High-intensity, low-frequency ultrasound treatment as sustainable strategy for innovative biomaterials with antioxidant activity from tomatoes, hemp, and hops by-products

by

Colleen Siobhan Celton

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Chemistry
Specialization in Food Science

Carleton University
Ottawa, Ontario

Abstract

Ultrasound is a novel green technology that has been shown to have a multitude of application. This study focused on ultrasound application as a green platform adding values from agricultural waste in two different contexts. Ultrasound was used to increase efficiency of extraction of bioactive compounds such as saponins and phenolics in tomato skin, hemp meal and hops flowers. Ultrasound was used to create stable emulsion gels from byproducts/leftovers of tomato skin, hemp meal and hops flowers to produce green biomaterials.

It was found that ultrasound treatment reduced extraction time for saponin and phenolic acid during tomato skin, hemp meal and/or hops flowers extraction from 24h to 30 min. The measured TPC for tomato, hemp and hops were also respectively 87.22±21.12, 147.39±16.92 g, 450.32±26.47 g of GAE/100g per sample for UAE extraction and for traditional extraction of respectively 89.14±11.61g, 159.42±28.20 and 460.95±48.57 g of GAE/100g of sample. Similar results were obtained from total saponin content. UAE and traditional extraction showed respective TSC of 1443.79±125.24 vs 1337.65 mg of DE/ 100g of sample for tomato, 1511.25±136.98 vs 1618.93±58.90 mg of DE/ 100g of sample for hemp meal extraction, 8037.83±885.45 vs 9847.34±2063.63 mg of DE/ 100g of sample for hops flower extraction. Influence of ultrasound was also shown to have no impact on antioxidative capacity of extract obtained from tomato skin, hemp meal and hops flowers.

Ultrasound treatment was shown to positively impact the overall microscopic structure and qualities of bioplastic such as water activity, % moisture, hardness, cohesiveness, resilience, and springiness index. This study suggests that ultrasound can be used as sustainable non-thermal method for extraction of active saponins and phenolics, enhancing their physico-chemical characteristic in bioplastic materials.

Acknowledgement

As is often said “Nothing worthwhile doing is ever easy” and as such writing my thesis was a very arduous endeavor. One that without the help and support of the people behind me would have been made much more laborious. As such, it would be distasteful of me to not recognize or acknowledge the support that was graciously offered to me throughout all this academic journey.

First, I would like to give my deepest thanks to my supervisor Farah Hosseinian who has been a source of constant support throughout my studies. I fail to imagine how I would have been able to meet the demands of my program without her valuable mentorship. Her unparalleled understanding and compassion helped me through many moments of doubt. Without her help, I fear I would have not been able to complete my work. I am deeply grateful for the opportunities she offered to me. I am also grateful to Rob Nicole, research professor from the bioindustrial process research centre (BPRC) at Lambton College, Sarnia, ON for providing us with samples, his recommendation and expertise.

I would also like to recognize the gracious help offered by Winnifred Akoetey. I can recognize now how much of a blessing her arrival in the lab was, as I would have undoubtedly faced many more struggles without her help. Her sharp wit helped me multiple times when faced with the common problems of the minutiae of the laboratory. Her companionship during the last few semesters of my program were also an immense source of aid.

Similarly, I am beholden to Kelly Dornan who on multiple occasions provided both knowledge and support when I was faced by the inevitable technical problems that so often plague scientific machinery.

I would also like to thank both Rana Haddad and Simon Caton for their help in lab. I was and still am greatly appreciative of the assistance they both provided.

I would like to also extend my thanks to Dr. Tyler Avis as well as Trinda Cripplin for helping in accessing equipment needed in the analysis and preparation of samples. To Jianqun Wang I am grateful for his help in obtaining scanning electron microscope pictures.

For the presence of Colleen Clarke, Dami Daramola and Keegan Harris I am also deeply appreciative as they all were a source of endless silliness and laughter. I am also sincerely grateful for they welcomed me into their office space as one of their own.

I would like to also thank Tasnime Soullane and Florence Dubois for their friendship and comfort in my tumultuous time. Their sustained presence helped me foster the belief that I would be able to finish this endeavour. I would also like to extend thanks to Aster and Mallow for they both provided me with cuddles in times of stress even if sometimes unwillingly.

To conclude I would like to express my deepest gratitude to my parents, Sophie and Eric Celton, for they were a source of constant emotional and mental support throughout this journey. I know that without their help I would never have accomplished even half of the goals that were set in front of me. I am and always will be deeply indebted to them for the help and protection they have always offered me.

To everyone who was part of my journey, Thank You.

Table of Contents

List of Tables

List of Figures

Abbreviations

AAPH - 2,2'-Azobis(2-amidinopropane) dihydrochloride

AUC - Area Under the Curve

DE - Diosgenin Equivalent

ELSD- Evaporating Light Scattering Detector

GAE - Gallic Acid Equivalent

HPLF- High Power Low Frequency

HPLF-US - High Power Low Frequency Ultrasound

HPLC - High Pressure Liquid Chromatography

ORAC - Oxygen radical absorbance capacity

PDA - Photodiode array detector

PLM - Polarized-light microscopy

TFC - Total Flavonoid Content

TPC - Total Phenolic Content

TPA - Texture Profile Analysis

TSC - Total Saponins Content

RPM - Rotation Per Minute

SEM - Scanning Electron Microscopy

UAE - Ultrasound Assisted Extraction

US - Ultrasound

24h - Samples extracted mechanically for 24h using acidified ethanol as solvent

US 30 min - Samples extracted using HPLF US for 30 minutes and acidified ethanol as a solvent

Chapter 1: Introduction

1.1 Ultrasound

Ultrasound waves are a form of acoustic waves. As such, ultrasound can be described as a form of energy. This energy travels through a process of compression and decompression which is known as adiabatic[1]. Therefore, ultrasonic waves can be defined by a set of characteristics. Namely, acoustic intensity, acoustic pressure, particle velocity and particle displacement. These characteristics can directly influence the acoustic velocity or the speed at which the ultrasound waves travel. In other words, the medium in which an ultrasound wave travels influences its attributes[2].

Ultrasound is often defined as a soundwave with a frequency higher than can be perceived by the human ear. This normally starts at a frequency of above 20 kilohertz or 20kHz. However, ultrasonic waves can have a frequency reaching up to a few hundred kilohertz[2]. Apart from frequency, intensity is an important descriptor of ultrasonic waves as it exhibits a broad range of effects. Higher Intensity creates direct mechanical action which can influence chemical reactions or lead to the breaking down of plant material.

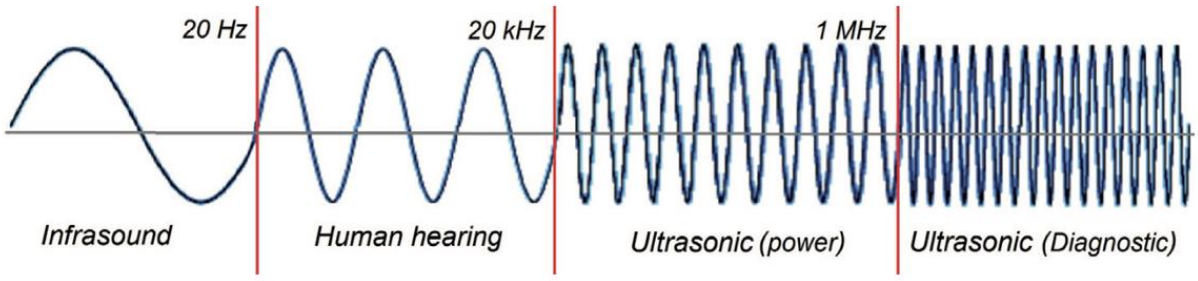


Figure 1.1: *Diagram of various acoustic waves frequencies*[3]

1.1.2 High Intensity Low Frequency Ultrasound or High-Power Low Frequency Ultrasound

The use of ultrasound in medical imaging is a well-studied field. However, recent research has shown novel use for ultrasound, more particularly the use of High Intensity Low frequency ultrasound. Here, High Intensity is defined as any intensity higher than 0.1 $W.cm^{-2}$ [4]. Low frequency is then roughly defined as any frequency between 20kHz and 40 kHz or alternatively 60 kHz. Throughout this research, the UIP500hd processor made by Hielscher a company from Germany was used. This Ultrasound probe was used at a frequency of 20 kHz and 90W meeting both criteria to be qualified of High Intensity Low Frequency Ultrasound.

Figure 1.2: *Ultrasonic processor UIP500hdT (20kHz, 500W) from Hielscher Ultrasonics*

The applications of High-Power Low Frequency Ultrasound are the result of proper exploitation of the combined effect of high Intensity and low frequency of ultrasonic waves on different mediums. According to literature, High intensity cause mechanical effects which can directly affect a multitude of physical processes. On the other hand, chemical processes appear to be influenced by exposure to high amplitude sound waves. This can lead to multiple mechanisms

of action which despite their opposite outcomes, can occur simultaneously. As such, heating, diffusion, agitation, cavitation, streaming, mechanical rupture, and chemical reactions can happen concurrently, allowing improvement in operations such as extractions or particle dispersion[4].

1.1.3 Cavitation: an important mechanism of action of Ultrasound

When using low frequency ultrasound, it is important to discuss the phenomenon of cavitation. The effectiveness of ultrasound in positively impacting a multitude of processes can often be led back to cavitation.

When applying ultrasound on a liquid, the acoustic waves create a cycle of compression and expansion of the molecules that make up the liquid. The expansion can also sometimes be referred to as the rarefaction portion of this cycle. During rarefaction, pressure in the liquid lowers and ultrasound waves are strong enough to influence the intermolecular forces present[5]. Cavitation happens when the pressure drops below a liquid's vapor pressure. This event leads to the formation of vapor bubbles. As pressure increases, these bubbles can collapse to generate shock waves. These shockwaves are stronger when close to their sources but weaken as they move farther away from their source. During transient, or inertial cavitation, the process is direct. Bubbles form increase in size and collapse in that order[3]. Another form of cavitation is also witnessed in ultrasound use: non-inertial cavitation. This type of cavitation is characterized by the same formation of bubbles resulting from lower pressure. However, the increase of pressure caused by the positive half of the pressure cycle created by ultrasonic waves, leads to the shrinking instead of the collapsing of the bubbles. These bubbles will go through a few cycles of growth and shrinkage before reaching a critical growth stage and collapsing. Alternatively, a bubble can also oscillate between a smaller size and its more critical bigger size, also referred to as its resonance size. This creates stable cavitation. As such, inertial cavitation always ends with the implosion of the bubble. However

non-inertial cavitation will create stable cavitation cycles. The process of cavitation takes on average 400 ms.

Lower frequency will often lead to bigger resonance sized bubbles as time between cycle allows for better bubble growth while simultaneously reducing the chances of the collapsing of the bubbles. As larger bubbles require more energy to grow, they proportionally also release more energy when imploding. Furthermore, higher frequency will lead to a higher quantity of bubbles being formed. When an acoustic cavitation bubble collapses close to a solid surface, it often does so in an asymmetric fashion. This can create high speed liquid jets that affect the solid surface. The speed of these liquids jets is approximately 111m/s[6]. The impact of those high-speed liquid jets leads to surface pitting. In addition to the production of high-speed liquid jets, the cavitation of bubbles also causes an increase in temperature linked to the high amount of energy released during the collapse of a bubble[7]. This process is illustrated in Figure 1.3.

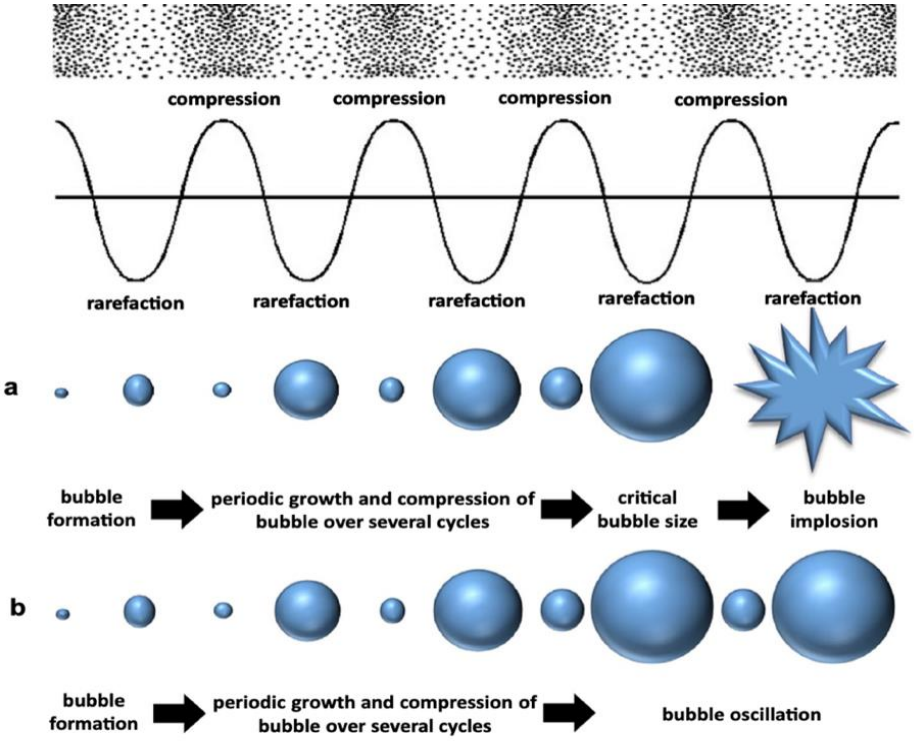


Figure 1.3: *Schematic of inertial (a) and non-inertial (b) cavitation from*[5]

1.1.4 Ultrasound Assisted extraction

Ultrasound Assisted extraction is as the terminology indicates, a type of extraction where ultrasound is used to promote better extraction of compounds from a sample material. The application of ultrasound can be direct, by using a probe which is in direct contact with the extraction medium/extracted media. Alternatively, the application can be indirect. This is done by using a sonication bath where the sample is in a vessel which is in contact with a water bath where sonication is applied. However, indirect application of ultrasound shows limitation in its ability to apply higher power. Direct application of ultrasound can generate power up to a hundred times higher than indirect application. Furthermore, sonication baths are more often limited in the frequency settings they can provide[8].

High-Power Low Intensity ultrasound treatments have been shown to have a positive impact on a variety of processes and have proven to increase the efficiency of extraction processes. Although easily achievable in a laboratory context, industrial scaling of extraction processes presents a multitude of problems. As such, UAE could be an important advancement[9]. UAE has been shown to reduce extraction time of a variety of food related compound including but not limited to polyphenols, anthocyanins, and polysaccharides. In these instances, UAE has been shown to reduce the amount of solvent needed, reduce the time needed, leading to a decrease in overall energy required when compared to traditional solvent extractions[10].

UAE's high efficiency has been attributed to the inherent properties of high energy low frequency ultrasonic waves. Cavitation plays an important role in this case. When cavitation bubbles implode, they create macro turbulences. These turbulences in turn lead to an increase in particle velocity and as such, stronger inter-particle collisions can be noticed. These strong inter-particle collisions affect the biomass that is being extracted by creating perturbation in the matrix

structure and exposing more material to the extraction solvent. Matrix changes can range from fragmentation to erosion which can lead to further detexturation/deterioration of the material. A deterioration of the matrix structure affects both eddy diffusion and internal diffusion by speeding up both of these processes[11]. Alternatively, sonoporation which is the creation of pores in a material due to ultrasound application, in conjunction with the sonocapillary effect of ultrasound application has been suggested as further mechanism of actions for UAE

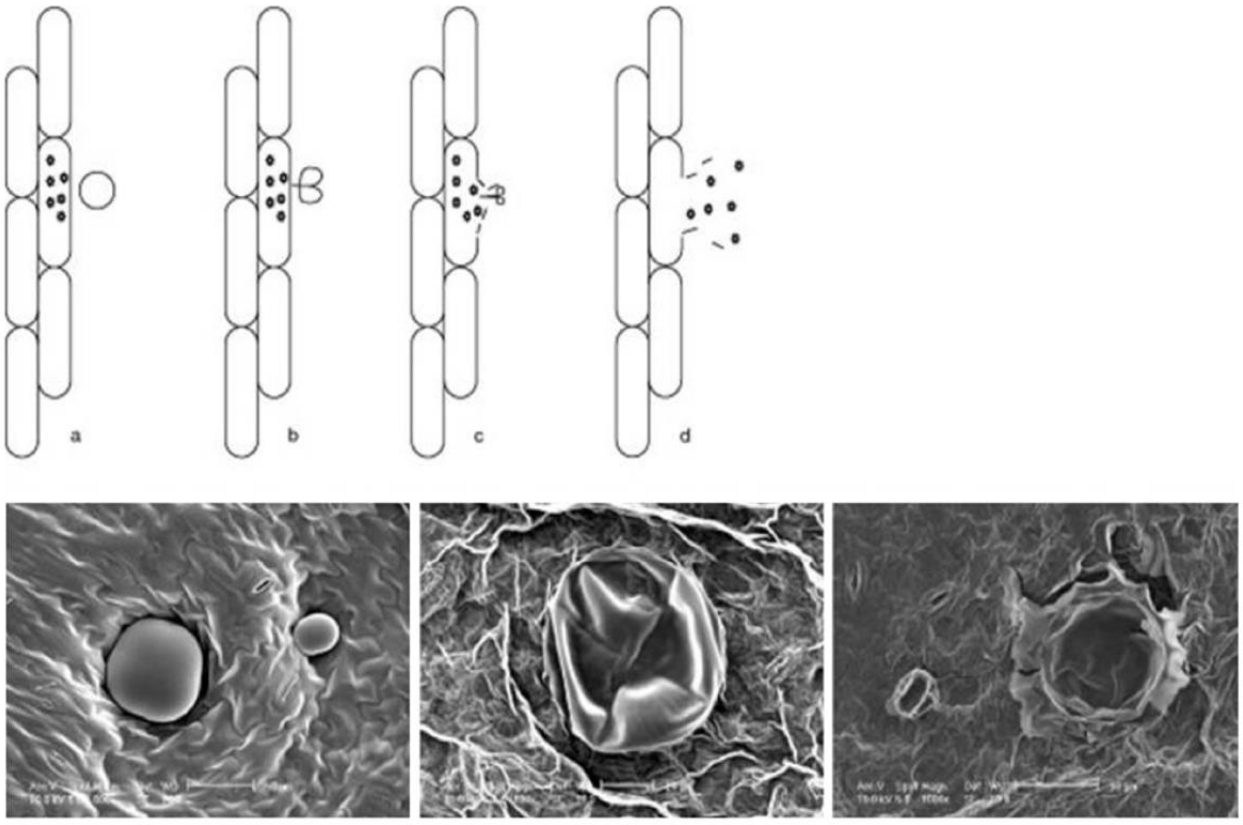


Figure 1.4: *Cavitation and its effect on plant cell (Top Schematic of effect of cavitation bubble on plant material, Bottom Cavitation-bubble collapsing, bottom Microscopic picture of bubble collapsing)*[8]

These theories seem to be confirmed by a study looking at ultrasound assisted extraction of menthol from peppermint. In this study, scanning electron microscope imaging of the plant material showed evidence of two mechanisms of actions as shown in Figure 1.4. First, the diffusion of menthol through the cuticle of the extracted material and secondly the exudation of menthol from the broken and damaged trichome of the plant[12].

Combined, these mechanisms suggest that High-Power Low Intensity ultrasound treatment during extraction would increase the efficiency of the extraction of various bioactive compounds from plant material. The increase in efficiency would be linked to both an increase in yield and diversity of compounds extracted as well as a decrease in the duration of extraction. These benefits of UAE have been proven in alkylresorcinols extraction from wheat bran and phenolic compounds extraction from a variety of plant materials such as peaches, grapes, or strawberries[13].

This capacity of ultrasound to increase the efficiency of extraction of bioactive compounds makes it valuable and presents ultrasound as a sustainable process. Ultrasound assisted extraction can allow for the replacement of organic solvents with less toxic alternatives. Furthermore, ultrasound assisted extraction can be performed with smaller amounts of solvent[14].

1.2 Phenolic Compounds

Phenolic compounds are a wide category of molecules that occur naturally in plants. Up to 4000 phenolic compounds have been identified and new compounds are still being identified[15]. Phenolics are generally the by-product of plant secondary metabolic pathways. They often are the end-product of two pathways: the shikimate pathway and the acetate pathway. As seen in Figure 1.5, through the acetate pathways, amino acids and carbohydrates are transformed by the intermediary compound of acetyl-CoA and Malonyl-CoA into single phenols. When passing through the shikimate pathway, carbohydrates are first transformed into shikimic acid by undergoing chemical change in the Calvin cycle before forming phenyl propanoids. Phenols resulting from the acetate pathways can then be combined with phenyl propanoids to form chalcone which then leads to the synthesis of flavonoids.

Phenolics have been found to be key in functions such as plant defense mechanism and plant growth/development. Phenolic compounds include compounds that range from flavors and pigments to signaling molecules and antibacterial/antifungal agents[16]. Some phenolic compounds such as flavonoids have been shown to influence honeybee response to promote or deter pollination[17]. Other phenolic compounds such as vanillic acid and caffeic acid have antibacterial properties which prevent the growth of a variety of bacteria like E.coli and K. pneumoniae[18].

Certain phenolic compounds are present in increased concentrations when plant tissues are under stress[19]. Stressors can be pathogen attacks, photoinhibition, trauma, drought, or temperature change[20]. Growth stages can also influence the concentration of a variety of phenolic compounds[21].

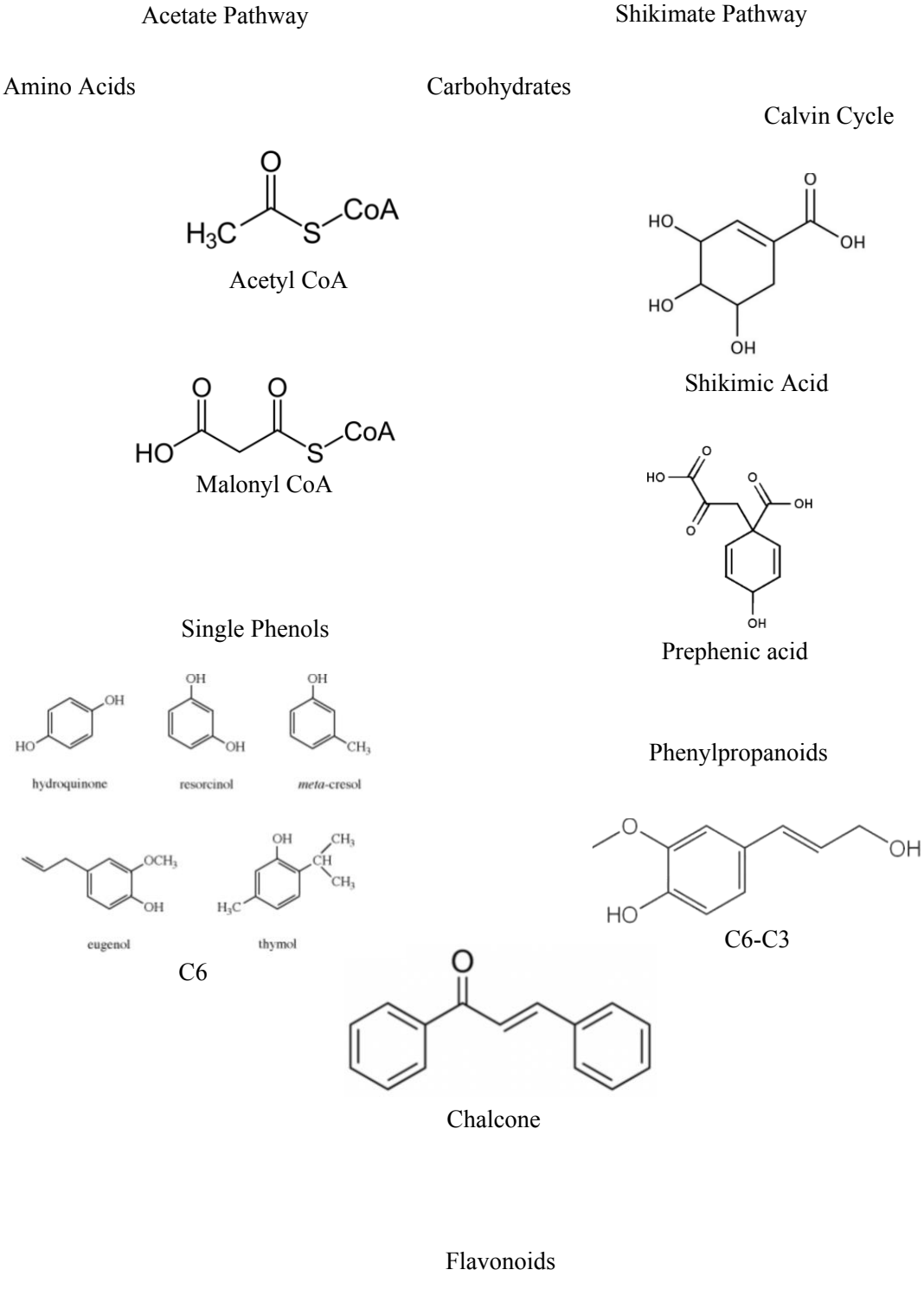


Figure 1.5: *Biosynthetic pathways of polyphenolic compounds synthesis*[22]

Phenolic compounds all share certain structural commonalities. They are composed of an aromatic ring bound to a hydroxyl group. Due to this configuration, the hydrogen present on the hydroxyl group is labile. This allows most phenolic compounds to act as weak acids.

