Evaluation of food matrix interactions and *in vitro* gastrointestinal digestion on the bioefficacy of polyphenols from blueberries (*Vaccinium* sp.)

by

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ABSTRACT

Evaluation of food matrix interactions and *in vitro* gastrointestinal digestion on the bioefficacy of polyphenols from blueberries (*Vaccinium* sp.)

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Bluberries (*Vaccinium* sp.) are rich in polyphenols that are responsible for lowering the risk of developing several chronic degenerative diseases. However, the effect of food matrix interactions on the bioaccessibility and bioavailability of polyphenols is not well understood. In this research free and complexed polyphenols found in blueberry extracts were characterized and their antioxidant activity as well as antiproliferative activities against colon cancer cells (HT-29) and normal colon cells (CRL-1790) were evaluated. The blueberry food matrix and different carbohydrate-rich synthetic matrices were characterized and their biological activities assessed alone and in complexed state with polyphenols. The degradation of polyphenols during their transit through the gastrointestinal tract (GIT) was evaluated using an *in vitro* digestion model. Biological activities of blueberry polyphenols and their parent metabolites produced during colonic fermentation were estimated by *in vitro* antioxidant assays and cell proliferation analysis using HT-29 and CRL-1790 cell lines. HPLC analysis revealed the presence of 7 phenolic compounds and 13 anthocyanins in all samples. Although the concentration of the polyphenols varied among the samples, free and complexed polyphenols showed significant antioxidant and antiproliferative activities. Polyphenol complexes were analyzed using transmission electron microscopy (TEM) revealing the presence of electron dense complexes ranging from 100 – 200
nm. Pectinase treatment disrupted the structure of the complexes, suggesting the pectin nature of the polyphenol complexes. The antioxidant- and antiproliferative activities of the blueberry food matrix alone was below 10% compared to almost 90% and 70% of free and complexed polyphenols, respectively. Polyphenols and anthocyanins were highly stable during simulated gastric digestion step with approximately 93% and 99% of recovery, respectively. The intestinal digestion process decreased the polyphenol- and anthocyanin- contents by 49% and 15 % respectively. During colonic digestion, the complex polyphenol mixtures were degraded to a limited number of phenolic compounds. Only acetylated anthocyanins were detected in low amounts after the colonic digestion process. After simulated colonic digestion, the isolated catabolites showed lowered antioxidant activity and cell growth inhibition potential. Understanding the interactions that occur among polyphenols and different food matrices may help to produce more stable foods with better bioavailability.

**Key words**: blueberries, food matrix, HT-29, CRL-1790, polyphenols, *in vitro* digestion, chemostat.
This Thesis is dedicated to Jesus and Mother Mary
to whom I owe my PhD
and to my beloved parents
for their endless love and ultimate support.

❤Los amo❤

Si para recobrar lo recobrado
tuve que haber perdido lo perdido,
si para conseguir lo conseguido
tuve que soportar lo soportado.

Si para estar ahora enamorada
fue menester haber estado herida,
tengo por bien sufrido lo sufrido
tengo por bien llorado lo llorado.

Porque después de todo he comprendido
que no se goza bien de lo gozado
sino después de haberlo padecido
porque después de todo he comprobado
que lo que tiene el árbol de florido
vive de lo que tiene sepultado.

*Sta. Teresa de Ávila*

“Let food be thy medicine and medicine be thy food” — Hippocrates
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CHAPTER 1
INTRODUCTION

1.1 General Introduction

Blueberries (Vaccinium sp.) have one of the highest amounts of phytochemicals among berry fruits. The most abundant phytochemicals in blueberries are polyphenols, with the anthocyanin group as the most predominant one. Anthocyanins are not only pigments, but they are also functional compounds that influence biological processes that maintain health in humans (Rolle and Guidoni, 2007; Llaudy et al., 2004; Stark et al., 2005). Some of the most significant functions of polyphenols include their ability to inhibit growth and cytotoxicity to various cancer cells lines (Kalt and Dufur, 1997; Kuo, 1998; Smith et al., 2000; Kang et al., 2003). In addition, anthocyanins have been shown to inhibit the activity of enzymes such as α-amylase and α-glucosidase in vitro, and their presence in the diet may help to reduce the indices of metabolic syndrome and prevent the development of obesity and diabetes (Shetty, 2001; Tsuda et al., 2003; McDugall and Stewart, 2005).

The biological activity of anthocyanins is influenced by the food matrix interactions with components such as carbohydrates, proteins, lipids, etc. Their release during digestion is critical for their ability to exert a biological function. The interactions between various components in the food to form interactive structures and how such structures affect the function of single components, is a subject of intense investigation. It has already been demonstrated that the food matrix has a very strong influence on both absorption and metabolism of functional compounds (Mazza and Kay, 2008). In order for a phytochemical, such as anthocyanins to exert its influence, it must necessarily reach the target organ at an optimal concentration effective for biological action (Holst and Williamson, 2004). Any physiological effects are dependent on sufficient bioavailability, a term that refers to the proportion of the nutrient that is digested,
absorbed and metabolized through biochemical pathways. The importance of physicochemical characteristics of functional compounds and their interactions with the food matrix become apparent when analyzing their implications in human health (During, 2008). This dissertation reports the results of investigations on the influence of the food matrix and the human gastrointestinal digestion on the stability and bioactivity of blueberry polyphenols.

To better understand the influence of the food matrix on the functional properties of blueberries, free polyphenols were separated from the complexed polyphenols using dialysis. Free- and complexed polyphenols were identified, quantified and tested for their in vitro antioxidant and antiproliferative activity (Chapter 4). To further elucidate the ultra-structure of polyphenol complexes isolated from blueberry, samples were treated with different ionic- and non-ionic surfactants, to evaluate if this treatment would release the polyphenols associated with the food matrix. Transmission Electron Microscopy (TEM) along with an enzymatic treatment using pectinase and cellulase were used to confirm the nature of the complexes. Also, the ability of free polyphenols to form complexes was studied by mixing pectin and cellulose with free polyphenols, and evaluating if these synthetic model matrices would simulate and affect the functional properties of the polyphenols (Chapter 5).

Previous research from our group has suggested that food matrix might play an important role in the stability of blueberry polyphenols during gastrointestinal passage, as well as in the bioaccessibility and bioavailability of their metabolites. To gain a deeper understanding of this possibility, blueberry samples were digested using an in vitro digestion system (Chapter 6). An in vitro gastrointestinal model comprising sequential chemostat fermentation steps simulating digestive conditions in the stomach, the small intestine and the colon, was used to investigate the breakdown of blueberry polyphenols. The catabolic products were isolated and biological effects tested using a normal colonic epithelial cell line (CRL-1790) and a human colorectal cancer cell line (HT-29).
1.2 Research hypothesis

The overall target of this study was to investigate the influence of the food matrix on the bioaccessibility and bioavailability of polyphenols from wild blueberry fruits. Furthermore, the aim was to evaluate the effect of the human digestive processes on the stability and bioavailability of blueberry polyphenols. Understanding the factors that govern the bioavailability and absorption of blueberry phytochemicals could help to develop storage and processing techniques that enhance the quality and functionality of blueberries.

**HYPOTHESIS:** The food matrix has an extensive effect on the stability, bioaccessibility and bioavailability of blueberry polyphenols.

**GOAL:** To increase the understanding of the food matrix and its influence in the stability, bioaccessibility, and bioavailability, which can potentially affect the biological action of blueberry polyphenols.

**OBJECTIVES:**

The following are the objectives of this research:

1. Isolate and characterize polyphenols that are free, and that are complexed with the food-matrices, and analyze their antioxidant properties, antiproliferative activity against colon cancer cells, and α-glucosidase-inhibitory activities (Chapter 4).

2. Assess the *in vitro* antioxidant and antiproliferative activity of polyphenols complexed with carbohydrate-rich matrices, and characterize the structure of the complexes (Chapter 5).
3. Evaluate the stability of blueberry polyphenols during passage through an *in vitro* human gastric digestion model, identify their metabolites and evaluate the changes in antioxidant function (Chapter 6).

4. Study the effect of *in vitro* digestion on the antiproliferative properties of blueberry polyphenols and their major identified metabolites on the growth and proliferation of normal colon epithelial cells (CRL 1790) and colon cancer cells (HT29) (Chapter 6).
CHAPTER 2
LITERATURE REVIEW

2.1 Blueberry

Since Neolithic times, edible berries have formed a part of the human diet, first as wild crops, and subsequently as cultivated species. Therefore, in many cultures throughout history, berries are likely to have been important sources of dietary components such as polyphenols, fiber, vitamin C, and folic acid that are now recognized as being essential for health (Rieger, 2006). Increased intake of berries is increasingly being seen as beneficial to health with numerous epidemiological studies suggesting that regular and long-term consumption of fruits and vegetables in sufficient amounts decreases the risk of developing chronic diseases such as heart disease, cancer, and lowers the rate of premature mortality (Pomerlau et al., 2003; Riboli and Norat, 2003). Since 1997 these benefits have been used in blueberry marketing campaigns (Strik, 2007).

Although there are approximately 400 species of blueberry (Vaccinium sp., family Ericaceae), the most important blueberries cultivated worldwide are the highbush (V. corymbosum), southern highbush (complex hybrids based largely on V. corymbosum and V. darrowi Camp.), rabbiteye blueberry (V. ashei) and the lowbush blueberry (native clones of Vaccinium angustifolium Ait. with some clones of Vaccinium myrtilloides Mich) (Camp, 1945). According to FAO, blueberries are produced commercially in 16 countries worldwide in about 72,643 Ha and increasing in cultivation yearly (Table 2.1).
Table 2.1 Production of blueberry crops worldwide in 2009

<table>
<thead>
<tr>
<th>Berry crop</th>
<th>Region</th>
<th>Area (Ha)</th>
<th>Production (tons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberry</td>
<td>Africa</td>
<td>9</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Asia</td>
<td>120</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>Europe</td>
<td>12,005</td>
<td>40,403</td>
</tr>
<tr>
<td></td>
<td>North America</td>
<td>59,995</td>
<td>269,581</td>
</tr>
<tr>
<td></td>
<td>Oceania</td>
<td>539</td>
<td>2,700</td>
</tr>
<tr>
<td></td>
<td>South America</td>
<td>25</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Worldwide</td>
<td>72,643</td>
<td>313,565</td>
</tr>
</tbody>
</table>

Source: FAO, 2009
The total production of blueberries in 2009 was approximately 313,565 tons, with the United States being the major producer at almost 54% of the world total production (Table 2.2). In Canada and the United States, most of the wild blueberries are managed and harvested for commercial production with more than 97% of the total production being processed. Among all types of blueberries, wild blueberries accounted for 68% of the total processed blueberries produced in North America and this level of the market share is expected to continue (Strik, 2007).

North American blueberries, both wild and cultivated, are grown throughout the Eastern and Northeastern U.S., Pacific Northwest, Wisconsin, Michigan, and much of Canada including British Columbia, parts of Quebec, Ontario and Nova Scotia (Camire, 2000). According to Agri-Food Canada (2010), Canada is the world's second-largest producer and exporter of cultivated (high-bush) blueberries and the world's largest producer of wild blueberries (low-bush). The commercial production of blueberries has increased in Canada over the years. Wild blueberry production (~45000 tons) in 2005 was estimated at 65 million dollars in farm gate value and the high-bush blueberry production (~26000 tons) at 73 million dollars. The total export market value of fresh and frozen blueberries was estimated at 259 million dollars. In addition, blueberries are also being processed into powder, juice, and smoothies that are becoming more and more commonly consumed foods in recent times. North America had a total low-bush blueberry production of 111,058 tons in 2003, and it is expected that the managed area of low-bush blueberries will increase 10% by 2013 (Strik, 2007)
Table 2.2 Top 20 highest producing blueberry countries for 2009.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Country</th>
<th>Production tons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>USA</td>
<td>166831</td>
</tr>
<tr>
<td>2</td>
<td>Canada</td>
<td>102750</td>
</tr>
<tr>
<td>3</td>
<td>Poland</td>
<td>11023</td>
</tr>
<tr>
<td>4</td>
<td>Germany</td>
<td>9940</td>
</tr>
<tr>
<td>5</td>
<td>Netherlands</td>
<td>5389</td>
</tr>
<tr>
<td>6</td>
<td>New Zealand</td>
<td>2700</td>
</tr>
<tr>
<td>7</td>
<td>Sweden</td>
<td>2576</td>
</tr>
<tr>
<td>8</td>
<td>Romania</td>
<td>2353</td>
</tr>
<tr>
<td>9</td>
<td>Lithuania</td>
<td>1794</td>
</tr>
<tr>
<td>10</td>
<td>Russian Federation</td>
<td>2000</td>
</tr>
<tr>
<td>11</td>
<td>Italy</td>
<td>1509</td>
</tr>
<tr>
<td>12</td>
<td>Ukraine</td>
<td>1000</td>
</tr>
<tr>
<td>13</td>
<td>Spain</td>
<td>924</td>
</tr>
<tr>
<td>14</td>
<td>Latvia</td>
<td>807</td>
</tr>
<tr>
<td>15</td>
<td>France</td>
<td>794</td>
</tr>
<tr>
<td>16</td>
<td>Uzbekistan</td>
<td>700</td>
</tr>
<tr>
<td>17</td>
<td>Portugal</td>
<td>250</td>
</tr>
<tr>
<td>18</td>
<td>Mexico</td>
<td>121</td>
</tr>
<tr>
<td>19</td>
<td>Morocco</td>
<td>60</td>
</tr>
<tr>
<td>20</td>
<td>Norway</td>
<td>44</td>
</tr>
</tbody>
</table>

*Source: FAO, 2009*
2.2 Chemical components of blueberry

Blueberries are one of the most popular berries found in the retail market. The fruit is distinguished for its moderately sweet, aromatic flavor, high dietary fiber and low fat. A considerable number of studies on the phytochemical content of ripe blueberries have been conducted showing that blueberries contain a wide variety of nutrients and other components such as vitamins, minerals, sugars, and organic acids (quinic, citric and malic acids) predominate in ripe blueberries (Talcott, 2007). The content and diversity of vitamins and minerals is shown in Tables 2.3 and 2.4. Buschway et al. (1983) evaluated the vitamin (niacin, riboflavin, thiamin vitamin C and vitamin A) and mineral (Ca, K, Mg, P, Al, B, Cu, Fe, Mn, Na, and Zn) content of blueberries. Gao and Mazza (1994) have reported the amount of simple and acylated anthocyanins, and other phenolics in low-bush and high-bush blueberries.

The content of sucrose in blueberries as well as other berries is influenced by several factors such as the degree of ripeness or duration of postharvest storage. However, blueberries contain an equimolar mixture of glucose and fructose. Starch is not significantly accumulated during growth; instead, the organic acids are concentrated into sugars and sucrose (Shaw, 1988). Starch is the primary carbohydrate during early berry development and later is converted to glucose and fructose during the ripening process and postharvest storage. Sucrose is a non-reducing sugar composed of α-D-glucopyranoside and β-D-fructofuranoside. Sucrose hydrolysis can occur by acid-catalyzed reactions or invertase activity during maturation and ripening. Total sugar content in berries is a good marker for consumer acceptability, but the presence of organic acids such as citric, malic and phenolic acids is also important, as it counterbalances the sweetness of ripen fruits (Talcott, 2007). Indeed, the organoleptic factors of blueberries such as sweetness, acidity, astringency and overall perception are measured based on the concentration of soluble sugars and the balance among acids, aroma, active volatiles and other
constituents such as polyphenols (Talcott, 2007). Organic acids are also helpful in the stabilization of ascorbic acid and anthocyanins in fresh and processed berries (Viljakainen et al., 2002). Composition and concentration of organic acids depend on cultivars, maturity and seasons (Kalt and Mc Donald, 1996). The content of glucose and fructose is nearly equal in low-bush blueberries and high-bush blueberries. However, the content of sucrose is very small in low-bush blueberries, compared to high-bush blueberries (Barker et al., 1963). The differences in the organic acid composition of different Vaccinium species suggest that blueberries may be distinguished based on their acid profiles (Ehlenfeldt et al., 1994).

The quality of fresh and processed blueberries is affected by the presence of various hydrolase and oxidase enzymes; these factors relate to the loss of colour and texture and the onset of browning. It has been reported that the presence of oxidizing enzymes is detrimental to colour, nutritional components and overall acceptability. Polyphenol oxidase (PPO; EC 1.12.18.1) or peroxidase (POD; EC 1.11.1.7) are the main compounds responsible for fruit browning, although their effect is often masked by the dark red-purple colour of the anthocyanins. PPO or POD in the presence of hydrogen peroxide catalyzes the oxidation of diphenolic compounds into quinones which eventually form brown pigments (Talcott, 2007). It has been reported that total activity of peroxidase in blueberries increased during the development of fruits, reaching a maximum in red berries. Most of the enzymes were ionically bound to cell walls and the enzyme activity throughout the berries during development (Miesle et al., 1991).

Cell-wall-degrading enzymes such as polygalacturonases (PG; EC 3.2.1.15) and endo-1,4- glucanases (EC 3.2.1.4) are another group of enzymes found in blueberries. Due to the high content of pectin, PG cleaves pectin chains affecting fruit texture.
Table 2.3 Vitamin content per 100 g of fresh blueberries from the U.S. Department of Agriculture Nutrient Database (Adapted from Talcott, 2007).

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Blueberry Value per 100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ascorbic acid (mg)</td>
<td>9.7</td>
</tr>
<tr>
<td>Thiamin (mg)</td>
<td>0.04</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>0.04</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>0.42</td>
</tr>
<tr>
<td>Panthothenic acid (mg)</td>
<td>0.12</td>
</tr>
<tr>
<td>Vitamin B₆ (mg)</td>
<td>0.05</td>
</tr>
<tr>
<td>Total folate (μg)</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin B₁₂ (μg)</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin A (IU)</td>
<td>54</td>
</tr>
<tr>
<td>α-Tocopherol (mg)</td>
<td>0.57</td>
</tr>
<tr>
<td>β-Tocopherol (mg)</td>
<td>0.01</td>
</tr>
<tr>
<td>γ-Tocopherol (mg)</td>
<td>0.36</td>
</tr>
<tr>
<td>Δ-Tocopherol (mg)</td>
<td>0.03</td>
</tr>
<tr>
<td>Vitamin K (μg)</td>
<td>19.3</td>
</tr>
</tbody>
</table>
**Table 2.4** Mineral content per 100 g of fresh blueberries from the U.S. Department of Agriculture Nutrient Database (Adapted from Talcott, 2007).

<table>
<thead>
<tr>
<th>Minerals</th>
<th>Blueberry Value per 100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg)</td>
<td>6</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>6</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>12</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>77</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>1</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>0.16</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>0.06</td>
</tr>
<tr>
<td>Manganese (mg)</td>
<td>0.34</td>
</tr>
<tr>
<td>Selenium (mg)</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Pectin modification is achieved by pectin methyl esterases (PME; EC 3.1.1.11), an ubiquitous enzyme that cleaves methyl esters from methoxylated pectins for subsequent depolymerization by PG. Glucanases are responsible for depolymerizing other components of the cell wall, including cellulose and hemicelluloses (Vicente et al., 2005; Talcott 2007). Polysaccharide depolymerization reactions will change the physical and chemical properties of the polymers, and it may be beneficial in increasing the levels of soluble polysaccharides. Soluble fiber has been linked to several benefits to human health. The consumption of blueberries has been associated with a reduction of serum cholesterol, and low-density lipoprotein (LDL) cholesterol. Fiber also enhances satiety and decreases the extent of sugar uptake from diet (Behall, 1986).

2.3 Blueberry phytochemicals

In recent years much attention has focused on the bioactive properties of natural compounds from plants. Blueberries are a rich source of phytochemicals such as polyphenols, which may afford protection and improve human health (Howard and Hager, 2007).

2.3.1 Polyphenols

Polyphenols are secondary metabolites produced by plants; their main role is to provide protection from UV light, colour, antimicrobial properties, and attraction of pollinators and seed dispersers (Strack et al, 1993). Chemically speaking, polyphenols are compounds that possess one or more aromatic rings bearing one or more hydroxyl substitutions (Machieux, 1990). In nature, more than 4000 polyphenols have been identified. Figure 2.1 shows the typical chemical structure of polyphenols found in nature.
Figure 2.1 Chemical structures of the main classes of polyphenols (redrawn from Scalbert and Williamson, 2000).
Polyphenols are produced via the pentose phosphate pathway, shikimate and phenylpropanoid pathways. Figure 2.2 shows a schematic diagram of the shikimate pathway. The building blocks of shikimic acid pathway are phosphoenolpyruvate and erythrose-4-phosphate, which together with acetate, forms the primary aromatic rings of many phenolic compounds. The polyphenol biosynthesis can be divided into two main parts: the phenylpropanoid, and the flavonoids biosynthesis. In the phenylpropanoid biosynthesis, several organic acids such as cinammic, coumaric, caffeic, ferrulic, and chlorogenic acids are formed from phenylanine via the shikimate pathway. A process involving three different enzymes: phenylalanine ammonia liase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumaryl-CoA ligase (4CL) converts phenylalanine to p-coumaryl-CoA, which is the main precursor of flavonoids, lignin and other phenyl propanoids. The portion of the benzopyran moiety of the flavonoid structure is formed from p-coumaric acid via p-coumaroyl CoA (Delgado-Vargas and Paredes-Lopez, 2003). Flavonoid synthesis requires the participation of a key enzyme called chalcone synthase (CHS), which catalyzes the condensation of three molecules of malonyl CoA with p-coumaroyl-CoA to form a chalcone structure. In the next step, chalcone is isomerized to flavonoid by the enzyme chalcone isomerase (CHI) in a steroespecific reaction. In a following reaction, flavonol hydrolases (F3H) convert the flavonoids to dihydroflavonoids, which in turn are converted to leucoanthocyanidins by the aid of dihydroflavonol 4-reductase (DFR). Next, leucoanthocyanidins are transformed to anthocyanidins by oxidation and dehydration, as well as the presence of anthocyanin synthase (ANS). Finally anthocyanidins are converted to anthocyanins by glycosylation in the presence of glucosyl transferase (3GT). It has been proposed that there is a step involving flavonoids methyl transferases (MT), and that the process of acylation of hexoses or pentoses in 3-position involves high specific acyl transferases such as 4-coumaryl or caffeoyl transferases (Delgado-Vargas and Paredes-Lopez, 2003).
Figure 2.2 Shikimate pathway. PAL: Phenylalanine ammonia lyase, C4H: cinnamate-4-hydroxylase, CoA: Coenzyme A, 4CL: 4 coumaroyl CoA ligase, 3M-CoA: 3 malonylCoA, ACHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavonol hydroxylase, DFR: dihydroflavonol-4-reductase, ANS: anthocyanin synthase, 3GT: glucosyl transferase, MT: methyl transferase. (Redrawn from Delgado-Vargas and Paredes-López, 2003; Stintzing and Carle, 2008).
In blueberries, the content of polyphenols depends on the species (high-bush vs. low-bush), cultivar, ripeness, season, region, field management practices, and postharvest storage (Prior, et al., 1998). Blueberries are rich in phenolic acid such as benzoic and cinamic acids. Figure 2.3 shows examples of benzoic acids found in blueberries—vanillin, syringic acid, gallic acid, protocatechuic acid, m-hydrobenzoic acid, p-hydrobenzoic acid, and ellagic acid. The cinamic acid derivatives found are chlorogenic acid, caffeic acid, ferulic acid, quinic acid, and coumaric acid (Azar et al., 1987; Kader et al., 1996, Taruscio et al., 2004). The polyphenols in blueberry are naturally esterified with other phenolic acids or sugars, meaning that most of the time they are found as glucosides such as quercetin glucoside, myriciten glucoside and kaempferol glucoside (Häkkinen et al., 1999).

2.3.1.1 Anthocyanins

Anthocyanins are water-soluble vacuolar pigments that are responsible for the purple and blue pigmentation in blueberries (Horbowicz et al, 2008). They are one of the largest and most important groups of secondary plant metabolites and belong to a subgroup of the polyphenols collectively known as flavonoids. Chemically speaking, anthocyanins are hydroxylated and methoxylated derivatives of the 2-phenylbenzopyrilum or flavylium cation. Their structure shown in Figure 2.4 is based on a C_{15} aglycone skeleton known as anthocyanidin, that consists of a chromane ring (A and C) bearing a second aromatic ring B in position 2. To obtain an anthocyanin, the anthocyanidin structures must be complemented by one or more sugars bonded at different hydroxylated positions (Harborne and Grayer, 1988; Brouillard et al., 1993).
Figure 2.3 Structure of selected phenolics present in blueberries a) benzoic acid, b) cinnamic acid, and c) Quinic acid derivatives. *5’ caffeoylquinic acid = chlorogenic acid (adapted from Macheix, 1990).
Figure 2.4 Basic structure of a) anthocyanidins and b) anthocyanins (adapted from Andersen and Jordheim, 2006).
The aglycone forms (anthocyanidins) are found in plants only in trace quantities because they are extremely unstable. In nature, anthocyanidins exist normally as glycosides of six common benzopyran structures (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin). Such glycosides can also form linkages with aromatic acids, aliphatic acids and methyl ester derivatives, thus about 539 different anthocyanins have been reported from plants. Usually anthocyanidin glycosides are 3–monoglycosides or 3,5–diglycosides formed by further linkages with glucose, galactose, rhamnose, xylose, arabinose and rutinose (Figure 2.5) (Clifford, 2000; Andersen and Jordheim, 2006). The sugar residues may be attached to organic acids such as cinnamic acid derivatives, and aliphatic acids such as acetic, malic, malonic, oxalic and succinic acids (Giusti and Wrolstad, 2003).

Several factors such as the number of hydroxyl groups, the degree of methylation, the nature and number of sugars attached to the flavylium cation and the nature and number of aliphatic or aromatic acids attached to the glycoside moiety in the molecule are distinctive characteristics of each individual anthocyanin (Jacob et al. 2012). The degree of hydroxylation and methylation of the B-ring is responsible for different patterns and properties among anthocyanins. The more the number of hydroxyl groups, the more bluish the shade, the more the number methoxyl groups the more reddish the color (Delgado-Vargas and Paredes-López, 2003). The color of the anthocyanins is also greatly influenced by pH, under acidic conditions (pH < 2) anthocyanins are red or orange, while under neutral conditions, they are colorless and in alkaline conditions (pH > 7) they change their color into blue. This occurs as a result the presence of eight conjugated double bonds that carry a positive charge (Delgado-Vargas and Paredes-López, 2003).
**Figure 2.5** Common sugars glycosylated to anthocyanidins (adapted from Mercadante and Bobbio, 2008).
Figure 2.6 shows the effect of pH on the chemical structure of anthocyanins. In edible plants, cyanidin is the most abundant anthocyanin with approximately 50% of the total anthocyanins, followed by pelargonidin with 12%, peonidin with 12%, delphinidin also with 12, petunidin with 7%, and malvidin with 7% (Kong et al., 2003). Overall, cyanidin is the most abundant aglycone in about 90% of the fruits (Prior, 2001).

In blueberry skin and flesh, delphinidin, cyanidin, petunidin, peonidin and malvidin monoglycosides (glycosides, galactosides and arabinosides) are the main anthocyanins. Because of the diversity in the glycosylation and acylation pattern, more than 25 anthocyanins have been identified in blueberries (Howard and Hager, 2007). The distribution of anthocyanins in five different blueberry genotypes have been reported by Cho et al. (2004), where delphinidin was the most abundant anthocyanin (27% - 40%), followed by malvidin (22% - 33%), petunidin (19% - 26%), cyanidin (6% - 14%) and peonidin (1% - 5%).

2.4 The potential health benefits of blueberry phytochemicals

2.4.1 Antioxidant Activity

Major degenerative diseases such as cancer, heart disease, type II diabetes, inflammation, ageing, and cognitive dysfunction are the result of oxidative stress (Lee and Lee, 2006; You et al., 2011). Several intervention studies have provided evidence which indicates that antioxidant activity of polyphenols may help through reducing/preventing oxidative stress in chronic diseases (Kraft et al., 2005, Sporman, et al., 2008; Chong et al., 2010). Although there are many phytochemicals in nature, fruits and vegetables that are rich in anthocyanins show a higher antioxidant activity compared to those rich in other type of polyphenols (Jacob et al. 2012).
Figure 2.6 Effect of pH on structural transformations of anthocyanins (adapted from Horbowicz et al., 2008).
The strong antioxidant activity of anthocyanins against free radicals has been already demonstrated in vitro. This property has been attributed to their structural characteristics that involve radical scavenging, metal chelation, and protein binding (Mazza and Kay, 2008). Anthocyanins can also, prevent lipid peroxidation in biomembranes because of the hydrophobic nature of the phenolic ring (Rice-Evans et al. 1996).

Several interventional trials using potent antioxidants such as β-carotene, vitamin A, vitamin C, vitamin E, and selenium have not shown effectiveness in preventing cancer in the general population or in patients with various diseases (Bjelakovic et al., 2007). However, studies using red blood cells in vitro and in vivo have shown that consumption of blueberry polyphenols is inversely related to oxidative stress in vivo (Youdim et al., 2000). In the midst of such contrasting results, it appears that maintaining the homeostasis by a non-physiological, excessive exogenous supply of antioxidants is the key to maintain the redox balance in healthy humans (Berger et al., 2012).

