Chapter 1

General Introduction

1 Flavonoids

1.1 General Information

Flavonoids are a subclass of polyphenols and thereby widespread plant secondary metabolites. Their key feature is a skeletal structure consisting of 15 carbon atoms showing a C_6-C_3-C_6 buildup, called diphenyl propanoid structure (Terahara, 2015). To date, more than 10,000 flavonoids are known (Veitch and Grayer, 2011). Based on the degree of unsaturation in their C_3 section and the position of the phenyl group they are divided into several subclasses, such as anthocyanins, flavanols, flavanones, flavonols, flavones, and isoflavones (Singh *et al.*, 2017). Flavonoids are dissolved in the cytosol inside the vacuoles of the plant cells and they mostly occur naturally as their respective glycoside which increases their water solubility as well as their physicochemical stability (Samanta *et al.*, 2011; Terahara, 2015).

Flavonoids show a variety of bioactivities in plants, as well as in bacteria and humans, wherefore, they have been referred to as Vitamin P in the past (D'Andrea, 2015; Panche *et al.*, 2016). The effects regarding the human organism will be dealt with separately in **chapter 1 paragraph 1.2.2**. Their role for the plant is largely as that of a mediator between the plant and its environment. The benefits for the plant are for example attracting animals in regard of seed dispersal, pollination or protecting against herbivores and microbial infestation (Vicente and Boscaiu, 2018). Furthermore, they protect the plant against biotic and abiotic stresses by acting as UV filters, and antioxidants (Panche *et al.*, 2016). Some flavonoids show a certain capacity to bond with proteins and thereby interact with enzymes or even inhibit them (Falcone Ferreyra *et al.*, 2012).

