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PhD THESYS

EFFECTS OF THERMAL PROCESSSING ON WILD BLUEBERRY ANTHOCYANINS AND THEIR ABSORPTION, METABOLISM, DISTRIBUTION AND EXCRETION IN SPRAGUE-DAWLEY RAT

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ABSTRACT

Anthocyanins, which are found in high concentrations in fruit and vegetable such as blueberries, may play a beneficial role in retarding or reversing the course of chronic degenerative diseases. Unfortunately, anthocyanins are labile compounds and susceptible to deterioration during processing and storage. For these reasons, the first objective of this study was to determine both the degradation kinetics of single anthocyanins contained in freeze-dried wild blueberry powder stored at different temperatures and total antioxidant activity (TAA). Preliminarily, wild blueberry powder was characterized for its content in anthocyanins, which was 1.8±0.1 mg/100 mg powder. Then, freeze-dried wild blueberry powder samples were stored at 25, 42, 60 and 80 °C for 49 days and degradation kinetics evaluated by the Arrhenius equation. At storage time-intervals of 3-4 days, anthocyanins and TAA were determined by liquid chromatography coupled to diode array and mass spectrometer detector (LC-DAD-MS) and Trolox Equivalent Antioxidant Activity (TEAC) method, respectively. Moreover, the Arrhenius equation was used to predict the shelf-life of ACNs and TAA at 4 °C. Results demonstrated that the degradation of ACNs followed a first-order kinetic. Total and single ACN decay occurred at all the temperatures but was slower at 25 °C compared to 60 and 80 °C. On the contrary, TAA was unaffected after storage at 42 °C for 49 days. In conclusion, wild-blueberry powder maintains the content of ACNs and TAA longer (up to 130 days) at 25 °C; however, storage at 4 °C represents the best way to preserve the nutritional quality of the product and delay decay. The second aim of this study was to investigate wild blueberry anthocyanins adsorption, metabolism, distribution in the plasma, liver, brain, and their excretion in urine and feces in rats fed a wild blueberry-enriched diet for 4 and 8 weeks. Moreover, we have evaluated anthocyanin transformation pathways by human and rat microflora. Thus, thirty-two rats were fed for 4 or 8 weeks with a control or a wild blueberry-enriched diet (24±5 mg/day of ANCs). Anthocyanin profile in plasma, urine, feces, brain, and liver was evaluated by LC-MS/MS, and significantly increased in urine and not in feces after 8 weeks on the wild blueberry diet compared to that in 4 weeks, but no anthocyanins were detected in plasma, liver, and brain samples either in the control or wild blueberry groups. Metabolites of ACNs were detected in the plasma, urine, feces, and tissues of both the groups, but the urinary excretion of hippuric acid increased significantly after 4 and 8 weeks of wild blueberry consumption. Thus, it seems that ACNs are metabolized by the intestinal microflora to respective phenyl-alkyl acids, which can be further metabolized to benzoic acid. In conclusion, ACNs are bioavailable in rats, and the extent of their metabolism and excretion is based on diet duration. The third aim of this research was to evaluated anthocyanin transformation pathways by human and rat microflora. Thus, anthocyanins standard and wild blueberry were incubated in anaerobic conditions for 48 h at 37 °C with rat microflora bacteria from stomach, small intestine and colon. At time zero, every hour for 8 hours and after 24 and 48 h of incubation an aliquot was collected in twice, diluted, centrifuged and the supernatants analyzed by LC-DAD-MS. The metabolic activity of the intestinal microflora from stomach was found, as expected, blander. Indeed, after 48 h of incubation the residual percentage of standard anthocyanins was 65±5 %, and peonidin-arabinoside appeared the most stable (74±3 %). On the contrary, in the batch containing the bacteria of the small intestine the residual anthocyanins were 70±6 and 11±7 % after 6 and 24 h, respectively. It should be highlighted that after 24 h were present only petunidin-glucoside (42±4 %) and peonidin-arabinoside (38±2 %). As expected, the colon bacteria showed the highest catabolic activity against anthocyanins. Indeed, the residual ACNs were 14±4 % after 6 h of incubation in anaerobic conditions, and no ACNs were found after 24 h incubation. Also in this experimental condition peonidin-ara (20±3 %) and petunidin-glc (18±4 %) were the more stable. Similar results were obtained by analyzing solutions containing wild blueberry. Indeed, anthocyanins were degraded mainly in the colon and after 3 and 6 h the residual amount were 33±4 and 18±7 %, respectively. Anthocyanins were not found after 24 h of incubation, and also in these trials the more stable anthocyanins were those esterified with acetic acid, thus confirming the protective effect of acyl groups. It should be underlined that cyanidin-galactoside, malvidin-

galactoside and delphinidin-galactoside were not detected already after 6 hours of incubation and also the percentages of the corresponding conjugated with glucose were low (3-7 %). At the parity of linked sugar, petunidin and peonidin were more stable than other anthocyanins. During the incubations new metabolites were formed and identified as the respective corresponding phenolic acids derived from the B-ring of the anthocyanidins. In case of malvidin-glucoside and peonidin-glucoside, syringic and vanillic acid were identified as the major degradation products respectively, and cyanidin-glucoside was degraded to protocatechuic acid. These metabolites were then degraded with consequent formation of benzoic acid, and regarding syringic acid the conversion was very slightly. Similar results were obtained incubating anthocyanins with human fecal microflora. Thus, the anthocyanin degradation to the corresponding phenolic acids seems to be a general pathway for these substances. The colon may be considered as an active site of metabolism in which bacteria contribute to the health effects of phenolic compounds. Although the degradation to the phenolic acid is an important pathway, it cannot explain the total disappearance of the anthocyanins during incubation. Indeed, calculated on the basis of our *in vitro* fermentation studies the amount of the formed phenolic acid reached up to a maximum of 40 % of the parent compound, suggesting the occurrence of anthocyanin degradation pathways not yet identified.

RIASSUNTO

Le antocianine, che si trovano in alte concentrazioni in frutta e verdura, soprattutto nei frutti di bosco come i mirtilli, possono svolgere un ruolo importante nel ritardare o invertire il corso delle malattie croniche degenerative. Purtroppo, le antocianine sono composti labili e suscettibile di deterioramento durante la lavorazione e lo stoccaggio. Per questo motivo, il primo obiettivo di questo studio era determinare sia la cinetica di degradazione delle singole antocianine che l'attività antiossidante totale (TAA) della polvere di mirtillo selvatico (*Vaccinium angustifolium*) freeze-dried conservato a diverse temperature. Preliminarmente, la polvere di mirtillo è stata caratterizzata per il suo contenuto di antocianine, che era di 1.8±0.1 mg/100 mg polvere. In seguito, i campioni di polvere di mirtillo selvatico sono stati conservati a 25, 42, 60 e 80 °C per 49 giorni e le cinetiche di degradazione valutate mediante l'equazione di Arrhenius. A intervalli di 3-4 giorni le antocianine e il TAA sono stati determinati rispettivamente mediante cromatografia liquida interfacciata a rivelatore a serie di diodi e spettrometro di massa (LC-DAD-MS) e metodica TEAC (Trolox Equivalent Antioxidant Capacity). Inoltre, l'equazione di Arrhenius è stata usata per prevedere la shelf-life delle antocianine e l'attività antiossidante totale a 4 °C. I risultati hanno dimostrato che la degradazione delle antocianine seguiva una cinetica di primo ordine e che la quantità di questi composti diminuiva a tutte le temperature testate. La diminuzione era più lenta a 25 °C rispetto a 60 e 80 °C, ma il TAA restava inalterato dopo conservazione a 42 °C per 49 giorni. In conclusione, polvere di mirtillo selvatico mantiene il contenuto di antocianine e il TAA più a lungo (fino a 130 giorni) se conservato a 25 °C e che comunque la conservazione a 4 °C rappresenta il modo migliore per conservare la qualità nutrizionale del prodotto e il ritardare la diminuzione delle antocianine . Il secondo obiettivo di questo studio era di valutare assorbimento, metabolismo, concentrazione in plasma, fegato, cervello e escrezione nelle urine e feci delle antocianine in ratti supplementati per 4 e 8 settimane con polvere di mirtillo selvatico. Inoltre, è stata valutata la trasformazione delle antociani effettuata dalla microflora umana e di ratto. Così, trentadue ratti sono stati alimentati per 4 o 8 settimane con dieta controllo o dieta arricchita di mirtillo selvatico (24±5 mg/giorno di antocianine). Il profilo qualitativo e quantitativo delle antocianine in plasma, urina, feci, cervello e fegato è stato valutato mediante LC-MS/MS, e queste aumentavano significativamente nelle urine e nelle feci dopo 8 settimane di dieta mirtillo selvatico, rispetto al trattamento di 4 settimane. Al contrario, le antocianine non sono state rilevate nel plasma, fegato e campioni di cervello nel gruppo di controllo. Metaboliti delle antocianine sono stati rilevati in plasma, urina, feci e tessuti di entrambi i gruppi, ma l'escrezione urinaria di acido ippurico aumentati significativamente dopo 4 e 8 settimane di consumo di mirtilli selvatici. Così, sembra che le antocianine siano metabolizzati dalla microflora intestinale con produzione dei rispettivi acidi fenil-alchilici, che possono essere ulteriormente metabolizzato ad acido benzoico. In conclusione, le antocianine sono biodisponibili nei ratti e l'entità del loro metabolismo ed escrezione è influenzata dalla durata della dieta. Il terzo obiettivo di questa ricerca è stato quello di valutare la trasformazione delle antocianine per opera della microflora umana e di ratto. Così, antocianine standard e polvere di mirtillo sono stati incubati in condizioni anaerobiche per 48 ore a 37 °C con microflora proveniente da stomaco, intestino tenue e colon. Al tempo zero, ogni ora per 8 ore e dopo 24 e 48 ore di incubazione,un'aliquota è stata raccolta in doppio, diluita, centrifugata e il surnatante analizzato mediante LC-DAD-MS. L'attività metabolica della microflora intestinale da stomaco è risultata, come previsto, piuttosto blanda. Infatti, dopo 48 ore di incubazione la percentuale residua di antocianine standard era del 65±5 % , e la peonidina-arabinoside è risultata la più stabile (74±3 %). Al contrario, nei batch contenenti microflora dell'intestino tenue, le antocianine residue erano rispettivamente il 70±6 e 11±7 % dopo 6 e 24 ore. Va sottolineato che dopo 24 ore di incubazione erano presenti solo la petunidina-glucoside (42±4 %) e la peonidina-arabinoside (38±2 %). Come previsto la microflora del colon ha mostrato la più alta attività catabolica verso le antocianine. Infatti, le antocianine residue erano il 14±4 % dopo 6 ore di incubazione in condizioni anaerobiche, ed erano non rilevabili dopo 24 h. Anche in queste condizioni sperimentali la peonidina-arabinoside (20±3 %) e la petunidina-glucoside (18 ±4 %) sono risultate le più stabili. Risultati simili sono stati ottenuti incubando

soluzioni contenenti mirtillo selvatico. Infatti, le antocianine erano degradate principalmente nei batch contenenti microflora del colon e in questi, rispettivamente dopo 3 e 6 h, le quantità residue erano il 33±4 e il 18±7 %. Le antocianine non sono state rilevate dopo 24 h di incubazione. Anche nel nostro studio le antocianine più stabili erano quelle esterificate con acido acetico, confermando così l'effetto protettivo dei gruppi alchilici. Va sottolineato che, dopo sole 6 ore di incubazione, cianidina-galattoside, malvidina-galattoside e delfinidina-galattoside erano completamente degradate e anche le percentuali dei corrispondenti coniugati con glucosio erano bassi (3-7 %). A parità di zucchero legato, petunidina e peonidina sono risultate le antocianine più stabili. Durante le incubazioni con microflora di ratto le antocianine sono state catabolizzate a derivati dell'acido benzoico, come l'acido vanillico, protocatecuico, siringico, etc. Questi metaboliti derivano dal catabolismo dell'anello B delle antocianine e perciò ogni antocianina produce uno caratteristico derivato dell'acido benzoico. Ad esempio, l'acido protocatecuico (3,4-diidrossi-benzoico) deriva dalla cianidina-glicoside, mentre l'acido siringico e vanillico rispettivamente dalla malvidina-glicoside e peonidina-glicoside. Questi metaboliti erano ulteriormente degradati dalla microflora con conseguente formazione di acido benzoico e nel caso dell'acido siringico la catabolizzazione era molto lenta. Risultati simili sono stati ottenuti incubando le antocianine con microflora fecale umana.

In conlusione, la degradazione delle antocianine ai corrispondenti acidi fenolici sembra essere una via generale per tali sostanze e il colon può essere quindi considerato come un sito attivo del metabolismo in cui i batteri contribuiscono agli effetti salutari dei composti fenolici. Sebbene la degradazione ad acidi fenolici sia una via metabolica importante, non spiega da sola la totale scomparsa delle antocianine durante l'incubazione con la microflora. Infatti, la quantità di acidi fenolici formata, calcolato sulla base dei nostri studi di fermentazione *in vitro*, giustifica fino a un massimo del 40 % del substrato iniziale. Questo dato suggerisce quindi la probabile presenza di vie di degradazione delle antocianine non ancora identificate.

0. PREFACE

As the most abundant antioxidants in our diets, polyphenols received increasing interest from consumers and food manufacturers both for their abundance in diets and biological activities. Among polyphenols, anthocyanins are important because of their higher consumption, which was estimated to be more than 100 mg/day. They are very widespread in fruits, vegetables and processed foods or beverages like juices and wines. The growing interest in anthocyanins was caused by the recognition of their potential health benefits. Epidemiological studies have suggested associations between the consumption of anthocyanin-rich wines and the prevention of coronary heart disease, which was known as the "French Paradox". Afterwards, a vast number of studies have been carried out on the potential benefits of anthocyanins on human health. To date, the potential health benefits of anthocyanins include radical scavenging, inhibition of lipoprotein oxidation and platelet aggregation, anti-inflammatory activity, reduction of capillary permeability and fragility, prevention of obesity, protecting on liver ischemia, anti-neoplastic and antitumor activity, improvement of eye vision, control of diabetes, and others. All the effects listed above are more or less related to the antioxidant mechanisms. However, there are two critical issues that may weaken the value of anthocyanins. First, although anthocyanins can have antioxidant effects in cell culture and other *in vitro* systems at relatively high concentrations, it is not clear whether concentrations can be reached *in vivo* at the tissue level to produce antioxidant effects. Numerous studies have suggested the low bioavailability of anthocyanins as indicated by the very low recovery in the plasma and urine after ingestion. Second, anthocyanins may not have sufficient stability to survive the physiological conditions during absorption and distribution. In any cell or tissue culture study using anthocyanins, one must be aware that at neutral pH, the anthocyanins may degrade. In addition, in the gastrointestinal tract and body tissues, enzymes such as β-glucosidase may also accelerate the degradation of anthocyanins. It is not clear whether anthocyanins remain intact in tissues long enough to act as antioxidants. Moreover, because anthocyanins are labile compound, they could be susceptible to deterioration during processing and storage. Accordingly, our study was designed to contribute information for answering the above uncertainties. Thus, degradation kinetics of single anthocyanins contained in freeze-dried wild blueberry powder stored at different temperatures and total antioxidant activity were determined. Than, anthocyanins in the urine, plasma, liver, brain, and feces of rats fed anthocyanin wild blueberry powder as well as control diet were analyzed to evaluate the their absorption, metabolism, and excretion *in vivo*. Wild blueberry was chosen as the additive in diet because was a good sources of antioxidants, yet contained a wide variety of individual anthocyanins, which might provide valuable information on the difference of individual anthocyanins. Unlike many other animal studies, in which relatively high intakes (up to 400 mg/kg BW) of anthocyanins were used to observe health-related responses in a short time, relatively low dose diet and long term feeding were employed in our study to simulate the normal intake of anthocyanins by humans. A focus of our study is the comparison of individual anthocyanin metabolim. It's not surprising that the chemical structure of anthocyanins will affect their biological properties. The number of hydroxyl groups, type of sugar moieties, as well as the acylated groups obviously can influence the polarity, size, and spatial conformations of individual compounds, and consequently have a certain impact on the bioavailability. Studies on the bioavailability of anthocyanins have been widely carried out, and differences among individual anthocyanins have been noticed but generally the studies didn't focus on the comparison of individual anthocyanins. Thus available information in this field is scarce. Our objective was to provide information for the screening of more bioavailable anthocyanins in foods and their catabolism after intake.

1. STATE OF THE ART

1.1 Blueberries

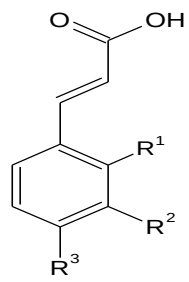
Since 2000 a series of international symposia on berry health benefits was began, especially to discuss the progress in research on consumption and nutritional health effects, which are highlighting the beneficial effects of these berries and increasing their popularity especially in North America (Seeram et al. 2008).The popularity of blueberry as a fruit and awareness of its beneficial effects has led to an expansion of the cultivated blueberry industry worldwide. Blueberry and derivative products have a wide range of uses. Blueberries are consumed not only as fresh fruits but also as frozen fruits, in bakery goods, fruit filling, in dried form in muffin mix, or in canned or preserved form.

Processed products of blueberries are widely popular, especially jam, syrups, fruit juices and beverages, and concentrates (Eck et al. 1988). Blueberry anthocyanins are also used as a natural food colorant (Espin et al. 2000) in some parts of the world (Bridle and Timberlake 1997), and extract can be used as a potential prebiotic (Molan et al. 2009). The popularity of blueberry with yogurt, and as a part of beverages with other berries such as cranberries, is expected to further increase the demand in the future. To meet the increasing demand, breeding experiments and genetic modifications have been considered to obtain higher yields and other desired characteristics in blueberries. However, blueberry is a seasonal crop for which processing and storage are important steps to maintain the availability of blueberry benefits year-round. During these processing steps there is a high possibility of loss of anthocyanins. Processing studies are taking place to minimize the nutrient loss by optimizing and examining the working parameters of the different processing steps (Yang and Atallah 1985). In these optimization studies, chemical analysis is an important part consisting of preparation of the sample, extraction and isolation, purification, and finally analysis. This can be done for identification of the compounds at different processing steps, quantification to detect the extent of loss, extraction from the by-products of different processing industries to recover the compounds (Lee and Wrolstad 2004) and adding them back as an additive into the final processed products. Analytical determinations are also required in the study of the distribution of the anthocyanins in different parts of the plant, which assist scientists working in the field of breeding in improving the genetic properties of blueberries. Analytical studies also help to better understand and modify the pathways of production of anthocyanins in a plant system, and to study the effects of various cultivation factors and their optimization. Analysis of different types of anthocyanins present in blueberries with their development pathways, their relative distribution in different parts of the plant, and variation according to the different seasons and growing conditions will be important for the continued development of the blueberry industry. These topics have been recently revised by Routray and Orsat 2011.

Blueberries are classified under the family “Ericaceae,” subfamily “Vacciniaceae,” genus “*Vaccinium*,” and subgenus “Cyanococcus” (Gough et al. 1994). The Ericaceae family comprises a large group of plants which are mainly woody shrubs that grow on acidic soils. The *Vaccinium* genus includes many popular berries consumed around the world including blueberries, huckleberries, cranberries, lingonberries, and bilberries. *Vaccinium* is speculated to be derived from the Latin word “vacca” meaning cow, because wild lingonberry otherwise known as cowberry is abundant in Sweden, the birth place of Linnaeus (Trehane et al. 2004). The plants in the genus

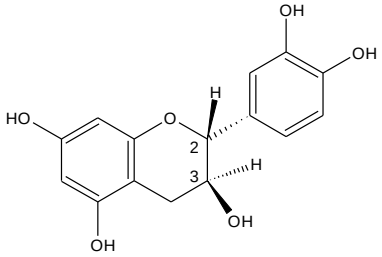
Vaccinium are dated back to the Cretaceous period, more than 100 million years ago, when they are believed to have developed and differentiated. After the Pleistocene glaciations, the tropical forms evolved into the temperate forms, which are now found predominantly in eastern North America. Plants under subgenus *Cyanococcus* expanded into the areas cleared after the ice sheets melted, where they hybridized and spread in the wild (Gough et al. 1994). The plants expanded further by dissemination of seeds by wild animals in their droppings and by spreading through rhizomes or underground runners. In today's world, blueberries are cultivated in North America (Canada and US.), China (Wang BC et al. 2010), Europe, and some countries of the southern hemisphere, such as Chile, Argentina, Uruguay, South Africa, New Zealand, and Australia (Lohachoompol et al. 2008). There are different kinds of blueberries and each has many local names. Some of them are wild-growing lowbush blueberries (*Vaccinium augustifolium*) and cultivated highbush blueberries. Northern highbush blueberry (*Vaccinium corymbosum*) is quite well known, while the rabbiteye blueberry (*Vaccinium virgatum*, also known as *Vaccinium ashei*) also falls under the category of highbush variety. In many other countries of the southern hemisphere such as Australia, the southern highbush blueberry is popular, which is a hybrid of the northern highbush blueberry and the rabbiteye blueberry. In European countries they have their own version of blueberries, known as bilberries (*Vaccinium myrtillus* L.) which belong to same genus and are similar to North American lowbush blueberries. The work reported in this thesis was focused on North American lowbush blueberries. Lowbush blueberries are native to Eastern Canada and the Northeast United States, where wild stands are commercially managed and harvested. Lowbush fruit is small compared to highbush or rabbiteye blueberries. Since stands of lowbush blueberries are made up of numerous wild clones, the commercial lowbush product is more heterogeneous than commercial highbush or rabbiteye blueberries. *Vaccinium angustifolium* is a low spreading deciduous shrub growing to 60 cm tall, though usually 35 cm tall or less. The leaves are glossy blue-green in summer, turning purple in the fall. The leaf shape is broad to elliptical. Buds are brownish red in stem axils. The flowers are white, bell-shaped, 5 mm long. The fruit is a small sweet dark blue to black berry. This plant grows best in wooded or open areas with well-drained acidic soils. In some areas it produces natural blueberry barrens, where it is practically the only species covering large areas. The *Vaccinium angustifolium* plant is fire-tolerant and its numbers often increase in an area following a forest fire. Traditionally, blueberry growers burn their fields every few years to get rid of shrubs and fertilize the soil. The state of Maine produces 25% of all lowbush blueberries in North America with 24.291 hectares under cultivation. Atlantic Canada contributes approximately half of the total North American wild lowbush annual production of 68.000 t. The pH of a blueberry fruit has been reported to range from 2.8 to 3.5, by Sapers et al. 1984, in the 11 cultivars examined. They reported titratable acidity to vary from 0.4 to 1.3% as citric acid. Sugars in blueberries have been found to be chiefly (in decreasing order) fructose, glucose, and sucrose (Kalt and McDonald, 1996; Eck et al. 1988). The main organic acids found in blueberries are citric, malic, quinic, chlorogenic, acetic, succinic, and shikimic acid; with citric acid being the most prevalent (Kalt and McDonald, 1996; Ehlenfeldt et al. 1994). The mineral and vitamin content of lowbush blueberries was reported by Bushway et al. 1983 and the total N and amino acid content was examined by Goueli et al. 1976. Blueberries contain also polyphenols, such as anthocyanins, flavonols, flavanols and alkyl-acids derivatives. These compounds have several functions within plants, such as protection from UV light, pigmentation, anti-fungal properties, attraction of pollinators and seed dispersers, and nodule production (Strack et al. 1994; Koes et al. 1994; Gould and Lee 2002). The appearance and flavor of berries, and berry products, are

influenced by phenolics. Structures of the phenolics found in blueberries are shown in figure 1.1. Phenolic acids such as benzoic and cinnamic acid derivatives are found in blueberries. The benzoic acid derivatives were vanillic, syringic, gallic, protocatechuic, m-hydroxybenzoic, phydroxybenzoic, and ellagic acid (Azar et al. 1987; Sellappan et al. 2002; Häkkinen et al. 1999; Amakura et al. 2000), while cinnamic acid derivatives were chlorogenic, caffeic, ferulic, p-coumaric, o-coumaric, and m-coumaric acid (Häkkinen et al. 1999; Azar et al. 1987; Sellappan et al. 2002). Chlorogenic acid was the major phenolic present in the fruit (Zheng and Wang 2003). Phenolic acids are rarely present as free acids, but rather are esterified with other phenolics, acids, or sugars (Herrmann 1989). Catechin, epicatechin and procyanidins (degree of polymerization found to range from 20 to 114) were found in lowbush blueberries (Gu et al. 2002) along with small amounts of quercetin-, kampferol-, and myricetin-glycosides (Zheng and Wang 2003).

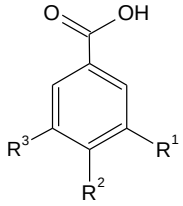


Cinnamic acids

Caffeic: R_2=R_3=OH, R_1=H
Ferulic: R_2=OCH_3, R_3=OH, R_1=H
p-Coumaric: R_3=OH, R_2=R_1=H
o-Coumaric: R_1=OH, R_2=R_3=H
m-Coumaric: R_2=OH, R_1=R_3=H

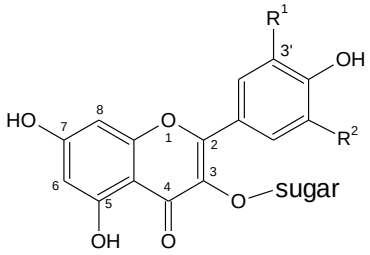


Catechin (2R, 3S)
Epicatechin (2R, 3R)



Benzoic acids

Gallic: R_1=R_2=R_3=OH
Protocatechuic: R_1=R_2=OH, R_3=H
Vanillic: R_1=OCH_3, R_2=OH, R_3=H
Syringic: R_1= R_3=OCH_3, R_2=OH



Flavonols
Quercetin: R_1=OH, R_2=H
Kaempferol: R_1=R_2=H
Myricetin: R_1=R_2=OH

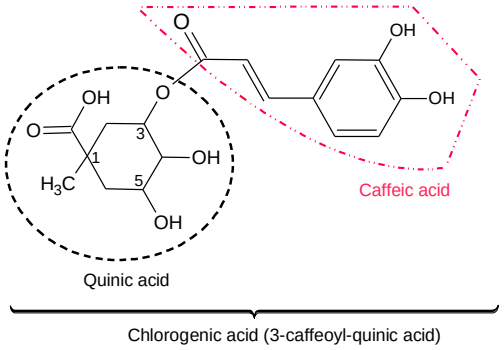


Figure 1.1. Structures of phenolic compounds reported to be present in lowbush blueberry.

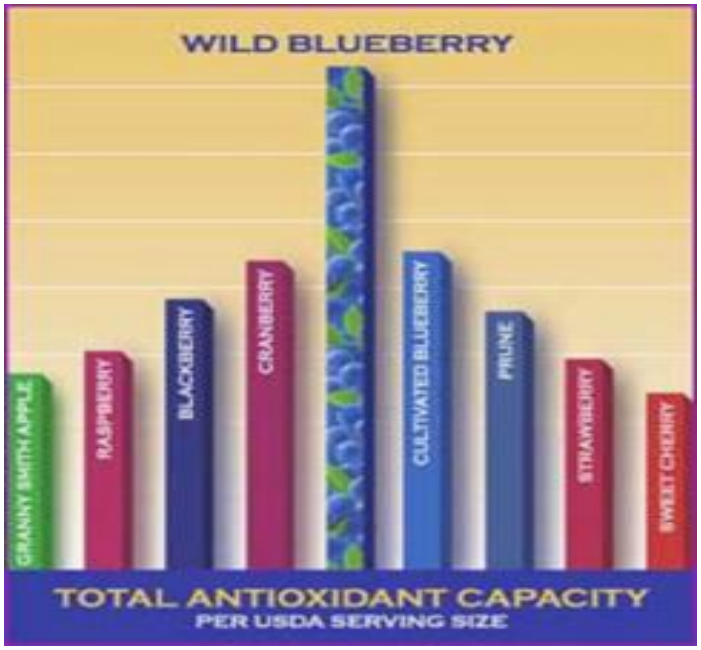


Figure 1.2. Total antioxidant activity of different berries and Apple.

The main class of polyphenols present in blueberry is formed by the anthocyanins. Fifteen different anthocyanins are commonly present in blueberry fruit: galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Mazza and Miniati, 1993; Kalt and Defour, 1997). The proportions of individual anthocyanins differ among cultivars, and stage of fruit maturation, and can also be influenced by environmental conditions (Mazza and Miniati, 1993; Ballington et al., 1987; Prior et al., 1998). Acylated anthocyanins (mainly acylated with acetic acid) have also been reported in lowbush blueberry (Prior et al., 2001).

Polyphenolics is the main group of substances responsible for potential health benefits in humans, anthocyanins in particular. These anthocyanins help to protect the body against oxidative stress and have in-vitro bioactivity relevant to several health issues, including cardio-vascular, liver disease, vision and eye health, brain aging, inflammation, etc. Wu et al. (2004), supported by USDA, showed that a one-cup serving of wild blueberries had more antioxidant capacity than a serving of cranberries, strawberries, raspberries, apples and even cultivated blueberries (Fig. 1.2). Moreover, wild blueberries have one of the highest antioxidant capacities due to their higher polyphenolics (anthocyanins) concentration. The same authors also concluded that on a per serving basis, wild blueberries have a higher antioxidant capacity, express as an ORAC value (Oxygen Radical Absorbance Capacity), than other fruit and berries (Fig. 1.3).

Anthocyanins are the natural colorants in blueberries, and gives typical deep purple color to blueberry. But the blueberry skin and "outer layer" of the pulp, contain a much higher amount of anthocyanins than the pulp of the blueberry (Gao and Mazza, 1994). There is up to a 320-fold difference in the anthocyanin concentration in blueberry skins (622 mg/100g) compared to blueberry pulp (1.9 mg/100g) in the species *Vaccinium angustifolium and Vaccinium corymbosum (Riihinen et al. 2008)*. Given the higher skin to pulp ratio of the smaller wild blueberry vs. the large highbush or cultivated blueberry, the wild blueberry has more anthocyanins by weight. Thus on an ORAC scale wild blueberries score higher than highbush or cultivated blueberries (Figure 1.4).

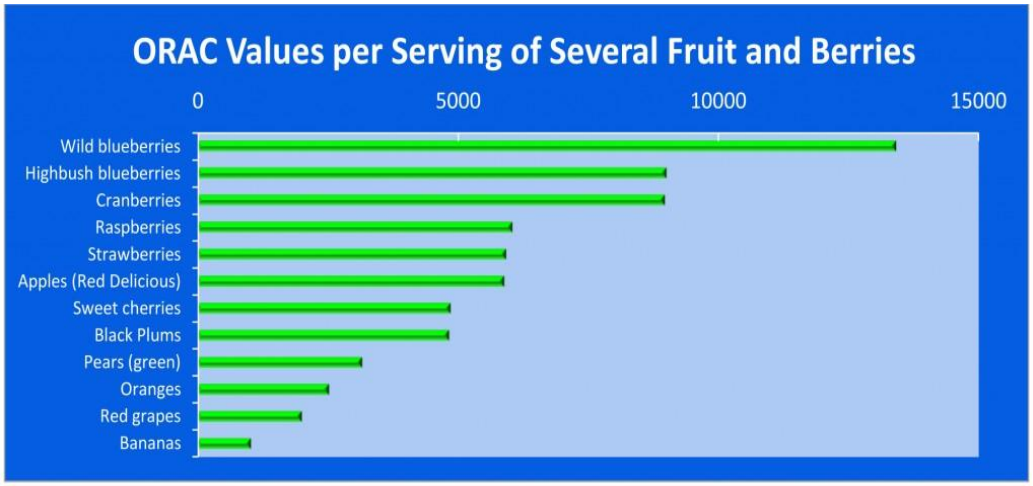


Figure 1.3. ORAC values of different berries and fruits.
Serving size according to the USDA National Nutrient Database for Standard Reference (www.ars.usda.gov/Services): 1 cup for Blueberries, Cherries, Raspberries, Strawberries, Grapes, and Cranberries; 1 fruit for Apples, Pears, Plums, Bananas, and Oranges.

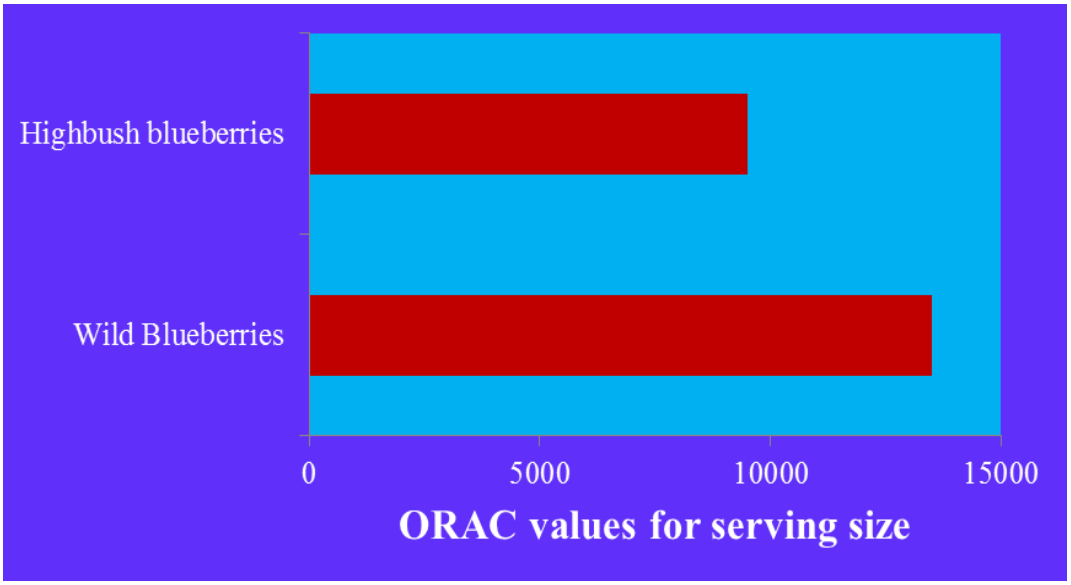


Figure 1.4. ORAC values of wild and highbush blueberries.
Serving size according to the USDA (www.ars.usda.gov/Services): 1 cup.

As mentioned in different reports and considering end uses of different antioxidant components, standardization can be done using different methos, such as the Oxygen Radical Absorbance Capacity (ORAC) (based on the hydrogen atom transfer mechanism), the Ferric Reducing

Antioxidant Power (FRAP), the Trolox Equivalent Antioxidant Capacity (TEAC), Total Radical-Trapping Antioxidant Parameter (TRAP) and the Folin-Ciocalteu (FC) (Joseph et al. 2007).

The TEAC and FRAP assays and FC method are electron transfer-based methods and give reducing capacity, whereas the FC method is generally expressed as total phenolic contents (Benzie and Strain 1996; Prior et al. 2005). The ORAC method has been found to be the most relevant to the human biological system and has been recommended to be superior to other similar methods, as "it uses an area-under-curve technique and combines inhibition time and inhibition degree of free radical action by an antioxidant into a single quantity" (Wang et al. 1997). According to Wang et al. 1997, based on ORAC activities, the anthocyanidin structure with the same hydroxylation pattern on the A and C rings, increased hydroxylation on B ring leads to the increase in the antioxidant capacity (3',4'-di-OH as compared to 3'-OH has higher ORAC capacity). Cyanidin was found to have higher ORAC capacity than malvidin and peonidin, but delphinidin, having 3 hydroxyl groups on the C ring, was an exception and was found to have lower capacity (Fig. 1.5). This was expected because of the probable decreasing effect of the 5-OH in the presence of 3',4'-OH (delphinidin) as compared to the presence of 3',4'-OH only (cyanidin). The effect of glucosylation varies with the type of aglycons and the type of sugar moiety (Wang et al. 1997). Also, pH has been reported to be a factor in the antioxidant capacity of anthocyanin extracts. Anthocyanin extracts with pH 1 were reported to have higher antioxidant capacity than extracts with pH 4 and 7 (Kalt et al. 2000). Fruit size has also been observed to be highly correlated with the anthocyanin content within *V. corymbosum L.*, but not in other Vaccinium species (Moyer et al. 2002). Smaller *V. corymbosum L.* berries contained more anthocyanins per unit volume. Anthocyanidins have been reported to have higher radical scavenging capacity than anthocyanins, where the radical scavenging capacity has been reported to decrease with an increase of number of sugar moieties (Wang and Stoner 2008). Other methods of analysis of antioxidant activity of blueberry extracts include tyrosine assay, galvinoxyl free radical quenching assay, lipid oxidation assay (Smith et al. 2000), and ferric reducing antioxidant power (Moyer et al. 2002). Antioxidative properties of blueberries include free radical scavenging, peroxide decomposition, singlet oxygen quenching, synergistic effects, and inhibition of enzymes (Wang et al. 2009).

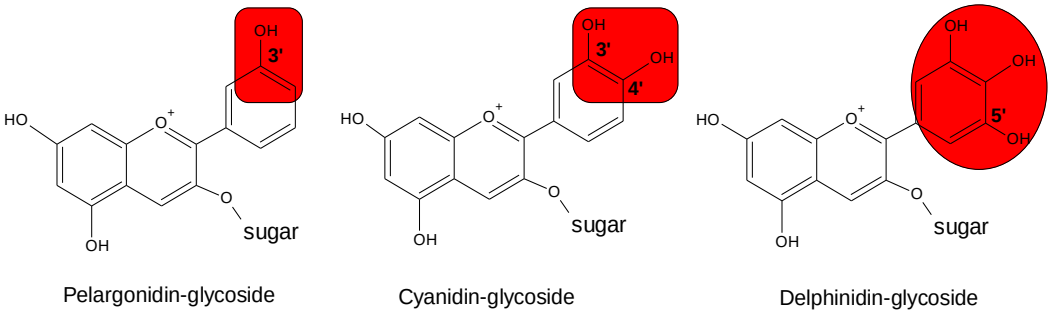


Figure 1.5. Chemical structures of pelargonidin-, cyanidin-, and delphinidin-glycoside.