### 2.4.2 Antiinflammatory activity

Inflammatory processes in cells are implicated in the initiation of tumor development and its progression. The microenvironment that surrounds tumors is rich in cytokines, chemokines and inflammatory enzymes (Balkwill and Mantovani, 2001; Hou et al, 2005). Anthocyanins have the capacity to target NF-κB pathway and cyclo-oxygenase-2 (COX-2) gene expression (Hou et al., 2005), and inhibit arachidonic acid catabolism leading its production of prostaglandins (Ferrandiz and Alcaraz, 1991). More specifically, delphinidin and cyanidin have been reported as the main compounds responsible for the inhibition of mitogen-activated-protein-kinase (MAPK) -mediated cyclooxygenase-2 (COX-2) expression (Hou et al. 2005).

Several in vitro trials have been performed to assess a reduction in inflammation using different berry extracts such as bilberries (Vaccinium myrtillus L.), blackberries (Rubus sp.),
blueberries (Vaccinium corymbosum L. cv Jersey), cranberries (Vaccinium macrocarpon Ait. cv Early Black), elderberries (Sambucus canadensis), raspberries (Rubus idaeus L.) and strawberries (Fragaria × ananassa Duch. cv Honeoye). Approximately 10% - 45 % of COX-1 and 5 % to 45 % of COX-2 were inhibited at levels of 125 μg/ ml of anthocyanins. These results are promising if they are compared to commercial pharmaceuticals such as naproxen and ibuprofen which inhibits approximately 50 % and 40 % of COX-1 and COX-2 respectively at a level of 10 μM (Seeram et al., 2001).

2.4.3 Antiproliferation

The anticarcinogenic effects of consuming high levels of fresh fruit and vegetables have been correlated with their polyphenol content. Polyphenols have been shown to inhibit proliferation of different types of cancer cell lines derived from organs such as colon, esophagus, lung, liver, mammary, and skin (Seeram et al., 2004, 2006). Among all polyphenols, anthocyanins have been demonstrated to have the greatest inhibitory effects on the growth of cultured tumor cells (Kamei et al. 1995; Hakimuddin et al. 2006, 2008). The anticarcinogenic effects of anthocyanins have been correlated directly to their antioxidant capacities in vitro that include radical scavenging and inhibition of oxidative process. If this was true, then vitamin C would have been an effective anticancer agent. However, there are several mechanisms including modulation of signal transduction processes and gene expression, that are involved in cancer prevention (Mazza and Kay, 2008; Jacob et al., 2012).

Cyanidin and delphinidin have been the most studied anthocyanins for their ability to inhibit cell growth and proliferation in vitro. In 2001 Meiers et al. showed that these two anthocyanins are able to shut off downstream signaling cascades in human epidermal carcinoma cell line A431, and therefore the epidermal growth factor receptor was inhibited. Wang and Mazza (2002) demonstrated that anthocyanins have the ability to modulate the
immune response in activated macrophages by affecting the expression of the tumor necrosis factor α (TNF-α), a cytokine involved in the inflammation pathway.

Inhibition of cell proliferation by anthocyanins is more pronounced in cancer cells compared to normal or immortalized cells. For instance, Hakimuddin et al. (2004) found that the flavonoids fractions of red wine showed selective cytotoxicity on MCF-7 cells with relatively low effect towards normal human mammary epithelial cells (HMEC) and non-tumorigenic MCF-10A cells. The proposed mechanism was associated with the inhibition of calcium signal transduction and down regulation of several pathways involved in tumorigenesis (Hakimuddin et al., 2004, 2006).

Anthocyanins have the capacity to block various stages of the cell cycle. HT-29 cells showed an arrest in the G1/G0 and G2/M phases when treated with extracts from chokeberry (Malik et al. 2003). The antiproliferative properties of many fruits and vegetables have been attributed to the mixture of diverse polyphenols found in whole fruits and juices. For instance, Seeram et al. (2004) analyzed the effect of total cranberry crude extracts and purified flavonols, anthocyanins and proanthocyanidins on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620), and prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines. Although the anthocyanin and the proanthocyanidin fractions exhibited significant inhibitory effect on almost all cell lines, when the fractions were combined the inhibitory effect against all cell lines is the highest (Seeram et al., 2004).

### 2.4.4 Control of metabolic syndrome and type II diabetes

Type II diabetes is a metabolic disorder associated with insulin resistance that is increasing worldwide. The number of cases is projected to increase from 171 million in 2000 to 366 million by 2030 (Wild et al., 2004).
Impaired insulin secretion and insulin resistance are associated with type II diabetes. Type II diabetes in turn can lead to chronic hyperglycemia, increased levels of oxidative stress and glycation of hemoglobin (Tourrel et al. 2002). Therapy that inhibits or reduce the oxidative process occurring in the cells could prevent or delay complications associated with diabetes. Preventive mechanisms include the expression of endogenous enzymes such as catalases and peroxidases, and intake of antioxidants including anthocyanins (Sancho and Pastore, 2012).

Anthocyanins possess anti-diabetic properties such as reducing the levels of glucose in the blood, increasing serum antioxidant status, increasing insulin secretion and decreasing insulin resistance (Kay and Holub, 2002; DeFuria et al., 2009; Prior et al., 2010). However, it has been suggested that anthocyanins can only alter the concentration of glucose, or insulin in plasma, when metabolic disorders are present (Basu et al., 2009; Prior et al., 2009).

In vivo studies with healthy human volunteers demonstrated that freeze dried wild blueberry powder increased the serum antioxidant status (Kay and Holub, 2002). Whole blueberry freeze dried powder, also reduced the insulin resistance in the adipose tissue, and increased the function of β cells in male C57BL mice fed with high fat diets (DeFuria et al., 2009, Prior et al., 2010). Research with cell lines, animal models and intervention trials in humans suggest that anthocyanins may be effective in preventing and controlling the effects of diabetes. In order to improve the effectiveness of polyphenols and anthocyanins in controlling this disease, it is important to understand the absorption and metabolism of anthocyanins that determine the effective levels of these compounds available in the blood plasma (Sancho and Pastore, 2012).

2.5 Bioavailability of blueberry polyphenols

Bioavailability needs to be assessed in order to evaluate the health benefits of any compound in humans. Also it is very important to evaluate the effects of the metabolites, in
addition to considering only the pure compound, since metabolites may also provide positive health effects (Manach et al., 2004; Wu et al., 2002).

There are several definitions for the term “bioavailability”; however the most commonly accepted definition establishes bioavailability as the proportion of the nutrient that is digested, absorbed and metabolized. Hence, it is not only important to know how much of a nutrient is present in a food or dietary supplement; but even more important to know how much of that is bioavailable (Srinivasan, 2001, Manach et al., 2005). On the other hand bioaccessibility refers to the amount of the nutrient that is released from the food matrix and becomes ready for absorption. The amount that becomes bioaccessible may be equal to or less than the amount that is liberated from the food matrix since: (i) it may not fully get absorbed/convert to absorbable species, (ii) some may become unabsorbable species, and (iii) some forms may interact with other components of the luminal contents and become unabsorbable (Stahl et al., 2002). Food components must be released from the food matrix and interact with the brush border cells of the small intestine for their absorption into the enterocyte by passive diffusion or active transport systems (Stahl et al., 2002). Bioavailability differs greatly from one polyphenol to another; so that the most abundant polyphenols in our diet are not necessarily those that are present at the highest concentrations, either unmetabolized as active metabolites in the target tissues.

Anthocyanins have been thought to differ from the common flavonoids since only intact anthocyanin glycosides were detected in urine and plasma (Felgines et al., 2002). The apparent bioavailability is consistently very low for polyphenols with often less than 0.1% of the ingested dose appearing in the urine even though bioabsorption occurs quickly following consumption (Wu et al., 2002; Bitsch et al., 2004). Previous research on the bioavailability of anthocyanins among humans who consumed doses of 150 mg to 2 g of anthocyanins showed that after such intakes, concentration of anthocyanins measured in plasma were very low, on the order of 10 –
50 nmol/L (Lapidot, 1998). One of the main factors that possibly affect anthocyanin bioavailability could be its structure. Anthocyanins naturally are derivatives of six common backbone structures that are glycosylated, and the glycosylations can be further modified with aromatic acids, aliphatic acids, and methyl ester derivatives (Clifford, 2000). Both glycosylation and acylation appear to affect bioavailability (Charron, 2009); however, acylation of anthocyanins plays a main role in stabilizing the color due to the possible intramolecular and/or intermolecular co-pigmentation, and self-association reactions (Giusti and Wrolstad, 2003; Stinizing and Carle, 2004).

Another aspect that could affect the bioavailability of anthocyanins is the fact that bioabsorption occurs quickly following consumption. Since \( t_{\text{max}} \) in plasma is 15 – 60 minutes and excretion is complete within 6 – 8 hours, absorption of the anthocyanins may occur even from the stomach (Passamoniti et al., 2003; McGhie et al., 2007). The absorption in the stomach is governed by the matrix in which the anthocyanins are attached to and this can have an important role in determining when and to what extent they are absorbed (Aguilera, 2005). This process is largely influenced by the physicochemical characteristics of the compound, the type of food matrix, the subcellular location of the compound in the plant tissue, and changes that occur during food processing. Therefore, the type of the food matrix greatly influences the bioaccessibility of the compounds (During, 2008). Nutrient absorption depends not only on the complete disruption of the cellular structure, but also on the presence of and interaction with other food components (Jacob and Paliyath, 2008). Plant derived bioflavonoids, typically associate with the protein fraction, and therefore, they are not available until the protein matrix is digested (Papadopoulou and Frazier, 2003).

The time required for digestive processes to liberate the anthocyanins from the plant matrix may account for the low levels of anthocyanin absorption (Charron et al., 2009). Disruption of the natural matrix or the microstructure created during processing may influence
the release, transformation, and subsequent absorption of anthocyanins in the digestive tract (McDougall et al., 2005a). Therefore, the food microstructure is an important factor in the release and bioavailability of several nutrients and has enormous consequences in determining the nutraceutical properties of foods used in the prevention and therapy of chronic degenerative diseases (Aguilera, 2005, Parada and Aguilera, 2007).

Several studies have demonstrated that anthocyanin bioavailability is low; nonetheless, it could have been underestimated, for 2 main reasons. The first one is that a number of studies have used detection methods that are based on the measurement of anthocyanins as red flavylium cations by HPLC or spectrophotometric methods. However, the flavilyum cation is unlikely to exist in vivo (McGhie and Walton, 2007) and so that their ability to predict in vivo activity is questioned for a number of reasons. The second one is that some of the studies are performed at non physiological pH and temperature, and none of them take into account the bioavailability, uptake and metabolism of the antioxidant compounds (Liu and Finley, 2005). Numerous methods have been proposed to study the individual processes of bioavailability of phytochemicals, most of them have originated from the pharmaceutical research sector, but may be applied and optimized for phytochemical studies. The absorption and metabolism of phytochemicals share similar principles and metabolic pathways as drugs. However, it may account for the fact that the food matrix is much more complex than the drugs and their carrier formulations, and there may be interactions and competition with other food constituents for common metabolic enzymes and transporters (Holst and Williamson, 2004).

2.5.1 Food matrix

Anthocyanins are rarely ingested on their own, but as a component of fruits or vegetables, or in meals containing other food components. Therefore, it is not clear how anthocyanins are absorbed and metabolized when ingested in foods containing multiple
structural matrices. Several factors such as the content of fat, protein, carbohydrate and fiber may alter digestion processes and subsequent absorption. Studies carried out by Mazza and co-workers have shown that there are significant differences in the absorption rates of anthocyanin when consumed either alone or with high fat meals (Kay et al., 2004, 2005; Mazza et al. 2002). In a study by Bitsch et al. (2004b) a synergistic effect of the glucose content and anthocyanins of red grape juice was proposed when the intestinal absorption of anthocyanins of red grape juice seemed to be improved compared to that in red wine. The relative urinary excretion of total anthocyanins was higher in volunteers that consumed red juice compared to those who consumed red wine. However, it has been shown that isolated polyphenols from grape juice are more effective in arresting the growth of tumor in mice than those isolated from red wine, when provided through gauge (Hakimuddin et al., 2004). This may suggest that it is the compositional difference that may influence the efficacy of dietary polyphenols.

2.5.2 Intestinal absorption

At present, the mechanisms by which the anthocyanins are absorbed from the gastrointestinal tract (GIT) are unclear. It remains to be determined whether anthocyanins are absorbed by passive diffusion or active transport mechanisms (Kay, 2006). After the ingestion, less than 1% of the dose is usually recovered in human serum and less than 5% in the inside fraction after in vitro digestion (McDougall et al., 2005a). Anthocyanins, unlike other polyphenols are recovered intact before their absorption (Wu et al., 2002). The causes for the poor absorption of anthocyanins are not clear. A possible reason is that many anthocyanin chemical structures are not efficiently hydrolyzed by β-glucosidases in the GIT resulting in a low absorption into the blood stream (Nemeth et al., 2003). Due to the fact that most polyphenols are too hydrophilic to cross the gut wall by passive diffusion, membrane carriers may be involved in the absorption mechanism. The sodium dependent absorption mechanism involved
in cinnamic and ferulic acid absorption in the rat jejunum is the only active transport mechanism that has been described (Ader et al., 1996). Several *in vitro* studies have demonstrated that phenolic compounds can be transported through the epithelium as glycosides by sugar transporters such as intestinal sodium-dependent glucose transporter (SGLT) (Hollman *et al*., 1995; Day *et al*., 1998; Morand *et al*., 2000). For instance, when sugars are supplemented along with anthocyanins, absorption of anthocyanins can be reduced, suggesting the role of SGLT in the absorption of anthocyanins (Mulleder *et al*., 2002).

Once inside the epithelial cells, cytosolic β-glucosidases can also hydrolyze these glycosides, and aglycones are formed after absorption. Aglycones can also be formed in the lumen by the action of membrane-bound lactase phlorizin hydrolase (LPH) and they may be absorbed passively through the epithelium (Scalbert and Williamson 2000). Most flavonoids such as quercetin, daidzein and genistein can be recovered in the plasma as aglycones. The organic anion carrier bilitranslocase (BLT) has been proposed by Passamonti *et al*. (2003) as a carrier protein to transport anthocyanin from the blood into the liver. This protein has shown high affinity towards anthocyandins; however, direct evidence for that is lacking in humans and further investigation is required (Passamonti *et al*., 2003; Kay, 2006). Figure 2.7 shows a putative mechanism of anthocyanin absorption in humans. The food matrix may also affect the absorption of polyphenols. Direct interactions between polyphenol and food components such as proteins or polysaccharides can occur, and may affect the absorption rate.
**Figure 2.7** Absorption of anthocyanins in humans. ANT: Anthocyanidin; Ant-gly: Anthocyanin 3-glucoside; Ant-G: Anthocyanin glucuronide; SGLT: sodium-dependent glucose transporter; LPH: lactate phlorizin hydrolase; CBG: cytosolic β-glucosidase; UDP-GT: UDP-glucoronosyl transferase; COMT: catechol-O-methyltransferase; SULT: sulfotransferase; BLT: Bilitranslocase (Adapted from Mazza and Kay, 2008; Passamonti et al., 2003).
Physiological conditions of the GIT, including pH changes, the indigenous microflora, biliary excretions and transit time may also influence the absorption of polyphenols. Digestive enzymes and carriers involved in the absorption and metabolism of polyphenols may be affected by some of the nutrients, or xenobiotics present in the food matrix (Manach et al., 2004). For instance, it has been hypothesized that the presence of alcohol in red wine may increase the absorption of polyphenols, because alcohol increases their solubility. However, studies suggest that the absorption of quercetin in rats is high but only when the concentrations of ethanol are more than 30% by volume, a concentration too high to be attained with a regular diet (Azuma et al. 2002). In addition, no significant differences were found in plasma concentrations of catechin metabolites when human subjects consumed red wine or dealcoholized red wine (Donovan et al., 1999).

2.5.3 Metabolism

Phytochemicals can be metabolized through the entire gastrointestinal tract (GIT). In the mouth they can be metabolized by resident microflora or salivary enzymes. In the stomach they may be affected by the acidic conditions. Within the small and large intestine, they are affected by pancreatin, brush border enzymes or microbial enzymes.

During the passage across the enterocytes, endogenous phase I and phase II enzymes metabolize several phytochemicals. While in the liver, hepatic phase I and phase II enzymes modify phytochemicals. Finally, they are completely metabolized in tissues via phase I and phase II enzymes. Changes in functional groups, partial or complete breakdown of structures or conjugation with other molecules are the main reactions involved in the metabolism of phytochemicals (Day et al., 2004).

Even though the mechanism of action of dietary phytochemicals such as anthocyanins has been established from in vitro studies, there is a lack of information about their
bioavailability, metabolism, tissue distribution and biological action in vivo. According to the current literature, it is believed that polyphenols are poorly bioavailable and extensively metabolized by colonic microflora. Metabolized compounds may remain in the body, accumulate in tissues and contribute to the beneficial effects that have been attributed to the anthocyanins themselves (Williamson and Clifford, 2010). Figure 2.8 shows the proposed pathway for the formation of anthocyanin metabolites in humans, modeled after studies involving recovery of metabolites in human urine and serum, after ingestion of cyanidin 3-glucoside (Mazza and Kay, 2008). Once absorbed by the epithelial cells, aglycones undergo a conjugation with glucuronic acid in the ileal epithelium or in the liver.

Methylation is one of the most studied pathways in the metabolism of flavonoids. Cyanidins are prone to O-methylation by catechol-O-methyltransferase (COMT) because of their catechol-like structure. In vivo and in vitro studies in hamsters have demonstrated that this reaction occurs primarily in the liver (Walle et al., 2001).

Glucuronidation is another reaction that can occur during the metabolism of polyphenols. Although at the present, neither the site of glucuronidation nor the UDP-glucuronosyltransferase (UDP-GT) has been well characterized, two pathways for the formation of cyanidin monoglucuronides have been proposed. The first possibility is the formation of cyanidin 3-glucuronide from cyanidin 3-glycoside by UDP-GT, and the other possibility is the hydrolysis of cyanidin 3-glycoside or peonidin 3-glycoside followed immediately by a glucuronidation in the intestine. Since aglycones of cyanidin and peonidin were found in rat plasma, the second possibility seems to be more probable in humans (Felgines et al., 2003; Wu et al., 2002; Mazza and Kay, 2008).
Figure 2.8 Putative pathway for the metabolism of anthocyanins in humans (redrawn from: Mazza and Kay, 2008).
Sulfonation has been proposed as another pathway in the metabolism of anthocyanins. Cyanidin sulfo-conjugate has been identified in human urine of volunteers that consumed strawberries (Felgines et al., 2003). Sulfo-conjugation of aglycones occurs in the intestine and liver by sulfotransferases (SULT) after the hydrolysis of the anthocyanin. Sulfation has long been recognized as an important mechanism in the detoxication of xenobiotics and toxic products, and may play an important role in the excretion of anthocyanins from the body through the urine. However, limitations in the identification and quantification methodologies have restricted the ability to measure these conjugates in biological fluids (Walle et al., 2001).

Hepatic metabolites (methylated, sulphated or glucuronidated conjugates) are returned to the luminal side via bile (enterohepatic circulation) (Nemeth et al. 2003; Scalbert and Williamson 2000). Phenolic compounds that enter the colon are unabsorbed glycosides, such as anthocyanins, and conjugates after ileal and hepatic metabolism via enterohepatic circulation (Scalbert and Williamson 2000). Polyphenol conjugates can also be enclosed in the food matrix and thus absorption can be prevented (Nielsen et al. 2003). When these compounds reach the caecum, they are subjected to the microbial metabolism. Many flavonoids undergo ring-fission, in which the B-ring is degraded and phenolic acids are formed (Scalbert and Williamson 2000; Scalbert et al. 2002; Rechner et al. 2002; Manach et al. 2004).

2.5.4 Microbial metabolism

Polyphenols may be fermented and that help to support microflora growth in the colon (Karle et al., 2006). It has also been suggested that the effects that occur in the GIT, particularly by digestive enzymes, may be partly responsible of the health benefits derived from a high intake of polyphenols (McDugall et al., 2005b). The colonic microflora may play a significant role in the metabolism of anthocyanins. Anthocyanins can reach the colon either by not being absorbed in the upper GIT or by enteropathic circulation (Kay, 2006). The colonic microflora
contains a wide variety of microorganisms including bacteria, archea and eukarya. However, bacterial species has been the most studied (Williamson and Clifford, 2010). Approximately 30% of the indigenous bacteria found in the colon is represented by *Bacteroides* spp., *Clostridium* spp., and *Eubacterium* spp., (Savage, 1977) and a significant amount of *Fusobacterium* spp., *Peptostreptococcus* spp., and *Bifidobacterium* spp. is found (Salminen et al., 1996). *Methanobrevibacter smithii* has been reported as the single methanogenic archeon while there is no reference of eukarya, such as protozoa on the ability to catabolize polyphenols (Bäckhed et al., 2005; Williamson and Clifford, 2010).

The colon is viewed as an anaerobic fermentator where nutrients such as polysaccharides, proteins, lipids, and also polyphenols can be transformed (Williamson and Clifford, 2010). The hydrolysis of glycosidic bonds between polyphenols and the sugar moieties does not occur by the action of the enzymes present in the stomach and small intestine. Therefore, it has been proposed that intact flavonoids can reach the colon (Scalbert and Williamson, 2000). In addition, human endogenous esterases that release phenolic acids do not exist; therefore, the colonic microflora is required to produce esterases and metabolize acylated flavonoids (Plumb et al., 1999). Colonic microorganisms metabolize polyphenols and produce a variety of catabolites. Depending on the chemical structure of polyphenols and their attached moieties such as sugar, organic acid, aliphatic acid, the main products in the colon are benzoic acid, protocatechuic acid, phenylacetic acid, phenyl and propionic acids (Williamson and Clifford, 2010). Walle et al. (2001) using $^{14}$C-quercetin demonstrated that quercetin is metabolized extensively by intestinal bacteria. Even though the fate of the aromatic rings was not studied, research showed that approximately 50% of the radioactive $^{14}$C was eliminated as CO$_2$.

Using an *in vitro* anaerobic fermentator, Aura (2005) demonstrated that human faecal flora deconjugates quercetin glycosides which later undergoes ring fission to generate phenolic
acids such as 3,4-dihydroxyphenylacetic acid and its derivatives. One of the microorganisms responsible for this reaction is *Eubacterium ramulus*. This bacteria is well known for its ability to deglycosylate and split the C-ring of flavonoids (Shneider and Blaut, 2000). Some *Clostridium* spp. posses the ability to cleave the C-ring of flavonoids and convert them to phenylacetic acid (Winter *et al.*, 1991). Anthocyanins can be converted by gut microflora, for instance, cyanidin 3-glucoside is hydrolyzed almost completely after 2 hours of incubation in the presence of human fecal slurries, while cyanidin 3-rutinoside was hydrolyzed by more than 60% (Aura, 2005).

*In vivo* studies have also demonstrated that after a 6 hour period of consumption of orange juice, protocatechuic acid is the major cyanidin-glucoside metabolite found in the bloodstream. This accounts for almost 44% of the ingested cyanidin-glucosides. A concentration of 2.0 nmol/g of protocatechuic acid was detected in human feaces collected one day after the consumption of orange juice, suggesting that colonic microflora metabolizes cyanidin glucosides (Vitaglione *et al.*, 2007). A study conducted by Felsigues *et al.* (2003) with human volunteers who ingested a meal containing 200 g of strawberries, revealed that the total excretion of all detected anthocyanin metabolites in the urine was 1.8% of the total pelargonidin 3-glucoside ingested. Also, pelargonidin 3-glucoside was metabolized to pelargonidin aglycone, three monoglucuronides and one sulfo-conjugate.

Anthocyanin diglycosides are also degraded when incubated with human faecal suspension. Fleschut *et al.*, (2006) reported that after a 2 hour period of incubation of cyanidin 3,5-diglucoside, more than 90% was hydrolyzed, and meanwhile partial hydrolysis generated cyanidin monoglucosides that underwent degradation, and protocatechuic acid accumulation was observed as well.
2.6 In vitro human digestion models for bioavailability estimation

Digestion is a very complex process that affects the bioaccessibility and bioavailability of food components. The food matrix plays a very important role in quantification of bioavailability and bioaccessibility of a particular compound (Versantvoort et al., 2005). Therefore, in past several years, in vitro models have been developed to study the human digestion process and understand the implications in absorption and metabolism of dietary compounds.

In vitro techniques have an important potential to study nutrient digestion and fermentation in the human hindgut. There are significant differences in the gastrointestinal tract among individuals and implementing an in vivo model becomes difficult and expensive (Coles et al., 2005). The purpose of the in vitro digestion models is to simulate in a simplistic manner the digestion processes that take place in the mouth, stomach and small intestine. Information obtained by these models is useful to investigate the bioaccessibility of compounds from their matrix and analyze their stability during their transit through the GIT (Versantvoort et al., 2005). Although the results obtained using these models differ from those found in vivo because of the complexity of the physicochemical and physiological events occurring in the human digestive system, in vitro digestion models are a useful alternative to animal and human models by rapidly screening food ingredients in a short time (Coles et al., 2005). The majority of the in vitro digestion models are based on the enzymatic digestion processes. Most models are based on incubation of the samples at 37 °C with digestive enzymes such as pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α-amylase, or lipase, along with bile salts and mucin (Hur et al., 2011).

2.6.1 In vitro enzymatic digestion

In vitro digestion methods are significantly influenced by several factors such as the nature of the sample, enzymatic activity, chemical composition, mechanical stress, and time. Although it is impossible to simulate all the conditions that are present during the digestion in vivo,
different techniques can be designed using specific enzymes to measure the initial rate of hydrolysis, and give more realistic digestibility values (Boissen and Eggum, 1991). When designing an *in vitro* digestion system, it is very important to take into account the characteristics of the enzymes, concentration, temperature, pH, stability, activators, inhibitors and time. It is also important to modify the conditions depending on the research and hypothesis, as there is no universal in vitro digestion method that can fully mimic the human digestion.

Although several enzymes participate in the *in vivo* digestion process, many *in vitro* digestion models use a single enzyme. Using a single purified enzyme, rather than a mixture is convenient and useful to predict the digestibility of defined nutrients. For instance, pepsin is used to analyze protein digestibility, while amylase and lipase are used to analyze starch and lipid digestibility, respectively (Boisen and Eggum, 1991). The use of single enzymes facilitates the standardization process, and enables comparisons between studies (Coles et al., 2005). Gil-Izquierdo and co-workers (2001) developed a very useful method to simulate *in vitro* physiological conditions in the stomach and small intestine. This method in turn is a modification of the first model proposed by Miller *et al.*, (1981) to estimate iron bioavailability. The alternative method established by Gil-Izquierdo *et al.* (2001) enables to analyze phenolic compounds from diverse sources such as juices, whole fruits and jams released from the food matrix and their metabolites during digestion.

### 2.6.2 *In vitro* colonic fermentation

To better simulate the digestion *in vitro*, it is necessary to consider not only the host-enzyme interactions, but also the microbial activity in the digestive tract. Microbial fermentation occurs in the lower intestine, hence in the case of polyphenols, it is recommended that digestibility be measured at the terminal ileum (Boissen and Eggum, 1991). The main types of *in
*vitro* fermentation models used to simulate human colonic fermentation includes continuous, semi-continuous and batch system (Coles *et al*., 2005). All the models share the same principle: the anaerobic incubation and growth of colonic microbiota, usually isolated from human faeces. The main differences among the models are the structure and volume of the fermentation vessel, composition of media, concentration of the inoculum, operating conditions and sampling techniques (Rumney and Rowland, 1992). In this case also the research question will dictate the type of colon model employed. For instance, batch models are widely used for studies of microbial metabolism of dietary fiber and isolated phenolic compounds. The typical incubation time for substrates in this type of systems is 24 h, using faecal slurry from healthy donors, a buffer, media and redox agents. A specific example of this type of models is the *in vitro* gas production technique which is similar to other *in vitro* fermentation batch methods. The only difference with this method is the fact that fermentation occurs in a syringe, flask or bottle. This allows working with live microbes and performing comparisons between substrates. Additionally, the gas production technique utilizes fewer amounts of sample and equipment and allows multiple end times of fermentation, if required. Other *in vitro* models destroy the sample to determine dry matter, while the gas production technique preserves the sample. The non-automated version of this model is relatively easy and can be set up easily in most standard laboratories (Coles *et al*., 2005).

The main drawback of this model is that during the incubation period as nutrients are consumed, products accumulate affecting the culture conditions (Campbell *et al*., 1992). These problems can be reduced by using a semi-continuous system. Moreover a multi-stage system is able to reproduce more accurately the heterogenicity of physiological conditions that occur in different parts of the colon (Allison *et al*., 1989).

