

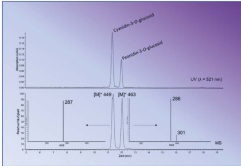
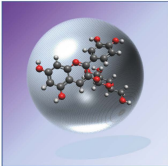



Sarah Straßmann (Autor)
Hemisynthesis of phenolic metabolites

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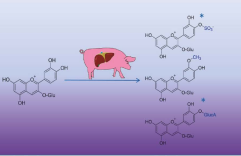
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Chapter 1

General Introduction

1 Polyphenols

Polyphenolic substances are ubiquitous secondary plant metabolites. As a part of this group of phytochemicals, they belong quantitatively to the most important compound classes in nature. They are the second most abundant group of all organic compounds after sugars, with a total share of about 30% of the total biomass on earth.¹ Most of them are produced by plants and are known, among others, as plant pigments or antioxidants in foods. Other producers are fungi and microorganisms. However, these play a minor role in human intake, which is about 1 g per day.² Polyphenols have gained general awareness due to their presence and positive image in so-called super fruits and super foods, which has led to an increased uptake by the population.³

1.1 Properties

As secondary plant compounds, polyphenols are not involved in the primary metabolism of the plant, yet they are essential for the survival of the plant. Their ability to absorb light of different wavelengths serves the plant as protection against harmful UV-B rays. Especially plants that are exposed to high levels of solar radiation increasingly incorporate polyphenols into the epidermis.⁴ There, the polyphenols act as a protective layer, thus preventing mutagenesis, cell death and the formation of oxygen radicals, for example.⁵ A further function is the signal effect. As color pigments, it is mainly anthocyanins that give the flowers and fruits a strong color and attract animals that are responsible for plant reproduction by dispersing the plant seeds or pollinating the flowers.⁶ Just as they can attract living organisms, polyphenols can also protect the plant from harmful organisms by forming physical or chemical barriers. They can repel predators by an anti-microbial effect, an astringent or bitter taste, or poor digestibility.^{5,7,8} Like the antioxidant effect, the anti-microbial properties are based on the inhibition of harmful



enzymes such as cellulases or pectinases, which can be released by microorganisms to destroy the plant cell wall.⁵

Furthermore, polyphenols that enter the soil after the plant has died can have a positive effect on decomposing microorganisms in the soil. The increased activity of the destructors results in a higher conversion of plant material and thus a higher nutrient density in the soil. Plants can also release polyphenols as allelochemicals to inhibit the growth of competing plants. The possible function of polyphenols as physiological regulators or messenger substances is also currently being discussed.⁵

1.2 Substance classes

The substance classes in which polyphenols occur are quite different and literature describes many possible ways of classifications.⁹ A grouping based on their chemical structure seems appropriate: They can be divided into tannins with more than 15 carbon atoms, hydroxycinnamic acids with 9 carbon atoms, and flavonoids with 15 carbon atoms. All these can in turn be divided into subclasses. As the compounds relevant for this work are the class of flavonoids, the others are neglected here.

Flavonoids often occur connected to one or more sugar or acid moieties. The structure of flavonoids is based on the flavan framework, which consists of two benzene rings (A and B) connected by a heterocyclic pyran ring (C) and thus is a C₆-C₃-C₆-structure.¹⁰ The class of flavonoids can be divided into subgroups such as, for example, flavanones like naringenin, flavonols like quercetin, or isoflavones like genistein. This division is created by varying the oxidation status of the C ring and the position of the B ring.¹¹ Since the focus of this work is on anthocyanins, only these will be discussed in the following sections.

