Subcritical water extraction and reaction of bioactive pectic polysaccharides

by

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Abstract

Natural pectic polysaccharides have drawn considerable attention as promising multifunctional materials for food, pharmaceutical and biomedical uses. Pectic polysaccharides production, however, involves the utilization of inorganic acids such as sulfuric, hydrochloric and nitric acids. The objective of this study was to isolate novel bioactive pectic polysaccharides from pomegranate *Punica granatum L*. biomass using a subcritical binary fluid system as well as to obtain pectin-based *sols* under subcritical water conditions prior to the production of pectin-based 2D films or 3D cryogel structures. Pomegranate pectic polysaccharides (PPs) were extracted using a semi-continuous subcritical water system at 100-140˚C, 50 bar and 5 mL/min with binary solvent systems (aqueous citric acid or aqueous ethanol) and, pectin-based *sols* were formed within a stirred-tubular subcritical water batch reactor at 100˚C and 70 bar. The variables evaluated were yield, galacturonic acid content, degree of esterification and anti-oxidant properties of isolated pomegranate pectic polysaccharides. Pectin-based *sols* were then used to obtain films and cryogels. The physico-chemical, mechanical, optical and functional properties of such gels were determined. These results showed that subcritical water and the above subcritical binary aqueous systems at 120˚C/50 bar and 30 min favored the extraction of bioactive pomegranate pectic polysaccharides with high galacturonic acid content (>70%) and degree of esterification (>60%). The total phenolic content and the anti-oxidant activity of extracted PPs were 152.9 mg GAE/g PPs and I %DPPH >87%, respectively. Pectin-based *sols* obtained with subcritical aqueous citric acid at 100˚C/70 bar led to flexible films with tensile strength, percent elongation and phenolics release capacity comparable to pectin-based films

produced by the traditional *sol-gel* method. Subcritical *sol* formation and freeze-dried *gel* integration led to lightweight and highly porous bioactive pectin-based cryogels.

Through this thesis, subcritical water technology has shown to be a versatile, environment-friendly and sustainable alternative that can be used for pomegranate biomass conversion into value-added products as well as in combination with other processes to obtain novel biocomposite materials.

***Keywords**: Bioactive films, pectic polysaccharides, pomegranate biomass, subcritical water.*

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ABBREVIATIONS

ANOVA	Analysis of variance
Aq	Aqueous solution
DE	Degree of esterification
DPPH	2,2-diphenyl-1-picrylhydrazyl assay
E%	Percent elongation at break
EOs	Essential oils
FTIR	Fourier transform infrared spectroscopy
GalA	Galacturonic acid
GAE	Gallic acid
GRAS	Generally recognized as safe
HDS	Honest significant difference
HM	High methoxyl
HPLC	High-performance liquid chromatography
HPAEC-PAD	High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection
LM	Low methoxyl
PFs	Pressurized fluids
PGb	Pomegranate biomass
PPs	Pomegranate pectic polysaccharides
RH	Relative humidity

RG-I	Rhamnogalacturonan type I
RG-II	Rhamnogalacturonan type II
sCW	Subcritical water
TS	Tensile strength
UV/Vis	Ultraviolet/visible light

Chapter 1: Introduction

1.1 Rationale

Natural pectic polysaccharides have drawn considerable attention as promising multifunctional materials for food, pharmaceutical and biomedical uses (Paulsen & Barsett, 2005; Mishra et al., 2014). The production of pectic polysaccharides, however, involves the utilization of inorganic acids, such as sulfuric, hydrochloric and nitric acids as well as the generation of large volumes of waste effluents that have to be treated before disposal. Considering that pectin production has increased in the last few years (42 000 MT of pectin produced in 2009) and, that the large world pectin producers (CPKelco, Danisco, Herbstreith & Fox and Carguill) are investing and expanding their industrial plants, the environmental impact associated to acid effluents and further water pollution is a serious concern (CPKelco, 2009). Thus, more eco-friendly technologies are imperative for pectin manufacture. The use of subcritical water technology, for example, has been reported as a green and versatile alternative for the extraction of a wide range of value-added compounds from biomass. As well described and reported by King et al. (2010) and Saldaña & Valdivieso-Ramírez (2015), subcritical water or pressurized hot water has been use for extraction/reaction to obtain polyphenols and carbohydrates from various agricultural residues (barley and lupin hull; potato and onion peel; wheat and corn bran; flax shives and livestock) as well as protein and aminoacids from cereals and animal sources (rice bran and soybean meal; fish meal, chicken residues, bovine serum albumin, hair and poultry feathers). Moreover, due to its potential application in various areas of research, the number of scientific publications on subcritical water extraction has dramatically increased since 2012 (Web of Science database). Although subcritical water

extraction of bioactive phytochemicals from biomass has been widely investigated and, in some cases patented, there are limited studies on subcritical water extraction of pectin from industrial residues.

As pectic polysaccharides or pectin is commercially produced mainly from juice and cider industrial co-products, there are a variety of fruit and agri-food industrial residues that can be potentially used as pectin sources, among them, pomegranate rind (1 000 000 MT/2007) (USAID, 2008). Pomegranate non-edible parts comprise about 60% of the fruit weight and, it is discarded after juice extraction. Recent studies have shown that pomegranate rinds and seeds have a high antioxidant activity compared to pomegranate juice (U. S. Patent No. 20150120, 2015). Hence, *in vitro* and *in vivo* human and mice studies have suggested that ethanolic extracts of pomegranate industrial co-products are promising sources of bioactive components, such as anthocyanins, anthoxanthins, alkaloids, glycosides and saponins, that might be responsible for the associated health benefits, such as prevention of cardiovascular diseases and cancer (Aviram & Rosenblat, 2012; Bhandary et al., 2012). Therefore, subcritical water technology can be investigated as a feasible and environment-friendly alternative for pomegranate biomass conversion into novel value-added products with potential applications as food ingredient, nutraceuticals and/or biocomposites production.

1.2 Hypothesis

- Subcritical water technology could favor solvency and extraction of bioactive pectic polysaccharides and polyphenols from pomegranate biomass without shortcommings associated to the conventional solid-liquid extraction process.
- Hydrogel formation of pectic/amino polysaccharides under subcritical conditions may lead to biocomposite materials with different physico-chemical properties.

1.3 Thesis objectives

The main objective of this thesis was to isolate novel bioactive pectic polysaccharides from pomegranate *Punica granatum L*. biomass using subcritical binary fluid systems as well as to obtain pectin-based *sols* under subcritical water conditions prior to the production of pectin-based 2D films or 3D cryogel structures. This was achieved through the following specific objectives:

- Study the effect of binary food grade and GRAS solvent systems, such as citric acid/water and ethanol/water, under subcritical conditions on the yield and physicochemical properties of isolated pomegranate pectic polysaccharides as well as the kinetics of the extraction.
- Understand the synthesis of pectin/chitosan/essential oils composite materials in subcritical water media.

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Chapter 2: Literature review[1]

2.1 Pectic polysaccharides

2.1.1 Structure

Pectin or pectic polysaccharides are collective names of a highly heterogeneous group of polymers that naturally occur in the middle lamella and primary cell walls of vascular plants (Van Buren, 1991; Mohnen, 2008; Voragen et al., 2009; Ghail et al., 2012). Although pectic polysaccharides are mostly related to turgor and mechanical strength of plant cell walls, they are essential for physiological processes such as growth, cell differentiation, water retention, ion transport, seed hydration and fruit ripening. Also, plant response against biotic stress as well as the lignification and enzyme modulation in plant tissues have been related to pectin domain (Walter, 1991; Voragen et al., 2009). As a highly reactive polysaccharide, the structure of pectin is one of the most complex among cell wall components (cellulose, hemicellulose and lignin) (Ochoa-Villarreal et al., 2012). Pectin structure comprises of numerous monosaccharide residues and different linkages owing to its dynamic interaction with other polymers within the cell wall. Such interactions are versatile and can change depending on the developing stage and the plant variety (Seymour & Knox, 2002; Mohnen, 2008).

[1] Aversion of this chapter was published as Saldaña, M. D. & Valdivieso-Ramírez, C. S. (2015). Pressurized fluid systems: Phytochemical production from biomass. *Journal of Supercritical Fluids, 96*, 228-244.)

Over the past years, different fragments of pectic polysaccharides have been identified as major pectin, including homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). Although the chemical composition differs among pectic polysaccharides, D-galacturonic acid (GalA) is the main and distinctive compound found in all pectic fragments (Salbu et al., 2012; Georgiev et al., 2012). As such, homogalacturonan is defined as a partially esterified linear polymer of α-(1-4) D-galacturonic acid and encompasses almost 60% of the plant pectic polysaccharides. Homogalacturonan fragments either partially methyl-esterified at position C_6 or O-acetyl-esterified at position O_2 or O_3 are found in plant cell walls. Citrus fruits and apple for example are rich in methyl-esterified homogalacturonan, while acetyl-esterified homogalacturonan occurs in beet, carrot and potato (Seymour & Knox, 2002). The substitution group type over the galacturonan backbone as well as its distribution led to pectic polysaccharides with different physico-chemical behavior (i.e. gel formation mechanism and rheology) (Seymour & Knox, 2002; Ochoa-Villarreal et al., 2012). On the other hand, rhamnogalacturonan-I is a branched pectic polysaccharide that embraces a number of saccharides and oligosaccharides attached to its backbone unit of alternating D-Galacturonic acid and L-Rhamnose ($[1\text{-}\alpha\text{-D-GalA-1, 2-}\alpha\text{-L-Rha -1-4-}]_n$). Among RG-I side chains, α-(1,5)-L arabinans, β-(1,4)-D galactants, arabinogalactans-I and II have been found (Kacurakova et al., 2000; Mohnen, 2008). Hence, RG-I with O-acetylated backbone at C_2 and/or C_3 has been reported (i.e. O-acetylated C_2 and C_3 apple RG-I) (Seymour & Knox, 2002). The abundance of RG-I (20-30%) has shown to be lower compared to homogalacturonan but higher than RG-II (0.5 to 8%). Rhamnogalacturonan-II, however, encompasses the most complex and branched structure

of pectic fragments. RG-II has a backbone of nearly nine α-(1-4) D-galacturonic acid units surrounded by four side branched chains, which include approximately 12 different and rare saccharides such as 2-O-methyl xylose, 2-O-methyl fucose, aceric acid, 2-keto-3-deoxy-D-lyxo heptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno octulosonic acid (Seymour & Knox, 2002; Ochoa-Villarreal et al., 2012). Moreover, the complexity of RG-II has been related to very important functional roles in cell walls, such as the control of boron concentration in plant, which is crucial for proper cell wall porosity, strength and integrity as well as for preventing plant growth defects (Perez et al., 2003). Figure 2.1 depicts a representation of native pectic fragments considering they are held together by covalent bonds (Perez et al., 2003).

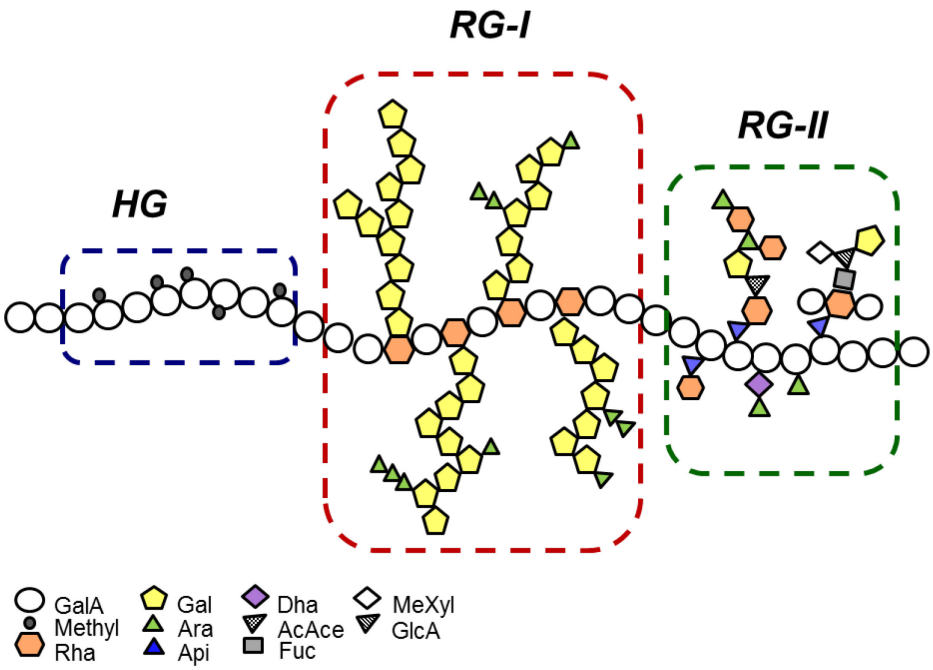


Figure 2.1. Scheme of pectic polysaccharides structure (adapted from Perez et al., 2003). GalA: galacturonic acid; Methyl: methyl group; Rha: rhamnose; Gal: galactose; Ara: arabinose; Api: apiose; Dha: 2-keto-3-deoxy-D-lyxo heptulosaric acid; AcAce: acetyl aceric acid; Fuc: fucose; MeXyl: methyl xylose; GlcA: gluconic acid.

2.1.2 Functionality

The medicinal properties of various plants have been recently attributed to their pectic polysaccharide content (Perez et al., 2013). Different biological activities have been reported for particular pectic fragments from selected plant sources as shown in Table 2.1 (Paulsen & Barsett, 2005).

Table 2.1 Biological activity of pectic fragments from selected plant sources (adapted from Paulsen & Barsett, 2005).

Source	Pectic fragment			Activity	Ref.
	HG	RG-I	RG-II		
Panax ginseng			x	Intestinal immune system modulation	Shin et al. (1997)
					Sun et al. (1994)
Plantago major		X		Pneumococcal infection-protector	Hetland et al. (2000)
Salvia officinalis		X		Mitogenic and comitogenic effect	Capek et al. (2003)
Tinospora cordifolia		X		Activation of B-cells	Chintalwar et al. (1999)
				Antioxidant	Subramanian et al. (2002)
Glycyrrhiza uralensis		X		Mitogenic effect	Hwang et al. (2003)
Apple peel	x	X		Inhibition of HT-29 cell proliferation	Ueno et al. (2008)
Orange and lemon peels	x	X		Inmunoestimulating activity	Georgiev et al. (2012)

2.2 Phenolic compounds

2.2.1 Structure

Phenolic compounds are heterogeneous class of secondary plant metabolites that according to their chemical structure can be either flavonoids (e.g., anthocyanins, flavan-3-ols, flavonols, flavones, isoflavonoids, flavanones, chalcones) or non-flavonoids (e.g., hydroxybenzoic and hydroxycinnamic acids, and stilbenes). Flavonoids comprise a C_6-C_3-C_6 structure, while C_6-C_1 and C_6-C_3 structures are commonly found in non-flavonoid components (Schieber & Saldaña, 2009; Saldaña et al., 2010; Wende & Beta, 2013). Table 2.2 depicts the structure and some physicochemical properties of selected phenolics.

Phenolic compounds are mainly present in fruits, cereals, vegetables and herbs, such as grapes, mango, pomegranate, citrus fruits, barley, rice, flax, potato, onion, olive, peppers, green tea, rosemary, oregano, ginseng, among others. The type of phenolics and their conjugates as well as their amount can differ markedly according to each plant or even the tissue of the same vegetable or fruit. For example, anthocyanins are the main polyphenols in the red grape skin, while flavan-3-ols are the major polyphenols in grape seeds (Makris et al., 2006). Also, their bioavailability changes due to the free, soluble and insoluble forms of phenolics found in plants. Thus, the total phenolic content (sum of individual phenolic acids) is commonly related to the antioxidant activity of the extracts.

Table 2.2 Structure and physico-chemical properties of selected bioactive compounds.

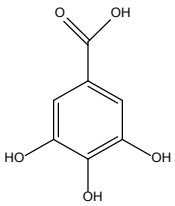
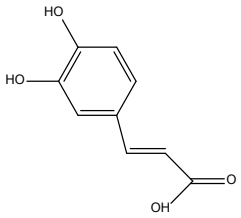
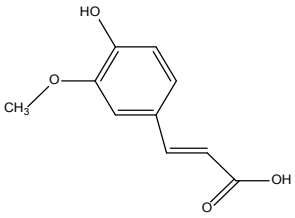
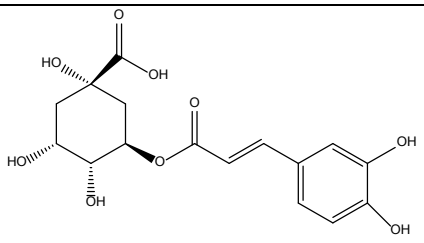
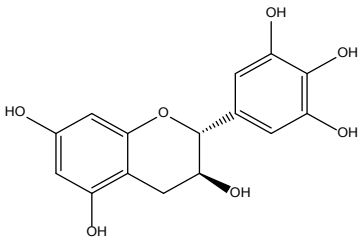
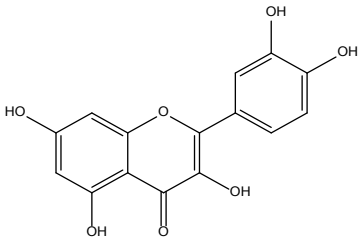
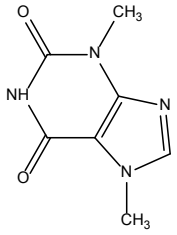
Group	Bioactive compound	Structure	Molecular weight (g/mol)	Solubility
Phenolic acids	Gallic acid		170	Hot H_2O
	Caffeic acid		180	Hot H_2O
	Ferulic acid		194	Hot H_2O
	Chlorogenic acid		354	Ethanol, acetone

Table 2.2 (*Continued*)

Group	Bioactive compound	Structure	Molecular weight (g/mol)	Solubility
Catechins	(+)-Gallocatechin		306	H_2O
Flavonoids	Quercetin		302	insoluble H_2O
Alkaloids	Theobromine		180	slightly soluble H_2O

2.2.2 Functionality

It has been proven that a diet rich in various classes of polyphenols decreases the risk of premature mortality from major clinical conditions, including cancer and heart disease (Duthie et al., 2003). Phenolic phytochemicals are known to exhibit several health beneficial activities, such as antioxidant, anti-inflammatory, anti-hepatotoxic, antitumor, and antimicrobial (Rice-Evans et al., 1996; Middleton et al., 2000; Revilla & Ryan, 2000; Podsedek, 2007). As antioxidants, phenolics act as reducing agents by

donating hydrogen, quenching singlet oxygen, acting as chelators of pro-oxidant metals and by trapping free radicals. Thus, these components have shown to be potential chemo-protective agents that can prevent or reduce cellular damage caused by the environmental oxidative stress (Stavric, 2007). The consumption of polyphenols from tea leaves (epigallocatechin) as well as in coffee extracts (cafestol+kahweol) for example have shown to inhibit the formation and growth of tumors (Inagake, et al., 1995; Hasegawa, et al., 1995; Stavric, 2007). Also, research on wine polyphenols (quercetin, rutin and resveratrol) has suggested that their antioxidant capacity can prevent low-density lipoprotein (LDL) oxidation and further to reduce the incidence of atherosclerosis and coronary heart disease (Stavric, 2007). As such, several studies have shown phenolics as relevant natural components for health promotion and disease prevention (Inagake, et al., 1995; Virgili & Scaccini, 2000; Ju & Howard, 2005). Therefore, their extraction and production have been actively investigated in the past few years (de Melo et al., 2014).

2.3 Essential oils

Essential oils are complex liquid mixtures, containing volatile aroma compounds from plants. They originate from the plant secondary metabolism and are contained within special secretory cells or plant tissues (Steiner, 2010). The aromatic constituents of essential oils are low molecular weight hydrocarbons (C_{10}-C_{15}) known as terpenes. Monoterpenes, sesquiterpenes as well as their oxygenated derivates, aliphatic aldehydes, alcohols and esters are responsible for their characteristic scent and therapeutic effect (Figure 2.2).

Current studies have shown that essential oils can be potentially used as natural agents for health promotion and food preservation because of their antibacterial, anti-

oxidant and anti-inflammatory properties (Amorati et al., 2013; Wei & Shibamoto, 2010). Sfeir et al. (2013) studied the antibacterial activity of eighteen essential oils against *Streptococcus pyogenes*, which is responsible for tonsillitis. Among them, the antibacterial activity of *Cinnamomum verum* essential oil was very similar to Amoxixillin. Also, Faleiro et al. (2012) found that essential oils can be potentially used to treat multidrug-resistant bacteria, such as *Staphylococcus aureus* and *Aeromonas hydrophila*. Besides, coriander, clove, oregano and thyme essential oils have proved to be effective antibacterial agents against food borne pathogens in meat products (Burt, 2004). Likewise, cinnamon, cardamom, clove and mint essential oils were effective against *Salmonella enteritidis* in dairy products (Sheeladevi & Ramamathan, 2012). On the other hand, their antioxidant activity has been also studied. Among the twenty-five essential oils tested by Wei and Shibamoto et al. (2008), thyme oil exhibited the greatest anti-oxidant activity, similarly to α-tocopherol. Tepe et al. (2004) also reported that radical scavenging and lipid peroxidation capacities of *Salvia cryptantha* and *Salvia multicaulis* essential oils were greater than ascorbic acid and butylated hydroxytoluene (BHT), suggesting their use as promising natural antioxidants for food applications. The anti-inflammatory, antimutagenic, antiproliferative, and detoxifying activities of a wide variety of essential oils have been described by Miguel (2010), Beum-So et al. (2013) and Bhalla et al. (2013). Therefore, production of pectin-based biocomposites loaded with essential oils could strategically deliver particular bioactive properties.

2.4 Pectin extraction methods

As pectic polysaccharides are tightly bound to other plant cell-wall biopolymers, extraction processes are needed to make them available for commercial uses.

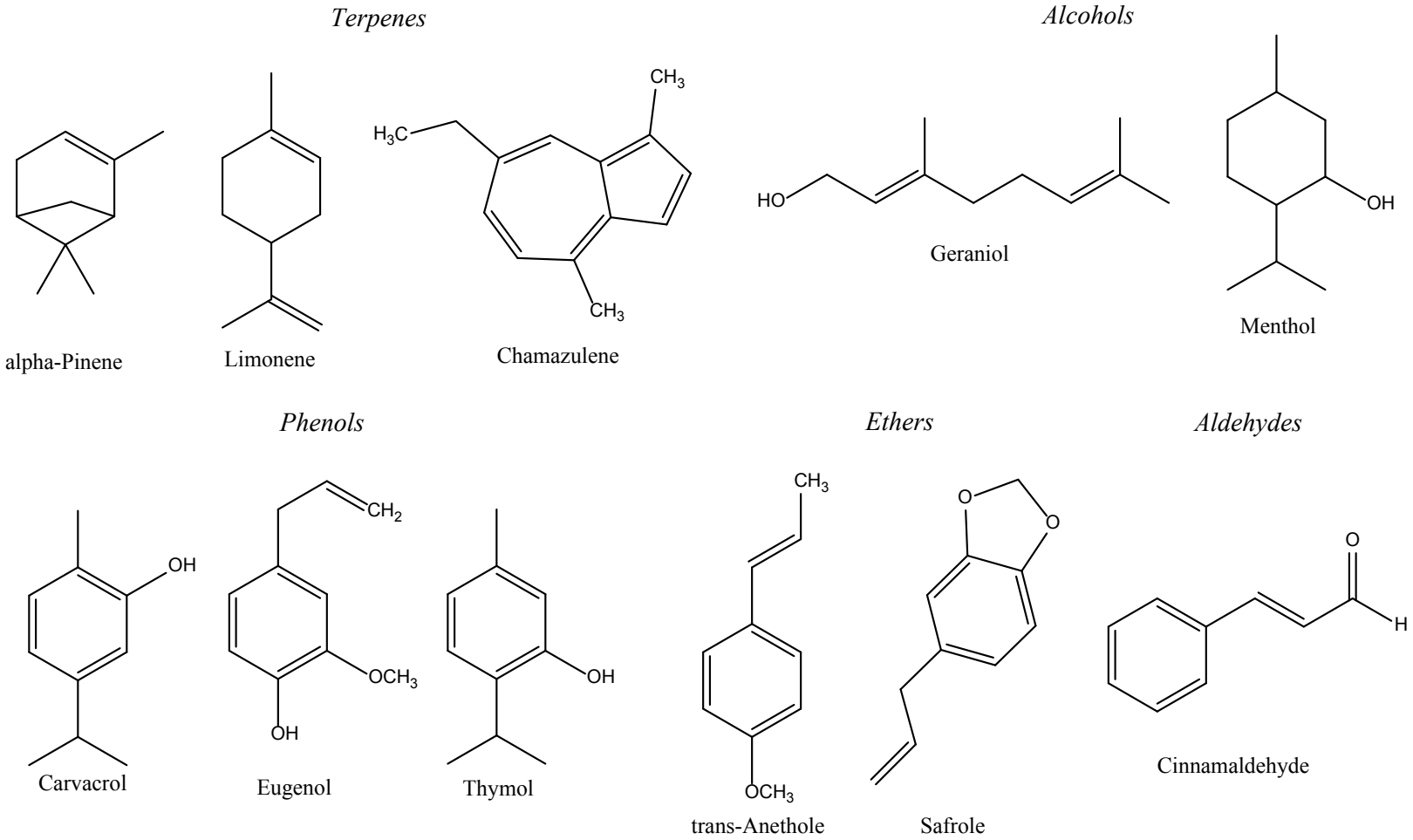


Figure 2.2 Chemical structure of some constituents in essential oils

Different extractants such as mineral acid solutions (hydrochloric, nitric and sulfuric acids), alkali solutions (potassium hydroxide and sodium carbonate) and calcium chelating agents (ethylenediaminetetraacetic acid and hexametaphosphate) have been used to isolate pectin from various plant sources (Walter, 1991). Acid solutions have shown to favor pectin extraction from fruit sources, while calcium-chelating agents are prone to extract pectin from vegetables, such as carrot, potatoes and beans (Walter, 1991).

In general, commercial pectin is isolated from industrial by-products, such as citrus peels, apple pomace or sugar beet chips. The industrial manufacture of pectin is a multi-stage process that involves acid-catalyzed hydrolysis at approximately $70^{o}C$, filtration, evaporation, ethanol-induced precipitation, purification, drying and milling processes (Figure 2.3). The resulting pectin, mainly homogalacturonan, is an odorless, white or yellowish and, water-soluble powder. The concentration of galacturonic acid residues in commercial pectin is often more than 65% and, its molecular weight ranges from 80 to 400 KDa. The structural features of isolated galacturonic acid residues, such as the degree of methyl-esterification and the distribution of these esters along the galacturonan backbone, are crucial elements that strongly determine the physical properties and final application of pectin. The degree of esterification (DE) is referred as the ratio of methyl esterified groups at C_6 position of galacturonic acid to the total galacturonic acid residues (Chen et al., 2014). Thus, high-methoxyl pectin (DE 55-75%) and low-methoxyl pectin (DE 20-45%) are commonly produced (Thakur et al., 1997; Seymour & Knox, 2002).

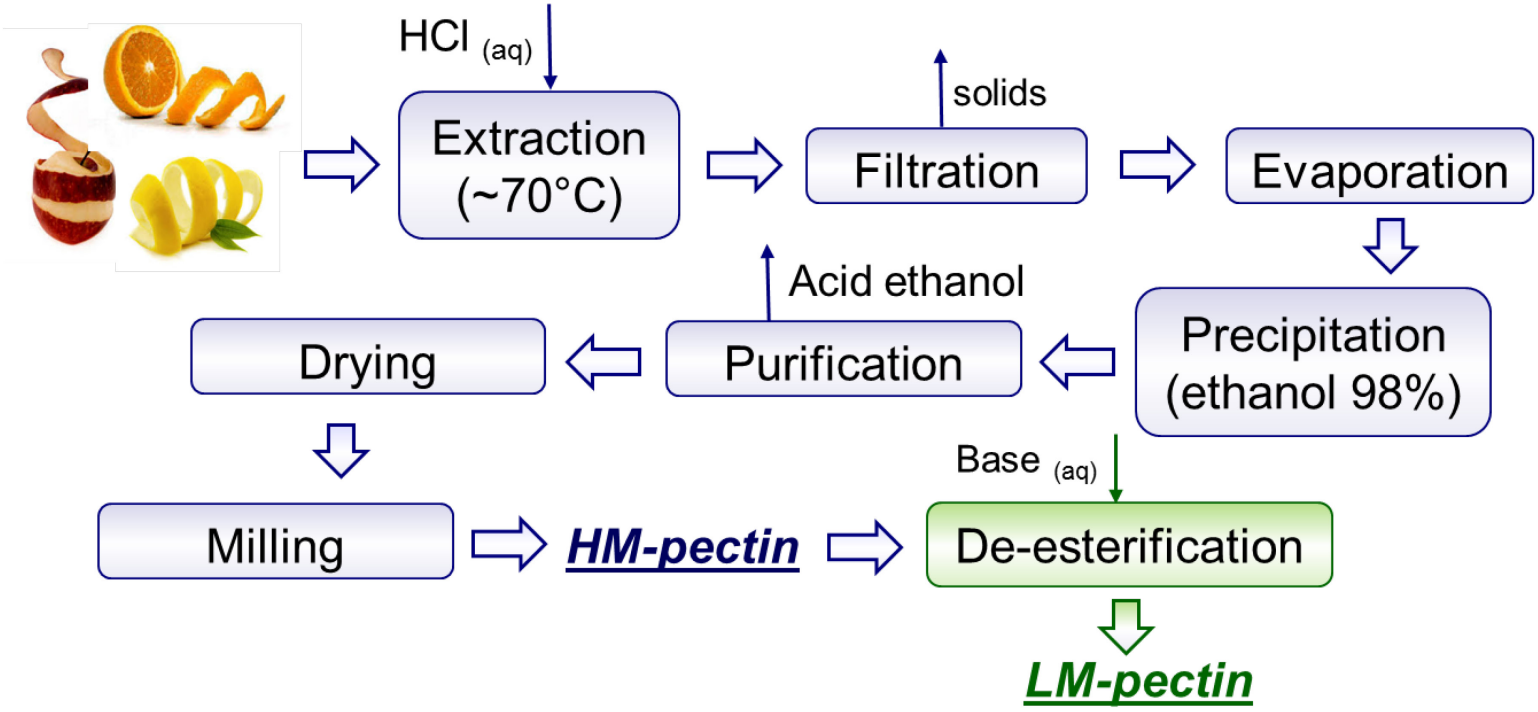


Figure 2.3 Pectin extraction flow chart (Adapted from CPKelco, 2005)

High-methoxyl pectin (HM) is obtained as described above, whereas low-methoxyl pectin (LM) requires a further controlled acid, alkaline or enzymatic ester hydrolysis. Also, modified low-methoxyl citrus pectin or amidated pectin can be found in the market. In addition, galacturonic acid residues with high content of O-acetyl-esterified groups extracted from sugar beet by-products are also part of current pectin fortafolio (Seymour & Knox, 2002; CPKelco, 2008). Figure 2.4 shows the characteristic D-galacturonic acid unit of each particular pectin type.

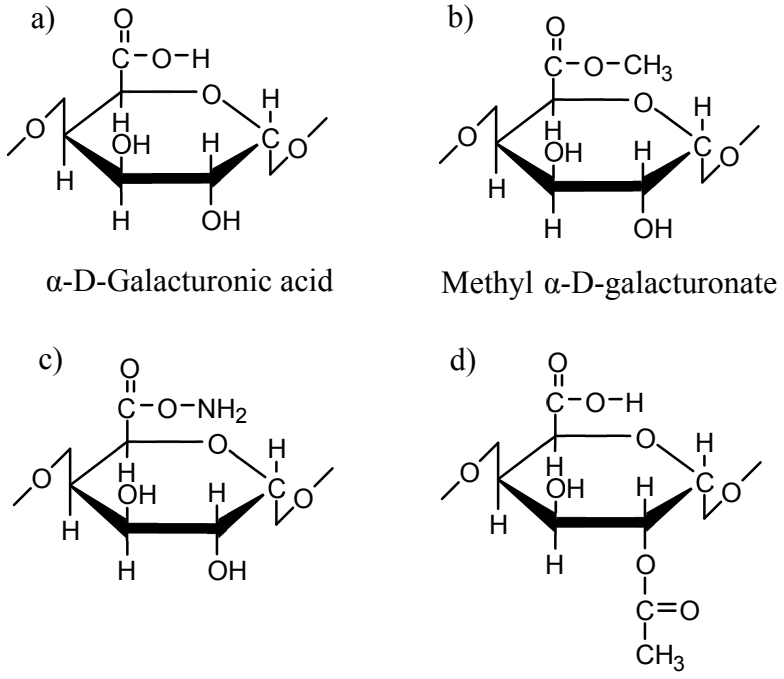


Figure 2.4 α-D-Galacturonic acid unit of: a) LM-pectin; b) HM-pectin; c) LM-amidated pectin and d) acetylated pectin (adapted from Seymour & Knox, 2002).

