DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: Food and Beverage Innovation and Management

MULTIVARIATE APPROACH FOR THE OPTIMIZATION OF POLYPHENOLS EXTRACTION IN POMEGRANATE PEELS

TYPE OF DISSERTATION: research

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INTRODUCTION AND AIM OF THE THESIS

In the last years, the consumption of the so called 'super foods' (both fresh and processed) has increased as a result of the higher consumer's demand.

In particular, the attention to - and consumption of – pomegranate (*Punica granatum*) has also rose, in the form of fresh fruit but also juice: pomegranate juice has been demonstrated to have a higher antioxidant activity than green tea and red wine (Gil MI, 2000).

Due to the structure of the fruit, in the juice production around 60-70% of the fruit is discarded. This amount of waste consists especially in peels (49-55%), rich in polyphenols, especially punicalagin, punicalin, gallic acid and ellagic acid.

Due to the high valuable compounds, juice companies have started looking for a way to utilize what is usually discarded.

These compounds in fact can be used in different ways: due to their antioxidant and antimicrobial activities, the peels can be used as natural additive for food preservation and quality enhancement.

This thesis aims to develop and optimize the extraction and the HPLC method for the quantification of the polyphenols usually found in the pomegranate peel.
The first three chapters will provide an introduction to the topic: the chapter 1 offers an overview of polyphenols, the chapter 2 presents a description of the pomegranate and the chapter 3 focus on food waste and valuable compound extraction technologies.
The following chapters focus instead on the materials and methods (chapter 4) used and the presentation of the results of the study (chapter 5).
The study presented in this thesis was divided into two crucial phases.
As first step the HPLC method was developed, starting with the establishment of parameters such as wavelength, flow rate, and composition of the mobile phase.
The second step of the research focused more on the polyphenol extraction. An experimental plan was defined: starting from defined variables (extraction time and temperature, ethanol concentration and solid - liquid ratio), the aim was to develop and define the most suitable method for a high-yield extraction.

CHAPTER 1 – POLYPHENOLS

Polyphenols are biologically active substances produced as secondary metabolites by the plant kingdom, consisting of over 500 molecules.

Being present in fruits and vegetables, they are an integral part of the human diet. Polyphenols are involved in the sensory and nutritional qualities of food and they are responsible of the color, flavor, odor and astringency of fruit and vegetables, which are involved in protecting plants against pathogens, ultraviolet radiations and extreme temperature.

It has been demonstrated that diets rich in polyphenol content reduced the risk of development of cardiovascular diseases, diabetes and antioxidant properties. Therefore, studies and applications in different foods have increased in recent years. Besides the health effects, the addition of polyphenols in technological processes can improve the general quality of the product, extending the shelf life, increasing the nutritional value and reducing the oxidation process.

1.1 Classification

Polyphenol's structure is characterized by the presence of at least one phenyl ring and one or more hydroxyl groups. According to the number of phenolic rings that they contain and the structural element bound to the rings, they can be classified into three main classes: phenolic acid, flavonoids and non-flavonoids. Each class can be then sub-divided into many subclasses.

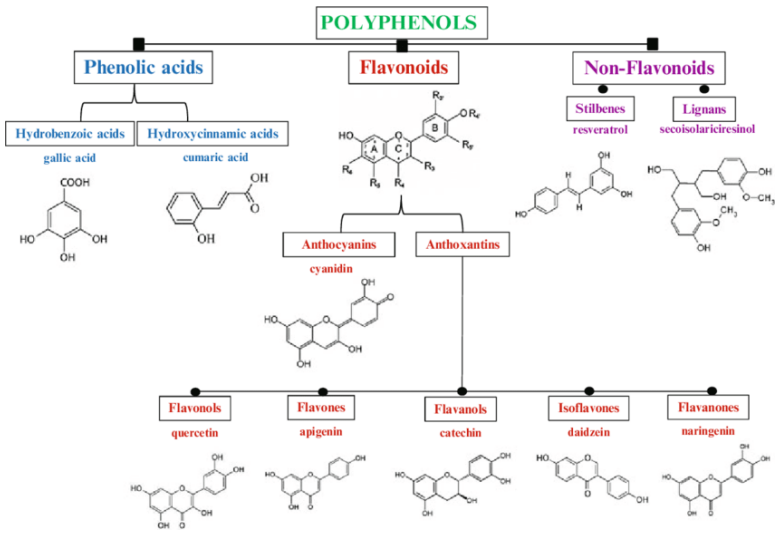


Figure 1 Polyphenols classification

Flavonoids are the most common class of polyphenols found in human diets, contributing to about two-thirds of all those ones ingested (Nishiumi, 2011). They are usually found in the peel and leaves due to the fact that their biosynthesis is stimulated by exposure to the light.

They have a common structure made of 2 aromatic rings bound together with 3 carbon atoms that form an oxygenated heterocyclic ring. According to the heterocycle involved, flavonoids can be divided into subclasses: flavonols, flavanones, flavonols, flavones, anthocyanins, and isoflavone.

Phenolic acids are the second most abundant class of polyphenols found in food following flavonoids. Phenolic acid structure consists in a single benzene ring, or phenolic group, with carboxylic acid group attached. When oxidized, they can develop a yellow color.

According to the group attached to the benzene ring, phenolic acids can be classified into derivatives of benzoic acid and derivatives of cinnamic acid. Hydroxycinnamic acids are more common with respect to the classes previously describes and caffeic, ferulic and sinapic acids belong to this group. The caffeic acid is mainly found in fruit and the content decrease as the ripeness stage increase. Ferulic acid is the most important phenolic acid in cereal grain, present in the aleurone layer and the pericarp, and it can represent 90% of the total polyphenol content.

Non flavonoids compounds are less common in human diet than flavonoids and phenolic acids and can be classified into lignans and stilbenes. The base structure of lignans is made up of two phenylpropane units linked together. Different classes of lignans are given by the different level of hydroxylation and addition of functional group side chain.

Stilbenes have a basic structure formed by two benzene rings joined by an ethylene bridge. Among stilbenes, resveratrol plays an important role in the plant for environmental stress and can be found mainly in wine.

1.2 Health benefits

High fruit and vegetable consumption is associated to a decrease in the risk of developing diseases. Referring to phenolic content in food, flavonoids are the main category described because they represent around two-thirds of the dietary phenols.

Polyphenols have antioxidant properties due to their hydroxyl group that is able to react with oxygen and nitrogen molecules, able to have pro-inflammatory effects.

Beyond the protective antioxidant behavior, polyphenols have also numerous beneficial effects. Studies recently demonstrated that polyphenols are involved in:

- lowering the risk of the development of cardiovascular disease (CVD);
- antitumor activity mainly for colon carcinogenesis;
- diabetes improvement;
- obesity risk reduction;
- improvement of visual function and protection of retinal pigment epithelial cells from UVB damage;
- improvement of memory performances in older adults;
- antinflammatory activity;
- antiallergic activity.

1.3 Polyphenols in foods

Polyphenols are widely distributed in plant kingdom, although there are some differences in quantity and composition depending on the species, so on the plant genetics. The species are not the only influencing parameter: pedoclimatic factors (soil characteristics, sun exposure…), cultural practices and ripening stage (phenolic acid content decrease by increasing ripening, while anthocyanin increase) influence the amount of polyphenols in plants. (Pandey, 2009). Also the stress conditions strongly influence their presence.
Fruits and some beverages such as tea, coffee and wine represent the main source of polyphenols, but legumes, cereals and vegetables provide a good amount.
The contents of polyphenols in a different class of food are given in table 1 (C. Manach, 2004).

Table 1. Polyphenols in food (C. Manach, 2004)

		Polyphenol content	
	Source (serving size)	**By wt or vol mg/kg fresh wt (or mg/L)**	**By serving mg/serving**
Hydroxybenzoic acids	Blackberry (100 g)	80-270	8-27
Protocatechuic acid	Raspberry (100 g)	60-100	6-10
Gallic acid	Black currant (100 g)	40-130	4-13
p-Hydroxybenzoic acid	Strawberry (200 g)	20-90	4-18
Hydroxycinnamic acids	Blueberry (100 g)	2000-2200	200-220
Caffeic acid	Kiwi (100 g)	600-1000	60-100
Chlorogenic acid	Cherry (200 g)	180-1150	36-230
Coumaric acid	Plum (200 g)	140-1150	28-230

Ferulic acid	Aubergine (200 g)	600-660	120-132
Sinapic acid	Apple (200 g)	50-600	10-120
	Pear (200 g)	15-600	3-120
	Chicory (200 g)	200-500	40-100
	Artichoke (100 g)	450	45
	Potato (200 g)	100-190	20-38
	Corn flour (75 g)	310	23
	Flour: wheat, rice, oat (75 g)	70-90	5-7
	Cider (200 mL)	10-500	2-100
	Coffee (200 mL)	350-1750	70-350
Anthocyanins	Aubergine (200 g)	7500	1500
Cyanidin	Blackberry (100 g)	1000-4000	100-400
Pelargonidin	Black currant (100 g)	1300-4000	130-400
Peonidin	Blueberry (100 g)	250-5000	25-500
Delphinidin	Black grape (200 g)	300-7500	60-1500
Malvidin	Cherry (200 g)	350-4500	70-900
	Rhubarb (100 g)	2000	200
	Strawberry (200 g)	150-750	30-150
	Red wine (100 mL)	200-350	20-35
	Plum (200 g)	20-250	4-50
	Red cabbage (200 g)	250	50
Flavonols	Yellow onion (100 g)	350-1200	35-120
Quercetin	Curly kale (200 g)	300-600	60-120
Kaempferol	Leek (200 g)	30-225	6-45
Myricetin	Cherry tomato (200 g)	15-200	3-40
	Broccoli (200 g)	40-100	8-20
	Blueberry (100 g)	30-160	3-16
	Black currant (100 g)	30-70	3-7
	Apricot (200 g)	25-50	5-10
	Apple (200 g)	20-40	4-8
	Beans, green or white (200 g)	10-50	2-10

	Black grape (200 g)	15-40	3-8
	Tomato (200 g)	2-15	0.4-3.0
	Black tea infusion (200 mL)	30-45	6-9
	Green tea infusion (200 mL)	20-35	4-7
	Red wine (100 mL)	2-30	0.2-3
Flavones	Parsley (5 g)	240-1850	1.2-9.2
Apigenin	Celery (200 g)	20-140	4-28
Luteolin	Capsicum pepper (100 g)	5-10	0.5-1
Flavanones	Orange juice (200 mL)	215-685	40-140
Hesperetin	Grapefruit juice (200 mL)	100-650	20-130
Naringenin	Lemon juice (200 mL)	50-300	10-60
Eriodictyol	Soy flour (75 g)	800-1800	60-135
Isoflavones	Soybeans, boiled (200 g)	200-900	40-180
Daidzein	Miso (100 g)	250-900	25-90
Genistein	Tofu (100 g)	80-700	8-70
Glycitein	Tempeh (100 g)	430-530	43-53
	Soy milk (200 mL)	30-175	6-35
Monomeric flavanols	Chocolate (50 g)	460-610	23-30
Catechin	Beans (200 g)	350-550	70-110
Epicatechin	Apricot (200 g)	100-250	20-50
	Cherry (200 g)	50-220	10-44
	Grape (200 g)	30-175	6-35
	Peach (200 g)	50-140	10-28
	Blackberry (100 g)	130	13
	Apple (200 g)	20-120	4-24
	Green tea (200 mL)	100-800	20-160
	Black tea (200 mL)	60-500	12-100
	Red wine (100 mL)	80-300	8-30

Phenolic may also influence the organoleptic properties of food: they can contribute to the bitterness, astringency, color, flavor, aroma, odor, and oxidative stability. Flavanols, flavonols and phenolic acids are primarily responsible for the tactile sensation of astringency and the bitter taste of food and beverages such as fruits, nuts, chocolate, tea, cider, wines, beer. (Milica Atanackoviš, 2012)

1.4 Food processing and polyphenols influence

In the food industry, polyphenols have an antimicrobial function with direct and indirect effects against pathogens (such as *L. monocytogenes*, *E. coli*, *Salmonella*, *S. Aureus*, *Campylobacter* and *C. perfringens*) and moulds, inhibition of spore germination, increase probiotic activity and antioxidant content, thus controlling also oxidative rancidity. They can be directly added into the product formulation, coated on the surface or incorporated in the packaging material.