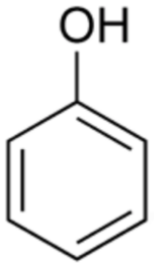


Figure 1.6: *Basic structure of phenolic compounds*

In certain chemical pathways, oligomerization and polymerization of phenolic compounds lead to the production of a complex compound. When more than one unit of the hydroxyl- bound phenyl ring is present in a molecule, the molecule is considered a polyphenol. It is important to note that most polyphenols are not polymers of individual phenols[16].

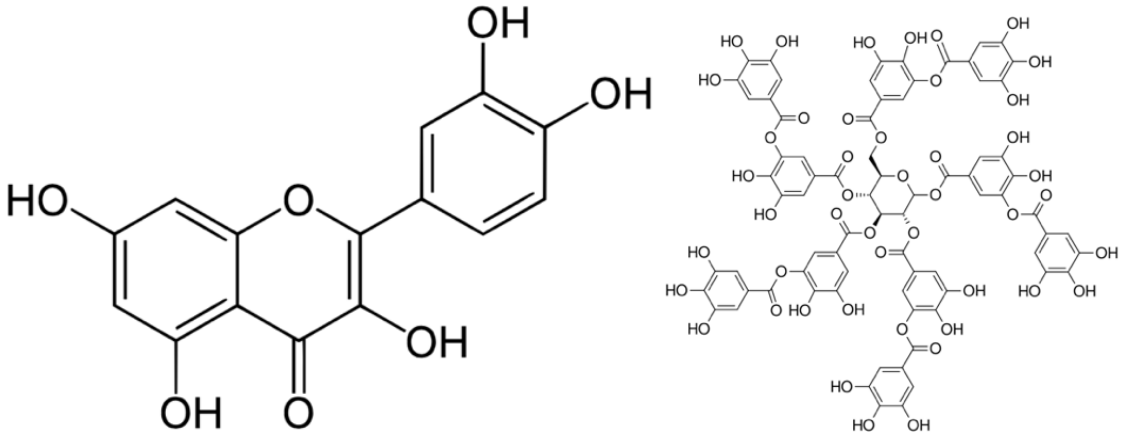


Figure 1.7: *Example of polyphenol structure Quercetin (left) and Tannic acid (right)*

Phenolic compounds are often classified based on the number of carbon rings/ carbon bound together as represented in Table 1.1.

Table 1.1: *Classifications of phenolic acids*[23]

Structure	Class	Example
C6	simple phenolics	Resorcinol
C6-C1	phenolic acids	Gallic acid, protocathechuic acid, vanillic acid
C6-C2	acetophenones, phenylacetic acids	2-hydroxyacetophenone, 2-hydroxyphenyl acetic acid
C6-C3	cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols, coumarins, isocoumarins and chromones	Cinnamic acid, p-coumaric acid, ferulic acid, Myristicin, Caffeic acid
C15	Flavans, flavones, flavanones, flavanonols, anthocyanidins, anthocyanins, chalcones, aurones and dihydrochalcones	Isoflavone, naringenin, taxifolin, leucocyanidin, quercetin, myricetin, Apigeninidin and Dihydrochalocone
C30	biflavonyls	Cupressuflavone
C6-C1-C6, C6-C2-C6	benzophenones, xanthones, stilbenes	Benzophenone
C6, C10, C14	quinones	Juglone
C18	betacyanins	Indicaxanthin
lignans, neolignans	dimers or oligomers	Pinoresinol
Lignin, tannin, Phlobaphenes	polymers	Tannic acid

Due to their structure, phenolic compounds are mostly water soluble because they are often bound to sugar molecules in forming glycosides. Rutin is a good example. It is composed of the flavonoid quercetin, aglycone portion, and the sugars, glucose and rhamnose as the glycone portion (Figure 1.8.) Rutin also shows a high-water solubility at 13mg/100mL.

As phenolic compounds include a variety of different compounds, the following research concentrated on a few categories: flavonoids and phenolic acids.

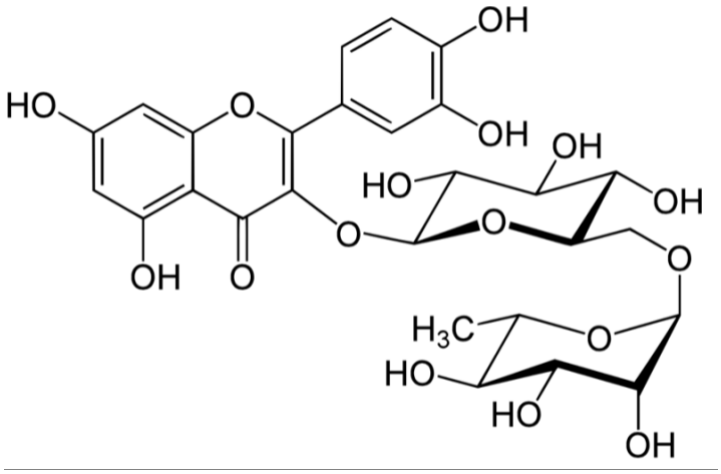


Figure 1.8: *Structure of Rutin*

1.2.2 Phenolic Acids

Phenolic acids generally possess a carboxyl group bound to a phenol ring. Compounds in this group are mostly derivatives of hydroxybenzoic acid, and cinnamic acids. They are often secondary metabolites resulting from the metabolism of cinnamic acid and other benzoic acid compounds. Hydroxybenzoic acids include p-hydroxybenzoic acid, gallic acid, catechuic and protocatechuic acid and vanillic acid. Cinnamic acid derivatives include chlorogenic acid, caffeic acid, o-coumaric acid and p-coumaric acid, sinapic acid and ferulic acid.

1.2.3 Flavonoids

Flavonoids refer to phenolic compounds which contain 15 carbon atoms in their structure[15]. Generated from a similar chalcone precursor, they are structurally and biologically linked. Depending on the saturation and oxidation of their carbon ring, they are often divided into subcategories. These subcategories are Flavone, flavonol, flavanone, flavan-3-ol, anthocyanidin and isoflavone[22]. Each category has a general structure depicted in Figure 1.9.

Flavonols are widely distributed in plants. Since all flavonols have a main structure of 3-hydroxyflavone, their diversity comes from the variation in the position of the hydroxyl group. More than 200 flavonols have been found to be present in their aglycone form, however, majority of flavonols are glycosylated. When glycosylated, the glycosidic bond is present on the 3rd carbon of the 3-hydroxyflavone backbone. Commonly linked sugars are glucose, rhamnose, galactose or glucuronic acid. Generally, sugars with the dextro optical configuration will participate in a b-linkage whereas L-sugars will participate in an αA bound-linkage[22].

Flavones are one of the least common categories of flavonoids. The main structure is based on function group variation on a backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) as can be seen in Figure 1.9[24].

Flavanones are ketones derived from flavone. They can be found in citrus as aglycones. However, when present as glycosides, glycosylation is often on the 7th carbon of the double phenol ring. Glucose and rhamnose are the two main sugars linked to flavone[15].

Flavan-3-ols or flavanols are the most abundant of flavonoid's subclasses. They have a 2-phenyl-3,4-dihydro-2H-chromen-3-ol backbone. Flavanols exist as monomers catechins and their polymerization products, proanthocyanidine. Like flavonols, the functional hydroxyl group can take several different positions which differentiate the flavanols as can be noted in both (+)-Catechin and (-)-epicatechin, some of the most studied flavanols[25].

Anthocyanidins are widely known as the family of molecules that give berries their bright colors. They are often monoglycosylated on the oxygen bridge.

Isoflavones are polyphenolic compounds which are less present in food. They as all other flavonoid sub-categories can also be found in glycone and aglycone form[16]. In the context of this

research, the standards used to identify flavonoids were myricetin, quercetin, apigenin, catechin, epicatechin, pyrogallol, epicatechin gallate, quercetin-3-beta glucoside and rutin.

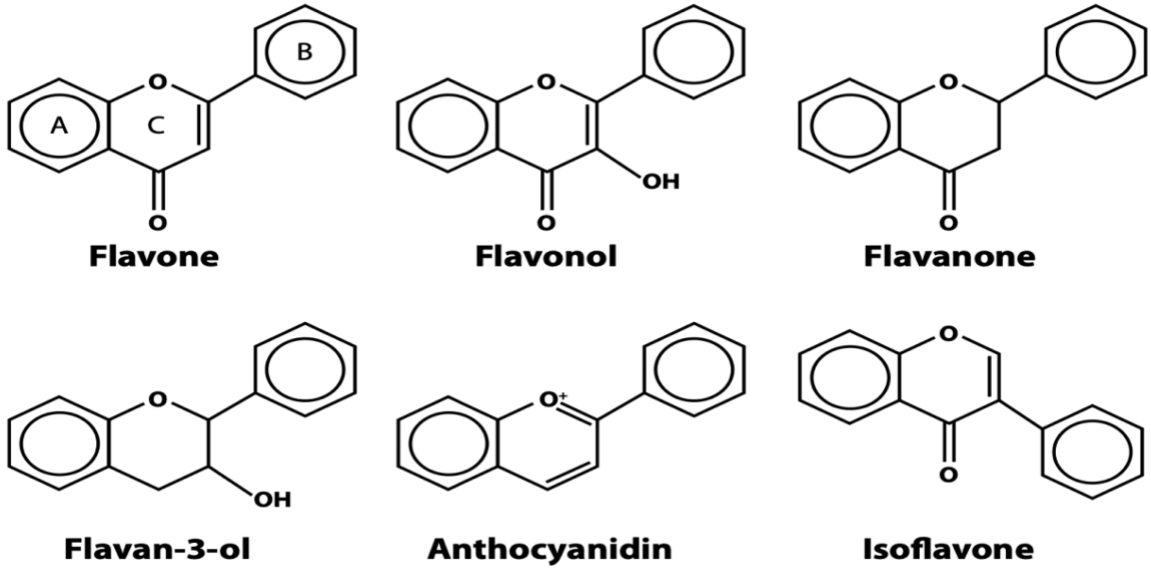


Figure 1.9: *Chemical Structure of flavonoid*[26]

1.2.4 Toxicology of phenolic compounds

Research indicates that consumption of phenolic compounds has a beneficial impact on overall human health. As such, the health promoting benefit of the consumption of fruits and vegetables are often linked to the phenolic compounds present. Ingestion of large amount of these compounds have been linked to a reduction in both animal and human of dyslipidemia and atherosclerosis, endothelial dysfunction and hypertension, platelet activation, thrombosis and the inflammatory process linked to cardiovascular issues[26].

Phenolic compounds have been theorized to positively impact different processes in the prevention of diseases. These compounds would work through two different modes of action: their antioxidant activity and their non-antioxidative cellular modulatory effects[27]. The antioxidant activity of phenolic compounds is the first mode of action to be scientifically researched. Directly,

phenolic compounds can act as radical scavengers allowing to reduce the cellular damage that can be caused due to oxidation[28]. However, they can also indirectly regulate oxidation through modulatory effects on a variety of pro and antioxidant enzymes.

Adding to their antioxidative properties, phenolic compound also shows antioxidant independent modulatory actions. Research has shown that phenolic compounds seem to regulate and influence different protein kinases and lipid kinases. These modulatory changes may influence further down the metabolic pathways such as altering the phosphorylation state of certain target molecules and/ or changing the regulation of gene expression[29]. For example, multiple phenolic compounds such as isothiocyanates and epicatechin are known to influence directly and indirectly the MAPK pathway[30]. The MAPK pathway being a key regulatory pathway which influences transcription. When proteins of these pathways undergo inappropriate changes, the affected cells can develop into cancerous ones. Furthermore, green tea extracts which are rich in phenolic compounds are known to have antitumor properties going as far as being able to prevent proliferation on cancer cell lines[31].

Through the modulation of various enzymes, phenolic compounds can drastically affect several cellular functions. Furthering those findings, flavonoid rich diets have been shown to influence gene expression as to beneficially impact overall health[32]. However, due to the complexity of the pathways influenced by phenolic compounds and the variety of compounds, it is hard to predict and control the exact outcome resulting from in vivo phenolic consumption.

1.2.5 Industry Application of Phenolic Compounds

Due to their inherent properties phenolic compounds offer a wealth of uses for a multitude of different industries. Through implementation and normalization of their use in the

pharmaceutical, packaging, cosmetic or food industry, phenolic compounds can bring value from plant material waste coming from the agricultural industry.

In the context of the food industry, phenolic compounds present two main uses: as a functional ingredient or as a preservative. For instance, they can be used directly as functional ingredients as to enhance the sensory quality and/or increase the nutritional value of a food stuff. Furthermore, due to their color changing capacities they can also be used as natural dies and colorants. Anthocyanins have been shown to be stable coloring agents through a variety of research in sport beverages, fresh sausages and yogurt drinks for example[33].

Alternatively due to their antioxidant properties and antimicrobial properties they can also be used as a natural preservative. For example, phenolic extract from grape pumace has been proven to negatively impact the growth of *E.coli* and *L.Innocua*[34].

Their antioxidant and antimicrobial properties are responsible for their use in packaging such as active packaging but also in cosmetics where they can act dually as therapeutic agents and preservatives.

1.3 Saponin

Saponins are a family of molecules which similarly to phenolic compounds result from the secondary metabolism of plants. Historically, Saponins got their name from the soapworth plant, which belongs to the genus Saponaria, a plant commonly used to make different forms of natural soaps. However, saponins have been found in chickpeas, mung beans, peanuts and a variety of other food plants[35]. The Isoprenoid pathway is often the first step of saponins precursor synthesis. However, most of these precursor molecules need to undergo further transformation through enzymatic pathways such as mevalonate and non-melavonate pathways which are both responsible for transforming squalene into either steroidal or triterpenoid saponins[36]. Saponins are found in a variety of plant structures however they are often present in higher concentration in plant roots. Due to their antimicrobial properties, saponins are theorized to help in the protection of plant roots from microbial infestation of plant tissues. As such, these compounds would act as chemical barriers to all soil born microbes[37]. Furthermore, saponins have demonstrated abilities in regulating plant growth. A variety of soyasaponins have been found to affect cellulose synthesis, increase the rate of growth of lettuce and even stimulate barley seed germination[38,39].

Compounds belonging to the saponins family are often composed of a sugar portion and a non-sugar portion which is often referred to as the aglycon portion. However common the term aglycon is, alternative denominations have also been in use. For instance, genin and sapogenin are also accepted terminologies to refer to the non-sugar moieties of the saponins[40].

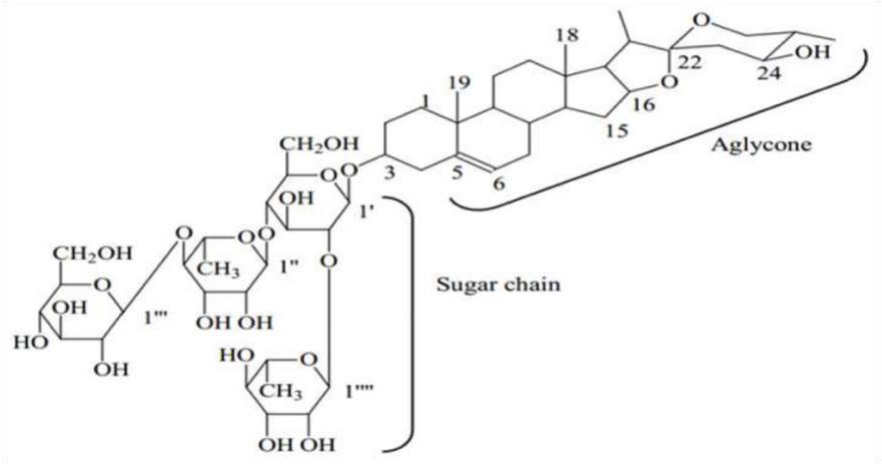


Figure 1.10: *Chemical Structure of Saponins (steroidal saponin)*[41]

The nature of this portion is often the first measure of classification. As such saponins are often divided in three major subgroups based on the nature of the aglycon portion: Triterpenoid saponins, steroid saponins and alkaloid saponins.

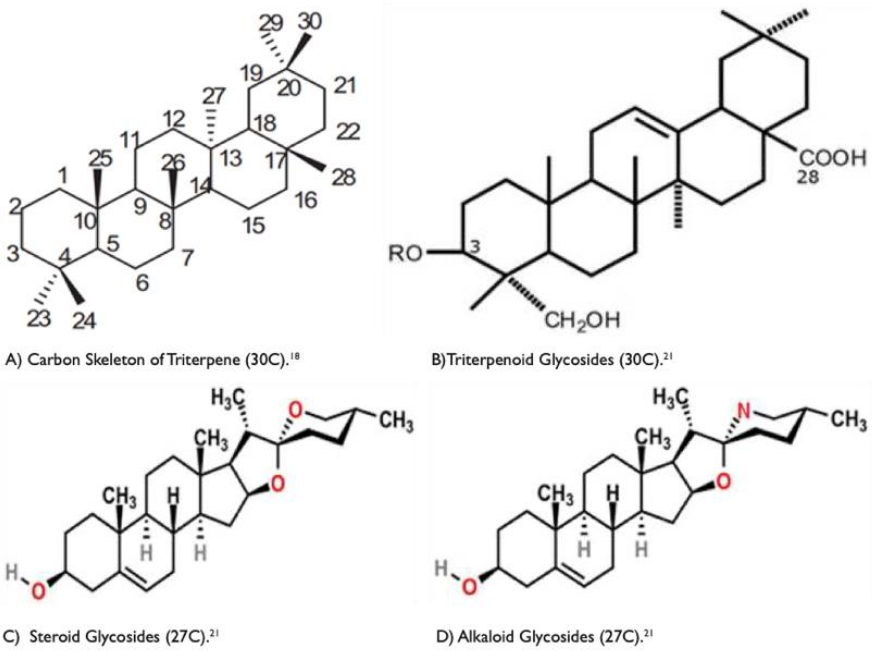


Figure 1.11: *Structure of the Different aglycon portion present in saponins*[41]

Triterpenoid Saponins show in their structure the presence of triterpenoid glycoside which is a molecule composed of 30 carbon atoms which creates the structure of three monoterpenes making this structural portion a pentacyclic compound. The compound also includes 4 different oxygen molecules which are present as part of a carboxyl, a hydroxyl group and an ester link as can be seen in Figure 1.11. Triterpenoid glycoside contrarily to both steroid and alkaloid glycoside do not contain spiro-carbon atoms[42]. The sugar moieties are attached to the triterpenoid portion through a glycosidic bond as can be noted in steroid and alkaloid saponins.

Steroid saponins contain as their aglycon portion a steroid glycoside. The steroid glycoside often presents lots of structural commonalities with triterpenoid glycoside and it is sometimes considered as a modified triterpenoid. It is composed of six carbon rings, a tetracyclic 6 carbon ring and a bicyclic 5 carbon ring. In total steroid glycosides are made up of 27 carbon atoms. The sugar moiety is often linked through a C-3 glycosidic bond as can be seen in Figure 1.11.

Alkaloid saponins are a variation on steroid saponins. They are structurally very similar both containing 27 carbons similarly distributed in rings. However, alkaloid saponins replace the pyranose ring present in steroid glycoside with a piperidine ring. They differ from triterpenoid glycoside in the same way that steroid saponins do by omitting in their structure the presence of a carboxylic group.

Circling back to the first subcategory of saponins, triterpenoid saponins can be further classified based on the number of sugar moieties present. As such if a saponins presents a triterpene aglycone and one sugar moiety it is referred as a monodesmosidic triterpenoid saponins. Similarly, if the triterpenoid saponins shows two sugar moieties it is referred to as a bidesmosidic terpenoid saponins[43]. Good examples of monodesmosidic triterpenoid saponins are hederagenin and oleanic acid. Steroid aglycon and alkaloid aglycon are mostly linked to a single sugar moiety. As for the

structure of the sugar moieties, a variety of pentoses and hexose sugars have been recorded. No preferential structure of the sugar has been noted as both D/L isomers and a/b anomers. Glucose, ribose, rhamnose, arabinose, xylose have been found to be the most common sugars in saponins.

1.3.2 Industry Application of Saponins

Contrary to phenolic compounds, industrial use of saponins is still very limited. However due to their antimicrobial properties, they show promising potential to limit toxic microbial development and as such, are used as preservative in both the food and cosmetic industry. One of saponins most interesting properties is their surfactant/emulsifier capacity which allows for the formation of foams and emulsions. As such, Quillija saponins are commonly used in the food industry to form stable emulsion with b-lactoglobulin and b-casein[44].

1.4 Agricultural Waste

Considering the reality of the climate crisis, environmental issues have grown in importance. Pollution and the many forms it might take, despite being an unwanted by-product of human activity, needs to be controlled and limited. One important source of pollution is intrinsically linked to the current methods of agricultural production[45]. In Canada, it is estimated that about 40% of the food produced in farms is not consumed every year[46]. However, this number doesn't account for the by-products linked to the processing of the other 60%. Although 50% of food waste happens in the consumer's home, the remaining half happens at different steps of the field-to-consumer chain. In that chain, up to 18% of waste stems from the processing/packaging stage[46].

Even more than environmental issues, finding value in waste is also a sound economical proposition as creating and implementing systems to process this waste could create a new economic sector. In Canada still, the difference in capital between the estimated market value of the food produced and the value of the food that reaches consumers, was estimated at 27 billion dollars in 2009[46].

As such, agricultural waste management has become an important topic of scientific research. Novel ways of finding value from the waste produced by the agricultural/food complex are needed to reduce the negative impact of this crucial sector of human activity. One interesting avenue of research has been to use food waste to formulate novel biomaterials.

1.5 Bioplastics

Bioplastic is a term often used to refer to material made from organic substances. However due to the lack of consensus on the exact terminology biobased material can also be referred to as biomaterial, biologically based materials and sometimes bio based materials[47]. Due to the variety of it the potential components from which a biobased material can be made of, they can exhibit an array of different rheological properties. It is for this reason that biobased materials can show a wide diversity of uses. Bioplastics more often include in their chemical structure polymeric molecules such as cellulose, starch, gelatin and chitosan to name a few[48].

Bioplastic materials are often composed of 4 types of ingredients which allows the formation of a stable material with plastic-like qualities. Biopolymer as the name indicates are made of polymeric molecules which are generally high molecular weight composed of repeating subunits[49]. Due to this configuration, polymers often have a secondary, tertiary and sometimes quaternary structure. They have the capacity when under the proper condition to undergo gelation where cross-linking will allow the formation of a 3-d network[50]. This process is crucial to creating bioplastics as this cross-linking network is responsible for the strength and integrity of the material[51].

1.5.2 Biopolymer

Bio polymers are polymeric molecules coming from bio sources. The sources can be microbial, plant based or animal. Common polymeric molecules used in bioplastics are starch, sodium alginate, gelatin, and chitosan.

1.5.3 Plasticizer

Plasticizers play an important role in the textural properties of a bioplastic. They often are responsible for four properties related to the flexibility of a material. They allow for lowering the

inelasticity of material at room temperature which simultaneously allows for stronger distortion without needing to heat the material to a higher temperature[52]. Plasticizers are also able to increase the elongation capacity of a bioplastic while increasing the toughness/impact strength at lower temperature. Plasticizer can be internal or external. External plasticizers are not bound to the polymers present in a bioplastic and as such can easily be lost form the bioplastic[53]. Oils can sometimes be used as external polymers, but they will often separate from the rest of the bioplastic structure given time. On the opposite spectrum are internal plasticizers who directly interact with the biopolymer. They will often bind to the polymer becoming an integral part of the final 3D structure. They are often bulky compounds which prevents the polymer chains from compacting too closely and providing better ability of molecular movement to the long polymeric chains[53]. Common plasticizers used in bioplastics are sorbitol, glycerol. These will often act as internal polymers when used in combination with starch and glycerine[54].

1.5.4 Additive

Additives encompass a wide variety of compounds that can be added for different reasons. They include colorant or any other material that provides bulkiness to the bioplastic.

1.5.5 Solvent

The solvent is often needed to provide the environment in which each of the other components can interact and rearrange themselves as to form a bioplastic. The most common solvent used is water in which all the other chemicals are dissolved more often at high temperature to ensure better homogeneity of the solution. Once the bioplastic is extruded/shaped, the solvent is dried out resulting in the final plastic-like material.

1.5.6 Advantages and disadvantages of Bioplastic/Bioplastics use: Replacement of plastic material

Bioplastics are of great interest for one main reason, they offer a sustainable alternative to the use of oil-based plastics. Oil-based plastic materials have rapidly become a mainstay material since its discovery in 1907 by a Belgian chemist. Part of our everyday life, plastics are ubiquitous and play an important function as a structural component of a lot of different objects which are now indispensable to human activity. In 2015, the global production of plastic was estimated at 350 million tons. A huge leap since the 1950s, where it was evaluated at 2 million tons[55]. Although plastics are so commonly used and are produced in enormous quantities, proper disposal systems are rarely put in place. Despite being a common material since the 50's, 30 years later incineration and recycling of plastic was almost negligible. To add to the problem, incineration generates greenhouse gases like carbon dioxide and recycling can be very resource intensive. Up to 2015, up to 55% of plastics were still disposed of mainly through landfills and although trends show a slow increase in the proportion of plastics that are properly disposed of through incineration and recycling a lot of environmental damage has and is still taking place[55]. Although plastics do undergo degradation processes through thermal, hydrolytically or UV-light induced processes, the by-product of these breakdowns are often toxic for the environment and can take when happening naturally thousands of years[56]. To add to this phenomenon, plastics that were buried in landfills often do not undergo full degradation processes producing further toxic chemicals[57]. As plastics do not properly biodegrade, they often break down and leak into the environment. Plastic breakdown will travel all the way to the ocean where they are either absorbed by the wildlife or end up at greater oceanic depths[58].

