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Anthocyanins and Copigments from fruits, vegetables and flowers: Characterization, Separation and Isolation by Membrane and Countercurrent Chromatography

Miriam A. Rodríguez-Werner

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To André and my parents

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Abbreviations

AcOH	Acetic acid
AF	Anthocyanin-fraction
AJ	Concentrated aronia juice
arab	arabinoside
AP	Aronia pomace
A20	Actiplants® Aronia 20 %
Conc.	Concentration
CCC	Counter-current chromatography
CH_3CN	Acetonitrile
CF	Copigment-fraction
CQA	Caffeoylquinic acid
Cy	Cyanidin
δ	Chemical shift in ppm (Parts per Million)
d	Doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DAD	Diode array detector
DEPT	Distortionless enhancement by polarisation transfer
Del	Delphinidin
diglc	diglucoside
DM	Dry matter
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
FW	Fresh weight
GAE	Gallic acid equivalent
gal	galactoside
glc	glucoside
H	Head
HCl	Hydrochloric acid
Hz	Hertz
HPLC	High-performance liquid chromatography
HSCCC	High-Speed Counter-current chromatography
i. D.	inside diameter
J	J-coupling in Hertz (s-1; Hz)

k	Partition coefficient
λ	Wavelength in nm
LC	Liquid Chromatography
LP	Lower phase
LSRCCC	Low Speed Rotary Countercurrent Chromatography
m	Multiplett
M	Molar
$[M]^+$	Pseudo-molecular ion (pos. Modus)
$[M-H]^-$	Pseudo-molecular ion (neg. Modus)
max	Maxima
MC	Membrane chromatography
MeOH	Methanol
min	Minutes
MS	Mass spectrometry
Mv	Malvidin
m/z	Mass-to-charge-ratio
n-BuOH	n-Butanol
n.d.	Not detected
nm	nanometer
NMR	Nuclear magnetic resonance
No.	Number
p. a.	per Analysis
Pg	Pelargonidin
Pn	Peonidin
ppm	Parts per million
Pt	Petunidin
q	quartet, quadruplet
rpm	Revolutions per minute
RP-18	Reversed-phase 18
rut	rutinoside
s	Singlet
samb	sambubioside
soph	sophoroside
T	Tail
t	triplet
tBME	*tert*-butyl methyl ether
TFA	Trifluoroacetic acid

TFA-d_1	Trifluoroacetic acid- d_1
TLC	Thin-layer chromatography
t_R	Retention time
UV	Ultraviolet
Vis	Visible
v/v	Volume ratio
xyl	xyloside

¡Ahí está el detalle! (Mario Moreno Cantinflas)

¡Lo sospeché desde un principio! (Roberto Gómez Bolaños)

1 Introduction and Intention

Colors and sensory perception have a relevant role with respect to products consumption. For example, colorants are used in the cosmetic industry in order to stimulate feelings such as freshness. In the food industry, consumers buy food according to color and quality. However, food coloration can change over time, after processing or due to addition of colorants.

Colorants are soluble or solubilized coloring agents. The colorants are classified into inorganic and organic colorants and both classes contain two subcategories: natural and synthetic. However, the regulations for the use of food colorants can vary from country to country. For instance, the synthetic colorant Amaranth (E123 or FD&C Red No 2), which gives red coloration, can be used only for defined purpose as food colorants in the European Union according to Regulation EC No.380/2012 (European Commission, 2012), while it is not permitted as food colorants in the U.S.A.
In the 1980´s, the use of tartrazine (Yellow No. 5), which is a synthetic colorant, caused controversy in the U.S.A. because it was related to hyperactivity disorder in children. In addition, this case forced an evaluation and investigation of diverse additives, which resulted in the Food Advisory Committee (FDA) no authorizing the use of Ponceau 4R, while yellow No. 5 and those authorized for use must be declared in food labeling because may cause allergic reactions. Quinolone yellow can only be used for cosmetic and drugs but not in food, while FD&C Red No. 40 and Yellow No. 6 can be used as colorants for food, drugs and cosmetic (Food Advisory Committee, 2011).
The food safety assessment has led to a change in the regulations and the establishment of accepted daily intakes (ADIs) based on scientific research. Moreover, the interest for removing and replacement of synthetic colorants has been increasing due to public scandals in previous years.
As a result, consumers have increased their preference for the consumption of natural products but also the impact of unfavorable publicity on reputation of companies, as was the case of Starburck Corporation US in 2012. Daelyn Fortney published on her website that some drinks of this company contained cochineal extract, which is not for vegans (Fortney, 2012). Subsequently, she started a petition to change the use of food colorants based on insects and thousands of people signed her online petition. The company reacted immediately on this petition and indicated that cochineal is approved by FDA and present no risik to the health. However, the company reviewed its ingredients and substituted cochineal extract by lycopene that is a tomato-based extract (Burrows, 2012a, 2012b).

Therefore, the search of natural colorant sources has raised concerns on the use of colorants which may led to allergic reactions. Nonetheless, natural colorants can be obtained from fruits or vegetables, as well as flowers (e.g. saffron, hibiscus, squash blossoms, etc.) that are used in the traditional cooking of some cultures. These sources contain natural pigments that give their characteristic coloration. For example, spinach contains chlorophylls that give a green coloration, while the orange-yellow color of

carrots is due to carotenoids and the red-purple colors from berries are due to the presence of anthocyanins.

The purpose of the present work is the characterization, separation and isolation of anthocyanins. These compounds can be used as natural food colorants and are present in many fruits, vegetables and flowers. The anthocyanins are responsible for the pink, magenta, red, purple and blue coloration. Several factors such as pH, temperature, metal ions, light, as well as copigmentation with other compounds like flavonoids can influence the stability of anthocyanins. Additionally, they are associated with significant bioactivities such as antioxidant, antimicrobial, antibacterial, anti-inflammatory, anti-hypertension, anti-diabetic, anti-cancinogenic, anti-cardiovascular, vision (Zhao, 2007; Gould et al., 2009; Wallace and Giusti, 2013; Warner, 2014).

In the first part of this work, a characterization of phenolic composition was performed by spectrophotometric and chromatographic methods in order to identify individual compounds, particularly, from blackberry, black chokeberry, sour cherry, black carrots, purple sweet potato, roselle and butterfly pea. Thus, their fingerprints were established for future authentication controls. Subsequently, the isolation of polyphenols was carried out on a large scale by solvent extraction and after concentration by adsorption chromatography, anthocyanin-rich extracts were obtained.

In the second part, the preparative separation of anthocyanins was achieved using two chromatographic methods. Firstly, the fractionation of polyphenols was carried out by membrane chromatography and the compounds were separated into two groups: anthocyanins and copigments. Secondly, the separation of concentrated extract, anthocyanin fraction or copigment fraction was performed on a large scale by countercurrent chromatographic techniques in order to isolate individual compounds in high purity and quantity.

Additionally, the application of preparative HPLC was used for a final purification of compounds. Finally, identification and structure elucidation of compounds were achieved by TLC, UV spectra, HPLC-DAD, HPLC-ESI-MS^n and NMR analyses.

2 General

2.1 Polyphenols

Metabolites are compounds synthesized by plants for both essential functions, such as growth and development of plants (primary metabolites), and non-essential functions, such as protecting plants (secondary metabolites). Primary metabolites like lipids, carbohydrates, proteins, fats, water, vitamins are present in all plants, and are therefore involved with the essential processes in the plant while secondary metabolites especially phenolic compounds, terpenoids, alkaloids and sulphur-containing compounds are present in specific plants and are involved with the non-essential processes. Secondary metabolites are of considerable interest as sources of pigments, flavors, drugs, antibiotics, pollinator attraction, insecticides and herbicides (Croteau et al., 2000; Crozier et al., 2006; Dewick, 2012).

Phenolic compounds are characterized by the presence of an aromatic ring bearing one or more hydroxyl groups while polyphenols contain two or several phenol rings and are present in fruits, vegetables and plants (Balasundram et al., 2006). They are considered as the most investigated metabolites for their biological activities and health benefits such as anti-inflamatory and antioxidant activities, therapeutic treatment of obesity and diabetes, and in treatment and prevention of cardiovascular and cancer diseases (Watson et al., 2014).

2.1.1 Classification of Polyphenols

Polyphenols can be classified, based on the number of carbons in their structure, into two main groups: non-flavonoids and flavonoids (Fig. 2.1). The non-flavonoids are further divided into hydroxybenzoic acids (which have a C6-C1-basic skeleton), hydroxycinnamic acids (with a C6-C3-basic skeleton) and stilbenes (which possess a C6-C2-C6-basic skeleton). The flavonoids are characterized by a C6-C3-C6 core structure, which consists of flavanones, flavones, flavonols, anthocyanidins and flavanols. The distribution of polyphenols is not similar in all foods of plant origin, considering that some polyphenol classes are present in a particular genus and species. As a result of this, the analysis of these compounds can help to confirm the authenticity of food products and to identify adulterations.

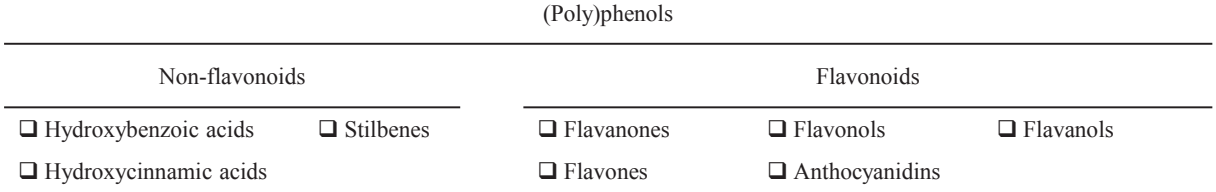


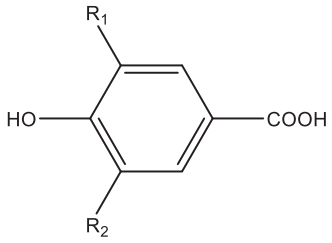
Fig. 2.1. Classes of polyphenols (Iriti and Faoro, 2009).

2.1.1.1 Non-flavonoids

This class of compounds is non-colored, but they are associated with the stability and increased color intensity in foods by inter- and intramolecular reactions. There are three subcategories: hydroxybenzoic acids, hydroxycinnamic acids and stilbenes. The most well-known stilbene is resveratrol and its glycosides which are present in grapes, particularly in seeds and roots of *Vitis vinifera*, *labrusca* and *Muscadine* grapes. However, derivatives of hydroxybenzoic and hydroxycinnamic acids are known as phenolic acids, although both groups present a carboxylic acid functionality. These phenolic acids are basic units and precursors for the biosynthesis of complex molecules like lignins, hydrolyzable tannins such as gallotannins (Rentzsch et al., 2009; Fraga, 2010).

2.1.1.1.1 Hydroxybenzoic acids

The most common hydroxybenzoic acids are *p*-hydroxybenzoic, protocatechuic, vanillic, gallic and syringic acids (Fig. 2.2), but they are generally present in bound forms (glycosides, glucose esters, etc.). Investigation on food composition revealed very low concentration of hydroxybenzoic acids in fruits like blackberries, blueberries, blackcurrants, redcurrants, sour cherries, sweet cherries, grape, grapefruit, plums and strawberries (Schuster and Herrmann, 1985; Macheix et al., 1990). Similarly, low concentrations are also present in some vegetables such as spinach, chicory, onions and cauliflowers, and in sweet potatoes and carrots. Gallic acid, on the other hand, is notably present in black and green tea, as well in nuts and spices like cloves (Herrmann, 1989; Rothwell et al., 2013).

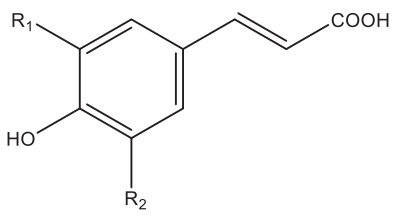


Compound	R_1	R_2
***p*-Hydroxybenzoic acid**	H	H
Protocatechuic acid	OH	H
Vanillic acid	OCH_3	H
Gallic acid	OH	OH
Syringic acid	OCH_3	OCH_3

Fig. 2.2. Chemical structure of hydroxybenzoic acids.

2.1.1.1.2 Hydroxycinnamic acids

Hydroxycinnamic acids consist of p-coumaric acid, caffeic acid, ferulic acid and sinapic acid (Fig. 2.3), and are more predominant than hydroxybenzoic acids.



Compound	R_1	R_2
***p*-Coumaric acid**	H	H
Caffeic acid	OH	H
Ferulic acid	OCH_3	H
Sinapic acid	OCH_3	OCH_3

Fig. 2.3. Chemical structure of hydroxycinnamic acids.

Ferulic acid is present in cereal grains, especially in wheat (0.15-72.21 mg/100 g FW) and maize (0.53 mg/100 g FW), but free forms of hydroxycinnamic acid are rarely found in foods. These acids are usually present in the form of hydroxyacid esters, for example quinic, shikimic and tartaric esters. Chlorogenic acid, which is commonly called 5-CQA, consists of an ester of caffeic acid and quinic acid, which is a principal hydroxycinnamic acid derivative in some fruits and coffee, while their isomers neochlorogenic and cryptochlorogenic acids are present in lower concentration. Nevertheless, hydroxycinnamic acids are also found as glycosylated derivatives, while some of them are linked with flavonoids, lignin, suberin and cutin. Food sources of hydroxycinnamic acids are fruits such as apples, berries, kiwi, lemon and stone fruits like apricots, cherries and peach. Furthermore, they are also found in olives, nuts, coffee, cocoa, cauliflower, root vegetables and tubers (Macheix et al., 1990; Manach et al., 2004; Rothwell et al., 2013).

2.1.1.2 Flavonoids

The flavonoid group is the largest group of polyphenols and contains numerous phenolic compounds, which have a chemical structure with three rings, two benzene rings (A- and B-ring) connected with a heterocyclic ring (C ring) as illustrated in Fig. 2.4.

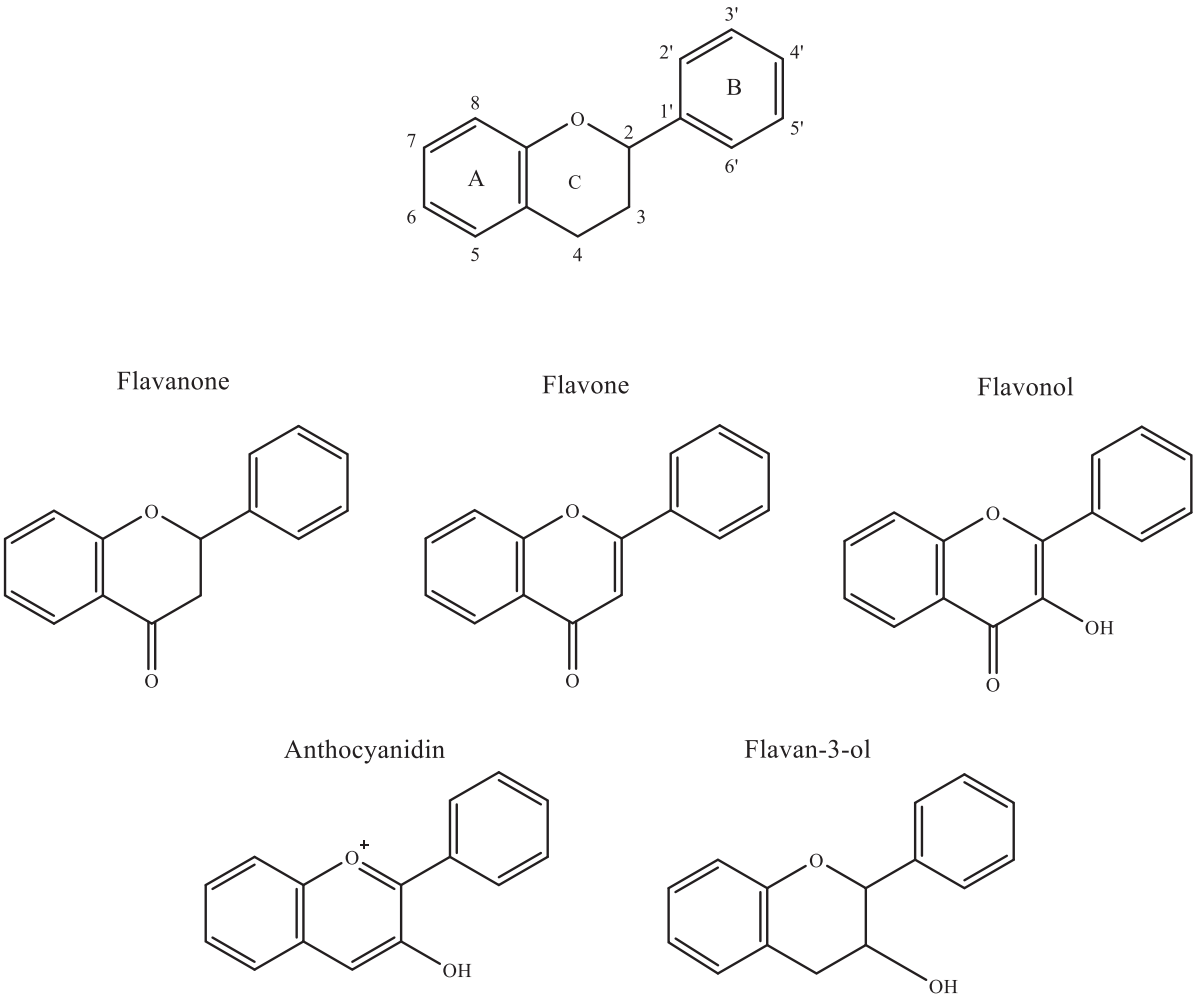
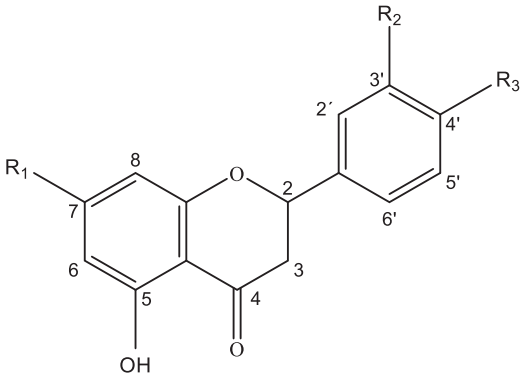


Fig. 2.4. Structures of principal flavonoids subgroups (Crozier et al., 2009; Iriti and Faoro, 2009).

Flavonoids in several plant foods are known to occur in form of aglycones, glycosides, and occasionally in acylated form. The classification of flavonoids is based on their chemical structure and substitution with their principal sub-classes being flavanones, flavones, flavonols, anthocyanidins and flavan-3-ols, which will be explained in more detail in the next subchapters due to their importance. Isoflavonoids are another minor sub-class of flavonoids, which occur in certain foods, especially in soybeans, alfalfa and peanuts (Yamane and Kato, 2012; Cobb, 2014). Isoflavones are the biggest subgroup of isoflavonoids and are phytoestrogens because of a slight estrogen activity. On the other hand, flavan-3,4-diols, dihydroflavonols, coumarins, aurones and chalcones are minor flavonoid subgroups (Crozier et al., 2009; Fraga, 2010). Besides, the flavonoid content in food can be influenced by several factors, such as food, variety, geographical location, weather, maturity, storage and industrial process.

2.1.1.2.1 Flavanones

This group contains a ketone group in the C-ring in position 4 with the rings A and B are usually substituted with hydroxy, methoxy or sugar groups. Content of flavanones has been reported in citrus fruits like grapefruit, lemon, orange and tangerine. Fig. 2.5 presents an overview of the common flavanones. Hesperetin has been found in lemon, orange and tangerine, while eriodictyol has been reported in lemon and naringenin in orange. In grapefruit, naringin is the principal flavanone, which gives a bitter taste (Scordino and Sabatino, 2014; Fraga, 2010).

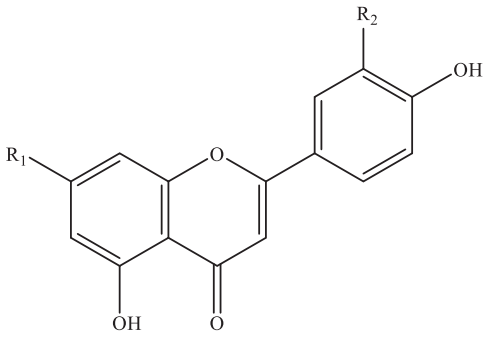


Compound	R_1	R_2	R_3
Naringenin	OH	H	OH
Eriodictyol	OH	OH	OH
Hesperetin	OH	OH	OCH_3
Eriocitrin	O-rutinoside	OH	OH
Naringin	O-neohesperidoside	H	OH

Fig. 2.5. Chemical structure of flavanones and some examples.

2.1.1.2.2 Flavones

The identification of flavones has been associated in herbs like oregano, marjoram, thyme, parsley, as well as in some green leafy vegetables such as celery and artichoke (Manach et al., 2004; Rothwell et al., 2013). Fig. 2.6 shows the typical chemical structure of flavones as well as apigenin and luteolin.

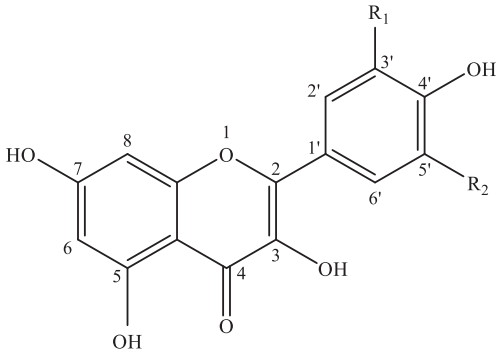


Compound	R_1	R_2
Apigenin	OH	H
Luteolin	OH	OH

Fig. 2.6. Structure of flavones.

2.1.1.2.3 Flavonols

Flavonols are characterized by their color, which varies from white to yellow, and are often present in form of flavonol aglycones and flavonol glycosides (Macheix et al., 1990; Fraga, 2010; Cobb, 2014). Several studies describe kaempferol, quercetin and myricetin as major flavonols (Fig. 2.7).



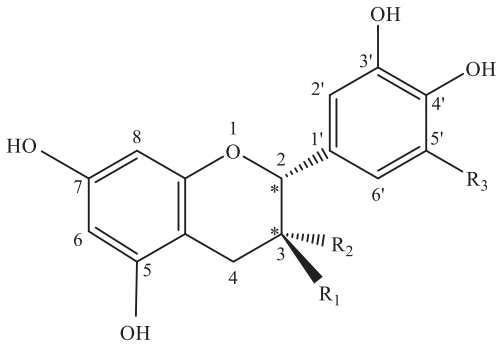
Compound	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH
Isorhamnetin	OCH_3	H

Fig. 2.7. Chemical structures of flavonol aglycones.

Sources of flavonols are fruits and vegetables, notably apple, grapes, berries, tomatoes, onion, broccoli and lettuce, and also in spices such as capers, and in tea and red wine. According to the literature, the glycosylation of flavonols is usually in the hydroxyl group in the C3 and on rare occasions in the hydroxyl group in position 7 and 4' such as kaempferol-7-*O*-glucoside in broad beans and quercetin 4'-*O*-glucoside in onions (Macheix et al., 1990; Rothwell et al., 2013). The most common flavonols in fruits are 3-*O*-monoglycosides, with glucose, galactose, arabinose and rhamnose being the most frequently found sugars. On the other hand, flavonols diglycosides are present in two forms, which depend on the attachment of glycosides. In the first instance, two glycosides are attached to the same carbon atom such as quercetin 3-*O*-rutinoside in apples. In the second case, the glycosides are attached to two distinct carbons, such as quercetin 3,4'-*O*-diglucoside in onions. Some examples of diglycosides are rutinoside, sophoroside, sambubioside and diglucoside. Several studies have been reported 3-rutinoside as the most frequent flavonol diglycoside. Flavonols triglycosides are rarely found in nature, for instance kaempferol 3,7,4′-tri-*O*-glucoside in saffron (Serrano-Díaz et al., 2014). Additionally, flavonols have also been identified as acylated derivatives with phenolic acids like *p*-coumaric, caffeic, ferulic, gallic and *p*-hydroxybenzoic acids (Harborne, 1988; Macheix et al., 1990; Zhao, 2007).

2.1.1.2.4 Flavan-3-ols: Catechins and Proanthocyanidins

This group of flavonoids includes catechins (monomeric form), oligomeric and polymeric proanthocyanidines (polymeric form), and proanthocyanidins (condensed tannins). The most common monomeric flavan-3-ols are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin (Fig. 2.8), which have been identified in fruits such as apricot, cherries, berries, apples, peach, nectarine and grapes, and also in tea and cocoa (Macheix et al., 1990; Crozier et al., 2010; Crozier et al., 2012; Spencer and Crozier, 2012).



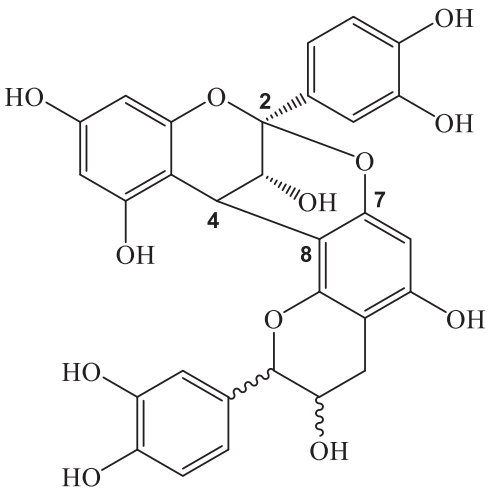
Compound	R_1	R_2	R_3
(+)-Catechin (2R, 3S)	OH	H	H
(-)-Epicatechin (2R, 3R)	H	OH	H
(+)-Gallocatechin (2R, 3S)	OH	H	OH
(-)-Epigallocatechin (2R, 3R)	H	OH	OH

Fig. 2.8. Chemical structure of flavan-3-ols.

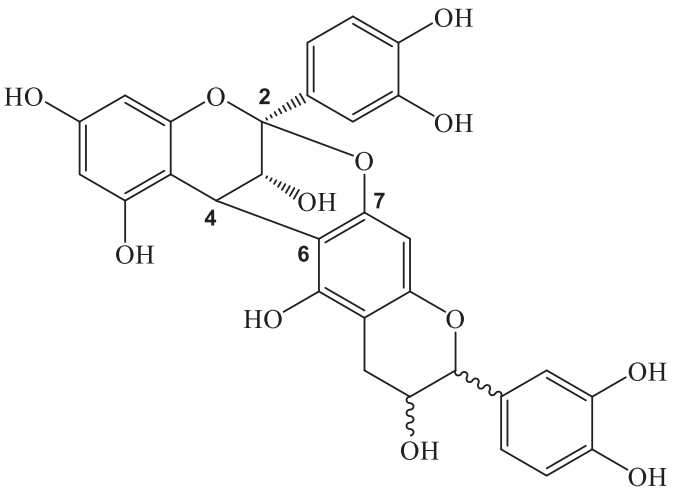
The combination of two or more flavan-3-ol monomers produces oligomers (dimers, trimers, tetramers, etc.) and polymers with unlimited number of monomers that could lead to complex chemical structures. The classification of proanthocyanidins conforms to the type of flavan-3-ol units. The principal class is procyanidins, which have units of (epi)catechin, the following class is prodelphinidins with (epi)gallocatechin units and then propelargonidins, which consist of (epi)afzelechin units. They are present in cranberries, black chokeberries, grape seed, cocoa, peanut, almond, tea and red wine (Crozier et al., 2012; Cobb, 2014).

The chemical structure of flavan-3-ols has stereogenic centers at C2 and C3, which leads to many stereoisomers. Furthermore, the stereochemistry can be *trans* (2R, 3S) or *cis* (2R, 3R), as in the case of (+)-catechin and (-)-epicatechin. In addition, when the flavan-3-ols are linked to position C4, a chiral center is present in C4 providing the option of more isomers. In the nomenclature of proanthocyanidins, the interflavanyl bond and its direction are expressed with parentheses such as carbohydrates and the orientation is described by α or β like in IUPAC rules. In view of the linkage between monomers, the classification of proanthocyanidins consist of two types: B- and A-types, but the abundance of B-type proanthocyanidins is higher than A-type (Andersen and Markham, 2006; Ferreira et al., 2010; Gu, 2012).

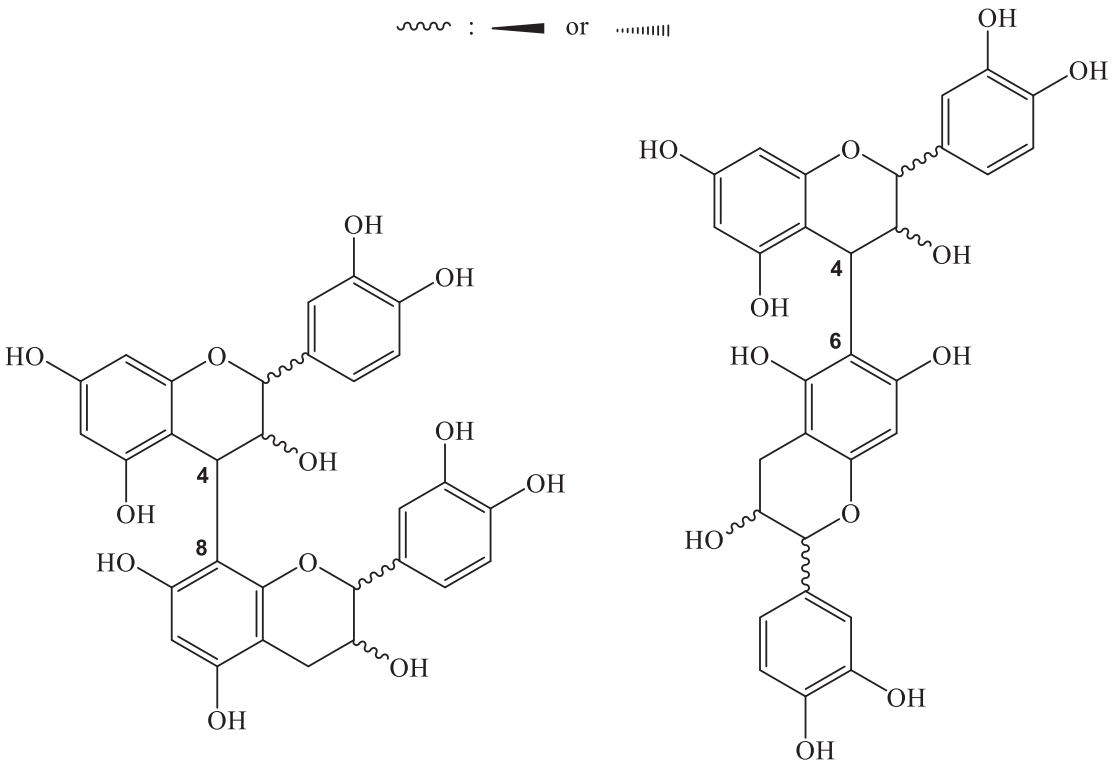
The B-type structures are linked through C4→C8 bond or C4→C6 bond, while A-type structures, which possess an interflavan connection with a double linkage, have usually an ether bond between C2→O7 and rarely between C2→O5, and are linked through C4→C8 bond or C4→C6 bond, as can be seen in Fig. 2.9 (Appeldoorn et al., 2009).



A-type dimer (2-*O*-7, 4-8) linkage



A-type dimer (2-*O*-7, 4-6) linkage



B-type dimer (4-*8*) linkage

B-type dimer (4-*6*) linkage

Fig. 2.9. Some chemical structures of A-type and B-type proanthocyanidins.

In order to identify catechins and proanthocyanidins, chromatographic methods are used such as normal phase HPLC using fluorescent or UV detection, because they have an UV-absorption at 280 nm (Gu, 2012). Furthermore, proanthocyanidins can be cleaved in an acidic medium to generate anthocyanidins (Ferreira et al., 2010; Yamane and Kato, 2012).

2.1.1.2.5 Anthocyanidins: Anthocyanins

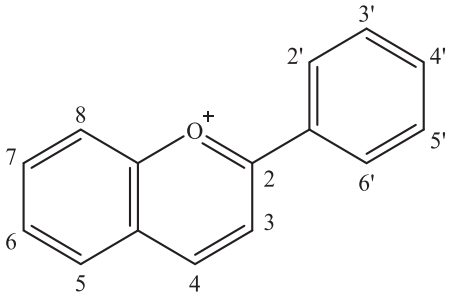
The main objective of this work is the identification, characterization, separation and isolation of anthocyanins from different foods, and for this reason, this section will be explained in more detail.

Anthocyanidins are derivatives of 2-phenylbenzopyrylium, also known as flavylium cation, because its chemical structure presents two double bonds in the C-ring and carries a positive charge. Anthocyanins, from the Greek words anthos (flower) and kyanos (dark blue), are water soluble pigments, which are composed of an aglycone (anthocyanidin) and a sugar. The pigments are responsible for the range of color from salmon pink to magenta or red and from violet to dark blue in fruits, vegetables, grains, roots, tubers, and leaves of plants and flowers. During the last years, interest in identification of anthocyanin sources has increased due to the importance of the use of natural colors in food and cosmetic industry as well as in pharmaceutical industry.

The classification of anthocyanidins depends on the variation in its chemical structure with variations in hydroxy, methoxy and acyl groups. Around 27 monomeric anthocyanidins have been described, but approximately 90% of them are composed of six anthocyanidins (common anthocyanidins): pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin, which differ in the substitution on the B-ring, as can be seen in Table 2.1. Cyanidin is the most predominant anthocyanidin (around 30%), then followed by delphinidin and pelargonidin (approximately 22% and 18%, respectively) (Andersen and Markham, 2006; Wallace and Giusti, 2013). Moreover, there are another three minor anthocyanidin groups: the first one is A-ring methoxylated in 5-O, 7-O or 5,7 di-O-position as rosinidin, hirsutidin, europinidin, etc. with the source of this class being mango peel from cultivar Tommy Atkins (Berardini et al., 2005). The second group consists of 6-hydroxylated anthocyanidins such as 6-hydroxypelargonidin, 6-hydroxycyanidin and 6-hydroxydelphinidin, which are described in flowers of Alstroemeria (Tatsuzawa et al., 2003) as well as in chinese bayberries, Myrica rubra (Suga et al., 2005). The final group are 3-deoxyanthocyanidins like apigeninidin, luteolinidin and tricetinidin, which do not have substitution at C3 position, and are present in sorghum (Awika et al., 2004), ferns and some bryophytes (Wallace and Giusti, 2013).

The glycosylation of anthocyanidins can occur in 3-, 5-, 7-, 3'- and 5'-positions. Glycosides are generally connected through O-linkages, but most of them exist in 3-position, as has been demonstrated in detailed reports of anthocyanins in fruits, vegetables and flowers (Harborne, 1988; Macheix et al., 1990; Mazza and Miniati, 1993; Andersen and Markham, 2006; Gould et al., 2009; Wallace and Giusti, 2013). The most frequent sugars, which are bonded to anthocyanidins, are monosaccharides and disaccharides, although trisaccharides are also present but in a smaller percentage. Nonetheless, studies of flowers containing anthocyanins have reported with up to seven glycosyl units in ternatin A1 from blue flowers of Clitoria ternatea and in cyanodelphin from the blue flower of *Delphinium hybridum* (Kondo et al., 1991; Yoshida et al., 2009).

Table 2.1. Structure of anthocyanidins.



2-Phenylbenzopyrylium

	Substitution Pattern						
Anthocyanidins	**3**	**5**	**6**	**7**	**3'**	**4'**	**5'**
Common Anthocyanidins							
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H
Cyanidin (Cy)	OH	OH	H	OH	OH	OH	H
Delphinidin (Del)	OH	OH	H	OH	OH	OH	OH
Peonidin (Pn)	OH	OH	H	OH	OCH_3	OH	H
Petunidin (Pt)	OH	OH	H	OH	OH	OH	OCH_3
Malvidin (Mv)	OH	OH	H	OH	OCH_3	OH	OCH_3
A-Ring Methoxylated Anthocyanidins							
5-*O*-MethylCy	OH	OCH_3	H	OH	OH	OH	H
7-*O*-MethylCy	OH	OH	H	OCH_3	OH	OH	H
7-*O*-MethylPn (Rosinidin)	OH	OH	H	OCH_3	OCH_3	OH	H
7-*O*-MethylDel	OH	OH	H	OCH_3	OH	OH	OH
7-*O*-MethylPt	OH	OH	H	OCH_3	OH	OH	OCH_3
7-*O*-MethylMv (Hirsutidin)	OH	OH	H	OCH_3	OCH_3	OH	OCH_3
5,7-Di-*O*-methylDep (Pulchellidin)	OH	OCH_3	H	OCH_3	OH	OH	OH
5,7-Di-*O*-methylPt (Europinidin)	OH	OCH_3	H	OCH_3	OH	OH	OCH_3
5,7-Di-*O*-methylMv (Canpensinidin)	OH	OCH_3	H	OCH_3	OCH_3	OH	OCH_3
6-Hydroxylated Anthocyanidins							
6-HydroxyPg	OH	OH	OH	OH	H	OH	H
6-HydroxyCy	OH	OH	OH	OH	OH	OH	H
6-HydroxyDel	OH	OH	OH	OH	OH	OH	OH
3-Deoxyanthocyanindins							
Apigeninidin (Ap)	H	OH	H	OH	H	OH	H
Luteolinidin (Lt)	H	OH	H	OH	OH	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH	OH
7-*O*-MethylAp	H	OH	H	OCH_3	H	OH	H
5-*O*-MethylLt	H	OCH_3	H	OH	OH	OH	H
6-Hydroxy-5-*O*-methylAp (Carajurone)	H	OCH_3	OH	OH	H	OH	H
6-Hydroxy-5,4'-di-*O*-methylAp (Carajurin)	H	OCH_3	OH	OH	H	OCH_3	H
6-Hydroxy-5-*O*-methylLt	H	OCH_3	OH	OH	OH	OH	H
6-Hydroxy-5,4'-di-*O*-methylLt	H	OCH_3	OH	OH	OH	OCH_3	H

Glucose, galactose, arabinose, xylose and rhamnose are typical monosaccharides, while sophorose, sambubioside and rutinoside are the most common disaccharides, as shown in Fig. 2.10. However, disaccharides like gentiobioside, robinobioside, lathyrose and neohesperidoside are rare.

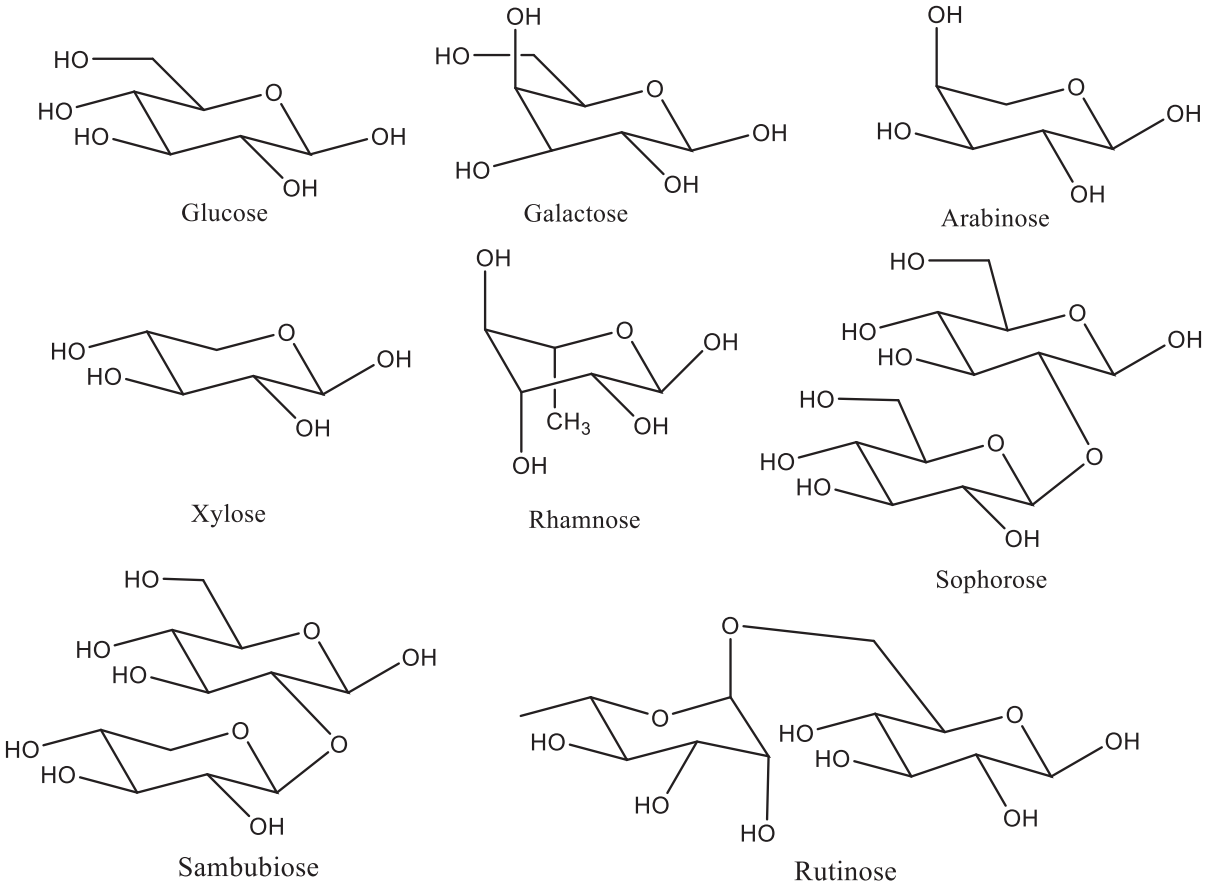
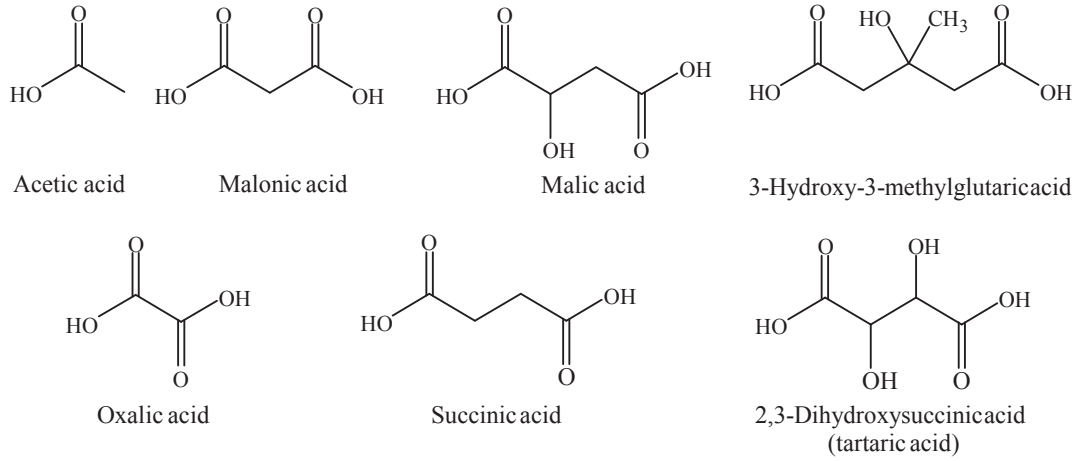


Fig. 2.10. Chemical structure of sugars, which usually occur in anthocyanins.

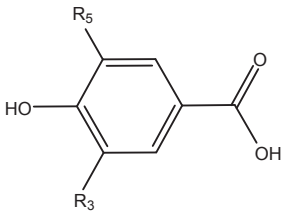
On the other hand, anthocyanins can be found acylated, and the acetylation occurs when an acyl group is inserted into anthocyanin structures by aliphatic and aromatic acids (Fig. 2.11). The most common aliphatic acyl group is malonic acid, even though acetic, malic, oxalic, succinic, tartaric and 3-hydroxy-3-methylglutaric acids are included only in specific foods. The acetylation by aromatic acids involve phenolic acids: hydroxybenzoic acids, such as *p*-hydroxybenzoic acid and gallic acid, and hydroxycinnamic acids, such as *p*-coumaric, caffeic, ferulic, sinapic and 3,5-dihydroxycinnamic acids. In the review of anthocyanins reported by Andersen and Markham (2006), *p*-coumaric and caffeic acids are the most frequently found aromatic acids in the majority of the cases, connected to the 6-position of glucose within a glycosidic moiety linked to the 3-position of the aglycone. Acetylated anthocyanins have been reported in red and purple potatoes, red cabbage, red onion, evergreen blackberries and flowers like *Clitoria ternatea* and orchids (Andersen and Markham, 2006; Hillebrand et al., 2009b; Wallace and Giusti, 2013).

Aliphatic acids



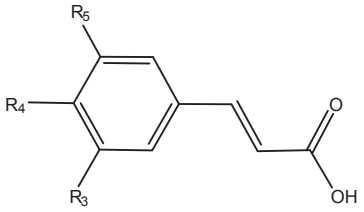
Aromatic acids

Hydroxybenzoic acids



	R_3	R_5
p-Hydroxybenzoic acid	H	H
Gallic acid	OH	OH

Hydroxycinnamic acids



	R_3	R_4	R_5
p-Coumaric acid	H	OH	H
Ferulic acid	OCH_3	OH	H
Caffeic acid	OH	OH	H
Sinapic acid	OCH_3	OH	OCH_3
3,5-Dihydroxycinnamic acid	OH	H	OH

Fig. 2.11. Structure of aliphatic and aromatic acids that can exist in anthocyanins.

The structure of anthocyanins depends especially on the pH. Anthocyanins can act as a pH indicators, because their coloration changes with pH values. Different equilibriums with different structures exist, depending on pH, as shown in Fig. 2.12. The flavylium cation (**AH**$^+$) presents a red color at a pH below three and is stable under strong acidic conditions. As a result, anthocyanins require an acidic media for their stabilization, but insofar as the pH is increased, two reactions are possible: firstly, a hydration in position 2 of **AH**$^+$ followed by a deprotonation, thus generating colorless carbinol pseudobases or hemiacetal forms (**B2** and **B4**). Because of a tautomerization in the carbinol pseudobase structure, a ring opening reaction can occur, giving yellow chalcone structures, which can exist as *trans* and *cis* isomers (**C$_Z$** and **C$_E$**). Secondly, the acidic hydroxyl group at **AH**$^+$ can lose a proton, giving violet quinoidal bases (**A$_{4'}$**, **A$_7$** and **A$_5$**), which can experience a deprotonation at pH 6-7 and generate bluish quinoidal anions (**A$^-_{4'7}$**, **A$^-_{75}$** and **A$^-_{54'}$**) (Cheminat and Brouillard, 1986; Mazza and Miniati, 1993; Andersen and Jordheim, 2013).

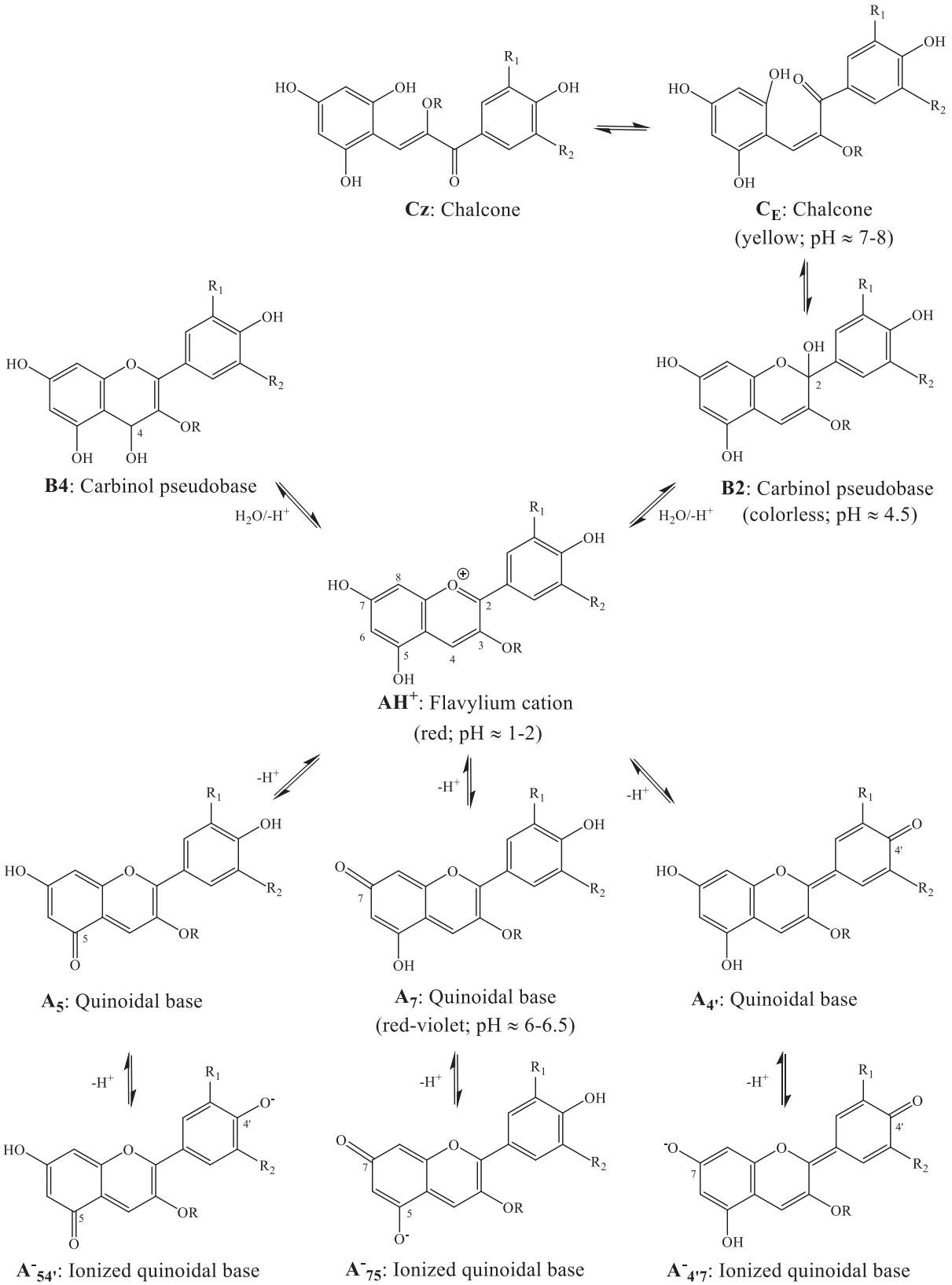


Fig. 2.12. Equilibrium forms of anthocyanidins. R = glycoside; R_1 and R_2 can be OH, CH_3 or OCH_3 groups, according to the type of aglycone.

The color of anthocyanins depends also on various other factors such as temperature, light, oxygen, methods for their extraction, concentration, type of aglycone, existence of other polyphenols,

polymerization, etc. Anthocyanins have specific positions on their structure that are susceptible to nucleophilic and electrophilic attacks. Moreover, they can react with another polyphenols and non-polyphenols such as flavonoids, copigments, metallic ions, etc. (Fig. 2.13), thus creating heterodimers, hemiketals, anthocyanins with sulfites, pyranoanthocyanins and chelation of anthocyanins by metals (Gould et al., 2009; Wallace and Giusti, 2013).

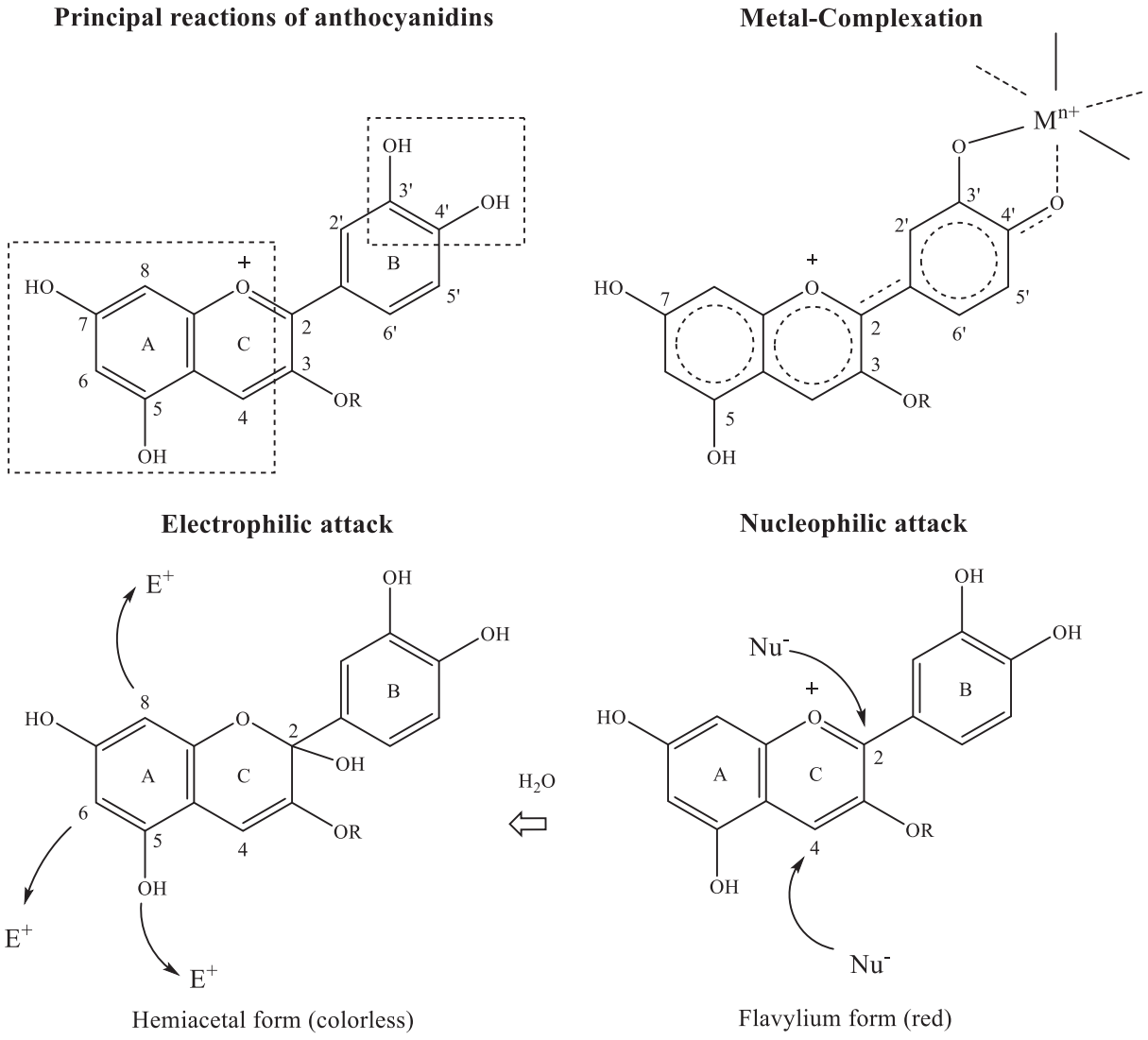


Fig. 2.13. Specific positions of anthocyanidins to react (Mateus and Freitas, 2009; Andersen and Jordheim, 2013).

Reactions on the A-Ring

Due to the presence of oxygen elements in their substituents in the 5-, 7 and 9- positions of the anthocyanin structure and that these groups are in meta position in relation to each other on the A-ring, there is a raised electron density at the carbons 6 and 8. The oxygen has pairs of electrons to be donated: as a result, they can contribute to resonance effect, thus the A-ring is influenced by nucleophilic character in carbon atoms in position 6 and 8, forming heterodimer compounds. Many examples of reactivity of carbon atom 8 are known, for instance reaction between anthocyanins and flavanols (mostly

catechins) leads to the formation of the flavanol-(4α→8)-anthocyanins, which are formed during food processing and storage. Catechin-(4-8)-Pg-3-glc, epicatechin-(4-8)-Pg-3-glc and (epi)afzelechin-(4-8)-Pg-3-glc, which are present in strawberries, are similar too (Lopes da Silva, F. et al., 2007), while catechin-Mv-3-glc and epicatechin-Mv-3-glc are found in wines (Salas et al., 2004). On the other hand, anthocyanin-flavonol and anthocyanin-flavone pigments are less common but they have been discovered in flowers like blue *Agapanthus*, blue-purple water hyacinth, leaves of *Oxalis triangularis*, orchids and pale-purple chive (Andersen and Markham, 2006).

Reactions on the C-Ring

The carbon atoms at position 2 and 4 of anthocyanins possess electrophilic character and therefore a nucleophilic attack is predominated in the C-Ring. The most well-known reaction occurs with water, which attack on C2 of flavylium cation to form a hemiketal form, while in the case of C4, reactions with SO_2 are very common, normally as bisulfite HSO_3^-, generating colorless adducts (Fig. 2.14). As example, SO_2 is used in treatments of juice and as preservative (Berké et al., 1998; Lee et al., 2002). Additionally, bisulfite reacts with monomeric anthocyanins, which are in their free form present as flavylium cation. Whereas there are no reactions with polymeric anthocyanins, the use of bisulfite has been applied to calculate the quantity of polymeric compounds in wine, juices and extracts (Berké et al., 1998; Bonerz et al., 2006).

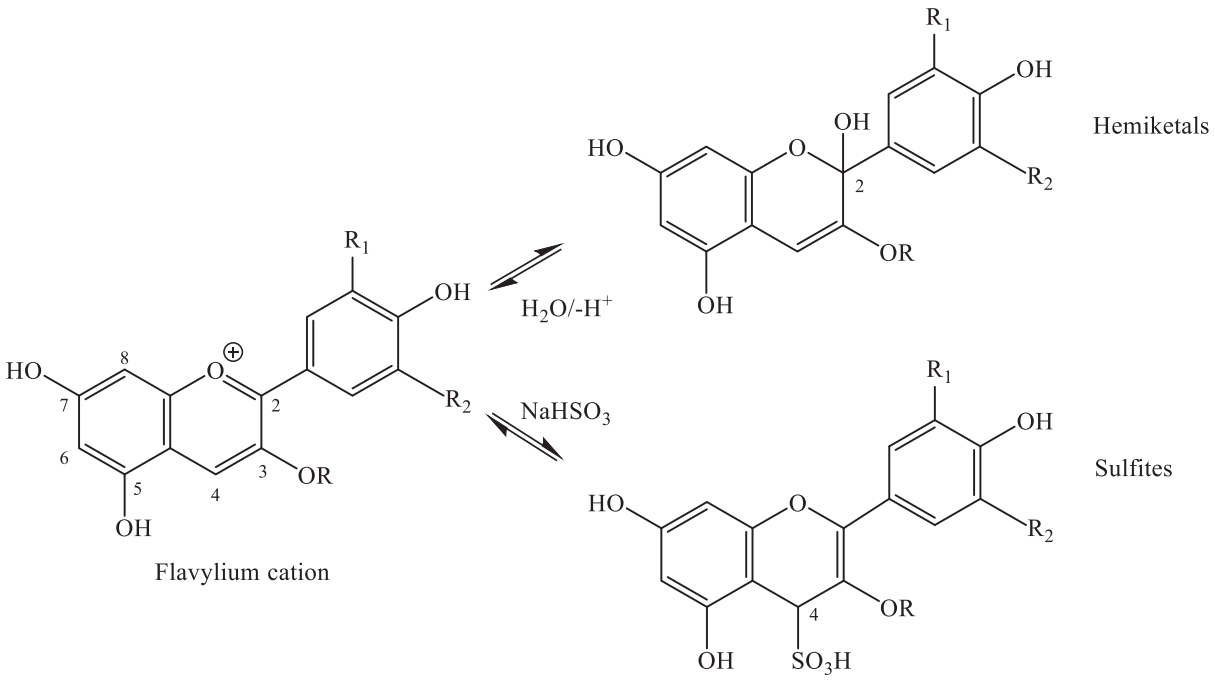


Fig. 2.14. Reaction on the C-Ring, generating hemiketals and sulfites. R = glycoside; R_1 and R_2 can be OH, CH_3 or OCH_3 groups, according to the type of aglycone.