Food processing techniques and storage influence the polyphenol content and bioavailability of food: processing and preservation induce physical and chemical changes to food resulting in impacts on the nutritional quality of products.

Among all the techniques, the heat treatment (boiling, frying, steaming, baking, roasting) may lead to harmful changes in fruit and vegetables. Heat causes cell rupture enhancing the availability of the components, but at the same time polyphenols are more prone to oxidation. The temperature used is directly related to the degree of degradation of polyphenols (Arfaoui, 2021). Oxidation by enzymes such as polyphenols oxidases and peroxidases influence the quality of food, the color and some organoleptic characteristics.

Moreover, new compounds can be generated impacting the quality attributes of food and beverages. They have low stability for denaturation by pH, light, oxygen, and enzymes, setting a limit for the use of the polyphenols as functional ingredients.

Processing and storage conditions can lead to chemical and structural changes in polyphenols, that can lead to instability due to modifications involving the hydroxyl group (esterification, alkylation, carboxymethylation, chelate formation).

A practical example can be the punicalagin, precursor of punicalin. During the degradation pathway, in fact, the punicalagin can be hydrolyzed in punicalin, which can be further hydrolyzed in ellagic acid, the last product of ellagitannin biodegradation, with the gallic acid as an intermediate (Cedra et al. 2003).

Although punicalagin, punicalin and ellagic acid have similar structural units (fig. 2) they have a different molecular size, polarity solubility, digestion and antioxidant ability (Y. Sun, 2017) (Brighenti, et al., 2021).

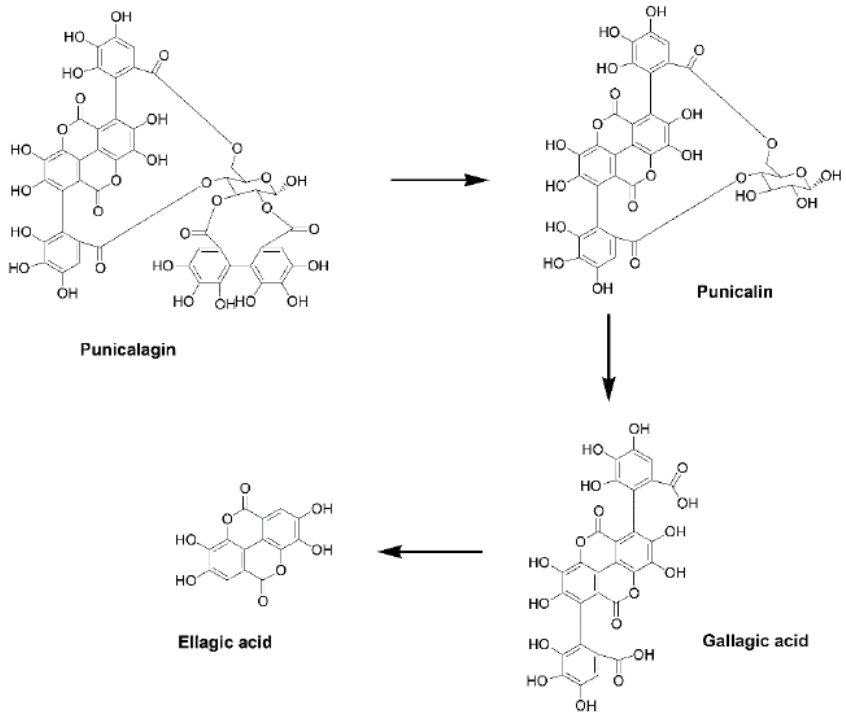


Figure 2 Degradation pathway of Punicalagin and related components

CHAPTER 2 – POMEGRANATE

Pomegranate belongs to the *Punicaceae* family, botanical name *Punica granatum* and is original from western Asia and the Mediterranean regions of Europe, present in subtropical and tropical areas. Nowadays it is cultivated mainly in Chile, Peru, Israel, Iran, Egypt, USA and Turkey. The different environmental conditions will affect the color, taste and antioxidant capacity of the fruit.

The pomegranate fruit has an outer and hard pericarp and an inner spongy mesocarp and the fruit wall where the seeds (called arils) attach. The fruits are used mainly as fresh or as juice but also as capsules and tablets, jam and jelly.

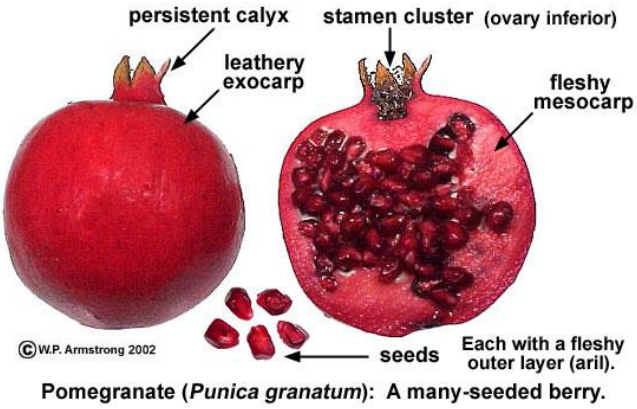


Figure 3 Pomegranate structure (Armstrong, 2002-2017)

In the last years, pomegranate has become a high value crop due to an increase of consumers' demand resulting from the potential health promoting benefits obtained through consumption.

The pomegranate juice has an antioxidant activity three times higher than those of red wine and green tea, thanks to its polyphenolic content (Gil MI, 2000). Even though the pomegranate juice has high antioxidant activity, the pomegranate fruit (usually discarded) are a richer source of natural antioxidants (Yunfeng Li, 2008). Peels, which represent about 49-55% of the total weight of the fruit, are discarded as a waste of processing, leading to a huge waste of resources (T. P. Magangana, 2020).

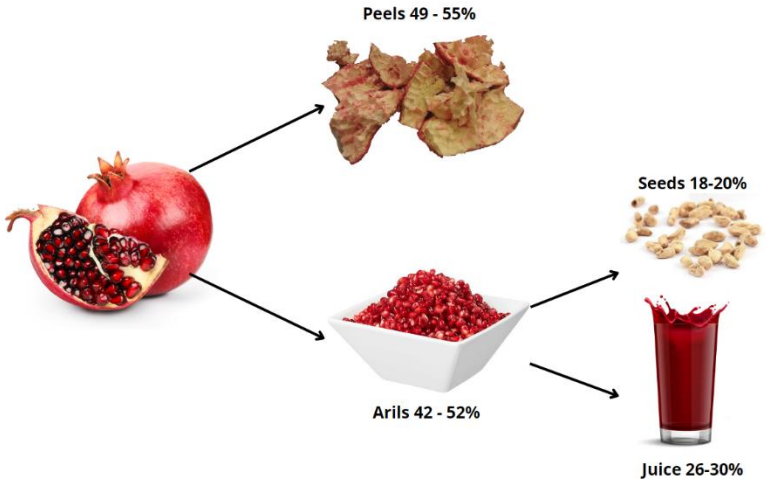


Figure 4. Pomegranate fruit composition (T. P. Magangana, 2020)

The production of pomegranate juice, as shown in figure 3, starts with the selection of the raw material: fruits have to be fully ripe and free from pests and diseases to allow a good final product with a good flavor. After a cleaning step, the fruits are crushed, sorted and deshelled to remove the peels. The seeds are then pressed to allow the juice extraction. The pressed pomegranate is then sieved to separate the seeds from the juice. The juice is then pasteurized and bottled.

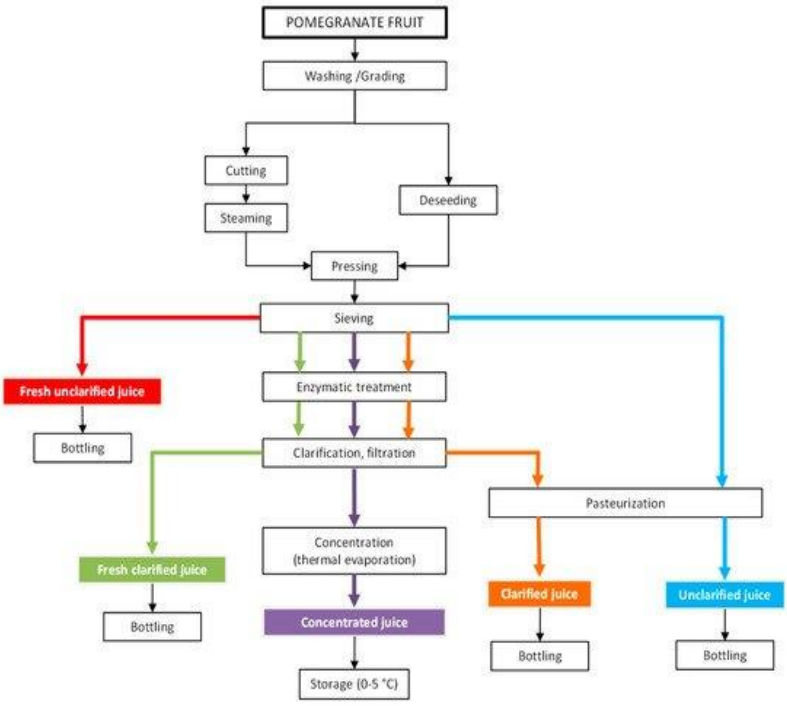


Figure 5 Pomegranate juice flow sheet (C. Conidi, 2020)

2.1 Phenolic composition

The main polyphenol constituents found in pomegranate husk include anthocyanins, ellagitannins (punicalagin, punicalin, gallic acid and ellagic acid), flavonoids, subclasses of the hydroxybenzoic acids (F. Lesa, 2017) (Deeba N. Syed, 2013) (Swapnil M. Chaudhari, 2013) , while in the pomegranate juice there is an higher quantity of anthocyanins like delhinidin, cyaniding and pelargonidin (Aloqbi, 2016).

In pomegranate peels the most abundant polyphenols are ellagitannins. Among all, punicalagin is the most abundant and it can reach 65,75 % of the total phenols in the peel (Sun Yu-qing, 2017).

This makes pomegranate wastes a good source of antioxidants isolates.

