

VALORIZATION OF *ACER SACCHARUM* LEAVES BIOMASS

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April 2024

A Thesis submitted to McGill University in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

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I would like to dedicate this thesis to every moment I lived, to every crisis I faced, to every success I achieved, to every lesson I learned, and to every good soul I met.....

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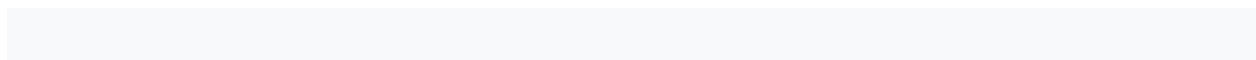
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List of Abbreviations

AOX	Antioxidant
ATR/FTIR	Attenuated total reflectance/Fourier transform infrared
BD	Bulk density
CI	Carr index
CTE	Catechin equivalent
CPAE	Cold plasma-assisted extraction
CIELab	Commission Internationale d'Eclairage
DW	Dry weight
DSC	Differential scanning calorimeter
DM	Dry matter
DOE	Design of experiment
DPPH	2,2-diphenyl-1-picrylhydrazyl
DESAE	Deep Eutectic Solvent-assisted Extraction
EAE	Electrically-assisted extraction
ESI	Electrospray ionization
EY	Encapsulation yield
EE	Encapsulation efficiency
EC	Emulsifying capacity
ES	Emulsifying stability
FC	Foaming capacity
FS	Foaming stability
FD	Fractal dimension
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GE	Green extraction
GC-MS	Gas chromatography
HPLC	High performance liquid chromatography
H ₀	Surface hydrophobicity
HE	Homogenization
HR	Hausner ratio
HVED	High voltage electrical discharge
HPAE	High pressure assisted extraction
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
LC	Loading capacity
MhPAE	Medium-to-high pressure assisted extraction
MAE	Microwave-assisted extraction
MS	Mass spectrometry

NIR-spectroscopy	Near infrared spectroscopy
OFAT	One factor at a time
OHC	Oil holding capacity
PCs	Phenolic compounds
PEFAE	Pulsed electric field assisted extraction
PAE	Pressure-assisted extraction
PDI	Poly dispersity index
RI	Refractive index
SFE	Super critical fluid extraction
SML	Sugar maple leaves
SMB	Sugar maple bark
SMF	Sugar maple fruit
SMFL	Sugar maple flower
SMT	Sugar maple twig
SMS	Sugar maple seed
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSF	Simulated salivary fluid
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPME	Solid-phase microextraction
SO	Surface opening
SEM	Scanning electron microscopy
SFE	Super critical fluid extraction
TD	Tapped density
TGA	Thermogravimetric analysis
TPC	Total phenolic content
TFC	Total flavonoid content
TOF-MS	Time of flight-mass spectrometer
TE	Trolox equivalent
UAE	Ultrasound-assisted extraction
UPLC	Ultra performance liquid chromatography
UV-Vis	Ultraviolet-visible
WHC	Water holding capacity

ABSTRACT

An essential component of the global strategy to attain sustainable and circular agri-food industrial economies involves the use of forestry biomass as feedstock to extract natural functional ingredients. The maple tree, a recognized symbol of Canada, makes up approximately 3% of Canada's total forests, produces huge amount of leafy biomass every year which are considered as waste and upon disposal pose some environmental issues.

Amongst the popular plant-based functional ingredients, phenolic compounds have seen increased demand in the food, nutraceutical, pharmaceutical, and cosmetic sectors. Extraction of phenolic compounds from leafy biomass was therefore investigated first with a comprehensive literature review. Based on this literature review, it may be concluded that the biomass of sugar maple (*Acer saccharum*) leaves can be a sustainable and renewable source of phenolic compounds. For this purpose, a maceration process was optimized using a full factorial design coupled to a response surface model for the extraction of phenolic compounds from sugar maple leaves, where characterization of sugar maple leaves was performed using colorimeter, scanning electron microscope, Fourier transform infrared spectroscopy, and liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry. It was found that sugar maple leaves contain a considerable amount of phenolic compounds having interesting antioxidant activities. Following this, the effect of the environmental conditions and plant morphological parts on the phenolic accumulation in sugar maple were investigated, followed by the encapsulation process of phenol rich sugar maple leaves extracts. It was established that apart from the phenolic compounds, sugar maple leaves can be additionally a potential source of plant-based protein. Therefore, alkaline-based homogenization pretreated ultrasound-assisted extraction, of protein from SML was performed together with their physical, structural, thermal, chemical, volatile, and functional attributes' characterization. It was found that sonication enhanced the particle breakdown and thermal stability as well as modified the protein structural conformation.

RÉSUMÉ

La stratégie mondiale visant à parvenir à une économie industrielle agroalimentaire durable et circulaire, devra inclure l'utilisation de la biomasse forestière comme matière première pour en extraire des ingrédients fonctionnels naturels. L'érable, un important symbole reconnu du Canada, représente environ 3 % du total des arbres du Canada, et il produit chaque année une grande quantité de biomasse feuillue qui est considérée comme un déchet et, une fois éliminée, peut présenter certains problèmes environnementaux.

Parmi les ingrédients fonctionnels d'origine végétale les plus populaires, les composés phénoliques connaissent une demande croissante de la part des secteurs alimentaire, nutraceutique, pharmaceutique et cosmétique. L'extraction de composés phénoliques à partir de la biomasse feuillue a donc d'abord été envisagée à l'aide d'une revue de la littérature. De cette revue, nous retenons que la biomasse feuillue de l'érable à sucre (*Acer saccharum*) peut être considérée comme une source renouvelable de composés phénoliques.

À cet effet, un procédé de macération a été optimisé grâce à un plan factoriel jumelé à un modèle de surface de réponse pour l'extraction des composés phénoliques des feuilles d'érable à sucre et la caractérisation a été effectuée par colorimétrie, microscopie électronique à balayage, spectroscopie par transformée de Fourier et chromatographie liquide et spectrographie de masse quadrupolaire.

Il a été constaté que les feuilles d'érable à sucre contiennent une quantité considérable de composés phénoliques ayant des activités antioxydantes. Par la suite, l'effet des conditions environnementales et des parties morphologiques de la plante sur l'accumulation phénolique dans l'érable à sucre a été étudié, suivi d'un procédé d'encapsulation des extraits de feuilles d'érable à sucre riches en phénol.

Il a été établi qu'outre les composés phénoliques, les feuilles d'érable à sucre peuvent également constituer une source potentielle de protéines végétales. Par conséquent, une extraction assistée par ultrasons prétraitée par homogénéisation alcaline, des protéines des feuilles d'érable a été réalisée ainsi que la caractérisation de leurs attributs physiques, structurels, thermiques, chimiques, volatils et fonctionnels. Il a été constaté que la sonication améliorait la rupture des particules et la stabilité thermique, ainsi que modifiait la conformation structurelle des protéines.

ACKNOWLEDGEMENTS

أَقْرَأُ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ

Iqra bismi rab bikal lazee khalaq

'Read in the name of your lord who created'

Alhamdulillah! I would like to begin by thanking IsDB-McGill Scholarship for granting the financial support to continue my Doctoral study at McGill University, Canada. I gratefully acknowledge the financial support of the Department of Bioresource Engineering, McGill University, for offering the Graduate Excellence Award and Fonds de Recherche du Quebec-Nature et Technologies (FRQnt), for offering the Doctoral Research Scholarship. I also would like to acknowledge the financial support of Natural Sciences and Engineering Research Council (NSERC).

I would like to thank my parents (Md. Noor Hossain & Rokeya Hossain) and family for their unconditional love and support that helped me reach where I am today. Words would be less to express my gratitude to my husband (Dr. Md. Hafizur Rahman Bhuiyan) for his continuous support, motivation, discussion, taking care of our daughter as well as myself. My heartfelt love to my brave girl (Iqra Yeasmen) for her understanding and patience. Mamma loves you a lot!

My heartfelt honor goes to my supervisor Dr. Valérie Orsat for her wisdom, instruction, guidance, motivation, energetic support, and student friendly nature throughout this PhD journey. Despite COVID pandemic and my first ever pregnancy journey, I never felt afraid because of her continuous support and guidance. I would also like to thank her for giving me the freedom to design my research project through which I have learned so many things.

I would like to express my heartiest gratitude to the members of my comprehensive committee, Dr. Vijaya Raghavan, Dr. Michael Ngadi, Dr. Saji George, and Dr. Marie-Josée Dumont for providing feedback and direction in shaping my project. I would also like to thank each of these professors, including Dr. Zhiming Qi for giving me access to their laboratory equipment.

My sincere thanks go to Mr. Yvan Gariépy for always been there whenever I needed. From equipment training to placing orders for chemicals, from technical discussion to problem solving, I got his spontaneous support all the time. On that note, I am grateful to Victoria Muise for showing her kindness with her expertise in setting up part of my experiment.

Last but not the least, I would like to show my sincere appreciation to my friends including Derek, Jolvis, Neha, Antar, Mina, Sofia, Sophie, Satwik, Estee, and Phillipe for making my life easier and enjoyable here at McGill University, Canada.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis introduces the possible conversion of forestry biomass namely *Acer saccharum* (sugar maple) leaves into functional ingredients for which pharmaceutical, nutraceuticals, cosmetics, and food industries have shown a great deal of interest. By combining the knowledge of green extraction (ultrasound-assisted extraction and microwave-assisted extraction), process optimization, and encapsulation, this PhD study attempts to assert this huge yearly available leafy biomass as a key contributor to the circular economy, to environmental sustainability, as well as to human health. The main highlights of added knowledge stemming from this whole work are as follows:

1. This work provides a comprehensive status of the recent progress, constraints, legislative framework, and future directions in green extraction and characterization of phenolic compounds from leaves.
2. This work unravels the major phenolic compounds present in sugar maple leaves biomass.
3. This work provides the optimum setup for maceration to extract phenolic compounds from sugar maple leaves biomass.
4. This study explains how the seasonal variation influences the phenolic biosynthesis throughout the annual growth cycle of leaves. Thus, providing useful insights into the optimum harvesting time of sugar maple leaves biomass in order to get maximum phenolic concentration while being in line with environmental sustainability.
5. Besides the leafy biomass, this work also provides information on the spatial distribution of the physicochemical, thermal, phytochemical, antioxidant, and antimicrobial attributes among different morphological parts namely flowers, fruits, seeds, barks, and twigs of sugar maple to give a complete view of the use of the different parts of sugar maple in purpose-based food ingredients.
6. This work demonstrates the efficient encapsulation method of phenol rich sugar maple leaves extract with their maximum retention, *in vitro* bioaccessibility, and shelf life.
7. This work demonstrates sugar maple leaves biomass as an alternative source of plant protein.

CONTRIBUTION OF AUTHORS

The format of this thesis is Manuscript-based, prepared by following the guidelines of the Faculty of Graduate and Postdoctoral Studies of McGill University. This thesis has generated eight (8) published manuscripts, co-authored by Nushrat Yeasmen and Valérie Orsat, where Yeasmen, N. is the principal author and Orsat, V. is the thesis supervisor. Their contributions are as follows:

Nushrat Yeasmen: Conceptualization, Experimental setup, Investigation, Data analysis, and Manuscript preparation.

Valérie Orsat: Supervision, Resources, Scientific guidance, and Manuscript reviewing & editing.

THESIS PUBLICATIONS IN PEER-REVIEWED JOURNALS

1. **Yeasmen, N., & Orsat, V. (2021).** Green extraction and characterization of leaves phenolic compounds: A comprehensive review. *Critical Reviews in Food Science and Nutrition*, 1-39. doi:10.1080/10408398.2021.2013771 [Chapter 2]
2. **Yeasmen, N., & Orsat, V. (2022).** Maximization of the recovery of phenolic compounds from sugar maple leaves. *Biomass Conversion and Biorefinery*, 1-16. doi:10.1007/s13399-022-02904-4 [Chapter 3]
3. **Yeasmen, N., & Orsat, V. (2023).** Phenolic mapping and associated antioxidant activities through the annual growth cycle of sugar maple leaves. *Food Chemistry*, 136882. doi:https://doi.org/10.1016/j.foodchem.2023.136882 [Chapter 4]
4. **Yeasmen, N., & Orsat, V. (2023).** Advanced separation of phytochemical and bioactive attributes from various morphological components of sugar maple (*Acer saccharum*) tree. *Food Bioscience*, 56, 103260. doi:https://doi.org/10.1016/j.fbio.2023.103260 [Chapter 5]
5. **Yeasmen, N., & Orsat, V. (2024).** Microencapsulation of ultrasound-assisted phenolic extracts of sugar maple leaves: Characterization, *in vitro* gastrointestinal digestion, and storage stability. *Food Research International*, 182, 114133. https://doi.org/10.1016/j.foodres.2024.114133 [Chapter 6]
6. **Yeasmen, N., & Orsat, V. (2024).** Pulsed ultrasound assisted extraction of alternative plant protein from sugar maple leaves: Characterization of physical, structural, thermal, electrical, and techno-functional properties. *Food Hydrocolloids*, 152, 109960. https://doi.org/10.1016/j.foodhyd.2024.109960 [Chapter 7]

And some additional manuscripts

7. **Yeasmen, N.**, Sharma, N., Bhuiyan, M. H. R., & Orsat, V. (2023). Ultrasound as a techno-functional modifier in food frying and bioactive compounds extraction. *Food Reviews International*, doi:10.1080/87559129.2023.2204156
8. **Yeasmen, N.**, Bhuiyan, M. H. R., & Orsat, V. (2021). Unravelling scientific research towards the green extraction of phenolic compounds from leaves: A bibliometric analysis. *International Journal of Food Science and Technology*, 56(10), 4893-4906. doi:10.1111/ijfs.15215

PART OF THIS THESIS PRESENTED AT SCIENTIFIC CONFERENCES

1. **Yeasmen, N.**, & Orsat, V. (2023/07). Spatial distribution of phenolic compounds and antioxidant attributes in *Acer saccharum*. American Society of Agricultural and Biological Engineers (ASABE) Annual International Meeting, Omaha, Nebraska, USA.
2. **Yeasmen, N.**, & Orsat, V. (2023/05). Unraveling scientific research towards the green extraction of phenolic compounds from leaves: A bibliometric analysis. Green Food Tech, Montreal, Quebec, Canada.
3. **Yeasmen, N.**, & Orsat, V. (2022/07). *Acer saccharum* leaves as source of bioactive compounds: Effect of harvesting time on phenolic content and antioxidant activity. Canadian Society for Bioengineering (CSBE) annual conference, Charlottetown, Prince Edward Island Canada.
4. **Yeasmen, N.**, & Orsat, V. (2021/07). *Acer saccharum* leaves: Effect of extraction parameters and pretreatment condition on the recovery of phenolic compounds and their antioxidant activities. American Society of Agricultural and Biological Engineers (ASABE) Annual International Meeting, USA.

CHAPTER 1: GENERAL INTRODUCTION

1.1. BACKGROUND

The genus *Aceraceae*, commonly known as maple, consists of more than 200 species which are distributed widely in temperate zones of the northern hemisphere. Among them *Acer saccharum* (sugar maple) is an ecologically and economically important deciduous hardwood species of eastern-North America (Geoffroy et al., 2017; St Clair et al., 2008). It grows best in a well-drained loamy soil rich in organic matter, although it can tolerate some sand, rock, or clay in the soil (Geoffroy, Fortin, & Stevanovic, 2017). The tree is dormant during the winter, while in the spring it starts to grow bright red twigs on the branches which will turn into flowers. The flowers are in panicles of five to ten together, which occur in early spring and the fruit is a pair of samaras (winged helicopter seeds) (Geoffroy, Fortin, & Stevanovic, 2017; St Clair, Sharpe, & Lynch, 2008). In the fall, green leaves turn yellow, orange, and red due to increasing contents of carotenoids and anthocyanin pigments and loss of chlorophyll (Apostolidis et al., 2012). Sugar maple tree is best known for the production of maple syrup, a natural sweetener obtained by concentrating the sap, which has been reported to have high nutritional values containing sugar, minerals, amino acids, vitamins, and phytochemicals (Bhatta et al., 2018). Maple syrup is commercially produced in Canada where 90% of its production comes from the province of Quebec (Sadiki & Martin, 2013; Yuan et al., 2011a). Currently there are 5340 maple farms in Canada, with 4776 maple farms originating from Quebec (AAFC, 2021).

Different parts of this species such as its bark, shoots, and sap have been used by native north Americans in the treatment of various ailments such as diarrhea, cramps, measles, backache, diuretic, cataracts, eyewash, and shortness of breath. This was attributed to the presence of a wide array of functional components like phenolic compounds (Bhatta et al., 2018; Bi et al., 2016). Despite the availability of scientific documentation identifying several antioxidant rich bioactive compounds, such as phenolic compounds (PCs) from sugar maple syrup, sap, bark, and wood (Li & Seeram, 2010; Yoshikawa et al., 2011; Yoshikawa et al., 2013; Yuan et al., 2011a), the biomass of sugar maple leaves (SML) is yet underexploited and considered as natural waste biomass which is why in urban and peri-urban areas, maple leaves are either ground to make compost or burnt directly. As a consequence, economic (disposal/handling of biomass represents a cost to the local government) and environmental problems (such as greenhouse gas emissions, air pollution, loss of soil moisture, loss of balance in ecosystem, and others) have evolved (Contreras et al., 2019;

Saini et al., 2019). According to NFI (2021), over the past ten years, maple generated biomass amounted to 1136 million tonnes in Canada. Given this exposition, recovery, and valorization of SML biomass would alleviate the waste disposal problems which in turn will reduce environmental pollution in addition to providing functional and healthy ingredients, such as PCs and proteins (Khan et al., 2023). Green leaves have been considered as an alternative protein source since the 1960s, that can be used as food and animal feed (Pérez-Vila et al., 2024; Zhang et al., 2014; Zhang et al., 2018a). The successful isolation of leafy protein with comparable techno-functionalities (Akyüz & Ersus, 2021; Cheng et al., 2021; Kaur & Bhatia, 2022; Vera-Salgado et al., 2022; Zhang et al., 2015) indicates the potentialities of forestry leaves biomass (e.g., SML) as an alternative protein source, which has not been fully explored yet.

In terms of sustainability, assessing the value of biomass-based raw materials has become an urgent need, therefore, there is a pressing need to evaluate forest residues that have no current economic value or may pose a threat to the environment (da Rosa et al., 2021). In this aspect, SML biomass, which represents 5% of the total maple tree, can be used for purposes that would be more profitable and ecofriendly. However, during the waste (SML biomass) to wealth conversion, the most profitable and sustainable challenges lie in the separation and recovery of the beneficial/useful compounds through extraction (Akhlish et al., 2021; Alanon et al., 2020; Cujic et al., 2016; Sandra et al., 2020). Therefore, it is of paramount importance to bring the information into light on how SML biomass can be sustainably and effectively managed to extract their intrinsic PCs and protein with maximum retention of their functional attributes.

Although extraction of natural origin compounds from waste products is a well-established procedure, the outcome i.e., yield recovery, is not always optimal because many methodologies are time, cost, and energy intensive. To address these limitations, development of a sustainable and resilient extraction technique by implementing the idea of green extraction is considered. The green extraction (GE) principle enlists the use of renewable plant sources, alternative and organic solvents, innovative extraction technologies, and process optimization while considering the near and far-future impact on the eco-system (Chemat et al., 2012; Choi & Verpoorte, 2019). To comply with the green principles, GRAS (generally recognized as safe) solvents should be used to guarantee environmentally friendly processes.

1.2. THESIS HYPOTHESES AND OBJECTIVES

1.2.1. Hypotheses

It is hypothesized that the sugar maple leaves (SML) biomass can be a promising source of phenolic compounds (PCs) and protein, (i) where green extraction methods, namely ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) could better be used to extract phenolics and protein with higher functionalities, and (ii) where harvesting time, plant morphological parts, and extracting conditions would have impact on the PCs and protein extraction efficiency and product quality.

1.2.2. Statement of research objectives

This thesis is focused on the valorization of sugar maple leaves biomass by combining the knowledge of green extraction (UAE and MAE), process optimization, and encapsulation. Towards achieving this goal, the specific objectives of this entire project are as follows.

1.2.2.1. Specific objectives

- (a) Optimization of maceration conditions for improving the extraction of PCs and antioxidant activities of SML biomass.
- (b) Investigation of the effect of seasonal dynamics on the phenolic composition and antioxidant activities of SML biomass.
- (c) Exploration of the spatial distribution of the physicochemical, thermal, phytochemical, antioxidant, and antimicrobial attributes among different morphological parts of SM.
- (d) Encapsulation and characterization of phenol rich SML extracts and quality assessment during storage.
- (e) Extraction of SML protein and assessment of its physical, structural, thermal, electrical, volatile, and functional qualities.

1.3. ORGANIZATION OF THESIS

This dissertation comprises nine chapters (introduction to bibliography) and appendix. Following introduction (Chapter 1), a comprehensive review on the current status of eco-friendly extraction of bioactive compounds from leaves along with their legislative framework was discussed in Chapter 2. In Chapter 3–7, the experiments and their results of specific objectives are presented. Connecting texts between the chapters are provided to clarify research transition and rationale between each experiment. Chapter 8 summarizes the general discussion and conclusion, drawn from this work, along with future recommendation that can be carried out further. This

thesis ends with a thorough bibliography (Chapter 9), permissions of all published articles, and other supplementary files. Throughout this thesis, The International System of Units (SI) is used.

CONNECTING TEXT TO CHAPTER 2

Chapter 2 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2021). Green extraction and characterization of leaves phenolic compounds: A comprehensive review. *Critical Reviews in Food Science and Nutrition*, 63(21), 5155-5193. doi:10.1080/10408398.2021.2013771

This chapter gives insights into the current status, constraints, and future directions in green extraction (GE) and characterization of phenolic compounds (PCs). In addition, a comprehensive summary of the application and advancement of GE techniques toward the extraction of PCs from leaves of different plant species is given. Finally, the legislative framework regarding the use/application of leaves PCs is discussed.

CHAPTER 2: GREEN EXTRACTION AND CHARACTERIZATION OF LEAVES PHENOLIC COMPOUNDS: A COMPREHENSIVE REVIEW

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2.1. ABSTRACT

Although containing significant levels of phenolic compounds (PCs), leaves biomass coming from either forest, agriculture, or the processing industry are considered as waste, which upon disposal, brings in environmental issues. As the demand for PCs in functional food, pharmaceutical, nutraceutical and cosmetic sector is escalating day by day, recovering PCs from leaves biomass would solve both the waste disposal problem while ensuring a valuable “societal health” ingredient thus highly contributing to a sustainable food chain from both economic and environmental perspectives. In our search for environmentally benign, efficient, and cost-cutting techniques for the extraction of PCs, green extraction (GE) is presenting itself as the best option in modern industrial processing. This current review aims to highlight the recent progress, constraints, legislative framework, and future directions in GE and characterization of PCs from leaves, concentrating particularly on five plant species (tea, moringa, stevia, sea buckthorn, and pistacia) based on the screened journals that precisely showed improvements in extraction efficiency along with maintaining extract quality. This overview will serve researchers and relevant industries engaged in the development of suitable techniques for the extraction of PCs with increasing yield.

Keywords

Alternative solvent assisted extraction, pressure assisted extraction, ultrasound assisted extraction, electrically assisted extraction, electromagnetically assisted extraction.

2.2. INTRODUCTION

Leaves, collectively known as foliage, are the lateral appendage of the vascular plant stem with or without stalks and functionally responsible for the plant photosynthesis. Apart from helping plants towards the storing of chemical energy for photosynthesis, leaves are also responsible for plant aesthetics. Regardless of being either deciduous or evergreen, once leaves detach from the plants it is considered as plant biomass (Madureira et al., 2020; Routray & Orsat, 2017; Selvamuthukumaran & Shi, 2017). Plant derived by-products range from 25-30%, with leaves being the major contributor, with on average 8 Mt/y of plant wastes being generated from agri-food processing industries globally (Ayala-Zavala et al., 2010; Selvamuthukumaran & Shi, 2017). The generated wastes are usually disposed-off by either burning or decomposing, giving rise to CO₂ emission, thereby, detrimental to the environment and causing wastage of natural resources, since leaves are one of the cheapest sources of phenolic compounds (Banozic et al., 2020;

Karageorgiou et al., 2017; Keshavarzi et al., 2020; Lama-Munoz et al., 2020; Rahmanian et al., 2015; Ueda et al., 2019; Wu et al., 2020).

Phenolic compounds (PCs), which are secondary metabolites found in plants with more than 8000 known structures identified so far, have been widely utilized in food, nutraceuticals and pharmaceutical industries due to their evident biological activities (Ajila et al., 2011; Banozic, Babic, & Jokic, 2020; Giacometti et al., 2018a; Gligor et al., 2019; Madureira et al., 2020; Patra et al., 2018; Wang & Zhang, 2021; Wang et al., 2020b; Zhang et al., 2020). For instance, natural bioactive compounds have been used to produce almost 60% of approved anti-cancer drugs (Zainal-Abidin et al., 2017). The demand for PCs in global markets was expected to reach USD 873.3 million in 2018 based on the study conducted by Transparency Market Research (Ameer et al., 2017b; TransparencyMarketResearch, 2016). Based on Grand View Research (2019), however, the global polyphenols market size was already worth USD 1.28 billion in 2018 and a Compound annual growth rate (CAGR) of 7.2% is expected to register from 2019 to 2025. According to them, the functional food and beverage industries are the largest sectors of the global market share (> 70%) of polyphenols followed by dietary supplements, cosmetics, and others (GrandViewResearch, 2019). Belwal et al. (2019b) also reported that the nutraceutical market share would increase through PCs derived biological materials. This trend towards the phenolic market might be due to the health consciousness of consumers and industrial growing interests for reusing and valorizing plant by-products and wastes respectively (Banozic, Babic, & Jokic, 2020; Bonifacio-Lopes et al., 2020; Odeleye et al., 2019).

Although valorization of several types of agricultural residues namely stalks, straws, hays, grass (Freitas et al., 2020; Parveen et al., 2008; Pinelli et al., 2008; Sanchez-Valdepenas et al., 2015); food processing wastes such as peels (Chan et al., 2009; Dailey & Vuong, 2015; Ferarsa et al., 2018; Karunanithi & Venkatachalam, 2019; Petkovska et al., 2016), pulps (Espada-Bellido et al., 2017; Mahdi et al., 2020; Maran et al., 2014a; Maran et al., 2014b; Marzuki et al., 2018; Mendez et al., 2014; Raja et al., 2014; Torres-Ossandon et al., 2018), seeds (Ismail et al., 2019; Natolino & Da Porto, 2020; Paludo et al., 2019; Rajha et al., 2020; Rodrigues et al., 2019; Zhu et al., 2019), pomace (Cheaib et al., 2018; Kheirkhah et al., 2019; Kumar et al., 2019; Nunes et al., 2018; Saad et al., 2019; Yammine et al., 2020), rinds (Baeri et al., 2018; Ebrahimi & Gashti, 2016; Feng et al., 2015; Geng et al., 2017; Ho et al., 2018; Mushtaq et al., 2015; Vigano et al., 2016), and stems (Alves et al., 2020; Apea-Bah et al., 2020; Nieto et al., 2020; Svarc-Gajic et al., 2018;

Tan & Ho, 2009; Wang et al., 2011; Yin et al., 2019; Yu et al., 2015), have gained considerable attention over the years, biomass obtained from leaves are comparatively underprivileged despite of their richness in PCs (Yeasmen et al., 2021). Till date only very few studies have been published to extract bioactive compounds from leaves (Alsaoud & Farid, 2020; Amaral et al., 2021; Chong et al., 2020; Djemaa-Landri et al., 2020; Dobrincic et al., 2020a; Essien et al., 2020; Gao et al., 2020; Goyeneche et al., 2020; Lopez-Hortas et al., 2020; Saifullah et al., 2020). In addition, considering the bottlenecks of conventional extraction methods for PCs, together with taking environmental sustainability and consumer demands into account, searching for eco-friendly, innovative, and cost-effective approaches, green extraction (GE) of PCs from leaves is gaining widespread attention among scientists as it is considered to be sustainable and eco-friendly.

To the best of our knowledge, no comprehensive literature on the valorization of leaf biomass for extracting their PCs through GE techniques has been carried out. Therefore, this review aims to establish the recent progress, constraints, legislative framework, and future directions for the extraction and characterization of PCs from leaves based on the published literature that studied the extraction efficiency while maintaining extract quality. Accordingly, the whole discussion of this paper is divided into four parts, namely (i) current status of GE techniques towards PCs extraction, (ii) synopsis of the characterization techniques of PCs, (iii) comprehensive summary of the application and advancement of GE techniques towards the extraction of PCs from leaves of different plant species, and (iv) regulation and challenges in the application of leaves PCs. The outcome of this review is to contribute to economic sustainability as well as provide environmental benefits in terms of plant waste valorization.

2.3. PART I: CONVENTIONAL EXTRACTION VS. GREEN EXTRACTION AND ITS IMPLEMENTATION STRATEGIES

The entire process of extracting PCs from plant matrices involves a series of steps starting with sample screening and selection followed by pretreatment, extraction, characterization, and mass production (Figure 2.1). Extraction is the key step and stands for the separation and recovery of desired compounds from the plant matrix without affecting their functionalities (Belwal et al., 2019a; Patra et al., 2018). Extraction is regulated by mass transfer phenomenon which is facilitated by the potential destruction of the plant cell walls (Florez-Fernandez et al., 2017). To maximize mass transfer of targeted compounds from sample matrix to solvent, several factors such as extraction time and temperature, particle size, solid-to-solvent ratio, pH, matrix type, solvent type

and polarity, extraction methods and their stability, among others, all playing a pivotal role (Putnik et al., 2016). Despite the fact that extraction was carried out in the form of maceration, percolation, digestion, decoction as far back as during the 11th century, followed by modern version viz. Soxhlet extraction during the 18th century, certain drawbacks, like time consumption, energy, cost, solvent, low yield of extract due to thermal degradation, inadequate execution of solvent recycling, have given rise to the development of extraction techniques that are greener and more cost-effective (Belwal et al., 2018; Zainal-Abidin et al., 2017). In addition, reducing the carbon footprint in processing industries, minimization of waste and (re)use of by-products are the most notable strategies declared by the United Nations in their 2030 agenda for sustainable development (UN, 2015). The EU (European Union) insisted on reducing the use of petrochemical solvents and volatile organic compounds as a part of their environmental policy targeted for 2010-2050 (EEA, 2013; Ivanovic et al., 2020). Since, green technology has been characterized as a way to ameliorate the production process by taking the near and far-future impact on the eco-system into account (Choi & Verpoorte, 2019), therefore, incorporation of “Green Concept” extraction phenomena would be worthy enough in favor of environment, industries, and consumers. According to Chemat et al. (2020), “Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product”.

As per Chemat *et al.* (2012) implementation strategy of GE on industrial scale should be in accordance with e^3 concept, i.e., environmentally friendly, economic, and efficient. The current review summarizes the current status of different green methods applied for the extraction of PCs from leaves (Table 2.1).

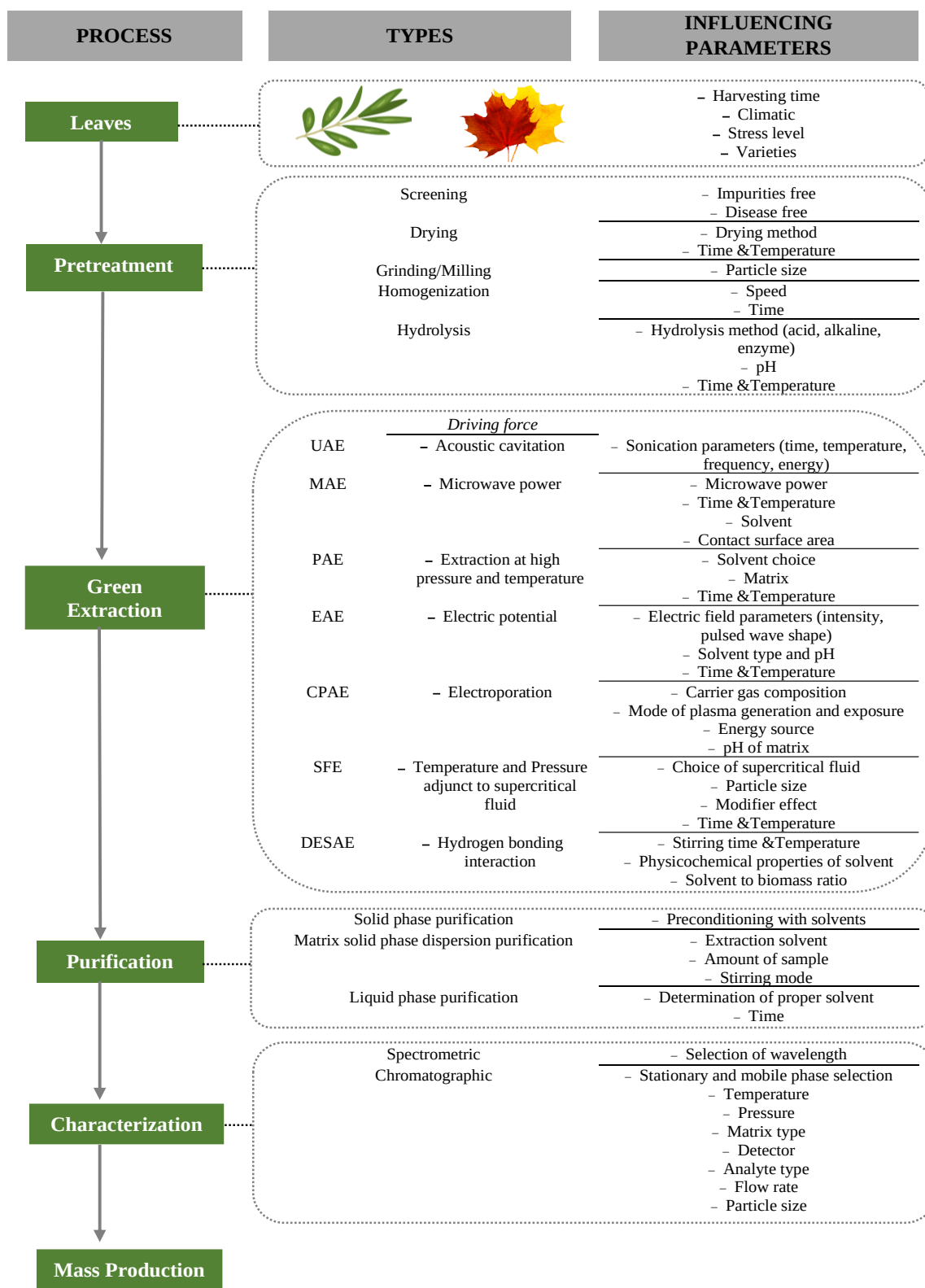


Figure 2.1: Green extraction protocol for the extraction of PCs from leaves. UAE: Ultrasound Assisted Extraction; MAE: Microwave Assisted Extraction; PAE: Pressure Assisted Extraction; EAE: Electrically Assisted Extraction; CPAE: Cold Plasma Assisted Extraction; SFE: Supercritical Fluid Extraction; DESAE: Deep Eutectic Solvent Assisted Extraction.

Table 2.1: Comparison of green extraction techniques applying for the recovery of phenolic compounds from leaves of different plant species.

Plant species	Target compounds	GE	Solvent used	Operating condition constants	Operating variables	Optimum condition/major findings	Yield	Reference
<i>Olea europaea</i>	Oleuropein	DES-MAE	CCLac, CCOx, CCTart, CCBut, CCEtg, CCXy, CCProp, CCMalt, CCU	-	T: 40-80°C It: 10-40 min %W: 0-70%	CCEtg T:79.6°C IT: 16.7 %W: 43.3%	10.58 mg/g DW	Alanon et al. (2020)
		DES-UAE	GFrSW, GFrW, GSW, FrSW, CCGW, CCFrW, CCSW, CCLac, CCMa, CCEtg, CCGly	t: 60 min SP: 140 W F: 37 KHz AED: 35 W/L	T: 55-75°C	CCFrW T:75°C	0.85 mg/g DW	Unlu (2021)
		UAE	Acetone	F: 37 KHz	T: 30-65°C St: 10-120 min Conc: 0-90%	T: 60°C St: 10 min Conc: 50%	100 mg/g DW	Irakli et al. (2018)
		UAE	80% Ethanol	F: 37 KHz SA: 70% T: 60°C	St: 1-5 min SM: pulsed-continuous L-S ratio: 15-25 mL/g	St: 4 min SM: continuous L-S ratio: 15 mL/g	13.39 mg/g DW	Giacometti et al. (2018b)
		UAE	80% Ethanol	T: 20°C SA: 100% SM: Continuous F: 20 KHz AED: 36 W/L	St: 5-60 min	St: 60 min	28.16 µg/mL	Rombaut et al. (2021)

<i>Ficus carica</i>	Caffeoylmalic acid;	UAE	Water	SA: 100% St: 10 min SP: 400 W	T: 10-70°C	T: 50°C	65.2 mg/g DW	Khemakhem et al. (2017)
		UAATPE	Ethanol	F: 35 KHz SP: 400 W t: 25 min	T: 30-50°C Conc.: 32-38%	T: 45°C Conc.: 35%	44.13 mg/L	Wang et al. (2020a)
		MAE	50% Ethanol	MP: 300 W	T: 45-80°C It: 2-15 min sa: 1.5-3 g	T: 80°C It: 2 min sa: 3 g	87.75 mg/g	Dobrincic et al. (2020b)
		UAE		SP: 400 W F: 24 KHz T: 60°C	St: 7-21 min SA: 50-100% sa: 1.5-3 g	St: 21 min SA: 100% sa: 3 g	80.91 mg/g	
		HPAE		Pr transmitting media: glycol solution	Pr: 300-500 MPa t: 1-10 min sa: 1.5-3 g	Pr: 500 MPa t: 5.5 min sa: 1.5 g	66.64 mg/g	
		UAE		F: 20 KHz SP: 450 W	SA: 10-50% t: 6-30 min Conc.: 50-90% T: 25-40°C	SA: 30% t: 25 min Conc.: 59% T: 40°C	22.61 mg/g	
		MAE	Solvent free	F: 2.45 GHz	MP: 250-350 W t: 2-3 min sa: 5-10 g	MP: 250 W t: 2 min sa: 5 g	0.060 ppm	Sahin et al. (2017)
		MAE	Ethanol		MP: 100-200 W It: 5-15 min Conc.: 80-100%	MP: 200 W It: 8 min Conc.: 80%	2.32%	Japon-Lujan et al. (2006b)
		PLE	Organic solvent	Pr: 10.3 MPa Solvent flow: 2 mL/min t: 110 min	T: 20-60°C Solvent: Ethyl acetate, acetone, ethanol (80% and 100%)	T: 60°C Solvent: 80% Ethanol	73.65 mg/g	Dalla Rosa et al. (2019)
		PLE	Ethanol		T: 50-200°C t: 5-20 min Conc.: 0-100%	T: 125°C t: 5 min Conc.: 100%	104 mg/g DW	Martin-Garcia et al. (2020)
		PLE	Ethanol	Pr: 1500 psi	T: 40-190°C Conc.: 14.6-100% No. of cycle: 1-3	T: 190°C Conc.: 57% No. of cycle: 1	26.1%	Xynos et al. (2014)
		-MAE	Surfactant SDS, CTMAB,	MP: 400 W	T: 30-50°C L-S ratio: 15-25 mL/g	T: 40°C L-S ratio: 19.95 mL/g	9.72 mg/g; 5.95 mg/g;	Yu et al. (2020)

	Psoralic acid-glucoside; Rutin; Psoralen; Bergapten		PEG8000, Tween80, TX100, TX114, Tween60		t: 5-15 min	t: 10.27 min	4.77 mg/g; 15.65 mg/g; 3.49 mg/g	
		DES-MAE	Gly:Xy:Fr (3:3:3)	MP: 250 W	T: 40-80°C L-S ratio: 5-25 mL/g t: 20-60 min	T: 50.58°C L-S ratio: 24.39 mL/g t: 43.50 min	6.48 mg/g; 16.34 mg/g; 5.21 mg/g; 15.22 mg/g; 2.47 mg/g	Wang et al. (2017)
<i>Juglans regia</i>	TPC	UAE	Ethanol	F: 35 KHz SP: 480 W T: 25°C	t: 8.17-91.83 min Conc.: 12.17-95.83% L-S ratio: 4.96-25.04 v/w	t: 51.28 min Conc.: 61% L-S ratio: 4.96 v/w	10125.4 mg GAE/L	Nour et al. (2016)
		MAE	Ethanol	-	T: 60-155°C t: 3-36.5 min Conc.: 0-100%	T: 107.5°C t: 3 min Conc.: 67.9%	209.1 mg/g DW	Vieira et al. (2017)
<i>Juglans regia</i>		UAE	Ethanol	SP: 120 W F: 40 KHz T: 40°C t: 80 min	Conc.: 0-98% L-S ratio: 5-20 L/Kg	Conc.: 50% L-S ratio: 20 L/Kg	29.26 mg GAE/g DW	Gavrilovic et al. (2018)
<i>Ziziphus jujuba</i>	TPC	UAE	Organic solvents	F: 20 KHz	Solvent nature: acetone; methanol; ethanol; water Conc.:25-100% Intensity: 25-100% L-S ratio: 50-300 v/w t: 1-15 min	Solvent nature: methanol Conc.:60% Intensity: 75% L-S ratio: 200 v/w t: 10 min	60 mg GAE/g DW	Zemouri-Alioui et al. (2018)
		UAE	Methanol	F: 20 KHz	Intensity: 20-100% Conc.: 40-100% t: 3-15 min	Intensity: 73.60% Conc.: 64.20% t: 13.27 min	60.98 mg GAE/g DW	Zemouri-Alioui et al. (2019)
<i>Camellia sinensis</i>	(+)-Catechin; (-)-Epicatechin; Epigallocatechin gallate	CPAE	Water	Pr: ATM	Plasma gas: Air; N ₂ P: 5-15 W t: 5-15 min	Plasma gas: N ₂ P: 15 W t: 15 min	0.48 mg/g 0.0013 mg/g 0.0007 mg/g	Keshavarzi et al. (2020)

<i>Origanum vulgare</i>	TPC	HVEDAE	Ethanol, Water	F=100 Hz Pulse duration: 400 ns	Plasma gas: Ar; N ₂ Voltage: 15-25 KV t: 3-9 min Conc.: 15-25%	Voltage: 25 KV t: 9 min Conc.: 25%	191.28 mg GAE/g	Nutrizio et al. (2020a)
<i>Annona squamosa</i>	TPC	PEFAE	Ethanol	-	EFS: 2-6 KV/cm Pulse No.: 100-300 Sp. Energy: 45-142 KJ/Kg t: 2.5-5 min	EFS: 6 KV/cm Pulse No.: 300 Sp. Energy: 142 KJ/Kg t: 5 min	281.28 mg GAE/g DW	Shiekh et al. (2021)
<i>Terminalia catappa</i>	TPC	PEFAE	Water	F: 1 Hz Electrical conductivity: 0.5 μ S Pulse: 50	EFS: 0.75-1.5 KV/cm	EFS: 0.75 KV/cm	241.40 mg GAE/g	Moghadda m et al. (2020)
<i>Ocimum americanum</i>	TPC	PEFAE	Ethanol	F: 1500 Hz	t: 10-20 s Voltage: 1-2 KV	t: 10 s Voltage: 1.5 KV	260.50 mg GAE/g	Sukardi et al. (2019)
<i>Fragaria × ananassa</i>	TPC	SFE	SC-CO ₂ + Ethanol	-	T: 308-333 K Pr: 15-30 MPa	T: 308 K Pr: 20 MPa	110.7 μ g/g sample	Sato et al. (2019)
<i>Prunus persica</i>	TPC	SFE	SC-CO ₂ + Ethanol	t: 60 min Flow: 15 g/min	T: 40-80°C Pr: 150-300 bar Conc.: 6-20%	T: 60°C Pr: 150 bar Conc.: 6%	79.92 mg GAE/g extract	Kazan et al. (2014)
<i>Beta Vulgaris</i>	TPC	SFE	SC-CO ₂ + Ethanol	Flow: 0.6 Kg/hr	T: 35-50°C Pr: 300-400 bar Conc.: 6-20%	T: 35°C Pr: 400 bar	3370.8 μ g GAE/ g DW	Goyenech e et al. (2020)

GE: Green extraction; MAE: Microwave assisted extraction; UAATPE: Ultrasound assisted aqueous two phase extraction; HP AE: High pressure assisted extraction; PLE: Pressurized liquid extraction; SFE: Supercritical fluid extraction; CP AE: Cold plasma assisted extraction; PEFAE: Pulsed electric field assisted extraction; HVEDAE: High voltage electrical discharge assisted extraction; DES: Deep eutectic solvent; CCLac: Choline chloride-Lactic acid; CCOx: CC-Oxalic acid; CCTart: CC-Tartaric acid; CCB ut: CC-1,4-Butanediol; CCEtg: CC-Ethylene glycol; CCXy: CC-Xylitol; CCProp: CC-1,2-Propanediol; CCMalt: CC-Maltose; CCU: CC-Urea; MeOH: Methanol; DW: Dry weight; GFrSW: Glucose-fructose-sucrose-water; GFrW: Glucose-fructose-water; GSW: Glucose-sucrose-water; FrSW: Fructose-sucrose-water; CCGW: CC-glucose-water; CCFrW: CC-fructose-water; CCSW: CC-sucrose-water; CCMa: CC-malonic acid; CCGly: CC-glycerol; It: Irradiation time; T: Temperature; SP: Sonication power; F: Frequency; AED: Acoustic energy density; St: Sonication time; SA: Sonication amplitude; SM: Sonication mode; L-S ratio: Liquid-solid ratio; MP: Microwave power; Pr: Pressure; sa: sample amount; EFS: Electric field strength.

2.3.1. Alternative solvent assisted extraction

As per the major principles of GE techniques, utilizing an alternative or agro-based solvent for the extraction of PCs would be the safest way. The solvent utilized should be nonflammable, nontoxic, environmentally acceptable and readily available. Water is considered as one of the most convenient and satisfactory solvents as per the EU and the United States Food and Drug Administration (USFDA), hence is used in the agricultural, cosmetic, pharmaceutical as well as food industries. However, water assisted extraction has some limitations such as selective extraction (only able to extract hydrophilic compounds), presence of impurities, and high cost. Therefore, there is a need to look for alternatives that are sustainable and eco-friendly (Zainal-Abidin et al., 2017).

2.3.1.1. Supercritical fluid-based extraction

Taking environmental safety and sustainability into account, supercritical (SC) fluids are considered to be the first generation of green solvents. The most commonly used supercritical solvent is carbon dioxide and is designated with GRAS status (generally recognized as safe) by the USFDA and the EU. SC fluids can minimize the generation of wastes, shorten time and improve the operating conditions (Ameer, Shahbaz, & Kwon, 2017b; Banozic, Babic, & Jokic, 2020; Okoliea et al., 2019). By definition, an SC fluid is a gas like compressible single homogenous fluid that forms above its critical region, i.e., critical temperature and pressure. Critical temperature (T_c) refers to the temperature at critical point beyond which a substance cannot exist as a liquid. On the other hand, critical pressure (P_c) is defined as the minimum quantity of pressure which is required to liquify a gas at its representative T_c . To summarize, critical point of a substance can be defined as the highest temperature and pressure where a substance can exist in a liquid-vapor equilibrium (Ajila et al., 2011; Demirbas, 2001). SC fluid is neither a liquid nor a gas, rather, it possesses intermediate features between gases and liquids. Density of SC fluids can easily be adjusted by changing either the temperature or pressure and the dissolving power of SC fluids highly depends on its density (Wang & Weller, 2006). Though the concept of supercritical fluid extraction (SFE) evolved over a hundred years ago in the chemical and petroleum industry for deasphalting of petroleum and removal of nicotine from tobacco (Demirbas, 2001), it was not until the end of 1970s that the application of SC fluids for the extraction of different chemical compounds from plants has gained interests among researchers (Banozic, Babic, & Jokic, 2020; Choi & Verpoorte, 2019). For example, decaffeination of coffee using SC-carbon dioxide (SC- CO_2), fractionation of essential oils from spices and butter oil from oil seeds were some of the successful industrial applications (Lu & Liu, 2020). Therefore, SFE is considered to be a

versatile extraction technique from lab scale (less than a gram of sample) to large industrial scale (tons of sample). Besides this, SFE offers good solubilization and recovery of thermally stable compounds, eliminates the time required for the concentration process unlike other solvent extraction methods and can be used for thermolabile compounds. In addition, quantification of volatile compounds immediately after extraction is possible with SFE without any losses (Ajila et al., 2011; Mandal et al., 2015; Wang & Weller, 2006).

2.3.1.1.1. Extraction principle and instrumentation of SFE

SC fluids usually take advantages of both solvation characteristics of liquids and mass transfer properties of gases during extraction. SC fluids are characterized as compressible, viscous with minimal surface tension, which makes SC fluids efficient extraction medium (Demirbas, 2001). SC-CO₂ based extractions are commonly employed for low polar compounds and non-polar compounds (e.g., lipids, terpenes) (Okoliea et al., 2019). However, to extract polar phenolic compounds, addition of an organic modifier, i.e., co-solvent (acetonitrile, water, ethyl ether, dichloromethane, methanol, ethanol, acetone) is required to enhance the solvation power of CO₂ (Ajila et al., 2011). Apart from addition of co-solvent, polarity and density of CO₂ can be manipulated by changing temperature/pressure for the extraction of polar PCs (Demirbas, 2001; Lefebvre et al., 2021).

Figure 2.2 presents a schematic diagram of SFE using CO₂ as SC fluid. The instrumentation of the process consists of a CO₂ reservoir, extraction vessel, CO₂-extract separator, and condenser. Briefly, biomass is loaded into the extraction vessel equipped with temperature and pressure controllers to maintain proper extraction conditions where supercritical CO₂ is pumped. In the extraction vessel, compounds of interest (PCs) are extracted using the desired solvation power of CO₂ by changing the temperature/pressure. Subsequently, the solution (extracted PCs together with fluid) moves into the CO₂-extract separator where PCs are separated and collected via a valve located in the lower part of the separators, followed by condensation and accumulation of CO₂ in the accumulator. The whole process of SFE continues in this cycle until maximum recovery of PCs is achieved (Ameer, Shahbaz, & Kwon, 2017b; Mandal, Mandal, & Das, 2015; Wang & Weller, 2006).

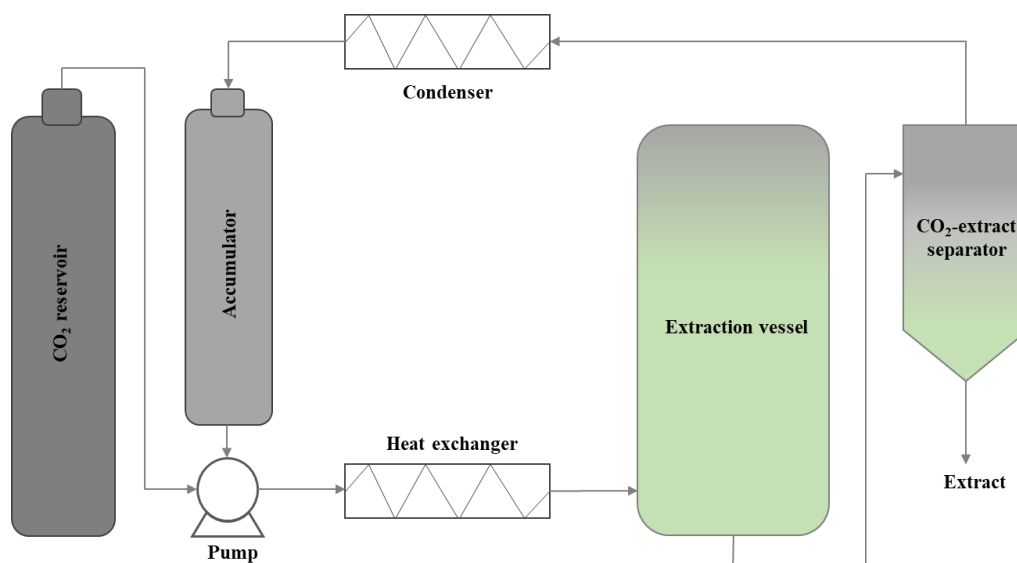


Figure 2.2: Schematic diagram of SFE cycle using CO₂ as SC fluid, adapted from Lefebvre, Destandau and Lesellier (2021); Wang and Weller (2006) with modifications. *SFE*: Supercritical Fluid Extraction; *SC*: Supercritical; *CO₂*: Carbon dioxide.

2.3.1.1.2. Influencing factors of SFE

As discussed earlier, a wide range of solvents can be used as SC fluids out of which CO₂ stands out due to its environmental and physicochemical features. Extraction of polar compounds with SC-CO₂ is difficult due to its non-polar nature and thus, water might be utilized for extracting polar compounds. Similarly, extraction of heat sensitive compounds with SC-water is not possible since most of PCs are thermolabile in nature. Therefore, for heat sensitive compounds, utilizing SC-CO₂ with modifications would prove beneficial (Wang & Weller, 2006).

Changing temperature and pressure above the critical points, modifies the gas and liquid like properties of SC fluids by which efficient penetration of solvent into the solid matrix will be possible, and, as a consequence extracts with high recovery rates will be achievable. Ethanol and methanol are commonly used as modifiers, with the former being utilized increasingly due to its lower toxicity. Despite the positive results in using modifiers, it can result in poor selectivity. Furthermore, utilizing co-solvents may require more energy to reach the SC state. Therefore, screening experiments should be performed before carrying out the extraction with SFE (Ameer, Shahbaz, & Kwon, 2017b).

The condition of the plant matrix (fresh or dried, particle size) is another influential factor which affects the efficiency of SFE of PCs. Although, fresh plant matrix is preferred in SC-CO₂ based extraction, its moisture content acts as a barrier to the extraction of PCs. To

circumvent this problem, coating of plant matrix with desiccants (silica gel, hydromatrix, Na_2SO_4 , MgSO_4) or dispersion of plant matrix can be suggested. However, the desiccants utilized should have a high water retention capacity, low cost, nonhazardous, consistent with sample, and should be able to resist high temperature and pressure (Lehotay & Lee, 1997).

Next to the matrix context, particle size is also one of the factors that have considerable influence on the recovery of PCs. It was observed that both higher and lower particle sizes lead to the unsatisfactory recovery of PCs (Ameer, Shahbaz, & Kwon, 2017b; Lang & Wai, 2001; Wang & Weller, 2006).

Extraction vessel geometry is another technical aspect which might cause changes in temperature and velocity profile during extraction and thus affect the selectivity and extraction kinetics. Furthermore, due to the partitioning coefficients of analytes between SC fluids and matrices, flow of solvent is an important consideration as higher flow rate is required for efficient extraction of targeted analytes from the solid matrices (Lefebvre, Destandau, & Lesellier, 2021; Mandal, Mandal, & Das, 2015).

2.3.1.1.3. Limitations of SFE

Despite the advantages offered by SFE for PCs, SFE requires extended time due to the slower rate of solute diffusion from the solid matrices. Furthermore, variation in the output with respect to process reproducibility and consistency may arise. With respect to instrumentation, high capital investment is necessary. In addition, with the compression of solvents, environmental issues for their disposal may arise (Ajila et al., 2011; Ameer, Shahbaz, & Kwon, 2017b).

2.3.1.2. Deep eutectic solvent-based extraction

Ionic liquids (ILs), a comparatively new class of sustainable solvents, introduced in 1914 have been significantly utilized for the extraction, separation, and purification of PCs. By definition, ILs are the mixture of molten salts where individual components (bulky and asymmetric organic or inorganic anions and organic cations) bind together via ionic bonds. Though certain key attributes such as high thermal and chemical stability, high conductivity, low volatility and vapor pressure distinguish ILs from conventional solvents, their applications were limited due to their toxicity, expensive synthesis for large scale applications, poor degradability, and potential health concerning effects (Choi & Verpoorte, 2019; Plechkova & Seddon, 2008; Wang et al., 2017; Zainal-Abidin et al., 2017).

To circumvent the limitations associated with ILs, a new generation of liquids characterized as “solvents of the 21st century” and named deep eutectic solvents (DESs) have been studied by Abbott et al. (2003) whose physicochemical properties are analogous to ILs,

except that DESs have been reported as nontoxic, easily accessible, chemically inert with water, biodegradable, recyclable, and affordable (Choi & Verpoorte, 2019; Wang et al., 2017). Unlike ILs, DES is formed via hydrogen bond by two or more components in certain molar ratios which upon mixing form a eutectic mixture that stays in liquid form and has a melting point far below than that of its initial constituents (Lu & Liu, 2020; Yang et al., 2018). DESs can be formed through the complexation of a halide salt acting as a hydrogen-bond acceptor (HBA) with a hydrogen-bond donor (HBD) and accordingly can be classified into four categories as follows (i) quaternary ammonium salt + metal halides, (ii) quaternary ammonium salt + hydrated metal halides, (iii) hydrated metal halide + amides or alcohols, and (iv) quaternary ammonium salt + HBD. However, some DESs have been introduced by combining molecules which are abundantly present in nature and named accordingly, are classified as natural deep eutectic solvents (NADESs) (Ivanovic, Razborssek, & Kolar, 2020; Lu & Liu, 2020; Zainal-Abidin et al., 2017). These natural molecules include a wide range of simple sugars (glucose, fructose, sucrose, etc.), amino acids (proline, glycine, etc.), quaternary ammonium compounds (choline, betaine, etc.), and organic acids (citric acid, malic acid, lactic acid, etc.). They are usually found inside or around the vesicles in plant cells and have been involved in transporting, storing, or solubilizing hydrophobic metabolites in living cells (Ruesgas-Ramon et al., 2017; Torres-Vega et al., 2020). From the extraction point of view, DESs/NADESs assisted extraction offers high extraction efficiency with increased stability of the extracted targeted compounds (Ivanovic, Razborssek, & Kolar, 2020).

2.3.1.2.1. Extraction principle and preparation of DESs/NADESs

The extraction principle behind DESs/NADEs assisted extraction of PCs is based on the targeted compound. Dissolution of cell wall due to similarities in polarities plays a crucial role behind the eutectic solvent assisted mass transfer from solid matrix to liquid phase. For instance, choline-DESs, comprising of different ranges of HBDs, have been utilized as an effective extraction media for the recovery of PCs from different matrices (Lu & Liu, 2020). With regard to organic acids used as HBD, higher number of hydroxyl (-OH) and carboxylic (-COOH) groups in solvent lowers the recovery of PCs in contrary to the number of proton (H^+) which facilitates the diffusion of PCs from the cell matrix to the solvent by loosening the cell tissues. With respect to alcohol and sugar based DESs, the molar ratio of quaternary ammonium compounds to the alcohol or sugar, chain length of methylene, branches in the HBD molecules, and number of -OH groups, all significantly affect the extraction of PCs (Lu & Liu, 2020; Wan Mahmood et al., 2019).

Tailor-made DESs/NADESs can be produced by adopting different methods by adjusting the process variables such as temperature and time depending on the nature of the compound. The commonly used methods for producing eutectic solvents include; evaporation method (Dai et al., 2013; Ruesgas-Ramon, Figueroa-Espinoza, & Durand, 2017), heating and stirring method (Ruesgas-Ramon, Figueroa-Espinoza, & Durand, 2017), freeze drying method (Gutiérrez et al., 2009) and ultrasound and microwave assisted methods (Ivanovic, Razborssek, & Kolar, 2020).

2.3.1.2.2. Influencing factors of DESs/NADESs

Among different physicochemical properties of DESs/NADESs, viscosity, melting point (MP), solubility, and polarity play a pivotal role for the efficient recovery of PCs from sample matrices. In terms of viscosity, higher value negatively affects the extraction of PCs, as it hampers the mass transfer phenomena. Besides Van der Waals and electrostatic interactions, formation of substantial hydrogen bonding network characterizes most of the DESs/NADESs as highly viscous solvents (viscosity > 100 centipoise) which restricts the movement of free species among them, consequently lowering the conductivity of DESs/NADESs as well (Zainal-Abidin et al., 2017). Nonetheless, this can be tailored by the addition of water in appropriate proportions (usually 20 to 40%) as observed by Ivanovic, Razborssek and Kolar (2020). It is worthy enough to mention that care must be taken in case of aqueous modifications of DESs/NADESs since an excess amount of water may limit the extraction yield by damaging hydrogen bonding interactions between targeted compounds and eutectic solvents (Lu & Liu, 2020; Ruesgas-Ramon, Figueroa-Espinoza, & Durand, 2017).

With respect to the lower MP of eutectic solvents, it is highly influenced by the ratio of HBD present in DESs/NADESs. In addition, DESs/NADESs have a greater dissolution potential thereby indicating their ability of donating and accepting protons and electrons (Wang et al., 2017; Zainal-Abidin et al., 2017). It was observed by Ruesgas-Ramon, Figueroa-Espinoza and Durand (2017) that hydrated DESs/NADESs showed 50–100 fold strong ability to dissolve flavonoids (e.g. rutin) in comparison to the water-based dissolution.

In terms of polarity, which is a variable characteristic of DESs/NADESs, it is dependent on the composition and molecular structure of HBD. For example, Abbott et al. (2011) reported increasing polarity of choline chloride based DESs as a function of the quaternary ammonium salt's concentration. In addition, temperature plays a critical role as increasing temperature results in decreasing polarity. To conclude, different combinations of starting components, i.e., HBD and HBA with varying molar ratios for the production of tailor-made DESs/NADESs offer great control over their physicochemical properties for the efficient separation and

extraction of compounds of interest (Duan et al., 2019; Ivanovic, Razborsek, & Kolar, 2020; Wang et al., 2017; Zainal-Abidin et al., 2017). Comparative ranges of some physicochemical properties of different extracting solvents are presented in Table 2.2.

Table 2.2: Comparative physicochemical properties of different solvents at 25⁰C and at critical conditions for SCFs.

Solvents	Polarity (Kcal/mol)	Density (gm/cm ³)	Viscosity (Poise)	References
Water	63.10	0.99	0.0091	(Demirbas, 2001; Duereh et al.,
OSs	37.50 - 55.70	0.6 - 1.6	0.02 - 0.003	2020; Ivanovic, Razborsek, &
SCFs	-	0.1 – 1.0	0.0001- 0.00001	Kolar, 2020; Jacquemin et al.,
ILs	49.9 - 52.5	1.24 - 1.50	0.69 - 1.15	2006; Ogihara et al., 2004;
DESS	44.81 - 58.49	1.04 - 1.63	0.19 - 23.46	Ruesgas-Ramon, Figueroa- Espinoza, & Durand, 2017; Smith et al., 2014)

OSs: Organic Solvents; SCFs: Supercritical Fluids; ILs: Ionic Liquids; DESS: Deep Eutectic Solvents

2.3.1.2.3. Limitations of DESs/NADESs

As mentioned above, DESs/NADESs possess higher viscosity with lower evaporation pressure and this seems to be a major barrier for their large-scale application. Lower evaporation pressure generates trouble specially in the event of dry extract production (Ivanovic, Razborsek, & Kolar, 2020).

2.3.2. Pressure assisted extraction

Pressure assisted extraction (PAE) is another noble approach that can be classified based on the intensity of applied pressure as follows (i) medium-to-high pressure assisted extraction (MhPAE) and (ii) high pressure assisted extraction (HPAE).

2.3.2.1 MhPAE

MhPAE is neither completely plant matrix dependent nor requiring any additional modifier as compared to SFE (Costa, 2016; Lehotay & Lee, 1997). In terms of application pressurized extraction started at the end of the 20th century basically in environmental analysis, for example, extraction of pollutants from natural matrices such as soil (Dean et al., 1996), foods (Lehotay & Lee, 1997) and sooner after had been used to extract secondary metabolites from medicinal plants (Benthin et al., 1999). The application of MhPAE towards PCs extraction was first evidenced in the work of Alonso-Salces et al. (2001a, 2001b).

Broadly, MhPAE blends traditional solvent extraction method (e.g., Soxhlet extraction) with the application of high pressure and temperature. The only way PAE differs from Soxhlet extraction is the applied temperature, i.e., in Soxhlet the solvent is heated at its boiling point while in MhPAE solvent is heated above the boiling point (T_b =boiling temperature, P_b =boiling pressure) but below the critical conditions (T_c , P_c). As a consequence, higher solvation power with efficient extraction can be achieved. Usually, the pressure and temperature in MhPAE

range from 1-21 MPa and 40-200⁰C respectively and this results in shorter extraction time as compared to other conventional methods (Ajila et al., 2011; Hidalgo & Almajano, 2017; Ong et al., 2006). Routray and Orsat (2012) have mentioned that MhPAE can minimize the extraction time up to 50%. Besides lowering extraction time, MhPAE utilizes minimum solvent for extraction. In addition, efficient and quicker extraction of targeted thermostable compounds from solid matrices is possible with MhPAE (Ajila et al., 2011; Ameer, Shahbaz, & Kwon, 2017b; Routray & Orsat, 2012; Tejedor-Calvo et al., 2020).

2.3.2.1.1. Extraction principle and instrumentation of MhPAE

MhPAE employs solvents at high temperature and pressure for enhancing the extraction rate of PCs. Elevated temperature increases the recovery of analytes during extraction because of increasing diffusion of the analytes and decreasing viscosity of the employed solvents. Facilitated diffusion of analytes from inner to outer surface of the solid matrix results from the disintegration of different bonding forces (Van der Waals forces, dipole attraction, hydrogen bond interactions) between the analytes and active sites of the matrices due to the application of high temperature. In addition, adhesive (analyte-matrix) and cohesive (analyte-analyte) interactions can be overcome by decreasing their activation energy with the application of thermal energy. On the other hand, elevated pressure maintains the solvent in liquid state at high temperature, forces solvent to flow through the solid matrix even into the small pores and through the filter wherever applicable and ensures the continuous flow of solvent consequently favoring mass transfer (Ameer, Shahbaz, & Kwon, 2017b; Lehotay & Lee, 1997; Ong, Cheong, & Goh, 2006).

Problems on selective extraction of compounds (i.e., polar compounds) with water can be overcome through MhPAE approach. As per the definition of MhPAE, water is heated above its atmospheric boiling condition ($T_b=100^0\text{C}$, $P_b=0.1\text{ MPa}$), but below its critical condition ($T_c=374^0\text{C}$, $P_c=22.1\text{ MPa}$) and is known as the pressurized hot water extraction (PHWE) method. Under this method, by varying temperature and pressure, water resembles the polarity of certain organic solvents which allows the extraction of both polar and nonpolar PCs. For example, methanol and ethanol have a dielectric constant (ϵ) of 33 and 24 respectively at 25⁰C while ϵ of water at the same temperature is 80. However, the dielectric constant of water can be modified at elevated temperature with enough pressure i.e., at 250⁰C with 5MPa, where the ϵ of water becomes 27 (Ong, Cheong, & Goh, 2006; Routray & Orsat, 2012).

