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PHYTOCHEMICAL CHARACTERIZATION, ANTIOXIDANT CAPACITY AND IN VITRO INHIBITION OF ADIPOGENESIS AND INFLAMMATION OF PHENOLIC EXTRACTS IN VACCINIUM FLORIBUNDUM AND ARISTOTELIA CHILENSIS

BY

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THESIS

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ABSTRACT

Interest in berries from South America has increased due to their potential health benefits. The objective of this study was to characterize the anthocyanins and proanthocyanidins total phenolics and antioxidant capacity (AC) of *V. floribundum* and *A. chilensis*, and evaluate, *in vitro*, the ability of their phenolic extracts to reduce adipogenesis, and lipid accumulation in 3T3-L1 adipocytes. The anti-inflammatory properties of these extracts on RAW 264.7 macrophages was also investigated. The berry of *A. chilensis* contained 45.7 mg/g DW (cyanidin-3-glucososide (C3G) equivalents) total anthocyanins. Seven main anthocyanin structures were identified in this berry of which delphinidin-3-glucoside was the main anthocyanin component in this berry. The berry and the commercial powder of *V. floribundum* contained 10.6 mg/g DW and 2.4 mg/g (C3G equivalents) respectively of total anthocyanins. Five main anthocyanin structures where identified of which delphinidin-3- arabinose and cyanidin-3-arabinose were the main anthocyanins present in the berry and in the commercial powder of *V. floribundum*. The berry of *A. chilensis* contained 4.0 mg/g DW (epicatechin equivalents) of total proanthocyanidins, while the berries and commercial powder from *V. floribundum* had a slightly higher concentration of 5.2 and 4.8 mg/g DW (epicatechin equivalents) respectively. *A. chilensis* contained mainly proanthocyanidin dimers (56%) and trimers (14%). *V. floribundum* contained trimers (68%) and in less proportion pentamers (16%) and hexamers (8%). The commercial powder contained a larger percentage of dimers (34%) and trimers (23%). The berries of *A. chilensis* and *V. floribundum* showed similar values of total phenolics; however, AC was higher in the berries of *A. chilensis*. The commercial powder of *V. floribundum* had a lower phenolic content and AC than the

freeze dried berry. Total phenolics and AC were highly correlated for *A. chilensis* and both the berries and the commercial powder of *V. floribundum* with R^2 values of 0.90, 0.86 and 0.78, respectively. Anthocyanins were more highly correlated with AC than proanthocyanins. Phenolic extracts of the two berries and the commercial powder inhibited lipid accumulation by 4.0 to 10.8% when adipocytes were treated at maturity and by 5.9 to 37.9% when treated throughout differentiation. Furthermore, a proanthocyanidin-enriched-fraction from *V. floribundum* significantly induced Pref-1 expression in preadipocytes. Phenolic extracts decreased the production of nitric oxide (3.7 - 25.5%) and prostaglandin E_2 (9.1 - 89.1%) and the expression of inducible nitric oxide synthase (9.8 - 61.8 %) and cycloxygenase-2 (16.6 - 62.0%) in lipopolysaccharide-stimulated RAW 264.7 macrophages. *V. floribundum* and *A. chilensis* phytochemicals limit adipogenesis and inflammatory pathways *in vitro*, warranting further *in vivo* studies.

To my family and friends

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CHAPTER 1
INTRODUCTION

Berries and their derived products have shown a positive impact on metabolic syndrome related conditions, including cardiovascular diseases, diabetes and inflammation (Juranic 2005, Seeram 2008). Their biological properties have been largely attributed to high levels of various phenolic compounds, as well as to the interactive synergies between their natural phytochemical components (Szajdek and Borowska 2008). Interest in berries from South America has increased in recent years mainly due to their potential health benefits, and growing consumer interest in novel exotic fruit selections in the market place.

Aristotelia chilensis is a fruit-bearing shrub that thrives in the temperate forests of central to southern Chile and western Argentina. It belongs to the Elaeocarpaceae family and is commonly known as "maqui." *A. chilensis* yields a small edible purple/black berry averaging 5 mm in diameter with typically 3-4 seeds. The leaves and fruits of *A. chilensis* have been used in folk medicine to treat a variety of ailments including sore throat, kidney pains, ulcers, fever, inflammation and diarrhea (Hoffmann 1991, Muñoz and others 1981). Reports concerning the phytochemical composition of the berry of *A. chilensis* have indicated the presence of phenolic acids, proanthocyanidins, anthocyanins as well as other flavonoids (Escribano-Bailon and others 2006, Céspedes and others 2010). The berry of *A. chilensis* has been shown to inhibit low-density lipoprotein (LDL) oxidation, to protect against intracellular oxidative stress in human endothelial cells and against acute isochemia/reperfusion *in vivo* in rat hearts (Miranda-Rottmann and others 2002, Céspedes and others 2008).

Vaccinium floribundum, commonly known as "mortiño," is a deciduous, spreading shrub that belongs to the family Ericaceae. It bears a round blue to nearly black edible berry of about 8 mm in diameter. This berry is found mostly in northern South America where it grows at elevations from 1800 to 3800 m (National Research Council 1989). In Ecuador, local communities have used this plant to treat various medical conditions such as diabetes and inflammation (de la Torre and others 2008), The chemical composition and the phenolic profile of *V. floribundum* has been reported, revealing predominantly quercetin, hydroxycinnamic acids and cyanidin-3-glucosides (Vasco and others 2009). There are no reports concerning the biological properties of this berry.

Within recent decades, the incidence of obesity has increased drastically (Ogden and others 2006) and has become a worldwide health concern due to its association with an increased risk of morbidity and mortality (Flegal 2005, Guh and others 2009). Obesity is a complex metabolic disorder that results from an imbalanced energy intake and energy expenditure leading to an increase in adipocyte size and number (Hirsh and Han 1969). The amount of adipose tissue can be reduced by inhibiting adipogenesis and fat deposition (Rayalam and others 2008). Adipogenesis is the cellular transition through which a fibroblastic cell first develops into a preadipocyte and finally into a mature adipocyte (Fernyhough and others 2005). Obesity is also associated with a chronic inflammatory state characterized by abnormal production of cytokines and the activation of inflammatory signaling pathways (Bastard and others 2006). Furthermore, chronic inflammation plays a crucial role in the development of metabolic disorders linked to obesity, including insulin resistance and arteriosclerosis (Arkan and others 2005, Van

Gaal and others 2006). Two important enzymes involved in activating the inflammatory response are inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2). iNOS and COX-2 can catalyze the synthesis of nitric oxide (NO) and prostaglandin E_2 (PG E_2) respectively, which in turn cause sepsis, sepsis shock, and systemic inflammatory response syndrome (Guha 2001). Therefore, the inhibition of the expression of these enzymes or of their products can help reduce inflammation and related conditions.

Berries are particularly rich sources of anthocyanins and proanthocyanidins which may help to ameliorate conditions related to obesity and inflammation. Studies have shown that proanthocyanidins can regulate adipocyte function and positively affect obesity management (Vogels and others 2004, Tebib 1996) as well inflammation (Terra and others 2009, Wang and others 2009). Anthocyanins have also shown to prevent the onset of obesity (Tsuda and others 2003, Prior and others 2009) and reduce inflammatory response (Wang and others 2008, Kraft and others 2008). The bioactive properties exerted by anthocyanins and proanthocyanidins have been attributed to diverse mechanisms of action including antioxidant capacity (Nijveldt and others 2001).

In this study, we investigated the *in vitro* antioxidant capacity (AC) of *V. floribundum* and *A. chilensis* berry extracts, characterized their anthocyanin and proanthocyanidin constituents, and evaluated the ability of their phenolic fractions to reduce adipogenesis, and lipid accumulation in 3T3-L1 adipocytes. In addition, the anti-inflammatory properties were evaluated *in vitro* using lipopolysaccharide-stimulated (LPS) RAW 264.7 macrophages by investigating the NO and PGE_2 production as well as iNOS and COX-2 expression. A commercial powder of *V. floribundum* was also evaluated and compared to the freeze dried berry extracts.

CHAPTER 2
LITERATURE REVIEW

I. Chemistry and biological properties of berries

A. Phenolic composition of berries

Berries contain micro and macronutrients such as sugars, vitamins and minerals as well as a broad diversity of secondary metabolites (Howard, Hager 2007). Among the latter, great interest has been shown towards phenolic compounds due to the various biological properties they have demonstrated. Anthocyanins and proanthocyanidins are two phenolic compounds present in berries that play an important role in the health related properties of these fruits (Juranic 2005, Seeram 2008).

Anthocyanins

Anthocyanins are a class of flavonoid compounds that are responsible for the red, orange, purple and blue colors in many fruits and vegetables. They are involved in the protection of plants against excessive light and microbial attack. The ability of anthocyanins to impart color is also important in attracting animals for pollination and seed dispersal (Kong and others 2003, Crozier and others 2006). These water soluble pigments are classified as flavonoids because of their $C_6C_3C_6$ carbon skeleton (**Figure 1**). Anthocyanidins are primarily present in plants joined to sugar moieties, where they are known as anthocyanins. The most common anthocyanidin structures are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. These anthocyanidins get their names according to what R_1 and R_2 substitutions are on the B-ring (Castaneda-Ovando and others 2009) (**Table 1**). Berries are some of the richest dietary sources of anthocyanins for humans having as high as 1480 mg of anthocyanins/100g fresh weight

(Wu 2004). Anthocyanins have shown to possess several biological properties such as antioxidant, anticancer and anti-inflammatory properties (Kong and others 2003)

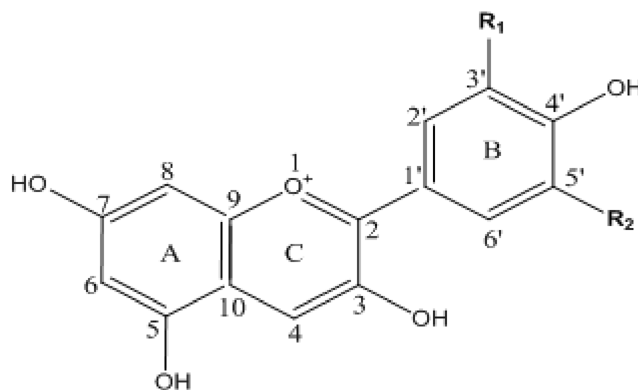


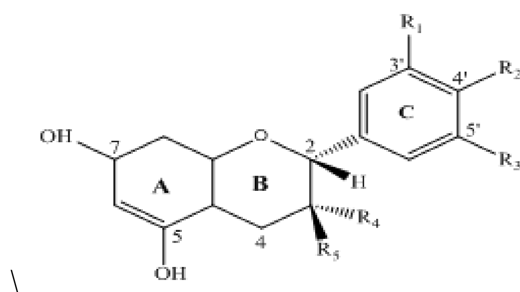
Figure 1. Basic chemical structure of an anthocyanidin.

Table 1. Chemical structure and color of the six common anthocyanidins (adapted from Seeram 2008).

Anthocyanidin	Abbreviation	R_1	R_2	Color
Pelargonidin	Pg	H	H	Orange
Cyanidin	Cy	OH	H	Orange-red
Peonidin	Pn	OCH3	H	Orange-red
Delphinidin	Dp	OH	OH	Red
Petunidin	Pt	OCH3	OH	Red
Malvidin	Mv	OCH3	OCH3	Bluish-red

Proanthocyanidins

Proanthocyanidins or condensed tannins are the second most abundant compounds after lignin in plants. They are mainly found in berries, beans, nuts, cocoa and wine and are responsible for the astringent character and bitterness of many of these foods (Rasmussen and others 2005). Proanthocyanidins oligomers and polymers are built with monomeric flavan-3-ols units. Oligomers are comprised of two to six monomeric units, while polymers can comprise up to 50 units. Monomeric flavan-3-ol units are linked through C4→C8 or through C4→C6 bonds; these are known as B-type bonds. In some cases an additional ether bond occurs between C2→C7 which is called an A-type linkage (Hummer and Schreier 2008). The most common flavan-3-ol monomeric units are (+) –catechin/(–)-epicatechin, (–)-gallocathechin/ (–)(epi)gallocathechin and (+)-afzelechin/(–)epiafzelechin, which form oligomers and polymers called procyanidins, prodelphinidins and propelargonidins respectively (Serrano and others 2009). **Figure 2** illustrates the structures of some flavan-3-ol monomeric units of proanthocyanidins.



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Flavan-3-ols	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**
Afzelechin	H	OH	H	H	OH
Epiafzelechin	H	OH	H	OH	H
Catechin	H	OH	OH	H	OH
Epicatechin	H	OH	OH	OH	H

Figure 2. Structures of flavan-3-ol monomeric units of proanthocyanidins.

Proanthocyanidins are widely distributed among many edible plants which makes them important in human diet (**Table 2).** The daily average intake of proanthocyanidins in the USA is about 53.6 mg/day excluding monomers and 57.7 mg/day including monomers (Gu and others 2004). Proanthocyanidins have shown to posses several biological properties that can potentially protect humans against cardiovascular disease, diabetes and cancer (Rasmussen and others 2005, Serrano and others 2009).

Table 2. Content of proanthocyanidins in common foods

Food	**Total**[a]
Blueberries	179.8 ± 50.8
Black-currants	147.8 ± 33.0
Cranberries	418.8 ± 75.3
Strawberries	145.0 ± 24.9
Apples	125.8 ± 6.8
Pears	31.9 ± 7.8
Plums	215.9 ± 50.7
Peaches	67.3 ± 20.9
Avocados	7.4 ± 4.3
Barley	74.2 ± 3.0
Pinto beans, raw	796.3 ± 58.7
Red kidney beans	563.8 ± 10.4
Pistachios	237.3 ± 52.0
Almonds	184.0 ± 48.2
Walnuts	67.3 ± 14.7
Peanuts	15.6 ± 2.3
Peanut butter	13.2 ± 5.2
Milk chocolate	246.0 ± 0.0
Black chocolate	192.0 ± 28.8
Beer	23.0 ± 2.0
Red wine	313.0 ± 5.0

[a]mg/100 fresh weight foods or mg/L beverages
(adapted from Rasmussen and others 2005).

B. Biological activities of berries and their phytochemicals

Antioxidant capacity

Reactive oxygen species (ROS) such as super oxide ion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), are produced in our body (Kaur and Kapoor 2001). These species react with and cause damage to lipids, proteins and DNA.
The damage to these macromolecules is in turn associated with increased risk of chronic diseases such as cancer and cardiovascular diseases. The human body posess different endogenous defense mechanisms that scavenge radicals and repair the oxidative damage. A diet rich in antioxidants can further contribute to this defense and aid in the prevention of several chronic conditions (Seifried and others 2007, Gutteridge 1993). Fruits and vegetables are a major source antioxidants. Some of the components in fruits and vegetables acting as antioxidant are phenolic compounds, fiber, and vitamins A, B, C, D (Kaur and Kapoor 2001). Among fruits, berries possess a high antioxidant capacity and this has been largely attributed to their high content of various phenolic compounds, vitamins and fiber (Li and others 2009). Among phenolic compounds in berries anthocyanins (Einbond and others 2004, Du and others 2008) and proanthocyanidins (Qa'Dan and others 2006) have shown to have strong antioxidant properties.

Obesity and inflammation

The incidence of obesity has increased drastically during the last decades (Ogden and others 2006) and is a worldwide health concern because it is associated with an increased risk of morbidity and mortality (Flegal 2005, Guh and others 2009). Obesity is a complex metabolic disorder that results from an imbalanced energy intake and energy expenditure leading to an increase in adipocyte size and number (Hirsch and Han 1969).

Adipose mass can increase when adipocytes increase in size due to higher triglyceride deposition. In addition, an increase in adipocyte number, arising from differentiation of precursor cells into adipocytes or adipogenesis, can also result in an increase in adipose mass. Therefore amount of adipose tissue can be reduced by inhibiting adipogenesis and fat deposition (Rayalam and others 2008).

Adipogenesis is a highly regulated cellular transition through which a pluripotent mesenchimal cell first develops into a preadipocyte and finally into a mature adipocyte (Fernyhough and others 2005) (**Figure 3**).

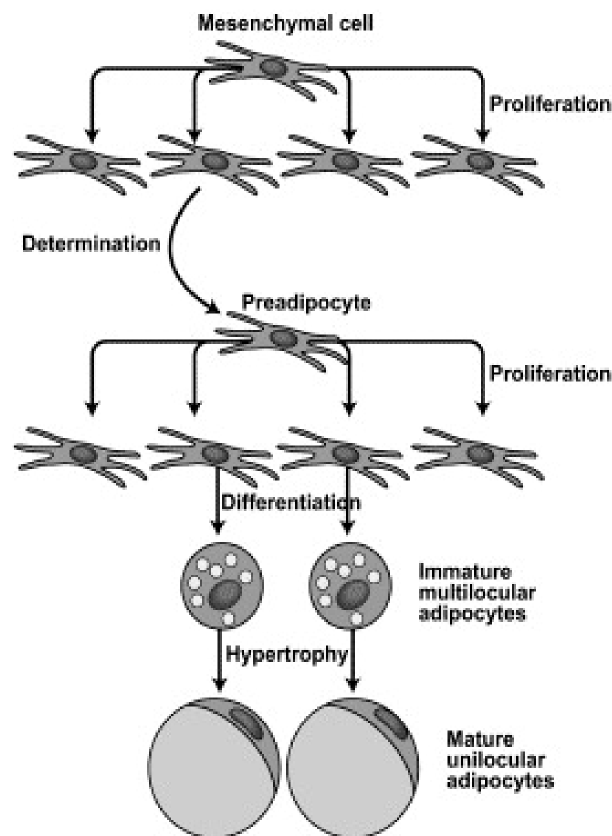

Figure 3. Differentiation of adipocytes from mesenchymal cells (taken from Avram and others 2007)**.**

Several inhibitors of adipogenesis have been reported and these include extracellular as well as intracellular molecules such as Preadipocyte factor-1 (Pref-1) (Harp 2004). Pref-1 also known as Delta-like protein 1 (Dlk1) is an epidermal growth factor –repeat containing transmembrane protein of 385 amino acids. This protein, upon cleavage by TNF-α converting enzyme (TACE), produces a 50 kD soluble form that inhibits adipogenesis through the activation of MAPK kinase/ERK pathway. By activating the MAPK kinase/ERK pathway, Pref-1 soluble form prevents the down regulation of the transcription factor SRY (sex determining region Y) – box 9 (Sox9), which is necessary for the expression CCAAT enhancer binding protein (C/EBP) β and δ. The expression of C/EBP β and δ is necessary to start the differentiation the process. Pref-1 is highly expressed in preadipocytes but decreases during differentiation process and is absent in mature adipocytes. Pref-1 reflects the degree of adipocyte differentiation *in vitro* and *in vivo* and therefore constitutes a good marker for adipogenesis (Sul 2009).

Obesity is associated with a chronic inflammatory state characterized by abnormal production of cytokines and other pro-inflammatory mediators and the activation of inflammatory signaling pathways (Bastard and others 2006). Furthermore, chronic inflammation plays a crucial role in the development of metabolic disorders linked to obesity, including insulin resistance and arteriosclerosis (Arkan and others 2005, Van Gaal and others 2006). In these inflammatory related diseases, macrophages secrete a variety of pro-inflammatory cytokines, pro-inflammatory enzymes and other inflammatory mediators. Two important enzymes involved in activating the inflammatory response are inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. iNOS and COX-2 can catalyze the synthesis of nitric oxide (NO) and prostaglandin E_2 (PG E_2)

respectively, which in turn can cause sepsis, sepsis shock and systemic inflammatory response syndrome (Guha 2001) (**Figure 4**). Therefore the inhibition of the expression of these enzymes or of their products can help to reduce inflammation and related conditions.

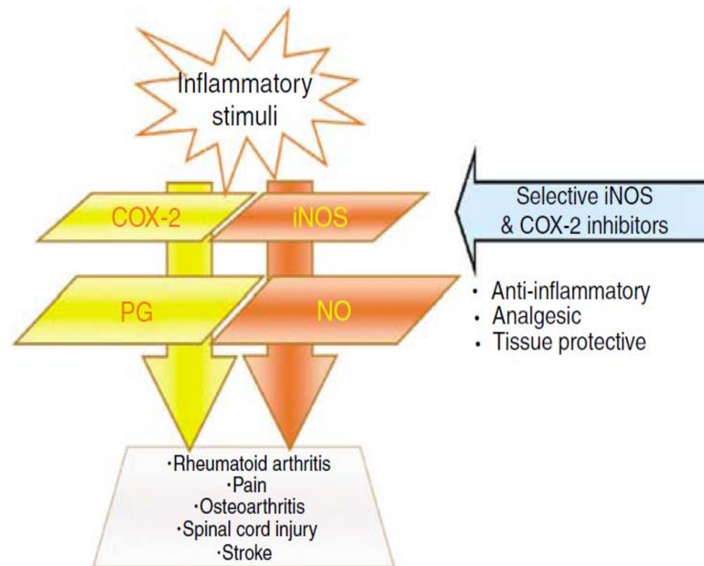

Figure 4. Role of iNOS and COX-2 in inflammation (taken from Cuzzocrea 2007).