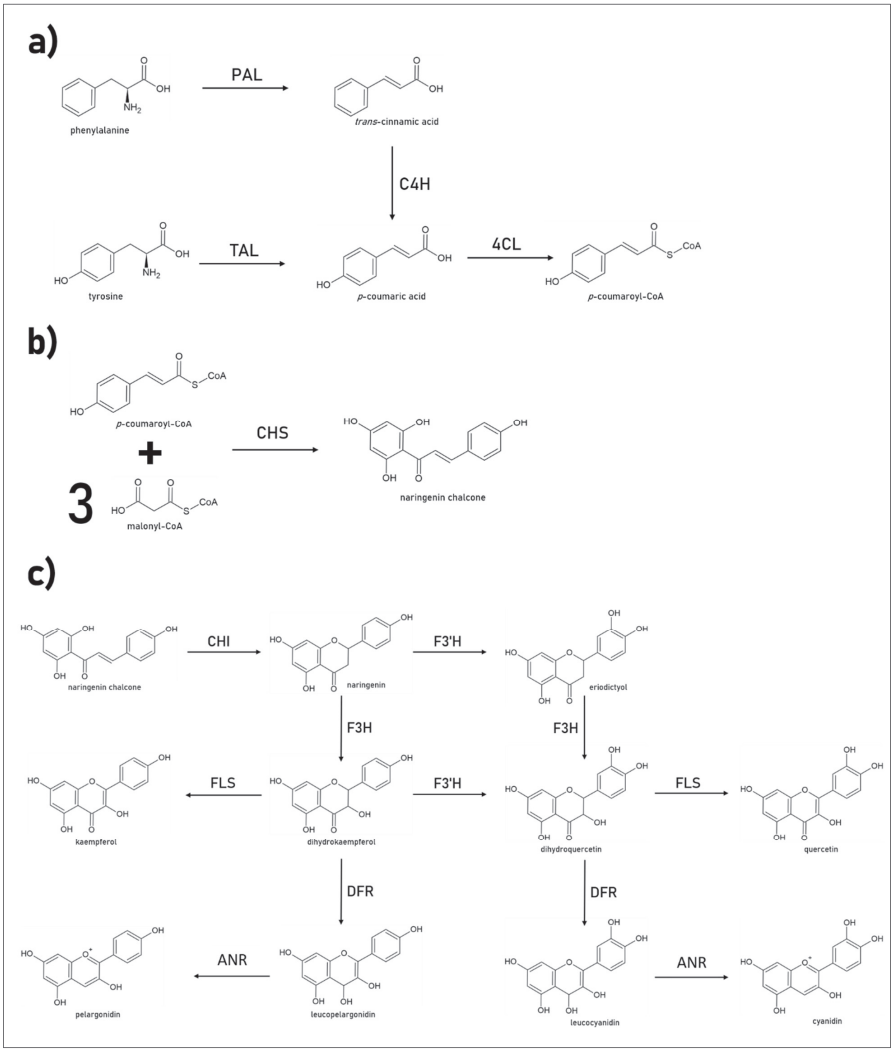

Figure 1-1. Flavonoid biosynthesis of flavonols and anthocyanidins. a) phenylpropanoid pathway, b) polyketide pathway, c) flavonoid pathway. (ANR = anthocyanidin reductase; CHI = chalcone isomerase; CHS = chalcone synthase; C4H = cinnamate 4-hydroxylase; FLS = flavonol synthase; F3H = flavanone 3-hydroxylase; F3'H = flavanone 3'-hydroxylase; PAL = phenylalanine ammonia lyase; TAL = tyrosine ammonia lyase; 4CL = 4-coumaroyl-CoA ligase)

The flavonoid biosynthesis (**Figure 1-1**) emanates from phenylalanine and malonyl-CoA, originating from either the shikimate pathway or the citric acid cycle respectively, and takes place in the cytosol (Tanaka *et al.*, 2008; Davies, 2009). Its initiation is catalyzed by the chalcone synthase and the thereby via the polyketide pathway produced intermediary chalcone (Singh *et al.*, 2017). The enzymes and their substrates form a super-molecular complex at the membrane of the endoplasmic reticulum. These enzymes can be divided into several subgroups, with the main enzymes belonging to cytochrome P450 oxydases (CYP450), glycosyl transferases and 2-oxoglutarate-dependent dioxygenases (Samanta *et al.*, 2011). Phenylalanine and tyrosine are metabolized to *p*-coumaroyl-CoA via the phenylpropanoid pathway (**Figure 1-1a**). Both *p*-coumaroyl-CoA and malonyl-CoA then enter the polyketide pathway (**Figure 1-1b**) which uses the malonyl-CoA as a condensing unit to elongate the C_2-chain of the *p*-coumaroyl-CoA (Singh *et al.*, 2017). The chalcone formed then passes through the flavonoid pathway (**Figure 1-1c**) and is enzymatically converted into the various flavonoid classes, such as flavonols and anthocyanidins. The flavonoid aglycones formed are then enzymatically glycosylated by the flavonoid 3-*O*-glycosyltransferases (Falcone Ferreyra *et al.*, 2012).

1.1.1 Flavonols

Flavonols are the most common and primordial class of flavonoids. They are found in higher concentrations in the outer tissue layers of plants and seeds (Hollman and Arts, 2000; Pollastri and Tattini, 2011; Falcone Ferreyra *et al.*, 2012). Mostly, flavonols occur as their corresponding 3-*O*-glucosides, 3-*O*-rutinosides, 3-*O*-rhamnosides, 3,4'-*O*-diglucosides, or rarely as 4'-*O*-glycosides (Terahara, 2015). Many different fruits and vegetables, such as onions, lettuce, kale, berries, apples, and grapes as well as tea, show rich flavonol contents demonstrating their galore occurrence in nature and thereby their fundamental role as part of the human diet (D'Andrea, 2015; Panche *et al.*, 2016). In addition to the general effects of flavonoids in plants mentioned in **chapter 1 paragraph 1.1**, flavonols show a hormonal effect in plants as well, for example by inducing the production of pollen in flowering plants

(Vicente and Boscaiu, 2018). Amongst the flavonols, quercetin shows the highest antioxidative potential and the highest rate of interactions with various proteins and enzymes in both plants and animal organisms (D'Andrea, 2015). Altogether, flavonols are the most crucial flavonoids in regard of plant stress responses (Pollastri and Tattini, 2011; Falcone Ferreyra *et al.*, 2012).