Schematic diagram of MhPAE equipment is presented in Figure 2.3a. Briefly, the sample matrix is placed in the extraction vessel which is surrounded by a heating oven. During the commencement of the extraction process, solvent is pumped into the extraction vessel

followed by heating and pressurizing the vessel at its desired temperature and pressure. Subsequently, the extract is removed from the vessel, cooled to room temperature, and collected for further analysis. After the completion of the extraction process, compressed gas (N₂) is purged through the extractor in order to remove the residual solvent left within the extractor. MhPAE can be carried out either in static or in dynamic mode. Sample in the static mode is extracted with a solvent until equilibrium is attained, followed by collection of analytes through rapid flushing with solvent and compressed gas. Similarly, in dynamic mode fresh solvent flows continuously through the sample resulting in faster extraction with higher yield. However, a combination of static and dynamic extraction is expected to deliver better results (Mandal, Mandal, & Das, 2015; Ong, Cheong, & Goh, 2006).

2.3.2.2. HPAE

HPAE is an emerging non-thermal, and eco-friendly way for extracting PCs. By definition, it is a completely pressure oriented extraction technology where the applied pressure usually ranges from 100-1000 MPa at room temperature. Although HPAE was first introduced in 1914 as a rising non-thermal technology in the food safety and preservation sector, it was not until 2004 that HPAE was first studied as an extraction method. HPAE was commonly utilized for extraction of thermolabile PCs. Since HPAE operates at high pressure, extraction can be performed with minimal side effects on the sample matrices subsequently producing quality products. HPAE requires lower amounts of solvent thereby ensuring environmental sustainability with minimal risk of pollution and has been approved by the USFDA. With respect to food safety, there is still no report on any adverse health effects related to HPAE technique (Giacometti et al., 2018a; Huang et al., 2013).

2.3.2.2.1. Extraction principle and instrumentation of HPAE

Based on the description of Irna et al. (2018), the extraction principle of HPAE follows both isobaric and Pascal's principles. The former one states that within a closed environment the applied pressure in a liquid medium is identical from all directions and is independent of any composition, size and shape of the product placed inside. The latter one indicates that while a static fluid of external power in a closed container is used to extract PCs, then any changes in pressure on the fluid will result in the transmission of equal pressure change to all parts of the fluid without any loss. By utilizing this identical pressure effect, HPAE increases cell permeability by causing damage to the plant cell membrane thereby providing enhanced penetration of the extraction solvent into the cells with changes in the concentration gradient and diffusivity of solvent. This results in increasing mass transfer while increasing the extraction rate and quality of yield (Huang et al., 2013). Briefly, HPAE principle is divided into

three stages namely: pressure boost-up stage, pressure maintenance stage and pressure relief stage. In the pressure boost-up stage, a large differential pressure is created between inside and outside of the plant cells and this causes the cell wall damage and cell deformation. The solvent will then permeate through the broken membranes into the cells and solubilize the cell components rapidly.

In the second stage, high preset pressure is maintained for a predefined period in order to balance the pressure between inside and outside of the cells. During this time, rapid penetration of extraction solvent within the cells occurs which causes the solubilization of active components into the solvents. Usually, this stage mostly determines the quantity of extraction.

Following the completion of pressure maintenance stage, the last stage, called pressure relief stage, is completed within a couple of seconds. In this phase, high pressure within the cells rapidly decreases to the atmospheric pressure while altering the non-covalent interactions (ionic bonds, hydrophobic interactions, hydrogen bonds), responsible for maintaining the polymeric structure of cell components. As a consequence, deformation in the cellular parts (cell wall, cell membrane, plasma membrane, nuclear membrane, microtubules, and vacuoles) occurs. Generally, the extraction efficiency depends on this pressure relief stage. Briefly, the shorter the stage is, the higher the formation of pores and fragmentation into the cell organelles leading to efficient extraction of compounds of interest (Giacometti et al., 2018a; Huang et al., 2013).

A typical HPAE system is presented in Figure 2.3b, which consists of a pressure reservoir, sample holder, a pressure generation system, and a temperature control device. During the extraction treatment, the sample together with the extraction solvent are placed in a sealed pressure vessel. Then the pressure is increased from the atmospheric pressure to the preset pressure within a very short time at room temperature. Subsequently, extraction process commences as per the mass transfer theory, i.e., rate of mass transfer is equivalent to the ratio between applied pressure and resistance to the mass transfer (Huang et al., 2013).

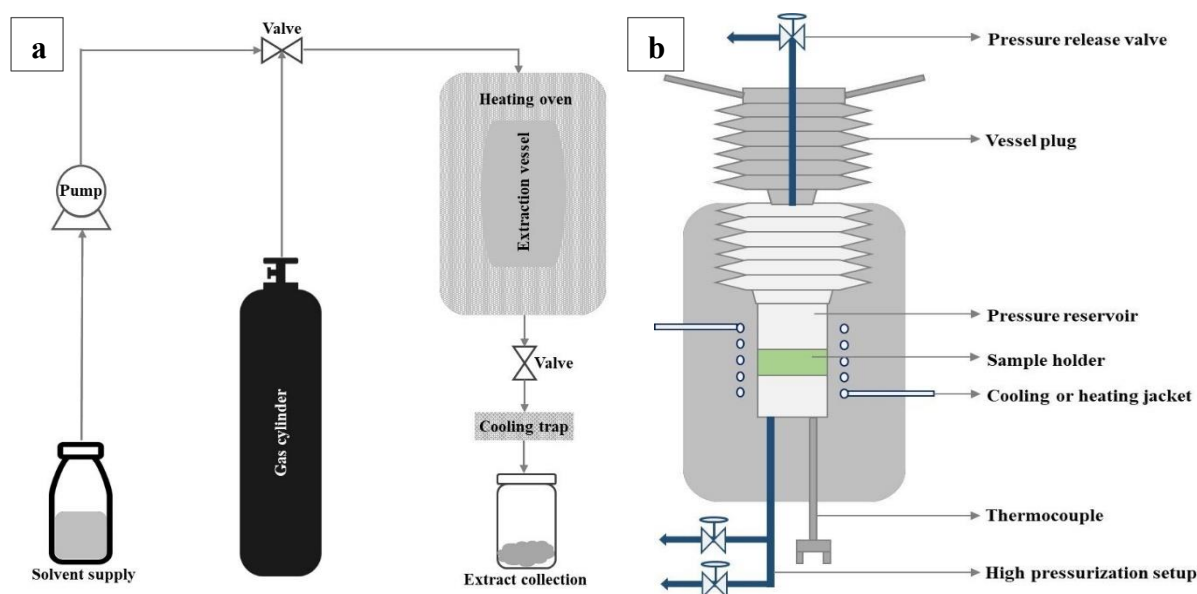


Figure 2.3: Schematic diagram of PAE equipment: **(a)** MhPAE, **(b)** HPAE, adapted from Huang et al. (2013); Mandal, Mandal and Das (2015); Ong, Cheong and Goh (2006) with modifications. *MhPAE: Medium-to-high Pressure Assisted Extraction; HPAE: High Pressure Assisted Extraction.*

2.3.2.3. Influencing factors of PAE

The extraction of analytes from solid matrices to the extracting solvent can be achieved via three steps namely, desorption of analytes from a solid particle, diffusion of analytes through the solvent located inside a particle pore, and transfer of the analytes to the bulk solvent (Sun et al., 2012). Each step depends on numerous factors namely temperature, pressure, time of extraction, type and flow rate of solvent, and nature of the solid matrix.

2.3.2.4. Limitations of PAE

Despite having advantages over conventional methods, careful attention should be paid for extraction of heat sensitive analytes utilizing MhPAE as it deals with high temperature. Elevated temperature may also facilitate the solubility of unwanted compounds along with the compounds of interest. Besides, heat induces reactions such as caramelization and the Maillard reaction which can produce toxic and unwanted compounds in the system. However, the choice of suitable solvent as a function of temperature, pressure, and reaction time might be the practical way to circumvent this heat induced problems (Plaza & Marina, 2019).

2.3.3. Ultrasound assisted extraction

Ultrasound assisted extraction (UAE) is a form of mechanically assisted extraction, since ultrasound is a mechanical wave which requires an elastic medium to spread. These acoustic waves have frequency ranges from 20kHz to 10MHz while the human audible range

is between 16-20kHz. Discovered at the end of 19th century, ultrasounds have been applied widely as a green technology in food processing, pharmaceutical, cosmetic, and nutraceutical industries (Lefebvre, Destandau, & Lesellier, 2021). Three physical parameters namely frequency (defined as the number of cycles per unit of time and expressed in Hz), power (defined as the ratio between the energy transported and the time considered and expressed in Watt) and wavelength (defined as the distance over which the shape of wave repeats, in other words wavelength is distance of one cycle and expressed in cm) are required to calculate the ultrasonic intensity (defined as the ratio of power across the unit area and expressed in W/cm²) (Medina-Torres et al., 2017; Pingret et al., 2013). The characteristic of ultrasound with the surrounding environment primarily depends on its frequency and energy level. The extraction mechanism of ultrasound occurs through the cavitation phenomena that results in cell wall disruption. This allows the permeation of solvent within the cell with release of intra-cellular compounds along with the solvent from the cell (Hidalgo & Almajano, 2017). UAE causes three main effects in the treated material namely mechanical (cavitation), thermal (increase of temperature), and physicochemical (ionization, increase of reaction rate) (Dolatowski & Stasiak, 2012). UAE is considered to be a green extraction technique as it is nondestructive, requires less time and solvent subsequently maximizing extraction yield and quality (Ameer, Shahbaz, & Kwon, 2017b).

2.3.3.1. Extraction principle and instrumentation of UAE

As mentioned earlier, the major driving force of UAE in liquid medium is acoustic cavitation which induces the formation, growth, and implosion of bubbles during the propagation of ultrasound waves in the liquid. When ultrasound is introduced through the liquid medium it induces a series of compression and rarefaction (expansion) phases having both positive and negative pressure respectively. During the compression phase, ultrasound is used to push the constitutive molecules together by attractive forces and during the rarefaction phase, the molecules tend to move apart from each other. The extent of the negative pressure depends on the homogeneity of the liquid. The attraction forces results in the generation of voids in the liquid and these voids are turned into the cavitation bubbles which grow since dissolved gases enter into those bubbles during the rarefaction phase. At a certain point, these cavitation bubbles will collapse onto a surface of solid material (i.e., plant matrices) and will produce a hot spot (temperature and pressure up to 5000K and 1000atm respectively). This hot spot will lead to the formation of a microjet close to the plant matrices, and thereby destroy the cell walls of the plant matrices with the release of the contents into the medium (Kumar et al., 2021; Medina-Torres et al., 2017; Pingret, Fabiano-Tixier, & Chemat, 2013).

The ultrasonic systems require two basic components namely transducer and emitter. The transducer generates ultrasound by converting electrical energy into sound energy whereas the emitter is used to irradiate and amplify the ultrasonic waves. The emitter is also known as the reactor. There are two types of reactors available namely ultrasonic bath and ultrasonic probe (Medina-Torres et al., 2017; Pingret, Fabiano-Tixier, & Chemat, 2013).

2.3.3.2. Influencing factors of UAE

There are many factors which govern the efficiency of UAE and are grouped under three major categories namely physical (ultrasonic power and frequency, intensity, shape and size of the reactors, amplitude), medium (solvent type, solid to solvent ratio, temperature, time, dissolved gases) and matrix parameters.

Cavitation effect has been attributed to the increase in ultrasonic power up to a certain limit with lower moisture content in the sample to ensure better solid-solvent contact. Since the ultrasound frequency influences the bubble resonance size it is assumed that the frequency is another important factor affecting the extraction process. Despite using the ultrasound in the frequency range of 20-100kHz, formation and intensity of cavitation in liquid medium decreases with the increase in frequency (Chemat et al., 2017). Amplitude, which is defined as the maximum height of the ultrasonic wave, plays an important role in the intensification of extraction of PCs. Increasing amplitude increases the number of compression and rarefaction cycles of ultrasonic waves which consequently increases the number of cavities therefore enhancing the extraction process. However, it should be noted that increasing amplitudes might lead to the fast deterioration of ultrasonic transducer which will result in poor transmission of ultrasound throughout the liquid. Similarly, intensity is another factor which increases the sonochemical effects during extraction (Medina-Torres et al., 2017; Pingret, Fabiano-Tixier, & Chemat, 2013). In terms of ultrasonic reactor design, presence of a dispersed phase will result in the attenuation of ultrasound wave, therefore the active part of ultrasound inside the extractor is restricted to a zone located in the vicinity of the ultrasound emitter which finally affect the whole extraction process (Wang & Weller, 2006).

Generally, a solvent having low vapor pressure is preferred in UAE, as the breakdown of the cavitation bubble is more profound in liquid medium in comparison to solvents with high vapor pressure. In this regard, temperature also plays crucial role, and for sonochemical effects, low temperature (20-70°C) is preferable since high temperature increases the solvent's vapor pressure. Although higher solid to solvent ratio favors mass transfer during UAE, it should be noted that the higher amount of solvent also requires post-extraction treatments like filtration and evaporation. Therefore, optimization is important to find out the right amount of solvent.

In addition, increasing sonication time increases the yield initially followed by a decrease with increase in time (Chemat et al., 2017; Medina-Torres et al., 2017).

In terms of plant matrix extraction, yields may vary due to the variation in matrix structure, physical status (dry or fresh), and chemical composition.

2.3.3.3. *Limitations of UAE*

Despite increased extraction of PCs using UAE, continuous exposure to high ultrasonic power or frequencies would result in the poor quality of extract due to the oxidation of compounds caused by the reactive OH radicals (Dzah et al., 2020; Medina-Torres et al., 2017).

2.3.4. Electrically assisted extraction

Both industry and academia have been looking for efficient non-thermal technologies for extraction of PCs with high quality. Development of electrically assisted extraction (EAE) using the principle of pulsed electric field, high voltage electrical discharge and cold plasma was considered to be an efficient non-thermal technology. EAE uses intense electric fields (kV/cm) to form temporary or permanent pores in the treated sample, thereby enhancing mass transfer subsequently leading to the release of intracellular compounds into the extraction solvent during extraction of PCs.

2.3.4.1. *Pulsed electric field assisted extraction*

Briefly, pulsed electric field assisted extraction (PEFAE) is a non-thermal method that utilizes electric potential generated from a high intensity electric field to disrupt cell membranes. Disruption of the cell membrane is caused by the formation of pores and this phenomenon is known as “electroporation” or “electropermeabilization” (Kovacevic et al., 2018b). Being minimally invasive by nature, PEFAE offers the advantage of avoiding unexpected structural changes in the biological materials. Discovery of bioelectricity by Luigi Galvani in 1791 and discovery of electroporation is considered to be the most crucial landmark associated with PEF. Subsequently, the most important breakthroughs using PEF were made between 1960s to 1970s. Technically, the concept of electroporation had been introduced by Weaver and Chizmadzhev (1996), however, the first application of PEF to disintegrate biomaterials was reported in 1961 by Doevenspeck (Vorobiev & Lebovka, 2012). Lopez et al. (2008) studied the extraction of PCs from Tempranillo grapes utilizing PEFAE for the first time. As an extraction technique, PEF is considered to be sustainable and possesses numerous advantages such as higher product yield, quality, purity, and process efficiency while requiring less time, lower operating temperature and reduced energy cost (Lloyd & Wyk, 2012; Martinez et al., 2020).

2.3.4.1.1. *Extraction principle and instrumentation of PEFAE*

Secondary metabolites like PCs are generally located in the inner glands of the plant cells either in free or bound form (i.e., not freely located in the cytosol, but strongly trapped in cell organelles like chloroplast or chromoplast). These are enveloped by the cell membrane which restricts the exchange of inter- and intra-cellular compounds. During extraction, the impact of PEF on biological materials is dominated by the loss of membrane thereby increasing its permeability, traditionally known as electroporation. Briefly, cell membrane consists of the phospholipid bilayer of which the inner part is hydrophobic in nature and the outer part is hydrophilic. This bilayer is a major barrier for the extraction of their inner compounds. There exists a difference between the exterior and the interior of the cell known as transmembrane potential (u_m) due to the presence of organic acids. For electroporation to occur, a threshold value of u_m is required, and electroporation occurs due to the increase of u_m caused by externally applied short and intense electric field. Therefore, the induced u_m provides free energy necessary for structural rearrangements of the membrane phospholipid bilayer thereby enabling hydrophilic pore formation. In addition, hydrophobic pores are also formed by spontaneous thermal fluctuations of membrane lipids leading to the release of both polar and non-polar molecules (Mandal, Mandal, & Das, 2015; Vorobiev & Lebovka, 2012).

PEFAE instrumentation consists of a high voltage pulse generator to produce the electrical pulse and a treatment chamber which holds the sample matrix and pulses are discharged. A schematic diagram of PEFAE is illustrated in Figure 2.4. It can be seen from the figure that the sample is placed within the chamber where the two plate electrodes (one is connected to the generator and the other to the ground) are separated by an insulator to avoid electrical flow from one another. To start with, electric field of medium to high intensity (0.1–50 kV/cm) and short duration (μ s to ms) is applied to the sample placed between the electrodes where the sample experiences either reversible or irreversible cell membrane degradation depending upon the applied electric field. Critical electric field strength (E_{crit}) is defined as the minimum field strength required to induce the formation of pores in the cell membrane. The cells thus undergo no permeabilization if E_{crit} is not exceeded, however, it might be reversibly permeabilized if E_{crit} is slightly exceeded followed by irreversible permeabilization. PCs which are located deep inside the cell matrix will be extracted by utilizing the solvent used as electroporating medium in PEFAE (Mandal, Mandal, & Das, 2015).

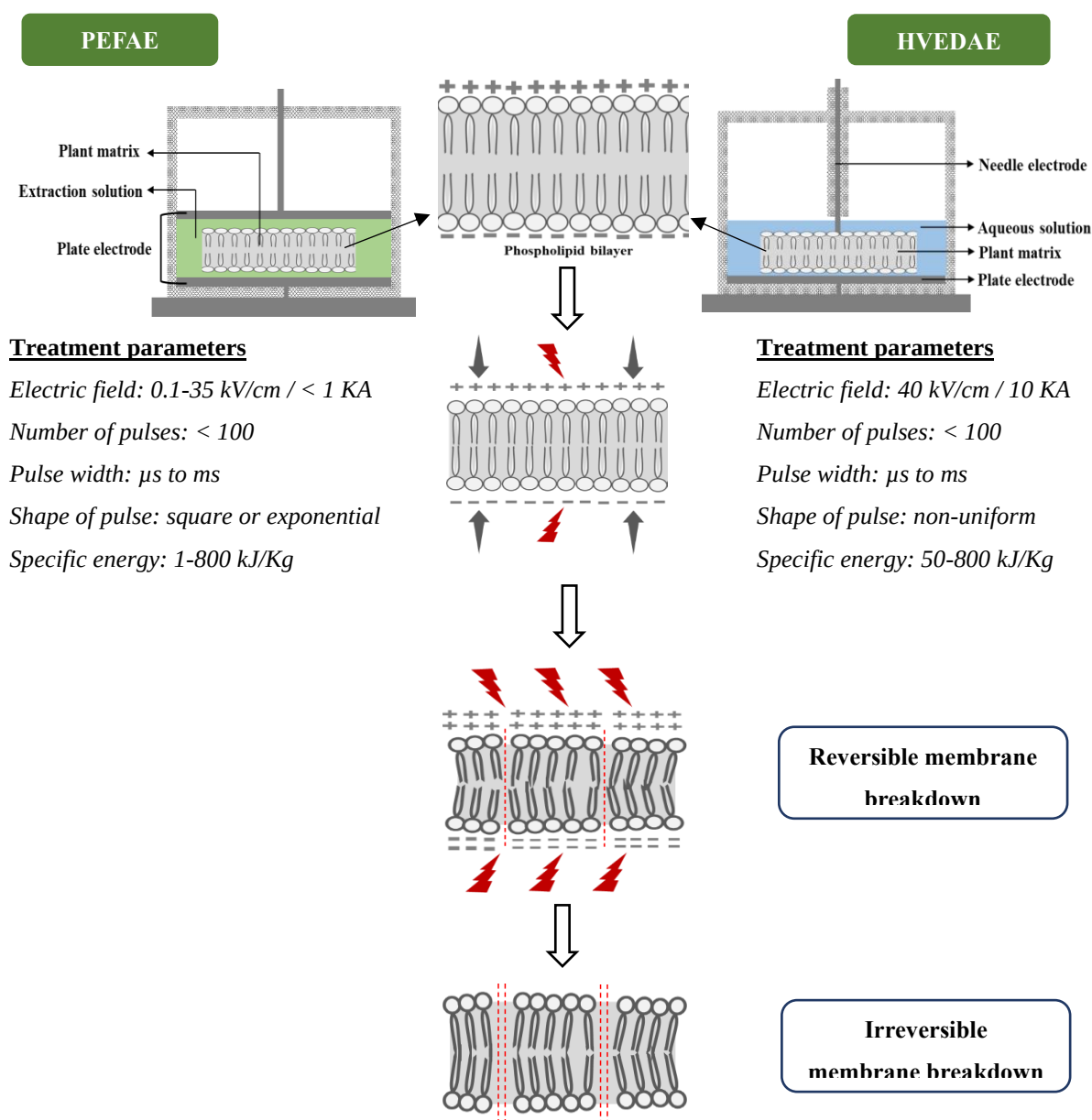


Figure 2.4: Schematic illustration of PEFAE and HVEDAE along with main treatment parameters. Adapted from Kovacevic et al. (2018b); Mandal, Mandal and Das (2015) with modifications. *PEFAE: Pulsed Electric Field Assisted Extraction; HVEDAE: High Voltage Electrical Discharge Assisted Extraction.*

2.3.4.2. High voltage electrical discharge assisted extraction

High voltage electrical discharge (HVED) was initially utilized for treating wastes generated from food industries and is analogous to the PEFAE. The advantages of using HVED is that it requires less solvent and energy while being sustainable and is a widely used extraction method (Barišić et al., 2020; Nutrizio et al., 2020b). HVED acts by the electrical breakdown in water, hence, this method has been utilized for the aqueous extraction of soluble biological materials (Boussetta et al., 2009).

2.3.4.2.1. Extraction principle and instrumentation of HVEDAE

The principle of HVEDAE involves ionization, with the application of high voltage along with high intensity and shorter pulse duration between two electrodes. The mechanism of HVEDAE requires the generation of electric pulse followed by electrical discharge and arcing. Application of electric field between the electrodes submersed in water will release electrons leading to electrification of water followed by formation of streamer (bombardment of electrons). The streamer will proceed from the positive to the negative electrode only if the electric field is intense. As soon as one of the streamer reaches the negative electrode, the electrical breakdown will take place. Besides, several associated secondary phenomena such as chemical reactions and physical processes (shock waves, bubbles cavitation, production of reactive species, creation of turbulence in the extracting liquid, among others) will occur. As a consequence, fragmentation of cellular structure with the release of intra-cellular compounds from plant matrices will occur (Boussetta et al., 2009; Loncaric et al., 2020; Puértolas et al., 2016).

Similar to PEFAE, basic instrumentation of HVEDAE consists of a high voltage pulse generator with some exceptions in the shape of pulses and geometry of the electrodes (Figure 2.4). The treatment chamber is particularly equipped with a needle electrode and a grounded plate electrode. As soon as the high voltage pulse comes in contact with the water, electrical discharges are generated by electrical breakdown within water (Puértolas, Koubaa, & Barba, 2016).

2.3.4.3. Influencing factors of EAE

The combination of electrical factors (electric field strength, pulse duration, frequency, specific energy of the treatment, electrode configuration), extraction factors (temperature, solvent concentration, and type), biological factors (sample matrices, cell geometry and size, hardness of the treated material, nature and location of the targeted compounds), and physicochemical factors (pH, conductivity of the treated sample matrices) control the effectiveness of PEFAE (Puértolas, Koubaa, & Barba, 2016).

2.3.4.4. Limitations of EAE

Due to the discrepancies between theoretical and experimental results with respect to the mechanism of membrane electroporation, PEFAE still requires in-depth study. In addition, the operating costs of PEFAE is high. Besides, there is also a possibility of formation of unwanted products after electrolysis of the sample matrix affecting the quality of the extracted compounds (Kumar et al., 2015; Martinez et al., 2020; Vorobiev & Lebovka, 2012). To

conclude, additional research is still required to build large scale generators and chambers for implementing HVEDA (Boussetta et al., 2009; Kovacevic et al., 2018b).

2.3.4.5. Cold plasma assisted extraction

Plasma, introduced by Irving Langmuir and Tonks in 1929, refers to a fourth quasi state of material which is comprised of a neutral charged collection of electrons, atoms, ions and molecules in their ground or excited states. Based on the temperature of electrons (T_e), plasma can be grouped into high temperature plasma ($T_e=10^6-10^8$ K) and low temperature plasma ($T_e=10^4-10^5$ K). The latter one is further classified into thermal plasma and non-thermal plasma based on the thermodynamic equilibrium between the electron and other reactive species (RS). Non-thermal plasma exhibits thermodynamic non-equilibrium and maintains its relatively low temperature ($< 40^\circ\text{C}$) because, the T_e is much higher than the neutral ions temperature (T_n) and whole gas temperature containing unionized species (T_g). The term non-thermal plasma is also referred to as cold plasma (CP) or high voltage electrical discharge plasma (Liao et al., 2017). Until the 21st century, advanced technologies like high hydrostatic pressure, pulsed electric field and ultrasound had been of prime interest to researchers as well as industries. However, recently with the invention of CP, the trend has changed for food-based research (Sharma & Singh, 2020). CP was first introduced in electronics and polymeric industries which is evident in the work of Ward et al. (1978). This was later introduced into the food sector, owing to the ample amount of RS generated by plasma for ensuring microbial food safety and was observed in the work done by Deng et al. (2006); Perni et al. (2008). Based on the positive outcome of the research conducted by Bao et al. (2020) and Keshavarzi et al. (2020), CP was considered to be an innovative solution for PCs' extraction. However, CP differs from HVEDA in such a way that CP generally introduces partially or completely ionized gas into a liquid, while HVEDA administers energy directly in the submerged liquid between electrodes (Barišić et al., 2020). CP assisted extraction has gained widespread attention owing to its non-thermal nature and generation of plasma at atmospheric pressure with energy efficient features. Therefore, CP assisted extraction (CPAE) has been suggested as a cost effective and sustainable solution (Shishir et al., 2020).

2.3.4.5.1. Extraction principle and instrumentation of CPAE

During the production of CP with pulsed rapid discharge voltages, occurrence of electrical breakdown induces both physical and chemical processes which will affect the cell walls and the cell membranes, subsequently releasing intracellular components. Two main mechanisms namely electroporation and electrostatic disruption are responsible for the physical and chemical processes respectively. Formation of the pores in the cell membranes is

induced during CPAE which paves the way of mass transfer, thereby favoring the extraction of PCs. This process is known as electroporation which is similar to PEFAE. The intensity of the electric field, however, depends on the distance of sample to be treated from the electrode. In addition to electroporation, electrostatic disruption also occurs disrupting the cell membranes along with etching on the cell surfaces. Electrostatic disruption occurs due to the bombardment of energetic particles (e.g., electrons, radicals, ions) on the cell surface (Giacometti et al., 2018a; Liao et al., 2017; Oh et al., 2016; Pragna et al., 2019).

With reference to the instrumentation, CP can be generated by various sources such as electrical discharges (dielectric barrier, corona, streamer, gliding arc, microwave discharge, plasma jets, radio frequency plasma, and nanosecond pulsed voltage) and shocks (electrically, chemically, and magnetically driven). Due to the application of CP in processing foods at low temperatures, CP induced by electrical discharges is of prime interest (Ekezie et al., 2017; Lukic et al., 2019). Dielectric barrier discharge (DBD) is the most commonly used source to produce CP as it is simple to construct, commercially available, able to produce a stable discharge with high concentration of RS. The schematic diagram of DBD CPAE treatment is presented in Figure 2.5.

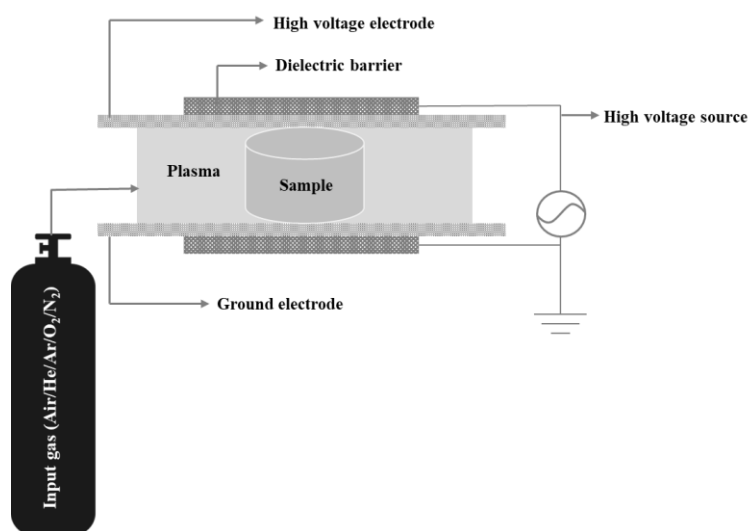


Figure 2.5: Schematic diagram of DBD plasma source during CPAE treatment. Adapted from Keshavarzi et al. (2020) with modifications. *DBD: Dielectric barrier discharge; CPAE: Cold Plasma Assisted Extraction.*

In this system, DBD plasma source includes two electrodes (high voltage electrode and ground electrode) covered by a dielectric material along with a sample holder. High voltage transformer is applied to deliver an output voltage (20-80 kV/cm) between the electrodes while the plasma forming gas (air/He/Ar/N₂/O₂) flows through the chamber producing plasma (Keshavarzi et al., 2020; Perinban et al., 2019).

2.3.4.5.2. *Influencing factors of CPAE*

Efficiency of CPAE has been affected by numerous extrinsic and intrinsic factors. Extrinsic factors refer to processing as well as surrounding variables which includes factors such as energy intensity (voltage and frequency), treatment time, flow rate, gas type, and exposure mode. Higher energy intensity has been related to the greater working efficiency of CP. Similarly, the frequency and input voltage also influence the generation of active species (the higher the intensity is, the greater the amount of RS). With respect to RS, the gas employed for processing eventually determines the nature and quantities of RS produced in the discharge. Direct exposure of plasma on the sample is preferred to non-direct exposure. However, care must be taken to ensure uniform exposure on the sample. In addition, surrounding factors such as pH, relative humidity, nature of sample matrix also substantially affect the efficacy of CP. For instance, increasing humidity favors the production of more hydroxyl ions and radicals thereby enhancing the effectiveness of CP activity. Concurrently, low pH increases the CP activity. In terms of sample matrix, solid and liquid sample interact variably with RS during CP treatment (Ekezie, Sun, & Cheng, 2017; Liao et al., 2017; Sarangapani et al., 2018). To conclude, the above-mentioned factors can significantly affect the outcome of CPAE efficiency.

2.3.4.5.3. *Limitations of CPAE*

Despite the positive outcomes of CPAE, reports on food quality loss, for example, loss of color, and changes in surface topography have become evident. Briefly, RS in the form of ions (H_3O^+ , O^+ , O^- , OH^- , N_2^+), reactive radicals ($\text{O}^\bullet$, $\text{OH}^\bullet$, $\text{NO}^\bullet$), and molecular species (O_3 , H_2O_2) in plasma may lead to several unwanted reactions. For instance, air plasma consists of more than 75 unique species which may result in 500 different chemical reactions (Pankaj & Keener, 2017; Shishir et al., 2020). In addition, CP might induce the degradation of phytochemicals which is due to the ability of phytochemicals to scavenge free radicals generated by CP (Kovacevic et al., 2018b). As mentioned in the previous section, different factors such as type of the plant matrix and plasma source, time and power of treatment, and differing active species may be responsible for these unexpected reactions induced by CP. Therefore, choosing a suitable combination of these factors for increasing the rate of extraction of PCs from plants is very promising for the food and pharmaceutical industry (Keshavarzi et al., 2020). CP, however, has not yet been employed in the extraction industry because of the minimal supporting research favoring CP.

2.3.5. Electromagnetically assisted extraction

Two types of oscillating perpendicular fields, called electric field and magnetic field, are collectively known as electromagnetic (EM) field, generating microwaves with frequencies and wavelengths ranging from 300 MHz to 300 GHz, and 1 to 0.001 m respectively. However, domestic and industrial microwaves usually operate at 2.45 GHz (at a particular wavelength of 12.2 cm) for avoiding the interferences with radio communications. In a nutshell, during microwave assisted extraction (MAE) conversion of EM energy into thermal energy causes the pressure build up inside the cell matrix thereby opening the cellular structure, leading to formation of pores and release of inter- and intra-cellular content. Microwave heating is volumetric, leading to faster warming of the sample matrices by keeping the temperature gradients minimal (Banozic, Babic, & Jokic, 2020; Destandau et al., 2013; Okoliea et al., 2019). It was the Canadian Department of the Environment (Environmental Technology Centre) that developed and patented the Microwave Assisted Process (MAPTM) in gas and liquid phases for the extraction of bioactive compounds from natural sources 1989 (Ameer, Shahbaz, & Kwon, 2017b; Destandau, Michel, & Elfakir, 2013). According to the literature, MAE of PCs has been in practice since 1997 and is already acknowledged as a safe, efficient, economic-, and environment- friendly method (Costa, 2016; Llompert et al., 1997; Okoliea et al., 2019).

2.3.5.1. Extraction principle and instrumentation of MAE

The extraction principle of MAE involves the conversion of EM energy into thermal energy in both the sample and the solvent via ionic conduction and dipole rotation. Ionic conduction refers to the electrophoretic migration of ions under the influence of an electric field. Alternatively, movement of polar molecules having dipole moments under the influence of an electric field is termed as dipole rotation. The larger the dipole moment of the molecule is, the larger the oscillation in the electric field. Disruption of weak hydrogen bonds is caused by the dipole rotation of molecules with release of heat. With respect to the dielectric properties of a material, combination of dielectric constant (ϵ) and dielectric loss factor (ϵ'') is considered to be of prime importance. The dielectric constant reflects the capacity of a molecule to be polarized under an electric field, indicating the ability of a material to absorb microwave energy. On the other hand, dielectric loss factor reflects the efficacy of the conversion of EM energy (or microwave energy) into heat. Briefly, the ability of a solvent or sample to absorb microwave energy followed by their conversion into heat is indicated by dissipation factor (a ratio of ϵ'' to ϵ). For instance, heating of a water molecule by microwave energy is initiated through the mechanism of dipole rotation followed by ionic conduction with the increase in

temperature. The gradual increase in temperature within the system (result of energy conversion) leads to dielectric loss (Destandau, Michel, & Elfakir, 2013; Mandal, Mandal, & Das, 2015; Routray & Orsat, 2012). Microwave heating differs from conventional heating, as heat and mass transfer occurs in the same direction with respect to microwave heating, whereas in conventional heating heat and mass transfer acts in opposite direction (Kala et al., 2016).

MAE of PCs can be performed with and without a solvent (Figure 2.6). Briefly, constitutive water and extracting solvent are hit by the electromagnetic beams, thereby, becoming energetic due to their dielectric properties. On the other hand, rupture of the cell walls is caused by the extreme internal pressure due to the rapid vaporization of the constitutive water. High temperature reduces the viscosity and increases the solubilization of solvent, thereby, allowing them to penetrate easily through the ruptured cell ducts, leading to higher extraction rates (Destandau, Michel, & Elfakir, 2013; Santos et al., 2019; Zin et al., 2020)

With respect to solvent free MAE (SFMAE) swelling of plant cells followed by the rupture of the glands occur by the internal heating of constitutive water. The PCs are extracted and collected in the flask through the sequence of evaporation and condensation of the distillates. In order to ensure the uniform temperature throughout the extraction vessel, the excess constitutive water is refluxed and restored to the plant material (Destandau, Michel, & Elfakir, 2013).

Instrumentation of MAE consists of two systems, namely closed extraction vessel (multi-mode) wherein the pressure and temperature are controlled, and open extraction vessel (mono-mode) operating at atmospheric pressure, which are commercially available (Ameer, Shahbaz, & Kwon, 2017b; Wang & Weller, 2006).

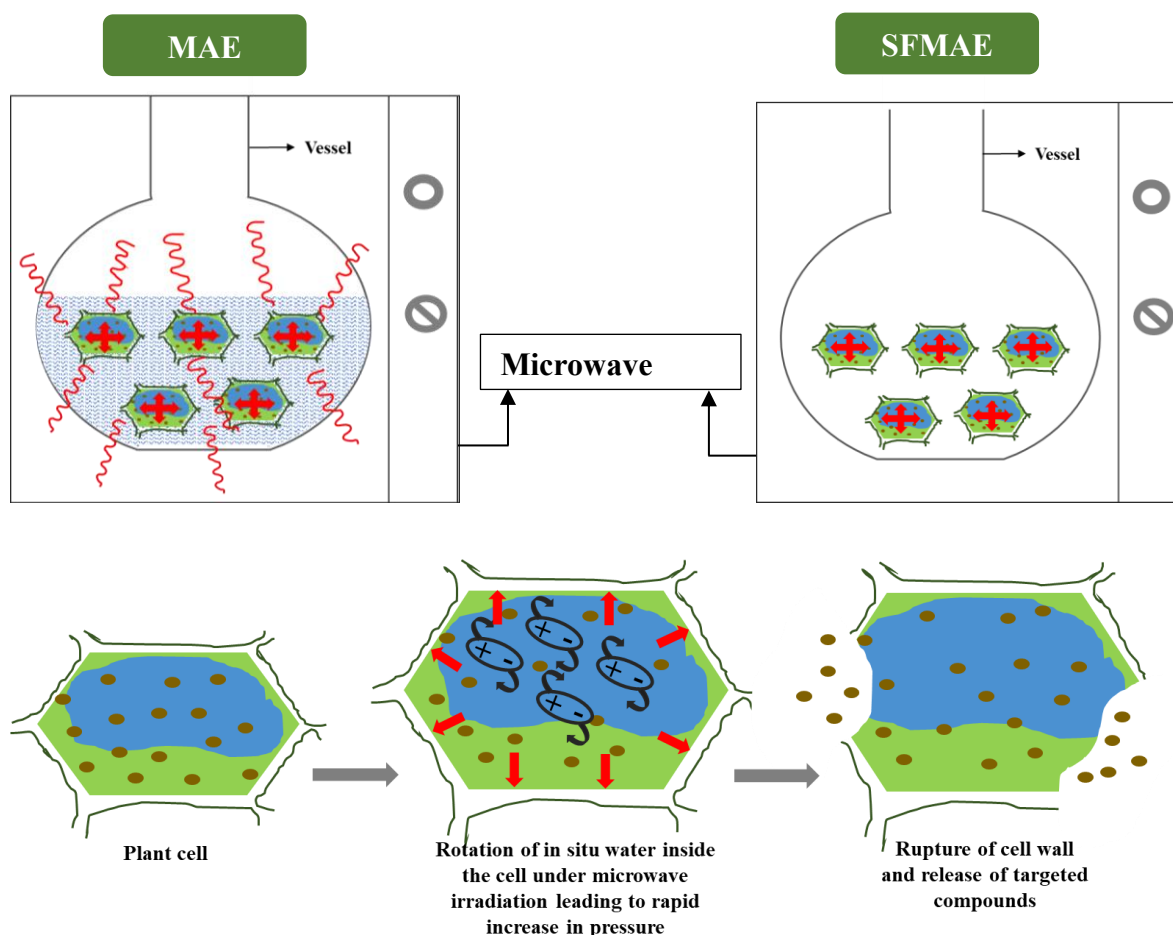


Figure 2.6: Schematic representation of MAE with and without solvent. Adapted from Destandau, Michel and Elfakir (2013) with modifications. *MAE: Microwave Assisted Extraction; SFMAE: Solvent Free Microwave Assisted Extraction.*

2.3.5.2. Influencing factors of MAE

Efficiency of MAE is strongly influenced by instrumental factors (microwave power level, agitation effect), extraction factors (type of solvent, time, temperature), and biological factors (sample matrix, contact surface area).

Conversion of thermal energy in the dielectric material to increase its temperature is controlled by the microwave power intensity. It also affects the interaction between the sample and the solvent leading to faster equilibrium rate. In brief, an increase in the yield of extracted compounds is observed with the gradual increase in microwave power level up to an optimum followed by decrease with increase in microwave power level. For instance, no significant impacts on the yield of bioactive components have been observed after the shifting of microwave power level from 500W to 1000W. Increase in microwave power leads to higher temperature, thereby heating the sample matrix because of localized interaction with

microwaves consequently leading to rapid breakdown of cell wall. During this process, unwanted compounds are also extracted, thereby decreasing the yield and product quality. To improve MAE efficiency, choice of low to moderate microwave power level is considered suitable (Bagade & Patil, 2019; Mandal, Mandal, & Das, 2015; Routray & Orsat, 2012).

Agitation during MAE reduces the barrier for mass transfer rate in the presence of inadequate extraction solvent. Furthermore, extraction rate of bound concentrated PCs can be improved by accelerated stirring thereby enhancing the extraction efficiency (Ameer, Shahbaz, & Kwon, 2017b).

During the extraction of PCs, microwave energy is used to promote the separation of PCs from the matrix. For this purpose, solvent with higher ϵ and dielectric loss is preferred, if the targeted analytes are thermally stable. Polar solvents have better microwave absorbing efficacy, thus either single polar solvent or binary mixture of polar solvents such as aqueous methanol or ethanol can be used (Ajila et al., 2011; Cristianini & Sanchez, 2020).

Extraction time is strongly related to the dielectric properties of extraction solvent. The quantity of the analytes increases with increasing extraction time. However, extracts with lower quality and yield are possible due to the disruption of the structural integrity of chemically active PCs within plant matrices (Bagade & Patil, 2019; Mandal, Mandal, & Das, 2015).

Temperature is another contributing factor affecting the efficiency of any extraction method. As mentioned earlier, high temperature brings in high solubility of targeted compounds in the extractant, reduces the viscosity of the extractant and increase their mobility as well as solubilizing power. This causes cellular pressure to build up resulting in cell rupture through which exchange of inter- and intra-molecular compounds with the extractant occurs. High temperature induced microwave power levels can degrade the product resulting in decrease of extraction yield and quality. Degradation temperature is different for every compound. For instance, it was observed that the compounds having more -OH groups are highly prone to degradation under higher temperature (Routray & Orsat, 2012).

Particle size of the plant matrices have significant influences on the efficiency of MAE. For instance, when the contact surface area of the plant material increases, the extraction efficiency increases. Briefly, the larger the surface area of a fine powder is, the higher the contact between the plant matrices and the solvent and higher the diffusion of compounds of interest out of the plant matrices. However, in certain cases of MAE with decreasing sample size, a considerable amount of energy is not absorbed but rather reflected. This reflected energy may damage the magnetron of the microwave. In addition, there is difficulty in the separation

of the extracted analyte from the solvent after the microwave treatment (de Castro & Priego-Capote, 2012; Mandal, Mandal, & Das, 2015; Wang & Weller, 2006).

In many cases, the amount of *in situ* moisture held inside the plant matrices can strongly enhance the microwave absorption and favors a high hydrolyzation tendency of solvent by affecting their polarity during microwave heating. This leads to increased heating of the moisture present inside the gland, and the vascular system of plant matrix continues to increase leading to localized heating. This will result in higher evaporation rates as well as the rise in internal pressure within the plant cell, leading to cell rupture which will allow the release of PCs from plant matrices. This phenomenon is related to the dielectric susceptibility of water. Effective MAE of thermolabile compounds from dried plant matrix can be performed by using solvent having lower ϵ or through the rehydration of dried plant material before extraction. In this case, the sample matrix interacts with the microwave and facilitates the extraction process while the extractant remains cold during the extraction. Moreover, the presence of moisture inside the plant matrix reduces the risk of PCs being oxidized (Ameer, Shahbaz, & Kwon, 2017b; Bagade & Patil, 2019; Kala et al., 2016).

2.3.5.3. Limitations of MAE

Extraction solvents are the main limiting factors for MAE. For instance, solvents incapable of absorbing microwave due to lack of significant dipole moment are usually not recommended. Additionally, post extraction steps like filtration or centrifugation is required in MAE for the removal of solid residues from the extracted compounds (Banozic, Babic, & Jokic, 2020; Mandal, Mandal, & Das, 2015).

2.4. PART II: CHARACTERIZATION TECHNIQUES

Following extraction, the extract is typically characterized for its active compounds. This usually includes both quantitative and qualitative information regarding extracted PCs, where the former one involves the determination of phenolic content present in the sample and the latter one is related to the identification of individual phenolic component in the matrix. Both qualitative and quantitative attributes are influenced by several factors such as biological (type, chemical nature, and size of matrix), processing (extraction condition, storage time, temperature), and assay method (standards, presence of impurities and interfering substances) (Ajila et al., 2011).

2.4.1. Quantitative characterization

Determination of plant phenolics is performed either by using the method of total content determination which is expressed in equivalent result or by determining the individual phenolic constituents. With regard to the total content determination, spectrophotometric

analysis using a UV spectrophotometer (190-600 nm) is the most commonly used technique due to its simplicity, low cost, and speed (Lopez-Fernandez et al., 2020). The working principle is based on Beer Lambert's law where the two attributes namely absorbance (A) and transmittance (T) are observed and calculated by using the equation (2.1).

$$A = \log (1/T) = \log (I_0/I_1) \dots\dots\dots (2.1)$$

Where, I_0 = intensity of the radiant energy coming to the sample

I_1 = intensity of the emerging light

Although absorbance is mostly used for the quantification purpose with respect to a standard, it is highly affected by the pH of the solution and the solvent applied (Moldoveanu & David, 2013). There is also a possibility of having overlapped spectral responses due to the reducing substances like ascorbic acid, and chlorophyll (Ajila et al., 2011; Naczk & Shahidi, 2006). In terms of specificity, spectrophotometric method cannot measure all the individual phenolic groups leading to the utilization of chromatographic techniques. Liquid chromatography (LC) is the most widely used technique as compared to gas chromatography (GC), column chromatography, thin layer chromatography, and flash chromatography. Besides quantification of individual PCs, LC can also be used to analyze the interaction of PCs with other compounds present in the food matrix (Diaz-de-Cerio et al., 2018a; Naczk & Shahidi, 2006; Patra et al., 2018). Depending on the particle size in the chromatographic column, LC can be categorized as HPLC (high performance liquid chromatography) (3-10 μm) or UPLC (ultra performance liquid chromatography) (less than 2.5 μm) (Moldoveanu & David, 2013). For the determination of different groups of PCs, optimization of the chromatographic parameters (selection of mobile and stationary phase, mass flow rate, particle size, temperature, pressure, pH, elution type, etc.), depending on the phenolic group (Naczk & Shahidi, 2006) is required. Identification as well as quantification is entirely dependent on the type of detector attached to the LC and the proper selection of standards. With respect to detectors, several types (mass spectrometry (MS), refractive index (RI), fluorescence, and ultraviolet-visible (UV-Vis)) have been utilized either individually or in series. Briefly, the detector is the component which is used to determine the analyte identity and/or concentration by converting a physical or chemical attribute into a measurable signal (Swartz, 2010). Parameters such as availability, reliability, reproducibility, specificity, type of analytes (molecular species of interest), properties of mobile phase and their type of elution (gradient or isocratic), mode of maintenance and response, all highly influence the selection of detectors (Moldoveanu & David, 2013; Swartz, 2010). Advantages and limitations of the detectors in LC are presented in Table 2.3. Apart from detector selection, standards (either external or internal) play a prime

role in the quantification of the phenolic groups. With respect to external standardization, a calibration curve is generated based on the chromatographic peak area against different concentrations of a known volume of pure compound identical to the compound of interest in the sample. The purpose of internal standardization is to verify the reproducibility, and to improve the loss of the analyte. An internal standard (IS) is generally absent in the sample, stable, available in the pure form, does not interfere with the analyte, compatible with the detector used, and elutes generally after the analyte. In the case of internal standardization, the analyte concentration is measured using the response factor (the ratio of response between the analyte and IS). To verify the accuracy of the chromatographic process, another type of IS namely chromatographic standard is added with the processed sample and injected into the LC system (Dolan, 2012; Moldoveanu & David, 2018; Moldoveanu & David, 2013).

2.4.2. Qualitative characterization

Qualitative characterization, particularly identification of PCs prior to quantification is very important. Due to the complexity in the phenolic structure, identification of PCs in the sample matrix might be a difficult task (Quirantes-Pine et al., 2013; Tang et al., 2020). LC is a preferred method as compared to other analytical methods like GC, attenuated total reflectance/Fourier transform infrared (ATR/FTIR), near infrared (NIR) spectroscopy, and FT-Raman spectroscopy for phenolic profiling due to its high sensitivity and resolution, short analysis time and lower solvent consumption (Pandey & Kumar, 2016). Sample extraction, purification and clean-up are the fundamental steps which ensure good recovery of the extract with low LOD (limit of detection) and LOQ (limit of quantification) limits (Lopez-Fernandez et al., 2020; Milevskaya et al., 2019). As mentioned earlier, detection and complete characterization of analytes of a full extract can be facilitated using appropriate detection systems either single or in series (Diaz-de-Cerio et al., 2018a). Appropriate choice of detectors is crucial, as some detectors cannot provide qualitative information (RI detector) or provide partial information (UV absorption) (Moldoveanu & David, 2013). Mass spectrometer (MS) or tandem mass spectrometer (MS/MS) coupled to HPLC has proved to be a very useful tool for rapid screening and characterization of PCs owing to high sensitivity, selectivity, specificity, accuracy, and shorter analysis time (Lopez-Fernandez et al., 2020; Pandey & Kumar, 2016; Rodríguez-Pérez et al., 2018; Tang, Dunshea, & Suleria, 2020). Structural elucidation and identification of PCs by LC-MS is based on the measurement of mass of the charged ions with respect to analyte produced by distinctive ion sources namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB), and matrix

assisted laser desorption ionization (MALDI) (Ajila et al., 2011; Moldoveanu & David, 2013). Due to the possibility of having co-elution from the isobaric compounds present in the real matrix, MS/MS has been preferred over MS in order to get a better structural information. With regard to QQQ/MS, prior to measurement, the generated ions are filtered based on their mass using either ion trap or triple quadrupole (QQQ), where the first quadrupole (Q1) separates the parent ion generated in the ion source which further undergoes fragmentation due to the interaction with collision cell in Q2 and finally Q3 separates those fragmented daughter ions. Besides QQQ-MS, there is another high-resolution mass analyzer called time of flight-mass spectrometer (TOF-MS) which offers better identification capacity of unknown compounds compared to QQQ-MS. Optimization of several MS/MS parameters including capillary voltage, collision energy, ion mode (positive or negative), dwell time before analysis, and fragmentation potential is crucial to enhance sensitivity along with the minimization of the matrix effect (Lopez-Fernandez et al., 2020; Moldoveanu & David, 2013; Pandey & Kumar, 2016; Quirantes-Pine et al., 2013; Rodríguez-Pérez et al., 2018). Table 2.4 summarizes the qualitative characterization of PCs found in different leaves using HPLC and related detection methods.

Table 2.3: Common HPLC detector properties.

Detector	Application range	Detection principle	Key attributes	MDQ	Linearity range	Limitations	Reference
UV-Vis	Universal	Detects compound of interest by measuring their absorbance as a function of single wavelength in the UV-Vis region (190-600 nm)	- Simple and low cost - User friendly - Compatible with elution gradient - Quantitative information - Partial qualitative information - Stable and reliable	ng	10 ⁵	- Requires chromophore bearing group - Possibility of having interference - Affected by solution pH and solvent - Universal only at low UV - Single wavelength at a time - Requires transparent solvent	Moldoveanu and David (2013); Swartz (2010)
DAD	Selective	Advance format of UV-Vis detector which detects compound of interest by measuring their absorbance simultaneously at different wavelength in the UV-Vis region (190-600 nm)	- Cheap and robust - High sensitivity that UV-Vis - Quantitative information - Detect a range of wavelength (190-600 nm) at a time - Evaluation of peak purity in the entire UV-Vis spectrum	µg	-	- Compound identification is based on retention time and UV spectra - Low detection and quantification limits in complex structure - Larger noise that UV-Vis	Lopez-Fernandez et al. (2020); Shah et al. (2018); Weon et al. (2011)
FLD	Very selective	Detects compound of interest by their emission of light at excited state results from the absorption of initial radiation	- Compatible with elution gradient - Sensitive and specific - Simple and low cost	pg	10 ⁴	- Requires fluorophore bearing group - Non-linearity due to self-absorption - Quenching effect by dissolved O₂ - Possibility of having interference - Affected by nature of solvent - Derivatization required	Moldoveanu and David (2013); Shah et al. (2018)
CLD	Very selective	Detects compound of interest by their emission of light due to chemical reaction	- Highly sensitive - Detect compounds by the emission of light because of chemical reaction - Energy level is the key attribute to cause the excitation of molecules - Interference by trace materials is nonexistent	amol	-	- Requires specific energy to occur chemical reaction - Derivatization required	Moldoveanu and David (2013)

RID	Universal	Detection is based on the deviation of the direction of light beam passing from one medium to another with a distinctive RI	- Quantitation of a wide range of analytes - Use monochromatic light beam - No requirement of chromophore or fluorophore groups - Require constant temperature maintenance	µg	10 ³	- Lower sensitivity - No qualitative information - Require constant temperature maintenance - Not compatible with gradient elution	Moldoveanu and David (2013); Swartz (2010)
MS	Universal	Detects analyte by measuring the mass of charged ions formed in the ion source such as electrospray ionization (ESI)	- High sensitivity and selectivity - Provide both qualitative and quantitative information - Provide structural elucidation of the analyte - Reproducibility and stability in results	ng	10 ⁵	- High cost - Time consuming - Sensitivity is highly dependent on the type of analyte and mobile phase composition - Sometimes derivatization is required - Requires special maintenance	Lopez-Fernandez et al. (2020); Moldoveanu and David (2013); Shah et al. (2018)

UV-Vis: Ultraviolet-Visible; DAD: Diode Array Detector; FLD: Fluorescence Detector; CLD: Chemiluminescence Detector; RID: Refractive Index Detector; MS: Mass Spectrometer; MDQ: Minimum Detectable Quantity; ng: nanogram; µg: microgram; pg: picogram; amol: attomole

Table 2.4: Application of HPLC for the qualitative characterization of PCs from different leaves.

Matrix (Leaves)	Major analytes	HPLC condition		Detection	Reference
		Stationary phase	Mobile phase		
<i>Capparis spinosa</i> L.	Phenolic acid (n=7) Flavonoids (n=10)	Analytical column: Aquasil C18 (150mm x 3mm, 3µm) Guard column: Aquasil C18 (10 mm x 3 mm, 3 µm)	A (0.1% formic acid in H ₂ O, v/v) and B (0.1% formic acid in methanol, v/v) Gradient elution for B: from 0 to 45 min, 10 to 100% B and 45 to 55min, 100% B	LC-ESI-MS	Yahia et al. (2020)
<i>Peumus boldus</i>	Phenolics (n=22)	Zorbax Eclipse XDB-C18 Narrow-Bore (150mm × 2.1mm; 3.5µm particle size)	A (water/formic acid/acetonitrile, 87:10:3, v/v/v) and B (acetonitrile/water/formic acid, 50:40:10, v/v/v). Gradient elution for B: (time, % of solvent B): 0 min, 3%; 10 min, 15%; 35 min, 40% B; 39–41 min, 100% B, and 47 min, 3% B	HPLC-PDA-IT-MS/MS	Torres-Vega et al. (2020)
<i>Silene vulgaris</i> subsp. macrocarpa	Phenolics (n=13)	Ascentis Express C18 (15cm x 4.6mm, 2.7µm particle size)	A (water/formic acid (99.9:0.1)) and B (acetonitrile/ formic acid (99.9:0.1)) Gradient elution for B: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, 70 min, 100% B, 71 min, 0% B	HPLC-PDA-ESI-MS	(Mohtia et al., 2020)
<i>Salvia miltiorrhiza</i>	Phenolics (n=5)	YMC-Pack ODS-A (250mm × 4.6mm, 5µm particle size)	A (0.1 % trifluoroacetic acid) and B (acetonitrile) Gradient elution for B: 0– 5 min, 10 % B; 5– 10 min, 10 %–12 % B; 10– 20 min, 12 %–15 % B; 20– 25 min, 15 %–20 % B; 25– 60 min, 20 %–30 % B; 60– 65 min, 30 %–100 % B; 65– 70 min, 100 % B; 70– 80 min, 100 %–10 % B.	HPLC-DAD	Hou et al. (2020)
<i>Rubus idaeus</i> L.	Bound phenolics	Zorbax Eclipse Plus C18 (250mm x 4.6mm, 5µm particle size)	A (0.1% formic acid-H ₂ O) and B (acetonitrile) Gradient elution for B: 0–5 min, 15% B; 5–10 min, 15–20% B; 10–20 min, 20–25% B; 20–30 min, 25–35% B; 30–40 min, 35–50% B; 40–55 min, 80% B; and 45–50 min, 15% B	HPLC-ESI-TOF-MS	Wang et al. (2019)
<i>Cecropia</i> species	Phenolic acid Flavonoids Flavonolignans	RP-18 Kinetex column (2.10mm ×100mm, 2.6µm particle size)	A (aqueous formic acid (0.1%, v/v)) and B (acetonitrile with 0.1% formic acid) Gradient elution for B: 10% B (0–5 min), 10–15% B (5–20 min), 15% B (20–30 min), 15–25% B (30–40 min), 25% B (40– 45 min), 25–40% B (45–55 min), 40% B (55–60 min), 40–100% B (60–65 min), 100% B (65–70 min), 100–10% B (70–75 min), 10% B (75–85 min)	RP-HPLC-DAD	Rivera-Mondragon et al. (2019)
<i>Sempervivum marmoreum</i> L.	Flavonoids (n=7)	Eclipse XDB-C18 (4.6mm × 150mm, 5µm particle size)	A (0.15%, phosphoric acid in H ₂ O: MeOH volume ratio 77:23 (pH 2)) and B (MeOH) Gradient elution for A: 3.6 min 100% A; linear–24.0 min; 80.5% A isocratic–30 min linear–60 min; 51.8% A-linear–67.2 min; 100% B	HPLC-DAD	Karabegovic et al. (2018)

<i>Acer rubrum</i>	Gallotannins (n=68) Flavonoids (n=25) Gallic acid (n=9)	Prodigy ODS column (150mm × 4.6mm, 5µm particle size)	A (0.1% formic acid/acetonitrile) and B (0.1% aqueous formic acid) Gradient elution for A: 5% A from 0 to 5 min, 5–16% A from 5 to 16 min, and 16–36% A from 16 to 76 min	UFLC-ESI-TOF-MS/MS	Li and Seeram (2018)
<i>Myrtus communis</i> L.	PCs (n=44)	Poroshell 120 EC-C18 analytical column (4.6mm × 100mm, 2.7µm particle size)	A (water with acetic acid (1%)) and B (acetonitrile) Gradient elution for B: 0 min, 2.5% B; 5 min, 10% B; 9 min, 15% B; 10 min, 16% B; 12 min, 18% B; 17 min, 20% B; 21 min, 50% B, 25 min, 75% B; 32 min, 100% B; 34 min, 100% B; 36 min, 2.5% B	HPLC-DAD-TOF-MS	Diaz-de-Cerio et al. (2018b)
<i>Annona cherimola</i> L.	Phenolic acids Flavonoids	Poroshell 120 EC-C18 (4.6 mm × 100 mm, 2.7µm particle size)	A (water with acetic acid (1%)) and B (acetonitrile) Gradient elution for B: 0 min, 0.8% B; 2.5 min, 0.8% B; 5.5 min, 6.8% B; 11 min, 14.4% B; 17 min, 24% B; 22 min, 40% B; 26 min, 100% B, 30 min, 100% B; 32 min, 0.8% B; 34 min, 0.8% B	HPLC-ESI-TOF/MS	Diaz-de-Cerio et al. (2018a)
<i>Morus nigra</i>	Phenolic acids (n=9) Flavonoids (n=4)	Phenomenex Gemini C18 column (250mm × 4.6mm, 57µm particle size)	A (methanol with 0.1% formic acid) and B (water with 0.1% formic acid) Gradient elution for A: 15% A and 85% B; 0–20 min, 15–30% A; 20–40 min, 30–45% A; 40–45 min, 45–50% A; 45–50 min, 50–55% A; 50–65 min, 55–70% A; 65–75 min, 70–100% A which was maintained for 5 min and returned to initial conditions in 10 min	HPLC-PDA	Radojkovic et al. (2018)
<i>Salvia officinalis</i> L.	Phenolic acids (n=5)	Zorbax ODS C18 column (250mm × 4.6mm, 5µm particle size)	A (3% formic acid in acetonitrile) and B (3% formic acid in water) Gradient elution: 25 min from 10% A in B to 40% A, then after 25 to 30 min from 40% A in B to 70% A, after 30 to 35 min from 70% A, in B to 10% A	HPLC-DAD	Putnik et al. (2016)
Cajanus cajan	Phenolics (n=14)	C18 column (50 mm 2.1 mm, I.D., 1.7 µm particle size)	A (0.1% formic acid aqueous solution), B (methanol) and C (acetonitrile) Gradient elution: 0–10 min, 80% A, 20% B isocratic; 10–18 min, 80–75% A, 20–25% B; 18–18.1 min, 75–77% A, 25–0% B, 0–23% C; 18.1–24 min, 77% A, 23% C isocratic; 24–24.1 min, 77–55% A, 0–45% B, 23–0% C; 24.1–34 min, 55–25% A, 45–75% B; 34–36 min, 25–16% A, 75–84% B; 36–40 min, 16% A, 84% B isocratic	HPLC-DAD	Wei et al. (2015)
<i>Moringa oleifera</i>	Phenolic acids (n=11) Flavonoids (n=12)	Zorbax Eclipse Plus C18 (150mm × 4.6mm, 1.8 µm particle size)	A (Acidified water (0.5% formic acid, v/v)) and B (acetonitrile) Gradient elution for B: 0 min, 5% B; 10 min, 35% B; 65 min, 95% B; 67 min, 5% B	HPLC-ESI-QTOF-MS	Rodriguez-Perez et al. (2015)
<i>Justicia secunda</i>	Flavonoids (n=2)	RP18 Acquity BEH column (10mm × 1mm, 1.7µm particle size)	A (water: formic acid, 99:1, v/v) and B (methanol: formic acid, 99:1, v/v). Gradient elution for B: 2% B from start to 1 min, 2–30% B from 1 to 10 min, 30% B from 10 to 12 min, 30–75% B from 12 to 25 min, 75–90% B from 25 to 30 min, and 90% B from 30 to 35 min	HPLC-ESI-MS/MS	Koffi et al. (2013)

<i>Prunus laurocerasus</i>	Flavonoids (n=7)	Eclipse column XDB-C18 (4.6mm x 150mm, 5µm particle size)	A (0.15% (w/v) phosphoric acid in H ₂ O: MeOH (77:23, v/v, pH = 2)) and B (methanol) Gradient elution: isocratic 0–3.6 min 100% A + 0% B; linear gradient in 24 min 80.5% A + 19.5% B; isocratic with 80.5% A + 19.5% B up to 30 min; linear gradient in 60 min 51.8% A + 48.2% B; linear gradient in 67.2 min 0% A + 100% B; followed by isocratic elution with 100% B for last 5 min	HPLC-UV	Karabegovic et al. (2013)
<i>Olea europaea</i>	PCs (n=36)	C18 Eclipse Plus analytical column (4.6mm × 150mm, 1.8 µm particle size)	A (water with acetic acid (0.5%)) and B (acetonitrile) Gradient elution: 0 to 10 min, 95% (A):5% (B) to 70% (A):30% (B); from 10 to 12 min, 70% (A):30% (B) to 67% (A):33% (B); from 12 to 17 min, 67% (A):33% (B) to 62% (A): 38% (B); from 17 to 20 min, 62% (A):38% (B) to 50% (A):50% (B); from 20 to 23 min, 50% (A):50% (B) to 5% (A):95% (B); from 23 to 25 min, 5% (A):95% (B) to 95% (A):5% (B); from 25 to 35 min, 95% (A):5% (B) to 95% (A):5% (B)	HPLC-ESI-MS/MS ²	Taamalli et al. (2012)
<i>Olea europaea</i>	PCs (n=48)	Poroshell 120 EC-C18 (4.6mm x 100mm, 2.7µm particle size)	A (Water with acetic acid 1%) and B (acetonitrile) Gradient elution for B: 0 min, 5%; 4 min, 9%; 7 min, 12%; 8 min, 15%; 9 min, 16%; 14 min, 20%; 15 min, 22%; 18 min, 38%; 19 min, 30%; 20 min, 31%; 21.50 min, 32%; 23 min, 34%; 24 min, 35%; 25.5 min, 40%; 27 min, 50%; 30 min, 100%; 35 min, 100%; 37 min, 5%	HPLC-DAD-ESI-TOF/MS	Alanon et al. (2020)

HPLC: High Performance Liquid Chromatography; UFLC: Ultra-Fast Liquid Chromatography; ESI: Electrospray Ionization; MS: Mass Spectrometer; MS/MS or MS²: Tandem Mass Spectrometer; TOF: Time Of Flight; PDA: Photo Diode Array; DAD: Diode Array Detector; IT: Ion Trap; UV: Ultraviolet; PCs: Phenolic Compounds

2.5. PART III: EXTRACTION OF PCS FROM LEAVES USING GE TECHNIQUES

The global market demand for PCs from natural sources has increased over the years. Considering environmental safety, advanced and efficient techniques for the extraction of natural active ingredients are becoming the prime focus of research. A set of five species of leaves, selected based on the beneficial health effects and available active ingredients are outlined in this section and presented in Figure 2.7.

