MICROWAVE-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM BROCCOLI (*Brassica oleracea*) BY-PRODUCTS (LEAVES AND STEMS), AND BROCCOLI EDIBLES (FLORETS)

By

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ABSTRACT

Food safety and the management of organic waste and loss, such as food waste, are problems of global interest. Fruit and vegetable by-products are listed as one of the main food wastes; among these wastes, there is a large number of vegetables of high global production, such as broccoli, however, broccoli florets are the ones that are commonly consumed in food, causing the broccoli by-products, such as stems, and leaves to be discarded. Broccoli by-products contain significant amounts of bioactive compounds, which are chemical compounds with beneficial properties for human consumption since they have antioxidant, antimicrobial, and anti-inflammatory properties. Among the bioactive compounds, phenolics are of great interest nowadays and their importance is growing.

Phenolic compounds can be recovered from broccoli by-products through extraction techniques. Microwave-assisted extraction is an unconventional extraction technique that allows various compounds to be recovered, such as phenolic compounds from plants, in short periods of time, with high efficiency. However, there is not enough research regarding the optimization and validation of microwave-assisted extraction to recover phenolic compounds from broccoli by-products.

Therefore, the purpose of this master's project was to perform the optimization of microwave-assisted extraction to recover phenolic compounds from broccoli by-products, which include stems and leaves, and compare them with the edible parts, known as florets. As a first step, the effect of three operational variables such as time, temperature, and methanol concentration in the microwave extraction process was evaluated, these variables were selected according to the literature review. The variables were optimized through a Response Surface Analysis to obtain the maximum total phenolic response of the broccoli stems, leaves, and florets. As a result, the optimal variables were determined to be 10 min, 73.27°C, and 80% methanol for broccoli leaves, 15.9 min, 74.45°C, and 74.54% methanol for broccoli stems, and 18.9 min, 75°C, and 80% methanol for broccoli florets.

The second stage of the project involved determining antioxidant activity, identifying the main phenolic acids in broccoli extracts, and comparing microwave and maceration extraction methods. Broccoli stems had the lowest antioxidant activity and total phenolic content, while leaves had the

highest. In addition, when compared to maceration extraction, microwave extraction increased phenolic production in all broccoli extracts. Finally, the last stage of this thesis consisted of the analysis of the behavior of acetone, water, and methanol, as polar compounds, and hexane, as a non-polar compound, in the microwave extraction process of broccoli stems, florets, and leaves. The results showed that polar solvents favor the extraction of phenolic compounds from broccoli extracts; however, some solvents such as methanol and acetone can degrade the compounds if they exceed 85°C of temperature.

In general, this project offers a comprehensive review of different extraction techniques of bioactive compounds from several fruit and vegetable by-products; In addition, it provides a broad view of the behavior of the main variables in the microwave extraction of phenolic compounds from broccoli, which serves as a basis for the extraction of phenols in this type of plants.

RÉSUMÉ

La sécurité alimentaire et la gestion des déchets et des pertes organiques, tels que les déchets alimentaires, est un problème d'intérêt mondial. Les sous-produits de fruits et légumes figurent parmi les principaux déchets alimentaires; parmi ces déchets, il y a un grand nombre de légumes de production mondiale élevée, comme le brocoli, cependant, les fleurons de brocoli sont ceux qui sont couramment consommés dans les aliments, ce qui entraîne le rejet des sous-produits du broccoli, tels que les tiges et les feuilles. Les sous-produits de brocoli contiennent des quantités importantes de composés bioactifs, qui sont des composés chimiques aux propriétés bénéfiques pour la consommation humaine car ils ont des propriétés antioxydantes, antimicrobiennes et anti-inflammatoires; les composés bioactifs les plus intéressants actuellement sont les composés phénoliques. Les composés phénoliques peuvent être récupérés à partir des sous-produits du brocoli grâce à des techniques d'extraction. L'extraction assistée par micro-ondes est une technique d'extraction non conventionnelle qui permet de récupérer divers composés, tels que les composés phénoliques des plantes, en peu de temps, avec une grande efficacité. Toutefois il y a un manque de recherche dans l'optimisation et la validation de l'extraction assistée par micro-ondes pour récupérer les composés phénoliques des sous-produits du brocoli.

Par conséquent, le but de ce projet de maîtrise était d'effectuer l'optimisation de l'extraction assistée par micro-ondes pour récupérer les composés phénoliques des sous-produits de brocoli, qui comprend les tiges et les feuilles, et les comparer avec les parties comestibles, appelées fleurons. Comme première étape, l'effet de trois variables opérationnelles telles que le temps, la température et la concentration de méthanol dans le processus d'extraction par micro-ondes a été évalué, ces variables ont été sélectionnées sur la base de la revue de la littérature. Les variables ont été optimisées par une analyse de surface de réponse pour obtenir la réponse phénolique totale maximale des tiges, des feuilles et des fleurons de brocoli. En conséquence, les variables optimales ont été déterminées comme étant 10 min, 73,27 °C et 80 % de méthanol pour les feuilles de brocoli, 15,9 min, 74,45 °C et 74,54 % de méthanol pour les tiges de brocoli, et 18,9 min, 75 °C et 80% de méthanol pour les fleurons de brocoli.

La deuxième étape du projet a consisté à déterminer l'activité antioxydante, à identifier les principaux acides phénoliques dans les extraits de brocoli et à comparer les méthodes d'extraction par micro-ondes et par macération. Les tiges de brocoli ont présenté l'activité antioxydante et la

teneur totale en composés phénoliques les plus faibles, tandis que les feuilles ont eu la plus élevée. De plus, par rapport à l'extraction par macération, l'extraction par micro-ondes a augmenté la production phénolique dans tous les extraits de brocoli. Enfin, la dernière étape de cette thèse a consisté en l'analyse du comportement de l'acétone, de l'eau et du méthanol, en tant que composés polaires, et de l'hexane, en tant que composé non polaire, dans le processus d'extraction par micro-ondes des tiges, fleurons et feuilles de brocoli. Les résultats ont montré que les solvants polaires favorisent l'extraction des composés phénoliques des extraits de brocoli; cependant, certains solvants tels que le méthanol et l'acétone peuvent dégrader les composés s'ils dépassent 85°C de température.

De manière générale, ce projet propose une revue complète des différentes techniques d'extraction des composés bioactifs de plusieurs sous-produits de fruits et légumes; En outre, il fournit une vision large du comportement des principales variables dans l'extraction par micro-ondes des composés phénoliques du brocoli, ce qui sert de base à l'extraction des phénols dans ce type de plantes.

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THESIS FORMAT AND ORGANIZATION

This thesis is presented in the form of papers published in a journal or suitable for journal publication. The thesis is divided into chapters, which consist of the following:

Chapter I presents the introduction of the research, focusing on the problem statement, hypothesis establishment, and covers the general and specific objectives of the research work. Secondly, Chapter II shows an overview of the green extraction techniques to obtain bioactive compounds from fruits and vegetables by-products, the literature review exhibits a general background on extraction techniques to recover bioactive compounds and their importance in food safety and food waste revaluation.

Subsequently, Chapter III presents a Microwave-assisted extraction optimization to maximize the total phenolic content from broccoli stems, leaves, and florets, making a characterization of the phenolic compounds, antioxidant activity evaluation, and comparation with maceration; furthermore, the total phenolic content of broccoli by-products (leaves and stems) and its edible parts (florets) is compared and discussed. Then, Chapter IV, shows the polar (methanol, acetone, and water) and non-polar (hexane) solvents behavior in the Microwave-assisted extraction of phenolic compounds from broccoli stems, leaves, and florets, this chapter discusses the relationship and importance of solvent selection, and temperature conditions in the extraction, to increase the total phenolic content of the extracts, and improve the microwave assisted extraction process. Finally, Chapter V presents the concluding remarks of the research, covering the general summary, conclusions, contributions to knowledge and future research recommendations.

CONTRIBUTION OF AUTHORS

This manuscript-based thesis follows the thesis preparation guideline of Graduate and Postdoctoral Studies of McGill University. The following manuscripts have been published or are in the process of being published:

- Sheila Lucía Rodríguez García & Vijaya Raghavan (2021): Green extraction techniques from fruit and vegetable waste to obtain bioactive compounds—A review. Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2021.1901651
- Sheila Lucía Rodríguez García & Vijaya Raghavan (2022): Microwave-Assisted Extraction of Phenolic Compounds from Broccoli (*Brassica oleracea*) Stems, Leaves, and Florets: Optimization, Characterization, and Comparison with Maceration Extraction. Recent Progress in Nutrition, DOI: 10.21926/rpn.2202011
- Sheila Lucía Rodríguez García & Vijaya Raghavan (2022): Polar and non-polar solvents behavior in Microwave-assisted extraction of phenolic compounds from Broccoli (*Brassica oleracea*) stems, leaves, and florets, (Submitted to Journal of Biologically Active Products from Nature).

The research work, which included a literature review, experiment design, experimental work, data analysis, and preparation of the manuscripts, was conducted primarily by Sheila Lucía Rodríguez García. Prof. G.S. Vijaya Raghavan, from the Bioresource Engineering Department at Macdonald Campus of McGill University, supervised the work, edited, and co-reviewed all the manuscripts. Mr. Yvan Gariepy provided technical and laboratory assistance during the experimental design and throughout the experimental runs. Most of the research, including the Microwave-Assisted extraction, High-Performance Liquid Chromatography analysis, phenolic, and antioxidant quantification, were performed in the Postharvest Technology Laboratory in the Macdonald Campus of McGill University. The Freeze-drying experiments were performed in Dr. Valerie Orsat's lab in Macdonald Campus of McGill University.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

°C	Degree Celsius
µg	Microgram
µL	Microlitres
µm	Micrometres
AA	Antioxidant activity
ABTS	2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
Ac	Control absorbance
ANOVA	Analysis of variance
As	Sample absorbance
BC	Bioactive compounds
CCRD	Central rotating composite design
cm	Centimetres
CO_2	Carbon dioxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl Hydrate
DW	Dry weight
EAE	Enzyme assisted extraction
FAO	Food and Agriculture Organization
FVW	Fruit and vegetable waste
g	Gram
GAE	Gallic acid equivalents
GHG	Greenhouse gas
GHz	Gigahertz
h	Hours
HPLC	High-Performance Liquid Chromatography
$K_2S_2O_8$	Potassium persulfate
kV	kilo-Volt
L	Litre
LLE	Liquid-liquid extraction
MAE	Microwave-assisted extraction
mg	Milligram
min	Minutes
mL	millilitres
mm	Millimetres
MMT	Million metric tons
MPa	Mega pascals
Na_2Co_3	Sodium carbonate
nm	Nanometres
Pc	Critical pressure
PEFE	Pulsed electric field extraction
pH	Potential of hydrogen
ppm	Parts per million
PTFE	Polytetrafluoroethylene
rpm	Rotations per minute
RSA	Response Surface Analysis
SD	Standard deviation
SFE	Supercritical fluid extraction
Tc	Critical temperature
TE	Trolox equivalents
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
v/v %	Percentage (volume per volume)
w/v	Weight per volume

CHAPTER I.
INTRODUCTION

Food waste is a major worldwide issue that contributes significantly to greenhouse gas (GHG) emissions (7% of total world GHG), an estimated 14% of the world's food is considered lost from harvest to the retail level (FAO, 2011, 2019a), exacerbating the problem of climate change, environmental sustainability, food safety, and nutrition for feeding the world population. Fruit and vegetable wastes (FVW) are one of the most significant food wastes, accounting for 0.5 billion tons per year of the overall food waste of 1.3 billion tons (FAO, 2011). It is vital to revalorize FVW since there is social, political, and environmental pressure to reduce, convert, and re-utilize FVW in consumer industries (Trigo et al., 2020). Broccoli is fifth in global output among fruits and vegetables; nevertheless, almost half of the overall broccoli production is considered an inedible portion, generally referred to as by-products, such as leaves and stems (Coman et al., 2020; FAO, 2018, 2019b; Sagar et al., 2018). Several studies have revealed that broccoli by-products contain significant amounts of bioactive compounds (BC) (Ares et al., 2013; Borja-Martínez et al., 2020; S. S. Ferreira et al., 2020; Liu et al., 2018; Rudra et al., 2015; Saini et al., 2019; Thomas et al., 2018).

Bioactive compounds (BC) are secondary metabolites that increase the general survival ability of plants and resolve their local environmental challenges, these compounds contain antioxidant, anticarcinogenic, antibacterial, and anti-inflammatory characteristics (Azmir et al., 2013; Hamzalioğlu & Gökmen, 2016; Saini et al., 2019; Soquetta et al., 2018). Carotenoids, flavonoids, phenolic compounds, and glucosinolates are some examples of BC (Azmir et al., 2013; Voung, 2017). Phenolic compounds have recently sparked interest, due to studies showing that vegetable by-products, including broccoli, contain high levels of them; furthermore, they exhibit antioxidant and antimicrobial properties, making them commercially valuable in a variety of industries, e.g., food, and pharmaceutical (al Jitan et al., 2018; Domínguez-Perles et al., 2010; N. Kumar & Goel, 2019; Luca et al., 2020; Thomas et al., 2018). Whereby, due to the large amounts of broccoli by-products generated in the world, the idea of reutilizing them for their value-added components was born.

Phenolic compounds can be extracted from plant materials with conventional, or non-conventional (also called green) extraction techniques (Ares et al., 2013; Chemat et al., 2012; Soquetta et al., 2018). Conventional extraction techniques, such as maceration, soxhlet, solid-liquid extraction, and hydro-distillation present several disadvantages compared with green extraction techniques, as they require larger quantities of solvent, they are longer processes, and they exhibit lower extraction efficiency (Azmir et al., 2013; Garavand et al., 2019; Soquetta et al., 2018). To overcome these problems, current trends in extraction of BC from plant sources, have been focused on the use of green extraction techniques, such as microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, and pulsed electric field extraction (Azmir et al., 2013; Chen & Chen, 2013; Md Salim, 2017; Vernès et al., 2019). The non-conventional extraction techniques, exhibit less energy consumption, lower extraction time, use fewer solvent quantities, use alternative solvents (such as water-alcohol mixtures), and reduce the unit operations (Azmir et al., 2013; Chemat et al., 2012). One of the most widely utilized green extraction procedures is microwave-assisted extraction (MAE), due to its several benefits, including high efficiency, minimal solvent volumes needed, and effective cell wall destruction in less time (Soquetta et al., 2018). MAE begins with solvent penetration into the plant matrix, then the electromagnetic waves break down the components, next, the solubilized components are transferred from the insoluble matrix to the bulk solution, finally, the liquid and residual solid phase gets separated (Panzella et al., 2020). The extraction yield will depend on several factors affecting the MAE process, such as temperature, solvent-solute affinity, time, solvent selection, and solvent concentration (Panzella et al., 2020; Soquetta et al., 2018).

This thesis proposes and discusses the optimal parameters and conditions for the MAE of broccoli by-products (leaves, and stems), and broccoli edibles (florets) to recover phenolic compounds, comparing the total phenolic content of the broccoli edibles and wastes in the MAE and in maceration extraction; furthermore, identifies the phenolic acids, evaluates the antioxidant capacity of the extracts, and discusses the main variables of MAE to make the phenol recovery process more efficient.

1.1 Problem Statement

The large amounts of FVW generated have prompted the development of new methods to reuse and revalorize the wastes. Among these wastes, broccoli is one of the most produced vegetables

worldwide, considering only the florets as edible parts (corresponding to 15% of the plant) and discarding the by-products such as stems and leaves (corresponding to 21%, and 47% of the plant respectively) (al Jitan et al., 2018). There are many healthy properties assigned to broccoli consumption due to its high content in mainly phenolic compounds (Domínguez-Perles et al., 2010). Whence, this vegetable has a significant economic and industrial importance across the globe. However, there is not much research based on the recovery of phenolic compounds from broccoli by-products, much less on new green extraction techniques such as MAE, which is a sustainable technique, since it is more efficient with less time, uses lesser solvents, and is more environmentally friendly. In addition, there is no establishment of the optimal conditions in MAE for broccoli by-products, nor a comparison of the extraction yield against conventional extraction methods, neither a solid base of the factors that affect the MAE for the recovery of phenolic compounds from broccoli by-products. Therefore, further research is needed in the establishment of the optimal MAE conditions to recover phenolic compounds from broccoli by-products.

This thesis will help to bridge knowledge gasp on the phenolic MAE from broccoli by-products, representing an alternative option to reuse and valorize these wastes, optimizing the resources and offering a sustainable solution for their utilization. Furthermore, MAE optimization will propose the most appropriate conditions to extract bioactive compounds, in this case, phenolics from broccoli by-products. Accordingly, this thesis, firstly evaluates several non-conventional techniques to extract BC from FVW, and the factors affecting the extraction; then, performs the MAE to extract phenolic compounds from broccoli by-products (leaves, and stems) and edibles (florets), optimizes the extraction to maximize the total phenolic content, compares against maceration, identifies the main phenolic acids in the broccoli extracts, and evaluates the antioxidant activity of the phenolic compounds, comparing the phenolic content between broccoli edibles and by-products; finally, discusses the effect of some variables, such as solvent selection, and temperature in the MAE of broccoli stems, leaves, and florets, to improve the extraction process.

1.2 Hyphothesis

The optimization of MAE from broccoli stems, leaves, and florets can maximize the total phenolic content response, it is expected that broccoli by-products such as leaves and stems, exhibit similar or higher amounts of phenolic content and antioxidant activity compared to broccoli florets. MAE

should exhibit higher phenolic extraction yield in less time compared to maceration. Moreover, the behavioral analysis of the extraction parameters, such as temperature, time, solvent selection, and solvent concentration will offer a strong foundation to improve the MAE process, and consequently select the most appropriate extraction parameters for broccoli by-products extraction.

1.3 Objectives

1.3.1 General objectives

The aim of this study is to evaluate the green extraction techniques to obtain BC from FVW, including the MAE optimization of broccoli stems, leaves, and florets, selecting the best parameters and conditions for the extraction to maximize the total phenolic content, through the evaluation of temperature, time, solvent, and antioxidant activity behavior.

1.3.2 Specific Objectives

I. Present a literature review of the green extraction techniques to obtain BC from FVW (Chapter II).
II. Optimize the MAE of broccoli stems, leaves, and florets, through Response Surface Analysis (RSA) with three operational variables (temperature, time, and solvent concentration), to maximize the total phenolic content (Chapter III).
III. Characterize the phenolic compounds of the optimized broccoli extracts, by identifying the main phenolic acids, and performing an antioxidant activity evaluation (Chapter III).
IV. Compare the broccoli extracts obtained by MAE and maceration extraction (Chapter III).
V. Evaluate the temperature and solvent behavior of polar (methanol, water, and acetone), and non-polar (hexane) solvents, in the MAE of broccoli stems, leaves, and florets to obtain phenolic compounds (Chapter IV).

Connecting text

Chapter II presents a comprehensive literature review of the non-conventional extraction methods to recover several bioactive chemicals from plant sources, mainly focusing on wastes from fruits and vegetables. This review has the objective of discussing the potential of FVW as a source of valuable components, creating conscience against food waste generation, and creating an understanding of current trends in extraction.

The content of BC of several fruit, and some vegetable wastes are discussed. Moreover, the properties of several bioactive chemicals are presented. Finally, the conventional, and non-conventional techniques in extraction are compared, analyzed, and discussed.

CHAPTER II.
GREEN EXTRACTION TECHNIQUES FROM FRUIT AND VEGETABLE WASTE TO OBTAIN BIOACTIVE COMPOUNDS: A REVIEW

2.1 Abstract

Food wastes imply significant greenhouse gas emissions, that increase the challenge of climate change and impact food security. According to FAO (2019), one of the main food wastes come from fruit and vegetables, representing 0.5 billion tons per year, of the 1.3 billion tons of total waste. The wastes obtained from fruit and vegetables have plenty of valuable components, known as bioactive compounds, with many properties that impact positively in human health. Some bioactive compounds hold antioxidant, anti-inflammatory, and anti-cancer properties and they have the capacity of modulating metabolic processes. Currently, the use of fruit and vegetable waste is studied to obtain bioactive compounds, through non-conventional techniques, also known as green extraction techniques. These extraction techniques report higher yields, reduce the use of solvents, employ less extraction time, and improve the efficiency of the process for obtaining bioactive compounds. Once extracted, these compounds can be used in the cosmetic, pharmaceutical, or food industry, the last one being focused on improving food quality.

2.2 Introduction

Food wastes imply significant greenhouse gas (GHG) emissions that increase the challenge of climate change and they also impact food security; an estimated 14% of the world's food is lost and wasted from the point of harvest to the retail level (FAO, 2019a). This has become a major global issue on environmental sustainability, food safety, and the necessity of feeding a world population that grows every day. The food that is wasted is associated with approximately 3.3 gigatons of CO_2 equivalent, corresponding to 7% of total global GHG emissions (FAO, 2011, 2019a).

The regional rate of food loss and waste in the world from post-harvest to distribution is shown in Figure 2.1; Central and Southern Asia are the main contributors of food loss with 20-21% of waste, while Australia and New Zealand only have an approximate of 5-6% of food wasted (FAO, 2019a). This rate variation is due to several factors such as climate conditions, transportation, pests, post-

harvest technologies available, and the level of economic and social development under which the food supply chain of each country operates.

The causes of food waste generation vary along the food supply chain; it is important to analyze these causes to determine where the food waste comes from and create strategies for its use or recovery. Some on-farm losses are due to the harvesting time, climate conditions, handling practice, and inadequate storage conditions (M. Wadhwa, 2013). Furthermore, during food processing and packaging the losses can be caused by the process itself that not only generates the desired products but also waste by inadequate facilities, human manipulation, or technical malfunction (Md Salim, 2017). At the retail level, food waste generation may be due to the limited shelf life of the products and the need for foods to meet specific quality and sensory standards.

One of the main food wastes is fruit and vegetable waste (FVW), representing 0.5 billion tons per year, of the 1.3 billion tons of total food waste (FAO, 2011). These losses are not only critical at the agricultural stage in developing countries, but they are also high at the processing plant stage (M. S. L. Ferreira et al., 2013; Md Salim, 2017).

Figure 2.2 shows that there are higher amounts of food waste in the fruit and vegetable group than for cereal and pulses; the wastes levels are high in perishable foods such as fruits and vegetables (FAO, 2019a). This can be explained since fruits and vegetables are commonly overproduced every season and without the proper utilization strategies, wastes can be generated. Furthermore, in the industry of food processing, the raw materials are converted into desirable products; however, some of them end up as wastes in amounts that vary from 15% to 30% (Md Salim, 2017). Based on this, it is expected that the food processing industry generates plenty amounts of FVW each year, due to the demand in consumption of a growing population.

It is important to benefit from FVW, since there has been social, political, and environmental pressure to reduce, reuse and valorize the FVW to their transformation, use in the food industry, and profitability (Trigo et al., 2020). The need for the utilization and transformation of the FVW into value-added products arises to prevent GHG emissions during decomposition and to reduce environmental and food security challenges.

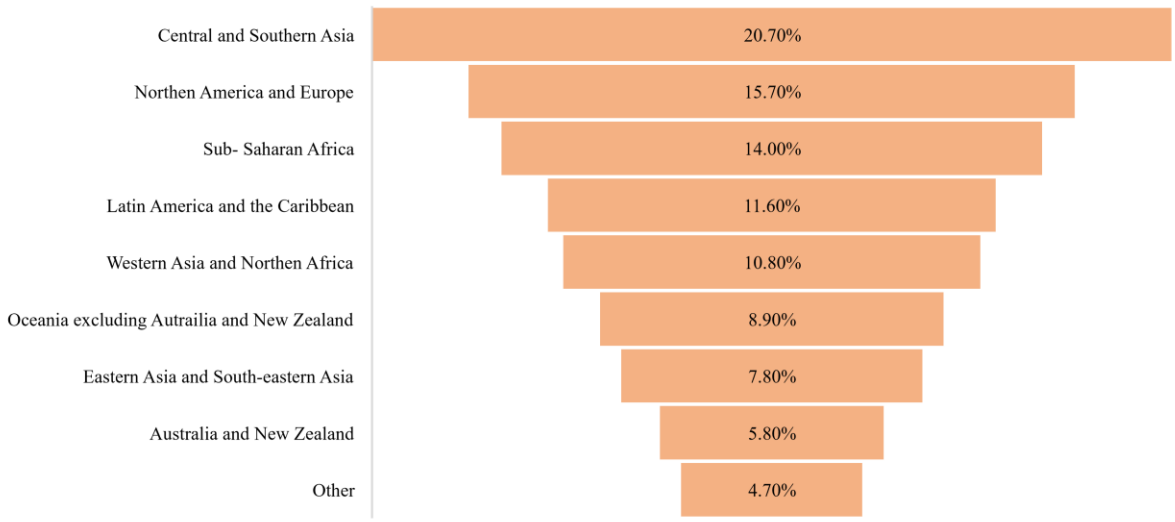


Figure 2. 1 Regional rate of food loss and waste in the world from post-harvest to distribution reported in 2016 (FAO, 2019a).