Although acid extraction of commercial pectin has led to relatively high yields, handling and disposal of large volumes of generated acid effluents are an environmental concern (Rovaris et al., 2008; Wang et al., 2014; Chen et al., 2014). Hence, extended extraction times, pectin depolymerization and reactor corrosion are some drawbacks associated with acid-catalyzed hydrolysis. Thus, alternative extraction methods that involve organic acids, ultrasound, microwave and subcritical water utilization have been proposed (Ueno et al., 2008; Vriesmann et al., 2012; Prakash Maran et al., 2014; Wang et al., 2014).

Subcritical water has been used in a wide range of applications since it was reported (King, 2006; Kubatova et al., 2001). The extractions of polycyclic aromatic hydrocarbons, herbicides and pesticides from soil and sediments as well as the extraction of flavors, fragrances and therapeutic compounds from plants and food have been earlier described (Smith, 2002; Tajuddin & Smith, 2005; Chienthavorn et al., 2007; Rodil & Popp, 2009; Carr et al., 2011). According to the Web of Science database, the number of articles and reviews published on subcritical water technology has increased exponentially in the last decade. Figure 2.5 and Figure 2.6 show the number of scientific publications and the growing areas of research on subcritical water extraction, respectively.

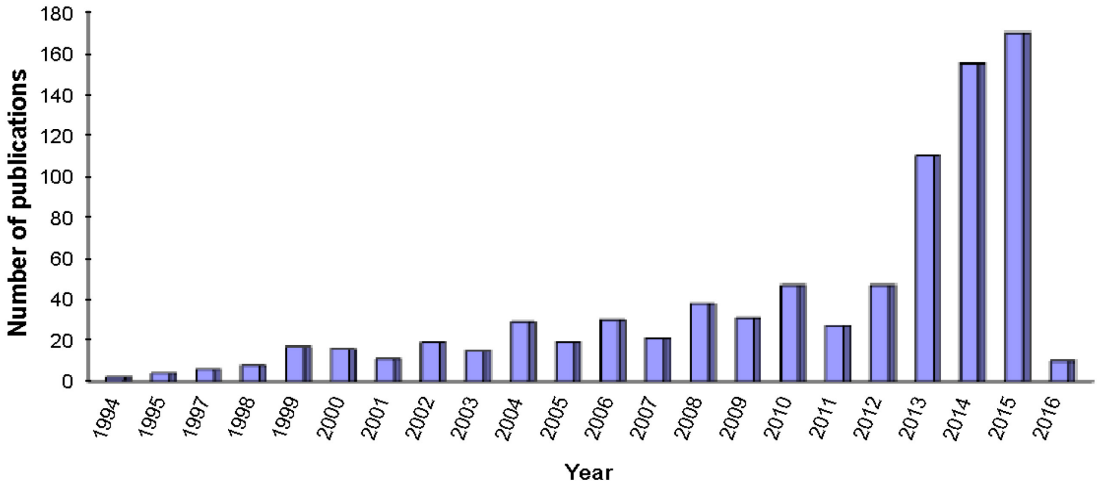


Figure 2.5 Number of scientific publications on subcritical water extraction (Total publications = 899, Source: Web of Science database 2015).

According to Figure 2.6a, Food Science and Technology is one of the most prominent areas of research on subcritical water utilization aside from the fields of Chemistry and Engineering. The extraction and production of phytochemicals have shown to be a mayor area of study. Several patents have been filed for potential use on food and pharmaceutical applications. Among them, the isolation of polyphenolic compounds from fruits and vegetables, production of essential oils from plant material and the extraction of phytochemicals from plant sources have been reported (King et al., 2010; Srinivas et al., 2011; de Melo et al., 2014; Saldaña & Valdivieso-Ramírez, 2015). Figure 2.6b depicts the current top 10 leading countries in terms of research on the application of subcritical water extraction.

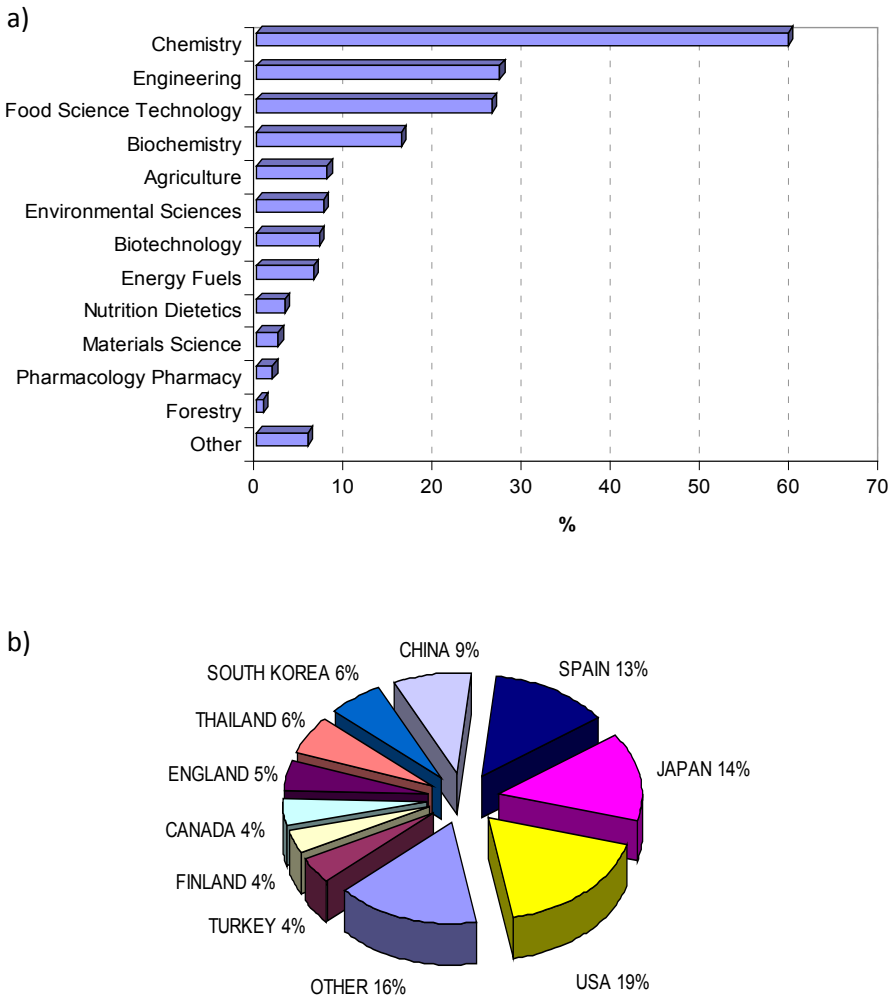


Figure 2.6 Graphical representation of subcritical water extraction by: a) research area and b) countries (Source: Web of Science database 2012).

Subcritical water or pressurized hot water is defined as water in the liquid phase under pressure (0.1-22 MPa) and temperatures higher than its boiling point (100°C) but below its critical temperature (374°C) (Figure 2.7). At subcritical conditions, the hydrogen-bonded lattice of water is disrupted and its physical and chemical properties are modified (King et al., 2003). Hence, in this condensed state, the diffusivity, permittivity, viscosity, density, solubility parameter, and surface tension of water can be tailored to induce solvency of a variety of compounds. For example, when water is heated up to

220˚C, its permittivity (ε) dramatically decreases and leads to a dielectric constant value close to select organic solvents (Table 2.3), favoring the extraction of non-polar chemicals. On the other hand, pressurized water at relatively low temperature (>100°C) induces the extraction of polar components (Sarkar et al., 2014). In addition, the use of pressurized fluids at the subcritical state, mainly water and water+ethanol, has shown to improve mass transfer rate, leading to short extraction times and high extraction yields compared to traditional solvent extraction (Mendiola et al., 2007; King et al., 2010; Herrero et al., 2013; Saldaña & Valdivieso-Ramírez, 2015).

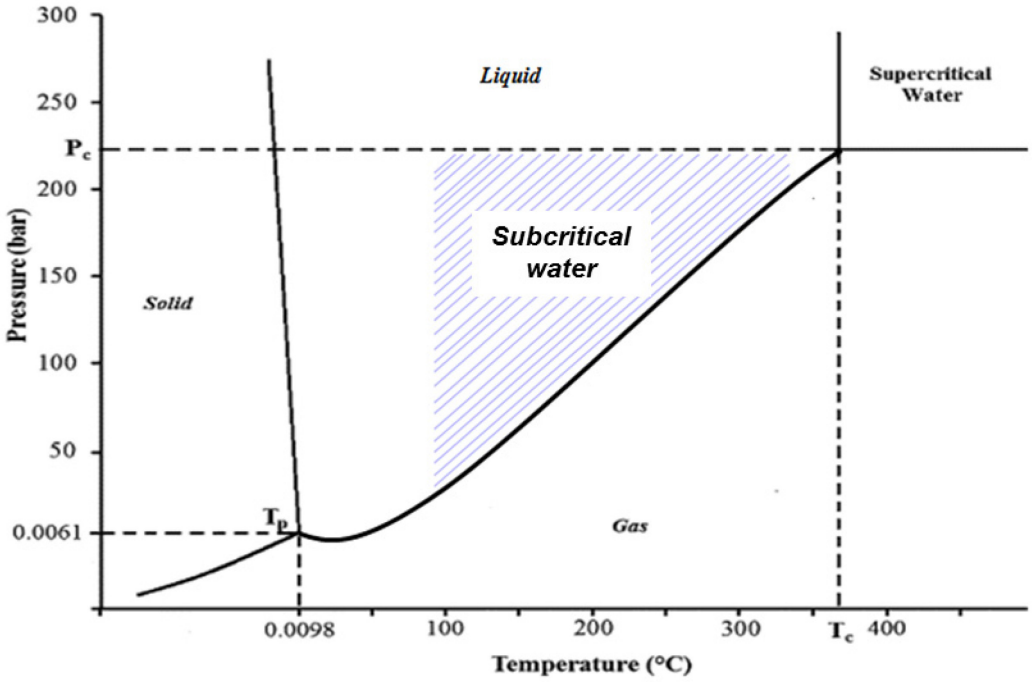


Figure 2.7 Phase diagram of water (T_c = critical temperature, 374°C; P_c = critical pressure, 221 bar; T_b = boiling point, 100°C).

Table 2.3 Physicochemical properties of water (The International Association for the Properties of Water and Steam, 2007)

Properties	Water	Subcritical water	Supercritical water	Superheated steam
	21°C 0.1 MPa	100-374°C 0.2-20 MPa	374-400°C 25-50 MPa	400°C 0.1 MPa
Density (kg/m^3)	1000	170-800	58-170	3
Dielectric constant (ε)	79	6-58	6-11	1
Ionic strength (pKw)	14	11-12	11-19	48

In general, subcritical water extraction is carried out within a high-pressure stainless steel apparatus. The main components of a subcritical water system are a water reservoir, a high-pressure pump to introduce water into the system, an oven for heating the aqueous media and, a stainless steel vessel where the extraction occurs. Associated components, such as controllers, manometers, a post extraction cooling system and, a collection flask for the extract are also required. A schematic of a typical subcritical water extraction system is shown in Figure 2.8.

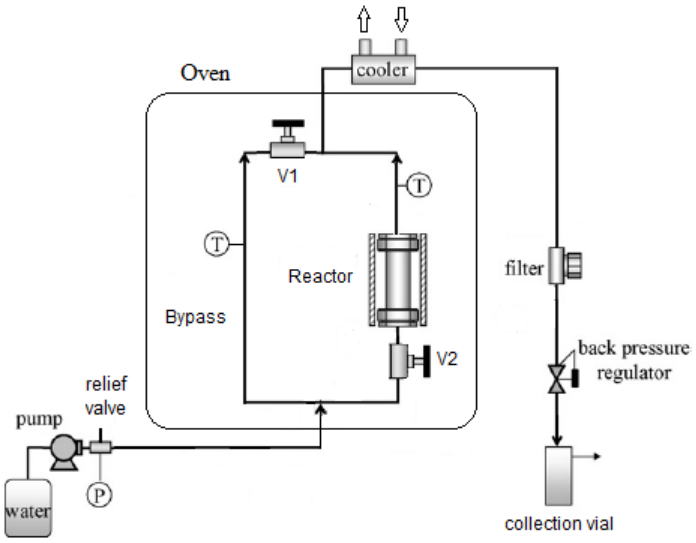


Figure 2.8 Typical subcritical water extraction system. P: pressure gauge, T: thermocouple, V: valve.

Although subcritical water technology has been used to obtain value-added products such as phenolics and polysaccharides from diverse agricultural biomass, there are only four studies reported on the extraction of pectin-containing substances under subcritical conditions. Ueno et al. (2008) were the first to isolate pectin from *Citrus junos* biomass with subcritical water. They recovered 80% of pectin with water at 160°C and 200 bar. Likewise, Wang et al. (2014) were able to extract pectin from citrus peel and apple pomace under subcritical conditions within 5 min. The highest pectin extraction yields for citrus peel (21.95, wt%) and applepomace (16.68%, wt%) were obtained at 120°C and 150°C, respectively. In addition, an ultrasound treatment followed by subcritical water extraction has also been reported to obtain pectic polysaccharides from sugar beet pulp (Chen et al., 2015). Under the reported optimal conditions (120°C, 107 bar and 30 min), the yield was 24.63%. Therefore, in this thesis, subcritical fluid systems will be further studied as a feasible method to obtain pectin with tailored physico-chemical properties suitable for biomedical applications.

2.5 Pectin sources and applications

2.5.1 Sources

As pectic polysaccharides are present in most dicotyledonous plants, their abundance in nature is quite high. Pectic polysaccharides concentrations of 0.1 to 30% have been found in the middle lamella of plant cell walls (Walter, 1991; Munarin et al., 2012). Various plant matrices, in particular, tropical fruits, have been studied as potential sources for food grade pectin manufacture (Table 2.4). Apple pomace and citrus peels, however, are currently used for commercial pectin production due to their high pectin content, high availability and final physico-chemical properties (i.e. water uptake, viscosity and gelling capacity) (Thakur et al., 1997; Munarin et al., 2012)

Table 2.4 Pectin sources (Adapted from Munarin et al., 2012; Yuliarti et al., 2015).

Source	Pectin Yield (%)	DE (%)
Apple pomace	2-19	22-80
Banana peel	2-12	49-80
Cacao pod husk	4-7	57
Carrots	7-9	34-58
Citrus fruits	6-26	56-80
Kiwifruit pomace	1.4-4.4	82-88
Guava peel	4-17	44-53
Passion fruit rind	4	10
Papaya peel	2-16	49-53
Sugar beet pulp	4-16	14-48

DE: degree of esterification, nr: no reported

The imperative need for biomass conversion and revalorization of agricultural co-products are mayor driven forces towards the utilization of non-traditional renewable sources for phytochemical production. As such, fruit residues like pomegranate rind could be further processed to obtain bioactive pectic polysaccharides.

Pomegranate *Punica granatum L.* is one of ancient fruits that has been used in traditional herbal medicine to treat and alleviate various ailments. The therapeutic and healing effects of pomegranate have been associated to its content of numerous phytochemicals. Recent studies have shown that polyphenols found in pomegranate juice can aid to prevent cardiovascular diseases, diabetes, Alzheimer's disease and cancer (Bhandary et al., 2012; Aviram & Rosenblat, 2013; U. S. Patent No. 20120276228, 2015). Among polyphenols, anthocyanins and anthoxanthins, and in particular punicalagin, have been associated to the strong antioxidant capacity of pomegranate juice. Also, pomegranate non-edible parts, rind and seeds, have shown potential bioactive properties. Hence, Aviram and Rosenblant (2013) reported that the extract of whole pomegranate fruit exhibited an increased antioxidant activity (20 fold) compared to pomegranate juice. Also, their *in vitro* and *in vivo* human and mice studies suggested that pomegranate co-products are promising sources of bioactive compounds to prevent lipid peroxidation and cardiovascular diseases (Aviram & Rosenblant, 2013).

In the last decade, pomegranate production, as an economic crop, has increased due to worldwide pomegranate juice demand. In 2007, the global pomegranate production was led by India (900.000 MT/year), Iran (800.000 MT/year) and the United States (110.000 MT/year) (USAID, 2008). As pomegranate juice content is about 40% of total fruit weight, the other 60% of the fruit is discarded as waste residue (Hasnaoui et al., 2014). Thus, the large amount of pomegranate biomass that is generated from juice production is a growing environmental and economic concern. Therefore, pomegranate biomass utilization for pectic polysaccharides and polyphenols production could be a

promising and economically attractive approach to facilitate its handling and final disposal.

2.5.2 Applications

Pectin has been widely used in diverse food applications, such as gelling, thickening and emulsifying agent. In recent years, however, it has been subject of study for pharmaceutical and biomedical applications due to its non-toxic, biocompatible and biodegradable properties.

As food ingredient, HM-pectin has been added to fruit-based products, such as jam and jellies to impart gel texture. Also, it has been used to enhance organoleptic properties, in particular mouthfeel, in a variety of fruit concentrates and beverages. On the other hand, LM-pectin has been added to non-fruit flavored products, such as chocolate fillings, confectionery, barbeque sausages and ketchup to enhance gel texture, mouthfeel, flavor release and the thixotropic behavior. Also, LM-pectin has shown to prevent protein aggregation at low pH and temperature, leading to more stable and long-shelf life acidified protein-based drinks (yogurt, milk-juice drinks and acidified soy beverages). Besides, LM-pectin has been used to enhance volume and moisture of bread as well as softness and freshness of frozen dough. Moreover, pectin was able to mimic a fat-like mouthfeel in low-fat and butter spreads, suggesting its potential use as fat replacement (CPKelco, 2005).

Also, current studies have shown that pectin exerts promising bioactive properties for disease prevention and treatment (Leclere et al., 2013; Ström et al., 2014; Mishra et al., 2014; Yapo & Gnakri, 2015). As dietary fiber, pectin is not digested in the small intestine but it is converted in the colon to short chain fatty acids (acetate, propionate and

butyrate), which has shown a boosting effect on immune functions as well as to prevent bacterial adhesion in the colon (Leclere et al., 2013). Also, pectin consumption has been associated to cardiovascular protection due to cholesterol reduction (diminishing either its absorption during digestion or its synthesis in the liver). In addition, pectin has shown to increase the viscosity of the chymus and, therefore delay glucose absorption and insulin production. Moreover, pectin has exhibited anti-tumor activity due to its binding effect to galectin-3 (protein involved in cancer), reducing its ability to promote cancer cell proliferation (Morris et al., 2011). On the other hand, pectin has been used as a detoxifying agent, in particular for metal poisoning, owing to its chelating effect. Likewise, pectin has shown to be effective for radio-isotope removal after radiation, as reported by Nesterenko et al. (2004). In their study, they found that a daily apple pectin intake of 10g per a time period of three weeks was able to reduce 62.6% of 137Cs (radio-isotope) load from Chernobyl children (Nesterenko al., 2004).

In addition, pectin-based materials, such as hydrogels, films, scaffolds and micro or nanoparticles, have been investigated for wound healing, tissue engineering and drug delivery (Bačáková et al., 2014; Mishra et al., 2014). As tissue repair and regeneration involve complex biological processes, biocompatible materials with non-toxic, non-mutagenic and non-immunogenic effect are required to induce and control cell adhesion, proliferation, differentiation and maturation (Bačáková et al., 2014). Coimbra et al. (2011), for example, obtained a biocompatible and non-cytotoxic scaffold of pectin and chitosan, in which human osteoblast cells were able to adhere and proliferate, suggesting potential applications for bone tissue engineering. Also, pectin gels have shown to control wounds exudates as well as to induce an acidic environment that protects tissue from

bacterial and virus infections (Munarim et al., 2012). Hence, treatment efficacy has been improved when antibiotics, anti-inflammatories or pain relievers were loaded into pectin hydrogels or scaffold systems (Mishra et al., 2012). Therefore, combining bioactive natural components, such as essential oils with pectin could lead to non-toxic bioactive materials that can be potentially used for food and biomedical applications (Benavides et al., 2012; Cutter, 2006).

2.6 Pectin gelation mechanism

It is generally accepted that the gelation mechanism of isolated pectins is governed by the esterification degree of homogalacturonan moieties (Yapo et al., 2015). This transition from liquid to gel suggests that pectin chains are highly cross-linked, forming a tangled and interconnected three-dimensional network that encloses substantial amounts of water and, possibly other polymers that are entrapped due to their smaller molecular size (Walter, 1991; Seymour & Knox, 2002). The network formation has mainly multiple lateral interactions between pectin chains (junction zones) rather than one-point linear associations (Seymour & Knox, 2002). There are particular conditions that induce gelation of either high-methoxyl pectin or low-methoxyl pectin. Gel formation of high-methoxyl pectins is favored at low pH (2.8-3.4) and a high concentration of soluble solids (mostly sucrose > 65%). Under those conditions, hydrophobic interactions between methyl groups as well as hydrogen bonding of the hydroxyl groups are increased. Gelation mechanism of low-methoxyl pectin, however, has been represented by the *egg-box* model (Yapo & Koffi, 2013). As such, junction zones are formed due to ionic cross-linking between deprotonated carboxylate ion groups (COO^-) of homogalacturonan moieties and calcium (Ca^{+2}), which are then strongly stabilized by hydrogen bonding, van

der Waals and electrostatic interactions as depicted in Figure 2.9 (Cabrera et al., 2008). Therefore, the strength and stability of formed egg-like boxes are related to the occurrence and distribution of methyl ester groups within the homogalacturonan moiety. In addition, the degree of esterification determines whether a pectin gel is thermally reversible or not. HM-pectin gels, in general, exhibit a thermo-reversible behavior where gel can be set, melted and set again, conversely, LM-pectin gels are not thermally reversible.

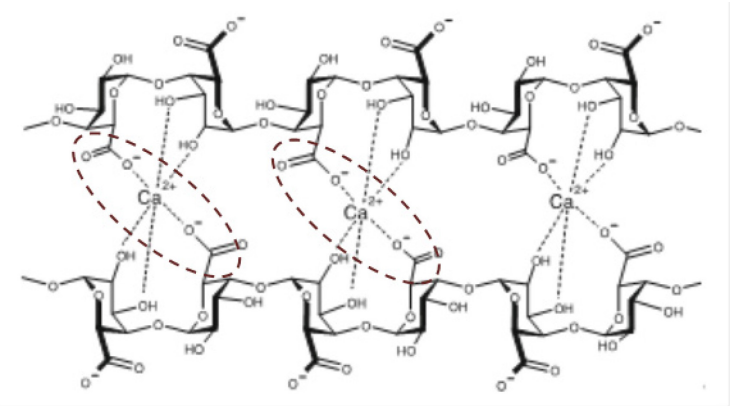


Figure 2.9 Scheme of the egg-box mechanism (adapted from Cabrera et al. 2008).

2.7 Pectin-based gels formation

In general, the *sol-gel* method has been used to produce gels from either natural or synthetic precursors. During this process, a stable suspension of colloidal particles in a liquid (*sol*) is obtained prior to *gel* formation (Schubert, 2015). Then, physical or chemical cross-link promoters are used to form a three-dimensional solid network surrounded by a continuous liquid phase (*gel*) (García-González et al., 2011; Schubert, 2015). Finally, the solvent of the resulting wet *gel* is removed by different drying methods to obtain materials with 2D (films) or 3D structures (aerogels or xerogels). Aerogels are formed when the solvent is removed by supercritical drying, while xerogels

are obtained by air-drying (García-González et al., 2011). Also, freeze-drying has been reported to manufacture highly porous materials, mainly scaffolds for tissue engineering applications. Such process involves a fast cooling of wet *gel* and further solvent removal by water sublimation, leading to cryogel formation (U. S. Patent No. 19980303, 1998).

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Chapter 3: Extraction of novel pectic polysaccharides from pomegranate *Punica granatum L.* biomass using subcritical fluids

3.1 Introduction

In recent years, subcritical water technology has emerged as a promising and ecologically attractive alternative for biomass conversion (King et al., 2010). The extraction of various bioactive compounds, such as polyphenols, anthocyanins, flavonoids and anthraquinones from agricultural residues (i.e. fruit peels and leaves, cereals husk and straw and sugarcane bagasse) using subcritical water or a water/ethanol system has been reported (King et al., 2010; Srinivas et al., 2011; Saldaña & Valdivieso-Ramírez, 2015). Although subcritical water extraction has led to diverse biomass utilization, there is numerous agricultural residues that could be explored for phytochemical production.

Among phytochemicals, pectic polysaccharides have been subject of scientific interest due to its wide range of applications. Pectic polysaccharides or pectin has been extensible used as a functional food ingredient to enhance texture, flavor and stability (CPKelco, 2005). Also, they have been used in nutraceuticals for detoxification, cholesterol reduction and weight management (Mishra et al., 2012). Hence, owing to its non-toxic, biocompatible and biodegradable properties, they have been considered for potential biomedical applications, such as wound dressings, scaffolds for cartilage and bone regeneration and, drug delivery systems (Mishra et al., 2012).

As pectic polysaccharides or pectin is commercially produced by acid hydrolysis, there are some disadvantages associated to acid waste generation, extended extraction times and long purification procedures. Thus, environment-friendly extraction

technologies are still required for pectin manufacture. In addition, such technologies could facilitate pectin production from various agricultural residues a part from traditional citrus peel and apple pomace. Hence, considering that the gradual shift in consumer's preferences towards health eating has increased the demand of fruit beverages with nutritional and medicinal value, non-traditional fruits (i.e. pomegranate) are being processed, increasing biomass availability.

In the last years, pomegranate *Punica granatum* production and utilization has increased because of its associated health benefits (antioxidant and anti-inflammatory effects) (Lansky & Newman, 2007). The current exporting marketing countries are India, Iran, Turkey and the United States. In 2007, around 2 000 000 MT were produced (USAID, 2008). As pomegranate juice content is about 30-42% of the fruit weight, seeds (3%) and rinds (65%) are commonly discarded. Therefore, pomegranate industrial residues, mainly the exocarp and albedo (Figure 3.1) are potential source of bioactive compounds, such as pectic polysaccharides and phenolics.

The objective of this study was to isolate for the first time pectic polysaccharides from pomegranate biomass using food grade and GRAS pressurized fluid systems. The parameters evaluated were the solvent system (water, citric acid/water and ethanol/water), temperature (100 to 140˚C) and extraction time (5 to 30 min). The responses were pectic polysaccharides yield, galacturonic acid content and degree of esterification. The functional properties of isolated pomegranate pectic polysaccharides (total phenolics content and inhibition %DPPH) were also assessed for potential biomedical and nutraceutical applications.

3.2 Materials and Methods

3.2.1 Materials

Ripen pomegranate *Punica granatum L.* fruits (La Calera-Peru) were bought from a local market (Superstore, Edmonton, AB, Canada). The fruits were water cleaned, cut opened and the arils taken apart. The resulting biomass (exocarp, mesocarp or albedo and inner carpellary membranes) shown in Figure 3.1 was frozen at -18˚C for 24 h and then freeze-dried. A centrifugal mill (Model ZM 200, Retsch, USA) was used to obtain homogeneous pomegranate biomass with a particle size of 1mm. The dry powder pomegranate biomass (PGb) was stored in sealed plastic bags at -18˚C for further use in the extractions.

Glass beads with a diameter of 22 mm were obtained from Fisher Scientific (Ottawa, ON, Canada). Chemical reagents and standards, such as D-galacturonic acid, polygalacturonic acid, citric acid, hydrochloric acid, sulfuric acid, glacial acetic acid (99.7%), ammonium hydroxide, 1,1-Diphenyl-2-picryl-hydazyl (DPPH, 99.9%), Folin-Ciocalteu' phenol reagent, and esterified citrus pectin were acquired from Sigma Aldrich (Oakville, ON, Canada). Distilled and de-gassed water from the Milli-Q system (Millipore, Bellerica, MA, USA) were used in these experiments.

A D-Glucuronic/D-Galacturonic kit assay from Megazyme (Megazyme International, Bray, Wicklow, Ireland) was used for galacturonic acid quantification.

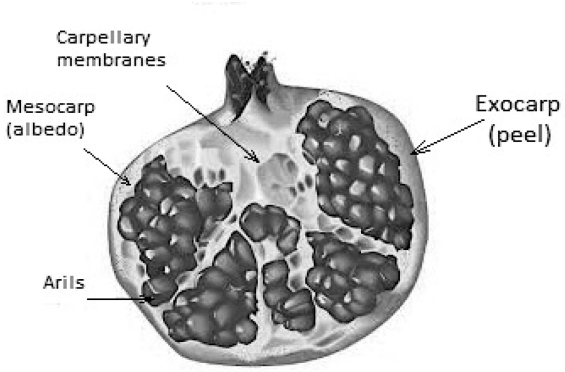

Figure 3.1 Pomegranate fruit anatomy.

3.2.2 Proximate compositional analysis

The contents of protein, fat, moisture, ash and total carbohydrates of PGb were determined. Protein content was estimated using a Leco TruSpec nitrogen analyser (Leco Instruments Ltd., Mississauga, ON, Canada). Corn and rye starch standards were run for calibration. Crude protein content was calculated using Equation 3.1 below:

$$Crude\ protein\ (\%) = 6.25 \times nitrogen\ content\ (\%) \qquad 3.1$$

Total fat content was analyzed according to the AOAC official method using a Goldfisch extraction unit (Labconco Co., Kansas, MO, USA). Total fat content was determined based on the following equation:

$$Fat\ content\ (\%) = \frac{mass\ of\ extracted\ fat}{mass\ of\ dry\ PGb} \times 100 \qquad 3.2$$

The moisture content was determined by a thermogravimetric method, which was performed within a moisture analyzer (Model MA45, Sartorius, IL, USA) that records the weight loss of the sample throughout the drying process.

The ash content was measured according to the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2005).

The total carbohydrate content of PGb was calculated by difference using Equation 3.3:

$$Total\ carbohydrates(\%) = 100\% - [protein(\%) + fat(\%) + moisture(\%) + ash(\%)] \quad 3.3$$

3.2.3 Pectic polysaccharides extraction

Pectic polysaccharides (PPs) were sequentially removed from pomegranate biomass (PGb) using Milli-Q water and a solution of hydrochloric acid 50 μM as solvents. The conventional method described by Pinheiro et al. (2008) and Georgiev et al. (2012) with some modifications was used as the extraction control for comparison and it is shown in **Figure 3.2**. Briefly, 4 g of PGb was mixed with 100 g of Milli-Q water, heated up to 94˚C and kept under constant stirring for 90 min. The water soluble PPs were removed from the suspension after centrifugation at 6500 rpm for 15 min. Then, ethanol (96%) at 4˚C was added to *supernatant A* (2:1 v/v ratio) to induce coagulation and separation of PPs from the solution. After 2 h, ethanol-insoluble PPs aggregates were concentrated by centrifugation at 6500 rpm for 15 min. The PPs aggregates were washed with acidified ethanol (0.5% citric acid) at 4˚C and centrifuged at 6500 rpm for 15 min to remove low molecular weight saccharides and minerals. Purified PPs were then rehydrated in 20 mL of Milli-Q water prior to freeze-drying, which was carried out for 3 days.

Water-insoluble solids (*residue A*) were further treated with 100 mL of 50 μM HCl for 60 min to extract acid-soluble PPs (*supernatant B*). PPs from the acid-soluble fraction were obtained following the procedure described in Figure 3.2 *precipitation B*, which involves precipitation, centrifugation, washing and freeze-drying processes.