Potential of berries to ameliorate obesity and inflammation

The consumption of berries has been shown to ameliorate several parameters related inflammation (Lehtonen 2010, DeFuria and others 2009, Larmo and others 2008) and obesity (Ruel and others 2005). Furthermore anthocyanins and proanthocyanidins, which are abundant in berries, have been shown to be beneficial for both conditions. Several studies *in vivo* have indicated that the consumption of proanthocyanidin- rich extracts is associated with a reduction in food intake and body weight (Vogels and others 2004, Tebib 1996) and with a modification in lipid profile and lipid metabolism (Tebib, and others 1994, Zern and others 2003, Del Bas and others 2004, Sugiyama and others 2007). Furthermore, it has been shown that proanthocyanidins can modulate adipose

differentiation *in vitro* (Pinent and others 2005b) and *in vivo* (Montagut and others 2007) and also to stimulate lipolysis in 3T3-L1 adipocytes (Pinent and others 2005a, Hasegawa 1999, Mochizuki, Hasegawa 2004). Consumption of anthocyanins has also prevented the onset of obesity in several animal studies (Prior 2008, Kwon and others 2007). In other reports, anthocyanins regulated adipocyte function by affecting adipocyte gene expression and adipocytokine expression (Tsuda and others 2005, Jayaprakasam and others, 2006, Tsuda and others 2004, Tsuda and others 2006). In addition anthocyanins and proanthocyanidins have been shown to possess anti inflammatory properties. Several studies have shown that anthocyanins reduce the levels of inflammatory mediators *in vitro* (Wang and others 2008, Kraft and others 2008, Pergola and others 2006, Tsoyi and others 2008) and *in vivo* (Park and others 2007, Shan and others 2009). Proanthocyanidins can also reduce inflammation by modulating cytokine expression, inhibiting pro-inflammatory enzymes, or other mechanisms (Terra and others 2009, Wang and others 2009, Ho and others 2007, Diouf, and others 2009). The bioactive properties exerted by anthocyanins and proanthocyanidins have been attributed to diverse mechanisms of action including antioxidant capacity, interaction with enzyme systems or other proteins, or others (Nijveldt and others 2001).

II. Berries from South America

International interest in berries found in South America has increased in recent years mainly due to their potential health benefits and increasing consumer interest in novel exotic fruit selections in the market place. The broad diversity of berry-producing plants native to this area includes species such as *Aristotelia chilensis* (Maqui), *Euterpe*

oleracea (Açaí), *Malpighia emarginata* (Acerola), *Ugni molinae* (Murta), *Fragaria chiloensis* (Frutilla), *Rubus glaucus* (Mora) and *Vaccinium floribundum* (Mortiño).

A. ***Aristotelia chilensis***

Ethnobotany and botanical description

Aristotelia chilensis is a dioecious fruit-bearing shrub that thrives in the temperate forests of central to southern Chile and western Argentina (Hoffmann 1991, Muñoz and others 1981) (**Figure 5A**). It belongs to the Elaeocarpaceae family and is commonly known as "maqui," "clon," "queldron" and "koelon" (Suwalsky and others. 2008). It is an evergreen shrub that may grow in "macales," or dense thickets, reaching 3-5 m in height (Hoffmann 1991). Maqui typically grows in moist, well-drained soils in either high or low light environments, but has consistently shown higher survival rates with exposure to high light (Lusk, Del Pozo 2002). *A. chilensis* yields a small edible purple/black berry averaging 5 mm in diameter and typically 3-4 seeds (Hoffmann 1991) (**Figure 5B**).

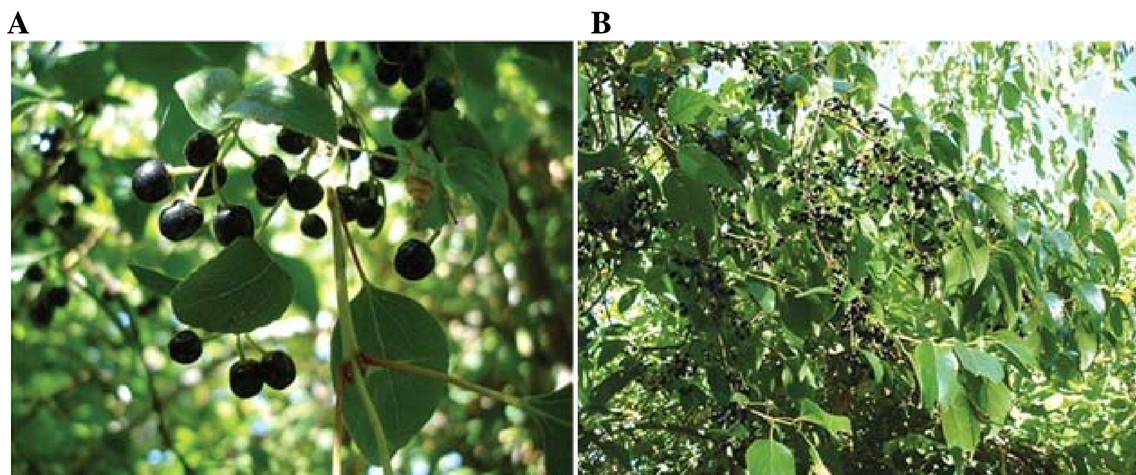

Figure 5. Photographs showing the berries (**A**) and the shrub (**B**) of *Aristotelia chilensis*. Reproduced courtesy of Guillermo Moreno Crisóstomo (http://www.nublenaturaleza.cl/articulos/flora/arboes/el-maqui/).

The leaves and fruits of *A. chilensis* have been used in folk medicine to treat a variety of ailments including sore throat, kidney pains, ulcers, fever, hemorrhoids, inflammation, diarrhea, lesions, migraines, and scars (Hoffmann 1991).

Phytochemistry

There are several reports concerning the chemical composition of *A. chilensis* (**Table 3**) that have indicated the presence of indole and quinoline alkaloids within leaf tissue (Watson and others 1989) and high levels of polyphenols in the berry (Miranda-Rottmann and others 2002, Céspedes and others 2008).

Table 3. Phytochemicals detected in leaves and berries of *Aristotelia chilensis*

Phytochemical constituent	Plant part used	References
• Alkaloids 8-oxo-9-dehydrohobartine 8-oxo-9-dehydromakomakine Aristone Aristotelinine Aristotelone Aristoteline	Leaves	Watson and others 1989 Bittner and others 1978 Gopalakrishna and others 1978 Bhakuni and others 1976
• Phenolics Delphinidin-3-sambubioside-5-glucoside Delphinidin-3,5-diglucoside Cyanidin-3- sambubioside -5-glucoside Cyanidin-3- sambubioside -5-glucoside Cyanidin-3,5,-diglucoside Delphinidin-3- sambubioside Delphinidin-3-glucoside Cyanidin-3- sambubioside Cyanidin-3-glucoside	Berries	Escribano-Bailon and others 2006, Céspedes and others 2010

The phytochemical profile of *A. chilensis* fruits has been reported, revealing mainly phenolic acids, proanthocyanidins, anthocyanins among other flavonoids (Escribano-Bailon and others 2006, Céspedes and others 2010).

Biological Properties

The berries and leaves of *A. chilensis* have demonstrated diverse biological properties that are mainly attributed to their rich phenolic content and antioxidant capacity (Miranda-Rottmann and others 2002, Céspedes and others 2010, Céspedes and others 2008). For instance, the maqui berry juice inhibits low-density lipoprotein (LDL) oxidation and protects human endothelial cells against intracellular oxidative stress, thus suggesting possible antiatherogenic properties (Miranda-Rottmann and others 2002) . Methanol extracts of mature maqui berries have also exhibited a protective effect against acute isochemia/reperfusion when performed *in vivo* in rat hearts. The fruit extracts likely prevented these harmful effects by reducing lipid oxidation and the concentration of thiobarbituric acid reactive substances (TBARS) (Céspedes and others 2008). Extracts from the maqui leaves have also shown potential nematicidal (Insunza and others 2001) and antiviral activities (Pacheco and others 1993) . Furthermore, aqueous extracts of *A. chilensis* leaves can induce alteration in human erythrocyte morphology by interacting with the membrane's outer phospholipid monolayer. These results suggest that the molecular mechanism of action of *A. chilensis* leaf extracts can be attributed to functional perturbation of cell membrane lipid bilayers (Suwalsky and others 2008). **Table 4** shows a summary of all the studies that have looked at the biological activity of the fruit and leaves of *A. chilensis*. In addition, the *A. chilensis* berries exhibit relatively high mineral levels; 100 g of maqui berries yield 27% recommended daily allowance (RDA) for

calcium, 28% RDA for potassium, and 70% RDA for iron; contrarily the plant exhibits low sodium levels. Thus, *A. chilensis* shows potential for use in dietary supplements or functional foods (Damascos and others 2008).

Table 4. Biological activities of leaves and berries of *Aristotelia chilensis.*

Part used	Biological Property	Model	Dose/ Effectiveness	Reference
Fruit	Cardio protective effect	Male Wistar Rats	Single dose methanol extract (10 mg kg^{-1})	Céspedes and others 2008
	Antioxidant activity	Human umbilical vein cells	10 µM GAE juice	Miranda-Rottmann and others 2002
		Human LDL from lipidemic blood donors	1 µM GAE juice	
Leaves	Nematicidal activity	*Xiphinema americanum sensu lato*	25 % 1 : 4 W/V aqueous extract	Insunza, and others 2001
	Anti viral activity	Herpes virus hominis type 2	IC_{50} 40 µg/mL hydroalcoholic extract	Pacheco and others 1993
	Alteration of human erythrocytes morphology	Human erythrocytes	1 mM GAE aqueous extract	Suwalsky and others 2008

IC_{50}, 50% inhibitory concentration; LDL, low-density lipoprotein; GAE, gallic acid equivalent.

Commercial use

A. chilensis is a widespread plant that is regionally collected and typically consumed fresh or used to make jam, tea, wine and juice (Damascos and others 2008). In addition it is commonly used as a natural dye due to its high content in anthocyanin pigments (Hoffmann 1991). Recently, the use of this plant has outgrown its traditional

borders, reaching new areas of commercialization. For instance, a topical formulation containing maqui berry extracts, with a high oxygen radical absorbance capacity (ORAC), has been developed and patented for the prevention and treatment of skin damage, particularly damage resulting from aging and sun exposure. Furthermore, functional beverages and dietary supplements containing Maqui berry extracts and featuring a patent pending Maqui Superberry™ are available in the market.

B. ***Vaccinium floribundum***

Ethnobotany and botanical description

Vaccinium floribundum is a deciduous, spreading shrub that belongs to the family Ericaceae (**Figure 6A**). It bears cylindrical pink flowers and round blue to nearly black edible berries (**Figure 6B**). This species is native to Ecuador and Peru but is also found in other countries in South and Central America (National Research Council 1989).

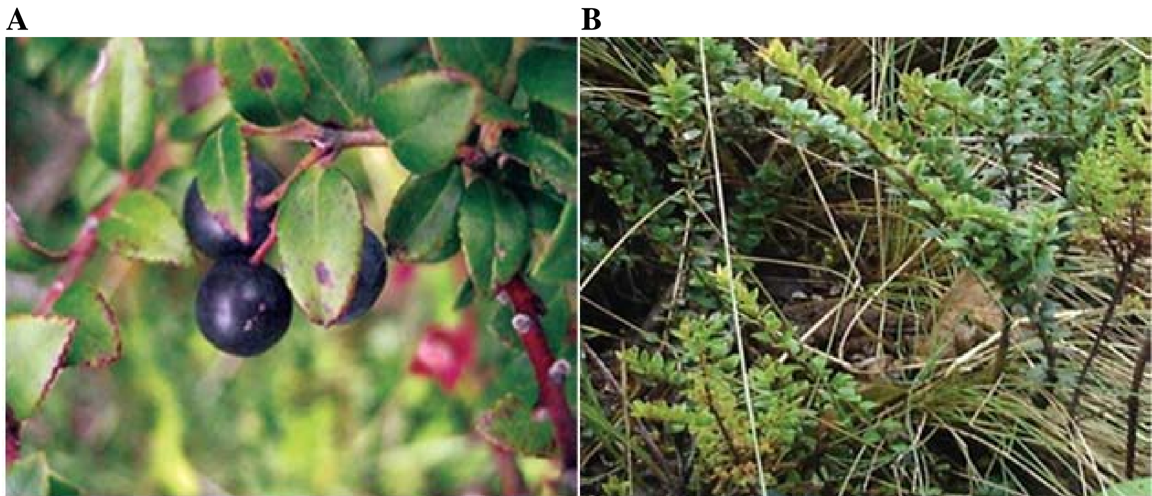

Figure 6. Photographs showing the berries (**A**) and the shrub (**B**) of *Vaccinium floribundum*.

V. floribundum berries are widely consumed in Ecuador as the fresh fruit or as processed products. Local communities also have used this plant to treat various medical conditions such as diabetes and inflammation (de la Torre L and others 2008).

Phytochemistry

The chemical composition and the phenolic profile of *V. floribundum* has been reported revealing predominantly quercetin, hydroxycinnamic acids and cyanidin-3-glucosides. Anthocyanins were shown to account for ~67% (345 mg cyanidin/100 g FW) of the total phenolic compounds with cyanidin derivatives the major components (~89%). Among the hydroxinnamic acids, chlorogenic and neochlorogenic together with caffeic and ferrulic acids were the predominant components. In addition, the total soluble phenolic content and the antioxidant capacity were reported as 882 mg gallic acid equivalent (GAE)/100 g fresh weight (FW) and 1200 mg Trolox equivalent/100 g FW, respectively (Vasco and others 2009).

Commercial use

V. floribundum does not currently exhibit widespread commercial use. Typically, it is gathered and sold in local marketplaces for raw consumption and small-scale processing. Jams, jellies and powders are prepared from the berries (de la Torre and others 2008). Although this plant and its derived products are primarily sold locally, it has shown promise for future commercial growth.

CHAPTER 3
SIGNIFICANCE OF RESEARCH

The incidence of obesity has increased drastically in the last decade and has become a worldwide health concern due to its association with an increased risk of morbidity and mortality. Obesity is closely associated with a chronic inflammatory state and this has been shown to play a crucial role in the development of metabolic disorders linked to obesity, including insulin resistance and arteriosclerosis. Research continues to focus on the role of diet, specifically in fruits and vegetables and their phytochemicals, as a preventive strategy that may be beneficial to treat health related conditions. *A. chilensis* and *V. floribundum* berries have been used in folk medicine to treat a variety of ailments. In addition, these two berries have been shown to contain a broad range of phenolic compounds, which have been associated with health beneficial properties. *A. chilensis* and *V. floribundum berries,* as well as their derived products have shown to have the potential to aid in the management of obesity and to ameliorate inflammation. This study provides the phytochemical characterization of *V. floribundum* and *A. chilensis* berries and investigates for the first time the ability of these berries and of their phytochemicals to reduce *in vitro* inflammation and obesity biomarkers.

CHAPTER 4
HYPOTHESIS AND OBJECTIVES

Hypothesis

V. floribundum and *A. chilensis* phenolic extracts have the potential to reduce inflammation and to inhibit adipogenesis and lipid accumulation.

Main objective

To characterize the anthocyanin and proanthocyanidin constituents, to investigate the antioxidant capacity (AC) of *V. floribundum* and *A. chilensis* berry extracts, and to evaluate the ability of their phenolic fractions to reduce adipogenesis, lipid accumulation and inflammation in *vitro*.

Specific objectives

1. To quantify and characterize the anthocyanin and proanthocyanidin constituents of *V. floribundum* and *A. chilensis.*
2. To determine the antioxidant capacity of *V. floribundum, A. chilensis,* and of their phenolic extracts.
3. To determine the effect of *V. floribundum* and *A. chilensis* phenolic extracts on adipogenesis by measuring lipid accumulation and Pref-1 expression *in vitro*.
4. To determine the effect of *V. floribundum* and *A. chilensis* phenolic extracts on lipid metabolism by measuring lipid accumulation *in vitro.*
5. To determine the effect of *V. floribundum* and *A. chilensis* phenolic extracts on NO and PGE_2 production and iNOS and COX-2 expression.

CHAPTER 5
MATERIALS AND METHODS

I. Materials

Ripe *Aristotelia chilensis* berries were collected in January 2009 from the Entrelagos region in Chile (S 40º 40' 48,5"/ W 72º 33' 43,3") (**Figure 7A**). Ripe *Vaccinium floribundum* berries were collected during late November 2008 in the grasslands of Simiatug, Ecuador (**Figure 7B**). The berries were cleaned by removing leaves, stems and damaged berries. The whole berries were freeze-dried, sealed in plastic bags, and shipped to our laboratory. In addition to the collected berries, a *V. floribundum* commercial powder (mortiño***;*** a natural product produced by Simiatug Samai) was purchased from a local market in Quito, Ecuador (**Figure 7C**). This powder was prepared by dehydrating fresh berry at low temperatures (< 45 ºC) and high ventilation. The berries and the commercial powder were stored at -80 ºC until usage.

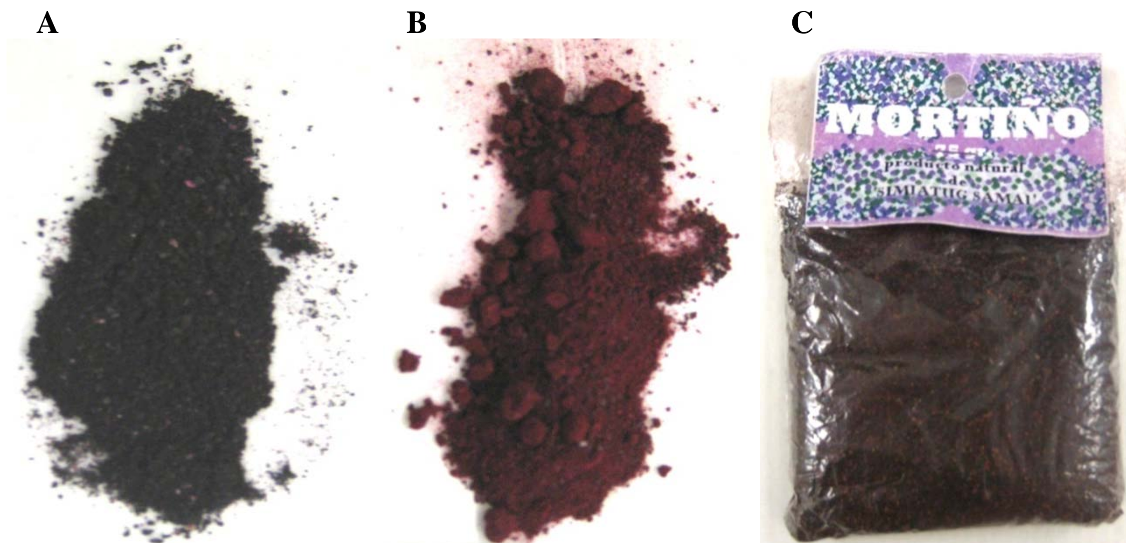

Figure 7. Photograph showing the freeze-dried *Aristotelia chilensis* (**A**) and *Vaccinium floribundum* (**B**) and the commercial powder of *Vaccinium floribundum* (**C**).

Swiss albino mouse 3T3-L1 fibroblasts, macrophage RAW 264.7 cell line and DMEM were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). Isobutylmethylxanthine (IBMX), dexamethasone (DEX), insulin, sodium pyruvate solution, penicillin (1000 U/mL), streptomycin (1000 U/ mL), sodium nitrite, sulfanilamide, N-1-(naphthyl)ethylenediamine- diHCl, LPS from *Escherichia coli* O55:B5, Trolox, Folin-Ciocalteu's phenol reagent, 2,2-azobis 2-amidinopropane dihydrochloride (AAPH), Amberlite XAD-7 resin, Oil Red O, C75 (≥ 98% purity), and epigallocatechin gallate (EGCG ≥ 95% purity) were purchased from Sigma–Aldrich (St. Louis, MO). Cyanidin-3-glucoside standard (≥ 97% purity) was purchased from Polyphenols Laboratories AS (Sandnes, Norway). Sephadex LH-20 was purchased from GE Life Sciences (Buckinghamshire, UK). Actin mouse mAb epitope mapping at the C-terminus of actin of human origin, COX-2 mouse mAb against amino acids 580 – 598 of human COX-2 and inducible iNOS mouse mAb epitope mapping at the C-terminus of mouse iNOS and DLK goat pAb epitope mapping at the C-terminus of human pref-1/DLK-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse IgG, horseradish peroxidase conjugate secondary antibody was purchased from GE Healthcare (Buckinghamshire, UK).

II. Methods

The complete experimental design of this study, including the preparation of the phenolic extracts, the phytochemical analysis and the biological assays is illustrated in **Figure 8.**

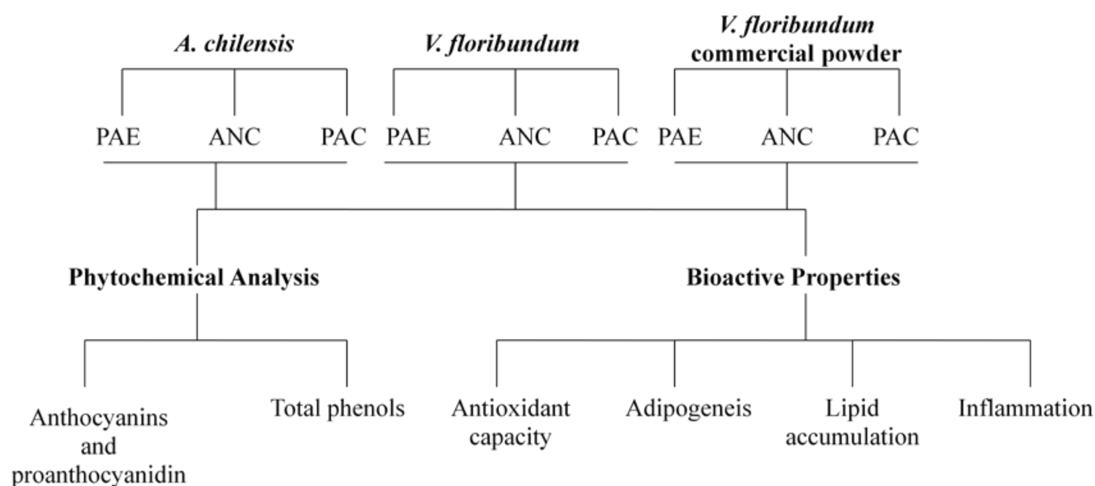

Figure 8. Experimental design. Abbreviations: PAE, post-amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction.