Reactions, including A- and C-Rings

Anthocyanins can react with organic molecules such as acetaldehyde, pyruvic acid, acetoacetic acid, vinyl-phenols, cinnamic acids, which are compounds with a carbonyl in a keto-enol equilibrium in

hydroalcoholic solutions, producing anthocyanins-pyruvic acid adducts, pyranoanthocyanins like Vitisin A and B, and pyranoanthocyanins-phenol pigments (Fulcrand et al., 1998; Mateus and Freitas, 2009; Wallace and Giusti, 2013). The reaction mechanism is a cycloaddition of these organic molecules at the C4 and the OH group in position C5 of the anthocyanins, followed by a dehydration and oxidation, thus generating the D-Ring, as can be seen in Fig. 2.15.

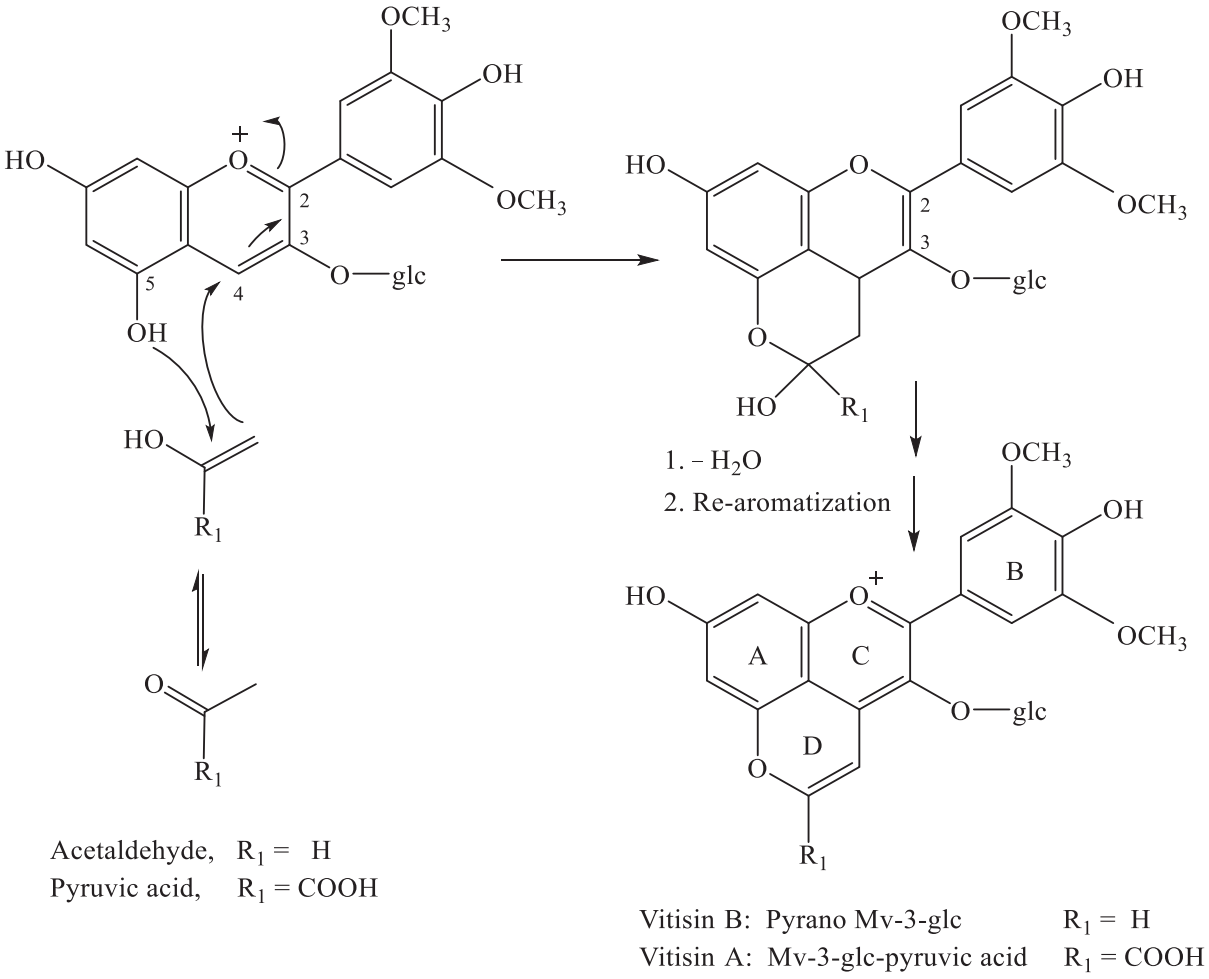


Fig. 2.15. The formation of pyranoanthocyanins: reaction between Mv-3-glc and carbonyl compounds.

Pyranoanthocyanins are frequently present in red wine, but they have also been detected in fermented and unfermented fruit juice as blood orange and black carrot juices (Hillebrand et al., 2004; Schwarz et al., 2004). The existence of pyranoanthocyanins increases during storage, fermentation and oxygenation processes. Moreover, the color of pyranoanthocyanins is more stable than in common anthocyanins, because the new D-Ring protects against nucleophilic attacks (Hillebrand et al., 2004; Rentzsch et al., 2007b).

Reactions on the B-Ring:

The presence of at least two adjacent hydroxyl groups on the B-Ring of anthocyanins like in cyanidin, delphinidin and petunidin facilitates the metal chelation. This is because these groups perform no similar tautomerization and persuade the electron density of the carbon atoms of ring B, thus producing the

metalloanthocyanins (Fig. 2.13). The metal complexation has been described in blue flowers like *Commelina communis*, *Salvia patens* and *Centaurea cyanus*, which contain metalloanthocyanins with magnesium and/or ferric ions (Fig. 2.16). Cammelinin is a metalloanthocyanin from the flower *Commelina communis* and presents six anthocyanin molecules of malonylawobanin (delphinidin 3-[6-(p-coumaryl)glucoside]-5-[6-[malonyl]glucoside]) with two magnesium ions (Mg^{2+}) and six flavones (flavocommelin). Moreover, it has been demonstrated that the magnesium ion can be substituted with Cd^{2+}, thus getting Cd-commelinin (Kondo et al., 1992; Shiono et al., 2008; Yoshida et al., 2009).

Protodelphin from *Salvia patens* is another metalloanthocyanin that contains six malonylawobanin (delphinidin 3-[6-(p-coumaryl)glucoside]-5-[6-[malonyl]glucoside]), six flavones (apigenin 7,4'-diglucosides) and two magnesium ions (Mg^{2+}). *In vitro* studies of the protodelphin resynthesis showed that the substitution of magnesium ions with other ions like $Ni^{2+,}$ Co^{2+}, Zn^{2+} and Cd^{2+} is possible (Takeda et al., 1994). On the other hand, there is the possibility that different ions exist in the metal complexation, such as in protocyanin from *Centaurea cyanus*, also called cornflower, where magnesium (Mg^{2+}) and ferric (Fe^{3+}) ions are present, and they are coordinated with six molecules of succinylcyanins, cyanidin 3-O-(6-O-succinylglucoside)-5-O-glucoside, and six molecules of apigenin-7-glucuronide-4'-[6-(malonyl) glucoside]. Additionally, another metal ions (Al^{3+}) can also coordinate in metalloanthocyanins (Yoshida et al., 2009).

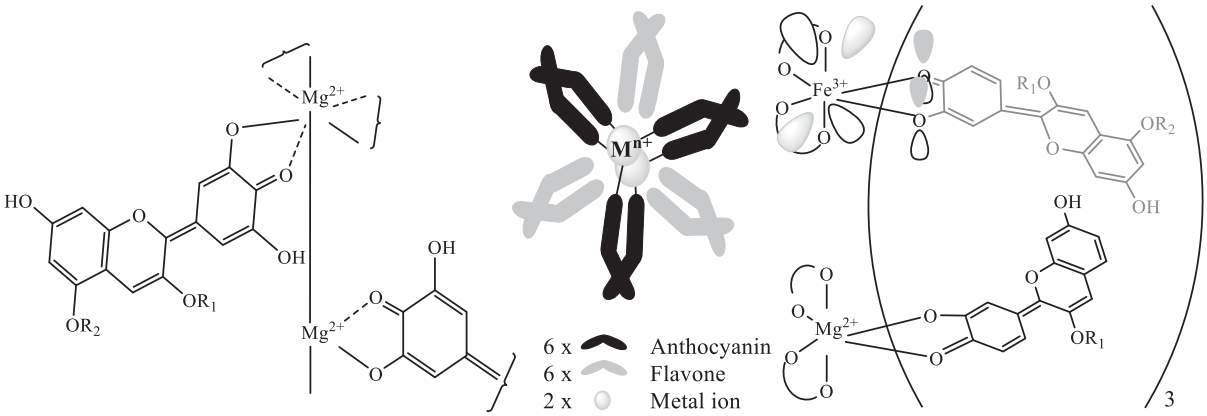


Fig. 2.16. Metalloanthocyanin: Representation of interaction between cation and anthocyanin in commelinin (left), self-assembly of metalloanthocyanins (middle) and protocyanidin (right) (Yoshida et al., 2009).

The blue coloration of flowers is currently related to the anthocyanins content of delphinidin-type like in cammelinin and protodelphin, but in the case of protocyanin there are anthocyanins of cyanidin type. Furthermore, the presence of metals generates bathochromic and hyperchromic shifts in their absorption spectra depending on the metal ion, their UV-Vis absorption spectrum can show maximum absorption at approximately 270-315 nm and 560-690 nm, and intensify the bluish color.

2.1.2 Biosynthesis of phenolics, polyphenols and anthocyanins

The biosynthesis of phenolic compounds and polyphenols involves diverse pathways that are well known, as can be seen in Fig. 2.17. Plants use the solar energy in the process of photosynthesis and turn

the solar energy into molecules of sugars by Calvin cycle, after which the sugars are broken down by glycolysis, producing pyruvates. On the other hand, the pentose phosphate pathway is a pathway that can yield NADPH, but also can convert glucose into five carbon sugars, called ribose, and into four carbon sugars, named erythrose (Karp, 2008). The reaction between erythose 4-phosphate and pyruvate and phosphoenolpyruate is the starting point of the shikimate pathway, which is implicated in the biosynthesis of amino acids and phenylpropanoids which are precursors of a wide range of phenolic and polyphenolic compounds in plants (Haslam, 1974; Dey and Harborne, 1997).

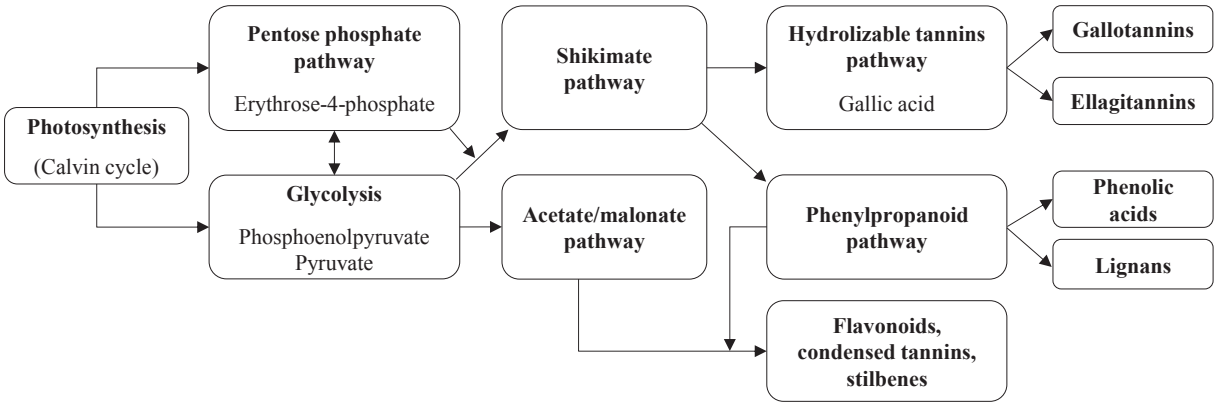


Fig. 2.17. Pathway for the biosynthesis of phenolics and polyphenols (modified from Salminen and Karonen, 2011).

The shikimate pathway is constituted of seven metabolic steps, as outlined in Fig. 2.18. At the beginning, there is a condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) producing 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) and inorganic phosphate (Pi), then an elimination of phosphate from DAHP yields 3-dehydroquinate (DHQ); subsequently there is a dehydration of DHQ to generate 3-dehydroshikimate (DHS) and the reduction of DHS produces shikimate. After this, there is a phosphorylation of shikimate to give shikimate 3-phosphate (S3P), then the addition of a second PEP is carried out and yields a condensation with S3P to produce 5-enolpyruvylshikimate 3-phosphate (EPSP) and inorganic phosphate. Finally the last step is the transformation of EPSP into chorismate, which is a precursor of aromatic amino acids such as phenylalanine, tyrosine and tryptophan (Seigler, 1998; Herrmann and Weaver, 1999).

Furthermore, it should be pointed out that 3-dehydro-shikimic acid, which is produced in the third step of shikimate pathway, can be involved in the production of gallic acid that plays a significant role in the synthesis of hydrolizable tannins such as gallotannins and ellagitannins (see also Fig. 2.20) (Macheix et al., 1990).

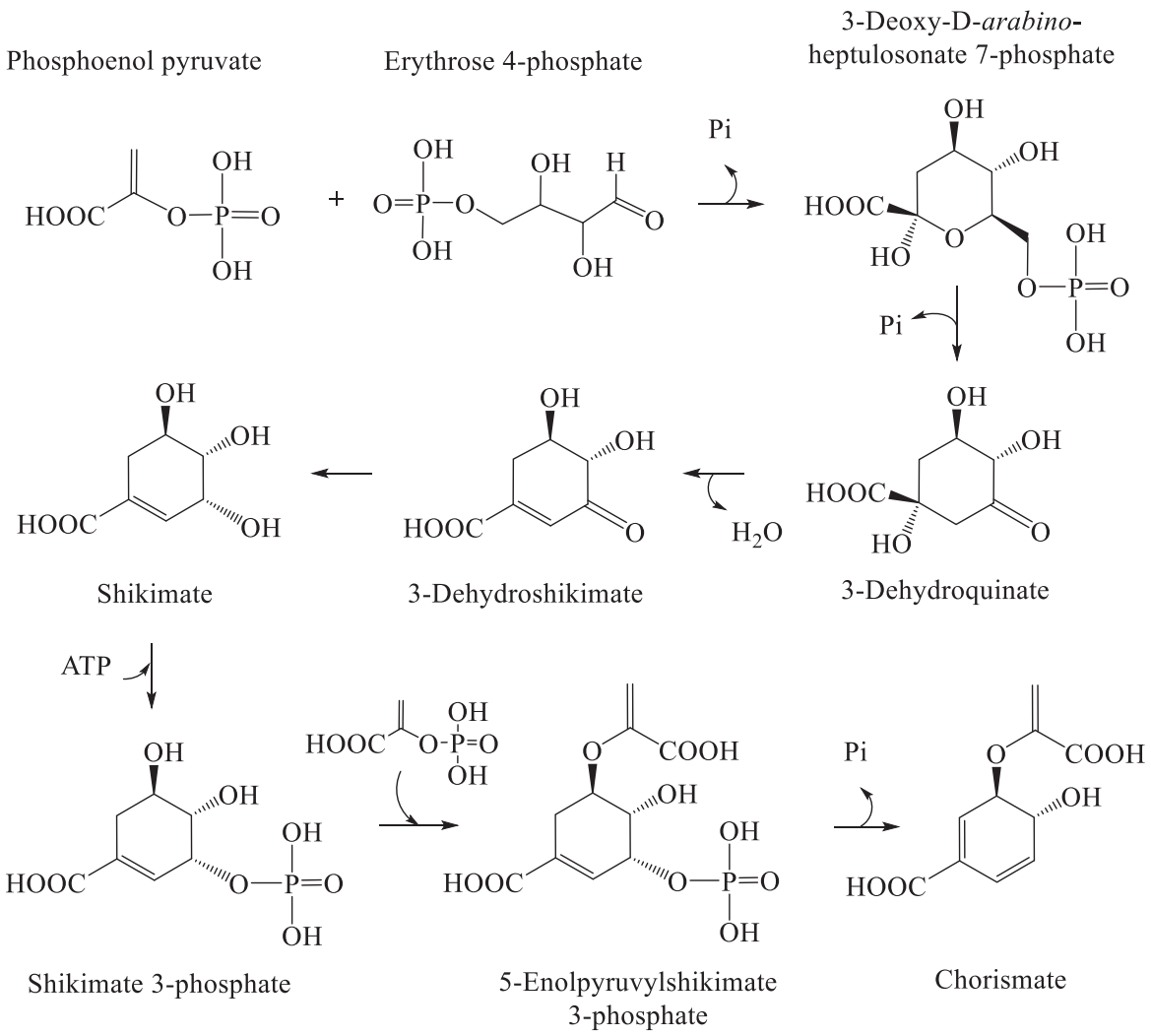


Fig. 2.18. The shikimate pathway (Herrmann and Weaver, 1999).

In the biosynthesis of amino acids (Fig. 2.19), the chorismate undergoes a [3,3]-sigmatropic Claisen rearrangement and generates prephenic acid by chorismate mutase (CM), then there are two pathways for the synthesis of phenylalanine. In the first pathway (via phenylpyruvate), the prephenate reacts with a prephenate dehydratase (PDT) to give phenylpyruvic acid, followed by the catalyzation of phenylalanine- (histidine) transaminase (EC2.6.58) to phenylalanine. The second pathway is via arogenic acid, where prephenic acid yields aroganic acid by aspartate-prephenate aminotransferase (PAT), and then the arogenate dehydratase (ADT) catalyzes the reaction, generating phenylalanine. The synthesis by phenylpyruvic acid has been described in bacteria, while the synthesis by arogenic acid has been reported in plants. In the case of tyrosine synthesis, there are also two routes, the first is via 4-hydroxyphenylpyruvic acid, where prephenic acid generates 4-hydroxyphenylpyruvic acid by prephenate dehydrogenase (PDH), and then generating tyrosine by prephenate dehydrogenase (TAT). The second route for tyrosine is via arogenic acid, which is catalyzed by the enzyme arogenate dehydrogenase (ADH) to give tyrosine (Keller et al., 1982; Bowsher et al., 2008).

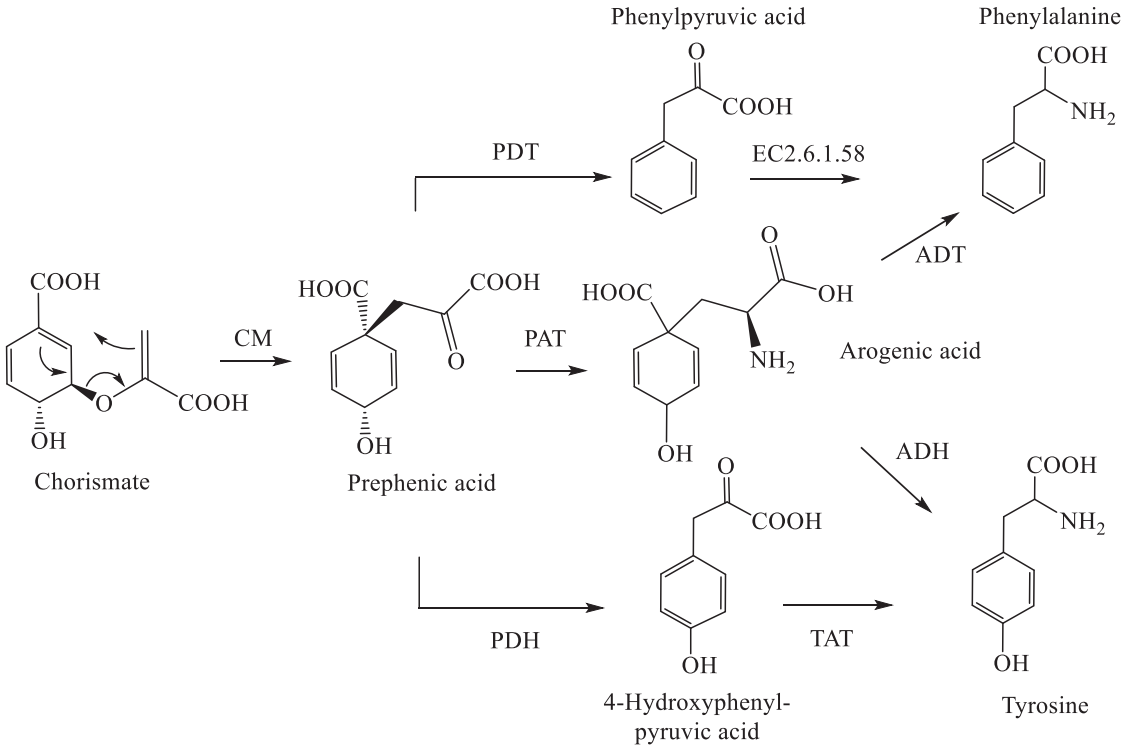


Fig. 2.19. Biosynthesis of phenylalanine and tyrosine (Keller et al., 1982).

In addition, the transformation of phenylalanine to *p*-coumaric acid-CoA is known as metabolic pathway of phenylpropanoids (Macheix et al., 1990) and it is required for the biosynthesis of flavonoids, condensate tannins, stilbenes, lignans and phenolic acids. This pathway has three steps, as can be seen in Fig. 2.20. Firstly, the phenylalanine is transformed to cinnamic acid by phenylalanine ammonia-lyase (PAL), secondly, it is converted into *p*-coumaric acid by cinnamate 4-hydroxylase (C4H), and thirdly, *p*-coumaric acid is catalyzed by p-coumarate:CoA ligase (4CL) giving *p*-coumaric acid CoA. Nevertheless, *p*-coumaric acid is an intermediate that can generate other hydroxycinnamic acids like caffeic, ferulic, 5-hydroxy-ferulic and sinapic acids, while *p*-coumaric acid CoA is an intermediate in the biosynthesis of phenolic compounds like chlorogenic acids that have been found in various fruits and vegetables. Moreover, it is noted that a wide variety of fruits contain hydroxycinnamic acid derivatives and are usually present in two types: hydroxyacid esters like quinic esters (caffeoylquinic, feruloylquinic and *p*-coumaroylquinic acids), shikimic and tartaric esters and hydroxycinnamic acids with sugars (Harborne, 1988; Macheix et al., 1990; Crozier et al., 2009).

Therefore, the non-flavonoids are yielded via shikimic and phenylpropanoid pathways. The benzoic acids can be produced from the shikimate pathway, particularly from 3-dehydro-shikimic acid to generate gallic acid and from phenylpropanoid pathway by a degradation of cinnamic acids. Besides, hydroxycinnamic acid derivatives are created from phenylpropanoid pathway, where this route involves reactions of hydroxylation and methylation. Furthermore, the stilbenes are generated from three malonyl-CoA and one CoA ester of a cinnamic acid at the beginning in the biosynthesis of flavonoids (Crozier et al., 2009; Rentzsch et al., 2009).

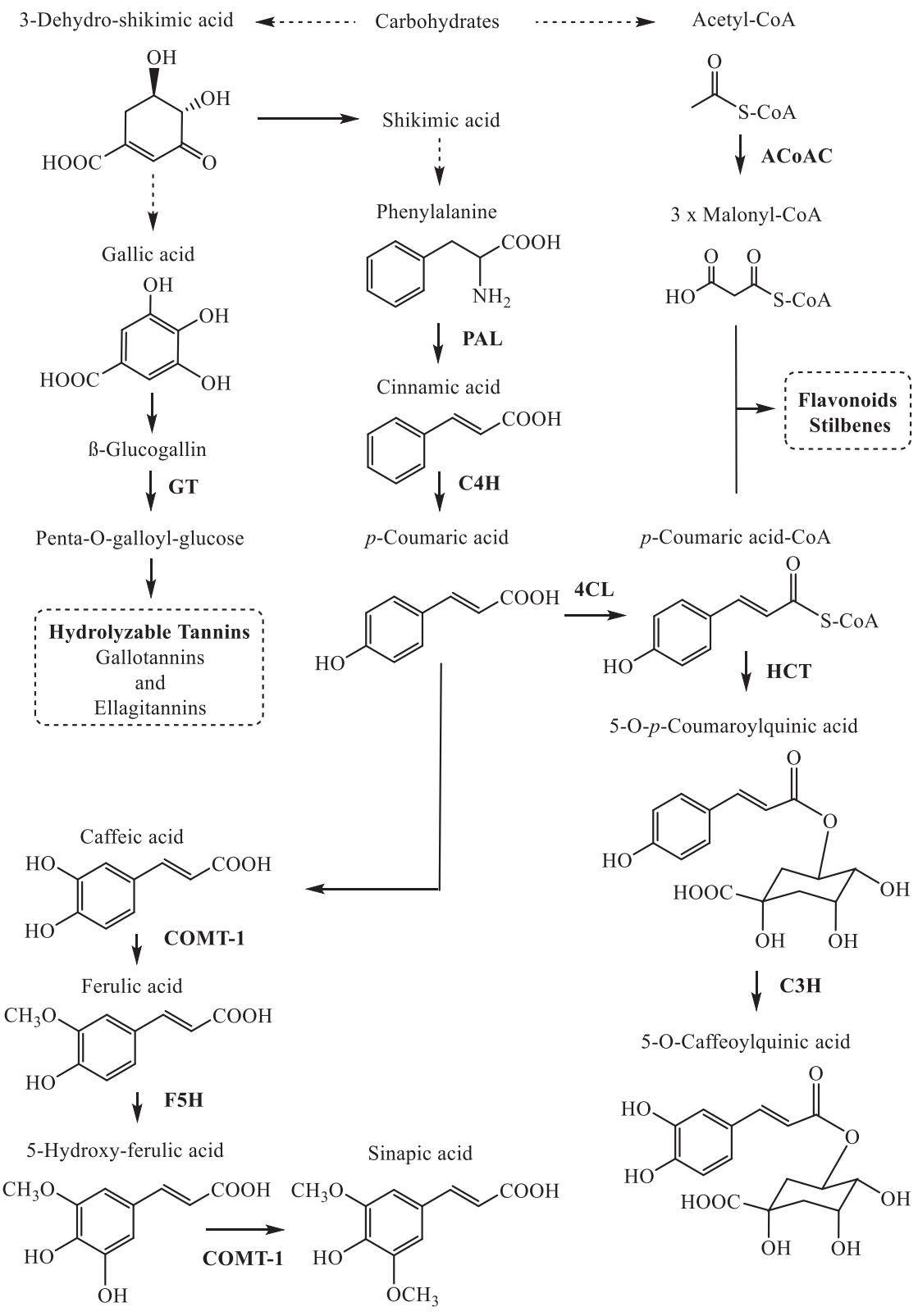


Fig. 2.20. Biosynthesis of phenolic acids and hydrolyzable tannins (Crozier et al., 2009).

On the other hand, the flavonoids are produced from two independent pathways (Fig. 2.21), first the phenylpropanoid pathway, where the phenylalanine generates *p*-coumaric acid-CoA and second the malonic pathway, where acetyl-CoA is transformed into malonyl-CoA by acetyl-CoA carboxylase (ACoAC). Subsequently, the reaction between three malonyl CoA and *p*-Coumaric acid-CoA yields a chalcone and its isomeric flavanone, which are intervening in synthesis of all flavonoids.

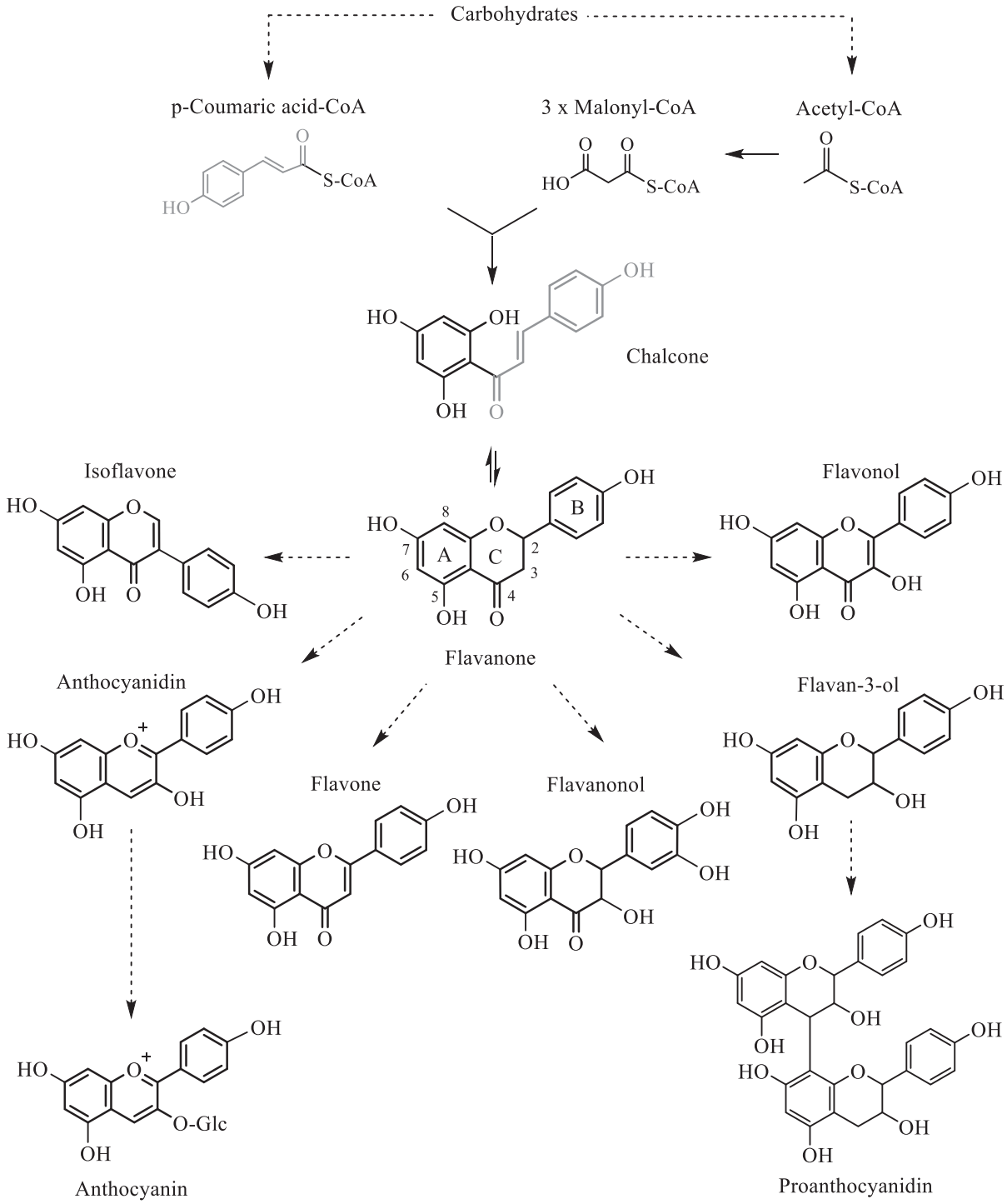


Fig. 2.21. Biosynthesis of flavonoids (Macheix et al., 1990).

The ring A of flavanone is provided from the condensation of the three molecules of malonyl-CoA units via the malonic pathway, while the aromatic B-ring and the three-carbon bridge (from C2 to C4) are yielded from the shikimate pathway. In the formation of flavonoids, some reactions such as hydroxylation, oxidation, methylation and glycosylation are carried out, producing changes in the different rings.

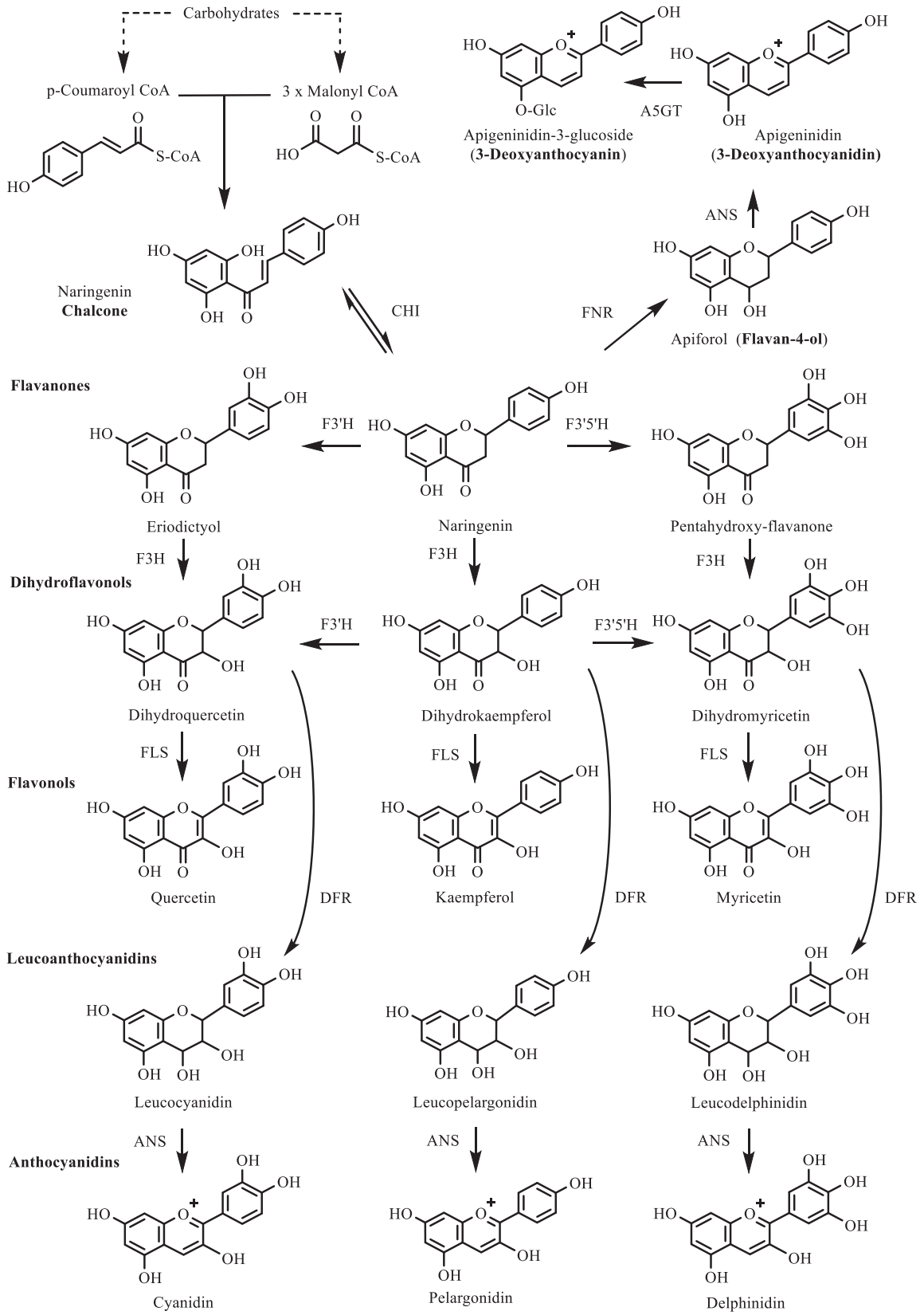
The formation of isoflavones takes place when a migration of aryl side chain of a flavanone/chalcone from C2 to C3 is carried out, for instance, the transformation of naringenin to genistein or from liquiritigenin to daizein (Harborne, 1988; Andersen and Markham, 2006).

Flavones and flavonols are formed from flavanone/chalcone, which are involved in a conversion of a saturated bond to an unsaturated bond between C2 and C3. Their chemical structures are different only with respect to the substitution in the 3ˈposition; where flavonols possess a hydroxyl group and flavones lack this substitution. In the biosynthesis of flavones, two flavone synthase enzymes (FNS) catalyze the reaction from flavanones to flavones, while in the case of flavonols, the reaction from flavanone to flavonols is catalyzed by flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) (Andersen and Markham, 2006).

The name of flavanonols is assigned to 3-hydroxyflavanones or dihydroflavonols, which contain a hydroxyl group in the C3 position. The dihydroflavonols like dihydroquercetin, dihydrokaempferol and dihydromyricetin are implicated in the biosynthesis of flavonols as intermediate product (Macheix et al., 1990; Bowsher et al., 2008). In addition, dihydroflavonols can generate flavan-3,4-diols by activity of dihydroflavonol 4-reductase (DFR), they are also called leucoanthocyanidins and can yield flavan-3-ols like catechin by leucoanthocyanidin reductase (LAR), and then generate proanthocyanidins (Tanner et al., 2003).

On the other hand, leucoanthocyanidins such as leucocyanidin, leucopelargonidin and leucodelphinidin are also associated with the biosynthesis of anthocyanidins by activity of anthocyanidins synthase (ANS), yielding cyanidin, pelargonidin and delphinidin, respectively, as can be seen in Fig. 2.22. In the case of 3-deoxyanthocyanin, their biosynthesis occur from naringenin flavonone to apirol by flavanone 4-reductase (FNR), subsequently apirol is catalyzed by ANS, producing 3-deoxyanthocyanidin, and finally 3-deoxyanthocyanidins give 3-deoxyanthocyanins by UDPG:anthocyanidins 5- *O*-glycosyl transferase (A5GT) (Gould et al., 2009).

Normally, anthocyanidins are not present as aglycones, however they are transformed in most case into their glucosides (anthocyanins) by UDPG:flavonoid 3-*O*-glucosyltransferase (UFGT), as shown in Fig. 2.23. Moreover, anthocyanins can undergo an acetylation by the action of anthocyanin acyl transferase (ACN) (Gould et al., 2009; He et al., 2010). Thus, the formation of anthocyanins shows a color variation in fruits, vegetables and flowers. Cyanidin derivatives are related to purplish-red color, pelargonidin derivatives to orange-red color and delphinidin derivatives to bluish-purple color (Cseke et al., 2006).



Enzymes

CHI	Chalcone isomerase	FNR	Flavanone 4-reductase	A5GT	UDPG:anthocyanidin 5-O-glycosyltransferase
F3'H	Flavonoid 3'-hydroxylase	F3H	Flavanone 3ß-hydroxylase	F3'5'H	Flavonoid 3'5'-hydroxylase
FLS	Flavonol synthase	DFR	Dihydroflavonol 4-reductase	ANS	Anthocyanidin synthase

Fig. 2.22. Biosynthesis of anthocyanidins and flavonols (Bowsher et al., 2008; Gould et al., 2009).

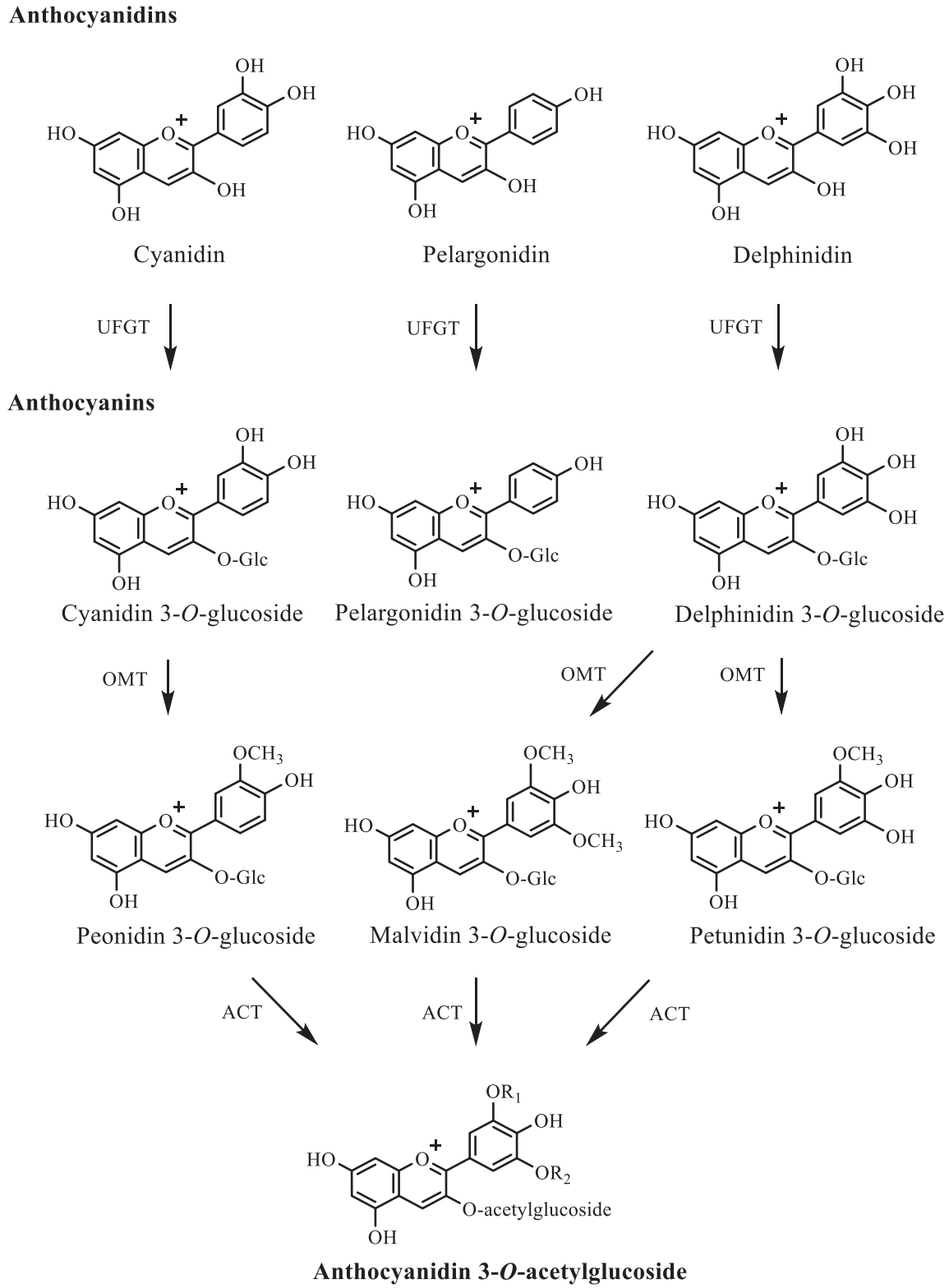


Fig. 2.23. Biosynthesis of anthocyanins (Gould et al., 2009; He et al., 2010).

2.1.3 Absorption, Bioactivity and Metabolism of anthocyanins

2.1.3.1 Anthocyanin absorption and metabolism

The human gastrointestinal tract (GTI) is constituted of the mouth, stomach, esophagus, small and large intestines, and is responsible for the food intake, food digestion, absorption of nutrients and excretion of indigestible and unabsorbed substances (Clancy and McVicar, 2009). Food experiments diverse environments in the GTI and the bioavailability of their compounds depend on the physiological properties of the GTI, such as pH, gastric emptying, transit time, microbiology and biochemistry, which can contribute to food and compounds degradation (McGhie and Stevenson, 2013). It is well known that organs of GTI present a variability of pH, where the pH value ranges from one to eight depending on the GTI organ (Pandit and Soltis, 2012; Koziolek et al., 2015). On the other hand, the stability of anthocyanins also depends on the pH value; consequently, the different environments of GTI organs can produce a stability or a degradation of anthocyanins (flavylium cations, quinonoidal or hemiacetal bases and chalcones) throughout the digestive system.

In food consumption, the anthocyanins are glycosylated in the majority of cases, while acetylated in fewer cases (Macheix et al., 1990; Mazza and Miniati, 1993; Andersen and Markham, 2006; Zhao, 2007; Gould et al., 2009; Warner, 2014). Before the food arrives in the stomach, it is mixed with the saliva, which subsequently break the food down. This process occurs in few minutes and results in degraded and undegraded anthocyanins moving to the stomach through the esophagus. The salivary pH is considered to be between 6.5 and 7, but food and drink can fluctuate the pH value (Pandit and Soltis, 2012; Koziolek et al., 2015). *Ex vivo* study on reaction of anthocyanin from blueberry, chokeberry, black raspberry, red grape, and strawberry extracts with human or artificial saliva after an incubation period at 37 °C has shown a partial degradation of anthocyanin glycosides but the decrease was not associated with formation of protocatechuic acid and phloroglucinol aldehyde. In addition, the degradation of delphinidin and petunidin derivatives was bigger than in the case of cyanidin, peonidin and malvidin derivatives (Kamonpatana et al., 2012). *In vivo* study using red grape or chokeberry juice in the mouth for 5 minutes has revealed that anthocyanins lost their stability due to microbial activity and quantity of secreted saliva in the time of retention in mouth. The study further showed that cyanidin 3-glucoside was transported to buccal epithelium in preference to other cyanidin 3-glycosides (Kamonpatana et al., 2014).

Anthocyanins glycosides arrive in the stomach and are mixed with gastric acid for a few hours, that allows some anthocyanins to be absorbed and subsequently pass through the gastric mucosa by a suggested process that includes interaction of anthocyanins with bilitranslocase (Passamonti et al., 2003). An additional property of bilitranslocase, an organic anion transporter in the gastric epithelium and in the liver, is the involvement of transportation of anthocyanins into the vascular endothelium, which enables anthocyanins to interact with the cardiovascular system (Ziberna et al., 2012). Moreover, previous reviews of anthocyanins absorption and metabolism in *in vitro* studies have reported that unencapsulated and encapsulated anthocyanins were stable in imitated gastric conditions in fasted and

fed state. Furthermore, there is a higher degradation of unencapsulated anthocyanins in simulated small intestine conditions than encapsulated anthocyanins (Oidtmann et al., 2012). This was expected, because the stomach has a low pH (1-3.5), in which anthocyanins are stable as flavylium cations, while the small intestine presents a higher pH (5.5-7.5). Studies with an *in situ* application of anthocyanins from blackberries into the rat stomach showed that anthocyanins were adsorbed rapidly and were detected in plasma from gastric vein and aorta (Talavéra et al., 2003).

Another part of anthocyanins is transferred from stomach into the liver by a suggested process that include bilitranslocase (Passamonti et al., 2002). The human liver has functions of detoxification and production of substances, in which reactions of methylation, glucuronidation and sulfatation are involved (van der Woude et al., 2004; Mazza and Kay, 2009). As a consequence, anthocyanins can be metabolized but some glycosylated and metabolized anthocyanins can return to the duodenum, the first part of small intestine, and enterohepatic circulation as bile whose pH value is alkaline, while another part of anthocyanins is able to move into organs and tissues. Talavera et al. (2003) demonstrated the existence of intact and methylated anthocyanins, as well as conjugated metabolites of Cy 3-glc, after administration of blackberry extract in rats. In addition, they suggested that Peo 3-glc, a methylated form of Cy 3-glc, is formed in the liver, because it was detected in high concentration in bile, but not detected in plasma.

Anthocyanins, which were not absorbed in the stomach, are transferred into the small intestine, where the pH is alkaline. Studies *in vitro* and *in vivo* have shown a degradation of Cy 3-glc into protocatechuic acid and phloroglucinaldehyde at physiological pH (Vitaglione et al., 2007; Kay et al., 2009), additionally the half-life of Cy 3-glc in the intestinal lumen was 120 min. (He et al., 2009). As a result, anthocyanins glycosides can undergo a deglycosylation or can be transformed into quinonoidal or hemiacetal bases or chalcones after crossing the intestine. Some anthocyanins are resistant to deglycosylation (e.g. anthocyanins with rutinosides) and should be undamaged (McGhie and Stevenson, 2013). In the process of absorption, compounds are transported directly into the blood and the principal area of absorption is the ileum, the last part of the small intestine that connects with the large intestine. Compounds that are not absorbed by the small intestine enter the colon or large intestine, where they can be degraded to sugar and phenolic acids and then eliminated in the human feces. Analysis of feces or fecal water confirmed the existence of phenolic acids after ingestion of isotopically labeled Cy-3-glc (Czank et al., 2013), raspberry supplementation (Gill et al., 2010) and red wine (Jiménez-Girón et al., 2013).

After passing of anthocyanins along the liver, metabolized and unmetabolized anthocyanins are also transported to the blood and go into the kidneys, which filter the blood though reactions like methylation and glucuronidation and also eliminate the waste from the body as urine (Mazza and Kay, 2009; McGhie and Stevenson, 2013). Studies *in vivo* have provided information about the anthocyanins after excretion into the urine. Original glycosylated anthocyanins were found in urine from elderly women after elderberry intake (Cao et al., 2001) but also in studies with males (20-45 years), who consumed extracts

from blueberry, boysenberry, black raspberry and blackcurrant (McGhie et al., 2003) and in persons with coronary artery disease (Milbury et al., 2010). Several studies have also identified metabolites of anthocyanins that are produced by reactions of methylation, glucuronidation and sulfatation. After strawberries intake, Pel 3-glc (the principal anthocyanin) was detected, and also monoglucuronides and sulfoconjugate of pelargonidin and pelargonidin (Felgines et al., 2003). Study with boysenberries, which contain cyanidin derivatives, has demonstrated the presence of monoglucuronides of peonidin, cyanidin and pelargonidin (Cooney et al., 2004). In the case of blackberry consumption, the anthocyanins in urine were principally methylated or glucuronidated conjugates. Pelargonidin contains a hydroxyl group on the B-ring, while cyanidin has two. Furthermore, pelargonidins are more acceptable to glucuronidation than to methylation (Felgines et al., 2003; Felgines et al., 2005). The transformation of cyanidin glycosides into glucuronide conjugates, methylated and oxidized derivative of cyanidin was observed in studies using a chokeberry extract. The studies also proposed the pathway for anthocyanin metabolites, where enzymes such as catechol-*O*-methyltransferase, S-adenosyl methionine, lactase-phlorizin hydrolase, β-galactosidase, uridine diphosphate glucuronosyltransferase, UDP-glucuronic acid and cytochrome P450 were associated (Kay et al., 2004). Fig. 2.24 summarizes the probable absorption and metabolism of anthocyanins in humans.

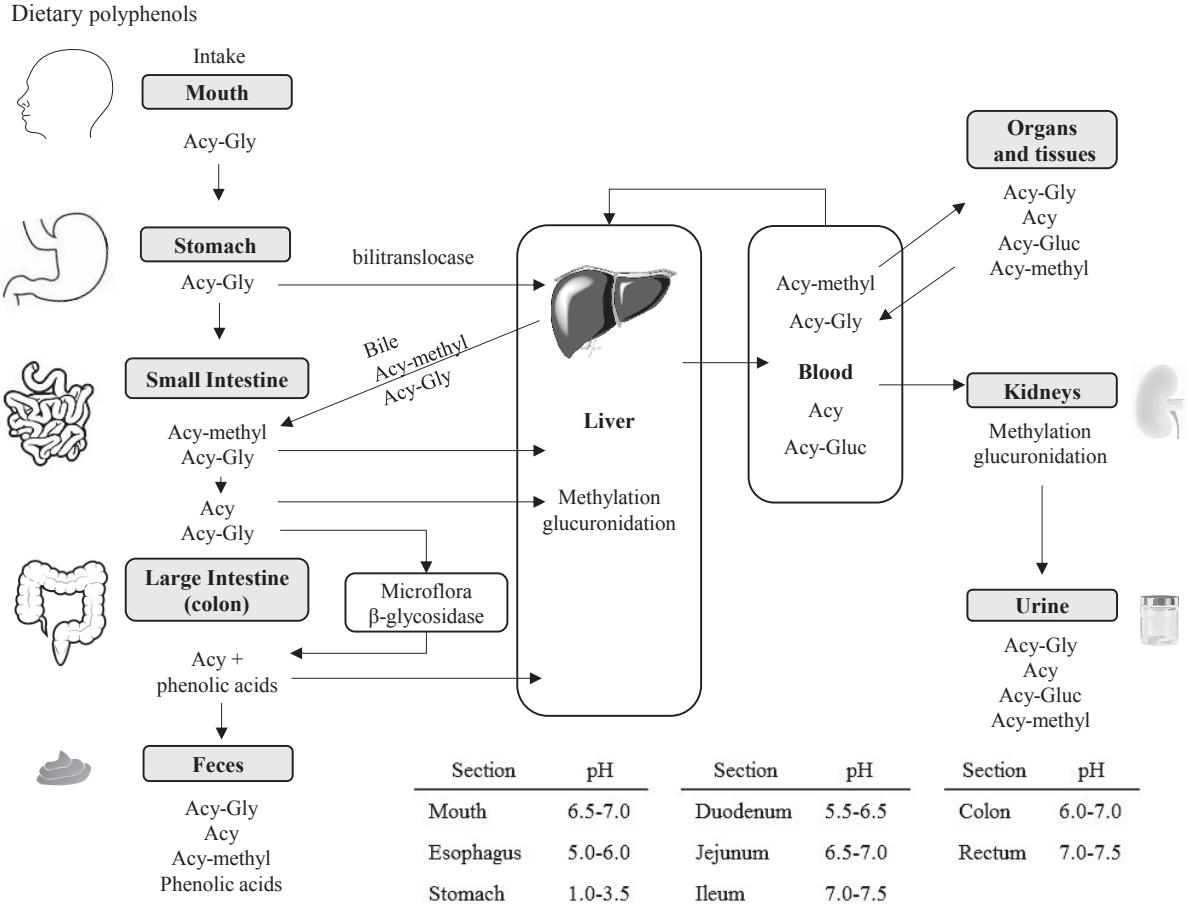


Fig. 2.24. Schematic of the probable anthocyanins bioabsorption (McGhie and Stevenson, 2013) and pH values of the different parts in the human gastrointestinal tract (Pandit and Soltis, 2012). Acy: Anthocyanidins; Acy-Gly: anthocyanin glycoside; Acy-methyl: methylated anthocyanidins; Acy-Gluc: anthocyanidin glucuronide.

Many factors such as intake dosage, chemical structure of anthocyanins, food matrix can influence the absorption of anthocyanins in the body. Additionally, the results of analysis may depend on the analytic procedure applied in the blood, urine and feces of human, animals, and cell models. Studies performed to date show that an increase of anthocyanin dosage is not always associated with an increment of anthocyanin absorption, as shown in Table 2.2 and 2.3, which summarize absorption of anthocyanins studies in human, animals and cell models.

Table 2.2. Pharmacokinetics of anthocyanins in humans and animals after consumption (Prior and Wu, 2006; Daayf and Lattanzio, 2009; Fernandes et al., 2015).

Model / Source	References	Type of source	Anthocyanin	Dose (mg)	C_{max} (nmol/L)	% Urinary Recovery	t_{max} (h)	Duration (h)
Human								
Elderberry	Cao and Prior (1999)	Extract		1500	100 µg/L	-	1.2	1
	Cao et al. (2001)	Extract		720	97.4	0.05	1.2	24
	Murkovic et al.(2001)	Dried juice		500	-	0.05	-	-
	Milbury et al. (2002)	Extract drink		720	97.4	0.08	1.2	24
	Wu et al. (2002)	Extract		720	97.4	0.08	1.2	24
	Mülleder et al. (2002)	Conc.		1900	-	0.03	-	6
	Bitsch et al. (2004 b)	Juice		3570	-	0.053	-	5
	Bitsch et al. (2004 c)	Extract		147	-	0.37	-	7
	Netzel et al. (2005)	Juice		361	-	0.033	1.5	7
				541	-	0.038	0.5	7
				722	-	0.04	1.0	7
	Frank et al. (2005b)	Conc. Juice		3570	-	0.05-0.16	1.0	24
	Frank et al. (2007)	Extract		278	-	0.39	-	7
		Extract		1852	-	0.27	-	7
	de Ferrars et al. (2014)	Capsules		500	-	-	-	3
Red grape	Lapidot et al. (1998)	Red wine		218	-	3.30	-	12
	Bub et al. (2001)	Red wine		68	1.4	0.03	0.3	6
		DRW		58	1.7	0.03	1.5	6
		Red grape		117	2.8	0.03	3.0	6
	Frank et al. (2003)	Juice		283	95.5	0.18	1.5	7
		Red wine		280	222.7	0.23	0.5	7
	Bitsch et al. (2004 a)	Juice		283	222.7	0.23	0.5	7
		Red wine		280	95.5	0.18	1.5	7
	Garcia-Alonso et al. (2009)	Extract		183.8	<10	0.05	1.6	24
			Pn 3-glc	12.4	0.8	0.06	-	24
			Del 3-glc	45.0	-	0.06	-	24
			Pt 3-glc	33.8	-	0.03	-	24
			Mv 3-glc	89.9	4.2	0.06	-	24
			Cy 3-glc	2.7	-	-	-	24
	Boto-Ordóñez et al. (2013)	DRW		272 mL	-	-	-	24
Black currant	Matsumoto et al. (2001)	Conc.		3.58	60.0	0.11	1.5	8
			Del 3-rut	1.68	73.4	0.11	1.8	8
			Cy 3-rut	1.24	46.3	0.098	1.5	8
			Del 3-glc	0.49	22.7	0.066	1.5	8
			Cy 3-glc	0.17	5.0	0.06	1.3	8
	Netzel et al. (2001)	Juice		153	-	0.02-0.05	-	5
	Rechner et al. (2002)	Extract		1000	-	0.007-0.133	-	7
	Nielsen et al. (2003)	Juice		1239	115.8	0.07	0.7	4
		Juice		716	35.6	0.05	0.7	4
	Bitsch et al. (2004 a)	Juice		145	-	0.04	-	7
	McGhie et al. (2003)	Conc.		345	-	0.029	-	7
Chokeberry	Kay et al. (2004)	Extract		1300	592	0.048	-	24
	Kay et al. (2005)	Capsules		721	96.1	0.15	2.8	24
	Wiczkowski et al. (2010)	Juice		0.8 mg/kg BW	32.7	-	1.3	24
Hibiscus	Frank et al. (2005a)	Extract		147	-	0.018	1.5	7
Sabdariffa L.			Cy 3-samb	62.6	3.8	-	1.5	7
			Dp 3-samb	81.6	2.1	-	1.5	7
	Frank et al. (2012)	Extract drink		130	29.1	-	-	24
Black	Stoner et al. (2005)	Dry	Cy 3-glc	135	4.8	-	1.1	1wk
Raspberry		Dry	Cy 3-samb	85.5	3.7	-	2.2	1wk
		Dry	Cy 3-rut	963	37.8	-	1.6	1wk
		Dry	Cy 3-xylrut	256.5	9.4	-	2.6	1wk
	Tian at al. (2006)	Freeze-dried		45g/day	1091 pmol/L	-	-	1wk

Table 2.2. Continued

Model / Source	References	Type of source	Anthocyanin	Dose (mg)	C_{max} (nmol/L)	% Urinary Recovery	t_{max} (h)	Duration (h)
Human								
Blueberry	Mazza et al. (2002)	Extract		1200	29.2	0.003	4.0	4
	Wu et al. (2002)	Extract		690	-	0.004	-	6
	McGhie et al. (2003)	Conc.		439	-	0.02	-	7
	Kalt et al. (2014)	Juice		216	-	0.08	-	24
Strawberry	Felgines et al. (2003)	Strawberry		77	-	1.90	-	24
Blackberry	Felgines et al. (2005)	Blackberry		431	-	0.16	-	24
Boysenberry	McGhie et al. (2003)	Extract		189	-	0.064	-	7
Bilberry/Lingonberry	Nurmi et al. (2009)	Purée		650	138	-	1.5	48
Cranberry	Ohnishi et al. (2006)	Juice		651	-	5.00	-	24
Blood orange	Vitaglione et al. (2007)	Juice		71	1.9	1.2	0.5	24
Mixed berries	Miyazawa et al. (1999)	Mixed berries		162	29.0		1.0	-
Purple sweet potato	Harada et al. (2004)	Beverage		311	0.0024	0.02	1.5	24
^{13}C5-Cy 3-glc	Czank et al. (2013)	Capsules		500	5970	5.37	10.25	48
Pig								
Elderberry	Wu et al. (2005)	Freeze-dried		228.1 µmol	-	0.13	-	24
Black currant	Wu et al. (2005)	Freeze-dried		139.9 µmol	-	0.0671	-	24
	Walton et al. (2006)	Conc.		100	0.09 µg/mL	-	2-4	8
Chokeberry	Wu et al. (2005)	Freeze-dried		228.8 µmol	-	0.0963	-	24
Marionberry	Wu et al. (2004b)	Freeze-dried		165.18 µmol	-	0.0883	-	24
Rabbit								
Blackcurrant	Nielsen et al. (2003)	Juice		117	780 ng/mL	0.04 (4h)	-	48
Mice								
Blackberry	Marczylo et al. (2009)		Cy 3-glc	500	25 µM	-	0.5	2
Bilberry	Sakakibara et al. (2009)	Extract		100	1.2 µM	1.88 (6h)	0.25	24
Rat								
Elderberry	Miyazawa et al. (1999)	Berries		320	1563 µg/L	-	0.25	4
Red grape	He et al. (2006)	Extract		64-72	33.2	<0.05	-	6
Black currant	Matsumoto et al. (2001)	Conc.		800 µmol	-	-	-	4
			Del 3-rut	-	580 ± 410	-	2.0	4
			Cy 3-rut	-	850 ± 120	-	0.5	4
			Cy 3-glc	-	840 ± 190	-	0.5	4
	Walton et al. (2009)		with water	250 mg/kg BW	0.37 µmol/L	-	0.25	24
			with oatmeal	250 mg/kg BW	0.20 µmol/L	-	1.0	24
	Jakobsdottir et al. (2014)			2253-2613 µg	0.25−0.75 µg/mL	-	-	5 days
Chokeberry	He et al. (2006)	Extract		64-72	52.5	<0.05	-	6
Black Raspberry	McGhie et al. (2003)	Extract		12.08	3.25 µg/mL	-	-	1
	He et al. (2009)	Extract		10		0.007-0.045	-	3
Blueberry	McGhie et al. (2003)	Extract		8.5	2.45 µg/mL	-	-	1
	Del Bò et al. (2010)	Extract		24		-	0.5	4-8 wk
Bilberry	Morazzoni et al. (1991)			400	2-3 µg/mL	-	0.25	2
	Ichiyanagi et al. (2006)	Extract		153	1.2 µM	-	0.25	8
	He et al. (2006)	Extract		64-72	17.3	<0.05	-	6
Red orange	Felgines et al. (2006)	Juice		2.8 µmol/day		0.081	-	0.75
Standards	Tsuda et al. (1999)	From purple corn	Cy 3-glc	900 µmol	0.31 µM	-	0.5	4
	Suda et al. (2002)	From Purple Sweet Potato	Pn 3-caf·soph-5-glc	146	50	-	0.5	2
	Ichiyanagi et al. (2005)	From purple black rise	Cy-3-glc	100	0.18 µM	-	0.25	8
	El Mohsen et al. (2006)		Pg	50 mg/kg BW	3.49 µM		0.25	2 or 18
	Matsumoto et al. (2006)		Del 3-rut	-	0.285 µmol/L	-	0.43	8
	Ichiyanagi et al. (2013)	Strawberry	Pg 3-glc	100 mg/kg BW	1.492	0.067	0.22	

DRW = dealcoholized red wine; BW = body weight; Conc. = concentrate, wk = week

Table 2.3. Absorption of anthocyanins in cell models (Fernandes et al., 2015).