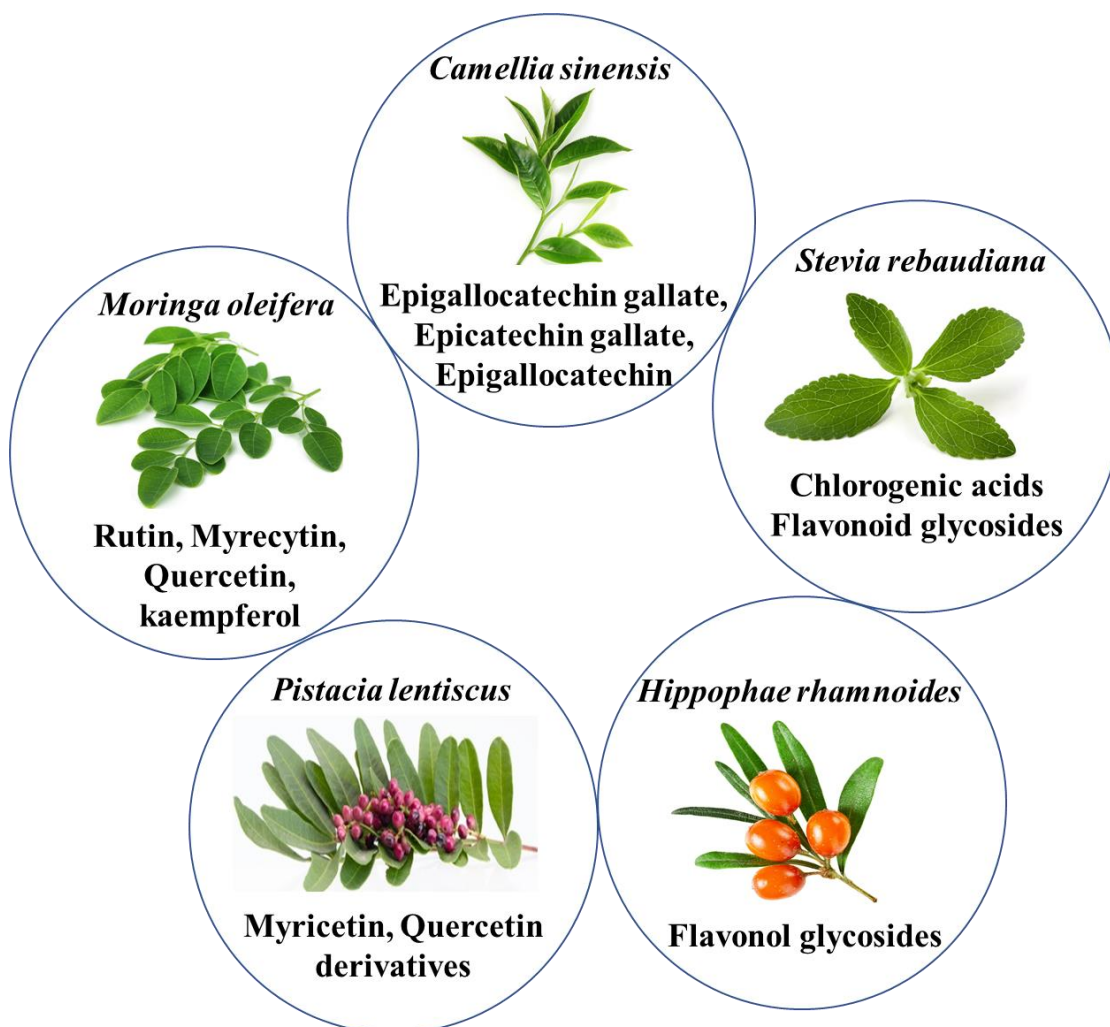


Figure 2.7: List of selected leaves and their predominant phenolic groups/compounds.

2.5.1. Camellia sinensis (Tea)

2.5.1.1. Plant details and importance

Owing to its phenomenal taste and outstanding health benefits, globally tea is one of the most frequently consumed beverages, next to water. It is estimated that roughly 3 million tons of tea is produced and consumed per year of which more than 0.12 million tons of teas are wasted as

spent tea leaves (Maran et al., 2015; Mukhtar et al., 2018). Tea is originally from China where it was first noticed approximately 5000 years ago, while its cultivation has gradually expanded to the Indian subcontinent and further into many tropical and sub-tropical regions across the world (Bharadwaz & Bhattacharjee, 2012; Nadiyah & Uthumporn, 2015). There are three major tea categories namely black, oolong, and green tea produced from the young green shoots of the single tea plant of two distinct species (*C. sinensis* or *C. assamica*) belonging to the same family of Theaceae. However, the difference among these categories exists in their processing techniques including withering, rolling, fermentation, drying, and packing of which fermentation plays the key role. Briefly, black tea undergoes complete fermentation while no fermentation is applied for green tea, oolong tea however lies in between, i.e. partially fermented (Nadiyah & Uthumporn, 2015). Although the chemical composition of tea changes significantly depending on the processing variations, nonetheless it is the PCs which are predominantly responsible for the pharmacological significance of tea irrespective of tea categories. In terms of PCs in tea leaves flavanols, flavonols, flavones, flavanones, anthocyanidins, and phenolic acids are present which collectively account for 10-30% of the dry weight (DW) of tea leaves. Among them the most abundant are flavanols which include catechin, epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and their corresponding stereoisomers. Following flavanols, the second most important subclass of flavonoids present in tea leaves is flavonol which accounts for approximately 13% of the total PCs present in tea leaves. Flavonols exist as *O*-glycosides form with a glycoside moiety at C3 position of aglycones such as quercetin, kaempferol and myricetin and to date more than 20 flavonol glycosides have been identified, for instance, kaempferol glycosides, myricetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucosyl-rhamnosyl-galactoside, among others (Guo et al., 2021; Liu et al., 2020). Next to the PCs, other valuable chemical compounds such as amino acids (2-5% of the DW of tea leaves), vitamins (B_{complex}, C, E), minerals, xanthic bases (caffeine, theophylline), volatile compounds (aldehydes, alcohols, esters, hydrocarbons, lactones), polysaccharides, and pigments (chlorophyll, carotenoids) are also present abundantly in tea leaves (Roshanak et al., 2016). The PCs present in tea leaves are well known for their antioxidant activities, which in turn can be linked with many human health benefits such as prevention of cancers, cardiovascular diseases, liver injury, microbial diseases, obesity, and diabetes mellitus. With respect to cancer, research has shown the inhibitory activity of tea PCs during every stage of carcinogenesis (initiation, promotion, and

progression) and this anticarcinogenic effect of tea PCs can be attributed to their antioxidative, anti-inflammatory as well as detoxification activities (Erugur et al., 2018; Roshanak, Rahimmalek, & Goli, 2016). Besides, an increasing demand for tea PCs has also been noticed in functional beverages, dietary supplements, functional foods, and cosmetics sectors (Liu et al., 2019).

2.5.1.2. Green extraction (GE) and characterization of PCs

A novel non-thermal technology using cold plasma, namely DBD atmospheric CPAE, was employed to increase the extraction rate of the total phenolic content (TPC) and its relevant antioxidant activities from green tea leaves. N₂ was selected as CP forming gas while other variables such as CP generation power (5-15 W) and CP generation time (5-15 min), at a constant frequency of 6 kHz were evaluated towards this phenol extraction. Based on the findings, authors suggested that N₂-based DBD-CPAE might be a suitable choice for the GE of PCs from natural sources as they were able to increase the TPC by 41.14% (913.894 mg gallic acid equivalent (GAE)/g of DW) and antioxidant activities by 41.06% (144.0 µM Trolox equivalent (TE)/g of DW) at the optimum conditions (15 W and 15 min) (Keshavarzi et al., 2020). Another non-thermal extraction technique, called PEFAE was applied for the extraction of PCs from fresh tea leaves of Kenya, suggested that electric field strength is an important controlling parameter in terms of electroporation of the plant cellular tissue. Maximum value for the extraction yield of 27% was obtained at the following conditions: number of pulses, N=30, pulse width, PD=0.05 s, pause between pulses, PBP=0.5 s, electric field strength=0.9 kV/cm, and total treatment time (product of N and PD applied to the system) =1.5 s. However, during the experiment the investigated variable ranges were electric field strength (0.1-1.1 kV/cm), N (10-50), PD (0.0001-0.1 s), and PBP (0.5-5 s). The results also indicated that, during the experiment increase in temperature did not exceed 10 °C which again supported PEFAE as a non-thermal extraction method (Zderic & Zondervan, 2016; Zderic et al., 2013). Liu et al. (2019) applied PEF as a pre-treatment for drying to enhance PCs extraction from fresh tea leaves, concluded that without significantly altering phenolic profile, PEF pre-treatment significantly increased the extraction yield besides reducing the extraction time and energy input.

Bindes et al. (2019) studied the influence of sonication temperature (22-83°C), tea-to-water ratio (12-73 g/L), and ultrasonic amplitude (23-77 %) using ultrasonic probe operated at 500 W and 20 kHz for 30 min to optimize the total phenol and flavonoids extraction from green tea leaves.

Based on the desirability function methodology, highest concentration of TPC and total flavonoid content (TFC) was obtained as 12318 mg GAE/L and 3774 mg rutin equivalent (RE)/L respectively, under the optimal UAE conditions (temperature of 77 °C, tea-to-water of 73 g/L, and amplitude of 77%). In another study 50 % ethanolic extracts obtained by UAE (sonication frequency 50 Hz, power 250 W, solvent-to-solid ratio 20:1 mL/g, time 90 min at 20-40°C) provided 29 % of the yield of PCs (Xi et al., 2009). Luo et al. (2020) screened the best DES (Choline chloride-glycerol at molar ratio of 1:2 having water content of 40 %) out of 12 combinations followed by the optimization of UAE conditions based on response surface methodology (RSM) using optimal DES as solvent towards the maximization of the extraction of TPC from green tea leaves. Highest TPC value of 243 ± 7 mg GAE/g DW was obtained at the optimal extraction conditions of solvent-to-solid ratio, 36:1 mL/g; ultrasonic power, 461.5 W; and sonication time, 21 min. Keeping these optimal extraction conditions as constant, a comparison between UAE-DES and UAE with ethanol was established, suggested that the combination of sustainable green solvents (DES) and UAE could be a prominent method for the extraction of antioxidant PCs from green tea leaves (Luo *et al.* (2020)).

To grow tea leaves, the initial 2-4 light green immature leaves and buds are preferably harvested once or twice a year during spring and/or autumn while leaving the rest of the leaves in the plant. Moreover, these mature discarded leaves need to be pruned timely to control their shape and size, to ensure the quality of tea leaves that will be harvested in the following year, thus creating a sizable amount of agricultural waste (Liu et al., 2020). Having said that, many researchers have focused on the utilization of these leaves towards the extraction of PCs. To that end, Sanz et al. (2020) studied the valorization of discarded tea leaves using a combination of clean technologies such as microwave hydrodiffusion and gravity (MHG), UAE, and PHWE at a constant solvent-to-solid ratio of 15:1 using distilled water as solvent. Starting with MHG as initial pre-treatment at a selected combination of irradiation power and time (100 W - 30 min; 200W - 10 min and 300 W - 10 min) was followed by three ways: UAE (ultrasound bath, 80°C, 80 kHz, time 15-60 min); PHWE (final temperature 140-200°C); and a sequence of the three aforementioned technologies. Based on the findings, maximum TPC (130 mg GAE/g extract) was obtained in the sequential combination of extraction methods, i.e. MHG-UAE-PHWE. Moreover, antioxidant capacity was measured based on the ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay where the antioxidant capacity ranged from 0.3-0.9 g TE/g extract. Pan et al. (2003)

optimized MAE conditions for the recovery of tea polyphenols and caffeine. Among various experimental conditions such as ethanol concentration (0-100%, v/v), MAE time (0.5-8 min), solvent-to-solid ratio (10:1-25:1 mL/g), and pre-leaching time (0-90 min), maximum recovery of tea polyphenols (30%, w/w) and caffeine (4%, w/w) was found while tea leaves pre-leached for 90 min followed by 4 min of irradiation with 50% (v/v) ethanol at solvent-to-solid ratio of 20:1 mL/g. The authors additionally showed that, to recover similar amount of tea polyphenols and caffeine, UAE (operated at 20-40°C) took 90 min; heat reflux extraction at boiling point of 85°C took 45 min; and maceration at room temperature (20°C) took 20 h.

Gadkari et al. (2015) optimized the effects of concentration (1.2-3.6%, w/w), extraction pressure (150-350 bar), extraction temperature (40-60°C), and solvent-to-solid ratio (100-200, w/w) during SC-CO₂ extraction of polyphenols from frozen green tea leaves. Both extraction pressure, temperature and solvent-to-solid ratio were found to be the most significant factors. Under the RSM optimized conditions (temperature, 50°C, pressure, 250 bar and solvent-to-solid ratio, 200), maximum yield of tea polyphenol was obtained as 4.20±0.27% (w/w). Furthermore, 722.68–848.09 mg EGCG/g of extractable solids extracted with SC-CO₂ was quantified by HPLC-UV proved SC-CO₂ as an effecting technique in obtaining extracts rich in EGCG in comparison to the conventional method where only 54.62 mg EGCG/g of extractable solids was separated. Another RSM based optimization of SC-CO₂ extraction conditions was studied by Maran et al. (2015) for the extraction of tea PCs. Apart from measuring PCs, antioxidant activity of the SC-CO₂ tea leaves extracts was also assessed by ABTS method. Maximum TPC of 131.24 mg GAE/100 ml, TFC of 194.60 mg quercetin equivalent/100 ml, total tannin content of 49.99 mg tannic acid equivalent/100 ml, and antioxidant activity of 262.23 µM TE/100 ml of extracts were found at optimum conditions of 188 bar extraction pressure, 50 °C temperature and 2.94 g/min co-solvent flow rate.

Liu et al. (2020) compared the phenolic profiles between young and old tea leaves of 30 samples using UHPLC-Q-Orbitrap-MS. A total of 60 PCs were identified. They reported a reduction of flavanols (1.7-fold) and phenolic acids (3.0-fold) and increase of flavonols (1.5-fold) with maturation of tea leaves. Moreover, they have identified particularly 14 differentiating PCs (rutin, EGC, EC, EGCG, ECG, kaempferol-hexosyl-hexoside, EGC-methylgallate, gallocatechin gallate, galloylquinic acid, kaempferol-pentosyl-deoxyhexosyl-*p*-coumaroylhexoside, trigalloyl-glucopyranose, glucogallic acid, quercetin-hexoside, and epiafzelechin gallate) responsible for the

differences between young and old tea leaves. *C. sinensis* leaves from Nilgiri hills sonicated for 2-3 min at 30°C in water bath were characterized by UHPLC-MS/SRM by Ramakrishnan and Rangiah (2016). They quantified 22 PCs out of which quinic acid, gallic acid, EGCG, EC, isoquercetin, astragalin, and isovitexin were highly quantifiable. According to them, presence of higher amount of EGCG, quinic acid, and gallic acid are primarily responsible for the uniqueness of Nilgiri tea. Mukhtar et al. (2018) obtained conjugates of gallic, chlorogenic, and caffeic acid as the major constituents in the maceration mediated liquid-liquid extracts of spent black tea leaves characterized by RP-HPLC-DAD.

2.5.2. *Moringa oleifera* (Moringa)

2.5.2.1. Plant details and importance

Moringa or drumstick is a rapidly growing perennial softwood tree of 10-12 m height and having tiny bipinnate or tripinnate leaves of 1-2 cm long (Oyeniran et al., 2020). Belonging to the Moringaceae family, moringa is comprised of around 13 species and among them *Moringa oleifera* is the most widely cultivated one. Although this species is native to the countries of sub-Himalayan regions (India, Pakistan, Bangladesh, and Afghanistan), nevertheless it can also be seen to grow in other tropical and sub-tropical regions (Arabia, south-eastern Asia, the Caribbean, South America, eastern, western, and central Africa) due to its ability to grow both in hot dry and humid lands as well as to survive in less fertile soils (Gonzalez-Romero et al., 2020). Moringa is also known as the miracle tree or the tree of life or all-purpose tree since from top to bottom all parts of this plant are utilized either as food, functional ingredients, medicine, or purifying agents where leaves are the most used part. Studies have reported that moringa leaves have been applied as folk medicine for the treatment of various diseases such as hypertension, diabetes, anemia, anxiety, hypoimmunity, typhoid, malaria fever, colitis, skin disease, and others. To this context, mentioned antidiabetic, anti-inflammatory, antidepressant, anticarcinogenic, antimicrobial, hepatoprotective, antioxidative, and neuroprotective functions of moringa leaves have been ascribed for its high content of bioactive compounds such as phenolic acids (gallic acid, chlorogenic acid, caffeic acid), flavonoids (glucosides, malonylglucosides, rutinoides, and acetylglucosides of quercetin, kaempferol and isorhamnetin), tannins, alkaloids, glucosinolates, isothiocyanates, saponins, ascorbic acid and carotenoids (Oldoni et al., 2021; Oldoni et al., 2019; Oyeniran, Ademiluyi, & Oboh, 2020; Vergara-Jimenez et al., 2017).

2.5.2.2. *GE and characterization of PCs*

GE of PCs from moringa leaves with the application of DES, an alternative solvent, composed of glycerol and sodium acetate (Gly-SA) was investigated by Karageorgou et al. (2017). For that purpose, optimization of Gly-SA concentration and molar ratio of Gly-to-SA was done following Box-Behnken experimental design. They found best yield of TPC (53.80 mg GAE/g of dry material) using 80% Gly-SA and 6:1 Gly-to-SA. However, an additional temperature effect on the extraction rate was observed suggesting that temperature above 50°C does not favor extraction. Following that observation, another set of experiment was conducted by similar group (Karageorgou et al., 2018) applying 2-hydroxypropyl β -cyclodextrin (CD) with Gly-SA to see the effectiveness of extraction of PCs from moringa leaves. In this aspect, the effects of the concentration of CD (1-9%), solvent-to-solid ratio (10-40 mL/g), and temperature (40-60°C) was optimized using RSM and results were compared with extraction by only Gly-SA and 80 % ethanol. Based on the findings, at optimized conditions (1% CD concentration, 40 mL/g solvent-to-solid ratio, and 40°C temperature), TPC obtained as 67.18 ± 5.04 mg GAE/g DW with Gly-SA-CD was 13.6% and 39.1% higher than that of TPC obtained with Gly-SA and 80 % ethanol respectively. Wu et al. (2020) screened the best DES (Proline-Glycerol) out of 13 combinations followed by the optimization of UAE conditions using optimal DES as solvent towards the maximization of the yield of TPC and TFC from moringa leaves. Accordingly, antioxidant activities (1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS⁺), OH⁻ free radical scavenging activity and ferric reducing and antioxidant power (FRAP)) were measured at those optimized conditions. Results revealed that the water content in DES significantly influenced TPC while ultrasonic time and solvent-to-solid ratio were less responsive. Optimal conditions were obtained at 37% water content in DES, 144 W ultrasonic power, and 40°C ultrasonic temperature where the maximum yields of TPC and TFC were obtained as 49.9 ± 2.6 mg GAE/g DW and 48.9 ± 5 mg RE/g DW respectively and in terms of antioxidant activities, 602.2 ± 79.7 μ mol TE/g DW for DPPH, 1189.2 ± 56.6 μ mol TE/g DW for ABTS⁺, 1108.6 ± 47.5 μ mol TE/g DW for OH⁻, and 229.6 ± 20.5 μ mol Fe(II)SE/g DW for FRAP were found.

Albarri et al. (2021) conducted research to clarify and understand the effects of MAE parameters such as extraction time (30-400 s), temperature (31-36°C), and power (200-500 W) on the kinetics and thermodynamics of the extraction of PCs from moringa leaves in favor of its

industrial applications. To this context, kinetic (activation energy, extraction rate) and thermodynamic (enthalpy, entropy, Gibbs free energy) parameters together with rate of diffusion, mass transfer coefficients and Biot number for the present system were estimated. Irrespective of extraction time they showed that all the estimated kinetic and thermodynamic parameters were increased with increasing temperature and power. In terms of TPC until it reaches an equilibrium, it continued to increase by power and temperature. For instance, starting with the extraction, i.e., in the first 30 s, TPC was quantified as 4.60, 5.01, 5.19, and 7.72 mg GAE/g of dry leaves against the microwave power of 200, 300, 400, and 500 W respectively. Whereas for each microwave power the equilibrium time was found as 400, 300, 200, and 100 s to reach the equilibrium TPC as 17.3, 19.16, 19.35, and 19.94 mg GAE/g of dry leaves respectively.

Untargeted metabolomic approach based on UHPLC-ESI-QTOF-MS allowed to annotate 291 compounds with a great abundance of flavonoids and phenolic acids in moringa leaves extracted with methanolic based homogenizer assisted extraction (Rocchetti et al., 2020). LC-MS based phenolic profiling of moringa leaves from three geographical regions (Haiti, Chad, and Southwestern Algeria), extracted with methanol confirmed that flavonoids followed by phenolic acids are the principal PCs in moringa leaves. Overall, 15 derivatives of quercetin (quercetin-3-*O*-glucoside, quercetin-3-*O*-(6''-malonyl) glucoside, quercetin-3-*O*-(X''-malonyl) glucoside, quercetin-3-*O*-glucoside-7-*O*-rhamnoside, methyl-*O*-quercetin-malonylglucoside), kaempferol (kaempferol-3-*O*-glucoside, kaempferol-3-*O*-malonylglucoside), apigenin (apigenin-C-glucoside, apigenin-*O*-glucoside), and ferulic acids (hexadecylferulate, 5-hydroxyferulic acid glucoside, diferuloyl-(5OH-feruloyl) spermidine) have been identified (Leone et al., 2015). Furthermore, Wu et al. (2020) characterized and quantified 11 flavonoids (quercetin, vicenin-2, rutin, orientin, hyperoside, kaempferol, kaempferol-3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside, apigenin, D-(+)-catechin, and (-)-epigallocatechin) and 3 phenolic acids (rosmarinic acid, gallic acid, and *p*-hydroxybenzoic acid) from moringa leaves extracted with DES-based UAE via HPLC-ESI-QTOF-MS/MS. Zhao et al. (2019) identified 7 flavonoids and 6 phenolic acids and derivatives by using UAE based extract analyzed with HPLC-DAD-MS method and this was in accordance with Rodriguez-Perez et al. (2015) who characterized 26 flavonoids and 11 phenolic acids and derivatives out of 59 bioactive compounds by using LC-ESI-QTOF-MS from moringa leaves extracted with 50% ethanol by UAE. On the other hand, LC-ESI-QTOF-MS based characterization of sequential extract of moringa leaves obtained from CO₂-expanded ethanol and PHWE

respectively showed similar type and number of phenolic acids and derivatives, but the lower numbers in case of flavonoids (total 17 compounds), implied that the extraction method affects the characterization of PCs, thus indicating the importance of choosing the right extraction method for an accurate and wider PCs characterization (Rocchetti et al., 2019; Rodriguez -Perez et al., 2016). It can also be evidenced from another study of Rodriguez-Perez et al. (2016), where they used two different extracts of moringa leaves obtained from the optimal MAE and PLE, which concluded that MAE allowed better recoveries of quercetin, kaempferol, and their glucosides derivatives while PLE was superior for the extraction of PCs with a higher number of -OH substituents such as kaempferol diglycoside and its acetyl derivatives.

2.5.3. *Stevia rebaudiana* Bertoni (Stevia)

2.5.3.1. Plant details and importance

Stevia, popularly known as candy leaf, sugar leaf or sweet leaf, is a member of the 950 genera belonging to the Asteraceae family (El-Mesallamy et al., 2018). Stevia is comprised of around 200 species out of which *Stevia rebaudiana* Bertoni is the most popular one. This perennial herb is native to the Amambay and Iguacu districts on the borders of Paraguay and Brazil and is cultivated commercially in a number of countries in Asia (Japan, China, Korea, India, Malaysia, Singapore, Taiwan, and Thailand), South America, and North America (Canada) (Zeng et al., 2013). It has been reported by Yilmaz et al. (2021) that, Brazil, Korea and Japan are the top countries that utilize stevia with China being the leading producer and exporter. Stevia leaves are rich in diterpenoid glycosides (stevioside, dulcoside A, rebaudioside A and D), which is a natural and dietetically valuable sweeteners that is about 50 to 300 times sweeter than sugar with zero calories. In addition, they are stable at a wide range of pH (Kaushik et al., 2010; Periche et al., 2016; Zeng et al., 2013). Stevia leaves have recently attracted a lot of attention in the medicinal and pharmaceutical industries due to presence of PCs with anti-inflammatory, diuretic, anti-hypertensive, immunomodulatory, antihyperglycemic, antitumor, and anti-diarrheal effects (Arriola et al., 2016; Lemus-Mondaca et al., 2016; Periche et al., 2016).

2.5.3.2. GE and characterization of PCs

Chlorogenic acids and flavonoids are the major polyphenol components present in *Stevia rebaudiana* leaves. Chlorogenic acids belong to the polyphenol family of esters which are formed by the reaction between quinic acid and trans-hydroxycinnamic acids viz. caffeic acid, ferulic acid, and *p*-coumaric acid. Flavonoids, on the other hand, are compounds having C6–C3–C6 structure

consisting of two aromatic rings. Chlorogenic acids possess excellent therapeutic and antioxidant properties (Karakose et al., 2015; Myint et al., 2020; Pacifico et al., 2019).

Yilmaz et al. (2021) optimized the extraction parameters using RSM for the extraction of PCs from *S. rebaudiana* leaves through maceration, MAE, and UAE. In this study, the optimized conditions for maceration (50% ethanol and 118 mL/g solvent-to-solid ratio) were fixed for UAE and MAE to optimize their extraction variables, i.e., extraction time and temperature. The results showed that the optimized conditions were obtained as 43 min and 50°C for UAE and 16 min and 51°C for MAE respectively. The TPC was observed to be 68.6 mg GAE/g and 67.8 mg GAE/g respectively and was higher when compared to macerated TPC. Another study, conducted by Yildiz-Ozturk et al. (2015) on the comparison of extraction efficiency between MAE and UAE found that UAE was superior to MAE in terms of TPC (86.47 mg GAE/g extract and 80.13 mg GAE/g extract respectively), TFC (126.70 mg quercetin/g extract and 116.16 mg quercetin/g extract respectively), and DPPH (92.40% and 91.39% respectively) in the ethanolic extracts of stevia. The optimum conditions for UAE were as follows, temperature of 90°C (40-90°C), time of 45 min (30-90 min), solid-to-solvent ratio of 1:15 g/mL (1:5, 1:10, 1:15 g/mL) and power of 75% (50-100 %) while for MAE the temperature was 90°C (40-90°C), time of 45 min (5-45 min), solid-to-solvent ratio of 1:10 g/mL (1:5, 1:10, 1:15 g/mL) and power of 400 W (300-500 W).

Ciulu et al. (2017) compared MAE with PLE using ethanol as a solvent to obtain extracts rich in PCs. Results represented PLE as a powerful tool to extract PCs from *S. rebaudiana* leaves at optimized conditions (50% ethanol and 200°C) with TPC of 79 ± 9 mg GAE/g dried leaves. For MAE, optimized operating conditions were obtained at 75% ethanol, 100°C and 20 min of extraction time. Another green technique called PHWE was employed towards the extraction of thermolabile compounds from *S. rebaudiana* leaves by Kovacevic et al. (2018a). Temperature was the most influencing parameter that showed higher recoveries of TPC and condensed tannins. The study also suggested PHWE as an efficient green tool for recovering hydrophilic to lipophilic antioxidant compounds as well as stevioside. Another PHWE study focusing on the effect of extraction parameters on the individual category of PCs (phenolic acids, flavanols, flavonols, and flavones) was carried out. According to this study, the most suitable extraction conditions for all phenolic categories was found to be at 10 min and 3 cycles while in terms of temperature, phenolic acids and flavanols were efficiently extracted at 130°C and flavones and flavonols at 160°C respectively (Sandra et al., 2020).

RSM and artificial neural network (ANN) modeling were compared for their estimation capabilities for building effective models with maximum response values (stevioside and TPC) based on the SC-CO₂ extraction. TPC of 25.76 mg GAE/g was observed at optimum SC-CO₂ extraction parameters (extraction temperature of 45°C, pressure of 225 bar and modifier concentration of 40%) by both RSM and ANN models (Ameer et al., 2017a).

Karakose, Muller and Kuhnert (2015) profiled chlorogenic acids and flavonoid glycosides of stevia leaves from seven different botanical varieties by using LC-TOF-ESI-MS/MS. Six chlorogenic acids namely 3-caffeoylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid and six flavonoid glycosides namely kaempferol, quercetin, apigenin, luteolin, kaemferol-7-*O*- β -D-glucoside, quercetin-3-*O*-beta-D-glucoside were identified. Another study utilized UHPLC-ESI-QqTOF-MS/MS for the phenolic profiling of stevia leaves and they successfully identified more than 50 PCs, out of which 33 were chlorogenic acids rich fractions and 22 were flavonoids rich fractions (Pacífico et al., 2019). Medina-Medrano et al. (2019) analyzed three stevia species called *S. origanoides*, *S. ovata* and *S. viscida* for their PCs via HPLC-DAD and found chlorogenic acid, caffeic acid, and quercetin in all species with chlorogenic acid as major compound ranging from 22.04 to 904.85 μ g/g DM. Recently, a total of 33 PCs of four major categories viz. phenolic acids, flavonols, flavones, and flavanols were observed in *S. rebaudiana* leave extracts using UPLC-ESI-MS/MS by Sandra et al. (2020). The main PCs were observed to be mono- and diacyl caffeoylquinic acids (chlorogenic acid, 3,5-dicaffeoilquinic, 4,5-dicaffeoilquinic) and quercetin glycosides (quercetin-rhamnoside, quercetin-3-rhamnoside, and quercetin pentoside).

2.5.4. *Hippophae rhamnoides* (Sea Buckthorn)

2.5.4.1. Plant details and importance

Sea buckthorn (*Hippophae* L.) is an economically and ecologically important plant which is widely utilized for its medicinal and nutritional value. Belonging to the Elaeagnaceae family, the most widely distributed species of sea buckthorn is *Hippophae rhamnoides*, further classified into nine subspecies (Heinaaho et al., 2006; Morgenstern et al., 2014). *H. rhamnoides* originates from China and grows naturally in Europe and North America. *H. rhamnoides* is nutritionally popular for its sweet and sour orange berries which are reported to contain a wide range of hydrophilic and lipophilic bioactive compounds such as carotenoids (lycopene, α -, β -, γ - carotene),

tocopherols, sterols, flavonoids, vitamins (B, C, E, k), minerals, tannins, essential fatty acids and lipids (Skalski et al., 2018; Sytarova et al., 2020).

2.5.4.2. GE and characterization of PCs

Notable concentrations of ellagitannins and flavonol glycosides have been found in sea buckthorn leaves at levels up to 100 mg/g DW and 11 mg/g DW respectively (Ma et al., 2019). Apart from flavonol derivatives (kaempferol, quercetin, kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside, and rutin), flavanol derivatives (catechin, epigallocatechin, gallocatechin, procyanidin monomer glycoside, procyanidin dimer aglycon, and hydrolysable tannin I-III) and phenolic acids (gallic acid, ferulic acid, caffeic acid) have also been identified in sea buckthorn leaves (Morgenstern et al., 2014).

Asofiei et al. (2016) studied the influence of different extraction parameters such as reactor type (biotage reactor, modified reactor), stirring rate (300-900 rpm), extraction time (50-1800 s), temperature (60-120°C), and aqueous ethanol concentration (0-50%) on the extraction of PCs from sea buckthorn leaves using MAE method. Based on the experimental observation, best results for MAE were obtained when using the modified reactor equipped with larger magnetic stirrer at 900 rpm, at a temperature of 90°C for 450 s with 50% ethanol and 1:20 solid-to-solvent ratio. Based on the quantification of individual PCs by HPLC-UV, catechin followed by gallic acid and rutin were the predominant PCs in sea buckthorn leaves. During the extraction process, MW power was held between 42.2-130.5 W and the average MW incident energy range was between 2290-24405 J where MW power of 105.5 W and MW incident energy of 9072 J was observed to be the best conditions giving highest concentration of TPC of 144 mg GAE/g DW. Another study by Asofiei et al. (2019), to observe the effect of solvent pre-heating strategy and flow rate (1.5-6 mL/min) on the extraction of PCs from sea buckthorn leaves by MAE, showed that solvent pre-heating recovered 80% more TPC from the sea buckthorn leaves. Furthermore, with solvent pre-heating, residence time decreased to 50 s without affecting the extraction efficiency. Besides, 6 mL/min of flow rate with solvent pre-heating was found to be optimum towards the extraction of PCs. The effect of solvent pre-heating can be attributed to the effect of temperature as higher temperature reduces the viscosity of the solvent and increases their mobility as well as solubilization power, thereby causing cellular pressure build-up which results in cell rupture. This, in turn, results in efficient exchange of inter- and intra-molecular compounds along with solvent (Routray & Orsat, 2012). Another study by Galan et al. (2017) recovered the microwave (200 W, 2.45 GHz) assisted

extract of sea buckthorn leaves with TPC and antioxidant activity of 162 mg GAE/g of plant material and 93.6 ± 0.5 $\mu\text{mol TE/g}$ of plant material respectively at the optimum microwave conditions of 50% aqueous ethanol, 1:20 solid-to-solvent ratio, temperature of 80°C, time of 450 s and stirrer rate of 1200 rpm. They also demonstrated that in a small scale system, the experimental parameters such as the bulk heating profile, reactor geometry, stirrer speed, solvent composition, extraction time, solid-to-solvent ratio, and energy requirement to heat a given amount of the same sample is the same for both MAE and conventional solvent extraction method.

Recently, an environment friendly approach, i.e., DES-based MAE of flavonoids (quercetin-3-*O*-glucoside, quercetin, rutin, kaempferol, and isorhamnetin) from sea buckthorn leaves was performed and compared with the traditional extraction methods by Cui et al. (2018). Based on the experimental observation and Box-Behnken design, extraction variables of DES-MAE such as DESs (12 kinds of DESs where choline chloride was used as hydrogen bond acceptor), liquid/solid ratio (15-25 mL/g), extraction temperature (45-75°C), and extraction time (10-20 min) were determined systematically. As per the optimized results, 1,4-butanediol/choline chloride (3/1, mol/mol) with liquid/solid ratio of 21 mL/g at 64°C for 17 min gave the maximum extraction yields of quercetin-3-*O*-glucoside, quercetin, rutin, kaempferol, and isorhamnetin of 1.821, 9.104, 8.972, 0.445, and 0.493 mg/g respectively, as compared to the 70 % ethanol-based MAE. During the experiment, the microwave power and frequency were fixed at 600 W and 2.45 GHz respectively.

Individual phenolic profiling from sea buckthorn leaves was done by HPLC-DAD and based on the obtained chromatogram, 18 PCs were identified namely flavonoids (rutin, epigallocatechin, catechin, epicatechin), phenolic acids (gallic acid, protocatechuic acid, neochlorogenic acid, 4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, ellagic acid, hydroxycinnamic acid, protocatechuic ethylester), and stilbenes (resveratrol) (Sytařova et al., 2020).

2.5.5. *Pistacia lentiscus* (Pistacia)

2.5.5.1. Plant details and importance

Pistacia belongs to the family called Anacardiaceae and consists of more than eleven species out of which *Pistacia lentiscus*, *Pistacia atlantica*, and *Pistacia terebinthus* can easily be distinguished for their characteristic attributes. *Pistacia* is native to the Mediterranean basin and is capable of withstanding higher temperature and solar irradiation (Ben Ahmed et al., 2016). It is

tolerant to drought, cold weather and resistant to calcareous soil (Dahmoune et al., 2014). Pistacia is also popular as an evergreen shrub. From the production of aromatic natural resin to medicinal ingredients, almost all the parts of the Pistacia plant (leaves, stems, root, fruit) have been widely used and are gaining widespread attention due to high concentrations of PCs (Garofulic et al., 2020).

2.5.5.2. GE and characterization of PCs

Pistacia leaves are considered as a rich source of PCs with the most abundant classes being phenolic acids (5-*O*-galloyl quinic acid and gallic acid), flavonoids (mainly myricetin and quercetin derivatives such as myricetin glucuronide, myricetin glycoside, myricetin 3-*O*-rutinoside, myricetin 3-*O*-rhamnoside, quercetin 3-*O*-rhamnoside), and hydrolysable tannins (galloylquinic acids and ellagitannins) (Ben Ahmed et al., 2016; Dahmoune et al., 2014; Dragović et al., 2020).

Deti et al. (2020) utilized UAE with a frequency and input power of 39kHz and 100W respectively to *Pistacia lentiscus* leaves with the aim of optimizing the extraction conditions by using Box–Behnken design while maximizing the yield of PCs and reducing the extraction time and solvent consumption. The study investigated the following extraction conditions namely, solvent (ethanol) concentration (50-75% v/v), solvent-to-solid ratio (0.06-0.1 L/g), extraction time (15-30 min), and temperature (5-25°C). The study revealed that the maximum yield of total phenols (51.3 ± 1.8 mg/g DW) and tannins (40.2 ± 1.4 mg/g DW) were obtained at 40% ethanol concentration with 0.12 L/g solvent-to-solid ratio in a ultrasonic bath operated at 50°C for 15 min. Dahmoune et al. (2014) reported that the optimized microwave power density and irradiation time were 17.86 W/mL and 60 s respectively based on the maximum phenolic yield of 185.69 ± 18.35 mg GAE/g DW from Pistacia leaves. However, they found that the maximum yield was obtained using 46% ethanol with 28:1 solvent-to-solid ratio during MAE. On the other hand, UAE had a maximum phenolic yield of 178.00 ± 19.80 mg GAE/g DW as compared to MAE. Scanning electron microscope (SEM) was used to compare the leave topography after MAE and UAE extraction and the results showed that MAE destroyed the leave tissues better than UAE. In another study on MAE, conducted by Garofulic et al. (2020), maximum TPC was obtained as 108.14 ± 2.12 mg GAE/g at the optimized MAE conditions (temperature: 69°C, microwave power: 512 W and irradiation time: 12 min).

LC-DAD-MS/MS was applied to characterize the PCs present in Pistacia leaves qualitatively. A total of 19 compounds have been identified and they belonged to three major classes namely flavonoids (myricetin-, quercetin-, and kaempferol-derivatives), gallotannins, and gallic acid derivatives (Detti et al., 2020). Similar kind of phenolic profiling was obtained while using UPLC-ESI-MS/MS in the study of Garofulic et al. (2020) and a total of 34 types of PCs were isolated.

2.6. PART IV: REGULATION AND CHALLENGES IN THE APPLICATION OF LEAVES PCS

Both functional foods (in the form of non-altered products/ fortified products/ enriched products/ altered products) and nutraceutical ingredients represent the most innovative, interesting, and promising areas in terms of the application of PCs. For example, in 2012 the global functional food market was estimated about USD 168 billion where Japan led the market share followed by USA and EU respectively (Khan et al., 2013). Currently, the estimated global market for functional foods is expected to increase by 7.9% by 2025 (TastesNatural, 2020).

According to Health Canada, “A *functional food* is similar in appearance to, or may be, a conventional food, which is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions” whereas “A *nutraceutical* is a product isolated or purified from foods that is generally sold in medicinal/supplemental forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against certain chronic diseases” (HealthCanada, 2002). However, within the EU food regulatory framework, neither the term functional food nor the term nutraceutical is defined, instead, each member of the EU has its own set of regulations and legislations governing the term “health foods”. When a new compound is going to be launched in the functional or nutraceutical market, it is always required a premarket approval based on the safety assessment by the corresponding regional committees such as the European Food Safety Authority (EFSA), USFDA, Australia and New Zealand Food Authority (ANZFA), Health Canada, Japanese Nutrition Improvement Law, etc. This implies that the inclusion of PCs in food legislation followed by their subsequent approval for commercial application always demand the completion and documentation of relevant risk assessment considering human health, which is a tedious and expensive task (Kalogianni et al., 2020).

To date, as a source of PCs, the EU has approved (EU, 1333/2008 and 1129/2011) the application of rosemary extract (E392), anthocyanin (E163), steviol glycosides (E960) as food additives and some flavoring agents to a certain concentration depending on the type of food products (EU, 2008, 2011a, 2011b; Kalogianni et al., 2020). Rosemary extract has also been approved for use in the food industry in China, Japan, US, Australia, and New Zealand (Kalogianni et al., 2020). Due to their strong antioxidant properties, green tea phenolic extracts have been seen in bread applications (Pasrija et al., 2015), olive leave extracts were seen in pork patties, sunflower oil, palm oil, and olive oil applications (Martillanes et al., 2017), and *Gingko biloba* leaves extract in pork meat (Lourenço et al., 2019). PCs can also be used as food additives in active food packaging (e.g., edible films, encapsulation) meaning it is considered acceptable that they will come into contact with foods (EU, 2009). For example, catechin and quercetin as PCs were used in ethylene-vinyl alcohol copolymer (EVOH) film for the packaging of oxygen sensitive foods, which resulted in the improvement of food stability (López-de-Dicastillo et al., 2010) whereas green tea extracts have been used in pork sausages which in turn ensured shelf life extension (Siripatrawan & Noipha, 2012). With reference to the approval of rosemary extract by the EU, the main PCs isolated from rosemary leaves extracts include coumaric acid, caffeic acid, gallic acid, luteolin, and isorhamnetin (Mulinacci et al., 2011; Tzima et al., 2021); from olive leaves extracts, the main PCs are luteolin, rutin, catechin, apigenin, and caffeic acid (Rahmanian, Jafari, & Wani, 2015). These PCs have also been isolated in the extract of leaves from stevia, moringa, sea buckthorn, and pistacia as presented in section 2.5. In addition, the extraction solvents used are another important consideration in the regulating bodies to ensure food safety while discussing the use of GE of PCs from leaves. The most used extractants namely ethanol, water, carbon dioxide is already in the List of Permitted Carrier or Extraction Solvents approved by HealthCanada (2015) and considered Generally Recognized As Safe (GRAS) by USFDA (2019).

In general, the purchasing behavior of functional foods and nutraceuticals are highly correlated with culture, lifestyle, packaging/marketing information, product branding, among others. Therefore, to ensure the success of the market of functional and nutraceutical products, combination of science, promotion media, industries, and government policies are highly and mutually influential. In addition, sensory analyses in terms of color, flavor, and appearance; market analyses in terms of price, demand, and health claims; and product promotion based on age groups with common and accessible vocabularies should be conducted to properly develop an acceptable

product image. In this aspect, package labelling with functional logos can play a vital role (Caleja et al., 2017; Kalogianni et al., 2020).

2.7. CONCLUSION AND FUTURE PERSPECTIVES

Leaves biomass coming from either forest, agriculture, or industry has been considered traditionally as waste which upon disposal brings in environmental issues. Leaves biomass can be a sustainable and cheap source of high-added value compounds such as phenolics, thereby possessing the potentiality to be used in the functional food and/or in pharmaceutical and nutraceutical industries. In this context, recovery and valorization of leave biomass would kill two birds with one stone: first, it will alleviate the waste disposal problems which in turn will reduce environmental pollution; second, it will address a societal need to source healthy ingredients, thus highly contributing to a sustainable food chain from both economic and environmental points of view. In line with recovering phenolic compounds (PCs) from leaves, the main constraints are the proper identification, isolation and extraction of PCs which represents a tedious task for the potential uses (i.e., the food, pharmaceutical and nutraceutical industries), therefore, calling for more detailed research on it. Considering environmental safety as well as industrial relevance (e.g., lower energy requirement, processing time, and solvent with high quality and safe end-products), green extraction (GE) concepts like microwave-, ultrasound-, pressure-, pulsed electric field-, high voltage electrical discharge-, cold plasma- assisted extraction, super critical fluid extraction, and deep eutectic solvent-based extraction would be best suited. This review started with detailing GE and characterization techniques of PCs followed by their reported application for the extraction of PCs and their characterization from leaves of five plant species, which will help to be of considerable use to researchers and relevant industries engaged in the development of suitable techniques for PCs extraction with increasing yield for future applications. In addition, existing regulation and challenges in support of PCs application have also been highlighted.

The documented GE techniques in support of PCs exploitation from leaves are entirely based on laboratory scales, therefore, are not yet ready for scaling up to the industrial level due to equipment and installation costs, differences in energy consumption among different extraction techniques, among others. In addition, the chemical stability of extracted compounds will need to be further addressed moving forward as it will be very important from the point of view of further processing stages such as encapsulation, fortification, etc. Furthermore, in addition to the stability, investigations on bioaccessibility and bioavailability of these PCs is of paramount importance to

get insights into their effective applications. Accordingly, the priority demand for further research must advance in the following directions:

- Investigations of the documented GE methods at pilot scales.
- Alleviating the technical barriers in terms of design and application of these extraction methods for future scaling up to the industrial level.
- Comparison of energy consumption among different GE methods based on the extraction of similar amount of phenolic compounds and yield from same biomass.
- Study on *in vitro* bioaccessibility followed by *in vivo* bioavailability of the extracted compounds.
- Chemical stability of extracted PCs and their interactions with other ingredients.

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CONNECTING TEXT TO CHAPTER 3

Chapter 3 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2022). Maximization of the recovery of phenolic compounds from sugar maple leaves. *Biomass Conversion and Biorefinery*, 1-16. doi:10.1007/s13399-022-02904-4

While reviewing the sources being used for the extraction of phenolic compounds, it was seen that along with different plant parts (fruits, seeds, barks, flowers, etc.), plant leaves can be a promising source of phenolics. Therefore, aiming to valorize the sugar maple leaves biomass, Chapter 3 focused on exploring the array of phenolic compounds present in sugar maple leaves. To this end, the most conventional extraction method (maceration) was applied to extract the phenolic compounds from sugar maple leaves.

Part of this research chapter was presented in a scientific conference.

Yeasmen, N., & Orsat, V. (2021/07). *Acer saccharum* leaves: Effect of extraction parameters and pretreatment condition on the recovery of phenolic compounds and their antioxidant activities. American Society of Agricultural and Biological Engineers (ASABE) Annual International Meeting, USA.

CHAPTER 3: MAXIMIZATION OF THE RECOVERY OF PHENOLIC COMPOUNDS FROM SUGAR MAPLE LEAVES

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3.1. ABSTRACT

A maceration process was optimized using a full factorial design coupled to a response surface model for the extraction of phenolic compounds (PCs) from sugar maple leaves (SML). As part of the extraction process, high-speed homogenization (HSH) as a pre-treatment condition, was investigated to promote the efficiency of maceration. Finally, characterization of SML was performed using colorimeter, SEM, FTIR spectroscopy, and LC-ESI-Q-TOF-MS/MS. The antioxidant activity of the extract was investigated as well using standard methods. Results showed that ethanol concentration and solid-to-solvent ratio had significant effects ($p < 0.05$) on the extraction of PCs and HSH can be considered as an effective pre-treatment towards maximizing phenolic compounds recovery from SML. Under the optimal conditions (ethanol concentration of 61.63% at a solid-to-solvent ratio of 1:38.22 g/mL), the experimental %total yield (28.57%), total phenolics (110.87 mg GAE/g DM), and total flavonoids (18.95 mg CTE/g DM) were in close agreement with the predicted values. A total of 81 PCs were tentatively identified including mostly phenolic acids and flavonoids and their derivatives from SML using LC-ESI-Q-TOF-MS/MS. What's more, a gallotannins pathway existing in SML from gallic acid to tetragalloyl-D-glucopyranose was proposed. The study suggested SML as a promising source of PCs which can be effectively extracted through maceration and be further used as natural antioxidants in the formulation of functional food systems.

Keywords

Antioxidant, FTIR, Leaves biomass, LC-ESI-Q-TOF-MS/MS, Maceration.

3.2. INTRODUCTION

Transition from a traditional to a functional diet is a challenging task and the scientific community is starting to bridge the communication and knowledge gaps to convince consumers in making this transition where functional food ingredients can help in improving some health outcomes. Searching through the literature on functional ingredients, in particular phenolic compounds, quite a number of biological activities including anti-allergenic, anti-inflammatory, anti-atherogenic, cardioprotective, anti-microbial, anti-thrombotic, and antioxidant effects have been reported to be exhibited by phenolic compounds (known as plant secondary metabolites synthesized through phenylpropanoid pathway) due to possessing many versatile molecular structures (DiNardo et al., 2019; Kumar et al., 2021; Martynenko & Chen, 2016). These biological activities have therefore prompted the use of phenolic compounds (PCs) by the food,

pharmaceutical, nutraceutical, and cosmetic sectors in place of synthetic compounds (Sim et al., 2019), and this importance can be seen on Grand View Research where the global market size of PCs in 2018 was already worth USD 1.28 billion and a compound annual growth rate (CAGR) of 7.2% is expected to register from 2019 to 2025 (GrandViewResearch, 2019; Yeasmen & Orsat, 2021).

Although PCs are widely available in different fruits and vegetables, yet similar compounds could be obtained from comparatively underprivileged resources where forestry biomass is of particular significance and the genus *Aceraceae* (commonly known as maple), in this context, could be a largely contributing representative. Among 200 species of maple which are widely distributed in the temperate regions of the northern hemisphere, *Acer saccharum* (sugar maple) species grows wild and is native to the eastern-North of North America. In addition to being ornamental, sugar maple is basically best known for the production of maple syrup, a widely consumed high antioxidant rich product produced from the sap of the sugar maple tree (Geoffroy et al., 2017; Honma et al., 2010). Despite the availability of scientific documentation on the identification of several antioxidant PCs from sugar maple syrup, sap, bark, and wood (Li & Seeram, 2010, 2011; Yoshikawa et al., 2011; Yoshikawa et al., 2013; Yuan et al., 2011), sugar maple leaves (SML) biomass is yet underexploited and considered as natural waste biomass which is why in urban and peri-urban areas, maple leaves are either ground to make compost or burnt directly. As a consequence, serious economic (disposal/handling of biomass represents a cost to the local government) and environmental problems (such as greenhouse gas emissions, air pollution, loss of soil moisture, loss of balance in ecosystem, and so many others) have been evolved (Clodoveo et al., 2022; Contreras et al., 2019; Saini et al., 2019). For example, according to FAOSTAT, burning of forestry biomass has increased by 9% over the last ten years in Canada which contributes 11% of the total emission of CO₂. However, the presence of PCs (flavonoids, flavones, and phenolic acids) in the leaves render the biomass residue as a potential source of natural antioxidants which, in literature, have been shown to retard the oxidative degradation of fat-containing food products, thereby improving their nutritional value and shelf-stability (FAOSTAT, 2020; Kaur et al., 2022; Sin et al., 2014; Yeasmen et al., 2021). In this context, recovery and valorization of SML biomass would provide double benefit: first, it will alleviate the waste disposal problems which in turn will reduce environmental pollution; second, it will address a societal need to source healthy ingredients, thus highly contributing to a sustainable food chain

from both economic and environmental points of view. However, during the waste (SML biomass) to wealth conversion, the most profitable and sustainable challenges lie in the separation and recovery of beneficial compounds through extraction, which, to the best of existing evidence, is missing in the literature (Akhlishah et al., 2021; Alanon et al., 2020; Cujic et al., 2016; Sandra et al., 2020).

Because of its simplicity and relative efficiency, maceration is one of the oldest yet the most frequently applied extraction method for recovering PCs (Uysal et al., 2019). To design an efficient extraction process in order to maintain extract's quality which will be used as components of the products of pharmaceutical, nutraceutical, cosmetic, and food industry, optimization of the extraction factors (matrix parameters, solvent type and concentration, solid-to-solvent ratio, particle size, extraction time and temperature) need to be investigated very carefully. To successfully do so, it is always recommended to carefully design the experiment where the obtained responses will be further optimized using statistical tool. Factorial designs have been widely used and validated as an adequate statistical approach considering multiple variable factors into the design while response surface (RS) models are a multivariate approach used to optimize the effects of multiple factors and their interactions on single or multiple variables (Hannachi et al., 2019). Furthermore, in searching for environmentally friendly extraction conditions, it is recommended to use solvents that are Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) (Garmus et al., 2015). High-speed homogenization (HSH) is an effective sample pre-treatment technique which helps to facilitate the destruction of fresh materials for better access to intracellular substances. It achieves this by creating a powerful shearing force which is produced by the inner and outer blades located in the homogenizer (Jiao et al., 2015; Pei et al., 2019).

To keep pace with United Nations Sustainable Development Goals such as to achieve the sustainable management and efficient use of natural bioresources by 2030 (UNSDGs, 2015), and to prioritize the ever-growing demand for PCs from both consumer and commercial applications, it is key to find a technique facilitating the sustainable and effective management of SML biomass. This work is originally contributing knowledge not yet reported in the literature with i) a factorial study to produce SML extract where optimization evaluated the entire maceration process; ii) the use of a pre-treatment applying HSH for enhancing the efficiency of maceration; iii)

characterization of the SML extract; and iv) use of LC-ESI-Q-TOF-MS/MS for the phenolic compounds' profiling of SML extracts.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Sugar maple (*Acer saccharum*) leaves were freshly harvested from the Morgan Arboretum located at McGill University's Macdonald campus in Sainte-Anne-de-Bellevue (Quebec, Canada, 45°25'50.1"N 73°57'8.2"W). To minimize the environmental variability, mix of both young and mature leaves were taken from different parts of selected maple trees. To ensure the homogeneity in the sample, leaves were sorted having no pigmentation, blemishes, or apparent disease.

All chemicals and reagents used were of analytical grade and were purchased from Fisher Scientific, Nepean, Ontario, Canada except for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), which was obtained from Sigma (Sigma-Aldrich, St-Louis, MO, USA). Prerequisite concentrations of chemicals were prepared using HPLC grade water in all experiments.

3.3.2. Processing of samples

Immediately upon picking, all the SML were removed from the stems and then thoroughly washed under running tap water followed by immediate freezing at -20 °C and stored until lyophilization. SML were lyophilized using a freeze drier (CHRIST, GAMMA 1-16 LSC, MBI, Kirkland, Canada) and ground using milling machine (Retsch GmbH, GM 200, Retsch-Allee 1-5, Germany) to decrease the particle size of the sample. Afterwards, leaves powder was sieved through sieve no. 1 to ensure the particle size of <0.1 mm and then stored in amber centrifuge tube at -80°C until being used for extraction and further analyses. The initial and final weight of respectively fresh and dried samples were measured to calculate the moisture content of the leaves for all the samples.

3.3.3. Design of experiments (DOE)

With the objective of maximizing phenolic recovery, this experimental design is comprised of one-factor-at-a-time (OFAT) design followed by a full factorial design.

3.3.3.1. OFAT (Screening) experiment

OFAT experiment was performed to screen the best solvent (acetone, ethanol, water, ethyl acetate, and hexane) and homogenization speed (5000, 10000, 15000, 20000, and 25000 rpm) towards the maximum phenolic recovery while solid-to-solvent ratio was kept at 1:50 g/mL.

3.3.3.2. Full factorial experiment

With the result obtained from the OFAT experiment, full factorial design was carried out as a new set of experiment based on the 4×3 factorial design (solvent concentration: 30%, 50%, 70%, and 100%; and solid-to-solvent ratio: 1:20 g/mL, 1:30 g/mL, and 1:50 g/mL) which was given as input in the ‘Custom Design’ menu of JMP (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, NC, USA). From there 36 runs (in triplicate) were created. *D-optimal* design (where the design was fully orthogonal with a D-efficiency of 100%) was used to sort out the best variables underlying any factor in support of efficient extraction. With the obtained best solvent concentration and solid-to-solvent ratio, duration of homogenization (1 min, 5 min, and 10 min) was analyzed as an OFAT experiment.

3.3.4. Extraction of phenolic compounds from SML

As per the DOE, Maceration was carried out in Erlenmeyer flask (125 mL) under magnetic stirring by using a shaker machine (Max^Q 4000, BransteadInternational, Iowa, USA) for 24 h at a constant stirring speed (200 rpm) and temperature (25°C). To minimize the light effect during extraction, all the Erlenmeyer flasks were wrapped with aluminum foil. After each extraction, the suspensions were centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Germany) at $8000 \times g$ for 15 min. Afterwards, the supernatants were vacuum filtered through 11 μ m filter paper (Whatman no 1) (Figure 3.1), transferred into an amber bottle and stored at -80°C until further analyses.

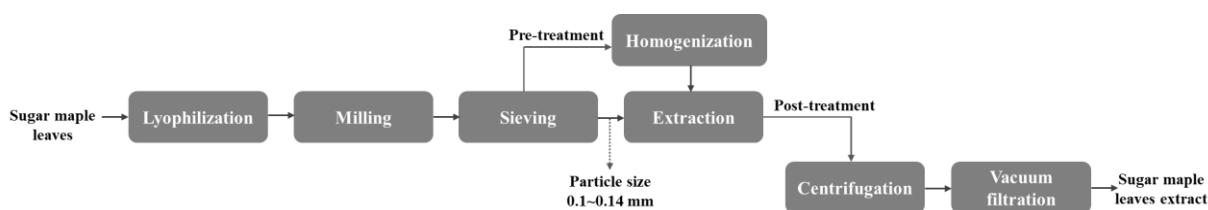


Figure 3.1: Schematic diagram of maceration procedure.

3.3.5. HSH assisted extraction

Prior to maceration, HSH as pre-treatment condition was carried out by a homogenizer (Homogenizer 850, Fisherbrand. USA), which is consisted of a controller, rotating cutter, and high-speed motor. The rotation speed of the inner cutter was regulated in the range of 5000–25000 rpm, and the homogenization time was in the range of 1-10 min. Afterwards, the extraction process took place in the similar manner of maceration.

3.3.6. Percentage total yield

Percentage total yield (%TY) of macerated SML extracts was calculated using the following equation (3.1):

$$\% \text{ TY} = \frac{\text{Dry mass of SML extract (g)}}{\text{Dry mass of SML powder (g) taken for extraction}} \times 100\% \dots\dots\dots (3.1)$$

3.3.7. Spectrophotometric quantification of phenolics

3.3.7.1. Total phenolic content

A spectrophotometric approach was used to quantify the total phenolic content (TPC) based on the method described by Singleton et al. (1999) with slight modification. First, 100 μL extract was mixed with 500 μL undiluted Folin-Ciocalteu reagent, to which, within 1 to 8 min, 1.5 mL of 20% (w/v) sodium carbonate was added and the volume of the solution was made up to 10 mL by adding HPLC grade water. This solution was vortexed for few minutes and incubated in the dark for 2 h at 25 C. The absorbance was measured at 760 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA). TPC in SML extract was quantified using gallic acid (0 – 2000 mg/L) standard calibration curve: $y = 0.0008x + 0.0284$, $R^2 = 0.998$ (with, y, absorbance at 760 nm; and x, concentration of TPC from the calibration curve) and calculated using equation (3.2):

$$\text{TPC} = \frac{C \times V}{m} \dots\dots\dots (3.2)$$

with, C, concentration of TPC from the calibration curve (mg/L); V, volume of solvent used in the extraction (L); and m, mass of the dried sample used (g). Results were expressed as mg gallic acid equivalent/g dry matter (mg GAE/g DM). All the analyses were done in triplicate and reported as mean $\pm$ standard deviation.

3.3.7.2. Total flavonoid content

Quantitative estimation of total flavonoid content (TFC) was done based on the method described by Jia et al. (1999). An aliquot of 1000 μL extract was thoroughly mixed with 300 μL of 5% sodium nitrite solution. After 5 min, 300 μL of 10% aluminum chloride solution was added to which 2000 μL of 1M sodium hydroxide solution was added after 6 min and the volume of the solution was made up to 10 mL by adding HPLC grade water. This solution was vortexed for few minutes and the absorbance was measured at 510 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA). Then, the concentration of TFC in the SML extract was calculated from the catechin (0 - 125 mg/L) standard calibration curve: $y =$

$0.0019x + 0.0014$, $R^2 = 0.998$ (with, y, absorbance at 510 nm; and x, concentration of TFC from the calibration curve) and calculated using equation (3.3):

$$\text{TFC} = \frac{C \times V}{m} \dots\dots\dots (3.3)$$

with, C, concentration of TFC from the calibration curve (mg/L); V, volume of solvent used in the extraction (L); and m, mass of the dried sample used (g). Results were expressed as mg catechin equivalent (mg CTE/g DM). All the analyses were done in triplicate and reported as mean $\pm$ standard deviation.

3.3.8. *In vitro* antioxidant activities of the SML extracts

3.3.8.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Electron donating ability of SML extracts was verified using DPPH assay based on the method described by Thaipong et al. (2006) with slight modification. To obtain the stock solution, 24 mg DPPH was diluted with 100 mL HPLC grade methanol and stored at -20°C until required. During the experiment, 100 μL SML extract was allowed to react with 3000 μL DPPH working solution (prepared by diluting the stock solution with HPLC grade methanol until getting the absorbance of 1.1 ± 0.02 unit at 515 nm wavelength) for 30 min in the dark. Then the absorbance was recorded at 515 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA). The standard curve ($y = -0.4159x + 0.633$, $R^2 = 0.991$; with, y, absorbance at 515 nm; and x, scavenging capacity of SML extract from the calibration curve) was linear between 0 and 1000 μM Trolox and results were expressed as μM trolox equivalent (μM TE/g DM). This assay was carried out in triplicate and mean $\pm$ standard deviation was computed.

3.3.8.2. FRAP (Ferric reducing antioxidant power) assay

The FRAP assay was done based on the method described by Benzie and Strain (1999) with little modification. The method is based on the reduction of ferric-TPTZ (2, 4, 6- tripyridyl-s-triazine) complex to the ferrous form at low pH. Briefly, working FRAP solution was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in the ratio of 10:1:1 followed by warming to 37 C before using. 100 μL SML extract was allowed to react with 3000 μL FRAP working solution at dark condition for 30 min. Absorbance of the colored mixture was recorded at 593 nm using plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA). The standard curve ($y = 0.9991x - 0.0104$, $R^2 = 0.992$; with, y, absorbance at 593 nm; and x, reducing capacity of SML extract from the calibration

curve) was linear between 0 and 1000 μM Trolox and results were expressed as $\mu\text{M TE/g DM}$. This assay was carried out in triplicate and mean $\pm$ standard deviation was computed.

3.3.9. Characterization of sample

3.3.9.1 Color indices

Commission Internationale d'Eclairage (CIELab) colour system was used to investigate the color indices of fresh leaves and was assessed using a spectrophotometer (CM-3500d, Minolta Spectrophotometer, Minolta Co. Ltd., Japan), calibrated with a standard white plate ($L = 98.80$, $a^* = -0.22$, and $b^* = -0.39$) and zero calibration cylinder (CIE $L^* = 0.09$, average reflectance = 0.01%). Color of leaves was determined in CIELab space (Illuminant D65, 10° viewing angle) under the reflection spectra between 400 and 700 nm. Measurements were taken in triplicate for all samples.

3.3.9.2. Scanning electron microscopy

The morphological changes of SML powder before and after extraction was examined by a scanning electron microscope (SEM, TM3000, Hitachi, Japan). Samples were mounted on aluminum stubs with double-sided carbon adhesive tabs and observed at a magnification of $\times 1000$. SEM micrographs were recorded at an accelerating voltage of 5 kV (to prevent sample modification due to heat generated at higher voltage) using auto contrast mode of imaging. To see the smoothness of leaves surface, SEM micrographs were analyzed by Java-based public domain image processing software i.e., Image J 1.52a (National Institute of Health, USA). Measurements were taken in triplicate for all samples.

3.3.9.3. Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transform infrared was applied to identify different characteristic functional groups in the SML. The IR spectra were obtained using an ATR-FTIR spectrometer (Thermo Scientific Nicolet iS5 FTIR, Thermo Fisher Scientific, U.S.A.) equipped with a fast recovery deuterated triglycine sulfate (DTGS) detector and a temperature controlled solid-state diode laser. The operation of the spectrometer, acquisition and manipulation of spectra was performed under the Omnic software (version 6.1, Nicolet, Madison, WI, USA). The obtained IR spectra were scanned across the wave numbers ranging from 4000 to 600 cm^{-1} by co-adding 32 scans at a resolution of 4 cm^{-1} . All spectra were rationed against the background spectrum of air where a new reference air background spectrum was taken in every 60 min of operation. The spectra were recorded as absorbance values and measurements were taken in triplicate for all samples.

3.3.9.4. LC-ESI-Q-TOF-MS/MS analysis of phenolic fraction

Separation and identification of SML phenolic compounds were performed using an Agilent 1290 Infinity II LC system coupled to the 6545 Q-TOF-MS (Agilent Technologies, Santa Clara, USA). To prepare the sample for chromatographic separation and identification, briefly, 0.5 g of SML powder was macerated with 15 mL of 70% ethanol ($\times 3$) on a shaker machine (Unimax 1010, Heidolph, Germany) for 24 h with a constant stirring speed of 200 rpm and temperature of 25 C. Suspensions were centrifuged (ST16R Centrifuge, Thermo Fisher Scientific, Leicestershire, UK) at 8000 $\times g$ for 15 min. Afterwards, the supernatants were vacuum filtered through 11 μm (Whatman no 1), evaporated and dissolved in 2 mL of 50% methanol. This solution was filtered through a 0.20 μm RC syringe filter, then transferred into an amber vial and stored at -80 C until analysis. The LC separation was conducted on a Poroshell 120 EC-C18 analytical column (Agilent Technologies; 3 mm $\times$ 100 mm, 2.7 μm) connected with a Poroshell 120 EC-C18 guard column (Agilent Technologies; 3 mm $\times$ 5 mm, 2.7 μm). The chromatographic conditions used were as follows: column temperature 40 C; injection volume 5 μL ; mobile phase, HPLC grade water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) with the following gradient program: 0–4 min, 2% B; 4–7 min, 2–20% B; 7–20 min, 20–100% B; 21–24 min, 100% B; 24–25 min, 100–2% B; 25–27 min, 2% B; and postrun for 3 min, at a flow rate of 0.3 mL/min.

The mass spectrometer electrospray capillary voltage was maintained at 3.5 kV, fragmentor voltage, 200 V; drying gas temp, 350 C; drying gas flow rate, 8 L/min; pressure on nebulizer, 40 psi; sheath gas temp, 400 C; sheath gas flow rate, 10 L/min. All Ions MS/MS was performed with collision energy of 0, 10, 20, 40 V. The mass spectra were acquired in both positive and negative ion modes. The mass-to-charge ratio (m/z) was scanned across the range of 100–1100. Suspect screening was performed with the MassHunter Profiling software series. In each ion mode, data were first aligned using Agilent MassHunter Profinder (B.10.0) with the “Batch recursive feature extraction” and a mass list of entities in each ion mode was obtained. The statistical comparison of the chemical profiles among groups was completed using MassHunter Profiler Professional (MPP, version B15.0).

3.3.10. Statistical analysis

Significant differences ($p < 0.05$) with 95% confidence interval among the mean values were analyzed applying ANOVA (analysis of variance) and Tukey HSD tests. Correlation between

phenols and antioxidant activity of SML extract was analyzed using Pearson correlation analysis. All the analyses were performed using the statistical software JMP (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, NC, USA) and graphical representations are developed with licensed version of Microsoft Excel 16 (Microsoft Corporation, USA).

3.4. RESULTS AND DISCUSSION

3.4.1 OFAT experiment

The total concentration of phenolics (TPC and TFC) extracted from the SML for 24 h with solvents including acetone, ethanol, water, ethyl acetate, and hexane at a solid-to-solvent ratio of 1:50 g/mL were compared to find out the best solvent type. Moreover, under the pre-treatment experiment, similar response was compared against the different homogenization speed ranging from 5000 to 25000 rpm using absolute ethanol. The ANOVA results and comparison of the mean values of TPC and TFC revealed that the type of solvent as well as homogenization speed had a statistically significant effect ($p < 0.05$) on the yield of extraction (Table 3.1).

Table 3.1: Total phenolic and total flavonoid content of SML extracts using different extracting solvents and homogenization speed. Different uppercase letters indicate significant differences based on 95% confidence interval ($p < 0.05$) where A-E and F-I show differences respectively in TPC and TFC among solvent types, J-L and M-O show differences respectively in TPC and TFC among homogenization speed.

		TPC (mg GAE/g DM)	TFC (mg CTE/g DM)
Solvent type (absolute)	Acetone	104.90±1.14 ^A	38.39±1.23 ^F
	Ethanol	89.11±1.36 ^B	29.06±1.69 ^G
	Water	68.75±0.88 ^C	15.03±0.13 ^H
	Ethyl acetate	41.25±1.09 ^D	15.67±1.57 ^H
	Hexane	3.03±1.63 ^E	1.10±1.65 ^I
Homogenization speed (rpm)	5000	86.88±0.15 ^K	23.98±0.58 ^O
	10000	96.28±1.71 ^J	27.97±0.15 ^M
	15000	86.84±1.77 ^K	26.42±0.21 ^N
	20000	84.57±0.54 ^K	26.01±0.37 ^N
	25000	75.64±0.66 ^L	24.41±0.44 ^O

TPC: Total phenolic content; GAE: Gallic acid equivalent; DM: Dry matter; TFC: Total flavonoid content; CTE: Catechin equivalent.

