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Study of the Extraction Process and Nutritional Benefits of Bioactive Compounds in Pomegranate Peel

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Study of the Extraction Process and Nutritional Benefits of Bioactive Compounds in Pomegranate Peel

By

XINGZHU WU
DISSERTATION

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Zhongli Pan, Chair

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Ph.D. is a journey of self-exploration to evoke the universe within me. I am ready for the new challenges and cannot wait to contribute what I have learned to the world.

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Abstract

Pomegranate (Punica granatum L.) is an ancient fruit and a commodity favored by people worldwide for its unique shape and flavor. Pomegranate peel is a major by-product from the juicing process with rich health-promoting bioactive compounds (mainly polyphenol content) but remains underutilized or disposed of. The main goal of this dissertation was to study the extraction of functional ingredients from pomegranate peel waste, evaluate their nutritional benefits, and develop value-added nutritional products. A novel green process for antioxidant extraction from wet pomegranate peel (WPP) was developed. The effects of various extraction conditions on polyphenol yield and quality were investigated, including particle size, time, temperature, and solvent ratios. WPP extraction at 20°C for 6 min with a solvent (water) ratio of 4:1 is recommended as an economic and sustainable process, resulting in 10.53% total phenolic yield (TPY) with 6.35 g g^{-1} tannic acid equivalent (TAE) and 88.93% punicalagin (most unique and potent polyphenol in pomegranate) purity. Plant bioactive compounds have demonstrated promising effects in promoting health. Therefore, the hypolipidemic effects of pomegranate peel powder (PPP) and pomegranate peel extract (PPE) were researched via Lakeview Golden (LVG) Syrian hamsters with a high-fat (20%) diet. PPP and PPE significantly improve obesity-related indices, including adjusting microbiota composition, increasing the microbiota diversity towards a leaner type, and down-regulating the expression of 2 genes (HMG-CoAR and LDLR) to reduce cholesterol ingestion and LDL uptake. However, further research on potential toxicity was required, as adverse plasma LDL-elevating effects were observed at a higher dose of PPP and PPE intake. As functional food fortified with plant bioactive compounds became more popular, the potential of PPE as ingredients for fortified food supplementation was evaluated. A PPE-

fortified Greek Style yogurt was developed using a top-down formulation method based on USDA dietary guidelines. According to the results from response surface methodology, the optimum formula for a 130 g (4.6 oz) product consisted of 10.4 g (8%) of protein and 77 g (59%) of PPE, which could satisfy the polyphenol daily need (1g per day) while maintaining pleasant product properties. An organic acid-based ultrasound-assisted co-extraction process of polyphenol and pectin was investigated for thorough WPP utilization and broader industry applications. Citric acid was applied for extraction since it's a common GRAS acid in food applications. Process conditions, including pH, extraction temperature, extraction time, ultrasound intensity, and solvent ratio, were evaluated via Box-Behnken design to study the effects on extraction performance, including polyphenol/pectin yield, antioxidant activity, and pectin characteristics. High methylated (Degree of methylation up to 79.66%) pectin yield was as high as 13.99%, which demonstrated that WPP could be utilized as a reliable pectin source. The extraction kinetics were characterized for future industry application guidance. In conclusion, this dissertation demonstrated the potential of pomegranate peel to be utilized as a source of healthy and bioactive compounds.

To the passion for food science.

Would you like some pomegranate?

Chapter 1 - Introduction

1.1. Research objectives

With the depletion of natural resources and growing populations, there is a great motivation for recovering valuable compounds from fruit and vegetable wastes and converting them into functional ingredients as a part of sustainable agricultural practices. Pomegranate is an important California commodity and pomegranate by-products are a large portion of the process. These by-products could be repurposed into valuable compounds, but they are not unutilized or underutilized, ending up in landfills or animal feeds. In this dissertation, physiochemical properties and extraction methods of bioactive compounds in pomegranate peel were comprehensively studied to determine the feasibility of converting these pomaces into healthful food ingredients. The research objectives were to (1) identify the physiochemical characteristics of pomegranate peel and assess different size reduction methods to obtain suitable peel particles for effective extraction by using water as a solvent; (2) evaluate the effects of extraction temperature, time, and water usage on the phenolic yield, phenolic composition, and antioxidant activity to optimize the extraction conditions; (3) study the bioactive properties and nutritional benefits of pomegranate peel powder and extract through animal models; (4) investigate the characteristics of food products developed with pomegranate peel powder and extract; and (5) study the co-extraction mechanism of pectin and polyphenol to improve extract yield and phenolic stability.

There are 6 chapters in this dissertation. Chapter 1 includes an introduction, research objectives, and literature review on the processing and waste utilization of pomegranate fruits, with a focus on the current waste management practices and effects of punicalagin, which is the most unique polyphenol in pomegranate.

In Chapter 2, the physicochemical properties of pomegranate fruits were investigated to explore the potential of recovering bioactive food ingredients from the inedible fruit peel. Phenolic extraction parameters and effects of wet and dried pomegranate peel were studied, aiming to increase the extraction sustainability with reduced cost. The chemical characteristics of the extracted components were also evaluated.

The study in Chapter 3 investigated the potential of developing functional food fortified with a value-added ingredient from pomegranate peel extract, specifically Greek Style Yogurt. Products with different levels of protein content and phenolic content were formulated to study the chemical and sensory properties, which provided solid guidance for future product development.

In the research of Chapter 4, the hypolipidemic properties of pomegranate peel and extract were investigated *in vivo* using hamster models. Currently, no literature is available on the functionality of complete pomegranate peel and extract. In this study, it was found that the pomegranate peel and extract were effective in lowering blood cholesterol in hamsters fed with high-fat diets.

The study in Chapter 5 studied the co-extraction mechanism of pectin and polyphenol for improved extract yield and phenolic stability. Ultrasound extraction of pectin and polyphenol in citric acid from pomegranate peel was tested and the corresponding physicochemical properties were evaluated. Unlike most of the research using inorganic acid for extraction, this research utilized GRAS organic acid and provided functional ingredients from fruit by-products without safety concerns.

Conclusions and future directions on reutilizing the pomegranate waste as food ingredients are detailed in Chapter 6.

1.2. Literature review

1.2.1. Production and processing of pomegranate fruits

Pomegranate (Punica granatum L.) belongs to the family *Punicaceae*. It has been grown since ancient times for its delicious fruit and as an ornamental plant for its red, orange, or occasionally creamy yellow flowers. The estimated global cultivation area for pomegranate is about 300,000 ha, with fruit production of 3.0 million metric tons (Kahramanoglu and Usanmaz, 2016). Spanish missionaries brought pomegranate to the Americas in the 1500s (LaRue, 1977; Pareek et al., 2015). Wonderful, a primary cultivar in the U.S., was discovered in Florida and brought to California in 1896. Since then, pomegranate has been grown abundantly in California and Arizona, where mild winters enable the fruits to reach the quality necessary for successful commercial production. In 2015, about 282,000 tons of pomegranate fruit were grown in California, with an economic value of $115.4 million (CDFA, 2014).

Pomegranate consists of three major parts (Figure 1.1), namely pomegranate peel (PP), pomegranate seed (PS), and pomegranate aril that is the flesh part for pomegranate juice (PJ) production. Teh (2016) studied the different fruit part distribution of 5 pomegranate cultivars (Wonderful, Molla Nepes, Parfianka, Purple Heart, and Vkusnyi) grown in California, observed a range of 38.33~50.38% PJ, 38.77~53.01% PP, and 7.71~12.10% PS. The study also determined the components (dry basis, d.b.) of PP and PS through proximate analysis. The peel portion consisted of 90.6~91.9% carbohydrate as the principal constituent, followed by 3.1~3.9% protein, 1.3~2.3% fat, and 3.3~4.3% ash or minerals. The PS contained higher proportions of protein (11.4~16.8%) and fat (15.2~20.6%) and included 60.5~71.8% carbohydrate and 1.6~2.5% minerals. PJ (wet basis, w.b.) consisted of 85% water, 10% total sugars, organic acids,

amino acids, and phenolics. Pomegranate exhibits a sweet, sweet-sour, or sour taste, which color ranges from white to pink and red (Tezcan et al., 2009).

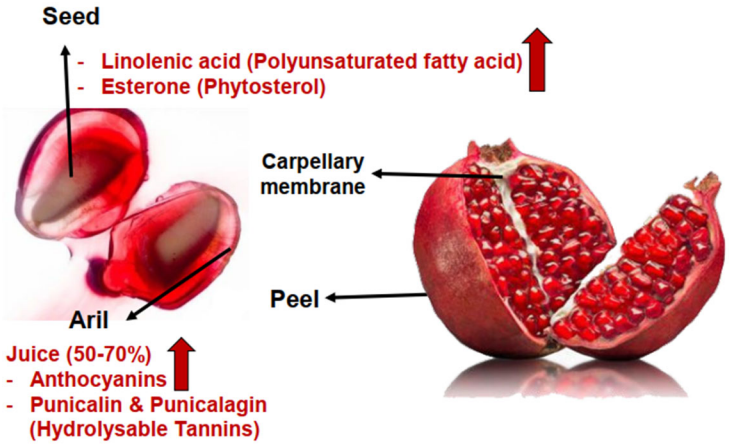

Figure 1.1 Pomegranate fruit and seed parts and their major chemical composition (Hegazi et al., 2021)

Traditional processes in the pomegranate juice industry squeezed the whole pomegranate, which had low yield, impurity, and bitter taste due to non-edible parts. Nowadays, novel techniques introduced a deseeding step at the beginning. Then the seed, aril, and juice are separated from the peel and squeezed, while the peel and the remaining pulp are discarded as wastes in landfills or used as animal feed. The juice stream continues to go through the processes of pasteurization, centrifugation, membrane process, storage, and quality assessment. Other juice extraction methods and their features were also discussed by (Hegazi et al., 2021) as shown in Figure 1.2.

PJ processing generates two types of solid by-products: peel and seeds. PP is non-edible and comprised mainly of bioactive compounds, such as hydrolyzable tannins (pedunculagin, punicalin, punicalagin, and ellagic and gallic acids) at concentrations ranging from 27 to 172 g kg^{-1} (Fischer et al., 2011), flavonoids (catechins, anthocyanins, and other complex flavonoids),

and complex polysaccharides (Ismail et al., 2014). Therefore, PP is an excellent source of phenolic compounds, tannins, flavonoids, sterols, fatty acids, dietary fiber, minerals, and vitamins.

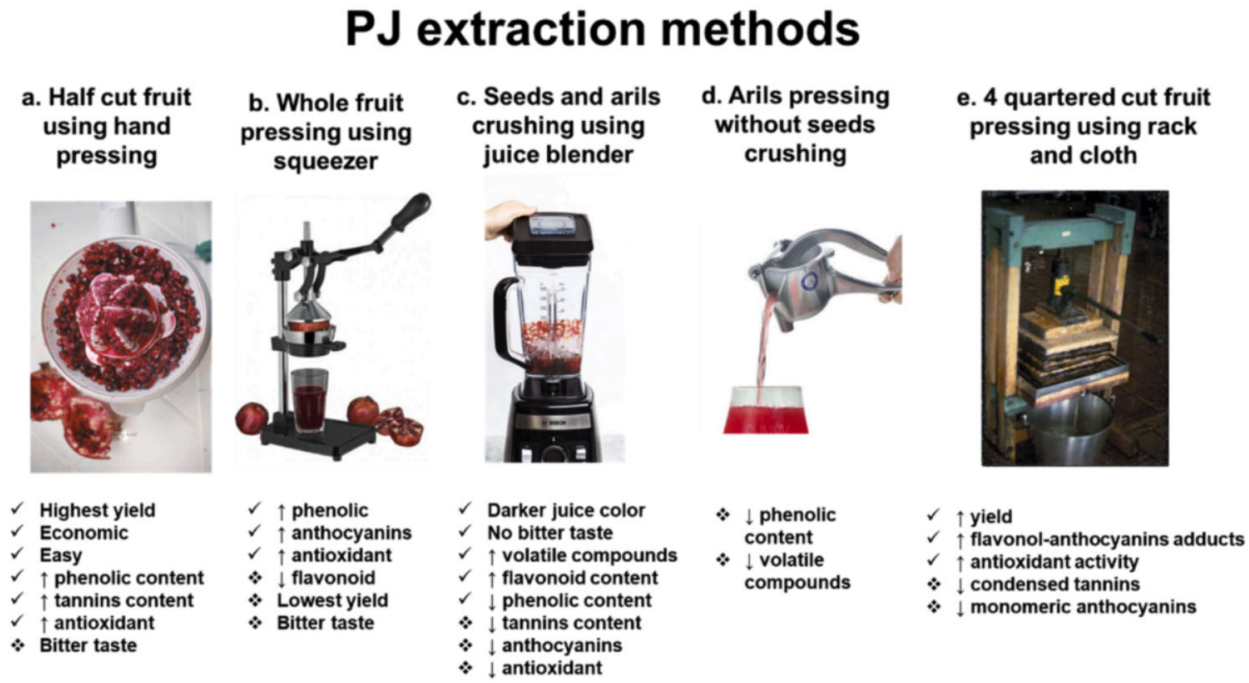

Figure 1.2 Different extraction methods of PJ and their features (Hegazi et al., 2021)

1.2.2. Pomegranate by-products

Due to the massive quantity and abundant bioactive compounds within PP, numerous research has been conducted to explore the potential utilization of PP.

Current practice: landfills or animal feed

Fruit and vegetable pomace has a long history of simply being disposed of in landfills or underutilized as fertilizers and soil conditioners. Several studies investigated the health potential of supplementing PP into animal feed. Modaresi et al., (2011) added 12% pomegranate seed pulp into the goat diets and observed increased polyunsaturated and conjugated fatty acids in goat milk. A feed with 1-2% PP lowered cholesterol levels and improved oxidative stability in the

harvested boiler chicken meats (Ahmed et al., 2015). Shabtay et al. (2008) supplemented PP in calves' diets and observed a significant increase in weight gain and blood antioxidant contents in the ruminants. Therefore, pomegranate pomace demonstrated great potentials for nutritional feed with improved health benefits in ruminant and chicken feed.

Due to the massive quantity of pomace, the convenience of disposal, and low realizable revenue from current waste utilization practices, processors and farmers have a low incentive to apply alternative waste management methods. With the pressure of climate change, energy shortage, and increasing nutritional needs, creating value-added products from the by-products would be an outstanding solution to incentivize novel waste practices.

Food additives and packaging materials

With an enormous amount and variety of polyphenols within the pomace, pomegranate by-products demonstrated great antioxidant and anti-microbial properties, which contributed to diverse application potentials as food additives and packaging materials.

Lipid oxidation is the principal deteriorative reaction during food processing and storage. It sharply reduces the product shelf life, destroys essential nutritional components, and generates toxic compounds which pose hazards to human health (Johnson & Decker, 2015). Synthetic antioxidants have been dominantly applied in the food industry to prevent oxidation, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Natural antioxidants are gaining attention as consumers prefer safe and natural ingredients (Gülçin, 2012). Topuz et al. (2015) incorporated alcoholic extract of PP into anchovy fish oil and observed a dose-dependent inhibitory effect on lipid oxidation, especially at a concentration of 500–1000 ppm. The antioxidant capacity of 500 ppm of PP extract (PPE) was comparable with that of 100 ppm (legal limits) of BHT, indicating that PPE could be applied as a potent antioxidant. Turgut et al., (2016) infused PPE into freshly minced beef at 5000/10000 ppm and compared it with 100 ppm BHT.

Their results demonstrated a lower thiobarbituric acid reactive substances (TBARS) value, peroxide formation, and other parameters, suggesting promising oxidation retarding effect of lipid and protein in pomegranate extract. However, they also observed a potential negative change of sensory value after extract addition, which required further research to quantitatively investigate the effects. Similar research also proved the preservative effects of pomegranate extract in burgers and cheese (Mahajan et al., 2015; Shahamirian et al., 2019). A more comprehensive review on the practical use of PP in meat products was reported by Smaoui et al., (2019).

PP may also prevent foodborne illness, which has been a worldwide safety concern. In the USA, there were millions of cases related to food contamination and foodborne outbreak each year, posing a severe threat to public health (Scallan et al., 2011). Traditional synthetic anti-microbial agents may have potential side effects, are expensive, and could induce drug resistance of microorganisms as their indiscriminate killing effects (Gutiérrez-del-Río et al., 2018). Natural anti-microbial agents are needed. Pomegranate is rich in polyphenols, which can inhibit bacterial growth by interacting with the sulfhydryl groups of bacterial cell wall proteins (Dey et al., 2012) and forming complexes, and then induce lysis (Akhtar et al., 2015). More applications of pomegranate by-products as anti-microbial agents could be found in Singh et al. (2019).

Biotechnological products

Biofuel is considered an ideal alternative to fossil fuel, as fossil fuels are experiencing a rapid depletion, uncertainty in the price, and contributing to significant environmental pollution (Höök & Tang, 2013). Biofuel is produced from biomass via thermochemical processes, including gasification, carbonization, pyrolysis, and direct combustion. Among all the methods, pyrolysis is considered the most viable due to its simplicity, cost-effectiveness, and wide range of final products (biochar, biogas, bioethanol). Siddiqui et al. (2019) studied the optimized

process parameters for biochar production from PP. Results showed that, at a temperature of 300 °C, the pyrolysis reaction time of 20 min and the particle size of 3 mm, biochar could be produced at a yield of 54.9% with an improved higher heating value (HHV) at 23.5 from 14.61 MJ/kg of parent biomass. Besides energy supply, PP-based biochar also demonstrated a desirable ability in eliminating inorganic compounds from water and CO_2 capture/storage (Jung et al., 2016; S. Jung et al., 2019). As for biofuel, Demiray et al. (2018) optimized bioethanol production from PP by *Saccharomyces cerevisiae* and *Pichia stipites.* They successfully increased the ethanol yield produced by S. cerevisiae up to 44.9%. Ethanol productivity (Q_p, g ethanol/L/h) and Ethanol yield ($Y_{P/S}$, g max ethanol/g consumed reducing sugar) of *S. cerevisiae* increased to 0.46 g/L/h and 0.43 g/g, respectively. These findings along with other similar research (Baghaie, 2018; Bhardwaj et al., 2020; Demiray et al., 2020) have demonstrated that PP is a promising biofuel source.

1.2.3. Polyphenol classification and punicalagin – unique polyphenol in pomegranate

Polyphenols, a family of molecules, are commonly found in fruits, vegetables, nuts, seeds, flowers, and tree bark. These components fundamentally are plant metabolites to attract pollinators, but research concluded multiple pharmaceutical effects, including antioxidant, anti-microbial, anti-cancer, etc (Cutrim & Cortez, 2018). This family derives from a fundamental polyphenol group (i.e. numerous hydroxyl radicals on aromatic rings). The structure ranges from simple elementary substances (phenolic acids, stilbenes) to complex polymerized molecules (tannins), contributing to a diversified classification profile (Tsao, 2010). Furthermore, sugar residues (monosaccharides, disaccharides, or oligosaccharides) can conjugate with the hydroxyl

groups of natural polyphenols through direct linkage of the sugar unit to an aromatic carbon, further diversifying the polyphenol structure (Bravo, 1998).

Based on the number of phenol rings and the binding components, polyphenols can be classified into the following 5 types of sub-groups: hydroxybenzoic acid, hydroxycinnamic acid, flavonoids, stilbenes, and lignans (Manach et al., 2004), as illustrated in Figure 1.3.

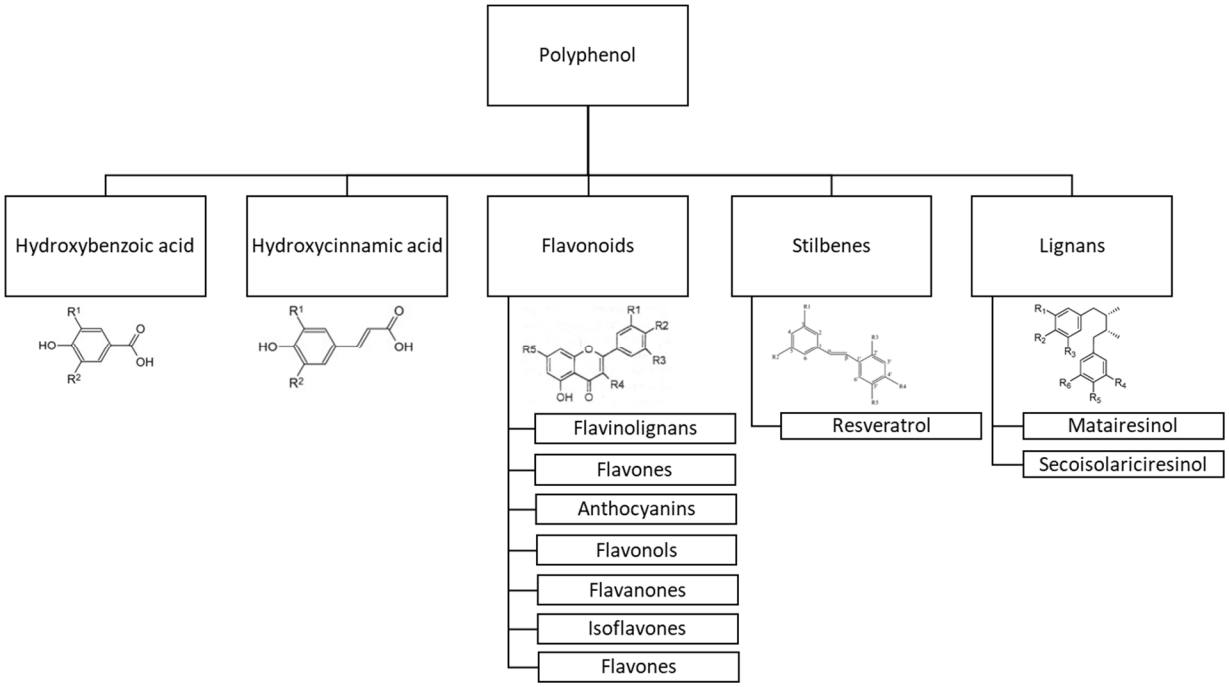

Figure 1.3 Classification and major sub-categories of polyphenols

According to Fischer et al., (2011), PP is an ideal source for polyphenol, as 48 types of polyphenols were detected within, including 9 anthocyanins, 2 gallotannins, 22 ellagitannins (predominant), 2 gallagyl esters, 4 hydroxybenzoic acids, 7 hydroxycinnamic acids, and 1 dihydroflavonol. Among the polyphenols in pomegranate, punicalagin (PU) is the most abundant (11~20 g/ kg dry matter) and unique water-soluble ellagitannin (ET) within. Besides PP and PJ, PUs are also commonly found in the leaves of *Lafoensia pacari* (Carneiro et al., 2016).

The synthesis pathway of PU is shown in Figure 1.4. Phenolic acids (PA) including two gallic acids (GA) and ellagic acid (EA) are combined to form gallagic acid. With glucose addition, gallagic acid can form punicalin (PC) and be further transformed into PU by adding an EA. The difference in the glucose carbon-1 induces two isomers of PU (α, β) (Reliene et al., 2015). The complexity synthesis of PU results in a large molecular weight (1084.71 g/mol) and rigid structure (Oudane et al., 2018). These features provided higher functionality with a large number of hydroxyl groups (Fischer et al., 2011) and a lower chance of degradation. A typical phenolic composition distribution in HPLC analysis is shown in Figure 1.5 (Wu et al., 2021).

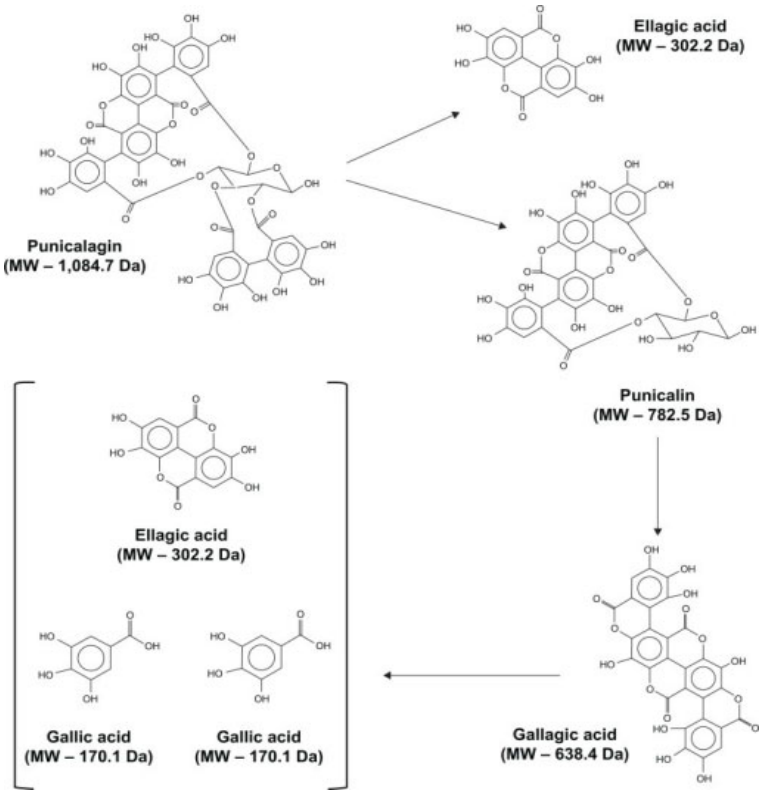

Figure 1.4 Synthesis pathway of punicalagin (Reliene et al., 2015)

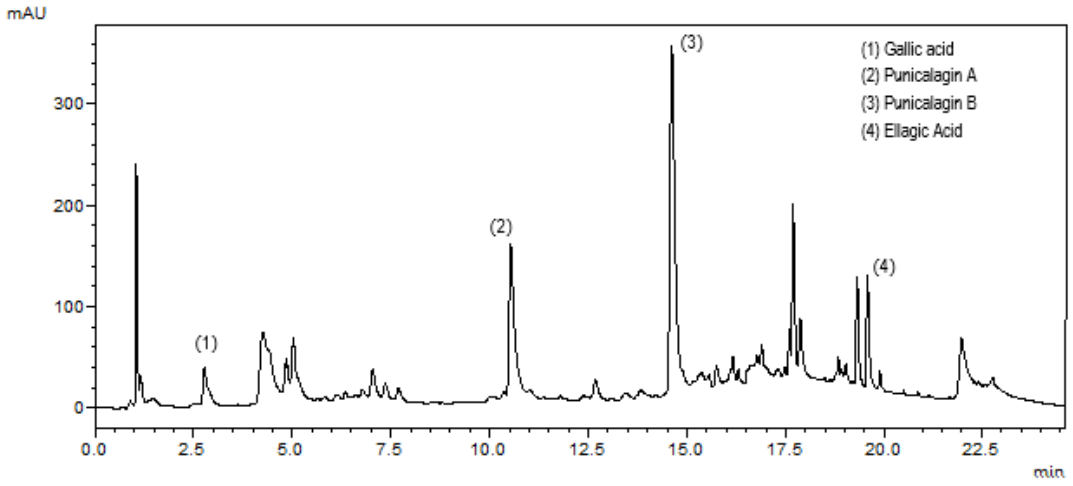

Figure 1.5 Separation of phenolic compounds in pomegranate peel by HPLC (Wu et al., 2021)

1.2.4. Effects of extraction methods on punicalagin

Extraction is a solvent-based process to separate desired substances from raw materials. Emerging extraction techniques of punicalagin are discussed below to reveal their effects on punicalagin quality and yields.

1.2.4.1. Ultrasound-assisted extraction (UAE)

Continuous ultrasound-assisted extraction (CUAE)

Ultrasound is a common industrial method to increase extraction efficiency in contrast to hot acid extraction. The intense vibration of ultrasound induces bubbles to break and forms instant high pressure, which is known as cavitation. Cavitation also generates mechanical effects, such as cell disruption and agitation of the extraction medium (Chemat et al., 2017).

Živković, Šavikin, Janković, Ćujić, & Menković (2018) studied the effect of extraction factors using ultrasound at 160 w power. The optimal extraction process condition was identified by using 59% ethanol at a ratio of 44 mL/g for 25 min at 80 ºC. It is verified with an extraction yield (EY) of 149.12 mg/g total phenolic content (TPC), including 18.05 mg/g PU, 61.93 ± 2.41

mg/g PC and 11.65 ± 0.42 mg/g EA. CUAE is an efficient extraction method with high EY. However, the PU retention was low, which might be due to the PU degradation induced by constant cavitation.

Pulsed ultrasound-assisted extraction (PUAE)

Compared to CUAE, PUAE has an intermediate working process known as the duty cycle. Therefore, it can reduce energy use, depreciation of the equipment, and erosion of the tip.