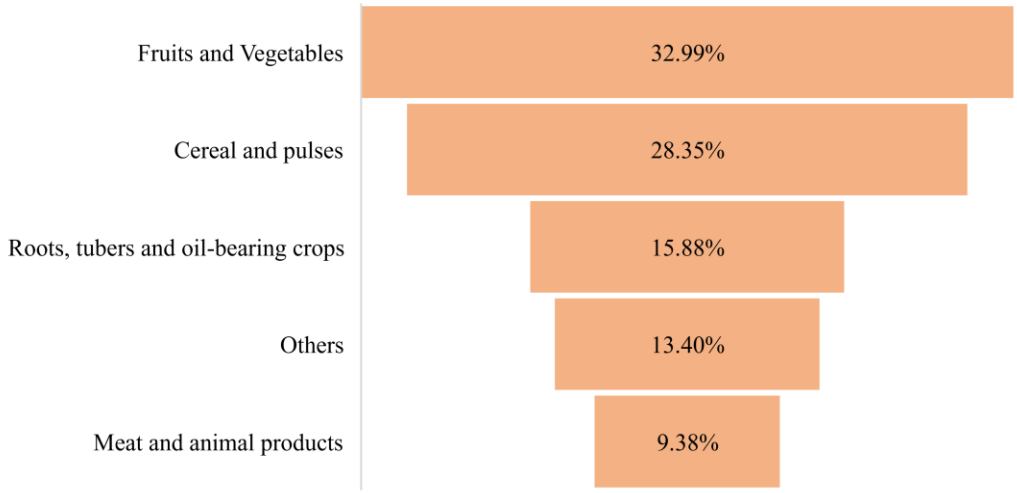


Figure 2. 2 Percentages by commodity groups of food loss and waste from post-harvest to distribution level from 2000 to 2017 (FAO, 2019a).

The wastes that can be obtained from fruits and vegetables are seeds, core, rag, peels, rind, vine, shell, skin, pomace, stones, and pods (M. Wadhwa, 2013; Saini et al., 2019; Trigo et al., 2020; Tylewicz et al., 2018). There are plenty of valuable components that are found in FVW, with many properties that impact positively in human health, these components are known as bioactive compounds (BC), such as carotenoids, alkaloids, glucosinolates, flavonoids, and phenolic acids.

Bioactive Compounds are extra-nutritional components that have the capacity of modulating the metabolic process, they also hold antioxidant, anti-cancer, anti-inflammatory, and anti-allergenic properties (Hamzalioğlu & Gökmen, 2016; Saini et al., 2019). The BC can be extracted from FVW with either conventional or non-conventional techniques. However, recent trends in extraction techniques are focused on finding solutions that minimize the use of solvents for the extraction of BC and still maintain good quality extracts and cost-effective production; this can be accomplished using green extraction or non-conventional techniques (Chemat et al., 2012; Soquetta et al., 2018). The effectiveness of the extraction of BC depends on a series of parameters and conditions in which the extraction is carried out. For example, the source of the bioactive compound, temperature, use of solvents, agitation, time, or pressure. Green extraction techniques, also called clean techniques, are the new methods for sustainable extraction for various compounds from FVW, since most of them use organic solvents, require less time for the extraction, and reduce the use of energy, which translates into a beneficial impact on the environment (Soquetta et al., 2018). Once the BC are extracted from FVW they can be added to food processing to improve the functional value in the food formula (Saini et al., 2019). According to the above, this review aims to delve into obtaining bioactive compounds by different green extraction techniques from fruits and vegetables wastes, remarking their importance and application in the food industry.

2.3 Bioactive compounds

For a better understanding of the origin of bioactive compounds (BC), it should be remembered that the biological plant system compounds are divided into two classes. The first class comprises primary metabolites, which are chemical substances with the purpose of development and growth, for example, carbohydrates, amino acids, proteins, and lipids. While the second class comprises secondary metabolites, which help the plant improve its overall survival capacity to thrive and resolve local obstacles, allowing it to engage with its environment (Azmir et al., 2013).

BC are secondary molecules, metabolites, or chemical substances, found in small amounts in plants and foods, such as vegetables, fruits, oils, grains, and nuts (Soquetta et al., 2018). These molecules have antioxidant, anticarcinogenic, antimicrobial, and anti-inflammatory properties, and they can modulate metabolic processes (Hamzalioğlu & Gökmen, 2016). The properties found in BC provide a positive impact on human health as they can reduce the risk of suffering plenty of diseases, such as cancer, heart disease, strokes, and diabetes (Santos et al., 2019).

There are two major classifications of BC, essential and non-essential. The essential group includes vitamins and minerals that maintain the specific biochemical process in the body; whereas the non-essential group encompasses phenolics, flavonoids, carotenoids, phytosterols, glucosinolates, saponins, alkaloids, and essential oils, which are responsible for the maintenance of optimal cellular health and enhance the action of other essential nutrients (Hamzalioğlu & Gökmen, 2016; Luca et al., 2020; Trigo et al., 2020). In recent years, the extraction of non-essential bioactive compounds from food waste sources has been studied to increase their yield production for food or drug synthesis applications.

In the plant kingdom large groups of BC can be distinguished, for example, the polyphenols (approximately 8000 types), alkaloids (approximately 12,000 types), terpenoids (approximately 25,000 types), and glucosinolates (approximately 200 types) (Azmir et al., 2013; Voung, 2017). Some of the main BC are described below.

2.3.1 Polyphenols

Polyphenols are present as one of the major plant secondary metabolites. They are mainly synthesized by the shikimic acid, pentose phosphate, and phenylpropanoid pathways. They contain one or more aromatic rings and one or more hydroxyl groups in their basic structure (Trigo et al., 2020; van Duynhoven et al., 2011). There are several classes of polyphenols, such as the phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and flavonoids (flavonols, flavanones, flavones, isoflavones, and anthocyanidin) that are ubiquitous in plants and are mainly present as glycosides (Sagar et al., 2018; Wiesner et al., 2016). Hydroxybenzoic acids include gallic, salicylic, p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids. On the other hand, hydroxycinnamic acids include ferulic, p-coumaric, chlorogenic, and sinapic acids (Luca et al., 2020). Polyphenols also exist in the form of polymers such as lignin, important for the plant structure, and tannins (gallotannins, ellagitannins, complex tannins, also known as hydrolyzable tannins, and condensed tannins, also called proanthocyanidins), which are involved in the plant defense (Marranzano et al., 2018; Sieniawska & Baj, 2017; Y. Zhao et al., 2014).

Table 2. 1 Examples of the main polyphenols found in plants and their properties.

Polyphenol class	Sub class	Examples	Biological Properties	References
Phenolic acids	Hydroxybenzoic acids	Gallic acid, Salicylic acid, Salicylaldehyde, Protocatechuic acid	Potent antioxidants, anti-inflammatory, antifungal, antiseptic properties	(Y. Zhao et al., 2014)
	Hydroxycinnamic acids	Coumaric acid, Caffeic acid, Ferulic acid, Sinapic acid, Quinic acid, Chlorogenic acid, Cichoric acid, Rosmarinic acid	Potent antioxidants, anticarcinogenic bioactivity, anti-inflammatory effects	(Bunzel, 2012; Y. Zhao et al., 2014)
Lignans		Lariciresinol, Pinoresinol, Isolariciresinol, Secoisolariciresinol diglucoside, Matairesinol	Reduce the risk of hormone-dependent cancers and antioxidant activity	(Y. Zhao et al., 2014)
Flavonoids	Flavones	Luteolin, Apigenin, Tangeritin	Anticarcinogenic activity, antibacterial, and antioxidant activity	(Lin et al., 2008; Shukla & Gupta, 2010)
	Flavonols	Quercetin, Kaempferol, Myricetin, Galangin	Encouraging blood flow and promoting cardiovascular health properties, anti-inflammatory activity, anticarcinogenic activity and antioxidant activity	(Chen & Chen, 2013; Patel et al., 2012; Perez-Vizcaino & Duarte, 2010)
	Flavanones	Hesperitin, Naringenin, Eriodictyol	Antioxidant, anticarcinogenic, and anti-inflammatory activities, and immune system modulator	(Cavia-Saiz et al., 2010; Chakraborty et al., 2012; Hamzalioğlu & Gökmen, 2016)
	Isoflavones	Genistein, Daidzein	Antioxidant activity and estrogen mimicking property	(Hamzalioğlu & Gökmen, 2016; Luca et al., 2020)
	Flavanols	Catechins, Gallocatechin	Antibacterial, anticarcinogenic and antimutagenic properties.	(Y. Zhao et al., 2014)
	Anthocyanidins	Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin, Malvidin	Antioxidant activity, anticarcinogenic, anti-inflammatory, anti-edema	(He & Monica Giusti, 2010)
Tannins	Condensed tannins (proanthocyanidins)	Procyanidins, Propelargonidins, Prodelphinidins	Cardio protection, cancer chemoprevention and antibacterial properties.	(J. Shi et al., 2003)
	Hydrolysable tannins	Gallotannins, Ellagitannins, other Complex tannins	Antioxidant activity, antibacterial properties	(Trigo et al., 2020; Y. Zhao et al., 2014)

Dependening on their chemical structure, polyphenols are antioxidant, anti-inflammatory, or anticarcinogenic. Consequently, the consumption of polyphenols is associated with the prevention of cardiovascular diseases, neurodegenerative diseases, and cancer (Wiesner et al., 2016; Y. Zhao et al., 2014). These effects in human health depend on the class of polyphenol, the amount consumed, the bioavailability, and the source from which it is extracted (Luca et al., 2020; Williamson, 2017; Y. Zhao et al., 2014). Table 2.1 shows a summary of the classes of polyphenols found in plants and the benefits they provide to human health.

2.3.2 Carotenoids

There are some lipid-soluble pigments found in the chloroplast and chromoplast of some plants, known as carotenoids (Trigo et al., 2020). These metabolites provide yellow, orange, or red colors to the plants, and they can be used as natural food products pigments (Cooperstone & Schwartz, 2016). There are approximately 700 carotenoids due to the modification of their basic chemical structure (Rodriguez-Amaya, 2015). However, they can be categorized into two main groups, oxygen-free (α-carotene, β-carotene, and lycopene) found in carrots, tomatoes, oranges, and pumpkins; and oxygen-containing xanthophylls (lutein, neoxanthin, and zeaxanthin), mainly found in dark green leafy vegetables (Trigo et al., 2020; Wiesner et al., 2016). Carotenoid consumption has been associated with the reduction of suffering several diseases, such as cancer, cardiovascular disease, and age degeneration; these compounds have important biological functions, in the provitamin A activity (for example, β-carotene, α-carotene, and β-cryptoxanthin), they also have antioxidant activity, protecting cells and tissues from oxidative damage, and the ability to enhance immune function. (Cooperstone & Schwartz, 2016; Wiesner et al., 2016). Due to the beneficial properties of carotenoids, they can be used in dietary supplements, or as colorants in food products, cosmetics, and animal fodder (Cooperstone & Schwartz, 2016).

2.3.3 Saponins

Saponins are steroids or polycyclic triterpene glycosides distributed in plants (Lacaille-Dubois & Wagner, 2000; Y. Zhao et al., 2014). Some of the several biological activities from saponins are the antitumor, hemolytic, anti-inflammatory, antifungal, antibacterial, antimicrobial, antiallergic, and hypoglycemic activities (Lacaille-Dubois & Wagner, 2000; Wiesner et al., 2016). There are

two major classifications for saponins: triterpenoids saponins (lupan, oleanane, ursan, and hopan) and steroid saponins (spirostan, dioscin, and furostan). Saponins are found in soybeans, peanuts, lentils, oats, asparagus, potatoes, green peppers, tomatoes, and onions; their content depends on many factors such as the age, the cultivar, or the geographical location of the plant (Wiesner et al., 2016)). Many steroidal and triterpenoidal saponins hold therapeutic potential, they exhibit cytotoxic and cytostatic, detergent, and cell permeability enhancing properties (Weng et al., 2011). Saponins can also form complexes with cholesterol in the gastrointestinal tract, which increases the excretion of cholesterol and a reduction in blood cholesterol level (Hill, 2003).

2.3.4 Alkaloids

Alkaloids are natural nitrogen organic compounds efficiently biosynthesized from amino acids or from amination and transamination reactions (Voung, 2017). They can be classified by their origin or their molecular precursors, such as pyridine (nicotine), tropane (cocaine), isoquinone (codein), purine (caffeine), and steroids (solanine) (Wiesner et al., 2016). The main groups of alkaloids are indole, pyrrolidine, tropane, quinolone, isoquinoline, and izidine (Zhao et al., 2014). Plant alkaloids have a wide range of pharmacological activities, such as antiviral, antimalarial, anti-inflammatory, antibacterial, and anticancer effects. Moreover, alkaloids increased the perception of flavor and taste in fruits and vegetables (Voung, 2017). However, some alkaloids in vegetables are toxic for humans, for example, different plant species possess numerous glycoalkaloids, some of them being toxic if consumed (Wiesner et al., 2016).

2.3.5 Glucosinolates

Glucosinolates are sulfur-containing glycosides molecules. They have been detected in all families of Brassicales and Capparales. Moreover, the main sources of glucosinolates are broccoli, cabbage, brussels sprouts, and cauliflower (C. Galanakis, 2020). Glucosinolates are hydrolyzed by the endogenic enzyme myrosinase, when the plant tissue is disrupted; once they are hydrolyzed, they release volatile products, such as isothiocyanates (Wiesner et al., 2016). The products from hydrolysis have different biological activities such as defense compounds and attractants. They also hold functions in human health as cancer-preventing agents, or in novel industries as biopesticides, and flavor compounds. (Hamzalioğlu & Gökmen, 2016). There are more than 130

glucosinolates, however, the most extensively studied glucosinolates are the aliphatic, ω-methylthioalkyl, aromatic, and heterocyclic (indole) glucosinolates (Fahey et al., 2001).

2.3.6 Phytosterols

Phytosterols are cholesterol-like molecules found in plant foods, with the highest concentrations occurring in vegetable oils (Gachumi et al., 2021). There are two main classes of phytosterols: sterols and stanols. The most common sterols in plants are sitosterol and campesterol. Stanols, on the other hand, comprise 10% of total phytosterols found in plants (Wiesner et al., 2016). Phytosterols are absorbed in trace amounts in human consumption, and they inhibit the absorption of intestinal cholesterol including recirculating endogenous biliary cholesterol, a key step in cholesterol elimination. Humans obtain all the phytosterol content in their blood and tissues from the diet (Gachumi et al., 2021). The greatest concentrations of phytosterols are found in unrefined plant oils, nuts, and olive oils. However, nuts, seeds, whole grains, and legumes are good sources of phytosterols as well (Wiesner et al., 2016). Phytosterol consumption can reduce cholesterol levels and prevent coronary heart disease, increase the activity of antioxidant enzymes, and reduce oxidative stress, they also can prevent cancer development. It is recommended to consume 2 g of phytosterols per day to stay healthy (Cabral & Klein, 2017).

2.3.7 Fatty acids

Fatty acids are considered essential bioactive compounds since the human body cannot synthesize them; they can be extracted from different plant sources to fortify the human diet due to their beneficial properties (Husain & Ahmad, 2020). Plant oils are good sources of fatty acids, and they come mostly from olive, palm fruit, and oilseeds such as sunflower, corn, walnut, and peanut; however, other fruits are good sources of fatty acids as well, for example, eggplant fruit, brussels sprouts, parsley, and avocado (Silva et al., 2020; Wiesner et al., 2016).

The fatty acids are mainly classified into saturated, monounsaturated, and polyunsaturated, for example, capric acid, oleic acid, and linoleic acid respectively; it has been shown that a monounsaturated fatty acids diet can reduce cholesterol levels and may prevent the risk of suffering from cardiovascular diseases (Wiesner et al., 2016). The fatty acids can be extracted from the plant source with different techniques, such as supercritical fluid, pulsed electric field, and microwave-assisted extraction (Balkrishna et al., 2021; Zamanhuri et al., 2021).

2.4 Fruit and vegetable waste as a source of bioactive compounds

Fruits and vegetables are necessary for a balanced diet, their consumption is associated with the prevention of cardiovascular and major diet-related chronic diseases. This can be explained by their content in different bioactive compounds (BC) (Soquetta et al., 2018). However, many of the fruits and vegetables produced globally end up as waste, most of them generated by industrial food processing and considered as by-products.

The amount of fruit and vegetables produced globally has been increasing due to the change in dietary habits and a growing population (Coman et al., 2020). The waste generated from fruits and vegetables represents 15 to 30% of the raw material used in the food processing industry (Md Salim, 2017; Sagar et al., 2018).

Only flesh or pulp is eaten in most fruits and vegetables; however, large quantities of phytochemicals and nutrients are still present in the waste. (Rudra et al., 2015). The high amounts of fruit and vegetable waste (FVW) need to be considered for their potential content on valuable components.

The wastes from fruit and vegetables contain several BC, such as phytochemicals (polyphenols and carotenoids) (Coman et al., 2020). These compounds can be extracted from FVW (Figure 2.3) with different extraction techniques and then, the extracted compound can be used in food, pharmaceutical, or cosmetic industries.

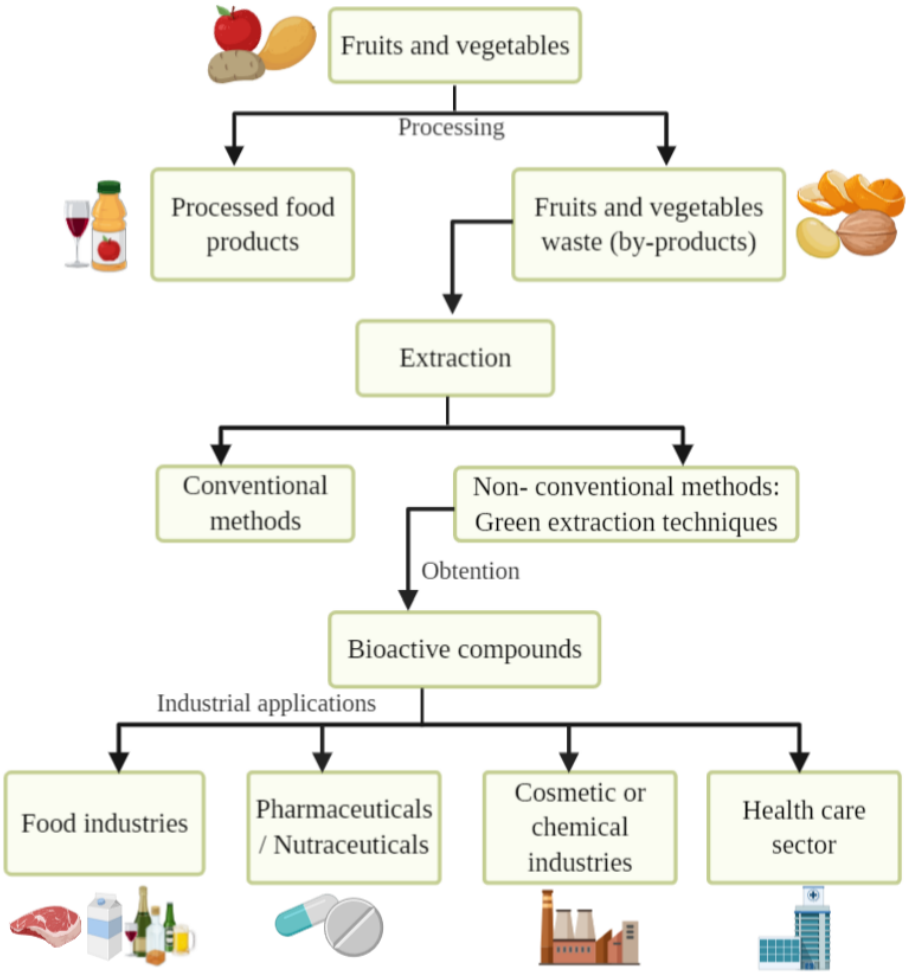


Figure 2. 3 Schematic representation of the use of fruits and vegetables waste to obtain bioactive compounds and their industrial applications.

FVW or food industrial by-products exist in the form of peels, kernels, pomace, seed fractions, stem, stones, pulp, and skin (Coman et al., 2020; Trigo et al., 2020; Tylewicz et al., 2018). Table 2.2 shows examples of FVW resulting from the most processed fruits and vegetables in the world and some BC that can be obtained from them. The most widely distributed BC available in FVW are phenolics and carotenoids (Trigo et al., 2020). However, the final BC content will depend on several factors such as fruit/vegetable variety, stage of maturation, the extraction method used, and the conditions of the extraction.

Table 2. 2. PART 1/2. Examples of bioactive compounds found in fruit and vegetable waste.

Fruits / Vegetables	Fruit and Vegetable Waste (FVW)	Approximate waste (%)	Bioactive compounds (BC)	Examples of approximate BC content (mg/ 100g) Dry Matter	References
Apple	Peel, pomace, seed	25 - 30	Phenolics: gallic acid, ferulic acid, caffeic acid, p-coumaric acid; Flavonoids	17.2 (gallic acid from peel) 303–306 (flavonoids from peel)	(Ajila et al., 2012; Coman et al., 2020; Jairath et al., 2016; Saini et al., 2019)
Banana	Peel	35	Phenolics: gallic acid, galangin; Flavonoids: quercetin, pro-anthocyanidins, catechin	29.2 (gallic acid from peel) 109.44 (galangin from peel) 196 (flavonoids from peel)	(Adiamo et al., 2018; Ajila et al., 2012; Anal et al., 2014; Saini et al., 2019)
Citrus	Peel, rag, seed	50	Phenolics: gallic acid, ferulic acid, vanillic acid, caffeic acid; Flavonoids: naringin, hesperidin, neohesperidin, diosmin, luteolin, sinensetin, rutin, kaempferol, quercetin; Carotenoids: Neochrome, lutein, β-cryptoxanthin, β-citraurin, luteoxanthin, cryp- tochrome, ξ-carotene	25.9 (gallic acid from orange peel) 29.2 (gallic acid from lemon peel) 206 (naringin from Bahia orange peel) 4117 (hesperidin from Bahia orange peel) 204 (carotenoid content from tangerine peel) 410 (carotenoid content from orange peel)	(Ajila et al., 2012; Deng et al., 2012; Pereira et al., 2017; Trigo et al., 2020)
Grape	Stem, skin, pomace, seed	15- 20	Phenolics: flavonol glycosides, anthocyanins, catechins, stilbenes; Carotenoids: lycopene	16.2 – 139 (phenolics from pomace) 25.96 (catechin from peel) 7 (lycopene from pomace)	(Deng et al., 2012)
Start Fruit	Peel, seed	10-20	Phenolics: catechin, epicatechin, ferulic acid, gallic acid, kaempferol	76.36 (catechin from peel) 52.92 (ferulic acid from peel) 4.36 (gallic acid from seed)	(Coman et al., 2020; Padayachee et al., 2017)
Mango	Peel, stones, kernel	60	Phenolics: gallic acid, chlorogenic acid, cyanidin 3-glucoside, kaempferol; Carotenoids	72.1 (gallic acid from kernel) 5.36 (gallic acid from peel) 49.20 (chlorogenic acid from peel) 37- 395 (carotenoids from peel)	(Deng et al., 2012; Saini et al., 2019)
Blueberry	Peel, leaves	10 – 20	Phenolics: epicatechin, catechin, cyanidin 3-glucoside, gallic acid, chlorogenic acid, anthocyanins; Flavonoids: quercetin, kaempferol, myricetin	61.16 (catechin from peel) 14.24 (gallic acid from peel) 113.4 (flavonoids from peel)	(Deng et al., 2012; Sagar et al., 2018; Saini et al., 2019; Song et al., 2013)

Table 2.2 PART 2/2. Examples of bioactive compounds found in fruit and vegetable waste.

Fruits / Vegetables	Fruit and Vegetable Waste (FVW)	Approximate waste (%)	Bioactive compounds (BC)	Examples of approximate BC content (mg/ 100g) Dry Matter	References
Guava	Peel, seed, leaves	10	Phenolics: catechin, galangin, homogentisic acid, gallic acid, kaempferol, cyanidin 3-glucoside	31.48 (catechin from peel) 5.56 (gallic acid from seed)	(Duarte et al., 2016; Trigo et al., 2020)
Pomegranate	Peel, mesocarp	40 – 45	Phenolics: ellagic acid, gallic acid, punicalin, punicalagin; Carotenoids	0.45 -168.3 (phenolics from peel) 0.32 (carotenoids from peel)	(Deng et al., 2012; Goula et al., 2017; Saini et al., 2019)
Avocado	Stones, peel	30 - 40	Phenolics: Cyanidin 3-glucoside, catechin, chlorogenic acid, homogentisic acid, gallic acid	7.20 (phenolics from peel) 8.39 (phenolics from stone)	(Ajila et al., 2012; Andres et al., 2017; Trigo et al., 2020)
Potato	Peel	30-35	Phenolics: chlorogenic, gallic, protocatechuic and caffeic acids; Flavonoids	2.33 – 14.031 (phenolics from peel) 3.31 (Flavonoids from peel)	(Kujala et al., 2000)
Tomato	Skin, core, pomace, seed	20	Phenolics: gallic acid; Carotenoids: lycopene	5.3 (gallic acid from peel) 13.8 (gallic acid from pomace) 1.32 (lycopene from pomace)	(Schieber, 2017; Vodnar et al., 2017)
Red beet	Peel, crown	15	Phenolics: Ferulic acids, tryptophane, p-coumaric acids, cyclodopa-glucoside derivatives	15.5 (phenolics from peel) 11.4 (phenolics from crown)	(Md Salim, 2017)
Carrot	Pomace	20	Phenolics; Flavonoids; Carotenoids: α-carotene, β-carotene	5.2 (phenolics from pomace) 8.5 (flavonoids from pomace) 4.6 (carotenoids from pomace)	(M. Wadhwa, 2013; Stoll et al., 2003)
Broccoli	Leaves and stalks	75	Phenolics; β-carotenoids	15.04 (phenolics from stalk) 3.02 (β-carotenoids from stalk)	(Coman et al., 2020)
Cauliflower	Leaves, steam	75	Phenolics: kaempferol, gallic acid, quercetin, ferulic acid	5.9 (phenolics from leaves)	(Mathew & Negi, 2017)
Garlic	Husk	15-30	Phenolics: ferulic acid, gallic acid; Flavonoids	56.26 (phenolics from husk) 0.617 (Flavonoids from husk)	(Coman et al., 2020)

2.4.1 Banana waste

Bananas are the most cultivated fruit around the world. Banana fruit production generates large quantities of waste such as rhizomes, fruit stalks, leaves, peels, and pseudo-stems, components containing useful BC. Banana waste is rich in phenolic acids (ellagic acid, vanillic acid), flavonoids (quercetin, pro-anthocyanidins, catechin), saponins, and sterols (campesterol, stigmasterol, β-sitosterol) (Sagar et al., 2018). Banana peels account for about 35% of the total fruit weight (Coman et al., 2020).