CHAPTER 3 - FOOD WASTES AND FOOD LOSSES

Food waste is an issue of global importance, related to social (e.g. healthy, equality), environmental (e.g. energy, climate change, availability of resources), and economic (e.g. resource efficiency, price volatility, increasing costs, consumption, waste management) impacts (Stenmarck, 2016).

According to FAO, a third of all food produced globally is lost or wasted every year. Food loss and waste has become an issue of great public concern: in EU, around 88 million tons of food waste are generated annually with associated costs estimated at 143 billion euros (Stenmarck, 2016).

Food loss refers to "*any food hat is discarded, incinerated or otherwise disposed of along the food supply chain from harvest/slaughter/catch up to, but excluding, the retail level, and does not re-enter in any other productive utilization, such as feed or seed*", while food waste refers to "*the decrease in the quantity or quality of food resulting from decisions and actions by retailers, food service providers and consumers*" (FAO, n.d.).

The by-product management becomes therefore essential. A by-product represents a product formed during the processing that may not count directly as a useful resource by its producers. However, they could still contain substances that can be used to develop new products with a market value.

Food wastes and by-products can represent a source of valuable components such as polysaccharides, proteins, fats, fibers, flavor compounds, polyphenols and other bioactive compounds, that can be used as natural antioxidants and functional ingredients.

For example, fruit skin and peels (e.g. from citrus and pomegranate) are rich in polyphenols and represent a huge amount of food waste. Therefore, the extraction and quantification of compound presence is essential for better management of wastes.

The recovery of food by-products allows more effective use of the resources (creating new opportunities), including the reduction of additional primary resources and the improvement of the environmental impact of the food industry. For this reason, the researchers and the food industries are more aware of the valorization and the management of food wastes, food losses and by-products.

3.1 Recovery of high value compounds from wastes

Food wastes, particularly fruit wastes from food processing, represent a good source of valuable compounds, such as polyphenols and other antioxidants.

According to the different raw material, the company has to choose the best extraction technique, considering that each technique could be better for a specific compound than another: a different technology may be preferred for each product.

The most used techniques at industrial level are known as "conventional extraction" technologies.

- The **solvent extraction** involves the extraction of a constituent from a solid or liquid feed by the use of a solvent. This is the most convenient techniques, the solvent acts as a carrier to transfer the molecules between different phases.
- To accelerate the compound extraction, **microwave-assisted extraction** (MAE) can be used: the electromagnetic waves penetrate in the material to make the solvent extraction faster.
- **Steam distillation** and **hydro distillation** are also used. Steam distillation is used to recover volatile compounds with high boiling point, using saturated or superheated steam as separation agent. This technique has the advantage to be solvent free, but at the same time sensitive compounds could be thermally degrades.
- The **supercritical fluid extraction** uses solvents as well, above or near the critical temperature and pressure: the properties of the fluid are between gas and liquid, so that it can better diffuse in the solid material, increasing the yield and making the process faster.

To better preserve the compounds from the degradation by elevated temperature and the use of solvents, innovative non thermal technologies can be also used.

- **Pulsed electric fields** (PEF) consists in the application of pulses of external electrical field of short duration (nanoseconds, milliseconds) and high electric field strength to a product placed between two electrodes. The pulses will cause a membrane electroporation to allow the easier release of the compounds entrapped in the cell membrane, as for the phenolic compounds in vinification or sugar extraction from sugar beet.
- **Ultrasound assisted extraction** (UAE) uses ultrasound waves (with frequencies ranging between 18 to 100 Hz) to alter the physical and chemical properties of plant material. They facilitate the extraction of some compounds by improving solvent penetration and enhance the mass transport by disrupting the plant cell walls, thus reducing the time and the use of solvent.

- **Hydrostatic Pressure Process** (HPP) is another non-thermal process, using high pressure to permeabilize the plant cells, increasing the efficiency and yields of extraction keeping in mind the type of solvent used, the target compound and the structure and composition of the matrix. Examples are the increase of beta carotene in tomato puree and juice extraction.

Even though emerging technologies have been proved to be fast and efficient and to increase the extraction yields, with some few exceptions, they are still not tested enough for industrial purposes. The application of these techniques has drawbacks on energy and operational costs, and they can lead to some modification to compounds. For example, proteins might be subjected to some modifications with the use of HPP.

Nowadays the most used techniques involve the use of a solvent. Then it became really important to optimize the extraction parameters to obtain high quality extract, with a special focus on the solvent selection and parameters as time and temperature. Among the most important parameters, there are: type of solvent, temperature and time of extraction and solid-liquid ratio.

The solvent has to be chosen according to the solubility of the target compound and polar organic solvents (methanol, ethanol, acetonitrile), water or a mixture can be used. The solvent choice and composition are really important: the directive 2009/32/CE is applied to the extraction solvents to define the conditions of use and the safety concern.

The temperature of extraction is an important parameter as well and it can influence the speed of extraction and the quality of the compounds. Using high temperature in solvent extraction increase the solubility: elevated temperatures are used to keep the viscosity low and increase the mass transfer, allowing the medium to pass in the solid substrate. However, a too high temperature can lead to extraction of non-desirable compounds and the degradation of some others. Temperatures higher than 70 °C cause polyphenol degradation. (Karishma Rajbhar, 2014).

Time can also influence the composition of the extract. Prolonging the extraction time will increase the total quantity of compound which had been extracted, but it also influences the quality of it, being the compounds more prone to the degradation.

Another important parameter is the proportion of sample: solvent used. Usually higher sample amount allows a higher extraction, even though if the ratio is too high it can lead to excessive extraction with subsequent difficulties of quantification.

So, not just the extraction technique has to be chosen, but also many related parameters have to be considered.

Those are important to maximize the production of the bioactive molecules and to define their quality.

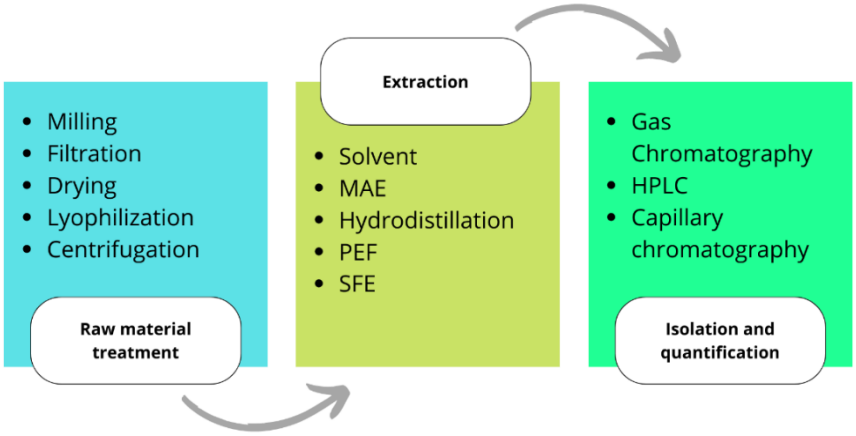


Figure 6. From raw material to quantification: main technologies

CHAPTER 4 – MATERIALS AND METHODS

1. Standard preparation

Stock solutions of Punicalagin (A+B) (Sigma Aldrich, St. Louis, MO, USA) and Punicalin (Sigma Aldrich, St. Louis, MO, USA) were prepared in MeOH: H_2O (80:20) solution.

Ellagic acid (Sigma Aldrich, St. Louis, MO, USA) was instead dissolved in HPLC water in the presence of 1 N of NaOH.

Different concentrations of standard were prepared. Punicalin, punicalagin, ellagic acid was injected at concentrations of 1000, 500, 400, 200, 100 ppm with a purity of 91%. Ellagic acid standard was injected at concentrations of 1000, 800, 400, 200, 100 ppm with a purity of 95%. Calibration curves where then obtained. A mixture of the standards was also injected.

2. Sample preparation

Frozen pomegranate peels have been provided by the juice producer company. The first step was the manual removal of the mesocarp for a better analysis.

Figure 7. Manual removal of the mesocarp

For the analysis, the peels have been treated in three different ways: two of them were dried at 40 and 60 °C using an air dryer, while the control sample was freeze dried. The dried peels were reduced then into a powder by using a mixer.

Figure 8. Peels ready to be dried

Figure 9. Dried peels

Figure 10. Grounded peels

Following the experimental plan, 1 g of the grounded sample was weighed and ethanol was added in the specific concentration (25, 50 or 75%) and volume (5, 15 or 25 ml). The samples were mixed by using a vortex and placed in a thermostat bath at different extraction temperatures (25, 35 and 45°C) at specific time (15, 30 or 45 minutes). Each sample was prepared following all the parameters combination reported in the table 2.

The extracts were then centrifuged. The supernatant was filtered through a 0.45 µm filter (CLARIFY-PTFE syringe filter) to remove particles. The filtrate is diluted by adding 500µl into 5 ml of ethanol of the same concentration as used for the extraction.

2.1 Sample drying

Pomegranate peel is high in moisture: it cannot be stored and subsequently used without a preservation method. Therefore, it is necessary to choose the right technique to preserve the quality of the compound composition. The drying process is a technique which aims at the moisture reduction, increasing then the shelf life. This process influences the physical and chemical composition of the product. It is necessary to select the best drying method, according to the product type, type of equipment, operation costs, drying conditions and efficiency.

For this research, samples were dried following three different conditions. The control sample was dried using the freeze drying, with the dehydration by sublimation of a frozen sample under vacuum conditions. It allowed to preserve the primary structure, aroma and flavor of the product.

Samples were also dried in the drying oven at 40°C and 60°C. Oven drying represent one of the most rapid and easiest method. Moreover, it easily accessible and economic, even though it may reduce the quality of the sample compared to the original state if drying temperature and time are too prolonged.

3. Experimental plan

To identify the best conditions for the polyphenols extraction and its optimization, an experimental plan was designed.

On the bases of a literature review, four main independent variables were considered as discrete factors:

- Temperature of extraction (25, 35 and 45 °C);
- Time of extraction (15, 30 and 45 minutes);
- Ethanol concentration (25, 50 and 75%);
- Solid - liquid ratio (1/5, 1/15 and 1/25).

The software JMP (version 11.0.0) was used to examine the different factors combinations to identify the best combination for higher efficiency extraction. The software generated 35 different trials (Table 4.1). The second-order polynomial equation was used to create and analyze the data matrix, according to the following equation:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i \neq j=1}^{3} b_{ij} X_i X_j$$

Equation 1 Second-order polynomial equation

Where:

- **Y** is the response variable (punicalin, punicalagin and ellagic acid);
- **Xi, Xj** represent the input variables (temperature and extraction time, EtOH concentration and solid-liquid extraction);
- **b0, bi, bii** and **bij** are the regression coefficients for the intercept, linear, quadratic and interaction terms.