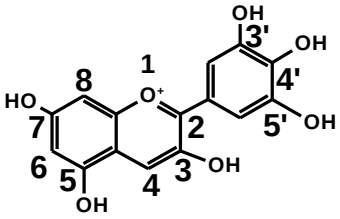
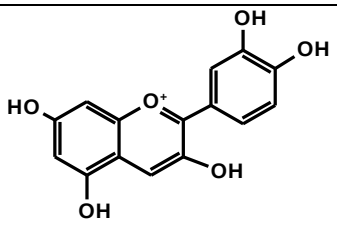
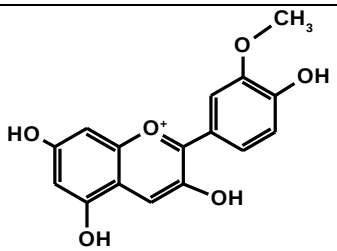
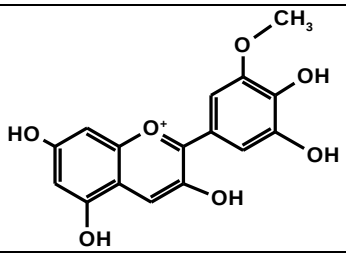
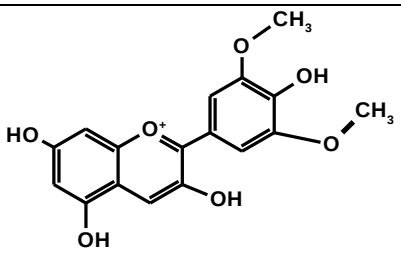
The cyanidin, due to the catechol moiety, shows the greatest ORAC capacity. Contrary to the expected, the introduction of an additional hydroxyl group on the ring B (delphinidin-) reduces the ORAC capacity. The pelargonidin, not present in blueberry, shows the lowest activity.

1.2. Blueberries anthocyanins

1.2.1. Chemical structure

Anthocyanins are glycosidic and acyl-glycosidic forms of anthocyanidins, which are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrilium (flavylium ions). The basic structure of anthocyanin is reported in figure 6 where it can be observed that all anthocyanins possess the characteristic $C_6C_3C_6$ skeletal structure, which is common for all flavonoids. The properties of the anthocyanins are dependent on the degree and pattern of hydroxylation and methoxylation of the skeletal structure. Anthocyanins are more stable than anthocyanidins and conversion of anthocyanins to anthocyanidins by ß-glycosidase leads to easier destruction of anthocyanidins by PPO (Buckow et al. 2010). Some anthocyanins are more vulnerable than others, hence knowledge regarding their composition in foods can help in the selection of proper storage and processing conditions, analytical studies for proper estimation, and extraction studies for maximum release, all with the least compound deterioration. The deterioration of anthocyanins depends on factors such as pH, enzymes, other supporting substrates, and temperature, the significance of which differs according to the structural properties of the anthocyanins, discrete from each other. The anthocyanins detected in blueberries are 3-glycosidic derivatives of cyanidin, delphinidin, malvidin, petunidin, and peonidin (Kader et al. 1996). The different anthocyanins also have different colors, which are affected by the pH. The structures of different anthocyanins present in blueberries are reported in table 1.1 with the colors associated with them. The most common derivatives determined are based on sugars such as glucose, galactose, and arabinose. In lowbush blueberry the anthocyanins were observed to be present in both nonacylated and acylated forms.
Acylation refers to the addition of acid (acyl) groups to the saccharides residues of anthocyanins. This kind of modification plays important roles in co-pigmentation of anthocyanins, which also functions in stabilization and has a blueing effect (Torskangerpoll and Andersen 2005). In blueberries the main acylated anthocyanins were the 6”-acetyl derivatives (Gao and Mazza 1995, Barnes et al. 2009).

Table 1.1 - Anthocyanins present in blueberries.

Aglycone	Structure	3-O-moiety	Color
Delphinidin		Galactose Glucose Arabinose 6”-acylated-glucose 6”-acylated-galactose	Blue-Red
Cyanidin		Galactose Glucose Arabinose 6”-acylated-glucose 6”-acylated-galactose	Orange-Red
Peonidin		Galactose Glucose Arabinose 6”-acylated-glucose 6”-acylated-galactose	Orange-Red
Petunidin		Galactose Glucose Arabinose 6”-acylated-glucose 6”-acylated-galactose	Blue-Red
Malvidin		Galactose Glucose Arabinose 6”-acylated-glucose 6”-acylated-galactose	Blue-Red

1.2.2. ACNs biosynthesis

Anthocyanins belong to the flavonoid group, which are polyphenolic compounds that come from the metabolic pathway in plants (Bloor and Abrahams 2002). As is shown in figure 1.6, anthocyanin comes later in the flavonoid synthetic pathway. The flavanones first undergo hydroxylation to produce dihydroflavonols. The next step catalyzed by DFR (dihydroflavonol 4-reductase) is quite

important for its determinant role in anthyocyanin types, thus, it should be of potential importance in the control of flower colors. The anthocyanins then undergo some secondary modifications, such as hydroxylation, glycosylation, methylation and acylation, to produce various anthocyanins (Winkel-Shirley 2001).

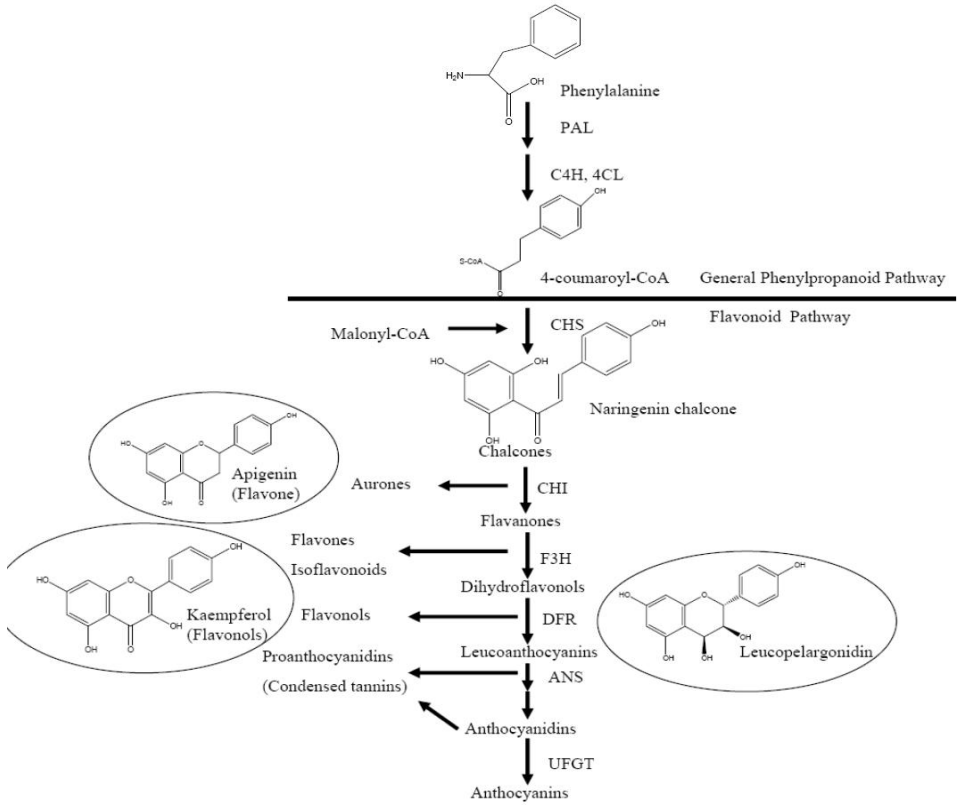


Figure 1.6. Biosynthesis pathway of anthocyanins.
Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP glucose-flavonoid 3-O-glucosyl transferase.

1.2.3. Daily Intake

Anthocyanins are widely ingested by humans, mainly deriving from fruits and red wines (Galvano et al. 2004). The worldwide annual consumption of anthocyanins has been estimated as 10,000 tons from black grapes alone (Clifford 2000). Of the various classes of flavonoids, the potential dietary intake of anthocyanins is perhaps the greatest, about 100 mg/day per person (Prior et al. 2004), which is much higher than the intake estimated for other flavonoids (23 mg/day per person), including quercetin, kaempferol, myricetin, apigenin, and luteolin (Cao and Prior 1999; Galvano et al. 2004). Depending on the nutritional habits, the daily intake of anthocyanins for individuals has been estimated to range from several milligrams to hundreds of milligrams per person. In the USA, average daily intakes were estimated at 215 mg per person during the summer and 180 mg per

person during the winter (Kühnau 1976). More recent studies suggest that the average anthocyanin consumption is in the order of 82 mg/day in Finland and 12.5 mg/day in the United States (Wu X. et al. 2006). Berry fruit are rich sources of dietary anthocyanins (Wada et al. 2002, Sirwoharn et al. 2004, Wu X. et al. 2004 Wu X et al. 2005) and can contribute to some tens to hundreds milligrams of anthocyanins in a single serving. Therefore, dietary choice can make a substantial impact on the amount of anthocyanin consumed. Many of the health benefits associated with berry fruit may be due to the high concentrations of anthocyanins that they contain. In this regard, regular consumers of red wine are likely to have significantly higher intake (Timberlake 1988). Moreover, the anthocyanin intake is increasing because extracts with high anthocyanin contents from fruits and vegetables like blueberry or elderberry are commercially available.

1.2.4. Major factors affecting ACNs stability

The color of anthocyanins is based on the fully conjugated 10-electron A-C ring π-system, with some contribution by the B ring as well. If that is disrupted, the color is lost as when anthocyanins are in higher pH medium or bleached by bisulfite (Waterhouse 2002). However, the structure in resonance is the cause of their instability, and consequently the groups attached to the structure (e.g. hydroxyl, methoxyl, glycosyl, and acyl) influence greatly the stability (Delgado-Vargas and Paredes-López 2003). Other factors like the pH, temperature, light, presence of other phenolic compounds, enzymes, metal ions, sugars, ascorbic acid, and oxygen etc. also have significant impact on the stability of anthocyanins (Shahidi and Naczk 2004b).

1.2.4.1. pH

In aqueous solution, anthocyanins undergo structural transformations that are pH-dependent (Figure 1.7), which had been studied and summarized by Brouillard et al. 1982. It has been found that four major anthocyanin forms exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone. At pH below 2, anthocyanins exist primarily in the form of the red flavylium cation. Flavylium ion in a slightly acidic or neutral aqueous solution results in the immediate formation of neutral and/or ionized quinonoidal bases (Takeoka and Dao 2002). The flavylium cation due of its positive charge is resistant to attack by electrophiles such as aldehyde and quinone (Clifford 2000). Rapid and almost complete hydration of the flavylium cation occurs almost exclusively at the C-2 position to give the colorless carbinol pseudobase at pH values ranging from 3 to 6. This can further equilibrate to an open form, the colorless chalcone pseudobase, at a slower rate. The generalized effect of pH on these equilibria for a non-acylated mono glucoside is illustrated in figure 1.8. Interestingly, under the same conditions the 3,5-diglycosides have a smaller concentration of the cationic form at any given pH value whereas acylated forms have noticeably more cation especially above pH 5 (Dangles et al. 1993). As pointed out by Brouillard in 1982, the hemiacetal form is most likely to be the target for attacking at slightly acidic pH. This was evidenced by later discovered anthocyanin-quinone adducts in the hemiacetal form detected by LC-MS.

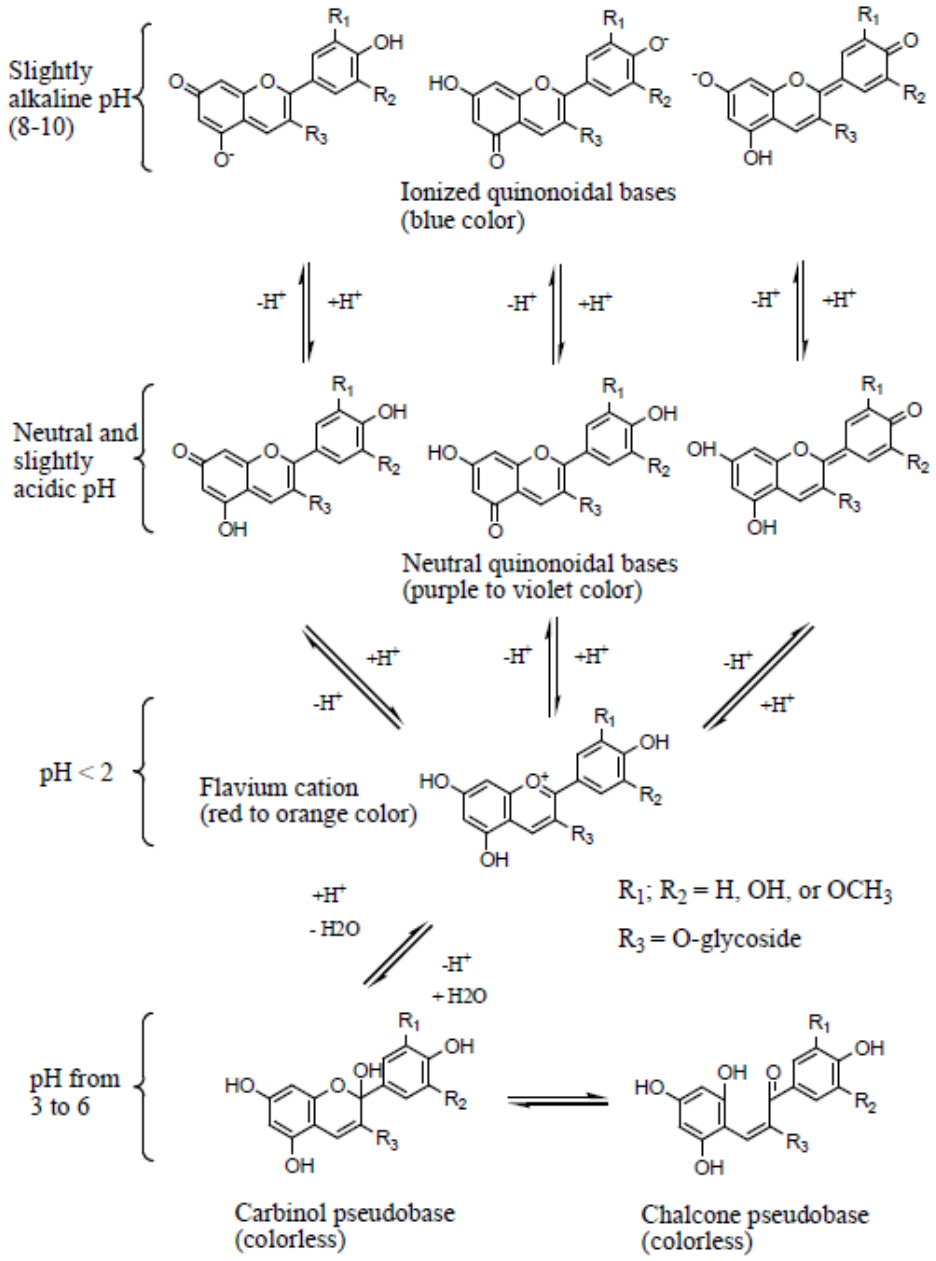


Figure 1.7. The various anthocyanin molecular structures generated under different pH conditions.

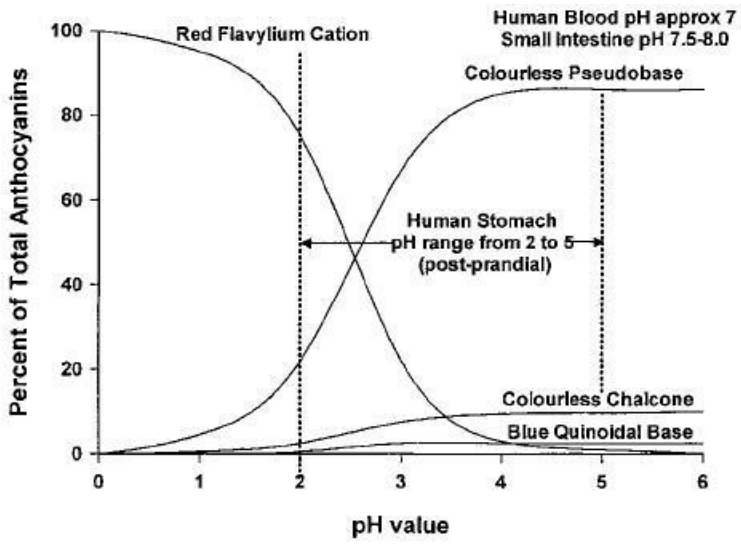


Figure 1.8. The pH effect on non-acylated glycosylated anthocyanins balance. (Brouillard 1982).

1.2.4.2. Acylation

It is well known that acylated anthocyanins, mostly with aromatic acyl groups, are more stable than the non-acylated ones (Phippen and Simon 1998, Mikanagi et al. 2000, Wu and Prior 2005). The acylated anthocyanins are more resistant to hydration (Fossen et al. 1998, Torskangerpoll and Andersen 2005), and the acyl moieties influence in inter-, intra-molecular co-pigmentation (Torskangerpoll and Andersen 2005). In addition to stabilization, the intermolecular co-pigmentation also has a blueing effect and increases the intensity of the color of anthocyanins. Intramolecular co-pigmentation was reported to stabilize more complex anthocyanins, for example, the ones poly-acylated with aromatic acids (Goto and Kondo 1991). Besides, anthocyanins also co-pigment with other aromatics like flavones and cinnamic acids (Fossen et al. 2007).

1.2.4.3. Copigmentation

Stability of anthocyanins can be enhanced though intramolecular or intermolecular copigmentation, which occurs by formation of weak bonds such as hydrogen bridges. Acylated anthocyanins containing two or more aromatic acyl groups may affect the color through a mechanism called intramolecular copigmentation (Mazza and Miniati 1993; Harborne and Williams 2001). Anthocyanins also interact with other flavonoids and related compounds to produce an increase in color intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic effect). Such a phenomenon is called intermolecular copigmentation, which can take place in acidic, neutral and even slightly alkaline aqueous solution (Mazza and Miniati 1993; Brouillard and Dangles 1994). The occurrence of copigmentation relies on at least two effects (Waterhouse 2002). First, the formation of the π-π complex causes changes in the spectral properties of the molecules in the flavylium form, resulting in hyperchromic shift and bathochromic shift (Giusti et al. 1999a). Secondly, the stabilization of the flavylium form by the π complex shifts the equilibrium to better favor the flavylium, thus boosting the proportion of anthocyanin molecules in the red-colored form (Figure 1.9).

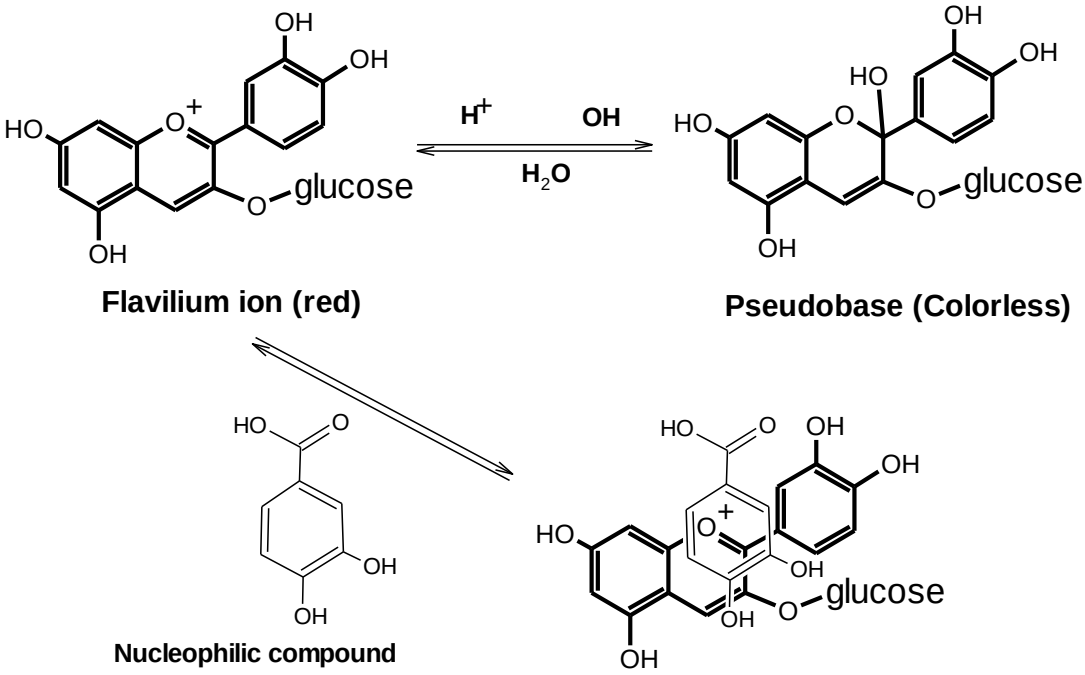


Fig. 1.9. Copigmentation of cyanidin-glucoside with an electron rich compound (Waterhouse 2002, adapted).

Several types of chemical groups were observed to induce anthocyanin copigmentation. Among those, flavonones, aurones, and flavonols show the most significant color modifications, including color intensity and lightness (Delgado-Vargas and Paredes-López 2003). Other phenomena contributing to the copigmentation involve the anthocyanin self-association (Clifford 2000) and metal complexation (Somaatmadja et al. 1964). Because of the self-association, anthocyanin absorbance in the solution doesn't follow Beer's law, especially at higher concentration. Copigmentation of anthocyanins usually results in improved stability, by protecting the colored flavylium cation from the nucleophilic attack of the water (Mazza and Miniati 1993; Delgado-Vargas and Paredes-López 2003). In terms of the intramolecular copigmentation, a sandwich type stacking of the aromatic residue of acyl groups with the pyrylium ring of the flavylium cation decreases hydration at C-2 and C-4 positions (Mazza and Miniati 1993). Moreover, intermolecular copigmentation also enhances the stability through intermolecular stacking (Clifford 2000).

1.2.4.4. Condensation

Unlike the copigmentation, the condensation occurs by formation of covalent bonds (Francis 1989; Fossen et al. 2000). Flavylium ions condense easily with amino acids, phloroglucinol, catechin, and other compounds to yield colorless flavones. This type of reaction may lead to condensation products, which can proceed to produce brown polymers. However, in some cases the condensation dramatically increases color density (Timberlake and Bridle 1977), indeed if anthocyanins interact with acetaldehyde the increase in color can be up to seven times.

1.2.4.5. Enzymatic system

Fresh blueberry fruits show an intense browning along with a color loss after crushing (Lee et al. 2002). Studies indicate that cytoplasmatic enzyme polyphenol oxidase (PPO) degrade polyphenolics present in the vacuole when the fruit is processed (Lee et al. 2002). PPO oxidizes polyphenolics to produce quinones (Jimènez et al. 1999). It should be emphasized that anthocyanins are not substrates for PPO. Indeed, they are the secondary quinones that induce the pigment degradation (Kader et al. 1997) producing brown pigments. In addition to the PPO, glycosidases are very important in anthocyanin stability because of a degradation effect producing unstable anthocyanidins (Skrede et al. 2000; Lee et al. 2002; Delgado-Vargas and Paredes-López 2003). Native peroxidase enzymes may be a cause of anthocyanin destruction in some berry commercial preparation (Skrede and Wrolstad 2002).

1.2.4.6. Other factors

Processing and storage under low temperature can improve the stability of anthocyanins (Delgado-Vargas and Paredes-López 2003). Temperature has been reported to induce a logarithmic destruction of pigment (Delgado-Vargas et al. 2000). When temperature is increased, the unstable formation of chalcone (Figure 1.7) is favored, and the chalcone is further degraded to brown products. Light is usually deleterious to anthocyanin components. Palamidis and Markakis (1975) reported that grape colorants in beverage had the half-life of 416 days in dark against 197 days in daylight at 20°C. Acylated anthocyanins are less affected by light, indeed slight difference on pigment stability were observed when exposed to light as compared to stored in dark (Giusti and Wrolstad 1996b). Oxygen and hydrogen peroxide can easily oxidize anthocyanins (Delgado-Vargas and Paredes-López 2003), but this mechanism is often accelerated by the presence of ascorbic acid. The mutual destruction between ascorbic acid and anthocyanin has been of great concern because of the ascorbic acid presence in fruit juice (Francis 1989). The interaction of ascorbic acid and oxygen may be mediated by H_2O_2 because one of the mechanisms of oxidation of ascorbic acid produces peroxide, and peroxide is known to bleach anthocyanins. Increased sugar level may affect the rate of anthocyanin destruction (Delgado-Vargas and Paredes-López 2003). Anthocyanins are very reactive toward metals, and they form stable complexes with tin, copper, and iron (Francis 1989) (Figure 1.10). For instance, cyanidin-3-glucoside forms a stable colored complex in the presence of aluminum ions at pH 5.5. Actually the addition of $AlCl_3$ is an analytical test for anthocyanidins which have catechol moiety (Cyanidin, petunidin and dephinidin) and those which do not (Peonidin and malvidin).

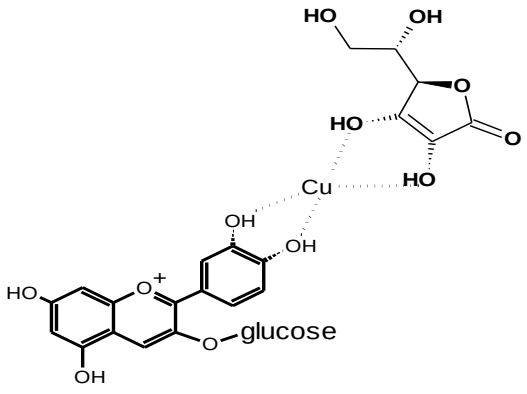


Figure 1.10. Suggested mechanism of anthocyanin-metal-ascorbic acid complexation.
(from Delgado-Vargas et al. 2000)

1.3. Factors conditioning the accumulation of anthocyanins

Cultivation practices are one of the main factors which affect the concentration level of anthocyanins in fruits and other vegetative parts when the plant is growing. The cultivation practices of blueberries required for healthy growth of the plant have been discussed in detail by many authors such as Trehane 2004, Gough 1994, and Eck 1988. They have discussed different cultivars, the important factors affecting blueberry cultivation, different types of diseases affecting the plant, proper site selection, soil requirements, climatic requirements, pest control, and other agronomic factors. Sites with slopes, which encourage drainage, and less windy areas are preferred for blueberry commercial cultivation. Well-drained and well-aerated soil with minimal required amount of water retention, especially in summer, and proper root anchorage are basic requirements in terms of soil characteristic, whereas optimum soil pH is expected between 4 and 5.2 to provide an ideal nutrient composition for proper growth and fruit-bearing (Trehane 2004).

Factors such as application of herbicides during spring, efficient irrigation management, and proper application of fertilizers have played a major role in increasing the production of blueberries in the last decades. Leaf analysis is generally employed to decide on the requirement of different minerals and the amount of fertilizers needed. The requirement of different essential elements as nutrients and micronutrients has been reported, but the relevance of the nutrient intake in terms of the rate of anthocyanin accumulation is not that clear, and there is no indication of the role of these nutrients in the biosynthesis of anthocyanins (Gough 1994; Trehane 2004). Similarly, there is no report of the correlation between optimized cultivation practices and the anthocyanin accumulation in the different parts of the plant. Organic farming, which is very much encouraged nowadays, has been recently encouraged to be used with blueberry cultivation (Drummond et al. 2009). Application of pine needles and organic manure has been suggested as a useful choice for providing blueberries the proper amount of nutrients required for growth and healthy fruit-bearing (Panicker et al. 2007). The amount of anthocyanins and total phenolics accumulated in different cultivars was either more or comparable in the case of organically grown as compared to conventionally grown rabbiteye blueberries (You et al. 2011). A similar study focused on the effect of different cultivation practices in the case of highbush blueberries and showed that the total anthocyanin content was significantly higher in organically cultivated blueberries (Wang SY et al. 2008).

This could be a potential area of research in the future and can contribute to the further development of commercial cultivation of blueberries. The season for blueberry cultivation spans May to September, in most parts of North America. In late July the fruit reaches peak ripeness with the entire fruit turning blue or black blue; and as the fruit turns from green to blue, the anthocyanin content of the fruit increases. The blue color of the fruits has been suggested as the best criteria of fruit maturity (Hall et al. 1972) and decision making regarding fruit-picking. Fruit-picking generally takes place during August and September in North America. The different phases of the blueberry growth cycle vary with the varieties of blueberry, and the time period of onset of these different phases also differs with temperature and variation of climatic conditions. All these factors together affect the total production which varies from year to year. The anthocyanins present in fruits and flowers, responsible for their color in many cases, act as an aid in pollination and seed dispersal (Harborne et al. 1975; Steyn et al. 2002). During fall season the lowering of temperature favors the production of anthocyanins in the senescing leaves. Anthocyanins in the leaves have been found to be a part of the defense mechanism against photoinhibition, along with other mechanisms in photorespiration and the cycle of xanthophylls (Hoch et al. 2001). Photoinhibition has been defined as “illumination of photosynthetic tissues in excess of the energy utilization potential of carbon reduction which can lead to a marked decrease in photosynthetic capacity” (Powles 1984). As suggested by many authors, the light screen hypothesis explains the function of foliar anthocyanins. Hoch et al. 2001 extended the light screen hypothesis by proposing that “autumnal anthocyanins protect senescing foliage from photoinhibitory irradiances, allowing the resorption of critical foliar nutrients to occur during a period of photosynthetic instability and deteriorating photoprotective

capacity." As observed in various plants (Nozzolillo et al. 1990) and also in the case of blueberries (Gough 1994; Trehane 2004), the rate of accumulation of anthocyanin varies with seasonal changes and their effects on growth patterns, and also with developmental patterns of different species, varieties, and plant cultivars (Steyn et al. 2002). Accumulation of anthocyanins in the vegetative parts of the plants has been discussed in detail by some authors along with the factors affecting this accumulation (Hoch et al. 2001; Steyn et al. 2002). In response to many stress factors, other than photoinhibition, such as decreased temperature, nutrient deficiency, and wounding or pathogen attack, the synthesis and increased accumulation of anthocyanin in plants has been observed which makes it a part of its built-in defense mechanism.

The increase in the level of anthocyanins in the vegetative part of the plant also subsequently affects the level of accumulation in the fruit. Breeding experiments have either focused on the increase of production of blueberries, sometimes by optimization of pollen load (Dogterom et al. 2000) or on the increase of the total amount of anthocyanins in fruits; with both approaches there is a good likelihood of a higher rate of consumption of phytochemicals (anthocyanins) by consumers. As color is correlated to the anthocyanins present in fruits like blueberries, during some studies with different *Vaccinium* species; albino fruits have been linked with a single recessive gene not likely to be preferred (Hancock et al. 2008). Total antioxidant capacity and total phenolic content, which includes anthocyanin content observed in blueberry progenies, have been found to be moderately heritable (Connor et al. 2002b; Scalzo et al. 2005) or in other cases from moderately to highly heritable (Scalzo et al. 2008b). Also, the variation in anthocyanin content with different species as well as cultivars is a well-known and established fact (Sapers et al. 1984; Kalt et al. 2001). During a study by Connor et al. 2002c, significant differences were found in anthocyanin content and antioxidant activity between the same cultivars grown in different locations and different cultivars grown in the same location, and also there was a difference in terms of year of harvest between the same cultivars grown in the same location, proving genotypic and environmental effects; and the effects of genotype have been reported to be stronger than environmental effects. Even though the direct evidence of increase of anthocyanins with progeny is not experimentally proven in the case of blueberry breeding studies, a significant increase is possible based on the moderate-to-high heritability, which might be evident in terms of increased total antioxidant capacity (Scalzo et al. 2005, 2008a) and careful biotechnological approach, which includes the tools of micropropagation, genetic engineering, and genetic fingerprinting (Serres et al. 1996). To constantly improve the breeding programs, oriented toward improvement of the product (blueberries rich in anthocyanins), preservation of germplasm with proper selection, evaluation, and dissemination would be important steps (Debnath 2009).

To establish a strong genetic base and ensure future use of the existing genetic resources, a thorough study of the genetic diversity could be helpful. Genes involved in anthocyanin biosynthesis have been identified and their activities have been traced during different developmental stages in the case of bilberries (Jaakola et al. 2002). Similar research applied to blueberries could help in narrowing the research focused on breeding programs specifically oriented toward the increase of anthocyanin content. Inter simple sequence repeat markers (a polymerase chain reaction generally used for amplification of a particular DNA sequence), based on molecular marker assay of the genomic sequence lying between adjacent repeating microsatellites, which are repeating sequences of base pairs of DNA (UN-FAO 2002) for genetic diversity studies, have been developed for lowbush blueberry (*Vaccinium augustifolium* Ait.), and they have been found helpful in differentiating among 43 lowbush blueberry clones (Debnath 2009). This can also be applied in the case of other blueberry varieties and cultivars which could help to build and preserve a gene pool from which parents with desirable characteristics such as high anthocyanin content could be selected. Study of the different agronomic factors affecting the cultivation and influencing the level of accumulation of anthocyanins in fruits is also helpful to provide a better product. During some studies, the combined effect of genotype and harvest year or time of cultivation was found to have a significant effect on total phenolic content, which includes anthocyanin. Hence, the study of the response of germplasms over several generations was found to play an important part in the

variation in phenolic content and the entire chemical composition of blueberry fruits (Scalzo et al. 2008b). The variation of anthocyanin content in lowbush blueberries between two growing seasons was found to be up to 30% (Kalt et al. 1999b) and in highbush blueberries and interspecific hybrid cultivars up to 35% to 40% (Connor et al. 2002a). Above-mentioned factors should be carefully considered while selecting blueberry samples for any type of analysis, characterization, and analytical studies especially *in vitro*, *in vivo*, or *ex vivo* studies. Cultivar selection and agronomic conditions influence the uniformity or variation in anthocyanins in the sample and their corresponding effects.

1.4. Health benefits of blueberry anthocyanins

Some of the beneficial effects of blueberry consumption are associated with anthocyanins ingestion and regarding this topic several published health reviews are available (Wang H. et al. 1997; de Pascual-Teresa and Sanchez-Ballesta 2008; Wang LS and Stoner 2008). The most commonly reported health effect of anthocyanins is their antioxidant activity. The incomplete reduction of oxygen during mitochondrial electron transport leads to the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (°OH) (Kalt 2005). The human body produces another free radical species, such as nitric oxide (NO) and peroxynitrite (ONOO), which are together known as reactive oxygen species (ROS) (Castro and Freeman 2001). Free radicals can also enter the body through UV radiation, air pollution, consumption of charred or rancid food, and cigarette smoking (Halliwell and Gutteridge 1999). Excessive ROS production lead to the imbalance of oxidants and antioxidants in the body carrying to oxidative stress, which could produce damage to tissues (Halliwell 1992), DNA, lipids, and proteins (Wang H. et al. 1996), and cause disorders such as cancer, diabetes, cardiovascular neurodegenerative diseases (Halliwell 1992; Castro and Freeman 2001). The body has its own defense mechanisms against ROS, which include enzymes (e.g. glutathione peroxidase), vitamins (e.g. E and C), hydrophilic and lipophilic radical scavengers. Anthocyanins could be included in the hydrophilic radical scavenger's category, which basically helps in the retardation of chain oxidation reactions of lipids (Castro and Freeman 2001). In addition to the antioxidant effect, many other health effects are associated with blueberries. Inhibition of proteosome activity of anthocyanins is reported to be contributing to the beneficial effects other than the antioxidant effect (Dreiseitel et al. 2008). Many of the beneficial effects which are associated with blueberries include anticancer, cardioprotective, and other properties which are either confirmed by assessing the bioactivity (Smith et al. 2000) through *in vitro*, *in vivo*, and *ex vivo* (Kay and Holub 2002) analyses. The different effects of blueberry or blueberry anthocyanins confirmed in human or animal studies are summarized in table 1.2. Many of the analyses are related to blueberry-enriched diets (Ahmet et al. 2009), blueberry extracts as a whole (Paredes-Lopez et al. 2010) as components specifically present in blueberries. Anthocyanins prevent cholesterol-induced atherosclerosis in rabbits (Kadar et al. 1979), reduce both the oxidative and inflammatory damage to microvascular endothelium (Youdim et al. 2002) that the possibility of occurrence of atherosclerosis (Kraft et al. 2005). Preventing atherosclerosis can lead to the prevention of cardiovascular dysfunction. Blueberry seems helpful against ischemic damage of heart (Ahmet et al. 2009). This is related to the antioxidant activity of anthocyanins at the cellular level. The blueberry diet increases the mitochondrial permeability transition reactive oxygen species threshold, which leads to an increase in cardio-myocytes survival (Ahmet and others 2009). Blueberry-rich diets also affected the biomechanical properties of the aorta in rats (Norton et al. 2005). Some other factors related to the prevention of cardiovascular disease include inhibition of vascular endothelial growth factor, protection of endothelial cells pro-inflammatory signaling (de Pascual-Teresa et al. 2010), and protection of membrane lipids from oxidation (Neto 2007). Regarding anticancer activity in animal and human, anthocyanins act according different mechanisms (Wang and Stoner 2008), which include phase II enzyme activation, anti-cell production, stimulation of apoptosis, anti-inflammatory effects, anti-angiogenesis production of fresh blood cells (angiogenesis), anti-invasiveness, and induction of differentiation (Wang LS and

Stoner 2008). *In vitro* analysis of the blueberry extract for different stages of carcinogenesis has also been reported (Bomser et al. 1996), which has been supported by *in vitro* analysis showing apoptosis of human cancer cells (Seeram et al. 2006).

Blueberry anthocyanins and their pyruvic acid adducts demonstrated anticancer potential in breast cancer cell lines during a study of the inhibition of cancer cell proliferation and cell anti-invasive and chemoinhibitor properties (Faria et al. 2010). Recently, the modulation of the PI3K/AKT/NFκB pathway has been explained as a reason of the antibreast cancer effects of blueberry extract (Adams et al. 2010). Anthocyanins were found to be effective against 2 colon cancer cell lines (HT-29 and Caco-2) in a study by Yi et al. 2005. Furthermore, the anthocyanin fraction was potentially effective in DNA fragmentation (Yi et al. 2005) and caspase-3 activity (Srivastava et al. 2007), leading to apoptosis. The effect was also correlated to the concentration of extracts (Olsson et al. 2004) and anthocyanins (Srivastava et al. 2007). Similar observations were found for cancerous cells in other studies such as human prostate cancer cells (Matchett et al. 2005, 2006), colon cancer cells (Zhao et al. 2004; Prior et al. 2008), and cervical cancer cells (Wedge et al. 2001).