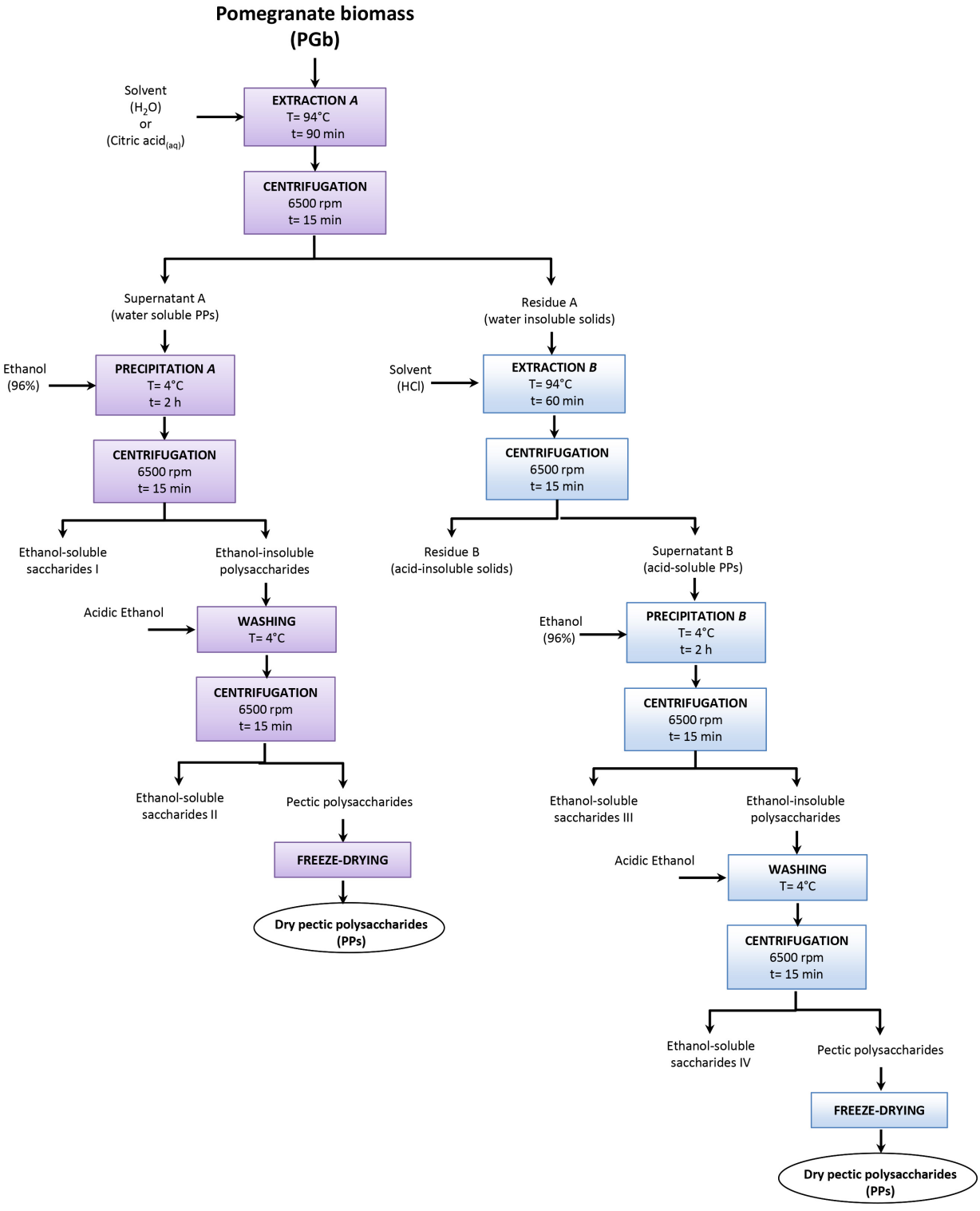

Figure 3.2 Flow-diagram for the sequential extraction of pectic polysaccharides from pomegranate biomass. A: aqueous extraction, B: acidic extraction. T: temperature and t: time.

3.2.3.1 Sequential acid extraction

The same extraction procedure described in Section 3.2.3 was followed. A two-step sequential extraction process was carried out to determine the pectic polysaccharides content from selected pomegranate biomass. Treatments I (MilliQ water and hydrochloric acid 50 μM) and, II (aqueous citric acid 50 μM and hydrochloric acid 50 μM) were used as extraction controls for comparison. The extraction parameters of PPs are shown in Table 3.1.

Table 3.1 Sequential extraction parameters of pomegranate pectic polysaccharides.

Treatment	Extraction parameters				
	Solvents	**Temperature (°C)**	**Pressure (bar)**	**Time (min)**	**Ratio (PGb/solvent, w/w)**
I	[a]H_2O	94	1	90	1/25
	[b]$HClb_{(aq)}$			60	
II	[a]Citric $acid_{(aq)}$	94	1	90 60	1/25
	[b]$HCl_{(aq)}$				

[a]: first step of sequential extraction; [b]: second step of sequential extraction.

3.2.3.2 Subcritical fluid extraction

Preliminary studies were performed on pectic polysaccharides extraction from various biomasses using subcritical water. As such, pectic polysaccharides were extracted from banana peel, cacao husk and pomegranate residues with pressurized fluids. Also, the effect of temperature (140 and 160˚C), solvent system (water, ethanol/water (20/80 v/v) and ethanol (96%)), pressure (50, 70 and 100 bar), flow rate (5 and 10 mL/min), PGb:glass bead ratio (2:35, 1:25 and 1:30, w/w) and extraction time (30 to 60 min) were evaluated following the algorithm presented in Figure 3.3. The high load of glass beads favored mass transfer. The responses for those variables were yield of PPs and galacturonic acid content (Table 3.4).

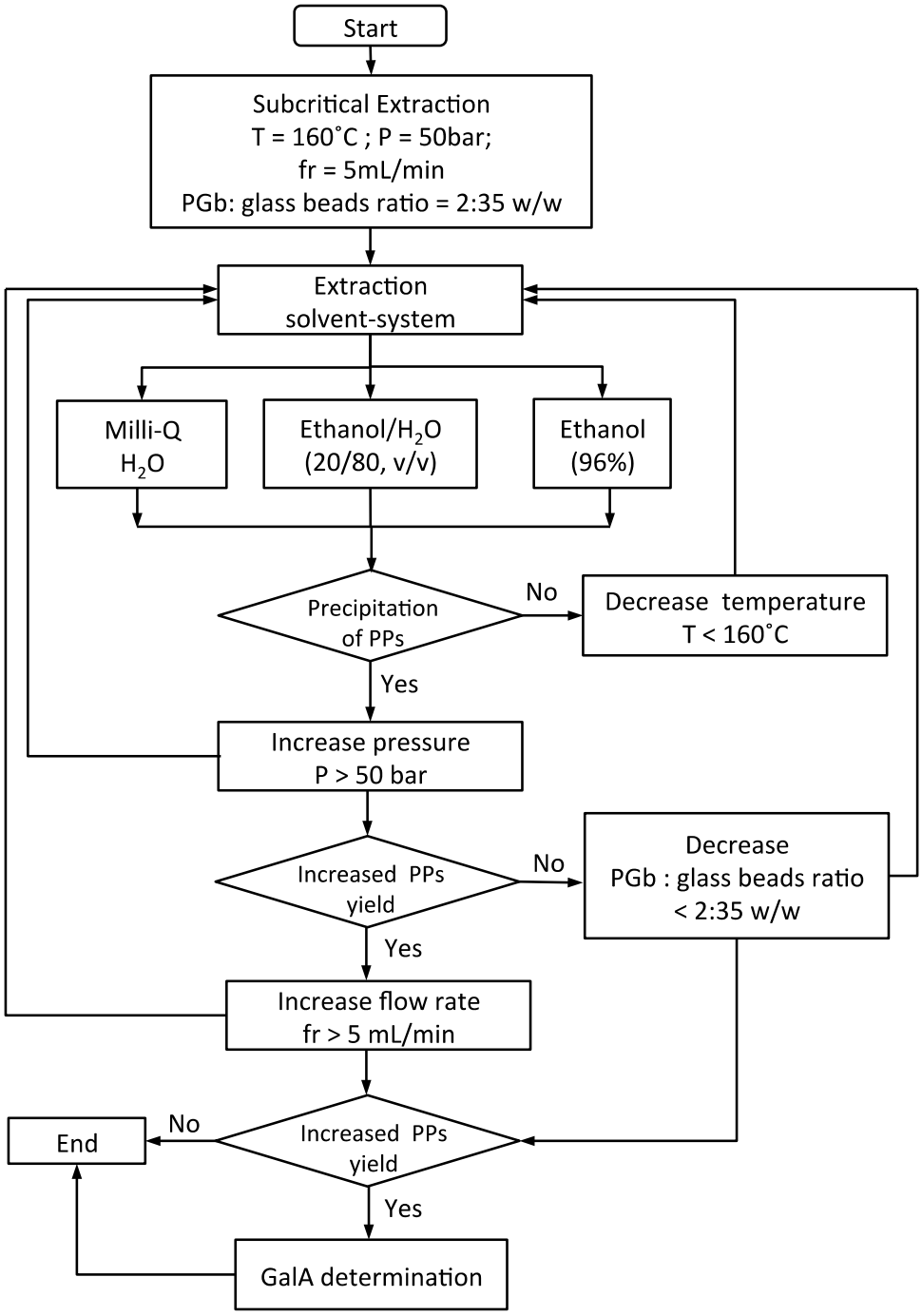

Figure 3.3 Algorithm for preliminary studies conducted on PPs extraction using subcritical fluids. T: temperature, P: pressure, fr: flow rate, PPs: pomegranate pectic polysaccharides and, GalA: galacturonic acid content.

Based on these preliminary studies, pomegranate biomass was selected for further PPs extraction and characterization because of its high pectic polysaccharides yield. In addition, variables such as pressure, flow rate and extraction time did not have influence on extraction yield and were set constant at 50 bar, 5 mL/min and 30 min, respectively (Brunner, 2009). The process parameters further evaluated were the binary solvent system (water, citric acid/water (0.2/100, w/w) and ethanol/water (20/80, v/v)) and temperature (100 to 140˚C).

The extraction process was carried out within a dynamic high-pressure extraction unit described by Singh and Saldaña (2011), with some modifications for fast heating and control (Figure 3.4). Basically, the unit was comprised of a water reservoir, a high-pressure pump (Model 305 pump, GILSON Inc, Guelph, ON, Canada), a convection oven (ED 115, BINDER, USA), a stainless steel vessel (2.54 cm O.D. x 10 cm length) with a heating band (Trutemp, Edmonton, AB, Canada), two K-type thermocouples (Trutemp, Edmonton, AB, Canada), a temperature controller (Thermomart, Toronto, ON, Canada), a digital pressure gauge (DPI 104, GE-Druck, Calgary, AB, Canada), a pressure relief valve setting (RVP, Parker Autoclave Engineers, Erie, PA, USA), a back pressure regulator (26-1700 Series, TESCOM, Elk River, MN, USA) and a cooling system (Swagelok Valve and Fitting Inc, Edmonton, AB, Canada).

In a typical experiment, first, powdered PGb and glass beads were loaded into the stainless steel reactor according to the experimental design (Table 3.5). Then, it was capped with sintered stainless steel porous discs (40μm) to avoid any clogging of the system. Stainless steel union reducing fittings (Swagelok Valve & Fitting Inc, Edmonton,

AB, Canada) were used to assemble and close the reactor. Milli-Q water or the selected solvent-system was then pumped from the reservoir into the reactor to reach the set temperature and pressure. Once the equilibrium was achieved, extracts of 50 mL each were collected every 10 min for 30 min. Then, the extracts were precipitated and freeze-dried according to the flowchart shown in Figure 3.5.

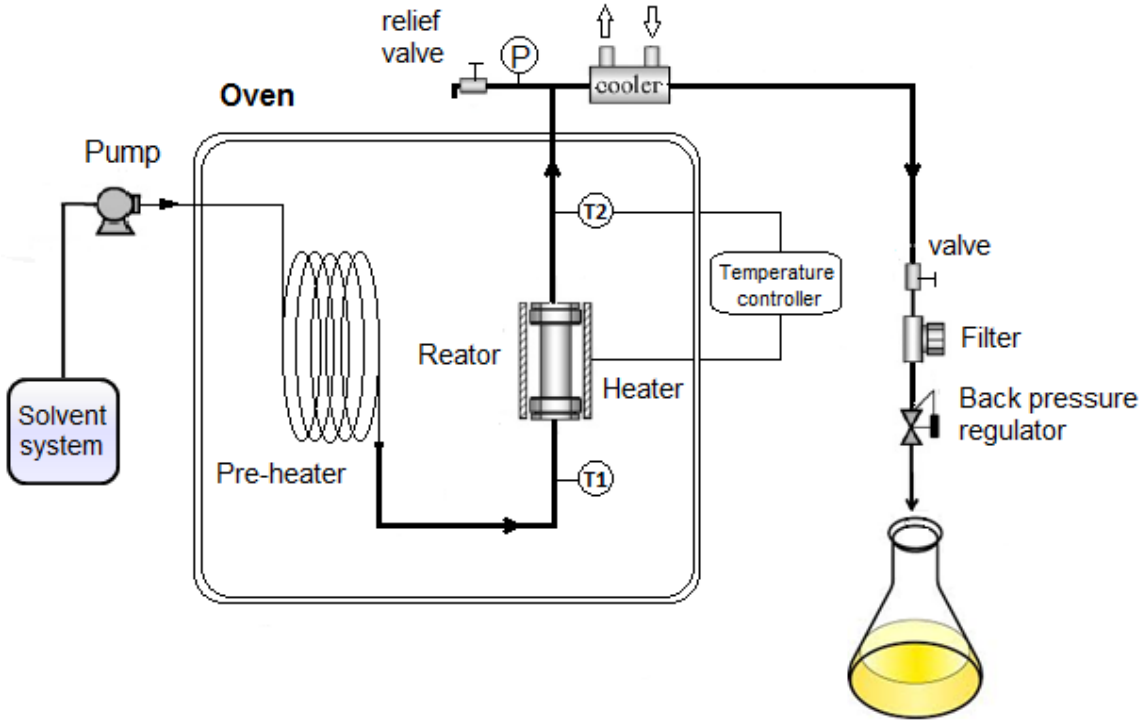

Figure 3.4 Semi-continuous subcritical fluid unit. T1 and T2: thermocouples, P: pressure gauge.

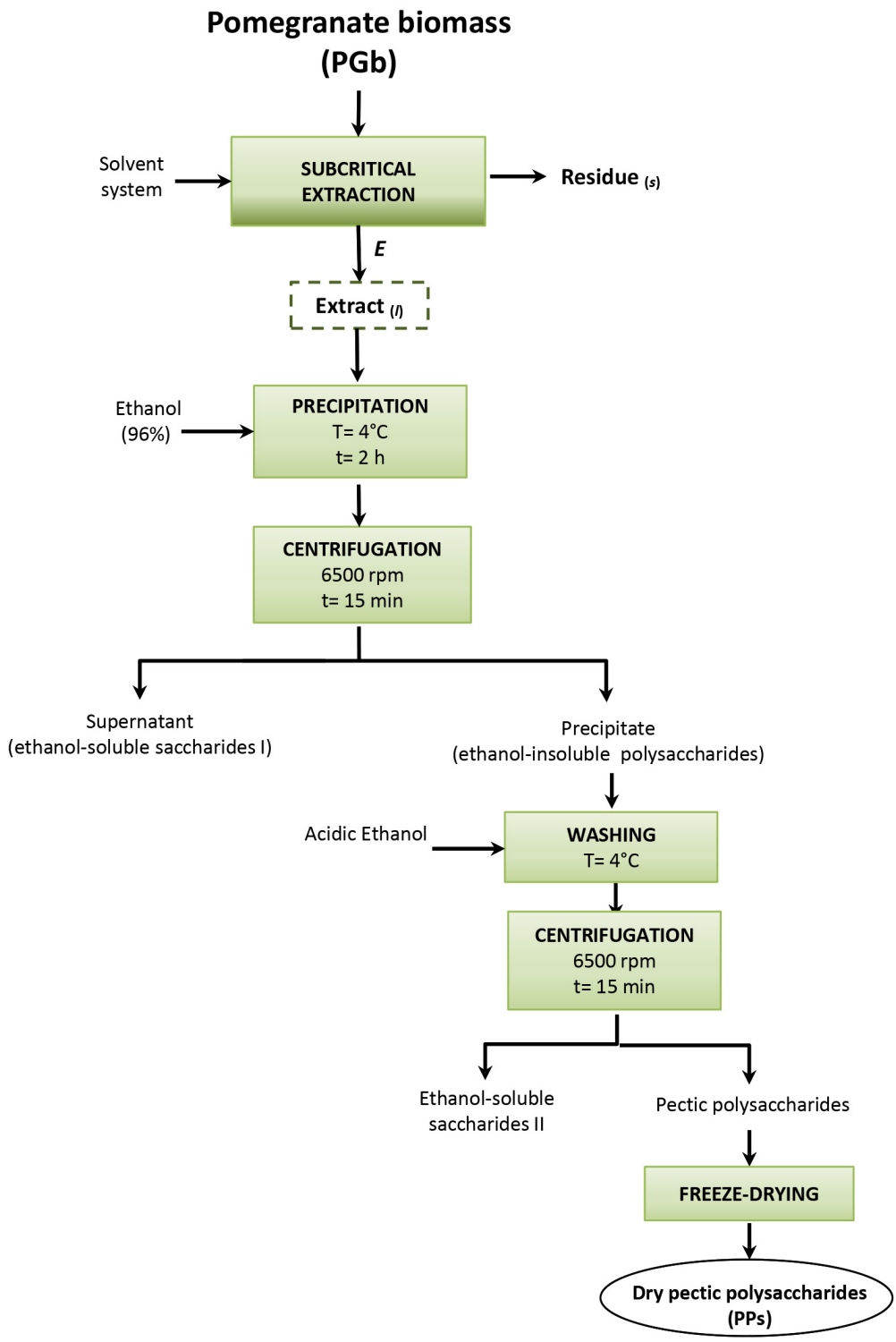

Figure 3.5 Flowchart for pomegranate pectic polysaccharides extraction using subcritical fluids.

3.2.4 Pectic polysaccharides yield

The yield of the extracted pectic polysaccharides was determined based on weight of freeze-dried ethanol-insoluble solids and the initial weight of dry PGb, as previously reported by Wang et al. (2014) and represented by Equation 3.4:

$$Yield\ (\%) = \frac{mass\ of\ alcohol\ insoluble\ solids}{mass\ of\ dry\ PGb} \times 100 \qquad 3.4$$

3.2.5 Pectic polysaccharides characterization

3.2.5.1 Degree of esterification

The degree of esterification (DE) of pectic polysaccharides was determined by titrimetric analysis as described by Pinheiro et al. (2008). Briefly, 100 mg of freeze-dried PPs was dispersed in 20 mL of Milli-Q water at 40˚C for 2 h under constant stirring. A 0.1 M NaOH solution was used to determine the carboxylate ionate groups of galacturonic acid in the sample. The volume of NaOH used to reach the end point for the titration (pH 7) was recorded as the initial titre (V_1). Then, 5 mL of 0.1 M NaOH was used to induce ester hydrolysis. After 2 h reaction under constant stirring, 5 mL of 0.1 M HCl was added and excess HCl was determined by titration with 0.1 M NaOH. The volume of NaOH was recorded as the saponification titre (V_2). The degree of esterification was calculated by Equation 3.5:

$$\%DE = \frac{V_2}{V_1 + V_2} \times 100 \qquad 3.5$$

3.2.5.2 Galacturonic acid content

The galacturonic acid (GalA) content of extracted PPs was assessed using a commercial D-Glucuronic/D-Galacturonic assay from Megazyme (Megazyme International, Bray, Wicklow, Ireland) as well as high-performance anion exchange chromatography using pulsed amperometric detection (HPAEC-PAD).

Acid hydrolysis of extracted PPs was carried out according to methodology of Emaga et al. (2012) prior to GalA determination. As such, 25 mg of PPs were hydrolyzed with 25 mL of 2 M H_2SO_4 at 94°C for 2h under constant stirring. Then, the reaction was stopped and neutralized with a solution of 7M NH_4OH to pH 7. The resulting solution was diluted up to 25 mL with Milli-Q water, centrifuged at 6000 rpm for 12 min and, filtered through a 0.22 μm Millipore membrane.

Hydrolyzed PPs were treated with uronate dehydrogenase (UDH) at 25°C for 10 min in presence of nicotinamide-adenine dinucleotide (NAD^+) to oxidize D-galacturonic acid to D-galactarate and, induce nicotinamide-adenine dinucleotide (NADH) formation. As the reduction of NAD^+ is stoichiometric with respect to D-galacturonic acid content, NADH was quantified at absorbance of 340 nm. The analysis was done in quadruplicates.

HPAEC-PAD analysis of GalA was performed using a Dionex DX-500 chromatographic system (Dionex Corp., Sunnyvale, CA, USA), following the method described by Di Cagno et al. (2006). Briefly, hydrolyzed PPs solutions (10 μL) were injected on a CarboPac PA-10 column (2 mm × 250 mm). Sodium hydroxide and sodium acetate solutions with different gradient elution sequences or patterns were used as mobile phases. Standard solutions of GalA from 0.25 to 7.5 g/L were prepared for quantification.

In addition, GalA content was indirectly determined using the titration volumes (V_1 and V_2) obtained from the previous degree of esterification assay (Section 3.2.5.1). Galacturonic acid content was calculated according to Equation 3.6:

$$\%\ GalA\ acid = 19.41 \times (V_1 + V_2) \qquad 3.6$$

3.2.5.3 Fourier transform infrared (FT-IR) spectroscopy analysis

The infrared absorption spectrum of freeze-dried pomegranate polysaccharides were collected using a Nicolet 8700 Fourier Transform Infrared Spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with Omnic software (version 7.1) and a Smart Speculator for Attenuated Total Refllection (ATR germanium crystal cell). The spectra was recorded from 350 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 128 scans.

3.2.5.4 Total phenolic content

Total phenolic content of freeze-dried pomegranate pectic polysaccharides as well as subcritical fluid extracts before precipitation was determined by colorimetric method using Folin-Ciocalteau reagent as described by Sarkar et al. (2014). Pomegranate pectic polysaccharides solutions with known concentration of 2mg/mL were prepared to measure the TPc. Liquid extracts were diluted with Milli-Q water (1/25 v/v) prior to analysis. Briefly, 40 μL of sample was dispersed in 3160 μL of Milli-Q water and then 200 μL of Folin-Ciocalteau reagent was added. The solution was vortex and let to react for 6 min. Saturated calcium carbonate solution (600 μL) was then added and vortex. The samples were incubated in darkness for 2 h. Finally, the absorbance of samples was measured at 765 nm within 1.5 mL plastic cuvettes in a spectrophotometer (6320D, Jenway, Bibby Scientific Ltd, Dunmow, Essex, UK). Standard solutions of gallic acid

were used for quantification purposes. All measurements were done in triplicates, including the blank.

3.2.5.4 Antioxidant activity

Total antioxidant activitynwas measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay as described by Wang et al. (2014). Pomegranate pectic polysaccharides solutions of 2mg/mL were prepared for the analysis. A sample of 100 μL was mixed with 900 μL of acetic acid buffer (0.1 M, pH 5.5) to adjust pH from 3.1 to 5.5. Then, 2 mL of 105 μM DPPH ethanolic solution was added, vortex for 10 s and, let to react in darkness for 2 h. The absorbance of samples was measured at 517 nm in a spectrophotometer (6320D, Jenway, Bibby Scientific Ltd, Dunmow, Essex, UK). The essay was performed in triplicates and the inhibition DPPH (%) was calculated according to the Equation:

$$Inhibition\ DPPH\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad 3.7$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

3.2.5.5 Electrical conductivity and pH determination

The electrical conductivity and pH of subcritical fluid extracts (fractions obtained at 10, 20 and 30 min) were determined at room temperature (23°C) within a pH/Conductivity meter (Fisher Scientific Accumet, Ottawa, ON, Canada). The cell conductivity constant was k=1 and, the uncertainties for the conductivity, pH and temperature measurements were ±10 µS/cm, pH±0.02 and ±0.1°C, respectively.

3.2.6 Statistical analysis

Subcritical fluid extractions at established operating conditions were performed in duplicate and, the analytical tests for pomegranate pectic polysaccharides characterization were done in triplicates. The effect of binary solvent systems and extraction temperature under subcritical fluid conditions on yield, degree of esterification, galacturonic acid content, total phenolic content and antioxidant activity of isolated pomegranate pectic polysaccharides were evaluated using a two-way analysis of variance (ANOVA). A significant difference between the treatments of $\alpha = 0.05$ was used. Differences between means were assessed by Tukey´s multiple range test ($p<0.05$) using Minitab software package v.17 (Minitab Inc., State Collegue, PA, USA).

3.3 Results and Discussion

3.3.1 Proximate composition analysis

Table 3.2 shows the proximate composition analysis of pomegranate biomass composed of exocarp, mesocarp and inner carpellary membranes. Total carbohydrates (45±2%) are the predominant component of freeze-dry pomegranate biomass (PGb). Other components are protein (5.61±0.03%), fat (0.68±0.08%) and ash (4.0±0.1 %).

The composition of pomegranate peel (exocarp-leathery rind) powder was reported by Ullah et al. (2012). Higher contents of protein, ash and fat were found in pomegranate peel powder compared to the freeze-dried pomegranate biomass. The differences between reported values are related to the different composition of the samples. Owing to the functionality of the exocarp that is protecting the fruit from pathogens and environmental stress, amino acids and essential oils can be found as part

of its structure. Thus, fat and protein contents could be concentrated in such a structure rather than in the mesocarp or inner carpellary membranes.

On the other hand, PGb had higher content of total carbohydrates compared to pomegranate peel powder probably due to the presence of mesocarp and inner carpellary membranes (spongy tissues where arils are attached). Further, Hasnaoui et al. (2014) found that pomegranate genotype and different environmental conditions led to pomegranate residues (exocarp + mesocarp) with different compositions. Among the 12 varieties studied, reported total dietary fiber, cellulose and lignin contents ranged from 33.10 to 62.09, 5.85 to 12.83 and 9.48 to 24.21 %, respectively. In addition, pomegranate peel has phenolic acids (i.e. vanillic, ferulic and syringic acids) and hydrolysable tannins (i.e. punicalagin, punicalin, gallagic acid and ellagic acid) that suggest its potential antioxidant, antibacterial and antifungal activities (Mushtaq et al., 2015; Aguillar et al., 2008; Ullah et al., 2012). Therefore, pomegranate residues are considered to be an important source for biomass conversion into value added products like soluble complex polysaccharides (pectin) and bioactive compounds (phenolic acids).

Table 3.2 Proximate composition analysis of pomegranate biomass.

Component	**% (w/w) dry basis**	
	Present study	**Ullah et al. (2012)**
Protein	5.61±0.03	8.7±0.1
Fat	0.68±0.08	5.00±0.14
Ash	4.0±0.1	9.4±0.1
Moisture	45±2*	4.00±0.22
Total carbohytrates	45±2	31.4±0.30

* % (w/w) wet basis.

3.3.2 Pectic polysaccharides yield

Although solvent systems, such as hot aqueous hydrochloric acid, sulfuric acid or nitric acid solutions with pH from 1.2 to 2.5 are commonly used for commercial citrus pectin manufacture (CPKelco, 2005; Pagan et al., 2001), alternative GRAS and environmental-friendly solvent systems have been explored to increase the extraction yield and overcome shortcomings associated with acid waste generation, handling and disposal (Naghshineh et al., 2013; Wang et al., 2014; Chen et al., 2015). Among those solvents, citric acid has shown to be a potential pectin extractant over mineral (H_2SO4, HCl) and organic acids. As such, Canteri-Schemin et al. (2005) used different extractants (i.e. malic, tartaric, citric, phosphoric, sulfuric, hydrochloric and nitric acids) to isolate pectin from apple pomace. The highest yield reported (13.8 %) was obtained when pectin was extracted with citric acid (6.2 % w/w) at 97°C for 150 min. Also, similar pectin extraction yields have been reported when fruit matrices (e.g. cacao husk and grape pomace) were treated with both, citric acid and hydrochloric acid solutions (Vriesmann et al, 2012); (Minjares-Fuentes et al., 2014). Hence, Rovaris Pinheiro et al. (2008) used citric acid (0.086% w/v) at 97°C for 60 min to obtain pectin from passion fruit peel with a degree of esterification (DE 78.6%), comparable to a hydrochloric acid extracted pectin (DE 73.2%).

Based on the above studies conducted on pectin extraction, aqueous citric acid 0.2% w/w (pH 2.6) was selected for further studies at subcritical water conditions as a solvent system for pectic polysaccharides extraction from pomegranate biomass.

Table 3.3 shows the results of pomegranate pectic polysaccharides yield obtained by the sequential extraction process (refer to Table 3.1). The total pectic polysaccharides

yield in pomegranate biomass ranged from 8.30±0.01 to 8.50±0.03g/100 g dry PGb, which is comparable to pectin yields obtained from banana peel (2-15%), cacao pod husk (4-11%), sugar beet pulp (4-16%) and sunflower (10-11%) yields (Munarin et al., 2012). However, the yield of PPs was low compared to apple (2-19%) and citrus fruits (6-26%) pectin yields (Munarin et al., 2012). Although there was no significant difference between water-soluble and citric acid-soluble PPs yields, treatment II led to the highest total pectic yield (8.50±0.03%). Thus, acid hydrolysis via aqueous citric acid could induce disruption of PGb matrix, exposing a higher surface area for $HCl_{(ac)}$ attack. In addition, Umeda et al. (2009) reported that a combination of diluted citric acid solutions (1-5%) and temperatures (25-80°C) enhanced hydrolysis of rice husk polysaccharides as well as the removal of metallic impurities by chelation reaction between citric acid carboxylate ion (COO^-) and ionized cations, such as Na^+, K^+ and Ca^{++}. Therefore, aqueous citric acid could favour the removal of calcium-linked pectic polysaccharides from pomegranate biomass (Manrique & Lajolo, 2002)

On the other hand, during the sequential extraction of PPs, the majority of pectic polysaccharides (77.6%) were extracted with either water or aqueous citric acid but not with hydrochloric acid (22.4%), revealing that pomegranate pectic polysaccharides might be linked to other cell wall components such as hemicellulose, phenolics and proteins in a particular fashion (Perez et al., 2003), rather than strongly attached to cellulosic materials by hydrogen bonds as proposed by Ueno et al. (2008). In fact, Ueno et al. (2008) reported that aqueous hydrochloric acid (50 μM) extracted the highest amount of pectic polysaccharides (72%) from *Citrus junos* compared to water (0.95%) or sodium hexametaphosphate (5.17%), suggesting that pectic polysaccharides from *Citrus junos*

were strongly hydrogen bonded to cellulose. As pectic polysaccharides are very dynamic cell wall components that change according to plant development and environmental conditions as well as from one tissue to another, their structure and solubility can be particular for selected plant or tissue, leading to a different pectin fragments. Thus far, the performed sequential extraction process showed that the structure of pomegranate biomass (mainly pericarp) differs from *Citrus junos* biomass and, pectic polysaccharides with different structural and functional properties should be expected (Seymour & Knox, 2002).

Table 3.3 Pomegranate pectic polysaccharides sequential extraction yield.

	Fraction yield (%)			Total yield (%)
	H_2O	**Citric Ac.**	**HCl**	
Treatment I	6.3 ± 0.3^{A}		2.0 ± 0.3	8.30 ± 0.01^{b}
Treatment II		6.60 ± 0.02^{A}	1.90 ± 0.01	8.50 ± 0.03^{a}

$^{A, a, b}$ Means in a column with different superscripts indicate significant difference ($p < 0.05$). Capital letters for solvents and, small letters for treatments.

Besides the sequential extraction process, subcritical water technology was investigated as an environment-friendly and sustainable alternative for pomegranate pectin extraction. Thus, the effect of temperature (140 and 160˚C), solvent system (water, ethanol/water (20/80 v/v) and ethanol 96%), pressure (50, 70 and 100 bar), flow rate (5 and 10 mL), PGb:glass beads ratio (2:35, 1:30 and 1:25, w/w) and extraction time (30 and 60 min) on PPs yield and galacturonic acid content were evaluated. PPs yield was qualitatively assessed based on ethanol precipitation, while galacturonic acid content was quantitatively determined using a commercial D-Glucuronic/D-Galacturonic assay from Megazyme. Screening results of such operating parameters are summarized in Table 3.4, parameters that exhibited a stronger influence on selected responses are noted with (++). Temperature, solvent system and extraction time were the most influencing parameters

on PPs extraction yield. Therefore, the effect of two binary solvent systems (citric acid/water and ethanol/water) and temperatures (100 to 140˚C) were further studied to improve yield, degree of esterification, galacturonic acid content and biological activity of pomegranate pectic polysaccharides. Pressure, flow rate, PGb:glass beads ratio and extraction time were set constant at 50 bar, 5 mL/min, 1:25 and 30 min, respectively (Table 3.5).

The extraction of pectic polysaccharides from PGb was favored using pressurized fluids at 120˚C. At a temperature of 140˚C, however, the extraction of pomegranate pectic polysaccharides had not occurred, suggesting depolymerization or β-elimination reactions (Chen et al., 2014)

Table 3.4. Screening of some operating parameters for pomegranate pectic polysaccharides extraction using subcritical fluids.