Table 2 Experimental design matrix

Run	Temperature	Time	[EtOH]	Solid:liquid
1	25	45	75	25
2	25	15	75	15
3	35	30	50	15
4	45	45	75	25
5	25	30	75	5

6	35	15	75	5
7	35	45	25	5
8	45	15	50	5
9	25	45	50	5
10	25	15	50	25
11	25	15	25	5
12	45	45	25	25
13	45	30	25	5
14	45	15	25	25
15	45	15	75	25
16	45	45	75	5
17	25	45	25	25
18	35	15	25	25
19	45	15	75	5
20	25	45	75	5
21	25	30	25	25
22	45	45	25	5
23	25	15	25	5
24	25	15	75	25
25	45	45	25	25
26	35	45	75	15
27	25	45	75	25
28	25	45	25	15
29	45	45	75	5
30	45	45	50	15
31	25	15	75	5
32	45	15	25	15
33	35	45	50	25
34	45	30	75	25
35	25	45	25	5

In the first 17 extractions, the samples treated with the three different drying techniques (freeze-dried, dried at 40°C and dried at 60°C) were used. When the results were analyzed, it was evident that the samples with the best results were those treated at 40°C with the best extraction yield of the polyphenols.

It was therefore decided to extend the experiment plan with further samples: 17 more extractions (runs from 18 to 35) were carried out using the sample dried at 40°C, with the different combinations of parameters (ethanol concentration, solid liquid concentration, extraction time and temperature).

4. HPLC method

Methods development experiments were conducted using a HPLC system (Agilent 1100 Series, Santa Clara, CA, USA) with a UV detector. Chromatography was achieved using a column C18 ZORBAX Eclipse Plus (3.0 x 150mm, 3.5µm).

Analyses were conducted at a flow rate of 0.7 mL/min and a sample injection of 20 µL and the signal was detected through the use of UV detector at a wavelength of 260 nm. For the finalized method, a biphasic mobile phase consisting of 0,15% H_3PO_4 in HPLC water (A) and 0,15% H_3PO_4 in acetonitrile (B) was utilized.

The elution conditions were as follows: isocratic elution 1% B, 0–1.5 min; linear gradient from 1% B to 4.5% B, 1.5–3.0 min; isocratic elution 4.5% B, 3.0–5.0 min; linear gradient from 4.5% B to 7.0% B, 5.0–8.5 min; isocratic elution 7.0% B, 6.5– 8.5 min; linear gradient from 7.0% B to 25% B, 8.5–13.75 min; to 90% B, 13.75–14.39 min; isocratic elution 90% B, 14.39 to 20.0 min; to 1% B, 20.0–24.0 min.

Table 3 shows the elution conditions, for a total run of 24.0 min.

Table 3 Gradient elution

Tempo (min)	**%A**	**%B**
0	99	1
1,50	99	1
3,00	95.5	4.5
5,00	95.5	4.5
8,50	93	7
13,75	75	25
14,39	10	90
20,00	10	90
24,00	99	1

For the identification and quantification of the compounds, calibration curves previously obtained with the standards of known concentrations have been used.

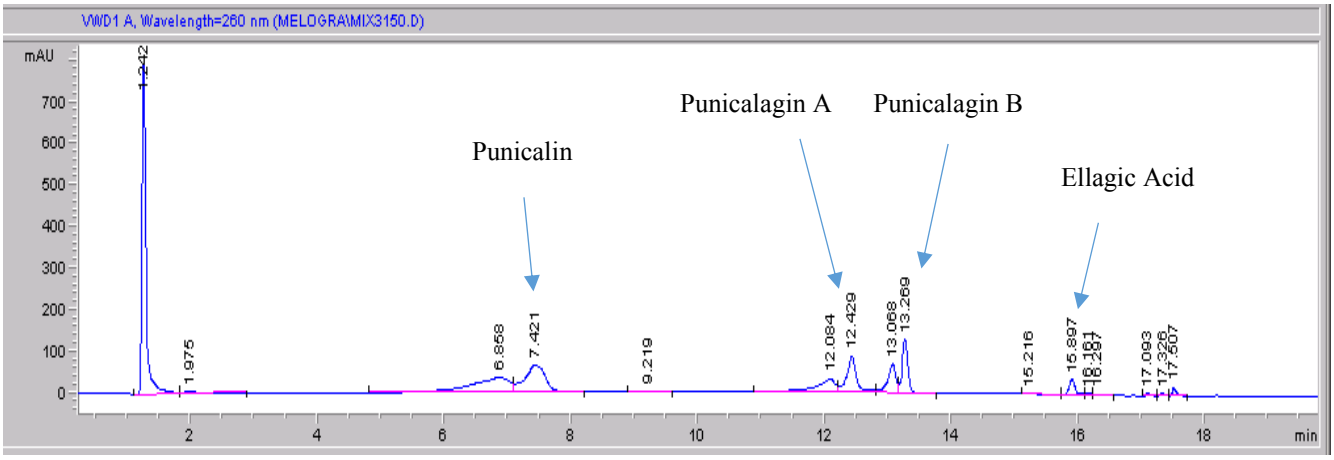
CHAPTER 5 – RESULTS AND DISCUSSION

1. Development and finalization of HPLC method

For the optimization of HPLC method different combinations of flow rate, wavelength detection and gradient elution were used.

After testing the different parameter combinations, a mix of standards was also injected. Figure 10 shows the chromatogram of the standard solution mix, which is important for defining the retention times of each compound to be identified in the peel samples.

Figure 11. Chromatogram of the sample mix 150 ppm



1.1 Flow rate

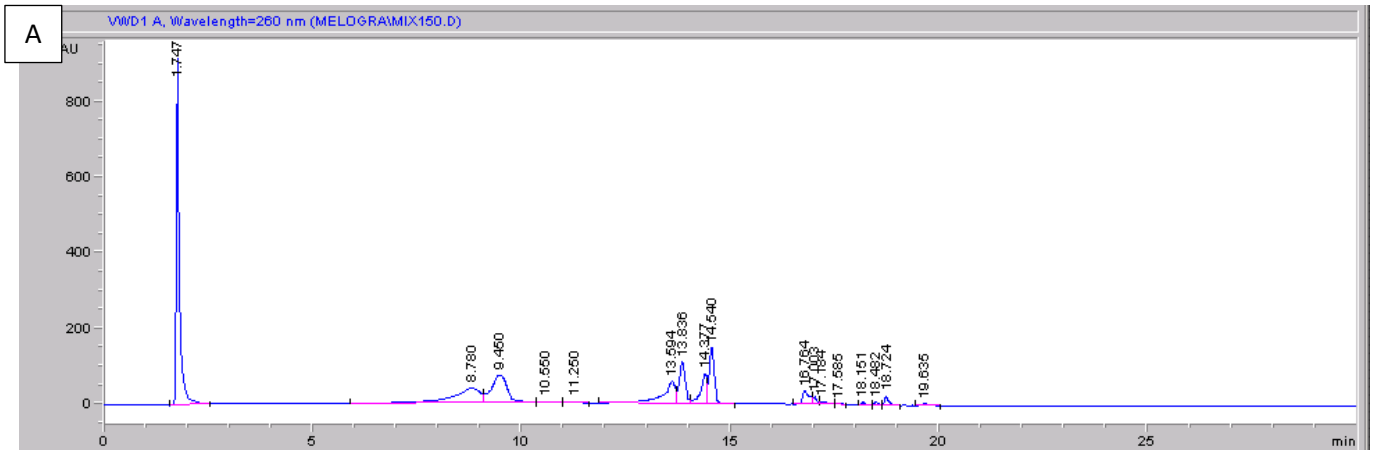
To identify and then quantify the different compounds, three different flow rates have been applied: 0.5 mL/min, 0.7 mL/min and 1.0 mL/min.

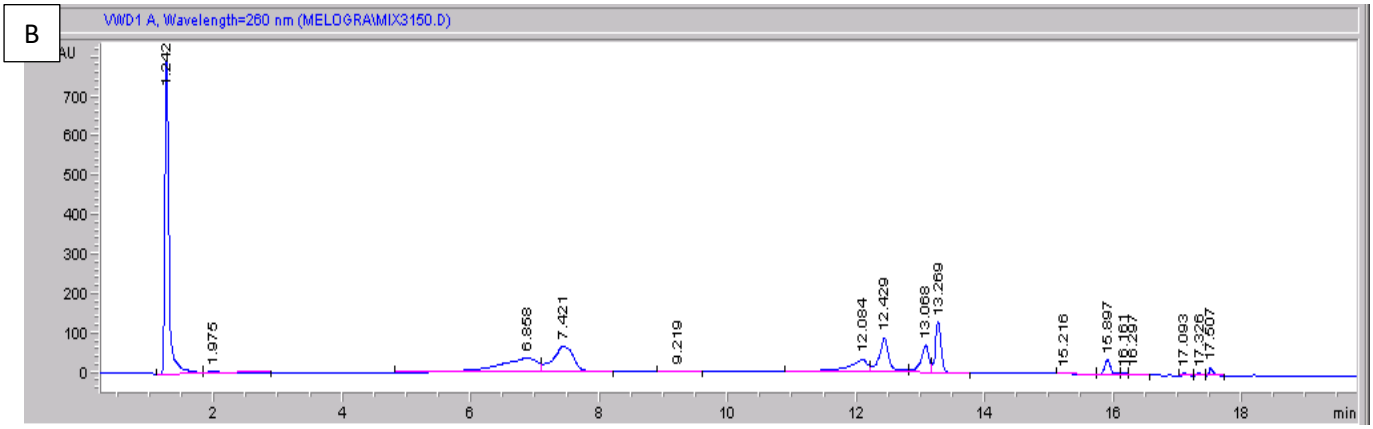
By changing the flow rate of the analysis can change the separation quality of the compounds.

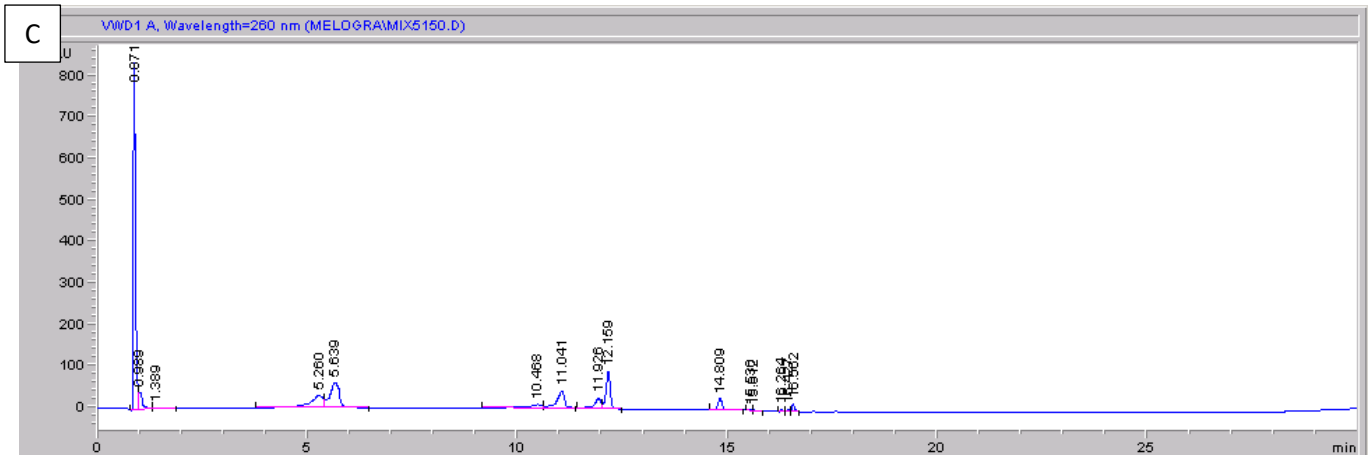
A high flow rate may affect the quality of the analysis: the analyte will do not have enough time to interact with the stationary phase, causing too narrow peaks or too close to each other. While a too low a flow rate can slow down the appearance of peaks on the chromatogram, often affecting their actual definition.