The simulated human intestinal microbial ecosystem (SHIME) model is an example of a 5-step multi chamber reactor that resembles the human intestinal digestion. The model consists
of two compartments that simulate the upper intestine (duodenum, jejunum and ileum), the third compartment simulates the caecum and ascending colon, the fourth compartment simulates the transverse colon, and the fifth compartment simulates the descending colon (Molly et al, 1993).

Another widespread in vitro model is the TNO human gastro intestinal tract (TIM) simulator. This is the latest continuous and anaerobic gastrointestinal model. It is a computer controlled in vitro system with peristaltic mixing, water absorption, and absorption of fermented substrates. The model consist of four computer-controlled chambers that simulates the conditions of stomach, duodenum, jejunum and ileum (TIM-1), and one chamber that simulates the conditions of the large intestine (TIM-2). The temperature, pH, peristaltic movements and absorption of water and fermentation products are monitored continuously when the substrate passes from one compartment to another (Minekus et al. 1999).

The applications of the TIM model include nutritional studies such as digestion of food, availability and interactions of food components. Also, it can be used in the food and pharmaceutical industries to develop novel and functional ingredients and analyze drug-nutrient interactions. One of the major drawbacks of this model is the complexity of the mathematical model; therefore, it is not possible to further analyze the results for predicting the fate of food components. Even more, the continuous flow models require high technical and scientific expertise, and they have high costs in terms of both hardware and running expenses (Spratt et al., 2005; Yoo and Chen, 2006).

Ideally an in vitro digestion method would be highly accurate and able to produce results in a short time. In most of the cases, the degree of accuracy is often sacrificed for rapid results. Inevitably, in vitro methods fail to match the accuracy of in vivo models because it is not possible to simulate the influx of endogenous compounds to the digestive system and their subsequent digestion and absorption. As well, the interaction between the host, the food and
the human microflora present in the GIT is difficult to replicate. In addition, errors increase when the complexity of the model increases (Fuller, 1991; Coles et al., 2005).

2.6.3 In vitro cell culture

Since every human reacts shows characteristic interactions when exposed to different food constituents, therefore, it is not practical and almost impossible to individually study such interactions (Ordovas and Corella, 2004). In vitro cell culture can offer advantages in terms of practicality and reproducibility of experiments. It can also be applied to medium to high-throughput screening of large number of compounds (Mortensen et al., 2008). One example is the incorporation of the Caco-2 cell line into different in vitro digestion models (Mortensen et al., 2008). The Caco-2 cell line is one of the most used models as an efficient tool for the absorption and permeability of bioactive compounds in foods and pharmaceutical preparations (Hur et al., 2011). Although Caco-2 is derived from human colorectal carcinoma, it retains many of the typical morphological features of human enterocytes (Pinto et al., 1983). The Caco-2 cell line was established in 1974 from human colon adenocarcinoma by Jorgen Fogh (Zweibaum et al., 1991), and has been used as a model system for studying intestinal absorption of drugs or functional compounds, evaluating dietary constituents, and contaminants, and testing for anticancer or cancer preventive phenolic compounds (Artrusson, 1990; Glahn et al. 1998; Giovanni et al., 1999).

Glahn et al. (1998) developed an in vitro digestion model that incorporates enzymatic digestion along with Caco-2 cell culture that is a rapid, low cost and an effective tool for screening iron bioavailability. This model has also been utilized by other researchers to predict iron bioavailability that correlates well with human studies. The Caco-2 cell line is well characterized, and the monolayers are able to differentiate into a structure resembling the
morphology of intestinal cells, expressing invertase and maltase; however, it does not secrete mucus (Williamson and Clifford, 2010).

HT-29 is another cell line that has been widely used in antiproliferative studies. HT-29 cell line is a pluripotent and moderately differentiated primary colon cancer cell line that was established in 1964 by Fogh. At a morphological level this cell line is homogeneous with subpopulations of cells with the capacity to differentiate. These cell line have been shown to consume high quantities of glucose (≈ 0.6 µmol• h⁻¹• mg⁻¹ protein) requiring approximately 25 mM glucose and daily medium change not to deprive the cells of glucose (Rousset et al., 1984; Zweibaum et al., 1985). Under such glucose supply and in the presence of serum (standard culture conditions), HT-29 populations have more than 95 % of undifferentiated cells, and during growth to postconfluence, the cells stack and do not polarize forming a multilayer (Fogh and Trempe, 1975). Functionally, they do not express any particular characteristics of epithelial intestinal cells, nonetheless, they are able to differentiate and undergo different intestinal patterns depending on modifications of the cell medium or the addition of inducers of differentiation (Zweibaum et al., 1991).

When mucus producing cell lines such as HT-29-MTX is incorporated into the in vitro digestion model a more accurate prediction data is obtained (Mahler et al., 2009). For instance, the transport of ferrulic acid was reduced by 23% using a co-cultured system of Caco-2 and HT-29-MTX. The supplied ferrulic acid was glucoronidated by the HT-29 cells alone, and hydroxycinammic acids were hydroxylated to dihydroxycinnamic acids. Dihydroferulic acid was more efficiently transferred to the basolateral side than dihydrocaffeic acid, in contrast to the system using rat inverted sacs (Poquet et al., 2008). Cillia et al. (2010) used an in vitro cell culture model after an in vitro digestion model to assess the antiproliferative effect of bioaccessible fractions of zinc-fortified fruit beverages against Caco-2 and HT-29 cell lines. Interestingly, they found that the fortification of the beverages with zinc decreased the soluble
polyphenol fraction before and after digestion; nonetheless, digested samples inhibited cell proliferation in both cell lines.

The CRL 1790 is a cell repository line designed as CCD841CoN which was isolated from female fetal tissue. The morphology of CRL 1790 cell line is similar to that of other mucosal lines. Although this cell line does not contain keratin and definitive evidence for epithelial character is lacking (Thompson et al. 1985). CRL 1790 cells can be incorporated in the in vitro systems to obtain more realistic information to study bioavailability and uptake of functional compounds. One of the advantages of these models is the fact that cells are of human origin and since cells are immortalized, experiments can be performed over a relatively long period without losing reproducibility (Anderberg and Artursson, 1993; Mortensen et al., 2008). In addition, the structure of these cell lines is indeed very similar to small intestine enterocytes, and the time course of the differentiation process mimics the situation found in the small intestine. However, these models have some limitations in regards to the fact that HT-29 and Caco-2 cell lines are not normal, but malignant and are not small intestinal, but colonic (Zweibaum et al., 1991).
CHAPTER 3
MATERIALS AND METHODS

In this chapter the general materials and methodologies used in this research are described. Details of the methods used in different stages of the research will be given in the following chapters.

3.1 Materials

3.1.1 Blueberries

Frozen wild blueberries (low-bush) obtained from the local supermarket were divided in two batches: 1) frozen blueberries and 2) blueberry freeze dried powder and stored at -20°C for further analysis. Blueberries from the same lot were used for all experiments.

A portion of the blueberries were freeze dried in a Modulyo D (Thermo Savant, USA) for 72 h. The temperature in the condensing chamber was controlled at -45 ± 5 °C and the pressure in the drying chamber was controlled at 100 Pa (1 mbar). The freeze dried samples at 2% moisture content (dry basis) were milled with a blender and stored in air tight containers at -20 °C.

3.1.2 Chemicals and reagents

All solvents were of HPLC grade. Methanol, peptone water, NaHCO₃, KH₂PO₄, and L-cysteine, and HCl were purchased from Fisher Scientific (Ottawa, Canada). Yeast extract was purchased from BD (New Jersey, USA). Casein and inulin (from Dahlia tubers) were purchased from Alfa Aesar (Massachusetts, USA), MgSO₄ and hemin were purchased from BDH (Pennsylvania, USA). Antifoam B silicone emulsion was purchased from J.T. Baker (Pennsylvania, USA). Anaerobe agar was obtained from Acumedia (Michigan, USA),
Defibrinated sheep blood was purchased from Hemostat Laboratories (California, USA). Formic acid, Folin Ciocalteu reagent, Na$_2$CO$_3$, gallic acid, 2,2-diphenyl-1 picrylhydrazyl (DPPH) radical, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), Nitrotetrazolium Blue chloride (NBT), Riboflavin, ethylenediaminetetraacetic acid (EDTA), methionine, p-nitrophenyl-α-D-Glucopyranoside, α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), pectin from apple, cellulose, pectinase from *Rhizopus* sp. (500 U/g solid), cellulase from *Trichoderma viride* (8560 U/g solid), Triton X-100, Tween 80, Sodium dodecyl sulfate (SDS), pepsin from porcine gastric mucosa (3802 units/mg protein), pancreatin from porcine pancreas (8x USP), bile salts, Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, trichloroacetic acid (TCA), sulforhodamine B (SRB), acetic acid, Tris base, CaCl$_2$, pectin (from citrus), xylan (from beechwood), arabinogalactan, NaCl, porcine gastric mucin (type II), menadione, and starch (from wheat, unmodified), and pectin from apple skin were purchased from SIGMA (Oakville, Canada). Cyanidin and delphinidin glucosides were purchased from Chromadex (Irvine, USA). Malvidin glucoside was obtained from from Extrasynthese (Geney, France). Human epithelial colorectal adenocarcinoma cell line (HT-29) and human normal epithelial colon cell line (CRL-1790) were obtained from the American Type Culture Collection (Rockville, USA). Minimal Essential Medium Eagle (MEME) was purchased from Hyclone, Thermoscientific (Ottawa, Canada). Phosphate buffer saline 10x (PBS), penicillin-streptomycin solution, and trypan blue were from Gibco (Bethesda, USA).

### 3.2 Methods

#### 3.2.1 Polyphenol extraction

The extraction of polyphenols is the very first step in the process of detecting and quantifying the total polyphenol content of a sample. The choice of the extraction method
depends on the nature of the material, the sample matrix in which the compounds are present, and the purpose of the extraction. A good extraction procedure should prevent degradation or alteration of the natural material and maximize the recovery of the compounds (Giusti and Jing, 2008). In several studies different solvents have been used to obtain high quantities of polyphenols closer to their natural state. For instance, 60 % methanol, n- butanol, cold acetone or mixtures of acetone, methanol, and water, or simply water are the most reported ones (Jackman et al, 1987). Although methanol is the most common solvent used, ethanol and water are preferred for food use to avoid the toxicity of methanolic solutions (Giusti and Jing, 2008).

For this research methanol and water were used as extraction solvents. Methanolic extracts allowed us to identify and quantify the majority of the polyphenols present in blueberries, and water on the other hand, simulated the state where anthocyanins are extracted naturally. One part of frozen blueberries was homogenized with two parts of solvent (50 g blueberries in 100 ml solvent) using a shear homogenizer (Brinkman Instruments, Westbury, NY) fitted with a Polytron PTA 10 probe during 5 minutes. The homogenates were centrifuged using a Beckman J2-21 centrifuge at 2700 x g for 20 min; the supernatants were collected and stored in amber tubes at -20 °C for further analysis.

3.2.2 Separation of free and complexed polyphenols

Blueberry extracts obtained after homogenization and centrifugations are rich in polyphenols and other components from the food matrix. In order to separate free polyphenols from the food matrix a conventional dialysis procedure was performed. Dialysis separates free small molecules from large or complexed molecules by allowing diffusion of the free molecules through a selectively permeable membrane. Figure 3.1 shows the typical setting used for dialysis. The sample to be dialyzed is loaded into a sealed dialysis membrane usually made of cellulose and immersed in a selected buffer. Small molecules equilibrate with the solutes across
the membrane. If the buffer solution moves across the membrane it can result in some sample dilution, generally increasing the sample volume inside the dialysis bag less than 50 % (Andrew et al., 1997). Blueberry extracts from the aqueous and methanolic crude extracts (10 ml) were transferred into a dialysis membrane reservoir (6 – 8 KDa cut-off, Spectrapor, Spectrumlabs, USA) previously soaked and rinsed with distilled water. The dialysis was carried out in 500 ml of distilled water at 4 °C for 24 hours. The dialyzed (complexed polyphenols) and the dialyzate (free polyphenols) were collected and used for the analysis.

3.2.3 Total polyphenol content

Blueberries are recognized as fruits with high amounts of polyphenols. The amount of polyphenols present in the different blueberry samples was estimated using the Folin-Ciocalteau (FC) assay, currently the standard protocol in food and wine analysis. Due to its high response to different dietary phenolic substances, this method is suitable for accurately measure total phenolic substances (Waterhouse, 2002). The assay is based on a chemical reduction of the FC reagent, which is a mixture of tungstate and molybdate in a highly basic medium (5- 10% Na₂CO₃). Phenolic compounds are oxidized in basic medium resulting in the formation of O₂⁻, which in turns reacts with molybdate and produce molybdenum oxide. The products of reduction have a maximum absorption at 765 nm and the intensity of the absorption is proportional to the concentration of phenols. The FC assay is not selective, it determines both polyphenols and monophenols; therefore, results are frequently expressed in Gallic Acid equivalents (GAE) (Singleton et al., 1999).
Figure 3.1 Making a dialysis chamber.
For blueberry samples, the total polyphenols content TPC was estimated using an adaptation of the microscale Folin-Ciocalteu method (Waterhouse, 2002). An aliquot of the extracts were mixed with 50% (v/v) ethanol to a final volume of 100 µl and 500 µl of distilled water along with 50 µl of FC reagent (2N). Then, 100 µl of 5% (w/v) sodium carbonate solution was added to each sample, vortexed, and incubated in the dark for 1 hour. Samples were mixed thoroughly after incubation and the absorbance was measured at $\lambda_{\text{max}} = 725$ nm using a spectrophotometer (Beckman Coulter DU 800, USA). A standard curve was generated using gallic acid with concentrations ranging from 0 to 200 µg/ ml. The polyphenol concentrations were expressed as weight of gallic acid equivalent per grams of fruit (fresh weight equivalent; the fresh weight of dry powder was converted to fresh weight).

### 3.2.4 Total anthocyanin content

Anthocyanins are highly affected by pH manifesting different absorbance spectra. The colored flavylium cation exists at pH 1, and the colourless hemiketal form exits at pH 4.5. The differential method (AOAC, 2005) measures the absorbance at these two different pH values and relies on structural transformations of the anthocyanin chromophore as a function of pH. Anthocyanins switch from a saturated bright red- bluish color at pH 1 to a colorless form at pH 4.5. Conversely, polymeric anthocyanins and others retain their color at pH 4.5 (Figure 3.2). Thus measurement of anthocyanins samples at pH 1 and 4.5 can remove the interference of other materials that may show absorbance at the 520 nm (Giusti and Wrolstad, 2001).

For the quantification of total anthocyanin content (TAC) aliquots (10 µl) of each sample were diluted with 490 µl of 0.025 M potassium chloride buffer (pH 1) and 495 µl of 0.4 M sodium acetate (pH 4.5) respectively. The absorbance of each dilution was measured at 500 and 700 nm against distilled water blank. The monomeric anthocyanin pigment concentration was expressed as cyanidin 3-glucoside equivalents according to the following equation:
\[
\text{TAC (mg cyan 3gluc/ml)} = \frac{(A \times MW \times DF)}{\varepsilon \times l}
\]  
Eq. 1

Where:
\[
A = (A_{520} - A_{700})_{pH=1} - (A_{520} - A_{700})_{pH=4.5}
\]  
Eq. 2

And:
\[
MW = 449.2 \text{ g/mol}
\]
\[
l = 1 \text{ cm}
\]
\[
\varepsilon = 26\,900 \text{ l/mol} \cdot \text{cm}
\]
\[
DF = \text{dilution factor}
\]

3.2.5 Alpha-Glucosidase Inhibition Assay

Alpha-glucosidase is a key enzyme involved in starch breakdown and absorption in the small intestine. This enzyme is needed by all animals to hydrolyze maltose to glucose. Inhibiting this enzyme can slow the breakdown of maltose and consequently reduce the absorption of glucose and the passage of carbohydrates into the bloodstream in vivo (Puls et al. 1977). As shown in Eq. 3, in vitro, α-glucosidase hydrolyzes the terminal, non reducing 1,4-linked α-D—glucose residues with release of α-D-glucose yielding a yellow color which can be measured at 405 nm. The enzyme activity is directly proportional to the rate of the reaction.

\[
p - \text{Nitrophenyl } \alpha - D - \text{ glucoside} \overset{\alpha - \text{glucosidase}}{\longrightarrow} \alpha - D - \text{ Glucose} + p - \text{Nitrophenol}
\]  
Eq. 3
Figure 3.2 Spectral characteristics of anthocyanins in pH 1.0 and 4.5 buffers, and the structure of the A) flavylium cation, and B) hemiketal forms. R=H or glycosidic substituent (Wrolstad et al., 2005).
In this research α-glucosidase inhibitory activity was measured according to a modified procedure described by Kwon et al. (2008). Blueberry samples were added into 1 mL cuvettes to obtain concentrations of 10, 25, 50 and 100 µg/mL of polyphenols and diluted with water to a sample volume of 50 µL. Plain water of equal volume was used as a control. Then 100 µL of 0.1 M sodium phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL) was added and the samples were incubated at 25°C for 10 min. After the reaction, 50 µL aliquots of 5 mM p-nitrophenyl-α-D-Glucopyranoside solution, prepared in 0.1 M sodium phosphate buffer (pH 6.9) were added to the tubes. The reaction mixtures $V_1 = 200$ µL in each cuvette were incubated at 25°C for 5 min. After incubation, 800 µL of plain water were added and absorbance readings were recorded at $\lambda_{\text{max}} = 405$ nm.

The inhibitory activity of the enzyme was expressed as percentage of inhibition and calculated as:

$$
\text{Alpha glucosidase inhibition (\%)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
$$

Eq. 4

3.2.6 In vitro digestion procedure

The main purpose of the in vitro digestion models is to simulate the conditions that occur in the stomach, upper intestine and colon. In the in vitro upper intestinal models, the removal of digestible components and the detection of changes in non-digestible ones are monitored.

For this study the gastrointestinal digestion was simulated according to a modification of Gil-Izquierdo et al. (2002). Samples of 50 g of frozen blueberry fruits were thawed and ground with 100 ml of distilled water using a Brinkman Homogenizer, fitted with a Polytron PTA 10 probe for 5 minutes. The pH was adjusted to 2 by addition of 10N HCl, then 15720 Units of pepsin were added and the samples incubated in a 37 °C shaking water bath (250 rpm). After 2
h incubation, aliquots (30 ml) were collected from each vessel and centrifuged at 27000 x g at 4 °C for 15 min. The supernatant was stored at – 20 °C for further analysis.

Digestion in the intestine was simulated by adjusting the pH of the remaining 20 ml from the gastric digestion to pH 7.5 with 0.5 N NaHCO₃. A solution of 2.5 ml of pancreatin (2 g/ L 8x USP specifications SIGMA) and 2.5 ml of bile salts (25 g/l) was added to each vessel and incubation was continued for an additional 2 h. At the end of the incubation period, aliquots of 10 ml were collected for the in vitro fermentation step; the pH of the remaining samples was adjusted to pH 2 and centrifuged as mentioned above. The supernatant was collected and stored at -20 °C for further analysis.

3.2.7 In vitro fermentation

3.2.7.1 Single-stage chemostat simulation of the human distal gut environment

An Infors Multifors bioreactor system (Infors, Switzerland) run as a chemostat was used for this work. A 400 ml culture was supported in a 500 ml vessel sparged with nitrogen gas (to maintain anaerobicity), and maintained at 37 °C and a pH of 6.9 – 7.0 (the latter by automated addition of 5 % (v/v) HCl, or 5 % (w/v) NaOH as required). Growth medium was continuously fed at a rate of 400 ml per day to give a retention time of 24 hours (Duncan et al., 2003; Van den Abdele et al., 2010). Prior to inoculation, the vessel was aseptically sampled to check for absence of contaminant growth on fastidious anaerobe agar supplemented with 5 % defibrinated sheep blood.

Chemostat growth medium was based on previous studies (MacFarlane et al., 1998; Lesmes et al., 2008) with several modifications. Media was prepared from 4 separate stock preparations as described below. To make 1L of media, 800 ml of preparation 1, 50 ml of
preparation 2, 50 ml of preparation 3, and 100 ml of preparation 4 were combined. Preparation 1 contained (per 800 ml): peptone water, 2 g; yeast extract, 2 g; NaHCO₃, 2 g; CaCl₂, 0.01 g; pectin, 2 g; xylan, 2 g; arabinogalactan, 2 g; starch, 5 g; casein, 3 g; inulin, 1 g; NaCl, 0.1 g. Preparation 2 contained (per 50 ml): K₂HPO₄, 0.04 g; KH₂PO₄, 0.04 g; MgSO₄, 0.01 g; hemin, 0.005 g; menadione, 0.001 g. Preparation 3 contained 0.5 g bile salts and 0.5 g L-cysteine HCl per 50 ml. Preparation 4 contained 4 g of porcine gastric mucin (type II) per 100 ml. Antifoam B silicone emulsion, 2.5 ml was added to each liter of prepared media. Media was stored at 4 °C for up to 2 weeks. Media preparations were checked for sterility by plating on fastidious anaerobe agar supplemented with 5% defibrinated sheep blood as above.

A defined community of 33 bacterial species, derived from a single healthy donor, was used for this work. Use of a defined community allowed for reproducible experiments and appropriate biological replicates, in comparison to fecal samples (which can vary donor-to-donor and cannot be easily characterized). The components of the defined community used for this work are described elsewhere (Petrof et al., 2013), but briefly, the mixture contained representatives of all 4 major phyla found in the human distal colon (Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria), and included strains of species that are known to be common residents of the human gut (e.g. Faecalibacterium prausnitzii, Bifidobacterium spp., Eubacterium rectale, Roseburia spp., Bacteroides spp. and Escherichia coli.) Freshly grown cultures of each of the 33 species components were inoculated into a prepared chemostat vessel and allowed to adjust to culture conditions for 24 h before the media pumps were switched on. Monitoring of the stability of the microbial community to steady-state was done using the method of McDonald et al. (submitted) briefly, by extracting gDNA from daily samples drawn from the vessel, and using this as template for amplification of the V3 region of the 16S rRNA genes, and subsequent separation of amplicons by %G+C content using denaturing gradient gel electrophoresis. Similarity indices of gel profiles were determined using
GeneDirectory software (Syngene), and moving window analysis was performed to ascertain the development and maintenance of steady state. At steady state, samples were removed from the vessel for fermentation experiments as described below.

Samples collected after the *in vitro* digestions from the upper intestine model were inoculated with aliquots of a sample drawn from the chemostat vessel at steady-state (described above), supporting a defined community of bacterial species representative of those found in the distal human gut, isolated from a single healthy donor. Five milliliters of digested sample was pre-reduced by overnight incubation under an anaerobic atmosphere (80 % N\textsubscript{2}, 10 % CO\textsubscript{2}, and 10 % H\textsubscript{2}) and then added to 200 µl of freshly drawn chemostat effluent, following which the sample was incubated at 37 °C for 12 h under anaerobic conditions as above. Following fermentation, the pH of each sample was adjusted to pH 2, and samples were centrifuged at 26940 x g, filtered through 0.2 µm polyethersulfone filters (Waters Corporation, MA, USA) to remove bacterial cells, and aliquots were stored at –20 °C for further analysis.

### 3.2.8 Solid Phase extraction

Blueberry samples are rich in polyphenols mixed with considerable quantities of extraneous materials such as pectins and sugars (Delgado-Vargas and Paredes-Lopez, 2003). Purification of the samples is a very important step for phenolics and anthocyanin characterization because interfering compounds prevent reliable HPLC separation and spectral information. One of the most common procedures to remove sugars and organic acids from the extract is by solid-phase extraction. Solid phase extraction (SPE) is a very simple and effective method of sample preparation. Components of interest present in the sample are isolated by loading the sample mixture to a solid chromatographic sorbent packed in a cartridge, usually a C\textsubscript{18} resin. The C\textsubscript{18} cartridge is composed by octadecylsilane activated with silica substrate which creates silyl-ether linkages. Anthocyanins and other phenolic compounds have high
affinity for the $C_{18}$ column and compete for binding sites. As it can be seen in Figure 3.3 a selective elution can be achieved by introducing a solvent such as water which removes the hydrophilic compounds such as acids and sugars. The cartridge will only retain the isolates if it is pre-conditioned with a moderately polar solvent such as methanol and then washed with water before loading the samples. After the sample is loaded to the cartridge, sugars, acids and other highly polar compounds can be removed with water, and finally polyphenols can be eluted by using alcohol (Rodriguez-Saona and Wrolstad, 2001).

A solid phase extraction (SPE) with Sep-Pak ® C18 (Waters Corporation, MA, USA) cartridge was used to clean and fractionate polyphenols according to Jacob et al. (2008). The columns were washed with water and polyphenols and anthocyanins eluted with 100% methanol. All the samples were concentrated to 1 mg/ ml of polyphenols, filtered with 0.45 μm nylon filters and used for LC-MS analyses.

3.2.9 Identification of individual polyphenols by HPLC-ESI-MS.

High performance liquid chromatography (HPLC) has become the method of choice for separation and characterization of anthocyanins due to its great resolution, short analysis time and easy quantitation compared to other analytical techniques. Reversed-phase chromatography offers also other advantages such as predictability of elution order based on polarity. The order of elution of the anthocyanins can be predicted based on their hydrophilicity/hydrophobicity characteristics. For instance, anthocyanins with free hydroxyl groups are more hydrophilic and elute faster than the hydrophobic anthocyanins with more methoxyl groups. Based on this, the elution of anthocyanins from a $C_{18}$ column generally follows the following pattern: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. The more glycosylations or hydroxylations a molecule has, the faster the elution from the column, conversely the more O-methylations and acylations, the slowest the elution time (Giusti and
Columns used for this analysis are almost exclusively reverse phase columns. C\textsubscript{18} stationary phase columns with internal diameters between 2.1 and 5 mm, and particle sizes ranging from 3 to 5 μm has been reported to be highly effective for the analysis of polyphenols using mass spectrometry coupled with liquid chromatography (Valls et al., 2009). The solvent system consists of a mobile phase composed of an aqueous medium and an organic medium (usually methanol or acetonitrile) that separates anthocyanins which later on can be detected by their absorbance at their visible wavelength maximum (Strack and Wray, 1989). Anthocyanins are usually detected between 480 and 500 nm by comparing retention times and UV-Vis chromatograms with commercial standards (Giusti and Jing, 2008). The lack of structural information provided by this detection method is a weakness. However, when it is coupled with mass spectrometry detectors, the identification and structural characterization of unknown compounds are notably improved (Flamini, 2003).

Mass Spectrometry (MS) analyses are used for the characterization of anthocyanins after they are isolated by HPLC. MS can be used to identify and quantify anthocyanins based on their differences in their charged fragments according to their mass-to-charge ratios. Typically, mass spectrometry is composed of an ion source, a mass analyzer and a detector. The ionization techniques reported elsewhere for the analyses of anthocyanins are atmospheric-pressure-chemical ionization (APCI) and the electrospray ionization (ESI). Generally APCI is used to ionize non-polar compounds that can undergo acid-base reaction in the gas phase. On the other hand, ESI is better for compounds that can be ionized in solution (McMaster, 2005). ESI in the positive mode is the unanimous choice for detecting anthocyanins, while the negative mode is used for detecting phenolic acids (Valls et al., 2009).
Figure 3.3 Solid-phase (C₁₈) purification of anthocyanins. The sample components (represented by differentially shaded circles) are resolved by subsequent wash steps as indicated. (Modified from Rodriguez-Saona and Wrolstad, 2001)
It should be noted that the choice of the mobile phase for the HPLC is critical, as buffers can interfere with the ionization process and influence significantly the sensitivity of the mass detectors. For instance phosphate buffers can quench the ionization process, thus formic acid instead of trifluoroacetic acid or ammonium acetate instead of phosphate buffers is recommended (Parsian, 2004). At the same time the column effluent rate and concentration of the mobile phase can affect the sensitivity of the mass detectors. For ESI is recommended to work with flow rates up to 2 ml/ min and no more than 1 % formic acid (Valls et al., 2009).

For this research, chromatographic analyses of the samples were performed on an Agilent 1100 series LC-MS. Separation was carried out using an X-Terra® MS C-18 (5μm, 150 x 3.0 mm, Waters Corporation, MA, USA). Anthocyanins were eluted with a gradient mobile phase formed by methanol (phase A) and 2.0% (v/ v) formic acid (phase B) at a flow rate of 0.8 ml/ min. The gradient used was as follows: 0 – 2 min, 93 % B; 2 – 30 min, 80% B; 30 – 45 min, 70% B; 45 – 50 min; 65 % B, 50 – 60 min, 50%; 60 – 65 min, 20%; 65 – 70 min, 93%. Detection was carried out at 520 and 260 nm. Electrospray ionization (ESI) was performed with an API-ESI mass spectrometer. Nitrogen was used as the nebulizing and drying gas, 12 l/ min at 350°C; ion spray voltage, 4000 V and fragmentor voltage, 80 V. ESI mass spectra were scanned from m/ z 250 to 700. Spectra were acquired in the positive and negative ion mode. A sample injection volume of 20 μl was used for all the samples. Identities of the compounds were obtained by matching their molecular ions (m/ z) obtained by LC-ESI-MS with literature data. Measurements were done from three independent experiments.