Depending on the individual diet, the daily consumption of flavonols in the western world is estimated to be about 20–50 mg with the majority being quercetin derivatives (Cermak and Wolffram, 2006; D'Andrea, 2015). Quercetin is a predestined study subject in bioavailability research as, in contrast to other flavonoids, flavonols like quercetin occur in plants in larger quantities as their glucuronide, one of the main human flavonoid metabolites. Thus, the imperatively needed reference substances can be isolated from plant material in sufficient quantities (D'Andrea, 2015; Kaiser *et al.*, 2019), whereas the chemical synthesis of flavonoid glucuronides or even sulfates is a challenging subject and, in this regard, the required amounts are hardly attainable (Zhang *et al.*, 2012; Schmitt *et al.*, 2019). This causes quercetin to be the best-studied flavonol (D'Andrea, 2015). Therefore, in **chapter 2**, quercetin-3-*O*-glucuronide was chosen as the target analyte for the conducted study to test whether enzymatic proteolysis is suitable as a step of sample preparation regarding flavonoid metabolite analyses in plasma. Further common flavonols are kaempferol, myricetin, fisetin, morin, rhamnetin, and isorhamnetin (Aherne and O'Brien, 2002; D'Andrea, 2015; Terahara, 2015).

1.1.2 Anthocyanins

Anthocyanins are the glycosylated derivatives of their aglycones, the so-called anthocyanidins, featuring a phenyl-2-benzopyrilium skeletal structure, also known as flavylium cation, bearing a positive charge (**figure 1-2**) with a chromane ring being substituted with a second aromatic ring in position 2 of the C_6-C_3-C_6 basic structure (Delgado-Vargas *et al.*, 2000). Thus, they are water-soluble plant pigments being responsible for the reddish, violet or blue color of many flowers, fruits — especially berries — and vegetables (Castañeda-Ovando *et al.*, 2009). In this role,

the anthocyanins are present in the plants outer tissue layers being dissolved in the cell vacuole, just as flavonols (Delgado-Vargas *et al.*, 2000).

As a plant pigment, anthocyanins are primarily responsible for the attraction of pollinators and seed distributors. They are able to protect the low-lying photosynthetically important plant tissues by absorbing sunlight in both the visible wavelength range and in the UV range, and thereby reducing the light stress on the plant. Furthermore, they are also important cellular antioxidants. Hence, anthocyanin biosynthesis is induced not just by stress conditions but also by pathogenic infections (Charron *et al.*, 2009; Zhang *et al.*, 2014).

To date, more than 635 anthocyanins have been scientifically recorded (Li *et al.*, 2017). The most common anthocyanidins (**figure 3-1**) are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin with cyanidin-3-*O*-glucoside being the prevalent anthocyanin in nature (Terahara, 2015; Kaiser *et al.*, 2020). As ingredients of red fruits and vegetables, red juices or red wine, and, nowadays, dietary supplements, they are common part of the daily diet. Depending on the individual's dietary habit and its origin, the amount consumed varies from just a few milligrams to more than 200 mg of anthocyanins per day (Wu *et al.*, 2006; Fang, 2014; Kalt *et al.*, 2017).

Anthocyanin color depends on several factors such as the pH, the substitution pattern, such as the degree of methylation or acylation, and even on the different bond glycosides (Panche *et al.*, 2016). The main glycosides of anthocyanidins are 3-*O*-glucosides, 3-*O*-galactosides, 3-*O*-arabinosides, 3-*O*-rhamnosides, and 3-*O*-xylosides. Furthermore, the sugar-moieties of anthocyanins can be esterified by different organic acids, such as coumaric, caffeic, ferulic, malonic, acetic, and *p*-hydroxy benzoic acid (Delgado-Vargas *et al.*, 2000; Zhang *et al.*, 2014).