Blueberry extract at various concentrations inhibited *Helicobacter pylori* (Chatterjee et al. 2004), which has been identified to be the causative organism of diseases such as duodenum ulcer and gastric cancer. Moreover, it was found that blueberry inhibited other bacteria such as *Enterococcus faecalis* and *Escherichia coli* (Ofek et al. 1991), *Lactobacillus* (Puupponen et al. 2001), *Salmonella enteritidis*, *Listeria monocytogenes* (Park et al. 2011), and *Citrobacter freundii* (Burdulis et al. 2009). *In vitro* studies also showed that blueberry extract is effective against the protozoan parasites *Giardia duodenalis* and *Cryptosporidium parvum*, which are mainly responsible for diarrhea (Anthony et al. 2007). Anthocyanins have been reported to be helpful against neurodegenerative disorders also for their antioxidant properties (Prior and Wu 2006). Other antioxidants along with anthocyanins present in blueberries can also be helpful against neurodegenerative disorders (Ramassamy 2006). Analysis of blueberry supplementation fed to aged rats showed that the effect of flavonoids to the brain was based on extracellular signal-related kinase, cAMP response-element-binding protein, and brain-derived neurotrophic factor and could be correlated to the improvement of spatial-working memory tasks after blueberry consumption (Williams et al. 2008).

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The crude extract of blueberries also showed analgesic and anti-inflammatory effects (Torri et al. 2007). In another study conducted on macrophages, the crude extract of blueberries was found to be effective against nitric oxide production, which is associated with inflammatory and cardiovascular disorders (Wang and Mazza 2002). Blueberries were found to be effective on adipocyte physiology and gene expression for adipose tissue macrophages (DeFuria et al. 2009). In the case of high-fat-diet-fed mice, diet supplementation with blueberry powder decreased adipocyte death and the inflammatory disorders that would lead to whole body insulin resistance. Another study on blueberry anthocyanins reported that they could control the cholesterol and triglycerides level in serum in the case of high-fat-diet-fed mice, better than the whole berries (Prior et al. 2009).

Blueberry juice was found to be effective for prevention of the onset of obesity in obesogenic high-fat-diet-fed mice, but not as effective as blueberry anthocyanins mixed with drinking water (Prior et al. 2010).

Table 1.2. Some studies in humans and animal that show beneficial effects of blueberry or blueberry anthocyanins.

Tested substance	Subject	Conclusions	Reference
Bluberry	Pig	Anthocyanins get accumulated in several parts of body including eyes and brain	Kalt et al. 2008
Bluberry-enriched diet	Rat	Protection against ischemic damage and prospective to avoid development of post-myocardial infarction heart failure	Ahmet et al. 2009
Bluberry-rich diet	Rat	"Suppressing α1-adrenergic receptor agonist-mediated contraction" and effect on vascular smooth	Norton et al. 2005
Bluberry	Human	Increase of postprandial serum antioxidant content in human volunteers	Kay and Holub 2002
Bluberry in diet	Human	Diet-induced amplification of *ex vivo* serum antioxidant content	Mazza et al. 2002
Bluberry	Rat	Decrease in oxidative DNA damage in liver of rats	Dulebohn et al. 2008
Bluberry extract	Mice	Inhibition of growth and metastatic potential of breast cancer cells	Adams et al. 2010
Bluberry	Rat	Effective against dextran sulfate sodium-induced colitis	Osman et al. 2008
Bluberry in feed	Rat	Effect on "spatial working memory"	Williams et al. 2008
Bluberry in diet	Rat	Reversal of age-related decrease in brain's "heat shock protein 70 mediated" neuroprotection	Galli et al. 2006
Bluberry in diet	Mice	Reversal of decline of age-related neuronal activity and behavioral aging	Joseph et al. 1999
Bluberry	Mice	Reduction of adipocyte death and inflammatory disorders leading to decrease in whole body insulin resistance	DeFuria et al. 2009
Purified berry ACNs	Mice	Prevention of dyslipidemia and obesity development	Prior et al. 2009

To underline, in this case, the major activity of blueberry anthocyanins as compared to other blueberry components. Anthocyanins have also been reported as potentially helpful in the prevention of diabetes (Ghosh and Konishi 2007). Moreover, the hypoglycemic activity of anthocyanin-rich extract from lowbush blueberry was found to be higher than for a lowbush blueberry phenolic extract showing the higher effectiveness of blueberry anthocyanins in this particular case (Grace et al. 2009).

1.5. Absorption, distribution, metabolism and excretion of anthocyanins

The bioavailability of anthocyanins differs from other flavonoids such as the flavonols (e.g. quercetin glycosides) in a number of key aspects. First, the apparent bioavailability is consistently very low across all studies with often less than 0.1% of the ingested dose appearing in the urine. Secondly, absorption occurs quickly following consumption. The t_{max} in plasma is 15-60 min, and excretion is complete within 6-8 h. These observations have led to the suggestion that anthocyanins are absorbed from the stomach. Moreover, anthocyanin glycosides appear to be absorbed, distributed into the circulatory systems and excreted in the urine. The most pressing issue for the

bioactivity of anthocyanins is to confirm the apparent low bioavailability. This will continue to be difficult to achieve, since anthocyanins exist in a number of different molecular structures and there are a number of potential metabolites that can be generated both in vitro and in the GIT. One approach to help to address this bioavailability issue is the use of labelled anthocyanins to estimate bioavailability and subsequent transportation, accumulation into various tissue, and excretion.
Most, if not all, studies on anthocyanin bioavailability and absorption have used detection methods that are based on the measurement of anthocyanins as red flavylium cations by HPLC. From the preceding discussion, it is clear that the flavylium cation form of anthocyanin is unlikely to exist in vivo. Consequently, all current assessments have indirectly measured anthocyanin bioavailability by relying on the measurement of the flavylium cation when it is probably the hemiketal and chalcone forms of anthocyanins that are likely e present in vivo and participate in absorption and metabolism reactions. As presented in figure 1.11, anthocyanin structures that fail to regenerate the flavylium cation because of in vivo metabolism or modification will not be detected by analytical methods based on HPLC separation and detection at 530 nm. Much progress towards understanding the bioavailability and bioactive behavior of anthocyanins might be made if selective and sensitive methods for determining the alternative molecular structures of anthocyanins (quinonoidal bases, hemiketals, and chalcones) were available. Currently, such methods do not exist.

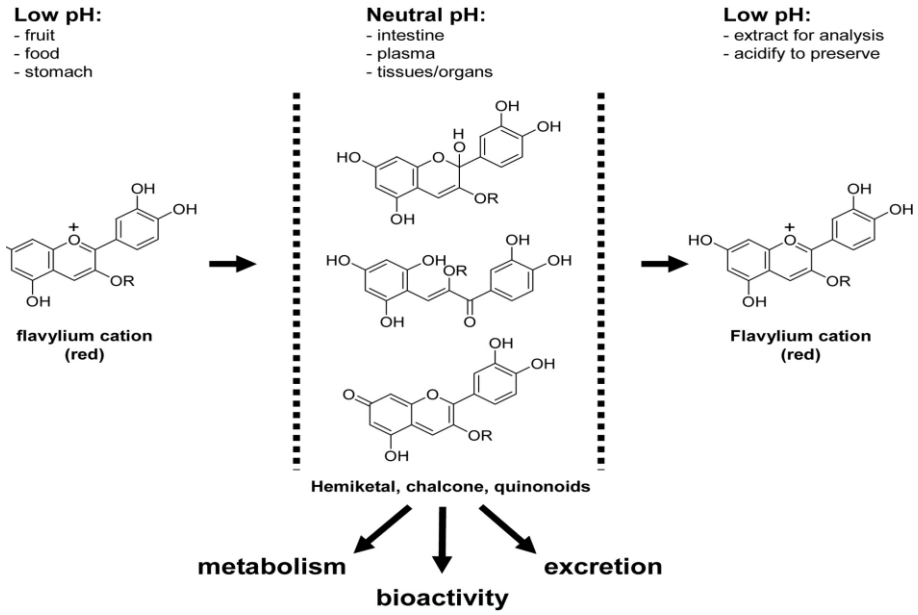

Figure 1.11. Schematic representing the different forms of anthocyanins during *in vivo* absorption. (from McGhie and Walton 2007).

1.5.1. Biovailability

To reveal the potential health effects of anthocyanins it is essential to understand their *in vivo* bioavailability and functions. Bioavailability studies involving anthocyanins were often performed with HPLC equipped with UV-Vis spectroscopy detector, and the detection mainly based on the colored flavylium cation form. In a study by McGhie et al. 2003 rats were fed with boysenberry extract, and their stomach was colored indicating the presence of the flavylium cation. On the

contrary, the small intestine seemed no colored, but after acidification it turned red indicating anthocyanins presence. Similarly, acidification of plasma, urine and liver tissue colorless anthocyanins were transformed to the colored flavylium cation form and detected by UV-Vis spectroscopy (McGhie et al., 2003; Tsuda et al., 1999; Miyazawa et al., 1999; Matsumoto et al., 2001; Cao et al., 2001; Felgines et al., 2002; Felgines et al., 2003; Cooney et al., 2004; Passamonti et al., 2005; Talavera et al., 2004). During the passage of anthocyanins through the gastrointestinal tract (GIT), they are exposed to different pH environments and might therefore exist at different forms (Fig. 1.11). The anthocyanin forms present in the different regions and tissues of the GIT, and eventually during absorption, are not well known (McGhie et al., 2003). It is possible that the flavylium cation exists only in the lumen of the stomach due to low pH, and the other forms are predominant lower down the GIT. McDougall et al. 2007 found that the anthocyanins were effectively stable under acidic gastric digestion conditions, but the total recovery after simulated pancreatic digestion was about 25% compared to around 100% recovery of phenol content. Acylated anthocyanins showed higher stability against pancreatic digestion than non-acylated forms, and anthocyanins with sinapic acid reduced the stability compared to the other hydroxycinnamic acids. They concluded that it is unlikely for the anthocyanins to reach the serum or survive long under serum conditions, and therefore they attributed the biological activities of anthocyanins could be carried out by their metabolites. Metabolites of anthocyanins such as methylated cyanidin-3-glucoside and their glucuronidated derivatives are found in urine, kidney and liver tissue (Tsuda et al. 1999; Wu X. et al. 2002; Talavera et al. 2004). Cyanidin-3-glucoside and its methylated derivatives appeared in the bile after about 25 minutes (Talavera et al. 2005). This supports the findings of Miyazawa et al. 1999, who reported a higher concentration of methylated cyanidin-3-glucoside in rat liver and a lower concentration in the plasma, indicating that these metabolites were excreted from the liver directly into the bile. In addition, sulfoconjugated cyanidin derivatives have been found in the urine (Felgins et al. 2005). Anthocyanidin sulfoconjugate formation requires hydrolysis of the anthocyanin to the aglycone followed by sulfoconjugation of the aglycone by sulfotransferases present in different tissues, including intestine and liver. The metabolic fate of anthocyanins may also differ according to their aglycone structure and the main metabolites of blackberry anthocyanins found in human urine were anthocyanidin monoglucuronides (Felgins et al. 2005). Anthocyanin concentration in plasma results from a balance between absorption and elimination (Passamonti et al. 2005). Both parameters are complex, because absorption may depend on gastrointestinal motility, blood flow, and the activity of membrane carriers. The removal rate of anthocyanins from the plasma depends on their uptake and metabolism in peripheral tissues, including excretion into bile and/or urine, and on the conversion between the different equilibrium forms of the anthocyanins. The presence of anthocyanins in the plasma could be related to their binding to proteins, which ensures their chemical stability. The variable levels of anthocyanins in the plasma may reflect individual differences with respect to the content of endogenous and exogenous competitors for protein-binding sites in the blood. The rate of anthocyanin breakdown in the plasma is also influenced by different active redox compounds present. The mean concentrations of anthocyanins in plasma are low but maybe adequate for antioxidant effect (Passamonti et al. 2005). The fact that cyanidin-3-glucosides and cyanidin-3,5-diglucosides are found in rats and human plasma, strongly confirms the ability of glycosides to cross the small intestine (Tsuda et al. 1999; Miyazawa et al. 1999). Matuschek et al. 2006 found that cyanidin-3-glucoside was mainly absorbed in the jejunum of the small intestine, which suggests involvement of an active transport mechanism. Tsuda et al. 1999 studied cyanidin-3-glucoside metabolism in the jejunal tissue of rats after direct stomach incubation, and they found in addition to the substrate, its aglycone and oxidation product such as 3,4-dihydroxybenzoic acid (protocatechuic acid). Cyanidin-3-glucoside was then rapidly detected in the plasma and its oxidation product was detected at concentrations eight times higher, while its aglycone was not found in the plasma. To date it is unclear whether anthocyanins can be transported by SGLT1 (Na-dependent glucose transporter) or they are hydrolyzed by the glycosidases. It could be that anthocyanins and anthocyanidins have the same transport system as quercetin-3-glucoside, but the fast degradation of

the unstable anthocyanidins prevents their detection. Other studies have demonstrated the possibility of anthocyanin absorption from the stomach (Passamonti et al. 2002, 2003). Bilitranslocase, an organic anion membrane carrier expressed in epithelial cells of the gastric mucosa, is suggested to be involved. Anthocyanins are rapidly absorbed following oral administration; the absorption through the gastric wall may provide an explanation (Matuschek et al. 2006). Interestingly Passamonti et al. 2005 found anthocyanins intact in rat brains just a few minutes after administration into the stomach. This is unexpected because of the presence of the blood-brain barrier which is thought to be impermeable to >98% of small, polar molecules occurring in the blood. The area of penetration has not been detected. Another study by Talavera et al. 2005 reports anthocyanins in rat brains after intake of anthocyanin rich diet, though with a different timing of the experiments. The probable metabolic process of anthocyanins has been described in detail by McGhie and Walton 2007, and discussion of the metabolic process of anthocyanins present in blueberries has also been reported by Wu X. et al. 2002. The number of human studies investigating the bioavailability of anthocyanins increased over the last 10 years and these are summarized in table 1.3.

Table 1.3. Human bioavailability studies (from McGhie and Walton, 2007).

Material	Anthocyanin dose[a] (total intake)	C_{max}[b]	t_{max}[c] (h)	Urinary excretion[d] (%)
Red wine (300 mL)	218 mg			5.10 (12 h)
Elderberry extract (25 g)	1.5 g	100 ng/mL	0.5	
Blackcurrant	236 mg	0.120 μmol/L	1.25–1.75	0.11 (8 h)
Elderberry juice (spray dried capsules)	180 mg	35 ng/mL	1	
Blackcurrant juice (200 mL)	153 mg			0.02-0.05 (5 h)
Red wine (500 mL)	68 mg Mv-3-glu[e]	0.0014 μmol/L	0.8	0.02 (6 h)
Dealcoholized red wine	56 mg Mv-3-glu[e]	0.0017 μmol/L	1.5	0.02 (6 h)
Red grape juice (500 mL)	117 mg Mv-3-glu[e]	0.0028 μmol/L	2.0	0.02 (6 h)
Elderberry (11 g)	1.9 g			0.003–0.012 (6 h)
Blueberry powder (100g)	1.2 g	0.029 μmol/L	4	
Elderberry extract (12 g)	720 mg	0.097 μmol/L	1.2	0.06 (24 h)
Elderberry extract (12 g)	720 mg			0.08 (4 h)
Blueberry (189 g)	690 mg			0.004 (6 h)
Red wine (400 mL)	180 mg	43 ng/mL	1.5	0.23 (7 h)
Red grape juice (400 mL)	284 mg	100 ng/mL	0.5	0.18 (7 h)
Blackcurrant juice	1.24 g	53 ng/mL	0.75	0.07 (4 h)
	0.72 g	16 ng/mL	0.75	0.05 (4 h)
	0.75 g	32 ng/mL	1.5	0.05 (4 h)
Blackcurrant concentrate (300 mL)	189 mg			0.06 (7 h)
Boysenberry concentrate (300 ml)	345 mg			0.03 (7 h)
Blueberry extract (300 mL)	439 mg			0.02 (7 h)
Strawberries (200 g)	76 mg			1.80 (24 h)
Chokeberry extract (7.1 g)	721 mg	0.096 μmol/L	2.8	0.15 (24 h)
Blackberries (200 g)	431 mg			0.16 (24 h)

a) Total anthocyanins if not stated otherwise.
b) Maximal plasma concentration.
c) Time to reach C_{max}.
d) % of intake.
e) Mv-3-glu = malvidin-3-glucoside.

1.5.2. Elimination

Elimination of anthocyanins is quite rapid ($t_{1/2}$ =1.5-3 h) compared to quercetin ($t_{1/2}$ =11-28 h), and accumulation is not likely to occur to any significant extent following normal dietary consumption (Kay, 2006). Studies identifying anthocyanins exclusively as un-metabolized parent compounds may result from several factors such as saturation of metabolic pathways following mega-dose interventions, insufficient extraction procedures, and misidentification as a result of insufficient detection methods (i.e. UV-Visible HPLC for identification) (Kay, 2006). He et al. 2006 have performed a long term (3 months) trial on rats, with a chokeberry-, bilberry-, and grape-enriched diet. In this study they observed a larger urinary excretion of methylated anthocyanins than in several shorter (less than 8-day adaptation) previously reported studies, suggesting the possible accumulation of anthocyanins in tissues or induction of methyltransferase. For the first time the occurrence of intact acylated anthocyanins in plasma and urine was demonstrated.
However, this study supports the finding by numerous researchers that anthocyanins have very low absorption, and it was suggested that anthocyanins in the gut content may influence GIT health without being delivered by the blood circulation system. He et al. 2006 observed high concentrations of possible metabolites in plasma, which remark the importance of further investigation of the significance of the accumulation of colonic metabolites and aglycone breakdown products.

1.5.3. Current knowledge

Currently, relatively little is known about how anthocyanins and compounds derived from them enter the body, distribute to tissues and exert beneficial health effects. Figure 1.11 is a representation of the absorption of anthocyanins based on current knowledge. Anthocyanin glycosides can be rapidly absorbed from the stomach after ingestion by a process that may involve bilitranslocase, and they enter the systemic circulation after passing through the liver. A portion of the anthocyanin is metabolized by methylation and glucuronidation reactions and some of the metabolites are transported to the intestine as bile. Anthocyanin glycosides that are not absorbed from the stomach move into the small intestine where, because of the higher pH they convert to a combination of hemiketal, chalcone, and quinonoidal forms. Further absorption appears to take place in the jejunum. The transport mechanism involved has not been identified, but if similar to flavonols, may involve hydrolysis of the glycosides by various hydrolases and absorption of the phenolic aglycone. Absorbed anthocyanins enter the systemic circulation after passage though the liver and may be metabolized. Anthocyanins that reach the colon are exposed to a substantial microbial population and may be degraded to sugar and phenolic components, with the phenolic components further degraded by disruption of the C-ring to yield phenolic acids and aldehydes. These products, derived from the ingested anthocyanins, may contribute to the health effect of anthocyanins either directly in the GIT or after absorption from the colon.
Much of the detail is missing about how anthocyanins are absorbed, how the variation of molecular structures consumed in food, and the forms generated in vivo contribute to the health benefits. Greater understanding will generate the potential for consumers to gain even more health benefits for high anthocyanin-containing foods such as berry fruit, than is currently the case.

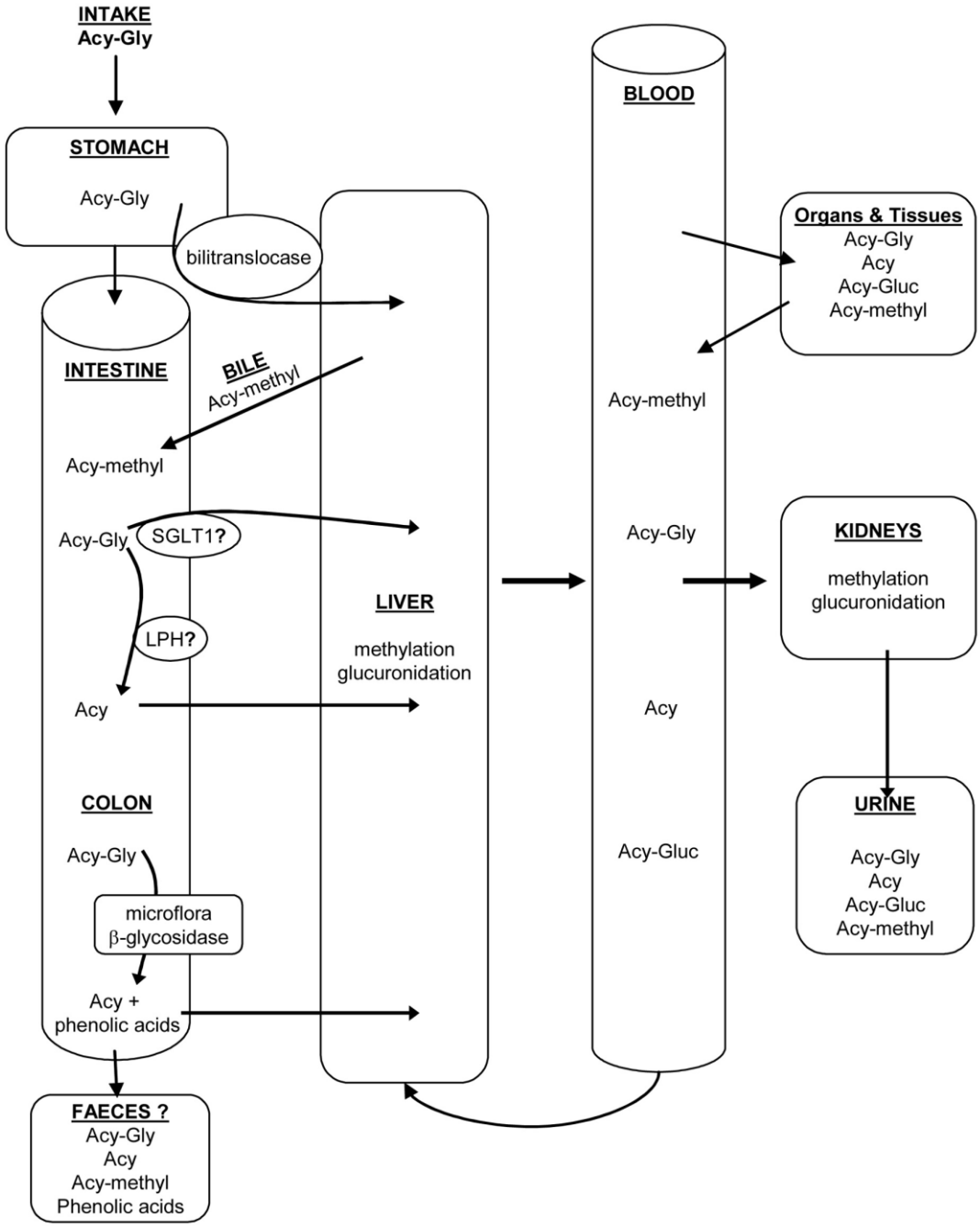

Figure 1.11. Schematic representation of absorption, distribution, metabolism and elimination of anthocyanins based on available information. Acy-Gly= anthocyanin glycosides; Acy = anthocyanin aglycone; Acy-methyl = methylated metabolite; Acy-Gluc = glucuronidated metabolite; LPH = lactase phloridzin hydrolase; SGLT1 = sodium dependent glucose transporter.

1.6. Analytical methods

Profiles of anthocyanins are distinctive in different fruits, and among different varieties of a given fruit. Many factors affect the total anthocyanin content in blueberry fruit, including species, cultivar, ripeness, season, growing region, yield, field management practices, and environmental factors. Total and individual anthocyanins can be monitored to study the influences of these factors (Mazza et al. 1995). The composition of the different species and varieties of the Vaccinium anthocyanins are distinct, and can be determined by High Performance Liquid Chromatography (HPLC) coupled to different detectors such as UV-Vis, photo-diode array (DAD), and mass spectrometer. (MS). Instead, the quantitative determination of anthocyanins may be performed with a benchtop spectrophotometer.

1.6.1. Liquid chromatography

Reversed-phase HPLC (RP-LC) coupled with photodiode array detection has been the most widely used tool for the identification, and quantification of anthocyanins. The different anthocyanins are separated according their different polarity, thus at equality of sugar linked the elution order is Mv> Peo> Pet> Cy> D. Unfortunately ACNs have similar UV-Vis spectra, thus by LC-DAD the identification is based only on retention times. At the contrary, LC-MS allows to identify the different anthocyanins according to their molecular weight and fragmentation patter (e.g. product ions). The latter option is particularly useful when anthocyanidins are poly-glycosylated or linked to sugars and organic acids such as acetic, caffeic, ferulic or p-coumaric. The probe most used for this type of analysis is the electrospray (ESI), which allows a "soft ionization" of the molecules so as to easily obtain their molecular weight. For anthocyanins, positive ions are extracted from the analyzer (e.g. quadrupole, time-of-flight). Because of their positive charge, anthocyanins (mostly in the flavylium form at low pH) generate a positive ion $[M]^+$ that can be easily measured by the mass spectrometer. Anthocyanins' positive charge allows the mass/charge ratio to correspond directly to the molecular weight of the anthocyanin. It should be underlined that despite MS specificity the chromatography remains essential because blueberry contains anthocyanin isomers, such as Mv-glc and Mv-gal, which cannot be distinguished from one another as they have the same mass/charge ratio. For example: D-3-glc and D-3-gal could be separated by HPLC, but would have the same *m/z* of 465. Anthocyanins quantification by LC can result in an underestimation of the amount present in a sample when using one standard for quantification. Quantification of anthocyanins in HPLC is determined by the peak area at a certain wavelength (520 nm), which is close to the maximum wavelengths (λmax) of individual anthocyanins. Typically, cyanidin-3-glucoside is selected as the external standard. Numerous studies show that depending on the anthocyanin chromophores, the maximum wavelength shifts slightly. For example, the max for malvidin-3-glucoside and cyanidin-3-glucoside are 534 and 523 nm, respectively, with 0.01% HC1 in methanol (Durst and Wroistad 2001). For anthocyanin quantification by LC-MS, intensity of mono-glycoside anthocyanins is linearly correlated to their relative molar ratios. But, for diglucoside anthocyanins, the relative molar ratios were reduced (one-fourth of the monoglucoside anthocyanins for equal concentration) (Wang and Sporns, 1999). Thus, to avoid underestimations we used different pure standard for ACNs quantification in FD-WB powder.

1.6.2. Total ACNs by differential pH method

A single pH method can be used when there are little or no interfering compounds in a sample. Anthocyanin content can be determined by measuring a sample's absorbance at its maximum wavelength. Anthocyanin absorbance is linear to the amount of pigment present (when absorption is within the linear region of the spectrophotometer). Anthocyanins typically absorb in the visible spectra of 490 to 550 nm region. This region is far from other interfering compounds; like phenolics

with maximum absorption in the 220-450 nm region. The presence of compounds, other than phenolics, can interfere with determination of the anthocyanin content of a sample. A differential method can be used with samples that contain interfering compounds such as anthocyanin degradation products (Giusti and Wrolstad, 2001). The pH differential method has shown to be simple, quick, and accurate to measure the total monomeric anthocyanin of a sample. The pH differential method has been used extensively by food technologists in assessing the quality of fresh, and processed fruits and vegetables. The method can be utilized for quantitative determination of total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and pH 4.5 (Figure 1.12). The use of the differential pH method is in research and quality control of anthocyanin containing fruit juices, wines, natural colorants, and other beverages.

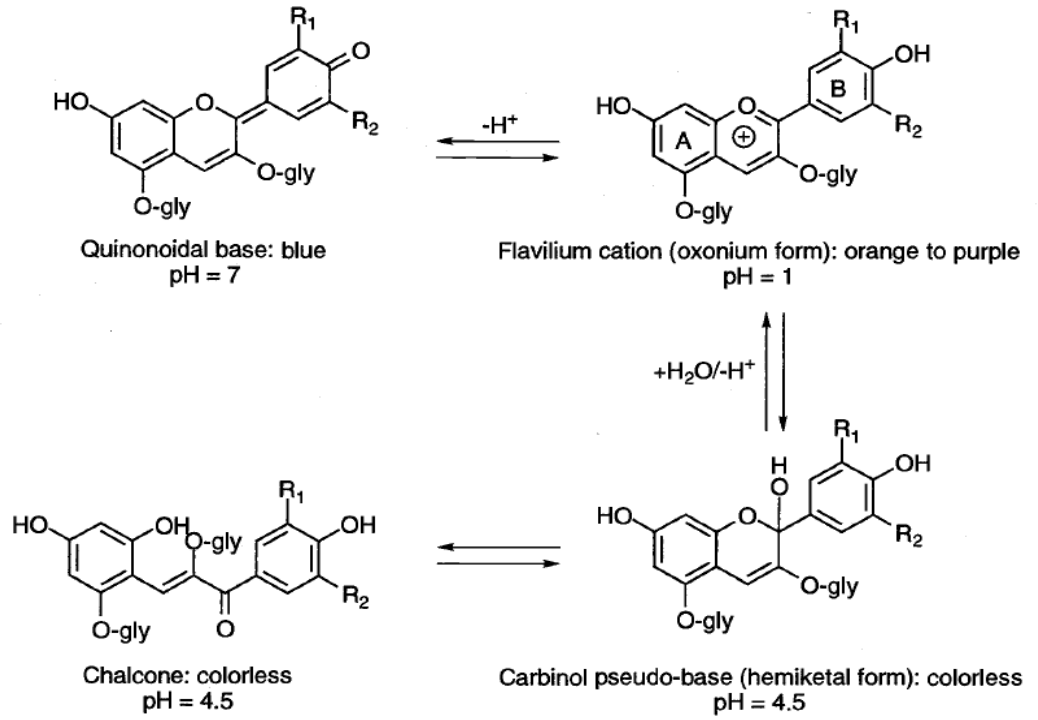

Figure 1.12. Anthocyanin structural forms present at different pH values.

The concept of measuring the amount of anthocyanin present in a sample (strawberries or strawberry preserves) by utilizing the change in absorbance at two different pH values (3.4 and 2.0) was first introduced by Sondheimer and Kertesz 1948. Since then, researchers have proposed using the pH values of 1.0 and 4.5 (Giusti and Wroistad 2001). Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored flavylium form at pH 1.0 and colorless hemiketal form at pH 4.5 (Figure 2.8). Thus the difference in absorbance at the pigments max lambda (520 nm) will be proportional to the pigment concentration. Figure 1.13 shows the spectra of blueberry anthocyanins in pH 1.0 and pH 4.5 buffers. To underline that, anthocyanins degraded in polymeric form are resistant to color change due to pH. Hence, polymerized anthocyanin pigments will not be measured in this method as they absorb at pH 4.5, as well as at pH 1.0.

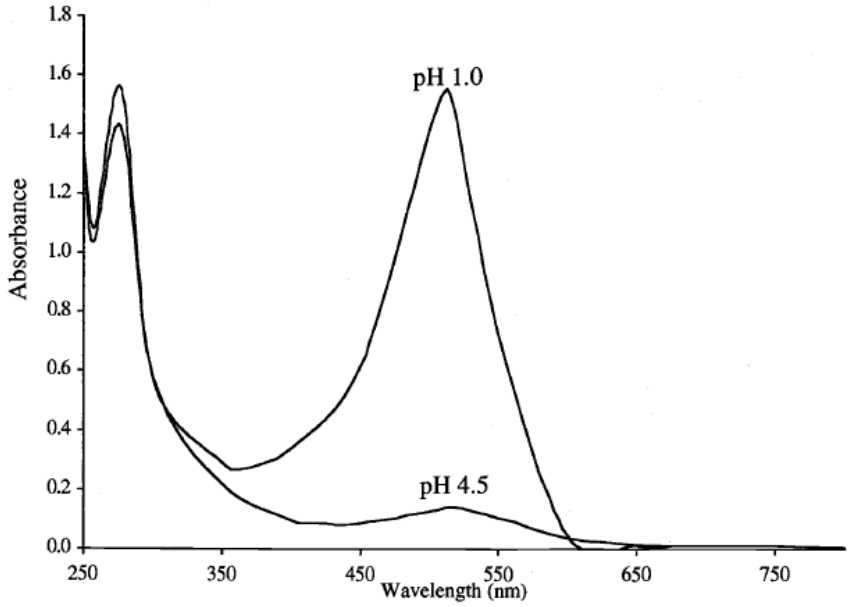

Figure 1.13. Spectral characteristics of FD-WB anthocyanins at pH 1.0 and pH 4.5 acetate buffer.

The limitations of the method are few. The presence of artificial colors or non-anthocyanin colorants can lead to confusion, but generally do not interfere adversely with the assay. Added colorants that might be encountered include Red No. 40, cochineal, and beet powder. Cochineal in high concentrations does lead to a reduction in measured anthocyanin content, but at low concentrations a quantitative total monomeric anthocyanin value can still be obtained.
Beet powder can be detected by its much higher at 550nm. Red 40 can be detected by its bright red color in the pH 4.5 treatment. Anthocyanins can be measured in the presence of these other colorants, if one measures at a wavelength of about 520 nm. The presence of ethanol in samples does not interfere with the assay at the levels typically encountered in wine (10-14%). It is customary to conduct spectral measurements at the wavelength of maximum absorbance and calculate pigment concentration using the molecular weight and molar extinction coefficient of the major anthocyanin present in the sample matrix. It is appropriate to modify the procedure by making measurements at the wavelength of maximum absorbance and calculating pigment concentration using the molecular weight of the major pigment in the sample matrix and its molar extinction coefficient, if known. Anthocyanin content of grape products, for example, should be determined as malvidin-3-glucoside.

2. EXPERIMENTAL

2.1. Chemicals

Standard of cyanidin (Cy), delphinidin (D), petunidin (Pet), peonidin (Peo), malvidin (Mv), their 3-O-glucoside (-glc), Cy-, Pet-, Peo-, Mv-3-O-galactoside (gal), and Cy- and Mv-3-O-arabinoside (-ara) were purchased from Polyphenols Laboratory (Sandes, Norway). Hydrochloric acid, potassium chloride, methanol, acetonitrile, phosphoric acid, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman- 2-carboxylic acid (Trolox) were purchased from Sigma (St. Louis, MO, USA). Water was obtained from Milli-Q apparatus (Millipore, Milford, MA). Freeze-dried wild Blueberry (Vaccinium angustifolium) (FD-WB) powder was provided by FutureCeuticals Company (Momence, IL, USA) and maintained at 4 °C.

2.2. Freeze-dried wild Blueberry (FD-WB) powder characterization

2.2.1. Sample preparation

Wild blueberry powder (250 mg) was placed in 40 mL of a solution methanol:1% TFA (30:70, v/v), sonicated for 10 min, and centrifuged at 1600 x g for 5 min. The supernatant was transferred into the flask and the volume adjusted to 50 ml, while the residue was extracted twice with 8 mL of solution methanol:1% TFA (30:70, v/v) and treated as described above. The supernatants were transferred into the flask and the volume adjusted to 10 ml.

2.2.2. LC-DAD-MS conditions

The LC system consisted of an ultra-performance liquid chromatography (UPLC) mod. Acquity (Waters, Milford, MA) equipped with a photodiode array detector (DAD) mod. E-lambda (Waters) and a triple quadrupole mass spectrometer mod. Quattromicro (Micromass, Beverly, MA). A 2.5 μm C_{18} Kinetex column (150x 4.6 mm, Phenomenex, Torrence, CA) maintained at 45°C was used. The flow-rate was 1.7 ml/min and the eluents were (A) 1% TFA and (B) acetonitrile/water (35:65, v/v). Fifty microliters were injected in the LC system. The flow-rate was split 5:1 before electrospray (ESI). The elution gradient was linear as follows: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32% B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90 % for 3 min. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 nm. The mass spectrometer operated in positive full-scan mode in the range 200-800 Da. The capillary voltage was set to 3.5 kV, the cone voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350 °C. Data were acquired by Masslinx 4.0 software (Micromass, Beverly, MA) with Quan-Optimize option for the fragmentation study.
Anthocyanin mother solutions were obtained by dissolving 10 mg of each standard in 10 ml of a solution of TFA/methanol (5:95, v/v). Working solutions were prepared by diluting the mother solution in a solution methanol: 1% TFA (10:90, v/v), and the calibration curves were in the range of 2-50 μg/ml. The concentration of the five ACNs not commercially available (D-gal, D-ara, Cy-ara, Mv-ara, and Pet-ara) was estimated using the calibration curve equation of the same anthocyanidin with different glycosylation. The acetylated ACNs were determined by the Cy-glc curve and the resulting data was corrected by their corresponding molecular weight ratios.

2.3. Evaluation of time and storage temperature on anthocyanin decay and antioxidant activity in FD-WB powder

2.3.1. Sample preparation

Sixty samples of one gram each were placed in zip-lock plastic bags for commercial products, sealed under vacuum and stored in the dark at four controlled temperatures (25, 42, 60 and 80°C). Wild blueberry powder was stored at -80°C until analysis.

2.3.2. Degradation studies

The thermal degradation of ACNs as well as the total antioxidant activity (TAA) of the FD-WB powder was investigated at 25, 42, 60 and 80°C for 49 days. Two samples of FD-WB powder (1 g each) were taken, based on the Accelerated Shelf Life Testing method at appropriate time intervals (3-4 days) for analyses. They were rapidly cooled and ACN extraction was performed for the determination of total and single ACNs concentration and TAA. All analyses were done in duplicate.

2.3.3. Extraction of ACNs from wild blueberry powder

Anthocyanin extraction was performed as follows: 50 mg of FD-WB powder was dissolved in 5 ml of methanol acidified with 1% of TFA and sonicated for 10 min. The suspension was centrifuged at 3000 x g for 15 min and the supernatant was recovered and the volume adjusted to 10 ml by methanol acidified with 1% of TFA.

2.3.4. Determination of total anthocyanins

The total content of ACNs was determined spectrophotometrically (Perkin Elmer Lambda 20, Waltham, MA) as described by Lee et al. 2005. Shortly, two aliquots of the extracted ACNs were diluted 1:10 in KCl 0.025 M at pH 1 and in CH_3COONa 0.4M at pH 4.5. The absorbance was measured twice for each sample and buffer at 520 and 700nm. The absorbance A (AU) was calculated as follows:

$A = (A_{520nm} - A_{700nm})$ at pH1 – $(A_{520nm} - A_{700nm})$ at pH4.5

The total ACN content was calculated as follows:

$$\frac{mg\ ACNS}{100g} = \frac{A\ \times MW\ \times W}{26900\ \times V\ \times DF}$$

Where: 26900= Cy-glc molar extinction coefficient (1/M), MW=Cy-glc molecular weight (449.2 Da), W=sample weight (g), V=volume (ml) and DF=dilution factor.