2 Anthocyanins

Anthocyanins play a crucial role among the flavonoids due to their positive charge. Hence, they are also referred to as flavylium salts, with chloride being the most common counterion in plants.¹² They are colorful and occur in plants in the vacuoles of different cells as blossom dye or as pigment in other parts of the plant, for example in berries like black currant, raspberries, or blackberries.⁶

The six mostly distributed anthocyanidins in nature are shown in **Figure 1**. They are distinguished by different hydroxy and methoxy substitution patterns at the B ring.¹³

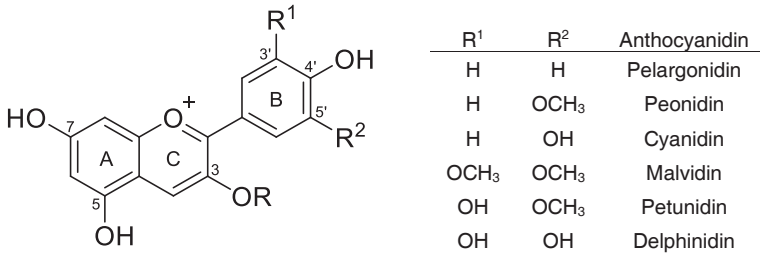


Figure 1: Anthocyanin backbone with R = sugar. If R = H, it represents an anthocyanidin.

The aglycone is called anthocyanidin. Anthocyanidins are very unstable concerning light, high temperatures, and high pH values, which is why they are glycosylated in plants, esterified with organic or other phenolic acids or copigmented with other flavonoids.¹⁴ Glycosylation on the C ring occurs most frequently. It is also observed on the two hydroxyl groups of the A ring, with position 5 being preferred.¹⁵ The monosaccharides glucose, galactose, rhamnose, xylose and arabinose are the most common sugar residues. Di- and trisaccharides and multiple substitutions may also occur. Furthermore, glycosylation at some of the carbon atoms is possible.⁶ In addition, the sugar residue may be esterified with aliphatic/aromatic acids.¹³ Glycosylation is generally associated with an increase in the physicochemical stability and water solubility of the anthocyanins. The acylation of the sugar residues with cinnamic acid or aliphatic acids even promotes these properties.¹⁶ According to He and Giusti, this increase in stability is attributed to the formation of intramolecular hydrogen bridge bonds.³

The focus of this work is on cyanidin, which is the most common anthocyanidin and cyanidin-3-O-glucoside as the most common anthocyanin.⁶

2.1 Chemical properties

Due to the substituted sugar residues, anthocyanins are water-soluble and are found in nature as pigments in the cell fluid of numerous plants, which has a slightly acidic environment. The color of the anthocyanins is exclusively due to the aglycone backbone, whose structural – and thus also color - appearance is strongly pH-dependent (**Figure 2**).¹⁷

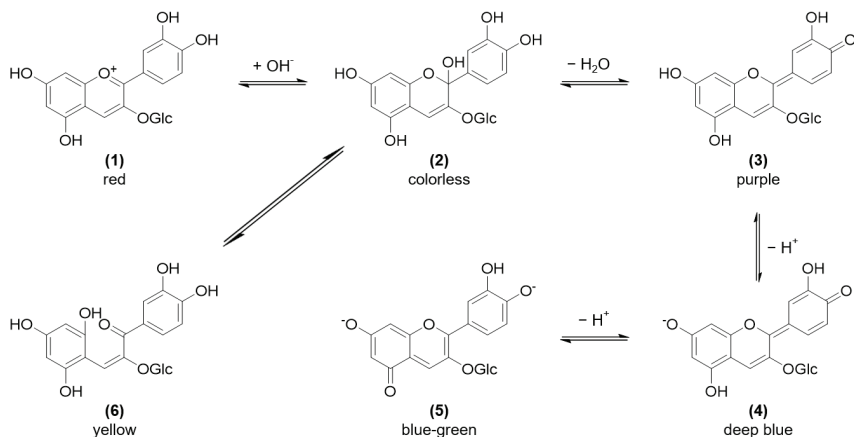


Figure 2: Structural formulae of cyanidin-3-O-glucoside at different pH values^{17,18}

For anthocyanidins, the formation of chromenol (**Figure 2**, compound (2)) is already critical because they can then be further hydrolyzed via the intermediate stage of an α -diketone (**Figure 3**, compound (7)) to an aldehyde (**Figure 3**, compound (8)). This originates from the A ring and is identical for all aglycons. In addition, a carboxylic acid (**Figure 3**, compound (9)) is formed, which is characteristic for the respective anthocyanidin. The ring opening and thus the transition into the chalcone structure is an endothermic reaction. The decomposition process can be accelerated considerably, especially by increasing the temperature.¹⁰