Response	Temperature (°C)		Solvent system			Pressure (bar)			Flow rate (mL/min)		PGb:glass beads ratio			Extraction time (min)	
	160	140	MilliQH_2O	Ethanol/water (20/80, v/v)	Ethanol (96%)	50	70	100	10	5	2:35	1:30	1:25	60	30
PPs yield[a]	-	++	++	++	-	+	-	-	-	+	-	+	+	+	++
GalA[b](%)	-	++	+	++	-	+	-	-	-	+	-	+	+	+	++

a: qualitative analysis; b: quantitative analysis; ++: stronger influence; +: weak influence; -: no influence.

Table 3.5 Operating parameters for pomegranate pectic polysaccharides extraction using subcritical fluids at 50 bar, 5 mL/min for 30 min.

Solvent System	Temperature (°C)
H_2O Citric acid/H_2O (50 µM) Ethanol/H_2O (20/80, v/v)	100
H_2O Citric acid/H_2O (50 µM) Ethanol/H_2O (20/80, v/v)	120
H_2O Citric acid/H_2O(50 µM) Ethanol/H_2O (20/80, v/v)	140

Pomegranate biomass: glass beads ratio (1/25, w/w); µM: micro molar.

Figure 3.6 shows the pomegranate pectic polysaccharides yield obtained with selected pressurized fluids at 100 and 120˚C, 50 bar, 5mL/min for 30 min. Although the effect of solvent system on PPs yield was not significantly different when temperature was kept constant at either 100 or 120˚C, the semi-continuous extraction of PPs with water at 100˚C was not possible. During the semi-continuous extraction at 100˚C, PPb was poorly solvated and remained inside the reactor chamber, leading to hard lump formation and a low mass transfer rate. PPs batch extraction at 100˚C, 50 bar and 30 min was low as illustrated in Figure 3.7a. The PPs content at 100˚C (3.9±0.4%) was determined based on solid residues remaining inside the reactor. On the other hand, the extraction of PPs at 100˚C within pressurized citric acid/H_2O or pressurized ethanol/H_2O was easily performed in a semi-continuous mode. Thus, modifying the pH with citric acid and adding ethanol as a co-solvent, improved solvation, cell wall disruption of PGb and mass transfer rate. As such, PPs yields of 6.0±0.7 and 6±1% were obtained with citric acid/H_2O and ethanol/H_2O within 30 min, respectively. Moreover, there was no significant difference between PPs yield obtained from the first step of Treatment II as a control (refer to Table 3.1) and yield obtained using the aqueous citric acid media at 100˚C and 50 bar (semi-continuous process, Figure 3.6).

Temperature significantly influenced PPs yield when compared to different solvent systems (Figure 3.6). The highest content of pectic polysaccharides from pomegranate biomass was obtained at 120˚C using any of the selected pressurized fluids. As such, pressurized H_2O, citric acid/H_2O and ethanol/H_2O led to PPs yields of 9.9±0.2, 10.8±0.6 and 10.4±0.9%, respectively. PPs yield obtained with pressurized citric acid/H_2O at 120˚C was approximately 55.5% higher than the yield obtained with the

same pressurized acid media at 100˚C. Pressurized citric acid/H_2O at 120˚C could induced the disruption of PPb cell wall layers (i.e. middle lamella, primary and secondary walls) via acid catalyzed hydrolysis of glycosidic bonds, leading to a high PPs extraction compared to pressurized citric acid/H_2O at 100˚C. Likewise, citric acid/H_2O media at 120˚C favored the elution of ions from cell wall as measured by the conductivity of the pectic liquid extracts (refer to Figure 3.5, *E*) compared to H_2O and ethanol/H_2O at 100˚C as is shown in Figure 3.8a. In addition, it can be highlighted that pressurized water at 120˚C exhibited similar hydrolytic capacity to citric acid/H_2O system (pH 2.6) and, the conductivity trend of pectic liquid extracts obtained with both solvents was quite similar as depicted in Figure 3.8b.

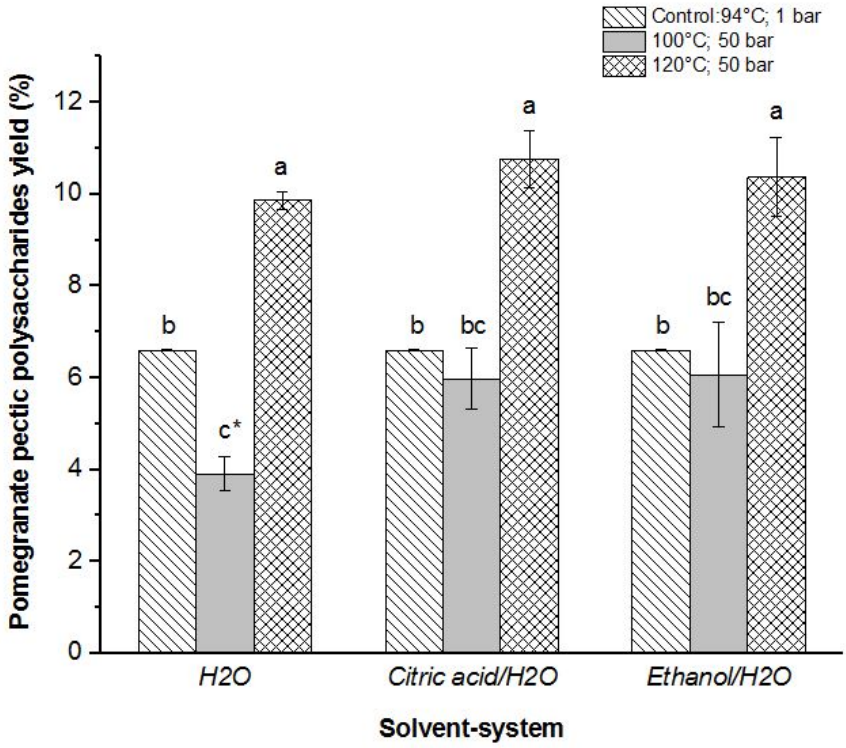

Figure 3.6 Extraction yield of pomegranate pectic polysaccharides obtained with pressurized fluids at 5 mL/min for 30 min. *PPs obtained by a batch process. Bars followed by different letters (a-c) are significantly different (Tukey's HDS, $p < 0.05$).

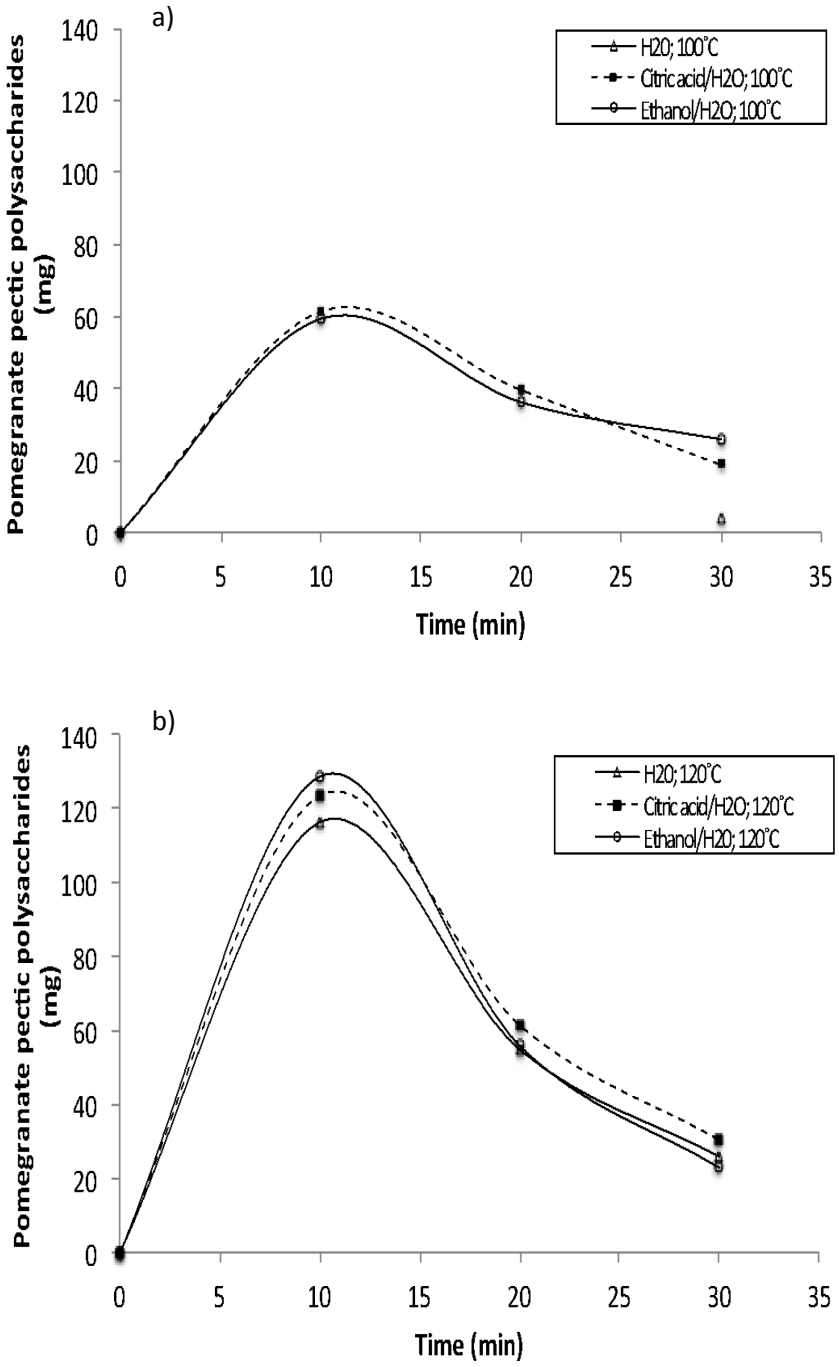

Figure 3.7 Extraction kinetics of pomegranate pectic polysaccharides using pressurized fluids at: a) 100˚C/50 bar (▲ PPs obtained by a batch process) and, b) 120˚C/50 bar.

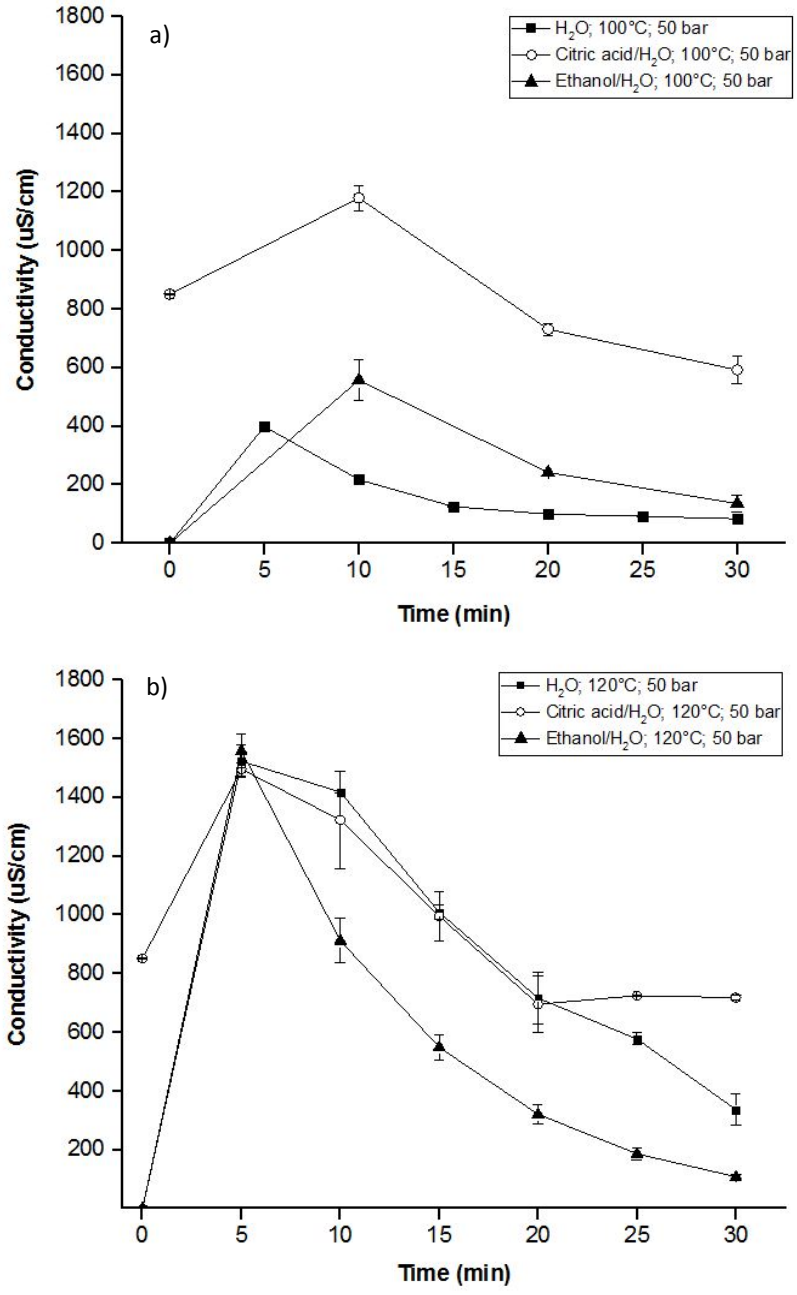

Figure 3.8 Conductivity of PPs liquid extracts obtained with pressurized fluids at: a) 100˚C and b) 120˚C.

Saldaña & Valdivieso-Ramirez (2015) and Plaza & Turner (2015) reported that physico-chemical properties of pressurized water can dramatically change at subcritical

temperatures (100<T<340˚C), for example self-ionization of water can be induced, leading to higher ionic product (K_w), favoring acid catalysis and further depolymerization reactions. Moreover, it has been reported that surface tension of water decreases when temperature increases, improving its wetting property and mass transfer rate. PPs yield obtained at 120˚C with water (9.9±0.2%) or citric acid/H_2O (10.8±0.6%) was not significantly different. Therefore, pressurized water at 120˚C could be potentially used over citric acid/H_2O solvent system for PPs extraction.

Although, pressurized ethanol/H_2O at 120˚C did not significantly increase PPs extraction yield, less hydrophilic compounds from primary cell walls, such as phenolics were simultaneously co-extracted. Recently, Benito et al. (2015) reported that during subcritical extraction of β-glucan, a water-soluble compound, from waxy barley, total phenolics extraction was induced when 5 - 20% v/v of ethanol was added as a co-solvent. Besides, they also found out that hot pressurized aqueous ethanol prevented β-glucan depolymerization, leading to high molecular weight β-glucan but low extraction yields (Benito et al., 2015). As pectic polysaccharides are also water-soluble compounds like β-glucan, pressurized ethanol/H_2O at 120˚C could prevent PPs further hydrolysis, leading to extracts with low conductivity as shown in Figure 3.8 b.

Pectic polysaccharides from pomegranate biomass were extracted at different operating conditions within batch and semi-continuous processes. When hot pressurized fluids were used as extractants, PPs extraction yield significantly increased with temperature. Pressurized solvent-systems at 120˚C extracted approximately 126% of PPs within 30 min compared to the control. The effect of operating conditions on chemical

characteristics of isolated pomegranate pectic polysaccharides is further discussed in the next sections.

3.3.3 Degree of esterification

The degree of esterification of pectic polysaccharides was determined to elucidate the effect of operating parameters on pectic polysaccharides structure and functionality by titrimetric analysis.

According to Figure 3.9 and Table A.1, the esterification degree of PPs extracted using pressurized fluids was significantly influenced by temperature and solvent system. Higher temperature led to higher esterification degree when the solvent system was kept constant. PPs extracted with pressurized ethanol/H_2O at 120˚C had the highest DE (72.3±0.7%). Although the pH of pectic liquid extracts was low at 120˚C (Figure 3.10 b) compared to 100˚C, PPs with higher DE were obtained at 120˚C rather than at 100˚C. It is known that de-esterification reaction is favored at high temperature and low pH (Krammer & Vogel, 2000; Stephen et al., 2006). However, there is a possibility that right after PPs extraction, new ester links were formed, in particular when ethanol was added as a co-solvent. In fact, an esterification reaction takes place when a carboxylic acid is treated with an alcohol under temperature and an acid catalyst. And, those requirements were met by water properties at subcritical conditions. Hence, at certain temperature and/or extraction time, de-esterification reaction could be induced and, beyond those conditions, esterification reactions could be favored. That can be the reason why either PPs with low esterification degree were obtained at 100˚C or PPs extracted with pressurized ethanol/H_2O at 100˚C exhibited the lowest DE (35±5%).

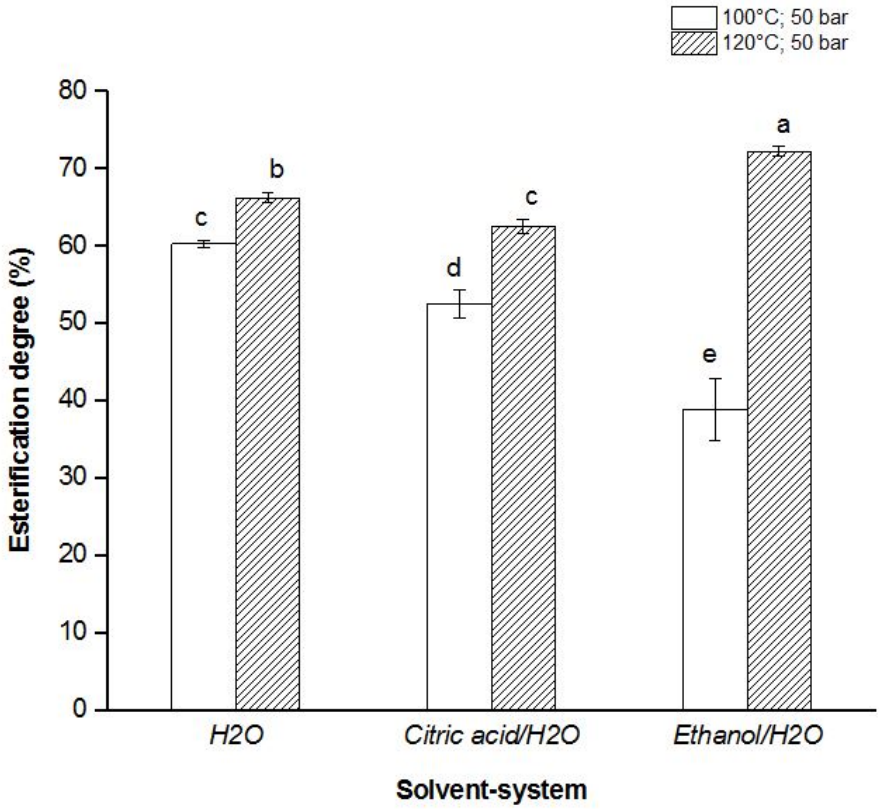

Figure 3.9 Esterification degree obtained by titration analysis of PPs extracted using pressurized fluids at 5mL/min for 30 min. Bars followed by different letters (a-e) are significantly different (Tukey's HDS, $p < 0.05$).

Pectic polysaccharides with both high and low methoxyl content were extracted from pomegranate biomass within the pressurized GRAS fluid systems. It was also shown that the use of co-solvents, such as citric acid and ethanol, can lead to value-added compounds within shorter processing time and less operational steps (Figure 3.5) compared to current methodologies that have been reported (Rovaris et al., 2008; Vriesmann et al., 2012).

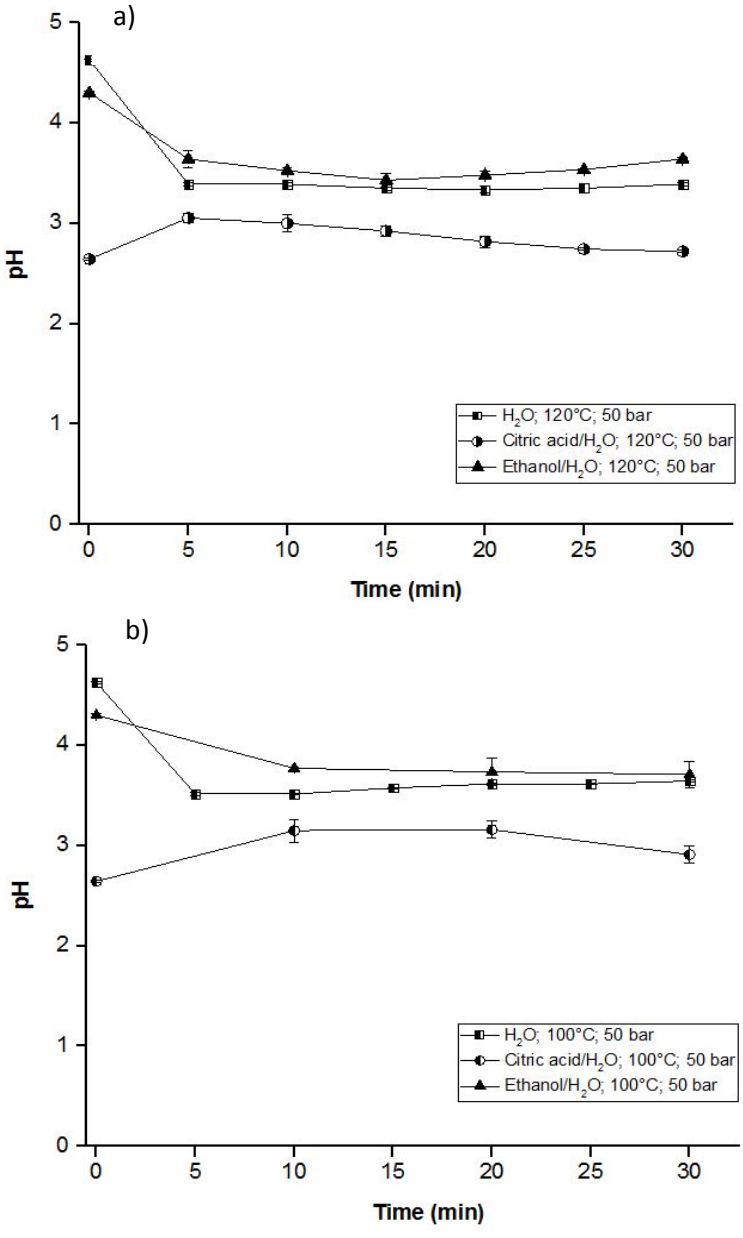

Figure 3.10 pH of pomegranate pectic liquid extracts obtained with pressurized fluids at: a) 100˚C and, b) 120˚C.

3.3.4 Galacturonic acid content

As pomegranate pectic polysaccharides were extracted and characterized for the first time in this thesis, acid hydrolysis was first used to cleavage pectic polymers. Acid type and concentration, temperature and reaction time are the main parameters for pectin hydrolysis. Garna et al. (2006) and Emaga et al. (2012) found out that sulfuric acid with concentrations between 1 and 2M at 100˚C and, reaction times from 2 to 6 h led to the highest recovery and the lowest degradation of GalA during hydrolysis of apple pectin (12% GalA content) and flaxseed pectin (10% GalA content). Therefore, PPs were hydrolyzed with 2M H_2SO_4 at 94°C for 2 h prior to galacturonic acid quantification.

Among analytical methods for galacturonic acid quantification, the colorimetric or spectrophotometric method is commonly used. However, error or overestimation in the final GalA content has also been reported, owing to monosaccharides and protein interferences (Filisetti-Cozzi & Capita, 199; Garna et al., 2006).

Following acid hydrolysis, galacturonic acid was determined by titration, spectrophotometry (D-Galacturonic assay from Megazyme) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Table 3.6 shows the GalA content of PPs from Treatments A and B (first stage) and commercial citrus pectic (HM). In general, GalA content of PPs was not influenced by solvent-system at 94˚C. Hence, the three quantitative techniques exhibited the same trend. However, GalA content measured by titration was significantly different from spectrophotometric and chromatographic methods. According to the titration method, GalA content of PPs was higher than that of commercial citrus pectin. Moreover, GalA content of pectic polysaccharides from pomegranate determined by titration was above

the minimum percentage required for food grade pectin (65%). Conversely, GalA contents determined by Megazyme assay or HPAEC-PAD were below the required percentage for both the extracted PPs (16-16.2%) as well as for the analyzed commercial citrus pectin (27-30%). Such results suggest that PPs and citrus pectin were either not fully hydrolyzed or the GalA released was partially degraded during the acid hydrolysis. Besides, the reported GalA content of PPs determined by spectrophotometry or chromatography was approximately 50% lower than that in commercial citrus pectin, suggesting that extracted PPs might have additional compounds that are different from pectic fragments. As different methods can lead to different results, titration and HPAEC-PAD analysis were carried out to determine GalA content for PPs extracted with pressurized fluids.

Table 3.6. Galacturonic acid content of pomegranate pectic polysaccharides quantified by different methods.

	Galacturonic acid content (%)		
	Titration	**Spectrophotometry (Megazyme assay)**	**Chromatography (HPAEC-PAD)**
PPs (Treatment I)	80±1	16±1	16.2±0.4
PPs (Treatment II)	79.6±0.4	15±1	15±2
Citrus pectin (HM)	66±1	27±1	30±2

According to Figure 3.11, GalA content of PPs obtained using pressurized fluids was significantly influenced by solvent system at 100°C. Titration method (Figure 3.11a) showed that pressurized citric acid/H_2O led to the highest GalA content (75.25±0.03%) at 100°C. Thus, pressurized citric acid/H_2O seems to favor extraction of homogalacturonan pectic fragment (GalA backbone longer than in R-GI and RG-II fragments) compared to H_2O and pressurized ethanol/H_2O at 100°C. Conversely, at 120°C, GalA content did not significantly changed for the three solvent-systems

evaluated. However, pressurized H_2O and pressurized ethanol/H_2O at 120°C led to a significantly higher GalA content compared to those obtained at 100°C for the same solvent-systems. Indeed, such pressurized systems at 120°C could induce cleavage of glycosidic and ester bonds in cell walls, leading to PPs with high GalA content. On the other hand, HPAEC-PAD analysis (Figure 3.11 b, Table A.1) showed that GalA content of PPs obtained with pressurized fluids was dramatically lower compared to GalA content determined by titration. However, with both methods, PPs with the highest GalA content (17$\pm$1 %) were obtained with pressurized citric acid/H_2O at 100°C. Such pattern could be considered to elucidate the selectivity of citric acid/ H_2O system towards the extraction of homogalacturonan pectic fragment. The lowest GalA content (9.7$\pm$0.6%) obtained at 120°C with pressurized ethanol/water could be also associated to a selectivity trend. Extraction of hairy pectic fragments with different structural backbone, such as RG-I and RG-II, could be favored under ethanol/H_2O media rather than H_2O or citric acid/ H_2O, which could lead to mayor variations in PPs functionality (Seymour & Knox, 2002).

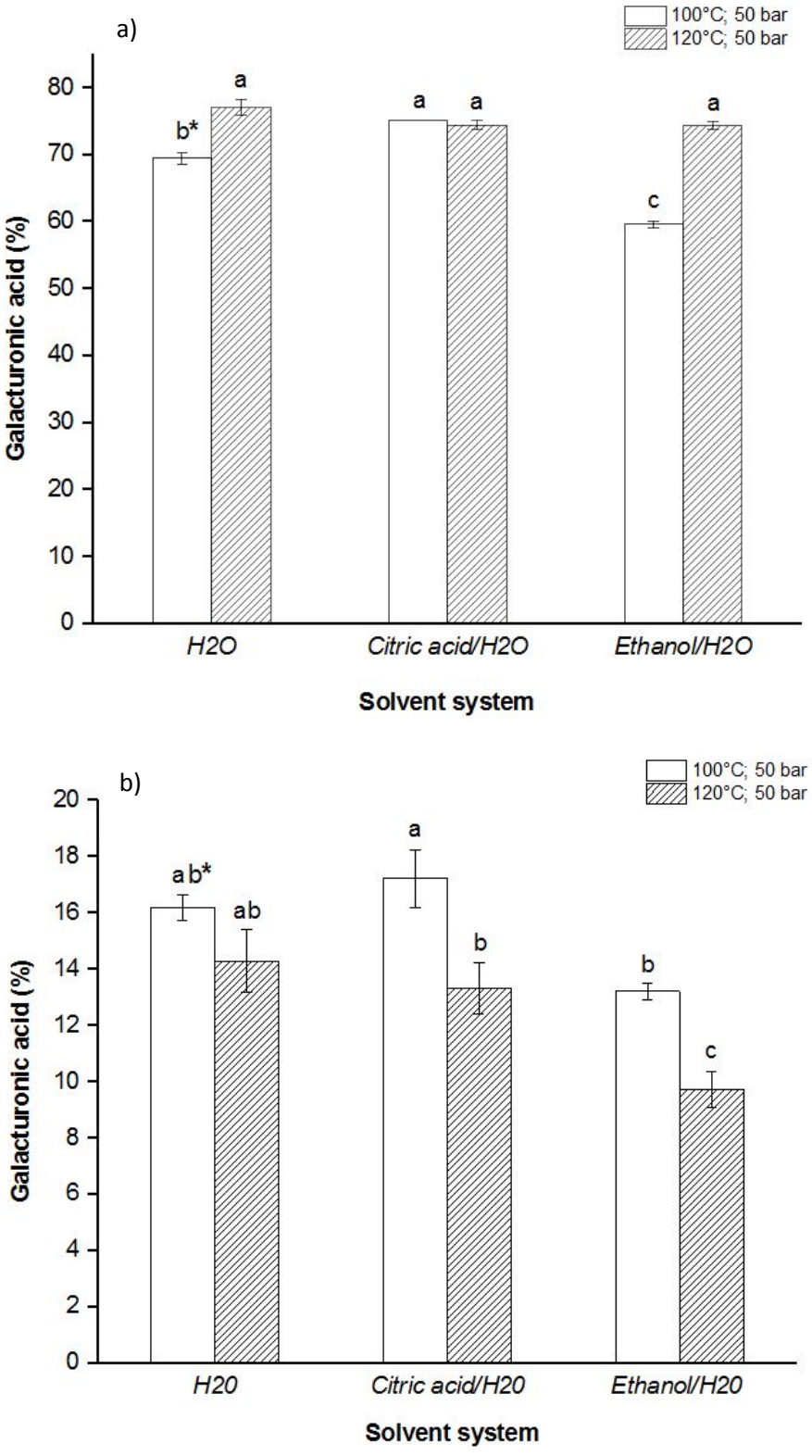

Figure 3.11 Galacturonic acid content of PPs obtained with pressurized fluids: a) Titration method and, b) HPAEC-PAD analysis. *PPs obtained by a batch process. Bars followed by different letters (a-c) are significantly different (Tukey's HDS, $p < 0.05$).

3.3.5 Fourier transform infrared (FT-IR) spectroscopy analysis

Figure 3.12 shows ATR/FT-IR spectroscopic analysis of PPs obtained at 120˚C with pressurized water or pressurized aqueous citric acid and, standards of polygalacturonic acid (86% purity) and citrus pectin (DE > 74%). As pectin spectra is unique in the 1200 to 1000 cm^{-1} infrared region, owing to the high homogalacturonan fragment content, the glycosidic linkage stretching vibration (C-O-C) as well as main stretching side groups (C-OH) and ring characteristic vibrations are useful for identification and comparison purposes. According to Figure 3.12a and b, pomegranate pectic polysaccharides that were extracted with either pressurized water or pressurized aqueous citric at 120˚C exhibited stronger bands at frequencies of 1726, 1600 and 1440 cm^{-1}, which are distinctive of carbonyl and carboxyl groups of homogalacturonan fragment (polygalacturonic acid) (Fellah et al., 2009). Also, peaks that correspond to pectin backbone vibration at 1143, 1101, 1053, 1022 and 979 cm^{-1} were identified. In fact, Figure 3.12c,d shows that polygalacturonic acid and high-methoxyl pectin standards exhibited stronger peaks at similar frequencies to those of extracted PPs. In addition, peaks related to (C-O-C) vibration of different sugar moieties, such as glucose, arabinose and galactose were also noted in the spectra of PPs, suggesting that PPs could also comprise other pectic fragments. In particular, those frequency bands could belong to monosaccharide moieties of the branched region of rhamnogalacturonan-I. The structure and composition of pectic samples were overall elucidated according to main band frequencies previously reported by Kacurakova et al. (2000) for individual cell wall polysaccharides and monosaccharides models as summarized in Table 3.7.