3.4.1.1. Effect of solvent type on phenolic recovery

All the tested solvents were capable of extracting phenolics from SML with TPC and TFC values ranging from 3.03 to 104.90 mg GAE/g DM and from 1.10 to 38.39 mg CTE/g DM, respectively (Table 3.1). Hexane, in both cases, provided the lowest phenolic recovery from SML. The variation in results as a function of the solvent used can be explained as phenolics are mostly soluble in organic solvents which are less polar than water, therefore, highly polar (e.g. water) or highly non-polar (e.g. hexane) solvent might not be a good fit for the better recovery of phenolics. Rafiee et al. (2011) reported that water used as the only solvent might result in the overestimation of phenolics as it yields the extract containing not only PCs but also other unwanted substances such as soluble protein, organic acids, sugars, among others due to their high solubility in water. Our results showed that, both acetone and ethanol worked better in comparison to the other solvents used in this study. Among other parameters, solubility of PCs in the extracting solvent is highly influenced by the polarity and molecular weight of the solvent. In this study, polarity and molecular weight of solvents followed order: water > ethanol > ethyl acetate > acetone > hexane compared to ethyl acetate > hexane > acetone > ethanol > water, respectively. In both cases acetone and ethanol placed in middle position, thereby, it can be anticipated that the nature of PCs present in SML are moderate to near high polar. Being in the *List of Permitted Carrier or Extraction Solvents* approved by HealthCanada (2015) and holding the GRAS label given by USFDA (2019), ethanol was considered as the optimum solvent for the subsequent experiments. However, according to Sim, Ong and Nyam (2019), absolute alcoholic solvents might cause the reduction of extraction yield therefore aqueous ethanol, as a moderately polar extracting medium, was adopted for further experiments. With this combination both glycosidic and non-glycosidic PCs can be extracted because molecular structure of water allowed it to be involved in the chemical reactions associated with oxygen-hydrogen bonds, whereas ethanol can participate in the reactions involving both oxygen-hydrogen and carbon-oxygen bonds (Sim, Ong, & Nyam, 2019). Knowing that the solubility of PCs in the extracting solvent is highly influenced by both the polarity and chemical composition of the solvent and PCs themselves, therefore, the extracting medium (ethanol), having similar chemical group (-OH) of the target compounds (phenol) would be best fit towards the maximum extraction efficiency. Moreover, the aqueous ethanol played a dual role as water is responsible for swelling of the plant matrix which consequently increased access to more contact surface area between the solvent and plant matrix where ethanol was responsible for disrupting the

existing bonds ensuring better mass transfer of the compounds between the solvent and plant matrix.

3.4.1.2. Effect of homogenization speed on phenolic recovery

According to Table 3.1, significantly higher ($p < 0.05$) phenolics (i.e., TPC of 96.28 ± 1.71 mg GAE/g DM and TFC of 27.97 ± 0.15 mg CTE/g DM) were observed at homogenization speed of 10000 rpm; increasing homogenization speed resulted in decreasing TPC and TFC values. This is because the increasing of shearing forces of the inner and outer blades of the homogenizer, with the increasing rotation speed of the homogenizer, is likely leading to the destruction of phenolic structures. While comparing with the unhomogenized results, homogenized ethanolic extracts of SML gave significantly higher TPC value based on the 95% confidence interval at a homogenization speed of 10000 rpm. Therefore, the optimum homogenization speed selected in the subsequent experiments was set to 10000 rpm.

3.4.2. Full factorial experiment

To determine the optimal levels of ethanol concentration (30-100% v/v) and solid-to-solvent ratio (1:20-1:50 g/mL) which will have significant influence on the yield of total phenolics (represented as TPC, TFC, and %TY), a full factorial (4×3 factorial) experiment was performed (Figure 3.2). Maximum contents of TPC, TFC, and %TY were recorded as 100.82 ± 0.38 mg GAE/g DM, 19.31 ± 0.28 mg CTE/g DM, and $29.62 \pm 2.04\%$ under the following experimental conditions: ethanol concentration of 70% and solid-to-solvent ratio of 1:30 g/mL. However, minimum contents of both %TY ($14.53 \pm 0.44\%$) and TPC (42.54 ± 0.41 mg GAE/g DM) were obtained when SML was extracted with 100% ethanol at a solid-to-solvent ratio of 1:20 g/mL. Our results are in accordance with previous literature reports (da Rosa et al., 2019; Elboughdiri, 2018; Krakowska et al., 2017; Zhao et al., 2019) in which the highest phenolic content from leaves of different plant species were recovered through maceration with the similar extraction conditions i.e., 70% ethanol at a solid-to-solvent ratio of 1:30 g/mL. Concerning solid-to-solvent ratio it can be assumed that at a ratio of 1:30 g/mL PCs were extensively extracted.

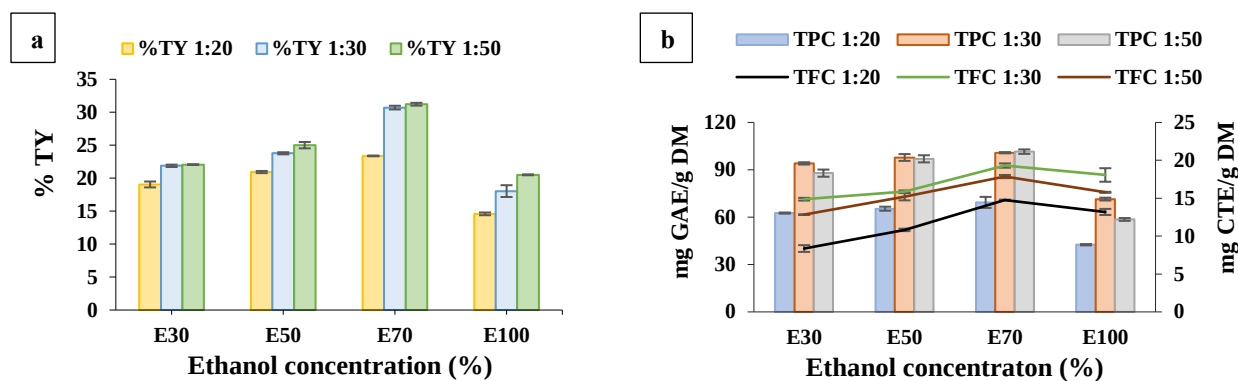


Figure 3.2: Effect of ethanol concentration and solid-to-solvent ratio on (a) %TY, (b) TPC, and TFC of SML extracts. All the presented data are expressed as mean $\pm$ SD of triplicate analyses. *TY*: Total yield; *TPC*: Total phenolic content; *TFC*: Total flavonoid content; *GAE*: Gallic acid equivalent; *CTE*: Catechin equivalent; *DM*: Dry matter; *E*: Ethanol; *SD*: Standard deviation.

3.4.2.1. Optimization of the extraction condition

A response surface model was used to optimize the extraction condition and the model summary is presented in Figure 3.3. The determination coefficient (R^2) values (Figure 3.3a-3.3c) from the regression analysis were 0.96, 0.90, and 0.71 for TPC, TFC, and %TY, respectively, which indicates a good fit of the model. Moreover, the adjusted determination coefficients (R^2_{adj}) for TPC, TFC, and %TY were respectively 0.95, 0.88, and 0.65, which were very close to R^2 . Overall, the regression coefficients from experimental data and the adjusted one were reasonably close to 1, indicating a high degree of correlation between the observed and predicted values for each response (Crupi et al., 2018). Ethanol concentration and solid-to-solvent ratio had a significant effect ($p < 0.05$) on the response of TPC and TFC except for %TY. Effect of solid-to-solvent ratio was found to be the most pronounced for every response. Moreover, concentration of ethanol was negatively correlated with both TPC and %TY i.e., higher the ethanol concentration, the lower the extraction yield. The two factor interactions (ethanol concentration*solid-to-solvent ratio) was significant only in the case of TPC. However, irrespective of responses (TPC, TFC, and %TY) both ethanol concentration and solvent ratio showed a quadratic effect which implies that at either extreme, the measured responses (TPC, TFC, and %TY) deviate from the maximum output. A common prediction profiler was created for the responses (TPC, TFC, and %TY) and the desirability function was included (Figure 3.3h). According to the prediction profiler at 86.42% desirability, the optimum value for TPC, TFC, and %TY were found as 111.09 mg GAE/g DM, 19.40 mg CTE/g DM, and 28.83% at a solid-to-solvent ratio of 1:38.22 g/mL with ethanol

concentration of 61.63%. A confirmatory experiment was performed under the optimal conditions where %TY, TPC and TFC were obtained as 28.57%, 110.87 mg GAE/g DM, and 18.95 mg CTE/g DM respectively, which were very close to the predicted values.

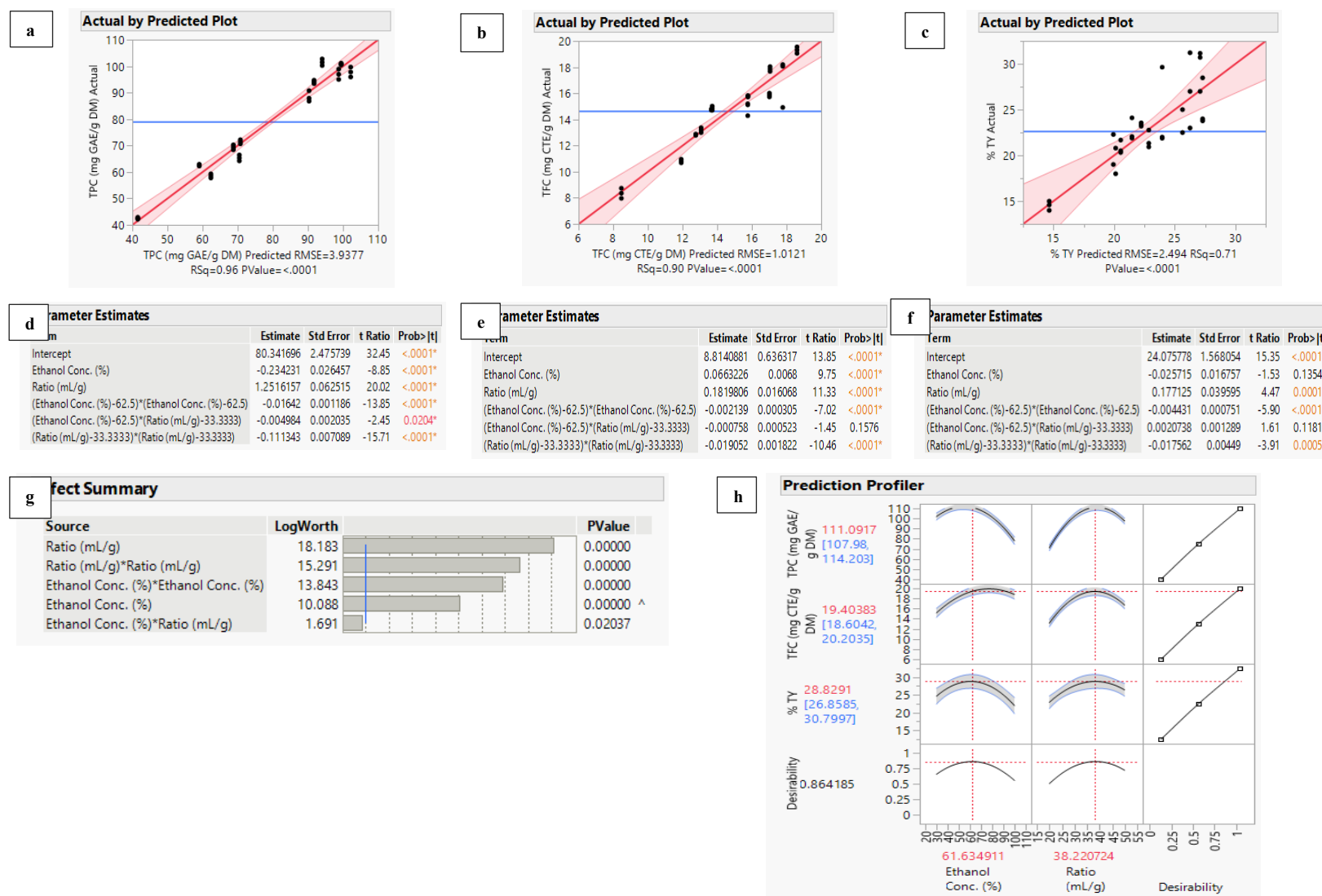


Figure 3.3: Summary of response surface model optimizing extraction condition for the recovery of phenolics from SML. Coefficient of determination (RSq) value for the response of TPC (a), TFC (b), %TY (c); parameter estimates for the response of TPC (d), TFC (e), %TY (f); effect summary of the model (g), and prediction profiler with 95% confidence interval for the response of TPC, TFC, and %TY (h). TY: Total yield; TPC: Total phenolic content; TFC: Total flavonoid content; GAE: Gallic acid equivalent; CTE: Catechin equivalent; DM: Dry matter.

3.4.2.2. Effect of homogenization duration on phenolic recovery

The results presented in Figure 3.4 illustrate that homogenization time plays a quadratic role during phenolic recovery; comparatively lower recovery of SML phenolics were obtained at either extreme (maximum or minimum) condition of homogenization duration. Based on these results, maximum %TY (27 ± 0.67 %), TPC (109.93 ± 1.7 mg GAE/g DM), and TFC (19.52 ± 0.46 mg CTE/g DM) were obtained when SML was homogenized for 5 min. Our results are in accordance with the findings of Xu et al. (2019) where they reported maximum yield of stevioside and rebaudioside from stevia leaves at 6 min while the homogenization time range was 2 to 12 min. The downward trend of phenolic recovery with extension of homogenization time is likely due to the thermal degradation of PCs caused by the high shearing forces where the shearing energy is dissipated as heat.

While comparing the results between with- and without- homogenization pre-treatment, under the same extraction conditions (70% ethanol and 1:30 g/mL solid-to-solvent ratio), HSH prior to maceration was found to offer significantly higher ($p < 0.05$) phenolic content. Therefore, HSH assisted maceration can be considered as an effective extraction condition towards the maximization of PCs recovery from SML.

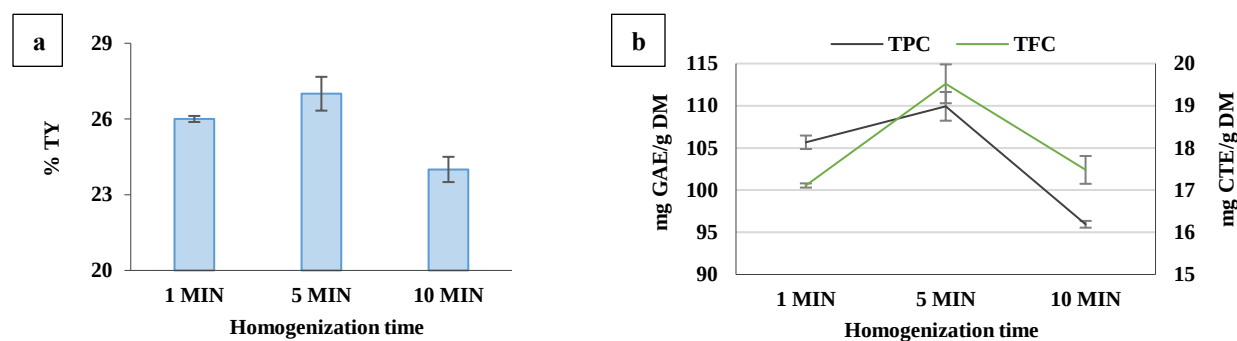


Figure 3.4: Effect of homogenization duration on (a) %TY, (b) TPC and TFC of SML extracts. All the presented data are expressed as mean $\pm$ SD of triplicate analyses. *TY*: Total yield; *TPC*: Total phenolic content; *TFC*: Total flavonoid content; *GAE*: Gallic acid equivalent; *CTE*: Catechin equivalent; *DM*: Dry matter; *MIN*: Minute; *SD*: Standard deviation.

3.4.3. In vitro antioxidant activity of the SML extracts and correlation analysis

The antioxidant potentiality of plants is attributed to the presence of three major groups: phenolics, vitamins (A, D, C, E), and carotenoids, which may act independently or in combination in varied mechanisms. Regarding phenolics, the antioxidant efficiency is mostly related to the

number of available hydroxyl groups present in their structure (Ozgen et al., 2006). *In vitro* antioxidant activity of SML extracts at a varying ethanol concentration and homogenization duration was measured using single electron transfer mechanism namely DPPH and FRAP assays (Figure 3.5). In both cases, significantly highest antioxidant activities were obtained using FRAP assay rather than DPPH assay (Figure 3.5a and 3.5b). Correlation analysis among TPC, FRAP, and DPPH assays were calculated where irrespective of ethanol concentration or homogenization time, FRAP assay showed highest positive correlation with TPC (Figure 3.5c and 3.5d). Explanation can be given as DPPH is a free radical which is lipophilic in nature, thereby mostly suitable for samples having lipophilic antioxidants. However, the discrepancy among the DPPH and FRAP results might be ascribed due to the complexity of the antioxidant reaction kinetics, time to reach the equilibrium condition (steady state), generation of new antioxidants through polymerization of PCs, type of extracting medium, calibration standards, and/or the nature of PCs present in SML extracts (Deng et al., 2014; Nongmaithema & Meda, 2017; Ozgen et al., 2006).

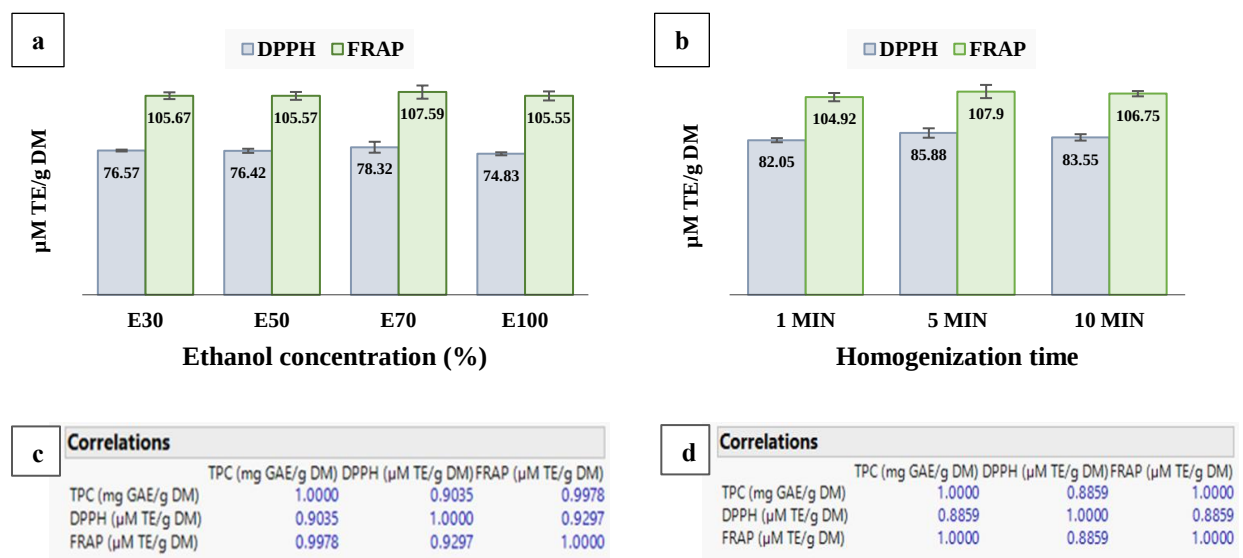


Figure 3.5: *In vitro* antioxidant activity of SML extracts using DPPH free radical scavenging potential and FRAP assay at a varying ethanol concentration (A) and homogenization duration (B). Pearson correlation coefficients (r) among TPC, DPPH and FRAP assay at a varying ethanol concentration (C) and homogenization duration (D). All the presented data are expressed as mean $\pm$ SD of triplicate analyses. E: Ethanol; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; MIN: Minute; μ M: Micro mol; TE: Trolox equivalent; TPC: Total phenolic content; GAE: Gallic acid equivalent; SD: Standard deviation.

3.4.4. Characterization of SML

3.4.4.1. SML color indices

The measured L (42.94 ± 0.83), a (-5.44 ± 0.22), b (9.52 ± 0.76) values of SML were indicative of more greenness color which in turn confirms the expected presence of chlorophyll pigments in tree leaves.

3.4.4.2. SML morphology by SEM

SEM photographs of SML before and after maceration are presented in Figure 3.6a and 3.6b. According to the micrograph, maceration caused damage to the leaf surface where extraction is more likely to occur due to solvent penetration into the cell, consequently facilitating the release of PCs from the cells into the extraction medium. Based on the SEM images, a surface plot was created (Figure 3.6c and 3.6d) using ImageJ software (version 1.52a, National Institutes of Health, USA) for which fractal dimension (FD) value was calculated by Box-counting algorithm (Rahimi & Ngadi, 2016). FD values (1.904 for non-macerated SML and 1.864 for macerated SML) indicate that the irregularity of cell walls and intercellular spaces in SML increased due to the loss of PCs during extraction (Sansiribhan et al., 2012). Accordingly, it can be concluded that non-macerated leaves had undamaged but rough surface whereas the macerated one contained smooth surface with porous/abrupted structure.

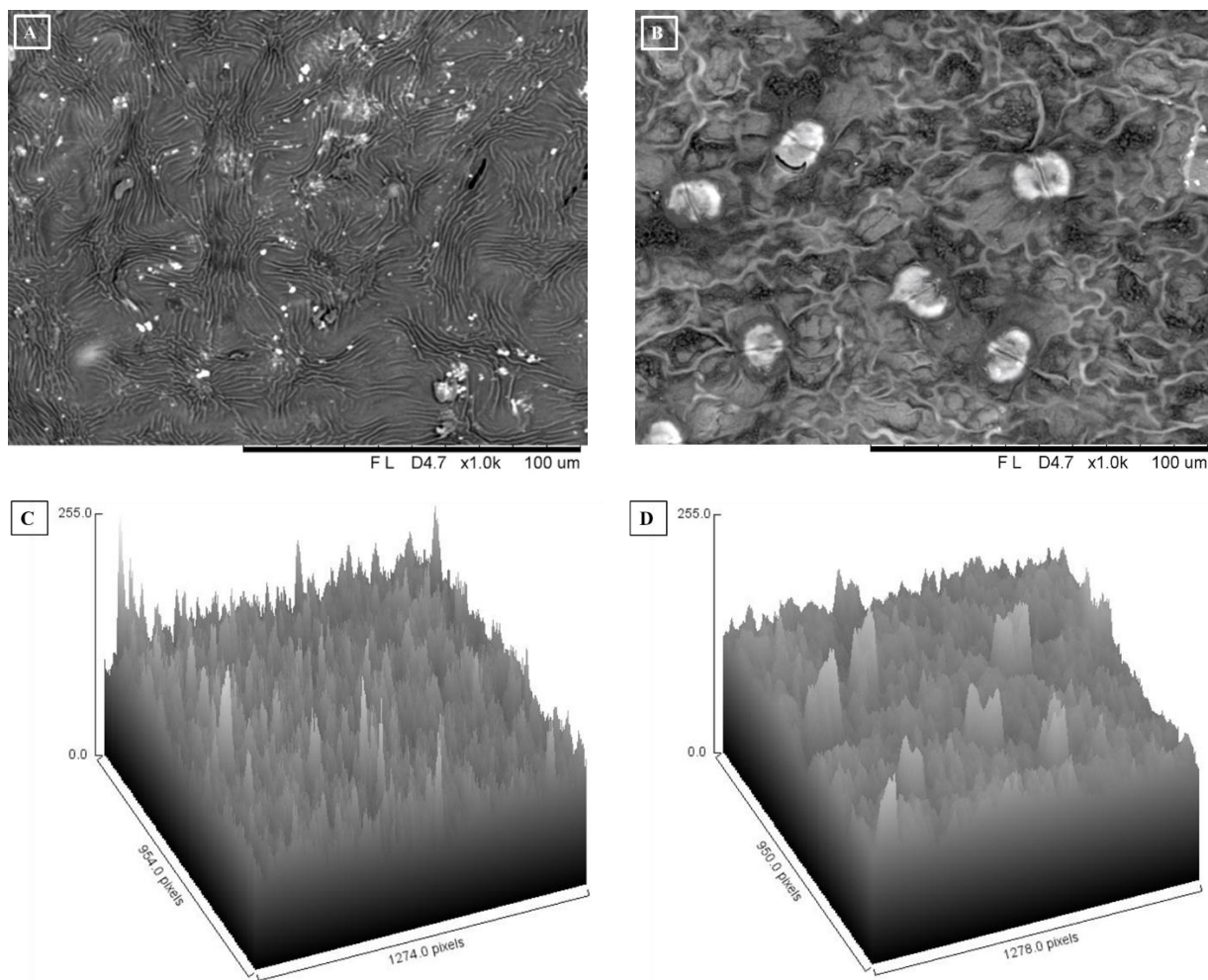


Figure 3.6: SEM images of SML at a magnification of 1000 $\times$ before (a) and after (b) extraction. Surface plot of SML (c) and after (d) extraction.

3.4.4.3. Identified functional groups in the extract

According to Figure 3.7, the observed FTIR spectra for SML before and after freeze drying indicates that freeze drying is an effective drying method leaving no chemical composition changed except for the removal of water. However, this FTIR spectrum can generate characteristic fingerprints of SML by determining the vibration of bonds present in the functional groups. Accordingly, seven characteristic absorption peaks ranging from 1097 cm^{-1} to 3385 cm^{-1} were obtained from SML at a threshold value of 0.1. The peak at 1097.26 cm^{-1} indicates the presence of secondary alcohol and/or ester group (Alara et al., 2018). The presence of alkoxy (C-O) group might be associated with the presence of a peak at 1162.95 cm^{-1} . Absorption band at 1642.67 cm^{-1} was assigned to the presence of amides while the presence of aldehyde, ketone and carboxylic acid can be associated with the peak at 1730.75 cm^{-1} . In addition, absorption peaks in the range of

2800 cm^{-1} to 3000 cm^{-1} indicate the existence of C-H stretch vibration that can be linked with the presence of lipids (Sim, Ong, & Nyam, 2019). Finally, the absorption band at 3385.38 cm^{-1} was assigned to the presence of an alcoholic or phenolic group due to presence of an -OH stretching vibration band (Alara, Abdurahman, & Ukaegbu, 2018). The identified functional groups in SML indicate the presence of phenols and flavonoids which further are associated with the antioxidant activity of the obtained SML extracts.

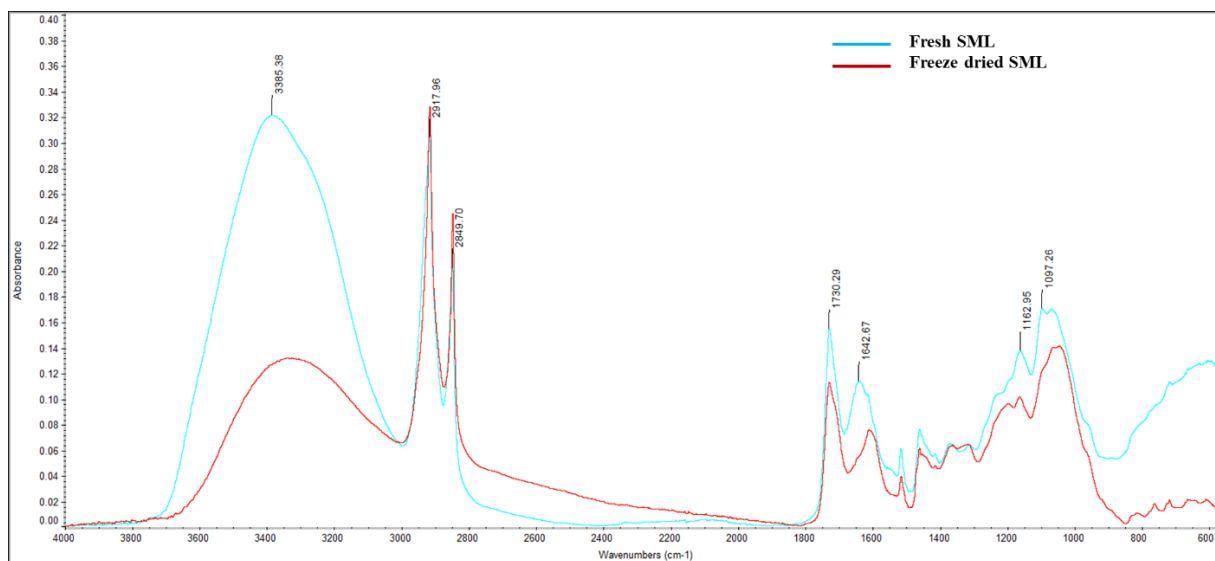


Figure 3.7: IR spectra of SML before and after freeze drying. *SML: Sugar maple leaves.*

3.4.4.4. Identified PCs by LC-ESI-Q-TOF-MS/MS

PCs in SML extracts were identified by LC-ESI-Q-TOF-MS/MS analyses (Table 3.2). The identification was based on the interpretation of their mass spectra given by TOF-MS and the data reported in HMDB 5.0 (Wishart et al., 2018) and Phenol-Explorer 3.6 (Rothwell et al., 2013). A total of 81 PCs were tentatively characterized, including some interesting compounds showing evidential pharmacological activities. For example, catechin, gallic acid, caffeic acid, coumaric acid, ferulic acid, myricetin have been reported to show anti-diabetic, anti-cancer, anti-inflammatory, cardioprotective, and anti-microbial activities (Belwal et al., 2019; Bhuyan & Basu, 2017; Cory et al., 2018; Kumar & Goel, 2019; Saltveit, 2018). Moreover, a gallotannins pathway is hypothesized to be present in sugar maple (*Acer saccharum*) leaves leading to the biosynthesis of galloyl-beta-D-glucopyranoside, digalloyl-beta-D-glucopyranose, trigalloyl-beta-D-glucopyranose, and tetragalloyl-D-glucopyranose from gallic acid. Yang et al. (2017) elucidated a pathway forming complex gallotannins in *Acer truncatum*

leaves where most of the mentioned galloylglucopyranoses were also found in sugar maple leaves. Hence, we assume that the gallotannins pathway present in *Acer truncatum* leaves and the one present in *Acer saccharum* leaves might be quite similar with each other.

Table 3.2: Chromatographic data regarding tentatively identified phenolic compounds in sugar maple leaves extract by LC-ESI-Q-TOF-MS/MS in both negative and positive ion mode.

Serial	t _R (min)	Formula	Neutral mass	Proposed compounds	Class	Sub-class
ESI-						
Phenolic acids and derivatives						
1	4.25	C ₇ H ₆ O ₅	170.0216	2,4,6-Trihydroxybenzoic acid	Phenolic acids	Hydroxybenzoic acid
2	7.04	C ₁₄ H ₁₆ O ₁₀	344.0751	Theogallin	Phenolic acids	Hydroxybenzoic acid
3	7.29	C ₁₉ H ₂₆ O ₁₅	494.1274	2-O-Galloylsucrose	Benzene and substituted derivatives	Benzoic acid and derivatives
4	7.52	C ₁₄ H ₁₄ O ₉	326.0638	Fertaric acid	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
5	7.56	C ₁₆ H ₂₀ O ₉	356.1111	1-O-Feruloylglucose	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
6	7.88	C ₁₄ H ₁₈ O ₁₀	346.0896	Methyl 6-O-galloyl-beta-D-glucopyranoside	Benzene and substituted derivatives	Benzoic acid and derivatives
7	7.92	C ₁₀ H ₁₀ O ₄	194.0581	Ferulic acid	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
8	8.43	C ₁₃ H ₁₂ O ₈	296.0532	Mono-trans-p-coumaroylmesotartaric acid	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
9	8.5	C ₂₀ H ₂₀ O ₁₄	484.0859	Hamamelitannin	Benzene and substituted derivatives	Benzoic acid and derivatives
10	8.71	C ₁₅ H ₁₆ O ₁₀	356.0748	Caffeic acid 3-O-glucuronide	Phenolic acids	Hydroxycinnamic acids
11	9.96	C ₁₄ H ₆ O ₈	302.0062	Ellagic acid	Phenolic acids	Hydroxybenzoic acid
12	10.67	C ₈ H ₈ O ₅	184.0373	Methyl 2,4,6-trihydroxybenzoate	Benzene and substituted derivatives	Benzoic acid and derivatives
13	11.02	C ₂₁ H ₂₄ O ₁₀	436.1365	Phenethyl 6-galloylglucoside	Benzene and substituted derivatives	Benzoic acid and derivatives
Flavonoids and derivatives						
14	7.35	C ₄₅ H ₃₈ O ₁₈	866.2051	Cinnamtannin A1	Flavonoids	Biflavonoids and polyflavonoids
15	7.93	C ₁₄ H ₁₀ O ₉	322.0343	Digallate	Flavonoids	Flavanols
16	7.96	C ₂₁ H ₂₀ O ₁₃	480.0912	Quercetin-3'-glucuronide	Flavonoids	Flavonols
17	8.30	C ₃₀ H ₂₆ O ₁₂	578.1421	ent-Epicatechin-(4alpha->6)-ent-epicatechin	Flavonoids	Biflavonoids and polyflavonoids
18	8.85	C ₂₁ H ₁₈ O ₁₃	478.0757	Quercetin 3-O-glucuronide	Flavonoids	Flavonols

19	9.36	C ₁₆ H ₁₂ O ₈	332.0534	4',5,6,7,8-Pentahydroxy-3'-methoxyflavone	Flavonoids	O-methylated flavonoids
20	9.38	C ₂₁ H ₂₄ O ₁₁	452.1326	Catechin 5-glucoside	Flavonoids	Flavonoid glycosides
21	9.52	C ₂₁ H ₂₀ O ₁₂	464.0965	Quercetin 3-galactoside	Flavonoids	Flavonoid glycosides
22	9.79	C ₂₈ H ₂₄ O ₁₆	616.1071	6"-O-Galloylquercimeritrin	Flavonoids	Flavonoid glycosides
23	10.15	C ₂₂ H ₁₈ O ₁₀	442.0908	7-Galloylcatechin	Flavonoids	Flavanans
24	10.26	C ₂₃ H ₂₂ O ₁₃	506.1067	Quercetin 3-O-(6"-acetyl-glucoside)	Flavonoids	Flavonoid glycosides
25	10.55	C ₂₁ H ₂₀ O ₁₁	448.1017	Astragalin	Flavonoids	Flavanols
26	10.62	C ₂₉ H ₂₂ O ₁₄	594.1014	3,5-Digalloylepicatechin	Flavonoids	Flavans
27	10.92	C ₃₂ H ₄₀ O ₁₈	712.2233	Glucoliquiritin apioside	Flavonoids	Flavonoid glycosides
28	11.01	C ₂₂ H ₂₂ O ₁₁	462.1173	6-Methoxyluteolin 7-rhamnoside	Flavonoids	Flavonoid glycosides
29	11.09	C ₂₁ H ₂₀ O ₁₀	432.1068	Kaempferol 3-O-alpha-L-rhamnofuranoside	Flavonoids	Flavonoid glycosides
30	11.51	C ₂₃ H ₂₆ O ₁₁	478.1484	4'-O-methyl(-)-epicatechin-5-O-beta-glucuronide	Flavonoids	Flavonoid glycosides
31	12	C ₃₇ H ₃₀ O ₁₈	762.1444	3'-Galloylprodelphinidin B2	Flavonoids	Biflavonoids and polyflavonoids
32	12.19	C ₁₅ H ₁₀ O ₇	302.0431	6-Hydroxykaempferol	Flavonoids	Flavonoid glycosides
Stilbenes						
33	11.66	C ₂₆ H ₃₂ O ₁₃	552.1856	(Z)-Resveratrol 3,4'-diglucoside	Stilbenes	Stilbene glycosides
Coumarins						
34	8.92	C ₁₆ H ₁₈ O ₁₀	370.0907	5-Hydroxy-6-methoxycoumarin glucoside	7-Coumarins derivatives and coumarin	Coumarin glycosides
Tannins						
35	7.82	C ₂₇ H ₂₄ O ₁₈	636.0933	1,3,4-Trigalloyl-beta-D-glucopyranose	Tannins	
36	8.77	C ₃₃ H ₂₄ O ₂₁	756.0819	Syzyginin B	Tannins	Hydrolyzable tannins
37	8.99	C ₂₇ H ₂₂ O ₁₈	634.0814	Sanguin H4	Tannins	Hydrolyzable tannins
38	9.68	C ₃₄ H ₂₈ O ₂₂	788.1086	2,3,4,6-Tetragalloyl-D-glucopyranose	Tannins	Hydrolyzable tannins
39	11.93	C ₂₉ H ₂₆ O ₁₅	614.1273	2-Cinnamoyl-1,6-digalloyl-beta-D-glucopyranose	Tannins	
Others						
40	2.53	C ₁₃ H ₁₆ O ₁₀	332.0746	6-Galloylglucose	Diazines	Pyrimidine and pyrimidine derivatives
41	7.09	C ₁₅ H ₂₀ O ₈	328.1157	Ethylvanillin glucoside	Organooxygen compounds	Phenolic glycosides
42	7.11	C ₁₆ H ₂₂ O ₉	358.1262	Phlorisobutyrophenone 2-glucoside	Organooxygen compounds	Phenolic glycosides
43	7.46	C ₉ H ₁₀ O ₇ S	262.0150	2-Hydroxy-4-methoxyacetophenone sulfate	5-Organooxygen compounds	Carbonyl compounds

44	7.94	C ₈ H ₈ O ₆ S	232.0043	2,4-Dihydroxyacetophenone 5-sulfate	Organooxygen compounds	Carbonyl compounds
45	8.06	C ₁₆ H ₁₈ O ₉	354.0950	Cis-5-Caffeoylquinic acid	Organooxygen compounds	Alcohols and polyols
46	8.42	C ₂₁ H ₂₂ O ₁₃	482.1056	3'-(2"-Galloylglucosyl)-phloroacetophenone	Organooxygen compounds	Phenolic glycosides
47	8.65	C ₁₆ H ₁₈ O ₈	338.1004	3-O-p-Coumaroylquinic acid	Organooxygen compounds	Alcohols and polyols
48	9.69	C ₃₅ H ₂₈ O ₂₂	800.1077	1,3,4,5-Tetra-O-galloylquinic acid	Organooxygen compounds	Alcohols and polyols
49	10.42	C ₂₈ H ₂₆ O ₁₇	634.1178	3'-(2",3"-Digalloylglucosyl)-phloroacetophenone	Organooxygen compounds	Phenolic glycosides
Serial	t _R (min)	Formula	Neutral mass	Proposed compounds	Class	Sub-class
ESI+						
Phenolic acids and derivatives						
1	3.11	C ₉ H ₈ O ₃	164.0487	Cis-p-Coumaric acid	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
2	4.60	C ₁₆ H ₁₆ O ₈	336.0844	3-Caffeoyl-1,5-quinolactone	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
3	6.12	C ₁₀ H ₁₀ O ₃	178.0632	4-Methoxycinnamic acid	Cinnamic acids and derivatives	Cinnamic acid
4	6.16	C ₂₁ H ₂₈ O ₁₂	472.1556	b-D-fructosyl-a-D-(6-O-(E))-feruloylglucoside	Cinnamic acids and derivatives	Cinnamic acid derivatives
5	6.78	C ₂₅ H ₃₁ NO ₁₂	537.1848	4-Demethylsimmondsin 2'-(E)-ferulate	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
6	7.44	C ₂₀ H ₂₆ O ₁₁	442.1492	1-O-E-Cinnamoyl-(6-arabinosylglucose)	Cinnamic acids and derivatives	Cinnamic acid esters
7	7.56	C ₁₄ H ₁₆ O ₆	280.0960	Gravolenic acid	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
8	8.97	C ₁₇ H ₂₂ O ₁₀	386.1206	1-O-Sinapoylglucose	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
9	9.20	C ₂₃ H ₂₀ O ₅	376.1330	Cinnatriacetin A	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
10	13.04	C ₁₆ H ₁₃ NO ₄	283.0840	Avenanthramide 1p	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
11	16.51	C ₄₈ H ₇₄ O ₈	778.5363	1,28-Octacosanediol diferulate	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
Flavonoids and derivatives						
12	1.60	C ₁₅ H ₁₄ O ₈	322.0675	Galocatechin-4beta-ol	Flavonoids	Biflavonoids and polyflavonoids
13	2.61	C ₂₂ H ₂₂ O ₆	382.1391	Kanzonol O	Isoflavonoids	Pyranoisoflavonoids
14	8.13	C ₂₈ H ₂₄ N ₂ O ₇	500.1596	Orcein	Flavonoids	O-methylated flavonoids

15	9.78	C ₂₇ H ₃₀ O ₁₆	610.1496	Quercetin 3-(2-glucosylrhamnoside)	Flavonoids	Flavonoid glycosides
16	11.2	C ₃₃ H ₄₄ O ₁₄	664.2720	2',7-Dihydroxy-4'-methoxy-8-prenylflavan 2',7-diglucoside	Flavonoids	Flavonoid glycosides
17	11.6	C ₃₅ H ₂₆ O ₈	574.1618	Viniferal	2-arylbenzofuran flavonoids	
18	11.6	C ₃₈ H ₂₂ O ₇	590.1350	4'-Hydroxyanigorootin	Isoflavonoids	Isoflavans
19	11.73	C ₂₀ H ₁₈ O ₁₂	450.0810	Myricetin 3-arabinoside	Flavonoids	Flavonoid glycosides
Coumarins						
20	1.60	C ₁₈ H ₁₈ O ₁₀	394.0885	9-Hydroxy-4-methoxypsoralen glucoside	9- Coumarins and derivatives	Coumarin glycosides
21	6.43	C ₁₅ H ₁₆ O ₈	324.0825	Mahaleboside	Coumarins and derivatives	Coumarin glycosides
22	6.73	C ₉ H ₆ O ₃	162.0313	3-Hydroxycoumarin	Coumarins and derivatives	Hydroxycoumarins
23	16.77	C ₄₈ H ₅₄ O ₈	758.3842	Marshdimerin	Coumarins and derivatives	Pyranocoumarins
Tannins						
24	2.83	C ₂₇ H ₁₈ O ₁₇	614.0557	Castacrenin A	Tannins	Hydrolyzable tannins
25	8.93	C ₃₄ H ₂₂ O ₂₂	782.0600	Emblicanin A	Tannins	Hydrolyzable tannins
26	9.37	C ₄₁ H ₃₀ O ₂₆	938.1025	Punicafolin	Tannins	Hydrolyzable tannins
27	9.48	C ₃₄ H ₂₄ O ₂₂	784.0743	Castanin	Tannins	Hydrolyzable tannins
Others						
28	3.45	C ₁₁ H ₁₄ O ₅	226.0834	3,4,5-Trimethoxyphenyl acetate	Phenol esters	
29	6.03	C ₉ H ₁₃ NO ₂	167.0931	3-Methoxytyramine	Phenols	Methoxy phenols
30	8.18	C ₁₃ H ₁₅ NO ₇	297.0845	Hexahydro-6,7-dihydroxy-5-(hydroxymethyl)-3-(2-hydroxyphenyl)-2H-pyrano[2,3-d]oxazol-2-one	Phenols	1-hydroxy-4-unsubstituted benzoids
31	12.61	C ₁₉ H ₂₀ N ₂ O ₃ S	356.1195	Pioglitazone	Phenol ethers	
32	16.04	C ₂₂ H ₂₆ N ₂ O	334.2047	Carnocin U I49	Phenol ethers	

3.5. CONCLUSION

Extraction of phenolic compounds (PCs) from sugar maple leaves (SML) through maceration was optimized using full factorial design. Full factorial experiment enabled us to determine the optimal extraction conditions as 61.63% ethanol concentration and 1:38.22 g/mL solid-to-solvent ratio for recovering maximum phenolic content from SML with high antioxidant activity. Effect of high-speed homogenization (HSH) as a pre-treatment condition was investigated where the results confirmed HSH as an effective pre-treatment technique to promote the maceration efficiency. In parallel, characterization of SML was done using colorimeter, SEM, FTIR and LC-ESI-Q-TOF-MS/MS. SEM images of SML indicated that macerated leaves contained smooth surface with porous structure whereas non-macerated one had undamaged but rough surface. FTIR showed the presence of PCs in the SML and the compounds were confirmed by LC-ESI-Q-TOF-MS/MS as 81 detectable PCs were isolated in the extracts. Our findings showed that, sugar maple leaves is a prominent source of bioactive compounds which reveals an alternative biological potential of this plant towards the development of newer functional foods, that could be useful for developing community economic, and consumer health. In addition, HSH assisted maceration was found as an effective and simple method for the exploitation of PCs from SML. In continuation, further studies will be focusing on the formulation of foodstuffs fortified with phenol rich SML extract and on their stability with time.

Acknowledgement

Authors are also thankful to Professor Saji George, Department of Food Science and Agricultural Chemistry, McGill University, for allowing the use of plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA) at no cost.

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CONNECTING TEXT TO CHAPTER 4

Chapter 4 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2023). Phenolic mapping and associated antioxidant activities through the annual growth cycle of sugar maple leaves. *Food Chemistry*, 136882. doi: <https://doi.org/10.1016/j.foodchem.2023.136882>

From Chapter 3, it was seen that sugar maple leaves are a good source of phenolic compounds. It was reported in the literature that phenolic biosynthesis has been shown to respond to a variety of environmental factors/stresses such as UV–B radiation, exposure to ozone, rainfall/drought, temperature, light, water availability, nutrient deficiencies, among others. Therefore, optimal harvesting time of respective plant parts is very influential to get maximum antioxidative phenolic concentrations. To this end, it can be hypothesized that knowledge on the seasonal variation in sugar maple leaves phenolics would inform their optimal harvesting time in favor of their valorization as functional ingredients in food industries. Thus, Chapter 4 unraveled the phenolic mapping of sugar maple leaves and their associated antioxidant capacities throughout the annual growth cycle of leaves.

Part of this research chapter was presented in a scientific conference.

Yeasmen, N., & Orsat, V. (2022/07). *Acer saccharum* leaves as source of bioactive compounds: Effect of harvesting time on phenolic content and antioxidant activity. Canadian Society for Bioengineering (CSBE) annual conference, Charlottetown, Prince Edward Island, Canada.

**CHAPTER 4: PHENOLIC MAPPING AND ASSOCIATED ANTIOXIDANT
ACTIVITIES THROUGH THE ANNUAL GROWTH CYCLE OF SUGAR MAPLE
LEAVES**

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4.1. ABSTRACT

Concentrations of antioxidant components (analyzed by HPLC–UV) and antioxidant attributes (assayed by radical scavenging and non–radical redox potential methods) of sugar maple leaves (SML) from different harvesting times were investigated. Moreover, measurements of colorimetry, SEM, and FTIR spectroscopy-based characterization of leaves composition, throughout the growth cycle, were performed. Results showed that the antioxidant activities of SML are strongly correlated with phenolic contents and significantly ($p<0.05$) varied with harvesting time where minimum amount of total phenolics (105.67 ± 13.16 mg GAE/g DM) and total flavonoids (3.27 ± 0.26 mg CTE/g DM) were found to be concentrated in Fall leaves. The absorption bands obtained from FTIR spectra revealed the presence of functional groups that have great significance towards the antioxidant activity of SML. Principal component analysis revealed that biosynthesis of maximum phenolic compounds in SML mostly occurs during the leaf expansion and growth phases. The obtained data provided a better understanding towards the effect of harvesting time on the phenolic mapping of SML in favor of its valorization into functional food ingredients.

Keywords:

Antioxidant components; Antioxidant attributes; FTIR; HPLC–UV; Microstructure; Mineral content; Seasonal variation.

4.2. INTRODUCTION

Amongst the available organic food products, maple syrup is the largest commercially produced and consumed natural sweetener in the world. It is prepared by concentrating the sap collected from certain maple species namely *Acer saccharum* (sugar maple) (Liu et al., 2017a; Liu et al., 2017b). Commercial production of maple syrup is predominantly located in North America where the Canadian maple syrup industry accounts for approximately 75% of the world's maple syrup production. In 2020, Canadian maple producers processed 14.3 million gallons of maple syrup which resulted in a total sale of USD 558.5 million (AAFC, 2021). The worldwide popularity of maple syrup benefits from its antioxidant, anti-inflammatory, anti-proliferative, and anti-mutagenic properties which are attributed to its diverse range of phenolic compounds (Rose et al., 2021). Apart from maple sap, other parts of sugar maple such as the bark (to treat diarrhea, cramps, measles, and diuretic), shoots (to treat cataracts and to be used as an eyewash), etc., have been used since ancient times as indigenous folk remedies in Canada and the United States due to their

high content of phenolic compounds (Bi et al., 2016). Therefore, it can be hypothesized that sugar maple leaves, otherwise known as maple biomass falling to the ground from the deciduous trees, can also be a potential source of extractable phenolic compounds (PCs) that can be utilized as natural functional ingredients in the food industry. The creation of sustainable and functional food products is of great interest to contribute to solving technological, health, and quality of life issues. In this context, application of zero-waste technologies for taking advantage of an unexploited plant-origin materials, *inter alia*, will help solve some of the problems inherent to organic waste handling while producing a valuable functional food ingredient. Zhao et al. (2022) showed that addition of guava leaf phenolics in chicken meatballs is a promising way to inhibit the formation of advanced glycation end products. In the study of Conte et al. (2021), incorporation of phenolic-rich extracts from olive leaves in gluten free breadstick formulations was shown to improve the functional and nutritional properties of the breadsticks with extended shelf-life. Santetti et al. (2021) studied the effect of yerba mate leaves addition in bread dough and found that it increases the dough's functionality which was attributed to the presence of biologically active compounds such as PCs in yerba mate leaves. Nowak et al. (2016) showed that incorporation of extracts from cherry and blackcurrant leaves in pork sausages resulted in the reduced formation of malondialdehyde with extended shelf life. This was due to the antioxidant properties of the phenol rich cherry and blackcurrant leaves extracts. All these effective applications of different leaves extracts in food products supports the venture of exploiting maple leaves as a resilient source of phenolic compounds, and by doing this, it will contribute to meeting the United Nations sustainable development goals (i.e., achieving the sustainable management and efficient use of natural bioresources by 2030) (UNSDGs, 2015; USDA, 2019).

Being a deciduous tree, the sugar maple growing cycle restarts every spring (March–May) with new leaves, flowers, and seeds. In the fall (September–November), green leaves turn yellow, orange, and red due to the metabolic production of carotenoids and anthocyanin pigments and the loss of chlorophyll (Apostolidis et al., 2012). By the end of the fall season, these colored leaves undergo senescence and consequently fall to the ground and the tree undergoes a dormancy period to prepare for winter (December–February) (MMPA, 2022; Routray & Orsat, 2014). It was reported that phenolic biosynthesis has been shown to respond to a variety of environmental factors/stresses such as UV–B radiation, exposure to ozone, rainfall/drought, temperature, light, water availability, nutrient deficiencies, among others (Apostolidis et al., 2012; Sultanbawa et al.,

2021; Yeshe et al., 2022). Therefore, optimal harvesting time of respective plant parts is very influential in order to get maximum antioxidative phenolic concentrations (Liu et al., 2017a; Liu et al., 2017b). In our previous work (Yeasmen & Orsat, 2022), LC–ESI–Q–TOF–MS/MS based phenolic profiling of SML was reported, yet little is known about the seasonal dynamics of major PCs and their associated antioxidant activities. However, it can be hypothesized that knowledge on the seasonal variation in SML phenolics would inform their optimal harvesting time in favor of its valorization as functional ingredients in food industries. To this end, the objective of this present study was to unravel the phenolic mapping of SML and their associated antioxidant capacities throughout the annual growth cycle of leaves. In addition, the effect of seasonal dynamics on the correlation between PCs and antioxidant capacities in SML was also elucidated. The outcome of this study will provide useful insights into the phytochemistry of this *Acer* species along with the developmental evolution of leaves, and consequently promoting its efficiency to be used as an interesting source of natural antioxidants.

4.3. MATERIALS AND METHODOLOGY

4.3.1. Chemicals

All the solvents used for the analytical chromatography were of HPLC grade with a purity of >99%. Acetonitrile (ACN), phosphoric acid (H_3PO_4), acetic acid (CH_3COOH), ethanol (EtOH), hydrogen chloride (HCl), methanol (MeOH), HPLC water, 2,4,6-tripyridyl-s-triazine (TPTZ), and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Fisher Scientific, Nepean, Ontario, Canada. Folin-Ciocalteu reagent (FCR), sodium carbonate (Na_2CO_3), Sodium Nitrite (NaNO_2), Aluminum chloride (AlCl_3), Sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), and neocuproine were purchased from Sigma-Aldrich, St-Louis, MO, USA. Copper (II) Chloride solution and Potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) were obtained from Acros Organics, Janssen Pharmaceuticaaan 3a, B2440 Geel, Belgium. Standards from phenolic acids including gallic acid, ellagic acid, sinapic acid, caffeic acid, ferulic acid, quinic acid, and tannic acid; flavonoids including catechin, epicatechin, and rutin; stilbene including resveratrol; and beta carotene were purchased from Sigma-Aldrich, St-Louis, MO, USA.

4.3.2. Sample collection and processing

Sugar maple leaves were freshly harvested from the Morgan Arboretum located at McGill University's Macdonald campus in Sainte-Anne-de-Bellevue (Quebec, Canada, 45°25'50.1"N 73°57'8.2"W). It is a natural forest stand of sugar maple trees where local cultivars, namely "Apollo" and "Columnare", are present. The sample collection site i.e., area encompassing sugar maple trees in the arboretum was grouped into five zones (east, west, north, south, and central). Within each zone, six healthy trees (free from external damage, diseases, insect attack) were selected ($5 \times 6 = 30$ trees) and SML were collected at plant heights ranging from 5–10 m above the ground. The selected sugar maple trees were chosen based on their diameter at human eye level to maintain the uniformity in tree age. All selected trees were in good condition without any visible signs of mechanical and infectious damage. SML collections were made on the last week of May (SML–M), July (SML–J), September (SML–S), and October (SML–O) in 2021, between 8:00 and 10:00 am from the same selected trees. Starting from May to September, sample was collected at an interval of 2 months, whereas last sample was collected at an interval of 1 month i.e., in October, hypothesizing that leaves senescence might have a significant impact on the biosynthesis of PCs. Known as a widely adapted tree, the sugar maple is a member of undisturbed tree community (Hagan et al., 2016). Therefore, likewise the studies of Apostolidis et al. (2012); Chang et al. (2019); Liu et al. (2017a); Liu et al. (2017b); Nour et al. (2014); Ozcan et al. (2019); Sytarova et al. (2020); Yang et al. (2018), and Zhang et al. (2018), sampling of one phenological season (2021) was conducted during this study to see the effect of seasonal dynamics on the phenolic composition of sugar maple leaves. Weather conditions at the sampling time and phenological stages of SML are summarized in Table 1. Afterwards, all the SML of different harvesting time were washed under running tap water and then freeze dried using a commercial freeze drier (CHRIST, GAMMA 1–16 LSC, MBI, Kirkland, Canada), followed by grinding of the leaves using a milling machine (Retsch GmbH, GM 200, Retsch-Allee 1–5, Germany) to ensure a particle size of <0.1 mm and then stored in amber tubes at -80°C until being used for further analysis.

Table 4.1: Phenophases and meteorological conditions at the sampling months of sugar maple leaves.

	SML-M	SML-J	SML-S	SML-O
Phenological stages	Leaf blooming, expansion	Intensive growth	leaf Leaf maturity	Leaf coloration, preparation for senescence
Maximum temperature (°C)	17	28	22	15
Minimum temperature (°C)	9	15	13	9
Sun hour (h)	268	286	254	212
Rain (mm)	59.2	125.2	27.2	39.4
UV index	3	5	3	2

SML-M: Sugar maple leaves collected in May; SML-J: SML collected in July; SML-S: SML collected in September; SML-O: SML collected in October.

4.3.3. Extraction of phenolic compounds

Free phenolic compounds were extracted from SML powder using 70% EtOH at a solid-to-solvent ratio of 1:30 g/mL for 24 h at a constant stirring speed (200 rpm) and temperature (25°C). These parameters were selected based on the results reported in a previous work (Yeasmen & Orsat, 2022). Following extraction, the suspensions were centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Germany) for 15 min at 8000 ×g. Finally, the supernatants were vacuum filtered through 11 µm filter paper (Whatman no 1) and stored at – 80°C. After each extraction, percentage total yield (%TY) of macerated SML extracts was calculated using the following equation (4.1):

$$\% \text{ TY} = \frac{\text{Dry mass of SML extract (g)}}{\text{Dry mass of SML powder (g) taken for extraction}} \times 100\% \dots\dots\dots (4.1)$$

4.3.4. Physicochemical characterizations

4.3.4.1. Color attributes

Color indices L* (lightness to darkness), a* (redness to greenness), and b* (yellowness to blueness) of all freeze-dried SML samples were measured at room temperature (25°C) using a spectrophotometer (CM-3500d, Minolta Spectrophotometer, Minolta Co. Ltd., Japan).

4.3.4.2. Microstructural analysis

Scanning electron microscope (SEM, TM3000, Hitachi, Japan) at a magnification of ×1000 was used to see the microstructural changes of SML powders from different harvesting times before and after extraction. Quantification of the microstructural changes was then performed by calculating the fractal dimension (FD) and surface opening (SO) of SEM micrographs of all samples. Following the approach of Rahimi and Ngadi (2016), FD of each microstructural image was calculated using a box counting method by Image J 1.52a (National Institute of Health, USA)

software, whereas the estimation of SO from SEM image was performed based on the approach as described by Rahimi and Ngadi (2015).

4.3.4.3. FTIR analysis

FTIR (Fourier transform infrared) spectra of SML powders of different harvesting times were recorded by an FTIR spectrometer (Thermo Scientific Nicolet iS5 FTIR, Thermo Fisher Scientific, USA) operated in attenuated total reflection (ATR) mode. Samples were placed in direct contact with the ATR-crystal and spectra acquisition was done at room temperature (25°C). FTIR spectra were collected in the region of 4000–600 cm^{-1} at a resolution of 4 cm^{-1} by co-adding 32 scans and expressed as absorbance values. All spectral measurements were normalized against the background air spectrum and every 60 min of operation, a new reference air background spectrum was taken.

4.3.4.4. Moisture content

Moisture content of fresh SML and freeze dried SML powders was determined using the official methodology of AOAC international (Horwitz, 2005). Briefly, each sample was air dried in an air-dryer (Thermo Scientific Precision Oven) at 105°C till constant weight and then was transferred into a desiccator to equilibrate for 30 minutes. Weight of the sample before and after drying was measured using a balance with a precision of ± 0.0001 g and moisture content was calculated on a dry weight basis (db).

4.3.4.5. Nitrogen content

Percentage nitrogen content was measured by nitrogen combustion method following the method by Yuliarti et al. (2021) and the analysis was performed using a VELP NDA-DUMAS analyzer (NDA 701, VELP Scientifica).

4.3.4.6. Mineral content

Potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), sulphur (S), manganese (Mn), and iron (Fe) were determined by digestion followed by inductively coupled plasma optical emission spectrometry (ICP-OES) analysis according to the method described by Nour, Trandafir and Cosmulescu (2014) with little modifications. Samples of approximately 0.4 g dry leaves were weighed in DigiPREP tubes, over which 5 mL concentrated TraceMetal grade Nitric Acid (~68%) and 2 mL hydrogen peroxide (30%) were added. This was placed on DigiPREP block heater at 95°C for 2 h to complete digestion. Following the digestion, the clear, colourless solution was transferred into a 50 mL volumetric flask and made up to the mark with deionized water. Finally,

ICP-OES (Thermo iCAP-6500 series Duo) was used throughout the analysis. The results were expressed in micrograms per gram of dry matter ($\mu\text{g/g DM}$).

4.3.5. Phytochemical characterizations

4.3.5.1. Total phenolic and flavonoid contents

The level of total phenolic content (TPC) and total flavonoid content (TFC) in the SML extracts of different harvesting time were assessed using spectrophotometric approach based on the methods reported respectively by Singleton et al. (1999) and Jia et al. (1999). TPC and TFC were expressed as mg gallic acid equivalent (mg GAE)/g DM and mg catechin equivalent (mg CTE)/g DM, respectively.

4.3.5.2. Quantification of individual phenolics

For the HPLC analysis, the SML extracts of varying harvesting times were filtered through a 0.20 μm PTFE syringe-filter (Fisher Scientific). HPLC analyses were performed on an Agilent 1100 Series HPLC system by following the methods of Rodríguez García and Raghavan (2022) and Yeasmen and Orsat (2022) with modifications to quantify the contents of 14 compounds (gallic acid, ellagic acid, sinapic acid, ferulic acid, tannic acid, quinic acid, caffeic acid, catechin, epicatechin, kaempferol, quercetin, rutin, resveratrol, and beta carotene). Reason behind selecting these individual compounds is based on the study of LC–ESI–Q–TOF–MS/MS based phenolic profiling of SML, where it was found that the studied compounds were present abundantly in SML among others (Yeasmen & Orsat, 2022). A quaternary gradient pumping system (G1311A, Agilent Technologies, Germany), an autosampler (G1313A, Agilent Technologies, USA, 100×2 mL vials) and a UV-detector (G1314A, Agilent Technologies, Germany) were used for these analyses. For the separation of compounds, a C18 column (Gemini, 150 mm $\times$ 4.60 mm, 5 μ , Torrance, USA) was used. Chromatographic conditions were set as: injection volume 5 μL , flow rate of 0.4 mL/min, HPLC grade water containing 0.1% formic acid (mobile phase A), HPLC grade acetonitrile containing 0.1% formic acid (mobile phase B), and detection was performed at 280 nm. The gradient used for the separation of ellagic acid, sinapic acid, ferulic acid, caffeic acid, quinic acid, catechin, epicatechin, and rutin was 10 min: 99% A and 1% B; 30 min: 50% A and 50% B; 40 min: 99% A and 1% B; and postrun for 15 min. For the separation of gallic acid, tannic acid, resveratrol, and carotene, the following gradient was used: 4 min: 98% A and 2% B; 7 min: 80% A and 20% B; 20 min: 100% B; 24 min: 100% B; 25 min: 98% A and 2% B; 27 min: 98% A and 2% B; and postrun for 15 min. The data were analyzed using the Agilent Chem Station

Software and the phenolic quantification was performed by the external standard method. Solutions of all standards (5–1000 $\mu\text{g/mL}$) were prepared with HPLC grade methanol where for all the calibration curves the coefficient of determination (R^2) values from the regression analysis were greater than 0.99. Amounts of phenolic compounds in the extracts were expressed as mg/g DM.

4.3.6. Antioxidant activity assays

Antioxidant activities of the SML extracts were analyzed through free radical scavenging (DPPH and ABTS) and non-radical redox potential (ferric reducing antioxidant power and cupric reducing antioxidant capacity) methods. Among them DPPH assay was performed by following the method described by Thaipong et al. (2006), a method based on the approach of Brand-Williams et al. (1995) and ferric reducing antioxidant power (FRAP) assay was performed based on the method given by Benzie and Strain (1999).

Cupric ion reducing antioxidant capacity (CUPRAC) of SML extracts was assayed based on the method described by Apak et al. (2004) with little modification. To a test tube, 1 mL each of copper (II) chloride (10 mM), ammonium acetate buffer (pH 7) and neocuproine (7.5 mM) solutions were taken to which 1.1 mL SML extract was added. The tube was stoppered and allowed to stand for 1.5 h in the dark. Absorbance of the colored mixture was recorded at 450 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA).

ABTS assay was performed as per the method described by Laporta et al. (2007) with little modification. ABTS radical cation (ABTS^+) was produced by reacting 10 mL ABTS stock solution (7 mM) with 10 mL 2.45 mM potassium persulfate in the dark at room temperature for 12–24 h before use. During the experiment, ABTS^+ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 unit at 734 nm wavelength. Afterwards, 100 μL SML extract was mixed with 3000 μL diluted ABTS^+ solution and immediately after 5 min the absorbance was recorded at 734 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA).

For all four assays, the antioxidant activities of SML extracts were calculated from the Trolox (0–1000 μM) standard calibration curve and results were expressed as $\mu\text{M TE/g DM}$.

4.3.7. Statistical analysis

All the measurements were taken in triplicate for all samples and reported as mean $\pm$ standard deviation. ANOVA and Tukey HSD tests were applied to analyze significant differences ($p < 0.05$) with 95% confidence interval among the mean values. Correlation between

phytochemicals and antioxidant activities of SML extracts was analyzed using Pearson correlation analysis. All the analyses were performed using the statistical software JMP (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, NC, USA) and graphical representations were developed with a licensed version of Microsoft Excel 16 (Microsoft Corporation, USA).

4.4. RESULTS AND DISCUSSION

4.4.1. Effect of seasonal dynamics on physicochemical properties of SML

4.4.1.1. *Color attributes*

Distinctive color changes of the leaves of deciduous plants along with their growth cycle has been reported in the literature (Routray & Orsat, 2014) which is also evident in this study particularly during leaf expansion (May) and during the onset of defoliation (October) (Table 4.2). SML collected in May was lighter while October leaves were found to have highest color index of “a” (redness to greenness). This might be due to their content in carotenoids (gives yellow color) and anthocyanin (gives red color) pigments that are synthesized during the growing season and towards the end of the leaf’s lifespan respectively (Apostolidis et al., 2012).

Table 4.2: Seasonal dynamics on the physicochemical characteristics of sugar maple leaves.