Kazemi, Karim, Mirhosseini, & Hamid (2016) optimized the PUAE process for higher TPC in pomegranate of Malas variety. At 105 W/cm^2 intensity level with 50% duty cycle for 10 min extraction, highest PU (146.58 mg/g) content was achieved within 20.66 mg/g EA and 0.053 mg/g GA, accounting for 87.6%. Extraction time and duty cycle were the most and least significant ultrasound variables for PU retention, respectively. PUAE required lower energy use and degradation at the same time as CUAE, indicating a lower degree of oxidation induced by the ultrasound and contacting with open air. On the other side, the conditions of PUAE were more material-dependent and required more time to optimize the conditions.

Combination with other techniques

Sumere et al. (2018) developed an EXTRACT-US system using ultrasound-assisted pressurized liquids extraction (UAPLE), which consisted of a 10 mL stainless steel extraction cell coupled to the ultrasonic horn (800 W, 19 kHz, 13 mm of diameter). The extraction process was evaluated based on 5 parameters: consecutive cycles, particle sizes, temperature, solvent, and power of ultrasounds. The highest EY was achieved using water at 70 ºC, with 43.27 ± 0.76 mg/g TPC, including 11.48 ± 0.21 mg/g α-PU and 24.55 ± 0.61 mg/g β-PU. Under pressurized liquids, UAE did not influence the extraction of TPC for small particles (0.68 mm), but 480 and 640 W powers improved the yields at large particle sizes (1.05mm). This research demonstrated

a promising effect on improving UAPLE on an industrial scale. At the same time, several challenging factors need to be considered in the future, including high pressure and high capital investment.

Santos et al. (2019) further developed the UAPLE system using expansion gas initial pressure (N2-Pi, 0/5/10 bar), a different combination of ultrasound power (0/200/400 W), system pressure (100/200/300 bar). The optimal extraction was achieved with 100 bar system pressure, 5 bar N2-Pi and 400 W, with 14.87 ± 0.36 mg/g α-PU, 37.13 ± 1.44 mg/g β-PU, and 1.11 ± 0.05 EA. N2-Pi influenced the process by facilitating the bubble formation and size expansion at the initial stage. However, 5 bar and 10 bar N2-Pi didn't make significant differences in phenolic yield and composition. It is worth noting that the conditions required a complex setup but less TPC compared to other techniques. Moreover, the increase in system pressure hampered the cavitation because the bubbles were required to reach a higher critical pressure for breakage.

1.2.4.2. Microwave-assisted extraction (MAE)

MAE is another common extraction method with reducing extraction time and solvent consumption. The heat and mass transfer processes take place at the same time and accelerate the overall extraction. Kaderides, Papaoikonomou, Serafim, & Goula (2019) compared the extraction performance of microwave-assisted extraction and the UAE. Results showed that the optimal MAE condition was using 60mL 50% aqueous ethanol per gram at 600 W power for 4 mins, with an extract yield of 199.4 mg/g GAE, PU yield of 143.64 mg/g, and radical scavenging activity of 94.91%. In contrast to 10 mins of UAE at 52 W power using 32.2mL water per gram peel at 35ºC, MAE achieved 1.7 times higher extract yield in a shorter processing time (4 mins), which was due to higher cell destruction but similar PU yield and scavenging activity. Limited research has focused on this topic so far. Researchers suggested a focus on the MAE-assisted

process to achieve a high yield of polyphenols as well as extract with great polyphenol profile and quality.

1.2.4.3. Enzymatic extraction

PU is sensitive to temperature and pH. Therefore, conventional hot acidic processing methods might increase the hydrolysis of PU and reduce its bioactivity.

Alexandre et al. (2019) proposed that cellulase and pectinase degraded the cell wall and released the intact PU along with pectin. They compared extraction under 300/600 MPa high pressure with that using 4% cellulase and pectinase. As result, enzymatic extraction yielded 1481.29 μg/g TPC with 62.9% PU, significantly higher than extraction at 300MPa (1634.71 μg/g TPC, 59.88% PU) and slightly lower than 600 MPa (1572.31 μg/g, 63.38% PU). Talekar, Patti, Vijayraghavan, & Arora, (2018) applied 55 U/g cellulases at a solvent ratio of 15 mL/g for 4h with 20 min ultrasound treatment. As the results, 84.8 mg/g PU was recovered, accounting for 71.2% of its TPC and was superior to the ones recovered by a conventional method (15 mL/g samples at 85 °C with 0.02 M aqueous HCl of pH 1.7 for 2 h, 52.2 mg/g PU for 42.4% of TPC). This result demonstrated reduced hydrolysis of PU under mild enzymatic extraction.

Since enzymes could be costly due to low recovery and reusability, biocatalyst became a more sustainable option. Biocatalyst immobilizes enzymes onto solid phase (such as magnetic) for selective enzyme recovery after the reaction. Talekar et al., (2019) experimented with extraction with magnetic nanocatalyst of $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ solutions, which yielded 64.2–66.5 mg/g PU (75.3–78.2% TPC). It was lower than their previous research using cellulase (Talekar et al., 2018). However, the nanocatalysts are recyclable and could reduce the cost of cellulase.

Compared to other processing methods, enzymatic extraction might have less TPC. On the other hand, it is desirable for PU retention. The usage of cellulase and pectinase also helped with pectin extraction, which further increased the value obtained from the waste process. More forms of enzymes and catalysts are expected to be developed for lower production costs.

1.2.4.4. Supercritical CO_2 extraction

Supercritical fluid (SCF) extraction is another extraction method with increasing popularity. It utilizes supercritical fluid, such as high-pressure water and CO_2, instead of chemical solvents that would cause drastic environmental impacts. (Pereira & Meireles, 2010; Sharif et al., 2014). Therefore, it is deemed a green and clean method for compound-specific extraction.

Bustamante et al. (2017) investigated one-passage supercritical CO_2 extraction of PP. The optimal extraction condition was 400 bar, 40 °C, and 20% ethanol, obtaining 8.94 mg/g TPC as of ellagic acid equivalent (EAG) with high antioxidant capacity (97.42 μg/g Trolox equivalent) and 9.5% PU retention (Bustamante et al., 2017). PU content increased with elevated pressure, due to higher fluid density and supercritical fluid solvent power, thus improving the dissolvability of polar compounds. However, the overall percentage of PU was low. Moreover, the scalability of supercritical CO_2 extraction is impossible due to the lack of molecular models of solutes diffusion (Ameer et al., 2017).

A summary of the processing methods is shown in Table 1.1, including extraction conditions, total phenolic content, and punicalagin content.

Table 1.1 Novel processing methods: conditions, total phenolic content, and punicalagin content

Processing Method	Extraction Condition	Total Phenolic Content	Punicalagin Percentage	Reference
Ultrasound-assisted extraction (UAE)				
Continuous ultrasound-assisted extraction (CUAE)	Temp: 20-80ºC/ Time: 5-65min Solvent: 10-50mL/g ethanol (10-90%)	149.12 mg/g GAE	12.1%	(Živković et al., 2018)
Pulsed ultrasound-assisted extraction (PUAE)	Time: 2/6/10min Ultrasound: 53/79/105w/cm^2 Duty Cycle: 50/70/90%	320.26 mg/g GAE	45.8%	(Kazemi et al., 2016)
Ultrasound-assisted pressurized liquids extraction (UAPLE)	Temp: 50-100ºC/ Time: 30min * 5cycles Pressure: 200-400bar Solvent: water, 30/50/70% ethanol Ultrasound: 0/160/320/480/640/800W	43.27 mg/g GAE	83.3%	(Sumere et al., 2018)
Ultrasound-assisted extraction using expansion gas	Temp: 40ºC/ Time: 20min Solvent: 0-20% ethanol System Pressure: 100/200/300bar N_2-Pi: 0/5/10bar Ultrasound power: 0/200/400W	53.11 mg/g GAE	97.90%	(Santos et al., 2019)
Microwave-assisted extraction (MAE)	Time: 0.5-4min Solvent: 10.0/17.3/35.0/52.7/ 60.0mL/g ethanol/methanol Microwave power: 100/173/ 350/527/600W	199.4 mg/g GAE	72.04%	(Kaderides et al., 2019)
Enzymatic extraction				
Cellulase and pectinase extraction with high pressure	Pressure: 300/600 MPa Time: 15 min Solvent: 62.5 mL/g water Enzyme: 4% cellulase and pectinase	1.63 mg/g GAE	59.9%	(Alexandre et al., 2019)
Cellulase extraction with ultrasound	Solvent: 5/15/25 mL/g, pH 5, 50 mM citrate buffer	119.01 mg/g GAE	71.2%	(Talekar et al., 2018)

	Time: 10/20/30 min ultrasound + 2/4/6 h cellulase Ultrasound: 150 W, 37 kHz Enzyme: 30/55/80 U/g			
Nanobiocatalyst of $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$	Temp: 50 ºC/ Time: 5h Solvent: 15mL/g, pH 5, 50 mM K-phosphate buffer Ultrasound: 150 W, 37 kHz Nanobiocatalyst: 100 U/g	85.30mg/g GAE	75.3–78.2%	(Talekar et al., 2019)
Supercritical CO_2	Temperature: 30-50 ºC Pressure: 200-400 bar Solvent: 0-20% ethanol CO_2 flow rate: 2.5 mL/min	8.94 mg/g EAG	9.5%	(Bustamante et al., 2017)

*GAE, gallic acid equivalent; EAG, ellagic acid equivalent.

1.2.5. Functionality of punicalagin

Tremendous health benefits of PU have been reported, such as anti-inflammation, anti-cancer, anti-oxidant, and cardiovascular disease prevention (Viuda-Martos et al., 2010). Processing methods affect the composition of bioactive phytochemicals, therefore further are reflected by the functionality. A thorough understanding of the biodegradation of PU will help to improve the processing in return. To be specific, this section will focus on the effect of PU on gut microbiota.

Similar to other ETs with a high molecular weight, such as proanthocyanidins, PU was reported to have low absorption through the gastro-intestine (GI) tract (Viuda-Martos et al., 2010). EA that hydrolyzed from PU before gastric digestion will be absorbed in the stomach. Then the remaining part of the PU will be degraded and absorbed in the alkaline intestinal environment (Espín et al., 2007). Gut microflora in the colon could also cleave the ester links from the PU to produce EA/GA.

Only less than 1% of free EA will be absorbed and metabolized in the stomach and small intestine (Espín et al., 2007), while the majority of the EA will arrive in the jejunum and be further transformed to different products in an order of urolithin D (tetrahydroxy-dibenzo pyranone), urolithin A (trihydroxy-dibenzo pyranone), urolithin A (dihydroxy-dibenzo pyranone) and finally urolithin B (mono-hydroxy-dibenzo-pyranone) (Gonzalez-Barrio et al., 2011). This metabolism relies on the sequential removal of hydroxyl groups by gut microbiota in the distal parts of the colon and will form urolithin aglycones (UA). After absorption, UA will undergo an extensive phase II metabolism (methylation, glucuronidation, sulfation, or a combination of these), which takes place within a large intestine wall (enterocytes) and/or within the hepatocytes

(Bialonska et al., 2010). The metabolites will further go through enterohepatic recirculation, enter the systemic circulation, and be excreted in urine (Espín et al., 2007).

In return, ETs also modify the composition of microbiota since they are toxic to certain bacteria. Several mechanisms were proposed. For example, ETs were able to form a complex with proteins, carbohydrates, or metal ions including Fe and Cu. The complex of ETs and cell wall proteins decreases cell wall permeability, which will reduce the substrate transportation among cells. At the same time, Enzyme activity will also be inhibited, since the formation of ETs and enzymes alters the structural conformation (Chung et al., 1998). Additionally, the stable complex of ETs and ion decrease metal ion availability to bacteria, which subsequently adversely affects the metalloenzymes activity and selectively inhibits the growth of bacteria (Goel et al., 2005).

Besides direct inhibition of bacterial growth, the degradation of ETs can significantly lower the intestinal pH with the production of PA, which will further alter the bacterial population. Puupponen-Pimia et al. (2005) investigated the pH lowering ability of berries and their phenolic extracts, which could bring down the liquid media (for intestinal pathogen growth) to pH 5 with the organic acids in the extract. Generally, lower pH is suitable for probiotic bacteria rather than pathogenic bacteria. For example, PUs, PCs, GA and EA were proven to significantly improve the growth of *Bifidobacterium breve* and *Bifidobacterium infantis*, while they also partially inhibited the growth of *Bifidobacterium animalis lactis* and *Bifidobacterium bifidum*. On the contrary, pathogens, such as *S. aureus*, *Clostridium perfringens*, *Clostridium clostridioforme*, *Clostridium ramosum,* and *Bacteroides fragilis,* were strongly inhibited with the presence of these polyphenols (Bialonska et al., 2009). Enhanced growth of *Bifidobacterium spp.* is associated with health benefits, including weight management under a high-fat diet and

cardiovascular disease prevention (R. K. Singh et al., 2017). A summary of the effects of punicalagin on gut microbiota is shown in Table 1.2.

Table 1.2 Effect of punicalagin on gut microbiota

Bacterium	Effect	Potential biological functions	Reference
Bifidobacterium infantis	Significantly improve the growth	Display anti-inflammatory activity in premature intestinal cells and decreases intestinal permeability.	(Underwood et al., 2015)
Bifidobacterium breve		Target tumors in athymic MF1 nu/nu mice bearing s.c. B16-F10 murine melanoma tumors.	(Arboleya et al., 2016)
Bifidobacterium animalis lactis	Partially inhibit the growth	Reduce the risk of infections in early childhood	(Taipale et al., 2016)
Bifidobacterium bifidum		Suppress allergic responses in mouse model and anti-inflammatory bowel disease	(Ku et al., 2016)
Bacteroides fragilis		Enterotoxigenic *Bacteroides fragilis* is associated with Inflammatory Diarrhea	(Sears et al., 2008)
Clostridium clostridioforme	Strongly Inhibit the growth	Associated with more serious or invasive human infections	(Finegold et al., 2005)
Clostridium ramosum		Promote high-fat-diet-induced obesity in gnotobiotic mouse models	(Woting et al., 2014)
Staphylococcus aureus		Gram-positive bacteria, a major pathogen for infections	(Harris et al., 2002)

Overall, the intact form of PU is desired to avoid significant biodegradation and antioxidant loss during the GI-tract digestion, which could further strengthen the gut microbiota and promote more health benefits.

This section discussed the health benefits of punicalagin including modifying the gut microbiota composition and possessing strong antioxidant activity. Figure 1.6 summarizes the effect on punicalagins by processing methods and transformation in GI track and by gut microbiota. Proper novel processing methods, such as ultrasonic-assisted method, microwave-

assisted method, enzymatic method, and supercritical fluid CO_2, showed promising potential in improving PU yield with high biological activity, but the effects on PU quality need to be further quantified. In the future, research should focus on developing processing methods to recover punicalagins with high yield, purity, and biological activity, while staying low cost, low time-consuming, and environmental-friendly.

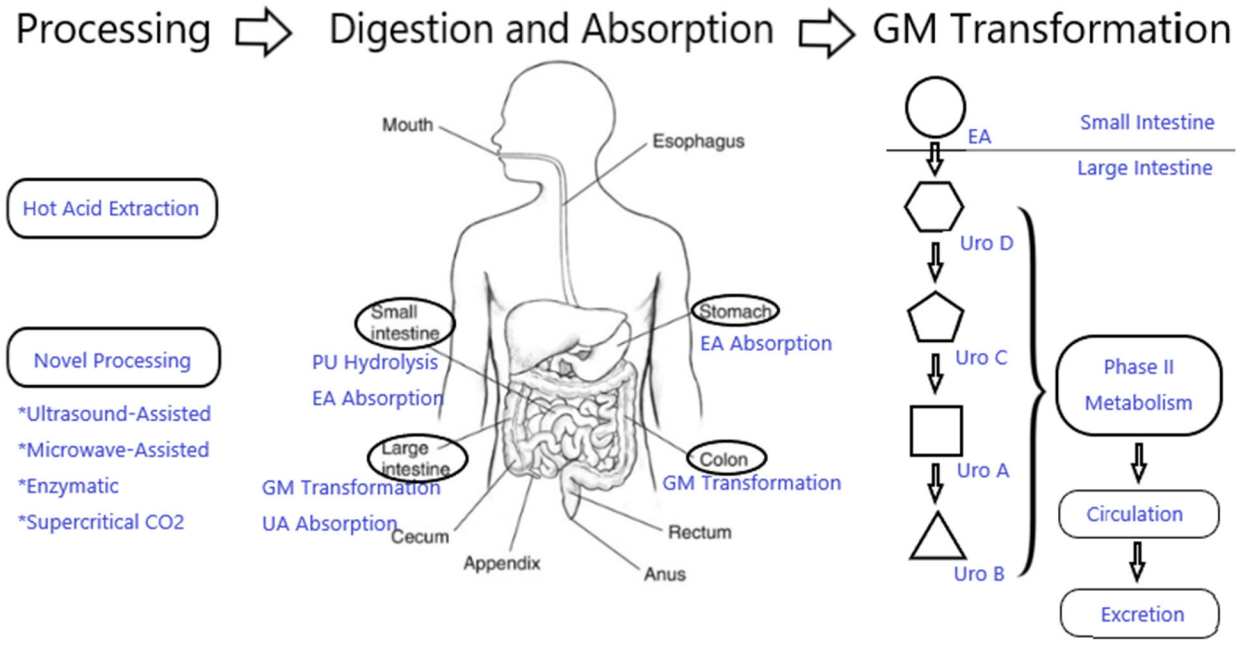

Figure 1.6 A schematic diagram of the relationship between processing methods, digestion and absorption, and gut microbiota transformation. Adopted from Mena, Calani, Bruni, & Del Rio, (2015). PU, punicalagin; EA, ellagic acid; GM, gut microbiota; UA, urolithin aglycones; Uro, urolithin.

1.2.6. References

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Chapter 2 - Physiochemical Properties of Pomegranate Peel and Extraction of Bioactive Compounds

2.1. Introduction

Research has shown that pomegranate fruit might be beneficial for its antioxidant, anti-mutagenic, and anti-hypertension activities and its ability to reduce liver injury (Al-Jarallah et al., 2013; Dhinesh and Ramasamy, 2016; Du et al., 1975; Gil et al., 1996; Lansky et al., 2000; Tsuda et al., 1994). It has also been studied for therapeutic purposes to alleviate ailments, such as colic, colitis-diarrhea, dysentery, leucorrhea, paralysis, and headache (Sadeghi et al., 2009; Schubert et al., 1999). Pomegranates are considered beneficial for curing chronic stomach ailments and are also known for their anti-inflammatory and anti-atherosclerotic activities against osteoarthritis, prostate cancer, heart disease, and HIV-I (Malik et al., 2005; Sumner et al., 2005).

High molecular weight polyphenols are the major high-value phytochemicals in pomegranate peel. They have demonstrated the likelihood of reducing risks of chronic diseases (Heber, 2011), including type-2 diabetes (Banihani et al., 2013) and cardiovascular diseases (Hamoud et al., 2014). Among all the polyphenols in pomegranate peel, gallic acid, ellagic acid, and punicalagin are most likely responsible for these health benefits (Aviram et al., 2008; Pai et al., 2011). Punicalagin has also demonstrated significant in vitro antioxidant activities (Fischer et al., 2011) with abundant hydroxyl groups, which can trap peroxyl radicals to reduce oxidation. Therefore, it is of great interest to extract punicalagin, ellagic acid, and gallic acid for nutrient fortification and other applications. Extraction is a mass transfer process influenced by the matrix properties of the plant part as well as the solvent, temperature, and time (Hernandez et al., 2009). The particle size and solvent type play an essential role in extraction. Particle size reduction

results in higher mass transfer efficiency and product yield, as smaller particles have a higher surface-to-volume ratio and less internal path (Adapa et al., 2011). The solvent type impacts extraction through different polarities and affinity to the compounds of interest (Alothman et al., 2009). However, a better understanding is needed of the effects of extraction conditions on antioxidant yield and antioxidant activity.

Most previous research on the extraction of bioactive compounds used organic solvents to improve the extraction rate. However, inappropriate use or recycling of organic solvents in food applications can cause pollution and raise safety concerns. Green extraction of natural products, focusing on modified extraction processes with reduced energy consumption, alternative solvents, and renewable natural products, is in demand (Armenta et al., 2015). Water is a universal solvent that is generally recognized as safe (GRAS). Therefore, deionized (DI) water was applied for extraction in this study.

Nearly all extraction studies of plant-based bioactive compounds have used dried material for extended research time (Azmir et al., 2013). However, drying increases energy and time consumption, in addition to the excessive preparation requirements. Therefore, a novel extraction approach using fresh wet pomegranate peel (WPP) was studied in this research.

2.2. Objectives

In summary, this work aimed to investigate the effects of extraction conditions on the isolation of bioactive compounds and compare the extraction rates of wet and dry pomegranate peel particles. The optimal extraction parameters were determined based on the antioxidant yield and activity to provide more insight for industrial-scale production.

2.3. Materials and methods

2.3.1. Raw pomace and chemicals

Pomegranate pomace of Wonderful variety was collected from SunnyGem LLC (Buttonwillow, Cal.). The wet pomace was stored at -18°C and thawed to room temperature before use. The chemicals used in the experiments, including Folin-Ciocalteu reagent, analytical standards of tannic, gallic, and ellagic acids, and 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma-Aldrich (St. Louis, Mo.). Methanol, HPLC-grade o-phosphoric acid (85), and analytical-grade sodium hydroxide and sodium carbonate were obtained from Fisher Scientific (Pittsburgh, Pa.). A mixture of punicalagin α and punicalagin β (51.54% α and 48.46% β) was purchased from ChromaDex Co. (Irvine, Cal.).

2.3.2. Preparation of peel particles

Pomegranate juice had been extracted by carving the peels and separating the peels from the arils. Therefore, the collected pomace contained about 96% (wt/wt, d.b.) peels and 4% (wt/wt, d.b.) seeds. Because the percentage of seeds in the pomace was low, further separation of seeds was not performed. The WPP was sliced into small pieces of less than 5 mm using a continuous-feed food processor (Hobart, Troy, Ohio). The sliced peel pieces were then ground into two groups of fine particles in a Comitrol processor (model 3600, Urschel Laboratories, Chesterton, Ind.) using 0.024 and 0.012 in. (~0.60 and 0.38 mm) cutting heads. The moisture contents of the two groups were determined by drying about 10 g of particles at 105°C in a hot-air oven until a constant weight was obtained (APHA, 1998).

To compare the polyphenol extraction yield and antioxidant activity of polyphenols produced from WPP and DPP, the WPP samples were dried using infrared radiation (IR) and hot air (HA). IR drying was performed by heating a single layer of peels at a surface temperature of

60°C, and HA drying was performed using hot air at 40°C until the moisture content was less than 10% (wt/wt, d.b.). DPP samples produced by IR and HA drying were milled in a sample mill (Stein M-2, Hoffman Manufacturing, Corvallis, Ore.) to less than 0.38 mm size for polyphenol extraction. A demonstration of the extraction process and the corresponding equipment was shown in Figure 2.1.

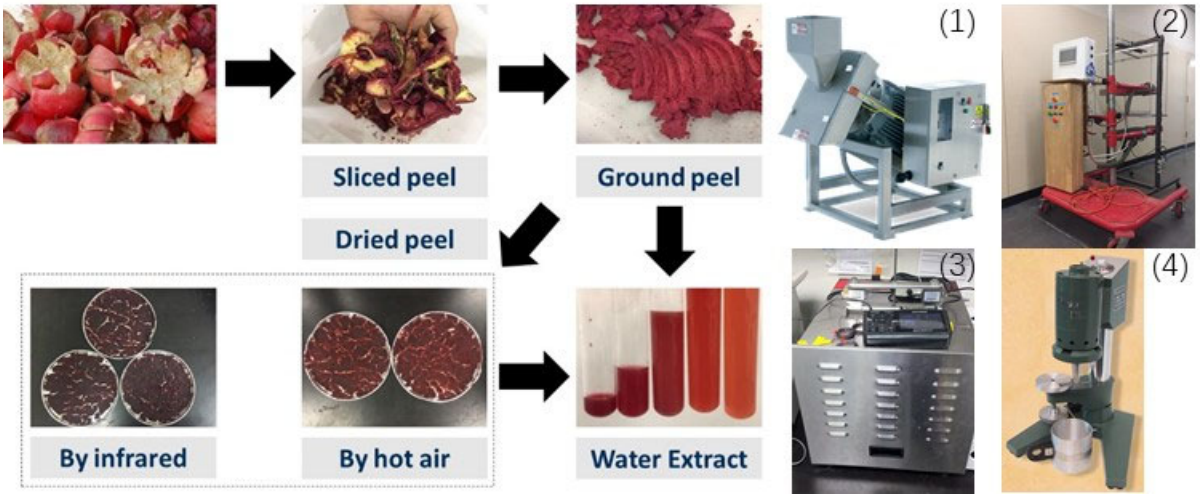


Figure 2.1 Demonstration of the extraction process and the corresponding equipment. (1). Comitrol processor; (2). Lab-scale IR dryer; (3). Lab-scale HA dryer; (4). Stein M-2 sample miller

2.3.3. Composition of wet and dried peel

Analytical measurements were used to determine the compositions of WPP and DPP. Ash content was measured by the gravimetric method by heating to 550°C (AOAC Method 942.05, AOAC, 1990). Crude fat was determined based on Randall modification of the standard Soxhlet extraction method (AOAC Method 2003.05, AOAC, 1990). Total protein content was quantified by nitrogen conversion using AOAC Method 990.03 (AOAC, 1990). Carbohydrate content was calculated by difference using Eq. 2.1:

$$\text{Carbohydrate} = \text{dry matter} - (\text{protein} + \text{fat} + \text{ash}) \quad (2.1)$$

Total dietary fiber and soluble and insoluble fiber were quantified using AOAC Method 991.43 (AOAC, 1990). Results were reported on a 100% dry basis of individual dry matter according to NFTA 2.2.2.5 (Shreve et al., 2006).

Table 2.1 Analytical measurements of the component in pomegranate peel

Composition	Method/Equipment	Reference
Dry matter (DM)	Oven drying at 105 ºC until constant weight	NFTA 2.2.2.5
Ash	The gravimetric method by heating to 550°C	AOAC 942.05
Crude fat-rinse	Randall modification of the Soxhlet extraction	AOAC 2003.05
Protein	Total nitrogen (N) conversion	AOAC 990.03
Carbohydrate	DM – (protein + fat + ash)	
Total dietary fiber (TDF)		
Soluble dietary fiber	Enzymatic-Gravimetric method	AOAC 991.43
Insoluble dietary fiber		

2.3.4. Effects of extraction parameters

Experiments were conducted using WPP particles of both sizes to determine the effects of single factors, including particle size, solvent ratio, extraction temperature, and extraction time, on the extraction of polyphenols. To study the effect of extraction time, 5 g of WPP was mixed with 40 mL of DI water and extracted at 20°C for 2, 3, 4, 5, and 6 min with a stirring speed of 1200 rpm. The effect of solvent ratio was investigated by extracting 5 g of WPP powder with 5, 10, 20, 30, and 40 mL of DI water for 6 min at 20°C. The effect of temperature was tested by adding 5 g of WPP powder to 40 mL of DI water and extracting for 6 min at temperatures of 20°C, 30°C, 40°C, 50°C, and 60°C. All extractions were conducted in triplicate using a Corning hot plate stirrer (Corning, N.Y.) properly shielded from light to avoid light-induced quality loss. After extraction, the extract mixture was separated by centrifugation at 4400×*g* at 4°C for 15 min (Centrifuge 5804R, Eppendorf, Hamburg, Germany). The liquid extract (supernatant) was collected for the determination of physicochemical qualities.

2.3.5. Assay analysis

2.3.5.1. Determination of phenolic content and yield

Total extract yield (TEY) was determined by drying 5 mL of liquid extract at 60°C under 20 kPa in a vacuum oven (Lindberg/Blue M VO1218C, Thermo Scientific, Columbia, Md.) and was expressed as g dry extract g^{-1} DPP. According to the Folin-Ciocalteu method (Li et al., 2006), total phenolic yield (TPY) in the extract was expressed as g tannic acid equivalent g^{-1} dry peel, while total phenolic content (TPC) was expressed as g tannic acid equivalent g^{-1} dry extract. To quantify the tannic acid equivalent, a 0.6 mL extract sample was mixed thoroughly with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent and 2 mL of 7.5% (wt/wt) Na_2CO_3 using a vortex mixer (Vortex-Genie 2, Scientific Industries, Bohemia, N.Y.). After 30 min of 25°C incubation of the mixed solution, the absorbance was measured at 760 nm using a UV spectrophotometer (UV-3600 Plus UV-Vis-NIR spectrophotometer, Shimadzu Scientific, Columbia, Md.). For each liquid extract, the tests were conducted in triplicate, and the absorbance was read three times for each sample. A reference blank was prepared using the procedure with DI water rather than liquid extract. TEY, TPY, and TPC were calculated using Eq. 2.2, 2.3, and 2.4, respectively:

$$\text{TEY (\%)} = \frac{W_2}{100W_1} \times 100 \quad (2.2)$$

$$\text{TPY (\%)} = \frac{CV}{100W_1} \times 100 \quad (2.3)$$

$$\text{TPC (\%)} = \frac{CV}{100W_2} \times 100 \quad (2.4)$$

where
W_1 = dry weight of sample (g)
W_2 = dry weight of extract (g)
C = phenolic concentration (g mL^{-1})
V = total volume of liquid extract (mL).

