Evaluation and Optimization of Pomegranate Peel Extract and Powder for Rice Milk Paneer Shelf Life

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Abstract:

Pomegranate peel byproduct contains various phenolic compounds with varying health benefits. Many extraction methods were developed and utilized for pomegranate peel extraction (PPE) with varying effect on its bioactive compounds. We hypothesized that micro wet milling (MWM) can be used to extract such compounds from fresh peel without drying to powder, as their particle size is reduced to micrometers. This will preserve the bioactive compounds as no heat treatment is involved in this proposed extraction method. To assess the MWM extraction process, response surface methodology (RSM) was used to determine the optimum extraction conditions. The main influential factors such as rotational speed (RS) 20–50 rpm, solid to solvent ratio (SS) 5–30 g/100 mL, and ethanol to water ratio (EW) 30–80% were optimized for total phenolic content (TPC), total anthocyanin content (TAC), punicalagin, ellagic acid (EA), gallic acid (GA), catechin, and epicatechin. The optimum operational conditions were 50 rpm of RS, 19.62 g/100 mL of SS, and 49.65% v/v of EW. The most signifcant infuential factor for pomegranate peel extraction was SS. The optimum phenolic compound values were 225.7 mg GAE/g dw of TPC, 3.1 mg/g dw of TAC, 71.6 mg/g dw of punicalagin, 6.9 mg/g dw of ellagic acid, 6.2 mg/g dw of gallic acid, 13.1 mg/g dw of catechin and 14.8 mg/g dw of epicatechin. Validation of the model and its comparison with the results obtained from the ultrasonic bath extraction method and scanning electron microscopy (SEM) revealed that MWM can be used to mill the peel into micro-scale particles making the extraction of pomegranate peel phenolics easier.

In the second part of the study, pomegranate peel powder was produced by spray drying after micro wet milling. The peel powder was produced with the purpose of using the fiber rich whole peel. The pomegranate peel was micro wet milled (MWM) to produce smaller particles increasing the extraction of phenolics in the whole peel slurry. The process parameters were adjusted to the optimum condition of MWM optimized in first part of the study. The MWM slurry was subjected to spray draying at 120, 140 and 160 °C. Maltodextrin as carrier agent, was used at 20%, 40% and 60% on a dry basis. The powder was optimized for total yield and analyzed for total phenolics content, total anthocyanin content, water activity,

color, solubility index, and water holding capacity. The morphology of powder was analyzed with scanning election microscopy. The results showed that the whole peel powder can be obtained with high yield and bioactive compounds.

In a comparative study, the peel extract was converted to powder using maltodextrin as wall material. The pomegranate peel phenolics were extracted using MWM. The extract was then concentrated in a rotary evaporator at 40°C to remove the excess ethanol from the extract. The spray dryer was adjusted to 120 °C as optimized for the whole peel powder. The wall material maltodextrin was chosen and integrated at 20%, 40%, and 60%. The powder was analyzed for its phytochemical and nutritional properties. Results revealed that the pomegranate peel extract powder has higher nutritional properties compare to whole peel powder. The nutritional properties of powder showed that the powder produced with minimum maltodextrin (20%) has higher TPC, TAC and antioxidants. While increasing the maltodextrin level increased the solubility and density of extract powder.

In the last chapter of this study, pomegranate peel powder was incorporated in soft cheese (paneer) as natural antioxidants and preservative. Paneer samples were stored in sterile zip lock plastic bags for 10 days at 4°C. The storage test was carried out with analysis of the physiochemical and biological properties of paneer. Our findings indicate that the addition of pomegranate peel powder increased the TPC and antioxidant properties of paneer. The total bacterial count shows that all the samples were under acceptable standard log of bacterial count. The bacterial count significantly decreased with higher in the pomegranate peel powder sample compared to the control sample. This verifies the antibacterial effect of the peel powder. There was no obvious defect detected in all the samples for 10 days storage while samples treated with pomegranate peel powder exhibits higher nutritional values and lower microbial counts.

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1.1 Introduction and literature review

1.1.1 Cultivation history

Pomegranate fruit (*Punica grantum*) originated and are native to the Near East region. It was cultivated in ancient Iran, Egypt, Afghanistan, India and later spread to the Mediterranean region, China and more. Traditionally, aside from being consumed, pomegranate fruit was used as a remedy for ailments since early history (Chandra *et al.*, 2010; Prakash and Prakash, 2011). About 3000 cultivars has been defined, the famous cultivar is 'Wonderful' which is growing worldwide. Pomegranate plant is shrub like tree, grows up to 9 m, has a long-life cycle, some of the plants are reported to lived up to 200 years, however the production start to decline after 15 years.

Afghanistan is one of the tenth high producer of pomegranate in the world. About 2% of horticultural products of the country is consisted of pomegranate production. The production is mainly from Balkh, Helmand, Nimroz, and Kandahar provinces. Kandahar province is the main producer of pomegranate, the annual production of Kandahari cultivar is approximately 20,000 metric tons. Pomegranate produced in Afghanistan is famous around the world especially in south Asian countries due to it distinguish qualities(Glozer and Ferguson, 2008).

1.1.2 Global statistics and market for pomegranate

According to the global market report (Market study report) for pomegranate it is valued at about $214.9 million USD in 2019 and is perceived to increase by an additional 6.1% by 2025.

1.1.3 Nutritional properties and composition

The growing popularity of pomegranate is mainly due to its nutritional values and with the increasing recent findings reports the therapeutic values of this fruit. Table 1; shows some of the major nutritional composition of the pomegranate fruit. Pomegranate fruit is commonly either consumed as fresh fruit or further processed to juice at food processing factories. However, its peel which accounts for 50 % of the whole fruits proportion is usually discarded (Al-Said, Opara and Al-Yahyai, 2009). As the global production and market for the fruit increases, it will also increase the peel waste. The peel consists of a variety of bioactive compounds recognized for its medicinal attributes. Therefore, many recent studies have focused on the phenolics composition of the peel and its antioxidant, antimicrobial, and antifungal activities.

Table 1: Nutritional composition of pomegranate fruit

Constituent	Unit	Value per 100 grams of edible portion
Water	g	80.97
Energy	Kcal	68
Protein	g	0.95
Total lipid	g	0.3
Ash	g	0.61
Carbohydrates by difference	g	17.17
Total dietary fiber	g	0.6
Total sugars	g	16.57
Calcium	mg	3
Iron	mg	0.3
Magnesium	mg	3
Phosphorus	mg	8
Potassium	mg	259
Sodium	mg	3
Zinc	mg	0.12
Copper	mg	0.07
Selenium	mcg	0.6
Vitamin C	mg	6.1
Thiamin	mg	0.03
Riboflavin	mg	0.03
Niacin	mg	0.3
Pantothenic acid	mg	0.596
Vitamin B6	mg	0.105
Total folate	mcg	6
Vitamin A	IU	108
Vitamin E	mg	0.6
Vitamin K	mcg	4.6

Note: This table is reproduced from (KV and D, 2016)

Table 2; shows the composition of pomegranate peel. Pomegranate peel consists of various components, such as dietary fibers, total sugars, protein, fat, moisture, and so forth.

1.1.4 Application in food

Many studies have reported the varying use of the peel for instances as a natural dye (granatonine) for cotton clothes as the peel contains alkaloid (N-methyl granatonine) which is responsible for its red color (Kulkarni SS *et al.*, 2011). Peel powder was supplemented in cookies, the results revealed that the nutritional properties was significantly improved in total dietary fibers from 0.32 to 1.96 g/100 g, total phenolics contents from 90.7–161.9 mg GAE/100 g, inorganic residues from 0.53–0.76 g/100 g as well as Ca, K, Fe and Zn levels. Its antioxidant activity was shown to be 50% and it reduced oxidative degradation during four months of storage (Ismail *et al.*, 2014). Similarly, the supplement of pomegranate peel powder in muffin cake, increased the total dietary fibers total phenolics, Mg, Ca, and K, and it also increased the hardness of the cake by 15% (Topkaya and Isik, 2019). The pomegranate peel powder was also incorporated in pan bread and the impact of the varying amounts of peel powder on the weight loss on thirty-six female albino adult rats were studied. Results showed that bread with 7.5% pomegranate peel powder reduced 47% of body weight in rats (E. F., 2014). The pomegranate peel extract was incorporated into the polyvinyl alcohol (PVA) composite film for preparing food packaging; results revealed that peel extract increased the antibacterial ability of the film encounter to *E. coli* and *S. aureus* bacteria (He *et al.*, 2019). The peel extract was incorporated in dairy cow's diet and results showed that the increase in peel extract reduced the protozoa population and decreased the NH3–N amount which resulted in an increase in microbial protein and milk yield and its quality (Abarghuei *et al.*, 2013). The pomegranate peel phenolics were extracted by ethanol, and the powder was produced with whey protein concetrate-70 (WPC-70) and skimmed milk powder (SMP). This powder was incorporated into curd for shelf-life extension, the results showed that the curd had reduced bacterial communities as shown by the bacterial count and the shelf-life was extended by 6 days compare to the control samples (Sandhya *et al.*, 2018). Pomegranate peel and seed extract was investigated in popular chicken meat revealed that the peel has higher antioxidant activities compared to seed and the shelf-life of chicken was improved by 2-3 weeks in chilled storage (Kanatt, Chander and Sharma, 2010).

Table 2: Composition of pomegranate peel

Component	Unit g/100 g	Reference
Total sugar	21.14	(Sumaiya, Jahurul and Zzaman, 2018)
Glucose	6.6	(Dafny-Yalin *et al.*, 2010)
Fructose	4.8	(Dafny-Yalin et al., 2010)
Total fiber	50.3	(Viuda-Martos *et al.*, 2012)
Soluble fiber	19.9	(Viuda-Martos et al., 2012)
Non soluble fiber	30.4	(Viuda-Martos et al., 2012)
Protein	10.9	(Viuda-Martos et al., 2012)
Ash	3.3	(Al-Rawahi *et al.*, 2013)
Moisture	8.8	(Al-Rawahi, Rahman, Guizani, & Essa, 2013)
Fat	1.3	(Al-Rawahi, Rahman, Guizani, & Essa, 2013)
Total soluble solid	7.6	(Al-Rawahi, Rahman, Guizani, & Essa, 2013)

The chemical composition of pomegranate peel is presented in Table 3; and the structure of some major compounds are shown in Figure 1 and 2. Several, studies reported the correlation of these compounds consisted in the peel with antioxidants, antibacterial, antifungal and antiproliferative properties. The ellagitannins and polyphenols content of pomegranate peel showed inhibitory abilities against some pandemic viruses such as H5N1 of influenza virus, HIV, Hepatitis B & C(Kotwal, 2008). Specific compounds such as ellagic acid reduces the proliferative activity of prostate cancer cells, gallic acid showed anti-carcinogenic activity in human cells, and punicalagin is correlated to antifungal activity (Bell and Hawthorne, 2008; Elango, Balwas and Padma, 2011; Rongai *et al.*, 2019). In addition, punicalin, catechin, epicatechin, anthocyanin and dietary fibers of the peel exhibited high antioxidant activities (Plumb *et al.*, 2002, 2017; Orak, Yagar and Isbilir, 2012; Hasnaoui, Wathelet and Jiménez-Araujo, 2014). The peel also contains other trace compounds such as; quinic acid, bis-HHDP-glucoside isomers, 2-O-galloylpunicalagin, digalloylpentoside, galloyl-HHDP-glucoside, apigenin-O-hexoside, luteolin-O-hexoside with antioxidant and anti-microbial properties (Negi, Jayaprakasha and Jena, 2003; Saad Sabbar Dahham, Mir Naiman Ali, 2010; Bachoual *et al.*, 2011; Song, Li and Li, 2016; Alexandre *et al.*, 2019).

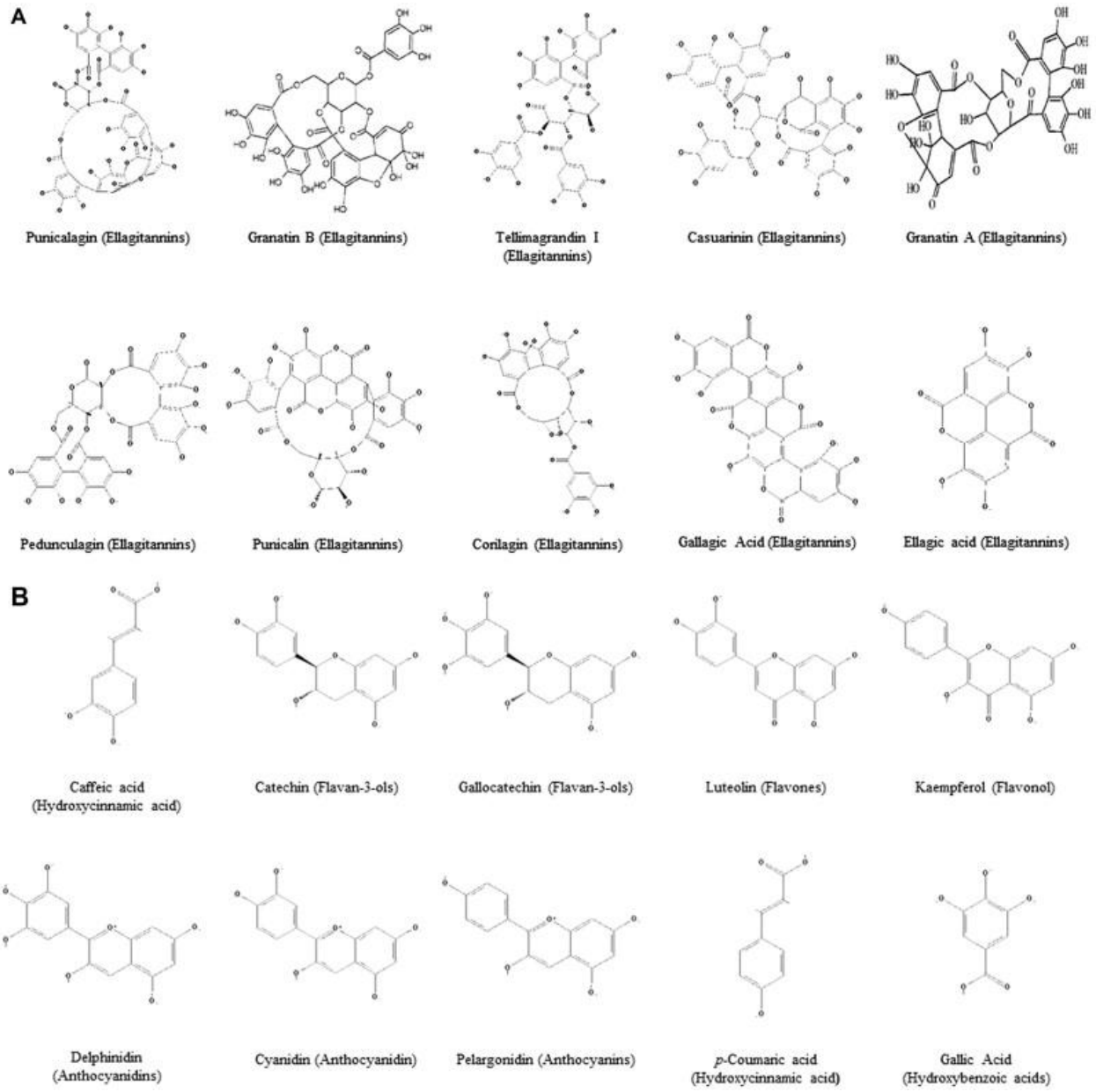


Figure 1. Chemical structures of certain compounds in pomegranate peel (Akhtar *et al.*, 2015).

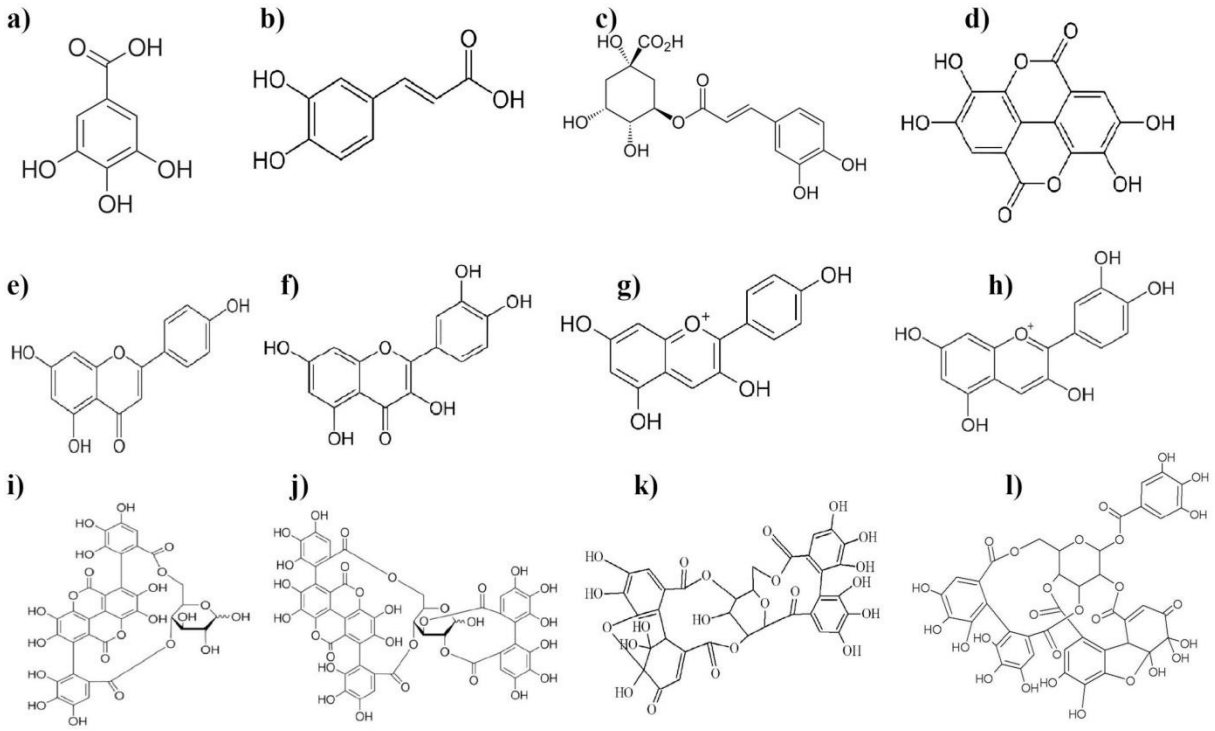


Fig.2. Chemical structures of major compounds found in the pomegranate peel (Singh *et al.*, 2018).

Gallic acid (a), caffeic acid (b), chlorogenic acid (c), ellagic acid (d), apigenin (e), quercetin (f), pelargonidin (g), cyanidin (h), punicalin (i), punicalagin (j), granatin A (k) and granatin B (l).

Table 3: Chemical composition of pomegranate peel

Component (Unit)	Amount	Reference
TPC mg QE/g	64.64	(Sumaiya, Jahurul and Zzaman, 2018)
Flavonoid mg QE/g	60.05	(More and Arya, 2019)
Hydrolyzable tannins mg TAE/g DW	262.7	(Saad *et al.*, 2012)
Condensed tannins mg CE/g DW	9.5	(Saad et al., 2012)
Gallic acid mg/g	0.053	(Kazemi *et al.*, 2016)
Ellagic acid mg/g	20.66	(Kazemi, Karim, Mirhosseini, & Abdul Hamid, 2016a)
Punicalin mg/g	65.67	(Živković *et al.*, 2018)
Punicalagin mg/g (α, β)	146.61	(Kazemi *et al.*, 2016)
Caffeic acid mg/100 g	17	(Zaki *et al.*, 2015)
Protocatchoic acid mg/100 g	58	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Vanillic acid mg/100 g	33	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
P-Cumaric acid mg/100 g	10	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Chlorogenic acid mg/100 g	493	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Ferulic acid mg/100 g	144	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Catechin mg/100 g	50	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Epicatechin mg/100 g	56	(Zaki, Abdelatif, Abdelmohsen, & Ismail, 2015)
Vitamin A (µg/gm)	14.06	(Kushwaha, 2013)
Zinc (µg/gm)	8.03	(Kushwaha, 2013)
Copper (µg/gm)	6.2	(Kushwaha, 2013)
Iron (µg/gm)	22.6	(Kushwaha, 2013)
Phosphorus (mg/kg)	33.96	(Kushwaha, 2013)
Magnesium (mg/kg)	1644.47	(Kushwaha, 2013)
Calcium (mg/kg)	645.70	(Kushwaha, 2013)
Potassium (mg/kg)	16237.41	(Kushwaha, 2013)
Sodium (mg/kg)	763.66	(Kushwaha, 2013)

1.1.5 Extraction of phenolic compounds

These bioactive compounds can be extracted via numerous methods such as pressurized, microwave-assisted, ultrasonic-assisted extractions (Çam and Hişil, 2010; Živković *et al.*, 2018; Kaderides *et al.*, 2019). Pomegranate peel phenolics were extracted with continuous high voltage electrical discharge (HVED) extraction system, results indicated that HVED can be used with high efficiency extraction with 196.7 ±6.4 mg/g phenolic contents (Xi, He and Yan, 2017). The pressurized liquids was combined with ultrasound to improve the extraction of phenolic compounds from pomegranate peel and the influence of solvent type, temperature, ultrasonic energy, particle size and number of cycle were measured for optimum conditions (Sumere *et al.*, 2018). Other known methods includes; continuous shaking extraction, maceration, hot water infusion, ethanol and acidified water, Soxhlet (Masci *et al.*, 2016; Ghosh, Chatterjee and Chalkroborty, 2019). The efficiency of these methods are based on the optimum yield of extraction conditions along with cost, energy, and time consumed. The main factors affecting the extraction process are; particle size of raw material, solid to solvent ratio, extraction temperature, and time (Wang, 2011). Both conventional and newest methods of phenolic extraction of pomegranate peel usually incorporates the drying process and dry milling to powder for smaller particles sizes before extraction. However, the drying process can affect bioactive compounds resulting in lower phenolics yield except when using a freeze-drier, which is expensive in comparison to the former (Mphahlele *et al.*, 2016; López-Vidaña, 2017).

Currently, the demand for environmental friendly extraction practices is sought-after due to global warming, resource sustainability and food security issues (Talekar *et al.*, 2019). Consequently, in this study, micro wet milling (MWM) process was used to extract the pomegranate peel phenolics. The MWM system with stone grinders can shorten the extraction process, as the extraction was carried out on micro milled slurry, avoiding drying and dry milling before extraction. The MWM was reported to produce smaller particle size (approximately 21 µm of D50) with adjusted milling rotational speed (RS), material feeding rate, and milling time (Li, Kokawa and Kitamura, 2018; Islam, Kitamura, *et al.*, 2020). Polyphenols extracted with pressurized extraction method stated that smaller particle size increases the extraction yield and decreases extraction time (Çam and Hişil, 2010; Qu, Pan and Ma, 2010). The effect of particle size was studied on tea and ginger powder extraction where smaller sized

particles showed greater results for total phenolic content (TPC) and antioxidant properties (Makanjuola, 2017). Smaller particle size facilitates the solvent to penetrate the plant cell wall and makes the extraction process easier by increasing the surface area leading to higher phenolics yield (Pătrăuţanu *et al.*, 2019). Ethanol (food grade) was used and mixed with water as a green extraction solvent. It increases the solvent polarity that is favorable in phenolics extraction (Venkataramanamma, Aruna and Singh, 2016). Solid to solvent ratio (SS) is another constraint which affects the extract yield and needs to be adjusted for the extraction solvent equilibrium (Wang, 2011). The process of pomegranate peel extraction was optimized through different experimental designs. Response surface methodology (RSM) was selected on the basis of simplicity and efficiency as the design tool for minimizing the experimental runs, time and energy consumption (Ali *et al.*, 2018). Currently, no studies have reported the use of MWM as an extraction process for pomegranate peel phenolic extraction.

1.1.6 Objectives

The objectives of this study are to determine the optimum conditions for MWM phenolics extraction of pomegranate peel by RSM design as a new extraction method. In addition, the extraction process was shortened through extraction of the peel extract without drying. The impact of RS, SS, and EW were determined for total phenolics content (TPC), total anthocyanin content (TAC), and the concentrations of punicalagin, ellagic acid, gallic acid, catechin, and epicatechin. The correlation of particle size and optimum extraction conditions for total and individual phenolics were also evaluated.

1.2 Material and methods

1.2.1 Material:

'Wonderful' cultivar pomegranate fruit (*Punica grantum*) imported from the USA and purchased from local market was used in this study. The aril (edible part including the seed) and pulp membranes were manually separated from the peel. The peel was vacuum packaged and stored at -60 °C until further use.

Figure 3. The primary process of pomegranate peel

1.2.2 System specification

The MWM system is primarily used for the milling of wet solid materials to micro sized particles. The system consists of two milling stones atop each other placed horizontality. The upper stone is fixed while the lower stone rotates clockwise by an electric motor. The stone rotational speed is controlled with the controller panel and rotates at a range of 20-50 rpm. Feeding rate is adjusted through a pump attached to the system. The gap size between the stones is adjusted with a controlling screw and the additional weight is attached to the alloy metal to keep the gap size by pressing. The sample was fed by a pump to the MWM system, and the milled material (slurry) was collected in a 1000 mL beaker. The MWM process was carried out at room temperature (Amini *et al.*, 2019).

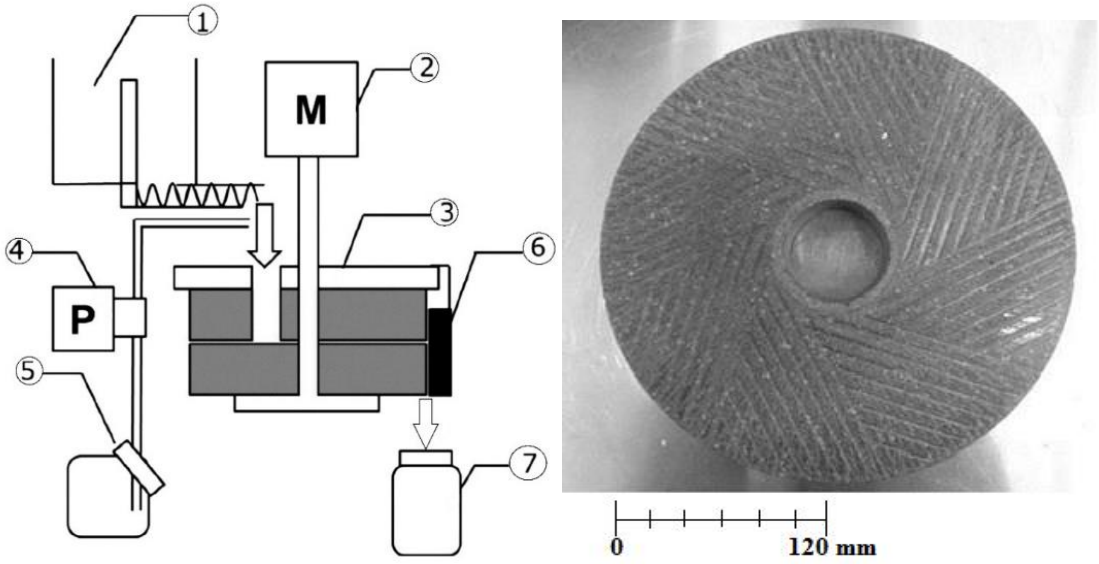


Figure 4. Schematic diagram of the micro wet milling system. (1) Rotary Rice Feeding equipment (2) Motor (3) Milling stone (4) Tubing Pump (5) Water tank (6) Rubber Spatula (7) Receiver. Picture of the lower stone of mill. (Koyama and Kitamura, 2014)

1.2.3 Extraction:

The frozen peel was thawed then cut to small pieces with a kitchen knife. The peel was mixed with solvent (ethanol and water at different ratios) and blended for 1 min with blender (Hamilton Beach-number, Japan). Afterwards, the mixture was fed to the MWM system at a feeding rate of 20 mL /min. The MWM system was adjusted to the required RS. The slurry was collected and then centrifuged at 6000 **g** for 10 min, the process flow diagram is shown in Figure 4. The supernatant (extract) was collected and stored at -60 °C until further analysis.