It is estimated that banana peel contains 928 mg /100g Dry Matter of total phenolic compounds, which is 25% more than the total phenolic content from the pulp. Furthermore, certain phytochemicals are found in peels in larger concentrations compared to the pulp, for example, the gallocatechin content is five times higher in the banana peel than in the pulp. The BC contained in the waste plays a major role in reducing cholesterol levels, lowering hyperglycemia, and have a strong antioxidant effect (Coman et al., 2020).

2.4.2 Apple waste

Apples are the third large production of fruits in the world, just after bananas and watermelons. The key waste produced during the processing of apple juice is apple pomace, which accounts for almost 30 percent of the whole fruit (Wolfe & Liu, 2003). The pomace is mainly composed of peels and flesh, comprising more than 90% of the fruit, followed by seeds comprising around 2 to 4%, and the stem is about 1% of the fruit. Those wastes are rich in polyphenols such as flavonoids, catechins, hydroxybenzoic acids, hydroxycinnamic acids, and their derivatives (ferulic acid, p-coumaric acid, caffeic acid, and chlorogenic acid) (Md Salim, 2017).

In the epidermis and the tissue just below the skin, the BC content in the apple is greater than in the middle portion of the fruit. (Gunes et al., 2019). The BC found in apple peels have plenty of benefits, for example, phloridzin may be used as a possible obesity therapeutic agent, and as an antihyperglycemic compound for diabetes mellitus (Md Salim, 2017; Tylewicz et al., 2018). Some experiments have been carried out to integrate defatted apple seeds into chewing gum to determine the dissolution kinetics of phloridzin; the findings of the analysis revealed that chewing gum may be an effective delivery material for phloridzin uptake (Grace et al., 2009).

2.4.3 Blueberry waste

The blueberry waste comprises peel, seeds, leaves, and in some cases pomace. The leaves are known to be lost during the mechanical harvesting of the fruit, the peel and seeds are commonly wasted once they enter a food processing step (as by-products), and the pomace is mainly obtained after the juice production. These wastes are rich sources of phenolic acids, flavonoids, and anthocyanins (Tylewicz et al., 2018). The anthocyanins are also known for their antioxidant properties and are mainly found in the blueberry peel (Sójka et al., 2013). For example, it is reported that the blueberry peel has more Total Anthocyanin Content (TAC, expressed as cyanidin-3-glucoside equivalents) compared with the pulp, the peel has between 102.7 to 504.5 mg/100 g TAC and the berry pulp has 27.5 to 102.2 mg/100 g TAC (Md Salim, 2017; Tylewicz et al., 2018). Furthermore, the total phenolic content (in gallic acid equivalents) for the blueberry peel is approximately 300.4 mg/ 100g, for pomace 119.3 mg/ 100g, and for the seeds 4.3 mg/ 100 g (Flores et al., 2015).

2.4.4 Citrus waste

The residue of citrus juice called citrus pomace, which represents 50-70% of the total fruit weight. It contains 60-65% of peel, 30-35% of internal tissues, and approximately 10% of seeds (Rezzadori et al., 2012). Citrus peel includes dietary fiber, polyphenols, essential oils, and vitamins in terms of bioactive capacity; the essential oil derived from orange peel, which is commonly used as an ingredient in food and drinks and in cosmetic factories, is the most useful citrus derivative (Papoutsis et al., 2018).

The citrus peel contains ferulic, p-coumaric, sinapic, caffeic, vanillic, gallic, and chlorogenic acid (Rafiq et al., 2018). Sweet oranges have the major total phenolic content in Gallic Acid Equivalent (1790 μg/g), followed by grapefruits (1550 μg/g), mandarin-ellendale (1211 μg/g), and lemons-yen ben (1190 μg/g) (Rafiq et al., 2018). On the other hand, citrus seeds have a higher antioxidant component than peels. The difference in compound composition between peels and seeds can explain this. For instance, hesperidin and eriocitrin are mainly present in lemon seeds, while lemon peels are rich in neoeriocitrin, naringin, and neohesperidinin (M. Wadhwa, 2013).

2.4.5 Grape waste

Fifty percent of the world's production of grapes goes into fresh and dried grapes (raisins), and the other half into winemaking (Schieber, 2017). The principal wine by-product is grape pomace, which consists of residual pulp or pomace, skins, stems, and seeds; the grape waste constitutes around 15-20% of the total grape weight (Machado & Domínguez-Perles, 2017). Depending on the variety, geographical origin of the crop, soil, atmosphere, and processing technology, grape waste has variability in quality and antioxidant activity. (Y. Zhao et al., 2014).

Grape pomace is rich in polyphenols such as phenolic acids, flavonols, and anthocyanins (Brenes et al., 2016). Furthermore, grape skins and seeds are particularly rich sources of proanthocyanidins (M. Wadhwa, 2013; Maier et al., 2009).

Grape seeds constitute 2-5% of the grape weight and about 38-52% (dry weight), of the grape pomace (Maier et al., 2009). Furthermore, they contain 4−6% of phenolics (mainly gallic acid, catechin, epicatechin, epicatechin gallate) and 12−17% of oil which is rich in linoleic acid-omega-6 fatty acid (J. Yu & Ahmedna, 2013).

Grape seed and peel extracts have beneficial effects on human wellbeing, such as radioprotective effects, hypertriglyceridemia prevention through insulin sensitivity enhancement, and anti-inflammatory effects (Vodnar et al., 2017). Grape waste extracts are also used in foodstuffs as natural antioxidants and antimicrobials (meat products and fruit juices) (M. Wadhwa, 2013).

2.4.6 Potato waste

It is estimated that waste produced from potato processing is around 33 to 35 percent of the fresh weight, the waste consists primarily of peel (Coman et al., 2020). A substantial proportion of this waste is used for the processing of animal feed and first-generation biofuels. However, potato peels, which make up a significant proportion of the waste from the potato industry, can be used as antioxidant natural sources (Sagar et al., 2018). The recovery of these valuable components from potato peel waste can provide a cheaper alternative to the antioxidants that are utilized in the food industries.

Potatoes could be considered a good source of polyphenols among the vegetables. Polyphenols are present in the flesh and peel; the peel contains more than half of all polyphenols in the vegetable (M. Wadhwa, 2013). For example, some studies have found that potato peel has a higher amount

of chlorogenic acid than the flesh (385 ± 50 µg/g dry weight of chlorogenic acid from peel and 21.9 ± 2.0 µg/g dry weight chlorogenic acid from flesh) (Coman et al., 2020).

2.4.7 Carrot waste

The carrot is composed by the body (83.19%), head (5.01%), and peel (14.19%). Orange carrots contain 200−1000 mg/kg dry matter of β-carotene. Furthermore, carrot waste still conserves a certain number of phenolic compounds such as carotenoids and anthocyanin. The carrot pulp has an approximate total phenolic content of 4.3 mg/100g dry matter (Sharma et al., 2012). On the other hand, carrot peel has about 6.96–9.02 mg/g of total polyphenols (M. Wadhwa, 2013). The carrot peel has polyphenols with antimutagenic and antitumor properties that fight against free radicals (Lu et al., 2019).

2.4.8 Tomato waste

The principal tomato wastes are the culled tomatoes and the tomato pomace. The tomato pomace is composed of peels, seeds, and by pulp that remains after processing, commonly known as pomace (Soquetta et al., 2018).

The tomato seeds and peels have a different composition. The peels are rich in carotenoids (lycopene and beta-carotene), and phenolic compounds; on the other hand, the seeds contain mostly high-quality proteins (with high lysine content) (Valdez-Morales et al., 2014). The key ingredient in tomato peels is lycopene, which accounts for 85 percent of the overall carotenoid content and the concentration in peels is 3-5 times greater than in fresh fruit, with an approximate content of 0.88- 4.2 mg/ 100g, depending on the variety, ripeness, and processing (Giacometti et al., 2018). Nevertheless, other phenolic compounds with important biological activities like caffeic, ferulic, and chlorogenic acids, and quercetin, were also identified and quantified in tomato peels (Dong et al., 2020; Saini et al., 2019; Soquetta et al., 2018).

2.5 Extraction techniques for obtaining bioactive compounds

There is a wide variety of BC found in the wastes from fruit and vegetables. To conduct the separation, identification, characterization, and appropriate extraction process of these compounds, it is important to know the origin and the methods that can be implemented for a specific plant matrix. In general, all extraction techniques have the same objectives, for example,

to extract the targeted BC from a complex plant matrix, to increase the sensitivity selectivity of the analytical method, to transform the BC by an effective identification and separation form, and to have reproducible methods of extraction. (Smith, 2003). There are many factors that can affect the extraction, such as pressure, temperature, plant matrix, and solvent used.

The BC can be extracted from FVW with either conventional (maceration, Soxhlet extraction, and hydro distillation) or non-conventional techniques (supercritical fluid, pulsed electric field, microwave extraction, and ultrasound extraction) (Garavand et al., 2019; Sagar et al., 2018). A comparison of some extraction techniques is shown in Table 2.3

2.5.1 Conventional extraction techniques

Conventional extraction techniques are considered as classical methods of extraction. The basic principle of these types of techniques is solvent extraction and an applied heat (Zhang et al., 2018). Some conventional extraction methods include Soxhlet extraction, hydro distillation, maceration, or solvent extraction; Table 2.4 shows a brief description of the conventional extraction methods. The solvent selection is critical in the extraction process. Alcohols (methanol and ethanol) are the most used solvents for BC extraction in conventional methods. Nevertheless, several solvents are employed, for example, chlorinated solvents, such as chloroform, carbon tetrachloride, and chlorobenzene, and non-chlorinated solvents, such as acetone, and acetonitrile (Tiwari, 2015). The solubility, cost, and security issues should be considered in the selection of the solvent, for example, solvents having similar polarity with the solute work better in the extraction. The bulk of polyphenols dissolve in polar solvents, while non-polar solvents such as hexane and acetone will help in the extraction of lipids and oils (Md Salim, 2017). A solvent with low toxicity, low boiling point, and quick mass transfer capability can be a good option.

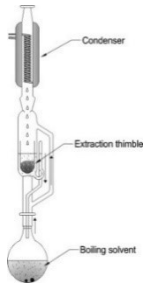
Table 2. 3- PART 1/2. Comparison of different extraction techniques for obtaining BC.

Technique classification	Name of extraction technique	Advantages	Disadvantages	BC that can be extracted	References
Conventional methods	Soxhlet	• Is the basic model technique for comparison and it is commonly used. • Best suited for small-scale industries.	• Requires large quantities of solvents. • Time consuming. • Low efficiency. • Not suitable for heat-sensitive ingredients.	Essential oils, lipids, fats, phenolics.	(Azmir et al., 2013; Garavand et al., 2019; Sagar et al., 2018)
	Hydro-distillation	• Simplest and oldest technique. • Has different classifications: steam, water, or hydro-diffusion distillation. • Used in small-scale industries. • Uses water to facilitate the extraction.	• Not suitable for heat-sensitive ingredients. • Time consuming. • Consume high energy levels.	Essential oils and phenolics.	(Azmir et al., 2013; Soquetta et al., 2018)
	Maceration	• Used in small scale extractions. • Inexpensive method.	• Time consuming. • Requires large quantities of solvents.	Essential oils and phenolics.	(Azmir et al., 2013; Garavand et al., 2019)
	Liquid-liquid extraction (LLE)	• Can be used at room temperature. • Standard, easy, and inexpensive method. • Suitable for liquid samples (by-products from beverages samples).	• Labor intensive. • Time consuming. • High degradation rate. • Requires hazardous chemicals.	Phenolic compounds.	(Sagar et al., 2018)
	Solid-phase	• Easy method. • High repeatability. • Faster separation rate tan LLE.	• Is more expensive than LLE. • Specific for polar compounds. • Unsuitable for volatile analytes.	Phytochemicals in medicinal plants and flavonoids.	(Garcia-Salas et al., 2010; Sagar et al., 2018)
Non-conventional methods or green extraction techniques	Emulsion liquid membrane	• Rapid extraction. • High efficiency. • High selectivity. • Capability of scaling up least use of toxic solvents. • Recyclability of membrane components. • Low energy consumption.	• Emulsion instability. • Emulsion leakage and swelling.	Wide variety of bioactive compounds: phenolics, flavonoids, and other phytochemicals.	(Garavand & Madadlou, 2014)

Table 2.3- PART 2/2. Comparison of different extraction techniques for obtaining BC.

Technique classification	Name of extraction technique	Advantages	Disadvantages	BC that can be extracted	References
Non-conventional methods or green extraction techniques	Supercritical fluid extraction (SFE)	• Better mass transfer. • Time saving. • Use of little amount of sample and organic solvent. • Recyclability of supercritical fluid. • Performed at room temperature.	• Not suitable for polar molecules. • Costly system.	Volatile compounds, sugars, phenolics, flavonoids, carotenoids.	(Md Salim, 2017; Nerome et al., 2015; Sagar et al., 2018)
	Microwave assisted extraction (MAE)	• Better extract quality. • High selectivity. • High extraction yield and less extraction time. • Cost-effective in comparison with Soxhlet. • Simply operable in comparison with SFE. • Short extraction time in comparison with UAE.	• Expensive equipment. • Operation is difficult compared to UAE. • Use organic solvents. • Non suitable for non-polar compounds. • Unsuitable for heat sensitive ingredients.	Polyphenols: flavonoids, phenolics, carotenoids.	(Chen & Chen, 2013; Sagar et al., 2018)
	Ultrasound assited extraction (UAE)	• Less energy and power usage. • Higher product yield. • Short processing time and less chemical usage. • Appropriated for thermo-sensitive ingredients.	• Proper optimization in ultrasound frequency. • Generation of free radicals at high sonication powers. • Solvent needed procedure. • Difficult to scale up for industrial uses.	Phenolics, lipids, carotenoids.	(Ameer et al., 2017; Azmir et al., 2013)
	Enzyme assited extraction (EAE)	• Uses water as solvent. • Suitable to separate bound compounds. • High extraction rate.	• Enzyme sensitivity. • Difficult to scale up to industrial applications. • Expensive price of enzyme for large volume of samples.	Essential oils and bounded phytochemicals.	(Sagar et al., 2018)
	Pulsed electric field extraction (PEFE)	• Short extraction time. • High efficiency. • Low energy consumption. • High final yield. • Reduced environmental impact in comparison with Soxhlet.	• High maintenance. • Accurate control parameters.	Phytosterols and polyphenols.	(Pourzaki et al., 2013)
	High hydrostatic pressure extraction	• Accelerated mass transfer. • Can be use with polar and non-polar compounds. • Do not utilize a heating process. • Low energy consumption. • High yield of extraction.	• Expensive equipment. • Difficulty of maintenance. • High pressure needed.	Polyphenols.	(Shinwari & Rao, 2018)

Table 2. 4. Description of the conventional extraction techniques.

Technique	Process description	Reference
Soxhlet extraction	**1.** A small dry sample is put into a thimble. **2.** Then, the thimble is set in a distillation flask containing the desired solvents (such as ethanol, methanol, chloroform, acetone, acetonitrile, or hexane). **3.** Once an overflow level is reached, the thimble-holder containing solvent-solute mixture is suctioned by a siphon. **4.** The siphon empties the solution into the distillation flask. **5.** This solvent carries the separated extracts into the bulk liquid. **6.** Solute (extracts) remains in the distillation flask and the solvent goes back into the plant tissue matrix. **7.** This procedure continues until the extraction is finished. Extraction time can be up to 48 hrs. Temperature ranges depend on the solvents used. 	(Azmir et al., 2013; Sagar et al., 2018; Zhang et al., 2018; Zygler et al., 2012)
Maceration	**1.** The sample is grinded into small particles, this increases the surface area, facilitating the proper mixing with the solvent. **2.** The solvent is added in a closed vessel (water, organic solvents, or their combination). **3.** The liquid is discarded, and then the solid residue is squeezed to recover the prepared solution. **4.** Finally, the prepared solution is separated from impurities with a filtration process. Agitation is used in this process to facilitate the extraction. This action promotes the diffusion and removes the concentrated solution from the sample surface and brings new solvent for increase the extraction yield.	(Azmir et al., 2013; Sagar et al., 2018; Soquetta et al., 2018)
Hydro-distillation	**1.** The sample is packed in a still compartment. **2.** Water is added and then boiled. **3.** At the same time (depending on the type of hydro-distillation), direct stream is injected into the sample. **4.** The hot water and the steam act as the extractor factors. **5.** Then a condenser cools the vapor mixture containing water and the compound of interest. **6.** The condensed mixture goes to a separator, to separate the compound from the water. The physicochemical processes involved are hydro diffusion, hydrolysis, and decomposition by heat. There are three types of hydro-distillation: the waster distillation, water and steam distillation and direct steam distillation. This process should be performed before drying of sample.	(Azmir et al., 2013; Garavand et al., 2019)
Reflux or solid liquid extraction	This process operates at a constant temperature with solvent repeatable evaporation and condensation: **1.** A dry sample is mixed with the solvent in a packed bed reactor or an agitated vessel. **2.** The liquid is boiled and condensed. The concentration reactants remain constant throughout the process. **3.** Followed by a centrifugation and filtration process to obtain the extract. Temperature ranges depend on the solvents used. The difference between this method of extraction and the Soxhlet extraction is that in Reflux extraction the condensates are collected while boiling so the amount of solvent remains constant. On the other hand, the Soxhlet process has a permeable beaker in between (that contains the sample), the liquid that evaporates is collected there, extract or dissolve the sample, and then is recycled back.	(Bandar et al., 2013; Chua et al., 2016; Md Salim, 2017; Zhang et al., 2018)

The particle size affects the efficiency of the extraction. Smaller particles enhanced the penetration of the solvents in them, however, if the size is too small the subsequent filtration process will be difficult (Zhang et al., 2018). The temperature also plays an important role, high temperatures enhance the solubility and diffusion; however, they can cause solvents loss, leading to impurities formation and possibly decomposition of thermosensitive compounds (Azmir et al., 2013).
The conventional methods have long processing times of extraction. However, increasing the time does not influence the extraction until the solvent balance is achieved within and outside the solid matrix (Zhang et al., 2018).

2.5.2 Green extraction techniques

To overcome the disadvantages of conventional extraction techniques, there are other extraction methods that exhibit shorter extraction times, high efficiency, and selectivity, as well as reduce the use of solvents. These techniques are called non-conventional or green extraction techniques (Mena-García et al., 2019). The name "green extraction" is due to the less energy consumption, renewable natural products, the reduction of hazardous substances, and less time in the extraction process. (Azmir et al., 2013; Chemat et al., 2012). These methods of extraction are the new trends for obtaining the BC from many plant sources including FVW, since they represent a sustainable option compared with conventional extraction techniques. Some examples of these techniques are microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and pulsed electric field extraction (PEFE). The non-conventional extraction techniques follow a series of principles, such as the innovation in the selection of renewable plant resources such as FVW, the use of alternative solvents, the reduction of energy, the production of co-products, obtaining a biodegradable and pure extract, and the reduction in the number of unit operations. Most organic solvents are volatile, flammable, and even toxic. The use of alternative solvents is a good option to replace the petrochemical solvents (Chemat et al., 2012). However, some alternative solvents have limitations, such as the high viscosity, and high boiling point. Table 2.5 shows some examples of alternative solvents that can be used in green extraction.

Table 2. 5. Alternative solvents for green extraction techniques (Chemat et al., 2012; Mena-García et al., 2019).

Solvent	Description	Extraction technique	Application	Solvent Power	Cost	Environmental Impact
Solvent Free	-	MAE and PEFE	Antioxidants, glucosinolates, essential oils, and pigments	Polar	Low	Medium
Water	It has polar nature for natural-water soluble compounds.	MAE, UAE, and SFE	Essential oils and aromas	Polar	Low	Low
CO_2	Non-flammable odourless gas produced during the burning of fossil fuels, by alcoholic fermentation.	SFE	Phenolics	Weakly polar and non-polar	Low	Low
Ethanol	Obtained from the fermentation of sugar-rich materials (sugar beet and cereals).	MAE and SFE	Pigments and antioxidants	Weakly polar	Medium	Low
Glycerol	By-product from the trans-esterification of vegetable oils.		Polyphenols, oils, and fats	Weakly polar	Low	Low
n-Hexane*	Petrochemical solvent.	MAE and SFE	Fats and oils	Non-polar	High	High

The extraction yield will depend on several factors, such as the process design, solvent selection, and type of matrix. The novel techniques of extraction can also be used as a pre-treatment or combined with alternative solvents to enhance the extraction. Table 2.6 shows examples of the implementation of some green extraction techniques.

Table 2. 6. Examples of BC extraction from FVW using green extraction techniques.

Extraction Technique	Concept	Variables	Source	BC extracted	References
Microwave-assisted (MAE)	Electromagnetic fields between 300 MHz to 300 GHz. The solvent penetrates the solid matrix by diffusion and the solute is dissolved.	500 W, 122 s of extraction time, solvent-to-solid ratio (25 mL/g) using acetone in water concentration. (51% v/v) 850 W and 2450 MHz in a domestic microwave oven	Citrus peel Carrot peel	Phenolics (12.20 mg/g Gallic Acid Equivalent Dry matter) β-carotene (58 mg/100 g Dry matter)	(Angiolillo et al., 2015; Chemat et al., 2012; Hiranvarachat & Devahastin, 2014; Nayak et al., 2015; Soquetta et al., 2018)
Ultrasound-assisted (UAE)	Sound wave between 20 kHz to 100 MHz. Cavitation to form pores that facilitate the leaching of organic compounds and inorganic plant matrix.	25 kHz – 150 kHz. 40 min and 0.764 W/cm^2	Apple pomace	Catechin (555 mg/100 g Dry weight)	(Azmir et al., 2013; Pingret et al., 2012; Rajha et al., 2015; Soquetta et al., 2018)
Pulsed electric field (PEFE)	Use of two electrodes. The pulse varies between 100-300 V/cm to 20-80 kV/cm. At room temperature.	3 kV/cm PEF, 30 pulses series at room temperature	Citrus peel	Phenolics (75% yield)	(Barba et al., 2016a; Corrales et al., 2008; Parniakov et al., 2014)
Supercritical fluid (SFE)	Changes in pressure and temperature, transforming the gas in the supercritical fluid.	CO_2, with ethanol as a co-solvent, 40-60°C and 350-500 bar	Grape seeds Grape skin	Polyphenols (10% yield) Polyphenols (25%)	(da Silva et al., 2016; Manna et al., 2015; Soquetta et al., 2018)
Enzyme-assisted (EAE)	Use of various enzymes such as cellulases and pectinases that hydrolyze the cell wall components, increasing cell wall permeability, resulting in the extraction.	Using Celluzyme, 2500 ppm Using Pectinolytic, 50°C, 5000 ppm	Citrus peel Grape pomace	Phenolics (65.7% yield) Polyphenols (98.1% yield)	(Kammerer et al., 2005; Li et al., 2006; Puri et al., 2011)

2.5.2.1 Microwave – assisted extraction (MAE)

Table 2. 7. Examples of optimum operating conditions for MAE.

Source	Optimal conditions	Yield of compound extracted	References
Grape skin	40 mL, 50% ethanol as solvent, 1 g of grape skin, 540W of microwave power and 3 min of extraction.	Polyphenols 13-16%	(H. B. Yu et al., 2014)
Grape skin	Presence of stirring, 100°C, 500 W of microwave power, 25 mL, 40% ethanol in water, 2 g of grape skin and 5 min of extraction.	Total anthocyanins 1.86 mg/g	(Liazid et al., 2011)
Mandarin peel	Solvent free, 2.5 g of peel, 339.19 W for 53 s of extraction.	0.86 mg/g of total phenolic content and 0.24 mg/g of total flavonoid content	(Şahin, 2015)
Lemon peel	Ethanol 48%, ratio 1:28 g/mL, 400 W and 123 s of extraction.	1574 mg/100 g of total phenolic content, gallic acid equivalent	(Dahmoune et al., 2013)
Lime peel	55% ethanol, 140W microwave power for 45 s with 8 repeats of the extraction step.	49 mg/g of total phenolic content, gallic acid equivalent.	(Rodsamran & Sothornvit, 2019)
Banana peel	Water as solvent, ratio 2:100 g/mL, 6 min of extraction, 960 W of microwave power.	50.55 mg/f of total phenolics	(Vu et al., 2019)
Tomato peel	1:10 solvent ratio at 400 W, using ethyl acetate as solvent, for 1 min.	Lycopene 13.59 mg/100g	(Ho et al., 2015)
Potato waste	60% ethanol, 80°C, for 2 min 1:40 g/mL solid-solvent ratio, 300 W of microwave power.	11 mg/g total phenolic content, gallic acid equivalent	(T. Wu et al., 2012)
Broccoli by-products	500 W of microwave power, 50 mL of water as solvent, 5 min of extraction.	317 µg/mg total phenolic content, gallic acid equivalent	(S. S. Ferreira et al., 2018)

Microwaves provide dielectric heating and solute dissolution; they are electromagnetic fields between 300 MHz to 300 GHz. Plenty of BC can be obtained through this extraction method, such as phenolics, carotenoids, and flavonoids (Soquetta et al., 2018). In MAE, the combination of heat and mass gradients is responsible for the acceleration and high extraction yield (Vernès et al., 2019). MAE begins with the solvent penetration into the plant matrix, then, with the aid of electromagnetic waves, the components break down, and the solubilized components are transferred from the insoluble matrix to the bulk solution, finishing with the liquid and residual solid phase separation (Panzella et al., 2020).