2.3.5.2. Determination of antioxidant activity

Antioxidant activity of the extract was determined as DPPH scavenging activity (DSA, g DPPH equivalent g^{-1}) using Eq. 2.5 (Qu et al., 2014a):

$$\text{DSA} = \frac{nV_t\left[C_c - (C_s - C_b)\right]}{C_t V_t} \quad (2.5)$$

where
C_c = DPPH conc. equivalent in control solution (g L^{-1})
C_s = DPPH conc. equivalent in sample solution (g L^{-1})
C_b = DPPH conc. equivalent in blank solution (g L^{-1})
n = dilution factor of liquid extract.

Liquid extract (60 μL) or DI water (control group) was mixed thoroughly with 3 mL of DPPH solution in methanol (0.05 g L^{-1}) using a vortex mixer and kept in a 25°C water bath for 20 min. Liquid extract (60 μL) was also mixed with 3 mL of methanol and used as a blank solution. Absorbance at 517 nm was noted. Three measurements were conducted for each liquid sample, and each test was replicated three times.

2.3.5.3. Phenolic composition of wet peel extract

The phenolic composition of wet peel extract was determined using a Prominence HPLC system with a photodiode array detector (PAD) (Shimadzu Scientific, Columbia, Md.). Chromatography was achieved using a 4.6 × 100 mm Kinetex 2.6 lm C-18 column (Phenomenex, Torrance, Cal.) equipped with a KrudKatcher Ultra in-line column filter. Instrument control and data acquisition were accomplished using Shimadzu LCsolution software. The HPLC method developed by Qu et al. (2012) was used with slight modification. Two mobile phases, including 0.1% (v/v) H_3PO_4 in HPLC water (A) and 0.1% (v/v) H_3PO_4 in acetonitrile (B), were filtered through Whatman 0.45 μm nylon membrane. The analysis was conducted at a

constant temperature of 30°C using a flow rate of 1.8 mL min^{-1} and a sample injection volume of 10 μL. Detector wavelengths of 270 nm for gallic acid, 254 nm for ellagic acid, and 378 nm for punicalagin α and β were used. The PAD detector was set to scan from 210 to 600 nm. The elution conditions were as follows: isocratic elution 1% B (0 to 4.5 min), linear gradient from 1% B to 4.5% B (4.5 to 9.0 min), isocratic elution 4.5% B (9.0 to 15.0 min), linear gradient from 4.5% B to 7.0% B (15.0 to 19.5 min), isocratic elution 7.0% B (19.5 to 25.5 min), linear gradient from 7.0% B to 25% B (25.5 to 41.19 min), to 90% B (41.19 to 43.17 min), and to 1% B (43.17 to 49.17 min).

Table 2.2 Parameters for HPLC analysis

Fixed Parameters		**Concentration gradient**	
Column	4.6 * 100 mm Kinetex 2.6μm C-18 column	Time (min)	Method
		0-4.5	Isocratic elution 1% B
		4.5-9.0	Linear gradient from 1% B to 4.5% B
Speed, temp	0.6 mL/min, 30 ºC	9.0-15.0	Isocratic elution 4.5% B
Injection	10 μL	15.0-19.5	Linear gradient from 4.5% B to 7.0% B
Phase A	0.1% (v/v) H_3PO_4 in HPLC water	19.5-25.5	Isocratic elution 7.0% B
		25.5-41.19	Linear gradient from 7.0% B to 25% B
Phase B	0.1% (v/v) H_3PO_4 in acetonitrile	41.19-43.17	Linear gradient from 25% B to 90% B
		43.17-49.17	Linear gradient from 90% B to 1% B
Detector	Photodiode array detector		
Responses		**Data Analyses/Outcome**	
Gallic acid	270 nm		
Ellagic acid	254 nm	ANOVA, test for significant differences ($p<0.05$)	
Punicalagin	378 nm		

2.3.6. Statistical analysis

The data were analyzed using ANOVA followed by Duncan's multiple range test in SAS (ver. 9.2., SAS Institute, Cary, N.C.), and significant differences among treatments were determined with a significance of $p < 0.05$. All experiments were replicated three times, and the

mean values are reported. Principal component analysis (PCA) was performed based on the correlation matrix of the values of the characteristics, which meant that the contribution of each variable was independent of the range of its values (Höft et al., 1999). In PCA, observations consisted of the average subsampling results from the 30 aforementioned extraction conditions at different extraction times, temperatures, and solvent ratios. Four vectors (F1 to F4) were estimated based on the eigenvectors of the correlation matrix of four indicators: total extract yield (TEY), total phenolic yield (TPY), total phenolic content (TPC), and DPPH scavenging activity (DSA).

2.4. Results

2.4.1. Effects of cutting heads on WPP particle size

The size distribution of the two groups of ground WPP obtained using two different cutting heads is shown in Figure 2.2. The group with large peel particles (LPP) had an average particle size of 0.60 mm, and the small peel particles (SPP) had an average size of 0.38 mm. The LPP had a moisture content of 70.37% ±0.27%, and the SPP had a moisture content of 70.53% ±0.60%. Two particle sizes (LPP and SPP) were used in the experiments to evaluate the effects of particle size on extraction yield and quality.

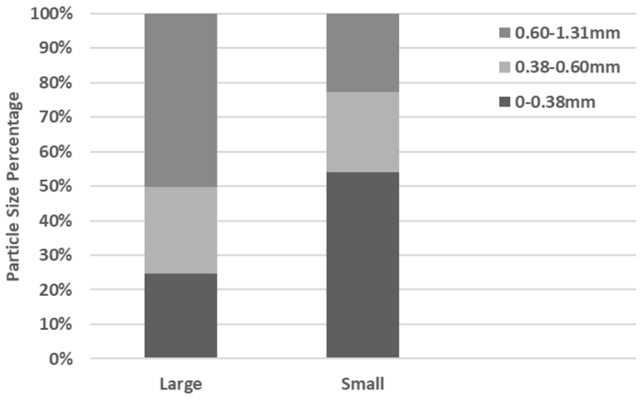


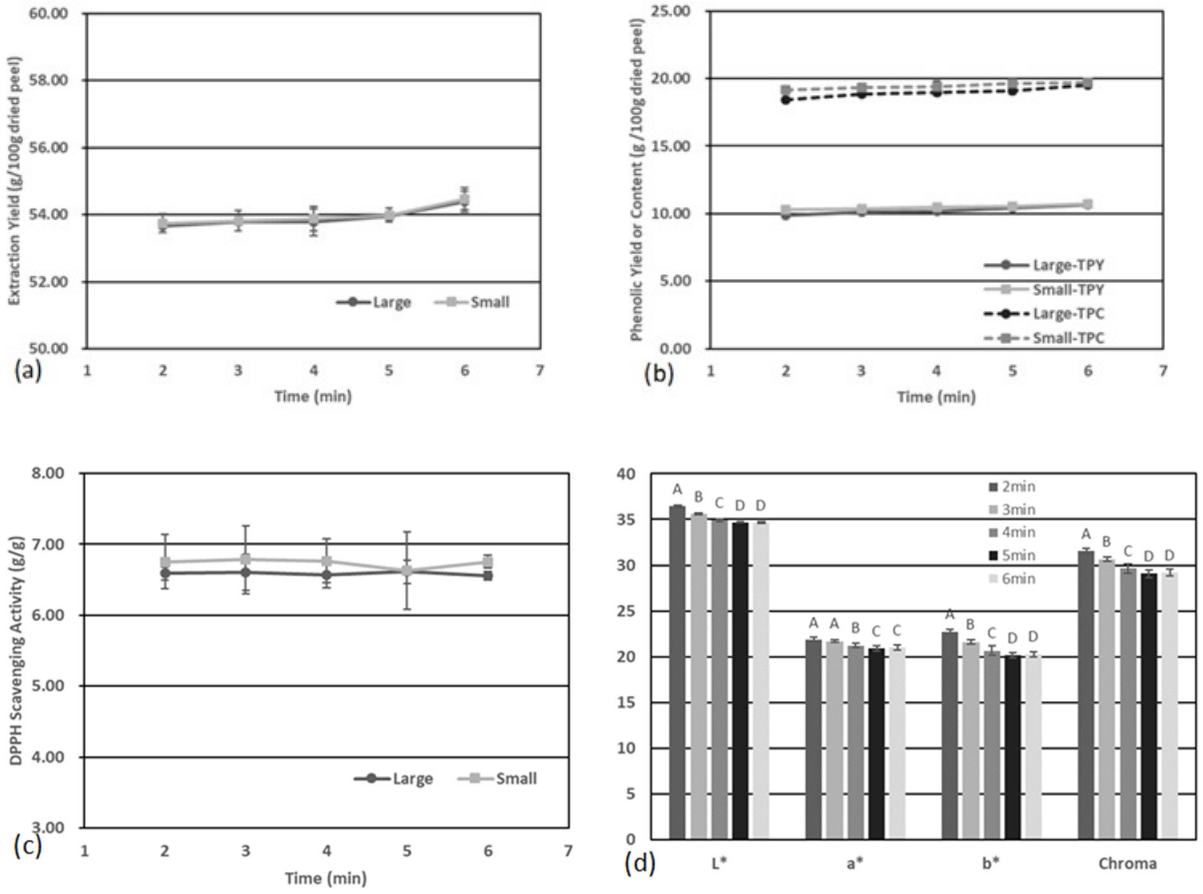
Figure 2.2 Size distribution of peel particles ground by large (0.024 in., ~0.60 mm) and small (0.012 in., 0.38 mm) cutting heads.

2.4.2. Effects of extraction time

The effects of extraction time on the TEY, TPY, TPC, DSA, and color of the extract are shown in Figure 2.3. When the extraction time was extended from 2 to 6 min, the TEY of SPP (Figure 2.3 a) increased slightly from 53.74% to 54.47%. The TPY and TPC (Figure 2.3 b) of SPP ranged from 10.28% to 10.71% and from 19.14% to 19.69%, respectively. The DSA (Figure 2.3 c) of the SPP extract ranged from 6.63 to 6.78 g g^{-1}. The TPY, TPC, and DSA were not significantly different among the extraction times. These results indicate that the extraction of antioxidants reached equilibrium after 2 min at 20°C. These findings are in agreement with the results of Wang et al. (2011), who reported that the TPY was 6.55%, 9.14%, and 11.92% after 2 min of extraction at 25°C, 60°C, and 95°C, respectively. The extraction of LPP resulted in similar TEY, TPY, TPC, and DSA values, and no significant difference in these values was found for extraction with LPP and SPP.

Increasing the extraction time significantly changed ($p<0.05$) the color characteristics of the liquid extracts of both SPP and LPP (figs. 2d to 2f). The lightness (L^*) of the extract, which is related to light transmission, decreased from 34.98 to 33.20. This result is consistent with the

findings of Nour et al. (2015), who reported a negative correlation between TPC and lightness. The chroma (C^*) value, which is used to quantitatively describe the intensity or saturation of the color, decreased from 31.88 to 29.09. Hue ($H°$) is a qualitative attribute representing colors, including red-purple (0°), yellow (90°), bluish-green (180°), and blue (270°). The $H°$ for both peel particle sizes decreased with increasing extraction time, indicating a color shift toward less intense red-purple due to the accumulation of phenolic compounds with increased time. The color characteristics of the SPP extract reached equilibrium after 4 min, while the LPP extract required 5 min. This result proves that the smaller particles had a more shattered cell structure and contained more liquid, which could be more easily extracted. To evaluate the effects of other parameters, an extraction time of 6 min was used in the subsequent experiments.



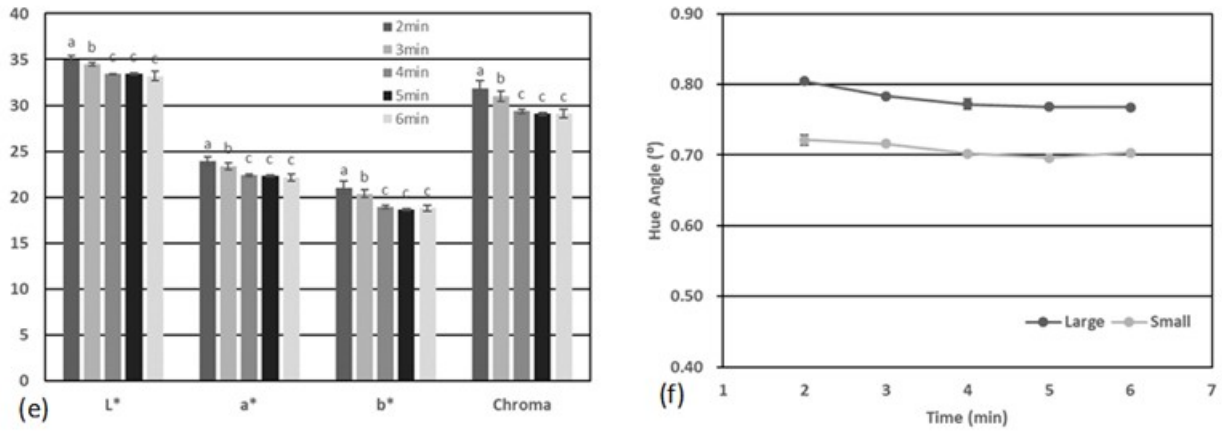
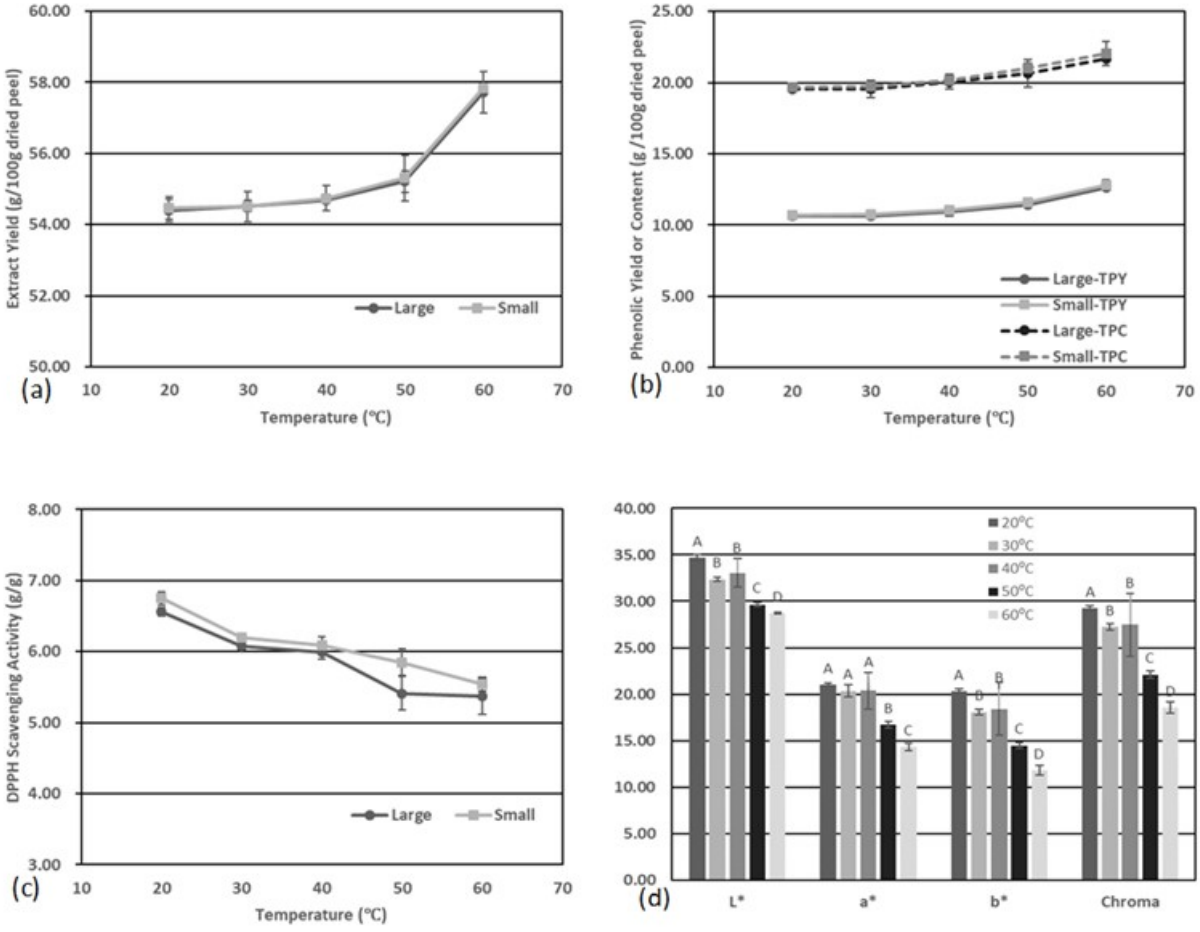


Figure 2.3 (a) Total extract yield, (b) total phenolic yield or content, (c) DPPH scavenging activity, (d and e) *L**, *a**, *b**, and chroma for large particles and small particles, and (f) hue angle of wet peel particle (WPP) extract for 2 to 6 min at 20°C with a solvent (water) ratio of 8:1. The tests are in triplicates. Within groups, the same letter means no significant difference according to LSD (0.05).

2.4.3. Effects of extraction temperature

The effects of extraction temperature on the TEY, TPY, TPC, DSA, and color of the extract are shown inFigure 2.4. As shown in Figure 2.4a, the TEY significantly ($p < 0.001$) increased from 54.47% to 57.83% when the temperature increased from 20°C to 60°C. The TPY and TPC (Figure 2.4 b) followed a similar trend as TEY and significantly increased ($p < 0.001$) from 10.71% to 12.80% and from 19.69% to 22.06%, respectively. This result demonstrated that extraction at higher temperatures promoted the solubility of phenolic compounds and the diffusion coefficient. Wang et al. (2011) reported similar findings and concluded that extraction was sensitive to a temperature increase in the range of 25°C to 95°C. On the contrary, the DSA (Figure 2.4 c) decreased from 6.75 to 5.54 g g^{-1} with the increase in temperature, which was consistent with the findings of Qu et al. (2010), who compared extractions at 25°C, 40°C, 60°C, 80°C, and 95°C and observed a significant drop ($p < 0.05$) in DSA from 6.2 to 3.1 g g^{-1}.

The L^* value decreased from 33.20 to 28.56 with an increase in the extraction temperature. This result agrees with the results of Qu et al. (2014b). The a^*, b^*, and C^* values also decreased as the extraction temperature increased from 20°C to 40°C, but no definite trend was observed when the temperature was above 40°C. The lower values of the color characteristics occurred because most of the phenolic compounds are heat-sensitive. No significant differences in the yield and color parameters were observed for extraction using LPP and SPP with the increase in extraction temperature. Therefore, to obtain a phenolic extract of desirable quality, including DSA and color characteristics, an extraction temperature of 20°C, which required less energy, was applied in the subsequent experiments.



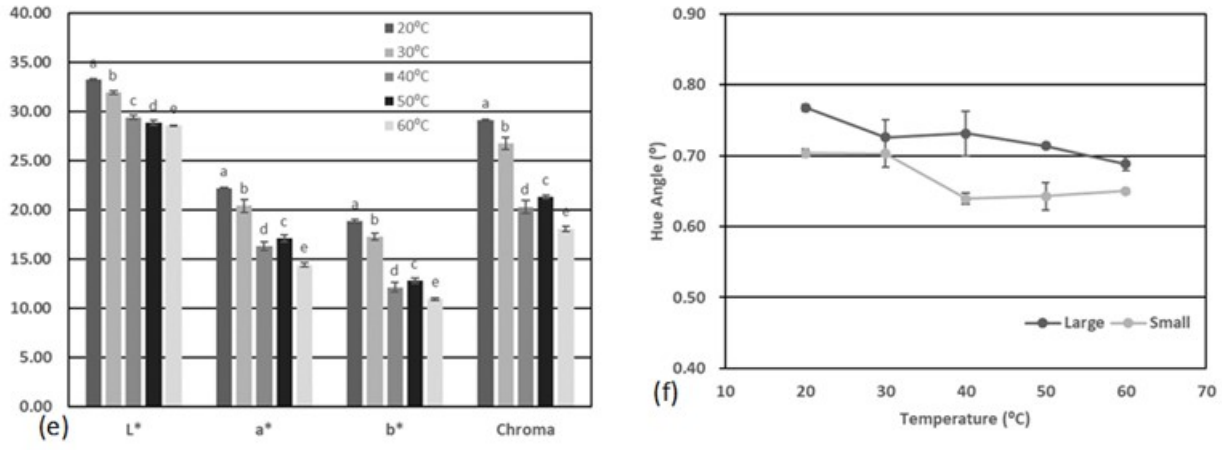


Figure 2.4 (a) Total extract yield, (b) total phenolic yield or content, (c) DPPH scavenging activity, (d and e) *L**, *a**, *b**, and chroma for large particles and small particles, and (f) hue angle of wet peel particle (WPP) extract at 20°C to 60°C for 6 min with a solvent (water) ratio of 8:1. The tests are in triplicates. Within groups, the same letter means no significant difference according to LSD (0.05).

2.4.4. Effects of solvent ratio

The effects of the ratio of solvent (water) to peel particles on the TEY, TPY, TPC, and DSA of the extract are shown in Figure 2.5. When the solvent ratio increased from 1:1 to 4:1, the TEY, TPY, and TPC significantly increased ($p < 0.001$) from 27.53% to 53.20%, from 4.42% to 10.53%, and from 16.06% to 19.53%, respectively. SPP achieved significantly higher TEY, TPY, and phenolic content than LPP, showing that the finer cell structure facilitated mass transfer consistent with Fick's law (Waterman and Sutton, 2003). The DSA was not significantly different between the two particle groups. Laroze et al. (2010) studied the extraction kinetics of polyphenols from raspberry pomace. They observed that small particles (0.15 to 1 mm) resulted in much higher extraction efficiency compared to large particles (1 mm) with methanol extraction at a 20:1 solvent ratio. The reason could be that smaller particles allowed the solvent

to access solutes with less resistance to mass transfer. Smaller particles were also related to more cell breakage, which promoted the release of phytochemicals.

Increasing the solvent ratio to greater than 20:1 resulted in a slightly increased TEY, but it was not significantly different, indicating that equilibrium was reached. A possible reason could be that a higher concentration gradient was built up with the increasing solvent ratio, which increased diffusion from the internal structure, thus increasing the extraction rate. Similar findings were observed for the extraction of phenolics from date seeds (Al-Farsi and Lee, 2008) and dried sage (Durling et al., 2007). The results demonstrated that a higher solvent ratio increased the antioxidant yield and content, with a limited effect on the DSA. The DSA was not significantly different regardless of the solvent ratio and peel particle size at this phase. A higher solvent ratio required more water consumption in the extraction and higher energy use for the concentration of the extract. In conclusion, a solvent ratio of 4:1, which resulted in relatively high TEY and TPY, was considered optimal in terms of solvent usage. Therefore, a solvent ratio at 4:1 is recommended for industrial applications. The LPP had an extraction rate comparable to the SPP, showing that the particle size had no significant effect on the extraction rate.

In summary, when using WPP for antioxidant extraction, higher TEY, TPY, and TPC can be achieved by increasing the extraction temperature, time, and solvent ratio. The DSA was independent of the extraction time and solvent ratio but decreased with the increase in extraction temperature. The two groups of peel particles, with average particle sizes of 0.60 and 0.38 mm, respectively, had no significantly different effects on the extraction of phenolics with varied extraction temperature, time, and solvent ratio. The highest TEY, TPY, and TPC obtained were 57.83%, 12.80%, and 22.06%, respectively. The DSA ranged from 5.37 to 6.35 g g^{-1}. The results indicated that extraction of phenolics from peel particles, produced by grinding to less than 0.6

mm with a large cutting head, at a temperature of 20°C for 6 min using a solvent (water) ratio of 4:1 could be the most economical and sustainable approach for industrial-scale production.

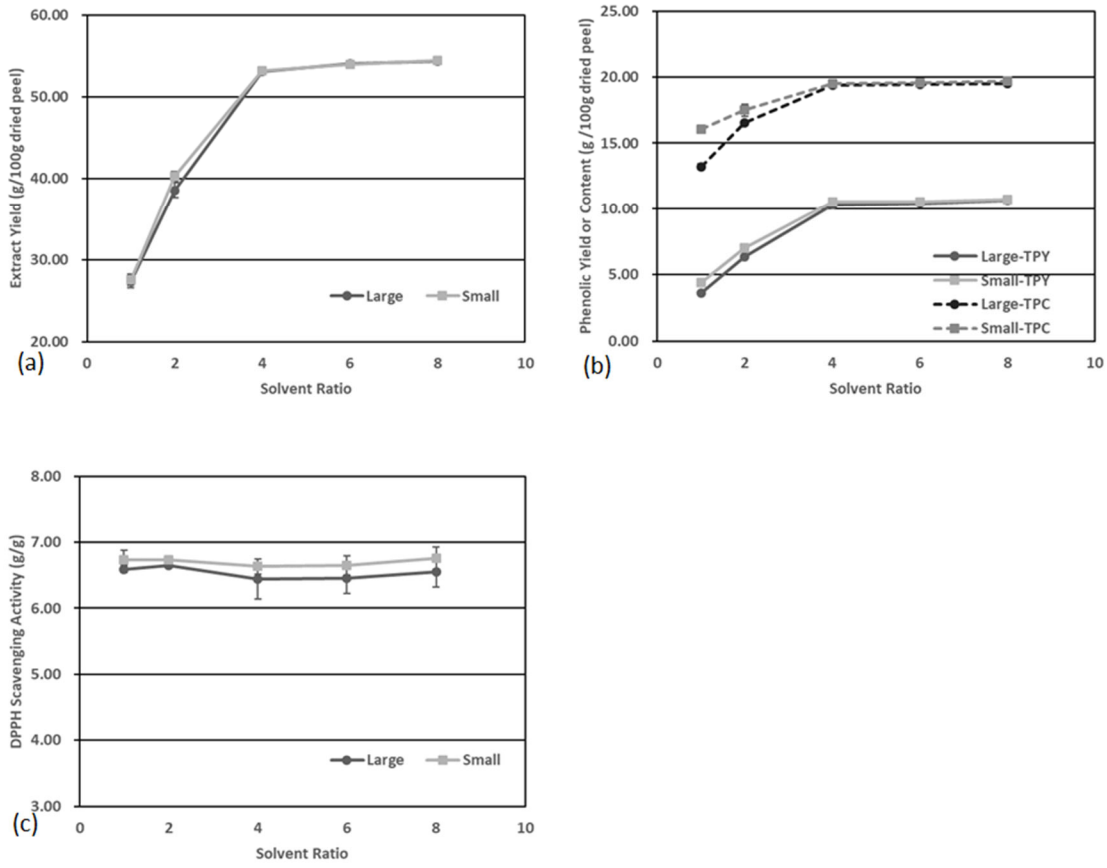


Figure 2.5 (a) Total extract yield, (b) total phenolic yield or content, and (c) DPPH scavenging activity of wet peel particles extracted at 20°C for 6 min with a solvent (water) ratio of 1:1, 2:1, 4:1, 6:1, and 8:1. Triplicates were tested and analyzed. Within groups, the same letter means no significant difference according to LSD (0.05).

2.4.5. Phenolic composition in extract

The effects of extraction conditions on the phenolic composition were investigated for the five groups of extraction conditions shown in Table 2.3. The parameters and standard curves of the HPLC analysis of the extract are shown in Table 2.4.