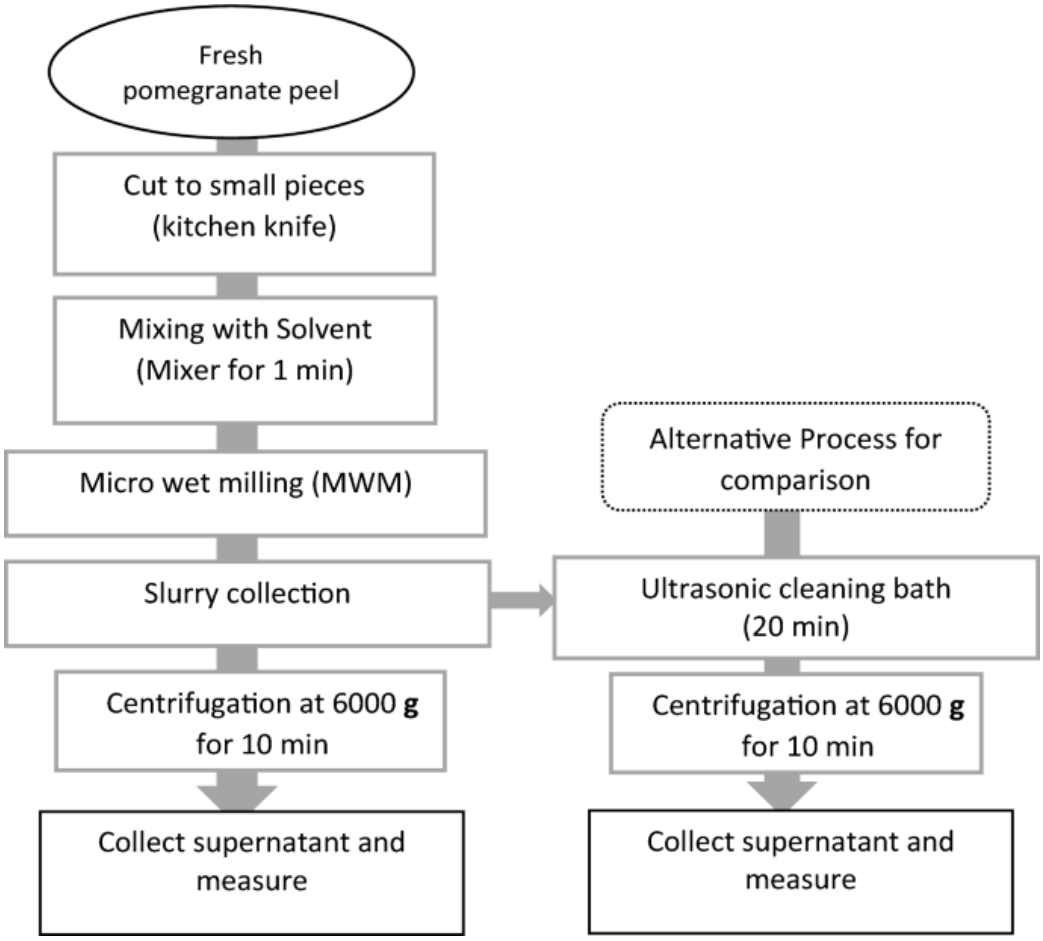


Figure 5. Flow diagram of MWM process for pomegranate peel phenolics extraction

1.2.4 Ultrasonic bath extraction

The ultrasonic bath extraction was carried out using an ultrasonic cleaning bath as a study previously reported with slight modifications (Živković *et al.*, 2018). The optimized MWM slurry was split into two parts. One part of the slurry was directly centrifuged, and the second part was extracted with an extended process of ultrasonic bath extraction for 20 min. The extracted slurry was centrifuged (6000 **g** for 10 min) and the supernatant was analyzed for total and individual polyphenols.

Figure 6. Image of ultrasonic cleaning bath

1.2.5 Experimental design

The central composite design (CCD) was developed to optimize the extraction of total and individual phenolics through a set of experimental runs. CCD was chosen to determine the optimum condition and maximize the extraction of phenolic compounds. Five influencing factors were identified through literature reviews and single factor experiments. Feeding rate, RS, SS, EW and particle size were chosen as the significant factors in the MWM process. The factors were then examined, and the most influential factors were selected for CCD design to minimize experimental runs. The most influential factors: RS, SS, and EW were aligned in polynomial equations. The RSM design comprised of 19 randomized runs with 3 replicates at the center points and each variable was tested at 5 levels. The design was optimized for seven responses: TPC, TAC, punicalagin, ellagic acid, gallic acid, catechin, and epicatechin. The real and coded values of RSM design are shown in Table 1. The model was design for each response as formulated in following equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{12} X_2^2 + \beta_{13} X_3^2 + \beta_{21} X_1 X_2 + \beta_{22} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the response, and X_1, X_2, X_3 are RS, SS, and EW, respectively. βo is the intercept and β_1, β_2, β_3 are the linear, β_{11}, β_{12}, β_{13} are the quadratic, and β_{21}, β_{22}, β_{23} are the interaction regression coefficients.

Table 4. The coded and actual values of RSM design

Runs	Coded values			Actual values		
	X_1	X_2	X_3	X_1	X_2	X_3
1	-	-	-	20	5	30
2	-	+	+	20	30	80
3	+	+	-	50	30	30
4	0	0	0	35	17.5	55
5	-	-	+	20	5	80
6	0	0	0	35	17.5	55
7	0	0	0	35	17.5	55
8	+	-	+	50	5	80
9	A	0	0	50	17.5	55
10	0	0	0	35	17.5	55
11	0	a	0	35	5	55
12	0	A	0	35	30	55
13	+	+	+	50	30	80
14	0	0	A	35	17.5	80
15	0	0	a	35	17.5	30
16	+	-	-	50	5	30
17	-	+	-	20	30	30
18	0	0	0	35	17.5	55
19	a	0	0	20	17.5	55

1.2.6 HPLC analysis

The individual phenolics were quantified with high performance liquid chromatography (HPLC) (Çam and Hişil, 2010). HPLC (LC-20A Prominence, SHIMADZU) with autosampler and degasser was used for individual

phenolics measurements. Data collection and analysis were accomplished with LabSolution LC/GC software. The samples extraction and separation were determined using a C18 VP-ODS (250 L × 4.6 mm) column. The mobile phase 'A' composed of 2% acetic acid in HPLC grade distilled water (DW) and mobile phase 'B' comprised of methanol at 1 mL min^{-1} flow rate. The elution program was set for the mobile phase as 5% B for 5 min, 5–70% B for 25 min, and 70–5% B for 10 min. The column temperature was kept at 35 °C and the spectra was recorded from absorbance at wavelengths of 280 nm and 378 nm, as shown in Figure 2a and b. Punicalagin and ellagic acid were quantified from the absorbance at 378 nm, while gallic acid, catechin and epicatechin were quantified from 280 nm chromatograms. Standards of punicalagin (500 µg/ L), ellagic acid, gallic acid, catechin, and epicatechin (1000 µg/L) were prepared and diluted accordingly. The samples and standards were filtered through filters of 0.45 µm pore size and 10 µL aliquots were injected in the HPLC.

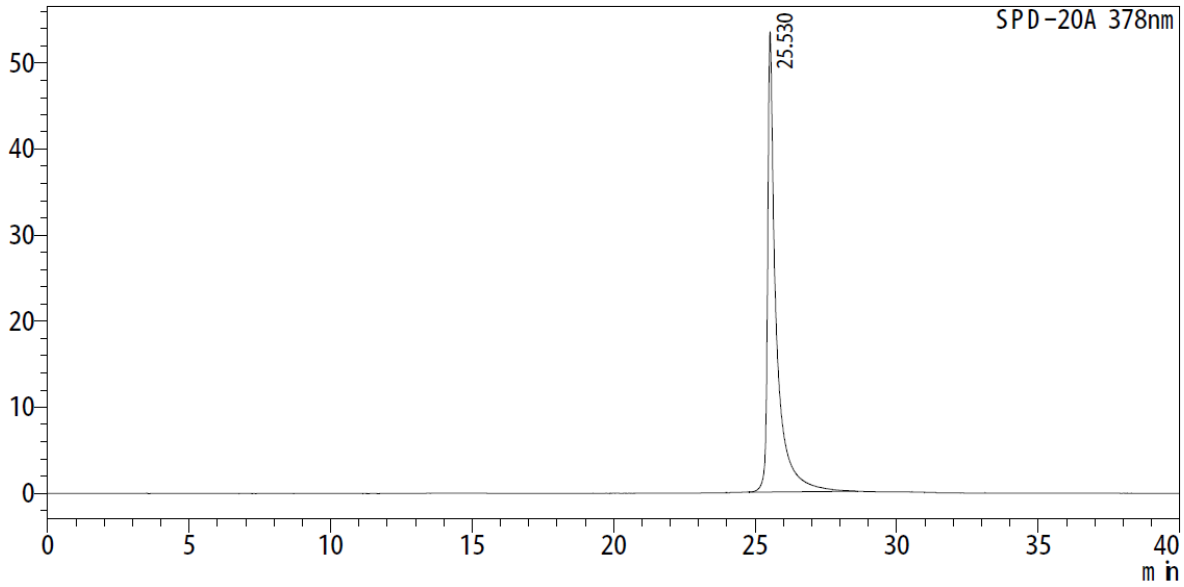


Figure 7. Standard of chromatogram of Ellagic acid

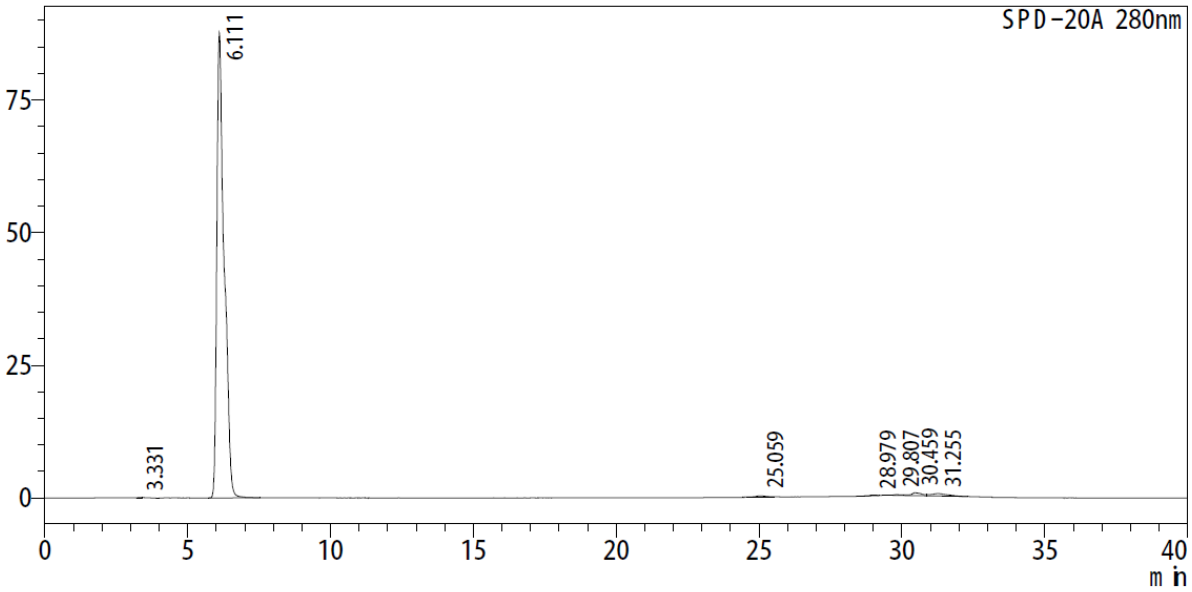


Figure 8. Standard of chromatogram of Gallic acid

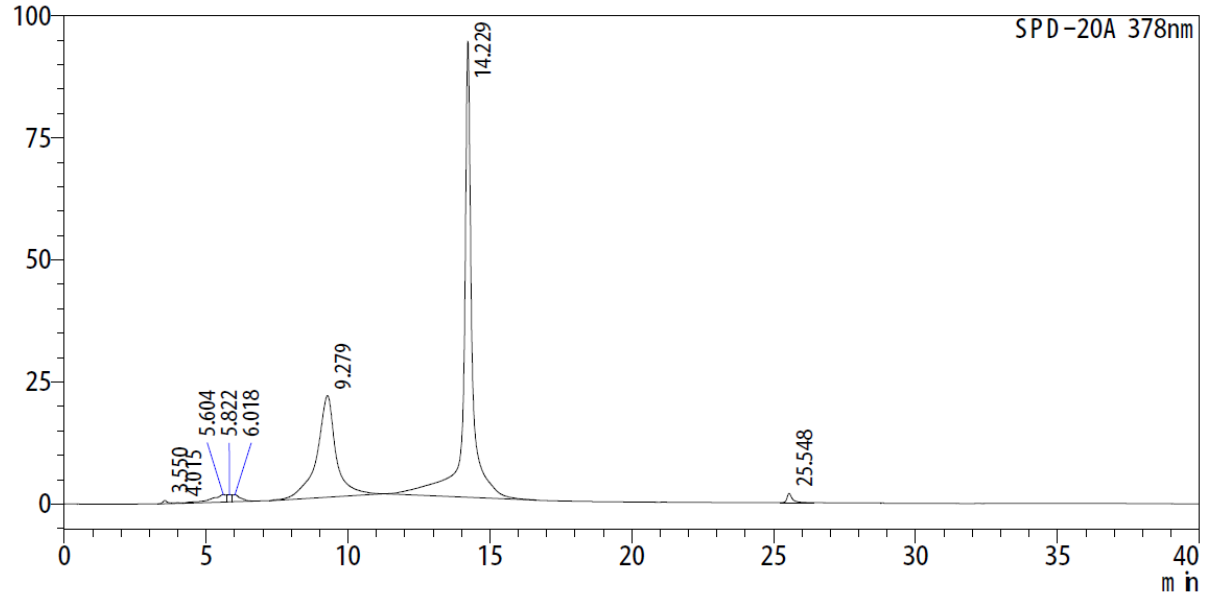


Figure 9. standard of chromatogram of Punicalagin α (9.279) punicalagin β (14.229)

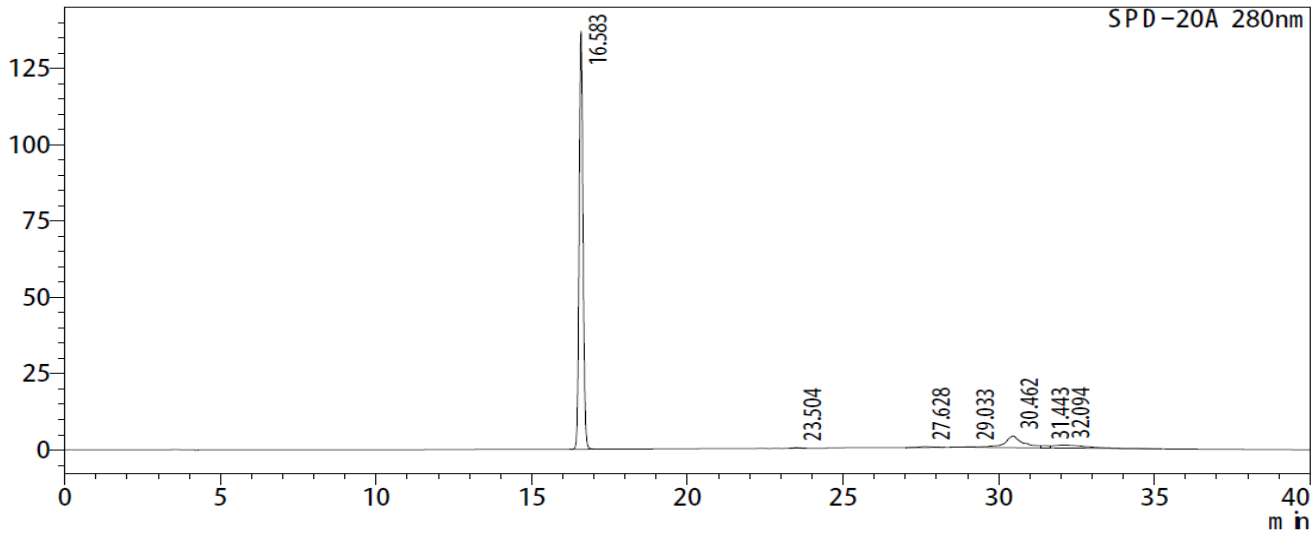


Figure 10. standard of chromatogram of catechin

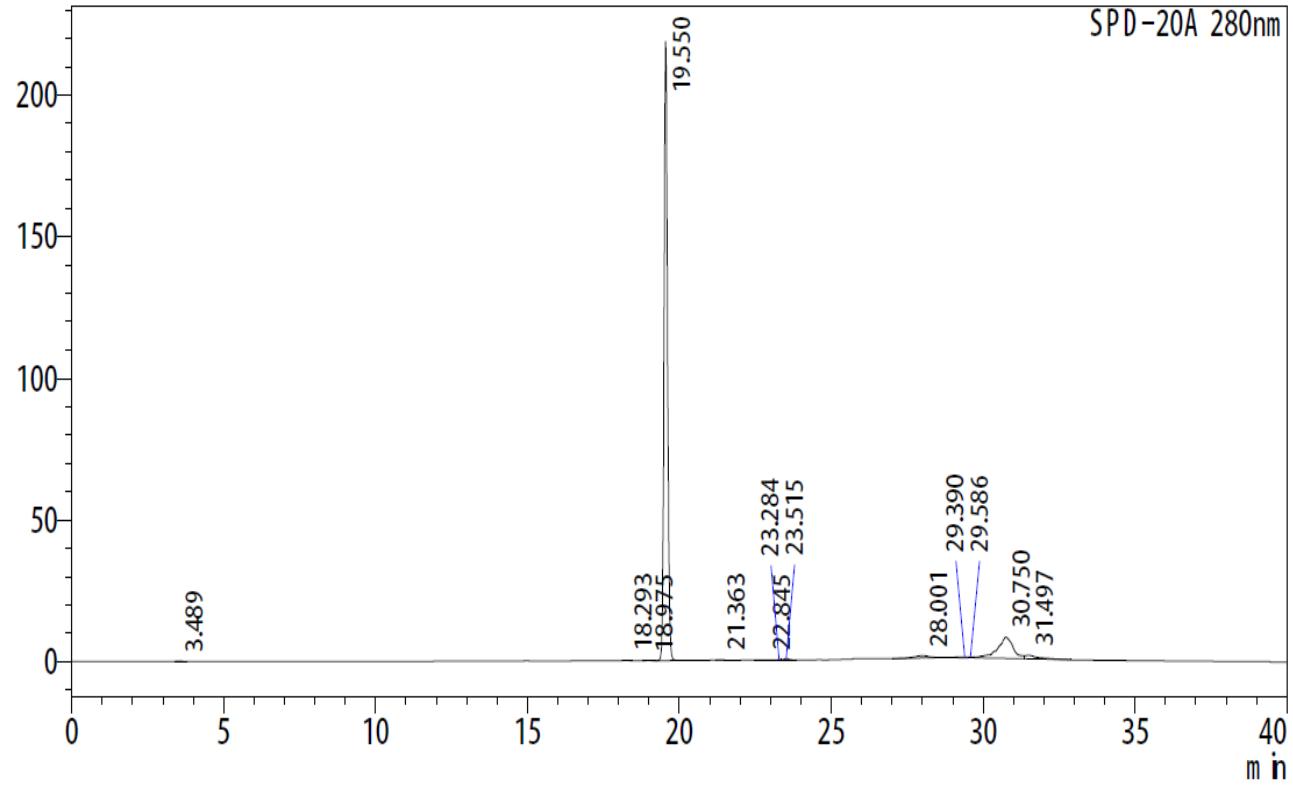


Figure 11. Standard of chromatogram of epicatechin (19.55)

1.2.7 Total Phenolic Contents (TPC)

TPC was determined accordingly (Hatami *et al.*, 2014) with slight modifications. The solutions of 10% Folin and Ciocalteu (FC) reagent was prepared with addition of 10 mL FC to 90 mL distilled water and 7.5% Na_2CO_3 were prepared 7.5 g of Na_2CO_3 in 100 mL distilled water. The pomegranate peel extract was diluted to 1/100. Sample aliquots of 0.5 ml were added and mixed with 2.5 ml of FC reagent then rested for 1 min. Afterwards, 2 ml of 7.5 % Na_2CO_3 was added and mixed then left to rested for 30 min in the dark, at room temperature. The absorbance was read at 760 nm using a spectrophotometer (JASCO V630 Japan). Gallic acid serial solution was prepared and measured following the same procedure. The standard curve was plotted the observance against the concentration of gallic acid.

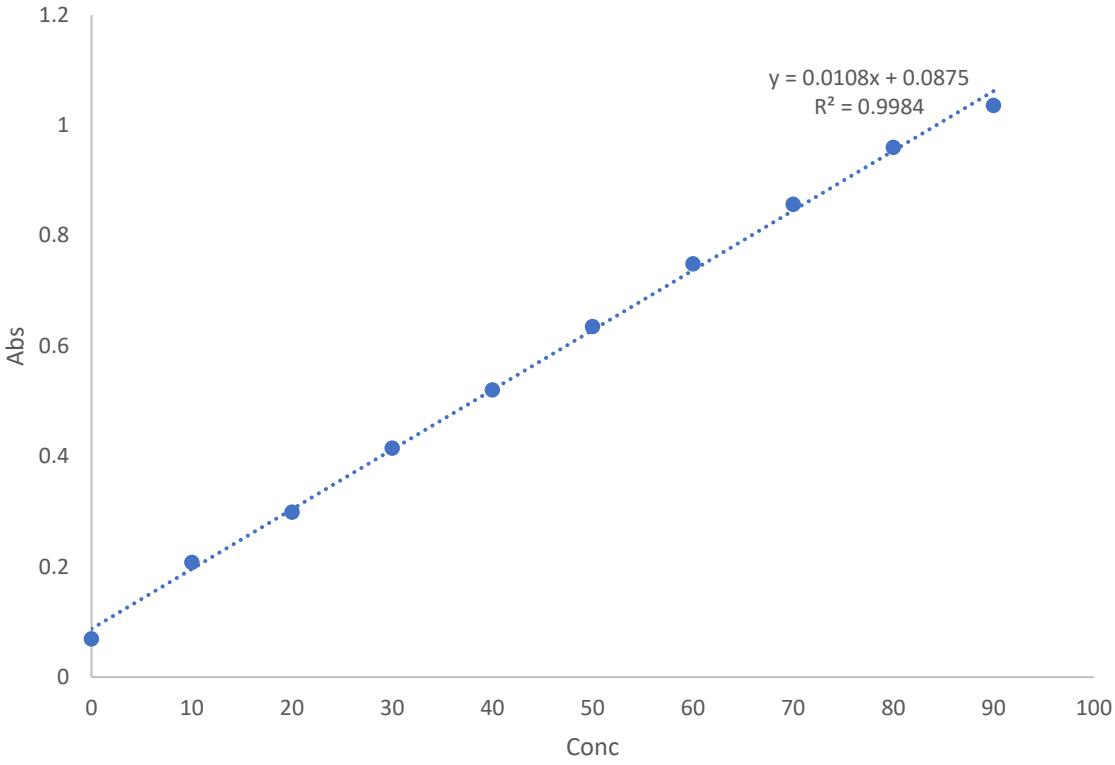


Figure 12. Gallic acid standard curve for TPC analysis

1.2.8 Total anthocyanin Contents (TAC)

TAC was measure with a pH differential method (Alexandre *et al.*, 2017) with slight modifications. A buffer solution of pH 1.0 was prepared weighting of 1.86 g KCl into a 1000 mL beaker and 980 mL water was added. The pH of the solution was adjusted to 1 by 6.3 mL of HCl the volume was filled to 1000 mL with distilled water. The second buffer solution of pH 4.0 was prepared with CH_3COONa. The amount of 54.43 sodium acetate (0.4M) was added to 1000 mL beaker into 960 mL of distilled water. The pH was adjusted to 4.5 by adding 20 mL HCL and the volume was filled to 1000 mL with distilled water. The samples were separately diluted with the buffer solutions by 1:2 ratio and kept at room temperature for 15 min. The absorbance was then read at 520 nm and 700 nm using a spectrophotometer (Jasco V-630, Tokyo, Japan). The results were calculated following the formula below and expressed as cynidin-3-glucoside equivalent (mg C3G/g).

$$A = (A_{520} - A_{700})\, pH1.0 - (A_{520} - A_{700})\, pH4.0 \quad (2)$$

$$TAC = \frac{A \times MW \times DF \times V_e \times 1000}{\varepsilon \times L \times M} \quad (3)$$

Where A are the absorbance values at 520 nm and 700 nm, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/ mol), DF is the dilution factor, V_e is the extraction volume in L, ε is the molar extinction coefficient of cyanidin-3-glucoside (26,900 L mol/ cm), L is the path length (1 cm), and M is the mass of peel extraction in g.

1.2.9 Particle size

The particle size was determined using a laser diffraction particle analyzer (SALD-2200, Shimadzu, Japan) in wet measurement mode (Koyama and Kitamura, 2014). Peel slurry after MWM was transferred with a transparent pipette and injected to the sampler channel. The sample was mixed by an attached ultrasonic probe and measured with high concentration system. The data was collected and analyzed using the WingSALDII software. The D50 value was chosen as particle size as the median diameter and is defined as the average particle size by mass.

1.2.10 SEM observation

The particle size and morphology were determined by field emission scanning electron microscopy (FE-SEM) (JSM-6330F, JEOL, Tokyo, Japan). The MWM samples were dried with a freeze drier, and the obtained powder was placed on stubs with double sided adhesive carbon tape. The samples were coated with a platinum-palladium in a sputter-coating unit (E-1045, Hitachi, Tokyo, Japan) under vacuum. The observation was performed (Islam *et al.*, 2017) with slight modifications at 5 kV at 100, 1000 and 1200 magnifications.

