Pressurized fluid extraction of anthocyanins from cranberry pomace and its use in bioactive food coatings for almonds

by

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ABSTRACT

About 94% of total cranberry production is mainly used by the juice industry, generating cranberry pomace, which is a rich source of anthocyanins. Pressurized fluids have been used to extract phytochemicals from different by-products. Such phytochemicals can be used in edible food coatings to prevent food deterioration reactions hence consumer rejection. The main objective of this study was to extract anthocyanins and total phenolics from cranberry pomace with pressurized fluids and use the extracts obtained in pectin and pectin+beeswax based coatings to prevent deterioration reactions of almonds. Pressurized fluid extractions were performed in a high pressure reactor using different solvents (water, ethanol, water+30-70%ethanol and water+5%citric acid) at 120–160ºC and 50–200 bar. Pressurized ethanol extractions were also performed at 50 bar and 40-100ºC. Spectrophotometric methods were used to determine total anthocyanin content (mg cyanidin 3-glucoside equivalent), total phenolic content (mg gallic acid equivalent) and antioxidant activity (µmol trolox equivalent). Individual anthocyanins were also quantified by HPLC-UV. Then, edible coatings, pectin based and pectin+beeswax based, were developed with the addition of cranberry extracts at ratios of 1:1 and 1:3 pectin:extract (w/w) and applied to almonds using the spraying method. Coated and uncoated almonds were stored at 40ºC and 50%RH for 90 days. Incipient rancidity of the coated and uncoated almonds was analyzed using a spectrophotometric method and almond fatty acid composition was analyzed using GC. High anthocyanin content was extracted using pressurized ethanol at 50 bar and 60-120ºC with an extraction range of 3.89-4.21 mgCy3GE/g d.w. with no significant difference between those conditions. High concentrations of cyanidin 3-arabinoside and peonidin 3-galactoside were obtained after all extractions. High total phenolic contents were obtained using pressurized ethanol30%+water at 140ºC (42.48±7.82 mg GAE/g d.w.) and 160ºC

(41.19±2.07 mg GAE/g d.w.). The use of pressurized ethanol resulted in better Pearson correlation value (P=0.84) between total anthocyanins and antioxidant capacity compared to pressurized water (P=-0.35). This last value suggests possible deterioration of anthocyanins into other phenolic compounds like phloglucinaldehyde and 4-hydroxybenzoic acid. The use of bioactive coatings on almonds had no significant impact in neither the fatty acid composition nor the incipient rancidity after storage at 40ºC and 50% RH for 90 days. Also, no significant difference was observed in incipient rancidity with a peroxide value range of 2.5-4.5 mEq/kg oil.

This thesis has shown that pressurized fluid extraction is an environmentally friendly alternative to extract anthocyanins and total phenolics from cranberry pomace and that the selectivity of anthocyanins with ethanol is higher compared to water and ethanol+water mixtures. Such extracts could be used as natural antioxidants or natural colorants. Also, the development and application of pectin and pectin+beeswax coating with cranberry extract was achieved. These bioactive coatings could be applied to nuts, fruits and candies.

Keywords: *Anthocyanins, cranberry pomace, pressurized fluids, almonds, bioactive coatings.*

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ABBREVIATIONS

A	Absorbance
ABTS	Radical scavenging assay using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AVIs	Anthocyanin vacuolar inclusions
BHT	Butylhydroxytoluene
BW	Beeswax
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	α-Linoleic acid
CMC	Carboxymethyl cellulose
Cy3GE	Cyanidin 3-glucoside equivalents
d.w.	Dry weight
ESI	Electron spray ionization
f.w.	Fresh weight
FRAP	Ferric reducing ability of plasma
GAE	Gallic acid equivalents
GC	Gas chromatography

GRAS	Generally recognized as safe
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography using an ultraviolet detector.
LDPE	Low density polyethylene
MANOVA	Multivariate analysis of variance
MS	Mass spectrometry
MW	Molecular weight
ORAC	Oxygen radical absorbance capacity
P	Pearson correlation value
p	p-value statistics
PE/EVOH/PP	Polypropylene/ethylene-vinyl alcohol/polypropylene
PET	Polyethylene terephthalate
PPO	Propylene oxide
PV	Peroxide value
RH	Relative humidity
sCW	Subcritical water
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
v/v	Volume/volume
w/w	Weight/weight

Chapter 1: Introduction & objectives

1.1. **Introduction**

In 2014, 98% of the worldwide cranberry (*Vaccinium oxycoccus*) production was mainly from the United States of America (58%), Canada (27%) and Chile (13%) (FAO, 2016). Cranberry production in Canada increased from 2007 (77,923 tons) to 2016 (175,066 tons) (Statistics Canada, 2016). This production increase is due to the increased consumption because of the potential health benefits reported when consuming cranberry juice, including cardiovascular benefits and the prevention of urinary tract infection (Yung et al., 2013; Maki et al., 2016). These health benefits can be attributed to the phenolic content found in cranberries (21.1±0.7 mg of gallic acid equivalent (GAE)/g dry weight (d.w.)), which is higher compared to strawberries (17.7±0.2 mg GAE/g d.w.), gooseberries (12.4±0.6 mg GAE/g d.w.), black currants (20.3±0.7 mg GAE/g d.w.) and red currants (12.6±0.2 mg GAE/g d.w.) (Kähkönen et al., 1999). Such phenolic compounds can be found at a higher concentration in the cranberry skin rather than the flesh, making the cranberry by-product from the juice industry, or cranberry pomace, a potential source of phenolic compounds (Brown et al., 2011).

Cranberry has mainly anthocyanins (39%), vitamin C (23%), procyanidin dimers (12%), flavonols (10%), chlorogenic acid (2%) and other unidentified peaks (14%) (Borges, Degeneve, Mullen and Crozier, 2009). The most abundant anthocyanin found in cranberry pomace is cyanidin 3–arabinoside (0.49±0.07 mg/g d.w.) followed by peonidin-3-arabinoside (0.27±0.01 mg/g d.w.), peonidin 3-galactoside (0.20±0.01 mg/g d.w.) and cyanidin 3-galactoside (0.13±0.00 mg/g d.w.) (White, Howard and Prior, 2009). Traditional solvent extraction has used

petrochemical solvents such as methanol+HCl (99:1. v/v) to extract anthocyanins from cranberry pomace with high anthocyanin extractions (4.51±0.11 mg Cy3GE/g d.w.) (Klavins L., Kviesis and Klavins M., 2017). However, those solvents are non-GRAS (generally recognized as safe) solvents and could be a potential hazard, leading to an additional removal step. Another method to obtain anthocyanin from cranberry pomace used supercritical CO_2 extraction at 80 bar and 60ºC, resulting in a low anthocyanin extraction (0.17 mg Cy3GE/g d.w.) (Laroze et al., 2010).

Recently, subcritical water extraction and pressurized hot fluid extraction have been used to extract phytochemicals from different sources such as raspberry pomace (Kryževičiūtė, Kraujalis & Venskutonis, 2016), grape pomace (Duba et al., 2015) and potato peel (Singh & Saldaña, 2011). This green processing technique consists in applying high pressure and high temperature to decrease electrostatic interaction between water molecules, resulting in a pH reduction (Plaza and Turner, 2015). A USA patent 9,084,948 (Mazza & Pronyk, 2015) reported a method of extraction using low polarized water to obtain phenolic compounds from cranberry pomace using only low polarity water but anthocyanin content was not reported.

Bioactive extracts can extend food shelf life by working synergistically with other food preservation techniques like food coatings, which are made of polysaccharides, lipids and proteins (Baldwin, 2007). Pectin, a natural polysaccharide found in the cell wall of various plants, is used due to its ability to form a gel (Cantu-Jungles, Lacomini, Cipriani and Cordeiro, 2017; Valdivieso-Ramirez, 2016). The gelling capacity of pectin depends on several factors such as temperature, pectin quality, pH, presence of other sugars and calcium ions (Bhat, Nagasampagi and Sivakumar, 2005). There are two types of pectin that can be used to form gels

in the food industry, low methoxyl and high methoxyl pectin (Thakur, Singh, Handa and Rao, 1997). High methoxyl pectin is used in low pH, producing a gel that does not remelt, while low methoxyl pectin calcium ions work independently of the pH and form a thermo reversible gel (Edwards, 2007).

The application of a pectin based coating can extend food shelf life. Strawberry shelf life was increased from 6 to 15 days when an edible active coating made of pectin, pullulan and chitosan with sodium benzoate and potassium sorbate was applied. However, the strawberries with the pectin based coating had no significant difference in ascorbic acid content after 15 days compared with pullulan and chitosan coatings (Trevino-Garza, Garcia, del Socorro Flores-Gonzalez and Arevalo-Niño, 2015). Also, the addition of ingredients in the coating can delay deterioration reactions. Beeswax, a by-product from the honey industry used as a texturizer, carrier and glazing agent, can be employed as an edible coating. A chitosan monolayer coating and a beeswax-chitosan-beeswax coating were applied to strawberries (Velickova et al., 2013). After 7 days of storage at 20ºC, a weight loss of 48% of the initial weight was observed in the control while a 37% loss and 23-33% loss were reported for chitosan and beeswax-chitosan-beeswax coatings, respectively. Other studies showed the use of beeswax as a promising component for edible coatings on Kashar cheese (Yilmaz and Dagdemir, 2012) and as a synergistic compound for other edible coatings of cherry tomato fruit (Fagundes, Palou, Monteiro and Perez-Gago, 2014) and raspberries (Perez-Gallardo et al., 2012).

Bioactive compounds previously extracted can be added to food edible coatings to prevent food deterioration reactions. Lipid oxidation, one of the most significant deterioration reactions

of high fat food products, can be inhibited with the use of antioxidant polyphenols. Earlier, lyophilized aqueous extract of cranberry inhibited 52.4% of lipid peroxidation of a linoleic acid emulsion (Kalin, Gülçin and Gören, 2015). Peroxidase value reduction has been reported in chilgoza nuts using gum cordia plant extract coating and cashew nuts using cashew tree gum coatings (Pinto et al., 2015; Haq et al., 2013). Also, lipid coatings have been explored to reduce oxygen interaction in mangos and extend their shelf life for 30 days (Soomro et al., 2013), and inhibit microbial growth in oranges (Njombolwana et al. 2013). Raspberry microbial spoilage was also reduced with a pectin coating enriched with essential oils (Guerreiro et al., 2015).

The preservation of high fat food products like nuts is crucial because of their potential lipid oxidation and because Canada does not grow nuts. In 2015, almonds had the highest trade in Canada with a value of $296,886,263 of which 99.21% was imported (Statistics Canada & US Census Bureau, 2016). Lipid oxidation, one of the most predominant deterioration reactions in nuts, can be reduced by two different pathway: i) preventing oxygen and moisture interaction by creating a barrier with the coating, ii) adding extracts rich in phenolics to the coating to prevent free radicals from oxidizing lipids. To the best of our knowledge, only one study is available about the extraction of anthocyanins and phenolic compounds from cranberry pomace using low pressurized water. There are no studies on the extraction of anthocyanins from cranberry pomace using pressurized hot fluids like ethanol, water ethanol mixtures and water citric acid mixtures, and the application of such extracts to a pectin and beeswax based food coating with the objective of preventing lipid oxidation in almonds.

1.2. Hypothesis

- Pressurized fluids can be suitable to extract anthocyanins and total phenolics from cranberry pomace.
- Some combinations of pressurized citric acid 5%+water or ethanol+water mixtures (30 and 70%) can solubilize better total anthocyanins than total phenolics, which can lead to a better correlation with total antioxidant capacity.
- The use of pectin and pectin+beeswax based food coatings with cranberry extract can delay deterioration reactions of almonds.

1.3. Thesis objectives

The main objective of this thesis was to optimize anthocyanin extraction from cranberry pomace using pressurized fluids to further develop a bioactive food coating for almonds. The specific objectives were to:

1. Study and optimize process parameters for the extraction of anthocyanins and total phenolics from cranberry pomace using pressurized fluids.
2. Evaluate the antioxidant capacity of the extracts obtained.
3. Use bioactive pectin and beeswax coatings for almonds to minimize lipid oxidation.

The first objective was to optimize anthocyanin extraction from cranberry pomace by a combination of temperature (120-160ºC), pressure (50 and 200 bar) and solvent (water, water+ethanol (30% and 70% (v/v)), ethanol and water+citric acid (5%) (w/w)). Total anthocyanin extraction was also evaluated using pressurized ethanol at 40-100ºC and 50 bar.

The second objective was to evaluate the antioxidant capacity of pressurized fluid extracts by correlating the total anthocyanin extraction and total phenolic content versus the antioxidant capacity.

The third objective was to evaluate the impact on lipid oxidation of fatty acid composition of treated almonds with different edible coatings (pectin, pectin+extract (1:1), pectin+extract (1:3, w/w), pectin+beeswax, pectin+beeswax+extract (1:1 w/w) and pectin+beeswax+extract(1:3 w/w)) after storage at 40ºC and 50% RH for 90 days.

The use of environmentally friendly pressurized fluids to extract anthocyanins from the cranberry juice industry's by-product contributes to the green extraction of valuable phytochemicals. The use of such extracts in edible food coatings could prevent nut deterioration reactions, which otherwise can lead to unpleasant flavors.

Chapter 2: Literature review

2.1. Fruits in Canada

In 2015, the total amount of fruit produced in Canada was 372,761 tons for apples, 182,965 tons for blueberries, 161,368 tons for cranberries, 87,959 tons for grapes and 22,520 tons for strawberries (Statistics Canada, 2016). Apples, blueberries, cranberries, grapes and strawberries were the top 5 fruits produced in Canada (Fig. 2.1). From 2011 to 2015, a production decrease of 14% and 11% were reported for apple and grapes, respectively, while strawberry production had a small increase of 0.2%. A significant increase was reported in the production of blueberries (58%) and cranberries (70%) (Statistics Canada, 2016).

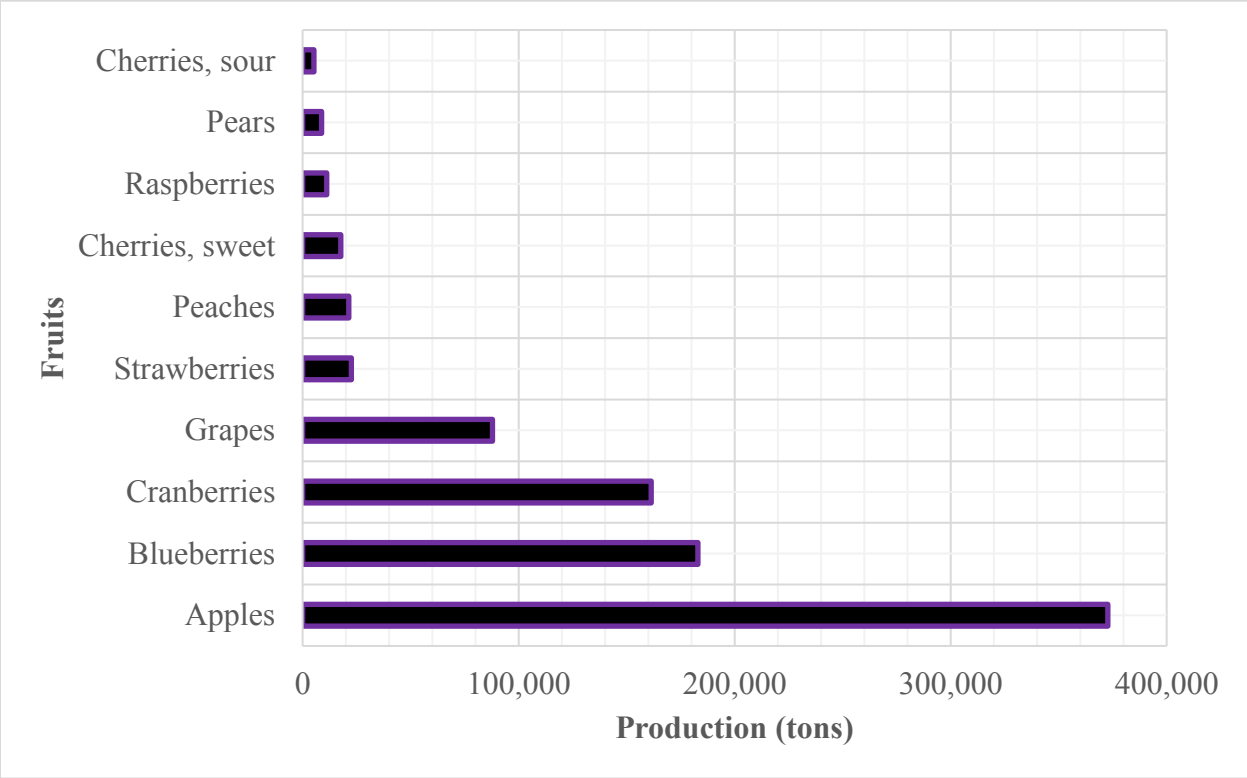


Figure 2.1. Total fruit production in Canada in 2015 (Statistics Canada, 2016).

2.1.1. Berries

While the production of blueberries and cranberries increased in Canada from 2008 to 2015, the production of cherries, raspberries, grapes and strawberries remained constant (Fig. 2.2).

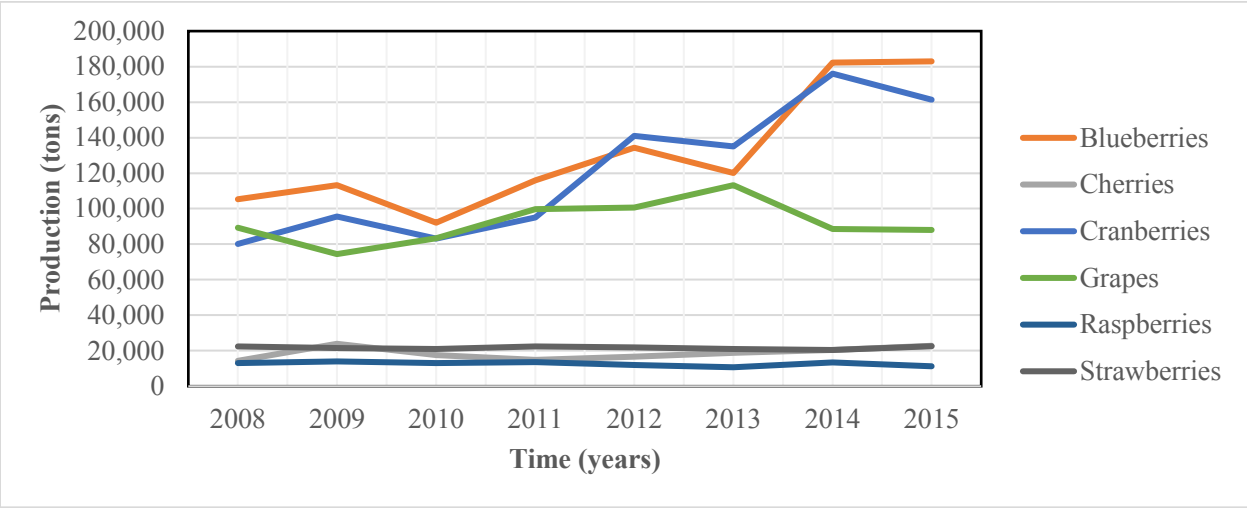


Figure 2.2. Berry production in Canada from 2008 to 2015 (Statistics Canada, 2016).

The distribution of the production of the three main berries, cranberries, blueberries and grapes, in Canada, correspond mainly to three provinces of Ontario, Quebec and British Columbia (Fig. 2.3). Other provinces such as Nova Scotia, New Brunswick and Prince Edward Island are considered minor contributors, however the prairies provinces, which includes Alberta, Saskatchewan and Manitoba, do not produce a significant amount of such berries. Other small market berries, like Saskatoon berries, are produced in the Prairies.

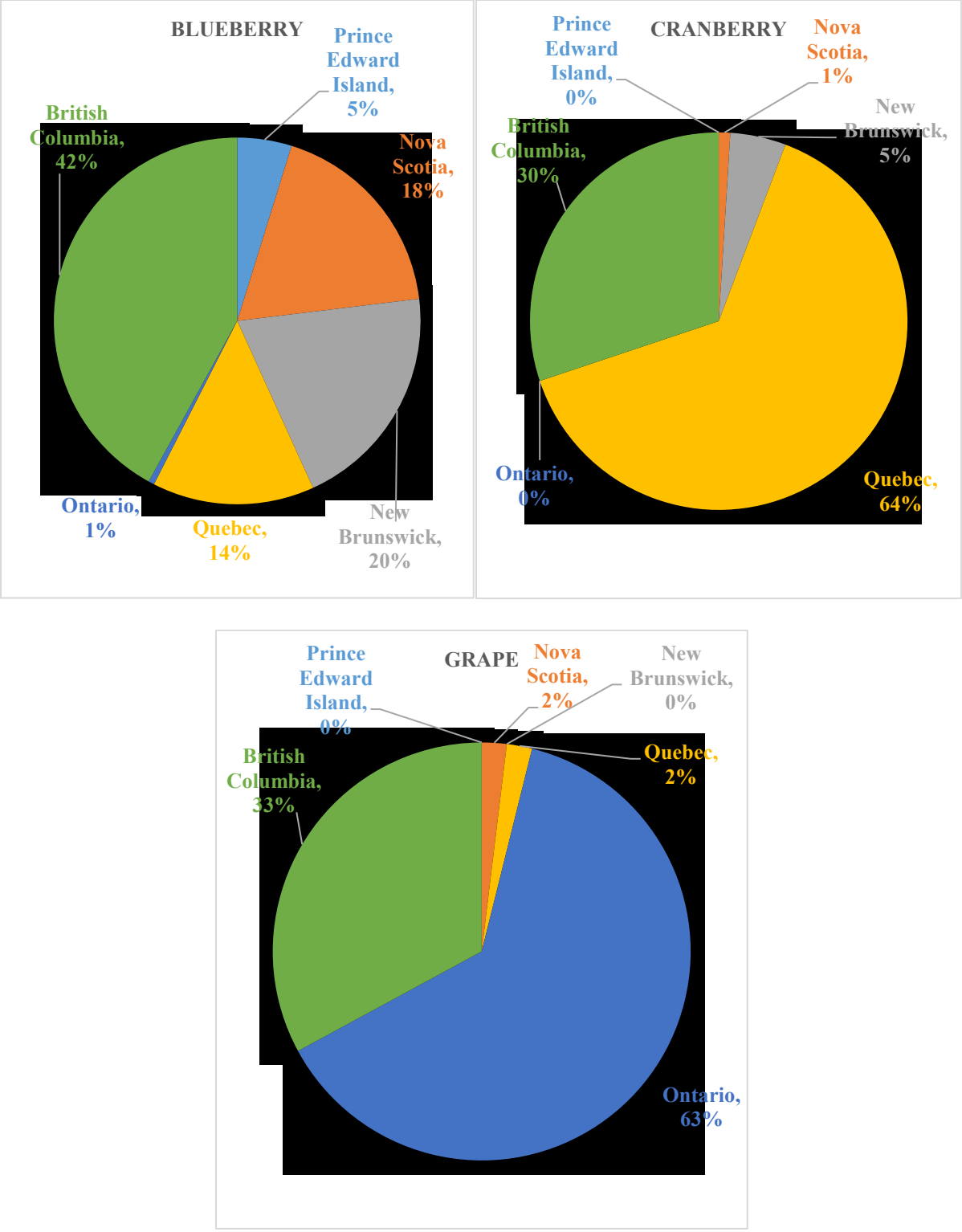


Figure 2.3. Canadian production distribution by province in 2015 (Statistics Canada, 2016).

2.2. Cranberry

2.2.1. Production and market status

By 2015, there was a total cranberry production of 161,368 tons on 7,369 hectares, with a farm gate value of CAD $11 million, mainly grown in Quebec and British Columbia with 64 and 30%, respectively (Statistics Canada, 2016). The USA produced higher amounts of cranberries in 2015 with a total of 856,300,000 tons, 560,010,000 tons of blueberries and 51,520,000 tons of blackberries (USDA, 2016). Figure 2.4 shows the market status of different berries in the USA.

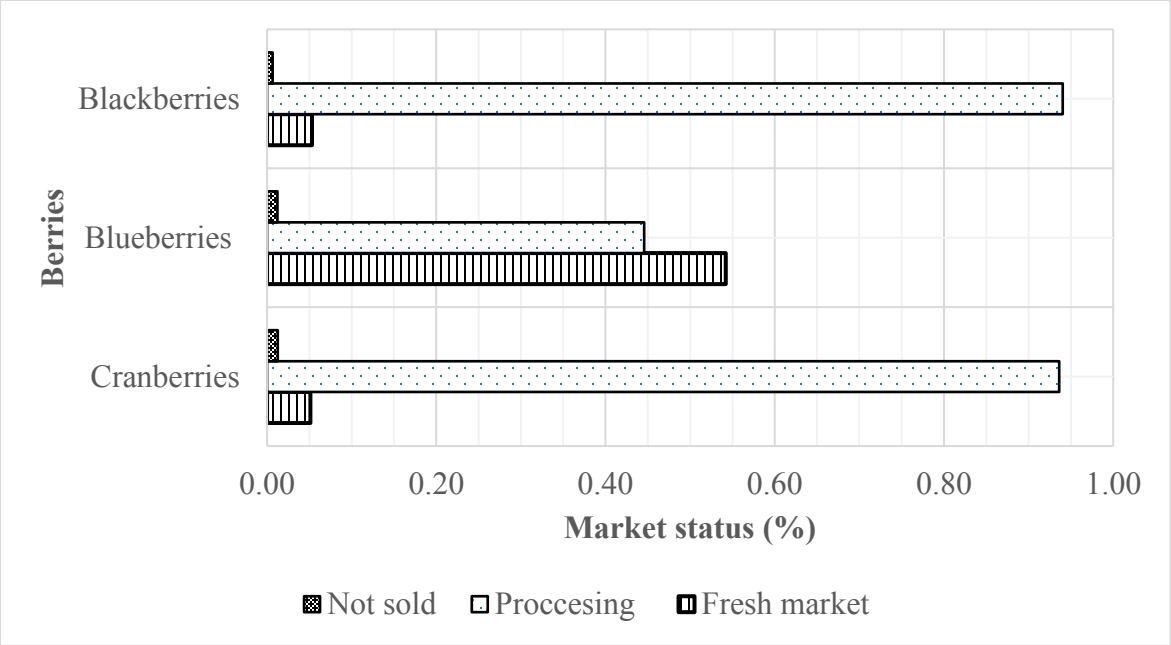


Figure 2.4. Market status for berries in the USA in 2015 (USDA, 2016).

2.2.2. Structure, classification and proximate composition

Cranberry (*Vaccinium macrocarpon*), also known as American cranberry (Fig. 2.5), is an evergreen tree producing pink flowers on upright shoots of 5-15 cm and eventually berries with a pear-shaped and shiny surface (Small, 2013).

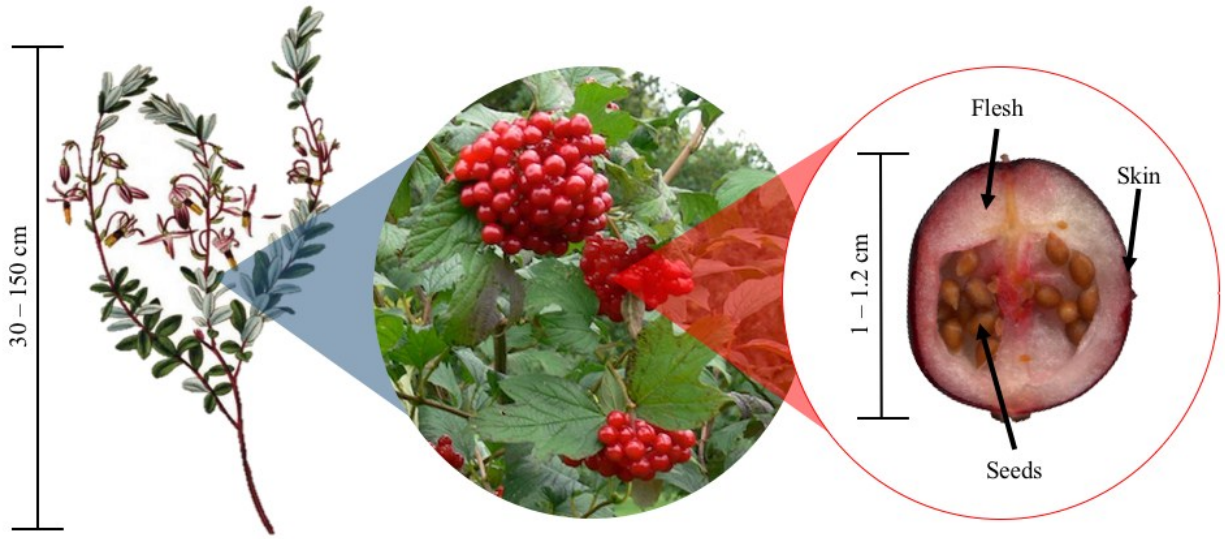


Figure 2.5. Cranberry (*Vaccinium macrocarpon*) tree and fruit (Adapted from Edwards, 1825; Shutterstock ©)

One of the most important characteristics of cranberries is their colour. Several colours in fruits are delivered by different pigment groups, such as chlorophylls, carotenoids, betalains and anthocyanins, the last one imparts red, blue and black hues to the fruit (Steyn, 2009). Cranberry compositional analysis is described in Table 2.1.

Table 2.1. Cranberry compositional analysis (USDA, 2016).

Macronutrient	Raw cranberry (USDA, 2016)
Moisture content (%)	87
Protein (%)	0.46
Fat (%)	0.13
Carbohydrate (%)	11.97

2.2.3. Uses

2.2.3.1. Food products

There are three main products obtained from cranberries (Fig. 2.6) as reported by Tokusoglu & Hall (2011).

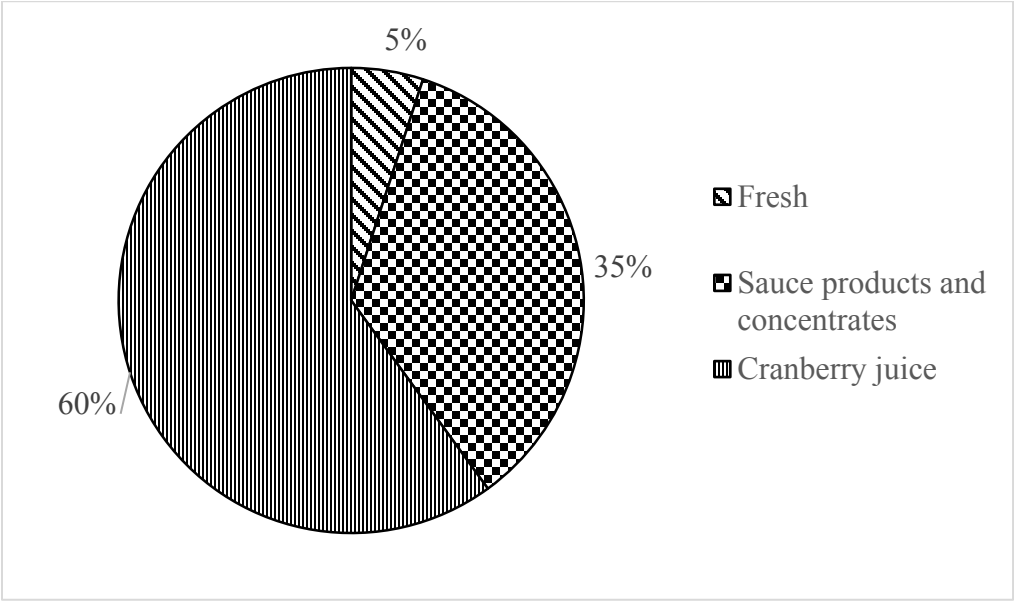


Figure 2.6. Cranberry product categories.

The main cranberry products consumed are cranberry cocktail juice, unsweetened cranberry juice, fresh cranberry and sweetened dried cranberries. Table 2.2 shows the nutritional facts of such products.

Table 2.2. Proximate composition for different cranberry final products (USDA, 2016).

	Cranberry juice cocktail	**Unsweetened cranberry juice**	**Fresh berry**	**Sweetened dried cranberries**
Water (%)	86.17	87.13	87.32	15.79
Protein (%)	0	0.39	0.46	0.17
Total lipid (fat) (%)	0.10	0.13	0.13	1.09
Carbohydrate (%)	13.52	12.20	11.97	82.80
Sugar, total (%)	11.87	12.1	4.27	72.56
Fiber, total dietary (%)	0	0.1	3.6	5.3
Energy in 100 g (kcal)	54	46	46	308

2.2.3.1.1. Cranberry juice production

There are three main juice extractions methods used in the cranberry juice industry that have cranberry pomace as a by-product. The first one uses a mechanical press to extract the juice where no heat is needed, preventing deterioration. The second one is mash depectinization, which consists in the addition of enzymes (approximately at 52ºC for 4-12 h) with the aim to reduce the fruit into a mash and then pressed. The last one is a countercurrent extraction of the sliced fruit and water, involving the use of a large screw. These processes yields are 75%, 100% and 90%, respectively (Girard and Sinha, 2006). Pectinase is obtained when fermenting *Asperillus niger* with carbon sources, such as glucose, sucrose and galacturonic acid (Solis-Pereira, Favela-Torres, Viniegra-González and Gutiérrez-Rojas, 1993).

Caillet, Côté, Doyon, Sylvain and Lacroix (2011) compared the chemopreventive effect change when processing cranberries into juice. Their juice production consisted in the addition of pectinase to cranberries followed by a milling and maceration steps at 55ºC and further pressing at 1.9 bar, then a clarification process (0.14 micron) and evaporation at 100ºC to reach 50º brix. A significantly lower chemopreventive effect was reported in cranberry juice concentrate followed by cranberry pomace, raw juice, clarified juice, mash and depectinazed mash, and fruit. The production of cranberry juice using an ultrafiltration membrane stacked into an electrodyalisis cell reported an increase of 34.8% in proanthocyanidins and 52.9% in anthocyanins (Bazinet, Cossec, Gaudreau and Desjardins, 2009).

2.2.3.2. Other applications

There are other applications of cranberry and cranberry extracts. Leusink et al. (2010) reported no impact of a diet with cranberry extract in poultry growth performance, meat quality and gut microflora. Different cranberry fruit extract concentrations (40, 80 and 160 mg of cranberry fruit extract/kg of feed) were fed to 1,200 chickens for 35 days without a significant effect in mortality, intestinal health and meat quality.

Another example of cranberry applications is cranberry supplements for human consumption. David Tournay, French biotechnologist and President of the European Association for the Valorization of Cranberry Extracts, said that the cranberry supplement market grew 16% between 2008 and 2009 while the food supplement market decreased by 6% (Byrne, 2009). Nonetheless controversy is found in the efficiency of bacterial anti-adhesion of different

commercial cranberry supplements, while some supplements are very potent not all of those had a relevant impact for urinary tract infection prevention (Chughtai, Thomas and Howell, 2016).

2.2.4. Cranberry pomace

2.2.4.1. Proximate composition

The cranberry juice industry has a valuable by-product known as cranberry pomace. A study of cranberry cultivars found that there are higher amounts of bioactive compounds within cranberry skin rather than flesh by comparing cultivar berry size. Total anthocyanins for different cultivars were quantified and the highest amount was found in Ben Lear (7.98±5.83 mg/g d.w.) followed by Bergman (7.02±1.75 mg/g d.w.), GH1 (6.05±2.51 mg/g d.w.), Pilgrim (3.28±1.88 mg/g d.w.) and Stevens (0.81±0.891 mg/g d.w.) (Brown, Murch and Shipley, 2011). Ben Lear and Bergman have the smallest fruit size and Pilgrim and Stevens have the largest fruit size, suggesting that high concentration of anthocyanins can be found in the skin. Cranberry pomace composition is reported in Table 2.3.

Table 2.3. Cranberry pomace proximate composition.

Parameter	Cranberry pomace (Ross et al., 2017)	Dried cranberry pomace (Park and Zhao, 2006)
Moisture (%)	68.37	4.0
Protein (%)	1.82	8.2
Fat (%)	1.39	1.2
Ash (%)	0.33	0.8
Carbohydrates (%)	28.02	85.8

Table 2.4 shows main anthocyanins reported in cranberry and cranberry pomace. The main anthocyanin contents differ from the raw and pomace cranberry. Differences between cranberry

pomace and organic cranberry pomace were also reported. White, Howard and Prior (2009) reported a total flavonol concentration of 3.58±0.16 mg/g d.w. Among them, quercetin (1.46±0.23 mg/g d.w.) had the highest value followed by myricetin (0.56±0.03 mg/g d.w.) and quercetin 3-benzoyl galactoside (0.28±0.03 mg/g d.w.).

Table 2.4. Main anthocyanins found in cranberry and cranberry pomace.

Main anthocyanins	**Freeze-dried cranberry (mg/g d.w.)** (Brown and Shipley, 2011)	**Cranberry pomace (mg/g d.w.)** (White, Howard and Prior, 2009)	**Organic cranberry pomace (mg/g d.w.)** (Ross et al. 2017)
Cyanidin-3-arabinoside	0.63±0.02	0.50±0.07	0.85±0.09
Peonidin-3-arabinoside	0.68±0.02	0.27±0.01	0.68±0.07
Peonidin-3-galactoside	1.82±0.05	0.20±0.01	1.58±0.16
Cyanidin-3-galactoside	1.11±0.03	0.13±0.002	1.20±0.13
Peonidin-3-glucoside	N/R	0.07±0.003	0.17±0.02
Cyanidin-3-glucoside	0.03±0.04	0.05±0.002	0.04±0.01
Total	N/R	1.21±0.06	4.75

N/R: not reported.

2.2.5. Bioactive compounds in cranberry

Cranberries are considered to have beneficial health components. For example, a reduction in weight gain and visceral obesity as well as a decrease in triglyceride accumulation and improved insulin sensitivity were observed when cranberry extract (200 mg/kg) was administrated daily to rats after 8 weeks (Anhe et al., 2015). Cranberry extract consumption led to a reduction of bacterial adhesion from 2.11 bacteria/urothelial cell to 0.28 bacteria/urothelial cell after 12 weeks while the placebo group increased from 1.81 to 2.14 bacteria/urothelial cell (Singh, Gautam and Kaur, 2016). Such components are represented mainly by phenolic acids, tannins, and flavonoids. Bioactive compounds in berries (Fig. 2.7) can be separated in two mayor groups: i) phenolic acids and ii) flavonoids.

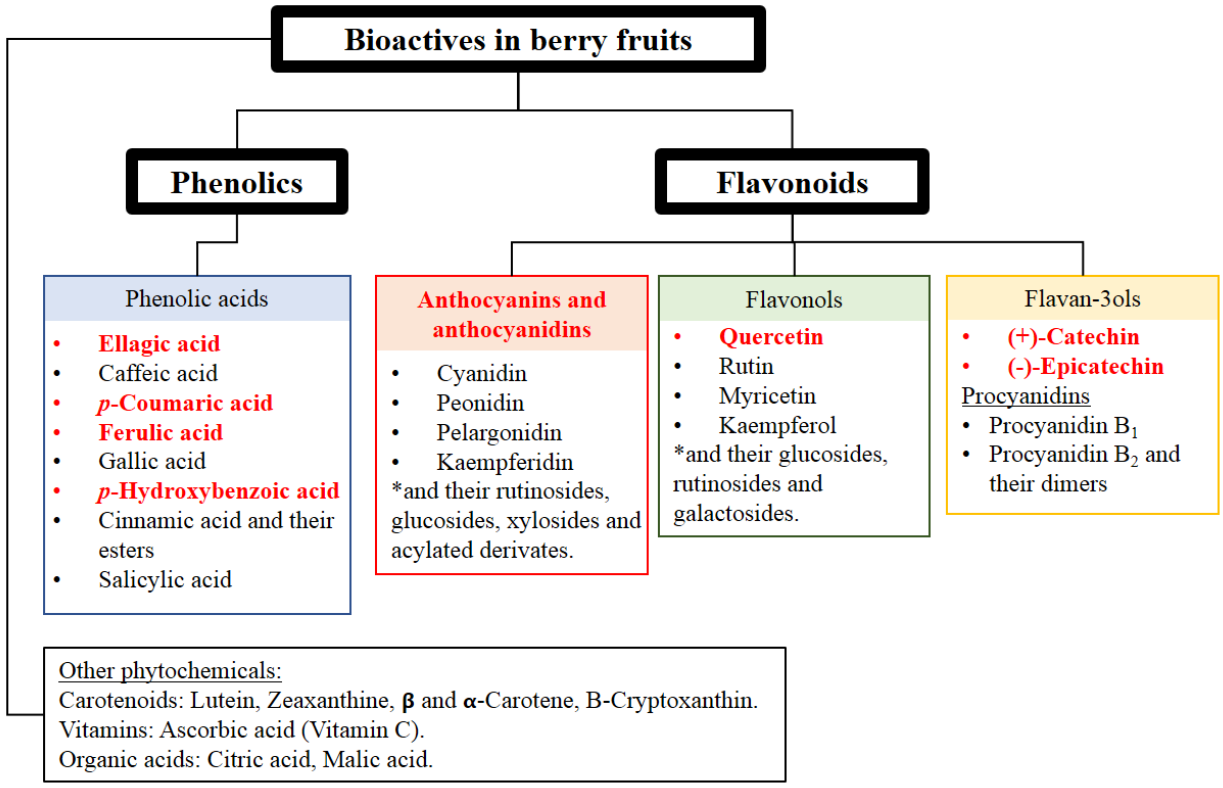
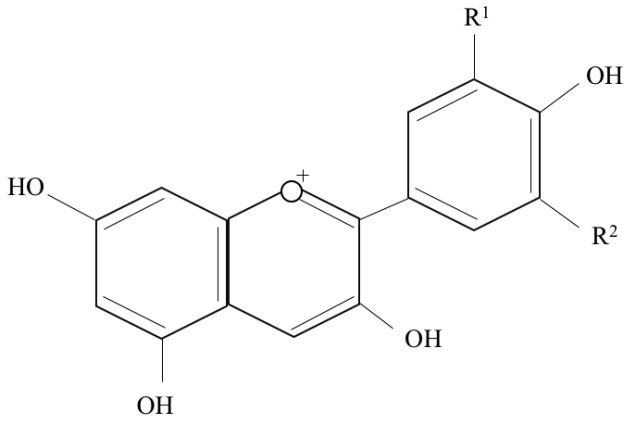


Figure 2.7. Bioactive compounds in berry. Bioactive compounds in cranberry are in red (Adapted from Tokusolgu and Stoner, 2011; Côte et.al., 2010).