Table 2.3 Groups of extraction conditions used in HPLC analysis

	G1	G2	G3	G4	G5
Time	6 min	6 min	6 min	6 min	2 min
Temp.	20°C	20°C	20°C	60°C	20°C
Ratio	1:1	4:1	8:1	8:1	8:1

Table 2.4 Parameters and standard curves of HPLC analysis

Standard	Retention Time (min)	λ (nm)	Equation ($y = ax + b$)	Adj-R^2
Gallic acid	2.772	270	$y = 12914.2x + 474352.9$	0.997
Ellagic acid	19.563	254	$y = 32581.9x - 1456409.3$	0.996
Punicalagin α	10.535	378	$y = 1791.3x - 41611.3$	0.995
Punicalagin β	14.613	378	$y = 4335.7x - 66014.9$	0.997

The chromatogram obtained from the HPLC, showing the peaks of major compounds in the peel extract, including gallic acid, ellagic acid, punicalagin α, and punicalagin β, is shown in Figure 2.5 a. The contents of the four major phenolic compounds produced by the five groups of extraction conditions (G1 to G5) are shown in Figure 2.5 b. The gallic acid content significantly improved, from 0.17 to 0.29 mg g^{-1} ($p < 0.05$), with the increase in solvent ratio from 1:1 to 4:1. Except for the group with a 1:1 solvent ratio (G1), the gallic acid contents obtained with the other groups with different solvent ratios varied from 0.27 to 0.35 mg g^{-1} and were not significantly different, regardless of the time and temperature. The ellagic acid content varied from 0.77 to 1.51 mg g^{-1}, with no significant differences among groups G3, G4, and G5. The solvent ratio mostly influenced punicalagin α. At the same extraction conditions, the punicalagin α content increased from 2.13 to 4.48 mg g^{-1} when the solvent ratio increased from 1:1 to 8:1. In addition to the solvent ratio (G1: 3.61 mg g^{-1}; G3: 8.28 mg g^{-1}), the extraction time and temperature affected the extraction of punicalagin β, which can be seen from the increased

punicalagin β content from 7.51 mg g^{-1} (G5) to 9.17 mg g^{-1} (G4). The punicalagin purity ranged in order of G2 > G4 > G5 > G3 > G1, and the corresponding values were 88.99%, 88.27%, 87.73%, 87.64%, and 85.93%, respectively.

Qu et al. (2012) compared the gallic acid, punicalagin α, punicalagin β, and ellagic acid concentrations of different pomegranate products. The results presented in this study showed significantly higher retention of punicalagin than the pomegranate peel extract using dried peel particles (50:1 solvent ratio, 25°C, 90 min) used by Qu et al. (2012). The WPP extraction in this study also achieved slightly higher phenolic concentrations compared to Langers 100% pomegranate juice.

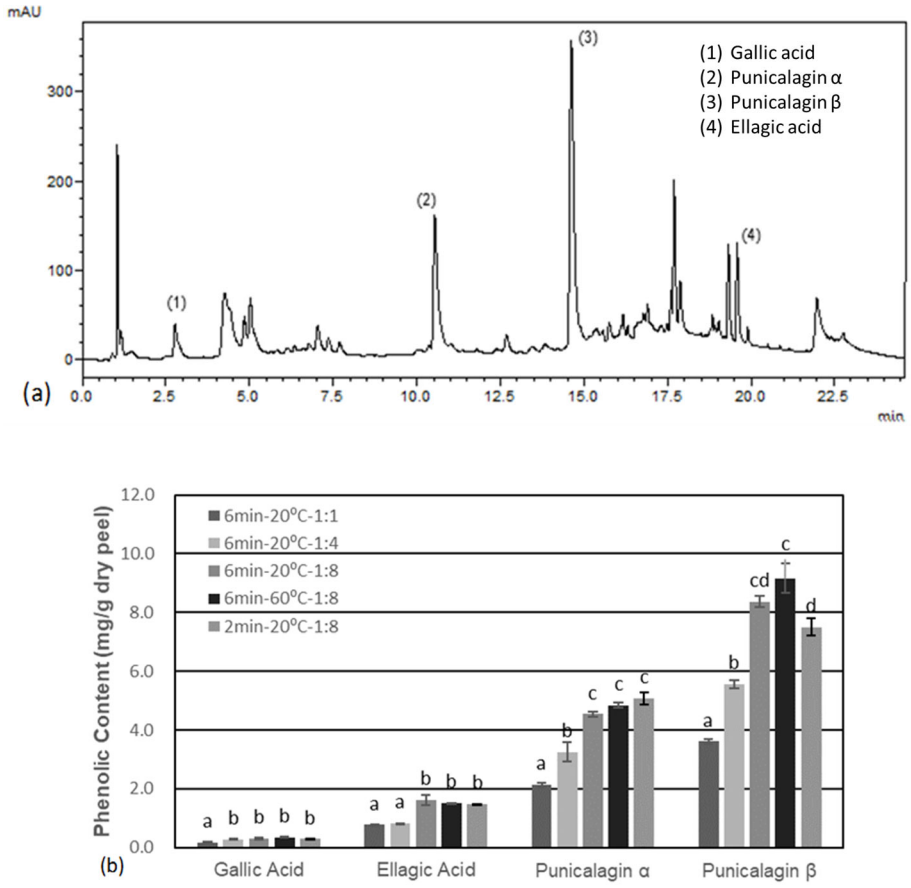


Figure 2.5 (a) HPLC analysis of pomegranate peel extract and (b) Composition of 4 major phenolic compounds in pomegranate peel extract. The tests were in triplicates. Within groups, the same letter means no significant difference according to LSD (0.05).

2.4.6. Composition, phenolic content, and quality of wet and dried pomegranate peel

To study the effects of drying, DPP was produced using HA drying and IR drying, and the peel compositions and phenolic extraction conditions were compared between DPP and WPP. The compositions of WPP and DPP on a dry basis are shown in Table 2.5 Composition of fresh and hot-air dried pomegranate peel. (g/100g, d.b). The WPP had higher contents of protein, ash, and crude fat than the DPP. The loss of these contents was reduced by avoiding the drying process. On the other hand, the HA DPP maintained higher total dietary fiber (TDF) at 25.44%, and the ratio of insoluble dietary fiber to soluble dietary fiber (IDF/SDF) was 8.84. This was higher than the values for WPP, which maintained 17.19% TDF and an IDF/SDF ratio of 4.93. Morais et al. (2016) compared the compositions of raw, freeze-dried, and oven-dried papaya peels. They reported similar findings for the differences in fiber content, but the differences were not statistically significant.

Table 2.5 Composition of fresh and hot-air dried pomegranate peel. (g/100g, d.b)

	DM	Protein	Ash	Crude Fat-Rinse	Carbohydrate	Total Dietary Fiber	Insoluble Dietary Fiber	Soluble Dietary Fiber
Fresh WPP	26.30 ±0.40	4.60 ±0.20	4.75 ±0.51	0.71 ±0.27	89.94 ±0.67	17.19	15.47	1.75
Hot-air DPP	82.23 ±0.06	4.43 ±0.06	4.18 ±0.12	0.56 ±0.03	90.83 ±0.19	25.44	21.16	4.28

Figure 2.6 shows the TEY, TPY, DSA, and color characteristics of IR DPP, HA DPP, and WPP. The DPP resulted in significantly lower TEY, TPY, and TPC than the WPP. The DSA values were similar for both DPP and WPP at about 6.41 g g^{-1}. In other words, extraction from WPP resulted in 10% more extract yield and 2.5% more TPY with similar DSA compared to DPP. Loizzo et al. (2016) investigated the phytochemical contents of extracts from fresh and processed peel and pulp. Compared to steamed, baked, and microwaved pulp, extraction with fresh pulp achieved up to 0.9% higher TEY and twice the TPC. Their results demonstrated that

extraction with fresh peel or pulp could reduce the phytochemical loss that occurs during the drying process. Similarly, Mphahlele et al. (2016) compared the bioactive compounds in fresh peel and hot-air dried peel at 40°C, 50°C, and 60°C.

As for color, extraction of WPP achieved significantly higher *L** (6.08%), *a** (38.71%), *b** (23.81%), and *C** (30.86%) values. The results demonstrated statistically higher retention of *L** (29.83%) and *a** (18.58%) values using WPP. According to Cadena et al. (2013), changed color characteristics indicate the formation of caramel-colored pigments resulting from nonenzymatic processes, which are related to lower sensory acceptance. Therefore, extraction from WPP could be a more suitable method for the extraction of polyphenols for use in food product development and supplementation.

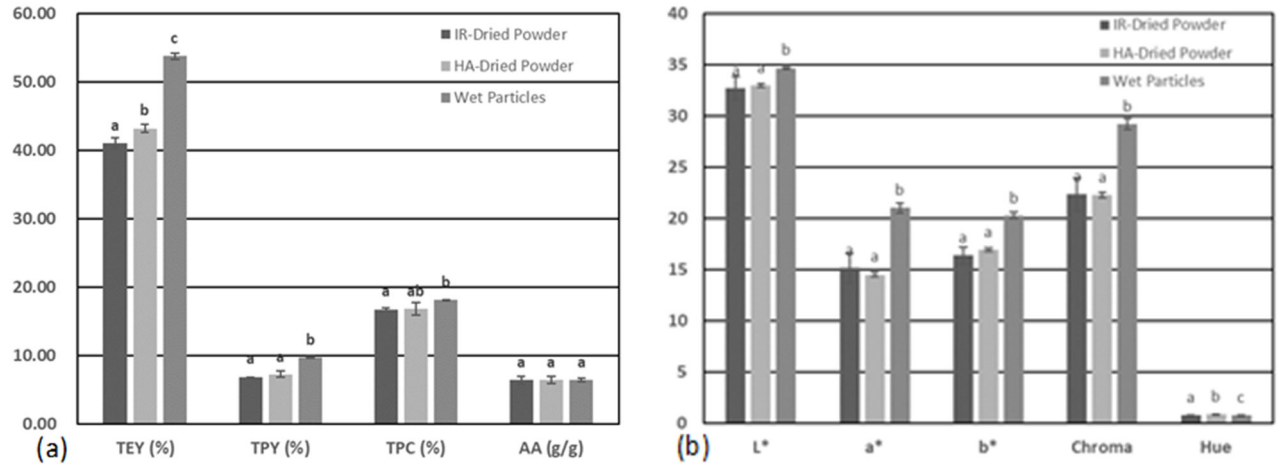


Figure 2.6 (a) Total extract yield (TEY), total phenolic yield (TPY), total phenolic content (TPC), and DPPH scavenging activity (AA), and (b) color characteristics of extracts from wet pomegranate peel, infrared (IR) and hot-air (HA) dried pomegranate peel. Extractions were performed at 20°C for 6 min with a solvent (water) ratio of 4:1. The tests are in triplicates.

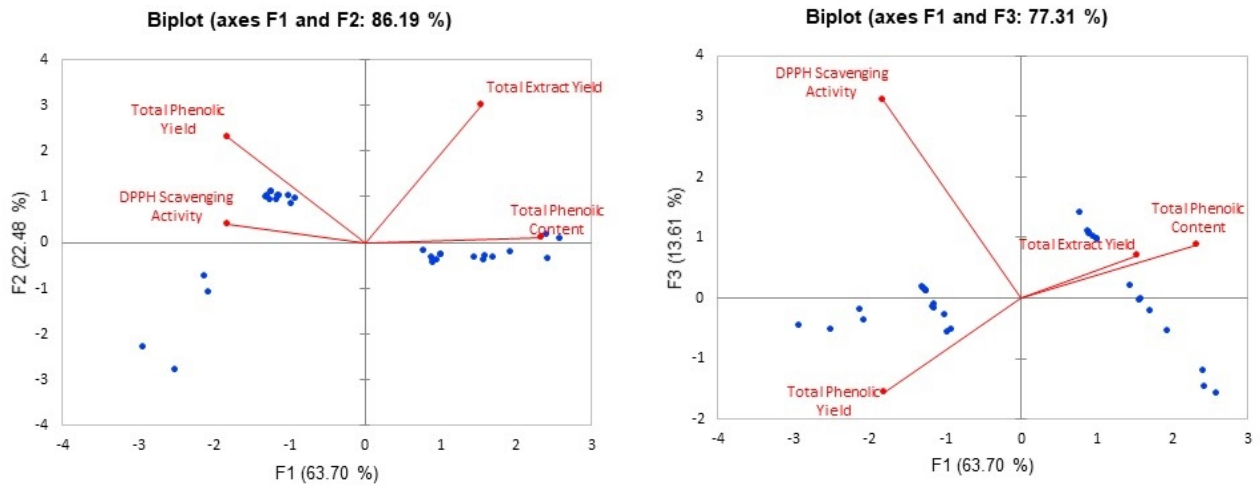
2.4.7. Principal component analysis

PCA was applied to explore the interdependence among variables. Observations consisted of the average subsampling results from the 30 aforementioned extraction conditions at different extraction times, temperatures, and solvent ratios. Four vectors (F1 to F4) were

estimated based on the eigenvectors of the correlation matrix of four variables: total extract yield (TEY), total phenolic yield (TPY), total phenolic content (TPC), and DPPH scavenging activity (DSA). The eigenvalues of F1 to F4 were 2.548, 0.899, 0.544, and 0.008, and the first three PCs accounted for 63.70%, 22.48%, and 13.61% of the sample variance, respectively, and represented 99.79% of the total variance in cumulation (Table 2.4). Biplots of the observations and variables (Figure 2.7) show the data distributions of F1-F2 and F1-F3. Confidence ellipses with 99% confidence intervals were used for each set of experiment groups, and no outliers were detected. PCA weighting scores of the four measured variables (TEY, TPY, TPC, and DSA) are listed in Table 2.6. Higher scores (absolute values) indicate a closer relationship between the variable and the factor. For instance, TEY and TPC were positively correlated with the F1 axis, whereas TPY and DSA were negatively correlated. In addition, TEY and TPY were positively correlated with the F2 axis, while DSA was negatively correlated with the F3 axis. Figure 2.7 also shows the data distribution for F1-F2 and F1-F3. For instance, the data point with a higher F1-axis value had higher TPC, which was extracted at 60°C for 6 min using a solvent ratio of 8:1. This was following the experimental results, indicating that PCA can be applied for future condition prediction.

Table 2.6 Factor loadings for interpretation of Figure 2.7

Variable	F1	F2	F3	F4
Total extract yield (TEY)	0.649	0.747	0.138	-0.039
Total phenolic yield (TPY)	-0.760	0.574	-0.302	0.045
Total phenolic content (TPC)	0.982	0.030	0.171	0.069
DPPH scavenging activity (DSA)	-0.764	0.102	-0.636	0.011
Eigenvalue	2.548	0.899	0.544	0.008
Explained variance	63.70	22.48	13.61	0.21



(a) (b)

Figure 2.7 Biplots of objects and vectors for (a) factors F1 and F2 and (b) factors F1 and F3 generated from correlation matrix PCA.

2.5. Conclusions

Extraction of bioactive compounds from waste fruit peel is an efficient approach to improve food system sustainability and industry profitability. This study developed a novel green process for antioxidant extraction from wet pomegranate peel (WPP) and investigated the effects of extraction conditions on polyphenol yield and quality, including phenolic composition, DPPH scavenging activity, and color characteristics. PCA condensed the multivariable analysis into three factors, which explained 99.79% of the variance and could be suitable for future process development. Three parameters, including drying preparation, extraction temperature, and solvent (water) ratio, significantly influenced the extraction rate. Considering water usage and energy consumption, WPP extraction at 20°C for 6 min with a solvent ratio of 4:1 is recommended as an economic and sustainable process, resulting in 10.53% total phenolic yield with 88.93% punicalagin purity.

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Chapter 3 - Plasma and Hepatic Cholesterol-lowering Effects of Pomegranate Peel and Extract

3.1. Introduction

Overweight is raising concern worldwide due to its high prevalence and various adverse health outcomes. According to a report from World Health Organization, an adult with Body Mass Index (BMI) between 25.0 to 30.0 is defined as overweight, and over 1.9 billion adults worldwide were overweight in 2016, accounting for 39% of the population (World Health Organization, 2018). Being overweight can further induce obesity when BMI increased beyond 30.0. These two statuses are major risk factors of physical and mental illness. Wilson et al. (2002) conducted 44-year follow-up research on 5209 participants aged 30 to 62 years from the Framingham cohort (Wilson et al., 2002). Results showed that being overweight was related to elevated cardiovascular risks, including hypertension (>26%), angina pectoris (>22%), and coronary heart disease (>15%). Calle and Kaaks reviewed obesity and obese-related epidemiological studies. An increased risk of cancers was noted from 1.2-2 folds, including colorectal, endometrial, kidney, and oesophageal cancer (Calle & Kaaks, 2004). BeLue et al. studied the relation between mental healthiness and overweight in youth aged 12 to years in different races and ethnicity (BeLue et al., 2009). Their results revealed that overweight white and Hispanic youth possessed a higher percentage of self-reported depression, anxiety, and other mental and behavioral problems.

Various causes contributed to being overweight. Among them, a calorie-dense and nutrient-poor diet is a major contributor along with a lack of exercise. Standard American Diet (SAD) is a typical western diet that includes excess natural and added carbohydrates, fats, and

sodium while lacking in consumption of fruits, vegetables, and whole grains (Grotto & Zied, 2010). Improving the diet pattern for weight control is in need.

Diet with the addition of phytochemicals, such as polyphenol, demonstrated positive health outcomes on weight management. Studies have suggested that polyphenol modulated the plasma and hepatic cholesterol in a few possible mechanisms, including (1) inhibiting enzymes related to intestinal carbohydrate digestion and glucose absorption (McDougall et al., 2005), (2) inhibiting pancreatic lipase activity and fat absorption from the intestine (Lei et al., 2007), (3) promoting β-oxidation of fatty acids (Hontecillas et al., 2009), (4) increasing bile excretion to eliminate the cholesterol (Saénz Rodriguez et al., 2002), and (5) regulating the gut microbiota towards a leaner composition (Most et al., 2017; Neyrinck et al., 2013).

Pomegranate peel is a common underused fruit by-product from the juicing process and consists of up to 53.01% of the fruit weight (Teh, 2016). The high molecular weight polyphenols in the pomegranate peel are the major high-value phytochemicals and have been proven associated with reduced risks of chronic diseases (Heber, 2011), including type 2 diabetes (Banihani et al., 2013) and cardiovascular diseases (Hamoud et al., 2014). Among all the polyphenols in pomegranate peel, gallic acid (monomer), ellagic acid (dimer), punicic acid, and punicalagin-α, -β are responsible for most health benefits (Aviram et al., 2008; Pai et al., 2011). It was worth noting that punicalagin is unique in pomegranate peel and demonstrated the greatest antioxidant activities with abundant hydroxyl groups, which can trap peroxyl radicals to reduce oxidation (Fischer et al., 2011). Therefore, pomegranate peel is a promising source for polyphenols.

By far, nearly all the studies of health benefits in pomegranate peel utilized liquid extractable polyphenol. Limited research focused on the pomegranate peel as a whole. Labib and Hossin characterized the effects of pomegranate peel powders (PPP) and extracts (PPE) on obese hypercholesterolemic rats solely from anthropometric and serum lipid. Insights of hepatic lipid profile were missing and the regulating mechanisms were not investigated in their study (Labib & Hossin, n.d.). Moreover, research has shown that the polyphenol and fiber in the food matrix might have a synergistic effect to promote health (Aprikian et al., 2003). Therefore, the objectives of this research were to compare the hypolipidemic properties of PPP and PPE and investigate the regulating mechanisms by supplementing the high-fat diets with different percentages of PPP and PPE to male Syrian hamsters.

3.2. Experimental section

3.2.1. Preparation of pomegranate peel particles and extract

Pomegranate peel of Wonderful variety was collected from a juicing plant located in Buttonwillow, California in October of 2017. The peel was processed based on Wu et al. (2021). In summary, the peel was sliced and ground into particles less than 0.6 mm. Then extract was obtained by mixing the peel particles with 4 times of water at 20 ºC for 6 mins, then filtered before administration to the hamsters. The male Syrian hamster was used since it possessed similarities of hepatic cholesterol and bile acid metabolism with humans compared to other rodents (Zhang et al., 2009). The compositions of peel powder and extract were listed in Table 2.5.

3.2.2. Administration detail

The study was approved by the Animal Care and Use Committee, Western Regional Research Center, USDA, Albany, CA, USA. 45 male golden hamsters (LVG strain, Charles River Laboratories, Hollister, CA) were acclimatized for 2 weeks. They were fed with Purina Rodent Laboratory Chow and individually raised in a 20−22 °C environment with relative humidity at 60 %, and 12 h alternating light/dark cycle. After that, 45 hamsters were randomly divided into five groups (n = 9 per group) for each diet as indicated in. A high-fat (HF) diet with 20% fat energy intake was set as a control group. 5 and 10% of lyophilized PPP (Low Peel/High Peel, LP/HP) were supplemented into the HF diet *ad libitum* to evaluate the dose effects. In correspondence, 2.5 and 5% of lyophilized PPE (Low Extract/ High Extract, LE/HE) addition were applied to investigate the effect of supplementation form. In this way, LP/LE group contained nearly 8.68 mg soluble phenolic compounds per kg body weight, and HP/HE group doubled the content. These dosages corresponded to approximately 70 mg (LP/LE) and 140 mg (HP/HE) per day in a 60 kg human according to the *Km* factor ratio of 5 and 37 for hamsters (80 g) and humans (60 kg), respectively (Nair & Jacob, 2016). Table 3.1 summarized the diet composition.

During 4 weeks of feeding, fresh food was provided to hamsters weekly. Their body weight and food intake were recorded weekly. Food efficacy, known as the weight gain per gram of diet consumption was calculated. At the end of the 4th week, hamsters were fasted for 14 h, anesthetized using isoflurane (Phoenix Pharmaceutical, St. Joseph, MO, USA), and sacrificed based on the procedures in the previous study (Kim et al., 2014). 5 mL blood was drained by cardiac puncture into EDTA rinsed syringes, then centrifuged at 2000 g, 4 °C for 15 mins to collect plasma and stored at -80 ºC. The liver, kidney, and epididymal adipose were excised,

weighed, and frozen in liquid nitrogen then stored at -80ºC. Feces were collected during the last day of feeding and stored at -20 ºC.

Table 3.1 Composition of different diets containing pomegranate peel and extract

Ingredients	High Fat Control	Low Peel (5%)	High Peel (10%)	Low Extract (2.5%)	High Extract (5%)
Fat 20%					
Butter	240.0	240.0	240.0	240.0	240.0
Corn Oil	300.0	300.0	300.0	300.0	300.0
Fish Oil	60.0	60.0	60.0	60.0	60.0
Cholesterol*	3.0	3.0	3.0	3.0	3.0
Pomegranate Peel	-	150.0	300.0	-	-
Pomegranate Extract	-	-	-	78.9	157.9
Fiber, 10%					
Cellulose (0.95 solids)	315.8	157.9	-	189.5	126.3
Protein 20%					
Casein (91.5% M.C)	658.3	658.3	658.3	658.3	658.3
Starch, Balance					
Corn Starch (90% M.C)	1493.3	1493.3	1493.3	1543.3	1526.7
Other:					
DL Methionine/Cystine	9.0	9.0	9.0	9.0	9.0
Choline Bitartrate	9.0	9.0	9.0	9.0	9.0
Mineral Mix	105.0	105.0	105.0	105.0	105.0
Vitamin Mix	30.0	30.0	30.0	30.0	30.0
Total Weight (g)	3223.4	3215.5	3207.6	3226.0	3225.2

3.2.3. Plasma, hepatic and fecal lipid analysis

Plasma lipoproteins, including very-low-density lipoprotein (VLDL)-, low-density lipoprotein (LDL)-, and high-density lipoprotein (HDL)- cholesterol, were determined using size-exclusion chromatography as described (Teh et al., 2019). Specifically, A cholesterol reagent (Roche Diagnostics, Indianapolis, IN, USA) was delivered using Hewlett-Packard (Agilent, Palo Alto, CA, USA) HPLC pump 79851-A at a flow rate of 0.2 mL/min. Lipoproteins were separated by injecting 15 µL of plasma onto the Superose 6HR HPLC column (Pharmacia

LKB Biotechnology, Piscataway, NJ, USA) in Agilent 1100 HPLC chromatography system. Then the lipoproteins were eluted with a solution containing 0.15 M NaCl and 0.02% sodium azide at a flow rate of 0.5 mL/min. Base signals of the peak areas were calibrated by applying bovine cholesterol lipoprotein standards (Sigma-Aldrich, St. Louis, MO). Total cholesterols (TC) in plasma were counted as the sum of the VLDL-, LDL-, and HDL-cholesterol concentrations, interpolating from the standard curve. Plasma triglyceride (TG) was quantified using an enzyme colorimetric assay kit (Sekisui Diagnostics PEI Inc., PE, Canada) based on the absorbance at 505 nm (Nanodrop 2000C spectrophotometer, Thermo Scientific, USA).

The liver and feces samples were ground in mortar and pestle, then the lipid was extracted with hexane/isopropanol (3:2, v/v) at 60 °C using Dionex ASE 350 accelerated solvent extractor (Thermo Scientific, Sunnyvale, CA). The lipid extracts were evaporated to dryness at 37 °C under nitrogen. Total hepatic and fecal lipid contents were calculated based on the weighed extracted lipid over liver weight. Hepatic lipids were re-dissolved in hexane/isopropanol solvent and mixed with Triton X-100. After drying and diluting the mixture in DI water, total cholesterol (TC) and free cholesterol (FC) were determined with enzymatic kits (Wako Chemicals, Richmond, VA), then esterified cholesterol (EC) was calculated based on the difference between TC and FC. Liver TG was measured using the same method for plasma TG.

3.2.4. Fecal microbiota analysis

The fecal microbiota was analyzed as described in our previous research (Kim et al., 2015) using next-generation sequencing (NGS) at the Genome Center Core Laboratory, University of California, Davis (Davis, CA, USA). Fecal microbiota was studied using real-time qPCR of feces (Xia et al., 2018). The DNA was amplified using the primers F319 (5′-

ACTCCTACGGGAGGCAGCAGT-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′), which targeted the V3 and V4 regions of bacterial 16S rRNA. Sequencing data corresponding to 300PE (55 hrs, 13.2 – 15 Gb per flowcell) was pre-processed using the MiSeq protocol and QiiME. Then the amplicon pools were assessed with Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). DADA2 and Phyloseq (Callahan et al., 2016; McMurdie & Holmes, 2013) were applied in data trimming, filtering, and visualization.

3.2.5. Hepatic gene expression using real-time PCR

Hepatic RNA was extracted using TRIzol and RNA purification kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Then GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) was applied to synthesize cDNA, which was further diluted 10 times with dH_2O. 1 μL of diluted cDNA of each liver sample underwent RT-PCR using SYBR green supermix (Bio-Rad, Hercules, CA, USA) with an Mx3000P instrument (Agilent, Cedar Creek, TX, USA). Cycle conditions were as follows: 95 °C for 5 min, 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Primers used for this study were shown in Table 3.2 (Bartley et al., 2010; Field et al., 2003; Kim et al., 2012). They were validated by PCR product sizes and no primer dimers were observed in gel electrophoresis of PCR products. Differences in hepatic mRNA expression were calculated after normalization to 36B4 mRNA expression.

Table 3.2 Sequences of PCR primers

Gene	Product Size (bp)	Primer pair	5′ primer sequence 3′
CYP51	195	forward	GAGAGAAGTTTGCCTATGTGCC
		reverse	TGTAACGGATTACTGGGTTTTCT
CYP7A1	154	forward	ACTGCTAAGGAGGATTTCACTCT
		reverse	CTCATCCAGGTATCGATCATATT
HMG-CoAR	132	forward	CAAGTGGTCCCACGAATGAAGAC
		reverse	ACGCTCCTTGAACACCTAGCATC
LDLR	128	forward	TGAGGAACATCAACAGCATAAAC

		reverse	ATCCTCCAGGCTGACCATCTGT
PPAR*a*	133	forward	CTCCACCTGCAGAGCAACCA
		reverse	CGTCAGACTCGGTCTTCTTGAT
SCD1	127	forward	GCCACCTGGCTGGTGAACAGTG
		reverse	GGTGGTAGTTGTGGAAGCCCTCG
SREBP-1c	93	forward	ATGAGCTGGAGCATGTCTTCAAA
		reverse	AGCTGGCAAATCAGAAAAACAAG

3.2.6. Statistical analysis

All data were expressed as means ± SE. Multiple comparisons of treatments were analyzed using the one-way analysis of variance (ANOVA) coupled with Duncan's multiple range test using the 2016 SAS (version 9.4, SAS Inc., Cary, NV, USA). Statistical analysis of differences in microbiome and RT-PCR were done by two-tailed student's t-test using the 2016 SAS. Pearson correlation coefficients were calculated in MATLAB 2019b version to detect the association of hepatic gene expression and types of bacteria with the hepatic cholesterol and physiological parameters.

3.3. Results and discussion

3.3.1. Body weights of hamsters

The body weight gains over 4-weeks of feeding were shown in Figure 3.1. All the diets elevated the total weight gain ranging from 18.14 to 33.82g, in order of HP, HF, HE, LP, and LE (Figure 3.1). The corresponding feed efficacy ratio was from 0.11 to 0.18 g/g. In both total weight gain and feed efficacy ratio, the HP diet was significantly lower while the LE diet was significantly higher compared to HF and HP diets ($p<0.05$). Possible reasons could be that the high fiber content in peel promoted earlier satiety (Marlett et al., 2002), reduced the digestion and absorption of lipid content. At the same time, liquid extract in LE and HE diets tended to

dissolve more reducing sugars from the pomegranate peel. Therefore, it promoted absorption efficiency and further weight gain. Sangüesa et al (2017) investigated the different adverse effects of glucose and fructose on the metabolism of female rats and observed that fructose had a greater impact on metabolic dysfunction (Sangüesa et al., 2016). The carbohydrate composition of the extract and peel could be examined in the future for a better understanding of the mechanisms.