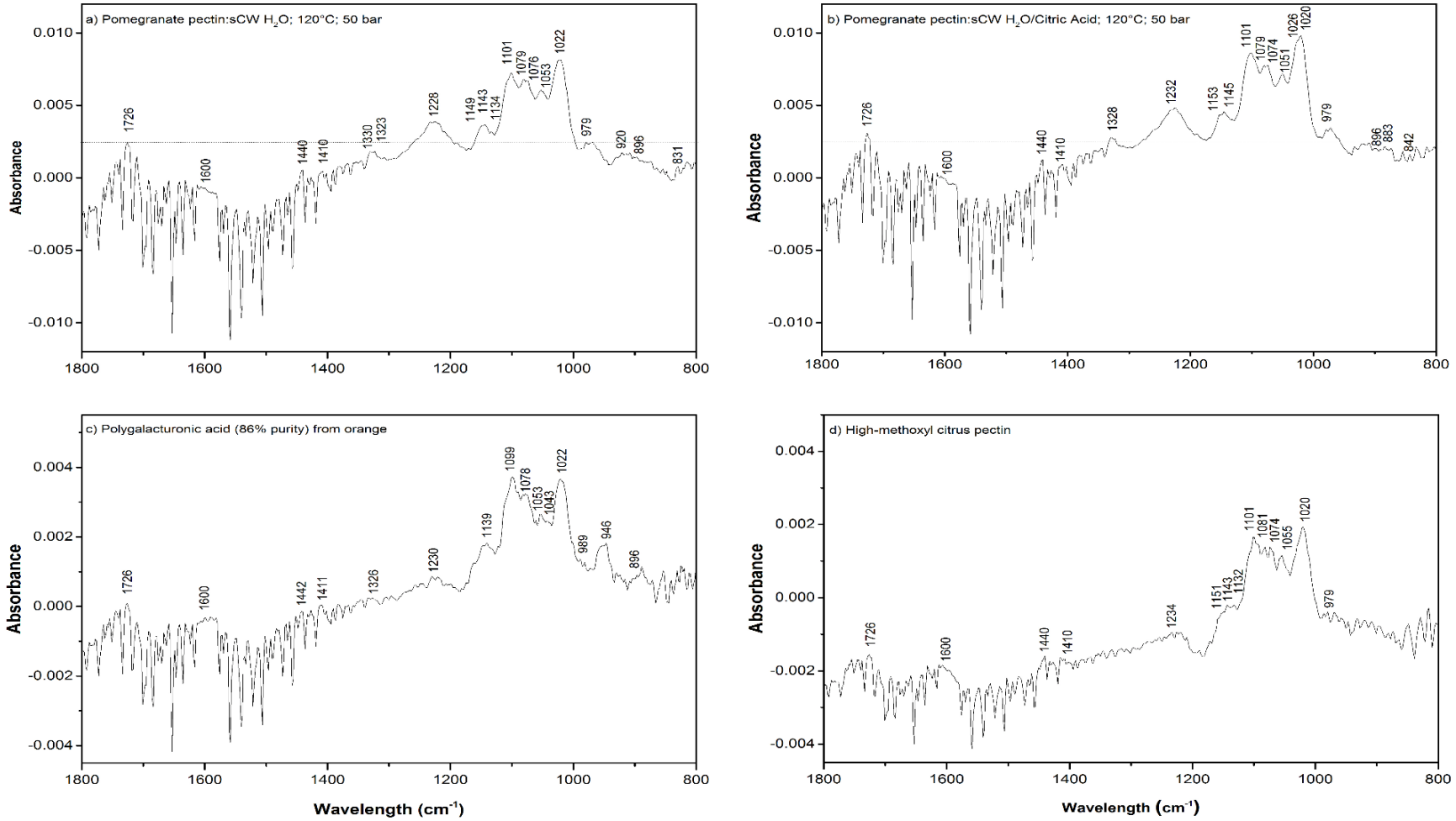

Figure 3.12 ATR-FTIR spectra of: a) PPs extracted with pressurized water at 120˚C and 50 bar, b) PPs extracted with pressurized aqueous citric acid at 120˚C and 50 bar, c) Polygalacturonic acid standard (86% purity) from orange and, d) High-methoxyl pectin standard (DE > 74%) from citrus.

Table 3.7. FT-IR absorption frequencies of cell wall polysaccharides and monosaccharides models reported by Kacurakova et al. (2000).

Cell wall polysaccharides	**FT-IR frequencies (cm^{-1})**				
	(C-OH)	**(C-O-C)**	**(C-C)**	**ring**	**(C_1-H)**
Homogalacturonan	1144	1100	1051	1022, 972	953
Rhamnogalacturonan	1150	nr	1070	1043,989	951,916,902
Galactan	1155	1134	1072	1038	nr
Arabinogalactan	Nr	nr	1074, 1078	nr	nr
Glucan	1151	1104	1078	1041, 1026	945
Xiloglucan	1153	1118	1078	1041	nr
Hexo and pentopyranoses					
Glucose	1079	1149	nr	1106	nr
Galactose	1078	1148	nr	nr	nr
Arabinose	1068	1143	1086	nr	nr

nr: not reported.

In addition, esterification degree of pectin has been studied according to the IR bands located between 1750-1730, 1630-1600 and 1440-1380 cm^{-1}, which denote ester carbonyl stretching (C=O), carboxylate ion asymmetric stretching (COO^-) and, ester asymmetric and symmetric stretching ($-CH_3$) vibrations, respectively. Szymanska-Chargot & Zdunek (2013) and Gnanasambandam & Proctor (2000) observed that FT-IR spectra of high-methoxyl pectin exhibited an increased band intensity and area around 1740 cm^{-1} (C=O) with a subsequent reduction of peak intensity assigned at 1600 cm^{-1} (COO^-). Also, Szymanska-Chargot & Zdunek (2013) reported that FT-IR spectra of low-methoxyl pectin did not show the corresponding $-CH_3$ band at 1440 cm^{-1}.

According to Figure 3.12a,b and Figure 3.13, PPs isolated with pressurized water, pressurized aqueous citric acid and pressurized aqueous ethanol at 120˚C exhibited

moderate absorption bands at 1726 cm^{-1} (C=O) and 1440 cm^{-1} ($-CH_3$) as well as a weak peak at 1600 cm^{-1} (COO^-), revealing that PPs with a high esterification degree were obtained. These results complement those previously obtained by the titration method (Figure 3.11a).

On the other hand, FT-IR analysis of PPs fractions obtained at different extraction times using pressurized aqueous ethanol at 120˚C (Figure 3.13) suggested that esterification and de-esterification reactions could be induced along the extraction process. Variations of peak intensities at 1726 and 1604 cm^{-1} were observed during the overall extraction time. Indeed, after 10 min extraction, de-esterification reactions were identified due to a sudden intensity decrease of carbonyl stretching (C=O) band located at 1737 cm^{-1} and, the subsequent increase on intensity of carboxylate ion asymmetric stretching (COO^-) band at 1604 cm^{-1}. Esterification reactions, however, were favored at long extraction times (>10 min). From Figure 3.13c and d, the highest intensity peak of carbonyl stretching (C=O) band was identified at 30 min of extraction. Therefore, pressurized aqueous ethanol at 120˚C showed to be a versatile GRAS solvent system that can induce not only PPs extraction within minutes but also favor pectin modification, avoiding the use of toxic chemical reagents and additional processing, such as amidation, sulfonation, grafting, etc.

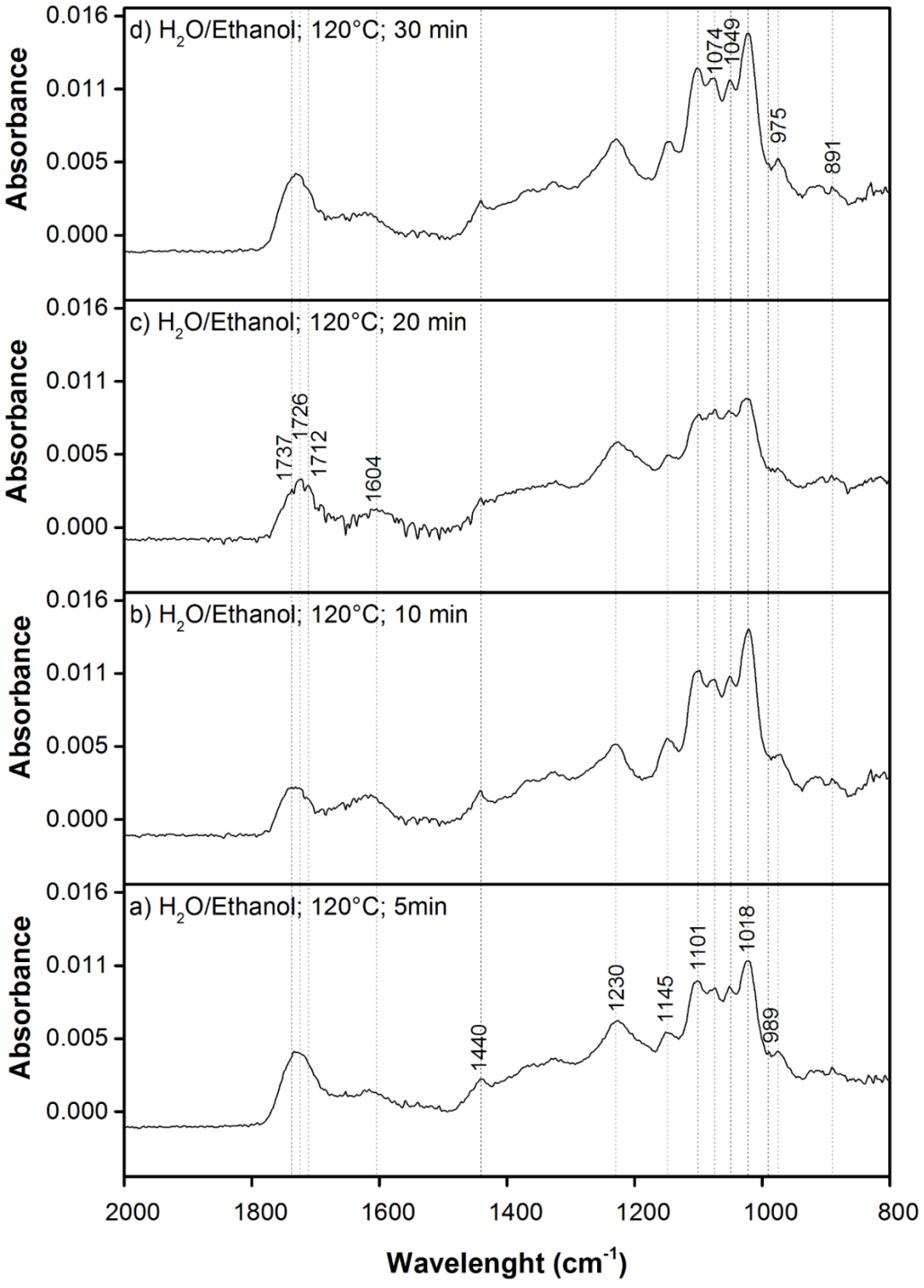

Figure 3.13. FT-IR spectra of PPs isolated using pressurized aqueous ethanol at 120˚C and extraction times of: a) 5 min, b) 10 min, c) 20 min and, d) 30 min.

3.3.6 Total phenolics content

Total phenolic content was determined for pomegranate pectic liquid extracts (*E*) obtained right after the subcritical fluid process (refer to Figure 3.5) as well as for the freeze-dried pomegranate pectic polysaccharides.

According to Figure 3.14, the total phenolic content of pectic liquid extracts was significantly influenced by the solvent-system and temperature. In general, pressurized ethanol/H_2O favored the extraction of phenolic compounds compared to pressurized H_2O or pressurized citric acid/H_2O systems. Pressurized ethanol/H_2O system could induce high solubility of phenols as well as lignocellulosic network cleavage, increasing its extraction (Zhang, 2015; Benito et al., 2015). In addition, Mushtaq et al. (2015) reported that the main phenolic acids of pomegranate peel isolated by enzyme-assisted supercritical CO_2 extraction were vanillic acid (108.36 μg/g extract), caffeic acid (75.19 μg/g extract), syringic acid (88.24 μg/g extract) and sinapic acid (3.58 μg/g extract). As is known, the solubility of those phenolic acids in water at normal conditions is low. Therefore, the addition of a co-solvent like ethanol under subcritical fluid conditions could increase solvency of polyphenols in water, favoring its recovery.

Various studies have shown that subcritical fluids (subcritical water or ethanol + water (5-20% v/v)) at high temperatures (135<T<180°C) have led to high recovery of phenolic compounds from agricultural biomass (i.e. potato peel, barley hull, grape pomace, lentil husk, flaxseed hull, etc.) (Singh & Saldana, 2001; Srinivas et al., 2011; Sarkar et al., 2014; Benito-Roman et al., 2015). For pomegranate biomass, however, the highest amount of total phenolics (368±5 mg GAE/g PGb) was extracted with pressurized ethanol/H_2O at 100°C rather than with pressurized ethanol/H_2O at 120°C.

Likewise, the removal of polyphenols from PGb with pressurized citric acid/H_2O was also favored at 100°C rather than at 120°C. In addition, pressurized H_2O at 120°C increased the removal of polyphenols compared to pressurized H_2O at 100°C. Hence, there was no significant difference ($p<0.05$) between total phenolic content extracted with either pressurized H_2O at 120°C or pressurized citric acid/H_2O at 120°C. It can be seen that at 120°C, physical and chemical properties of pressurized H_2O notably change, inducing not only solvency of less polar components like phenolic acids but also hydrolytic reactions and biomass conversion (Saldaña & Valdivieso-Ramírez, 2015). Also, from Table 3.8, it can be inferred that phenolic compounds that have been extracted from pomegranate peel are prone to dissolve in less polar solvents like methanol and ethanol at low temperatures (21 to 55°C) rather than in water over the same temperature range (Orak et al., 2012; Fawole et al., 2012; Mushtaq et al., 2015). Using pressurized fluids at high temperatures (>100°C) could induce pomegranate polyphenols, mainly anthocyanins degradation (Monrad et al., 2010) and, this could be the reason why higher total phenolic content was achieved at 100°C rather than at 120°C.

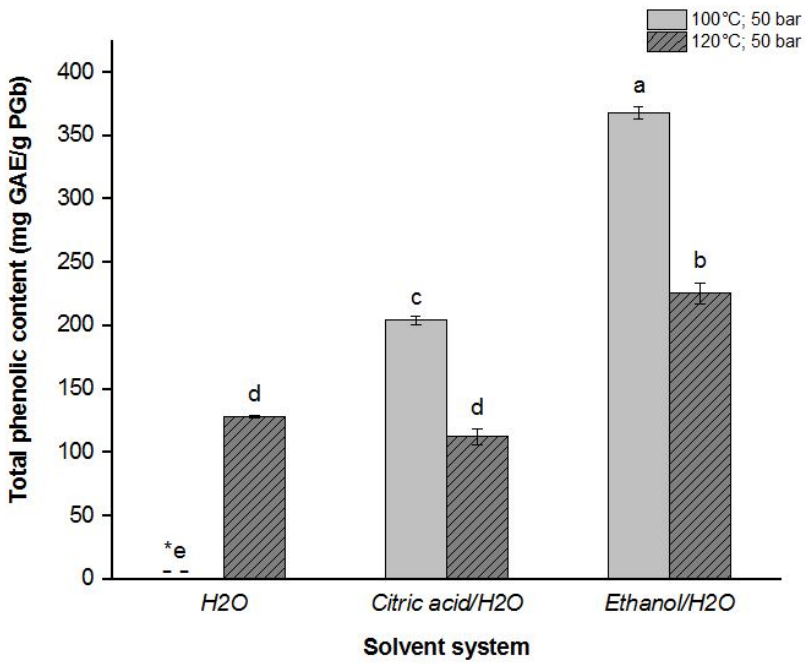

Figure 3.14 Total phenolic content of pomegranate liquid extracts obtained using pressurized fluids. *PPs obtained by a batch process. Bars followed by different letters (a-e) are significantly different (Tukey's HDS, $p < 0.05$).

Overall, water and binary solvent-systems under subcritical conditions led to similar or higher polyphenols recovery compared to conventional solid-liquid solvent extraction or combined technologies like enzyme-assisted supercritical CO_2 extraction (Table 3.8). Hence, pressurized ethanol/H_2O and pressurized citric acid/H_2O at 100°C could be a feasible and sustainable alternative to obtain phenolic compounds from similar matrices as PGb without additional pre-treatments (i.e. enzymatic hydrolysis prior to extraction) and, shortcomings associated to large volumes of toxic solvents and extended extraction times.

The total phenolic content of freeze-dried pomegranate pectic polysaccharides was determined to elucidate their potential bioactivity. The total phenolic content was calculated based on either the pomegranate biomass (PGb) or isolated pomegranate pectic polysaccharides (PPs) as shown in Figure 3.15.

Table 3.8. Total phenolic content of pomegranate pectic liquid extracts obtained by different extraction methods.

Source	Extraction method	Operating conditions			TPc (mg GAE/g DPb)	Ref.
		Temperature (°C)	Solvent system	Time		
Pomegranate peel (*Hicaznar* variety)	S-L	21	Ethanol (98%) Methanol (97%)	12 h	132.3±0.4 168.0±0.4	Orak et al. (2012)
Pomegranate peel (*Molla de Elche* variety)	S-L/ ultrasound	21	Methanol/H_2O (80% v/v)	60 min	179±5	Fawole et al. (2012)
Pomegranate peel (Pakistan)	EASCFE	55	Pectinase/protease/ cellulose/SC-CO_2+Ethanol	120 min	302±8	Mushtaq et al. (2015)
	EASE	55	Pectinase/protease/ Cellulose/Ethanol/H_2O (80% v/v)	120 min	279±7	Mushtaq et al. (2015)
Pomegranate biomass (Peru)	**PFs***	**100**	**Pressurized ethanol/H_2O (20% v/v)**	**30 min**	**368±5**	**Present study**
			Pressurized citric acid/H_2O (0.2 %)		**204±3**	**Present study**

S-L: solid-liquid solvent extraction; EASCFE: Enzyme-assisted supercritical CO_2+Ethanol; EASE: Enzyme-assisted solid-liquid solvent extraction; PFs*: Pressurized fluids at optimum temperature (present study); DPp: dry pomegranate peel; a: TPc (mg GAE/mL extract).

According to Figure 3.15a and Table A.2, the total phenolic content (mg GAE/g PGb) remaining as part of pectic polysaccharides chains after ethanol-precipitation and purification was quite low. In fact, there was an opposite correlation between phenolic content in pectic extracts (*E*) and phenolic content in pomegranate pectic polysaccharides (PPs) powder. Total phenolic content of 17.6±0.1, 16.5±0.2 and 15.1±0.2 were obtained for pressurized ethanol/H_2O, citric acid/H_2O and H_2O at 120°C, respectively. As pressurized ethanol/H_2O and pressurized citric acid/H_2O at 100°C led to pectic liquid extracts with higher content of free phenolics, less phenolics remained bonded to PPs at the same conditions. Conversely, pressurized H_2O at 120°C led to pectic liquid extracts with low content of polyphenols but PPs with more phenolic compounds (31.61%) compared to PPs extracted with pressurized ethanol/H_2O at 100°C.

Figure 3.15b shows that overall values of total phenolic content were high when calculated according to extracted PPs (mg GAE/g PPs). Temperature had a significant influence on PPs phenolic content, in particular, when pressurized H_2O or pressurized citric acid/H_2O system was used. Then, PPs with total phenolic contents of 169±2, 153±3 and 153±2 (mg GAE/g PPs) were reported for pressurized ethanol/H_2O, citric acid/H_2O and H_2O at 120°C, respectively. Hence, PPs extracted with pressurized H_2O or pressurized citric acid/H_2O at 120°C did not exhibit significant difference with respect to total phenolic content. However, PPs with the highest content of polyphenols were extracted with pressurized ethanol/H_2O at either 100 or 120°C.

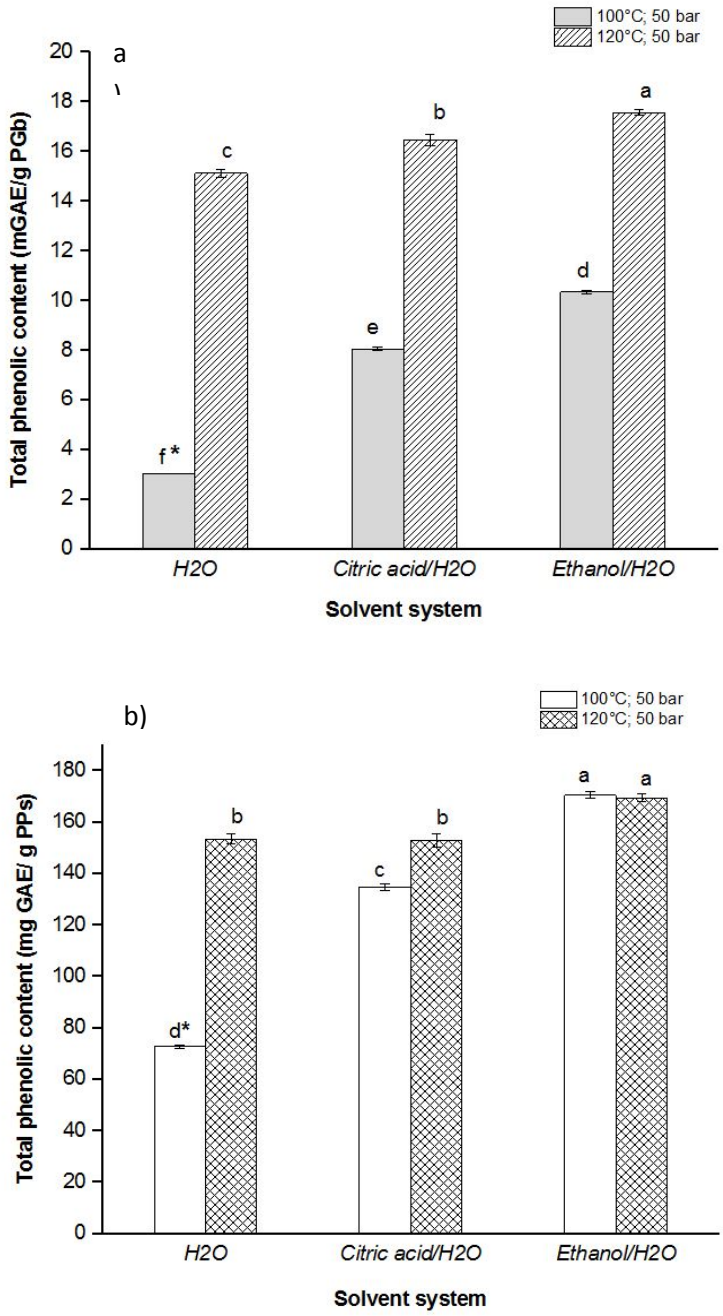

Figure 3.15 Total phenolic content of pomegranate pectic polysaccharides obtained using pressurized fluids: a) TPc (mg GAE/g PGb) and, b) TPc (mg GAE/g PPs). * PPs obtained by a batch process. Bars followed by different letters (a-d) are significantly different (Tukey's HDS, $p < 0.05$).

3.3.7 Antioxidant activity

Total antioxidant activity of freeze-dried pomegranate pectic polysaccharides was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay on PPs solutions with a concentration of 2mg/mL. As shown in Figure 3.16 and Table A.2, the extraction temperature significantly influenced the antioxidant activity of PPs. PPs with the highest inhibition percentage of DPPH activity (I% DPPH > 89.7%) were obtained at 120°C with any of the selected pressurized solvent-systems. Conversely, Wang et al. (2014) found that pressurized H_2O at 140°C led to apple pomace and citrus peel pectic polysaccharides with slight higher IC_{50} (less I% DPPH) due to a potential reduced proton donation from hydroxyl group of monosaccharides units and carboxyl group from GalA units caused by extended pectin hydrolysis. There was no significant difference between I% DPPH of PPs extracted with pressurized citric acid/H_2O and pressurized ethanol/H_2O at 100°C. Although PPs with the lowest I% DPPH (87.30$\pm$0.80%) were obtained with pressurized H_2O at 100°C, it was higher than I% DPPH reported for apple pomace and citrus peel pectic polysaccharides (60%) at the same concentration of 2mg/mL. Wang et al. (2014) and Georgiev et al. (2012) reported that apple pomace and citrus peel pectic polysaccharides were further evaluated for inhibitory effect on cell proliferation and inmunostimulating activity, respectively. Results showed that pectic polysaccharides from apple pomace significantly inhibit HT-29 (colon cancer) cell proliferation where the exerted proliferation inhibition rate was 76.45%. Also, citrus peel pectic polysaccharides (1.25 mg/mL) showed anti-complementary activity of 30% based on hemolysis inhibition of mutton erythrocytes (Georgiev et al., 2012). Therefore, pomegranate pectic polysaccharides with higher I% DPPH seems to be a promising bioactive component for health promotion.

In addition, radical scavenging capacity assay was assessed for commercial HM-pectin and LM-pectin at the same concentration of 2mg/mL for comparison purposes. Results showed that the average I% DPPH of extracted PPs (89.2%) was 2.6 times more than the I% DPPH of commercial pectins (33.8%). Therefore, PPs obtained using pressurized fluids might have a promising role for halting oxidative stress and cell damage.

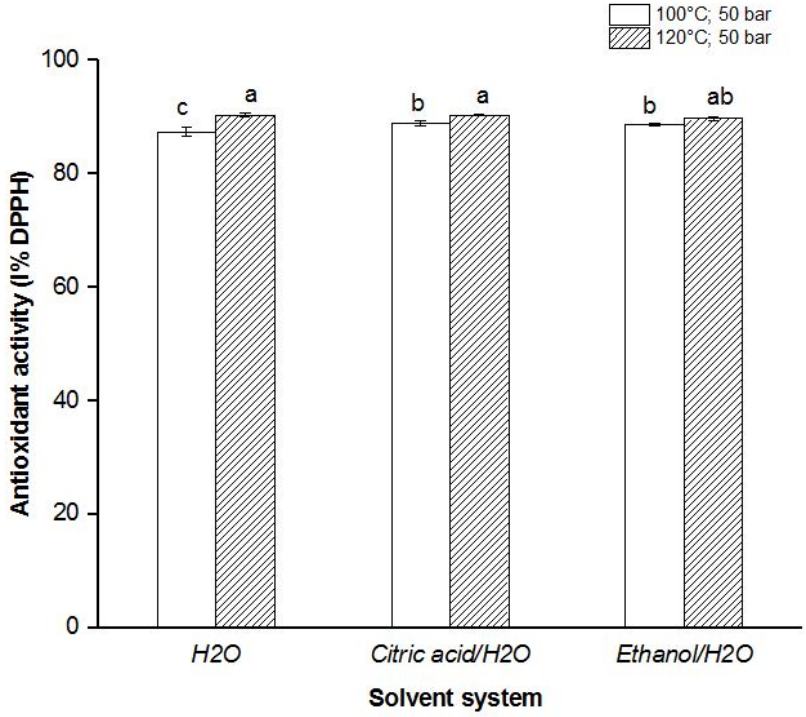

Figure 3.16 DPPH radical scavenging activity of pomegranate pectic polysaccharides obtained using pressurized fluids. Bars followed by different letters (a-c) are significantly different (Tukey's HDS, $p < 0.05$).

3.4 Conclusions

- For the first time, pressurized aqueous citric acid was used to extract pectic polysaccharides from pomegranate biomass. Pressurized aqueous citric acid at 120°C, 50 bar and 30 min led to bioactive pomegranate pectic polysaccharides with galacturonic acid content (74.4±0.7%) and degree of esterification (63±1%) comparable to commercial citrus pectin.

- Temperature had a significant influenced on pomegranate pectic polysaccharides extraction yield. The PPs extraction yield obtained at 120˚C, 50 bar and 30 min using either pressurized water or pressurized binary solvent systems was approximately 80% higher than the yield obtained with the conventional solid-liquid method.
- The esterification degree of PPs was significantly influenced by temperature and pressurized solvent systems. PPs extracted with pressurized ethanol/H_2O at 120˚C had the highest DE (72.3±0.7%).
- Pressurized citric acid and aqueous ethanol systems at 100˚C and 50 bar favored the co-extraction of phenolic compounds from pomegranate biomass. A PGb extract with a total phenolic content of 368±5 mg GAE/g PGb was obtained with pressurized ethanol/H_2O at 120˚C, 50 bar and 30 min.
- Pressurized citric acid and aqueous ethanol at 120˚C and 50 bar showed to be versatile GRAS solvent systems that can be potentially used for pomegranate pectic polysaccharides extraction and modification, avoiding the use of toxic chemical reagents and additional processing.
- The homogalacturonan pectic fragment was identified by FT-IR of isolated pomegranate pectic polysaccharides. FT-IR analysis suggested that pomegranate pectic polysaccharides could also embrace Rhamnogalacturonan-I pectic fragment.
- DPPH radical scavenging activity of pomegranate pectic polysaccharides obtained with pressurized fluids at either 100 or 120°C and 50 bar was significantly higher (I% DPPH > 89%) than those of commercial citrus pectin (I% DPPH = 33.8%).

3.5 Recommendations

Some recommendations for future studies are:

- Liquid extracts of pomegranate biomass obtained with pressurized citric acid and aqueous ethanol at 100˚C and 50 bar could be further analyzed as potential sources of bioactive compounds, such as anthocyanins, flavonoids, tannins and saponins.
- A combination of chemical and enzymatic treatments could be used to hydrolyze pectic polysaccharides before HPAEC-PAD analysis for galacturonic acid determination.
- Studies on pectic polysaccharides rheology could lead to optimize extraction parameters towards a particular pectic product.
- Studies on antibacterial and antifungal activities and, the minimum inhibitory concentration of isolated pomegranate pectic polysaccharides could be further determined to evaluate potential applications.
- The toxicity of pectic extracts obtained with pressurized fluids as well as the purified pomegranate pectic polysaccharides could be assessed to determine whether they can be used as food grade products or in biomedical applications.
- Studies on the structure of pomegranate pectic polysaccharides could be conducted using NMR to elucidate the main moieties related to its potential biological activities.
- A screening of potential anticancer properties of isolated pomegranate pectic polysaccharides could be done in particular using the MTT Cell Proliferation Assay.

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Chapter 4: Synthesis of bioactive pectin-based gels using subcritical water technology

4.1 Introduction

Nowadays, the production of functional bio-based materials for industrial applications is a growing area of research. Natural polysaccharides, such as cellulose, starch, pectin, chitosan and carrageenan, have been studied due to their non-toxic and biodegradable properties. Among them, pectin has drawn considerable attention as a promising material for food, pharmaceutical and biomedical uses (Mishra et al., 2014; Chen et al., 2014). However, no studies with integration of biomass conversion from agro-industrial residues to pectin-based *gel* formation have been reported. Different pectin-based structures such as films, hydrogels, aerogels and micro/nanoparticles have been produced for coating, encapsulation and drug delivery systems (Yoshimura et al., 2005; Liu et al., 2007; Kumar et al., 2012; Marras-Marquez et al., 2015).

Although pectin-based structures have shown advantageous responses over synthetic substrates, its remarkable hydrophilic nature has been associated to low mechanical strength as well as to an early drug release. Chemical modifications of pectin structure by amidation, thiolation, sulfonation, cross-linking and, grafting have been reported to address those limitations. Mishra et al. (2012), for example, synthetized pectin hydrogels with improved gelling, film forming capacity and extended time of drug delivery at colonic conditions by amidation and cross-liking reactions with ethanolamine and glutaraldehyde. Also, Fares et al. (2010) reported that N-isopropylacrylamide grafted pectin hydrogels could be potentially used for colon targeted modulated-theophylline release. Besides, physical modifications like blending of natural polysaccharides have led to pectin-based materials with enhanced mechanical and functional properties. As such, Liu et al. (2007) obtained flexible pectin/fish gelatin/soy bean

protein composite films with high strength and low water solubility compared to pure pectin films. Also, Coimbra et al. (2011) used a blend of pectin/chitosan to obtain a non-toxic and biocompatible polyelectrolyte complex cryogel for potential bone tissue engineering. Hence, a combination of chemical and physical pectin modification has been also used to obtain tailored pectin-based gels. Mishra et al. (2011) formed a glutaraldehyde cross-linked pectin/gelatin hydrogel with superior tensile strength and thermal stability for wound dressing applications. Although the *sol-gel* method is conventionally used to produce either pectin or other polysaccharide-based gels, the use of emerging and sustainable technologies could induce *gel* formation without the shortcomings associated to the use of toxic cross-linking reagents and extended reaction times. Obtaining pectin-based gels with high bioactivity, biocompatibility and water stability could lead to functional and effective products suitable for internal healing and external tissue repair (Munarin et al., 2012).

In this study, subcritical water technology was explored as a novel *sol* formation process to synthesize pectin-based 2D films and 3D cryogels. Essential oils from clove and star anise as well as chitosan were also loaded into such biogels to enhance their functional properties for potential biomedical applications. In addition, an *in situ* $CaCl_2$ cross-linking process was used to induce water stability in synthesized 2D and 3D structures.