Furthermore, one of the main culprits of plastic production is the sector of packaging, which is responsible for the production of around 151 million tons yearly, highlighting the need for more biodegradable replacement options[55]. One of plastics' main appeal is its perennity due to lack of biodegradability which in most packaging situation is not a necessity as packing is a transient need before a product reaches a consumer. The food industry is one of the industries that heavily relies on plastic packaging to prolong food shelf life. As such, finding alternative to plastics such as biobased material/bioplastic is an important sector of any research in Food Science.

Biobased materials are very promising because they have the capacity to exhibit similar properties as plastics but also because they can be readily broken down and biodegraded, thus, preventing the leakage of environmentally toxic compounds. Bioplastics are made of plant materials. As such, plant materials are renewable sources making them a great alternative to petroleum-based materials. Petroleum being a non-renewable resource.

However, bioplastics show some limitations and disadvantages. One worry linked to the development and normalization of biobased material would be the need to increase land usage to produce the quantities of plant materials needed[59]. This could be partially mitigated by using raw plant materials from agricultural wastes. One other issue often mentioned is the need for a specific environment for proper biodegradation processes to happen. Proper biodegradation of bioplastics happens through 3 steps. First biodeterioration during which microorganism grows on the surface of the material leading to changes in its chemical composition. This process is followed by bio-fragmentation where the same microorganism will start to break down the long polymeric chains that forms the bioplastic until finally assimilation happens. Assimilation resulting in the breakdown of bigger molecules to produce nutrients, carbon and water which are reabsorbed by the environment[47]. If issues arise during one of these stages, improper biodegradation can happen,

leading to the potential release of greenhouse gases such as methane[60]. However, finding alternative environmental alternatives is often more about harm reduction than complete harm deletion. For this reason, bioplastics show promise in replacing plastic material.

1.6 Emulsions

An emulsion is a colloid composed of two non-miscible liquids or fluids. One fluid is often dispersed in the second fluid forming microdroplets in a continuous phase. Due to the chemical incompatibility between the two fluids, which are often water and oils, an emulsion will seek to reduce the interfacial area between both phases[61]. Reducing this area lowers the interfacial tension and can be helped through the presence of a compound that reduces the surface tension between the two fluids. This compound is often referred to as a surfactant. Surfactants are often amphiphilic. Their chemical structure allowing them to dissolve/interact with both water and oil. This affinity with both liquids allows the creation of a barrier around the droplet formed from one liquid in the continuous phase of the second liquid[62]. As such, depending on the surfactant molecule and the proportion of each phase, very stable emulsions can be formed. Emulsions are sometimes qualified as metastable as they can be very stable if they don't undergo important disturbance such as high changes in temperature. Certain emulsion can be stable for more than one year.

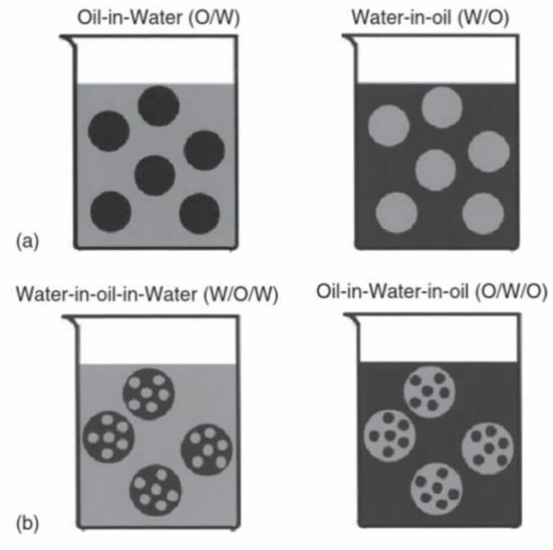


Figure 1.12: *Different Types of Emulsionns*[63]

There are four main types of emulsion, water in oil emulsions (w/o), oil in water emulsion (o/w), oil in water in oil (o/w/o) and finally water in oil in water (w/o/w) which both belong to a subcategory of emulsions called double emulsions. A Common example of w/o emulsion are margarine where the continuous phase is oil and lecithin or another molecule that can act as a stabilizer. O/W emulsion includes most cosmetic creams and foods such as milk and salad dressings\. It is easy to see that emulsions are ubiquitous in both the cosmetic industry and the food industry where they find many uses.

1.6.2 Double emulsions

Double emulsions, as was mentioned above, are divided into two types W/O/W and O/W/W. IN the case of these types of emulsions, an interesting phenomenon can be noted. Double emulsions are made up of colloids dispersed inside of another colloidal as can be seen in Figure 1.13. To create a double emulsion, it is important to create a primary emulsion that will then be dispersed in the main continuous phase. HPLF ultrasound has been shown to help with this process[64].

When looking at the droplets of emulsions formed in the main phase, three different types of droplets can be observed as can be seen in Figure 1.13. The first type would be type A where one main droplet of the liquid A is dispersed in a droplet of the liquid B which is in turn dispersed in a continuous phase of liquid A. The next type of observe droplets is type B. In these droplets of liquid B can be observed multiple droplets of liquid A meaning that the original droplets behave as a secondary continuous phase for the liquid A. Finally in type C, we can observe that the droplets have reach a significant size leading to the encapsulation of multiple droplets of liquid A into a thin layer of liquid B which is dispersed in the continuous layer of liquid A[65].

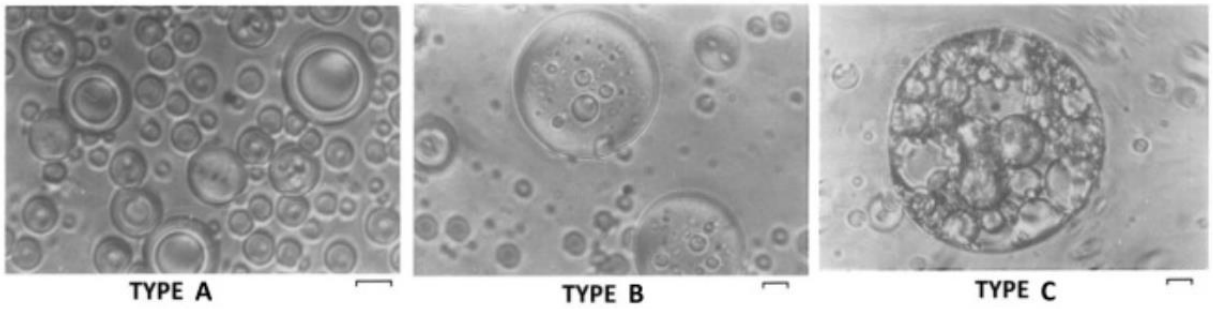


Figure 1.13: *Different types of droplets present in double emulsions*[65]

Double emulsions are useful as they show promising ability in fat reduction in food but also in pharmaceutics where they can be used to control the rate of release of certain compounds[66].

1.5.3 Hydrocolloid gels and emulsions

In the context of this research, as to allow the formulation of biomaterials made from agricultural waste (tomato skin, hops flowers and hemp meal) both sodium alginate and gelatin were used as surfactants. These two compounds are considered hydrocolloids as they both present the ability to form gels[67]. As such hydrocolloids have been defined as long polymers which form gels (sometimes called viscous dispersions) when dispersed in water. They can be of various sources which include microbial, plant, animal and alguae[68]. Hydrocolloid can be used in multiples ways. They can be structuring agents, delivery carrier, bioactive ingredients, or stabilizer.

In a gel, a complex 3D network of molecules will be formed resulting from the intertwining and cross-linking between long hydrocolloid molecules. Structurally, three structures are the most common in gel-networks: aggregate, double helix and aggregated double helix[69]. Multiple gelling mechanisms have been observed leading to the creation of these interactions. These mechanisms are ionotropic gelation, diffusion setting, internal gelation, cold-set gelation and heat-set gelation. A good example of ionotropic gelation is sodium alginate where addition of calcium ions will lead to cross-linking between the sodium alginate solution. Cold-set gelation results from the heating

of a solution to facilitate the absorption of the wanted hydrocolloids, as per instance gelatin, and the subsequent cooling allowing gel formation[70].

When present in low concentrations, hydrocolloids can lead to the destabilization of an emulsion. Due to the lack of absorbing hydrocolloid, the coalescence of the droplets will increase, affecting the stability of the emulsion. However, a slight increase in the hydrocolloid concentration impedes the formation of the emulsion as the viscoelasticity of the different phases increases. This is a direct result of increased flocculation of the droplets which creates a gel like network. However, when dealing with a significant increase of the concentration of hydrocolloid, a stable emulsion can be reached as the emulsion becomes gel-like, where trapped matrix of hydrocolloid structured water can be noticed[51]. To properly stabilize an emulsion, further shearing needs to be applied as to ensure that each droplet is properly encapsulated by hydrocolloid molecules as can be seen in Figure 1.14.

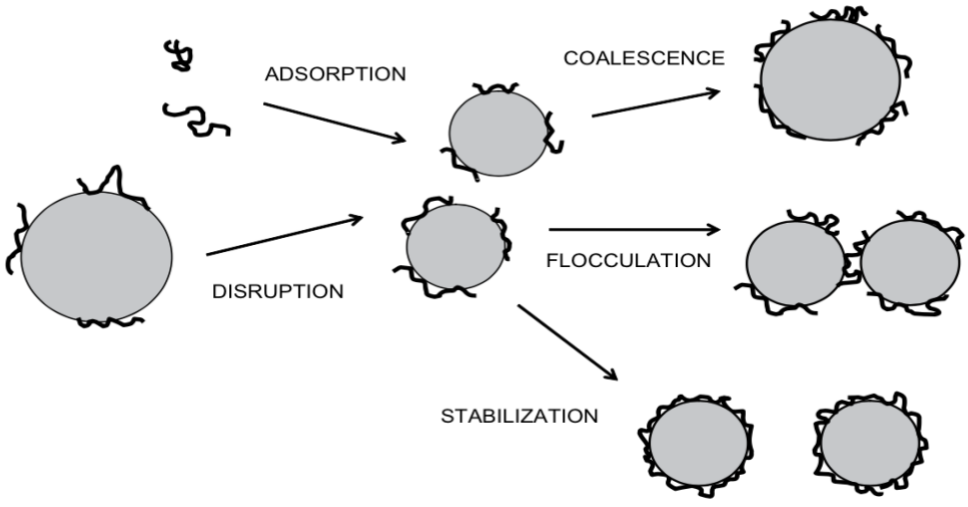


Figure 1.14: *Schematic of hydrocolloid absorption/disruption and the different outcome of droplet/hydrocolloid matrix*[51]

1.7 Texture Analyzer

Texture Profile Analysis is a mechanical method designed to systematically measure a variety of quantitative properties relating to the texture of a multitude of materials. Mainly used in the context of quantifying the textural properties of food products, Texture Profile Analysis was developed in the 1960 by a researcher at General Food[71].

This test was previously performed by a texturometer but modern testing has evolved into using a texture analyzer. This instrument uses a strain gauge sensing element and an appropriate software which records the change in force needed as a probe is lowered into uniformly shaped food samples. TPA is composed of two cycles[72]. Through both cycles a probe is lowered into a food sample and removed while the forces needed in relation to time are recorded. This creates a graph as can be seen in Figure 1.15. As the General Food original Texturometer was adapted from an MIT denture tenderometer, these cycles of lowering and rising of the probe are said to mimic the mastication process of a human mouth[73]. The aim this design was to attribute quantitative values to the sensory properties of food[74].

Interpretation of the curve produced by a texture analyzer allows to quantify the following mechanical parameters: Hardness, Elasticity/Springyness, Brittleness/Fracturability, Chewiness, gumminess and viscosity. All these parameters were defined in the following paper, The Texturometer – A New Instrument for Objective Texture Measurements, in which the method of texture Profile Analysis was first presented. From the 7 parameters defined, 5 are direct measurements from the curve produced during testing.

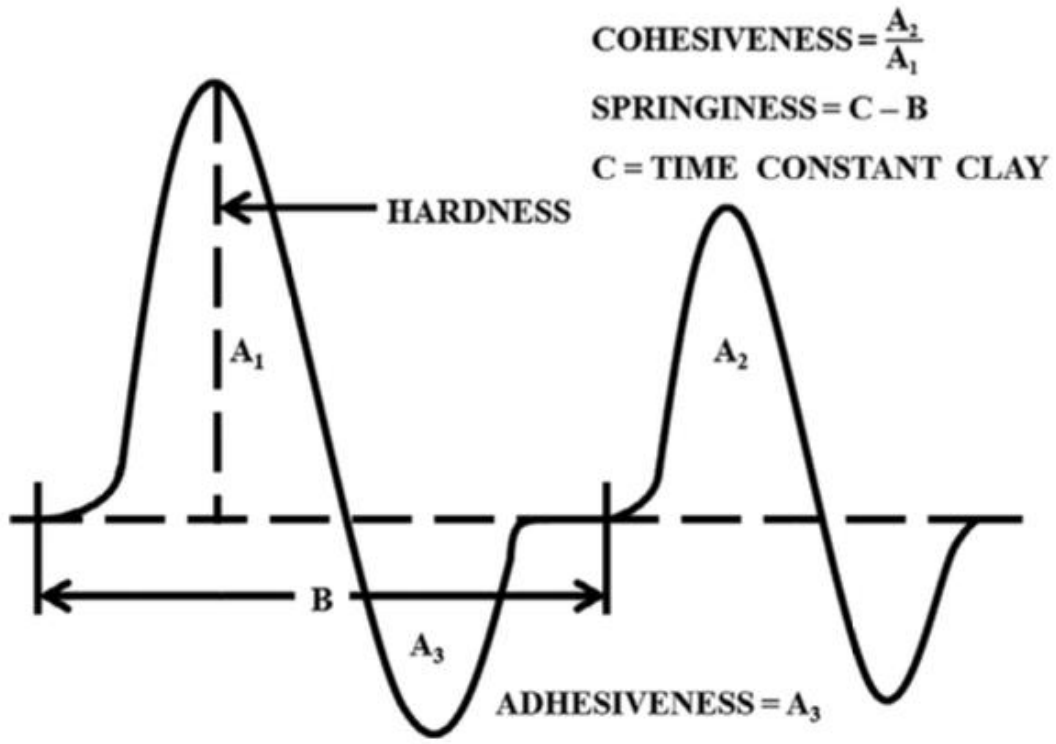


Figure 1.15 : *Typical Instrumental TPA* [75]

Hardness is defined in the context of texture analysis as the peak force needed in the first cycle of compression performed during a TPA. Hardness will often be normalized to a one-volt input. Consequently, the quantitative values associated with hardness are a direct indication of the resistance of a material to plastic deformation.

Adhesiveness measures the capacity of a material to cling to the probe and the needed force to separate the probe from the sample. It is represented mathematically by the area under the curve of the negative peak following the first cycle of compression.

Cohesiveness is a measurement directly proportional to the forces needed to overcome the internal bond of a sample. This measurement is obtained by calculating the ratio between the area under the curve of the peaks produced through the first and second cycle of compression. As a result, cohesiveness is unitless.

Elasticity or Stringiness as it has been renamed in other publications, is the height recovered by sample after the plastic deformation performed during the first cycle of the TPA.

Brittleness/Fracturability is an indicator of the force needed to induce a fracture of break in a sample. Finally, from the previously mentioned parameters both gumminess and chewiness can be calculated which are respectively the product of cohesiveness and hardness for gumminess and the product of gumminess and springiness. Although viscosity can be measured in fluids using TPA, it is often more appropriate to use a viscometer[75].

As can be deduced, although TPA provides us with a wealth of textural quantitative data, not every single one of those values are an appropriate quantifier of certain samples' textural properties. For example, when testing gel-like food samples such as puddings, fracturability cannot be measured as no "fractures" can be induced in the sample.

Texture Profile Analysis is a useful method of analysis as it allows researchers to gather descriptive quantitative information about any substance. TPA presents two main advantages flexibility and objectivity[76]. As such this method can be used in a variety of contexts, from its intended use on most food products to any substance with textural properties[77]. However, despite the low instrumental error linked to the use of a texture analyzer, heterogeneity of material can greatly increase the variability in the results obtained leading to significant difference in textural characteristics between samples[75]. TPA is a method designed for testing on food samples, however due to its ability to be performed on almost any sample material it can be a cheap and effective way to obtain textural information about most material.

1.8 Hypothesis and Objectives

Throughout the course of this research, the following Hypothesis were made:

1. High power low frequency ultrasound treatment increases the efficiency of phenolics and saponins extraction from tomato, hemp, and hops
2. High-power low frequency ultrasound can positively impact the characteristics of biomaterials made from tomato, hemp, and hops

Throughout this research the objectives were as follows:

1. Extract tomato, hemp and hops using traditional and high-power low frequency ultrasound
2. Characterize active compounds present in the above plant material focusing on phenolic and saponin compounds in the extract
3. Evaluate the antioxidant capacity of the above extracts
4. Develop innovative emulsion gels/double emulsion for biomaterials/bioplastics made from tomato, hemp and hops using high-power low frequency ultrasounds
5. Characterize the physical and rheological properties of the developed biomaterials/bioplastics

Chapter 2: Ultrasound Assisted Extraction of saponin and phenolic compounds from Tomato, Hops and Hemps

2.1 Abstract

Traditional extraction for most bioactive compounds from plant material has been found to be too long. As such UAE has been proposed as an environmentally safe and highly efficient alternative to traditional extraction[13]. In this study multiple plant materials, tomato skin, hemp meal and hops flowers were extracted using 5% acetic acid in ethanol as solvent as to compare UAE and traditional 24h extraction. UAE was performed by applying HPLF-US for 30 minutes on solvent and source material matrix. Using RV-HPLC-PDAD, phenolic acid and flavonoid composition was analyzed. Alternatively, saponin composition was analyzed using RV-HPLC-ELSD. TPC and TFC did not show difference in concentration between UAE samples and traditionally extracted samples. The measured TPC for tomato, hemp and hops were also respectively 87.22±21.12, 147.39±16.92 g, 450.32±26.47 g of GAE/100g per sample for UAE extraction and for traditional extraction of respectively 89.14±11.61g, 159.42±28.20 and 460.95±48.57 g of GAE/100g of sample. Values for TFC were measured to be 19.92±2.24 mg of QE/g of sample, 15.08±1.37 mg of QE/g of sample for tomato UAE, 31.75±6.30 mg of QE/g of sample for traditional hemp UAR, 51.31±3.52 mg of QE/g of sample for traditionally hemp extracted samples, TFC, 171.10±2.66 mg of QE/g of sample for the UAE hop extraction and 131.80±13.79 mg of QE/g of sample for traditional extraction of hop flowers. Similar results were obtained from total saponin content. UAE and traditional extraction showed respective TSC of 1443.79±125.24 vs 1337.65 mg of DE/ 100g of sample for tomato, 1511.25±136.98 vs 1618.93±58.90 mg of DE/ 100g of sample for hemp meal extraction, 8037.83±885.45 vs 9847.34±2063.63 mg of DE/ 100g of sample for hops flower extraction. Antioxidant activity did

not seem to be influenced by the method of extraction as ORAC values did not show significant difference. This study suggests that UAE could be an efficient alternative to traditional extraction of bioactive compounds mainly phenolic compound and saponins when using a green solvent such as 5% acetic acid in ethanol.

2.2 Introduction

Plant materials are an important source of bioactive compounds such as phenolic compounds and saponins. Both families of compounds have been shown to have interesting functionality when used as ingredient in food, cosmetics and/or drugs. Furthermore, resulting from improper management, overproduction or simply necessity the food/agricultural industry produces large amounts of plant materials which end up as waste. As such, one way of bringing value from plant waste produced through food production would be to find ways to efficiently extract compounds such as phenolic acid and saponins, using environmentally friendly techniques and solvents. Ultrasound assisted extraction is a green technique that has become of interest for its capacity to increase extraction efficiency. High-Power Low-Frequency ultrasound application has been shown to decrease the time needed for extraction of a variety of samples through use of cavitation and detexturation of samples. Through application of micro jet forces ultrasound breaks down the surface of plant matrix allowing for the different compounds to dissolve in the solvent in a more efficient matter than with regular stirring. It is for these reasons that this study will aim at evaluating the impact of High-Power Low-Frequency ultrasound on the extraction process of various plant material considered as waste (tomato skin, hemp meal and hops flowers) compared to traditional extraction methods. The impact of UAE was then quantified through characterization and quantifying of both saponins (using of RV-HPLC-ELSD) and phenolic compounds (using of RV-HPLC-PDAD) as well as evaluating its impact on antioxidant capacity through ORAC.

2.3 Materials and Methods

2.3.1Materials

Tomato skin, Hemp meal and Hops flowers were provided by Agriculture and Agri-Food Canada (AAFC) London Ontario and Lambton College, Sarnia, ON. Acetonitrile, HPLC grade methanol and acetic acid were obtained from Fisher Chemical (Ontario, Canada). Ethanol (100%) was from Commercial Alcohols (Ontario, Canada). Acetic acid was from Aldrich Chemical Company INC. Flavonoid, Phenolic compound standard, Aluminum Chloride powder and Folin-Ciocalteux's reagent were ordered from Sigma-Aldrige (Ontario, Canada) as was Sodium Acetate and Sodium Carbonate Anhydreous were from Bioshop (Ontario, Canada). Saponin standard was purchased from Biopurify (Sichuan, China).

2.3.2 Traditional extraction of tomato skin, hemp meal and hops flowers

10g of grinded tomato skin, hemp meal or hops flowers were each extracted for 24h with 5% acetic acid in ethanol using traditional mechanical stirring. Stirring was set to 500 RPM. Each extraction was performed in triplicates. Once the extraction was completed, the solution was filtered using Whatman double filter paper (number 5). The samples were then completely dried to obtain the yield before being redissolved in methanol for a total volume of 25mL.

2.3.3 Ultrasound assisted Extraction of tomato skin, hemp meal and hops flowers

10g of grinded tomato skin, hemp meal or hops flowers were each extracted for 30 minutes using HPLF ultrasound. The ultrasound probe was submerged and set to a power of 90W and a frequency of 20 kHz. Once the extraction was completed, the solution was filtered using Whatman double filter paper (number 5). The samples were then completely dried to obtain the yield before being redissolved in methanol for a total volume of 25mL. This created solution with a

concentration of 10g of defatted sample/25mL of methanol. Each extraction was performed in triplicates.

2.3.4 Phenolic Analysis using HPLC-PDAD

Methanolic solution, prepared in 2.3.3 with concentration of 10g of defatted sample/25mL of methanol, were loaded in the carrousel of an HPLC-PDAD machine. The samples were analysed by a Waters e2695 HPLC system equipped with aa 2998 Photodiode array detector (PDA) (Waters, Milford, MA, USA) and the Empower Software. Phenolic compounds and flavonoids were separated using a reversed-phase column Synergi-Max-RP column (250X4.6mm, 5 mm). 10 mL were injected at a temperature of 23°C. The column temperature was set at 35oC. The flow rate was maintained at 1 mL/min. The total run time was 50 min. Two mobile phases were used, solvent A: 0.05% formic acid/water (v/v) and solvent B: 100% acetonitrile. The solvent gradient was as follows:

0-35 minutes: 90% Solvent A, 10% solvent B

35-40minutes: 50% solvent A, 50% solvent B

40-50minutes: 90% solvent A, 10% solvent B

Detection of phenolic and flavonoids was done at 280nm and 320nm. Identification of compounds was done by comparing retention time to commercially obtained standards shown in Table 7. Standard calibration curves were generated using a mixture of the phenolic compounds and flavonoids at 5 different concentrations.

2.3.5 Total Phenolic Content

A 60g/L solution of sodium carbonate was prepared by dissolving 3.505g of monohydrated Na2CO3 with 50mL of ddH2O in a 50mL volumetric flask. A 10-fold dilution of Folin-Ciocalteux reactant was prepared by diluting in a 25mL volumetric flask 2.5mL of Folin-Ciocalteux reagent

with ddH2O. A Ferulic acid standard of a concentration of 0.5mg/mL was prepared by dissolving 0.0050g of Ferulic acid in 10mL ddH2O and vortexing the solution. Concentration of 0.25mg/mL, 0.125mg/mL, 0.0625mg/mL and 0.03125mg/mL were prepared by doing a serial dilution of the standard of concentration 0.5mg/mL using 500mL of solution diluted in 500mL of ddH2O. X4, X8 dilution of the Methanolic solution, prepared in section 2.3.3, were performed for each of the samples by doing a serial dilution using 500ml of solution diluted in 500mL methanol. 50mL of all diluted extracts and standards were placed in 1.5mL amber Eppendorf tubes. 475mL prepared Folin-Ciocalteux reagent dilution was added to each Eppendorf tube. A blank was prepared by adding 700mL of Folin-Ciocalteux reagent in an empty Eppendorf tube. Each sample was vortexed in the order in which the Folin-Ciocalteux reagent was added. After 5 minutes 475mL of 60g/L sodium carbonate was added to each tube in the same order that the Folin-Ciocalteux reagent was added. After 2hours, each tube was vortexed. 200mL of each mixture was plated in a microplate. Absorbance was read at 725nm using Biotek Cytation Hybrid Multi-Mode Reader (Biotek, Winooski, VT, USA). The TPC values were expressed as mg of gallic acid per 100g of samples

Standard curve using the absorbance of the different gallic acid concentrations was generated and total phenolic content was calculated from the triplicates. This method was based on the following article[78]. Antioxidant functionality resulting from phenolic content and other bioactive compounds was measured as explained in section 2.3.9 through the ORAC assay.