2.2.5.1. Anthocyanins and anthocyanidins

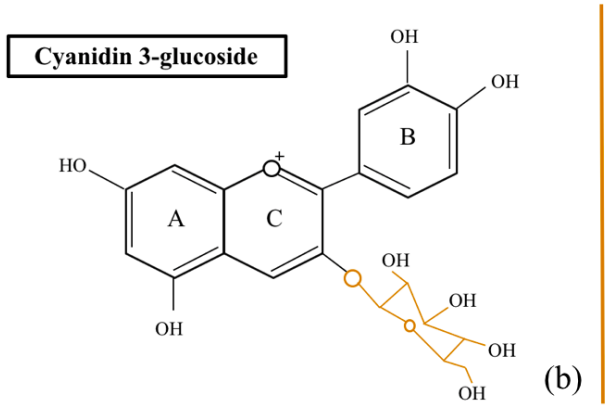
The difference between anthocyanidins and anthocyanins is that anthocyanidins refers to the non-glycosylated molecule and anthocyanins refer to anthocyanidins attached to a sugar molecule. Anthocyanins are compounds responsible for colours, ranging from pink to red, purple and blue. Anthocyanins are water-soluble glycosides of anthocyanidins. The most common glycoside is the 3-glycoside and if a second sugar is attached, it is bounded to the 5-hydroxyl position (Vermerris & Nicholson, 2006). Plenty of anthocyanin pigments have been identified but most anthocyanin types are divided in three structures based on the number of hydroxyl

groups on the B-ring: pelargonidin, cyanidin and delphinidin (Deroles, 2008). The chemical structure of the six different anthocyanidin molecules that occur in nature are shown in Figure 2.8, with their absorption maxima.



Anthocyanin	Radical		Adsorption (nm)
	R^1	R^2	λ_{max}
Pelargonidin	-H	-H	503
Cyanidin	-OH	-H	517
Peonidin	$-OCH_3$	-H	517
Delphinidin	-OH	-OH	526
Petunidin	$-OCH_3$	-OH	526
Malvinidin	$-OCH_3$	$-OCH_3$	529

(a)



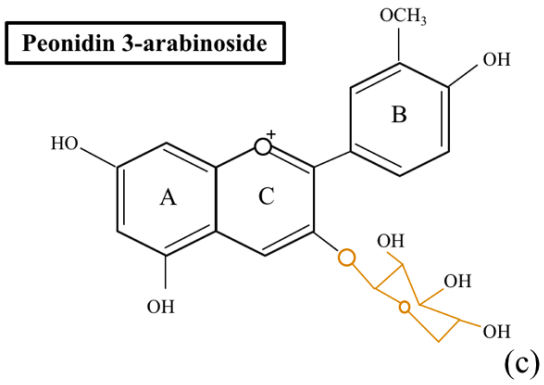


Figure 2.8. Chemical structures of: (a) anthocyanidins with their maxima absorption maxima of the corresponding 3-glucoside at pH 3 (Adapted from Coulate, 2009), (b) Cyanidin 3-glucoside, and (c) Peonidin 3-arabinoside.

Anthocyanin colour change depending on the pH of the media they are exposed to. Figure 2.9 shows the wavelength absorbance at different pH. Colour intensity was gradually lost when rising pH, strong blue colours can be found at high pH values (Coultate, 2009).

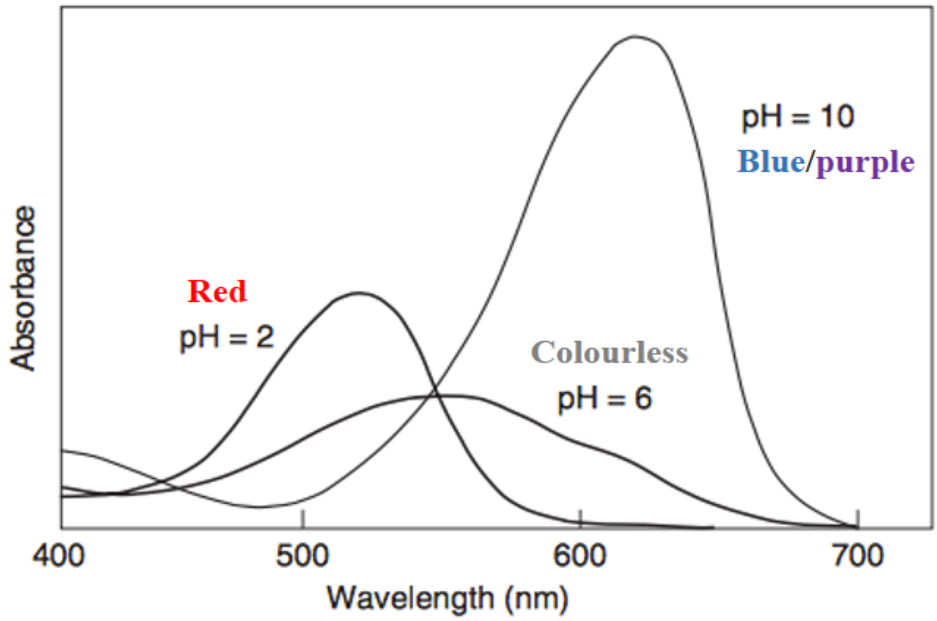


Figure 2.9. Effect of pH on the absorption spectrum of anthocyanins (Adapted from Coultate, 2009).

2.2.5.1.1. Functionality and stability

Anthocyanin stability can be influenced by various factors. Storage conditions, including temperature, oxygen and light exposure, are the most important factors related to anthocyanin stability. At temperatures below 20ºC, a positive effect in total anthocyanin and total phenolic contents in cranberries was reported by Wang and Stretch (2001), who studied the storage of cranberries (cv Ben Lear) for three months and reported an increase in both anthocyanin content and phenolic content. The initial anthocyanin content was 0.25±0.01 mg Cy3GE/g which

increased depending on the storage temperature with values of 0.46±0.05 mg Cy3GE/g (0ºC), 0.55±0.03 mg Cy3GE/g (5ºC), 0.62±0.04 mg Cy3GE/g (10ºC), 0.77±0.04 mg Cy3GE/g (15ºC) and 0.66±0.02 mg Cy3GE/g (20ºC). Initial total phenolic content was 1.37±0.03 mg GAE/g, which increased to 1.40±0.03mg GAE/g (0ºC), 1.43±0.03 mg GAE/g (5ºC), 1.60±0.03 mg GAE/g (10ºC), 1.92±0.03 mg GAE/g (15ºC) and 1.85±0.05 mg GAE/g (20ºC). Also, the total content of anthocyanin in cranberries depends on its ripeness stage, where mature berries have a darker appearance and with nearly four times more anthocyanins compared with light colored berries (Ozgen, Palta, & Smith, 2002).

2.2.5.1.1.1. Temperature

It is important to consider anthocyanin stability when exposed to high temperature. Sadilova, Carle and Stintzing (2007) studied the effect of exposing pigment isolates from strawberry, elderberry and black carrot at 95ºC up to 4 hours. Strawberry showed an initial anthocyanin content of 171±1.86 (mg Cy3GE/L) that decreased to 129.43±4.88 (mg Cy3GE/L) in the first hour and to 40.39±2.86 (mg Cy3GE/L) in 4 hours. A similar behavior was observed in elderberry and black carrot anthocyanin content, with a total decrease in 4 hours from 194.09±5.52 (mg Cy3GE/L) to 45.62±1.39 (mg Cy3GE/L) and from 185.66±2.39 (mg Cy3GE/L) to 42.77±2.31 (mg Cy3GE/L), respectively.

2.2.5.1.1.2. pH

The pH also affects anthocyanin stability. Acidic conditions can be achieved naturally due to the presence of organic acids, such as citric acids in cranberry. While cranberry bioactive compounds are 39% anthocyanins and 22.6% vitamin C, blueberry bioactive compounds are 84% anthocyanins and 14% flavonols (Borges, Degeneve, Mullen and Crozier, 2009). Cranberry extracts obtained with water:methanol (85:15 v/v), acetone:methanol:water (40:20:20, v/v/v) and methanol:water:acetic acid (85:15:0.5, v/v/v) at a pH of 2.5 had a higher free radical scavenging capacity of 1.99±0.03, 2.12±0.01, and 2.13±0.03 mmol trolox equivalent/mg d.w. compared with extracts obtained at a neutral pH of 7 with 1.39±0.01, 0.60±0.01 and 0.63±0.02 mmol trolox equivalent/mg d.w., respectively (Caillet, Cote, Doyon, Sylvain & Lacroix, 2011).

2.2.5.1.1.3. Enzymes

Two main enzymes can be found in cranberries. Endo-polygalacturonase enzyme, also known as pectin depolymerase (which soften the cell wall) which is inactivated at 100ºC after 35 min (Arakji and Yang, 1969). Total glucosinolates can also be found in cranberries. These enzymes were significantly reduced in red cabbage when blanched (94-96ºC), boiled and steamed with reductions of 64, 38 and 42%, respectively. The thermal negative impact in total anthocyanin content (114±5 mg Cy3GE) on red cabbage was also reported after blanching (81.9±1.3 mg Cy3GE), boiling (88.5±2.7 mg Cy3GE), and steaming (77.7±3.2 mg Cy3GE) (Volden et al., 2008).

2.2.5.2. Phenolic acids

One of the most important bioactive components of berries are phenolic compounds or phenols, which aromatic compound contains hydroxyl groups directly attached to the nucleus and can be classified as monohydric, dihydric and trihydric phenols based on the number of hydroxyl groups (Fig. 2.10).

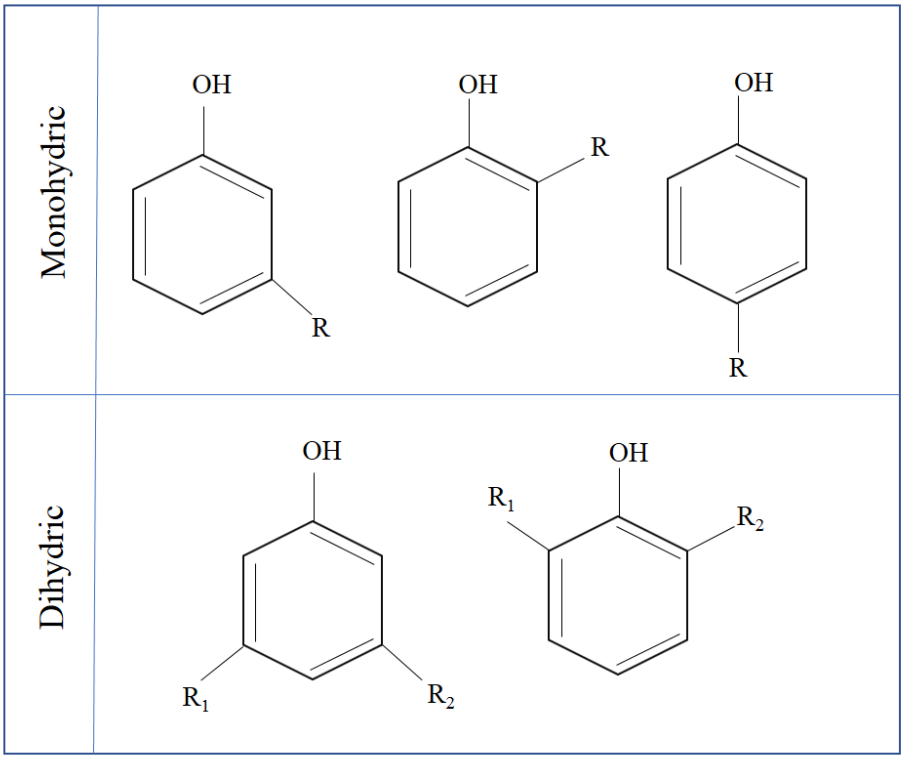


Figure 2.10. Chemical structures of simple phenolic compounds (Adapted from Vermerris and Nicholson, 2006).

It has been reported that the most abundant phenolic acids in cranberries are benzoic acid (4.7g/kg fresh weight) followed by *p*-coumaric acid (0.25 g/kg fresh weight) and sinapic acid (0.21 g/kg fresh weight) (Zuo, Wang and Zhan, 2002). The primary activity of benzoic acid

found in berries is to prevent yeast and molds. A study that analyzed different cranberry genotypes found that benzoic acid content increased when fruit is ripening. After 52 days, the genotype US88-1 increased from 0.0030±0.0006 mg/g fresh weight to 0.0275±0.0010 mg/g fresh weight (Tadych et al., 2015).

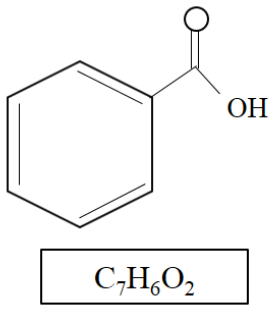


Figure 2.11. Chemical structure of benzoic acid.

2.2.5.3. Flavonoids

The second major group of bioactive compounds are flavonoids. Flavonoid includes a C_6-C_3-C_6 structure. Depending on the linkage of the aromatic ring with the benzopyrano, it can be divided into three classes: flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans) and neoflavonoids (4-benzopyrans) (Grotewold, 2006). Anthocyanins are flavonoids found in berries, including cranberry.

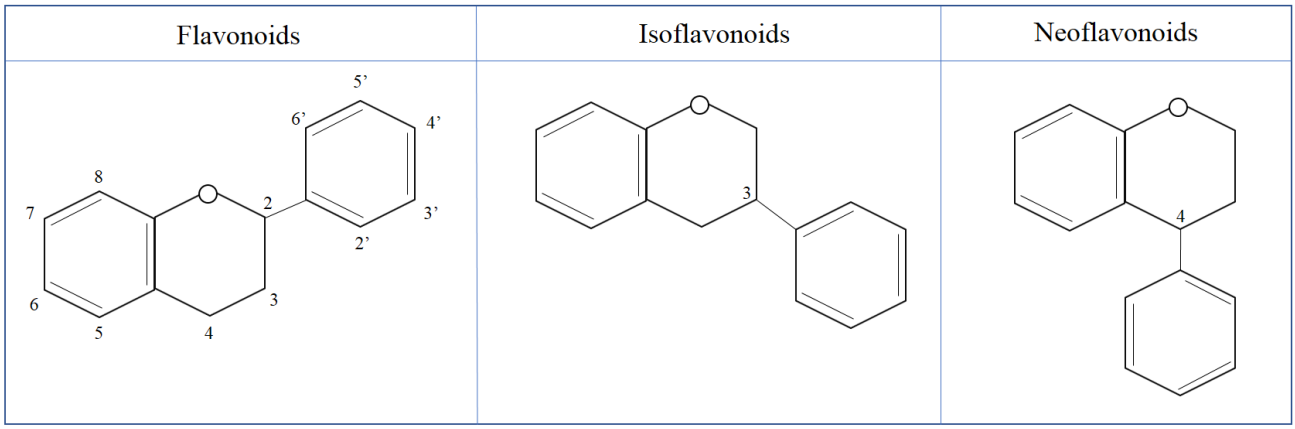


Figure 2.12. Flavonoid chemical structures.

2.2.6. Analytical methods to determine bioactive compounds

A variety of analytical methods have been used to identify polyphenols from various berries (Table 2.5). The most common technique to quantify total phenolics, total anthocyanins and total proanthocyanins use a spectrophotometer, which measures the interaction of ultraviolet (UV), visible and infrared (IR) radiation with a material in a solution. An spectrophotometer can measure spectral reflectance, transmittance, absorbance, emitance, scattering and flourescence (Germer, Zwinkels and Tsai, 2014).

Table 2.5 shows various analytical techniques used to identify bioactive compounds from black currant, cranberry seeds, fresh cranberry, cherry, apples and fresh strawberry. In those studies, total anthocyanins were commonly identified using the pH differential method. Folin-Ciocalteu was also used frequently to identify total phenolic content, including phenolic acids. However, both phenolic acids and total anthocyanins can be better quantified using high liquid chromatography.

Table 2.5. Analytical methods used to quantify bioactive compounds from fruit sources.

Source	Objective	Analysis	Analytical method	Reference
Black caraway, carrot, cranberry and hemp seed oils	Determine potential application of seed oils by evaluating antioxidant capacity	Antioxidant capacity	ORAC, ABTS, DPPH	Gorinstein et al. (2010)
		Total phenolics	Folin-Ciocalteu	
Cranberry and cherry	Determination of anthocyanins	Anthocyanins	HPLC-ESI-MS	Karaaslan and Yaman (2016)
Grapes	Characterization of five grape varieties grown in Turkey	Total phenolics	Folin-Ciocalteu	Karasu et al. (2016)
		Total flavonoids	Colorimetric	
		Total anthocyanins	pH differential	
Malay apple fruit	Antioxidant activity and bioactive compounds	Total anthocyanins	pH differential	Nunes et al. (2016)
		Anthocyanins	HPLC-DAD-MS/MS	
		Total phenolics	Folin-Ciocalteu	
		Antioxidant capacity	FRAP, DPPH	
		Antioxidant capacity	Colorimetric	
		Phenolic acids	HPLC	
		Phenolic acids	HPLC-MS	
Strawberry	Compare phenolic composition and antioxidant capacity between achenes seeds and raw fruit before and after simulated digestion.	Total phenolics	Folin-Ciocalteu	Ariza et al. (2016)
		Total flavonoids	Colorimetric	
		Total anthocyanins	pH differential	
		Phenolic acids	HPLC	
		Anthocyanins	HPLC	

HPLC: High-performance liquid chromatography, ESI: electrospray ionization, MS: mass spectrometry, ORAC: Oxygen radical absorbance capacity, radical scavenging assay using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH), and FRAP: Ferric reducing ability of plasma.

The Folin-Ciocalteu reagent, which is a spectrophotometric technique, is commonly used to determine the total phenolic content. Such technique works by mixing an extract with the Folin-Ciocalteu reagent and sodium carbonate solution. Once the reaction takes place after approximately two hours, the absorbance is measured at 765nm. Such value is then compared with a calibration curve of different gallic acid concentrations and the total phenolic content is reported as gallic acid equivalents.

Total monomeric anthocyanin content can be quantified by the pH differential method (AOAC official method 2005). Such technique exposes the extract to a pH 1.0 buffer (potassium chloride, KCl and hydrochloric acid, HCl) and to a pH 4.5 ($CH_3CO_2Na \cdot 3H_20$ and HCl). Both solutions are measured at two different absorbances of 520 nm and 700 nm. The calculation of anthocyanin pigment concentration is expressed as cyanidin-3-glucoside equivalent using equation 2.1.

$$Anthocyanin\ pigment\ \left(\frac{mg\ C3GE}{L}\right) = \frac{A*MW*DF*10^3}{\varepsilon*1} \quad (2.1)$$

where:

$A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$

Molecular weight (MW) = 449.2 g/mol of cyanidin-3-glucoside (Cy3GE)

DF = Dilution factor

ε = 26,900 molar extinction coefficient in (L/mol cm of Cy3GE)

10^3 = conversion factor from g to mg

Table 2.6 shows different amounts for total anthocyanins form berry sources using the pH differential method.

Table 2.6. Total anthocyanin content of berries using the pH differential method.

Berry (solvent, temperature)	Total anthocyanin content (mg Cy3GE/ g fresh weight)	Reference
Blueberries (Acetone 80%, 60ºC)	0.53	Wang, Jung, Tomasino and Zhao (2016)
Blueberries (Methanol 80%, 70ºC)	0.49	
Cherries (Acetone60%, 60ºC)	0.04	
Cherries (Methanol 60%, 70ºC)	0.05	
Byrsonima ligustrifolia (Acetone 30% + methanol 60.9% + water 9.1%, N/R)	1.85±0.07	Sampaio et al. (2015)
Blueberry wine pomace (Ethanol 70% + hydrochloric acid 0.01% + water 29.99% , 60ºC)	4.11±0.01	He et al. (2016)
Cranberry (Bergman) (Methanol 99.9% + hydrochloric acid 0.01%, 40ºC)	0.73±0.002	Borowska, Mazur, Kpciuch and Buszewski (2009)
Cranberry (Ben Lear) (Methanol 99.9% + hydrochloric acid 0.01%, 40ºC)	0.52±0.001	
Wild cranberry (Methanol 99.9% + hydrochloric acid 0.01%, 40ºC)	0.43±0.001	

N/R: not reported.

Another method to quantify anthocyanins, which is more accurate than the pH differential method, is high performance liquid chromatography (HPLC). This method consists in the injection of a liquid sample solution with a mobile phase into a column. Once the retention time of the sample solution is obtained this is compared with the retention time of a specific standard. Lee et al. (2016) reported the anthocyanin content of different berries and compared the difference after analyzing anthocyanins using HPLC and the pH differential method, finding a similar trend but significantly different anthocyanin amounts for both blueberry and cranberry

(Fig. 2.13). They concluded that the discrepancy observed is when the anthocyanin's glycone is not a monosaccharide.

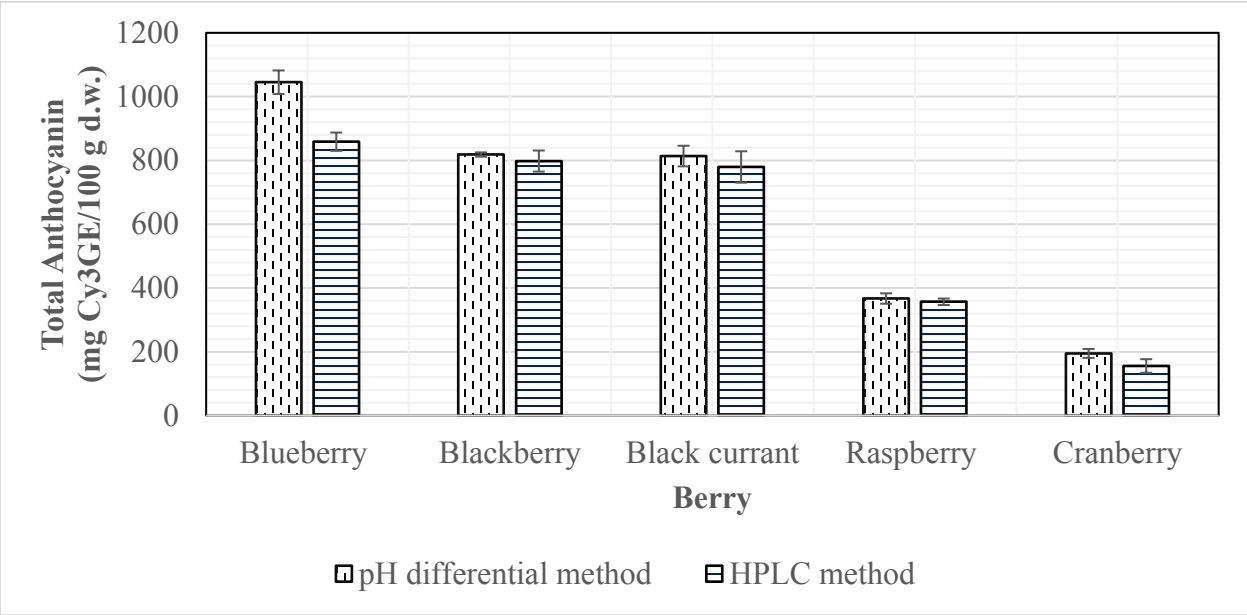


Figure 2.13. Comparison of anthocyanin analysis using the pH differential method and HPLC (Adapted from Lee et al., 2016).

There are two common methods used to quantify the antioxidant capacity of extracts by measuring the absorbance change with a spectrophotometer. A common method is the ferric reducing ability of plasma (FRAP), which is based on the change of coloured ferrous tripyridyltriazine complex, a subsequent reaction of the reduction of a colored ferric complex to ferrous ion at low pH (Griffiths, 2016). After the reaction occurs, the sample is analyzed with a spectrophotometer and the absorbance at 593nm is recorded. A second method to analyze antioxidant capacity is the trolox equivalent antioxidant capacity (TEAC) assay. In this method, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS is converted to colored radical

cation ($ABTS^{+}$) and then mixed with the sample to further analyze the decrease of absorbance at 734nm, which is expressed as trolox equivalents (Wada, Kishikawa, Kuroda and Nakashima, 2008).

Total antioxidant capacity of berries depends on total phenolic content, including total anthocyanin and vitamin C contents. Figure 2.14 shows the amounts of vitamin C (mg/100 g fresh weight (f.w.)) and total anthocyanins (mg Cy3GE/100 g f.w.) where total anthocyanin is reported as cyanidin-3-glucoside equivalent.

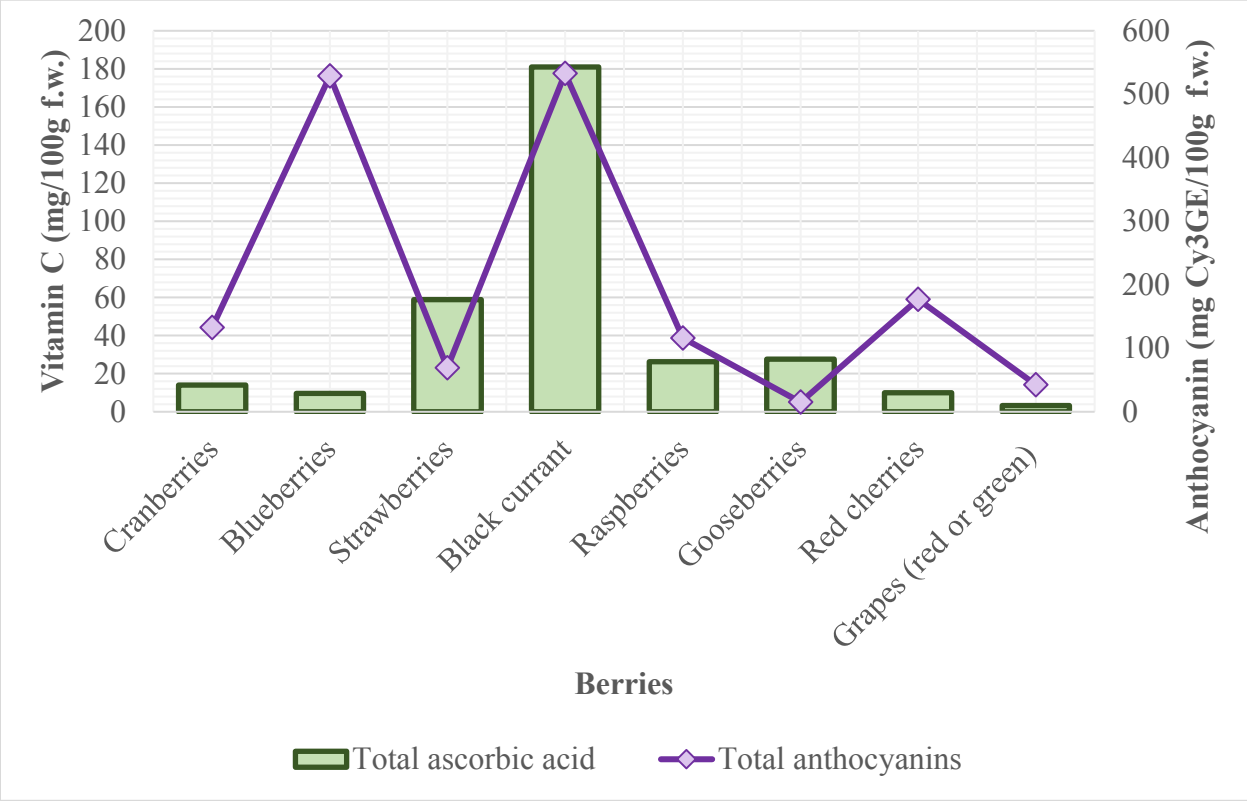


Figure 2.14. Total vitamin C and total anthocyanins in berries (Adapted from USDA, 2016; Wu et al., 2006).

2.3. Extraction methods of phenolic compounds

2.3.1. Conventional extraction of phenolic compounds

Conventional methods of phenolic extraction use petrochemical solvents (Table 2.7). This technique has high yields, but it also has some disadvantages. Environment and Climate Change Canada (2013) defined volatile organic compounds (solvents) as a precursor pollutant as well as sulphur dioxide, nitrogen oxides and gaseous ammonia, which together with particulate matter cause adverse health effects. Since 1999, the government of Canada took actions to control volatile organic compounds. Therefore, the use of petrochemical solvents to extract phenolics for food applications is restricted because they are non-GRAS solvents. After the extraction, the solvent must be removed completely, involving additional processing time.

Table 2.7 shows total phenolic content extracted from apple pomace, grape skin, stems, and seeds, peach, canola and black beans. Differences between the studies included the solvent type used and their mixtures and concentrations, sample solvent ratio, temperature and time of extraction.

Table 2.7. Extraction of total phenolic compounds from various sources.

Source (g)	Solvent (mL)	Processing conditions	Total phenolic content	Reference
Apple pomace (100g)	Methanol (500mL)	T= 37ºC t= 40 min.	3.05±0.82 mg GAE/g d.w.	Zhang et al. (2016)
	Acetone (500mL)		2.15±0.35 mg GAE/g d.w.	
	Ethyl acetate (500mL)		2.51±0.42 mg GAE/g d.w.	
	Chloroform (500mL)		1.62±0.23 mg GAE/g d.w.	
Milled white grape skin (1g)	First, Methanol:water (80:20 v/v) (10mL) Second, Acetone:water (75:25, v/v) (10mL)	T= room temperature t= 3 h.	~0.14 mg GAE/g d.w.	Sá et al. (2014)
Entire white grape skin (1g)			~0.04 mg GAE/g d.w.	
Milled white grape stems (1g)			~0.18 mg GAE/g d.w.	
Entire white grape stems (1g)			~0.14 mg GAE/g d.w.	
Milled white grape seeds (1g)			~0.42 mg GAE/g d.w.	
Fresh white grape seeds (1g)			~0.16 mg GAE/g d.w.	

GAE: gallic acid equivalents.

Table 2.7 continued.

Source	Solvent	Processing conditions	Total phenolic content		Reference
Peach cultivar "spring belle" (20g) Peach cultivar "Cardinal, dixired and red top" (20g) Peach cultivar "Flavorcrest and Romea" (20g)	Double extraction Acetone:water (60:40, v/v) (200mL)	T= room temperature + agitation t= 2 hrs.	81.5	mg chlorogenic acid CAE/g d.w.	Mokrani et al. (2016)
			34.3 - 37.9	mg CAE/g d.w.	
			19.8-23.1	mg CAE/g d.w.	
Canola (10g)	Acetone:water (70:30, v/v) (50mL)	T= 60ºC + ultrasound t= 15 min	12.35±0.76	µmol catechin equivalent (CE)/g d.w.	Chandrasekara et al. (2016)
	Methanol:water (80:20 v/v) (50mL)		21.27±0.13	µmol CE/g d.w.	
Black beans (10g)	Acetone:methanol:water (7:7:6 v/v) (50mL)		21.91±0.63	µmol CE/g d.w.	
	Acetone:water (70:30, v/v) (50mL)		11.64±0.31	µmol CE/g d.w.	
	Methanol:water (80:20 v/v) (50mL)		7.54±0.85	µmol CE/g d.w.	
	Acetone:methanol:water (7:7:6 v/v) (50mL)		7.65±0.55	µmol CE/g d.w.	

CAE: chlorogenic acid, and CE: catechin equivalent.

2.3.2. Pressurized fluids

An alternative method to extract anthocyanins and phenolic compounds is the use of subcritical water and pressurized fluids. Subcritical water extraction consists in exposing water to temperatures between 100ºC and 374ºC, and under enough pressure to remain in the liquid state (Saldaña and Valdivieso-Ramirez, 2015: Monrad, Howard, King, Srinivas, and Mauromoustakos, 2010). Other solvents, such as ethanol and their mixtures with water, can be used as fluids. When the solvent is exclusively water, it is denominated as subcritical water (sCW) and when there is a mixture of solvents, it is known as pressurized fluids. Figure 2.15 shows the water phase diagram, including the subcritical and supercritical regions.

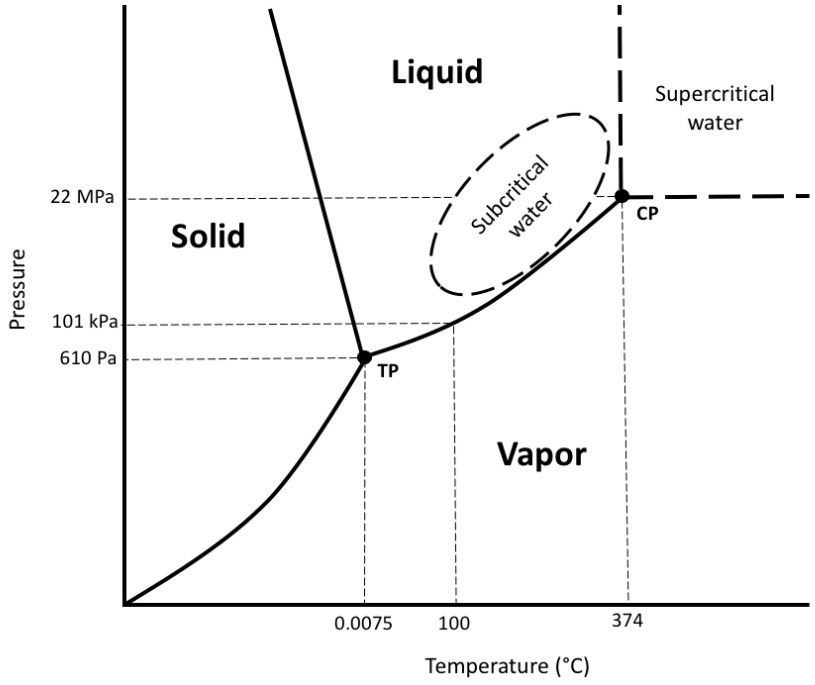


Figure 2.15. Water phase diagram.

Table 2.8 summarizes studies that used subcritical water extraction as a technique to obtain bioactive compounds from food by-products, such as fermented grape pomace, black chokeberry

pomace, lupin hull, mango peel, onion skin, potato peel and winery waste. Most studies reported total phenolics but also hemicellulose and lignin were reported from lupin hull and quercetin from onion skin. Optimal temperature, pressure, time and sample:solvent ratio conditions vary depending on the matrix used.

Table 2.8. Extraction of bioactives from plant-based matrices using subcritical water (sCW).

Source	sCW conditions	Bioactive compound	Highest yield	Reference
Fermented grape pomace (5g)	150ºC 10.33 bar 5 min 1:10 sample:solvent	Total phenolics	4.2 mg GAE/g d.w.	Vergara-Salinas et al. (2013)
Black chokeberry pomace (1g)	110ºC 103 bar min 1:11 sample:solvent (w/v)	Total phenolics	183±2.75 mg GAE/g	Brazdauskas, Montero, Venskutonis, Ibañez, and Herrero (2016)
Lupin hull (3g)	180-260ºC 10-20MPa 2-1 mL/min 200mL	Hemicellulose Lignin	86 mg/g 10 mg/g	Ciftci and Saldaña (2015)
Mango peels	180ºC Pressure not clear. 90 min, pH 4 Solid water ratio as 1:40 (w/v)	Total phenolics	50.25 mg GAE/g d.w.	Tunchaiyaphum et al. (2013)
Onion skin	165ºC 15 min 1.5:2.5 (onion skin: diatomaceous earth)	Quercetin	16.29±0.75 mg/g	Ko et al. (2011)
Potato peel (10g)	180ºC 60 bar 2 mL/min 30min	Total phenolics	81.83 mg GAE /100 g	Singh & Saldana (2011)
Winery waste (2g)	140ºC 116 bar 1-2 mL/min 100 min	Total phenolics	31.69 mg GAE/g d.w.	Aliakbarian et al. (2012)

GAE: Gallic acid equivalent.

2.3.2.1. Solvent properties

By exposing water to subcritical conditions its molecule is modified, hence its physiochemical properties are modified. Figure 2.16 shows the effect on water molecules at high temperatures (0-400ºC) and 250 bar. At a temperature of ~35ºC, pH drops, self-diffusivity increases and both viscosity and surface tension decreases. It can be suggested that at these conditions water acts as an extraction solvent.

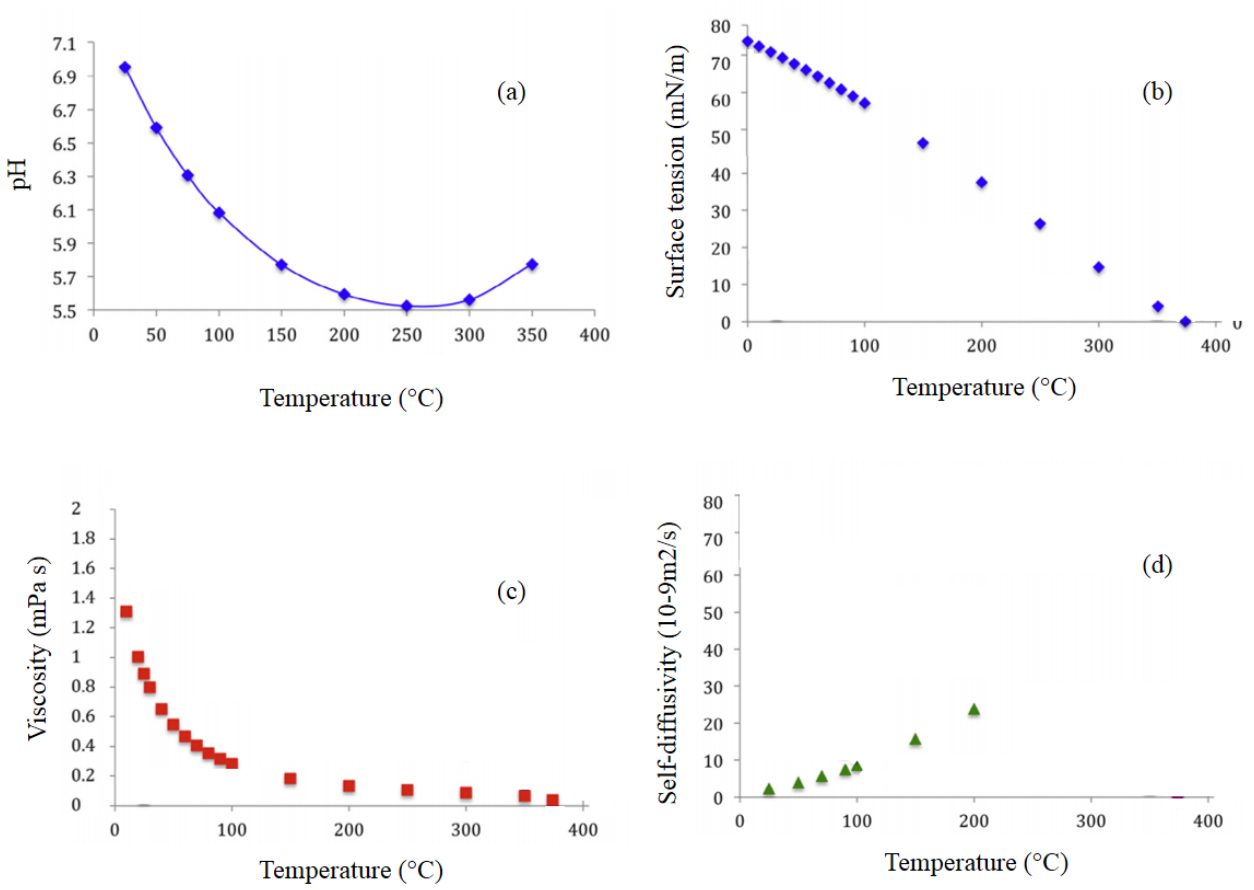


Figure 2.16. Water physicochemical properties as function of temperature: (a) Water pH at 250 bar, (b) Surface tension at saturation pressure, (c) Viscosity at saturation pressure and (d) Self-diffusivity at saturation pressure (Adapted from Plaza and Turner, 2015).