Model/ Source	References	Model	Type of Source	[Anthocyanin]	Transport efficiency		Duration (h)
Cell Model							
Red grape	Oliveira et al. (2015)	MKN-28	Red wine	50 µg/mL	Apical pH 5.0	5.56	3
	Kuntz et al. (2015)	Caco-2	Grape extract	50 µmol/L	≈ 1%		4
	Faria et al. (2009)	Caco-2	Skin extract	200 µg/mL	1.77 %		2
Black currant	Steinert et al. (2008)	Caco-2	Extract	180 µM	≈ 11%		1.33
Blueberries	Yi et al. (2006)	Caco-2	Extract	50 µg/mL	≈ 3-4% < 1% in Del 3-glc		2
	Liu et al (2014)	Caco-2	Extract	50 µg/mL	1.59-4.22%		2
Açaí	Pacheco-Palencia et al. (2010)	Caco-2	Cy-3-rut, Cy-3-glc	50–500 µg/mL	0.5%–4.9%		2
	Cardona et al. (2015)	Caco-2	Cy 3-glc		1.38%		
			Cy 3-rut		1.06%		
Strawberry	Kosińska et al. (2015)	Caco-2	Extract	10 mg/mL			2
Sour cherry	Toydemir et al. (2013a)	Caco-2	Fruit	16.7 mg /mL	0.011		6
			Nectar	16.7 mg /mL	0.034		6
Standards	Zou et al. (2014)	Caco-2	Cy 3-glc	10 µmol/L	2.41		2
				20 µmol/L	1.45		2
				40 µmol/L	0.76		2
	Zhang et al. (2014)	Caco-2	Cy 3-glc	37.5 µM	2.51 µg		1
			Encapsulated Cy 3-glc	37.5 µM	2.74 µg		1
	Faria et al. (2014)	BBB		100 µM			18
		hCMEC/D3	Del 3-glc	-	12%		
			Cy 3-glc	-	16%		
			Mv 3-glc	-	20%		
			4'Me-Del 3-glc	-	18%		
			4'/3'Me-Cy 3-glc	-	19%		
	Fernandes et al. (2012)	MKN-28	Commercial standards	500 µM	Apical pH 3.0	Apical pH 5.0	3
			Del 3-glc	-	6.38%	6.95%	
			Cy 3-glc	-	7.96%	7.06%	
			Mv 3-glc	-	10.44%	8.25%	

Results of pharmacokinetics studies demonstrated that anthocyanins have a structural transformation over time but some anthocyanins maintain their structure in some cases too. Cell culture studies in gastric (MKN-28), intestine (Caco-2 and HT-29) and blood-brain barrier (BBB) models showed the transport efficiency of anthocyanins, which increases with the time of incubation. In gastric model, the range was between 5% and 10% (Fernandes et al., 2012; Oliveira et al., 2015), while the intestine models was between 1% and 11% (Yi et al., 2006; Steinert et al., 2008; Faria et al., 2009; Pacheco-Palencia et al., 2010; Toydemir et al., 2013a; Liu et al., 2014; Zhang et al., 2014; Zou et al., 2014; Cardona et al., 2015; Kosińska-Cagnazzo et al., 2015; Kuntz et al., 2015). Additionally, the transportation of anthocyanins, other flavonoids (flavan-3-ols and flavonols) and their metabolites were evaluated in a BBB model, where metabolites had a higher efficiency than the native flavonoids (Faria et al., 2014).

On the other hand, anthocyanins and glucuronide-conjugates are predominant in blood between 0 and 5 hours, than there is an increase of methyl-conjugates between 6 and 24 hours after intake. Nevertheless, other flavonoids are chiefly present as metabolites in blood and urine (Scalbert and Williamson, 2000; van der Woude et al., 2004; Walle et al., 2005; Barron, 2009; Williamson and Stalmach, 2012; Gonzales et al., 2015). The concentration of anthocyanins in plasma is in the range from nM to µM, while the percentage of urinary recovery is between 0.003 to 5.37% (Table 2.2). Metabolites of anthocyanins like glucuronide- and methyl-conjugates have mainly been found in human studies (Wu et al., 2002,; Bitsch et al., 2004c; Kay et al., 2004; Kay et al., 2005; Tian et al., 2006; Vitaglione et al., 2007; Garcia-Alonso et al., 2009; Frank et al., 2012; Kalt et al., 2014) and also in

animal studies (Wu et al., 2004b; Talavéra et al., 2005; Wu et al., 2005; Ichiyanagi et al., 2006; Walton et al., 2009; Jakobsdottir et al., 2014), while on fewer occasions as sulfo-conjugates at low concentrations after strawberries and blackberries consumption (Felgines et al., 2003; Felgines et al., 2005). In addition, there is a degradation of anthocyanins to phenolic compounds like protocatechuic acid as well as hydroxycinnamic and hydroxybenzoic acids (Vitaglione et al., 2007; Boto-Ordóñez et al., 2013).

2.1.3.2 Bioactivity of Anthocyanins

Anthocyanins attribute to numerous biological activities and diverse health benefits such as antioxidant, anti-inflammatory, anti-carcinogenic, antiatherogenic, antibacterial, antiviral, and antidiabetic activities; including gastric, neuro and skin protective effects. They have also been found to improve vision and to protect against ultraviolet radiation. Therefore, the interest in anthocyanins has risen in the past years, as well as consumption of anthocyanins, identification of anthocyanins sources and the large-scale isolation.

2.1.3.2.1 Antioxidant activity

Antioxidant is "a molecule that protects a biological target against oxidative damage" (Halliwell, 2011). Natural antioxidants are present in the body or foods and are classified as enzymatic and non-enzymatic (Fig. 2.25). Nevertheless, it is possible to produce synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG). In addition, antioxidants perform their functions by diverse mechanisms such as donating electrons, co-antioxidants, gene expression regulation and metal ion chelating (Lobo et al., 2010).

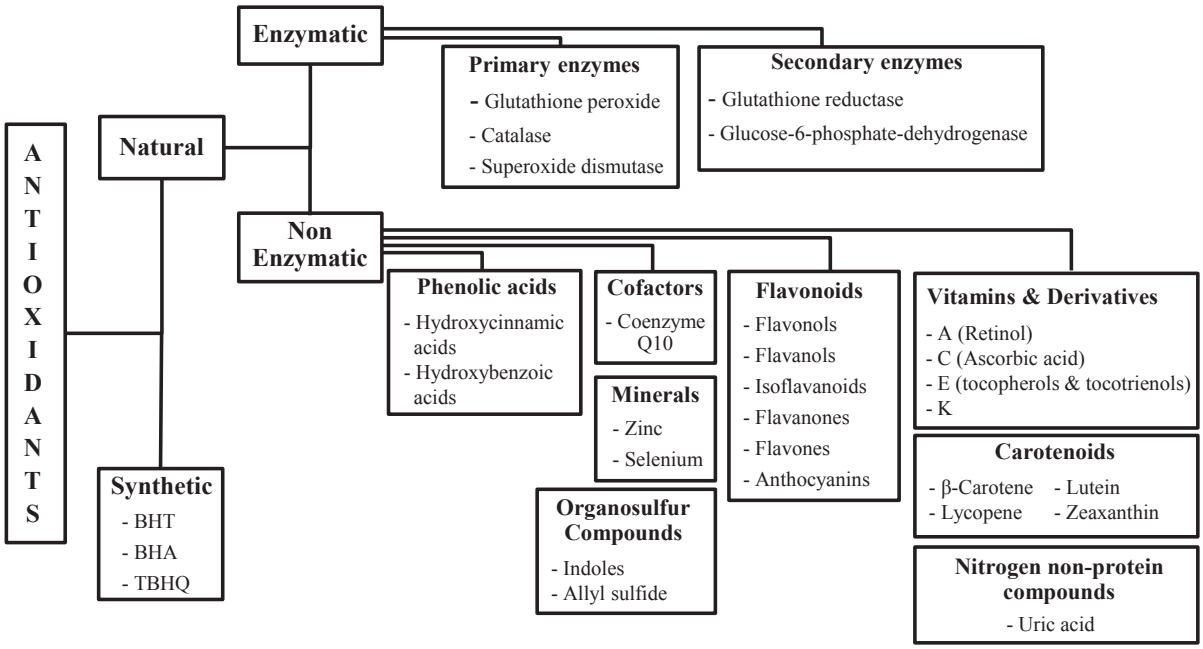


Fig. 2.25. Classification of antioxidants (Carocho and Ferreira, 2013).

Several studies have demonstrated that the oxidative damage is related to free radicals. The free radicals have one or more unpaired electrons and can donate or accept an electron; consequently, they are extremely reactive and can act as oxidant or reductant. There are different reactive species: radicals

such as superoxide ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($RO_2^{\cdot}$), nitric oxide ($^{\cdot}NO$), nitrogen dioxide ($^{\cdot}NO_2$) and alkoxyl radical ($LO^{\bullet}$); and non-radicals, which are oxidizing agents and can convert to radicals, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), lipid hydroperoxide (LOOH) and nitrous acid (HNO_2).

In addition, they are also classified according to their reactivity: reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chlorine species (RCS), reactive bromine species (RBS) and reactive sulphur species (RSS), but ROS and RNS are predominant in the human body. The balance between free radicals and antioxidants is necessary for physiological functions, for example in defense system. Nonetheless, if there are low defenses levels, the body can suffer immunodeficiency. Conversely, if there is an excess of radicals in the human body, it produces oxidative stress, which generates pathogenesis of diseases and can lead to chronic and degenerative diseases (Lobo et al., 2010; Wallace and Giusti, 2013; Halliwell and Gutteridge, 2015). Fig. 2.26 shows an overview of human diseases associated with oxidative stress.

The production of free radicals is attributed to two types of sources, firstly endogenous sources, which originated from one part of an organism, tissue or cell, for example in mitochondria, endoplasmic reticulum, peroxisomes respiratory chain and as a result of stress. Secondly, exogenous sources relating to the environment such as air pollution (cigarette smoke, exhaust fumes and industrial contaminants), ozone pollution, non-ionizing radiation (ultraviolet, microwaves, infrared, etc.), water pollution, drugs (immunosuppressant and narcotic drugs, etc.), alcoholism, xenobiotics (herbicides, pesticides, toxins, etc.) and chemicals (heavy metals, solvents, etc.) (Valko et al., 2007; Mani, 2015).

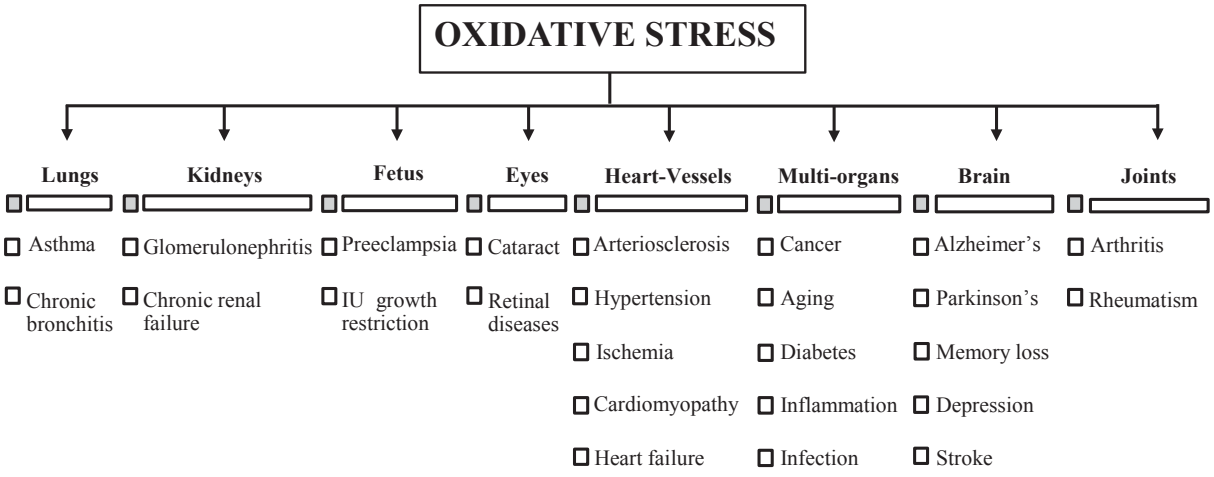


Fig. 2.26. Diseases induced by oxidative stress in humans (Pham-Huy et al., 2008).

However, the total antioxidant capacity does not reflect in one assay, because they need the lipophilic and hydrophilic capacity. There is a variety of *in vitro* methods to evaluate the antioxidant capacity. For example trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), total antioxidant potential (TRAP), ferric reducing/antioxidant power (FRAP), copper reduction (CUPRAC), total oxidant scavenging capacity (TOSC), low-density lipoprotein oxidations (LDL-oxidations), β-carotene bleaching (BCB), chemiluminescence (CL),

photochemiluminescence (PCL) and electron spin resonance (ESR) assays, which deactivate the radicals. These assays are based on mechanisms such as hydrogen atom transfer (HAT) or single electron transfer (SET) and in some cases, both mechanisms are used. The results of antioxidant activity can vary depending on the used method and free radical as reactant, but HAT assays are mostly applicable to human biology and foods (Prior et al., 2005).

On the other hand, due to published information of the European Food Safety Authority (EFSA) about acceptable daily intakes (ADIs) and the possible toxicity of some synthetic antioxidants beyond these ADIs, the popularity and interest in natural antioxidants have increased in recent times (Carocho and Ferreira, 2013; Shahidi and Ambigaipalan, 2015). There has been increasing evidence in the recent years on the potential health benefits of polyphenols in foods, due to their antioxidative activity. An evaluation of antioxidant activity measurement using 25 diverse common fruits consumed in the United States (US) of America by cellular antioxidant activity assay (CAA) demonstrated that pomegranate and berries such as blackberries, blueberries and raspberries had a high CAA, while bananas and melons had the lowest activity (Wolfe et al., 2008). *In vitro* and *in vivo* studies have documented that anthocyanins exhibit antioxidant properties (Wang, 1997; Wang et al., 2012; Wallace and Giusti, 2013; Del Bò et al., 2015b). It has been proven by TEAC, ORAC and TRAP assays that after consumption of anthocyanins, the antioxidant capacity in serum increases (Cao et al., 2001; Mazza et al., 2002). A comparison of methods (ABTS, DPPH and ORAC) in 50 popular US foods (fruits, vegetables and beverages) showed that results of ABTS assay had higher values than the results of DPPH assay. In addition, cherry, plums, blueberry, strawberries, red cabbage and red wine, which are associated with anthocyanins, have high antioxidative capacity (Floegel et al., 2011). A study calculated the theoretical total antioxidant capacity by HPLC-CUPRAC and HPLC-TEAC, and compared it with spectrophotometric methods using standards and foods. Additionally, there was a concordance with theoretical expectations and TEAC coefficients, which were found in the following order: Cy > Cy 3-glc > Cy 3-rut > Dp > Pg > Pn > Mv > Pn 3-glc, where cyanidin had the highest TEAC coefficient, while peonidin 3-glucoside presented the lowest value (Başkan et al., 2015). On the other hand, there is a high correlation between total phenolic content and antioxidant capacity (Connor et al., 2002).

2.1.3.2.2 Anti-carcinogenic effects

Cancer in humans involves a complex process of cellular and molecular changes. Furthermore, oxidative stress produces an imbalance that is associated with oxidative DNA damage and cancer growth. DNA damage, mutations and modified gene expression have been observed in various tumors and play an important role in carcinogenic process (Valko et al., 2007). An excess of free radicals has been found to exhibit cancer promoting effect, especially ROS, which are attributed to cancers such as breast, glioma, colon, liver, pancreas, prostate, bladder, ovarian and melanoma (Afanas'ev, 2011).

Because of antioxidative capacity of anthocyanins, they act as scavengers of ROS, thus reducing the DNA damage. The presence of hydroxyl groups in ring C and ring B of anthocyanins give the radical scavenging activity, and this activity is higher in anthocyanidins than their specific anthocyanins (Wang

and Stoner, 2008). A scavenging capacity comparison of juices from blackberry, blueberry, cranberry and strawberry showed the ability to scavenge radicals like $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ and $^1O_2$, resulting in blackberry having the highest percent inhibition of these radicals, followed by strawberry (Stoner et al., 2008). Studies in apples peel demonstrated a high contribution of anthocyanins on hydrogen peroxide scavenging in contrast to other phenolic compounds in apples (Bi et al., 2014).

Anthocyanin extracts from fruits or vegetables and their isolated anthocyanins have been used to analyze the inhibitory or antiproliferative effect in different cancers, using human and animal cell lines and *in vivo* animal model tumor systems. Up to now, *in vitro* studies have been tested in cancer cell lines for colon (HT-29, HCT116, SW620 and CaCo-2), lung (LXFL529L, H460 and NCI), stomach (AGS), leukaemia (U937, HL60, Jurkat and Raw264), valvar (A431), uterine (HeLa S3), prostate (RWPE-1, RWPE-2 and 22Rv1), mammary (MCF-7 and RBA), glioblastoma (SF-268), oral (KB and CAL27) and skin (JB6) (Cooke et al., 2005). In addition, several factors can influence the results like cell culture type, concentration of extract, stability of compound in various media, treatment time, free radical type and absorption of compounds (Seeram et al., 2006). Furthermore, studies has been carried out containing factors such as radical scavenging activity, stimulation of phase II detoxifying enzymes, reduction of cancer proliferation, inflammation, apoptosis, invasiveness, differentiation and angiogenesis (Wang and Stoner, 2008). The effect of inhibition in the formation of cancers may be attributable to two mechanisms: firstly a modification in redox status and secondly variation of basic cellular functions (Warner, 2014). The results have shown that anthocyanins can reduce and may prevent the risk of various diseases. For example, investigations of anthocyanins extracts from bilberry, chokeberry and grape showed an inhibition in multiple biomarkers of colon cancer in rats (Lala et al., 2006). In addition black chokeberries are known to possess a higher antioxidant activity than another fruits (Kulling and Rawel, 2008), and may reduce oxidative stress and the risk of cancer, particularly in breast (Olas, 2014). In case of cherries, they reduced cell growth in human colon cancer cell, especially in cell lines HT-29 and HCT 116 (Kang et al., 2003). Red grape have an effect on colon cancer reduction in animal models (Mendes Silva et al., 2015), as well as in human colorectal carcinoma cell line like HCT116 (Signorelli et al., 2015). *In vitro* study of bilberry showed a reduction of ROS levels in Caco-2 cells and oxidative DNA damage in HT-29 cells (Juadjur et al., 2015). Blackcurrant and blueberry presented an antiproliferative potential on tumor cell lines of mouse skin melanoma (B16F10), human ovarian carcinoma (A2780) and human cervical cancer (HeLa) (Diaconeasa et al., 2015). In other studies of berry juices, blackcurrant showed the highest inhibition effect in other cancer cell lines like human melanoma (Fem X), human colon cancer (LS 174), human breast carcinoma (MCF-7), human prostate carcinoma (PC-3) (Konić-Ristić et al., 2011), and a pro-apoptotic effect on leukaemia Jurkat cells (León-González et al., 2015). On the other hand, cranberries present anti-cancer properties and are involved in different cancer types such as ovarian, cervical, breast, prostate, oral, esophageal, gastric, colon, lung and melanoma due to the wide variety of polyphenol content (Katsargyris et al., 2012). Because of this, a diet rich in anthocyanins such as berries (cherries, red grapes, plums, prunes, pomegranate), red

cabbage, red onions, purple sweet potates, purple potaoes, black carrots and eggplant is recommended. The World Health Organization (WHO) recommends also a consumption of 400g of fruit and vegetables per day and has promoted a campaign "5 A Day" in order to prevent chronic non-communicable diseases (NCDs) like diabetes, cancers and cardiovascular diseases (WHO, 2003).

2.1.3.2.3 Anti-inflammatory effects

The inflammation is a biological response of an organism to tissue damage that are caused by bacteria, chemicals or trauma. Swelling, pain, redness, heat and loss function are signs of inflammation. Furthermore, there are two classes of inflammation: acute and chronic inflammation. Additionally chronic inflammation can play a role in numerous degenerative pathologies such as hypertension, insulin resistance and dyslipidemia (Mena et al., 2014). An acute inflammation involves a sequence of events like production of inflammatory mediators, vascular permeability, vasodilatation, increase of blood flow, oedema, pavementing, phagocytosis, production of anti-inflammatory molecules, hemodynamic changes, production of resolution mediators and restoration of tissue (Linton, 2012; Halliwell and Gutteridge, 2015). Chemical mediators such as prostaglandins are involved in the inflammatory response and are produced from arachidonic acid by cyclooxygenase (COX) isoenzymes (Ricciotti and Fitzgerald, 2011). The inhibition of arachidonic acid metabolism was preferred to anti-inflammatory mechanism (Ferrándiz and Alcaraz, 1991). The anti-inflammatory activity of polyphenols was recognized at the first step of transformation of arachidonic acid, that can be regulated by phospholipase A2 (Mitjavila and Moreno, 2012). Another proposed mechanism is related to the prostaglandin synthase cyclooxygenase activity, where COX-2 products have a contribution in the reduction of inflammation and tumorigenesis (Hou et al., 2005b; Ricciotti and Fitzgerald, 2011). There are inflammatory markers such as C-reactive protein (CRP), interleukins (IL-1, IL-6, IL-8, IL-12 and IL-16), tumor necrosis factor (TNF)-α and transcription factor nuclear-κB (NF-κB), which are measurable (Zhang and An, 2007; Cassidy et al., 2015). An anthocyanin and flavonoid rich diet is related to anti-inflammatory effects, because they produce inhibitory effects on biomarkers of inflammation (Cassidy et al., 2015). Studies in red grapes and red wine consumption showed a decrease of TNF-α and IL-6 levels in plasma (Zern et al., 2005) and a reduction of expression of markers in chronic intestinal inflammation (Angel-Morales et al., 2012). On the other hand, anthocyanins from bilberry caused mRNA biomarkers suppression of acute inflammation such as COX-2, iNOS and IL-β (Esposito et al., 2014). In the case of anthocyanins, anti-inflammatory activity has been linked with the *ortho*-position of two hydroxy groups on the B-ring, because LPS-induced COX-2 protein was inhibited by cyanidin and delphinidin, while peonidin, pelargonidin and malvidin did not produce an inhibition effect (Hou et al., 2005b). Human studies have revealed that the impact of anthocyanins from tart cherry (Lynn et al., 2014), bilberry (Karlsen et al., 2007), blueberry (Basu et al., 2010; Riso et al., 2013), blackcurrant (Karlsen et al., 2007; Hassellund et al., 2013), purple carrot (Wright et al., 2013), pomegranate (Asgary et al., 2014) and cranberry (Dohadwala et al., 2011) on inflammation markers have showed a positive effect in patients.

2.1.3.2.4 Anti-cardiovascular effects

Chronic inflammation plays an important role in cardiovascular diseases (CVD) like hypertension, coronary heart and cerebrovascular diseases. There are different causes of CVD, but the principal is from atherosclerosis, that is averted by good diet. Arteriosclerosis occurs when the blood vessel present thickening and hardening. The atherosclerosis is the most common type of arteriosclerosis and results in the formation of atheroma that are plaques in the intima of artery walls. Consequently, the elasticity of blood vessels can be lost and blood flow is restricted (Maciejko, 2004). Several studies have demonstrated that polyphenols intake can reduce the CVD risk, improving the vascular function and regulating inflammation, based on clinical markers of CVD in humans such cholesterolemia, blood pressure, endothelial and platelet functions (Habauzit and Morand, 2012). A comparison of metabolic effects of flavonoids was tested in mice fed with a high fat diet, especially apigenin (flavone), epicatechin (flavan-3-ol), hesperetin (flavanone), quercetin (flavanol) and extract of anthocyanins from bilberries and blackcurrant. The results showed a reduction of risk of CVD by all flavonoids (17-29%), particularly by quercetin (Hoek-van den Hil et al., 2015). The efficiency of bioactive compounds in treatment and prevention of CVD have been reviewed based on diverse human studies, which were obtained from databases like MEDLINE, LILACS, PubMed and all randomized controlled trials. Effects on blood pressure, cholesterol, triacylglycerol, low and high-density, carbohydrate metabolism, inflammation, endotherial function and oxidative stress were compared. As a result, polyphenols like anthocyanins, catechols, isoflavones, flavonols, flavanols and stilbenes, showed beneficial effects for CVD (Rangel-Huerta et al., 2015). Oxysterols oxidized like 7-ketocholesterol (7-KC) and are in high levels in plaques of atherosclerosis and in plasma of patients with CVD. Treatment with Cy 3-glc upregulated cholesterol transporters (ABCG1 and ABCA1) expression in human aortic endothelial cells and has lead to the inducement of 7-KC mass efflux, and decrease in oxidative stress and apoptosis (Wang et al., 2012). Atherosclerosis chemo-protective activities of anthocyanins have resulted in the activation of Nrf2-ARE, reducing the generation of pro-inflammatory mediators (Aboonabi and Singh, 2015). Study with blueberry showed a reduction of lipid accumulation in THP-1-derived macrophages (Del Bò et al., 2015a). *Hibiscus sabdariffa* L. is related to positive effects in CDV such as decrease blood pressure and total cholesterol, as well as increase high-density lipoprotein cholesterol (Hopkins et al., 2013).

2.1.3.2.5 Antidiabetic effects

Diabetes mellitus is characterized by having persistent high blood glucose levels (hyperglycemia) and excretion of glucose in urine, because there are a defective production of insulin and/or an inefficient use of insulin. The pancreas has clusters of cells called islets that contain β-cells, which make the hormone insulin and deliver it into the blood. Insulin plays a role in the regulation of metabolism, which include ROS (Halliwell and Gutteridge, 2015). The principal classes of diabetes are type 1 and type 2, but there are other minority classes of diabetes such as gestational diabetes and diabetes caused by specific genes, drugs, infections, etc. An extended period with chronic hyperglycemia is related to

damage, dysfunction and problems in organs like blood vessels, nerves, kidneys, heart and eyes. Some diabetic patient can present more than one class. Type 1 diabetes represents 5% to 10% of diabetes cases and presents a fault or lack of insulin secretion, because of problems in the pancreatic islet cells. Additionally, this diabetes is insulin dependent and is identified by marker testing like islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD). It is typical in childhood diabetes. Type 2 diabetes represents 95% to 90% of all diabetes cases. It presents normal or high blood insulin levels and there is an insulin resistance, where cells are not able to use insulin adequately. This type of diabetes is insulin-independent, commonly in adults, who have obesity, high-calorie diet and do not exercise (American Diabetes Association, 2010).

In vitro study tested the capacity of anthocyanins from fruits to stimulate insulin secretion from pancreatic β cells (INS-1 832/13) in different concentrations. Cy 3-glc and Del 3-glc had the most efficient insulin secretagogues, Cy 3-gal, Cy, Del, Pg, Mv and Pn had minor effects (Jayaprakasam et al., 2005). An animal study has shown a good contribution of Cy 3-glc from purple corn to the reduction of hyperglycemia and insulin sensitivity through the decrease of retinol binding protein 4 expression in diabetic mice (Sasaki et al., 2007). Human studies showed anti-diabetic effects of anthocyanins including prevention of free radicals and lipid peroxidation, decrease of blood lipids and hemoglobin A1c (HbA1c), increase of insulin secretion and improvement of insulin resistance (Guo and Xia, 2014). Anthocyanin-rich food is associated to type 2 diabetes due its anti-oxidant and anti-inflammatory effects, as well as the protection of pancreatic β-cells against glucose toxicity (Xiao and Högger, 2015). Diabetic patients have a positive effect on blood glucose and cardiovascular risk factors after 6 weeks consumption of sour cherry juice (Ataie-Jafari et al., 2008). Black chokeberry extract reduces marker levels such as blood pressure, serum endothelin (ET-1), lipids, uric acid and oxidative stress status (GSH-Px, SOD, TBARS) in patients with metabolic syndrome (Broncel et al., 2010). A study with 58 diabetic patients, who consumed purified anthocyanin for 24 weeks, showed a reduction of dyslipidemia and prevention of insulin resistance (Li et al., 2015). In addition, an improvement in insulin action was observed after strawberry consumption and a reduction of anti-inflammatory markers in overweight patients (Edirisinghe et al., 2011).

2.1.3.2.6 Neuroprotective effects

Oxidative stress, excitotoxicity, neuroinflammation and apoptosis are associated with many neurodegenerative disorders. Over the years, there are more evidences for neuroprotective effects of anthocyanins, in connection with Alzheimer, Parkinson, aging, ischemia and brain injury (Ross et al., 2013). Alzheimer's disease (AD) is the most popular type of dementia and a neurodegenerative illness (Lu and Bludau, 2011). A characteristic of AD is the production of senile plaques in the nervous system, these plaques are constituted by amyloid beta peptide (Aβ) and hyperphosphorylation of tau protein (Serrano-Pozo et al., 2011). *In vivo* and *in vitro* studies using black soybean anthocyanins displayed neuroprotective effects on Aβ-induced neurotoxicity in HT22 cells and adult male rats (Badshah et al., 2015). Cy 3-glc from tart cherries was used against permanent middle cerebral artery occlusion

(pMCAO) in mice, subsequently a reduction of superoxide levels was observed and blocked the apoptosis inducting factor (Min et al., 2011). Treatment with purple sweet potato anthocyanins provided a reduction on Aβ toxicity in PC12 cells, intracellular ROS production, calcium influx, mitochondria dysfunction and inhibition of cell apoptosis, thus it suggest that purple sweet potato may be used for treatment of AD (Ye et al., 2010). Likewise, other neurodegenerative disorder is Parkinson's disease (PD), which is associated with a deficit of dopamine neurons in the midbrain. Human study showed that a dietary flavonoid consumption is related to lower risk of PD, especially anthocyanins (Gao et al., 2012). Extracts from blackcurrant, blueberry, grape seed, hibiscus and mulberry, which are rich in anthocyanins and other polyphenols, have been tested in a cell model of PD. As a result, a suppression of neurotoxic effect of rotenone, which causes the death of dopaminergic cells, was observed (Strathearn et al., 2014). Another clinical research using berries such as blackcurrant, blackberry, bilberry, strawberry and mulberry have demonstrated that they can prevent neurodegenerative diseases due to the content of anthocyanins, flavonols, flavanols and phenolic acids, which reduce the oxidative stress and inflammation (Subash et al., 2014).

2.1.3.2.7 Antibacterial, antimicrobial and antiviral activity

Most of phenolic compounds exhibit antibacterial and antimicrobial properties. Cisowska et al. (2011) has reviewed numerous studies focused on antimicrobial activity of anthocyanins, where pure anthocyanins and extracts containing anthocyanins were tested for microbial properties. As a result, they have shown an antimicrobial activity against human pathogens and diverse microbes. Moreover, there is more susceptibility of gram-positive bacteria to anthocyanins than gram-negative bacteria. Antibacterial properties of blackberry against periodontopathogens were demonstrated on ten different oral bacteria (González et al., 2013). Studies of diverse *Hibiscus sabdariffa* extracts have demonstrated an inhibition of bacterial growth for *E. coli, S. enteritidis, S. aureus* and *M. luteus* (Borrás-Linares et al., 2015). Pomegranate has an inhibitory effect on bacteria growth of two human pathogens, *E. coli* and *S. aureu* (Pagliarulo et al., 2016). *Aronia melanocarpa* showed a bacteriostatic activity against *S. aureus* and *E. coli*, as well as an antiviral activity against influenza A virus (Valcheva-Kuzmanova and Belcheva, 2006). Antiviral effects of anthocyanins have been reported in several studies. For example, blackcurrant against influenza virus (Knox et al., 2003), red fleshed potatoes inhibited influenza viruses A and B (Hayashi et al., 2003), mulberry presented antiviral activity on foodborne viral infection (Lee et al., 2014b), extracts from strawberry, raspberry, bilberry and lingonberry inhibited the replication of CV-B1 and influenza A virus (Nikolaeva-Glomb et al., 2014).

2.1.3.2.8 Gastro protective effects

Animal studies demonstrated gastroprotective effects of anthocyanins. Black chokeberry was administrated in rats with acute gastric hemorrhagic lesions and the results showed antiulcerative activities (Matsumoto et al., 2004). Protective effect of Cy 3-glc from purple corn was analyzed in ethanol-induced gastric lesions in rats, consequently the gastric lesions in rats decreased after treatment with Cy 3-glc (Li et al., 2008). Similarly a reduction of gastric lesions was observed after administration

of strawberry (Alvarez-Suarez et al., 2011) and bilberry (Ogawa et al., 2011). Human studies provided a protective effect of cyanidin (IdB 1027) compared with aspirin-induced gastric mucosal damage (Barzaghi et al., 1991). Study with human epithelial gastric AGS cells and wild raspberry reported a protective effect against H_2O_2 and methylglyoxal-induce damage in AGS cells (Jiménez-Aspee et al., 2016).

2.1.3.2.9 Skin protective effect

Some skin disorders are erythema, inflammation, sunburn, cancer and skin aging. External lesions due to Ultraviolet (UV) radiation exposure, diet and smoking can cause skin aging. Anthocyanins can prevent the oxidative skin damage such as skin cancer, UV-induced erythema, because they are antioxidants (Rojo et al., 2013). Photochemopreventive effects of delphinidin on UVB-mediated oxidative stress were observed in human keratinocyte (HaCaT) cells and mouse skin (Afaq et al., 2007). Similar results have shown that cell models with bilberry that have a protective effect against UVA and UVB-induced damage, reducing oxidative stress markers in HaCaT cells (Calò and Marabini, 2014). Cy 3-glc has also a potential efficacy against UVB-induced inflammation, which is associated with skin cancer and skin diseases (Pratheeshkumar et al., 2014). Pomegranate products showed a decrease in UVB-induced damage, which is a cause of photoagaing and skin cancer (Afaq et al., 2009). Anthocyanin rich extract from strawberry has a protective effect on human dermal fibroblasts with a reduction of apoptotic and dead cells (Giampieri et al., 2014).

2.1.3.2.10 Effect on vision

Ocular dysfunctions are myopia, cataract, vascular pathologies, vascular inflammation, retinopathies and glaucoma. There is an association between anthocyanins and vision, because an improvement of vision was observed after anthocyanin intake. The formation of cataract, cataractogenesis, is a multifactorial process, which can be formed due to diabetes and oxidative stress. The flavonoids intake has shown preventive and inhibitory effects on cataractogenesis and visual functions (Stefek, 2011; Durga et al., 2014; Field et al., 2014; Nabavi et al., 2015). After bilberry intake, positive effects on myopic eyes and nocturnal vision was detected (Canter and Ernst, 2004; Lee et al., 2005; Kamiya et al., 2013), additionally effect of anthocyanins from bilberry with human corneal limbal epithelial (HCLE) cells has been investigated and results suggest a beneficial effect on physiological renewal and homeostasis of corneal epithelial cells (Song et al., 2010). On the other hand, glaucoma factors are elevated intraocular pressure (IOP), and high myopia and heredity. Anthocyanins from bilberry and maritime pine bark decreased the IOP in patients with ocular hypertension (Steigerwalt et al., 2010), also anthocyanins from blackcurrant showed benefits for patients with glaucoma (Ohguro et al., 2013). Studies indicate beneficial properties of anthocyanins for diabetic and vision diseases like diabetic retinopathy, cataracts, vision loss and retinal degeneration (Ghosh and Konishi, 2007; Tremblay and Kalt, 2013; Nabavi et al., 2015).

2.2 Raw materials

2.2.1 Blackberry (*Rubus fruticosus*)

The blackberry, which belongs to the *Rosaceae* family and the *Rubus* genus, is a dark purple aggregate fruit that is constituted of smaller fruits named drupes (Fig. 2.27). The color of blackberries changes from green to red and then to black as ripe fruit. Blackberries are sometimes confused with raspberries; while blackberries have a core, raspberries possess a hollow center.

Fig. 2.27. Blackberry plant (left, © Wolfgang Dirscherl/pixelio.de) and blackberry fruits (right).

Blackberries are sorted according to the cane architecture into three classes: erect, semierect and trailing cultivars. Erect blackberries are either thorny like Brazos, Tupy, Cherokee, or thornless such as Navaho and Arapaho. Examples of semierect blackberries are Chester Thornless, Thornfree, Loch Ness and Čačanska Bestrna. Trailing blackberries are Marion, Silvan and Thornless Evergreen (Zhao, 2007; Badenes and Byrne, 2012; Strik and Finn, 2012). The production of blackberries was estimated at 20,035 ha and 154,643 tons in 2005, which increased by 45% in comparison with the production in 1995. Additionally, it is estimated that blackberry production may increase to 27,032 ha worldwide in 2015 (Strik et al., 2007). Its production is not so big in comparison with other berries like blueberries (420,379 tons), cranberries (540,259 tons) and red raspberries (578,233 tons) in 2013 (FAO, 2013). The world highest producer of blackberry in 2005 were North America, particularly the U.S.A. and Mexico, then followed by Europe. In particular Serbia alone produced 90% of the production in Europe. China was the third highest producer of blackberry (Strik et al., 2007; Strik et al., 2008). Furthermore, blackberries are treated in the food industry as fresh, frozen, freeze-dried, canned, puree or juice for their commercialization.

On the other hand, blackberry is a source of anthocyanins and other polyphenols. The total phenolic content varies from 114 to 1056 mg/100 g FW with anthocyanins being the principal compounds, followed by procyanidins, flavonols, ellagitannins, ellagic and phenolic acids (Zhao, 2007; Kaume et al., 2012). Blackberries are associated with beneficial effects on health, such as antioxidant (Elisia et al., 2006; Hassimotto et al., 2008) and anti-cancer effects (Cooke et al., 2006; Folmer et al., 2014; Zia-Ul-Haq et al., 2014).

2.2.2 Black chokeberry (*Aronia melanocarpa*)

Chokeberries are commonly known as aronia berries and belong to the *Rosaceae* family and *Amygdaloideae* subfamily. They originated from the eastern part of North America and East Canada, and were introduced to Europe at the beginnings of the 20th century. Differences among Aronia species are by the berry color and foliage pubescence (Fig. 2.28). *Aronia arbutifolia* produces red berries, while *Aronia melanocarpa* has black berries. *Aronia prunifolia* is a hybrid of the other two cultivars and yields purple berries (Small and Catling, 2005; Knudson, 2009; Brand, 2010). The most popular cultivars are Nero and Viking followed by Aron, Rubina and Hugin (Strigl et al., 1995a; McKay, 2004).

Fig. 2.28. From left to right: Aronia arbutifolia, Aronia prunifolia, Aronia melanocarpa Nero and Viking (Source: with permission of Aronia ORIGINAL Naturprodukte GmbH).

Black chokeberries possess approximately a diameter of 6-13 mm and a weight of 0.5-2 g, as well as an umbel, which can have between 10 and 30 berries. The harvest of black chokeberries is made between August and September and can produce from five to twelve tons per hectare, depending on the age of the plants and cultivar (Rousseau and Bergeron, 2003; McKay, 2004). Currently, important producing countries are Poland, Russia, Lithuania, Bulgaria and Slovenia, although the Aronia planting began in the 1970s in Germany. The chokeberry harvest increased from 38,000 tons in 2004 to 58,000 tons in 2013 in Poland (ARR, 2014). The principal commercial product of black chokeberry is juice, even though chokeberries are also processed as wine, syrup, tea, jam or as flavor in other products like yogurt. In recent years, its popularity is increasing in the market, because black chokeberries are rich in polyphenols, which are associated with positive health benefits, due to their high antioxidant capacity (Wu et al., 2004a; Oszmiański and Wojdylo, 2005; Kapci et al., 2013). In addition, black chokeberries contain vitamin C, B1, B2, B6, niacin and pantothenic acid, as well as diverse phenolic compounds such as procyanidins, anthocyanins, flavonols and chlorogenic acids (Kulling and Rawel, 2008; Esatbeyoglu et al., 2010; Esatbeyoglu and Winterhalter, 2010). Clinical studies have proven that an intake of Aronia juice or Aronia extracts can reduce the cholesterol concentration in patients with hypercholesterolemia (Duchnowicz et al., 2012), produced a decrease of clot formation (Sikora et al., 2012) and reduction of breast cancer (Kędzierska et al., 2013; Olas, 2014). On the other hand, other studies showed a reduction of plasma triglycerides and plasma glucose in diabetic rats after consumption of chokeberry (Valcheva-Kuzmanova et al., 2007), as well as hepatoprotective effects (Valcheva-Kuzmanova et al., 2004) and reduction of oxidative stress (Faff and Frankiewicz-Jóźko, 2003).

2.2.3 Sour cherries (*Prunus cerasus* L.)

Cherries are stone fruits, belonging to the *Rosaceae* family and *Prunus* genus, which are native to Europa and Asia (Serradilla et al., 2015). Cherries are commonly marketed as fresh, frozen, dried, juice, nectar, puree, canned and jam. According to Food and Agriculture Organization of the United Nation (FAO), around 2.3 million tons of cherries were produced in the world in 2013; with Europe producing 36.7%, Asia 43.4% and America 18% of cherries. However, the major producer was Turkey (494,325 tons), subsequently the USA (301,205 tons), Iran (200,000 tons), Italy (131,175 tons) and Uzbekistan (100,000 tons) (FAO, 2013). The most popular cherry species are the sour (*Prunus cerasus* L.) and sweet (*Prunus avium* L.) cherries (Stéger-Máté, 2012) (Fig. 2.29). The sour cherries production was approximately 1.35 million tons in 2013, with Europa being the largest producer of sour cherries (63.2%) (FAO, 2013).

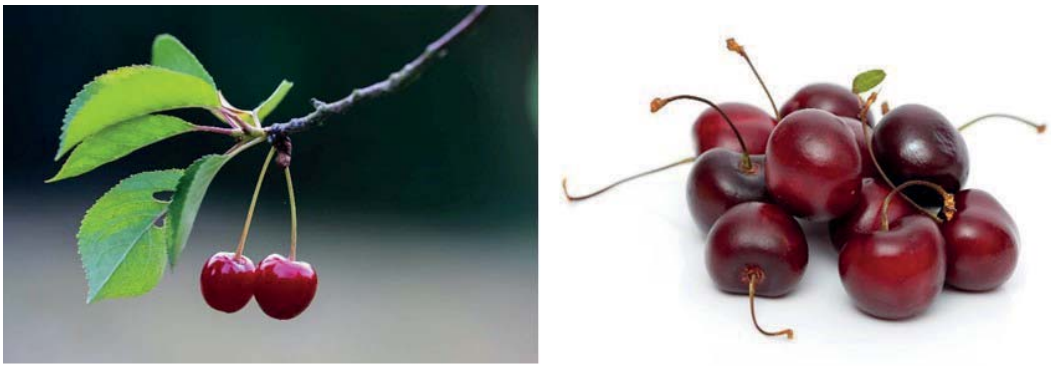


Fig. 2.29. Sweet cherries (© Johannes Heide/pixelio.de) and sour cherries (© Tim Reckmann/pixelio.de).

Sour cherries are divided in two groups, according the color of fruit flesh: Amarelle cherries, which are light colored, and the second group are Morello cherries that possess a dark red flesh (Brown et al., 1996). Some cultivars are Montmorency, Northstar, Meteor, English Morello and Early Richmond. The frequently cultivated sour cherry in Germany and in Europa is Morello cherries and the harvest is between July and August for sour and sweet cherries. Similarly, sweet cherries are classified in two groups concerning their color: yellow and dark sweet cherries. Some sweet dark varieties are Bing, Lapins, Sweetheart, Burlat and Van (Serradilla et al., 2015). On the other hand, cherries are characterized by sweetness due to its fructose and glucose content, while other cherries are described by sourness because of the presence of organic acids like L-malic acid (Usenik et al., 2008). Moreover, cherries are rich in phenolic compounds like hydroxycinnamic acids, anthocyanins and flavan-3ols (Chaovanalikit, 2003), while their composition can be influenced by ripening, industrial processing and storage (Gonçalves et al., 2004a; Gonçalves et al., 2004b; Serrano et al., 2009; Toydemir et al., 2013b). Several studies of cherries have demonstrated health properties like antioxidant activity (Jakobek et al., 2007; Haddad et al., 2013), a decrease of oxidative stress after intake of sour cherry juice (Traustadóttir et al., 2009), as well as benefits against muscle pain (Kuehl et al., 2010), insomnia (Garrido et al., 2010; Pigeon et al., 2010; Howatson et al., 2012), and an inhibitory effect on colon cancer (Kang et al., 2003; Bobe et al., 2006).

2.2.4 Black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.)

Carrots (*Daucus carota*) are root vegetables, and are cultivated in tropical and subtropical areas. The carrots and turnips production was around 37 million tons in 2013 with China being the main producer. In addition, their production has shown an annual growth rate of approximately 3.58% as reported by FAO. Currently the most popular carrot is orange, but there are also white, yellow, red and purple carrot varieties. With exception of white carrots, all colored carrots present a carotenoid profile (Surles et al., 2004). Orange carrots contain α- and β-carotene, while yellow carrots present lutein. The red carrots are characterized by lycopene, while purple-yellow and purple-orange contain particularly β-carotene and anthocyanins (Arscott and Tanumihardjo, 2010). Based on their roots and foliages, there are two principal types of cultivated carrots: the eastern or anthocyanin carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) with branched roots, which are purple or yellow, and the western or carotene carrots (*Daucus carota* ssp. *sativus* var. *sativus*) with unbranched roots that are orange, red or white (Hanelt, 2001; Stolarczyk and Janick, 2011). The origin and domestication of eastern carrots is from Afghanistan (Mackevic, 1929). Between the 11th and 14th century, they spread to Mediterranean countries, then between the 14th and 17th century to Asia (Banga, 1957). On the other hand, western carrots originated from Turkey and showed up between the 16th and 17th century by combination of yellows forms (Stolarczyk and Janick, 2011). Nowadays, black carrots are cultivated in Turkey and Middle East countries and their consumer demand is increasing mainly as source of natural food colorants (Kammerer et al., 2004b). Furthermore, black carrots have anthocyanins including acylated anthocyanins, which improved their stability in comparison with non-acylated anthocyanins (Malien-Aubert et al., 2001), as well as diverse phenolic acids such as hydroxycinnamic acid derivatives (Kammerer et al., 2004a). The interest in black carrots is growing because several studies have demonstrated their antioxidant activity (Stintzing et al., 2002a; Leja et al., 2013; Koley et al., 2014), as well as an antiprofileration activity of black carrot anthocyanins on human colon cancer cells (HT-29) (Sevimli-Gur et al., 2013), leukaemia (HL-60) (Netzel et al., 2007), breast (MDA-MB-231) and prostate (PC-3) (Sevimli-Gur et al., 2013).

In this work, black carrots cultivated in Germany were used, especially cultivars Antonina, Deep Purple, Purple Haze and Purple Sun (Fig. 2.30). Deep Purple carrots are extremely dark, while Antonina carrots are more yellow inside. In contrast, Purple Haze and Purple Sun have an orange inner flesh.

Fig. 2.30. Black carrots cultivars: Antonina, Deep Purple, Purple Haze and Purple Sun (From left to right).

2.2.5 Purple sweet potato (*Ipomoea batatas* L.)

Sweet potatoes (*Ipomoea batatas*) are storage roots from the family and genus *Convolvulaceae* and *Ipomoea*, respectively, which are often confused with potatoes (*Solanum tuberosum*) or yams (*Dioscorea sp.*) (Loebenstein and Thottappilly, 2009). The sweet potato originated from Central and South America, and spread to tropical and subtropical zones (Woolfe, 1992; Huaccho and Hijmans, 2000). They are an important basic commodity, after sugar cane, maize, rice, wheat, potatoes and cassava, especially in Asia and Africa. According to FAO, the world production in 2014 of sweet potatoes was 104,453,966 tons with Asia, in particular China, being the principal producer (75.3%), followed by Africa (20.2%). On the other hand, sweet potatoes are a source of starch, sugars, dietary minerals like calcium, magnesium, potassium and sodium, vitamins such as vitamin A, thiamin, riboflavin, niacin, B6, B12 and C (Burri, 2011; Mohanraj and Sivasankar, 2014; USDA, 2015). Depending on carotene or anthocyanin content, there are diverse sweet potato varieties with yellow, white, orange or purple flesh (Teow et al., 2007; Bradshaw, 2010; Cuevas Montilla et al., 2011). In this thesis, purple sweet potatoes (PSP) were analyzed due to the anthocyanin content (Fig. 2.31).

Fig. 2.31. Purple Sweet Potato from China (left and in the middle) and the distribution of anthocyanin pigmentation in sweet potatoes (right) by Huamán (1992).

Depending on the variety of sweet potatoes, the distribution of anthocyanin pigments can vary, as shown in Fig. 2.31, right. For example they can be present in the cortex or in the flesh area as scattered spots, diverse rings, covering all or most of the flesh (Huamán, 1992). PSP are consumed as fresh or fermented juice, alcoholic beverages and jams. In addition, PSP are associated with natural health-promoting compounds like phenolic acids and anthocyanins (Truong et al., 2007; Zhu et al., 2010), specially acylated anthocyanins that are more stable than non-acylated anthocyanins (Cevallos-Casals and Cisneros-Zevallos, 2004). Several studies using PSP have demonstrated biological activities like radical-scavenging (Oki et al., 2003; Philpott et al., 2004; Teow et al., 2007), anti-inflamatory (Zhang et al., 2009; Wang et al., 2010), antiproliferative effects on cancer cells (Konczak-Islam et al., 2003; Lim et al., 2013; Sugata et al., 2015), anti-diabetic (Zhao et al., 2013), effect on the uric acid level (Hwa et al., 2011; Zhang et al., 2015), hepatoprotective (Choi et al., 2009; Choi et al., 2011; Hwang et al., 2011) and neuroprotective effects (Cho et al., 2003; Wu et al., 2008; Lu et al., 2010; Sasaki et al., 2013).

2.2.6 Roselle (*Hibiscus sabdariffa* L.)

Fig. 2.32. Roselle plant from Mexico (left and in the middle, by R. René Rodríguez Arzaba) and its dark red calyces.

Roselle (Fig. 2.32), also known as Jamaica tea flower in Latin America, belongs to *Malvaceae* family and *Hibiscus* genus which originate from Africa (Lim, 2014). It is an annual shrub with branches, which can grow up to 1-3 m in height. In addition, it is cultivated in hot climate, particularly in Mexico, Egypt, Iran, India, Philippines, Malaysia, Thailand, Taiwan, Nigeria and West African countries (Farooqi and Sreeramu, 2004; Lim, 2014). Their steams are used to produce fiber as a substitute for jute (Singhaa and Thakura, 2008) but the most consumed parts are their calyces. *Hibiscus sabdariffa* L. calyces are traditionally exposed to sunlight or using drying systems in order to be dehydrated (Meza-Jiménez et al., 2009), then they are used to make beverages, specially "agua de Jamaica", which is a cold and dark red beverage made of water extracts from Roselle calyces, sweetening it with sugar and is very popular in Latin America. Other uses are as tea, syrups, liquors, jellies, desserts, wine and as food colorant (Ismail et al., 2008; Bolade et al., 2009; Lim, 2014).

Hibiscus sabdariffa contains volatile compounds, principally linalool and α-terpineol (Pino et al., 2006). Furthermore, it is a source of polysaccharides, organic acids like hibiscus and citric acids. Anthocyanins and other flavonoids, which give its intensive dark red color, are also present (Palé et al., 2004; Segura-Carretero et al., 2008; Rodríguez-Medina et al., 2009; Obouayeba et al., 2014).

In vitro and *in vivo* studies using *Hibiscus sabdariffa* have demonstrated cardioprotective, anti-hypertension and hypolipidemic effects (Herrera-Arellano et al., 2007; McKay et al., 2010; Hopkins et al., 2013). Moreover, *Hibiscus sabdariffa* produces a reduction of salt output in the urine after intake (Kirdpon et al., 1994) and possess antimicrobial (Jung et al., 2013; Higginbotham et al., 2014), and antioxidant activity (Tsai et al., 2002; Galicia-Flores et al., 2008; Cisse et al., 2012; Frank et al., 2012a) and has contributed to anticarcinogenic properties in leukemia (Chang et al., 2005; Hou et al., 2005a; Lin et al., 2007; Tsai et al., 2014) and gastric carcinoma cells (Lin et al., 2007). Its protective properties have been summarized in diverse reviews (Ali et al., 2005; Lin et al., 2011; Da-Costa-Rocha et al., 2014; Guardiola and Mach, 2014; Mahadevan et al., 2014; Cid-Ortega and Guerrero-Beltrán, 2015).

2.2.7 Butterfly pea (*Clitoria ternatea* L.)

Fig. 2.33. *Clitoria ternatea* L.: seeds (left), flowers (in the middle) and dried flowers (right).

Clitoria ternatea is a leguminous plant with bluish purple flowers (Fig. 2.33) belonging to the *Fabaceae* family. It is native to Asia but some authors have assigned its origin to Ternate Island, Indonesia (Gupta et al., 2010). *C. ternatea* grows in tropical and subtropical countries but primarily in Southeast Asia, India and China. It is also called Butterfly pea, Blue pea, Asian pigeonwings, Cordofan pea, Aparajita and Conch flower. The plant of *C. ternatea* can grow up to 3 m in height with pinnate leaves from 3 to 5 cm long and yields flat pods, 6-12 cm long, with up to 10 seeds. Its blue flowers with yellow tones are 3 cm wide and 2-4 cm long. Its roots, seed and leaves are frequently used in the Ayurvedic medicine (Mukherjee et al., 2008) but its flowers are utilized as food colorant (Terahara et al., 1998). For example extracts of *C. ternatea* are added to rice or are used to prepare blue and purple-red drinks like “nam dok anchan” which is a typical drink in Thailand.

C. ternatea flowers contain acylated anthocyanins, which are characterized for its high stability and are known as “Ternatins”. The ternatins are based on delphinidin 3,3’,5’-tri-*O*-β-glucoside and are classified in A, B, C and D groups. The simplest form of ternatin is Ternatin C5 and the largest form is Ternatin A1 with a molecular weight of 2108.8 g/mol. Ternatins are responsible for the blue coloration of its petals (Kondo et al., 1990; Terahara et al., 1990; Terahara et al., 1998; Kazuma et al., 2004). Moreover, diverse flavonols glycoside such as the glycosides of kaempferol, myricetin and quercetin were also found in *C. ternatea* flowers (Kazuma et al., 2003; Hassan et al., 2014). Consequently, *C. ternatea* is a potential source of anthocyanins. For this reason, flowers of *C. ternatea* from Thailand were used for the isolation of anthocyanins and copigments in this research.

In addition, *C. ternatea* flowers have been associated with medicinal properties and have shown antioxidant activity in an evaluation of 15 red and blue flowers, as well as a high anthocyanin concentration in relation to red flowers (Vankar and Srivastava, 2010). *In vitro* studies with anthocyanins of *C. ternatea* showed a platelet aggregation inhibitory activity in rabbits (Honda et al., 1991), a positive effect on hyperglycemia and oxidative stress (Sharma and Majumder, 1990; Talpate et al., 2013), as well as an anti-inflammatory activity (Suganya et al., 2014).