A. Preparation of phenolic-rich extracts and fractions

The process used to prepare the phenolic extracts and fractions is illustrated in **Figure 9**

Preparation of the phenolic-rich extract

Berry extraction and fractionation were performed based on procedures developed by Grace and others (2009). The freeze dried berries and the commercial powder were blended with 80% ethanol acidified with 0.3% trifluoroacetic acid (TFA). The extract was then filtered through cotton, followed by Whatman # 4 then #1 filter paper with the aid of suction. The collected hydro-alcoholic extract was evaporated using a rotary evaporator at a temperature not exceeding 40 °C. The aqueous concentrate was partitioned with ethyl acetate (4 × 500 mL) to remove lipophilic material. The aqueous layer was retained and loaded onto an Amberlite XAD-7 column (30 × 10 cm) preconditioned with acidified water, (0.3% TFA). The resin was washed thoroughly with

acidified water (0.3% TFA, ~3 L) to remove free sugars, pectins, and phenolic acids. The polyphenolic mixture was then eluted with acidified ethanol (0.3% TFA), evaporated, and freeze-dried to yield post-amberlite extract (PAE).

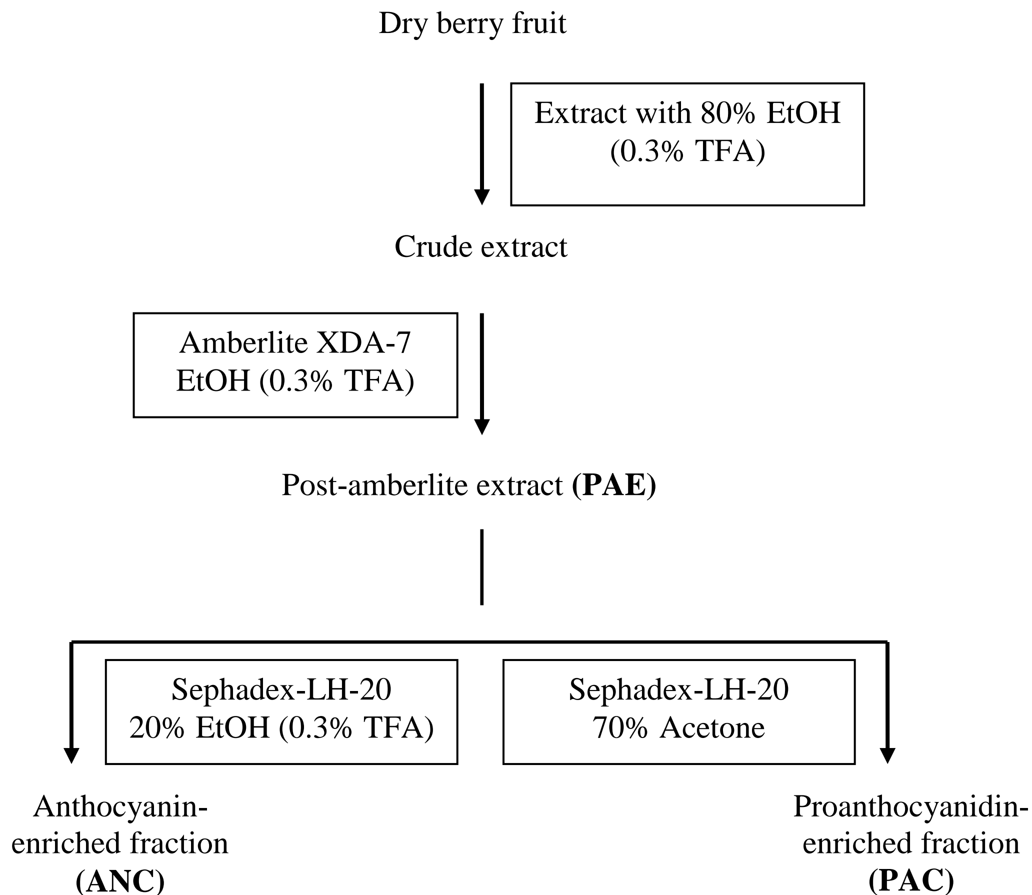

Figure 9. Flow chart showing extraction and anthocyanin and proanthocyanidin enrichment process. Abbreviations: PAE, post-amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction. Adapted from Grace and others 2009.

Preparation of anthocyanin and proanthocyanidin-enriched fractions

The enriched fractions were prepared by placing 2 g of the PAE on a Sephadex LH-20 column (30 × 3 cm). Anthocyanins were obtained from an isocratic elution of

20% aqueous ethanol acidified with 0.3% TFA. Two anthocyanin fractions of 200 ml each were collected for the two berries and the commercial powder. The column was then washed with 70% aqueous acetone to elute polymeric proanthocyanidins. Two proanthocyanidins fractions of 200 ml each were collected for the two berries and the commercial powder. All fractions were concentrated and freeze-dried to yield an anthocyanin-enriched-fraction 1 (ANC 1), anthocyanin-enriched-fraction 2 (ANC 2), proanthocyanidin-enriched-fraction 1 (PAC 1) and proanthocyanidin-enriched-fraction 1 (PAC 2). For the following analysis only anthocyanin-enriched-fraction 1 (ANC 1) and proanthocyanidin-enriched-fraction 1 (PAC 1) were used, and will be referred as ANC and PAC throughout the text.

B. Chemical analysis

Total polyphenol content

Total phenolic content was measured using the Folin-Ciocalteu method, adapted to a micro-assay, from the method described by Chandra and de Mejia (Chandra, de Mejia 2004). Briefly, to a 96-well flat bottom plate, 50 μL of 1 N Folin-Ciocalteu's phenol reagent and 50 μL of either sample, standard or blank were added; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na_2CO_3. The solution was then allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multi-well plate reader (Biotek, Winooski, VT). Results were expressed as catechin mg equivalents, using the standard curve $y= 0.011x - 0.071$, $R^2 = 1$.

Anthocyanin and proanthocyanidin analysis

Anthocyanin separation was conducted on an 1100 HPLC (Agilent Technologies, Santa Clara, CA) using a reverse phase Supelcosil-LC 18 column (250 mm × 4.6 mm ×

5 μm) (Supelco, Bellefonte, PA). Samples were prepared by dissolving 5 mg of extract or enriched-fractions in 1 mL of methanol and filtering through 0.45 μm nylon filters (Fisher Scientific, Pittsburg, PA) before injection. The mobile phase consisted of 5% formic acid in H_2O (A) and 100% methanol (B). The flow rate was held constant at 1 mL/min with a step gradient of 10, 15, 20, 25, 30, 60, 10, and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47, and 60 min, respectively. The same instrumentation was used to separate proanthocyanidins. Mobile phase consisted of 94.9% H_2O, 5% acetonitrile and 0.1% formic acid (A) and of 94.9% acetonitrile, 5% H_2O and 0.1% formic acid (B). The flow rate was held constant at 1 mL/min with a step gradient of 0, 5, 15,30,60, 90, 0 and 0% of solvent B at 0, 3, 40, 45, 50, 55, 47, and 60 min, respectively. Anthocyanins and proanthocyanins were detected at 520 nm and 280 nm using diode array detector (DAD), respectively. Chemstation software (Agilent Technologies Inc, Santa Clara, CA) was used for both protocol control and data processing.

The HPLC-ESI-MS analyses were made with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (m/z 150-2000), with a photodiode array (PDA) detector (200-600 nm), version 1.2, autosampler version 1.2, and Xcalibur software for data processing. The HPLC separations were carried out on a C-18 reversed-phase column (150 mm, 2.1 mm i.d., particle size 5 μm, 90Å) (VYDAC, Western Analytical, Murrieta, CA, USA). The analysis was carried out using mobile phase consisting of 94.9% H_2O, 5% acetonitrile and 0.1% formic acid (A) and of 94.9% acetonitrile, 5% H_2O and 0.1% formic acid (B), with a step gradient of 5, 30, 60, 90, 90, 5 and 5% of solvent B at 0, 40, 45, 50, 55 and 60 min. A constant rate flow rate of 200 μL/min and an injection

volume of 10 µL; were employed. Samples were prepared by dissolving 5 mg of extract or enriched-fraction in 1 mL of methanol and filtering through 0.45 µm nylon filters before injection. Acquisition of LC-PDA-MS data was performed and processed using XCalibur Qual Browser v1.4 software (Thermo Electron Corp., Waltham, MA).

The total anthocyanin content of the two berries (*A. chilensis* and *V. floribundum*) and the commercial powder was calculated as cyanidin-3-glucoside equivalent (C3G equivalent) Three concentrations of the standard at 0.25 mg, 0.5 mg, and 1.0 mg/mL were used to quantify the anthocyanins using peak areas measured by HPLC at 520 nm. The identification of anthocyanins was based on comparison with published data (Chandra, de Mejia 2004), MS spectral data and comparison to reference standards. In the same way, the total proanthocyanidin content of the two berries and the commercial powder was calculated as epicatechin equivalents from the peak area measured at 280 nm, with the exclusion of 520 nm wavelength peaks which were anthocyanins.

C. Biochemical analysis

Antioxidant Capacity

Antioxidant capacity (AC) was measured by the oxygen radical absorbance capacity (ORAC) assay (Prior et al 2003, Dávalos and others 2001). Briefly, aliquots of 20 µL sample (0.1 mg/mL), Trolox standard dissolved in 75 mM phosphate buffer pH 7.4 or 75 mM phosphate buffer pH 7.4 blank were added to a 96-well black walled plate. This was followed by the addition of 120 µL 17 nM fluorescein. The plate was then incubated for 15 min at 37 ˚C and then 60 µL of 153 mM AAPH were added. The plate was read in a Synergy 2 multi-well plate reader (Bio-Tek), at 37 ˚C, sensitivity 60, read every 2 min for 120 min with excitation 485 and emission 582 nm. Results were

expressed as mmol Trolox equivalents, using the standard curve y = 0.23x + 1.11, R^2 = 0.99.

D. Biological activities

Adipogenesis and lipid metabolism

Cytotoxicity assay

All treatments were assayed for cytotoxicity before lipid quantification or Pref-1 assays. CellTiter 96®AQueous One Solution was used to determine the number of viable cells according to the manufacturer's manual (Promega, Madison, WI). Briefly, the CellTiter 96®AQueous One Solution (20 µL) was added to 100 µL of media containing wells (with cells) and then the plate was incubated in a 5% CO_2 incubator at 37 ˚C. After 2 h, absorbance was measured at 515 nm with a 96-well plate reader (Biotek® Instruments, Winooksi, VA). Cell viability was calculated using the following equation:

$$A_{treatment\ 515\ nm}/A_{control\ 515\ nm} * 100 = \%\ cell\ viability$$

Cell culture and treatments

The 3T3-L1 preadipocytes were seeded at 3 x 10^4 cells/well in 24-well plates and cultured in DMEM containing 10 mM sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin and 10% FBS (FBS/DMEM medium). For pre-adipocyte differentiation, two days after reaching 100% confluence, the cells were stimulated with FBS/DMEM medium containing 167 µM insulin, 0.5 M IBMX, and 1 M DEX for 2 days. Cells were then maintained in FBS/DMEM medium with 167 nM insulin for another 2 days, followed by culturing with FBS/DMEM medium for an additional 4 days, at which time

up to 90% of cells were mature adipocytes with accumulated fat droplets. To study the effect of the phenolic extracts on lipid metabolism, mature adipocytes received a single treatment of 100 μM (C3C or epicatechin equivalents) PAE, ANC and PAC of the two berries and the commercial powder, 10 days after the initiation of differentiation for 48 h. After this period, lipid accumulation was quantified (**Figure 10**).

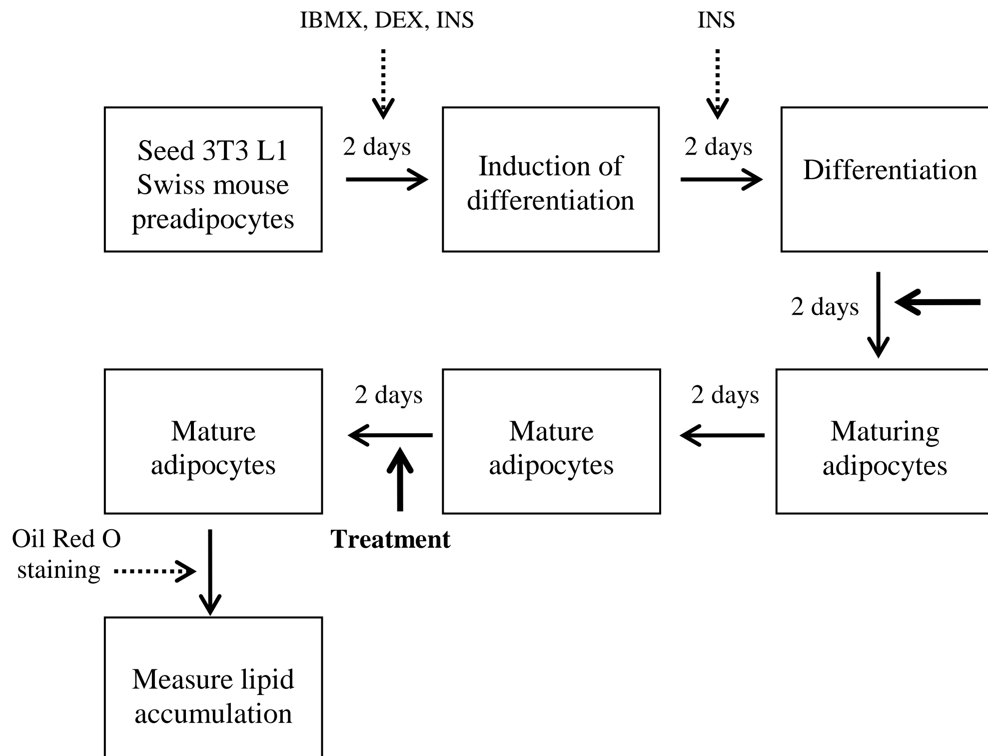

Figure 10. Diagram showing differentiation, treatments and red oil assay timeline when adipocytes received a single treatment at mature stage. Abbreviations: IBMX, 3-isobutyl-1 methylxanthine; DEX, Dexamethasone; INS, Insulin.

To monitor the effect of these extracts on adipogenesis, adipocytes were treated throughout the differentiation process at days 2, 4, 6, 8 and 10. Twelve days after the adipocytes were seeded and treated throughtout the differentiation, lipid accumulation

was quantified (**Figure 11**). In addition, EGCG and C75 (100 µM) were used as positive controls. All treatments were dissolved in 0.01% DMSO. The concentrations used were based on preliminary data from a cytotoxicity assay (**Appendix J)**, making sure that cell viability was at least 80%.

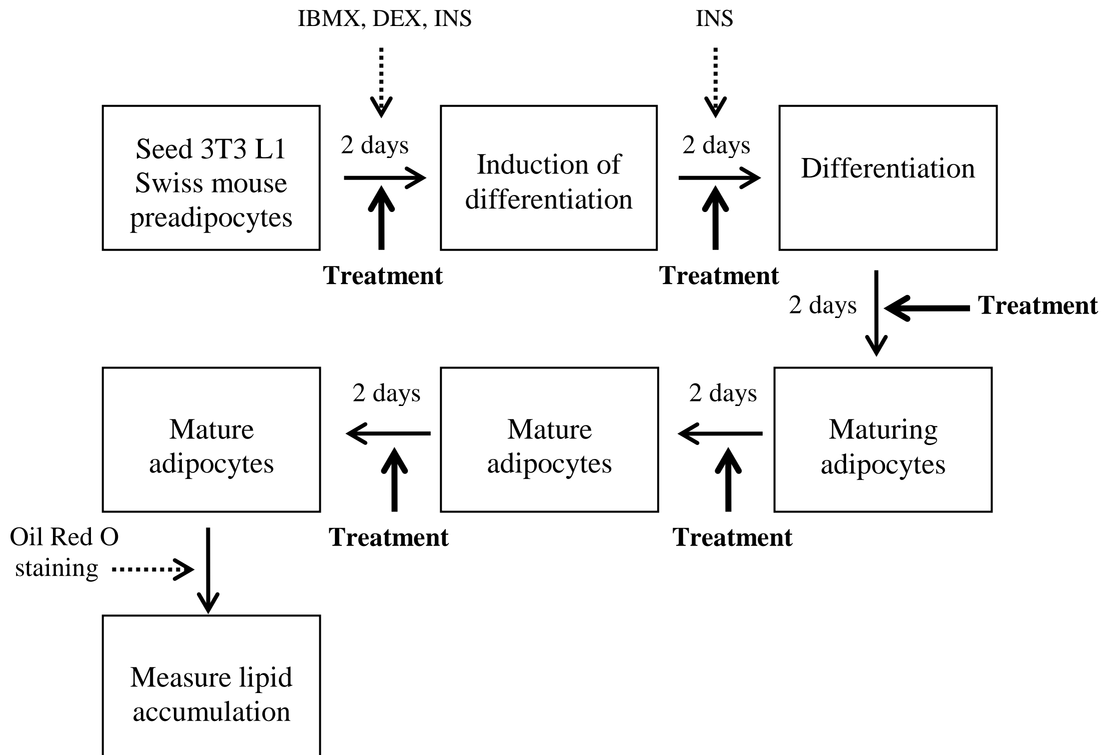

Figure 11. Diagram showing differentiation, treatments and red oil assay timeline when adipocytes were treated throughout the differentiation process. Abbreviations: IBMX, 3-isobutyl-1 methylxanthine; DEX, Dexamethasone; INS, Insulin.

Lipid quantification by Oil Red O assay

Briefly, treated mature adipocytes were washed with Dulbecco's phosphate buffered saline (DPBS) and fixed with 10% formalin (in DPBS) in 24-well plates for 1 h. Then, cells were washed with 60% isopropanol and allowed to air dry. The Oil Red O

stock solution (6:4 v/v with water) was added to lipid droplets for 1 h. After Oil Red O lipid staining, cells were washed with water four times and were air dried. Oil Red O dye was eluted by adding 100% isopropanol and then incubating at room temperature for 10 min. The absorbance of the resulting eluant was measured at 510 nm using a Synergy 2 multi-well plate reader (Bio-Tek). Inhibition of lipid accumulation in adipocytes was calculated using the following equation:

$$(A_{control,\ 510\ nm} - A_{treatment,\ 510\ nm})/A_{control,\ 510\ nm} * 100 = \%\ \text{inhibition of lipid accumulation}$$

Measurement of Pref-1 expression by Western blotting

Preadipocytes were seeded at a concentration of 1.0×10^5 cells/well in 6- well plates and cultured with FBS/DMEM. After 24 h, the cells were treated with100 µM (C3C or epicatechin equivalent) PAE, ANC and PAC of the two berries and the commercial powder for 24 h. The concentrations used were based on preliminary data from a cytotoxicity assay (**Appendix K**), making sure that cell viability was at least 80%. Treated preadipocytes were lysed in sample loading buffer (Laemmli buffer with 5% b-mercaptoethanol) and sonicated using an ultrasonic cell disruptor from Misonix Inc. (Farmingdale, NY). After lysis, the cell lysates were boiled for 5 min and separated via electrophoresis on 4–20% Tris-HCl ready gels (Bio Rad Laboratories). Gels were run on a PowerPac 300 (Bio-Rad) at 200 V for 30 min. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 0.1 % SDS) using Western sandwich assembly for 1 h at 4 ºC using 110 V. The membrane was then blocked with 5% non-fat dry milk (NFDM)

in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at 4 ºC. After blocking, the *membrane was washed with TBST and incubated with 10 mL of anti-pref-1 goat* polyclonal IgG antibody (1:2000 in TBST with 1% NFDM) at 4 ºC overnight. The membrane was washed again and incubated with 10 mL of ECL anti-goat IgG horseradish peroxidase conjugate (1:1000 in TBST with 1% NFDM) for 3-4 h at room temperature. The membranes were washed a final time in TBST. Pref-1 expression was visualized using chemiluminescent reagent (GE Life Sciences, Piscataway, NJ) following manufacturer's instructions. The membrane picture was taken with a Kodak Image station 440 CF (Eastman Kodak Company, New Haven, CT). Pref-1 expression was calculated as the ratio between Pref-1 and actin band intensity.

Inflammation

A diagram showing the cell culture, treatments and measurement of NO and PGE_2 production and iNOS and COX-2 expression in RAW 264.7 macrophages is shown in **Figure 12.**

Cytotoxicity assay

All the cell treatments were assayed for cytotoxicity before performing any inflammatory marker assay. CellTiter 96®AQueous One Solution was used (Promega, Madison, WI) as mentioned previously.

Cell culture and treatments

Macrophage cell line RAW 264.7 were seeded at 2 x 10^5cells/well in 6- well plates and cultured in FBS/DMEM medium at 37 °C in 5% CO_2/95% air. After 48 h of incubation, cells were treated with 100 µM (C3C or epicatechin equivalent) of PAE, ANC and PAC of the two berries and the commercial powder and 1 µg/mL of LPS for

24 h. In addition, quercetin (100 µM) was used as a positive control. Treatments were dissolved in 0.01% DMSO. The concentrations used were based on preliminary data from a cytotoxicity assay (**APPENDIX L**), making sure that cell viability was at least 80%. After 24 h treatment, the spent medium was collected and analyzed for NO and PGE_2. Cell lysates were used to study the effect of the phenolic extracts on the expression of COX-2 and iNOS.