2.3.2.2. Typical sCW extraction system

A typical subcritical extraction system mainly consists of a heating system, a pump and a reactor where the extraction occurs (Fig. 2.17). The aimed temperature can be reached with the support of an oven and a heating band. The pressure can be increased with a high-pressure pump, which is controlled by a pressure regulator. Once the solvent is pumped, it is heated up by a heat exchanger usually installed inside the oven. After the extraction occurs, the extract leaves through a cooling system and is further collected.

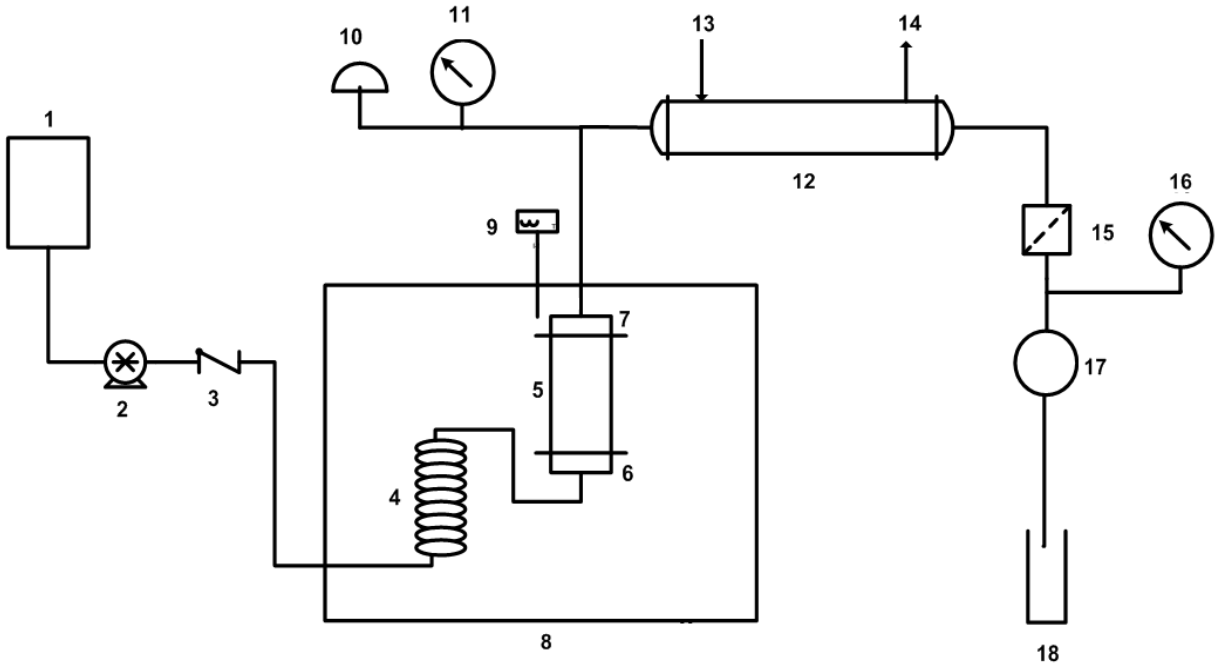


Figure 2.17. Subcritical water extraction system, 1. Water tank, 2. HPLC pump, 3. Check valve, 4. Pre-heating section, 5. Extraction vessel, 6. and 7. Reducers, 8. Oven, 9. Thermometer, 10. Pressure relief valve, 11 and 16. Pressure gauges, 12. Cooling system, 13. Water in, 14. Water out, 15. Filter, 17. Back pressure regulator, and 18. Collection vial.

2.3.2.3. Advantages and disadvantages of extraction techniques

There are some advantages and disadvantages using pressurized fluid extraction, which are summarized in Table 2.9.

Table 2.9. Comparison of three phenolic extraction techniques.

	sCW extraction	Pressurized fluid extraction	Traditional solvent extraction
Solvent used	Water	Mixture of water with other GRAS solvents.	Noxious solvents: methanol, ethyl acetate, acetone, chloroform, hexane, etc.
Extraction time	1 – 60 min	1 – 60 min	> 60 min
Advantages	Green technology, time efficient.	Sometimes higher yields than sCW, time efficient.	Simple technique and cheap.
Disadvantages	Restricted to thermal sensitivity of phytochemicals.	Organic solvent residue in extract.	Needs organic solvents, solvent residue in extract, longer extraction time.

GRAS: Generally recognized as safe. sCW: subcritical water.

2.3.3. Extraction of phytochemicals from cranberry pomace

Table 2.9 shows that different extraction methods have been reported to obtain anthocyanins and total phenolics from cranberry pomace including traditional solvent extraction, ultrasound, microwave, supercritical CO_2 and subcritical water. Total anthocyanins and total phenolics were better extracted with pressurized low polarity water and water+ethanol mixtures than with microwave and supercritical CO_2 extraction. Total anthocyanin extraction with supercritical CO_2 was not reported as anthocyanins are polar and CO_2 is non-polar. The sample solvent ratio also influenced the extraction of both anthocyanins and phenolics.

Table 2.10. Extraction of total anthocyanin and total phenolic from cranberry pomace using various extraction methods.

Source (sample:solvent ratio)	Extraction method	Conditions	Total anthocyanins (mg Cy3GE/g d.w.)	Total phenolics (mg GAE/g d.w.)	Reference
Cranberry pomace (0.5:50, sample:solvent, w/v)	Solvent extraction	Methanol + HCl 1% (v/v), T= N/R, t= N/R.	4.51±0.11	48.0±1.40	Klavins, Kviesis and Klavins (2017)
		Acetonitrile 49.5% + trifluoroacetic acid (TFA) 0.5% + water 50% (v/v/v), T= N/R, t= N/R.	2.28±0.06	38.4±1.20	
		Ethanol 70% + water 29% + HCl 1% (v/v/v), T= N/R, t= N/R.	2.04±0.05	34.3±0.90	
	Microwave	10 min heat up at 600W, reach 80ºC and held for 20 min (solvent: ethanol 96% + TFA 0.5% + unclear solvent 3.5%, v/v/v)	0.054±0.01	10.9±0.40	
	Ultrasound	360W ultrasound at 30ºC (solvent: ethanol 96% + 0.5% TFA + 3.5% unclear solvent, v/v)	1.47±0.04	16.8±0.70	
Cranberry pomace (1:4, sample solvent w/v)	Solvent extraction (acetone:water:acetic acid 70:29:1, v/v/v) + drying + separation process with ethanol (200mL ethanol)+ elutions with different solvents (1L)	Ethanol 70%, water 30% (v/v), T= N/R, t= N/R.	~1.4	~53.73	Rupasinghe, Neir and Parmar (2016).
		Ethanol 80%, water 20% (v/v), T= N/R, t= N/R.	~1.25	~53.73	
		Acetone 50%, water 50% (v/v), T= N/R, t= N/R.	~1.25	~8.95	

N/R: not reported.

Table 2.10 Continued.

Source (sample:solvent ratio)	Extraction method	Conditions	Total anthocyanins (mg Cy3GE/g d.w.)	Total phenolics (mg GAE/g d.w.)	Reference
Organic cranberry pomace (1:5 substrate:solvent ratio)	Solvent extraction	Ethanol 80%, water 20% vigorously mixed for 1 hour.	4.46±0.17	24.87±0.66	Ross et al. (2017)
Depectinized cranberry pomace (5:1, wet pomace: solvent)	Blend + solvent extraction	Ethanol+water (1/1, v/v), T= 80ºC, t= 2 h.	N/R	34.45±2.65	Roopchand et al. (2013)
(10:1, wet pomace: solvent)		Ethanol+water (1/1, v/v), T= 80ºC, t= 2 h.		57.86±7.23	
Cranberry pomace (10g)	Supercritical CO_2 extraction	80 bar and 60ºC	N/R	0.17	Laroze et al. (2010)
		100 bar and 60ºC		0.11	
		200 bar and 60ºC		0.11	
		300 bar and 60ºC		0.08	
Cranberry pomace (1:7.5, w/v)	Low polarity water extraction	T= 150ºC P= 52 bar Flow rate= 5mL/min	N/R	134.67	Mazza & Pronyk (2015)
		T= 120ºC P= 52 bar Flow rate= 10mL/min	N/R	122.4	

N/R: not reported.

2.4. Food coatings

Edible coatings are defined as a thin layer of edible material placed on a food, while edible film is a thin layer of edible material placed on a film or between food components. Coatings can be applied by methods such as dipping, spraying or brushing. Edible coatings can help to prevent an ripening of fruits by delaying fruit respiration, transpiration, and ethylene production. The main mechanism that ripening is delayed is by creating a barrier between the fruit and the air, controlling the migration of water. Such barriers are aimed to have neutral organoleptic properties, be clear, transparent, odourless and tasteless for them not to be detected. Coatings can also improve fruit appearance, by creating brilliance in the surface and maintaining color (Guilbert, Gontars and Cuq, 1995).

Besides preventing fruit ripening by forming a barrier, there are other advantages of applying food coatings to preserve food. Edible coatings act as a physical and mechanical protector, preventing damage from physical impact, pressure, vibrations and other factors (Park, Byun, Kim, Whiteside and Bae, 2005). Also, using nature-based materials to preserve food rather than petro-based plastics make the use of edible coatings an environmentally friendly option (Garcia-Ibarra, Sendon and Rodriguez-Bernaldo, 2003). Moreover, such coatings can be used as a functional coating, meaning that they can transport bioactive compounds to help prevent microorganism from growing or enzymatic reactions.

2.4.1. Main components of films and coatings

Several coating components are used to generate a film or a coating. Polysaccharides, proteins and lipids are the main components used to create a network and then applied as a coating. They

can be used alone or combined, depending on the purpose of the coating. Table 2.11 shows materials previously reported as food coatings, such as protein, polysaccharides and lipids. Most food coatings use mixtures of polysaccharides and proteins.

Table 2.11. Materials used for food coatings.

Macromolecule	Material	Reference
Protein	Collagen, gelatin, casein, corn zein, whey protein, soy protein, egg white protein, wheat gluten, fish myofibrillar protein, sorghum protein, cottonseed protein, pea protein, rice bran protein, peanut protein, keratin.	Ogur and Erkan (2015)
Polysaccharides	Modified cellulose, low methoxyl pectin.	Guerreiro, Gago, Faleiro, Miguel and Antunes (2015)
	High methoxyl pectin.	Maftoonazad and Ramaswamy (20080
	Chitosan.	Wang and Gao (2013)
	Pea starch.	Mehyar, ElAssi, Alsmairat and Holley (2014)
	Xanthan gum.	Sharma and Rao (2015)
Lipids	Beeswax.	Shahid and Abbasi (2011)
	Carnauba wax.	Njombolwana et al. 2013
	Sunflower wax.	Soomro, Sherazi and Sheikh (2013)
	Resins.	Meighani, Ghasemnezhad and Bakhshi (2015)

Some plasticisers, such as glycerol and Tween 80, are used to assist the creation of a network. Bioactive compounds, such as essential oils and other extracts, are used to provide functional properties to the coating. Table 2.12 summarizes the different materials used to develop coatings and their impact in different food products. Most of the food coatings were applied to fruits, such as apples, avocadoes, mango and strawberries. Few studies were conducted for cheese and nuts.

From the three most commonly coating methods, the dipping method was the most studied. Among the emulsifiers, glycerol was the one selected in most of the studies.

Table 2.12. Summary of coating materials, application methods and effect on food products.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Fruits						
Lipid	Semperfresh ™	Not mentioned	Zucchini	Brushing	Moisture barrier.	Avena-Bustillos, Krochta, Saltveit, Rojas-Villegas, and Sauceda-Pérez (1994)
Lipid	Carnauba wax	Not mentioned	Naval oranges and Valencia oranges	Brushing	Mold protection and sporulation inhibition.	Njombolwana et al. (2013)
Lipid	Sunflower wax	Not mentioned	Mango	Dipping or cold wax method	Increase shelf life (30 days), microbial growth inhibition, quality prolongation.	Soomro, Sherazi and Sheikh (2013)
Lipid	Bee wax	Not mentioned	Sweet orange	Not clear	Maintenance of weight loss, firmness, total sugars and ascorbic acid.	Shahid and Abbasi (2011)
Lipid	Chitosan, carnauba wax, resin wax	Not mentioned	Pomegranate	Dipping and brushing	Lower respiration rate, weight loss, maintain bioactive quality.	Meighani, Ghasemnezhad and Bakhshi (2015)
Lipid	Wax	Not mentioned	Valencia oranges and Marsh grapefruit.	Brushing	Moisture barrier, reduction of weight loss.	Hagenmaier and Baker (1995)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Polysaccharides	Sodium alginate, pectin and essential oils.	Calcium chloride	Raspberries	Dipping	Increase shelf life. Essential oils reduced microbial spoilage.	Guerreiro, Gago, Faleiro, Miguel and Antunes (2015)
Polysaccharides	Pectin, potassium sorbate, sodium benzoate, nisin, oleic acid, Tween-80	Glycerol, Tween 80	Fresh-cut persimmon	Not clear	Browning inhibition, inhibit microbial growth.	Sanchis et al. (2016)
Polysaccharides	Low methoxyl pectin, vitamin C.	Not mentioned	Dried papayas.	Immersion	Higher vitamin C retention during drying and storage.	Canizares and Mauro (2015)
Polysaccharides	High methoxyl pectin, sorbitol, beeswax	Not mentioned	Avocados	Immersion	Reduction of weight loss and respiration rate.	Maftoonazad and Ramaswamy (2008)
Polysaccharides	Xanthan gum, cinnamic acid.	Not mentioned	Fresh-cut Asian pears.	Dipping	Retardation of oxidative browning and shelf life extension.	Sharma and Rao (2015)
Polysaccharides	Chitosan, Tween 80, acetic acid	Tween 80	Strawberries	Immersion	Extend shelf life, maintain fruit quality and control decay.	Wang and Gao (2013)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Polysaccharides	Chitosan, acetic acid	Not mentioned	Sweet cherry	Dipping	Reduction of water loss, respiration rate and changes in color.	Petriccione et al. (2015)
Polysaccharides	Chitosan, glacial acetic acid, Tween 80	Tween 80	Guava	Dipping	Reduction on firmness, weight loss and increase antioxidant ability.	Hong, Xie, Zhang, Sun and Gong (2012)
Polysaccharides	Pea starch, zein protein, carnauba wax	Glycerol	Palm fruits (Khalal)	Dipping	Shelf life extension from 7 to 14 days.	Mehyar, ElAssi, Alsmairat and Holley (2014)
Protein and lipids	Corn zein, citric acid and ethanol	Glycerin	Tomatoes	Dipping	Ripening delayed for 6 days with coatings of 5 mm and 15 mm.	Park, Chinnan and Shewfel (1994)
Protein	Whey protein concentrate	Glycerol	Frozen strawberries	Dipping	Maintain quality attributes when freezing.	Soazo, Pérez, Rubiolo and Verdini (2015)
Protein	Whey protein, zataria multiflora extract and glycerol	Glycerol	Pears	Immersion	Shelf life improvement. Preservation of the amount of total soluble solids.	Javanmard, Ojnordi and Esfandyari (2012)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Protein	Corn zein, cysteine, ascorbic acid and jamun leaves extract	Not mentioned	Jamun fruits	Dipping	Decrease weight loss, accumulation of sugars and ripening.	Baraiya, Rao and Thakkar (2015)
Protein	Galactomannan, collagen and glycerol	Glycerol	Apples and mangos	Brushing	28% less O_2 consumption and 11% less CO_2 production.	Lima et al. (2010)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Dairy products						
Protein, polysaccharides and essential oils	Sorbitol, whey protein isolate and ginger essential oil	Alginate	Kashar cheese	Dipping	Water vapor permeability increase and microorganism inhibition (*E.coli O157:H7)* after 30 days with essential oil (2.93±0.54 cfu/g) compared to control (5.10±0.93 cfu/g).	Kavas, Kavas, and Saygili (2016)
Protein and polysaccharides	Chitosan, chestnut starch and different antimicrobial substances (*Cornus officinalis* fruit extract, pine needle essential oil and nisin)	Glycerol	Bod Ljong cheese	Dipping	Decrease water loss and lipid oxidation. Antimicrobial activity observed in coatings with bioactive substances.	Mei, Guo, Wu, and Li (2015)
Protein, lipid and polysaccharides	Zein, ethanol, glycerol, oleic acid and xanthan gum.	Glycerol	Brazilian cheese (Minas Padrao)	Brushing	Decrease in weight loss (30%). Prevented microbial growth for 50 days whereas control samples lasted 21 days. Coated cheese was 124% harder, 30% proteolysis decrease and color change.	Peña-Serna, Penna and Lopes Filho (2016)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Protein	Chitosan and whey protein.	Not mentioned	Göbek Kashar cheese	Not clear	Coated samples were preferred by panelists. Lower mold counts in coated samples.	Yangilar (2015)
Nuts						
Polysaccharide, protein and lipid	Pea starch, whey protein isolate and carnauba wax.	Glycerol	Walnuts and pine nuts.	Immersion	Decrease lipid oxidation by remaining peroxide value below acceptance (20 meq O_2/kg oil) for more than 6 months while control had above acceptance after 4 months.	Mehyar, Al-Ismail, Han and Chee (2012)
Polysaccharide	Carboxymethyl cellulose (CMC) with jujube extract, pomegranate extract and tocopherol	Glycerol	Roasted peanuts and roasted-sonicated peanuts	Immersion	Reduction of oxidation after 12 weeks of 14.5 and 19.7% with jujube extract and pomegranate extract, respectively.	Wambura, Yang and Mwakatage (2010)

Table 2.12 Continue.

Coating type	Coating material	Plasticizer	Food product	Application method	Effect of coating	Reference
Polysaccharide	Starch, cashew tree gum and montmorillonite-type nanoclays	Glycerol and Tween 80	Cashew nuts	Immersion	Decrease of texture changes by reducing moisture absorption, water activity, peroxide value and acidity for 120 days.	Pinto et al. (2015)
Polysaccharide	CMC, peanut skin extract and BHT	Glycerol	Almonds	Not clear	After 126 days, a reduction in peroxide value was observed in coated samples with BHT of 2.00 meq O_2/kg oil, while control had 3.90 meq O_2/kg oil.	Larraui et al. (2016)
Polysaccharide	Prickle pear syrup and dried solid mix (sucrose, salt and corn starch)	Not mentioned	Roasted almonds	Not clear	Peroxide value remained <1 meq O_2/kg oil for coated samples after 60 days while uncoated sampes had >2 meq O_2/kg oil	Gayol, Soliani, Quiroga, Nepote and Grosso (2009)

2.4.1.1. Polysaccharides

Polysaccharide based edible coatings are made of several materials that can be grouped as: starch, non-starch carbohydrates, gums and fibers. Pectin is a natural polysaccharide that can be found between cell wall of many plants and it is used in food products due to its ability to form a gel. The gelling capacity of pectin depends on several factors such as temperature, pectin quality, pH, presence of other sugars and calcium ions (Bhat, Nagasampagi and Sivakumar, 2005). Two types of pectin can be used in the food industry, low methoxyl and high methoxyl pectin. High methoxyl pectin is used in low pH media, producing a gel that does not remelt while low methoxyl pectin calcium ion works independently of the pH, forming a thermo reversible gel (Edwards, 2007).

2.4.1.2. Proteins

Edible protein coatings can be divided in two groups: plant origin protein and animal origin protein. Such coatings can be created from protein isolates or concentrates. Protein structure (secondary, tertiary and quaternary) can be easily modified by different treatments such as heat denaturation, pressure, irradiation, acid, alkali, mechanical treatments, salts, metal ions, chemical hydrolysis, enzymatic treatment and chemical cross-linking (Han and Gennadios, 2005). Once the protein is modified and a coating is created, differences in the coating behavior can be observed depending on the nature of the protein. Ogur and Erkan (2015) analyzed the positive properties of protein based films including soy protein isolate, whey powder protein, egg white powder protein, wheat gluten, corn zein, cattle gelatin, rainbow trout protein and Atlantic mackerel protein. Gelatin presented the highest tensile force of 5.27±0.559 N and light

transmission of 63.30±0.01% followed by collagen with 4.15±0.198 N in tensile force and Atlantic mackerel protein with 39.35±0.01% in light transmission. Whey protein presented the highest oxygen permeability with 322±0.01 mL/mm/day, followed by Atlantic mackerel protein with 281±0.01 mL/mm/day and wheat gluten with 218.00±0.01 mL/mm/day.

2.4.1.3. Lipids and waxes

Lipids are common materials used in fruit coatings. Most lipids or resins used for such purpose are soft solids at room temperature and can easily melt. Using wax as a fruit coating can restrict the transport of oxygen, carbon dioxide and ethylene by partially or completely plugging pores of citrus fruits and providing a glossy appearance (Shellhammer and Krochta, 1997). Wax also provides an improvement in water repellency and moisture vapor resistance, which not only prevents fruits from drying but also improve the appearance by adding gloss to oranges, lemons, limes and grapefruits (NPCS, 2006).

2.4.1.3.1. Beeswax

A natural wax produced by honeybees (C*era alba*) is beeswax. Such wax, which is solid at room temperature, is composed of mixed esters of long chain alcohols (C_{26-32}) and fatty acids and hydroxyl fatty acids of chain length of 16-26 (Spiess, 1992). Beeswax is used in the food as a glazing agent, stabilizer, texturizer and carrier. A total production of 66,173 tonnes of beeswax was produced in 2014 in the world (FAO, 2006). Table 2.13 shows characteristics of beeswax, including its melting temperature, peroxide value and solubility. Beeswax can be white and yellow. White beeswax, which undergoes a blanching process, is free from rancidity and yellow

beeswax presents a honey like odor, easy to break when cold, with a dull, granular, noncrystalline fracture when broken (FCC, 1981). Waxes contain about 31-55% unsaponifiable matter, while fats contain 1-2% (Miller, 1928). Composition of beeswax is shown in Table 2.14, being monoesters the highest compounds found.

Table 2.13. Characteristics of beeswax (Adapted from FAO, 2006).

Characteristic	Range
Melting range	62-65ºC
Acid value	17-24
Peroxide value	No more than 5
Saponification value	87 -104
Solubility	Insoluble in water; sparingly soluble in ethanol; very soluble in ether.

Table 2.14. Composition of beeswax (Hepburn, Pirk and Duanghakdee, 2014)

Constituent fraction	Amount (%)
Hydrocarbons	14
Monoesters	35
Diesters	14
Triesters	3
Hydroxy monoesters	4
Hydroxy polyesters	8
Acid esters	1
Acid polyesters	2
Free acids	12
Free alcohols	1
Unidentified	6
Total	100

2.4.1.4. Emulsifier/Plasticizer

Plasticizers, used in food coatings, help softening the rigid structure of the coating, improving flexibility and extensibility. Casariego et al. (2008) studied the wettability of a chitosan food coating by adding glycerol and sorbitol as plasticizers. By increasing the concentration of chitosan and plasticizers, there was a decrease in the wettability values and adhesion coefficients. Plasticizers tend to attract water molecules and form a large complex with water. Common hydrophilic compounds used as plasticizers are glycerol, sorbitol and polyethylene glycol (Zaritzky, 2010).

2.4.2. Coating application methods

There are three main coating methods to apply an edible coating to food products: dipping, spraying and brushing. The dipping method consists in immersing the food product into the liquid coating briefly and further drying. The brushing method consists in applying the coating with the use of a brush. Lastly, the spraying method consists in applying the coating with the use of a nozzle.

Coating methods impact coating properties. A significant thickness difference was observed when coating mozzarella cheese by dipping or spraying methods. Dipping presented higher coating thickness (chitosan: 66μm, sodium alginate: 71.9μm, soy protein isolate: 81.8μm), while spraying presented a thinner coating (chitosan: 30.6μm, sodium alginate: 54.2μm, soy protein isolate: 68.5μm) (Zhong, Cavender and Zhao, 2014).

2.4.3. Shelf life

Factors regulating deterioration reactions must be considered to extend food shelf life. Depending on the food products, different deterioration reactions can be observed. As an example, deterioration reactions in fruits are related to respiration rate, are affected by temperature, atmospheric gas composition and ethylene presence. Raza et al. (2013) studied the impact of temperature on mangos from Pakistan where their respiration rate was doubled at 12ºC and 14ºC compared with mangos stored at 10ºC, concluding that temperature influenced the respiration rate.

On the other hand, high fat food products undergo deterioration reactions such as hydrolytic rancidity and oxidative rancidity. Hydrolytic rancidity occurs when triglycerides react with water molecules and glycerol is separated from the fatty acids. Oxidative rancidity or autoxidation is related to the number of unsaturated fatty acids present and, when exposed to heat, light and enzymes, free radicals are produced (Vaclavik and Christian, 2008).

2.4.4. Bioactive coatings

New research in the edible coating field has focused in adding an extra value to such coatings. One of the developments is to use a coating that not only acts as a barrier to slow down fruit deterioration but also prevents microorganisms from growing or delays oxidative reactions. Tayel, Moussa, Salem, Mazrou and El-Tras (2016) studied the effect of adding plant extracts to a fruit coating to prevent fungal growth. Cress seed extract was the most efficient in inhibiting and inactivating fungal strains followed by extracts from pomegranate peels and olive leaves. On the other hand, chitosan coatings enriched with rosemary, onion, cranberry, garlic and capsicum

reduced polyphenoloxidase activity after 5 days of storage with reductions of 7, 42, 74, 17 and 33%, respectively (Ponce, Roura, Del Valle and Moreira, 2008). The use of essential oils in edible coatings has a bioactive function. For example, oregano oil in a concentration of 0.1% (w/w) in an edible film made of apple pure solution had an inhibitory zone (colony free parameter) for *E.coli O157:H7* of 1.4mm. In contrast, cinnamon oil and lemongrass oil had inhibitory zones of <1mm (Rojas-Grau et al., 2006).

As reported in the literature, large amounts of cranberry pomace are produced by the juice industry, containing bioactives like anthocyanins. Such compounds could be extracted using traditional solvents, however there are limited applications of the extracts because of the presence of non-GRAS solvents in the final product. Therefore, this thesis focused on the green extraction of anthocyanins and total phenolics using pressurized fluids (GRAS) in a semi-continuous system at 50-200 bar and 40-160ºC. Extracts were characterized using the pH differential method, Folin-Ciocalteu method, FRAP (antioxidant capacity) and HPLC-UV. The use of these extracts with pectin and pectin + beeswax coating was also studied. Coatings were applied to almonds to prevent deterioration reactions like lipid oxidation. Almond oil of coated samples was analyzed using gas chromatography and incipient rancidity after storage.

Chapter 3: Pressurized fluid extraction of anthocyanins from cranberry pomace.

3.1. Introduction

Cranberries, one of the most highly consumed berries worldwide, are mainly grown in the Americas. In 2014, 98% of the total cranberry production was from the United States of America (58%), Canada (27%) and Chile (13%) with the remaining 2% from Belarus (1%), Azerbaijan (<1%) and other eastern European countries (<0.37%) (FAO, 2016). Canadian cranberry production has increased from 95114 tons in 2011 to 161368 tons in 2015 (Statistics Canada, 2015). This representative increase in cranberry production could be related to its potential health benefits when consumed. More specifically, numerous research studies relate the consumption of cranberry products (e.g. cranberry juice and cranberry capsules) with the prevention and treatment of urinary tract infections (Durham, Stamm & Eiland, 2015; Caljow et al., 2014). Its potential to benefit humans health is related to the phenolic compounds found in cranberries. Health Canada approved the claim of antioxidants in food labeling with the following legend "source of antioxidants" or "source of antioxidants that help protect against the oxidative damage caused by free radicals" (Health Canada, 2017).

Phenolic compounds can be generally grouped in two, phenolic acids and flavonoids (anthocyanins). Anthocyanins contribute 39% of total antioxidant capacity in cranberries followed by vitamin C (23%), procyanidin dimers (12%), flavonols (10%) and chlorogenic acid (2%) (Borges, Degenve, Mullen and Crozier, 2009). Anthocyanin chemical structure consists of a glycosylation of a sugar with an anthocyanidin. Because of the variety of anthocyanidins (e.g. cyaniding, peonidin and delphinidin) and sugars (e.g. glucose, arabinose and galactose), various anthocyanin configurations are found. The main anthocyanins found in cranberry are peonidin-3-

galactoside (47.5%), peonidin-3-arabinoside (30%), cyanidin-3-galactoside (11.4%) and cyanidin-3-arabinoside (11.1%) (Lee et al., 2016). Anthocyanins are pigment molecules that provide the red color of cranberries. This pigment can be found in both the pulp and the skin of the berries. Higher concentrations of anthocyanins are found in the cranberry skin than in the pulp. The smallest berry size cultivars Ben Lear (70-90 cup counts) and Bergman (65-80 cup count) had the highest anthocyanin content (7.98±5.83 and 7.02±1.75mg Cy3GE/g dry weight (d.w.), respectively) and the largest berry size cultivars Pilgrim (46-66 cup count) and Stevens (50-60 cup count) had the lowest anthocyanin content (3.28±1.88 and 2.81±0.81mg Cy3GE /g d.w., respectively) (Brown, Murch and Shipley, 2011). Cranberry skin and seeds, known as cranberry pomace, are by-products from the cranberry juice industry. Such industry consumes 60% of the total cranberry production (Tokusoglu and Hall, 2011). Cranberry pomace is a good anthocyanin source not only because of its high availability due to the high cranberry consumption of the cranberry juice industry, but also because of the anthocyanin concentration found in cranberry skin.

To remove anthocyanins and phenolic compounds from berries, there are two main extraction techniques. The first one uses petrochemical based solvents such as methanol, acetone, ethyl acetate and chloroform (Sa et al., 2014; Zhang et al., 2016). The extraction of anthocyanins from cranberry pomace using methanol + HCl (98:1, v/v) resulted in 4.51±0.11 mg Cy3GE/g d.w. and lower values (2.28±0.06 mg Cy3GE/g d.w.) were obtained using acetonitrile 49.5%+trifluoroacetic acid 0.5%+water 50% (v/v) (Klavins L., Kviesis and Klavins M., 2017). The use of this petrochemical extraction technique has disadvantages as it requires extended

extraction times and there is solvent residue in the final extracts, which are toxic and limit the extracts application.

The second extraction technique is an environmentally friendly alternative known as "pressurized fluids" and consists in the exposure of a fluid to pressures above 6 bar and a temperature above 100ºC in the case of water. At such conditions, the main physicochemical changes include an increase in both ionization and self-diffusivity and a decrease in surface tension (Saldaña and Valdivieso-Ramirez, 2015; Herrero, Cifuentes and Ibañez, 2006). This green technique has been applied to extract bioactive compounds found in different sources such as grape pomace (Vergara-Salinas et al., 2012), bilberry (Babova, Occhipinti, Capuzzo & Maffei, 2016), onion skin (Ko et al., 2011), winery waste (Aliakbarian et al., 2012), and mango peels (Tunchaiyaphum, 2013) among others.

To the best of our knowledge, the USA patent 9,084,948 in 2015 uses only pressurized low polarity water to extract phytochemicals from sources such as grape pomace, cranberry pomace and hemp meal (Mazza & Pronyk, 2015). Their study include temperature ranges from 85-150ºC where the highest total phenolic yield (172.84%, wt product/wt available unclear, possibly obtained from traditional extraction) was obtained at 150ºC with a flow rate of 5mL/min, 7.5:5 solvent:solid ratio and pressures of 20-50 bar. They did not report anthocyanin yield but stated that desirable anthocyanins were eliminated above 110ºC. There are no studies that extract anthocyanins from cranberry pomace using pressurized fluids such as water, ethanol, water+ethanol (30 and 70%) and water+citric acid (5%). The main objective of this study was to extract anthocyanins and total phenolics from cranberry pomace using pressurized fluids at

processing conditions of temperature and pressure. The antioxidant activity of the liquid extracts was also evaluated in relation to the total anthocyanin and total phenolic contents.

3.2. Materials and methods

3.2.1. Materials

Cranberry pomace was obtained after juice extraction of cranberry purchased from a local grocery store (Safeway, Edmonton, AB, Canada).

Chemical reagents used such as ethanol (99.9%, HPLC grade), chloroform (99.9%, HPLC grade), methanol (99.9%, HPLC grade), Folin Ciocalteau's phenol reagent (2M), glacial acetic acid, gallic acid standard (99.9% purity), Fe_2SO_4 (98% purity), potassium chloride, tripyrodyltriazine, ferric chloride, HCl (37%), sodium acetate were purchased from Sigma Aldrich (Oakville, ON, Canada). Glass beads (3mm) were purchased from Fisher Scientific Co. Ltd (Toronto, ON, Canada).

3.2.1.1. Sample preparation

Frozen cranberries (Compliments brand, Edmonton, AB, Canada) obtained from a grocery store were stored at -18°C. Frozen cranberries were used over fresh cranberries to prevent deterioration and to use the same lot number to prevent variation. After the cranberries were defrosted at 4°C for 48 hour, cranberry juice was extracted with a conventional speed juice extractor (Hamilton Beach 67900, Southern Pines, NC, USA). Cranberry pomace (cranberry skin and seeds) after juice extraction was collected, and stored at -18°C in thin layers in aluminum

containers. Frozen cranberry pomace was freeze dried (Labconco FreeZone® 12 liter, Kansas city, MO, USA) at a vacuum of 0.280 mbar and -53°C for 7 days. Freeze dried cranberry pomace was milled (Retsch®ZM200, Dusseldorf, North Rhine-Westphalia, Germany) to a particle size of 0.5mm. Dried cranberry pomace was milled to homogenize the sample. Dried sample was stored at -18°C until further use.

3.2.2. Proximate compositional analysis

3.2.2.1. Moisture

Moisture content determination was performed in triplicate by the gravimetric method (AOAC, 2000). A total of 5.5±0.5 g of cranberry pomace were placed inside a previously dried aluminium tray and further placed in a convection hot air oven (Memmert 100 – 800, Büchenbach, Germany) at 105°C for 25 hours. After reaching a constant weight, the samples were placed in a desiccator for cooling. After the samples cooled down, they were weighed in a balance (Citizen scale CX165, Cumming, GA, USA). Equation 3.1 was used to calculate the moisture content:

$$Moisture\ content\ (\%) = 100 - \left(\frac{Dried\ sample\ weight}{Initial\ sample\ weight} * 100\right) \quad (3.1)$$

3.2.2.2. Ash

Ash content was determined in triplicate by the incineration of the dried sample at 550°C (AOAC, 2000). A muffle furnace (model F-A1730, Thermolyne corporation, Chula Vista, CA,

USA) was used to incinerate the samples in crucibles overnight. After the incineration, the samples were placed in dessicators to cool down and were weighed in a balance (Citizen scale CX165, Cumming, GA, USA). The ash content was calculated using the following equation:

$$Ash\ (\%) = (1 - Moisture\ content\ ) * \left(\frac{Incinerated\ sample\ weight}{Inicial\ dried\ sample\ weight} * 100\right) \qquad (3.2)$$

3.2.2.3. Protein

Protein content was determined by nitrogen content using the Leco TruSpec nitrogen analyzer (Leco instruments Ltd., Mississauga, ON, Canada). A total of 0.1g was weighed and placed into an aluminum fold cone and further sealed and pressed. The sample was placed in the loading head to be processed. The combustion occurred in a sealed chamber under atmospheric air free environment inside a furnace at 950ºC using pure oxygen, where the thermal conductivity was analyzed to quantify the nitrogen content (%). Rye flour (Leco Corporation, Saint Joseph, MI, USA) was used to calibrate the equipment.

3.2.2.4. Fat

Fat content was determined following the methodology reported by Folch (1957), with minor modifications. A solution of 2:1 (v/v) chloroform:methanol was placed inside a 50mL beaker and mixed. A total of ~0.2g of cranberry pomace was placed inside a tube with a Teflon tap (100x13mm) and 10mL of the solution were added. Closed tubes were placed in a shaker (Lab-line instruments Inc 3540, Melrose Park, IL, USA) for 20 min. The mixed solution was vacuum filtered (Maxkold VP2200, North East London, UK) using a 125mm filter (Whatman® No 1001 125, Maidstone, UK) to remove solids from the solvent. The solid-free solvents were placed in

another test tube and 2mL of a solution of 0.3% NaCl in water were added. Test tubes were vortexed for 1 min and further centrifuged for 5 min at 2000 rpm. The upper methanol+NaCl solution layer was removed and the lower layer, containing chloroform and lipids, was placed in an aluminum tray. Then, the tray was placed in a heating tray inside a fume hood to evaporate the solvent. The dried aluminum tray was placed inside an oven (Memmert 100 – 800, Büchenbach, Germany) at 100ºC for 15 min to remove any chloroform traces. The final weight was measured and fat content was calculated.

3.2.2.1. Carbohydrate

Total carbohydrate content of cranberry pomace was calculated by subtracting the ash content (dry basis), protein content (dry basis) and fat content (dry basis) from the total dry solids.

3.2.3. Extraction method

3.2.3.1. Traditional solvent extraction

The solvent extraction was performed following the methodology described by Brown and Shipley (2011). The extraction solvent was made by mixing 98% methanol and 2% HCl (v/v) in a 100 mL beaker. Approximately 0.25 g of freeze dried and milled cranberry pomace was weighed and placed in a 50 mL test tube with a Teflon cap. Then, 20 mL of the acidified methanol solution were added to the test tubes. Test tubes were vortexed for 10 seconds and then placed in an ultrasonic bath of 5.7L (Fisher Scientific, Brightwaters, NY, USA) for 15 min at ambient temperature and vacuum filtered (Maxkold VP2200, North East London, UK) using 125mm filters (Whatman® No 1001 125, Maidstone, UK). The solid-free solvent was placed in

a 25mL volumetric flask and brought to total volume of 25mL using the same extraction solvent. Liquid extracts were stored at 4ºC until further analysis.

3.2.3.2. Pressurized fluid extraction

The pressurized fluid extraction system used in this study is the same used earlier by Ciftci & Saldaña (2015). Briefly, 2g of freeze dried cranberry pomace and 25 grams of 3 mm glass beads were filled into the reactor. The pressurized fluid extraction was performed in a semi-batch extraction system (Fig 3.1), which consisted of a HPLC pump (Reaxus 6010R, Teledyne, Lincon, NE, USA), an oven (Binder 06-94265, Tuttlingen, Germany) to preheat the system, an extraction cell (Swagelok. Edmonton, AB, Canada), a reactor heating jacket (TruTemp, Edmonton, AB, Canada), a back-pressure regulator (Swagelok, Edmonton, AB, Canada), a cooling system (Swagelok, Edmonton, AB, Canada), a filter (Swagelok, Edmonton, AB, Canada) and a safety check valve (Swagelok, Edmonton, AB, Canada). Extractions were performed at 120, 140 and 160°C, and 50 and 200 bar. The solvents used were Milli-Q water + ethanol (30 and 70%) (v/v), ethanol, and Milli-Q water + citric acid 5% (w/w). The reactor volume was 20 mL and the flow rate for the extractions remained constant at 5 mL/min and samples were collected every 5, 10, 20 and 30 minutes. All extractions were performed at least in duplicates. Collected samples were stored at -18°C until further analysis.