2.3 Preparative techniques for separation

2.3.1 Countercurrent Chromatography

Countercurrent chromatography (CCC) is a liquid-liquid partition chromatography using two immiscible solvent phases in which one act as stationary phase and the other one as mobile phase. Furthermore, this technique is based on Nernst's distribution law in which a distribution of solute between immiscible solvents can be observed. In contrast to liquid chromatography (LC), CCC does not employ any solid support as stationary phase and as a result, it avoids the irreversible adsorption on the stationary phase. Other advantages of CCC are that it can separate compounds in high purity and quantity, as well as it shows a good resolution and reproducibility (Conway, 1990; Ito and Conway, 1996). CCC systems can perform in two modes: hydrostatic equilibrium system (HSES) and hydrodynamic equilibrium system (HDES), as shown in Fig. 2.34.

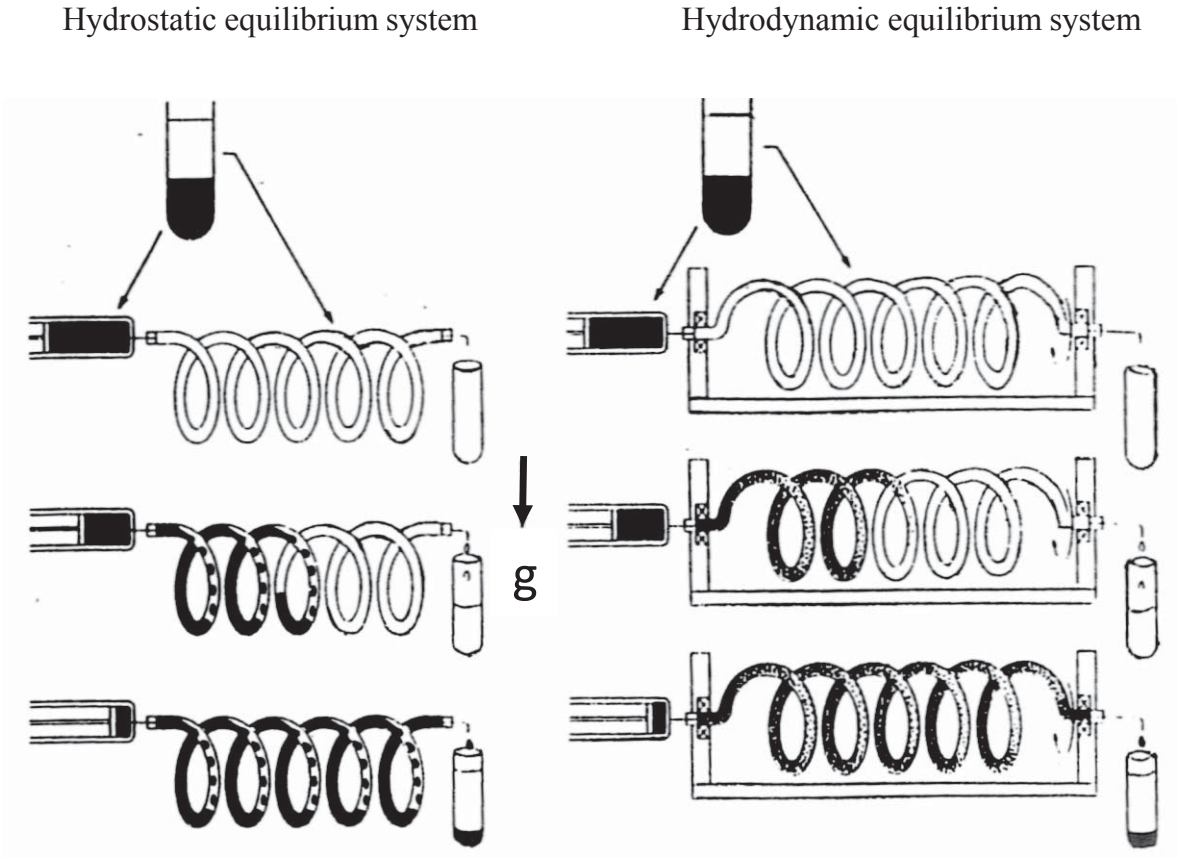


Fig. 2.34. Basic model systems for CCC: hydrostatic (left) and hydrodynamic (right) equilibrium systems (Ito and Conway, 1986).

Hydrostatic Principle: In this case, fictitious forces such as gravitational and centrifugal forces can retain the stationary phase. Droplet CCC and Rotation Locular CCC by gravitation force or Centrifugal Partition Chromatography, Gyration Locular CCC and Toroidal-Coil CCC by centrifugal force are all examples of this principle. The hydrodynamic equilibrium system uses a stationary coiled tube that is filled with one phase of a two-phase solvent system then the other phase is introduced at one end of the

coil, flows to the first phase on the front side of coil, then combines and goes up the coil to the next turn. This procedure is repeated constantly leaving stationary portions of the first phase in all coils. The introduced solute is separated according to their partition coefficients (Ito and Conway, 1984).

Hydrodynamic Principle: This principle is similarly to hydrostatic principle but the difference is that the coiled tube is moved under slow rotation around its axis (Fig. 2.34, right) based on Archimedes' screw force. The mobile phase is introduced at the head of the coiled tube, then flows and mixes with the stationary phase and the hydrodynamic equilibrium occurs. The half of stationary phase is retained in the coiled tube while continuously mixed with the mobile phase. Consequently, there are more efficient partition processes (Ito and Conway, 1984).

CCC techniques has been developed over the years and according to the type and motion of coil, various types of instruments had emerged. For example, Fig. 2.35 shows the development of High Speed Countercurrent Chromatography, which has previously be called "Multi-layer coil CCC".

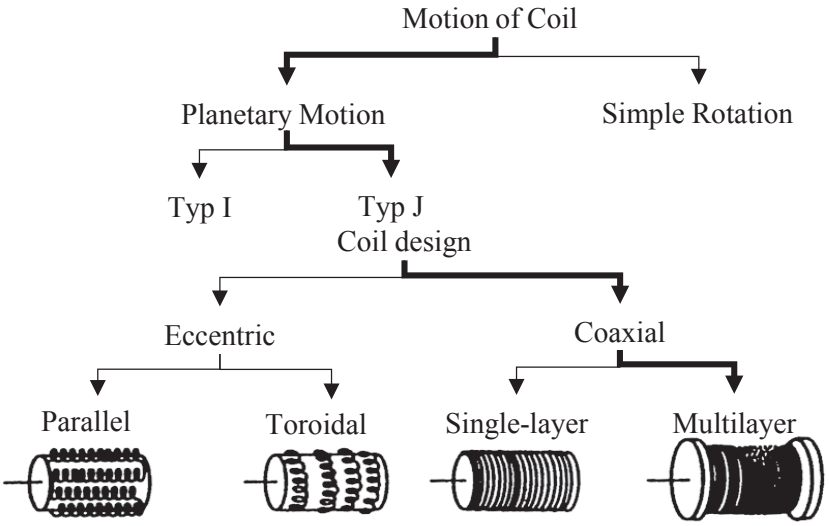


Fig. 2.35. Search for the HSCCC (Ito and Conway, 1986).

Moreover, there is an established terminology to understand the possible CCC modes. The beginning of coiled tube, which is in the same direction as the rotation, is known as head (H) and the end is called tail (T) (Ito, 1984). For instance, Fig. 2.36 shows the head and tail in helical coils or columns.

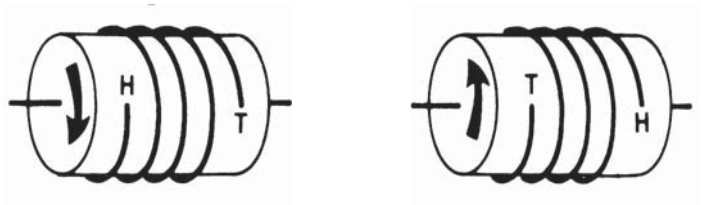


Fig. 2.36. Head (H) to Tail (T) orientations of helical column in synchronous planetary motion. They are determined by the handedness of the coil, right or left (Ito, 1984).

On the one hand, Fig. 2.37 shows the distribution of the two solvents phases in a rotating coil. If the hydrodynamic equilibrium system (HDES) uses a slow rotation of the coil it is called basic HDES, whereas using a critical rate is called unilateral HDES (Ito and Conway, 1986).

When a closed coil contains two immiscible phases in equal volume in the case of basic HDES (Fig. 2.37, A), each phase is distributed around half space of the coil. In the case of basic HDES, either phase is eluted from head to tail causing a decrease of the other phase in the coil (Fig. 2.37, B). If the flow rate of the mobile phase is increased, the retention of the stationary phase decreases in the coil. Consequently, this low retention of stationary phase limits the use of high flow of mobile phase (Ito and Conway, 1986). On the other hand, in the case of unilateral HDES two solvent phases are separated in a different way in a closed coil (Fig. 2.37, C). One phase (head phase) occupies the head side and the other one (tail phase) occupies the tail side of the coil. If the tail phase (lower phase) is introduced at the head, it will move to the tail. Similarly, if the head phase (upper phase) is introduced at the tail side, it will move to the head. Therefore, there are two possible elution modes (Fig. 2.37, D): First, the normal elution mode or "Head to Tail" in which the coil is completely filled with the head phase and then the tail phase is pumped through the head side. The second mode is called reversed elution mode or "Tail to Head" in which the coil is totally filled with the tail phase then the head phase is pumped through tail side. The retention of stationary phase in the coil is high in both cases (Ito and Conway, 1984).

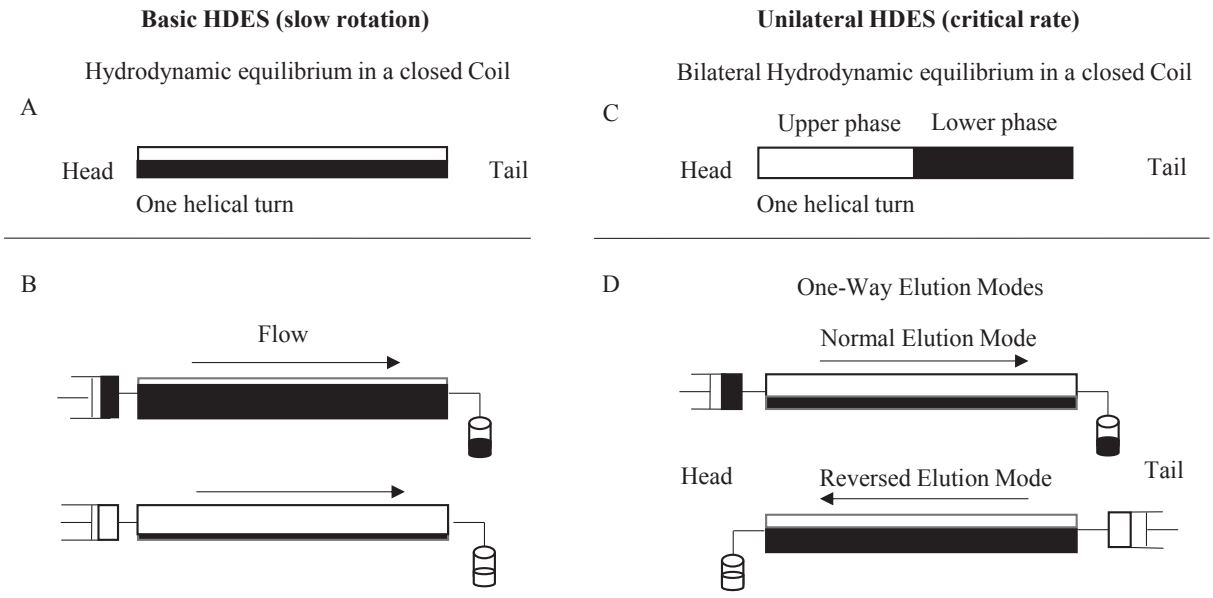


Fig. 2.37. Principle unilateral hydrodynamic equilibrium system (Ito and Conway, 1986).

Other way to eluate all the injected analytes from the column is the use of Elution-Extrusion mode. In the elution, some analytes are eluted from the coil by mobile phase, while other analytes are retained in the stationary phase. The stationary phase can be extruded out of the coil using stationary phase (Berthod et al., 2003). The efficiency of CCC separations can be influenced by many factors such as selection of two-phase solvent system, elution mode, stationary phase retention, solution of sample, flow rate and revolution speed (Ito and Conway, 1996).

2.3.1.1 High-Speed Countercurrent Chromatography (HSCCC)

High-Speed Countercurrent Chromatography is a CCC system using a multilayer coil column in which its tubing are winded by coaxially form around a spool shaped holder doing multiple coiled layers (see Fig. 2.35). Additionally, it is subjected to type J coil synchronous planetary motion (Fig. 2.38) in which the coil rotates around its axis and rotates around the centrifuge axis at the same angular velocity (ω) and direction. Using one or more coils and depending on size tubing, HSCCC can be used on analytical, semipreparative or preparative scale. In this work, a preparative HSCCC with triple coil was used.

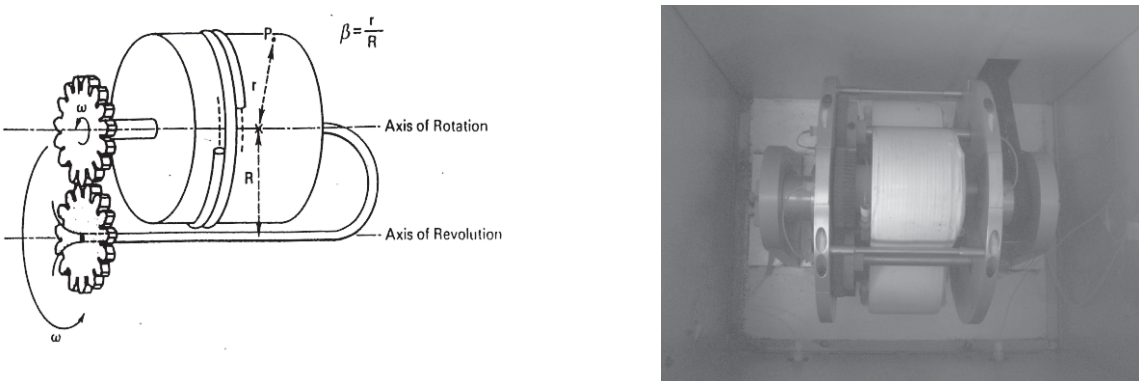


Fig. 2.38. Single-coil design (left) (Ito and Conway, 1986) and HSCCC with multilayer triple-coils (right).

HSCCC system includes preparative pump, an injection valve with loop, CCC column or coil, detector for monitoring of eluates and fraction collector as shown in Fig. 2.39.

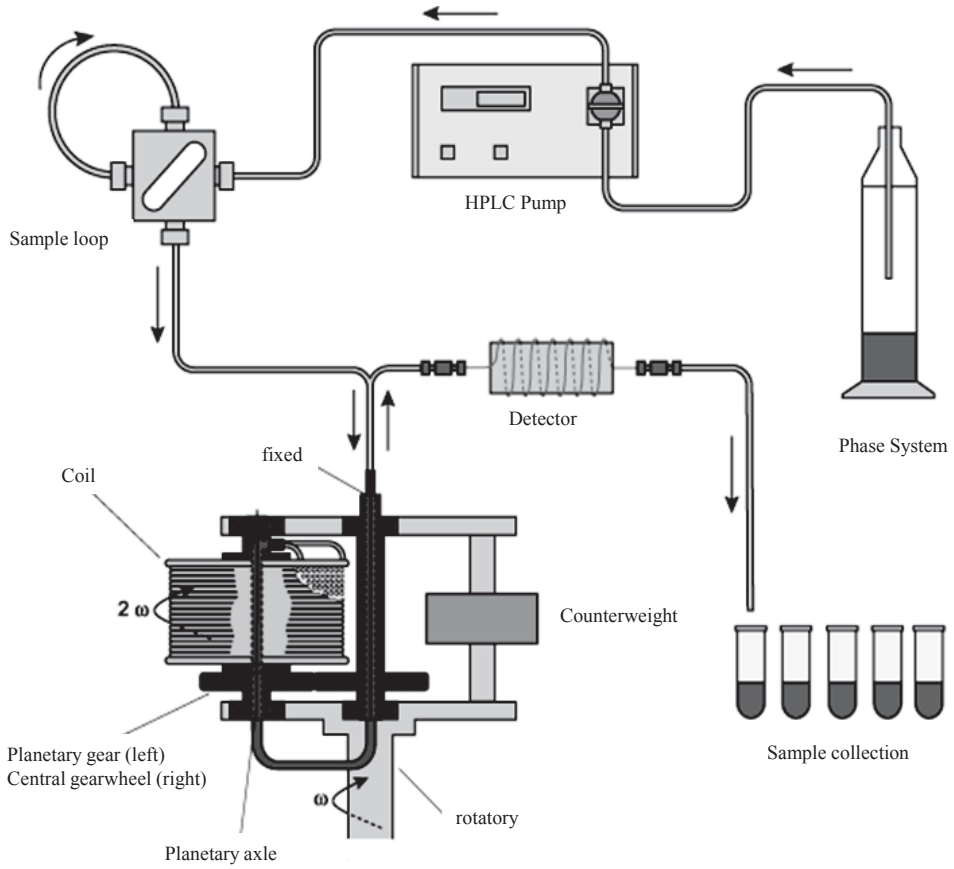


Fig. 2.39. Single-Coil CCC system (modified from Sutherland, 1987).

For the appropriate two-phase solvent system with a variation of polarity, several combinations have been developed in diverse scales, such as the "Oka" (mixtures of n-hexane-EtOAc-n-BuOH-MeOH-water), the "HBAW" (mixtures of n-heptane-n-butanol-ACN-water), the "Arizona" (mixtures of n-heptane-EtOAC-MeOH-water), acetone-based solvents (n-heptane-toluene-acetone-water), "ChMWat" (mixtures of chloroform-methanol-water) and "HEMWat" (hexane-EtOAc-MeOH-water) (Renault et al., 2002). Ito (2005) had suggested some rules to select good condition for HSCCC and presented a similar solvent system scale based on mixture of hexane-EtOAc-MeOH-n-BuOH-water and polar series of tBME-n-BuOH-ACN-water. The solvent system should be evaluated in order to meet some requirements. For instance, the stability and solubility of analyte(s) in solvent system, the formation of two phases with adequate volume ratios, the acceptable retention of stationary phase (between 50 and 90%), as well as an appropriate partition coefficient (K) of analytes.

$$K_{U/L} = \frac{C_U}{C_L}$$

K = Partition coefficient
C_U = solute concentration in the upper phase
C_L = solute concentration in the lower phase

If analytes are soluble in mobile phase, the partition coefficient is ≤ 1, whereas if they are soluble in stationary phase, K is ≥ 1. The K value should be between 0.5 and 1 for a good CCC separation. If the K value is higher than 1, the separation will require long time (Ito, 2005).

2.3.1.2 High Performance Countercurrent Chromatography (HPCCC)

HPCCC technique is a current development of CCC methods based on liquid-liquid partitioning with a J-type coil which is rotated in a planetary motion. Nevertheless, its separations are carried out in a lower volume column and in shorter time than in HSCCC.

It has been demonstrated that the g-level parameter (g) can influence the stationary phase retention and separation performance. While HSCCC creates many times the acceleration of the gravity of earth (54-80 x g) and works with a speed rotation between 750-1000 rpm, HPCCC separations are performed at 1600 rpm (240 x g) thus reducing the separation time (Guzlek et al., 2009; Wei et al., 2011). Moreover, HPCCC instruments can also be used in analytical, semi-preparative and preparative scale (Guzlek et al., 2009; Wei et al., 2011). For separations of black chokeberry and butterfly pea, we used a Spectrum-HPCCC instrument (Fig. 2.40).

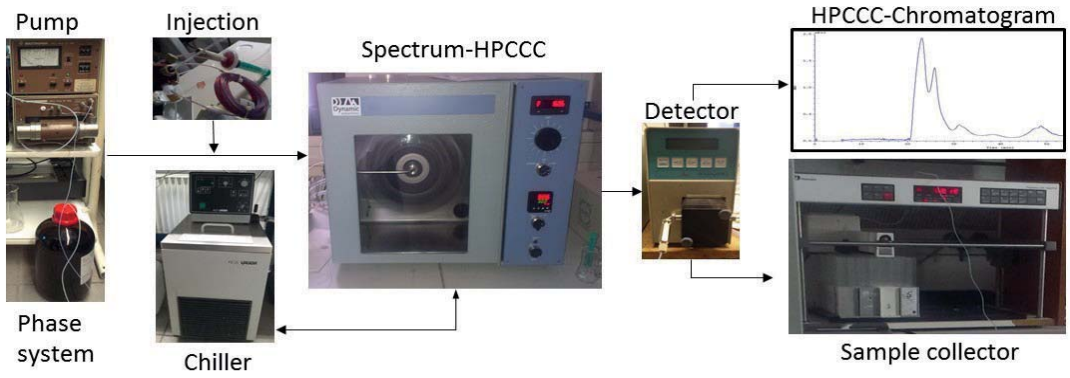


Fig. 2.40. Spectrum HPCCC system which can be used in analytical or semipreparative scale.

2.3.1.3 Low-Speed Rotary Countercurrent Chromatography (LSRCCC)

In the development of CCC systems, a variety of prototypes emerged for large-scale separations. Du et al. (2000) developed a slow speed rotary countercurrent chromatography apparatus with a 10 L column and separated 150 g of tea extract obtaining 40 g of epigallocatechin gallate with a purity of 92.7%. Du et al. (2005) furthermore separated 400 g of crude extract of *S. armeniacae* by slow rotary countercurrent chromatography with a 40 L column yielding 221 g of amygdalin (94% purity).

Low-Speed Rotary Countercurrent Chromatography is based on a hydrodynamic equilibrium and uses a multilayer coil column (Fig. 2.41). In LSRCCC systems, the use of convoluted tubing is required because it provides higher retentions of stationary phase than the standard wall tubing (Du et al., 2000). In addition, eight elution modes are possible according to the choice of three factors: first, the selection of mobile phase, lower phase (L) or upper phase (U), second, the rotation direction, Tail to Head (T) or Head to Tail (H) and third the selection of flow direction, from inner to outer (I) or from outer to inner (O). The modes are lower phase pumping from the inner tail terminal (L-I-T), lower phase pumping from the inner head terminal (L-I-H), upper phase pumping from the outer tail terminal (U-O-T), upper phase pumping from the outer head terminal (U-O-H), lower phase pumping from the outer tail terminal (L-O-T), lower phase pumping from the outer head terminal (L-O-H), upper phase pumping from the inner tail terminal (U-I-T) and upper phase pumping from the inner head terminal (U-I-H).
On the other hand, preliminary studies have shown that L-I-T, L-I-H, U-O-T and U-O-H presented a good retention of stationary phase, while L-O-T, L-O-H, U-I-T and U-I-H had a lower retention of stationary phase (Ito et al., 2003; Köhler, 2006).

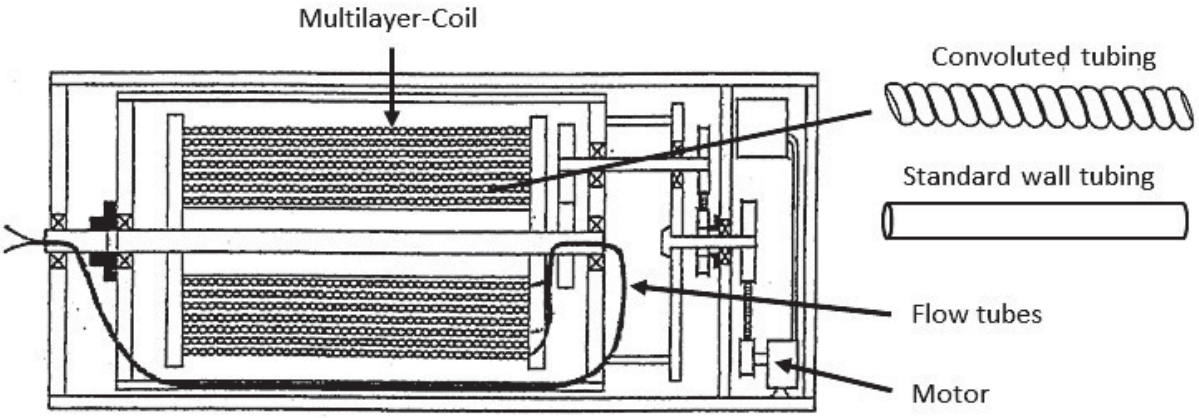


Fig. 2.41. Low-Speed Rotary Countercurrent Chromatography system (Du et al., 2000).

In the present study, a LSRCCC Prototype (Pharma-Tech Research Corp., Baltimore, MD, USA) with a column capacity of 5.5 L was used. The convoluted tubing had a diameter of 8.5 mm and was 107 m long. This apparatus consists of a single coil that can rotate around its axis at a slow speed between 0 and 100 rpm. Because of its large volume capacity, it is possible to use higher amounts of sample than in HSCCC.

2.3.2 Membrane chromatography

Over the past few years, the use of membrane chromatography or membrane adsorbers in separation and purification processes is expected to continue growing in contrast to traditional column chromatographic techniques because of its significant advantages such as its simple operation, short processing time, easy scale up, automation, as well as the high throughput (Warner and Nochumson, 2002). In contrast, packed bed columns depend on the diffusion of molecules into the pores of beads and have limited flow, which has an influence on the binding capacity. In large scale, a large bed volume is required for a high product throughput and they involve more packed bed and high cost (Lim et al., 2007). Furthermore, packed bed columns present a lower binding capacity for larger molecules like DNA, proteins and virus (Warner and Nochumson, 2002).

Membrane chromatography, in particular, contains microporous membranes with functional ligands attached to their surface and is characterized by the lack of pore diffusion. This absence produces lower pressure drops and the dynamic binding capacity of membrane adsorbers can be many times greater than in conventional packed bed chromatography. Consequently, it can use faster flow rates and its processing time can be reduced (Zeng and Ruckenstein, 1999). According to their functional group, they are either available such as anion exchange (e.g. quaternary ammonium), cation exchange (e.g. sulfonic acid), affinity (e.g. protein-A) or hydrophobic interactions (e.g. polyvinylidene fluoride). Currently, there are a diverse membrane adsorbers based on ion-exchange, affinity, hydrophobic interactions, reversed phase and multistage chromatography available (Charcosset, 1998).

In the biopharmaceutical industry, these membrane adsorbers have been developed for small scale and industrial scale applications due to the reduction of large process volume, rapid and simplified isolation. Moreover, membrane chromatography has already been used for the purification and concentration of proteins, viruses, viral particles, monoclonal antibodies and oligonucleotides (Zeng and Ruckenstein, 1999; Zhou and Tressel, 2005; Giovannoni et al., 2008; Ghosh et al., 2014; Madadkar and Ghosh, 2016).

Juadjur and Winterhalter (2012) applied membrane chromatography to separate the anthocyanins of bilberry from copigments on a preparative scale based on ion-exchange chromatography principle, particularly cation exchange chromatography, where ionic compounds are retained by the stationary phase. Conversion of anthocyanins into positively charged flavylium cations by acidification is possible (see section 2.1.1.2.5 and Fig. 2.12). Using a cation exchange membrane adsorber, the anthocyanin flavylium cations are retained on the membrane adsorber surface, whereas compounds, which are not positively charged, are not retained and pass the membrane unretained. In this way, anthocyanins can be separated selectively from other polyphenols. This procedure includes adsorption, washing, elution and regeneration steps. After membrane chromatography, an isolation of 3.71 g of pure anthocyanin fraction from 10 g bilberry extract was obtained.

In this work, adsorptive membrane chromatography was applied to separate the anthocyanins of diverse foods from other polyphenols on a preparative scale. The membrane chromatography system

(Fig. 2.42) requires a peristaltic pump to inject the solvents or sample. As pre-column, a filter capsule Sartopore 2300 with a double layer membrane from Sartorius Stedim Biotech (Göttingen, Germany) was used in order to protect the membrane adsorber. The pre-column was connected with a Sartobind S IEX 150 mL membrane adsorber from Sartorius Stedim Biotech (Göttingen, Germany). Sartobind S IEX 150 mL membrane adsorber is a strong acidic cation exchanger with sulfonic acid groups ($R\text{-}CH_2\text{-}SO_3^-$), binding capacity 3.9 g, 8 mm bed height and a membrane area of 5500 cm^2. The stabilized cellulose membrane adsorber contains a net-like structure (Fig. 2.43).

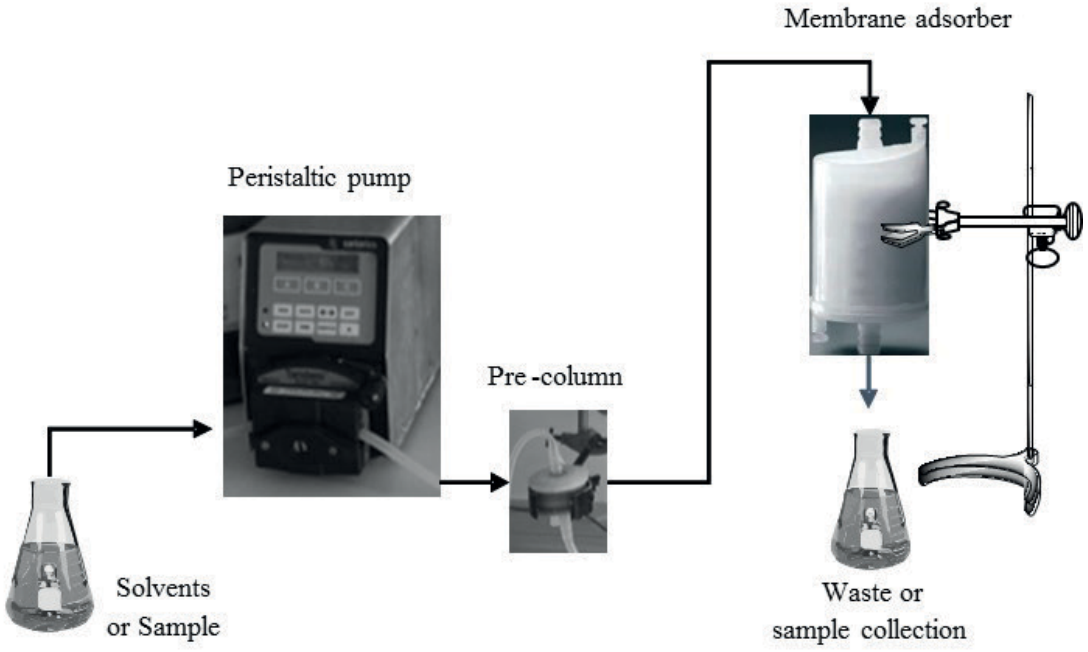


Fig. 2.42. Membrane chromatography system.

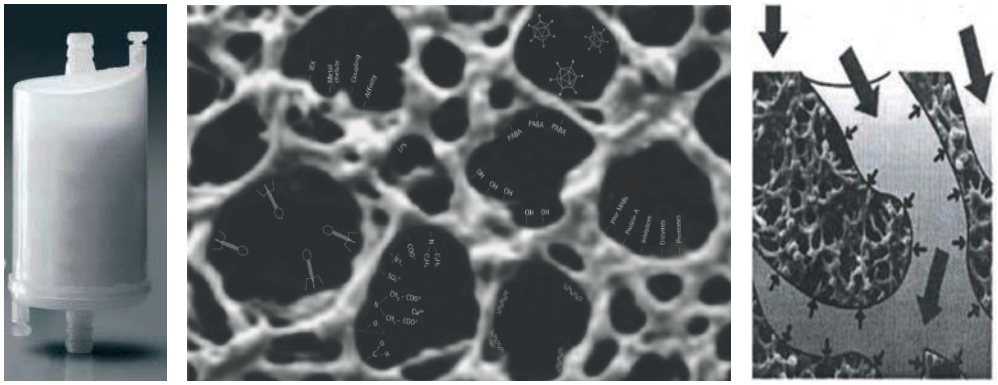


Fig. 2.43. Sartobin S IEX 150 mL membrane adsorber capsule (left), microporous structure with pore size 0.45-3µm (at the middle) and flow path (right) (Source: Sartorius AG, Göttingen, Germany).

An advantage of this technique is that it prevents the use of perflurorinated reagents such as TFA and PFPA, which are toxic, in comparison to other preparative separation applications such as CCC. Hence, the isolated compounds can be used for biological assays.

3 Results and Discussion

3.1 Working outline

Because of the association of anthocyanins with health benefits such as prevention of degenerative diseases, anthocyanins, which are found in many fruits, vegetables and flowers, have attracted more attention in recent years. Futhermore they are important natural pigments, which present a variety of color, from orange-red to blue-dark violet. The purpose of this work was to extract, characterize, separate and isolate anthocyanins from various fruits, vegetables and flowers by diverse chromatographic techniques, as shown in Fig. 3.1.

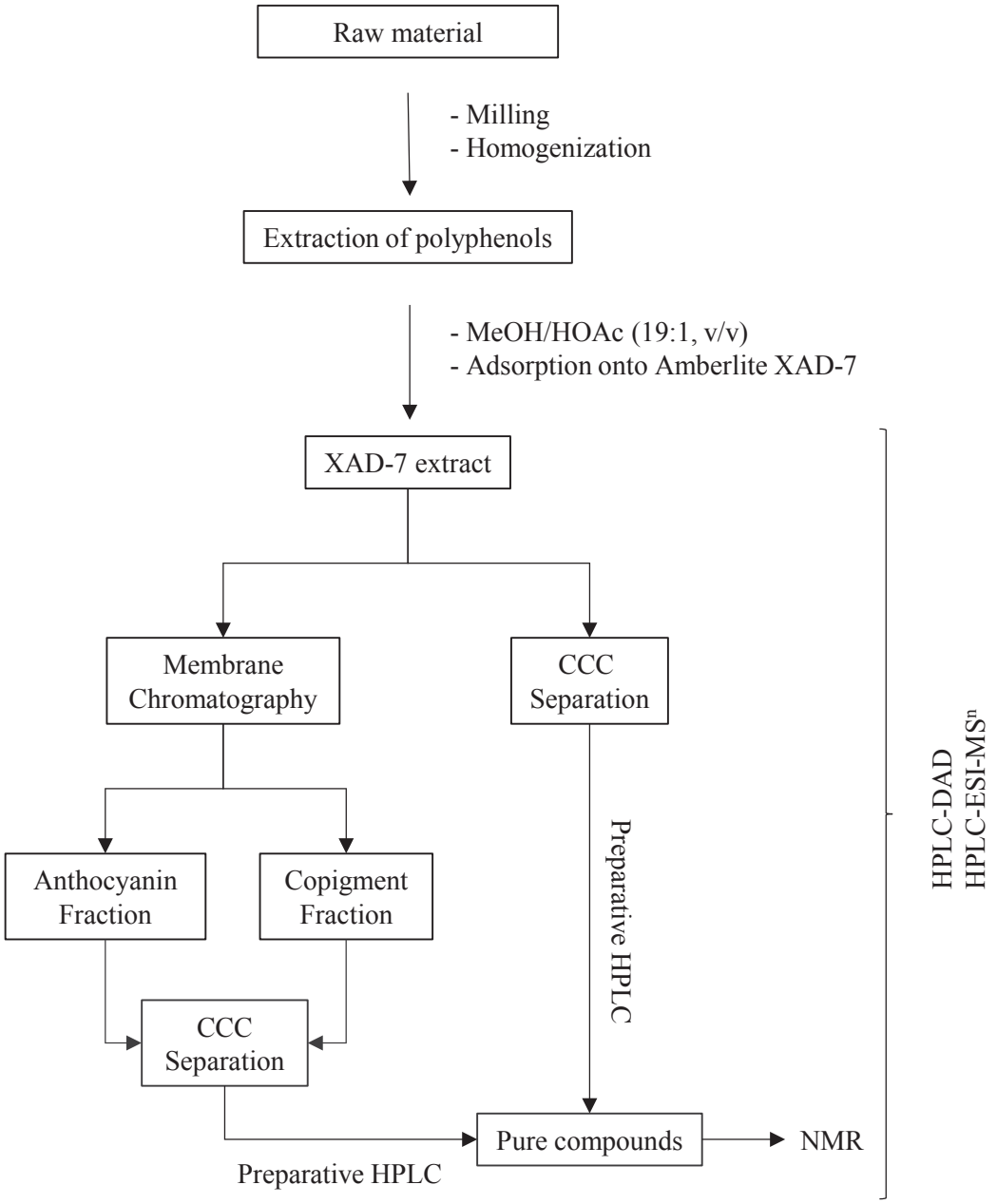


Fig. 3.1. Schema of work.

The raw material was cut into small pieces and homogenized, then extracted with methanol/acetic acid (19:1, v/v) for 8 hours. After filtration, the raw extract was concentrated in vacuo in order to evaporate the solvents after which the crude extract was applied onto an Amberlite XAD-7 column to concentrate the polyphenols and to eliminate sugars, proteins and salts. The column was then washed with water and a mixture of methanol/acetic acid (19:1, v/v) was used for the elution of polyphenols. After that, the solvents were evaporated by rotary evaporator, and the concentrated XAD-7 extract was redissolved in water and freeze-dried.

In our work, the preparative isolation of anthocyanins from XAD-7 extracts was performed by means of the application of two different chromatographic techniques: membrane chromatography and countercurrent chromatography. On the one hand, polyphenols of XAD-7 extracts were separated by CCC, but in some fractions anthocyanins were mixed with copigments. Consequently, a preparative separation by HPLC was necessary to obtain pure compounds. On the other hand, an alternative method emerged, i.e. membrane chromatography in which XAD-7 extracts are fractionated into two groups: an anthocyanin fraction, which is free of other copigments, and a copigment fraction that is free from anthocyanins. After fractionation, anthocyanin or copigment fraction were separated by CCC in order to isolate pure compounds. HPLC-DAD, HPLC-ESI-MS^n and NMR analysis were carried out for the identification and characterization of pure compounds. Moreover, the anthocyanin profiles from diverse fruits were analyzed in our study in order to check their authenticity.

3.2 Blackberry

3.2.1 Chemical composition

Blackberry is a rich source of polyphenols such as anthocyanins, ellagitannins, flavonols, procyanidins, ellagic and phenolic acids. Moreover, its black-red color is due to the presence of anthocyanins. According to anthocyanin aglycone type, fruits can be classified into categories such as cyanidin/peonidin, pelargonidin or multiple anthocyanins groups. Blackberry is assigned as a fruit of the cyanidin/peonidin group because its anthocyanin profile is mainly composed of compounds based on the cyanidin aglycone (Fang, 2015).

In this work, as shown in HPLC-chromatogram at 520 nm in Fig. 3.2 and Table 3.1, cyanidin-3-*O*-glucoside is the main pigment (around 80%, Peak 1), followed by cyanidin 3-*O*-(6"-dioxalyl-glucoside) with approximately 7 % (Peak 4). Additionally, cyanidin-3-*O*-xyloside (Peak 2) and cyanidin 3-*O*-(6"-malonyl-glucoside) (Peak 3) were found as minor pigments. Depending on the blackberry cultivars and other factors like year of cultivation, influence of climate, ripeness, processing, etc., the total anthocyanin content can vary from 70 to 326 mg/100 g berries and from 12 to 107 mg/100 g single strength juice (Fan-Chiang and Wrolstad, 2005; Kaume et al., 2012).

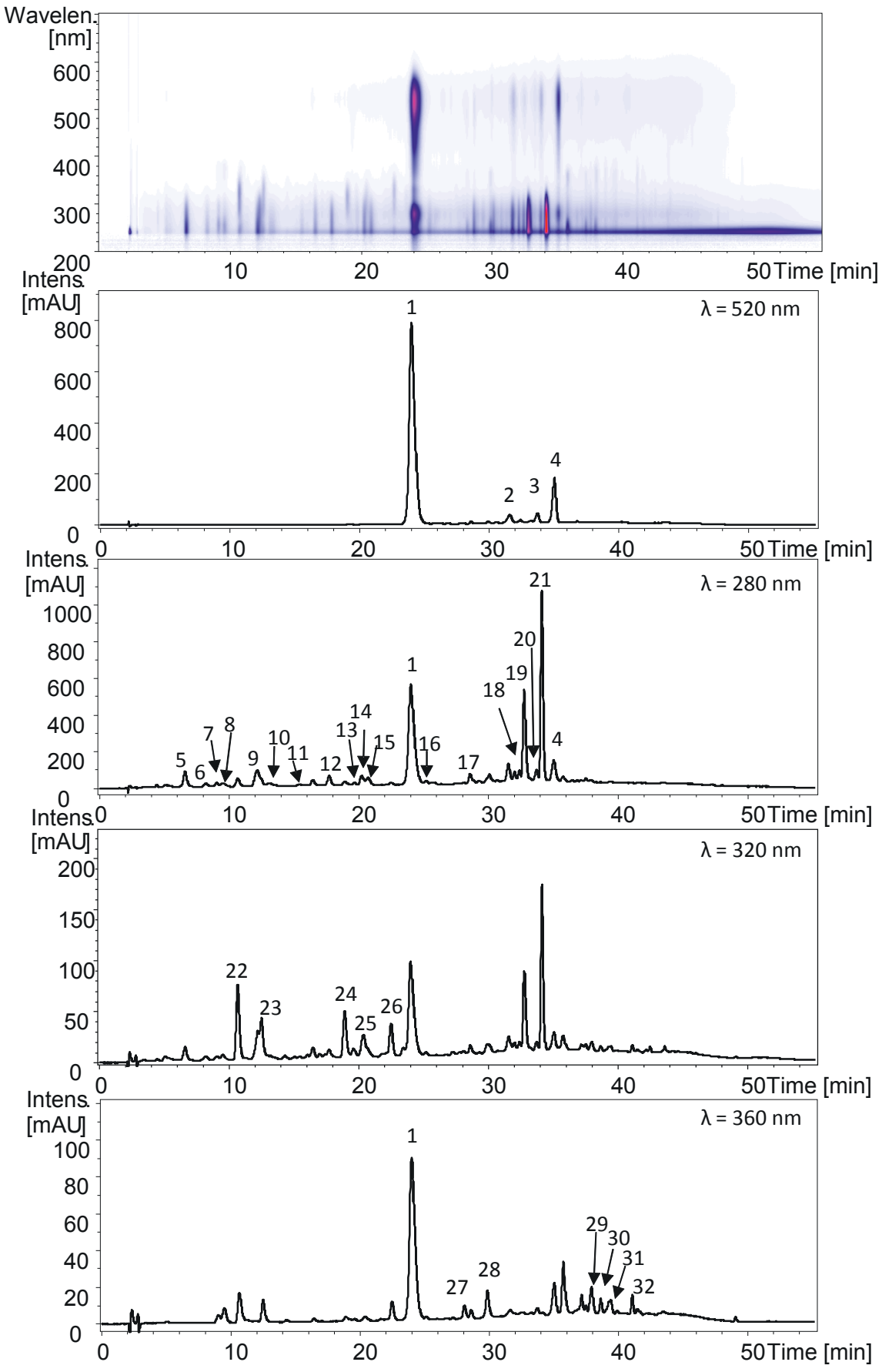


Fig. 3.2. HPLC-DAD and HPLC-Chromatograms at 520 nm, 280 nm, 320 nm and 360 nm of blackberry. Peak numbering according to Table 3.1, 3.2 and 3.3.

Table 3.1. Anthocyanin content and LC-MS data of anthocyanins in blackberry.

Peak No.	Anthocyanins	Anthocyanin content (mg Cy-3-glc/100 g)	$[M]^+$	Fragment ions (*m/z*)
1	Cy-3-*O*-glc	67.17 ± 3.26	449	287
2	Cy-3-*O*-xyl	2.68 ± 0.16	419	287
3	Cy-3-*O*-(6"-malonyl-glucoside)	1.98 ± 0.07	535	287
4	Cy-3-*O*-(6"-dioxalyl-glucoside)	5.95 ± 0.50	593	287

Peak numbering according to HPLC-chromatogram at 520 nm (Fig. 3.2)

The anthocyanin profile of blackberries depends on the cultivars but the main anthocyanin is cyanidin-3-*O*-glucoside in all varieties, while in some cases there is the presence or lack of cyanidin-3-*O*-rutinoside, and cyanidin-3-*O*-xyloside, cyanidin-3-*O*-(6"-malonyl-glucoside) and cyanidin-3-*O*-(6"-dioxalyl-glucoside) are detected as minor compounds (Stintzing et al., 2002b; Fan-Chiang and Wrolstad, 2005).

For example, Marion blackberry, which is a most important trailing blackberry, contains cyanidin-3-*O*-glucoside (70-78%) as the main pigment, followed by cyanidin-3-*O*-rutinoside (19-27%). The minor compounds were cyanidin-3-*O*-(6"-malonyl-glucoside) (≈1%), cyanidin 3-*O*-(6"-dioxalyl-glucoside) (2-3%) and only traces of cyanidin-3-*O*-xyloside. In the case of Evergreen blackberry, all the five anthocyanins are present, but their quantity varies: cyanidin-3-*O*-glucoside (80-83%), cyanidin-3-*O*-rutinoside (3-4%), cyanidin-3-*O*-xyloside (7%), cyanidin-3-*O*-(6"-malonyl-glucoside) (4-5%) and cyanidin-3-*O*-(6"-dioxalyl-glucoside) (3-4%). However, Kotata blackberry are characterized by the presence of only two anthocyanins, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, in addition this variety presented the highest cyanidin-3-*O*-glucoside content (90-95%) in all blackberry varieties, while cyanidin-3-*O*-rutinoside content was between 5 and 10% (Fan-Chiang and Wrolstad, 2005).
On the other hand, hybrides of blackberry and raspberry like boysenberry (*Rubus ursinus* × *idaeus*) and loganberry (*Rubus loganbaccus*) are characterized by the presence of cyanidin-3-*O*-sophoroside, cyanidin-3-glucosylrutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (Mazza and Miniati, 1993; McGhie et al., 2006; Kaume et al., 2012).

One of the most distinguishing features of blackberry is the presence of ellagitannins, which are also present in some fruits such as raspberries, strawberries and pomegranate (Clifford and Scalbert, 2000; Quideau, 2009). Ellagitannins are hydrolyzable tannins, which are characterized by the 6'6-dicarbonyl-2,2',3,3',4,4'-hexahydroxybiphenyl moiety, commonly known as HHDP group.
In blackberries, Hager et al. (2008) described ellagitannins including monomeric forms (e.g. casuarictin) to polymeric forms such as sanguiin H-6 (dimer), lambertianin A (dimer), lambertianin C (trimer) and lambertianin D (tetramer). In this work, the identification of ellagitannins by HPLC-DAD-ESI was carried out and some ellagitannins were found (Table 3.2).

Table 3.2. LC-MS data of ellagitannins in blackberry.

Peak No.	t_R (min)	Compound	$[M-H]^-$	Fragments ions (*m/z*)
5	6.9	Pedunculagin isomer	783	481, 301, 275
6	8.5	Ellagitannin derivative	951	907, 783, 633, 301
7	9.2	Castalagin/vescalagin isomer	933	783, 631, 451 301
8	9.8	Castalagin/vescalagin isomer	933	783, 631, 451, 301
9	12.4	Pedunculagin isomer	783	481, 301, 275
10	13.4	Ellagitannin derivative	951	907, 783, 605, 301
11	15.7	Ellagitannin derivative	951	907, 783, 605, 301
12	17.9	Pedunculagin isomer	783	617, 469, 301
13	19.8	Galloyl-HHDP glucose isomer	633	463, 301
14	20.5	Ellagitannin derivative	1251	1235, 1100, 933, 783, 702, 633, 469
15	21.1	Ellagitannin derivative	858	783, 631, 481, 301
16	25.4	Ellagitannin derivative	951	907, 783, 605, 301
17	28.8	Pedunculagin isomer	783	633, 469, 301
18	32.5	Ellagitannin derivative	1251	1100, 1019, 935, 783, 633, 469
19	33.5	Lambertianin C isomer	1401	1251, 1100, 935, 783, 633, 469
20	33.9	Sanguiin H-2	1103	935, 633, 469, 301
21	34.4	Galloyl-bis-HHDP glucose isomer	935	898, 783, 633, 469, 301

Peak number according to HPLC-chromatogram at 280 nm (Fig. 3.2); HHDP: hexahydroxydiphenoyl

The loss of HHDP, galloyl units, galloylglucose and ellagic acid were observed. Subsequently, many ellagitannins like galloyl-HHDP glucose ($[M-H]^-$ at *m/z* 633), pedunculagin isomers ($[M-H]^-$ at *m/z* 783), castalagin/vescalagin isomers ($[M-H]^-$ at *m/z* 933), galloyl-bis-HHDP glucose ($[M-H]^-$ at *m/z* 935), sanguin H-2 ($[M-H]^-$ at *m/z* 1103) and lambertianin C ($[M-H]^-$ at *m/z* 1401) were identified in accordance with previous studies of blackberries (Hager et al., 2008; Kaume et al., 2012; Kolniak-Ostek et al., 2015). Moreover, compounds 6, 10, 11 and 16 presented $[M-H]^-$ at *m/z* 951 with fragments ions at *m/z* 907 (loss of carboxyl group, 44u), 783, 605 and 301. These compounds were also found by Hager et al. (2008) but not identified. A suggestion is that they could be trigalloyl-HHDP glucose isomers like in the case of pomegranate (Mena et al., 2012).

Other detected ellagitannins in blackberries are lambertianin D, sanguiin H-6, sanguiin H-6 without or with gallic acid moiety, sanguiin H-10 and methyl ellagic acid pentose conjugate (Hager et al., 2008; Gasperotti et al., 2010; Kaume et al., 2012).

As shown in Table 3.3 and Fig. 3.2, some phenolic acids were detected in blackberry such as neochlorogenic acid (3-CQA), as well as caffeoyl glucose that presented $[M-H]^-$ *m/z* 341 with fragments ions of *m/z* 179 and two compounds with $[M-H]^-$ *m/z* 325 and fragment ions *m/z* 145 and 163 that are identified as p-coumaroyl hexoses. A ferulic acid derivative was present with $[M-H]^-$ *m/z* 355 and fragment ion *m/z* 193 (Kolniak-Ostek et al., 2015). Additionally traces of protocatechuic acid, 3-feruloylquinic acid and some flavan-3-ols like (-)-epicatechin and procyanidin dimer B1 were observed. On the other hand, flavonols are present in blackberry, as shown in HPLC-chromatogram at 360 nm (Fig. 3.2), principally quercetin derivatives such as quercetin 3-glucuronide, quercetin 3-rhamnoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside and quercetin- 3-*O*-[6''-(3-hydroxy-3-methylglutaroyl)]-β-galactoside (Cho et al., 2004; Cho et al., 2005).

Table 3.3. Phenolic acids, flavonols and flavan-3-ols in blackberry.

Peak No.	Compound	$[M-H]^-$	Fragment ions (*m/z*)
22	3-CQA	353	191, 179, 135
23	Caffeoyl glucose	341	179, 161, 135
24	p-Coumaroyl hexose	325	163, 145
25	p-Coumaroyl hexose	325	163, 145
26	Ferulic acid derivative	355	193
*	3-Feruloylquinic acid	367	193, 173, 134
*	Protocatechuic acid	153	109
27	Quercetin 3-glucuronide	477	301
28	Quercetin 3-rhamnoside	477	301
29	Quercetin 3-*O*-rutinoside	609	463, 301
30	Quercetin 3-*O*-galactoside	463	301
31	Quercetin 3-*O*-glucoside	463	301
32	Quercetin 3-*O*-[6''-(3-hydroxy-3-methylglutaroyl)]-β-galactoside	607	463, 301
*	(-)-Epicatechin	289	245
*	Procyanidin dimer B1	577	559, 451, 425, 407, 289

*traces; Peak number according to Fig. 3.2 at 320 nm and 360 nm

Depending on the variety, blackberry can contain other flavonols like kaempferols. Kaempferol-3-*O*-rutinoside (*m/z* 593), kaempferol-3-*O*-glucuronide (*m/z* 461), kaempferol-3-*O*-glucoside-rhamnoside-7-*O*-rhamnoside (*m/z* 739) and kaempferol-3-*O*-pentoside (*m/z* 417) are present in wild blackberry (Oszmiański et al., 2015).

3.2.2 Preparative separations

3.2.2.1 Membrane chromatography

Blackberry (8 kg) were used for the extraction of anthocyanins. The blackberries were cut and blended for 3 min after which the anthocyanins were extracted according to method 1 (see section 4.3) and polyphenols were concentrated onto an Amberlite XAD-7 column (see section 4.4). After extraction, the solvents were removed by vacuum evaporation and 27.8 g XAD-7 extract of blackberry was obtained. Subsequently, part of the blackberry XAD-7 extract (10 g) was separated into an anthocyanin fraction and a copigment fraction by means of membrane chromatography (Fig. 3.3) according to Juadjur and Winterhalter (2012). At the beginning, the copigment fraction presented a yellow color and it was orange at the end due to concentration of compounds, while the anthocyanin fraction had an intensive dark-purple color.

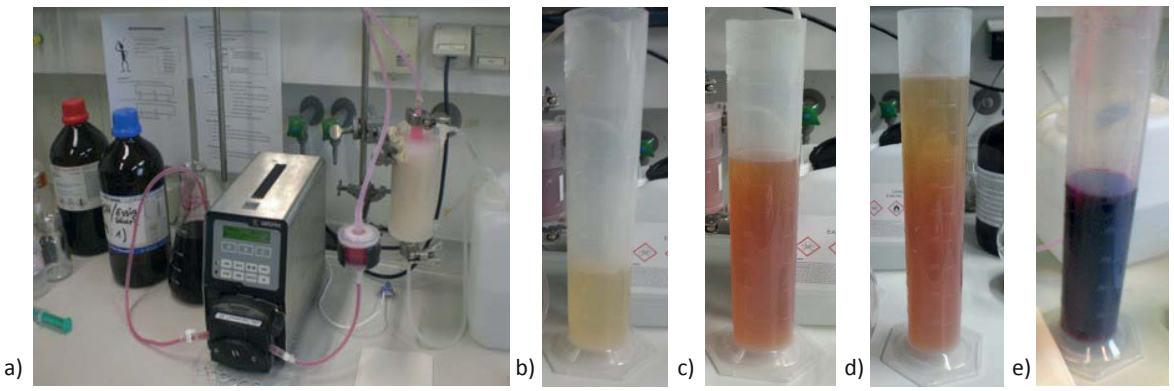


Fig. 3.3. Membrane Chromatography overview (a), fraction collection of copigments (b-d) and anthocyanins (e).

In order to monitor the separation, HPLC-DAD analyses were carried out (Fig. 3.4), as well as the monitoring of chromatograms at 280 nm and 520 nm (Fig. 3.5). It is well-known that anthocyanins have absorption maxima at 280 nm and 520 nm, while in the case of copigments absorption maxima are between 200 nm and 400 nm. Consequently, a comparison of chromatograms at 520 nm can demonstrate if there is a successful separation after membrane chromatography. In this case, a lack of anthocyanins is shown in the HPLC-chromatogram at 520 nm of the copigment fraction. In addition, high quantities of anthocyanin fraction (5.56 g) and copigment fraction (3.6 g) were obtained after separation and a polymeric fraction was eluted in small quantity (0.84 g). After membrane chromatographic separation, the concentration of compounds in each fraction, anthocyanins or copigments, was higher than in the original extract. As a result, minor compounds could be better detected after separation. In case of anthocyanin fraction, a cyanidin derivative (Peak 5, Fig. 3.5) was detected in traces in the original extract

but after separation, its concentration was higher. This compound presented $[M]^+$ *m/z* 491 and fragment ion *m/z* 287 which could be a cyanidin-3-(acetyl)hexose or cyanidin-3-(oxaly)pentose.

The membrane chromatographic method allowed a good separation into two groups of phenolic compounds, anthocyanins and copigments. Moreover, this method is appropriate for the isolation of anthocyanins or copigments on a large-scale that can be used for biological testing and to investigate biological effects of these groups.

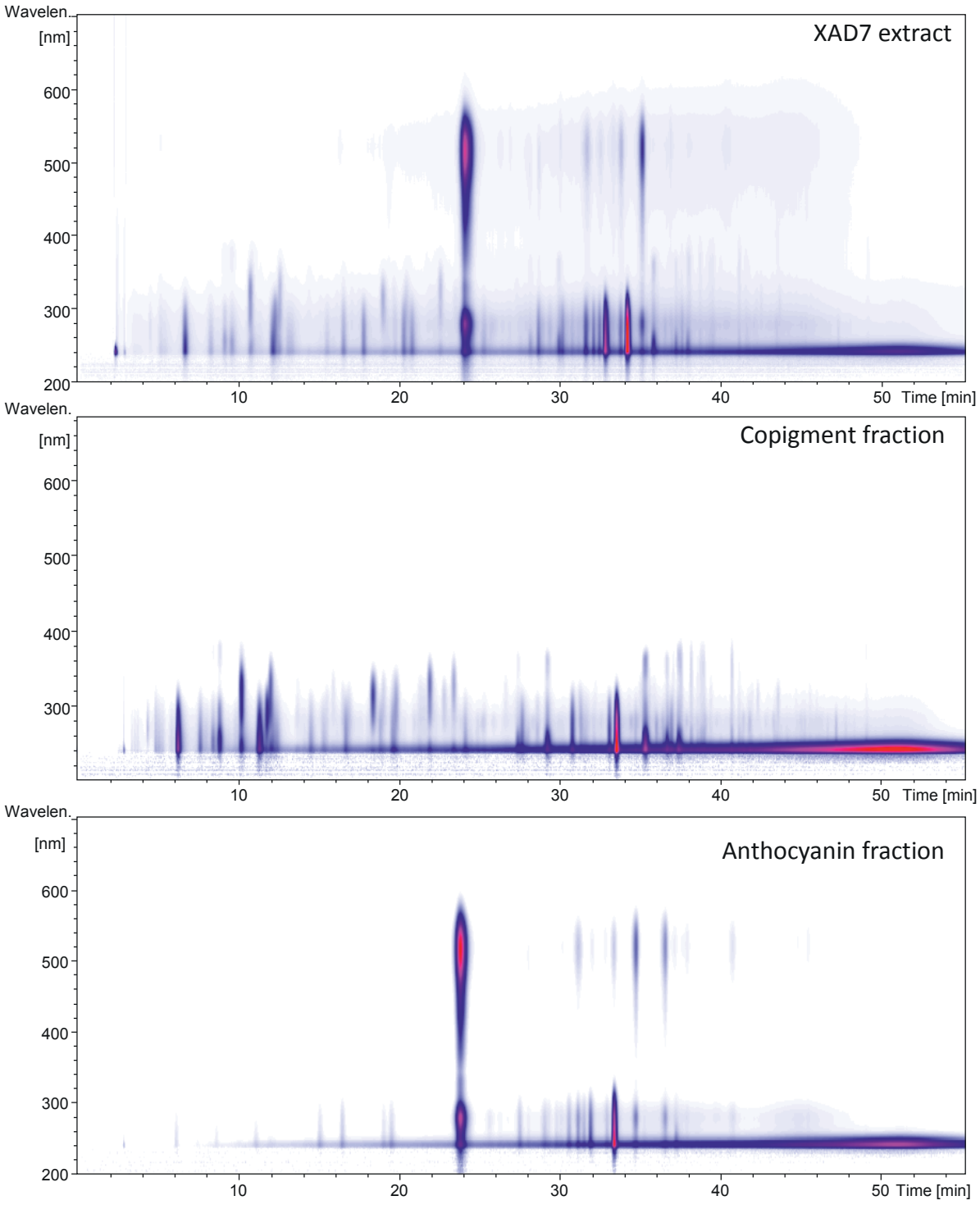


Fig. 3.4. HPLC-DAD analyses of a separation of a blackberry extract by Membrane Chromatography.