2.3.5. Determination of Total Antioxidant Activity (TAA)

The TAA was determined by the Trolox Equivalent Antioxidant Capacity (TEAC) assay as described by Pellegrini et al 2003. The TEAC assay measures the ability of antioxidants to take away a radical cation in both lipophilic and hydrophilic environments. In particular, the method is based on the ability of antioxidant molecules to quench the long-lived ($ABTS^{\bullet+}$), a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water-soluble vitamin E analog. The addition of antioxidants to the preformed radical cation reduces it to ABTS, determining a decolorization. A stable stock solution of ($ABTS^{\bullet+}$) was produced by reacting a 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use (Pellegrini et al. 1999). At the beginning of the analysis day, an ($ABTS^{\bullet+}$) working solution was obtained by the dilution in ethanol of the stock solution to an absorbance of 0.70±0.02 AU at 734 nm. The TAA was determined spectrophotometrically by a Perkin-Elmer Lambda 20, and results expressed as TEAC in mmol of Trolox eq /100 g DW.

2.3.6. Determination of single ACNs

The liquid chromatography (LC) system was an Alliance mod. 2695 (Waters, Milford, MA) equipped with a photodiode array detector (mod. 2998, Waters). The separation was carried out by a C_{18} Kinetex column (150 x 4.6 mm, 2.6 µm, Phenomenex, Torrence, CA) maintained at 45°C. The flow-rate was 1.7 ml/min and the eluents were (A) 1% H_3PO_4 and (B) acetonitrile/1% H_3PO_4 (35:65, v/v). The elution gradient was linear as follows: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32% B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90 % for 3 min. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 nm. Calibration curves ranged from 2 to 50 µg/ml; the working solution was obtained diluting the stock solution (1 mg/ml in methanol acidified with 0.1% TFA) with 1% TFA. Each analysis was carried out in duplicate. The concentration of the five ACNs not commercially available (D-gal, D-ara, Cy-ara, Mv-ara, and Pet-ara) was estimated using the calibration curve equation of the same anthocyanidin with different glycosylation. The acetylated ACNs were determined by the Cy-glc curve and the resulting data was corrected by their corresponding molecular weight ratios.

2.3.7. Degradation kinetic studies

The thermal degradation of ACNs was performed according to the method reported by Kechinski et al. 2010. Degradation is a temperature-dependent process, as described by the Arrhenius equation:

$$K = K_0 \times e^{\frac{-Ea}{RT}}$$

Thus, Arrhenius' equation gives the dependence of the rate constant K of a chemical reaction on the temperature T.
Where: k_0=frequency factor (1/s), E_a=activation energy (J/mole), R=universal gas constant (8.314 J/mol x K), and T=absolute temperature (K).
The coefficient Q_{10} expresses ACN degradation when the temperature is increased to 10 °C and it is calculated as follows:

$$Q_{10} = \frac{K_{at\,T2}}{K_{at\,T1}}^{\frac{10}{T2-T1}}$$

2.3.8. Statistical analysis

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Analysis of variance (ANOVA) with type of treatment as the dependent factor was used to evaluate the variations of ACNs and TAA. One-way ANOVA was performed to determine the variation among the samples stored at different temperatures. Differences between means were evaluated by the Least Significant Difference (LSD) test. Differences were considered significant at $P \leq 0.05$.

2.4. Absorption, metabolism, distribution and excretion of anthocyanins from FD-WB in rats

2.4.1. Chemicals

Standard of cyanidin (Cy), delphinidin (D), petunidin (Pet), peonidin (Peo), malvidin (Mv), their 3-O-glucoside (-glc), Cy-, Pet-, Peo-, Mv-3-O-galactoside (gal), and Cy- and Mv-3-O-arabinoside (-ara) were purchased from Polyphenols Laboratory (Sandes, Norway). Gallic acid (GA), 3,4-dihydroxybenzoic acid (DHBA), benzoic acid (BA), 4-hydroxybenzoic acid (4HBA), hippuric acid (HA), syringic acid (SA), vanillic acid (VA), 3-O-methyl-gallic acid (3-MG), and 3,4-dimethoxy benzoic acid (VA, veratric acid, internal standard) were purchased from Chromadex (Irvine, CA). Cyanidin-3,5-diglucoside (CydG), as ACN's internal standard (IS), was purchased from Sigma (St. Louis, MO). Potassium chloride, hydrochloric acid, methanol, acetonitrile, formic acid, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q apparatus (Millipore, Milford, MA). Freeze-dried wild blueberry powder was purchased from FutureCeuticals Company (Momence, IL, US) and utilized within 6 months. The dietary ingredients were purchased from Laboratory Piccioni (Gessate, Mi, Italy) where diets were pelleted. The composition of experimental diets is summarized in table 3.1. All diets were prepared fresh on a weekly basis and stored at 4°C until use.

Table 3.1. Composition of experimental diet.

Ingredient	Diet	
	AIN-93[a]	AIN-93 with 8% FD-WB
Casein	200	200
L-Cystine	3.0	3.0
Sucrose	100	30.6
Corn starch	397.5	397.5
Soybean oil	70	70
Fiber (cellulose)	50	50
Mineral Mix	35	35
Vitamin mix	10	10
Choline bitartrate	2.5	2.5
FD-WB	0	69,4

[a]AIN-93 is the standard rat diet.

2.4.2. Experimental design *in vivo*

Thirty-two male SD rats (28 days old, about 90 g) were purchased from Charles River Laboratories (Calco, Co, Italy) and were housed in metal cages in a controlled temperature (21-23 °C), humidity (40-60%), and light (from 7 a.m. to 7 p.m.).
Animals were divided into four groups of 8 rats each and were fed a control (AIN93) (C) or wild lowbush blueberry diet (WB) (C + 8% w/w blueberry powder substituting for dextrose) for a period of four or eight weeks. Tap water and diet were provided ad libitum. Diet consumption was measured daily (20±4 g), a dose equivalent to 24±5 mg/day of ANCs (about 48 mg/kg bodyweight). The protocol was approved by the University of Milan Animal Care and Use Committee.

2.4.3. Gathering and tissue preparation

Animals were anesthetized in a chamber with diethyl ether. Blood samples were collected from the abdominal aorta into a vacutainer containing heparin (0.2 mg/ml) as anticoagulant, and the plasma was obtained by centrifugation at 250 x g for 15 min at 4 °C. Samples were acidified with a hydrochloric acid solution (1% final concentration) to preserve anthocyanin stability, centrifuged at 4500 x g for 10 min, and the supernatants stored at -80 °C in aliquots (1ml) for no longer than 2 months. Animals were perfused via the portal vein with physiologic salt solution (PSS) (1.15% KCl), and organs such as the brain and liver were removed; feces and urine were collected and stored at -80 °C until analysis. Animals were placed and fed in metabolic cages (Tecniplast, Varese, Italy) two days before euthanization (one day for acclimatization and another one for the collection of 24 h urine and feces). Urine was collected in tubes containing 0.2 ml of hydrochloric acid solution (10% v/v). Distilled water was added to give a final concentration of 1% HCl.

2.4.4. Purification method development

2.4.4.1. Feces

The extraction method was designed based on reported procedures for cecal content anthocyanins extraction (Felgines et al. 2002). An aliquot of about 100 mg was taken from each frozen fecal sample, and placed in a 40 ml centrifuge tube containing different amount of FD-WB (5-20 mg). Then, 20 ml of 1% TFA in water was added, and tissue homogenizer used to homogenize the spiked feces. The suspension was sonicated for 5 min and centrifuged at 4 °C for 10 min at 1650 x g to precipitate water insoluble content. The supernatant was taken and the residue was extracted twice with 10 ml of extracting solvent in the same way. The combination of supernatants was carefully evaporated in a rotovapor at 30°C. During the evaporation, pressure was carefully controlled to avoid boiling. After evaporation to almost dry, the solution was diluted with small amount of acidified water, and then applied to a 3 ml Oasys solid phase extraction (SPE) cartridge (Waters) pre-conditioned with three volume of methanol containing 1% TFA followed by six volume of water containing 1% TFA. After washing with three volume of water containing 1% TFA, anthocyanins were eluted with two volume of methanol containing 1% TFA. The eluate was carefully evaporated, and then dissolved in 10 ml of a solution methanol:1% TFA (30:70, v/v). The experiment was done by duplicate.

2.4.4.2. Plasma and urine

Plasma purification method was adapted from Matsumoto et al. 2001. Plasma or Urine (1 ml) were diluted to 3 ml with water containing 1 μg anthocyanins from FD-WB and IS (CydG). The resulting solution was loaded on a 3 ml HLB Oasys 100 mg cartridge (Waters) pre-activated with methanol

(3 ml) and then washed with water (6 ml). The loaded SPE cartridge was washed with 3 mL of 1% TFA in water, and the ACNs were eluted by 3 ml of methanol containing 1% TFA. The eluate was dried under vacuum, the residue dissolved in 50 µl of a solution methanol:1% TFA (10:90, v/v), and the solution was centrifuged at 2000 x g for 1 min, Then, 20 µl were injected into UPLC-MS/MS system for analysis.

2.4.5. Anthocyanin extraction from biological samples

One milliliter of urine or plasma was diluted with 2ml of water or 4 ml 1% TFA, respectively, containing 2.5 ng of IS (CydG); the resulting solution was loaded on a 3 ml HLB Oasys 100 mg SPE cartridge (Waters) pre-activated with methanol (3 ml) and then washed with water (5 ml). The loaded SPE cartridge was washed with 3 mL of 1% TFA in water, and the ACNs were eluted by 3 ml of methanol containing 1% TFA. The eluate was dried under vacuum, the residue dissolved in 50 µl of a solution methanol:1% TFA (10:90, v/v), and the solution was centrifuged at 2000 x g for 1 min, and 20 µl were injected into UPLC-MS/MS system for analysis. Fecal samples were defrosted, and an aliquot of 0.5 g was homogenized in the Potter homogenizer (IKA-Werke, Staufen, Germany) using 4 ml of a solution of CH_3OH:1%TFA (20:80, v/v) containing 500 ng of the IS (CydG). Samples were centrifuged at 3645 x g for 10 min and the residue extracted twice. Supernatants were collected and brought to a final volume of 10 ml for the analysis. Brain or liver samples (1 g) were homogenized in the Potter homogenizer (IKA-Werke) with 3 ml of a solution of CH_3OH:1%TFA (10:90, v/v) containing 8 ng of the IS (CydG); the mixtures were then centrifuged at 3645 x g for 10 min and the residue extracted twice. Supernatants were collected and brought to a final volume of 10 ml. Samples (3 ml) were purified and concentrated by SPE as described above.

2.4.6. Anthocyanin determination in biological samples

The chromatographic system was an Acquity UPLC (Waters) coupled to a model Quattromicro triple quadrupole mass spectrometer. The analyses were carried out in gradient mode by a 1.7 µm C_{18} Kinetex column (150 x 2.1 mm, Phenomenex) maintained at 45 °C, and the flow-rate was 0.5 mL/min. The eluents were (A) 1% TFA and (B) CH_3CN/1% TFA (35:65), and the gradient was as follows: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32% B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90 % for 3 min. Routine analyses were carried out in multiple reaction monitoring (MRM) mode, monitoring the following transitions: $(m/z)^+$ 449 → 287 Cy-gal and Cy-glc; 465 → 303 D-gal and D-glc; 463 → 301 Peo-gal and Pet-glc; 479 → 317 Pet-glc and Pet-gal; 493 → 331Mv-gal and Mv-glc; 419 → 287 Cy-ara; 433 → 301 Peo-ara; 435 → 303 D-ara; 449 f 317 Pet-ara; 463 → 331 Mv-ara; and the acetylated (-ac) forms of Peo, Pet, Mv (505 → 301 Peo-glc-ac; 521 → 317 Pet-glc-ac; 535 → 331 Mv-glc-ac).
The capillary voltage was set to 3.5 kV, and the cone voltage and collision energy were specific for each compound; source and desolvation temperatures were 130 and 350 °C, respectively. The argon for the fragmentation in the collision cells was 3.2×10^{-3} mbar. The calibration curves were prepared in 1% TFA:methanol (90:10, v/v) in the range of 2-40 ng/ml. Acetylated forms were assayed using the calibration curves of the un-acetylated standard compounds, and their amounts were then normalized by molecular mass ratios.

2.4.7. Phenolic acid extraction from biological samples

Plasma (1 ml) was diluted with water (4 ml) containing veratric acid (10 ng) as IS and treated as the plasma ACN extraction with the following modifications. The SPE cartridge was washed with 3 mL of 0.1% acetic acid instead of 1% TFA.
Urine samples (0.2 ml) were treated with 1.8 ml of 0.1% acetic acid solution, centrifuged at 3600 x g for 10 min, and the supernatant used for analysis.

Brain, liver (1 g), and fecal samples (0.5 g) were homogenized in the Potter homogenizer (IKA-Werke, Staufen, Germany) with 3 and 5 ml, respectively, of H_2O:CH_3OH:acetic acid (80:20:0.1, v/v/v) solution containing veratric acid as the IS (1 μg), the mixtures were centrifuged at 3600 x g for 10 min, and the residue was extracted twice. Supernatants were collected and brought to a final volume of 10 ml.

2.4.8. Phenolic acid analysis in biological samples

The analyses were carried out with the UPLC-MS/MS system in gradient mode by a 1.8 μm C_{18} HSS column (100 x 2.1mm, Waters) maintained at 50 °C. The flow-rate was 0.5 ml/min, and the injection volume was 2 μl. The eluents were (A) 0.1% CH_3COOH and (B) acetonitrile. The gradient was as follows: 5 to 15% B in 1.5 min, from 15 to 25%B in 1 min, from 25 to 30% B in 0.5 min, and then from 30 to 50% B in 0.5 min. Analyses were carried out in an ESI negative mode monitoring the following transitions: $(m/z)^-$ BA 121 → 77; 4HBA 137 → 93; DHBA 153 → 109; GA 169 →125; HA 178 →134; VA 167 →152; SA 197 →182; IS 181 → 137.
Capillary voltage was fixed at 3.0 kV, source and desolvation temperature at 130 and 350 °C, respectively, and argon pressure at 1.2×10^{-3} mbar, while cone voltage and collision energy were specific for each compound.
The mother solutions were prepared dissolving 10 mg of each standard in 10 ml of methanol. The calibration curves were prepared in 0.1% acetic acid:methanol (80:20, v/v) in the range of 5-400 ng/ml.

2.4.9. Validation Method

Cyanidin-3,5-diglucoside (Cy-dG) or veratric acid was used as the internal standard to correct the loss of anthocyanins or phenolic acids during sample preparation, respectively. Calibration curves were constructed for each standard at five concentration levels, and two independent determinations were performed at each concentration. The LC-MS/MS methods were validated for linearity, lower limit of quantification (LOQ) and detection (LOD), peak purity, precision, and repeatability. Limit of quantification (S/N ratio of 6) and limit of detection (S/N ratio of 3) were determined by serial dilution of standards in the different biological matrixes. The accuracy (matrix effect) was evaluated according to Matuszewski et al. 2003. Specifically, three sets with different concentrations of anthocyanins (2, 10, and 40 ng/ml) or phenolic acids (5, 25, 100, 200, and 400 ng/ml) and IS (50 ng/ml) were prepared. The first set consisted of standards plus IS; the second set was represented by a biological matrix (plasma, urine, liver, or brain) containing standards and IS subjected to extraction treatment and SPE purification, and the third was obtained adding the standards and IS after extraction and purification of the different biological matrix. All of the tests and LC-MS/MS analyses were carried out in triplicate. Peak purity and identity were confirmed by LC-MS/MS experiments. Intra- and inter-day precision of the assay was verified by analyzing sample sets 3 times on five consecutive days. Repeatability was confirmed by evaluating standard deviations of the retention times. Calibration curves were constructed for each standard at five concentration levels, and three independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs.

2.4.10. Statistical Analysis

The STATISTICA software (Statsoft Inc., Tulsa, OK) was used for statistical analysis of data. A two-way ANOVA analysis was used to compare the effect of diet (FD-WB vs C) and diet duration (4 vs 8 weeks) on anthocyanins and phenolic acid concentration in the plasma and different rat tissues (liver and brain). The values are presented as the mean ± standard deviation. A value of $p < 0.05$ was considered statistically significant

2.5. Experimental design *in vitro*

2.5.1. Stability test

Cyanidin-3-glc, Cy-3-ara, Peo-3-glc, Peo-3-ara, Pet-3-glc, D-3-glc, Mv-3-glc, Cy, Mv, Pet, Peo at concentrations of 90 μM and FD-WB (10 mg/ml) in 0.1M phosphate buffer (pH 7) at 37 °C were incubated into an anaerobic cabinet (Forma Scientific) under a $N_2/H_2/CO_2$ atmosphere (85:10:5, v/v/v). After defined times (0, 20, 40, 60, 120, 180, 240, 300, 360, 420, 480 min and 24, 48, 72 h) an aliquot of 0.5 ml was taken from the incubation mixture and analyzed by UPLC-DAD-MS.

2.5.2. Incubation with rat microflora from stomach, small and large intestine

Four male SD rats (200-220 g) were purchased from Charles River Laboratories (Calco, Co, I) and were housed as described above (2.4.2.). Animals were fed with a standard diet (AIN93) for a period of 1 week. Tap water and diet were provided ad libitum. The protocol was approved by the University of Milan Animal Care and Use Committee. Animals were anesthetized in a chamber with diethyl ether and stomach and intestine removed, tied at the ends, stored in anaerobic bags (Generbag Anaer, bio-Merieux), cooled at +4°C, delivered to the laboratory within 30 min after collection and immediately introduced into an anaerobic cabinet (Forma Scientific, Marietta, OH, US) under a $N_2/H_2/CO_2$ atmosphere (85:10:5, v/v/v). The intestine was then stretched, cut into two pieces corresponding to small intestine (duodenum, jejunum and ileum) and large intestine (cecum and colon), and from these were cut two pieces 10 cm each. Then, stomach and the two pieces of intestine were turn inside out and scraped in 10 ml of reduced brain hearth infusion modified medium (BHI) containing ACNs standard (Cy-glc, Peo-glc, Pet-glc, D-glc, M-glcn Peo-ara and Cy-ara, 40 μg/ml) and FD-WB (20 mg/ml) and incubated in anaerobic cabinet at 37 °C for 48 h. At time zero, every hour for 8 hours and after 24 and 48 h of incubation 0.4 ml were collected in twice and diluted to 1 mL with a solution of methanol:2%TFA (30:70, v/v). The mixture was sonicated for 5 min, centrifuged at 1000 x g for 1 min and the supernatants stored at -20°C before LC-DAD-MS analysis. The control suspensions (medium, medium plus microflora, medium plus FD-WB) were also incubated under the same operative conditions and treated as described in 2.5.1. Anthocyanins were determined by LC-DAD-MS as described above (2.2.2).

2.5.3. Incubation with human faecal microflora (HFM)

In the morning stool specimens obtained from healthy volunteers, who had not taken any antibiotics for at least the previous six months, were collected in anaerobic bags, cooled at +4°C, delivered to the laboratory within 2 h after collection and immediately introduced into an anaerobic cabinet (Forma Scientific) under a $N_2/H_2/CO_2$ atmosphere (85:10:5, v/v/v). A faecal sub-sample of 1.5 g was homogenized, suspended in 30 ml of reduced brain hearth infusion modified medium (BHI) and 10 ml of the suspension were pipetted in 90 ml of BHI containing FD-WB (20 mg/ml) or standard ACNs (Cy-glc, Peo-glc, Pet-glc, D-glc, M-glcn Peo-ara and Cy-ara, 40 μg/ml). The mixture was incubated in anaerobic cabinet at 37°C for 72 h, treated as described in 2.5.2 and analysed by UPLC-DAD-MS. The control suspensions (medium, medium plus microflora, medium plus FD-WB) were also incubated under the same operative conditions and treated as described above.

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3. RESULTS AND DISCUSSION

3.1. Freeze-dried wild blueberry powder characterization

3.1.2. Solvents comparison

Methanol (Frøytlog et al. 1998; Torskangerpoll et al. 2001), acetone (Giusti et al. 1996a, Rodriguez-Saona and Wrolstad 2001), water, and organic-inorganic mixture solvents (Ju and Howard 2003a; Ju and Howard 2003b) have been widely used for anthocyanin extraction. Preliminarily, we compared the extraction efficiency of four solvent systems: aqueous methanol (20-80%, v/v), water, methanol, and acetone.
All solvents were acidified with 1% of TFA (Torskangerpoll et al. 2001) to preserve anthocyanins. Anthocyanins were extracted following procedure described in 2.2.1, and calibration curve (2.2.2) was used to evaluate the concentration. Total anthocyanins recovered from FD-WB samples were calculated by LC-DAD method and spetrophotometrically. Results were summarized in figure 3.1.

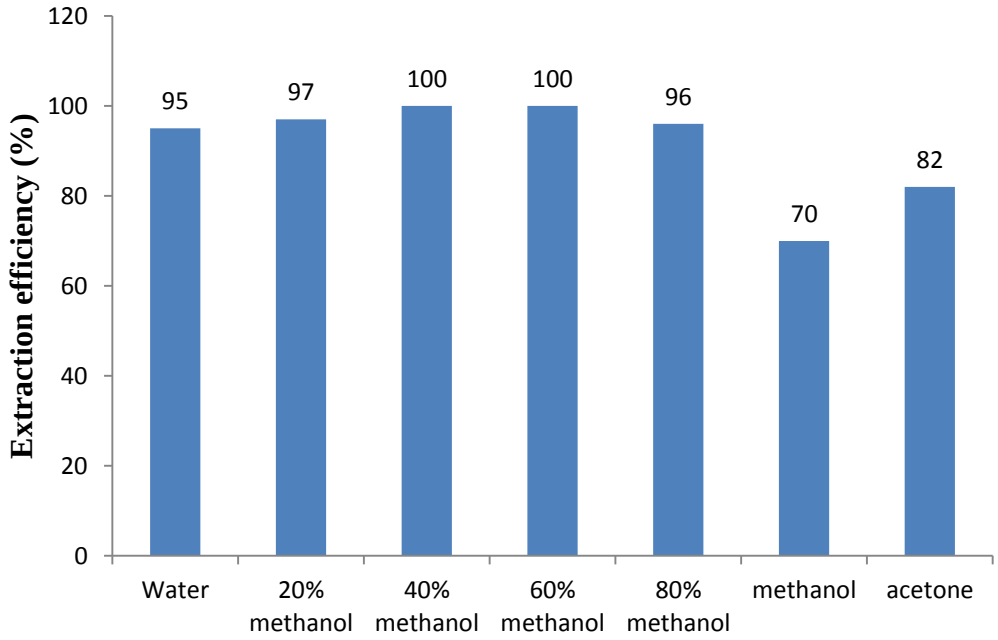

Figure 3.1. Relative efficiency of different solvents on FD-WB anthocyanin extraction.
Values are the mean of two replications.

Figure 3.1 clearly shows that the extraction efficiency with water or aqueous methanol (40-80%) gave the highest percentage of extraction. This was reasonable since the distribution of more or less hydrophilic anthocyanins in FD-WB powder. In addition, methanol helped to precipitate proteins and insoluble matter during centrifugation. Traditionally, organic solvents were preferred for anthocyanin extraction from fruits and vegetables (Giusti et al. 1996a; Harborne 1998; Eder 2000; Takeoka and Dao 2002), partly because organic solvents have the advantage of easily penetrating the cell membranes and consequently extracting anthocyanins, the vacuolar pigments (Delgado-Vargas and Paredes-López 2003). Water is a good alternative for aqueous methanol, not only because it is an environmental friendly solvent, but also because water extraction can be directly

applied to the C18 cartridge, and therefore save a step of evaporation. Moreover, we found water extraction gave the lowest RDS%. Regarding LC analysis, methanol percentages higher than 40% caused a worsening of the chromatographic profile. Thus, we chose acidified aqueous methanol (70:30, v/v) to extract FD-WB anthocyanins.

3.1.3. Sample preparation

Three subsequent extractions were applied to extract all the anthocyanins present in the FD-WB sample. For the first and second extraction the calculated recovery was about 94.1±2.0 and 5.9±0.3 %, respectively. Anthocyanins were not detected in the third extract.

3.1.4. Method validation

Calibration curves were constructed for each standard at five concentration levels and three independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs. The accuracy (recovery) of the extraction for anthocyanins from spiked FD-WB samples was 98±3. The precision of the method was tested by both intra-day (n=5) and inter-day (5 days, n=5) reproducibility, and the coefficient of variation was below 5.4 %. Limit of quantification (LOQ) and detection (LOD) were 2 μg/ml and 0.8 μg/ml, respectively. Regarding repeatability, a maximum relative standard deviation of 3 % was observed for triplicate injections.

3.1.5. LC-DAD-MS analysis

Liquid chromatography combined with photo-diode array and tandem mass spectrometry detector is a reliable approach to identify anthocyanin fractions in complex food matrix such as blueberry. The analytical method used allowed for the separation of 21 ACNs, 15 glycosylated anthocyanidins and 6 acetylated derivatives within 45 min (Fig. 3.2).
The peak identity was established by co-chromatography (peaks 1-3, 5-9, and 11-15), on-line UV-Vis spectra comparison, and molecular ion and product ion evaluation. The MS and MS/MS data (at lower collision energy values, 15-25 eV), combined with the UV-Vis and chromatographic behavior suggest that peaks 16-21 were the acetylated forms (D-glc-ac, Cy-glc-ac, Pet-glc-ac, Mv-gal-ac, Peo-glc-ac, and Mv-glc-ac).
As an example, malvidin-glucose-acetate (peak 21) yielded under low collision energy as a main fragment the ion with $(m/z)^+$ 493 accounting for $[M\text{-acetate}]^+$ accompanied by minor ions with $(m/z)^+$ 331 (Malvidin) arising from $[M\text{-acetate-glucose}]^+$ (Fig. 3.3). At higher collision energy only the fragment with $(m/z)^+$ 331 was produced. A similar fragmentation pattern was observed for the other acetyl derivatives.

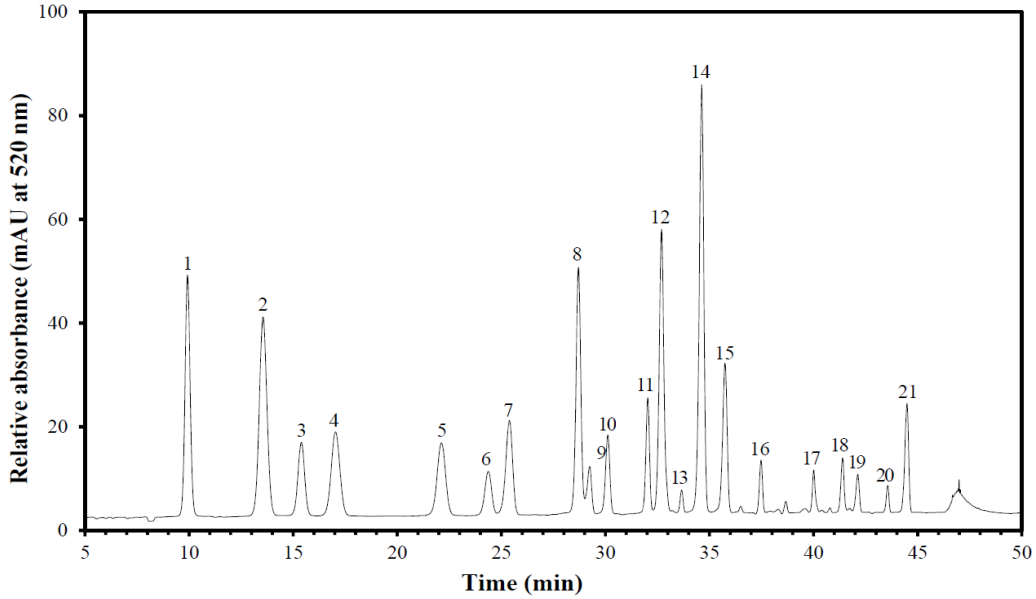

Fig. 3.2. Typical chromatogram of FD-WB powder integrated at 520 nm. The ACNs were: (1) D-gal, (2) D-glc, (3) Cy-gal, (4) D-ara, (5) Cy-glc, (6) Pet-gal, (7) Cy-ara, (8) Pet-glc, (9) Peo-gal, (10) Pet-ara, (11) Peo-glc, (12) Mv-gal, (13) Peo-ara, (14) Mv-glc, (15) Mv-ara, (16) D-glc-Ac, (17) Cy-glc-Ac, (18) Pet-glc-Ac, (19) Mv-gal-Ac, (20) Peo-glc-Ac, and (21) Mv-glc-Ac.

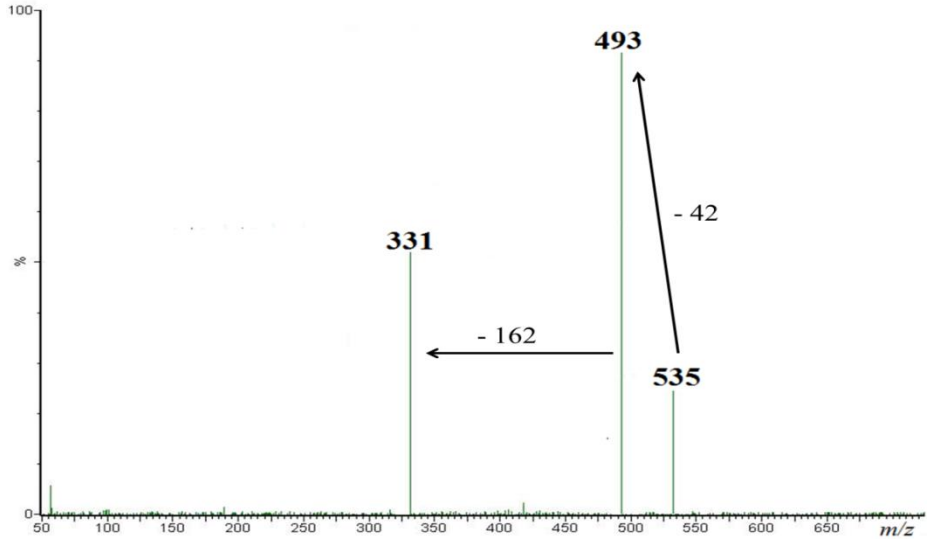

Figure 3.3. Fragmentation pattern of the peak 21 (MW 535 Da) obtained at lower collision energy. The ions product with $(m/z)^+$ 493 and $(m/z)^+$ 331 correspond to malvidin-glucoside and malvidin, respectively.

Triplicate analyses were performed on the freeze-dried WB powder. Twenty-one different ACNs were detected, and their total amount was 1.8±0.1 mg/100 mg with the mean relative standard deviation (RSD%) lower than 6.0 % for concentrations from 0.5 to 20 µg/ml. The main ones were Mv-3-glc, Mv-3-gal, D-gal and D-glc, and these four compounds represented about 38% of the total amount of ACNs. Peonidin-ara and Peo-glc-ac were in lower concentrations. Table 3.1 reports the peak identity and the mean ACN content in freeze-dried wild blueberry powder.

Table 3.1. Anthocyanins in FD-WB blueberry powder and their MS and MS/MS characteristics.

Peak	$[M]^+$	Product ion	Compound	%	mg/100 mg
1	465	303	delphinidin-galactose (D-gal)	8,3	0,15
2	465	303	delphinidin-glucose (D-glc)	8,0	0,14
3	449	287	cyanidin-galactose (Cy-gal)	3,5	0,06
4	435	303	delphinidin-arabinose (D-ara)	3,6	0,06
5	449	287	cyanidin-glucose (Cy-glc)	4,0	0,07
6	479	317	petunidin-galactose (Pet-gal)	3,0	0,05
7	419	287	cyanidin-arabinose (Cy-ara)	4,1	0,07
8	479	317	petunidin-glucose (Pet-glc)	8,5	0,15
9	463	301	peonidin-galactose (Peo-gal)	2,4	0,04
10	449	317	petunidin-arabinose (Pet-ara)	3,7	0,07
11	463	301	peonidin-glucose (Peo-glc)	3,6	0,06
12	493	331	malvidin-galactose (Mv-gal)	9,1	0,16
13	433	301	peonidin-arabinose (Peo-ara)	1,3	0,02
14	493	331	malvidin-glucose (Mv-glc)	13,1	0,24
15	463	331	malvidin-arabinose (Mv-ara)	3,9	0,07
16	507	303	delphinidin-glucose-acetyl (D-glc-ac)	3,3	0,06
17	491	287	cyanidin-glucose-acetyl (Cy-glc-ac)	2,8	0,05
18	521	317	petunidin-glucose-acetyl (Pet-glc-ac)	3,1	0,06
19	535	331	malvidin-galactose-acetyl (Mv-gal-ac)	2,5	0,05
20	505	301	peonidin-glucose-acetyl (Peo-glc-ac)	1,1	0,02
21	535	331	malvidin-glucose-acetyl (Mv-glc-ac)	7,1	0,13
			Total ACNs	100,0	1,8

Repeatable quantification of anthocyanins was performed with different standards and not by the calibration curve of Cy-glc because the latter method underestimates the content of some compounds, such as delphinidin glycosides, by about 27% (Kähkönen et al. 2003). Moreover, the sugar moiety affects the LC-DAD online response; for example, there was a 16% decrease in the content of the cy-gal and cy-ara if they were quantified as glucoside equivalents instead of using the corresponding standard (Wang et al. 2000). Therefore, the comparison of the contents of blueberry anthocyanidin glycosides to the contents in the literature is complicated owing to analytical differences.

3.1.6. Total ACNs by differential pH method

The total monomeric anthocyanin content of FD-WB was 1.9 mg/100 mg (mean value, RSD%< 5%) determined by the pH differential method as cyanidin-3-glucoside (ε = 26.900 and MW= 449). This was lower than the values of 2.3 and 2.2 mg/100 mg reported for blueberries by Prior et al. 1998, 2001, as well as 3.2 mg/100 mg reported by Ehlenfeldt and Prior, 2001.
The results obtained with the spectrophotometric method were in agreement with those obtained by LC-DAD method.

3.2. Effect of time and temperature on anthocyanin and antioxidant activity of the FD-WB powder

Anthocyanins (ACNs) are bioactive compounds present in many edible plants that include berries and blood orange. Several studies investigated the ACNs activities suggesting anti-inflammatory and anti-carcinogenic properties, promotion of lipid profile and vasodilation. Despite the high amounts of ACNs introduced through the diet, their bioavailability is the lowest between flavonoids and little is known about the changes in equilibrium due to temperature. Many foods which contain ACNs are heated to temperatures from 50 to 150 ºC prior to consumption and this process can greatly influence ACNs content in the final product. For this reason ACN chemical stability is the main focus of many recent studies. The aim of this work was to evaluate the stability of freeze-dried lowbush wild blueberry (FD-WB) exposed to temperatures (25-80ºC) in absence of light. FD-WB was chosen for the high content, about 1.6%, and type of ACNs present. To evaluate the effect of temperature, the FD-WB was incubated for two months and every four days samples were withdrawn and extracted by acidic methanol. The solutions were analyzed by UHPLC-DAD-MS. The isothermal kinetic parameters were calculated according to the Arrhenius model.
The decay of total ACNs was evaluated at four different temperatures (25, 42, 60 and 80°C) for 49 days and the raw and normalized data are reported in figure 3.4a and 3.4b, respectively. On the whole, a significant difference ($P \leq 0.0001$) on ACN content was detected for each temperature studied, and as expected, time and degree of ACN decay was dependent on temperature. In fact, the ACN decay occurred slowly up to 3% at day 14 at 25°C and 42°C while it was faster, achieving about 60 and 85% decay at day 3 at 60 and 80°C, respectively.

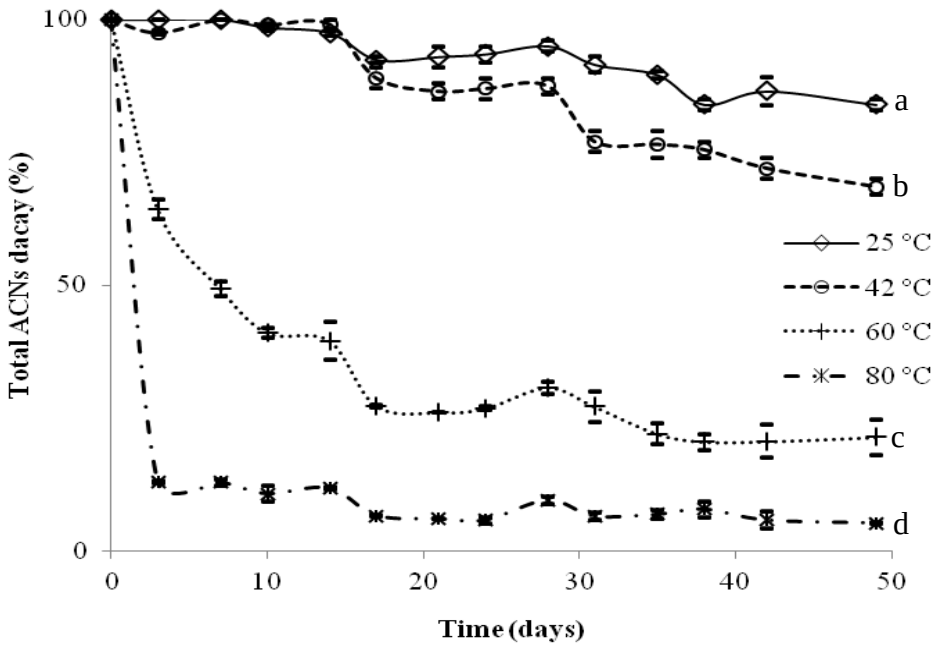

Figure 3.4a. Decay (%) of total ACNs in the WB powder stored at (a) 25°C, (b) 42°C, (c) 60°C and (d) 80°C. Curves with different letters are significantly different at $P \leq 0.05$.
ACN (-): anthocyanin, ACN-Ac (○): Acetylated anthocyanin.