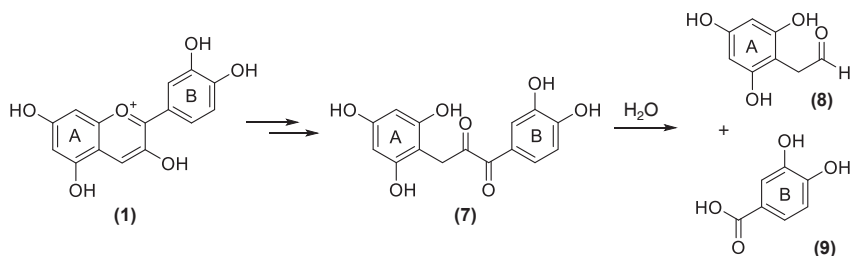


Figure 3: Decomposition of cyanidin at higher pH values¹⁸

2.2 Physical properties

Anthocyanins show characteristic UV absorption spectra, which can be used for identification and confirmation purposes during analysis. The spectra of anthocyanins, present in the form of flavylium cations (**Figure 2**, compound (1)), are characterized by two maxima depending on



the respective solvent: One is located at 265 - 280 nm, while the other is in the visible range at 465 - 560 nm and is very pronounced. The UV absorption spectra of anthocyanins are pH-dependent: If no conjugated aromatic system is present at an pH value about 4.5 (structure (2), **Figure 2**), no maximum is observed at 465 - 560 nm.¹⁹ This characteristic is illustrated in **Figure 4**.

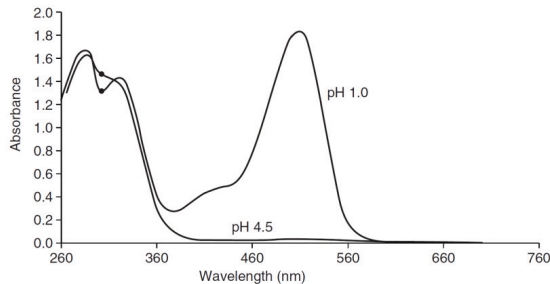


Figure 4: UV-Vis spectra of acylated pelargonidin-3-sophoroside-5-glucoside derivatives at pH 1.0 and 4.5.¹⁹

Structural differences can be identified using the UV spectrum. Thus, 3-monoglucosides can be differentiated from anthocyanins with a further sugar residue at the C-5 position. The monoglucosides have a shoulder at about 440 nm or before their second absorption maximum.²⁰ Conjugated groups as well as pH changes lead to shifts of the absorption maxima. Cruz and co-workers¹⁷ investigated the changes of the absorption maximum of cyanidin-3-O-glucoside-7-O-glucuronide with increasing pH values. Moreover, certain conjugated groups may have additional absorption maxima, e.g., conjugation with acylated hydroxycinnamic acid leads to a third maximum at about 310 nm.²¹ Glycosylation and methylation at the B ring cause a blue shift (shift to lower wavelengths, also: hypsochromic shift), while with increasing hydroxylation as well as inter- and intramolecular copigmentation a red shift (shift to higher wavelengths, also: bathochromic shift) is observed.²¹ This knowledge found application in this work.

2.3 Physiological properties

It has been known for many centuries that a high proportion of vegetable food is healthy. In the past two decades, this traditional knowledge has been scientifically verified, with the result that the consumption of larger quantities of fruit and vegetables leads to a reduction in the incidence of cardiovascular diseases, cancer, osteoporosis, or diabetes.³ Besides vitamins and minerals, mainly polyphenols have been identified as the cause of this positive health effect. Dietary antioxidants, such as anthocyanins, are able to increase the antioxidant capacity of the serum, which can provide low density lipoprotein (LDL) oxidation and thus prevent