3.2.10 DPPH radical scavenging assay

DPPH radical scavenging assay is the oldest method for indirectly determining antioxidant activity of natural materials and has been a method of choice for fruit and vegetable extracts. DPPH• is considered a stable radical because of the spare electron over the molecule
preventing it to dimerize as other free radicals do. This delocalization provides a deep violet coloration characterized by an absorption maximum of 520 nm. The DPPH test is based on the reaction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) with H-donors including phenolics (Roginsky and Lissi, 2005). When samples that can donate a hydrogen atom are mixed with DPPH•, it is converted into a reduced form of DPPH along with the loss of its characteristic violet colour, which is reported as a function of the antioxidant activity. Figure 3.4 shows the schematic representation of the reaction between the DPPH• radical phenolics. The DPPH• reacts with phenols (ArOH) by a direct abstraction of phenol H-atom (Eq. 5), and by an electron transfer process from ArOH or its phenoxide anion (ArO•) to DPPH• (Eq. 6).

\[
\text{ArOH} + \text{DPPH}^* \rightarrow \text{ArO}^* + \text{H} - \text{DPPH} \quad \text{Eq. 5}
\]

\[
\text{ArO}^* + \text{DPPH}^* \rightarrow \text{products} \quad \text{Eq. 6}
\]

Total antioxidant activity of blueberry crude extracts and dialyzed fractions was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to Molyneux (2004). A 0.2 ml aliquot of phenolic aqueous extract (to give a final total phenol content of 10, 25, 50, 100 µg/ml or protein content of 0, 10, 20, 30 and 40 µg/ml) was mixed with 0.8 ml of 0.1 mM ethanolic DPPH, vortexed well and incubated at room temperature for 30 min and absorbance measured at \(\lambda_{\text{max}} = 517\) nm. The control sample was 1 ml of 95% ethanol. Antioxidant activity was expressed as % DPPH scavenging, calculated using the following equation:

\[
\text{DPPH radical scavenging (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \quad \text{Eq. 7}
\]
Figure 3.4 Schematic representation of the reaction between the DPPH$^*$ radical phenolics.
3.2.11 Superoxide anion radical scavenging assay

Superoxide anion $O_2^-$ is a very reactive oxygen species that has been implicated in reactions in both physiological and disease states (Freeman and Capro, 1982). The autoxidation and inactivation of small molecules such as reduced flavins, and thiols is the main generator of intracellular free radicals. The activity of certain oxidases, cyclooxygenases, lipoxygenases, dehydrogenases and peroxidases is a common source of $O_2^-$ (Lawrence et al., 1987). In the laboratory, singlet oxygen is generated by photosensitization reactions. If certain molecules such as riboflavin are illuminated with light of a given wavelength (400 nm) they absorb energy and the molecule gets into an “excited state”. The excitation energy is then transferred onto adjacent oxygen molecules, converting it to the singlet state while the photosensitizer molecule returns to the ground state (Halliwell and Gutteridge, 1985a, b).

In the assay, superoxide ions are generated by illuminating a solution of riboflavin in the presence of L- methionine. Riboflavin then re-oxidizes and simultaneously reduces oxygen to $O_2^-$, when it reacts with a detector molecule such as nitro blue tetrazolium (NBT) it forms a coloured solution of NBT-diformazan which has an absorption maximum at 560 nm (Eq. 8). Superoxide dismutase and phenolic compounds have been demonstrated to reduce the superoxide ion concentration lowering the NBT-diformazan formation (Halliwell and Gutteridge, 1985a, b). Thus, the reduction in NBT-formazan products can be used as a measure of the chemicals present in the solution with superoxide inhibiting ability

$$NBT + O_2^* \rightarrow \text{Diformazan} \quad \text{Eq. 8}$$

The superoxide anion activity of different blueberry samples was determined as described by Madamachi et al., 1994. The assay mixture (900 µl) contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 45 µM NBT, 100 µl of the
blueberry extract (0, 10, 25, 50, 100 μl/ml) and 1 ml of Riboflavin (2 μM). The reaction was initiated by illuminating the tubes under a 15 W fluorescent lamp. The reaction was terminated after 15 min by turning off the fluorescent lamp. Samples that were covered and incubated in the dark served as blanks. The ability of blueberry extracts to inhibit the photochemical reduction of NBT was measured at 560 nm as:

\[
\text{Superoxide radical scavenging (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100
\]

Eq. 9

3.2.12 Hydroxyl radical scavenging assay

In the laboratory, hydroxyl radicals can be generated by the reaction of iron-EDTA complex with hydrogen peroxide in the presence of ascorbic acid and deoxyribose to malonaldehyde that reacts with thiobarbituric acid (TBA) upon heating at low pH. When hydroxyl scavengers such as phenolics are added to the mixture, they compete with the deoxyribose for providing the hydrogen ions to Fe-EDTA and reducing the ascorbic acid levels. This result in a reduction of TBA, and a reduction in the pink chromagen formation (Halliwell et al., 1987). The sugar deoxyribose is degraded in the presence of hydroxyl radicals generated by irradiation or by Fenton systems. Malonaldehyde (MDA) is formed when the resulting mixture is heated under acidic conditions, and it may be detected by its ability to react with TBA to form a pink chromophere that has an absorption maximum of 532 nm. When a \( \text{Fe}^{2+} \)-EDTA chelate in incubated with deoxyribose at pH 7.4, hydroxyl radicals are formed and if they are not scavenged by the EDTA they can react with the deoxyribose as following:

\[
\text{Fe}^{2+} + \text{EDTA} + O_2 \leftrightarrow \text{Fe}^{3+} - \text{EDTA} + O^-_2
\]

Eq. 10

\[
2O^-_2 + 2H^+ \rightarrow H_2O_2 + O_2
\]

Eq. 11
This assay was based on the method described by Halliwell et al. (1987). Different volumes of blueberry extracts (so as to obtain a total phenol concentration of 10, 25, 50, 100 µg/ml) were added to 690 µl of 2.5 mM 2-deoxyribose (made up in 10 mM phosphate buffer, pH 7.4) and 100 µl of 1 mM ferric chloride (made up in 1.04 mM MEDTA). The samples were kept in a water bath at 37°C and reaction was started by adding 100 µl of 1mM ascorbic acid and 10 µl of freshly prepared 0.1M H₂O₂. After 20 minutes, the reaction was stopped by adding 1 ml of 2.8% cold trichloroacetic acid (TCA) and 0.5 ml of 0.5% thiobarbituric acid (TBA). The samples was then boiled for 8 minutes, cooled and finally the absorbance measured at λ<sub>max</sub> = 532 nm.

The hydroxyl radical scavenging capacity blueberry polyphenols was expressed as % scavenging relative to the control:

\[
Hydroxyl \text{ radical scavenging} \, (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100
\]

3.2.13 Cell culture

3.2.13.1 Cell lines

HT-29 cells were cultured in 25 cm² flasks with DMEM medium supplemented with 10% (v/v) FBS, 200 mM L-glutamine and 1% (v/v) penicillin/streptomycin. CRL-1790 cells were cultured in 25 cm² flasks with MEM supplemented with FBS, glutamine and penicillin/streptomycin as above. Both cell lines were incubated at 37 °C in a humidified chamber including 5 % CO₂. Medium was changed 2-3 times per week.
3.2.13.2 Cell Proliferation Assay

HT-29 cells seeded at $2.0 \times 10^3$ cells per well and CRL-1790 seeded at $1.0 \times 10^3$ cells per well in 96-well plates were allowed to incubate for 24 hr. At time 0, blueberry extracts, stomach digests, intestinal digests, and colon digests (1 mg GAE/ ml) were diluted in growth media to obtain polyphenol concentrations of 10, 25, 50, 75 or 100 μg/ ml and dilutions were immediately added to prepare HT-29 or CRL-1790 cells. Negative control cells were supplied with growth media, and blank wells contained growth media only.

The sulforhodamine B (SRB) assay is one of the most widely used methods for cell density determination. It is based on the measurement of cellular protein content. The assay relies on the ability of SRB dye to bind to protein components of the cell. Cells are fixed to tissue culture plates by adding cold tricholoracetic acid (TCA). SRB dye is a bright-pink aminoxanthane compound with two sulfonic grups that binds to basic amino-acid residues under mild conditions, and dissociate under basic conditions. Therefore the amount of dye extracted from stained cells is directly proportional to the protein content in the cell mass, and it can be measured by spectrophotometry at a wavelength of 510 nm. The sensitivity of this test is very high allowing the assay to be carried out in 96- well plates and detect densities as low as 1,000 – 2,000 cells per well (Skehan et al., 1990; Vichai and Kirtikara, 2006).

Cell proliferation assays were carried out according to a modification of the method of Vichai and Kirtikara (2006) using HT-29 and CRL-1790 cells. Briefly, after 24 and 48 h incubation, spent media was replaced with media free from any additives or growth factors. Cells were fixed to the bottom of the wells by the addition of 50 μl of 50% (w/v) TCA at 4°C for 1 hr. TCA and media were then removed from the wells and cells were washed 5 times with 300 μl QHPLC-grade water to remove residual TCA, and media, following which cells were dried using a blow drier set to cool. SRB dye (0.4% dissolved in 1% (v/ v) acetic acid, 50 μl) was added to each well and incubated at room temperature for 30 min. Wells were washed 5 times
with 1% (v/v) acetic acid to remove any unbound dye and dried as above. Incorporated dye was then solubilized in 100 μl 10 mM Tris buffer for 15 min at room temperature with agitation. Optical density at 570 nm was measured for each well using a Synergy™ HT Multi Detection Microplate Reader (Bio-Tek Instruments) and KC4 version 3.03Power Report™ Bio-Tek® software was used to analyze the data.

3.2.14 Statistical Analysis

Statistical analyses were conducted using Prism software, version 4 (Graph Pad) and SPSS version 12.0. Analysis of variance (ANOVA) and Tukey’s post-test analyses of three independent experiments were conducted to evaluate the level of significance. P values < 0.05 were considered to be significant.
CHAPTER 4

ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF FREE AND COMPLEXED BLUEBERRY (Vaccinium angustifolium) POLYPHENOLS

ABSTRACT

Blueberries are a rich source of dietary polyphenols with several linked health-benefits. Biological activity of polyphenols is influenced by their rate of absorption which, in turn is highly affected by food matrix interactions. This study evaluated the effect of the blueberry food matrix on the antioxidant activity, and assessed their potential antiproliferative properties using two colon cell lines, HT-29 and CRL 1790, the former being a colorectal cancer cell line and the latter being a normal colon cell line. Free and complexed polyphenols were obtained by dialyzing methanolic-extracted and water-extracted blueberries. Polyphenols and anthocyanins were isolated using solid phase extraction, and quantified by LC-MS. Total polyphenols ranged from 1.6 to 3.6 mg of gallic acid equivalent (GAE)/g fruit, and total anthocyanins ranged from 0.4 to 1.8 mg of cyanidine 3-glucoside equivalents (cyan 3-gluc)/g fruit. Seven phenolic compounds and 13 anthocyanins were identified in the extracts from both frozen and freeze dried blueberries. All aqueous samples showed significant antioxidant and antiproliferative activities. Crude extracts containing free polyphenols and polyphenols complexed to the food matrix showed high free radical scavenging activities (DPPH, hydroxyl and superoxide) at concentrations of 100 µg/ml. Although free polyphenols showed a lower degree of free radical scavenging, their antiproliferative activity, and α-glucosidase enzymatic inhibition was higher than complexed polyphenols. These findings suggest that interactions between polyphenols and the food matrix play an important role in the bioefficacy of blueberry polyphenols.

Key words: Blueberries, food matrix, antioxidant, antiproliferative
4.1 Introduction

Blueberries (*Vaccinium angustifolium*) are widely produced in Eastern Canada and North Eastern U.S, and are becoming a popular berry fruit due to their functional properties. In Canada and the United States, most of the wild blueberries are managed and harvested commercially with more than 97% of the total production being processed (Strik, 2007). Blueberries have drawn increased interest due to their high concentration of polyphenols (Prior *et al*., 1998).

Dietary polyphenols are secondary metabolites that show biological properties such as antioxidant, anticarcinogenic, or antimutagenic activities (Papadopoulou and Fraizer, 2004; Williamson and Manach, 2005, Wang and Stoner, 2008). Blueberries are rich in polyphenols such as anthocyanins. (Kyle and Duthie, 2006). Anthocyanins can be present as monoglycosides (e.g. galactoside, glucoside and arabinosides) of five aglycones (delphinidin, cyanidin, petunidin, peonidin and malvidin). Since each aglycone may be glycosylated and/ or acylated by different sugars and phenolic acids and aliphatic acids, about 539 different anthocyanins have been reported from plants (Andersen and Jordheim, 2006). Besides being responsible for their intense red-bluish colours in berries, they are believed to provide protection against oxidative stress induced by free radicals (Jackman and Smith, 1996). Since oxidative activities have been identified as a causative factor for the development of many chronic diseases, high consumption of anthocyanin-rich foods may prevent their occurrence (Cho *et al*, 2004).

Inhibitory effects of purified plant phenolics and extracts on mutageniesis and carcinogenesis have been published elsewhere (Meyers *et al*., 2003; Yi *et al*., 2005). Some studies have focused on the antioxidant and chemopreventive effects of single compounds used at high concentrations (Zhao *et al*, 2004). Although this information may help identifying its mechanisms of action, the additive or synergistic effects provided by all the components present
in whole fruits cannot be observed by such approach (Liu, 2003). Since functionality of foods is highly influenced by the macromolecular organization of food and the interactions between food components, experiments performed on pure components may not reflect a realistic situation (Aguilera et al., 2000; Coates et al., 2007).

Functionality of foods is usually evaluated based on individual components and the potential effect of food matrix is rarely considered (Turgeon and Rioux, 2011). It is essential to understand the influence of the food matrix on the release of the functional compounds in order to develop value-added products with enhanced health promoting functions. Studies addressing total polyphenols and anthocyanins from complex blueberry mixtures are rare; therefore, the objective of this work was to identify free and complexed polyphenols present in blueberries and analyze their in vitro antioxidant capacity and antiproliferative activity on colon cancer cells. Comparing such properties of different polyphenol fractions from blueberries may help to better understand the role of polyphenol complexes in nutrition and health. A colon cancer cell line (HT-29) widely employed as a model of colorectal cancer was used along with a normal colon cell line (CRL-1790) to assess and compare the antiproliferative effect of free and complexed polyphenols extracted from frozen and freeze dried blueberry powder.

4.2 Materials and Methods

4.2.1 Fruits

Frozen wild blueberries (low-bush) obtained from the local supermarket were divided into two batches: 1) frozen blueberries and 2) blueberry freeze dried powder and prepared following the specifications mentioned in 3.1.1.
4.2.2 Chemicals and reagents

All solvents were of HPLC grade. Methanol was purchased from Fisher Scientific (Ottawa, Canada). Formic acid, Folin Ciocalteu’s Reagent, Gallic Acid, 2,2-diphenyl-1 picrylhydrazyl (DPPH) radical, (+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), Nitrotetrazolium Blue chloride (NBT), Riboflavin, Ethylenediaminetetraacetic acid (EDTA), Methionine, 2-deoxyribose, ferric chloride, ascorbic acid, p-nitrophenyl-α-D-Glucopyranoside, α-glucosidase from Saccharomyces cerevisiae (EC 3.2.1.20), trichloroacetic acid (TCA), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, surflorhodamine B (SRB), acetic acid, and Tris base were purchased from Sigma Aldrich (Oakville, Canada). Cyanidin and delphinidin glucosides were purchased from Chromadex (Irvine, USA) and malvinidin glucoside from extrasynthese (Geney, France). Human epithelial colorectal adenocarcinoma cell lines HT-29 and human normal epithelial colon cell line CRL-1790 were obtained from the American Type Culture Collection (Rockville, USA). Minimal Essential Medium Eagle (MEME) was purchased from Hyclone (Thermoscientific, Canada). Phosphate buffer saline (PBS) 10 x, penicillin-streptomycin solution and trypan blue were from Gibco (Bethesda, USA).

4.2.3 Polyphenol extraction

Frozen blueberries (F) (50 g) and freeze dried powder (P) (6 g) were ground with 100 ml of water (FW, PW) or 100 ml of methanol (FM, PM) using a Brinkman Homogenizer, fitted with a Polytron PTA 10 probe for 5 minutes. The homogenates were centrifuged using a Beckman J2-21 centrifuge at 2700 x g for 20 min; the supernatants were collected in amber tubes and stored at -20 °C for further analysis. See section 3.2.1 for more details.
4.2.4 Separation of free and bound polyphenols

Blueberry extracts from the crude extract (10 ml) were loaded on a Spectra/por molecular porous membrane (6 – 8 KDa cut off). Dialysis was carried out against 500 mL of distilled water at 4 °C for 24 hours. The dialyzed (complexed polyphenols) and the dialyzate (free polyphenols) were collected and used for further analysis. See section 3.2.3 for more details.

4.2.5 Total polyphenol content

Total polyphenol content of the samples was estimated by using the Folin–Ciocalteau method. See section 3.2.4 for more details.

4.2.6 Total anthocyanin content

Total anthocyanin content of blueberry samples was estimated following the pH differential method described in AOAC (2005). See section 3.2.5 for more details.

4.2.7 Solid Phase extraction

A solid phase extraction (SPE) with Sep-Pak ® C18 (Waters Corporation, MA, USA) cartridge was used to purify and fractionate polyphenols according to Jacob et al. (2008). All the samples were concentrated to 1 mg/ ml of polyphenols, filtered with 0.45 μm nylon filters and placed into a LC auto sampler vials for LC-MS analyses. See 3.2.9 for more details.

4.2.8 Identification of individual polyphenols by LC-ESI-MS.

Chromatographic analysis of anthocyanins isolated from the crude extracts, the dialyzed and the dialyzate fractions were performed using liquid chromatography (LC) and Mass Spectrometry (MS). See section 3.2.10 for more details.
4.2.9 DPPH radical scavenging assay

Total antioxidant activity of blueberry crude extracts and dialyzed fractions was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to Molyneux (2004). Vitamin C and TROLOX were used as standards See section 3.2.10 for more details.

4.2.10 Superoxide anion radical scavenging assay

The superoxide anion activity was determined as described by Madamachi et al., 1994. Vitamin C and TROLOX were used as standards. See section 3.2.11 for more details.

4.2.11 Hydroxyl radical scavenging assay

This assay was based on the method described by Halliwell et al. (1987). TROLOX was used as standard. See section 3.2.12 for more details.

4.2.12 Alpha glucosidase inhibition

See section 3.2.5 for details.

4.2.13 Cell proliferation assay

See section 3.2.13 for details.

4.2.14 Statistical Analysis

One was analyses of variance (ANOVA) and multiple comparison tests were conducted as described in Section 3.2.14.
4.3 Results

4.3.1 Polyphenol composition of blueberry extracts

Blueberry extracts were prepared from frozen blueberries and blueberry freeze dried powder using methanol or water as solvents. The total polyphenol and anthocyanin content are presented in Table 4.1. Total polyphenol content ranged from 1.6 – 3.6 mg GAE/ g and total anthocyanin content ranged between 0.4 to 1.8 mg cyan 3-gluc/ g fresh fruit.

4.3.2 Identification and quantification of phenolic compounds

The polyphenols from the dialyzed and dialyzate fractions obtained using 6–8 kDa molecular weight cut-off dialysis membrane were analyzed to compare the effectiveness of free and complexed polyphenols on the antioxidant and antiproliferative activity of blueberry polyphenols. The identification of individual phenolics and anthocyanins is reported in Table 4.2.

The HPLC chromatograms of the methanolic extracts revealed the presence of 16 major phenolic compounds (Figure 4.1). Identities of the compounds were obtained by matching their molecular ions (m/ z) obtained by LC-ESI-MS with published data. The content of individual polyphenols in different blueberry samples extracted with water and methanol is shown in Table 4.3 and Table 4.4 respectively. As reported earlier, chlorogenic acid was the major phenolic acid component in blueberries (Gu et al., 2002, Kalt et al. 2008), approaching nearly 50% of the total phenolic components in the methanolic and aqueous crude extracts and the dialyzates. Quercetin arabinoside was the major flavonoid glucoside, present in both crude extract and dialyzate. In addition, flavonoids such as myricetin, quercetin arabinoside and syringetin galactoside were also present in the methanolic and aqueous crude extracts. Aqueous crude extracts contained low amounts of isorhametin and 4-O feruloylquinic acid (chromatograms not shown). Surprisingly, no caffeic and p-coumaric acids were detected in any fractions. Both
methanolic and aqueous dialyzed fraction contained nearly 25% of the polyphenols present in the crude extract, having most of the components typically present in the crude extract and the dialyzate.

The amounts of individual anthocyanins identified in all samples are shown in Table 4.5 and 4.6, respectively. Anthocyanins included arabinosides, glucosides and galactosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Delphinidin-3-galactoside, delphinidin-3-glucoside, peonidin-3-galactoside, and peonidin-3-arabinoside could be observed among the anthocyanins in significant amounts. Glucoside and galactoside of malvidin were also present in high amounts. Acetoyl glucosides of delphinidin and malvidin were also observed in significant amounts. Surprisingly, no coumaroyl glucosides of any anthocyanins were observed in any of the extracts. The anthocyanin that was present in the highest concentration was delphinidin 3-glucoside.

In both aqueous and methanolic extract, the dialyzed extract contained most of the anthocyanins in low amounts, totaling to nearly 20% of the total anthocyanins present in the crude extract. The dialyzed extract showed a composition that was both qualitatively and quantitatively different than that one of the crude extract or dialyzate.
Table 4.1 Total polyphenol content and total anthocyanin content of different blueberry extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>Total phenol*</th>
<th>Total Anthocyanin**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen blueberry</td>
<td>Water</td>
<td>1.662 ± 0.033a</td>
<td>0.401 ± 0.042c</td>
</tr>
<tr>
<td>Frozen blueberry</td>
<td>Methanol</td>
<td>3.203 ± 0.077b</td>
<td>1.805 ± 0.057d</td>
</tr>
<tr>
<td>Blueberry freeze dried powder</td>
<td>Water</td>
<td>2.001 ± 0.153a</td>
<td>0.473 ± 0.175c</td>
</tr>
<tr>
<td>Blueberry freeze dried powder</td>
<td>Methanol</td>
<td>3.635 ± 0.190b</td>
<td>1.778 ±0.156d</td>
</tr>
</tbody>
</table>

Notes: Means with different letters are statistically significant (p< 0.05). Mean ± SEM n= 3.  
*Expressed in gallic acid equivalents (GAE), (mg GAE/ g fruit).  
** Expressed in cyanidin 3-glucoside equivalents (cyan 3-gluc), (mg cyan 3-gluc/ g fruit).
Table 4.2 Identification of Polyphenols from Blueberry crude extracts.

<table>
<thead>
<tr>
<th>Peak</th>
<th>tr\textsuperscript{a} (min)</th>
<th>m/z</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$15.75 \pm 0.21$</td>
<td>353</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>2</td>
<td>$22.00 \pm 0.25$</td>
<td>465</td>
<td>Delphinidin 3-galactoside</td>
</tr>
<tr>
<td>3</td>
<td>$24.91 \pm 0.23$</td>
<td>465</td>
<td>Delphinidin 3-glucoside</td>
</tr>
<tr>
<td>4</td>
<td>$26.91 \pm 0.21$</td>
<td>435</td>
<td>Delphinidin 3-arabinoside</td>
</tr>
<tr>
<td>5</td>
<td>$28.70 \pm 0.21$</td>
<td>449</td>
<td>Cyanidin 3-glucoside</td>
</tr>
<tr>
<td>6</td>
<td>$30.27 \pm 0.20$</td>
<td>479</td>
<td>Petunidin 3-galactoside</td>
</tr>
<tr>
<td>7</td>
<td>$33.30 \pm 0.24$</td>
<td>463</td>
<td>Peonidin 3-galactoside</td>
</tr>
<tr>
<td>8</td>
<td>$35.05 \pm 0.17$</td>
<td>449</td>
<td>Petunidin 3-arabinoside</td>
</tr>
<tr>
<td>9</td>
<td>$36.77 \pm 0.23$</td>
<td>493</td>
<td>Malvidin 3-galactoside</td>
</tr>
<tr>
<td>10</td>
<td>$39.03 \pm 0.19$</td>
<td>433</td>
<td>Peonidin 3-arabinoside</td>
</tr>
<tr>
<td>11</td>
<td>$40.56 \pm 0.18$</td>
<td>493</td>
<td>Malvidin 3-glucoside</td>
</tr>
<tr>
<td>12</td>
<td>$45.15 \pm 0.17$</td>
<td>463</td>
<td>Malvidin 3-arabinoside</td>
</tr>
<tr>
<td>13</td>
<td>$47.44 \pm 0.20$</td>
<td>507</td>
<td>Delphinidin 6-acetyl,3-glucoside</td>
</tr>
<tr>
<td>14</td>
<td>$49.06 \pm 0.53$</td>
<td>535</td>
<td>Malvidin 6-acetyl,3-glucoside</td>
</tr>
<tr>
<td>15</td>
<td>$52.98 \pm 0.17$</td>
<td>433</td>
<td>Quercetin arabinoside</td>
</tr>
<tr>
<td>16</td>
<td>$55.54 \pm 0.11$</td>
<td>507</td>
<td>Syringetin 3-O-galactoside</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Retention time, the result represents the mean ± SD (n= 3).
Figure 4.1 HPLC profile of methanolic blueberry polyphenols, detected at 260 nm. a) crude extracts, b) dialyzed fraction, c) dialyzate fraction. Refer to table 1 for the identification of each numbered peak.
Table 4.3 Polyphenol composition (mg/ g fruit) of a) Crude extract, b) Dialyzed fraction and the c) Dialyzate fraction obtained from blueberry frozen fruits and blueberry freeze dried powder extracted with water.

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Crude extracts&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Dialyzed&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Dialyzate&lt;sup&gt;A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frozen fruit</td>
<td>Powder</td>
<td>Frozen fruit</td>
</tr>
<tr>
<td>Isorhametin</td>
<td>0.028 ±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.024 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tr</td>
</tr>
<tr>
<td>Myricetin</td>
<td>0.024 ±0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.025 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.031 ± 0.014&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.216 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.195 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.062 ± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-O-feruloyquinic acid</td>
<td>0.041 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.043 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.036±0.006&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quercetin arabinoside</td>
<td>0.091 ± 0.021&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.023 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.076 ± 0.008&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quercetin a-l-rhamnoside</td>
<td>0.019 ± 0.015&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.021 ± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tr</td>
</tr>
<tr>
<td>Syringetin 3-o-galactoside</td>
<td>0.020 ± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.021 ± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tr</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>0.440 ± 0.040&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td><strong>0.407 ± 0.022&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td><strong>0.145 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</strong></td>
</tr>
<tr>
<td>Total polyphenols&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.600± 0.100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.000±0.040&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.570±0.020&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A</sup>Means with different letters in the same row are statistically significant (p< 0.05). Mean ± SEM. n= 3.<br><sup>B</sup>Total phenolic content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses.<br><sup>C</sup>Total polyphenol content estimated by Folin-Ciocalteau assay and expressed in gallic acid equivalents (GAE), (mg GAE/ g fruit). Tr = traces.
Table 4.4 Polyphenol composition (mg/ g fruit) of a) Crude extract, b) Dialyzed fraction, and the c) Dialyzate fraction obtained from blueberry frozen fruits and blueberry freeze dried powder extracted with methanol.

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>SOLVENT: Methanol</th>
<th>a) Crude extracts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dialyzed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dialyzate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frozen fruit</td>
<td>Powder</td>
<td>Frozen fruit</td>
<td>Powder</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isorhametin</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Myricetin</td>
<td>0.024 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.020 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.015 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003 ± 0.001&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.379 ± 0.015&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.350 ± 0.033&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.071 ± 0.011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.033 ± 0.010&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-O-feruloyquinic acid</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Quercetin arabinoside</td>
<td>0.029 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.032 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.046 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.027 ± 0.018&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quercetin a-l-rhamnoside</td>
<td>0.170 ± 0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.094 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tr</td>
<td>0.108 ± 0.013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Syringetin 3-o-galactoside</td>
<td>0.027 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.029 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.087 ± 0.012&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>TOTAL</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td><strong>0.629 ± 0.037&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td><strong>0.589 ± 0.045&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td><strong>0.142 ± 0.029&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td><strong>0.085 ± 0.040&lt;sup&gt;b&lt;/sup&gt;</strong></td>
</tr>
<tr>
<td>Total polyphenol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.230±0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.63±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.970±0.020&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.800±0.020&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means with different letters in the same row are statistically significant (p< 0.05). Mean ± SEM. n= 3. <sup>b</sup>Total phenolic content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses. <sup>c</sup>Total polyphenol content estimated by Folin-Ciocalteau assay and expressed in gallic acid equivalents (GAE), (mg GAE/ g fruit). Tr = traces.
Table 4.5 Anthocyanin composition of the a) Crude extract, b) Dialyzed and the c) Dialyzate blueberry frozen fruits and blueberry freeze dried powder extracted with water.