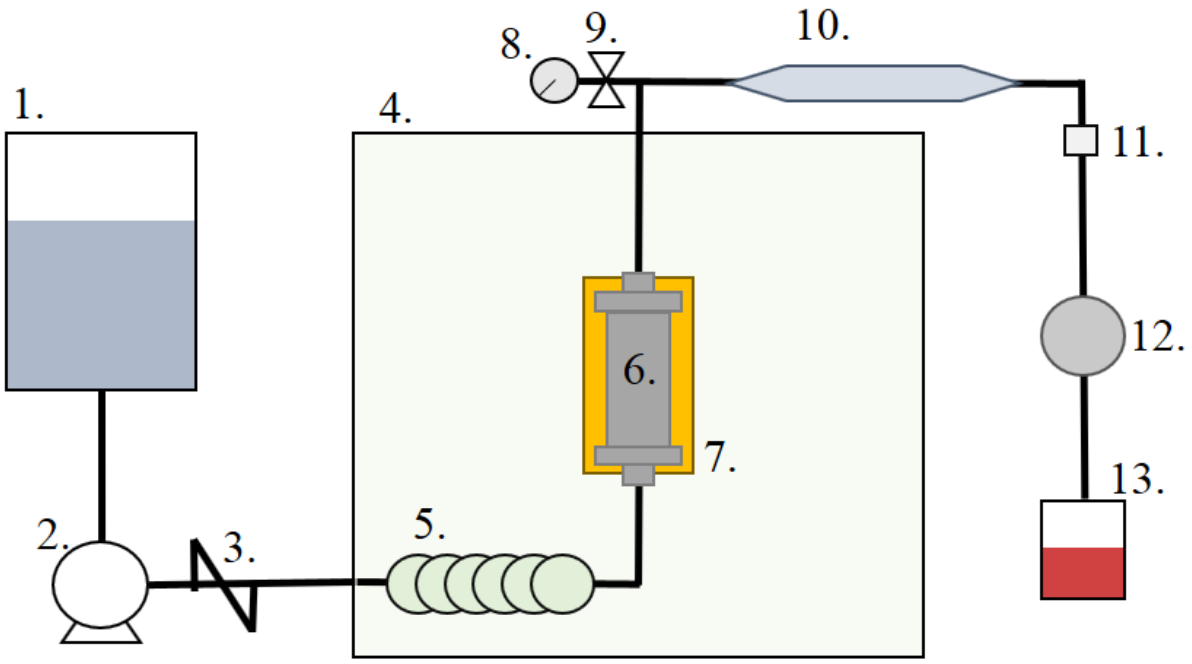


Figure 3.1. Pressurized fluid extraction system. 1. Water tank, 2. HPLC pump, 3. Check valve, 4. Oven, 5. Pre-heating section, 6. High pressure reactor, 7. Heating jacket, 8. Manometer, 9. Check valve, 10. Cooling system, 11. Filter, 12. Back pressure regulator, and 13. Collection vial.

3.2.4. Characterization of liquid extracts

3.2.4.1. pH and conductivity measurements

The pH and conductivity of the extracts were measured using an Excel XL20 pH/conductivity meter (Fisher Scientific Accumet, Brightwaters, NY, USA) at room temperature.

3.2.4.2. Total anthocyanin content

Total anthocyanin content was calculated using the pH differential method (AOAC 2005). Two buffer solutions were prepared at different pH values of 1 and 4.5. Briefly, the acidic buffer solution was prepared by mixing 1.86g of KCl with 980 mL of distilled water and the pH was further adjusted to 1.0 (±0.05) using HCl. The second solution with a pH of 4.5 was prepared by mixing 54.43g $CH_3CO_2Na \cdot H_2O$ with 960mL of distilled water and the pH was also adjusted using HCl. Cranberry liquid extracts were mixed with each buffer solution in a ratio of 1:1 (v/v) extract:buffer. Dilutions were done if needed.

Absorbance (A) was measured with a spectrophotometer at two wavelengths of 510 and 700nm for each solution at pH 1 and pH 4.5. Anthocyanin content is expressed as milligrams of cyanidin-3-glucoside equivalent (Cy3GE) and calculated with the following equation:

$$Anthocyanin\ pigment\ \left(\frac{mg\ C3GE}{L}\right) = \frac{A*MW*DF*10^3}{\varepsilon*1} \quad (3.3)$$

where:

$A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$

Molecular weight (MW) = 449.2 g/mol of cyanidin-3-glucoside (Cy3GE)

DF = Dilution factor

ε = 26900 molar extinction coefficient (L/mol cm of Cy3GE)

3.2.4.3. Total phenolic content

Total phenolic content was analyzed following the methodology reported by Singleton and Rossi (1965) with some minor modifications. From the extract solution, 0.04mL were mixed with 3.16mL of distilled water and vortexed for 10 seconds. Folin-Ciocalteau's phenol reagent

were added (0.2mL) and vortexed for 10 seconds. After 6 minutes of reaction, 0.6mL of sodium carbonate solution were added followed by 10 seconds of vortex. Samples were stored for two hours in a dark place inside 1.5mL plastic cuvettes. The absorbance was measured using a spectrophotometer (Jenway 6230D, Stone, Staffordshire, UK). All extracts were analyzed at least in duplicates. A calibration curve of gallic acid solutions was generated and total phenolics were expressed as milligrams of gallic acid equivalent per two grams of dried cranberry pomace.

3.2.4.1. Anthocyanin determination by HPLC-UV

Individual anthocyanins were calculated by HPLC-UV (Shimadzu prominence 20, Kyoto, Japan), using the methodology reported by Brown and Shipley (2011). Briefly, a volume of 10 µL of solution was injected into an analytical column (5C18-PAQ, 4.6 x 150 mm) at 25ºC with a UV detector at 520 nm. The total run time was 35 minutes per sample and the mobile phases used were A: water+phosphoric acid (99.5:5, v/v), and B: water+acetonitrile+glacial acetic acid (50:48.5:0.5, v/v/v). Calibration curves were performed using cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and peonidin 3-galactoside standards (Polyphenols, Sandnes, Norway) diluted in methanol+HCl (98:2, v/v).

3.2.4.2. Antioxidant capacity by ferric reducing antioxidant assay

Ferric reducing antioxidant assay (FRAP) analysis was performed following the methodolody reported by Benize and Strain (1996) with minor modifications. This method is based on the reduction of Fe^{3+} complex of tripyridyltriazine Fe $(TPTZ)^{3+}$ to a blue coloured Fe^{2+} complex Fe $(TPTZ)^{2+}$ by antioxidants in an acidic medium. Solution "A", a buffer solution with pH=3.6 of

0.3M acetate, was made by adding 0.2019g of glacial acetic acid and 0.0324g of sodium acetate trihydrate in 1L of milli-Q water. The pH of the solution was measured; if the pH was higher than 3.6, it was adjusted by the addition of drops of glacial acetic acid. A second solution "B" was made by mixing 765mg of TPTZ in 250mL of a HCl 40mmol/L solution. A third solution "C" was made by adding 1324mg of $FeCl_3 \bullet 6H_20$ in 250 mL of water. The FRAP reagent solution was prepared by mixing the solutions A, B and C in a volume ratio of 10:1:1, respectively. A liquid extract of 100µL was mixed with 300µL of water and 3000µL of the working FRAP solution, vortexed for 3 seconds and placed in a water bath at 37ºC for 30 min. Absorbance was measured using a spectrophotometer at 593nm. A calibration curve was used to calculate the antioxidant capacity using an aqueous solution of Trolox at 2400µMol/L. The FRAP content was reported as µMol of trolox equivalent/L (µMol TE/L).

3.2.5. Statistical analysis

The software used for the statistical analysis was Minitab 17 (State College, PA, USA). Total phenolic extraction and total anthocyanin extraction were analyzed as two independent responses using a multivariate analysis of variance (MANOVA).

3.3. Results and discussions

Proximate composition analysis of cranberry pomace is shown in Table 3.1. Moisture content of cranberry pomace was 86.29±0.22% and whole cranberry was 88.61±0.34%. These close values suggest that the amount of water removed by the juice extractor was ~2.3%. Ross et al. (2017) reported the moisture content of cranberry pomace (68.37±1.56%) and blueberry pomace

(63.61±2.15%) after a juice extraction using a hydraulic rack and press with stages of 69, 138 and 207 bar. In this study no press was applied after the juice extraction, hence little moisture reduction was observed. However, the initial moisture content does not impact the total anthocyanin extraction values because they are reported as total anthocyanin per gram of dried weight. There is approximately 12.51% of carbohydrates. Cranberry pomace carbohydrates are mainly 82.29% insoluble fiber, 7.16% soluble fiber and 10.55% other carbohydrates (White, Howard and Prior, 2010).

Differences in the proximate compositional analysis could be attributed to the variety of cranberry cultivars, even though only few of them are used commercially such as Ben Lear, Bergman, Crowleys, Howes, Early black, McFarlin, Pilgrim, Searles and Stevens (Stewart, 2005). Besides the differences between cultivars, other factors influence cranberry chemical composition such as irrigation system (Samson, Fortin, Pepin and Caron, 2016), number of uprights (fruit shoots) per square meter (Szwonek et al., 2016) and the water table depth below soil surface (Pelletier et al., 2015).

Other factors influence the initial content of anthocyanins available for extraction from cranberries, such as cultivar variations, agricultural factors and maturity stage. Viskelis et al. (2009) studied the anthocyanin content variation between cultivars at progressive ripening stages. At the beginning of ripening, total anthocyanin contents were 0.17 mg cyaniding-3-rutinoside (Cy3RE)/g d.w. for cultivar “Stevens”, 0.37 mg Cy3RE/g d.w. for cultivar “Pilgrim”, 0.27 TAcy mg/100 g for cultivar “Ben Lear” and 0.3 mg Cy3RE/g d.w. for cultivar “Black Viel”, and overripe berries had an increase of anthocyanins with values of 8.12, 10.5, 12.6 and 15.6 mg

Cy3RE/g d.w., respectively. These results suggest that anthocyanin content increased during fruit ripening and the ripening stage is crucial for anthocyanin extraction.

Table 3.1. Proximate compositional analysis of cranberry.

Macronutrient	Cranberry pomace (This study)	Cranberry pomace (Ross et al., 2017)	Raw cranberry (USDA, 2016)
Moisture content (%)	86.29±0.220	68.37	87
Ash (%)	0.20±0.009	0.33	-
Protein (%)	0.47±0.004	1.82	0.46
Fat (%)	0.53±0.030	1.39	0.13
Carbohydrate (%)	12.51	28.08	11.97

Figure 3.2 shows the total anthocyanin extraction using a range of temperatures (120-160ºC), pressures (50-200 bar) and solvents (water, ethanol, ethanol30%+water, ethanol70%+water, citric acid5%+water). By increasing the concentration of ethanol, an increase of total anthocyanin extraction yield was observed. At 120ºC and 50 bar, high anthocyanin extraction was obtained with pressurized ethanol (3.89±0.19 mg Cy3GE/g dry weight (d.w.)) followed by 70% ethanol (2.91±0.23 mg Cy3GE/g d.w.), 30% ethanol (1.03±0.05 mg Cy3GE/g d.w.), 5% citric acid (0.86±0.20 mg Cy3GE/g d.w.) and water (0.39±0.04 mg Cy3GE/g d.w.).

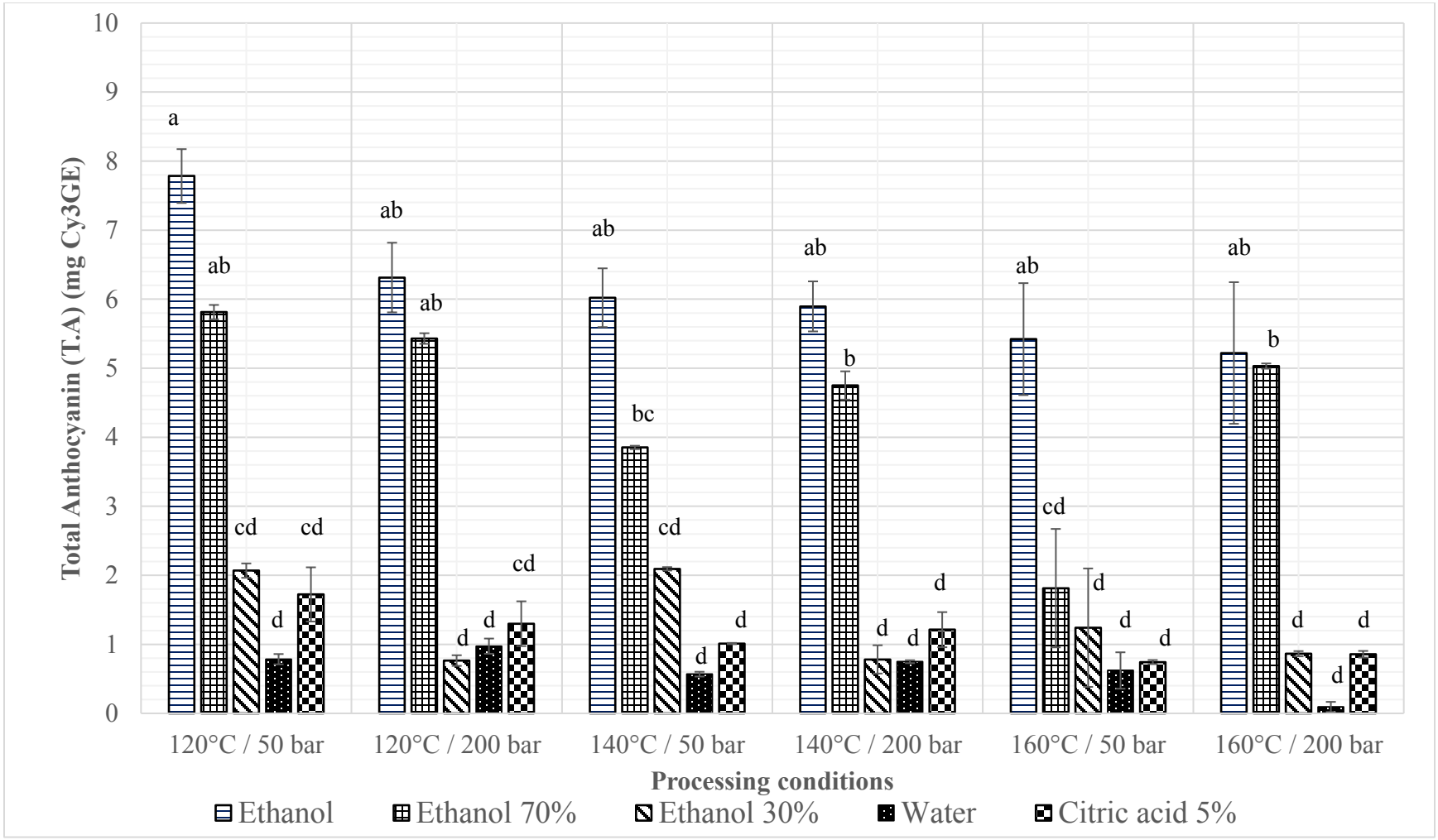


Figure 3.2. Total anthocyanin extraction using pressurized fluids at different processing conditions. Letters a-d correspond to difference between all values ($p<0.05$).

Main effects and interactions of processing factors are reported in Table 3.2. Temperature and solvent had a significant impact ($p<0.05$) in anthocyanin extraction, unlike pressure. However, pressure and solvent together showed a significant impact in total anthocyanin extraction.

Table 3.2. Effect and interaction of processing condition for total anthocyanin extraction using pressurized fluids.

MANOVA	Test statistic	F	Num	Denom	p
Temperature	0.415	13.523	2	38	<0.05
Pressure	0.007	0.282	1	38	0.60
Solvent	0.941	150.922	4	38	<0.05
Temperature*Pressure	0.145	3.234	2	38	0.05
Pressure*Solvent	0.324	4.546	4	48	<0.05
Temperature*Solvent	0.236	1.467	8	38	0.20

Figure 3.3 shows the relation between total anthocyanin extraction and pH with pressurized ethanol concentration. Pressurized fluid pH significantly increased when increasing the ethanol concentration. The increase of ethanol concentration increases the total anthocyanin extraction from cranberry pomace as the dielectric constant of ethanol (25.02±0.02 at 20ºC) is lower compared to the dielectric constant of water (79.99±0.04 at 20ºC) (Mohsen-nia, Amiri and Jazi, 2009). Earlier, Oancea, Stoia and Coman (2012) reported that anthocyanin extraction from blueberries was significantly greater using ethanol + water 50% (v/v) (10.45 mg Cy3GE/g d.w.) compared to water (0.23 mg Cy3GE/g d.w.).

Also, assisting the extraction with ultrasound at 20 kHz and 50% amplitude, a higher anthocyanin extraction yield from purple potato (moisture content basis unclear, moisture content of sweet potato 77.29% (USDA, 2016)) was observed with 70% ethanol + water

(1.60±0.0005mg Cy3GE/g d.w.) and a lower yield with 50% ethanol + water (0.44±0.02mg Cy3GE/g d.w.) after 5 minutes. However, after 120 min of sonification treatment for both ethanol water concentrations, total anthocyanin decrease of 62% and 65% were observed for the 70% ethanol+water and 50% ethanol+water, respectively (Mane, Bremner, Tziboula-Clarke and Lemos, 2015).

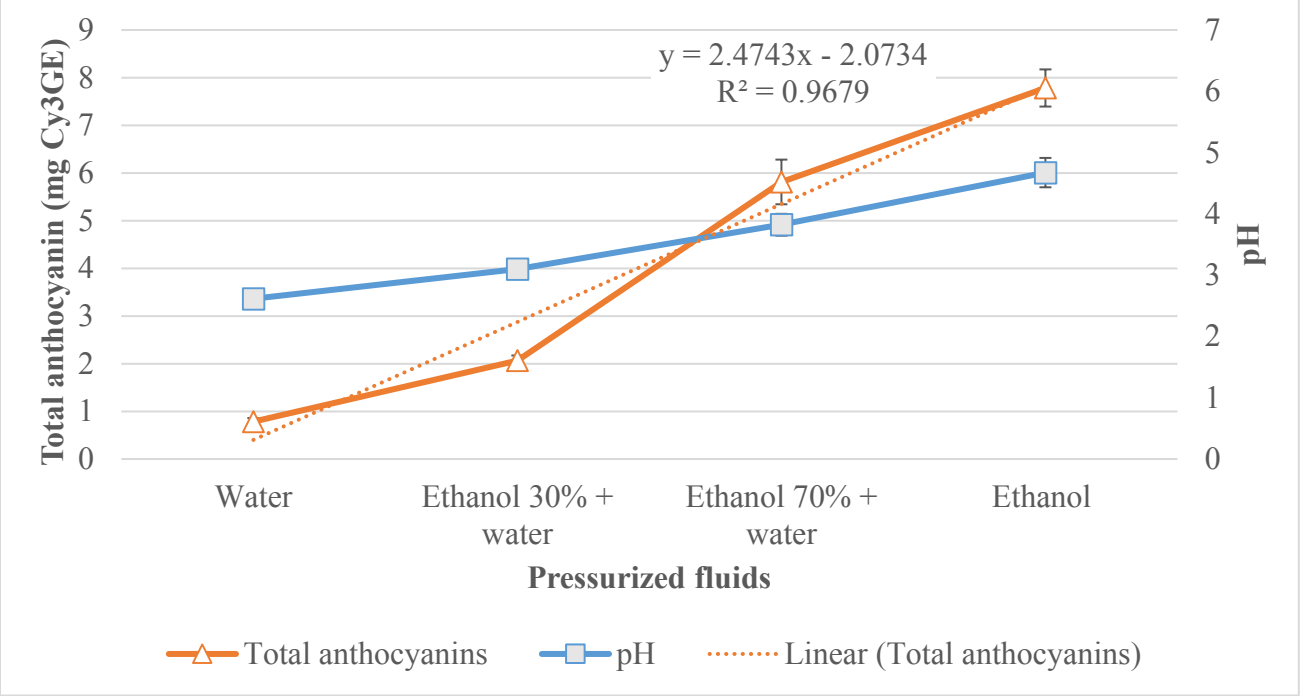


Figure 3.3. pH and total anthocyanin extraction using pressurized aqueous ethanol concentrations, pressurized ethanol and pressurized water at 120ºC and 50 bar.

Figure 3.4 shows total anthocyanin extracted from cranberry pomace using pressurized fluids (water, water+ethanol 30%, water+ethanol 50%, ethanol and water + citric acid 5%) at 50 bar and temperatures of 120, 140 and 160ºC. There is a significant impact ($p<0.05$) varying temperature in total anthocyanin extraction with a higher anthocyanin extraction trend observed

at lower temperatures. This behaviour was also observed in the extraction of anthocyanins from grape pomace, which ideal extraction temperature ranged from 80-120ºC and non-ideal temperatures included 40-60ºC and 140ºC (Monrad, Howard, King, Srinivas and Mauromoustakos, 2010).

Sui, Dong and Zhou (2014) reported that temperature and pH had a crucial role in anthocyanin deterioration rate, whereas anthocyanin deterioration rate increases when increasing both temperature (100-165ºC) and pH (2.2-6). The deterioration rate at 165ºC and pH 6 was 14 times higher than the one at 100ºC and pH 2.2. Total anthocyanin contents of blueberry purée of three cultivars, Bluecrop, Jersey and Earliblue, were quantified at 4ºC (7.90±0.198, 12.83±0.34 and 11.47±0.39 mg total anthocyanin/g d.w.) and 100ºC (5.74±0.13, 8.81±0.32 and 6.99±0.262 mg anthocyanin/g d.w.), showing that there is a decrease in anthocyanin content at 100ºC compared to 4ºC (Zorenc, Veberic, Stampar, Koron and Mikulic-Petkovsek, 2017). The content of cyanidin 3-glucoside under pH 1, pH 4 and pH 7 was reported after 60 days of dark storage at 10ºC (Fossen, Cabrita and Andersen, 1998). Initial absorbance values were 2.06 (pH 1), 0.70 (pH 4) and 0.72 (pH 7). After 60 days, the absorbance values were 2.21, 0, and 0, respectively, suggesting a higher stability in acidic media. Anthocyanins in acidic media of pH 3 showed better retention percentage after 19 days at 25ºC (3.1±0.02%) than at pH 4 (0.4±0.03%) (West and Mauer, 2013). In this study, the addition of citric acid 5% to pressurized water had no significant impact in total anthocyanin extraction as pH is ~2.

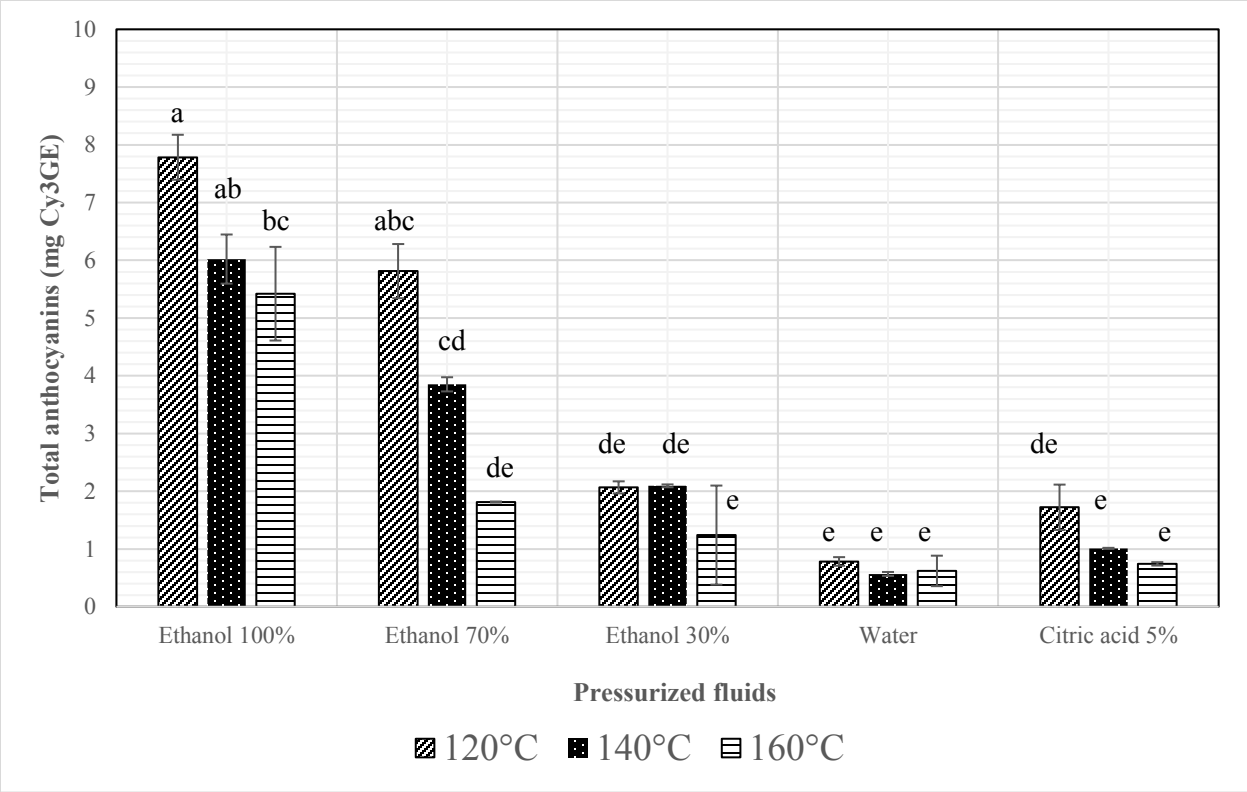


Figure 3.4. Total anthocyanin extraction using pressurized fluids (50 bar) at 120, 140 and 160ºC (Letters a-e correspond to differences between all values).

As the best temperature to extract anthocyanins from cranberry pomace was 120ºC (Fig. 3.4), extractions at lower temperatures and 50 bar using pressurized ethanol were performed (Fig. 3.5).

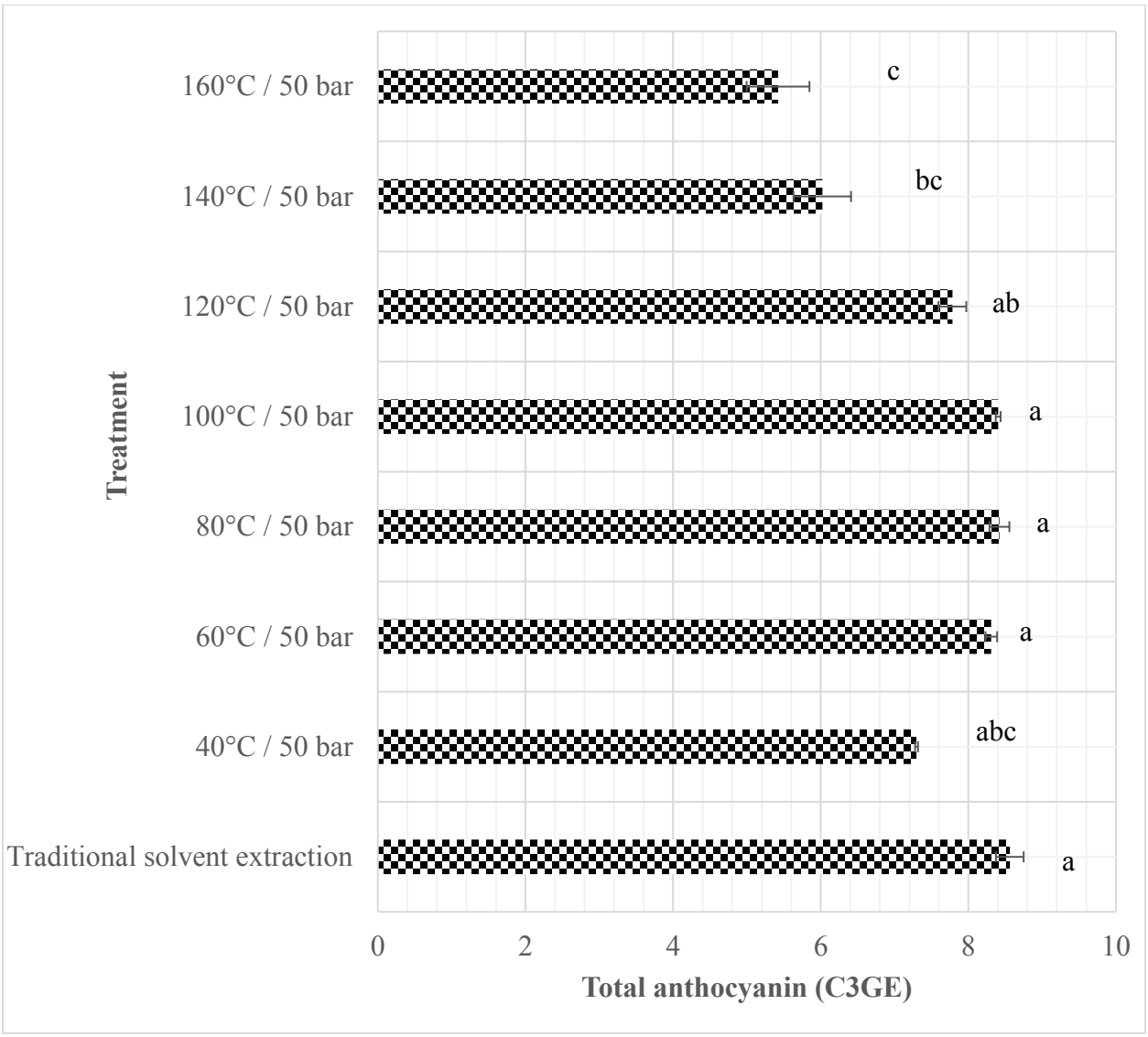


Figure 3.5. Total anthocyanin extraction using pressurized ethanol and traditional solvent extraction (98% MeOH + 2% HCl) at ambient temperature and pressure. Letters a-c correspond to difference between all values ($p<0.05$).

As shown in Figure 3.5, total anthocyanin extraction using pressurized ethanol at 50 bar and temperatures of 60-120ºC resulted in a similar extraction (3.89-4.21 mgCy3GE/g d.w.) compared to the traditional solvent extraction using acidified methanol at atmospheric pressure and ambient

temperature (4.28±0.01 mgCy3GE/g d.w.). Also, the anthocyanin extraction content obtained is higher than those reported in the literature, including ultrasound extraction (1.47±0.04 mgCy3GE/g d.w.) and microwave extraction (0.054±0.01 mgCy3GE/g d.w) (Klavins, Kviesis and Klavins, 2017). Similar anthocyanin results (4.46±0.01 mgCy3GE/g d.w.) were also reported using solvent extraction (ethanol 80%+water) and vigorous mixing on a previously pressed cranberry pomace (Ross et al., 2017). Their juice extraction process, which consisted in a juice extraction and press, enhance the availability of anthocyanins hence resulting in similar results. In this study, cranberry pomace was not pressed.

Individual anthocyanins such as cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and peonidin 3-galactoside were also quantified using HPLC-UV (Figs. 3.6a,b and 3.7). On the other hand, Fig. 3.6c and d compares the chromatograms obtained using HPLC-UV from pure standards to analyze cranberry products (Brown and Shipley, 2011) with chromatograms obtained in this study. Similar peaks were identified in both chromatograms. No significant differences in cyanidin 3-galactoside and cyanidin 3-glucoside extraction were observed among the extraction conditions, including pressurized water, pressurized ethanol 30%+water, pressurized ethanol 70%+water at 50 bar and 120ºC, pressurized ethanol at 50 bar and 80 and 120ºC, and traditional solvent extraction using acidified methanol. High cyanidin 3-arabinoside and peonidin 3-galactoside were obtained using traditional solvent extraction (6.17±0.12 mg/g d.w., 7.21±0.25 mg/g d.w., respectively), pressurized ethanol at 50 bar and 80ºC (7.82±0.08 mg/g d.w., 6.75±0.20 mg/g d.w., respectively) and 120ºC (7.35±0.05 mg/g d.w., 6.17±0.19 mg/g d.w., respectively). Also, anthocyanin extraction increasing trend was observed when increasing pressurized ethanol concentration at 120ºC and 50bar. Cyanidin 3-arabinoside

and peonidin 3-galactoside were found in high concentrations in the cranberry extracts, however peonidin 3-arabinoside was not quantified because of unavailability of the standard. White, Howard and Prior (2009) reported the anthocyanin profile in cranberry pomace where cyanidin 3-arabinoside predominated (41% of total anthocyanins) followed by peonidin 3-arabinoside (22%), peonidin 3-galactoside (17%), cyanidin 3-galactoside (11%), peonidin 3-glucoside (6%) and cyanidin 3-glucoside (4%). Ross et al. (2017) also reported the anthocyanins profile in cranberry pomace, where peonidin 3-galactoside predominated with 33.24%, followed by cyanidin 3-galactoside with 25.31%, cyanidin 3-arabinoside with 17.92%, peonidin 3-arabinoside with 14.26%, peonidin 3-glucoside with 3.53%, malvidin 3-arabinoside with 1.34%, cyanidin 3-glucoside with 0.86%, petunidin 3-arabinoside with 0.51%, delphinidin 3-galactoside with 0.38% and some unidentified peaks. In both studies, cyanidin 3-arabinoside, peonidin 3-galactoside, cyanidin 3-galactoside and peonidin 3-arabinoside remained as the four most abundant anthocyanins in cranberry pomace.

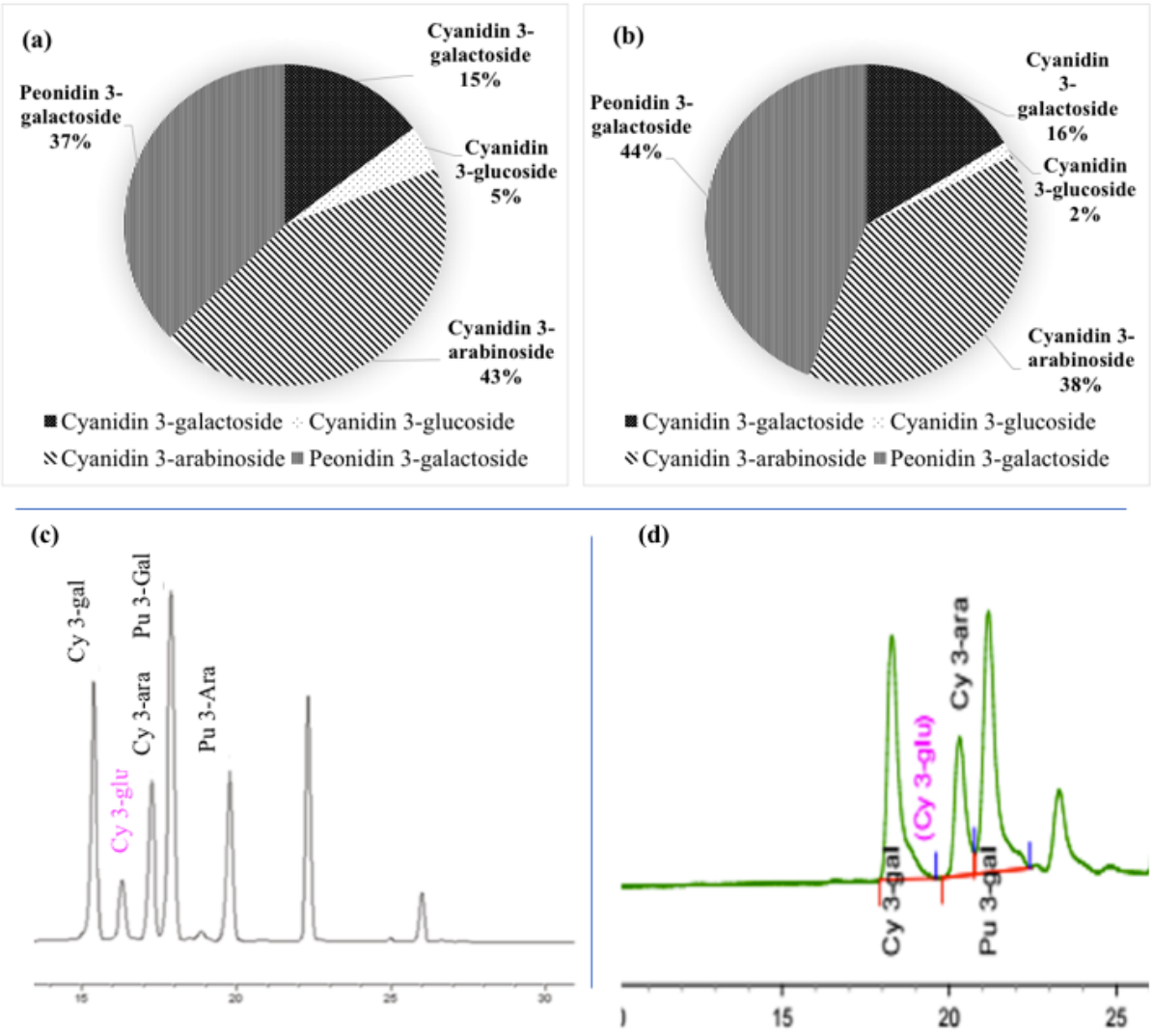


Figure 3.6. HPLC-UV: (a) data quantification of extracted anthocyanins with pressurized ethanol at 80ºC/50 bar, (b) data quantification after traditional solvent extraction using acidified methanol at room temperature and pressure, (c) chromatogram of pure standards for cranberry products (adapted from Brown and Shipley, 2011), and (d) chromatogram of a liquid extract obtained using pressurized ethanol at 120ºC and 50 bar from cranberry pomace (this study). Cy 3-gal: cyanidin 3-galactoside, Cy 3-glu: cyanidin 3-glucoside, Cy 3-ara: cyanidin 3-arabinoside, Pu 3-gal: peonidin 3-galactoside and Py 3-ara: peonidin 3-arabinoside.

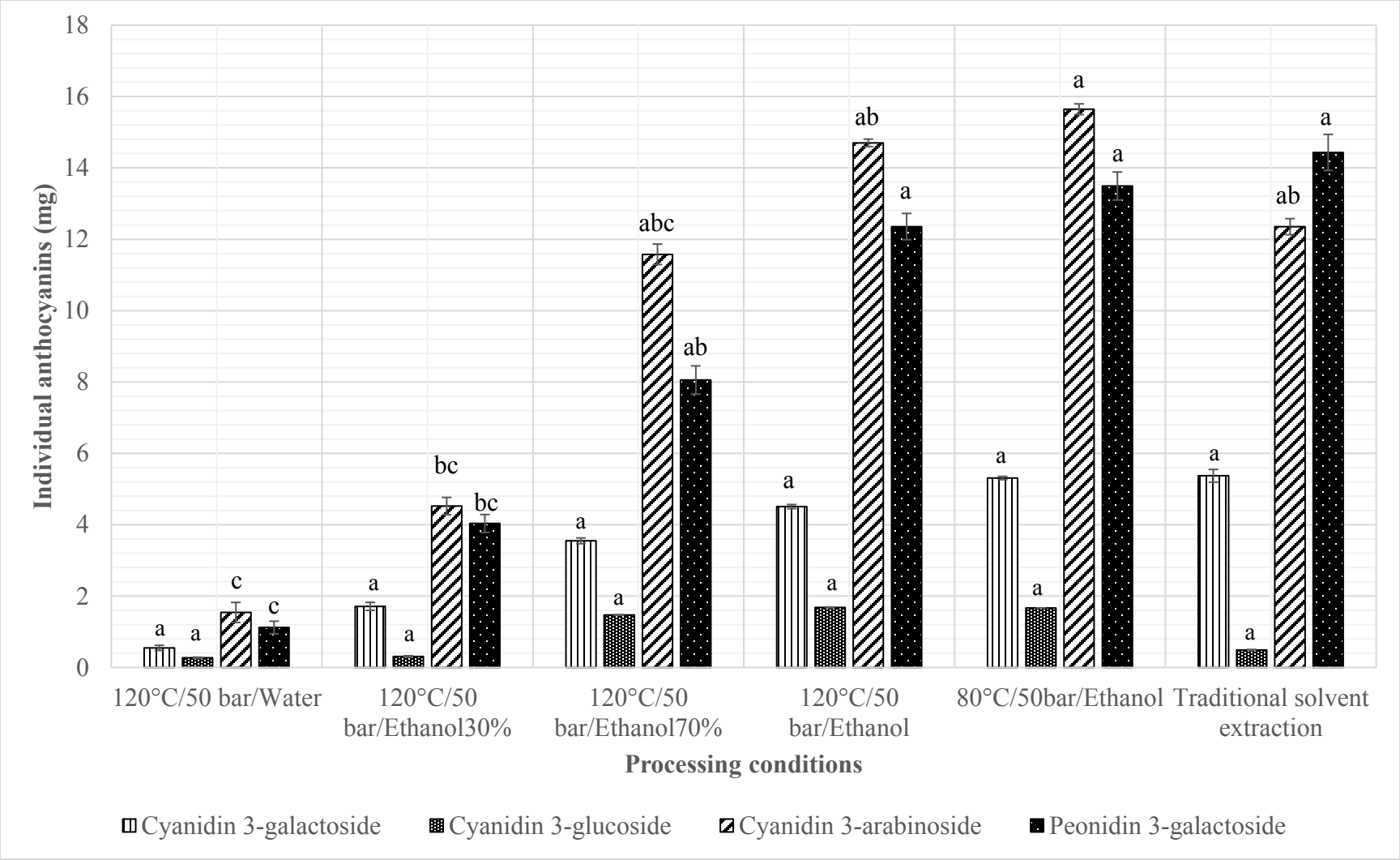


Figure 3.7. HPLC-UV anthocyanin quantification at various processing conditions investigated. Letters a-e correspond to difference between each individual anthocyanin and not between all anthocyanins ($p<0.05$).