Category	Characteristics	SML–M	SML–J	SML–S	SML–O
Color	L*	47.02±0.57 ^A	42.50±3.54 ^B	49.94±0.83 ^B	42.21±1.20 ^B
	a*	−4.00±0.09 ^B	−6.48±0.04 ^B	−5.44±0.22 ^B	17.47±3.07 ^A
	b*	12.05±0.025 ^A	8.45±0.06 ^B	9.52±0.76 ^{AB}	7.34±2.06 ^B
Microstructural analysis	FD (BE)	1.785±0.12 ^{Aa}	1.763±0.02 ^{Aa}	1.751±0.23 ^{Aa}	1.787±0.02 ^{Aa}
	FD (AE)	1.863±0.03 ^{Aa}	1.802±0.04 ^{ABa}	1.755±0.02 ^{Ba}	1.758±0.01 ^{Ba}
	%SO (BE)	45.39±0.14 ^{Ac}	42.35±0.05 ^{Cc}	42.64±0.33 ^{Cc}	43.29±0.03 ^{Bc}
	%SO (AE)	64.63±0.01 ^{Ab}	53.84±0.03 ^{Bb}	52.16±0.01 ^{Db}	53.71±0.12 ^{Bb}
FTIR peak area calculation	3700–3000 cm ^{−1}	20.87	17.35	15.89	14.25
	3000–2870 cm ^{−1}	1.19	0.92	0.94	1.10
	2870–2835 cm ^{−1}	0.19	0.15	0.15	0.20
	1743–1680 cm ^{−1}	0.61	0.31	0.34	0.51
	1680–1560 cm ^{−1}	2.39	2.70	2.71	2.15
	1480–1420 cm ^{−1}	0.78	0.52	0.55	0.45
	1420–1280 cm ^{−1}	2.07	1.29	1.36	1.21
	1280–1175 cm ^{−1}	1.78	1.04	0.94	0.73
	1175–1135 cm ^{−1}	0.19	0.23	0.16	0.17
	1135–925 cm ^{−1}	13.09	12.56	12.88	11.69
	%Moisture (wb)	68.07	63.89	57.55	50.41
	%Nitrogen (db)	0.13	1.74	2.05	1.88
Mineral content	K (µg/g DM)	12447.07±0.251 ^A	8162.21±0.087 ^B	6486.78±0.127 ^D	6491.14±0.221 ^C
	Ca (µg/g DM)	7627.69±0.146 ^D	17964.55±0.271 ^C	19610.12±0.420 ^B	31982.95±0.737 ^A
	Mg (µg/g DM)	2418.32±0.044 ^D	4496.83±0.031 ^A	3188.06±0.063 ^C	4325.43±0.129 ^B
	P (µg/g DM)	3635.79±0.027 ^C	3668.46±0.058 ^B	3356.96±0.044 ^D	4049.08±0.039 ^A
	S (µg/g DM)	2299.49±0.021 ^B	2354.77±0.038 ^A	2052.94±0.030 ^C	1621.41±0.022 ^D
	Mn (µg/g DM)	125.57±0.002 ^D	205.22±0.001 ^C	296.70±0.005 ^A	242.76±0.005 ^B
	Fe (µg/g DM)	79.93±0.001 ^D	111.48±0.004 ^B	105.77±0.006 ^C	132.39±0.008 ^A

SML–M: Sugar maple leaves collected in May; SML–J: SML collected in July; SML–S: SML collected in September; SML–O: SML collected in October; FD: Fractal dimension; SO: Surface opening; BE: Before extraction; AE: After extraction; wb: Wet weight basis; db: Dry weight basis; DM: Dry matter.

Note: 1. The values are presented as average value±standard deviation; 2. Different uppercase letters in a row indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective characteristics of different harvesting time; 3. Lowercase letter (a) and (b–c) indicate significant differences based on 95% confidence interval ($p < 0.05$) respectively in FD and SO values between before and after extraction.

4.4.1.2. Surface morphology

Surface morphology also known as surface structure or surface topography, is an essential physical feature of solid materials indicating their behavior during processing (such as drying) and extraction and can be examined using advanced microscopic techniques such as SEM (Rahimi & Ngadi, 2016). Accordingly, micrographs of freeze dried SML powders from different harvesting times with and without extraction are shown in Figure 4.1. For all samples, differences in the micrographs are clearly visible between before and after extraction conditions indicating that

maceration caused damage to the leaf microstructure, thereby facilitating the release of PCs from the cells into the extraction medium (Yeasmen & Orsat, 2022). However, to quantify these microstructural damages in the leaves, surface fractal dimension (FD) and surface opening (SO) of each SEM image were calculated (Table 4.2). The word “fractal” corresponds to the Latin verb “frangere” meaning “to create irregular fragments”, or “to break” whereas fractal dimension is interpreted as the surface roughness index (Rahimi & Ngadi, 2016). On the other hand, SO quantifies the extent of holes, cracks, and crevices to delineate between ruptures in the surface caused by drying and extraction. Regardless of harvesting time and extraction effect, there was little significant difference ($p<0.05$) in the FD values of SML. This could be attributed to the freeze-drying effect which resulted in averaging of the intracellular spaces and surface irregularities into the cell walls during the loss of moisture. However, to know the difference between drying and extraction effects on the surface microstructures, SO values might be helpful as they clearly showed substantial differences between macerated and non-macerated SML powder while in terms of seasonal effect, significantly higher ($p<0.05$) percentage of surface opening ($64.63\pm0.01\%$) was observed in the May samples. This is related to the leaf’s physical condition as May is the month of SML blooming and expansion for which leaves in this month are still immature and softer. Moreover, this increased SO also favored the extraction efficiency which can be seen in the later discussion. It is therefore possible to conclude that both the freeze-drying and extraction methods caused surface roughness/irregularities while the leaves subjected to extraction had more surface ruptures/damages/pores.

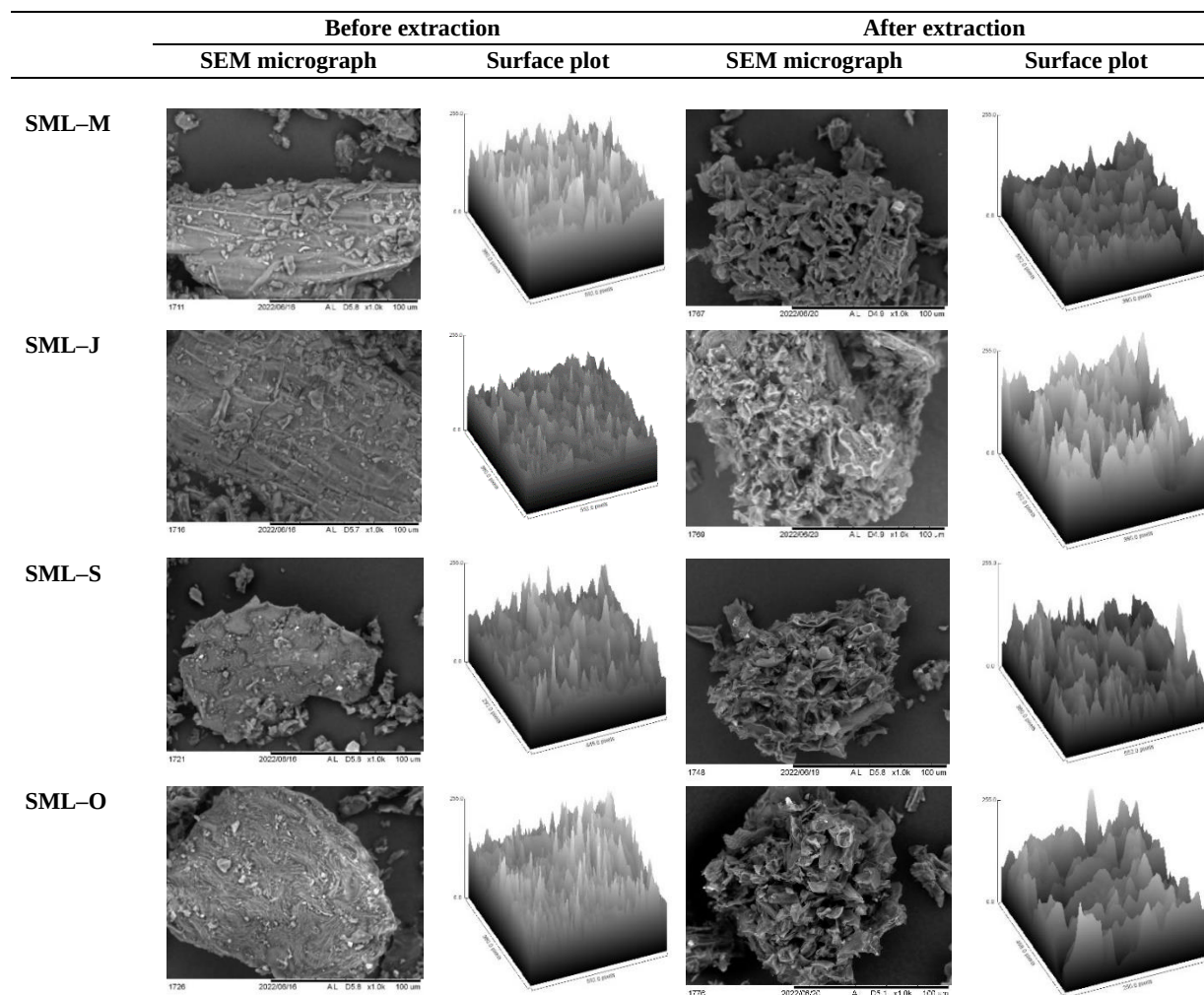


Figure 4.1: SEM images and surface plots of sugar maple leaves powder of different harvesting time at a magnification of $\times 1000$ before (left) and after (right) extraction. *SML-M*: Sugar maple leaves collected in May; *SML-J*: SML collected in July; *SML-S*: SML collected in September; *SML-O*: SML collected in October.

4.4.1.3. FTIR analysis

FTIR was used to illustrate the effect of seasonal dynamics in the change of chemical compositions of SML. The FTIR generated spectra (Figure S1a) can be regarded as the biochemical fingerprints of the sample. The bands observed between $4,000$ and 600 cm^{-1} displayed ten fingerprint regions based on the obtained peaks: $3700\text{--}3000$, $3000\text{--}2870$, $2870\text{--}2835$, $1743\text{--}1680$, $1680\text{--}1560$, $1480\text{--}1420$, $1420\text{--}1280$, $1280\text{--}1175$, $1175\text{--}1135$, and $1135\text{--}925\text{ cm}^{-1}$ (Figure S1b–S1k). Wavenumber ranges of FTIR peaks and associated functional groups in SML were identified by comparing with the literature (Table 3) from where, six fingerprint regions (3700--

3000, 1680–1560, 1480–1420, 1420–1280, 1280–1175, and 1175–1135 cm^{-1}) are associated with the presence of phenolic groups. However, to see the effect of seasonal dynamics in the change of chemical compositions of SML, FTIR band intensities were calculated in terms of peak area in the above-mentioned regions (Table 4.3). SML–M showed higher band intensities in most of the discussed regions indicating that it contained more PCs, while the SML–J sample was showed to contain more flavonoids. Thus, the observed characteristic fingerprints of SML at varying collection times reflected that seasonal variations have an effect on the functional characteristics associated with phenolic compounds.

Table 4.3: Wavenumber ranges of FTIR peaks and associated functional groups of sugar maple leaves.

Wavenumber range	Assigning functional group	Possible chemical group/compound	Reference
3700–3000 cm^{-1}	Stretching vibrations of O–H and N–H	Water, alcohols, phenols , carbohydrates, peroxides, carotenoids	(Alara et al., 2018; Chou et al., 2020; Hssaini et al., 2022; Seregelyj et al., 2019; Sim et al., 2019; Thummajitsakul et al., 2020; Yeasmen & Orsat, 2022)
3000–2870 cm^{-1}	Stretching vibrations of C–H	Lipid, carbohydrate	(Alara, Abdurahman, & Ukaegbu, 2018; Seregelyj et al., 2019; Sim, Ong, & Nyam, 2019; Yeasmen & Orsat, 2022)
2870–2835 cm^{-1}	C–H stretch	Polysaccharides, lipids, carbohydrates	(Thummajitsakul et al., 2020)
1743–1680 cm^{-1}	C=O band stretching	Aldehyde, ketones, ester, carboxylic acid	(Hssaini, Razouk, & Bouslihim, 2022; Seregelyj et al., 2019; Yeasmen & Orsat, 2022)
1680–1560 cm^{-1}	Stretching vibrations of O–H, C–O–C, and C=O, N–H bending vibrations	Protein, amino acid, ether, flavonoids	(Alara, Abdurahman, & Ukaegbu, 2018; Chou et al., 2020; Sim, Ong, & Nyam, 2019; Sultanbawa et al., 2021; Thummajitsakul et al., 2020)
1480–1420 cm^{-1}	Bending vibrations of C–H, N–H and primary or secondary O–H	Phenol	(Alara, Abdurahman, & Ukaegbu, 2018; Chou et al., 2020; Thummajitsakul et al., 2020)
1420–1280 cm^{-1}	C–H, bending of carboxylic acid, amine (C–N),	Tannins, flavonoids , glycosides, saponins	(Alara, Abdurahman, & Ukaegbu, 2018; Seregelyj et al., 2019; Sim, Ong, & Nyam, 2019)
1280–1175 cm^{-1}	Stretching vibration of C–O, stretching of phosphodiester	Nucleic acid, hydroxyflavonoid , aldehyde, ketones, ester	(Alara, Abdurahman, & Ukaegbu, 2018; Chou et al., 2020; Sim, Ong, & Nyam, 2019)
1175–1135 cm^{-1}	C–O and O–H stretching vibration	Carbohydrate, phenol	(da Rosa et al., 2019; Hssaini, Razouk, & Bouslihim, 2022; Yeasmen & Orsat, 2022)
1135–925 cm^{-1}	C–H bending vibration, C–O stretching vibrations	Starch, ester, secondary alcohol, carbohydrate	(Alara, Abdurahman, & Ukaegbu, 2018; Thummajitsakul et al., 2020; Yeasmen & Orsat, 2022)

4.4.1.4. Moisture and mineral contents

Seasonal variations in moisture and mineral contents of SML are given in Table 4.2. During the initial development of the leaves in the month of May, the moisture content was found higher (68.07%) which lowered with the growing season and was lowest in October (50.41%). Moisture contents of plant tissues are influenced by variation in rates of water uptake, dry weight, or both where the former one depends on the water availability in the environment (Zhang & Zhou, 2019). In Canada, following the winter season, the month of May experiences a lot of ice/snow melting which might cause greater water availability in the soil. As a result, there is a high chance of plant's increasing water uptake, consequently, increasing moisture content in the plant tissues such as leaves. Routray and Orsat (2014) also observed highest moisture content in Canadian blueberry leaves in their May samples which was found correlated to the ice/snow melting conditions in Canada. Along the growth season, July leaves had the second highest moisture content (63.89%) which again can be attributed to the water availability in the soil from the summer rain (Table 4.1). On contrary, lower moisture content of SML in October can be related to the plant's gained dry weight as with seasonal progress, plants continue to store energy in their different tissues/organ (leaves, bark cells, roots, etc.) resulting in the increase of dry weight.

Plants assimilate minerals from their growing environment where assimilation of minerals is influenced by several factors including cultivation techniques, nutrient status, and environmental stresses (Nour, Trandafir, & Cosmulescu, 2014). Therefore, it is essential to have a knowledge of the mineral composition in plant extract. Since the sugar maple trees of this study were grown under the same environmental conditions, the obtained results thus enabled us to study more precisely the influence of the harvesting time on the mineral compositions. The composition of macro elements (K, Ca, Mg, P, and S) and trace elements (Mn and Fe) determined for the SML extracts of varying harvesting time are reported in Table 4.2. The most abundant mineral was calcium (ranged between 7627.55 and 31983.69 $\mu\text{g/g DM}$), followed respectively by potassium (ranged between 6486.65 and 12447.32 $\mu\text{g/g DM}$), magnesium (ranged between 2418.27 and 4496.86 $\mu\text{g/g DM}$), phosphorus (ranged between 3356.92 and 4049.12 $\mu\text{g/g DM}$), and sulphur (ranged between 1621.39 and 2354.81 $\mu\text{g/g DM}$). Reports on minerals present in sugar maple sap, syrup, and bark are available while data on analyses of minerals present in SML are insufficient. Bhatta et al. (2018) reported the presence of same studied minerals (K, Ca, Mg, P, Mn, and Fe) in sugar maple bark where except for K (30250 $\mu\text{g/g DM}$), all other minerals were in lower

concentrations than that of the currently studied SML extracts. Ball (2007) reported that maple syrup predominantly contains K (1300–3900 µg/g DM) and Ca (400–2800 µg/g DM), with remaining minerals in low concentrations. Minerals are essential for physiological processes such as tissue growth, development of bone, among others. They also act as cofactors of various enzymes such as PAL (phenylalanine ammonialyase), C4H (cinnamate-4-hydroxylase), C3H (4-coumarate-3-hydroxylase (monooxygenase)), CHS (chalcone synthase), F3H (flavanone-3-hydroxylase), DFR (dihydroflavonol reductase), and LAR (leucoanthocyanidin reductase), that regulate the biosynthesis of PCs following the shikimate pathway (Bhatta et al., 2018; Oney-Montalvo et al., 2020; Santos-Buelga et al., 2023). With respect to the harvesting times, a significantly higher content of K was found in SML–M sample. SML–J registered maximum contents of Mg and S, while SML–S registered maximum content of Mn. It can be correlated to the temperature effect as an increase in temperature would generate vapor pressure deficit that might increase transpiration, consequently increasing uptake of minerals in trees that would be driven by mass flow (Fernando et al., 2016; Kohler et al., 2019). However, SML–O registered significantly higher contents of Ca, P, and Fe.

4.4.2. Effect of seasonal dynamics on the phytochemical properties and *in vitro* bioactivities of SML

4.4.2.1. Extraction yield, total phenolics, total flavonoids, and individual PCs

Results of extraction yield, total phenolics, total flavonoids, and individual phenolic compounds are given in Table 4.4. Significantly higher ($p < 0.05$) extraction yield was obtained from SML–M ($57 \pm 3.24\%$) while SML–S gave a lower extraction yield ($30 \pm 2.05\%$). These differences can be supported by varying leaf's SO after extraction as discussed earlier.

Regarding total phenolics and flavonoids, significant changes ($p < 0.05$) have been observed throughout the harvesting time which seems to be caused by fluctuations in environmental conditions such as temperatures, UV index, rainfalls, duration of sunshine, soil nutrient conditions, among others (Bernal et al., 2013; de Oliveira et al., 2023; Oney-Montalvo et al., 2020). As can be seen in Table 4, contents of total phenolic in SML extracts of varying harvesting time were in the range of 105.67 ± 13.16 – 192.74 ± 3.15 mg GAE/g DM, which is in line with the previously reported value of total phenolic content (110.87 mg GAE/g DM) from macerated sugar maple leaves by Yeasmen and Orsat (2022). In addition, based on the reported yields of total phenolics from other macerated leaves' extracts such as: olive leaves (57 – 115 mg GAE/g DM) (da Rosa et al., 2019);

thyme leaves (142.08 mg GAE/g DM) (Palmieri et al., 2020); bitter melon leaves (20.66 mg GAE/g DM) (Uysal et al., 2019); herbal teas (0.2–72.42 mg GAE/g DM) (Jin et al., 2016); purpleblow maple leaves (34.07–89.96 mg GAE/g DM) (Yang et al., 2018), it can be said that the total phenolics in SML are comparable to other leaves' extracts. Concerning seasonal dynamics, young leaves (SML–M) yielded a significantly higher total phenolic content of 192.74 ± 3.15 mg GAE/g DM (Table 4.4), which can be explained from the hypothesis of photoprotection as mentioned by Zhang et al. (2018). Briefly, due to possessing an immature chloroplast structure and a reduced utilization ability of absorbed photons in the photosynthetic apparatus, young leaves require more photoprotection than do mature leaves. In addition, this increasing phenolic concentration in the SML–M sample can be attributed to its lower contents of Ca, Mg, and Mn, as these minerals in lower contents are associated with the accumulation of NO_3 and PO_4 , two structural nutrients known as the precursors for phenol biosynthesis (Oney-Montalvo et al., 2020). This can also be related to the lower nitrogen and higher potassium content of SML–M (Table 4.2), which triggered the biosynthesis of phenolics. Likewise, Sauvesty et al. (1993) found a correlation between the phenolic and nitrogen contents in sugar maple where high humidity of the soil reduced the availability of nitrogen which results in a stress reaction on the sugar maple tree, while regulation of PAL, C4H, and C3H enzymes, which are responsible for the biosynthesis of phenolic acids, were seen to increase their activity in presence of a high concentration of potassium (Oney-Montalvo et al., 2020). Concerning the total flavonoid content, SML–J gave the highest flavonoid content (34.34 ± 0.66 mg CTE/g DM). After a brief weather survey from spring to fall months of the sampling location (Morgan Arboretum, Sainte-Anne-de-Bellevue, Canada) during 2021, it was found that the month of July 2021 was under environmental stresses by experiencing maximum average temperature (28°C) and UV-index (5). This environmental condition could be a factor contributing to the accumulation of higher flavonoid content in the summer specimens i.e., SML–J as concentrating flavonoids represents the protection against excessive UV radiation (Saltveit, 2018). Moreover, in July 2021, plants were exposed to stronger sunlight for a longer duration (286 sun hour) which might cause photooxidative stress in leaves and favor the upregulation of flavonoid compounds in the photosynthetic organ (such as leaves) as a defensive mechanism. Flavonoids can alleviate photooxidative damage by limiting the formation of reactive oxygen species (ROS) in chloroplasts through metal chelation (Mira et al., 2002), eroding the flux of damaging solar wavelengths (Zhang et al., 2016). Lower phenolic and flavonoid contents of

SML were obtained from the SML–S sample. One of the reasons behind this lower concentration might be due to the Mn toxicity effect as overaccumulation of foliar Mn (Table 4.2) could reduce the carboxylation efficiency (Fernando, Marshall, & Lynch, 2016). Apostolidis et al. (2012) observed a similar trend in phenolic accumulation in red and sugar maple leaves as a function of the time of harvest i.e., autumn/fall samples had lower phenolic contents in comparison to others. Following September, during the onset of defoliation i.e., in the month of October, SML were seen to contain sustained high phenolic and flavonoid contents.

To know how seasonal variations particularly affect the biosynthesis of the phytochemicals in SML, carotene, tannic acid, ellagic acid, gallic acid, ferulic acid, sinapic acid, quinic acid, caffeic acid, catechin, epicatechin, kaempferol, quercetin, rutin, and resveratrol were quantified individually (Table 4.4). Selection of compounds were based on their significant antioxidant activity as well as other health benefits (such as carotene which known as an important vitamin source for human; gallic acid known to perform excellent activities for neuropsychological, gastrointestinal, cardiovascular, and metabolic disorders; caffeic acid which possesses strong anti-inflammatory and anticancer effects; ferulic acid known for its strong antioxidant, prooxidant, and antibacterial activities; quinic acid known for its anti-inflammatory properties; sinapic acid and rutin known to be neuroprotective agents; ellagic acid for its effectiveness in reducing obesity and ameliorating obesity-mediated metabolic complications, such as insulin resistance, type 2 diabetes, and atherosclerosis; kaempferol for its strong anticancer potential; quercetin for its strong antibiotic potential; and catechins and resveratrol for their associated anti-obesogenic effects (Batoool et al., 2022; Dong et al., 2022; Kang et al., 2016). Quinic acid followed by ellagic acid are found to be the most abundant phenolic acids, whereas rutin followed by catechin are the two most abundant flavonoids in all SML samples. Moreover, flavanols (i.e., catechin and epicatechin), flavonols (i.e., kaempferol and quercetin) and stilbene (i.e., resveratrol) have been seen to concentrate in the SML–J sample which again is directly connected to a responsive protection against UV radiation (Saltveit, 2018).

Table 4.4: Seasonal dynamics on the phytochemical compositions of sugar maple leaves.

Attributes	SML–M	SML–J	SML–S	SML–O
TY (%)	57±3.24 ^A	45±2.07 ^B	30±2.05 ^C	40±1.33 ^B
TPC (mg GAE/g DM)	192.74±3.15 ^A	172.81±5.94 ^B	105.67±13.16 ^C	165.30±5.48 ^B
TFC (mg CTE/g DM)	29.66±0.44 ^B	34.34±0.66 ^A	23.27±0.26 ^C	24.31±0.36 ^C
Phenolic acids (mg/g DM)				
Gallic acid	5.87±1.70 ^A	0.46±0.10 ^B	0.17±0.10 ^B	0.17±0.11 ^B
Ellagic acid	10.51±4.28 ^A	6.18±0.89 ^A	5.02±1.02 ^A	7.30±1.01 ^A
Sinapic acid	ND	ND	0.44±0.12 ^A	ND
Ferulic acid	2.27±0.55 ^A	1.47±0.41 ^A	1.38±0.10 ^A	1.58±0.28 ^A
Tannic acid	1.95±0.70 ^A	0.66±0.56 ^{AB}	0.42±0.60 ^B	ND
Quinic acid	793.83±3.25 ^A	156.32±5.5 ^C	140.46±1.2 ^D	212.49±2.8 ^B
Caffeic acid	9.58±0.41 ^B	3.14±0.55 ^A	1.61±0.30 ^D	2.90±0.10 ^C
Flavonoids (mg/g DM)				
Catechin	4.16±2.57 ^B	11.97±0.04 ^A	2.84±0.03 ^B	14.39±0.05 ^A
Epicatechin	0.03±0.01 ^D	3.29±0.20 ^A	1.61±0.03 ^C	1.90±0.02 ^B
Kaempferol	ND	0.39±0.11 ^A	ND	ND
Quercetin	ND	1.70±0.20 ^A	0.71±0.02 ^B	0.30±0.01 ^C
Rutin	39.56±5.38 ^A	18.12±1.69 ^B	6.79±1.47 ^C	5.21±1.44 ^C
Stilbenes (mg/g DM)				
Resveratrol	ND	0.12±0.01 ^A	ND	ND
Beta carotene (mg/g DM)	55.19±0.03 ^A	32.01±0.50 ^B	19.33±0.40 ^C	9.51±0.05 ^D

CTE: Catechin equivalent; DM: Dry matter; GAE: Gallic acid equivalent; ND: Not detected; SML–M: Sugar maple leaves collected in May; SML–J: SML collected in July; SML–S: SML collected in September; SML–O: SML collected in October; TY: Total yield; TPC: Total phenolic content; TFC: Total flavonoid content.

Note: The values are presented as average value ± standard deviation. Different uppercase letters (A–D) in a row indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective phenolic composition of different harvesting time.

4.4.2.2. *In vitro* bioactivities of SML

PCs are considered as potential antioxidant compounds where their antioxidant activities depend on the number and arrangements of the hydroxyl groups (Albuquerque et al., 2021). Noteworthy to mention that plant antioxidant activities could be influenced by a number of factors such as the presence of diverse chemical compounds which could be further affected by harvesting time and environmental conditions. Besides, synergistic or antagonistic effects of these compounds might also play a crucial role in the resulting antioxidant activities (Sytarova et al., 2020). Various *in vitro* methods are available for evaluating plant bioactivities. However, they are based on different principles and mode of actions, thus, might present some limitations on reflecting the real antioxidant potential when applied individually. Hence, it is of great importance to assess antioxidant activities with different methods to achieve a concrete and global understanding by including a broader scope of bioactive compounds with various antioxidant mechanisms (Yang et al., 2018). As illustrated in Figure 4.2, seasonal variation of the antioxidant activities of SML were assayed by both ROS/radical scavenging (DPPH assay and ABTS assay) and non-radical redox

potential based methods (FRAP assay and CUPRAC assay). Antioxidant potentiality of SML ranged from 25.10 ± 0.41 to 203.30 ± 17.51 $\mu\text{M TE/g DM}$ which could be attributed to the differences in harvesting month, phenolic compositions, and in the antioxidant measurement assays. Overall, all the specimens showed considerable antioxidant potential whereas the SML-S had the lowest antioxidant activity.

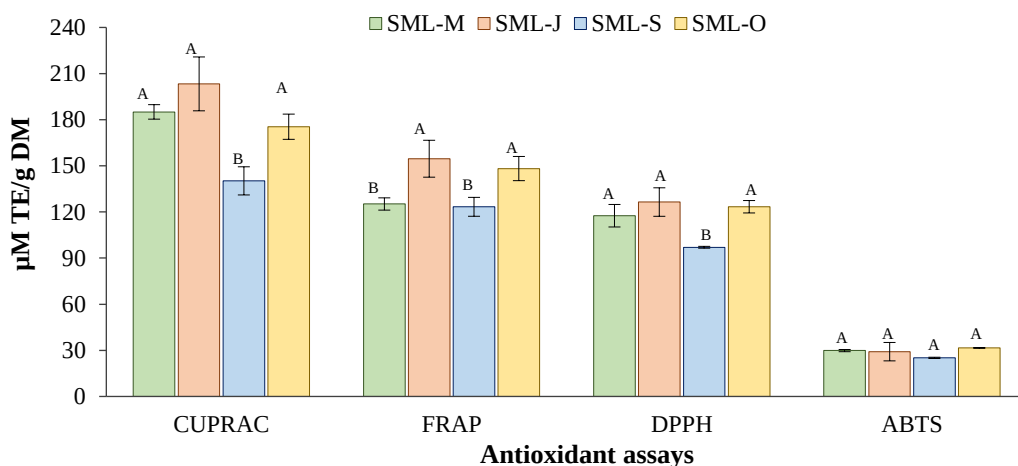


Figure 4.2: Seasonal changes of antioxidant capacities in sugar maple leaves. For each antioxidant assays, different uppercase letters indicate significant differences based on 95% confidence interval ($p < 0.05$) in the harvesting time.

Possible impacts of analyzed phenolic contents on the resulting antioxidant activities established by CUPRAC, FRAP, DPPH, and ABTS assays were assessed by regression analysis using Pearson correlation coefficients and are summarized in Table 4.5. Based on the CUPRAC assay, both phenolics and flavonoids seem to be the most potent antioxidants as could be concluded from extreme correlations between TPC and CUPRAC ($r^2 = 0.8683$, $p < 0.05$) and between TFC and CUPRAC ($r^2 = 0.8815$, $p < 0.05$). Among four antioxidant assays, DPPH assay showed strong correlation with other assays such as with CUPRAC ($r^2 = 0.9247$, $p < 0.05$), with ABTS ($r^2 = 0.8801$, $p < 0.05$), and with FRAP ($r^2 = 0.8027$, $p < 0.05$). DPPH is a stable lipophilic free radical which adopts a chemical reaction mechanism where a single electron transfer is involved in the reduction of given compounds. Furthermore, the DPPH method may also involve hydrogen atom transfer during the reaction.

Table 4.5: Pearson correlation coefficients (r) among phenolic contents and antioxidant assays at varying harvesting times.

Correlations	TPC	TFC	CUPRAC	FRAP	DPPH	ABTS
TPC (mg GAE/g DM)	1.0000	0.6463	0.8683*	0.3598	0.8430	0.8261
TFC (mg CTE/g DM)	0.6463	1.0000	0.8815*	0.4723	0.6445	0.2653
CUPRAC (μ M TE/g DM)	0.8683	0.8815	1.0000	0.6766	0.9247	0.6859
FRAP (μ M TE/g DM)	0.3598	0.4723	0.6766	1.0000	0.8027	0.5732
DPPH (μ M TE/g DM)	0.8430	0.6445	0.9247	0.8027	1.0000	0.8801
ABTS (μ M TE/g DM)	0.8261	0.2653	0.6859	0.5732	0.8801	1.0000

ABTS: 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CTE: Catechin equivalent; CUPRAC: Cupric ion reducing antioxidant capacity; DM: Dry matter; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalent; TPC: Total phenolic content; TFC: Total flavonoid content; TE: Trolox equivalent.

Note: The correlations are estimated by Row-wise method.

4.4.3. Principal component analysis

Principal component analysis (PCA) was applied to different leaf samples as a function of phenolic compositions, antioxidant activities, and environmental factors *viz.* temperature, UV index, nitrogen content, solar exposure. Accordingly, a biplot of PCA is obtained (Figure 4.3) which narrates the relationships between the samples (SML from different harvesting times) and between quality attributes (phenolic compositions, antioxidant activities, and environmental factors) of the samples. First two components 1 and 2 (PC1 and PC2, respectively) explained 79.6% of the variance of the experiment where PC1 explained 43.2% and PC2 explained 36.4%. PCA reveals that SML-M is mostly correlated with phenolic acids (except for sinapic acid and caffeic acid) and carotene whereas SML-J is correlated with flavonoids with the exception of rutin. Therefore, it can be assumed that biosynthesis of maximum phenolic compounds in SML mostly occurred during the leaf expansion and growth phases which is positively correlated with the extent of environmental stresses namely temperature, UV index, and hours of solar exposure. However, there is a negative correlation between nitrogen content and phenolic acids biosynthesis. With respect to the antioxidant activities, these are mostly influenced by the positive correlation among CUPRAC, DPPH, ABTS, major phenolic acids, and carotene. In other words, the antioxidant activity measured by CUPRAC and DPPH assays is mostly supported by total flavonoids while the antioxidant activity reported by the ABTS assay, is supported by phenolic acids.

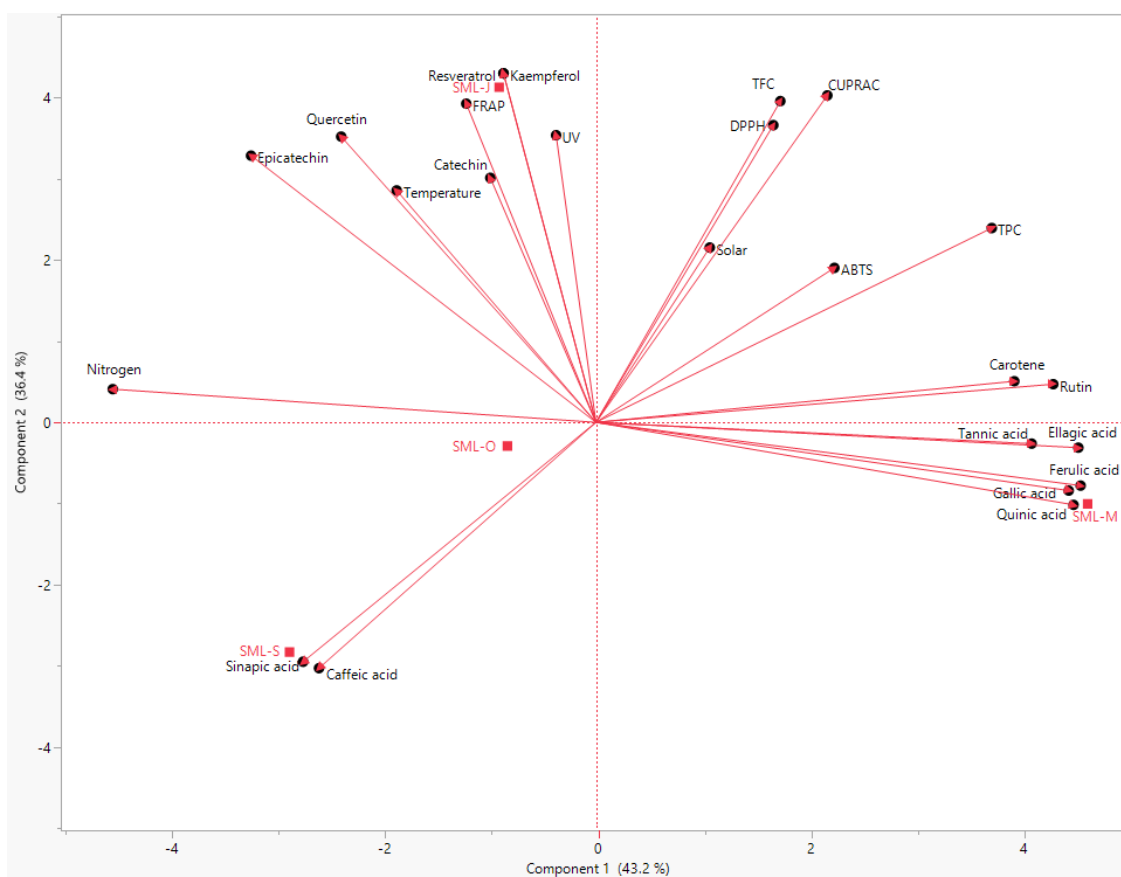


Figure 4.3: Principal component analysis of sugar maple leaves collected in different time as a function of phenolic compositions and environmental factors. *SML–M*: Sugar maple leaves collected in May; *SML–J*: SML collected in July; *SML–S*: SML collected in September; *SML–O*: SML collected in October.

4.5. CONCLUSION

Nowadays, forestry biomass like sugar maple leaves are recognized to be a cheap and versatile source of bioactive compounds that can be utilized for the ever-growing functional food ingredient sector. However, the bioactive composition of leaves varies throughout their life cycle due to differences in environmental and weather conditions where the seasonal temperature is likely to play an important role. Distinctive color changes in sugar maple leaves (SML) were observed during leaf expansion (May) and during the onset of leaf defoliation (October) along with its annual growth cycle. Microstructural analysis confirmed that both the freeze-drying and extraction methods caused surface roughness/irregularities whereas leaves which were subjected to extraction had more surface ruptures/damages/pores. All seasonal SML samples contained considerable phenolics, flavonoids, and exhibited high antioxidant capacity as can be seen from

the FTIR spectra and HPLC based quantification. The antioxidant activity can be related to the contents of both flavonoids and phenolics. Among the quantified bioactive compounds, quinic acid, ellagic acid, rutin, and catechin presented higher contents compared to others. Moreover, principal component analysis was efficient to show the effect of seasonal dynamics on the phenolic mapping of SML, giving evidence of positive effect of higher temperature, UV index, solar radiation and negative effect of nitrogen content on the phenolic biosynthesis. To conclude, SML provides a wide array of application scope, for instance, if the target is to fortify food products with high mineral containing ingredients, then SML–O would be the best choice. On the other hand, taking antioxidant potentialities into account SML collected in May to July should be preferred. However, since all the leave samples irrespective of collection time contained considerable amount of phenolic compounds and minerals with higher antioxidant activities, this study suggests that sugar maple leaves should preferentially be collected during the defoliation period giving full emphasis on environmental sustainability.

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CONNECTING TEXT TO CHAPTER 5

Chapter 5 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2023). Advanced separation of phytochemical and bioactive attributes from various morphological components of sugar maple (*Acer saccharum*) tree. *Food Bioscience*, 56, 103260. doi: <https://doi.org/10.1016/j.fbio.2023.103260>

Apart from the environmental conditions (Chapter 4), the accumulation and concentration of phenolic compounds vary with other factors including the type of plant, cultivar, parts of plant namely leaf, fruit, stem, twig, root, flower, seed, etc. and phase of their ontogenesis, as well as the function of phenolic compounds in plants' lifecycle. Hence, Chapter 5 comprehensively investigated the spatial distribution of the physicochemical, thermal, phytochemical, antioxidant, and antimicrobial attributes among different morphological parts (leaves, flowers, fruits, seeds, barks, and twigs) of sugar maple tree.

Part of this research chapter was presented in a scientific conference.

Yeasmen, N., & Orsat, V. (2023/07). Spatial distribution of phenolic compounds and antioxidant attributes in *Acer saccharum*. American Society of Agricultural and Biological Engineers (ASABE) Annual International Meeting, Omaha, Nebraska, USA.

**CHAPTER 5: ADVANCED SEPARATION OF PHYTOCHEMICAL AND BIOACTIVE
ATTRIBUTES FROM VARIOUS MORPHOLOGICAL COMPONENTS OF SUGAR
MAPLE (*ACER SACCHARUM*) TREE**

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5.1. ABSTRACT

This work provides data on the advanced extraction of phenolic compounds from different morphological parts (flower, leaf, fruit, twig, seed, and bark) of the sugar maple tree, information which is still limited. Additionally, it explores the operating parameter of a fundamental advanced extraction unit operation (microwave-assisted extraction), considered environment friendly. Results based on the FTIR spectra, spectrophotometric and HPLC analyses showed that sugar maple's morphological parts can be dynamic candidates to be used as either natural preservative or antioxidant additive for application in food, nutraceuticals, and pharmaceutical industries. Yields of antioxidant components such as total phenols, total flavonoids, condensed tannins, and extractable tannins in sugar maple ranged from respectively 35.77–136.55 mg GAE/g DM, 10.51–47.33 mg CTE/g DM, 5.33–127.33 mg CTE/g DM, and 32.21–110.35 mg GAE/g DM. In addition, different morphological parts of sugar maple could also be used as purpose-based food ingredients, other than as antioxidant additives. For instance, leaf can be utilized as a promising source for protein ($21.16\% \pm 0.83\%$) extraction, the bark was determined to have high carbohydrate ($87.27\% \pm 0.33\%$) content, while seeds showed higher ash (inorganic nutrients) ($7.84\% \pm 0.07\%$) and fat ($6.12\% \pm 0.44\%$) contents. Thermogravimetric analysis showed that leaf followed by bark have considerable thermal stability while the opposite condition was seen in the case of maple fruits.

Keywords

ATR-FTIR, Biorefinery, Differential scanning calorimeter (DSC), HPLC-UV, Microwave-assisted extraction (MAE), Sustainability, Thermogravimetric analysis (TGA).

5.2 INTRODUCTION

Taking advantage of the advancements in the chemical industry, food industries started to use many chemical/synthetic additives in food products to improve their shelf life. However, the use of synthetic additives in food products has now left a growing concern about their risks to human health and the environment (Martiny et al., 2021). Given the concerns, interest in alternative plants extracts with health-promoting potential has increased remarkably in the functional food sectors as a replacement of synthetic compounds, for instance, synthetic antioxidants (e.g., BHA, BHT, PG, and TBHQ) and synthetic antimicrobial agents (e.g., benzoate, sorbate, nitrite, sulfite, etc.). According to the United States Food and Drug Administration Code of Federal Regulation, “antioxidants are the substances which participate in physiological,

biochemical, or cellular processes that inactivate free radicals or prevent free radical-initiated chemical reactions”. Apart from the consumers increasing demand for “clean label” natural ingredients, the raising safety concerns about synthetic antioxidants also necessitate the search for effective alternatives. Phenolic compounds are classified as primary antioxidants that can delay or inhibit the initiation step or interrupt the propagation step of oxidation by scavenging the free radicals (Shahidi & Ambigaipalan, 2015). The antioxidant potential of phenolic compounds mainly depends on the number and position of the hydroxyl groups in their molecular structure (Liu et al., 2023). Since phenolics are plant-produced secondary metabolites, different plant parts may have higher concentrations, and such particular plant organs should be harvested in priority to provide highest cumulative contents of phenolic compounds. However, when in doubt of which organ contains the highest concentration of phenolics, the entire plant is harvested which may not be the most environmentally sustainable (Mamphiswana et al., 2010).

Linked with selecting appropriate harvestable plant organ(s), it was reported that the contents of phenolic compounds in plants vary among tissues and organs (Yang et al., 2018a). For instance, total phenols, total extractable tannins and non-precipitable phenols were found higher in the order of stem bark > bark of branches > twig extracts of red maple (Royer et al., 2011). Gao et al. (2022) found higher phenolic and flavonoid contents in the flower bud extract followed by, respectively, leaves extract and fruit extract of *Vaccinium dunalianum* Wight (an evergreen shrub of northern India and Western China). Based on the study of Medic et al. (2021), the highest content of phenolic compounds in walnut plant material was in the order of main root > buds > leaves > bark > petioles. According to them, the root showed the highest content of phenolic compounds compared to other tissues, and this was correlated with their higher content of hydroxybenzoic acids and naphthoquinones. The maximum content of secondary metabolites was noted in the flower and seeds of *Rumex crispus*, both in the flowering and fruiting period where stems were characterized by a minimum content of the studied substances. The antioxidant activity of the studied parts was in the following order: the generative part (flowers, seeds) > leaves > root > stem (for flowering and fruiting stages) (Feduraev et al., 2019). Sytarova et al. (2020) found the phenolic content in sea buckthorn greater in leaves than in berries. Higher total phenolic content, total tannin content and antioxidant activity were observed in *Cassia fistula* bark extracts compared to other plant parts’ extracts (stem, leaf, and root) (Tzekiat & Chiang, 2013).

Acer saccharum, commonly known as sugar maple (SM), is an ecologically and economically important deciduous hardwood species of the eastern-north of North America. It belongs to the family of Sapindaceae with an average height of 25-35 m. Sugar maple grows best in a well-drained loamy soil rich in organic matter, although it can tolerate some sand, rock, or clay in the soil (Geoffroy et al., 2017). The tree is dormant during the winter, while in the spring it starts to grow bright red twigs on the branches which will turn into flowers later. The flowers are in panicles of five to ten together, which occur in early spring and the fruit is a pair of samaras (winged helicopter seeds) (Geoffroy, Fortin, & Stevanovic, 2017; St Clair et al., 2008). In the fall, green leaves turn yellow, orange, and red due to increasing contents of carotenoids and anthocyanin pigments and loss of chlorophyll (Apostolidis et al., 2012). Sugar maple tree is best known for the production of maple syrup, a natural sweetener obtained by concentrating the sap, which has been reported to have high nutritional values containing sugar, minerals, amino acids, vitamins, and phytochemicals (Bhatta et al., 2018). Other than maple syrup, different parts of this species such as barks, shoots, and sap have been used by native north Americans in the treatment of various ailments such as diarrhea, cramps, measles, diuretic, cataracts, eyewash, and shortness of breath. This was attributed to the presence of a wide array of functional components like phenolic compounds (Yeasmen & Orsat, 2023). However, from the economic viewpoint, up to now the interest in sugar maple has been limited to maple syrup production (Yeasmen & Orsat, 2023). Nevertheless, it can also make a potential place in other rising sectors such as functional food industries, nutraceutical industries, etc., due to containing considerable amounts of phenolic compounds as reported by Bhatta et al. (2018); Geoffroy, Fortin and Stevanovic (2017); and Yeasmen and Orsat (2022).

In terms of phenolics' exploitation, extraction is the key step which involves the separation of target compounds from plant matrices. To do so, plant cell walls need to be essentially destroyed to ensure proper mass transfer of desired compounds from the plant matrix to the extractant (Yeasmen & Orsat, 2021). Considering the bottlenecks of conventional extraction methods such as excessive requirement of time, energy, cost, and solvent, green extraction methods like microwave-assisted extraction (MAE) can be very promising towards the extraction of phenolic compounds. It is known to speed up the extraction process with less energy and solvent requirement and cause minimum degradation of target compounds while leaving little to no carbon footprint (Martiny et al., 2021; Routray & Orsat, 2014). The extraction principle of MAE involves

the conversion of electromagnetic energy into thermal energy in both the sample and the solvent via ionic conduction and dipole rotation (Yeasmen & Orsat, 2021). Information related to the thermal and physicochemical properties of powders is important for the development of novel food products with desirable attributes, better stability, and prolonged shelf life (Manju et al., 2021). In this aspect, many techniques have been developed for the characterization of powders/extracts, for instance colorimetry, Fourier transform infrared (FTIR) spectral analysis, thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) are some effective techniques used for detailing the color, types of functional groups, thermal stability, and glass transition temperature (T_g), respectively.

The main goal of this work is to comprehensively investigate the spatial distribution of the physicochemical, thermal, phytochemical, antioxidant, and antimicrobial attributes among different morphological parts (leaves, flowers, fruits, seeds, barks, and twigs) of sugar maple trees. The assessment of such aspects will provide useful insights which could be of great interest for the more comprehensive use of sugar maple as a profitable as well as sustainable source of natural ingredients in the food industry.

5.3. MATERIALS AND METHODOLOGY

5.3.1. Chemicals

Folin-Ciocalteu reagent (FCR), sodium carbonate (Na₂CO₃), Sodium nitrite (NaNO₂), Aluminum chloride (AlCl₃), Sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferric chloride (FeCl₃.6H₂O), NaCl, peptone, plate count agar, yeast extract, and neocuproine were purchased from Sigma–Aldrich, St-Louis, MO, USA. Acetonitrile (ACN), acetic acid (CH₃COOH), ethanol (EtOH), hydrogen chloride (HCl), methanol (MeOH), HPLC water, 2,4,6-tripyridyl-s-triazine (TPTZ), yeast extract, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), copper (ii) chloride (CuCl₂) were purchased from Fisher Scientific, Nepean, Ontario, Canada. Standards of gallic acid, ellagic acid, coumaric acid, sinapic acid, caffeic acid, ferulic acid, catechin, epicatechin, quercetin, luteolin, and rutin were purchased from Sigma–Aldrich, St-Louis, MO, USA. Bacteriological peptone and standard plate agar were purchased from Oxoid, UK. All the solvents used for analytical chromatography were of HPLC grade.

5.3.2. Sample collection and preparation of the dried powders

Samples of SM leaves (SML), flowers (SMFL), fruits (SMF), seeds (SMS), barks (SMB), and twigs (SMT) without any visible signs of mechanical and infectious damage were harvested in the two consecutive years of 2021 and 2022, from the Morgan Arboretum located at McGill University's Macdonald campus in Sainte-Anne-de-Bellevue (Quebec, Canada, 45°25'50.1"N 73°57'8.2"W). It is a natural forest stand of sugar maple trees. Details of tree selection are reported in Yeasmen and Orsat (2023). Regarding sample collection, twigs and flowers were collected in April, barks were collected in July, fruits were collected in September at the ripening stage, seeds were collected in October while leaves were collected in May, July, and September. Immediately after the collection of all the respective plant parts, they were thoroughly washed under running tap water followed by freeze drying (HARVEST RIGHT, USA). Afterwards, freeze-dried samples were ground using a milling machine (Retsch GmbH, GM 200, Retsch-Allee 1-5, Haan, Germany). The produced sample powders (particle size < 0.1 mm) of different morphological parts were kept in brown sealed glass bottles at -20°C until further analyses.

5.3.3. Physicochemical characterizations

5.3.3.1. Color indices

Color indices L* (lightness to darkness), a* (redness to greenness), and b* (yellowness to blueness) of all freeze-dried SML, SMFL, SMF, SMB, SMT, and SMS samples were measured at room temperature (25°C) using a colorimeter (KONICA MINOLTA, CR-300, Japan).

5.3.3.2. Attenuated total reflectance- Fourier transform infrared (ATR-FTIR) spectroscopy

FTIR spectra of freeze-dried SML, SMFL, SMF, SMB, SMT, and SMS samples were recorded using an ATR-FTIR spectrometer (Thermo Scientific Nicolet iS5 FTIR, Thermo Fisher Scientific, Madison, WI, U.S.A.) accessorized with a fast recovery deuterated triglycine sulfate (DTGS) detector. All the spectra were recorded in the wave range of 4000 to 600 cm⁻¹ region with 40 scans at a resolution of 4 cm⁻¹. Smoothing of spectra was performed using Omnic software (version 6.1, Nicolet, Madison, WI, USA) (Yeasmen & Orsat, 2022).

5.3.3.3. Moisture and ash contents

Moisture and ash contents of all plant parts were determined using the official methodology of AOAC 925.10 (Horwitz, 2005). Briefly, all sample powders were air dried in an air-dryer (Thermo Scientific Precision Oven, USA) at 105°C till constant weight and then transferred into

a desiccator to equilibrate for 30 minutes. Weight of the samples before and after drying was measured using a balance with a precision of ± 0.0001 g. About 2 g of oven dried sample was ignited with a gas burner until white smoking came-off and then placed in a muffle furnace (THERMOLYNE, Thermo-Scientific FB1315M, USA) at 700°C and ignited for 2 h. Weight of the samples before and after ignition were taken using a balance with a precision of ± 0.0001 g.

5.3.3.4. Crude protein content

The crude protein content was estimated by nitrogen combustion method using a Dumas Nitrogen Analyzer (NDA 701, VELP Scientifica), where the protein content of SM leaves, bark, fruit, flower, twig, and seed powders were obtained by multiplying the nitrogen value by a factor of 6.25 (Yuliarti et al., 2021).

5.3.3.5. Crude fat content

Crude fat contents of SML, SMFL, SMF, SMB, SMS, and SMT powder were determined by following Randall's method with little modifications. The method was outlined by the manufacturer (VELP SCIENTIFICA) for VELP SER 148/6 Solvent Extractor (VELP, 2013). Around 3 g sample of each was placed in thimbles of a VELP SER 148 solvent extraction unit (Velp Scientifica, Usmate, Italy) where petroleum ether was introduced in a glass cylinder kept at a pre-set temperature condition of 130°C. Complete fat extraction process was performed in three consequent steps of 90 min: immersion (30 min), washing (30 min), and recovery (30 min). Afterwards, fat content was calculated by measuring the glass cylinder before and after the fat extraction.

5.3.3.6. Carbohydrate content

Estimation of carbohydrate content was performed based on the subtraction method (Bello et al., 2023). This is achieved by subtracting the sum of the percentages of moisture, ash, fat, and protein from 100.

5.3.3.7. Fiber content

SM samples were also analyzed for %ADF (acid detergent fiber), %NDF (neutral detergent fiber), and %ADL (acid detergent lignin) using the batch procedures outlined for an Ankom²⁰⁰ Fiber Analyzer by the manufacturer (Ankom Technology, Macedon, NY) (ANKOM). %NDF consists of hemicellulose, cellulose, and lignin; %ADF consists of cellulose and lignin; and %ADL consists of lignin only. Therefore, %Cellulose and %Hemicellulose was calculated by the following equations (5.1) and (5.2) respectively.

$$\% \text{Cellulose} = \% \text{ADF} - \% \text{ADL} \dots\dots\dots (5.1)$$

$$\% \text{Hemicellulose} = \% \text{NDF} - \% \text{ADF} \dots\dots\dots (5.2)$$

5.3.4. Thermal characterizations

5.3.4.1. Thermogravimetric analysis

Thermal stabilities of the freeze-dried SML, SMFL, SMF, SMB, SMS, and SMT samples were measured using a TGA Q50 (TA Instruments, USA). Around 11 ± 1 mg of sample powder was placed in a platinum pan and the experiment was conducted from 25 to 700°C under nitrogen atmosphere at a heating rate of $5^\circ\text{C}/\text{min}$ (Manju et al., 2021). The resulting degradation profiles were plotted as %weight loss and its first-time derivative as a function of temperature.

5.3.4.2. Differential scanning calorimetric analysis

Differential scanning calorimeter (TA, DSC250, Delaware, USA) was used to measure the glass transition temperature (T_g). Prior to the measurement, DSC instrument was calibrated with indium in a closed aluminum pan. Following calibration, about 7 ± 1 mg powdered samples of SML, SMB, SMFL, SMF, SMS, and SMT were placed in a hermetic aluminum pan and an empty aluminum pan was used as a reference. DSC curves were obtained under nitrogen atmosphere ($50 \text{ mL}/\text{min}$). All the scans were taken from -40 to 300°C at a heating rate of $5^\circ\text{C}/\text{min}$ and T_g was calculated by using a half-height technique in the transition region. All the obtained thermograms were analyzed for their respective onset ($T_{g_{\text{on}}}$), mid ($T_{g_{\text{mid}}}$), and end point ($T_{g_{\text{end}}}$) glass transition temperatures (Jaya & Das, 2009).

5.3.5. Extraction of phenolics

5.3.5.1. Microwave-assisted extraction (MAE)

The MAE of phenolic compounds from SM samples was performed in a microwave unit (MINI-WAVE, SCP Science, Baie-d'Urfé, Quebec, Canada) using the previously screened temperature (90°C) with a duration of 10 min. Mini-wave is a closed-vessel microwave unit with a forced air ventilation system for cooling. The frequency and power of irradiation were 2.45 GHz and 1000 W, and the duration of irradiation included ramp time of 5 min (time to reach the set temperature) followed by the holding time (actual time of the sample being irradiated at set temperature). The module includes a non-rotating digestion rack which holds six equidistant and radially constructed quartz tubes of 75 mL. The magnetron is located below the floor to ensure uniform distribution of the microwave energy across the digestion chamber. In addition, six

infrared sensors are located on the side walls of the oven to provide real-time temperature monitoring of each sample temperature (da Rosa et al., 2019).

5.3.5.2. Maceration extraction (ME)

Macerated extracts of SM morphological parts (leaves, bark, fruit, flower, twig, and seed) were obtained by using a shaker machine (MaxQ 4000, Branstead International, Dubuque, Iowa, USA) where 70% EtOH at a solid-to-solvent ratio of 1:30 g/mL was applied for 24 h at a constant stirring speed of 200 rpm and temperature of 25°C.

After the extraction processes, the obtained extracts containing the solvent and sample powder mixture were subjected to centrifugation (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany) for 15 min at $8000 \times g$ (temperature of sample at speed was 25°C), followed by the vacuum filtration using Whatman 1 filter paper (Sigma Aldrich, St. Louis, USA).

5.3.6. Phytochemical characterizations

5.3.6.1. Total phenolic and total flavonoid contents

Total phenolic contents (TPC) and total flavonoid contents (TFC) of all microwave-assisted and macerated extracts were measured by following the methods of Singleton et al. (1999) and Jia et al. (1999), respectively. For TPC, briefly, 100 μ L extract was mixed with 500 μ L undiluted Folin-Ciocalteu reagent, to which, 1.5 mL of 20% (w/v) Na_2CO_3 was added. Finally, the volume of the solution was made up to 10 mL by adding HPLC grade water. This solution was vortexed for a few minutes and incubated in the dark for 2 h at 25°C. The absorbance was measured at 760 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, Salzburg, Austria). For TFC, 1000 μ L extract was thoroughly mixed with 300 μ L NaNO_2 (5% w/v) solution. After 5 min, 300 μ L AlCl_3 (10% w/v) solution was added to which 2000 μ L of NaOH (1M) solution was added after 6 min and the volume of the solution was made up to 10 mL by adding HPLC grade water. This solution was vortexed for few minutes, and the absorbance was measured at 510 nm using a plate reader (SpectraMax® i3x, Multi-mode micro- plate reader, Molecular Devices, Salzburg, Austria). Results of TPC were expressed in mg gallic acid equivalent per gm dry matter (mg GAE/g DM) while catechin was used as the standard to express the results of TFC (mg CTE/g DM).

5.3.6.2. Condensed tannin and extractable tannin contents

Condensed tannin contents were determined using vanillin assay by following the method reported by Saad et al. (2019). To a test tube 0.5 mL extract solution was mixed with 3 mL of 4% vanillin-methanol solution followed by adding 1.5 mL concentrated hydrochloric acid. The mixture was allowed to stand for 15 min in the dark and the absorbance was recorded at 500 nm using a plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, Salzburg, Austria). Condensed tannins were expressed as mg CTE/g DM.

Total extractable tannins (TET) were estimated by precipitating insoluble tannins with polyvinyl(polypyrrolidone) (PVPP) as described by Royer, Diouf and Stevanovic (2011). Briefly, 110 mg PVPP was added to 1 mL extract and the mixture was vortexed thoroughly followed by the centrifugation at $\times 3000$ g for 10 min (temperature of sample at speed wat 25°C) to precipitate the insoluble tannins. After that, the mixture was filtered through a 0.22- μ m filter to obtain the supernatant which corresponds to the non-precipitable phenols (NPP). The phenol content of NPP was measured by Folin–Ciocalteu’s method as mentioned in section 5.3.6.1. and the total extractable tannins (TET) were calculated by using equation (5.3) and expressed as mg GAE/g DM.

$$\text{TET} = \text{TPC} - \text{NPP} \dots\dots\dots (5.3)$$

5.3.6.3. HPLC-UV-based quantification of phytochemicals

Quantification of gallic acid, ellagic acid, sinapic acid, ferulic acid, caffeic acid, coumaric acid, catechin, epicatechin, quercetin, and rutin contents were performed using Agilent 1100 Series HPLC system designed with a C18 column (Gemini, 150 mm $\times$ 4.60 mm, 5 μ , Torrance, USA), an autosampler (G1313A, Agilent Technologies, USA, 100 $\times$ 2 mL vials), a quaternary gradient pumping system (G1311A, Agilent Technologies, Germany), and a UV-detector (G1314A, Agilent Technologies, Germany). Chromatographic conditions were set by following the description of Yeasmen and Orsat (2023): injection volume 5 μ L, flow rate of 0.4 mL/min, HPLC grade water containing 0.1% formic acid (mobile phase A), HPLC grade acetonitrile containing 0.1% formic acid (mobile phase B), and detection was performed at 280 nm. The gradient used for the separation of ellagic acid, sinapic acid, ferulic acid, caffeic acid, quinic acid, catechin, epicatechin, and rutin was 10 min: 99% A and 1% B; 30 min: 50% A and 50% B; 40 min: 99% A and 1% B; and postrun for 15 min. For the separation of gallic acid, tannic acid, and resveratrol, the following gradient was used: 4 min: 98% A and 2% B; 7 min: 80% A and 20% B; 20 min: 100% B; 24 min:

100% B; 25 min: 98% A and 2% B; 27 min: 98% A and 2% B; and postrun for 15 min. The data were analyzed using the Agilent Chem Station Software and the phenolic quantification was performed by the external standard method and the results were expressed as $\mu\text{g/g DM}$.

5.3.7. Antioxidant activity analysis

The antioxidant capacity of SM extracts obtained through MAE and ME was evaluated through measuring ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), and DPPH free radical scavenging activities based on the methods given by Benzie and Strain (1999), Apak et al. (2004), and Brand-Williams et al. (1995), respectively. Under the CUPRAC assay, to a test tube 1 mL each of CuCl_2 (10 mM), ammonium acetate buffer (pH 7) and neocuproine (7.5 mM) solutions was taken to which 1.1 mL extracts of leaves, bark, fruit, flower, twig, and seed was added. The tube was stoppered, allowed to stand for 1.5 h in the dark, and then the absorbance of the colored mixture was recorded. For DPPH assay, at first, stock of DPPH solution was prepared by diluting 24 mg DPPH with 100 mL HPLC grade methanol and then stored at -20°C until required. During the experiment, 100 μL all the extracts were allowed to react with 3000 μL DPPH working solution (prepared by diluting the stock solution with HPLC grade methanol until getting the absorbance of 1.1 ± 0.02 unit at 515 nm wavelength) for 30 min in the dark followed by recording the absorbance. The working FRAP solution was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in the ratio of 10:1:1 followed by warming to 37°C before using. Afterwards, 100 μL of leaves', bark's, fruit's, flower's, twig's, and seed's extract was allowed to react with 3000 μL FRAP working solution at dark condition for 30 min and then the absorbance was recorded.

A plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, Salzburg, Austria) was used to measure the absorbance at 593 nm, 450 nm, and 515 nm for FRAP, CUPRAC, and DPPH, respectively. Trolox was used to prepare standard curves for all three types of assays and the results are expressed as $\mu\text{mol TE/g DM}$.

5.3.8. Antimicrobial activity

Antimicrobial (antibacterial and antifungal) activities of macerated and microwave-assisted extracts of SMFL, SMF, SMB, SML, SMT, and SMS were tested by disc diffusion method against *Escherichia coli* (ATCC 10798) and *Saccharomyces cerevisiae* (ATCC 9763) (Deng et al., 2014; Taylor et al., 1995). *E. coli* was chosen because it is known as the best indicator of fecal contamination. From the point of food safety, the presence of *E. coli* signifies a more positive

assumption of microbial hazard (Martiny et al., 2021). On the other hand, *S. cerevisiae* is known as one of the most heat-resistant yeast species while it can grow at 2–10°C, thus can also be significant in the spoilage of refrigerated foods (Fleet, 2011). Sterilized blank discs (4.6 mm diameter) were punched from filter paper sheets and dipped into the obtained extracts of SM sample followed by drying in the laminar flow. Afterwards, petri plates containing plate count agar were inoculated with *E. coli* and *S. cerevisiae* cultures where the discs containing sample extracts and negative control were aseptically placed. Then the plates were incubated at 37°C (INCUSHAKE, Benchmark, Korea) for 24 h except for fungi (incubated for 48 h). Blank disc impregnated with EtOH was used as negative control. The diameters of the inhibition zones were measured in mm using slide calipers.

5.3.9. Statistical analysis

All the measurements were taken in triplicate for all samples and reported as mean $\pm$ standard deviation. One-way ANOVA and Tukey HSD tests were applied to analyze significant differences ($p < 0.05$) with 95% confidence interval among the mean values. Graphical representations were developed with licensed version of Microsoft Excel 16 (Microsoft Corporation, USA) and all the analyses were performed using the statistical software JMP (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, NC, USA).

5.4. RESULTS AND DISCUSSION

5.4.1. Physicochemical characterizations

5.4.1.1. Color attributes

Distinctive color changes in terms of lightness, redness to greenness, and yellowness to blueness have been observed in different parts of sugar maple (Table 5.1). SMS was found significantly ($p < 0.05$) lighter than the others. Except SMB, all the other sugar maple parts namely SMFL, SMS, SMF, SMT, and SML were more towards the greenness which is ascribed due to the presence of chlorophyll pigment (Li et al., 2018; Ohmiya et al., 2014). However, the redness value (10.96 ± 0.10) of SMB can be correlated with carotenoids, which gives yellow/orange color (Yang et al., 2017).

Table 5.1: Physicochemical and thermal properties of different lyophilized morphological parts (flower, twig, fruit, bark, seed, and leaf) of sugar maple.

Attributes	SMFL	SMT	SMS	SML	SMB	SMF
<u>Color attributes</u>						
L*	66.53±1.31 ^{AB}	63.23±1.93 ^{BC}	69.47±0.81 ^A	58.15±3.10 ^C	45.83±1.55 ^D	61.79±1.58 ^{BC}
a*	-15.74±0.32 ^A	-6.68±0.17 ^C	-1.12±0.03 ^D	-8.57±0.45 ^B	10.96±0.10	-7.30±0.34 ^C
b*	49.15±1.15 ^A	27.99±0.25 ^C	33.02±0.28 ^B	21.56±2.96 ^D	26.43±0.69 ^C	26.44±1.19 ^C
<u>% Proximate composition</u>						
Moisture	7.41±0.04 ^A	3.44±0.02 ^F	5.32±0.01 ^C	6.32±0.04 ^B	4.49±0.01 ^E	4.94±0.02 ^D
Ash	4.93±0.03 ^{BC}	4.7±0.23 ^C	7.84±0.07 ^A	4.65±0.19 ^C	5.19±0.17 ^B	4.81±0.05 ^C
Crude fat	3.10±0.24 ^{BC}	2.80±0.09 ^{CD}	6.12±0.44 ^A	3.60±0.14 ^B	0.34±0.17 ^E	2.62±0.09 ^D
Crude protein	14.61±2.16 ^B	3.75±0.72 ^C	14.69±1.51 ^B	21.16±0.83 ^A	2.54±0.13 ^C	10.38±0.29 ^B
Carbohydrate	69.80±2.34 ^C	85.14±0.92 ^A	65.77±1.81 ^C	64.08±1.04 ^C	87.27±0.33 ^A	77.17±0.39 ^B
Cellulose	11.35±2.38 ^B	10.62±0.09 ^B	26.18±2.01 ^A	15.75±1.63 ^B	27.15±0.13 ^A	29.65±0.01 ^A
Hemicellulose	1.12±0.02 ^C	0.85±0.06 ^C	3.07±1.13 ^{BC}	6.56±1.52 ^{AB}	4.83±0.32 ^{ABC}	8.86±1.21 ^A
Lignin	2.14±0.52 ^D	1.05±0.06 ^D	13.37±0.08 ^{BC}	16.46±1.30 ^B	40.60±0.03 ^A	10.28±0.92 ^C
<u>% Weight loss obtained by thermogravimetric analysis</u>						
Stage 1	11.21	10.97	10.26	7.36	10.16	11.52
Stage 2	52.76	53.62	43.74	58.86	46.02	54.28
Stage 3	10.61	10.95	13.82	11.92	16.53	9.14
RM at 700°C	25.42	24.46	32.18	21.86	27.29	25.06
<u>Glass transition temperatures (T_g) obtained by differential scanning calorimetry</u>						
T _{g on} (°C)	19.96	26.74	31.60	35.50	42.66	24.93
T _{g mid} (°C)	35.92	42.09	51.39	54.39	62.18	42.47
T _{g end} (°C)	52.89	58.63	72.24	77.05	84.15	61.01

Note: The values are presented as average value±standard deviation. All the chemical composition is based on dry matter. Different uppercase letters (A–F) in a row indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective attributes among different morphological parts of sugar maple.