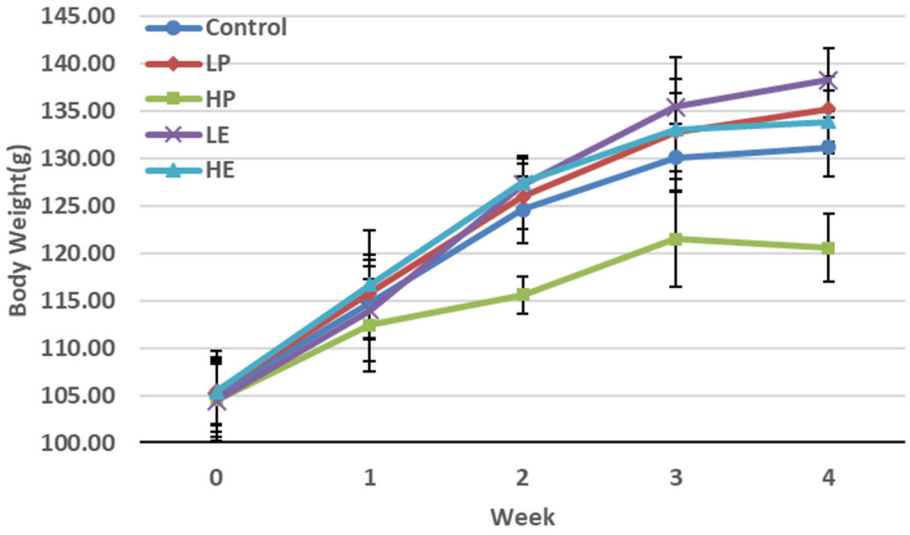


Figure 3.1 Bodyweight gains of hamsters over 4 weeks of feeding

As shown in Table 3.3. Compared to the HF diet, HP and HE diets reduced the liver weights by 22.0% ($p<0.05$) and 9.0% ($p>0.05$), while LP and LE diets increased that ($p>0.05$) by 2.1% and 11.9%, respectively. The corresponding liver to body weight ratio was 3.10±0.08, 3.25±0.12, 3.61±0.15, 3.65±0.09, and 3.76±0.16. HP and HE diets significantly lowered the liver to body weight ratio, indicating a less lipid accumulation in the liver under these two diets. Adipose weights exhibited an increasing trend in HP, HF, LE, LP, and HE, with the values at 1.15±0.05, 1.40±0.10, 1.48±0.13, 1.53±0.12, and 1.54±0.10 g. At the same time, the corresponding adipose to body weight ratios were 0.96±0.04, 1.06±0.06, 1.05±0.06, 1.12±0.05,

and 1.15±0.06. HP diet achieved significantly lowered adipose to body weight ratio while the other four diets were not statistically different. It is in line with research from Lei et al., (2007) who observed that pomegranate leaf extract at the dose of 800 or 400 mg/kg induced genital/perirenal/inguinal adipose pads of CD-1 mice under a high diet (incorporated with 35% lipid, including 15% saturated fat and 20% unsaturated fat). It indicated that the HP diet reduced fat storage in hamsters.

Table 3.3 Diet consumption, body weight gain, feed efficacy, organ weight, and lipid contents of hamsters feed with different diets supplemented with pomegranate peel and extract

Diets	HF	LP	HP	LE	HE
Consumed diet (g)	174.73±3.05	180.13±7.23	169.88±8.02	184.05±6.85	177.79±4.28
Weight gain (g)	26.10±2.14^{a}	29.74±2.44^{ab}	18.14±2.40^{c}	33.82±2.51^{b}	28.48±2.06^{ab}
Feed efficiency (g gain/g diet)	0.15±0.01^{a}	0.16±0.01^{ab}	0.11±0.01^{c}	0.18±0.01^{b}	0.16±0.01^{ab}
Liver (g)	4.80±0.23^{bc}	4.90±0.35^{bc}	3.75±0.14^{a}	5.24±0.38^{c}	4.37±0.26^{ab}
Adipose (g)	1.40±1.53^{ab}	1.53±0.12^{b}	1.15±0.05^{a}	1.48±0.13^{b}	1.54±0.10^{b}
Kidney (g)	0.48±0.01	0.49±0.01	0.50±0.02	0.50±0.02	0.50±0.01
Liver/body (%)	3.65±0.09^{b}	3.61±0.15^{b}	3.10±0.08^{a}	3.76±0.16^{b}	3.25±0.12^{a}
Adipose/body (%)	1.06±0.06^{ab}	1.12±0.05^{ab}	0.96±0.04^{a}	1.05±0.06^{ab}	1.15±0.06^{b}
Blood glucose (mg/dL)	82.94±6.49^{c}	73.81±6.41^{b}	68.11±5.27^{a}	82.39±3.30^{c}	68.00±4.51^{a}
Hepatic lipid (g/100g liver)	7.49±0.22^{a}	6.98±0.23^{ab}	6.31±0.26^{bc}	7.58±0.37^{a}	5.94±0.26^{c}
Fecal lipid (g/100g feces)	1.55±0.30^{cd}	1.01±0.23^{b}	0.57±0.20^{a}	1.19±0.13^{bc}	1.72±0.22^{d}

* Within rows, means followed by the same letter are not significantly different ($p>0.05$).

3.3.2. Lipoprotein and triglycerides in plasma and liver

Among the 5 diet groups, the plasma TG content varied from 68.59 to 167.29 mg/dL, 2.60%, 44.37%, and 16.78% of the decrease in TG was observed in LP, HP, and HE diets, while the LE diet increased the TG by 35.67%. VLDL ranged from 20.42 to 36.57 mg/dL. Compared with the control HF diet, LP, HP, and HE diets lowered the VLDL by 12.7%, 28.9%, and 20.5%, indicating a potential amelioration on the plasma lipid profile. On the contrary, the LE diet increased the VLDL by 27.3%. Plasma LDL ranged from 35.63 to 93.43 mg/dL, surprisingly LP, HP, LE, and HE diets increased LDL content by 83.3%, 162.2%, 44.0%, and 61.5% relative to the HF diet. Plasma HDL was from 111.72 to 134.41 mg/dL, with a reduction of 11.9%, 13.5%, 11.8%, and 16.9% in LP, HP, LE, and HE diets. The ratio of LDL/HDL ratio ranged from 0.28 to 0.89, correspondingly. Most previous research on hypolipidemic effects of pomegranate demonstrated the LDL-lowering effects of PPP and PPE (Abdel-Rahim et al., 2013; El-Hadary & Ramadan, 2019; Esmael et al., 2015; Labib & Hossin, n.d.; Sadeghipour et al., 2014) Surprisingly apparent adverse LDL-elevating effects were observed in this study, which could be ascribed to potential toxicity to the liver. Chuang et al. (2011) investigated the hypolipidemic effects of different angiocarp parts of *Alpinia zerumbet* and observed the same adverse effects when feeding the Syrian hamsters with 5% *Alpinia* seed powder and oil, which means optimum safe dosage should be lower than 5%. Theoretically, PPE ingestion at the concentration up to 2000 mg/kg body weight (In the present study: 8.68 mg/kg) should be considered safe (Das & Singh, 2014; Joseph et al., 2013). Therefore, for this wonderful variety of PPP and PPE, further study needs to be conducted to identify the optimum safe dosage.

As for hepatic lipid composition, hepatic TG was found to be in the range of 6.63 to 9.54 mg/g. Hamsters fed with HP diets had the lowest TG content. Total cholesterol can be

categorized as free cholesterol (mostly embedded in cell membranes) and cholesterol-ester (esterified form to be delivered into plasma). HP and HE diets significantly lowered the free cholesterol from 7.16±0.32 to 5.26±0.47 and 5.17±0.23 mg/g liver. Cholesterol-esters ranged from 0.62 to 2.50 mg/g, while total cholesterol was from 5.03 to 8.92 mg/g. HE and HP diets demonstrated the highest reduction in cholesterol parameters, which could be explained by the ingestion of ellagic acid and fiber (Liu et al., 2015). To the best of our knowledge, this is the first study that investigated the change of free cholesterol and cholesterol ester in hamsters induced by ingestion of PPP and PPE addition.

Hepatic lipid content was in the range of 5.94 to 7.58 g/100g liver. HE and HP groups demonstrated the lowest liver weight, indicating less liver fat storage. Fecal lipid content ranged from 0.57 (HP) to 1.72 g/100g liver, suggesting that the HP diet had the lowest fecal fat secretion.

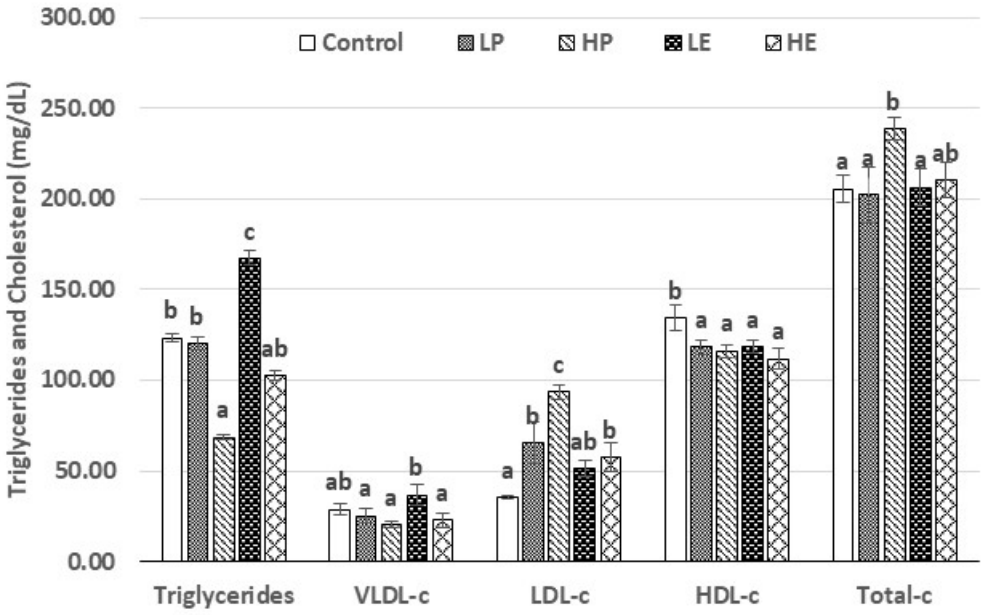


Figure 3.2 Plasma cholesterol and triglycerides of hamsters fed with different diets. Within groups, the same letter means no significant difference according to LSD (0.05).

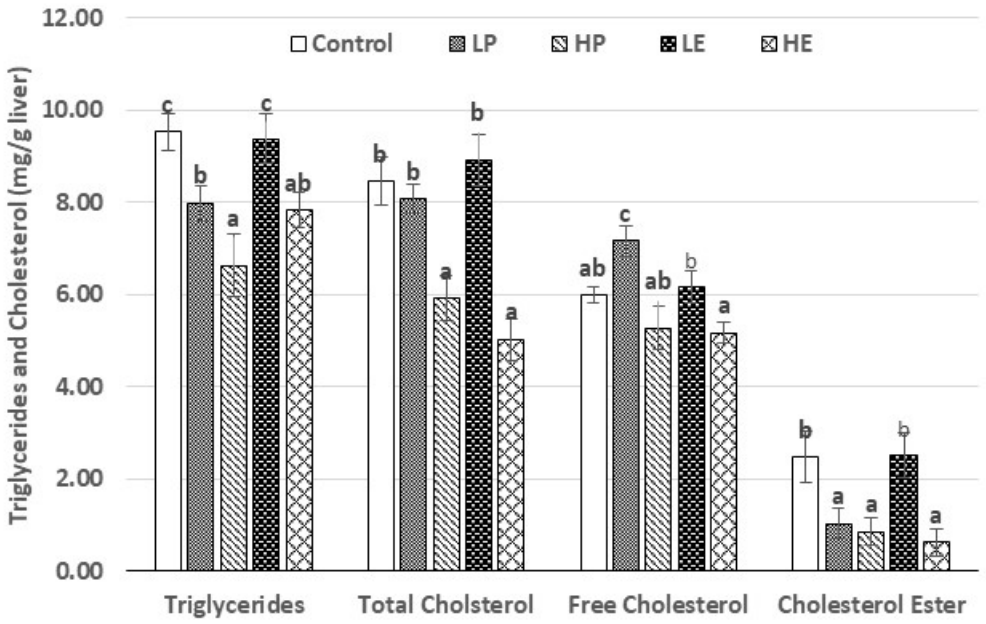


Figure 3.3 Hepatic cholesterol and triglycerides of hamsters under different diets. Within groups, the same letter means no significant difference according to LSD (0.05).

3.3.3. Expression of hepatic genes related to cholesterol, bile acid, and fatty acid metabolism

As unusual LDL-elevating effects in plasma after PPP and PPE ingestion were observed, real-time qPCR was applied to investigate the potential mechanisms of diets modulating the lipid and cholesterol metabolism. As shown in Figure 3.4, the expression level of CYP7A1 (cytochrome P450 7A1), a gene that controls bile acid synthesis rate from cholesterol, was increased by 2.1-folds ($p<0.05$) in the HP diet, while that was reduced to 0.86-, 0.91- and 0.90-fold ($p<0.05$) in LP, LE, and HE diets. HMG-CoAR (3-hydroxy-3-methylglutaryl CoA reductase, rate-limiting step) and Cyp51 (lanosterol 14α-demethylase) are two important genes in cholesterol biosynthesis. Compared with the control diet, LP, HP, LE, HE demonstrated the same up-regulating pattern in these two genes -- 1.03-, 1.51-, 1.11- and 1.18-folds for HMG-CoAR ($p>0.05$), as well as 1.18-, 1.72-, 1.25- and 1.22-folds for Cyp51 ($p<0.05$), respectively. LDL

receptor (LDLR) facilitates the hepatic LDL uptake from circulation. In the present study, LP and HP diets up-regulated LDLR expression by 1.18- and 1.38-folds ($p<0.05$), while that was slightly down-regulated by 0.95-fold ($p>0.05$) in both LE and HE diets. These findings were in line with hepatic LDL cholesterol -- all the reformulated diets prompted hepatic cholesterol synthesis. HP diet increased bile acid synthesis while other diets decreased it. Peel-formulated diets elevated LDL uptake and extract-formulated ones alleviated it. For peel-formulated diets, bile acid synthesis was dominating in lowering hepatic cholesterol, whereas for extract-formulated diets it lowered LDL uptake.

PPARα (Peroxisome proliferator-activated receptor alpha) was an essential transcription factor regulating fatty acid β-oxidation. It was up-regulated in LP and HP diet-fed hamsters by 1.04- ($p>0.05$) and 1.61- ($p<0.05$) folds. In contrast, a lower expression of 0.90- and 0.93-fold of PPARα was observed in LE and HE diets ($p>0.05$). SREBP-1c (Sterol regulatory element-binding proteins) is a gene encoding transcription factor for fatty acid synthesis (glycolysis and lipogenesis). It targets SCD-1 (stearoyl-CoA desaturase-1) to catalyze the synthesis of monounsaturated fatty acids, which is a substrate for TG synthesis and storage. LP diet slightly up-regulated the expression of SREBP-1c by 1.07-folds, while HP, LE, and HE diet down-regulated that by 0.74-, 0.85- and 0.92-fold ($p>0.05$). All the diets significantly reduced SCD-1 expression level by 0.42-, 0.24-, 0.73- and 0.61-fold ($p<0.05$). These results indicated that all the formulated diets induced lower uptake of fatty acids. Peel-enhanced diet slightly promoted fatty acid β-oxidation in a dose-dependent manner, while extract-enhanced diet mitigated that, which was aligned with liver TG levels and hepatic lipid contents.

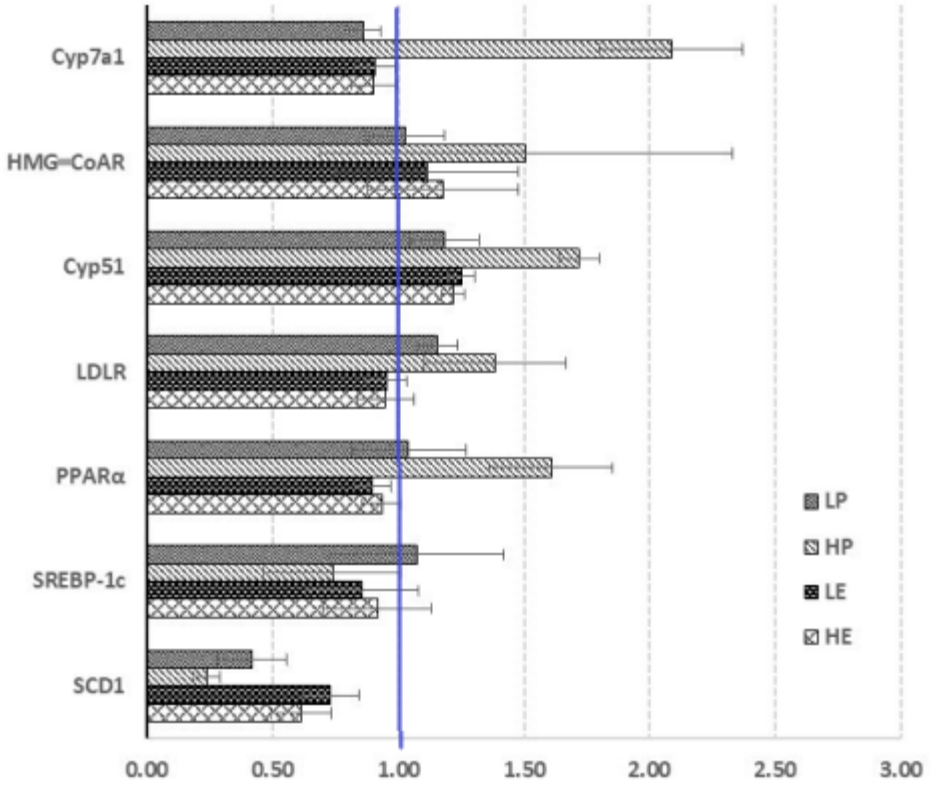


Figure 3.4 Hepatic gene expression change of hamsters fed with different diets

3.3.4. HP and HF diets shifted fecal microbiota towards a leaner phenotype

To identify the microbiota-changing effects of different diets on lipid metabolism, relative abundance (RA) was assessed at the phylum level (Table 3.4 and Figure 3.5). Human gut microbiota is generally dominated by the bacterial phyla *Bacteroidetes* and *Firmicutes* (The Human Microbiome Project Consortium., 2012). Besides these, *Proteobacteria* is considered correlated for the variation of the functionality of gut microbiota (Bradley & Pollard, 2017). Compared to the control HF diet, HE and HP diets reduced the relative abundance of Firmicutes by 9.33% and 18.3% while increasing the RA of Bacteroidetes by 43.1% and 41.9% and nearly triplicated the RA of Proteobacteria. The corresponding ratio of *Firmicutes*/ *Bacteroidetes* (F/B) dropped by 39.4% and 42.4%, with an increase of *Proteobacteria*/ *Bacteroidetes* (P/B) ratio by

89.6% and 105.1%, indicating that a shift of fecal microbiota towards leaner phenotypes. *Verrucomicrobia* was boosted to 4.0% and 8.5% for HP and HE diets, which was non-detectable for the HF diet. Similar observations of *Verrucomicrobia* increase were found in formulated diets with PPE (George et al., 2019), cranberry (Anhê et al., 2015), and black raspberry (Pan et al., 2017), which could be attributed to the abundant polyphenol content. At the same time, *Cyanobacteria* was elevated from 0.3% in the HF diet to 2.3% in HE and shown of associated with improved gut health (Jiao et al., 2019; McFadden et al., 2015). To the best of our knowledge, no other research reported the change of *Verrucomicrobia* and *Cyanobacteria* after PPP incorporation in hypolipidemic diets. Further metagenomics studies are required to understand how *Verrucomicrobia* and *Cyanobacteria* modulate the lipid metabolism pathways.

Table 3.4 Fecal microbiota changes of hamsters fed with different diets

Diets	**HF**	**HP**	**HE**
Bacteria phylum			
Firmicutes	81.80±4.13 a	74.16±1.52 ab	66.83±8.66 b
Proteobacteria	0.37±0.10	1.15±0.20	1.13±0.49
Bacteroidetes	13.38±2.71	19.14±2.44	18.99±4.09
Others	4.44±1.78	5.54±2.18	13.05±6.02
F/B ratio	6.86±1.24	4.16±0.56	3.95±1.11
P/B ratio	0.03±0.01	0.06±0.01	0.07±0.03

***** Within groups, the same letter means no significant difference according to LSD (0.05).

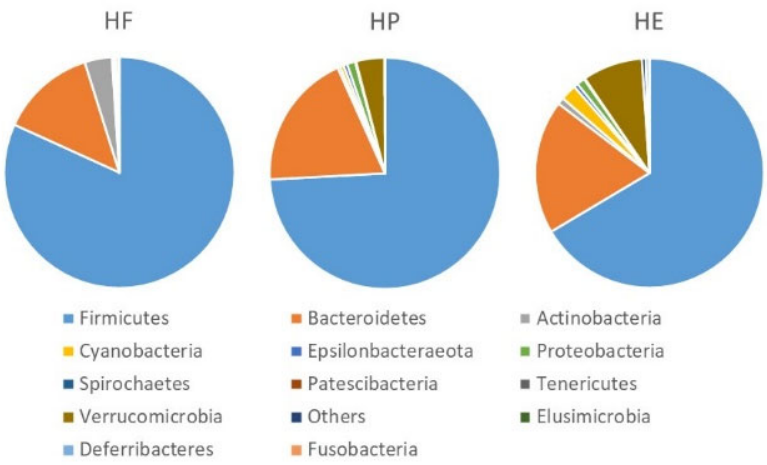
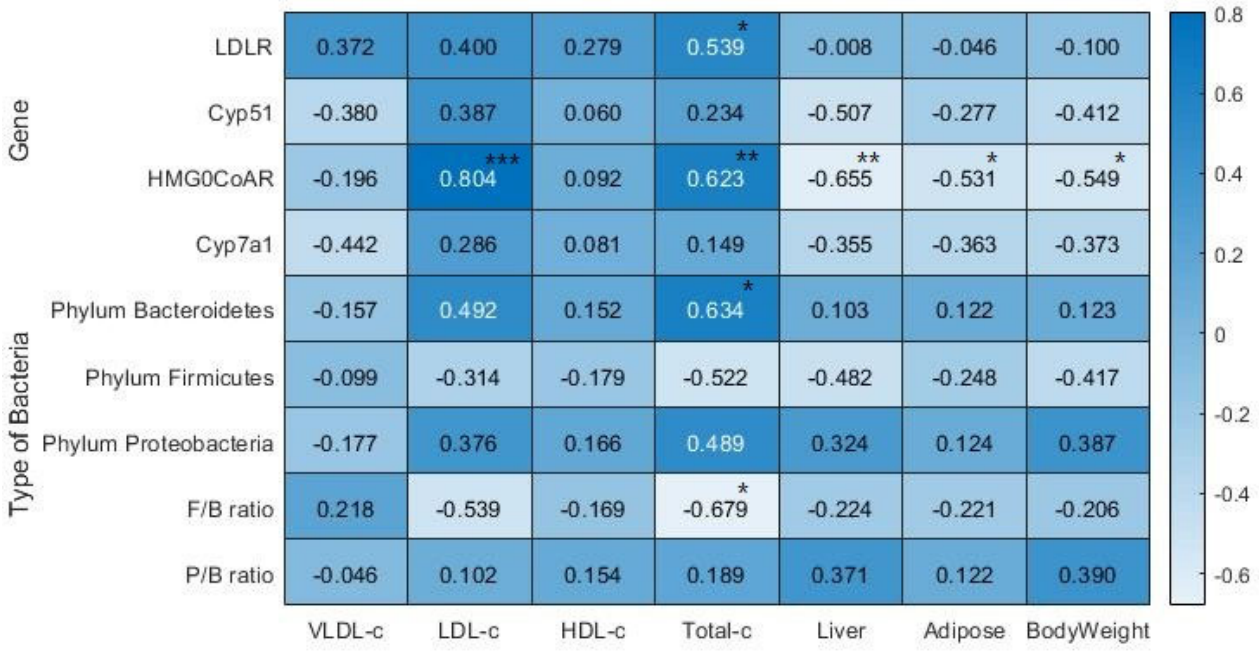


Figure 3.5 Fecal microbiota of hamsters fed with HF, HP, and HE diets

3.3.5. Pearson correlations among physiological parameters, plasma cholesterol, hepatic genes, and types of bacteria

As shown in Figure 3.6, the expression of hepatic HMG-CoAR was significantly correlated with obesity-related indices, with a positive correlation with plasma concentrations of the total- ($p<0.01$) and LDL-cholesterol ($p<0.001$), and a negative correlation with liver ($p<0.01$), adipose ($p<0.05$) and body weight ($p<0.05$). The expression of LDLR also exhibited a significant positive correlation with total cholesterol. This was consistent with previous research from Teh et al. (2019), who concluded that HMG-GoAR and LDLR were the two major regulating factors in meditating hypo cholesterol effects of hamsters fed with fruit and vegetable seed meals.

As for types of bacteria, total plasma cholesterol exhibited a significant positive correlation with the phylum *Bacteroidetes*, and a significant negative correlation with the F/B ratio in response, suggesting that the increase of *Bacteroidetes* attributed to the decrease of F/B ratio and played a role in elevating total cholesterol level.



n = 16. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3.6 Pearson correlations between plasma cholesterol concentrations and expression of hepatic genes

3.4. Conclusions

Pomegranate peel, a commonly underutilized by-product with high phenolic and fiber content, was incorporated into the hypolipidemic diet in the form of powder and extract (8.68 or 17.36 mg/kg/day body weight) to investigate its hypoalipidemic potential. PPP and PPE containing a rich mixture of phytonutrients demonstrated sufficient effects to suppress weight gain, hepatic lipid profile and ameliorate the symptoms of metabolic syndrome in Golden Lakeview Golden (LVG) Syrian hamsters with HF diet-induced obesity. These observations can be at least partially explained by hepatic metabolism changes and changes in gut microbiota composition. In this study, PPP and PPE lowered *Firmicutes* and boosted *Bacteroidetes, Verrucomicrobia, and Cyanobacteria* to lower the F/B ratio, as well as increased microbiota

diversity. These indices were significantly correlated with obesity-related indices, indicating that microbiota might play an important role in the hypolipidemic effects of PPP and PPE. 2 hepatic genes (HMG-CoAR and LDLR) were closely related to modulating the plasma and lipid profile, suggesting the ingested cholesterol and LDL uptake level were crucial metabolic changes. However, adverse plasma LDL-elevating effects were observed in a higher dose of PPP and PPE intake, which required further study on the potential toxicity.

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Chapter 4 - Greek Style Yogurt Fortified with Pomegranate Peel Extract

4.1. Introduction

Since the 21st century, society has an increasing awareness of health and is switching to healthier lifestyles and eating habits (Fernandes et al., 2019). Several sectors for product development are responsible for the change, such as food industries, researchers, health professionals, and regulatory authorities (Prakash et al., 2017). In this context, functional foods have great potential. Functional foods represent the portion of the human diet that could provide health benefits and reduce the risk of chronic diseases beyond nutrition. Polyphenol-containing products are a common type of functional food with proven health benefits, such as protecting against certain cancers, cardiovascular diseases, type 2 diabetes, osteoporosis, pancreatitis, gastrointestinal problems, lung damage, and neurodegenerative diseases (Fraga et al., 2010; J. B. & P., 2014; Martin - Peláez et al., 2013; Rossi et al., 2008). According to Scalbert & Williamson (2000), 1 g of daily consumption of polyphenols in long term is suggested to fulfill all the aforementioned health benefits of polyphenols. U.S. dietary guidelines recommended daily food intake to satisfy certain nutrient needs (Figure 4.1 Healthy U.S.-style eating pattern at the 2000-calorie level with daily or weekly amounts from foot groups subgroups and components. (DeSalvo et al., 2016)). However, polyphenols are not included and only 552 mg of polyphenol is satisfied through the recommended diet based on our calculation (Table 4.1).