MAE can be performed with or without the use of solvent; ethanol in combination with water is the most used, due to its good capacity to absorb microwave energy and good compound solubilization. The factors that affect the MAE are the choice of microwave power, the temperature of extraction, time, and amount of solvent (Panzella et al., 2020; Soquetta et al., 2018). It is reported that the ratio of solvent-solid between 10:1 to 20:1, is optimal for this extraction. Furthermore, particle sizes for the solute between 100 μm to 2 mm are typically used and the sample can be pre-soaked in the solvent to improve the extraction efficiency (E. Davis, 2018).

MAE can be classified into two systems, closed and open systems. Closed systems are associated with high pressure while the open system is employed under atmospheric operating pressure. Nevertheless, the open system can also run at high pressure. In a closed MAE system, the extraction is carried out in a sealed vessel with different microwave radiations, the high pressure and temperature allow faster extraction times due to the solvent's ability to absorb the microwave energy. However, closed systems are susceptible to volatile compound losses. The most common system used in BC extraction is the open MAE system due to its higher sample throughput, solvent addition, and atmospheric system conditions (Chan et al., 2011a).

The heat transfer from microwave radiation allows the moisture in the cell to evaporate, resulting in an increase in the pressure within the plant matrix. This pressure splits the cell membranes and enables the penetration of the solvent to expel the BC, dissipating the heat volumetrically within the irradiated medium (Vernès et al., 2019). A solvent's ability to consume and transform microwave energy to heat depends on the dissipation factor (tan δ), as denoted in Eq. (1.1):

$$\tan(\delta) = \frac{\varepsilon'}{\varepsilon''} \qquad (1.1)$$

Where ε 'is the solvent's dielectric constant or relative permittivity, representing its capacity to be polarized by an electric field, and ε" is the dielectric loss factor associated with the conversion of electromagnetic energy into heat efficiency. Based on that, polar solvents have a high dielectric loss, which means they absorb microwaves intensely (Vernès et al., 2019). It is possible to change the dielectric property of a solvent by mixing it with other solvents to increase extraction (Md Salim, 2017).

There are several cases that prove the efficiency for MAE, for example, a study for isolating hesperidin from citrus skin at 1 kW, 2.45 GHz of power with 140°C for 8 min, using ethanol as solvent, reported an 86.8% (47.7 mg/g) of hesperidin yield, almost 10% more efficient than

conventional extraction (Inoue et al., 2010). It is reported that mango peel has 1.5 to 6 times more phytochemicals and antioxidant power after 15 min of the MAE, compared to conventional solvent extraction (10 h), and required less extraction time (Dorta et al., 2013). More examples of optimum operating conditions for MAE are listed in Table 2.7. The frequencies that are commonly used in microwave ovens (for domestic uses and laboratories) are 0.915 GHz and 2.45 GHz (Garavand et al., 2019).

2.5.2.2 Ultrasound – assisted extraction (UAE)

The UAE, also called sonication, uses a sound wave from 20 kHz to 100 MHz, this wave travels through a medium and creates compression and expansion, producing a cavitation phenomenon (Garavand et al., 2019). The cavitation process involves the generation, growth, and collapse of tiny bubbles; when the bubbles exceed a critical diameter, they break down, inducing a high amount of energy that converts the kinetic motion into heat. It is estimated that bubbles have a temperature of approximately 4700°C and 1000 atm of pressure. The materials that have a cavitation effect are liquid and liquid-containing solids (Azmir et al., 2013).

The physical phenomena of UAE can be described with two steps: the diffusion through the cell wall, and the rising of the cell material, after the cell wall breakdown. For this extraction techniques the moisture, particle size, solvent, temperature, time of sonication, and pressure are the main factors to consider for an effective extraction (Vernès et al., 2019). For BC extraction frequencies between 20 to 100 kHz, and 80 to 200 W of power are used, therefore, cavitation is produced when the pressure applied to the medium decreases below the liquid-vapor saturation pressure (Vernès et al., 2019). The ultrasound can be applied directly to the medium, with higher intensities, or with indirect methods, such as an ultrasonic water bath, until the waves enter the sample (Soquetta et al., 2018). It is reported that at low frequencies from 20 to 40 kHz the yield of extraction is higher, also low temperatures enhance cavitation and viscous solvents reduce it (Tiwari, 2015). The combination of extraction techniques can be used to enhance the yield of extraction. For example, SFE of 50°C with 25 mPa of pressure, followed by a UAE, with 400 W of power, using ethanol and water as a co-solvent, leads to a global phenolic extraction yield of 9.87%, twice the performance of only using SFE for blackberry bagasse (Pasquel Reátegui et al., 2014). Another study shows that a 97.4% extraction yield of lycopene from tomato paste can be obtained with

microwaves (98W) delivered to an ultrasonic bath, operating at 40 kHz and 50W, with 365 s of extraction time and a temperature of 86.4°C, using ethyl acetate as a solvent; in comparison, an 89.4% lycopene yield can be obtained by only using UAE (Lianfu & Zelong, 2008). These results imply that a combined extraction method could be more efficient for obtaining bioactive compounds.

UAE have several advantages, such as the reduction in time, energy and power usage, less thermal degradation, and a good-quality extract, for example, there is a study that shows a 2.3-to-3-fold increase in total phenolic content from grape pomace in the temperature range of 20 to 50°C for 2.5 min using UAE at 55 kHz, water as a solvent, and 22.9 W/cm^2 compared to conventional extraction (González-Centeno et al., 2015).

2.5.2.3 Pulsed electric field extraction (PEFE)

The different extraction techniques work in destroying the cell membrane structure for the liberation of BC. The PEFE (pulsed electric field extraction) is a non-thermal process that consists of a living cell suspension, which is then subjected to an electric field, an electrical potential then passes across the cell membrane, splitting the molecules in the cell membrane according to their charge. Once the transmembrane potential of 1 V is reached, the charged molecules create pores in the weakest areas of the membrane, increasing the membrane permeability and causing electroporation (Azmir et al., 2013; Sagar et al., 2018).

The extraction yield depends on various parameters, such as the field strength, the energy input, number of pulses, temperature, and plant matrix. It is estimated that 500 and 1000 V/cm of the electric field, during 10^{-4} to 10^{-2} s can break the cell membrane for the releasing of BC, with little temperature increment, this can reduce the degradation of thermolabile components. PEFE is often used as a pre-extraction treatment, and it can be combined with other extraction techniques to enhance the final extraction yield (Azmir et al., 2013). The PEFE of anthocyanins from grape skin at 3 kV/cm of pulsed electric fields showed a high selectivity after 1 h of extraction, using this technique the extraction yield is 50% higher than conventional extraction methods (Corrales et al., 2008). Another study shows that PEFE can be applied to obtain a 39% yield of carotenoids from tomato peel, using 5 kV/cm of intensity during 300 μs, with a mixture of hexane, acetone, and ethanol (50:25:25) as solvent (Luengo et al., 2014). Some phenolic acids, such as chlorogenic, ferulic, and salicylic acids are found in apple pomace under a PEFE treatment with optimal

conditions of 2 kV/cm of electric field intensity, 500 μs of extraction time, and 12.5% w/v solid to water ratio, obtaining a total phenolic yield content 37% higher than using conventional extraction technique (Lohani & Muthukumarappan, 2016). PEFE enhances the extraction of BC from FVW as it is less time consuming, it uses less solvent, and lower temperatures of extraction compared with conventional extraction techniques; those advantages make PEFE a good method for industrial application (Giacometti et al., 2018; Saini et al., 2019). Usually, PEFE operates at room temperature for less than 1 s (Barba et al., 2016b). In certain situations, however, the application of electric fields at room temperature is not adequate, so pulsed ohmic heating may be used through ionic motions in the series to raise the temperature (Soquetta et al., 2018).

2.5.2.4 Enzyme – assisted extraction (EAE)

The enzyme-assisted extraction (EAE) can be employed as a pre-extraction or extraction method for obtaining BC. The plant cell wall is destructed, and the bounded BC attached to the carbohydrate and lipid chains are released (Garavand et al., 2019). This process is done under the action of some enzymes, such as cellulase, pectinesterase, hemicellulase, fructosyltransferase, pectinase, α-amylase, and protease in the solvent extraction. EAE enters the green extraction techniques category due to the natural origin of enzymes and water, instead of using hazardous solvents (Garavand et al., 2019). EAE is employed when the plant matrix compounds are preserved by hydrogen or hydrophobic bonding in the polysaccharide-lignin network and are not accessible to remove using a solvent in a traditional extraction process. The factors that affect EAE are the moisture content, the particle size of the material, chemical composition of the plant matrix, type and dosage of the enzyme, solvent amount, time, and temperature (Azmir et al., 2013).

This technique has been studied for the extraction of several BC found in FVW, such as the phenolic extraction (18-20 mg/g) from grape seeds, using pectinase (Štambuk et al., 2016) and phenolic extraction (0.152 mg/g) from apple pomace, using Pectinex® (Oszmiański et al., 2011). EAE as other extraction techniques can be combined to enhance extraction yield. For example, a combination of EAE and SFE can separate almost twice the total phenolic content from pomegranate peel than conventional methods, the process begins with a pre-treatment with pectinase, protease, cellullase, and viscozyme, followed by supercritical CO_2, using ethanol as solvent (Mushtaq et al., 2015).

2.5.2.5 Supercritical fluid extraction (SFE)

Until gas and liquid stages do not occur as a separate process, the critical point of a material is reached, in other words, the critical point of a substance occurs when the densities of liquid and vapor are the same (when the substance is above its critical temperature (T_C) and pressure (P_C)). When a material is exposed to a temperature and pressure above the critical limit, the supercritical condition takes place. In this state, the fluid acquires gas/liquid properties of diffusion, viscosity, and density (Md Salim, 2017). Those properties make the extraction of the BC possible in a short time. In supercritical fluid extraction (SFE) the analyte is distributed in two different stages: the separation and stationary phase (Md Salim, 2017).

SFE is a process in which an oven contains a mobile phase (normally CO_2, ethane, propane, butane, water, or pentane), which is pumped until it pressurizes the gas, then a vessel with co-solvent is also pumped to the extraction vessel, working at high pressure (Azmir et al., 2013; Panzella et al., 2020; Saini et al., 2019). CO_2, is the most used solvent for SFE, having a critical temperature and pressure of 31°C and 74 bars, it offers stable working conditions for pressures between 100 to 450 bar. However, due to CO_2 low polarity, the extraction is limited to mostly non-polar compounds. To overcome this limitation a chemical modifier can be added to enhance the polarity, such as ethanol, methanol, water, and acetone (Panzella et al., 2020; Soquetta et al., 2018).

Bioactive Compounds can be extracted using this technique with higher extraction yields, for example, 79% of catechin yield extraction can be obtained from grape seeds using CO_2 and methanol as a modifier (40%) (Ashraf-Khorassani & Taylor, 2004). Or a 61% of lycopene yield (7.19 mg/g) at 86°C, 34.47 MPa, 500 mL of CO_2 at 2.5 mL/min, from tomato seeds and skin (Rozzi et al., 2002).

2.6 Food industry applications

Bioactive Compounds can be used in various applications, such as in the cosmetic, pharmaceutical, or food industry. The last one being of recent interest in food improvement. Although the quality and properties of the final food products can be enhanced using BC, the selection of BC concentration becomes very important to ensure the fortification and sensory attributes of the final product (Trigo et al., 2020). Due to the beneficial health properties of BC, they can be used in the food industry to retard lipid oxidation and added to fresh or processed meats to improve color

stability, retard the appearance of off-flavors and prevent oxidative rancidity (Fontana et al., 2013); BC can also be added in the formulation of cheese products to improve antioxidant activities (Giroux et al., 2013). Some crude and powdered BC extracts are even useful against *E. coli* and *S. aureus* in vegetable soup, due to their antimicrobial properties (Sagdic et al., 2012). Table 2.8 shows some examples of the uses of BC in the food industry.

One of the most used BC extracted from FVW are phenols, which can preserve the stability of some vegetable and animal fats and could represent an environmentally friendly substitution of synthetic antioxidants, such as butylated hydroxytoluene and propyl gallate (Veneziani et al., 2017). Furthermore, phenols can be used as disinfectants in food and chemical industries since they have antimicrobial properties against *E. coli, S. aureus, P. aeruginosa,* and, *B. subtilis* strains (Azaizeh et al., 2011; Yangui et al., 2009). However, extracted phenols must pass a check on the antibacterial spectrum, to define the optimal dose and not affect the sensory properties of the final foods and still maintain their preservative action (Veneziani et al., 2017). Other widely used compounds are anthocyanins and carotenoids, which are natural coloring agents. However, the FVW source from which they come is important to maintain the stability of the BC in the food formula. For example, grape waste anthocyanins are less stable compared to potato, carrot, or radish waste anthocyanins (Lavelli et al., 2017).

In summary, the BC are used to increase the antioxidant activity, antimicrobial effect, maximize total phenolic content and enhance some sensory attributes of final foods as coloring agents (Marranzano et al., 2018). The addition of these compounds can be by crude extract, powder, or nanoencapsulation; however, the method of addition will depend on the application to which it is directed. For example, cellular antioxidant activity is reported to be higher in encapsulated BC, than nonencapsulated ones, which suggests an important role in delivering these compounds through the biological membranes (Fontana et al., 2013). Furthermore, the functionality and activity of the BC in the food will depend on external conditions such as temperature, oxygen, light exposure, ionic strength, pH, and as mentioned earlier, the method of addition (Lavelli et al., 2017).

Table 2. 8. Examples of BC applications in the food industry.

Food Category	BC Application	References
Animal products (meat, poultry, and fish)	• Reducing degree of deterioration by antioxidant properties, in lipid and proteins. The concentration varies between 5 to 1000 mg/100 g, at cold temperatures from 4°C to -18°C. • Antimicrobial potential. • Enhance of sensory attributes (colour, odour, texture, and taste)	(Andres et al., 2017; Kryževičūtė et al., 2017; Nishad et al., 2018; Pal et al., 2017; Trigo et al., 2020)
Dairy products (cheese, butter, ice cream, yoghurt, and milk)	• Retarding lipid oxidation. • Antimicrobial potential (16, 25, and 29% lower counts for total microorganisms, psychrophilic, yeasts and molds, respectively). • Improve phenolic content. • To enhance probiotic growth. • Enhance of sensory attributes.	(Costa et al., 2018; Mahajan et al., 2015; Sandhya et al., 2018; Trigo et al., 2020; Vital et al., 2018)
Beverages (Juices)	• Improvement of total phenolic content and antioxidant activity. • Enhance odour and colour.	(Kulichová et al., 2018; Trigo et al., 2020)
Bakery products (white wheat flour, cakes, cookies, muffins)	• Improves antioxidant activity and enhance total phenolic content. • Enhance of carotenoid content to improve sensory attributes. • Reduce calorie content. • Enhance dietary fiber content.	(Ordóñez-Santos et al., 2016; Pathak et al., 2017; Quiles et al., 2018; Trigo et al., 2020)
Candies	Increase in total phenolic content and antioxidant activity.	(Cappa et al., 2015; K. Kumar et al., 2017)
Mustard		(L. Davis et al., 2018)
Pasta		(Pasqualone et al., 2017)

2.7 Conclusion and Recommendations

The study and application of FVW have become very important in varied sense, such as to fight against the carbon footprint and greenhouse gas emissions that mainly come from these wastes. Some by-products from fruits and vegetables have even more BC content than the whole fruit, but commonly the fruit and vegetable by-products are considered as wastes and discarded. These wastes are still rich in plenty of BC, mainly phenolics, flavonoids, and carotenoids, that have antioxidant, antimicrobial, and coloring properties and can be used in pharmaceutical or food industries to enhance the properties and final quality of foods, and retard food spoilage.

The BC can be extracted from the FVW with several methods, being the green techniques the most recent trends of extraction, which compared with the conventional methods have an equivalent and even better-quality extract, higher yields of extraction, less time of extraction, reduce the use

of solvents, and reproducible and repeatable efficiencies. Furthermore, the use of green techniques is a sustainable option for the extraction of BC, as it is possible to work with green solvents such as water and in some cases even without any solvent. The combination of more than one green extraction technique has better performance results in terms of yield of extraction and extract purity. The most widely used green extraction techniques are micro-wave assisted, and ultrasound-assisted extraction, due to their several advantages such as the efficient cell wall disruption in less time, and the mature and developed process both in the laboratory and industry level.

The use and application in the food industry of the extracted BC will depend on the dosage of BC extract, the delivery method or method of addition (powder, encapsulation, crude), the fruit and vegetable by-product matrix, and external factors in food processing. Furthermore, the use of BC extracts in food improvement must pass from an *in vitro* to an *in vivo* analysis to check the bioavailability, bioactivity, formula stability, and food safety before promoting it to the consumer. More experimentation must be carried out to select the appropriate extraction processes to meet the BC needs that can be applied in food fortification or other uses.

Connecting text

Chapter II discussed the main trends in green BC extraction, such as microwave, ultrasonic, supercritical fluid, and pulsed electric field extractions, from some vegetable and fruit wastes. It is noted that MAE, and UAE are of interest due to their advantages in terms of time, costs and extraction efficiency compared to conventional extraction techniques. However, MAE optimization has not received enough attention, despite its advantages.

As observed in the literature review, there is minimal information about the MAE in broccoli wastes. *Brassica oleracea*, also known as broccoli, is a member of the Brassicaceae (Cruciferae) family, and it is one of the top 5 vegetables produced globally. It is estimated that the floret corresponds to 15%, the root 17%, the stem 21%, and the leaves 47% of the broccoli plant (al Jitan et al., 2018). Furthermore, more than 40% of waste is generated from the broccoli harvest, encompassing mainly stalks and leaves (Coman et al., 2020)

Therefore, Chapter III addresses the optimization of MAE to recover phenolic compounds from one of the high global importance vegetable wastes, such as broccoli. The main operation variables (time, temperature, methanol concentration) in the broccoli by-products extraction are evaluated and optimized by RSA, to maximize the phenolic response. Furthermore, antioxidant capacity and phenolic composition are discussed. This chapter also shows a comparison in the response and phenolic yield of MAE and maceration. Chapter III has been published in the Journal Recent Progress in Nutrition.

CHAPTER III.
MICROWAVE-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM BROCCOLI (*Brassica oleracea*) STEMS, LEAVES, AND FLORETS: OPTIMIZATION, CHARACTERIZATION, AND COMPARISON WITH MACERATION EXTRACTION

3.1 Abstract

Microwave-assisted extraction (MAE) to obtain phenolics from vegetable wastes has been of recent interest. Broccoli is one of the most globally produced vegetables, and around 43% of the harvest is considered waste. Thus, given the significant quantity of broccoli waste generated, the objective of this work was to optimize the MAE, to maximize the total phenolic content (TPC) from broccoli by-products (leaves and stems) and broccoli florets. The Response Surface Analysis was used in the optimization model to evaluate the impacts of methanol concentration, time, and temperature, and their interactions on the TPC of the broccoli extracts. The optimal MAE conditions were found to be 74.54% (methanol), 15.9 min, and 74.45°C for broccoli stems; 80% (methanol), 10 min, and 73.27°C for broccoli leaves; and 80% (methanol), 18.9 min, and 75°C for broccoli florets. Under these conditions, the broccoli leaves exhibited the highest TPC (1940.35 ± 0.794 µg GAE / g DW), followed by the florets (657.062 ± 0.771 µg GAE / g DW) and stems (225.273 ± 0.897 µg GAE / g DW). The antioxidant activity of the broccoli extracts was evaluated under the optimal conditions by DPPH and ABTS assays, and the same behavior was observed in both studies, the broccoli leaves exhibited the highest antioxidant activity, among florets and stems. In addition, vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids in the broccoli extracts were identified and quantified using HPLC. Furthermore, MAE was found to increase the phenolic yield by up to 45.70% for broccoli leaves, 133.57% for broccoli florets, and 65.30% for broccoli stems, in less time compared with maceration extraction. MAE proved to be an efficient and sustainable technique to obtain phenolics from broccoli by-products, which can constitute a viable solution for valorizing broccoli wastes.

3.2 Introduction

Food waste is a major worldwide concern in terms of environmental sustainability, food safety,

and the need to feed the growing global population (Rodríguez García & Raghavan, 2021). Among the food wastes, fruits and vegetables represent around 33% of the total food waste from the post-harvest to the distribution level (FAO, 2019a). Broccoli (*Brassica oleracea L.* var. Parthenon) ranks in the top 5 most-produced vegetables in the world, with 24.17 million metric tons (MMT), the main global producers are China, India, USA, and Mexico (FAO, 2019a; Sagar et al., 2018). However, it is estimated that approximately 43% of the total broccoli harvest is considered waste, such as leaves and stems (Coman et al., 2020). The large amounts of broccoli waste have a negative effect on the agricultural environment and food security. Some studies have revealed that significant amounts of bioactive compounds and essential nutrients are present in the broccoli by-products, such as phenolic compounds, glucosinolates, flavonoids, carotenoids, and sterols (Ares et al., 2013; Borja-Martínez et al., 2020; S. Kumar et al., 2022; Reguengo et al., 2022). The bioactive compounds are extra-nutritional components with the capacity of modulating metabolic processes, and some of them hold antioxidant, anti-microbial, or anti-inflammatory properties (Hamzalioğlu & Gökmen, 2016; Saini et al., 2019). Among the bioactive compounds, phenolic acids have been of recent interest, due to their antioxidant and antimicrobial properties, which make them have a commercial value in different industries, including cosmetics, pharmaceuticals, and food (al Jitan et al., 2018; N. Kumar & Goel, 2019; Rodríguez García & Raghavan, 2021). The enormous range of health advantages and industrial applications of phenolic acids has prompted scientists to enhance extraction and purification strategies for these naturally given compounds (N. Kumar & Goel, 2019). Furthermore, the use of phenolic acids in food enhancement has sparked a lot of interest, since they can be used to retard lipid oxidation and can be added to fresh or processed meats to improve color stability, retard the appearance of off-flavors, and prevent oxidative rancidity (Fontana et al., 2013; Rodríguez García & Raghavan, 2021). The evaluation of phenolic acids properties, such as antioxidant activity demonstrates the high potential and added value of these compounds for further industries applications.

The main phenolic acids found in broccoli are caffeic acid, chlorogenic acid, neochlorogenic acid, gallic acid, ferulic acid, and sinapic acid (Borja-Martínez et al., 2020; Liu et al., 2018). The phenolics can be obtained from broccoli by-products, using different extraction techniques: conventional (Soxhlet, maceration, hydro-distillation), or non-conventional (microwave-assisted, ultrasound-assisted, supercritical fluid). However, recent trends in extraction techniques are focused on finding solutions that minimize the time, and use of solvents for phenolic extraction

and still maintain high-quality extracts; this can be accomplished using green extraction or non-conventional techniques (Ares et al., 2013; Chemat et al., 2012; Kaderides et al., 2019; Soquetta et al., 2018).

Green extraction techniques are also known as non-conventional techniques since they employ organic solvents, take less time to extract, and use less energy, all of which have a positive influence on the environment (Soquetta et al., 2018). The microwave-assisted extraction (MAE) is one of the most used, due to its several advantages such as the efficient cell wall disruption in less time, less use of solvent, high selectivity, cost-effective in comparison with maceration, and the mature and developed process both in laboratory and industry level; thus, the microwaves provide dielectric heating and solute dissolution (Rodríguez García & Raghavan, 2021; Sagar et al., 2018). The comparison between conventional and non-conventional methods of extraction, becomes crucial to enrich and propose a solid base to choose the most appropriate extraction method to extract phenolics from broccoli waste. Generally, methanol/water, and methanol/ethanol/water mixtures are commonly used for extracting phenolics from broccoli by MAE (Ares et al., 2013). However, there are few examples and experimentation on MAE of broccoli by-products; more experimentation needs to be carried out to establish the proper parameters for phenolic extraction by MAE from broccoli wastes. The successful phenolic green extraction from broccoli by-products may represent an alternative option to reuse and valorize the vegetable wastes, optimizing the resources and offering a sustainable solution for waste utilization. Some comparisons between the total phenolic content from different broccoli parts have demonstrated that the leaves and stems have similar content and antioxidant activity to the edible parts of the broccoli (florets) (Borja-Martínez et al., 2020; Drabińska et al., 2018). To our knowledge, as far as broccoli by-products extraction of phenolics, (Ares et al., 2013; Borja-Martínez et al., 2020; Domínguez-Perles et al., 2010; S. S. Ferreira et al., 2020; Hwang & Lim, 2015; Jokić et al., 2012; Thomas et al., 2018) have done similar research; however, those studies are not optimizing MAE methodologies, nor comparing the key desirable attributes between the edible broccoli parts (florets), broccoli by-products (leaves, and stems), and conventional extraction methods. According to the above, the aim of this research is to perform the optimization of the microwave-assisted extraction of broccoli by-products: leaves and stems, and broccoli edibles: florets, to maximize the total phenolic content; identify the main phenolic acids, evaluate their antioxidant activity (with DPPH and ABTS radical scavenging activity analyses), and

compare against conventional extraction methods, in this case: maceration. Response Surface Analysis (RSA) is employed in the optimization model to evaluate the impacts of solvent concentration, time and temperature, and their interactions on the total phenolic content of the broccoli extracts. It is expected that the broccoli leaves, and stems exhibit a similar o higher amount of total phenolic content, and antioxidant activity compared with the broccoli florets. Furthermore, the MAE should have a higher phenolic extraction yield compared to maceration extraction in less time. This study is focused on phenolic acid identification through High-Performance Liquid Chromatography (HPLC) due to the properties and commercial value of these specific compounds.