The optimum temperature range to extract anthocyanins from cranberry pomace in this study was from 60 to 120ºC at 50 bar. There are three possible reasons: i) the difference between the critical points of ethanol (241ºC, 63 bar) and water (374ºC, 220 bar) and their boiling temperatures (ethanol: 78.73ºC, and water: 100ºC) to reach the subcritical region with ethanol faster compared to water, resulting in a better anthocyanin extraction; ii) anthocyanins have high thermal sensitivity, leading to degradation at temperatures above 120 ºC. Fischer, Carle and Kammerer (2013) reported total anthocyanin losses from 76 to 87% after heating pomegranate juice to 90ºC for 5 hours, and iii) the particle size of the sample, which was relatively small compared to the original pomace. Significantly higher total phenolic extraction yields were observed with a particle size of 1 to 0.75 mm (14.9 and 15.4 mg GAE/g d.w., respectively) compared to larger particle size of 2, 3 and 6 mm (11.8, 11.4 and 10.5 mg GAE/g d.w., respectively) from dried chokeberry (Cujuc et al., 2016). Also, freeze-drying the cranberry pomace might have influenced positively the anthocyanin extraction. Freeze dried blueberries under pressurized ethanol extraction showed a slight but not significant increase of 9% compared to the total anthocyanin extraction from fresh blueberries under the same conditions (Paes, Dotta, Barbero and Martinez, 2014). A semi-continuous system rather than batch system was used in this study, resulting in a short exposure time to pressure and temperature, which could have prevented deterioration of anthocyanins.

Anthocyanins in cranberries are higher concentration in the skin (17.02±0.67 mg Cy3GE/g d.w.) compared with the flesh (1.01±0.06 mg Cy3GE/g d.w.) (Grace, Massey, Mbeunkui, Yousef and Lila, 2012). A microscopic study suggests that anthocyanins can be found in the outer fruit cell layer and inside the vacuole within the cell. Figure 3.8a and b shows microscopic images of

freeze dried cranberry pomace, the residue after a pressurized ethanol at 120ºC and 50 bar. The outer cells of Fuji apples had higher anthocyanin concentration that decreases inward to the flesh (Fig. 3.8c and d) (Bae, Kim, Kim, and Lee, 2006). The vacuole is a membrane bound organelle that stores water and water-soluble metabolites, including sugars and organic acids (Hodson and Bryant, 2012). Within the vacuole, there are "free" anthocyanins and smaller groups of anthocyanins, which are denominated as anthocyanin vacuolar inclusions (AVIs). Mizuno, Hirano and Okamoto (2015) compared the anthocyanin content in the whole skin tissues with the AVIs of three different grape cultivars. Their results showed that higher amount of AVIs (size 5-10μm) can be found in the grape's epidermis (Cultivar: Pione, epidermis: 381 AVIs per mm^2; Cultivar: Cabernet Sauvignon, epidermis: 96 AVIs per mm^2, hypodermis: 0 AVIs per mm^2; Cultivar: Red Port, epidermis: 827 AVIs per mm^2, hypodermis: 0 AVIs per mm^2) and that approximately 50% of total anthocyanins from cultivars Cabernet Sauvignon and Red Port and 70% of cultivar Pione are acylated.

Both AVIs and vacuole grape membranes were studied and there was no significant difference between their compositions. The rupture of the membrane can facilitate the total anthocyanin extraction. At high concentrations of ethanol (>30.5%), there was desorption of the lipid molecules of the phospholipid membranes and the formation of micelle-like structures were observed (Gurtovenko and Anwar, 2009). This ethanol impact explains why the highest anthocyanin extraction content was observed at higher ethanol concentrations (Fig. 3.3).

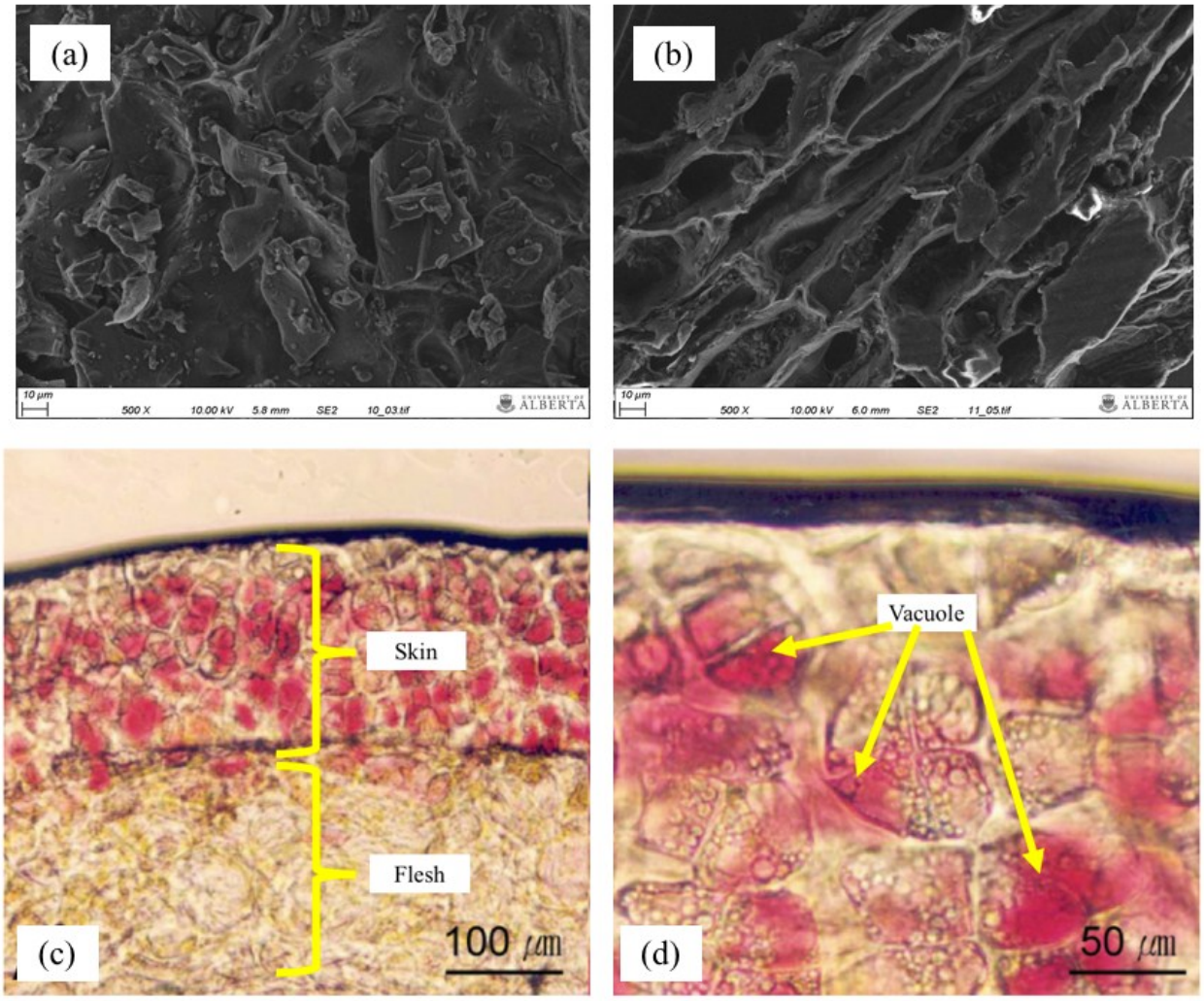


Figure 3.8. Scanning electron microscope images (10μm) for (a) freeze dried cranberry pomace and (b) sample residue after pressurized ethanol extraction at 120ºC and 50bar. Light microscopy of fresh fuji apple skin: (c) 100μm and (d) 50μm. (Adapted from Bae et al., 2006).

When comparing pressurized water with pressurized aqueous citric acid (5%), little differences in total anthocyanins extract were observed (Fig 3.2). Higher anthocyanins were obtained using pressurized aqueous citric acid, however that difference was not significant. This result could be attributed to the decrease of pH in the pressurized citric acid 5%+water from ~5 (milli-Q water) to 2.06±0.06 and the consequent co-pigmentation of anthocyanin with citric acid.

The co-pigmentation of anthocyanins, which refers to the association between pigments and organic molecules, causes stabilization and light absorption increase effect of anthocyanins. In the presence of phenolic compounds such as flavonols and hydroxycinnamic acids with anthocyanin (malvidin 3-glucoside), the formation of new pigments over time like xanthylium structures and pyranoanthocyanins resulted in a color change (Gomez-Miguez et al., 2006). The addition of catechin and caffeic acid to intensify red grape wine colour impacted with an increase of 10% and 60%, respectively, explaining that caffeic acid enables more pigment to be dissolved, resulting in a more intense color due to co-pigmented anthocyanins rather than free anthocyanins (Darias-Martin, Carrillo and Diaz, 2001). Paes, Dotta, Barbero and Martínez (2014) extracted anthocyanins from freeze-dried blueberries where no significant difference was observed using pressurized ethanol (2.57±0.04 mgCy3GE/100g) and 100% acidified water pH 2 (undefined reagent used to lower pH) (2.63±0.01 mgCy3GE/100g), but, a significant difference was obtained using pressurized 50% ethanol + acidified water (1.10±0.10 mgCy3GE/100g). These results suggest that the use of citric acid lowered the extracts pH but very little co-pigmentation was obtained, which could be related to the low citric acid concentration used. A 260% colour enhancement was obtained when adding rosmarinic acid in a molar ratio of 1:100 (malvidin 3-glucoside:rosmarinic acid) and a 150% colour enhancement corresponded to a lower ratio of 1:10 (Eiro and Heinonen, 2002). However, the use of pressurized 5% citric acid aqueous solution led to the filter breakage within the reactor after some of the experiments. Because of the low pH value the solvent had, the extracts looked red as shown in Figure 3.9.

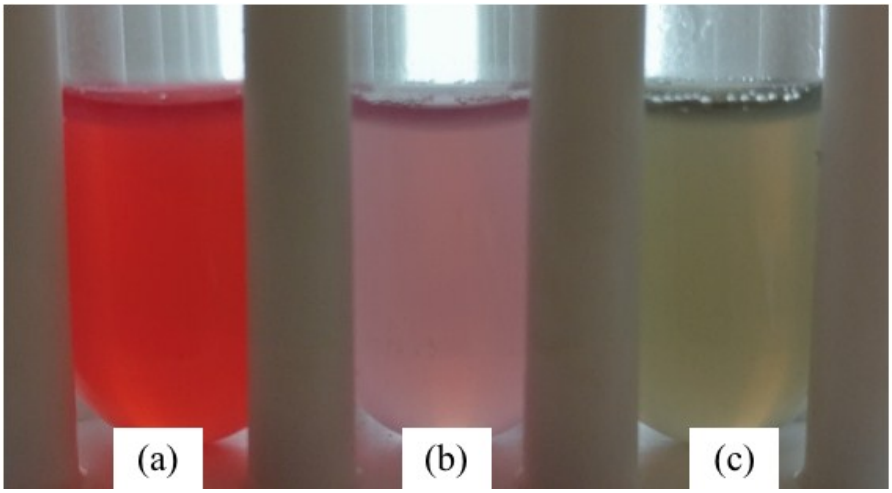
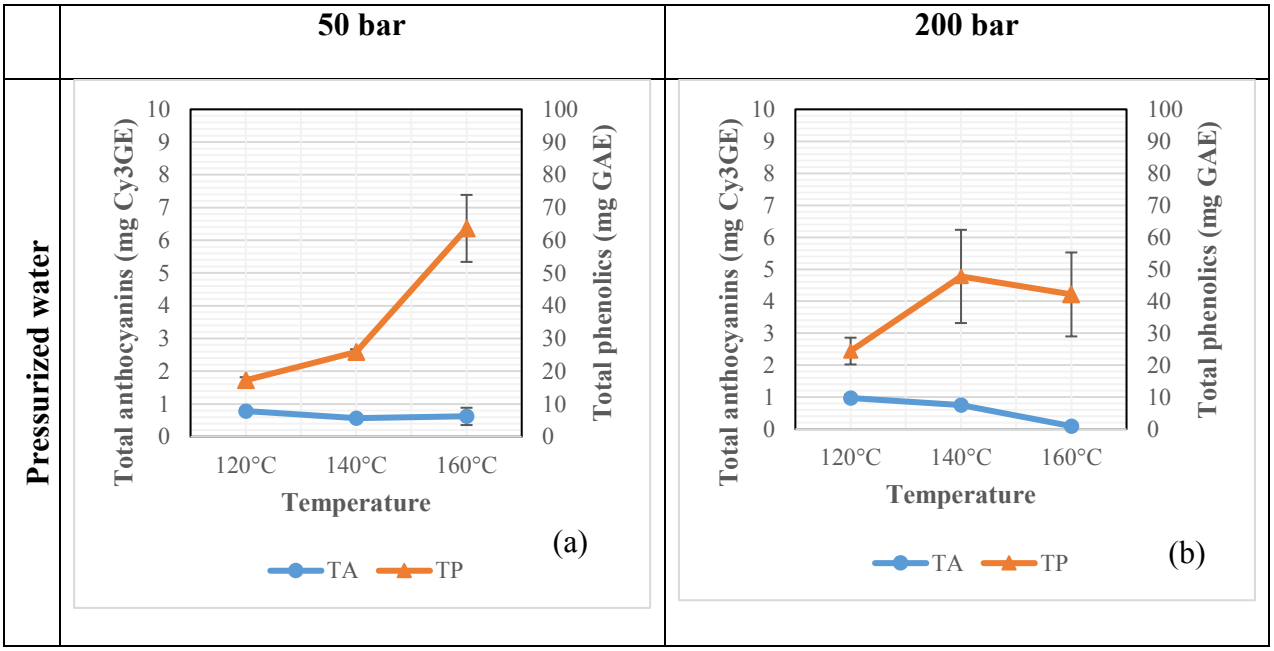


Figure 3.9. Pressurized cranberry liquid extract (0.5 mL) diluted with 1.5 mL of: (a) pH 1 (HCl acid solution), (b) pH 4.5 (sodium acetate solution adjusted with HCl), and (c) pH 7 (sodium chloride solution) buffers.

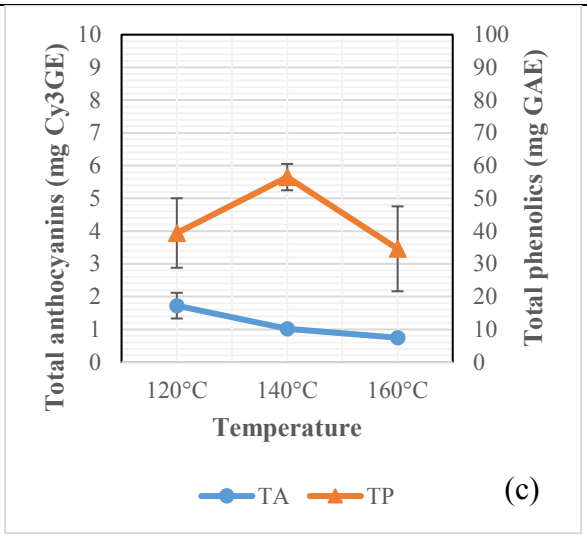
Conn, Franco and Zhang (2010) studied anthocyanin storage sites in grapes and found that there was a decrease of 25% in the number of AVIs and an increase in average AVI volume of 50% after 2 hours of a cell bombardment assay (refers to the bombarding of 1µm of gold particles to induce extraction), suggesting that the increase of volume corresponds to the fusion of smaller AVI groups. For this reason, it can be inferred that the vacuole size expands over time, as higher amounts of anthocyanins must be stored hence a darker colour is developed.

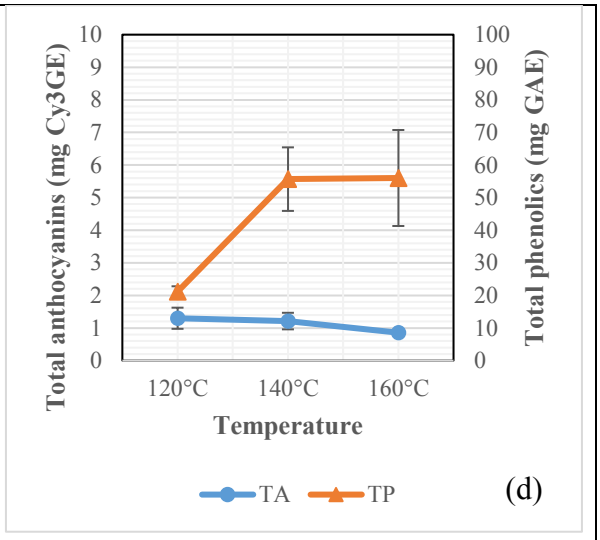
Figure 3.9 shows total phenolics and total anthocyanins extracted using pressurized fluids at temperatures and pressures. Similar phenolic contents, with no significant difference among them, were obtained at 140ºC/50 bar/ethanol (42.28±7.82 mg GAE/g d.w.), 160ºC/50 bar/30% ethanol (41.17±2.07 mg GAE/g d.w.) and 160ºC/200 bar/70% ethanol (39.18±1.64 mg GAE/g d.w.). Overall, using pressurized ethanol+water mixtures increased the extraction of total

phenolics. Singh and Saldaña (2011) reported similar results after extracting phenolic compounds from potato peel using subcritical water, where the best extraction temperature was 180ºC (5.03 mg GAE/100 g wb) and lower extractions were reported at 160ºC (3.84 mg GAE/100 g wb) and 200ºC (2.73 mg GAE/100 g wb). Moreover, a significant increase of total phenolic extraction from pomegranate using subcritical water was reported at 220ºC (48.55 mg GAE/g d.w.) than at 80ºC (4.39 mg GAE/g d.w.) (He et al., 2012). While total phenolic overall trend followed an increase when increasing temperature (Fig. 3.10, a, f-j), total anthocyanin showed an opposite trend when exposed to high temperatures (Fig. 3.10, b-e, g, i-j).

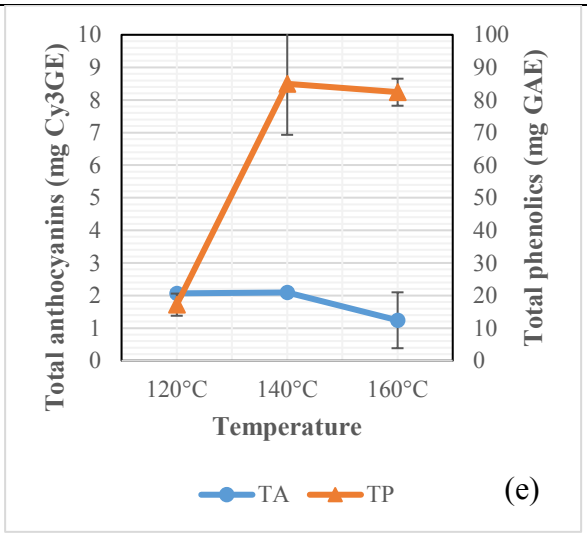


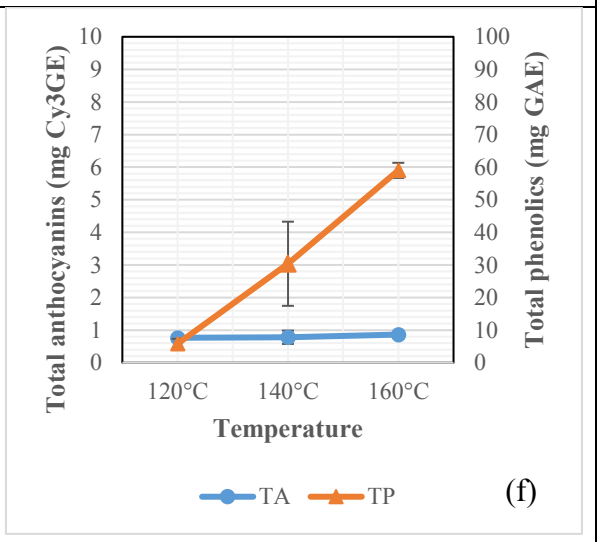
Pressurized citric acid (5%)



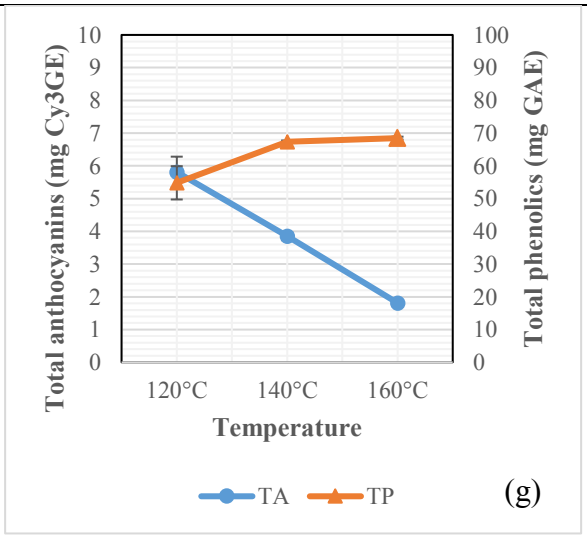


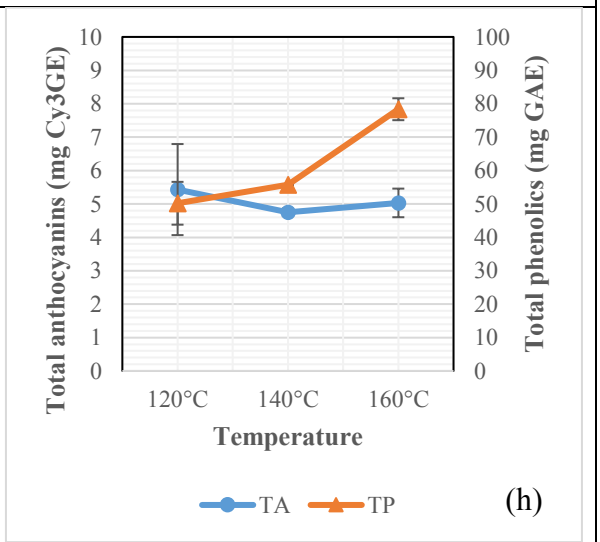
Pressurized ethanol (30%)





Pressurized ethanol (70%)





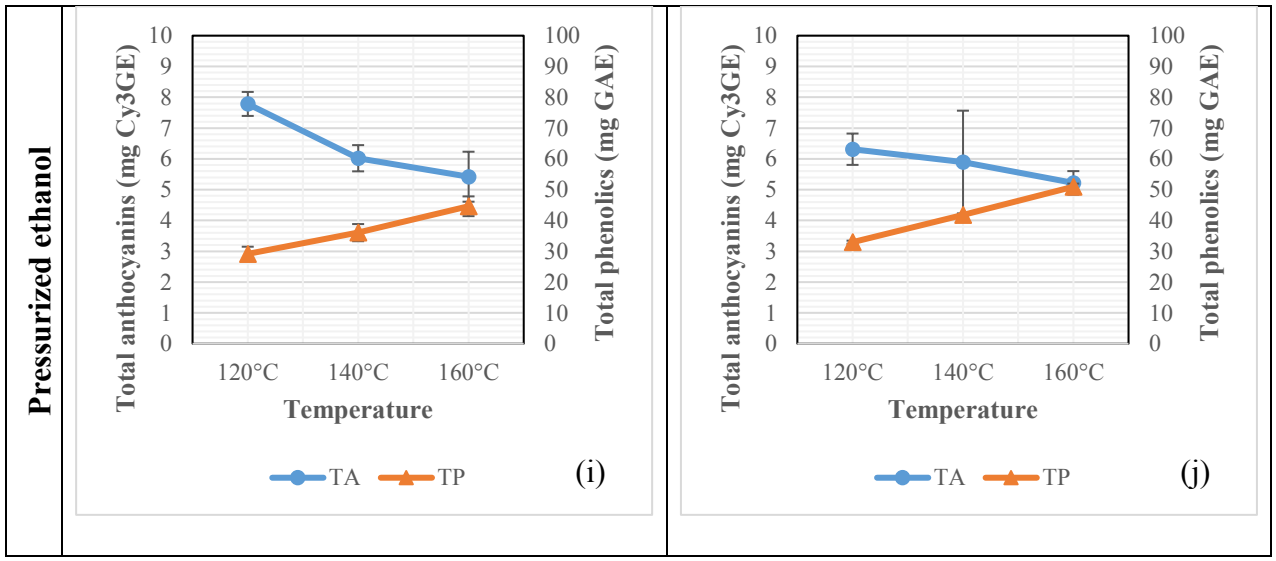


Figure 3.10. Total phenolics and total anthocyanins extracted using pressurized fluids at 120-160ºC, 50-200 bar and water, ethanol, ethanol30%+water, ethanol70%+water and citric acid5%+water. TA: total anthocyanins, and TP: total phenolics.

A significant difference in the total phenolic extraction was obtained using pressurized fluids, temperatures and the interactions of solvent, pressure and temperature (Table 3.3).

Table 3.3. Statistical analysis of total phenolic extraction.

MANOVA	Test statistic	F	Num	Denom	p
Temperature	0.45771	12.660	2	30	<0.05
Pressure	0.08171	2.669	1	30	0.113
Solvent	0.57096	9.981	4	30	<0.05
Temperature*Pressure	0.61574	24.036	2	30	<0.05
Pressure*Solvent	0.40098	5.020	4	30	<0.05
Temperature*Solvent	0.54605	4.511	8	30	<0.05

The two highest phenolic extraction were obtained using pressurized aqueous ethanol 30%+water at 140ºC (42.48±7.82 mg GAE/g d.w.) and 160ºC (41.19±2.07 mg GAE/g d.w.) (Fig.

3.10). Thermal degradation of anthocyanins and quantification of phenolic degradation products was reported by Sadilova, Stintzing and Carle (2006). Their study included the exposure of anthocyanins from strawberries, elderberries and black carrots to heating at 95ºC and pH 1 for 7 hours. After three hours, black carrot showed the highest loss (62%) followed by strawberry (59%) and elderberry (50%). After 7 hours, all samples showed a big loss (0.69, 0.25 and 0.34%, respectively) and strawberries had an increase from 0 to 13.75±1.18 µg 4-hydroxybenzoic acid /mL and from 0 to 5.81±0.54 µg phoroglucinaldehyde/mL and black carrots had an increase from 0 to 18.44±0.26 µg protocatechuic acid/mL and 0 to 3.57±0.58 µg phloglucinaldehyde/mL. Such thermal degradation exposure led to the degradation of anthocyanins into phloglucinaldehyde (cyanidin, pelargonidin), 4-hydroxybenzoic acid (pelargonidin) and protocatechuic acid (cyanidin). Thermal degradation mechanism suggested by Patras, Burnton, O'Donnell and Tiwari (2010) is shown in Fig. 3.11.

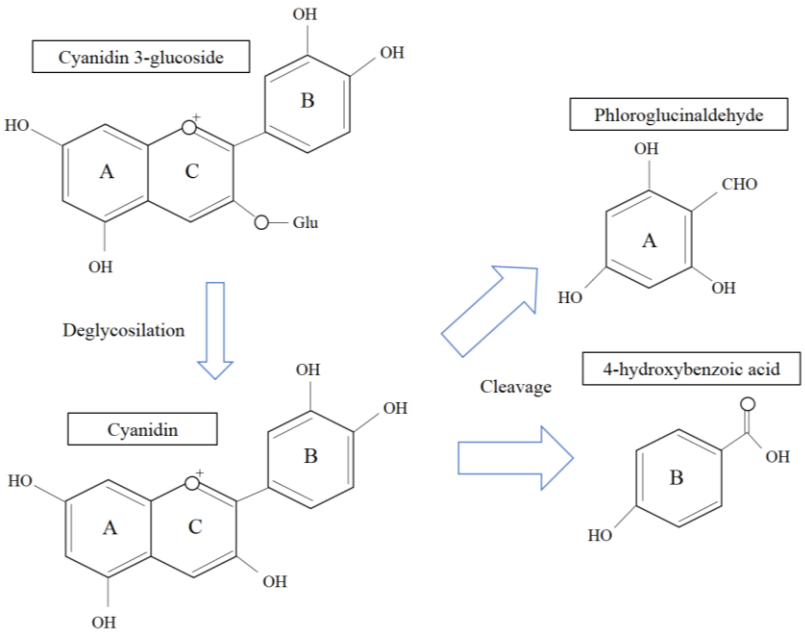


Figure 3.11. Thermal degradation mechanism of cyaniding 3-glucoside (Adapted from Patras, Burnton, O'Donnell and Tiwari, 2010).

These phenolic compounds complex molecular structures can be soluble or insoluble in a solvent. Lou, Hsu and Ho (2014) reported the extraction of both total phenolics and total flavonoids from calamondin peel using concentration ranges of water and ethanol of 50-95%. When increasing the ethanol concentration from 80% to 95%, a decrease in total phenolic content was obtained from 17.43±0.84 mg GAE/g d.w. to 12.85±0.1 mg GAE/g d.w., respectively, and an increase in total flavonoids content from 3.01±0.18 quercetin equivalents (QE) mg/g d.w. to 3.97±0.16 QE mg/g d.w. The use of pressurized ethanol is more selective to extract anthocyanins. Ethanol also resulted in a lower total phenolic extraction compared to pressurized water+ethanol mixtures (Fig. 3.12).

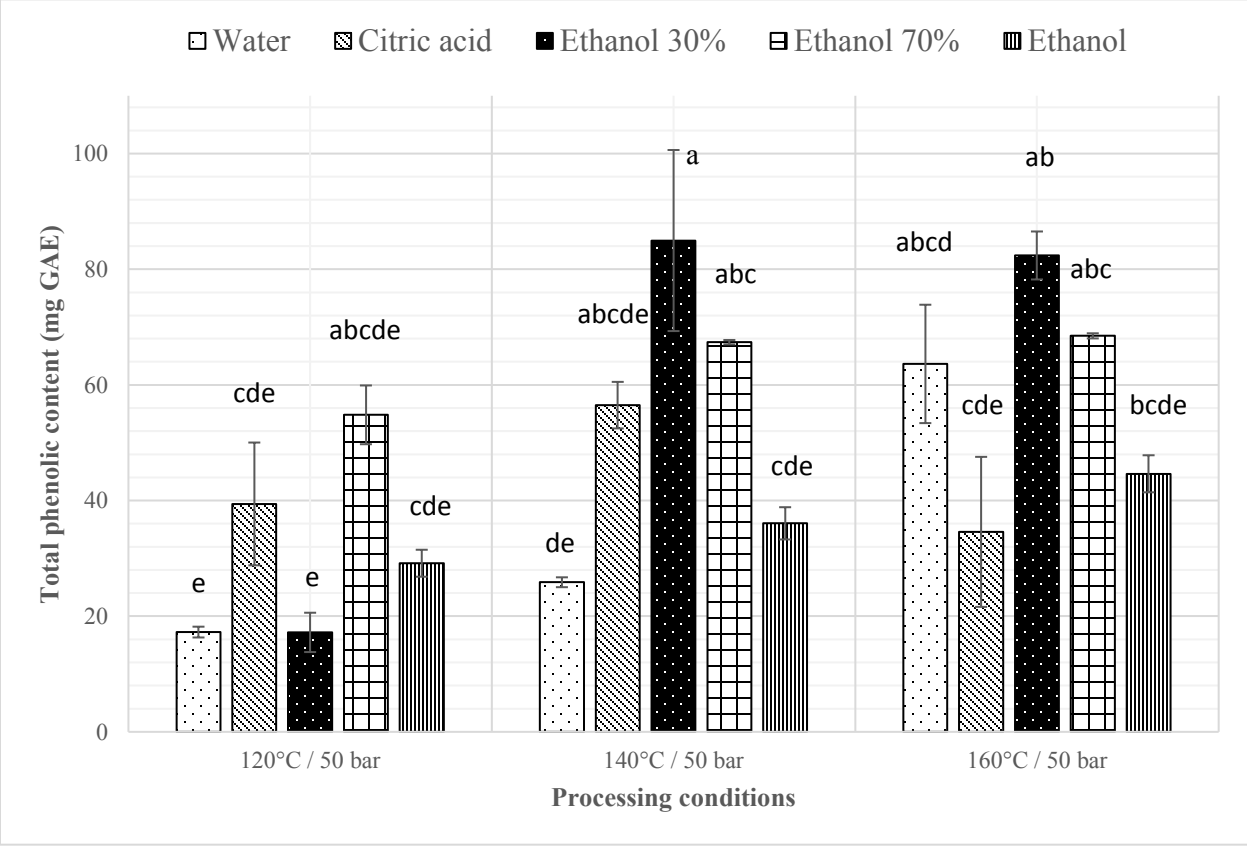


Figure 3.12. Total phenolic extraction using pressurized fluids at 50 bar and 120, 140 and 160ºC. temperatures. Letters a-e correspond to difference between all values ($p<0.05$).

Extraction rates of the ideal conditions to obtain total anthocyanins (120ºC and 50 bar) and total phenolics (160ºC and 50 bar) are shown in Fig. 3.13. Total anthocyanins extraction from cranberry pomace predominated within the first 10 minutes and total phenolics followed a similar trend at 140ºC and 50 bar. In contrast, using pressurized water at 160ºC and 50 bar, low amounts of anthocyanins were obtained and total phenolic extraction predominated in the first 10-25 minutes of extraction. This behavior could be attributed to the different extraction temperatures of 120ºC (Fig. 3.13b) and 160 ºC (Fig. 3.13b), resulting in degradation of anthocyanins for 160 ºC.

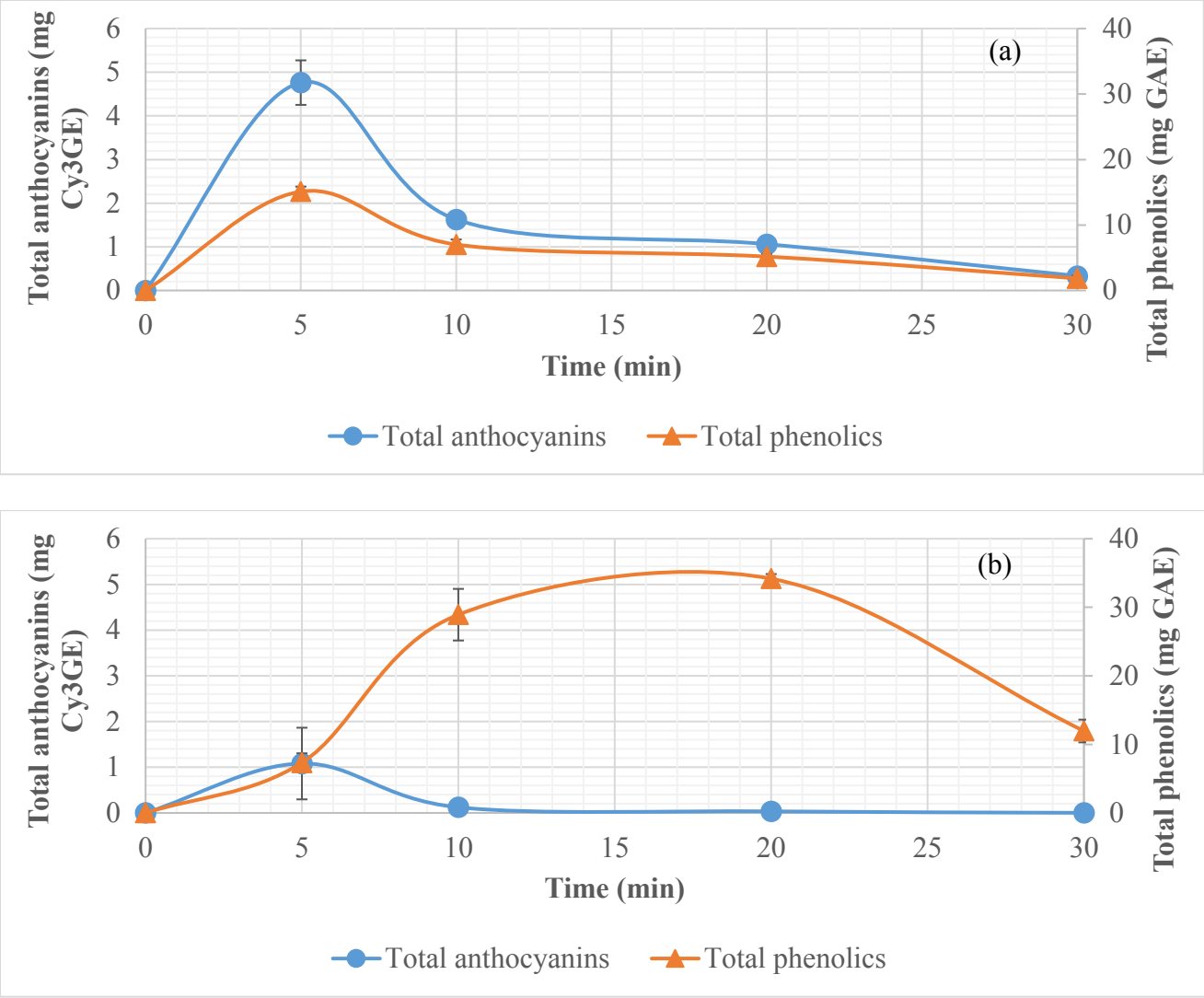


Figure 3.13. Total anthocyanin and total phenolic extraction rates using: (a) pressurized ethanol at 120ºC and 50 bar, and (b) pressurized ethanol 30%+water at 160ºC and 50 bar.

Figure 3.14 shows the regression between total antioxidant capacity (FRAP) and total phenolics and total anthocyanins using pressurized fluids. Pressurized water extraction showed a low regression coefficient (R^2=0.12) between total anthocyanin and FRAP. However, pressurized ethanol extraction showed a high regression coefficient (R^2=0.71) between total anthocyanin and FRAP. Higher pearson correlation values (Table 3.4) were observed between

antioxidant capacity and total phenolic content regardless of the pressurized fluid used. An increase in pearson correlation value was observed for total anthocyanins vs FRAP using pressurized ethanol (P=0.84) followed by ethanol 70%+water (P=0.34) and citric acid 5%+water (P=0.07). On the other hand, a high regression was obtained between FRAP vs total phenolic content for both pressurized water extraction (R^2=0.94) and pressurized ethanol extraction (R^2=0.90). Total phenolic and total anthocyanins from elderberry showed a higher antioxidant capacity correlation coefficient when analyzed using FRAP assay (P=0.84 and 0.85, respectively) compared to DPPH assay (P=0.82 and P=0.70, respectively) (Özgen, Scheerens, Reese and Miller, 2010). Brito, Areche, Sepúlveda, Kennelly and Simirgiotis (2014) reported linear correlation between total phenolic content from Chilean berry extracts analyzed by the FRAP assay (R^2=0.98) compared to the DPPH assay (R^2=0.67).

Table 3.4. Pearson correlation (P) values for total antioxidant capacity (FRAP) vs. total phenolic content (TPC) and total anthocyanin (TA).