Table 4.1 Calculation of polyphenol intake from the USDA dietary guideline

Food Group	Recommended Consumption per Day	Equivalent Weight (g)	Phenolic Content (mg/100g)	Consumed Phenolic Content (mg)	Reference
Vegetable					
Green beans	1 cup	150	8.3	12.45	(Preti et al., 2017)
Spinach	½ cup	30	103.9	31.17	(Ambrose et al., 2016)
Baked Potato	1 medium	225	28.5	64.13	(Scalbert & Williamson, 2000)
Fruit					
Canned peach	½ cup	115	26.5	30.48	(Durst & Weaver, 2013)
Banana	1 large	118	1	1.18	(Rothwell et al., 2013)
Orange	1 small	96	0.5	0.48	(Park et al., 2014)
Grain					
Bread	6 oz	168	1.65	277.20	(Yu et al., 2013)
Dairy					
Yogurt	3 cups	0.709 (L)	39.08 (mg/L)	27.71	(Trigueros et al., 2014)
Protein food	5 ½ oz		0	0	
		Total Daily Consumption (mg)		444.80	

Healthy U.S.-Style Eating Pattern at the 2,000-Calorie Level, With Daily or Weekly Amounts From Food Groups, Subgroups, & Components

Food Group[a]	Amount[b] in the 2,000-Calorie-Level Pattern
Vegetables	**2½ c-eq/day**
Dark Green	1½ c-eq/wk
Red & Orange	5½ c-eq/wk
Legumes (Beans & Peas)	1½ c-eq/wk
Starchy	5 c-eq/wk
Other	4 c-eq/wk
Fruits	**2 c-eq/day**
Grains	**6 oz-eq/day**
Whole Grains	≥ 3 oz-eq/day
Refined Grains	≤ 3 oz-eq/day
Dairy	**3 c-eq/day**
Protein Foods	**5½ oz-eq/day**
Seafood	8 oz-eq/wk
Meats, Poultry, Eggs	26 oz-eq/wk
Nuts, Seeds, Soy Products	5 oz-eq/wk
Oils	**27 g/day**

Figure 4.1 Healthy U.S.-style eating pattern at the 2000-calorie level with daily or weekly amounts from foot groups subgroups and components. (DeSalvo et al., 2016)

Yogurt is a popular fermented dairy product known for its high nutritional value, especially the significant content of proteins and essential minerals, such as calcium. Greek Style Yogurt (GSY) is a type of nutrient-dense yogurt with increasing popularity among consumers. According to Statista (Gullen & Plungis, 2013), from 2015 to 2020, the consumption of GSY in the U.S. significantly improved 50%, worth $3.7B and accounting for 52% of the U.S. yogurt market share. Compared to regular yogurts, GSY contains a higher solids content and is often perceived as being less acidic. The nutritional information commonly claims “twice the amount of protein as in regular yogurt” (Kilara & Chandan, 2013). However, they are never considered a

source of functional foods. Therefore, it is of great interest to investigate the GSY fortified with polyphenol ingredients for improved polyphenol intake and increased consumption of dairy foods (Allgeyer et al., 2010).

El-Said et al., (2014) evaluated the antioxidant activities and physical properties of stirred yogurt fortified with the extracts of oven-dried and solar-dried pomegranate peel. Results showed that increasing the percentage of the added pomegranate peel extract (PPE, up to 20%) statistically increased antioxidant activities and reduced the viscosity without significantly affecting the sensory attributes. However, since stirred yogurt has lower protein content and viscosity than GSY, a modified manufacturing process is needed for GSY. Kharchoufi et al. (2017) investigated quality changes of GSY fortified with pomegranate juice (PJ) and arils (PA) during 18 days of storage. Researchers directly incorporated PJ and PA into GSY and quantified the total phenolic content, antioxidant activity, and sensory 1, 6, 12, and 18 days. This research demonstrated that the addition of PJ and PA in fermented GSY could significantly increase the antioxidant content and activity without negatively altering the sensory quality. However, incorporation of PPE in GSY prior to fermentation was not discussed, which could provide unique product characteristics and health benefits, as research has shown that bacteria could transform polyphenolic compounds into smaller units for increased extractability and stability (El-Said et al., 2014; Sun-Waterhouse, 2011).

The texture is an important sensory attribute that affects consumer preferences (Szczesniak, 2002). According to Ozcan (2013), several main processing parameters influence the yogurt texture, including (1). fortification level and materials used, (2). stabilizers type and usage levels, (3). fat content and homogenization conditions, (4). milk heat treatment conditions, (5). starter culture (type, rate of acid development, and production of EPS), (6). incubation

temperature (influences growth of starter cultures, gel aggregation, bond strength), (7). pH at breaking, (8). cooling conditions and (9). handling of product post manufacture (e.g. physical and temperature abuse).

Based on these considerations, this study aimed to investigate the effects of different contents of protein and PPE on the sensory, nutritional, and functional attributes of GSY. Response surface methodology (RSM) with the multi-response statistical technique was applied to optimize a yogurt formulation.

4.2. Objectives

The objective of this study was to develop a top-down formulation incorporating pomegranate peel extract into Greek Style yogurt before fermentation for increased antioxidant content and activity while maintaining suitable texture.

4.3. Materials and methods

4.3.1. Raw materials and chemicals

The chemicals used in the experiments, including Folin-Ciocalteu reagent, analytical standards of tannic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, Mo.). Sodium carbonate was obtained from Fisher Scientific (Pittsburgh, Pa.). Milk powder was from the local market. Yogurt starter consisting of *S. thermophilus* and *L. bulgaricus* was purchased from Natural Probiotic Selection (London, UK)

4.3.2. Experimental design: GSY formulation

The manufacturing process was modified from El-Said et al. (2014). To study the effects of protein and phenolic addition, A modified response surface methodology (RSM, alpha =2,

repeat =2) with the central composite design was applied with 5 levels of protein content (4, 6, 8, 10, 12 %) and phenolic extract (52, 59, 66, 73, 80 g) to form a 130g mixture. 2 times of water was used to extract the phenolic content from pomegranate peel. Protein was incorporated from skim milk powder and boiled water was added to make up 130g of weight. After homogenization, the mixture was fermented at 37 ºC for 36 h.

USDA dietary guideline-recommended 24oz (equal to 855g) of daily normal yogurt (protein content: 7.33g/100g) for protein enrichment. Therefore, based on the recommended protein content, we calculated that 420 grams of GSY could replace normal yogurt for daily intake (calculation as shown below). To achieve daily phenolic consumption gap (0.555g), a final product with the phenolic concentration 0.555g/420g = 1.321mg/g is desired.

$$\frac{Normal\ yogurt\ protein}{Greek\ yogurt\ protein} = \frac{Greek\ yogurt\ amount}{Normal\ yogurt\ amount}$$

$$\frac{3.6g/100g}{7.33g/100g} = \frac{x}{855g}$$

$$x = 420g$$

The independent variables used in the design were protein content (X1, %), phenolic extract weight (X2, g), while the dependent or response variables were total phenolic content (Y1, mg/130g), DPPH scavenging activity (Y2, g/g), pH (Y3), syneresis (Y4, %). The range for each variable was determined from the preliminary single factor tests. 21 experiments were conducted randomly (Table 4.2) to analyze the response pattern and to establish models.

Experimental data were fitted into a linear, 2FI, and second-order polynomial model. Corresponding regression coefficients were calculated. The generalized equations to predict the optimal point were explained as follows:

Linear: $Y=b_0+b_1X_1+b_2X_2$ (4.1)

2FI: $Y=b_0+b_1X_1+b_2X_2+b_{12}X_1X_2$ (4.2)

Quadratic:

$$Y=b_0+b_1X_1+b_2X_2+b_{11}X_1^2+b_{22}X_2^2+ b_{12}X_1X_2 \quad (4.3)$$

In Eq. 4.1, 4.2, and 4.3, the coefficients of the polynomial terms were represented by b_0 (constant term); b_1 and b_2 (linear effects); b_{11} and b_{22} (quadratic effects); and b_{12} (interaction effects). Significant terms in the model for each response were found by analysis of variance (ANOVA) and significance was judged by the F-value calculated from the data (Eren & Kaymak-Ertekin, 2007).

Table 4.2 Modified central composite response surface design with experimental results

Standard Order	Run Order	Independent Variables		Dependent Variables							
		X1	X2	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
		Protein Content (%)	Extract Weight (g)	TPC (mg/130g)	DSA (g/g)	pH (Y_3)	Syneresis (%)	Firmness (g)	Cohesiveness (g)	Consistency (g*s)	Viscosity (g*s)
8	1	10 (1)	73	66.67	4.82	4.82	10.78	1.80	0.55	10.22	0.30
10	2	4 (-2)	66	134.40	2.92	3.89	38.80	0.44	0.15	4.46	0.55
20	3	8 (0)	66	82.15	5.41	4.53	22.11	1.50	0.61	16.00	2.21
12	4	12 (2)	66	56.16	6.56	5.14	0.00	2.88	1.70	29.47	4.70
17	5	8	66	84.53	3.98	4.40	21.83	1.23	0.42	13.31	1.02
14	6	8	52	61.23	4.72	4.51	20.61	0.81	0.25	8.47	0.90
1	7	6 (-1)	59	93.15	3.77	3.92	22.93	1.47	0.39	8.22	0.84
2	8	6	59	94.20	3.39	3.92	31.10	0.81	0.21	7.81	0.71
5	9	6	73	110.46	2.75	3.85	32.42	0.87	0.21	8.47	0.80
11	10	12	66	60.85	6.21	5.09	0.30	2.67	1.07	25.29	2.98
6	11	6	73	115.15	3.45	3.87	30.90	0.95	0.27	8.52	0.95
19	12	8	66	118.03	3.20	4.25	21.36	1.55	0.63	15.99	2.58
4	13	10	59	74.10	5.76	4.97	3.93	1.41	0.51	14.60	1.68
9	14	4	66	115.04	3.40	3.79	42.02	0.40	0.11	3.92	0.40
16	15	8	80	102.98	3.22	4.38	20.65	1.39	0.51	13.91	1.85
21	16	8	66	82.81	5.24	4.03	13.91	1.60	0.56	16.92	2.11
15	17	8	80	96.12	4.52	4.59	15.62	1.88	0.68	18.41	2.57
7	18	10	73	76.12	5.39	4.98	8.82	1.94	0.58	18.74	1.75
3	19	10	59	63.87	4.71	4.99	4.54	1.77	0.70	18.88	2.10
13	20	8	52	56.44	6.18	4.52	16.75	0.96	0.35	10.33	1.13
18	21	8	66	67.29	6.70	4.19	17.57	1.75	0.73	19.21	2.53

4.3.3. Analysis of GSY quality

After incubation, GSY went through a compression test using TA.XT2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK) with TA-4 probe for texture evaluation. The test parameters were 2 mm/s for pre-speed and test speed, 2 mm/s for post-speed, and 20 g of trigger force. Firmness (g), consistency (g*s), viscosity (g*s), and cohesiveness (g) were recorded as positive peak force, positive peak area, negative peak area (absolute value), and the negative peak force (absolute value). A demonstration of texture data collection is shown in Figure 4.2.

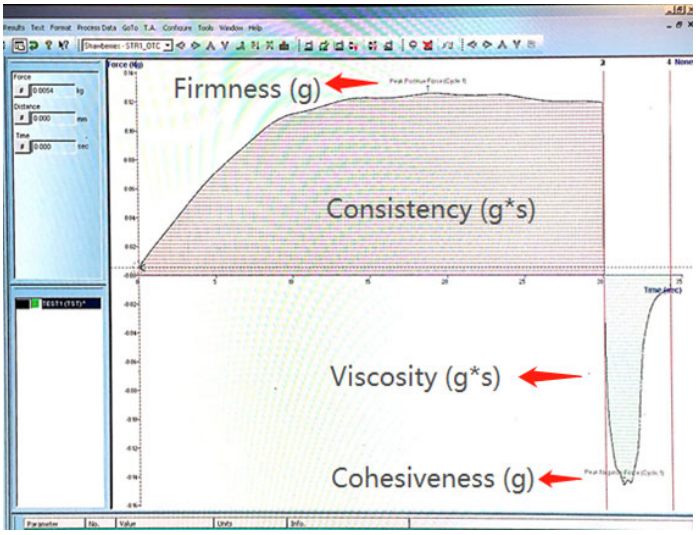


Figure 4.2 Demonstration of texture profile analysis

Syneresis is the proportion of free whey in the yogurt samples, which is highly correlated with yogurt stability (Zainoldin & Baba, 2009). 10g yogurt was centrifuged at 5000 rpm, 4 ºC for 20 min. Then the syneresis was calculated based on Eq. 4.4:

$$\text{Syneresis (\%)} = \frac{W_1}{W_2} \times 100 \qquad (4.4)$$

where

W_1 = weight of supernatant (g)
W_2 = weight of Greek-style yogurt (g)

The measurements of phenolic content and antioxidant activity were modified from Trigueros et al. (2014). 20g yogurt was mixed with 30mL acidified methanol and incubated at 4 ºC overnight. Then the mixture was centrifuged at 5000 rpm for 5 mins and the supernatant was obtained. Total phenolic yield (TPY) in the extract was expressed as g tannic acid equivalent (TAE) or gallic acid equivalent (GAE) per g dry peel, while the antioxidant activity of the extract was determined as DPPH scavenging activity (DSA, g DPPH equivalent g^{-1}) using Eq. 4.6.

$$\text{TPY}\,(\%) = \frac{CV}{100W_1} \times 100 \tag{4.5}$$

where

W_1 = dry weight of sample (g)
W_2 = dry weight of extract (g)
C = phenolic concentration (g mL^{-1})
V = total volume of liquid extract (mL).

$$\text{DSA} = \frac{nV_t\left[C_c - \left(C_s - C_b\right)\right]}{C_t V_t} \tag{4.6}$$

where

C_c = DPPH conc. equivalent in control solution (g L^{-1})
C_s = DPPH conc. equivalent in sample solution (g L^{-1})
C_b = DPPH conc. equivalent in blank solution (g L^{-1})
n = dilution factor of liquid extract.

To quantify the tannic acid equivalent, a 0.6 mL extract sample was mixed thoroughly with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent and 2 mL of 7.5% (wt/wt) Na_2CO_3 using a vortex mixer (Vortex-Genie 2, Scientific Industries, Bohemia, N.Y.). After 30 min of 25°C incubation of the mixed solution, the absorbance was measured at 760 nm using a UV spectrophotometer (UV-3600 Plus UV-Vis-NIR spectrophotometer, Shimadzu Scientific, Columbia, Md.). To measure the DPPH scavenging activity, liquid extract (60 μL) or DI water

(control group) was mixed thoroughly with 3 mL of DPPH solution in methanol (0.05 g L^{-1}) using a vortex mixer and kept in a 25°C water bath for 20 min. Liquid extract (60 μL) was also mixed with 3 mL of methanol and used as a blank solution. Absorbance at 517 nm was noted. Three measurements were conducted for each liquid sample, and each test was replicated three times. For each liquid extract, the tests were conducted in triplicate, and the absorbance was read three times for each sample. A reference blank was prepared using the aforementioned procedure with DI water rather than liquid extract.

4.3.4. Statistical analysis

The design of experiments, analysis of the results, and prediction of the responses were carried out using Design-Expert Version 9.0 (Stat-Ease, 2014). Comparisons of means were performed by one-way ANOVA (analysis of variance) followed by Tukey's test ($p < 0.05$). The optimum models of response variables were selected based on backward model selection.

4.4. Results and discussion

4.4.1. Fitting of response surface models

Levels of RSM variables and corresponding response results are shown in Table 4.2. ANOVA analyses for the model responses are exhibited in Table 4.3. For all the responses, linear models achieved significant fitting ($p < 0.0007$), suggesting a great regression on the experimental data. Lack of fit for fitted models, including TPC, DSA, syneresis, firmness, and viscosity, was found to be not significant ($p > 0.05$), indicating the reliability in predicting future experimental outcomes. Other parameters, including R^2, adjusted-R^2, coefficient of variation (C.V.), prediction error sum of squares (PRESS), and adequate precision were calculated to

Table 4.3 Evaluation of the fitted linear models for Y1 to Y8 responses

Sources	Response Variables							
	TPC (mg/ 130g)	**DSA (g/g)**	**pH**	**Syneresis (%)**	**Firmness (g)**	**Cohesiveness (g)**	**Consistency (g*s)**	**Viscosity (g*s)**
Model (p value)	<0.0001*	0.0002*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0007*
Lack of Fit (p value)	0.6781ns	0.8794ns	0.0315*	0.4155ns	0.0519ns	0.0396*	0.0402*	0.0501ns
R-Squared	0.7822	0.6093	0.8649	0.9336	0.8164	0.6546	0.7234	0.4654
Adj R^2	0.7580	0.5659	0.8578	0.9301	0.7959	0.6364	0.7088	0.4373
C.V. %	13.16	18.15	3.90	16.53	20.12	39.97	25.52	48.65
PRESS	2937.47	15.46	0.69	222.44	2.09	1.21	288.59	15.57
Adequate Precision	15.42	10.72	29.18	43.26	18.73	15.88	18.65	10.76

* Terms are significant ($p < 0.05$); ns means that terms are not significant ($p > 0.05$).

evaluate the model adequacy, as shown in Table 4.3. The R2 and adjusted-R2 ranged from 0.4373 to 0.9336, implying a variety in model adequacy. The low C.V. values (3.90–48.65%, criteria: C.V. < 100%) indicated a low variability of the models. For the different models of the same response, lower values of PRESS indicate a model that predicts well. In this research, the PRESS values ranged from 0.69 to 2937.47. Adequate precision measures the signal-to-noise ratio and a ratio greater than 4 is considered great (Borror et al., 2002). For all the fitted models, adequate precision values were between 10.72 and 43.26, which was desirable.

Table 4.4 Estimated regression coefficients and significance of each variable

Response		**Intercept**	**Linear effects**	
			X1	**X2**
TPC	Coefficient	71.54	-8.27	1.22
	p-value		<0.0001*	0.0016*
DSA	Coefficient	4.18	0.42	0.04
	p-value		0.0001*	0.0803
pH	Coefficient	2.86	0.19	-
	p-value		<0.0001*	-
Syneresis	Coefficient	60.62	-5.22	-
	p-value		<0.0001*	-
Firmness	Coefficient	-1.83	0.25	0.02
	p-value		<0.0001*	0.0411*
Cohesiveness	Coefficient	-0.51	0.13	-
	p-value		<0.0001*	-
Consistency	Coefficient	-6.50	2.54	-
	p-value		<0.0001*	-
Viscosity	Coefficient	-1.02	0.33	-
	p-value		<0.0001*	-

* Terms are significant ($p < 0.05$); ns means that terms are not significant ($p > 0.05$).

4.4.2. Effect of protein and extract content on GSY quality

For each response, linear, 2FI, and quadratic models were built. Models of the highest adjusted-R^2 value without aliasing were selected, and all the responses could be extrapolated by linear models. Linear effects of protein content were significant ($p < 0.0001$) on all response

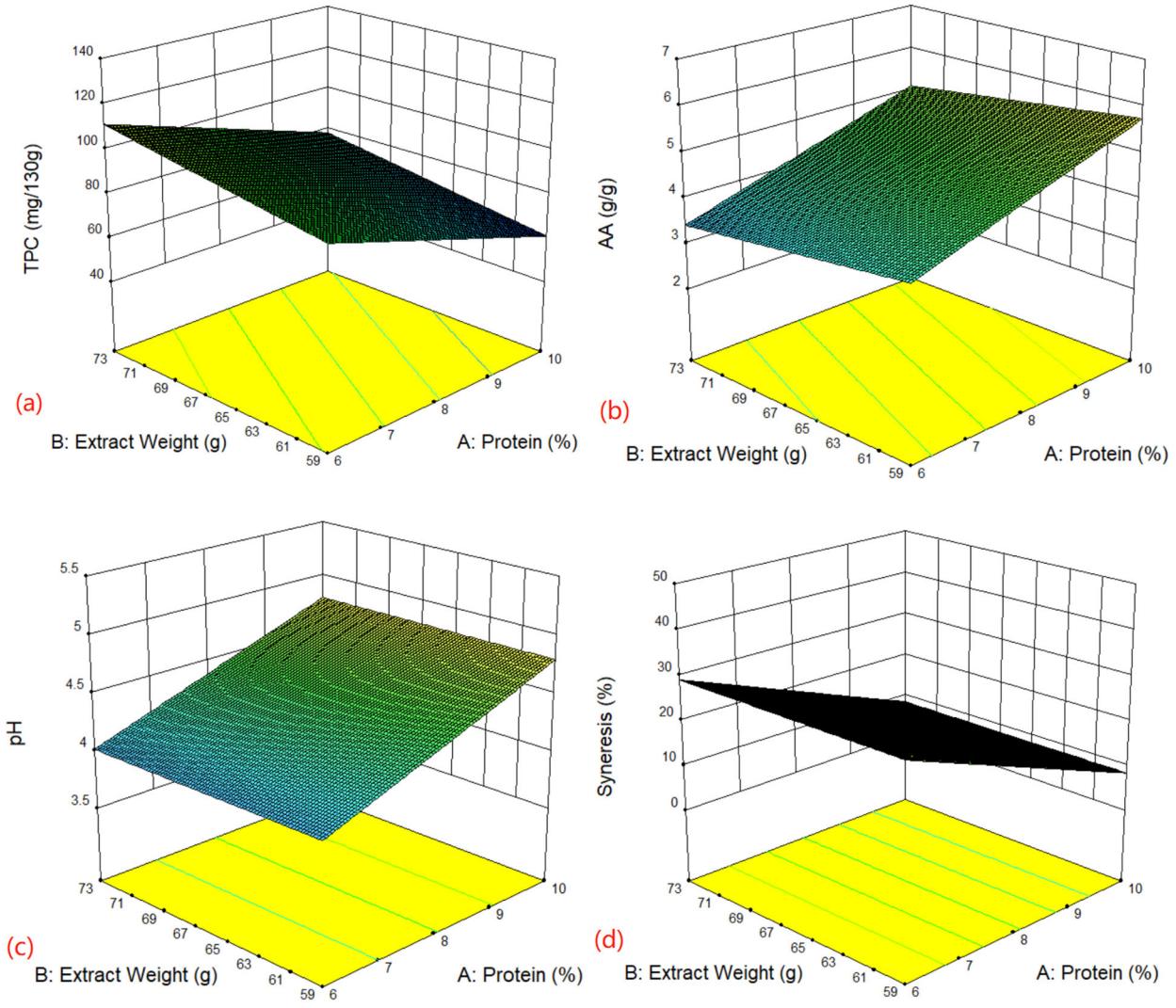
variables, while that of extract content were only related to TPC ($p = 0.0016$), firmness ($p < 0.0411$) and DSA ($p=0.0803$, insignificant). The corresponding coefficients along with respective p-values were listed in Table 4.4.

3D response surface graphs were generated to visualize the interaction effects of protein and extract content on GSY characteristics (Figure 4.3). TPC (Figure 4.3 a) increased with lower protein content and extract addition. This finding was in line with previous research. Trigueros et al., (2014) incorporated pomegranate juice into yogurt (PGY) and observed the polyphenol-protein interaction. After formulation, their PGY contained 40% of juice (TPC: 707.25 mg GAE/L) and presented 241.44 mg GAE/L of TPC, which meant 85.35% of the theoretically expected. They also evaluated the TPC of PGY permeate after 1-day storage and concluded a TPC of 111.92 mg GAE/L. indicating nearly 54% of the TPC remained interacting with milk proteins. An increase in DSA (Figure 4.3 b) was observed with higher protein and extract content. The same pattern was found in the study carried out by Jiménez et al., (2008)

As expected, syneresis decreased with enhanced protein content, which led to a dense yogurt matrix microstructure and enhanced denaturation of whey protein (Sodini et al., 2004). Usually, lower syneresis equals to longer shelf-life. However, a grainy texture should be avoided when enriching the milk base with high protein.

pH and all the texture properties were positively correlated with protein content while not affected by the extracted content. *S. thermophilus* and *L. bulgaricus* in yogurt starter were able to produce exopolysaccharides (EPS) during fermentation and improve yogurt texture. According to Sodini et al., (2004), higher solid content was correlated with stronger EPS interaction with casein, therefore a stronger texture could be formed.

Considering all the parameters and visual appeals (Figure 4.4), the optimum formulation should consist of 8% of protein and 77g of extract for a 130 g yogurt.



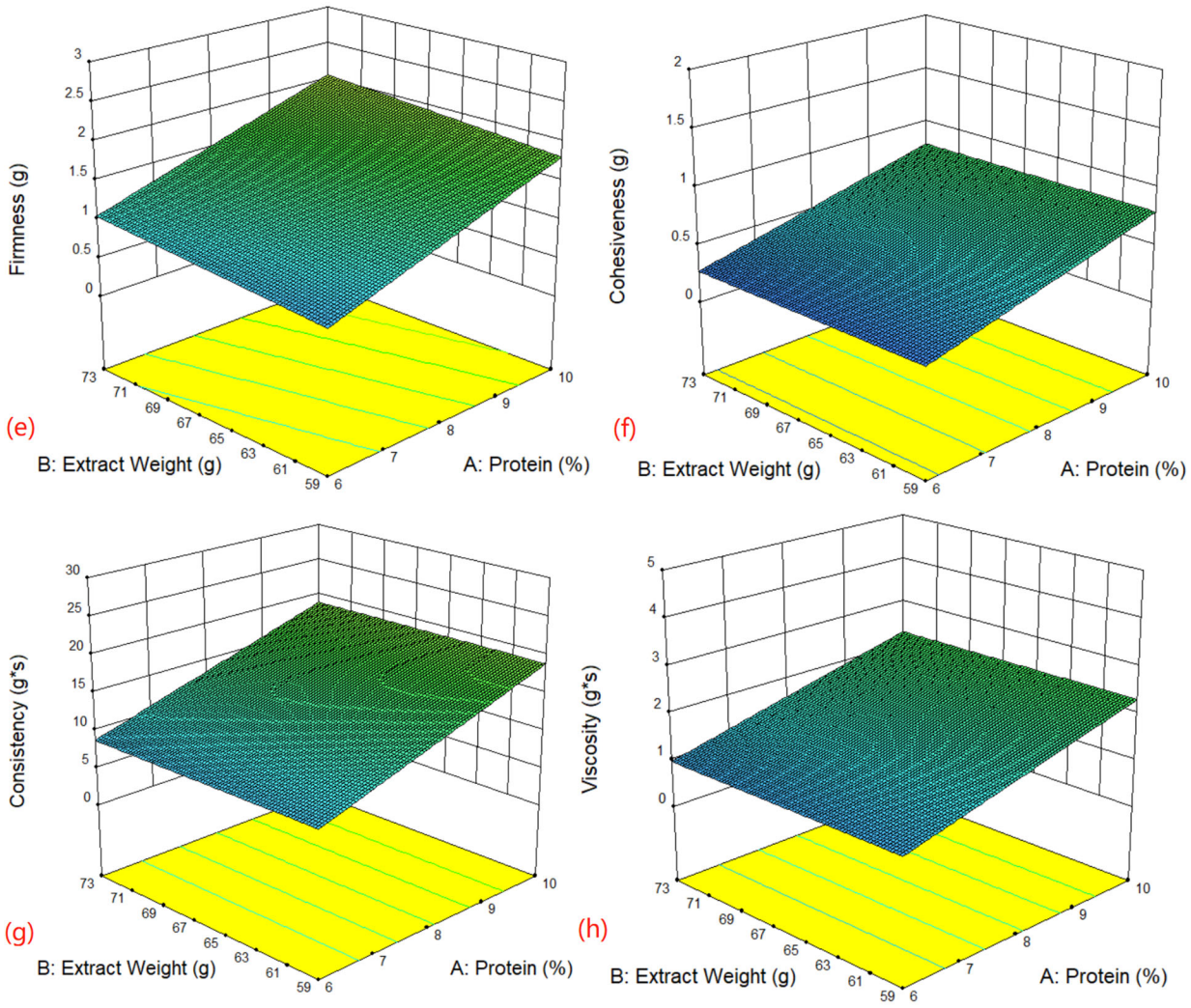


Figure 4.3 The effect of phenolic content and protein addition on the physio-chemical characteristics of fortified Greek-style yogurt. (a) Total phenolic content; (b) Antioxidant activity; (c) pH; (d) Syneresis; (c) Firmness; (d) Cohesiveness; (g) Consistency; (h) Viscosity

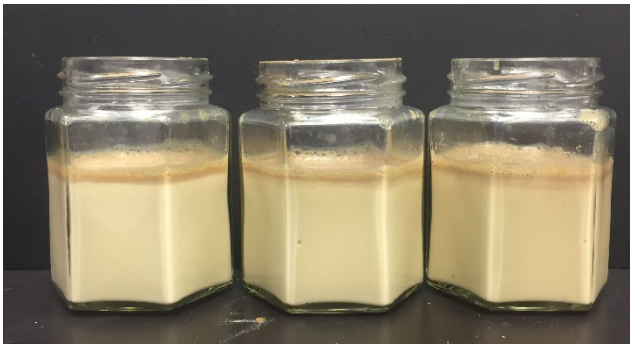


Figure 4.4 Example of Greek-style yogurt with 8% of protein content

4.5. Conclusions

In this study, a modified response surface methodology was applied to investigate the effects of protein and pomegranate peel extract on the physiochemical characteristics of Greek Style Yogurt, including total phenolic content, DPPH scavenger activity, pH, syneresis, firmness, cohesiveness, consistency, and viscosity. Enhanced protein content affects all the characteristics while extract content only affects TPC, DSA, and firmness. Based on product quality and visual appeals, the optimum formulation should consist of 8% of protein and 77g of extract for a 130 g yogurt in this study. Further research is needed to analyze the product cost and explore the bioavailability of polyphenol within the fortified GSY for better mass production guidance.