4.2 Materials and Methods

4.2.1 Materials

Low-methoxyl pectin Classic CU 701 (GalA content: 86% and, degree of esterification: 34%) was kindly provided by Herbstreith & Fox (Neuenburg/Wurtt, Germany). Chitosan (Deacetylated chitin, deacetylation degree: 75-85%) was obtained from Sigma Aldrich (Oakville, ON, Canada). Star anise *Illicium verum* essential oil (*trans*-Anethole > 85%) and clove *Eugenia*

caryophyllanta essential oil (Eugenol > 80%) were purchased from a local market (Simply Health, Edmonton, AB, Canada).

Chemical reagents and standards, such as calcium chloride anhydrous (>93%), citric acid (>99.5%, ACS grade), sodium trihydrate (99%), acetate glacial acetic acid (99.7%), 1,1-Diphenyl-2-picrylhydrazyl (DPPH)(99.9%), ethanol (>98%), Folin-Ciocalteu's phenol reagent, sodium carbonate anhydrous (>99%), *trans*-anethole (>98.0%) and eugenol (>99%) were acquired from Sigma Aldrich (Oakville, ON, Canada). Glycerol (>95% purity, certified ACS grade) was purchased from Fisher Scientific (Ottawa, ON, Canada). Distilled water from Milli-Q system (Millipore, Bellerica, MA, USA) was used.

4.2.2 Novel synthesis of essential oil-loaded pectin/chitosan films

Essential oil-loaded pectin/chitosan films were synthesized by the *sol-gel* method using calcium chloride as the cross-linking agent. The essential oil was loaded in the pectin *sol* prior to pectin-based *gel* formation. An innovative approach for pectin/chitosan-essential oil *sol* formation was carried out using subcritical water technology. The traditional *sol* formation at 1 bar (atmospheric pressure) and 70˚C was also performed for comparison purposes. After *gel* formation (cross-linking process of pectin *sol* with aqueous $CaCl_2$), essential oil-pectin/chitosan hydrogels were cast onto plastic round flat base molds to obtain bioactive pectin films. The hydrogel formulations are shown in Table 4.1. In addition, pectin based films were *in situ* cross-linked with aqueous $CaCl_2$ to modify physico-chemical properties of the films. On the other hand, a freeze-drying process was also conducted to obtain essential oil-pectin/chitosan cryogels. The new process used for essential oil-loaded pectin/chitosan biomaterials is summarized in Figure 4.1.

Table 4.1 Hydrogel formulation.

Hydrogel	**Formulation ratios (w:w)**				
	Pectin:water	**Glycerol:pectin**	**EOs:pectin**	**Chitosan:pectin**	**$CaCl_{2\ (aq)}$: pectin**
Pectin	3:100	9:6	0:0	0:0	7:3
Pectin-EOs	3:100	5:6	2:3	0:0	7:3
Pectin-EOs/chitosan	3:100	5:6	2:3	1:8	7:3

EOs: essential oil blend (clove [2 $\mu L/mL_{blend}$] + star anise [10 $\mu L/mL_{blend}$]); $CaCl_{2\ (aq)}$: 1% calcium chloride aqueous solution.

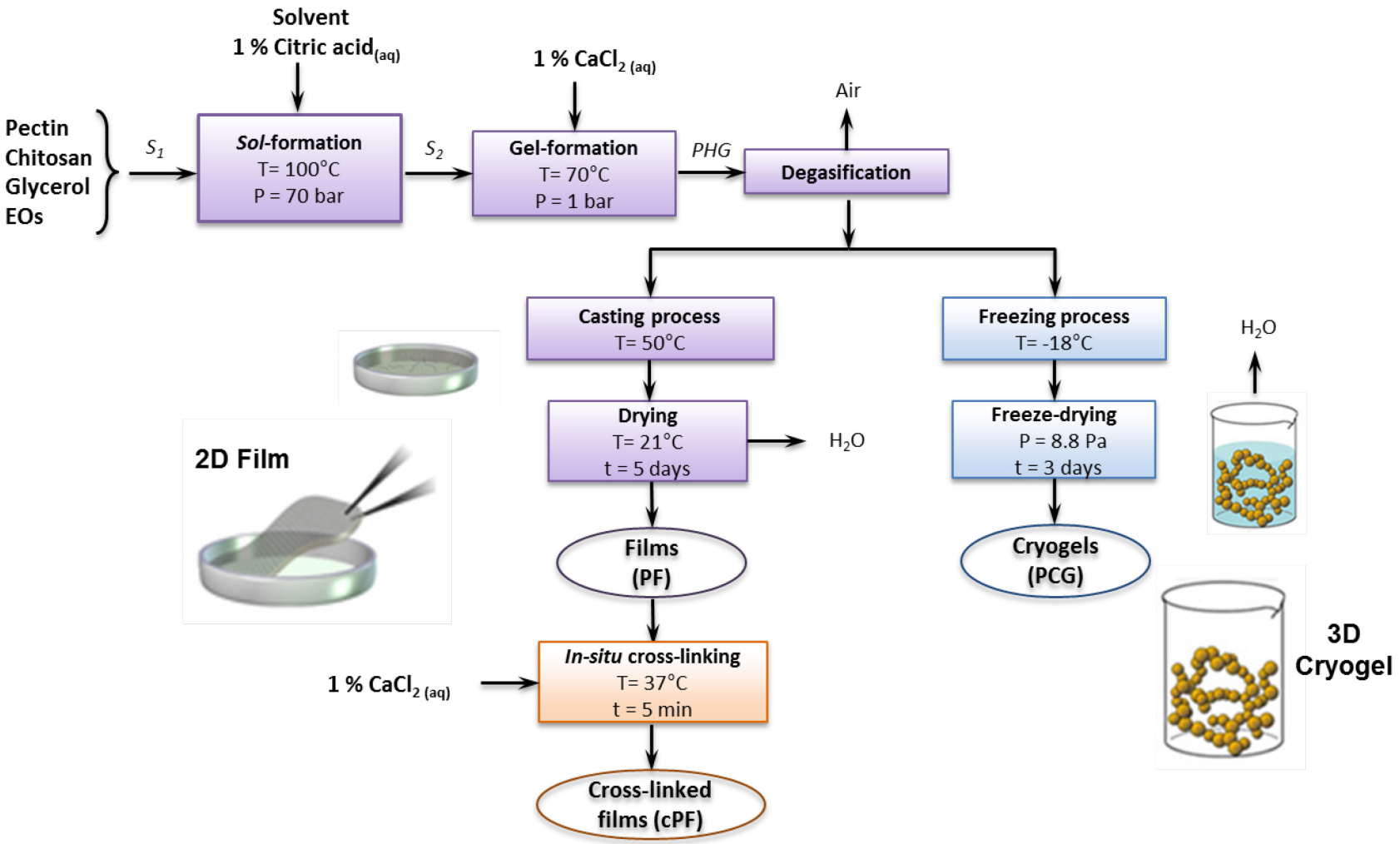

Figure 4.1 Flowchart for production of essential oil-loaded pectin/chitosan biomaterials. T: temperature; P: pressure; PHG: pectin hydrogel; PF: pectin film; PCG: pectin cryogel; cPF: cross-linked pectin film; EOs: clove and star anise essential oils; S_1: pectin + chitosan+glycerol+Eos and, S_2: 1% citric acid $_{(aq)}$.

A subcritical batch system with a continuous stirred-tubular reactor was used to obtain the essential oil-pectin/chitosan *sol* as shown in Figure 4.2. The method previously described by Zhang (2015) for bioactive starch films with modifications was followed. Briefly, low-methoxyl pectin (6 g) was dissolved in aqueous citric acid (200 mL, 1% w/w) at 70˚C within a blender. Chitosan (0.75 g), glycerol (5 g) and essential oil (4 g) were then added to the pectin solution and mixed for 10 min to obtain a multicomponent and homogeneous solution (S_1). Next, the tubular reactor loaded with the solution S_1 was connected to the subcritical unit system. Milli-Q water was pumped to discard any remaining oxygen inside the system to prevent oxidation or overpressure along the process. Once the system was purged and the reactor was completely filled with water, valve was closed and, both double helix stirrer switch and heating system were activated. A temperature-sensing element (thermopar) placed inside the reactor, two band-heaters and a temperature controller were used as the heating mechanism to keep temperature at constant set conditions. After reaching the set temperature, the pump was turned on to increase pressure up to 70 bar according to operating parameters. The reaction time for essential oil-pectin/chitosan *sol* at set temperature and pressure was 10 min. Once the reaction time ended, the system was turned off and the reactor was immediately quenched to 50˚C by cold running water. The reactor was then unplugged from the system and the resulting pectin based *sol* (S_2) was poured down into a 500 mL Buchner flask for *gel* formation and degasification.

For hydrogel formation, solution S_2 was heated up to 70˚C and aqueous $CaCl_2$ (1% w/w) was added dropwise under continuous stirring to induce the cross-linking process. Then, vacuum was used to remove air bubbles that were trapped inside the essential oil-pectin/chitosan hydrogel (PHG) due to the stirring process. A 10 min degasification step was used for PHG prior to casting.

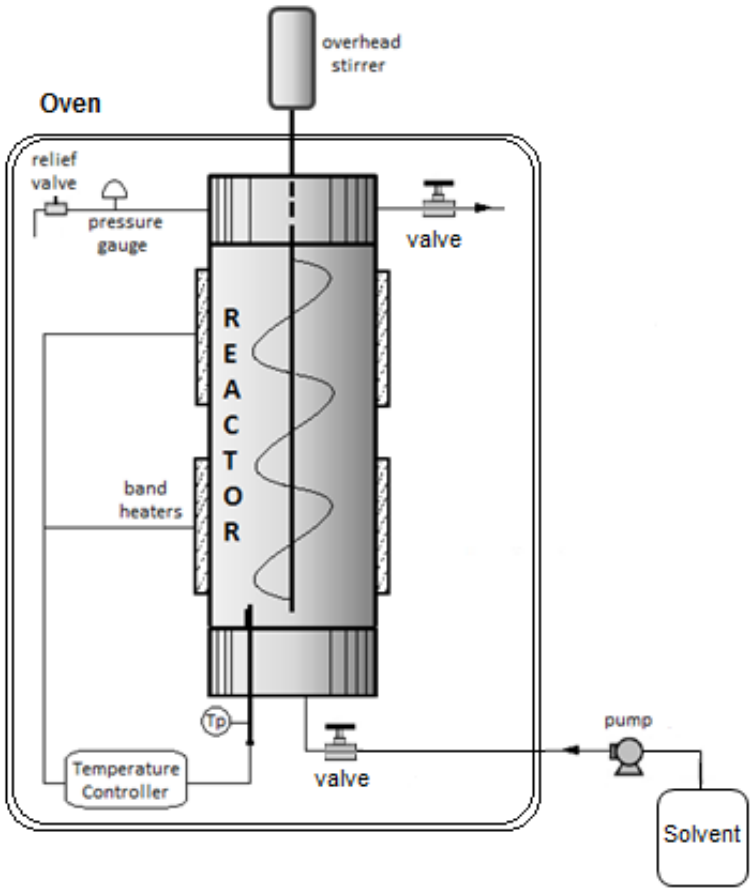

Figure 4.2 Batch subcritical fluid unit system. Tp: thermopar.

Degassed essential oil-pectin/chitosan hydrogels (70 g) were cast onto a 15 cm diameter plastic petri dish and dried at room temperature (21˚C) for 5 days. Then, pectin/chitosan-essential oil films (PF) were removed from the molds and stored at 25˚C and 30% relative humidity for further physicochemical characterization (Zhang, 2015).

Pectin/chitosan-essential oil films were *in situ* cross-linked as described by Penhasi & Meidan (2014) with some modifications. PF were cut in rectangle shaped samples (1.5 cm x 0.5 cm) and immersed into 10 mL of aqueous $CaCl_2$ (1% w/w) at 37˚C for 5 min. Then, the cross-linked pectin/chitosan-essential oil films (cPF) were dried in an oven (Model 655G, Fisher

Scientific Iso Temp oven, Toronto, ON, Canada) at 30˚C for 48 h. Dried cPF were stored at 25˚C and 30% relative humidity.

Besides, essential oil-pectin/chitosan hydrogel samples (15 g) were frozen at -18˚C for 24h and then freeze-dried for 36 h in order to obtain highly porous and lightweight cryogels. A freeze-drier system (Labonco, Kansas City, MO, USA) at 8.8 Pa and a collector temperature of -44˚C was used to remove the solvent from hydrogels by water sublimation. Essential oil-pectin/chitosan cryogels (PCG) were kept in plastic-sealed containers at room temperature for further analysis and characterization.

4.2.3 Film characterization

4.2.3.1 Fourier transform infrared (FT-IR) spectroscopy analysis

The infrared absorption spectrum of pectin-based films were obtained following the procedure described by Fellah et al. (2009) and Cabello et al. (2015) using a Nicolet 8700 Fourier Transform Infrared Spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with Omnic software (version 7.1) and a Smart Speculator for Attenuated Total Reflection (ATR germanium crystal cell). The spectra was recorded from 350 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 128 scans.

4.2.3.2 Film thickness

Film thickness was measured according to the procedure proposed by Zhang (2015) using a digital micrometer (Model 534-552A, Mitutoyo, Tokyo, Japan) with a 0.0001 mm precision. An average thickness value of 6 measurements tested in random points of the film was obtained for each film. Such value was further used to determine the tensile strength and percent of elongation at break of the films.

4.2.3.3 Mechanical properties

Tensile strength (TS) and percent elongation at break (% E) of pectin-based films were determined according to the ASTM D882 (Standard Test Method for Tensile Properties of Thin Plastic Sheeting) method. A texture analyzer (5960 Dual Column Table top Testing System with Instron Bluehill Software, Instron, MA, USA) equipped with a 5N load cell was used for the test. The crosshead speed and the initial separation between the grips were set at 4 mm/min and 30 mm, respectively (Zhang, 2015). Briefly, rectangle shaped film strips (5cm x 1cm) were carefully placed aligned with the direction of pull and tighten in the testing machine grips prior to the analysis. Then, load versus extension data was automatically recorded and further processed by the software, leading to direct tensile strength and percent of elongation at break values.

4.2.3.4 Swelling index

The swelling index (SI) of pectin-based films was determined according to the method reported by Cabello et al. (2015) with some modifications. Square shaped dried films (2cm x 2cm) were accurately weighed (W_1) using an analytical balance (Mettler Toledo, Mississauga, ON, Canada) and, then immersed into airtight plastic centrifuge tubes filled with 50 mL of Milli-Q water. Films were kept at 25˚C for 24 h. After this time, samples were removed from the liquid media and placed over weighing paper to withdraw excess of water. The final weight (W_2) of swollen samples was recorded and the SI was calculated using Equation 4.1:

$$\%SI = \frac{W_2 - W_1}{W_1} \times 100 \qquad 4.1$$

where, W_1 = weight (g) of dried film, and W_2= weight (g) of swollen film.

4.2.3.5 Solubility

Solubility of pectin-based films within water or a phosphate buffer solution with pH 7.4 was assessed following the same procedure described in Section 4.2.3.4 for swelling index determination with an additional agitation process prior to drying. As such, tighten centrifuge tubes were shaken at 180 rpm for 24 h. Then, film residues were filtered from the solution and dried in a convection oven at 105˚C for 48h. The final weight of film residues was recorded as W_3. The solubility of films was calculated according to Equation 4.2.

$$\%WS = \frac{W_1 - W_3}{W_1} \times 100 \qquad 4.2$$

where, W_1 = weight (g) of dried film, and W_3= weight (g) of dried film residues.

4.2.3.6 Transparency

Transparency of pectin-based films was determined according to the ASTM D1746 (Standard Test Method for Transparency of Plastic Sheeting) specification. A spectrophotometer (6320D, Jenway, Bibby Scientific Ltd, Dunmow, Essex, UK) was used for the test. The methodology early described by Zhang (2015) was followed. Briefly, rectangle shaped film strips (5cm x 1cm) were carefully placed inside the cell cuvette and the % transmittance at 600nm was measured. The transparency value was calculated using equation 4.3:

$$Transparency\ value = \frac{-(\log T_{600})}{x} \qquad 4.3$$

where, T_{600} is the fractional transmittance at 600 nm and x is the thickness of films in mm. A high value represents a low transparency of the film.

4.2.3.7 Gloss

Gloss of pectin-based films was analyzed according to the ASTM D523 (Standard Test Method for Specular Gloss) method. A flat surface gloss meter (GM 268, M&I instruments,

Mississauga, ON, Canada) was used for the measurements. The gloss at 60˚ angle geometry of film samples was measured in triplicates. A highly polished plane black glass with an assigned specular gloss value of 100 was used as the standard. The resulting measurements were expressed in gloss units (GU).

4.2.3.8 Total phenolic content

A solid-liquid extraction was carried out to determine the amount of phenolic compounds released from pectin-based films. First, 0.2 g of the selected film was finely cut into pieces to increase surface area and then extracted with 8 mL of aqueous ethanol (50 % v/v) under constant stirring for 24 h (Zhang, 2015). The resulting mixture (solvent + partially dissolved film) was centrifuged at 4100 rpm for 20 min and filtered. The filtrate was collected for further analysis of total phenolic content using the colorimetric method and Folin-Ciocalteu reagent as described in Chapter 3, Section 3.2.4.5.

4.2.3.9 Antioxidant activity

The antioxidant activity of pectin-based films was measured on the filtrate obtained from the solid-liquid extraction described in Section 4.2.3.8 for total phenolic determination. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay was used to calculate the inhibition DPPH percentage of film extracts following the same methodology described in Chapter 3, Section 4.2.3.1.

4.3 Screening of pectin-based cryogels properties

4.3.1 Bulk density

Bulk density of cryogels was calculated as the ratio of mass to volume using equation 4.4:

$$\delta = \frac{m}{v} \tag{4.4}$$

where, δ is the cryogel density in g/cm^3, m is the mass in g and, v is the volume in cm^3 of the cryogel calculated based on its diameter and length.

4.3.2 Porosity

Porosity of cryogels was determined according to the solvent replacement method previously describe by Zhang (2015). Briefly, cryogels with 2 cm diameter and 0.5 cm length were dipped in 45 mL of ethanol (98%) for 24 h. After that, samples were removed from the liquid media and placed over weighing paper to withdraw excess of water. Porosity was calculated using equation 4.5:

$$Porosity\,(\%) = \frac{(m_2 - m_1)}{\delta_{ethanol} \times v} \times 100 \tag{4.5}$$

where, m_1 is the mass in g of the cryogel before ethanol immersion, m_2 is the mass in g of the cryogel after ethanol immersion, δ is the density of ethanol equal to 0.789 g/cm^3 and, v is the volume in cm^3 of the cryogel.

4.3.3 Total phenolic content

A solid-liquid extraction was carried out to determine the amount of phenolic compounds released from pectin-based cryogels. Cryogel samples of 0.2 g were extracted with 8 mL of aqueous ethanol (50 % v/v) under constant stirring for 24 h (Zhang, 2015). The filtrate was

collected for further analysis of total phenolic content using the colorimetric method and Folin-Ciocalteu reagent as described in Chapter 3, Section 3.2.4.5.

4.3.4 Antioxidant activity

The antioxidant activity of pectin-based cryogels was measured on the filtrate obtained from the solid-liquid extraction described in the above Section 4.2.4.3 for total phenolic determination. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay was used to calculate the inhibition DPPH percentage of film extracts following the same methodology described in Chapter 3, Section 4.2.3.1.

4.4 Statistical analysis

Film and cryogel synthesis at established operating conditions were performed in duplicates and all the analysis were performed in triplicates. The effect of gel formation under subcritical fluid conditions as well as the *in situ* $CaCl_2$ cross-linking on physico-chemical, mechanical and functional properties of films and cryogels were evaluated using a two-way analysis of variance (ANOVA). A significant difference between the treatments of $\alpha = 0.05$ was used. Differences between means were assessed by Tukey´s multiple range test ($p<0.05$) using Minitab software package v.17 (Minitab Inc., State College, PA, USA).

4.5 Results and discussion

Pectin film results are discussed based on final physico-chemical (thickness, swelling index, solubility, water activity), mechanical (tensile strength and percent elongation at break), optical (transparency and gloss) and functional (antioxidant activity) properties.

4.5.1 Physico-chemical properties

Figure 4.3 shows the FT-IR spectra and characteristic absorption peaks of LM-pectin (Classic CU 701), glycerol, essential oils (clove + star anise, Table 4.1) and chitosan. FT-IR

spectrometry was also used to elucidate functional groups and bonding in synthesized essential oil-loaded pectin/chitosan films.

The FT-IR spectra of LM-pectin exhibited characteristic peaks at ranged frequencies of 3000-2800 cm^{-1} (C-H stretching), 1740-1730 cm^{-1} (C=O, esterified), 1620-1400 cm^{-1} (COO^-, asymmetric and symmetric stretching) and 1330 cm^{-1} (ring vibration). In addition, absorption bands between 1300-800 cm^{-1} are referred as the fingerprint of pectin (Fellah et al., 2009). Such absorption region includes functional groups relative to pectin backbone vibration at 1144 cm^{-1} (O-C-O, asymmetric stretching) for glycosidic linkage, 1102 cm^{-1} (C-O stretching and C-C stretching) pectin ring, 1010 cm^{-1} (C2-C3, C2-O2 and C1-O1) as well as at 954 cm^{-1} (C-O bending), which are very close to those frequencies reported by Fellah et al. (2009) and Gnanasambandam et al. (2000).

For glycerol, peaks at 3312 and 2935-2883 cm^{-1} have been reported due to O-H and C-H stretching, respectively (Vodnar et al., 2013). In this study, however, these peaks appeared at 3266, 2933 and 2879 cm^{-1}, respectively. Also, frequency bands at 1400, 1100, 1050 and 995 cm^{-1} were assigned for secondary alcohol, primary alcohol, C-O and C-C stretching, respectively (Vodnar et al., 2013). In this study, peaks at frequencies of 1415, 1110, 1027 and 995 were registered for the same functional groups, respectively.

As essential oils are complex and multicomponent mixtures, principal absorption peaks of main bioactive components from clove (eugenol) and star anise (anethole) essential oils were identified on the FT-IR spectrum. Wang & Sung (2011) reported that eugenol, isoeugenol and acetyl eugenol exhibited significant peaks at 1610 (C=C aromatic band), 1440 ($-CH_2$ vibration), 1032 (C-H) and 960 cm^{-1} (*trans* C-H), respectively. In addition, absorption frequencies of 2924 ($-CH_3$), 1653 (C=C), 1609 (phenyl ring) and 1245 cm^{-1} (C-O-C) were found in anethole

(Mohammed, 2009). For chitosan, FT-IR spectra exhibited characteristic bands at 2923, 1650, 1592 and 1381 cm^{-1} due to C-H aliphatic stretching vibrations, amide-I, amide-II and amide-III, respectively (Jana et al., 2014). Besides, chitosan fingerprint has been located between 1156 and 890 cm^{-1} as shown in Figure 4.3d.

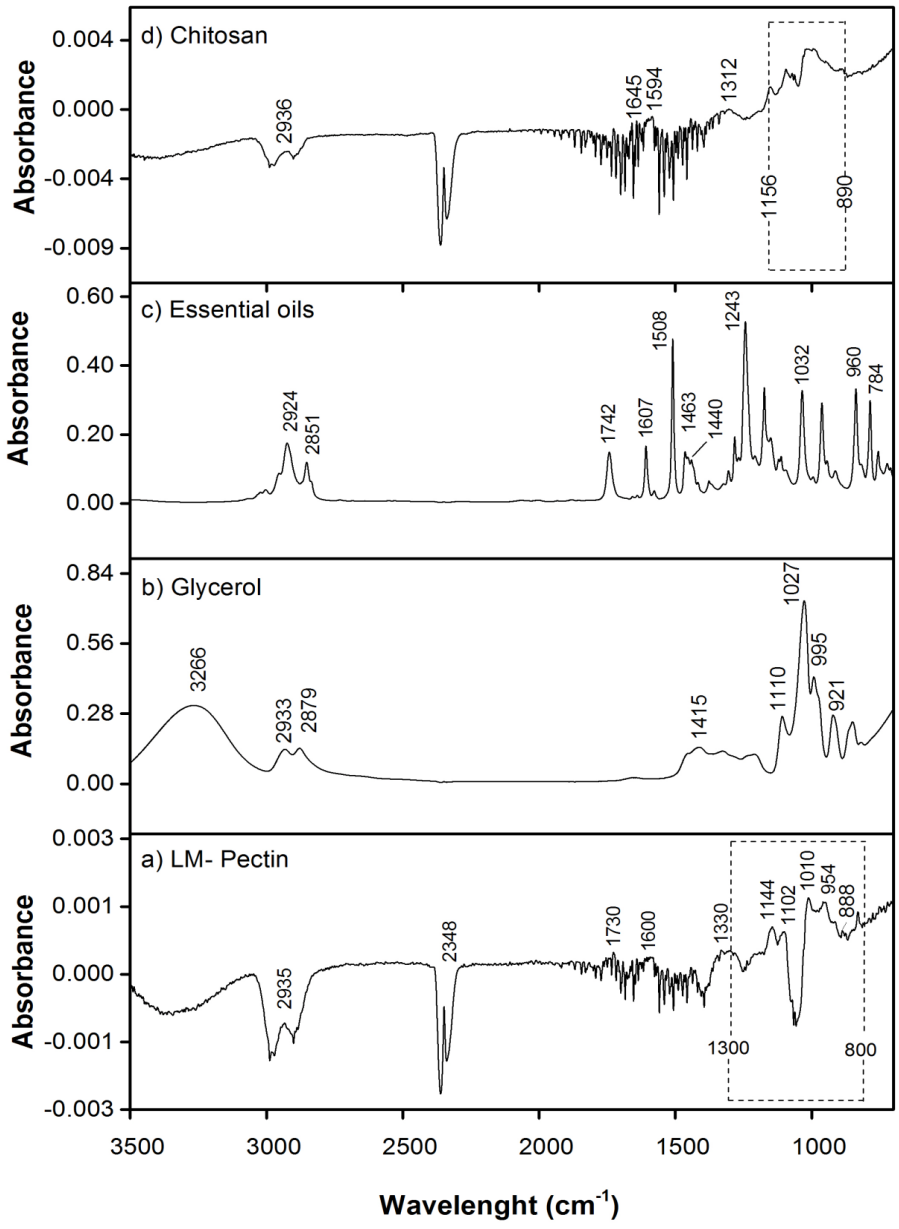

Figure 4.3 FT-IR spectra of film precursors: a) LM-Pectin, b) Glycerol, c) Essential oils and d) Chitosan.

Figure 4.4 depicts FT-IR spectra of synthesized pectin-based films at 70˚C and 1 bar. The absorption frequencies of pectin film (Figure 4.4a) revealed that there was a strong interaction between glycerol and pectin due to ester and hydrogen bonding formation (Figure 4.5). As LM-pectin has a low esterification degree (DE 20-45%), the carboxylate ion (COO^-) could be prone to react with the alcohol group (-OH) of glycerol, favoring ester linkages (Figure 4.5a). Indeed, pectin film spectra (Figure 4.4a) showed high peak intensity at 1730 cm^{-1} due to ester groups (C=O) and a low absorption of COO^- ion group located at 1600 cm^{-1} compared to the standard pectin (Figure 4.3a). Such intensity changes in both peaks can be attributed to esterification reactions. Recently, Cabello et al. (2015) also reported that the addition of glycerol to pectin films increased the IR band area ratio C=O:COO^- from 1 to 1.35 due to ester formation.

On the other hand, the hydrogen atom (H^+) of the alcohol group from glycerol could be attracted to the electronegative charged oxygen of galacturonic acid glycosidic bond (C-O-C), leading to hydrogen bond formation and pectin backbone modification (Figure 4.5b). In fact, the spectra relative to pectin fingerprint showed particular variations. As such, the absorption band at 1144 cm^{-1} assigned to pectin glycosidic linkage (C-O-C) was not found when compared to pectin standard spectrum due to either new interactions or overlapping bands. Hence, additional peaks emerged in pectin film spectra at 1030 and 1110 cm^{-1}, corresponding to C-O and primary alcohol groups of glycerol, respectively. Moreover, the slight shift in absorption band of C-O group from 1027 to 1030 cm^{-1} as well as its higher intensity compared to glycerol spectrum (Figure 4.3b) emphasizes that some changes have taken place in the environment surrounding the pectin molecule.

Although glycerol is a well-known plasticizer agent, there is not much information about its precise interaction with LM-pectin. The above-mentioned bonding interactions could be

responsible for disrupting molecular connectivity between pectin chains, favoring bonding motion and plasticity of pectin gel network (Cabello et al., 2015). This information could be used to determine the stoichiometric amount of glycerol required to enhance specific mechanical properties of pectin-based materials.

Likewise, FT-IR spectra of synthesized essential oil-loaded pectin films (Figure 4.4b) exhibited changes in shape and intensity of peaks compared to the pectin film. Moderated bands at 1730 (C=O, ester) and 1440 cm^{-1} ($-CH_3$) could be attributed to esterification reactions between the alcohol group (-OH) of eugenol (phenolic compound) and carboxylate ion groups (COO^-) of pectin. Although the absorption bands of essential oil-loaded pectin film and pectin film were similar in the region between 1230 and 921 cm^{-1}, increased peak intensities appeared at 1230 (C-O) and 1110 (-OH) cm^{-1}, suggesting potential interactions with different aromatic constituents of essential oils (i.e. monoterpenes, sesquiterpenes, aliphatic aldehydes, alcohols and esters). A slight shift of C-H group of glycerol from 2879 to 2850 cm^{-1} accompanied by an increased absorption of C-H group of pectin at 2940 cm^{-1} could be triggered by anethole functional groups, in particular $-CH_3$ group at 2924 cm^{-1}. According to Figure 4.4c, essential oil-loaded pectin/chitosan film showed the highest peak intensity at 1730 cm^{-1} (C=O, esterified) among the pectin-based films investigated (Figure 4.4a,b). The changes in shape and intensity of such peak could be attributed to chitosan due to electrostatic interaction between positively charged amine group (NH^{+3}) of chitosan and carboxylate anion of pectin (COO^-) (Coimbra et al., 2011).

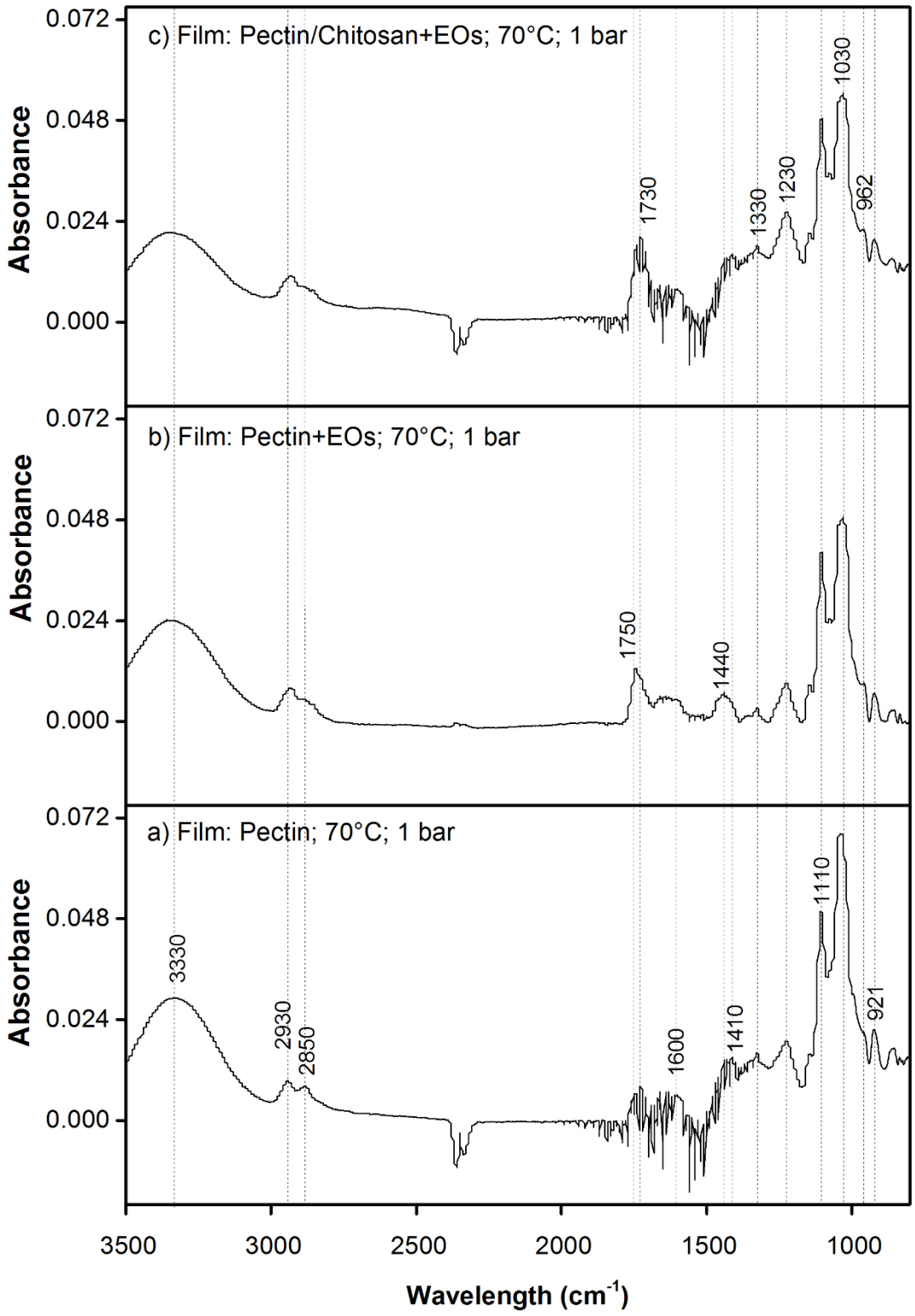

Figure 4.4 FT-IR spectra of pectin based films synthesized at 70˚C/1 bar: a) pectin film, b) essential oil-loaded pectin film and, c) essential oil-pectin/chitosan film.