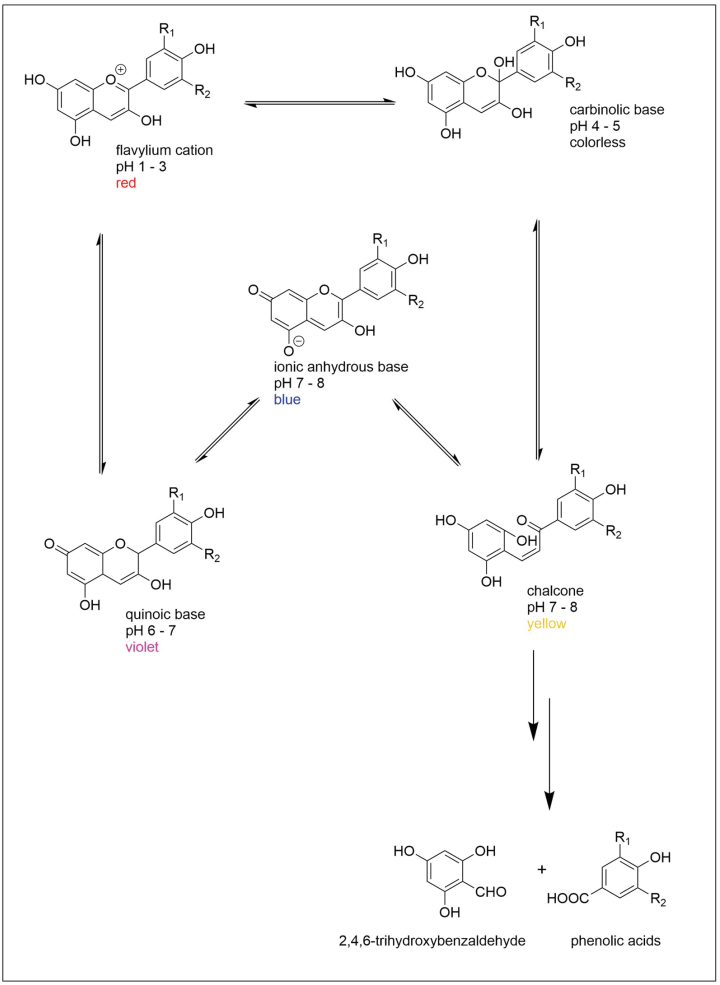

Figure 1-2. pH-dependent structural changes and degradation of anthocyanidins.

Unlike flavonols, anthocyanins do not naturally occur as glucuronides in plants, which means that the necessary reference substances first have to be synthesized with great difficulty (Schmitt *et al.*, 2019). A synthesis of these substances in sufficient quantities is currently hardly feasible so that the glucosides are often used alternatively as a reference substance for quantification purposes (Schmitt *et al.*,

2019; Kaiser *et al.*, 2020). Therefore, the glucosides and the aglycones of cyanidin, delphinidin, malvidin, peonidin and petunidin but not their phase II metabolites have been used as target analytes in the study presented in **chapter 3**.

Contrary to flavonols and other flavonoids, anthocyanins show a minor physicochemical stability (Clifford, 2000; Panche *et al.*, 2016). Their stability depends on various factors, such as the pH value, the temperature, the exposure to light, the surrounding matrix constituents, and their own substitution pattern (Fang, 2014). The latter is the reason only anthocyanins, but not anthocyanidins, are present in nature in free form (Delgado-Vargas *et al.*, 2000). Even small changes in the pH result in fundamental structural changes in anthocyanins (**figure 1-2**), which leads to increasing instability with increasing pH (Fang, 2014; Kaiser *et al.*, 2020).