RM: Residual mass; SMB: Sugar maple bark; SMF: Sugar maple fruit; SMFL: Sugar maple flower; SML: Sugar maple leaf; SMS: Sugar maple seed; SMT: Sugar maple twig.

5.4.1.2. FTIR characterization

ATR-FTIR analysis based on the sample's absorption of infrared light at various wavelengths is used to assess the molecular composition and structure of the sample. It can simultaneously collect high-resolution data over a wide spectral range within a short duration (Guo et al., 2018). FTIR spectra of SMFL, SMT, SML, SMF, SMS, and SMB are presented in Figure 5.1. In all the samples, bands from 3600–3000 cm^{-1} are associated with the stretching vibrations of O–H and N–H in water, carbohydrates, phenols, and carotenoids (Chou et al., 2020; Hssaini et al., 2022; Seregelyj et al., 2019; Thummajitsakul et al., 2020). Bands that ranged from 3000–2800 cm^{-1} are assigned to the stretching vibrations of C–H in lipids, carbohydrates, and polysaccharides (Zhang et al., 2020). For SMB, SMS, SMF, and SMFL, a band at 2359 cm^{-1} indicates the presence of unsaturated C=C vibrations (Babatunde & Umoru, 2022). The peaks at 1744–1715 cm^{-1} were produced by C=O band stretching and indicate the presence of ester and carboxylic acid (Seregelyj et al., 2019). Peaks at 1652–1606 cm^{-1} are likely due to the strong COO–asymmetric stretching

indicating the presence of carbohydrates; stretching vibrations of O–H, C–O–C, and C=O, N–H bending vibrations are indicating the probable presence of protein, amino acid, ether, and flavonoids (Alara et al., 2018; Guo, Ai, & Cui, 2018). C–N stretching and N–H bending from amide II group were seen to happen in the 1537–1513 cm^{-1} range (Bunaciu et al., 2014). Given the exposition of protein, of which the secondary structure in FTIR spectra is mostly reflected by the coexistence of amide I (1700–1600 cm^{-1}) and amide II (1575–1480 cm^{-1}) bands (Bunaciu, Fleschin, & Aboul-Enein, 2014), it is evident that all the studied parts of SM contain protein. Different peaks present in the region of 1446–1026 cm^{-1} are attributed to the stretching vibrations of C–O and O–H; bending vibrations of C–H, N–H and primary or secondary O–H; and bending of carboxylic acid which are the indications of the presence of phenols, tannins, flavonoids, carbohydrate, starch, and ester (Alara, Abdurahman, & Ukaegbu, 2018; Chou et al., 2020).

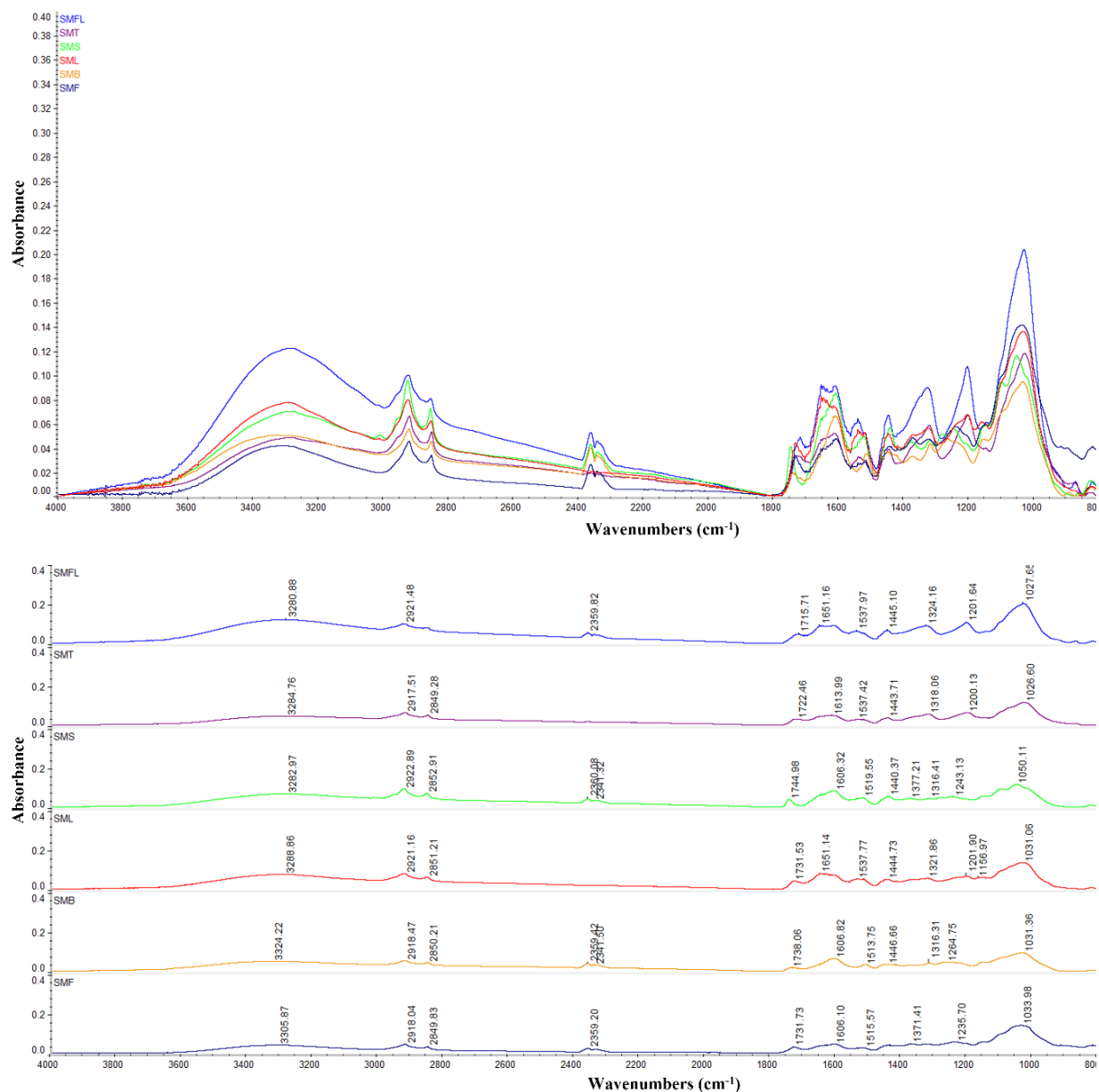


Figure 5.1: FTIR spectra of SMFL, SMF, SMB, SML, SMT, and SMS powders. *SMB: Sugar maple bark; SMF: Sugar maple fruit; SMFL: Sugar maple flower; SML: Sugar maple leaf; SMS: Sugar maple seed; SMT: Sugar maple twig.*

5.4.1.3. Proximate composition

Estimation of proximate composition is significant to assess the quality of raw material as it gives an overview of the quantity of macromolecules present in a sample (Bello et al., 2023). Data associated with the proximate composition of freeze-dried sugar maple's morphological parts (SMFL, SMS, SMF, SMT, SML, and SMB) are given in Table 5.1. The moisture contents of all freeze-dried powders were in the range of 4.49 ± 0.01 to $7.67 \pm 0.04\%$ which is desirable, as low

moisture content increases the product's shelf life by providing storage stability (Bhatta et al., 2018). However, differences in moisture levels in varying morphological parts of SM are also evident from the FTIR spectra in the region of 3600–3000 cm^{-1} and $\sim 1600 \text{ cm}^{-1}$ (Figure 5.1). Drying can disrupt the cellular matrix of the material which in turn favors the phenolic compounds' extraction efficiency. For instance, microstructural analysis of Yeasmen and Orsat (2023) showed the minimal significant differences ($p < 0.05$) in the fractal dimension values of SML subjected to freeze-drying followed by maceration and without extraction (freeze-dried only). The removal of moisture tends to increase the concentration of nutrients and the ash content. From Table 5.1, it can be seen that SMS followed by SMB have the highest ash contents, therefore, they could be a good source of inorganic nutrients i.e., minerals (Bhatta et al., 2018). However, their high ash content can be correlated with the higher fiber compositions where ash (e.g., minerals) may be bound to the fibers (Zhao et al., 2022). TGA analysis (discussed in section 5.4.4.1.) also confirmed the same trend of ash depositions in different sugar maple parts by the residual mass obtained at 700°C. In terms of fat, SMS (6.12%±0.44%) followed by SML (3.60%±0.14%) gave the higher fat contents while SMFL was third with a fat content of 3.10%±0.24%. These results show a similar trend with the study of Manju et al. (2021), where 13.63%±0.32%, 3.95%±0.12%, and 2.10%±0.03% fat contents were reported for moringa seed, leaf, and flower, respectively. On the other hand, SMB has the lowest fat content of 0.34%±0.17% which is in agreement with the study of Bhatta et al. (2018) where 0.43%±0.12% fat was reported in SMB extracts. Protein content was determined in SML as 21.16%±0.83% which is 8.3-folds, 5.6-folds, 2.0-folds, 1.5-folds, and 1.4-folds higher than in SMB, SMT, SMF, SMFL, and SMS, respectively. In addition, by comparing with the commercial plant protein sources like soybean (~31%) and pea (21-33%), it can be said that sugar maple leaves have a great potential of becoming a promising and sustainable source of protein which can support the current transition of the human diet from animal-based to plant-based (Shrestha et al., 2023; Yu et al., 2023). Carbohydrate, an essential part of human diet, was the most abundant macronutrient in all the studied samples. Comparing six aerial parts of sugar maple, carbohydrates were significantly higher ($p < 0.05$) in SMB (87.27%±0.33%) and SMT (85.14%±0.92%) than in the others. As expected, SMB contains higher cellulose (27.15%±0.13%) and lignin (40.60%±0.03%) content. Lignin, which is responsible for providing mechanical support, accounts for about 40% of the organic carbon in the biosphere (Saltveit, 2018). According to the proximate composition, different parts of sugar maple could be used as purpose-based food

ingredients. For instance, SML can be utilized as a promising source for protein extraction, while SMB was determined to have high carbohydrate content, and SMS showed higher ash (inorganic nutrients) and fat contents.

5.4.4. Thermal characterizations

5.4.4.1. Thermal analysis by TGA

TGA was performed to assess the thermal stability of SM tree parts (bark, leaf, twig, seed, fruit, and flower) and the results are depicted in Figure 5.2A. Moreover, the thermal decomposition temperatures of the analyzed samples were determined from the first derivate function of the TGA curve (Figure 5.2B). As can be seen from Figure 5.2, all the studied SM samples showed three stages of thermal decomposition. Stage 1 was observed in the temperature range of 25 to 200°C, which is attributed to the moisture evaporation and partial loss of some phenolic compounds (e.g., epicatechin, resveratrol, myricetin) (Kala et al., 2016; Kumar et al., 2023; Liazid et al., 2007). The overall mass loss during the first stage was <11.52% (Table 5.1). Moreover, it can be said that up to the temperature of 200°C, dried SML followed by SMB had considerable thermal stability while the opposite condition was seen in case of SMF. The major mass loss up to 58.86% (Table 5.1) took place in stage 2 (200 to 400°C), which was associated with the loss of cellulose, hemicellulose, some parts of lignin, pectin, and other phenolic compounds (e.g., catechin, kaempferol, epicatechin, myricetin, quercetin, gallic acid, ferulic acid, sinapic acid, caffeic acid) (Ahmad et al., 2021; Jeyakumar et al., 2020; Liazid et al., 2007). Further, stage 3 (400 to 700°C) indicated the mass loss of complex compounds (e.g., lignin) and formation of char (Gullon et al., 2018). Char residues that remained at 700°C correspond to the non-pyrolyzable compounds (Gullon et al., 2018) and represented respectively 25.42%, 24.46%, 32.18%, 21.86%, 27.29% and 25.06% of SMFL, SMT, SMS, SML, SMB, and SMF.

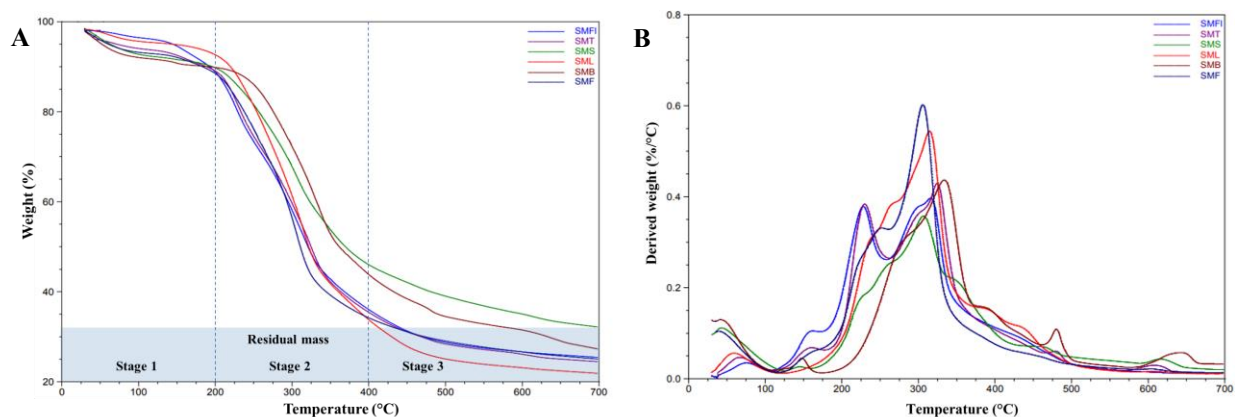


Figure 5.2: TGA (A) and DTG (B) curves of SMFL, SMF, SMB, SML, SMT, and SMS powders at a heating rate of 5°C/min. *DTG: Derivative thermogravimetric analysis; SMB: Sugar maple bark; SMF: Sugar maple fruit; SMFL: Sugar maple flower; SML: Sugar maple leaf; SMS: Sugar maple seed; SMT: Sugar maple twig; TGA: Thermogravimetric analysis.*

5.4.4.2. Tg analysis by DSC

Glass transition temperature (Tg) indicates the zone where the hard, solid, or amorphous system changes the properties from a glassy state to a soft, rubbery state (Zhang et al., 2020). It is one of the important properties associated with a powder's shelf stability. In powdered samples, when Tg is reached, a sequence of deleterious events such as stickiness, caking, and agglomeration might occur, and this can be explained from the point of molecular mobility. For example, when powder is stored above Tg, the mobility of molecules is increased due to decrease of powder's viscosity, consequently caking and stickiness of powders happen. On the other hand, stability of the powder can be ensured at temperatures at or below Tg (Masilungan-Manuel, 2017; Zhang et al., 2020). Thus, it is very crucial to know the Tg of a powder sample to prevent these undesirable phenomena. DSC thermograms of SMB, SMFL, SMS, SMT, SML, and SMF powders are presented in Figure 5.3, whereas the glass transitions of all samples are presented in Table 5.1 with parameters indicating their onset, midpoint, and endpoint, so the width of the transitions is clear. In case of sugar maple aerial parts, the onset Tg ranged from 19.96 to 42.66°C, the midpoint Tg from 35.92 to 62.18°C, and the endpoint Tg from 52.89 to 84.15°C. Amongst all, SMB had the highest Tg while SMFL showed the lowest Tg. This can be explained from the moisture and macronutrients present in SMB as Tg is highly dependent on the composition of the studied samples (Mahato et al., 2019). Moisture is negatively correlated with Tg and acts as a plasticizer (Manju et al., 2021; Staniszewska et al., 2021), whereas content of lignocellulosic fiber has a

positive impact on Tg (Zhao et al., 2021). In addition, Tg is highly influenced by carbohydrate molecules counting on its great affinity to make hydrogen bond with the available moisture, consequently increasing viscosity (Mahato, Zhu, & Sun, 2019). To this end, among all the morphological parts, significantly ($p < 0.05$) lower moisture level and higher carbohydrate as well as fiber content of the SMB sample experienced the highest Tg.

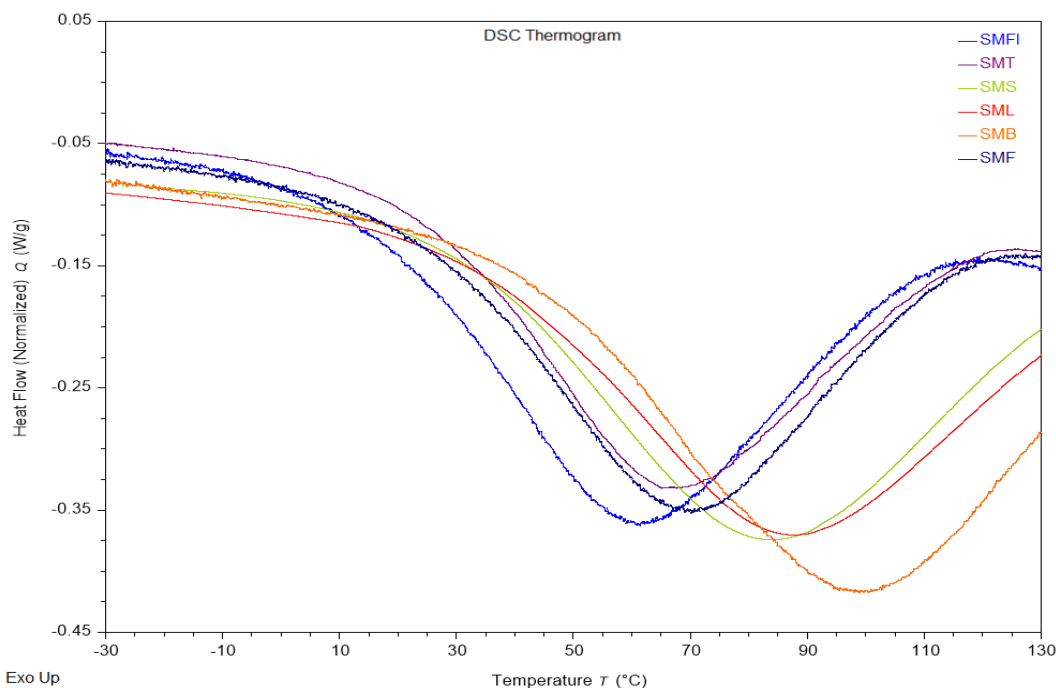


Figure 5.3: DSC thermograms of SMFL, SMF, SMB, SML, SMT, and SMS powders. *DSC: Differential scanning calorimetry; Derivative thermogravimetric analysis; SMB: Sugar maple bark; SMF: Sugar maple fruit; SMFL: Sugar maple flower; SML: Sugar maple leaf; SMS: Sugar maple seed; SMT: Sugar maple twig.*

5.4.5. Phytochemical composition

Phenolic compounds represent a class of secondary plant metabolites that include several thousand compounds of different structures (from simple phenolic acids to complex tannins) (Feduraev et al., 2019). Biosynthesis of phenolic compounds in plants is a dynamic process. Briefly, phenolic compounds are synthesized via two metabolic pathways, namely the shikimic acid pathway, where phenylpropanoids are formed, and the malonic acid pathway. The shikimate pathway converts simple carbohydrate precursors into aromatic amino acids namely phenylalanine (PHE), tryptophan, and tyrosine. PHE is the precursor for phenylpropanoid synthesis, which starts with the phenolic acids formation and subsequently ends with the flavonoids in most of the

vascular plants (Zuiter, 2014). Phenolic acids are hydroxylated derivatives of cinnamic and benzoic acids. Hydroxycinnamic acids (C6–C3) result from the deamination of PHE by the phenylalanine ammonia lyase (PAL) enzyme to produce trans-cinnamic acid which, through hydroxylation, yields coumaric acid with the action of cinnamate 4-hydroxylase enzyme. Thus, the enzyme PAL presents itself as an important contributor behind the synthesis of phenolic compounds, whose expression is controlled by the plant's developmental stage, organ type, and a wide variety of environmental factors including microbial elicitors and UV light (Santos-Buelga et al., 2023). However, coumaric acid leads to the formation of the rest of hydroxycinnamic acids such as caffeic acid, ferulic acid, and sinapic acid, through a series of hydroxylation and methylation reactions. On the other hand, hydroxybenzoic acids (C6–C1) can occur from the intermediates of shikimate or phenylpropanoid pathways and are involved in the synthesis of gallic acid and its derived tannins. Furthermore, a series of reactions reduces the carboxylic group of coumaric, ferulic, and sinapic acid into their corresponding alcohols i.e., coumaryl, conferyl, and sinapyl alcohol, respectively. These three alcohols are known as monolignols, precursors of lignans and lignins (Zuiter, 2014). Condensation of one molecule of coumaroyl-CoA and three molecules malonyl-CoA (derived from citrate produced by the Krebs cycle) leads to the formation of naringenin chalcone and subsequently to naringenin, with the action of chalcone synthase and chalcone isomerase enzymes, respectively. Starting from naringenin, with the action of several enzymes such as flavone synthase, isoflavone synthase, flavonol synthase, anthocyanidin synthase, anthocyanidin reductase, etc., the synthesis of flavonoids' subsequent classes, namely flavonols (quercetin, rutin, etc.), flavanols (catechin, epicatechin, etc.), flavones (luteolin, etc.), anthocyanins (malvidin, pelargonidin, etc.), and so forth, usually takes place (Del Mondo et al., 2022). Now the accumulation and concentration of phenolic compounds vary with numerous factors including the plant itself (type of plant, cultivar, phase of its ontogenesis, parts of plant namely leaf, fruit, stem, twig, root, flower, seed, etc.), the function of these compounds in plants' lifecycle, the agricultural practices (cultivation techniques, cultivation area, etc.) as well as the edaphoclimatic conditions being boosted by abiotic (UV radiation, temperature, drought, rainfall, ozone, sun hour, soil condition, etc.) and biotic (fungal attack, bacterial attack, etc.) stresses (Feduraev et al., 2019; Santos-Buelga, González-Paramás, & González-Manzano, 2023). This study aims to investigate the biosynthesis of phenolic compounds in various plant organs of sugar maple.

Results on the total phenolic contents and contents of various classes of (poly)phenols in macerated and microwave-assisted extracts of different morphological parts of sugar maple are presented in Table 5.2, which indicate that plant parts as well as the extraction techniques greatly influence the (poly)phenolic profile of the extracts. In general, MAE seems a good candidate for “green extraction” as it provided comparable and to some extent the best extraction yields, while comparing with maceration. This can be explained by a high mass transfer due to the volumetric heating capacity of MAE (Yeasmen & Orsat, 2021). Our results demonstrate the richness in phenolic compounds (35.77–135.55 mg GAE/g DM) of different sugar maple tree parts. To compare between the extraction methods, MAE yielded higher contents of total phenol (TP), total flavonoid (TF), total extractable tannin (TET), and condensed tannin (CT) in SMB, SMF, and SML while maceration provided extracts of SMS richer in TPC, TFC, and CTC. Furthermore, it was seen that microwave-assisted extracts of all plant parts showed higher contents of phenolic acids (gallic acid, ferulic acid, sinapic acid, ellagic acid, caffeic acid, and coumaric acid). This can be attributed to the volumetric heating effect produced by microwave energy because of the interaction between microwave energy and solvent molecules. As a result, abrupt increase in temperature and internal pressure takes place inside the plant material, therefore rupturing cell walls which leads to a faster release of cell compounds into the solvent (da Rosa et al., 2019). Although phenolic acids can be found in free forms, they are most frequently linked to other compounds such as carbohydrates, protein, organic acids, flavonoids, as well as bounded to plant cell wall polymers (cellulose, lignin, cutin). Thermal treatment has been seen to release these bound phenolic acids, thus enriching their availability in the extracts (Santos-Buelga, González-Paramás, & González-Manzano, 2023). Bryngelsson et al. (2002) found the increasing content of coumaric and ferulic acid in oats due to an autoclaving process. Moreover, thermal treatment could liberate oligomers from their native polymers (repeating subunits of the oligomers). For example, hydrolysable tannins (gallotannins, ellagitannins), which are the derivatives of gallic acid and ellagic acid, were seen to depolymerize due to the heat treatment in *Acer truncatum* leaves and flowers (Yang et al., 2018b). Similar rising trends of gallic acid and ellagic acid in SMFL and SMT were observed in the present study due to the thermal effect (MAE) while comparing to maceration. Note that SMFL and SMT were dominated by TET which are in accordance with the study of Lachowicz et al. (2020). Apart from the hydrolysable tannin, based on the composition and structures, there is another group of tannins called condensed tannin (CTC). Among all, SMS,

SMB, and SML were seen to contain significantly higher CTC which is justified since condensed tannins protect plant seeds and leaves from animals and herbivores by providing an undesirable astringency flavor (Zuiter, 2014). Moreover, condensed tannins exclusively contain epicatechin units which were also found in higher contents in the above-mentioned plant parts (Table 5.2).

Diets rich in phenolic compounds have been catching attention, increasingly day by day, counting on their number of health beneficial properties such as antioxidant, antihypertensive, antihyperglycemic, anti-inflammatory, and so forth (Ribeiro et al., 2023). Studies on the dietary intake of phenolic compounds indicate that phenolic acids contributed around one third of the dietary intake of total phenols, while flavonoids accounted for two thirds (Santos-Buelga, González-Paramás, & González-Manzano, 2023). Scalbert and Williamson (2000) showed that fruits (fresh, juice, concentrate), beverages (wine, tea, coffee, chocolate), and to a lesser extent vegetables and cereals are the dietary sources of phenolic compounds most consumed around the world. Since the world population continues to be increasing, it is high time to look for alternatives that could fulfill the increasing demand for agricultural commodities. Talking about the bright sides of phenolic compounds, forestry biomass can be an effective alternative to fruits and vegetables. Forestry biomass including residues (leaves, barks, stumps, stems, and branches) and processing waste (logs and sawdust), account for around 4.6 billion tons annually of which 60% goes to energy generation, 20% to industrial ‘round wood’ and the remaining 20% being primary production losses that remain in-field to decay (UNEP, 2013). Based on the above analyses, it can be said that different morphological parts of sugar maple, representing 3% of Canadian’s forests, contain a considerable amount of phenolics including flavonoids, phenolic acids, and tannins. From a sustainable and resilient viewpoint, maple biomass, namely leaves, can be a promising source of phenolic compounds as several studies showed that leaves contained higher phenolic fraction than the respective tree’s fruits (Bujor et al., 2018; Kubola & Siriamornpun, 2008; M’Rabet et al., 2017). In this study we also found higher TPC (88.39–102.04 mg GAE/g DM), TFC (13.92–14.47 mg CTE/g DM), TET (73.55–86.39 mg GAE/g DM), CTC (31.03–67.46 mg CTE/g DM) in SML in comparison to SMF (TPC: 37.09–42.19 mg GAE/g DM; TFC: 11.83–17.89 mg CTE/g DM; TET: 32.21–39.37 mg GAE/g DM; CTC: 29.19–44.01 mg CTE/g DM).

Table 5.2: Phytochemical compositions, antioxidant, and antimicrobial activities of macerated and microwave-assisted extracts of sugar maple flower, leaf, twig, fruit, bark, and seed.

Attributes	SMFL		SMT		SMS		SML		SMB		SMF	
	ME	MAE	ME	MAE	ME	MAE	ME	MAE	ME	MAE	ME	MAE
TPC	132.89±1.77 ^{Ba}	132.34±2.17 ^{Ca}	129.82±0.06 ^{Ba}	128.36±1.38 ^{CHa}	136.55±0.09 ^{Aa}	127.24±2.29 ^{Fb}	88.39±0.12 ^{Cb}	102.04±1.50 ^{Ja}	35.77±0.27 ^{Db}	48.06±1.36 ^{Ja}	37.09±0.00 ^{Db}	42.19±0.19 ^{Ka}
TFC	10.86±0.02 ^{Da}	10.78±0.07 ^{Ka}	10.51±0.23 ^{Da}	10.55±0.14 ^{Ka}	47.33±0.77 ^{Aa}	34.32±2.08 ^{Gb}	13.92±0.14 ^{Ca}	14.47±0.24 ^{Ja}	21.91±0.02 ^{Bb}	28.12±0.14 ^{Ha}	11.83±0.27 ^{Db}	17.89±0.52 ^{Ja}
CTC	13.12±0.40 ^{Da}	5.33±0.04 ^{Kb}	7.35±0.23 ^{Fb}	12.06±0.33 ^{Ja}	126.98±0.16 ^{Aa}	127.53±0.77 ^{Ga}	31.03±1.05 ^{Cb}	67.46±1.31 ^{Ha}	39.25±2.90 ^{Bb}	46.73±1.96 ^{Ja}	29.19±0.93 ^{Cb}	44.01±1.62 ^{Ja}
NPP	24.74±0.58 ^{Bb}	26.96±0.42 ^{Ha}	19.20±0.14 ^{Cb}	27.35±0.68 ^{Ha}	43.03±0.56 ^{Aa}	31.54±0.49 ^{Gb}	14.71±0.12 ^{Db}	15.65±0.34 ^{Ja}	0.56±0.07 ^{Fb}	4.12±0.13 ^{Ja}	4.85±0.10 ^{Ea}	2.82±0.07 ^{Kb}
TET	108.18±0.99 ^{Ba}	105.38±1.77 ^{Ca}	110.35±0.53 ^{Aa}	101.00±1.37 ^{Fb}	53.67±0.59 ^{Db}	95.70±1.81 ^{Ja}	73.55±0.30 ^{Cb}	86.39±1.23 ^{Ja}	35.62±0.74 ^{Eb}	43.94±1.31 ^{Ka}	32.21±0.13 ^{Fb}	39.37±0.24 ^{La}
Flavonoids (µg/g DM)												
Catechin	401.80±4.10 ^{Ea}	361.18±1.18 ^{Lb}	ND	754.28±5.21 ^{Ka}	6729.76±29.62 ^{Ab}	6897.43±22.74 ^{Ga}	683.93±2.94 ^{Db}	846.79±1.71 ^{Ja}	1483.54±9.04 ^{Bb}	1707.97±10.77 ^{Ha}	776.19±30.93 ^{Cb}	914.86±3.33 ^{Ja}
Epicatechin	ND	ND	122.84±2.49 ^{Fb}	221.67±3.93 ^{Ka}	7050.64±36.88 ^{Ab}	8135.51±44.44 ^{Ga}	1145.62±7.08 ^{Ca}	1010.91±10.59 ^b	1380.72±10.26 ^{Bb}	1619.50±11.17 ^{Ha}	213.10±1.17 ^{Db}	293.89±4.88 ^{Ja}
Rutin	5223.02±81.55 ^{Bb}	6004.93±17.52 ^{Ga}	ND	ND	6998.72±945.28 ^{Aa}	3418.12±839.25 ^{Hb}	ND	ND	ND	ND	1818.08±2.63 ^{Cb}	1829.25±2.26 ^{Ja}
Quercetin	5366.66±24.09 ^{Aa}	5168.65±22.72 ^{Cb}	ND	105.33±5.22 ^{Ha}	ND	ND	ND	ND	ND	ND	186.02±1.86 ^{Ba}	137.05±9.24 ^{Fb}
Phenolic acids (µg/g DM)												
Gallic acid	3831.94±4.74 ^{Bb}	4928.51±6.96 ^{Ga}	2428.87±6.36 ^{Cb}	4369.92±20.5 ^{Ha}	7780.09±28.92 ^{Aa}	1.03±0.03 ^{Kb}	51.37±0.41 ^{Db}	1948.18±1.84 ^{Ja}	28.06±0.34 ^{Db}	73.23±14.42 ^{Ja}	19.59±0.83 ^{Db}	46.56±0.74 ^{Ja}
Caffeic acid	ND	ND	ND	ND	124.68±8.74 ^{Ba}	147.62±23.67 ^{Ha}	757.88±2.07 ^{Aa}	759.45±1.40 ^{Ga}	ND	ND	4.27±5.88 ^{Ca}	4.86±2.98 ^{Ja}
Ellagic acid	27180.54±2322.16 ^{Bb}	41209.58±115.43 ^{Ga}	34767.90±23.31 ^{Ab}	37378.05±202.55 ^{Ha}	ND	ND	9552.15±24.35 ^{Cb}	11334.26±21.30 ^{Ja}	236.03±6.33 ^{Da}	233.59±1.20 ^{Ka}	ND	1490.28±19.25 ^{Ja}
Sinapic acid	ND	1484.99±33.68 ^{Ga}	ND	ND	ND	ND	ND	ND	ND	ND	318.34±24.09 ^{Aa}	419.13±112.36 ^{Ha}
Ferulic acid	36595.19±4230.07 ^{Aa}	9679.68±44.07 ^{Ib}	13573.97±514.42 ^{Bb}	17357.08±233.54 ^{Ha}	42.18±14.15 ^{Ca}	37.89±1.60 ^{Ka}	17766.79±62.25 ^{Bb}	24033.29±114.27 ^{Ga}	16.03±3.66 ^{Ca}	26.60±7.46 ^{Ka}	4178.02±50.30 ^{Ca}	3892.08±373.07 ^{Ja}
Coumaric acid	ND	28757.62±31.65 ^{Ha}	ND	3432.88±81.34 ^{Ga}	ND	ND	ND	12202.10±59.13 ^{Ja}	ND	ND	ND	155.25±81.62 ^{Ja}
In vitro antioxidant activities (µmol TE/g DM)												
FRAP	103.35±0.17 ^{Aa}	103.44±0.61 ^{Ga}	100.08±0.68 ^{Ba}	100.14±0.45 ^{Ja}	103.41±0.58 ^{Aa}	102.09±0.72 ^{CHa}	102.26±0.67 ^{Aa}	101.42±0.52 ^{HJa}	99.02±0.51 ^{Ba}	99.76±0.73 ^{Ja}	98.73±1.02 ^{Bb}	101.46±0.58 ^{HJa}
CUPRAC	91.37±0.5 ^{BCb}	98.49±0.26 ^{Ga}	88.82±0.03 ^{Db}	95.12±0.47 ^{Ga}	92.96±0.07 ^{Ab}	96.44±0.43 ^{Ga}	90.82±0.01 ^{Cb}	98.32±0.6 ^{Ga}	91.14±0.68 ^{BCa}	95.67±2.23 ^{Ga}	91.82±0.15 ^{Bb}	96.90±0.84 ^{Ga}
DPPH	88.21±0.35 ^{Aa}	88.52±0.04 ^{Ga}	85.97±0.23 ^{Ca}	85.52±0.1 ^{Ja}	87.17±0.27 ^{Ba}	85.48±0.4 ^{Ib}	86.11±0.24 ^{Ca}	85.72±0.27 ^{Ja}	86.03±0.54 ^{Ca}	85.61±0.1 ^{Ja}	87.37±0.17 ^{ABa}	87.07±0.04 ^{Ha}
Antimicrobial activities (zone of inhibition, mm)												
Antibacterial	7.55±0.29 ^{Ab}	11±0.12 ^{Ca}	8.95±0.87 ^{Aa}	9.75±0.29 ^{GHa}	6.80±0.35 ^{Ab}	8.3±0.12 ^{Ja}	8.15±0.64 ^{Aa}	9.25±0.17 ^{HJa}	ND	ND	8.0±0.23 ^{Aa}	8.0±0.58 ^{Ja}
Antifungal	7.50±0.46 ^{Aa}	8.70±0.23 ^{Ga}	7.60±0.58 ^{Aa}	9.25±0.29 ^{Ga}	ND	ND	7.05±0.29 ^{ABa}	6.15±0.06 ^{Ha}	ND	ND	5.85±0.06 ^{Ba}	6.20±0.12 ^{Ha}

Note: The values are presented as average value±standard deviation. Different uppercase letters (A–F) and (G–L) in a row indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective attributes among different morphological parts of sugar maple for maceration and microwave-assisted extraction respectively. Lowercase letters (a–b) in a row indicate significant differences ($p < 0.05$) between the extraction methods for each morphological part.

CTC: Condensed tannin content (mg CTE/g DM); NPP: Non-precipitable phenols (mg GAE/g DM); SMB: Sugar maple bark; SMF: Sugar maple fruit; SMFL: Sugar maple flower; SML: Sugar maple leaf; SMS: Sugar maple seed; SMT: Sugar maple twig; TET: Total extractable tannin (mg GAE/g DM); TPC: Total phenolic content (mg GAE/g DM); TFC: Total flavonoid content (mg CTE/g DM).

5.4.6. Potential of sugar maple's aerial parts as an antioxidant additive and correlation analyses between phenolic content and antioxidant attributes

Antioxidant capacity of each different parts of sugar maple tree is expressed as number of equivalents of trolox (Table 5.2). The assays are based on the transfer of one electron including the CUPRAC test and the FRAP test, as well as the transfer of both a hydrogen atom and an electron, including the DPPH test. Although all the morphological parts showed antioxidant potentialities ranging from 85.48 ± 0.4 to 103.44 ± 0.61 $\mu\text{mol TE/g DM}$, however, SMS and SMFL (extracted by both extraction techniques) had the maximum antioxidant capacities. These differences might be due to the variation in sampling parts, phytochemical compositions as well as the extraction and measurement techniques. Since phenolics are known as natural antioxidants with an array of compounds, we established the correlation between the phenolic constituents (TPC, TFC, TET, CTC, and individual phenolic compounds) of SM's extracts and their scavenging as well as reducing activities, with a regression analysis using Pearson correlation coefficients (data not shown). In general, considerable correlations ($>50\%$) were found between antioxidant components (TPC, gallic acid, sinapic acid, coumaric acid) and antioxidant capacities. On the other hand, rutin and quercetin individually were mostly correlated ($>70\%$) with the antioxidant assays (FRAP and DPPH).

5.4.7. Potential of sugar maple's aerial parts as a food preservative

Macerated and microwave-assisted extracts of SM were tested for their antimicrobial activities where the measurements of inhibition zones against selected microorganisms are given in Table 5.2. As expected, control treatment (EtOH) had no inhibitory effect on any of the tested microorganisms. Except for SMB, almost all the morphological extracts showed some inhibitory actions against bacteria and fungi where significantly higher antimicrobial activities were given by SMFL and SMT extracts with the diameters of the zone of inhibition ranging between 7.55 and 11 mm. The antimicrobial activities of plant extracts may reside in a variety of components namely flavonoids, phenolic acids, tannins, stilbenes, lignan, and so on (Deng et al., 2014). They exert antimicrobial activity by several mechanisms, such as by inhibiting the nucleic acid synthesis, damaging the cytoplasmic membrane, inactivating cells by reducing the fluidity of outer and inner layers, and so forth (Ribeiro et al., 2023). As per Table 5.2, since extracts of all the morphological parts contain significant phenolic fractions (TPC, TFC, or tannins), it is now obvious that almost all the extracts showed antimicrobial activities. To explain insightfully, the presence of

hydrolysable tannin, gallic acid, caffeic acid, coumaric acid, and quercetin have been reported to have effectiveness against bacteria and fungi, when applied to various products (Deng et al., 2014; Kaczmarek-Szczepanska et al., 2023). Significant presence ($p < 0.05$) of these compounds in SMFL and SMT extracts can also be correlated with their antimicrobial actions. As per the report of Delisle-Houde and Tweddell (2020), SMB extracts appeared less toxic against bacteria, namely *Pseudomonas cichorii* and *Xanthomonas campestris* pv. *vitians* while SML seem to have comparable toxicity against those species. Furthermore, irrespective of the tested microorganisms, there was no significant difference in antimicrobial activities of the extracts obtained by MAE and ME except for SMFL and SMS (antibacterial). In case of SMFL and SMS extracts, MAE offered significantly higher ($p < 0.05$) extraction efficiency of phenolic compounds in comparison to ME (Table 5.2).

5.5. CONCLUSION

Acer species namely *Acer saccharum* (sugar maple) is economically important for the commercial production of maple syrup. In addition, bark and leaf extracts of sugar maple were reported by several studies as a potential source of phenolic contents with the purpose of their use in pharmaceutical industries. Nevertheless, the possibility of presence of bioactive compounds in other morphological parts of sugar maple, with the exploration of their use as food ingredients, had yet to be explored. Thus, the detailed study performed in the present report was aimed at exploring the potential use of different sugar maple parts as food additives in the food industry, exploiting their antioxidant and antimicrobial activities. Findings of this research demonstrated that aerial parts (flower, fruit, seed, bark, twig, and leaf) of sugar maple can supply compounds with bioactive potential, that can act as preservative and antioxidant additives. When comparing the performance of MAE and maceration, microwave-assisted extraction provided comparable, and to some extent the best results, for the extraction of phenolics. However, using resources that are circularly sustainable might be an effective choice to create a beneficial impact on the agri-food economy. Among all the studied parts of sugar maple biomass, leaves can be chosen as a sustainable source where overexploitation is believed not to be an issue as leaves can be easily grown back by the tree in the subsequent growth season. In addition to providing a phenolic fraction, sugar maple leaves can also be a cheap source of plant protein, thus, can be used as a valuable resource and should not be discarded as simple biomass residues. We anticipate a real biorefinery potential for sugar maple biomass through the integration of the advanced separation

units (microwave-assisted extraction/ultrasound-assisted extraction/pulsed electric field-assisted extraction/cold plasma-assisted extraction, etc.), in terms of either phenolic compounds and/or protein into the existing sugar maple industry.

Acknowledgements

The authors are grateful to Professor Michael Ngadi (Department of Bioresource Engineering, McGill University) for allowing the use of the differential scanning calorimeter (TA, DSC250, USA) and Professor Saji George (Department of Food Science and Agricultural Chemistry, McGill University) for allowing the use of the plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA) at no cost. Authors are also thankful to Mr. Yvan Gariepy (Professional associate, Department of Bioresource Engineering, McGill University) and Mohammed Antar (PhD Candidate, Department of Plant Science, McGill University) for their technical assistance.

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CONNECTING TEXT TO CHAPTER 6

Chapter 6 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2024). Microencapsulation of ultrasound-assisted phenolic extracts of sugar maple leaves: Characterization, *in vitro* gastrointestinal digestion, and storage stability. *Food Research International*, 182, 114133. <https://doi.org/10.1016/j.foodres.2024.114133>

Based on the promising results obtained in the previous chapters, sugar maple leaves offered themselves as a sustainable and renewable source of phenolic compounds and a natural antioxidant. However, the chemical structure of phenolic compounds may reduce their effectiveness against oxidation when added to food products because of their unsaturated bonds. To address this bottleneck, Chapter 6 focused on the encapsulation of phenol rich sugar maple leaf extracts followed by their physicochemical, thermal, functional, structural, and bioaccessibility characterizations along with the storage stability of encapsulated microparticles.

**CHAPTER 6: MICROENCAPSULATION OF ULTRASOUND-ASSISTED PHENOLIC
EXTRACTS OF SUGAR MAPLE LEAVES: CHARACTERIZATION, *IN VITRO*
GASTROINTESTINAL DIGESTION, AND STORAGE STABILITY**

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6.1. ABSTRACT

Sugar maple leaves (SML), usually considered residue plant biomass and discarded accordingly, contain a considerable amount of phenolic antioxidants. In this study, SML phenolics were extracted employing both advanced (homogenization pretreated ultrasound-assisted extraction) and conventional (maceration) methods followed by their encapsulation by freeze drying and spray drying using a combination of maltodextrin and gum arabic as coating agents. Detailed physicochemical analyses revealed that the encapsulated microparticles had high solubility (> 90%) and encapsulation efficiency (> 95%), acceptable thermal stability with good handling properties. Phenolic compounds were completely released from microparticles during simulated gastric conditions. The microparticles influenced the bioaccessibility of more than 43% of the phenolic fraction in the intestinal phase. The antioxidant capacity of the microparticles was preserved during storage. These findings suggest the effectiveness of the microencapsulation process for producing high quality microparticles of SML phenolic extracts and the possibility of their use in the food, nutraceutical, bio-pharmaceutical sectors.

Keywords:

Acer saccharum leaves, antioxidant, bioactive compounds, microparticles, quality.

6.2. INTRODUCTION

In 2023, the global food antioxidant market size is estimated at USD 1.6 billion and is projected to reach USD 2.2 billion by 2028 with an expectation of compound annual growth rate (CAGR) of 6.2% from 2023 to 2028 (M&M, 2023). Antioxidants, from synthetic or natural sources, and in dry or liquid forms, have been used in different food products (meat and poultry, bakery and confectionery, fats and oil, fish, among others) and packaging materials (GVR, 2022a). Owing to the growing awareness of health, the side effects of synthetic antioxidants, and stricter regulations set by the food safety bodies (FDA, EU, etc.) about manufacturing and usages of synthetic antioxidants, natural antioxidants are becoming increasingly popular over the projected years (Conte et al., 2021; da Rosa et al., 2020; Maqsood, Benjakul, & Shahidi, 2013; Nowak et al., 2016). Compared to other natural antioxidants, plant secondary metabolites namely phenolic compounds (PCs) stand out, because of their capacity for scavenging free radicals, quenching reactive oxygen species, inhibiting oxidative enzymes, chelating metal ions, besides interacting with bio membranes as well (Maqsood, Benjakul, & Shahidi, 2013). The credit behind these possible antioxidant mechanisms of PCs goes to their chemical structure highlighting the number

and position of the hydroxyl groups present in that structure (Maqsood, Benjakul, & Shahidi, 2013; Yeasmen & Orsat, 2023a). However, the same chemical structure of PCs may reduce their effectiveness against oxidation when added to food products because their unsaturated bonds. These unsaturated bonds are susceptible to degradation by either oxidation, condensation, or polymerization when exposed to oxygen, light, heat, enzymatic activity, and pH differences (Mzoughi et al., 2023; Papoutsis et al., 2018; Pashazadeh et al., 2021). It can be hypothesized that microencapsulation can address these bottlenecks by preserving the quality and stability of PCs in the environment to which they are exposed. The encapsulation process consists of trapping compounds of interest (such as phenolic extracts) in a solution of wall/ encapsulating/ coating material, followed by drying (Kalkan et al., 2017). Apart from the controlled release of entrapped compounds, during food processing and pharmaceutical application, encapsulation additionally gives the option of masking the unwanted taste and flavor of the extracts (Saifullah et al., 2023; Saikia, Mahnot, & Mahanta, 2015). It also improves the shelf life of the encapsulants and minimizes their evaporation and reaction with other food ingredients (Saifullah et al., 2023; Ahmadian, Niazmand, & Pourfarzad, 2019). Several factors such as type of coating material, drying methods, solution homogeneity, core-to-coating ratio, extracts type, among others, play on the efficacy of the encapsulation process. Widely used coating materials for encapsulation include maltodextrin, gums, protein conjugates, chitosan, cyclodextrins, and pectin. As of the recent studies, mixture of coating materials such as maltodextrin (MD) and gum arabic (GA) showed higher efficiency during the encapsulation of phenolic extracts (da Silva Júnior et al., 2023; Mzoughi et al., 2023; Papoutsis et al., 2018). MD has the ability to protect encapsulated material from oxidation and light by forming amorphous glassy matrices during the encapsulation process. Due to the higher water solubility of MD, it favors the atomization of liquid during spray drying by lowering the viscosity of feed (Saikia, Mahnot, & Mahanta, 2015). On the other hand, GA is known for its surface-active characteristics as well as protection for reactive compounds (Cilek et al., 2012). On the other hand, spray drying, freeze drying, extrusion, coacervation, co-crystallization, ionic gelation, and emulsion are some of the widely used encapsulation methods (Carreón-Hidalgo et al., 2022; Pashazadeh et al., 2021; Saikia, Mahnot, & Mahanta, 2015). Another variable, namely quality of the phenolic extracts, is depending on the extraction methods employed for the production of phenolics. Several eco-friendly extraction techniques, with or without pretreatment conditions, are being used for the extraction of PCs and among them,

ultrasound-assisted extraction showed comparatively better extraction efficiency (da Rosa et al., 2019; Mzoughi et al., 2023).

To address the escalating demand for phenolic compounds from different sectors (food, nutraceutical, and pharmaceutical industries) while being in line with the United Nations Sustainable Development Goals (SDGs), especially in target 12.2 (to achieve the sustainable management and efficient use of natural bioresources) and 12.5 (to substantially reduce waste generation through prevention, reduction, recycling and reuse by 2030) (UNSDGs, 2015), scientific communities have been working on the valorization of agri-food wastages. To this extent, encapsulation of bioactive compounds and their controlled delivery is one of the approaches that has been studied extensively e.g., microencapsulation of phenolic compounds from cashew apple pomace (Andrade et al., 2023), from tea leaf wastewater (Ravichai & Muangrat, 2019); from cocoa pod husk (Nguyen et al., 2021); from maize waste (Pashazadeh et al., 2021); from plum peels (Mzoughi et al., 2023); from coffee husk (Silva et al., 2024); from ciriguela peel (da Silva Júnior et al., 2023), among others. While wastage from agri-food sectors is getting attention towards their valorization in terms of bioactive compounds' extraction, another big sector of biomass/wastages derived from forest is being overlooked. Forestry biomass includes residues such as stumps, branches, barks, stems, and leaves and processing waste such as logs and sawdust which accounts for around 4.6 billion tons annually of which 60% goes to energy generation, 20% to industrial 'round wood' and the remaining 20% being primary production loss that remains in-field to decay (UNEP, 2013). Forestry biomass used in power plants to produce energy causes huge increase in carbon emissions compared to coal and other fossil fuels. However, these forestry biomasses such as leaves are underutilized source of material as such there has been little activity focusing on a comparatively 'low-carbon' route for their valorization (Tripathi et al., 2019). To this extent, previous studies (Yeasmen & Orsat, 2022; Yeasmen & Orsat, 2023a, 2023b) showed that biomass of *Acer saccharum* (sugar maple) leaves can be utilized as a sustainable source of PCs with excellent antioxidant capacity. Sugar maple is an ecologically and economically important deciduous hardwood species of eastern-North of North America (Geoffroy et al., 2017; St Clair et al., 2008). It grows best in a well-drained loamy soil rich in organic matter, although it can tolerate some sand, rock, or clay in the soil (Geoffroy, Fortin, & Stevanovic, 2017). To the best of existing evidence, there is a research gap in the literature regarding the encapsulation of phenolic extracts of the biomass of sugar maple leaves followed by their functional behavior during simulated

gastrointestinal digestion and storage time. Thus, the aim of this study was (i) to microencapsulate phenol rich sugar maple leaf extracts, obtained via conventional and advanced extraction methods, (ii) to characterize the phenolic microparticles for their physicochemical, thermal, functional, structural, and bioaccessibility, and (iii) to verify the storage stability of the phenolic microparticles.

6.3. MATERIALS AND METHODOLOGY

6.3.1. Chemicals and processing of sample

Chemicals used in the study were of analytical grade and purchased from Fisher Scientific, Nepean, Ontario, Canada; Sigma–Aldrich, St–Louis, MO, USA; and Oxoid, UK. All the standards were purchased from Sigma–Aldrich, St–Louis, MO, USA and the solvents used for analytical chromatography were of HPLC grade.

A. saccharum leaves were harvested in the two consecutive years of 2021 and 2022, from the Morgan Arboretum located in Sainte-Anne-de-Bellevue, Quebec, Canada, 45°25'50.1"N 73°57'8.2"W by following the sampling process of Yeasmen and Orsat (2023b). Immediately after the collection of the samples, they were thoroughly washed under running tap water. Following freeze drying (HARVEST RIGHT, USA), the samples were ground using a milling machine (Retsch GmbH, GM 200, Retsch-Allee 1-5, Haan, Germany). The produced leaf powders (particle size < 0.1 mm) were kept in brown sealed glass bottles at -20°C until further uses/analyses.

6.3.2. Extraction of phenolic compounds from sugar maple leaves (SML)

SML were extracted for PCs in aqueous ethanol (70% v/v) with a solid-to-solvent ratio of 1:30 g/mL using two conditions. Macerated extract of SML was obtained by using a shaker machine (MaxQ 4000, Branstead International, Dubuque, Iowa, USA) for 24 h at a constant stirring speed of 200 rpm and temperature of 25°C. Ultrasound-assisted extraction (UAE) was carried out using ultrasonic probe system (SONICS Vibra cell™, VCX 500, 500 W, 20 kHz, Newtown, Connecticut, USA) with optimized conditions (Energy: 120000 J, Amplitude: 60%, Pulse (ON:FF): 5:5, Temperature: 25°C, and Time: 5 min) based on the screening experiments. Prior to the UAE, SML powder was pretreated by homogenization (Homogenizer 850, Fisher-brand, USA) for 5 min at a homogenization speed of 10000 rpm.

After the extraction processes, all the obtained extracts were subjected to centrifugation (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany)

for 15 min at 8000 ×g (temperature of sample at speed was 25°C). Then the supernatants were vacuum filtered using Whatman 1 filter paper (Sigma Aldrich, St. Louis, USA) and stored at -80°C in amber bottle until subsequent analyses.

6.3.3. Bioactive characterization of SML extract

Total phenolic contents (TPC) of all SML extracts were measured based on the Folin-Ciocalteu method as reported by Singleton, Orthofer and Lamuela-Raventos (1999). Total flavonoid contents (TFC) were measured by following the method of Jia, Tang and Wu (1999). Results of TPC were expressed in mg gallic acid equivalent per gm dry matter (mg GAE/g DM) while catechin was used as the standard to express the results of TFC (mg CTE/g DM).

The antioxidant capacity of SML extracts obtained through above mentioned extraction conditions were evaluated through cupric reducing antioxidant capacity (CUPRAC) based on the methods given by Apak et al. (2004). A plate reader (SpectraMax® i3x, Multi-mode microplate reader, Molecular Devices, USA) was used to measure the absorbance at 450 nm and trolox was used to express the equivalent result as µmol TE/g DM.

6.3.4. Microencapsulation process

SML extracts were concentrated under vacuum in a rotary evaporator (RE-201D Rotary Evaporator, 1200 W, Zhengzhou, Henan, China) at 40°C to remove the ethanol and then freeze dried (LABCONCO, Kansas City, Missouri, USA) for 48 h. The freeze-dried extract powder was then dissolved in deionized water to make a solution of 10% w/w (10 °Brix) and subjected for microencapsulation where combination of maltodextrin (MD) and gum arabic (GA) was used as coating material. 10 g of coating material (MD:GA = 60:40, w/w) was dissolved in 90 g of deionized water to form a solution of 10% w/w coating material (10 °Brix). Afterwards, SML extract solution and coating solution were mixed to obtain core to coating ratio of 1:20 by weight. The mixtures were homogenized for 2 min at 20,000 rpm in a homogenizer (Homogenizer 850, Fisher- brand, USA) and divided into two lots. One lot was freeze dried and the second lot was spray dried.

6.3.4.1. Freeze drying

The homogenized mixture was frozen overnight at -20°C and then freeze dried for 48 h. Microencapsulates thus obtained after drying were ground in a mortar and pestle and stored in a sealed glass jar at 4°C until further analysis.

6.3.4.2. *Spray drying*

Spray drying was performed using a Buchi mini spray dryer (Mini spray dryer B-290, Buchi Corporation) at the following conditions: Inlet temperature of 180°C; outlet temperature of 88±5 °C; pump rate of 50%; aspiration rate of 100%; liquid feed rate of 12 mL/min; and compressed air flow rate of 45 m³/h. The obtained microparticles were stored in a sealed glass jar at 4°C until further analysis.

Bioactivities of freeze- and spray-dried microparticles were determined as described in section 6.3.3. For these analyses, each sample was prepared by dissolving 100 mg powder in 10 mL deionized water. The mixture was vortexed for 5 min and then filtered through 0.45 µm PTFE syringe-filter (Fisher Scientific).

6.3.5. Yield determination, encapsulation efficiency, and loading capacity

Microencapsulation yield (%EY) is an important indicator determining the effectiveness of encapsulation process. On the other hand, encapsulation efficiency (%EE) refers to the potential of the coating materials to encapsulate and retain the core material inside the microparticles (Mzoughi et al., 2023). %EY and %EE, were calculated using the equation (6.1) and (6.2), respectively (Mzoughi et al., 2023; Saikia, Mahnot, & Mahanta, 2015).

$$\%EY = \frac{\text{Microparticles after freeze/spray drying (g)}}{\text{Solids in the feed solution (g)}} \times 100\% \dots\dots\dots (6.1)$$

$$\%EE = \frac{\text{CPC-SPC}}{\text{CPC}} \times 100\% \dots\dots\dots (6.2)$$

with, CPC, core phenolic content (mg GAE/g DM) and SPC, surface phenolic content (mg GAE/g DM). CPC and SPC were determined using the Folin-Ciocalteu method described in section 6.3.3. Sample extraction method outlined by Cilek et al. (2012) was used with modifications. For the CPC, 100 mg of microparticles was dispersed in 1 mL ethanol, acetic acid and water (50:8:42 v/v). The mixture was then vortexed (Corning LSE vortex mixer, Mexico) for 1 min, then centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany) for 3 min at 3000 ×g at 20°C. The supernatant was then filtered through 0.45 µm filter. For the SPC, 100 mg of sample was dispersed in 1 mL of ethanol and methanol (1:1 v/v) mixture and then vortexed, centrifuged, and filtered. Loading capacity (%LC) was calculated based on equation (6.3) (Ahmadian, Niazmand, & Pourfarzad, 2019).

$$\%LC = \frac{\text{CPC-SPC}}{\text{Weight of microencapsulated powder}} \times 100\% \dots\dots\dots (6.3)$$

6.3.6. Microparticles characterizations

6.3.6.1. Moisture content, water solubility index, and hygroscopicity

The moisture contents of microparticles were determined using the official methodology of AOAC 925.10 (Horwitz, 2005). Water solubility index (%WSI) and hygroscopicity were determined following the methods of Ahmadian, Niazmand and Pourfarzad (2019) and Mzoughi et al. (2023), respectively. Briefly, 0.4 g of microparticles were mixed with 4.8 mL of deionized water and the mixture was vortexed for 1 min. The resulting suspension was incubated at 30 °C for 15 min. The suspension afterwards was centrifuged for 5 min at 3000 ×g at 20°C and the supernatant was dried at 105 °C for 24 h. %WSI was calculated based on the ratio of dried supernatant in the primary 0.4 g sample. Hygroscopicity (%H) of the microparticles was determined by taking 1 g of microparticles in a glass desiccator prepared with NaCl saturated salt solution (~75% RH) and placing it for 7 days at 25°C. After one week %H was estimated based on the proportion of adsorbed water.

6.3.6.2. Bulk density, tapped density, Carr index, and Hausner ratio

Bulk density, tapped density, Carr index, and Hausner ratio were measured according to the method described by Akyüz and Ersus (2021); Saifullah et al. (2023). A known amount of microparticles was transferred to a 50 mL graduated cylinder to the volume line without applying pressure. The bulk density (kg/m³) was determined by the ratio of the sample weight (g) to the volume (mL). For the tapped density, the same graduated cylinder was tapped 100 times. The tapped density (kg/m³) was calculated by the ratio of the sample weight to the tapped volume. The Carr index (CI) and Hausner ratio (HR) were calculated by equation (6.4) and (6.5), respectively.

$$CI = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100\% \dots\dots\dots (6.4)$$

$$HR = \frac{\text{Tapped density}}{\text{Bulk density}} \dots\dots\dots (6.5)$$

6.3.6.3. Particle size distribution

Particle size distribution was analyzed using the NanoBrook Omni particle size analyzer (NanoBrook Omni, Brookhaven Instruments Corporation, Holtsville, NY, USA) based on the dynamic light scattering (DLS) technique. The microparticles (25 g/L) were dispersed in absolute ethanol as a carrying fluid and subjected to ultrasonication using an ultrasound bath (BRANSON 1200, Danbury, CT, USA) for 2 min to increase the particle dispersibility. Following sonication, the sample was again vortexed and subjected to particle size distribution analysis. The

measurements of the diluted samples (0.2°Brix) were made at 90° angle and carried out at 25°C after 120 s equilibration time. A refractive index of 1.357 was used for the measurements. The results were reported as intensity-weighted effective mean particle diameter ($D_{i, \text{effective}}$), poly dispersity index (PDI), and intensity percentiles D_i (0.1), D_i (0.5), and D_i (0.9).

6.3.6.4. Chromatic and morphological analysis

Chromatic attributes namely L^* (0: dark, 100: light), a^* (-, green; +, red), and b^* (-, blue; +, yellow) of all microparticles were measured at room temperature (25°C) using a colorimeter (KONICA MINOLTA, CR-300, Japan). Before the measurements, the colorimeter was calibrated accordingly. Morphological characteristics of the microparticles was examined using a SEM (TM3000, Hitachi, Japan). Operating conditions were 15 kV at a magnification of 1000×.

6.3.6.5. Thermal analysis

Thermogravimetric analysis (TGA) was performed for microparticles to evaluate thermal stability using a TGA Q50 (TA Instruments, USA). The thermogravimetric curves of the samples were obtained by heating around 11±1 mg of sample powder in a platinum pan up to 700°C under nitrogen atmosphere at a heating rate of 5°C/min. Data visualization and analysis of TGA curves and first-order derivatives of TGA curves (DTGA) were performed using the TA Universal Analysis software (Universal Analysis 2000, version 4.5A, Build 4.5.0.5). Glass transition temperatures (T_g) of phenolic extracts, coating materials, and encapsulated microparticles were determined using differential scanning calorimeter (TA, DSC250, Delaware, USA) by following the procedure of Yeasmen & Orsat (2023a). About 7–8 mg sample were placed in an aluminum pan, which was then hermetically sealed using a mechanical device. Similarly, an empty aluminum pan was sealed to use it as a reference. Prior to the measurement, DSC instrument was calibrated with indium in a sealed aluminum pan. Following calibration, in the DSC chamber sample was rapidly cooled to -40°C at a cooling rate of 10°C/min, held isothermally for 3 min. Afterwards, sample was heated to 200°C at a heating rate of 5 °C/min. DSC data were recorded and analyzed by DSC-Trios 5.1.1v software (TA Instruments, USA).

6.3.7. SPME/GC–MS characterization of volatile fraction

Qualitative flavor profiling of SML extracts and the microcapsules was performed by solid-phase microextraction/gas chromatography coupled to mass spectrometry (SPME/GC-MS) based on the method described by Chang et al. (2022) with slight modification. Briefly, 0.50 g of SML powder/dry extract/encapsulated microparticles were transferred into a 10 mL glass vial and

sealed with a rubber septum. Vials were incubated for 10 min at 60°C in a heating bath. Afterwards, carboxen®/polydimethylsiloxane coated SPME fiber needle (75µm, Supelco-504831, Sigma-Aldrich, Darmstadt, Germany) was inserted into the vial. Prior to the insertion, SPME fiber was conditioned at 250°C for 30 min in GC (Hewlett Packard HP 5890, Las Vegas, NV, USA). Then the grey fiber was exposed to the headspace for 1h to adsorb volatiles at 60°C. After exposure, the fiber was retracted into the needle and transferred into the injector port of GC (Agilent 7683 Series G2613A Injector, China) and kept there for 3 min at 250°C for fiber desorption.

Agilent 6890N Network Gas Chromatograph (G1530N, USA) coupled to Agilent 5973 Network Mass Selective Detector (MSD G2570A) was used for GC-MS analysis. Separation was done in a DB-Wax column (30 m × 0.25 mm, 0.25 µm film thickness—Agilent J&W GC column, USA).

The temperature gradient was as follows: starting temperature 40°C, followed by an increase of 45°C /min to 85°C, then by 3°C /min to 135°C and held for 1 min, next by 1°C /min to 160°C and held for 1 min, afterwards by 9°C /min to 200°C, and lastly by 45°C /min to 250°C and held for 12 min. The carrier gas was Helium (flow rate 2 mL/min). The column effluent was transferred to the mass detector, operated in electron impact (EI) ionization mode at 70 eV. Ion source temperature was 230°C. Selecting ion monitoring mode was applied with scan time and segments setting from 4 to 63 min and scanning m/z from 40 to 350.

The volatile compounds were identified by analyzing raw data acquired by GC–MS using Agilent MSD Productivity ChemStation (E.02.02.1431). The min match score > 80 and area > 1% were the main criteria applied for identifying the compounds.

6.3.8. *In vitro* simulated gastrointestinal digestion

In order to see the percentage release and bioaccessibility of the microencapsulated phenolic extracts, static *in vitro* simulated digestion, composed of oral, gastric, and intestinal phases, was performed following the protocol of Minekus et al. (2014). Before starting, stock solution of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared in a concentration of 1.25×.

For the *oral phase*, a final ratio of 50:50 (w/v) of microencapsulated powder to SSF was targeted. To achieve that, 2.5 g microparticles was taken to which 1.75 mL SSF electrolyte stock solution, 0.25 mL of 1500 U/mL salivary α-amylase (Type IX-A EC 3.2.1.1, Sigma, made up by 1.76 mg/mL in stock SSF), and 12.5 µL of 0.3M CaCl₂(H₂O)₂ were added. Finally, 487.5 µL of

water was added for a total volume of 5 mL of salivary juice where pH was maintained at 6.8. To mimic the oral mastication, all the above-mentioned solutions including microparticles were introduced in a 15 mL centrifuge tube containing magnetic coated stirrer which was then incubated at 37°C for 2 min.

The *stomach digestion* was simulated on the oral bolus (5 mL) by the addition of 3.75 mL of SGF stock electrolyte and 0.8 mL of 25,000 U/mL porcine pepsin stock solution (pepsin from porcine gastric mucosa EC 3.4.23.1, 3,200–4,500 U/mg protein, Sigma). Afterwards, 2.5 μ L of 0.3M $\text{CaCl}_2(\text{H}_2\text{O})_2$ was added, followed by the addition of 0.1 mL of 1M HCL to reduce the pH to 3.0, and 347.5 μ L of water necessary to reach a final volume of 10 mL of gastric juice. Finally, the mixture was incubated at 37°C for 2 h with an agitation of 50 rpm in a shaker machine (MaxQ 4000, Branstead International, Dubuque, Iowa, USA).

The *intestinal phase* of digestion was performed on the gastric chyme (10 mL) by the addition of 5.5 mL of SIF electrolyte stock solution, 2.5 mL of 800 U/mL pancreatin from porcine pancreas (8X USP, P/N P7545, Sigma-Aldrich), 1.25 mL of 160 mM fresh bile (B8631 porcine from Sigma-Aldrich), 20 μ L of 0.3M $\text{CaCl}_2(\text{H}_2\text{O})_2$, 0.075 mL of 1M NaOH, and 655 μ L of water for a total volume of 20 mL of intestinal juice. The mixture (pH 7) was incubated at a set temperature of 37°C for 2 h with an agitation of 50 rpm in a shaker machine (MaxQ 4000, Branstead International, Dubuque, Iowa, USA).

In all three phases (oral, gastric, and intestinal), the final ratio of 50:50 (v/v) (oral: microencapsulated powder to SSF; gastric: oral bolus to stomach fluids; and intestinal: gastric chyme to small intestinal fluids) was targeted individually. A blank without the samples was carried out under the same conditions to eliminate the interference of the chemical components during the digestion process. At each digestion step, 2.5 mL of each sample, including the control, were collected and centrifuged at $8000 \times g$ for 15 min, and the supernatants were stored at -20°C for further analysis.

6.3.8.1. Percentage release of PCs and their bioaccessibility

Percentage release of PCs at each stage (oral, gastric, and intestinal) was measured based on the Folin-Ciocalteu method (Section 6.3.3) and was calculated based on the ratio of digested phenols to the microencapsulated phenols (Ahmadian, Niazmand, & Pourfarzad, 2019). Bioaccessibility (BC) of PCs is represented by the percentage of TPC released in the intestinal phase (Silva et al., 2024).