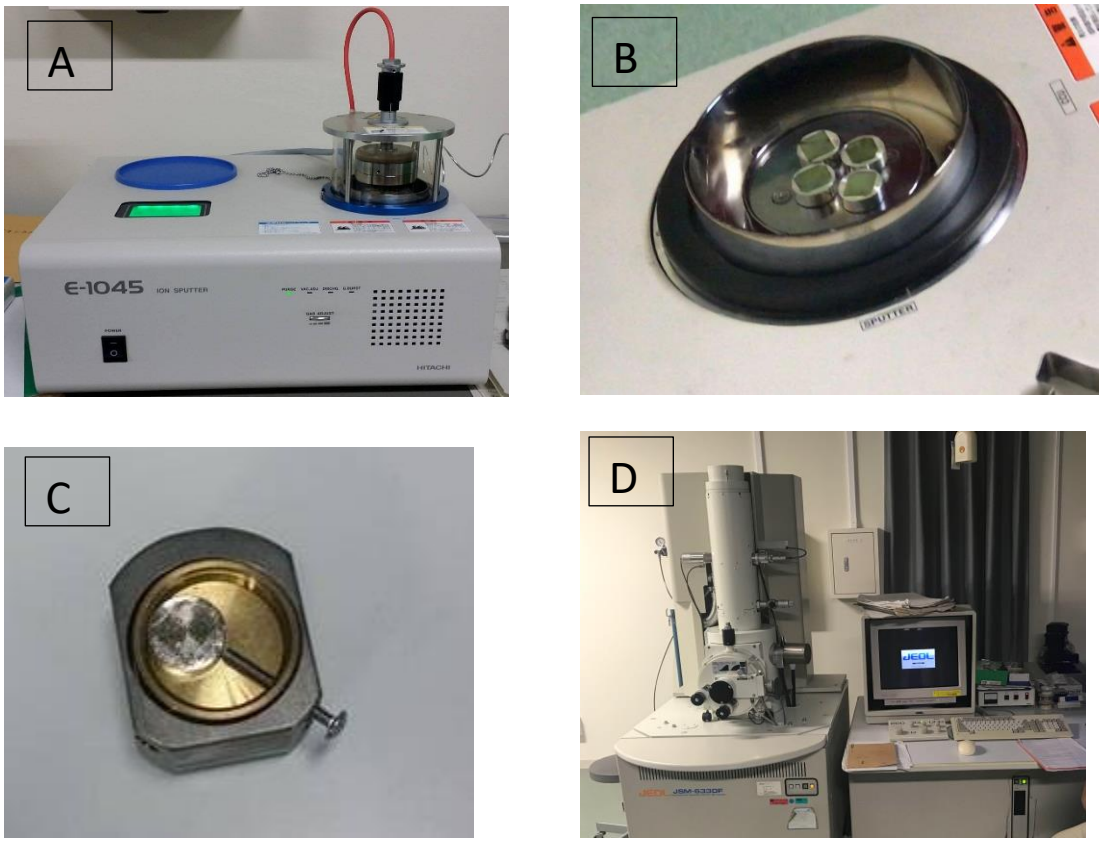


Figure 13. SEM observation process A (E-1045, Hitachi), B (Aluminum stubs), C (stubs holder), D (SEM system)

1.2.11 Statistical analysis and model adequacy

The software JMP 2.0.1 (SAS Institute Japan Ltd., Tokyo, Japan) was used for experimental design and analysis of the data. Analysis of variance (ANOVA) was used for the significant of independent variables and their interactions at $p < 0.05$. Student's *t*-test was applied for the comparison between predicted and experimental obtained values. The adequacy of the model was evaluated with coefficient of determination (R^2) and the insignificance of p values for lack of fit tests. The correctness of the model was verified by obtaining the predicted values through experiment in optimum predicted condition.

1.3 Results and discussion

1.3.1 Comparison of extraction characteristics between MWM and ultrasonic bath extraction with MWM

The MWM system mills the wet material to the degree where the particle surface area increases, facilitating the extraction process of plant cell compounds (Islam *et al.*, 2017). Hence, the MWM system was used to mill fresh pomegranate peel with solvents for phenolics extraction without prior drying. The influential factors: RS, SS and EW were tested for maximum extraction. In the MWM system, varying RS produced various particles sizes which affected the extract yield (Li, Kokawa and Kitamura, 2018). Similarly, SS showed significant effect on the pomegranate peel extract (Kaderides *et al.*, 2019). The type and combination of different solvent amounts has influence on the extract yield by regulating the solutions polarity, increasing the solubility of specific compounds, and mass transfer kinetics (A. Singh *et al.*, 2014; Kaderides *et al.*, 2019). RSM was used for the above three factors influence and interactions on optimum extraction values. Pomegranate peel extract obtained with MWM was compared with ultrasonic bath extraction to validate that the extraction process is possible with MWM. The results are presented in Table 3. There were no significant differences among MWM and MWM plus 20 min ultrasonic bath extractions. This indicated that the extraction process was completed with MWM alone and the extract yield did not increase with further ultrasonic extraction. Studies have mentioned that ultrasonic extraction can disrupt the cell providing a large surface area contact between solvent and plant material and leads to more phenolics extraction (Kaderides, Goula and Adamopoulos, 2015). However, plant cell disruption has already occurred when MWM was

used. The particle size reduction after MWM shown in (Figure 14&15) indicates that the minimal particle size obtained was 11.45 µm. Similarly, the observations by SEM showed complete cellular disruption after MWM. The surface of the peel particle before MWM was firmly packed with particle size of 247.6 µm reducing to 11.44 µm after MWM. The SEM images after MWM shows structural changes in the plant cell that seems like clusters of fragmented and very thin fibers Figure 15bc. Similar results was observed in a previous study after microwave-assisted extraction (Kaderides *et al.*, 2019).

Table 5. Comparison of MWM and MWM plus ultrasonic bath extraction and predicted versus experimental obtained values.

	Punicalagin mg/g dw	Gallic acid mg/g dw	Ellagic acid mg/g dw	Epicatechin mg/g dw	Catechin mg/g dw	TAC mg/g dw	TPC mg GAE/g dw
MWM	47.99±2.94^{A}	5.78±1.08^{A}	2.95±1.26 ^{A}	9.68±4.45 ^{A}	12.28±2.08 ^{A}	191.03± 0.32 ^{A}	2.92±32.58 ^{A}
MWM+ Ultrasonic bath	46.67±5.70^{A}	5.98±1.09 ^{A}	3.09±0.90 ^{A}	10.25±5.16 ^{A}	12.48±2.29 ^{A}	203.95± 0.73 ^{A}	2.97±30.96 ^{A}
Predicted values	70.8^{a}	14.03 ^{a}	8.5^{a}	21.53 ^{a}	12.21 ^{a}	4.19 ^{a}	251.99 ^{a}
Experimental values	71.57±4.23^{a}	6.2±1.30^{a}	6.89±2.58 ^{a}	14.8±1.30 ^{a}	13.15±2.65 ^{a}	3.1±0.5 7 ^{a}	225.7±15.65 ^{a}

Significant differences between values in the same column of sections are indicated by different letters ($p < 0.05$)

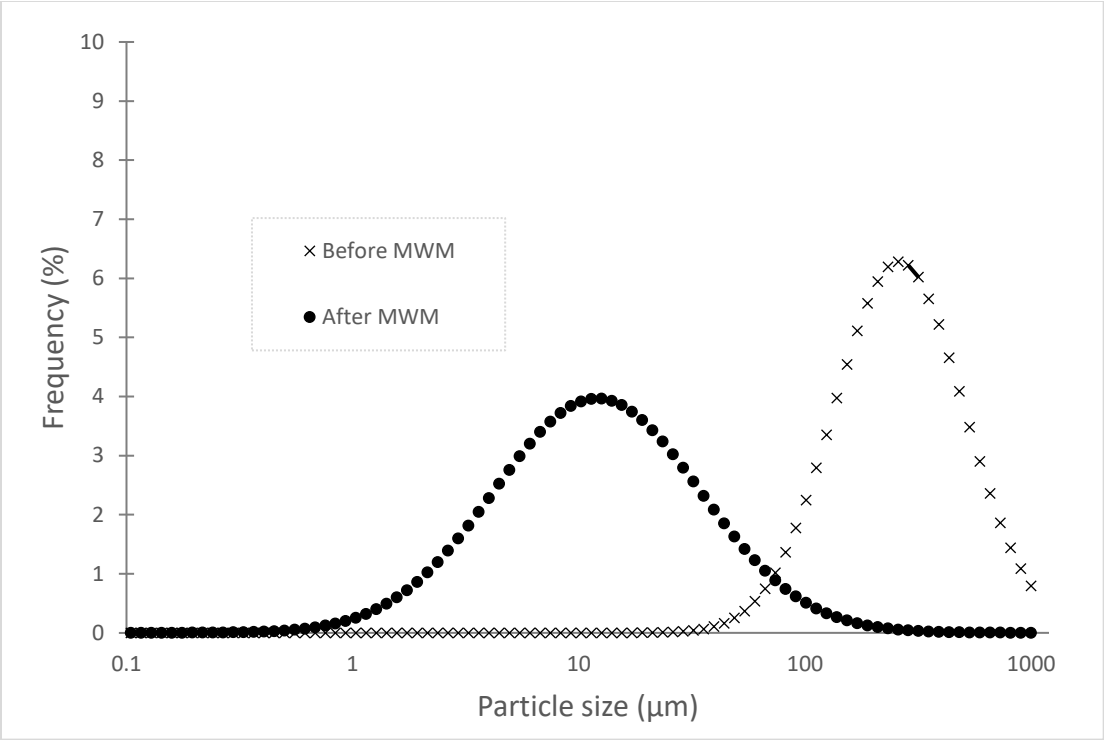


Figure 14. Particle size distribution of pomegranate peel before and after MWM process

a

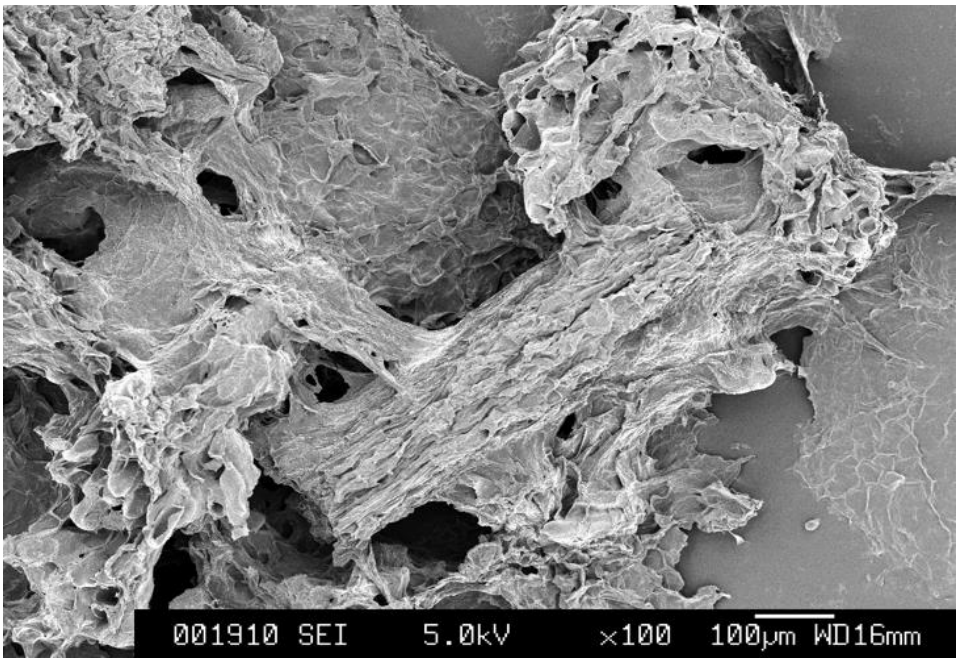
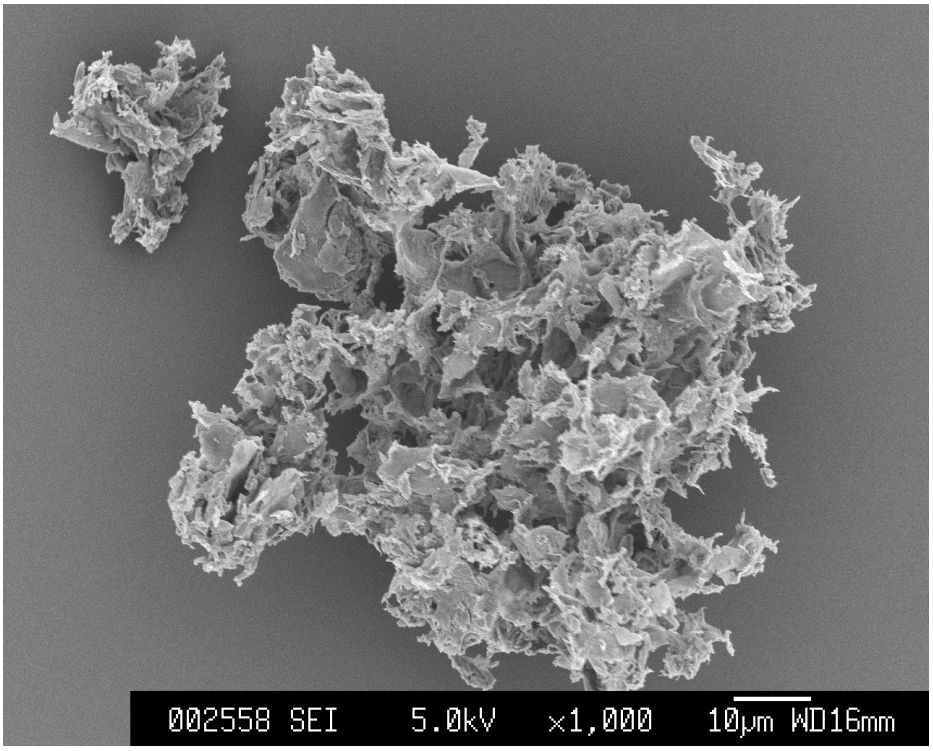


Figure 15. SEM images of pomegranate peel (a) at (x100) before MWM

b



c

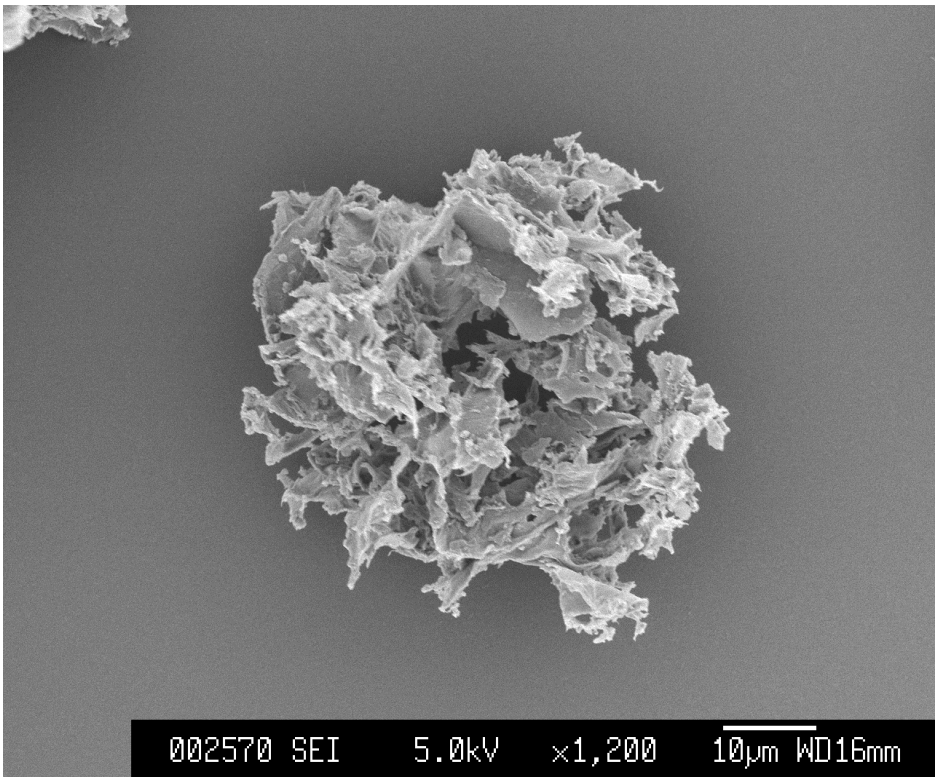
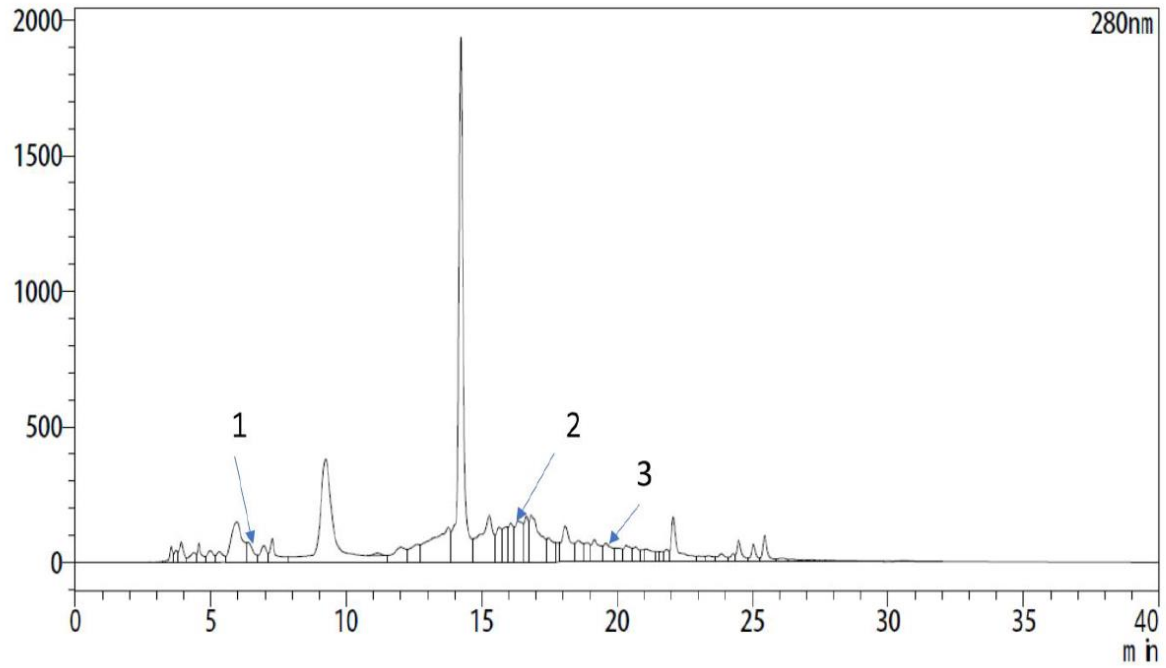
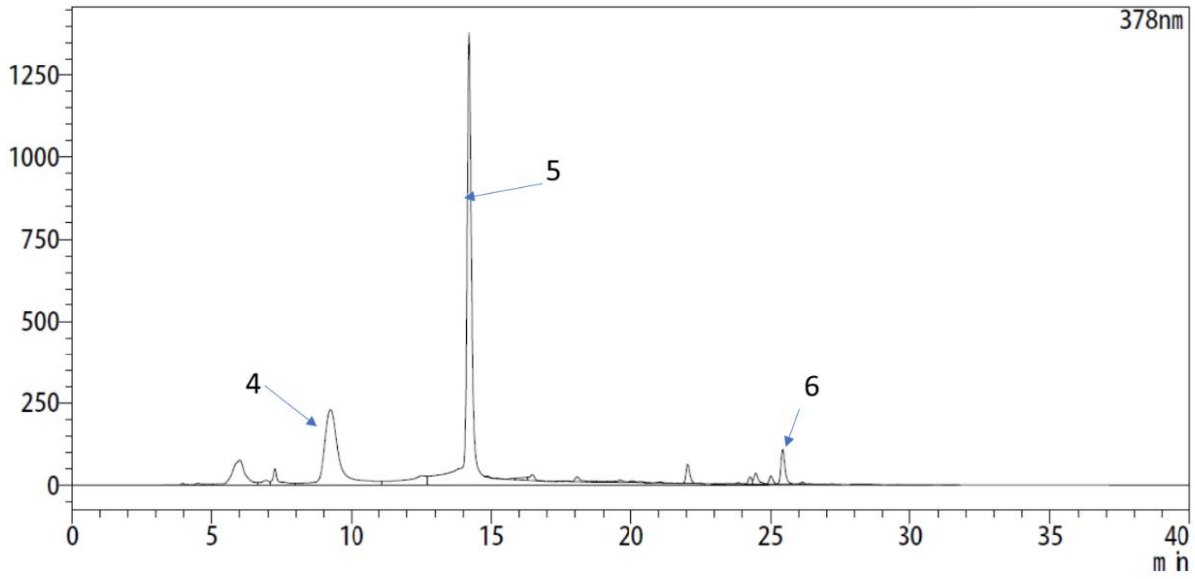


Figure 16. SEM images of pomegranate peel (b), (c) at (x1000) and (x1200) after MWM process at optimum conditions



a



b

Figure 17. ab Shows the HPLC chromatogram recorded at 280 nm and 378 nm, Peak 1. Gallic acid, 2. Catechin, 3. Epicatechin, 4. α-Punicalagin, 5.β-Punicalagin, 6. Ellagic acid.

1.3.2 Optimization and validation procedures for MWM extraction

The RSM model fitness adequacy was determined with the R^2 value and the significant of lack of fit values. For all the responses, the lack of fit results was not significant, indicating the fitness of the model shown in Table 2. The R^2 results for punicalagin, gallic acid, epicatechin, ellagic acid, catechin, and TAC were indicating the fitness of the model. The model validation was obtained by running the experiments following the predicted conditions, Table 3. The predicted and experimental values obtained were not significantly different except for gallic acid. Its obtained results were less than the predicted values due to the change in its forms, specifically, since ellagic acid is a dimeric derivative of gallic acid produced from hexahydroxydiphenic acid (ellagitannins) hydrolysis in aqueous solution (M. Singh *et al.*, 2014).

Table 6. ANOVA results for MWM process of the effects and responses

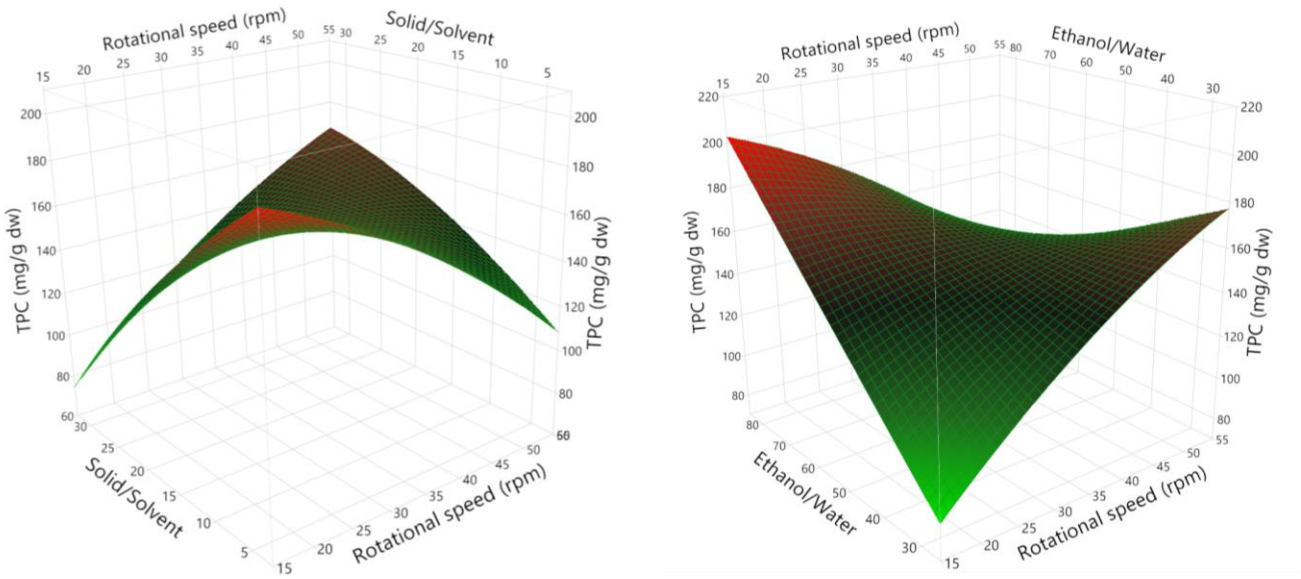
		Main effect			Quadratic effect			Interaction effect			
		X1	X2	X3	X_1^2	X_2^2	X_3^2	X1X2	X1X3	X2X3	Lack of fit
Punicalagin	P-Value	0.8798	0.6657	0.3765	0.2048	0.6606	0.6615	0.7051	0.7364	0.7372	0.1823
	F-Value	0.0241	0.1982	0.8566	1.8403	0.2047	0.2035	0.1517	0.1205	0.1199	
Gallic acid	P-Value	0.0385	0.0001	0.0584	0.6170	0.1881	0.1937	0.0006	0.2522	0.2583	0.3534
	F-Value	5.6744	117.563	4.5656	0.2663	1.9962	1.9420	24.754	1.4968	1.4562	
Ellagic acid	P-Value	0.7099	0.1470	0.1343	0.5063	0.0525	0.9106	0.5618	0.3943	0.5427	0.1008
	F-Value	0.1465	2.4710	2.6545	0.4751	4.8357	0.0133	0.3600	0.8005	0.4002	
Epicatechin	P-Value	0.5803	0.0153	0.9454	0.1466	0.3213	0.4775	0.1390	0.3943	0.5427	0.3508
	F-Value	0.3266	8.5181	0.0049	2.4765	1.0889	0.5445	2.5837	0.8005	0.4002	
Catechin	P-Value	0.9967	0.0841	0.5526	0.7211	0.2773	0.6376	0.1833	0.3385	0.6792	0.1911
	F-Value	3.6781	0.3777	0.1349	1.3204	0.2359	2.0442	0.1044	1.0217	0.1825	
TAC	P-Value	0.7604	0.9287	0.2391	0.0743	0.1749	0.5787	0.3996	0.4387	0.7574	0.6336
	F-Value	0.0982	0.0084	1.5674	3.9691	2.1327	0.3293	0.7740	0.6565	0.1014	
TPC	P-Value	0.8145	0.5175	0.4024	0.1844	0.1138	0.5526	0.6231	0.2784	0.6921	0.5325
	F-Value	0.0580	0.4500	0.7647	2.0327	3.0030	0.3775	0.2572	1.3308	0.1673	

Significant impact of factors is indicated by values of (p<0.05)

1.3.3 Effect of MWM extraction variables on TPC

TPC of the MWM extract varied from 92.33 to 381.59 mg GAE/ g dw. These results were comparable with previous findings during extraction with pulsed ultrasound-assisted method (Kazemi *et al.*, 2016). The optimum condition for maximized extraction was predicted by RSM as RS 50 rpm, SS 12.62 g/100 mL, and EW of 50% v/v. The highest TPC was obtained with RS 50 rpm, SS 17.50 g/100 mL, and EW of 55.% v/v, whereas the lowest was achieved at RS, 20 rpm, SS 17.50 g/100 mL, and EW of 55.% v/v. The influence of individual parameters and its dominance was estimated by RSM surface graphs. The values of two parameter at the central design was fixed and dynamically the third parameter was changed to determine the influence of each factor. The most influential parameter on TPC extraction was Solid to Solvent ratio (SS) which affect the TPC yield negatively. As the TPC

amount decreases with increasing SS (Fourati *et al.*, 2019). Ethanol to Water ratio (EW) also showed a similar tendency to decrease TPC yield, but not to the same extent as SS. On the contrary, TPC yield increased with increasing RS. A similar trend reported that TPC increased with ethanol ratio up to 50% and then decreased with continual increase in ethanol ratio (Venkataramanamma, Aruna and Singh, 2016). The response surface plot shows the interaction between RS and SS had positive effect on TPC yield while the interaction of SS and EW exhibited negative effect (Figure 18 a, b). It has been reported that the TPC increased until the solution reached equilibrium on extracted phenolics content and then decreased with further increase in solid ratio (Wang, 2011). The interaction of the main and quadratic effects did not influence the extraction of TPC significantly.