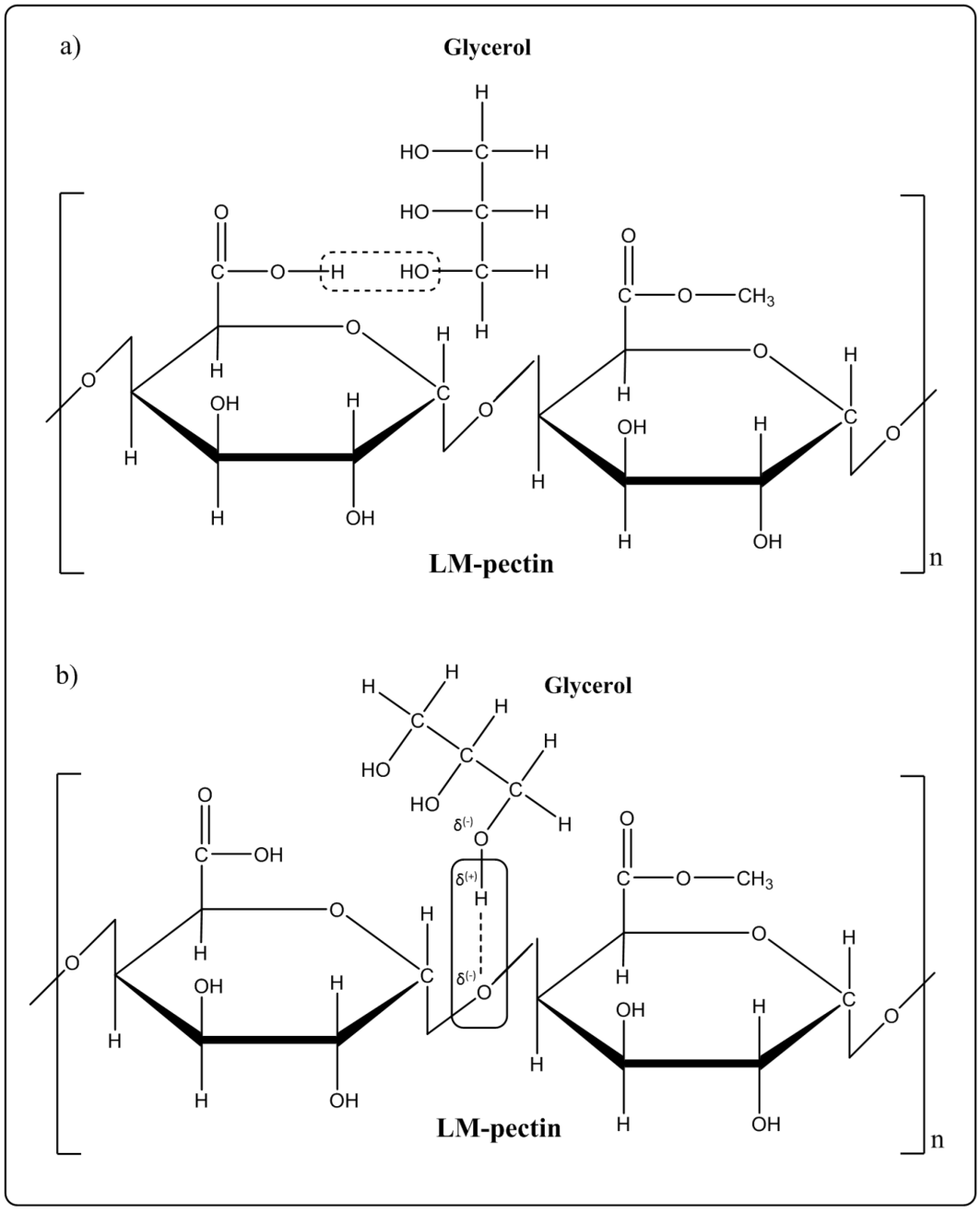

Figure 4.5 Schematical representations of LM-pectin and glycerol interactions during hydrogel formation: a) ester linkage and, b) hydrogen bonding.

FT-IR spectra of pectin-based films synthesized at 100˚C/70 bar (Figure 4.6) also suggested that there was a molecular interaction between *sol* components, in particular between pectin and glycerol due to esterification reactions and hydrogen bonding (Figure 4.5). As such, a sharp peak characteristic of carbonyl group (C=O, ester) was identified at 1739 cm^{-1} along with the reduced intensity of primary alcohol (OH) band of glycerol located at 1105 cm^{-1}, revealing formation of ester linkages. At subcritical conditions, however, pectin film spectra exhibited an overall shift of stretching absorption bands towards lower frequencies compared to pectin film obtained at 70˚C/1 bar (Figure 4.4), suggesting increased interactions and complexation between molecules. Slightly shifts were also registered in absorption peaks of C-H (2921 cm^{-1}), C=O (1739 cm^{-1}) and OH (1105 cm^{-1}) groups due to possible hydrogen bond formation at 100˚C/70 bar, leading to compounds with low IR absorption frequencies.

4.5.2 Solubility

Solubility of pectin-based films synthesized at 100˚C/70 bar was determined using MilliQ water at 37 °C as well as a phosphate buffer solution (pH 7.4) to explore potential biomedical applications (Table 4.2). As pectin is a highly hydrophilic polysaccharide, pectin-based films obtained at either 70˚C/1 bar or 100˚C/70 bar showed 100% solubility in both solvents, water and phosphate media. Although pectin-calcium cross-linking was induced during gel formation, it was not enough to prevent hydration and dissolution of films after 24 h of being in contact with water under constant stirring. The *in situ* $CaCl_2$ cross-linking process, however, decreased the solubility of films obtained at 100˚C/70 bar. The hydrophobicity of pectin-based films that were *in situ* cross-linked with 1% $CaCl_{2(aq)}$ for 5 min was significantly high (> 15.5%) compared to not cross-linked pectin-based films that were completely soluble in water (Table 4.2).

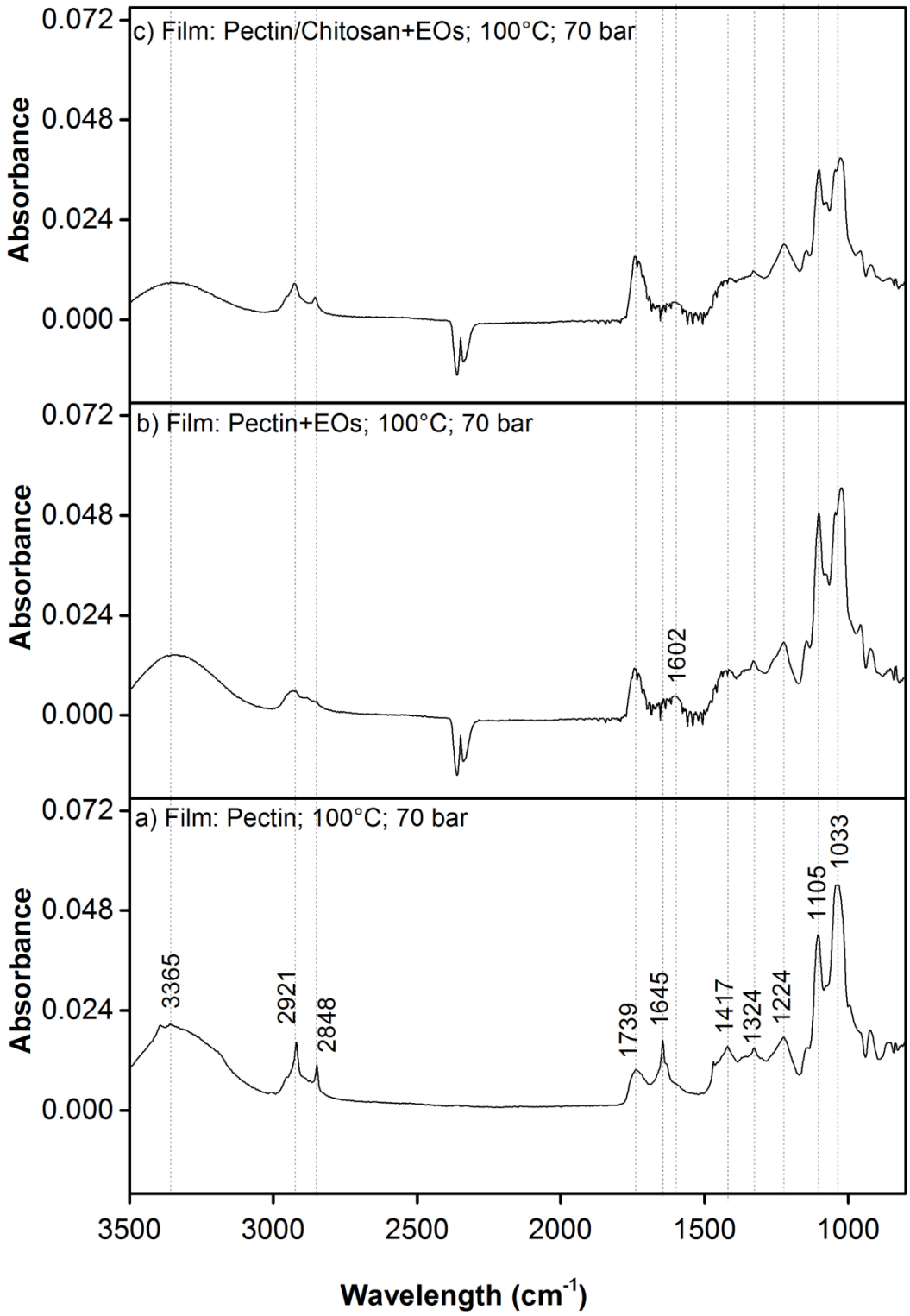

Figure 4.6 FT-IR spectra of pectin based films synthesized at 100˚C/70 bar: a) pectin film, b) essential oil-loaded pectin film and, c) essential oil-pectin/chitosan film.

In addition, Penhasi & Meidan (2014) found that high concentrations of $CaCl_2$ solution (up to 10%) and long cross-linking reaction time (1-30 min) can lead to very low water-soluble pectin films (1.75%). However, they reported that those cross-linking parameters could also trigger a precipitation reaction and calcium phosphate formation (Penhasi & Meidan, 2014). As such, the synthesized and *in situ* cross-linked pectin-based films were completely soluble in the 50 mM phosphate solution, suggesting that either the induced degree of pectin-calcium cross-linking was low and not able to hinder the absorption and diffusion of phosphate solution into the film core or in effect, calcium phosphate was formed, inducing the dissolution of films.

Table 4.2 Solubility of $CaCl_2$ cross-linked pectin-based films synthesized at 100˚C/70 bar.

System	Cross-linking parameters		Solubility test parameters		Solubility	Ref.
	$CaCl_2$ (%)	Reaction time (min)	Phosphate sol. [mM]	Exposure time (h)	(%)	
LM-pectin	10	30	0	20	1.75	Penhasi & Meidan (2014)
			25		21.8	
			50		41.9	
LM-pectin[a]	1	5	0	24	18±1	This study
			50		100	
LM-pectin+EOs[a]	1	5	0	24	17±2	This study
			50		100	
LM-pectin+EOs/ chitosan[a]	1	5	0	24	16±1	This study
			50		100	

[a]Films obtained at 100˚C/70 bar and *in situ* $CaCl_2$ cross-linked. EOs: clove and star anise essential oils.

4.5.3 Swelling index

According to Table 4.3, swelling index of $CaCl_2$ cross-linked pectin and essential oil-loaded pectin films synthesized at 100˚C/70 bar were comparable to swelling index exhibited by of acetone/glutaraldehyde cross-linked LM-pectin films obtained by Cabello et al. (2015) using the conventional casting method at 40˚C/atmospheric pressure. Conversely, the addition of chitosan during *sol* formation dramatically reduced pectin films water uptake from 560±47 to 243.1±11.02%. Even though the chitosan:pectin ratio in such films was quite low (1:8), hydrophobicity and ionic bounding of chitosan to pectin gel network could hinder or prevent water diffusion into the film core, reducing water absorption. Similarly, Marudova et al. (2004) reported a cross-linking effect between chitosan oligomers and low-methoxyl pectin (DE: 36%) due to a polyelectrolyte complex formation, where opposite charged polymers are attracted and bound together.

Table 4.3. Swelling index of $CaCl_2$ cross-linked pectin-based films synthesized at 100˚C/70 bar.

Film-system	[a]Glycerol (w/w %)	Swelling index (%)	Ref.
LM-pectin	4.5	559.6±47	This study
LM-pectin+EOs	2.5	532.9±50	This study
LM-pectin+EOs/chitosan	2.5	243.1±11	This study
LM-pectin	5	500±10	Cabello et al. (2015)

[a]Glycerol percentage (w/w) based on 100 g of pectin solution. EOs: clove and star anise essential oils.

Subcritical *sol* formation could be used as a potential green technology to produced pectin-based materials as it induces high molecular interaction and cross-linking effects, reducing the need of toxic chemical catalyzers as well as reducing the reaction time.

4.5.4 Mechanical properties

Mechanical properties, such as tensile strength (TS) and percent elongation at break (% E) of pectin-based films synthesized at 70˚C/1bar, 100˚C/70 bar as well as after *in situ* $CaCl_2$ cross-linking, are shown in Figure 4.7 and Figure 4.8, respectively.

Figure 4.7 shows that tensile strength of films was not significantly influenced by the addition of essential oil or chitosan when the operating conditions were kept constant either at 70˚C/1 bar or at 100˚C/70 bar. The tensile strength of essential oil-pectin/chitosan films, however, was significantly different when obtained at 70˚C/1 bar or at 100˚C/70 bar. The tensile strength of the essential oil-pectin/chitosan film synthesized at 70˚C/1 bar was significantly higher (18.3±0.2 MPa) than its homologous (8.4±0.2 MPa) obtained at subcritical conditions So, pectin/chitosan particle formation could be favored at 100˚C/70 bar, leading to films with an irregular and less smooth surface that failed easily during the tensile test.

In addition, the Young's modulus of essential oil-pectin/chitosan film (161±8MPa) produced at subcritical conditions (100˚C/70 bar) was between the Young's modulus of pectin film (138.5±13.4 MPa) obtained at 70˚C/1 bar and the Young's modulus of the essential oil-pectin/chitosan film (237±11MPa) obtained at 70˚C/1 bar (Figure 4.8). As the Young's modulus (E) is the slope of the stress-strain curve and describes the tensile elasticity, stiff materials exhibit high Young's modulus while flexible materials have low Young's modulus. Then, flexible and plastic essential oil-pectin/chitosan bioactive films were formed from the subcritical *sol* at 100˚C/70 bar. Moreover, those films could be potentially used in similar applications as pectin films obtained by the conventional casting method at 70˚C/1 bar.

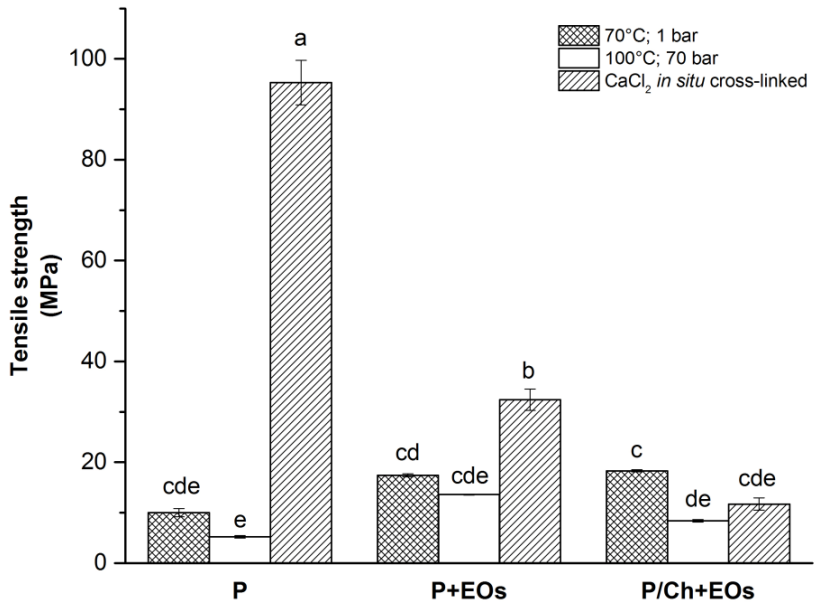

Figure 4.7 Tensile strength of synthesized pectin-based films. Bars followed by different letters (a-e) are significantly different (Tukey's HDS, $p < 0.05$).

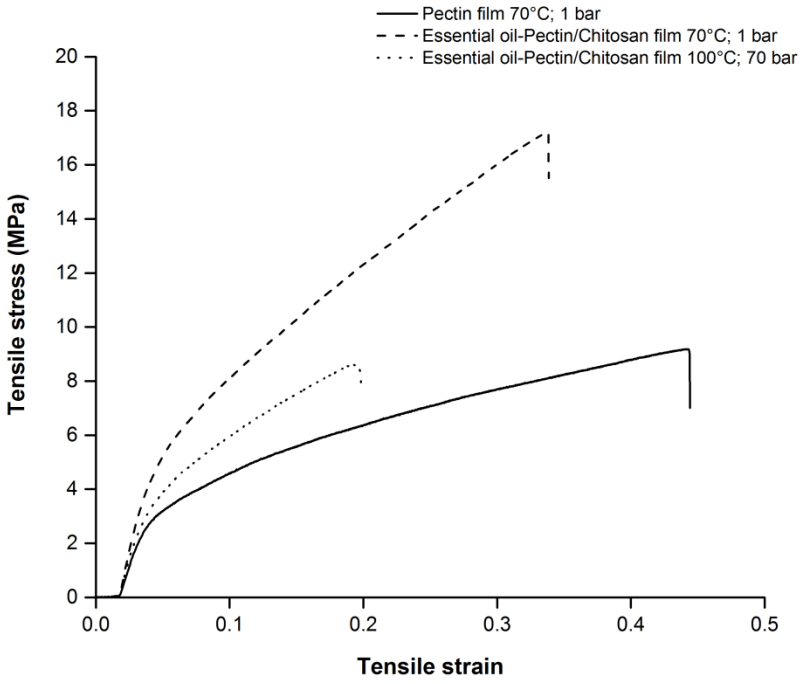

Figure 4.8 Stress-strain curve for essential oil-loaded pectin/chitosan films.

On the other hand, the *in situ* $CaCl_2$ cross-linking process dramatically increased the tensile strength of overall pectin-based films obtained at 100˚C/70 bar. As such, the tensile strength of pectin film increased from 5.2±0.2 to 95±4 MPa. The reported tensile strength for essential oil-loaded pectin and essential oil-loaded pectin/chitosan films were 32±2 and 12±1 MPa, respectively. Although *in situ* $CaCl_2$ cross-linking induced high tensile strength on films, it also led to deformations of film geometry. Essential oil-pectin and essential oil-pectin/chitosan films exhibited moderate deformations compared to the pectin film as depicted in Figure 4.9. Hence, those film deformations could be associated to crack initiation and propagation and, therefore to low tensile strength values for cross-linked essential oil-pectin and essential oil-pectin/chitosan films.

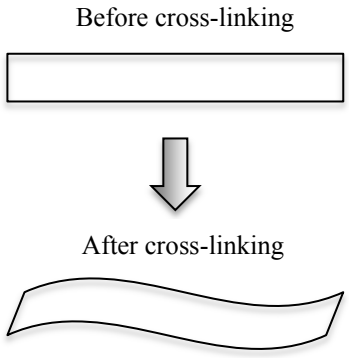

Figure 4.9 Scheme of film geometry before and after *in situ* $CaCl_2$ cross-linking process.

Figure 4.10 shows that operating conditions significantly influenced the percent of elongation at break of pectin-based films. Overall, films with higher percent of elongation at break were obtained at 70˚C/1 bar rather than at 100˚C/70 bar. For example, recorded percent of elongation at break of pectin films synthesized at 70˚C/1 bar and 100˚C/70 bar were 48±5 and 27±1%, respectively. Likewise, the percent of elongation at break of essential oil-loaded pectin films followed the same trend 28±2 (70˚C/1 bar) and 17.7±0.7 (100˚C/70 bar), respectively. It is

hypothesized that, during *sol* formation, subcritical conditions could induce new interactions between components (pectin, glycerol and essential oils), leading to film structures with interesting mechanical properties i.e. lower percent of elongation at break but similar tensile strength. On the other hand, the addition of essential oils or glycerol reduction from 4.5 to 2.5% significantly decreased the percent of elongation at break of pectin films obtained by conventional casting method. Meanwhile, there was not significant difference between percent of elongation at break of films synthesized at subcritical conditions suggesting that essential oils could enhance both mechanical and functional properties. Although the *in situ* $CaCl_2$ cross-linking process did not enhance or reduce the percent of elongation at break when a 5N force was applied to the essential oil-loaded pectin and the essential oil-loaded pectin/chitosan films synthesized at subcritical conditions, the percent of elongation at break when the same force was applied to the cross-linked pectin film obtained at 100˚C/70 bar, dramatically decreased, owing to inverse relationship between tensile strength and elongation percent.

Pectin-based *sols* obtained under subcritical conditions led to the production of films with high percent of elongation at break compared to various LM-pectin systems where toxic cross-linking agents were used as shown in Table 4.4.

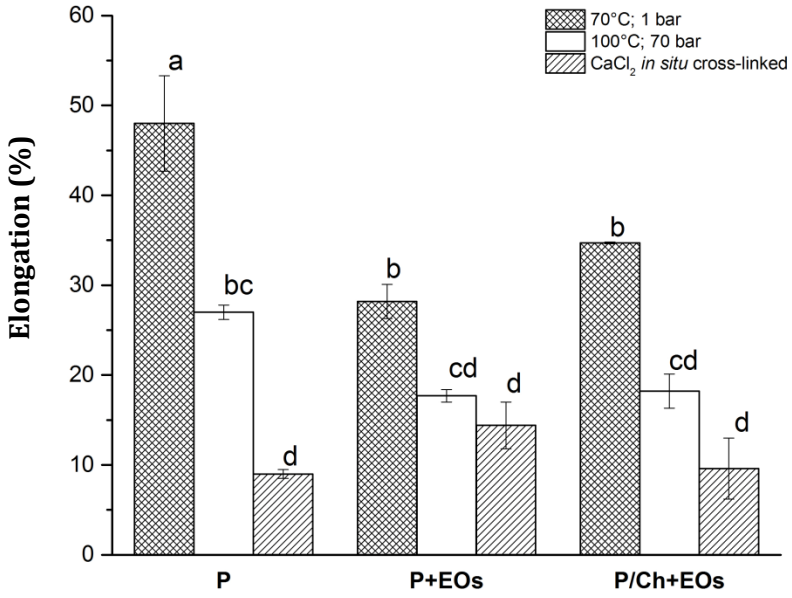

Figure 4.10 Elongation percentage of pectin-based films obtained at different pressure and temperature conditions. Bars followed by different letters (a-d) are significantly different (Tukey's HDS, $p < 0.05$).

Table 4.4. Mechanical properties of cross-linked pectin films.

System	Hydrogel formulation (%)			Mechanical properties		Ref.
	Pectin	Glycerol	Cross-linker	TS (MPa)	E (%)	
LM-pectin	2.5	1.25	1% $CaCl_2$	22±2	6±1	Galus & Lenart (2013)
LM-pectin	2	5	5% Glutaraldehyde	4.5±1.3	72±1.	Cabello et al. (2015)
LM-pectin	2	nr	10% $CaCl_2$	5.4	15	Penhasi & Meidan (2014)
HM-pectin/gelatin	2	20	0.5 M Periodic acid	48.3±0.7	4.6±1.2	Gupta et al. (2014)
Chitosan/CEO	Nr	1.5	nr	29. 2 ±2.2	3.6±0.4	Ojagh et al. (2010)
LM-pectin[a]	3	4.5	1 % $CaCl_2$	95±4	9.0±0.3	This study
LM-pectin+EOs[a]	3	2.5	1 % $CaCl_2$	32±2	14±2	This study
LM-pectin+EOs/ chitosan[a]	3	2.5	1 % $CaCl_2$	12±1	10±2	This study

LM: low methoxyl pectin; HM: high methoxyl pectin; CEO: cinnamon essential oil; EOs: clove and star anise essential oils; a: Films synthesized at 100˚C/70 bar and $CaCl_2$ cross-linked; TS: tensile strength; E: percent elongation at break; nr: not reported.

4.5.5 Optical properties

Figure 4.11 shows that the optical properties of pectin based-films were significantly influenced by the addition of essential oils and chitosan as well as by the *sol* formation method, respectively. In general, essential oil-loaded pectin/chitosan films synthesized either at 70˚C/1 bar or at 100˚C/70 bar had the highest transparency values (Figure 4.11a), where higher values represent lower transparency of the films. The emulsifying properties of pectin and essential oils and, the polyelectrolyte complex formation between pectin and chitosan could potentially cause a reduction of film transparency. Besides, *sol* formation under subcritical conditions led to pectin-based films with significantly lower transparency (high transparency value) compared to films obtained with the conventional method. However, transparency of essential oil-loaded pectin film synthesized at 100˚C/70 bar did not follow such a trend, suggesting that a reduction of the emulsion droplet size could occur due to the use of high-pressure, causing a subsequent increase of film transparency. Thus, transparency of oil-loaded pectin films could be potentially enhanced under subcritical conditions.

On the other hand, essential oil-pectin/chitosan films obtained at subcritical conditions exhibited the lowest transparency, indicating stronger electrostatic interactions between pectin and chitosan. Furthermore, the formation of core-shell particles was observed in the *sol* obtained at 100˚C/70 bar. A water core-shell pectin/chitosan particles could be formed due to enhanced physico-chemical properties of water at subcritical state as well as due to molecular reorganization according to hydrophilicity of compounds as schematized in Figure 4.12a.

Also, essential oil core-shell pectin/chitosan particles were formed after the addition of $CaCl_2$ due to potential molecular reorganization or interactions in the film forming solution as

shown in Figure 4.12b. These core-shell particles could be used for loading either water-soluble or oil-soluble bioactives for potential applications in the food, cosmetic and biomedical areas.

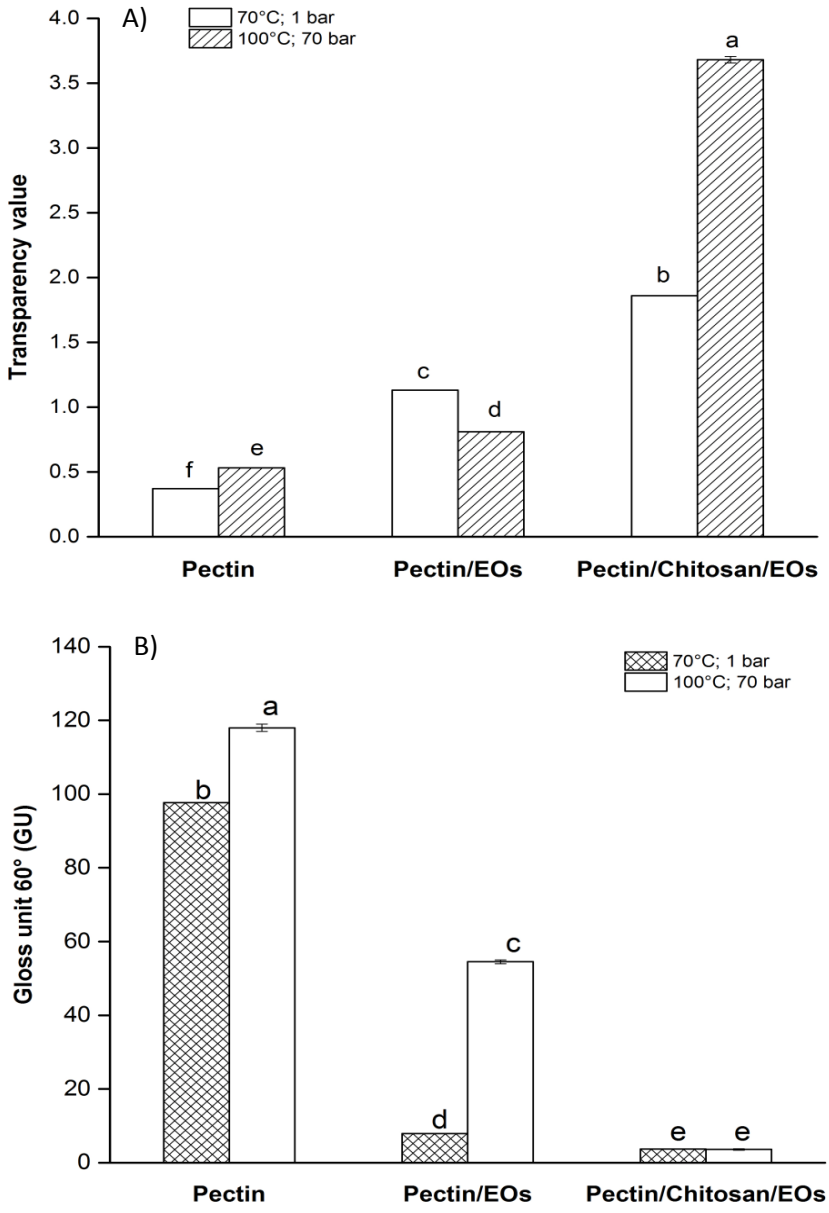

Figure 4.11 Optical properties of pectin-based films: a) transparency and, b) gloss. EOs: clove and star anise essential oils. Bars followed by different letters (a-f) are significantly different (Tukey's HDS, $p < 0.05$).

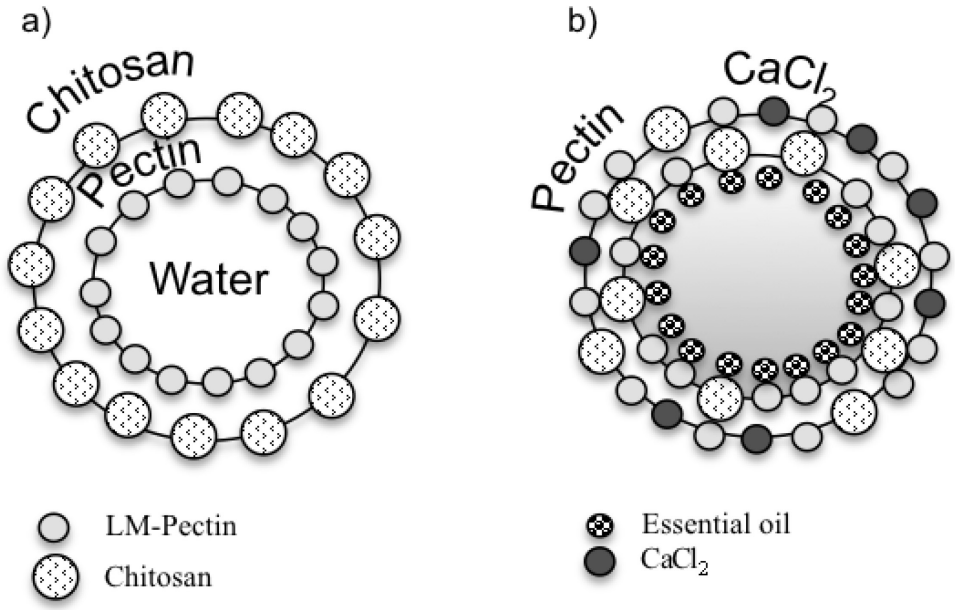

Figure 4.12 Schemes of core-shell particles formed at 100˚C/70 bar: a) water core-shell pectin/chitosan particle and, b) essential oil core-shell pectin/chitosan particle.