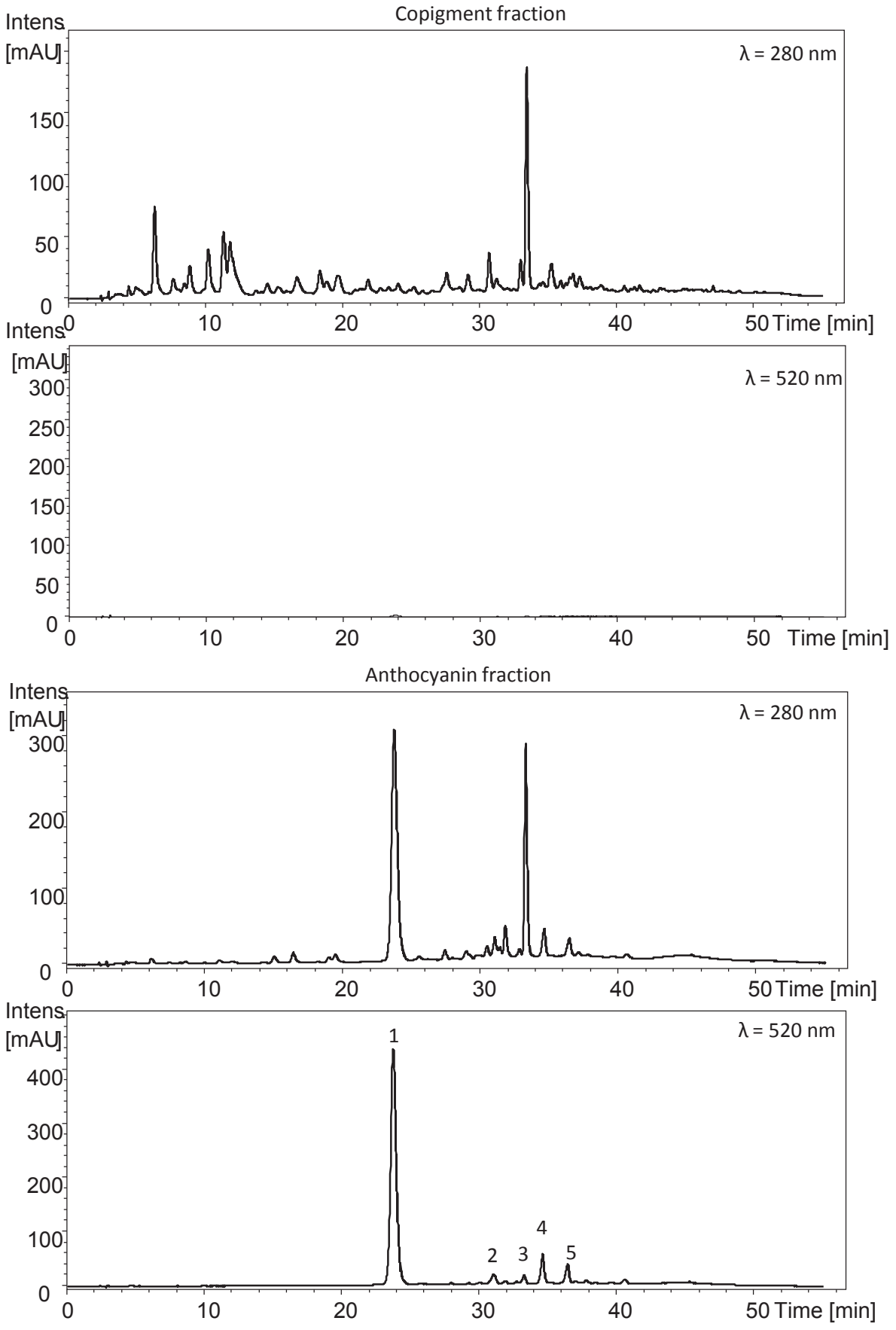


Fig. 3.5. HPLC-Chromatograms at 280 nm and 520 nm of copigment fraction and anthocyanin fraction. Peak numbering according (peak 1-4) to Table 3.1 and peak 5 is a cyanidin-derivative.

3.2.2.2 Countercurrent Chromatography

Low-Speed Rotary Countercurrent Chromatography (LSRCCC)

Another possibility for the isolation of anthocyanins on a preparative scale is the use of Countercurrent Chromatography. In this study, a separation of blackberry extract was carried out on a large scale by LSRCCC in order to isolate reference compounds. A variety of wild growing blackberries, which contained cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside as main compounds, was separated by HSCCC (Schwarz et al., 2003) and by LSRCCC (Köhler, 2006). In both cases, the separation was performed with a solvent system consisting of tBME:n-BuOH:ACN:H_2O (2:2:1:5; v/v/v/v) acidified with 0.1 % TFA and the elution mode was Head to Tail.

In this case, the same solvent system was used in which the lighter phase was used as the stationary phase and the aqueous phase as mobile phase. The used blackberry XAD-7 extract contained cyanidin-3-*O*-glucoside as main pigment, followed by cyanidin-3-*O*-(6"-dioxalyl-glucoside), cyanidin-3-*O*-xyloside and cyanidin-3-*O*-(6"-malonyl-glucoside) as minor compounds, as well as ellagitannins and flavonols (Fig. 3.6). For the separation, 35.5 g of blackberry XAD-7 extract was dissolved in 300 mL of two solvent system (1:1, v/v) and was injected in a LSRCCC system with a 5.6 L column (Fig. 3.7). The flow rate was set at 4 mL/min and fractions were collected every 12 min. The LSRCCC separation was monitored at 520 nm (Fig. 3.8). After elution, the coil was collected in eight fractions (C1-C8: coil fractions) and the identification of compounds was made by TLC, HPLC-DAD and HPLC-ESI-MS^n.

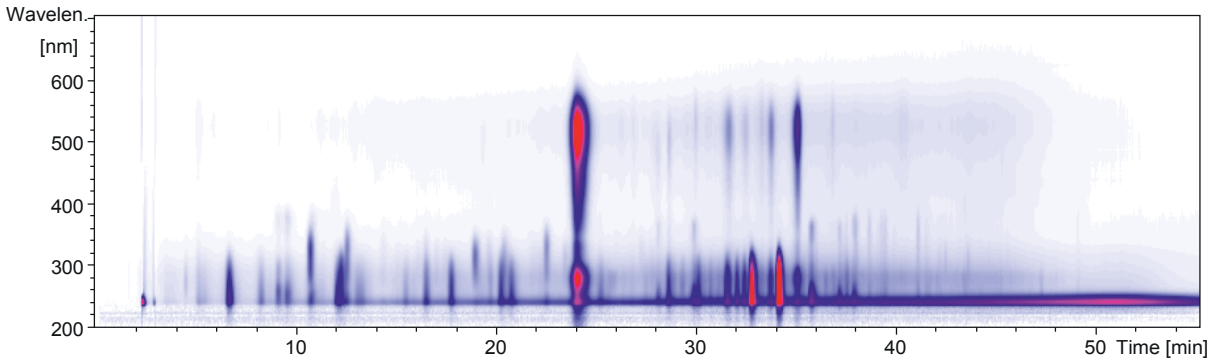


Fig. 3.6. HPLC-DAD chromatogram of blackberry XAD-7 extract before separation.

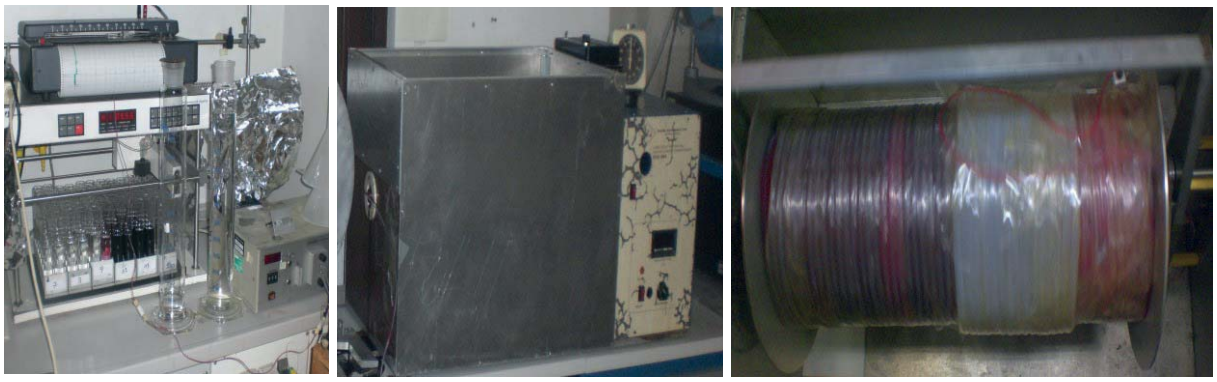


Fig. 3.7. LSRCCC system: Detector, fraction collector and plotter (left), column and motor (at the middle) and coil with blackberry sample (right).

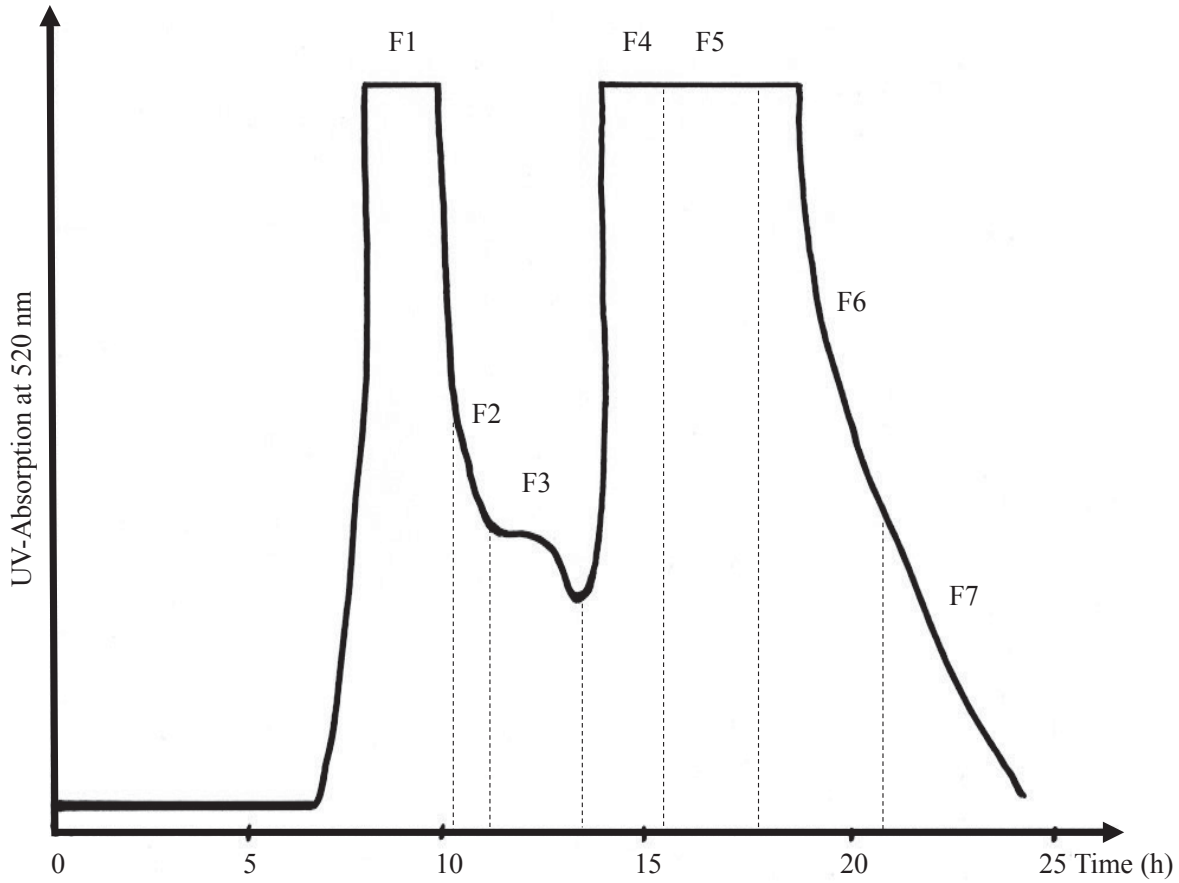


Fig. 3.8. LSRCCC chromatogram at 520 nm of blackberry XAD-7 extract.

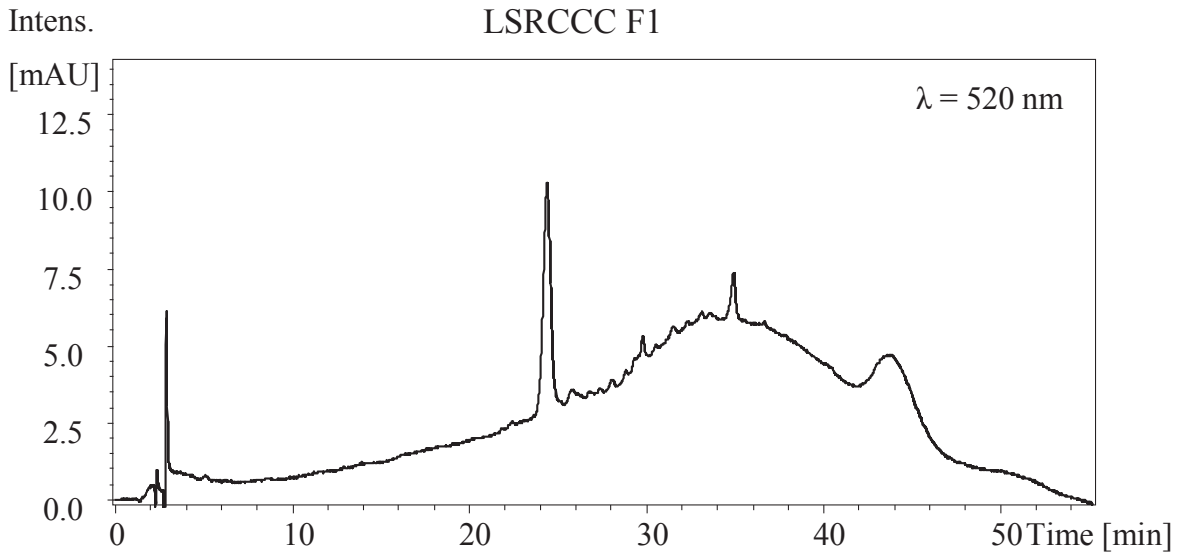


Fig. 3.9. Polymeric fraction of blackberry after LSRCCC separation.

It is known that anthocyanins can be differentiated from ellagitannins and copigments by HPLC analysis due to the different UV-absorption. After identification of compounds, fraction F1 (5.29 g) showed a broad hump (Fig. 3.9), which consists of a polymeric fraction and ellagitannins with high molecular weight, $[M-H]^-$ at *m/z* = 1250 and $[M-H]^-$ at *m/z* = 1087.

Lambertianin C isomer (*m/z* = 1401) was obtained in a high purity and quantity (87%, 1.34 g) in fraction F2 but also was detected in F1 and F3. Castalagin/vescalagin isomers and pedunculagin isomers were found in F3. The main anthocyanin of blackberry, cyanidin-3-*O*-glucoside, was obtained in F4 (2.52 g) in 98% purity at 520 nm, but it was also eluted from F4 to F7. Pedunculagin isomers (*m/z* = 783) eluted together with cyanidin-3-*O*-glucoside in F5 (4.92 g). Other ellagitannins and phenolic acids such as pedunculagin isomers (*m/z* =783), caffeoyl glucose (*m/z* = 341), ferulic acid derivative (*m/z* = 355), galloyl-HHDP glucose isomer (*m/z* = 633) and galloyl-bis-HHDP glucose isomer (*m/z* = 935) were present with cyanidin-3-*O*-glucoside in F6 (3.17 g) and F7 (0.67 g).

At the beginning of coil collection, cyanidin 3-*O*-(6"-dioxalyl-glucoside) was eluted in low concentration together with galloyl-bis-HHDP glucose isomer (*m/z* = 935) in C1 (1.17 g) but cyanidin 3-*O*-(6"-dioxalyl-glucoside) was identified in 80 % purity in fraction C2 (0.92 g) and 60% purity in fraction C3 (1.47 g). Cyanidin-3-*O*-xyloside was found in C2 (traces), C3 (22%) and C4 (34%), while cyanidin-3-*O*-(6"-malonyl-glucoside) was detected in C2 (20%), C3 (13%) and C4 (11%). Neochlorogenic acid (3-CQA) was detected in C1, C2 and C3, while cryptochlorogenic acid (4-CQA), (-)-epicatechin and 3-p-coumaroylquinic acid were found in C4. Gallic acid was identified in C5. On the other hand, quercetin 3-*O*-rutinoside (*m/z* = 609) and a quercetin-derivative (*m/z* = 433) eluted in C3 and C4, while C6 and C7 contained quercetin-3-*O*-[6''-(3-hydroxy-3-methylglutaroyl)]-β-galactoside, quercetin-3-glucuronide, quercetin-3-rhamnoside, quercetin 3-galactoside and quercetin 3-glucoside. Protocatechuic acid was present in C7 and C8. The identity and distribution of compounds after LSRCCC separation of blackberry extract are summarized in Table 3.4.

Table 3.4. Distribution of phenolic compounds from blackberry after LSRCCC separation.

Fraction No.	Compound	$[M]^+$	$[M-H]^-$
1, 2	Polymeric fraction		
1	Ellagitannin derivatives		1250
1	Ellagitannin derivative		1087
1, 2, 3	Lambertianin C isomer		1401
3	Castalagin/vescalagin isomers		933
3	Unknown		858
3, 5, 6, 7	Pedunculagin isomers		783

Table 3.4. Continued.

Fraction No.	Compound	$[M]^+$	$[M-H]^-$
4, 5, 6, 7, C1	Cy-3-*O*-glc	449	
6, 7	Caffeoyl glucose		341
6, 7	Ferulic acid derivative		355
6, 7	Galloyl-HHDP glucose isomer		633
6, 7, C1, C5	Galloyl-bis-HHDP glucose isomer		935
C1, C2, C3, C4	Cy-3-*O*-(6"-dioxalyl-glucoside)	593	
C2, C3, C4	Cy-3-*O*-(6"-malonyl-glucoside)	535	
C2, C3, C4	Cy-3-*O*-xyl	419	
C1, C2, C3	3-CQA		353
C3, C4, C5	Ellagitannin derivatives		951
C3, C4	p-Coumaroyl hexose		325
C3	3-Feruloylquinic acid		367
C3, C4	Sanguiin H-2		1103
C3, C4	Quercetin-derivative		433
C3, C4	Quercetin 3-*O*-rutinoside		609
C4	4-CQA		353
C4	(-)-Epicatechin		289
C4	3-p-Coumaroylquinic acid		337
C5	Gallic acid		169
C6, C7	Quercetin-3-*O*-[6’’-(3-hydroxy-3-methylglutaroyl)]-β-galactoside		607
C6, C7	Quercetin 3-glucuronide		477
C6, C7	Quercetin 3-rhamnoside		477
C6, C7	Quercetin 3-*O*-galactoside		463
C6, C7	Quercetin 3-*O*-glucoside		463
C7, C8	Protocatechuic acid		153

C1-C8: coil fractions

High-Speed Countercurrent Chromatography (HSCCC)

The fractions F5 and F6 were separated by HSCCC in Head to Tail elution mode using tBME:n-BuOH:ACN:H_2O (1:3:1:5; v/v/v/v), which is a more polar solvent system than that used in LSRCCC separation, acidified with 0.1 % TFA. For a satisfactory HSCCC separation, the sample loading required some considerations. The injected sample should not exceed 0.7 g because if it is bigger, compounds will elute together in a broad peak. After HSCCC separation, cyanidin-3-*O*-glucoside was obtained in pure form (Fig. 3.10).

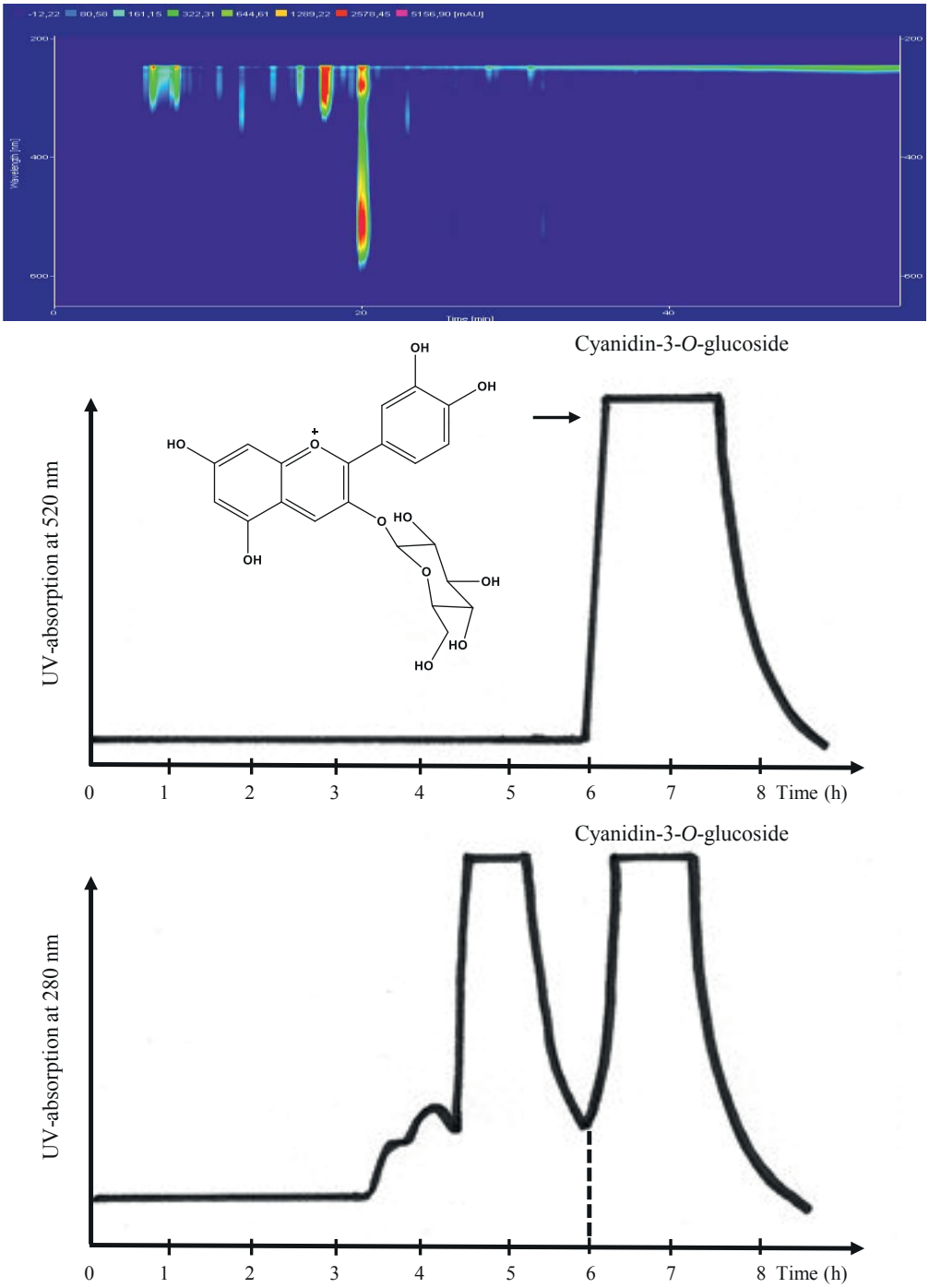


Fig. 3.10. DAD-Contour-Plot Chromatogram of LSRCCC Fraction 5 before HSCCC separation (at the top) and HSCCC separation of LSRCCC Fraction 5 at 520 nm (in the middle) and 280 nm (at the bottom).

3.3 Black chokeberry

3.3.1 Chemical composition

According to some factors such as cultivar, location, year, climate and maturity, the total phenol content of berries can vary. Nonetheless, black chokeberries or *Aronia melanocarpa* berries possess one of the highest total phenolic content in comparison to other berries (Määttä-Riihinen et al., 2004; Mikulic-Petkovsek et al., 2012a). In addition, the consumption of aronia berries has increased in recent years due to its high antioxidant activity which is related with health-promoting activities (Kulling and Rawel, 2008; Denev et al., 2012).

Aronia melanocarpa berries present a dark violet color that is due to the presence of anthocyanins, especially anthocyanin glycosides (Strigl et al., 1995b; Chandra et al., 2001) but its berries also contain phenolic acids, flavonols, proanthocyanidins and polymeric proanthocyanins (Oszmiański and Wojdylo, 2005; Slimestad et al., 2005; Esatbeyoglu and Winterhalter, 2010). In contrast to other fruits, *Aronia melanocarpa* is characterized by its high polymeric procyanidin content, around 1578.8 mg/100 g DW in juice, 5181.6 mg/100 g DW in berries and 8191.6 mg/100 g DW in pomace as well as its high radical scavenging activity (Oszmiański and Wojdylo, 2005).

In this study, the identification of individual anthocyanins and phenolic compounds of *Aronia melanocarpa* juice was performed to determine its fingerprint by HPLC-DAD and HPLC-ESI-MS^n. Anthocyanins of *Aronia melanocarpa* were identified at 520 nm, as shown in Fig. 3.11. The main anthocyanins are cyanidin-3-*O*-galactoside (Peak 1) and cyanidin-3-*O*-arabinoside (Peak 3), with around 60.7% and 28.9% of total anthocyanin content, respectively, while the minor anthocyanins are cyanidin-3-*O*-glucoside (Peak 2) and cyanidin-3-*O*-xyloside (Peak 4), which accounted for 5.7% and 4.6% of the total anthocyanins, respectively. In addition, LC-MS analyses provided information on its molecular and fragment ions (Table 3.5). Cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside presented the pseudomolecular ion at *m/z* 449 together with the fragment ion at *m/z* = 287 corresponding to $[M\text{-galactose}]^+$ or $[M\text{-glucose}]^+$, respectively. The fragmentation pattern of cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-xyloside showed a pseudomolecular ion at *m/z* = 419 and fragment ion at *m/z* = 287, which were assigned to $[M\text{-arabinose}]^+$ or $[M\text{-xylose}]^+$, respectively. Consequently, *Aronia melanocarpa* is characterized by the content of anthocyanins based on the cyanidin aglycone.

On the other hand, hydroxycinnamic acids were detected at 280 and 360 nm. The LC-ESI-MS^n analyses demonstrated the presence of three chlorogenic acids. According to Clifford et al. (2003), chlorogenic acids can be identified and differenced by fragmentation patterns. In this case, neochlorogenic acid (Peak 5) and chlorogenic acid (Peak 6) were found in a ratio of about 1:1 and they are the main hydroxycinnamic acids, while cryptochlorogenic acid (Peak 7) is a minor compound.
The flavonols were monitored at 360 nm and several compounds (Peak 9 to peak 17) showed a fragment ion at *m/z* = 301 in LC-ESI-MS^n analyses that is characteristic for quercetin derivatives.

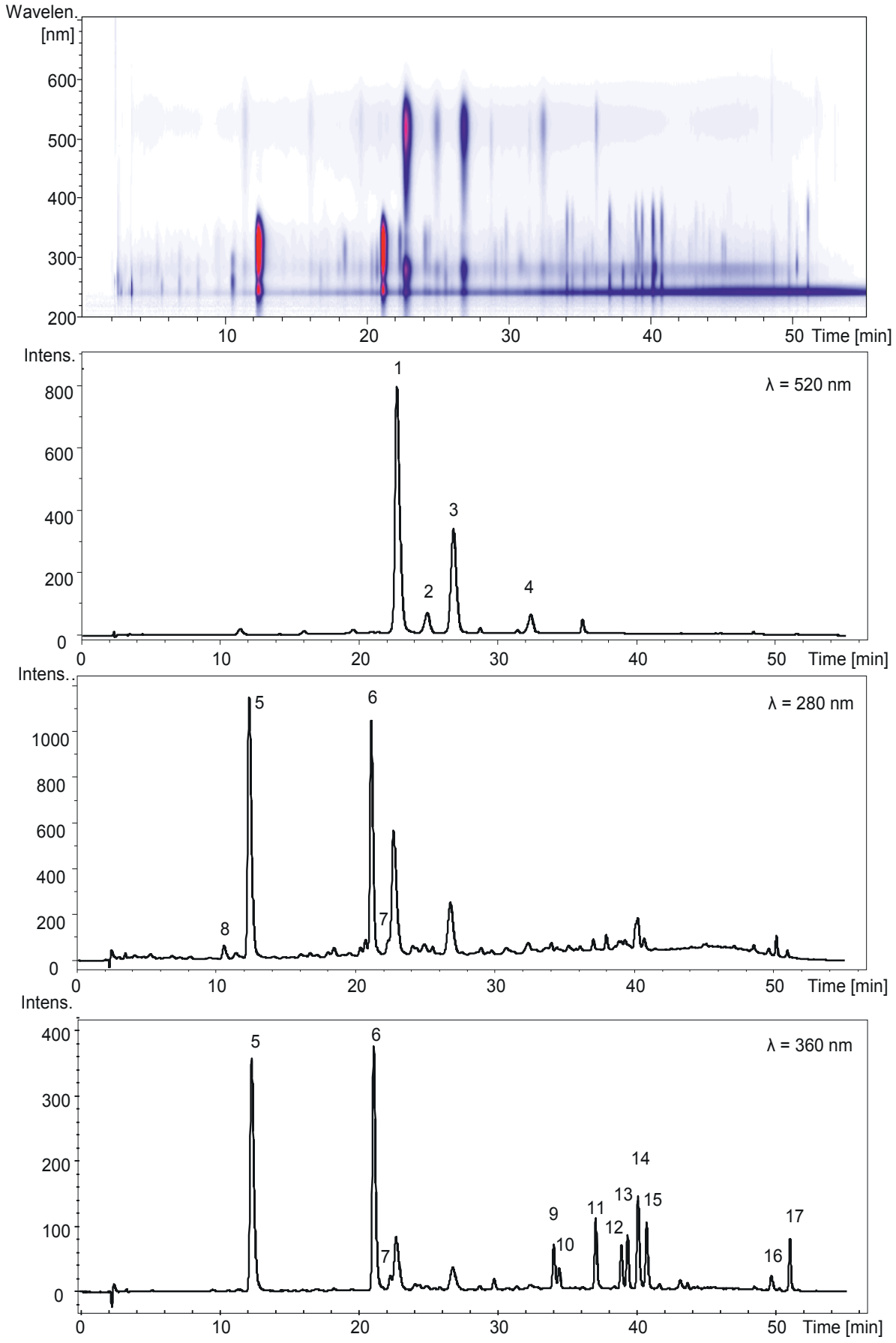


Fig. 3.11. DAD-Contour-Plot Chromatogram and HPLC Chromatograms at 520 nm, 280 nm and 360 nm of *Aronia melanocarpa* juice. Peak numbering according to Table 3.5.

Table 3.5. LC-ESI-MS^n data of polyphenols in *Aronia melanocarpa* juice.

Peak	Compound	$[M]^+$	$[M-H]^-$	Fragment ions (m/z)
1	Cyanidin 3-galactoside	449		287
2	Cyanidin 3-glucoside	449		287
3	Cyanidin 3-arabinoside	419		287
4	Cyanidin 3-xyloside	419		287
5	Neochlorogenic acid (3-CQA)		353	191, 179, 135
6	Chlorogenic acid (5-CQA)		353	191, 179, 161
7	Cryptochlorogenic acid (4-CQA)		353	191, 179, 173, 135
8	Protocatechuic acid		153	109
9	Quercetin dihexoside Nr.1		625	301
10	Quercetin dihexoside Nr.2		625	301
11	Quercetin 3-vicianoside		595	301
12	Quercetin 3-robinobioside		609	301
13	Quercetin 3-rutinoside		609	301
14	Quercetin 3-galactoside		463	301
15	Quercetin 3-glucoside		463	301
16	Quercetin derivative		601	465, 301
17	Quercetin		301	
*	Coumaroyl quinic acid ester Nr. 1		337	191, 173, 163, 119
*	Feruloylquinic acid Nr. 1		367	193, 161
*	Coumaroyl quinic acid ester Nr. 2		337	191, 173, 163
*	Feruloylquinic acid Nr. 2		367	193, 191
*	Eriodictyol-7-O-glucuronide		463	287, 151
*	Procyanidin dimer		577	559, 451, 425, 407, 289
*	(-)-Epicatechin		289	245
*	Unknown (Quercetin-pentoside)		433	301
*	Unknown (Gambiriin derivative)		579	289, 245
*	Unknown (Quinic acid + phloroglucinol)		317	299, 255, 207, 191, 163
*	Dicaffeoylquinic acid ester Nr. 1		515	353, 191
*	Dicaffeoylquinic acid ester Nr. 2		515	353, 191
*	Procyanidin C1 trimer		865	847, 739, 577, 289
*	Eriodictyol		287	151
*	Unknown Luteolin-derivative Nr. 1		465	285, 241
*	Unknown Luteolin-derivative Nr. 2		435	285, 241

*Traces

Based on literature data, quercetin-3-O-vicianoside (Peak 11), quercetin-3-*O*-robinobioside (Peak 12), quercetin-3-*O*-rutinoside (Peak 13), quercetin-3-*O*-galactoside (Peak 14), quercetin-*O*-3-glucoside (Peak 15) and quercetin (Peak 17) were identified (Slimestad et al., 2005; Zhao, 2007; Mikulic-Petkovsek et al., 2012a). Peak 16 showed a molecular ion $[M-H]^-$ at *m/z* = 601 and fragment ions at *m/z* = 465 and 301 corresponding to a quercetin derivative. Additionally, two compounds (Peak 9 and 10) presented a molecular ion $[M-H]^-$ at *m/z* = 625 with a fragment ion at *m/z* = 301, which corresponds to the loss of two hexose units. Their chemical structures are assigned as quercetin dihexoside and they were also detected by Hillebrand (2004) and Mikulic-Petkovsek et al. (2012b).

Furthermore, *Aronia melanocarpa* juice presented traces of coumaroyl quinic acid esters, feruloylquinic acids, eriodictyol-7-*O*-glucuronide, procyanidin dimer and trimer, (-)-epicatechin, unknown quercetin-pentoside, unknown gambiriin derivative, dicaffeoylquinic acid esters, eriodictyol and unknown luteolin-derivatives. A brief outline of phenolic composition in *Aronia melanocarpa* juice is summarized in Table 3.5, together with their LC-ESI-MS^n data.

Nonetheless, traces of other anthocyanins were also reported in *Aronia melanocarpa* like pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-arabinoside, which had a molecular ion at *m/z* 433 $[M]^+$ and *m/z* 403 $[M]^+$, respectively, and in both cases presented a fragment ion at *m/z* 271 corresponding to pelargonidin aglycone (Wu et al., 2004a). In case of flavonols, Mikulic-Petkovsek et al. (2012b) also identified myricetin glycosides, isorhamnetin glycosides and kaempferol glycosides such as myricetin-3-*O*-galactoside (*m/z* 479 $[M-H]^-$, fragment *m/z* 317), myricetin-3-*O*-glucoside (*m/z* 479 $[M-H]^-$, fragment *m/z* 317), isorhamnetin-3-*O*-galactoside (*m/z* 477 $[M-H]^-$, fragment *m/z* 315), isorhamnetin-3-*O*-glucoside (*m/z* 477 $[M-H]^-$, fragment *m/z* 315) and kaempferol-3-*O*-galactoside (*m/z* 447 $[M-H]^-$, fragment *m/z* 285) in low concentration between 0.2 and 1.5 mg/kg. In addition, some flavones were also described in other cases. For example, apigenin 7, 4'-di-*O*-rhamnoside (*m/z* 561 $[M-H]^-$, fragments *m/z* 415, 269) and isorhamnetin rhamnosylhexoside isomers (*m/z* 623 $[M-H]^-$), that may be due to chokeberry leaves in which they have been reported (Lee et al., 2014a). The presence of apigenin 6,8-di-C-glucoside (*m/z* 593 $[M-H]^-$, fragments *m/z* 503, 473) may be due to a blend of chokeberry and lemon juice (Gironés-Vilaplana et al., 2012).

In comparison with *Aronia arbutifolia* (red chokeberry) and *Aronia prunifolia* (purple chokeberry), *Aronia melanocarpa* is distinguished from other aronia species by the fruit color, foliage pubescence (Small and Catling, 2005; Brand, 2010) and its phenolic composition (Rugină et al., 2012; Taheri et al., 2013; Wangensteen et al., 2014). Nevertheless, the information of the differences among them in individual polyphenol content is limited. Taheri et al. (2013) described the highest anthocyanin content for *Aronia melanocarpa*, followed by *Aronia prunifolia* and the lowest anthocyanin content was found in *Aronia arbutifolia*. However, Rugină et al. (2012) and Wangensteen et al. (2014) reported the highest anthocyanin content for *Aronia prunifolia* (366 or 737 mg/100 g FW, respectively), followed by *Aronia melanocarpa* (176 mg/100 g FW for Aron, 277 mg/100 g FW for Viking, 252 mg/100 g FW for Moskva, 249 mg/100 g FW for Hugin and 447 mg/100 g FW for Nero). Furthermore, the total procyanidin content

also follows the same tendency in which *Aronia prunifolia* had 855.13 mg epicatechin/100 g FW, while *Aronia melanocarpa* Aron cultivar presented 354.94 mg epicatechin/100 g FW and Viking cultivar contained 627.57 mg epicatechin/100 g FW (Rugină et al. 2012).

All Aronia species presented cyanidin-3-*O*-galactoside as main anthocyanin, followed by cyanidin-3-*O*-arabinoside and the minor compound was cyanidin-3-*O*-glucoside, while the presence or a lack of cyanidin-3-*O*-xyloside was described in some cases. However, the content of individual anthocyanins varied among Aronia species and some significant percentage differences of anthocyanin content were found. *Aronia prunifolia* and *Aronia melanocarpa* presented cyanidin-3-*O*-galactoside in a range between 63% and 77% of the total anthocyanins, while *Aronia arbutifolia* showed the highest content (86-97%). In the case of cyanidin-3-*O*-arabinoside, this pigment was in a range of 21% and 34% in *Aronia prunifolia* and *Aronia melanocarpa*, whereas it was in a range from 3% to 13% in *Aronia arbutifolia* (Taheri et al., 2013). In addition, traces of cyanidin-3-*O*-glucoside were presented in *Aronia prunifolia*, as well as in Moskva and Hugin cultivars of *Aronia melanocarpa* (Wangensteen et al., 2014). A lack of cyanidin-3-*O*-xyloside and neochlorogenic acid were observed in *Aronia arbutifolia*, while cyanidin-3-*O*-xyloside ranges from traces to 2% in *Aronia prunifolia* (Taheri et al., 2013). In Hugin cultivars of *Aronia melanocarpa* it was present as trace (Wangensteen et al., 2014). In reference to Aronia species, future analytical studies should be able to clarify their individual phenolic composition.

The aim of this work was to examine the anthocyanin content of *Aronia melanocarpa* products in which compounds were separated by preparative techniques. The quantification of anthocyanins in aronia juice, pomace and an extract made from aronia juice is presented in Table 3.6. The results showed a high anthocyanin content in these aronia products. *Aronia melanocarpa* pomace is a by-product of aronia berries after pressing and consists of skins, flesh fruit and seeds. Skin, flesh and leaf stalks are around 45% of the total pomace, while seeds and peels are around 30 % and 25% contains shredded skins, fruit flesh and crushed seeds (Sójka et al., 2013). The pomace of *Aronia melanocarpa* is a rich source of anthocyanins, chlorogenic acids, quercetin derivatives and protocatechuic acid, as shown in Fig. 3.12. Consequently, *Aronia melanocarpa* pomace can be recovered and utilized to extract bioactive compounds.

Table 3.6. Anthocyanin content in *Aronia melanocarpa* products.

Anthocyanin	Aronia Juice [mg/100 mL]*	Aronia Pomace [mg/100 g]*	A20 extract [mg/100 g]*
1) Cyanidin-3-*O*-galactoside	319.2	519.9	13632.4
2) Cyanidin-3-*O*-glucoside	44.4	39.6	786.9
3) Cyanidin-3-*O*-arabinoside	142.0	197.1	6572.3
4) Cyanidin-3-*O*-xyloside	31.8	29.8	858.5

*Calculated as mg of cyanidin-3-glucoside per 100 g of sample or 100 mL of sample, respectively

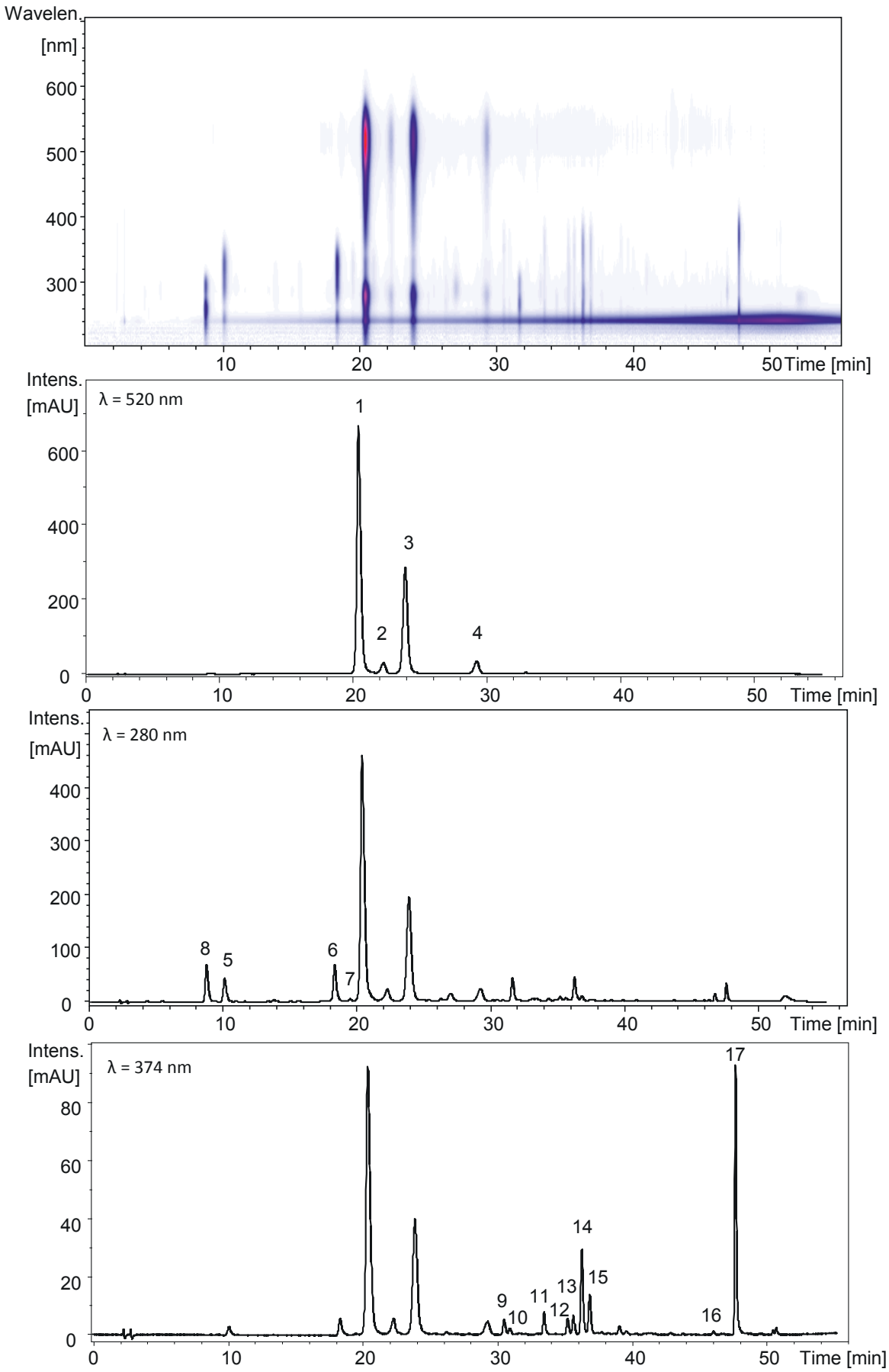


Fig. 3.12. DAD-Contour-Plot chromatogram and HPLC-chromatograms of *Aronia melanocarpa* pomace at 520 nm, 280 nm and 374 nm. Peak numbering according to Table 3.5.

3.3.2 Preparative separations

3.3.2.1 Membrane chromatography

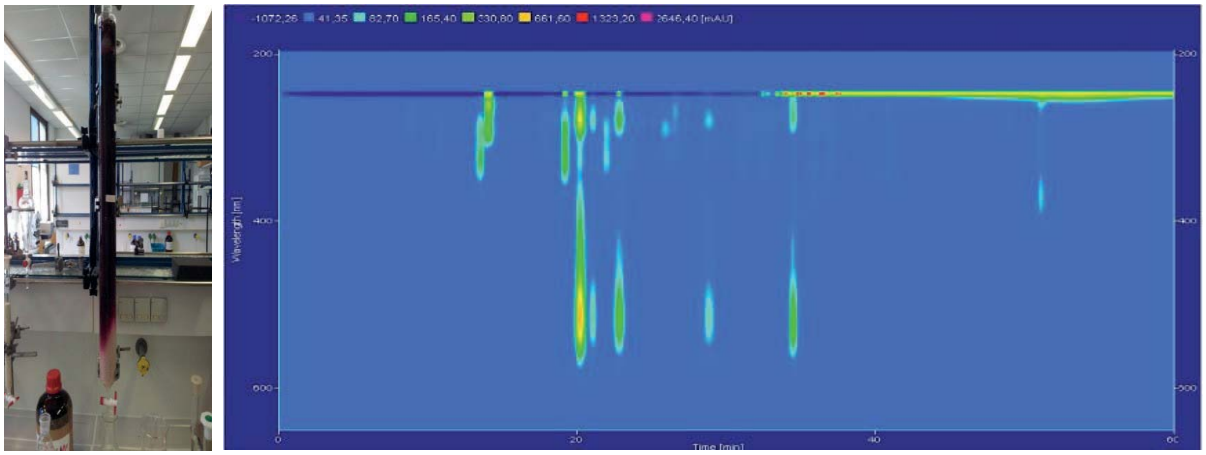
The purpose of this work was the extraction of polyphenols from *Aronia melanocarpa* products, in particular pomace as well as an extract made from aronia juice (A20), and the subsequent preparative separation of polyphenols by membrane chromatography and CCC techniques.

Aronia melanocarpa pomace is a waste product after processing of aronia berries into juice and it is characterized by a higher phenolic composition (10583.27 mg/100 g DW), higher polymeric procyanidin content (8191.58 mg/100 g DW) and higher antioxidant activity compared to aronia berries and juice (Oszmiański and Wojdylo, 2005). Moreover, the procyanidins present in *Aronia melanocarpa* are mainly B-type and are based on (-)-epicatechin units (Wu et al., 2004a; Oszmiański and Wojdylo, 2005; Esatbeyoglu and Winterhalter, 2010). Beside this, aronia pomace can be used as source of polyphenols, especially anthocyanins (Table 3.6 and Fig. 3.12).

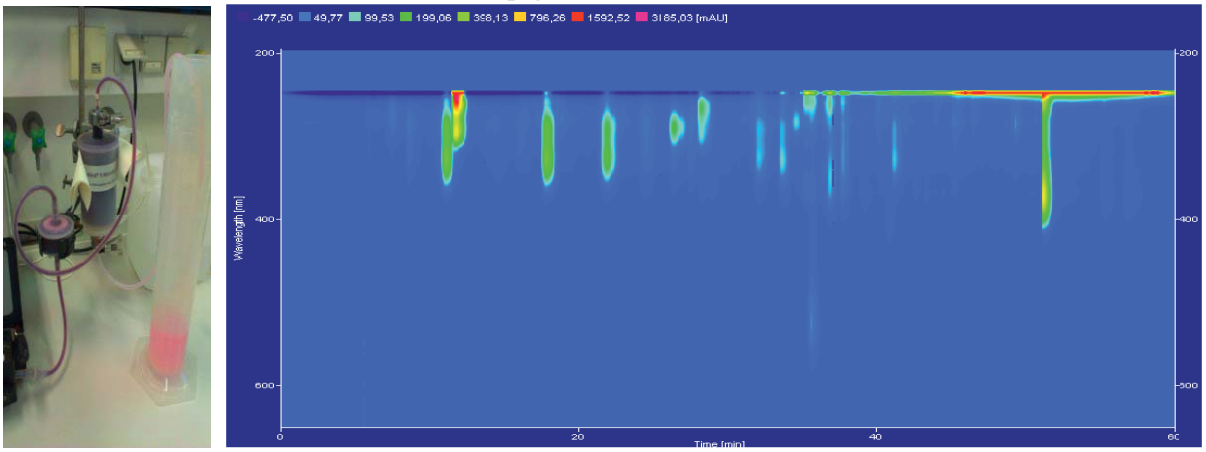
Therefore, *Aronia melanocarpa* pomace or extract A20 (500 g) was defatted three times with n-hexane and dichloromethane (3x1L). Then the polyphenols were extracted according to method 1 (see section 4.3). After extraction, polyphenols were concentrated by using adsorption chromatography (see section 4.4) and a polyphenol-rich extract, aronia pomace XAD-7 extract or A20 XAD-7 extract, was obtained. Prior to the separation of the extract by membrane chromatography in anthocyanin and copigment fraction, a separation of the polymeric procyanidins by solvent precipitation was required (see section 4.5). Afterwards, the XAD-7 extract was fractionated by membrane chromatographic method (see section 4.6.5) (Juadjur and Winterhalter, 2012). During the fractionation, samples of 2 mL of the solution were collected and subsequently analyzed by HPLC-DAD. As a result, the analyses of anthocyanin fraction showed a complete absence of copigments at 280 nm, but the co-pigment fraction contained small amounts of anthocyanins, which were detected at 520 nm. Because of an exceedance of the maximum capacity sample load of the membrane adsorber, a repetition of the membrane chromatographic method was necessary for this fraction. After a second membrane chromatographic separation, a comparison of DAD-Contour-Plot chromatograms of the original extract XAD-7 and both separated fractions showed a successful separation, because the copigment fraction contained no anthocyanins (Fig. 3.13). Additionally, HPLC chromatograms of copigment fraction from A20 or pomace (AP) at 520 nm confirmed the lack of anthocyanins (Fig. 3.14), while HPLC chromatograms of anthocyanin fraction at 280 nm and 520 nm detected only cy-3-gal, cy-3-glc, cy-3-arab and cy-3-xyl. A separation of 10 g of A20 XAD-7 extract was performed, yielding 4.81 g of copigment fraction and 3.74 g of anthocyanin fraction. Similarly, 10 g of aronia pomace XAD-7 extract were separated, generating 6.04 g of copigment fraction and 3.05 g of anthocyanin fraction. The yields obtained are summarized in Table 3.7.

In conclusion, a preparative separation of XAD-7 extracts was carried out successfully by membrane chromatography obtaining high quantities of anthocyanin and copigment fractions. In addition, these fractions were used for isolation of individual compounds in the next section by HSCCC and HPCCC.

XAD-7 Extract



Copigments Fraction



Anthocyanins Fraction

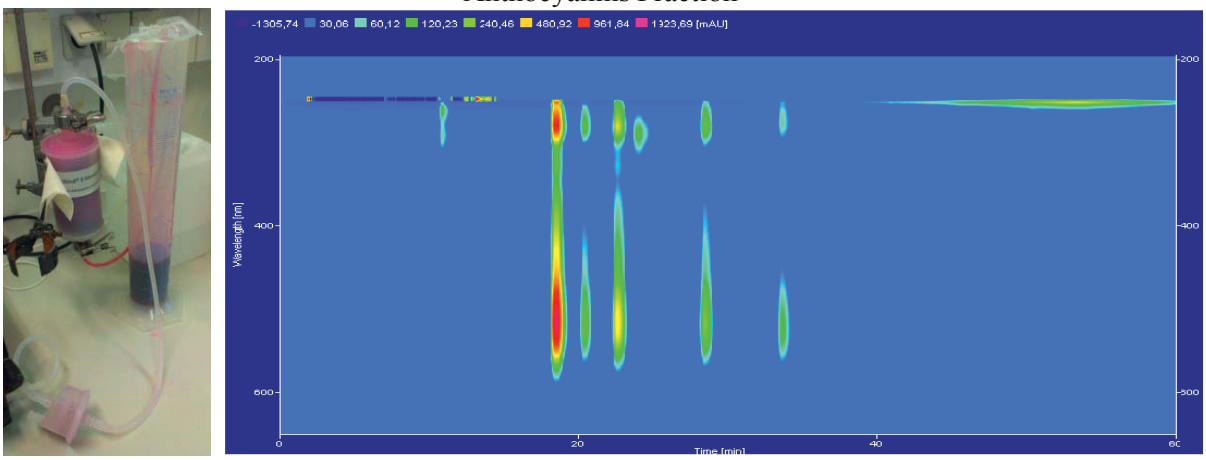


Fig. 3.13. DAD-Contour-Plot Chromatograms of *Aronia melanocarpa* pomace XAD-7 extract (at the top), copigment fraction (at the middle) and anthocyanin fraction (at the bottom).

Table 3.7. Separation of *Aronia melanocarpa* XAD-7 extracts by Membrane Chromatography.

Sample	XAD-7 Extract (g)	Copigment-Fraction (g)	Anthocyanin-Fraction (g)	Polymer Fraction (g)
A20	10.00	4.81	3.74	1.46
AP	10.00	6.04	3.05	0.91

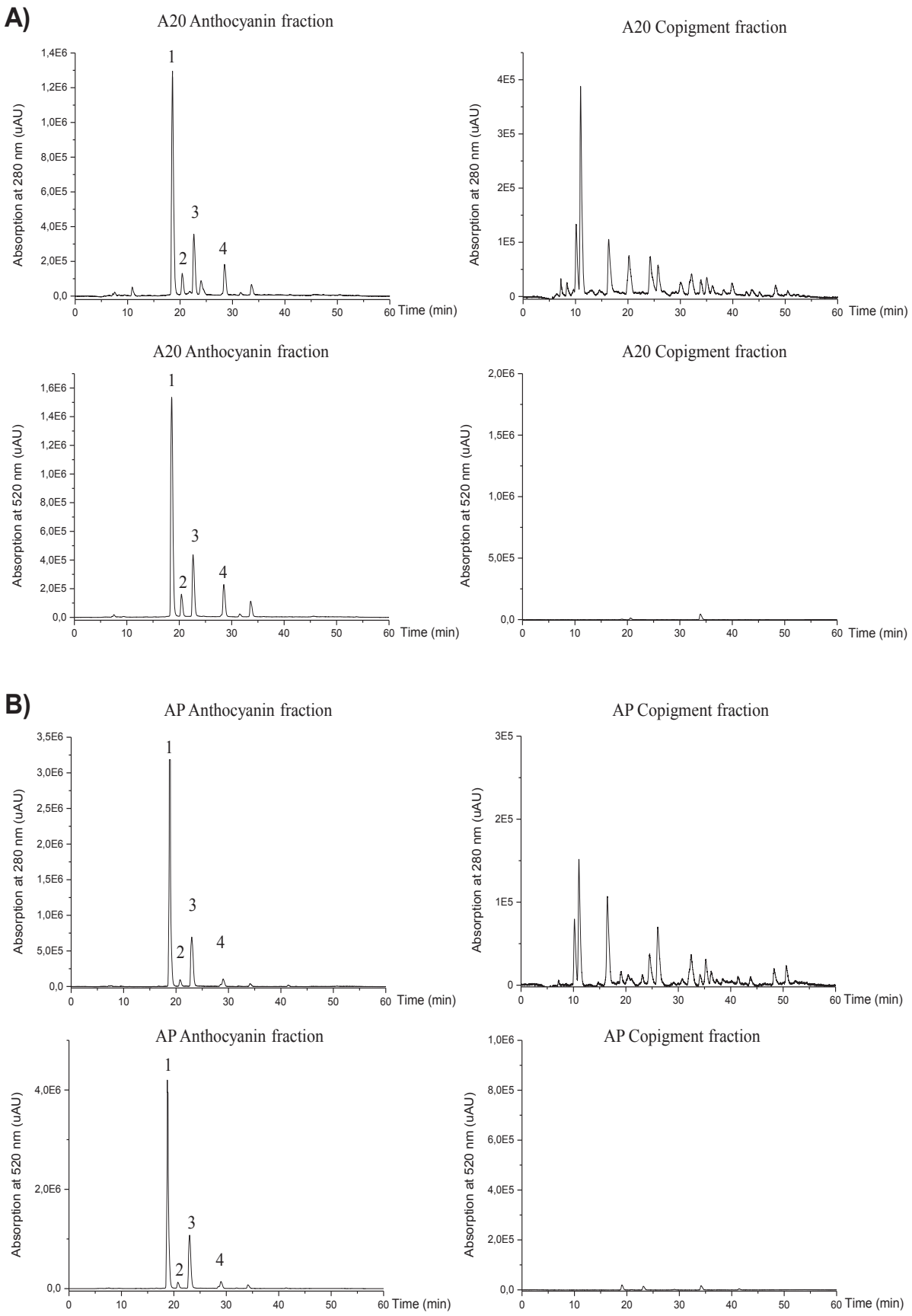


Fig. 3.14. HPLC chromatograms of anthocyanin fraction and copigment fraction from *Aronia melanocarpa* juice extract (A) and pomace (B) at 520 nm and 280 nm. Peak numbering according to Table 3.5.

3.3.2.2 Countercurrent Chromatography

After separation of XAD-7 extracts into a copigment and an anthocyanin fraction of *Aronia melanocarpa* pomace or extract A20 by membrane chromatography, the application of CCC techniques allowed the separation of individual compounds. The obtained fractions were analyzed by thin layer chromatography, HPLC-PDA and LC-ESI-MS^n in order to identify suitable compounds. Then a purification of compounds was performed by preparative HPLC and finally ^1H and ^{13}C NMR analyses of pure anthocyanins or copigments provided detailed information about their structures.

HSCCC Separation of copigment fraction of *Aronia melanocarpa* pomace

The copigment fraction of *Aronia melanocarpa* is mainly characterized by the presence of chlorogenic acids and quercetin derivatives. Based on previous studies on HSCCC separations of chlorogenic acids from Robusta coffee (Kaiser, 2014), the HSCCC separation of copigment fraction (1 g) was carried out using a two solvent system consisting of n-hexane/ethyl acetate/n-butanol/formic acid/water (1:2:2:0.8:5, v/v/v/v/v) in elution and extrusion mode. The HSCCC chromatogram was monitored at 280 nm as shown in Fig. 3.15.