3.3 Materials and Methods

3.3.1 Sample preparation

The broccoli (*Brassica oleracea*) florets, stems, and leaves were collected from a local market: Chez Robin in Montreal, Quebec. The broccoli by-products were cut into small pieces (3 to 4 cm) and separated from each other, 500 g of each category (florets, leaves, and stems). Then the materials were lyophilized in a Freeze-Dryer (Labconco Catalog No. 7670520, Serial No. 091017338G, USA), and they were ground to a fine powder with the help of a commercial blender (Retsch, Knife Mill Grindomix GM 200) at 5000 rpm for 1 min. Finally, the materials were stored at -20°C until further analysis. The sample preparation was based on previous research on broccoli samples (Domínguez-Perles et al., 2010; M. Shi et al., 2019; Thomas et al., 2018).

3.3.2 Maceration extraction

Two and a half gram of each of the previously treated broccoli samples, consisting of stems, leaves, and florets were extracted with 4 different solvents: 50 mL methanol (80% v/v), 50 mL methanol (40% v/v), 50 mL methanol (60% v/v), and 50 mL of distilled water (methanol free), in 100 mL closed flasks. The maceration was carried out at room temperature for 24 h, with constant agitation at 250 rpm. The maceration extraction process was an improvement based on (Domínguez-Perles et al., 2010; M. Shi et al., 2019; Thomas et al., 2018) previous studies. Then, the mixture was centrifuged (Centrifuge, Sorvall Legend X1R- Thermo Scientific) for 20 min at 10350 rpm, and 4°C. Finally, the supernatant was filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific), then the aqueous phase was stored at -20°C until further analysis.

3.3.3 Microwave-assisted extraction (MAE) and optimization

The MAE of all the broccoli by-product samples was carried out using a Mini WAVE Digestion Module (SCP Science Canada) that operates at a frequency of 2.45 GHz using 6 cylindrical quartz reactor vessels of 50 mL. In the MAE experiments, 2.5 g of each of the previously treated broccoli samples were extracted in 50 mL of solvent, with a liquid solid ratio of 20:1. Table 3.1 shows the variables and the levels proposed in the experimental designs. The solvent selected for the MAE was methanol (at different concentrations, see Table 3.1), which has been proved to be one of the best polar solvents for phenolic extractions (Jokić et al., 2012; Thomas et al., 2018). The parameters such as time and temperature were established, monitored, and controlled in the Mini Wave Digestion module set up. After each extraction, the mixture was centrifuged (Centrifuge, Sorvall Legend X1R- Thermo-Scientific) for 20 min at 10350 rpm, and 4°C. Finally, the supernatant was filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific), then the aqueous phase was stored at -20°C until further analysis.

In total 3 MAE optimizations were done, one for each category: broccoli stems, leaves, and florets. Response Surface Analysis (RSA) modeling technique with Central Composite Rotatable Design (CCRD) was selected for the experiment design of the Microwave-assisted extraction (MAE) to evaluate the effect of temperature (°C), time (min), and solvent concentration (% v/v) in the total phenolic content (TPC) of the broccoli by-products.

The experimental design was based on the CCRD with 20 experimental runs for each optimization, including 6 central points, 8 factorial runs, and 6 axial points. The choice of the axial runs (α) gives the design the rotatable aspect, where α represents the extreme values (low and high). For the three variables (temperature, time, and solvent concentration), the value of α is 1.682. The CCRD design uses least-squares regression to fit the experimental data to a quadratic model.

Rotatability was the criteria for choosing RSA, due to two factors: the TPC optimization purpose, and the position of the optimum values which was unknown before the experiments; it was reasonable to select a design that allowed for equal precision and reasonable distribution of the data points (Montgomery, 2017). Furthermore, the modeling technique used offers the advantage of reducing time and expenses (B. Wu et al., 2022). In all the experiments, the three independent variables (methanol concentration, time, and temperature) were correlated in the range to maximize TPC response of the broccoli extracts.

The RSA of the data, and optimization of the models were performed using Design Expert software (version 13.0 Stat-Ease Inc. Minneapolis, MN, USA). The ANOVA (Analysis of variance), the variables (methanol concentration, time, and temperature), and the responses (TPC) under the optimized conditions were validated using the same software.

Table 3. 1. Experimental variables and levels used in Central Composite Rotatable Design.

Variables	Variable	Levels				
		-α	**-1**	**0**	**+1**	**+ α**
Solvent concentration (methanol % v/v)	A	26.36	40	60	80	93.63
Time (min)	B	6.59	10	15	20	23.40
Temperature (°C)	C	48.18	55	65	75	81.81

3.3.4 Determination of total phenolic content (TPC)

To quantify the TPC from the different extracts, the Folin-Ciocalteu method was followed, with minor adaptations from (Kabir et al., 2015; Pan et al., 2012; Patras et al., 2011; Şahin, 2015). A mixture consisting of 100 µL of the extract, 475 µL of distilled water and 100 µL of Folin-Ciocalteu reagent, was stored in the dark for 5 min at room temperature, then 1325 µL of 75g/L sodium carbonate (Na_2CO_3) were added into the mixture, homogenized, and incubated at room temperature in darkness for 2 h. The absorbance was measured at 765 nm. The same procedure was used with gallic acid (0 to 100 ppm) as standard compound.

The sample concentration (µg gallic acid equivalent (GAE) /mL) was calculated based on the standard gallic acid calibration curve. The TPC results are expressed in µg gallic acid equivalent (GAE) / g dry weight (DW).

3.3.5 Phenolic acids characterization

The phenolic acids were characterized for the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A high-performance liquid chromatography (HPLC) method was used to characterize the phenolic acids, with some changes from (Kabir et al., 2015; Pan et al., 2012; Şahin & Şamli, 2013). The calibration solutions were made with standards of vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids, in aliquots, in concentrations between 0 to 100 ppm diluted in 0.1% formic acid and distilled

water 99.9% (v/v). Before the HPLC method, all the samples, including the standards, were filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific).

The HPLC (Agilent 1100 Series) used a C18 column (Gemini, 5µ 150 × 4.60 mm), a mobile phase A: formic acid 0.1% + 99.9% water (v/v), and a mobile phase B: formic acid 0.1% + acetonitrile 99.9% (v/v). The flow rate was 0.4 mL/min, with an injection volume of 5 µL, and 40 °C. The gradient was as follows: 99% A, 1 % B for 10 min; 50% A, 50% B, for 20 min; and then 99% A, 1% B, for 10 min. The absorbance was set at 330 nm. The identification and quantification of the phenolic compounds were performed by comparing the retention time of pure standards solutions.

3.3.6 Determination of antioxidant activity (AA)

3.3.6.1DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) radical scavenging method

The AA was determined through an DPPH assay, based on (Aires et al., 2017; Kabir et al., 2015; Şahin, 2015) experimentations for the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A methanolic-DPPH stock solution was prepared (0.048 mg/mL), and 5 aliquots of diluted extracts to facilitate the quantification.

Then a mixture consisting in 500 µL of extract and 500 µL of the DPPH-methanolic solution was vortexed and incubated in the dark for 30 min at room temperature. Finally, the absorbance was measured at 517 nm. The control sample consisted in 500 µL of methanol and 500 µL of the DPPH-methanolic solution. To quantify the percentage of inhibition in the samples, the equation (Eq. 3.1) was used, which is the mean inhibitory concentration, that is, the concentration of antioxidant compounds that can inhibit 50% of the DPPH radical.

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100\% \qquad \text{(Eq. 3.1)}$$

Where A_c is the control absorbance and A_s the sample absorbance. Finally, a standard commercial Trolox calibration curve was developed (0 to 100 ppm) to quantify the AA in the sample, and the same procedure of the DPPH assay was followed. The AA in the samples is expressed in µg of Trolox Equivalents (TE) / g of dry weight (DW) of the sample. The analyses were made in triplicates for each broccoli sample.

3.3.6.2 ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging method

The antioxidant activity was also determined by an ABTS assay, based on (Dahmoune et al., 2015; Drabińska et al., 2018; Oldoni et al., 2016; Shannon & Motha, 2015) experimentations for the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A mixture of 7 mmol/L ABTS and 140 mmol/L potassium persulfate ($K_2S_2O_8$) was stored in the dark at 25 °C for 14 h. Then, the ABTS radical solution was diluted to the absorbance level of 0.70 ± 0.02 at 734 nm, using an aqueous methanol solution (67% v/v).

Then a mixture consisting of 20 µL of the broccoli extracts (the extracts were diluted to facilitate the analysis), and 2 mL of the ABTS radical solution was stored in the dark for 6 min at room temperature. Finally, the absorbance was measured at 734 nm. A mixture of 2 mL of the ABTS radical solution and 20 µL of methanol was used as control.

Finally, a standard commercial Trolox calibration curve was developed (0 to 100 ppm) to quantify the AA in the sample, and the same procedure of the ABTS assay was followed. The AA in the samples is expressed in µg of Trolox Equivalents (TE) / g of dry weight (DW) of the sample. The analyses were made in triplicates for each broccoli sample.

3.4. Results

3.4.1 Optimization: Microwave-assisted extraction (MAE) of broccoli samples: stems, leaves and florets

3.4.1.1 Analysis of responses models: Response Surface Analysis (RSA)

RSA with CCRD was used to evaluate the effect of three variables: methanol concentration (v/v %), extraction time (min), and temperature of the extraction (°C), on the total phenolic content (TPC) of the broccoli samples. In all the experiments, the three independent variables were correlated to maximize TPC response. The surface response model obtained for the TPC was of second order for the three analyses (broccoli stems, leaves and florets), the results of the analysis are shown in Table 3.2. For all the models, the results were significant at *p*-values <0.05, and the models were focused on maximizing the adjusted R^2 and the predicted R^2, in general a greater R^2 suggests a better fit for the model.

Table 3. 2. Reduced mathematical models for the total phenolic content (TPC) response and its evaluation parameters based on Fit Summary and Model Summary Statistics of the broccoli samples.

Broccoli Samples	Model[a]	Model[b]	R^2	Adjusted R^2	Predicted R^2	Std. Dv.	Sequential[c] *p*-value	Lack of fit[c] *p*-value
Stems	$Y = -2.81\,A^2 - 2.75BC + 23.58A + 5.60B + 12.17C + 191.81$	$Y = -0.007029A^2 - 0.054935BC + 2.02245A + 4.69122B + 2.04101C - 53.70021$	0.9907	0.9823	0.9748	3.11	*	ns
Leaves	$Y = -8.12A^2 - 1.90B^2 + 35.11A + 7.75B + 16.79C + 1908.51$	$Y = -0.020293A^2 - 0.076083B^2 + 4.19045A + 3.83297B + 1.67921C + 1580.61459$	0.9950	0.9905	0.9829	3.37	***	ns
Florets	$Y = -2.50B^2 - 2.50C^2 + 30.26A + 6.67B + 15.07C + 609.28$	$Y = -0.100193B^2 - 0{,}025048C^2 + 1.51319A + 4.33981B + 4.76289C + 272.17951$	0.9912	0.9833	0.9429	3.82	*	ns

[a] Final Equation in Terms of Coded Factors; [b] Final Equation in Terms of Actual Factors; [c] the *p*-value results are indicated as follows: ns: $p > 0.05$; *: $0.05 < p < 0.01$; **: $0.01 < p < 0.001$; ***: $p < 0.001$; Y: total phenolic content (TPC) expressed in µg gallic acid equivalent (GAE) / g dry weight (DW); A: Methanol concentration (%); B: Time (min); C: Temperature (°C).

In the case of the broccoli stems model, only the methanol concentration was significant for the quadratic terms (Table 3.2), the reduction of the whole quadratic model was carried out since there were some insignificant terms. Furthermore, in the Analysis of variance (ANOVA) for the reduction of quadratic model, the F-value was 184.52, and *p*-value was < 0.0001 which provide

evidence that the model is significant. Figure 3.1 shows the RSA for the TPC in the broccoli stems model. The graph corresponds to the interaction between the most significant variable, methanol concentration, with the other two variables: time and temperature. In Figure 3.1A, the interaction between methanol concentration and time during the MAE is shown. The experimental runs showed that the maximum TPC (224.174 µg GAE/ g DW) was obtained using the highest values of methanol concentration (80% and 93.63% v/v) for 20 min. In Figure 3.1B, the interaction between methanol concentration and temperature is observed. Although in the model, the temperature is not significant in quadratic terms, it affects the TPC; at higher temperatures (65°C, and 75°C) the TPC increases, compared to the lower temperatures.

The RSA for the broccoli leaves samples showed that the methanol concentration and time were significant in quadratic terms (Table 3.2). The temperature affected the quadratic model in terms of lineal behavior. Due to the presence of several insignificant terms, the quadratic model was reduced. The ANOVA for the reduced quadratic model showed a Model F-value of 396.21, and *p*-value was < 0.0001 which implied that the model was significant; the Lack of Fit was not significant, so that the model fits. Figure 3.2 shows the RSA for the TPC in the broccoli leaves model. Figure 3.2A indicates the interaction between methanol concentration, and time during the MAE. The experimental runs exhibited that the maximum TPC (1960.12 µg GAE/ g DW) was obtained at a methanol concentration of 80% (v/v), for 20 min; the behavior is similar to the broccoli stems model (Figure 3.1A). In general, the broccoli leaves presented higher amounts of TPC compared with the broccoli stems, which agrees with the established values by (Ares et al., 2013; Domínguez-Perles et al., 2010; Hwang & Lim, 2015; Liu et al., 2018). Figure 3.2B shows the interaction between methanol concentration, and temperature. In the experiments, the 75°C temperature exhibited the highest amount of TPC at 80% (v/v) methanol concentration. In Figure 3.2C, the interaction between time and temperature is shown; between 15 min and 20 min at 75°C, the TPC increases, compared to the lower times.

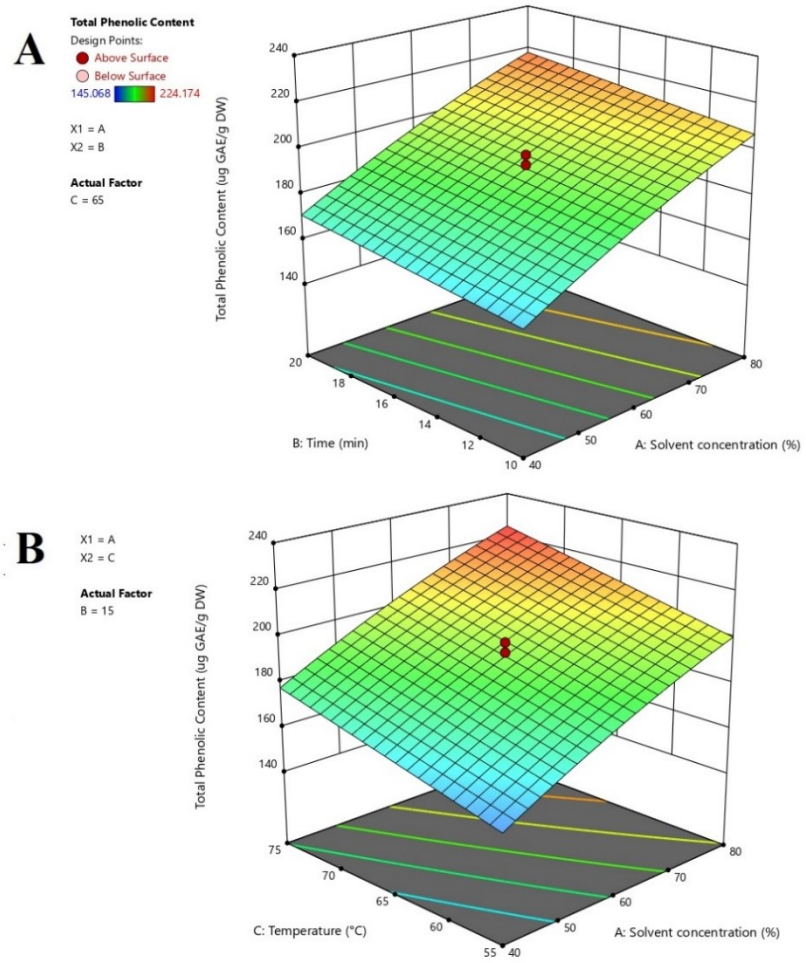


Figure 3. 1. 3D Broccoli stems Response Surface plot of total phenolic content (TPC). (A) Interaction AB, Solvent concentration (methanol %), and time (min). (B) Interaction AC, Solvent concentration (methanol %), and temperature (°C).

In the broccoli florets model, the time and temperature of extraction were significant in quadratic terms (Table 3.2). However, the methanol concentration shows a linear behavior in the quadratic model. The model was reduced, due to the presence of insignificant terms. The ANOVA for the reduced quadratic model exhibited a Model F-value of 193.07, and a p-value < 0.0001, providing support for the model to be significant. Figure 3.3 presents the RSA for the TPC in the broccoli florets model. Figure 3.3A indicates the interaction between time, and temperature during the MAE. The experimental runs showed that the highest TPC (668.049 µg GAE/ g DW) was obtained at 65°C for 15 min. Figure 3.3B exhibits the interaction between time, and methanol concentration; in the experiments, the highest TPC was obtained at 93.63% (v/v) of methanol concentration.

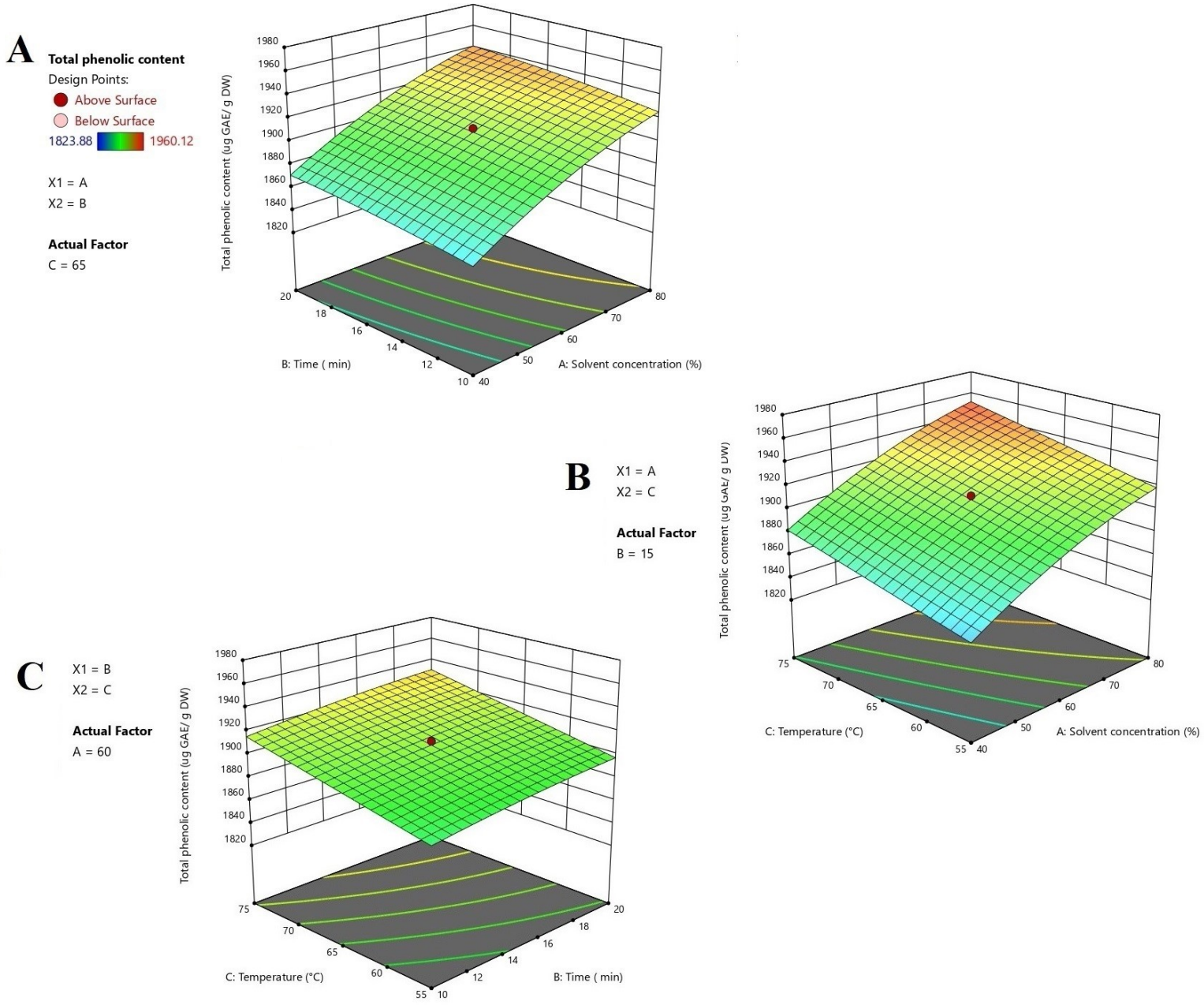


Figure 3. 2. 3D Broccoli leaves Response Surface plot of total phenolic content (TPC). (A) Interaction AB, Solvent concentration (methanol %), and time (min). (B) Interaction AC, Solvent concentration (methanol %), and temperature (°C). (C) Interaction CB, Time of extraction (min), and temperature (°C).

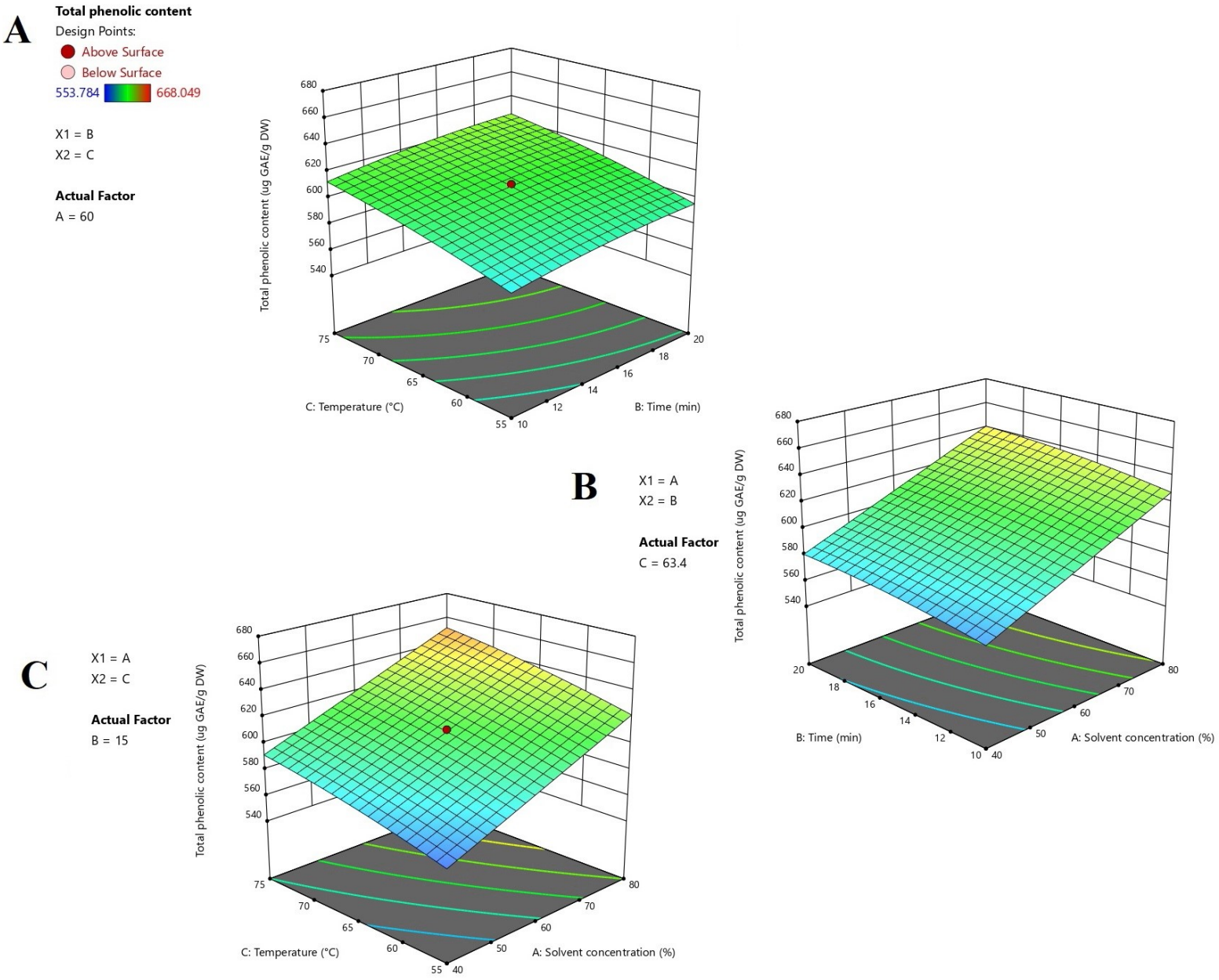


Figure 3. 3. 3D Broccoli florets Response Surface plot of total phenolic content (TPC). (A) Interaction BC, Time (min) and temperature (°C) of the extraction. (B) Interaction AB, Solvent concentration (methanol %), and time (min). (C) Interaction AC, Solvent concentration (methanol %), and temperature (°C).