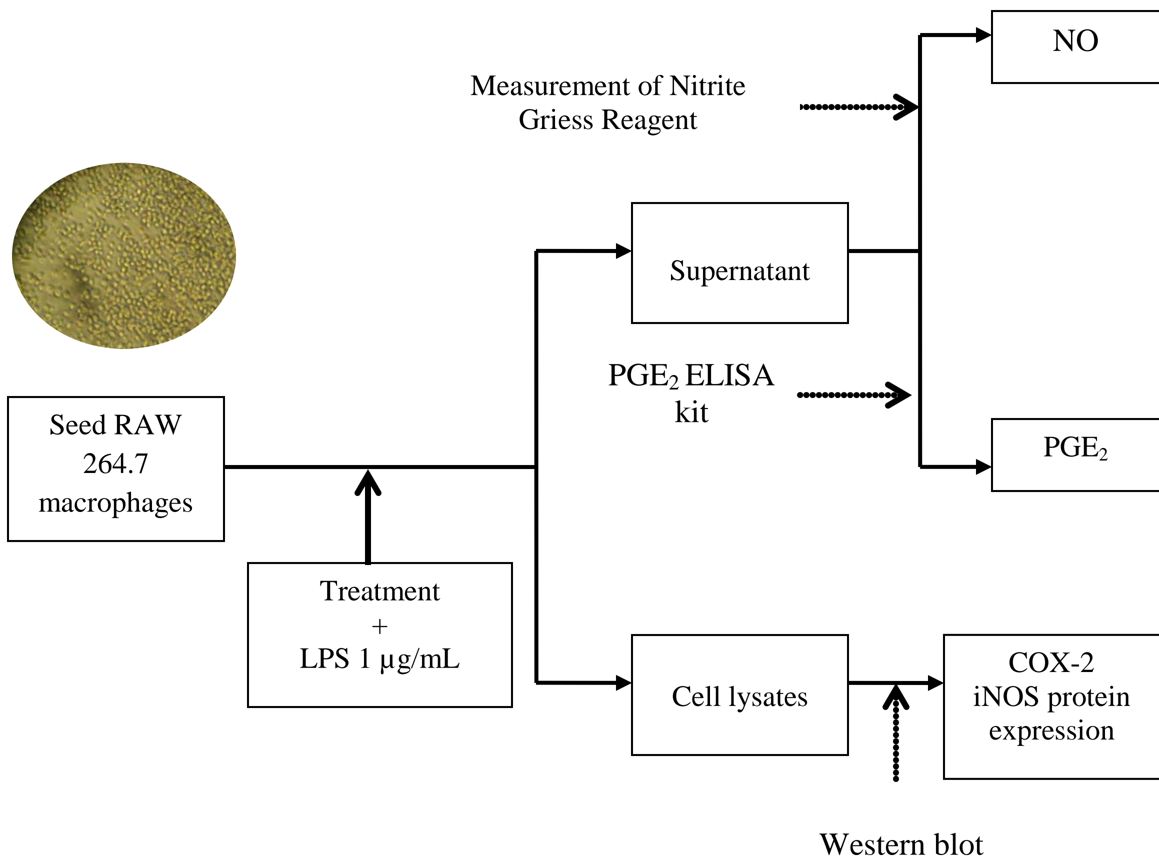

Figure 12. Diagram showing cell culture, treatments and measurement of NO and PGE_2 production and iNOS and COX-2 expression in RAW 264.7 macrophages. Abbreviations: NO, nitric oxide; PGE_2 prostaglandin E2; iNOS, inducible nitric oxide synthase; COX-2, cycloxygenase-2; LPS, lipopolysaccharide.

Measurement of NO and PGE_2 production

NO production was determined by measuring the level of nitrite accumulation (the stable metabolite of NO) in the spent medium. For nitrite measurement, 100 µL of the spent medium was plated in 96-well plate and an equal amount of Griess reagent (1% w/v sulfanilamide and 0.1% w/v N-1 (naphthyl) ethylenediamine-diHCl in 2.5% v/v H_3PO_4) was added. The plate was incubated for 5 min and the absorbance measured at 550 nm. The amount of NO was calculated using the following sodium nitrite standard curve $y = 0.14x + 0.09$, $R^2 = 0.99$. For PGE_2 measurement, PGE_2 ELISA kit monoclonal was used following manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Measurement of iNOS and COX-2 expression by Western blotting

COX-2 and iNOS expression were determined in cell lysates. The primary antibodies used were COX-2 or iNOS mouse monoclonal antibodies (1:200) and antimouse IgG horseradish peroxidase conjugate as the secondary antibody (1:1000). All other steps were preformed as described above. The expression of these enzymes was calculated as the ratio between COX-2 or iNOS and actin band intensity.

E. Statistical analysis

Data were expressed as means of at least two independent replicates. Results were compared by one-way analysis of variance (ANOVA) using the proc GLM function of Statistical SAS version 9.2 (SAS Inst. Inc., Cary, NC). Group means were considered to be significantly different at $p < 0.05$. Mean separation was achieved through least significant difference (LSD) procedure in SAS.

CHAPTER 6
RESULTS AND DISCUSSION

I. Phenolic composition and antioxidant capacity

Table 5 shows the total anthocyanins, total proanthocyanidins, total phenolics and antioxidant capacity of *A. chilensis* berries and its phenolic extracts. **Table 6** shows the total anthocyanins, total proanthocyanidins, total phenolics and antioxidant capacity of *V. floribundum* berries, the commercial powder and their phenolic extracts.

Table 5. Total anthocyanins, total proanthocyanidins, total phenolics and antioxidant capacity of *Aristotelia chilensis* berries and phenolic extracts.

	Anthocyanins (%) [1]	**Proanthocyanidins (%)** [2]	**Total phenolics (mg/g)** [3]	**Antioxidant capacity (mmol/g)** [4]	**Yield** [5]
DB	4.6 ± 0.1 [6] [c]	0.4 ± 0.3 [6] [c]	53.3 ± 5.8 [7] [d]	0.3 ± 0.1 [7] [d]	NA
PAE	58.4 ± 0.7 [b]	5.1 ± 0.4 [b]	632.6 ± 5.8 [b]	7.5 ± 0.1 [b]	7.8
ANC	79.8 ± 1.7 [a]	≥ 0.1 [d]	573.7 ± 13.0 [c]	9.5 ± 0.3 [a]	2.9
PAC	≥ 0.1 [d]	49.3 ± 3.2 [a]	973.2 ± 36.9 [a]	9.8 ± 0.5 [a]	0.6

[1]% expressed as cyanidin-3-glucoside equivalents; [2]% expressed as epicatechin equivalents; [3]mg/g of catechin equivalent estimated by Folin Ciocalteu; [4]mmol/g of Trolox equivalents estimated by ORAC; [5]g extract or enriched-fraction obtained per 100 grams of dried berry; [6]Data from phenolic-rich extracts was converted to freeze-dried fruit weight basis; [7]The dried sample was homogenized in 80% ethanol, kept overnight at 4 ºC and centrifuged. The ethanolic extract was used to measure TP and AC. (NA) Not applicable. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Values within a column followed by different letters are significant at $p \leq 0.05$. Abbreviations: DB, dry berry; PAE, post-amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction.

Table 6. Total anthocyanins, total proanthocyanidins, total phenolics and antioxidant capacity of *Vaccinium floribundum* freeze-dried berries, the commercial powder and their phenolic extracts.

	Anthocyanins (%) [1]	Proanthocyanidins (%) [2]	Total phenolics (mg/g) [3]	Antioxidant capacity (mmol/g) [4]	Yield [5]
	Freeze-dried berries				
FB	1.1 ± 0.1 [6] [c]	0.5 ± 0.1 [6] [c]	53.0 ± 1.6 [7] [e]	0.2 ± 0.01 [7] [f]	NA
PAE	11.1 ± 0.5 [b]	5.3 ± 0.5 [b]	524.4 ± 4.5 [d]	8.3 ± 0.4 [c]	9.8
ANC	15.7 ± 0.2 [a]	≥ 0.1 [d]	711.2 ± 21.2 [c]	10.6 ± 0.9 [a]	2.1
PAC	≥ 0.1 [d]	54.3 ± 2.4 [a]	869.2 ± 15.1 [b]	9.2 ± 0.3 [b]	0.4
	Commercial powder				
CP	0.2 ± 0.1 [7] [c]	0.5 ± 0.03 [7] [c]	18.1 ± 0.2 [f]	0.1 ± 0.03 [7] [g]	NA
PAE	2.3 ± 0.6 [b]	4.6 ± 0.3 [b]	495.6 ± 9.1 [7] [d]	3.3 ± 0.1 [d]	10.2
ANC	4.3 ± 0.6 [a]	≥ 0.1 [d]	927.2 ± 7.6 [a]	10.1 ± 0.1 [a]	0.1
PAC	≥ 0.1 [d]	49.2 ± 1.1 [a]	788.1 ± 3.2 [b]	4.9 ± 0.4 [d]	0.8

[1]% expressed as cyanidin-3-glucoside equivalents; [2]% expressed as epicatechin equivalents; [3]mg/g of catechin equivalent estimated by Folin Ciocalteu; [4]mmol/g of Trolox equivalents estimated by ORAC; [5]g extract or enriched-fraction obtained per 100 grams of dried berry; [6]Data from phenolic-rich extracts was converted to freeze-dried fruit weight basis; [7]The dried sample was homogenized in 80% ethanol, kept overnight at 4 ºC and centrifuged. The ethanolic extract was used to measure TP and AC. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Values within a column followed by different letters are significant at $p \leq 0.05$. Statistical analysis was performed independently for the freeze dried and the commercial powder of *V. floribundum.* Abbreviations: freeze-dried berry (FB), dry post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC), commercial powder (CP). Not applicable (NA).

A. Anthocyanins

The berry of *A. chilensis* contained seven main anthocyanin structures: delphinidin-3-sambubioside-5-glucoside (*m/z*: 759), delphinidin-3,5-diglucoside (*m/z*: 627), cyanidin-3-sambubioside-5-glucoside (*m/z*: 743), delphinidin-3- sambubioside (*m/z*: 597), delphinidin-3-glucoside (*m/z*: 465), cyanidin-3-sambubioside (*m/z*: 581), cyanidin-3-glucoside (*m/z*: 449). This was in agreement with previous reports (Escribano-Bailon and others 2006, Céspedes and others 2010, Vasco and others 2009, Grace and others 2009). An HPLC chromatogram illustrating these structures in the PAE is shown in **Figure 13** and the peak assignment and the concentration of individual anthocyanins in the PAE and in the ANC for *A. chilensis* is presented in **Table 7**. Delphinidin 3-glucoside (peak 5) was the main anthocyanin component present in this berry. HPLC analysis indicated that berries from *A. chilensis* contain 45.7 mg/g DW (C3G equivalents). This value is higher than that previously reported by Escribano-Bailon and others and Céspedes and others (Escribano-Bailon and others 2006, Céspedes and others 2010) who reported on average about 2.5 mg/g DW (C3G equivalents). This difference in anthocyanin concentration in part may be explained by differences in the time and place of collection of *A. chilensis* berries in the previous studies. The berries analyzed in this report were collected at the most southern location in Chile. The time and place of harvest can influence growing conditions, which have been shown to affect the composition of these fruits (Ortega-Regules and others 2006, Howard and others 2003). The differences in the method of extraction used in previous studies may also explain this difference in anthocyanin concentration.

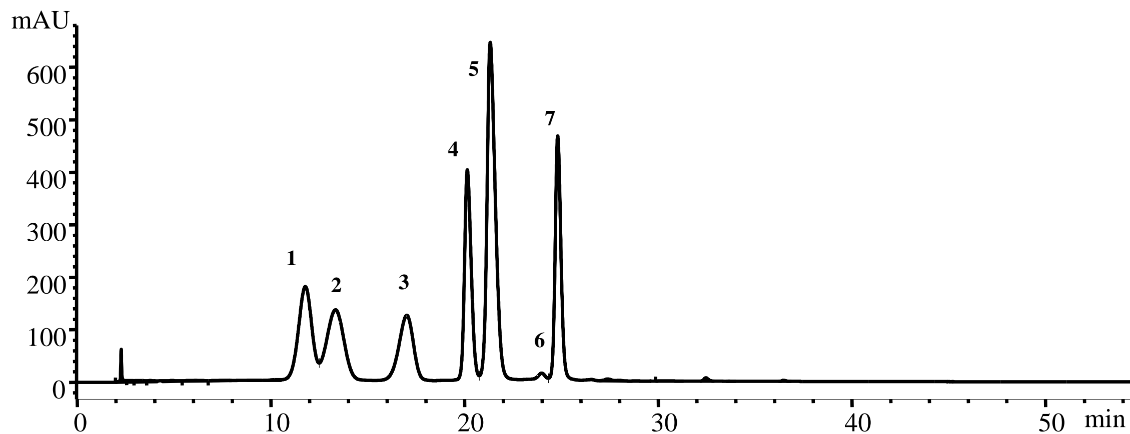

Figure 13. HPLC chromatogram of the post-amberlite extract (PAE) of *Aristotelia chilensis.* The identities of the compounds associated with the anthocyanin peaks shown here are given in **Table 7.**

Table 7. Identification and content of anthocyanins in the post-amberlite extract (PAE) and anthocyanin-enriched-fraction (ANC) of *Aristotelia chilensis* using HPLC and ESI-MS at 520 nm.

Peak	Anthocyanin	(m/z)	PAE (%)	ANC (%)
1	**Delphinidin-3-sambubioside-5-glucoside**	759/597/465/303	8.4	11.9
2	**Delphinidin-3,5-diglucoside**	627/465/303	7.5	11.0
3	**Cyanidin-3-sambubioside-5-glucoside**	743/581/449/287	6.1	8.7
4	**Delphinidin-3-sambubioside**	597/303	9.3	12.9
5	**Delphinidin-3-glucoside**	465/303	17.5	22.3
6	**Cyanidin-3-sambubioside**	581/287	0.3	0.4
7	**Cyanidin-3-glucoside**	449/287	9.3	12.5
	Total		**58.4**	**79.7**

Percentages were calculated as cyanidin-3-glucoside equivalents. Abbreviations: post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC).

Berries and commercial powder of *V. floribundum* contained the same anthocyanin profiles (**Figure 14A, B**). Five anthocyanins were identified in the berries of *V. floribundum*: delphinidin-3-galactoside (*m/z* 465), cyanidin-3-galactoside (*m/z* 449), delphinidin-3-arabinose (*m/z* 435), cyanidin-3-glucoside (*m/z* 449), and cyanidin-3-arabinose (*m/z* 419). This was in agreement with previous studies. Delphinidin-3-arabinose (peak 2) and cyanidin-3-arabinose (peak 5) were found to be the main anthocyanins in this berry.

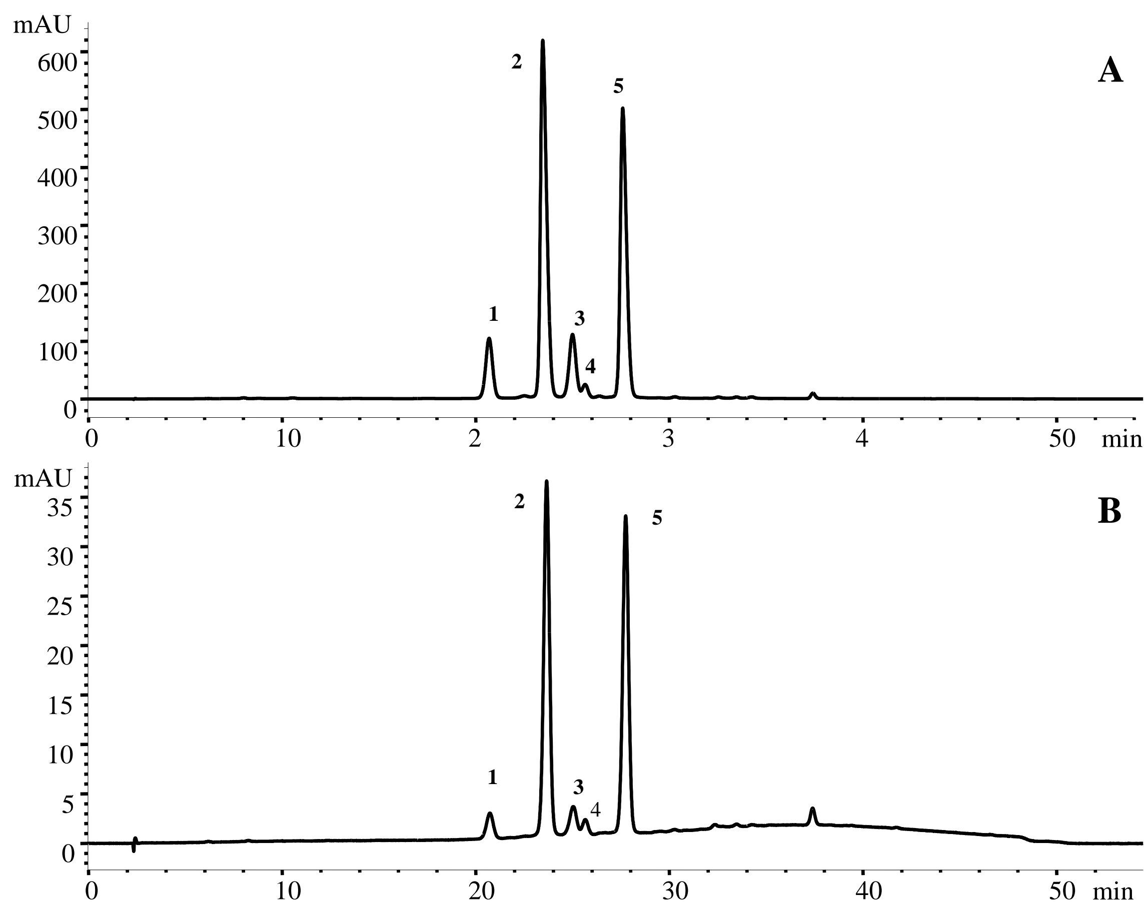

Figure 14. HPLC chromatograms of the post-amberlite extract (PAE) of *Vaccinium floribundum* (A) and the commercial powder of *Vaccinium floribundum* (B). The identities of the compounds associated with the anthocyanin peaks shown here are given in **Table 8**.

The individual concentrations of anthocyanins present in the PAE and ANC of the berry and the commercial powder of *V. floribundum* are presented in **Table 8**. HPLC analysis indicated that the total anthocyanin content of this berry was 10.6 mg/g DW (C3G equivalent) which is comparable to the value previously reported (Vasco and others 2009). The commercial powder of *V. floribundum*, although maintaining the same anthocyanin profile, showed a lower anthocyanin content (2.4 mg/g DW C3G equivalents) as compared with the freeze dried berry. This was consistent with several studies that indicated that processing of anthocyanin- containing foods can lead to anthocyanin degradation (Yue 2008).

Table 8. Identification and content of anthocyanins in the post-amberlite extract (PAE) and in the anthocyanin-enriched-fraction (ANC) of the freeze-dried (F) and the commercial powder (C) of *Vaccinium floribundum* using HPLC and ESI-MS at 520 nm.

Peak	**Anthocyanin**	**(m/z)**	**PAE (%)**		**ANC (%)**	
			F	**C**	**F**	**C**
1	**Delphinidin-3-galactoside**	465/303	1.0	0.1	1.4	0.3
2	**Cyanidin-3-galactoside**	449/287	4.8	0.8	7.0	2.9
3	**Delphinidin-3-arabinose**	435/303	1.0	0.2	1.4	0.1
4	**Cyanidin-3-glucoside**	449/287	0.4	0.2	0.4	0.2
5	**Cyanidin-3-arabinose**	419/287	3.9	0.9	5.5	0.8
	% Total		**11.1**	**2.2**	**15.7**	**4.3**

Percentages were calculated as cyanidin-3-glucoside equivalents. Percentages were calculated as cyanidin-3-glucoside equivalents. Abbreviations: post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC).

The fruit of *A. chilensis* showed a higher concentration as well as a more diverse anthocyanin profile, when compared to the fruit of *V. floribundum*.

B. Proanthocyanidins

The berry of *A. chilensis* contained 4.0 mg/g DW (epicatechin equivalents), while the freeze dried berries and commercial powder from *V. floribundum* had a slightly higher concentration of 5.2 and 4.8 mg/g DW (epicatechin equivalents), respectively. HPLC-MS analysis revealed a series of proanthocyanidins ranging from dimers to hexamers. The proportion of proanthocyanidin oligomers and polymers varied among the two berries and the commercial powder. To estimate the relative ratio of the proanthocyanidins, the HPLC-MS peaks were summed, and each peak was expressed as a percentage of the total sum (Yousef and others 2004). Based on this estimation, *A. chilensis* contained mainly dimers (56%) and trimers (14%). Small MS peaks of proanthocyanidin tetramers, pentamers, and hexamers were also detected. *V. floribundum* contained trimers (68 %) and in less proportion pentamers (16 %) and hexamers (8%). The commercial powder contained a larger percentage of dimers (34%) and trimers (23%) (**Table 9**). **Figure 15** illustrates the UV chromatogram, at 280 nm absorption and the ESI/MS spectra of the PAE **(A)** and the PAC **(B)** of *V. floribundum.* In the PAE, anthocyanins are present at a higher proportion in comparison with proanthocyanidins oligomers and polymers (**Figure 15A**). After the enrichment process (**Figure 15B**) the proportion of proanthocyanidins increases displaying a series of proanthocyanidins ranging from dimers to hexamers. In the UV chromatogram of the PAC (**Figure 15B**) a large hump characteristic of proanthocyanidins can be visualized.