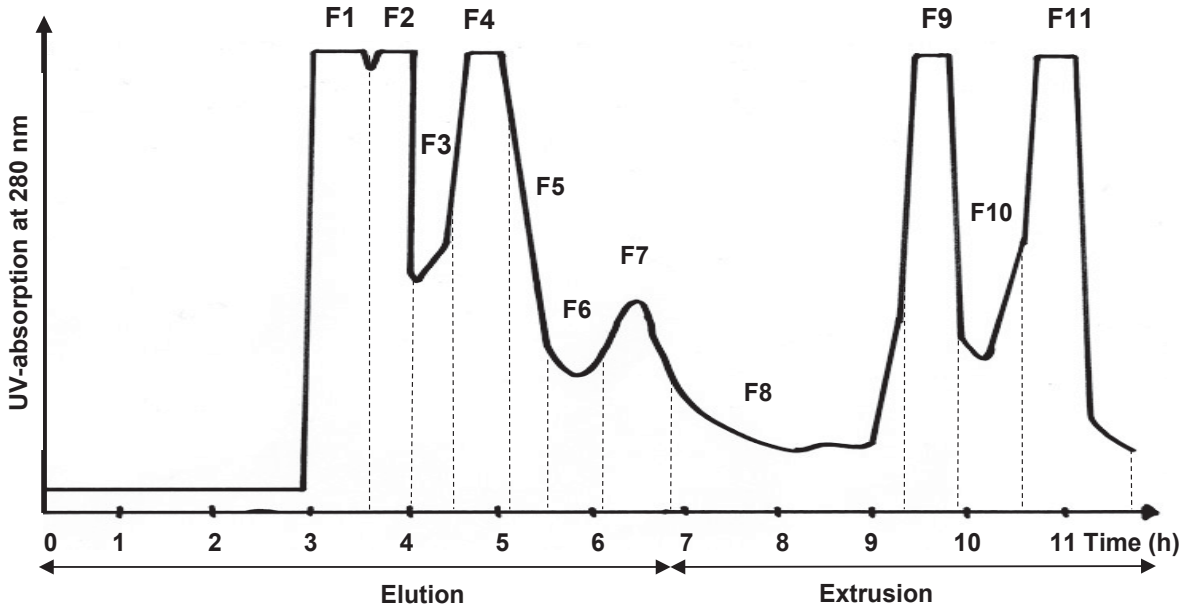


Fig. 3.15. HSCCC chromatogram of the copigment fraction of *Aronia melanocarpa* pomace at 280 nm.

HSCCC separation allowed the purification of copigments in high quantities and purity. Quercetin-3-vicinanoside, quercetin dihexoside No. 1 and No. 2 were identified in fraction 1 (F1, 129 mg). Neochlorogenic acid eluted in fraction 2 (F2, 43 mg) in a high purity of 86%. Fraction 3 (F3) contained quercetin 3-robinobioside and quercetin 3-rutinoside being also present in low concentration in fraction 4 (F4). The chlorogenic acid (49 mg) could be isolated from F4 in a high purity of 87%. Eriodictyol-7-*O*-glucuronide (78%) eluted in fraction 5 (F5), whereas cryptochlorogenic acid and two feruloylquinic acids, 3-p-coumaroylquinic acid ester No. 1 and 5-feruloylquinic acid No. 2 were identified in fraction 6 (F6). Quercetin-3-galactoside and quercetin-3-glucoside were obtained in fraction 7 (F7, 26.1 mg), while quercetin-3-galactoside, quercetin-3-glucoside and two coumaroyl quinic acid esters eluted

together in fraction 8 (F8), as well as traces of protocatechuic acid, which were also detected in previous research of aronia (Hillebrand, 2004). Protocatechuic acid (79.8 mg) was obtained with a purity of 71% in fraction 9 (F9). In fraction 10 (F10), a mixture of protocatechuic acid, unknown quercetin derivatives and quercetin were detected, while in fraction 11 (F11), quercetin was identified in minor purity of 58%.

HSCCC separation of copigment fraction from A20

In this case, 700 mg of A20 copigment fraction were fractionated with a solvent system consisting of tert-butyl methyl ether/acetonitrile/water (2:2:3, v/v/v). HSCCC separation was performed in elution and extrusion mode and was monitored at 280 nm (Fig. 3.16). The separation yielded 16 fractions.

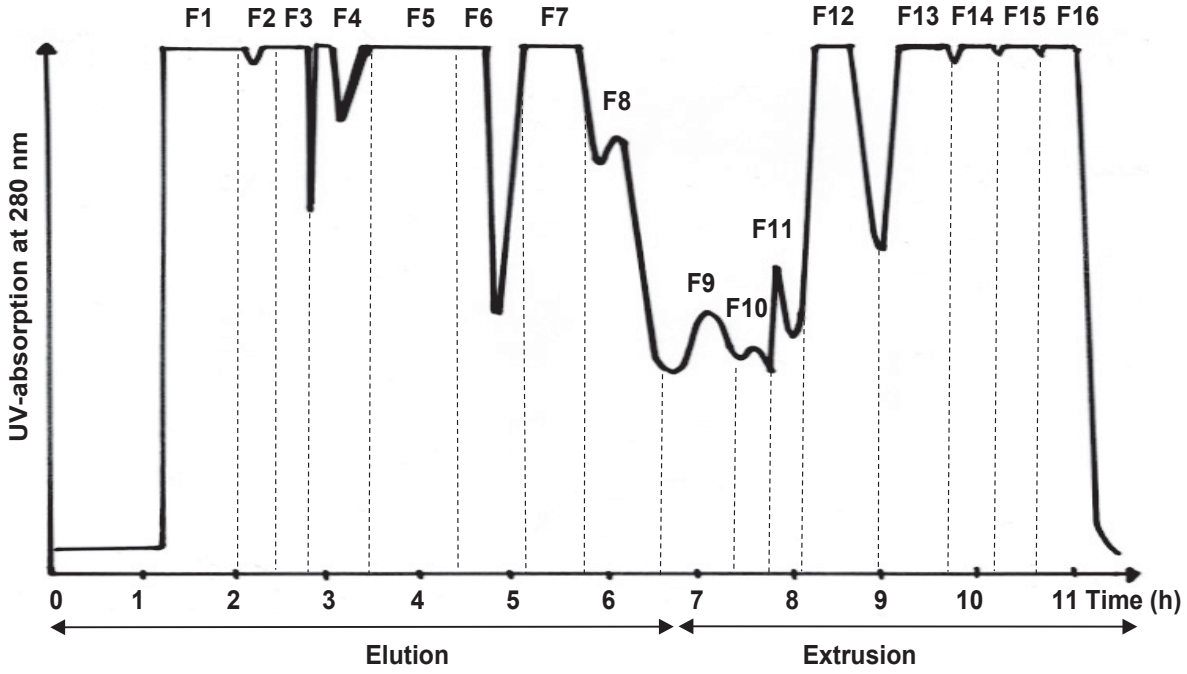


Fig. 3.16. HSCCC chromatogram of the copigment fraction of *Aronia melanocarpa* juice extract at 280 nm.

The elution of compounds was two hours earlier than for the HSCCC separation of AP copigment fraction, because the used solvent system was more polar compared to the solvent system used for AP copigment fraction (n-hexane/ethyl acetate/n-butanol/formic acid/water (1:2:2:0.8:5, v/v/v/v/v)). The most polar compounds were detected in the elution and the less polar eluted in the extrusion mode with the stationary phase. A similar trend of elution in the case of HSCCC separation of AP copigment fraction was observed. At the beginning, a mixture of quercetin-3-vicinanoside, quercetin dihexoside No. 1 and No. 2, neochlorogenic acid and eriodictyol-7-*O*-glucuronide were detected in F1 (93 mg), additionally traces of unknown luteolin-derivates (*m/z* = 465, $[M-H]^-$; fragment ions *m/z* = 285, 241) were present. Quercetin-3-robinobioside, quercetin-3-rutinoside and small quantities of neochlorogenic acid were identified in fraction 2 (30 mg), while neochlorogenic acid (34 mg) with a high purity of 86% eluted in fraction 3. Then cryptochlorogenic acid and feruloylquinic acids were obtained in fraction 4

(34 mg). Chlorogenic acid was isolated with a high purity (88%) in fraction 5 (51.5 mg), but it was also detected along with traces of procyanidin trimer (*m/z* = 865, $[M-H]^-$; fragment ions *m/z* = 847, 739, 577, 289), feruloylquinic acid (*m/z* = 367, $[M-H]^-$; fragment ions *m/z* = 193, 191) and di-caffeoylquinic acid ester (*m/z* = 515, $[M-H]^-$; fragment ions *m/z* = 353, 191) in fraction 6 (21 mg). These compounds were reported in previous studies of *Aronia melanocarpa* (Esatbeyoglu, 2011).

Quercetin-3-galactoside and quercetin-3-glucoside were found in fraction 7 (31 mg), whereas coumaroylquinic acid esters and feruloylquinic acids were detected in fraction 8 (24 mg). Dimeric procyanidins with a molecular ion at *m/z* 577 $[M-H]^-$ and fragment ions at *m/z* = 559, 451, 425, 407, 289 were identified in fraction 9 (10.6 mg) and fraction 10 (10.5 mg). Fraction 12 (36 mg) contained protocatechuic acid and an unknown quercetin derivative with a molecular ion *m/z* = 433 $[M-H]^-$ and fragment ion *m/z* = 301, which was described as quercetin-pentoside or quercetin 3-*O*-arabinopyranoside in the literature data (Esatbeyoglu, 2011; Mikulic-Petkovsek et al., 2012b). Protocatechuic acid (94.4 mg) was isolated in a higher purity (84%) in fraction 13. A di-caffeoylquinic acid ester, which presented a molecular ion at *m/z* 515 $[M-H]^-$ and fragment ions at *m/z* = 353, 191, 178 was found in F14. Fraction 15 was a mixture of compounds, while quercetin was identified in F16.

HSCCC separations of copigment fractions from *Aronia melanocarpa* pomace and juice extract presented a similar order of elution than a previous LSRCCC separation of a *Aronia melanocarpa* pomace XAD-7 extract (Esatbeyoglu, 2011). In addition, many copigments could be isolated in a high purity and sufficient amount for subsequent NMR analyses that confirmed their chemical structures.

HSCCC separation of anthocyanin fraction from A20

Anthocyanins are water-soluble pigments, consequently series of polar solvent systems are required for the HSCCC separations. Ito (2005) suggested a polar system based on tert-butyl methyl ether/n-butanol/acetonitrile/water or to start with n-butanol/water for methanolic extracts and anthocyanins. According to that and previous analyses of anthocyanins distribution in solvent systems, the separation of anthocyanin fraction (700 mg) from A20 extract was carried out by using a solvent system tert-butyl methyl ether/acetonitrile/water (3:1:3, v/v/v) acidified with 0.1% TFA. Anthocyanins were detected at 520 nm (Fig. 3.17) and HSCCC separation yielded six fractions. Cyanidin-3-*O*-galactoside (128 mg) was isolated with a high purity (87%) in fraction 1, while a mixture of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-xyloside was detected in fraction 2 (339 mg) and fraction 3 (30 mg). However, cyanidin-3-*O*-arabinoside could be isolated from fraction 4 and cyanidin-3-*O*-xyloside from fraction 5 (F5).

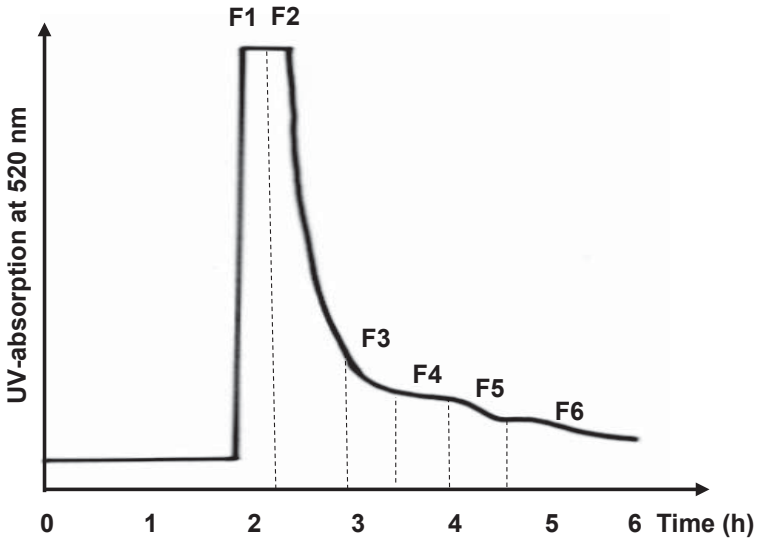


Fig. 3.17. HSCCC chromatogram of the anthocyanin fraction of *Aronia melanocarpa* juice extract at 520 nm.

HSCCC separation of anthocyanin fraction from *Aronia melanocarpa* pomace

In the case of anthocyanin fraction from *Aronia melanocarpa* pomace, a solvent system consisting of ethyl acetate/ethanol/water (5:2:3, v/v/v) acidified with 0.1% TFA was utilized. This solvent system is more polar than the used solvent system for anthocyanin fraction from A20 extract. Five hundred milligram of anthocyanin fraction were separated and anthocyanins were detected at 520 nm (Fig. 3.18). After HSCCC separation, 62 mg of cyanidin-3-*O*-galactoside was isolated with a high purity (91%) from fraction 1. Cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside were detected in fraction 2 and 3 in which cyanidin-3-*O*-galactoside content was reduced as fractions were eluted. Nevertheless, cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-xyloside were eluted but cyanidin-3-*O*-arabinoside was the main compound in fraction 4. Cyanidin-3-*O*-xyloside was identified in fraction 5.

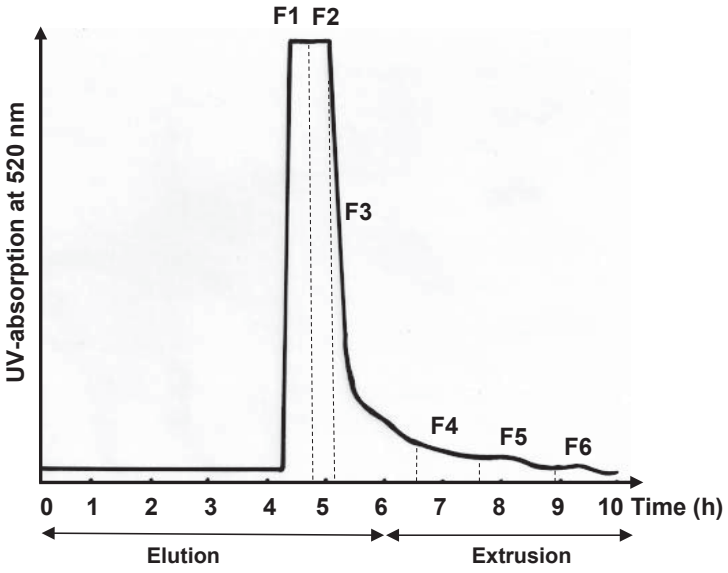


Fig. 3.18. HSCCC chromatogram of the anthocyanin fraction from *Aronia melanocarpa* pomace at 520 nm.

HPCCC separation of anthocyanin fraction from A20

Anthocyanins of *Aronia melanocarpa* present similar structure. For instance, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside contain similar molecular weight, their LC-ESI-MS^n data show the pseudomolecular ion at *m/z* 449 with the fragment ion at *m/z* 287. Their structures are different in one hydroxyl group (OH) in the hexose ring, consequently both compounds present similar polarity. Cyanidin-3-*O*-arabinoside and cyanidin-3-*O*-xyloside follow the same trend. They have a pseudomolecular ion at *m/z* 419 and fragment ion at *m/z* 287. Because of similar polarity of anthocyanins, the separation of these isomers was improved taking into account factors such as concentration of sample injected, polarity of solvent systems and retention of stationary phase. The separation of 250 mg anthocyanin fraction from A20 was performed using solvent system ethyl acetate/n-butanol/water (2:8:10, v/v/v) acidified with 1% formic acid by semi-preparative HPCCC system in head to tail mode. The flow rate was set at 3 mL/min, the revolution speed was 1600 rpm. In addition, a reduction of solvent system volume was carried out from around 2 L to 300 mL, as well as the separation time decreased from hours to minutes. Anthocyanins were monitored at 520 nm, as shown in Fig. 3.19. Fraction 1 (27 mg) contained a mixture of four minor compounds. All of them presented a fragment ion at *m/z* 287 corresponding to cyanidin aglycone and were identified as cyanidin derivatives for the first time. The first compound presented *m/z* 737 $[M]^+$ with fragments ions at *m/z* 575, 423, 239 and 287. The second compound had *m/z* 611 $[M]^+$ and fragments ions at *m/z* 449 and 287. The third compound presented *m/z* 581 $[M]^+$ with fragments ions at *m/z* 449 and 287. The fourth compound showed *m/z* 1025 $[M]^+$ with fragments ions at *m/z* 863, 737, 575, 423, 329 and 287. NMR analyses of these four compounds were not possible due to the small quantities obtained. However, cyanidin-3-*O*-galactoside could be isolated in a high purity of 98% in fraction 2 (56 mg), subsequently cyanidin-3-*O*-glucoside eluted in a purity of 41% in fraction 3 (8.3 mg). In fraction 4 (17 mg), cyanidin-3-*O*-arabinoside was identified in a high purity of 81% and cyanidin-3-*O*-xyloside eluted in F5 (purity: 42%).

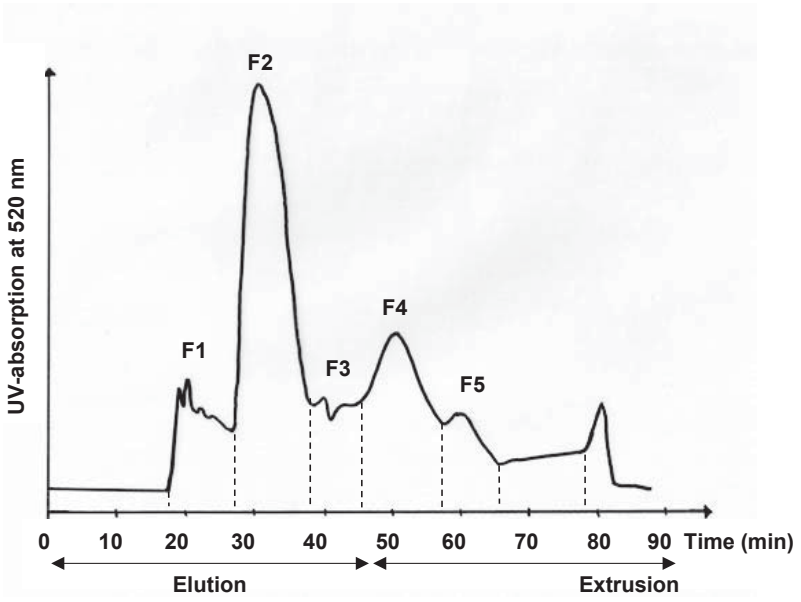


Fig. 3.19. HPCCC chromatogram of anthocyanin fraction from *Aronia melanocarpa* A20 extract at 520 nm.

3.4 Sour cherries

3.4.1 Chemical composition

Sour or tart cherry (*Prunus cerasus L.*), which is a stone fruit, is one of the most produced cherry species worldwide. In recent years, studies on anthocyanins and polyphenols in general have revealed their antioxidant activity and their possible use in the prevention and treatment of diseases such as cancer, diabetes and cardiovascular diseases, which are generated by oxidative stress. Sour cherries are a source of phenolic compounds like phenolic acids, anthocyanins, flavonols and flavan-3-ols. In the work described here, a characterization of phenolic composition in Actiplants® Cherry Purevital extract, which is a high-purified sour cherry extract, was performed, as well as a comparison with sour cherry products. Subsequently the separation and isolation and large scale of polyphenols by membrane chromatography was carried out.

As shown in Fig. 3.20, the results confirmed the presence of anthocyanins that have absorption maxima at 280 nm and 520 nm. In particular sour cherries are characterized by cyanidin-3-(2^{G}-glucosylrutinoside) as major anthocyanin with around 64% of total anthocyanins (Peak 3), followed by cyanidin-3-rutinoside (Peak 7) with approximately 15%, while sweet cherries are characterized by the presence of cyanidin-3-rutinoside and cyanidin-3-glucoside as main anthocyanins (Mozetič and Trebše, 2004; Mozetič et al., 2006).

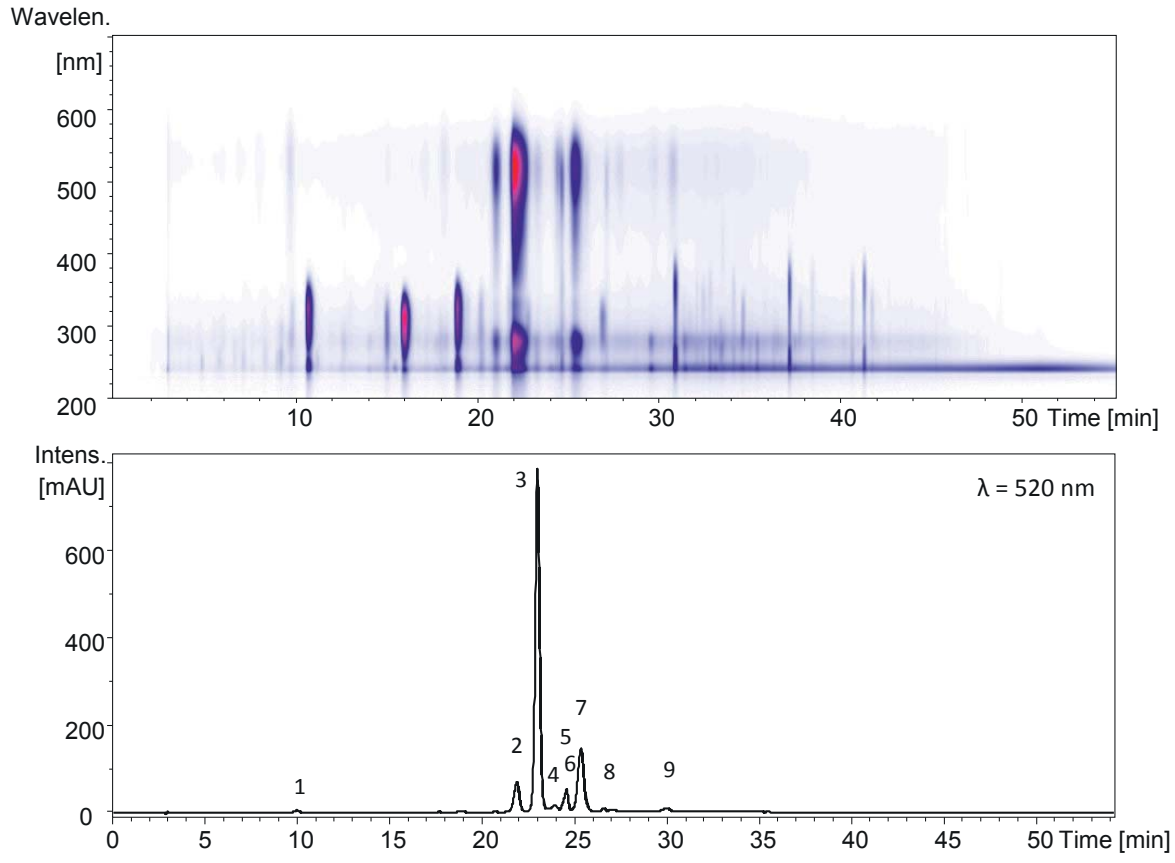


Fig. 3.20. HPLC-DAD-Contour-Plot chromatogram and Base Peak UV chromatogram at 520 nm of sour cherry extract Actiplants® Cherry Purevital. (For peak numbers see Table 3.8).

Other detected anthocyanins were cyanidin-3-sophoroside (Peak 2), cyanidin-3-glucoside (Peak 4), cyanidin-3-(2^G-xylosylrutinoside) (Peak 5), peonidin-3-*O*-rutinoside (Peak 9), as well as a low concentration of flavan-3-ol-cyanidin-3-(2^G-glucosylrutinoside) (Peak 1), 5-carboxypyranocyanidin-3-*O*-(2^G-glucosylrutinoside) (Peak 6) and 5-carboxypyrano-cyanidin-3-rutinoside (Peak 8). Table 3.8 gives a summary of anthocyanins detected, retention times and LC-MS data of Actiplants® Cherry Purevital.

Table 3.8. LC-MS^n data of anthocyanins in sour cherry extract Actiplants® Cherry Purevital.

Peak	t_R (min)	Compound	$[M]^+$	Fragment ions (*m/z*)
1	10.1	Flavan-3-ol-cyanidin-3-(2^G-glucosylrutinoside)	1045	899, 575
2	22.2	Cyanidin-3-sophoroside	611	287
3	23.2	Cyanidin-3-(2^G-glucosylrutinoside)	757	287
4	24.2	Cyanidin-3-glucoside	449	287
5	24.5	Cyanidin 3-(2^G-xylosylrutinoside)	727	581, 287
6	24.8	5-Carboxypyranocyanidin-3-*O*-(2^G-glucosylrutinoside)*	825	355
7	25.4	Cyanidin-3-*O*-rutinoside	595	449, 287
8	26.9	5-Carboxypyrano-cyanidin-3-rutinoside	663	517, 355
9	30.1	Peonidin-3-*O*-rutinoside	609	463, 301

*traces

A flavan-3-ol-cyanidin-3-(2^G-glucosylrutinoside) was detected, which is described as condensation product of a flavan-3-ol (catechin or epicatechin) and its concentration can increase during storage. The flavan-3-ol-anthocyanins have been reported in strawberries and black currant juices (Fossen et al., 2004; McDougall et al., 2005). Additionally, two pyranoanthocyanins were identified (Fig. 3.21). These compounds are formed from the reaction between pyruvic acid and anthocyanins. In the present case, 5-carboxypyranocyanidin-3-*O*-(2^G-glucosylrutinoside) is generated by reaction of the principal compound cyanidin-3-(2^G-glucosylrutinoside) with pyruvic acid in sour cherries (Bonerz et al., 2007; Rentzsch et al., 2007a).

According to Symrise (2013), one gram of Actiplants® Cherry Purevital extract contains anthocyanins from approximately 72 cherries, which are approximately equivalent to 305 g of fresh fruit and possess a high concentration of anthocyanins. The quantification of anthocyanins is given in Table 3.9, calculated as mg cy-3-glc/30 g extract, corresponding to the content of an "Actiplants® Cherry Purevital extract" packet. In addition, the anthocyanin content of this extract was compared with other sour cherry products like direct juice, juice (45% fruit content) and frozen sour cherries. As a result, Actiplants® Cherry Purevital extract presented the highest anthocyanins content, while the juice with 45% fruit content showed the lowest concentration of anthocyanins. In all cases, cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-rutinoside were the main anthocyanins that characterize the sour cherry.

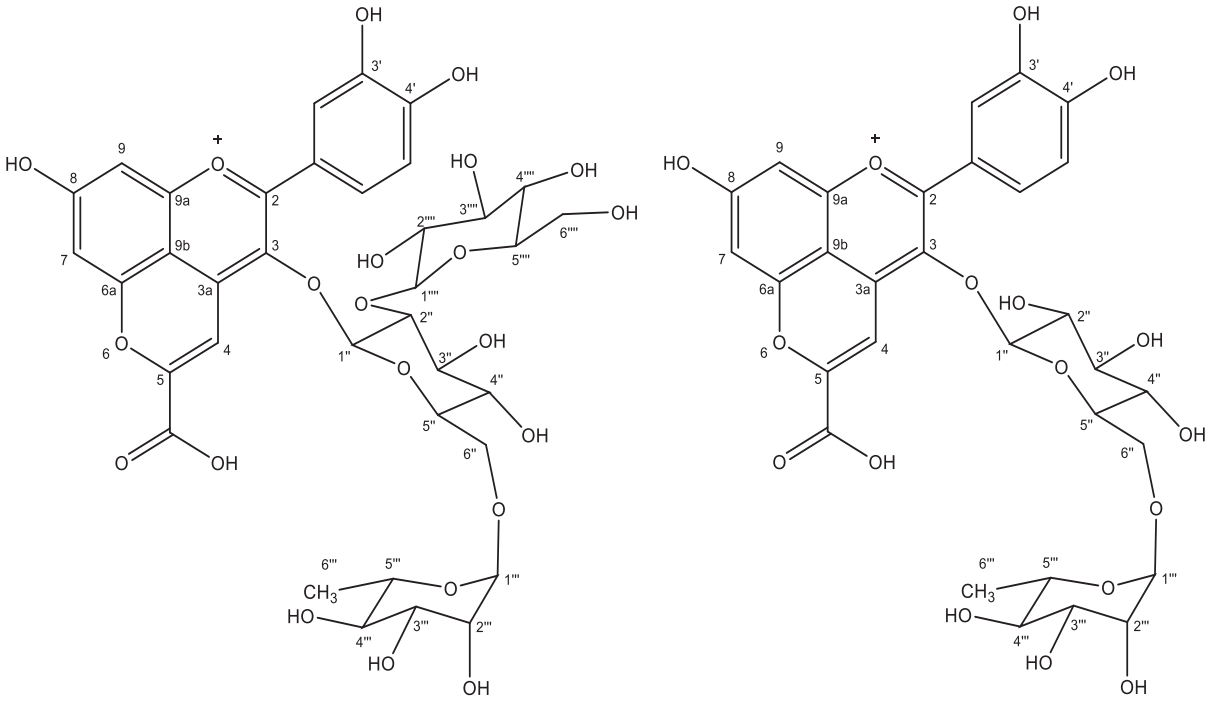


Fig. 3.21. 5-Carboxypyrano-cyanidin-3-(2^{G}-glucosylrutinoside) (left) and 5-Carboxypyrano-cyanidin-3-rutinoside (right).

Table 3.9. Anthocyanin content in cherry products.

	Anthocyanin Content *			
Compound	Actiplants® Cherry Purevital (mg/30g)	Frozen cherries (mg /L)	Direct Juice (mg /L)	Juice (45%) (mg /L)
Flavan-3-ol-cyanidin-3-(2^{G}-glucosylrutinoside)	502.37	-	-	-
Cyanidin-3-sophoroside	1901.90	46.41	11.02	4.5
Cyanidin-3-(2^{G}-glucosylrutinoside)	18523.77	553.7	95.12	31.35
Cyanidin-3-glucoside	676.11	-	-	-
Cyanidin 3-(2^{G}-xylosylrutinoside)	1079.36	27.01	11.33	5.77
5-Carboxypyranocyanidin-3-*O*-(2^{G}-glucosylrutinoside)	-	22.74	7.02	4.39
Cyanidin-3-rutinoside	4365.29	135.94	22.22	9.24
5-Carboxypyrano-cyanidin-3-rutinoside	241.15	-	-	-
Peonidin-3-rutinoside	375.73	-	-	-

*Calculated as mg of cyanidin-3-glucoside

In order to detect compounds like copigments, the extract was analyzed at 280 nm, 320 nm and 360 nm, as shown in Fig. 3.22. Previous reports of cherries described phenolic acids such as hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives, as well as some kaempferol derivatives,

quercetin derivatives and flavan-3-ols have also been detected (Chaovanalikit and Wrolstad, 2004; Stéger-Máté, 2012; Toydemir et al., 2013b).

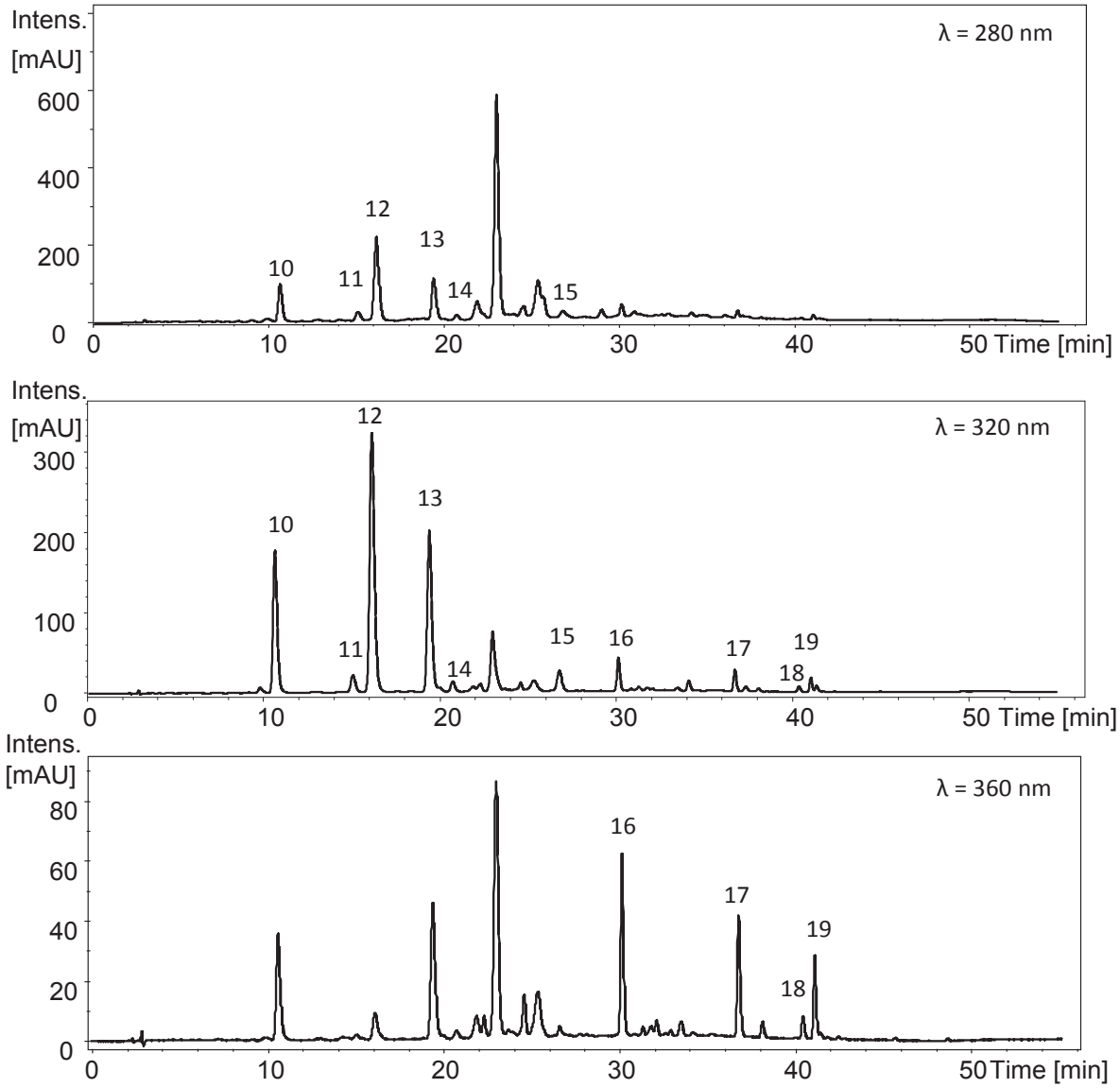


Fig. 3.22. Base Peak UV Chromatogram at 280 nm (at the top), 320 nm (in the middle) and 360 nm (at the bottom) of sour cherry extract. Peak numbering according to Table 3.10.

Actiplants® Cherry Purevital extract presented a high concentration of 3-caffeoylquinic acid (3-CQA, peak 10) and 5-caffeoylquinic acid (5-CQA, peak 13), as well as a low concentration of 4-caffeoylquinic acid (4-CQA, Peak 14) that were detected at 320 nm. To identify these compounds, a comparison with authentic standards was achieved, additionally chlorogenic acids are characterized by $[M-H]^-$ *m/z* 353 and they could be differentiated by their fragmentation according to the hierarchical scheme for LC-MS^n identification of chlorogenic acids (Clifford et al., 2003). Other main phenolic acid was 3-p-coumaroylquinic acid (3-p-CoQA, peak 12) and as minor compounds 5-p-CoQA (Peak 11) and 4-p-CoQA (Peak 15) were detected with $[M-H]^-$ *m/z* 337, as well as traces of di-QCA that had $[M-H]^-$ *m/z* 515 (Wojdyło et al., 2014; Nowicka et al., 2015). Their LC-MS data are shown in Table 3.10 and

the quantification of phenolic acids is presented in Table 3.11. Moreover, sour cherries contain flavonols that were identified at 360 nm. Particularly quercetin-3-glucosylrutinoside (Peak 16), quercetin-3-rutinoside (Peak 17), kaempferol-3-rutinoside (Peak 18) and isorhamnetin-3-rutinoside (Peak 19). Significant amounts of isorhamnetin-3-rutinoside have been reported in sour cherries Balaton and Montmorency varieties (Macheix et al., 1990; Kirakosyan et al., 2009; Stéger-Máté, 2012). The quantification of flavonols is shown in Table 3.12. Furthermore, flavan-3-ols, like (+)-catechin and (-)-epicatechin were detected as traces, which could not be quantified. However, the content of (-)-epicatechin was higher than (+)-catechin, which is a characteristic tendency of sour cherry and the same trend has been reported in sweet cherry (Macheix et al., 1990). Traces of three procyanidin isomers, which had a molecular ion at *m/z* 577 $[M-H]^-$ and fragment ions at *m/z* = 407, 425, 451 and 289, were detected corresponding to procyanidin B1, B2 and B5 or B7, while traces of procyanidin C1 presented a molecular ion at *m/z* 865 $[M-H]^-$ and fragment ions at *m/z* = 739, 713, 695, 577, 575, 451, 425, 407 and 287. It is known that sour cherries like Montmorency variety and cherry pits contain procyanidins (Chaovanalikit, 2003; Capanoglu et al., 2011). A summary of phenolic acids, flavonols and flavanols is shown in Table 3.10.

Table 3.10. LC-MS data of copigments in Actiplants® Cherry Purevital extract.

Peak	Compound	$[M-H]^-$	Fragments ions (*m/z*)
10	3-caffeoylquinic acid (3-CQA)	353	191, 179, 173, 135
11	5-p-coumaroylquinic acid (5-p-CoQA)	337	163, 191
12	3-p-coumaroylquinic acid (3-p-CoQA)	337	163
13	5-caffeoylquinic acid (5-CQA)	353	191, 179, 173, 135
14	4-caffeoylquinic acid (4-CQA)	353	191, 179, 173, 135
15	4-p-coumaroylquinic acid (4-p-CoQA)	337	173, 163
16	Quercetin-3-glucosylrutinoside	771	609, 301
17	Quercetin-3-rutinoside	609	463, 301
18	Kaempferol-3-rutinoside	593	285
19	Isorhamnetin-3-rutinoside	623	315, 300, 271, 255
	diCaffeoylquinic acid (di-QCA)*	515	353, 191, 179, 135
	Procyanidin B1*	577	407, 425, 451, 289
	(+)-catechin*	289	245
	Procyanidin B2*	577	407, 425, 451, 289
	(-)-epicatechin*	289	245
	Procyanidin C1*	865	739, 713, 695, 577, 575, 451, 425, 407, 287
	Procyanidin B5 or B7*	577	407, 425, 451, 289

*Traces; Peak numbering according to Fig. 3.22.

Table 3.11. Quantification of chlorogenic acids in Actiplants Cherry Purevital.

Peak	Compound	Conc. (mg 5-CQA/g extract)	Conc. (mg 5-CQA/30g extract)
10	3-caffeoylquinic acid (3-CQA)	66.43	1992.79
11	5-p-coumaroylquinic acid (5-p-CoQA)	1.19	35.82
12	3-p-coumaroylquinic acid (3-p-CoQA)	78.13	2343.80
13	5-caffeoylquinic acid (5-CQA)	109.06	3271.78
14	4-caffeoylquinic acid (4-CQA)	1.91	57.25
15	4-p-coumaroylquinic acid (4-p-CoQA)	10.35	310.38

* determined as mg chlorogenic acid (5-CQA) at 324 nm; Peak numbering according to Fig. 3.22 and Table 3.10.

Table 3.12. Quantification of flavonols in Actiplants Cherry Purevital.

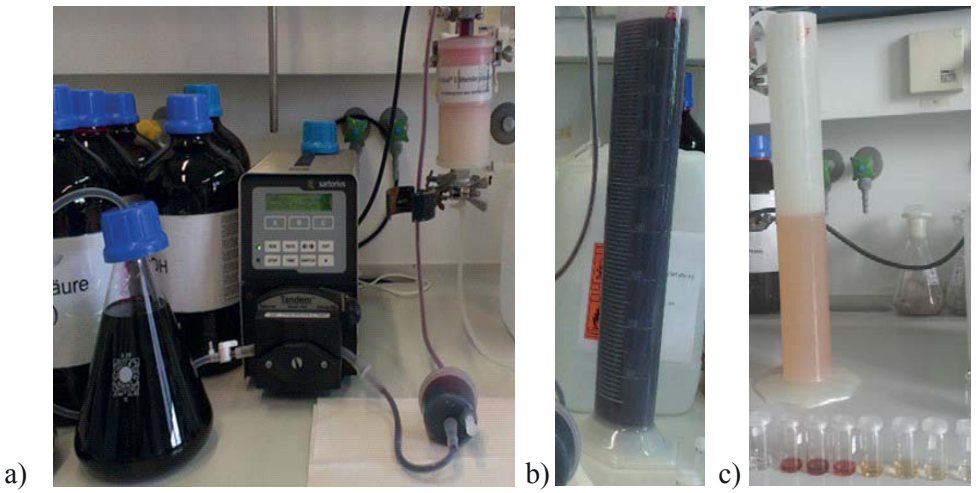
Peak	Compound	Conc. (mg quercetin/g extract)	Conc. (mg quercetin/30g extract)
16	Quercetin-3-glucosylrutinoside	7.57	227.24
17	Quercetin-3-rutinoside	4.59	137.68
18	Kaempferol-3-rutinoside	0.11	3.22
19	Isorhamnetin-3-rutinoside	3.06	91.97

Concentration calculated as mg quercetin at 374 nm; Peak numbering according to Fig. 3.22 and Table 3.10.

3.4.2 Preparative separations

3.4.2.1 Membrane chromatography

The separation of polyphenols from sour cherry extract was performed by membrane chromatography (see section 4.6.5). Actiplants® Cherry Purevital extract (10 g) was dissolved in MeOH:Acetic acid (19:1, v/v) and then loaded on the membrane adsorber (Fig. 3.23). Whereas anthocyanins were retarded, copigments were eluted obtaining the copigment fraction. After that, anthocyanins were eluted with a mixture of aqueous 1M NaCl solution and methanol (1:1, v/v). Subsequently the identification of individual compounds was carried out by HPLC-DAD and HPLC-ESI-MS^n analyses. Due to the high concentration of anthocyanins in Actiplants® Cherry Purevital extract, the maximal capacity of the membrane adsorber was exceeded in the first separation, consequently a second separation was necessary in order to remove anthocyanins of the copigment fraction. In addition, the content of the Actiplants® Cherry Purevital extract packet was 30 g, therefore the separation was done in triplicate. After that, the membrane chromatographic method yielded a fraction rich in anthocyanins and a fraction rich in copigments (Fig. 3.24) as in the case of bilberry described by Juadjur and Winterhalter (2012). The yield of the anthocyanin fraction was 11.19 g, while 12.92 g were obtained as copigment fraction (Tab. 3.13).



a) b) c)

Fig. 3.23. a) Sample loading of Actiplants® Cherry Purevital extract b) elution of anthocyanins and c) elution of copigments after second separation.

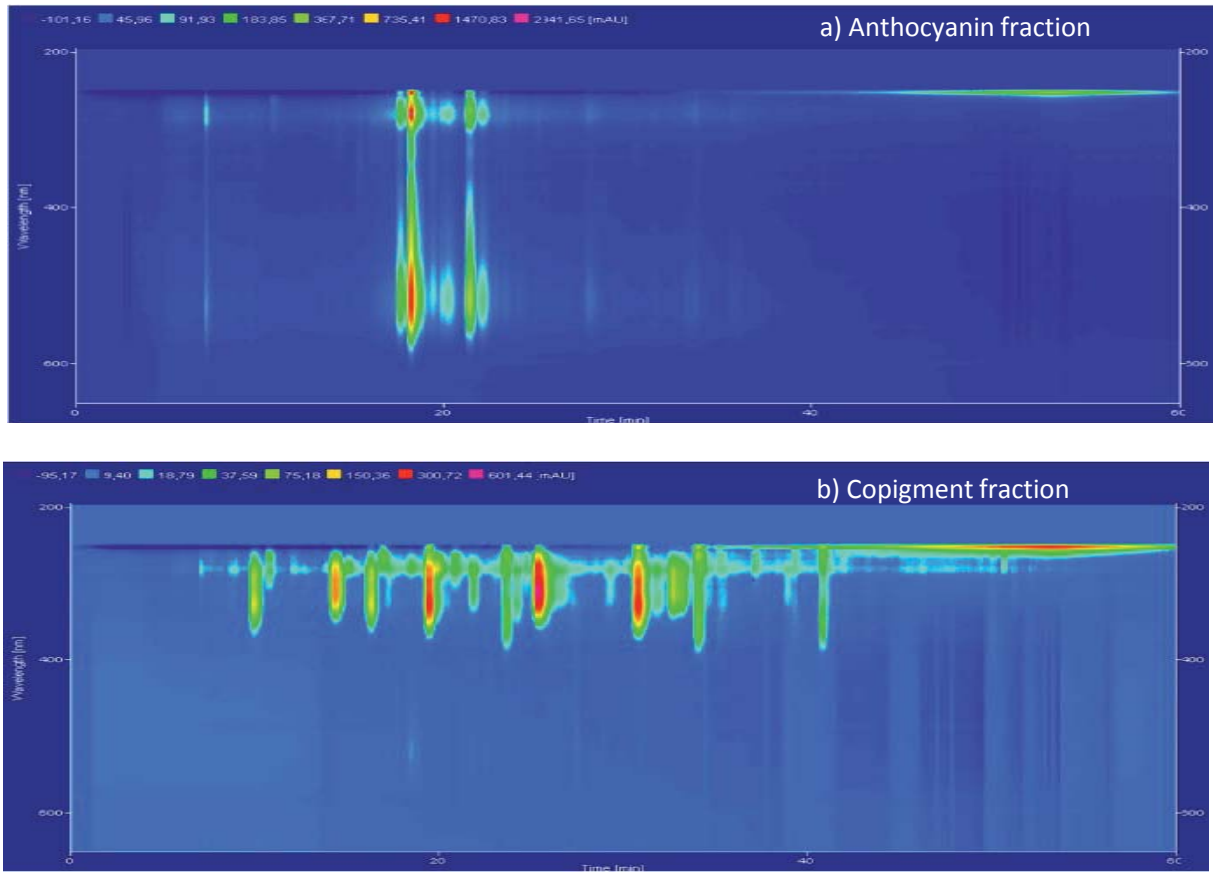


Fig. 3.24. DAD-Contour-Plot chromatogram of the anthocyanin fraction (a) and of the copigment fraction (b) from Actiplants® Cherry Purevital extract.

Table 3.13. Amount of obtained fractions from sour cherry extract after membrane chromatography.

Actiplants® Cherry Purevital (g)	Copigment-Fraction (g)	Anthocyanin-Fraction (g)	Polymer Fraction (g)
30	12.92	11.19	5.89

Anthocyanins can be differentiated from copigments, because they have absorption maxima at 280 nm and 520 nm, while copigments absorb between 200 nm and 400 nm. Therefore, a comparison of HPLC-DAD chromatograms at 280 nm and 520 nm can verify the presence or lack of anthocyanins in the copigment fraction. In the case of anthocyanin fraction (Fig. 3.25), anthocyanins like 5-carboxypyrano-cyanidin-3-rutinoside (peak 8) and peonidin-3-O-rutinoside (Peak 9) could be better identified, because their concentration increased, while the rest of peaks shows the same tendency as before.

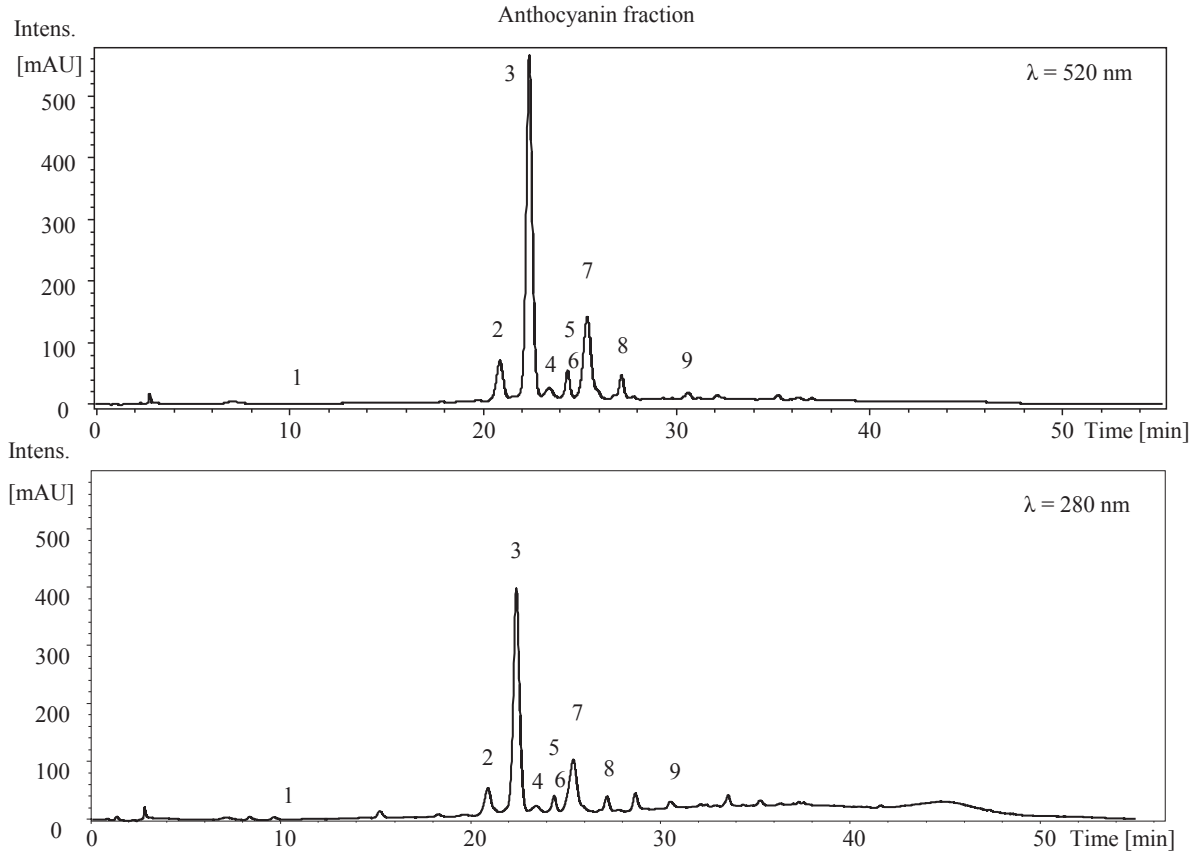


Fig. 3.25. Base Peak chromatogram at 520 nm and 280 nm of the anthocyanin fraction from sour cherry extract. For peak numbers see Table 3.8.

In the case of copigments, Fig. 3.26 shows that the copigment fraction presented a lack of anthocyanins at 520 nm. Consequently, the separation has been successfully performed. Some compounds such as (+)-catechin, (-)-epicatechin, procyanidins B1, B2 and C1 were detected better than before separation because their concentration has increased. In addition, some feruloylquinic acids (peak 20 and 21), which presented $[M-H]^-$ *m/z* 367, were detected at 320 nm in the copigment fraction (Fig. 3.27). Peak 20 was identified as 3-feruloy quinic acid (3-FQA), which had fragment ions at *m/z* 193 and 161, while Peak 21 is assigned as 5-feruloy quinic acid (5-FQA) (Macheix et al., 1990; Chaovanalikit, 2003).

Studies of sour cherries showed anti-inflammatory effects and that their polyphenols are involved in the prevention of degenerative diseases. The applied method allows the isolation of individual group of anthocyanins or copigments on a large scale, which are available for further biological studies with the

aim of identifying in which of the groups there is a significant anti-inflammatory effect or a positive effect against these diseases.

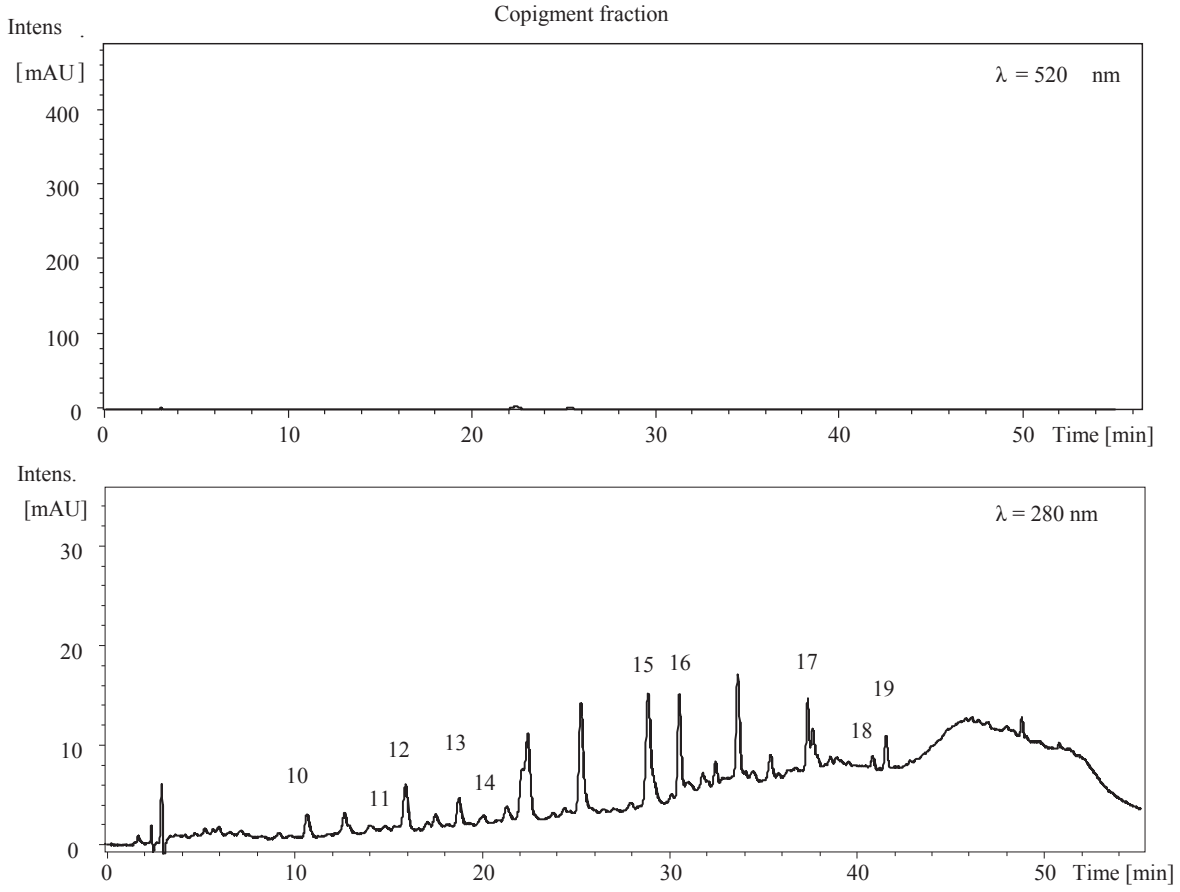


Fig. 3.26. Base Peak chromatogram at 520 nm and 280 nm of the copigment fraction from sour cherry extract. Peak numbering according to Table 3.10.

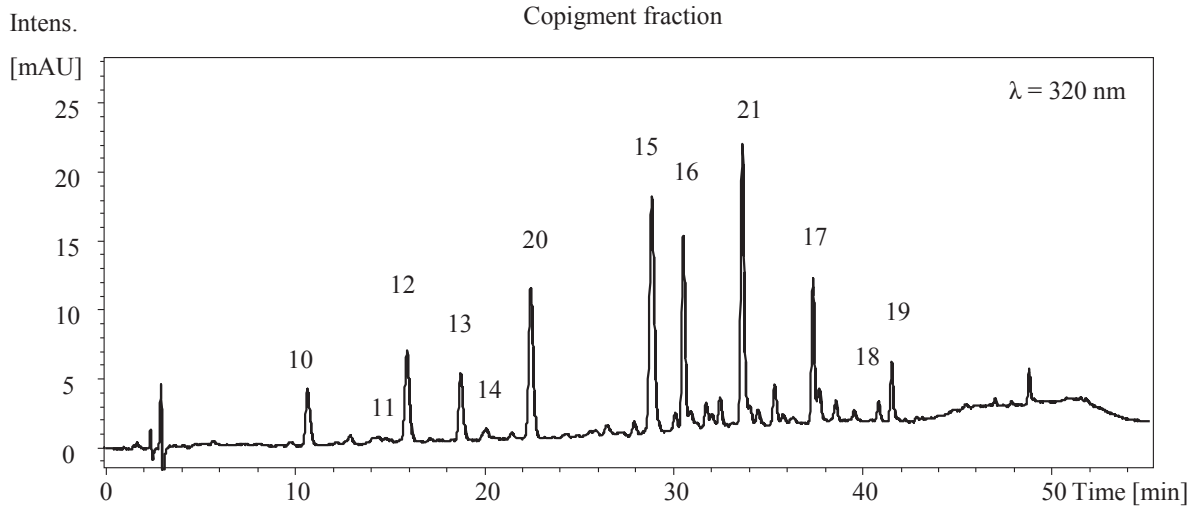


Fig. 3.27. Base Peak Chromatogram at 320 nm of the copigment fraction from sour cherry extract. Peak numbering (Peak 10-19) according to Table 3.10, while peak 20 and peak 21 are 3-FQA and 5-FQA, respectively.

3.5 Black carrots

3.5.1 Chemical composition

Black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) which comprises varieties such as Antonina, Deep Purple, Purple Sun, Purple Haze and Beta Sweet were cultivated in Osnabrück (Germany). The black carrot varieties, Antonina and Deep Purple, present a dark purple coloration with a white flesh, while Purple Sun contains an orange flesh (Fig. 3.28). The varieties Purple Haze and Beta Sweet are less darkened and a large part of them is orange. Black or purple carrots contain anthocyanins that give their characteristic dark color but are also source of phenolic acids, as shown in their DAD-Contour-Plot chromatograms (Fig. 3.28, left). In order to identify the characteristic anthocyanin profile of these black carrots, HPLC chromatograms of black carrots were recorded at 520 nm (Fig. 3.28, right), as well as HPLC-ESI-MS^n analyses were carried out. In concordance with the literature (Kammerer et al., 2004b; Schwarz et al., 2004; Elham et al., 2006; Montilla et al., 2011; Algarra et al., 2014; Gras et al., 2015), their anthocyanins are mainly based on cyanidin glycosides that displayed a fragment ion at *m/z* 287, which is specific for the cyanidin aglycone. Subsequently peonidin and pelargonidin derivatives were detected as minor compounds that have a fragment ion at *m/z* 301 corresponding to peonidin aglycone or a fragment ion at *m/z* 271 corresponding to pelargonidin aglycone, respectively. Some anthocyanins are acylated with phenolic acids such as hydroxycinnamic and hydroxybenzoic acids, thus enhancing the pH-stability (Malien-Aubert et al., 2001).