2.3.6 Total flavonoid Content

The Total flavonoid content was measured through colorimetric assay[79]. Methanolic solution, prepared in 2.3.3 with concentration of 10g of defatted sample/25mL of methanol, were diluted by a factor of 5 before being tested. A solution of aluminium chloride of concentration 0.1mM was made by dissolving mg of aluminium chloride in 10mL of water. A solution of sodium

acetate of 0.1nM concentration was also prepared. Standard concentration of quercetin was prepared from a stock solution of 1mg/mL (0.01mg/mL, 0.02mg/mL, 0.04mg/mL, 0.05mg/mL, 0.06mg/mL, 0.08mg/mL and 0.1mg/mL). In an Eppendorf tube 1mL of standard or sample dilutions were added, followed by 50 ml of aluminum chloride solution and 50 mL sodium chloride solution. Each tube was vortexed for 5 seconds before platting 200mL of final solution

2.3.7 Saponin analysis using HPLC-ELSD

A methodology for the analysis of saponin was developed from *Tenon, M and al*[80]. Samples prepared in section 2.3.3 by dissolving the total dried extract in 25mL of methanol loaded in the carrousel of an HPLC-PDAD machine. The samples were analyzed by a Waters e2695 HPLC system equipped with a w2424 Evaporating Light Scattering Detector (ELSD) (Waters, Milford, MA, USA) and the Empower Software. Saponin standards were separated using a reversed-phase column Synergi-Max-RP column (250X4.6mm, 5 mm). 10 mL were injected at a temperature of 25°C. The column temperature was set at 40oC4. The flow rate was maintained at 1 mL/min. The total run time was 50 min. Two mobile phases were used, solvent A: 0.1% formic acid/water (v/v) and solvent B: 0.1%formic acid/acetonitrile (v/v). The solvent gradient was set at the start to 98% solvent A, 2% solvent B followed by:

0-40 minutes: 2% Solvent A, 98% solvent B

40-45minutes: 98% solvent A, 2% solvent B

Nebulizer was set to 67% of its power level and the drift tube was set to a temperature of 55°C. Detector were set to a gain of 500 and gas pressure was set to 51.0 psi. Identification of saponin compounds was done by comparing retention time to commercially obtained standards shown in Table 2.5. Standard calibration curves were generated using a mixture of the saponin compounds at 5 different concentrations.

2.3.8 Total Saponin Content

Total saponin Content was measured using a modified version of the method found in *Navarro del Hierro, J and al*. and *Makkar, H. P. S* [81,40]. A solution of vanillin was prepared by dissolving 800mg of vanillin in 10mL of ethanol. A standard solution of diosgenin was prepared by diluting 10mg of diosgenin in 10mL of ethanol. This solution was then further diluted to obtain 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL and 0.5mg/mL concentration of diosgenin. In an Eppendorf tube, 100mL of standard or sample dilution were pipetted followed by 100mL of the vanillin solution (0.8% w/v). 1000mL of a solution of 70% sulfuric acid was then added to each Eppendorf tubes. Each solution was vortexed for 5 seconds before incubating at 60°C for 10 minutes. Each tube was then cooled in an ice bath before being platted on a microplate. Absorbance was read at 544nm using Biotek Cytation Hybrid Multi-Mode Reader (Biotek, Winooski, VT, USA).

Standard curve using the absorbance of the different diosgenin concentration was generated and total saponin content were calculated from that those standard curves for each sample triplicates.

2.3.9 Oxygen radical Absorbance Capacity

Oxygen Radical Absorbance Capacity assay was based on pre-existing protocols adapted from[82]. A buffer solution was prepared by mixing 351ml of 0.75M K2HPO4 monobasic stock solution with 603ml of 0.75M K2HPO4 dibasic stock solution. 100mL of this solution was then diluted to 1L to make a buffer solution of 75mM. This buffer solution was then used to prepare the Trolox standard and the fluorescein solution. To prepare the fluorescein solution 0.01125g of fluorescein was dissolved in a 25mL volumetric flask using the previously prepared buffer solution. A stock solution for the standard dilution of Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid) was then prepared by dissolving 0.0026g of Trolox in 10mL of buffer solution. This stock solution was then further diluted in buffer to obtain the following concentrations: 50 µM, 25 mM, 12.5mM and 6.25mM.

On a microplate 20mL of Trolox standard or sample solution were platted individually in a well. Methanolic samples prepared in section 2.3.4 were diluted by a factor of 10000 in ORAC buffer before being plated. 120mL of fluorescein was then added to each well and incubated at 37°C after placing a film on the microplate. A blank of 20mL of buffer solution was also plated. A few minutes before the end of the incubation period, a solution of AAPH was prepared by dissolving 0.21375g of 2,2'-Azobis(2-amidinopropane) dihydrochloride in 5mL of ORAC buffer solution. After incubating for 30 minutes, 60mL of AAPH solution was added on each individual plate. Absorbance was recorded each second for 60 minutes to create a curve of the absorbance in relation to time using a Biotek Cytation Hybrid Multi-Mode Reader (Biotek, Winooski, VT, USA). The area under the curve of different concentrations of Trolox was then used to create a linear regression of the standard. This linear regression was then used to evaluate the oxygen radical absorbance capacity of each sample in mg of Trolox/ g of samples. ORAC gives indication of the functionality properties of our extract. This functionality is partially resulting from the presence of phenolic compounds which were measured as described in section 2.3.5., by the total phenolic content/TPC assay.

2.3.10 Statistical Analysis

Results were analyzed using IBM SPSS Statistics software (IBM corp, Armonk, New York, USA). Significant difference between triplicate values (3 indivitual runs) was determined using a statistical analysis of variance (ANOVA), using one-way ANOVA. A P-value inferior to

0.05 indicated a significant difference between the triplicate values. The mean values were then compared using Duncan's Multiple Range with a=0.05.

2.4 Results and Discussions

2.4.1 Standard Used for RV-HPLC-PDAD analysis of Phenolic Compounds

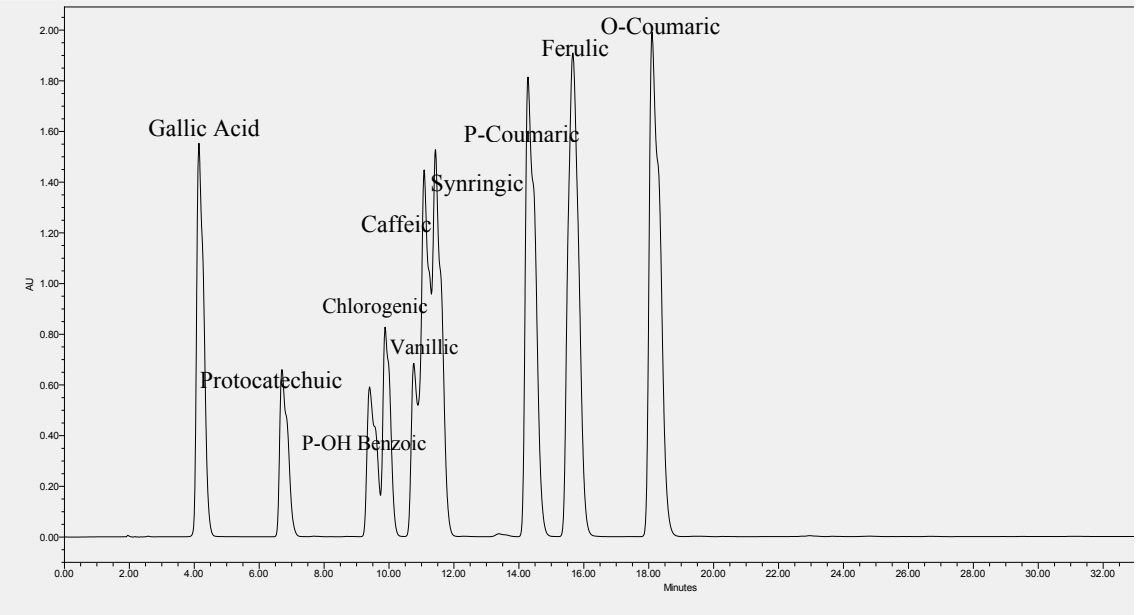


Figure 2.1: *RP-HPLC-PDA Chromatogram of Phenolic acid standard mixture at 280 nm.*

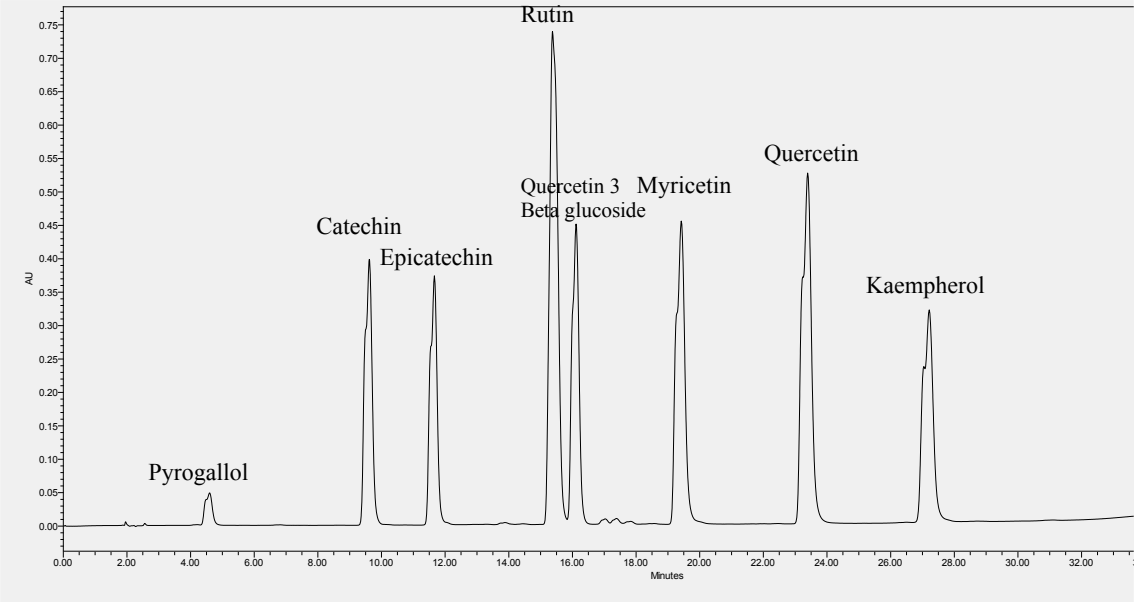


Figure 2.2: *RP-HPLC-PDA Chromatogram of Flavonoid standard mixture at 320nm*

***Table 2.1**: Retention time of Phenolic Acids standards analyzed through RP-HPLC-PDAD at 280nm*

Phenolic Acid Standard	Retention time (min)
Gallic	4.375
Proto catechuic	6.771
P-oh-benzoic	9.510
Chlorogenic	9.951
Caffeic	11.203
Vanillic	10.889
Syringic	11.558
P-coumaric	14.402
Ferulic	15.654
O-coumaric	18.098

***Table 2.2:** Retention time of Flavonoid standards analyzed through RP-HPLC-PDA at 320nm*

Flavonoid Standard	Retention time (min)
Pyrogallol	4.597
Catechin	9.606
Epicatechin	11.652
Rutin	15.385
Quercetin 3 beta glucoside	16.137
Epicatechin gallate	15.483
Myricetin	19.412
Quercetin	23.397
Kaempherol	27.210

2.4.2 Effect of UAE on yield of extract

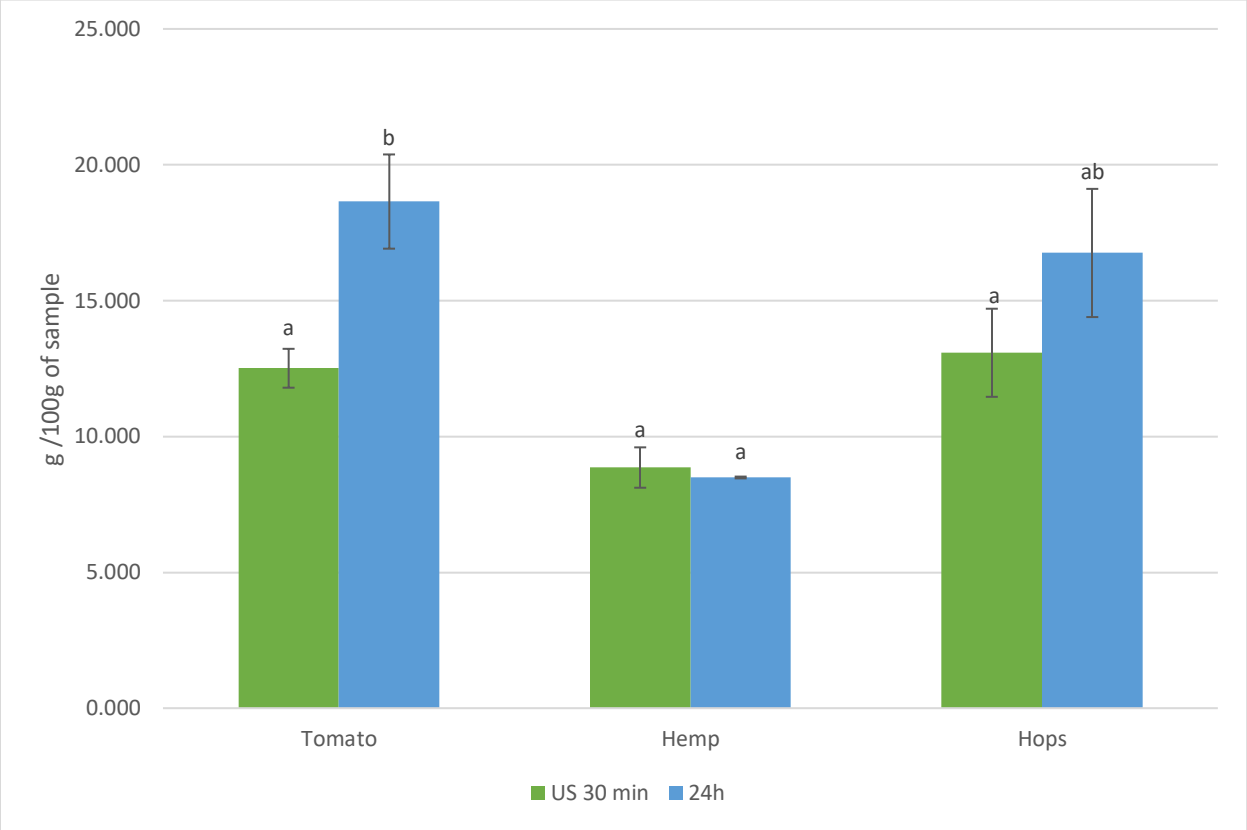


Figure 2.3: *Average Yield in g of final extract per 100g of dried sample resulting from the 24h mechanic and 30 minutes HPLF-US ethanolic extraction of Tomato Skin, Hemp meal and Hops Flowers. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

The percentage yield of final extracts using machinal stirring for 24h and 30 minutes HPLF-US treatment as extraction methods were compared for each of the samples obtained as seen in Figure 2.3. Tomato skin extraction showed a yield of 12.51±0.51g/100g of sample when extracted using US (US 30 min) and 18.65±3.0 g/100g of sample when extracted using traditional mechanical extraction (24h). Hemp meals extraction had a yield of 8.86±0.55 g/100g of sample and 8.49±0.01 g/100g of sample when respectively extracted using US treatment for 30 min and

mechanical extraction for 24h. When looking at the extraction of samples of hops flowers, the average yield for US treatment was of 13.08±2.63 g/100g of sample and the average yield for traditional mechanical 24h extraction was of 16.75±5.56 g/100g of sample for both the hops sample extraction and the hemp sample extractions, there was no statistical difference ($P>0.05$) between the 24h mechanical extraction and the 30 minutes US extraction. Tomato on the other hand had a significantly higher yield for the 24h mechanical extraction when compared to the sample treated with US. In the case of hemp meal and hops flowers, it can be said that 30 minutes US treatment produced a similar yield to 24h mechanical stirring. Tomato skin samples however, produced a final yield when extracted for 24h with mechanical stirring than when extracted for 30 minutes using HPLF-US. However, when looking at the difference between the sample types, there was no significant difference in final yield between tomato skin, hemp meal and hops flowers indicating that for this extraction methodology yield seemed independent of sample type.

2.4.3 Effect of UAE on phenolic content

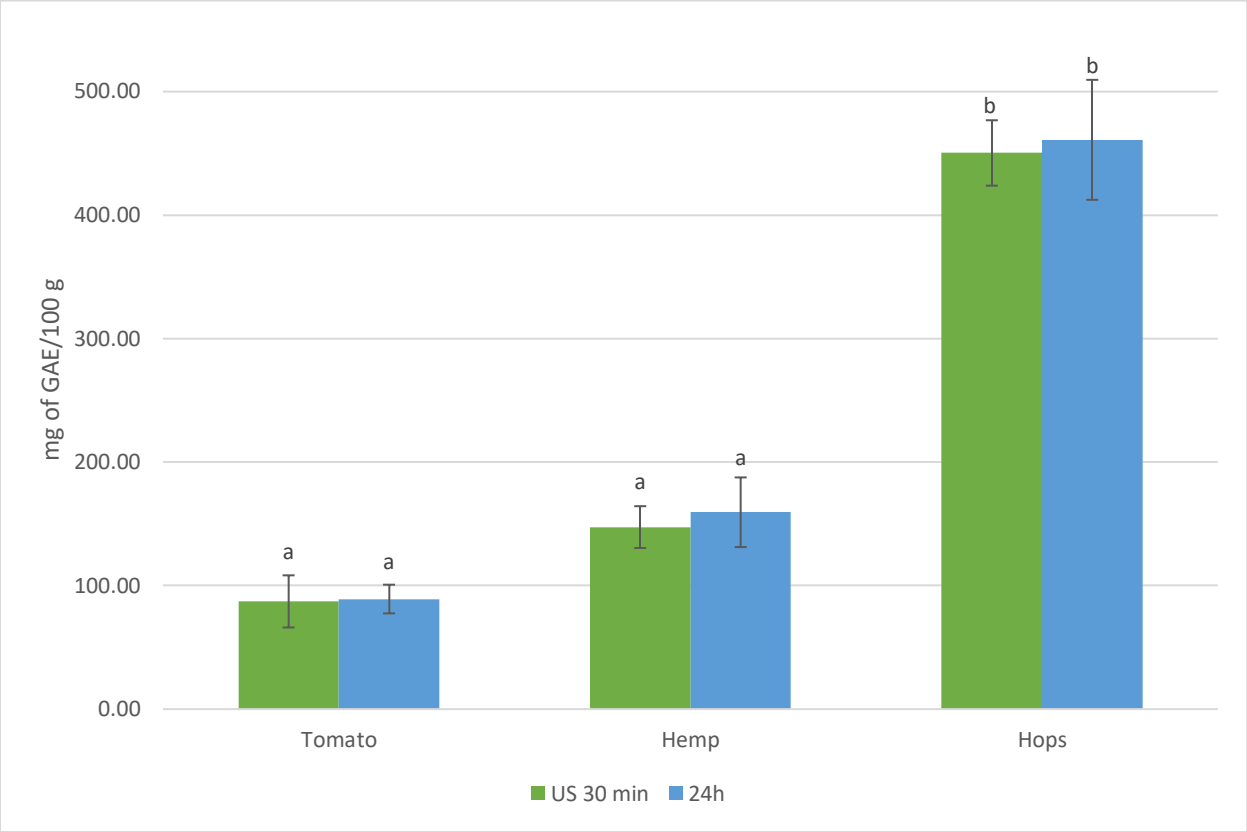


Figure 2.4: *Total Phenolic Content in mg of gallic acid equivalent per 100g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

The TPC (total phenolic content) of ultrasound extracted (US 30 min) and traditional mechanically extracted (24h), tomato skin, hemp meal and hops flowers are shown in Figure 2.4. Tomato skin extracted with US had a TPC of 87.22±21.12 g of GAE/100 g of sample similarly the same sample extracted using mechanical stirring had a TPC of 89.14±11.61g of GAE/100g of sample. Hemp samples when respectively extracted using 30 minutes ultrasound treatment and

24h mechanical stirring had an average TPC of 147.39±16.92 g of GAE/100g and 159.42±28.20 g of GAE/100g respectively. The average TPC accounted for each of the hops flower extraction was respectively for the US extraction and the 24h extraction 450.32±26.47 g of GAE/100g of sample and 460.95±48.57 g of GAE/100g of sample. There was no significant difference ($p>0.05$) between the US treatment (US 30 min) and 24h extraction (24h) for any of the 3 samples (tomato, hemp, and hops). However, there was a significant difference in average TPC between the Hop flower extraction and the tomato and hemp extractions. No significant difference was found between tomato skin TPC and hemp meal TPC. As such, hops flower can be said to have a higher TPC than both hemp meal and tomato skin. Furthermore, from the lack of significant difference mentioned between the TPC associated to US and traditional extraction of any of the sample, it can be mentioned that UAE doesn't influence the TPC when compared to a 24h traditional extraction.

Table 2.3: *Phenolic Profile in mg/g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM[*] with differing subscript letter representing statistical difference*

Sample	Gallic mg/g	Protocatechuic mg/g	P-OH Benzoic mg/g	Chlorogenic mg/g	Vanillic mg/g	Syringic mg/g	P-Coumaric mg/g	Ferulic mg/g	O-Coumaric mg/g
Tomato US 30min	5.02±0.09^{a}	4.83±0.09^{a}	6.17±0.36^{a}	5.38±0.05^{a}	-	10.95±0.2^{a}	19.68±0.01^{a}	4.59±0.07^{a}	-
Tomato 24h	4.89±0.03^{a}	4.86±0.01^{a}	6.24±0.47^{a}	5.50±0.06^{a}	-	10.90±0.02^{a}	19.68±0.01^{a}	4.29±0.02^{a}	-
Hemp US 30min	-	28.12±0.60^{b}	2.44±0.04^{b}	3.00±0.07^{b}	-	-	9.99±0.01^{b}	2.15±0.003^{b}	-
Hemp 24h	-	29.40±0.31^{c}	8.78±0.08^{c}	3.49±0.01^{b}	1.35±0.04^{a}	4.61±0.03^{b}	10.03±0.01^{b}	2.45±0.02^{b}	-
Hops US 30min	3.14±0.09^{b}	12.79±0.24^{d}	19.04±1.32^{c}	17.90±0.44^{d}	11.82±0.09^{b}	13.43±0.35^{a}	13.70±0.04^{c}	8.00±0.16^{c}	5.68±0.21
Hops 24h	3.20±0.20^{b}	7.92±0.34^{e}	10.60±0.96^{d}	12.50±0.96^{e}	5.67±0.31^{c}	10.60±2.32^{a}	13.78±0.28^{c}	6.90±0.59^{c}	4.48±0.19

[*] Caffeic acid was also analyzed for but was not found in detectable concentration in any sample and as such was not included in the table.

Table 2.3 compounds the concentration of the different phenolic acid present in each of the plant extracts analyzed. Tomato skin extracted using US (Tomato US 30 min) and tomato skin extracted using traditional methods (Tomato 24h) show the same phenolic acid profile. Gallic acid, Protocatechuic acid, hydroxybenzoic acid, chlorogenic acid, syringic acid, p-coumaric and ferulic acid were all present in both extracts. Furthermore, no statistical difference ($p>0.05$) can be noted for the concentration of any of the phenolic present in both samples. Hemp extract on the other hand show significant difference in both composition and concentration of phenolic acids. 24 hours extracted hemp sample (Hemp 24h) shows a higher diversity of phenolic acid when compared to the 30 minutes US extracted samples. As such, two more phenolic acid are present in the 24h extract vanillic acid and syringic acid. In both sample, chlorogenic acid, p-coumaric,and ferulic acid were present in concentration that were not shown to be statistically different. On the other hand, the concentration of protocatechuic acid and hydroxybenzoic acid was shown to be statistically different ($p<0.05$) in both hemp extract. The 24 hours extraction showing higher average concentration of both these compounds. Hops flower extract had similar phenolic acid profile. In both extracts the following phenolic acid were detected: gallic acid, protocatechuic acid, hydroxybenzoic acid, chlorogenic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid and o-coumaric acid. Gallic acid, p-coumaric acid, syringic acid and ferulic acid were all found to be similar concentration in both 24h hop extracts (Hops 24h) and US hop extracts (Hops US 30 min), meaning there was no statistical difference between the concentration resulting from the analysis. Alternatively, protocatechuic acid, hydroxybenzoic acid, chlorogenic acid and o-Coumaric acid were present in statistically higher concentration in both the 24h hop extract and the US extract. As such hops showed the most diversity in phenolic acid composition between all 3 plant samples. UAE extraction for both tomato skin and hops flower seemed to increase the

concentration of phenolic compound extracted. However, the same cannot be said for the extraction of Hemp where traditional method showed overall higher diversity and concentration of phenolic. This could indicate a need to increase time of extraction for hemp sample as to increase the efficiency of US.