Pressurized Fluid	FRAP vs. TPC	FRAP vs. TA
Water	0.97	-0.35
Citric acid5%+water	0.70	0.07
Ethanol30%+water	0.79	-0.02
Ethanol70%+water	0.79	0.34
Ethanol	0.95	0.84

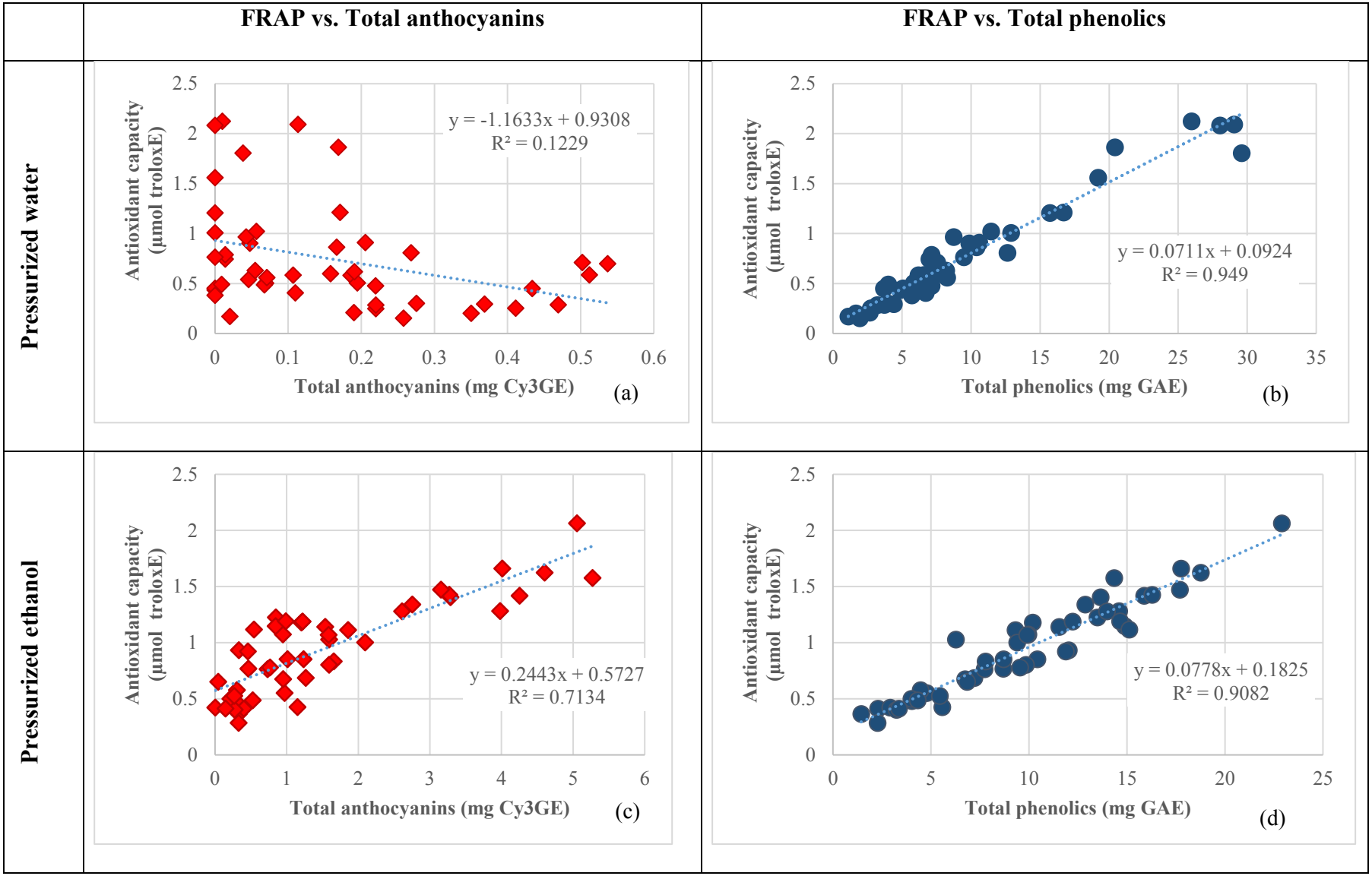


Figure 3.14. Regression between FRAP vs. total anthocyanins and total phenolics extracted using different pressurized fluids.

Figure 3.15 shows total anthocyanin and total phenolic extractions with pressurized fluids (water, ethanol, water+ethanol 30 and 70%) at 120ºC and 50bar. The superior pressurized fluid to extract anthocyanins was ethanol. However, to extract both anthocyanins and phenolics, the pressurized fluid of the solvents evaluated was ethanol 70%+water.

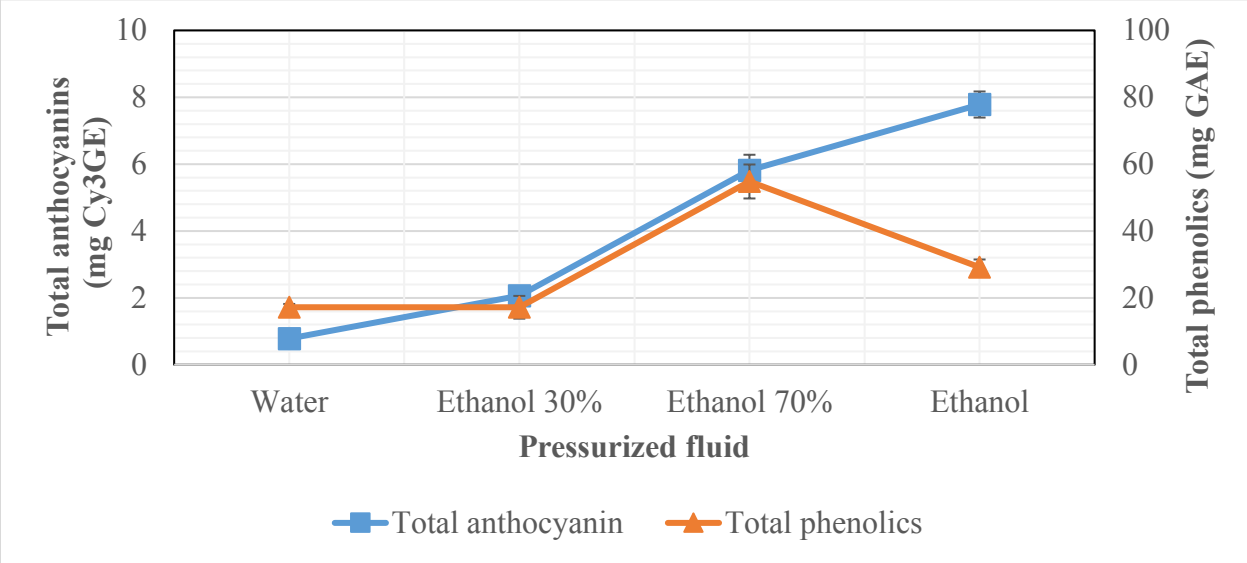


Figure 3.15. Total anthocyanin and total phenolic extraction from cranberry pomace using pressurized fluids at 120ºC and 50bar.

3.4. Conclusions and recommendations

3.4.1. Conclusions

- Extraction of anthocyanins and phenolics was possible using pressurized water, pressurized ethanol, pressurized aqueous citric acid 5%, pressurized aqueous ethanol 30% and pressurized aqueous ethanol 70%.

- By MANOVA analysis, both temperature and solvent had a significant impact ($p<0.05$) for total anthocyanin extraction but pressure was not significant at the range of processing conditions studied.
- Total anthocyanin extraction rate obtained using pressurized fluids followed a similar trend in which most of the extraction occurred within the first 10 min. When anthocyanins were not degraded, total phenolics follow a similar extraction rate over time.
- High anthocyanin extraction was obtained at 50 bar using pressurized ethanol at 60ºC (4.15±0.07 mgCy3GE/g d.w.), 80ºC (4.21±0.01 mgCy3GE/g d.w.), 100ºC (4.20±0.09 mgCy3GE/g d.w) and 120ºC (3.89±0.19 mg Cy3GE/g dry weight (d.w.)) with no significant differences between them.
- The anthocyanins, cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and peonidin 3-galactoside were identified and quantified after pressurized fluid extraction from cranberry pomace using HPLC-UV.
- The highest total phenolic extraction was obtained using pressurized water+ethanol 30% at 50 bar and 140ºC (42.48±7.82 mg GAE/g d.w.) and 160ºC (41.19±2.07 mg GAE/g d.w.) with no significant difference between them.
- Optimum conditions to extract both total anthocyanins and total phenolics were 50 bar and 120ºC using water+ethanol 70% and a flow rate of 5mL/min at the investigated conditions.
- A better regression value and Pearson correlation value were obtained between FRAP and total anthocyanin extraction using pressurized ethanol (R^2=0.71, P=0.84) compared to pressurized water (R^2=0.12, P=-0.35), meaning that pressurized water extracts are less stable as anthocyanins had no influence on total antioxidant capacity.

3.4.2. Recommendations

- The study of the impact that different drying pre-treatments such as hot air, vacuum drying infrared radiation drying and nitrogen has on the extraction of anthocyanins from cranberry pomace should be considered.
- The quantification of proanthocyanins in cranberry extracts and their stability should also be assessed for further applications.
- The use of pressurized water with the addition of other organic acids (such as gallic acid, ferulic acid and caffeic acid) in a pressurized extraction system to extract anthocyanins from cranberry pomace could be studied to increase anthocyanins stability.
- Stability of the cranberry extracts with the addition of phenolic acids could be evaluated at storage conditions, including temperature and light exposure for further applications in the food industry.
- Further cranberry extract drying as a preservation method and the stability of the dried extracts should be studied to ensure its application in the future.
- Cranberry extracts can be used to substitute artificial colourants in hard candies, coated candies and sport beverages.

Chapter 4: Bioactive food coatings for almonds based on cranberry extract, pectin and beeswax.

4.1. Introduction

There are three main preservation methods applied currently with the objective of extending a food's shelf life. The first one refers to the addition of preservatives to the food product, which functions as an antimicrobial or an antioxidant preservative. Sorbic acid, sodium benzoate and sodium nitrite are some antimicrobial agents (Stanojevic, Comic, Stefanovic and Solujic-Sukdoal, 2003). Some antioxidant agents include water-soluble vitamin C (L-ascorbic acid), fat soluble vitamin E (α-tocopherols), synthetic antioxidants (e.g. butylated hydroxytoluene) and phenolic acids (e.g. gallic acid and caffeic acid) (Brewer, 2011). The second preservation method includes the use of a thermal process to inhibit deterioration reactions in food products, hence extend their shelf life. Such thermal process includes pasteurization and sterilization. Lastly, the packaging of food products prevents food's interaction with the environment. Packaging materials are inedible; however, there are some edible coatings that protect the food products from deteriorating (Baldwin, 2007).

Food coatings are edible layers that attach to the food product. It has been proven that edible and non-edible coatings like wood resin wax can extend shelf life by preventing the respiration rate of pomegranates from 35.5±0.8 mg CO_2/kgh (control) to 25.2±0.9 mg CO_2/kgh at 4.5ºC after 120 days (Meighani, Ghasemnezhad & Bakhshi, 2015). Also, the application of a methyl cellulose based coating forms a barrier on avocados between the fruit and the environment, hence a reduction in the fruit respiration rate is observed, which increases avocado shelf life from 6 to 10 days (Maftoonazad and Ramaswamy, 2005). A reduction in deterioration reactions due to

the application of food coatings have been reported in strawberries using a chitosan-based coating (Wang and Gao, 2013) and Kashar cheese using a whey protein based coating (Kavas N, Kavas G, and Saygili, 2016).

The reduction of deteriorating reactions after using edible food coatings lead to shelf life increase. The three main pathways are: i) respiration decrease, ii) transpiration decrease, and iii) oxygen and moisture barrier. The application of edible coatings to extend nut's shelf life has been studied in roasted peanuts (Wambura, Yang and Mwakatage, 2010), cashew nuts (Pinto et al., 2015) and roasted almonds (Gayol, Soliani, Quiroga, Nepote and Grosso, 2009). Almond oil fatty acid profile is mainly composed of monounsaturated fatty acids (C18:1 (60.93±0.03%), C16:1 (0.66±0.00%)) followed by polyunsaturated fatty acids (C18:2 (29.21±0.00%) and C18:3 (0.10±0.00%)) and saturated fatty acids (C14:0 (0.06±0.00%), C16:0 (7.36±0.02%), C18:0 (1.56±0.01%) and C20:0 (0.06±0.00%)) (Venkatachlam and Sathe, 2006). Miraliakbari and Shahidi (2008) reported the stability of solvent extracted almond oil at 60ºC after 12 days where the peroxide value of the oil increased from 0.040 to 0.335 meq O_2/kg oil.

The addition of bioactive compounds such as peanut skin extract (Gayol, Soliani, Quiroga, Nepote and Grosso, 2009) and ginger essential oil (Kavas N, Kavas G, and Saygili, 2016) has also been reported in edible food coatings. Park and Zhao (2006) reported the influence of sorbitol or glycerol on mechanical and water barrier properties of edible films based on low methoxyl pectin with cranberry pomace extract (0.50 and 075% w/w). The addition of sorbitol to the edible film showed a tensile strength of 8.1 MPa and elongation of 13.7%, and water vapor permeability of 68.5 g mm/m^2 day kPa and the use of glycerol showed a tensile strength of 6.9

MPa, elongation of 12.9% and water vapor permeability of 73.2 g mm/m^2 day kPa, with no significance difference among films. On the other hand, Lozano-Navarro et al. (2017) reported the antimicrobial impact when adding 0.5% (final weight) of cranberry and blueberry extract to chitosan and starch based films. Inoculated chitosan+starch films showed total aerobic mesophilic bacteria of 2.5±2.1 colony forming units (CFU) and fungi of 6.5±5.0 CFU. Those same films with cranberry extract had <1±0 CFU for aerobic mesophilic bacteria and fungi and films with blueberry extract showed lower results with 0.5±0.7 CFU for aerobic mesophilic bacteria and 1.5±0.7 CFU for fungi. To the best of our knowledge, there is no information about the addition of cranberry pomace extract in a pectin based and pectin + beeswax based food coating to prevent lipid peroxidation in almonds. The objective of this study was to use a bioactive edible coating, based on pectin, beeswax and cranberry extract, in almonds to extend shelf life by preventing fat deterioration reactions.

4.2. Materials and methods

4.2.1. Materials

Almonds (Whole almonds, Kirkland, USA) were obtained from a grocery store (Costco, Edmonton, AB, Canada) and stored packaged with low density polyethylene at ambient temperature. Coating ingredients include low methoxyl pectin (CP Kelpo, Atlanta, GA, USA), bleached beeswax (Sigma-Aldrich, Oakville, ON, Canada), Tween ® 80 (Sigma-Aldrich, Oakville, ON, Canada), glycerol ≥ 99.5% (Sigma-Aldrich, Oakville, ON, Canada) and Milli-Q water. Other reagents used were acetone HPLC grade 99.8% (Fisher Scientific, Hampton, New Hampshire, USA), ammonium thiocyante (Sigma-Aldrich, Oakville, ON, Canada), ferrous ammonium sulfate (Sigma-Aldrich, Oakville, ON, Canada), methanolic HCl (Supelco, Sigma-

Aldrich, Oakville, ON, Canada), anhydrous sodium sulphate (Sigma-Aldrich, Oakville, ON, Canada), and hexane 97.0% HPLC (Fisher Scientific, Hampton, NH, USA).

4.2.2. Coating preparation and application

Coating formulations are described in Table 4.1. Pectin was used as the main component because it was previously extracted in our lab using sCW (Valdivieso-Ramirez, 2016). Firstly, water was heated to 70ºC and the low methoxyl pectin was added slowly until full homogenization. High methoxyl pectin was not considered because its not flexible (Edwards, 2007). After pectin was solubilized completely in water, glycerol and Tween 80 were added. When beeswax was part of the formulation, the wax was added after the plasticizer and the emulsifier, assuring that the temperature remained at 70ºC. Ethanolic cranberry extract (concentration of 210.6 mg Cy3GE/L) was added after full homogenization of the rest of the ingredients and the temperature was increased to 80ºC to evaporate the ethanol of the extract. The extract was added at 1:1 (w/w) and 1:3 (w/w) pectin:extract weight ratio. Then, coatings were homogenized at power level 3 for 2 minutes with a homogenizer (Heidolph DIAX 900, Sigma-Aldrich, Oakville, ON, Canada).

Table 4.1. Formulation of pectin and pectin + beeswax based bioactive coatings by weight.

Coating ingredient	Pectin	Pectin + E(1:1) (w/w)	Pectin + E(1:3) (w/w)	Pectin + BW	Pectin + BW + E(1:1) (w/w)	Pectin + BW + E(1:3) (w/w)
Water (g)	96.5	95.9	94.7	93.0	92.4	91.2
Pectin (g)	2	2	2	2	2	2
Glycerol (g)	1.5	1.5	1.5	1.5	1.5	1.5
Beeswax (g)	-	-	-	2	2	2
Tween 80 (g)	-	-	-	1.5	1.5	1.5
Cranberry extract (g)	-	2	6	-	2	6

BW: beeswax, and E: extract.

After the coating solution was cooled down, they were sprayed onto the almonds from various angles to cover them completely. A second sprayer was used with a solution of 3% w/w calcium chloride in water for the pectin to create the networking and convert into a gel (Edwards, 2007). Coated samples were placed inside an oven at 65ºC for 6 minutes, then removed to be turned around to be coated on the missing side and dried as described previously. A total of 525 almonds were coated in 105 groups, containing 5 coated almonds. All coatings were developed and applied in triplicate. The thickness of the coating was not measured in this study, but this measurement could be performed by cutting the almond in half, and visualizing it using a microscope.

Uncoated and coated samples were stored at 40ºC and 50%RH following Larraui et al. (2016) methodology with minor modifications. A control group of almonds obtained the day of the coating applications were also stored at the same temperature and relative humidity. Coated almonds and uncoated almonds were collected to determine their fatty acid composition and incipient rancidity after 7, 14, 30, 60 and 90 days. Other temperatures and RH were not evaluated in this study but temperatures up to 50ºC and RH up to 80% are suggested for future studies.

Mechanical press of the almonds was performed to extract the almond oil, which was stored at -18ºC for further analysis. Almond oil was thawed and filtered using syringe filters of 0.45µm PTFE (Whatman ® puradisc) for further fatty acid composition analysis and incipient rancidity.

4.2.3. Proximate compositional analysis

Proximate compositional analysis of almonds for moisture, ash, protein, fat and carbohydrate was performed using methods described in Chapter 3, Section 3.2.3.

4.2.4. Characterization of coated and uncoated almonds

4.2.4.1. Fatty acid content

The fatty acid content was analyzed using gas chromatography (GC, Bruker Scion 456-GC, Massachusetts, USA). The sample injection volume was 0.2µL in a SP 2560 Fused Silica Capillary Column (100m x 0.25mm x 0.2µm film thickness). Helium was used as a carrier gas and a FID detector was used with a constant flow rate of 1 mL/min. Filtered almond oil was weighed (~2mg) in a 13mm x 100mm test-tube with a Teflon-lined cap and 2mL of methanolic HCl was added. The test tube was closed tightly to prevent evaporation and heated in a water bath at 60ºC with frequent mixing every 20 minutes for a total time of 120 min. The test tubes were removed from the water bath and cooled at room temperature for 20 min. Then, 2 mL of Milli-Q water and 3 mL of hexane were added. The test tube was shaken vigorously for 1 minute using a vortex and centrifuged for 3 minutes at 380.12*xg*. The majority of the upper hexane layer was transferred to a second test tube, where ~50mg of anhydrous sodium sulfate were added to prevent water contact with the GC column. The second test tube was further centrifuged for 2

minutes at 380.12 *xg* and approximately 1 mL was transferred to a GC vial that was stored at 4ºC until further analysis.

4.2.4.2. Incipient rancidity

Lipid rancidity was determined spectrophotometrically using the ferric thiocyanate method described by Lips, Chapman and McFarlane (1943) with minor modifications. Briefly, 250 mL of acetone (HPLC $\geq$ 99.8%) were placed into a beaker, closed and further weighed. Such weight represented 96% of the final weight and the other 4% corresponded to Milli-Q water. Ammonium thiocyanate, weighed at a concentration of 0.4% of the total solution weight (100%), was diluted in the 4% of Milli-Q water for 10 minutes. The total of ammonium thiocyanate solubilized in water was added to the acetone solution and mixed for 10 minutes. Ferrous ammonium sulphate was added to the solution at a concentration of 0.1% w/w, mixed and further stored in the dark for 2 hours with frequent shaking every 30 min. Filtered almond oil (~100µL) was weighed in a test-tube and 9 mL of the prepared solution were added. The test tube was placed in a hot water bath at 70 – 80ºC until the first bubble was formed and then placed in a water bath at 50ºC for 10 min. The intensity of the colour was measured with a spectrophotometer (Jenway 6230D, Stone, Staffordshire, UK) at an absorbance of 485nm. Total peroxides (TP, mEq/kg, milliequivalent of peroxide per kilogram of fat) were calculated using the following equation:

$$TP = \frac{(A*B)}{(C*55.84)} \quad (4.1)$$

where:

A = micrograms of Fe^{+++} in fat – micrograms of Fe^{+++} in the blank reagent,

B = volume of the extract (1 mL),

C = weight of the sample (g),

55.84 = equivalent weight of iron (MW).

4.2.4.1. Statistical analysis

Data were analyzed using Minitab 17 (State Collage, PA, USA) software previously described in Chapter 3, Section 3.2.5.

4.3. Results and discussion

Proximate compositional analysis of the almonds used is shown in Table 4.2. A total amount of protein of 26.81±0.06% and fat of 44.41±0.45% were obtained. Protein content in this study is higher than those reported by Venkatachalam and Sathe (2006) and USDA (2016). Fat content obtained in this study is similar to the one reported by Venkatachalam and Sathe (2006). However, there is a difference with the fat content reported by the USDA (2016). There was also reported fat content variation within almonds Lopez-Ortiz et al. (2008) reported the difference in fat composition for 4 cultivars grown in different locations and years. The cultivar "garrigues" grown in Cordoba showed a higher fat content in 2005 (46.6±0.5%) compared to those grown in 2004 (42.5±1.0%). In contrast, that same cultivar grown in Alicante showed a high fat content in 2004 (52.0±0.5%) and a low fat content in 2005 (44.7±0.5%). The fatty acid composition of almonds, which mainly corresponds to oleic acid (C18:1) and linoleic acid (C18:2), has a significant variation depending on the location, year and cultivar (Sathe et al., 2008). Yada, Lapsley and Huang (2011) also reported a large fat variation in almonds, depending on the

location they were grown, in which almonds grown in Spain had 40–67% fat, almonds grown in the USA (California) had 35–66% fat, and almonds grown in Greece had 56–61% fat.

Table 4.2. Proximate compositional analysis of almonds.

Macronutrient	This study (%)	Almonds (%) (Venkatachalam and Sathe, 2006)	Raw almonds (%) (USDA, 2016)
Moisture content	4.80±0.09	9.51±0.08	4.41
Ash	3.16±0.04	2.48±0.05	-
Protein	26.81±0.06	19.48±0.51	21.15
Fat	44.41±0.45	43.36±0.62	49.93
Carbohydrate (by difference)	21.81	25.17	21.55

The weight change of uncoated and coated almonds during storage at 40ºC and 50% RH for up to 90 days is shown in Fig. 4.1. All samples showed a similar behavior in which samples dried in the first 14 days and then moisture content started to increase slightly after 30 days, however no result obtained after treatment had any significant difference ($p<0.05$). The behavior of coated samples showing a higher weight change compared to the control could be related to the drying of the coating material which had water up to the first ~14 days followed by moisture absorption of the environment up to 90 days. The uncoated almonds also showed the same trend with a slight moisture loss followed by a slight moisture gain (<0.01%). Galus, Turska and Lenart (2012) studied the impact of both pectin and glycerol concentrations in water sorption and their results showed a lower water vapour sorption in the film with 3.5:1.75 pectin:glycerol ratio with an increase from 0.24 to 1.33 g water/g d.m compared to other films with a ratio of 2.5:1.75 pectin:glycerol from 0.23 to 2.18 g water/g d.m and a 2.5:0.75 ratio from 0.19 to 1.59 g water/g d.m. The significance of this last study, however, remains unclear as no statistical analysis was performed. Saini and Sharma (2016) reported the rehydration ratio increase of uncoated dried

pineapple (11.53%) and pectin based coated dried pineapple (8.27%) packed in a laminated 30 µm thick low density polyethylene (LDPE) pouch stored at 75% relative humidity for 18 months. They explained that their result could be related to the fact that the uncoated dried pineapple may have absorbed moisture from the atmosphere.

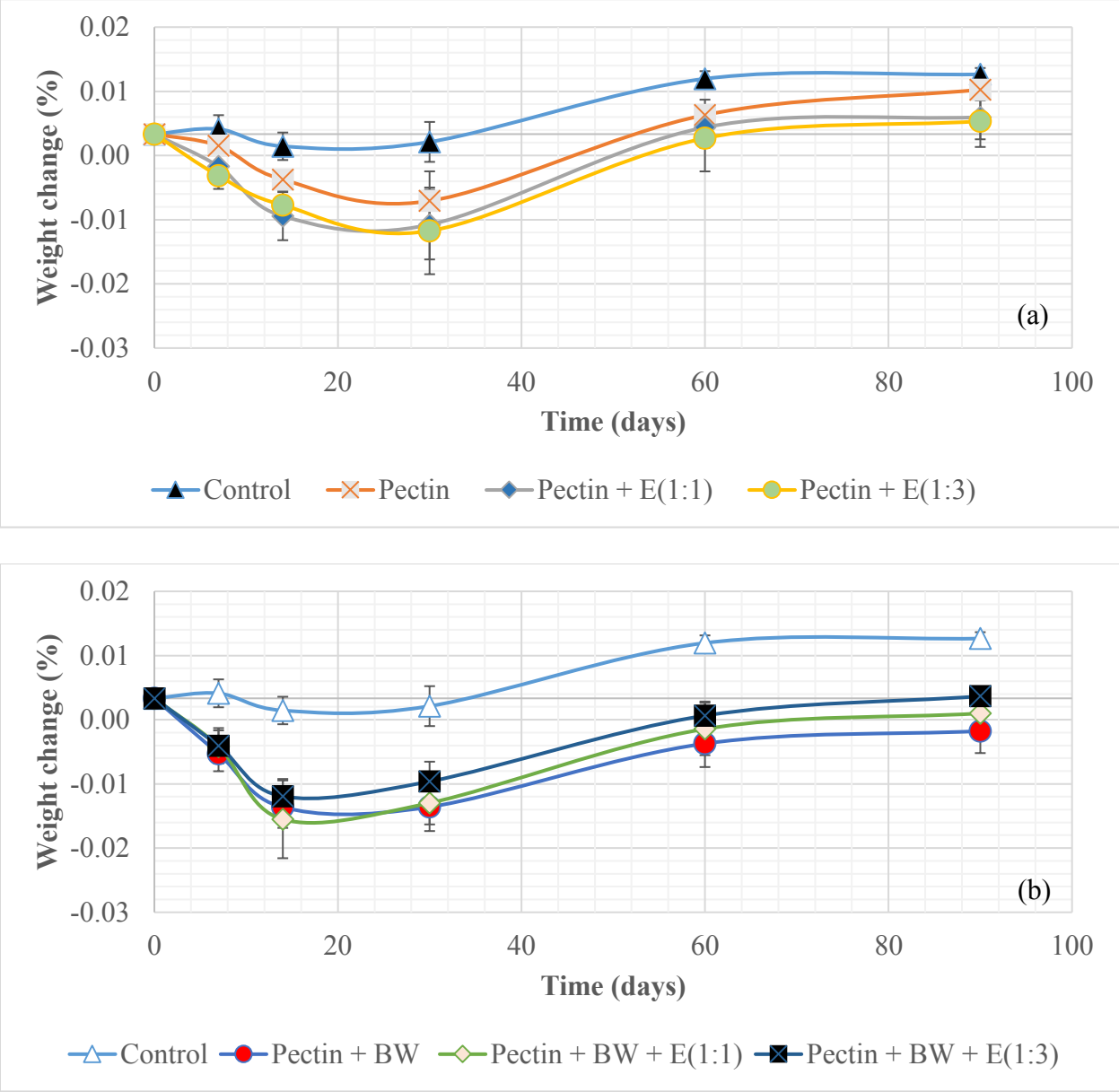
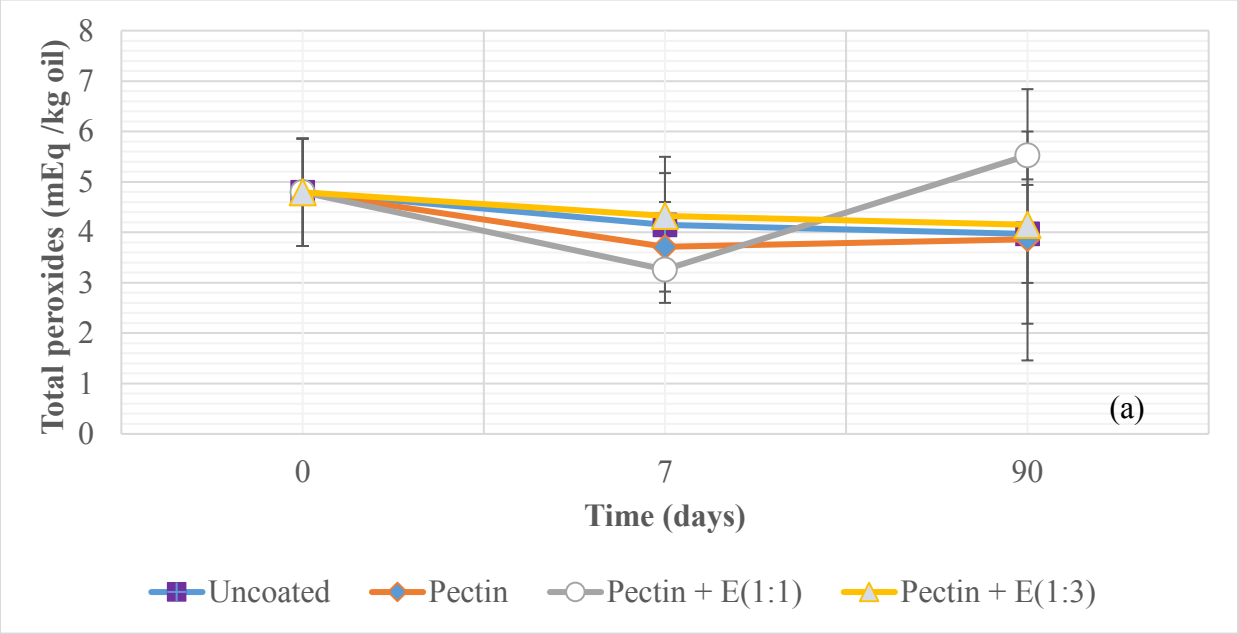


Figure 4.1. Uncoated and coated almond ((a) Pectin based; and (b) Pectin+beeswax based) weight change during storage time at 40ºC and 50% RH for 90 days.

Fig. 4.2 shows the total peroxide values obtained for coated and uncoated samples. No significant peroxide value (PV) change was observed over time up to 90 days (Fig. 4.2). Larrauri et al. (2016) reported the changes in peroxide values of roasted almonds and coated roasted almonds with carboxymethyl cellulose (CMC), CMC + butylhydroxytoluene (BHT) and CMC + peanut skin extract (0.2%) stored at 40ºC but relative humidity was not reported. Almond initial PV was 0.58 meq O_2/kg oil. After 128 days, the peroxide value of the uncoated almonds increased to 3.90 meq O_2/kg oil. The CMC + BHT and the CMC + peanut skin extract coatings increased to 2.69 meq O_2/kg oil while the CMC coated almond increased to 2.57 meq O_2/kg oil. Mehyar, Al-Ismail, Han and Chee (2012) reported the mechanical properties of an edible food coating based on whey protein + pea starch + carnauba wax and its impact on walnut shelf life. Their accelerated shelf life analysis was performed at 50ºC (relative humidity not reported) with an initial peroxide value of ~2.5 meq O_2/kg oil that increased drastically after two and five days with values of ~17 and ~20 meq O_2/kg oil, respectively. Another walnut shelf life study reported peroxide values of ~20 meq O_2/kg oil after 10-12 months at 20ºC. Walnuts were packaged with low-density polyethylene (LDPE), polyethylene terephthalate (PET) and polypropylene/ethylene-vinyl alcohol/polypropylene (PE/EVOH/PP). But, at 4ºC, peroxide values were below ~16 meq O_2/kg oil after 12 months (Mexis et al., 2009). The high peroxide values could be attributed to the amount of unsaturated fatty acid walnuts have compared to almonds. Walnuts fatty acid composition is 49.93-54.41% of C18:2, 22.63-27.27% of C18:1 and 14.32-17.82% of C18:3 (Dogan and Akgul, 2005) while almond fatty acid composition is 29.21±0.00% of C18:2, 60.93±0.03% of C18:1, 7.36±0.02% of C16:0 and 1.56±0.01% of C18:0 (Venkatachlam and Sathe, 2006).



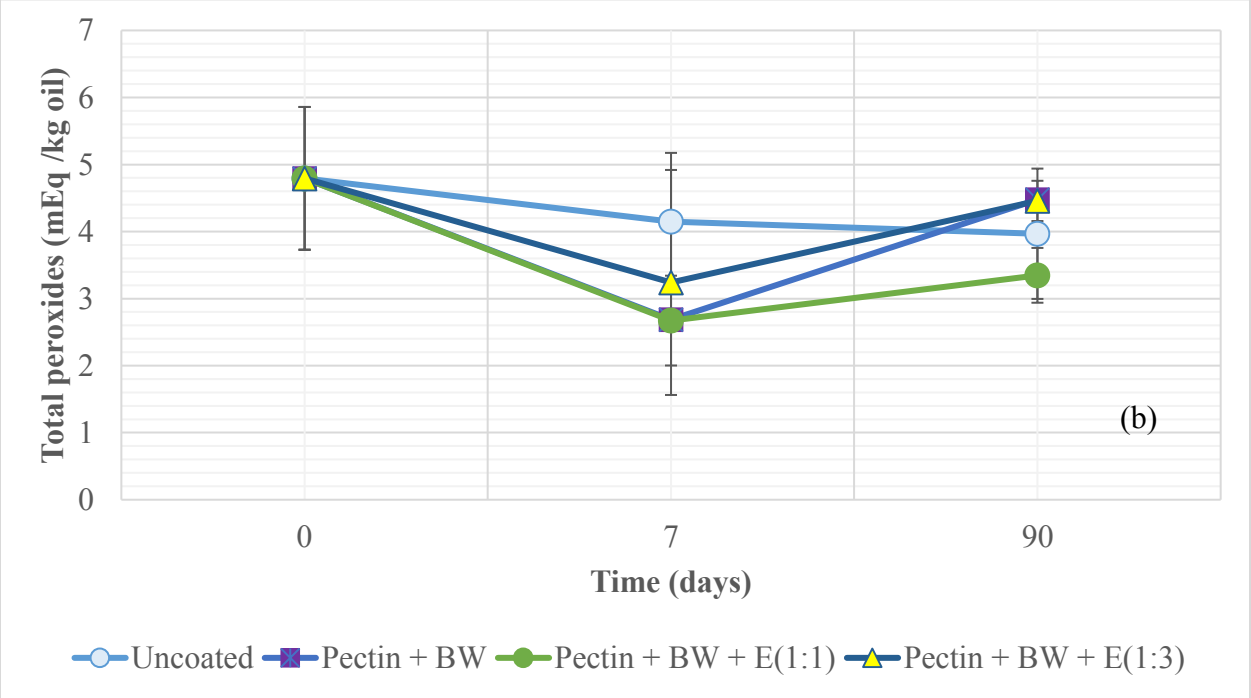


Figure 4.2. Total peroxide values of different coatings: (a) Pectin based; and (b) Pectin+beeswax based.

No significant differences in total peroxide value were observed with any of the coating treatments up to 90 days (Fig. 4.2). Lin et al. (2012) studied the impact of blanching and critical storage conditions (4.4–37.8ºC and 45–95% RH) in almond deterioration. For their experiment that lasted 500 days, they reported a positive Pearson correlation value between free fatty acids and moisture content at 21.1ºC and 95% RH in whole California blanched packed samples (P=0.80) and nonpareil whole raw packed samples (P=0.94). On the other hand, the shelf life of steam peeled almonds (at 98ºC for 2 min) packed under vacuum with transparent films and metallized films was studied by Sensi et al. (1991). A decrease in percentage of oleic acid (C18:1) at 20ºC after 546 days was observed for all packaging materials, including transparent film from 81.72 to 69.86%, metallized film from 79.74 to 66.59% and metallized film under nitrogen from 79.58 to 61.27% and an increase in linoleic acid (C18:2) was observed in transparent film under vacuum from 12.44 to 17.34%, metallized film under vacuum from 12.6 to 16.63% and metallized film under nitrogen from 13.50 to 17.57%.

No significant difference was observed between uncoated and coated almonds in both oleic (C18:1) (Fig. 4.4) and linoleic acid (C18:2) after 90 days (Fig. 4.5, Table 4.3). Probably the coated and uncoated almonds had not started a lipid oxidation cycle, hence no peroxide value and change on fatty acid profile was observed. A similar trend was observed in most of the treatments with an increase of C18:1 and a decrease of C18:2 in the uncoated, pectin, pectin + E(1:3), pectin+beeswax and pectin+beeswax+E(1:1). The chemical structures of oleic acid (C18:1) and linoleic acid (C18:2) are shown in Fig. 4.3. In contrast, coated almonds with pectin+E(1:1) and pectin+beeswax+E(1:3) followed an opposite trend with a slightly increase in C18:2 and a slightly decrease in C18:1. Zacheo, Cappello, Gallo, Santino and Cappello (2000)

reported the change of almond fatty acids stored at 20ºC for 2 years and a significant increase in oleic acid (C18:1) from 71.1 to 76.5%, 69.8 to 74.3%, 61.3 to 70.3% and 74.5 to 77.5% and a decrease in linoleic acid (C18:2) from 20.1 to 15.1%, 18.3 to 14%, 26.2 to 17.0% and 15.1 to 13.8% for cultivars Fra Giulio G, Padula di R, Desmayo L and Sannicandro, respectively.

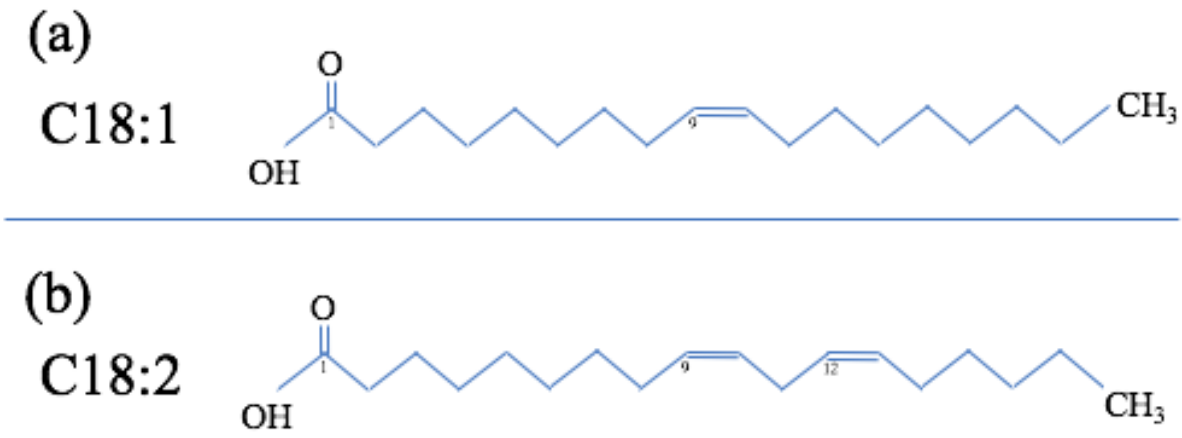


Figure 4.3. Chemical structures for: (a) oleic acid and (b) linoleic acid.