In addition, Figure 4.11b shows that pectin and essential oil-loaded pectin films obtained at subcritical conditions exhibited a significant increase of gloss compared to their analogues produced at 70˚C/1 bar. High temperature (100˚C) and pressure (70 bar) could induce pectin hydration and interaction with glycerol and essential oils, leading to very homogeneous solutions prior to hydrogel formation. Hence, pectin and essential oil-pectin films with smoother surface and high gloss (> 60 GU) were obtained at subcritical conditions.

4.5.6 Functional properties

Figure 4.13 shows the amount of phenolic compounds released from pectin-based films obtained using both conventional casting method and subcritical fluid technology as well as after the additional *in situ* $CaCl_2$ cross-linking process.

According to Figure 4.13, total phenolics released from essential oil-loaded pectin (18.7±0.2 mg GAE/g film) and essential oil-loaded pectin/chitosan (18.6±0.4 mg GAE/g film)

films synthesized at 70˚C/1 bar were significantly higher compared to their analogues obtained at 100˚C/70 bar. Accordingly, the lower amount of phenolic compounds was released from essential oil-loaded pectin (8.7±0.3 mg GAE/g film) synthesized under subcritical fluid conditions, owing to induced ester bonding between eugenol from clove essential oil and carboxylate group of pectin as shown by the FT-IR spectra (Figure 4.6). The addition of chitosan, however, enhanced phenolics released (11.8±0.3 mg GAE/g film) from films obtained at 100˚C/70 bar, suggesting that chitosan exerted a hindering effect for phenolic-pectin interaction. Likewise, the *in situ* cross-linking process could favor the ionic cross-linking between deprotonated carboxylate ion groups of pectin and calcium (egg-box mechanism), limiting the phenolic-pectin binding and therefore favoring their release.

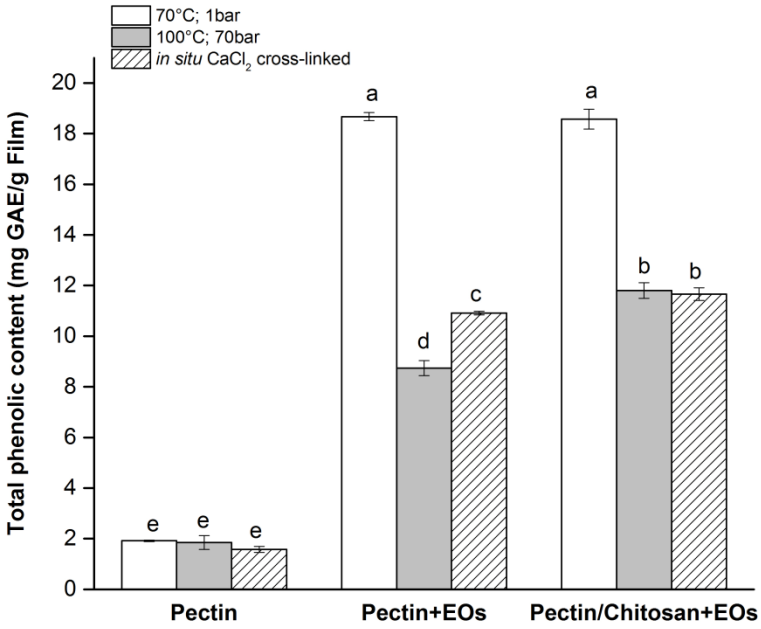

Figure 4.13 Total phenolic content of pectin-based films. EOs: clove and star anise essential oils. Bars followed by different letters (a-e) are significantly different (Tukey's HDS, $p < 0.05$).

Figure 4.14 shows that there was a direct relationship between the inhibition DPPH percentage and the total phenolic content of pectin-based films. As such, the addition of essential oils or essential oils and chitosan at 70˚C/1 bar led to films with the highest inhibition DPPH percentage (> 80%). On the other hand, the inhibition DPPH percentage of essential oil-loaded pectin (62±1%) and cross-linked essential oil-loaded pectin (70.2±0.8%) films synthetized at subcritical fluid conditions were the lowest. This reduction of DPPH inhibition could be related to the volatility of essential oils and esterification reactions at subcritical fluid conditions. Although there was a difference between DPPH inhibition of pectin-based films obtained at 70˚C/1 bar or at 100˚C/70 bar, subcritical fluid technology showed to be a novel and feasible alternative to produce bioactive films, in particular, for potential wound dressing materials, where a controlled release rate is desired.

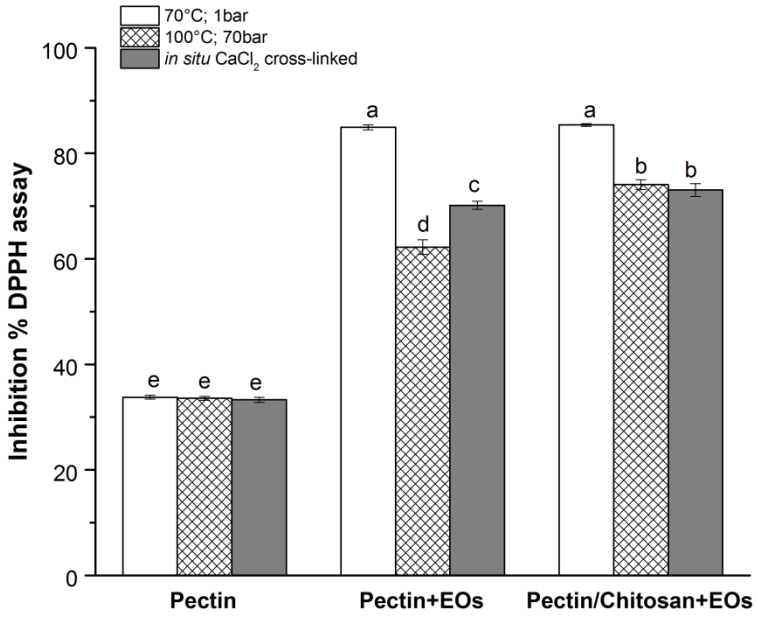

Figure 4.14 Inhibition DPPH percentage of pectin-based films. EOs: clove and star anise essential oils. Bars followed by different letters (a-e) are significantly different (Tukey's HDS, $p < 0.05$).

4.6 Screening of pectin-based cryogels properties

Pectin-based cryogels were also assessed based on FT-IR analysis, density, porosity, phenolics release and DPPH inhibition.

4.6.1 Physico-chemical properties

Figure 4.15 shows FT-IR spectra of essential oil-loaded pectin/chitosan cryogels obtained at 70˚C/1 bar, 100˚C/70 bar as well as after *in situ* $CaCl_2$ cross-linking process. Essential oil-pectin/chitosan cryogel obtained at 100˚C/70 bar exhibited strong intensity peaks at 3330 cm^{-1} (OH, stretching), 1726 cm^{-1} (C=O, esterified) and 1230 cm^{-1} (C-C) compared to intensity of the peaks of essential oil-loaded pectin/chitosan synthesized at 70˚C/1 bar, suggesting that esterification reactions are favored when subcritical water and freeze drying process are combined (Figure 4.15a and b). Moreover, the intensity bands of such peaks were stronger in the cryogels rather than in their analogue films (Figure 4.6). On the other hand, FT-IR spectra of *in situ* essential oil-loaded pectin/chitosan cryogels showed that there was a strong interaction between pectin and $CaCl_2$, that led to a dramatic decrease on peaks intensity at 1726 cm^{-1} (C=O, esterified), 1102 cm^{-1} and 1027 cm^{-1} as well as no peak formation at 3330 cm^{-1} (OH, stretching) and 1230 cm^{-1}. Thus, cryogels with different physical characteristics, such as bulk density and porosity were expected for each condition.

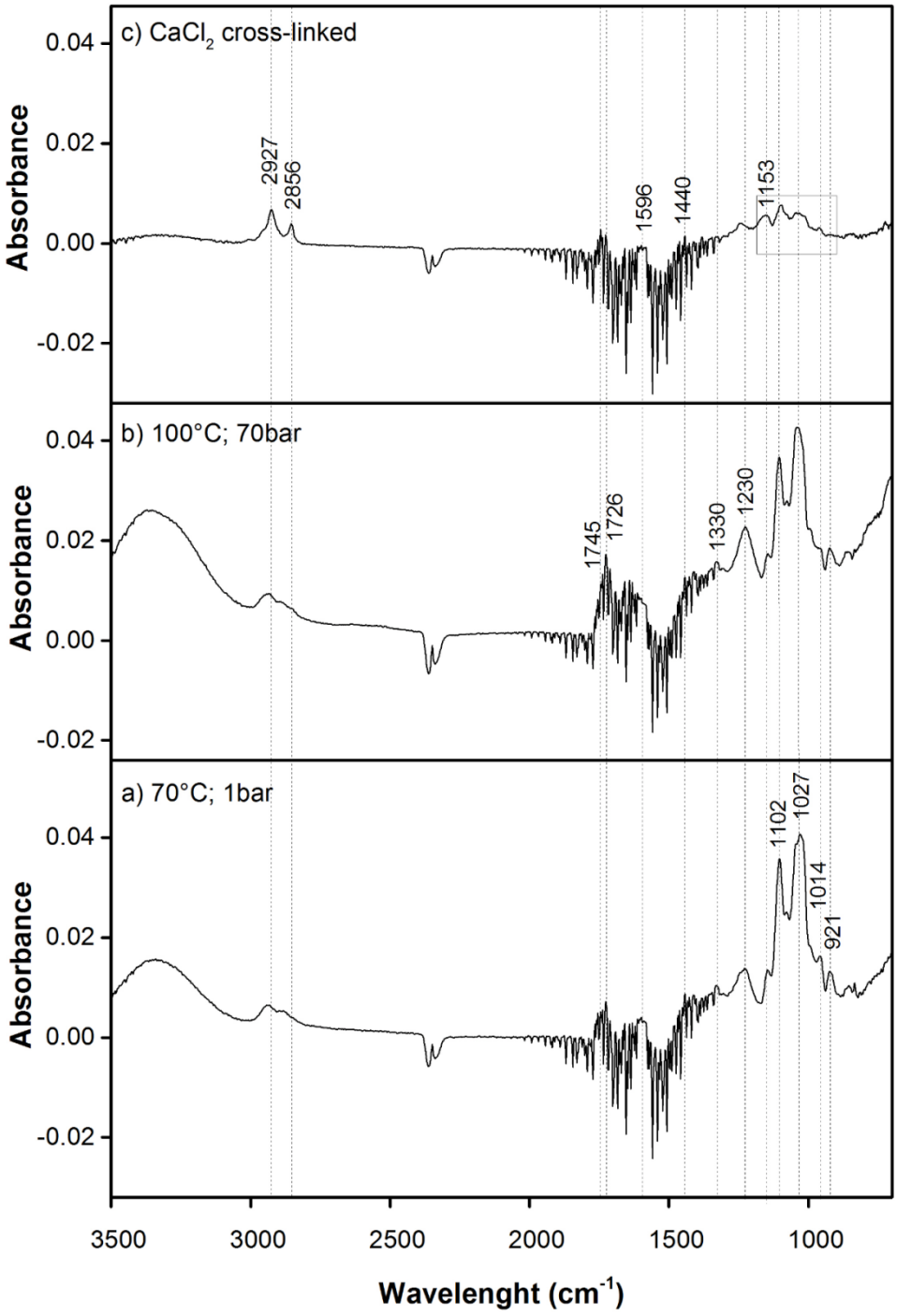

Figure 4.15. FT-IR spectra of essential oil-loaded pectin/chitosan cryogels obtained at: a) 70˚C/1 bar, b) 100˚C/70 bar and, c) *in situ* $CaCl_2$ cross-linked.

Figure 4.16 shows that bulk density of pectin-based cryogels did not change significantly when temperature and pressure increased from 70 to 100˚C and from 1 to 70 bar, respectively. Likewise, the addition of essential oil and chitosan did not have a significant effect on density of pectin-based cryogels, mainly due to their ratios in the formulation (Table 4.1). Lightweight cryogels with densities from 0.031 to 0.046 g/cm^3 were obtained either at 70˚C/1 bar or 100˚C/70 bar. Moreover, densities of obtained pectin-based cryogels were lower than the density of LM-pectin aerogels (0.077±0.002 g/cm^3) produced by Tkalec et al. (2015) using supercritical CO_2 drying. The *in situ* $CaCl_2$ cross-linking significantly increased the bulk density of essential oil-pectin (0.056±0.003 g/cm^3) and essential oil-pectin/chitosan (0.067±0.003 g/cm^3) cryogels. The high surface area of cryogels could favor pectin-$CaCl_2$ cross-linking, leading to calcium-enriched cryogels with a compact structure. Thus, *in situ* $CaCl_2$ cross-linking could be a feasible method to obtain porous biodegradable materials for potential biomedical applications, such as scaffolds for bone regeneration.

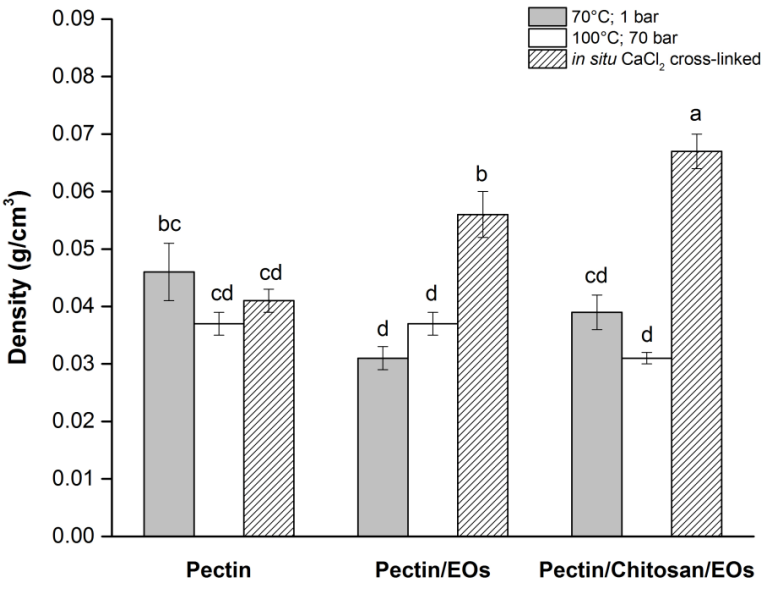

Figure 4.16 Density of pectin-based cryogels. Bars followed by different letters (a-d) are significantly different (Tukey's HDS, $p < 0.05$).

According to Figure 4.17, porosity of pectin-based cryogels was also significantly influenced by the *in situ* cross-linking process, leading to sponge-like materials with decreased porous size. Besides, the recorded porosity of cryogels obtained at either 70˚C/1 bar or 100˚C/70 bar was high (> 80%) and, comparable to polyvinyl alcohol (PVA) scaffolds with a ranged porosity of 40-80% (Nanda et al., 2013).

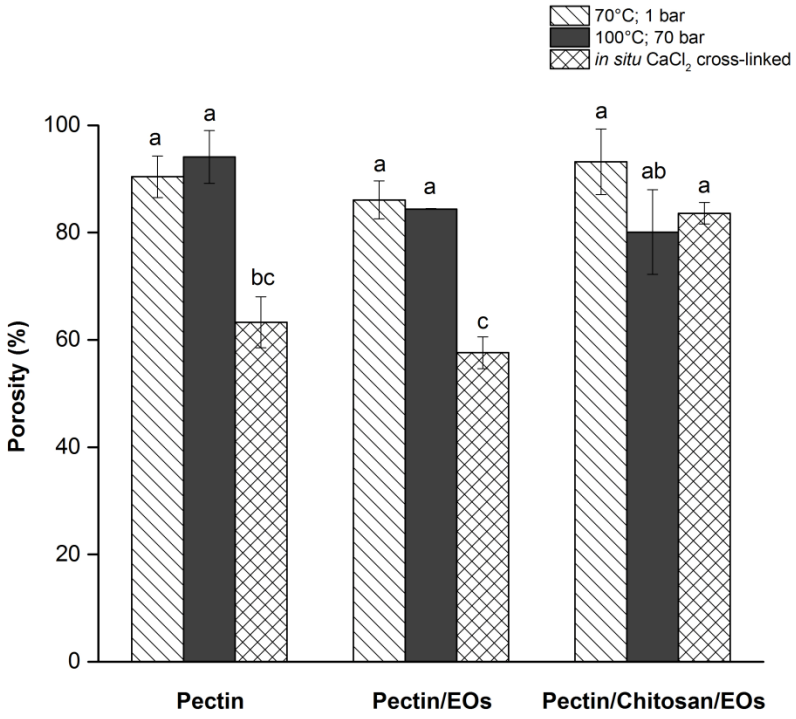

Figure 4.17 Porosity of pectin-based cryogels. Bars followed by different letters (a-c) are significantly different (Tukey's HDS, $p < 0.05$).

4.6.2 Functional properties

Figure 4.18a shows that cryogel formation by freeze-drying process favored phenolic release from pectin-based gels obtained at either 70˚C/1 bar or at 100˚C/70 bar compared to their film analogues (Figure 4.13). As cryogels are tridimensional highly porous structures, diffusion of entrapped solutes is commonly enhanced. Although changing from 2D to 3D structure could increase phenolics extraction, the *in situ* $CaCl_2$ cross-linking could also influence such

extraction. Indeed, the phenolic content released from the essential oil-pectin cryogel increased 62.8% compared to its film analogue obtained at the same temperature and pressure conditions. Likewise, the increased phenolic content released from the cross-linked essential oil-pectin/chitosan cryogel compared to its film analogue (Figure 4.13) was 70.62%. Therefore, different solvent exchange processes applied to hydrogels can lead to the production of bioactive gels with different features and applications.

Figure 4.18b shows the antioxidant activity according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay of pectin-based cryogels synthesized at 70˚C/1 bar, 100˚C/70 bar as well as after *in situ* $CaCl_2$ cross-linking process. As mentioned earlier, the tridimensional structure of cryogels enhances mass transfer phenomena, favoring extraction and reaction mechanisms, leading to gels with higher DPPH inhibition percentage. Thus, the *in situ* cross-linking process significantly increased the DPPH inhibition capacity of essential oil-loaded pectin-based cryogels. Hence, the recorded DPPH inhibition percentage of cross-linked essential oil-pectin and cross-linked essential oil-pectin/chitosan cryogels compared to their cross-linked analogues 2D structures increased from 70 to 89% and 73 to 86% (Figure 4.14), respectively.

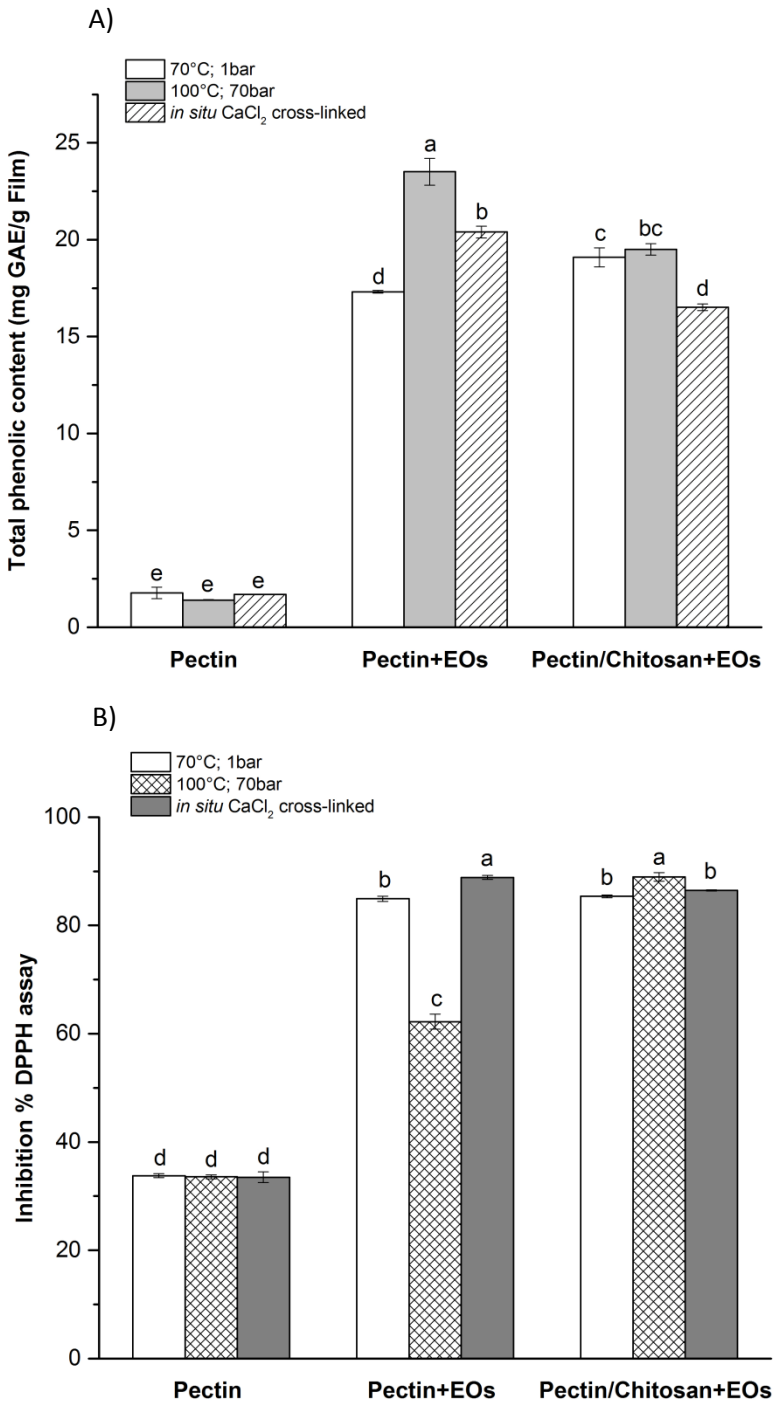

Figure 4.18 Functional properties of pectin-based cryogels: a) Total phenolics content and, b) Antioxidant activity according to the DPPH inhibition. Bars followed by different letters (a-d) are significantly different (Tukey's HDS, $p < 0.05$).

4.7 Conclusions

- For the first time, subcritical water technology was used as a novel approach to *sol* formation prior to the synthesis of pectin-based biomaterials. The essential oil-pectin-based *sol* obtained with subcritical aqueous citric acid at 100˚C/70 bar led to flexible films with physico-chemical, mechanical and functional properties comparable to pectin-based films produced by the traditional *sol-gel* method.
- The influence of glycerol on low methoxyl-pectin hydrogel formation was mainly due to esterification or hydrogen bond formation as OH groups from glycerol were prone to react with the carbonyl group (C=O) and/or the glycosidic bond (C-O-C) of homogalacturonan pectin fragment. Furthermore, such interactions were favored during *sol* formation at 100˚C/70 bar.
- *Sol* formation under subcritical conditions could be considered as a feasible alternative to produce pectin-based films with a controlled time of bioactive release, without shortcomings associated to the use of cytotoxic cross-linking reagents.
- The proposed process integration, subcritical *sol* formation and freeze-dried *gel*, could be potentially used to obtain lightweight and highly porous bioactive pectin-based cryogels for tissue repair and regeneration.
- The *in situ* $CaCl_2$ cross-linking process significantly increased the tensile strength of formulated pectin-based films. The pectin film synthesized at 100˚C/70 bar exhibited the highest tensile strength (95 ± 4 MPa).
- The solubility of pectin-based film in water was significantly influenced by *in situ* $CaCl_2$ cross-linking process. The solubility of pectin-based films in water decreased approximately 84% compared to those that were not *in situ* cross-linked.

4.8 Recommendations

Recommendations for future studies are:

- Determine the optimal ratio of chitosan/pectin to obtain 2D and 3D biocomposites with improved tensile strength and limited solubility on aqueous phosphate media to facilitate biomedical applications.
- Study the effect of pH from 3 to 7 on subcritical pectin/chitosan *sol* formation, considering the p*Ka* values of pectin (3.5-4.5) and chitosan (6.2-7).
- Evaluate and characterize pectin/chitosan microparticle formation under subcritical conditions as in the present study water core-shell pectin/chitosan and essential oil core-shell $CaCl_2$ cross-linked pectin/chitosan particles were co-obtained at 100˚C/70 bar.
- Studies on $CaCl_2$ cross-linking kinetics as function of time and $CaCl_2$ concentration for selected systems (chitosan:pectin ratio and essential oil:pectin ratio) could lead to bioactive composites with tailored solubility suitable for internal or external use.
- Determine the antimicrobial and antifungal properties of formulated essential oil-loaded pectin and essential oil-loaded pectin/chitosan films and cryogels.
- Measure the tensile and compressive strength of pectin-based cryogels for potential tissue engineering applications.
- Determine the cell toxicity of pectin-based biomaterials according to the MTT Cell proliferation assay.
- Examine the bioadhesive property of pectin-based films and cryogels to membranes or skin.

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Appendix

Table A1. Pomegranate pectic polysaccharides characterization.

Run #	Yield (%)		Average*	Degree of esterification (%)		Average*	GalAc content (%)		Average*	GalAc content HPAEC (%)		Average*
	Analysis 1	Analysis 2		Analysis 1	Analysis 2		Analysis 1	Analysis 2		Analysis 1	Analysis 2	
Subcritical conditions: 100°C/50 bar												
1	4.18	3.64	3.91 b	60.65	60.00	60.32 c	68.91	70.00	69.45 b	16.49	15.90	16.17 ab
2	6.45	5.52	5.98 b	53.90	51.30	52.55 d	75.30	75.30	75.25 a	17.90	16.50	17.22 a
3	5.26	6.88	6.07 b	38.70	39.00	38.85 e	59.20	60.00	59.61 c	13.40	13.00	13.21 b
Subcritical conditions: 120°C/50 bar												
4	9.73	9.99	9.86 a	65.80	66.70	66.24 b	77.90	76.20	77.04 a	15.10	13.50	14.29 ab
5	11.21	10.34	10.77 a	61.80	63.20	62.50 c	73.90	75.00	74.43 a	14.00	12.70	13.32 b
6	9.77	10.98	10.37 a	72.70	71.80	72.26 a	74.70	73.90	74.32 a	9.30	10.20	9.71 c

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A2. Functional properties of isolated pomegranate pectic polysaccharides.

Run #	Total phenolic content (mg GAE/g PPs)			Average*	DPPH Inhibition (%)			Average*
	Analysis 1	Analysis 2	Analysis 3		Analysis 1	Analysis 2	Analysis 3	
Subcritical conditions: 100°C/50 bar								
1	72.70	73.20	72.30	72.733 d	86.50	88.10	87.20	87.26 c
2	134.70	136.10	133.30	134.70 c	89.40	88.70	88.50	88.86 b
3	170.40	169.10	171.70	170.40 a	88.50	85.50	88.80	87.60 b
Subcritical conditions: 120°C/50 bar								
4	151.70	155.40	153.10	153.40 b	90.00	90.70	90.30	90.33 a
5	150.30	153.10	155.40	152.93 b	90.30	90.10	90.30	90.23 a
6	168.30	168.70	171.10	169.36 a	89.50	89.60	90.00	89.70 ab

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A3. Mechanical properties of pectin-based films.

Run #	Tensile strength (MPa)		Average*	Elongation (%)		Average*
	Analysis 1	Analysis 2		Analysis 1	Analysis 2	
70°C /1 bar						
1	10.80	9.20	10.00 cde	51.70	44.30	48.00 a
2	17.20	17.70	17.45 cd	29.60	26.80	28.20 b
3	18.50	18.10	18.30 c	34.60	34.80	34.70 b
Subcritical conditions: 100°C /70 bar						
4	5.40	5.00	5.20 e	27.50	26.40	26.95 bc
5	13.70	13.50	13.60 cde	18.20	17.30	17.75 cd
6	8.60	8.10	8.35 de	19.50	16.80	18.15 cd
***in situ* $CaCl_2$ cross-linking**						
7	99.60	90.90	95.25 a	8.60	9.30	8.95 d
8	34.60	30.30	32.45 b	12.50	16.20	14.35 d
9	10.40	12.90	11.65 cde	12.00	7.20	9.60 d

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A4. Functional properties of pectin-based films.

Run #	Total phenolic release (mg GAE/g film)			Average*	DPPH Inhibition (%)			Average*
	Analysis 1	Analysis 2	Analysis 3		Analysis 1	Analysis 2	Analysis 3	
70°C /1 bar								
1	1.94	1.94	1.89	1.923 e	33.50	33.50	34.20	33.73 e
2	18.49	18.81	18.72	18.67 a	85.40	84.40	85.00	84.93 a
3	18.34	18.34	19.02	18.56 a	85.10	85.50	85.60	85.40 a
Subcritical conditions: 100°C /70 bar								
4	2.11	1.88	1.57	1.853 e	34.00	33.30	33.50	33.6 e
5	8.72	9.05	8.45	8.74 d	60.80	63.50	62.40	62.23 d
6	11.56	12.15	11.69	11.8 b	73.20	73.90	75.10	74.06 b
***in situ* $CaCl_2$ cross-linking**								
7	1.5	1.67	1.67	1.613 e	33.70	33.40	32.70	33.26 e
8	10.93	10.98	10.84	10.91 c	71.00	69.90	69.60	70.16 c
9	11.38	11.7	11.88	11.65 b	73.40	74.00	71.70	73.03 b

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A5. Optical properties of pectin-based films.

Run #	Transparency value			Average*	Gloss (GU)			Average*
	Analysis 1	Analysis 2	Analysis 3		Analysis 1	Analysis 2	Analysis 3	
70°C/1 bar								
1	0.37	0.37	0.37	0.371 f	97.60	97.70	97.70	97.66 b
2	1.13	1.14	1.14	1.134 c	7.90	8.00	7.90	7.933 d
3	1.86	1.86	1.87	1.864 b	3.80	3.70	3.70	3.733 e
Subcritical conditions: 100°C/70 bar								
4	0.53	0.53	0.53	0.5313 e	117.00	118.00	119.00	118.0 a
5	0.81	0.81	0.81	0.810 d	54.70	54.00	54.90	54.53 c
6	3.71	3.69	3.66	3.683 a	3.50	3.80	3.60	3.633 e

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A6. Physico-chemical properties of pectin-based cryogels.

Run #	Bulk density (g/cm^3)		Average*	Porosity (%)		Average*
	Analysis 1	Analysis 2		Analysis 1	Analysis 2	
70°C /1 bar						
1	0.049	0.043	0.046 b	93.20	87.70	90.45 ab
2	0.032	0.030	0.031 b	88.60	83.60	86.10 ab
3	0.040	0.037	0.038 b	97.50	88.90	93.20 a
Subcritical conditions: 100°C /70 bar						
4	0.036	0.038	0.037 b	97.60	90.70	94.15 a
5	0.032	0.033	0.032 b	84.40	84.30	79.50 abc
6	0.032	0.031	0.031 b	85.70	74.50	80.10 abc
***in situ* $CaCl_2$ cross-linking**						
7	0.042	0.039	0.040 b	66.60	59.90	63.25 c
8	0.059	0.053	0.075 a	59.70	55.40	73.90 bc
9	0.069	0.065	0.070 a	82.20	85.00	83.60 ab

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Table A7. Functional properties of pectin-based cryogels.

Run #	Total phenolic release (mg GAE/g cryogel)			Average*	DPPH Inhibition (%)			Average*
	Analysis 1	Analysis 2	Analysis 3		Analysis 1	Analysis 2	Analysis 3	
70°C /1 bar								
1	1.73	2.09	1.51	1.78 e	33.50	34.20	33.50	33.73 d
2	17.34	17.37	17.23	17.31 d	84.40	85.00	85.40	84.93 b
3	18.88	18.73	19.64	19.08 c	85.50	85.60	85.10	85.40 b
Subcritical conditions: 100°C /70 bar								
4	1.39	1.43	1.39	1.40 e	33.30	33.50	34.00	33.60 d
5	22.72	23.93	23.89	23.51 a	63.50	62.40	60.80	62.23 c
6	19.34	19.27	19.82	19.47 bc	89.40	89.40	88.10	88.96 a
***in situ* $CaCl_2$ cross-linking**								
7	1.69	1.69	1.69	1.69 e	32.30	34.20	33.90	33.46 d
8	20.05	20.59	20.55	20.39 b	89.20	88.80	88.50	88.83 a
9	16.31	16.64	16.57	16.50 d	86.50	86.50	86.50	86.50 b

*Means in a column followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)