2.4.4 Effect of UAE on flavonoid content

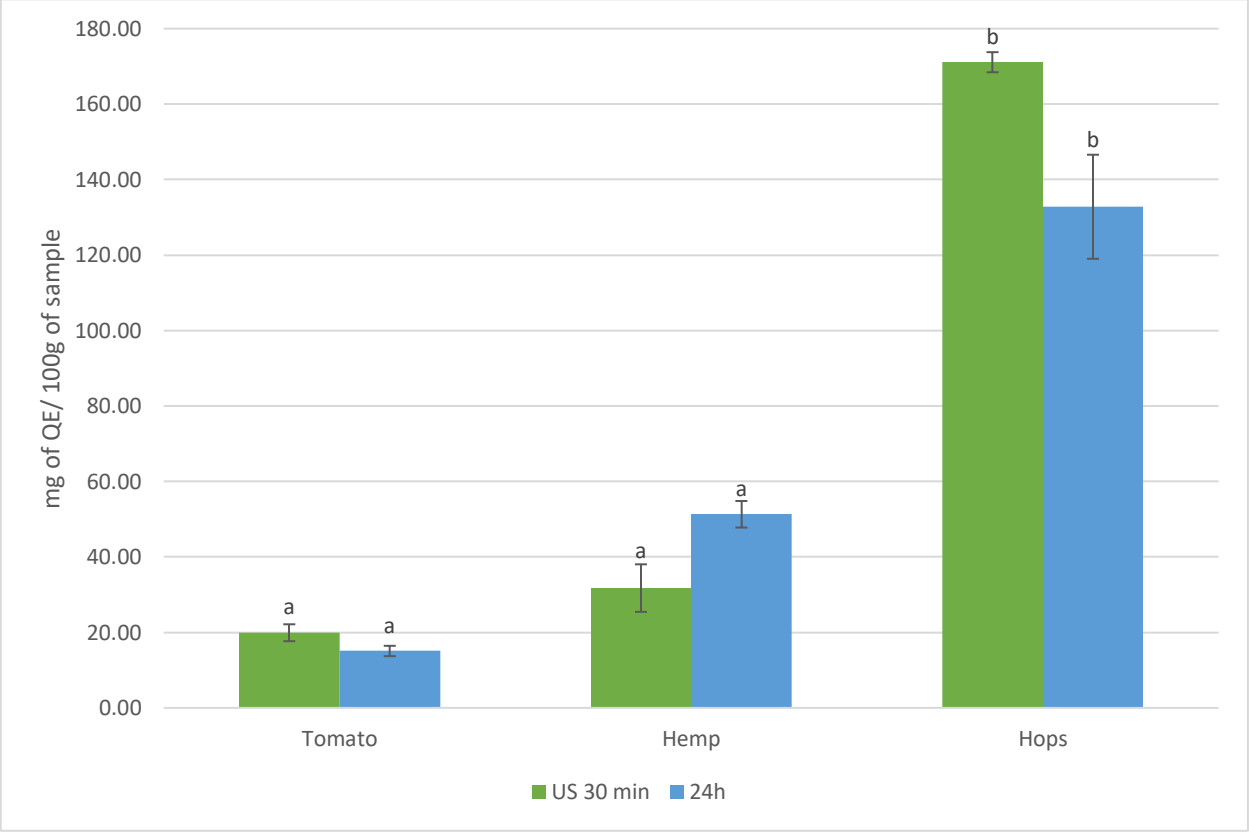


Figure 2.5: *Total Flavonoid Content in mg of quercetin equivalent per 100g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

The total flavonoid content in tomato skin, hemp meal and hops flower are shown in Figure 2.5. Values for the TFC of US extracted tomato, 24h extracted tomato, US extracted hemp and 24h

extracted hemps were observed to be respectively 19.92±2.24 mg of QE/g of sample, 15.08±1.37 mg of QE/g of sample, 31.75±6.30 mg of QE/g of sample and 51.31±3.52 mg of QE/g of sample. Tomato skin and hemp meal extractions showed no statistical difference ($p > 0.5$). However, both hop sample extraction had significantly higher TFC, 171.10±2.66 mg of QE/g of sample for the US extraction and 131.80±13.79 mg of QE/g of sample for the 24h extraction, when compared to all other extractions. This is in accordance with the results in Figure 2.4, which showed significantly higher results for the hops extraction. Flavonoid being a subcategory of phenolic content it is logical to see that samples with significantly higher phenolic concentration also showed a significantly higher concentration of flavonoids. Finally, there was no significant difference in TFC between 24h and US extracts of any of the sample tomato, hemp or hops samples indicating that TFC was not influenced by the difference in extraction techniques

.

Table 2.4: *Flavonoid Profile in mg/g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM[*] with differing subscript letter representing statistical difference*

Sample	**Pyrogallol** mg/g	**Catechin** mg/g	**Rutin** mg/g	**Myricetin** mg/g	**Quercetin** mg/g	**Kaempherol** mg/g
Tomato US 30min	-	6.27±0.04^{a}	4.07±0.21^{a}	-	2.96±0.05^{a}	-
Tomato 24h	-	6.25±0.23^{a}	3.56±0.25^{a}	-	2.93±0.02^{a}	-
Hemp US 30min	-	2.03±0.28^{b}	1.85±0.15^{b}	-	3.95±0.6^{b}	6.87±0.86^{a}
Hemp 24h	-	3.22±0.12^{c}	1.80±0.01^{b}	-	6.00±0.2^{c}	5.68±.19^{a}
Hops US 30min	23.01±0.67	23.19±0.56^{d}	10.18±0.84^{c}	10.67±0.29^{a}	15.12±0.05^{d}	15.03±0.87^{b}
Hops 24h	-	21.66±0.54^{e}	14.11±0.67^{d}	11.28±0.30^{a}	13.46±0.4^{f}	13.11±2.16^{b}

[*] Epicatechin was also analyzed for but was not found in detectable concentration in any sample and as such was not included in the table.

The flavonoid profile of the various tomato skin, hemp meal and hop flower extract are all summarized in Table 2.4. Tomato skin extracts showed the same flavonoid profile even when extracted using US (Tomato US 30 min) or using traditional method of extraction for 24h (Tomato 24h). Catechin, Rutin and Quercetin were all found in both tomato extracts. Moreover, the concentrations of each of those phenolic compounds were not statistically different. Hemp on the other hand showed a significant difference when it came to the concentration of the various flavonoid identified in both US extract (Hemp US 30 min) and the traditionally extracted extract (Hemp 24h). Both extracts showed the presence of the same 4 flavonoids: catechin, rutin, quercetin and kaempherol. All these compounds were found in higher concentration in the 24h extract aside from kaempherol. Consequently, kaempherol concentration was not found to be statistically different between US extracts and the 24h extracts. Hops flower extracts showed difference in the flavonoid profile. As such, the US extract (Hops US 30 min) had a broader diversity of flavonoid showing the presence of pyrogallol, catechin, rutin, myricetin, quercetin and kaempherol. Comparatively, pyrogallol was not found to be present in the 24h extracts. However, the concentration of catechin and quercetin was found to statistically higher in the US extract. Rutin concentration appeared to be significantly lower in the US extracted sample than the 24h extracts. The concentrations of myricetin and kaempherol of both hop extracts were not found to be statistically different. Additionally, Hops was found to have the highest diversity of flavonoids of the three plant samples. Although, the total flavonoid content did not seem to be affected by US treatment when compared to a 24h traditional extraction method, the flavonoid composition seems to change when looking at hops and hemp extracts. This could be remedied by performing longer US extraction as to ensure further cavitation and sonoporation of the sample which would logically lead to further leaching of bioactive compounds still present in the plant matrix.

2.4.6 Effect of UAE on saponin content

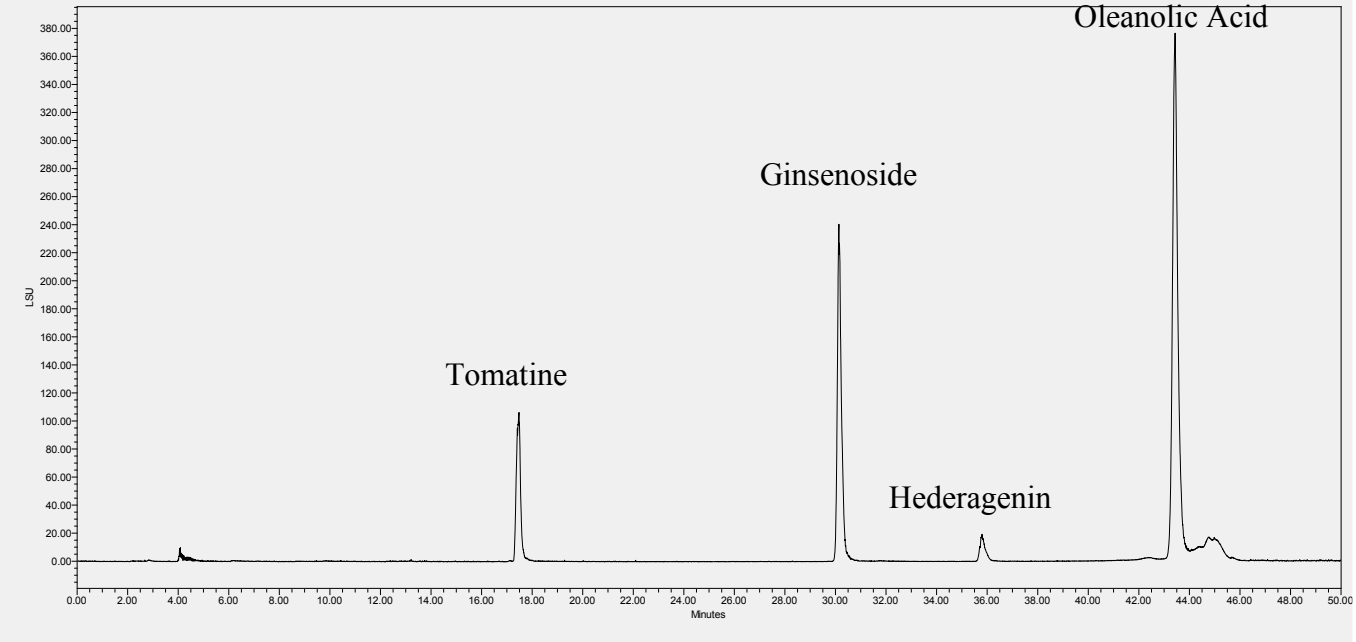


Figure 2.6: *RP-HPLC-ELSD Chromatogram of Saponin standard mixture*

Table 2.5: *Retention time of Saponin used in standards mixture and analyzed through RP-HPLC-ELSD*

Standard	Retention time (min)
Tomatine	17.451
Ginsenoside	30.135
Hederagenin	35.794
Oleanolic Acid	43.410

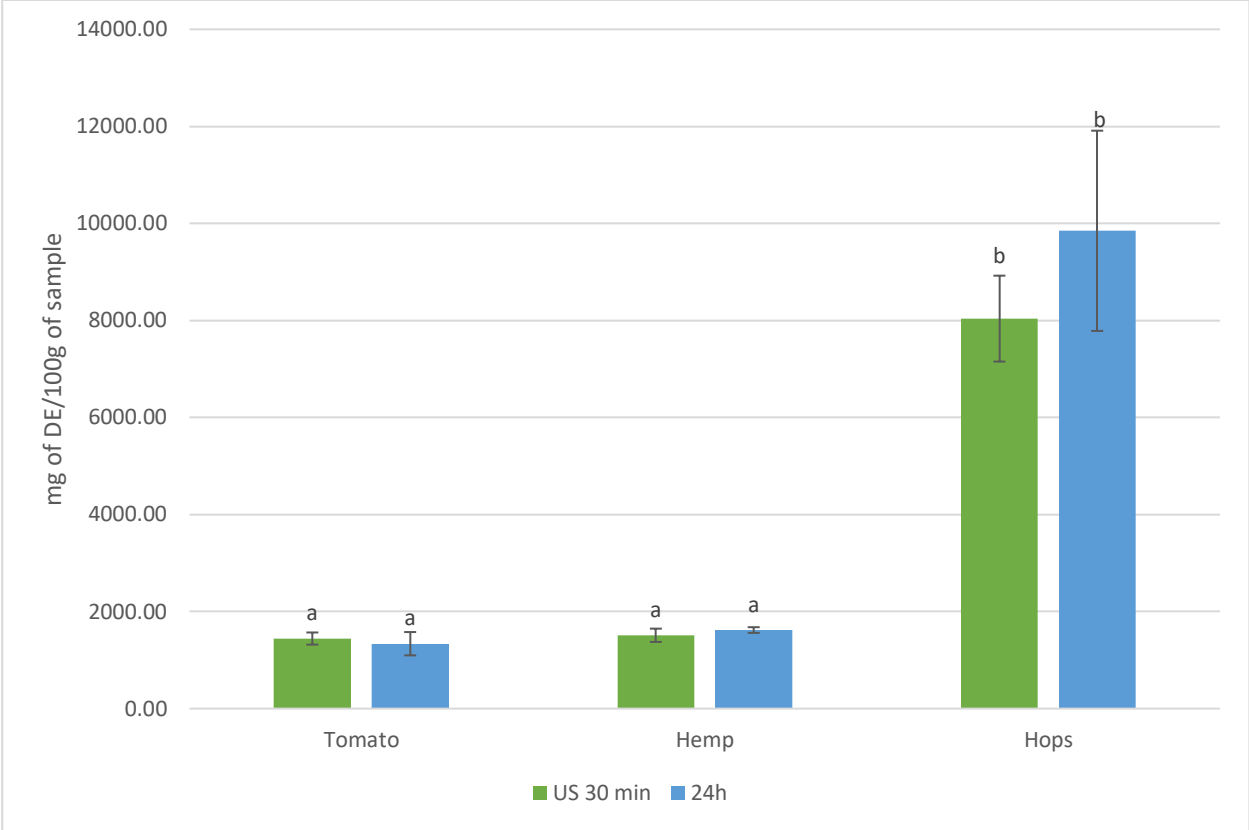


Figure 2.6: *Total Saponin Content in mg of diosgenin per 100g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

The total saponin content was measured for each of the samples (tomato skin, hemp meal and hops flowers) extracts. Tomato samples when extracted using US (US 30 minutes) had an average TSC of 1443.79±125.24 mg of DE/ 100g of sample compared to 1337.65 mg of DE/ 100g of sample when extracted using traditional methods (24h). There was no significant difference found between the TSC of US extracts and the 24h extracts ($p > 0.05$). Hemp extracts also did not show any significant difference in TSC when extracted with US vs the traditional method, the measured TSC was respectively found to be 1511.25±136.98 mg of DE/ 100g of sample and 1618.93±58.90 mg of DE/ 100g of sample. Hops extracts similarly showed no significant

difference between TSC of the US extraction and the traditional extraction where values were respectively 8037.83±885.45 mg of DE/ 100g of sample and 9847.34±2063.63 mg of DE/ 100g of sample. Both Hops extraction had total saponin concentration significantly higher than hemp and tomato extracts ($p<0.05$). However, between the different methods of extractions, there was no significant difference meaning that ultrasound treatment for 30 minutes did not influence the final concentration of saponin when compared to 24h traditional extraction.

Table 2.6: *Saponin Profile in mg/g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

Sample	**Tomatine** mg/g	**Ginsenoside Rb3** mg/g	**Oleanic Acid** mg/g	**Hederagenine** mg/g
Tomato US 30min	1476.6±243.2[a]	-	211.4±37.7[a]	-
Tomato 24h	1415.7±69.7[a]	-	157.1±16.1[a]	-
Hemp US 30min	-	-	692.9±8.8[b]	-
Hemp 24h	-	-	667.7±33.6[b]	-
Hops US 30min	-	43.4±5.5[a]	105.2±5.5[a]	2331.3±105.9[a]
Hops 24h	-	29.2±0.8[b]	353.7±93.5[c]	7225.6±1842.1[b]

Table 2.5 shows the concentration of various saponin present in the different extracts. Tomatine was found only in tomato extracts. Tomato extracts show the presence of tomatine and oleanic acid. There was no significant difference in the values obtained from US extraction when compared to traditional extraction for either the concentration of tomatine and oleanic acid. However, tomato is the only sample in which tomatine was detected. Alternatively, oleanic acid

was detected in all samples. However, in hemp sample oleanic acid was the only saponin detected. Furthermore, there was no statistical difference between the concentration of oleanic acid between the extract obtained from US and the extract obtained from traditional extraction. Hops on the other hand showed a wider variety of saponin when compared to the other samples. Ginsenoside Rb3, oleanic acid and hederagenin were all found to be present in hops extracts. Additionally, the concentration of each of these saponin was found to be statistically different between the US extracts (Hops US 30 min) and the traditionally extracted extracts (Hops 24h). Ginsenoside Rb3 was found to be higher in the US extracts whereas the 24h extracts showed higher concentration of both hederagenin and oleanic acid. These results seem to indicate that when extracting Hemp meal and Tomato skin for saponin, using US treatment is more efficient than 24h extraction as it allows to reduce the time needed to perform the extraction. However, it seems that in the case of Hops flower, US treatment might be insufficient to fully extract all the saponin present. An increase in US treatment time would likely show an increase in extract concentration. These findings seem to confirm that US application could increase the efficiency of extraction on tomato skin, hemp meal and hops flower for saponin compounds.

2.4.7 Effect of UAE on extract antioxidant activity

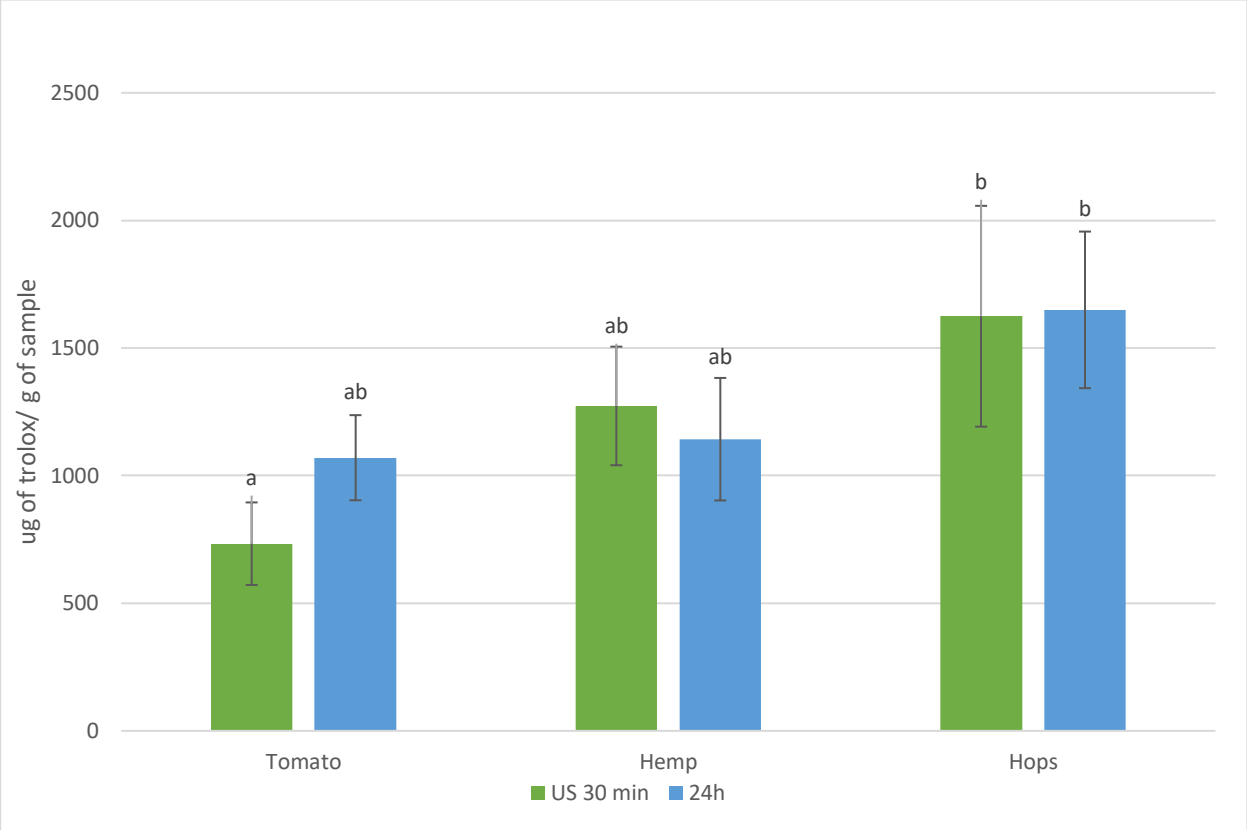


Figure 2.7: *Oxygen radical absorbance capacity in mg of Trolox/g of dried grinded Tomato Skin, Hemp Meal and Hops Flower after 24h mechanical extraction and HPLF-US 30 minutes extraction. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

The Oxygen radical absorbance capacity (ORAC) was measured for each extraction method and each sample. Tomato extracts showed values of 733.44±161.73 mg of Trolox/g of sample and 1070.28±166.83mg of Trolox/g of sample for the US extraction and the traditional extraction. When it comes to hemp, those values were respectively 1273.16±240.06 mg of Trolox/g of sample and 1142.86±240.06 mg of Trolox/g of sample. Hops on the other hand showed values of 1624.59±432.66 and 1649.57±306.86 mg of Trolox/g of sample for the US extracts and 24h extracts. No significant difference was found between the 24h extracted tomato sample, the

hemp extracts, and the hops extracts. Furthermore, no significant difference was found between the tomato extracts and the hemp extracts. Overall, it can be said tomato and hemp and hops extracts had similar antioxidant activity despite the differences in TPC mentioned in 2.1.3. The method of extraction did not seem to influence ORAC as there was no significant difference between US extracted sample results and 24h extracted sample results. This also corresponds to the conclusion reached in 2.1.3 signifying similarly that US extraction did not influence the antioxidant activity of the extracts obtained from hops flower, tomato skin and hemp meal.

2.5 Conclusion

Report from this study seems to suggest that UAE increases the efficiency of biologically active compounds (phenolics and saponin) in tomato skin, hemp, and hops. However, when looking at hemp meal extraction, results seem to indicate that longer ultrasound treatment would be needed to fully extract phenolic compounds. As such phenolic compound diversity was higher in 24h extraction of hemp meal despite both no significant difference in TPC (147.39±16.92 and 159.42±28.20 g of GAE/100g) between both extraction methods. Alternatively, in the case of hops flower extracts, UAE was more efficient in the extraction of phenolics than the extraction of saponins. As saponin diversity was increased in traditional extraction despite similar TSC (8037.83±885.45 and 9847.34±2063.63 mg of DE/ 100g of sample). Similarly, it should be mentioned that longer HPLF-US treatment should also facilitate saponin extraction in hops flowers. This seems to demonstrate the value of HPLF-US as a viable treatment solution to reduce the time of extraction (24h to 30 minutes) when seeking to extract phenolic compounds and/or saponin. Both molecular families show important uses as functional food in the food industry, cosmetic industry and or pharmaceutical industry.

Chapter 3: Influence of HPLF-US treatment on bioplastics

3.1 Abstract

Bioplastics made from previously extracted left over of tomato skin, hemp meal and hops flower were formulated as to evaluate the impact of US treatment. As such, 30-minute US treatment was applied and resulting bioplastics were compared to non-treated bioplastic samples. Bioplastic treated with US were found to have different surface structure and gel-structure when analyzed using PLM and SEM. Overall US treated samples had less air bubbles in their gel structure and seem to show slight surface cavitation. These structural changes seemed to also affect the rheological properties of our bioplastic as differences were noted in hardness, resilience, springiness index, cohesiveness, water activity and % moisture. Lower hardness and increase resilience, springiness and cohesiveness seemed to indicate improvement in US treated bioplastic's rheological ability. Furthermore, despite increased % moisture, water activity was lower in US-treated samples indicating a potential lower microbial growth.

3.2 Introduction

Plastic is the term used to refer to materials which are made of synthetic polymer made from oil derivative. Due to their composition, plastics are known to cause environmental damage as they take years to decompose. As plastics have multiple uses, its production is estimated at 8.3 billion metric tons[83]. However up to 80% of it ends up in landfill and by extent end up leaking in the environment[83]. To remedy to this situation novel material are constantly developed. Bioplastics or sometimes referred to as biomaterials are one of such alternatives. Bioplastic are made from biological material which allows them to biodegrade making them a sustainable and more environmentally friendly option. Often time, bioplastic relies on the presence of biopolymer who can create a 3D network lending strength and cohesiveness to the product[47]. Ultrasound treatment

has been shown to allow the development of stronger double emulsion[84]. As such we hypothesize that ultrasound treatment could improve the quality of bioplastic through creating more stable gel/emulsion complex. As such bioplastic formulations were developed as to incorporate agricultural waste material (tomato skin, hemp meal and hops flower) as bulking agent of the biomaterial and US treatment was applied to identify the effect on resulting products. Texture profile analysis of the bioplastics was performed to identify change in the rheological properties of the biomaterial. Scanning electron microscopy was also used to identify the surface structure of bioplastic. To further get information on gel structure and effect of ultrasound treatment, Polarized Light Microscopy images were obtained.

3.3 Material and methods

3.3.1 Materials

Glycerol, Phosphoric acid, and calcium hydroxide were obtained from Fisher Chemical (Ontario, Canada). Gelatin was received from Sigma-Aldrige (Ontario, Canada). Sodium Alginate was sourced from Landor Trading Co. (Quebec, Canada). Casein Sodium was ordered from MP biomedicals (CA, USA).

3.3.2 Developing Bioplastic formulation

Bioplastic were formulated based on previous research[85]. After testing multiple formulation based on the previously mentioned work from Macarena Freire certain changes were applied. Certain ingredients were added while other were changed. As such whey powder was replaced by casein as to provide a more stable surfactant. An increase in sodium alginate and addition of gelatin as a secondary gelling agent allowed the formulation of a biomaterial with a cohesive overall structure that was able to be extruded. Furthermore, canola oil was added to act as secondary plasticizer. Role and mass of each ingredient are outlined in Table 3.1.

Table 3.1: *Overall formulation of bioplastic per 100g*

	Mass (g)	**Role**
Biowaste Material	15	Bulking Agent/ Additive
Casein	9	Biopolymer
Calcium Hydroxide	0.1	Plasticizer
Canola Oil	8	Plasticizer
Glycerol	8	Plasticizer
Phosphoric Acid	2	Plasticizer
Gelatine	3	Biopolymer
Sodium Alginate	0.5	Biopolymer
Water	54.4	Solvent

15g of biowaste material, either tomato skin, hemp meal and hops flower previously extracted in part 2.3 were mixed with 9g of casein and 0.1 g of calcium hydroxide. Once the homogenous, 8g of canola oil, 8g of glycerol and 2 g of phosphoric acid were mixed in. In a separate beaker, 3g of gelatin were mixed with 0.5g of sodium alginate. Boiling water was then added to the gelatin/sodium alginate mixture before being homogenized until completely dissolved. The gelatin mixture was then mixed with the biowaste material preparation until a cohesive mass was formed. This preparation was then separated into two equal 50g portions. The first portion was separated into aliquots of 10g before being extruded. The second portion was treated with 30 minutes of HPLF US before being separated in aliquots of 10g and being extruded. All samples were left to dry for 24h under a fume hood before testing. This procedure was repeated for each of the three types of biowaste materials: tomato skin, hemp meal and hops flowers.