Figure 3.3C shows the interaction of temperature, and methanol concentration; at higher temperatures and concentrations the TPC increases. The broccoli stems, leaves, and florets models

exhibited similar behaviors; in the experiments for the three models, the highest amounts of TPC were found in methanol concentrations ≥ 80% (v/v), between 15 min to 20 min, and temperatures between 65°C to 75°C, which agrees with the established values by (Ares et al., 2013; Jokić et al., 2012; Liu et al., 2018).

3.4.1.2 Optimization and validation of the total phenolic content (TPC) responses

The model optimization and validation of the TPC responses for the broccoli stems, leaves and florets were made to streamline the MAE process and propose the optimal and most efficient parameters (methanol concentration, time, and temperature) all in the range to maximize the TPC, through RSA. The optimal values calculated for the independent parameters using Design Expert 13 software are presented in Table 3.3. These values were estimated using the mathematical models shown in Table 3.2. For the validation of the adequacy of the model, triplicate experiments were carried out under optimized MAE conditions and the observed values of TPC responses were obtained. The values of TPC in the broccoli stems, leaves, and florets are very close to those estimated with the model (see Table 3.3). As a result, the model proved its capacity for prediction. According to (Montgomery, 2017) the lower the Relative Standard Deviation (RSD) value, the more precise the data collection is.

Table 3. 3. Optimization and validation values of the total phenolic content (TPC) responses for the broccoli samples*

Broccoli Sample	Optimized conditions			Predicted TPC (µg GAE/ g DW)	Observed TPC (µg GAE/ g DW) **	RME (%)	RSD (%)
	Methanol concentration (% v/v)	Time (min)	Temperature (°C)				
Stems	79.54	15.9	74.45	224.206	225.273 ± 0.897	0.48	0.48
Leaves	80.00	10.0	73.27	1939.73	1940.350 ± 0.794	0.03	0.11
Florets	80.00	18.9	75.00	655.82	657.062 ± 0.771	0.18	0.33

TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight. RME: Relative Mean Error; RSD: Relative Standard Deviation *: all the predicted solutions presented a Desirability of 1.0; **: Each value was expressed by mean ± SD.

3.4.2 Antioxidant Activity (AA) evaluation by DPPH and ABTS assays of the validated broccoli samples: stems, leaves, and florets

The DPPH and ABTS radical scavenging methods were used to measure the AA of the validated broccoli extracts (the samples under the optimal MAE conditions). The results of the mean total AA evaluation are shown in Table 3.4; the AA in the samples is expressed in µg of Trolox Equivalents (TE) / g of dry weight (DW) of the sample.

Table 3. 4. ABTS and DPPH radical scavenging activities (AA values) for the validated broccoli samples.

Broccoli Sample	TPC (µg GAE/ g DW)	AA in DPPH assay (µg TE/ g DW)	AA in ABTS assay (µg TE/ g DW)
Stems	225.273 ± 0.897[a]	193.110 ± 0.415[c]	212.118 ± 0.213[a]
Leaves	1940.350 ± 0.794[b]	632.057 ± 0.087[a]	1034.220 ± 0.324[b]
Florets	657.062 ± 0.771[c]	290.973 ± 0.669[b]	452.169 ± 0.093[c]

TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight; AA: antioxidant activity; TE: Trolox equivalents; DPPH: 2,2-diphenyl-1-picrylhydrazyl hydrate; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Each value was expressed by mean ± SD; Same letters in the same column refer to means not statistically different ($p > 0.05$).

In the broccoli extracts the TPC was significantly correlated with DPPH assay ($p < 0.001$, r=0.897). The AA with the ABTS assay was also correlated with the TPC ($p < 0.001$, r=0.858). The other authors (Domínguez-Perles et al., 2010; Hwang & Lim, 2015) have reported similar correlations between the TPC and AA for broccoli extracts. Broccoli by-products such as leaves, and stems contain high total phenolics and show high and similar activities compared with broccoli florets. The AA of all the broccoli extracts was higher in the ABTS assay compared with the DPPH assay, among DPPH and ABTS analyses, the broccoli leaves extracts had the highest AA and TPC followed by florets, and stems (see Table 3.4).

3.4.3 Phenolic Acids Characterization: Application of HPLC Method to the validated broccoli samples: stems, leaves, and florets

The identification and quantification of phenolic acids in all the validated broccoli extracts were based on calibration curves of external standards (vanillic, sinapic, caffeic, chlorogenic, ferulic,

gallic, neochlorogenic, and p-coumaric acids). The analyses were made in triplicates of each sample. The results of the phenolic acid characterization are shown in Table 3.5. Among the individual phenolic acids in the extracts, chlorogenic, neochlorogenic and ferulic acids were quantifiable in sufficient amounts in the three broccoli samples (stems, leaves, and florets). Caffeic and p-coumaric acids were quantifiable in the broccoli leaves extracts. Gallic acid and vanillic acid were quantifiable in the broccoli stems and florets extracts, while sinapic acid was only quantifiable in the broccoli leaves and florets extracts.

Table 3.5 Phenolic acids identification and quantification for the validated broccoli samples.

Phenolic acid	**Retention time (min)**	**Broccoli sample concentration (µg /mL)**		
		Stems	**Leaves**	**Florets**
Caffeic	27.333	ns	1.959 ± 0.042	ns
Chlorogenic	26.051	0.869 ± 0.011	2.153 ± 0.005	1.001 ± 0.004
Ferulic	30.224	21.920 ± 0.004	23.845 ± 0.021	21.954 ± 0.084
Gallic	21.127	17.127 ± 0.023	ns	21.736 ± 0.014
Neochlorogenic	24.958	8.789 ± 0.031	12.148 ± 0.008	9.020 ± 0.032
p-coumaric	29.680	ns	5.502 ± 0.063	ns
Sinapic	29.935	ns	9.149 ± 0.045	2.065 ± 0.055
Vanillic	27.339	17.582 ± 0.011	ns	29.171 ± 0.067

y: Area (mAU*s) milli-Absorbance Units; x: Concentration of the phenolic compound (µg/mL); ns: not significant, low concentrations unable to quantify; Each value was expressed by mean ± SD.

Figure 3.4 shows the phenolic acid chromatograms of the broccoli extracts. Figure 3.4A represents the chromatogram for the broccoli stem extract, in this chromatogram 5 phenolic acids were identified in sufficient amounts; ferulic acid was found to be in highest amount, followed by vanillic acid, gallic acid, neochlorogenic acid, and chlorogenic acid (see Table 3.5). Figure 3.4B shows the phenolic acids chromatogram of the broccoli leaves extract, where 6 phenolic acids were identified: ferulic acid being in the highest concentration, followed by neochlorogenic acid, sinapic acid, p-coumaric acid, chlorogenic acid, and caffeic acid.

The phenolic acid chromatogram of the broccoli florets extract is shown in Figure 3.4C. In the broccoli florets extract, 6 phenolic acids were identified; vanillic acid (Table 3.5) was found in the

highest amounts, followed by ferulic acid, gallic acid, neochlorogenic acid, sinapic acid, and chlorogenic acid.

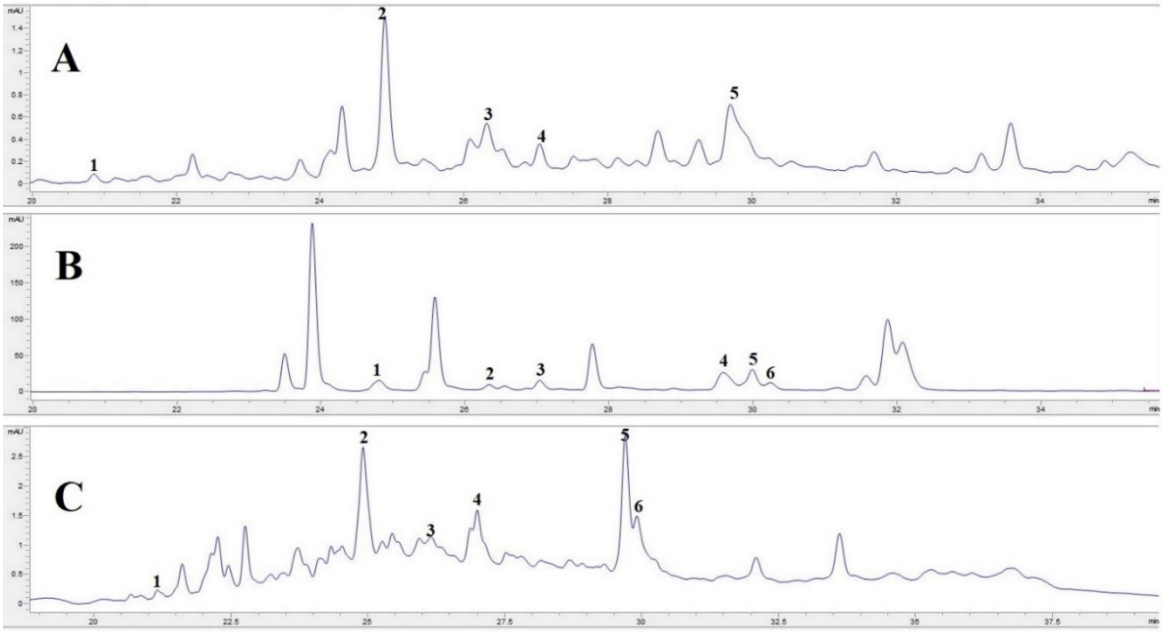


Figure 3.4 Phenolic acids chromatograms of the validated broccoli extracts (stems, leaves, and florets). (A) Chromatogram of the broccoli stems extract: 1- gallic acid, 2- neochlorogenic acid. 3- chlorogenic acid, 4-vanillic acid, 5- ferulic acid. (B) Chromatogram of the broccoli leaves extract: 1- neochlorogenic acid, 2-chlorogenic acid, 3- caffeic acid, 4- p-coumaric acid, 5- sinapic acid, 6- ferulic acid. (C) Chromatogram of the broccoli florets extract: 1- gallic acid, 2- neochlorogenic acid, 3- chlorogenic acid, 4- vanillic acid, 5- sinapic acid, 6-ferulic acid.

3.4.4 Comparison between the MAE validated broccoli sample extracts and Maceration broccoli sample extracts

The total concentration of phenolic compounds extracted from the broccoli stems, leaves, and florets, with methanol concentrations of 80% (v/v), 60% (v/v), 40% (v/v), and methanol free (distilled water) were compared after 24 h of extraction. The comparison of the TPC between a common maceration extraction and the MAE from the validated broccoli samples, is shown in Table 3.6. The TPC is expressed in µg gallic acid equivalent (GAE) / g dry weight (DW). The optimized MAE process increased the total phenolic yield in the broccoli stems by 0.49%, 9.04%, 19.18%, and 65.30% in comparison to the maceration extraction with methanol concentrations of

80 %, 60 %, 40 %, and distilled water as solvents, respectively. In the case of broccoli leaves, the total phenolic yield increased by 13.20 %, 13.49 %, 25.07 %, and 45.70 % using MAE in comparison with maceration extraction, under the previously mentioned methanol concentrations. Finally, the MAE process increased the total phenolic yield in the broccoli florets by 89.23 %, 99.32 %, 107.62 %, and 133.57 %, in comparison with maceration extraction with the same methanol concentrations.

Table 3.6 Comparison of total phenolic content (TPC) of broccoli samples (stems, leaves, and florets) with maceration method and the validated broccoli extracts with MAE.

Extraction method	**Methanol concentration (% v / v)**	**Time**	**Temperature (°C)**	**TPC of broccoli samples (µg GAE / g DW)**		
				Stems	**Leaves**	**Florets**
Maceration	80.00	24 h	24.00	224.174 ± 0.922	1714.011 ± 1.223	347.228 ± 0.956
	60.00			206.595 ± 0.721	1709.616 ± 0.946	329.649 ± 1.567
	40.00			189.016 ± 1.188	1551.404 ± 0.792	316.465 ± 1.683
	0.00*			136.278 ± 1.034	1331.664 ± 1.834	281.306 ± 0.871
MAE	79.54	15.9 min	74.45	225.273 ± 0.897	-	-
	80.00	10 min	73.27	-	1940.350 ± 0.794	-
	80.00	18.9 min	75.00	-	-	657.062 ± 0.771

* Methanol free, distilled water used as solvent; TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight; MAE: Microwave-assisted extraction; Each value was expressed by mean ± SD.

In terms of extraction time, the highest TPC results in the broccoli extracts were also observed in MAE with the optimal conditions 10 min to 18.9 min, compared with 24 h in maceration extraction. MAE has been demonstrated to be a rapid extraction technique in comparison to a 24 h maceration extraction for obtaining phenolics from the broccoli samples in a short period of time. The results (Table 3.6) showed the highest TPC at the highest methanol concentration (80% v/v) in maceration for the three broccoli categories, which supports the results obtained in MAE.

3.5 Discussion

3.5.1 Optimization: Microwave-assisted extraction (MAE) of broccoli extracts

The MAE process begins with the solvent penetration (in this case methanol) into the broccoli samples, then the components break down with the help of electromagnetic waves, the solubilized compounds are moved from the insoluble matrix to the bulk solution, and the liquid and residual solid phase are separated (Panzella et al., 2020; Rodríguez García & Raghavan, 2021). The microwave radiation absorption in the extraction system enhanced the heat buildup of the extraction solution, resulting in the dissolution of phenolics into the solution for 15 to 20 minutes (see Table 3.3), the same behavior was observed by (Kaderides et al., 2019; Touati et al., 2021) in MAE for different plant samples. In the experiments, the increase in temperature, and solvent concentration enhanced the TPC extraction of all the broccoli by-products. The same behavior was presented in the study of (Jokić et al., 2012) for lyophilized broccoli samples (by-products not specified), with optimal MAE conditions of 71.51°C, for 17 min, methanol concentration of 72.06% v/v, and 160 W. However, it is demonstrated that prolonged exposure times (more than 20 min) at high temperatures degrade the phenolic compounds, reducing the extraction yield in the microwave field (Kaderides et al., 2019).

According to Table 3.3 results, the broccoli leaves exhibited the highest amount of TPC (1940.35 ± 0.794 µg GAE/ g DW) under the optimal conditions, followed by the broccoli florets (657.06 ± 0.771 µg GAE/ g DW), and finally the broccoli stems (225.27 ± 0.897 µg GAE/ g DW). Other authors reported similar results with different extraction methods on broccoli samples; (S. S. Ferreira et al., 2018) reported 317 µg GAE/ mL of TPC in broccoli stems using Microwave hydro diffusion and gravity assisted extraction. (Thomas et al., 2018) presented a TPC of 5.4 mg GAE/ g DW, for a mixture of broccoli stems and leaves. (Liu et al., 2018) reported a TPC of 4.14 mg GAE/ g DW for broccoli leaves, 2.51 mg GAE/ g DW for broccoli florets, and 1.41 mg GAE/ g DW for broccoli stems, using solid liquid extraction. (Hwang & Lim, 2015) presented a TPC of 1310 mg GAE/ 100 g DW for broccoli leaves, 215.6 mg GAE/ 100 g DW for broccoli stems, and 528.9 mg GAE/ 100 g DW for broccoli florets, using maceration.

Some authors reported higher amounts of TPC in the broccoli extracts, compared with the results of this study, this can be explained since the conditions of the extraction vary in several ways, such as the type of extraction (the present study focuses in MAE, compared with convention methods,

such as solid liquid extraction and maceration), the solvent concentration (this experiment used aqueous methanol in different concentrations, compared with the use of absolute methanol in (Liu et al., 2018) experiments), time (the conventional extraction methods require more extraction time compared with green extraction such as MAE), and temperature. At higher solvent concentrations the TPC increases (Jokić et al., 2012; Rodríguez García & Raghavan, 2021); However, the purpose of MAE is to reduce the use of solvent in less extraction time to obtain bioactive compounds from the plant samples.

Although the values mentioned vary depending on experimental conditions and extraction methods, in general the broccoli leaves exhibited higher amount of TPC, followed by the florets and stems. It has been observed that in general the peels and outer parts of fruits and vegetables (in this case the leaves), present a higher amount of polyphenol content, since this part of the plants are exposed to an aggressive or stressful environment, secondary metabolism is induced, resulting in increased phenolic compound production (Faller & Fialho, 2010). Moreover, MAE has been demonstrated to be a good extraction method to obtain phenolics in less time, and at lower temperatures compared to the other extraction methods, such as maceration, Soxhlet extraction, and ultrasound-assisted extraction (Chen & Chen, 2013; Dahmoune et al., 2015; Sagar et al., 2018).

3.5.2 Antioxidant Activity (AA) evaluation and phenolic acids identification of the validated broccoli extracts

3.5.2.1 DPPH and ABTS radical scavenging methods

Any compound that delays or inhibits oxidative damage to a target molecule is considered as an antioxidant. Antioxidant molecules such as phenolic acids scavenge free radicals, inhibiting the oxidative pathways that contribute to degenerative diseases (Mahdi-Pour et al., 2012). In the case of the DPPH assay, the DPPH radical is reduced in the presence of antioxidants, which causes the solution to fade. The methanolic solutions acquire a violet color characterized at 517 nm. (Dahmoune et al., 2015; Gutiérrez Avella et al., 2008). On the other hand, the ABTS test compares antioxidants to a Trolox standard in terms of their capacity to scavenge the ABTS produced in aqueous phase (Ratnavathi & Komala, 2016).

Among DPPH and ABTS analyses (see Table 3.4), the broccoli leaves extract had the highest AA (632.057 ± 0.087 DPPH µg TE/ g DW; 1034.220 ± 0.324 ABTS µg TE/ g DW) and TPC followed by florets (290.973 ± 0.669 DPPH µg TE/ g DW; 452.169 ± 0.093 ABTS µg TE/ g DW), and stems (193.110 ± 0.415 DPPH µg TE/ g DW; 212.118 ± 0.213 ABTS µg TE/ g DW). The activities of the broccoli by-products in terms of DPPH and ABTS radical scavenging were considerably different. However, the AA was higher in the ABTS assay in all the broccoli extracts compared with the DPPH assay. It is demonstrated that the DPPH decolorizing process was not promoted by the components of the extracts, and so had limited activity, compared with ABTS assay, which agrees with the established assay by (Gunes et al., 2019).

According to (Wijngaard et al., 2009) the outer regions of most fruits and vegetables exhibit higher AA, since antioxidants play a protective role in them. In this study the TPC was significantly correlated with the AA with the DDPH and ABTS assays. The phenolic content is directly related to the AA (Kabir et al., 2015), so it is expected to have a higher AA in the broccoli leaves compared to the florets and stems. Broccoli extracts using MAE showed a direct correlation between the TPC and AA responses, the higher the TPC, the higher the AA; moreover, it is reported that MAE exhibits higher AA compared to the other extraction methods, such as ultrasound assisted extraction, and Soxhlet extraction (Dahmoune et al., 2015). However, in the literature there are contrasting statements about positive or negative correlations between the TPC and AA (Gunes et al., 2019; Hwang & Lim, 2015). This could be explained by the different kinetic profiles of phenolic compounds against the DPPH and ABTS radicals, such as, the need for a longer reaction time, other unspecified reactions between the phenolic compounds, as well as other radical reagent parameters like pH, temperature, and solvent choice (Dahmoune et al., 2015; Gunes et al., 2019).

A study made by (M. Shi et al., 2019) showed an AA of 77.84 mg TE/ g DW, for a mix of broccoli leaves and stems, and 51.06 mg TE/ g DW for broccoli florets. Another study by (Radošević et al., 2017) showed an AA of 67.32 mg TE/ g DW for broccoli florets. (Hwang & Lim, 2015) studied different broccoli cultivars and found that the AA of the leaves was the highest and that of the stems was the lowest. The mentioned studies exhibited the same behavior of AA as this study since leaves presented the highest AA. All the validated broccoli samples presented antioxidant activity; the antioxidant potential of the broccoli extracts might be attributed to the vegetable natural antioxidants (Domínguez-Perles et al., 2010; Hwang & Lim, 2015).

Broccoli by-products (leaves and stems) have a similar profile to their edible counterparts (florets); the experiments not only revealed that they include phenolics, but they also exhibited higher AA (leaves), which increase their chances of being employed for extraction of bioactive chemicals, particularly those linked to key health benefits. (Aires et al., 2017) stated that samples extracted with acidic methanol at 70°C had greater AA, which corresponded to those with higher polyphenol concentration, which agrees with the results of this experiment, since the optimal conditions for MAE extraction exhibited that at 80% v/v methanol concentration, 74.45 °C (stems), 73.27 °C (leaves), and 75 °C (florets) the TPC and AA were the highest.

3.5.2.2 Phenolic acids identification

Vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids were identified in the validated broccoli extracts of leaves, stems, and florets (see Table 3.5). (M. Shi et al., 2019) also identified neochlorogenic acid (7.2 mg / g DW), chlorogenic acid (0.2 mg / g DW), and caffeic acid (trace level) in a mix of broccoli leaves and stems extract. Another study in broccoli by-products: leaves and stems, (Aires et al., 2017) showed that the predominant phenolics in the broccoli extracts were neochlorogenic acid (124.7 µg / g DW), chlorogenic acid (128.0 µg / g DW), sinapic acid (80.8 µg / g DW), and ferulic acid (88.2 µg / g DW). (Domínguez-Perles et al., 2010) found chlorogenic acid (112.44 mg / g DW), and sinapic acid (9.85 mg / g DW) in different cultivars of broccoli leaves, and chlorogenic acid (8.63 mg / g DW) in broccoli stems. (Salama et al., 2021) studied dried broccoli florets ethanol extracts and found that gallic acid had the highest concentration (3884.59 µg / g DW) followed by chlorogenic acid (140.60 µg / g DW); other phenolic acids detected were ellagic acid, cinnamic acid, and syringic acid. (Fernández-León et al., 2012) studied fresh broccoli florets and found that the predominant phenolic acids in the samples were gallic acid (1.80 mg /100 g Fresh Weight (FW)), chlorogenic acid (1.38 mg /100 g FW), and sinapic acid (1.25 mg / 100 g FW). The phenolic acid concentrations vary due to several factors, such as the extraction conditions, types of extraction, genetic, agronomic, and environmental factors, that enhance the final concentration (Vallejo et al., 2003).

3.5.3 Comparison between MAE and Maceration of the broccoli extracts

In both extraction techniques (MAE and maceration), the broccoli leaves exhibited the highest amount of TPC followed by the florets, and stems (see Table 3.6). The extraction conditions for

obtaining the greatest amount of TPC in maceration were the same for all the broccoli samples: 80% (v/v) methanol concentration, 24 h, and room temperature; under these conditions, the broccoli leaves exhibited 1714.011 ± 1.223 µg GAE / g DW of TPC, followed by florets 347.228 ± 0.956 µg GAE / g DW, and stems 224.174 ± 0.922 µg GAE / g DW.

The solvent concentration is similar to the optimal conditions found in MAE, which range from 79.54 to 80% (v/v) of methanol. The highest amounts of TPC were found in the highest solvents concentrations, which agrees with other studies (Jokić et al., 2012). However, in MAE the time varies between 10 to 18.9 min, against the 24 h maceration extraction, remarking the time efficiency of the MAE process. Temperature is another important factor in the extractions, in the optimized MAE temperatures between 73.26 and 75°C were employed, against room temperature in maceration; due to the long period of extraction in maceration, it is not recommended to employ high temperatures, since prolonged exposure to high temperatures reduces the phenolic extraction yield due to the breakdown of the chemical active structures of phenolic compounds (Pimentel-Moral et al., 2018).