6.3.9. Storage stability

Storage stability of spray-dried and freeze-dried microparticles was performed for 50 days in two temperature conditions (refrigerated and ambient). All the powders were kept in airtight brown glass containers. TPC, TFC, and antioxidant activity were measured at every 10-days interval.

6.3.10. Statistical analysis

The experiments were performed in triplicate and reported as mean $\pm$ standard deviation of the data obtained. The results were analyzed using the test of variance (ANOVA), followed by Tukey's Honestly Significance Difference (HSD) test to see the significant differences ($p < 0.05$) with 95% confidence interval among the microparticles. Moreover, the parametric Student's t-test ($p < 0.05$) was applied to compare each characterizing attribute between spray-dried and freeze-dried microparticles as well as to compare each treatment before and after digestion. ANOVA, Tukey HSD, and Student's t-tests were performed using the statistical software JMP (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, NC, USA).

6.4. RESULTS AND DISCUSSION

6.4.1. Bioactive properties of sugar maple leaves' extracts

Sugar maple leaves (SML), commonly treated as plant biomass residue, were extracted for their phenolic compounds (PCs) through conventional (maceration) and advanced (homogenization pretreated ultrasound-assisted extraction) approaches and results are shown in Table 6.1. Paired Student's t-test was performed to statistically compare the differences between the yields of TPC, TFC, and antioxidant activity (AOX) from the two extraction methods. For all the attributes, UAE gave significantly ($p < 0.05$) higher TPC (123.78 ± 1.34 mg GAE/g DM), TFC (19.17 ± 0.20 mg CTE/g DM), and AOX capacity (241.19 ± 1.21 μ M TE/g DM) than the values obtained from maceration. The reason behind this higher efficiency of ultrasound can be linked with its extraction mechanism, commonly known acoustic cavitation phenomena. Homogenization used as a pretreatment additionally affected the higher extraction efficiency of ultrasound. Sonication is a method of transferring ultrasonic waves in the liquid medium. Due to the pressure differences, propagation of this wave generates cavitation bubbles that upon implosion release a huge amount of mechanical and kinetic energy (Solanki, Desai, & Parikh, 2018). Consequently, high turbulence occurs in the bubble collapsing zone that results in the disruption/damage of plant cell wall and improvement of solvent penetration through that disrupted cell wall (Palmieri et al.,

2020). This is how the increased extraction efficiency of UAE can be explained. Moreover, the short duration (5 min) of UAE in comparison to maceration (24 h) indicates sonication as an energy efficient and environment friendly extraction method. Compared to maceration, Jovanović et al. (2017); Safdar, Kausar and Nadeem (2017); and Saifullah et al. (2020), observed higher extraction efficiency of PCs with effective antioxidant activities from thyme (TPC: 32.7 mg GAE/L; TFC: 16.7 mg CE/L), mango peel (TPC: 67.58 mg GAE/g of extract), and lemon scented tea leaves (TPC: 98.91 ± 1.20 mg GAE/g DM; TFC: 76.12 ± 0.79 mg CTE/g DM; AOX: $5534.8776.12 \pm 0.79$ 19.56 mM TE/g DM), respectively, when using UAE.

Table 6.1: Phytochemical properties of sugar maple leaves' extracts and encapsulation performance.

Attributes	M1	M2	M3	M4
TPC (mg GAE/g DM)	123.78 ± 1.34^A		119.91 ± 1.68^B	
TFC (mg CTE/g DM)	19.17 ± 0.20^A		18.39 ± 0.10^B	
AOX (μ M TE/g DM)	241.19 ± 1.21^A		232.39 ± 0.87^B	
%EY	93.33 ± 1.47^{Aa}	62.86 ± 0.73^{Bb}	89.48 ± 0.68^{Aa}	65.08 ± 1.83^{Bb}
%EE	96.80 ± 0.22^{Ba}	97.14 ± 0.31^{Ba}	95.31 ± 0.07^{Cb}	99.07 ± 0.01^{Aa}
%LC	18.34 ± 0.20^{Ba}	18.03 ± 0.15^{Ba}	20.02 ± 0.32^{Aa}	12.44 ± 0.11^{Cb}

Note: The values are presented as average value $\pm$ standard deviation. Different uppercase letters (A–D) in a row based on the Tukey's HSD test indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective attributes among the microparticles. Lowercase letters (a–b) in a row based on the Student's t-test indicate significant differences ($p < 0.05$) between spray-dried and freeze-dried microparticles.

M1: Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microcapsules of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. AOX: Antioxidant activity; EE: Encapsulation efficiency; EY: Encapsulation yield; HE: Homogenization; LC: Loading capacity; MAE: Microwave-assisted extraction; ME: Maceration; TFC: Total flavonoid content; TPC: Total phenolic content; UAE: Ultrasound-assisted extraction.

6.4.2. Yield of microparticles, encapsulation efficiency, and loading capacity

Encapsulation yield (%EY) is an important indicator for determining the encapsulation performance. It indicates the relationship between the amount of microparticles obtained after the encapsulation process and the feed was given for microencapsulation. Moreover, to determine the potential of the coating material for the entrapment of core materials (PCs), knowledge of the encapsulation efficiency (%EE) and loading capacity (%LC) is essential. In this study, microencapsulation of SML extracts was performed by freeze drying and spray drying processes, where freeze drying was found to give significantly higher ($p < 0.05$) %EY than the spray drying (Table 6.1). Reason behind the lower yield of microparticles for spray drying can be explained from the point of higher adhesion of the particles to the drying chamber, while in case of freeze-drying, the phenolic extracts and coating materials were dried without atomization and heat exposure, therefore, the dried powder can be directly collected from the process container (Saikia,

Mahnot, & Mahanta, 2015). Comparatively less %EE was observed in freeze dried microparticles in comparison to the spray dried microparticles (Table 6.1). Ahmadian et al. (2019) found significantly higher %EE (~86%) for spray dried microparticles of saffron petal phenolic extract with varying combination of maltodextrin and pectin than the freeze dried powder (~72%). Drying technique applied for encapsulation influences the encapsulation efficiency as well as loading capacity. In this study, after freeze drying all the microparticles were crushed in a mortar, which might result in the breakage of the particle wall and thus, may increase the amount of surface phenolic content. However, for the %LC, spray dried microparticles gave comparatively higher values which again might be attributed to the available phenolic content on to the surface due to the large surface area-to-volume ratio of the particles resulted from the atomization process. However, varying combination of coating material together with the variation in spray drying conditions (inlet temperature, aspiration rate, etc.) might help to understand the effect of drying condition towards the encapsulation process.

6.4.3. Physical and physicochemical properties of encapsulated powders

6.4.3.1. Moisture content

Moisture content is one of the important parameters affecting the products' quality. It is directly related to the stickiness, caking, and aggregate condition of the powder. Moreover, thermal properties of powders are affected by the amount of moisture present in the powder since water is acting as a plasticizing agent (Mzoughi et al., 2023). The moisture content of microparticles in this study was between 2.26–4.48% (Table 6.2) where maltodextrin (MD) and gum arabic (GA) were used as coating agent for encapsulating phenolic extracts. The type of coating material is known to influence the moisture content of the final product since different carrier agents interact with moisture and relative humidity in different ways (Silva et al., 2024). MD is best known for its low hygroscopic nature and it provides low resistance to mass transfer during drying, thus, the removal of moisture during the drying process becomes fast (Ahmadian, Niazmand, & Pourfarzad, 2019; Nguyen, Tran, & Le, 2021). On the other hand, GA is a good emulsifier with good film forming properties (Ravichai & Muangrat, 2019). Therefore, the combination of MD and GA can be considered as a good coating material against moisture uptake, which was reflected in our study and in the findings of Nguyen, Tran and Le (2021); Papoutsis et al. (2018). However, the moisture content of spray-dried microparticles (3.87%–4.38%) was found significantly higher ($p < 0.05$) in comparison to the freeze-dried particles (2.26%–2.43%). These results are in the range with the

findings of Andrade et al. (2023); da Silva Júnior et al. (2023); Ravichai and Muangrat (2019); Saikia et al. (2015), who found moisture content of 2.79%–4.35% for the freeze dried microparticles while for spray dried microparticles, moisture content of 3.83%–4.98% was observed. The differences can be attributed from the working function of these two drying techniques. In spray drying, atomization of the feed solution (mixture of phenolic extracts and coating materials) at a higher temperature might form a crust layer around the particles that might result in the lower mass transfer (Ahmadian, Niazmand, & Pourfarzad, 2019). On the other hand, freeze drying implies the sublimation of ice under low pressure, so that it helps to remove the bound water through desorption (Nguyen, Tran, & Le, 2021). In addition, slow freezing at -20°C might result in relatively large pore channels towards the outer layer, which may enhance the mass transfer and consequently lower moisture content in the freeze dried microparticles (Nguyen et al., 2021).

6.4.3.2. Water solubility index (WSI)

WSI was high for all the samples with values ranging from 89.80 to 92.28% and indicates that these microparticles of phenolic extracts can be reconstituted easily and instantly (Mzoughi et al., 2023). This high solubility can be correlated with the high solubility of maltodextrin as mentioned earlier. Apart from coating material, particles solubility also depends on the drying process. From Table 6.2, it can be seen that freeze-dried microparticles have significantly higher solubility index than spray-dried ones. This can be connected to the higher moisture content of the spray dried products. Presence of moisture in powder results in the formation of agglomeration with the consequences of comparatively lower solubility of the particles (Ahmadian, Niazmand, & Pourfarzad, 2019).

6.4.3.3. Hygroscopicity

Hygroscopicity characterizes the propensity for a material to absorb or adsorb moisture from the surrounding environment and is dependent on the particle size, temperature, and the chemical mixture in each particle (Araújo & Pena, 2020; Russell, 2014). High hygroscopicity values result in the formation of caking which is undesirable for powder materials. The hygroscopicity values were higher in spray-dried samples (Table 6.2). The spray-dried encapsulates appear very fine and their surface area to volume ratio was comparatively higher which enables them to absorb more moisture. Apart from the encapsulation techniques, it was found that phenolic extracts obtained through the homogenization pretreated UAE method are

comparatively more hygroscopic in contrast to the macerated extracts. Because of sonoporation and acoustic cavitation, the results of ultrasonication might extract protein molecules along with PCs as phenolics can interact with proteins (Yu et al., 2023). Protein has hygroscopic properties due to their improved water holding capacity, consequently the encapsulated ultrasound-assisted phenolic extracts can possess a more hygroscopic nature (Peng et al., 2023). As an approximation, Kemp (1999) mentioned that under conditions between 40% to 90% relative humidity at 25°C, if a material picks up less than 1% moisture by mass, then it is denoted as a non-hygroscopic material. On the other hand, under the same condition if the materials absorb more than 5% moisture, it is considered as a hygroscopic material. Based on our findings, the sample showed hygroscopic nature, therefore, it should be well protected from moisture and air during storage and transportation.

6.4.3.4. Density, flowability, and cohesiveness

Bulk density and tapped density are important powder properties during their processing, packaging, storage, and transportation (Akyüz & Ersus, 2021). There is a possibility of oxidation in the material having lower bulk density because of having more air space between the particles. Consequently, storage stability of those particles would be less than those having a greater bulk density (Pashazadeh et al., 2021). Typically powders prefer to have bulk densities between 320 to 400 kg/m³ (Mzoughi et al., 2023). As can be seen in Table 6.2, bulk densities of the microparticles in this study fell more or less within that range (284 to 364 kg/m³). While comparing between the encapsulation techniques, powders produced by spray drying provided significantly higher ($p < 0.05$) bulk density (M2: 364.75 ± 2.60 kg/m³ and M4: 303.85 ± 4.44 kg/m³) than those of freeze-dried powders (M1: 284.17 ± 0.96 kg/m³ and M3: 237.65 ± 2.72 kg/m³). This can be explained from the particle size as spray-dried powders have lower particle diameter compared to the freeze-dried powders, however, this particle size distribution will be discussed more elaborately later in this section. Not far from our findings, Saikia, Mahnot and Mahanta (2015) and Pashazadeh et al. (2021) have found the similar trend i.e., freeze-dried products have lower density than spray-dried products. Density information is also important to determine the powders' flowability and cohesiveness which can be determined from the values of Carr index (CI) and Hausner ratio (HR), respectively. Jinapong, Suphantharika and Jamnong (2008) interpreted values of CI and HR, and categorized the powder as flowable (very good, good, fair, bad, very bad) and cohesive (low, intermediate, high) based on the values of CI and HR. CI and HR values of microencapsulated

SML phenolic extracts fall between the range of 11.71 to 17.44% and of 1.13 to 1.21 which indicates our phenol rich microparticles' flowability are “very good” to “good” while possessing low cohesiveness.

6.4.3.5. Chromatic properties

Color represents in many aspects an indicator of a product's quality. Color parameters of encapsulated phenolic powders were determined as darkness to lightness (L^*), greenness to redness (a^*), and blueness to yellowness (b^*) values (Table 6.2). Since for all the treatments, the same combination of MD and GA was used, the effect of coating material on the chromatic values of the microparticles can therefore be omitted. Based on Table 6.2, microparticles of phenolic extracts obtained from ultrasound treatment have significantly higher ($p < 0.05$) a^* and b^* values in comparison to the microparticles of macerated phenolic extracts. The greenness values ($-a^*$) here can be correlated with the presence of chlorophyll pigment whereas existence of carotenoids can be linked with the yellowness (b^*) values of the studied encapsulated phenolic extracts (Yeasmen & Orsat, 2023b). Known as photosynthetic pigments, both chlorophyll and carotenoids are sitting together in the chloroplast and are associated with the photosynthesis process of capturing light (Lichtenthaler, 1987). During UAE, the sonication results in cavitation in the sample matrix that might cause the breakdown of cell organelles and the release of pigments. Moreover, prior to the UAE, sugar maple leaves powder in extracting solvent was homogenized which again created the mechanical shear between the particles, thus helping in extracting more phytochemicals (Yeasmen & Orsat, 2022). However, compared to freeze drying, spray drying generated significantly ($p < 0.05$) lighter powders which is the resultant of decreased greenness and yellowness values during the spray drying operation. This might be due to the non-enzymatic (heat mediated) degradation of chlorophyll and carotenoids pigments (Kardile, Nanda, & Thakre, 2020; Lípová et al., 2010). Moreover, among the spray dried microparticles (sonicated and macerated extracts), b^* value of macerated microparticle (3.04) was obtained significantly lower ($p < 0.05$), which in addition may be correlated with the lower extraction of carotenoid pigment.

Table 6.2: Microencapsulated powder properties.

Attributes	M1	M2	M3	M4
Moisture (%)	2.43 ± 0.06 ^{Bb}	3.87 ± 0.18 ^{Aa}	2.26 ± 0.17 ^{Bb}	4.38 ± 0.30 ^{Aa}
WSI (%)	92.28 ± 0.01 ^{Aa}	92.05 ± 0.04 ^{Ab}	91.24 ± 0.07 ^{Ba}	89.80 ± 0.24 ^{Cb}
Hygroscopicity (%)	14.50 ± 0.45 ^{Bb}	19.10 ± 0.41 ^{Aa}	10.87 ± 0.37 ^{Db}	12 ± 0.18 ^{Ca}
Bulk density (kg/m ³)	284.17 ± 0.96 ^{Cb}	364.75 ± 2.60 ^{Aa}	237.65 ± 2.72 ^{Db}	303.85 ± 4.44 ^{Ba}
Tapped density (kg/m ³)	321.88 ± 3.61 ^{Cb}	417.15 ± 3.30 ^{Aa}	287.86 ± 2.48 ^{Db}	361.82 ± 2.10 ^{Ba}
Carr index (%)	11.71 ± 0.69 ^{Ba}	12.56 ± 0.07 ^{Ba}	17.44 ± 0.23 ^{Aa}	16.03 ± 0.74 ^{Aa}
Hausner ratio	1.13 ± 0.01 ^{Ba}	1.14 ± 0.00 ^{Ba}	1.21 ± 0.01 ^{Aa}	1.19 ± 0.01 ^{Aa}
Chromatic parameters				
L*	77.32 ± 0.30 ^{Bb}	90.75 ± 1.28 ^{Aa}	77.27 ± 8.65 ^{Bb}	97.71 ± 0.27 ^{Aa}
a*	-3.69 ± 0.05 ^{Aa}	-2.69 ± 0.05 ^{Bb}	-0.84 ± 0.17 ^{Ca}	-0.03 ± 0.40 ^{Db}
b*	25.68 ± 0.33 ^{Aa}	14.09 ± 0.70 ^{Bb}	18.29 ± 1.91 ^{Ca}	3.04 ± 1.22 ^{Db}
				
Particle size distribution				
D _{i, effective} (nm)	1894.46 ± 121.5 ^{Aa}	408.68 ± 3.10 ^{Bb}	3179.68 ± 212.71 ^{Aa}	448.91 ± 6.66 ^{Bb}
D _i (0.1)	1411.94 ± 29.26 ^{Aa}	215.91 ± 8.63 ^{Bb}	1418.24 ± 136.53 ^{Aa}	270.35 ± 12.98 ^{Bb}
D _i (0.5)	2667.85 ± 41.94 ^{Aa}	358.02 ± 55.39 ^{Bb}	3179.68 ± 212.71 ^{Aa}	448.91 ± 6.66 ^{Bb}
D _i (0.9)	5040.94 ± 54.07 ^{Ba}	599.55 ± 159.72 ^{Cb}	7182.38 ± 326.06 ^{Aa}	746.05 ± 13.69 ^{Cb}
PDI	0.28 ± 0.01 ^{Aa}	0.12 ± 0.02 ^{Bb}	0.33 ± 0.03 ^{Aa}	0.17 ± 0.02 ^{Ba}

Note: The values are presented as average value ± standard deviation. Different uppercase letters (A–D) in a row based on the Tukey's HSD test indicate significant differences based on 95% confidence interval ($p < 0.05$) in the respective attributes among the microparticles. Lowercase letters (a–b) in a row based on the Student's t-test indicate significant differences ($p < 0.05$) between spray-dried and freeze-dried microparticles.

M1: Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; M2: Spray-dried microparticles of phenolic extracts obtained from HE+UAE; M3: Freeze-dried microparticles of phenolic extracts obtained from ME; M4: Spray-dried microparticles of phenolic extracts obtained from ME. HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction; WSI: Water solubility index.

6.4.4. Particle size distribution and surface morphology of encapsulated powders

6.4.4.1. Particle size distribution

Intensity-weighted particle size distribution of freeze-dried and spray-dried microparticles are shown in Figure 6.1 and Table 6.2. Particle size distribution of both conditions showed monomodal distribution with an effective diameter range of 408.68 to 3179.68 nm and is within the range of 1420 to 8730 nm, observed by Silva et al. (2024) and Cilek et al. (2012) for microparticles of phenolic extracts from organic coffee husk and sour cherry pomace, respectively.

However, the variation in the diameter range against the reported ones might be due to the differences in size calculation techniques i.e., surface-weighted (or Sauter) mean diameter (Cilek et al., 2012) and volume-weighted (or De Brouckere) mean diameter (Silva et al., 2024). Along with the effective diameter, significantly lower D_i (0.1), D_i (0.5), and D_i (0.9) were observed with spray-dried powders than that of freeze-dried ones which is in agreement with the findings of da Silva Júnior et al. (2023). Another important parameter called poly dispersity index (PDI) indicates the spreading/broadness of particle size distribution. Ranging between “0” to “1”, particles having PDI close to “0” indicate monodisperse particles while PDI close to “1” is termed as polydisperse particles (Mzoughi et al., 2023). As can be seen from Table 6.2, the PDI values are less than 0.33, thus, it can be concluded that the microparticles produced in this study are in between the monodisperse and polydisperse ranges.

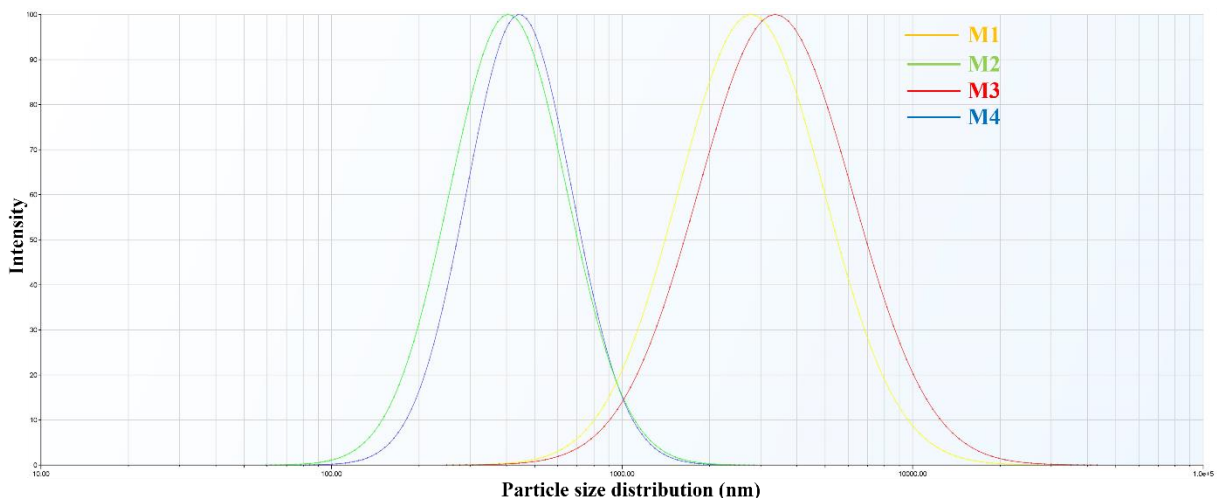


Figure 6.1: Intensity-weighted lognormal particle size distribution of encapsulated microparticles.

M1: Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction.

6.4.4.2. Morphological characteristics

In terms of surface morphology, Figure 6.2 displays the microscopic images of encapsulated SML phenolic extracts. Overall, freeze-drying resulted in a sawdust-like shape with sharper edges while relatively spherical shaped microparticles were produced by spray drying process, confirming the observations of other authors (Andrade et al., 2023; da Silva Júnior et al.,

2023; Kalkan et al., 2017; Pashazadeh et al., 2021). However, those spheres have been seen to have few deformations (dent and indentation) which might be due to the faster moisture evaporation rate followed by immediate cooling during the spray drying operation. Due to the exposure of feed solution to the higher air inlet temperature, vacuoles might be formed within the particles which become inflated because of build-up of vapor pressure. However, since the atomization process lasts for a very short time, the vacuoles might deflate and shrivel followed by the formation of dry skin, if the existing moisture does not evaporate properly (Mzoughi et al., 2023). This confirms the results of the comparatively higher moisture retention in spray-dried particles in contrast to the freeze-dried ones (Table 6.2).

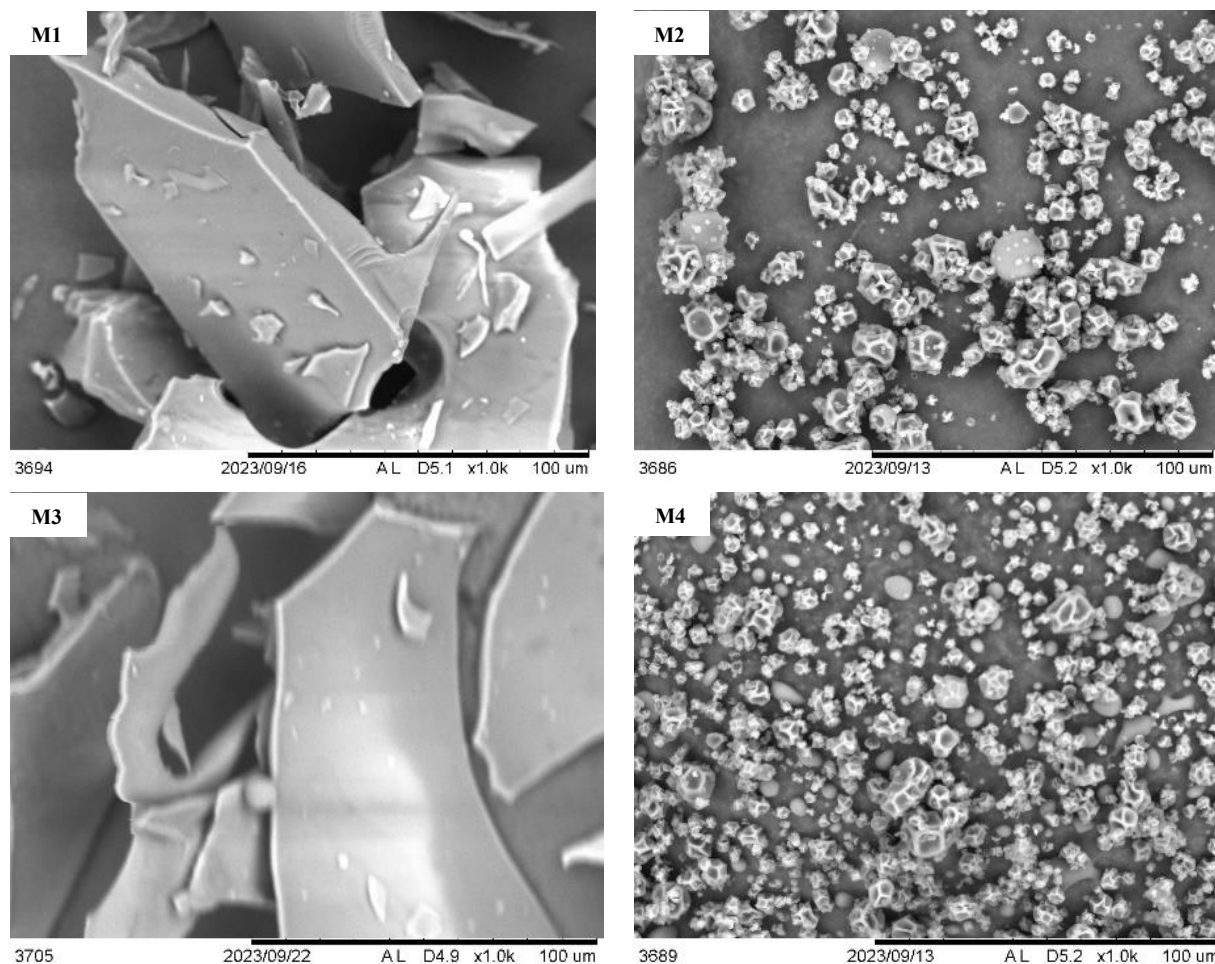


Figure 6.2: Morphological characteristics of freeze-dried and spray-dried microcapsules at a magnification of 1000×. **(M1)** Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **(M2)** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **(M3)** Freeze-dried microparticles of phenolic extracts obtained from ME; **(M4)** Spray-dried microparticles of phenolic extracts obtained from ME. *HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction.*

6.4.5. Thermal properties of encapsulated powders

6.4.5.1. Analysis of the TGA curves

Weight loss of the microparticles and phenolic extracts as a function of temperature is represented by the TGA curves shown in Figure 6.3 (a). Three main stages of weight loss were observed. For microparticles, three degradation stages at 25–220°C; 220–325°C; and 325–700°C correspond to the loss of moisture and phenolic compounds; degradation of cellulose, hemicellulose, protein, and fat (if any); and degradation of lignin and formation of char (Bikaki,

2019; Chen et al., 2018; Yeasmen & Orsat, 2023a). During the first stage, it can be additionally seen that weight loss of freeze-dried microparticles with temperature was comparatively lower than for the spray-dried microparticles and this can be related to the higher moisture content (Table 6.2) of spray-dried powders. It is worthy to mention that microencapsulation delays the thermal degradation of the encapsulated phenolic extracts due to the protective nature of the wall materials which is also evidenced in the TGA graphs (Figure 6.3), as the degradation stages happen quite earlier in the extracts without encapsulation. These results are in accordance with the findings of Ahmadian, Niazmand and Pourfarzad (2019) for the microencapsulation of saffron petal's phenolic extracts with maltodextrin and pectin as coating agents. The DTGA curve, shown in Figure 3 (b), also confirms this result where the maximum decomposition temperature increased in the microencapsulated phenolic extracts compared to the nonencapsulated phenolic extracts. Overall, microencapsulated phenolic extracts exhibited higher thermal stability than for the nonencapsulated ones, while the freeze-dried microparticles showed the highest thermal stability, greater than for the microparticles produced by spray drying.

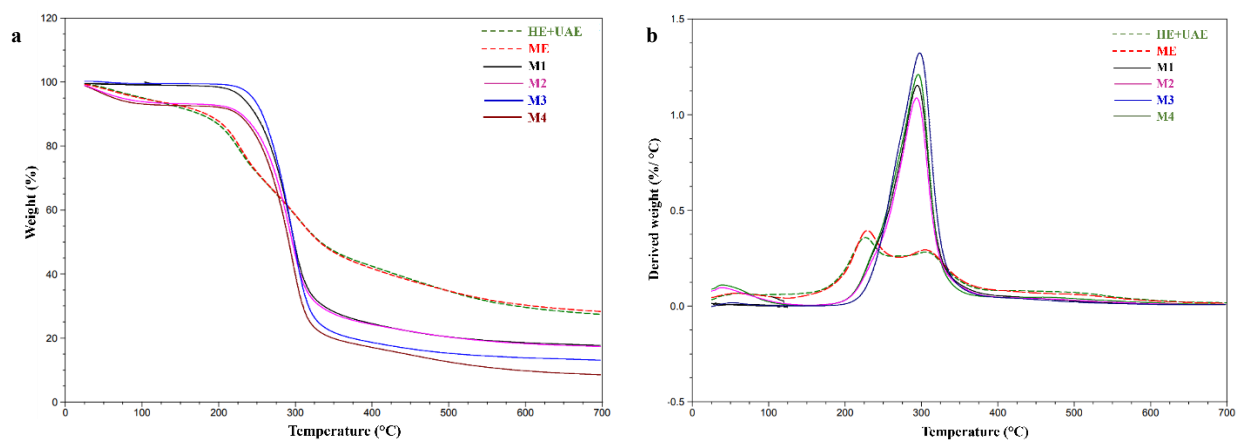
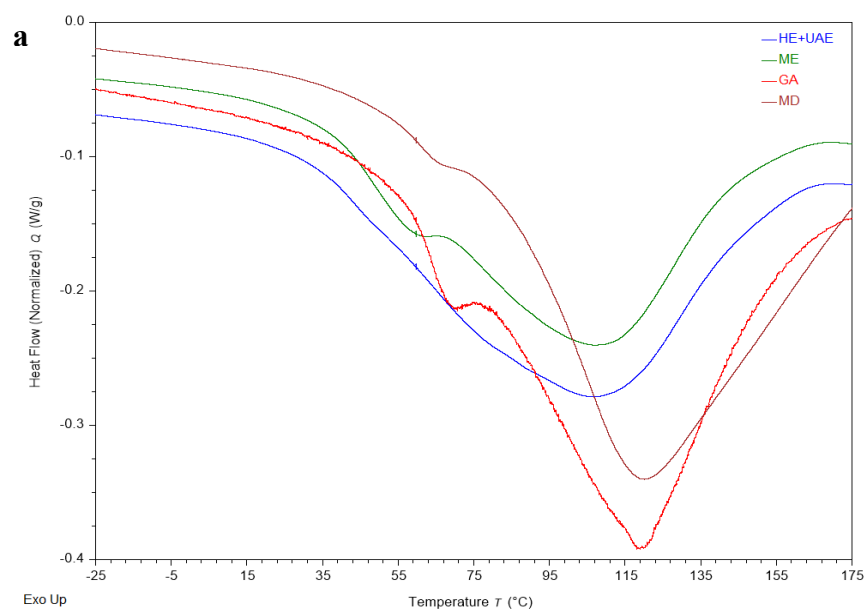


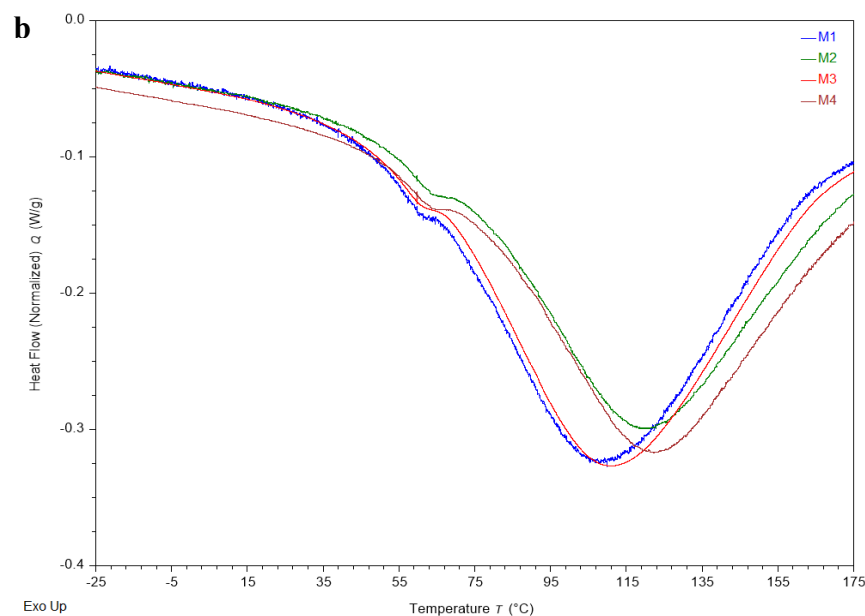
Figure 6.3: (a) TGA and (b) DTGA curves of phenolic extracts (dotted lines) and freeze-dried and spray-dried microparticles (solid lines). **M1:** Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. DTGA: Derivative thermogravimetric analysis; HE: Homogenization; ME: Maceration; TGA: Thermogravimetric analysis; UAE: Ultrasound-assisted extraction.

6.4.5.2. Analysis of the DSC thermograms

To see the effectiveness of the encapsulation processes, in terms of thermal stability, the DSC analysis was carried out for the microparticles produced under spray and freeze-drying conditions, and accordingly the produced DSC thermograms are presented in Figure 6.4. The T_{gon} values for phenolic microparticles ranged between 44.70 to 50.44°C suggesting the effectiveness of the encapsulation process as the glass transition temperatures of microparticles were close to the values of the encapsulating agents. Moreover, the encapsulation process increased the glass transition temperatures of the microparticles while comparing the T_{gon} values for phenolic extracts (35.66 to 37.57°C) (Figure 6.4a). All the thermograms, except for macerated phenolic extracts, showed two endothermic peaks. The first one ranged between 61.78 to 82.74°C, might be attributed to the starch gelatinization temperature (Lund & Lorenz, 1984), while the second endothermic peaks between 101.86 to 119°C, might be related to the water evaporation (Mzoughi et al., 2023). Based on the findings, it can be suggested that the phenolic microparticles should be stored at a temperature below 35.66°C to remain stable.



Sample	T _{gon} (°C)
HE+UAE	35.66
ME	37.57
MD	47.19
GA	54.47



Sample	T _{gon} (°C)
M1	44.79
M2	48.86
M3	46.41
M4	50.44

Figure 6.4: DSC thermograms of phenolic extracts and coating agents (a); microparticles (b). **M1:** Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. DSC: Differential scanning calorimetry; GA: Gum Arabic; HE: Homogenization; MD: Maltodextrin; ME: Maceration; TGA: Thermogravimetric analysis; UAE: Ultrasound-assisted extraction.

6.4.6. SPME headspace volatiles

The effect of encapsulation on masking unwanted aroma characteristics was investigated using SPME/GC-MS-based qualitative characterization. Based on the findings, out of other volatile fractions, both SML powder and its sonicated and macerated phenolic extracts were seen to contain 2-hexanal and 3-hexen-1-ol compounds which are combinedly responsible for the green, leafy, and grassy odor (Jirovetz, Smith, & Buchbauer, 2002; Turner et al., 2021). However, based on the set criteria of identifying the headspace volatiles, these two compounds were not detected in the microcapsules suggesting the effectiveness of encapsulation process against masking the unwanted flavor.

6.4.7. Effect of *in vitro* digestion on the release and bioaccessibility of phenolic compounds

The use of static *in vitro* digestion models using digestive enzymes (α -amylase, pepsin, and pancreatin), bile salts, electrolytes, and mimicking pH condition using HCL or NaOH, in each phase of the digestive process observed in human body, is helpful to understand the natural phenomena of the digestion process on encapsulated PCs. Moreover, by doing this, it will help to understand the effect of encapsulating materials on the bioaccessibility of core components (PCs). Regarding the bioaccessibility of PCs, it can be defined as the fraction of phenolics which is released into the gastrointestinal (GI) tract through the digestion process and becomes available for intestinal absorption (Rousseau et al., 2019). Along with the environment of the GI tract (e.g., pH), the release percentage of nutrients also depends on other factors namely the chemical nature of wall materials and their resistance or susceptibility to digestive enzyme (Saifullah et al., 2023; Saikia, Mahnot, & Mahanta, 2015). Figure 6.5 shows the percentage release of microparticles along with their bioaccessibility fraction. Starting with the oral phase, it was found that microparticles of sonicated phenolic extracts provided more release of TPC than that of macerated microparticles. The differences between the release percentage of TPC can be linked with the quality of phenolic extracts which upon sonication treatment might be containing more quantity of free PCs. Phenolics in plants can occur in free and bound (soluble and insoluble) forms where free phenolics are usually soluble in polar aqueous/organic solvent. Since free phenolics do not interact both chemically and physically with other molecules, they are easy to extract. On the other hand, bound phenolics require some additional forces to become accessible in the extracting solvent (Alu'datt et al., 2017; Wang et al., 2020). In addition, microparticles with higher loading capacity (Table 6.1) offered significantly ($p < 0.05$) higher % release of TPC and it might be due

to the hydrolysis effect from the salivary enzyme (α -amylase), that is responsible for the breakdown of maltodextrin. Irrespective of the type of microparticles, release percentage of TPC in gastric phase increased significantly ($p < 0.05$) compared to the oral phase, indicating maximum release of these compounds due to the effect of acidic medium. Moreover, spray-dried particles in the stomach phase accessed more TPC which is consistent with their lower loading capacity (Table 6.1). This can be additionally attributed to the higher surface area-to-volume ratio due to the lower particle size (Table 6.2) of spray dried microparticles, thus, more encapsulated phenolic extracts that got release in the stomach phase compared to the freeze-dried powder. Ahmadian et al. (2019) also reported that in stomach phase, spray dried microcapsules containing saffron petal phenolic extract have a higher release percentage in comparison with freeze-dried samples. Regarding intestinal digestion, a decrease in the TPC percentage was observed in all treatments compared to the values in the stomach phase which might be due to the degradation of PCs by intestinal enzymes (Silva et al., 2024). Saifullah et al. (2023) also observed the lower release and stability of lemon myrtle leaves phenolics in the simulated intestinal environment than the gastric environment. Finally, MD and GA coated sugar maple leaves phenolic extracts experienced their bioaccessibility from the microparticles (represented by the % of TPC released in the intestinal phase for absorption) in the range of 43.34 to 49.30%. However, digestion study encompassing non-encapsulated phenolic extract besides encapsulated ones with different coating agents would give more insights into the bioaccessibility of encapsulated phenolic extracts.

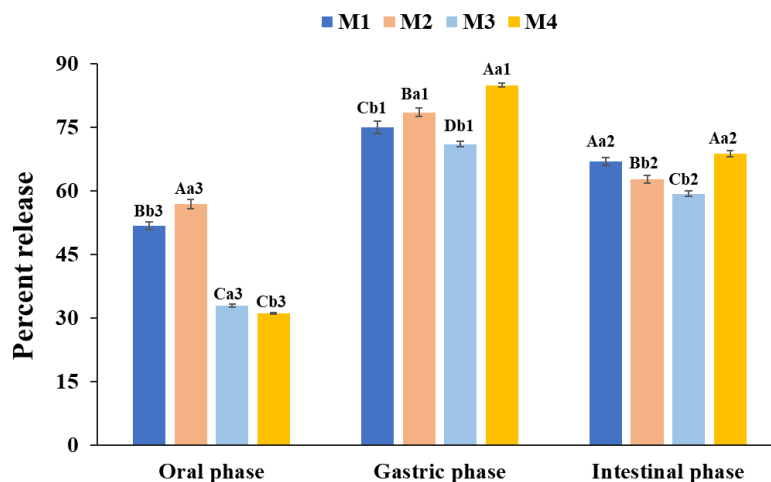


Figure 6.5: Release and bioaccessibility percentage of phenolic compounds in simulated gastrointestinal tract. Results are given as mean $\pm$ deviation. Different uppercase letters (A–D) based on the Tukey’s HSD test indicate a significant difference ($p < 0.05$) between encapsulations (M1, M2, M3, and M4) for each digestion phase while different numbers (1–3) indicate a significant difference ($p < 0.05$) between the digestion phase for each encapsulation. Lowercase letters (a-b) based on the Student’s t-test indicate a significant difference ($p < 0.05$) between the spray-dried and freeze-dried microparticles for each digestion phase. **M1:** Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. DTGA: Derivative thermogravimetric analysis; HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction.

6.4.8. Storage stability of encapsulated powders

In order to simulate the storage conditions of microparticles for possible industrial/commercial applications, samples of phenolic microparticles were studied for storage stability for 50 days. For every 10 days interval, samples were taken in triplicates and analyzed for their TPC, TFC, and antioxidant capacity, and results are illustrated in Figure 6.6. Overall, TPC and TFC values of both spray-dried and freeze-dried microparticles remained almost constant till one month, then showed a slight increase. This can be, on the one hand, due to the structural changes of PCs, or due to the hydrolysis of conjugated phenols (da Silva Júnior et al., 2023; Mzoughi et al., 2023). On the other hand, the increasing content might be the overestimation of

phenolics as TPC was determined based on the Folin-Ciocalteu method which is associated with the reduction of central molybdenum ion in Folin-Ciocalteu reagent by the phenolic antioxidants (Munteanu & Apetrei, 2021). Now, owing to the contribution of other possible non-phenolic reducing agents present in the system, results in the form of TPC might be overestimated. Linking with the non-phenolic reducing agents, maltodextrin which was used as coating material, has a reducing power (Klinjapo & Krasackoopt, 2018). Moreover, the sonochemical effect of ultrasound might produce reactive radicals which again may contribute to the reduction of molybdenum ion in the Folin-Ciocalteu reagent (Buettner & Schafer, 2000; Yeasmen et al., 2023). Concerning the storage stability of the antioxidant microparticles, it can be seen from Figure 6.6 (c) that microparticles of ultrasound-assisted phenolic extracts showed relatively higher retention of antioxidant capacity over the storage period, thus, suggesting the effectiveness of sonication upon the extraction of quality extracts. Based on the storage analysis, it can be said that both the storage conditions for microparticles can be safe for a month without much significant changes.

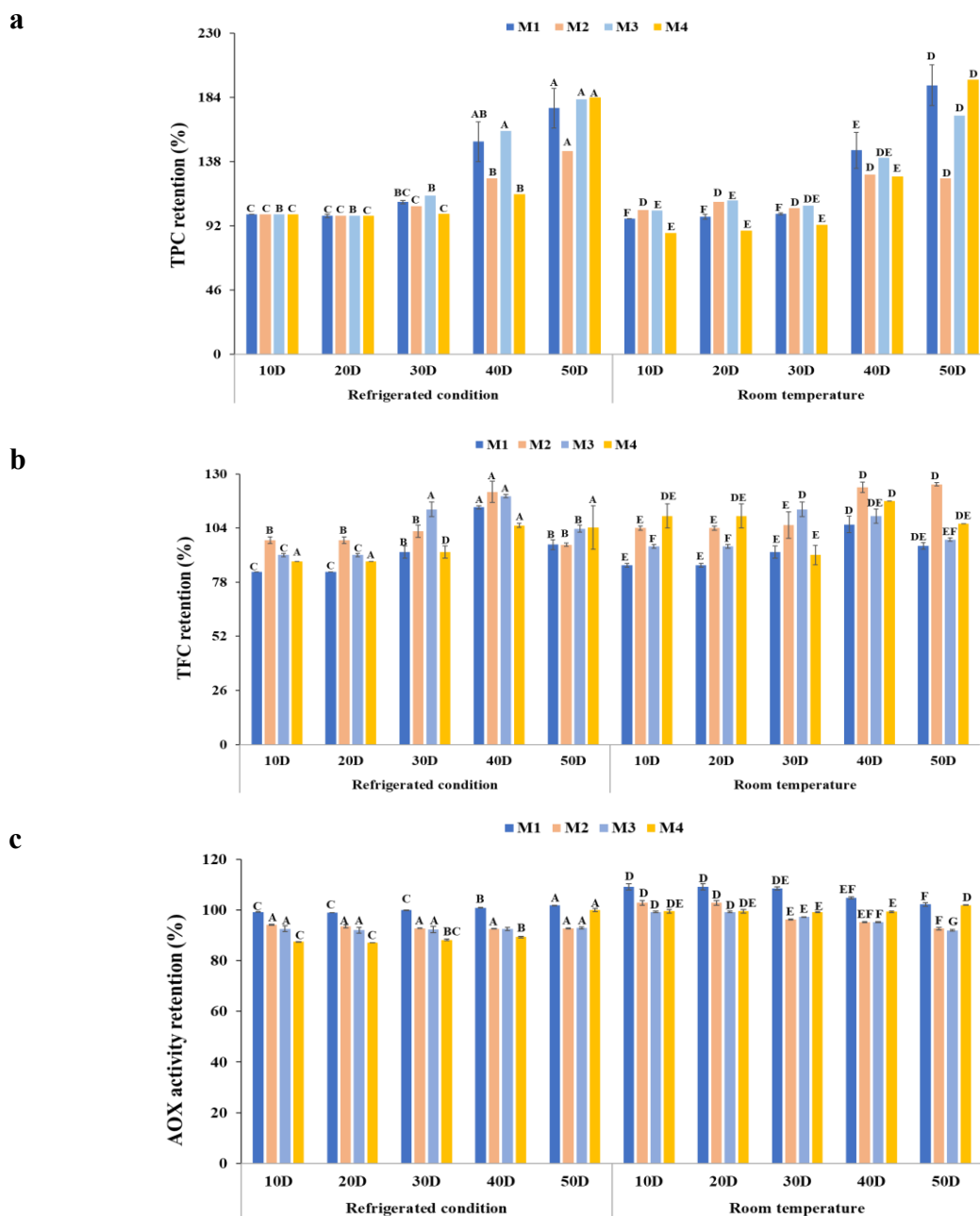


Figure 6.6: Changes in the TPC retention (**a**), TFC retention (**b**), and AOX activity retention (**c**) of the microparticles during storage for 50 days at refrigerated and room temperature. Results are given as mean $\pm$ deviation. Different uppercase letters (A–C) and (D–G) based on the Tukey’s HSD test indicate a significant difference ($p < 0.05$) among the storage days for each type of microparticles in refrigerated and room temperature, respectively. **M1:** Freeze-dried microparticles of phenolic extracts obtained from HE+UAE; **M2:** Spray-dried microparticles of phenolic extracts obtained from HE+UAE; **M3:** Freeze-dried microparticles of phenolic extracts obtained from ME; **M4:** Spray-dried microparticles of phenolic extracts obtained from ME. AOX: Antioxidant; HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction; TPC: Total phenolic content; TFC: Total flavonoid content.

6.5. CONCLUSION

In this study, phenolic compounds from the forestry biomass of sugar maple leaves (SML) were extracted via ultrasound-assisted extraction and the bioactivities of phenolic extracts were compared with the macerated extracts followed by their microencapsulation. Results showed that, UAE, known as an eco-friendly extraction method, gave better phenolic profile with higher antioxidant capacity compared to conventional maceration. Encapsulated microparticles were found with elevated glass transition temperature and with the absence of unwanted volatile fraction. *In vitro* digestion study showed greater release of PCs in the stomach phase, whereas spray dried microparticles gave more release percentage than the freeze dried encapsulated phenolic extracts. Storage stability of the microparticles showed the preservation of antioxidant capacity of SML phenolic compounds and suggested that the phenolic microparticles can be stored for a month without affecting their stability. However, for a more precise understanding in the shelf-life study, profiling of individual phenolic compounds is recommended.

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CONNECTING TEXT TO CHAPTER 7

Chapter 7 is already published and can be cited as:

Yeasmen, N., & Orsat, V. (2024). Pulsed ultrasound assisted extraction of alternative plant protein from sugar maple leaves: Characterization of physical, structural, thermal, electrical, and technological properties. *Food Hydrocolloids*, 152, 109960. <https://doi.org/10.1016/j.foodhyd.2024.109960>

In the previous chapters (3,4,5, and 6) it was seen that sugar maple leaves biomass is not only a good source of phenolic compounds but also contains a comparable amount of protein. To address the current rising demand of alternative plant protein, it was hypothesized that sugar maple leaves would be a renewable and sustainable source of alternative plant proteins. To this end, in Chapter 7 an effort was attempted to valorize sugar maple leaves in terms of extracting protein by ultrasound-assisted extraction based on alkaline extraction-isoelectric precipitation method. The extracted protein fractions were further characterized for their physical, structural, thermal, electrical, volatile, and functional properties.

**CHAPTER 7: PULSED ULTRASOUND ASSISTED EXTRACTION OF ALTERNATIVE
PLANT PROTEIN FROM SUGAR MAPLE LEAVES: CHARACTERIZATION OF
PHYSICAL, STRUCTURAL, THERMAL, ELECTRICAL, AND TECHNO-
FUNCTIONAL PROPERTIES**

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7.1. ABSTRACT

Plant proteins are gaining in popularity and are increasingly being considered as an alternative to animal protein, thus a sustainable, yet comparable source of plant protein is highly recommended. To this end, the current study was conducted to investigate the structural, thermal, physical, and functional attributes, as well as the volatile profile of sugar maple leaves (SML) protein. To this end, SML protein was extracted by homogenization (10000 rpm, 5 min) pretreated ultrasound-assisted extraction (120000 J, 60% amplitude, 5:5 pulse, 25°C, and 15 min) at varying pH (8–10) and compared with conventionally extracted SML protein. Among the different protein extracts, sonicated extract (pH9) provided a good protein yield (up to 7%) having higher protein content (210 mg Bovine serum albumin/g dry matter), thermal stability (onset of denaturation temperature:114°C), and functional properties (solubility, emulsion capacity, water holding capacity, and antioxidant activity). On contrary, the characteristic green aroma, caused by 3-hexen-1-ol and nonanal, was found higher in sonicated protein extracts than that of macerated extracts. Having said that, this study showed the versatile properties of SML protein fractions that can be used as plant-based food ingredients.

Keywords

Alternative plant protein, biomass valorization, emerging technologies, protein quality, sustainability.

7.2. INTRODUCTION

The global population is projected to reach its peak at nearly 11 billion by the end of the 21st century with an increasing demand for protein (Hadidi, Aghababaei, & McClements, 2023; Masijn et al., 2023). However, the growing protein demand will result in a large volume of greenhouse gas emissions as long as it is associated with the increase in the production of protein of animal origin, the most common protein source due to possessing balanced nutritional quality, good digestibility, and functionality (Ortega et al., 2024). Consequently, plant-based eating habits have become more popular by taking the concerns of environmental, sustainability, and animal welfare together with the lessor of COVID-19 pandemic into account (Ou et al., 2023). Increasing health and fitness trends, growing vegetarianism and veganism trends, and inadequate consumer acceptance of food grade insect proteins are additionally some of the other driving forces behind this escalating demand for plant-based protein. According to Markets and Markets (MAM) and National Research Council Canada (NRCC), the global plant-based protein market alone stood at

\$10.8 billion in 2022 supported by a CAGR of 6.7% and is projected to reach \$17.4 billion by 2027 (MAM, 2022; NRCC, 2022). As a reflection, a rising research trend related to plant-based protein/animal protein alternatives has been observed where usually various legumes and pulses are subjected for protein extraction (Arshad, Anwar, Pasha, Ahmed, & Aadil, 2022; González-Félix et al., 2023; Hewage et al., 2024; Li et al., 2023; G. Loushigam & A. Shanmugam, 2023; Wintersohle, Kracke, Ignatzy, Etzbach, & Schweiggert-Weisz, 2023). The comparable amino acid composition of leaf protein concentrate can also complement the effort of addressing the increasing protein demand (Pérez-Vila, Fenelon, Hennessy, O'Mahony, & Gómez-Mascaraque, 2024; Zhang, Sanders, & Bruins, 2014). Since most of the consumable amino acids are synthesized in plant leaves, leaf protein has been suggested as an additional protein source dating back to the 1960s (Akeson & Stahmann, 1965; Gerloff, Lima, & Stahmann, 1965), however, potentiality of this sector as a protein source is the least explored. Maple leaves, usually considered residue plant biomass, can be a dynamic contributor for the extraction of protein. Yeasmen and Orsat (2023a) reported a 21.16% protein concentration in maple leaf (SML) which is comparable to commercial plant protein sources like soybean (~31%) and pea (21–33%). Based on the Canadian forest inventory, approximately 3.1% of Canada's total forests by volume are composed of native Canadian maple species of which the best-known being the sugar maple (*Acer saccharum*). Sugar maple is a deciduous and one of the largest hardwood trees with an average height of 25-37 m (Godman, Yawney, & Tubbs, 1990; NaturalResourcesCanada, 2022). Therefore, it can be hypothesized that sugar maple leaves (SML) would be a renewable and sustainable source of alternative plant proteins.

Protein as a macromolecule is prominent for both biological and techno-functional properties where the former one is associated with their physiological and nutritional behavior and the later one is related to their physicochemical (color, texture, solubility) and functional (stability, gelling, foaming, emulsifying, thickening, viscosity, etc.) behaviors (Pojić, Mišan, & Tiwari, 2018). These techno-functional properties feature the possible application area (food, pharmaceutical, and cosmetic industries) of the extracted protein (Chakravorty & Das, 2023; Vera-Salgado et al., 2022). Regarding protein's functionality, extraction methods have significant impact on the techno-functional properties of protein (Hadidi, Aghababaei, & McClements, 2023; Pojić, Mišan, & Tiwari, 2018). Among different approaches such as salt-, enzyme-, deep eutectic solvents-assisted extraction (Akyüz & Ersus, 2021; Fang, Chang, Ohm, Chen, & Rao, 2023;

Hewage et al., 2024; Lone et al., 2023; Singh et al., 2023), alkaline-assisted extraction followed by isoelectric precipitation is the most applied protein isolation methods from plant sources and this can be conducted conventionally (maceration) and non-conventionally (advanced extraction methods). Being affordable, safe, effective, and ecologically friendly, the ultrasound-assisted extraction method has become popular nowadays especially with the extraction of protein (Chakravorty & Das, 2023; Lone et al., 2023; Geetarani Loushigam & Akalya Shanmugam, 2023; Naik, Natarajan, Modupalli, Thangaraj, & Rawson, 2022; Ortega et al., 2024; Singh et al., 2023; Vera-Salgado et al., 2022). In addition to increasing the percentage yield, ultrasound has been reported to enhance the functional characteristics of protein when compared to the conventional extraction (Chakravorty & Das, 2023; Geetarani Loushigam & Akalya Shanmugam, 2023). Ultrasound during its propagation into the liquid media with the changes of pressure, results in the formation of cavitation bubbles followed by their implosion (Bhat et al., 2022). Consequently, related physical effects, including micro-turbulence, shockwaves, microjets, and particle collisions take place near the plant matrix that result in higher extraction yield through surface peeling, erosion, or sonoporation (Li, Yang, Coldea, & Zhao, 2021; Roobab et al., 2023). Moreover, it has been demonstrated that the effectiveness of UAE towards the isolation of plant protein is maximized when it is accompanied with other techniques (Hadidi, Aghababaei, & McClements, 2023). Homogenization-assisted alkaline extraction has been found to improve the protein functionality (Burger & Zhang, 2019). Thus, it can be hypothesized that combination of ultrasound and homogenization method would increase the efficiency of alkaline extraction-isoelectric precipitation for the isolation of SML proteins.

To the best of existing evidence, the literature is currently lacking information on the application of advanced extraction methods to obtain protein concentrate from sugar maple leaves. Hence, this study concentrated on the homogenization-pretreated ultrasound-assisted alkaline extraction of SML proteins followed by their characterizations. For this, the objectives of this study were (i) to extract SML protein using homogenization pretreated ultrasound-assisted extraction, and (ii) to characterize the physical, structural, thermal, electrical, volatile, and functional properties of the extracted SML protein.

7.3. MATERIALS AND METHODOLOGY

7.3.1. Sample preparation and ultrasound treatment

Samples of sugar maple leaves (SML) were collected from a natural forest stand (Morgan Arboretum, Sainte-Anne-de-Bellevue, Quebec, Canada). Immediately after the collection, healthy leaves without any visible injuries and insects attack were screened and thoroughly washed under running tap water. Then the leaves were dried using a freeze dryer (Martin Christ, GmbH, Osterode am Harz, Lower Saxony, Germany) with a condenser temperature of -55°C . Freeze-drying was carried out at 10°C for 48 h under 0.85 mbar pressure. Afterwards, freeze-dried samples were ground to a size of < 0.1 mm using a milling machine (Retsch GmbH, GM 200, Retsch-Allee 1-5, Haan, Germany) and kept in brown sealed glass bottles at -20°C until further uses/analyses.

Homogenization pretreated alkaline based extraction of SML protein was conducted in a probe ultrasonic processor (SONICS Vibra cellTM, VCX 500, 500 W, 20 kHz, Newtown, Connecticut, USA) (Figure S2). SML powder was added to distilled water in a 1:30 ratio (w/v), and the pH of the solution was adjusted to 8–10 using 1M NaOH (Chang et al., 2022). Then the pH adjusted SML powder solution was homogenized (Homogenizer 850, Fisher- brand, USA) for 5 min at 10000 rpm. Immediately afterwards, the homogenized solution was subjected to ultrasonication, where the pulsed ultrasound-assisted extraction conditions were as energy: 120000 J, amplitude: 60%, pulse: 5 (ON):5 (OFF), temperature: 25°C , and time: 15 min. During the sonication process, air channel was connected to the ultrasound apparatus to keep the instrument cold. Moreover, the entire sonication procedure was carried out in ice water and the temperature of the solution was measured at each OFF-pulse state using a digital thermometer (KT800 IPX7, Waterproof Digital Thermometer, China). The average recorded temperature was $23 \pm 2^{\circ}\text{C}$. Afterwards, the sample was centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany) for 15 min at $8000 \times g$ (temperature of sample at speed was 25°C). The pH of collected supernatant was adjusted to 4.5 using 1M HCl (Geetarani Loushigam & Akalya Shanmugam, 2023) for protein precipitation, followed by centrifugation at $8000 \times g$ for 15 min at 25°C . After discarding the supernatant, the residues were redispersed in water to which pH was neutralized by NaOH (1M). Finally, the extracted soluble protein fraction was freeze-dried (LABCONCO, Kansas City, Missouri, USA) for 48 h and collected in an air-tight amber bottle and stored at 4°C for further study.

To compare with the ultrasound treated sample, a conventional alkaline based extraction method was conducted at pH 9 for 30 min following the same steps of isoelectric precipitation and sample collection.

7.3.2. Characterization of SML protein properties

7.3.2.1. Protein content and yield

Spectrophotometric (at 595 nm) measurement of total crude protein in SML powder and protein extracts were done following the method of Bio-Rad protein assay which is based on the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as standard, and the results were expressed as mg BSA/g DM. The extraction yield was determined by taking the ratio of grams of SML protein extracts per 100 g of dry SML powder (Pérez-Vila, Fenelon, Hennessy, O'Mahony, & Gómez-Mascaraque, 2024).

7.3.2.2. Color profile

Chromatic attributes namely L* (0: dark, 100: light), a* (-, green; +, red), and b* (-, blue; +, yellow) of SML protein powders were measured at room temperature (25°C) using a chroma meter (KONICA MINOLTA, CR-300, Minolta Co., Ltd., Tokyo, Japan). Before the measurements, the colorimeter was calibrated against a white reference tile (L*: 97.36, a*: 0.09, and b*: 2.81), which additionally was used as a white reference for comparison among the protein extracts. The total color difference (ΔE^*) and whiteness index (WI) were calculated using equations (7.1) and (7.2), respectively (Gökkaya Erdem, Dıblan, & Kaya, 2019).

$$\Delta E^* = [(L^*_{\text{reference}} - L^*_{\text{sample}})^2 + (a^*_{\text{reference}} - a^*_{\text{sample}})^2 + (b^*_{\text{reference}} - b^*_{\text{sample}})^2]^{1/2} \dots\dots\dots (7.1)$$

$$WI = 100 - [(100 - L^*_{\text{sample}})^2 + (a^*_{\text{sample}})^2 + (b^*_{\text{sample}})^2]^{1/2} \dots\dots\dots (7.2)$$

7.3.2.3. Fourier-transformed infrared (FTIR) analysis

FTIR was used to characterize the secondary structure changes of extracted protein fraction using an ATR-FTIR spectrometer (Thermo Scientific Nicolet iS5 FTIR, Thermo Fisher Scientific, Madison, Wisconsin, USA) accessorized with a fast recovery deuterated triglycine sulfate (DTGS) detector. IR spectra in the 600-4000 cm^{-1} range were collected at a resolution of 4 cm^{-1} using 40 scans and reported as absorbance values. All the spectral measurements were normalized against the background air spectrum. The collected spectra were analyzed by generating the second derivative of the IR spectra followed by the Gaussian curve peak fitting in an amide I region (1600-1700 cm^{-1}). This was carried out by using OriginPro 2023b software (10.0.5.157, OriginLab Corporation, Northampton, MA, USA).

7.3.2.4. *SDS-PAGE electrophoretic profile*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), according to the described method of Laemmli (1970), was used under reducing condition to determine the molecular mass of the proteins extracted from SML, with modifications. 5 μ L sample (0.5% w/v) was mixed with 2 $\times$ Laemmli sample buffer (4.75 μ L) and β -mercaptoethanol (0.25 μ L) at a 1:1 ratio. Then the sample was heated at 95°C for 5 min in a dry bath incubator (Fisher Scientific, USA) followed by cooling at room temperature. Precast gel (10%, Mini-PROTEAN TGX Gels) was loaded with 10 μ L protein ladder (PageRuler™ Unstained Protein Ladder, 10-200 KDa) along with 10 μ L of each protein sample and analyzed in a vertical electrophoresis unit (Bio-Rad, Mini-PROTEAN® II Electrophoresis Cell, Hercules, California, USA). Before the analysis, the inner chamber of the electrophoresis unit was filled completely, and the outer chamber was filled partially with the running buffer. Electrophoresis was conducted at a constant current of 120V for around 40 min. After electrophoresis, the gel was stained with Coomassie stain followed by removing the excess stain with destaining solution (10% acetic acid, 40% water, and 50% methanol). Finally, the images of the clear gel were taken using a portable camera (Samsung Galaxy S20 FE).

7.3.2.5. *Density*

A known amount (4 g) of freeze-dried protein extract was transferred to a 50 mL graduated cylinder to the volume line without applying pressure. The bulk density (g/cm^3) was determined by the ratio of the sample weight (g) to the volume (mL). For the tapped density, the same graduated cylinder was tapped 100 times. The tapped density (g/cm^3) was calculated by the ratio of the sample weight to the tapped volume. The Carr index (CI) and Hausner ratio (HR) were calculated by equations (7.3) and (7.4), respectively.

$$\text{CI} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100\% \dots\dots\dots (7.3)$$

$$\text{HR} = \frac{\text{Tapped density}}{\text{Bulk density}} \dots\dots\dots (7.4)$$

7.3.2.6. *Scanning electron microscopy (SEM)*

Surface images of the protein extracts were taken using a SEM (TM3000, Hitachi, Japan) where the operating conditions of 15 KV electron power, auto focus function and composition mode of imaging at a magnification of 150–500 $\times$ were used. Prior to scanning, samples were mounted on an aluminum stub (32 mm holder size) with double-sided carbon adhesive tabs in a

vacuum condition. Once the sample was properly aligned as per the visual image on computer screen, then the micrographs were quickly scanned in order to prevent the charging and heating effect that could lead artifacts to micrographs (Bhuiyan & Ngadi, 2024). All the micrographs were exported as 8-bit Joint Photographic Experts Group (JPEG) file.

7.3.2.7. Thermal properties

Thermogravimetric analysis (TGA) of SML protein powder was performed using a thermogravimetric analyzer (TGA Q50, TA Instruments, New Castle, Delaware, USA) and the denaturation temperature (T_d) of each protein sample was measured using a differential scanning calorimeter (TA, DSC250, New Castle, Delaware, USA) according to our previous report (Yeasmen & Orsat, 2023a). For TGA analysis, 10–11 mg of sample was taken in a platinum pan and heated up to 700°C under nitrogen atmosphere at a heating rate of 5°C/min. Data visualization and analysis of TGA curves was performed using the TA Universal Analysis software (Universal Analysis 2000, version 4.5A, Build 4.5.0.5). For T_d determination, about 7–8 mg sample was placed in an aluminum pan, which was then hermetically sealed using a mechanical device. Similarly, an empty aluminum pan was sealed to use it as a reference. Prior to the measurement, DSC instrument was calibrated with indium in a sealed aluminum pan. Following calibration, in the DSC chamber sample was rapidly cooled to -40°C at a cooling rate of 10°C/min, held isothermally for 3 min. Afterwards, sample was heated to 200°C at a heating rate of 5 °C/min. DSC data were recorded and analyzed by DSC-Trios 5.1.1v software (TA Instruments, USA).

7.3.2.8. SPME/GC–MS characterization of volatile fraction

Qualitative flavor profiling of SML extracts and the microcapsules was performed by solid-phase microextraction/gas chromatography coupled to mass spectrometry (SPME/GC-MS) based on the method described by Chang et al. (2022) with slight modification. Briefly, 0.50 g of SML protein extracts were transferred into a 10 mL glass vial and sealed with a rubber septum. Vials were incubated for 10 min at 60°C in a heating bath. Afterwards, carboxen®/polydimethylsiloxane coated SPME fiber needle (75µm, Supelco-504831, Sigma-Aldrich, Darmstadt, Germany) was inserted into the vial. Prior to the insertion, SPME fiber was conditioned at 250°C for 30 min in GC (Hewlett Packard HP 5890, Las Vegas, NV, USA). Then the grey fiber was exposed to the headspace for 1h to adsorb volatiles at 60°C. After exposure, the fiber was retracted into the needle and transferred into the injector port of GC (Agilent 7683 Series G2613A Injector, China) and kept there for 3 min at 250°C for fiber desorption.

Agilent 6890N Network Gas Chromatograph (G1530N, USA) coupled to Agilent 5973 Network Mass Selective Detector (MSD G2570A) was used for GC-MS analysis. Separation was done in a DB-Wax column (30 m × 0.25 mm, 0.25 µm film thickness—Agilent J&W GC column, USA).