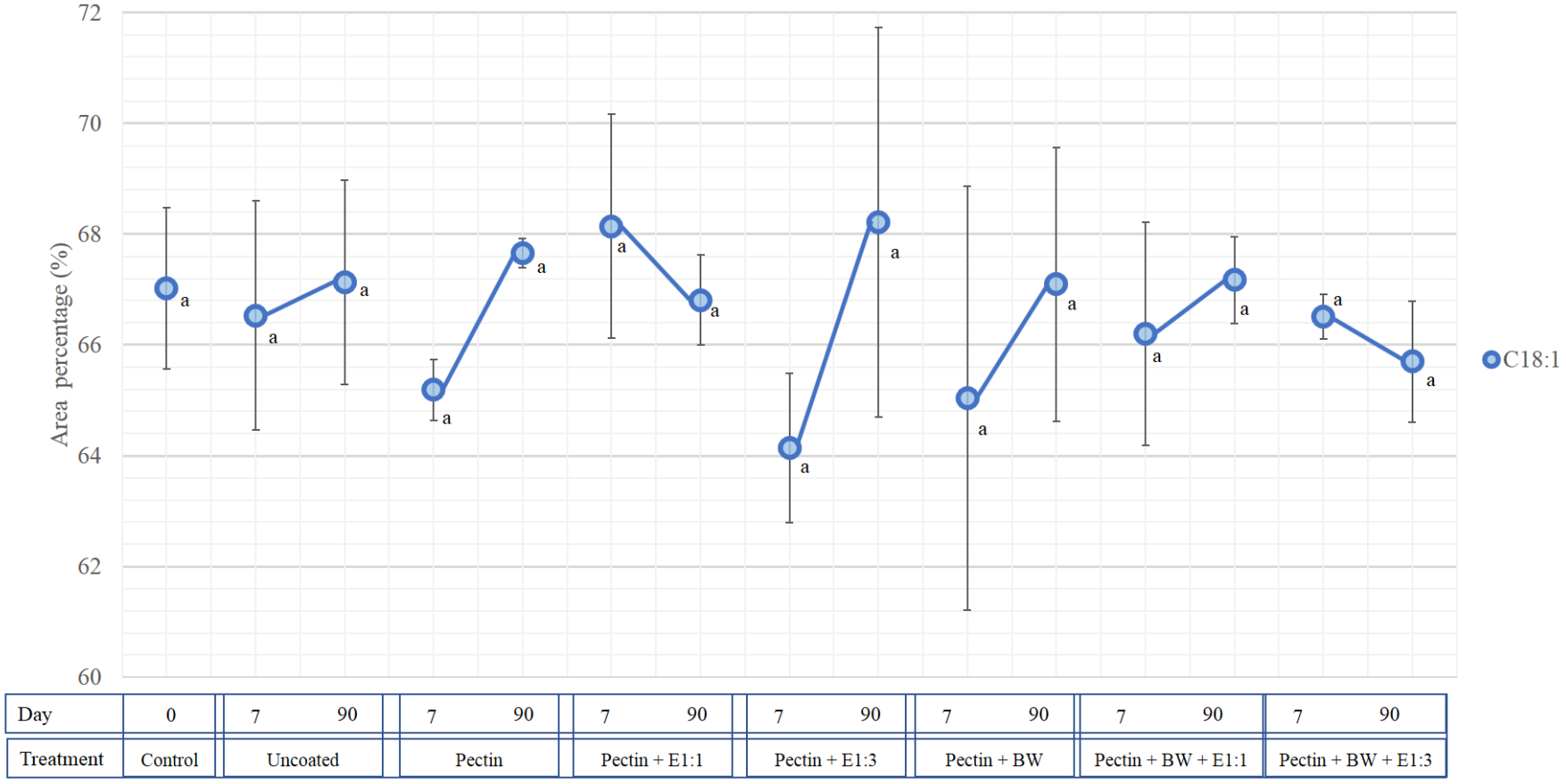
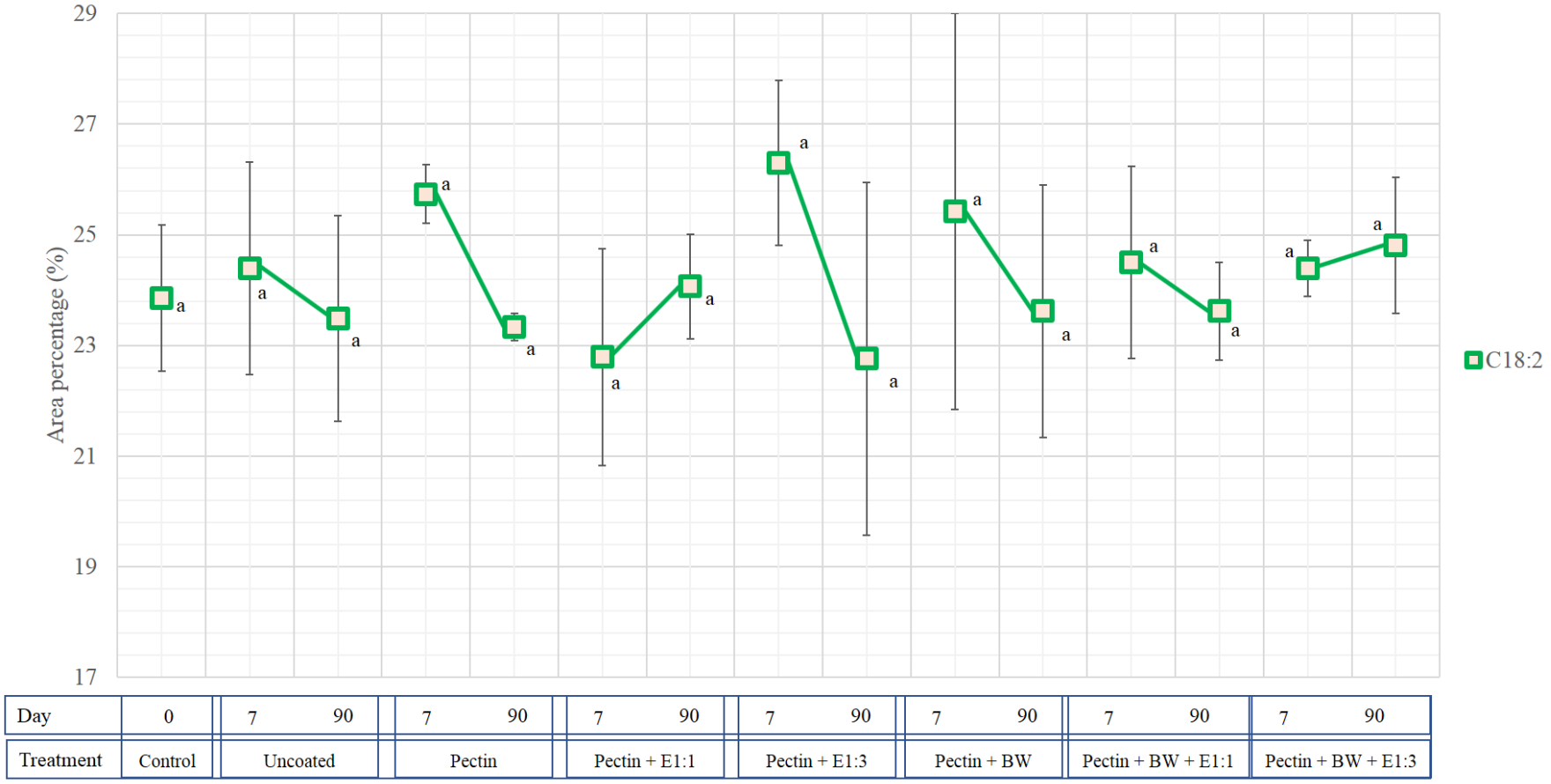


Figure 4.4. Fatty acid C18:1 area percentage over time after different coating treatments.



Day	0	7	90	7	90	7	90	7	90	7	90	7	90	7	90
Treatment	Control	Uncoated		Pectin		Pectin + E1:1		Pectin + E1:3		Pectin + BW		Pectin + BW + E1:1		Pectin + BW + E1:3	

Figure 4.5. Fatty acid C18:2 area percentage over time after different coating treatments.

Table 4.3. One-way ANOVA analysis for GC area percentage of unsaturated fatty acids.

		Source	DF	Adj SS	Adj MS	F-value	p-value
Time (0, 7 and 90 days)	C18: 2	Time	2	14.96	7.48	1.74	**0.19**
		Error	42	180.32	4.30	-	-
		Total	44	195.28	-	-	-
	C18: 1	Time	2	15.13	7.56	1.49	**0.24**
		Error	42	213.75	5.09	-	-
		Total	44	228.87	-	-	-
Different coating treatments	C18: 2	Treatment	14	82.28	5.88	1.20	**0.32**
		Error	30	146.59	4.89	-	-
		Total	44	228.87	-	-	-
	C18: 1	Treatment	14	66.50	4.75	1.11	**0.39**
		Error	30	128.78	4.29	-	-
		Total	44	195.28	-	-	-

There are two main reasons attributed to the long shelf life of almonds. Firstly, almonds contain tocopherols, which could extend their shelf life. Vitamin E or tocopherols, which can be quantified using high performance liquid chromatography, have antioxidant capacity that functions as a peroxy free radical scavenger (Eitenmiller, Landen and Ye, 2016). Kodad et al. (2014) studied the composition of 44 Spanish almond cultivars where they found that cultivars had a large range of α-tocopherol (313.0–616.1 mg/kg oil), with oil contents of 50.58-64.95% and oleic acid in total oil of 64.97–79.59%. They explained that the variation between cultivars could be related to both the cultivar's nature and environment. Secondly, due to multiple cases of *Salmonella enterica* serotype Enteritidis, almonds now must undergo a pre-treatment before they reach the final customer. A total of 29 patients infected with *Salmonella enterica* in the USA and Canada were identified between 2003 and 2004 in which raw almonds were implicated, this led to a recall from the producer of approximately 5.9 million kilograms (CDC, 2004). Because of such implication, the USDA 981.442 b required since September 1, 2007 that almond producers included a treatment that achieves a minimum of 4-log reduction of *Salmonella* bacteria in

almonds prior to shipment (USDA, 2007). Two main pasteurization treatments are used to reduce the bacterial count of almonds: steam pasteurization and propylene oxide (PPO) treatment. The PPO treatment consists in placing the almonds in a chamber under vacuum (27 inch of Hg) and the PPO fumigant is injected at a concentration of 46.5 g PPO/m^3 for 4 hours. Then, there are 4-14 aeration cycles and a post-ventilation step at 38-43ºC for 2 days or above 15ºC for 5 days (Almond board of California, 2008). While PPO is being used in the USA, the use of such chemical is not approved in Canada due to its potential to cause cancer (Canadian Food Inspection Agency, 2014). However, neither of those pasteurization treatments are identified in the labeling of almonds, leading to confusion among consumers. It is unclear the treatment almonds that used in this study underwent but it is probably a steam treatment.

A thermal treatment of almonds can change its shelf life behavior. The main objective of such treatment is to lower its *Salmonella* count. As an alternative to the use of saturated steam (>100ºC), the application of superheated steam (200ºC) for 15 or 30 seconds can achieve a 5 log reduction of *Escherichia coli* O157:H7, *Salmonella Typhymurium*, *Salmonella Enteritidis* and *Listeria monocytogenes* on almonds and in-shell pistachios (Ban and Kang, 2016). Also, 18% of inactivation of lipoxygenase is obtained by exposing almonds to 55ºC for 2 minutes and a 73% of inactivation after 10 minutes treatment (Buranasompob et al., 2007). This thermal treatment minimizes the development of oxidative rancidity in almonds. In this study, coated almonds were dried at 65ºC for 12 minutes, leading to lipoxygenase inactivation, explaining the stability of almonds at 40ºC and 50%RH up to 90 days.

Xiao et al. (2014) reported the volatile generated (Table 4.4) after a roasting process at 138ºC, which was applied to activate initial oxidation. They explained that the increase of straight chain aldehydes and alcohols corresponded to heat induced oxidation during roasting. The influence of storage conditions at 35ºC and 65% RH for 168 days in the volatile composition of roasted almonds resulted in a complete loss of both 2-Methylbutanal starting at ~4000 ng IS equivalent/g after 20 weeks and 1-Methylthio-2-propanol from ~290 ng IS equivalent/g after 24 weeks and a significant increase of acetic acid of >10 ng/g (Lee et al., 2014). The roasting process not only initiated almond oxidation but it also increased volatile compounds, which can be quantified by GC-MS (Leal Davila, 2013) to study roasted almond shelf life. Irradiated packed almonds stored at 20ºC for 12 months and exposed to light showed an increase of peroxide value from 0.45±0.14 to 13.36±0.98 meq O_2/kg oil with PET//LDPE package and 1.0 kGy and from 0.94±0.09 to 19.79±1.12 meq O_2/kg oil with PET/LDPE package and 3.0 kGy compared to the non-radiated control that increased from 0.17±0.03 to 9.20±0.44 meq O_2/kg oil (Mexis, Riganakos and Kontominas, 2011).

Table 4.4. Volatile compositional change in almonds after different roasting times at 138ºC (Adapted from Xiao et al., 2014).

	Compound	Raw	Roated almonds (28 min)	Roasted almonds (38 min)
Aldehydes and ketones	2-Methylbutanal (ng/g)	14.3±0.3	1468.6±25.7	6573.7±257.0
	3-Methylbutanal (ng/g)	32.4±0.5	911.4±50.9	4268.9±381.8
	Benzaldehyde	2934.6±272.5	368.8±41.2	331.9±65.4
	Hexanal (ng/g)	422.6±97.9	983.0±133.7	1140.8±3.8
Pyrazines	2-Methylpyrazine (ng/g)	ND	4.1±0.3	26.5±1.8
	2,5-Dimethylpyrazine (ng/g)	11.4±0.5	16.2±0.6	66.5±0.4
Alcohols	1-(Methylthio)-2-propanol (ng/g)	12.8±1.3	247±23.9	325.0±53.1
	1,2-Propanediol (ng/g)	269.1±2.5	789.4±72.3	647.0±73.8

4.4. Conclusions and recommendations

4.4.1. Conclusions

The use of pectin and pectin+beeswax based bioactive coatings on almonds had no significant difference in peroxide value after storage at 40ºC and 50%RH up to 90 days possibly because the lipid oxidation stage was not activated.

No significant difference was observed between the initial almond fatty acid composition and the fatty acids after storage at 40ºC and 50%RH for 90 days. This could be attributed to the enzyme inactivation that happened after the drying process of the coated almonds and/or the treatment almonds had after harvest. The presence of vitamin E (α-tocopherol), a natural antioxidant, also prevented the degradation of unsaturated fatty acids.

4.4.2. Recommendations

- As only one temperature and RH were evaluated for coated almonds, bioactive coated almonds should be analyzed for at least two years at a wide range of temperature (45-90ºC) and relative humidity (30-90%).
- Because unsaturated fatty acids lose their double bonds through β-oxidation, other nuts with higher unsaturated fatty acids could be analyzed to ensure that the coatings can be used in different products, such as walnuts and pine nuts.
- Roasting the almonds before the application of the coating can activate lipid oxidation, hence faster oxidation to measure fatty acid composition during shelf life.
- Volatile analysis of the roasted almonds can be performed by GC-MS as these compounds relate to the flavour profile of almonds.
- Bioactive coatings of cranberry extract and pectin could also be used in other food matrices such as fruits and confections, because of its antioxidant activity potential.

Chapter 5: Conclusions and recommendations

The following conclusions are based on the major findings of this research.

5.1. Conclusions

5.1.1. Pressurized fluid extraction

The first part of this thesis focused on the pressurized fluid extraction of anthocyanins and total phenolics from cranberry pomace using solvents (water, ethanol, mixtures of water+ethanol and 5% citric acid+water), temperatures (120-160ºC) and pressures (50 and 200 bar). Also, total anthocyanins were extracted from cranberry pomace using pressurized ethanol (50 bar and 40-160ºC).

Temperature variation and solvent type had a significant ($p<0.5$) impact in both total anthocyanin and total phenolic extraction. No significant difference ($p>0.05$) was reported changing pressure for both total phenolics and total anthocyanin, however there was a significant difference ($p<0.5$) analyzing pressure and solvent together. In addition, a significant difference ($p<0.5$) analyzing pressure and temperature together was observed for total phenolic extraction but not for total anthocyanin extraction. Also, total anthocyanin was mostly extracted within the first 10 with phenolic compounds obtained after 10 minutes. For all temperatures and pressures studied, the best solvent to extract anthocyanins was pressurized ethanol. In addition, at 120ºC, the ideal solvent for total phenolic extraction was ethanol 30%+water while above 140ºC, the ideal solvents were ethanol 30%+water and ethanol 70%+water.

The extraction of total anthocyanins using pressurized ethanol at 60-100ºC and 50 bar resulted in 4.15-4.21 mgCy3GE/g d.w, which could be an alternative method to traditional solvent extraction using acidified methanol at ~25ºC at 1 bar (4.28±0.01 mgCy3GE/g d.w.). The ideal anthocyanin extraction using only pressurized ethanol at 80ºC and 50 bar resulted in

4.21±0.01 mgCy3GE/g d.w. Studies reported total anthocyanin extraction of 4.51±0.11 mgCy3GE/g d.w. using acidified methanol (99:1, methanol:HCl v/v), 2.28±0.06 mgCy3GE/g d.w. using acetonitrile+trifluoroacetic acid+water (49.5:0.5:50, v/v) and 2.04±0.05 mgCy3GE/g d.w. using ethanol+water +HCl (70:29:1, v/v) (Klavins, Kviesis and Klavins, 2017).

Pressurized ethanol at 160ºC and 50 bar extracted 30.3% lower anthocyanin content and 53% higher total phenolic content compared to pressurized ethanol at 120ºC and 50 bar,. This could be attributed to the depolymerization of anthocyanins into other phenolic compounds such as phloroglucinaldehyde and 4-hydroxylbenzoic acid at high temperatures. Similar extraction rate trends were observed between total anthocyanin extraction and total phenolic extraction using pressurized ethanol at 120ºC and 50 bar while different extraction rates were observed using pressurized water at 160ºC and 50 bar. Because of these trends and the high pearson correlation value between antioxidant capacity and total anthocyanins (P=0.94) using pressurized ethanol, it is suggested that pressurized ethanol had high anthocyanin selectivity compared to phenolics.

5.1.2. Bioactive coating of almonds

The second part of the research described in this thesis used the extracts obtained in the first study into a food coating to prevent deterioration reactions in almonds. Such coatings were either pectin based or pectin+beeswax based, which were applied using the spraying method. Coated and uncoated almonds were stored at 40ºC and 50% relative humidity for up to 90 days.

- After 90 days of storage at 40ºC and 50% RH, no significant differences were observed for uncoated and coated almond's fatty acid profile and peroxide values, suggesting that lipid oxidation was not initiated at such storage conditions.

- A general increasing trend in the almonds was observed for fatty acid C18:1 and a decreasing trend for C18:2 in most treatments with the exception of pectin+extract (1:1, w/w), and pectin+beeswax+extract (1:3, w/w) which had a slightly opposite behaviour. This suggests that those coatings might have prevented fatty acid change better however there was no significant difference between all samples.
- The presence of natural antioxidants, like α-tocopherol (313.0–616.1 mg/kg oil), and the inactivation of lipoxygenase due to the coating drying process could influence the almond stability for up to 90 days.

5.2. Recommendations

The following recommendations from this research are for further studies.

5.2.1. Pressurized fluid extraction

The impact of blending and drying methods (e.g. vacuum drying, nitrogen drying and air drying) of cranberry pomace before pressurized fluid extraction should be considered. Images of the samples using scanning electron microscope to study the impact in the cell structure is recommended before and after the pre-treatment and the extraction. Controlling the possible ripening of cranberry pomace at different conditions (temperature and relative humidity) before drying and extraction is suggested.

The relation between proanthocyanins and anthocyanins should also be studied in the extracts and over different storage conditions. The addition of gallic acid, ferulic acid and caffeic acid in pressurized fluid extractions should be included due to the potential of these acids to interact with sugar of the anthocyanin, hence increasing anthocyanin storage stability. The stability of extracts in liquid form and freeze-dried powder is also recommended.

5.2.2. Bioactive coating for almonds

The impact of storage conditions of uncoated and coated almonds at a wider temperature range of 45-90ºC and relative humidity of 30-90% should be considered for at least 2 years because of almond stability due to natural antioxidant presence and required thermal treatment applied by producers before almond shipment. Obtaining untreated samples from almond industry suppliers is highly recommended. With the objective of activating lipid oxidation, a heating treatment (135ºC for ~ 30 min) to almonds is suggested for further quantification of volatile compounds by GC-MS initially and over time.

Because of the bioactive coating potential to prevent deterioration reactions, the application of pectin based edible coatings with cranberry extracts should also be studied in other high fat products such as walnuts, pine nuts, cheese and chocolate. The same coatings can also be applied to other food products such as apples and strawberries. Different coating processes such as dipping and brushing should also be studied to compare application efficiency.

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APPENDIX A. Pressurized fluid extraction of anthocyanins from cranberry pomace.

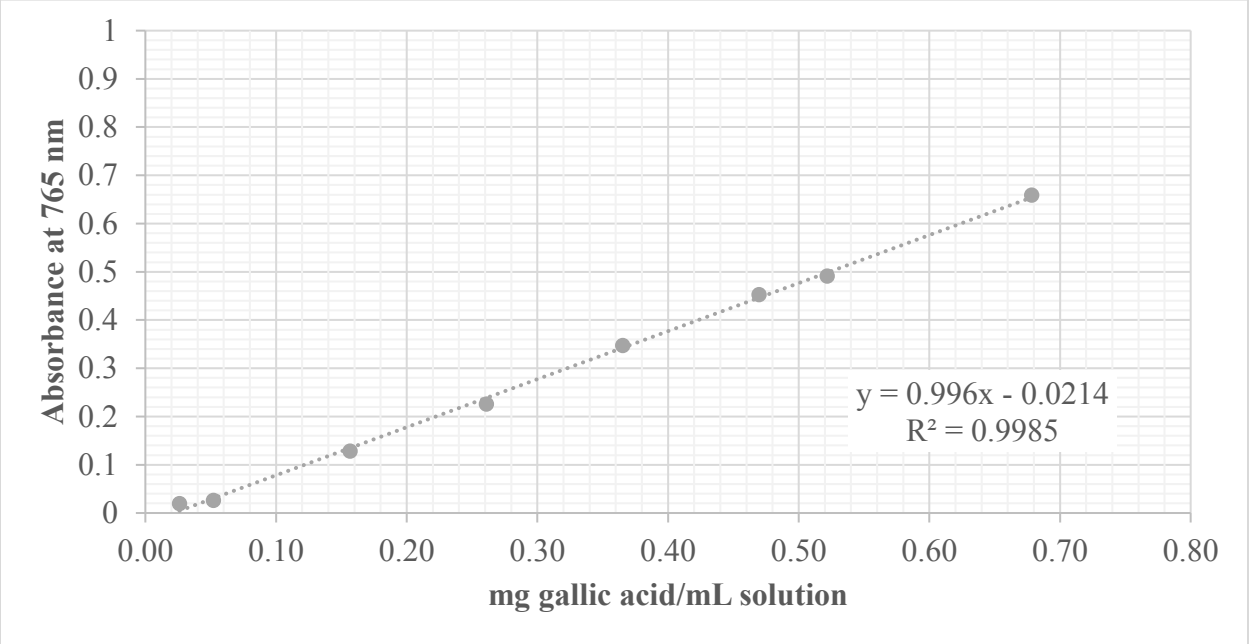

Figure A1. Gallic acid calibration curve to determine total phenolic content.

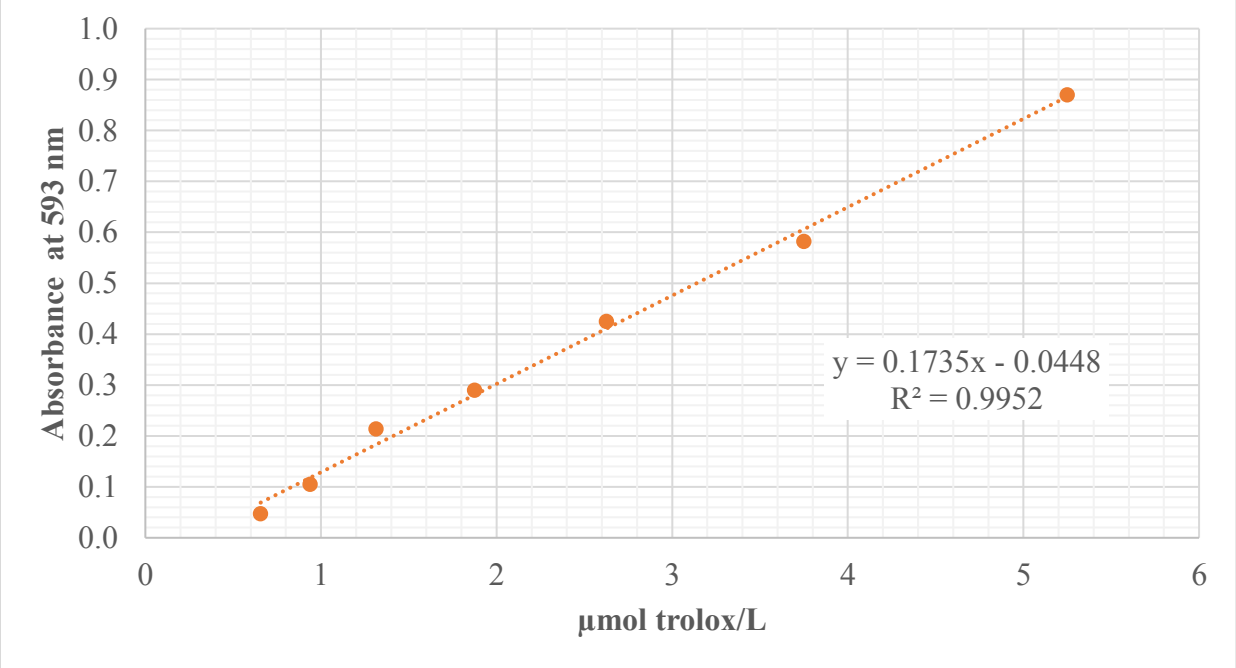

Figure A2. Antioxidant activity calibration curve for FRAP analysis.

Table A1. Steps to calculate total anthocyanins using the pH differential method (AOAC 2005) of sample collected in the first 5 minutes of pressurized ethanol at 50 bar and 80ºC.

Step 1. Measure absorbance at 520 and 700 nm of the extract diluted in two buffers, one at pH of 1 and the other one at pH of 4.5.

pH	Absorbance (nm)	
	520	**700**
pH 1	0.826	0.172
pH 4.5	0.316	0.145

Step 2. Calculate A value

$$A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$$

$$A = (0.826\text{-}0.172)_{pH1.0} - (0.316\text{-}0.145)_{pH\ 4.5} = 0.483$$

Step 3. Know the dilution factor and other values.

$$Anthocyanin\ pigment\ \left(\frac{mg\ C3GE}{L}\right) = \frac{A * MW * DF * 10^3}{\varepsilon * 1}$$

Molecular weight (MW) = 449.2 g/mol of cyanidin-3-glucoside (Cy3GE)
DF = 20
ε = 26900 molar extinction coefficient (L/mol cm of Cy3GE)

Step 4. Substitute values in the equation

$$Anthocyanin\ pigment\ \left(\frac{mg\ Cy3GE}{L}\right) = \frac{0.483 * 449.2 * 20 * 10^3}{26900 * 1}$$

Anthocyanin pigment = 161.31 mg Cy3GE/L

Step 5. Multiply by the final volume of the sample.

$$\left(161.31\ \frac{mg\ Cy3GE}{L}\right)\ (0.025\ L) = 4.03\ mg\ Cy3GE$$

Table A2. Total anthocyanin, total phenolic and antioxidant capacity of cranberry pressurized extracts at different conditions.

Solvent	Temperature (ºC)	Pressure (bar)	Total anthocyanins (mg Cy3GE)			Total phenolics (mg GAE)			Antioxidant capacity (mol troloxE)		
			Exp 1	Exp 2	Average	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average
Water	120	50	0.86	0.71	0.78±0.07d	18.19	16.32	17.25±0.93ef	1.43	1.36	1.40±0.04fgh
		200	0.86	1.08	0.97±0.11d	20.22	28.59	24.41±4.19cdef	1.66	1.96	1.81±0.15efgh
	140	50	0.60	0.53	0.57±0.03d	26.72	25.02	25.87±0.85cdef	2.68	1.72	2.20±0.48efgh
		200	0.73	0.77	0.75±0.02d	62.33	33.17	47.75±14.58abcdef	5.19	3.08	4.13±1.05abcdef
	160	50	0.88	0.36	0.62±0.26d	53.39	73.86	63.63±10.23abcd	4.75	5.31	5.03±0.28abc
		200	0.16	0.00	0.09±0.07d	23.92	60.31	42.11±18.20abcdef	2.07	4.66	3.36±1.29bcdefg
Citric acid 5% + water	120	50	1.33	2.12	1.72±0.39cd	28.79	50.03	39.41±10.62abcdef	1.31	2.54	1.93±0.61efgh
		200	1.62	0.97	1.30±0.32cd	22.79	19.48	21.14±1.66def	0.77	0.42	0.60±0.18h
	140	50	1.00	1.02	1.01±0.01d	52.47	60.52	56.50±4.02abcde	2.36	2.97	2.66±0.30cdefgh
		200	1.47	0.96	1.21±0.25d	65.43	45.92	55.68±9.75abcde	1.96	1.53	1.75±0.21efgh
	160	50	0.71	0.77	0.74±0.03d	21.61	47.56	34.58±12.97bcdef	1.36	1.79	1.57±0.21fgh
		200	0.90	0.81	0.86±0.05d	70.75	41.29	56.02±14.73abcde	1.55	2.95	2.25±0.70defgh
Ethanol 30% + water	120	50	2.17	1.96	2.07±0.10cd	20.60	13.80	17.20±3.40ef	1.90	1.49	1.70±0.21fgh
		200	0.84	0.69	0.77±0.08d	7.33	4.29	5.81±1.52f	1.20	0.92	1.06±0.14gh
	140	50	2.12	2.07	2.09±0.02cd	100.62	69.31	84.96±15.66a	5.68	4.55	5.12±0.57abc
		200	0.58	0.99	0.78±0.20d	43.28	17.45	30.36±12.91cdef	2.49	3.31	2.90±0.41cdefgh
	160	50	2.10	0.38	1.24±0.86d	86.53	78.24	82.38±4.15a	5.28	4.67	4.97±0.31abcd
		200	0.90	0.83	0.86±0.04d	56.70	61.34	59.02±2.32abcde	5.88	5.71	5.80±0.08ab

*Means in a column followed by the same letter are not significantly different.

Table A2. Continued.

Solvent	Temperature (ºC)	Pressure (bar)	Total anthocyanins (mg Cy3GE)			Total phenolics (mg GAE)			Antioxidant capacity (mol troloxE)		
			Exp 1	Exp 2	Average	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average
Ethanol 70% + water	120	50	5.34	6.28	5.81±0.47ab	49.74	59.90	54.82±5.08abcde	6.07	5.87	5.97±0.10ab
		200	6.79	4.07	5.43±1.36ab	56.62	43.80	50.12±6.41abcdef	6.02	4.43	5.23±0.79abc
	140	50	3.97	3.73	3.85±0.12bc	67.76	67.01	67.39±0.38abc	5.98	5.41	5.69±0.29ab
		200	4.87	4.63	4.75±0.12b	55.90	55.63	55.77±0.14abcde	7.08	6.05	6.56±0.52a
	160	50	1.82	1.80	1.81±0.1cd	68.92	68.03	68.48±0.45abc	6.95	5.73	6.34±0.61a
		200	5.46	4.60	5.03±0.43b	75.08	81.64	78.36±3.28ab	6.93	6.14	6.54±0.40a
Ethanol	120	50	8.17	7.39	7.78±0.39a	26.82	31.49	29.16±2.33cdef	3.52	2.96	3.24±0.28bcdefgh
		200	6.82	5.81	6.31±0.50ab	32.44	33.46	32.95±0.51bcdef	3.62	3.36	3.49±0.13bcdefg
	140	50	5.60	6.45	6.02±0.43ab	38.85	33.27	36.06±2.79bcdef	3.81	3.21	3.51±0.30bcdefg
		200	6.26	5.53	5.90±0.36ab	40.16	43.50	41.83±1.67abcdef	4.18	3.92	4.05±0.13abcdef
	160	50	6.23	4.61	5.42±0.81ab	41.42	47.83	44.62±3.21abcdef	3.97	3.96	3.96±0.01abcdef
		200	6.25	4.19	5.22±1.03ab	51.33	50.57	50.95±0.38abcdef	4.64	4.24	4.44±0.20abcde

Means in a column followed by the same letter are not significantly different.

Table A3. Total anthocyanin extraction using pressurized ethanol at 50 bar and different temperatures compared with traditional solvent extraction using acidified methanol.

Extraction	Temperature (°C)	Exp 1	Exp 2	Total Anthocyanin (mg C3GE)
Pressurized ethanol	40	7.22	7.38	7.30±0.08abc
	60	8.18	8.45	8.31±0.14a
	80	8.39	8.45	8.42±0.03a
	100	8.22	8.59	8.40±0.19a
	120	8.17	7.39	7.78±0.39ab
	140	5.60	6.45	6.02±0.43bc
	160	6.23	4.61	5.42±0.81c
Acidified MeOH	Room temperature	8.58	8.54	8.56±0.01a

Means in a column followed by the same letter are not significantly different.

Table A4. Individual anthocyanins from cranberry pomace extracts using HPLC-UV.

Temperature	Pressure	Solvent	Anthocyanin	Exp 1	Exp 2	Average (mg)
120ºC	50 bar	Water	Cyanidin 3-galactoside	0.62	0.48	0.55±0.07a
			Cyanidin 3-glucoside	0.29	0.25	0.27±0.02a
			Cyanidin 3-arabinoside	1.82	1.26	1.54±0.28c
			Peonidin 3-galactoside	1.30	0.95	1.12±0.18c
		Ethanol30 % +water	Cyanidin 3-galactoside	1.83	1.60	1.71±0.11a
			Cyanidin 3-glucoside	0.29	0.33	0.31±0.02a
			Cyanidin 3-arabinoside	4.77	4.28	4.52±0.24bc
			Peonidin 3-galactoside	4.29	3.79	4.04±0.25bc
		Ethanol70 % +water	Cyanidin 3-galactoside	3.47	3.63	3.55±0.08a
			Cyanidin 3-glucoside	1.47	1.48	1.47±0.01a
			Cyanidin 3-arabinoside	11.29	11.87	11.58±0.29abc
			Peonidin 3-galactoside	7.66	8.45	8.06±0.40ab
		Ethanol	Cyanidin 3-galactoside	4.45	4.57	4.51±0.06a
			Cyanidin 3-glucoside	1.67	1.70	1.68±0.01a
			Cyanidin 3-arabinoside	14.81	14.60	14.70±0.10ab
			Peonidin 3-galactoside	11.99	12.72	12.36±0.37a
80ºC			Cyanidin 3-galactoside	5.26	5.36	5.31±0.05a
			Cyanidin 3-glucoside	1.67	1.67	1.67±0.00a
			Cyanidin 3-arabinoside	15.49	15.79	15.64±0.15a
			Peonidin 3-galactoside	13.89	13.10	13.49±0.39a
Room temperature	Room pressure	Methanol+ HCl	Cyanidin 3-galactoside	5.55	5.19	5.37±0.18a
			Cyanidin 3-glucoside	0.48	0.51	0.49±0.01a
			Cyanidin 3-arabinoside	12.58	12.13	12.35±0.23ab
			Peonidin 3-galactoside	14.94	13.93	14.43±0.51a

Letters correspond to difference between each individual anthocyanin and not between all anthocyanins.

Table A5. pH and conductivity of extracts at 5, 10, 20 and 30 min.

Solvent	Temperature (ºC)	Pressure (bar)	Extraction time (min)	pH	k (mS/cm)
Water	120	50	5	2.52±0.1	123.71±38.79
			10	2.56±0.02	145.35±5.66
			20	2.54±0.01	136.15±9.05
			30	2.53±0.06	130.45±7.15
		200	5	2.57±0.04	157.3±17
			10	2.64±0.01	156.35±5.35
			20	2.72±0.02	111±4.91
			30	2.7±0.02	100.6±2.8
	140	50	5	2.67±0.05	177.35±24.15
			10	2.66±0.04	141.3±20.4
			20	2.67±0.04	112.3±3.6
			30	2.68±0.02	100.3±2.5
		200	5	2.57±0.04	199.05±10.15
			10	2.59±0.02	199.55±1.45
			20	2.59±0.03	165.1±43.4
			30	2.7±0.15	129.55±77.96
	160	50	5	2.6±0.01	198.05±15.15
			10	2.7±0.01	228.1±12.5
			20	2.63±0	228.8±1.3
			30	2.73±0.03	110.35±2.45
		200	5	2.55±0.05	189.9±2.8
			10	2.61±0.01	209.95±6.75
			20	2.59±0.01	204.75±8.06
			30	2.51±0.01	133.2±11.9

Table A6. Continue.

Solvent	Temperature (ºC)	Pressure (bar)	Extraction time (min)	pH	k (mS/cm)
Citric acid 5% + water	120	50	5	2.14±0.01	1196±74
			10	2.13±0.03	1090.5±9.51
			20	2.1±0	1177.5±2.5
			30	2.12±0.02	973±117
		200	5	2.15±0.02	1079±22
			10	2.12±0.02	1130±4
			20	2.11±0.04	1269.5±22.5
			30	2.15±0.05	1171.5±64.5
	140	50	5	2.12±0.02	1063.5±42.51
			10	2.11±0	1044±41
			20	2.12±0.02	1150±34
			30	2.13±0.01	1289±4
		200	5	2.02±0.05	1171±99
			10	1.98±0.01	1218.5±100.5
			20	1.94±0	1357.5±139.51
			30	1.93±0.01	1378.5±158.51
	160	50	5	2.1±0.01	998.5±11.5
			10	2.09±0.02	1077.5±37.5
			20	2.08±0.01	1160.5±39.5
			30	2.08±0.01	1217±37
		200	5	2.09±0.09	1194.5±27.5
			10	2.04±0.06	1283.5±286.5
			20	2.02±0.07	1153±141
			30	1.97±0.02	1175.5±69.5

Table A6. Continue.

Solvent	Temperature (ºC)	Pressure (bar)	Extraction time (min)	pH	k (mS/cm)
Ethanol	120	50	5	4.35±0.02	7.63±1.11
			10	4.61±0.12	5.1±0.76
			20	4.36±0.07	3.42±0.6
			30	4.58±0.37	1.41±0.3
		200	5	4.49±0.04	7.87±1.28
			10	4.64±0.07	7.48±0.34
			20	4.75±0.03	5.2±0.21
			30	4.83±0.04	2.86±0.06
	140	50	5	4.42±0.04	7.77±0.31
			10	4.52±0.04	6.85±0.02
			20	4.49±0.08	4±0
			30	4.66±0.09	2.43±0
		200	5	4.55±0.1	9.58±0.51
			10	4.71±0.14	7.96±0.04
			20	4.96±0.12	4.69±0.1
			30	5.14±0.01	3.09±0.23
	160	50	5	4.53±0.09	8.66±0.5
			10	4.56±0.31	6.47±1.35
			20	4.61±0.22	3.67±0.49
			30	5.15±0.03	1.57±0.14
		200	5	4.45±0.05	9.5±1.01
			10	4.7±0.17	7.25±1.14
			20	5±0.11	4.25±1.49
			30	5.17±0.09	1.83±0.52

Table A6. Continue.

Solvent	Temperature (ºC)	Pressure (bar)	Extraction time (min)	pH	k (mS/cm)
Ethanol 30% + water	120	50	5	3.17±0.04	165.9±23.31
			10	3.19±0.13	151.85±48.65
			20	3.25±0.13	57.32±20.1
			30	3.27±0.22	36.45±12.8
		200	5	3.1±0.12	57.5±6.33
			10	3.15±0.14	54.13±6.71
			20	3.16±0.11	24.06±0.23
			30	3.24±0.01	16.53±2.22
	140	50	5	3.03±0.01	175.1±29
			10	3.04±0.01	210.7±43.7
			20	3.04±0.05	83.6±8.73
			30	3.14±0.02	42.9±4.67
		200	5	2.99±0.01	92.69±19.51
			10	2.97±0.01	82.61±6.13
			20	3.07±0.02	38.74±0.81
			30	3.13±0.05	31.91±0.02
	160	50	5	3.14±0.08	102.06±15.24
			10	3.17±0.09	124.55±14.55
			20	3.22±0.02	63.26±6.81
			30	3.2±0.06	51.75±10.33
		200	5	3.03±0.1	88.58±3.25
			10	2.84±0.2	101.8±1.1
			20	2.99±0.04	31.32±1.99
			30	2.93±0.06	31.28±0.97

Table A6. Continue.