Overall, there is a significant increase in the total phenolic yield using MAE as a method to extract phenolics, compared to the maceration extraction, other authors reported the same behavior comparing both techniques (da Rosa et al., 2019; Garavand et al., 2019; Rafiee et al., 2011; C. N. Zhao et al., 2018). MAE was found to increase the phenolic yield by up to 45.70% for broccoli leaves, 133.57% for broccoli florets, and 65.30% for broccoli stems. Moreover, higher TPC was obtained by the MAE method compared to the maceration approach, further confirming its high efficiency. Similar results were also reported in comparing MAE with conventional extraction techniques in extracting polyphenols from other plant samples (da Rosa et al., 2019; Vernès et al., 2019; C. N. Zhao et al., 2018). Among other advantages of MAE, some studies have demonstrated that MAE exhibit better quality extracts, high selectivity, and cost-effectiveness in comparison with maceration extraction (Chen & Chen, 2013; Sagar et al., 2018)

3.6 Conclusions

The effect of three variables: methanol concentration, temperature, and time, in MAE to extract phenolic acids from Broccoli stems, leaves, and florets, was evaluated and then optimized through RSA methodology, with CCRD as an upgrading technique to maximize the TPC. A second-order polynomial regression model with high reliability was obtained for the three broccoli samples, the

optimal extraction conditions were: 74.54% (methanol concentration), 15.9 min, and 74.45°C for broccoli stems; 80% (methanol concentration), 10 min, and 73.27°C for broccoli leaves; and 80% (methanol concentration), 18.9 min, and 75°C for broccoli florets. The TPC values obtained under the optimal MAE conditions were: 225.273 ± 0.897 µg GAE / g DW, 1940.35 ± 0.794 µg GAE / g DW, and 657.062 ± 0.771 µg GAE / g DW, for the broccoli stems, leaves, and florets respectively. The results showed that the broccoli by-products (leaves and stems) contain significant amounts of phenolic compounds. The broccoli leaves not only exhibited higher amounts of phenolic content compared to the florets, but also higher antioxidant activity: 632.057 ± 0.087 DPPH µg TE/ g DW; 1034.220 ± 0.324 ABTS µg TE/ g DW (broccoli leaves), 290.973 ± 0.669 DPPH µg TE/ g DW; 452.169 ± 0.093 ABTS µg TE/ g DW (broccoli florets), and 193.110 ± 0.415 DPPH µg TE/ g DW; 212.118 ± 0.213 ABTS µg TE/ g DW (broccoli stems).Therefore, the broccoli by-products can constitute a viable solution for repurposing and valorizing broccoli wastes. Moreover, MAE remarkably increased the TPC, and the phenolic yield values of the broccoli extracts compared to the maceration extraction in a shorter period. MAE proved to be an efficient green extraction technique to obtain phenolics. Furthermore, several phenolic acids were identified in the broccoli by-products, with HPLC method, such as vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids. Both the food and cosmetic industries are increasingly interested in using phenolic extracts as antioxidants, and broccoli by-products might be one of them. However, further extensive investigations of prospective industrial uses, as well as economic considerations, should be done in the future for the industrial application of broccoli by-product extracts.

Connecting text

Chapter III showed the importance of broccoli and its waste, due to its high phenolic content, it was shown that broccoli leaves contain a greater phenolic content and exhibited greater antioxidant activity, while the stems had the lowest phenolic responses. In addition, the optimal conditions for the development of the MAE were described and the parameters and their behavior in the extraction were discussed.

In the following Chapter IV, the behavior of different solvents in the microwave extraction process of broccoli by-products is discussed. Hexane was used as a nonpolar solvent, while the polar solvents used were acetone, water, and methanol. In addition, the behavior of the temperature increase is observed and discussed using the different solvents in MAE for the phenolic recovery of the stems, florets, and leaves of broccoli. This chapter has been submitted to the Journal of Biologically Active Products from Nature.

CHAPTER IV.
POLAR AND NON-POLAR SOLVENTS BEHAVIOR IN MICROWAVE-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM BROCCOLI (B*rassica oleracea*) STEMS, LEAVES, AND FLORETS.

4.1 Abstract

Broccoli (*Brassica oleracea*) is one of the most consumed and produced vegetables in the world, almost half of the harvested plant, ends up as waste, such as leaves and stems. However, the broccoli by-products still contain significant amounts of phenolic compounds, thus, the objective of this study was to perform the Microwave-assisted extraction (MAE) of broccoli leaves, stems, and florets, using different types of polar solvents (methanol, acetone, and water), and non-polar solvent (hexane), in increasing temperatures from 55°C to 95°C, to compare the total phenolic content (TPC), and antioxidant activity (AA) by DPPH assay of the extracts. The polar solvents exhibited higher amounts of TPC and AA in all the extracts, compared with hexane. Methanol presented the highest TPC and AA values, under the same temperature conditions, among other solvents. Broccoli leaves exhibited the highest TPC (3643.328 ± 0.033 µg GAE/ g DW at 85°C with methanol) followed by broccoli florets (1428.349 ± 0.038 µg GAE/ g DW, at 95°C with methanol), and broccoli stems (1002.053 ± 0.012 µg GAE/ g DW, at 85°C with methanol). Furthermore, the TPC presented a positive correlation with the AA. In terms of the temperature, at higher temperatures the TPC increased in all extracts; however, in the case of methanol and acetone, beyond 85°C the TPC decreased, due to a phenolic compound degradation. Broccoli by-products (leaves and stems) should be revalued since through MAE they proved to contain significant amounts of phenolic compounds and exhibited AA.

4.2 Introduction

Broccoli (*Brassica oleracea* L. var. Parthenon) is one of the most consumed and produced vegetables globally, their wastes represent almost half of the harvested plant, including stems and leaves, which represent a threat to the environment and food security (FAO, 2019a; Sagar et al., 2018). These by-products still contain significant amounts of valuable components, also known as bioactive compounds (BC), which have antioxidant and antimicrobial properties (S. S. Ferreira et

al., 2020; Liu et al., 2018; Rudra et al., 2015). Hence, broccoli has a significant economic and industrial importance across the globe, not only the edible parts of the broccoli (florets) are useful, but also the by-products (leaves, and stems), as the BC in them can be used in food, cosmetic or pharmaceutical industries due to their beneficial properties (Marranzano et al., 2018; Sagar et al., 2018; Soquetta et al., 2018).

There are some BC found in broccoli, such as phenolic acids, flavonoids, carotenoids, and saponins. Among these compounds phenolics have been of recent interest due to their antioxidant, antimicrobial, and anti-inflammatory properties (Garcia-Salas et al., 2010; Rodríguez García & Raghavan, 2021). Phenolic compounds can be obtained from broccoli wastes with either conventional or green extraction techniques (Rodríguez García & Raghavan, 2021; Sagar et al., 2018; Soquetta et al., 2018). Microwave-assisted extraction (MAE) is a green extraction technique to obtain BC that has been recently studied due to its less solvent consumption, shorter extraction time, and higher extraction yield in comparison with conventional methods, such as maceration, and Soxhlet extraction (Garavand et al., 2019; Soquetta et al., 2018). MAE can extract polar and non-polar compounds from solid matrices (Sun & Kee Lee, 2002), this is an important feature since phenols have one or more hydroxyl groups (polar component) connected directly to an aromatic ring (non-polar part), that stereochemistry differentiates them based on polarity variance (C. M. Galanakis et al., 2013). Temperature, solvent-solute affinity, plant matrix, duration of the extraction, and solvent selection are only a few of the variables that might influence MAE efficiency (Hernández et al., 2009; Zhang et al., 2018).

The phenolic extraction from fruits and vegetables by-products has traditionally been done with a mixture of methanol and water; however, ethanol, acetone and n-hexane have also been used (Ares et al., 2013; Rodríguez García & Raghavan, 2021). In MAE the phenolic extraction yield depends on the nature of the solvent, as the diffusion coefficient and dissolution rate of the compounds in a plant matrix influence the extraction until they reach equilibrium concentration inside the solvent (C. M. Galanakis et al., 2013). Non-polar solvents such as hexane, and petroleum ether are preferred for the recovery of some phenolic terpenes, whereas low molecular weight molecules and phenolic acids can be efficiently recovered using solvents like diethyl ether and ethyl acetate (C. M. Galanakis et al., 2013). Nevertheless, most organic solvents are flammable, volatile, and even toxic; thus, alternative solvents, such as water or methanol are a viable option for replacing petrochemical solvents (Chemat et al., 2012). Additionally, more polar solvents, such as hydro-

alcoholic combinations, have been found to extract flavonoid glycosides and larger molecular weight phenols (Ares et al., 2013; C. M. Galanakis et al., 2013; Sagar et al., 2018). The total phenolic content and therefore the antioxidant activity of the broccoli by-products will depend on several factors, such as the solvent selection, solvent-solute affinity, and temperature. To our knowledge, as far as broccoli by-products extraction of phenolics, only (Ares et al., 2013; Borja-Martínez et al., 2020; Hwang & Lim, 2015; Jokić et al., 2012; Rodríguez García & Raghavan, 2022; Thomas et al., 2018) have done similar research; however, those studies are not focused on the comparison of polar and non-polar solvents, through microwave extraction, nor do they compare the phenolic content of broccoli by-products and the edible parts of the plant.

Thus, the objective of this research is to evaluate the MAE of broccoli stems, leaves, and florets to obtain phenolic compounds, compare the Total Phenolic Content (TPC), and Antioxidant Activity (AA) of each sample, using different polar (water, acetone, and methanol), and non-polar solvents (hexane), at different extraction temperatures. It is expected that polar solvents exhibit higher amounts of TPC in the broccoli samples and that temperature affects the TPC behaviour in degrading phenolic compounds at higher temperatures. A knowledge base for the preference of phenolic compounds among several solvents would be very helpful for the recovery of phenolics from broccoli by-products. Furthermore, the relationship between the solvent selection, temperature conditions, phenolic content, and antioxidant activity of the different broccoli parts (stems, leaves, and florets) will provide a solid base to improve the MAE process. The effective MAE from broccoli by-products may constitute an option for repurposing and valorizing broccoli wastes, therefore conserving resources and providing a long-term waste management solution.

4.3 Materials and Methods

4.3.1 Sample Preparation

The stems, leaves, and florets of broccoli (*Brassica oleracea*) were purchased from Chez Robin in Montreal, Quebec. 500 g of each type of broccoli by-products were chopped into tiny pieces and isolated from one another (stems, leaves, and florets). The components were then lyophilized in a Freeze-Dryer (Labconco Catalog No. 7670520, Serial No. 091017338G, USA) and ground to a fine powder in a commercial blender (Retsch, Knife Mill Grindomix GM 200) for 1 minute at 5000 rpm. Finally, the materials were kept at -20°C. The sample preparation was based on a prior

broccoli sample study (Rodríguez García & Raghavan, 2022).

4.3.2 Microwave Assisted Extraction (MAE)

The broccoli samples were extracted utilizing a Mini WAVE Digestion Module (SCP Science Canada) that runs at a frequency of 2.45 GHz and uses six 50 mL cylindrical quartz reactor containers. In the MAE experiments, 2.5 g of each of the previously treated broccoli samples were extracted in 50 mL of pure solvent, with a liquid-solid ratio of 20:1. The polar solvents used for the extraction were: methanol, water, and acetone, whereas the non-polar solvent was hexane. The extraction was carried out at 5 different temperatures: 55°C, 65°C, 75°C, 85°C, and 95°C. The experiments were done in triplicates, and the extraction time was 15 min for all of them. After each extraction, the mixture was centrifuged for 20 min at 10350 rpm and 4°C (Centrifuge, Sorvall Legend X1R- Thermo Scientific). Finally, a 0.20 µm PTFE syringe-filter (Fisher Scientific) was used to filter the supernatant, and the aqueous phase was kept at -20°C until further analysis.

4.3.3 Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu technique was used to determine the phenolic content of the broccoli extracts, based on previous research (Rodríguez García & Raghavan, 2022). A mixture of 100 µL of extract, 475 µL of distilled water, and 100 µL of Folin-Ciocalteu reagent was stored in the dark for 5 min at room temperature, then 1325 µL of 75 g/L Na_2CO_3 were added to the mixture, homogenized, and incubated in the dark for 2 h at room temperature. At 765 nm, the absorbance was measured, and the findings were represented as µg gallic acid equivalent (GAE) / g dry weight (DW). The TPC was calculated by multiplying the 50 mL of solvent used by the concentration of the sample, over the grams of the dry weight of each sample. The same procedure was followed for the gallic acid standard (0-100 ppm). The sample concentration was estimated based on the standard gallic acid calibration curve.

4.3.4 Antioxidant Activity (AA) evaluation

For each sample category: broccoli stems, leaves, and florets, the AA was evaluated using a 2,2-Diphenyl-1-Picrylhydrazyl Hydrate radical DPPH test based on (Rodríguez García & Raghavan, 2022). The AA was calculated for the broccoli extracts that exhibited the highest TPC of each

solvent used, that is, 5 DPPH tests for leaves, 5 for stems, and 5 for florets (for hexane, water, acetone, and methanol). All measurements were done in triplicate.

To enable measurement, a methanolic-DPPH stock solution (0.048 mg/mL) was prepared. Then, a mixture of 500 µL of extract and 500 µL of DPPH-methanolic solution was vortexed and incubated at room temperature for 30 min in the dark. At 517 nm, the absorbance was finally measured. 500 µL of methanol and 500 µL of DPPH-methanolic solution made up the control. The mean inhibitory concentration, or the concentration of antioxidant compounds that may inhibit 50% of the DPPH radical, was calculated dividing the difference between the absorbance of the control and the absorbance of the sample over the absorbance of the control.

Finally, to assess the AA in the samples, a standard commercial Trolox calibration curve was created (0-100 ppm), and the DPPH test protocol was followed. The amount of AA in the samples was measured in µg Trolox equivalents (TE) / g of dry weight (DW).

4.5 Results

4.5.1 Total Phenolic Content (TPC) in broccoli samples: stems, leaves, and florets, under Microwave Assisted Extraction (MAE)

The TPC was calculated after the MAE for the broccoli extracts of stems, leaves, and florets, the results are shown in Table 4.1. In general broccoli leaves exhibited the highest TPC, regardless of the solvent used, followed by florets and stems; however, the extracts that used polar solvents (acetone, water, and methanol) showed a higher TPC compared to the non-polar solvent (hexane). Under the same MAE conditions (temperature, and time) the highest values of TPC were reported using methanol as solvent, followed by water, acetone, and lastly hexane, in all broccoli samples, regardless of the category.

Figure 4.1 shows the TPC behavior of the broccoli extracts using different solvents at different temperatures. In the case of broccoli leaves (Figure 4.1-A), the highest TPC value (3643.328 ± 0.033 µg GAE /g DW) was reported using methanol as solvent, at 85°C, for 15 min. Under the same temperature and time conditions, the highest TPC values were obtained for the broccoli leaves with other solvents, such as acetone (1010.843 ± 0.067 µg GAE /g DW), and hexane (52.776 ± 0.043 µg GAE /g DW); however, in the case of water the highest TPC value (2491.891 ± 0.022 µg GAE /g DW) was found at 95°C for 15 min. The hexane exhibited the lowest phenolic yield

compared to the rest of the solvents; compared to hexane, methanol increased the phenolic yield by 98.55%, water by 97.88%, and acetone by 94.77%.

On the other hand, the highest TPC value (1002.050 ± 0.012 µg GAE /g DW) for the broccoli stems extracts (Figure 4.1-B) was found using methanol at 85°C for 15 min. Acetone and hexane exhibited the highest amount of TPC (465.888 ± 0.132 µg GAE /g DW, and 39.592 ± 0.075 µg GAE /g DW respectively) under the same temperature, and time conditions; water presented the highest TPC (672.443 ± 0.086 µg GAE /g DW)) at 95°C for 15 min. Hexane exhibited the same behavior as the broccoli leaves, it showed the lowest phenolic yield; in comparison, methanol increased the phenolic yield by 96.04%, water by 94.11%, and acetone by 91.50%.

Table 4. 1. Total phenolic content (TPC) of broccoli extracts, using different solvents, at different temperatures*

Temperature	**TPC (µg GAE/ g DW)**			
(°C)	**Hexane**	**Acetone**	**Water**	**Methanol**
55	30.802 ± 0.066^{A}	338.438 ± 0.066^{A}	479.072 ± 0.051^{A}	509.836 ± 0.014^{A}
	39.592 ± 0.089^{B}	391.176 ± 0.078^{B}	821.866 ± 0.046^{B}	3173.084 ± 0.074^{B}
	30.802 ± 0.049^{C}	386.781 ± 0.231^{C}	496.651± 0.114^{C}	751.55 ± 0.176^{C}
65	30.802 ± 0006^{A}	360.412 ± 0.133^{A}	492.256 ± 0.031^{A}	553.784 ± 0.009^{A}
	43.987 ± 0.018^{B}	650.469 ± 0.106^{B}	843.840 ± 0.016^{B}	3278.560 ± 0.010^{B}
	35.197 ± 0.005^{C}	470.282 ± 0.045^{C}	514.230 ± 0.028^{C}	988.869 ± 0.034^{C}
75	35.197 ±0.076^{A}	435.124 ± 0.022^{A}	509.836 ± 0.113^{A}	646.074 ± 0.047^{A}
	48.382 ±0.051^{B}	694.417 ± 0.094^{B}	1169.056 ± 0.005^{B}	3335.692 ± 0.055^{B}
	43.987 ±0.096^{C}	505.441 ± 0.138^{C}	566.968 ± 0.023^{C}	1393.190 ± 0.031^{C}
85	39.592 ± 0.075^{A}	465.888 ± 0.132^{A}	654.864 ± 0.098^{A}	1002.053 ± 0.012^{A}
	52.776 ± 0.043^{B}	1010.843 ± 0.067^{B}	2316.098 ± 0.075^{B}	3643.328 ± 0.033^{B}
	48.382 ± 0.097^{C}	549.389 ± 0.114^{C}	1177.845 ± 0.081^{C}	1472.297± 0.025^{C}
95	39.592 ± 0.005^{A}	443.914 ± 0.048^{A}	672.443 ± 0.086^{A}	826.261 ± 0.101^{A}
	52.776 ± 0.027^{B}	668.048 ± 0.069^{B}	2491.890 ± 0.022^{B}	3282.954 ± 0.005^{B}
	48.382 ± 0.033^{C}	496.651 ± 0.206^{C}	1327.268 ± 0.051^{C}	1428.349 ± 0.038^{C}

^{A}: Broccoli stems; ^{B}: Broccoli leaves; ^{C}: Broccoli florets; GAE: gallic acid equivalent; DW: dry weight; * Each value was expressed by mean ± SD.

Figure 4.1-C shows the TPC behavior of the broccoli stems extracts, at 85°C, for 15 min, methanol, acetone, and hexane exhibited the highest TPC (1472.297 ± 0.025 µg GAE /g DW, 549.389 ± 0.114 µg GAE /g DW, and 48.382 ± 0.097 µg GAE /g DW respectively); whereas water exhibit the highest TPC (1327.268 ± 0.051 µg GAE /g DW) at 95°C, for 15 min. However, hexane showed the lowest TPC compared with the rest of the solvents, methanol increased the phenolic yield by 96.71% compared with hexane, water by 96.35%, and acetone by 91.19%.

Figure 4.2 represents the solvents behavior in the TPC of the broccoli extracts under different temperatures. Hexane behavior is shown in Figure 4.2-A, the TPC values are very similar between the broccoli samples (leaves, stems, and florets), the absorbance values vary between 0.061 to 0.066, and the phenolic yield decreases by more than 90% compared to the rest of the solvents; however, the highest TPC is also reported in the broccoli leaves, followed by broccoli florets, and broccoli stems. In terms of temperature, at higher temperatures the TPC increases; nevertheless, beyond 85°C the TPC remains constant in the hexane behavior.

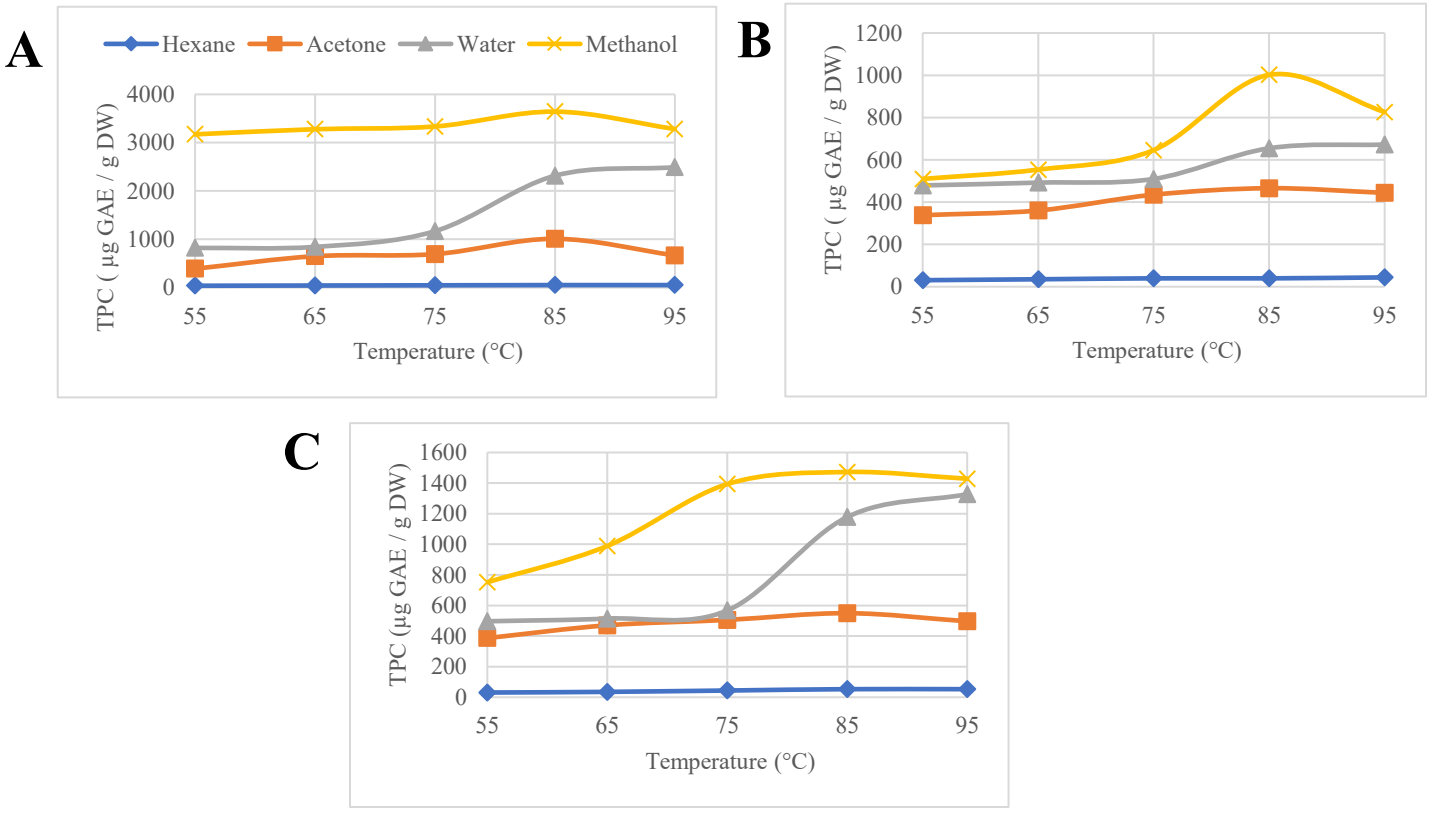


Figure 4. 1. Total Phenolic Content (TPC) behavior of the broccoli extracts using different solvents at various temperatures; **A:** TPC behavior of broccoli leaves extracts; **B:** TPC behavior

of broccoli stems extracts; **C:** TPC behavior of broccoli florets extracts; GAE: gallic acid equivalent; DW: dry weight.

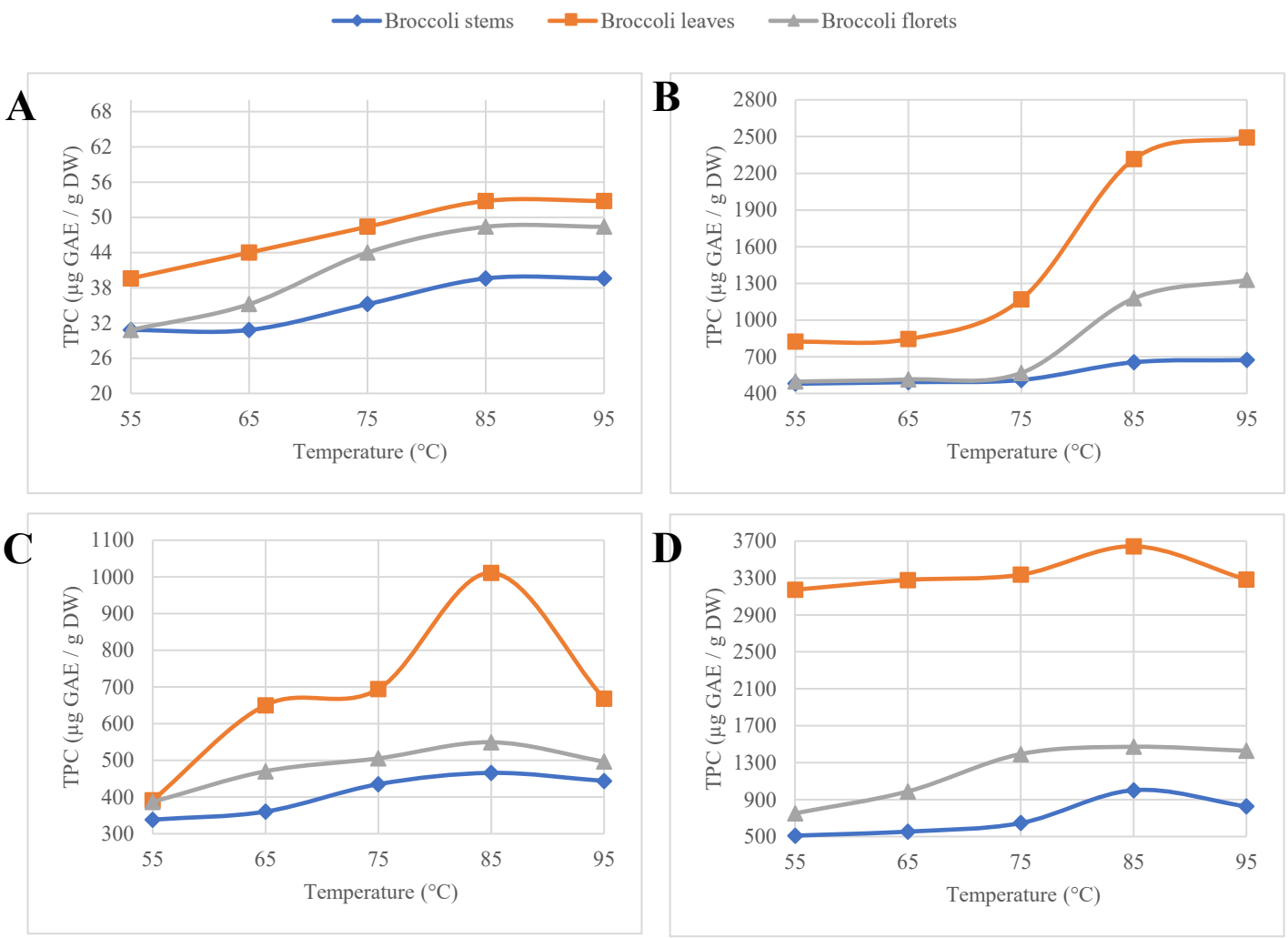


Figure 4. 2. Solvent behavior in the Total Phenolic Content (TPC) of broccoli extracts at different temperatures; **A:** Hexane; **B:** Water; **C:** Acetone; **D:** Methanol; GAE: gallic acid equivalent; DW: dry weight.