1.2 Nutritional Importance of Flavonoids

Other than their ubiquitous occurrence in food hypothesizes, flavonoids and their metabolites could only be detected in very small concentrations (nmol/L) in the fluids of the human body. Yet, it is believed that they play a vital role in health care via nutrition (Gleichenhagen and Schieber, 2016), as they provide many health-promoting effects that have been proven *in vivo* (**Chapter 1 Paragraph 1.2.1**). Furthermore, the phase II metabolism of flavonoids is extensively but not entirely studied and most of the resulting metabolites are known to date (**Chapter 1 Paragraph 1.2.2**). Therefore, the present work deals with the development of new state of the art methods to assess flavonoid derivatives levels in blood and urine.

1.2.1 Health-promoting Effects

In the recent past, countless epidemiological, *in vitro* or *in vivo* studies on animals and humans have dealt with the health-promoting effects of flavonoids. Several properties could be attributed to the flavonoids, including the potential to reduce the risk of neurodegenerative or cardiovascular diseases, preventing high blood pressure, type 2 diabetes, atherosclerosis and osteoporosis or to attenuate their etiopathology (Ross and Kasum, 2002; Melo-Filho *et al.*, 2014; Panche *et al.*, 2016; Williamson,

2017). Flavonoids can also demonstrably modulate energy metabolism, reduce endothelial dysfunction, and lower blood pressure or the level of LDL cholesterol in the blood (Williamson, 2017). In summary, they have been shown to possess the following health-promoting effects: anti-carcinogenic, anti-tumoral, anti-inflammatory, anti-oxidative, anti-mutagenic, and anti-viral properties (Ross and Kasum, 2002; Panche *et al.*, 2016; Gramza-Michałowska *et al.*, 2017; Singh *et al.*, 2017), as well as antibacterial, antilipidemic, hepatoprotective, and even anti-obesity properties (Smeriglio *et al.*, 2016; Vicente and Boscaiu, 2018). In addition, flavonoids cause a positive impact on the colon microbiome, including promotion of the growth of desired bacteria and inhibiting the undesirable (Stevens and Maier, 2016; Tian *et al.*, 2019). Hence, flavonoids have recently become an integral part of nutraceutical, medicinal, cosmetic, and pharmaceutical applications (Panche *et al.*, 2016).

In vitro, anti-cholinesterase activity for quercetin was demonstrated as it inhibited both acetylcholinesterase and butyrylcholinesterase. This leads to quercetin being an interesting agent in Alzheimer's research and treatment (Panche *et al.*, 2016). Anthocyanins are of interest regarding the research and treatment of Alzheimer's and dementia, too, since several neuroprotective properties in animals and humans were evidenced in different studies. The mechanism of action in combating Alzheimer's is most likely different for quercetin and anthocyanins, as quercetin reduces the synthesis of Alzheimer's amyloid protein, which significantly delays the course of the disease, whereas anthocyanins are believed to directly affect the neurocytes (Panche *et al.*, 2016; Li *et al.*, 2017). For example, anthocyanins prevent dopaminergic cell death in the brain by influencing the mitochondrial functions (Li *et al.*, 2017). However, both anthocyanins and flavonols can cross the blood-brain barrier *in vitro* and *in vivo* (Youdim *et al.*, 2003; Andres-Lacueva *et al.*, 2005).

Furthermore, anti-tumoral effects were demonstrated for anthocyanins using *in vitro* and *in vivo* studies (Vicente and Boscaiu, 2018). By influencing elementary cell processes such as mitosis, apoptosis, general cell cycle, autophagy, and biochemical

metabolic processes, cancer growth could be prevented or at least inhibited by anthocyanins and their phase II metabolites (Smeriglio *et al.*, 2016; Li *et al.*, 2017). This has been proven *in vitro* and *in vivo* for many different cancers. Anthocyanins impact several major intra- and intercellular signaling pathways by influencing factors such as Wnt/β-catenin, mitogen-activated as well as AMP-activated protein kinases, and the nuclear factor κB (Li *et al.*, 2017). *In vitro*, quercetin can also trigger apoptosis by affecting the mitochondria of the cancer cells. Other anti-cancer activities of quercetin and its derivatives are primarily due to their high antioxidative potential and are reflected in the protection of DNA and RNA against radical compounds like superoxides and peroxynitrite (Ross and Kasum, 2002; D'Andrea, 2015; Panche *et al.*, 2016).