cardiovascular diseases such as arteriosclerosis.³ Based on model experiments, it is also assumed that a diet rich in anthocyanins may have positive effects on the loss of cognitive abilities and neurodegenerative diseases with increasing age.¹³ The neuroprotective efficacy is based in particular on the fact that anthocyanins are able to cross the blood-brain barrier of pigs and rats and are thus possibly also centrally effective.²² Anthocyanins from berry fruits are also believed to have a positive effect on vision in several ways.²³ On the one hand, they improve night vision by increasing the generation of retinal pigments and, on the other hand, they are considered to help prevent glaucoma, retinitis pigmentosa and cataracts.¹³ Anthocyanins are also said to possess an antidiabetic activity.²³ The antioxidant anthocyanins help to protect pancreatic cells from glucose-induced oxidative stress.²³ It is also believed that anthocyanin uptake might improve the function of adipocytes, thereby preventing metabolic syndrome or obesity.³ It has been observed that all inflammatory parameters can effectively be reduced by a sufficiently high anthocyanin concentration.³ Therefore anthocyanins are also supposed to have an anti-inflammatory effect¹³. The anticarcinogenic activity of anthocyanins is attributed to additive effects of several mechanisms. Among the mechanisms involved are antimutagenic activity, inhibition of oxidative DNA damage, inhibition of carcinogenic activation, stimulation of phase II enzymes for detoxification, stimulation of cell cycle arrest, inhibition of COX-2 enzymes, induction of apoptosis and anti-angiogenesis.³ In particular, cyanidin and delphinidin have been shown to inhibit the growth of certain tumor cells, whereas the respective glucosides have been shown to be inactive in this regard.⁶

2.3.1 Antioxidativity and radical scavenging properties

Due to their numerous OH groups on aromatic rings, polyphenols are rich in electrons and therefore good nucleophiles. This means they can be easily oxidized and therefore used as antioxidants.²⁴ Phenols can release hydrogen ions. This is possible via radical or acid-base reactions. The aromatic ring can then stabilize the resulting compound well by resonance. Harmful biological radicals such as singlet oxygen, hydroxyl- and peroxy radicals as well as nitrogen radicals, can thus be intercepted by them. Reactive oxygen species (ROS) can otherwise lead to oxidative damage to DNA, proteins, and lipids.¹

There are various mechanisms by which anthocyanins unfold their antioxidative potential. For example, due to their electron-donating properties, they can directly intercept free radicals and ROS²⁴. Anthocyanins indirectly support the body's own antioxidative defense mechanisms by, for example, increasing the glutathione content in the cells²⁵, inhibit enzymes that are responsible for the formation of free radicals or regenerate other antioxidant substances such as α -tocopherol.²⁶⁻²⁸



2.3.2 Bioavailability

In order for a substance to exert an effect in the body, it needs to be absorbed into the blood and must be transported to its site of action. This process is called bioavailability. Knowledge of the bioavailability provides a crucial basis for assessing the physiological significance of bioactive food ingredients. Without any knowledge about the behavior of a substance in the body, it is almost impossible to make a statement about its efficacy. In order to investigate this, studies have to be carried out that explore every single way a molecule may pass in the body. The bioavailability of a nutrient can be strongly influenced by factors such as other food ingredients in the gastrointestinal tract, but also the change of the substance by metabolism.²⁹ In animal model studies, anthocyanins are detectable in the blood just a few minutes (6-20) after oral administration.³⁰ There are several assumptions about their uptake by humans and animals. One concludes that a part is already absorbed in the stomach as intact glycosides with the help of bilitranslocase and enters the bloodstream via the liver.³¹ Another study shows absorption in the jejunum. It takes place actively via the sodium-dependent glucose co-transporter SGLT1.³² In contrast, the aglycones can diffuse passively through the mucosa because they are more hydrophobic.¹¹ Consequently, anthocyanins can either be taken up via active mechanisms or they are absorbed passively by enzymes such as β -glucosidase converting them into their corresponding anthocyanidins.³³ Anthocyanins that have not been absorbed up to that point can be hydrolyzed by the colon microbiota. The resulting aglycons are broken down to phenolic acids, among other compounds, which can then pass through the intestinal wall.³⁴