Water behavior in the TPC of the broccoli extracts is shown in Figure 4.2-B, broccoli leaves exhibited the highest TPC, followed by florets and stems. Furthermore, considering the same temperature conditions, broccoli leaves presented approximately double TPC content as broccoli stems and florets. TPC increases with increasing temperature, since the highest values of TPC in

the broccoli extracts were obtained at the highest temperature of 95°C and the lowest at 55°C. Figure 4.2-C shows the acetone behavior in the TPC of the samples, considering the same temperature conditions, broccoli stems exhibited the lowest TPC, and broccoli leaves the highest. The temperature enhances the TPC until it reaches 85°C, at temperatures above 85°C the TPC decreases, this behavior is presented in the three broccoli categories, leaves, stems, and florets.
The broccoli extracts that used methanol (Figure 4.2-D) exhibited higher amounts of TPC compared with the rest of the solvents. Under the same temperature conditions, broccoli leaves presented more than triple TPC than broccoli stems, and more than double TPC than broccoli florets. The lowest TPC was observed at 55 °C (the lowest temperature), as the temperature increases the TPC does, until reaching 85 °C, beyond that temperature the TPC decreases in the broccoli extracts regardless of the category.

4.5.2 Antioxidant Activity (AA) of broccoli samples: stems, leaves, and florets

The DPPH radical scavenging method was used to measure the AA of the broccoli extracts. The results of the mean total AA evaluation are shown in Table 4.2; the AA in the samples is expressed in µg of Trolox Equivalents (TE)/g of dry weight (DW) of the sample.

Table 4. 2. DPPH radical scavenging activities (AA values) for the broccoli extracts*

Broccoli extract	**AA (µg TE / g DW)**			
	Hexane	**Acetone**	**Water**	**Methanol**
Stems	55.789 ± 0.136	113.206 ± 0.035	162.010 ± 0.109	193.110 ± 0.096
Leaves	166.794 ± 0.115	180.191 ± 0.022	201.244 ± 0.087	210.813 ± 0.128
Florets	83.062 ± 0.092	124.211 ± 0.078	176.364 ± 0.028	205.550 ± 0.051

AA: antioxidant activity; TE: Trolox equivalents; DW: dry weight; * Each value was expressed by mean ± SD.

In the broccoli extracts the TPC was significantly correlated with DPPH assay ($p < 0.001$, $r = 0.886$). Some authors have presented similar correlations of TPC and AA in broccoli extracts (Domínguez-Perles et al., 2010; Hwang & Lim, 2015; Rodríguez García & Raghavan, 2022). Considering the same solvent selection, broccoli leaves exhibited the highest AA, followed by

florets, and stems. Among solvents methanol showed the highest AA results, followed by water, acetone, and finally hexane, regardless of the broccoli category.

4.6 Discussion

MAE (Mini-Wave operating at 2.45 GHz) consists of electromagnetic waves, those microwaves are made up of an electric and a magnetic field that oscillates at frequencies 2.45 GHz perpendicular to each other (Chan et al., 2011b). According to the findings, microwaves penetrate broccoli samples and generate heat by interacting with bioactive components for 15 minutes; other authors report comparable MAE timeframes for extracting phenolic chemicals (Chan et al., 2011b; S. S. Ferreira et al., 2018; Kaderides et al., 2019; Rodríguez García & Raghavan, 2022). The phenolic extraction yield depends on several factors, such as the time, solvent selection/concentration, and temperature. Under the same temperature, and solvent conditions, broccoli leaves exhibited the highest TPC (3643.328 ± 0.033 µg GAE/ g DW at 85°C with methanol) among broccoli florets (1428.349 ± 0.038 µg GAE/ g DW at 95°C with methanol), and stems (1002.053 ± 0.012 µg GAE/ g DW at 85°C with methanol) (Table 4.1 and Figure 4.1), other authors reported similar TPC behavior, in the extraction of phenolic compounds from broccoli, since generally the outer parts of the plants, such as leaves, are exposed to a stressful environment, resulting in the induction of the second metabolism (Faller & Fialho, 2010), and therefore increasing the response of phenolic production. (Rodríguez García & Raghavan, 2022) reported 1940.35 µg GAE/ g DW for broccoli leaves, 657.06 µg GAE/ g DW for broccoli florets, and 5.27 µg GAE/ g DW for broccoli stems. (Liu et al., 2018) reported 4140 µg GAE/ g DW for broccoli leaves, 2510 µg GAE/ g DW for broccoli florets, and 1410 µg GAE/ g DW for broccoli stems, (Hwang & Lim, 2015) presented 215.6 mg GAE/ 100 g DW for broccoli stems, 1310 mg GAE/ 100 g DW for broccoli leaves, and 528.9 mg GAE/ 100 g DW for broccoli florets.

The variation in the results among authors is due to the different methods of extraction, time, temperature, and solvent selection/concentration employed, e.g., (Liu et al., 2018) employed solid liquid extraction, at 75°C, with methanol as solvent; (Rodríguez García & Raghavan, 2022) used MAE, with methanol at lower concentrations (80 % v/v), and lower temperatures (73-75°C); (Hwang & Lim, 2015) employed maceration extraction with 80% methanol, for 24 h. Although the conventional extraction methods, such as maceration and solid-liquid extraction have some advantages in the highest recovery of TPC, and use of large amount of sample, they have several

disadvantages compared with MAE, as they need significant amounts of solvent and longer extraction times (Sun & Kee Lee, 2002).

4.6.1 Effect of solvent selection on the Total Phenolic Content (TPC) of broccoli extracts

It is known that MAE may be used to extract polar and ionic molecules as well as non-polar analytes from solid matrices (Rodríguez García & Raghavan, 2021). In the experiment (Figure 4.1), polar solvents (methanol, water, and acetone) proved to be more efficient to increase the TPC in the broccoli extracts compared with non-polar solvents (hexane), this can be explained by the dielectric properties of the solvents. The dissipation factor (determined by the dielectric constant of the solvent (ε'), which reflects its capacity to be polarised by an electric field, and the dielectric loss factor, which is related to the conversion efficiency of electromagnetic energy into heat), determines the ability of a solvent to absorb microwave energy by converting it to heat (Rodríguez García & Raghavan, 2021; Vernès et al., 2019). The greater the dielectric constant for a given frequency, the more thermal energy is generated, and faster the heating (Sun & Kee Lee, 2002). As a result, polar solvents have high dielectric constants, meaning that they strongly absorb microwaves; solvents such as water (ε'=76.7 at 25°C and 3 GHz), methanol (ε'=32.63 at 25°C and 3 GHz), and acetone (ε'=21.01 at 25°C and 3 GHz), are preferred for enhancing microwave energy absorption, compared with the non-polar solvents, such as hexane (ε'=1.89 at 25°C and 3 GHz) (Sun & Kee Lee, 2002).

The higher the solvent's dielectric constant, the faster it heats up under microwave irradiation. Therefore, hexane, having such a low dielectric constant, is not heated up, so the extraction of phenols is not efficient, resulting in the lowest TPC values. Methanol and water were the best solvents for increasing the TPC in the broccoli extracts. Other authors, (Jokić et al., 2012; Sagar et al., 2018; Thomas et al., 2018) also prefer to use methanol, and water, to enhance the absorption of the microwave energy; however, due to their naturally high boiling temperatures, some solvents with high dielectric constants, such as water, make the following concentration step difficult, due to polar co-extractives, and low extraction selectivity (C. M. Galanakis et al., 2013; Sun & Kee Lee, 2002).

Although water has the largest dielectric constant among the other solvents, its dissipation factor is low (Mendes et al., 2016), hence water absorbs microwave energy at a faster rate than the system dissipates heat. These effects explain the "superheating" effect that occurs when water is employed

as the only solvent. The solubility of phenolic compounds in the different solvents can also be explained by their stereochemistry (C. M. Galanakis et al., 2013), (the polar and non-polar fragments inside their molecules) and intermolecular forces (mostly hydrogen bonds) between them and the solvents. For example, the hydroxyl groups of phenols can form hydrogen bonds with the electronegative oxygen of methanol, water, and acetone explaining why they prefer polar protic solvents (methanol, water) over polar aprotic solvents (acetone) (C. M. Galanakis et al., 2013).

(Zaidel et al., 2019) states that the selected solvent should have a high dielectric constant and be able to absorb a great amount of microwave radiation that can heat solvents like acetone, methanol, and water since they are sufficiently polar. Hexane for example, is not an appropriate solvent for MAE since it is a non-polar solvent and has low dielectric constant; mixtures of water and hexane may help to increase the heating rate and speed up the extraction process.

4.6.2 Effect of temperature variation on the Total Phenolic Content (TPC) of broccoli samples

One of the most important criteria to achieve high TPC following the MAE is temperature. When MAE is carried out in closed containers, as in this work, the temperature can increase above the solvent's boiling point, resulting in quicker and more effective extraction. (Mendes et al., 2016). However, longer exposure times (above 20 minutes) at high temperatures, destroy the phenolic compounds, lowering the extraction yield in the microwave field (Kaderides et al., 2019). That was the case with the methanol, and acetone, at higher temperatures the TPC increased in all broccoli categories, florets, stems, and leaves (Figure 4.2-C, and Figure 4.2-D), but beyond 85°C the TPC decreases, indicating a phenolic degradation, due to higher temperatures. In the case of the water no phenolic degradation was observed, (Figure 4.2-B), since the TPC continued to increase as the temperature increased, and the highest TPC value was reported at 95°C.

(Mendes et al., 2016) reported similar findings using water and methanol as solvents and observing a decrease in phenolic yield with increasing temperatures in a MAE optimization. (Xiao et al., 2008) also discovered that temperatures beyond 110°C (with different concentrations of alcohols as solvents) decreased the phenolic extraction yield. A study made by (Liazid et al., 2007), showed the stability of phenolic compounds during MAE with methanol as the extraction solvent and temperatures ranging from 50 to 175 °C; the study demonstrates that as the temperature rises, the

phenolic compounds degrade significantly, and MAE is viable for extracting phenolics at temperatures equal to or less than 100 °C for 20 min.

It is common that higher temperatures enhance the phenolic extraction, since as the temperature rises, surface tension and solvent viscosity decrease, facilitating sample wetting and matrix penetration; however, higher temperatures may cause phenolic compounds to degrade, lowering extraction yield efficiency and bioactivity, furthermore, many phenolic compounds can be readily hydrolyzed and oxidized at higher temperatures. (Ghafoor et al., 2019; Mendes et al., 2016). The phenolic degradation due to high temperatures varies depending on the solvent used, the boiling point of the solvent, and their interaction in the microwave system (Sun & Kee Lee, 2002). In the case of hexane (Figure 4.2-A), the TPC was slightly affected by the increment of temperature; however, beyond 85°C the TPC remained constant.

(C. M. Galanakis et al., 2013) studied the behaviour of water, methanol, acetone (polar solvents), and diethyl ether (non-polar solvent), in the recovery of phenolics, and reported that phenolic coefficients increased in polar protic solvents (methanol, water), and aprotic solvent (acetone), as a function of temperature, but not varied in non-polar solvents, which agrees with the results reported in this study. (Maillard & Berset, 2002) proposed three methods to explain phenolic behaviour at high temperatures. When the lignin connections between phenolics are disrupted, the insoluble phenolic compounds may be liberated. Second, high temperatures can cause lignin to breakdown, releasing additional phenolic compounds, this might explain why the TPC rises as the temperature of the extractions rises. Finally, the phenolic compounds may be thermally degraded at higher temperatures. (Chan et al., 2011b) suggest adding vacuum pressure in MAE to reduce the related boiling temperature of the solvent, which reduces the danger of thermal degradation and oxidation of the active compounds. The highest temperature that a MAE system may run at is governed by the boiling point of the solvents at atmospheric pressure, this is an important factor to avoid thermal degradation of the bioactive compounds (Zaidel et al., 2019).

4.6.3 Antioxidant Activity (AA) evaluation of broccoli extracts

According to Table 4.2, the broccoli leaves presented the highest AA, followed by broccoli florets, and broccoli stems, this can be explained since the antioxidants play a protective role in the outer regions of broccoli, so the leaves may present higher TPC, which is related to the antioxidant potential of the plant to protect it to a stressful environment (Wijngaard et al., 2009). Other authors

reported similar behaviors of AA, (Rodríguez García & Raghavan, 2022) reported an AA of 632.057 µg TE/ g DW for broccoli leaves, 290.973 µg TE/ g DW for broccoli florets, and 193.110 µg TE/ g DW for broccoli stems, using the DPPH method. According to (M. Shi et al., 2019) a mix of broccoli leaves, and stems has an AA of 77.84 mg TE/ g DW, whereas broccoli florets have an AA of 51.06 mg TE/ g DW.

A direct link between antioxidant activity and phenolic content of plant extracts has been discovered in several research (Hatami et al., 2014; Tung et al., 2011). Free radicals are scavenged by antioxidant molecules such as phenolic acids, and flavonoids, blocking the oxidative pathways that lead to degenerative illnesses (Mahdi-Pour et al., 2012). Methanol extracts exhibited the highest AA, among other solvents, which was directly correlated to the TPC, hence the methanol extracts have a higher ability to scavenge free radicals compared to water, acetone, and hexane extracts.

According to (Ghafoor et al., 2019) the phenolic compounds are directly associated to AA rather than flavonoids or other compounds; however, they are not the only ones responsible of the AA of broccoli extracts. Temperature, location, genotype, plant part, and season of cultivation can all affect the chemical composition of broccoli and therefore the AA. The positive correlation between TPC and AA support the theory that phenolic molecules may be the primary contributors to the AA of broccoli extracts.

4.7 Conclusions

Using pure methanol, water, acetone, and hexane as solvents, the MAE of broccoli by-products (stems and leaves) and edible portions (florets) was realized to get the TPC and AA of the extracts at temperatures ranging from 55°C to 95°C. The polar solvents (methanol, water, and acetone) proved to recover higher amounts of TPC and AA, compared with the non-polar solvent (hexane) in all broccoli extracts, under the same temperature conditions, this behaviour was due to the dielectric properties of the solvents, the higher the solvent's dielectric constant, the faster it heated up under microwave radiation, thus, polar solvents have higher dielectric constants compared with non-polar solvents, so they enhanced the microwave energy absorption resulting in higher phenolic extraction yields. In all broccoli extracts, methanol extracts had the greatest TPC and AA, followed by water, acetone, and hexane. Broccoli leaves had the greatest levels of TPC and AA (3643.328 ± 0.033 µg GAE/ g DW, and 210.813 ± 0.128 µg TE / g DW respectively, at 85°C with methanol)

followed by broccoli florets (1428.349 ± 0.038 µg GAE/ g DW, and 205.550 ± 0.051 µg TE / g DW respectively, at 95°C with methanol), and broccoli stems (1002.053 ± 0.012 µg GAE/ g DW, and 193.110 ± 0.096 µg TE / g DW respectively, at 85°C with methanol). The TPC and AA of broccoli extracts were shown to have a positive correlation, with the greater the TPC, the higher the AA. TPC values in the extracts rose as the temperature in MAE increased; however, phenolic compounds were thermally degraded in methanol and acetone at temperatures over 85°C for all the broccoli extracts. Not only the broccoli florets proved to contain significant amounts of TPC and AA, but also the broccoli by-products (leaves, and stems), this represents an alternative for repurposing and valorizing broccoli by-products. The chemical structure and polarity of phenolic compounds of each broccoli sample determines their extractability and efficiency in the different types of solvents, the effects of temperature, and time of extraction; therefore, a future approach can be an optimization study under different MAE conditions, to obtain the optimal values among different solvent, solvent concentrations, and temperatures.

Connecting text

Chapter IV showed that polar solvents enhanced the total phenolic response in the broccoli extracts, hexane exhibited the lowest antioxidant activity and phenolic response, while methanol exhibited the highest. Furthermore, temperatures above 85°C (if using methanol, or acetone) cause thermal degradation of the phenolic compounds.

Finally, Chapter V, summarizes and discusses the global and final findings of the project, it also presents the contribution to knowledge of this thesis, and the future research recommendations.

CHAPTER V.
CONCLUDING REMARKS

5.1 General Summary and conclusions

The main purpose of this thesis consisted of a literary review of the unconventional techniques for the extraction of bioactive chemicals, especially from by-products from fruits and vegetables. Methanol concentration, temperature, and time were evaluated in the MAE process to extract phenolic compounds from broccoli florets, stems, and leaves. Furthermore, the behavior of various solvents such as hexane (non-polar), methanol, acetone, and water (polar) at different raising temperatures was evaluated.

Firstly, in Chapter II, a bibliographic review of the main unconventional extraction techniques, also known as green extraction techniques, to extract different BC from FVW, was discussed; the revaluation of the by-products represents an alternative for sustainable waste management and promoting food security. Several BC, extracted from plant sources (including fruits and vegetables by-products) such as phenolics, carotenoids, sterols, and flavonoids, are used in different consumer industries for their antioxidant, antimicrobial and even coloring properties, for example, to retard the spoilage of certain foods or improve their sensory attributes. Green extraction techniques such as MAE, UAE, and SFE have been studied to make the recovery process of BC from FVW more efficient, since they present more advantages in terms of cost, time, degradation of thermolabile compounds, and amount of solvent used, compared to conventional techniques such as maceration or soxhlet extraction. Among the non-conventional extraction techniques, MAE presents an efficient extraction of BC, in a very short period, with high recovery rates (Chapter II). However, there are several factors affecting the BC recovery in the MAE process, such as time, temperature, solute-solvent affinity, solvent concentration, among others, therefore, this chapter laid the foundations for the establishment of the following steps of the research project.

Among the FVW, broccoli (*Brassica oleracea*), is a vegetable of global importance with a high waste level, including leaves and stems, which still contain a significant amount of BC, mainly phenolic compounds, in Chapter III, the optimization of the operation variables of the MAE was presented to recover phenolic compounds the broccoli leaves, stems, and florets; the operational variables were selected according to what was established in the literature review (Chapter II). Temperature, methanol concentration, and time were the operational variables selected for the

optimization of the MAE, an RSA with CCRD was used for the analysis and optimization of the process. The three variables were put in range to maximize the TPC of the three broccoli extracts (including leaves, stems, and florets), a second-order polynomial regression model was obtained, and the optimum operational variables were (methanol concentration, time, and temperature respectively): 80%, 10 min, 73.27°C (broccoli leaves), 74.54%, 15.9 min, 74.45°C (broccoli stems), and 80%, 18.9 min, 75°C (broccoli florets); under these conditions the broccoli leaves (1940.35 ± 0.794 µg GAE/g DW TPC, 632.057 ± 0.087 DPPH µg TE/g DW, and 1034.220 ± 0.324 ABTS µg TE/g DW) exhibited the greatest TPC and AA amounts, followed by the broccoli florets (657.062 ± 0.771 µg GAE/g DW TPC, 290.973 ± 0.669 DPPH µg TE/g DW, and 452.169 ± 0.093 ABTS µg TE/g DW), and broccoli stems (225.273 ± 0.897 µg GAE/g DW TPC, 193.110 ± 0.415 DPPH µg TE/g DW, and 212.118 ± 0.213 ABTS µg TE/g DW). Generally, the superficial or external parts of the plants (e.g., leaves) tend to present a greater number of phenolic compounds, since its secondary metabolism is induced due to stress responses related to environmental conditions (Chapter III), which generates an increase in the production of BC, including phenolic compounds, and therefore, a greater antioxidant activity, related to these compounds (Faller & Fialho, 2010). Moreover, MAE remarkably increased the TPC, and the phenolic yield values of the broccoli extracts compared to the maceration extraction in a shorter period. MAE proved to be an efficient green extraction technique to obtain phenolics. Furthermore, several phenolic acids were identified in the broccoli by-products, with HPLC method, such as vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids.

Finally, Chapter IV, discussed the behavior of some of the main factors affecting the MAE process of the broccoli samples, such as the temperature and solvent selection. In Chapter IV, the MAE of broccoli leaves, stems, and florets was carried out, with temperatures from 55°C to 95°C, using different polar (water, methanol, and acetone) and non-polar (hexane) solvents. Under the same temperature conditions, the polar solvents (acetone, water, and methanol) exhibited higher TPC and AA than hexane. This behavior was caused by the dielectric characteristics of the solvents; the higher the dielectric constant of the solvent, the quicker it heated up under microwave radiation (Sun & Kee Lee, 2002; Zaidel et al., 2019); hence, polar solvents having larger dielectric constants than non-polar solvents exhibit greater extraction yields. Methanol proved to exhibit the highest amount of TPC and AA among the rest of the solvents, 3643.328 ± 0.033 µg GAE/ g DW, 210.813 ± 0.128 µg TE / g DW respectively, at 85°C for broccoli leaves; 1428.349 ± 0.038 µg GAE/ g

DW, 205.550 ± 0.051 µg TE / g DW respectively, at 95°C for broccoli florets; and 1002.053 ± 0.012 µg GAE/ g DW, 193.110 ± 0.096 µg TE / g DW respectively, at 85°C for broccoli stems; furthermore, regardless of solvent selection, the broccoli leaves exhibited the greatest TPC and AA, and the broccoli stems the lowest, under the same temperature conditions. TPC in the extracts increased with the increase of temperature in the extraction; however, phenolic compounds were thermally degraded in methanol and acetone at temperatures over 85°C for all broccoli extracts. The polarity and chemical structure of phenolic compounds in each broccoli sample impacted their extractability and effectiveness in different types of solvents, as well as the effects of temperature and extraction time.

The research presented in this thesis demonstrated the effect of time, temperature, solvent concentration, and solvent selection in MAE of broccoli stems, leaves, and florets to recover phenolic compounds, it is demonstrated that the broccoli by-products exhibit not only similar TPC, and AA compared with broccoli edibles, but also broccoli leaves exhibited the highest phenolic content and AA among the extracts, regardless of the solvent selection, suggesting a potential method for repurposing broccoli wastes. Moreover, the optimization of the MAE process establishes a solid foundation for the extraction of phenolic compounds from broccoli waste, thus the main objective of this research was met.

5.2 Contribution to knowledge

This study emphasizes the main non-conventional techniques of recovery of different bioactive chemicals from alternative plant sources, such as vegetable and fruit by-products, offering a comprehensive analysis of the main sources of bioactive compounds among FVW, the different secondary metabolism compounds found in them, and the trends in the extraction. Furthermore, this thesis proposes that methanol concentration, temperature, and time, are crucial to improving and optimizing the MAE to maximize the total phenolic content from broccoli leaves, stems, and florets.

MAE optimization of broccoli leaves, stems, and florets was performed, demonstrating that broccoli leaves exhibit greater amounts of phenolic content and antioxidant activity compared to the edible broccoli parts, maximizing their potential and use in further applications, such as food, cosmetic, or pharmaceuticals industries. Moreover, MAE exhibited higher phenolic yields in less time in comparison with maceration extraction, for the broccoli extracts. Polar solvents proved to

be more efficient for the phenolic compound recovery from broccoli samples compared to non-polar solvents, such as hexane; moreover, it is recommended that temperatures beyond 85°C are avoided if methanol or acetone are used as extraction solvents in MAE.

Finally, the recovery of phenolic compounds and antioxidant activity evaluation from the broccoli leaves, and stems is an example of the great potential of reuse and vegetable by-product management, which could be a viable option for revalorization due to high waste generation of broccoli, and its worldwide production.

5.3 Future work recommendations

Although this thesis provides different contributions to the knowledge of MAE and its optimization to extract phenolic compounds from broccoli stems, leaves and florets, more research is needed, some recommendations for future research are listed below:

- The optimization of the parameters of temperature, extraction time, and methanol concentration in the MAE to maximize the response of total phenolic content in the broccoli extracts, was carried out through RSA; however, it is recommended to add other types of polar solvents as variables to the optimization process for future studies and observe their behavior in the total phenolic response.
- It is necessary to compare the MAE process against other non-conventional techniques of extraction, such as UAE, and SFE, to provide a better understating of the phenolic behavior in the broccoli stems, leaves, and florets, and to establish the main variables and differences in the extraction processes.
- It is recommended to extend the analysis of the bioactive compound composition of broccoli by-products, by analyzing the flavonoid, carotenoid, and sterol content, after the MAE.
- It is recommended to follow the purification process of broccoli extracts, and observe their bioactivity, bioavailability, and stability for further applications in consumer industries, such as food or cosmetic.

CHAPTER VI. REFERENCESS

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