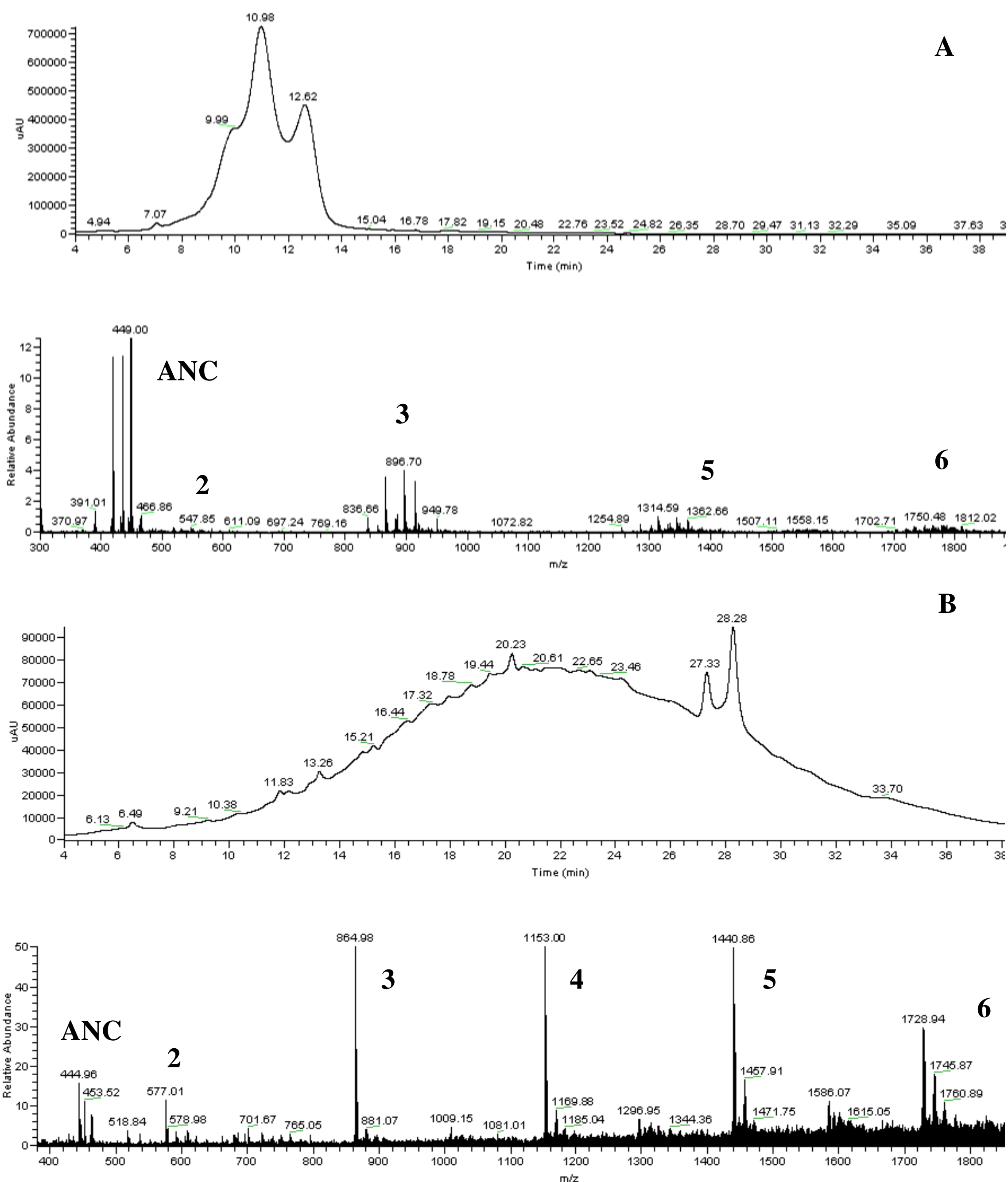

Figure 15. HPLC –ESI/MS chromatogram of the post-amberlite extract (PAE) (**A**) and the proanthocyanidin-enriched-fraction (PAC) (**B**) of *V. floribundum* including UV chromatogram with 280 nm absorption and ESI/MS spectra. The large bold numbers indicate the average degree of polymerization from dimers to hexamers and ANC refers to anthocyanins.

C. Antioxidant capacity

The berries of *A. chilensis* and *V. floribundum* showed similar values of total phenolics expressed as catechin equivalents (**Tables 5 and 6**). However, the AC measured as oxygen radical scavenging capacity and expressed as Trolox equivalents, was higher in the berries of *A. chilensis* indicating that the constituents of this berry have more AC. When comparing the berry and the commercial powder of *V. floribundum*, results indicate that the phenolic content and the AC of the commercial powder decreases and this is likely to be a consequence of processing. Total phenolics and AC correlated for *A. chilensis* and both the *V. floribundum* berries and commercial powder with R^2 values of 0.90, 0.86 and 0.78, respectively. Anthocyanins were more highly correlated with AC than proanthocyanins.

II. Adipogenesis and lipid metabolism

Figure 16 shows the percent inhibition of lipid accumulation for both the single treatment on mature adipocytes and treatment throughout their differentiation process with 100 µM (C3C or epicatechin equivalents) of the phenolic extracts (PAE, ANC and PAC) from the two berries and the commercial powder, and at 100 µM of positive controls (EGCG and C75).

Lipid accumulation was inhibited from 4 to 11% when adipocytes received a single treatment and from 6 to 38% when adipocytes were treated throughout the differentiation process. The highest lipid accumulation inhibition (38%) was observed when adipocytes were treated throughout the differentiation process with PAC from *V. floribundum*. **Figure 17** shows 3T3-L1 adipocytes after treatment with PAC from *V. floribundum* in comparison to the negative control. It can be visualized that most cells

remain as preadipocytes **(Figure 17A)** as compared with the mature adipocytes seen in the control (**Figure 17B**).

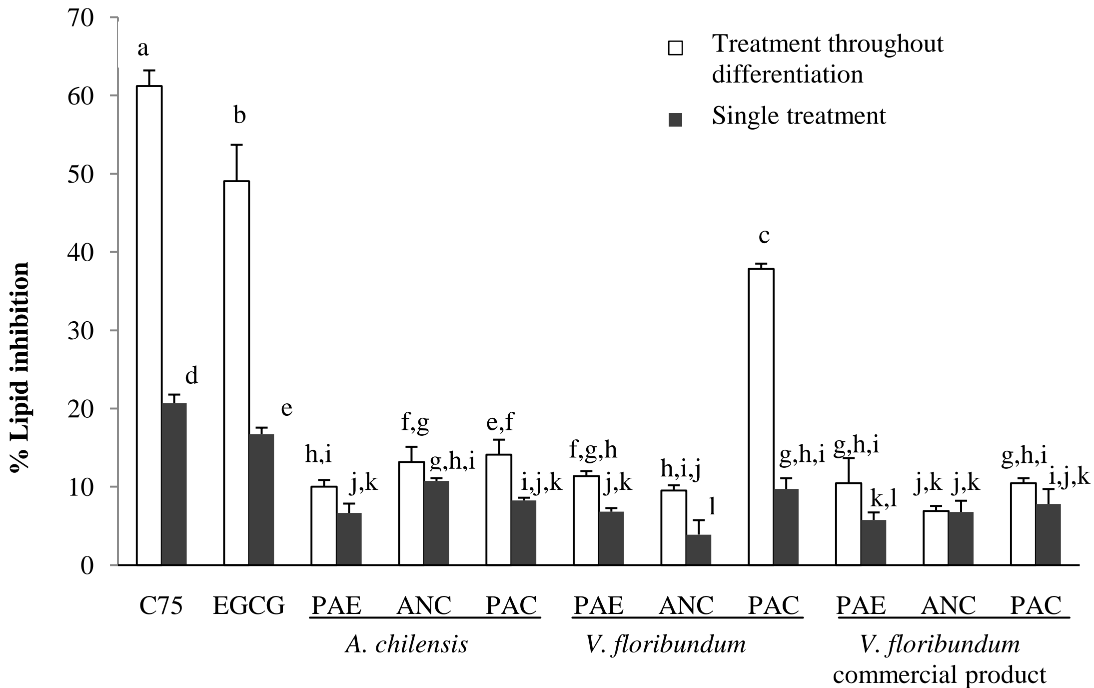

Figure 16. Effect of phenolic extracts (PAE, ANC and PAC) of the two berries and the commercial powder and positive controls (EGCG and C75) on lipid accumulation inhibition, when 3T3-L1 adipocytes were treated throughout the differentiation process and when adipocytes received a single treatment at the mature stage. 3T3-L1 adipocytes were treated with 100 µM of each phenolic extract (equivalent C3C or epicatechin) and positive controls for 48 h at 37 ºC in a humidified 5% CO_2 incubator. Cells were harvested 10 days after initiation of differentiation and lipid quantification was performed by Oil Red O assay. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Different letters indicate significant difference, $p \leq 0.05$. Abbreviations: ECGC, epigallocatechin-3-gallate; PAE, post- amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction.

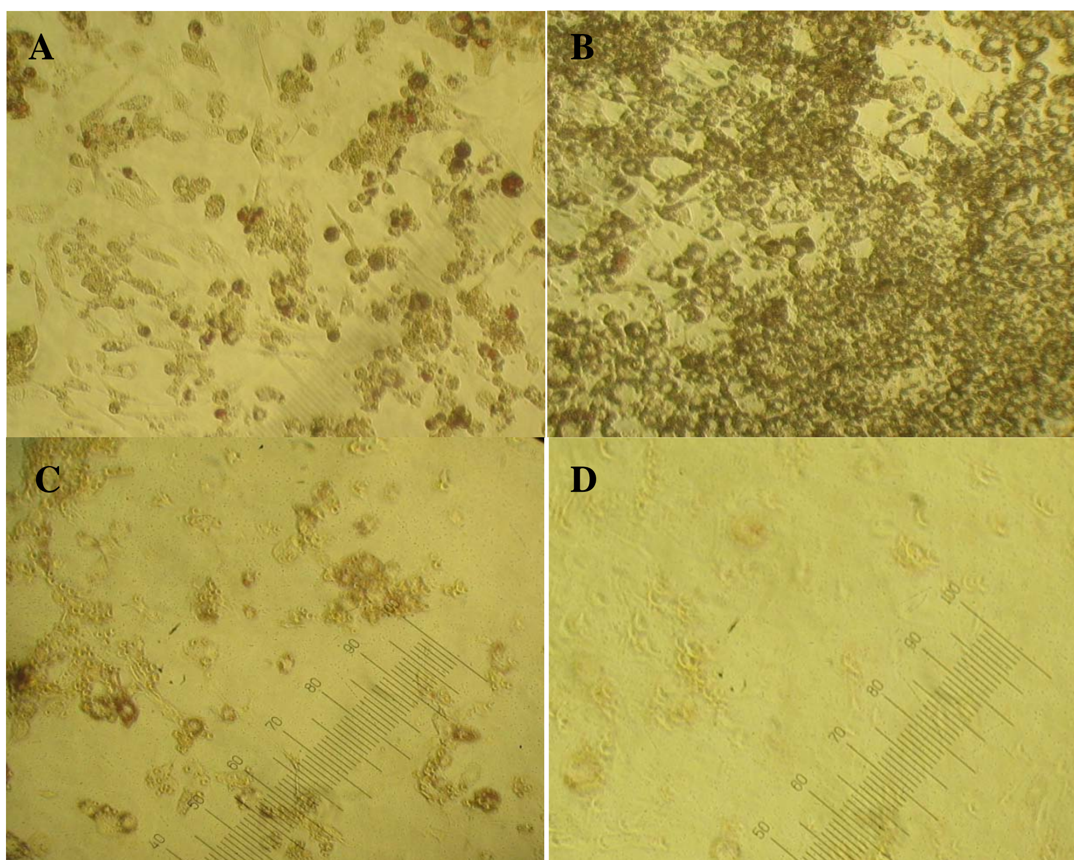

Figure 17. Visualization of 3T3-L1 mature adipocytes when treated with 100 µM equivalent epicatechin of PAC from *Vaccinium floribundum* throughout the differentiation process **(A)** as compared to the negative control (no PAC treatment) **(B),** and treatment with 100 µM ECGC (epigallocatechin 3-gallate) **(C)** and 100 µM C75 (α-methylene-γ-butyrolactone) (**D**)**.** Images were photographed at 20X magnification**.**

As shown in **Figure 16**, lipid accumulation inhibition was higher when adipocytes were treated continuously throughout the differentiation process. To further study the effect of these extracts on adipogenesis, Pref-1 expression was analyzed. The ability of a phenolic extract to induce or maintain the expression of Pref-1 can indicate its potential to inhibit adipogenesis (Wang and others 2006). The effect of the phenolic extracts on Pref-1 expression in 3T3-L1 preadipocytes when treated for 24 h is shown in **Figure 18**. The PAC from *V. floribundum* showed a significant increase ($p < 0.05$) in Pref-1 expression in comparison with the control. The PAC of *A. chilensis* and of the

commercial powder also induced Pref-1 expression, although these treatments were not statistically different than the control ($p > 0.05$).

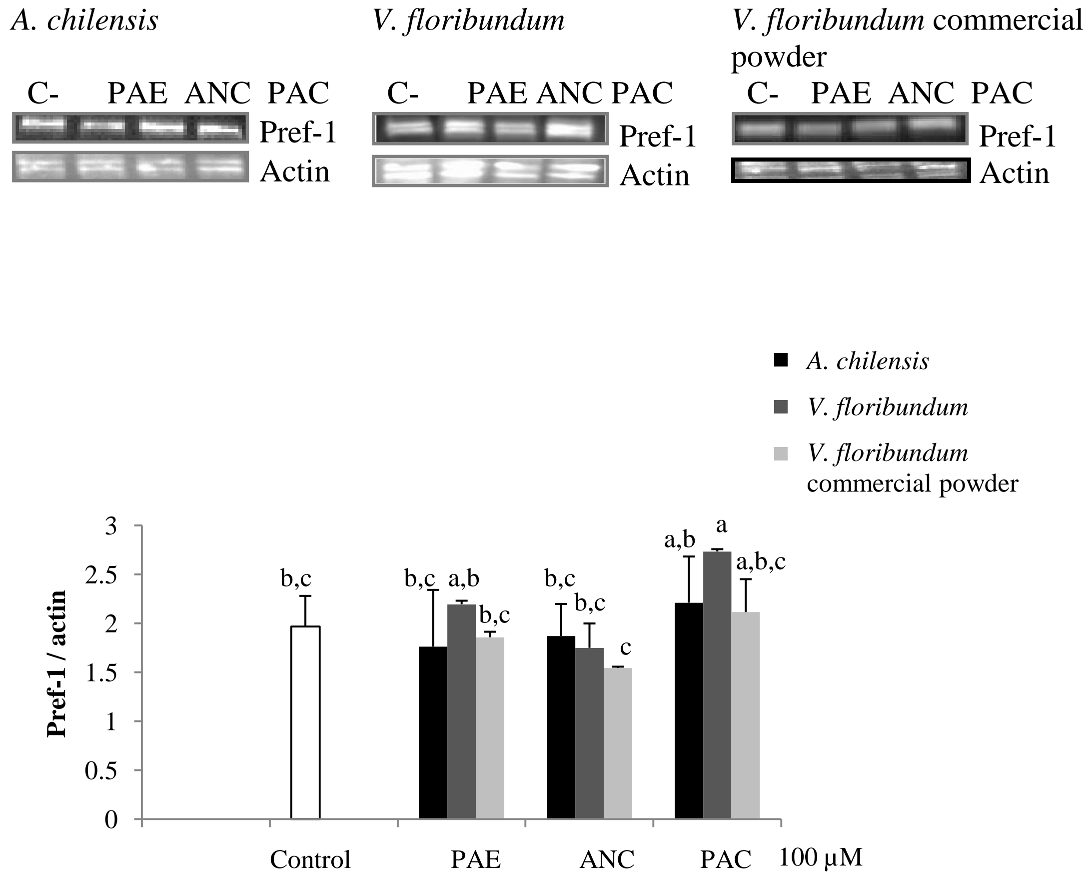

Figure 18. Effect of phenolic extracts (PAE, ANC and PAC) of the two berries and commercial powder on Pref-1 expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with 100 μM of each phenolic extract (C3C or epicatechin equivalents) for 24 h at 37 ºC in a humidified 5% CO_2 incubator. Cells were harvested 24 h after treatment and Pref-1 expression was analyzed by Western blotting. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Different letters indicate significant difference, $p \leq 0.05$. Abbreviations: Post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

The present study showed that the phenolic extracts of the two berries were more efficient in reducing lipid accumulation *in vitro* through the inhibition of adipogenesis than through regulation of lipid metabolism at the mature stage of adipocytes. It was also shown that PAC of *V. floribundum* was potent in inhibiting adipogenesis (38%), reaching inhibition levels close to that of EGCG (49%), a known adipogenesis inhibitor (Hwang and others 2005) . This observation is supported by previous studies that have shown that proanthocyanidin rich extracts inhibit differentiation *in vitro* (Pinent and others 2005b) and *in vivo* (Montagut and others 2007). In addition, the PAC of *V. floribundum* induced the expression of Pref-1 in preadipocytes indicating that this is one of the potential mechanisms by which it inhibits adipogenesis. This is in accordance with other studies which show that proanthocyanidins can induced Pref-1 expression in 3T3-L1 (Pinent and others 2005b). The PAC of *A. chilensis* and commercial powder of *V. floribundum* also showed a modest inhibition (14 %) in adipogenesis as well as an elevation in Pref-1 expression; nevertheless their effects were not as potent as PAC of *V. floribundum*. HPLC-MS analysis performed in this study indicated that PAC of *V. floribundum* had a higher degree of polymerization than *A. chilensis* and the commercial powder. Several studies have demonstrated that higher molecular weight proanthocyanidins have a greater biological activity than smaller weight forms (Santos-Buelga, Scalbert 2000, Scalbert 2000, Schmidt and others 2006). Thus, the higher degree of polymerization of proanthocyanidns found in *V. floribundum* PAC may partially explain its higher inhibitory effect on adipogenesis. Nevertheless, further characterization of proanthocyanidins is needed to better understand the relation between the proanthocyanidins structure and their role in adipogenesis.

III. Inflammation

Figure 19 shows that nitrite production was significantly inhibited by four of the nine phenolic extracts as compared to positive control ($p \leq 0.05$).

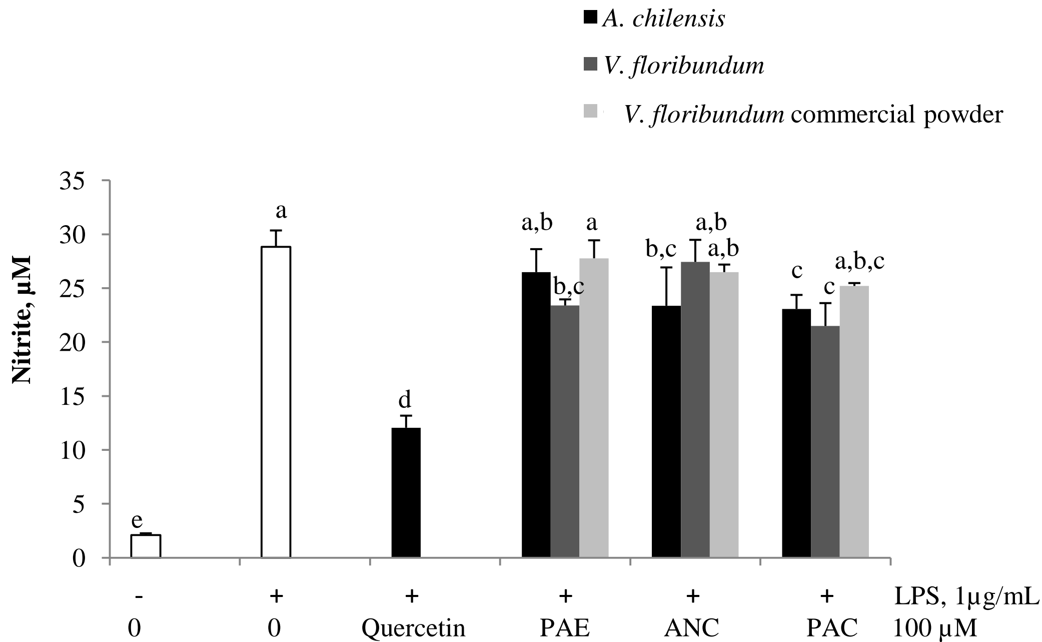

Figure 19. Effect of 100 µM (C3C or epicatechin equivalents) of phenolic extracts (PAE, ANC and PAC) of the two berries and commercial powder on nitrite production in LPS-stimulated RAW 264.7 macrophages. Quercetin was used as a positive control. The data represent the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p \leq 0.05$. Bars indicate SD. Abbreviations: post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

PAC fractions of both berries showed the highest inhibitory effect. In addition, all the phenolic extracts inhibited iNOS expression from 9.8-61.8% (**Figure 20**). PAC from *A. chilensis* showed the highest inhibitory effect (61.8%), which is comparable to the effect exerted by quercetin, a potent anti-inflammatory agent. To further understand the

role of these phenolic extracts on inflammation, the production of PGE_2 and the expression of COX-2 were also determined on LPS stimulated RAW 264.7 macrophages.