The peaks of the 1.0 ml/min flow rate appeared to be too wide. As a result, it was decreased in order to have more defined peaks: first at 0.7, then 0.5 mL/min. After different trials, the best flow rate for the detection resulted in 0.7 ml/min.

Figure 12 Chromatograms obtained by using three different flow rates (A=0.5 ml/min; B= 0.7 ml/min; C= 1.0 ml/min)







1.2 Wavelength detection

Wavelength choice is an important factor for the UV-HPLC detection to measure the response of the analyte and influence the detector response, results and reproducibility. It depends on the spectra of the standards of each compound of interest, but also it changes according to the nature of the solvent, the pH of the solution and the temperature. The wavelength is strictly related to the kind of compound and it's related to its absorbance.

According to the literature, the best wavelengths for the polyphenols for our interest were 260 and 380 nm. Several tests were carried out to identify the best wavelength for the compounds also based on the retention times and peaks obtained.

After different trials, detection at 260 nm wavelength offered high response and the best option for the detection conditions of punicalin, punicalagin A and B and ellagic acid compared to 380 nm wavelength.

1.3 Mobile phase acidification

For the optimization of the method, three different levels of acidified phase have been tested. While the solution B was kept as acetonitrile, the solution A consisted in HPLC water, with the addition of 0.1% H_3PO_4, 0.15% H_3PO_4 or 0.2% H_3PO_4.

The different amount of H_3PO_4 is directly related to the ability of polyphenols to be detected in an acidic condition by the HPLC, related also to the type of HPLC column used. In fact, according to the column we used, the C18 ZORBAX Eclipse Plus (3.0 x 150mm, 3.5µm), the pH limit was 2.

The mobile phases must be fully compatible with the compounds we want to detect, to increase the selectivity of the extraction. The mobile phase has to be chosen according to the nature of the analyte.

Lowering the pH was essential for the detection, as polyphenols are more stable at low pH, for this reason 0.15% was selected as the best mobile phase dilution.

2. Calibration curves

Punicalin, punicalagin (A and B), ellagic acid standards have been injected following the HPLC method.

Calibration curves were then obtained plotting the peak areas of the analyte versus its concentration.

Punicalin, punicalagin (A and B), ellagic acid standards were injected at concentrations of 1000, 500, 400, 200, 100 ppm with a purity of 91%. Ellagic acid standards were injected instead at concentrations of 1000, 800, 400, 200, 100 ppm with a purity of 95%.

After each run, the area under the curves of the chromatogram were collected and the calibration curve was obtained for each standard.

Table 4 Areas for the standards calibration curve

Standard	ppm	Area	Retention time (min)	Standard purity
Punicalin	1000	67110.5	6.763	91%
	500	30407.1	6.587	
	400	24063.8	6.505	
	200	12080.1	6.606	
	100	6035.7	6.555	
Punicalagin A	1000	41713	12.056	91%
	500	21980.3	12.058	
	400	16087.7	11.838	
	200	7080.6	11.853	
	100	2982.1	11.890	
Punicalagin B	1000	30571.9	13.018	91%
	500	17283.9	13.030	
	400	13693	12.915	
	200	5870.9	12.895	
	100	2687.7	19.925	
Ellagic Acid	1000	4152.2	15.616	95%
	800	3198.2	15.868	
	400	1221.2	15.766	
	200	553.7	15.762	
	100	66.5	15.800	

The areas where then plotted with the ppm concentrations of the standards. Calibration curves, that can be seen in the figures 12, 13, 14 and 15, were finally obtained.

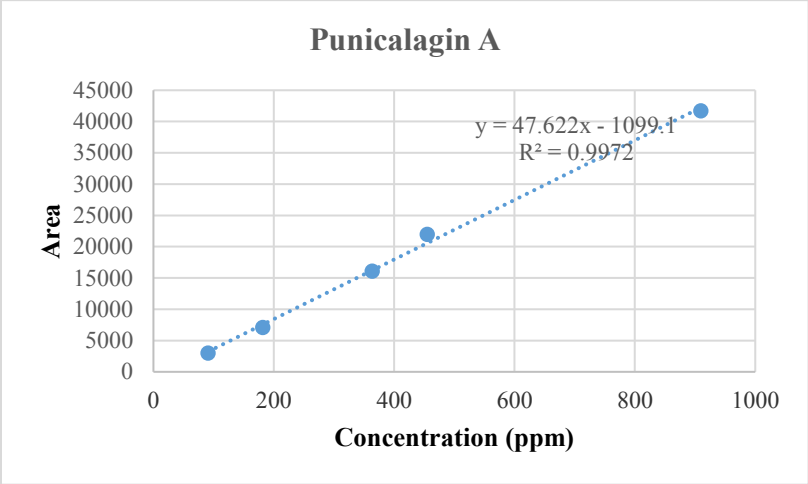


Figure 14 Punicalagin A: calibration curve

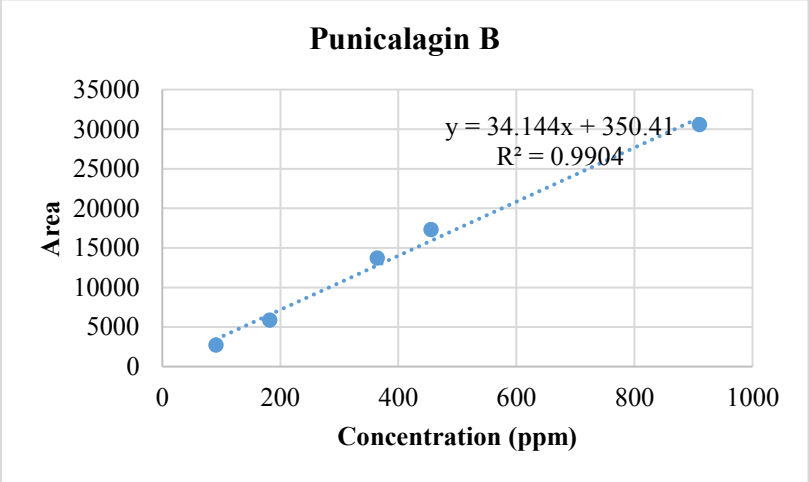


Figure 13 Punicalagin B: calibration curve

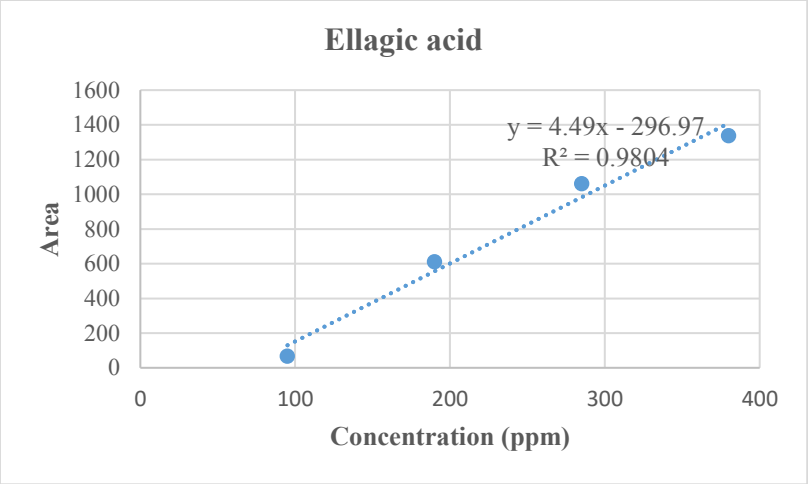


Figure 15 Ellagic Acid: calibration curve

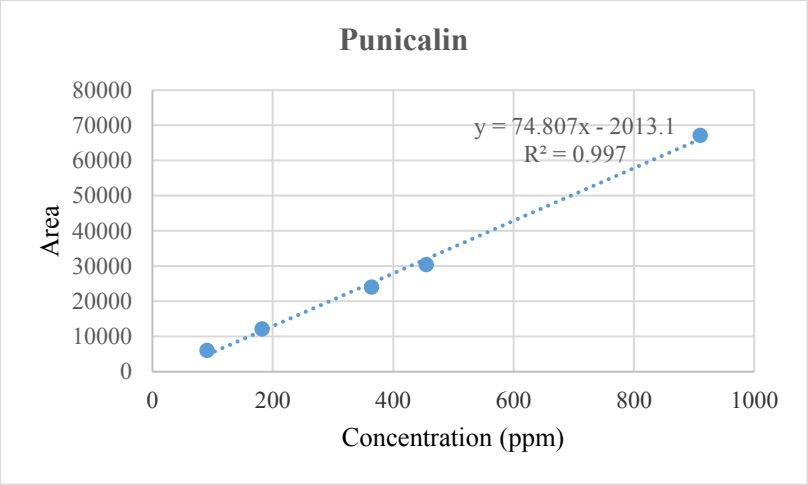


Figure 16 Punicalin: calibration curve

3. Effect of the extraction factors

After performing the 35 runs provided by the experimental plan (see table 2), the areas of the peaks were analyzed and translated into a polyphenol concentration. This allowed to understand the effects of the extraction parameters (extraction temperature, extraction time, ethanol solvent concentration, solid: liquid ratio) and the drying temperature of the peel samples.

The values obtained are shown in the table 5.

Table 5 Punicalin, punicalagin (A+B), ellagic acid content of pomegranate peel samples (control - 40°C - 60°C).