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>Frozen fruit</th>
<th>Powder</th>
<th>Dialyzed a</th>
<th>Frozen fruit</th>
<th>Powder</th>
<th>Dialyzed a</th>
<th>Frozen fruit</th>
<th>Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin 3-galactoside</td>
<td>0.007 ± 0.001 a</td>
<td>0.009 ± 0.003 a</td>
<td>Tr</td>
<td>0.009 ± 0.001 a</td>
<td>0.014 ± 0.005 a</td>
<td>0.011 ± 0.003 a</td>
<td>0.018 ± 0.005 a</td>
<td></td>
</tr>
<tr>
<td>Delphinidin 3-glucoside</td>
<td>0.015 ± 0.003 a</td>
<td>0.016 ± 0.003 a</td>
<td>Tr</td>
<td>0.010 ± 0.003 b</td>
<td>0.029 ± 0.032 b</td>
<td>0.017 ± 0.004 c,b</td>
<td>0.004 ± 0.004 a,b</td>
<td></td>
</tr>
<tr>
<td>Delphinidin 3-arabinoside</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Cyanidin 3-glucoside</td>
<td>0.016 ± 0.003 a</td>
<td>0.017 ± 0.002 a</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.010 ± 0.003 b</td>
<td>0.029 ± 0.032 b</td>
</tr>
<tr>
<td>Petunidin 3-galactoside</td>
<td>0.012 ± 0.003 a,b</td>
<td>0.012 ± 0.002 a,b</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.104 ± 0.003 b</td>
<td>0.017 ± 0.004 a,b</td>
<td>0.004 ± 0.004 b,a</td>
</tr>
<tr>
<td>Peonidin 3-galactoside</td>
<td>0.019 ± 0.004 a</td>
<td>0.020 ± 0.002 a</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.061 ± 0.012 a,b</td>
<td>0.038 ± 0.015 b</td>
<td>0.007 ± 0.007 b</td>
</tr>
<tr>
<td>Petunide 3-arabinoside</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.055 ± 0.005 a</td>
<td>0.055 ± 0.007 b</td>
<td>0.006 ± 0.006 a</td>
</tr>
<tr>
<td>Malvidinid 3-galactoside</td>
<td>0.077 ± 0.011 a</td>
<td>0.056 ± 0.011 a,b</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.061 ± 0.012 a,b</td>
<td>0.038 ± 0.015 b</td>
<td>0.007 ± 0.007 b</td>
</tr>
<tr>
<td>Peonidin 3-arabinoside</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.017 ± 0.005 a</td>
<td>0.013 ± 0.006 a</td>
<td>0.006 ± 0.006 a</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>0.072 ± 0.007 a</td>
<td>0.055 ± 0.007 b</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.017 ± 0.005 a</td>
<td>0.013 ± 0.006 a</td>
<td>0.006 ± 0.006 a</td>
</tr>
<tr>
<td>Malvidin 3-arabinoside</td>
<td>0.024 ± 0.004 a</td>
<td>0.017 ± 0.003 b</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>0.017 ± 0.005 a</td>
<td>0.013 ± 0.006 a</td>
<td>0.006 ± 0.006 a</td>
</tr>
<tr>
<td>Delpinidin 6-acetyl.3-glucoside</td>
<td>0.095 ± 0.014 a</td>
<td>0.135 ± 0.010 a</td>
<td>0.029 ± 0.008 b</td>
<td>0.044 ± 0.010 b</td>
<td>0.101 ± 0.022 a</td>
<td>0.132 ± 0.028 a</td>
<td>0.007 ± 0.007 b</td>
<td>0.008 ± 0.007 b</td>
</tr>
<tr>
<td>Malvidin 6-acetyl 3-glucoside</td>
<td>0.116 ± 0.010 a</td>
<td>0.120 ± 0.014 a</td>
<td>0.054 ± 0.018 b,c</td>
<td>0.035 ± 0.033 b</td>
<td>0.101 ± 0.036 c</td>
<td>0.101 ± 0.020 c</td>
<td>0.007 ± 0.007 b</td>
<td>0.008 ± 0.007 b</td>
</tr>
<tr>
<td>TOTAL a</td>
<td>0.453 ± 0.056 a</td>
<td>0.455 ± 0.052 a</td>
<td>0.083 ± 0.025 b</td>
<td>0.079 ± 0.006 b</td>
<td>0.472 ± 0.089 a</td>
<td>0.427 ± 0.070 a</td>
<td>0.007 ± 0.007 b</td>
<td>0.008 ± 0.007 b</td>
</tr>
<tr>
<td>TA c</td>
<td>0.36±0.054 a</td>
<td>0.473±0.016 a</td>
<td>0.066±0.006 b</td>
<td>0.068±0.013 b</td>
<td>0.304±0.015 c</td>
<td>0.348±0.022 c</td>
<td>0.007 ± 0.007 b</td>
<td>0.008 ± 0.007 b</td>
</tr>
</tbody>
</table>

a Means with different letters in the same row are statistically significant (p< 0.05). Mean ± SD n= 3. b The total anthocyanin content calculated from the sum of total anthocyanin peak areas obtained by HPLC analyses c TA = Total anthocyanin content estimated by the pH differential assay and expressed in cyanidin 3-glucoside equivalents (cyan 3-gluc), (cyan 3-gluc/ g fruit). Tr = traces
<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>a) Crude extracts</th>
<th>Dialyzed</th>
<th>Dialyzate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frozen fruit</td>
<td>Powder</td>
<td>Frozen fruit</td>
</tr>
<tr>
<td>Delphinidin 3-galactoside</td>
<td>0.138 ± 0.008a</td>
<td>0.121 ± 0.010a</td>
<td>0.018±0.003b</td>
</tr>
<tr>
<td>Delphinidin 3-glucoside</td>
<td>0.219 ±0.010a</td>
<td>0.168 ± 0.013b</td>
<td>0.033 ± 0.007c</td>
</tr>
<tr>
<td>Delphinidin 3-arabinoside</td>
<td>0.075 ± 0.002a</td>
<td>0.057 ± 0.004b</td>
<td>Tr</td>
</tr>
<tr>
<td>Cyanidin 3-glucoside</td>
<td>0.068 ± 0.003a</td>
<td>0.054 ± 0.004b</td>
<td>Tr</td>
</tr>
<tr>
<td>Petunidin 3-galactoside</td>
<td>0.094 ± 0.006a</td>
<td>0.076 ± 0.006b</td>
<td>0.0165 ± 0.003c</td>
</tr>
<tr>
<td>Peonidin 3-galactoside</td>
<td>0.129 ± 0.022a</td>
<td>0.107 ± 0.008a,b</td>
<td>0.025 ± 0.003c</td>
</tr>
<tr>
<td>Petunidin 3-arabinoside</td>
<td>0.032 ± 0.006a</td>
<td>0.025 ± 0.002b</td>
<td>Tr</td>
</tr>
<tr>
<td>Malvinidin 3-galactoside</td>
<td>0.180 ± 0.017a</td>
<td>0.132 ± 0.011b</td>
<td>0.034 ± 0.006c</td>
</tr>
<tr>
<td>Peonidin 3-arabinoside</td>
<td>0.181 ± 0.007a</td>
<td>0.115 ± 0.009b</td>
<td>0.034 ± 0.006c</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>0.119 ±0.019a</td>
<td>0.091 ± 0.008a</td>
<td>0.028 ± 0.006b</td>
</tr>
<tr>
<td>Malvidin 3-arabinoside</td>
<td>0.027 ± 0.002a</td>
<td>0.094 ± 0.124a</td>
<td>Tr</td>
</tr>
<tr>
<td>Delpinidin 6-acetyl.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucoside</td>
<td>0.191 ± 0.015a</td>
<td>0.177 ± 0.103a</td>
<td>0.034 ± 0.008b</td>
</tr>
<tr>
<td>Malvidin 6-acetyl 3-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucoside</td>
<td>0.154 ± 0.097ab</td>
<td>0.159 ± 0.027ab</td>
<td>0.053 ± 0.001b</td>
</tr>
<tr>
<td>TOTAL</td>
<td><strong>1.608 ± 0.146ab</strong></td>
<td><strong>1.372 ± 0.112a</strong></td>
<td><strong>0.283 ± 0.055c</strong></td>
</tr>
<tr>
<td>TA</td>
<td>1.819±0.029a</td>
<td>1.778±0.051a</td>
<td>0.135±0.023c</td>
</tr>
</tbody>
</table>

Means with different letters in the same row are statistically significant (p< 0.05). Mean ± SD n= 3. The total anthocyanin content calculated from the sum of total anthocyanin peak areas obtained by HPLC analyses. TA = Total anthocyanin content estimated by the pH differential assay and expressed in cyanidin 3-glucoside equivalents (cyan 3-gluc), (cyan 3-gluc/ g fruit). Tr = traces.
4.3.3 Antioxidant activity

Antioxidant activities of free and complexed blueberry polyphenols were evaluated to observe if any deviations in the antioxidant activity occurred due to compositional differences in each fraction. The ability to scavenge DPPH, superoxide and hydroxyl radicals of the two crude extracts, dialyzed fractions and dialyzate fractions, was assessed to evaluate the antioxidant activity. Different concentrations of total polyphenols (0 – 50 µg/ ml for dialyzate fractions and 0 -100 µg / ml for crude extracts and dialyzed fractions) were used to obtain a dose-response curve (Figures 4.2 – 4.4) and then IC$_{50}$ values were calculated by non-linear regression using Graph Pad Prism 4.0. The values are summarized in Table 4.6.

Figure 4.2 shows the DPPH radical scavenging capacity of the polyphenols present different in the different blueberry extracts. Crude extracts, dialyzed fractions, and the dialyzate fractions showed a similar scavenging potential compared to Vitamin C and Trolox with a maximum of 95 – 98% at 100 µg/ ml for all the polyphenols obtained from frozen blueberries. By contrast, the polyphenols from blueberry freeze dried powder showed a slightly lower scavenging potential with a maximum inhibition of 90% at 100 µg/ ml for the polyphenols recovered from the dialyzed fraction. The polyphenols recovered from the dialyzate fraction showed a lower antioxidant activity with a 40% inhibition at 100 µg of polyphenols per ml. DPPH scavenging capacity of frozen and freeze dried samples increased with increasing levels of polyphenols in both crude extracts and dialyzed fractions; however the efficiency of scavenging by methanolic extracts was higher than the aqueous extracts.
### Table 4.7 IC\(_{50}\) *values of free and bound blueberry polyphenols on the antioxidant activity*

<table>
<thead>
<tr>
<th>Radical</th>
<th>Extraction solvent</th>
<th>Crude extract*</th>
<th>Dialyzed*</th>
<th>Dialyzate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frozen Blueberry</td>
<td>Blueberry Powder</td>
<td>Frozen Blueberry</td>
</tr>
<tr>
<td>DPPH</td>
<td>Water</td>
<td>36.99±0.23(^a)</td>
<td>33.16±1.07(^ {a,b})</td>
<td>30.42±0.95(^ {a,b})</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>14.26±0.33(^ {c,d})</td>
<td>21.63±1.08(^ {b,c,d})</td>
<td>15.74±1.27(^ {c,d})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin C</td>
<td>8.607±0.51(^d)</td>
<td>9.70±0.53(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TROLOX</td>
<td>14.23±0.63(^c,d)</td>
<td>20.54±3.70(^ {A,B,C})</td>
</tr>
<tr>
<td>Superoxide</td>
<td>Water</td>
<td>15.98±1.4(^ {A,B})</td>
<td>34.2±2.14(^ {B,C,D})</td>
<td>10.17±0.59(^A)</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>6.22±0.82(^a)</td>
<td>33.42±0.28(^ {B,C,D})</td>
<td>9.70±0.53(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin C</td>
<td>280.2±44.59(^G)</td>
<td>80.24±8.43(^ {a,b})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TROLOX</td>
<td>226.2±47.0(^F)</td>
<td>40.06±11.7(^ {B,Y})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TROLOX</td>
<td>5.07±0.024(^{c})</td>
<td>20.54±3.70(^ {A,B,C})</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>Water</td>
<td>79.73±5.05(^ {a,g})</td>
<td>73.08±6.03(^B)</td>
<td>171±20.58(^ {G,Y})</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>12.10±0.90(^B)</td>
<td>63.08±16.36(^ {a,b})</td>
<td>80.24±8.43(^ {a,b})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TROLOX</td>
<td>5.07±0.024(^{c})</td>
<td>20.54±3.70(^ {A,B,C})</td>
</tr>
</tbody>
</table>

*Means with different letters in the same row are statistically significant (p< 0.05). Mean ± SEM n= 3.
Figure 4.2 Effect of free and bound polyphenols extracted with methanol from a) frozen blueberries and b) blueberry freeze dried powder on the radical scavenging of DPPH. Mean ± SEM (n=3).
The superoxide radical scavenging potential of the polyphenols present in the blueberry crude extracts, the dialyzed fractions and the dialyzate fractions are shown in Figure 4.3. All samples showed a dose-dependent increase in superoxide scavenging ability. However, blueberry samples had higher superoxide scavenging capacity compared to Vitamin C and Trolox. Approximately 90% and 80% of inhibition was obtained at a level of 100 µg /ml while using polyphenols from frozen and freeze dried samples, respectively. Only 20% of inhibition was obtained at the same level of Vitamin C and Trolox. The polyphenols in the dialyzate fractions showed a lower scavenging potential compared to that of the crude extracts and dialyzed fractions in both frozen and freeze dried samples.

The hydroxyl radical scavenging capacity of the polyphenols present in the blueberry crude extracts, the dialyzed fractions and the dialyzate fractions are also shown in Figure 4.4. All samples showed an increasing scavenging potential at a level of 10 µg/ ml reaching the plateau after 25 µg/ ml. Freeze dried samples showed higher levels of hydroxyl scavenging compared to frozen samples, reaching the same inhibition as TROLOX (approximately 90%) at 100 µg of total polyphenols per ml in the methanolic crude extracts. Surprisingly, dialyzed samples had the lowest hydroxyl scavenging capacity compared to that of the crude extracts and dialyzate fractions in both frozen and freeze dried blueberries.

4.3.4 Alpha glucosidase inhibition

Blueberry extracts also showed high inhibitory activity towards alpha-glucosidase (30 – 70%). As can be seen in Figure 4.5, methanolic extracts (FW, PM) had the highest enzymatic inhibition compared to aqueous extracts and catechin. Neither gallic acid (data not shown) nor catechin inhibited the α-glucosidase activity more than 20 % at a concentration of 100 µg/ ml. Polyphenols from the dialyzed fractions showed the highest inhibitory activity towards α – glucosidase (Figure 4.6) compared to those from the crude extracts (Figure 4.5) and the
dialyzate fractions (Figure 4.7). The highest specific inhibition of 3.7 (inhibition/µg polyphenol) was observed in dialyzed methanol extract of fresh fruits (FM) with nearly 90% inhibition/ 25 µg. The dialyzed extracts of fresh blueberry, and powdered blueberry showed specific inhibition ranging from 2 (FW), 1.3 (PM) and 1 (PW) (Figure 4.6). Figure 4.7 shows the effect of different concentrations of free polyphenols collected in the dialyzate fractions that were analyzed for their enzymatic inhibition. Surprisingly free polyphenols did show a low enzymatic inhibition ranging from 10 – 15 % which was lower than the inhibition obtained with catechins.
**Figure 4.3** Effect of free and bound polyphenols extracted with methanol from a) frozen blueberries and b) blueberry freeze dried powder on the radical scavenging of superoxide radical. Mean ± SEM (n=3).
Figure 4.4 Effect of free and bound polyphenols extracted with methanol from a) frozen blueberries and b) blueberry freeze dried powder on the radical scavenging of hydroxyl radical. Mean ± SEM (n=3).
**Figure 4.5** Percentage of inhibition of α-glucosidase of blueberry crude extracts. Where:

- **FW, FM,** PW, PM, Catechin, F: frozen blueberry, P: Freeze dried blueberry powder, W: water extraction, M: Methanol extraction. n=3 independent experiments.
Figure 4.6 Percentage of inhibition of α-glucosidase of blueberry dialyzed fractions. Where: FW, FM, PW, PM, Catechin, F: frozen blueberry, P: Freeze dried blueberry powder, W: water extraction, M: Methanol extraction. n=3 independent experiments.
Figure 4.7 Percentage of inhibition of α-glucosidase of blueberry dialyzate fraction. Where:

- FW, FM, PW, PM, Catechin, F: frozen blueberry, P: Freeze dried blueberry powder, W: water extraction, M: Methanol extraction. n=3 independent experiments.
4.3.5 Antiproliferative activity

The aqueous and methanolic polyphenolic extracts from frozen blueberry and blueberry freeze dried powder were dialyzed and the fractions tested against the colon cancer cell line (HT-29) and the normal colon cell line (CRL-1790). The cells were incubated in the presence and absence of free and complexed blueberry polyphenols (dialyzed and dialyzate fractions). Polyphenols were added to obtain concentrations ranging from 0 - 100 µg total polyphenols/ml to the medium and proliferation was evaluated using the SRB binding assay. A dose-response curve was generated for each sample. The values are expressed as percent change relative to an untreated control (only medium without polyphenols) (Figure 4.8 and Figure 4.9). All samples exhibited significant inhibitory effects on the growth of both HT-29 and CRL-1790 cells in a dose-dependent manner. Results clearly indicated that all samples exhibited significant inhibitory activities on the growth of both HT-19 and CRL-1790 cells in a dose-dependent manner. Crude extracts showed the highest inhibition of HT-29 with IC$_{50}$ values ranging from 31 to 75 µg/ml. The corresponding IC$_{50}$ values for the dialyzed and dialyzate samples varied between 53 and 248 µg/ml, and 53 and 201 µg/ml, respectively (Table 4.7). Freeze dried samples showed no significant difference in the IC$_{50}$ values compared to the polyphenols samples from frozen blueberries, demonstrating that the freeze drying process did not alter the antiproliferative properties of blueberry polyphenols. From Figure 4.8 it can be seen that methanolic extracts significantly inhibited the growth of HT-29 in a time and dose-dependent manner. At high concentrations, the growth of CRL-1790 was also inhibited, after 24 (data not shown) and 48 h of exposure to 100 µg/ml of total polyphenols. Polyphenols from frozen blueberries showed 90% and 70% growth inhibition for HT-29 and CRL-1790 cells, while the polyphenols from freeze dried blueberry powder inhibited the growth of the cell line at 95% and 60%, respectively.
Table 4.8 IC\textsubscript{50} *values of free and bound blueberry polyphenols on the proliferation of epithelial colon cancer cells (HT-29) and normal epithelial colon cells (CRL-1790).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Solvent</th>
<th>48 h</th>
<th>Crude extract</th>
<th>Dialyzed</th>
<th>Dialyzate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Frozen blueberry</td>
<td>Blueberry Powder</td>
<td>Frozen Blueberry</td>
</tr>
<tr>
<td>HT-29</td>
<td>Water</td>
<td>58.12±2.214 \textsuperscript{a,A}</td>
<td>75.18±4.57 \textsuperscript{e,A}</td>
<td>193.3±16.71 \textsuperscript{b,C}</td>
<td>248.3±7.382 \textsuperscript{f,C}</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>45.43±2.416 \textsuperscript{c,G}</td>
<td>31.11±1.86 \textsuperscript{g,G}</td>
<td>127.4±9.844 \textsuperscript{d,I,J}</td>
<td>52.96±1.45 \textsuperscript{h,I}</td>
</tr>
<tr>
<td>CRL-1790</td>
<td>Water</td>
<td>37.16±5.372 \textsuperscript{j,B}</td>
<td>65.32±15.04 \textsuperscript{m,B}</td>
<td>38.34±1.81 \textsuperscript{j,D}</td>
<td>95.7±1.5 \textsuperscript{m,D}</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>58.89±1.2 \textsuperscript{k,G}</td>
<td>200±44.38 \textsuperscript{n,H}</td>
<td>135.9±4.333 \textsuperscript{l,J}</td>
<td>211.8±39.2 \textsuperscript{n,J}</td>
</tr>
</tbody>
</table>

*IC\textsubscript{50} values (µg/ ml) were calculated by non-linear regression using Graph Pad Prism 4.0. The results are the mean ± standard error from three independent analyses. Different letters represent statistically significant differences (p<0.05).
Figure 4.8 Antiproliferative activities of a) frozen- and b) freeze dried- methanolic blueberry extracts on HT-29 cells after 48 h. Mean ± SEM (n=3).
Figure 4.9 Antiproliferative activities of a) frozen- and b) freeze dried- methanolic blueberry extracts on CRL-1790 cells after 48 h. Mean ± SEM (n=3).
4.4 Discussion

The amount of total polyphenols in both, aqueous and methanolic crude extracts are consistent with those reported elsewhere for wild blueberry (Grace et al., 2009). Since polyphenols are compartmentalized in plant tissues they need to be mechanically released before they can be absorbed. Although most of them are water soluble, high molecular weight phenols may be insoluble (Stahl et al., 2002). In this research, the amount of polyphenols extracted with methanol was significantly higher than that extracted with water. The freeze drying process did not affect total polyphenol recovery, and there were no significant differences among frozen blueberry samples and freeze dried blueberry powder.

The total anthocyanin contents in crude extracts reported in this study are also consistent with previous reports (Nicoué et al., 2007; Horbowicz et al., 2008; Grace et al., 2009; You et al., 2011). The results indicated that methanolic extracts had a higher amount of polyphenols, specifically anthocyanins, compared to the water extracts. Since anthocyanins are polar molecules, they are more soluble in polar than in non polar solvents. Different solvents such as 0.001% HCl in methanol, ethanol or water, methanol acidified with citric acid, water acidified with acetic acid and water with 1000 ppm of SO\textsubscript{2}\textsuperscript{-} have shown efficiency and high yields; however, this methods have been compromised by safety concerns (Delgado-Vargas et al., 2000). As well, these extraction methods do not represent the natural conditions occurring \textit{in vivo}, in which phenolic compounds are subjected to a number of physical and chemical changes during their gastro intestinal tract (GIT) transit and are released from the food matrix (Gil-Izquierdo et al., 2001, 2003). In this study we used methanol, which provides a better extraction of polyphenols, and water, which provides a condition where polyphenols are extracted. Two different crude extracts were then obtained with different compositions. Anthocyanins are known to be very reactive compounds and readily degrade, or react with other constituents in mixtures to form colorless or brown compounds; this occurs due to the presence of oxygen and various
enzymes, and as a result of high temperature processing (Jackman et al., 1987). For the experiments described in this chapter, special care was taken with the samples in order to avoid an overexposure to light, oxygen and high temperatures. Blueberry extracts were kept in the dark during storage (-20 °C), thawing and anthocyanin quantification (25 °C). Therefore, light and temperature did not have any influence in the measurements.

Previous studies have shown that blueberry fruits contained the monoglycosides (glucosides, galactosides and arabinosides) of delphinidin, cyanidin, petunidin, peonidin and malvidin (Wu and Prior, 2005). The percentage distribution of monomeric anthocyanins as reported by Cho et al. (2004), in five different genotypes of blueberry was delphinidin (27-40%), malvidin (22-33%) petunidin (19-26%) cyanidin (6-14%) and peonidin (1-5%). In nature the common anthocyanins are either 3- or 3,5-glycosides, and when the number of sugar residues is higher than three, it is likely to find them attached to the benzopyran ring with alternating sugar and acyl linkages (Francis, 1989). Simple and acylated anthocyanins have been found in some lowbush cultivars (Gao and Mazza; 1994; Wu and Prior, 2005). Overall, the profile of polyphenols in this study is consistent with those reported earlier in the literature (Kader et al., 1996; Sellappan et al., 2002; You et al. 2011; Wang et al., 2012). The crude extracts had the highest content of polyphenols followed by dialyzate and dialyzed fractions.

Although most of the anthocyanins were recovered in the dialyzed fraction the total recovery (dialyzae and dialyzed fraction) was not 100%, most probably because of some binding to the dialysis membrane that resulted in reduced recovery. Some of the polyphenols naturally bound to the matrix may be released during the dialysis process, and may account for the increase observed in the total anthocyanin content of the dialyzate fraction. Recovery of polyphenols after dialysis was substantial in the dialyzate fraction, including approximately 80% of individual phenolics and anthocyanins. As reported earlier, certain phenolic compounds from
blueberries that are not normally detectable can become detectable as a consequence of the fractionation and freeze drying process (Yi et al 2005).

Among different antioxidants, anthocyanins have been shown to inhibit lipid peroxidation more effectively than other classic antioxidants (Narayan et al., 1999; Ramirez- Tortosa et al., 2001; Prior, 2003). However, it has been reported earlier that the ability of different phytochemicals including polyphenols to act as antioxidants depends on their structure, and the presence of hydroxyl groups in the molecules (Harbone, 1986; Rice-Evans and Miller, 1996). Hence, substitution patterns of the aromatic ring not only give different colours, but also change antioxidant capacity of the molecules. For instance, anthocyanins with only one hydroxyl group in the R2 position such as pelargonidin, malvidin, and peonidin have shown a lower antioxidant activity compared to cyanindin which has three hydroxyl groups (Wang et al., 1997). Antioxidant potential of complex mixtures of polyphenols can also be affected by several physical parameters during food processing and gastrointestinal digestion that affect their native antioxidant structure (Cilla et al., 2008). When cell components are liberated during processing, they interact with other molecules present in the food matrix. Although it is generally believed that the food matrix may not affect the functionality of polyphenols, molecular interactions between polyphenols and other components present in the food matrix ultimately may alter their bioefficacy (Saura-Calixto and Rubio-Diaz, 2007; Jacob and Paliyath, 2008). For instance, 34 – 40% of polyphenols in red wine seem to be complexed to dietary fibre (Saura-Calixto and Rubio-Diaz, 2007). In another study, nearly 25 % of polyphenols present in grape juice concentrate were found to be confined in lipid vesicles (Jacob and Paliyath, 2008).

Besides having high in vitro antioxidant activity, different fruit extracts have been studied for their ability to inhibit α-glucosidase, a key enzyme involved in the digestion of starch into sugars. Inhibition in the activity of carbohydrate-hydrolyzing enzymes has the potential to lower sugar absorption, and consequently, may provide an additional tool for the management of
blood sugar levels by preventing post-prandial hyperglycemia, and thus control the onset of type II diabetes (Block, et al., 1992; Serdula, et al., 1996; Shetty, 2001). The efficacy of the polyphenols in binding with proteins and subsequent enzymatic inhibition could be attributed to the fact that polyphenols are multidentate ligands, capable of binding simultaneously (via different hydroxyl groups) to more than one site of the protein (enzyme) (Freitas et al., 2003). In this research the dialyzed fractions showed the highest inhibitory activity towards α-glucosidase. The highest specific inhibition of 3.7 (inhibition/µg polyphenol) was observed in dialyzed methanol extract of fresh fruits (nearly 90%/25 µg). The dialyzed extracts of fresh blueberry, and powdered blueberry showed specific inhibition ranging from 2 (FW), 1.3 (PM) and 1 (PW). Neither gallic acid (data not shown) nor catechin inhibited the α-glucosidase activity more than 20 % at 100 µg/ml.

It has been previously hypothesized (McDugall and Stewart 2005) that the inhibitory effectiveness of berry fruit extracts against α-glucosidase was related to their anthocyanin content. In this work, where different fractions were tested based on total polyphenol content, it was shown that although the total anthocyanin concentrations were lower in the dialyzed fractions. These results suggest that the complexed polyphenols or their interactions with other components of the food matrix enhance their ability to inhibit α-glucosidase activity. It may also be possible that other components in the dialyzed fraction maybe responsible for the inhibition. Interactions between the food matrix and polyphenols have been hypothesized to affect the functional properties of grape juice (Jacob and Paliyath, 2008). Further analysis of the composition of the food matrix is needed to clarify the nature of such interactions.

This work clearly demonstrated that the quantification of polyphenols and evaluation of their in vitro antioxidant activity is an effective tool to screen the quality and integrity of samples containing these important bioactive compounds; however, it cannot predict the bioefficacy of the food in vivo. The use of transformed cell lines is widely used in vitro to obtain more
meaningful biological information. Anthocyanins have been shown to reach the animal intestine in higher levels after oral supplementation (Manach et al., 2004). It has been previously demonstrated that the proliferation of human colon cancer (HT-29) cells was inhibited 50% when incubated with anthocyanin-rich extracts from different fruits (Zhao et al., 2004), and therefore, in this study all samples were analyzed for their antiproliferative activity on HT-29 cells.