3.3.3 Texture Profile Analysis

Texture Profile Analysis was performed on the biomaterials using a CT3 Brooksfield Texture Analyzer. Texture Analyzer was set to the following settings:

Test Type: TPA	Recovery Time: 60 s
Target Deformation: 0.15 cm	Pretest Speed: 0.02 cm/s
Trigger Load: 6.8g	Data Rate: 10 points/sec
Test Speed: 0.05 cm/s	Probe: TA3/100
Return Speed: 0.05 cm/s	Fixture: TA-RT-KI
# of cycles: 2	Load Cell: 4500

Hardness, Springness, Cohessiness, Resilience and Adhessiveness were measured. Each test was repeated to obtain triplicates.

3.3.4 Water Activity

1g of biomaterial samples were analyzed using an Aqualab Model Series 3 water activity meter (Decagon Devide Inc, WA, US) . Each sample was measured as to obtain triplicates values.

3.3.5 Scanning Electron Microscopy

Small section of 0.7mm of thickness of the biomaterials were cut. The surface of each biomaterial section was coated with gold. The gold coted samples were then sent to be processed following the information below:

Company of SEM: TESCAN

Condition of SEM: 10 kv at 10-3 Pa

Place: Carleton Nano Imaging Facility

SEM pictures at the following focus were taken. Each sample was pictured, and the most representative picture was selected from each triplicate.

3.3.6 Polarized Light Microscopy

Small sections were cut and positioned on a glass plate before being observe at an objective of X10. Each picture was taken in triplicate and the most representative picture was selected from each triplicate.

3.3.7 % Moisture

In a crucible, 1g of sample bioplastic was weighted. The Crucible with the samples was then put in an oven at 110oC for 4 hours. Each crucible was then weighted and % Moisture was calculated using the following equation:

$$\%\ Moisture = \frac{(initial\ weight\ of\ sample + crucible) - (final\ weight\ of\ sample + crucible)}{(initial\ weight\ of\ sample + crucible) - (weight\ of\ crucible)}$$

Each testing was done as to obtain triplicate for each sample bioplastic.

3.3.8 Statistical Analysis

Results were analyzed using IBM SPSS Statistics software (IBM corp, Armonk, New York, USA). Significant difference between triplicate values was determined using a statistical analysis of variance ANOVA. A P-value inferior to 0.05 indicated a significant difference between the triplicate values. The mean values were then compared using Duncan's Multiple Range with a=0.0

3.4 Results and Discussion

3.4.1 Overall appearance of bioplastics

Table 3.1: *Overall appearance of bioplastic made from tomato, hemp and hops wit ultrasound treatment and no ultrasound treatment*

	US Treatment	No US Treatment
Tomato		
Hemp		
Hops		

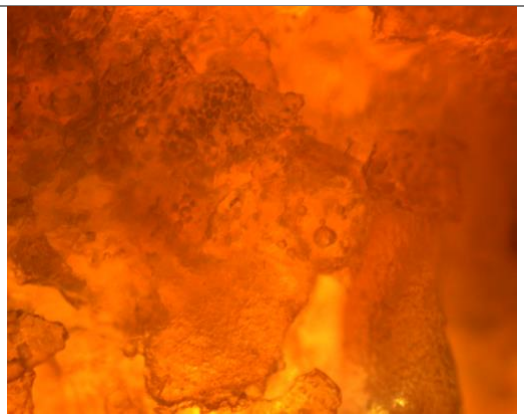
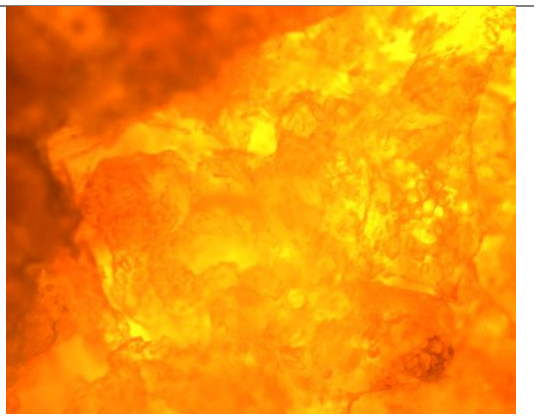
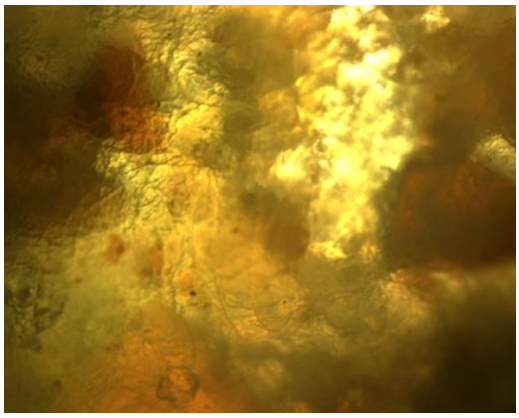
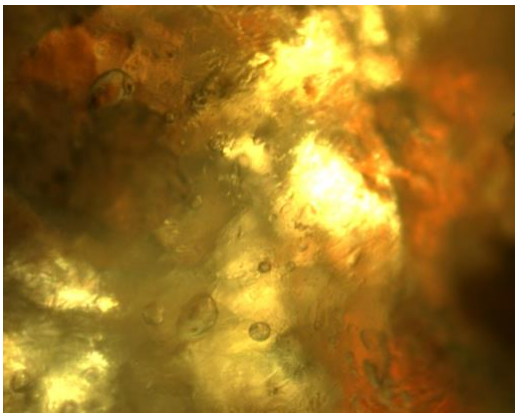
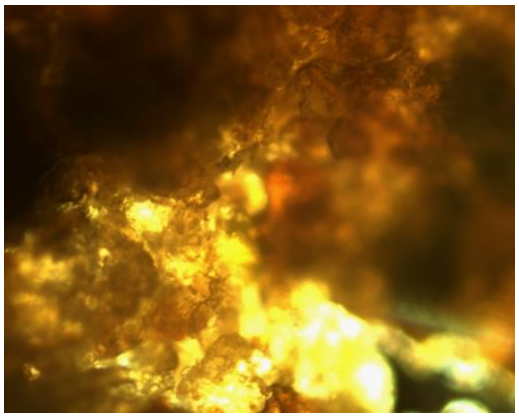
Overall appearance of each biomaterial sample is organized in Table 3.1. When looking at each sample, little difference is found between the physical appearance of samples treated with US and samples treated without ultrasound. The tomato-based bioplastic samples were red in color and mostly uniform in appearance with densely distributed tomato particles all throughout the sample. The tomato-based bioplastic sample also had a smooth surface. However, the US treated tomato biomaterial was slightly oily toward the center of the sample surface when compared to the non-US treated tomato biomaterial. The hemp-based biomaterial showed common traits to the tomato-based biomaterial. They were mostly uniform albeit of a brown/green color. The surface of both hemp-based biomaterials was smooth and uniform with evenly distributed particles of hemp meal throughout the bioplastic sample. Furthermore, similarly to the tomato-based biomaterial samples the hemp-based biomaterial treated with US showed a clearly visible oily sheen situated on the center surface of the biomaterial. This oily sheen could potentially be canola oil separating from the bioplastic structure. As canola oil was used as an external plasticizer it is possible that US treatment increased the rate of separation of canola oil from the biopolymer structure[53]. The hops-based bioplastics were of a brown color and did not show any differentiation in appearance between US treated samples and non-treated samples. Overall, the hops-based bioplastics were much drier in appearance and did not present a smooth surface. Particles of hops were also of much more varied sizes as can be seen in Table 3.1

The difference in overall color/ appearance of each sample size can be directly linked to the base bioplastic used as bulking agent. Tomato skin gave which was of orangish/red color gave the biomaterial a bright red color, hemp meal which was of a dark green-brown color and hops flower were of light brown color led to a similarly colored bioplastic.

Overall, US treatment seemed to influence the overall appearance of both hemp and tomato sample but did not seem to influence the appearance of hemp samples.

3.4.2 Polarized ight microscopy image of biomaterial

Table 3.2: *Polarized Light microscopy image of bioplastic made from tomato, hemp and hops with ultrasound treatment and no ultrasound treatment under a magnification of 10*

	US Treatment	No US Treatment
Tomato		
Hemp		
Hops		

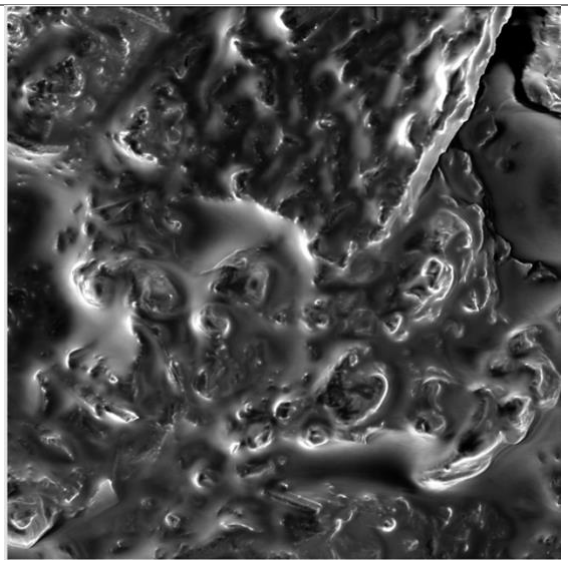
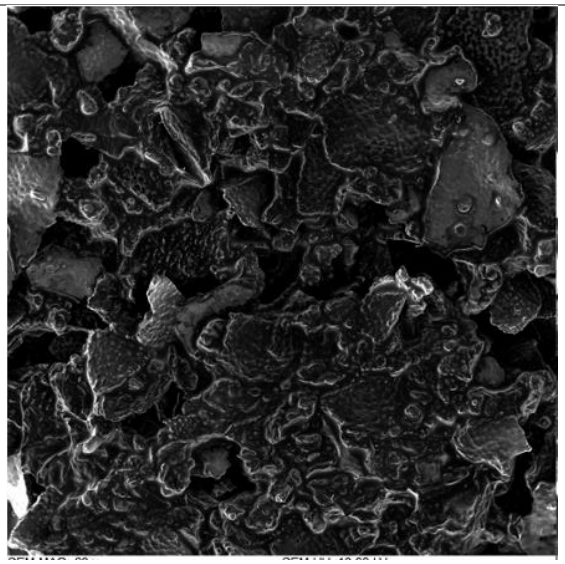
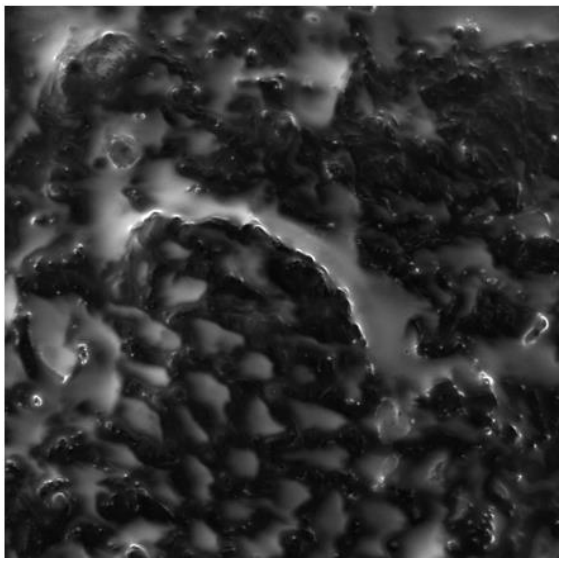
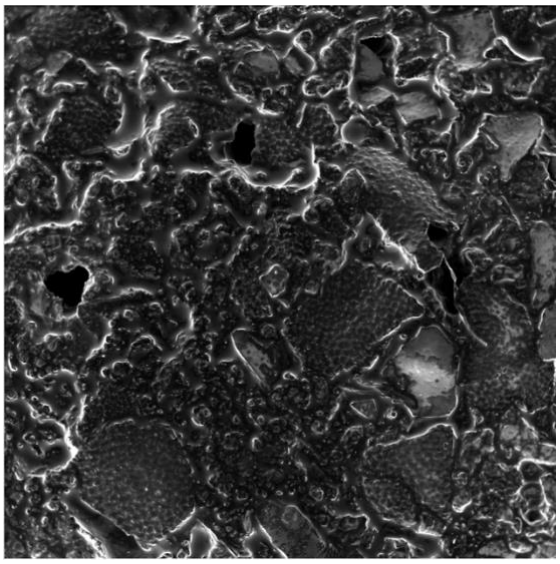
Polarized Light Microscopy was used to obtain the image shown in Table 3.2. Looking at the tomato-based bioplastic it is clear to see the inclusion of tomato particles on the US treated samples. The non-treated tomato bioplastic shows clusters of bubble trapped in the matrix of the biopolymer. However, when looking at the US treated bioplastic these clusters are replaced by a few single bubbles distributed unevenly.

Similarly, the hemp-based biomaterial clearly showed the inclusion of hemp particles in the biopolymer structure. When looking at the non-US treated hemp-based bioplastic, many bubbles can be seen close to the surface of the biopolymer structure. This density of bubbles clearly diminishes when the bioplastic is treated with US as can be seen on the hemp-based bioplastic sample.

Hops-based bioplastic had a different appearance from the other samples that were looked at under PLM. In both, US-treated and non-treated samples can be seen the inclusion of hops particle. No bubbles seem to be caught in the matrix of the biopolymer and the overall appearance of the biopolymer seems jagged and rougher. Furthermore, US treatment increased the roughness of the biopolymer structure when looking at the PLM structure. Small crater like structures seem to be present at the surface of the biopolymer gel hinting at US induced cavitation. On the non-treated sample, we can also notice a particle which is only partially covered by the biopolymer gel created indicating that the proportion of hops/biopolymer gel would need to decrease.

3.4.3 Scanning electron microscopy image of biomaterials

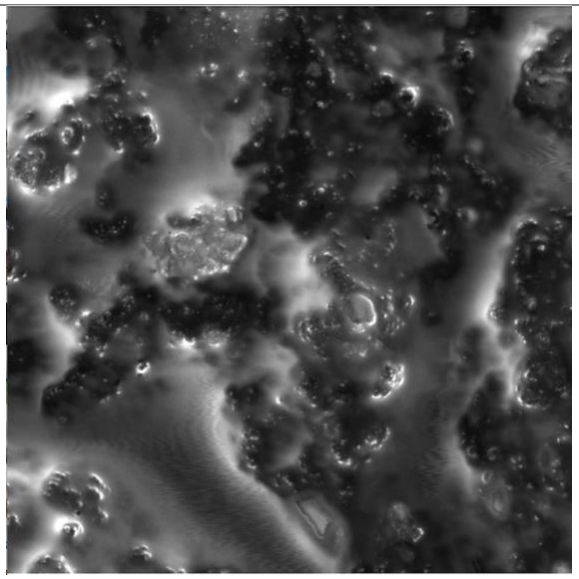
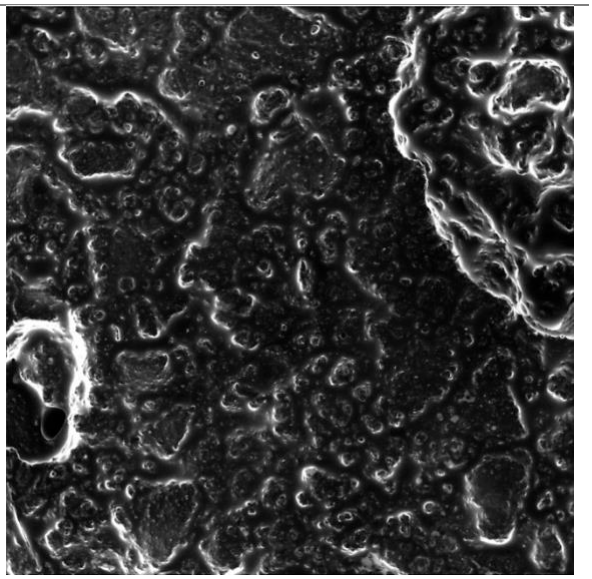
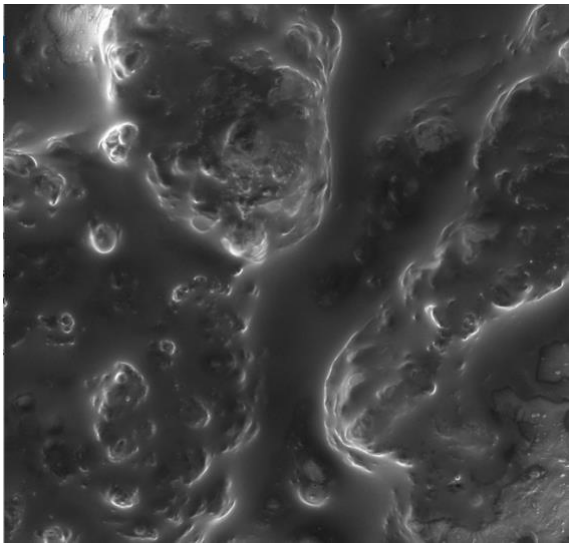
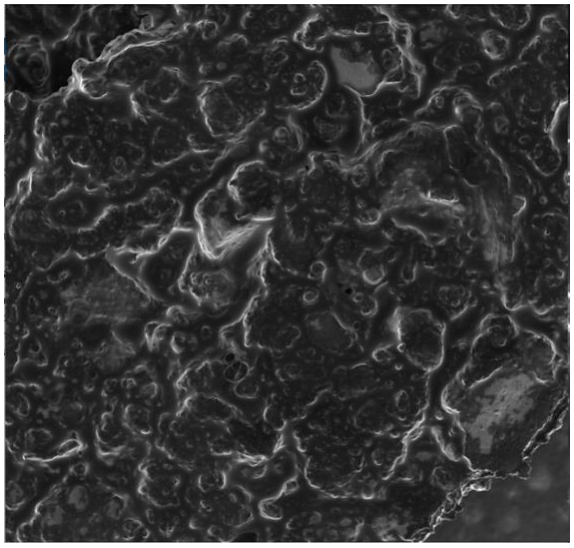
Table 3.3: *Scanning Electron Microscopy image of biomaterial made from tomato skin with ultrasound treatment and no ultrasound treatment under magnification of 400 and 60.*

	400X	60x
Tomato US		
Tomato No US		

Scanning Electron Microscopy pictures were obtained of the surface of our tomato bioplastic at a magnification of 400 and 60. Looking at the 60x magnification, it can be noticed that the surface of the bioplastic treated with US present a far rougher appearance than the bioplastic sample not treated with US. This can be noticed to a further extent when looking at the

400X magnification pictures obtained from both bioplastic samples. US-treated samples showed clear micro crater showing potential sign of microcavitation resulting from the US treatment. Comparatively, the sample that was not treated with US showed a more uniform surface.

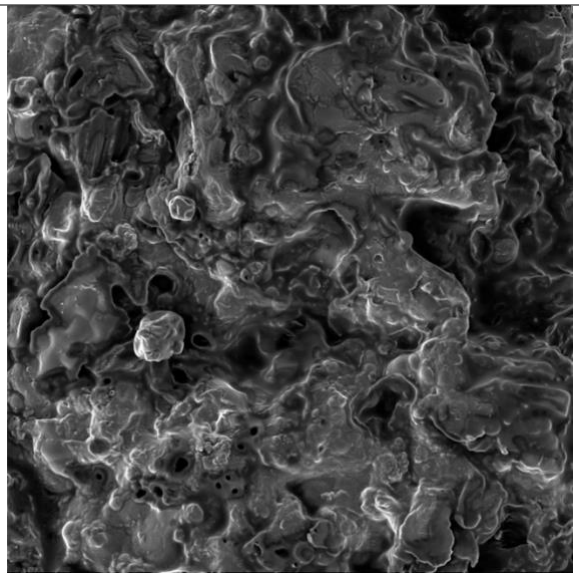
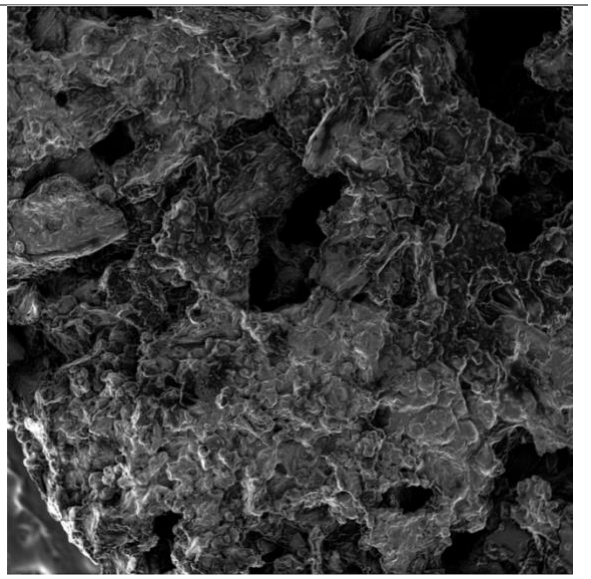
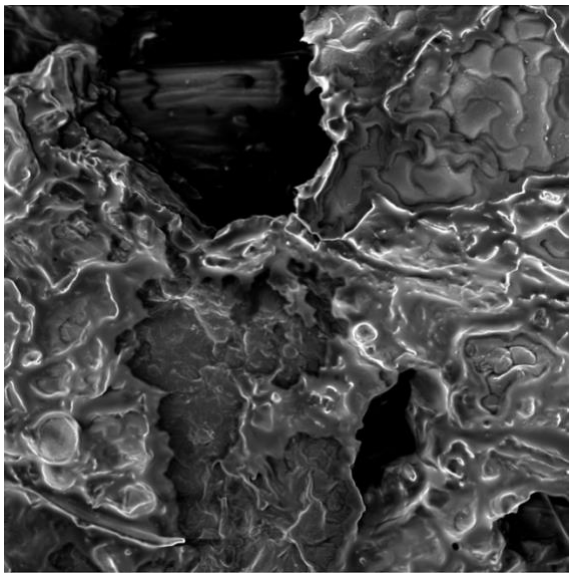
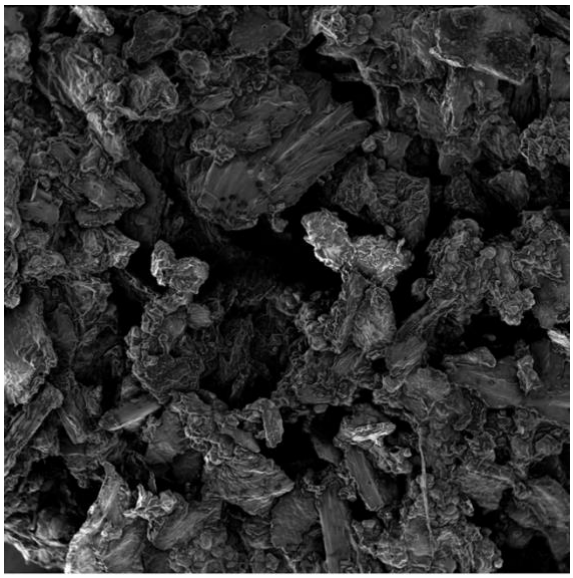
Table 3.3: *Scanning Electron Microscopy image of biomaterial made from hemp meal with ultrasound treatment and no ultrasound treatment under magnification of 400 and 60.*

	400X	**60x**
Hemps US		
Hemps No US		

Similarly, to the tomato samples presented in Table 3.3, Hemp-based biopolymer samples showed some clear difference in appearance PLM. When looking at 60x magnitude, not much difference can be seen in the surface of the biomaterial made from hemp meal. However, at a

magnitude of 40x, little crater can be noticed in higher density and appearance on the US-treated samples when compared to the non-US treated samples. Furthermore, the non-treated sample showed a more uniform and smoother surface than the US-treated samples. These results seem to support the hypothesis that microcavitations happens on the surface of our US treated biomaterials.

Table 3.5: *Scanning Electron Microscopy image of biomaterial made from hops flower with ultrasound treatment and no ultrasound treatment under magnification of 400 and 60.*

	400X	**60x**
Hops US		
Hops No US		

Hops bioplastics deviate in appearance the most strongly from both tomato-based bioplastics and hemp-based bioplastics. At a magnitude of 60x, the surface of both US treated bioplastic and non-treated bioplastic appears to be rough and jagged. This seems to indicate the lack of biopolymer to coat completely the hops flower particles. This seem to be directly reinforced by the appearance of an uncoated fiber of cellulosic material in the 400x magnification picture of non-treated hop-based bioplastic. However, looking at the US treated sample, small tunnel like structure seem to be present. These structures do not seem to be present in the non-treated sample. These little tunnels could be the result of sonoporation/cavitation on the barely coated cellulosic material of hops flowers. This would further justify the claim of cavitation and sonoporation affecting the surface of our US treated bioplastic as has been mentioned for both tomato-based bioplastic and hemp-based bioplastics.