Solvent	Temperature (ºC)	Pressure (bar)	Extraction time (min)	pH	k (mS/cm)
Ethanol 70% + water	120	50	5	3.77±0.02	34.65±1.67
			10	3.79±0.09	28.63±0.16
			20	3.82±0.02	10.08±1.27
			30	3.85±0.2	4.96±1.03
		200	5	3.83±0.15	25.11±2.87
			10	3.73±0.18	27.65±1.48
			20	3.73±0.23	10.2±0.9
			30	3.5±0.09	5.64±1.11
	140	50	5	3.83±0.03	22.75±0.01
			10	3.8±0.01	32.02±1.92
			20	3.87±0.17	13.04±0.83
			30	4.04±0.12	5.31±0.36
		200	5	3.89±0.04	36.92±1.52
			10	3.82±0.21	34.67±1.25
			20	3.99±0	11.93±1.75
			30	3.82±0.03	5.73±0.31
	160	50	5	3.79±0.41	35.5±26.58
			10	3.4±0.16	50.15±29.51
			20	3.59±0.01	14.4±0.7
			30	3.83±0.22	5.24±0.16
		200	5	3.71±0.28	55.47±6.51
			10	4.07±0.18	26.49±2.95
			20	4.19±0.2	10.05±3.81
			30	4.15±0.25	5.3±0.29

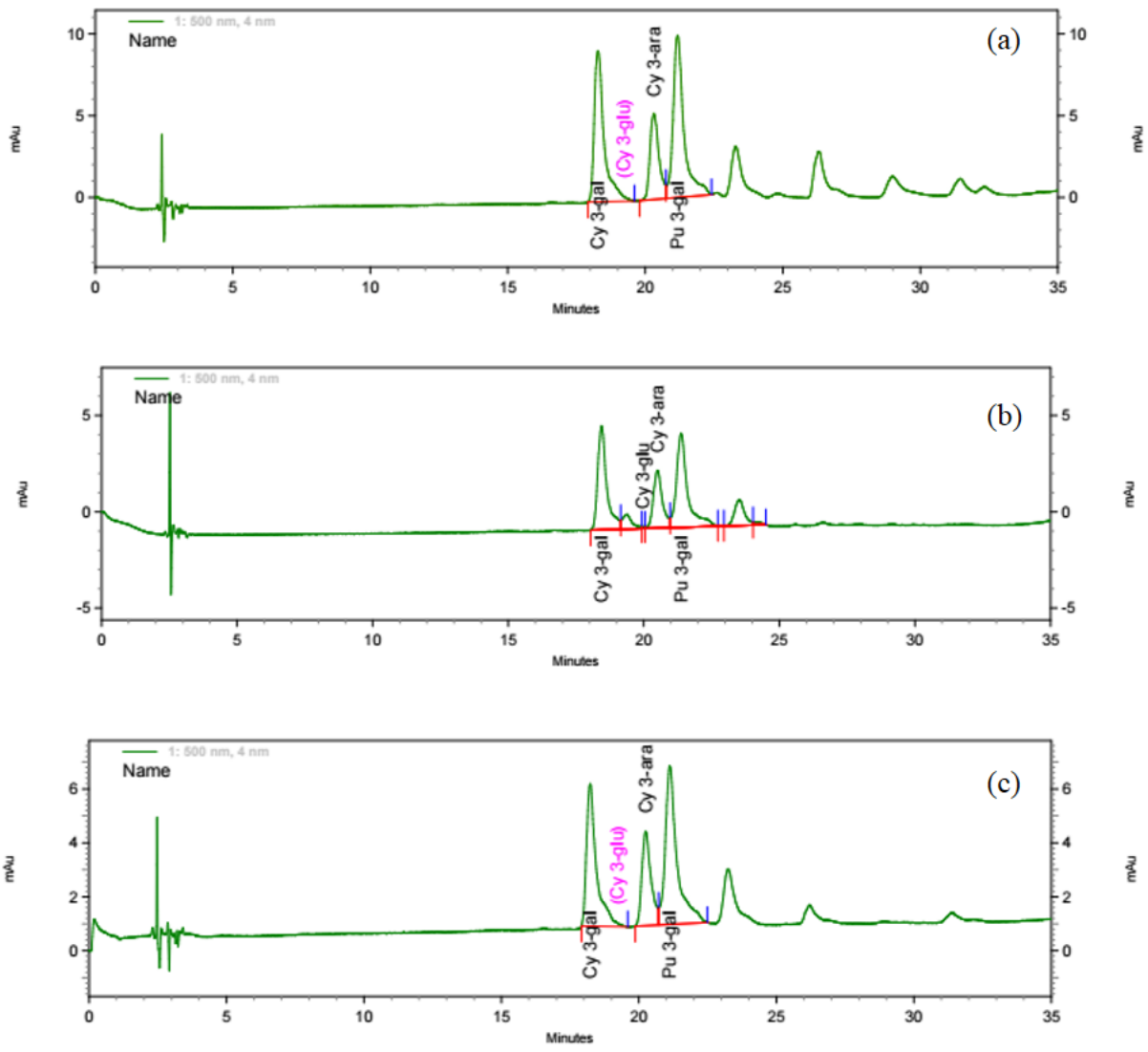

Figure A3. Individual anthocyanins chromatograms using HPLC-UV from cranberry pomace obtained by: (a) traditional solvent extraction (MeOH+HCl), (b) pressurized water at 120ºC and 50 bar for 5 minutes, and (c) pressurized ethanol at 120ºC and 50 bar for 5 minutes.

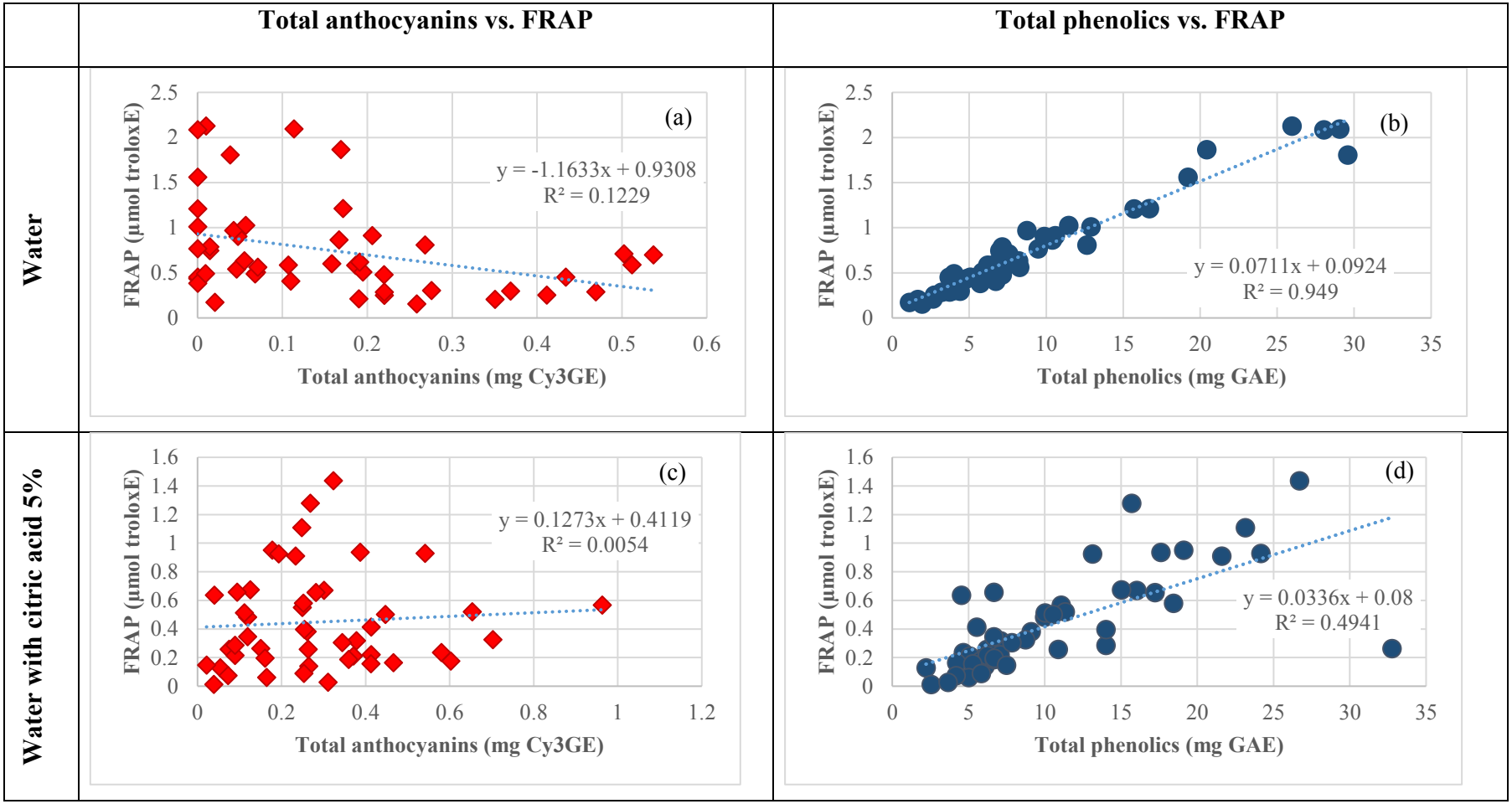

Figure. A.4. Regression between FRAP vs. total anthocyanins and total phenolics extracted using different pressurized fluids

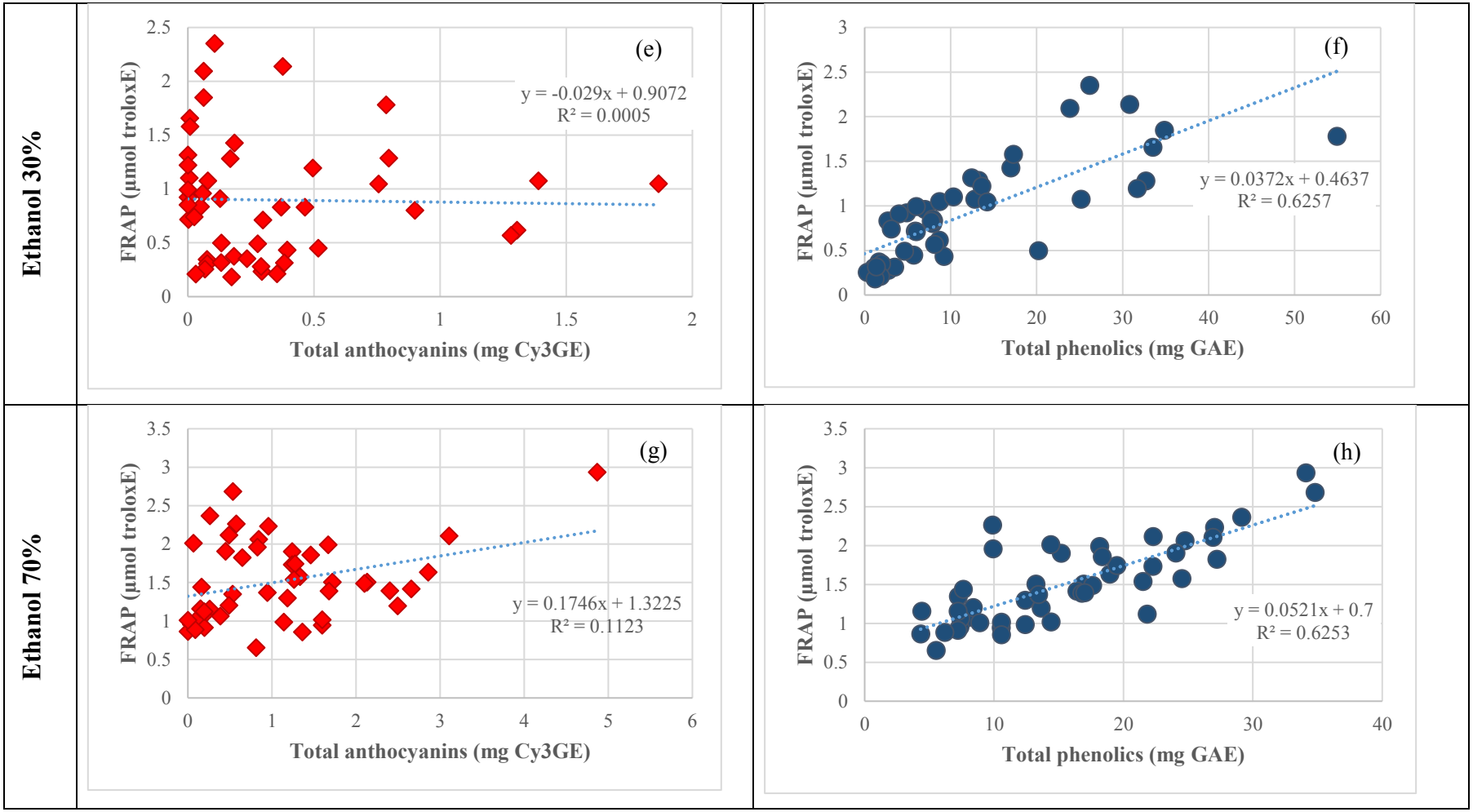

Figure. A.4 Continue.

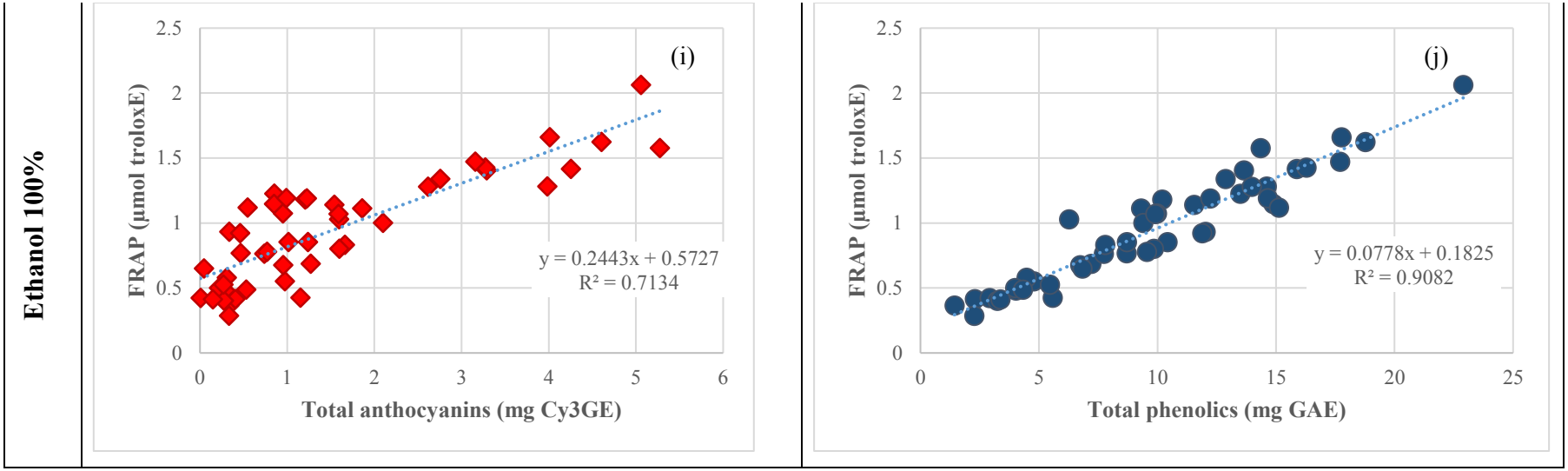

Figure. A.4 Continue.

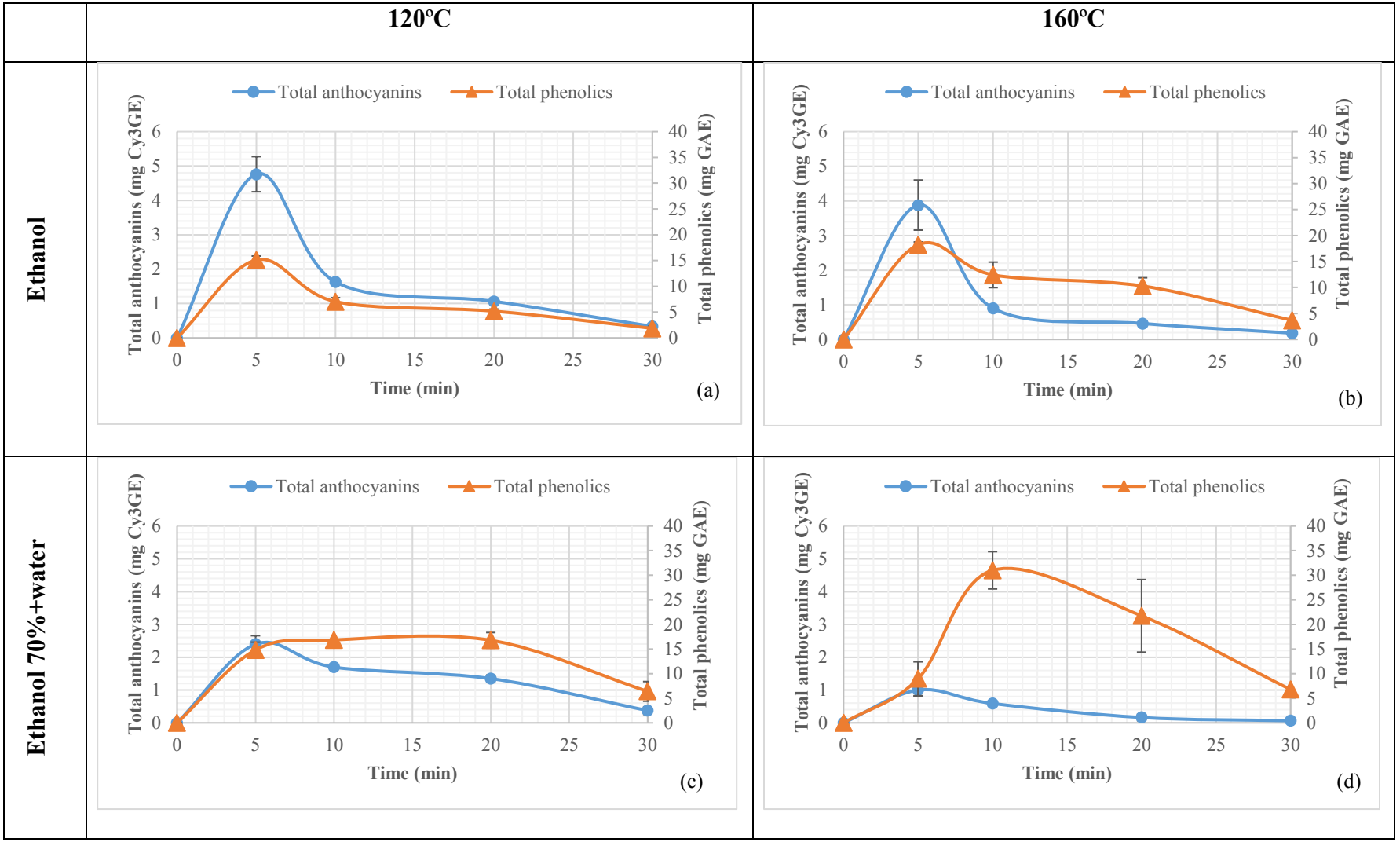

Figure. A.5. Total anthocyanin and total phenolic extraction rate using different pressurized solvents and temperature at 50bar.

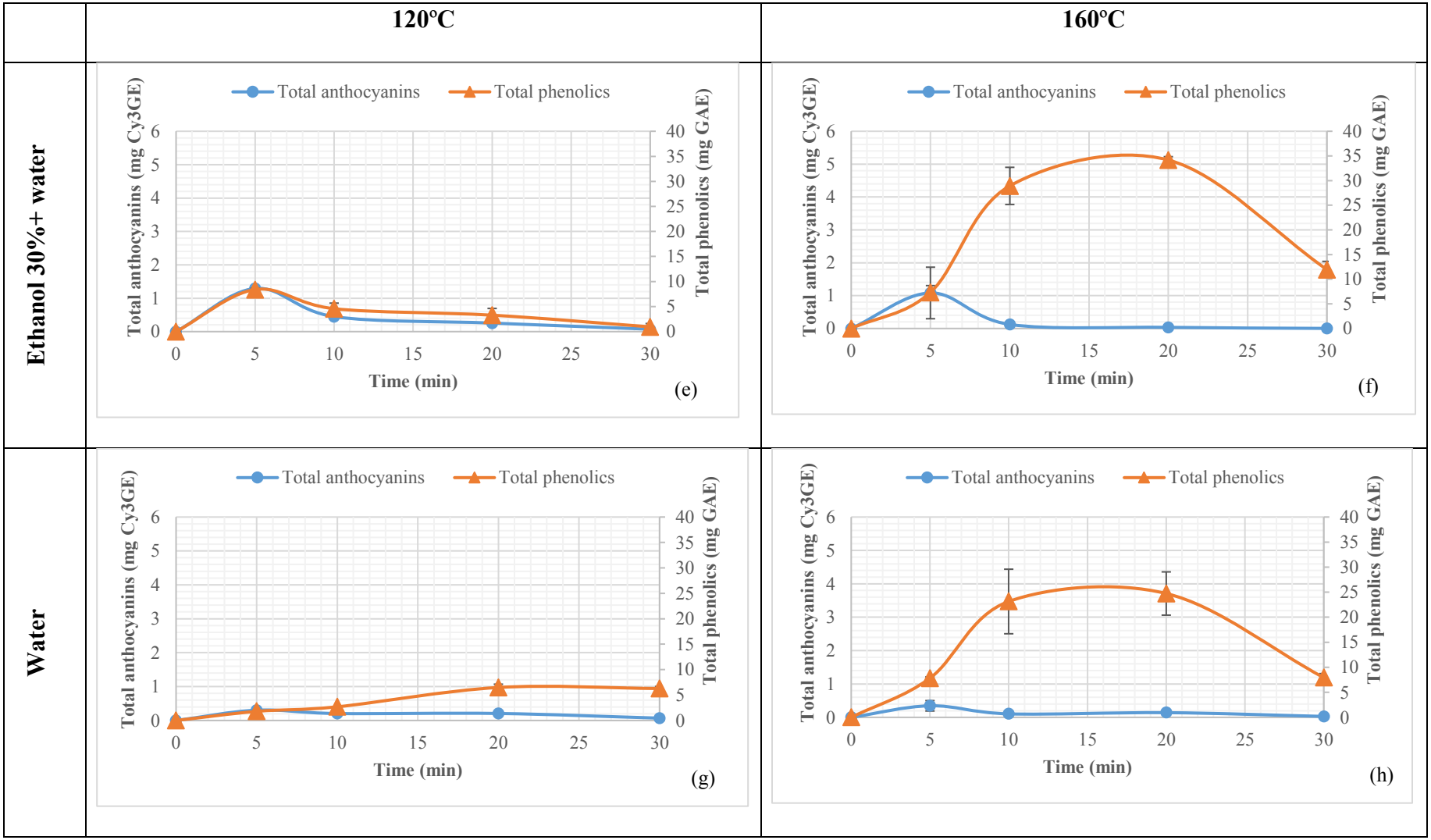

Figure. A.5 Continue.

APPENDIX B. Bioactive food coatings based on cranberry extract, pectin and beeswax for almonds.

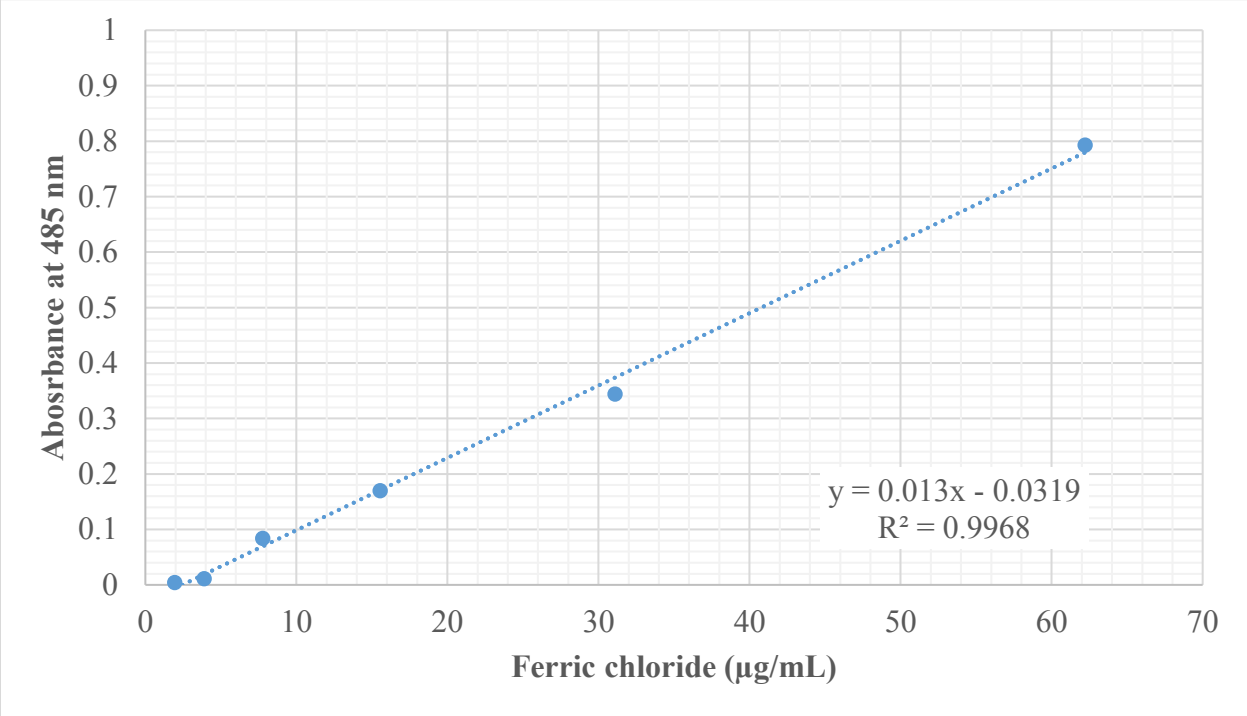

Figure B1. Incipient rancidity calibration curve.

Table B1. Uncoated and coated weight variation percentage over time.

Day 7							
Treatment	**Triplicate**						**% Change**
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	
Uncoated	6.5432	6.5591	6.3169	6.3227	6.2639	6.2541	0.0040±0.0106abc
Pectin	6.8206	6.8119	6.7575	6.7565	6.8831	6.8640	-0.0096±0.0074abc
Pectin + E(1:1)	6.8373	6.8209	5.9958	5.9768	6.7306	6.6908	-0.0251±0.0105abc
Pectin + E(1:3)	7.0725	7.0527	6.8588	6.8185	6.2404	6.2018	-0.0329±0.0093abc
Pectin + BW	7.3716	7.3405	6.6460	6.6035	6.2265	6.1700	-0.0439±0.0104abc
Pectin + BW + E(1:1)	6.7822	6.7584	6.5620	6.5260	7.2438	7.1836	-0.0400±0.0151abc
Pectin + BW + E(1:3)	6.3060	6.2851	6.3232	6.2910	6.3502	6.2971	-0.0354±0.0133abc
Day 14							
Treatment	**Triplicate**						**% Change**
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	
Uncoated	6.1582	6.1375	6.1617	6.1652	6.4225	6.4130	-0.0089±0.0099abc
Pectin	6.8341	6.7988	6.3705	6.3395	6.7590	6.7181	-0.0357±0.0041abc
Pectin + E(1:1)	6.5104	6.4662	6.2027	6.1203	6.5816	6.5248	-0.0611±0.0159abc
Pectin + E(1:3)	6.3384	6.2999	6.8699	6.7989	6.6317	6.5745	-0.0556±0.0133abc
Pectin + BW	6.8322	6.7664	6.9086	6.8162	6.4614	6.3630	-0.0855±0.0142bc
Pectin + BW + E(1:1)	6.9725	6.9126	6.3693	6.2787	6.8795	6.7452	-0.0949±0.0305c
Pectin + BW + E(1:3)	6.9091	6.8447	6.2823	6.2132	5.8117	5.7316	-0.0712±0.0066abc

Means in a column followed by the same letter are not significantly different.

Table B1. Continued.

Day 30							
Treatment	**Triplicate**						**% Change**
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	
Uncoated	6.1318	6.1259	6.5157	6.5287	6.3653	6.3414	-0.0053±0.0151abc
Pectin	6.6037	6.5842	6.1893	6.1271	6.5227	6.4552	-0.0497±0.0251abc
Pectin + E(1:1)	6.4764	6.4447	6.3819	6.2936	6.6360	6.5499	-0.0687±0.0262abc
Pectin + E(1:3)	6.8531	6.8213	6.6799	6.5683	6.5409	6.4584	-0.0753±0.0330abc
Pectin + BW	6.7831	6.7208	6.2677	6.1694	6.5888	6.5029	-0.0822±0.0149bc
Pectin + BW + E(1:1)	6.5706	6.5054	6.9344	6.8253	6.6285	6.5548	-0.0827±0.0190bc
Pectin + BW + E(1:3)	6.6911	6.6411	6.8760	6.7868	6.1234	6.0697	-0.0643±0.0177abc
Day 60							
Treatment	**Triplicate**						**% Change**
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	
Uncoated	6.7325	6.7749	6.2553	6.2905	6.2443	6.2929	0.0421±0.0055a
Pectin	6.7746	6.8016	6.4416	6.4400	6.6677	6.6886	0.0154±0.0123abc
Pectin + E(1:1)	6.4757	6.5028	7.2061	7.2019	6.3871	6.3794	0.0051±0.0156abc
Pectin + E(1:3)	6.2642	6.2958	6.4156	6.3979	6.4864	6.4623	-0.0034±0.0249abc
Pectin + BW	6.7149	6.7051	6.6636	6.6127	6.7379	6.6910	-0.0359±0.0185abc
Pectin + BW + E(1:1)	6.3901	6.3946	6.6866	6.6516	6.6138	6.5720	-0.0241±0.0204abc
Pectin + BW + E(1:3)	6.4525	6.4499	6.1753	6.1667	6.2528	6.2261	-0.0126±0.0102abc

Means in a column followed by the same letter are not significantly different.

Table B1. Continued.

Day 90							
Treatment	**Triplicate**						**% Change**
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	
Uncoated	6.7584	6.8059	6.8421	6.8854	6.4312	6.4833	0.0476±0.0036a
Pectin	6.3568	6.3879	6.6702	6.6966	6.6877	6.7348	0.0349±0.0089ab
Pectin + E(1:1)	6.6749	6.7132	6.3006	6.3044	6.0753	6.0737	0.0135±0.0177abc
Pectin + E(1:3)	6.6061	6.6442	6.0802	6.0820	6.5888	6.5793	0.0101±0.0203abc
Pectin + BW	6.2086	6.1886	6.6800	6.6731	6.6556	6.6070	-0.0252±0.0174abc
Pectin + BW + E(1:1)	6.3591	6.3660	6.9556	6.9301	6.9506	6.9309	-0.0128±0.0141abc
Pectin + BW + E(1:3)	6.4526	6.4521	6.3022	6.3078	6.6142	6.6136	0.0015±0.0029abc

Means in a column followed by the same letter are not significantly different.

Table B2. Uncoated and coated peroxide value over time.

Day 0				
	Triplicate			**Peroxide value (mEP/kg oil)**
Untreated (initial)	6.03	3.43	4.91	4.79±1.07a
Day 7				
	Triplicate			**Peroxide value (mEP/kg oil)**
Uncoated	3.10	5.54	3.80	4.15±1.03a
Pectin	4.78	3.75	2.61	3.71±0.89a
Pectin + E(1:1)	2.99	2.62	4.17	3.26±0.66a
Pectin + E(1:3)	3.82	5.95	3.22	4.33±1.17a
Pectin + BW	2.15	3.07	2.84	2.69±0.39a
Pectin + BW + E(1:1)	2.16	3.62	2.24	2.67±0.67a
Pectin + BW + E(1:3)	2.36	5.59	1.77	3.24±1.68a
Day 90				
	Triplicate			**Peroxide value (mEP/kg oil)**
Uncoated	5.31	3.05	3.54	3.97±0.97a
Pectin	6.01	1.94	3.63	3.86±1.67a
Pectin + E(1:1)	5.30	6.18	5.09	5.52±0.47a
Pectin + E(1:3)	3.52	1.21	7.71	4.15±2.69a
Pectin + BW	4.19	3.12	6.12	4.48±1.24a
Pectin + BW + E(1:1)	3.50	2.79	3.75	3.35±0.41a
Pectin + BW + E(1:3)	4.84	4.41	4.11	4.46±0.30a

BW: Beeswax; E: cranberry extract. Means in a column followed by the same letter are not significantly different.

Table B3. Fatty acid composition (area percentage) for coated and uncoated almonds.

		Oleic acid (C18:1)				Linoleic acid (C18:2)			
Day	**Treatment**	**Triplicate**			**%**	**Triplicate**			**%**
0	Untreated (initial)	64.96	68.06	68.04	67.02±1.46a	25.73	22.99	22.87	23.86±1.32a
7	Uncoated	68.30	63.63	67.65	66.53±2.07a	22.55	27.05	23.59	24.39±1.92a
	Pectin	64.80	60.27	65.21	63.43±2.24a	25.74	29.77	25.49	27.00±1.96a
	Pectin + E(1:1)	64.66	64.97	65.94	65.19±0.55a	26.05	26.17	25.00	25.74±0.53a
	Pectin + E(1:3)	67.27	63.37	67.30	65.98±1.85a	23.36	26.93	23.56	24.62±1.64a
	Pectin + BW	67.92	70.72	65.79	68.14±2.02a	22.76	20.40	25.21	22.79±1.96a
	Pectin + BW + E(1:1)	70.14	67.06	66.11	67.77±1.72a	21.23	23.74	24.96	23.31±1.55a
	Pectin + BW + E(1:3)	63.42	66.02	62.96	64.13±1.35a	27.48	24.20	27.23	26.30±1.49a
90	Uncoated	73.08	66.67	64.90	68.22±3.52a	18.39	23.98	25.91	22.76±3.19a
	Pectin	69.63	66.56	65.81	67.33±1.65a	21.00	23.97	24.82	23.26±1.64a
	Pectin + E(1:1)	68.74	63.60	68.94	67.09±2.47a	22.03	26.85	21.98	23.62±2.28a
	Pectin + E(1:3)	67.92	67.75	66.42	67.37±0.67a	22.99	23.45	24.09	23.51±0.45a
	Pectin + BW	66.07	67.71	67.75	67.17±0.78a	24.86	23.10	22.90	23.62±0.88a
	Pectin + BW + E(1:1)	66.37	67.95	65.03	66.45±1.19a	24.48	22.75	25.85	24.36±1.27a
	Pectin + BW + E(1:3)	64.17	66.69	66.23	65.70±1.09a	26.40	23.41	24.63	24.81±1.23a

Means in a column followed by the same letter are not significantly different.

Table B3. Continued.

Day		C16:0				C18:0				C18:1 11c			
	Treatment	**Triplicate**			**%**	**Triplicate**			**%**	**Triplicate**			**%**
0	Untreated (initial)	6.60	6.14	6.55	6.43±0.21a	1.45	1.38	1.09	1.30±0.15a	1.27	1.44	1.45	1.39±0.08ab
7	Uncoated	6.34	6.76	6.19	6.43±0.24a	1.38	1.09	1.13	1.20±0.13a	1.43	1.47	1.44	1.45±0.02ab
7	Pectin	6.68	7.27	6.61	6.85±0.30a	1.38	1.33	1.20	1.31±0.08a	1.40	1.35	1.49	1.41±0.06ab
7	Pectin + E(1:1)	6.52	6.28	6.49	6.43±0.11a	1.24	1.04	1.15	1.14±0.08a	1.53	1.54	1.43	1.50±0.05ab
7	Pectin + E(1:3)	6.62	6.70	6.44	6.59±0.11a	1.07	1.52	1.17	1.25±0.19a	1.69	1.48	1.52	1.56+0.09a
7	Pectin + BW	6.46	6.30	6.50	6.42±0.08a	1.44	0.96	1.06	1.15±0.20a	1.42	1.61	1.44	1.49±0.08ab
7	Pectin + BW + E(1:1)	5.98	6.53	6.37	6.29±0.23a	1.37	1.36	1.25	1.33±0.05a	1.29	1.31	1.31	1.30±0.01ab
7	Pectin + BW + E(1:3)	6.50	6.91	6.94	6.78±0.20a	1.17	1.49	1.40	1.35±0.14a	1.44	1.39	1.48	1.43±0.04ab
90	Uncoated	6.04	6.81	6.64	6.49±0.33a	1.16	1.13	0.98	1.09±0.08a	1.33	1.42	1.58	1.44±0.10ab
90	Pectin	6.49	6.62	6.56	6.56±0.05a	1.40	1.36	1.30	1.35±0.04a	1.49	1.50	1.51	1.50±0.01ab
90	Pectin + E(1:1)	6.38	6.95	6.49	6.61±0.25a	1.36	1.14	1.21	1.24±0.09a	1.49	1.46	1.38	1.44±0.04ab
90	Pectin + E(1:3)	6.47	6.22	6.73	6.47±0.21a	1.25	1.22	1.39	1.29±0.07a	1.37	1.36	1.38	1.37±0.01ab
90	Pectin + BW	6.29	6.37	6.65	6.44±0.15a	1.40	1.54	1.32	1.42±0.09a	1.38	1.27	1.39	1.35±0.05b
90	Pectin + BW + E(1:1)	6.46	6.54	6.39	6.46±0.06a	1.25	1.30	1.38	1.31±0.05a	1.44	1.46	1.36	1.42±0.05b
90	Pectin + BW + E(1:3)	6.73	7.04	6.49	6.75±0.22a	1.13	1.34	1.21	1.23±0.09a	1.57	1.52	1.44	1.51±0.05ab

Means in a column followed by the same letter are not significantly different.

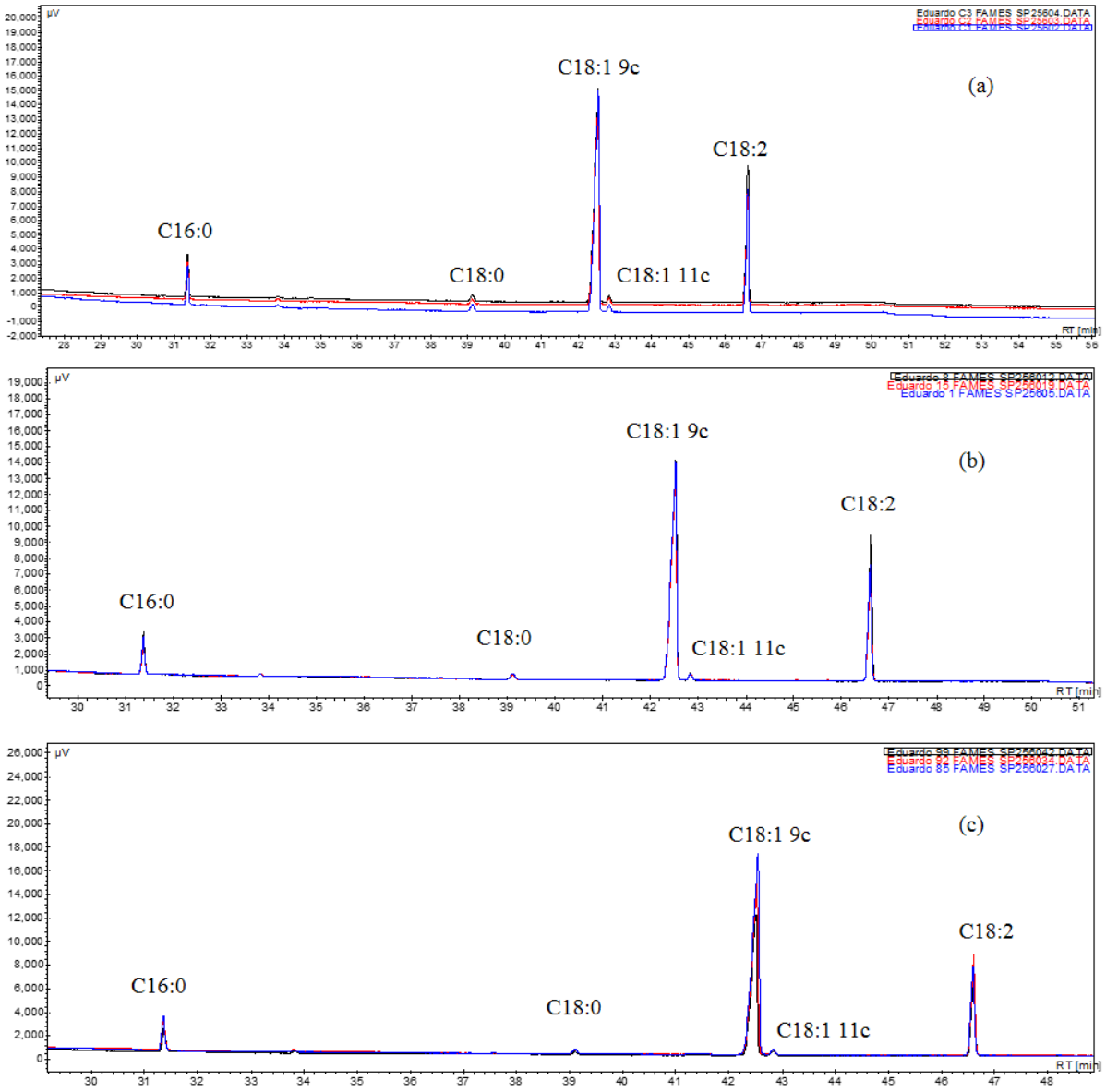

Figure B2. Chromatograms of uncoated almond fatty acids after storage at 40ºC and 50% RH at: (a) 0 day, (b) 7 days, and (c) 90 days.