In this study we observed that that blueberry polyphenols inhibit in a dose-dependent manner HT-29 cells, even when the polyphenols were complexed with the food matrix. These results clearly indicate that the complexes formed with polyphenols are not hindering the bioeffectivity of the molecules. However, the interactions between polyphenols and other component of the blueberry matrix may affect the cell absorption, thus; affect the cell proliferation in vitro. A previous study, focusing on the differences between crude and anthocyanin-rich extracts reported IC\textsubscript{50} values for crude extracts at 1000 to 3000 µg/ ml and for anthocyanin-rich extracts at 15 to 50 µg/ ml. The proliferation of human colon cancer (HT-29) cells was inhibited 50% when incubated with anthocyanin-rich extracts from different berry fruits at levels of 25-75 µg/ ml after 48 h of incubation. The same concentrations of the anthocyanin-rich extracts did not show a significant toxicity towards normal colon cells (NCM460) (Zhao et al., 2004). In our study we also used CRL-1790 cell line to mimic the effect of the different blueberry fractions on colon normal cells. We observed that blueberry polyphenols reduced approximately 90 % of the growth of HT-29 and 70% of the cell growth of CRL-1790 when both cell lines were treated with the dialyzate fraction containing free polyphenols. By contrast, when the polyphenols were complexed with the food matrix their antiproliferative activity was significantly reduced. Complexed polyphenols reduced approximately 50% of the growth of HT-29 cell line and only 20% of the growth of CRL-1790 cell line. This behavior may be beneficial in vivo because in one hand complexation allows polyphenols to reduce the growth of cancer cells,
and on the other when polyphenols are complexed with the food matrix they may not damage normal cells in the same extent as when they are free.

4.5 Conclusions

The interactions between polyphenols and the food matrix play an important role in the bioactivity of blueberries. Although the presence of a supramolecular complex did not reduce the antioxidant and α-glucosidase activities of blueberry polyphenols, it slightly reduced their ability to inhibit the growth and proliferation of HT-29 cells. The present results also demonstrated that polyphenols from the crude extracts and the dialyzate fractions inhibited the growth and proliferation of the normal colonic epithelial cells (CRL-1790) to a lower extent than cancer cells. In addition, when polyphenols were present as a complex their ability to affect the cell growth of normal colonic epithelial cells was significantly reduced. These results suggest that different fractions of polyphenols obtained in fresh or processed blueberry products may provide multiple effects such as antioxidant activity, regulation of glucose homeostasis and antiproliferative functions, and to different extents depending on their complexed or free state. The presence of a food matrix may positively affect the stability of polyphenols in the gastrointestinal tract, as well as the extent of absorption and their metabolism in vivo. A better understanding of which compounds form complexes with the polyphenols is needed, as well as their stability in the gastrointestinal tract. In addition, more in vivo animal and human intervention studies are needed to obtain relevant information about the bioavailability and metabolism of phenolic compounds in blueberries. This in turn will help to establish adequate dietary parameters for the consumption of polyphenols.
CHAPTER 5

EFFECT OF THE FOOD MATRIX ON THE BIOEFFICACY OF BLUEBERRY (*Vaccinium angustifolium*) POLYPHENOLS

ABSTRACT

In recent years a number of studies have provided evidence of the beneficial health effects of a diet rich in polyphenols. Although many phytochemicals have been shown to improve human’s health, the interactions between phytochemicals and the food matrix are not well understood. In the present study, we tried to better understand such interactions by removing polyphenols from blueberries and comparing the bioactive properties of the food matrix by itself to the bioactive properties of free polyphenols. Crude extracts of blueberry were dialyzed to separate free from complexed polyphenols. The dialyzed fraction containing the complexed polyphenols was also further treated with surface active compounds, namely, Triton X-100, SDS, and Tween 80. There was no decrease in total polyphenol content after the addition of surfactant suggesting that polyphenols are not complexed lipid vesicles easily disrupted by surfactants. Transmission Electron Microscopy (TEM) revealed the presence of electron dense complexes ranging from 100 – 200 nm in the dialyzed fractions of blueberry crude extracts. Pectinase (1 U/ ml) treatment disrupted the structure of such complexes, suggesting that pectin is present in the blueberry complexes containing polyphenols. On the other hand cellulases did not have any effect on the microstructure of the complexes. The antioxidant and antiproliferative activity of the blueberry food matrix alone was below 10% when compared to almost 90% and 70% of the free and complexed polyphenols, respectively. In addition, model mixtures were prepared containing blueberry polyphenols and carbohydrates (pectin and cellulose). The presence of polyphenols with these polysaccharides did not affect the antioxidant activity of
blueberry polyphenols but it showed a significant decrease in the antiproliferative activity on HT-29 cells.

**Key words:** food matrix, blueberries, nano-vesicles, HT-29, TEM

### 5.1 Introduction

In the last decades there has been an increased interest on phytochemicals such as polyphenols and anthocyanins. The interest rose from the fact that phytochemicals in the diet can be a natural alternative to prevent and reduce the severity of several chronic diseases such as cardiovascular diseases and some types of cancers (Williamson and Manach, 2005). The effectiveness of a bioactive compound is affected by the bioaccessibility, and this in turn is greatly influenced by the food matrix. A food matrix refers to the continuous medium that may be of cellular origin, where the nutrient or bioactive compounds are contained, especially in fruits and vegetables; or may originate on microstructures produced during processing (Aguilera and Stanley, 1999; Parada and Aguilera, 2007). Food matrices containing plant polyphenols may vary from relatively simple structures where the bioactive compound is dissolved to more complex structures encapsulating nutrients. Polyphenols can be trapped in different sub-cellular locations of the cells, for instance, anthocyanins are uniformly dissolved in the vacuolar solution of epidermal cells of many plants, while in certain species they are found in discrete regions of the cell vacuoles called anthocyanplasts (Peket and Small, 1980). Polyphenols have been reported to be transiently bound to food matrices and interactions reduce extraction efficiency, but it may protect the more labile anthocyanins from degradation during digestion (Markham et al., 2000; McDougall et al., 2005b).

The degree of complexity of the food matrix governs the accessibility in the gastrointestinal tract (Castenmiller and West, 1998). Recent studies appear to demonstrate that the state of the
matrix or the microstructure of processed foods may affect the nutritional response of certain foods in vivo (Parada and Aguilera, 2007). In addition, food processing can affect bioaccessibility of polyphenols by releasing them from the food matrix. For example, preparation methods such as juicing, blending, chopping, and heating may improve the bioavailability of the bioactive compounds by weakening the interactions to the cell wall of plant tissues (During, 2008). However, during processing, some polyphenols such as anthocyanins when are exposed to harsh conditions (pH, temperature, and light) may be easily destroyed (Delgado-Vargas et al., 2000). Differences in concentration within plant tissues, cell wall structure, glycosylation and binding within the food matrix can then affect their stability and bioavailability (Balasundram et al., 2006).

The effect of the food matrix on the bioavailability of polyphenols has not been examined in detail. There is an extensive variability of information reported in the bioavailability and bioefficacy of polyphenols as it relates to human digestion, and this may suggest that the bioavailability of different phenolic groups is limited (Milbury et al., 2002). The objective of this research was to analyze the physicochemical and bioactive properties of different blueberry food matrices and the functionality of polyphenols. Understanding the role of the blueberry food matrix and their interaction with polyphenols is necessary to develop products with enhanced biofunctionality.

5.2 Materials and Methods

5.2.1 Fruits

Frozen wild blueberries (low-bush) from the same batch were obtained from the local supermarket.
5.2.2 Chemicals and Reagents

All solvents were of HPLC grade. Methanol and sulfuric acid were purchased from Fisher Scientific (Ottawa, Canada). Folin Ciocalteu’s Reagent, Gallic Acid, 2,2-diphenyl-1 picrylhydrazyl (DPPH) radical, pectin from apple skin, cellulose, pectinase from \textit{Rhizopus} sp. (400 – 800 U/g solid), cellulase from \textit{Trichoderma viride} (8560 U/g solid), carbazole, Triton X-100, Tween 80, Sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), Dubelcco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, surforhodamine B (SRB), acetic acid, and Tris base were purchased from Sigma Aldrich (Oakville, Canada). Human colorectal epithelial adenocarcinoma cell line HT-29 was obtained from the American Type Culture Collection (Rockville, USA). Phosphate buffer saline (PBS) 10 x, penicillin-streptomycin solution and trypan blue were from Gibco (Bethesda, USA).

5.2.3 Polyphenol extraction

Frozen blueberries (50 g) were ground with 100 ml of water as mentioned earlier in section 3.2.1.

5.2.4 Separation of free and complexed polyphenols

Free and complexed polyphenols were obtained by dialyzing 10 ml of blueberry crude extracts overnight. Polyphenol complexes were obtained from the dialyzed fraction while free polyphenols were obtained from the dialyzate fraction. (See section 3.2.3 for more details).

5.2.5 Solid phase extraction

Free polyphenols were recovered after dialysis by solid extraction of the dialyzate fraction. See section 3.2.8 for more details. Aliquots of 10 ml containing free polyphenols recovered after
the solid phase extraction were placed under nitrogen at 60 °C during 30 minutes to remove the methanol, then polyphenols solubilized in water and stored at 4°C for further analysis.

5.2.6 Total polyphenol content

Total polyphenol content of the samples were estimated by using the Folin–Ciocalteau method. See section 3.2.4 for more details.

5.2.7 Food matrix isolation

The polyphenols were removed from whole blueberries (50 g) by soaking (5x) in 50 % v/v ethanol (100 ml), and (1x) with distilled water (100 ml) for 1 h. To verify the absence of polyphenols, samples were dialyzed over night and Folin-Ciocalteau assay performed on the extracts. Samples containing the food matrix were stored at -20 °C until further analysis.

5.2.8 Recovery of polyphenols from the complexes

An aliquot (2ml) of complexed polyphenols obtained from the dialyzed samples were transferred again into a new dialysis membrane reservoir (6- 8 KDa cut off). The second dialysis was carried out in 250 ml of 0.1 % (v/ v) Triton x-100, sodium dodecyl sulfate (SDS), and Tween 80, respectively. Polyphenol leakage was monitored by quantifying the total polyphenols in the dialyzed samples during 12 h.

5.2.9 Pectin content

The analysis of the pectin content in the food matrix and crude extracts was carried out using a colorimetric assay according to Taylor (1993). Briefly, 200 µl of the samples were placed in 16 X 150 mm borosilicate tubes. Three ml concentrated sulfuric acid were added, followed by the addition of 100 µl of 0.1 % (w/ v) carbazole reagent (made up in 100% ethanol). Then tubes
were incubated at 60°C in a water bath for 1 h and cooled to room temperature in another water bath. Finally, the absorbance values of the samples were read using a spectrophotometer at 530 nm against water blank. A stock solution of pectin from apple was used at concentrations ranging from 0 – 200 µg/ml to generate a standard curve.

5.2.10 Interactions between free polyphenols and a pectin-cellulose based food matrix

Free polyphenols recovered from the dialyzate fraction, purified by solid phase extraction and solubilized in water were mixed with different carbohydrate solution. Aliquots of samples containing free polyphenols (1mg/ml) were incubated for 30 min in different carbohydrate solutions. Solution 1: 0.1% w/v pectin, solution 2: 0.25% w/v pectin, solution 3: 0.5% w/v pectin, solution 4: 0.1% w/v cellulose, solution 5: 0.25% w/v cellulose, solution 6: 0.5% w/v cellulose, and solution 7: 0.5% w/v cellulose-pectin in a 1:1 weight ration. After incubation period, samples were centrifuged using a Beckman J-21 centrifuge at 2700 x g for 20 minutes. Supernatants were collected and total polyphenols quantified using the Folin-Ciocalteau assay.

5.2.11 Recovery of free polyphenols from a pectin-cellulose based food matrix

Aliquots containing polyphenols mixed in solutions containing 0.5% w/v pectin and cellulose; respectively, were dialyzed overnight and polyphenols released from the matrix recovered by solid phase extraction. Recovered polyphenols were solubilized in water and quantified using the Folin-Ciocalteau assay.

5.2.12 Micro-structure of blueberry polyphenols

Transmission Electron Microscopy (TEM) was used to characterize the micro-structure of the polyphenol complexes and to determine the stability of polyphenol-food matrix complexes subjected to various treatments. A 450 µl sample containing complexed polyphenols
were treated with 1 U/ ml pectinase and 1 U/ mL cellulase and incubated for 10 min, respectively. Then, samples were spotted onto formvar-coated 200-mesh copper grids for 1 min. The grid was blotted dry and stained with 1% w/v uranyl acetate for 30 s. The excess of stain was removed and the grid was examined using a Leo 912 B transmission electron microscope. Analyses were performed in triplicate and representative images are shown in the results section.

5.2.13 DPPH radical scavenging assay

Total antioxidant activities of blueberry free polyphenols and polyphenols mixed in pectin and cellulose based solutions were measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to Molyneux (2004). See section 3.2.10 for more details.

5.2.14 Cell proliferation

The antiproliferative activities of polyphenol-complexes were estimated by the Sulforhodamine B assay. See section 3.2.13 for details.

5.2.15 Statistical Analysis

One-way analyses of variance (ANOVA) and multiple comparison tests were conducted as described in Section 3.2.14.

5.3 Results

5.3.1 Polyphenol composition of blueberry complexes

Blueberries were ground with water and the crude extracts obtained were subjected to dialysis. Polyphenols from each fraction were recovered by solid phase extraction and quantified. Table 5.1 shows the total amount of polyphenols in the crude extract (6.16 mg of
gallic acid equivalents (GAE), after dialysis. Approximately 28% (1.75 mg of GAE) and 55% (3.36 mg GAE) were recovered in the dialyzed and dialyzate fractions, respectively.

Folin-Ciocalteu assay was also performed to samples containing the food matrix alone. Results showed that the amount of polyphenols in the food matrix was below the detection limit (Table 5.1). Therefore any positive response obtained in further analysis of the food matrix cannot be attributed to the influence of polyphenols.

5.3.2 Stability of the blueberry polyphenol complexes

It has been previously hypothesized that polyphenol complexes in blueberries may be vesicular in nature. Hence, to test this hypothesis, dialysis against surfactant solutions such as Triton X-100, Tween 80 or sodium dodecyl sulfate (SDS) was conducted, as the presence of surfactant would rupture the lipid vesicular membrane surrounding the polyphenol complexes. This would result in a decrease of the total polyphenol content in the dialyzed fraction and higher recovery of free polyphenols. Figure 5.1 shows the amount of polyphenols that was quantified after 12 h dialysis, and it was clearly shown that the concentration of polyphenols remained unaffected after dialysis in the presence of different surfactants.
### Table 5.1  Recovery of Total polyphenols after dialysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Blueberries</th>
<th>Blueberry food matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>6.16 ± 0.37&lt;sup&gt;a&lt;/sup&gt; (100%)</td>
<td>BDL</td>
</tr>
<tr>
<td>Dialyzed (complexed polyphenols)</td>
<td>1.75 ± 0.16&lt;sup&gt;b&lt;/sup&gt; (28.4%)</td>
<td>BDL</td>
</tr>
<tr>
<td>Dialyzate (Free polyphenols)</td>
<td>3.36 ± 0.07&lt;sup&gt;c&lt;/sup&gt; (54.5%)</td>
<td>BDL</td>
</tr>
</tbody>
</table>

Notes: Means with different letters are statistically significant (p< 0.05). Mean ± SEM n= 3. *Expressed in gallic acid equivalents (GAE), (mg GAE) BDL = below detection limit
Figure 5.1 Effect of Triton X-100, SDS, and Tween 80 on the total polyphenol content of dialyzed blueberry samples.
5.3.3 Pectin content of blueberry extracts

The amount of pectin in different blueberry fractions is shown in Figure 5.2. There were no significant differences in the pectin content among crude extracts, complexed polyphenols and the food matrix. The average pectin content in all samples was approximately 1.5 mg/g of blueberries.

5.3.4 Microstructure of the blueberry polyphenol complexes

The recovery of polyphenols in the dialyzed material (i.e. not released during dialysis) was substantial, and it may suggest that blueberry polyphenols are present in supramolecular structures complexed to large molecular weight components. Transmission electron microscopy (TEM) was conducted to better evaluate the microstructure of such complexes. A positive staining using uranyl acetate allows the detection of electron-dense materials. Figure 5.3 shows representative images of the complexes found in crude extracts, dialyzed fraction, dialyzed fraction, food matrix, and dialyzed food matrix. Nanostructures with an electrodense interface from 50 – 200 nm were detectable in all samples. While in the crude extract (Figure 5.3a), the dialyzed fraction (Figure 5.3b), the food matrix (Figure 5.3d), and the dialyzed food matrix (Figure 5.3e) these vesicle like structures were always present, they were absent in the dialyzate fraction (Figure 5.3c). It was hypothesized that the polyphenols complexes may contain carbohydrates. To test this hypothesis, polyphenol complexes were subjected to a treatment with carbohydrate degrading enzymes. TEM images of dialyzed samples treated with pectinase and cellulase enzymes are shown in Figure 5.4. While after the addition of pectinase (Figure 5.4b) the electrodense periphery of the vesicles seemed to disappear, and the vesicular complexes showed a much lighter staining of the aggregates, in the case of the cellulase treated samples, the vesicle appearance remained very similar to that of the original samples (see Figure 5.4c and a).
**Figure 5.2** Pectin content of different in blueberry food matrix and blueberry crude extracts.
Figure 5.3 Representative transmission electron micrograph (TEM) of vesicles complexes from whole blueberry extracts stained with uranyl acetate. a) crude extract, b) dialyzed fraction, c) dialyzate fraction. Control samples containing the food matrix alone were also analyzed d) food matrix crude extract, e) food matrix dialyzed fraction.
Figure 5.4 Transmission electron micrographs of a) complexed polyphenols from blueberries before (a) or after treatment with b) 1 U/ml pectinase, and c) 1 U/ml cellulase. The samples were stained with uranyl acetate, and the images are representative.
To better understand the ability of free polyphenols to form complexes with the food matrix, synthetic model matrices made of pectin and cellulose were mixed with free polyphenols and their structure was analyzed using TEM. Representative images are shown in Figure 5.5. It is clear from these images that the structures present in the blueberry extracts are pectinaceous in nature, and that in the presence of free polyphenols, these structures become more electron dense. As the synthetic complexes in the pectin samples had similar shape and size as the natural complexes found earlier in blueberry polyphenols complexes and blueberry food matrix (Figure 5.3), confirming the pectin nature of the aforementioned complexes. In contrast, cellulose based matrices were not able to form nano-structures.

5.3.4.1 Recovery of free polyphenols from different synthetic food matrices

To evaluate the ability of different carbohydrate-rich matrices to complex with polyphenols, different concentrations of pectin and cellulose were mixed with 1 mg of free polyphenols. The mixtures were then incubated for 30 min at room temperature, centrifuged, and polyphenols quantified using the Folin-Ciocalteau assay. As shown in Figure 5.6, there were no significant differences in the recovery of polyphenols among samples with different concentrations of pectin, and cellulose, respectively. Surprisingly, when a mixture of pectin-cellulose was used (1:1 ratio) there was a significant decrease in the recovery of total polyphenols.
Figure 5.5 Transmission electron micrograph (TEM) of free polyphenol-carbohydrate mixtures. Where: a), c) and e) carbohydrates with no free polyphenols; b), d), and f) corresponding mixtures. a) and b) 0.5 % w/v pectin; c), d) 0.5 w/ v cellulose; and e), f) 0.5% w/ v pectin cellulose.
Figure 5.6 Effect of different carbohydrate-rich food matrices on the total polyphenol content obtained from blueberries.
To confirm that free polyphenols were complexed with pectin and cellulose after mixing, the mixtures were dialyzed overnight. Polyphenols that were not complexed with the carbohydrate-rich matrices were recovered in the dialyzate fraction. On the other hand, complexed polyphenols would remain in the dialyzate fraction. Table 5.2 shows the effect of the dialysis process on the recovery rate of polyphenols complexed with different carbohydrate-rich food matrices. A fixed amount of polyphenols (1.2 mg) was mixed with different solutions: 0.5 % w/v pectin, 0.5 % w/v cellulose, and 0.5 % w/v pectin-cellulose, and total polyphenols were quantified. When free polyphenols were mixed with pectin and cellulose, 100% of the polyphenols were detected by the Folin-Ciocalteau assay (See also Figure 5.6). By contrast, when polyphenols were mixed with a solution containing pectin and cellulose, the FC assay was able to detect only 83% of the initial amount of polyphenols mixed with the solution. After the dialysis process, the amount of polyphenols recovered from the mixtures of polyphenols containing pectin and cellulose, respectively, were similar to the control sample containing only polyphenols (0.1 mg, 7.7%). The amount of polyphenols recovered from the mixture containing pectin and cellulose inside the dialysis bag was 0.08 mg, representing 6.2% of the total polyphenols mixed with the matrix.

The results shown in Table 5.2, clearly demonstrated that the majority of the polyphenols in the mixtures were in the form of free polyphenols as indicated by the high recovery in the dialyzate fraction. Approximately of 92% of the polyphenols in the control sample were recovered, while 86%, 91% and 97% of the polyphenols in the pectin, cellulose, and pectin-cellulose mixtures were recovered. The amount of polyphenols that was not recovered may be complexed with the matrices or lost during the purification step.
Table 5.2 Effect of the dialysis process on the polyphenol content of different carbohydrate-rich matrices.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original extract</th>
<th>Dialyzed</th>
<th>Dialyzate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPC* (mg)</td>
<td>Recovery (%)</td>
<td>TPC* (mg)</td>
</tr>
<tr>
<td>Free polyphenols (FP)</td>
<td>1.3 ± 0.05a</td>
<td>100</td>
<td>0.10 ± 0.003b</td>
</tr>
<tr>
<td>FP- Pectin (P)</td>
<td>1.3 ± 0.03b</td>
<td>100</td>
<td>0.10 ± 0.002b</td>
</tr>
<tr>
<td>FP-Cellulose (C)</td>
<td>1.3 ± 0.06c</td>
<td>100</td>
<td>0.10 ± 0.006b</td>
</tr>
<tr>
<td>FP-P- C</td>
<td>1.0 ± 0.04d</td>
<td>82.88</td>
<td>0.08 ± 0.001e</td>
</tr>
</tbody>
</table>

Notes: concentration of the carbohydrate-rich matrices: 0.5 % w/ v pectin, 0.5 % w/ v cellulose, 0.5% w/ v pectin-cellulose. Means with different letters are statistically significant (p< 0.05). Mean ± SEM n= 3. *Expressed in gallic acid equivalents (GAE), (mg GAE).
5.3.4.2 Antioxidant activity

The ability of free and complexed polyphenols to scavenge DPPH radicals was assessed in the dialyzed and dialyzate fractions. The total polyphenol concentration in all samples varied from 0 to 100 μg/ml and the testing was based on the polyphenols concentration (Figure 5.7). Control samples containing the food matrix alone (after the extraction of the polyphenols) were also tested at corresponding levels of the samples containing polyphenols. For those samples, the concentrations were based on the amount of the food matrix compounds (i.e. pectin, cellulose, pectin-cellulose) or the amount of natural food matrix (i.e. extracts obtained from blueberries whose polyphenols were removed by extensive soaking with 50% v/v ethanol).

Samples containing polyphenols showed a dose-dependent increase in DPPH scavenging ability (Figure 5.7). Free polyphenols reached almost 90% scavenging of DPPH radicals, followed by the different mixtures containing free polyphenols and pectin or cellulose at various concentrations with approximately 80% scavenging of DPPH radicals. Naturally complexed polyphenols showed a lower scavenging ability of DPPH that reached 75%.

The natural food matrix obtained from blueberries before and after dialysis showed a scavenging ability of DPPH of 10%. The different solutions containing food matrices made of pectin or cellulose reached only 5% inhibition. These results suggest that the antioxidant properties of blueberries are attributable to the polyphenols, and that the matrix does not provide any synergistic effects. Figure 5.8 shows the effect of dialysis process on the antioxidant activity of free polyphenols mixed with 0.5% (v/w) pectin and/or cellulose solutions. Complexed polyphenols collected in the dialyzed fraction showed approximately 40% DPPH scavenging activity at the highest concentration (100μg/ml), while the original mixtures and the dialyzate containing free polyphenols showed approximately 90% inhibition.
Figure 5.7 Antioxidant activity of blueberry free polyphenols mixed with different carbohydrate-rich food matrices. Concentrations of all samples were standardized at 100 µg of total polyphenols per ml of solution or 100 µg of food matrix per ml of solution, respectively.
Figure 5.8 Antioxidant activity of blueberry polyphenols mixed with 0.5% (w/v) pectin or/and cellulose solutions a) before; and b), c) after dialysis. Concentrations of all samples were standardized at 100 µg of total polyphenols per ml of solution.
5.3.4.3 Antiproliferative activity

Antiproliferative activities of the blueberry polyphenols and different food matrices were evaluated using the colon cancer cell line HT-29. The cells were seeded in 96-well plates at an initial concentration of $2 \times 10^3$ cells per well and allowed to proliferate during 24 hours. After the initial incubation period cells were treated with different concentrations of free and complexed polyphenols ranging from $0 – 100 \ \mu g/ ml$ and the cell proliferation estimated after 24 h and 48 h as the amount of protein bound to sulphorodamine dye. Control samples containing plain media and the food matrices were run in parallel to monitor any positive effects caused by the matrices. The values are expressed as percent change with respect to untreated controls.

As shown in Figure 5.9 free polyphenols at the highest concentration (100 \mu g/ ml) inhibited approximately 80% of the cell growth of HT-29 cells. In contrast, when the same amount of polyphenols was naturally complexed with the blueberry food matrix there was only 50% inhibition. Both samples containing 100 \mu g/ ml of the food matrix and the dialyzed food matrix showed only 10% and 30% cell growth after 24h and 48 h incubation period, respectively.

Figure 5.10 shows the antiproliferative activities of polyphenols mixed with different synthetic food matrices. All samples showed a dose-dependent decrease in cell growth before and after dialysis. Both polyphenol mixtures and complexed polyphenols with the synthetic matrices obtained after dialysis, reduced significantly the cell growth of HT-29 cells compared to control samples containing only pectin and/or cellulose at the same concentrations (Figure 5.11).
Figure 5.9 Antiproliferative activities of blueberry free polyphenols, complexed polyphenols and food matrices on HT-29 cells after 24 and 48 hours. Concentrations of samples were standardized at 100 µg of polyphenols per ml of solution or 100 µg of food matrix per ml of solution, respectively.
Figure 5.10 Antiproliferative activities of free polyphenols mixed with 0.5% (w/v) pectin or cellulose, before and after dialysis, on HT-29 cells after 24 and 48 hours. Concentrations of all samples were standardized at 100 µg of polyphenols per ml of solution.
Figure 5.11 Antiproliferative activities of 0.5% (w/v) pectin, 0.5% (w/v) cellulose, 0.5% (w/v) pectin-cellulose, on HT-29 cells after 24 and 48 hours. Concentrations of all samples were standardized at 100 µg of carbohydrates per ml of solution.
5.4 Discussion

The beneficial health-related properties of high intake of blueberries have been widely reported. In this study we characterized the potential health effects provided by the food matrix in blueberry extracts. Synthetic matrices of pectin or/ and cellulose were used to elucidate the interactions that occur between polyphenols and the food matrix, and their effects on bioactivity. Although it is generally assumed that the food matrix in crude extracts may not exert an influence on the availability of polyphenol, it has recently been found that interactions occur between polyphenols and polysaccharides in wine (Saura-Calixto and Diaz-Rubio, 2007) or polyphenols and carbohydrates-lipids in grape juice (Jacob and Paliyath, 2008). Such interactions may have an impact on the delivery and absorption of polyphenols in vivo. Therefore, when the biological effects of fruits and vegetables are estimated, the effects of such interactions within the food matrix should be considered for obtaining more realistic conclusions. Several steps are involved before the bioactive compounds, such as polyphenols, reach the target organ. Processing, on the one hand, plays an important role by making the bioactive compounds more accessible, however, significant losses can occur due to over exposure of such compounds to unfavourable processing conditions such as the light, oxygen, moisture, etc. Digestion also affects the structure of the food. Mastication reduces the size and enzymes present in the saliva start the digestive process by breaking down the carbohydrates. In the stomach, acid pH and enzymatic activity of pepsin and amylase continues breaking down polysaccharides into sugars. Finally, when polyphenols reach the intestine, polyphenols may remain complexed with the natural food matrix present in blueberries or may be bound to other compounds from the diet. The present study explores the composition of the blueberry matrix, and compares it with different carbohydrate-rich synthetic matrices. Results showed that nearly 28% of the polyphenols in blueberry crude extracts are naturally complexed with the food matrix. These results are in agreement with previous studies (Jacob and Paliyath, 2008)
demonstrating that approximately 25% of non dialyzable polyphenols in grapes were bound to carbohydrates and lipids. In this work polyphenol complexes in the dialyzed fraction were further extracted using surfactants. Samples were treated with a non ionic detergent Triton X-100 to examine the possible inclusion of polyphenols in lipid containing vesicles. This treatment did not further extract polyphenols. Complexed polyphenols were also treated with sodium dodecylsulfate (SDS). This is an anionic detergent known to disrupt non-covalent association of proteins. Even with this detergent, the complexes did not show further disruption. Treatment with Tween 80, a gentle surfactant that helps in minimizing non-specific binding and removing unbound moieties, also did not affect the complexes. These results may suggest that the polyphenol in blueberry are not complexed with simple lipids or proteins structures.