In the case of Antonina, Deep Purple and Purple Sun, cyanidin-3-xylosyl(feruloylglucosyl)galactoside (Peak 5) was found as the main anthocyanin with a pseudomolecular ion at *m/z* 919, while Purple Haze and Beta Sweet presented cyanidin-3-xylosyl(sinapoylglucosyl)galactoside (Peak 4, $[M]^+$ *m/z* 949) together with cyanidin-3-xylosyl(feruloylglucosyl)galactosid (Peak 5) as the main anthocyanins. Cyanidin-3-xylosyl-glucosyl-galactoside (Peak 1, $[M]^+$ *m/z* 743) was identified as minor compound in all varieties, as well as cyanidin-3-xylosyl-glucoside (Peak 2, $[M]^+$ *m/z* 581) but this was the second main anthocyanin in Antonina variety. Cyanidin-3-xylosyl(p-hydroxybenzoylglucosyl)galactoside (Peak 3, $[M]^+$ *m/z* 863) was present in small amounts in Antonina, Purple Haze and Beta Sweet, while cyanidin-3-xylosyl(coumaroylglucosyl)galactoside (Peak 6, $[M]^+$ *m/z* 889) was identified in all black carrots with the exception of Purple Sun. On the other hand, Deep Purple carrot presented traces of peonidin-3-xylosyl-galactoside (Peak 8) with a molecular ion $[M]^+$ *m/z* 595. Traces of peonidin-3-xylosyl(sinapoylglucosyl)galactoside (Peak 9, $[M]^+$ *m/z* 963) and peonidin-3-xylosyl(feruloylglucosyl)-galactoside (Peak 10, $[M]^+$ *m/z* 933) were found in Beta Sweet carrot. In the case of Antonina, small amounts of pelargonidin-3-xylosyl(feruloylglucosyl)galactoside (Peak 7) were detected with $[M]^+$ *m/z* 903. Moreover, the highest anthocyanin content was present in black carrots Deep Purple and Antonina, which possess a dark color, while Beta Sweet contained the lowest amounts of anthocyanins. The identification of anthocyanins and their mass spectrometric data are summarized in Table 3.14, as well as the quantification of anthocyanins in Table 3.15.

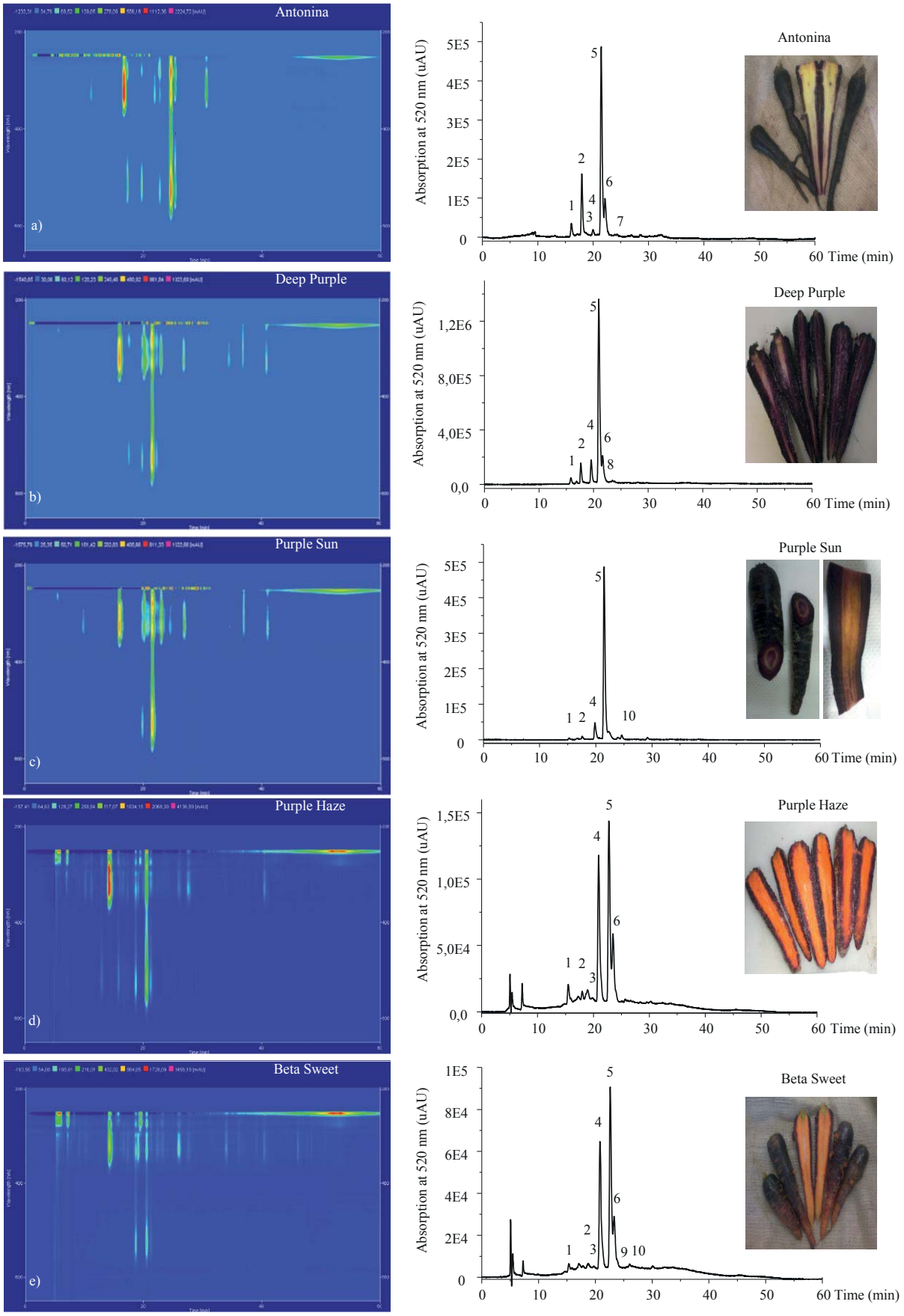


Fig. 3.28. DAD-Contour-Plot chromatograms (left) and HPLC chromatograms at 520 nm (right) for detection of anthocyanins of black carrots: a) Antonina, b) Deep Purple, c) Purple Sun, d) Purple Haze and e) Beta Sweet. Peak numbering according to Table 3.14.

Table 3.14. LC-MS data of the main polyphenols in black carrots.

Peak	Compound	$[M]^+$	Fragment ions (*m/z*)
1	Cyanidin-3-xylosyl(glucosyl)galactoside	743	287
2	Cyanidin-3-xylosylgalactoside	581	287
3	Cyanidin-3-xylosyl(p-hydroxybenzoylglucosyl)galactoside	863	287
4	Cyanidin-3-xylosyl(sinapoylglucosyl)galactoside	949	287
5	Cyanidin-3-xylosyl(feruloylglucosyl)galactoside	919	287
6	Cyanidin-3-xylosyl(coumaroylglucosyl)galactoside	889	287
7	Pelargonidin-3-xylosyl(feruloylglucosyl)galactoside	903	271
8	Peonidin-3-xylosylgalactoside	595	301
9	Peonidin-3-xylosyl(sinapoylglucosyl)galactoside	963	301
10	Peonidin-3-xylosyl(feruloylglucosyl)galactoside	933	301

Table 3.15. Quantification of anthocyanins in black carrots.

Peak	Compound	Concentration (mg/kg)*				
		Deep Purple	Antonina	Purple Sun	Purple Haze	Beta Sweet
1	Cy-3-xyl-(glc)-gal	38.30	29.8	11.19	25.68	6.2
2	Cy-3-xyl-gal	93.60	112.5	15.46	10.39	**
3	Cy-3-xyl-(p-hydroxybenzoyl-glc)-gal	-	19.6	-	5.95	**
4	Cy-3-xyl-(sinapoyl-glc)-gal	114.00	8.3	51.79	43.25	43.7
5	Cy-3-xyl-(feruloyl-glc)-gal	874.00	325.4	422.45	242.21	55.0
6	Cy-3-xyl-(coumaroyl-glc)-gal	166.70	84.5	-	37.41	17.3
7	Pg-3-xyl-(feruloyl-glc)-gal	-	9.7	-	-	-
8	Pn-3-xyl-gal	**		-	-	-
9	Pn-3-xyl-(sinapoyl-glc)-gal	-	-	-	-	**
10	Pn-3-xyl-(feruloyl-glc)-gal	-	-	**	-	**

*calculated as mg cyanidin-3-glucoside equivalent per kg carrots at 520 nm.
** Traces.

With respect to the characterization of individual phenolic acids, Fig. 3.29 shows HPLC chromatograms of black carrots at 280 nm in which chlorogenic acid (Peak 12) was found as predominant copigment in all black carrot varieties, but also neochlorogenic acid (Peak 11) and cryptochlorogenic acid (Peak 13) were detected. These chlorogenic acids are characterized by the presence of a pseudomolecular ion $[M-H]^-$ at *m/z* 353 and can be differentiated by $LC\text{-}MS^n$ fragmentation (Clifford et al., 2003).

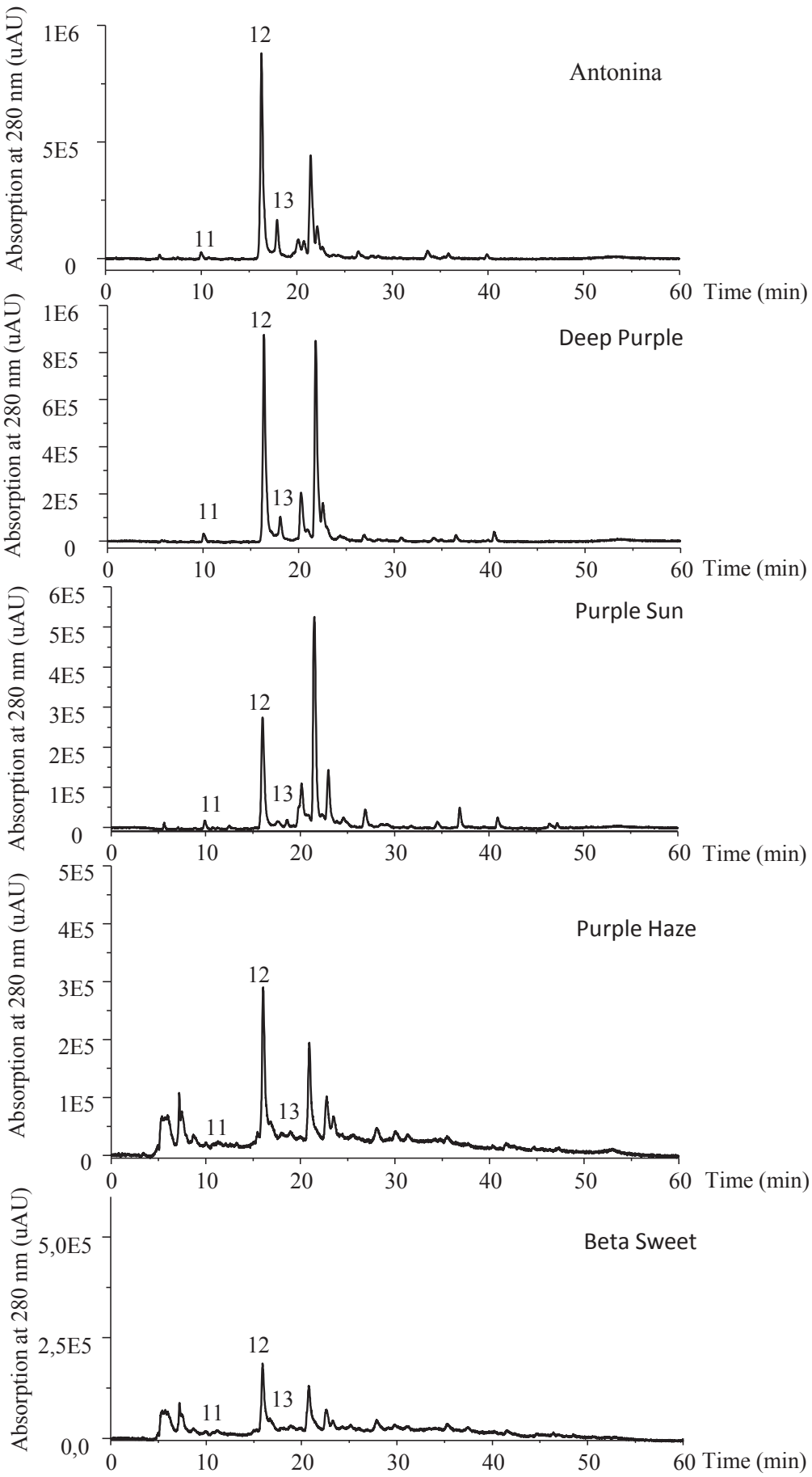


Fig. 3.29. HPLC chromatograms of black carrots at 280 nm. For peak numbering, cf. Table 3.16.

Moreover, Antonina variety presented the highest quantity of chlorogenic acids, while Beta Sweet had the smallest quantity (Table 3.16). In addition, these results coincide with previous studies of black carrots in which 5-CQA was detected as the most abundant phenolic acid (Alasalvar et al., 2001; Kammerer et al., 2004a; Suzme et al., 2014).

Table 3.16. LC-MS data and quantification of main chlorogenic acids in black carrots.

Peak	Compound	$[M-H]^-$	Fragment ions (*m/z*)	Concentration (mg /100g)*				
				DP	A	PS	PH	BS
11	3-CQA	353	191, 179, 135	10.23	6.89	14.16	4.95	5.24
12	5-CQA	353	191, 179	61.66	95.03	59.19	78.54	23.41
13	4-CQA	353	191, 179, 173, 135	15.86	5.63	15.11	4.81	5.02

* mg chlorogenic acid (5-CQA) per 100 g carrot at 324 nm; DP = Deep Purple; A= Antonina; PS = Purple Sun; PH = Purple Haze; BS = Beta Sweet.

Nonetheless, Alasalvar et al. (2001) and Kammerer et al. (2004a) detected p-coumaric, caffeic and ferulic acids as minor compounds. For example, compounds with a $[M-H]^-$ ion at *m/z* 367 and fragment at *m/z* 191 were found to correspond to feruloylquinic acids (3-,4- or 5-FQA). Additionally, dicaffeoylquinic acids such as 3,5-di-CQA and 4,5-di-CQA were identified with $[M-H]^-$ ion at *m/z* 515, as well as diferuloylquinic acids like 3′,4′-diferuloylquinic acid and 3′,5′-diferuloylquinic acid with a $[M-H]^-$ ion at *m/z* 543. In addition, p-coumaroylquinic acids (3-pCoQA and 5-pCoQA) were present with a $[M-H]^-$ ion at *m/z* 337. However, there is limited information about individual phenolic acids and future studies are required for the identification, isolation and confirmation of their chemical structure.

3.5.2 Preparative separations

3.5.2.1 Membrane chromatography

Black carrots (around 900 g) were washed, dried, cut and then their polyphenols extracted according to method 1 (see section 4.3). After adsorption onto Amberlite XAD-7 column (see section 4.4), elution yielded around 2-3 grams of extracts. Antonina XAD-7 extract was constituted of 34% anthocyanins and 66% copigments, while Deep Purple XAD-7 extract contained 53% anthocyanins and 47% copigments. In the case of Purple Sun XAD-7 extract, 51% were anthocyanins and 49% copigments.

On the other hand, two grams of black carrot XAD-7 extract from Antonina, Deep Purple or Purple Sun cultivars were separated into an anthocyanin and a copigment fraction by membrane chromatography (see section 4.6.5). After this procedure, a comparison of DAD-Contour-Plot chromatograms of the XAD-7 extract, anthocyanin fraction and copigment fraction was carried out in order to verify their separation (Fig. 3.30). Additionally, the comparison of HPLC chromatograms at 520 nm and 280 nm of anthocyanin fraction and copigment fraction from Deep Purple (Fig. 3.31),

Antonina (Fig. 3.32) and Purple Sun carrot (Fig. 3.33) also demonstrated that their copigment fractions had a complete lack of anthocyanins.

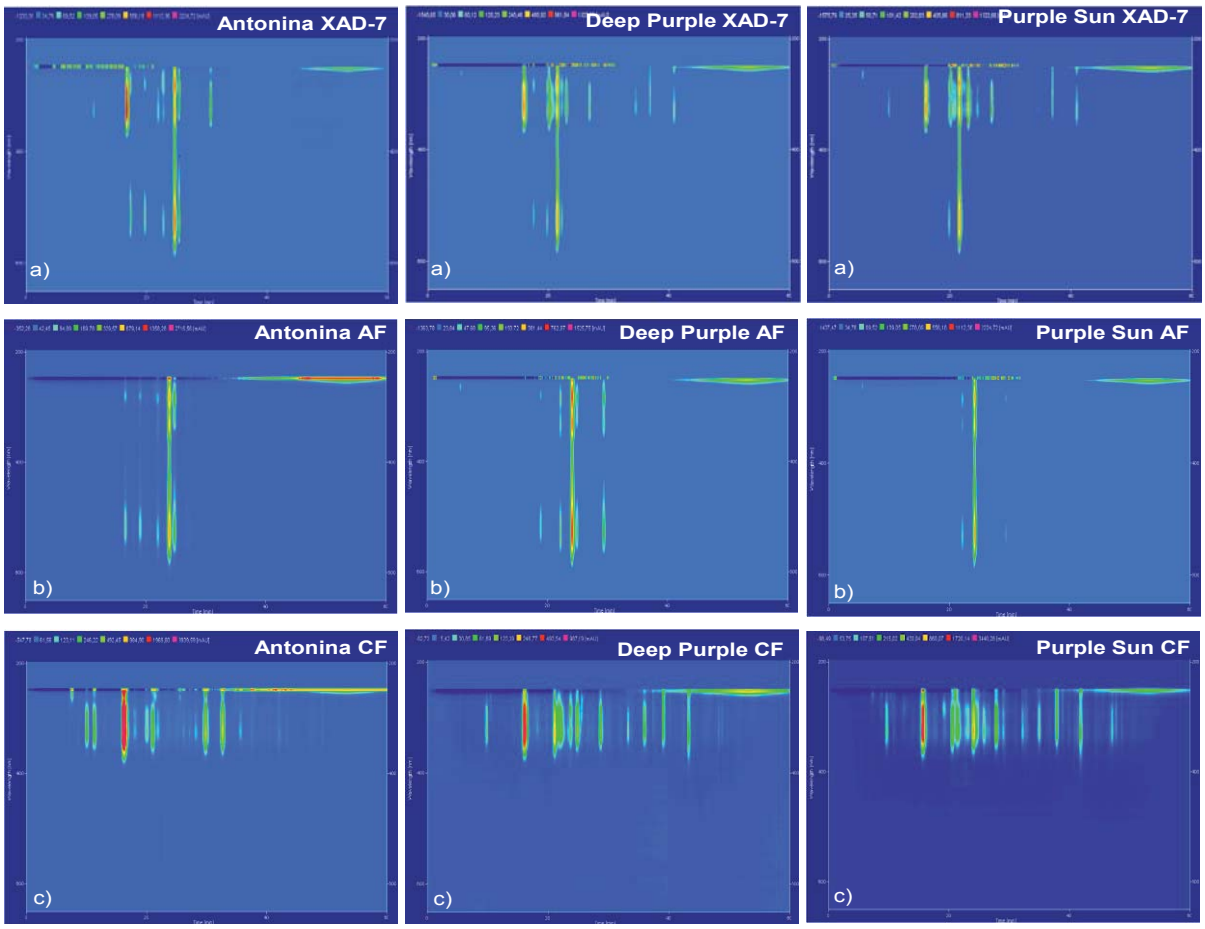


Fig. 3.30. DAD-Contour-Plot chromatograms of the XAD-7 extract, anthocyanin fraction and copigment fraction of Antonina, Deep Purple and Purple Sun carrots.

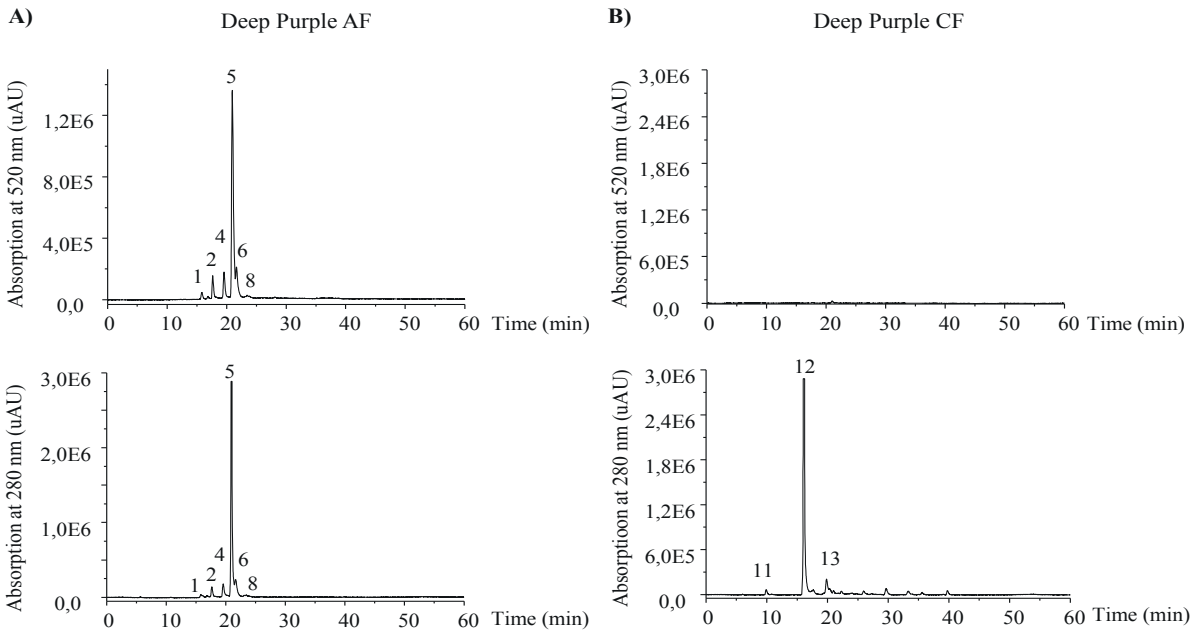


Fig. 3.31. HPLC chromatograms of anthocyanin fraction (A) and copigment fraction (B) from Deep Purple black carrot at 520 nm and 280 nm. Peak numbering according to Table 3.14 and Table 3.16.

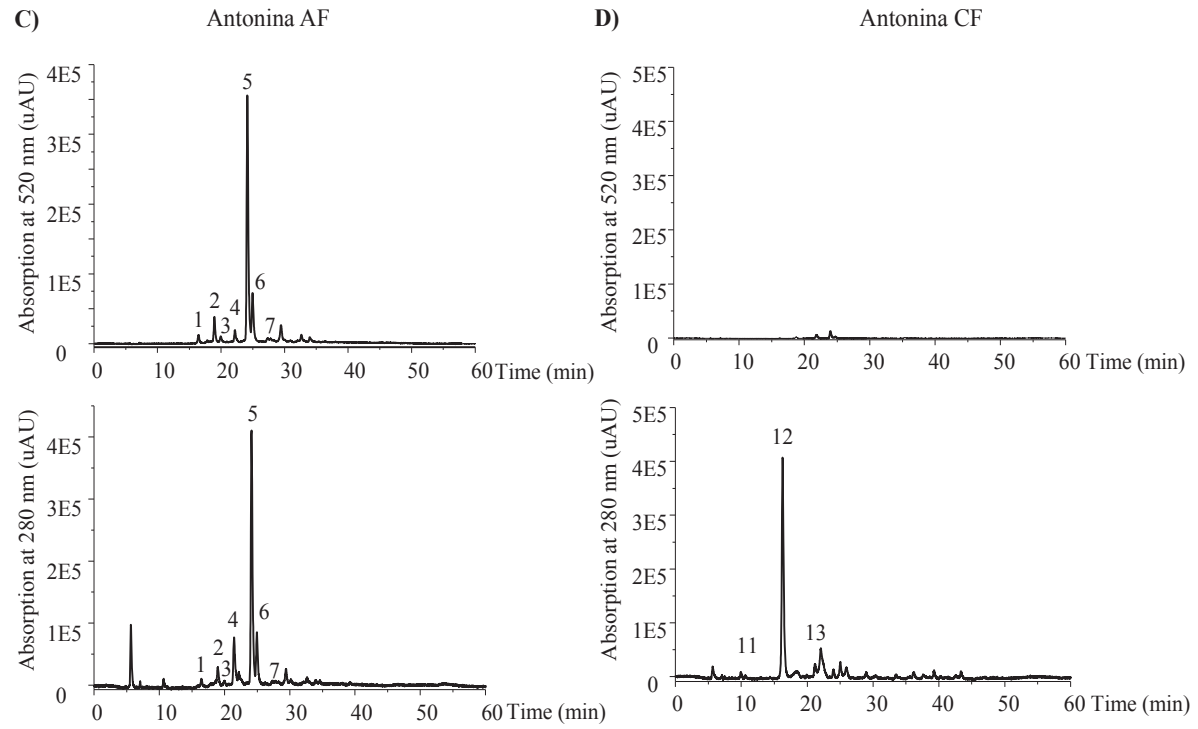


Fig. 3.32. HPLC chromatograms of anthocyanin fraction (C) and copigment fraction (D) from Antonina black carrot at 520 nm and 280 nm. Peak numbering according to Table 3.14 and Table 3.16.

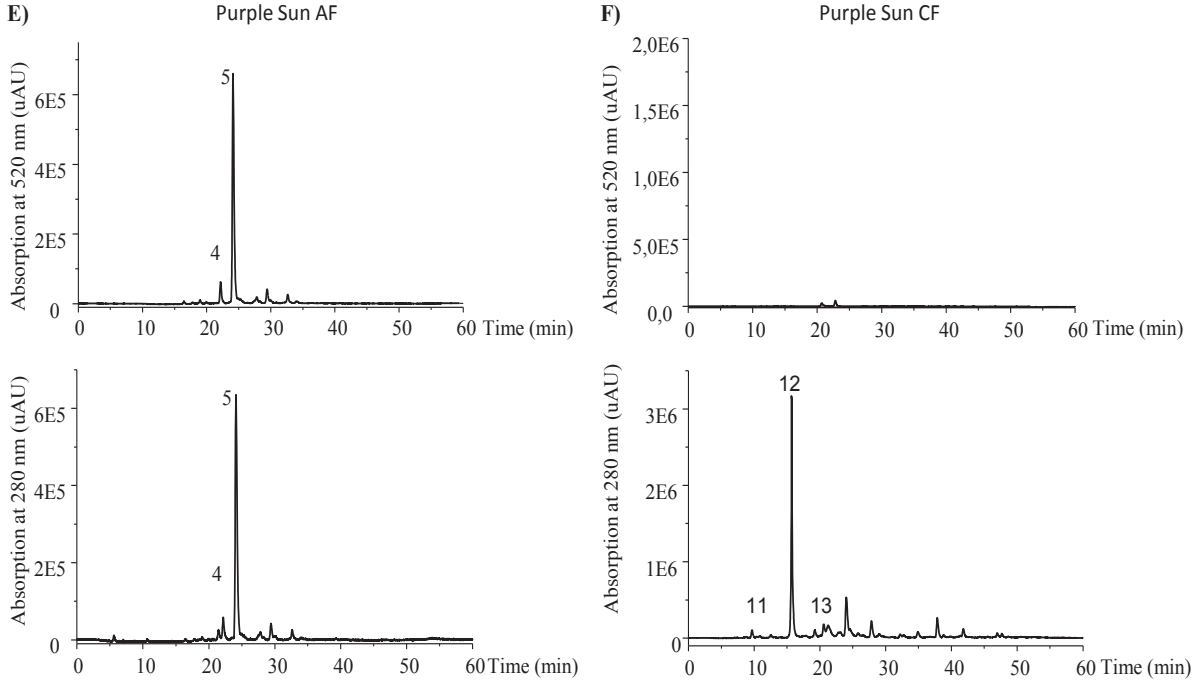


Fig. 3.33. HPLC chromatograms of anthocyanin fraction (E) and copigment fraction (F) from Purple Sun black carrot at 520 nm and 280 nm. Peak numbering according to Table 3.14 and Table 3.16.

3.5.2.2 Countercurrent Chromatography

After separation of black carrot XAD-7 extracts into a copigment fraction and an anthocyanin fraction by membrane chromatography, the separation and isolation of compounds were performed by HSCCC. The obtained fractions provided anthocyanins or copigments in a high purity. Moreover, the fractions were analyzed by TLC, HPLC-DAD and LC-ESI-MS^n in order to identify compounds.

HSCCC separation of anthocyanin fraction from black carrots

Previous studies showed a separation of black carrot XAD-7 extracts that were obtained from juice (500 mL) or concentrates. The XAD-7 extract obtained from juice was separated by HSCCC (Schwarz et al., 2004), while 15 g of XAD-7 extract obtained from concentrates was fractionated by LSRCCC (Hillebrand et al., 2009a). In both cases, a two solvent system consisting of tert-butyl methyl ether/n-butanol/acetonitrile/water (1:3:1:5, v/v/v/v) acidified with 0.1% TFA was used.

In this case, approximately 500 mg of anthocyanin fraction from Antonina, Deep Purple or Purple Sun carrots were fractionated by HSCCC using the same solvent system. The separation was carried out in Head to Tail mode with a rotational speed of 900 rpm. The flow rate was 3 mL/min and the fraction collector was set at 4 min/tube. Anthocyanins were monitored at 520 nm, as shown in Fig. 3.34.

At the beginning of the HSCCC separation, a non-acylated anthocyanin was eluted and presented a pseudomolecular ion at *m/z* 743 and fragments ion at *m/z* 287. This compound corresponds to cyanidin-3-xylosyl(glucosyl)galactoside. Subsequently, an acylated anthocyanin with sinapic acid eluted and was identified as cyanidin-3-xylosyl(sinapoylglucosyl)galactoside with a pseudomolecular ion at *m/z* 949 and fragments ion at *m/z* 287. This anthocyanin was present in high quantity in Deep Purple and Purple Sun, while it was present in small amounts in Antonina.

Cyanidin-3-xylosyl(feruloylglucosyl)galactoside was identified as the main anthocyanin with a pseudomolecular ion at *m/z* 919 and fragments ion at *m/z* 287 in the three varieties. According to Schwarz et al. (2004), the elution of the main anthocyanin was between 3.3 and 4 hours with a flow rate of 3.5 mL/min, while in our case the flow rate was reduced and the main anthocyanin eluted approximately between 3.6 and 4.5 h and was obtained in a high purity (~71-90%).

Additionally, in the same fraction co-eluted cyanidin-3-xylosylgalactoside ($[M]^+$ at *m/z* 581 and fragment ion at *m/z* 287) in the three black carrot varieties. However, in the case of Antonina anthocyanin fraction, cyanidin-3-xylosyl(p-hydroxybenzoylglucosyl)galactoside ($[M]^+$ at *m/z* 863 and fragment ion at *m/z* 287) was also detected in this fraction. At the beginning of the extrusion, an acylated anthocyanin with coumaric acid was found in Antonina and Deep Purple anthocyanin fractions and presented a pseudomolecular ion at *m/z* 889 and fragment ion at *m/z* 287 corresponding to cyanidin-3-xylosyl(coumaroylglucosyl)galactoside. The Antonina anthocyanin fraction contained pelargonidin-3-xylosyl(feruloylglucosyl)galactoside ($[M]^+$ at *m/z* 903 and fragment ion at *m/z* 271) in small amount in the same fraction. However, this pelargonidin compound was not detected in Deep Purple and Purple Sun anthocyanin fractions. On the other hand, a peonidin derivative with a pseudomolecular ion at *m/z*

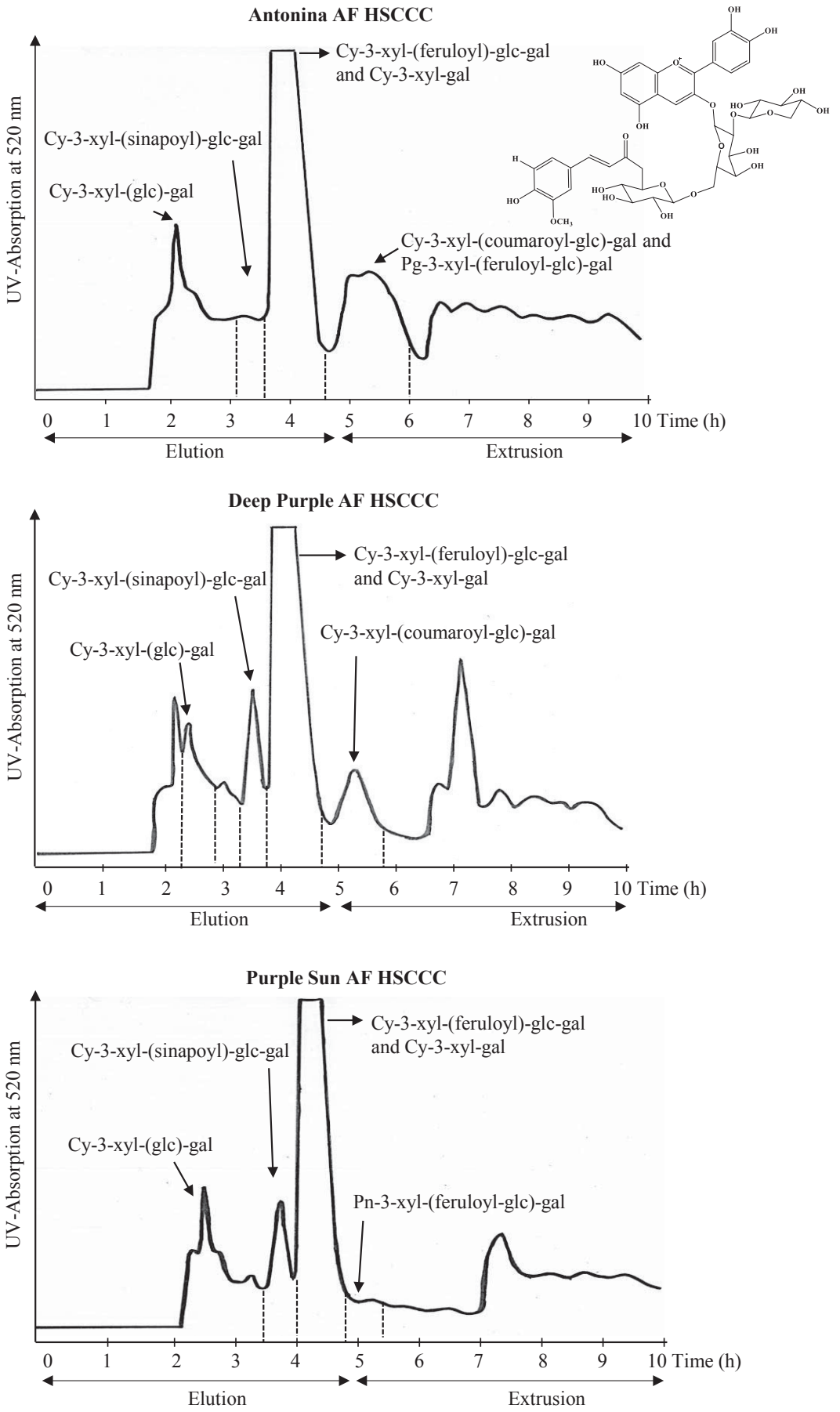


Fig. 3.34. HSCCC chromatograms of anthocyanin fraction from Antonina, Deep Purple and Purple Sun carrots at 520 nm and chemical structure of the main anthocyanin.

933 and fragment ion at *m/z* 301 was present in Purple Sun anthocyanin fraction and corresponding to peonidin-3-xylosyl(feruloylglucosyl)galactoside. The elution tendency of compounds coincide with previous separation of complete carrot extracts, anthocyanins and copigments (Schwarz et al., 2004; Hillebrand et al., 2009a; Cuevas Montilla, 2011).

After HSCCC separations, small quantities of other anthocyanins were detected that have not been previously described. For example, two compounds with a pseudomolecular ions $[M]^+$ at *m/z* 1081 and *m/z* 1051 were detected between cyanidin-3-xylosyl(glucosyl)galactoside and cyanidin-3-xylosyl(sinapoylglucosyl)galactoside. These compounds presented a fragment ion at *m/z* 287 corresponding to cyanidin derivatives. In the extrusion, other minor anthocyanins were found with a pseudomolecular ion $[M]^+$ at *m/z* 889, $[M]^+$ at *m/z* 817, $[M]^+$ at *m/z* 961, $[M]^+$ at *m/z* 787, $[M]^+$ at *m/z* 757, $[M]^+$ at *m/z* 491. All of them had a fragment ion at *m/z* 287 that corresponds to cyanidin aglycone. Because of their low amount present, they could only be identified as cyanidin derivatives based on their UV spectrum and ESI-MS^n spectrum.

HSCCC separation of copigment fraction from black carrots

For the isolation of the main chlorogenic acids present in the copigment fraction of Antonina, Deep Purple or Purple Sun carrots, 300 mg were fractionated by HSCCC. The solvent system consisted of ethyl acetate/ethanol/water (5:2:3, v/v/v) and the separation was carried out in Head to Tail mode at 900 rpm. The flow rate was set at 3mL/min and the fraction collection was 4 min per tube. HSCCC chromatograms were monitored at 280 nm, as shown in Fig. 3.35. Neochlorogenic acid (3-CQA) eluted first, followed by chlorogenic acid (5-CQA) and cryptochlorogenic acid (4-CQA) in the three black carrot varieties. The three chlorogenic acids presented a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 353 and were differentiated by LC-MS^n fragmentation according to Clifford et al. (2003). The 4-CQA presents a MS^2 base peak at *m/z* 173, while 3-CQA and 5-CQA have a MS^2 base peak at *m/z* 191, additionally the distinction between 3-CQA and 5-CQA is done by intensity of the secondary peak at *m/z* 179 (3-CQA>>5-CQA). After elution of 3-CQA, 5-CQA and 4-CQA, other phenolic acids such as p-coumaric, caffeic and ferulic acids were detected as minor compounds.

In concordance with the literature data (Alasalvar et al., 2001; Kammerer et al., 2004a), mixture of some feruloylquinic acids with a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 367 and fragment at *m/z* 191 were present. Dicaffeoylquinic acids were identified with $[M–H]^-$ at *m/z* 515 and they may be 3,5-di-CQA and 4,5-di-CQA. Moreover, two compounds with a pseudomolecular ion $[M–H]^-$ at *m/z* 543 were found, which were tentatively identified as 3′,4′-diferuloylquinic acid and 3′,5′-diferuloylquinic acid. Also p-coumaroylquinic acids (3-pCoQA and 5-pCoQA) were present with a $[M\text{-}H]^-$ ion at *m/z* 337. However, because of limited information about phenolic acids in black carrots, the purification of HSCCC fractions for their isolation and future studies such NMR are required for the confirmation of their chemical structure.

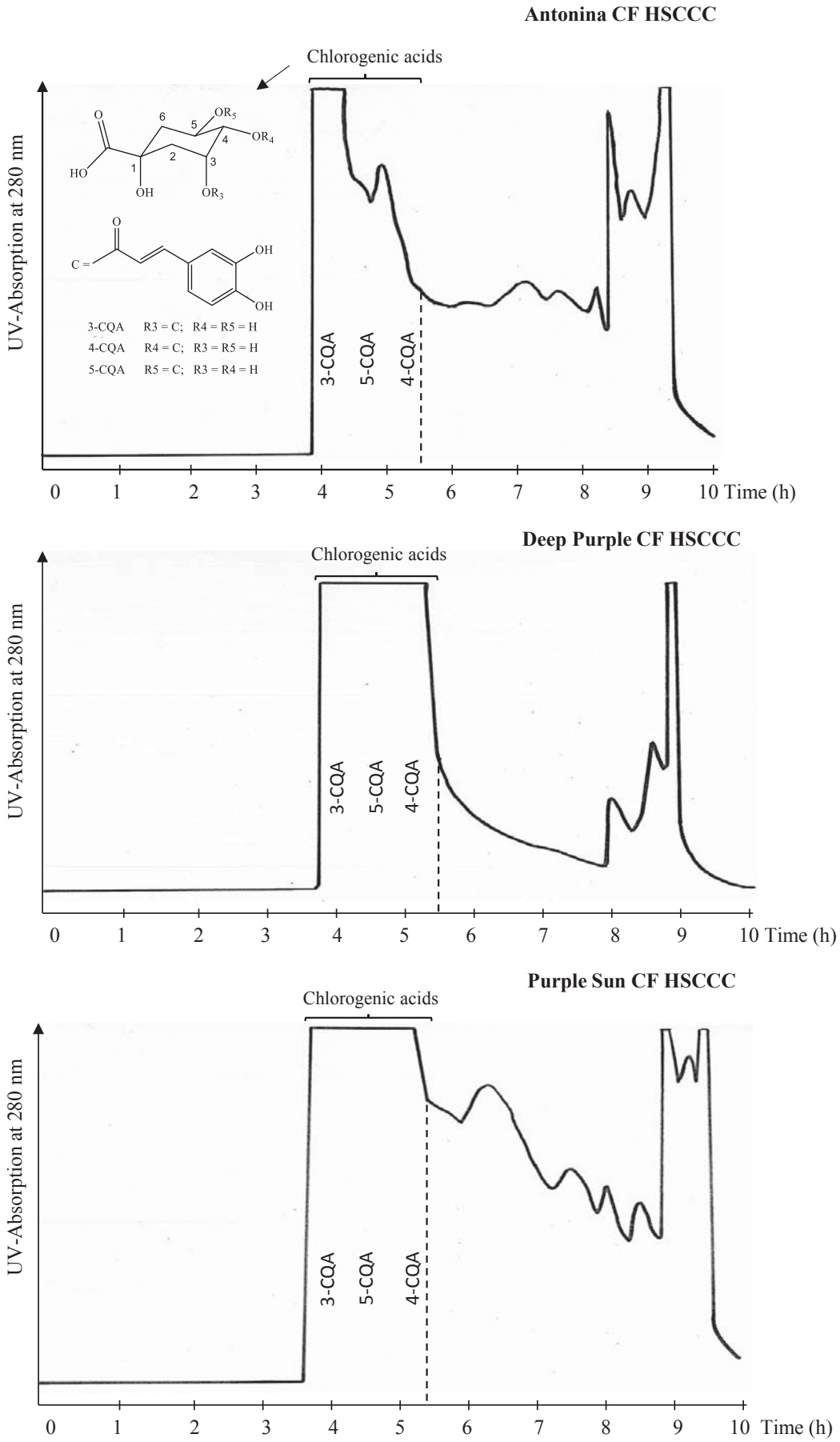


Fig. 3.35. HSCCC Chromatograms of copigment fraction from Antonina, Deep Purple and Purple Sun carrots at 280 nm.

3.6 Purple sweet potato

3.6.1 Chemical composition

In this work, polyphenols of Chinese Purple sweet potatoes (*Ipomoea batatas* L.) (PSP) were identified by HPLC-DAD and HPLC-ESI-MS^n. PSP contain many anthocyanins that have adsorption maxima at 280 nm and 520 nm, as shown in the HPLC DAD plot chromatogram (Fig. 3.36, top), while their copigments were detected in the range from 200 nm to 400 nm. The HPLC chromatogram at 520 nm (Fig. 3.36, middle) displays the characteristic anthocyanin profile of Chinese PSP, in which Peak 11, 13, 14 and 15 were identified as major anthocyanins. Peak 11 presented a molecular ion at $[M]^+$ at *m/z* 1055 and fragment ions at *m/z* 893, 449 and 287 that correspond to cyanidin aglycone and was identified as cyanidin-3-(6"-caffeoyl-6"'-p-hydroxybenzoylsophoroside)-5-glucoside based on literature data. On the other hand, Peak 13, 14 and 15 showed a fragment ion at *m/z* 301 that is characteristic for peonidin-based anthocyanins. Peak 13 had molecular ion $[M]^+$ at *m/z* 1111 and fragment ions at *m/z* 949, 463, 301, corresponding to the loss of a glucose and dicaffeoyl-sophoroside moiety. Thus it was identified as peonidin-3-(6",6"'-dicaffeoylsophoroside)-5-glucoside. Peak 14 ($[M]^+$ at *m/z* 1069 and fragment ions at *m/z* 907, 463 and 301) has been identified as peonidin-3-(6"-caffeoyl-6"'-p-hydroxybenzoylsophoroside)-5-glucoside, while Peak 15 ($[M]^+$ at *m/z* 1125 and fragment ions at *m/z* 963, 463, 301) is peonidin-3-(6"-caffeoyl-6"'-feruloylsophoroside)-5-glucoside. Zhang et al. (2015) also detected peonidin-3-(6",6"'-dicaffeoylsophoroside)-5-glucoside (Peak 13), peonidin-3-(6"-caffeoyl-6"'-p-hydroxybenzoylsophoroside)-5-glucoside (Peak 14) and peonidin-3-(6"-caffeoyl-6"'-feruloylsophoroside)-5-glucoside (Peak 15) as major anthocyanins in Chinese purple sweet potato.

On the other hand, only one non-acylated anthocyanin was found, Peak 1 with a molecular ion $[M]^+$ at *m/z* 787 and fragments ions at *m/z* 625, 463, 301. It was identified as peonidin-3-sophoroside-5-glucoside due to the loss of glucosyl moieties. However, the detected main anthocyanins were present in acylated form. Monoacylated anthocyanins with caffeic, ferulic or p-hydroxybenzoic acid were identified. For example, cyanidin-3-(6"'caffeoylsophoroside)-5-glucoside (Peak 3, *m/z* at 935), peonidin-3-(6"caffeoylsophoroside)-5-glucoside (Peak 12, *m/z* 949) and cyanidin-3-(6"caffeoylsophoroside)-5-glucoside (Peak 9, *m/z* at 935) presented a loss of a glucose and caffeoylsophorose residue, as well as cyanidin or peonidin aglycone moieties. In the case of cyanidin-3-p-hydroxybenzoylsophoroside-5-glucoside (Peak 2, *m/z* at 893) and peonidin-3-p-hydroxybenzoylsophoroside-5-glucoside (Peak 5, *m/z* 907), they produced the losses of a glucose and a sophorose monoacylated with p-hydroxybenzoic acid, whereas cyanidin-3-feruloylsophoroside-5-glucoside (Peak 7, *m/z* at 949) and peonidin-3-feruloylsophoroside-5-glucoside (Peak 8, *m/z* 963) showed the loss of a glucose and a sophorose monoacylated with ferulic acid. As diacylated anthocyanins with caffeic, ferulic or p-hydroxybenzoic acid were deteted cyanidin-3-(6",6"'-dicaffeoylsophoroside)-5-glucoside (Peak 10, *m/z* at 1097) and the main anthocyanins (Peak 11, 13, 14 and 15). Other two compounds, Peak 4 with *m/z* 1111 and Peak 6 with *m/z* 949 could be assigned only as peonidin-derivatives.

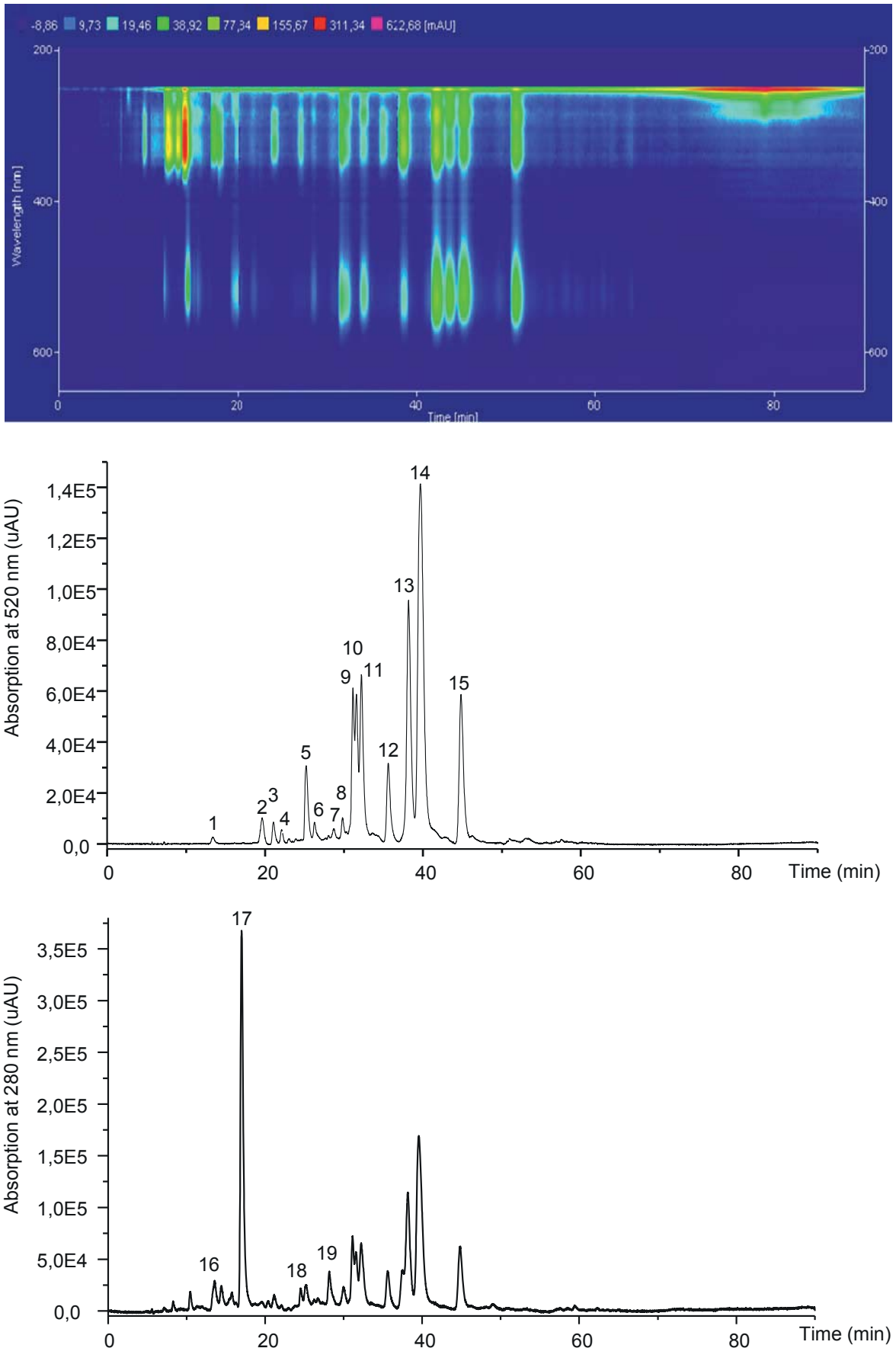


Fig. 3.36. HPLC DAD Plot chromatogram of Chinese PSP (top) and HPLC chromatograms at 520 nm (middle) and 280 nm (bottom). Peak numbering according to Table 3.17 and Table 3.18.

Similar anthocyanins were found in purple sweet potatoes from Korea, especially in Borami, Mokpo 62, Shinzami, and Zami varieties (Kim et al., 2012; Lee et al., 2013), as well as from Japan, in particular Chiran Murasaki, Purple Sweet, Tanegashima Murasaki and Naka Murasaki cultivars (Cuevas Montilla, 2011). In addition, Purple Sweet potatoes can be classified as cyanidin-type such as Tanegashima Murasaki and Naka Murasaki cultivars or peonidin-type like Chiran Murasaki and Purple Sweet cultivars, according to the anthocyanin content. The anthocyanins present in Chinese PSP were based approximately 70% on peonidin and 30% on cyanidin aglycone, respectively. Therefore, Chinese purple sweet potatoes could be assigned as peonidin-type due to their high content of peonidin derivatives. Table 3.17 summarizes the anthocyanin profile of Chinese PSP, LC-MS data as well as their quantification.

Table 3.17. Mass Spectrometric data and quantification of Anthocyanins from Chinese PSP.

Peak No.	Compound	$[M]^+$ (*m/z*)	Fragments (*m/z*)	Conc.* (mg/100 g PSP)
1	Pn-3-soph-5-glc	787	625, 463, 301	2.40 ± 0.10
2	Cy-3-p-hydroxybenzoylsoph-5-glc	893	731, 449, 287	4.94 ± 0.12
3	Cy-3-(6'''caffeoylsoph)-5-glc	935	773, 449, 287	3.78 ± 0.17
4	Pn-derivative	1111	949, 463, 301	3.14 ± 0.10
5	Pn-3-p-hydroxybenzoylsoph-5-glc	907	745, 463, 301	10.16 ± 0.10
6	Pn-derivative	949	787, 463, 301	4.61 ± 0.14
7	Cy-3-feruloylsoph-5-glc	949	787, 449, 287	3.62 ± 0.09
8	Pn-3-feruloylsoph-5-glc	963	801, 463, 301	4.12 ± 0.13
9	Cy-3-(6"caffeoylsoph)-5-glc	935	773, 449, 287	14.57 ± 0 14
10	Cy-3-(6",6'''-dicaffeoylsoph)-5-glc	1097	935, 449, 287	14.36 ± 0.18
11	Cy-3-(6"-caffeoyl-6'''-p-hydroxybenzoylsoph)-5-glc	1055	893, 449, 287	20.47 ± 0.23
12	Pn-3-(6”caffeoylsoph)-5-glc	949	787, 463, 301	10.88 ± 0.13
13	Pn-3-(6”, 6'''-dicaffeoylsoph)-5-glc	1111	949, 463, 301	32.78 ± 0.17
14	Pn-3-(6"-caffeoyl-6'''-p-hydroxybenzoylsoph)-5-glc	1069	907, 463, 301	68.36 ± 0.32
15	Pn-3-(6"-caffeoyl-6'''-feruloylsoph)-5-glc	1125	963, 463, 301	21.86 ± 0.39

*Calculated as cyanidin-3-O-glucoside (Cy-3-glc) equivalents at λ 520 nm.
Cy: cyanidin; Pn: peonidin; glc: glucoside; soph: sophoroside;

Furthermore, the HPLC chromatogram at 280 nm (Fig. 3.36, at the bottom) shows the presence of some phenolic acids in Chinese PSP. Chlorogenic acid (Peak 17, $[M-H]^-$ at *m/z* 353) was detected as main copigment, while two feruloylquinic acids (Peak 18 and peak 19, respectively) were found as minor compounds with $[M-H]^-$ at *m/z* 367. Peak 16 had $[M-H]^-$ at *m/z* 341 and fragment ions at *m/z* 281, 179 and 251 but it could not be identified. In addition, three di-*O*-caffeoylquinic acids with $[M-H]^-$ at

m/z 515 were present. According to Clifford et al. (2003), Truong et al. (2007) and Zhu et al. (2010), they were identified as 3,4-diCQA, 3,5-diCQA and 4,5-diCQA. Table 3.18 shows an overview of identified copigments in Chinese PSP and their LC-MS data.

Table 3.18. LC-MS data of Copigments in Chinese PSP.

Peak No.	Compound	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)
16	Unknown	341	281, 179, 251
17	5-CQA	353	191
18	FQA-derivative	367	191
19	5-FQA	367	179, 135, 161
**	3,4-diCQA	515	353, 173, 335
**	3,5-diCQA	515	353, 191
**	4,5-diCQA	515	353

** minor copigments
CQA: caffeoylquinic acid; FQA: feruloylquinic acid; diCQA: di-*O*-caffeoylquinic acid.

Due to limited knowledge of phenolic acids in purple sweet potatoes, more studies are necessary to identify and characterize their chemical structures.

3.6.2 Preparative separations

3.6.2.1 Membrane chromatography

For an isolation of anthocyanins and copigments of PSP on a large scale, several steps like extraction and concentration of polyphenols are required when a membrane chromatographic separation has to be performed. First, seven kilos of purple sweet potatoes (Fig. 3.37, a-b) were washed with water, dried and cut in small pieces including their peels. Then the polyphenols were extracted using method 2 (see section 4.3). PSP were blanched with hot water at 100 °C for 3 min, then a solution of water/hydrochloric acid (19:1, v:v) was added and cooled at 0 °C for 3 hours, subsequently stored at room temperature for 8 h (Fig. 3.37, c-d). After extraction, filtration was performed in order to remove the solid material. The raw extract was put onto an Amberlite XAD-7 column (for deteils see section 4.4, Fig. 3.37, e). As a result, a polyphenolic enriched XAD-7 extract was obtained (30.3 g). Afterwards, a separation of PSP polyphenols in two groups, anthocyanins and copigments, was carried out by adsorptive membrane chromatography (see section 4.6.5, Fig. 3.38) but due the high anthocyanin content in PSP, a second membrane chromatographic procedure was necessary. Consequently, the PSP XAD-7 extract (10 g) was separated in an anthocyanin (4.96 g) and a copigment fraction (5.04 g). The HPLC-DAD chromatograms of both fractions (Fig. 3.39), as well as the comparison of their HPLC chromatograms at 520 nm and 280 nm (Fig. 3.40) confirmed a good separation.

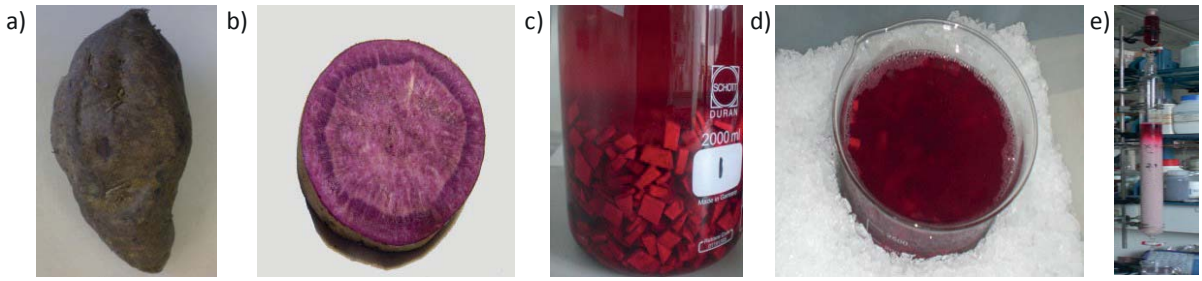


Fig. 3.37. Purple Sweet potatoes (a-b), extraction of polyphenols from purple sweet potato (c-d) and isolation of PSP polyphenols by Amberlite XAD-7 column chromatography (e).

Fig. 3.38. Separation of purple sweet potato XAD-7 extract by membrane chromatography (left), copigment fraction elution (at the middle) and anthocyanin fraction elution (right).

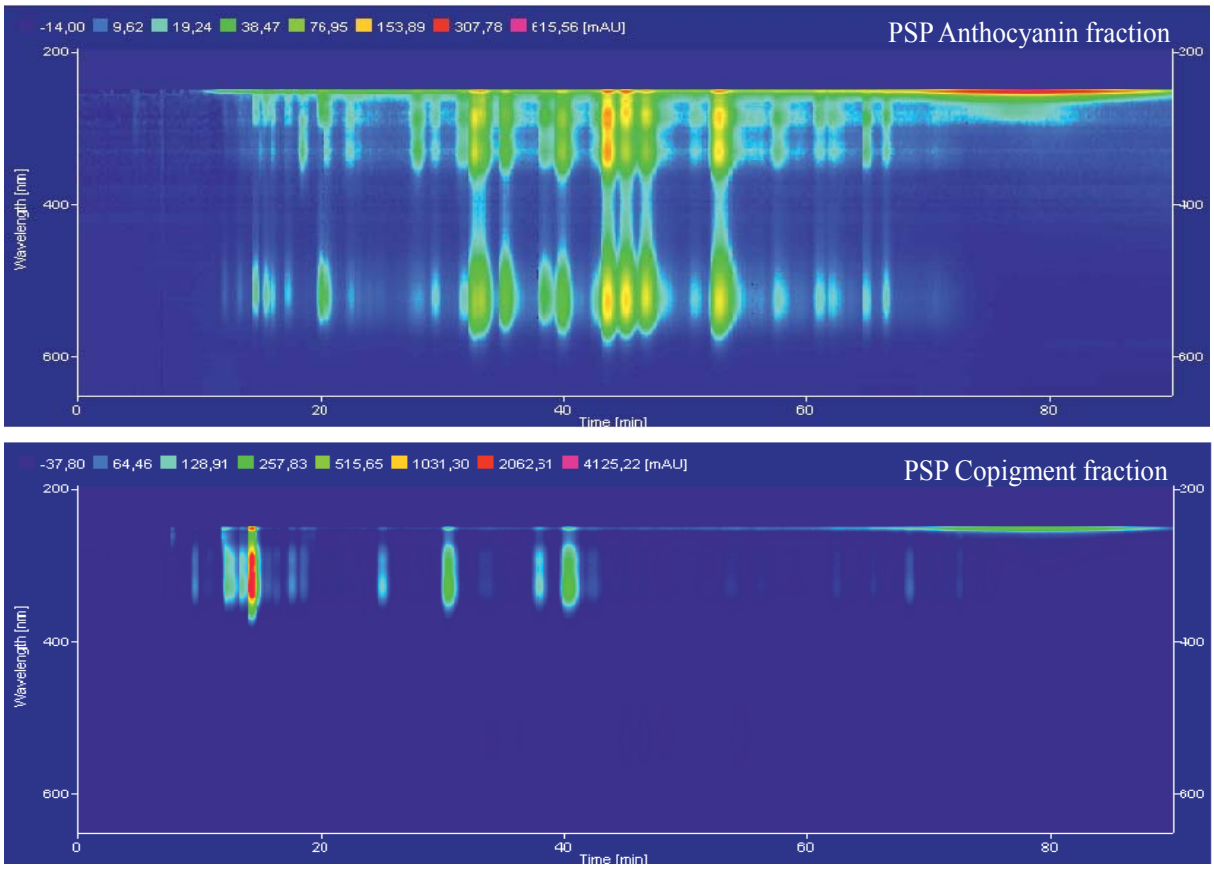


Fig. 3.39. HPLC DAD Plot chromatograms of PSP anthocyanin fraction and PSP copigment fraction.