The antioxidant capacity of the flavonols and the anthocyanins has further positive effects on the human organism. However, both depend on the particular molecular structure of the individual substance, as a higher degree of hydroxylation is associated with a higher antioxidative capacity (D'Andrea, 2015; Smeriglio *et al.*, 2016). Flavonol aglycones show superior properties than their derivatives (Ross and Kasum, 2002). However, evidence was found that quercetin-3-*O*-glucuronide, in particular, showed the highest activity in the target tissues, and, thus, the phase II metabolism of flavonoids leads to an increase in effectiveness (D'Andrea, 2015). Quercetin derivatives and anthocyanins, especially derivatives of delphinidin and cyanidin, can chelate metal ions, which could otherwise catalyze the formation of oxygen radicals (Delgado-Vargas *et al.*, 2000; Ross and Kasum, 2002). Regarding anthocyanins, an anthocyanin – metal ion – ascorbic acid complex is also known in this connection (Delgado-Vargas *et al.*, 2000). Due to its low hydrophilicity, quercetin aglycone is a particularly good inhibitor of lipid peroxidation (Ross and Kasum, 2002; D'Andrea, 2015). On the other hand, anthocyanins might play a preventive role concerning fibrosis, liver diseases or vascular failure. Since they can also pass the blood-retina barrier, anthocyanins also reduce the photooxidation of retinal pigments that are essential for eyesight. However, the data available of

improving night vision is contradictory and, therefore, such an effect may be doubted (Smeriglio *et al.*, 2016).

Furthermore, certain parts of the anti-inflammatory activities of flavonols and anthocyanins are caused by their antioxidative capacity. For example, these substances reduce the inflammatory immune response by disposing the resulting radical compounds. A major part of the anti-inflammatory properties of the flavonoids are due to the inhibition of cyclo-oxygenase 2 and the associated abatement of the immune response by inhibiting prostaglandin formation. This effect is much more distinct for flavonols than for anthocyanins (Panche *et al.*, 2016).

Anthocyanins have been well studied regarding palliating metabolic disorders. For example, *in vivo* they can protect pancreatic cells from glucose-induced oxidative stress in the case of obesity. They also modulate the insulin sensitivity and the adipocytokine secretion of adipocytes (Norberto *et al.*, 2013). Anthocyanins showed antidiabetic activity *in vitro* and in animal models. In this regard, there is also epidemiological evidence regarding humans, but this still needs to be proven by *in vivo* studies (Smeriglio *et al.*, 2016; Li *et al.*, 2017). Furthermore, they were able to lower LDL cholesterol levels in the blood in several studies and decreased glucose uptake in the small intestine by about 60% by interfering with the cellular glucose transporters (Kamiloglu *et al.*, 2015; Panche *et al.*, 2016).

Additionally, anthocyanins and flavonols possess anti-viral capacities. While quercetin directly affects the reproduction and infectivity of viral DNA and RNA, anthocyanins can reduce the viral shell's stability (Panche et al., 2016; Gramza-Michałowska et al., 2017). Moreover, *in vivo*, quercetin derivatives can reduce platelet aggregation and thrombus formation, and decrease blood pressure (Brüll *et al.*, 2015; Williamson, 2017). They can also improve the effectiveness of antibiotic therapies by negatively affecting the antibiotic resistance of bacteria. Flavonols are also said to prevent kidney stones and hyperuricaemia by affecting the activity of xanthine oxidase and thus inhibiting the uric acid production (Panche *et al.*, 2016).