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Chapter 5 - Co-extraction of Pectin and Polyphenol for Improved Extract Yield and Phenolic Stability

5.1. Introduction

5.1.1. Pectin – useful polysaccharides from the plant cell wall

5.1.1.1. Characteristics and applications

Pectin is widely found in the middle lamella layers between plant cells (Figure 5.1), forming a primary cell wall during plant growth and development (Cosgrove & Jarvis, 2012; Picot-Allain et al., 2020). It is a family of heterogeneous polysaccharides consisting of α-1,4-D-galacturonic acid (GalA), L-rhamnose (Rha), D-galactose (Gal), L-arabinose (Ara), and other 13 different monosaccharides through 20 different linkages (Kaya et al., 2014; Wang et al., 2018). The pectin backbone primarily consists of D-GalA residues (~70%) linked at α-1,4 positions. Based on the abundance of side chains, pectin can be divided into "smooth", homogalacturonan, and "hairy" regions, namely rhamnogalacturonan I, rhamnogalacturonan II, xylogalacturonan, and apio-galacturonan (Figure 5.2). A comparison of these regions is listed in Table 5.1 (Chomto & Nunthanid, 2017; Picot-Allain et al., 2020). The backbone unit, GalA, can be partially esterified with a methyl group or converted into the carboxylic acid amide with ammonia. (Wang et al., 2018). Based on the degree of methyl-esterification and acetylation, pectin can be divided into high-methoxyl pectin (HMP), low-methoxyl pectin (LMP), and amidated pectin as shown in Table 5.2.

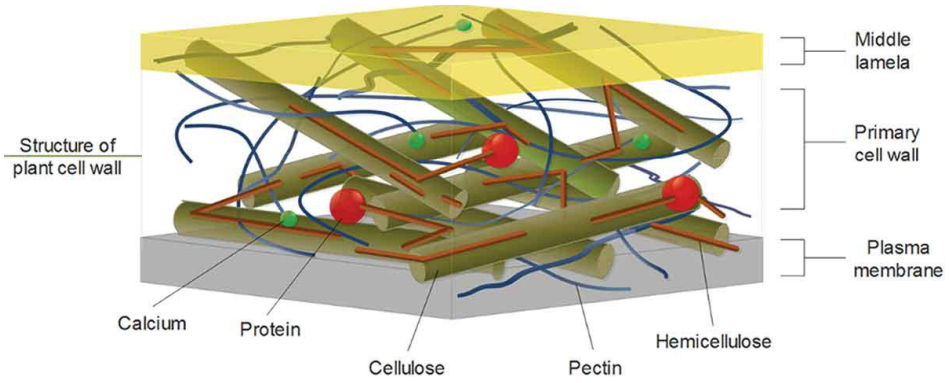

Figure 5.1 Demonstration of plant cell wall structure (Dranca & Oroian, 2018)

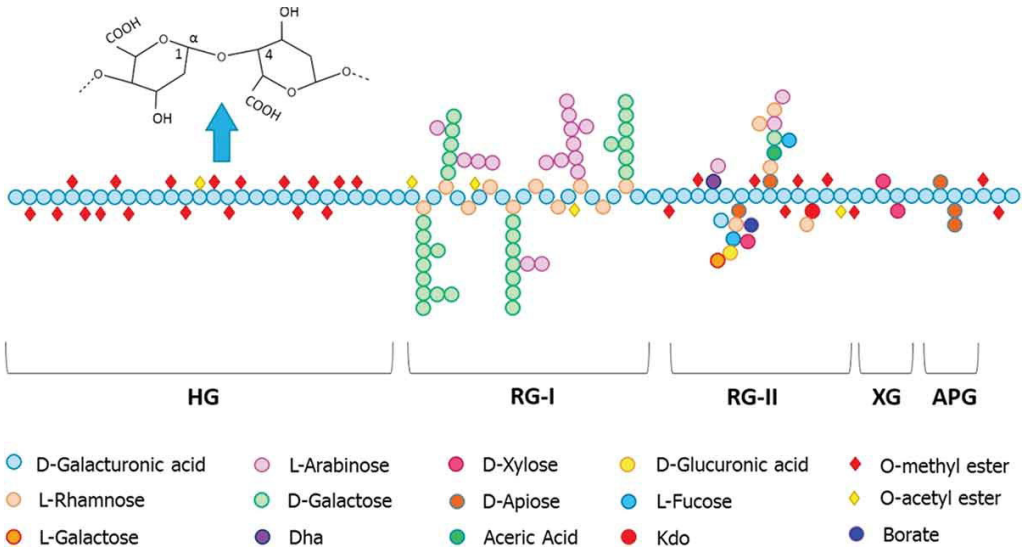

Figure 5.2 Regions of pectin and their compositions (Wang et al., 2018)

Table 5.1 Categorization of pectin region (Chomto & Nunthanid, 2017; Picot-Allain et al., 2020)

Name	Backbone and the linkage	Sidechain and the linkage
Homo-galacturonan (~65%)	α-1,4-linked GalA units; Methyl methylation at C-6 carboxyl; acetylation at O-2 or O-3	No side chains
Rhamno-galacturonan I (20~35%)	(1–4)-α-d-GalA-(1,2)-α-L-rhamnose unis, partially O-acetylated on C-2 and/or C-3 by α-L-rhamnose	~half are linear and branched α-L-arabinose and β-D-glucuronic acid. A-L-fucose, 4-O-methyl β-D glucuronic acid, as ferulic and coumaric acid may be present

Rhamno-galacturonan II (0.5~8%)	α-1,4-linked GalA units, partially methyl esterified at C-6 with galactosyl units	11 to 12 rare sugars including apiose, 2-O-methyl-l-fucose, 2-keto-3-deoxy-d-manno-2-octulosonic acid, 2-O-methyl-D-xylose, 3-C-carboxy-5-deoxy-l-xylose, and 3-deoxy-d-lyxo-2-heptulosaric acid; over 20 glycosidic linkages to backbone
Xylo-galacturonan Apio-galacturonan	α-1,4-linked GalA units	Xylose or apiose at the C-2 or C-3 via β-glycoside bond

Table 5.2 Categorization of pectin, adopted from Wang et al. (2018)

Name	Reactions	Characteristics	Dominating Interaction	Usage
High-methoxyl pectin (HMP)	Methylation with methanol (>50% galacturonic acid)	>60% sugar and soluble solid content; Extracted at pH <3.5	Hydrogen bonds & hydrophobic interactions	In conventional jams and marmalades with high sugar content
Low-methoxyl pectin (LMP)	Methylation with methanol, (<50% galacturonic acid)	10-70% soluble solids content; Extracted at pH from 2.6 to 7.0	Ionic bridges between calcium ions and the ionized carboxyl groups	The gelling agent, thickening agent, and stabilizer
Amidated pectin	Acetylation: galacturonic acid forms a carboxylic acid amide	Similar to LMP; Thermo-reversible	Similar to LMP; more tolerant of Ca^{2+}	Increases the stabilizing and emulsifying effects

5.1.1.2. Functionality

Besides the aforementioned significant industrial benefits, pectin has functional properties as dietary fiber, prebiotics, and fat replacer, as well as in antiglycation, antioxidant, and antibacterial. As a viscous soluble fiber, pectin is associated with lowering blood serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), without changing high-density lipoprotein cholesterol (HDL-C). By increasing satiety, pectin can help weight control by reducing food consumption. (Gunness & Gidley, 2010). Due to its complex structure, pectin is a common drug formulation agent for oral delivery, in the form of tablets, gels, hydrogels, beads, aerogels, coated, and

compression-coated dosage (Chomto & Nunthanid, 2017). It is especially beneficial for colon-specific drugs as the bioactive compounds, mostly short-chain fatty acids, and can be released after hydrolyzation of pectinolytic enzymes and microorganisms in the colon (Wong et al., 2011). The rhamnogalacturonan type I fragments within the pectin also have lower molecular weight to function as an anticancer down-regulator of galectin-3, which is a pro-metastatic protein in many cancer cells. Pectin has also been used to nano-encapsulate bioactive compounds, thereby enhancing shelf life and stability.

5.1.1.3. Regulation

Pectin is considered a safe food substance through various government food agencies. Even though its usage and composition are regulated under different food additive laws, the Food and Drug Administration (FDA) of the U.S. considers it GRAS. At the Joint FAO/WHO Expert Committee Report on Food Additives and in the European Union, no numerical acceptable daily intake (ADI) has been set, as pectin is considered safe. Its abundant benefits stimulated great demands and the global pectin market size was valued at USD 964.1 million in 2015 (Petkowicz & Williams, 2020).

5.1.1.4. Sources

Current industrial sources of pectin include citrus peels, apple pomace, and sugar beet pulp. Expansion of pectin resources is in demand to accommodate the market growth. Recently, there is a growing trend of pectin sources from food wastes, such as banana peels, grapefruit peels, papaya peels, and kiwifruit pomace (Happi et al., 2008; Koubala et al., 2014; Maxwell et al., 2016; Xu et al., 2014; Yuliarti et al., 2015).

Little is known about the pectin in pomegranate peel. Since pomegranate peel may consist of more than 50% of pomegranate fruit, the investigation of pectin extraction in pomegranate peel will fulfill the increasing pectin demand and reduce the pollution of the wasted peel.

5.1.2. The interaction between pectin and polyphenol

5.1.2.1. Mechanism

Polyphenol compounds can bind with the cell wall polysaccharides (CPSs) to strengthen the structure (Wang et al., 2018). In return, CPSs can interact with polyphenols to modify their bio-accessibility, bio-availability, and bio-efficacy (Holland et al., 2020; Ribas-Agustí et al., 2018). Liu et al., (2020) summarized multiple research that has been done to investigate the binding effects between pectin and polyphenol. Non-covalent bonds are dominant in the polyphenol-pectin interactions, such as hydrogen bonds, hydrophobic interactions, electrostatic interactions. These weak bonds are sensitive to the intrinsic properties of polyphenol and pectin (composition, hydrophobic groups, and structures, etc.), as well as environmental conditions (processing temperature, storage condition, etc.).

Table 5.3 Selective review on potential binding mechanisms of polyphenol and pectin, adopted from X. Liu et al. (2020)

Polysaccharides	Polyphenol	Binding mode					References
		Hydrogen bond	Hydrophobic interactions	Electrostatic interactions	Π–π stacking	Others	
Apple, citrus, and sugar beet pectins	Strawberry extracts (E-1, E-2) (83.6% and 81.7% total anthocyanins, respectively)	√					Buchweitz et al., (2013b)
Apple and citrus pectins	Apple procyanidins: degree of polymerization (DP) 9 and DP30	√	√				Watrelot et al., (2013)
Citrus peel pectins	Tannins (DP26 and DP5)	√	√				Mamet et al., (2018)
Apple, citrus, and sugar beet pectins	Purified black currant extract comprising anthocyanins and non-anthocyanin phenolics (PP-E), and purified anthocyanin (ACN-E)	√	√	√			Buchweitz et al., (2013)
Citrus pectins	Cyanidin-3-O-glucoside	√	√	√	√		Fernandes et al., (2020)
Blueberry pectins	Anthocyanins	√	√	√	√		Koh et al., (2020)
Blueberry pectin-rich fractions	Anthocyanins			√		Anthocyanin intermolecular stacking	Lin et al., (2016)
Sugar beet pectin (SBP)	Anthocyanins	√	√			Van der Waals forces	Larsen et al., (2019)

5.1.2.2. Benefits

Considering the binding property of pectin and polyphenols, a co-extraction of pectin and polyphenol should be considered because of the following advantages: (1) Boosted stability of polyphenol and overall antioxidant activity; and (2) Enhanced unique and beneficial characteristics for developing novel food products.

As for stability, Oszmiański et al. (2009) proposed a cooperative hydrogen bond or hydrophobic interactions existed between the oxygen atom of pectin and the phenolic hydroxyl group. A natural pectin coating is speculated as less loss of polyphenols was observed due to the limited oxygen contact. The strength of pectin-polyphenol interaction is mainly determined by the non-covalent bonds, including hydrogen bonds, ionic bonds, and hydrophobic forces.

Regarding the quality improvement, Hayashi et al., (2005) found the astringency of gallate-type polyphenol was reduced by the addition of pectin. A similar finding was reported in persimmons (Taira et al., 1997).

5.1.3. Extraction of pectin

5.1.3.1. Thermal

Pectin polymer degradation is a fundamental step of extraction. The common degradation approaches include chemical hydrolysis, heating, radiation, and enzymatic reaction. Acid hydrolysis (pH < 3.0) on pectin was one of the most conventional chemical degradation methods. Thibault et al. (1993) studied the mild acid hydrolysis (0.1 M HCl, 80 ºC) of apple, beet, and citrus pectin. Inorganic solvents, including HCl and HNO_3, have been widely used in the industry. However, they raised concerns in food applications and were difficult to recover, thus

causing undesired pollution and high processing cost (Gogate & Prajapat., 2015). Novel technologies with reduced pollution are in demand.

5.1.3.2. Non-Thermal

Physical technologies, such as the non-thermal process, were superior due to their high efficiency, reduced pollution and cost, and ease of control. Ultrasound as a novel non-thermal technology has been widely applied in food industries, including extraction, preservation, emulsification, filtration, and cutting (Chemat et al., 2017). Successful ultrasound applications in pectin extraction include citrus (Zhang et al., 2015), apple (Liu et al, 2013), and sweet potato (Ogutu & Mu, 2017).

Ultrasound extraction combined with acid hydrolysis was reported to further increase the pectin yield. Muñoz-Almagro et al. (2017) studied the pectin degradation under ultrasound power of 400 W (20 kHz, 2s pulse on 1s pulse off) in the presence of nitric acid and citric acid (CA). The combined approach achieved higher pectin degradation than ultrasound or acid individually. In the meantime, no significant differences were found between using CA and nitric acid under ultrasound. Zhang et al., (2013) concluded the same findings when comparing the combined extraction with CA and hydrochloric acid. Since CA is a common food-grade ingredient that increases polyphenol stability (Kuyu et al., 2018), the technique of combining ultrasound and CA should be considered to extract pectin along with stable polyphenol.

Enzyme extraction is another option for non-thermal pectin recovery. It relies on the enzymes that selectively degrade the cell wall composition to release the pectin, such as cellulases, hemicellulases, and proteases (Fissore et al., 2009). Compared with traditional thermal processes, enzyme extraction can achieve selective catalysis reaction (i.e: hydrolysis),

reduce the solvent needed or increase higher yield with the same solvent (Puri et al., 2012). However, the major challenges for industrialization include high cost and high sensitivity to processing parameters (temperature, nutrient accessibility, etc.) (Adetunji et al., 2017).

5.2. Objectives

This study aimed to investigate the influences of ultrasound-assisted co-extraction of pectin and polyphenol from wet pomegranate peel using citric acid.

5.3. Materials and methods

5.3.1. Chemicals

Folin-Ciocalteu reagent, analytical standards of tannic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), galacturonic acid, sulfuric acid, and methanol were purchased from Sigma-Aldrich (St. Louis, Mo.). Sodium carbonate and copper (II) sulfate were obtained from Fisher Scientific (Pittsburgh, Pa.). Glacier acetic acid and ethanol were collected from VWR (Radnor, Pa.).

5.3.2. Co-extraction using ultrasound under citric acid

Co-extraction parameters and measurements are shown in Table 5.4 and Figure 5.3. A QSonica-S4000 sonicator (Qsonica L.L.C, Newtown, CT) was utilized for ultrasound treatment. The ultrasound intensity, solvent ratio, extraction time, extraction temperature, and CA pH were expected to be the major factors in co-extraction. The effects of these factors were studied based on Box–Behnken design, a type of response surface methodology that statistically evaluated main effects, interaction effects, and

quadratic effects of the formulation. A randomized experimental design comprising of 46 runs was listed in Table 5.5, while -1/0/1 corresponds to the levels of different factors. The following quadratic equation was established to evaluate the effects of different extraction factors:

$$Y = \beta_0 + \sum_{i=0}^{5} \beta_i X_i + \sum_{j=0}^{5} \beta_{ii} X_j^{\ 2} + \sum_{i=0}^{5} \sum_{j=0}^{5} \beta_{ij} X_i X_j \qquad (5.1)$$

Y: the dependent variable;
β_0: constant;
β_i: regression coefficients computed from the observed experimental values of Y;
X_i are the coded levels of independent variables;
$X_i X_j$ and x_i^2 represent the interaction and quadratic terms

After the treatment, the mixture was centrifuged at 5000rpm for 4 mins. (Zhu et al., 2015). The supernatant was split into two portions. One portion was used for quality measurement (Analysis-A), including total phenolic yield (TPY), DPPH scavenging activity (DSA), and color index (L*, lightness; a*, red-green; b*, yellow-blue). The other portion was mixed with 98% ethanol overnight to wash out impurities. The mixture was centrifuged and mixed with 98% ethanol 2 more times to complete the washout. Then the pectin was collected in centrifuge tubes and hot-air dried at 30 ºC overnight for pectin yield and other characteristics (Analysis-B).

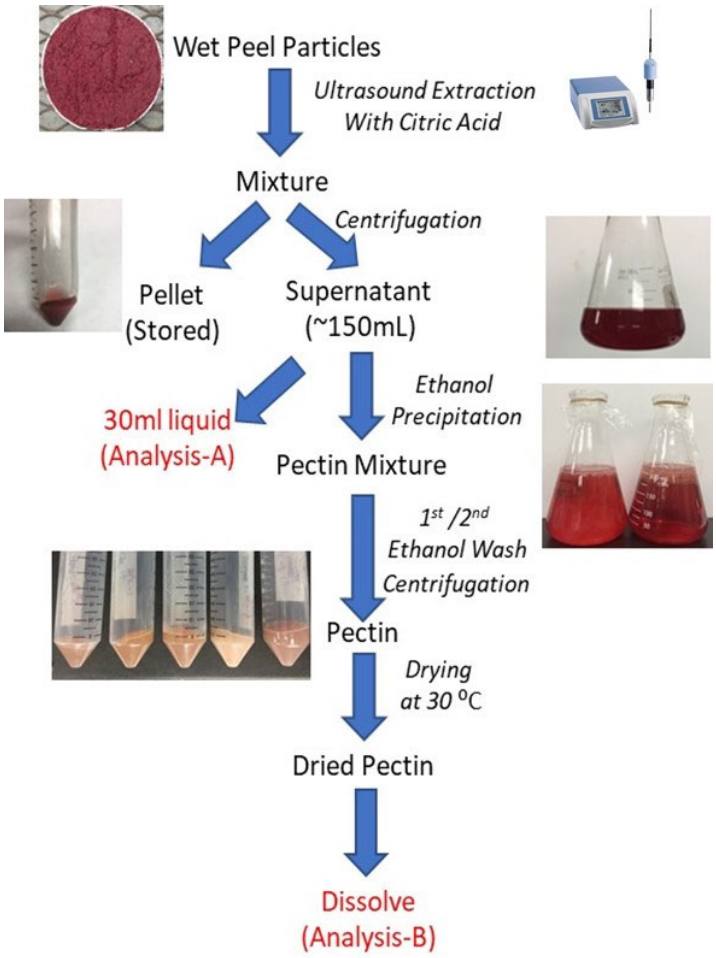

Figure 5.3 Co-extraction and analysis flow diagram

Table 5.4 Co-extraction parameters

Fixed Parameters		**Responses**
Solvent Type	Citric Acid	Pectin Yield, TPY, DSA, Color
Ultrasound model	QSonica-S4000	Galacturonic content
Container	150 mL beaker	Degree of methylation/
Variables		Degree of acetylation
Solvent Ratio	10/20/30	Chemical bonds (FTIR)
Ultrasound Intensity	10/50/90 %	
Temperature	10/50/90 ºC	**Data Analyses/Outcome**
Time	10/50/90 min	Response surface methodology, quadratic model
pH	1.4/3/4.4	ANOVA, test for significant differences ($p<0.05$)

5.3.3. Co-Extraction Kinetics

After the quadratic equations for each parameter were established, The solvent ratio/ultrasound intensity/pH that achieved the highest pectin yield was fixed for co-extraction kinetic study. According to Pan et al. (2012), the second-order rate law is ideal to identify the kinetic parameters of ultrasound-assisted co-extraction, including second-order extraction rate constant (k, L/g min), which could be expressed as:

$$\frac{d(PC_t)}{dt} = k(PC_e - PC_t)^2 \qquad (5.2)$$

PCe: Equilibrium phenolic concentration (g/L) or pectin yield (%)
PCt: Total phenolic concentration (g/L) or pectin yield (%) in the liquid extract at a given extraction time t

Based on the integration law under the boundary conditions ($t = 0 \rightarrow t$, $PC_t = 0 \rightarrow PC_t$), Eq. 5.2 could be written as Eq. 5.3 or linearized form Eq. 5.4:

$$PC_t = \frac{(PC_e)^2 kt}{1+kt\,(PC_e)} \qquad (5.3)$$

$$\frac{t}{PC_t} = \frac{1}{k(PC_e)^2} + \frac{t}{PC_e} \qquad (5.4)$$

The initial extraction rate, h (g/L min), when t -> 0, could be defined as:

$$h = k(PC_e)^2 \qquad (5.5)$$

The h, PCe, and k were determined by Eq. 5.4 using Origin 2021 (Originlab Corporation, Northampton, MA) and Eq. 5.5.

5.3.4. Analysis of co-extract quality and interaction

Besides TPY, DSA, and pectin yield, several other parameters were also evaluated to investigate the quality change of co-extraction through the extraction process at different

temperatures, including galacturonic acid (GalA) content, degree of methoxylation (DM), and acetylation (DA) of pectin.

GalA content was analyzed by hydrolyzing with sulfuric acid (Wang et al., 2016). 2 mg of pectin was mixed with 0.5 mL of DI water and 3 mL of concentrated sulfuric acid. After 15 mins of hydrolyzation in boiling water, the solution was cooled down in an ice bath. The solution was further mixed with 0.1mL of 0.15 % carbazole ethanol, incubated at 25 °C for 30 mins, and diluted 5 folds. The absorbance at 530 nm was recorded and compared with standard GalA.

DM and DA are two major indicators of pectin accessibility. Following the method modified by Wang et al. (2017), DM and DA were quantified based on the molar percentage of methanol or acetic acid content from the GalA content. 5 mg of pectin was reacted with 0.5 mL of 0.2 mM $CuSO_4$ for saponification, while 0.25 μM of isopropanol was added as internal standard. After 1 hr of incubation at 4 °C, the mixture was centrifuged at 8000 rpm for 5 min and adjusted to a pH of 2. HPLC system with C-18 column and refractometer function was applied for analysis, where the isocratic elution was with 4 mM sulfuric acid solution at a flow rate of 0.8 mL/min. A demonstration of major peaks, including methanol, acetone, and isopropanol, is shown in Figure 5.4.

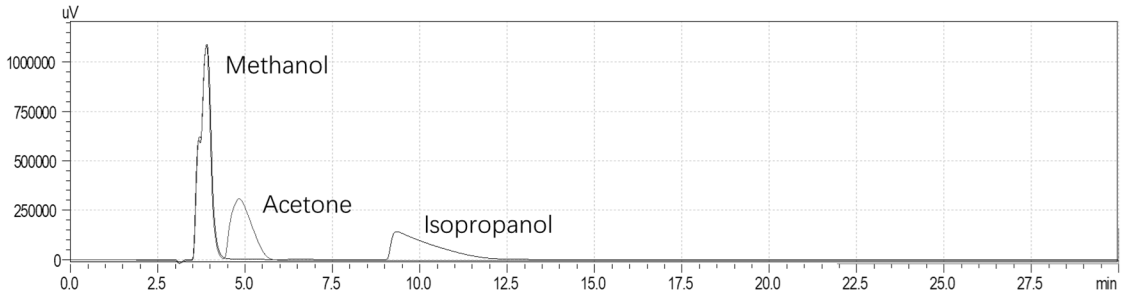

Figure 5.4 HPLC analysis on the degree of methylation and acetylation

To speculate the chemical bonds involved in the co-extract, Fourier Transform Infrared (FTIR) spectroscopy analysis of the mixture was recorded by a Nicolet 6700 FTIR spectrometer (Thermo Electron Co., USA) at the absorbance mode with the frequency ranging from 4000 to 400 cm^{-1} and a resolution of 4 cm^{-1}.

5.3.5. Statistical analysis

The design of experiments, analysis of the results, and prediction of the responses were carried out using Design-Expert Version 11.0 (Stat-Ease, 2019). The optimum models of response variables were selected based on backward model selection. Results were plotted using Origin 2021 (Originlab Corporation, 2021).

Table 5.5 Experimental conditions of pectin extraction from pomegranate peels with citric acid, and corresponding responses. Values in brackets are coded according to the central composite design.

Run	Independent Variables					Dependent Variables						
	X1 Ultrasound Intensity (%)	X2 Solvent Ratio	X3 Time (min)	X4 Temperature (°C)	X5 pH	Y1 TPY (%)	Y2 DSA (g/g)	Y3 L*	Y4 a*	Y5 b*	Y6 Pectin Yield (%)	Y7 Energy (kJ)
1	50 (0)	20 (0)	50 (0)	55 (0)	3 (0)	5.11	6.56	38.23	14.38	20.15	2.51	101.42
2	50	30 (1)	10 (-1)	55	3	6.92	7.30	41.68	14.38	23.92	1.21	14.22
3	90 (1)	20	50	55	4.4 (1)	16.04	2.62	33.93	13.59	13.20	4.23	100.48
4	90	30	50	55	3	8.12	6.57	39.84	12.27	19.77	4.05	99.36
5	50	20	10	25 (-1)	3	14.13	3.14	39.87	13.64	18.71	1.80	14.30
6	10 (-1)	20	10	55	3	4.26	2.69	42.3	13.64	26.43	0.27	7.09
7	50	20	50	55	3	5.11	6.56	38.23	14.38	20.15	2.46	101.42
8	50	30	50	55	1.6 (-1)	9.96	5.17	36.61	18.30	17.46	5.11	66.15
9	10	20	50	85 (1)	3	6.46	1.43	34.47	15.33	19.37	4.89	33.49
10	50	20	50	55	3	5.11	6.56	38.23	14.38	20.15	2.45	101.42
11	10	20	50	55	4.4	15.78	2.61	35.30	16.05	15.01	0.18	34.42
12	90	20	50	25	3	13.07	3.11	36.63	11.93	17.93	5.20	113.2
13	50	20	50	25	1.6	19.59	3.91	37.53	21.73	17.61	6.67	77.91
14	10	20	50	55	1.6	8.40	1.12	36.49	21.13	16.25	5.15	35.80
15	50	10 (-1)	50	25	3	4.85	4.67	35.41	14.83	15.40	1.17	69.04
16	90	20	90 (1)	55	3	6.84	5.32	38.18	18.59	20.08	4.90	178.78
17	50	10	50	55	1.6	6.38	2.62	33.01	19.52	12.23	8.34	63.81
18	90	20	50	55	1.6	8.04	3.56	35.02	18.94	14.32	9.53	106.48
19	90	10	50	55	3	6.12	3.48	34.66	16.18	13.72	5.10	101.02
20	50	20	50	25	4.4	13.94	3.12	33.88	9.45	8.99	0.61	73.85
21	50	20	50	55	3	5.11	6.56	38.23	14.38	20.15	2.51	101.42
22	10	30	50	55	3	7.36	6.60	42.39	16.22	22.72	0.26	36.72
23	50	10	10	55	3	4.51	4.79	37.29	15.33	19.95	2.06	14.28
24	50	20	10	85	3	6.53	6.14	37.87	16.04	20.99	5.21	10.21
25	50	30	50	85	3	4.62	6.62	36.01	13.03	13.3	7.91	52.15
26	50	30	50	25	3	15.69	7.47	40.54	13.16	17.02	1.56	68.82

27	50	10	50	85	3	4.98	3.23	32.17	14.29	10.03	4.91	50.82
28	50	20	10	55	4.4	15.10	2.77	35.25	15.20	13.40	1.07	13.43
29	50	20	50	55	3	5.11	6.56	38.23	14.38	20.15	2.46	101.42
30	50	20	50	55	3	5.11	6.56	38.23	14.38	20.15	2.53	101.42
31	50	20	50	85	1.6	8.32	3.83	32.51	14.38	9.87	8.90	53.63
32	50	30	90	55	3	8.48	6.40	38.47	17.03	19.52	4.93	116.71
33	50	30	50	55	4.4	17.55	3.56	37.64	13.71	13.43	0.40	64.29
34	50	10	50	55	4.4	13.15	1.56	32.72	14.00	8.59	1.08	63.59
35	90	20	10	55	3	7.02	5.50	38.29	14.96	21.20	2.44	21.56
36	50	20	90	85	3	9.30	2.03	32.13	16.83	10.60	8.63	105.74
37	50	20	90	25	3	16.25	6.83	36.04	15.48	17.29	3.22	123.87
38	50	10	90	55	3	5.91	3.23	34.03	14.29	14.30	3.96	118.4
39	50	20	90	55	4.4	16.47	2.39	33.36	13.98	12.40	2.34	114.02
40	50	20	90	55	1.6	16.02	2.39	34.14	23.93	15.73	9.36	115.51
41	50	20	10	55	1.6	10.28	2.48	36.96	16.21	19.06	7.43	13.01
42	10	10	50	55	3	5.78	3.69	37.61	19.52	17.06	0.34	35.59
43	90	20	50	85	3	4.52	6.39	38.83	11.93	14.47	9.16	80.71
44	50	20	50	85	4.4	18.71	2.07	34.18	15.33	11.80	3.44	53.03
45	10	20	90	55	3	12.08	3.50	37.47	19.65	17.21	0.71	63.37
46	10	20	50	25	3	11.55	6.30	42.04	17.38	21.92	0.35	38.07

L^* (lightness) axis indicates ‘0’ is black and ‘100’ is white.

a^* (red-green) axis indicates positive values represent red and ‘0’ is neutral and negative values indicate green.

b^* (yellow-blue) axis indicates positive values; they are yellow when positive; negative value represents blue and ‘0’ is neutral.