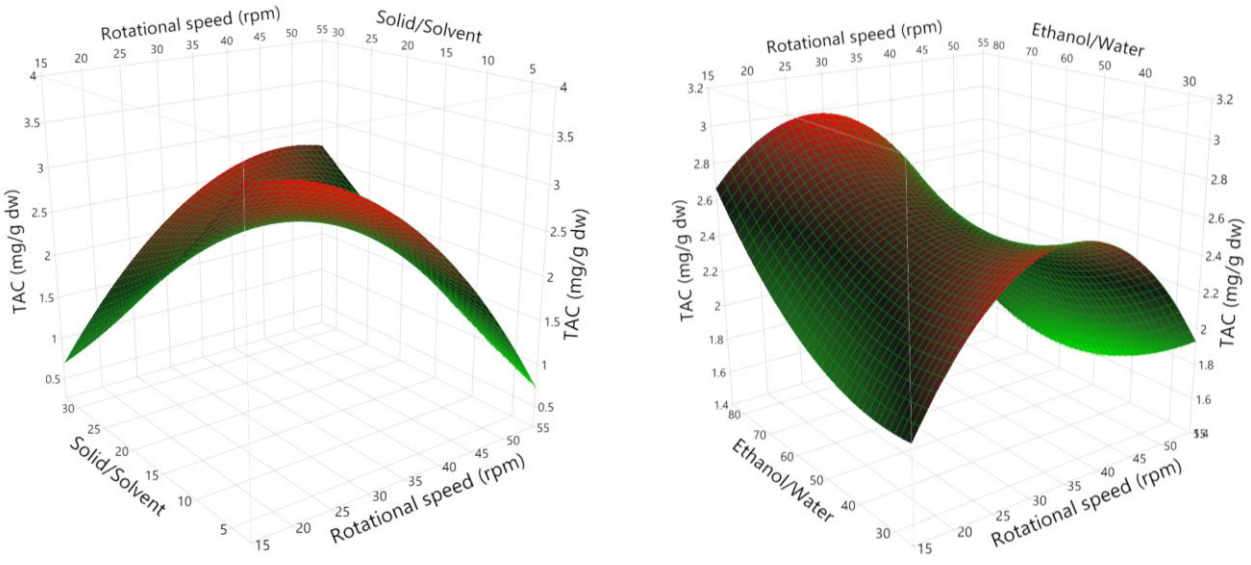


a b

Figure 18. Response surface graphs shows the effects of extraction factors on responses; (a,b) TPC

1.3.4 Effect of MWM parameters on Total anthocyanin Content (TAC)

Anthocyanins are important component of flavonoids as they are secondary metabolites of plants found in different pigments (Zhao *et al.*, 2013). Anthocyanin content of pomegranate peel is reported to have various health benefits and antioxidant activities (Sharifiyan *et al.*, 2016). In this study, TAC was measured with pH differential method. The method is suitable for numeric amount of TAC where its structure changes in a reversible transformation between pH 1.0 and 4.5 (Inácio *et al.*, 2013). TAC varied between 0.8 to 4.14 mg C3G/g. These results were higher than those previously reported from three different pomegranate cultivars in South Africa (Fawole, Makunga and Opara, 2012). The higher amount may be attributed to the heatless MWM extraction process. A loss of 46% in anthocyanin content was observed at 90 °C in sour cherry juice (Szalóki-Dorkó1 *et al.*, 2015). The highest TAC was obtained under the following MWM conditions: RS 35 rpm, SS 17.5 g/100 mL and EW 55% (v/v). SS was the most influential factor that affected the TAC yield negatively. However, the increase in EW decreased TAC and RS did not show any effect on TAC yield. Similarly, the TAC amount increased with increasing ethanol ratio of up to 60% maximum (Bae *et al.*, 2017). The interaction of both RS with SS and SS with EW showed positive effect on TAC amount whereas RS with EW showed negative effect, as shown in Figure 19 c, d.

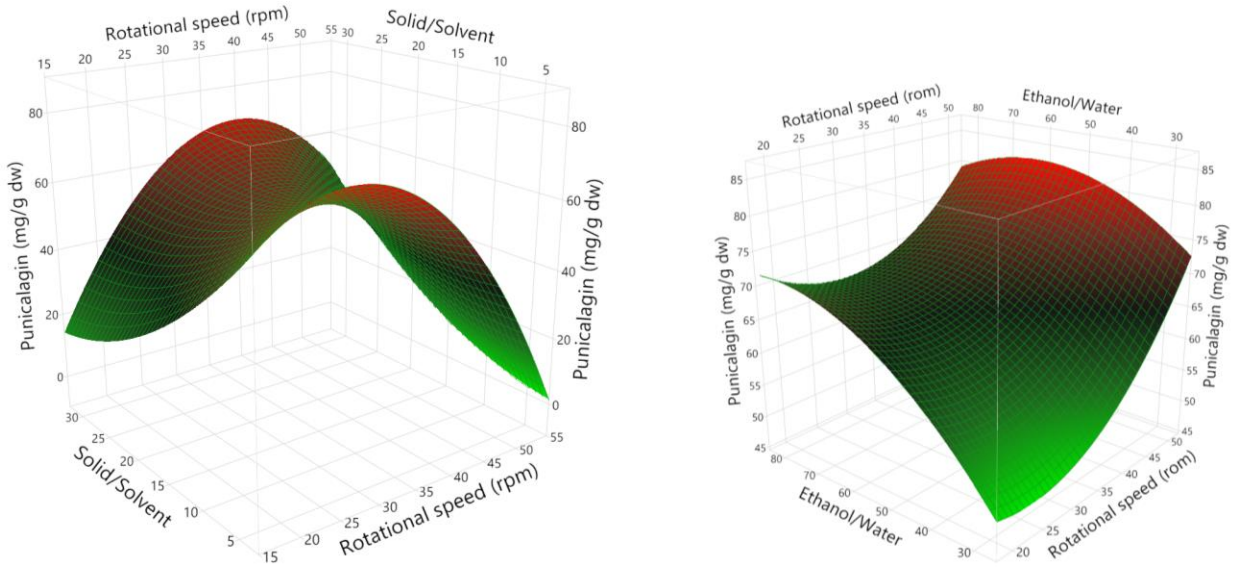


C d

Figure 19. Response surface graphs shows the effects of extraction factors on responses; (c,d) TAC

1.3.5 Effect of MWM parameters on punicalagin

Punicalagin is one of the ellagitannins and is found in two forms alpha and beta. About 50% of the pomegranate peels antioxidant properties is related to this compound (Fiechtner, 1996). In this study, the total punicalagin was calculated as the sum of alpha and beta punicalagin. The lowest punicalagin amount was 18.63 mg/ g and the highest was 91.94 mg/ g; similar results were reported by Lu (Lu, Ding and Yuan, 2008). The optimum conditions for MWM extraction predicted by RSM were RS 38.9 rpm, SS 26.23 g/100 mL, and EW of 80.00 % v/v. The highest punicalagin amount 91.94 mg/g dw was produced with RS 35 rpm, SS 17.50 g/100mL, and EW of 55% conditions. The dominant factors were in order of SS>RS>EW. SS increases the extraction rate until it reaches equilibrium (Qu, Pan and Ma, 2010). In addition, the profile of individual phenolics in pomegranate peel varied among different fruit cultivars (Fawole, Makunga and Opara, 2012; Khalil *et al.*, 2017). The interaction between RS with SS was positive and RS with EW exhibited negative effects on punicalagin amount, as shown in Figure 20 e, f.

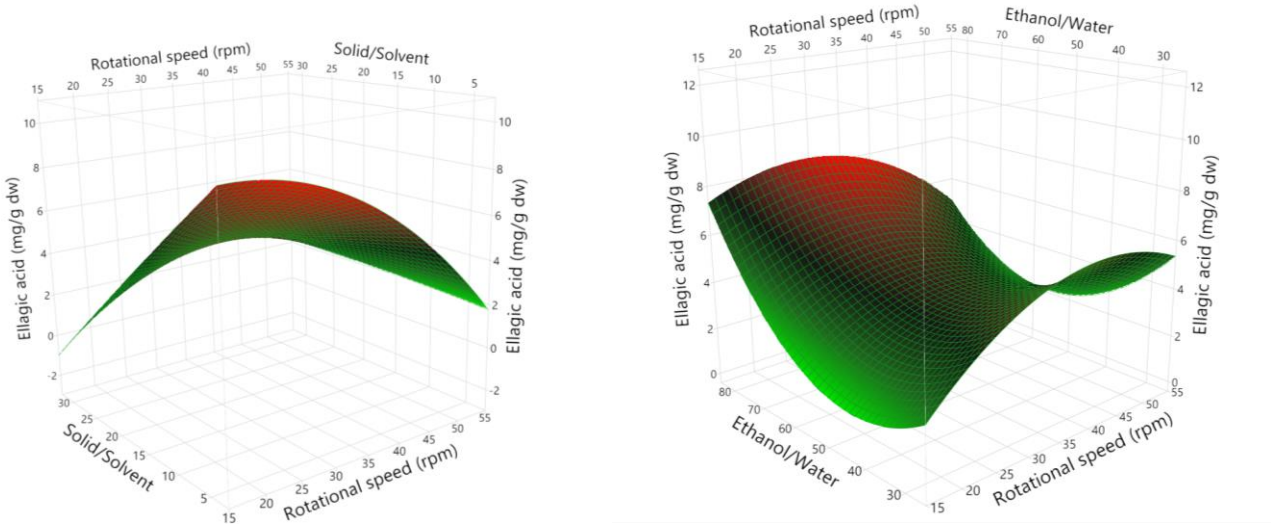


e f

Figure 20. Response surface graphs shows the effects of extraction factors on responses; (e,f) Punicalagin,

1.3.6 Effect of MWM parameters on Ellagic acid (EA)

Ellagic Acid (EA) is one the major compounds found in pomegranate peel extract, and it derives from gallic acid. Ellagic Acid is a natural phenolic compound with antioxidant and anti-proliferative properties (Bell and Hawthorne, 2008). The lowest quantity of EA extracted was 1.82 mg/g dw under RS 20 rpm, SS 5g /100mL, and EW 30% conditions. The highest amount was 12 mg/g dw obtained by RS 20 rpm, SS 5 g/100mL and EW ratio of 80% v/v. This indicates that the increase in ethanol ratio significantly increases the EA extracted amount. The solubility of EA was reported to be significantly lower in water compared to phosphate buffer and methanol (Bala *et al.*, 2006). Similarly, 11.85 mg/g EA was obtained by stirring in ethanol for 24 h and an increase of 20 fold was observed when the extraction was completed with ethanol in contrast to aqueous extraction (Masci *et al.*, 2016). The most influential factor was SS; indicating its increase reduces the EA amount. Followed by RS then EW influenced the EA amount. The interaction of RS with SS showed positive effect while RS with EW and SS with EW showed negative effect on EA as presented in Figure 21 g, h.

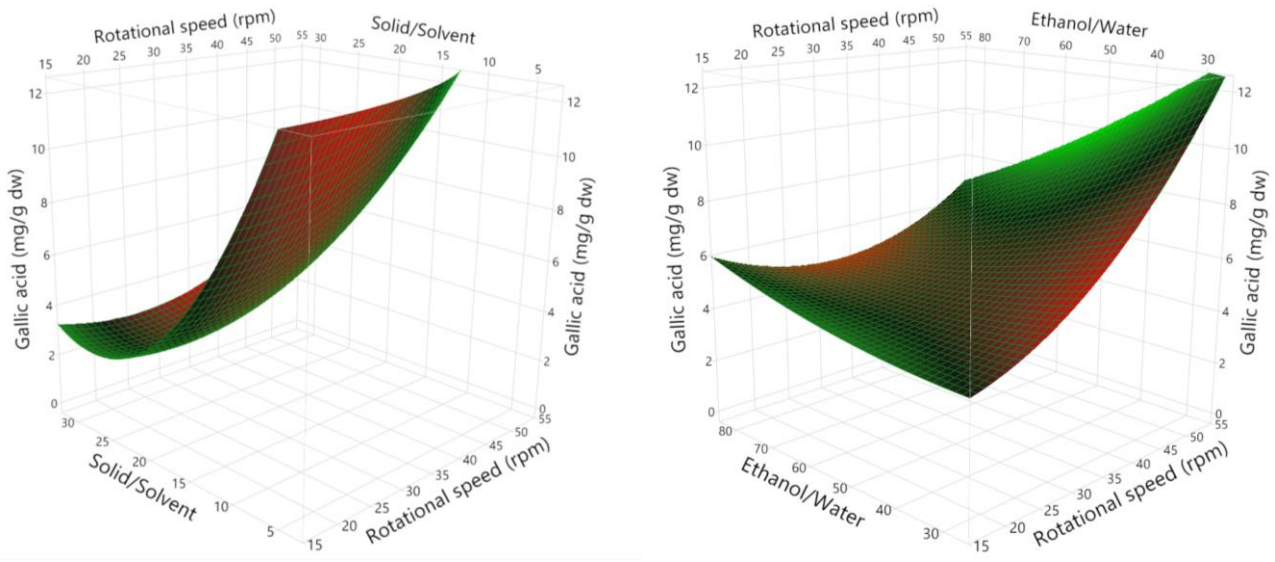


g h

Figure 21. Response surface graphs shows the effects of extraction factors on responses; (g,h) Ellagic acid

1.3.7 Effect of MWM parameters on Gallic acid (GA)

Gallic Acid (GA) is another phenolic compound of pomegranate peel. GA has antioxidant and anti-carcinogenic properties (Elango, Balwas and Padma, 2011). The RSM results showed that GA amounts extracted from pomegranate peel were in the ranges of 2.36 to 22.84 mg/g dw. The highest amount was obtained at RS of 50 rpm, SS of 5 g/100 mL, and EW of 30%. These results were higher than those in Lintong Jingpitian pomegranate peels (Li *et al.*, 2015). The most prominent factor was SS which showed negative effects on GA extracted amount followed by RS. The least effective factor on GA extraction was EW. The interaction between RS with SS and RS with EW showed negative effects meanwhile the quadratic effect showed positive effects as shown in Figure 22, I and j.

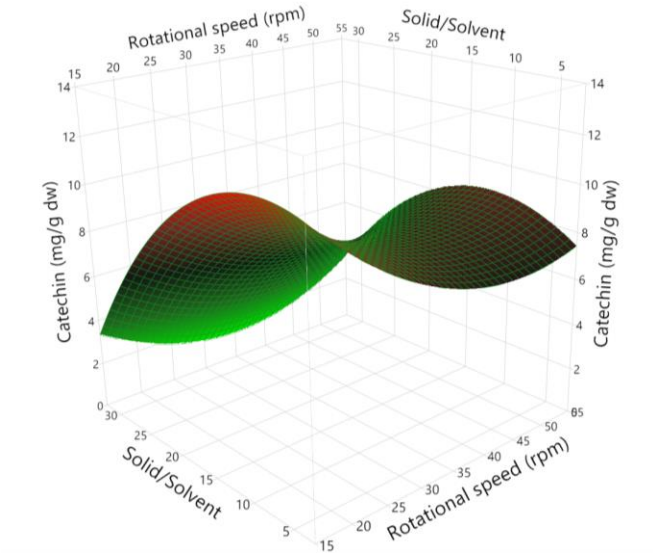


i j

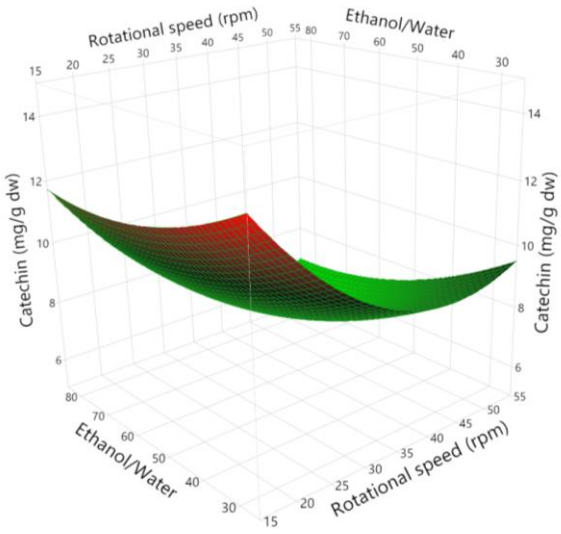
Figure 22. Response surface graphs shows the effects of extraction factors on responses; (i,j) Gallic acid

1.3.8 Effect of MWM parameters on catechin and epicatechin

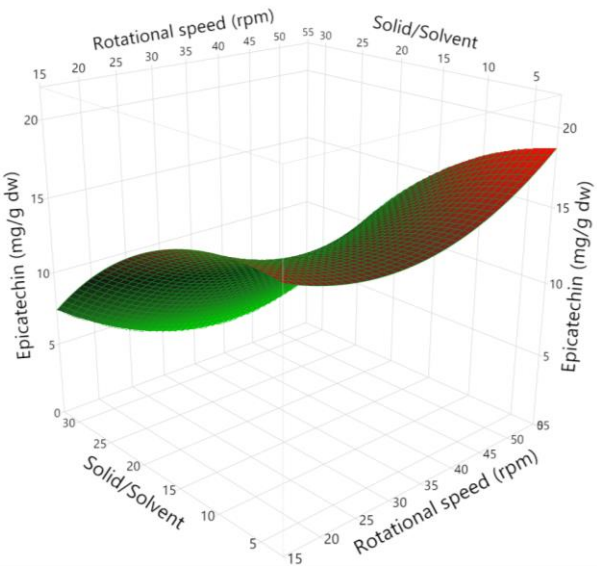
Catechin and epicatechin are flavones produce by plants as secondary metabolites. Several studies have reported that there are the antioxidant activities and other beneficial health properties of these flavones (Colomer *et al.*, 2016). The catechin amount of MWM extraction was attained at a range of 2.30 to 12 mg/g dw. The highest amount was obtained at RS of 20 rpm, SS of 5g/100 ml, and EW of 80 % ethanol. The lowest was obtained at RS of 20 rpm, SS of 30 g/100 ml, and EW of 80% ethanol. Similar results were obtained from 10 batches of pomegranate collected from different orchards (Li *et al.*, 2015). The optimum catechin extraction from green tea was reported at 70% ethanol concentration (Vural *et al.*, 2020). The most influential factor was SS which affected the catechin amount negatively. The interaction of RS with SS and RS with EW showed insignificant and negative effects on catechin extraction as presented in Figure 22 k and l. In contrast, epicatechin extraction values were from a range 1.40 to 19.68 mg/ g dw, where the highest epicatechin content was obtained at RS of 50 rpm, SS of 5g/100 ml and EW of 30% ethanol. These results were higher comparing to those reported by other studies (Mphahlele *et al.*, 2016). SS was the most influential factor in epicatechin extraction. The interaction of RS with SS, RS with EW and the quadratic effects showed negative effects but insignificant on the epicatechin extraction in Figure 23 m and n.



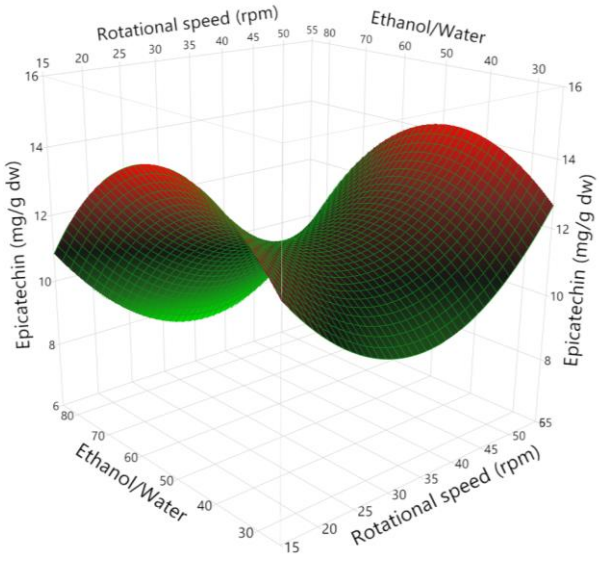
k



l



m



n

Figure 23. Response surface graphs shows the effects of extraction factors on responses; (k,I)Catechin, (m,n) Epicatechin

1.3.9 Correlation of particle size and phenolic content extraction

The principal component analysis results showed in figure 24. The correlation of phenolics content and particle size revealed that particle size has negatively correlated with all the phenolics except punicalagin. The results indicated that smaller particles size attributed with higher phenolics extraction.

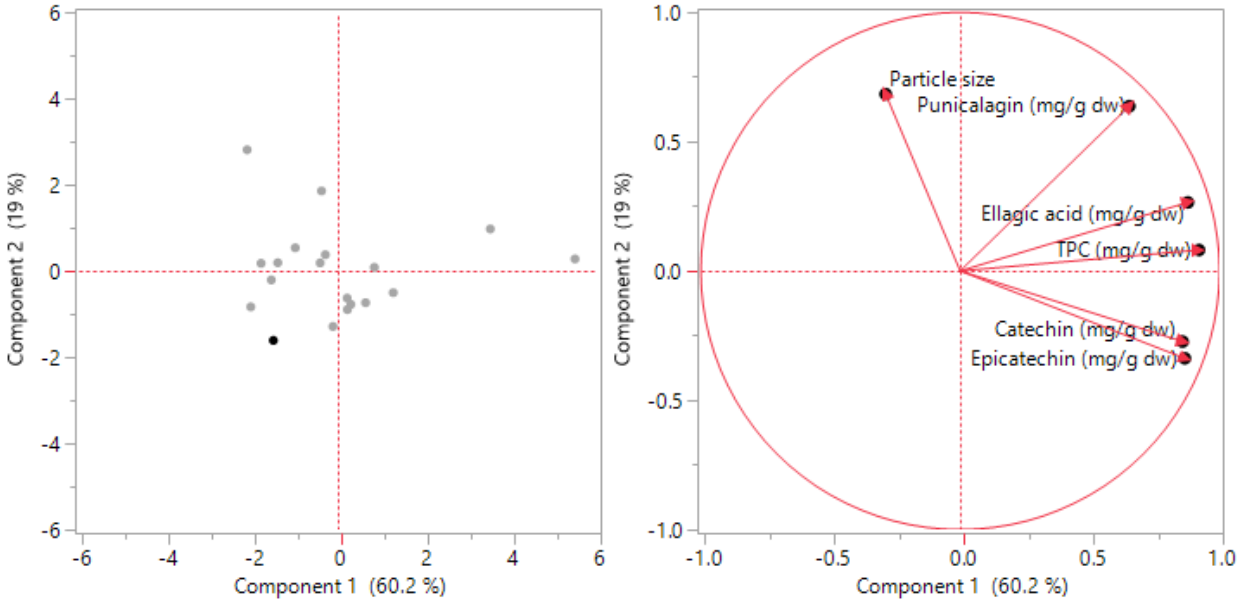


Figure 24. Principle component analysis of particle size and phenolics content.

1.4 Conclusion

A new extraction method for pomegranate peel phenolic extraction was proposed using MWM to produce micro-scale particle size. The MWM system was able to produce smaller particles of 11.44 µm, easing the extraction process by solvents. The process was optimized by RSM with three factors: RS, SS, and EW. The optimum response values were; TPC of 225.7 mg GAE/g dw, TAC of 3.1 mg/g dw, punicalagin of 71.6 mg/g dw, EA of 6.9 mg/g dw, GA of 6.2 mg/g dw , catechin of 13.1 mg/g dw and epicatechin of 14.8 mg/g dw. The optimum operational conditions were RS of 50 rpm, SS of 12.62 g/100 mL, and EW of 49.65% ethanol. The validated this extraction method with an extra 20 min ultrasonic bath extraction did not increase the extraction of phenolics from the peel, revealing that the extraction was completed with the MWM method alone. We concluded that MWM could be utilized in pomegranate peel phenolic extraction process to produce micro-sized particles and extract phenolics from fresh peel without drying or processing to powder. Further investigations are needed to determine the effects of MWM on heat-sensitive and trace compounds. And the proposed method can be used and optimize in different plant material for phenolics extraction.

Chapter 2

Pomegranate peel powder making with spray drying

2.1 Introduction

Plant byproducts are commonly produced during processing. These byproducts contain different bioactive compound and phenolics. Pomegranate is one of those fruits that is consumed or processed to juice by processing plants and its' peel is a byproduct. Pomegranate peel contain high bioactive compounds with anti-inflammatory and anti-infective properties (Ismail, Sestili and Akhtar, 2012). The peel showed high antioxidant activities in different assays such as the antioxidant activities with β-carotene bleaching test showing 58% higher than seed and juice (Derakhshan *et al.*, 2018), 70% of DPPH and ABTS antioxidant activities (Malviya *et al.*, 2014) .

The extraction process of pomegranate peel involves extracting of its total phenolics content included in the matrix of the peel. The peels' phenolics content contains dietary fibers which settles as residue during the extraction process. This residue is usually discarded throughout the extraction process. The total dietary fibers were reported to be 33.10 to 62 per 100 g from 12 different cultivars the higher neutral sugar contents were Arabinose and xylose (Hasnaoui, Wathelet and Jiménez-Araujo, 2014).

Dietary fibers are considered an essential part of the diet for its health-related properties. The dietary fibers are reported to alleviate type 2 diabetes (Zhao *et al.*, 2018). The complexity of fibers and their individual effects are addressed; that fibers improve digestibility, reducing cholesterol level and blood pressure, help weight loss and prevention from colonel cancers (Fuller *et al.*, 2016). Pomegranate dietary fibers showed two and half fold higher cholesterol absorption capacity compare to lemon, grapefruit, lemon albedo, and tiger nuts fibers (López-Marcos *et al.*, 2015).

The utilization of the peel powder in food products can have benefits like extending the shelf-life, masking of astringency, stability through gastrointestinal track and stability as food additives (Kaderides and Goula, 2019). The drying process comprises of dehydration of solid contained solutions. Although, drying methods has been

proposed from traditional drying to spray drying and some of the drying methods are quite expensive such as freeze drying (Patil, Chauhan and Singh, 2014).

The spray drying process convert the liquid samples into the smaller droplets from where it dries along hot drying gas and further collected into cyclone and receiver as powder. Nevertheless, the major problems to be assess during spray drying process is stickiness, degradation of bioactive compounds, and poor powder yield. To overcome such problems many researchers suggested using different carrier agents such as maltodextrin, gum arabic, starch sodium octenyl succinate, whey protein concentrate, and egg albumin (Du *et al.*, 2014). Using combine wall material such as maltodextrin, β-cyclodextrin, whey protein isolate and ,gum arabic showed better results as a wall material for blueberry anthocyanin extract (Tao *et al.*, 2017). Wall materials have emulsifying, film making and drying properties while synthetic wall material sometimes can change the flavor and is also an unnatural additive. However, natural fibers produced from byproducts can be used as a cheap and natural source of wall material as orange juice byproduct was used to produce new wall material(Kaderides and Goula, 2017).

In this study, pomegranate whole peel after Micro Wet Milling (MWM) extraction was used to produce a high fiber and bioactive retaining powder. Maltodextrin was used as an encapsulation material in the spray drying process of the peel powder. The powder was evaluated and compared with the extracted phenolic peel powder for its physio-chemical and functional properties.

2.2 Material and methods

2.2.1 Sample preparation

Pomegranate peel wonderful cultivar was purchased from the local market. The peel was separated manually by hand from the edible portion (aril and membranes). The obtained peel was cut into small pieces using a kitchen knife and mixed with the extraction solvent mixed for one minute using (Hamilton Beach-number, Japan) mixer. The coarse slurry was then subjected to micro wet milling (MWM). The MWM system conditions was adjusted to the previously optimized conditions consisting of the feeding rate, rotational speed 50 rpm, solid to solvent ratio12.62 g/100 mL, and ethanol to water ratio 49.65 %. The MWM milled slurry was collected and concentrated using a rotatory evaporator to remove excessive ethanol at 40 ℃.