3.4.4 Percentage Moisture of biomaterials

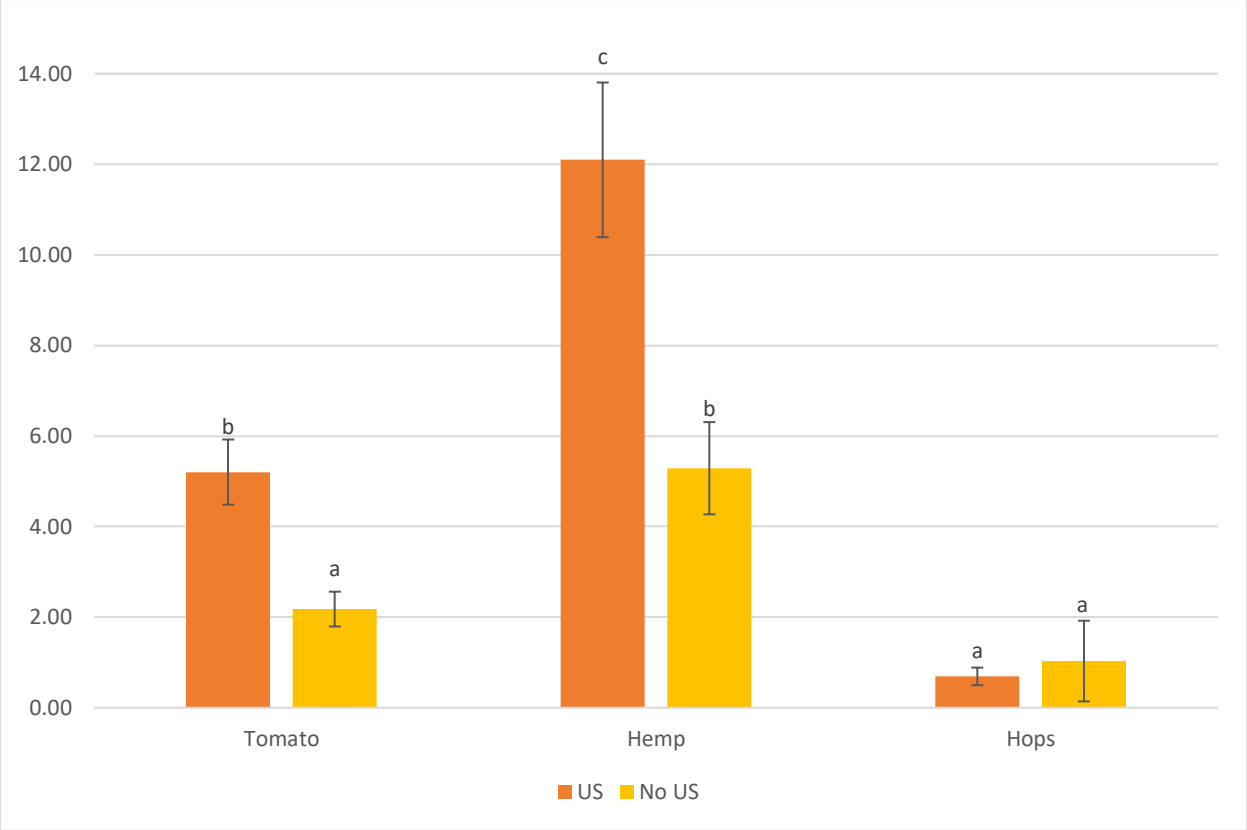


Figure 3.1: *% moisture in biomaterial made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

% Moisture was measured for samples of biomaterial and results were in Figure 3.1. Tomato-based based sample treated with US had a 5.20±0.72% moisture. On the other hand, non-treated Tomato-base sample (Tomato No US) showed a percentage moisture of 2.18±0.38. These values were found to be significantly different ($p<0.05$) when compared to each other. Similarly, a statistical difference was found between both Hemp-based bioplastic sample treated with US (Hemp US) and non-treated (Hemp No US). The respective values were 12.10±1.71% and 5.29±1.02 for US treated samples and non-ultrasound treated samples. Hops is the only sample where US treatment did not seem to influence the % moisture as there was no statistical difference

between US treated samples (Hops US) which had a value of 0.69%±0.19 and the non-treated samples (Hops No US) with a value 1.03±0.89%.

3.4.5 Water activity of biomaterials

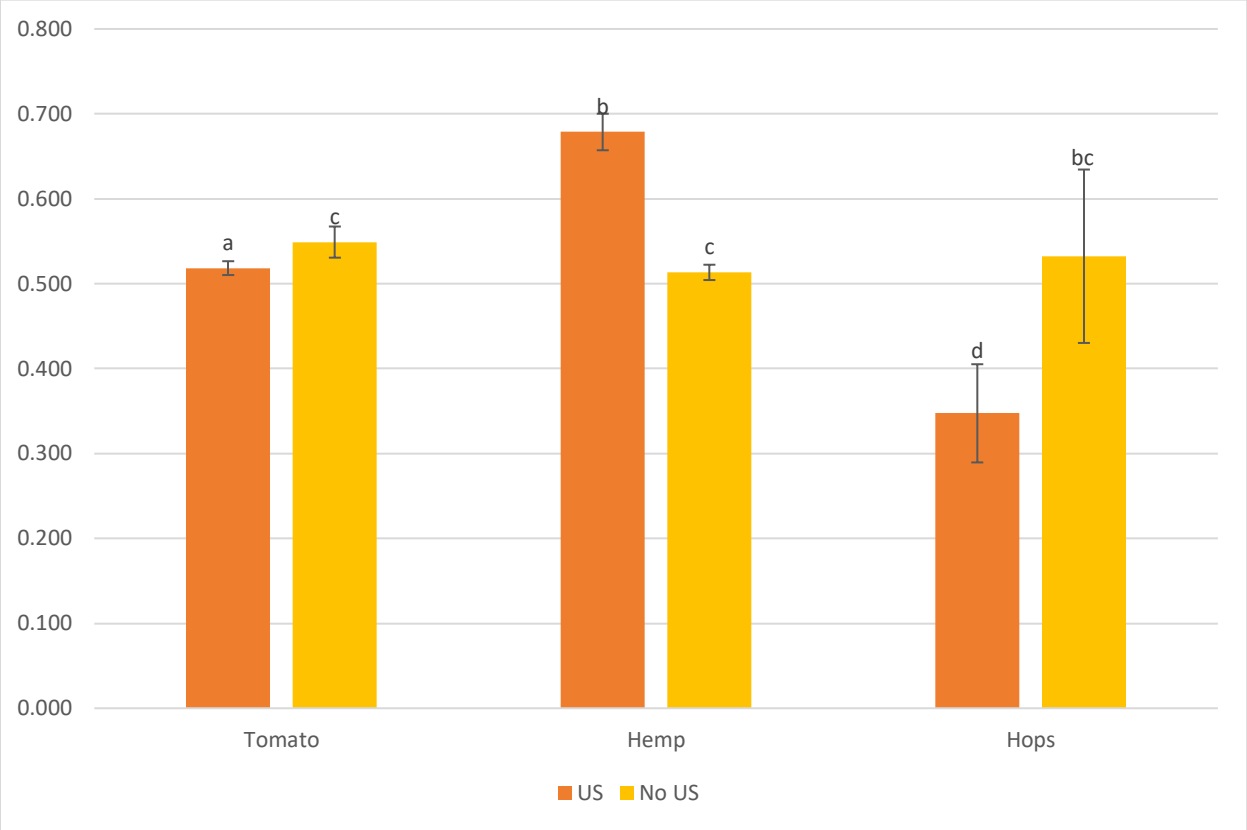


Figure 3.2: *Water activity in plastic made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

Water activity was measured for each sample bioplastic treated with US and non-treated. Results are compiled in the graph shown in Figure 3.2. Water activity was measured at 0.518±0.008 for US treated tomato-based bioplastic samples (Tomato US) and 0.549±0.018 for non-treated tomato-based bioplastic samples (Tomato No US). These values were statistically different indicating that US treatment decreased the water activity of our tomato-based bioplastic. This effect was also noticed in hops-based bioplastic where US treated samples had a water activity

of 0.347±0.058 and non-treated samples had a water activity of 0.532±0.102. These values were also found to be statistically different ($p<0.05$). Hemp-based biomaterial did not show any significant difference ($p>0.05$) in water activity values as values where respectively 0.679±0.022 and 0.513±0.009 for US (Hemp US) treated samples and non-treated hemp samples (Hemp No US).

3.4.5 Texture profile analysis of biomaterials

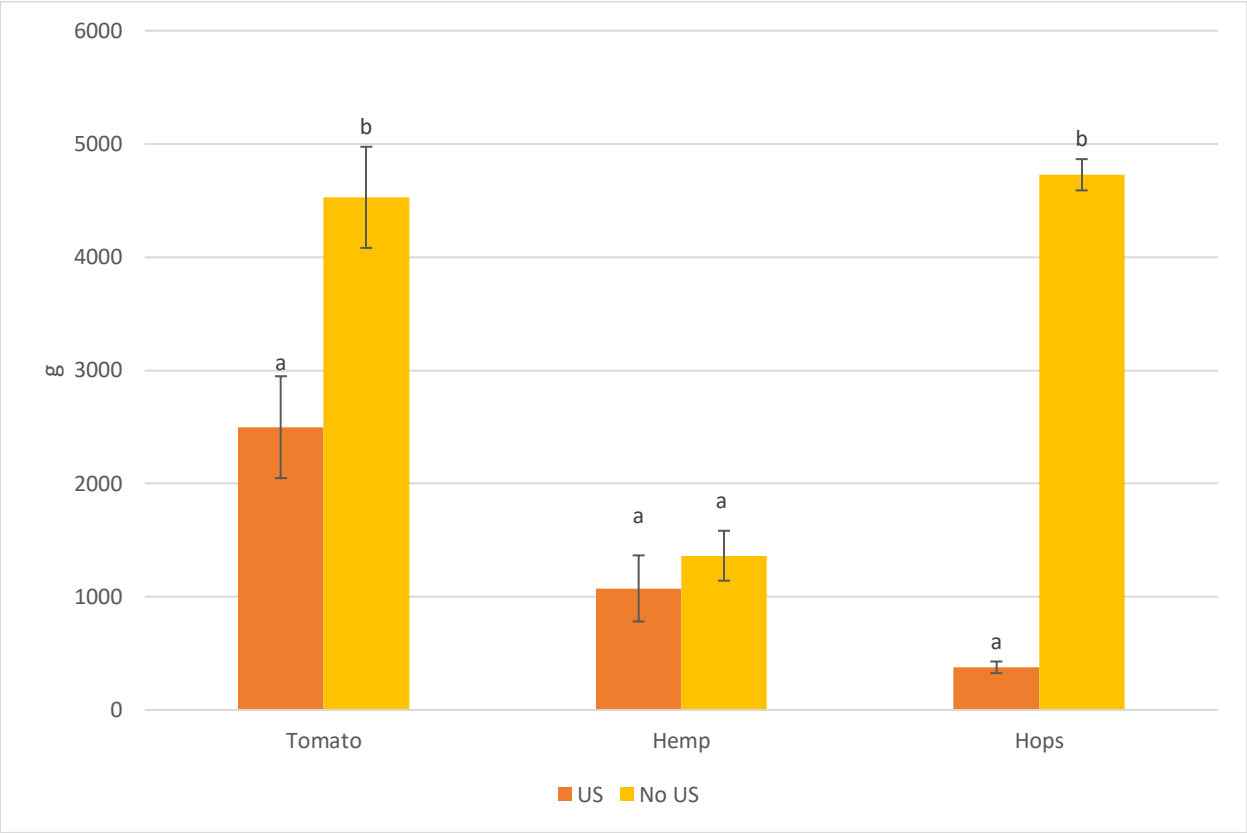


Figure 3.3: *Hardness of biomaterial made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

Hardness of tomato, hemp and hop based biomaterial treated with US and non-treated is shown in Figure 3.3. Tomato-based biomaterial had a hardness of 2498±450g when treated with US and 4529±447g when non-treated with US. The values were statistically different ($p<0.05$)

indicating that US treatment seemed to reduce the hardness of tomato-based bioplastic. This can also be noticed on hops-based bioplastics where hardness was statistically lower when treated with US (hops US) than when not treated with US (hops NO US). The hardness measured were respectively 377±52g and 4728±138g. The measure of hardness for hemp-based biomaterial was not statistically different between US treatment which had a hardness of 1074±291g and 1363±221g. These results seem to hint at the fact that US treatment leads to an overall reducing of hardness of samples of bioplastic.

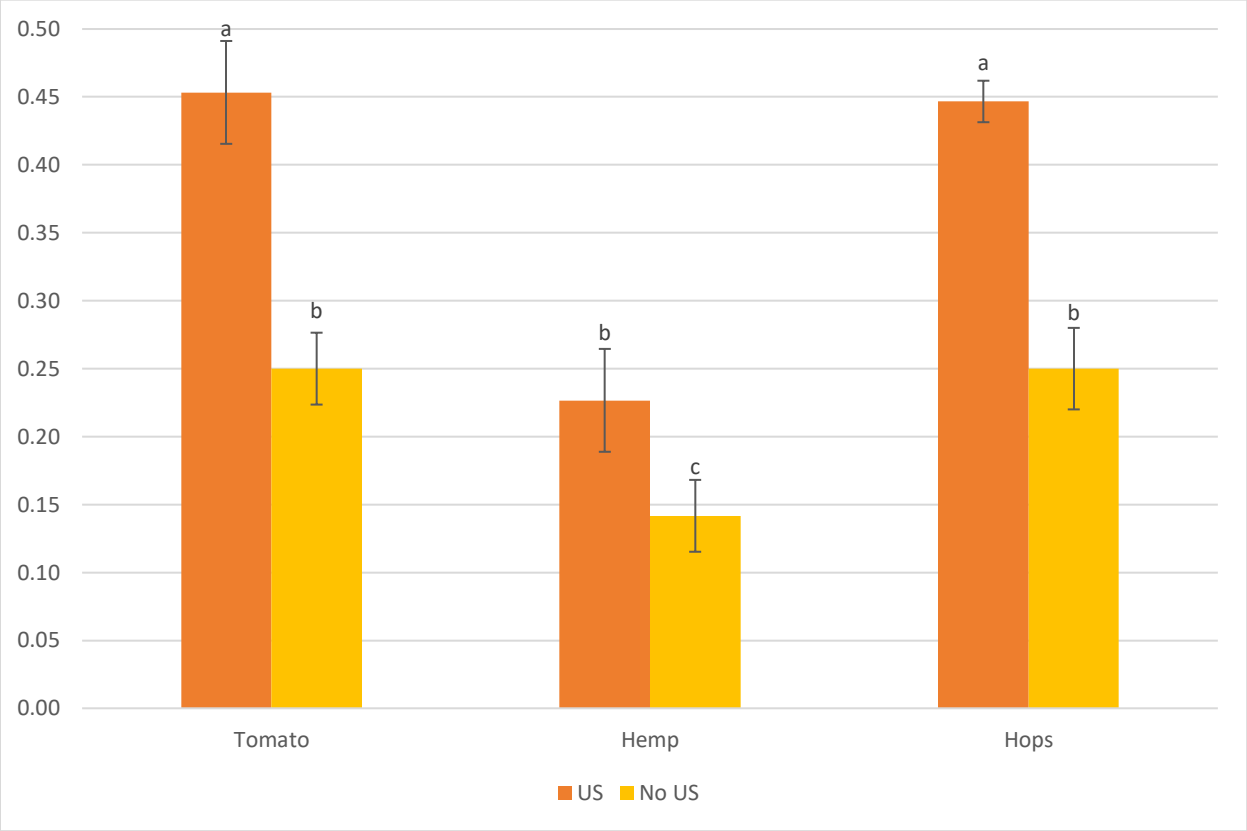


Figure 3.4: *Resilience in bioplastic made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

Resilience was measured and data was compiled in the graph shown in Figure 3.4. Tomato-based samples were found to have value of 0.45±0.04 and 0.25±0.03 for US treated sample (Tomato US) and Non-treated samples (Tomato No US). Hemp based samples showed values of

0.23±0.04 for US treated samples (Hemp US) and 0.14±0.03 for non-treated samples (Hemp No US). Hops based sample had values of 0.45±0.02 (Hops US) when treated with US and 0.25±0.02 when non-treated (Hops No US). All samples were statistically different when comparing the US treated sample to the non-US treated samples. Furthermore, a clear trend can be noticed where US treated samples had higher resilience than their non-US treated samples. This seems to indicate that US treatment increased the resilience of our biomaterials.

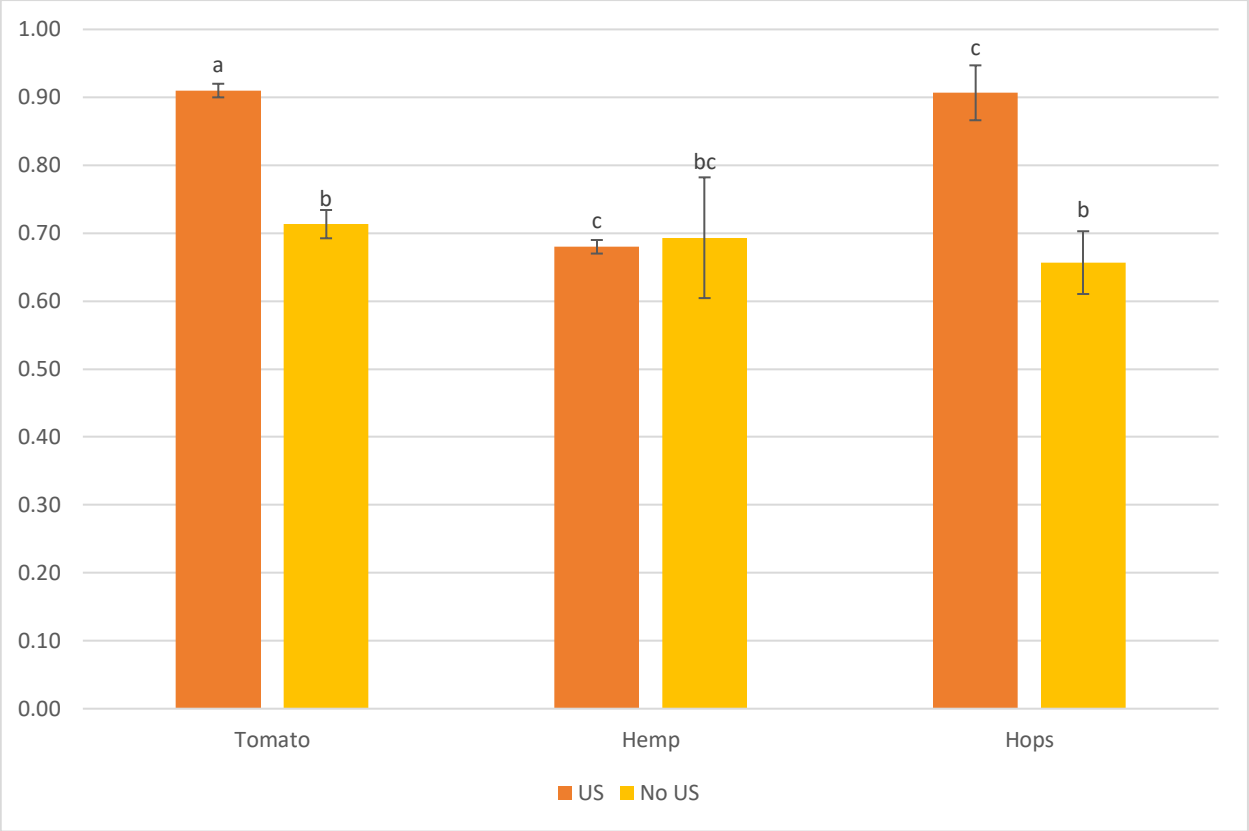


Figure 3.5: *Cohesiveness in bioplastics made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letters representing statistical difference*

The Cohesiveness of each bioplastic sample was measured as to compare the influence of ultrasound treatment and lack of ultrasound treatment as can be seen in Figure 3.5. Tomato-based bioplastics were found to have a cohesiveness of 0.91±0.01 for the US-treated samples (Tomato US) and 0.71±0.02 for the non-US treated samples (Tomato No US). These values were also found

to be statistically different ($p<0.05$). On the other hand, hemp-based bioplastics did not show statistically different values ($p>0.05$) with 0.68±0.01 and 0.69±0.09 for the US-treated samples (Tomato US) and the non-US treated samples (Hemp No US) respectively. However, similarly to the tomato-based samples, hops-based samples showed an increase in cohesiveness when US was applied. The values measured were 0.91±0.04 for the US-treated hops samples (Hops US) and 0.66±0.05 for the non-US treated hops samples (Hops No US). These sample as indicated in the graph were found to be significantly different ($p>0.05$).

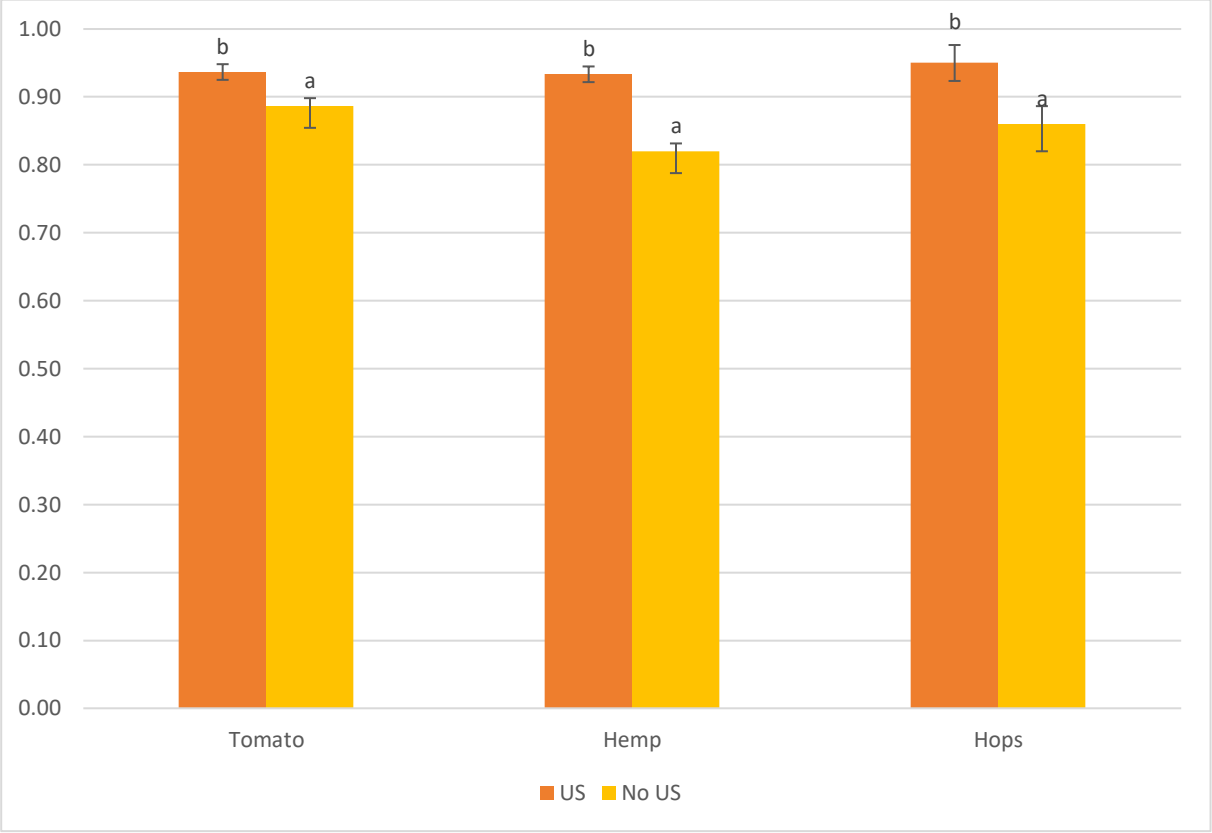


Figure 3.6: *Springiness index in bioplastic made from tomato skin, hemp meal and hops flowers with and without ultrasound (US, NO US) treatment. Results are represented as mean values ± SEM with differing subscript letter representing statistical difference*

As can be seen in Figure 3.6, springiness index was measured for each individual bioplastic sample. Tomato-based bioplastic showed a springiness index of 0.94±0.01 for the US treated samples (Tomato US) and 0.89±0.03 for the non-treated samples (Tomato No US). Statistical

difference ($p<0.05$) was calculated between those two samples indicating that US treatment did influence the springiness index of the biomaterial.

Furthermore, in the case of both Hemp-based bioplastic and hop-based bioplastic, the springiness indexes were found to be statistically different ($p<0.05$) between US treated samples and non-treated samples. As such, Hemp-based bioplastic showed a springiness index of 0.93±0.01 and 0.82±0.03 for respectively US treated samples (Hemp US) and non-treated samples (Hemp No US). When looking at Hops-based biomaterial the values measured for the US treated samples (Hops US) and non-treated samples (Hops No US) were found to be 0.95±0.03 and 0.86±0.04. For both Hemp-based bioplastic and Hops-based bioplastic results seem to show that US treatment increases the springiness index of our bioplastic. Springiness is a measurement of a material to regain its form. As such, it can be said that US treatment increases the capacity of our bioplastic to regain form after deformation. The base material used for the bioplastic did not seem to greatly influence the springiness index of our bioplastic as no statistical difference was found between the US/Non-US treated tomato samples, the non-US treated hemp sample and the non-US treated hops samples. Similarly, no statistical difference was found between the US-treated hemp samples and the US-treated hops samples.

3.5 Conclusion

Bioplastic formulated in this study showed a textural difference based on the bulking agent used (tomato skin, hemp meal or hops flowers). Ultrasound treatment was proven to have an impact on the microstructure of these bioplastic. This impact can be perceived as positive as it improves most rheological properties of these bioplastic samples. Hardness was decreased while resilience, cohesiveness and springiness were increased indicated that US treated bioplastic were overall better resistant to deformation and by extension were more flexible and elastic. This could also be due to an improved capacity at keeping moisture trapped in the gel matrix of the biomaterial

as % moisture present in biomaterial treated with US was also higher. However, despite an increased %moisture, the water activity of the bioplastic was lower when treated with US indicating that US treated bioplastic would be less susceptible to microbial growth appearing on their surface. Overall US treatment has been shown to have positive effects on the development of bioplastics made from agricultural waste materials.