Run	Sample	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
1	Control	0.68	4.73	7.06	12.71	0.61
	40°C	0.69	5.18	7.02	13.10	0.67
	60°C	0.69	4.13	7.92	12.89	0.48
2	Control	0.42	3.82	4.86	9.35	0.72
	40°C	0.42	3.45	4.66	8.69	0.68
	60°C	0.42	4.70	4.95	10.32	0.87
3	Control	0.60	6.51	5.05	13.17	1.15
	40°C	0.77	6.27	5.53	13.41	1.00
	60°C	0.48	6.51	5.30	13.05	1.13
4	Control	0.00	5.31	6.59	12.25	0.81
	40°C	0.00	5.73	5.66	11.65	1.01
	60°C	0.00	5.48	5.85	11.63	0.94
5	Control	0.16	3.15	1.46	4.96	1.94
	40°C	0.22	3.50	1.27	5.17	2.34
	60°C	0.18	3.08	1.38	4.81	1.98
6	Control	0.17	3.13	1.25	4.85	2.20
	40°C	0.14	3.10	0.98	4.40	2.78
	60°C	0.16	3.17	1.35	4.91	2.10
7	Control	0.27	6.73	1.72	9.54	3.37
	40°C	0.32	6.41	1.83	9.25	2.98
	60°C	0.25	5.87	1.82	8.55	2.84
8	Control	0.51	4.87	1.38	7.66	2.58

	40°C	0.23	5.30	1.50	7.80	3.07
	60°C	0.31	4.84	1.67	7.53	2.44
9	Control	0.29	4.17	1.13	6.26	2.93
	40°C	0.38	5.95	1.05	8.26	4.18
	60°C	0.20	5.27	1.06	7.50	4.20
10	Control	0.97	7.15	5.29	14.67	1.14
	40°C	0.74	6.94	5.11	13.78	1.19
	60°C	0.69	7.19	6.01	14.87	1.07
11	Control	0.19	6.14	2.08	9.09	2.71
	40°C	0.34	7.61	2.02	10.83	3.22
	60°C	0.29	7.03	1.88	10.04	3.23
12	Control	0.78	12.42	4.82	19.34	2.22
	40°C	0.83	14.67	5.00	21.88	2.52
	60°C	0.83	12.57	5.92	20.99	1.86
13	Control	0.19	13.29	1.29	16.38	8.98
	40°C	0.16	12.11	1.07	14.06	9.86
	60°C	0.17	10.88	1.13	13.48	8.37
14	Control	0.93	11.13	8.44	21.37	1.19
	40°C	0.92	9.03	5.58	16.02	1.39
	60°C	0.80	8.21	9.73	19.42	0.78
15	Control	0.96	9.79	5.98	16.89	1.41
	40°C	0.92	9.17	4.39	14.64	1.73
	60°C	0.00	7.64	7.60	15.43	1.01
16	Control	0.00	4.93	1.22	6.30	4.05
	40°C	0.16	7.11	1.16	8.61	5.36
	60°C	0.18	6.95	1.22	8.55	4.99
17	Control	0.75	13.05	5.86	20.26	1.97
	40°C	0.00	14.73	5.76	21.56	2.56
	60°C	0.00	12.59	4.99	19.05	2.52
18	40°C	0.75	12.59	9.08	22.68	1.28
19	40°C	0.33	11.36	2.61	14.57	3.87
20	40°C	0.41	11.24	2.67	14.59	3.64
21	40°C	0.73	14.21	6.10	21.35	2.08
22	40°C	0.47	13.99	2.14	17.40	5.36

23	40°C	0.46	14.21	2.63	17.99	4.60
24	40°C	0.67	4.00	6.74	11.46	0.54
25	40°C	0.73	13.47	6.22	20.72	1.94
26	40°C	0.67	7.81	9.18	17.70	0.79
27	40°C	0.66	5.29	5.36	11.38	0.88
28	40°C	0.74	15.95	5.89	23.02	2.40
29	40°C	0.41	8.61	2.31	11.55	3.16
30	40°C	1.06	12.81	7.39	22.18	1.52
31	40°C	0.33	6.13	3.70	10.29	1.52
32	40°C	0.80	14.19	2.30	17.59	4.57
33	40°C	0.74	9.36	5.64	16.29	1.47
34	40°C	0.66	8.53	3.03	12.33	2.31
35	40°C	0.43	10.64	1.53	13.67	5.42

3.1 Drying temperature

To find the optimal drying temperature of the peels, the concentrations of the compounds in each run can be compared. The run was performed three times: one for the control sample freeze – dried, and the other two for the samples dried at 40°C and 60°C.

The three runs followed all the same operational parameters (extraction time and temperature, ethanol concentration, solid liquid concentration), with the only variable of the drying temperature of the peels.

As we can see for example comparing the first run (table 6), the highest concentrations of punicalin, punicalagin A and B can be found in the peels dried at 40 °C, even though it has the lowest concentration of ellagic acid.

Table 6 Fist run: polyphenols content comparison

Run	Drying temperature	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
1	Freeze dried	0.68	4.73	7.06	12.71	0.61
1	40°C	0.69	5.18	7.02	13.1	0.67
1	60°C	0.69	4.13	7.92	12.89	0.48

Analyzing also the other runs, it can be notices that the polyphenol composition also follows the same trend (punicalin and punicalagin A and B higher in the sample dried at 40°, lower ellagic acid in the sample dried at 60°).

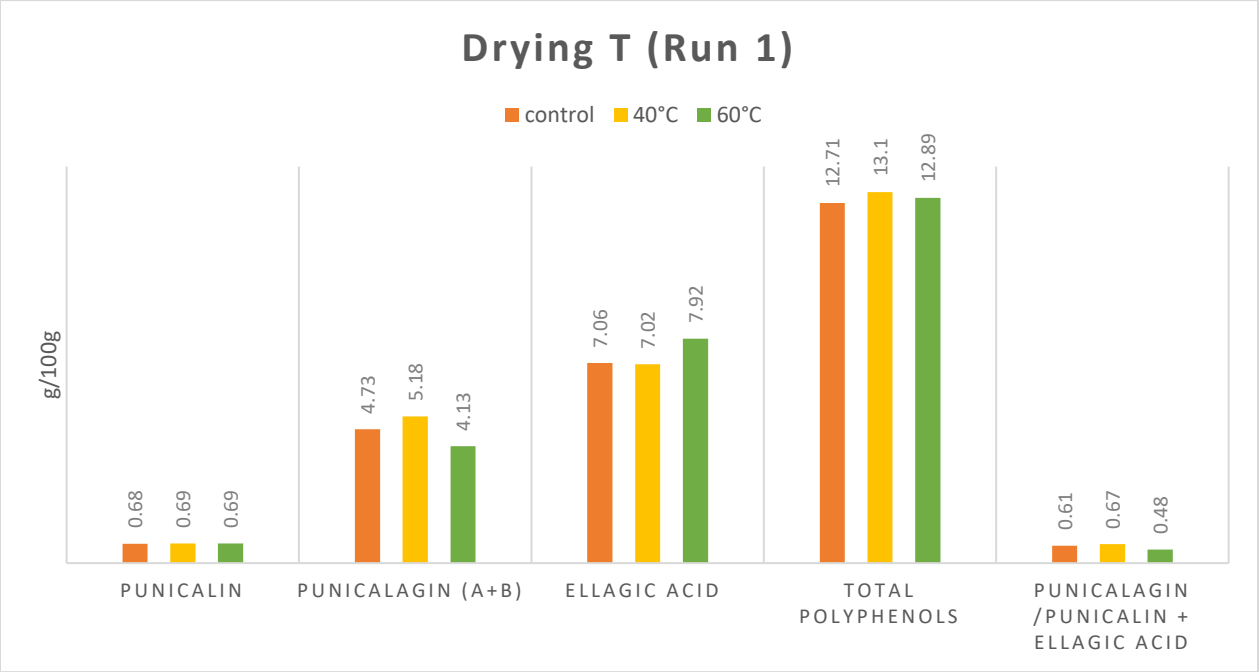


Figure 17 Effect of the drying temperature in the run 1

The higher drying temperature gave us the higher content of punicalin and ellagic acid compared to the original form punicalagin. This is strictly due to the degradation state of the original compound, the punicalagin that can be hydrolyzed into punicalin and then ellagic acid. Higher temperature treatment shifts the ratio in favor of the hydrolysis products.

The most efficient drying temperature in terms of extraction efficiency appeared to be the 40°C treatment. It is also the less costly temperature among the ones used, being as a consequence, the most appropriate also at industrial level.

3.2 Extraction temperature

An increase in temperature can increase the mass diffusivity, increasing the rate at which the solute can diffuse into the solvent and decreasing the viscosity of the solvent.

The application of high temperature can be positive for some type of extraction, but for some other can represent a damage. Some compounds are prone to oxidation at high temperature and long extraction time, thus leading to the decrease of the stability of some phenolic compounds.

Extraction temperatures used for our analysis are 25, 35 and 45 °C. To identify the best extraction temperature, we can compare the results obtained in the runs 1 and 4 for the sample dried at 40°C.

The runs 1 and 4 used the following extraction parameters: time of extraction 45 min, ethanol concentration 75% and solid- liquid ratio 1/25. The only different parameter was the temperature of extraction, in the first run 25°C, in the fourth 45°C.

Table 7 runs comparison for the best extraction temperature

Run	Extraction temperature (°C)	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
1	25	0.69	5.18	7.02	13.1	0.67
4	45	0	5.73	5.66	11.65	1.01

As we can see in the table 7, a lower extraction temperature allowed a lower punicalagin and ellagic acid extraction. While, a lower temperature allowed a higher punicalin A and B levels.

3.3 Extraction time

The effect of the different extraction time was investigated in the range of 15-45 minutes.

To understand the effect of the extraction time, the runs 4 and 15 can be compared. The independent variables were kept constant in the two runs compared, were the temperature of extraction was 45 min, the ethanol concentration 75% and solid-liquid ratio 1/25 (0,04 g/ml).

The influence of the extraction time can be seen in the table 8, which shows that less time in the run 15 allowed a higher punicalin and punicalagin (A+B) extraction, and a lower ellagic acid, with an overall higher total polyphenols yield. Prolonged extraction time may lead to oxidation, degradation of some compounds of interest.

Table 8 Runs comparison for the best extraction time

Run	Extraction time (min)	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
4	45	0	5.73	5.66	11.65	1.01
15	15	0.92	9.17	4.39	14.64	1.73

3.4 Ethanol concentration

Water, methanol, ethanol and their combinations are the most suitable solvents used for the extraction of phytochemicals from pomegranate peels. In this analysis, ethanol was used as extraction solvent, in three different concentrations: 25%, 50%, 75%. It is a relatively low cost, easy to find and recognized as green solvent.

The effect of ethanol concentration could be understood by comparing the runs 9 and 35. The three runs have the same parameters for drying temperature of the sample of 40°C, extraction time 45 minutes, extraction temperature 25 °C and solid: liquid ratio 1/5. The only difference consisted in the ethanol concentration: 50% in the number 9, 25% in the run 35.

The run 35, in which a lower concentration of ethanol was used, showed a higher concentration of punicalin, punicalagin (A and B) and overall total polyphenols percentage. As a result, 25% of ethanol concentration was chosen for the experiment optimization.

Table 9 Runs comparison for the best extraction temperature

Run	[EtOH]	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
9	50	0.38	5.95	1.05	8.26	4.18
35	25	0.43	10.64	1.53	13.67	5.42

3.5 Solid - liquid ratio

To analyze the effect of the ethanol concentration, runs 1 and 20 can be compared. The two runs had the same operational factors for the sample dried at 40°C, with temperature of extraction of 25 °C, time of extraction 45 min and ethanol concentration 75%. The run 1 had as solid - liquid ratio 25, while the run 20 had 5.

Table 10 Runs comparison for the best solid - liquid ratio

Run	Solid Liquid ratio	Punicalin g/100g	Punicalagin (A+B) g/100g	Ellagic acid g/100g	Total polyphenols (%)	Punicalagin /punicalin + ellagic acid
1	25	0.69	5.18	7.02	13.1	0.67
20	5	0.41	11.24	2.67	14.59	3.64

The results show that the compounds extracted are strictly related to the amount of solids used. Higher solid - liquid ratio allowed an overall higher extraction of phenolic compounds. A high solid-liquid ratio can increase the diffusion of the components into the solvent accelerating the mass transfer.

3.6 Polyphenol content: optimal conditions

After a comparison of all the extractions, it was possible to highlight the best parameters condition. Each compound has specific requirement, that can be seen in the table 11.

The table report the best values found for the compounds of our interest and the related time and temperature of extraction, ethanol concentration and solid:liquid ratio. All the samples analyzed that allowed those results turned up to be the ones dried at 40°C.