2.2.2 Spray drying process

The concentrated slurry obtained after the evaporation of excess ethanol, was mixed with different ratio of maltodextrin, and subjected to a laboratory-scale spray drier (SD-1000, EYELA) Figure 25. The spray dryer was adjusted to three different temperatures 120 ℃, 140 ℃, and 160 ℃, the dry airflow rate was constant at 0.6–0.8 m^3/ min, flow rate of 200 mL/h, and the spray pressure of 12 kPa. The powder was collected from the collector bottle and stored in a desiccator until further analysis.

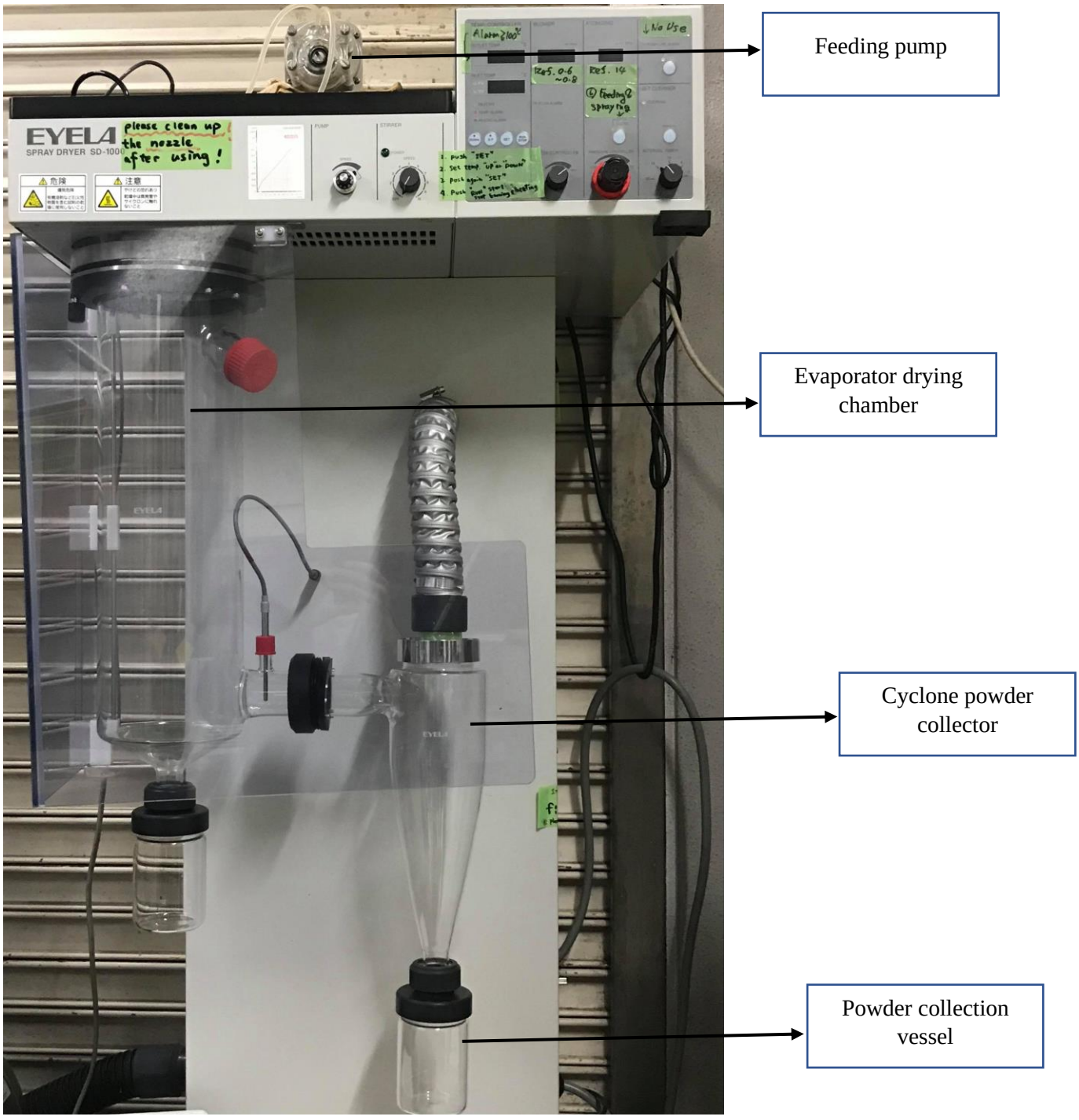


Figure 25. Spray dryer

2.2.3 Total phenolic content (TPC)

TPC was determined with the Folin-ciocalteu method. The reagents Folin & Ciocalteu (FC) reagent and Na_2CO_3 were prepared by adding 10 mL of FC reagent plus 7.5 g Na_2CO_3 and made up to 100 mL. An aliquot of 0.5 mL added to 2.5 ml of FC reagent and vortexed for 1 min. Subsequently, 2 ml of 7.5 % Na_2CO_3 was added and vortexed and the samples was rested for 30 min in the dark, at room temperature. The absorbance was read at 760 nm with a Spectrophotometer (JASCO V630 Japan). Standard curve was created from a serial dilution of gallic acid using the same procedure. The retention percentage phenolics content was calculated with following formula.

$$\text{Retention \% of TPC} = \frac{TPC\ of\ powder}{TPC\ of\ feed\ sample} \times 100 \quad (4)$$

2.2.4 Total anthocyanin content (TAC)

The total anthocyanin content was measured using the pH differential method. The pH 1.0 buffer solution was prepared with (0.025M) KCl and the pH was adjusted to pH 1 with HCl. The buffer solution pH 4.5 was prepared with 0.4M sodium acetate where the pH was adjusted with acetic acid to pH 4.5. The samples were extracted and diluted with a mixture of the buffer solutions (pH 1.0 and pH 4.5 solutions) at a ratio of 1:2. The samples were rested at room temperature in the dark for 30 minutes. The absorption was read at 520 and 700 nm using a spectrophotometer (JASCO V630 Japan). The TAC content was calculated from following formula.

$$A = (A520 - A700)\ pH1.0 - (A520 - A700)\ pH4.0 \quad (5)$$

$$TAC = \frac{A \times MW \times DF \times Ve \times 1000}{\varepsilon \times L \times M} \quad (6)$$

2.2.5 Antioxidant activities

The antioxidant activities were determined using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activates on the powder extracts according to (Sabokbar and Khodaiyan, 2016) with slight

modifications. The known amount of powder was diluted and extracted in an ultrasonic bath for 20 mins afterward the sample was serially diluted. The aliquant of 0.1 mL of each diluted sample was added to 3.9 mL of 0.1mM DPPH solution. The solution was vortexed for 1 min and stored at room temperature in the dark for 30 mins. The absorbance was measured at 515nm using a spectrophotometer (Jasco V-630, Tokyo, Japan). The IC50 values were calculated with the equations obtained from standard curve using the concentration against the percentage of inhibitions. Where the inhibition percentage was calculated form following equation.

$$PI = \frac{Ab - As}{Ab} x\ 100 \quad (7)$$

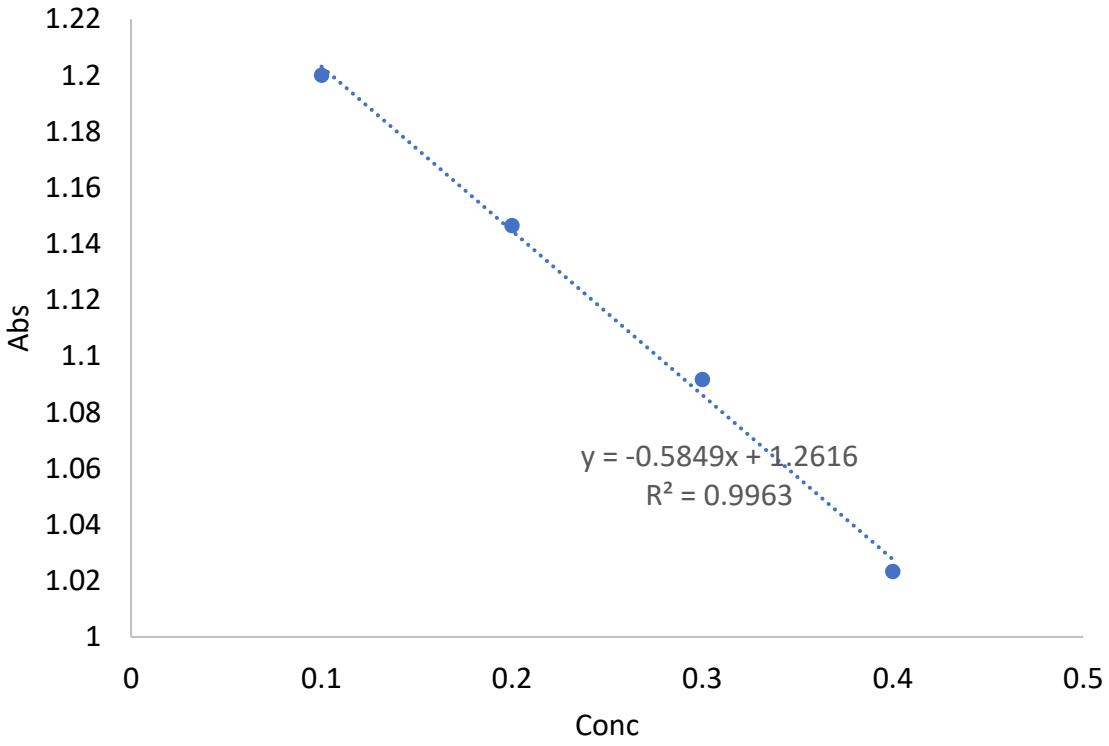


Figure 26. Standard curve for determination of IC 50 values

2.2.6 Yield of PPE spray dry powder

The yield was calculated according to the method mentioned by (Islam, Ayami, *et al.*, 2020) and weight of and dry content of feed samples were determined by scale weigh and oven drying. The collected powder was again subjected to weight and dry content measurement. The calculations were carried out with following formula.

$$Y = \frac{TSp \times \mathrm{Wp}}{TSs \times \mathrm{Ws}} \times 100 \quad (8)$$

Y: Yield

TSp : Solid content of powder

Wp: Weight of powder

TSs: Solid content of feed sample

Ws: Weight of feed sample

2.2.7 Solubility index

Solubility of the powder was measured by suspending 2.5 g of the powder in 30 mL of water. The suspension was vortexed for 1 minute and placed in a water bath at 37 °C for 30 minutes, followed by 20 minutes centrifugation at 3500 rpm. The supernatant was collected, and oven dried until the weight is constant after checking multiples times. The solubility was calculated as follow.

$$WSI(\%) = \frac{Dried\ supernatant\ weight}{Initial\ sample\ weight} \times 100 \quad (9)$$

2.2.8 Water holding capacity (WHC)

The water holding capacity was determined based on the water binding capacity of the powder. One gram of powder was dispersed in 3 mL of water and rested at room temperature for 12 hours. The suspension was centrifuged at 5000 g for 10 minutes and the supernatant was collected and weighed. The total moisture was determined by oven drying and the WHC was calculated with following formula.

$$WHC = \frac{Total\ weight\ of\ water\ in\ sample - weight\ of\ water\ released}{total\ weight\ of\ powder} \quad (10)$$

2.2.9 Water activity

Water activity is the thermodynamic measures of unbounded water in food samples as vapor pressure. Water activity is measures by the scale of 0.0 no water and 1 represents pure water. The higher water activity leads to faster deterioration of powder because of microbial growth. The water activity index of powder was measured using the water activity meter (Novasina Labmaster-aw, Switzerland).

2.2.10 Bulk and tapped density

The bulk density was measured by weighting the powder sample and pouring it gently in a 10 mL empty measuring cylinder. The weight and volume of the powder was recorded, and the density of the powder calculated g/mL volume. Afterward, the cylinder containing the powder sample was tapped for 2.5 mins with 60 taps/ minute using (A.B.D-72, Tsutsui physics and, chemistry instrument Co., Ltd. Japan) and the final volume was recorded, and the tapped density was calculated as g/mL. The cohesiveness of powder was calculated from balk and tapped density using following formula no (11). Meanwhile, the flowability of powder was calculated from formula no (12) as Carr Index (CI) (Islam, Ayami, *et al.*, 2020).

$$HR = \rho T/\rho B \quad (11)$$

$$\text{CI}(\%) = \frac{\rho T - \rho B}{\rho T} \times 100 \quad (12)$$

2.2.11 Moisture content and dry matter

The moisture contents were determined by oven drying. The powder samples were weight in aluminum cups and dried over night at 105 °C until constant weight and the moisture content were calculated from following formula.

$$\text{MC}\% = \frac{Wet\ wieght - Dry\ weight}{Wet\ weight} * 100 \quad (13)$$

$$DM\% = 100 - Moisture\ conter\% \quad (14)$$

2.2.12 Color parameters (Total color different)

The powder color was determined using Colorimeter (CR-200, Minolta Co., Japan). The color values were presented as Hunter color (*L, *a, *b). The (+*L , -L) values represent lightness and darkness, (+a, *-a) represents redness and greenness and (*+b, *-b) represent yellowness and blueness. The Chroma value which shows the color intensity and total color differences were calculated with following formulas.

Hue angle (Ø) =180+ArcTan(b/a) (15)

Chroma = $(a^{*2} + b^{*2})^{1/2}$ (16)

$$\Delta E = \sqrt{\Delta L\ *^2 + \Delta a\ *^2} + \Delta b\ *^2 \quad (17)$$

2.2.13 SEM observations

The powder morphology and particles were determined with field emission scanning electron microscopy (FE-SEM). The powder samples were placed on double adhesive tape which was stocked on metal stubs and dried at 40 °C. The samples were coated with platinum-palladium in a sputter-coating system (E-1045, Hitachi, Tokyo, Japan) under vacuum. The sample was observed at 5 Kv for 500 and 100 magnifications. The particle size was calculated from SEM images using image j (ImageJ bundled with 64-bit Java 1.8.0_172, NIH, 2021) software.

2.2.14 Statistical analysis

The software JMP 2.0.1 (SAS Institute Japan Ltd., Tokyo, Japan) was used for experimental design and analysis of the data. ANOVA was used for the significant of independent variables and their interactions at $p < 0.05$. Tueky test was applied for the comparison among different MD level samples. The adequacy of the model was evaluated with coefficient of determination (R2) and the insignificance of p values for lack of fit tests.

2.3 Results and discussions

2.3.1 The factorial design optimization

The whole pomegranate peel powder was produced with spray drying at 3 level of inlet temperature and 3 level of maltodextrin. The temperatures range was set to 120-160 °C as the lower temperature is favorable for heat-sensitive components. Yet, the lower temperature also results for high moisture contents which case for the shorter shelf-life(T.A. Tran and V.H. Nguyen, 2018). The maltodextrin (MD) level was chosen as 20%, 40% and 60% on dry basis. The MD level lower than 20% resulted a very low yield as the feed sample was stuck to the chamber wall. The experiment was designed with full factorial design as the full factorial design allow to examine the impact of factors, as well as the interaction between the factors. The coded and actual values of factorial design are shown in table 7. The analysis of full factorial design revealed that the inlet temperature has no significant impact on the powder properties. Where, the maltodextrin level significantly affects the powder properties table 8. There were no significant interactions detected between the factors. Similarly, results were reported by (Jafari, Ghalegi Ghalenoei and Dehnad, 2017) where they used 25, 35, and 45% maltodextrin and inlet temperatures were 124-43 °C. The powder was further analyzed and compared for the three level of maltodextrin concentrations. The R^2 values showed a good correlation between the predicted and actual values while the insignificance of F value shows fitness of the experimental design.

Table 7. Experimental design (Full factorial design) runs and factors value.

Runs	TEMP	MD
1	140	60
2	120	60
3	120	20
4	120	40
5	160	20
6	140	20
7	120	20
8	120	60
9	160	40
10	140	40
11	120	40
12	160	60

Table 8. ANOVA results for spray drying of the effects and responses.

Parameters	Factors				
		Temperature	MD %	Interaction	R^2
TPC	P-Value	0.0759	0.0013*	0.0574	0.99
	F-Value	6.8700	123.69	8.2160	
TAC	P-Value	0.0668	0.0893	0.0841	0.95
	F-Value	7.6116	6.0099	6.1305	
IC 50	P-Value	0.8213	0.8235	0.8934	0.35
	F-Value	0.2103	0.2073	0.2494	
Yield %	P-Value	0.4958	0.0571	0.3994	0.89
	F-Value	0.8946	8.6197	1.4341	
Solubility %	P-Value	0.0340*	0.0011*	0.1965	0.99
	F-Value	12.785	138.54	3.0041	
WHC	P-Value	0.6652	0.0487*	0.7603	0.89
	F-Value	0.4685	9.7485	0.4704	
Density	P-Value	0.7338	0.2078	0.6436	0.76
	F-Value	0.3437	2.7750	0.6953	

2.3.2 Effect of maltodextrin and temperature on TPC

Pomegranate peel is a rich source of phenolic contents. Folin-Ciocalteu is an antioxidant which measures the reactive ability of reducing capacity of plant derived antioxidants. The TPC value were obtained from 75.09-111.54 mg GAE/g the results shown in Table 9. The TPC was significantly different among the samples where the highest TPC was obtained from the 20% powder samples. The increase in MD level resulted in lower TPC content. This may be due to higher dilution of phenolics content in higher MD levels. Similar results were reported from whole mandarin powder with different ratio of maltodextrin (Islam, Ayami, *et al.*, 2020). However these TPC results were higher than those reported for pomegranate peel powder that were freeze dried with maltodextrin and b-cyclodextrin incorporated (Sharayei, Azarpazhooh and Ramaswamy, 2020). The micro wet milling process might have played a role in increasing the extracted phenolics as it produces smaller particles (Khan *et al.*, 2021). The retention of TPC was slightly higher in the 20% and 40% samples but there was no significant different among the sample. The increase in inlet temperatures, resulted in a slight decrease in TPC amount but the decrease was not significant due to most of the phenolic in the peel being thermally stable. Meanwhile, the drying time interval is very short during spray drying which leaves no significant impact on the powder properties (Çam, Içyer and Erdoğan, 2014).

2.3.3 Effect of maltodextrin and temperature on Total Anthocyanin Content

Anthocyanin are water soluble flavonoids, and they are secondary metabolites of plants found in different pigments. Total Anthocyanin Content (TAC) was measured with pH differential method, suitable for numeric quantification. The structure of anthocyanin changes in a reversible transformation between pH 1.0 and 4.5 (Inácio *et al.*, 2013). TAC obtained were in a range of 10.04-15.98 mg C3 E/g. Results are presented in Table 9. There was no significant different among the three levels of maltodextrin (MD). A slight decrease was observed when the temperature increased from 120 °C to 160 °C. Similar trend was observe and justified as the anthocyanin content is very sensitive to processing parameters especially temperature intensity and heating duration (Jafari, Ghalegi

Ghalenoei and Dehnad, 2017). The degradation of anthocyanin was not significant as the drying sample is exposed to these high temperature for a short period of time in drying chamber (Santiago *et al.*, 2016).

2.3.4 Effect of maltodextrin and temperature on antioxidant activities (IC50)

Pomegranate peel possess many phenolics compounds and the antioxidant ability of peel was reported higher compare to the pulp and seed of the fruit (Li *et al.*, 2006). The antioxidant capacity of powder was determined with DPPH. The DPPH activities are the scavenging capacity of plant derives antioxidants. The antioxidant capacity was measured quantitively (IC 50 values) as the known amount of powder sample is required inhibiting 50% of the radicals. The lower the IC50 value, the higher the antioxidant capacity. The lowest IC50 values were obtained with the 20% MD samples. The IC50 values slightly increased with increasing levels of MD but the impact of MD level was insignificant. Different temperatures also had an insignificant impact on the IC50 values shown in Table 9. The IC50 values were reported as 6.12 μg/mL from the peel extract which were higher compare to the powder samples and the DPPH values showed a strong correlation with phenolics content (Vikas Dadwal, Shriya Bhatt, Kanika Sonkhla, 2017). One study informed (Zhong *et al.*, 2016) that the smaller particle size increases the surface area which leads to faster realize of polyphenols and flavonoids from plant cell and increases the antioxidant activities of extracts. The higher antioxidant ability of pomegranate peel powder in this study was mainly due to the micro sized particles by the MWM process before spray drying.

Table 9. Nutritional properties of whole pomegranate peel powder

	TPC mg GAE/g db	Retention %	TAC mg C3E/g	Yield %	IC50 µg/mL
MD 20%	110.24a	57.84a	13.82a	29.11a	3.53a
MD 40%	96.99b	62.54a	14.23a	26.61ab	3.58 a
MD 60%	84.06c	55.66 a	12.03a	35.87b	3.86 a

Significant differences between values in the same column of sections are indicated by different letters ($p < 0.05$)

2.3.5 Physiochemical properties

Physiochemical properties of a powder are important characteristics that are relevant in its sensory, storage or shelf life and in its incorporation in other food products. The physiochemical properties of pomegranate whole peel powder are presented in Table 10.

2.3.6 Density of the powder

The bulk density represents the mass of powder that includes the particles and the porosity in a volume. The bulk density of the whole peel powder slightly increased with the increasing MD level, but the overall impact was insignificant. Similarly, results from the extract powder of mountain tea increased 13% with increase in carrier agent and the inlet temperature showed no significant impact (Şahin Nadeem, Torun and Özdemir, 2011). The increase in bulk density, maybe a result of the reduction in moisture content. The higher MD level allows the powder to packed tightly, or increase in the solid concentration of the feed samples increases with MD level (Tewa-Tagne, Briançon and Fessi, 2007). Higher bulk density is favorable in packaging and shipping of powder as it decreases the oxidation of powder by decreasing the powder porosity (Santhalakshmy *et al.*, 2015). The tapped density is the ratio of occupied volume by a mass after certain tapping. The trend for tapped density was similar to bulk density the lowest density was in 20% and the higher was obtained in 60% MD samples. Similar results were obtained from whole mandarin powder with spray drying (Islam, Ayami, *et al.*, 2020). The cohesiveness and flowability of the powder were determined from both the bulk and tapped density. The cohesiveness and flowability of powder is presented in Table 10 as Hausner ratio (HR) and Carr index (CI). It shows that a value of HR greater than 1.2 indicates low, a value from 1.2 to 1.4 specifies intermediate, and less than 1.4 indicates high cohesiveness. Meanwhile, Carr Index (CI) greater than 15 indicates very good, greater than 20 shows good, greater than 35 shows fair, between 40 and 45 shows bad, and smaller than 45% shows very bad flowability. The cohesiveness and flowability of the powder slightly increased with MD level but there was no significant difference among the samples. The cohesiveness and flowability of the powder showed good cohesiveness and flowability. Similar results were reported by (Shishir *et al.*, 2014) that the HR and CI values increased with increasing in maltodextrin level

and temperature. The increase in flowability of the 40% and 60 % MD samples could be due to the decrease in moisture content. The increase in moisture content moreover increases the cohesiveness among the powder particles (Caliskan and Dirim, 2016).

2.3.7 Solubility index

Solubility index is an important parameter in powders, as it determines the reconstitution of powder in aqueous medium. Solubility of the powder can be affected by the initial composition of the feed sample, wall material, feeding rate, and air flow rates (Jafari, Ghalegi Ghalenoei and Dehnad, 2017). The solubility results in Table 10, shows that the powder solubility increases with MD levels. A significant difference in solubility index among the different level MD level samples was observed. Inlet air temperatures and MD level had significant impact on the powder solubility index, but their interaction was insignificant. Similar results were obtained by (Muzaffar, Dinkarrao and Kumar, 2016) that the inlet air temperature was the most prominent factor to impact the solubility of spray dried pomegranate juice powder. They stated that the solubility index of pomegranate juice powder with MD as a carrier agent was from 91-98% which is higher compared to this study. The reason for the lower solubility of the whole peel powder is the presence of insoluble dietary fibers. These results were comparable with powder from persimmon pulp with five different carrier agents (Du *et al.*, 2014).

2.3.8 Water holding capacity (WHC)

The water holding capacity of a powder is its ability to retain water after submerging in water and subjected to an external centrifugal force. The water holding capacity of powder mainly depends on the fiber contents where the particle size of the fibers play an important role in water holding capacity (Sangnark and Noomhorm, 2003). The water holding capacity results are presented in Table 10, the WHC reduced with increase in MD level due to the higher MD is more water soluble and it also substitutes for a part of the fiber content. There was no significant different between sample 20 % and 40 % MD level, but the samples were significantly different with 60% MD level. The water holding capacity of the whole peel powder was three folds higher compared to the freeze dried pomegranate juice with maltodextrin as carrier agent (Adegoke Olusesan Adetoro 1, 2020). The fiber content plays

important role in WHC, meanwhile the higher soluble fiber content such as pectin and gums accounts for higher water holding capacity (Viuda-Martos *et al.*, 2012). On the contrary, (Chiou and Langrish, 2007) identified that the insoluble content of fiber have more absorption capacity in regards to phenolics, making it a strong carrier agent compared to soluble fibers.

Table 10. The physiochemical properties of pomegranate peel powder

	Bulk density g/mL	Tapped density g/mL	Hausner ratio (HR)	Carr index	WHC mL/g	MC %
MD 20%	0.179[a]	0.225 [a]	1.258 [a]	20.437 [a]	2.407 [a]	7.57[a]
MD 40%	0.175 [a]	0.232[b]	1.333 [a]	24.737 [a]	2.302 [a]	7.55[a]
MD 60%	0.217 [a]	0.286 [b]	1.323 [a]	24.209 [a]	2.046[b]	5.21[b]

Significant differences between values in the same column of sections are indicated by different letters ($p < 0.05$)

2.3.9 Color parameters

The color properties of powder is a sensory factor for consumer acceptance. It is correlated to the phenolics compound presence in the original sample (Li *et al.*, 2014). The results of color parameters are presented in Table 11, where the lightness (L*) of powder increased with increase in MD level being that maltodextrin owns white color, while the a* value slightly decreased and the b* value increased in 40% of sample and further decreased in 60% MD samples. The sample were not significantly different in comparison of the color values. For allocation of the exact color and intensity, the hue angle and Chroma values were calculated. The results revealed that the hue angle was around 70° and the color intensity was obtained was around 22. The hue angle and Chroma value interpretation shows that the powder holds slightly deep yellow tone. The value was slightly lower than those reported from pomegranate juice powder with gum Arabic as carrier agent. However, the (a*, b*) value were higher in present study. The a* value shows the redness of the powder is mainly associated to the anthocyanin content of the powder (Santiago *et al.*, 2016).

Table 11. Color parameters of pomegranate peel powder

	L*	a*	b*	Chroma Values	Hue angle
MD 20%	69.54 [a]	7.23 [a]	21.33 [a]	22.54 [a]	71.07 [a]
MD 40%	72.77 [a]	6.95 [a]	22.08 [a]	23.15 [a]	72.49 [a]
MD 60%	74.24 [a]	6.54 [a]	19.33 [a]	20.43 [a]	70.96 [a]

Significant differences between values in the same column of sections are indicated by different letters ($p < 0.05$).

