1±0.33	3.24±0.03
1,3DG 32:2	1±0.09	3.25±0.15
TG 48:1	1±0.03	3.32±0.04
CE 16:3	1±0.01	3.38± 0.52
TG 42:0	1±0.01	3.42±0.02
TG 55:5	1±0.04	3.42±0.02
TG 51:1	1±0.01	3.45±0.08
1,2 DG 36:5	1±0.01	3.52±0.16
TG 44:0	1±0.1,3	3.91±0.03
TG 48:0	1±0.03	4.32±0.05
PI 36:4	1±0.03	4.33±0.02
TG 48:3	1±0.05	4.49±0.2
TG 53:2	1±0.02	4.89±0.14
CE 19:0	1±0.19	4.95±1.36
TG 44:2	1±0.03	4.96±0.06
PI 34:2	1±0.15	5.52±0.26
PI 34:1	1±0.03	5.66±0.25
PI 34:0	1±0.06	5.74±0.02
CE 18:2	1±1	5.84±1.09
PI 36:3	1±1	6.82±0.57
TG 44:3	1±0.03	6.9±0.21

Species detected higher in MO		
Identification	MP	MP
TG 50:4	1±0.03	1.11±0.01
TG 56:4	1±0.01	1.11±0.06
PC 33:4	1±0.05	1.1,2±0.04
PC 35:0	1±0.02	1.1,3±0.05
1,2 DG 35:5	1±0.04	1.14±0.06
TG 53:6	1±0.03	1.17±0.01
TG 52:4	1±0.03	1.18±0.04
TG 51:3	1±0.04	1.24±0.03
TG 52:2	1±0.04	1.28±0.02
1,3 DG 32:0	1±0.07	1.35±0
TG 54:3	1±0.08	1.38±0.1
TG 54:6	1±0.01	1.42±0.21
PC 34:0	1±0.01	1.43±0.05
PC 34:1	1±0.03	1.46±0.05
PC 35:3	1±0.05	1.47±0.19
1,3 DG 34:0	1±0.05	1.51±0.07
PC 34:2	1±0.02	1.53±0.04
TG 49:2	1±0.04	1.53±0.07
PC 33:5	1±0.08	1.54±0.31
TG 51:5	1±0.03	1.55±0.07
PI 36:2	1±0.02	1.71±0.01
PI 36:1	1±0.19	1.71±0.11
1,2 DG 34:2	1±0.05	1.72±0.02
TG 52:3	1±0.05	1.75±0
TG 55:4	1±0.02	1.75±0.09
1,3 DG 34:1	1±0.04	1.77±0.07
TG 49:3	1±0.03	1.78±0.15
1,2 DG 32:1	1±0.04	1.81±0.04
PE 35:1	1±0.05	1.81±0.43
TG 56:6	1±0.01	1.84±0.07
1,2 DG 35:6	1±0.09	1.86±0.08
TG 46:2	1±0.02	1.87±0.05
PI 36:0	1±0.03	1.93±0.23
TG 55:2	1±0.04	1.96±0.01
PC 33:3	1±0.04	1.96±0.41
TG 46:1	1±0.02	1.97±0.02
TG 50:5	1±0.2	2.05±0.42
PC 32:1	1±0.01	2.08±0.08

Species detected uniquely in MP	Species detected uniquely in MO	
TG 51:4	MG 18:0	MG 22:6
TG 53:0	LPC 22:0	LPE 18:0
TG 51:0	PC 40:2	LPC 18:3
1,3 DG 35:4	LPC 19:0	22:0-Sitosterol
1,3 DG 36:5	PC 36:6	1,3 DG 36:6
TG 51:2	TG 52:6	TG 51:4
MG 20:5	PC 38:8	1,3 DG 35:4
TG 52:6	PC 34:4	TG 51:0
	LPE 18:2	TG 51:2
	PC 36:5	1,3 DG 36:5
	PC 38:7	TG 53:0

CHAPTER 5

5 Conclusions and recommendations

5.1 Conclusions

Optimisation of a green extraction system namely pressurized hot water extraction proved to be a powerful tool capable of producing extracts rich in bioactive compounds from *Moringa oleifera* plant. Pressurized hot water was optimised for a near complete recovery of vitamin C, kaempferol and quercetin from *Moringa oleifera* leaves. In order to understand the effect of the processing conditions, two modelling approaches, namely RSM and ANN were used. ANN model was found to be superior to the RSM model in terms of various statistical errors such as AAD, RMSE, MAE, χ^2 and R^2 when predicting the recovery of vitamin C, kaempferol and quercetin. The optimum conditions were found to be 91°C, 60 min and 0.5 ml min⁻¹ for a predicted maximum value of 3.92 ± 0.3 , 4.74 ± 0.32 and 0.357 ± 0.19 g per 100 g of kaempferol, quercetin and vitamin C.

Moringa oleifera has been found to be an important source of macro and micro-elements. Pressurized hot water extraction was optimised for the recovery of Moringa extract rich in macro-elements and less micro-elements. The optimum conditions for a satisfactory amount of macro and micro-elements were 90°C and 60 minutes' extraction time.

A one step particle formation using an on-line process for pressurised hot water extraction and drying of plant extracts, based on the use of SC-CO₂ as dispersant, was optimised. With this process, dried complex extracts were obtained as a fine powder of particle size smaller than 4 µm in diameter. The WEPO process developed in this work could be considered as a suitable and promising process for

obtaining, in only one step, fine and dried particles with unchanged antioxidant capacity, directly from *Moringa oleifera* leaves.

The recovery of high value oil from seeds of *Moringa* species was optimised using ultra-high-pressure supercritical extraction (UHSPE). The optimum conditions required to obtain the highest extracted amount of oil were found to be 80 MPa and 57°C. At these conditions, the amount of oil extracted reached 398 mg per g of seeds. The addition of co-solvent such as ethanol increased the extracted amount of polar lipids. UHPSFC analysis revealed that *Moringa* seed oil was composed of TGs, DGs, MGs, campesterol and stigmaterol ester, FAs, PCs, LPCs, PE, LPE, PI and PS. The results showed that phospholipids were more abundant in *Moringa oleifera* than *Moringa peregrina*. Furthermore, *Moringa peregrina* seeds had a higher content of oil than *Moringa oleifera* seeds.

5.2 Recommendations

In view of the results obtained in this study, could be recommended that:

- ✓ Pressurized hot water extraction be used to extract other bioactive compounds such as alkaloids, saponins and carbohydrates from plant.
- ✓ The amount of powder lost during WEPO process be recovered by applying an electrode deposition in the spraying nozzle and increasing the size of the drying chamber.
- ✓ Ultra-high-pressure supercritical extraction can be applied in medium scale production of high quality of oil from seeds.