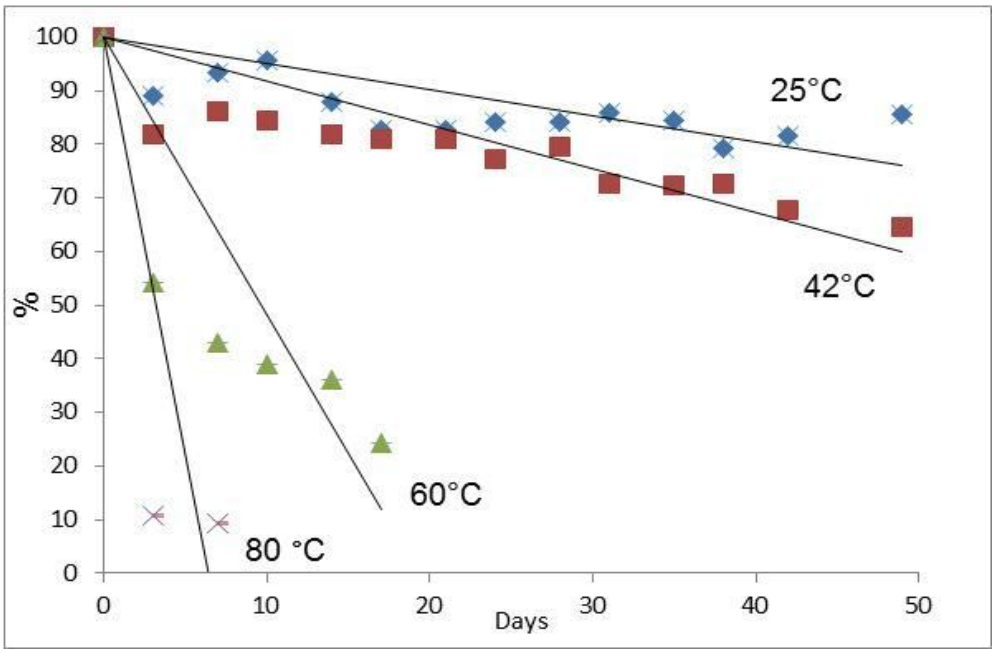

Figure 3.4b. Normalized degradation kinetic curves of total ACNs in the WB powder stored at 25, 42, 60 and 80 °C.

Also visually the products stored at lower temperatures (25, 40 °C) were different from those stored at higher temperatures (65 and 80 °C). The latter, even after a few days, lost the typical red-purple color showing conversely a brownish-yellow coloring, likely due to oxidations and/or condensation reactions with other phenolic compounds (Figure 3.5). However, the gradual degradation of red color was visually observed in all systems, even in those stored at lower temperatures.

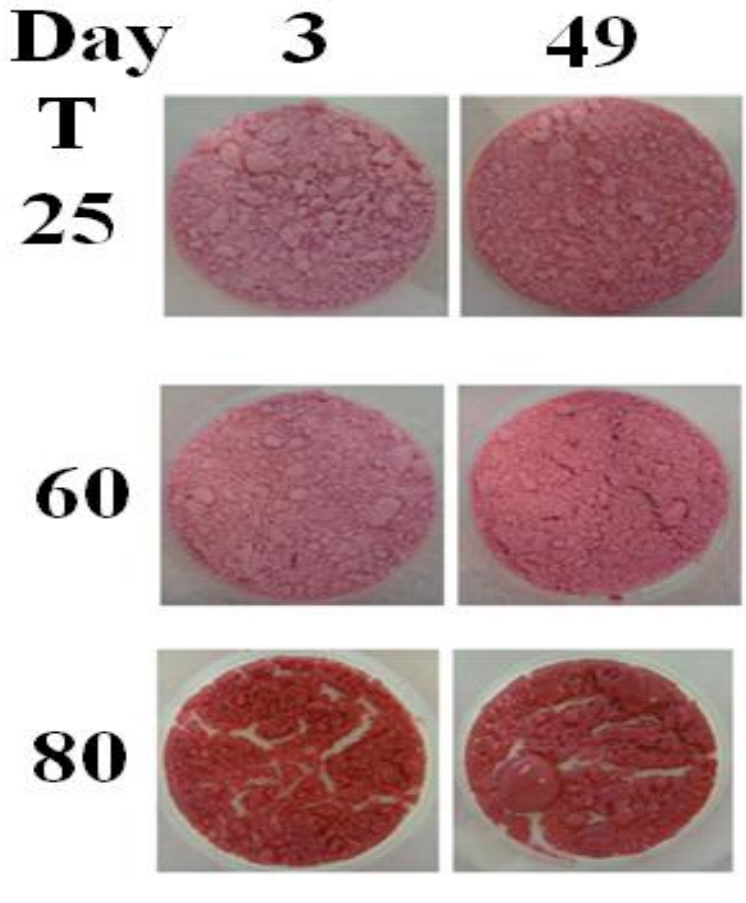

Figure 3.5. Images of FD-WD samples after 3 and 49 days of storage at 25, 60 and 80 °C.

The quantification of the single ACNs allowed for the calculation of the decay slope (mean ± SD) in the WB powder (Fig. 3.6). The reduction in ACN content at 80°C was higher than 90% after 3 days only, thus the data of single ACNs at this temperature were not used to evaluate their degradation rate. The quantification of the single ACNs allowed for the calculation of the decay slope (mean ± SD) in the WB powder (Fig. 3.6). The reduction in ACN content at 80°C was higher than 90% after 3 days only, thus the data of single ACNs at this temperature were not used to evaluate their degradation rate. The slopes calculated at 25, 42 and 60 °C showed that the degradation rate followed a first-kinetic order. This trend was in accordance to that observed by several researchers

on different juices, such as blood orange, blackberry, and blueberry juices and red wine (Kechinski et al. 2010, Kirca et al. 2003, Wang et al. 2007, Romero et al. 2000).
Each compound displayed its own specific decay, related to the sugar binding and the storage temperature. Moreover, it seems that the ACNs bound to glucose, exhibited a faster degradation rate than those bound to galactose (data not shown). For all the ACNs, the correlation indices (R^2) were higher than 0.90, demonstrating a direct correlation between ACN concentration decrease and storage time. Good correlation indices were also found for the acetylated forms (R^2>0.81), which seems more stable that the correspondent glycosides.

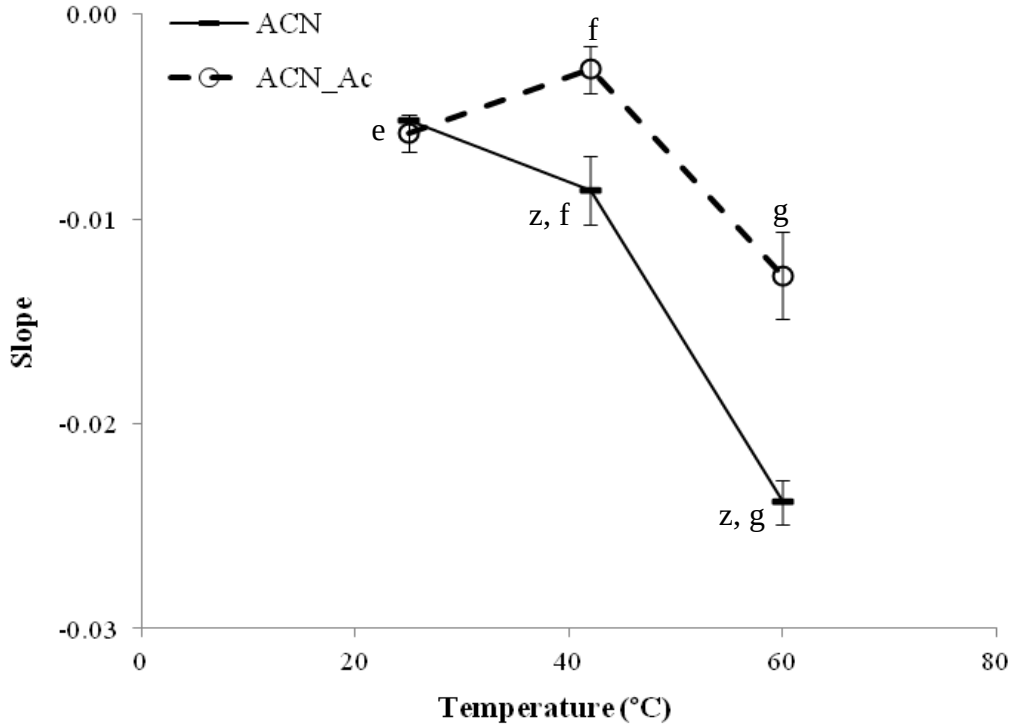

Figure 3.6. Effect of temperature on slope (mean±SD) for glycosylated and acetylated ACN degradation in FD-WB powder stored at 25, 42, 60°C.
[z] Data between curves (ACN vs ACN_Ac) at 42 and 60°C are significantly different at P≤ 0.05
[e, f, g] Data between points (25, 42 and 60°C) of the same curves are significantly different at P≤ 0.05.
ACN (-): anthocyanin, ACN_Ac (○): Acetylated anthocyanin

The linear regression approach allows also for the calculation of the reaction rate constant (*k*). A direct relationship between *k* values and temperature was found (Figure 3.7), confirming the major effect of temperature on ACN degradation.

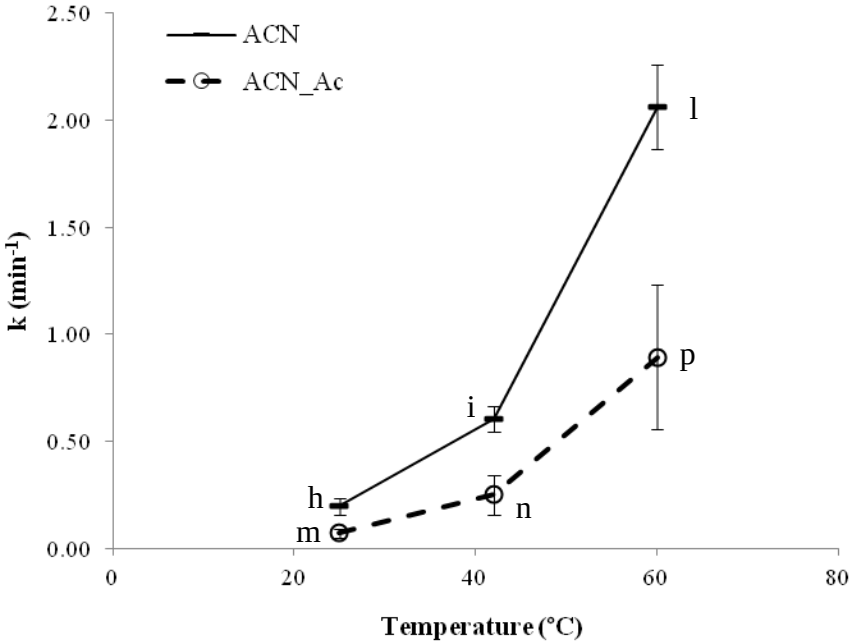

Figure 3.7. Effect of temperature on reaction rate constant (*k*) slope (mean±SD) for single ACN and ACN_Ac degradation in WB powder stored at 25, 42 and 60 °C.
h, i, l, m, n, p Data between curves (ACN *vs* ACN_Ac) and within temperatures (25, 42 and 60°C) of the same curves are significantly different at P≤0.05.
ACN (-): anthocyanin, ACN_Ac (○): Acetylated anthocyanin

The values of E_a and half-life of total and single ACNs are reported in table 3.2. The value of E_a for the total ACNs was about 58 kJ/mol. This data is lower than that reported by Kechinski et al. 2010, which found a value of about 80 kJ/mol in blueberry juice. The difference may be due to the different type of tested product, suggesting that ACNs contained in the WB powder are more susceptible to temperature than that in the juice. This could be attributable to a matrix effect and/or a different pH (pH 4 or lower in case of juice) that maintains ACN stability. Considering single ACNs, as already observed from the slope values (Table 3.2), the ACNs linked to galactose such as Cy-gal, Mv-gal, Pet-gal, and Peo-gal have values of E_a higher than 70 kJ/mol. This implies that in the WB powder, the galactosylated ACNs are more heat-stable. These data are in accordance with those reported by Scibsz et al. 2009, which hypothesized a possible protective effect of galactose compared to glucose.
On the contrary, the data reported by Buckow et al. 2010 for blueberry juice, delphinidin glycosides were not the compounds decaying faster with increased temperature. Indeed, in our product the most temperature labile compounds were Pet-glc (E_a= 18.1 kJ/mol) and Cy-ara (E_a=39.0 kJ/mol) as reported in table 3.2. The possible relation between their chemical structure, such as the number of the hydroxyl groups or the glycosylation degree or the acylated form and heat stability was studied by several researchers (Rice-Evans et al. 1996, Cevallos-Casals et al. 2004, Patras et al. 2010). Unfortunately, the data reported in literature are often contradictory (Howard et al. 2012).
For example, Trôst et al. 2008 reported that ACN stability in blueberry-aronia nectar stored for over 207 days at 30°C was higher for Cy- and Peo-, and lower for Pet-, Mv- and D-glycosides. In regard to conjugated sugars, the ranking order was glucoside>galactoside>arabinoside from the most to the least stable. The greater stability of ACNs bound to glucose and galactose compared to arabinose

was proposed to be due to steric hindrance which results larger for the hexose sugars. On the contrary, Ichiyangi et al. 2001 reported that the ranking order was arabinoside > galactoside > glucoside from the most to the least stable.
From our observations, the relative amount of a single ACN did not affect its heat stability. Indeed, Pet-gal is one of the compounds present in lower amount in the WB powder but with the highest E_a (84.1 kJ/mol) (Table 1). Among the acetylated forms, Pet-glc-Ac is the most heat sensitive (E_a=7.8 kJ/mol), while Cy-glc-Ac is the compound most heat resistant (E_a= 84.3 kJ/mol).
In addition to the degradation rate, the half-life time ($t_{1/2}$) was calculated by the Arrhenius equation for the single and total ACNs in relation to the investigated temperatures (Table 4.2).
The $t_{1/2}$ values obtained for the total ACNs decay were 139, 39, 12, and 4 days at 25, 42, 60 and 80°C (data not shown), respectively. Large differences in the $t_{1/2}$ value existed among the single compounds stored at same temperatures (Table 3.2), as well as at different temperatures.

Table 3.2. Activation energy (E_a) and half-life ($t_{1/2}$) of total and individual ACNs of the FD-WB powder stored at 25, 42 and 60 °C.

Compound	**Ea (kJ / mol)**	**$t_{1/2}$ (days)**		
		25 °C	*42 °C*	*60 °C*
Total ACNs	58.3	139	39	12
Individual ACNs				
D-gal	57.8	212	60	18
D-glc	45.4	131	49	19
D-ara	64.8	256	62	16
Cy-gal	72.2	460	95	21
Cy-glc	55.7	234	69	22
Cy-ara	39.0	117	49	21
Mv-gal	73.5	608	122	27
Mv-glc	55.8	162	48	15
Mv-ara	65.3	261	63	16
Pet-gal	69.8	374	81	19
Pet-glc	18.1	86	58	40
Pet-ara	51.4	611	199	69
Pet-gal	84.1	549	87	15
Acetylated ACNs				
D-glc-Ac	62.0	625	161	45
Cy-glc-Ac	84.3	1948	310	54
Mv-gal-Ac	27.1	936	519	296
Mv-glc-Ac	62.1	295	76	21
Pet-glc-Ac	51.3	542	177	61
Pet-glc-Ac	nd	nd	nd	nd

The $t_{1/2}$ value ranged from 86 to 611 days at 25°C, from 48 to 199 days at 42°C, and from 15 to 69 days at 60°C. Thus, storage at room temperature (25°C) can induce important loss of some ACNs such as Pet-glc, Cy-ara and D-glc even though for most of them the $t_{1/2}$ value is much higher than 150 days (Table 3.2). Moreover, the acetylated forms were generally more resistant than only glycosylated compounds for all the temperatures considered. Additionally, our study found considerable changes for the different acetylated ACNs, whose $t_{1/2}$ values were from few days to 1948, 519, and 296 days at 25, 42 and 60°C, respectively (Table 3.2).
The Q_{10} values for the total and single ACNs at the temperatures investigated are presented in table 3.3. The Q_{10} values for total ACN and for each ACN decreased as temperature increased.

Table 3.3. Q_{10} values for the Total and Individual ACNs of the FD-WB powder stored at different temperatures.

Compound	**Temperature (°C)**		
	25 to 35	*42 to 52*	*60 to 70*
Total ACNs	2.1	2.0	1.8
Individual ACNs			
D-gal	2.1	2.0	1.8
D-glc	1.8	1.7	1.6
D-ara	2.3	2.15	2.0
Cy-gal	2.6	2.3	2.1
Cy-glc	2.1	1.9	1.8
Cy-ara	1.7	1.6	1.5
Mv-gal	2.6	2.4	2.2
Mv-glc	2.1	1.9	1.8
Mv-ara	2.4	2.2	2.0
Pet-gal	2.5	2.3	2.1
Pet-glc	1.3	1.2	1.2
Pet-ara	2.0	1.8	1.7
Peo-gal	3.0	2.7	2.4
Acetylated ACNs			
D-glc-Ac	2.3	2.1	1.9
Cy-glc-Ac	3.0	2.7	2.4
Mv-gal-Ac	1.4	1.4	1.3
Mv-glc-Ac	1.1	1.1	1.1
Pet-glc-Ac	2.0	1.8	1.7
Peo-glc-Ac	nd	nd	nd

In particular, for the single ACNs the highest values were observed for the low temperatures (25-35 °C), while lower Q_{10} values were observed for the higher temperatures (42-52 °C and 60-70 °C). This may be attributed to a molecular change, such as ACN polymerization, that occurs at the high temperatures and may decrease the rate of ACNs degradation (Kechinski et al. 2010). Moreover, since most of the Q_{10} values were about 2.0, the increase of temperature by 10 °C approximately doubled the decay rate (Table 3.4). In contrast to our results, Kechinski et al. 2010 observed a higher Q_{10} value (4.3 at the range from 40 to 50 °C) in highbush blueberry juice, probably due to the high content of water in juice with respect to the powder.

The values of activation energy (E_a), half-life ($t_{1/2}$) and Q_{10} were calculated for TAA of the FD-WB powder stored at different temperatures (Table 3.4). The TAA showed values of E_a (52.3 kJ mol^{-1}) and Q_{10} comparable to that obtained for the total ACNs, while the value of $t_{1/2}$ was higher, ranging from 130 days at 60 °C to 1200 days at 25 °C.

Table 3.4. Activation energy (E_a), half-life ($t_{1/2}$) and Q_{10} values of total antioxidant activity (TAA) of FD-WB powder Stored at 25, 42 and 60 °C.

Ea	***$t_{1/2}$* (days)**			***Q_{10}***		
(kJ/ mol)	*25 °C*	*42 °C*	*60 °C*	*25 to 35°C*	*42 to 52 °C*	*60 to 70 °C*
52.31	1212	387	131	2.0	1.8	1.7

Additionally, the Arrhenius equation was used to predict the shelf-life of total ACNs when stored at 4 °C. Under these experimental conditions, the half-life time for total ACNs is up to 829 days and for TAA more than 10 years.

The logarithmic reduction kinetics of total ACNs (A) and TAA (B) of the WB powder stored at different temperatures are reported in figure 3.8. The TAA and the content of ACNs decreased with increasing temperature but the reduction of the TAA does not seem directly correlated to that of the ACNs. Indeed, no significant difference (p=0.89) was observed in TAA values at 25 and 42 °C. Moreover, the logarithmic decrease of TAA at 80 and 60 °C (1.5 and 0.3) was lower in comparison to the logarithmic reduction of total ACN content (2.5 and 1.5). This result is not surprising since it has been reported that at higher temperatures (i.e. 60 and 80 °C) Maillard and caramelization reactions occur and the generated products show an increase of TAA (Tsai et al. 2005). These reactions can also occur in presence of hexoses and in absence of the aminic group (Al-Zubaidy et al. 2007). The reduction of total ACNs and the maintenance of antiradical activity have been described for several processed blueberry products (Brownmiller et al. 2008, 2009, Syamaladevi et al. 2012, Tsai et al. 2004). This is probably due to the formation of antioxidant polymers, such as low molecular weight procyanidins or the formation of degradation products of ACNs or phenolic acids which show antioxidant activity as well (Tsai et al. 2004, Oliveira et al. 2010, Seeram et al. 2001, Howard et al. 2012).

In our study, the initial TAA value of the WB powder was 58.5 mmol Trolox eq /100 g DW of product, similar to the data (52.9 mmol Trolox eq /100 g DW) obtained from fresh wild blueberry by Kalt et al. 2000. These results further confirm the importance of freeze drying process to preserve TAA. In fact, after storage for 50 days, the TAA was 48.7, 49, 41.9 and 22.5 Trolox eq TE/100 g of product, stored at 25, 42, 60 and 80 °C, respectively.

In summary, the degradation of ACNs in freeze-dried WB powder followed a first-order kinetic, thus its storage at room temperature (25 °C) reduced ACN content less in comparison to other temperatures. The decrease of single ACN monomers may be attributed to the formation of ACN polymers through a mechanism which is not well understood.

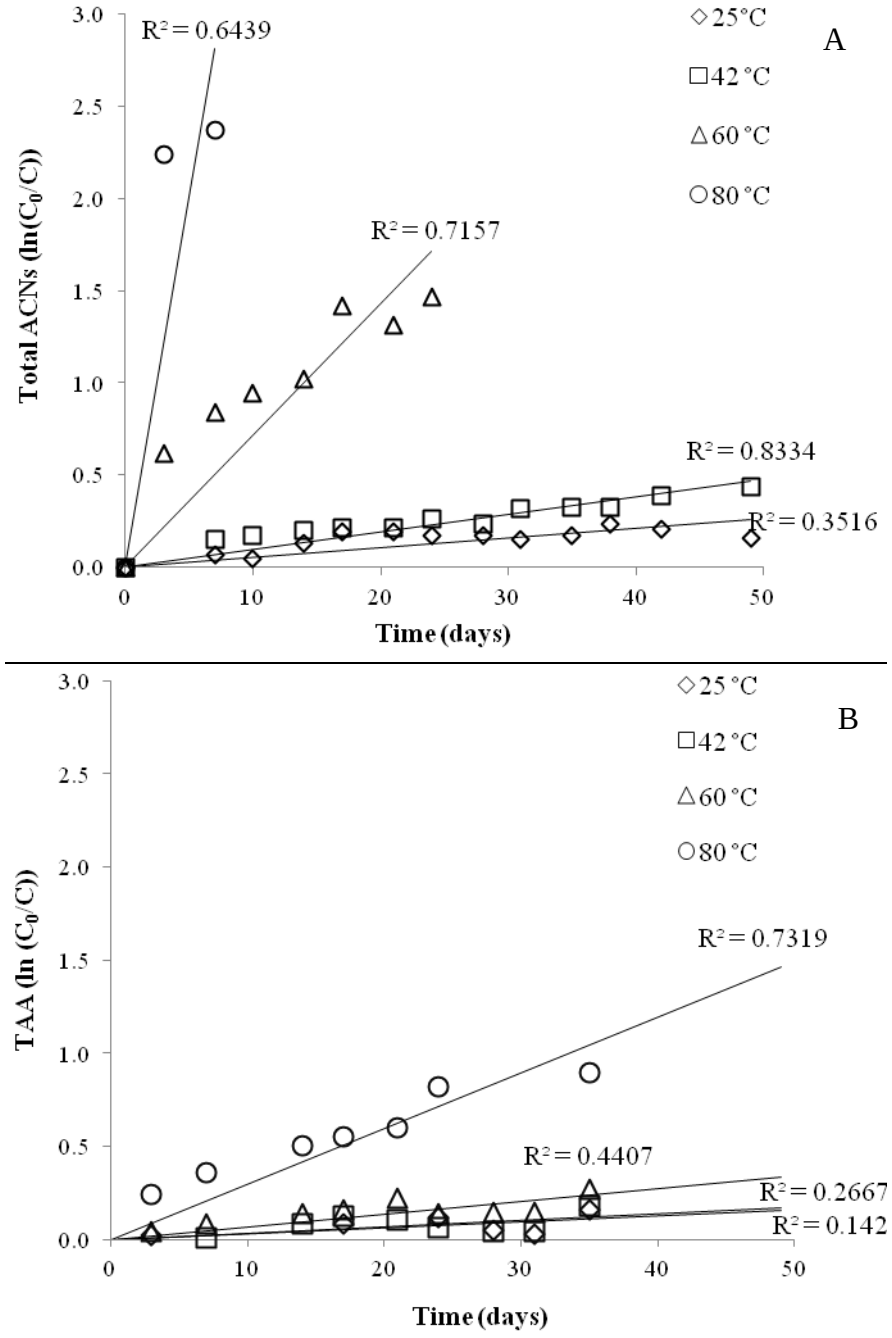

Figure 3.8. Logarithmic reduction kinetics of (A) total ACNs and (B) TAA in FD-WB powder stored at 25, 42, 60 and 80 °C.

For a better understanding, in figure 3.9 was reported the trends of total ACNs decay and TAA decrease in FD-WB powder during storage at different temperatures (25, 42, 60 and 80 °C).

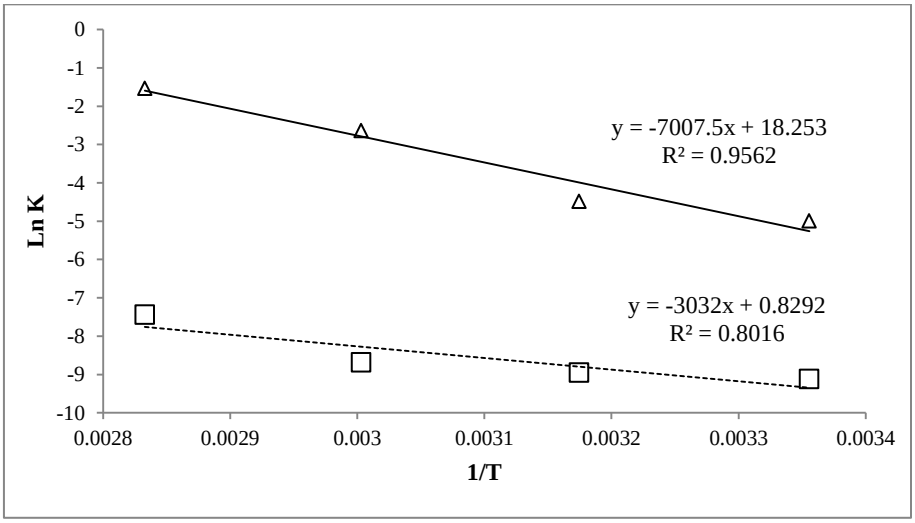

Figure 3.9. Decrease of total ACNs (Δ) and TAA (□) in FD-WB powder stored at different temperatures (25, 42, 60 and 80 °C).
(□): total antioxidant activity, (Δ): total anthocyanin

The TAA of the FD-WB powder was almost unchanged after storage at 42 °C for 50 days, suggesting that other compounds (e.g. fiber, polymers, Maillard reaction products) affect its antioxidant power. The use of this freeze-dried WB powder for food ingredients may be important since the content of ACNs and the TAA are maintained longer, up to 130 days at 25 °C, in comparison to other blueberry products. At the same time, storage of FD-WB powder would maintain stability of ACNs. This could be very important for scientists that use FD-WB powder as feed ingredient in animal and human studies and for the food industry.
The results from the present study have provided detailed information regarding the stability of anthocyanins in FD-WB powder, which was strongly dependent on temperature.
Increasing temperature during storage increased the degradation rate constants of the investigated anthocyanins. In order to keep the anthocyanin degradation rate as lower as possible, it is recommended that FD-WB powder should be kept at refrigeration temperatures.

3.2.1. Determination of unknown compounds

We reported that ACN decayed occurred quickly up to 85% at day 3 at 80 °C, while it was slower at other lower temperatures tested. Simultaneously, the appearance of a polar compound (RT 2.4 min) with UV_{max} of 284 nm and molecular weight of 128 Da was detected (Figure 3.10).

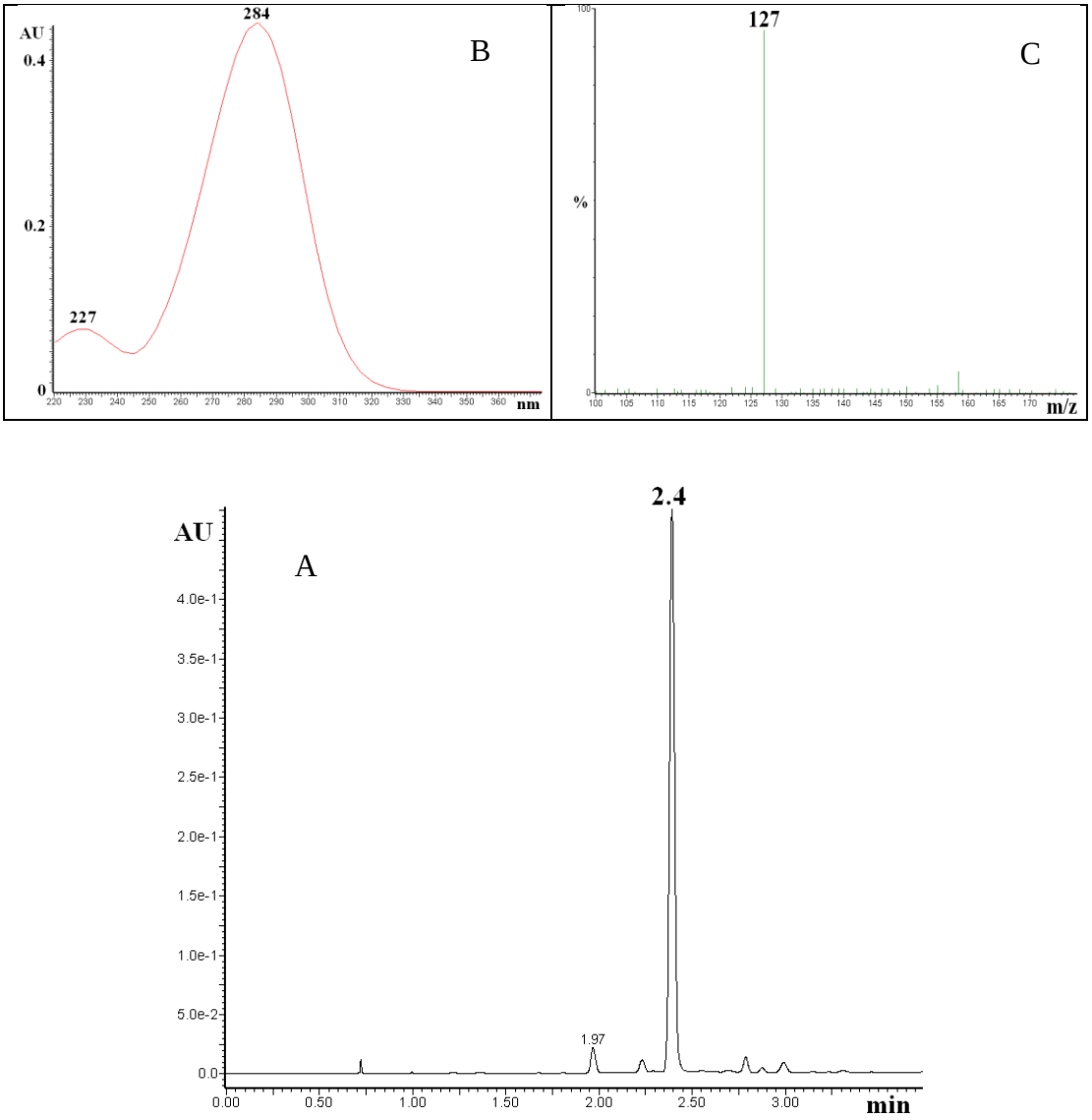

Fig. 3.10. Typical chromatogram (A), integrated at 284 nm, of FD-WB powder incubated at 80 °C for 7 days. UV (B) and negative MS spectrum (C) of the peak with RT 2.4 min.

UPLC coupled to a High-Resolution MS (LC-ESI-TOF), which operated in negative mode, was employed to confirm the identity of the unknown compound with RT 2.4 min. The resolution was 20.000, and the MS spectrum of the unknown compound showed ions with *m/z* 127,0380, corresponding to the deprotonated molecule $[M-H]^-$. The obtained value was in agreement with the empirical formula $C_6H_6O_3$, which provided two structural formulas corresponding to phloroglucinol and 5-hydroxy-methylfurfural (5-HMF). The identity of the unknown compound was then verified

by co-chromatography against reference standards and the data obtained allowed to establish that the peak with RT 2.4 min corresponds to 5-HMF (Figure 3.11).

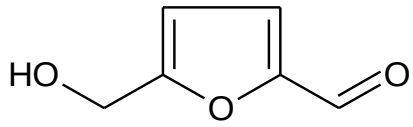

Fig. 3.11. Chemical structure of the 5-hydroxy-methylfurfural (5-HMF).

The 5-HMF was then quantified against external standard in the samples incubated at 80°C for 28 days, and the figure 3.12 reports the formation of 5-HMF and the decay of anthocyanins.
From the analysis of the obtained results, it seems that anthocyanin disappearance is correlated with the 5-HMF formation. In particular, the amount of 5-HMF produced shown a plateau in correspondence of the ACNS disappearance. Overall, it seems there is an inverse correlation between 5-HMF formation and ACNs decay or vice versa. It is known that in acidic solutions the most probable compounds derived from sugars are furfural and 5-hydroxymethylfurfural (Shinoda et al. 2005), which in turn were proved to be responsible for the highest degradation when added to the blackberry anthocyanin buffer solution at pH 3.45 at 24 °C.

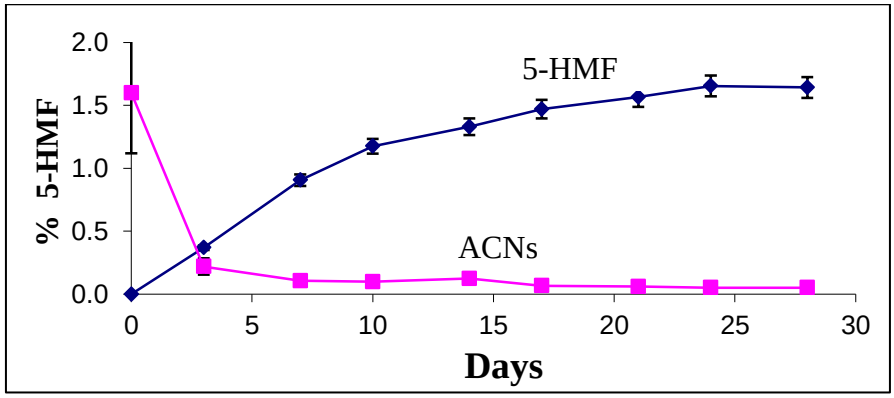

Figure 3.12. Kinetics of 5-HMF and Total FD-WB ACNs during stability experiments (80 °C). Data are reported as means ± s.d.

5-Hydroxymethylfurfural is an organic compound derived from dehydration of certain sugars, such as fructose and glucose (Rosatella et al. 2011). FD-WB contains high percentage of fructose and glucose, equal to 37±0.5 and 32±0.2%, respectively, thus the total percentage of monomeric sugar in FD-WB is about 70%. Therefore, if the 5HMF derived from the sugars present in the FD-WB his amount in samples incubated at 80 °C should be significantly higher, considering that stoichiometric ratio between sugar and 5-HMF is approximately 1.
High sugar concentration and reduced A_w in fruit has an overall protective effect on anthocyanins (Wrolstad et al. 1990, Erlandson and Wrolstad 1972). Dried anthocyanin powders in hermetically sealed containers at A_w lower than 0.3 are relatively stable at room temperature for several years

(Bronnum-Hansen and Flin, 1985). Above a threshold level such as 100 ppm, sugars and their degradation products accelerate the degradation of anthocyanins (Calvi and Francis, 1978), and fructose, arabinose, lactose and sorbose have greater degradative effects on anthocyanins than glucose, sucrose or maltose (Tinsley and Bockian, 1960). The rate of anthocyanin degradation is associated with the rate at which the sugar is degraded to furfural-type compounds (Markakis *et al.*, 1957), such as furfural and 5-hydroxymethylfurfural (HMF). These compounds are derived not only from Maillard reaction but also from oxidation of ascorbic acid, polyuronic acids, or anthocyanins themselves. Anthocyanin degradation in the presence of furfural and HMF is temperature dependent and more obvious in natural systems, such as fruit juice, and 0xygen enhances the degradative effects of all sugars and sugar derivatives (Jackman and Smith, 1996).
To test the possible contribution to the antioxidant activity of the FD-WB stored at different temperatures, it was determined the TAA of standard solutions of 5-HMF. The results obtained showed clearly that, the amount present in the products tested, the 5-HMF has no antioxidant activity. Thus, the antioxidant activity of FD-WB powder stored at different temperatures must be sought in other metabolites, such as for example the condensation products.
About the 5HMF ingested with the intake of blueberry products not well preserved, it remains to determine what its effects on human health (Figure 3.13).

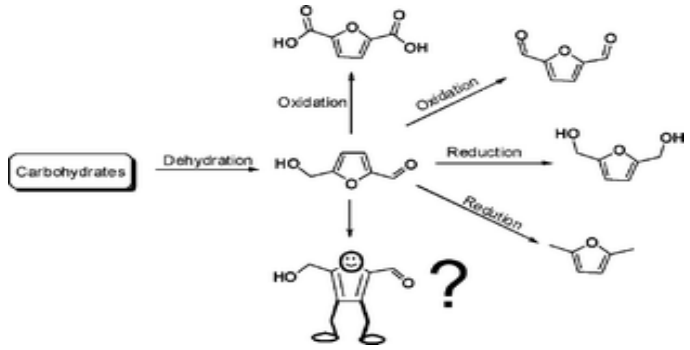

Figure 3.13. Formation of 5-HMF and its possible degradation products in human.

3.3. Anthocyanin absorption, metabolism, and distribution from a FD-WB in Sprague-Dawley rat

Through the years, anthocyanin biological activity has been overlooked given their poor absorption in the gastrointestinal tract (GIT). It has been only in the last decade that methods have become available that allow for detection and quantitation of anthocyanins in biological samples. Unlike many other flavonoids, anthocyanins are not well degraded by gut microflora, judging by the visual color of feces from animals fed anthocyanin rich diets (Brouillard 1982). Because of the close contact between gut content and colon epithelium cells, direct influence of anthocyanins to gut health including colon cancer is plausible. Actually, our hypothesis is supported by the fact that to date all of the cancers that may be inhibited by anthocyanins are related to the GIT. Breast cancer, on the other hand, was found not effectively inhibited by anthocyanins. Felgines et al. 2002 were probably among the first to report anthocyanin availability in the cecal contents. However to date there is no established method available for analysis of fecal anthocyanins. In our study we developed such a method based on reported procedures for cecal content anthocyanin extraction (Felgines et al. 2002) as well as the fecal quercetin extraction (Aura et al. 2002) in order to systematically study anthocyanins in the gut contents.
Only few studies have investigated the metabolic fate of ACNs introduced through food sources by analyzing the distribution of ACNs and their metabolites in plasma and tissues and their excretion in urine and feces following long periods of supplementation. Since ACNs are rapidly metabolized, the majority of studies have taken the pharmacokinetic approach (i.e., one dose, acute study) that has the advantage of allowing one to track the compounds under study in the short term, but the disadvantage is that one is not able to examine their absorption, metabolism, and excretion after long-term exposure. This is the first study that has investigated the time effect (i.e., 4 vs 8 weeks) of exposure to ACNs on the absorption, metabolism, and distribution of ACNs and their metabolites following the consumption of wild blueberries powder.