2.3.10 Microstructure of powder (SEM observations)

The SEM photos shown in Figure 20 demonstrates that the spray dried powder exhibited similar spiracle shapes. The mean particle size of 20% MD samples were 3.5 µm, 40% MD samples were 3.8 µm and 60% MD were 4.1 µm Figures 29, 30, 31 respectively. The particle size increased with the higher maltodextrin level owing to the increase in viscosity of the feed sample, as an increase in MD also increases the viscosity of sample (Tonon, Brabet and Hubinger, 2008). The powder with 20% maltodextrin showed smother surface compare 40% and 60% maltodextrin samples. The maltodextrin percentage of 20% was adequate to encapsulate the peel extract along with the fiber contained in the whole peel. While a very small portion of 20% MD samples were shown in Figure 27 b, were uncovered by the wall material. The increase in maltodextrin level, the surface of particle shrunken due to maltodextrin moved to the surface area from inside of the particle. Similar results were reported and described as the surface are shrinking it cases for less contact and strength between the particles (Jafari, Ghalegi Ghalenoei and Dehnad, 2017). The SEM photos also revealed that powder produced with spray drying have smoother particle surfaces and the particle were scattered.

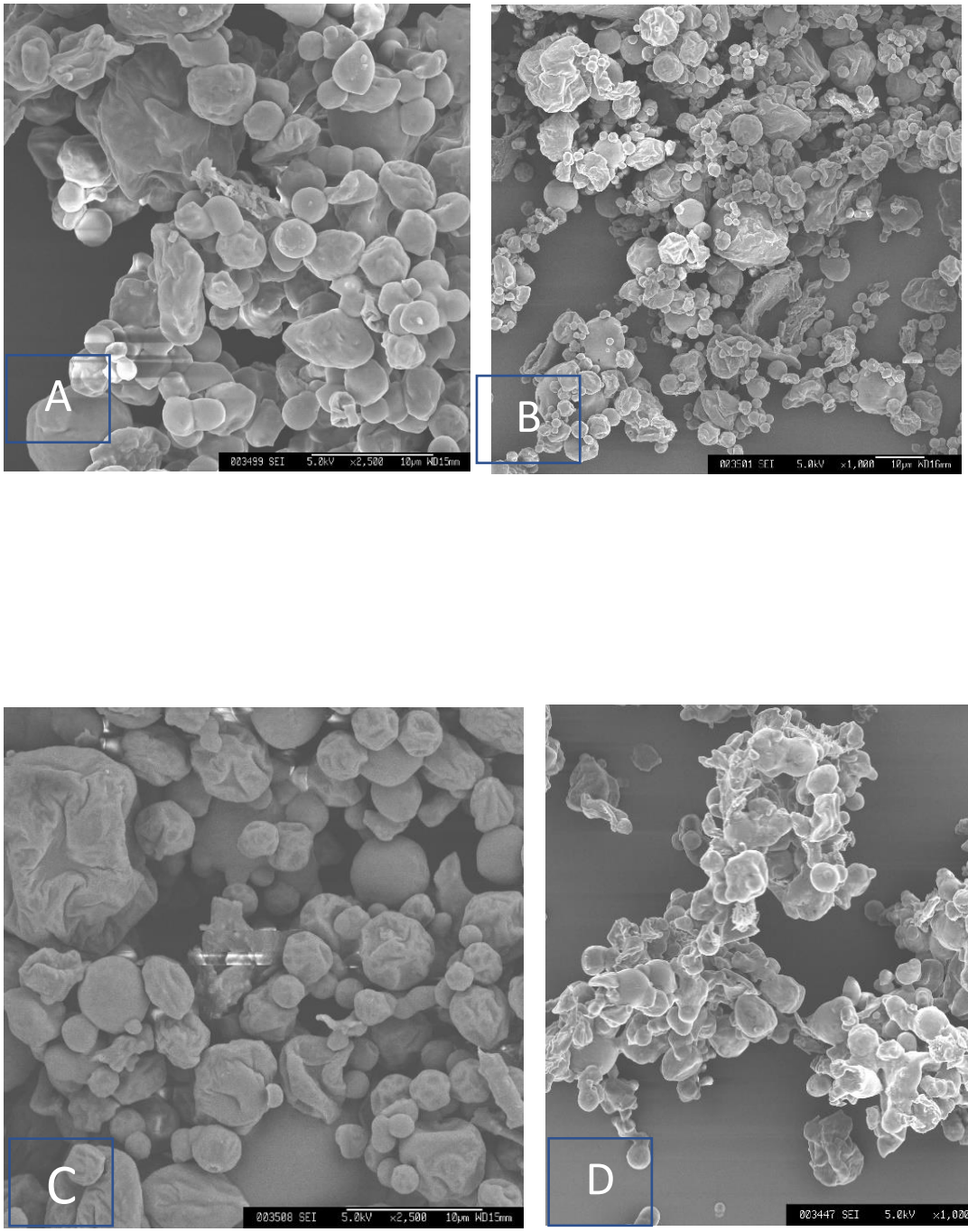


Figure 27. SEM images of pomegranate peel powder (a & b) 20% MD, (c & d) 40% MD

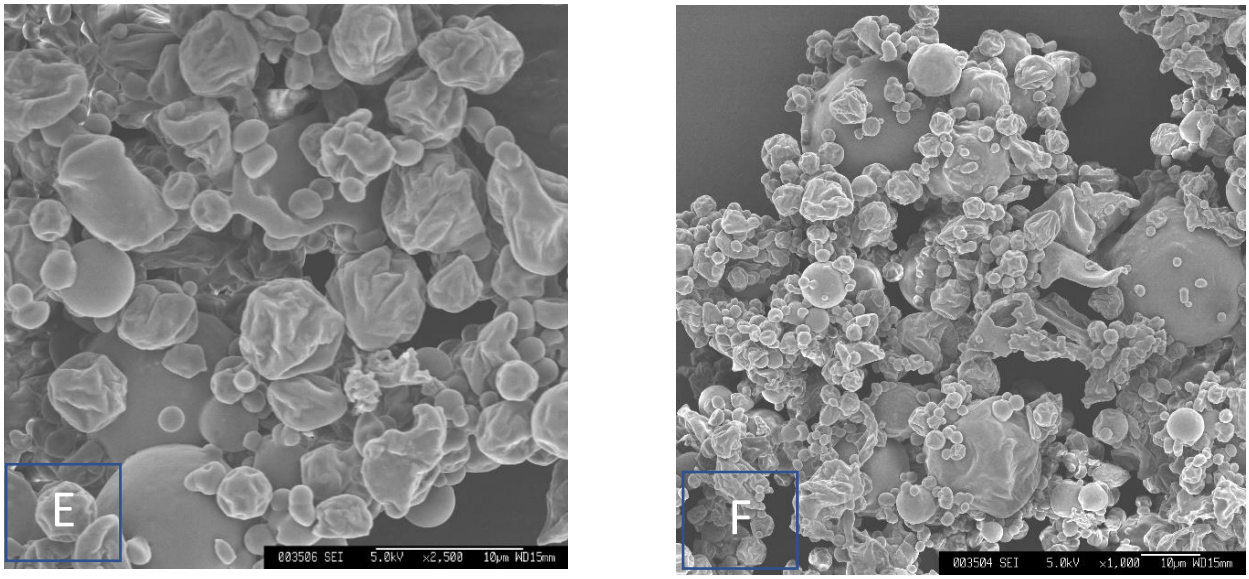


Figure 28. SEM images of pomegranate peel powder (e & f) 60% MD.

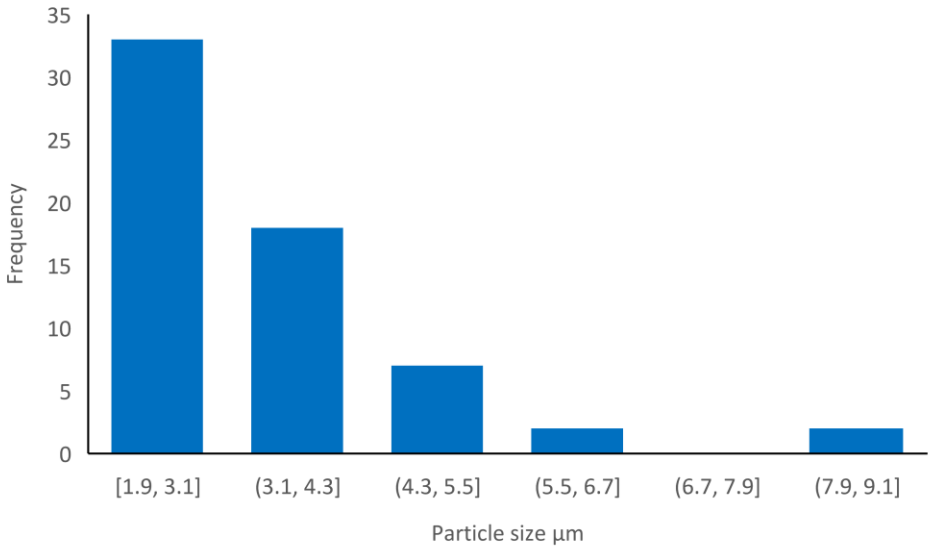


Figure 29. Particle size distribution of 20% MD powder

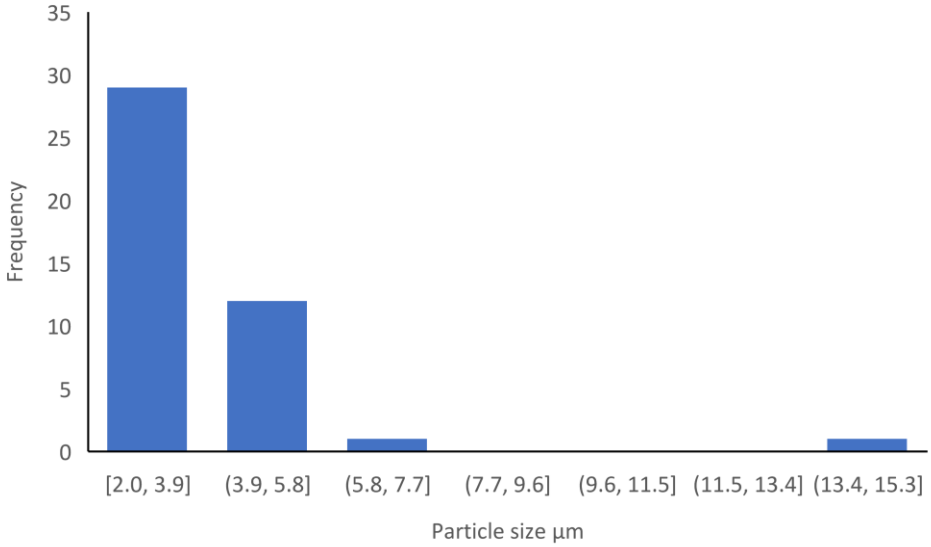


Figure 30. Particle size distribution of 40% MD powder

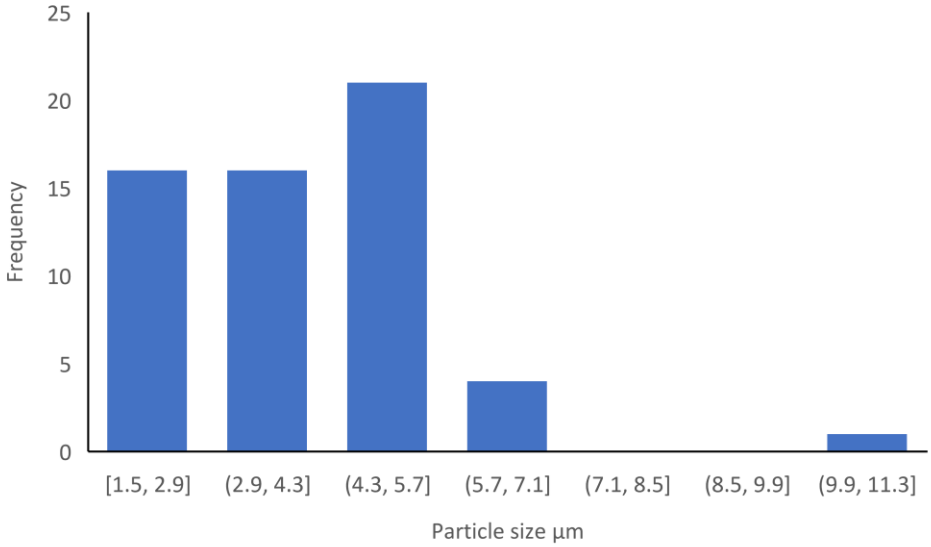


Figure 31. Particle size distribution of 60% MD powder

2.4 Conclusion

Pomegranate is a rich source of phenolic compounds and the extraction of these phenolics were optimized in the first part of the study. The incorporation of the liquid extract directly into food products is an unfavorable practice and therefore the extract conversion to powder is required beforehand. The pomegranate whole peel was micro wet milled, and the extracted slurry containing high fiber content was converted to powder with spray drying. The spray drying process was optimized with full factorial design. Maltodextrin level and inlet temperatures was tested as the influential factors during spray drying process. The process was optimized for total phenolic contents, total anthocyanin contents, yield of powder and antioxidant activities. The powder was also examined for physiochemical and microstructure properties. The results showed that the whole peel powder can be produce with spray drying process when coupled with a prior micro wet milling process. The most influenced factor was maltodextrin level while variation in temperature did not influenced the powder significantly. In conclusion the powder produced with 20% maltodextrin and 120 °C inlet temperatures showed higher nutritional and physical properties.

Chapter 3

Pomegranate peel extract powder with spray drying

3.1 Pomegranate peel extract powder

In chapter tow, the powder was produced from whole pomegranate peel with spray dry. In this chapter the powder was produced from the pure extract after centrifugation. The pomegranate peel was extracted with MWM process as described in the first part of the study. The extracted slurry was centrifuged for 10 minutes to obtain pure phenolic extract. The extract was then rotatory evaporated at 40 °C to remove excess ethanol for safety reasons (flammable) as the spray drying process operates at high temperatures. Maltodextrin (MD) was used as wall material, which was optimized in the second part of the study, at 20%, 40%, and 60% dry basis. The spray dryer was operated at 120 °C as the variation in temperature was not significant from our previous findings. The obtained powder was analyzed for its nutritional and physiochemical properties. The process flow diagram is shown in Figure 23.

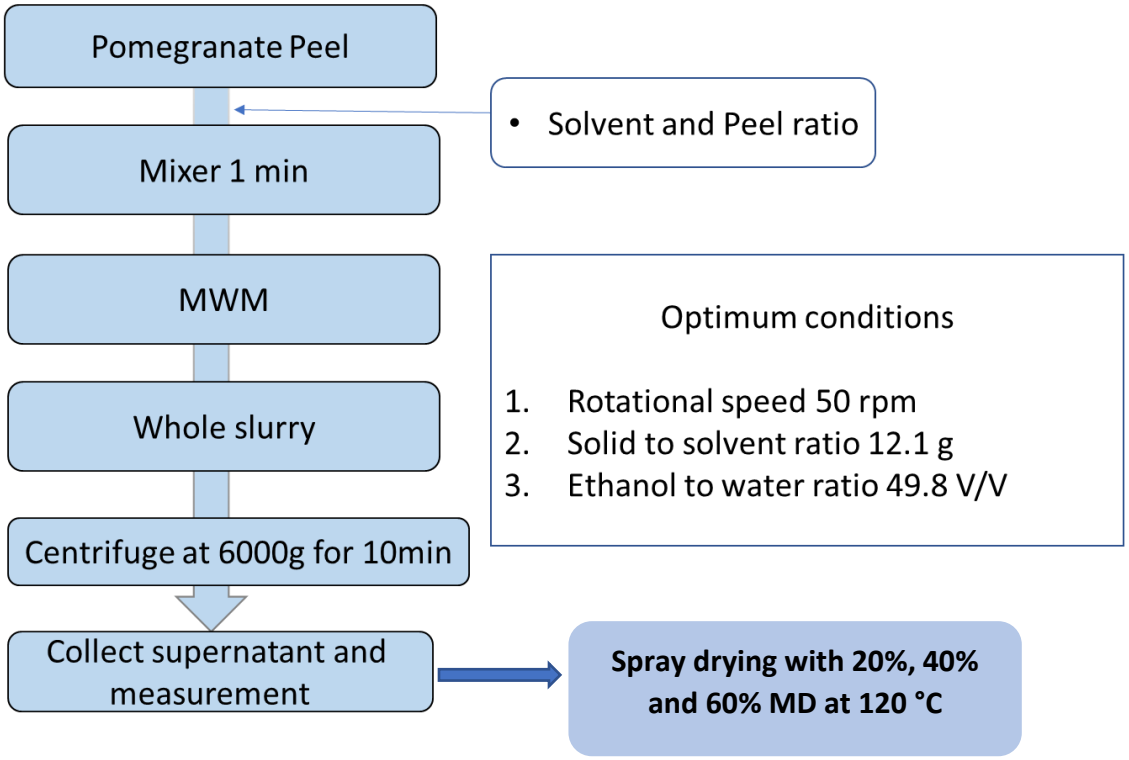


Figure 32. Flow diagram of process of producing pomegranate peel extract powder with spray dry.

3.2 Results and discussions

3.2.1 Total phenolic content (TPC) of extract powder

The highest TPC obtained was 167.81 mg GAE/g from 20% maltodextrin samples. The increased in maltodextrin concentration, decreased the TPC amount. The decrease in TPC was largely a result of the substitution of the extract with higher MD amounts in the powder, as MD lacks TPC . Similar results were obtained by (Çam, Içyer and Erdoğan, 2014) from pomegranate peel extract powder. Overall, the TPC contents were higher compare to the whole peel powder. The increase was due to the extract was concentrated and the fiber part of the peel was discarded before spray drying process compare to the whole peel powder.

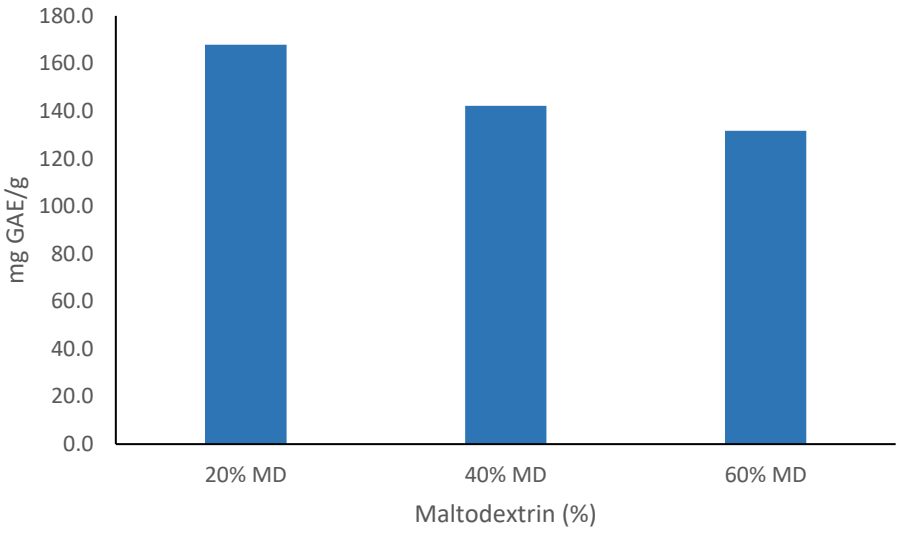


Figure 33. TPC amount of peel extract powder with maltodextrin as wall material

3.2.2 Total anthocyanin content (TAC) of extract powder

The anthocyanin content of pomegranate peel is heat sensitive and unstable during processing. The major anthocyanin contents identified in the pomegranate peel was in order delphinidin 3,5-diglucoside, yanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, delphinidin 3-glucoside, a cyanidin–pentoside–hexoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside and a cyanidin-pentoside (Fischer, Carle and Kammerer, 2011). Maltodextrin and other wall material are essential in the spray drying process to avoid the powdered material from sticking to the drying chamber and to preserve heat sensitive components. The peel extract powder showed similar trend of TAC to TPC. The TAC amount decreased with increase in MD amounts. The highest TAC was obtained in 20% maltodextrin concentration. Similar decline was observed on spray dried pomegranate juice powder (Jafari, Ghalegi Ghalenoei and Dehnad, 2017).

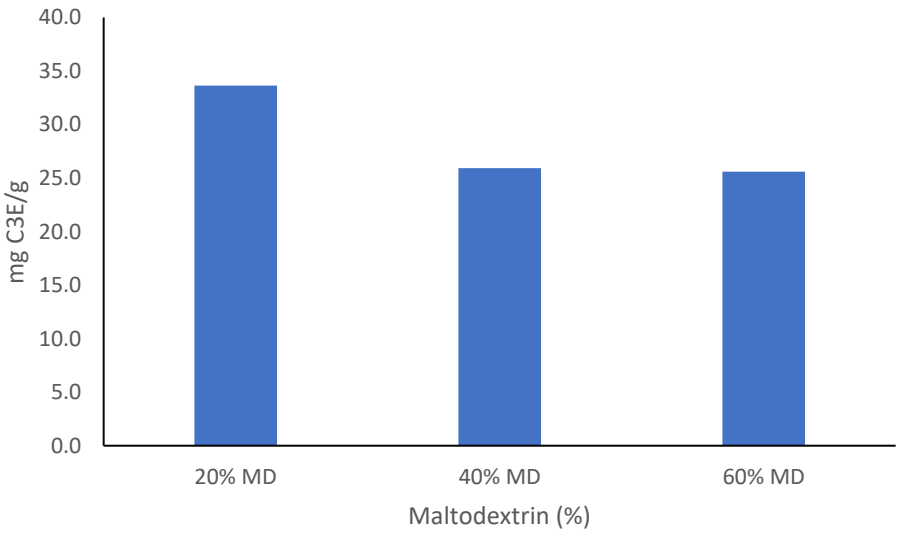


Figure 34. TAC contents of pomegranate peel extract powder

3.2.3 Antioxidant activities of the extract powder

The antioxidant activities were measured with DPPH free radical scavenging activities. The radical scavenging activities were quantified as the peel powder inhibited half of the radical activities (IC50). The IC50 values showed similar trend as TPC and TAC. The amount of the IC50 increased with the increase in MD level. The lower the IC50 value the higher the inhibitory activity of the powder. The IC50 value of whole peel powder was insignificantly different equated to the extract powder. Despite the higher TPC and TAC in extract powder, the whole peel showed better antioxidant activities. The fiber content accounts for the higher retaining ability of fiber components in the whole peel powder. These results were lower compared to the freeze dried pomegranate peel powder (Azarpazhooh *et al.*, 2019).

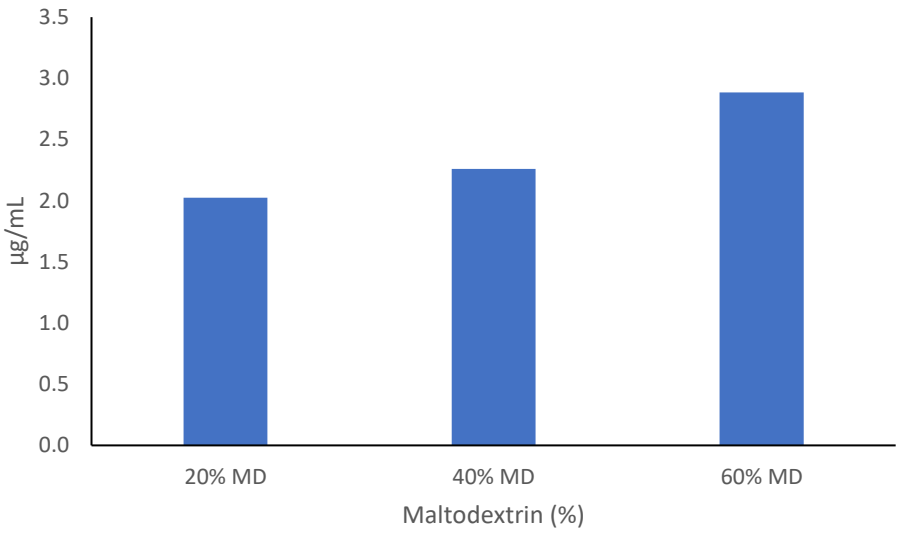


Figure 35. Antioxidant activity of pomegranate peel extract powder with varying maltodextrin amounts.

3.2.4 Physiochemical properties of extract powder

The physiochemical properties are important from consumers perception and for the products storage period. The results in Table 12 shows that the bulk density of the powder is lowest at the 40% MD and the highest was observed at the 60% MD sample with 20% MD sample being slightly similar to it. Similar trend was observed in tapped density, HR and CI ratios. The water activity was not significantly different among the samples. The lower water activity of the powder samples signifies longer shelf-life and storage stability.

Table 12. Physiochemical properties of pomegranate peel extract powder

	Bulk density g/mL	Tapped density g/mL	HR	CI	aw
20% MD	0.143	0.164	1.147	12.787	0.069
40% MD	0.127	0.149	1.169	14.471	0.063
60% MD	0.146	0.188	1.286	22.210	0.066

3.2.5 Color parameters of extract powder

The color of a powder is one of the key parameters to determine its acceptability by the consumers. The L* value of the color shows the lightness of the product. The L* value close to 100 indicates lightness while values close to 0 is the darkness of the product. Results show that the powder was lighter in color. The value of L* increased with the increase in MD level as maltodextrin has a white color. The a* value of the color represents red and green color. On the X axis, the a* value is expressed from right to left, positive a* indicates redness while negative a* value represents the intensity of green color. The a* value results showed a positive number implying slight redness in color of the extract powder. The a* values slightly decreased with decreasing MD level validating the results from TPC and TAC which also reduced. The reduction of TPC and TAC with a* value is mainly a result of many of these components especially TAC having a red color. The b* values is represented on the Y axis as blue and yellow. Positive b* values show yellowness, and negative values show blueness. The b* value for the color shown in Table 13 was a positive number. The change in b* value was significant. To locate the exact value of the product color the chroma vale and hue angle were calculated. The chroma value shows the intensity of the color while the angle determines the exact location of the color on the axis. The chroma values was around 20 and the hue angle was 60. These results have determined that the powder is white with yellowish tone in color.

Table 13. The color parameters of pomegranate peel extract powder

MD Level	L	a	b	Chroma Value	Hue angle
20% MD	74.48	11.91	18.57	22.06	57.31
40% MD	76.21	10.45	17.98	20.79	59.83
60% MD	76.27	10.04	18.13	20.73	61.02

3.2.6 Solubility of extract powder

The solubility of powder is measuring the reconstitution of powder in water. The solubility of powder increased with an increasing MD concentration. The solubility of extract powder was higher compare to the whole peel powder due to the whole peel powder consisting of some insoluble fibers. The higher solubility of extract powder was due to the extract being water soluble and the maltodextrin has a higher solubility in water. Similar results were reported from pomegranate fruit juice powder (Jafari, Ghalegi Ghalenoei and Dehnad, 2017) .

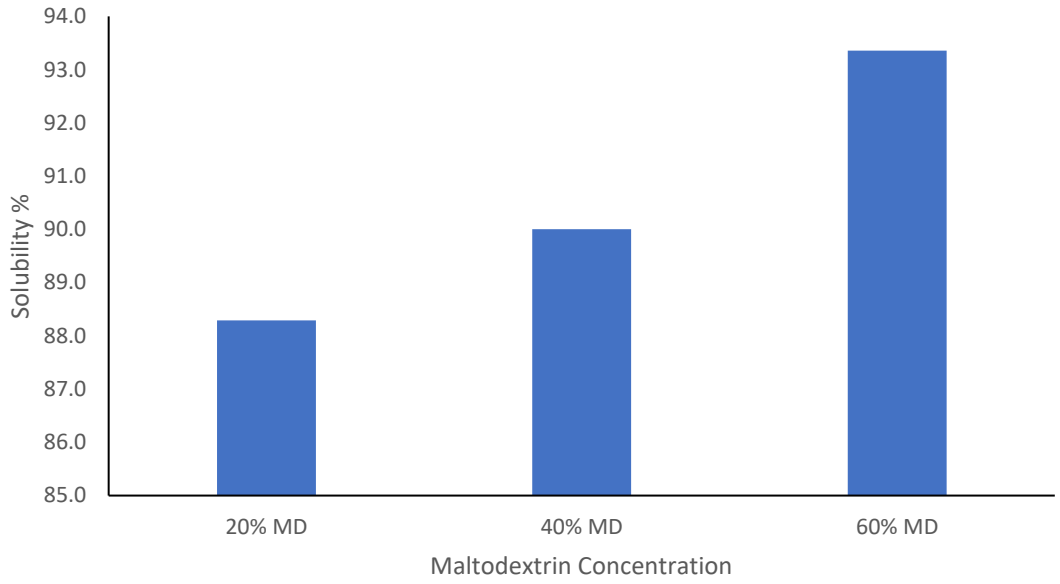


Figure 36. Water solubility of pomegranate peel extract powder with varying maltodextrin amounts.