Previous observations have shown that a crude extract of blueberry rich in polyphenols is also rich in pectin and other high molecular weight carbohydrates. The amount of pectin present in the crude extracts and their respective dialysis fractions was quantified and the results demonstrated that both crude extracts and dialyzed fractions contained considerable amounts of pectin. Previous studies (Jacob and Paliyath, 2008) suggested the existence of confined and discreate areas containing electron-dense complexes that may arise from a complex formation between pectic polysaccharides and vesicles containing polyphenols. In the present study, the TEM analyses revealed the presence of electron-dense complexes. To determine the nature of potential carbohydrate moieties involved in the formation of macromolecular complexes, the dialyzed juice was subjected to a treatment with carbohydrate-degrading enzymes. The pectic nature of the polyphenol complexes found in blueberry extracts was confirmed by the disruption of the complexes when treated with pectinase (1 U/ ml). By contrast, the integrity of the complexes was not affected by the treatment with cellulase (1 U/ ml). Thus, may be hypothesizes that anthocyanidins conjugated with sugars may interact with galacturonic acid chains of pectin through their sugar moiety.
To better understand the nature of the vesicular structures that may have originated from membrane inclusions during extraction and that would have complexed with polyphenols, free polyphenols recovered from the dialyzate fraction were mixed with different concentrations of pectin and cellulose, the most common carbohydrates found in the cell wall of fruits. We found that, when a fixed amount of blueberry free polyphenols were mixed with 0.1%, 0.25% and 0.5% (w/v) pectin or cellulose-rich, the same amount of total polyphenols was detected within the mixtures. However, when pectin and cellulose were combined in the same matrix a reduction in the detection of the polyphenols occurred. These results may suggest that the pectin-cellulose mixture may interact with polyphenols reducing the ability of the Folin Ciocalteu reagent to react with the phenolic compounds. The same behavior was observed when a fixed amount of total polyphenols were added to 0.5% (w/v) pectin or/and cellulose solutions. However, it was demonstrated that less than 10% of the free polyphenols remained complexed within the food matrix after dialysis. The recovery of total polyphenols was more than 95% in the pectin-cellulose solution, compared to 86% and 91% in the pectin and cellulose solutions, respectively.

To avoid any overestimation of the bioactivity of blueberry crude extracts it was necessary to monitor the in vitro antioxidant and antiproliferative activities of the food matrix alone. In previous studies we have found that the composition of polyphenols in the complexed form is similar to the composition in the crude extracts and free polyphenols recovered from the dialyzate fraction. However, it was observed that there were differences in the antioxidant activities and ability to affect cell proliferation of HT-29 cells. This suggested that the food matrix may have contributed to such reduction as a consequence of the interactions of the polyphenols with pectin or cellulose (Chapter 4). Therefore, for this study the food matrix was isolated and tested for its antioxidant activity. It was shown that the food matrix alone, as well as the synthesized food matrices used in the experiments did not have a considerable antioxidant activity. When free polyphenols were mixed with different concentrations of pectin or/and
cellulose their scavenging capacity of the DPPH radical was similar to free polyphenols. Thus, when polyphenols are mixed even with high amounts of pectin or cellulose, their antioxidant activity does not decrease. However, when the mixtures containing the synthetic matrices were dialyzed, and the samples containing true complexed polyphenols were tested in the same concentration range towards DPPH radicals, they showed a lower scavenging potential compared to the original mixtures. This may be due to complex formations involving key hydroxyl groups of the polyphenols with the polygalacturonic acid moieties of the pectin that make polyphenols less amenable to scavenge free radicals.

In spite of the fact that there are numerous reports of inhibition of HT-29 cell growth in vitro by purified anthocyanin-rich extracts or crude extracts (Zhao et al., 2004; Flis et al., 2012), this is the first study that compares the effect of complexed polyphenols and the food matrix on colon cancer cells. It is of great interest to understand the effect of the food matrix and other compounds that interact with the polyphenols to develop processing techniques that take advantage of such interactions and lead the production of high quality and value added products.

In previous studies, free and complexed polyphenols obtained from blueberry extracts exhibited varying degrees of inhibition of colon cancer and normal cells respectively (Chapter 4). In this study we observed that all samples showed a dose-dependant decrease in the cell growth of HT-29 cells. Although, the blueberry food matrix did not show a considerable effect on the inhibition of HT-29 cells growth; there was a significant decrease in the inhibitory activity of the samples when polyphenols were naturally complexed with the blueberry food matrix. By contrast, the various mixtures containing free polyphenols and the synthetic food matrices showed the same profile before and after the samples were dialyzed. These results suggest that the strong interactions between polyphenols and the blueberry food matrix may reduce the
bioavailability of polyphenols before they reach the colon cancer cells, while there are no specific interactions between pectin and cellulose and blueberry polyphenols, and that these matrices do not affect bioaccessibility and bioavailability.

5.5 Conclusions

The blueberry food matrix plays an important role in the bioavailability of polyphenols. The present research clearly demonstrated that the matrix is not affected by treatment of surfactant. In addition, it was possible to conclude that when polyphenols are mixed with other carbohydrates such as pectin or cellulose they do not form complexes to the same extent, as they are released during dialysis and they behave, on cell cultures, as if they were free. However, it is possible to hypothesize that the naturally complexed polyphenols may be more stable during the digestion process \textit{in vivo} and may become bioavailable by the time they reach the colon. Understanding the changes that occur during the digestion may be also interesting as a basis for the processing or formulation of products that combine the positive effect of blueberry polyphenols and carbohydrate rich food matrices.
CHAPTER 6

STABILITY AND BIOLOGICAL ACTIVITY OF WILD BLUEBERRY (Vaccinium angustifolium) POLYPHENOLS DURING SIMULATED IN VITRO GASTROINTESTINAL DIGESTION*

ABSTRACT

Wild blueberries are rich in polyphenols and have several potential health benefits. Understanding the factors that affect the bioaccessibility and bioavailability of polyphenols is important for evaluating their biological significance and efficacy as functional food ingredients. Since the bioavailability of polyphenols such as anthocyanins is generally low, it has been proposed that metabolites resulting during colonic fermentation may be the components that exert most of the health benefits. In this study, an in vitro gastrointestinal model comprising sequential chemostat fermentation steps simulating digestive conditions in the stomach, small intestine and colon was used to investigate the breakdown of blueberry polyphenols. The catabolic products were isolated and biological effects tested using a normal human colonic epithelial cell line (CRL 1790) and a human colorectal cancer cell line (HT 29). The results showed a high stability of total polyphenols and anthocyanins during simulated gastric digestion step with approximately 93% and 99% of recovery, respectively. The intestinal digestion process decreased the polyphenol- and anthocyanin- contents by 49% and 15 % respectively, by comparison to the non-digested samples. During colonic digestion, the complex polyphenol mixtures were degraded to a limited number of phenolic compounds such as syringic, cinnamic, caffeic, and protocatechuic acids. Only acetylated anthocyanins were detected in low amounts after the colonic digestion process. After simulated colonic digestion, the isolated catabolites showed lowered antioxidant activity and cell growth inhibition potential. Results suggest that colonic fermentation decreases the biological activity of blueberry polyphenols.
**Key words:** wild blueberry, polyphenols, *in vitro* digestion, chemostat, bioavailability, HT-29, CRL-1790

* Submitted to Food Chemistry

6.1 Introduction

Blueberries are rich in polyphenols such as anthocyanins. Anthocyanins are watersoluble nutraceutical compounds with potential to prevent chronic degenerative diseases through their biological functions, of which antioxidant capacity may be one of the most significant (Kalt and Dufur, 1997). They have been reported to be growth-inhibiting and cytotoxic to cancer cells (Kuo, 1998; Smith *et al.*, 2000; Kang *et al.*, 2003). In addition, they have the potential to inhibit the activity of enzymes such as α-amylase and α-glucosidase; enzymes that can help reduce the indices of metabolic syndrome, and the prevention of obesity and type II diabetes (Tsuda *et al.*, 2003; McDugall and Stewart, 2005). The putative evidence supporting the biological activity of polyphenols has been obtained from *in vitro* experiments, cell culture studies and experiments using mammalian systems. However, the concentrations of these compounds used *in vitro* and cell culture experiments are usually higher than that would be obtained through a normal diet, as well as in a biological form that may not be normally present in the food (Day *et al.*, 2004).

Despite the presence of high amounts of polyphenols present in the ingested food, only a very small portion (ranging from 0.5 – 1%) is actually absorbed (Lapidot, 1998; McGhie and Walton, 2007). However, biotransformations involving catabolic breakdown, methylation, deglycosylation etc., have been suggested to enhance bioavailability (McGhie and Walton, 2007). Several *in vitro* studies have demonstrated that phenolic compounds can be transported through the intestinal epithelium as glycosides by sugar transporters (Hollman *et al.*, 1995; Day
et al., 1998; Morand et al., 2000). Following absorption into epithelial cells, β-glucosidase can hydrolyze these glycosides to form aglycones. Aglycones can also be formed in the lumen by the action of membrane-bound lactase phlorizin hydrolase (LPH), aglycones produced in this way are absorbed passively through the epithelium (Scalbert and Williamson 2000) whereupon they undergo conjugation within the ileal epithelium or the liver. Hepatic metabolites (methylated, sulfated or glucuronidated conjugates) are returned via the enterohepatic circulation (in bile) to the gut lumen (Nemeth et al., 2003; Scalbert and Williamson, 2000). Thus, phenolic compounds that enter the colon consist largely of unabsorbed glycosides and conjugates that have been cycled through ileal and hepatic metabolism (Scalbert and Williamson 2000). Polyphenol conjugates can also be enclosed in the food matrix and would thus be unavailable for absorption (Nielsen et al. 2003); on reaching the caecum, these compounds are subjected to metabolism by members of the gut microbiota. For example, many flavonoids undergo ring-fission, where the B-ring is degraded resulting in the formation of several phenolic acids (Scalbert and Williamson 2000; Scalbert et al. 2002; Rechner et al. 2002; Manach et al., 2004).

As it is complicated to conduct in vivo studies on the metabolic transformation process during gastrointestinal tract (GIT) digestion, the system has been simplified into separate segments using chemostats. In vitro digestion models are invaluable tools for simulation of the conditions that occur in the GIT as accurately as possible in a sequential and compartmentalized fashion. In addition, the colon models help to elucidate the role of microbiota in the metabolism of non-digestible compounds such as dietary fiber, components bound to the fiber such as the polyphenolic compounds, as well as resistant starch. Several studies have been performed to evaluate the stability and absorption of diet-derived phenolics in the lower gastrointestinal tract (Aura et al., 2002). However, studies on the mode of digestion of macromolecular complexes formed by the food matrices with polyphenols are limited; most work
has focused on analysis of pure polyphenols and their parent metabolites, and possible effects of the food matrix on digestive processes are not considered (Aura et al., 2005; Hidalgo et al., 2012). Blueberries have a wide variety of polyphenols that interact with, and may be protected by, the food matrix during the early gastrointestinal passage. Such interactions may affect their stability, absorption and cellular uptake, but provide a valuable nutritional resource to the colon.

Previous studies have shown that polyphenols exist in free and complexed state, especially to pectic moieties to form nanostructures. Such complexed states may influence the bioavailability of polyphenols (Jacob and Paliyath, 2008). The aim of this research was to evaluate the stability and functional properties of blueberry polyphenols that exist in free and complexed states in extracts, and their catabolic breakdown using in vitro models that simulates the conditions that occur in the GIT. Along with the evaluation of their effects on human colonic epithelial cells in culture, this study will enable us to understand the factors that govern the stability, bioavailability and absorption of blueberry phytochemicals. In turn, this will help in the development of storage and processing techniques designed to enhance the quality and functionality of blueberries in the context of improving human health.

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

All solvents were of HPLC grade. Methanol, peptone water, NaHCO₃, KH₂PO₄, and L-cysteine HCl were purchased from Fisher Scientific (Ottawa, Canada). Yeast extract was purchased from BD (New Jersey, USA). Casein and inulin (from Dahlia tubers) were purchased from Alfa Aesar (Massachusetts, USA), MgSO₄ and hemin were purchased from BDH (Pennsylvania, USA). Antifoam B silicone emulsion was purchased from J.T. Baker (Pennsylvania, USA). Anaerobe agar was obtained from Acumedia (Michigan, USA),
Defibrinated sheep blood was purchased from Hemostat Laboratories (California, USA). Formic acid, Folin Ciocalteu reagent, Na$_2$CO$_3$, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), Nitrotetrazolium Blue chloride (NBT), Riboflavin, ethylenediaminetetraacetic acid (EDTA), methionine, pepsin from porcine gastric mucosa (3802 units/mg protein), pancreatin from porcine pancreas (8x USP), bile salts, Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, trichloroacetic acid (TCA), sulforhodamine B (SRB), acetic acid, Tris base, CaCl$_2$, pectin (from citrus), xylan (from beechwood), arabinogalactan, NaCl, porcine gastric mucin (type II), menadione, and starch (from wheat, unmodified) were purchased from SIGMA (Oakville, Canada). Cyanidin and delphinidin glucosides were purchased from Chromadex (Irvine, USA). Malvidin glucoside was obtained from from Extrasynthese (Geney, France). Human epithelial colorectal adenocarcinoma cell line (HT-29) and human normal epithelial colon cell line (CRL-1790) were obtained from the American Type Culture Collection (Rockville, USA). Minimal Essential Medium Eagle (MEME) was purchased from Hyclone, Thermoscientific (Ottawa, Canada). Phosphate buffer saline 10x (PBS), penicillin-streptomycin solution, and trypan blue were from Gibco (Bethesda, USA).

6.2.2 Fruits

Frozen wild blueberries (low-bush) belonging to the same lot were obtained from the local supermarket and were stored at -20 °C for further extractions and analysis.

6.2.3 In vitro digestion procedure

Gastrointestinal digestion was simulated according to a modification of Gil-Izquierdo et al. (2002). See section 3.2.6 for details.
6.2.4 Single-stage chemostat simulation of the human distal gut environment

A chemostat model was used to perform a single stage fermentation procedure. See section 3.2.7 for full details.

6.2.5 In vitro fermentation

Samples collected after the in vitro digestions from the upper intestine model were inoculated with aliquots of a sample drawn from the chemostat vessel at steady-state (described in section 3.2.7.1) supporting a defined community of bacterial species representative of those found in the distal human gut, isolated from a single healthy donor.

6.2.6 Extraction of phenolic components from blueberry digests

Polyphenol-rich extracts after each step of the in vitro digestion were obtained by a solid phase extraction (SPE) using Sep-Pak C18 cartridges (Waters Corporation, MA, USA). The columns were washed with water and polyphenols and anthocyanins eluted with 100% methanol. All the samples were concentrated to 1 mg/ ml of polyphenols, filtered with 0.45 μm nylon filters and placed into a LC auto sampler vials for LC-MS analyses. (See 3.2.8 for more details)

6.2.7 Identification of individual polyphenols by LC-ESI-MS

Chromatographic analysis of anthocyanins isolated from the crude extracts, the dialyzed and the dialyzate fractions were performed using liquid chromatography (LC) and Mass Spectrometry (MS) (See section 3.2.9 for more details).
6.2.8 DPPH radical scavenging assay

Total antioxidant activity of blueberry crude extracts and dialyzed fractions was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to Molyneux (2004). See section 3.2.10 for more details.

6.2.9 Superoxide anion radical scavenging assay

The superoxide anion activity was determined as described by Madamachi et al., 1994. See section 3.2.11 for more details.

6.2.10 Cell culture

See section 3.2.13 for details.

6.2.11 Statistical Analysis

One-way analyses of variance (ANOVA) and multiple comparison tests were conducted as described in Section 3.2.14.

6.3 Results

6.3.1 Polyphenol composition of blueberry extracts

Anthocyanins were the major polyphenols of blueberry extracts, these being the pentose and hexose derivatives of delphinidin, malvidin, cyanidin, peonidin and petunidin (Fig 6.1a, peaks 2-12; Table 6.1). Table 6.1 enumerates the peaks and the molecular mass of the ions detected in the crude extract and the samples subjected to in vitro digestion and fermentation
steps, and Table 6.3 summarizes the changes in anthocyanin and phenolics composition during in vitro fermentation.

Delphinidin- and malvidin acetoxyl-glucosides (peaks 13 and 14, Fig 6.1a) were detected in significant amounts. In crude extracts, chlorogenic acid was the most abundant phenolic acid (peak 1, Fig 6.1a, Table 6.1). Over 65% of the anthocyanins occurred as hexoside, pentoside and acetoxyl glucoside of malvidin (Table 6.3). Delphinidin-3-acetoxyl glucoside occurred at nearly 30% of the total anthocyanins (Table 6.3). Quercetin arabinoside and syringetin glucoside were also found in lower amounts.

Anthocyanins are generally considered to be very unstable compounds; thus to determine in detail their stability during their passage through the GIT, blueberry extracts were digested in vitro and their degradation tracked by LC-ESI-MS analyses. No major qualitative or quantitative changes were observed in the phenolics composition after simulated gastric digestion (Fig 6.1a, b; Table 6.3).

After intestinal digestion of the gastric digest, there is a clear disappearance of the absorbance of the anthocyanin peaks (Fig 6.1c) indicating ring cleavage of most of the anthocyanins as the pH of the incubation medium changes from acidic (pH 2) to alkaline (pH 8) conditions. However, peaks 13 and 14 corresponding to delphinidin- and malvidin-6-acetoxyl 3-glucosides respectively, were relatively stable to the changes in pH and showed much less degradation. After simulated colonic digestion, most of the anthocyanins were degraded except delphinidin- and malvidin-6-acetoxyl 3-glucoside, which were still present at nearly 50% of their initial levels (Figure 6.1d).

6.3.2 Recovery of total polyphenols and anthocyanins after in vitro digestion

In order to evaluate the stability of polyphenols during their passage through the GIT, blueberries were ground and incubated with pepsin to simulate digestion by the stomach;
pancreatin to simulate digestion in the small intestine; and fermented with a defined community of human fecal microbiota to simulate colonic fermentation. After each step of the in vitro digestion process samples were centrifuged to remove debris. In order to remove free sugars and other compounds that could interfere with subsequent analysis, the polyphenols in the supernatant were purified by a C18 solid phase cartridge. Finally polyphenols were quantified by UV/Vis spectrometry. Figure 6.2 shows the absorption spectra of crude extract, stomach digested, intestine digested and colon fermented blueberry extracts, showing structural changes that occur during various steps of the digestion/fermentation processes. Anthocyanins show a characteristic absorption spectrum with a peak at 260 nm arising from the absorption of the phenyl ring (B ring) and at 520 nm, arising from the absorption of the benzopyran ring (A ring) (Fossen and Anderson, 2006).

Anthocyanins extracted after in vitro digestion that simulates gastric and intestinal conditions showed a very similar absorption spectrum as was obtained for the crude extract. A slight reduction in the 520nm absorption peak observed in the crude extract (pH 3.5) and the small intestine digestion simulation extract may have been due to changes in sample pH. After colonic fermentation, the catabolites showed a complete loss of 520 nm absorption peak that may be due to the cleavage of the benzopyran ring, and retained the absorption at 260 nm characteristic to the phenyl rings, suggesting the presence of simple phenolic components.
Table 6.1 Identification of polyphenols and parent metabolites from blueberries

<table>
<thead>
<tr>
<th>Peak</th>
<th>(t_R^a) (min)</th>
<th>m/z</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.75 ± 0.21</td>
<td>353</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>2</td>
<td>22.00 ± 0.25</td>
<td>465</td>
<td>Delphinidin 3-galactoside</td>
</tr>
<tr>
<td>3</td>
<td>24.91 ± 0.23</td>
<td>465</td>
<td>Delphinidin 3-glucoside</td>
</tr>
<tr>
<td>4</td>
<td>26.91 ± 0.21</td>
<td>435</td>
<td>Delphinidin 3-arabinoside</td>
</tr>
<tr>
<td>5</td>
<td>28.70 ± 0.21</td>
<td>449</td>
<td>Cyanidin 3-glucoside</td>
</tr>
<tr>
<td>6</td>
<td>30.27 ± 0.20</td>
<td>479</td>
<td>Petunidin 3-galactoside</td>
</tr>
<tr>
<td>7</td>
<td>33.30 ± 0.24</td>
<td>463</td>
<td>Peonidin 3-galactoside</td>
</tr>
<tr>
<td>8</td>
<td>35.05 ± 0.17</td>
<td>449</td>
<td>Petunidin 3-arabinoside</td>
</tr>
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<td>9</td>
<td>36.77 ± 0.23</td>
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<td>Malvidin 3-galactoside</td>
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<td>10</td>
<td>39.03 ± 0.19</td>
<td>433</td>
<td>Peonidin 3-arabinoside</td>
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<td>11</td>
<td>40.56 ± 0.18</td>
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<td>Malvidin 3-glucoside</td>
</tr>
<tr>
<td>12</td>
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<td>Malvidin 3-arabinoside</td>
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<tr>
<td>13</td>
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<tr>
<td>14</td>
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<td>535</td>
<td>Malvidin 6-acetyl,3-glucoside</td>
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<td>Quercetin arabinoside</td>
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<tr>
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<td>Syringetin 3-O-galactoside</td>
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<td>4.72 ± 0.20</td>
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<td>Rhamnetin</td>
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<td>7.40 ± 0.46</td>
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<td>Hippuric acid</td>
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<td>10.44 ± 0.48</td>
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<td>Cinnamic acid</td>
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<td>12.92 ± 0.031</td>
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<td>Caffeic acid</td>
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<td>15.21 ± 0.063</td>
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<td>Protocatechuic acid</td>
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<tr>
<td>23</td>
<td>19.59 ± 0.027</td>
<td>432</td>
<td>Kaempferol 3-rhamnoside</td>
</tr>
</tbody>
</table>

\(a\) Retention time, the result represents the mean ± SD (n=3).
Figure 6.1 Chromatogram of analytical HPLC (260 nm) of blueberry polyphenols from a) Crude extracts, b) Stomach digestion, c) Intestinal digestion, d) Colonic fermentation. Refer to Table 1 for the identification of each numbered peak.
6.3.3 Changes in the polyphenol composition during in vitro digestion

The effect of the in vitro gastric, intestinal digestion and the colonic fermentation on the total polyphenol and anthocyanin content is presented in Table 6.2. The initial amounts of polyphenols and anthocyanins present in the blueberry samples (at pH 2) were 2.14 mg GAE/ g fruit and 1.48 mg cyan3-gluc/-mg fruit, respectively. The recovery of polyphenols and anthocyanins after the gastric digestion was approximately 94% and 97%, respectively, and after the intestinal digestion only 49% and 17% respectively. After the colonic fermentation, major qualitative changes in composition of the phenolic components were noticeable including seven new peaks identified as syringic acid, rhamnetin, hippuric acid, cinammic acid, protocatechuic acid, caffeic acid, and kaempferol rhamnoside (Fig 6.1d). The final recovery of polyphenols after colon fermentation was approximately 42 % and 1.5 % of the initial polyphenols and anthocyanins, respectively. During the fermentation process a set of control samples was run in order to monitor the background and thus to prevent overestimation of polyphenol degradation due to interactions between polyphenols and media. A 10% loss of polyphenols was observed when samples were incubated with sterile media alone suggesting that only minimal loss occurred due to non-specific degradation (data not shown).

The qualitative changes in the content of various phenolic components during the simulated digestion processes and colonic fermentation are shown in Table 6.3. There were no significant qualitative and quantitative differences in the phenolics and anthocyanin content between crude extract and that subjected to simulated digestion under gastric conditions, except for quercetin arabinoside and syringetin-3-glucoside. After subjecting the phenolic components (after gastric digestion) to simulated digestion under intestinal conditions (higher pH, pancreatin), significant declines were observed in the quantitative profile of several phenolic components. All the anthocyanin components showed a significant decline during incubation under intestinal conditions. Among phenolic acids, chlorogenic acid was stable to digestion both
under gastric and intestinal conditions, and its level remained constant during colonic fermentation.

The levels of various phenolic compounds formed during colonic fermentation are shown in Table 6.4. Chlorogenic acid, quercetin arabinoside and syringetin-3-galactoside are originally present in the blueberry crude extracts. The levels of these components declined during the colon fermentation irrespective of whether the source of polyphenols that were used for fermentation were blueberry crude extract or intestinal digest.

The final recovery of polyphenols after colon fermentation was approximately 42 % and 1.5 % of the initial polyphenols and anthocyanins, respectively. During the fermentation process a set of control samples was run in order to monitor the background and thus to prevent overestimation of polyphenol degradation due to interactions between polyphenols and media. A 10% loss of polyphenols was observed when samples were incubated with sterile media alone suggesting that only minimal loss occurred due to non-specific degradation (data not shown).

Crude extract was used as a control to observe any potential effect of food matrix on bacterial action during fermentation. In both cases, the relative amounts of several new phenolic acids formed during fermentation were relatively similar except for cinnamic acid, protocatechuic acid, caffeic acid and chlorogenic acid. The acetylated anthocyanins were transformed to a lesser extent and their levels remained similar to that observed in the intestinal digest.
**Figure 6.2** UV/visible spectrum of blueberry extracts before and after *in vitro* digestion.
Table 6.2 Total polyphenol and anthocyanin content in blueberry samples before and after in vitro digestion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Phenolics $^A$</th>
<th>% Recovery</th>
<th>Total Anthocyanins $^B$</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Crude extract)</td>
<td>2.14 ± 0.11$^a$</td>
<td>100</td>
<td>1.48 ± 0.06$^A$</td>
<td>100</td>
</tr>
<tr>
<td>Gastric digestion (pH 2) 2 h, 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>2.12 ± 0.44$^a$</td>
<td>99.1</td>
<td>1.49 ± 0.23$^A$</td>
<td>101.4</td>
</tr>
<tr>
<td>Crude extract + pepsin</td>
<td>2.00 ± 0.14$^a$</td>
<td>93.5</td>
<td>1.43 ± 0.08$^A$</td>
<td>96.8</td>
</tr>
<tr>
<td>Intestine digestion (pH 7.5) 2h, 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric digest</td>
<td>1.05 ± 0.21$^b$</td>
<td>49.1</td>
<td>0.22 ± 0.05$^B$</td>
<td>15.0</td>
</tr>
<tr>
<td>Gastric digest + pancreatin</td>
<td>1.05 ± 0.28$^b$</td>
<td>49.1</td>
<td>0.25 ± 0.036$^B$</td>
<td>17</td>
</tr>
<tr>
<td>Colon fermentation (pH 7.5) 12h, 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.92 ± 0.045$^c$</td>
<td>42.9</td>
<td>0.15 ± 0.01$^C$</td>
<td>10.1</td>
</tr>
<tr>
<td>Intestine digest</td>
<td>0.94 ± 0.18$^c$</td>
<td>43.9</td>
<td>0.045 ± 0.002$^D$</td>
<td>3.0</td>
</tr>
<tr>
<td>Intestine digest + chemostat</td>
<td>0.91 ± 0.11$^c$</td>
<td>42.5</td>
<td>0.023 ± 0.010$^D$</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Note: Numbers in columns followed by different letters are significantly different (p < 0.05). Mean ± SEM of three independent experiments.
$^A$ Total polyphenol content (mg GAE/ g fruit).
$^B$ Total anthocyanin content (mg cyan 3-gluc/ g fruit).
Table 6.3 Polyphenol composition (mg/ g fresh weight of fruit equivalent) of blueberry a)