3.3.1. Growth Rate and Body Weight

Control and FD-WB rat groups exhibited the same growth rate at 4 and 8 weeks. The final mean body weights were 351±21 g for the FD-WB group and 354±32 g for the control group at 4 weeks, and 459±21 g for the FD-WB group and 444±20 g for the control group at 8 weeks respectively. No statistically significant difference in body weight or food intake was detected between the diet groups either at 4 or at 8 weeks of diet duration.

3.3.2. Validation Method

The lower limit of detection (LLOD) was 1 ng/mL for Mv-glc, 4 ng/mL for D-glc, and about 2 ng/mL for the other anthocyanins. Regarding phenolic acids, the LLOD was 2 ng/mL for BA and IS and about 4 ng/mL for the other acids. The accuracy of the extraction for anthocyanins and phenolic acids was 89±4 and 91±4 %, respectively. The precision of the method was tested by both intraday (n=3) and interday (5 days, n=3) reproducibility, and the coefficient of variation was below 12.5 %. Regarding repeatability, a maximum relative standard deviation of 5.2 % was observed for triplicate injections. Due to the presence of the flavylium ion at lower pH values, the positive ESI ion mode was selected as it was more sensitive for FD-WB ACNs determination in biological sample. The anthocyanins fragmentation was obtained at low values of collision energy, which gave the product ions corresponding to $[M]^+$. In figure 3.14 was reported the MS and MS^2 of the cyanidin-glucoside. Also for the other anthocyanins their respective aglycones were the main products of fragmentation.

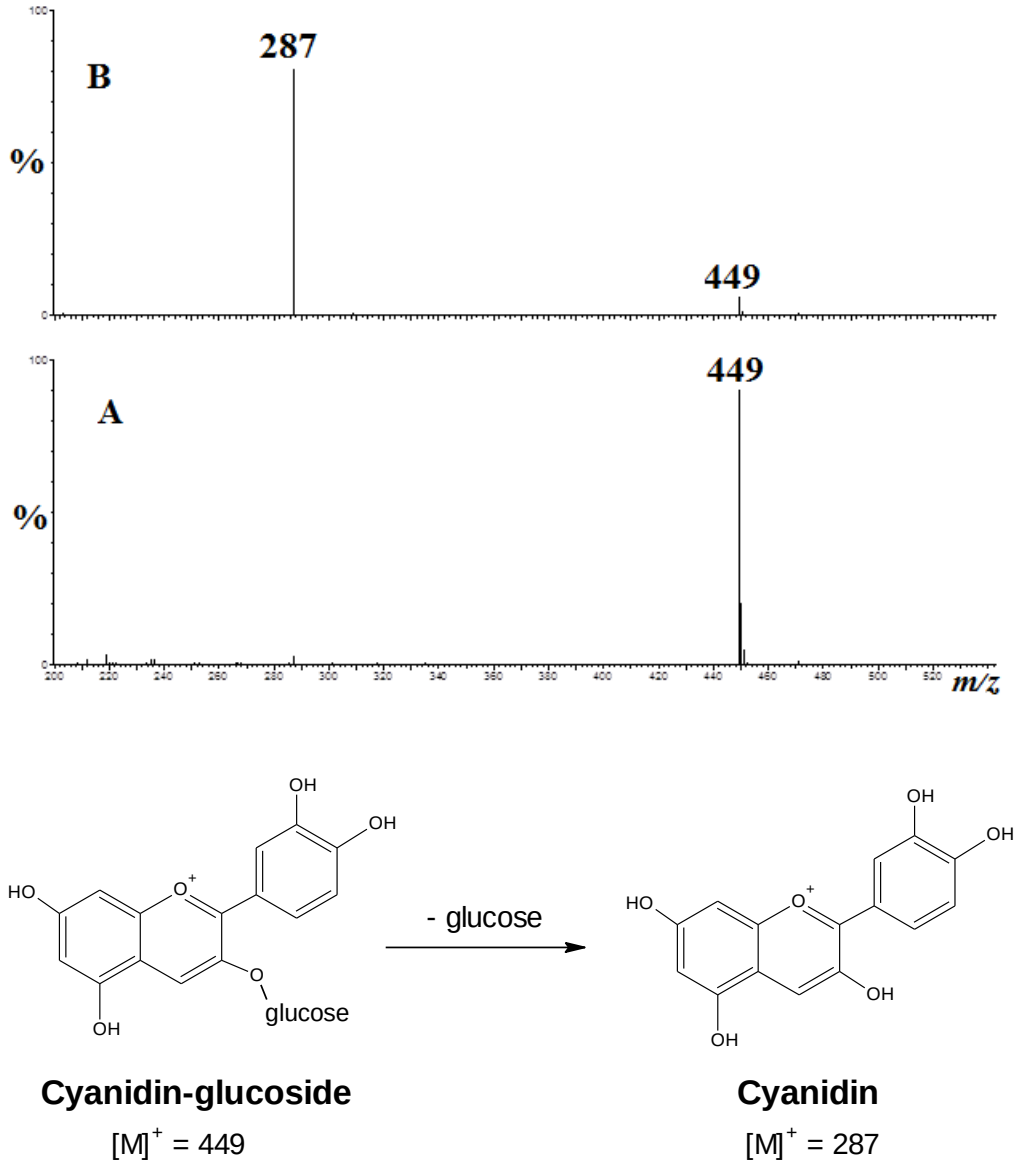

Figure 3.14. Mass spectrum (A) and (B) fragmentation pattern of cyanidin-glucoside obtained at collision energy of 20 eV.

The negative ESI ion mode was selected as it was more sensitive for GA, 4-HBA, DHBA, SA, VA, HA and BA, and gave a better fragmentation. The collision-induced dissociation (CID) spectrum of DHBA gave a typical product ion with m/z 109 corresponding to $[M\text{-}COO]^-$ and $[M\text{-}COO]^{\bullet-}$, respectively. In the same way, the other acids fragmentation gave product ions corresponding to $[M\text{-}COOH]^-$ and $[M\text{-}COO]^{\bullet-}$, and in figure 3.15 MS and MS^2 spectra of DHBA are reported. Thus, these compounds gave common product ions resulting from homolytic cleavage with formation of a radical product, such as $[M\text{-}COO]^{\bullet-}$, and this behavior was presumably related to the stability of the

derived benzyl radical. On the contrary, the heterolytic cleavage produce the fragment corresponding to $[M\text{-}COO]^-$, and it should be highlighted that heterolytic cleavage is predominant, above all at lower collision energy. Also hippuric acid gave product ions with $(m/z)^-$ 134 corresponding to $[M\text{-}COO]^-$, suggesting that a rearrangement takes place during the fragmentation.

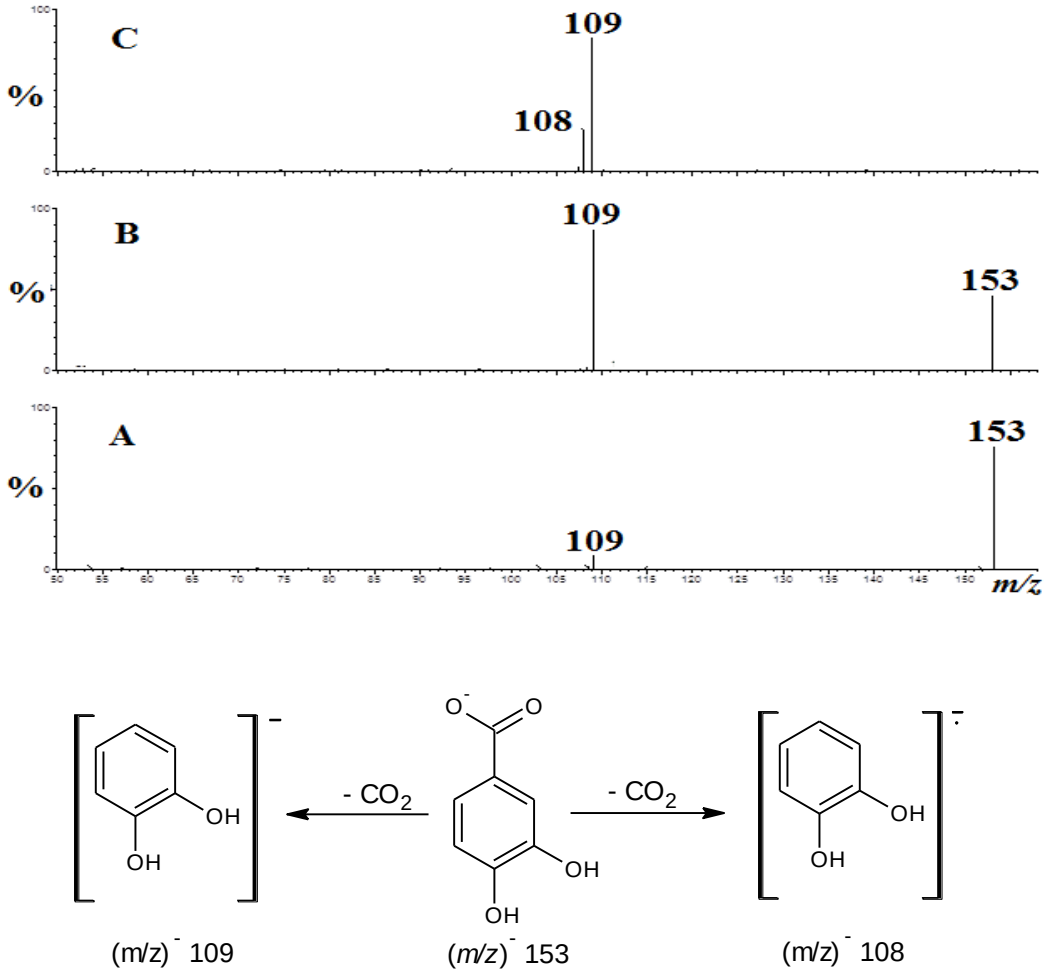

Figure 3.15. Mass spectrum (A) and fragmentation pattern of DHBA obtained at (B) lower and (C) higher different collision energy.

3.3.3. Evaluation of ACNs in the Plasma, Liver, Brain, Urine, and Feces

Anthocyanins were not detected in plasma, tissues, urine, and feces in control group (C) and, at time zero, in the FD-WB group. At the time of collection urine samples showed no red coloration, but immediately became red light after acidification with TFA. This color change was attributed to the re-equilibration of the chemical forms of anthocyanin in acidic pH; in fact under neutral pH condition ACNs were predominantly in colorless pseudobase form, but under acidic pH most anthocyanins converted to the red colored flavylium form. ACNs were found in the urine of the FD-WB group and not of the C group after 4 or 8 weeks of dietary treatment, and their concentrations are reported in table 3.5. Total ACNs in urine significantly increased after 8 weeks of FD-WB consumption (1990±929 ng/24 h) with respect to 4 weeks (887±299 ng/24 h). In particular, we obtained significantly higher differences after 8 weeks of FD-WB consumption ($p<0.05$) for the following ACNs: Cy-gal, Cy-glc, Pet-gal, Peo-gal, Pet-ara, Mv-gal, and Mv-glc-ac. Total ACN content in the feces after 4 and 8 weeks of FD-WB treatment was 77±43 μg/24 h and 55±40 μg/24 h, respectively, with main ACNs, Peo-gal, Pet-ara, and Peo-glc-ac. Individual ACN concentrations decreased at 8 weeks with respect to 4 weeks, but the differences were not statistically different. Three different acetylated forms were also identified in the feces: Mv-glc-ac, Pet-glc-ac, and Peo-glc-ac. Anthocyanins were not detected in the plasma, liver, and brain samples after 4 or 8 weeks of dietary treatment either in the C or WB diet groups.

3.3.4. Determination of phenolic acids in biological sample

The amount of phenolic acid in plasma (ng/ml), urine and feces (μg/24 h) of rats after the consumption of a C or a FD-WB diet for either 4 or 8 weeks was reported in table 3.6. No significant differences were detected in total plasma metabolite concentrations between the C and the FD-WB groups after 4 and 8 weeks of diet treatment. Hippuric acid (HA) was the main metabolite present in the urine of the C and FD-WB groups, and its concentration significantly increased ($p<0.05$) after 8 weeks of FD-WB consumption compared to that after 4 weeks. Hippuric acid concentration in the C group was significantly lower compared to that of the FD-WB group either after 4 or 8weeks of dietary treatment (Table 3.6). Lower amounts of GA and SA were detected in the urine of the FD-WB group which slightly increased from 4 to 8 weeks, whereas DHBA significantly increased after 8 weeks of FD-WB diet. 4-HBA was also present in the C group but was higher ($p<0.05$) in the FD-WB group. Benzoic acid was not detected in the urine possibly due to its poor solubility or/and rapid conjugation with glycine to produce HA. The principal phenolic acid present in feces after 4weeks of FD-WB consumption was DHBA (7±2 μg/24 h), while after 8 weeks, a significant reduction ($p<0.05$) was observed, indicating that this compound may be metabolized to BA by intestinal microflora. Indeed, after 8 weeks of FD-WB intake, BA remained unmodified. Some metabolites such as 4-HBA and HA were also present in the C groups, but they were lower and significantly different ($p<0.05$) from those found in the FD-WB group at four weeks. Benzoic acid was the main metabolite present in the liver and brain with other phenolic acids present in trace amounts. Total concentration of BA in the liver was 41±7 μg/g in the C groups and 43±5 μg/g and 48±7 μg/g after 4 and 8 weeks of FD-WB supplementation, respectively. Regarding the brain, the total amount of phenolic acids was 69±0.6 μg/g in the C groups and 65±4 μg/g and 63±3 μg/g after 4 and 8 weeks of FD-WB intake, respectively.

Table 3.5. Anthocyanin content in urine and feces of Sprague-Dawley Rats after 4 and 8 Weeks on the FD-WB Diet[a].

ACN	Urine (ng/24h)		Feces (μg/24h)	
	4 weeks	8 weeks	4 weeks	8 weeks
D-gal	42.7 ± 16.4	84.5 ± 61.7	0.8 ± 0.6	0.8 ± 0.8
D-glc	45.4 ± 20.4	81.4 ± 67.8	0.9 ± 0.8	0.9 ± 1.0
Cy-gal	74.3 ± 27.6[a]	219.2 ± 149.8[b]	0.9 ± 0.8	1.1 ± 1.1
D-ara	60.9 ± 24.6	123.9 ± 93.8	1.1± 0.8	1.4 ± 1.6
Cy-glc	45.5 ± 17.9[a]	108.5 ± 80.0[b]	0.6 ± 0.4	1.2 ± 1.1
Pet-gal	61.3 ± 18.7[a]	153.5 ± 33.8[b]	2.4 ± 0.8	2.6 ± 2.3
Cy-ara	29.2 ± 13.6	46.9 ± 25.5	1.0 ± 0.8	1.3 ± 1.1
Pet-glc	38.4 ± 13.6	60.1 ± 31.1	1.9 ± 0.9	2.3 ± 2.2
Peo-gal	192.1 ± 57.5[a]	553.7 ± 157.0[b]	6.6 ± 2.1	9.6 ± 6.0
Pet-ara	157.6 ± 42.2[a]	323.9 ± 146.3[b]	9.1 ± 2.6	11.5 ± 8.1
Mv-gal	55.4 ± 12.1[a]	98.0 ± 14.3[b]	nd	nd
Peo-glc	16.4 ± 9.0	25.3 ± 14.2	nd	nd
Mv-glc	4.3 ± 3.0	13.3 ± 19.3	2.5 ± 2.3	4.7 ± 5.3
Peo-ara	7.7 ± 4.0	15.5 ± 8.5	2.4 ± 2.0	0.8 ± 0.4
Mv-ara	5.6 ± 5.1	9.8 ± 6.2	3.6 ± 2.5	1.2 ± 0.8
Mv-glc-ac	50.1 ± 13.1[a]	72.3 ± 19.7[b]	6.0 ± 4.1	2.2 ± 1.2
Pet-glc-ac	nd	nd	5.1 ± 3.8	1.9 ± 0.9
Peo-glc-ac	nd	nd	31.7 ± 18.1[b]	11.9 ± 5.9[a]
Total	886.8 ± 298.8[a]	1989.6 ± 929.2[b]	76.7 ± 43.5	55.4 ± 39.9

[a] Data are expressed as the means ± standard deviation. For urine, means between groups not having the same letters are statistically different at $p<0.05$.
nd: not detectable.

Table 3.6. Amount of phenolic acid in plasma (ng/mL), urine and feces (μg/24 h) of SD rats after 4 and 8 Weeks on control and FD-WB Diets[a].

Phenolic acids	4 weeks		8 weeks	
	Control	WB diet	Control	WB diet
	Plasma (ng / ml)			
GA	nd	nd	nd	nd
SA	nd	nd	nd	nd
DHBA	nd	nd	nd	nd
4HBA	4.0 ± 0.4	5.0 ± 1.2	3.8 ± 0.3	3.3 ± 0.7
BA	430.6 ± 64.1	489.8 ± 54.4[a]	408.1 ± 90.5	319.4 ± 39.3[b]
HA	14.0 ± 13.6	29.6 ± 12.4	35.3 ± 13.6	31.2 ± 4.6
Total	448.6 ± 78.1	524.4 ± 68.0[a]	447.2 ± 104.4	353.9 ± 44.6[b]
	Urine (μg / 24h)			
GA	nd	136.4 ± 22.2	nd	159.8 ± 39.7
SA	nd	nd	nd	721.4 ± 801.1
DHBA	nd	122.3 ± 12.9[a]	nd	182 ± 67.8[b]
4HBA	564.1 ± 294.9[a]	2749.4 ± 1353.1[b]	617.5 ± 63.1[a]	3760.9 ± 1956.1[b]
BA	nd	nd	nd	nd
HA	301.8 ± 124.9[a]	13020.1 ± 7190.1[b]	653.9 ± 239.1[c]	25230.2 ± 4730.1[d]
Total	302.4 ± 125.2[a]	13023.1 ± 7191.4[b]	654.5 ± 239.2[c]	25234.8 ± 4733.5[d]
	Faeces (μg / 24h)			
GA	nd	1.6 ± 0.7[a]	nd	0.7 ± 0.5[b]
SA	nd	nd	nd	nd
DHBA	nd	6.7 ± 2.4[a]	nd	1.6 ± 1.1[b]
4HBA	0.7 ± 0.3[b]	2.1 ± 1.5[a]	1.0 ± 0.5[b]	0.7 ± 1.1[b]
BA	nd	1.9 ± 2.1	nd	2.0 ± 4.3
HA	0.6 ± 0.2[b]	23.9 ± 42.1[a]	1.3 ± 0.9[c]	10.0 ± 19.3[ac]
Total	1.3 ± 0.5	36.2 ± 48.8	2.3 ± 1.4	14.9 ± 26.3

Figure 3.16 shows an example of phenolic acids peaks detected by UPLC-MS/MS in a feces sample obtained 8 weeks after the daily intake of FD-WB.

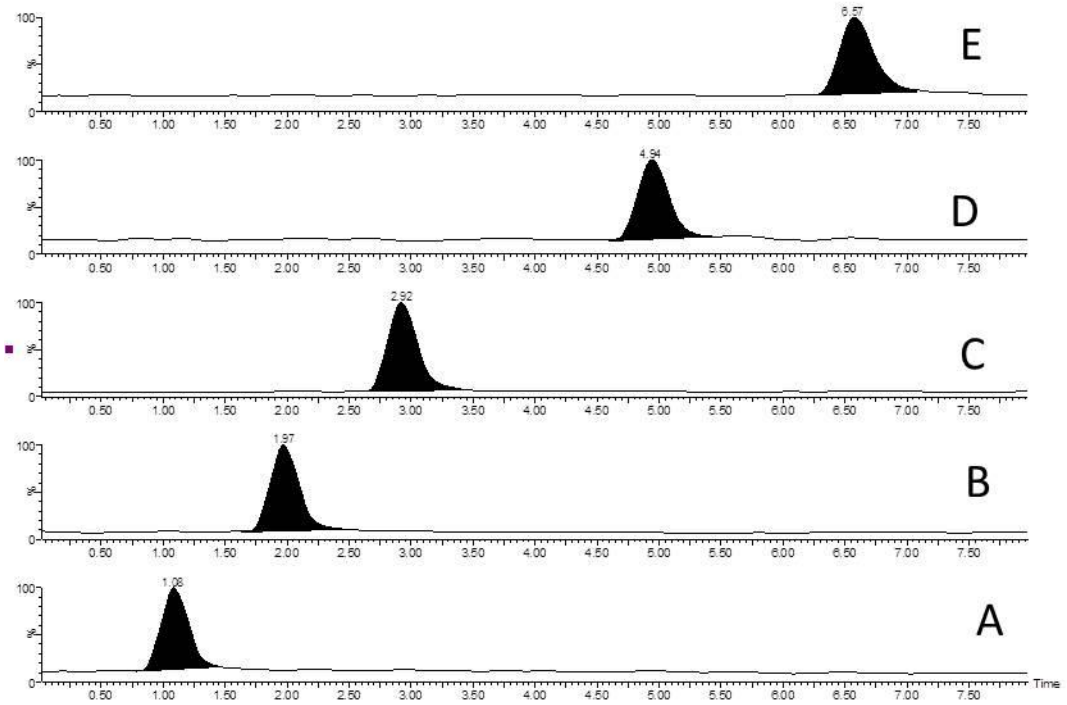

Fig. 3.16. Typical UPLC-MS/MS chromatograms of a feces sample collected 8 weeks after the daily intake of 24±5 mg of FD-WB anthocyanins.
A: GA, RT 1.1 min, $(m/z)^-$ 169 → 125
B: DHBA, RT 2.0 min, $(m/z)^-$ 153 → 109
C: 4HBA, RT 3.0 min, $(m/z)^-$ 137 → 93
D: HA, RT 4.90 min, $(m/z)^-$ 178 →134
E: BA, RT 6.6 min, $(m/z)^-$ 121 → 77

3.3.5 Conclusion

Data obtained in the present study confirm previous observations on ACN absorption and include some new information on ACN metabolism as well as their metabolites. The bioavailability of ACNs has been investigated in different human and animal pharmacokinetic studies demonstrating that the maximum level of ACNs in serum is found between 2 and 4 h after consumption and very low concentrations are reached (Mazza et al. 2002, Matsumoto et al. 2001). The rapid absorption of ACNs could be dependent and mediated by specific enzymes such as bilitranslocase present in the stomach (Passamoti et al. 2003). From these studies, the time/rate of absorption of ACNs does not seem to be different between rats and humans. In a recent study, ACNs were not detected in the plasma of rats at 3, 6, and 24 h after a meal containing blackberry powder (Felgines et al. 2002). This is also in accordance with data by Kalt et al. 2008, where no anthocyanins were detected in the plasma of pigs supplemented with blueberry powder after 18-21 h of fasting. Accordingly, in the present study, we also could not detect anthocyanins in the plasma of rats since blood samples were obtained from animals that consumed the last blueberry-enriched meal 3-4 h before euthanization. Thus, the above data confirm the rapid absorption and metabolism of ACNs.

Urinary excretion is often used to assess ACN absorption and metabolism. Matsumoto et al. indicated that ACN 3-glycosides can be excreted in urine as intact forms in rats within 4 h from ingestion (Matsumoto et al. 2001, 2006). The excretion of ACNs after ingestion of cranberry juice was also investigated in humans; ACN urinary levels reached a maximum, between 3 and 6 h after consumption (Ohnishi et al. 2006). Similarly in another human study, urinary levels of ACNs reached a maximum concentration after 4-8 h of black raspberry consumption and decreased during the following 8-12 h (Tian et al. 2006). These data suggest that ACNs are excreted rapidly with kinetics that appears to be comparable in rats and humans. In the present study, the content of ACNs in the 24 h urine of rats fed a FD-WB diet for 4 and 8 weeks was reported. Studies in which a short-term (10-12 days) feeding with blackberry or red orange juice were performed, ACNs were excreted in the urine as intact forms, while no aglycones or conjugated forms were detected (Felgines et al. 2002, 2006). Anthocyanin content in the urine significantly increased at 8 weeks compared to that at 4 weeks (i.e., Cy-gal, Cy-glc, Pet-gal, Peo-gal, Pet-ara, Mv-gal, and Mv-glc-ac). This observation may be explained by saturation of storage.

Anthocyanins that were not absorbed or excreted by bile were detected in the feces. Peo-gal, Pet-ara, and Peo-glc-ac were the dominant excretory products after WB consumption. An increase (but not significant) of ACNs was detected in the feces of rats fed for 8 weeks compared to those for 4 weeks particularly for D-ara, Cy-glc, Pet-gal, Cy-ara, Pet-glc, Peo-gal, Pet-ara, and Mv-gal. Interestingly, Peo-gal and Pet-ara were among the major ACNs excreted not only in the urine but also in the feces. Since their content in the WB powder was very low, the presence in urine and feces may be due to the methylation of Cy and D as reported by different studies (Felgines et al. 2002, Prior et al. 2004). Differences in the type of monosaccharide present in the molecule may affect absorption and ACN metabolism (Kurilich et al. 2005, Wu et al. 2002, McGhie et al. 2003).

It is known that ACNs and polyphenols in general can be metabolized and transformed by intestinal microflora to phenolic acids (Wu et al. 2009, Vitaglione et al. 2007). These compounds can be further absorbed and detected in the blood following ACN intake. In fact, intestinal microflora exhibits a significant hydrolytic potential since they can cleave glycosidic bonds and generate degradation products such as aglycons and phenolic acids that are metabolized by the liver (Toromanovic et al. 2008). Vitaglione et al. 2007 demonstrated that 3,4-dihydroxy-benzoic acid (protocatechuic or DHBA) was the main metabolite present in the bloodstream and was excreted in the feces of human volunteers consuming one liter of blood orange juice providing mainly Cy-glc. In the present study, we detected different phenolic acids in the plasma and in particular, BA. Since BA was also present in the control rats, we may postulate that benzoic acid is not only related to ACN metabolism but its metabolism may depend on many other factors such as intake of fiber or amino acids and the type of intestinal microflora (El Mohsen et al. 2006, Aura et al. 2008, Gonthier et al. 2003, Visser et al. 2002). Moreover, our results suggest that the concentration of BA (but also HA) in the plasma was not dependent on the duration of the dietary treatment and cannot be considered a good marker of ACN absorption under our present experimental conditions.

Recently, Nurmi et al. 2009 studied the metabolism of berry ACNs to phenolic acids in subjects consuming bilberry-lingonberry puree. The excretion of phenolic acids was demonstrated at 4-6 h after the consumption of the puree. The principal anthocyanin metabolites detected in the urine were homovanillic and VA. In our study, the principal ACN metabolite in the urine was HA; this metabolite is produced in the liver through a conjugation of glycine with aromatic phenolic acids such as BA (Gonthier et al. 2003). Hippuric acid represents the final product of the metabolic pathway of ACNs but also of amino acids and fiber. In the present study, HA concentration in the urine significantly increased after 4 and 8 weeks of blueberry consumption with respect to the control group. These results confirm that HA represents an important product of ACN metabolism. The BA, a partially insoluble compound, is absorbed and conjugated with glycine by liver microsomes to produce the more polar compound HA, which is easily excreted in the urine. In fact, unlike benzoic acid, HA did not reach a steady-state in the urine but significantly increased at 8 weeks with respect to 4 weeks. This increase in excretion could be attributed to an increase in the extent of absorption, saturation of storage, or to an activation of detoxification systems. Thus, the

presence of HA in the urine could be related to ACN absorption. This hypothesis is well supported by the fact that the HA content of the control group at 4 and 8 weeks was much lower ($p<0.05$) than that of the WB group.
In the feces, the principal phenolic acids were DHBA at 4 weeks and HA at 8 weeks. This is in agreement with data obtained in humans by Vitaglione et al. 2007.
Traces of BA were also present in the liver and brain of all diet groups, while no ACNs were detected. Talavera et al. 2005 reported that the methylated and glucuronidated forms were the main metabolites present in the liver, while just traces of native ACNs were found. The native forms of ACNs were instead found in the brain of rats that were sacrificed 3 h after the beginning of the last meal composed of blackberry extract in contrast with our study in which no ACNs were detected.
We may conclude that ACNs from a WB diet are bioavailable and are detected in the feces and urine of rats after 4 and 8 weeks; this process is enhanced on the basis of the duration of diet exposure. However, since they are rapidly metabolized and our animals were sacrificed 3-4 h from the last meal, we were not able to detect their native forms in the plasma, liver, or brain tissues. Phenolic acids and in particular HA were the principal metabolites detected in all analyzed tissues. The amount of metabolites increased with time of exposure to the ACNs, suggesting a modulatory effect on metabolic pathways or an increased efficiency in absorption/excretion in the older animals (8 vs 4 weeks). Additionally, HA (the ultimate product of ACNs degradation) detected in urine could represent a potential marker of ACN absorption under our experimental conditions.
Overall, the data obtained in vivo suggest that FD-WB anthocyanins are extensively metabolized by the intestinal micro flora, which mainly produces benzoic acid. This metabolite is then reabsorbed by the enterohepatic circulation and, as sparingly soluble in water, conjugated with glycine and excreted in the urine as hippuric acid (Figure 3.17). The reaction intermediates are represented by benzoic acids derived from the oxidation of the ring B of anthocyanins. The reaction intermediates are represented by benzoic acids such as GA, 4HBA, DHBA, VA and SA derived from the oxidation of the ring B of anthocyanins. The intermediates are heavily metabolized to benzoic acid which, after conjugation with glycine, is excreted in the urine. However, more studies will be necessary to understand the metabolic fate of ACNs.

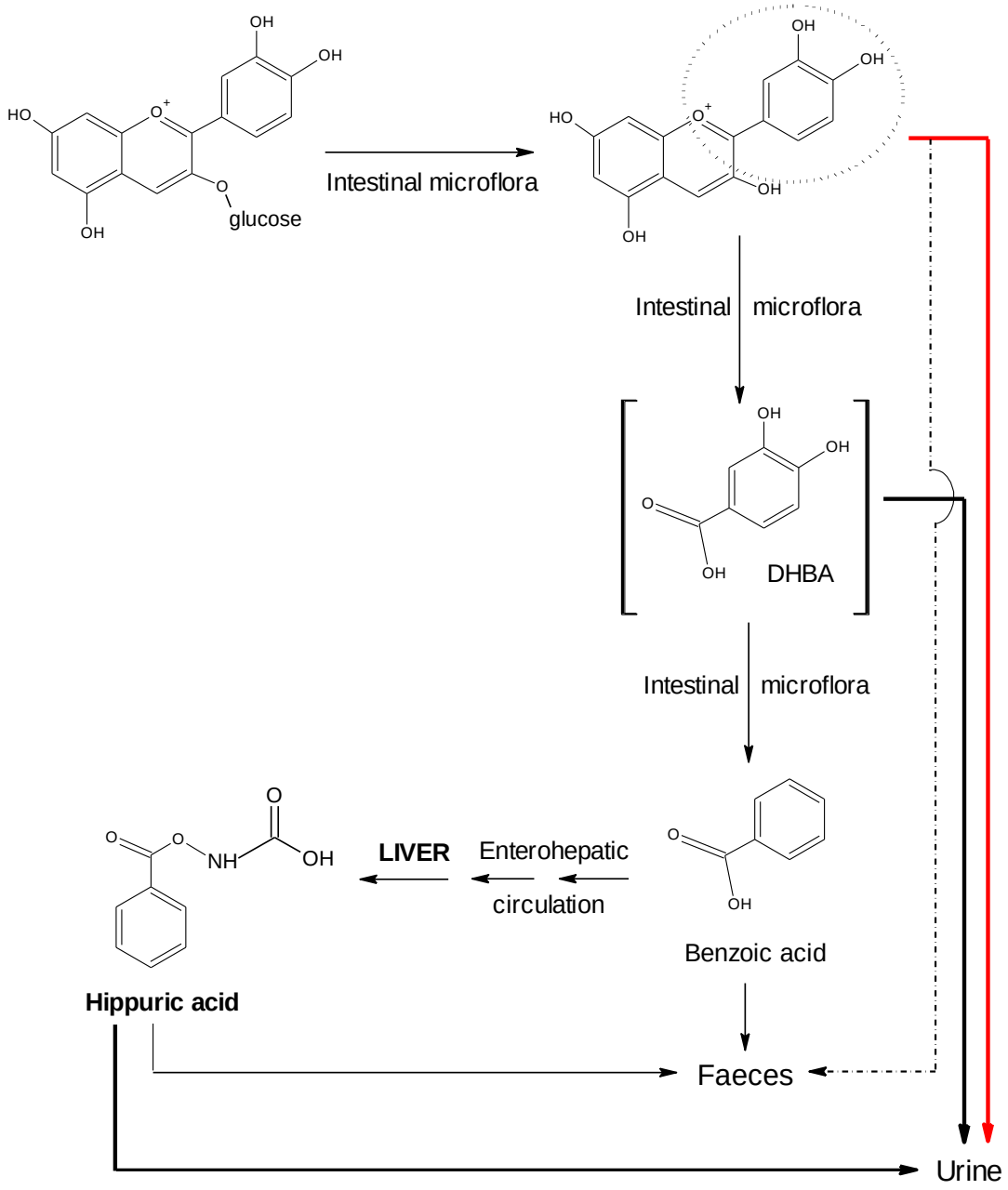

Figure 3.17. Schematic representation of metabolism and elimination of cyanidin-glucoside based on obtained results. The darkest lines are the main routes of elimination.

3.4. Biotransformation of FD-WB anthocyanins *in vitro*

Until now, most of the research on the biological activities of anthocyanins with regard to their potential health effects has been performed *in vitro*. For that reason, research in the last years focused on the bioavailability and biotransformation of anthocyanins, which could be major determinants of the biological activity of these compounds *in vivo*. The bioavailability of ACNs was investigated in humans after the consumption of anthocyanin-rich food like blackcurrant juice (Netzel et al. 2001), red grape juice and red wine (Bub et al. 2001), elderberries and blueberries (Wu X et al. 2002) and strawberries (Felgines et al. 2003). **One consistent result of these studies is that the systemic bioavailability of anthocyanins is very poor.** Only 0.02-1.8 % of the ingested anthocyanin doses were excreted unchanged or as phase II metabolites in the urine of the volunteers. For example, Felgines et al. 2003 found that pelargonidin-3-glucoside, which was ingested by volunteers with a meal containing 200 g of strawberries, was metabolized to three mono-glucuronides, one sulfo-conjugate and the aglycone pelargonidin. The total urinary excretion of all detected anthocyanin metabolites was 1.8 % of Pelargonidin-3-glucoside ingested. Thus, the question was: **what happened to more than 98% of the anthocyanins in the human body?**
In the present study, we investigated the biotransformation of anthocyanins by using two *in vitro* models. Firstly, we determined the metabolism of anthocyanins in the gut by incubation of pure anthocyanins (Mv-glc and Cy-glc) and FD-WB with suspensions of human faecal microflora under anaerobic conditions. Secondly, we incubated pure anthocyanins (Mv-glc and Cy-glc) and FD-WB with rat microflora obtained from different sections of the intestine. The analysis of the incubation extracts was carried out by UPLC-DAD-MS and UPLC-MS/MS techniques.

3.4.1. Stability test

Anthocyanidins are unstable compounds in neutral media. Thus, to determine their stability we incubated Cy-3-glc, Cy-3-ara, Peo-3-glc, Peo-3-ara, Pet-3-glc, D-3-glc, Mv-3-glc, Cy, Mv, Pet, Peo at concentrations of 90 μM and FD-WB (10 mg/ml) in phosphate buffer (pH 7) at 37 °C in anaerobic conditions and followed the degradation by UPLC-DAD-MS analysis.
After 20 min the amount of incubated ACNs drastically reduced and simultaneously appeared several peaks not present at time zero. For example, the representative chromatogram of the degradation process of Mv is shown in figure 3.18. Peak 1-5 has an anthocyanin-like absorption spectrum indicating that the flavylium structure is still present. The positive MS spectra, which were obtained at lower cone voltage (Fig. 3.19), showed molecular ions with *m/z* 677 (peak 1-3), 683 (peak 2) and 675 (peak 5). These peaks were probably dimerization products of two Mv units, because at higher pH the anthocyanidins in neutral media is the reactive quinoidal base. In addition to these dimers, some of which were also detected in the case of all other anthocyanidin, small amounts of the corresponding phenolic acid (DHBA for Cy and SA for Mv) was detected. Similar results were obtained by analyzing solutions containing FD-WB.

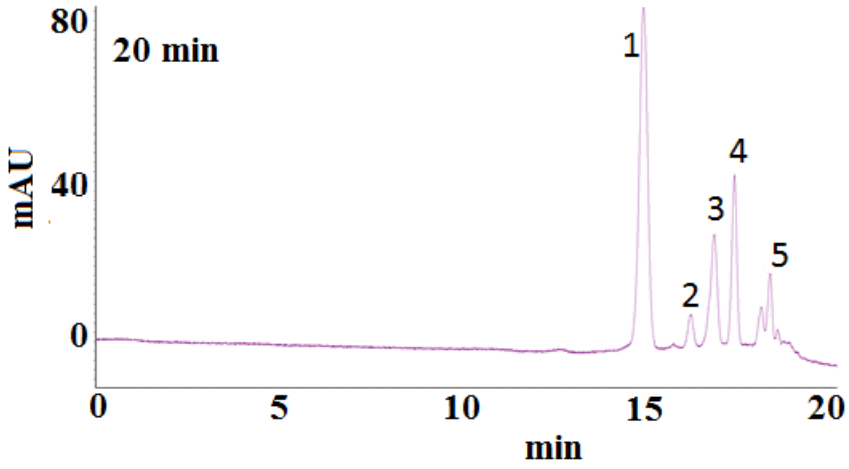

Figure 3.18. LC chromatogram of the degradation process of Mv monitored at 520 nm.