3.3 Summary

The powder was produced from pomegranate peel extract using maltodextrin as wall material. The pomegranate peel was thawed and cut to small parts using kitchen knife. The peel was then mixed with solvent and feed to the MWM. The MWM was able to produce smaller particles which enable the peel phenolics to extract in the solvents. The extract was then centrifuged the supernatant was collected and concentrated in a rotatory evaporator. The concentrated extract was mixed with maltodextrin to avoid the sticking of feed sample on the spray drying chamber. The powder was collected and analyzed for its nutritional and physiochemical properties. The powder showed higher TPC, TAC and antioxidant activities compare to whole peel powder.

Chapter 4

Soft cheese (Paneer) making with fermented rice milk and pomegranate peel powder as a natural preservative additive.

4.1 Introduction

Dairy products is one of the largest sectors in the world food market. It has maintained its place on the market for centuries due to its natural production and its nutritious value. Milk and dairy products contain micronutrients such as minerals and vitamins, which contribute to multiple and different vital functions in the organism (Gaucheron, 2011). However, malabsorption of lactose commonly known as lactose intolerance is a concern for many dairy products consumers (Casellas *et al.*, 2016). This has led to the food dairy industry to invent or manufacture alternative dairy products that are lactose intolerance friendly but still maintaining its nutritious value and taste. Cheese is a dairy product produced by the coagulation of milk protein (Casein) which contains milk protein and fats and a minimal amount of lactose. The lower lactose amount makes cheese a lactose intolerance friendly dairy product.

Paneer is a soft variant of cheese and is obtained by heat and acid coagulant of milk. Paneer is famous in South Asian countries such as Pakistan, India, Bangladesh, Afghanistan, and Iran. Paneer is served as breakfast or used in culinary dishes (Kumar *et al.*, 2014). Paneer is usually made from buffalo milk and can also be made from cow or goats' milk. Paneer has a white color, spongy texture with a mildly acidic flavor. Paneer has a low amount of lactose and a high percentage of fat content especially when prepared with buffalo milk ("KANAWJIA, S.K.; SINGH, S," 1996). Paneer has a high moisture content (50%-60%) which leads to high bacterial growth and a shorter shelf-life. The shelf-life of paneer is only one day at room temperature and 7 days at refrigerator temperature (Venkateshaiah *et al.*, 2011).

Table 14. Chemical composition of Paneer

Chemical composition of Paneer						
	Constituents (%)					
Type of milk used for Paneer making		Fat	Protien	Lactose	Ash	Reference
Buffalo milk (3.5% fat)	56.99	18.1	18.43	-	-	Chawla et al. (1987)
Buffalo milk (4% fat)	54.05	23.27	16.78	2.69	2.2	Chawla et al. (1987)
Buffalo milk (5% fat)	56.77	22.3	-	-	-	Bhattacharya et al. (1971)
Buffalo milk (5% fat)	52.75	25.64	15.62	2.68	2.14	Kumar et al. (2008b)
Buffalo milk (6% fat)	54.76	25.98	-	-	-	Bhattacharya et al. (1971)
Buffalo milk (5.8% fat)	50.72	27.13	17.99	2.29	1.87	Rajorhia et al. (1984)
Buffalo milk (5.5% fat)	55.19	23.8	17.99	-	-	Chawla et al. (1987)
Buffalo milk (5.8% fat)	54.1	23.5	18.2	2.4	1.8	Sachdeva and Singh (1987)
Buffalo milk (6% fat)	50.98	27.97	14.89	2.63	2.08	Kumar et al. (2008b)
Whole buffalo milk	51.52	27.49	17.48	2.28	2.18	Das and Ghatak (1999)
Cow milk (3.5% fat)	55.97	18.98	20.93	2.01	1.24	Mistry et al. (1992)
Cow milk (5% fat)	53.9	24.8	17.6	-	-	Singh and Kanawjia (1988)
Cow milk (4.5% fat)	55.26	24.15	18.43	-	-	Syed et al. (1992)
Buffalo milk and soya milk (50:50)	54.6	18.33	19.81	-	1.68	Babaje et al. (1992)

Source: this table is reproduced from Paneer - A soft Indian cheese variant: A review (Kumar et al., 2014a)

The growing popularity in Paneer production in the last few decades have led to multiple attempts to improve the quality of Paneer and add value to Paneer product. To develop new types of Paneer such as recombined and reconstituted milk paneer (Pal, 2010), dietary fiber enriched low fat paneer (Kandeepan and Sangma, 2015), soy paneer (A Masoodi, 2014), filled paneer, vegetable impregnated paneer and Ultrafiltration(UF)paneer.

Acidified and cultured whey *L. acidophilus* was used as Paneer coagulant at coagulation temperatures 90°C and 80°C. The results showed the yield of Paneer decreased with increasing coagulation temperatures. Cultured whey had good effects on the yield of Paneer which increased respectively (Deshmukh *et al.*, 2009).

Paneer has a higher fat content of about 25% of its mass and is unhealthy to human health when consumed in large quantities overtime. Therefore, several studies were interested in developing low-fat Paneer such as paneer prepared from yak milk. A study mentioned, low fat paneer can be produced from yak milk having 1% fat with acceptable texture for consumption (Kandeepan and Sangma, 2011).

Study was conducted to produce Paneer from peanuts milk, the peanut milk was made from peanuts by soaking in water for 8 hr then the skin was removed and grinded. Milk was coagulated with 1% calcium sulphate solution. Paneer samples were vacuum packed and refrigerated at 6°C. Peanut Paneer evaluation showed good results up to 45 days at 6±1 storage temperature (Chauhan *et al.*, 2015).

Similar product was developed with egg yolk, natural antioxidants, and salt. The samples were dehydrated and stored in polyester pouches at 27±2 °C for 6 months. The results revealed from sensory evaluation that rehydrated yolk paneer had very good texture and was very close to fresh samples before dehydration during storage tell 6 months (Pawar, Das and Modi, 2012).

Meanwhile, many attempts have been made to enhance the shelf-life of Paneer such as adding various spices (Eresam, Pinto and Aparnathi, 2015), essential oil in processing (Khatkar *et al.*, 2017), vacuum packaging (Venkateshaiah *et al.*, 2011), dipping in bran solution, bacteriocin and more. Study conducted for extension of shelf life of Paneer incorporation of hurdle technology and MAP packaging. The hurdle technology was used as NaCl and citric acid for lowering the moisture content and pH of Paneer with MAP packaging under (CO_2: N_2) by 50:50%

conditions. The result reported that the shelf life of Paneer was extended from 1 day to 12 days at room temperature and from 6 days to 20 days under refrigeration temperature (Venkateshaiah *et al*., 2011).

The effect of low temperature storage on Paneer shelf life was studied. Paneer samples were stored at -13 °C and -32 °C and examined for their physiochemical and sensory properties. One study stated that paneer samples can be stored for 120 days at -13 and -32°C and the sensory results were acceptable after frying (Arora & Gupta, 1980).

Paneer has a high moisture content which can be good breeding ground for bacterial leading to quick spoilage of the product. Therefore, some selective spices with known antimicrobial properties such as black pepper, cardamom, cinnamon, and clove were added to paneer samples at 0.2, 0.4, 0.6, 0.8 and 1.0 % by wt and stored at 7±1 °C. After sensory evaluations the control samples were acceptable for up to 7 days, samples with black paper were acceptable up to 14 days, 21 days with cinnamon, and cardamom inclusive samples for more 28 days of storage (Eresam et al., 2015).

A similar study evaluated the addition of turmeric to paneer samples for shelf-life extension. Turmeric was added at it different amounts from 0.2, 0.4, 0.6, 0.8 and 1.0 % by weight. The samples were stored for 12 days at 7±1 °C The samples of paneer with 0.6 % turmeric by weight of predictable yield of paneer stay satisfactory for up to 12 days on storage at 7±1 °C. The present study reported, that adding turmeric in paneer at the rate higher than 0.6 % by mass of expected yield of paneer had reduced sensory score. Addition of turmeric at the rate of 0.6 % by weight of projected yield of paneer extended its shelf life for up to 12 days of storage at 7±1 °C (Buch, Pinto, & Aparnathi, 2012).

The use of clove essential oil in paneer processing resulted in the decline in moisture content and increase free fatty acid and titratable acidity when the paneer was stored at 8±1 ºC. Sensory evaluation showed that paneer samples treated with clove essential oil was acceptable for 10 days storage (Khatkar et al., 2017a).

The evaluation of the effect of plant essential oils for paneer shelf-life extension with cinnamon essential oil used along with different packaging materials such as LLD/ BA*/Nylon-6/BA*/LDPE (110 micron) (*poly

binding agent), Unprinted metalized polyester-LDPE laminates and LDPE pouch. The shelf-life evaluation with different packaging reported, the control paneer samples exhibited only 10 days shelf life with metalized polyester, 8 days with nylon and 5 days with LDPE, whereas cinnamon added samples resulted 18 days of shelf life with metalized polyester, 14 days with nylon and 9 days with LDPE at 8±1ºC. (Khatkar, Ray, & Kaur, 2017b)

For this study, fermented rice milk was used as coagulant instead of citric acid to make soft cheese (Paneer). Rice milk is produced from brown rice with the Micro Wet Milling (MWM) system. The advantages of this system were the production of less heat during operation and rice milk making from uncooked rice was made possible. MWM system rice can mill in very small particles size less than 2μm which can be serve as a rice milk beverage. (Koyama & Kitamura, 2014)

Whereas rice is a staple food in many Asian countries including Japan. However, recently there has been a decline in its consumption. Therefore, there is a need for new techniques to develop new recipes not only for the consumers consumption and health but also considering the utilization of food resources.

Brown rice is highly nutritious, low in calories compared to white rice because white rice is produced from brown rice by removing the outside hull which contains additional nutrients. Brown rice is gluten-free and is high in fiber. On the other hand, white rice kernel removal process loses a substantial amount of vitamins B and other essential elements such as manganese (Mn), phosphorus (P), Iron (Fe), dietary fibers, and essential fatty acids. Brown rice can be utilized for rice milk production that can be used for to make a variety of commercial or subsistence food preparation. Rice milk prepared from whole brown rice, is healthier and is a rich source of fiber which is consider good for Paneer structure and texture. (Jessie, 2015)

Rice milk is a liquid beverage made from milled rice grains where the mixture ratio of rice and water is 1: 2. Rice milk is mostly made from brown rice instead of white rice obtained after removal of the hull of brown rice. This means rice milk is rich in nutrients from brown rice. Rice milk contains vitamins, minerals, fibers, and protein which are ideal for vegetarian people (Nakamura, Kitamura, & Kokawa, 2016).

Rice Milk can be taken as a drink without pasteurization. This preserves all the nutrients contained in its natural state also avoiding starch gelatinization and saccharification from producing more glucose.

Rice grain is commonly made of starch which existed in granular shape. Starch structures differ in the ratio of amylose and amylopectin among plant species. A major part of rice milk consists of starch and lactic acid bacteria (LAB) that only use simple sugars such as glucose. Therefore, it needs to convert starch to glucose beforehand. Rice milk produced from raw rice grains contain bacteria (unpasteurized or unsterilized). Consequently, rice milk is subjected to sterilization at 90°C for 30 mins. Along the sterilization process, rice milk was gelatinized. Gelatinization is a process whereby starchy food is subject to wetting combined with heating. In gelatinization process, intermolecular bonds of rice starch break down and water absorbs into molecules in an irreversible manner. Gelatinization makes starches transparent and viscous which appears in gel form. (Santos & Perez, 2013)

Rice milk gelatinization forms a gel like structure, it became more viscous and denser. Since rice milk needs to be in a liquid state for fermentation it is subject to the liquefaction process for fermentation after gelatinization. Liquefaction is the partially hydrolysis of starch which makes starch less viscous and become liquidy using amylase enzyme. (Linko & Javanainen, 1996) Subsequently, rice milk is saccharified with the addition of glucoamylase. Saccharification is the process where complex compound such as starch breaks down to simple sugars glucose and fructose in the presence of enzyme or acids. Starch must be converted to reducing sugar before fermentation. In some rare cases, lactic acid bacteria can utilize direct polysaccharides without conversion of starch to simple sugars. Bacteria such as *Lactobacillus amylovorus, Lactobacillus amylophilus* (Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008).

Fermentation for Lactic Acid (LA) production is commonly carried out by addition of lactic acid bacteria. Fermentation is the process where living organisms consumes sugar and produce organic acids, alcohol and so on. Conventionally, fermentation is done after a long process of gelatinization and liquefaction of starch at high temperatures (90-130 degrees Celsius) for 15 minutes followed by enzymatic saccharification to glucose. Therefore, saccharification of starch and fermentation can be done simultaneously to avoid energy and time consumption.

Studies showed, SSF (Simultaneous Saccharification and Fermentation) is simple for fermentation of starchy material to Lactic Acid (LA) and ethanol (C_2H_5OH) etc. (Linko & Javanainen, 1996) In the present study, both conventional and SSF methods were compared for rice milk lactic acid fermentation.

As reported in literature, during the simultaneous saccharification and fermentation (SSF) process *Lactobacillus planetarium* bacteria, produces lactic acid mostly in the first 24 h of fermentation which reduces the pH. This suppresses the growth rate of lactic acid bacteria. Lactic acid bacteria were suppressed at 4.5-5 pH and the maximum growth was observed at pH 6-6.8 when barley starch was fermented for lactic acid production. The fermenting pH also affects the saccharification process at pH 4.5 is the optimum pH for α-amylase activities. (Tanaka et al., 2006) Therefore, in this study we used $CaCo_3$ for pH buffering.

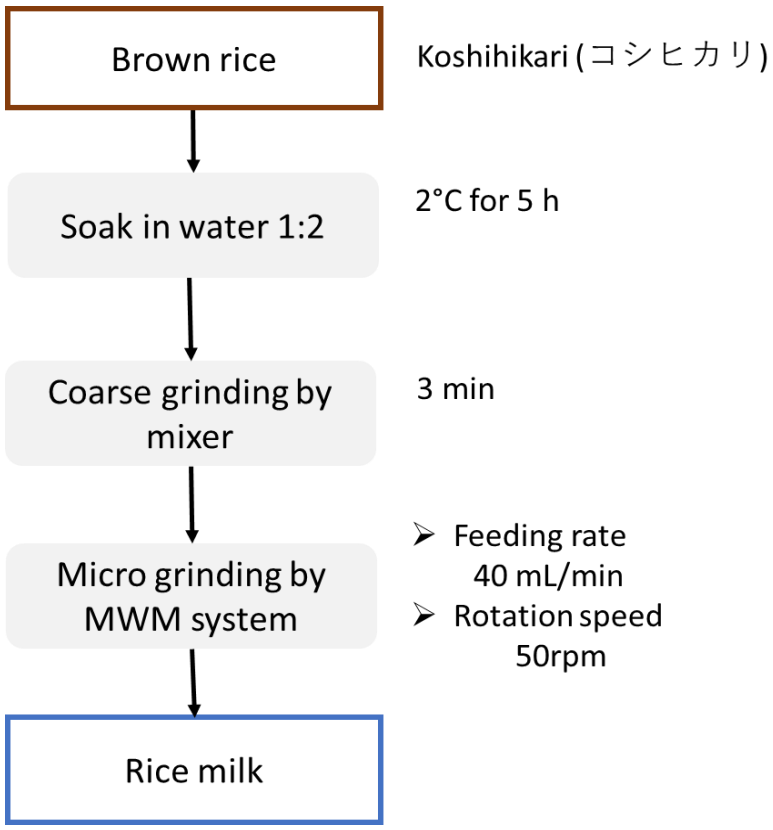
Another study stated that saccharification of starch at varying pH, temperature, and inhibitors such as citric acid, propionic acid, acetic acid, ethanol, and lactic acid showed minimum inhibitory effects. The fermentation conditions are different with the different species of bacteria the optimum conditions for SSF were established as 45°C and pH 5.5. *L. delbrueckii* was used for fermentation and suggested the productivity could be increased by incorporating simultaneous removal of lactate from the broth or increasing the growth associated production of lactate. (Anuradha, Suresh, & Venkatesh, 1999a)

The objectives of this study were to produce paneer (soft cheese) with fermented rice milk. Furthermore, pomegranate peel extract powder was incorporated as natural preservatives and antioxidants. The impact was study during storage at 4°C with analyzing the physicochemical and biological properties of paneer.

4.2 Material and methods

4.2.1 Production of rice milk

In this study rice milk was produced from brown rice. Brown rice was soaked in water with a 1:2 ratio and kept in 2°C for 5 h. Rice was then coarse grinded with a mixer (Hamilton Beach) for 3 mins to the Micro Wet Milling (MWM) machine to utilize rice milk. Grounded rice was grinded by the MWM system where the feeding was 40 mL/min and the rotational speed was 50 rpm (Nakamura et al., 2016). The rice milk was then subjected to gelatinization, liquification, saccharification and fermentation processes. Before rice milk pasteurization and gelatinization 0.1% α-amylase enzyme was added to avoid rice adherent to the pot. Rice milk was Then heated to 90 °C and kept at same temperature for 30 minutes.



Fiugure 37. Flow diagram of rice milk producing

4.2.2 Total bacterial count

For the total bacterial count was carried out on culturing the sample on 3M Petri film, samples were diluted $10^{10}$with 0.9% saline solution then cultured and incubated at 37°C for 48 h and counted.

4.2.3 Preparation of paneer

Paneer was prepared with the method stated by (Kumar et al., 2014a). Dairy milk (1000mL) was heated to 88°C for 5 min, cooled to 85°C, and 100 mL of fermented rice milk was added as the coagulant. The mixture was rested for 5 min and was drained using a muslin cloth to separate whey and paneer curd. Curd was then pressed by two 2 kg-weights and was cut into 15 g cubic shapes. Samples were stored at refrigerator temperature for 10 days and were analyzed for physicochemical and biological properties.

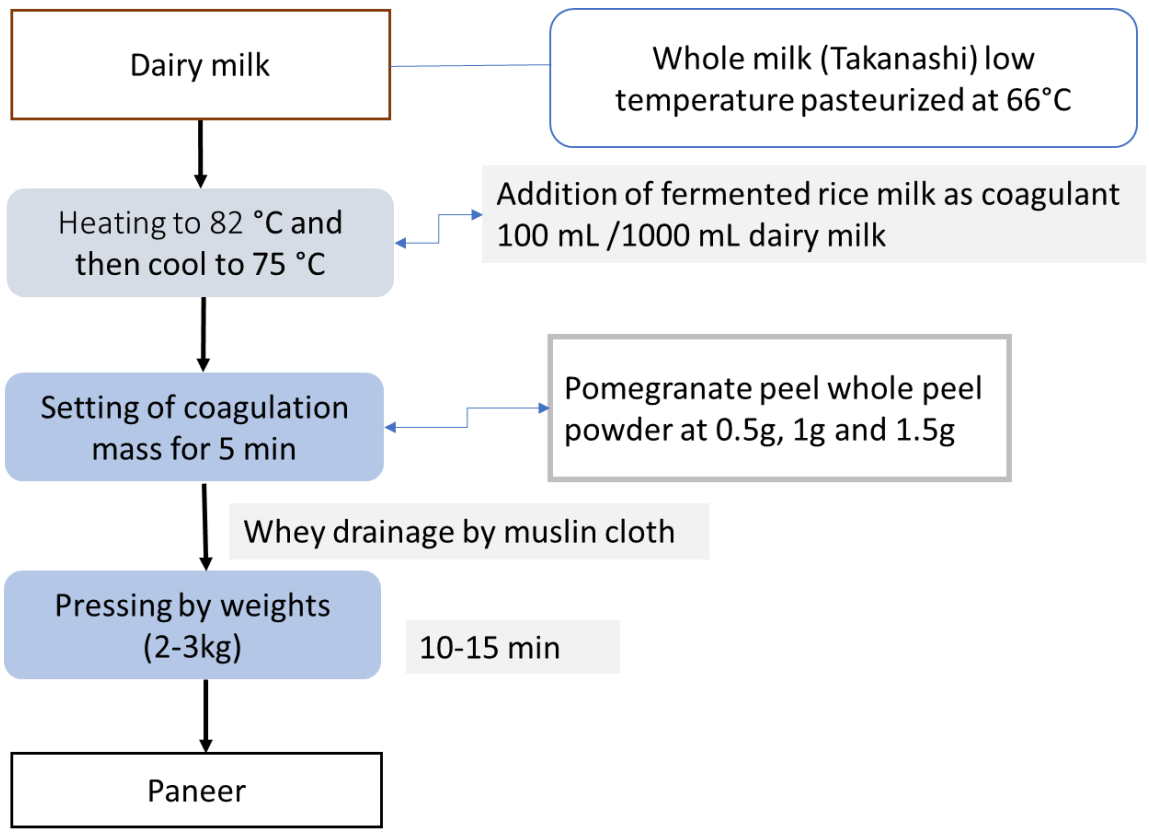


Figure 38. Flow diagram of making paneer.

4.2.4 Moisture content

Ten grams of samples were measured and placed in an oven for 24 h at 105°C. The moisture content was calculated with.

$$moisture\ content\ (\%) = \frac{(W_1 - W_2) * 100}{W_1} \quad (18)$$

4.2.5 Paneer yield

where w_1 and w_2 are the weights of the samples before and after drying, respectively. Total yield was calculated as

$$Yield\ (\%) = \frac{W_{milk} + W_{ricemilk} - W_{whey}}{W_{milk} + W_{ricemilk}} * 100 \quad (19)$$

4.2.6 Determination of ash and fat content

Ash content was determined using the official method (AOAC Methods 900.02 A, 920.117) where 2 g sample was measured and placed in an oven for 6 h at 550°C. Ash content was calculated as:

$$Ash\ content\ (\%) = \frac{W_4 * 100}{W_3} \quad (20)$$

where W_3 and W_4 are the weights of the sample before and after heating, respectively (Maurice,2010).

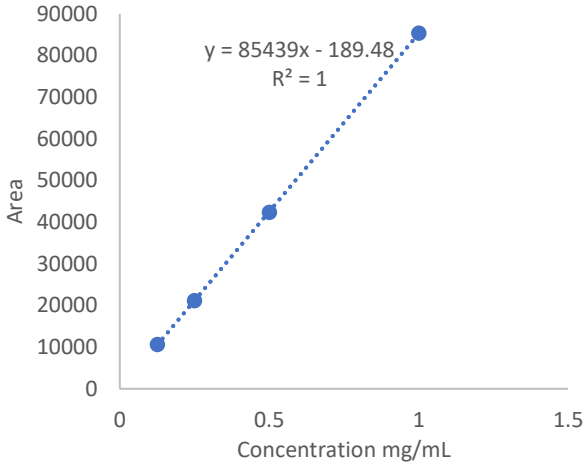
Paneer fat content was determined by the Gerber method ISI. 1977. (KEVINE OTIENO, 2018) in first step 5 g of Paneer samples, 10 ml sulfuric acid, 5 ml distilled water, and 1 ml isoamyl alcohol were added to Gerber bottles. In the second step, a robber locks were inserted and shake until the Paneer samples dissolved in the solution. Thirdly, the samples were centrifuged for five minutes. Finally, the samples were put in water bath for five minutes and the fat content was read on the bottles upper side which numbered to show the percentage of fat content in samples.

4.2.7 Determination of free fatty acids

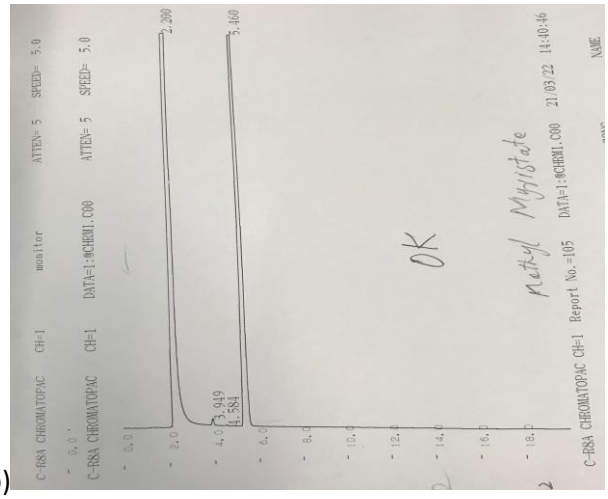
The fat content of paneer was extracted with Soxhlet method. The paneer sample was first mixed with cellulose powder and mashed to smaller particles(Aghav *et al.*, 2014). The mashed paneer was then placed in

extraction container and extracted with hexane as extraction solvent. The hexane was evaporated with rotary evaporator at 40°C attached with suction pump.

The oil sample 50 mg was then methyl ester using esterification kit. The upper layer of the sample was collected and injected to GC-14B / C-R8A (SHIMAZU). The GC program was set to INJE: 260°C, DET:260°C, COLM-INIT-210°C, TIME:20 min and the final temperature was set 210°C. Nitrogen was used as carrier gas and the injection volume was set to 1 µL. The free fatty acid was calculated from a standard curve created from the area of standards of free fatty acids.