The temperature gradient was as follows: starting temperature 40°C, followed by an increase of 45°C /min to 85°C, then by 3°C /min to 135°C and held for 1 min, next by 1°C /min to 160°C and held for 1 min, afterwards by 9°C /min to 200°C, and lastly by 45°C /min to 250°C and held for 12 min. The carrier gas was Helium (flow rate 2 mL/min). The column effluent was transferred to the mass detector, operated in electron impact (EI) ionization mode at 70 eV. Ion source temperature was 230°C. Selecting ion monitoring mode was applied with scan time and segments setting from 4 to 63 min and scanning m/z from 40 to 350. The volatile compounds were identified by analyzing raw data acquired by GC–MS using Agilent MSD Productivity ChemStation (E.02.02.1431). The min match score > 80 and area > 1% were the main criteria applied for identifying the compounds.

7.3.2.9. Surface hydrophobicity (H_0)

Spectrophotometric determination of the surface hydrophobicity of SML protein extracts was performed based on the Coomassie Brilliant Blue (G-250) (CBBG) dye binding principle given by Cao, Zhao and Xiong (2016). Briefly, 5% (w/v) protein solution was prepared in 20mM phosphate buffer (pH 6). Afterwards, 300 µL of 0.1 mg/mL CBBG (prepared in ultrapure water) was added to the 1.2 mL of 5% (w/v) protein solution and 1.2 mL of phosphate buffer solution (control sample). Then the sample and control were vortexed for 3 min followed by centrifugation (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany) at 2000 ×g for 10 min. Absorbance of the obtained supernatant was collected at 585 nm using a spectrophotometer (Ultrospec 1000, Biochrom, Cambridge, England) and surface hydrophobicity was calculated by equation (7.5).

$$\text{CBBG bound } (\mu\text{g}) = 30 \mu\text{g} \times \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \dots\dots\dots (7.5)$$

7.3.2.10 Water and oil holding capacities

Determination of water holding capacity (WHC) and oil holding capacity (OHC) was done following the method of Wang, Zhang, Xu and Ma (2020) with slight modification. 0.2 g SML protein powder was mixed with 6 mL distilled water (for WHC) and sunflower oil (for OHC), incubated for 10 min at 30°C, and centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher

Scientific, GmbH, Osterode am Harz, Germany) for 10 min at 8000 ×g. After removing the supernatant, the residue was weighed and WHC/OHC were calculated and expressed as the ratio of oil/water absorbed per gram of the tested sample.

7.3.2.11. Foaming property

Foaming capacity (FC) and stability (FS) of SML protein fraction was measured by homogenizing (Homogenizer 850, Fisher- brand, USA) 15 mL of 1% protein solution in water at 20000 rpm for 2 min (Wang, Zhang, Xu, & Ma, 2020). FC was measured by determining the foam volume at 0 min while FS was measured based on the foam volume after 30 min.

7.3.2.12. Emulsifying capacity and stability

15 mL of 1% (w/v) of SML protein solution, prepared in distilled water (pH 7.0), was mixed with 5 mL sunflower oil, and homogenized (Homogenizer 850, Fisher- brand, USA) for 2 min at 10000 rpm. About 50 µL of emulsions were drawn from the bottom at 0 min and 30 min and diluted with 5 mL of 0.1% SDS solution. The absorbances were measured at 500 nm in a spectrophotometer (Ultrospec 1000, Biochrom, Cambridge, England). The emulsion capacity (EC) and emulsion stability (ES) was calculated by equations (7.6) and (7.7), respectively (Cattan et al., 2022; Naik, Natarajan, Modupalli, Thangaraj, & Rawson, 2022).

$$EC (m^2/g) = \frac{2 \times 2.303 \times A \times DF}{C \times L \times \theta \times 10000} \dots\dots\dots (7.6)$$

$$ES (\%) = \frac{EC \text{ at "30" min}}{EC \text{ at "0" min}} \times 100\% \dots\dots\dots (7.7)$$

With, A, Absorbance; DF, Dilution factor (100); C, Concentration of sample (g/mL); L, optical path length of cuvette (1.25 cm); θ, Oil fraction (0.25).

7.3.2.13. Total antioxidant capacity

Total antioxidant capacity of SML protein extracts was estimated based on the cupric reducing antioxidant capacity (CUPRAC) method (Apak, Guclu, Ozyurek, & Karademir, 2004). A plate reader (SpectraMax® i3x Platform, Multi-mode microplate reader, Molecular Devices, Sunnyvale, California, USA) was used to measure the absorbance at 450 nm and Trolox equivalent was used to express the equivalent result as µmol TE/g DM.

7.3.2.14. Protein solubility and zeta potential

Protein solubility was measured according to Chang et al. (2022) with few modifications at varying pH conditions. The pH of 1% (w/v) protein solution was adjusted in the range of 2–10 using NaOH (1M) and HCl (1M). After adjusting pH, the solution was kept at room temperature

($25 \pm 1^\circ\text{C}$) for 1h while the pH was checked every 20 min. Afterwards, the solutions were centrifuged (Sorvall Legend X1R Centrifuge, Thermo Fisher Scientific, GmbH, Osterode am Harz, Germany) for 10 min at $10000 \times g$. The obtained supernatant was tested for the total protein content using Bradford dye-binding assay (Bradford, 1976) and the results were expressed as mg BSA/g DM. Supernatants were additionally used to measure the zeta potential using the NanoBrook Omni instrument (NanoBrook Omni, Brookhaven Instruments Corporation, Holtsville, New York, USA) based on the phase analysis light scattering (PALS) technique.

7.3.3. Statistical analysis

The experimental data were analyzed based on the one-way analysis of variance (ANOVA) using the JMP software (JMP Pro, Version 15.1.0, SAS Institute Inc., Cary, North Carolina, USA). Tukey HSD test was applied to separate the means and to establish significant differences ($p < 0.05$) within the protein extracts.

7.4. RESULTS AND DISCUSSION

7.4.1. Physicochemical properties

7.4.1.1. Protein content and yield

SML protein was extracted based on the alkaline extraction-isoelectric precipitation through maceration as the conventional method and homogenization pretreated ultrasound-assisted extraction as the advanced method. The starting SML biomass had a crude protein content of 43.32 ± 0.08 mg BSA/g DM, that increased around 2.69- to 4.85-fold in the macerated and sonicated protein extracts. While comparing between the extraction methods, sonication provided extracts with significantly ($p < 0.05$) higher protein content than maceration, and so did the protein yield (4.50–8.25%). Although in both maceration and ultrasonication methods, alkaline extraction-isoelectric precipitation approach was applied, however, the ineffective cell disruption can be linked with the lower recovery of protein content for maceration (Pérez-Vila, Fenelon, Hennessy, O'Mahony, & Gómez-Mascaraque, 2024). Knowing that, plant protein are sometimes covalently linked with other macromolecules such as carbohydrates, cellulose, hemicellulose, pectin, etc., that inhibit the release of protein from plant matrices as well as lower their availability in the extracting medium, thus requires an extraction method that is capable of effectively disrupting the cell matrix (Hadidi, Aghababaei, & McClements, 2023). Ultrasonic waves in the alkaline medium generate cavitation, turbulence, and shear forces near the plant matrix that results in the disruption of plant tissue and facilitates protein extraction (Zia, Khan, & Medina Meza, 2023). Regarding the

effect of pH, the alkaline condition of pH9 gave significantly higher protein content (210.21 ± 0.08 mg BSA/g DM) in comparison to pH8 (195.01 ± 0.08 mg BSA/g DM) and pH10 (185.28 ± 0.16 mg BSA/g DM). Similar observations, i.e. higher protein content was obtained when pH of the alkaline extraction was neither too low nor too high, were reported by Hou et al. (2017), Wang, Zhang, Xu and Ma (2020), and Kaur and Bhatia (2022) for protein extraction from respectively rice residue, pea, and radish leaf. Under strong alkaline condition, protein might undergo denaturation, aggregation, Maillard reaction, and hydrolysis, therefore becoming less available (Hadidi, Aghababaei, & McClements, 2023). However, strong alkaline condition in this study i.e., pH10 provided significantly higher protein yield (8.25%) which might be due to the coextraction of other conjugated plant matrix compounds (carbohydrate, mineral, fiber, etc.) as mentioned earlier in this section. Gerliani, Hammami and Aider (2019) found pH10 as more effective to disintegrate the fiber-protein matrix, thus increasing extractability of protein-carbohydrate was obtained from soybean meal.

Table 7.1: Protein content and chromatic properties of sugar maple leaves and their extracted protein.

Attributes	SML	ME_pH9	HE+UAE_pH8	HE+UAE_pH9	HE+UAE_pH10
Protein (mg BSA/g DM)	43.32 ± 0.08^E	116.61 ± 0.08^D	195.01 ± 0.08^B	210.21 ± 0.08^A	185.28 ± 0.16^C
% Yield	—	3.48 ± 0.03^B	4.50 ± 0.58^B	6.75 ± 0.29^A	8.25 ± 0.29^A
L*	91.18 ± 0.14^{AB}	92.55 ± 0.22^A	85.11 ± 2.67^B	88.53 ± 0.61^{AB}	80.56 ± 1.92^C
a*	-7.66 ± 0.07^C	-10.01 ± 0.16^C	-12.36 ± 1.15^{BC}	-16.00 ± 1.02^B	-24.60 ± 1.67^A
b*	6.42 ± 0.15^B	3.27 ± 0.53^B	15.79 ± 1.28^A	15.41 ± 0.17^A	19.99 ± 1.73^A
ΔE^*	10.42 ± 0.09^C	11.12 ± 0.03^C	20.84 ± 1.96^B	22.38 ± 0.15^B	34.42 ± 1.12^A
WI	86.67 ± 0.13^A	87.03 ± 0.09^A	75.89 ± 1.95^B	74.72 ± 0.00^B	62.73 ± 1.03^C
BD (g/cm ³)	—	0.13 ± 0.01^B	0.19 ± 0.02^{AB}	0.31 ± 0.01^A	0.26 ± 0.05^{AB}
TD (g/cm ³)	—	0.23 ± 0.03^B	0.23 ± 0.03^B	0.45 ± 0.01^A	0.38 ± 0.07^{AB}
T _{on} (°C)	67.3	90.15	111.67	114.25	110.14
T _d (°C)	85.02	103.15	123.8	121.74	114.61
T _{end} (°C)	105.2	105.6	131	129.33	121.10

Note: In a row, different uppercase letters (A–E) indicate the significant difference based on the 95% confidence interval among the SML powder and protein extracts.

BD: Bulk density; BSA: Bovine serum albumin; DM: Dry matter; HE: Homogenization; ME: Maceration; SML: Sugar maple leaf; TD: Tapped density; T_{on}: Onset denaturation temperature; T_d: Denaturation temperature; T_{end}: Endset denaturation temperature; UAE: Ultrasound-assisted extraction; WI: Whiteness index.

7.4.1.2. Color and whiteness index

As per the Commission Internationale d'Eclairage (CIE Lab) color system, L* indicates the lightness (100) to darkness (0), a* measures the greenness (when negative) to redness (when positive) with gray value (when zero), and b* measures the blueness (when negative) to grayness

(when zero) to yellowness (when negative) of a sample (Kaur & Bhatia, 2022). Among other parameters, the application array of any plant extracts, concentrates, or isolates in food systems largely depends on its color (Naik, Natarajan, Modupalli, Thangaraj, & Rawson, 2022). Note that, color profile (L^* , a^* , and b^*) of any powder material may vary according to their extraction/isolation condition (extraction method, pH), their particle size, and color pigments present in the system (Akyüz & Ersus, 2021). As per the whiteness index, it can be observed that sonicated protein extracts are relatively darker than the SML powder and macerated extract (Table 7.1). This can be attributed to the coextraction of chlorophyll and carotenoid pigments along with the protein by the action of cavitation and bubble implosion during ultrasound-assisted extraction. In the studies of Chakravorty and Das (2023) and Naik, Natarajan, Modupalli, Thangaraj and Rawson (2022), similar changes in the chromatic values were observed in the sonicated protein extracts from *Parkia timoriana* seed and *Momardica charantia* seed, respectively. Moreover, protein extracts from extreme alkaline condition (HE+UAE_pH10) has significantly higher ($p < 0.05$) green values (-24.60 ± 1.67), and this might be due to the fact that chlorophyll pigment is more stable at higher pH (Koca, Karadeniz, & Burdurlu, 2007). While comparing with one of the commercial green protein sources i.e., green pea protein isolate, the total color differences (ΔE^*) of HE+UAE_pH8 (20.84 ± 1.96) and HE+UAE_pH9 (22.38 ± 0.15) were in line with the ΔE^* values of green pea protein isolates (19.3–20.3) obtained by García Arteaga et al. (2021). This indicates the potential acceptance of SML protein extracts as a food ingredient.

7.4.1.3. Bulk density (BD) and tapped density (TD)

The results of BD and TD of different protein extracts are shown in Table 7.1. The density property of powdered material is important from the point of transportation, packaging, and storage. The BD and TD of ME_pH9 were 0.13 g/cm^3 and 0.23 g/cm^3 ; HE+UAE_pH8 0.19 g/cm^3 and 0.23 g/cm^3 ; HE+UAE_pH9 0.31 g/cm^3 and 0.45 g/cm^3 ; and HE+UAE_pH10 0.26 g/cm^3 and 0.38 g/cm^3 respectively. Sonicated protein extracts were not significantly different from each other for BD and TD while macerated extracts gave lower values for both the densities. This might be due to the comparatively large particle size of macerated protein extracts which was also evident in the scanning electron micrographs and SDS gel. Naik, Natarajan, Modupalli, Thangaraj and Rawson (2022) found similar BD of 0.41 and TD of 0.54 from the sonicated protein extracts of bitter melon seed. Based on the Carr index (ranging from 18.29 to 43.75), flowability of sonicated protein extracts were expressed as “fair” by comparing the reference values given by Jinapong,

Supphantharika and Jamnong (2008). Information on powders' flowability is important to improve their handling properties, and is inversely proportional to the cohesiveness of powders, meaning the more the powder is cohesive, the less they will be flowable (Akyüz & Ersus, 2021). This cohesiveness can be determined by Hausner ratio, which ranged between 1.2 to 1.7 in this study.

7.4.1.4. Surface hydrophobicity (H_0)

H_0 is often used to evaluate the protein conformational changes and has an effect on the protein functional properties. It indicates the amount of hydrophobic groups exposed on the protein surface that was originally buried inside (Wang et al., 2021). Figure 7.1a shows the surface hydrophobicity of protein extracts obtained by different methods at a varying pH. Among the extrinsic factors (ionic strength, temperature, pH, etc.), pH is very important for the change in protein conformation and surface charge (Hadidi et al., 2024). Proteins have a low/no net charge at pH close to their isoelectric region that promotes protein-protein interaction and precipitation due to the absence of electrostatic repulsion. On the other hand, under alkaline or acidic conditions, proteins have negative or positive surface charges that promotes electrostatic repulsion between protein molecules (Shevkani, Singh, Chen, Kaur, & Yu, 2019). The H_0 of HE+UAE_pH10 was significantly higher ($p < 0.05$) indicating the unfolding of the protein molecule and exposure of hydrophobic groups, originally hidden inside the protein molecules, at higher alkaline condition due to the action of sonication. Moreover, exposure of more hydrophobic amino acids might result in protein aggregation and precipitation, and this was also evident during the gel electrophoresis analysis for HE+UAE_pH10 extract. In contrast, HE+UAE_pH9 showed significantly lower H_0 , thus indicating the structure of this protein extract possibly as a globular form where some of the hydrophobic components are present in the core of the protein (Cattan et al., 2022). While investigating the effect of pH(3–11) on the UAE of whey protein isolate, Gao et al. (2019) found significantly higher H_0 in extreme alkaline condition i.e., at pH11. They explained these increasing hydrophobic residues to protein surfaces due to the unfolding of protein molecules resulting from their electrostatic interactions.

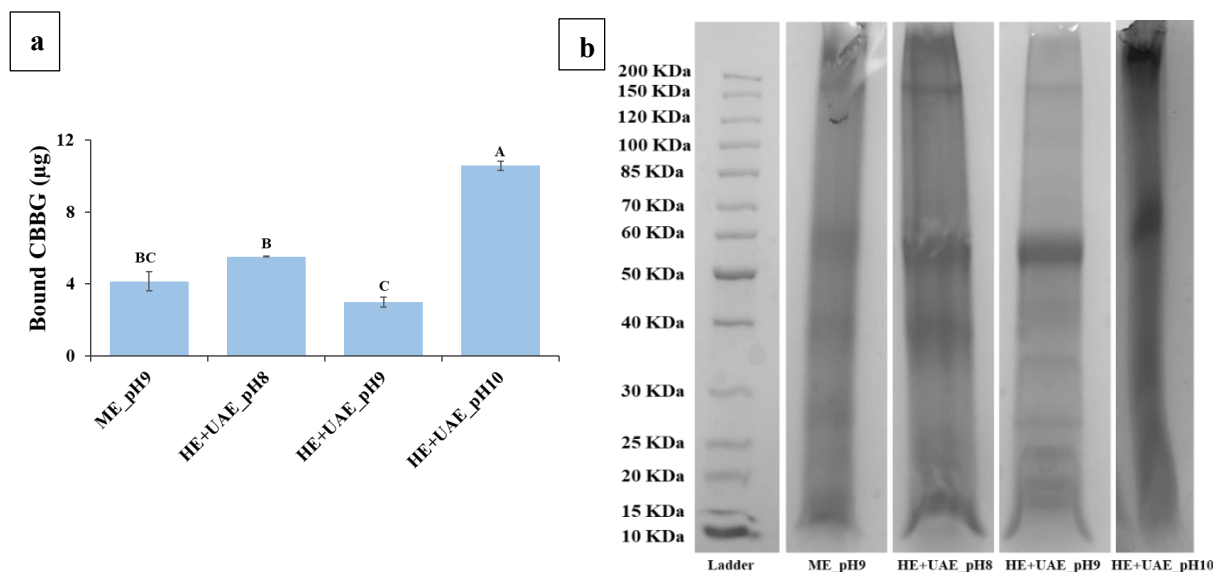


Figure 7.1: (a) Surface hydrophobicity of sugar maple leaves protein extracts. Different uppercase letters (A–C) indicate the significant difference ($p < 0.05$). (b) SDS-PAGE profile of sugar maple leaves protein extracts. *HE*: Homogenization; *ME*: Maceration; *UAE*: Ultrasound-assisted extraction.

7.4.1.5. Molecular mass

The proteins obtained by maceration (pH9) and ultrasonication at varying pH (8–10) were characterized using SDS-PAGE under reducing condition (Figure 7.1b). The most distinctive band for all the samples was around 55kDa, which is associated with the large subunit of RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) (Yu, Kleuter, Taghian Dinani, Trindade, & van der Goot, 2023). RuBisCo is considered as the most abundant soluble plant protein in the world that meets the FAO requirements for essential amino acids (Ortega et al., 2024; Pérez-Vila, Fenelon, Hennessy, O'Mahony, & Gómez-Mascaraque, 2024; Pérez-Vila, Fenelon, O'Mahony, & Gómez-Mascaraque, 2022). As expected, sonicated extracts gave an intense band in comparison to the macerated extract, suggesting the efficiency of UAE towards the extraction of protein. Another band near 40kDa was observed in all the extracts except HE+UAE_pH10, which indicates the proteolytic subunit of large RuBisCo. Pérez-Vila, Fenelon, Hennessy, O'Mahony and Gómez-Mascaraque (2024) also observed the similar band in the leaf protein concentrate from ryegrass. Bands in the range of 25–35kDa in protein extracts of pH9 are associated with the thylakoid membrane (Yu, Kleuter, Taghian Dinani, Trindade, & van der Goot, 2023). The small subunit of RuBisCo at around 12–17 kDa was present in HE+UAE_pH9 and in ME_pH9 with a lesser extent, indicating the solubility of this unit at pH9. Furthermore, bands at around 150–200 kDa which

were observed in all the extracts might be related to the aggregation state of protein (Geetarani Loushigam & Akalya Shanmugam, 2023) or might be due to the protein-phenol covalent interaction which is favored by high pH (Yu et al., 2023).

7.4.1.6. Surface morphology

The microscopic images of extracted SML protein fractions are depicted in Figure 7.2 at a magnification of 150× and 500×. All the protein extracts showed irregular flakes like structures, attributed to the freeze drying effect applied for the drying of the protein extracts obtained through maceration and ultrasonication. Similar observation was reported by Hewage et al. (2024) for fava bean protein isolates. Sonication followed by homogenization produced comparatively smaller particles in comparison to the non-sonicated one (ME_pH9), suggesting the breakdown of the plant matrix by the application of mechanical forces (Geetarani Loushigam & Akalya Shanmugam, 2023). This mechanical force in the form of shear force and microstreaming current can effectively break down the hydrogen bonding, hydrophobic and electrostatic interactions in aggregated proteins, thus leading to the effective reduction of the particle size (Hadidi, Aghababaei, & McClements, 2023).

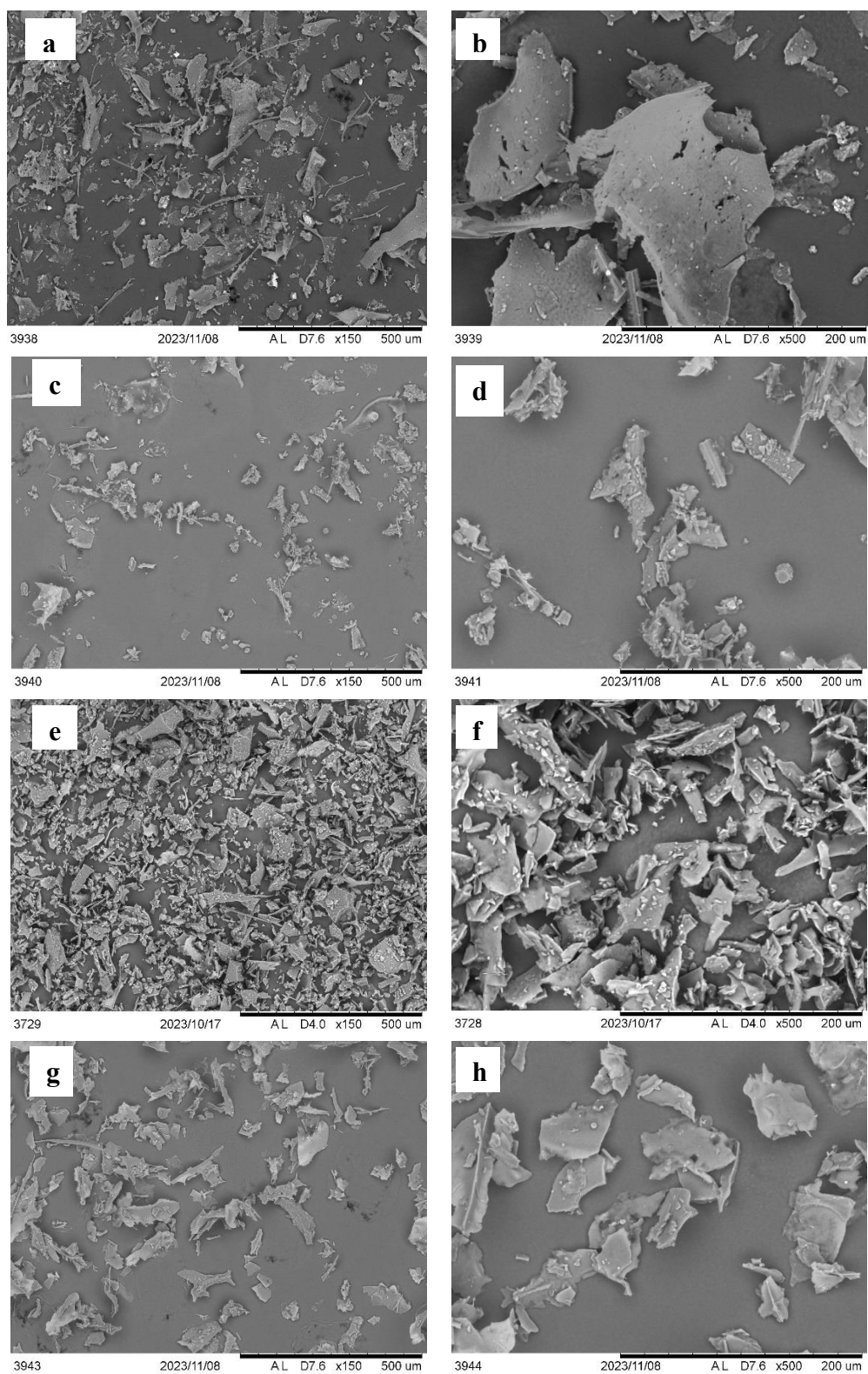


Figure 7.2: Scanning electron micrographs of sugar maple leaves protein extracts: (a-b) ME_pH9, (c-d) HE+UAE_pH8, (e-f) HE+UAE_pH9, and (g-h) HE+UAE_pH10. *HE*: Homogenization; *ME*: Maceration; *UAE*: Ultrasound-assisted extraction.

7.4.1.7. Changes in secondary structures

Based on the FTIR spectra of all SML protein extracts, coexistence of amide I ($1700\text{--}1600\text{ cm}^{-1}$) and amide II ($1575\text{--}1480\text{ cm}^{-1}$) bands indicates the presence of protein in all extracts. However, among these bands amide I has long been the most widely used to analyze the protein secondary structure which is composed of β -sheet ($1612\text{--}1640\text{ cm}^{-1}$ and $1670\text{--}1690\text{ cm}^{-1}$), random coils ($1640\text{--}1650\text{ cm}^{-1}$), α -helix ($1650\text{--}1660\text{ cm}^{-1}$), and β -turn ($1660\text{--}1670\text{ cm}^{-1}$ and $1690\text{--}1700\text{ cm}^{-1}$) (Dong, Wang, & Raghavan, 2021; Perinban, Orsat, & Raghavan, 2023; Yu et al., 2023). Changes in secondary structures of SML protein extracts were analyzed by processing amide I peak into the second derivative of FTIR spectra followed by peak fitting procedure (Hewage et al., 2024), and illustrated in Figure 7.3(a–d). The percentages of secondary structural components of each protein extract were calculated based on the band area of the second derivative spectrum (Figure 7.3e). As can be seen, SML protein extracts revealed those Gaussian bands whose wavenumbers corresponded to α -helix (1651.08 , 1659.62 , 1654.53 , 1650.93 , and 1656.47 cm^{-1}), β -sheet (1673.45 , 1613.49 , 1681.83 , 1616.13 , 1613.93 , and 1680.87 cm^{-1}), β -turn (1666.89 , 1661.21 , 1669.87 , and 1666.7 cm^{-1}), and random coils (1647.14 , 1644.46 , 1641.47 , and 1647.27 cm^{-1}). In addition, the majority of the secondary structures of protein extracts were α -helix followed by random coils, β -sheet, and β -turn. Since all the sonicated SML protein extracts went the same ultrasonic treatment, therefore, the differences in the percentage of secondary structures might be attributed to the differences in pH of the extraction medium. For example, pH9 increased the level of β -structures and random coils at the expense of α -helix than the other pH conditions (pH8 and pH10). This lowering α -helix levels in pH9 indicates the unordering of protein rigid structure by breaking the hydrogen bond, and by doing this it facilitates the protein molecules flexibility (Gao et al., 2019). Overall, it can be said that the combination of sonication and alkaline treatment weakened the ordered and regular structures of proteins and led to secondary structural changes. However, based on the structural changes the applicability of protein extracts might change. For instance, β -structures (β -sheet and β -turn) are more thermally stable and denature at higher temperatures in comparison to the α -helix content (Shevkani, Singh, Chen, Kaur, & Yu, 2019). Therefore, both SML protein extracts obtained in pH9 can be incorporated into the products that will go under heat treatment (e.g., baked goods, plant-based meat alternatives, snacks, etc.). On the other hand, there is a positive correlation between digestive enzymes and β -turn and random coil while β -sheet is negatively correlated with digestive enzymes (Shevkani, Singh, Chen, Kaur,

& Yu, 2019; Zhou et al., 2019). To this aspect, sonicated SML extracts irrespective of pH medium in this study can be used as nutritional supplements or can be applied to the food products of vulnerable groups (e.g., infant, old-aged nutrition), since they contain lesser content of β -sheets.

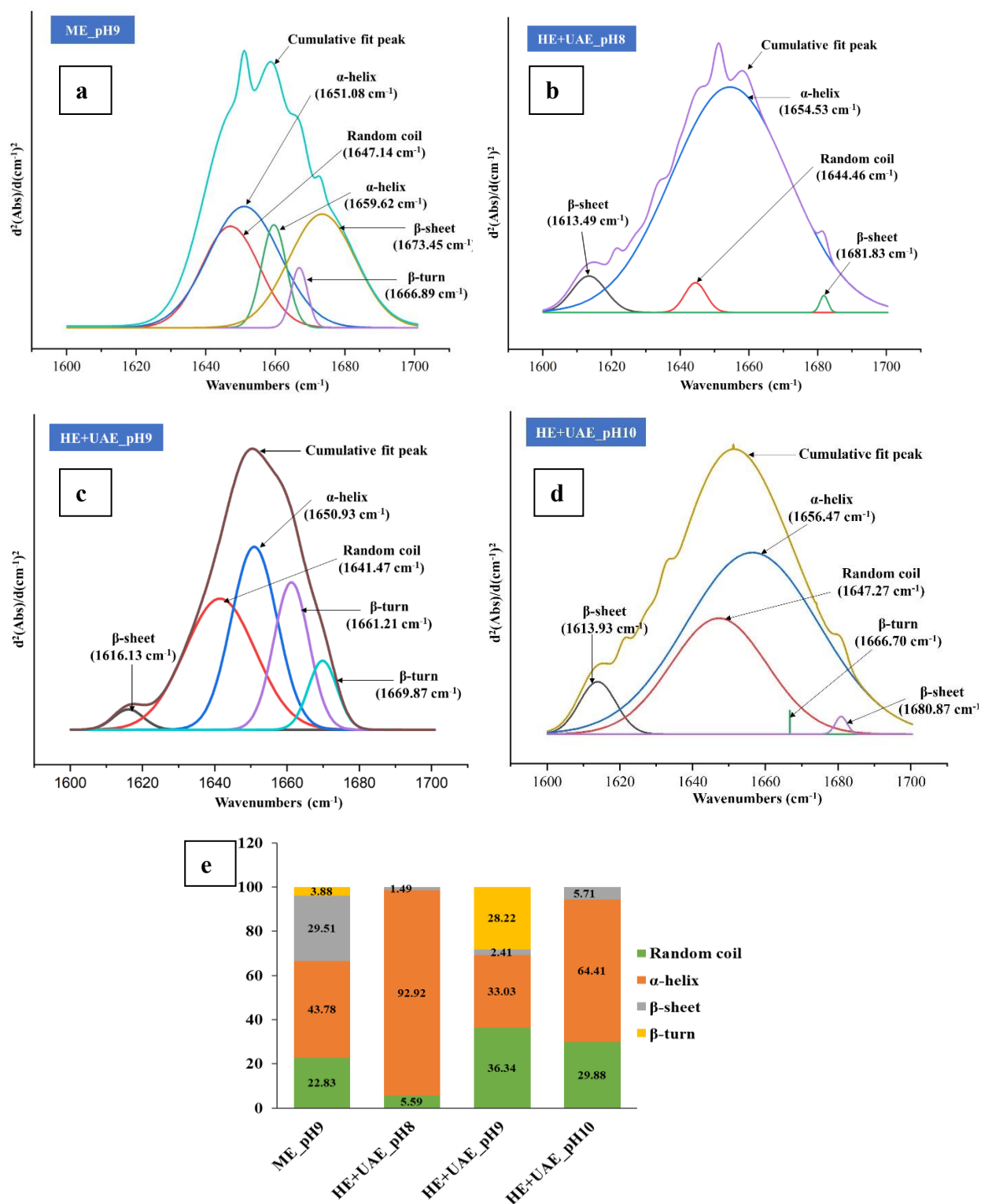


Figure 7.3: (a–d) Structural changes in the protein extracts of sugar maple leaves analyzed by peak fitting of Amide I (1600–1700 cm⁻¹) zone in FTIR spectra and (e) quantification of secondary structure. HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction.

7.4.2. Thermal properties and weight loss characteristics of SML protein

DSC is often applied to study the thermal property of proteins in terms of their stability against denaturation or aggregation while experiencing continuous heat treatment (Wang et al., 2021). Protein denaturation is an endothermic process as it involves the dissociation of weak intramolecular bonds (hydrogen bond) and in some cases, covalent (disulfide) bonds (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). Onset temperature (T_{on}), denaturation temperature (T_d), and endset temperature (T_{end}) of native, ultrasound treated, and macerated SML protein are presented in Table 7.1. Differences were observed in the denaturation temperatures of native, macerated, and sonicated protein extracts, wherein sonication increased T_d of SML proteins (from 67.3°C to 114.25°C). Similar observations were reported by Chakravorty and Das (2023) for *P. timoriana* seed protein ($T_{d,sonicated\ extract}$: 84°C and $T_{d,macerated\ extract}$: 80.4°C) and by Wang et al. (2021) for pecan protein (from 80.5°C to 110.1°C). This suggests that ultrasound could possibly dissociate the protein aggregates and provide a stable structure of SML protein by exposing the hydrophobic groups on the protein surface that are deeply buried inside the protein molecule (Li, Yang, Coldea, & Zhao, 2021; Wang et al., 2021).

Thermogravimetric analysis is widely used to determine the thermal stability of protein extracts with an increase in temperature. Similar trend of weight loss for both the native and protein extracts was observed in Figure 7.4. Compared to the SML powder and macerated protein extracts, the weight loss for the sonicated protein extracts was higher. This can be correlated with the structural modification (breakdown of higher molecular weight protein into lower molecular weight) of protein caused by ultrasound. This finding is in accordance with the results of Mir, Riar and Singh (2019) for high intensity ultrasound treated album seed protein isolates. Overall, the weight loss occurred in three steps: 25–200°C, 200–450°C, and 450–700°C, where the former step is possibly associated with the water evaporation and the removal of low molecular weight volatile fraction (Yeasmen & Orsat, 2023a). Weight loss in the second step might be attributed to the breakdown of inter- and intra-molecular hydrophobic interactions, hydrogen bonds, and electrostatic interactions, and to some extent the breakdown of covalent bonds in the protein structure. Finally, the weight loss in the third stage of SML protein extracts indicated the char formation after experiencing oxidation under the air flow (Mir, Riar, & Singh, 2019).

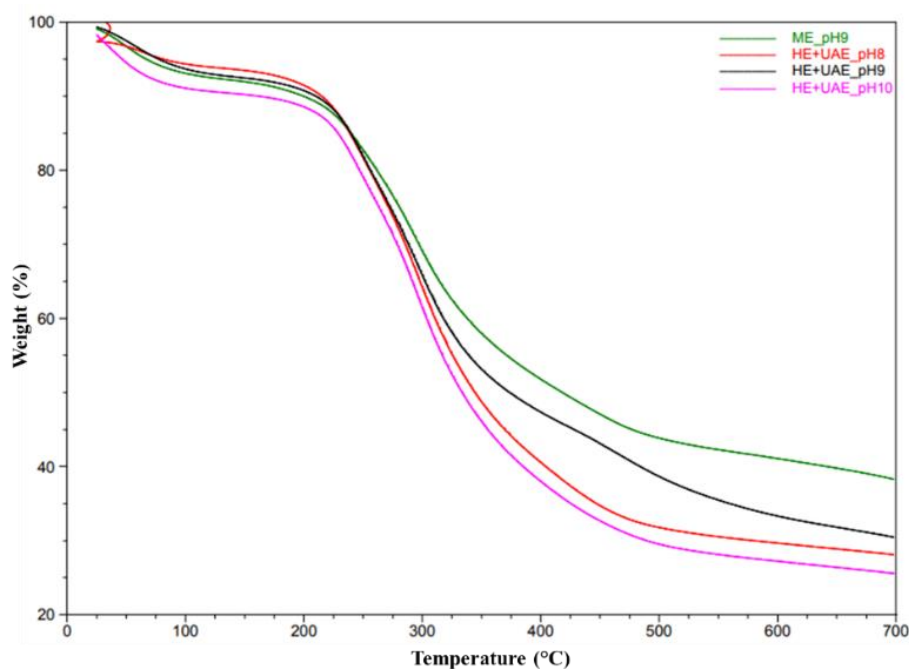


Figure 7.4: Weight loss characteristics of macerated and sonicated SML protein extracts. *HE*: Homogenization; *ME*: Maceration; *SML*: Sugar maple leaf; *UAE*: Ultrasound-assisted extraction.

7.4.3. Volatile fraction

Upon crushing, plant leaves emit a strong odor which is most likely dominated by alcohol, ketone, aldehyde, and ester compounds (Jirovetz, Smith, & Buchbauer, 2002). These aromatic compounds are released from the damaged membranes due to the action of lipoxygenase (LOX) enzyme on lipids and further might be trapped into the hydrophobic pocket of the protein molecule, thus creating the characteristic leafy flavor (Chang et al., 2022). This sharp smell of leaf, on the other hand, acts as a defensive mechanism against predators. Based on the preset screening criteria (concentration higher than 1.0%, calculated as % peak area using GC-MS with a DB-Wax column), a total of 9 compounds including two aldehydes and four alcohols were identified (Table 7.2). Among the identified compounds, the characteristic green aroma, caused by 3-hexen-1-ol and nonanal, was found higher in sonicated protein extracts than in that of macerated extracts. This can be correlated to the ultrasonic efficiency to break down the cell structure, consequently, favoring more action of LOX towards the cellular lipids (Khalil et al., 2023). However, information on the aroma profile of leaf protein extracts will help to understand their possible application arena as well as masking of unwanted compounds, if necessary.

Table 7.2: Aroma compounds of sugar maple leaf protein extracts characterized by SPME/GC-MS.

Identified compounds	Odor characteristics	Peak area			
		ME_pH9	HE+UAE_pH8	HE+UAE_pH9	HE+UAE_pH10
Cyclohexasiloxane	Odorless	700893	1448184	3359321	34925
Benzene	Sweet, petroleum like	2755734	9808317	11784952	201235
Acetic acid	Vinegar	1274240	129467	269399	–
Benzaldehyde	Bitter, almond	177202	847414	705025	59856
Benzyl Alcohol	Herbal	109421	142899	83225	–
3-Hexen-1-ol	Grass-green	107534	1835672	5138404	507640
Cyclohexanol	Camphor	20727	62165	352655	–
Nonanal	Green, fruity	–	4404708	–	396239
Phenylethyl Alcohol	Rose like	–	282923	77538	19839

HE: Homogenization; ME: Maceration; UAE: Ultrasound-assisted extraction.

7.4.4. Techno-functional properties

The analysis of the physico-structural properties (color, SEM, surface hydrophobicity, FTIR analysis, and DSC thermograms) of SML protein extracts indicated that ultrasound could modify the compact structure of protein by leading to conformational changes, while comparing with macerated protein structures. As a result, the protein structure became more flexible which was believed to influence the functional properties of protein (Wang et al., 2021). Functional properties including fluid binding capacity, foaming capacity, emulsifying capacity, solubility, and gelling capacity make plant protein attractive to be applied as food ingredients.

7.4.4.1. Fluid binding capacity

Fluid binding capacity indicates the amount of adsorbed/absorbed water/oil per gram of protein. It is an important parameter for controlling the retention and release of fluids from foods to ensure their desirable texture and mouthfeel (Hadidi, Aghababaei, & McClements, 2023). As can be seen from Table 7.3, extreme alkaline condition (pH10) gave inverse results for fluid (WHC and OHC) binding capacity. The improved OHC could be related to the exposure of hydrophobic groups that was discussed above. Generally, proteins with higher hydrophobicity tend to have a better fat absorption capacity as the non-polar side chains of amino acids can easily interact with the aliphatic chain of lipids (Li, Yang, Coldea, & Zhao, 2021). Moreover, the suitability of protein application in a particular food sometimes depends on the ratio of WHC and OHC. Note that, the existence of non-protein substances (phenol, carbohydrate, lipid, etc.) interacting with protein might change the fluid binding capacity, thus indicating the proper isolation of protein from the plant matrix.

Table 7.3: Functional properties of sugar maple leaves protein extracted by maceration and ultrasonication.

Attributes	ME_pH9	HE+UAE_pH8	HE+UAE_pH9	HE+UAE_pH10
WHC (g/g)	3.52 ± 0.89 ^A	3.93 ± 0.94 ^A	4.19 ± 0.95 ^A	1.04 ± 0.05 ^B
OHC (g/g)	8.70 ± 0.63 ^A	9.66 ± 1.58 ^A	3.63 ± 0.33 ^B	11.31 ± 0.11 ^A
FC (%)	40.60 ± 0.69 ^B	51.95 ± 0.06 ^A	32.79 ± 0.63 ^C	50.89 ± 0.51 ^A
FS (%)	74.80 ± 0.23 ^C	87.23 ± 0.65 ^A	80.90 ± 1.04 ^B	83.86 ± 0.64 ^{AB}
EC (m ² /g)	10.72 ± 0.02 ^B	9.82 ± 0.20 ^C	11.94 ± 0.02 ^A	7.97 ± 0.03 ^D
ES (m ² /g)	84.66 ± 0.06 ^{AB}	62.71 ± 1.39 ^C	81.67 ± 0.10 ^B	85.38 ± 0.17 ^A
AOX (mM TE/g DM)	1.18 ± 0.001 ^B	1.24 ± 0.003 ^A	1.24 ± 0.001 ^A	1.14 ± 0.001 ^C

AOX: Antioxidant capacity; DM: Dry matter; EC: Emulsifying capacity; ES: Emulsifying stability; FC: Foaming capacity; FS: Foaming stability; HE: Homogenization; ME: Maceration; OHC: Oil holding capacity; UAE: Ultrasound-assisted extraction; WHC: Water holding capacity.

7.4.4.2. Foaming capacity (FC) and foaming stability (FS)

Foaming capacity is important in both food manufacturing and drug formulations (Li, Yang, Coldea, & Zhao, 2021). As shown in Table 7.3, FC increased up to 51% in sonicated extracts while comparing with the extracts obtained from maceration. This increase in FC can be attributed to the partial unfolding of protein structure upon sonication treatment (Wang, Zhang, Xu, & Ma, 2020). Consequently, sonication exposed more hydrophobic groups in the protein surface which enables protein to spread on the air-water interface. FS was also found higher in sonicated extracts, which is rational, as FS depends on protein concentration, hydration and molecular interactions (Li, Yang, Coldea, & Zhao, 2021).

7.4.4.3. Emulsifying capacity and stability

Plant protein nowadays has gained potential attention to be used as an emulsifying agent as protein has the capacity to rapidly adsorb to oil droplet surfaces. It is capable of retarding creaming by reducing the interfacial tension among the oil droplets. Moreover, it can prevent the flocculation of oil droplets by providing strong steric and electrostatic repulsive forces between the droplets (Hadidi, Aghababaei, & McClements, 2023). Sonicated extract at pH10 gave significantly lower EC (7.97 ± 0.03 m²/g) while a lower pH offered significantly higher EC (11.94 ± 0.02 m²/g) and that could be connected with the higher protein concentration, reduced molecular weight, and lower H_0 of HE+UAE_pH9 extract (Cattan et al., 2022).

7.4.4.4. Antioxidant (AOX) capacity

SML protein extracts expressed antioxidant capacity, that was evaluated based on the measurement of cupric ion reduction method. Among the protein extracts, the least AOX capacity

was exhibited by HE+UAE_pH10 extracts (1.14 ± 0.001 mM TE/g DM). The obtained AOX capacity might be related to the co-extraction of non-protein molecules such as phenolic compounds along with protein extraction. Previous studies reported that SML contained a good amount of phenolic compounds having antioxidant properties (Yeasmen & Orsat, 2023a, 2023b). Bioactivity of antioxidant compounds is influenced by pH which is also evident in this study. In the study of Yu et al. (2022), the antioxidant capacity of aqueous phenolic extracts of orange peel was higher at pH7 while lower at pH10.

7.4.4.5. Protein solubility and zeta potential

Protein solubility is an important functionality for the successful incorporation of protein in varying aqueous-based food products including beverages and plant-based milk. For other protein functionalities, namely emulsifying and foaming properties, water solubility of protein is additionally considered as a prerequisite property (Chang et al., 2022). Due to providing higher protein content along with good physicochemical, thermal, and functional properties, the percentage water solubility of HE+UAE_pH9 extract was measured as a function of pH (2–10) and shown in Figure 7.5. In addition to water solubility at different pH, surface charge in the form of zeta potential was also measured (Figure 7.5). Results showed that protein solubility increased with the increasing pH where higher solubility was observed at pH9. The increasing solubility of sonicated protein extracts can be due to alteration of protein conformation in a way that ultrasonication might expose the hydrophilic parts of amino acids. Consequently, the higher electrostatic interactions resulted in protein-water interaction, thus increasing the protein solubility (Naik, Natarajan, Modupalli, Thangaraj, & Rawson, 2022). Similar observation was also obtained in the study of Chang et al. (2022). However, at pH10 solubility further decreased which might be linked with protein aggregation due to containing more surface hydrophobic groups (Figure 7.1). Regarding the zeta values, the protein solubility curve showed a good correlation with the zeta potential curve. Zeta values of protein solution under alkaline condition ranged between -17 to -26 mV, indicating a comparable electrical stability of SML protein fraction while compared with green pea (> -20 mV) (Chang et al., 2022), cowpea (-44 mV) (Geetarani Loushigam & Akalya Shanmugam, 2023).

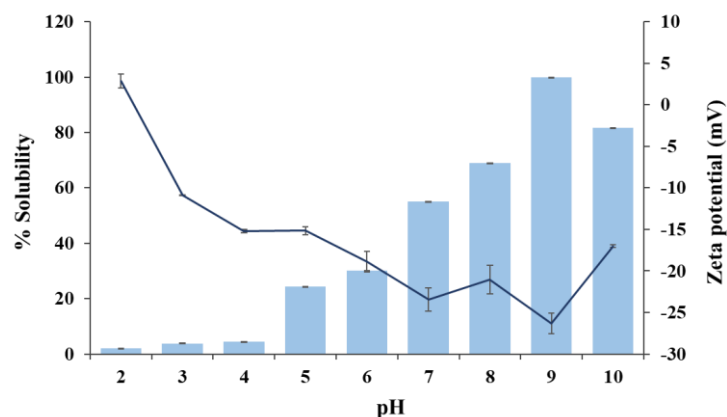


Figure 7.5: pH-dependence of percent protein solubility (%) and surface charge (zeta potential) at pH 2–10 of sugar maple leaves protein (HE+UAE_pH9). *HE: Homogenization; UAE: Ultrasound-assisted extraction.*

7.5. CONCLUSION

This study showed the combined effects of alkaline pH and ultrasound on the extraction of plant protein from sugar maple leaves. Then the extracted SML protein fractions were characterized for their physicochemical, thermal, volatile, and functional properties. Sonication enhanced the particle breakdown and modified the protein structural conformation, confirmed by the SEM, SDS-PAGE, and FTIR studies. Moreover, increasing thermal stability was observed in the sonicated extracts. In terms of protein functionalities, no major significant differences were observed between sonicated and macerated extracts, but the role of pH was seen to have an impact on functionalities. Overall, ultrasound treated protein extracts obtained at pH9 gave good solubility, electrical stability, emulsion capacity ($81.67 \pm 0.10 \text{ m}^2/\text{g}$), water holding capacity ($4.19 \pm 0.9 \text{ g/g}$) as well as antioxidant properties ($1.24 \pm 0.001 \text{ mM TE/g DM}$). On the other hand, appreciable foaming and oil holding capacity were obtained with protein fractions extracted at pH8 and pH10. Having said that, this study showed the versatility of SML protein fractions to be used as functional food ingredients. However, there is a possibility of being attached/extracted with other non-digestible macromolecules, therefore further studies on the *in vitro* digestibility of SML protein is recommended.

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CHAPTER 8: GENERAL DISCUSSION, CONCLUSION, AND FUTURE STUDIES

8.1. GENERAL DISCUSSION

Transition from an economy largely based on fossil resources to one based on renewable raw materials processing, in terms of sustainability, is a challenging task. Among all the known sustainable resources (geothermal, solar, wind), only plant biomass derived from agriculture and forestry represents an accessible and renewable source of bioactive compounds (e.g., phenolics, protein) (Talmaciu et al., 2015). Approximately 3% of Canada's total forests are composed of native Canadian maple species of which the best-known being the sugar maple (*Acer saccharum*). Considered as waste biomass in the autumn, sugar maple leaves have been either ground into compost or burned directly which results in serious economic (disposal of biomass represents a cost to the government) and environmental (such as CO₂ emissions, air pollution, loss of soil moisture, loss of balance in ecosystem, and others) concerns (Saini, Panesar, & Bera, 2019). On the other hand, the largely available low-cost maple biomass can be a key contributor to the circular economy since it contains phenolic compounds (PCs) and proteins for which pharmaceutical, nutraceuticals, cosmetics, and functional food industries have shown a great deal of interest (Ameer, Shahbaz, & Kwon, 2017b; Belwal et al., 2019b; Gil & Tuberoso, 2021). To this end, the crucial stage involves extracting valuable components, which entails effectively separating and retrieving them from plant matrices (Belwal et al., 2018; Lopez-Fernandez et al., 2020). At the same time, the main challenge lies in the isolation of plant-based ingredients from the plant matrix as they are interlinked with other macromolecules such as carbohydrates, cellulose, and hemicellulose. Regarding protein, poor solubility, foaming, emulsifying, and gelling capabilities, and the presence of anti-nutritional substances (e.g., pectin, cellulose) with a noticeable unpleasant flavor, are restricting the use of plant protein in food preparations and these are even intensified by the rigorous conventional protein extraction conditions (Loushigam & Shanmugam, 2023).

The goal of this study was to valorize the sugar maple leaves (SML) biomass by combining the knowledge of green extraction (UAE and MAE), process optimization, and encapsulation. The general and specific objectives of this entire project are as follows. To know the extent of phenolic compositions in the SML and to optimize their extraction in terms of solvent type, concentration, solid-to-solvent ratio, homogenization time and speed. Throughout this study, the physicochemical characterization of macerated SML extracts together with the LC-ESI-Q-TOF-MS/MS-based phenolic profiling were performed.

Phenolic composition varies along with variable factors namely temperatures, UV index, rainfall, duration of exposure to sunshine, soil nutrient conditions, among others. Therefore, a study was conducted to see how seasonal dynamics influence the constituents of PCs in sugar maple leaves over their growth cycle where the conclusion was drawn relying on the thorough investigation associated with their physical, microstructural, chemical, mineral, phytochemical, and bioactivity analyses.

Apart from the environmental conditions, it was reported that phenolic composition varies as a function of the plant parts. Thus, a study on the spatial distribution of PCs, antioxidant, and antimicrobial attributes in the microwave-assisted extracts of different sugar maple parts was conducted and compared with the extracts obtained through conventional extraction (i.e., maceration). Additionally, the thermal behavior of different parts was investigated based on thermogravimetric and differential calorimetric analysis.

There is a possibility of phenolic extracts' degradation by either oxidation, condensation, or polymerization when exposed to oxygen, light, heat, enzymatic activity, and pH differences (Mzoughi et al., 2023; Papoutsis et al., 2018; Pashazadeh et al., 2021). To address this concern, an a study concentrated on the microencapsulation of ultrasound-assisted SML phenolic extracts which were compared with conventionally macerated extracts. The encapsulated microparticles were then examined for their physical, physicochemical, structural, thermal, volatile, and *in vitro* bioaccessibility attributes. Storage stability of the encapsulated extracts was additionally investigated for 50 days.

In addition to phenolic compounds, SML biomass was also utilized to extract protein. To achieve this objective, homogenization pretreated ultrasound-assisted extraction of protein fraction was performed, where the extracted protein fractions were analyzed to see changes in the secondary structures, surface hydrophobicity, density, surface morphology, volatile fractions, as well as the techno-functional properties (fluid binding capacity, foaming capacity and stability, emulsion capacity and stability, antioxidant activities).

Overall, the body of this whole work showed the potential to utilize SML biomass based on its richness in phenolic compounds and proteins. While the experiments were accomplished on a laboratory scale, data obtained from this experimental body of evidence indicate that there is a huge potential for forestry biomass to contribute to the circular economy.

8.2. CONCLUSION

This PhD study aimed at two major objectives: (i) isolation and characterization of PCs and protein from SML biomass using conventional and green extraction methods, and (ii) encapsulation of phenol rich SML extracts and their stability assessment. To fulfill these objectives five research experiments were designed and conducted. The findings of these experiments are summarized below.

After a comprehensive review on the leaves and their PCs, this PhD study started its first experiment to explore the array of PCs present in collected SML. To this end, the most conventional extraction method (maceration) was applied to extract the PCs from SML. A full factorial experiment enabled us to determine the optimal extraction conditions as 61.63% ethanol concentration and 1:38.22 g/mL solid-to-solvent ratio for recovering maximum phenolic content from SML with high antioxidant activity. Effect of high-speed homogenization (HSH) as a pre-treatment condition was investigated where the results confirmed HSH as an effective pre-treatment technique to promote the maceration efficiency. In parallel, characterization of SML was done using colorimeter, SEM, FTIR and LC-ESI-Q-TOF-MS/MS. SEM images of SML indicated that macerated leaves contained smooth surfaces with porous structure whereas non-macerated one had undamaged but rough surfaces. FTIR showed the presence of PCs in the SML and the compounds were confirmed by LC-ESI-Q-TOF-MS/MS as 81 detectable PCs were isolated in the extracts. Our findings showed that, sugar maple leaves are a prominent source of bioactive compounds which reveals the biological potential of this plant towards the development of newer functional ingredients, that could be useful for developing localized community economic opportunities, and consumer health. In addition, HSH assisted maceration was found as an effective and simple method for the exploitation of PCs from SML.

Once it was evident from the first research chapter that SML is a good source of PCs, the second experiment was set to unravel the phenolic mapping of sugar maple leaves and their associated antioxidant capacities throughout the annual growth cycle of SML. As per the results, distinctive color changes in sugar maple leaves (SML) were observed during leaf expansion (May) and during the onset of leaf defoliation (October) along with its annual growth cycle. Microstructural analysis confirmed that both the freeze-drying and extraction methods caused surface roughness/irregularities whereas leaves which were subjected to extraction had more surface ruptures/damages/pores. All seasonal SML samples contained considerable phenolics,

flavonoids, and exhibited high antioxidant capacity as can be seen from the FTIR spectra and HPLC based quantification. The antioxidant activity can be related to the contents of both flavonoids and phenolics. Among the quantified bioactive compounds, quinic acid, ellagic acid, rutin, and catechin presented higher contents compared to others. Moreover, principal component analysis was efficient to show the effect of seasonal dynamics on the phenolic mapping of SML, giving evidence of positive effect of higher temperature, UV index, solar radiation and negative effect of nitrogen content on the phenolic biosynthesis. To conclude, SML can provide a wide array of applications as ingredients, for instance, if the target is to fortify food products with high mineral containing ingredients, then SML–O would be the best choice. On the other hand, taking antioxidant potentialities into account, SML collected from May to July should be preferred.

Next to the effect of environmental conditions, the third experiment was conducted to comprehensively investigate the spatial distribution of the physicochemical, thermal, phytochemical, antioxidant, and antimicrobial attributes among different morphological parts (leaves, flowers, fruits, seeds, barks, and twigs) of the sugar maple tree. Aerial parts (flower, fruit, seed, bark, twig, and leaf) of sugar maple can supply compounds with bioactive potential, that can act as preservative and antioxidant additives. When comparing the performance of microwave-assisted extraction and maceration, microwave-assisted extraction provided comparable, and to some extent the best results, for the extraction of phenolics. Using resources that are circularly sustainable might be an effective choice to create a beneficial impact on the agri-food economy. Among all the studied parts of sugar maple biomass, leaves can be chosen as a sustainable source where overexploitation is believed not to be an issue as leaves can be easily grown back by the tree in the subsequent growth season. In addition to providing a phenolic fraction, sugar maple leaves can also be a cheap source of plant protein, thus, can be used as a valuable resource and should not be discarded as simple biomass residues. Moreover, the residue generated after each extraction can further be valorized to increase the sustainability of the process. We anticipate a real biorefinery potential for sugar maple biomass through the integration of advanced separation steps (microwave-assisted extraction/ultrasound-assisted extraction/pulsed electric field-assisted extraction/cold plasma-assisted extraction, etc.), in terms of either phenolic compounds and/or protein into the existing sugar maple industry supply chain.

In the microencapsulation study, comparatively higher microencapsulation yield and loading capacity were obtained in freeze-dried microparticles. Both tested encapsulation methods

provided flowable microparticles with low cohesiveness. Particle size distribution of both conditions showed monomodal distribution with a poly dispersity index of less than 0.33, indicating that the microparticles produced in this study are in between the monodisperse and polydisperse conditions. DSC thermograms showed the effectiveness of the encapsulation process by indicating the rise in glass transition temperatures in all the microparticles. SPME/GC-MS-based qualitative characterization of volatile fraction indicates the effectiveness of the encapsulation process against masking the unwanted flavor. *In vitro* digestion study showed greater release of PCs in the stomach phase, whereas spray dried microparticles gave more release percentage than the freeze dried encapsulated phenolic extracts. Storage stability of the microparticles showed the preservation of antioxidant capacity of SML phenolic compounds and suggested that the phenolic microparticles can be stored for at least a month without affecting their stability.

To address the current rising demand for alternative plant proteins, the last experiment was conducted to extract the SML protein based on the alkaline-based homogenization pretreated ultrasound-assisted extraction method. Study showed that sonication enhanced the particle breakdown and modified the protein's structural conformation, confirmed by the SEM, SDS-PAGE, and FTIR studies. Moreover, increasing thermal stability was observed in the sonicated extracts. In terms of protein functionalities, no major significant differences were observed between sonicated and macerated extracts but the role of pH was seen to have an impact on functionalities. Overall, ultrasound treated protein extracts obtained at pH9 gave good solubility, electrical stability, emulsion capacity ($81.67 \pm 0.10 \text{ m}^2/\text{g}$), water holding capacity ($4.19 \pm 0.9 \text{ g/g}$) as well as antioxidant properties ($1.24 \pm 0.001 \text{ mM TE/g DM}$). On the other hand, appreciable foaming and oil holding capacity were obtained with protein fractions extracted at pH8 and pH10. Having said that, this study showed the versatility of SML protein fractions to be used as functional food ingredients. However, there is a possibility of being attached/extracted with other non-digestible macromolecules, therefore further studies on the *in vitro* digestibility of SML protein is recommended.

8.3. RECOMMENDATIONS FOR FUTURE WORK

This PhD study showed that the biomass of sugar maple leaves is a promising source of phenolic compounds and protein, where the extraction of phenolic compounds and protein was performed with the help of electromagnetic and ultrasonication techniques. However, while discussing and concluding the research findings, several questions are stemming from these investigations that should be considered in future studies. The following studies are recommended to gain further insights into the biorefinery process of forestry biomass.

1. Life cycle assessment of the emerging technologies for the extraction of phenolic compounds to find out the most suitable one.
2. Stability assessment of the encapsulated phenolic extracts within a real food matrix.
3. Impact of processing and storage conditions on the bio-chemical conversion of the phenolic compounds in food product applications.
4. Application of leafy protein isolate to develop plant-based meat analogue.
5. Assessment of the protein functionality in real food applications.
6. Potential exploration of the utilization of the residual solids obtained after extraction.

CHAPTER 9: BIBLIOGRAPHY

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Phenolic mapping and associated antioxidant activities through the annual growth cycle of sugar maple leaves

Author: Nushrat Yeasmen, Valérie Orsat

Publication: Food Chemistry

Publisher: Elsevier

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Advanced separation of phytochemical and bioactive attributes from various morphological components of sugar maple (*Acer saccharum*) tree

Author: Nushrat Yeasmen, Valérie Orsat

Publication: Food Bioscience

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Microencapsulation of ultrasound-assisted phenolic extracts of sugar maple leaves: Characterization, in vitro gastrointestinal digestion, and storage stability

Author: Nushrat Yeasmen, Valérie Orsat

Publication: Food Research International

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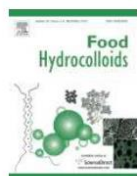
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Pulsed ultrasound assisted extraction of alternative plant protein from sugar maple leaves: Characterization of physical, structural, thermal, electrical, and techno-functional properties

Author: Nushrat Yeasmen, Valérie Orsat

Publication: Food Hydrocolloids

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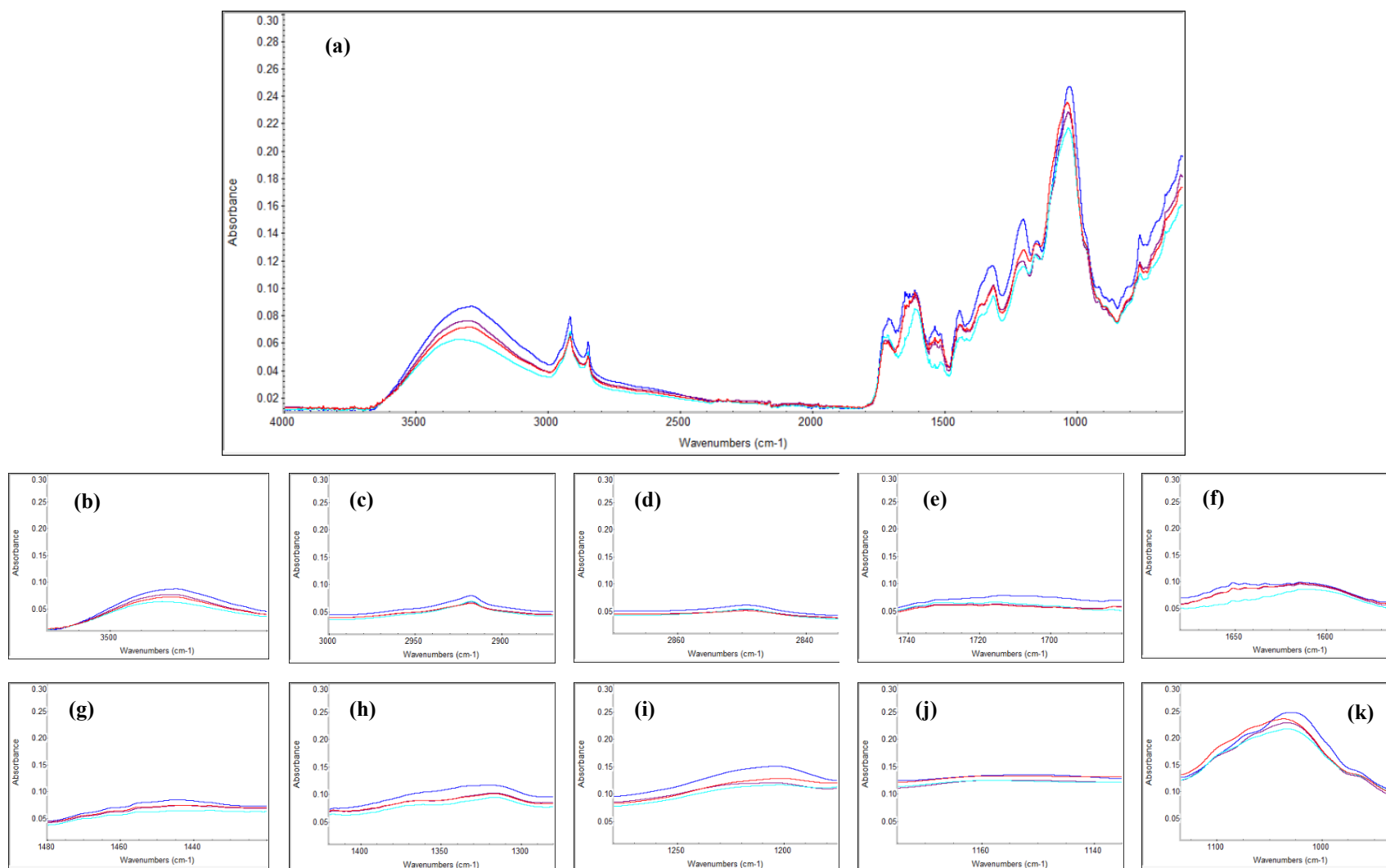


Figure S1: FTIR spectra of sugar maple leaves of different harvesting time (a); Integrated FTIR spectra of the major wavenumbers: 3700-3000 cm⁻¹ (b), 3000-2870 cm⁻¹ (c), 2870-2835 cm⁻¹ (d), 1743-1680 cm⁻¹ (e), 1680-1560 cm⁻¹ (f), 1480-1420 cm⁻¹ (g), 1420-1280 cm⁻¹ (h), 1280-1175 cm⁻¹ (i), 1175-1135 cm⁻¹ (j), and 1135-925 cm⁻¹ (k).

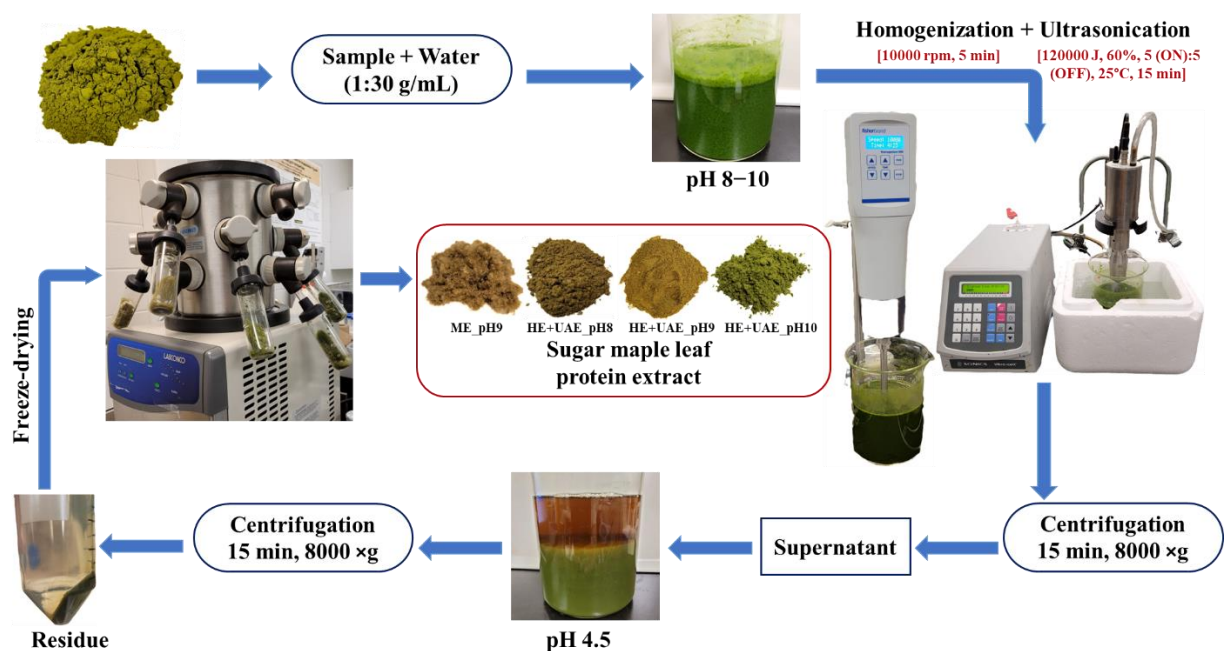


Figure S2: Schematic overview of the homogenization pretreated alkaline based pulsed ultrasound-assisted extraction of sugar maple leaves' protein.