The stability of the anthocyanidins is influenced by the B-ring substituents. It seems that hydroxy or methoxy groups decrease the stability of the aglycone in neutral media. In contrast to their aglycones, Mv-glc and Cy-glc are more stable under neutral pH conditions. The reason for the different behavior could be that the sugar moieties prevent the degradation of the highly unstable α-diketone intermediates to the phenolic acid and the aldehyde component. Furthermore it is most likely that dimerisation of the anthocyanidins is prevented by the steric demanding sugar moieties. The solutions at neutral pH were colorless but after acidification returned colored, indicating that the flavylium ion was the predominant form in solution.

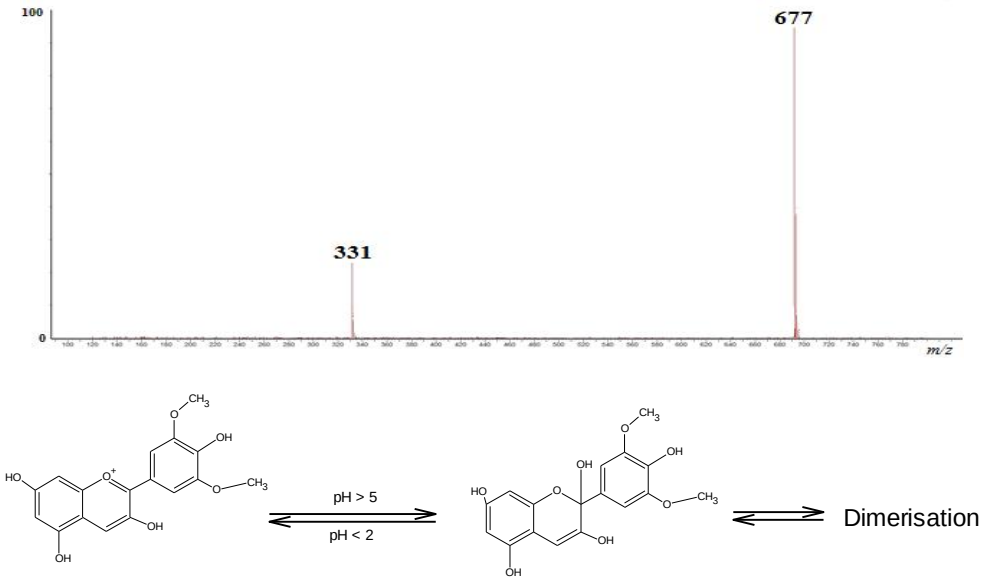

Figure. 3.19. LC-MS spectra of the degradation products of malvidin formed at pH 7. The ions with *m/z* 331 correspond to malvidin, which are derived from spontaneous fragmentation of the ion with *m/z* 677.

3.4.2. Degradation of ACNs by human fecal microflora (HFM)

To obtain ACNs degradation kinetics we used faecal diluted mixtures, because the dilutions normally used to evaluate the degradation of other flavonoids caused a too fast disappearance of the ACNs. The incubation of Cy-3-glc, Cy-3-ara, Peo-3-glc, Peo-3-ara, Pet-3-glc, D-3-glc, Mv-3-glc, Cy, Mv, Pet and Peo in anaerobic conditions with human faecal flora resulted in a rapid decrease of the parent compounds. After 1 hour, more than 90% of all compounds were degraded. In control incubations only a slight decrease, in the first hour, could be observed suggesting that the microflora is primarily responsible for the degradation process. In figure 3.20 was reported the degradation kinetic of Cy-glc. No aglycones were found for any of the anthocyanin glycosides used under any of the collection times assayed. Anthocyanin glycosides must have been hydrolyzed by enzymes of fecal microbiota by cleavage of the 3-O-glycosidic linkage; the released aglycones formed transitorily could have been degraded into the corresponding phenolic acids, thus aglycones are very unstable under physiological conditions in the intestine at neutral pH. However, Keppler and Humpf 2005 detected small amounts of aglycones after 2 h of incubation with pig gut microbiota.
During the incubations new metabolites were formed and identified as the respective corresponding phenolic acids derived from the B-ring of the anthocyanidins. In case of Mv-glu and Peo-glc, syringic and vanillic acid were identified as the major degradation products respectively, and Cy-glc was degraded to protocatechuic acid. These metabolites were then degraded with consequent formation of benzoic acid, and regarding syringic acid the conversion was very slightly. Thus, the degradation of ACNs to the corresponding phenolic acids seems to be a general pathway for these substances. According to other studies (Keppler and Humpf 2005, Fleschhut et al. 2006), incubation of gut microbiota with Mv-glc and D-glc led to the formation of syringic and gallic acid, respectively. However, in a previous study where D-glc was incubated with pig gut microbiota, gallic acid was not found, indicating that this phenolic acid could have been metabolized (Forester et al. 2008). The rate of degradation and transformation of the tested compounds differed in batch cultures inoculated with samples of different fecal donors. Therefore, the individual composition of intestinal microbiota seems to be an important factor as regards the degradation of ACNs to phenolic compounds. Consequently, the colon may be considered as an active site of metabolism in which bacteria contribute to the health effects of phenolic compounds.
Although the degradation to the phenolic acid is an important pathway, it cannot explain the total disappearance of the ACNs during incubation. Calculated on the basis of our *in vitro* fermentation studies the amount of the formed phenolic acid reached up to a maximum of 40% of the parent compound, suggesting the occurrence of ACNs degradation pathways not yet identified.

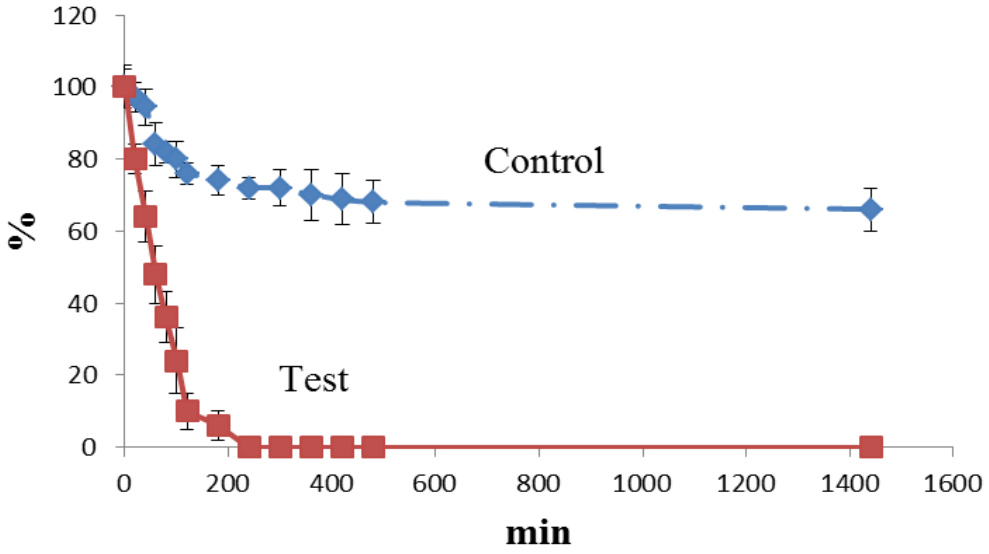

Figure 3.20. Degradation kinetics of Cy-glc during fermentation experiment using human faecal sample. Data are showed as means±s.d.

Incubations of anthocyanin aglycones in buffer solution and cell culture media show that these substances are unstable under neutral pH conditions. ACNs degrade via the α-diketone intermediate with the formation of a phenolic acid and an aldehyde, or they could dimerise via the quinoid anhydrobase. The different molecular weights of the detected Mv dimers (Fig. 3.18) indicate that their structures may consist of two different Mv monomer units (flavylium cation, hemiketal quinoid base and α-diketone), which possess different molecular weights. The chemical instability of aglycones must therefore be taken into consideration when the biological properties of these compounds are investigated in *in vitro* studies, because it can be assumed that the degradation products also contribute to the observed biological effects. In contrast to the anthocyanidins the anthocyanin mono-glycosylated and even more the acylated anthocyanins are rather stable over a wide pH range. Moreover, it seems that glucosides are metabolized quicker that corresponding arabinosides (Fig. 3.21), and more generally the arabinosides seems more stable than glucosides.
Similar results were obtained by analyzing solutions containing FD-WB. In particular, it seems that the more stable ACNs were those esterified with acetic acid. Moreover, it seems that the ACNs bound to glucose, exhibited a faster degradation rate than those bound to galactose and arabinose. At the parity of linked sugar, petunidin and peonidin seems more stable than malvidin, cyanidin and delphinidin, and the latter was generally the least stable.

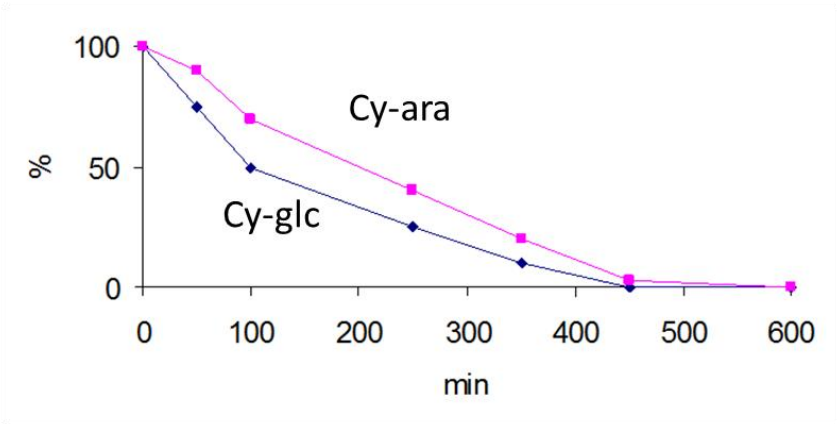

Figure 3.21. Degradation kinetic of cyanidin-glucoside and cyanidin-arabinoside obtained by diluted mixture of HFM. Data are presented as means.

A major pathway of the degradation process is the formation of the phenolic acid derived from the B-ring of the anthocyanin skeleton. Thus, it can be concluded that the first step of the bacterial biotransformation is the cleavage of the sugar moiety leading to the formation of the anthocyanin aglycone. The aglycone could be further metabolized by the bacteria or degraded by a chemical reaction without the action of bacteria via the quinoid anhydrobase to the phenolic acid. However, this pathway cannot explain the whole fate of the anthocyanins, which disappeared during the incubation with HFM. Thus, a possible degradation pathway could be the reaction of the quinoid anhydrobase with reactive groups in macromolecules like proteins. Indeed, this type of reaction has been described for various quinone structures. In contrast to anthocyanins, phenolic acids seem stable against further metabolism by human gut microflora, and benzoic acid was the main end product. Our results are comparable with those reported by other authors (Hsu et al. 1990), who found a degradation of protocatechuic acid during incubation with *Clostridium thermoaceticum* with production of catechol. Summarizing, anthocyanins were degraded by enzymes of the gut bacteria releasing the aglycones. The pH of the intestine is in the range of pH between 6 and 7, and in the colon can reach about 8; therefore the aglycone does not exist as a flavylium cation in the gut but undergoes a pH dependent structural change to the unstable quinoid base and to the chalcone. These intermediates could either be degraded to the phenolic acid and aldehyde or react with macromolecules. On the whole, the *in vitro* reactions can be important also for the *in vivo* studies and could help to understand and explain the poor bioavailability of anthocyanins observed in human intervention studies

3.4.3. Degradation of ACNs by rat microflora (RM)

The aim of this study was evaluate the role of the gastrointestinal microbiota of the rat in the biotransformation of anthocyanins to benzoic acid derivatives. Microbial activity was tested on FD-WB and individual anthocyanins more representative of this fruit. The assessments were carried out covering both the disappearance of anthocyanins that the formation of the respective metabolites. Thus, intestinal microbial cultures were set up using the contents of different gastrointestinal tracts (stomach, small intestine and large intestine) of Sprague-Dawley rats fasted for 12 hours. Figure 3.22 reports the degradation kinetic of ACN pure compounds carried out by bacteria from stomach, small intestine and colon. As already pointed out (HFM study), no aglycones were found for any of the anthocyanin glycosides tested. The benzoic acid derivatives were found only in samples containing bacteria from the colon. This data confirms that the catabolism of ACNS to phenolic

acids occurs mainly in the colon. Also in this case the amount of phenolic acid cannot explain the total disappearance of the ACNs during incubation. Indeed, the amount of the formed phenolic acid in the batch containing bacteria from colon reached up to a maximum of 30 % of the parent compound.

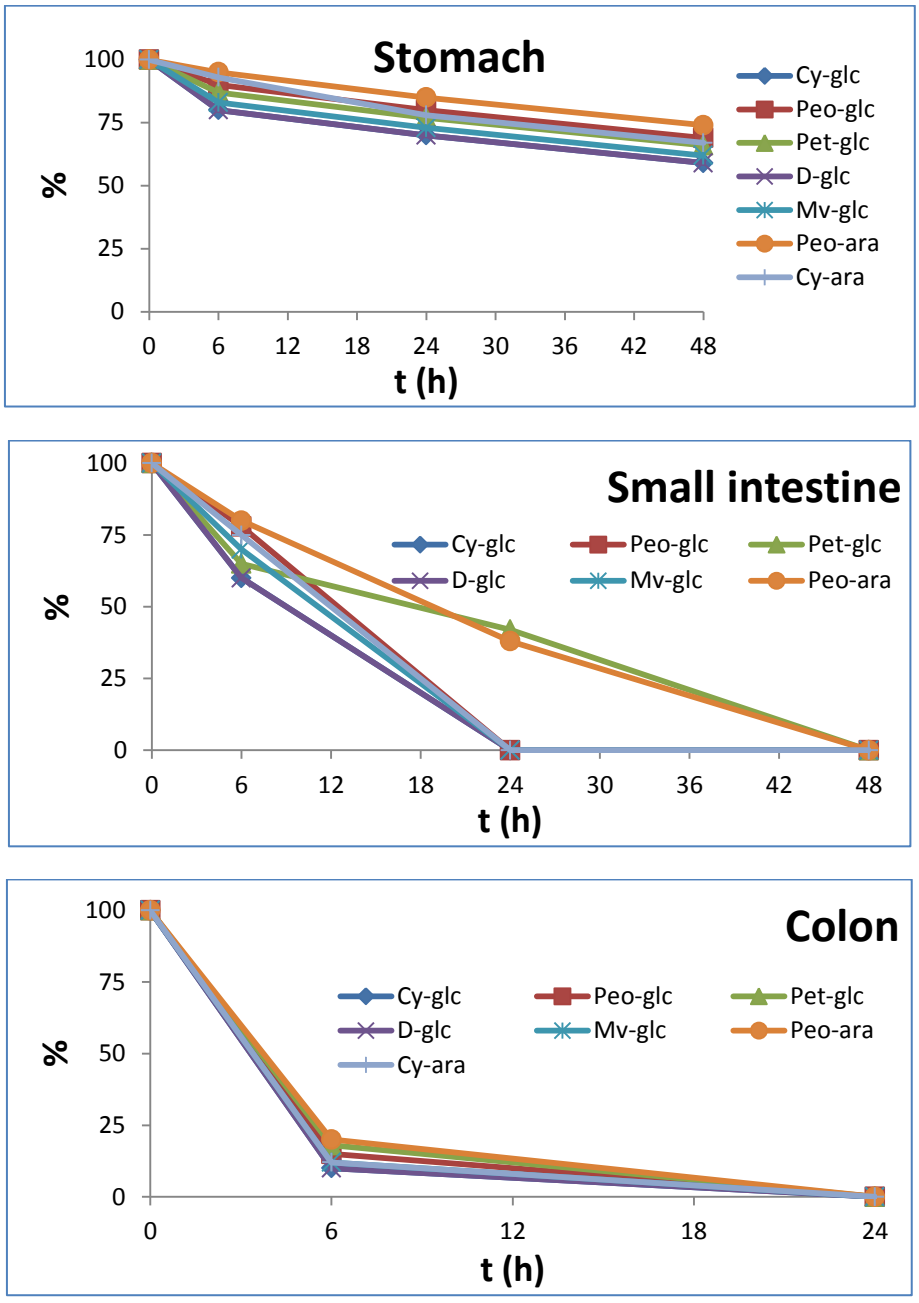

Figure 3.22. Kinetics of ACNs standard solution during fermentation by bacteria taken from stomach, small intestine and colon of rats. Data are presented as means.

The metabolic activity of the intestinal microflora from stomach was found, as expected, blander. Indeed, after 48 h of incubation the residual percentage (RP) of anthocyanins was 65±5 % (mean±sd) (Fig. 3.23), and Peo-ara appeared the most stable (74±3 %). On the contrary, in the batch containing the bacteria of the small intestine the residual anthocyanins were 70±6 and 11±7 after 6 and 24 h, respectively (Fig. 3.23). It should be highlighted that after 24 h were present only Pet-glc (42±4) and Peo-ara (38±2 %).
As expected, the colon bacteria showed the highest catabolic activity against anthocyanins. Indeed, the residual ACNs were 14±4 % after 6 h of incubation in anaerobic conditions (Fig. 3.23), and no ACNs were found after 24 h incubation. Also in this experimental condition Peo-ara (20±3 %) and Pet-glc (18±4 %) were the more stable.

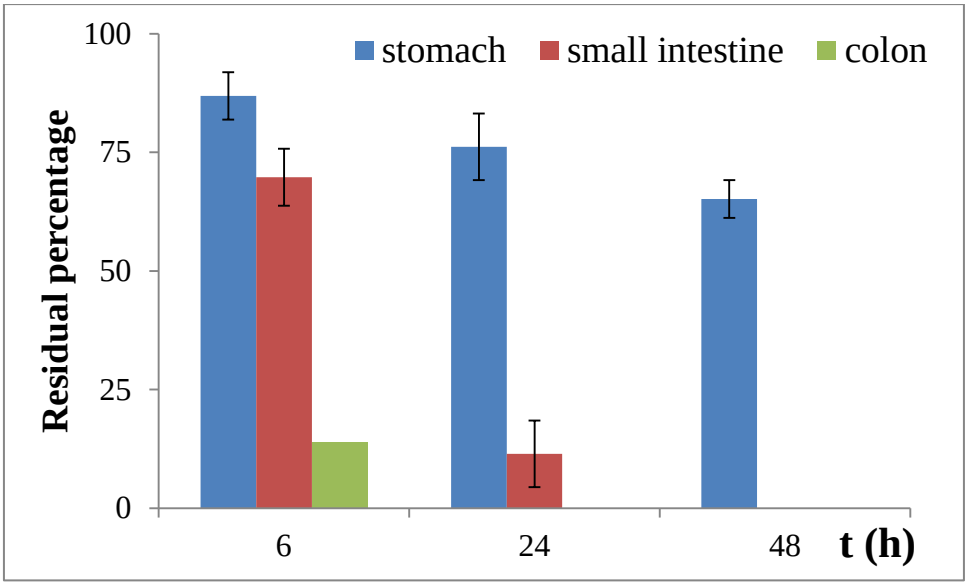

Figure 3.23. Residual percentage of total ACNs incubated in anaerobic conditions with bacteria from stomach, small intestine and colon. (Data are reported as means±s.d.).

Similar results were obtained by analyzing solutions containing FD-WB. The ACNS were degraded mainly in the colon and after 3 and 6 h the residual amount were 33±4 and 18±7 %, respectively. ACNs were not found after 24 h of incubation, and also in these trials the more stable ACNs were those esterified with acetic acid (Figure 3.24), thus confirming the protective effect of acyl groups. It should be underlined that Cy-gal, Mv-gal and D-gal were not detected already after 6 hours of incubation and also the percentages of the corresponding conjugated with glucose were low (3-7 %). At the parity of linked sugar, petunidin and peonidin were more stable than other ACNs.

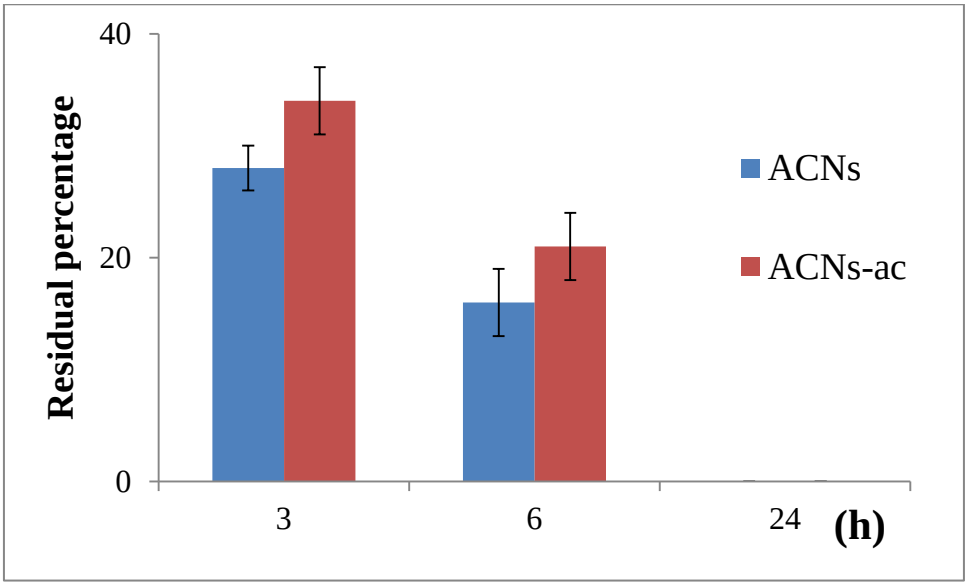

Figure 3.24. Residual percentage of ACNs incubated in anaerobic conditions with bacteria from colon. (Data are reported as means±s.d.).
ACNs: total anthocyanins-monoglycosylated,
ACNs-ac: total anthocyanins-monoglycosylated-acetylated.

4. References

Adams, L.S.; Phung, S.; Yee, N.; Seeram, N.P.; Li, L.; Chen, S. Blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway. Cancer Res. 2010, 70(9), 3594-605.

Ahmet, I.; Spangler, E.; Shukitt-Hale, B.; Juhaszova, M.; Sollott, S.J.; Joseph, J.A.; Ingram, D.K.; Talan, M. Blueberry-enriched diet protects rat heart from ischemic damage. PLoS One 2009, 4(5934), 1–10.

Al-Zubaidy, M. M. I.; Khalil, R.A. Kinetic and prediction studies of ascorbic acid degradation in normal and concentrate local lemon juice during storage. Food Chem. 2007, 101, 254-259.

Amakura, Y.; Umino, Y.; Tsuji, S.; Tonogai, Y. Influence of jam processing on the radical scavenging activity and phenolic content in berries. J. Agric. Food Chem. 2000, 48, 6292-6297.

Anthony, J.P.; Fyfe, L.; Stewart, D.; McDougall, G.J.; Smith, H.V. The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts, in vitro. Methods 2007, 42(4), 339-48.

Aura, A.M.; O'Leary, K.A.; Williamson, G.; Ojala, M.; Bailey, M.; Puupponen-Pimiä, R.; Nuutila, A.M.; Oksman-Caldentey, K.M.; Poutanen, K. Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. J. Agric. Food Chem. 2002, 50(6), 1725-30.

Aura, AM.; Microbial metabolism of dietary phenolic compounds in the colon. Phytochem. Rev. 2008, 7, 407-429.

Azar, M.; Verette, B.; Brun, S. Identification of some phenolic compounds in bilberry juice *Vaccinium myrtillus*. J. Food Sci. 1987, 52, 1255-1257.

Ballington, J.R.; Ballinger, W.E.; Maness, E.P. Interspecific differences in the percentage of anthocyanins, aglycones, and aglycone-sugars in the fruit of seven species of blueberries. J. Amer. Soc. Hort. Sci. 1987, 112, 859-864.

Barnes, J.S.; Nguyen, H.P.; Shen, S.; Schug, K.A. General method for extraction of blueberry anthocyanins and identification using high-performance liquid chromatography-electrospray ionization ion-trap time-of-flight mass spectrometry. J. Chromatogr. A 2009, 1216(23), 4728-35.

Benzie, I.F.F. and Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 1996, 239(1), 70-76.

Bloor, S.J.; Abrahams, S. The structure of the major anthocyanin in Arabidopsis thaliana. Phytochemistry 2002, 59, 343-346.

blueberry and muscadine phenolic extracts. J Food Sci 76(2):M101–5.

Bomser, J.; Madhavi, D.L.; Singletary, K.; Smith, M.A.L. In vitro anticancer activity of fruit extracts from *Vaccinium* species. Planta Medica 1996, 62, 212-216.

Bridle, P.; Timberlake, C.F. Anthocyanins as natural food colours: selected aspects. Food Chem. 1999, 58(1-2), 103-109.

Bronnum-Hansen, K. and Flink, J.M. Anthocyanin Colourants from Elderberry (*Sambucus nigra* L.). 3. Storage Stability of the Freeze Dried Product. J. Food Tech. 1985, 20: 725-733.

Brouillard, R. and Dangles, O. Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging? Food Chem. 1994, 51, 365-371.

Brouillard, R. Chemical structure of anthocyanins. In Anthocyanins as food colors. Markakis, P., Eds.; Academic Press, New York, 1982; pp. 1-40.

Brownmiller, C.; Howard, L.R.; Prior, R.L. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. J. Food Sci. 2008, 73, H72-H79.

Brownmiller, C.; Howard, L.R.; Prior, R.L.; Processing and storage effect on proantocyanidin composition and concentration of processed blueberry products. J. Agric. Food Chem. 2009, 57, 1896-1902.

Bub, A.; Watzl, B.; Heeb, D.; Rechkemmer, G.; Briviba, K. Malvidin-3-glucoside bioavailability in humans after ingestion of red wine,dealcoholized red wine and red grape juice. Eur. J. Nutr. 2001, 40, 113-120.

Buckow, R.; Kastell, A.; Terefe, N.S.; Versteeg, C. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. J. Agric. Food Chem. 2010, 58, 10076-10084.

Burdulis, D.; Sarkinas, A.; Jasutiene, I.; Stackevicene, E.; Nikolajevas, L.; Janulis, V. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. Acta Pol. Pharm. 2009, 66(4), 399-408.

Bushway, R.J., McGann, D.F.; Cook W.P.; Bushway, A.A. Mineral and vitamin content of lowbush blueberries *(Vaccinium angustifolium* Ait.) J. Food Sci. 1983, 48(6), 1878-1880.

Calvi, J.P. and Francis, F.J. Stability of Concord Grape (*V. labrusca*) Anthocyanins in Model Systems. J. Food Sci. 1978, 43(5), 1448-1456.

Cao, G.; Prior, R.L. Anthocyanins are detected in human plasma after oral administration of an elderberry extract. Clin. Chem. 1999, 45 (4), 574-576.

Castro, L. and Freeman, B.A. Reactive oxygen species in human health and disease. Nutrition (Burbank, Los Angeles County, CA) 2001, 17(2), 161-5.

Cevallos-Casals, B.A.; Cisneros-Zevallos, L. Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. Food Chem. 2004, 86, 69-77.

Chatterjee, A.; Yasmin, T.; Bagchi, D.; Stohs, S.J. Inhibition of *Helicobacter pylori* in vitro by various berry extracts, with enhanced susceptibility to clarithromycin. Mol. Cell Biochem. 2004, 265(1), 19-26.

Clifford, M.N. Anthocyanins - nature, occurrence and dietary burden. J. Sci. Food Agric. 2000, 80, 1063-1072.

Connor, A.M., Luby, J.J.; Hancock, J.F., Berkheimer, S.; Hanson, E.J. Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. J. Agric. Food Chem. 2002c, 50(4), 893-898.

Connor, A.M., Luby, J.J.; Tong, C.B.S.; Finn, C.E., Hancock, J.F. Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. J. Am. Soc. Hortic. Sci. 2002a, 127(1), 89-97.

Connor, A.M.; Luby, J.J.; Tong, C.B.S.; Finn, C.E.; Hancock, J.F. Variation and heritability estimates for antioxidant activity, total phenolic content, and anthocyanin content in blueberry progenies. J. Am. Soc. Hortic. Sci. 2002b, 127(1), 82-88.

Cooney, J.M.; Jensen, D.J.; McGhie, T.K. LC-MS identification of anthocyanins in boysenberry extract and anthocyanin metabolites in human urine following dosing. J. Sci. Food. Agric. 2004, 84, 237-245.

Dangles, O.; Saito, N.; Brouillard, R. Anthocyanin intramolecular copigment effect. Phytochemistry. 1993, 34, 119-124.

de Pascual-Teresa, S. and Sanchez-Ballesta, M.T. Anthocyanins: from plant to health. Phytochemistry Rev. 2008, 7(2), 281-299.

de Pascual-Teresa, S.; Moreno, D.A., Garcıa-Viguera, C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. Int. J. Mol. Sci. 2010, 11(4), 1679-703.

Debnath, S.C. Development of ISSR markers for genetic diversity studies in *Vaccinium angustifolium*. Nordic J. Bot. 2009, 27(2), 141-148.

DeFuria, J.; Bennett, G.; Strissel, K.J.; Perfield, J.W.; Milbury, P.E.; Greenberg, A.S.; Obin, M.S. Dietary blueberry attenuates whole-body insulin resistance in high-fat-fed mice by reducing adipocyte death and its inflammatory sequelae. J. Nutr. 2009, 139(8), 1510-1516.

Delgado-Vargas, F. and Paredes-López, O. Anthocyanins and betalains. In Natural Colorants for Food and Nutraceutical Uses. CRC Press, Boca Raton, 2003; pp 167-219.

Delgado-Vargas, F.; Jiménez, A.R.; Paredes- López, O. Natural pigments: carotenoids, anthocyanins, and betalains – characteristics, biosynthesis, processing and stability. Crit. Rev. Food Sci Nutr. 2000, 40, 173-289.

Dogterom, M.H., Winston, M.L., Mukai, A. Effect of pollen load size and source (self, outcross) on seed and fruit production in highbush blueberry cv.'Bluecrop' (*Vaccinium Corymbosum*; Ericaceae). Am. J. Bot. 2000, 87(11), 1584-1591.

Dreiseitel, A.; Schreier, P.; Oehme, A.; Locher, S.; Rogler, G.; Piberger, H.; Hajak, G.; Sand, P.G. Inhibition of proteasome activity by anthocyanins and anthocyanidins. Biochem. Biophys. Res.Commun. 2008, 372(1), 57-61.

Drummond, F.; Smagula, J.; Annis, S.; Yarborough, D. 2009. Organic wild blueberry production. Bulletin 852 (Maine Agricultural and Forest Experiment Station. Available from http://www.umaine.edu/mafes/elec%5Fpubs/bulletins/b852.pdf.

Dulebohn, R.V.; Yi, W.; Srivastava, A.; Akoh, C.C.; Krewer, G.; Fischer, J.G. Effects of blueberry (*Vaccinium ashei*) on DNA damage, lipid peroxidation, and phase II enzyme activities in rats. J. Agric. Food Chem. 2008, 56(24), 11700–6.

Durst, R.W. and Wroistad, R.E. 2001. Unit F1.3: Anthocyanins. Separation and characterization of anthocyanins by HPLC. In: Wrolstad RE, editor. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons. p 1-13.

Eck, P. 1988. Blueberry science. New Brunswick, N.J.: Rutgers University Press.

Eder, Pigments In Food Analysis by HPLC. Nollet, M.L.L. Eds.; Marcel Dekker, New York, 2000; pp. 845-880.

Ehlenfeldt, M.K. and Prior, R.L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. J. Agric Food Chem. 2001, 49, 2222-2227.

Ehlenfeldt, M.K.; Meredith, F.I.; Ballington, J.P. Unique organic acid profile of rabbiteye vs. highbush blueberries. Hort. Science. 1994, 29, 321-323.

El Mohsen, MA.; Marks, J.; Kuhnle, G.; Moore, K.; et al. Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats. Br. J. Nutr. 2006, 95, 51-58.

Erlandson, J.A. and Wrolstad, R.E. Degradation of Anthocyanins at Limited Water Concentration. J. Food Sci. 1972, 37, 592-595.

Espin, J.C.; Soler-Rivas, C.; Wichers, H.J.; Garcia-Viguera, C. Anthocyanin-based natural colorants: a new source of antiradical activity for foodstuff. J. Agric. Food Chem. 2000, 48(5), 1588-1592.

Faria, A.; Pestana, D.; Teixeira, D.; de Freitas, V.; Mateus, N.; Calhau, C. Blueberry anthocyanins and pyruvic acid adducts: anticancer properties in breast cancer cell lines. Phytother. Res. 2010, 24(12), 1862-1869.

Felgines, C.; Talavera, S.; Gonthier, M.P.; Texier, O.; Scalbert, A.; Lamaison, J.L.; Remesy, C. Strawberry anthocyanins are recovered in urine as glucuroand sulfoconjugates in humans. J. Nutr. 2003, 133, 1296-1301.

Felgines, C.; Talavera, S.; Texier, O.; Besson, C.; Fogliano, V.; Lamaison, J.L.; la Fauci, L.; Galvano, G.; Rémésy, C.; Galvano, F. Absorption and metabolism of red orange juice anthocyanins in rats. Br. J. Nutr. 2006, 95(5), 898-904.

Felgines, C.; Talavera, S.; Texier, O.; Gil-Izquiedo, A.; et al. Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans, J. Agric. Food Chem. 2005, 53, 7721-7727.

Felgines, C.; Texier, O.; Besson, C.; Fraisse, D.; Lamaison, J.L.; Remesy, C. Blackberry anthocyanins are slightly bioavailable in rats. J. Nutr. 2002, 132, 1249-1253.

Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. Eur. J. Nutr. 2006, 45, 7-18.

Forester, S. C.; Waterhouse, A. L. Identification of cabernet sauvignon anthocyanin gut microflora metabolites. J. Agric. Food Chem. 2008, 56, 9299-9304.

Fossen, T.; Cabrita, L.; Andersen, O.M. Colour and stability of pure anthocyanins infuenced by pH including the alkaline region. Food Chem. 1998, 63(4), 435-440.

Fossen, T.; Rayyan, S.; Holmberg, M.H.; Nimtz, M.; Andersen, O.M. Covalent anthocyanin-flavone dimer from leaves of Oxalis triangularis. Phytochemistry 2007, 68, 652-662.

Fossen, T.; Slimestad, R.; Øvstedal, D.O.; Andersen, Ø.M. Covalent anthocyaninflavonol complexes from flowers of chive, Allium schoenoprasum. Phytochemistry. 2000, 54, 317-323.

Francis, F.J. Food colorants: anthocyanins. Crit. Rev. Food Sci. Nutr. 1989, 28(4), 273-314.

Frøytlog, C.; Slimestad, R.; Andersen, Ø.M. Combination of chromatographic techniques for the preparative isolation of anthocyanins - applied on blackcurrant (*Ribes nigrum*) fruits. J. Chromatogr. A. 1998, 825, 89-95.

Galli, R.L.; Bielinski, D.F.; Szprengiel, A.; Shukitt-Hale, B.; Joseph, J.A. Blueberry-supplemented diet reverses age-related decline in hippocampal HSP70 neuroprotection. Neurobiol. Aging 2006, 27(2), 344-350.

Galvano, F.; Fauci, L.L.; Lazzarino, G.; Fogliano, V.; Ritieni, A.; Ciappellano, S.; Battistini, N.C.; Tavazzi, B.; Galvano, G. Cyanidins: metabolism and biological properties. J. Nutr. Biochem. 2004, 15, 2-11.

Gao, L. and Mazza, G. Characterization of acetylated anthocyanins in lowbush blueberries. J. Liq Chromatogr. 1995, 18(2), 245-259.

Gao, L. and Mazza, G. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J. Food Sci. 1994, 59, 1057-1059.

Ghosh, D. and Konishi, T. Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Asia Pac. J. Clin. Nutr. 2007, 16(2), 200–208.

Giusti, M.M. & Wroistad, R.E. (2001) Unit F1.2: Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In, Current Protocols in Food Analytical Chemistry. RE Wroistad (Ed.) New York: John Wiley & Sons. p 1-13.

Giusti, M.M.; Rodríguez-Saona, L.E.; Griffin, D.; Wrolstad, R.E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. J. Agric. Food Chem. 1999b, 47, 4657-4664.

Giusti, M.M.; Rodríguez-Saona, L.E.; Wrolstad, R.E. Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. J. Agric. Food Chem. 1999a, 47, 4631-4637.

Giusti, M.M.; Wrolstad, R. E. Characterization of red radish anthocyanins. J. Food Sci. 1996a, 61 (2), 322-326.

Gonthier, M. P.; Verny, M. A.; Besson, C.; Remesy, C.; Scalbert, A. Chlorogenic acid bioavailability largely depends on its metabolism by gut microflora in rats. J. Nutr. 2003, 133, 1853-1859.

Goto, T. and Kondo, T. Structure and molecular stacking of anthocyanins –flower color variantion. Ang. Chemie- Int. Ed. Eng. 1991, 30: 17-33.

Goueli, S.A. 1976. Biochemical and physiological studies of the developing lowbush blueberry fruit (*Vaccinium angustifolium* Ait.). Diss. Abstr. B 37(6) 2822: Order no. 76-28672.

Gough, R.E. 1994. The highbush blueberry and its management. New York, N.Y.: Food Products Press.

Gould, K.S. and Lee D.W. (Eds.). 2002. Anthocyanins and Leaves. The Function of Anthocyanins in Vegetative Organs (Vol. 37). Advances in Botanical Research. London: Academic Press.

Grace, M.H.; Ribnicky, D.M.; Kuhn, P.; Poulev, A.; Logendra, S.; Yousef, G.G.; Raskin, I.; Lila, M.A. Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, *Vaccinium angustifolium* Aiton. Phytomedicine 2009, 16(5), 406-415.

Gu, L.; Keim, M.; Hammerstone, J.F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R.L. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. J. Agric. Food Chem. 2002, 50, 4852-4860.

Häkkinen, S.; Heinonen, M.; Karenlampi, S.; Mykkanen, H.; Ruuskanen, J.; Törrönen, R. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res. Intl. 1999, 32, 345-353.

Hall, I.V.; Forsyth, F.R.; Aalders, L.E.; Jackson, L.P. Physiology of the lowbush blueberry. Econ. Bot. 1972, 26(1), 68-73.

Halliwell, B. Reactive oxygen species and the central nervous system. J. Neurochem. 1992, 59(5), 1609-1623.

Halliwell, B. and Gutteridge, J.M.C. Ed. 1999. Free Radicals in Biology and Medicine. 3rd edition. Oxford University Press. Oxford, NY.