Crude extract, b) \textit{in vitro} stomach digests, and c) \textit{in vitro} intestine digests. Twenty µg equivalents of polyphenols were injected in each of the experimental sets.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Crude extract (^a)</th>
<th>Stomach (^a)</th>
<th>Intestine (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.291 ± 0.027(^a)</td>
<td>0.315 ± 0.048(^a)</td>
<td>0.345 ± 0.094(^a)</td>
</tr>
<tr>
<td>Quercetin arabinoside</td>
<td>0.113 ± 0.001(^a)</td>
<td>0.167 ± 0.027(^a)</td>
<td>0.084 ± 0.021(^a)</td>
</tr>
<tr>
<td>Syringetin-3-galactoside</td>
<td>0.108 ± 0.015(^a)</td>
<td>0.045 ± 0.007(^b)</td>
<td>0.049 ± 0.012(^b)</td>
</tr>
<tr>
<td>TOTAL(^b)</td>
<td>0.512 ± 0.026(^a)</td>
<td>0.527 ± 0.087(^a)</td>
<td>0.478 ± 0.125(^b)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>100</td>
<td>102.9</td>
<td>93.4</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delphinidin 3- galactoside</td>
<td>0.107 ± 0.032(^a)</td>
<td>0.106 ± 0.014(^a)</td>
<td>0.00472 ± 0.0002(^b)</td>
</tr>
<tr>
<td>Delphinidin 3- glucoside</td>
<td>0.180 ± 0.053(^a)</td>
<td>0.175 ± 0.023(^a)</td>
<td>0.0519 ± 0.0051(^b)</td>
</tr>
<tr>
<td>Delphinidin 3- arabinoside</td>
<td>0.057 ± 0.021(^a)</td>
<td>0.059 ± 0.080(^a)</td>
<td>Tr.</td>
</tr>
<tr>
<td>Cyanidin 3-glucoside</td>
<td>0.059 ± 0.007(^a)</td>
<td>0.082 ± 0.011(^a)</td>
<td>0.0064 ± 0.001(^b)</td>
</tr>
<tr>
<td>Petunidin 3- galactoside</td>
<td>0.108 ± 0.020(^a)</td>
<td>0.104 ± 0.014(^a)</td>
<td>Tr.</td>
</tr>
<tr>
<td>Peonidin 3- galactoside</td>
<td>0.150 ± 0.031(^a)</td>
<td>0.148 ± 0.020(^a)</td>
<td>0.003 ± 0.008(^b)</td>
</tr>
<tr>
<td>Malvidin 3-galactoside</td>
<td>0.270 ± 0.055(^a)</td>
<td>0.232 ± 0.030(^a)</td>
<td>0.025 ± 0.007(^b)</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>0.250 ± 0.043(^a)</td>
<td>0.239 ± 0.030(^a)</td>
<td>0.023 ± 0.007(^b)</td>
</tr>
<tr>
<td>Malvidin 3-arabinoside</td>
<td>0.107 ± 0.022(^a)</td>
<td>0.110 ± 0.016(^a)</td>
<td>0.009 ± 0.002(^b)</td>
</tr>
<tr>
<td>Delphinidin 6-acetyl 3-glucoside</td>
<td>0.200 ± 0.037(^a)</td>
<td>0.183 ± 0.026(^a)</td>
<td>0.068 ± 0.0001(^b)</td>
</tr>
<tr>
<td>Malvidin 6-acetyl 3-glucoside</td>
<td>0.260 ± 0.040(^a)</td>
<td>0.234 ± 0.03(^a)</td>
<td>0.097 ± 0.002(^b)</td>
</tr>
<tr>
<td>TOTAL(^c)</td>
<td>1.78 ± 0.35(^a)</td>
<td>1.67 ± 0.22(^a)</td>
<td>0.235 ± 0.026(^b)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>100</td>
<td>93.8</td>
<td>18.3</td>
</tr>
</tbody>
</table>

Notes: Identification based on MS and published data and chromatographic standards. Numbers in rows followed by different letters are significantly different (p < 0.05).
\(^A\) Mean ± SEM; n= 3.
\(^B\) Total phenolic content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses.
\(^C\) Total anthocyanin content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses.
Tr = traces
Table 6.4 Phenolic composition (mg g⁻¹ fruit) of blueberry extracts after *in vitro* colon fermentation. Twenty µg equivalents of polyphenols were injected in each of the experimental sets.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fermentation of Crude extracts A</th>
<th>Fermentation of Intestine digests A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenolics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringic acid</td>
<td>0.048 ± 0.007⁩⁺</td>
<td>0.029 ± 0.013⁩⁺</td>
</tr>
<tr>
<td>Rhamnetin</td>
<td>0.027 ± 0.004⁩⁺</td>
<td>0.027 ± 0.001⁩⁺</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>0.026 ± 0.002⁩⁺</td>
<td>0.032 ± 0.021⁩⁺</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>Tr</td>
<td>0.023 ± 0.001⁩⁺</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.024 ± 0.002⁩⁺</td>
<td>0.037 ± 0.007⁩⁺</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.078 ± 0.01⁩⁺</td>
<td>0.034 ± 0.009⁩⁺</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.093 ± 0.010⁩⁺</td>
<td>0.135 ± 0.027⁩⁺</td>
</tr>
<tr>
<td>Kaempferol 3-rhamnoside</td>
<td>0.03 ± 0.002⁩⁺</td>
<td>0.019 ± 0.003⁩⁺</td>
</tr>
<tr>
<td>Quercetin arabinoside</td>
<td>0.059 ± 0.002⁩⁺</td>
<td>0.070 ± 0.039⁩⁺</td>
</tr>
<tr>
<td>Syringetin-3-galactoside</td>
<td>0.044 ± 0.02⁩⁺</td>
<td>0.105 ± 0.003⁩⁺</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>0.430 ± 0.02⁩⁺</td>
<td>0.527 ± 0.081⁩⁺</td>
</tr>
<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delphinidin 6-acetoyl 3-glucoside</td>
<td>0.109 ± 0.028⁩⁺</td>
<td>0.097 ± 0.023⁩⁺</td>
</tr>
<tr>
<td>Malvidin 6-acetoyl 3-glucoside</td>
<td>0.051 ± 0.002⁩⁺</td>
<td>0.094 ± 0.016⁩⁺</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>0.160 ± 0.057⁩⁺</td>
<td>0.191 ± 0.038⁩⁺</td>
</tr>
</tbody>
</table>

Notes: Identification based on mass spectroscopy, published data and chromatographic standards.

Numbers in rows followed by different letters are significantly different (p < 0.05).

A Mean ± SEM n= 3.

B Total phenolic content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses.

C Total anthocyanin content calculated from the sum of total polyphenol peak areas obtained by HPLC analyses.

Tr = traces
6.3.4 Antioxidant Activity

Polyphenols are strong antioxidants, and the antioxidant capacity may change due to the chemical transformations that occur during GIT transit. To evaluate these possible changes, the antioxidant activity of phenolic components isolated from the crude extract and digested extracts was assessed using the DPPH- and superoxide- radical scavenging assays (Table 6.5).

DPPH radical scavenging and superoxide radical scavenging activities of the crude extracts, and those subjected to gastric and intestinal digestion showed much higher activity on a specific activity basis when compared to vitamin C and TROLOX. Crude extracts and samples subjected to gastric digestion conditions showed the highest DPPH radical scavenging activity. After digestion under gastric conditions, the antioxidant activity of the crude extract did not show a significant reduction.

The polyphenols obtained after digestion under gastric conditions were further subjected to digestion under conditions that simulate intestinal conditions. A significant reduction (< 50%) in DPPH scavenging activity was observed after digestion under intestinal conditions. After simulated colon fermentation, the DPPH radical scavenging activity was almost completely lost, as the spent media by itself showed similar levels of DPPH radical scavenging activity. Sterile media did not show any antioxidant activity.

6.3.5 Antiproliferative activity

Blueberry crude extracts were evaluated for their antiproliferative activity on human colon cancer cells (HT-29) and normal human epithelial colon cells (CRL-1790), before and after in vitro digestion. Cell proliferation was analyzed after 24 h and 48 h of exposure to media containing increasing levels of phenolic components from crude and digested blueberry extracts using the SRB assay. Cell proliferation in both HT-29 and CRL-1790 cell lines were inhibited in a dose-dependent manner. The results showed that the growth-inhibitory effects of phenolic
components from crude extract and stomach digests were significantly higher than that from the phenolic components obtained after simulated intestinal digestion and those isolated from simulated colon fermentation (Figure 6.3a and 6.3b). Crude extracts inhibited cell growth of HT-29 and CRL-1790 by 90% and 60%, respectively. By contrast, intestine digests and fermented samples caused only a 40% growth inhibition on both cell lines. Authentic cyanidin 3-glucoside and delphinidin 3-glucoside also inhibited proliferation of HT 29 cells more efficiently than that of the CRL 1790 cells (Figures 6.3c and 6.3d). However, a common anthocyanin metabolite protocatechuic acid did not show any antiproliferative activity on HT-29 cells and approximately 20% cell growth inhibition of CRL-1790.

6.4 Discussion

The biological effects of consuming a diet rich in anthocyanins are widely recognized. However, there are several factors such as the mechanisms involved in the metabolic breakdown of anthocyanins, the influence of the food matrix on their bioavailability, and the stability of the flavilium cation during GIT transit etc., that are less understood. Previous works on the stability of anthocyanins have been focused mainly on the chemical properties of the pigments or the recovery of anthocyanins after in vitro digestion (Ansen et al., 1972; Stinizing et al., 2002; Gil-Izquierdo et al. 2001, 2002; Perez-Vicente et al., 2002; Bermudez-Soto et al., 2007). Several factors such as the source and binding to the food matrix may affect the bioavailability of polyphenols (Nielsen et al., 2003). Food microstructure is an important factor in the release and bioavailability of several nutrients, and this has important consequences in assessing the nutraceutical properties of foods used in the prevention and therapy of chronic degenerative diseases such as cancer, diabetes and metabolic syndrome (Holst and Williamson, 2004; Aguilera, 2005; Parada and Aguilera, 2007).
Table 6.5 DPPH and superoxide radical scavenging capacity of different blueberry polyphenols expressed as % quenching.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Quenching/10 μg polyphenols</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH</td>
<td>Superoxide</td>
</tr>
<tr>
<td>Crude extract</td>
<td>46.89 ± 9.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.06 ± 8.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stomach</td>
<td>39.40 ± 6.16&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>62.13 ± 9.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intestine</td>
<td>19.88 ± 5.30&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>39.87 ± 1.35&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon (control)</td>
<td>14.30 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.04 ± 3.53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>17.54 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.34 ± 4.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Media</td>
<td>Bdl.</td>
<td>2.37 ± 1.47&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spent media</td>
<td>10.98 ± 0.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.55 ± 2.16&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>26.05 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.16 ± 0.42&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TROLOX</td>
<td>22.24 ± 2.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.29 ± 2.56&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: Numbers in columns followed by different letters are significantly different (p < 0.05). Mean ± SEM of three independent experiments. Bdl. = below detection limit.
Figure 6.3 Antiproliferative activities of blueberry polyphenols, protocatechuic acid, cyanidin 3-glucoside and delphinidin 3-glucoside on HT-29 and CRL-1790 cells, during in vitro digestion. Mean ± SEM (n=3).
Studies on the nutraceutical properties of the digested food fractions enriched in anthocyanins, the chemical nature and the biological properties of the metabolites and the influence of food matrix components are scarce. Therefore, it is important to understand how the process of digestion affects polyphenols structure and stability, as this, in turn, affects their bioaccessibility and their possible beneficial effects on cells of the gut epithelium (Rios et al., 2002). In the present study, we incorporated in vitro simulated gastrointestinal digestion systems along with a colon fermentation step to analyze the stability of blueberry anthocyanins and their metabolites. During in vitro digestion studies, different fruit phytochemicals undergo structural modifications and even degradation (Tavares et al., 2012). It is believed that metabolites generated during GIT transit and polyphenols that are released from the food matrix can be better absorbed through the epithelial cells of the gut (Saura-Calixto et al., 2007), and this in turn may provide enhanced beneficial effects in the human body (Bermudez-Soto et al., 2007). Therefore, the antioxidant and antiproliferative effects of such components were also evaluated in this study.

Changes in the structural properties of anthocyanins were reflected in their absorption characteristics and that of the resulting products, as this was predominant after the simulated intestinal digestion and colonic fermentation. It has been well established that anthocyanin absorption is dependent on pH due to reversible structural changes in the benzopyran ring structure. Under acidic conditions the flavylium ion is favored and distinguishable by the characteristic absorption maximum at 520 nm. Structural transformations of anthocyanins occur under alkaline conditions where mixtures of pseudobase and quinoidal structures are observed. Because of these properties, the pH value of anthocyanin containing processed is maintained at pH values below 4 (Delgado-Vargas et al., 2000; Martson and Hostettmann, 2006). To obtain reproducible results and prevent an over or underestimation of polyphenols before and after the in vitro digestion process as a consequence differences in the anthocyanin structures, the pH of
all the samples was adjusted to pH 2 prior analyses. The present study suggests that structural transformations occur during intestinal transit indicative of benzopyran ring cleavage under alkaline conditions and further catabolism during colonic fermentation results in the formation of several simple phenolic components, apart from those that are present in the original extract such as chlorogenic acid. After the gastric digestion, where the anthocyanins are reduced to nearly 50% of the original levels, the anthocyanin levels drop to nearly 10-15% during intestinal digestion and to 2-3 % during colon fermentation. While most of the simple anthocyanins were degraded during this transit, the acetylated derivatives of delphinidin and malvidin showed relatively high stability during the gastrointestinal transit, showing levels at nearly 50% of their original levels. Thus, fruits containing high levels of the acetylated derivatives are likely to provide the highest levels of anthocyanins into the colon. The presence of enzymes such as pepsin during the preceding simulated stomach digestion, and pancreatin during the simulated intestinal digestion, did not appear to have any effect in destabilizing anthocyanins through their potential effects on proteinaceous food matrix. Human system is devoid of enzymes such as pectinases and cellulases which enables the transit of pectin and cellulose components through the GIT. Therefore, food matrix interactions between anthocyanins and these carbohydrate molecules may have a significant enhancing effect on the bioavailability of anthocyanins in the colon, where such macrocomplexes are degraded, and where it may exert protective effects.

Results obtained in this study showed high stability of the blueberry polyphenols under the gastric conditions and, overall, are in agreement with previously reported in vitro studies on pomegranate juice (Perez-Vicente et al., 2002); raspberries (Mc Dougall et al., 2005); commercial chokeberry juice concentrate (Bermudez-Soto et al., 2007), and red grapes (Tagliazucchi et al., 2010). Control samples carried out without the enzymes showed similar stability, demonstrating that structural changes were mostly a consequence of the physical conditions of the assay and not due to an enzymatic degradation. After the incubation with
pancreatin and bile salts under alkaline conditions, a significant amount of the blueberry polyphenols, especially anthocyanins was lost. Interestingly, among all the anthocyanins found in the crude extracts, malvidin glucosides, delphinidin 6-acetyl 3-glucoside and malvidin 6-acetyl 3-glucoside showed high stability during the in vitro intestinal digestion. The results obtained in this research support previous findings and indicated that blueberry anthocyanins are stable in acidic conditions that simulate gastric digestion whilst they are unstable under alkaline conditions that resemble the intestine (Tsuda et al., 1999; Bermudez-Soto et al., 2007). It has been reported that the stability of anthocyanins in vitro is dependent on different factors such as the nature and number of sugars attached to the aglycon and the number of acids linked to the glucoside. Nonacylated 3,5-diglucosides had lower stability compared to the 3-monoglucosides and that the anthocyanins containing aromatic acyl groups showed higher stabilities than unacylated anthocyanins (Ansen et al., 1972; Stinizing et al., 2002). For instance, Perez-Vicente et al., (2002) have also reported high stability of anthocyanins from pomegranate juice during gastric digestion and a reduction of approximately 97% in the total anthocyanin content during the intestinal digestion. We recovered approximately 50% of total anthocyanins after in vitro intestine digestion. This discrepancy may be attributed to the difference in food matrices along with the fact that blueberries contain more 3-monoglucosides and 3,5-acylated anthocyanins which are chemically more stable than anthocyanins found in pomegranates (Galvano et al., 2004).

Most of the investigation on the effect of human fecal microbiota has been conducted using pure or isolated flavonoid compounds. These models have been helpful in the identification of the role of fecal microbiota enzymes. Several reports have shown their hydrolytic activity when incubated with anthocyanin 3-glucosides (Aura et al., 2002; Aura et al., 2005, Hidalgo et al., 2012). To simulate a more natural condition, anthocyanins that are complexed with food matrices were subjected to an in vitro fermentation step using a chemostat
model. Dividing the digestion process in two parts enabled the evaluation of the stability of blueberry anthocyanins during their passage through the upper digestive tract, and the colon. Formation of new compounds as a result of the metabolic break down of the polyphenols was also followed. The results showed that significant amounts of phenolic acids such as syringic acid, caffeic acid, protocatechuic acid, cinnamic acid, rhamnetin, and kaempferol-3-rhamnoside were formed after the colon fermentation. Similar observations were made by Hidalgo et al. (2012), who reported syringic acid as the major product from the degradation of malvidin 3-glucoside by fecal bacteria, while a mixture of various anthocyanins produced gallic, syringic and p-coumaric acids. We also observed that chlorogenic acid and quercetin arabinoside were not affected by in vitro gastric conditions. After in vitro fermentation a decrease in the content of chlorogenic acid was observed, along with the formation of caffeic acid. Caffeic acid has been reported as the major product resulting from the hydrolysis of chlorogenic acid by colonic microbiota (Gonthier et al., 2003; Lafay et al., 2006). Aglycones of anthocyanins were not detected (Bermudez-Soto et al., 2007; Hidalgo et al., 2012, Aura et al., 2005) at any point of the in vitro simulation of the human gastrointestinal digestion.

Antioxidant activities of polyphenol-rich foods have been compared and correlated to their potential in vivo efficacy (Lila, 2004). In this study we evaluated the scavenging potential of blueberry polyphenols and metabolites towards DPPH-, and superoxide-free radicals during the in vitro digestion process. DPPH is regarded as a stable synthetic radical suitable to measure the antioxidant activity of fruits and vegetables in vitro. However, under in vivo conditions, superoxide is one of the most predominant free radical species (Sánchez-Moreno, 2002). Estimations based on these two assays can provide a reliable estimation of the effect of in vitro digestion on the antioxidant activity of blueberry polyphenol components. We observed that the scavenging of DPPH and superoxide radicals was not reduced during the simulated in vitro gastric digestion, but was significantly reduced (approximately 50%) during the in vitro intestinal
digestion and to very low levels during in vitro colon fermentation. Similar observations were made by Tagliazzuchi et al., (2010) with red grape polyphenols. Therefore, most of the antioxidant benefits of polyphenols must come from those that are absorbed prior to the colonic fermentation. Polyphenols show detectable antioxidant activity, even at a concentration of 2.5 µg/ml under in vitro conditions (Jacob et al., 2008, 2012). Thus, stabilization of anthocyanins through food matrix interactions may help to provide sufficient levels for absorption during gastrointestinal transition.

The transition of blueberry polyphenols through the GIT may help to release polyphenols from the food matrix progressively and prepare them to exert their biological effects in the colon (Serrano et al., 2007). The GIT is continuously exposed to toxic agents including free radicals, lipid hydroperoxidases and carcinogens (Halliwell et al., 2000).

When polyphenols reach the colon either they are absorbed intact through the epithelium, or they are metabolized by the colonic microbiota, or simply they may encounter prooxidant or toxic species resulting from bacterial metabolism (Rios et al., 2003; Jenner et al., 2005; Serrano et al., 2007). The ability to counteract free radicals may contribute to the ability of dietary polyphenols to protect against colon cancer (Halliwell et al., 2000). In this study, we used a colon cancer cell line HT-29 and a normal colon cell line CRL-1790 to evaluate the effect of in vitro digestion on the bioactive properties of blueberry polyphenols. We observed that in vitro gastric digestion did not reduce the antiproliferative activity of blueberry polyphenols on both cell lines compared to crude extracts. However, after in vitro intestinal digestion and colonic fermentation a significant reduction was observed. When pure cyanidin 3-glucoside and delphinidin 3-glucoside were tested on these cell lines, they showed a similar antiproliferative activity as the blueberry digests. When protochatecuic acid, the major phenolic acid formed during the colonic fermentation, was incubated with HT-29 and CRL-1790 cells, it did not show significant antiproliferative activity. These results suggest that once metabolized, the antioxidant
and antiproliferative activities of blueberry anthocyanins are reduced. The concentration of some phenolic acids formed in the colon has been reported to be higher than the parent flavonoids (Rechner et al., 2002). Thus, it is most likely that any biological effect provided by blueberry anthocyanins in vivo is an implication of the anthocyanins absorbed during their transit through the gastric and intestinal system and less so due to the microbial metabolites. As well, the higher stability of acylated anthocyanins to fermentation may serve as a source of anthocyanins in the colon. Therefore, consumption of fruits or products rich in acylated anthocyanins may provide enhanced benefits to colon health. In this study, we used a defined microbial community of fecal bacteria to allow us to simulate colonic fermentation, and whilst there are benefits to this approach (including chemostat stability and culture reproducibility), it is likely that variations in results would be seen if a native fecal community had been used. Additionally, as we begin to understand the variations among gut microbiota populations between different people (Human Microbiome Project Consortium, 2012), the inevitable question arises as to whether these differences would influence polyphenol breakdown in divergent ways. Although beyond the scope of the present study, determining the bioactivity and stability of polyphenols fermented by different gut microbial communities is a future research goal of our laboratory.

Many of the earlier studies on the chemopreventive properties of purified dietary polyphenols were conducted using high levels of polyphenols that may not exist in human body (Zhang et al. 2008; Chen et al., 2009). While polyphenols from grape show very high antiproliferative activity at low µM concentrations in breast cancer cells (Hakimuddin et al., 2004, 2006, 2008; Jacob et al., 2008), it appears that blue berry polyphenols require at least a fold higher level to achieve similar levels of cytotoxicity as grape polyphenols. If this difference arises due to the origin of the cell lines (breast vs colon), this may suggest that some form of cancer may be more susceptible to physiologically lower levels of polyphenols, or a qualitatively
different composition of polyphenols. These aspects need further investigation. As well, these studies do not take into account other factors such as interactions among compounds and/or the food matrix, as well as possible additive or synergistic effects (Coates, et al., 2007). However, this study provides a more realistic scenario for the evaluation blueberry polyphenols and their potential benefits during their transit in the GIT.

6.5 Conclusions

To our knowledge, this is the first time that the stability of wild blueberry polyphenols during passage through the GIT was investigated. The approach proposed could represent a rapid and simple tool for evaluating the stability and bioaccessibility of polyphenols from fresh and processed polyphenol-rich foods. Our study provides new insights about the biotransformation of blueberry anthocyanins and their interactions with the food matrix. The above results indicate that the in vitro digestion process decreases the antioxidant and antiproliferative activity of blueberries significantly. Blueberry phenolic compounds that reach the colon still may have antioxidant properties that may be directly associated with their disease preventive properties. Metabolites such as protocatechuic acid, hippuric acid and caffeic acid appeared after bacterial fermentation with a defined community of human colonic bacteria and may be responsible for the biological effects obtained by incorporating blueberries in the diet. However, more in vivo studies are needed to correlate data and establish appropriate guidance for the consumption of polyphenol-rich foods.
CHAPTER 7
GENERAL CONCLUSIONS

Blueberries are of great interest due to their high content of anthocyanins that have been shown to have several biological activities and functionalities in food. This has resulted in a worldwide increase in the production of value-added products from blueberries with high amounts of natural antioxidants. At the value added products containing natural antioxidant are increasingly popular as the consumer are increasing the demand for health promoting foods. Although the information about the metabolism and absorption of anthocyanins is limited, there is a wide variety of anthocyanin-rich products in the market. In most cases, those functional foods such as juices, jams, yogurts, snacks, etc., are made from fresh or processed blueberries. This trend has increased the production and commercialization of blueberries worldwide. However, it is important to consider that anthocyanins are not the only compounds found in blueberries. In order to develop high quality functional foods it is essential to understand the role of other compounds and their interactions with the food matrix that may affect or improve the stability and delivery of anthocyanins in the body.

The overall aim of this research was to identify the polyphenols that are present in blueberries and determine the extent of their interaction with the food matrix. In addition, the effect of the food matrix and the gastrointestinal digestion on the stability and bioavailability of blueberry polyphenols were investigated.

In the first part of this study free polyphenols were separated from the food matrix and characterized. Their biological activities were similar to the crude extracts reported elsewhere. Although the composition of the polyphenols obtained in all the fractions after dialysis were similar to the polyphenols in the crude extracts, we could observe that dialyzed fractions contained a low amount of polyphenols, indicating that only a portion of the polyphenols is
complexed with the food matrix. Complexation did not significantly affect the antioxidant activity of polyphenols as evaluated by their ability to scavenge DPPH, hydroxyl, and superoxide. In addition, complexed polyphenols were demonstrated to be more effective in inhibiting the activity of α-glucosidase compared to polyphenols from the crude extracts and from the dialyzate fraction. This would suggest that maintaining the complexed state through food matrix interactions in blueberries has the potential to reduce postprandial glucose spike and help regulate glucose metabolism in the body. This may help prevent the development of obesity and its onset of type II diabetes.

The polyphenols obtained from different fractions showed considerable antiproliferative activities. Although complexation slightly reduced the ability of polyphenols to inhibit the growth and proliferation of HT-29 cells, there was still a considerable dose-dependent inhibition. Polyphenols from crude extracts and dialyzate fractions showed lower IC$_{50}$ values towards human HT-29 colon cancer cells compared to complexed polyphenols. We could also observe that polyphenols from blueberry crude extracts and dialyzate fractions inhibited the growth and proliferation of the normal colonic epithelial cells (CRL-1790) to a lower extent than cancer cells (30% in normal cells to 70% in cancer cells), however when polyphenols are complexed only 30% of the cells are inhibited, compared to almost 70% when cells are treated with free polyphenols. These results suggest that different fractions of polyphenols obtained in processed products or those formed during consumption of fresh blueberries may provide multiple effects such as antioxidant activity, regulation of glucose homeostasis and antiproliferative functions. The strong interaction between the food matrix and the polyphenols may positively affect the stability of polyphenols in the gastrointestinal tract.

In the second part of this research, the food matrix was characterized and compared to model systems prepared with a synthetic mixture of blueberry polyphenols and pectin and/or cellulose, the common components of blueberries. Of course, these were very simple models
systems, as the natural food matrix contains several compounds, most probably all playing a role in the formation of the supramolecular structures interacting with the polyphenols. The polyphenols from whole blueberries were removed using 50% (v/v) ethanol extensively until the fruits were colorless. The colorless fruits were ground and crude extracts collected. To assure that there were no polyphenols present in the samples, the crude extracts of the food matrix were dialyzed. Testing the food matrix with no polyphenols showed that the compounds present in the food matrix did not have a significant effect on the in vitro antioxidant towards DPPH free radicals and the growth and proliferation of HT-29 cells. In order to understand the interactions between polyphenols and the food matrix, free polyphenols were mixed with different solutions containing pectin and/or cellulose to observe whether the polyphenols were able to complex to the same extent as they are observed within the natural food matrix in processed fruits. After analyzing the microstructure of the extracts and quantifying the amount of pectin in the blueberry extracts we found a considerable amount of pectin. In addition, we also used cellulose to evaluate if free polyphenols could also complex with cellulose in vitro. Results showed that, in presence of pectin and cellulose, polyphenols did not lose their bioactivity. The polyphenols were not complexed with the mixtures after dialysis, most of the polyphenols were recovered as free polyphenols and only a small amount (<10%) was recovered in the dialyzed samples with the carbohydrates. The polyphenols that were artificially complexed to pectin and cellulose showed the same antioxidant and antiproliferative activities as the original mixtures. This behavior may suggest that there may be different types of interactions between polyphenols and the food matrix. Polyphenols showed strong interactions when they are naturally complexed with the food matrix and weak interactions when they are artificially complexed with carbohydrates. The strong interactions between polyphenols and the food matrix may occur during processing or consumption steps. The vesicles observed during ultrastructural analysis of complexed polyphenols suggest that polyphenols may have originated from microvesiculation.
during the extraction and grinding process. In this study we also observed a reduction in the ability of naturally complexed polyphenols to scavenge free radicals. This may be a consequence of complex formations involving hydroxyl groups, reducing their ability to exchange protons. On the other hand artificially complexed polyphenols did not lose their ability to scavenge free radicals, suggesting that hydroxyl groups of the polyphenol structures may not participate in such complexation process. Consequently, these findings may be an advantage while designing a food matrix to encapsulate anthocyanins to incorporate into foods.

In the third part of this research, we evaluated the effect of the digestion process on the stability and bioavailability of blueberry polyphenols. As it is complicated to conduct *in vivo* studies to obtain the metabolic compounds after each step of the digestion, we used an *in vitro* gastrointestinal model. The *in vitro* model used for this study comprised a sequential incubation with pepsin and pancreatin, followed by a chemostat fermentation step to simulate the conditions in the stomach, small intestine and colon, respectively. This allowed us to follow changes in the relative composition and degradation of blueberry polyphenols. The composition of the samples collected after digestion in the presence of pepsin and pancreatin were not different from the one of the original extracts. There was a slight decrease in the amount of anthocyanins after the simulated intestine digestion. Surprisingly, acylated anthocyanins were more stable under the intestinal conditions and were recovered in considerable amounts. The structural changes that anthocyanin-3-glucosides suffer during the incubation under alkaline conditions reduced the spectrophotometric detection of the anthocyanins. However, such structural changes did not suppress the bioactivity of the compounds. Samples collected after fermentation showed a considerable antioxidant and antiproliferative activities. Metabolites such as protocatechuic acid, hippuric acid and caffeic acid appeared after bacterial fermentation with a defined community of human colonic bacteria, and may be responsible for the biological effects obtained. However, the metabolites produced during the fermentation process, such as
protocatechuic acid did not show any effect on growth and proliferation of HT-29 cells. Thus, it appears that although the antioxidant activity was retained in the metabolites after fermentation, some of the key health regulatory properties such as anticancer activity is lost. Therefore, anthocyanins absorbed during the GIT transit as well as their metabolites provide a combined role in health regulatory properties. To the best of our knowledge this is the first investigation on the stability of blueberry polyphenols during passage through the gastrointestinal tract. The approach proposed in this thesis could be applied to monitor and evaluate the stability and bioaccessibility of polyphenols from fresh and processed polyphenol-rich foods. The contribution of this dissertation to the field states the basis to develop processing techniques that fully take advantage of the functional properties of blueberries. However, in vivo investigations are required to determine the biological activities of polyphenol complexes and their parent metabolites in relation to their probable circulating concentrations.
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