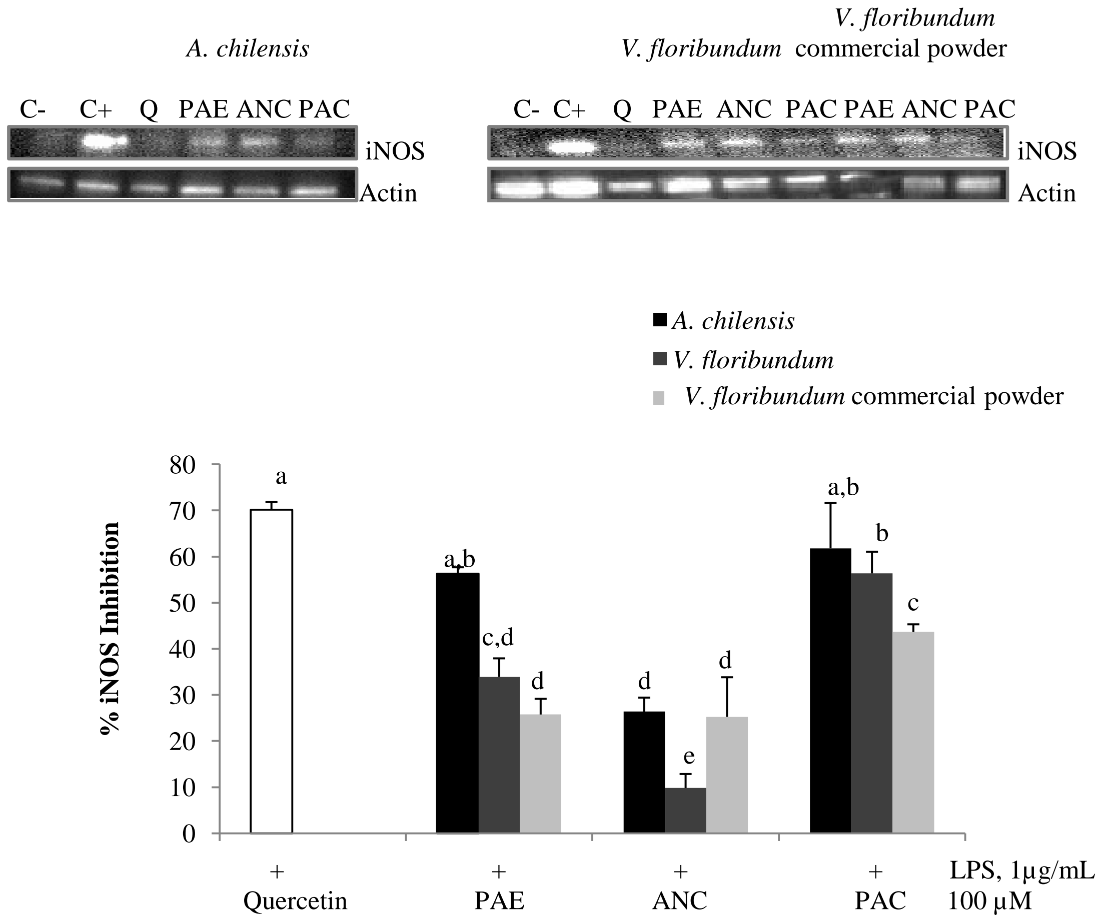

Figure 20. Effect of 100 µM (C3C or epicatechin equivalents) of phenolic extracts (PAE, ANC and PAC) of the two berries and commercial powder on iNOS protein expression by Western blot in LPS-stimulated RAW 264.7 macrophages. Quercetin was used as a positive control. The data represent the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p \leq 0.05$. Bars indicate SD. Abbreviations: Post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

Figure 21 and **Figure 22** show the effect of the phenolic extracts of the two berries and the commercial powder on the production of PGE_2 and the expression of COX-2. **Figure 21** shows eight of the nine phenolic extracts significantly suppressed the

production of PGE_2 as compared to the positive control ($p \leq 0.05$). ANC fraction from *V. floribundum* showed the highest inhibition (89.1%), which was comparable to that of quercetin. In addition, all the phenolic extracts inhibited the expression of COX-2 at levels that ranged from 16.6 to 62.0% (**Figure 22**). The PAC fraction of the commercial powder and the ANC fraction from *V. floribundum* showed the highest inhibitory effect (62 and 58% respectively).

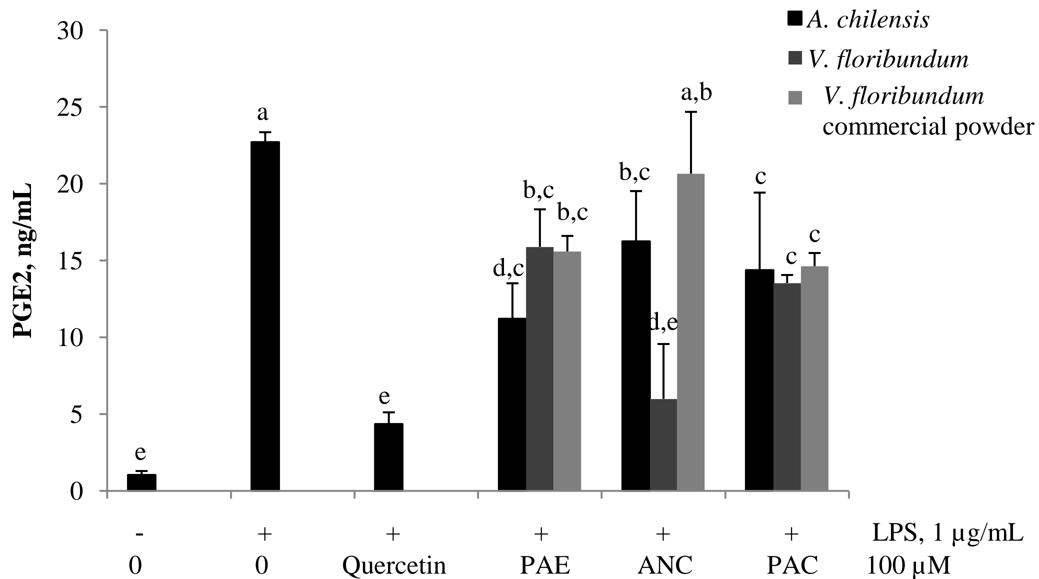

Figure 21. Effect of 100 µM (C3C or epicatechin equivalents) of phenolic extracts (PAE, ANC and PAC) of the two berries and commercial powder on PGE_2 production in LPS-stimulated RAW 264.7 macrophages. Quercetin was used as a positive control. The data represents the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p \leq 0.05$. Bars indicate SD. Abbreviations: Post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

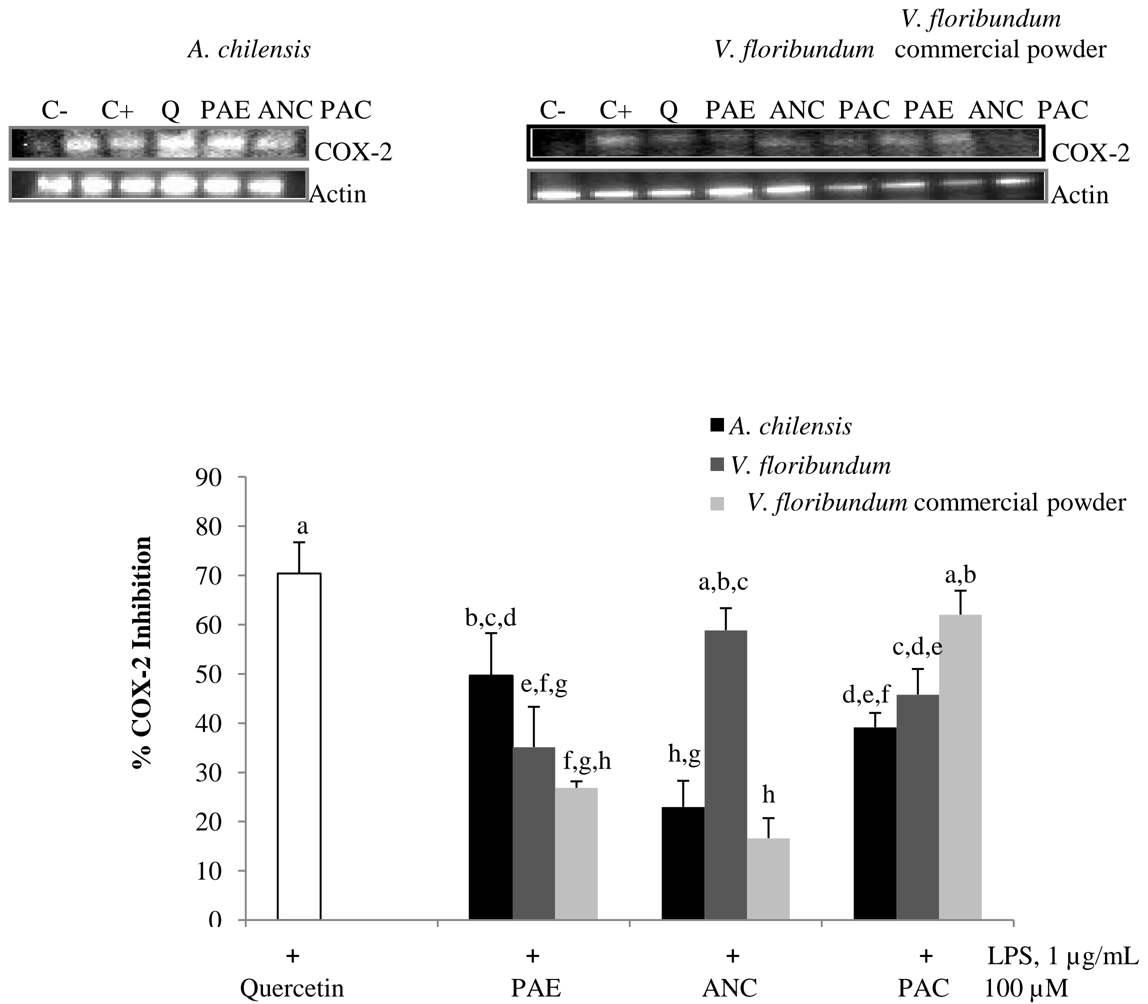

Figure 22. Effect of 100 µM (C3C or epicatechin equivalents) of phenolic extracts (PAE, ANC and PAC) of the two berries and commercial powder on COX-2 protein expression by Western blot in LPS-stimulated RAW 264.7 macrophages. Quercetin was used as a positive control. The data represents the mean ± SD from at least two independent studies and at least triplicate analyses. Different letters indicate significant difference, $p \leq 0.05$. Bars indicate SD. Abbreviations: post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

The results of this study showed that the phenolic extracts from the two berries and the commercial powder decreased inflammation *in vitro* by inhibiting LPS-induced iNOS /NO and COX-2/PGE_2 pathways in macrophages. This is in accordance with several other studies that have shown berries possess anti-inflammatory properties (Defuria and others 2009). Furthermore, anthocyanins have been shown to reduce the levels of inflammatory mediators *in vitro* (Kraft and others 2008, Wang and others 2008, Pergola and others 2006) and *in vivo* (Park and others 2007, Shan and others 2009, Tsoyi and others 2008) . Proanthocyanidins have also been shown to ameliorate inflammation by modulating cytokine expression, inhibiting pro-inflammatory enzymes, or other mechanisms (Terra and others 2009, Wang and others 2009, Ho and others 2007, Diouf, and others 2009).

CHAPTER 7
SUMMARY AND INTEGRATION

Interest in berries from South America has increased in recent years mainly due to their potential health benefits, and growing consumer interest in novel exotic fruit selections in the market place. The present study characterized the anthocyanins and proanthocyanidins of *V. floribundum* and *A. chilensis*, total phenolics and antioxidant capacity, and evaluated, *in vitro*, the ability of their phenolic extracts to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes.

In accordance with previous studies, seven main anthocyanins were identified in the berry of *A. chilensis* and five anthocyanins in the berry of *V. floribundum*. The berry of *A. chilensis* showed a higher concentration as well as a more diverse anthocyanin profile, when compared to the fruit of *V. floribundum*. The commercial powder of *V. floribundum*, although maintaining the same anthocyanin profile, had a lower anthocyanin content as compared with the freeze dried berry. Total proanthocyanidins measured as epicatechin equivalents, were lower in the berry of *A. chilensis* when compared to the freeze dried berries and commercial powder from *V. floribundum.* HPLC-MS analysis revealed a series of proanthocyanidins ranging from dimers to hexamers in both berries and in the commercial powder.

The berries of *A. chilensis* and *V. floribundum* showed similar values of total phenolics, but the AC was higher in the berries of *A. chilensis*, indicating that these berries possess more antioxidant constituents. The commercial powder of *V. floribundum* had a lower phenolic content and AC when compared to the freeze dried berry and this is likely to be a consequence of processing. Total phenolics of the two berries and of the

commercial product showed a high correlation with AC. Anthocyanins were more highly correlated with AC than proanthocyanidins.

The phenolic extracts from the berries from *A. chilensis* and *V. floribundum*, and the commercial powder of *V. floribundum* inhibited lipid accumulation, adipogenesis and inflammatory mediators *in vitro*. PAC fraction from *V. floribundum* markedly inhibited adipogenesis in 3T3-L1 adipocytes by increasing the expression of Pref-1 in preadipocytes. Furthermore, the phenolic extracts inhibited expression of LPS-induced iNOS/NO and COX-2/PGE_2 pathways in RAW264.7 macrophages. The potency of these extracts to reduce inflammation *in vitro* depends on their phytochemical composition. The results of this study show that *A. chilensis* and *V. floribundum* contain phytochemicals that limit adipogenesis and lipid accumulation in fat cells and inflammatory pathways in macrophages *in vitro* and therefore further research *in vivo* should be conducted on these berries.

CHAPTER 8
CONCLUSIONS

- The total anthocyanin content in the berry of *A. chilensis* was 45.7 mg/g DW (C3G equivalents). Seven main anthocyanin structures were identified: delphinidin-3-sambubioside-5-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, delphinidin-3- sambubioside, delphinidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-glucoside. Delphinidin-3-glucoside was the main anthocyanin component in this berry.
- The total anthocyanin content in the berries and in the commercial powder of *V. floribundum* was 10.6 mg/g DW and 2.4 mg/g (C3G equivalents) respectively. Five main anthocyanin structures were identified: delphinidin-3-galactoside, cyanidin-3-galactoside, delphinidin-3-arabinose, cyanidin-3-glucoside, and cyanidin-3-arabinose. Delphinidin-3-arabinose and cyanidin-3-arabinose were the main anthocyanins present in this berry.
- The berry of *A. chilensis* contained 4.0 mg/g DW (epicatechin equivalents), while the berries and commercial powder from *V. floribundum* had a slightly higher concentration of 5.2 and 4.8 mg/g DW (epicatechin equivalents), respectively.
- *A. chilensis* contained mainly proanthocyanidin dimers (56%) and trimers (14%). *V. floribundum* contained trimers (68%) and in less proportion pentamers (16%) and hexamers (8%). The commercial powder contained mainly dimers (34%) and trimers (23%).
- The berries of *A. chilensis* and *V. floribundum* showed similar values of total phenolics , but the AC was higher in the berries of *A. chilensis* .
- The commercial powder of *V. floribundum* had a lower phenolic content and AC than the freeze dried berry and this is likely to be a consequence of processing.

- Total phenolics and AC correlated for *A. chilensis* and both the *V. floribundum* berries and commercial powder with R^2 values of 0.90, 0.86 and 0.78, respectively.
- Anthocyanins were more correlated with AC than proanthocyanins.
- Phenolic extracts inhibited lipid accumulation by 4.0 to 10.8% when adipocytes were treated at maturity and 5.9 to 37.9% when treated throughout differentiation.
- The proanthocyanidin-enriched-fraction from *V. floribundum* significantly induced Pref-1 expression in preadipocytes.
- Phenolic extracts decreased the production of nitric oxide (3.7 - 25.5%) and prostaglandin E_2 (9.1 - 89.1%) and the expression of inducible nitric oxide synthase (9.8 - 61.8%) and cycloxygenase-2 (16.6 - 62.0%) in lipopolysaccharide-stimulated RAW 264.7 macrophages.
- *V. floribundum* and *A. chilensis* phytochemicals modulate adipogenesis and inflammatory pathways *in vitro*, warranting further *in vivo* studies.

CHAPTER 9
FUTURE STUDIES

This study presented a detailed examination of the antioxidant capacity of *V. floribundum* and *A. chilensis* berry extracts, and determined the potential of their phenolic fractions to reduce adipogenesis, lipid accumulation and inflammation *in vitro*.

Further animal studies and human trials should be performed in order to better understand how these berries and their phenolic compounds are absorbed and behave in the human body. Toxicological studies of the berry extracts are also important in order to assess their safety for human consumption. Once the efficacy and safety of the extracts of these berries have been properly evaluated and validated, these can be incorporated in diverse products to increase their value or use as supplements for human diet.

In this study a commercial powder of *V. floribundum* was also evaluated and compared to the freeze dried berry. Further studies can look at different drying conditions for the elaboration of this commercial powder and suggest a process that will minimize phenolic degradation and that will preserve the bioactive properties of the fresh berry. Studies looking at other ways the berries of *V. floribundum* and *A. chilensis* can be processed would be valuable commercialization and increasing the value of these berries.

CHAPTER 10
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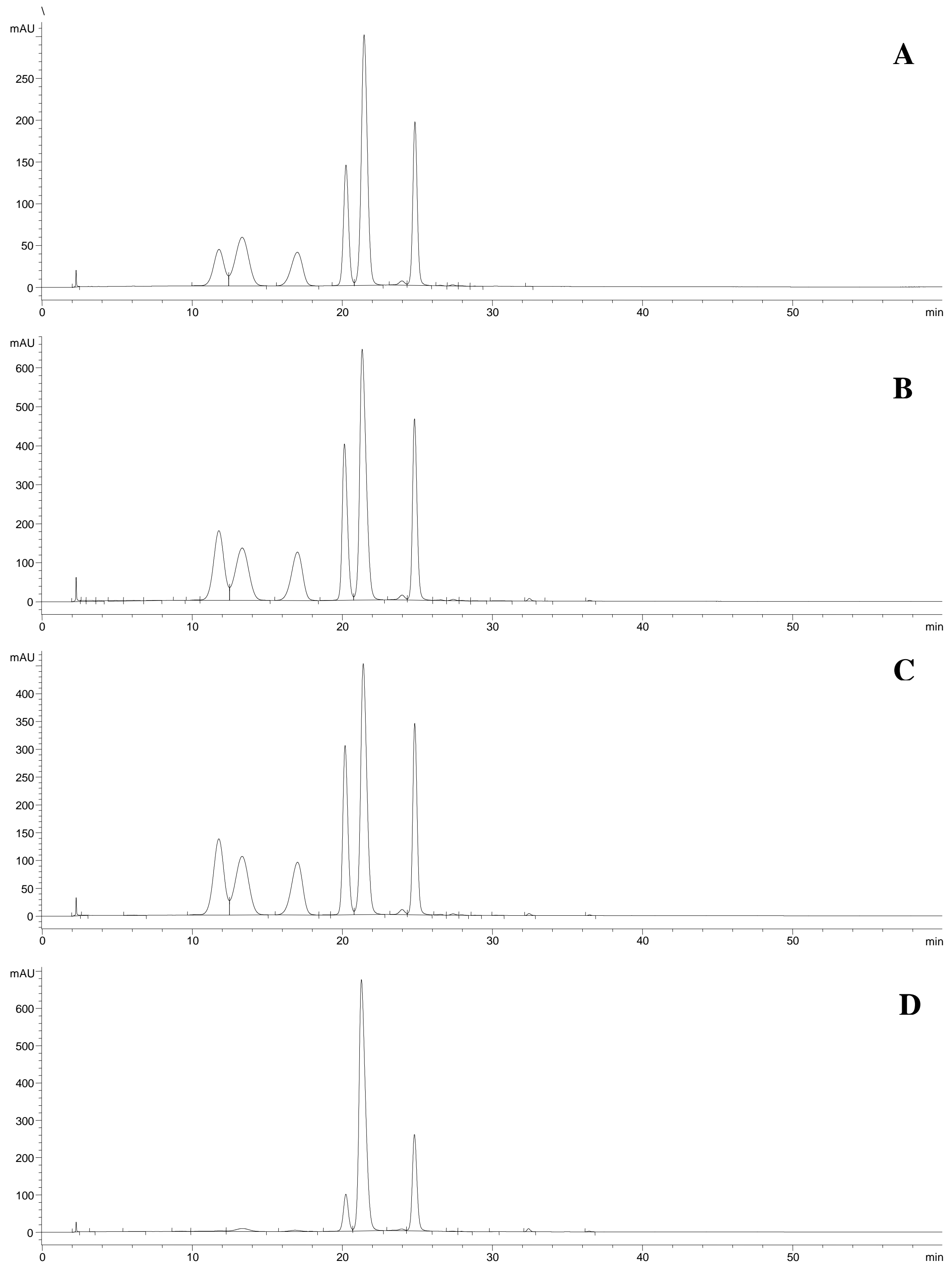
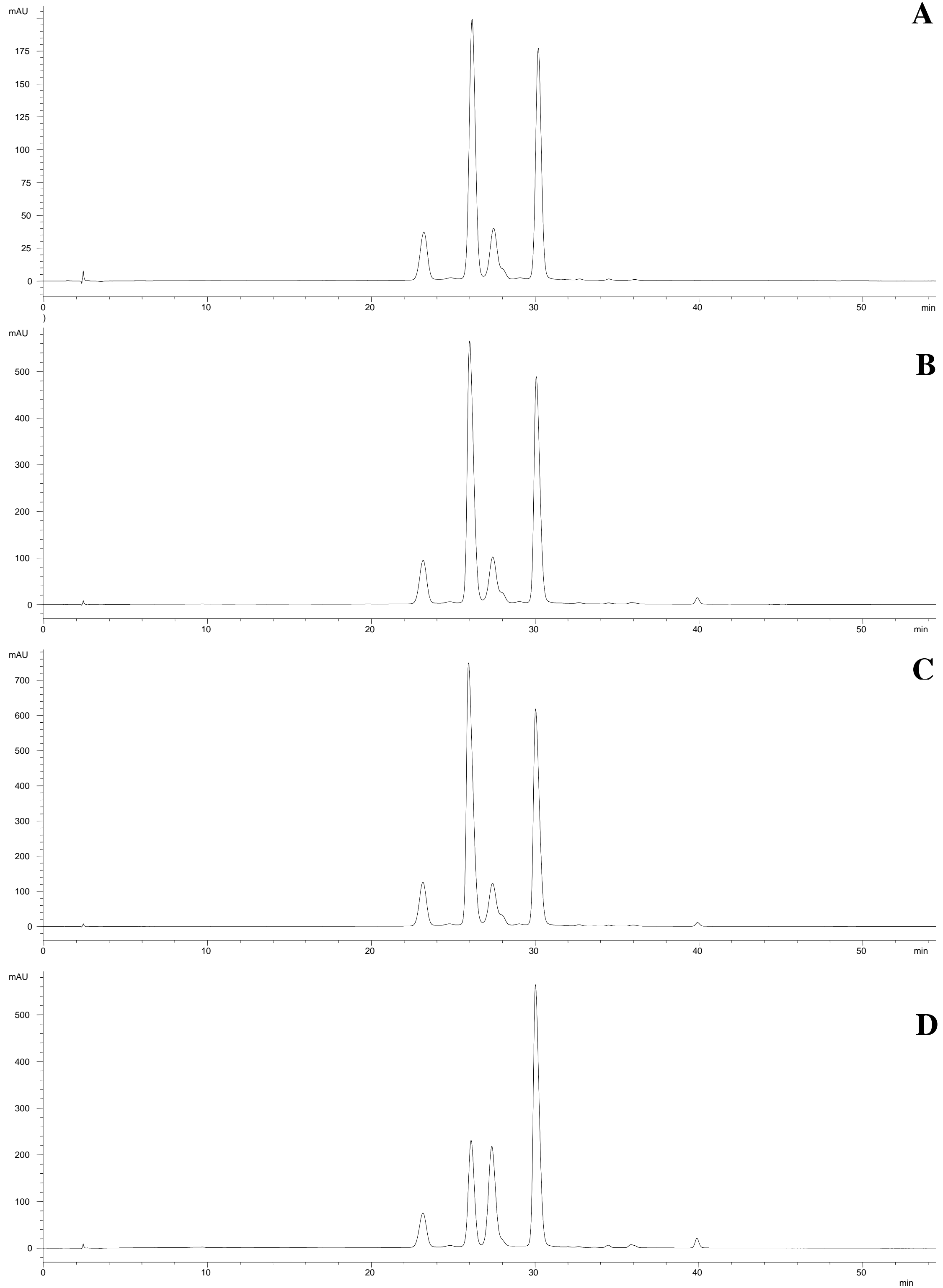
APPENDIX A