Table 5.6 Regression coefficients (for the coded values) and statistical parameters of the models.

Terms	TPY (%)		DSA (g/g)		Pectin Yield (%)		L*		a*		b*		Energy (kJ)	
	Coef.	p	Coef.	p	Coef.	p	Coef.	p	Coef.	p	Coef.	p	Coef.	p
Intercept	5.40		6.38		2.70		37.91		14.50		20.79		101.42	
X_1	-0.12	0.80	0.54	<0.01	2.03	<0.01	-0.79	<0.01	-1.28	<0.01	-1.33	<0.01	32.32	<0.01
X_2	1.69	<0.01	1.40	<0.01	-	-	2.27	<0.01	-0.62	0.09	2.24	<0.01	0.12	0.84
X_3	1.41	0.01	-0.17	0.28	1.04	<0.01	-1.61	<0.01	1.27	<0.01	-2.28	<0.01	51.77	<0.01
X_4	-2.85	<0.01	-0.43	0.03	2.03	<0.01	-1.49	<0.01	-0.03	0.94	-1.53	<0.01	-8.71	<0.01
X_5	2.48	<0.01	-0.27	0.07	-2.95	<0.01	-0.38	0.05	-2.68	<0.01	-1.61	<0.01	-0.95	0.12
X_1X_3	-2.00	<0.01	-	-	-	-	1.18	<0.01	-	-	2.03	<0.01	25.24	<0.01
X_1X_4	-	-	2.04	<0.01	-	-	2.44	<0.01	-	-	-2.24	<0.01	-6.98	<0.01
X_1X_5	-	-	-0.61	0.05	-	-	-	-	-	-	-	-	-	-
X_2X_4	-2.80	<0.01	-	-	-	-	-	-	-	-	-	-	-	-
X_2X_5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
X_3X_4	-	-	-1.96	<0.01	-	-	-	-	-	-	-	-	-3.51	0.01
X_3X_5	-	-	-	-	-	-	-	-	-2.24	<0.01	-	-	-	-
X_4X_5	4.01	<0.01	-	-	-	-	1.33	<0.01	3.31	<0.01	2.64	<0.01	-	-
X_1^2	-	-	-1.22	<0.01	-	-	0.82	<0.01	1.05	0.02	-	-	-14.72	<0.01
X_2^2	-	-	-	-	-	-	-	-	-	-	-2.37	<0.01	-18.98	<0.01
X_3^2	2.16	<0.01	-1.02	<0.01	-	-	-	-	1.21	0.01	-	-	-18.19	<0.01
X_4^2	3.19	<0.01	-0.73	<0.01	1.52	<0.01	-1.13	<0.01	-0.82	0.08	-3.46	<0.01	-20.33	<0.01
X_5^2	6.63	<0.01	-2.78	<0.01	1.54	<0.01	-2.92	<0.01	1.73	<0.01	-5.63	<0.01	-17.78	<0.01
R^2	0.91		0.93		0.91		0.94		0.86		0.93		1.00	
F reg	24.64		29.71		62.29		31.67		18.40		27.96		977.15	

TEY: total phenolic yield; DSA: DPPH scavenging activity (g/g); X_1, X_2, X_3, X_4, and X_5: ultrasound intensity, solvent ratio, extraction time, extraction temperature, and pH (respectively), in coded values ranging from −1 to 1 (according to Table 5.5). Regression terms of $p<0.05$ are statistically significant.

5.4. Results

5.4.1. Box-Behnken results for major factor identification

Box-Behnken design is an effective response surface methodology to evaluate the main effects of among multiple factors. Levels of variables and corresponding response results are shown in Table 5.5. Regression coefficients and statistic parameters for each model are exhibited in Table 5.6. Significant fitting was found in all responses ($p < 0.01$), suggesting the effectiveness of regressions.

TPY varied from 4.26 to 19.59% and was favored by higher ultrasound intensity, solvent ratio, extraction time, pH, and lower temperature. DSA was elevated from 1.12 g/g (g DPPH equivalent g^{-1}) to 7.47 g/g at higher ultrasound intensity and solvent ratio but with lower extraction time, pH, and temperature. The results were consistent with the research from Pan et al. (2012), which studied the effects of continuous and pulsed ultrasound-assisted on pomegranate polyphenol extraction using 25 °C water. They observed a positive relationship between phenolic yield and ultrasound intensity as well as extraction time. Peak TPY (14.8%) was achieved at 100% ultrasound intensity (the equivalent of 59.2 W/cm^2) after 60 mins of extraction. Acidic pH promoted the dissolve of phenolic acid compounds, until to a limit that the polyphenol compounds will be degraded into other molecules (Amyrgialaki et al., 2014; Chen et al., 2014). In some conventional extraction research, high temperature tended to increase the TPY, due to the chemical reactions subjected to the heat treatment, including polymerization and the release of polyphenol compounds (Chen et al., 2014; Wu et al., 2021). However, Çam & Hışıl (2010) and Sood & Gupta (2015) concluded the negative correspondence of TPY towards high temperature due to heat-degradation, which resonated with the results hereby.

L*, a*, and b* values represent the perceptual lightness, redness to greenness, and blueness to yellowness. L* and b* values were positively correlated with solvent ratio, while negatively correlated with ultrasound intensity, extraction time, extraction temperature, and pH. A higher a* value indicated the solution was redder, which was mainly due to the anthocyanins in solutions (Larsen et al., 2019). Higher a* value was observed at longer extraction time, lower intensity, extraction temperature, solvent ratio, and pH, indicating that there were anthocyanins extracted and accumulated, while less degradation happened. Similar findings were concluded in grape pomace (M. Librán et al., 2013) and blueberry (Koh et al., 2020). Figure 5.5 showed the co-extract color at pH of 1.6 and pH of 3.0.

Figure 5.5 Extract of pH 1.6 (left) and pH 3.0 (right)

Pectin yield (0.18-9.53%) demonstrated a linear positive dependence relative to ultrasound intensity, extraction time, and temperature, as well as acidic pH. Ultrasound treatment and high temperature stimulated the de-polymerization of pectin from the cell wall (Wang et al., 2018). Moorthy et al. (2015) utilized a 130W ultrasonic device on an Indian variety of pomegranate peel and obtained up to 24.18% of pectin yield. They also concluded the optimal pectin extraction pH to be 1.6 since, at this condition, the acidic hydrolysis of the insoluble pectin constituents into soluble pectin reached maximum pectin recovery. However, a lower pH value beyond 1.6 could cause the aggregation of pectin, therefore, contributing to a lower pectin

yield. Pereira et al. (2016) researched pectin extraction from dried pomegranate peel using citric acid and yielded from 2.81% to 8.74. Similar responses of pectin yield to the extraction temperatures (70 °C to 90 °C), times (40 mins to 150 mins), and pH (2 to 4) were concluded. The energy usage for the co-extraction was also recorded to guide the ultrasound-assisted extraction process design.

5.4.2. Co-extraction performance on polyphenol and pectin characteristics

After determining the major factors for co-extraction, pH 1.6 and ultrasound intensity of 90% were selected for the co-extraction kinetic study. Co-extraction using citric acid was conducted at 2, 10, 20, 30, 60, and 90 mins with 3 temperature levels: 25, 55, and 85 °C. For comparison, HCl at pH 1.6 was also utilized as a solvent to co-extract at 85 °C.

To specifically understand the effects of different parameters on co-extraction, phenolic concentration (g/L) could be a better indicator for kinetic study, as it excluded the evaporation of solution induced by high heat. Phenolic concentration was the highest in CA+85 °C group (1.23-1.83 g/L) and the lowest in CA+25 °C group (0.99-1.28 g/L). Citric acid was a more effective extraction solvent compared with HCl, but the temperature was the dominating factor, like HCl, 85 °C (1.26-1.76 g/L) achieved a higher equilibrium concentration than CA+55 °C (1.19-1.51 g/L). These values were lower than the results from Pan et al. (2012), who applied ultrasound for polyphenol extraction in pomegranate peel using water and observed equilibrium concentration up to 2.8g/L. This indicated that acidic pH might interfere with the releasing of certain polyphenol components in source materials, such as phenolic acid. DSA varied from 3.09 g/g (g DPPH equivalent g^{-1}) to 5.66 g/g. No significant trend was observed at different conditions.

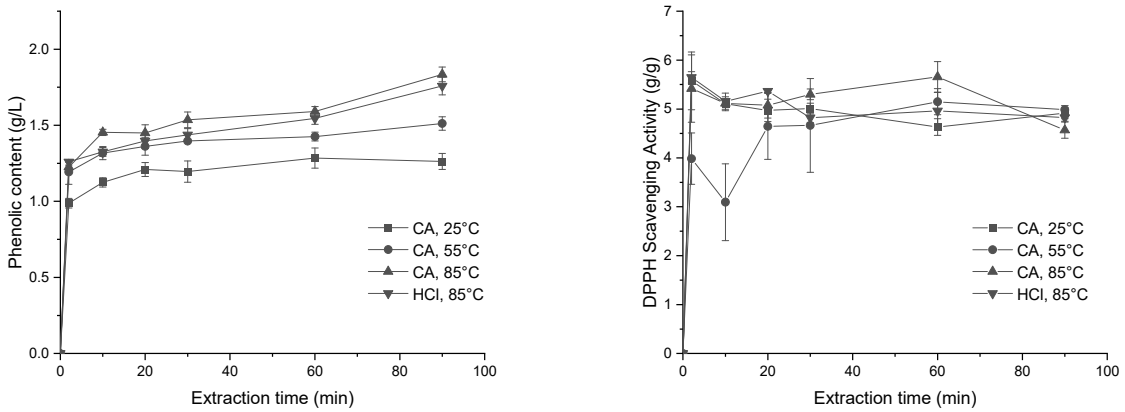

Figure 5.6 Comparison of total phenolic concentration (left) and DPPH scavenging activities (right) of co-extract from pomegranate peel at different extraction conditions

GalA content ranged from 47.78% to 83.20% and increased with higher temperature and longer extraction time. Pereira et al. (2016) applied conventional heating extraction of pomegranate peel pectin using citric acid and received similar GalA content (80.95±3.05%). Citric acid-extracted pectin tended to have a higher level of GalA than the HCl-extracted one since citric acid could selectively extract pectin chains rich in homogalacturonans, which further released GalA (Minjares-Fuentes et al., 2014; Umaña et al., 2019).

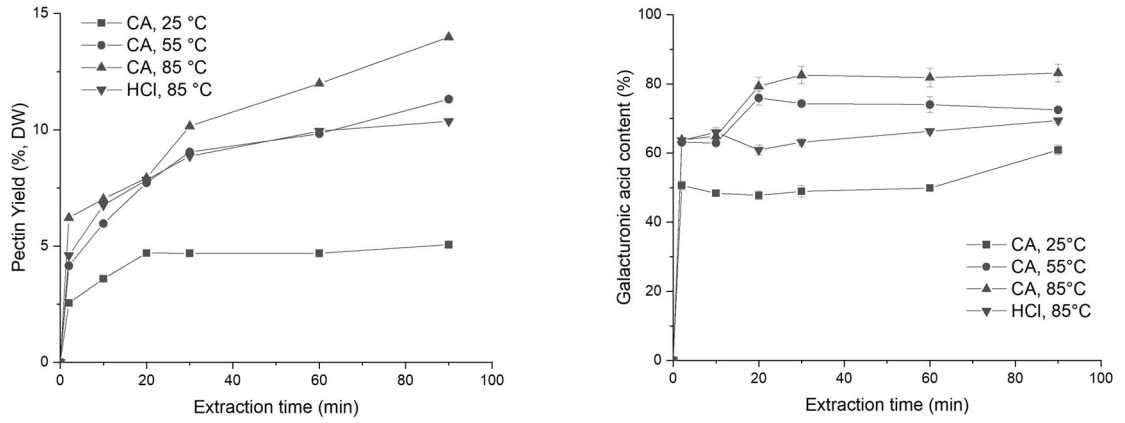

Figure 5.7 Pectin yield and galacturonic acid content of pectin extracted at different conditions

DM is the ratio of methylated GalA to the total amount of GalA. It is an important pectin property for applications in the industry. The initial DM depended on the material intrinsic properties and DM reached an equilibrium of de-methylation (a first-order reaction related to temperature and pH) at different processing conditions (Andersen et al., 2017). As shown in the current study (Figure 5.8), the degree of methylation (DM) varied from 53.31% to 79.66%. For each condition, no significant change of DM over-extraction was observed. CA-extracted pectin had higher DM (64.18% to 79.62%) compared with the HCl-extracted ones (53.31% to 62.00%), as HCl is a strong acid and could induce higher de-methylation. It was similar to research findings from Muñoz-Almagro et al. (2017), who observed a lower DM in nitric acid-extracted pectin. Except for extraction with CA at 25 °C, other extractions showed the tendency of decreasing DM relative to higher extraction time and temperature, which was in agreement with Andersen et al. (2017). Pereira et al. (2016) researched CA extraction of pectin in oven-dried pomegranate, at a temperature of 70 to 90 °C and pH 2 to 4 for 40 to 150 mins. They observed a set of DM ranging from 47.18 (80 °C, pH 2, 95 mins) to 71.45% (74 °C, pH 3.6, 62 mins), while pectin yield and DM were negatively correlated in CA-extracted extracts from pomegranate peel. The current study demonstrated the same tendency.

The percentage of GalA that is acetylated at positions O-2 and O-3 could be expressed as DA. DA is a major factor in pectin emulsifying properties. Hereby, DA ranged from 6.19% to 10.68% and slightly decreased with higher temperature and longer extraction time. The results were lower than research from Zhuang et al. (2019), who found 12% to 15% of DA in 3 Chinese var. of pomegranate peel. Shakhmatov et al. (2019) also obtained a high-DM (75%) and high-DA (15%) pectin from lyophilized pomegranate *Punica granatum.* However, the DA in the current study was still higher than that of some common pectin sources, including citrus (1% to

2%) and apple (2% to 4%) (J.-S. Yang et al., 2019). This indicated pomegranate peel had the potential to be applied as an effective emulsification agent, which was in agreement with the study from X. Yang et al., (2018).

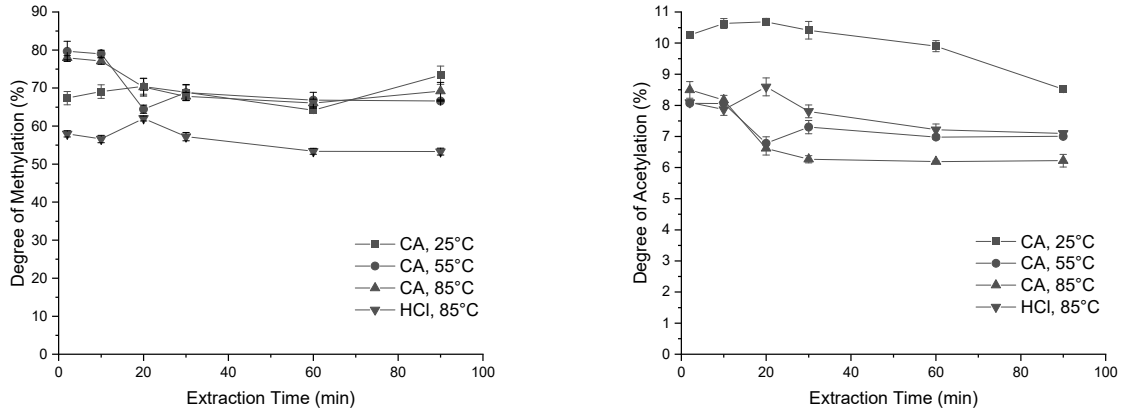

Figure 5.8 Degree of methylation and acetylation of pectin extracted at different conditions

Figure 5.9 demonstrated the FTIR spectra of pectin extracted at the aforementioned 4 different conditions. Absorption at different wavenumber reflects certain chemical structures -- Table 5.7 lists out some of the most discussed structures in the pectin study. The bands at 3406 cm^{-1} and 2930 cm^{-1} indicated the O–H stretching and the C–H stretching of the CH2 group, respectively. Similar patterns were observed by Pereira et al. (2016) in pomegranate peel. Absorbance at 1743 cm^{-1} and 1634 cm^{-1} represented the methylated and free GalA. DM could therefore be determined by the ratio of methylated GalA based on absorbance. The calculated values were (CA+25°C: 75.52%; CA+55°C: 68.68%; CA+85°C: 70.64%; HCl+85°C: 55.61%), which was close to the experimental data (CA+25°C: 73.38%; CA+55°C: 66.59%; CA+85°C: 69.21%; HCl+85°C: 53.31%).

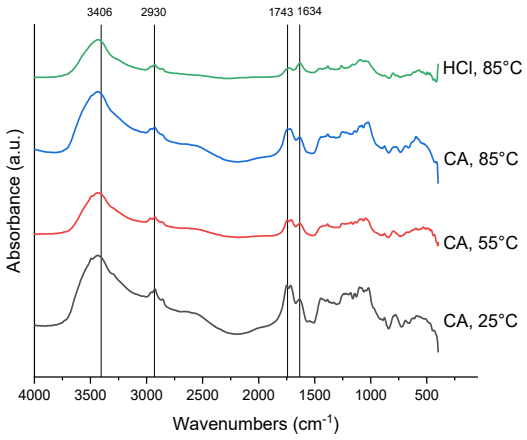

Figure 5.9 FTIR spectra of pectin extracted from pomegranate peel using citric acid and HCl at 25/55/85 °C for 90 mins.

Table 5.7 The chemical structures corresponded to different wavenumbers in FTIR

Wavenumber (cm^{-1})	Structure	Reference
3406	O-H stretching	Chen et al., (2012); Ogutu & Mu, (2017); Pang et al., (2018); Pereira et al., (2016); Wang et al., (2016); X. Yang et al., (2018)
2930	O-H stretching of CH_2 group	
1743	C-O stretching of COOR	
1634	C-O stretching of COO^-	
Fingerprint region below 1500	Couple C–C, C–O–C, and C–OH vibration modes of the carbohydrate ring and glycosidic linkage	
1486 to 1250	CH deformation vibration, 3 peaks within associated with COO^-, $COOCH_3$ and $CO\text{-}NH_2$	
1439	O-CH_3 deformation	
1354	O-H bending	
1225	-CH_3CO stretching	
1148.7	Asymmetric C-O-C stretching, indicating the presence of O-CH3	
1012	C-O-H deformation	
969	C-O bending	
919	Rocking mode of -CH3	
884	-CCH and -COH bending at the C-6 position	

5.4.3. Co-extraction kinetics on phenolic concentration and pectin yield

Figure 5.10 Comparison of extraction rate reciprocal (t/Ct) of polyphenol concentration (left) and pectin yield (right) from pomegranate peel over different extraction times (t) using

citric acid and HCl at different temperatures (°C). It exhibited the second-order model for co-extraction of polyphenol content (g/L) and pectin yield (%) in the linearized form. The kinetic parameters, including initial extraction rate (h), extraction rate constant (k), and equilibrium phenolic concentration (PCe), were determined by plotting t/PCt vs. extraction time (Table 5.8). With a high coefficient of determination (R^2=0.972-0.999) and great fitting of experimental data (Table 5.8), these second-order models were sufficient to describe co-extraction kinetics. This suggested that there could be two phenomena involved in co-extraction, including the dissolution and degradation of polyphenol (Pan et al., 2012) and pectin (Umaña et al., 2019; Xu et al., 2014; Zaid et al., 2019). Theoretically, the k parameter should increase with higher temperature as heat accelerate the mass transfer (Andersen et al., 2017; Sucheta et al., 2020). However hereby when increasing the temperature, the extraction rate constant dropped while the equilibrium level elevated for both polyphenol concentration and pectin yield. This finding was consistent with research from Patil & Akamanchi (2017), who extracted camptothecin from *Nothapodytes nimmoniana* using 76.4 to 191 Wcm^{-2} of ultrasound at 30 to 60 °C. Goula (2013) also extracted oil from pomegranate seeds using 130W ultrasound from 20 to 80 °C and observed a similar trend. In all these studies, the k parameter was negatively correlated to an equilibrium level. It was speculated that during ultrasound-assisted extraction, multiple processes (heat transfer, cavitation, etc) could jointly affect the extraction rate and result in various responses.

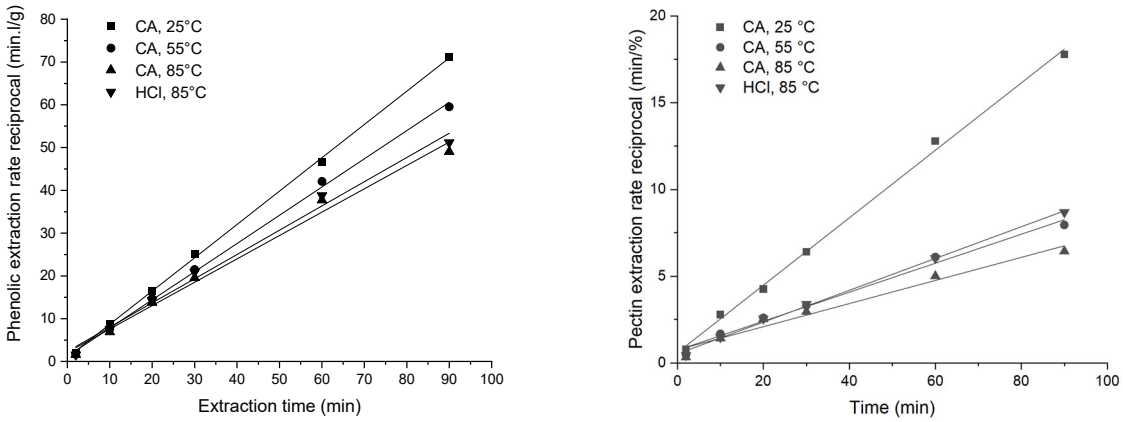

Figure 5.10 Comparison of extraction rate reciprocal (t/Ct) of polyphenol concentration (left) and pectin yield (right) from pomegranate peel over different extraction times (t) using citric acid and HCl at different temperatures (°C)

Table 5.8 Comparison of kinetic parameters of polyphenol concentration and pectin yield from pomegranate peel extracted using citric acid and HCl at different temperatures (°C)

	Condition (acid type, temperature)	**Initial extraction rate, h (%/min)**	**Extraction rate constant, k (%/min)**	**Equilibrium phenolic concentration, PCe (g/L)**	**R^2**
Phenolic Concentration (g/L)	CA, 25 °C	1.089	0.660	0.733	0.999
	CA, 55 °C	0.839	0.366	0.594	0.998
	CA, 85 °C	0.470	0.140	0.756	0.990
	HCl, 85 °C	0.422	0.136	0.620	0.990
	Condition (acid type, temperature)	**Initial extraction rate, h (%/min)**	**Extraction rate constant, k (%/min)**	**Equilibrium pectin yield, PCe (%)**	**R^2**
Pectin Yield (%)	CA, 25 °C	1.683	0.064	5.143	0.998
	CA, 55 °C	1.365	0.010	11.959	0.989
	CA, 85 °C	1.322	0.006	15.011	0.972
	HCl, 85 °C	1.910	0.016	10.913	0.997

5.5. Conclusions

In this chapter, a process of citric acid-based ultrasound-assisted co-extraction of pectin and polyphenol was established. The corresponding characteristics of extraction parameters and products were evaluated. Box-Behnken experimental design was applied first to study the major impacting factors on extraction performance, including pH, extraction temperature, extraction time, ultrasound intensity, and solvent ratio. After that, co-extraction kinetics of citric acid (25, 55, 85 °C) and HCl (85 °C) were investigated. The potential bonding mechanism of polyphenol and pectin in the extract for improved stability was also discussed based on FTIR results.

In the future, further characterization of pectin is expected to improve the utilization in food applications, such as monosaccharide composition, intrinsic viscosity, and zeta-potential. The effects of ultrasound on polyphenol composition could also be assessed, as pomegranate peel contains some of the most unique ellagitannins (punicalagins, punicalins, etc.). Similar to research from Buchweitz et al (2013), a simplified model solution that contains major polyphenols and pectin in pomegranate peel will also be helpful to understand the bonding mechanism within.

5.6. References

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Chapter 6 - Conclusions and Future Work

In this dissertation, the utilization potential of pomegranate peel was researched in a systematic approach, including designing extraction processes of functional ingredients, evaluating nutritional benefits via animal models, and developing value-added nutritional products. Major findings were listed as follows:

A novel green process for antioxidant extraction from wet pomegranate peel (WPP) was developed (Chapter 2). The effects of processing conditions on extraction performance, including particle size, extraction time, extraction temperature, and solvent ratio, were investigated. WPP extraction at 20°C for 6 min with a solvent ratio of 4:1 is recommended as an economic and sustainable process, resulting in 10.53% total phenolic yield (TPY) with 6.35 g g^{-1} tannic acid equivalent (TAE) and 88.93% punicalagin purity. Principal component analysis (PCA) condensed the multivariable analysis into 3 factors, which explained 99.79% of the variance and could be suitable for future process development. A thorough analysis of how polyphenol composition changes during extraction could be implemented in the future to provide more guidance for industrial applications.

The hypolipidemic effects of pomegranate peel powder (PPP) and pomegranate peel extract (PPE) towards a high-fat (20%) diet were researched (Chapter 3) via animal study. PPP and PPE demonstrated promising health-promoting effects by adjusting microbiota composition, increasing the microbiota diversity towards a leaner type, and regulating the expression of 2 hepatic genes (HMG-CoAR and LDLR) to reduce cholesterol ingestion and LDL uptake. However, further research on potential toxicity is required, as adverse plasma LDL-elevating effects were observed at a higher dose of PPP and PPE intake.

A PPE-fortified Greek Style yogurt was developed to boost polyphenol consumption based on current USDA dietary guidelines (Chapter 4). Product nutritional properties and textural characteristics were linearly correlated with protein content and extract addition. The optimum formula for a 130g (4.6 oz) product consisted of 10.4g (8%) of protein and 77g (59%) of PPE, which could satisfy the polyphenol daily need without compromising product properties. A shelf-life study with proper flavoring work would be beneficial for further product commercialization.

An ultrasound-assisted co-extraction process of polyphenol and pectin using citric acid was investigated for thorough WPP utilization and broader industry applications (Chapter 5). The effects of process conditions on extraction performance were evaluated, including pH, extraction temperature, extraction time, ultrasound intensity, and solvent ratio. High methylated (Degree of methylation up to 79.66%) pectin yielded up to 13.99%. The product properties and extraction kinetics were characterized and proved the applicability of citric acid (organic acid) for an improved co-extraction process. In the future, more characteristics are expected to be identified to study the interaction mechanism of pectin and polyphenol during extraction. Therefore, the process could be more controlled for the production of certain compounds.