2.3.3 Absorption, distribution, metabolization and excretion (ADME)

It is known that, though their bioavailability is low, the human organism largely metabolizes dietary flavonoids to different conjugates that further appear in the bloodstream. Through this biotransformation the organism makes substances more hydrophilic to excrete them more efficiently. All of the following steps of the metabolism are highly dependent on various factors such as age, gender, genotype, microbiome and the composition and processing of the ingested food.²⁹ A recapitulation of the ADME process is illustrated in **Figure 5**. At each of the described points, it is possible that the body builds substances that can have a different effect than the original compounds. They can then, for example, change the effects described in section 2.3 or even cause them themselves. Therefore, more research concerning these metabolites is needed.



2.3.3.1 Mouth and stomach

Already in the mouth some polyphenols are deglycosylated and hydrolyzed by the oral microbiota. In the stomach, the polyphenols are further hydrolyzed by the acidic environment. Smaller phenolic acids can already be absorbed in the stomach, while some larger compounds reach the intestine completely unchanged.³⁵ Anthocyanins are the only group of flavonoids that are absorbed in this stage and are thus the first that reach the bloodstream.³⁶

2.3.3.2 Small intestine

Monomeric flavonoids such as catechin can be absorbed unchanged by passive diffusion into the enterocytes.³⁷ In general, however, polyphenols are found in food as esters, glycosides or polymers. Due to their higher molecular weight, these cannot be absorbed directly, but are first depolymerised by the enzymes of the microflora. The glycosidic bond is cleaved and the aglycones are broken down to smaller molecules.³⁸⁻⁴¹ The smaller molecules are then also absorbed into the enterocytes by passive diffusion. Some of the glycosidically bound polyphenols are not degraded but taken up via D-glucose transporter proteins.⁴¹ In the enterocytes, the absorbed phenolic compounds are glucuronidated by the UDP-glucuronosyltransferase and subsequently absorbed by the liver cells.^{42,43} Some studies have shown that the absorbed aglycones in the enterocytes are not only glucuronidated but also partly methylated there and in the case of quercetin also sulfated.⁴⁴⁻⁴⁶ Sulfation, glucuronidation and methylation are part of the biotransformation of xenobiotics in the human body. To excrete xenobiotics more effectively, water solubility and molecular weight are increased in two phases. In phase I a polar functional group is attached to the foreign substance by hydrolysis, oxidation, or reduction. These are catalyzed by enzymes like oxygenases, dehydrogenases, cytochrome P450 or esterases. In vitro experiments demonstrated that anthocyanins do not undergo phase I metabolism by cytochrome P450 enzymes.⁴⁷ In phase II, the water solubility and molecular weight of the foreign substance is further increased by sulfation and glucuronidation, among others. Methylation additionally reduces the pharmacological activity of foreign substances.⁴⁸⁻⁵⁰ The phase II reactions are described in detail in section 2.3.4.

2.3.3.3 Colon (microbiome)

Only 5-15% of the flavonoids are absorbed in the small intestine; the majority reaches the large intestine. Here they are intensively metabolized by the intestinal bacteria. Dependent on the concentration, they are toxic to many intestinal bacteria and may thus change the spectrum of the intestinal biota. However, the microorganisms also produce new compounds like small phenolic acids or other aromatic catabolites, which in turn can be absorbed into the blood, be metabolized, and can have effects on health. This bidirectional relationship is the subject of



current research. It is also investigated whether the catabolites may have a higher health-promoting effect than the phase II metabolites of intact anthocyanins.²⁹ To study this in humans, reference substances are urgently needed to investigate their effects in cell experiments or in vivo.

2.3.3.4 Liver

The liver plays an important role in the detoxification of the body. Xenobiotics and toxic substances can be converted and broken down. These can then be excreted through the urine or bile. Nutrient-rich blood from the intestine is supplied via the portal vein to the liver. Nutrients as well as drugs and other substances are absorbed and metabolized. Most of the compounds are already glucuronidated in the enterocytes, but some compounds also reach the liver unmetabolized and are glucuronidated there. A wide variety of metabolic reactions of unreacted, already metabolized or hydrolyzed compounds can take place here.^{42,43,49} Methylation is carried out by O-methyltransferases, glucuronidation by UDP-glucuronosyltransferases and sulfation by sulfotransferases.^{49,51} The enzymes and their reactions are described more in detail in section 2.3.4.