Table 11 Best extraction conditions for each phenolic acid

	Run	Extraction time (min)	Extraction temperature (°C)	[EtOH]	Solid:liquid ratio	Value (g/100g)
Punicalin	30	45	50	45	15	1.06
Punicalagin	28	45	25	25	15	15.95
Ellagic Acid	26	45	75	35	15	9.18
Total polyphenols	28	45	45	25	15	23.02
Punicalagin /punicalin + ellagic acid	13	30	25	45	5	9.86

Punicalin appeared to be higher in the run 30, with a value of 1.06 mg/ per dry weight, where the extraction time was 45 minutes, temperature 50 °C, ethanol concentration 45% and solid-liquid ratio 1:15.

On the other hand, the highest value for the *punicalagin* has been showed in the run 28 (temperature of extraction 25°C, time of extraction 45 min, ethanol concentration 25%, solid – liquid ratio 1:15), with a content of 15.95 g/ 100g.

The best yield for the *ellagic acid* using the sample dried at 40°C has been showed in the run 26, with 9.18 g/100g (temperature of extraction 75°C, time of extraction 45 min, ethanol concentration 35%, solid – liquid ratio 1:15).

Even though each compound has his best extraction parameters, the *total polyphenol content* has been recorded in the runs 28 (temperature 45°C, time 45 min, ethanol concentration 25% e solid-liquid 1:15) with a value of 23.02 g/100g.

The ratio *punicalagin/ (punicalin + ellagic acid*) refers to the level hydrolysis of the initial polyphenol (punicalagin) has been transformed into hydrolysis products (punicalin and ellagic acid). Higher it is, more of the original compound can be found, and less of the hydrolysis products. So, where the punicalagin levels are high, the punicalin and ellagic acid levels will be lower.

3.7 Extraction optimization

After finding the best conditions among the standard ones provided and reported in the literature, by using the software JMP version 11.0.0 it was possible to obtain more specific values.

The best conditions to maximize the extraction yield for the specific polyphenols and for the total polyphenols content were the following (Table 11):

Table 12. Optimal conditions parameters

Control 17 runs	Temperature (°C)	Time (min)	[EtOH] (%)	solid: liquid	Estimated value (g/100g)	Desirability
Punicalagin (A+B)	25	33	25	1:25	15.20	0.9971
Punicalin	45	15	43	1:21	1.08	0.9961
Ellagic acid	40	15	25	1:22	8.64	0.9378
Total polyphenols	45	27	25	1:25	23.15	0.9934
Punicalagin/punicalin +ellagic acid	45	35	25	1:5	8.11	0.7976

40°C 17 runs						
Punicalagin (A+B)	25	32	25	1:25	13.11	0.9033
Punicalin	45	15	43	1:25	1.08	0.9962
Ellagic acid	40	15	25	1:22	8.64	0.9378
Total polyphenols	25	38	25	1:25	22.73	0.9888
Punicalagin/punicalin +ellagic acid	45	35	25	1:5	8.65	0.8501
60°C 17 runs						
Punicalagin (A+B)	25	35	25	1:25	13.75	0.9964
Punicalin	39	15	25	1:25	0.93	0.9929
Ellagic acid	43	15	25	1:25	9.92	0.9729
Total polyphenols	25	15	25	1:20	24.91	0.9989
Punicalagin/punicalin +ellagic acid	45	36	25	1:5	7.37	0.8053
40°C 35 runs						
Punicalagin (A+B)	25	45	25	1:19	15.89	0.8743
Punicalin	45	15	45	1:21	1.08	0.9011
Ellagic acid	33	15	25	1:23	8.13	0.7786
Total polyphenols	25	45	25	1:20	23.62	0.9226
Punicalagin/punicalin +ellagic acid	45	30	25	1:5	7.33	0.7215

The conditions reported in the table 12 have been provided by the software JMP on the basis of the concentrations of compounds previously obtained and reported in the table 5.

The maximization estimate has been carried out initially on the first 17 runs, considering the samples freeze-dried, dried at 40°C and dried at 60°C. Then it was carried out also on the total 35 runs with the sample dried at 40°C.

The table provide the ideal extraction conditions for each compound in terms of extraction time and temperature, ethanol concentrations and solid/liquid ratio. Following the specific suggested parameters, the estimated value provided should be reached as a result of a maximum optimization of the extraction. Being an estimate, it is necessary to consider a certain level of desirability: closer to 1 the desirability is, more

possibility we have to obtain the reported value. “Desirability” is related to the possibility to obtain that value. Higher value (close to 1) means more likely to occur.

The experimental plan showed that it is possible to optimize the extraction parameters to increase the yield. The optimal conditions were identified as:

- Higher solid: liquid ratio;
- Short extraction time;
- Low extraction temperature;
- Low ethanol concentration.

Moreover, the best drying temperature of the sample peels appeared to be 40 °C, which is also optimal for the industrial application, with less energy consumption compared to the freeze-drying process.

A freeze-drying procedure could be in fact beneficial from one side (helping to maintain the compounds), but at the same time it would require too much time and money for the huge amount of peels to treat: almost half of the pomegranate fruit is represented by peels that are wasted.

Yan et al. (Linlin Yan, 2017) conduced a research on the phenolic profiles and the antioxidant activities on six different Chinese pomegranate cultivars. They analyzed physicochemical characteristics, phenolic contents, and antioxidant capacities of the juices, seeds, and peels of the provided varieties and then compared them to show the differences.
The peels, after the manual removal from the fruits, were lyophilized, grounded, and extracted with 80% methanol–water solution. Then the samples were analyzed by using the UPLC-DAD with the C18 column (2.1 mm id × 50 mm, 1.8 μm), and 0.2% formic acid water solution as eluent A and acetonitrile as eluent B. Results showed in the table 13 are provided in mg/g of dry weight.

As can be seen in the table 11, among the six cultivars SD-QP possessed the highest contents of punicalin, (A and B), punicalagin, and ellagic acid, while SD-BLC possessed the lowest. This makes the cultivar the most ideal for the phenolic industrial preparations.

A and B punicalagin was the dominant polyphenol, accounting for more than 90% of the phenolic in all of the tested cultivars (range, 17.8–114.5 mg/g of dry weight) followed by punicalin, ellagic acid, and gallic acid.

Table 13 phenolic content of the pees of six pomegranate cultivars (Linlin Yan, 2017)

Cultivars	Phenolic compounds contents (mg g^{-1} DW)				
	Gallic acid	Punicalin	Punicalagin	Ellagic acid	Total contents
SD-BLC	0.12 ± 0.00^{f}	0.75 ± 0.01^{f}	17.80 ± 0.05^{e}	0.39 ± 0.00^{f}	19.05 ± 0.04^{e}
SD-DMY	0.56 ± 0.01^{b}	1.84 ± 0.01^{b}	52.77 ± 1.18^{d}	1.59 ± 0.01^{b}	56.76 ± 1.19^{d}
SD-QP	1.44 ± 0.01^{a}	5.42 ± 0.03^{a}	114.52 ± 3.64^{a}	3.04 ± 0.01^{a}	124.42 ± 3.61^{a}
XJ-HT	0.55 ± 0.00^{c}	1.09 ± 0.00^{e}	75.18 ± 1.57^{b}	0.86 ± 0.04^{d}	77.68 ± 1.54^{b}
XJ-KS	0.35 ± 0.00^{e}	1.25 ± 0.01^{d}	76.48 ± 0.60^{b}	1.34 ± 0.02^{c}	79.42 ± 0.61^{b}
XJ-PYM	0.39 ± 0.00^{d}	1.73 ± 0.01^{c}	61.30 ± 1.18^{c}	0.80 ± 0.01^{e}	64.23 ± 1.19^{c}

Mean value ± SD (n = 3), different letters in each column indicate significant differences at $p < 0.05$.

It's interesting to notice that the polyphenol content of each sample (and variety) is strictly related to the color of the peel of the fruits used (table 12). As previously noticed, the cultivar SD-QP presented the highest phenolic content. And we can see that the skin of this cultivar is the more red-brown.

Table 14 Fruit characteristics of the six Chinese pomegranate cultivars (Linlin Yan, 2017)

Cultivars	Fruit skin colour	Fruit weight (g)	Fruit diameter (mm)	100-aril weight (g)	Aril yield (%)	Juice yield (%)
SD-BLC	White	356 ± 26^{bc}	92 ± 3^{b}	74 ± 10^{a}	62 ± 6^{a}	50 ± 2^{b}
SD-DMY	Yellow green	611 ± 79^{a}	96 ± 7^{a}	57 ± 10^{b}	58 ± 5^{ab}	48 ± 2^{a}
SD-QP	Red brown	361 ± 31^{b}	97 ± 2^{a}	32 ± 5^{e}	57 ± 5^{ab}	42 ± 1^{d}
XJ-HT	Rosy	285 ± 24^{d}	87 ± 3^{c}	38 ± 12^{c}	56 ± 7^{bc}	42 ± 1^{d}
XJ-KS	Dark red	330 ± 31^{bcd}	93 ± 3^{b}	35 ± 6^{d}	59 ± 6^{ab}	47 ± 1^{c}
XJ-PYM	Rosy	362 ± 84^{b}	97 ± 7^{a}	33 ± 9^{e}	51.7 ± 5^{c}	36 ± 1^{e}

Mean value ± SD (n = 10) followed by different letters in each column indicate significant differences at $p < 0.05$.

The values obtained and showed in the table 13 presents differences from the values obtained in this thesis, with lower values compared to the ones obtained in the present study.

For example, while the average value of the punicalin content in this research is 0.201 g/100g of dry weight, in our study the punicalin content for lyophilized samples appeared to be (in the ideal conditions) 1.08 g/100g of dry weight (variation of 81.48%). The average punicalagin content appeared to be 6.58 g/100g, lower compared to the 15.20 g/100g of the optimized method (variation of 56.71%). And the average ellagic acid in the research was 0.134 g/100g, while in our study the optimal value showed to be 8.64 g/100g (variation of 98.50%).

Also *Lu at al.* (Lu, 2008) performed an analysis focused on the punicalagin content. Freeze dried samples of pomegranate peels were extracted with 40% of ethanol by using a LC method. The table 15 shows the total content of punicalagin in the 16 different varieties of pomegranate husks. The highest content was found in the husk of a pomegranate named red ruby from Shanxi province (12.15 g/100g), which is almost three times the lowest content in the peel of a drugstore in Guangdong province (3.98 g/100g). Data showed

significant differences in the content of punicalagin among husks from various areas. Here, the higher content is close to the ideal value of punicalagin we found (12.15 g/100g compared to 15.20g/100g).

Table 15 Total punicalagin content in various pomegranate husks analyzed by Lu et al.

Pomegranate husk accession		Total content of punicalagins in husk (mg g^{-1})
Province	Area or name of variety	
Xinjiang	Black husk	56.5 ± 0.8
Shangdong	Binzhou	80.5 ± 1.3
Shanxi	Lintong	66.0 ± 0.3
Sichuan	Panzhihua	82.7 ± 0.1
Sichuan	Huili	80.3 ± 0.1
Shanxi	Fengxiang	75.9 ± 0.3
Yunnan	Dali	80.8 ± 0.5
Anhui	Huaiyuan	89.8 ± 0.1
Xinjiang	White husk	92.5 ± 0.8
Hunan	Changsha	44.9 ± 0.2
Yunnan	Mengzi	47.8 ± 0.8
Shangdong	Taishan	97.6 ± 0.6
Shanxi	Red ruby	121.5 ± 0.2
Western	Soft seed	111.0 ± 0.9
Heibei	Drug store	97.3 ± 0.8
Guangdong	Drug store	39.8 ± 0.3
Mean		82.4

Values represent mean ± SD, $n = 3$

Why can we find such huge different values?