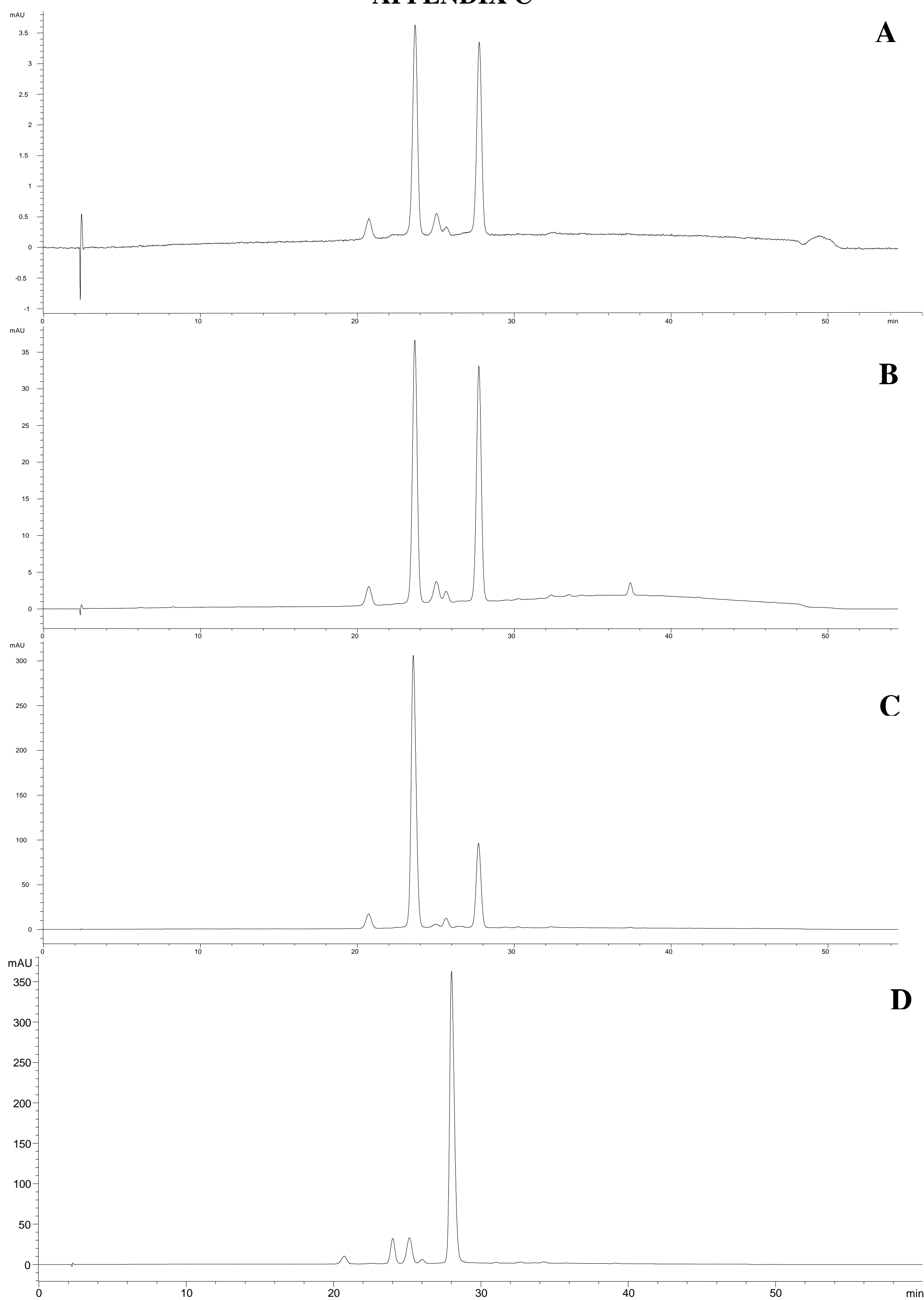
Chromatographic-guided enrichment for anthocyaninis in *A. chilensis* (520 nm). Crude extract (A), post- amberlite extract (B), anthocyanin-enriched-fraction (C), anthocyanin-enriched-fraction 2 (D).

APPENDIX B



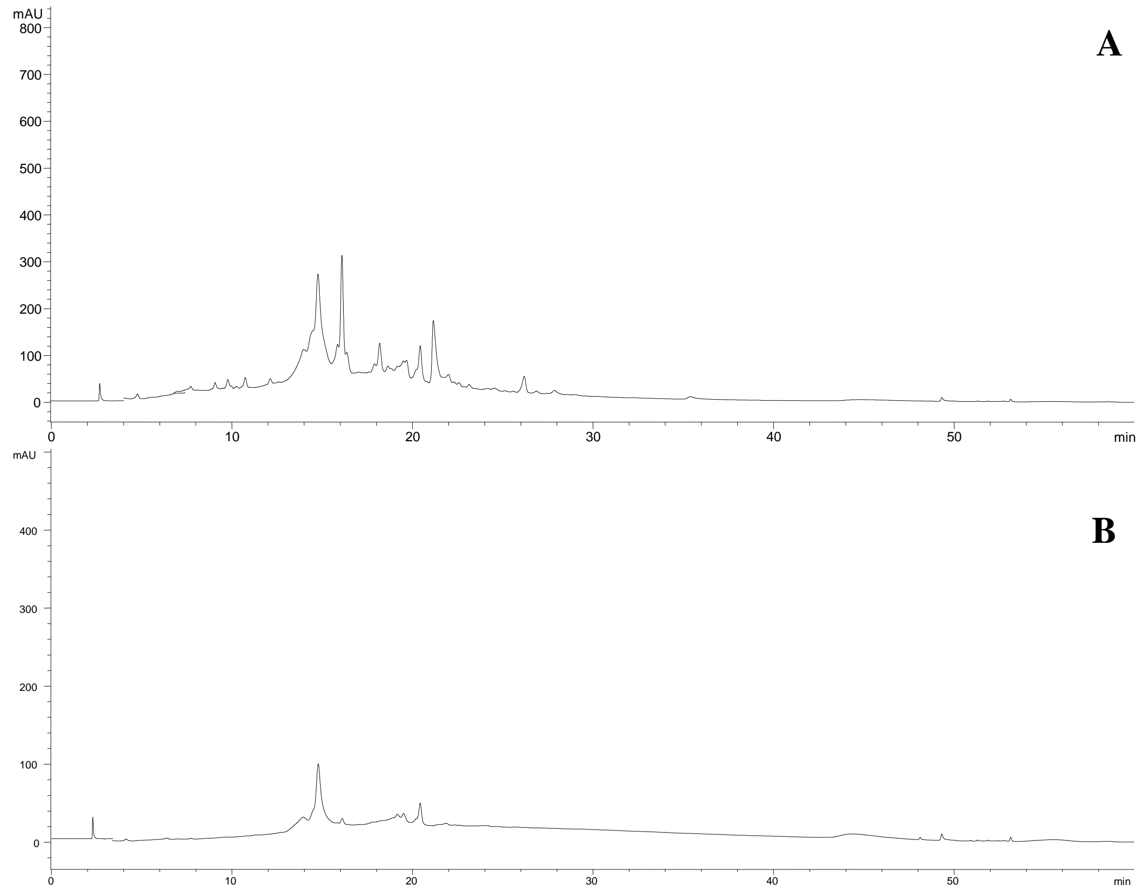
Chromatographic-guided enrichment for anthocyaninis in *V. floribundum* (520 nm). Crude extract (A), post- amberlite extract (B), anthocyanin-enriched-fraction 1 (C), anthocyanin-enriched fraction 2 (D).

APPENDIX C



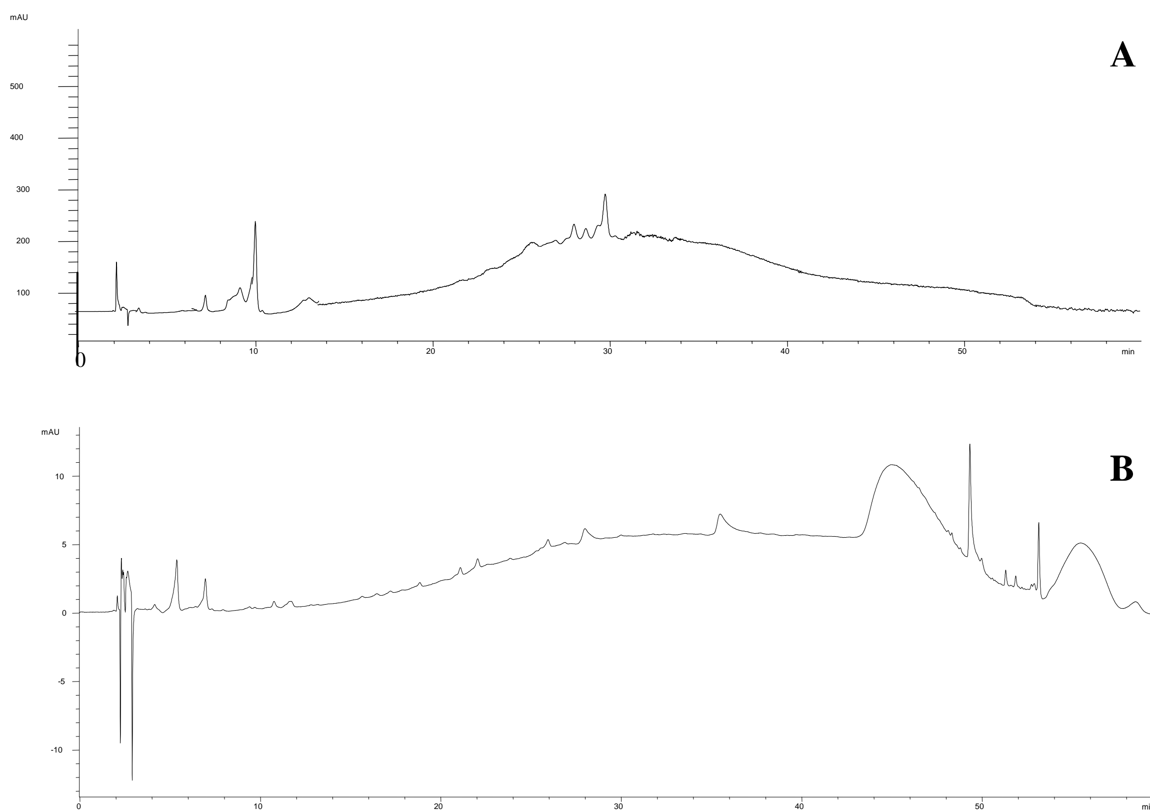
Chromatographic-guided enrichment for anthocyaninis in the *V. floribundum* commercial powder (520 nm). Crude extract (A), post- amberlite extract (B), anthocyanin-enriched-fraction 1 (C), anthocyanin-enriched-fraction 2 (D).

APPENDIX D



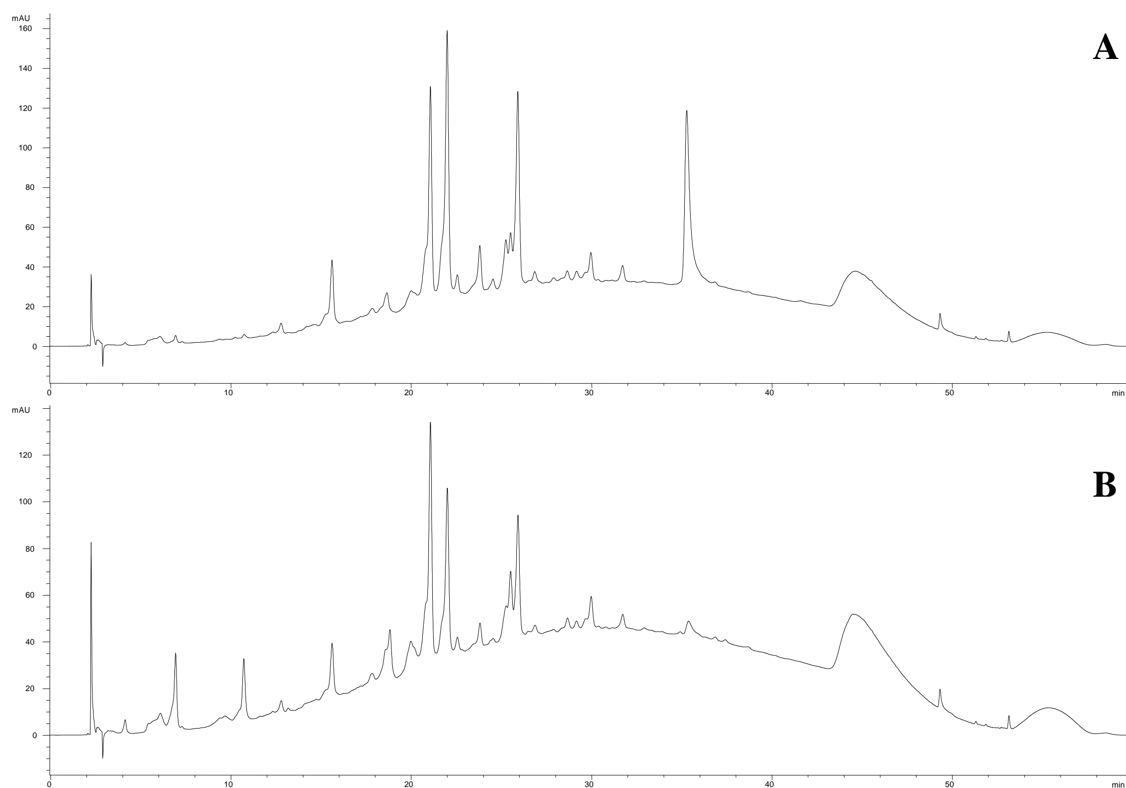
HPLC chromatogram (280 nm) of the proanthocyanidin-enriched-fraction1 (**A**) and proanthocyanidin-enriched-fraction 2 (**B**) of *A. chilensis.*

APPENDIX E



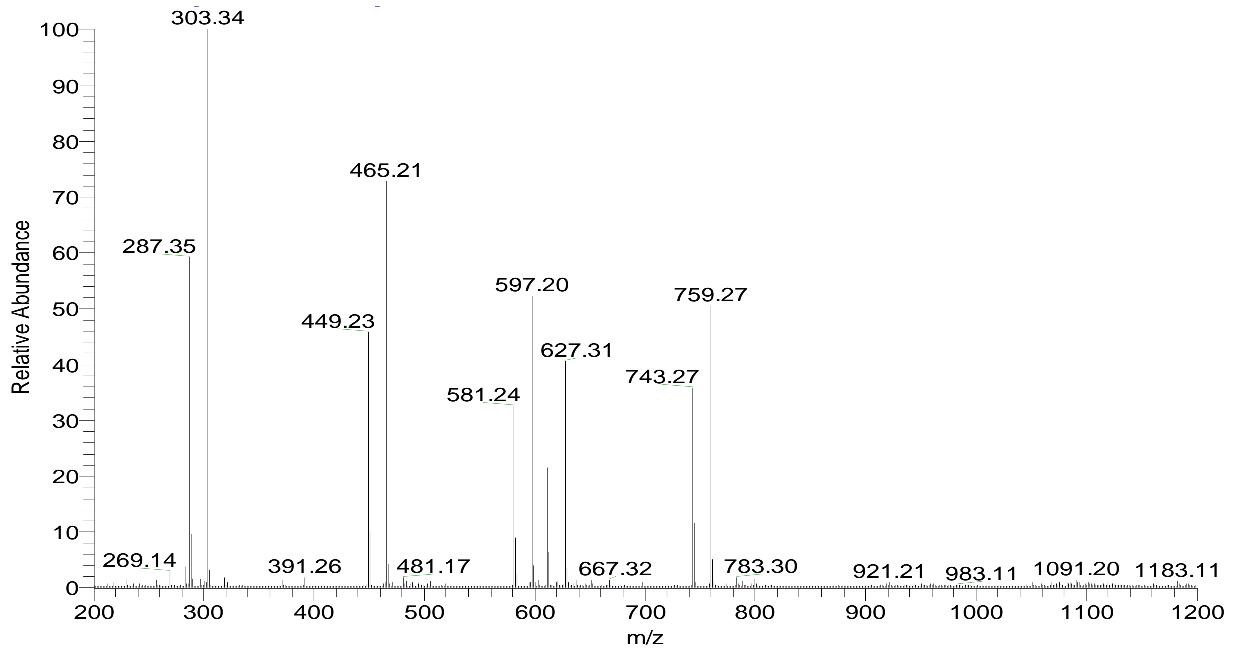
HPLC chromatogram (280 nm) of the proanthocyanidin-enriched-fraction1 (**A**) and proanthocyanidin-enriched-fraction 2 (**B**) of *V. floribundum.*

APPENDIX F



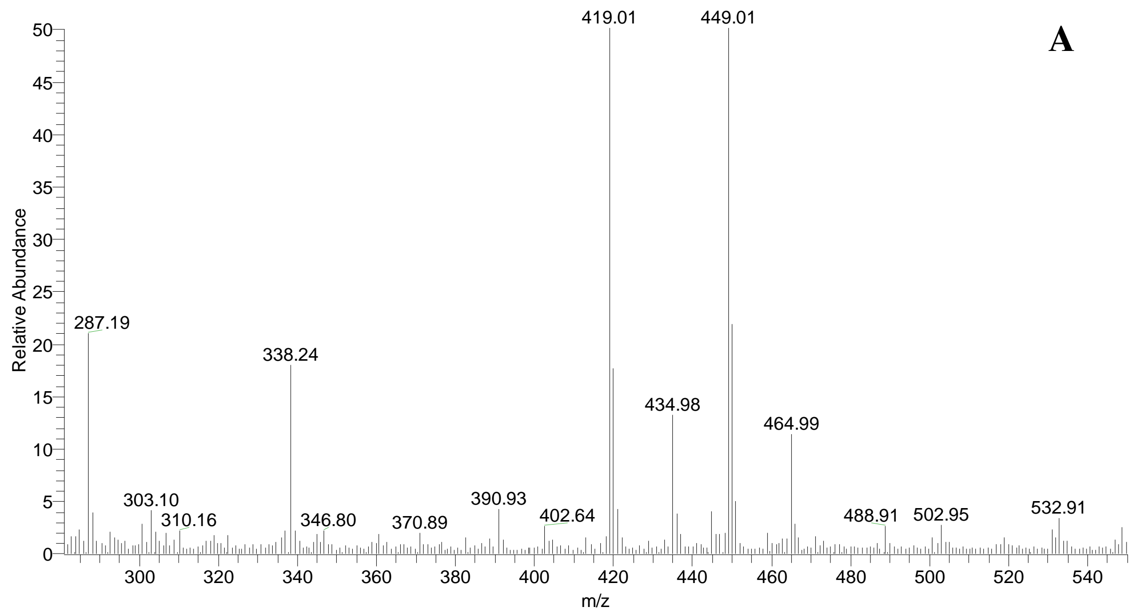
HPLC chromatogram (280 nm) of the proanthocyanidin-enriched-fraction1 (**A**) and proanthocyanidin-enriched-fraction 2 (**B**) of *Vaccinium floribundum* commercial powder.