Hancock, J.F.; Lyrene, P.; Finn, CE.; Vorsa, N.; Lobos, G.A. 2008. Blueberries and cranberries. In: Hancock JF, editor. Temperate fruit crop breeding germplasm to genomics. Dordrecht, the Netherlands: Springer Science & Business Media B.V. p. 115-150.

Harborne, J.B. Phenolic Compounds in phytochemical methods - a guide to modern techniques of plant analysis 3rd edition. Chapman & Hall, New York, 1998; pp. 66-74.

Harborne, J.B.; Mabry, T.J.; Mabry, H. 1975. The flavonoids. London: Chapman & Hall.

Harborne, J.B.; Williams, C.A. Anthocyanins and other flavonoids. Nat. Prod. Rep. 2001, 18, 310-333.

He. J.; Magnuson, B.A.; Lala, G.; Tian, Q.; Schwartz, S.J.; Giusti, M.M. Intact anthocyanins and metabolites in rat urine and plasma after 3 months of anthocyanin supplementation. Nutr. Cancer. 2006, 54(1), 3-12.

Herrmann, K. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci. Nutr. 1989, 28, 315-347.

Hsu, T.; Daniel, S.L.; Lux, M.F.; Drake, H.L. Biotransformation of carboxylated aromatic compounds by the acetogen Clostridium thermoaceticum: Generation of growth-supportive CO_2 equivalents under CO_2-limited conditions. J. Bact. 1990, 172, 212-217.

Hoch, W.A.; Zeldin, E.L.; McCown, B.H. Physiological significance of anthocyanins during autumnal leaf senescence. Tree Physiol. 2001, 21(1), 1-8.

Howard, L.R.; Prior, R.L.; Liyanage, R.; Lay, J.O. Processing and storage effect on berry polyphenols: challenges and implications for bioactive properties. J. Agric. Food Chem. 2012, 60, 6678-6693.

Ichiyanagi, T.; Oikawa, K.; Tateyama, C.; Konishi, T. Acid mediated hydrolysis of blueberry anthocyanins. Chem. Pharm. Bull. 2001, 49, 114-123.

Jaakola, L.; Maatta, K.; Pirttila, A.M.; Torronen, R.; Karenlampi, S.; Hohtola, A. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. Plant Physiol. 2002, 130(2), 729-739.

Jackman, R.L. and Smith, J.L. 1996. Anthocyanins and betalains, pp. 244-280. In: G.A.F. Hendry and J.D. Houghton (eds.). Natural Food Colorants. Blackie Academic & Professional, New York.

Jiménez, M. and García-Carmona, F. Oxidation of the flavonol quercetin by polyphenol oxidase. J. Agric. Food Chem. 1999, 47(1), 56-60.

Joseph, J.; Carey, A.; Brewer, G.; Lau, F.; Fisher, D. Dopamine and A-induced stress signaling and decrements in Ca^{2+} buffering in primary neonatal hippocampal cells are antagonized by blueberry extract. J. Alzheimer's Dis. 2007, 11(4), 433-446.

Joseph, J.A.; Shukitt-Hale, B.; Denisova, N.A.; Bielinski, D.; Martin, A.; McEwen, J.J.; Bickford, P.C. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J. Neurosci. 1999, 19(18), 8114-8121.

Ju, Z.; Howard, L.R. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J. Agric. Food Chem. 2003b, 51, 5207-5213.

Ju, Z.; Howard, L.R. Improved extraction of procyanidins from grape seeds with near critical mixtures of carbon doxide and aqueous solvents. Poster presented at Ann. Mtg., Inst. Food Tech., Chicago, Illinois, July 14-18.

Kadar, A.; Robert, L.; Miskulin, M.; Tixier, J.M.; Brechemier, D.; Robert, A.M. Influence of anthocyanoside treatment on the cholesterol-induced atherosclerosis in the rabbit. Paroi Arterielle 1979, 5(4), 187-205.

Kader, F.; Rovel, B.; Girardin, M., Metche, M. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum*, L.). Food Chem. 1996, 55(1), 35-40.

Kader, F.; Rovel, B.; Girardin, M.; Metche, M. Mechanism of Browning in Fresh Highbush Blueberry Fruit (*Vaccinium corymbosum* L). Partial Purification and Characterization of Blueberry Polyphenol Oxidase J. Sci. Food Agric. 1997, 73, 513-516.

Kähkönen, M.P.; Heinämäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: isolation, identification and antioxidant activities. J. Sci. Food Agric. 2003, 83, 1403-1411.

Kalt, W. and McDonald, J.E. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 1996, 121, 142-146.

Kalt, W. Effects of production and processing factors on major fruit and vegetable antioxidants. J. Food Sci. 2005, 70(1), R11-R19.

Kalt, W.; Blumberg, J. B.; McDonald, J. E.; Vinqvist-Tymchuk, M. R.; Fillmore, S.A.; Graf, B.A.; O'Leary, J.M.; Milbury, P.E. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. J. Agric. Food Chem. 2008, 56, 705-712.

Kalt, W.; McDonald, J.E.; Donner, H. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. Food Chem. Toxicol. 2000, 65, 390-393.

Kalt, W.; McDonald, J.E.; Ricker, R.D.; Lu, X. Anthocyanin content and profile within and among blueberry species. Can. J. Plant. Sci. 1999b, 79(4), 617-624.

Kalt, W.; Ryan, D.A.J.; Duy, J.C.; Prior, R.L.; Ehlenfeldt, M.K, van der Kloetm SP. Interspecific variation in anthocyanins, phenolics, and antioxidantcapacity among genotypes of highbush and lowbush blueberries (Vaccinium section Cyanococcus spp.). J. Agric. Food. Chem. 2001, 49(10), 4761-4767.

Kay, C.D. and Holub, B.J. The effect of wild blueberry (*Vaccinium angustifolium*) consumption on postprandial serum antioxidant status in human subjects. Br. J. Nutr. 2002, 88(04), 389-397.

Kay, C.D. Aspects of anthocyanin absorption, metabolism and pharmacokinetics in humans. Nutr. Res. Rev. 2006, 19, 137-146.

Kechinski, C.P.; Ramos Guimarães, P.V.; Zapata Noreña, C.P.; Tessaro, I.C.; Ferreira Marczak, L.D. Degradation kinetics of anthocyanins in blueberry juice during thermal treatment. J. Food Sci. 2010, 75, C173-C176.

Keppler, K.; Humpf, H. U. Metabolism of anthocyanis and their phenolic degradation products by the intestinal microflora. Bioorg. Med. Chem. 2005, 13, 5195-5205.

Kirca, A.; Cemeroğli, B. Degradation kinetics of anthocyanins in blood orange juice and concentrate. Food Chem. 2003, 81, 583-587.

Koes, R.; Quattrocchio, F.; Mol, J. The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays. 1994, 16, 123-132.

Kraft, T.F.B.; Schmidt, B.M.; Yousef, G.G.; Knight, C.T.G.; Cuendet, M.; Kang, YH..; Pezzuto, J.M.; Seigler, D.S.; Lila, M.A. Chemopreventive potential of wild lowbush blueberry fruits in multiple stages of carcinogenesis. J. Food Sci. 2005, 70(3), S159–S166.

Kuhnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet. 1976, 24, 117-191.

Kurilich, A.C.; Clevidence, B.A.; Britz, S.J.; Simon, P.W.; Novotny, J.A. Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from raw and cooked purple carrots. J. Agric. Food Chem. 2005, 53, 6537-6542.

Lee, J.; Durst, R.W.; Wrolstad, R.E. Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments. J. Food Sci. 2002, 67 (5), 1660-1667.

Lee, J.; Durst, R.W.; Wrolstad, R.L. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J. AOAC Intl. 2005, 88, 1269-1278.

Lee, J.; Wrolstad, R.E. 2004. Extraction of anthocyanins and polyphenolics from blueberry processing waste. J. Food Sci. 2004, 69(7), 564-573.

Lohachoompol, V.; Srzednicki, G.; Craske, J. The change of total anthocyanins in blueberries and their antioxidant effect after drying and freezing. J. Biomed. Biotechnol. 2004, 5, 248-252.

Markakis, P.; Livingston, G.E.; Fellers, C.R. Quantitative Aspects of Strawberry Pigment Degradation. Food Res. 1957, 22, 117-129.

Matchett, M.D.; MacKinnon, S.L.; Sweeney, M.I.; Gottschall-Pass, K.T.; Hurta, R.A.R. Blueberry flavonoids inhibit matrix metalloproteinase activity in DU145 human prostate cancer cells. Biochem. Cell Biol. 2005, 83(5), 637-643.

Matchett, M.D.; MacKinnon, S.L.; Sweeney, M.I.; Gottschall-Pass, K.T.; Hurta, R.A.R. Inhibition of matrix metalloproteinase activity in DU145 human prostate cancer cells by flavonoids from lowbush blueberry (*Vaccinium angustifolium*): possible roles for protein kinase C and mitogen-activated protein-kinase-mediated events. J. Nutr. Biochem. 2006, 17(2), 117-125.

Matsumoto, H.; Ichiyanagi, T.; Lida, H.; Ito, K.; Tsuda, T.; Hirayama, M.; Konishi, T. Ingested delphinidin-3-rutinoside is primarily excreted to urine as the intact form and to bile as the methylated form in rats. J. Agric. Food Chem. 2006, 54, 578-582.

Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; et al., Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J. Agric. Food Chem. 2001, 49, 1546-1551.

Matuschek, M.C.; Hendriks, W.H.; McGhie, T.K.; Reynolds, G.W. The jejunum is the main site of absorption for anthocyanins in mice, J. Nutr. Biochem. 2005, 17, 31-36.

Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 2003, 75, 3019-3030.

Mazza, G. and Miniati, E. 1993. Anthocyanins in Fruits, Vegetables, and Grains. Boca Raton, FL.: CRC Press Inc. p 87-92.

Mazza, G. Anthocyanins in grapes and grape products. Crit. Rev. Food Sci. Nutr. 1995, 35, 341-371.

Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J. Agric. Food Chem. 2002, 50, 7731-7737.

McDougall, G.J.; Gordon, S.; Brennan, R.M.; Stewart, D. Anthocyanin-flavanol condensation products from black currant (*Ribes nigrum L.*). J. Agric. Food Chem. 2005, 53, 7878-7885.

McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolised by both humans and rats. J. Agric. Food Chem. 2003, 51, 4539-4548.

McGhie, T.K. and Walton, M.C. The bioavailability and absorption of anthocyanins: Towards a better understanding. Mol. Nutr. Food Res. 2007, 51, 702-713.

Mikanagi, Y.; Saito, N., Yokoi, M.; Tatsuzawa F. Anthocyanins in flowers of genus Rosa, sections Cinnamomeae ("Rosa), Chinenses, Gallicanae and some modern garden roses. Biochem. Syst. Ecol. 2000, 28, 887-902

Miyazawa, T.; Nakagawa, K.; Kudo, M.; Muraishi, K.; Someya, K.; Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. J. Agric. Food Chem. 1999, 47, 1083-1091.

Molan, A.L.; Lila, M.A.; Mawson, J.; De, S. In vitro and in vivo evaluation of the prebiotic activity of water-soluble blueberry extracts. World J. Microbiol. Biotechnol. 2009, 25(7), 1243-1249.

Moyer, R.A.; Hummer, K.E.; Finn, C.E.; Frei, B.; Wrolstad, RE. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. J. Agric. Food Chem. 2002, 50(3), 519-525.

Neto, C.C. Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. Mol. Nutr. Food Res. 2007, 51(6), 652-664.

Netzel, M.; Strass, G-; Janssen, M.; Bitsch, I.; Bitsch, R. Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice. J. Environ. Pathol. Toxicol. Oncol. 2001, 20, 89-95.

Norton, C.; Kalea, A.Z.; Harris, P.D.; Klimis-Zacas, D.J. Wild blueberry-rich diets affect the contractile machinery of the vascular smooth muscle in the Sprague-Dawley rat. J. Med. Food. 2005, 8(1), 8-13.

Nozzolillo, C.; Isabelle, P.; Das, G. 1990. Seasonal changes in the phenolic constituents of jack pine seedlings (*Pinus banksiana*) in relation to the purpling phenomenon. Can. J. Bot. 1990, 68(9), 2010-2017.

Nurmi, T.; Mursu, J.; Heinonen, M.; Nurmi, A.; Hiltunen, R.; Voutilainen, S. Metabolism of berry anthocyanins to phenolic acids in humans. J. Agric. Food Chem. 2009, 57, 2274-2281.

Ofek, I.; Goldhar, J.; Zafriri, D.; Lis, H.; Adar, R.; Sharon, N. Anti-*Escherichia coli* adhesin activity of cranberry and blueberry juices. New Engl. J. Med. 1991, 324(22), 1599.

Ohnishi, R.; Ito, H.; Kasajima, N.; Kaneda, M.; Kariyama, R.; Kumon, H.; Hatano, T.; Yoshida, T. Urinary excretion of anthocyanins in humans after cranberry juice ingestion. Biosci. Biotechnol. Biochem. 2006, 70, 1681-1687.

Oliveira, C.; Amaro, L. F.; Pinho, O.; Ferreira, I. M. Cooked blueberries: anthocyanin and anthocyanidin degradation and their radical-scavenging activity. J Agric Food Chem. 2010, 58, 9006-9012.

Olsson, M.E.; Gustavsson, K.E.; Andersson, S.; Nilsson, A.; Duan. R.D. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. J. Agric. Food Chem. 2004, 52(24), 7264-7271.

Osman. N.; Adawi, D.; Ahrnè, S.; Jeppsson, B.; Molin, G. Probiotics and blueberry attenuate the severity of dextran sulfate sodium (DSS)-induced colitis. Dig. Dis. Sci 2008, 53(9), 2464-73.

Palamidis, N. and Markakis, P. Stability of grape anthocyanin in a carbonated beverage. J. Food Sci. 1975, 40, 1047.

Panicker, G.K., Sims, C.A.; Spiers, J.M.; Silva, J.L.; Matta, F.B. 2007. Effect of worm castings, cow manure, and forest waste on yield and fruit quality of organic blueberries grown on a heavy soil. Paper presented at the II International Symposium on Human Health Effects of Fruits and Vegetables; Houston, Tex.

Paredes-Lopez, O.; Cervantes-Ceja, M.L., Vigna-Perez, M.; Hernandez-Perez, T. Berries: improving human health and healthy aging, and promoting quality life, a review. Plant Foods Human Nutr. 2010, 65, 299-308.

Park YJ, Biswas R, Phillips RD, Chen J. Antibacterial activities of blueberry and muscadine phenolic extracts. J. Food Sci. 2011, 76(2), 101-105

Passamonti, S.; Vanzo, A.; Vrhovsek, U.; Terdoslavich, M.; et al. Hepatic uptake of grape anthocyanins and the role of bilitranslocase. Food Res. Int. 2005, 38, 953-960.

Passamonti, S.; Vrhovsek, U.; Mattivi, F. The interaction of anthocyanins with bilitranslocase, Biochem. Biophys. Res. Commun. 2002, 296, 631-636.

Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. FEBS Lett. 2003, 5, 210-213.

Patras, A.; Brunton N. P.; O'Donnell, C.; Tiwari, B. K. Effect of thermal processing in anthocyanin stability in foods; mechanisms and kinetics of degradation. Trends Food Sci. Technol. 2010, 21, 3-11.

Pellegrini, N.; Re, R.; Yang, M.; Rice-Evans, C.A. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying the 2,2'-azobis(3-ethylenebenzothiazoline-6-sulfonic) acid radical cation decolorization assay. Methods Enzymol. 1999, 299, 379-389.

Pellegrini, N.; Serafini, M.; Colombi B.; Del Rio, D.; Salvatore, S.; Bianchi M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assay. J. Nutr. 2003, 58, 2491-2497.

Phippen, W.B. and Simon, J.E. Anthocyanins in Basil (*Ocimum basilicum* L.). J. Agric. Food Chem. 1998, 46, 1734-1738.

Powles, S.B. Photoinhibition of photosynthesis induced by visible light. Ann. Rev. Plant Physiol. 1984, 35(1), 15-44.

Prior, R.L. 1998. Antioxidant capacity and health benefits of fruits and vegetables: blueberries, the leader of the pack. Proc. 32 Ann. Open House North Carolina Blueberry Council. 32, 3-12.

Prior, R.L. Absorption and Metabolism of Anthocyanins: Potential Health Effects. In Phytochemicals Mechanisms of Action; Meskin M.S., Bidlack W.R., Davies A.J., Lewis D.S., Randolph R.K., Eds.; CRC Press: Boca Raton, FL, 2004; pp 1-19.

Prior, R.L. and Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. Free Rad. Res. 2006, 40(10), 1014-28.

Prior, R.L.; Cao, G.; Martin, A.; Sofic, B.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W;, Krewer, G.; Mainland, C.M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species. J. Agric. Food Chem. 1998, 46, 2686-2693.

Prior, R.L.; Lazarus, S.A.; Cao, G.; Muccitelli, H.; Hammerstone, J.F. Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) using high-performance liquid chromatography/mass spectrometry. J. Agric. Food Chem. 2001, 49, 1270-1276.

Prior, R.L.; Wilkes, S.E.; Rogers, T.R.; Khanal, R.C.; Wu, X.; Howard, L.R. Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. J. Agric. Food Chem. 2010, 58(7), 3970-3976.

Prior, R.L.; Wu, X.; Gu, L.; Hager, T.; Hager, A.; Wilkes, S.E.; Howard, L.R. Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet. Mol. Nutr. Food Res. 2009, 53(11), 1406-1418.

Prior, R.L.; Wu, X.; Gu, L.; Hager, T.J.; Hager, A.; Howard, L.R. Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. J. Agric. Food Chem. 2008, 56(3), 647-53.

Prior, R.L.; Wu, X.; Schaichs, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 2005, 53(10), 4290-302.

Puupponen-Pimia, R.; Nohynek, L.; Meier, C.; Kahkonen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K.M. Antimicrobial properties of phenolic compounds from berries. J. Appl. Microbiol. 2001, 90(4), 494-507.

Ramassamy, C. Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. Eur. J. Pharmacol. 2006, 545(1), 51-64.

Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Structure-antioxidant activity relationship of flavonoids and phenolic acid. Free Rad. Biol. Med. 1996, 20, 933-956.

Riihinen, K.; Jaakola, L.; K¨arenlampi, S.; Hohtola, A. Organ-specific distribution of phenolic compounds in bilberry (*Vaccinium myrtillus*) and 'northblue' blueberry (*Vaccinium corymbosum* x *V. angustifolium*). Food Chem. 2008, 110(1), 156–60.

Rodríguez-Saona, L.E.; Wrolstad, R.E. Unit F1.1: Anthocyanins. Extraction, isolation and purification of anthocyanins. In Current Protocols in Food Analytical Chemistry; Wrolstad, R.E., Ed.; Wiley: New York, 2001.

Romero, C.; Bakker, J. Effect of storage temperature and pyruvate on kinetics of anthocyanins degradation, visitin A derivate formation, and color characteristics of model solution. J. Agric. Food Chem. 2000, 48, 2135-2141.

Rosatella A.A.; Simeonov, S.V.; Frade, R.F.M.; Afonso, C. 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological Properties, Synthesis and Synthetic Applications. Green Chem. 2011, 13, 754-793.

Routray, W.; Orsat, V. Blueberries and Their Anthocyanins: Factors Affecting Biosynthesis and Properties. Compr. Rev. Food Sci. Food Safety. 2011, 10, 303-320.

Sapers, G.M.; Burgher, A.M.; Phillips, J.G.; Jones, S.B.; Stone, E.G. Color and composition of highbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 1984, 109, 105-111.

Scalzo, J.; Miller, S.; Edwards, C.; Meekings, J.; Alspach, P. 2008b. Variation in phytochemical composition and fruit traits of blueberry cultivars and advanced breeding selections in New Zealand. Paper presented at the IX International Vaccinium Symposium, Corvallis, Oregon, USA.

Ścibisz, I.; Mitek, M. Effect of processing and storage conditions on phenolic compounds and antioxidant capacity of highbush blueberry jams. Pol. J. Food Nutr. Sci. 2009, 59, 45-52.

Seeram, N. P.; Bourquin, L. D.; Nair, M. G. Degradation products of cyanidin glycosides from tart cherries and their bioactivities. J. Agric. Food Chem. 2001, 49, 4924-4929.

Seeram, N.P. Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. J. Agric. Food Chem. 2008, 56(3), 627-629.

Seeram, N.P.; Adams, L.S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H.S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. J. Agric. Food Chem. 2006, 54(25), 9329-9339.

Sellappan, S.; Akoh, C.C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. J. Agri. Food Chem. 2002, 50, 2432-2438.

Serres, J.; Battino, M.; Costantini, E.; Mezzetti, B. Breeding and biotechnology for improving berry nutritional quality. BioFactors 2005, 23(4), 213-220.

Serres, R.A.; Zeldin, E.L.; McCown, B.H. 1996. Applying biotechnological approaches to *Vaccinium* improvement: a review. Paper presented at the VI International Symposium on *Vaccinium* Culture, Maine, Orono, USA.

Shahidi, F.; Naczk, M. Contribution of phenolic compounds to flavor and color characteristics of foods in phenolics in food and nutraceuticals. CRC press, Boca Boton, 2004b; pp. 443-461.

Shinoda, Y.; Komura, H.; Homma, S.; Murata, M. Browning of model orange juice solution: Factors affecting the formation of decomposition products. Biosci. Biotech. Biochem. 2005, 69, 2129-2137.

Siriwoharn, T.; Wrolstad, R.E.; Finn, C.E.; Pereira, C.B. Influence of cultivar, maturity, and sampling on blackberry (Rubus L. hybrids) anthocyanins, polyphenolics, and antioxidant properties, J. Agric. Food Chem. 2004, 52, 8021-8030.

Skrede, G.; Wrolstad, R.E. Flavonoids from berries and grapes In Functional Foods: Biochemical and Processing Aspects. Vol 2. Shi, J.; Mazza, G.; Maguer, M.L. Eds. CRC press, Boca Boton, 2002; pp. 71-133.

Skrede, G.; Wrolstad, R.E.; Durst, R.W. Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). J. Food Sci. 2000, 65 (2), 357-364.

Smith, M.A.L.; Marley, K.A.; Seigler, D.; Singletary, K.W.; Meline, B. Bioactive properties of wild blueberry fruits. J. Food Sci. 2000, 65(2), 352-356.

Somaatmadja, D.; Powers, J.J.; Hamdy, M.; Anthocyanins. VI. Chelation studies on anthocyanins and other related compounds. J. Food Sci. 1964, 29, 655-660.

Sondheimer, E. and Kertesz, Z.I. Anthocyanin pigments. Colonimetric determination in strawberries and strawberry perserves. Anal. Chem. 1948, 20, 245-248.

Srivastava, A.; Akoh, C.C.; Fischer, J.; Krewer, G. Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. J. Agric. Food. Chem. 2007, 55(8), 3180-3185.

Steyn, W.J.; Wand, S.J.E.; Holcroft, D.M.; Jacobs, G. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist 2002, 155(3), 349-361.

Strack, D.; Wray, V. 1994. The Anthocyanins. In: Harborne J.B. editor. The Flavonoids, London, UK: Chapman & Hall. p 1-22.

Syamaladevi, R.M.; Andrews, P.K.; Davies, N.M.; Walters, T.; Sablani, S.S. Storage effects on anthocyanins, phenolics, and antioxidant activity of thermally processed conventional and organic blueberries. J. Sci. Food Agric. 2012, 92, 916-924.

Takeoka, G.; Dao, L. Anthocyanins. In methods of analysis for functional foods and nutraceuticals. Hurst, W.J. Eds. CRC press, Boca Boton, 2002; pp. 219-241.

Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; et al. Anthocyanins are efficiently absorbed from the small intestines in rats, J. Nutr. 2004, 134, 2275-2279.

Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J.L.; Remesy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. J. Agric. Food Chem. 2005, 53, 3902-3908.

Tian, Q.; Giusti, M.M.; Stoner, G. D.; Schwartz, S.J. Urinary excretion of black raspberry (*Rubus occidentalis*) anthocyanins and their metabolites. J. Agric. Food Chem. 2006, 54, 1467-1472.

Timberlake, C. F. and Bridle, P. Anthocyanins: colour augmentation with catechin and acetaldehyde. J. Sci. Food Agric.1977, 28, 539-544.

Timberlake, C.F. The biological properties of anthocyanins. Nat. Assoc. Colourers. Quart. Inf. Bull. 1988, 4-15.

Tinsley, I.J. and Bockian, A.H. Some Effects of Sugars on the Breakdown of Pelargonidin-3-Glucoside in Model Systems at 90°C. Food Res. 1960, 25, 161-173.

Toromanovic, J.; Kovac-Besovic, E.; Sapcanin, A.; Tahirovic, I.; Rimpapa, Z.; Kroyer, G.; Sofic, E Urinary hippuric acid after ingestion of edible fruits. Bosn. J. Basic. Med. Sci. 2008, 8, 38-43.

Torri, E.; Lemos, M.; Caliari, V.; Kassuya, C.A.L.; Bastos, J.K.; Andrade, S.F. Anti-inflammatory and antinociceptive properties of blueberry extract (*Vaccinium corymbosum*). J. Pharm. Pharmacol. 2007, 59(4), 591-596.

Torskangerpoll, K.; Andersen, Ø.M. Colour stability of anthocyanins in aqueous solutions at various pH values. Food Chem. 2005, 89, 427-440

Torskangerpoll, K.; Chou, E.; Andersen, Ø.M. Separation of acylated anthocyanin pigments by high speed CCC. J. Liq. Chrom. Rel. Tech. 2001, 24 (11-12), 1791-1799.

Trehane, J.. Blueberries, cranberries, and other vacciniums. 2004. Portland: Timber Press.

Trost, K.; Golic-Wondra, M.; Pros□ek, M.; Milivojevic, L. Anthocyanin degradation of blueberry-aronia nectar in glass compared with carton during storage. J. Food Sci. 2008, 73, S405-S411.

Tsai, O.; Huang, H. Effect of polymerization on the antioxidant capacity of anthocyanins in Roselle. Food Res. Int. 2004, 37, 313-318.

Tsai, O.; Huang, H.; Huang, T. Relationship between anthocyanin patterns and antioxidant capacity in mulberry wine during storage. J. Food Qual. 2005, 27, 497-505.

Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-O-B-D-glucoside in rats, FEBS Lett. 1999, 449, 179-182.

Tsuda, T.; Horio, F.; Uchida, K.; Aoki, H.; Osawa, T. Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. J. Nutr. 2003, 133, 2125-2130.

Vissers, M.D.; Zock, P.L.; Roodenburg, A. J.C.; Leene, R.; Katon, B. Olive oil phenols are absorbed in humans. J. Nutr. 2002, 132, 409-417.

Vitaglione, P.; Donnarumma, G.; Napolitano, A.; Galvano, F.; Gallo, A.; Scalfi, L.; Fogliano, V. Protocatechuic acid is the major human metabolite of cyanidin-glucoside. J. Nutr. 2007, 137, 2043-2048.

Wada, L.; Ou, B.; Antioxidant activity and phenolic content of Oregon caneberries. J. Agric. Food Chem. 2002, 50, 3495-3500.

Wang, B.C.; He, R.; Li, Z.M. The stability and antioxidant activity of anthocyanins from blueberry. Food Technol. Biotechnol. 2010, 48(1), 42-49.

Wang, C.Y.; Chen, C.T.; Wang, S.Y. Changes of flavonoid content and antioxidant capacity in blueberries after illumination with UV-C. Food Chem. 2009, 117(3), 426-431.

Wang, H.; Cao, G.; Prior, R.L. Oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 1997, 45(2), 304-309.

Wang, H.; Cao, G.; Prior, R.L. Total antioxidant capacity of fruits. J. Agric. Food Chem. 1996, 44(3), 701-705.

Wang, J. and Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-activated RAW 264.7 macrophages. J. Agric. Food Chem. 2002, 50(4), 850-857.

Wang, J. and Sporns, P. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. J. Agric. Food Chem. 1999, 47, 2009-2015.

Wang, J.; Kalt, W.; Sporns, P. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. J. Agric. Food Chem. 2000, 48, 3330–3335.

Wang, L.S. and Stoner, G.D. Anthocyanins and their role in cancer prevention. Cancer Lett. 2008, 269(2), 281-290.

Wang, S.Y.; Chen, C.T., Sciarappa, W.; Wang, C.Y.; Camp, M.J. Fruit quality, antioxidant capacity, and flavonoid content of organically and conventionally grown blueberries. J. Agric. Food. Chem. 2008, 56(14), 5788-5794.

Wang, W.D.; Xu, S.Y. Degradation kinetics of anthocyanins in blackberry juice and concentrate. J. Food Eng. 2007, 82, 271-275.

Waterhouse, A.L. Wine Phenolics. Ann. N.Y. Acad. Sci. 2002, 957, 21-36.

Wedge, D.E.; Meepagala, K.M.; Magee, J.B.; Smith, S.H.; Huang, G.; Larcom, L.L. Anticarcinogenic activity of strawberry, blueberry, and raspberry extracts to breast and cervical cancer cells. J. Med. Food 2001, 4(1), 49-51.

Williams, C.M.; El Mohsen, M.A.; Vauzour, D.; Rendeiro, C.; Butler, L.T.; Ellis, J,A.; Whiteman, M.; Spencer, J.P.E. Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Free Rad. Biol. Med. 2008, 45(3), 295-305.

Winkel-Shirley B. Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiol. 2001, 126: 485-493.

Wrolstad, R.E.; Skrede, G.; Lea, P.; Enersen, G. Influence of Sugar on Anthocyanin Pigment Stability in Frozen Strawberries. J. Food Sci. 1990, 55(4) 1064-1065, 1072.

Wu, X. and Prior, R.L. Identification and Characterization of Anthocyanins by High-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry in Common Foods in the United States: Vegetables, Nuts, and Grains. J. Agric. Food Chem. 2005, 53, 3101-3113.

Wu, X. and Prior, R.L. Systematic identification and characterization of anthocyanins in HPLC-ESI-MS/MS in common foods in the United States: fruits and berries, J. Agric. Food Chem. 2005, 53, 2589-2599.

Wu, X.; Beecher, G.R.; Holden, J.M.; Haytowitz, D.; et al. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption, J. Agric. Food Chem. 2006, 54, 4069-4075.

Wu, X.; Beecher, G.R.; Holden, J.M.; Haytowitz, D.B. Lipophilic and Hydrophilic Antioxidant Capacities of Common Foods in the United States, J. Agric. Food Chem. 2004, 52, 4026-4037.

Wu, X.; Cao, G.; Prior, R.L. Absortpion and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. J. Nutr. 2002, 132, 1865-1871.

Wu, X.; Gu, L.; Prior, R.L.; McKay, S. Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity, J. Agric. Food Chem. 2004, 52, 7846-7856.

Wu, X.; Pittman, H.E.; Hager, T.; Hager, A.; Howard, L; Prior, R.L. Phenolic acids in black raspberry and in the gastrointestinal tract of pigs following ingestion of black raspberry. Mol. Nutr. Food Res. 2009, 53, 76-84.

Yang, C.S.T.; Atallah, W.A. Effect of four drying methods on the quality of intermediate moisture lowbush blueberries. J. Food Sci. 1985, 50(5), 1233-1237.

Yi, W.; Fischer, J.; Krewer, G.; Akoh, CC. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. J. Agric. Food Chem. 2005, 53(18), 7320-7379.

You, Q.; Wang, B.; Chen, F., Huang, Z,: Wang, X.; Luo, P.G. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. Food. Chem. 2011, 125, 201-208.

Youdim, K.A.; McDonald, J.; Kalt, W.; Joseph, J.A. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J. Nutr Biochem. 2002, 13(5), 282-288.

Zhao, C.; Giusti, M.M.; Malik, M.; Moyer, M.P.; Magnuson, B.A. Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. J. Agric. Food Chem. 2004, 52(20), 6122-6128.

Zheng, W. and Wang, S.Y. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J. Agric. Food Chem. 2003, 5 1, 502-509.

LIST OF ABBREVIATIONS

3-MG, 3-O-methyl-gallic acid;
4HBA, 4-hydroxybenzoic acid;
ABTS, 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt;
ACN(s), anthocyanin(s);
AIN-93, standard rat diet;
ASLT, accelerated shelf-life testing;
a_w, water activity;
BA, benzoic acid;
BHI, brain hearth infusion;
Cy, cyanidin;
Cy-ara, cyanidin-arabinoside;
CydG, Cyanidin-3,5-diglucoside;
Cy-gal, cyanidin-galctoside;
Cy-glc, cyanidin-glucoside;
Cy-glc-Ac, acetylated cyanidin-glucoside;
D, delphinidin;
DAD, photodiode array detector;
D-ara, delphinidin-arabinoside;
D-gal, delphinidin-galactoside;
D-glc, delphinidin-glucoside;
D-glc-Ac, acetylated delphinidin-glucoside;
DHBA, 3,4-dihydroxybenzoic acid;
DW, Dry weight;
Ea, activation energy;
ESI, Electrospray ionization;
FD-WB, Freeze dried wild blueberry;
GA, Gallic acid;
GIT, Gastro-intestinal tract;
HA, hippuric acid;
HFM, human faecal microflora.
HPLC, high-performance liquid chromatography;
IS, Internal standard;
LC, liquid chromatography;
LC-ESI/MS, liquid chromatography coupled with electrospray ionization and mass spectrometry;
LC-MS/MS, liquid chromatography-tandem mass spectrometry;
LLOQ, lower limit of quantification;
LOD, limit of detection;
LSD, least significant difference;
MRM, multiple reaction monitoring;
MS, mass spectrometer;
MS/MS, mass/mass;
Mv, malvidin;
Mv-ara, malvidin-arabinoside;
Mv-gal, malvidin-galctoside;
Mv-gal-Ac, acetylated malvidin-galctoside;
Mv-glc, malvidin-glucoside;
Mv-glc-Ac, acetylated malvidin-glucoside;
Nd, not detectable;
Peo, peonidin;

Peo-ara, peonidin-arabinoside;
Peo-gal, peonidin-galctoside;
Peo-glc, peonidin-glucoside;
Peo-glc-Ac, acetylated peonidin-glucoside;
Pet, petunidin;
Pet-ara, petunidin-arabinoside;
Pet-gal, petunidin-galactoside;
Pet-glc, petunidin-glucoside;
Pet-glc-Ac, acetylated petunidin-glucoside;
PSS, physiologic salt solution;
RP, Residual percentage;
RSD, relative standard deviation;
SA, syringic acid;
SD, Sprague-Doyle;
SPE, solid phase extraction;
$t_{1/2}$, half-life time;
TAA, total antioxidant activity;
TEAC, Trolox equivalent antioxidant capacity;
TFA, Trifluoroacetic acid;
Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman- 2-carboxylic acid;
UPLC, ultra-performance liquid chromatography;
VA, 3,4-dimethoxy benzoic

PUBBLICATIONS

Gardana C, Canzi E, Simonetti P.
R(-)-O-desmethylangolensin is the main enantiomeric form of daidzein metabolite produced by human *in vitro* and *in vivo*.
J. Chrom. B - Accepted

Sri Harsha PS, **Gardana C**, Simonetti P, Spigno G, Lavelli V.
Characterization of phenolics, in vitro reducing capacity and anti-glycation activity of red grape skins recovered from winemaking by-products. Bioresour Technol. 2013; 140: 263-8.

Varoni EM, Vitalini S, Contino D, Lodi G, Simonetti P, **Gardana C**, Sardella A, Iriti M.
Effects of red wine intake on human salivary antiradical capacity and total polyphenol content.
Food Chem Toxicol. 2013; 8: 289-94.

Fracassetti D, Del Bo' C, Simonetti P, Gardana C, Klimis-Zacas D, Ciappellano S.
Effect of Time and storage temperature on anthocyanin decay and antioxidant activity in wild blueberry (*Vaccinium angustifolium*) powder. J Agric Food Chem. 2013; 61(12): 2999-3005

Vitalini S**, Gardana C**, Simonetti P, Fico G, Iriti M.
Melatonin, melatonin isomers and stilbenes in Italian traditional grape products and their antiradical capacity. J Pineal Res. 2013; 54(3): 322-33.

Bò CD, Simonetti P, **Gardana C**, Riso P, Lucchini G, Ciappellano S.
Horse meat consumption affects iron status, lipid profile and fatty acid composition of red blood cells in healthy volunteers. Int J Food Sci Nutr. 2013; 64(2): 147-54

Del Bo' C, Riso P, Brambilla A, **Gardana C**, Simonetti P, Bertolo G, Klimis-Zacas D, Porrini M.
Blanching improves anthocyanin absorption from highbush blueberry (*Vaccinium corymbosum L.*) purée in healthy human volunteers: a pilot study. J Agric Food Chem. 2012; 60(36): 9298-304.

Gardana C, Barbieri A, Simonetti P, Guglielmetti S.
Biotransformation strategy to reduce allergens in propolis. Appl Environ Microbiol. 2012; 8(13): 654-8.

Vitalini S, Gardana C, Zanzotto A, Simonetti P, Faoro F, Fico G, Iriti M.
The presence of melatonin in grapevine (Vitis vinifera L.) berry tissues. J Pineal Res. 2011; 51(3): 331-7.

Gardana C, Simonetti P.
Evaluation of allergens in propolis by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2011; 25(11): 1675-82.

Vitalini S, **Gardana C**, Zanzotto A, Fico G, Faoro F, Simonetti P, Iriti M.
From vineyard to glass: agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. J Pineal Res. 2011; 51(3): 278-85

Pellegrini N, Chiavaro E, **Gardana C**, Mazzeo T, Contino D, Gallo M, Riso P, Fogliano V, Porrini M.
Effect of different cooking methods on color, phytochemical concentration, and antioxidant capacity of raw and frozen brassica vegetables. J Agric Food Chem. 2010; 58(7): 4310-21

Gardana C, Scaglianti M, Simonetti P.
Evaluation of steviol and its glycosides in Stevia rebaudiana leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. J Chromatogr A. 2010; 1217(9): 1463-70.

Del Bò C, Ciappellano S, Klimis-Zacas D, Martini D, **Gardana C**, Riso P, Porrini M.
Anthocyanin absorption, metabolism, and distribution from a wild blueberry-enriched diet (*Vaccinium angustifolium*) is affected by diet duration in the Sprague-Dawley rat.
J Agric Food Chem. 2010; 58(4): 2491-7

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