Differences in the amount of compounds is related certainly to pre-harvest factors as genotype, climate and altitude, maturity status and cultural practices (as irrigation, fertilization…) and to postharvest factors as storage temperature and humidity and technological treatments (Rebogile R. Mphahlele, 2014)

But such huge difference is also related to technical and operational parameters, so type of extraction, solvent and type of technique used. In fact, although a peel may have high quantities of polyphenols, these must be extracted and identified in the best possible way.

The experimental plan presented in this thesis aimed to maximize the extraction of compounds from the skins, and this appears to be clear if we compare the results with the values presents in the literature. The results obtained from the combination of the different extraction parameters, and the HPLC method optimization confirmed the initial objective of the thesis.

CONCLUSIONS AND FUTURE PROSPECTS

This thesis has highlighted the conditions for a better extractability from the pomegranate fruit peels, which is a rich source mainly of polyphenols but also vitamins and minerals.

Due to high polyphenols levels, waste peels represent a huge opportunity in the pharmaceutical, nutraceutical and food industries: bioactive compounds can be easily integrated into different products, representing also a good economic and environmentally friendly scenario. Based on the results obtained the better extraction in terms of polyphenol content was obtained in pomegranate peels treated at 40°C. Moreover, each polyphenol requires specific parameters for the optimal extraction. Maximum release of punicalagin A+B was achieved at 25°C for 32 minutes by using an ethanol concentration of 25% and 1:25 of solid: liquid ratio. In the same way the punicalin required 1:25 solid: liquid ratio, even though 45°C for 15 minutes by using 43% of ethanol concentration were necessary. Finally, the ellagic acid should be better extracted at 40°C for 15 minutes with a 25% of ethanol concentration and 1:22 of solid-liquid ratio.

The conditions applied in the laboratory for the analysis could also be easily applied at the industrial level as it does not require technologies or practices with high extraction costs.

Solvent extractions in fact it's one of the easiest extraction practices. According also to the compounds of interest, the solvent used can be changed. Moreover, the solvents have relatively low costs and this makes this type of extraction one of the best for the company. It represents a good way to optimize the extraction yield and to balance the overall costs for a big scale.

Also to be considered is the large production of waste. As reported by Magangana (T. P. Magangana, 2020), the peel accounts for about half the weight of the pomegranate fruit. It therefore becomes necessary to use techniques that are in any case inexpensive and simple but still effective.

The presented research represents a first step toward the waste valorization. The next ones will involve the use of more emerging practices: it has been demonstrated that the use of ultrasound increase by 24% the recovery of polyphenols extraction compared to the conventional solvent extraction (Zhongli Pan, 2011).

Nevertheless, the use of some technologies results too expensive for an industrial use and, most important, may lead to the degradation of some compounds.

The results obtained in this research showed us that the valorization of pomegranate peels is possible also applying the conventional solvent extraction and that is actually possible to recover them.

It is also necessary to point out that it is essential not only the extraction process, but also the storage of the peels (temperature, humidity, treatments…) (Rebogile R. Mphahlele, 2014) . In fact, after the process, peel wastes have to be stored in the best way possible to avoid compound degradation. The compounds can undergo to oxidative and hydrolysis reaction, causing a depletion in quality. Being a great source of polyphenols, the peels should represent a huge opportunity to reuse what is usually considered waste but can actually be used in various ways: for the food processing (to preserve the oxidative stability of food products and to inhibit the growth of some bacteria), in the packaging for their antimicrobial activity or also as food supplement. (Maryam Pirzadeh, 2020).

BIBLIOGRAPHY and SITOGRAPHY

- Aloqbi, Akram & Ulfat, Maham & Yousr, Marwa & Grace, Mary & Lila, Mary & Howell, Nazlin. (2016). Antioxidant Activity of Pomegranate Juice and Punicalagin. *Natural Science*. 08. 235-246. 10.4236/ns.2016.86028.
- Arfaoui L. Dietary Plant Polyphenols: Effects of Food Processing on Their Content and Bioavailability. Molecules. 2021 May 16;26(10):2959.
- Ascacio-Valdés, Juan & Aguilera-Carbó, Antonio & Buenrostro Figueroa, Jose & Prado, Lilia Arely & Rodriguez, Raul & Aguilar, Cristobal. (2016). The complete biodegradation pathway of ellagitannins by *Aspergillus niger* in solid-state fermentation. *Journal of basic microbiology*.
- Atanackovic, M. & Cvejic, Jelena & Bukarica, Ljiljana & Veljovic, M. & Despotovic, Sasa & Pecić, Sonja & Gacesa, B. & Leskosek, Ida. (2012). Quantitative determination of total anthocyanins and flavonoids in natural products obtained from grapes and malt.
- Beconcini, Denise & Felice, Francesca & Fabiano, Angela & Sarmento, Bruno & Zambito, Ylenia & Di Stefano, Rossella. (2020). Antioxidant and Anti-Inflammatory Properties of Cherry Extract: Nanosystems-Based Strategies to Improve Endothelial Function and Intestinal Absorption. *Foods* (Basel, Switzerland).
- Brighenti V, Iseppi R, Pinzi L, Mincuzzi A, Ippolito A, Messi P, Sanzani SM, Rastelli G, Pellati F. Antifungal Activity and DNA Topoisomerase Inhibition of Hydrolysable Tannins from *Punica granatum L*. *International Journal of Molecular Sciences*. 2021 Apr 17;22(8):4175.
- Chaudhari, Swapnil & Patel, Kalyani & Badole, Sachin. (2013). *Punica granatum* (Pomegranate Fruit): In Cancer Treatment. 10.1016/B978-0-12-398456-2.00106-7.
- Claudine Manach, Augustin Scalbert, Christine Morand, Christian Rémésy, Liliana Jiménez, Polyphenols: food sources and bioavailability, *The American Journal of Clinical Nutrition*, Volume 79, Issue 5, May 2004, Pages 727–747.
- Cravotto, Giancarlo & Mariatti, Francesco & Gunjević, Veronika & Secondo, Massimo & Villa, Matteo & Parolin, Jacopo & Cavaglià, Giuliano. (2018). Pilot Scale Cavitational Reactors and Other Enabling Technologies to Design the Industrial Recovery of Polyphenols from Agro-Food By-Products, a Technical and Economical Overview. *Foods*. 7. 130.
- Conidi C, Drioli E, Cassano A. Perspective of Membrane Technology in Pomegranate Juice Processing: A Review. *Foods*. 2020; 9(7):889.
- Galanakis, Charis M. 2021. "Functionality of Food Components and Emerging Technologies" *Foods* 10, no. 1: 128.

- Les, Francisco & Carpéné, Christian & Arbones-Mainar, Jose & Decaunes, Pauline & Valero, Marta & Lopez, Victor. (2017). Pomegranate juice and its main polyphenols exhibit direct effects on amine oxidases from human adipose tissue and inhibit lipid metabolism in adipocytes. *Journal of Functional Foods*. 33. 323-331.
- Lu, Jingjing & Ding, Ke & Yuan, Qipeng. (2008). Determination of Punicalagin Isomers in Pomegranate Husk. *Chromatographia*. 68. 303-306.
- Jia Li, Guoliang Wang, Chen Hou, Jianke Li, Ying Luo & Baicun Li (2019) Punicalagin and ellagic acid from pomegranate peel induce apoptosis and inhibits proliferation in human HepG2 hepatoma cells through targeting mitochondria, *Food and Agricultural Immunology*, 897-912.
- Kammerer, Dietmar & Kammerer, Judith & Valet, Regine & Carle, Reinhold. (2014). Recovery of polyphenols from the by-products of plant food processing and application as valuable food ingredients. *Food Research International*. 65. 2-12. 10.1016/j.foodres.2014.06.012.
- Khan, Muhammad & Ahmad, Khalil & Hassan, Sadia & Imran, Muhammad & Ahmad, Nazir & Xu, Changmou. (2017). Effect of novel technologies on polyphenols during food processing.
- Magangana TP, Makunga NP, Fawole OA, Opara UL. Processing Factors Affecting the Phytochemical and Nutritional Properties of Pomegranate (*Punica granatum L.*) Peel Waste: A Review. Molecules. 2020 Oct 14;25(20):4690.
- Manach, Claudine & Scalbert, Augustin & Morand, Christine & Rémésy, Christian & Jimenez, Liliana. (2004). Polyphenols: Food source and bioavailability. *The American journal of clinical nutrition*. 79. 727-47.
- María I. Gil, Francisco A. Tomás-Barberán, Betty Hess-Pierce, Deirdre M. Holcroft, and Adel A. Kader, 2000, Antioxidant Activity of Pomegranate Juice and Its Relationship with Phenolic Composition and Processing*, Journal of Agricultural and Food Chemistry* 48 (10), pp. 4581-4589
- Maryam Pirzadeh, Nicola Caporaso, Abdur Rauf, Mohammad Ali Shariati, Zhanibek Yessimbekov, Muhammad Usman Khan, Muhammad Imran & Mohammad S. Mubarak (2020): Pomegranate as a source of bioactive constituents: a review on their characterization, properties and applications, *Critical Reviews in Food Science and Nutrition*
- Mphahlele, Rebogile & Fawole, Olaniyi & Stander, Marietjie & Opara, Umezuruike. (2014). Preharvest and postharvest factors influencing bioactive compounds in pomegranate (Punica granatum L.)—A review. *Scientia Horticulturae*. 178. 114–123.
- Nishiumi S, Miyamoto S, Kawabata K, Ohnishi K, Mukai R, Murakami A, Ashida H, Terao J. 2011 Jun 1 Dietary flavonoids as cancer-preventive and therapeutic biofactors. *Frontiers in bioscience (Scholar edition)* ;3(4):1332-62.

- Pan Z, Qu W, Ma H, Atungulu GG, McHugh TH. Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel. *Ultrason Sonochem*. 2011 Sep;18(5):1249-57.
- Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*. 2009 Nov-Dec;2(5):270-8.
- Qu, Wenjuan & III, Andrew & Pan, Zhongli & Ma, Haile. (2012). Quantitative determination of major polyphenol constituents in pomegranate products. *Food Chemistry*. 132. 1585–1591.
- Stenmarck, Åsa & Jensen, Carl & Quested, Tom & Moates, Graham. (2016). Estimates of European food waste levels.
- Sun, Yu-qing & Tao, Xin & Men, Xiao-ming & Xu, Zi-wei & Wang, Tian. (2017). In vitro and in vivo antioxidant activities of three major polyphenolic compounds in pomegranate peel: Ellagic acid, punicalin, and punicalagin. *Journal of Integrative Agriculture*. 16. 1808-1818.
- Syed DN, Chamcheu JC, Adhami VM, Mukhtar H. Pomegranate extracts and cancer prevention: molecular and cellular activities. Anticancer Agents *Medical Chemistry*. 2013 Oct;13(8):1149-61.