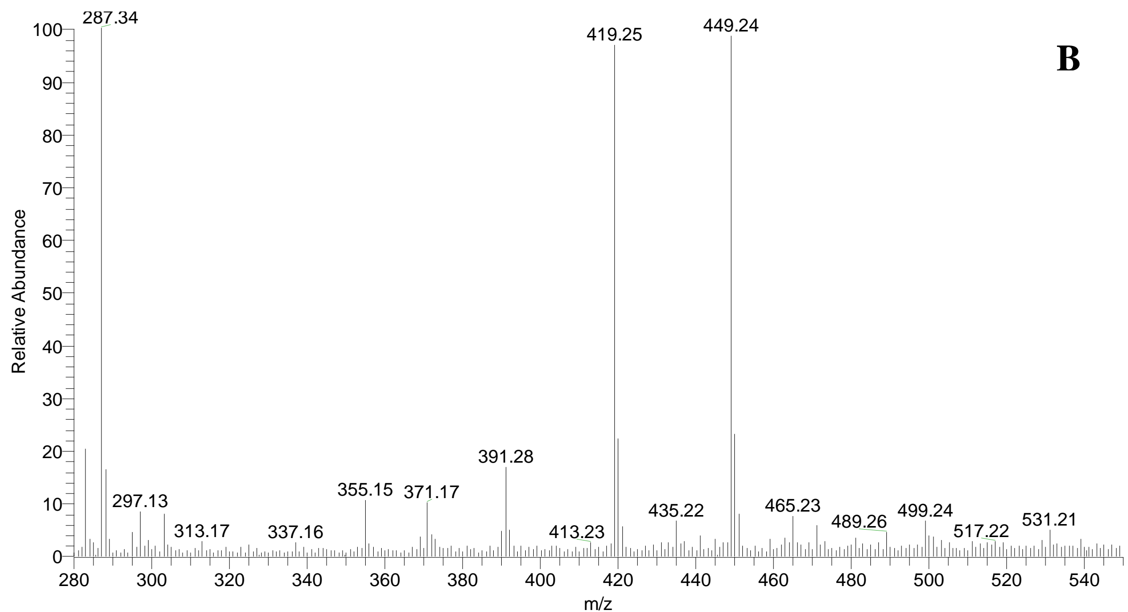
APPENDIX G



LC-MS ion spectrum showing the molecular ion [m^+] (m/z) of the major anthocyanins in the berry of *A. chilensis:* delphinidin-3-sambubioside-5-glucoside (*m/z*: 759), delphinidin-3,5-diglucoside (*m/z*: 627), cyanidin-3-sambubioside-5-glucoside (*m/z*: 743), delphinidin-3-sambubioside (*m/z*: 597), delphinidin-3-glucoside (*m/z*: 465), cyanidin-3-sambubioside (*m/z*: 581), cyanidin-3-glucoside (*m/z*: 449).

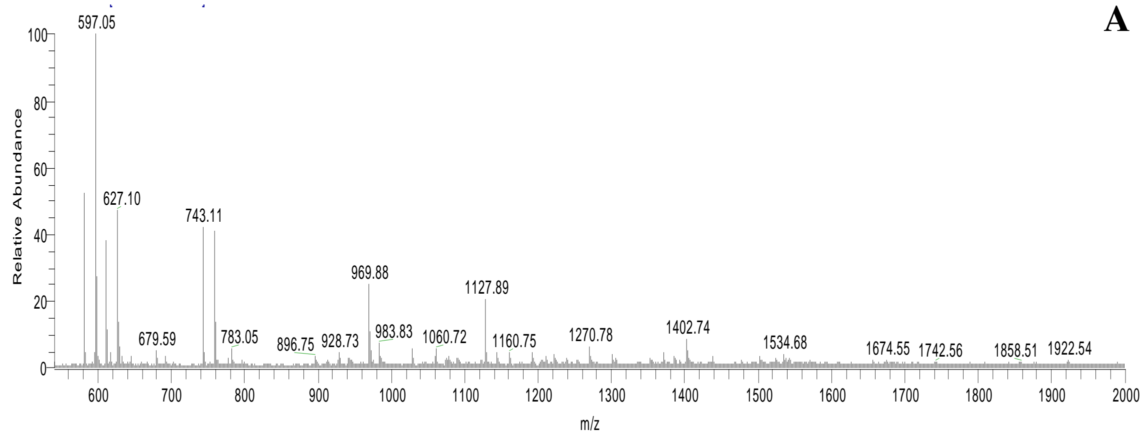
APPENDIX H

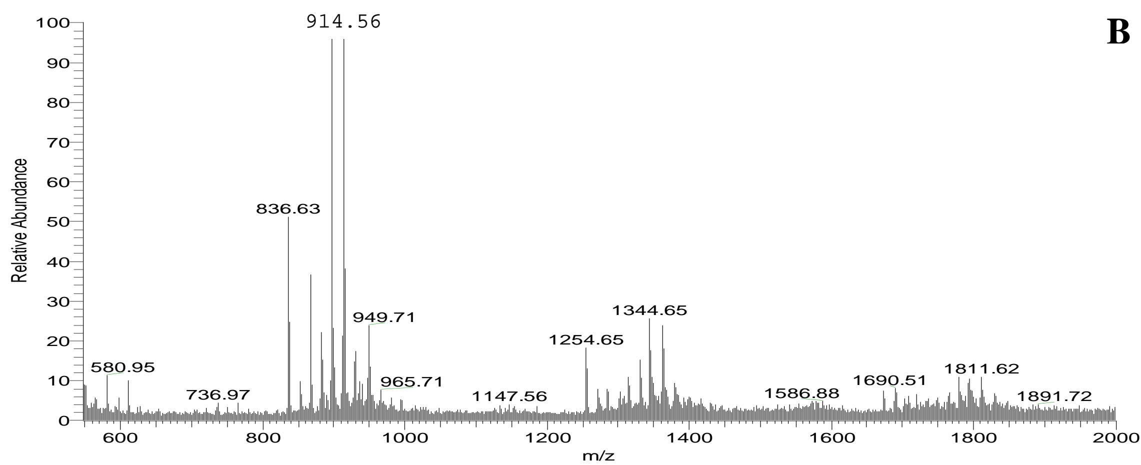


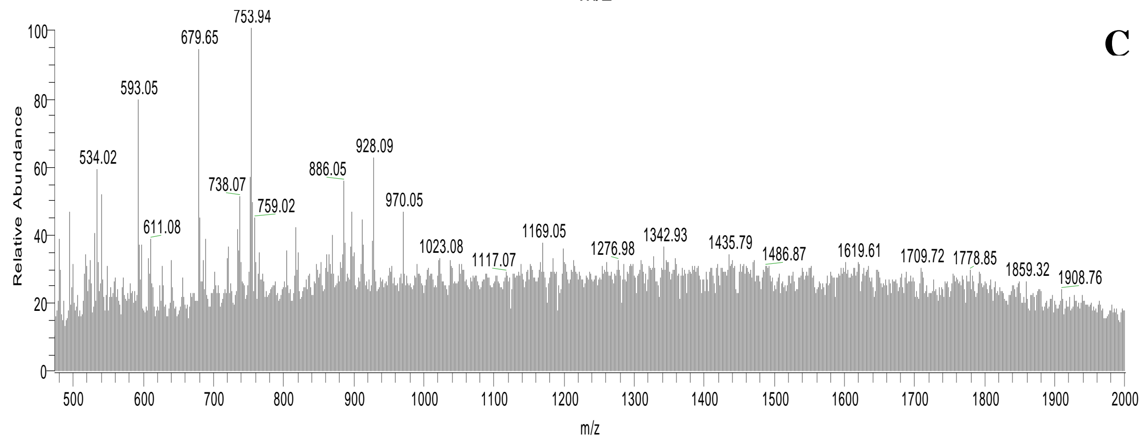


LC-MS ion spectrum showing the molecular ion [m^+] (m/z) of the major anthocyanins in the berry of *V. floribundum* (**A**) and in the commercial powder (**B**): Delphinidin-3-galactoside (*m/z*: 465), cyanidin-3-galactoside (*m/z*: 449), delphinidin-3-arabinose (*m/z*: 435), cyanidin-3-glucoside (*m/z*: 449), cyanidin-3-arabinose (*m/z*: 419).

APPENDIX I

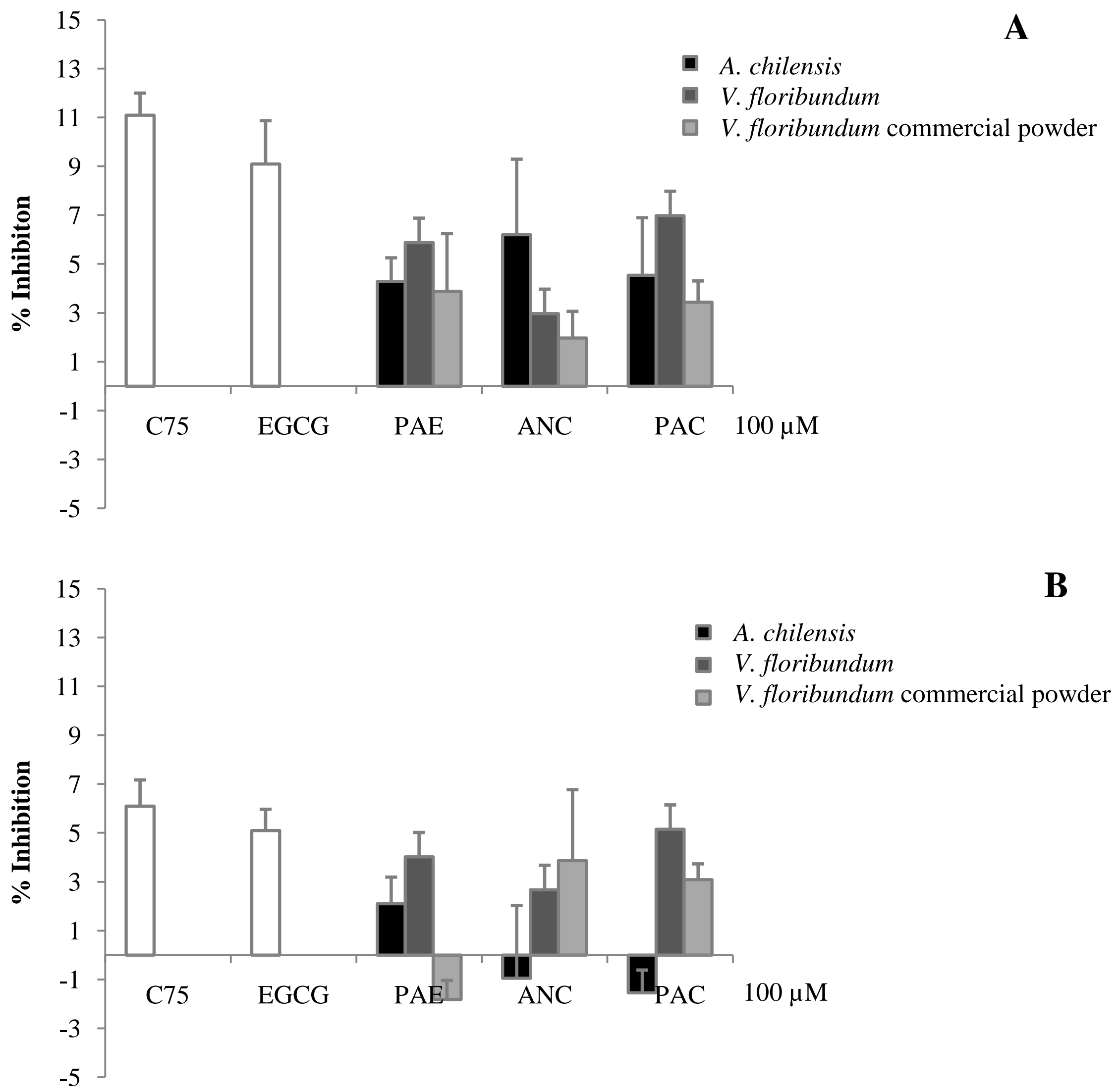






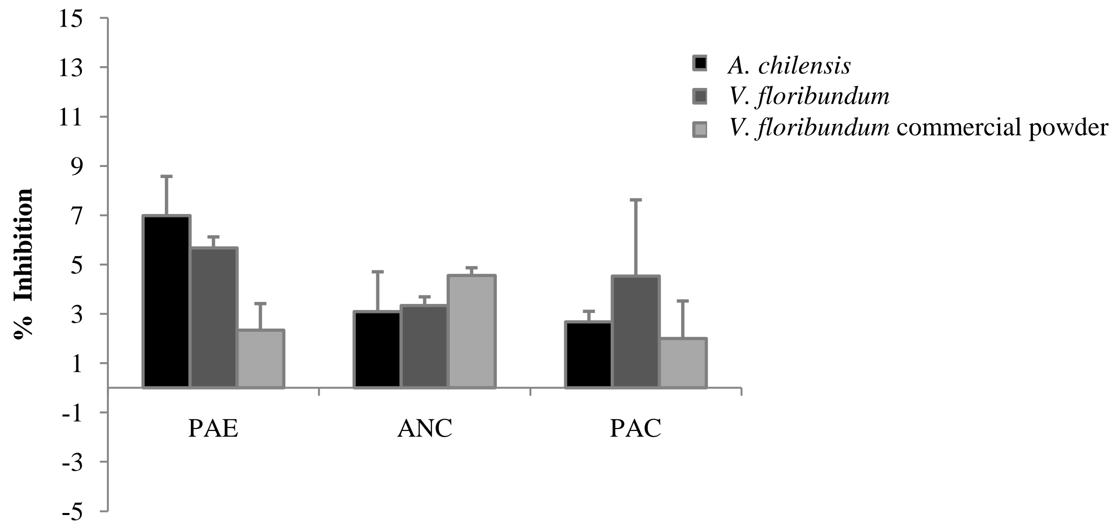
LC-MS ion spectrum showing the the molecular ion $[m^+]$ (m/z) of the major proanthocyanidins oligomers and polymers in the berry of *Aristotelia chilensis* (A) *Vaccinium floribundum* (B) and the commercial powder of *Vaccinium floribundum* (C)

APPENDIX J



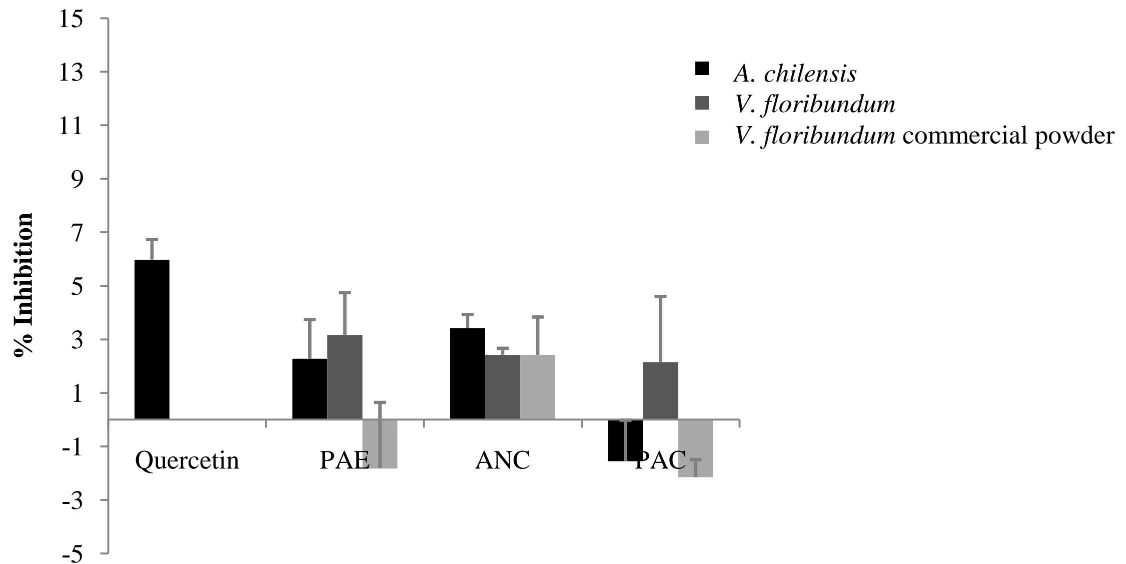
Inhibition of cell viability (%) by phenolic extracts (PAE, ANC and PAC) of the two berries and the commercial powder and positive controls (EGCG and C75) when 3T3-L1 adipocytes were treated throughout the differentiation process (**A)** and when adipocytes received a single treatment at mature stage (**B**). 3T3-L1 adipocytes were treated with 100 µM of each phenolic extract (equivalent C3C or epicatechin) and positive controls. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Abbreviations: ECGC (epigallocatechin-3-gallate), post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

APPENDIX K



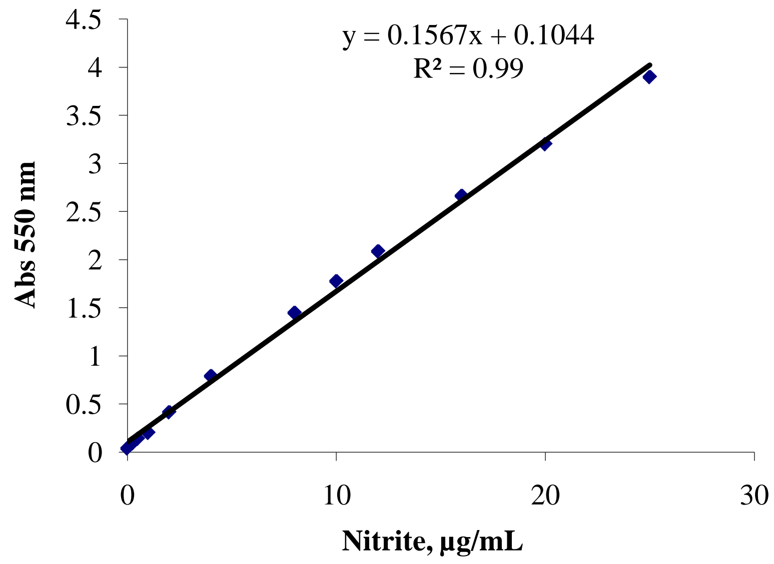
Inhibition of cell viability (%) by phenolic extracts (PAE, ANC and PAC) of the two berries and the commercial powder when 3T3L1 preadipocytes were treated with 100 µM of each phenolic extract (C3C or epicatechin equivalents) and control for 24 h at 37 ºC in a humidified 5% CO_2 incubator. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Abbreviations: post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

APPENDIX L



Inhibition of cell viability (%) by phenolic extracts (PAE, ANC and PAC) of the two berries and the commercial powder and positive control (quercetin) when RAW 264. macrophages were treated with 100 µM of each phenolic extract (C3C or epicatechin equivalents) and control for 24 h at 37 ºC in a humidified 5% CO_2 incubator. The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis. Abbreviations: post- amberlite extract (PAE), anthocyanin-enriched-fraction (ANC) or proanthocyanidin-enriched-fraction (PAC).

APPENDIX M



Standard curve used to measure nitric oxide production in RAW 264.7 macrophages.

CURRICULUM VITAE

Maria Elisa Schreckinger

EDUCATION

- University of Illinois at Urbana-Champaign; GPA: 3.70/4.0
  Masters of Science in the Division of Nutritional Sciences, Expected Graduation May 2010
- Universidad San Francisco de Quito, Quito, Ecuador; GPA: 3.81/4.0
  Bachelor of Science in Biotechnology, May 2008
- Virginia Tech, Blacksburg, VA; GPA: 3.43/ 4.0
  Exchange program, 2005-2006

TECHNICAL SKILLS

- HPLC/HPLC-MS
- Total polyphenol content
- Antioxidant capacity
- SDS-PAGE electrophoresis
- Western blot analysis
- Cell culture
  - 3T3L1 adipocytes
  - RAW 264.7 macrophages
- Excel, Word, Power Point, SAS

PUBLICATIONS

- Schreckinger, ME; Lotton J; Lila, MA; Gonzalez de Mejia, E. Berries from South America: A comprehensive review on chemistry, health potential and commercialization. *J. Med. Food* **2010**, *13,* 1–14.

- Schreckinger, ME; Wang J; Yousef G; Lila, MA; Gonzalez de Mejia, E. Antioxidant capacity and *in vitro* inhibition of adipogenesis and inflammation by phenolic extracts of *Vaccinium floribundum* and *Aristotelia chilensis* (submitted to the Journal of Agriculture and Food Chemistry).

WORK EXPERIENCE

Research Assistant University of Illinois, Division of Nutritional Sciences (August 2008-Present)

- Conducting research on the chemistry and the metabolic enhancing properties of berries from South America.

*Lab Assistant at Universidad San Francisco de Quito***,** Laboratory of Microbiology and Molecular Biology. (June- September 2007).

- Assist in the identification of pathogenic *Escherichia coli* strains in an epidemiological study preformed in a northern region of Ecuador.

Lab Technician at Virginia Polytechnic Institute and State University, Plant Pathology, Physiology and Weed Science Department. (June – August 2006)

- Assist in current research about the evolution of the plant pathogen *Pseudomona syringae*.

Hospital Carlos Andrade Marin, Department of Microbiology. Quito, Ecuador, June – July 2005

- Learn to utilize different techniques and equipment to make microbiological analysis of urine, blood and tissue samples.

SELECTED AWARDS AND ACTIVITIES

- **Global Institute for BioExploration Scholarship** awarded in 2008 to 2010.
- **Scholarship** for students of Science at Universidad San Francisco de Quito, awarded in 2003 to 2008.
- **Dean's List and President's List** for five semesters at Universidad San Francisco, Quito, Ecuador.
- **Scholarship** at Virginia Tech, Fall 2005- Spring 2006, granted through the VT-Ecuador Student Exchange Program.
- **Secretary of the Ecuadorian Association** at University of Illinois at Urbana-Champaign

RELATED COURSE WORK

- Biochemical Nutrition
- Bioactive Components in Food
- Plant Secondary Metabolism
- Advanced Food Microbiology
- Principles of Nutrition
- Applied Statistical Methods I
- Food Safety: Biotechnology and GMOs
- Epigenetic Aspects of Human Diseases
- Plant Biochemistry

FOREIGN LANGUAGE SKILLS

- Native Spanish, fluent English, intermediate Italian, basic French.