Chapter 4: Conclusion & further research

4.1 Conclusion

HPLF-US can have a positive influence on a variety of processes and shows great potential for use in the food industry. This research focuses on utilizing HPLF-US in two different contexts: the extraction of bioactive compounds from agricultural waste and the development of bioplastics from agricultural waste materials. Multiple studies have shown the positive impact that HPLF-US can have on extraction efficiency of a variety of compounds present in plant materials. This study comes to support these findings by showcasing the influence of UAE on saponin and phenolic content. As such, this study demonstrates the ability of UAE to reduce extraction time from 24h to 30-minutes when seeking to extract phenolic acid and/or saponin compounds. Furthermore, UAE was shown to not significantly influence the antioxidant ability of these extracts when compared to traditional method of extraction. Here UAE showed its ability to diminish the overall time needed to extract phenolic and saponin from samples.

Adding to its extraction ability HPLF-US also has been reported to influence microscopic structure of emulsion-gels. This was further demonstrated here, where bioplastic treated with HPLF-US demonstrated change in emulsion-gels structure and improved rheological properties. However, further analysis is required notably testing for microbial growth. Other testing needed would include the evaluation of the stability of the rheological properties of the developed biomaterials over an extended period.

Overall US treatment shows great potential in the context of extracting bioactive compounds as it is a relatively cheap technology to implement. In addition to its overall low cost, it is also demonstrated to be a green technology. Utilization of US in the context of biomaterial development also seems to bring value as it clearly improves the rheological properties of biomaterial developed.

4.2 Further research

In this research the diversity of use of HPLF-US was demonstrated. However other utilizations could be explored.

Furthermore, more in depth research could be performed in ways that utilize the extracts obtained from chapter 2 of this research. Both phenolic acids and saponin are known to have antimicrobial properties[86]. This could give us a new avenue of research for functional use of the obtained extracts. As such, phenolic compounds and saponin extracts could be utilized as antimicrobial agents in the development of multiple food products.

Additionally, deepening of the research on bioplastic development could be performed. Testing for microbial activity, solubility or evaluating the influence of HPLF-US on different steps of the bioplastic formulation could lead to further understanding of the influence of HPLF-US on gel complexes and emulsions.

References

(1) K., P. Acoustic Wave. *Acoust. Waves - From Microdevices to Helioseismology* 2011, No. March. https://doi.org/10.5772/17765.

(2) O'Brien, W. D. Ultrasound-Biophysics Mechanisms. *Prog. Biophys. Mol. Biol.* 2007, *93* (1–3), 212–255. https://doi.org/10.1016/j.pbiomolbio.2006.07.010.

(3) E.A. Mohammed, M.; R. Alhajhoj, M. Importance and Applications of Ultrasonic Technology to Improve Food Quality. In *Food Processing*; IntechOpen, 2020. https://doi.org/10.5772/intechopen.88523.

(4) Yao, Y.; Pan, Y.; Liu, S. Power Ultrasound and Its Applications: A State-of-the-Art Review. *Ultrason. Sonochem.* 2020, *62* (July 2019). https://doi.org/10.1016/j.ultsonch.2019.104722.

(5) Izadifar, Z.; Babyn, P.; Chapman, D. Ultrasound Cavitation/Microbubble Detection and Medical Applications. *J. Med. Biol. Eng.* 2019, *39* (3), 259–276. https://doi.org/10.1007/s40846-018-0391-0.

(6) Luque-García, J. .; Luque de Castro, M. . Ultrasound: A Powerful Tool for Leaching. *TrAC Trends Anal. Chem.* 2003, *22* (1), 41–47. https://doi.org/10.1016/S0165-9936(03)00102-X.

(7) Chatel, G.; Colmenares, J. C. Sonochemistry: From Basic Principles to Innovative Applications. *Top. Curr. Chem.* 2017, *375* (1), 8. https://doi.org/10.1007/s41061-016-0096-1.

(8) Picó, Y. Ultrasound-Assisted Extraction for Food and Environmental Samples. *TrAC - Trends Anal. Chem.* 2013, *43*, 84–99. https://doi.org/10.1016/j.trac.2012.12.005.

(9) Virot, M.; Tomao, V.; Le Bourvellec, C.; Renard, C. M. C. G.; Chemat, F. Towards the Industrial Production of Antioxidants from Food Processing By-Products with Ultrasound-Assisted Extraction. *Ultrason. Sonochem.* 2010, *17* (6), 1066–1074. https://doi.org/10.1016/j.ultsonch.2009.10.015.

(10) Chemat, F.; Rombaut, N.; Sicaire, A. G.; Meullemiestre, A.; Fabiano-Tixier, A. S.; Abert-Vian, M. Ultrasound Assisted Extraction of Food and Natural Products. Mechanisms, Techniques, Combinations, Protocols and Applications. A Review. *Ultrason. Sonochem.* 2017, *34*, 540–560. https://doi.org/10.1016/j.ultsonch.2016.06.035.

(11) Vilkhu, K.; Mawson, R.; Simons, L.; Bates, D. Applications and Opportunities for Ultrasound Assisted Extraction in the Food Industry - A Review. *Innov. Food Sci. Emerg. Technol.* 2008, *9* (2), 161–169. https://doi.org/10.1016/j.ifset.2007.04.014.

(12) Shotipruk, A.; Kaufman, P. B.; Wang, H. Y. Feasibility Study of Repeated Harvesting of Menthol from Biologically Viable Mentha x Piperata Using Ultrasonic Extraction. *Biotechnol. Prog.* 2001, *17* (5), 924–928. https://doi.org/10.1021/bp010074u.

(13) Herrera, M. C.; Luque De Castro, M. D. Ultrasound-Assisted Extraction for the Analysis of Phenolic Compounds in Strawberries. *Anal. Bioanal. Chem.* 2004, *379* (7–8), 1106–1112. https://doi.org/10.1007/s00216-004-2684-0.

(14) Medina-Torres, N.; Ayora-Talavera, T.; Espinosa-Andrews, H.; Sánchez-Contreras, A.; Pacheco, N. Ultrasound Assisted Extraction for the Recovery of Phenolic Compounds from Vegetable Sources. *Agronomy* 2017, *7* (3). https://doi.org/10.3390/agronomy7030047.

(15) Mabry, H.; Harbone, J. .; Mabry, T. J. *Flavonoids*, 1st ed.; Chapman, Ed.; London, 1975.

(16) Vermerris, W.; Nicholson, R. *Phenolic Compound Biochemistry*, 1st ed.; Springer, D., Ed.; Springer Netherlands: Dordrecht, 2006. https://doi.org/10.1007/978-1-4020-5164-7.

(17) Hernández, I. G.; Palottini, F.; Macri, I.; Galmarini, C. R.; Farina, W. M. Appetitive Behavior of the Honey Bee Apis Mellifera in Response to Phenolic Compounds Naturally Found in Nectars. *J. Exp. Biol.* 2019, *222* (2). https://doi.org/10.1242/jeb.189910.
(18) Aziz, N. H.; Farag, S. E.; Mousa, L. A.; Abo-Zaid, M. A. Comparative Antibacterial and Antifungal Effects of Some Phenolic Compounds. *Microbios* 1998, *93* (374), 43–54.
(19) Treutter, D. Significance of Flavonoids in Plant Resistance: A Review. *Environ. Chem. Lett.* 2006, *4* (3), 147–157. https://doi.org/10.1007/s10311-006-0068-8.
(20) Kalevitch, M.; Borsari, B. Phenolic Cycle in Plants and Environment. *Mech. Landsc. Rehabil. Sustain.* 2012, No. January 2003, 75–78. https://doi.org/10.2174/978160805168711101010075.
(21) Lynn, D. G.; Chang, M. Phenolic Signals in Cohabitation: Implications for Plant Development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1990, *41* (1), 497–526. https://doi.org/10.1146/annurev.pp.41.060190.002433.
(22) Escarpa, A.; Gonzalez, M. C. An Overview of Analytical Chemistry of Phenolic Compounds in Foods. *Crit. Rev. Anal. Chem.* 2001, *31* (2), 57–139. https://doi.org/10.1080/20014091076695.
(23) Harborne, J. B. *Biochemistry of Phenolic Compounds*; Harborne, J. B., Ed.; Academic Press: London, 1964.
(24) Grotewold, E. *The Science of Flavonoids*; Grotewold, E., Ed.; Springer New York: New York, NY, 2006. https://doi.org/10.1007/978-0-387-28822-2.
(25) Harborne, J. B. Comparative Biochemistry of the Flavonoids. *Phytochemistry* 1967, *6* (12), 1643–1651. https://doi.org/10.1016/S0031-9422(00)82897-6.
(26) Kyselova, Z. Toxicological Aspects of the Use of Phenolic Compounds in Disease Prevention. *Interdiscip. Toxicol.* 2011, *4* (4), 173–183. https://doi.org/10.2478/v10102-011-0027-5.
(27) Schewe, T.; Steffen, Y.; Sies, H. How Do Dietary Flavanols Improve Vascular Function? A Position Paper. *Arch. Biochem. Biophys.* 2008, *476* (2), 102–106. https://doi.org/10.1016/j.abb.2008.03.004.
(28) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant Properties of Phenolic Compounds. *Trends Plant Sci.* 1997, *2* (4), 152–159. https://doi.org/10.1016/S1360-1385(97)01018-2.
(29) Kong, A. N. T.; Yu, R.; Chen, C.; Mandlekar, S.; Primiano, T. Signal Transduction Events Elicited by Natural Products: Role of MAPK and Caspase Pathways in Homeostatic Response and Induction of Apoptosis. *Arch. Pharm. Res.* 2000, *23* (1), 1–16. https://doi.org/10.1007/BF02976458.
(30) Yu, R. Activation of Mitogen-Activated Protein Kinases by Green Tea Polyphenols: Potential Signaling Pathways in the Regulation of Antioxidant-Responsive Element-Mediated Phase II Enzyme Gene Expression. *Carcinogenesis* 1997, *18* (2), 451–456. https://doi.org/10.1093/carcin/18.2.451.
(31) Yan, Y. S. [Effect of Chinese Green Tea Extracts on the Human Gastric Carcinoma Cell in Vitro]. *Zhonghua Yu Fang Yi Xue Za Zhi* 1990, *24* (2), 80–82.
(32) Guarrera, S.; Sacerdote, C.; Fiorini, L.; Marsala, R.; Polidoro, S.; Gamberini, S.; Saletta, F.; Malaveille, C.; Talaska, G.; Vineis, P.; Matullo, G. Expression of DNA Repair and Metabolic Genes in Response to a Flavonoid-Rich Diet. *Br. J. Nutr.* 2007, *98* (3), 525–533. https://doi.org/10.1017/S0007114507725151.
(33) Albuquerque, B. R.; Heleno, S. A. 8.; Oliveira, M. B. P. P.; Barros, L.; Ferreira, I. C. F. R. Phenolic Compounds: Current Industrial Applications, Limitations and Future Challenges. *Food Funct.* 2021, *12* (1), 14–29. https://doi.org/10.1039/d0fo02324h.

(34) Takó, M.; Beáta Kerekes, E.; Zambrano, C.; Kotogán, A.; Papp, T.; Krisch, J.; Vágvölgyi, C. Plant Phenolics and Phenolic-Enriched Extracts as Antimicrobial Agents Against. *Antioxidants* 2020, *9* (2), 1–21.
(35) Fenwick, D. E.; Oakenfull, D. Saponin Content of Food Plants and Some Prepared Foods. *J. Sci. Food Agric.* 1983, *34* (2), 186–191. https://doi.org/10.1002/jsfa.2740340212.
(36) Faizal, A.; Geelen, D. Saponins and Their Role in Biological Processes in Plants. *Phytochem. Rev.* 2013, *12* (4), 877–893. https://doi.org/10.1007/s11101-013-9322-4.
(37) Morrissey, J. P.; Osbourn, A. E. Fungal Resistance to Plant Antibiotics as a Mechanism of Pathogenesis. *Microbiol. Mol. Biol. Rev.* 1999, *63* (3), 708–724. https://doi.org/10.1128/MMBR.63.3.708-724.1999.
(38) Macías, F. A.; Simonet, A. M.; Galindo, J. C. G. Bioactive Steroids and Triterpenes from Melilotus Messanensis and Their Allelopathic Potential. *J. Chem. Ecol.* 1997, *23* (7), 1781–1803. https://doi.org/10.1023/B:JOEC.0000006451.19649.a0.
(39) Osbourn, A.; Goss, R. J. M.; Field, R. A. The Saponins – Polar Isoprenoids with Important and Diverse Biological Activities. *Nat. Prod. Rep.* 2011, *28* (7), 1261. https://doi.org/10.1039/c1np00015b.
(40) Makkar, H. P. S.; Siddhuraju, P.; Becker, K. Saponins; 2007; pp 93–100. https://doi.org/10.1007/978-1-59745-425-4_16.
(41) Ashour, A. S.; El Aziz, M. M. A.; Gomha Melad, A. S. A Review on Saponins from Medicinal Plants: Chemistry, Isolation, and Determination. *J. Nanomedicine Res.* 2019, *7* (4), 282–288. https://doi.org/10.15406/jnmr.2019.07.00199.
(42) Mohamed, M.; El-Aziz, A.; Ashour, A. S.; Madbouly, H. A.; Sadek, A.; Melad, G.; Sdig, K.; Kerikshi, E. Investigations on Green Preparation of Heavy Metal Saponin Complexes. *J. Water Environ. Nanotechnol* 2017, *2* (2), 103–111. https://doi.org/10.22090/jwent.2017.02.005.
(43) Güçlü-Üstündağ, Ö.; Mazza, G. Saponins: Properties, Applications and Processing. *Crit. Rev. Food Sci. Nutr.* 2007, *47* (3), 231–258. https://doi.org/10.1080/10408390600698197.
(44) *Application and Characterization of Surfactants*; Najjar, R., Ed.; InTech, 2017. https://doi.org/10.5772/65591.
(45) Loehr, R. C. *Agricultural Waste Management*; Elsevier, 1974. https://doi.org/10.1016/B978-0-12-455250-0.X5001-4.
(46) Gooch, M.; Felfel, A.; Marenick, N. Food Waste in Canada. *Value Chain Manag. Cent.* 2010, No. November, 1–16.
(47) *Bioplastics for Sustainable Development*; Kuddus, M., Roohi, Eds.; Springer Singapore: Singapore, 2021. https://doi.org/10.1007/978-981-16-1823-9.
(48) Flieger, M.; Kantorová, M.; Prell, A.; Řezanka, T.; Votruba, J. Biodegradable Plastics from Renewable Sources. *Folia Microbiol. (Praha).* 2003, *48* (1), 27–44. https://doi.org/10.1007/BF02931273.
(49) *Biopolymer Composites in Electronics*; Elsevier, 2017. https://doi.org/10.1016/C2014-0-04575-3.
(50) Schiessel, H. Polymer Physics. In *Biophysics for Beginners*; Jenny Stanford Publishing, 2013; pp 74–103. https://doi.org/10.1201/b16269-5.
(51) Dickinson, E. Hydrocolloids as Emulsifiers and Emulsion Stabilizers. *Food Hydrocoll.* 2009, *23* (6), 1473–1482. https://doi.org/10.1016/j.foodhyd.2008.08.005.
(52) Sothornvit, R.; Krochta, J. M. Plasticizers in Edible Films and Coatings. In *Innovations in Food Packaging*; Elsevier, 2005; pp 403–433. https://doi.org/10.1016/B978-012311632-1/50055-3.
(53) Tyagi, V.; Bhattacharya, B. Role of Plasticizers in Bioplastics. *MOJ Food Process.*

Technol. 2019, *7* (4), 128–130. https://doi.org/10.15406/mojfpt.2019.07.00231.
(54) Al-Hassan, A. A.; Norziah, M. H. Starch-Gelatin Edible Films: Water Vapor Permeability and Mechanical Properties as Affected by Plasticizers. *Food Hydrocoll.* 2012, *26* (1), 108–117. https://doi.org/10.1016/j.foodhyd.2011.04.015.
(55) Geyer, R.; Jambeck, J. R.; Law, K. L. Production, Use, and Fate of All Plastics Ever Made. *Sci. Adv.* 2017, *3* (7). https://doi.org/10.1126/sciadv.1700782.
(56) Gewert, B.; Plassmann, M. M.; MacLeod, M. Pathways for Degradation of Plastic Polymers Floating in the Marine Environment. *Environ. Sci. Process. Impacts* 2015, *17* (9), 1513–1521. https://doi.org/10.1039/C5EM00207A.
(57) Arvanitoyannis, I. S. Waste Management for Polymers in Food Packaging Industries. In *Plastic Films in Food Packaging*; Elsevier, 2013; pp 249–310. https://doi.org/10.1016/B978-1-4557-3112-1.00014-4.
(58) Eriksen, M.; Lebreton, L. C. M.; Carson, H. S.; Thiel, M.; Moore, C. J.; Borerro, J. C.; Galgani, F.; Ryan, P. G.; Reisser, J. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS One* 2014, *9* (12), e111913. https://doi.org/10.1371/journal.pone.0111913.
(59) Weiss, M.; Haufe, J.; Carus, M.; Brandão, M.; Bringezu, S.; Hermann, B.; Patel, M. K. A Review of the Environmental Impacts of Biobased Materials. *J. Ind. Ecol.* 2012, *16*, S169–S181. https://doi.org/10.1111/j.1530-9290.2012.00468.x.
(60) Emadian, S. M.; Onay, T. T.; Demirel, B. Biodegradation of Bioplastics in Natural Environments. *Waste Manag.* 2017, *59*, 526–536. https://doi.org/10.1016/j.wasman.2016.10.006.
(61) Length, D. C.; Huang, Y.; Wang, R.; Wu, Y.; Beverages, T. M.; Makhoul-mansour, M. M.; Eric, C. Emulsions : Basic Principles. 1999.
(62) Friberg, Stig, Larsson, Kare, Sjoblom, J. *Food Emulsions*, 4th ed.; CRC Press, 2003.
(63) *Emulsions, Foams, Suspensions, and Aerosols*; Schramm, L. L., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2014. https://doi.org/10.1002/9783527679478.
(64) Silva, M.; Anh Bui, T. H.; Dharmadana, D.; Zisu, B.; Chandrapala, J. Ultrasound-Assisted Formation of Double Emulsions Stabilized by Casein-Whey Protein Mixtures. *Food Hydrocoll.* 2020, *109* (July), 106143. https://doi.org/10.1016/j.foodhyd.2020.106143.
(65) Iqbal, M.; Zafar, N.; Fessi, H.; Elaissari, A. Double Emulsion Solvent Evaporation Techniques Used for Drug Encapsulation. *Int. J. Pharm.* 2015, *496* (2), 173–190. https://doi.org/10.1016/j.ijpharm.2015.10.057.
(66) Giroux, H. J.; Robitaille, G.; Britten, M. Controlled Release of Casein-Derived Peptides in the Gastrointestinal Environment by Encapsulation in Water-in-Oil-in-Water Double Emulsions. *LWT - Food Sci. Technol.* 2016, *69*, 225–232. https://doi.org/10.1016/j.lwt.2016.01.050.
(67) BeMiller, J. N. Hydrocolloids. In *Gluten-Free Cereal Products and Beverages*; Elsevier, 2008; pp 203–215. https://doi.org/10.1016/B978-012373739-7.50011-3.
(68) Lu, W.; Li, X.; Fang, Y. Introduction to Food Hydrocolloids. In *Food Hydrocolloids*; Springer Singapore: Singapore, 2021; pp 1–28. https://doi.org/10.1007/978-981-16-0320-4_1.
(69) Djabourov, M.; Nishinari, K.; Ross-Murphy, S. B. *Physical Gels from Biological and Synthetic Polymers*; Cambridge University Press: Cambridge, 2013. https://doi.org/10.1017/CBO9781139024136.
(70) Burey, P.; Bhandari, B. R.; Howes, T.; Gidley, M. J. Hydrocolloid Gel Particles: Formation, Characterization, and Application. *Crit. Rev. Food Sci. Nutr.* 2008, *48* (5),

361–377. https://doi.org/10.1080/10408390701347801.
(71) Peleg, M. The Instrumental Texture Profile Analysis Revisited. *J. Texture Stud.* 2019, *50* (5), 362–368. https://doi.org/10.1111/jtxs.12392.
(72) BREENE, W. M. Application of Texture Profile Analysis To Instrumental Food Texture Evaluation. *J. Texture Stud.* 1975, *6* (1), 53–82. https://doi.org/10.1111/j.1745-4603.1975.tb01118.x.
(73) Pons, M.; Fiszman, S. M. Instrumental Texture Profile Analysis with Particular Reference to Gelled Systems. *J. Texture Stud.* 1996, *27* (6), 597–624. https://doi.org/10.1111/j.1745-4603.1996.tb00996.x.
(74) Bourne, M. And Mastication. *J. Texture Stud.* 2004, *35*, 125–143.
(75) Friedman, H. H.; Whitney, J. E.; Szczesniak, A. S. The Texturometer—A New Instrument for Objective Texture Measurement. *J. Food Sci.* 1963, *28* (4), 390–396. https://doi.org/10.1111/j.1365-2621.1963.tb00216.x.
(76) Brandt, A.; Coleman, J. A. Method.
(77) Trinh, K. T.; Glasgow, S. On The Texture Profile Analysis Test. *Chemeca* 2012, No. October, 749–760.
(78) Gunenc, A.; HadiNezhad, M.; Farah, I.; Hashem, A.; Hosseinian, F. Impact of Supercritical CO2 and Traditional Solvent Extraction Systems on the Extractability of Alkylresorcinols, Phenolic Profile and Their Antioxidant Activity in Wheat Bran. *J. Funct. Foods* 2015, *12*, 109–119. https://doi.org/10.1016/j.jff.2014.10.024.
(79) Mathur, R.; Vijayvergia, R. Determination of Total Flavonoid and Phenol Content in Mimusops Elengi Linn. *Int. J. Pharm. Sci. Res.* 2017, *8* (12), 5282–5285. https://doi.org/10.13040/IJPSR.0975-8232.8(12).5282-85.
(80) Tenon, M.; Feuillère, N.; Roller, M.; Birtić, S. Rapid, Cost-Effective and Accurate Quantification of Yucca Schidigera Roezl. Steroidal Saponins Using HPLC-ELSD Method. *Food Chem.* 2017, *221*, 1245–1252. https://doi.org/10.1016/j.foodchem.2016.11.033.
(81) Navarro del Hierro, J.; Herrera, T.; García-Risco, M. R.; Fornari, T.; Reglero, G.; Martin, D. Ultrasound-Assisted Extraction and Bioaccessibility of Saponins from Edible Seeds: Quinoa, Lentil, Fenugreek, Soybean and Lupin. *Food Res. Int.* 2018, *109* (April), 440–447. https://doi.org/10.1016/j.foodres.2018.04.058.
(82) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem.* 2002, *50* (16), 4437–4444. https://doi.org/10.1021/jf0201529.
(83) Brooks, A. L.; Wang, S.; Jambeck, J. R. The Chinese Import Ban and Its Impact on Global Plastic Waste Trade. *Sci. Adv.* 2018, *4* (6), 1–7. https://doi.org/10.1126/sciadv.aat0131.
(84) Dornan, K. Odd Chain Phenolic Lipids in Liposomes as a Delivery System in Food Using High Power Ultrasound. 2021.
(85) Freire, M. Innovative Bio-Packaging Material from Banana Peel with Potential to Improve a Sustainable Environment. Carleton University: Ottawa 2019.
(86) Wallace, R. J. Antimicrobial Properties of Plant Secondary Metabolites. *Proc. Nutr. Soc.* 2004, *63* (4), 621–629. https://doi.org/10.1079/pns2004393.

Appendix

Table 1: Trial Formulation for biomaterials

	FORMULA 1	FORMULA 2	FORMULA 3	FORMULA 4	FORMULA 5	FORMULA 6	FORMULA 7	FORMULA 8	FORMULA 9
	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)
BIOLOGICAL WASTE	30	30	30	30	30	30	30	30	30
WHEY	10	10	10	10	10	10	10	10	5
GELATINE	0	0	0	0	0	0	0	0	0
GLYCEROL	6	6	6	6	6	6	6	6	6
PHOSPHORIC ACID	2	2	0	0	2	2	2	2	0
CANOLA OIL	0	0	0	0	5	5	5	10	0
WATER	45	50	47	45	50	50	45	41	47
SODIUM ALGINATE	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	1.2
CALCIUM HYDROXIDE	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	2.8
CASEINE	0	0	0	0	5	5	5	5	10

Table 2:Trial Formulation for biomaterials (cont.)

	FORMULA 10	**FORMULA 11**	**FORMULA 12**	**FORMULA 13**	**FORMULA 14**	**FORMULA 15**	**FORMULA 16**	**FORMULA 17**
	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)
BIOLOGICAL WASTE	30	30	30	30	12.39	7	15	18
WHEY	10	10	0	5	4.13	4.13	0	0
GELATINE	0	0	0	0	1.24	1.24	3	3
GLYCEROL	6	6	6	6	8.26	3.72	8	8
PHOSPHORIC ACID	2	2	2	0	1.65	0.83	2	2
CANOLA OIL	5	5	5	0	2.07	2.06	8	8
WATER	40	50	50	47	24.69	24.8	54.4	54.4
SODIUM ALGINATE	0.6	0.6	0.6	1.2	0.24	0.24	0.5	0.5
CALCIUM HYDROXIDE	1.4	1.4	1.4	2.8	1.15	0.58	0.1	0.1
CASEINE	5	5	15	10	0	0	9	9