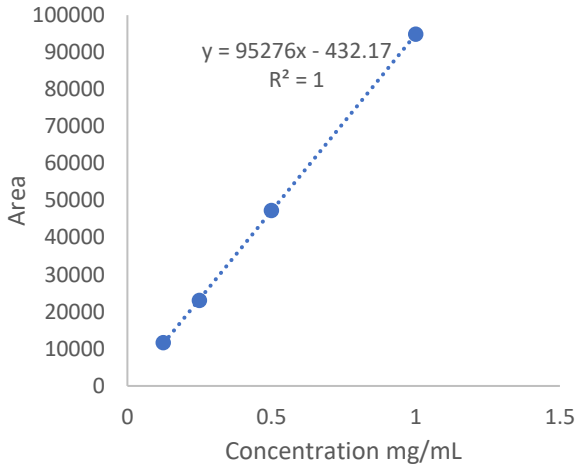
(a)



(b)

Figure 39. (a) Methyl Myristate standard curve, (b) GC chromatogram of methyl myristate

(c)



(d)

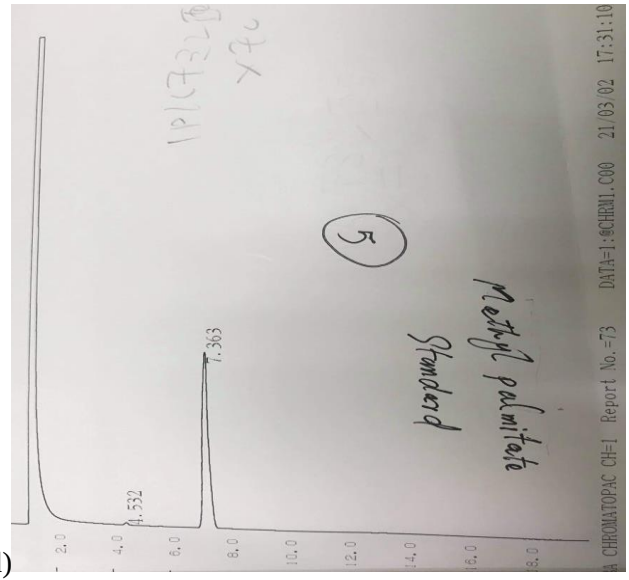
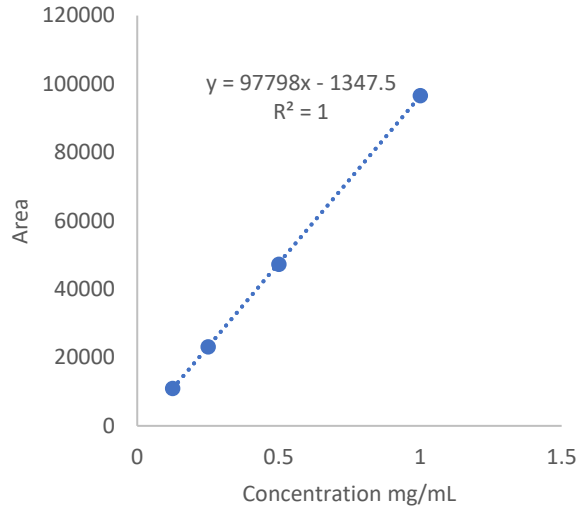


Figure 40.(c) Methyl Palmitate standard curve, (d) GC chromatogram of methyl palmitate

(e)



(f)

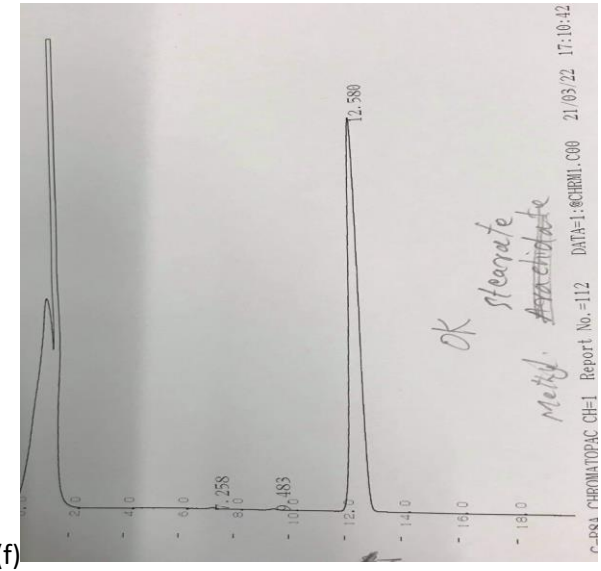
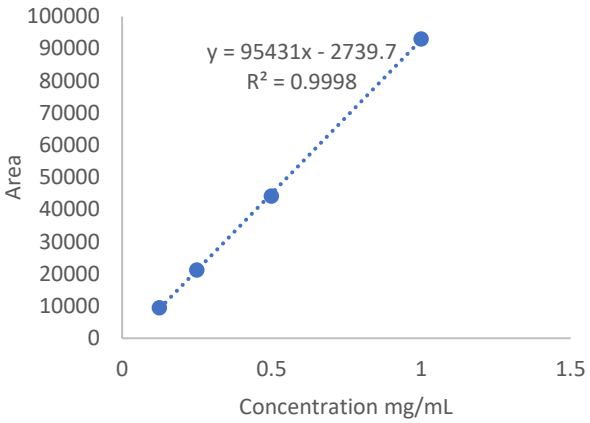
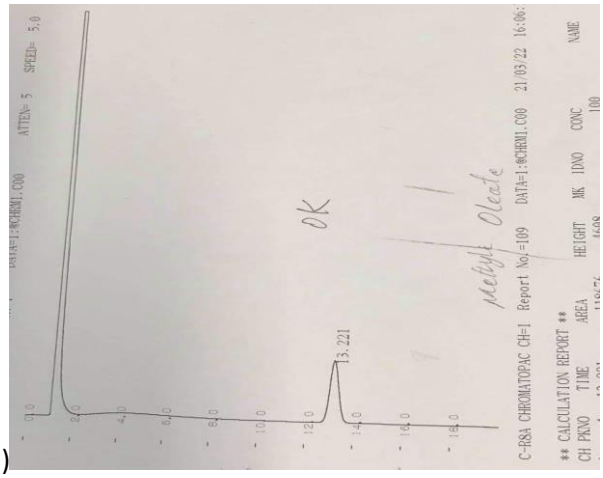


Figure 41. (e) Methyl Stearate standard curve, (f) GC chromatogram of methyl stearate



(g)



(h)

Figure 42. (g) Methyl Oleate standard curve, (h) GC chromatogram of methyl oleate

4.3 Results and discussions

4.3.1 Physiochemical properties of paneer

Paneer produced with rice milk as coagulant showed higher yield compare to paneer produced with citric acid, lactic acid, tartaric acid as coagulant. The obtained yield was 36.36 % where the reported yield from different coagulants were from (16-20%) the paneer was prepared with buffalo milk (Manan and Sharma, 2018). Four kinds of samples were prepared with rice milk as coagulant and pomegranate peel powder as additives. The first kinds of samples were prepared as control (C) without peel powder, second type was prepared with 0.5g of pomegranate peel powder (S1), third samples were prepared with 1g peel powder (S2), and the last kind of samples were prepared with 1.5g peel powder (S3). The pomegranate peel powder additives were added per 1 kg of dairy milk used. The paneer samples were packaged in LDPE zip locks bags and stored at 4°C.

Ash content determines the amount of minerals in food, the ash content of paneer samples prepared with 100 mL rice milk was 1.49% which was higher compare to paneer prepared solely from cow milk 1.18%. Thus, the fat content of paneer samples prepared with 100 mL rice milk was 7.5% and the fat content of paneer prepared from cow milk was 8.7% which was higher to rice milk incorporated paneer.

4.3.2 Moisture content

The moisture content of food determines that different food has different moisture contents and can deciding standard for its shelf life and microbial activities. Other food properties such as texture, taste, appearance, and stability are dependent on the amount of moisture content contained in food.

Different dairy products have various amounts of moisture content depending on the processing methods and type of products. Paneer is one of the dairy products which contain a high amount of moisture content. The moisture contained in Paneer is high due to the heat and acid expansion of casein micelles which holds moisture. Normally, Paneer has 40 % to 55% of moisture content in its structure which affect its shelf life.

Results are shown in Table 15, the moisture content of control samples were lower compared to samples with the addition of pomegranate peel powder. The moisture content slightly decreased during storage period. The reason for reduction in moisture content was probably due to the bacterial count which was higher in day 5 of storage. Additionally, the expulsion of moisture content from the product to its surroundings causes a reduction in its moisture contents. Similar results were reported by another study (Perveen, Alabdulkarim and Arzoo, 2011).

Table 15. Moisture content (%) of paneer storage

Samples/Storage period	DAY 1	DAY 5	DAY 10
C	58.57±0.01	56.39±0.32	56.80±0.36
S1	61.20±0.24	60.56±0.64	59.28±0.47
S2	61.93±0.10	60.06±0.85	61.66±0.78
S3	63.37±0.70	63.29±0.08	62.51±0.23

4.3.3 Total bacterial count during storage

Paneer is very perishable dairy product it contains high moisture content which leads to a good environment for bacteria growth and spoilage. The microbial growth of paneer also depends on the making process, handling, and storage conditions. The microbial analysis during storage at 4±1°C shown in figure 42. The total bacterial count decreased during storage in all the samples. The initial bacterial count was around 4.3 log CFU which decreased to 3.8 in control and 3.09 in S3 (1.5g pomegranate peel powder for 1000 mL milk) samples. The decrease in treated samples with pomegranate peel powder was higher compare to control sample. The decrease in paneer samples were observed to decrease in day 5 of storage and increased with further storage. Similarly, results were reported that bacterial growth was higher in control sample compare to cinnamon treated samples (Khatkar *et al.*, 2017). There was obvious defect observed in paneer samples for 10 days storage and the bacterial growth was lower than standard limit for paneer spoilage which is more than 5.5 log of CFU (FSSAI/2010).

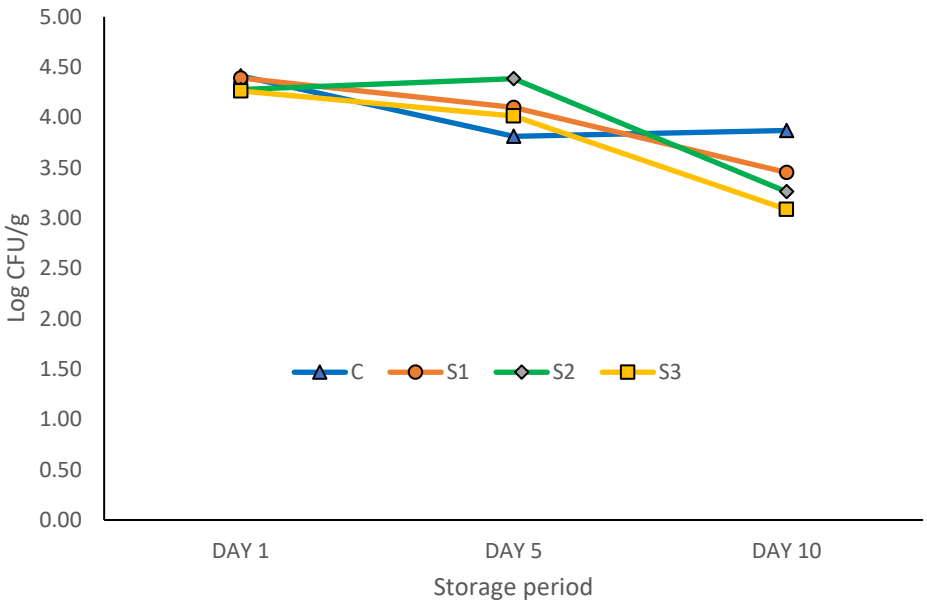


Figure 43. bacterial growth of paneer samples during storage

4.3.4 Acidity during storage

The acidity of paneer samples was measured as total lactic acid during storage (Figure 43). The lactic acid amount increased during the storage in all the samples. The increase in lactic acid was higher in control (C) and S1 samples compare to S2 and S3 samples. The increase of lactic acid during storage is probably a result of the higher bacterial activities in these samples. Comparably, another study revealed that lactic acid increased during storage from 0.45 % to 0.61% in a 16 days storage period (Chaudhary et al., 2019). The lower lactic acid in S2 and S3 samples is indication of the antimicrobial activities of pomegranate peel extract.

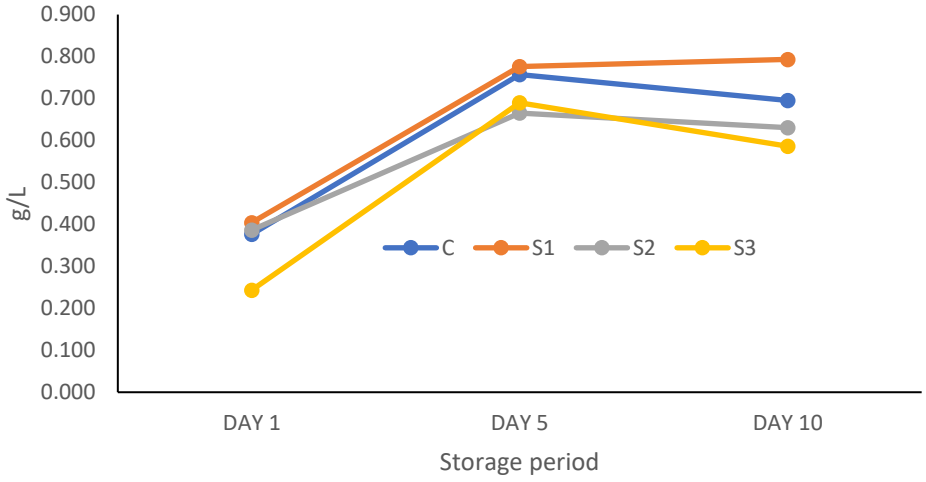


Figure 44. the total lactic acid amount during paneer storage

4.3.5 Color properties of paneer samples during storage

The first parameter in food evaluation is color which has an important role in the customer's acceptance and desirability. The measurement of color also affects the food quality and can detect defect level in food products to a certain extent. The Lab color characteristics of paneer prepared with fermented rice milk and pomegranate peel powder as additives shown in table 15. The L* value of color which indicates the lightness of samples and was

higher in the control samples. But there was not significantly impact of storage period on paneer color characteristics. The a* value of color which represents the redness and greenness of product. The negative value of a* shows that product has slightly red and green color. The a* value was lower in samples containing the pomegranate peel powder indicates more redness and anthocyanin color of whole pomegranate peel powder. Meanwhile, the b* values of color represent blue/yellow color of product. The b* values sightly increased but significantly during storage, which indicated that the paneer samples are going to form yellow and appears to decay by forming more yellow color. The color parameters of paneer samples did not change significantly, indicated that samples are not decayed for 10 days storage.

Table 16. The color characteristics of paneer samples during storage

Day 1				Day 5				Day 10			
	L	a	b		L	a	b		L	a	b
C	86.91	-1.58	11.80	C	85.59	-1.76	13.43	C	86.96	-1.91	14.21
S1	80.51	-0.59	13.02	S1	81.48	-0.24	11.15	S1	85.93	-2.06	12.22
S2	81.89	-0.44	12.87	S2	82.58	-0.23	12.58	S2	83.92	-0.89	13.27
S3	81.41	-0.47	13.33	S3	82.14	-0.62	10.54	S3	81.77	-0.73	13.85

4.3.6 Total phenolics content of paneer

The total phenolics content of paneer samples during storage are shown in Figure 44. The overall TPC contents were lower compared to the pomegranate peel extract or whole peel powder. The smaller amount of whole peel powder used during the paneer processing is responsible for lower TPC. The TPC amount was higher in sample order of; control > S1>S2>S3 due to the variation in the usage of peel powder. The total phenolic contents slightly decreased during storage, but the decrease was insignificant. The higher TPC contents were correlated to the lower

bacterial activities as shown in S3 samples. Thus, similar results were reported, using pomegranate peel powder in curd by (0.5, 1 & 1.5%) indicating that the curd with higher peel powder was resistant to microbial growth with higher antioxidant activities compare to the control samples (Sandhya *et al.*, 2018).

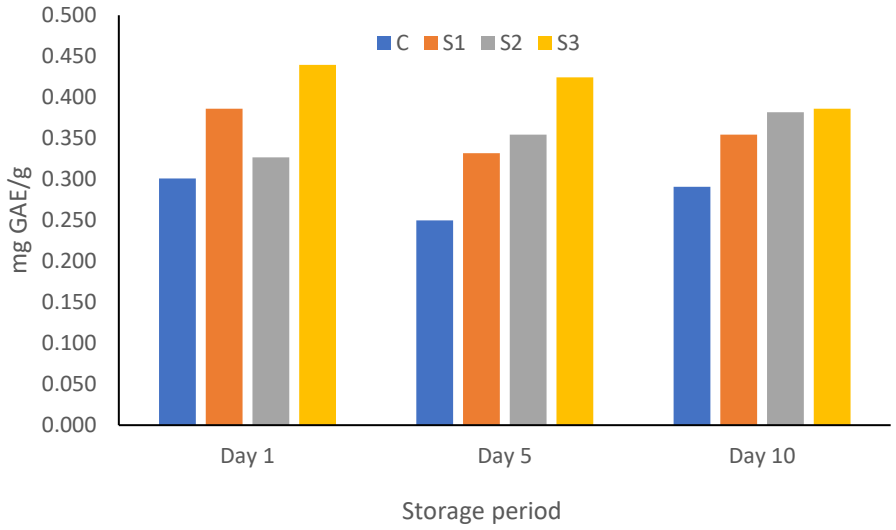


Figure 45. Total phenolics content of paneer during storage

4.3.7 DPPH scavenging activities of Paneer

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) antioxidant assay is used for scavenging the free radical upon on electron transfer which produces violet color in methanol solution. The antioxidant scavenging activities are shown in Figure 45. The antioxidant activity was higher in samples treated with pomegranate peel powder compare to the control samples. The results are also in accordance with TPC, as TPC of pomegranate peel powder exhibits higher antioxidant activities. There was no significant change in the antioxidant activities for 10 days storage of paneer. These results agreed with those reported by (Genitha Immanuel, 2014) where they used

pomegranate peel, lemon and orange extract as natural antioxidant additives in paneer. The pomegranate peel showed higher antioxidant activities with the ability to prevent peroxide compared to lemon and orange.

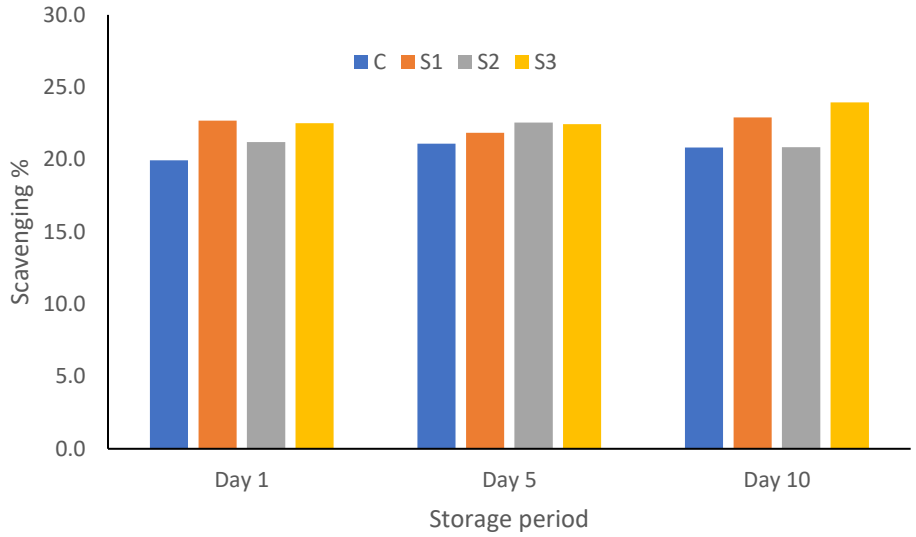


Figure 46. The antioxidant activities of paneer during storage

4.3.8 Free fatty acid

The free fatty acid profile was created with GC-FID using methyl esterification kit. The results presented in Table 16 shows that control samples have higher free fatty acids at the initial stage of storage in comparison to pomegranate powder treated samples. The samples with higher pomegranate peel powder showed lower free fatty acids which indicates the antioxidant activities of pomegranate peel powder. On the contrary, to the previous studies the free fatty acids reduced during the storage period, the free fatty acid was reported to increase from 0.17 % to 0.54 % at 10 days of storage when paneer was packed in LDPE bags (Khatkar *et al.*, 2017). The decrease in free fatty acids can be justify as the high bacterial count in the control samples will utilize free fatty acid as energy source; and the oxidation of free fatty acids can cause deformation and reduction of free fatty acid which produce ketones and aldehydes (Liu, Liu and Chen, 2019). The GC chromatograms of free fatty acids are shown in (Figure 36 ab).

Table 17. Fatty acid profile of paneer

	Area											
	Day1				Day 5				Day 10			
Retention Time	**C**	**S1**	**S2**	**S3**	**C**	**S1**	**S2**	**S3**	**C**	**S1**	**S2**	**S3**
2.187	12888	5389	4296	3252	4221	4195	2304	550	2621	2651	13671	8605
2.371	1177	420	303	214	315	342	158	ND	ND	ND	926	651
3.041	14496	6625	5284	4168	5087	5289	2971	949	3723	3776	17981	10920
4.643	46537	21985	17414	13858	15986	17106	9492	3148	12687	12588	62658	36829
5.066	5028	2283	1766	1365	1411	1663	903	301	1123	1172	5877	3417
5.572	2835	1351	1014	ND	ND	756	ND	ND	ND	ND	3655	1861
5.913	3793	1771	1395	1009	1079	1227	670	ND	874	846	5111	2775
7.66	121881	59089	47018	38429	41344	45431	25518	8463	38576	34821	172289	103334
8.178	7180	3125	2484	1984	2242	2386	1395	ND	ND	ND	8628	5135
13.322	34478	16620	12922	10619	9774	12149	6561	1641	10109	9572	50265	30555
13.978	106505	47644	37625	29408	32360	35068	20061	6470	37767	28162	129458	86346
15.535	16199	6564	5498	3890	6047	5266	2900	ND	10949	ND	19061	15085

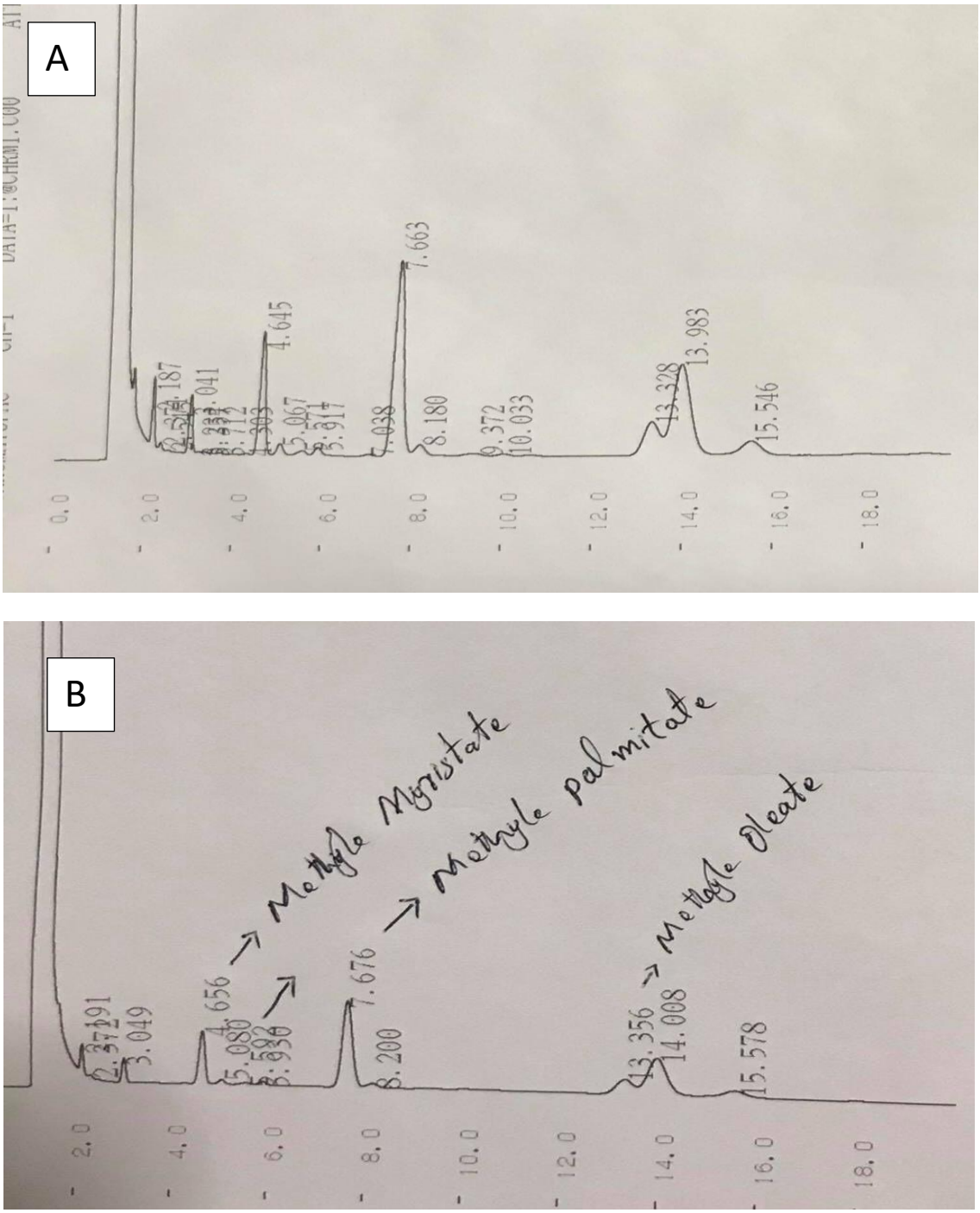


Figure 47. Chromatogram of fatty acids (A, control) , (B, S1)

4.4 Conclusion

Pomegranate peel as a byproduct rich in bioactive compounds was used as natural preservatives in paneer for enhancing the shelf life. The optimum operational conditions were 50 rpm of RS, 19.62 g/100 mL of SS, and 49.65% v/v of EW. The most significant influential factor for pomegranate peel extraction was SS. The optimum phenolic compound values were 225.7 mg GAE/g dw of TPC, 3.1 mg/g dw of TAC, 71.6 mg/g dw of punicalagin, 6.9 mg/g dw of ellagic acid, 6.2 mg/g dw of gallic acid, 13.1 mg/g dw of catechin and 14.8 mg/g dw of epicatechin.

The peel powder was produced with the purpose of using the fiber rich whole peel. The pomegranate peel was micro wet milled (MWM) to produce smaller particles the MWM slurry was subjected to spray draying at 120, 140 and 160 °C. Maltodextrin as carrier agent, was used at 20%, 40% and 60% on a dry basis.

Pomegranate peel powder was incorporated in soft cheese (paneer) as natural antioxidants and preservative. The storage test was carried out with analysis of physiochemical and biological properties of paneer. The results shows that addition of pomegranate peel powder increased the TPC and antioxidant properties of paneer. The color properties of paneer sample during storage did not changes significantly, while there was a slight decrease in moisture content. The total bacterial count shows that all the samples were under acceptable standard log of bacterial count. Where the bacterial count significantly decreased with higher pomegranate peel powder samples compare to control sample, which indicated the antibacterial effect of the peel powder. The free fatty acids decreased in all the samples but the decreased in control samples were higher compared to the peel powder treated sample. This was due to the antioxidant capacity of pomegranate peel powder. There was no obvious defect detected in all the samples for 10 days storage while samples treated with pomegranate peel powder exhibits higher nutritional values and lower microbial counts. The results showed that pomegranate peel can used in many food products as natural antioxidant and preservative. More studies are need to find out the peel impact in different food products as well to find out and correlate these bioactive compounds with their specific impact on the food properties.

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- Rasool Khan Amini, Md Zohurul Islam, Yutaka Kitamura, and Mito Kokawa. Utilization of Fermented Rice Milk as a Novel Coagulant for Development of Paneer (Soft Cheese), Foods, Volume (8)-8,399 (August 2019)

- Rasool Khan Amini, Md Zohurul Islam, Yutaka Kitamura, Mito Kokawa, Victoria Faith Eseese. Impact of Micro Wet Milling process on pomegranate peel phenolics extraction using multi-response optimization, Journal of Food Measurement and Characterization, (February 2021)

Conference:

- Rasool Khan Amini, Md Zohurul Islam, Yutaka Kitamura, and Mito Kokawa. Optimization and Evaluation of Pomegranate Peel Extract by Micro Wet Milling Using Response Surface Methodology International Joint Conference on JSAM and SASJ, and CIGR VI Technical Symposium joining FWFNWG and FSWG Workshops, Hokkaido, Japan. Sep 3-6, 2019

- Rasool Khan Amini, Md Zohurul Islam, Yutaka Kitamura, and Mito Kokawa. Optimization and Evaluation of Pomegranate Peel Extract by Micro Wet Milling Using Response Surface Methodology. The society of Agricultural Structures, Japan, Chiba University, Japan. February 27, 2020

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