2.3.3.5 Blood

The metabolized phenols are now released from the stomach, intestine and liver into the blood, where they bind to plasma proteins such as human serum albumin (HSA).¹⁴ The polyphenol metabolites can reach the cells via the bloodstream. By binding to the HSA and the cells, the availability of the phenolic metabolites may be reduced.^{52,14} Through the association to proteins, the resulting metabolites or even the sensitive anthocyanin aglycones may be protected from degradation.²²

2.3.3.6 Cells

Possibly an altered pH value or interactions with the cell membrane can induce the dissolution of the phenol-HSA complex. Whether any phenolic compounds can be taken up into the cells depends on their form, e.g., methylated flavan-3-ols are taken up into cells, whereas glucuronidated flavan-3-ols are not.³⁵ Binding to the target cells probably occurs via a hydrogen bond between the polar phospholipid head of the cell membrane and the hydroxyl group of the phenol, whereby a higher number of hydroxyl groups on a phenol could provide a stronger bond.¹⁴ It is still unclear whether certain phenols only bind to specific target cells. However, in animal experiments a large number of metabolites in various concentrations have been found in almost all organs.¹⁴ Some mainly non-polar, lipophilic phenolic compounds can even pass the blood-brain barrier.³⁵

2.3.3.7 Excretion

The presence of a high level of methylated anthocyanins in rat liver and the low level of these derivatives in plasma suggests that these metabolites may be excreted directly from the liver into the bile, as previously suspected. The final site anthocyanins pass before excretion is the kidney.

Here, metabolism also occurs, with methylation appearing to be the main pathway. However, UGT has also been detected in kidney tissue and glucuronides have been detected accordingly.⁵³

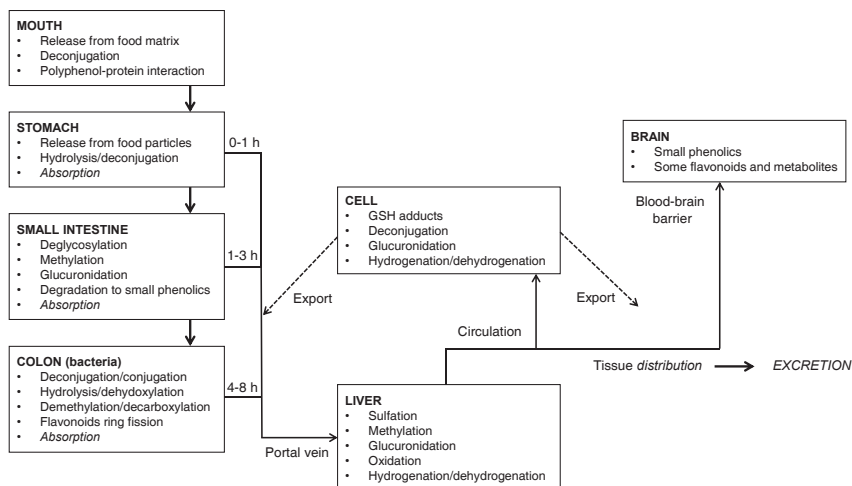


Figure 5: Absorption, distribution, metabolism and excretion of polyphenols³⁵

2.3.4 Phase II metabolism

Phase II enzymes, such as UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT), catalyze the functionalization of activated or nucleophilic compounds, such as anthocyanins, by conjugation with electrophilic molecules such as glucuronic acid or sulfate residues. (Figure 6) This allows inactivation of these compounds, which usually become more polar and thus may be more easily excreted via the kidneys and bile. Enzymes like the catechol-O-methyltransferases (COMT) are mainly used for the masking of reactive functional groups (e.g. -SH, -OH and -NH).