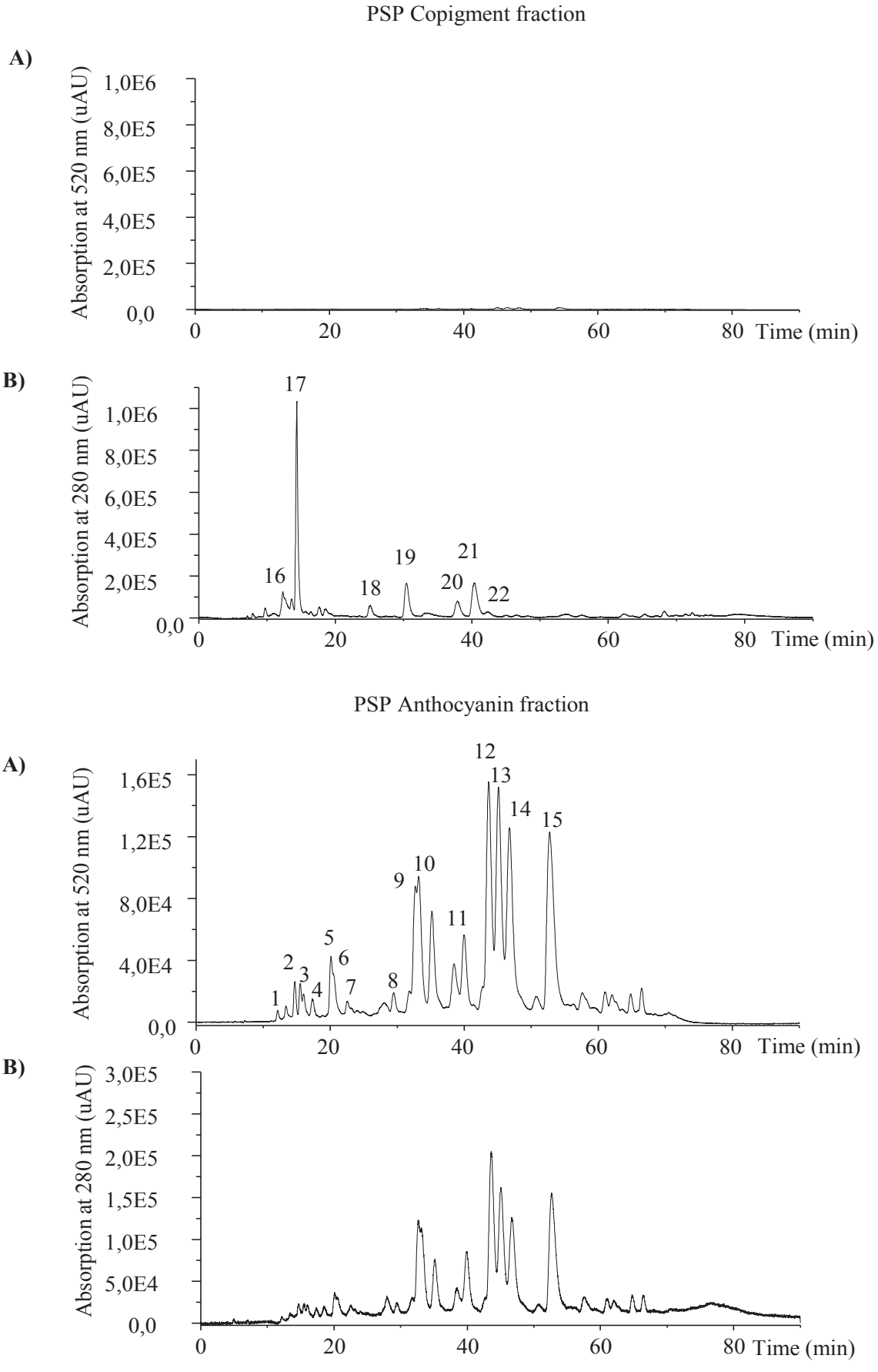


Fig. 3.40. HPLC-DAD chromatograms at 520 nm (A) and 280 nm (B) of copigment and anthocyanin fraction. For peak numbering, see Table 3.17 and Table 3.19.

The absence of anthocyanins in the copigment fraction provided a more precise information about the phenolic acids of PSP. Chlorogenic acid (5-CQA) was the main copigment, followed by 3,5-di-O-caffeoylquinic acid (3,5-diCQA). Additionally, the presence of feruloylquinic acid (5-FQA) was confirmed, as well as other two di-*O*-caffeoylquinic acids (3,4-diCQA and 4,5-diCQA). Table 3.19 presents a summary of LC-MS data and the quantification of phenolic acids at λ 324 nm.

Table 3.19. LC-MS data and quantification of phenolic acids present in copigment fraction after separation by membrane chromatography.

Peak No.	Compound	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	Conc.* (mg/100 g PSP)
16	Unknown	341	281, 179, 251	2.72 ± 0.10
17	5-CQA	353	191	106.74 ± 0.87
18	FQA-derivative	367	191	7.18 ± 0.14
19	5-FQA	367	179, 135, 161	22.49 ± 0.25
20	3,4-diCQA	515	353, 173, 335	10.91 ± 0.17
21	3,5-diCQA	515	353, 191	28.62 ± 0.23
22	4,5-diCQA	515	353	3.54 ± 0.12

* calculated as chlorogenic acid (5-CQA) equivalents at λ 324 nm.
CQA: caffeoylquinic acid; FQA: feruloylquinic acid; diCQA: di-O-caffeoylquinic acid.

3.6.2.2 Countercurrent Chromatography

A separation of anthocyanin and copigment fraction was performed by HSCCC. The isolated compounds were identified by TLC, HPLC-DAD and HPLC-DAD-ESI-MS^n and further purified by preparative HPLC.

HSCCC separation of anthocyanin fraction of PSP

After membrane chromatography, the anthocyanin fraction of PSP was fractionated by HSCCC. Previous studies for preparative separations of PSP extracts by HSCCC used a solvent system based on a mixture of tert-butyl methyl ether, acetonitrile and water acidified with TFA (Qiu et al., 2009; Cuevas Montilla et al., 2010; Lu et al., 2011). In this case, 700 mg of PSP anthocyanin fraction were separated with a solvent system consisting of tert-butyl methyl ether/acetonitrile/water (1:3:1:5, v/v/v/v), acidified with 0.1% TFA. The HSCCC separation was performed in elution mode with a flow rate of 3 mL/min and a rotational speed of 900 rpm. Fractions were collected every 4 min and anthocyanins were monitored at 520 nm. As a result, four fractions and a coil residue were obtained as shown in the HSCCC chromatogram (Fig. 3.41).

At the beginning of the separation, polymeric pigments were eluted in fraction 1 (F1, 52 mg). The fraction 2 (F2, 131 mg) contains pn-3-(6”caffeoylsoph)-5-glc (compound 12, $[M]^+$ at *m/z* 949) and cy-3-(6"caffeoylsoph)-5-glc (compound 9, $[M]^+$ at *m/z* 935) as main compounds. Subsequently, cy-3-

(6",6"'-dicaffeoylsoph)-5-glc (compound 10, $[M]^+$ at *m/z* 1097), pn-3-(6",6"'-dicaffeoylsoph)-5-glc (compound 13, $[M]^+$ at *m/z* 1111), pn-3-(6"-caffeoyl-6"'-feruloylsoph)-5-glc (compound 15, $[M]^+$ at *m/z* 1125), as well as cy-3-(6"-caffeoyl-6"'-feruloylsoph)-5-glc ($[M]^+$ at *m/z* 1111) were isolated in fraction 3 (F3, 101 mg). Pn-3-(6"-caffeoyl-6"'-p-hydroxybenzoylsoph)-5-glc (compound 14, $[M]^+$ at *m/z* 1069) and cy-3-(6"-caffeoyl-6"'-p-hydroxybenzoylsoph)-5-glc (compound 11, $[M]^+$ at *m/z* 1055) were detected in fraction 4 (F4, 132 mg). After elution, the coil residue (47 mg) was collected and it contained a mixture of five minor anthocyanins: pn-3-soph-5-glc (compound 1, $[M]^+$ at *m/z* 787), cy-3-p-hydroxybenzoylsoph-5-glc (compound 2, $[M]^+$ at *m/z* 893), cy-3-(6"'caffeoylsoph)-5-glc (compound 3, $[M]^+$ at *m/z* 935), pn-3-p-hydroxybenzoylsoph-5-glc (compound 5, $[M]^+$ at *m/z* 907) and pn-3-feruloylsoph-5-glc (compound 8, $[M]^+$ at *m/z* 963). The trend of anthocyanins elution was in concordance with previous separations of PSP extracts (Montilla et al., 2010).

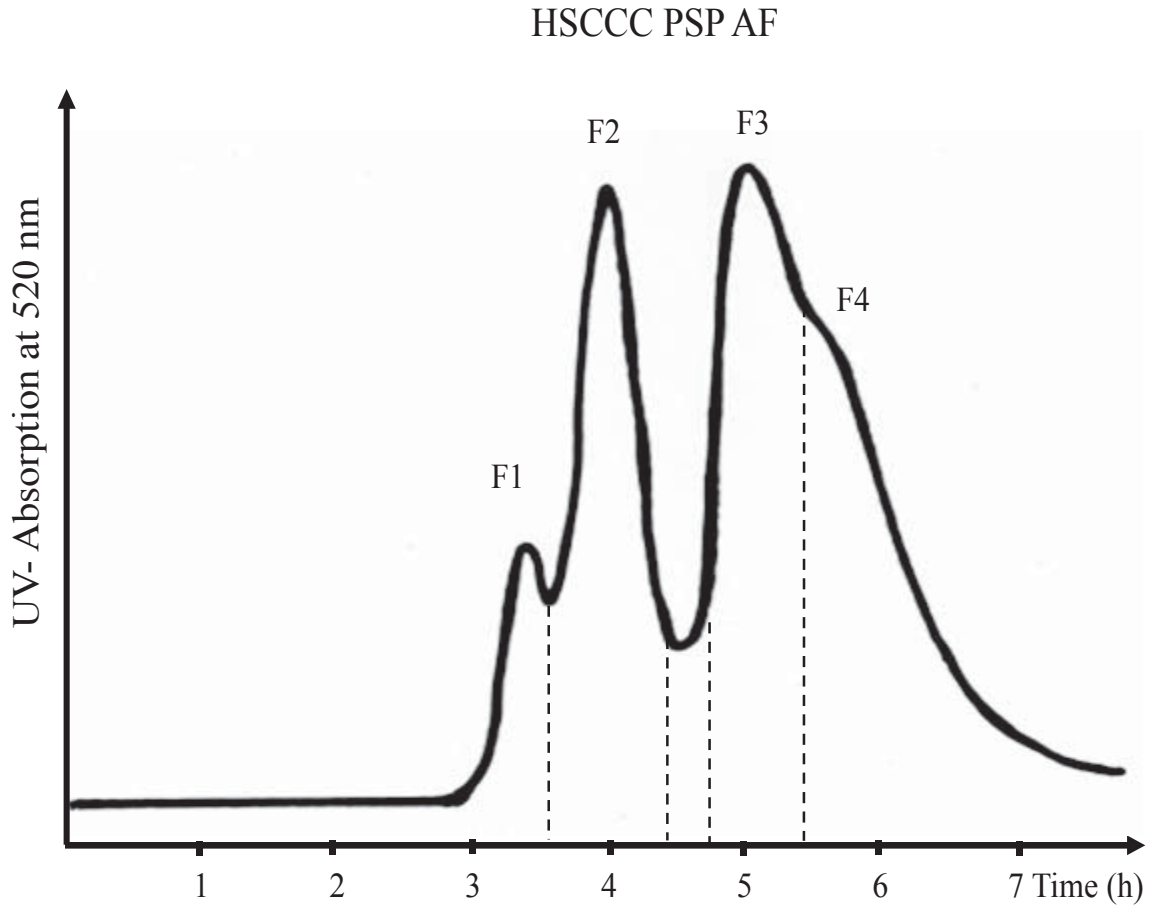


Fig. 3.41. HSCCC chromatogram of PSP anthocyanin fraction at 520 nm.

The anthocyanin fraction contains several acylated anthocyanins and due to their complexity and high anthocyanins content, some HSCCC parameters such as flow rate of mobile phase and concentration of load sample have an influence on the separation. These aspects should be taken into consideration and according to Qiu et al.(2009), a low flow rate (1.5 mL/min) yielded a good separation.

HSCCC Separation of copigment fraction of Purple Sweet potato

PSP copigment fraction (600 mg) was fractionated by HSCCC using a solvent system composed of tert-butyl methyl ether/acetonitrile/water (1:3:1:5, v/v/v/v), acidified with 0.1% TFA. The HSCCC separation was performed in Head to Tail mode with a flow rate of 3 mL/min and 900 rpm. Fractions of 12 mL were collected and copigments were monitored at 280 nm (Fig. 3.42). The separation yielded ten fractions and a coil residue.

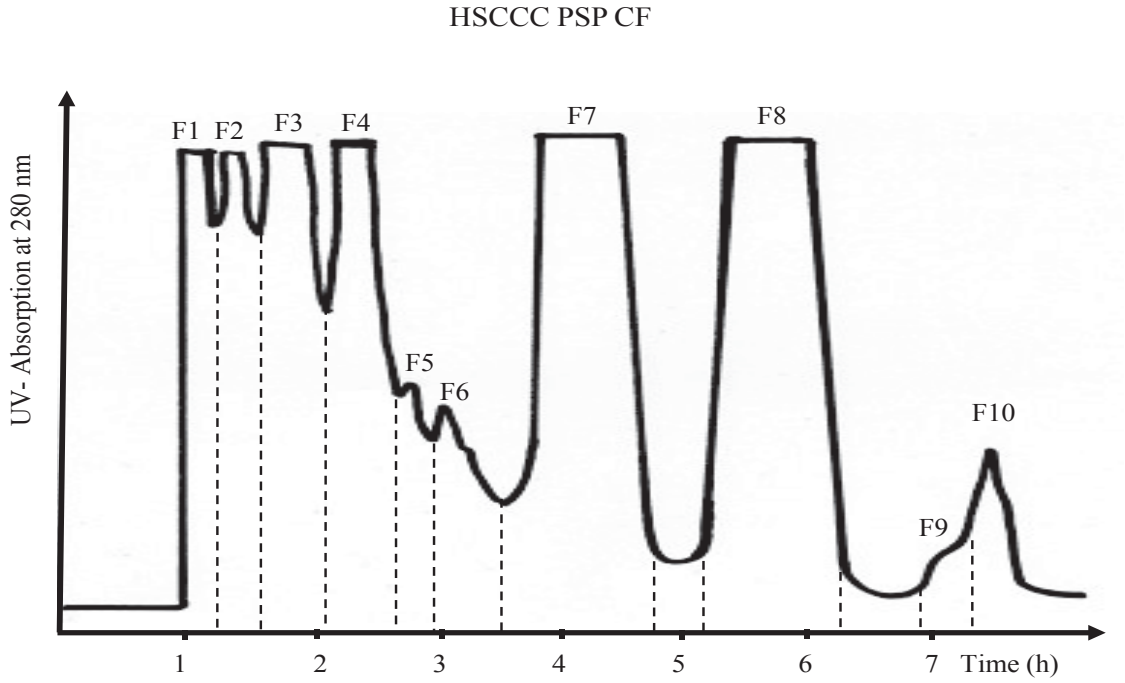


Fig. 3.42. HSCCC chromatogram of PSP copigment fraction at 280 nm.

After separation, the main copigment, chlorogenic acid, was isolated in high purity (96%) in fraction 7 (F7, 141 mg). Subsequently, two feruloylquinic acids (*m/z* 367) eluted in fraction 8 (F8, 49 mg) in which one corresponds to 5-FQA, while other FQA derivative was found in fraction 9 (F9, 2 mg). The three di-*O*-caffeoylquinic acids (3,4-diCQA, 3,5-diCQA and 4,5-diCQA) were isolated in the coil residue (148 mg). On the other hand, minor compounds were detected in F1-F6 and F9-F10. Unknown compounds (*m/z* 503 and *m/z* 399) and a caffeoylquinic acid (*m/z* 353) were isolated in fraction 1 (F1, 54 mg). Afterward a caffeoyl hexose eluted in fraction 2 (F2, 21 mg) that displayed a molecular ion at *m/z* 341 and fragment ions at *m/z* 179 and 135 corresponding to [M-H-hexose] and [M-H-hexose-CO_2], respectively. This was also detected in fraction 3 (F3, 44 mg) together with two isomers. Fraction 4 (F4, 25 mg) contained a caffeoylquinic acid derivative that had a molecular ion $[M-H]^-$ at *m/z* 677 and fragments ions at *m/z* 515 and 353. It was tentatively identified as 3,4,5-triCQA that was reported in leaves and roots of sweet potatoes (Islam, 2006; Truong et al., 2007). Other unknown compounds were found in fraction 5 (F5, 3 mg, *m/z* 679), fraction 6 (F6, 13 mg, *m/z* 693, *m/z* 691 and *m/z* 367) and fraction 10 (F10, 8 mg, *m/z* 381). In conclusion, the major copigments of purple sweet potaoes could be isolated in high quantities and purity using HSCCC for the first time.

3.7 Roselle

3.7.1 Chemical composition

In the present work, the identification of phenolic compounds from Mexican *Hibiscus sabdariffa* L., China variety, was performed by HPLC-DAD and HPLC-DAD-ESI-MS^n. The presence of anthocyanins and copigments in *Hibiscus sabdariffa* L. was confirmed by its HPLC DAD plot chromatogram from 200 nm to 600 nm (Fig. 3.43). For the detection of anthocyanins, HPLC-DAD chromatogram was monitored at 520 nm and its anthocyanin profile showed four anthocyanins. Their MS/MS spectra produced fragment ions at *m/z* 303 and at *m/z* 287 corresponding to anthocyanins based on delphinidin aglycone or cyanidin aglycone, respectively. Peak 1 and Peak 3 were the most abundant pigments, representing 58% and 32% of total anthocyanins, respectively. Peak 1 was identified as delphinidin-3-*O*-sambubioside also called hibiscin that showed a molecular ion $[M]^+$ at *m/z* 597 and fragment ion at *m/z* 303 corresponding to [M-Xyloside-Glucose]$^+$. Peak 3 corresponded to cyanidin-3-*O*-sambubioside also called gossypicyanin that had a molecular ion $[M]^+$ at *m/z* 581 and fragment ion at *m/z* 287 corresponding to the loss of a sambubiose moiety. Minor compounds were detected: delphinidin-3-*O*-glucoside (Peak 2) with a molecular ion $[M]^+$ at *m/z* 465, as well as cyanidin 3-*O*-glucoside (Peak 4) with a molecular ion $[M]^+$ at *m/z* 449. In both minor compounds detected, there was a loss of a sugar molecule. A summary of the detected anthocyanins in Mexican hibiscus is reported in Table 3.20.

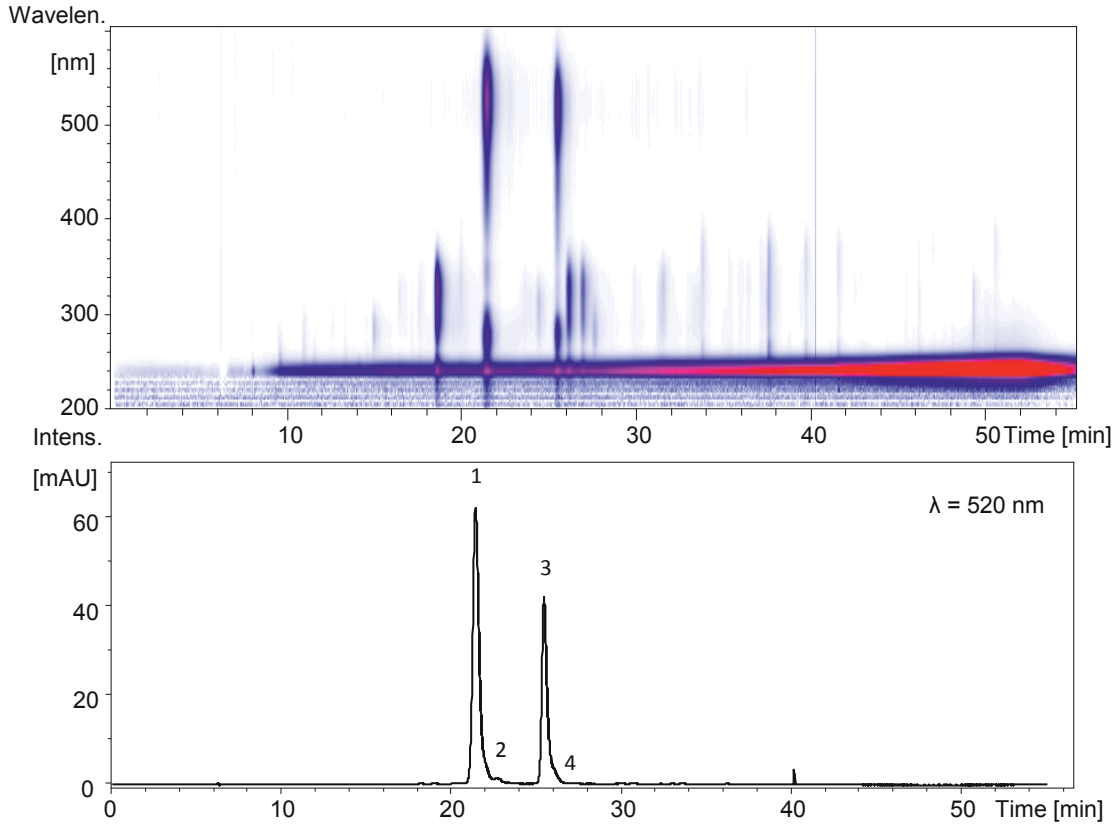


Fig. 3.43. HPLC DAD plot chromatograms of *Hibiscus sabdariffa* L. from 200 nm to 600 nm and HPL-DAD chromatogram at 520 nm.

Table 3.20. LC-MS data and quantification of anthocyanins *Hibiscus sabdariffa* L. from Mexico.

Peak No.	Compound	$[M]^+$ (*m/z*)	Fragment ions (*m/z*)	Concentration (mg/100g)*
1	Del-3-samb	597	303	247.35
2	Del-3-glc	465	303	6.50
3	Cy-3-samb	581	287	137.47
4	Cy-3-glc	449	287	7.31

*calculated as cyanidin-3-glucoside at 520 nm.

Several authors confirmed the presence of delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside as major pigments, as well as delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside as minor pigments in hibiscus (Rodríguez-Medina et al., 2009; Peng et al., 2011; Da-Costa-Rocha et al., 2014; Cid-Ortega and Guerrero-Beltrán, 2015). However, Segura-Carretero et al. (2008) also detected cyanidin-3-*O*-rutinoside (*m/z* 595) and cyanidin-*O*-3,5-diglucoside (*m/z* 611) as minor compounds in *Hibiscus sabdariffa* L. from Senegal, while Hsieh et al. (2008) reported other two unknown compounds with molecular ions at *m/z* 769 and at *m/z* 787 as minor anthocyanins in *Hibiscus sabdariffa* L. from Taiwan. On the other hand, the total anthocyanin content of *Hibiscus sabdariffa* L. can vary between 170-1500 mg/100 g depending on parameters such as maturity, cultivar and extraction method using polar solvents in particular water, ethanol, methanol and acidified alcoholic solvents (Segura-Carretero et al., 2008; Ramírez-Cortés et al., 2011; Salinas-Moreno et al., 2012; Cid-Ortega and Guerrero-Beltrán, 2015). In order to determine the optimal harvest time for hibiscus, Ramírez-Cortés et al. (2011) showed the variation of anthocyanin content during maturation in China, Reina and Criolla varieties, which had grown in Mexico, and they suggested that calyces should be harvested between 20-24 days after flowering, while Salinas-Moreno et al. (2012) reported a higher anthocyanin content in deep red calyces (Sudan, Colima 6 and China varieties) compared to light red calyces (Colima 3, Colima 5, Colima 7 and Coneja varieties) that were cultivated in Mexico.

Furthermore, other flavonoids such as phenolic acids were detected in *Hibiscus sabdariffa* L. (Fig. 3.44). The HPLC-DAD chromatogram at 320 nm showed the presence of neochlorogenic acid (Peak 5), chlorogenic acid (Peak 6) and cryptochlorogenic acid (Peak 7) that were identified as main copigments. Nonetheless, some minor copigments were found such as coumaroylquinic acids (Peak 11 and Peak 13, *m/z* 337), feruoylquinic acids (Peak 12 and Peak 15, *m/z* 367), 5-caffeoylshikimic acid (Peak 16, *m/z* 335) and N-feruloyltyramine (Peak 21, *m/z* 312).

Moreover, some flavonols could be detected in HPLC-DAD chromatogram at 360 nm. The results of mass spectrometric analysis in negative mode showed that these compounds presented fragment ions at *m/z* 301, *m/z* 285 or *m/z* 317 which are characteristic for quercetin-, kaempferol- or myricetin-derivatives, respectively. The identification of flavonols was based on UV spectra and HPLC-DAD-ESI-MS^n analysis. Therefore, *Hibiscus sabdariffa* L. contained myricetin-3-arabinogalactoside (Peak 14, *m/z* 611), quercetin-3-sambubioside (Peak 17, *m/z* 595), quercetin-3-rutinoside (Peak 18, *m/z* 609),

quercetin-3-glucoside (Peak 19, *m/z* 463), kaempferol-3-rutinoside (Peak 20, *m/z* 593) and quercetin (Peak 22, *m/z* 301). Additionally, protocatechuic acid (Peak 8) and a protocatechuic acid glucoside (Peak 10) were also detected as minor compounds.

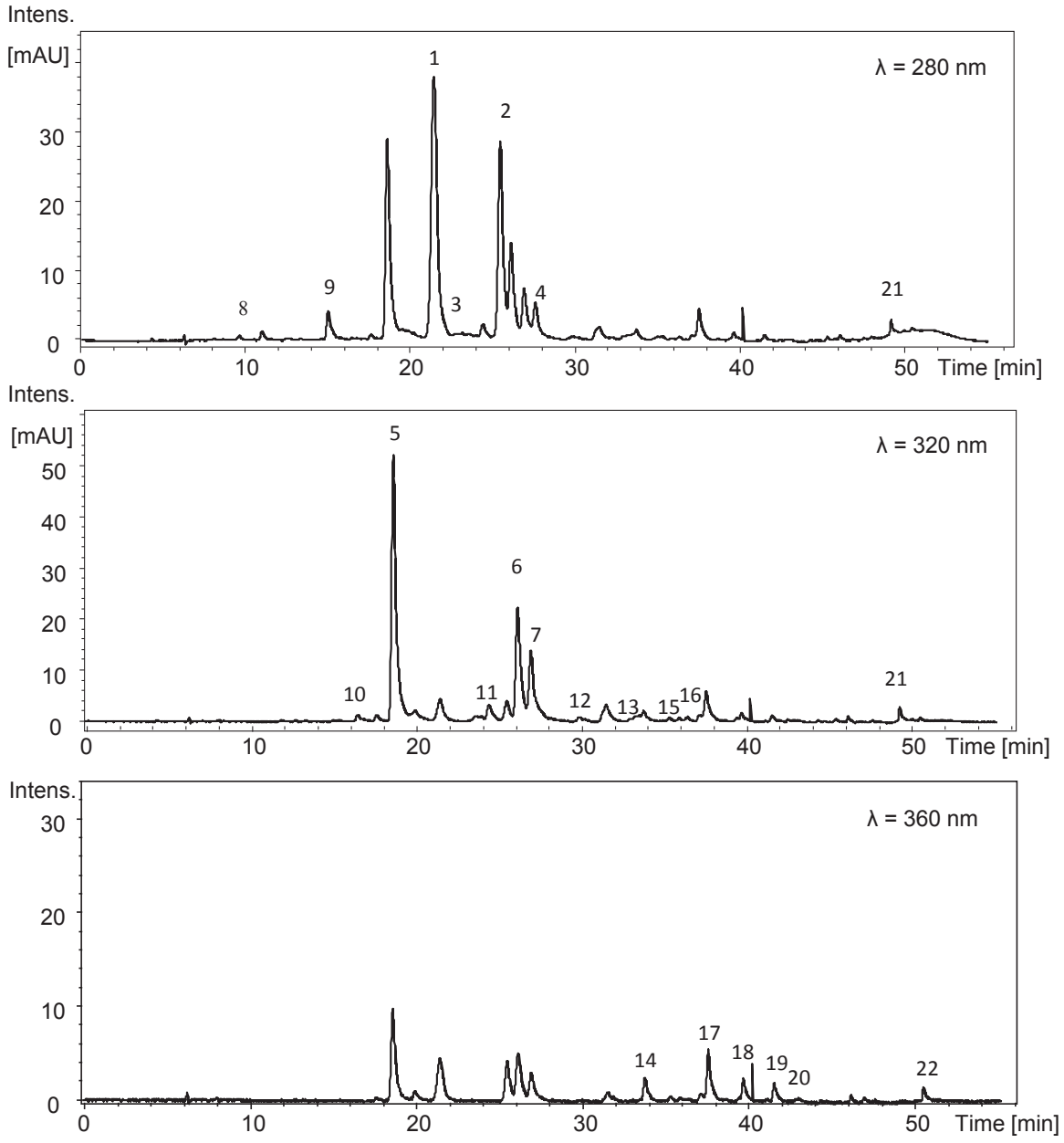


Fig. 3.44. HPLC-Chromatograms of *Hibiscus sabdariffa* L. at 280 nm, 320 nm and 360 nm. Peak numbering according to Table 3.21.

Two compounds did not show absorbance in the UV/Vis region, but they were detected by LC-MS in the negative mode. One had a molecular ion at *m/z* 207 and fragments ions at *m/z* 189 and 127, which correspond to the loss of a water molecule and subsequently a loss of a water molecule and CO_2. This compound was identified as hydroxycitric acid. The second compound had a *m/z* 189 with a fragment ion at *m/z* 127 and corresponded to hibiscus acid. Both compounds have been reported in previous studies (Rodríguez-Medina et al., 2009; Beltrán-Debón et al., 2010; Peng et al., 2011).

An overview on the detected phenolic compounds in *Hibiscus sabdariffa* L. is shown in Table 3.21.

Table 3.21. LC-MS data of phenolic compounds in *Hibiscus sabdariffa* L.

Peak No.	Compound	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)
5	3-CQA	353	191, 179, 135
6	5-CQA	353	191
7	4-CQA	353	173, 179, 135, 191
8	Protocatechuic acid	153	108
9	Unknown		
10	Protocatechuic acid glucoside	315	153, 109
11	Coumaroylquinic acid	337	163, 191, 119
12	FQA-Derivative	367	161, 193, 133, 335
13	Coumaroylquinic acid	337	173, 191, 163
14	Myricetin-3-arab-gal	611	316, 317
15	FQA-Derivative	367	179, 191, 135, 161
16	5-Caffeoylshikimic acid	335	161, 135
17	Quercetin-3-samb	595	463, 445, 300, 301
18	Quercetin-3-rut	609	301
19	Quercetin-3-glc	463	301
20	Kaempferol-3-rut	593	285
21	N-Feruloyltyramine	312	178, 135
22	Quercetin	301	179, 151
*	Hydroxycitric acid	207	189, 127
*	Hibiscus acid	189	127

*These compounds do not absorb in the UV region.

3.7.2 Preparative separations

3.7.2.1 Membrane chromatography

In order to isolate polyphenols of *Hibiscus sabdariffa* L. (HSL), 250 g of dried hibiscus calyces were extracted with 2.5 L of a mixture of methanol:acetic acid (19:1, v/v) for 8 h. Then the plant material was removed by filtration (Fig. 3.45). After extraction and evaporation of solvents, polyphenols were concentrated by adsorption chromatography on an Amberlite XAD-7 column (see section 4.4). In this way, 5.3 g of an enriched anthocyanin HSL XAD-7 extract were obtained (Fig. 3.45, e).

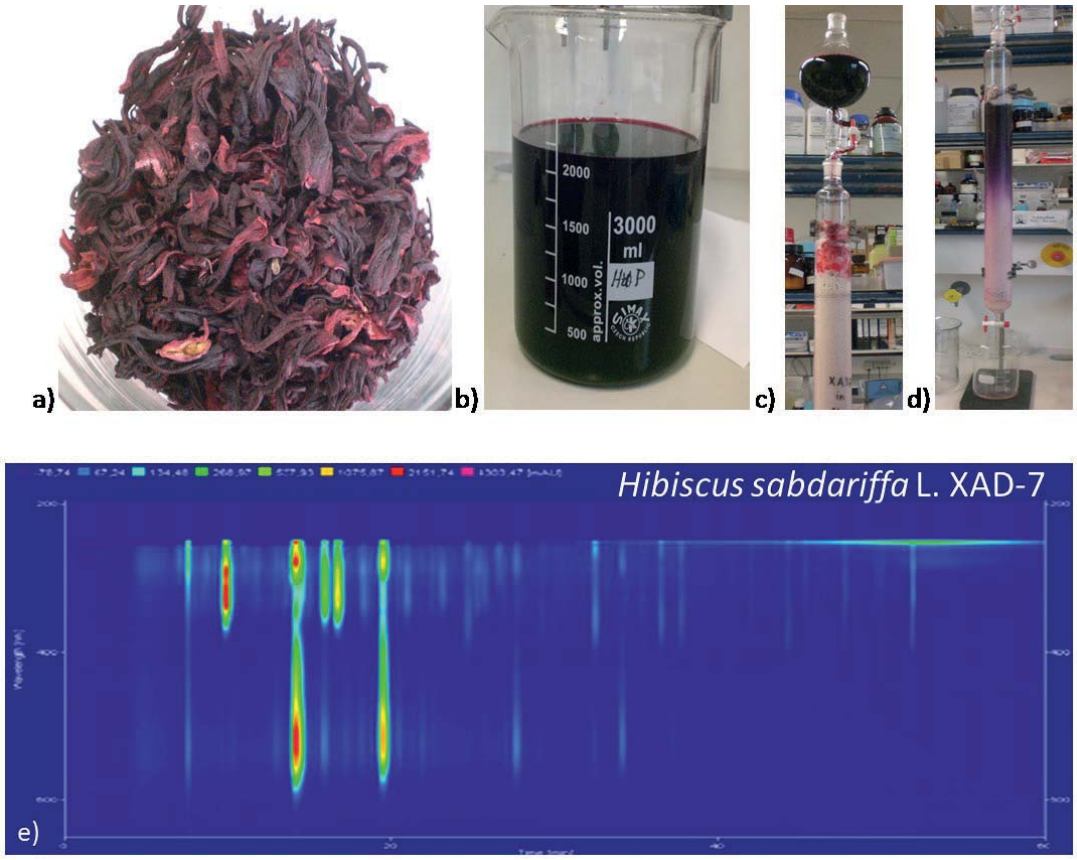


Fig. 3.45. *Hibiscus sabdariffa* L.: a) dried calyces, b) extraction of polyphenols, c-d) concentration of polyphenols onto XAD-7 column and e) HPLC DAD plot chromatogram of HSL XAD-7 extract.

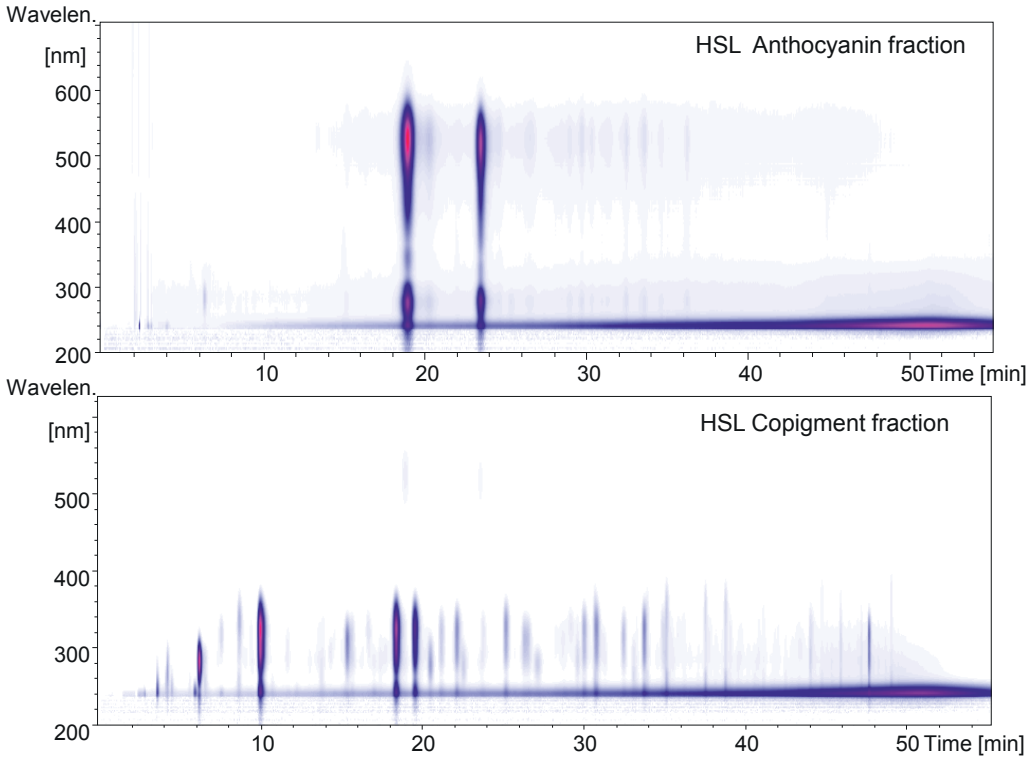


Fig. 3.46. HPLC DAD plot chromatograms of HSL anthocyanin fraction and HSL copigment fraction after separation by membrane chromatography.

Afterward, a fractionation of HSL XAD-7 extract (4 g) was performed by membrane chromatography (see section 4.6.5), generating an anthocyanin fraction (HSL AF, 1 g) and a copigment fraction (HSL CF, 2.5 g). To verify the separation after membrane chromatography, HPLC-DAD analyses of both fractions, HSL AF and HSL CF, were carried out. The HPLC DAD Plot chromatograms of HSL anthocyanin fraction (Fig. 3.46), as well as their HPLC chromatograms at 520 nm and 280 nm (Fig. 3.47) demonstrated that anthocyanins were successfully separated from copigments.

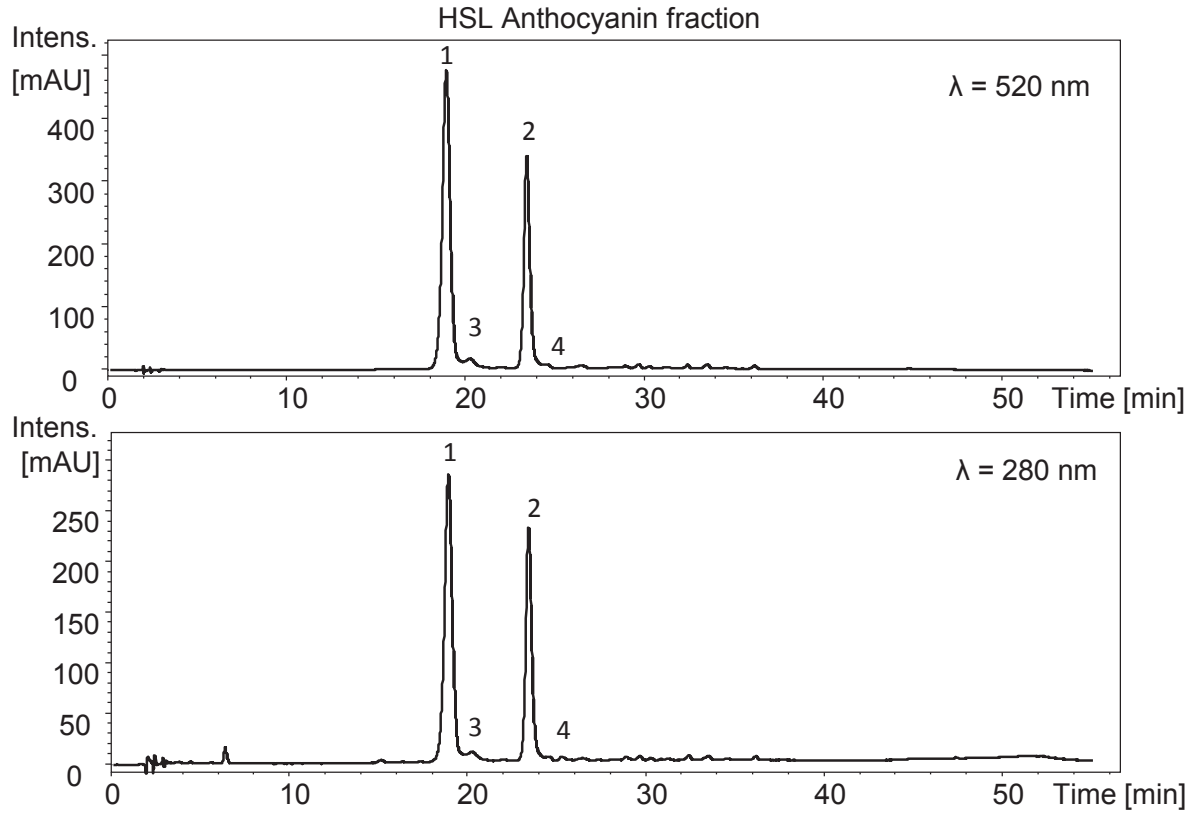


Fig. 3.47. HPLC chromatogramms of HSL anthocyanin fraction at 520 nm and 280 nm. For peak numbering, see Table 3.22.

Moreover, the HPLC chromatogram of copigment fraction at 520 nm (Fig. 3.48) was free of anthocyanins, confirming the separation of polyphenols of HSL XAD-7 extract into two groups: anthocyanins and copigments. Juadjur (2012) separated 6 g HSL XAD-7 extract and required a repetition of the membrane chromatographic process in order to separate anthocyanins. In this case, 4 g HSL XAD-7 extract required only one separation, because the maximum binding capacity of the membrane adsorber was not exceeded.

In addition, an enrichment of copigments was observed after membrane chromatography. As a result, some minor compounds, which were identified at 320 nm and 360 nm, were present in higher concentration, such as myricetin-3-arabinogalactoside, quercetin-3-sambubioside, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-rutinoside and quercetin, as well as phenolic acids like

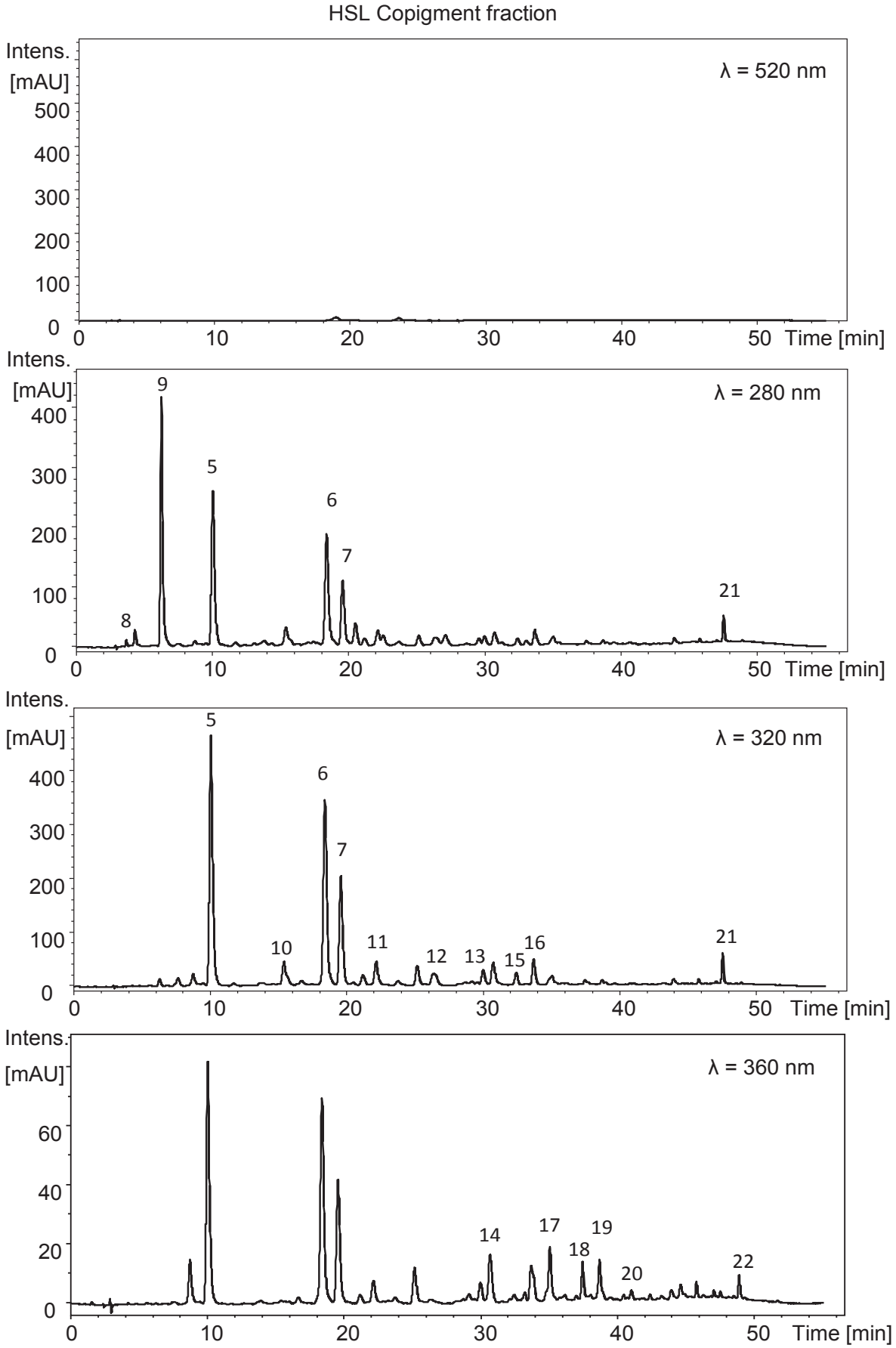


Fig. 3.48. HPLC-DAD chromatogramms of HSL copigment fraction at 520 nm, 280 nm, 320 nm and 360 nm. For peak numbering, see Table 3.22.

coumaroylquinic acids, feruoylquinic acids, 5-caffeoylshikimic acid and N-feruloyltyramine. Table 3.20 summarizes the detected phenolic compounds in the copigment fraction of Mexican hibiscus.

Table 3.22. LC-MS data of phenolic compound in *Hibiscus sabdariffa* L. after membrane chromatography.

Peak No.	Compound	$[M]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)
1	Del-3-samb	597		303
2	Cy-3-samb	581		287
3	Del-3-glc	465		303
4	Cy-3-glc	449		287
5	3-CQA		353	191, 179, 135
6	5-CQA		353	191
7	4-CQA		353	173, 179, 135, 191
8	Protocatechuic acid glucoside		315	153, 109
9	Unknown		503	415, 339, 207
10	Coumaroylquinic acid		337	163, 191, 119
11	FQA-Derivative		367	161, 193, 133, 335
12	Coumaroylquinic acid		337	173, 191, 163
13	FQA-Derivative		367	161, 191, 135
14	Myricetin-3-arab-gal		611	316, 317
15	5-O-Caffeoylshikimic acid		335	161, 135
16	FQA-Derivative		367	179, 191, 135, 161
17	Quercetin-3-samb		595	463, 445, 300, 301
18	Quercetin-3-rut		609	301
19	Quercetin-3-glc		463	301
20	Kaempferol-3-rut		593	285
21	N-Feruloyltyramine		312	178, 135
22	Quercetin		301	179, 151
*	Hydroxycitric acid		207	189, 127
*	Hibiscus acid		189	127

*These compounds do not absorb in the UV region.

3.7.2.2 Countercurrent Chromatography

Polyphenols of HSL XAD-7 extract were separated by means of HSCCC and the obtained fractions were analyzed by TLC, HPLC-DAD and LC-ESI-MS^n. In order to determine the chemical structure of the major compounds in *Hibiscus sabfdarifa* L., isolated compounds were purified by preparative HPLC, then ^1H and ^{13}C NMR spectroscopy were used to identify the chemical structure of pure compounds.

HSCCC Separation of Hibiscus XAD-7 extract

The fractionation of 500 mg of HSL XAD-7 extract was performed by HSCCC. Based on a previous separation of anthocyanins, the solvent system consisted of tert-butyl methyl ether/acetonitrile/water (1:3:1:5, v/v/v/v), acidified with 0.1% TFA at a flow rate 3 mL/min. Degenhardt et al. (2000) separated anthocyanins from Roselle by HSCCC using a different volume ratio (2:2:1:5, v/v/v/v) and a flow rate of 5 mL/min.

In addition, the elution of compounds was monitored at 520 nm and 280 nm in this case, as shown in Fig. 3.49. Anthocyanins have absorption maxima at 280 nm and 520 nm, while copigments absorb between 200 and 400 nm. Consequently, copigments were only detected at 280 nm, while anthocyanins were found in both chromatograms. After separation, seventeen fractions were collected.

A polymeric fraction (F1, 139 mg) eluted at the beginning of HSCCC separation, then the main anthocyanin, delphinidin-3-*O*-sambubioside was isolated in a high purity of 97% and high quantity (59 mg) in fraction 3, while cyanidin-3-*O*-sambubioside eluted with a purity of 96% in fraction 4 (47 mg). The minor anthocyanin delphinidin 3-*O*-glucoside was found in fraction 6, while cyanidin-3-*O*-glucoside was isolated in fraction 7. Another compound, which was detected at 520 nm, presented a molecular ion at *m/z* 623 and fragment ion at *m/z* 287 corresponding to a cyanidin-derivative but due its low quantity it could not be identified.

On the other hand, neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid, which are the main copigments of *Hibiscus sabdariffa* L., were detected in high concentration in fraction 6 (40 mg), fraction 8 (4 mg) and fraction 9 (28 mg), respectively. The flavonols quercetin-3-sambubioside and quercetin-3-rutinoside co-eluted with cyanidin-3-*O*-glucoside in fraction 7 (12 mg). A mixture of other minor copigments such as quercetin-3-glucoside, kaempferol-3-rutinoside and quercetin were found at the end of HSCCC separation.

In conclusion, four anthocyanins from *Hibiscus sabdariffa* L. were successfully separated by HSCCC, as well as the main chlorogenic acids.

Hibiscus sabdariffa L. XAD-7

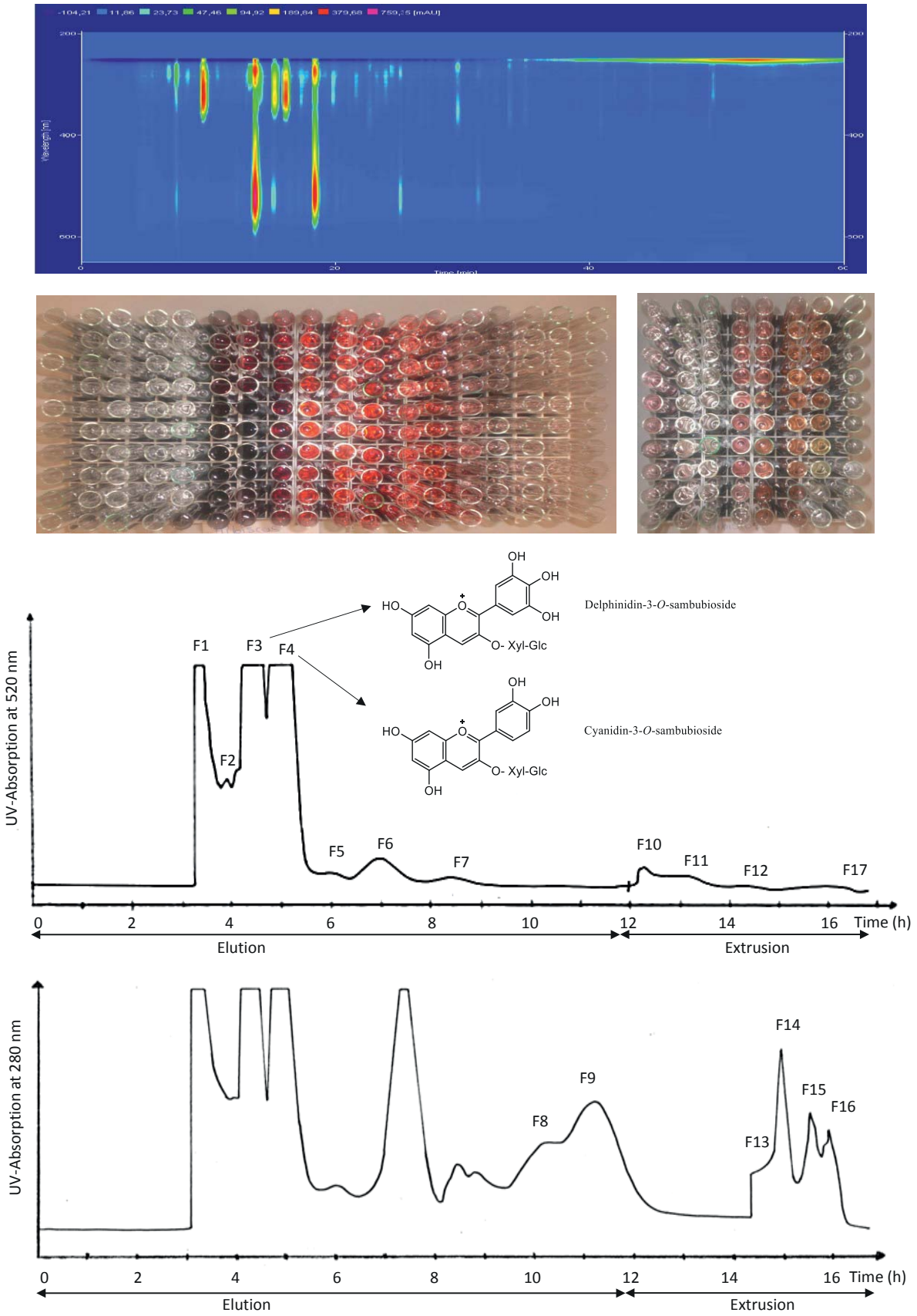


Fig. 3.49. HSCCC separation of *Hibiscus sabdariffa* L. XAD-7 extract at 520 nm and 280 nm.

3.8 Butterfly pea

3.8.1 Chemical composition

Butterfly pea (*Clitoria ternatea* L.) flowers are characteristic for their blue coloration, and their pigments are used as natural food colorant in some countries such as Thailand, Indonesia and Malaysia. The purpose of this study was to characterize and isolate anthocyanins and copigments of *Clitoria ternatea* L. flowers from Thailand. The blue color of *Clitoria ternatea* L. is due to the presence of glycosylated and acylated anthocyanins, especially polyacylated anthocyanins called ternatins and preternatins.

Anthocyanins were identified at 520 nm (Fig. 3.50) and sixteen anthocyanins were found. The UV-spectra of ternatins and preternatins presented an absorption maximum between 523 nm to 550 nm. In addition, they were identified by HPLC-ESI-MS^n and displayed high molecular ions. Ternatins are based on delphinidin-3-O-(6”-O-malonyl)-β-glucoside, while preternatins are considered precursors for ternatins because they are demalonylated analogues of ternatins. Ternatins and preternatins have 3’, 5’-side chains with alternative D-glucose and p-coumaric acid units (Fig. 3.51 and Fig. 3.52).

Consequently, ternatins produced mainly fragments ions by the losses of malonylglucose unit (248 Da), glucose (162 Da) and *p*-coumaric acid (146 Da), while preternatins displayed fragment ions by losses of glucose and p-coumaric acid units. The aglycone cation was detected at *m/z* 303, corresponding to delphinidin. As major anthocyanins peak 10 (*m/z* 1637), peak 11 (*m/z* 993), peak 12 (*m/z* 1475) and peak 13 (*m/z* 1783) were detected. Peak 10 was identified as ternatin B2 ($C_{75}H_{81}O_{41}^+$), while peak 12 was ternatin D2 ($C_{69}H_{71}O_{36}^+$) and peak 13 corresponded to ternatin D1 ($C_{84}H_{87}O_{43}^+$). In the case of peak 11 and peak 7 (*m/z* 1167), they could be characterized as delphinidin derivative due to the presence of delphinidin aglycone. The following minor compounds were detected: preternatin A3 (Peak 1, *m/z* 1405), ternatin C4 (Peak 2, *m/z* 1183), ternatin A3 (Peak 3, *m/z* 1491), preternatin B4 (Peak 4, *m/z* 1243), ternatin B4 or ternatin C1 (Peak 5 *m/z* 1329), ternatin C3 (Peak 6, *m/z* 1021), ternatin D3 (Peak 8, *m/z* 1167), preternatin B2 (Peak 9, *m/z* 1551) and preternatin D1 (Peak 14, *m/z* 1698). Moreover, traces of preternatin C4 (*m/z* 1097) and delphinidin-3-(6"-malonyl)glucoside (*m/z* 551) were found. However, ternatin A1 (*m/z* 2107), A2 (*m/z* 1799), B1 (*m/z* 1945), B3 (*m/z* 1637), C2 (*m/z* 1491), C5 (*m/z* 875) have also been reported in *Clitoria ternatea* L (Terahara et al., 1990; Terahara et al., 1998; Kazuma et al., 2003; Yoshida et al., 2009; Nair et al., 2015). These compounds were absent in the present sample. Table 3.23 summarizes the LC-MS data of detected anthocyanins in *Clitoria ternatea* L. The quantification of anthocyanins was performed at 520 nm and the total anthocyanin content was 120.64 mg/g dried flowers by HPLC, calculated as delphinidin-3-glucoside equivalents. *Clitoria ternatea* L. has a high anthocyanin content in comparison with other red and blue flowers (Vankar and Srivastava, 2010; Suppadit et al., 2011). Additionally, studies on thermal stability demonstrated that anthocyanins of *Clitoria ternatea* L. are more stable compared to other common anthocyanins extracts from fruits or monomeric anthocyanins (Lee et al., 2011).

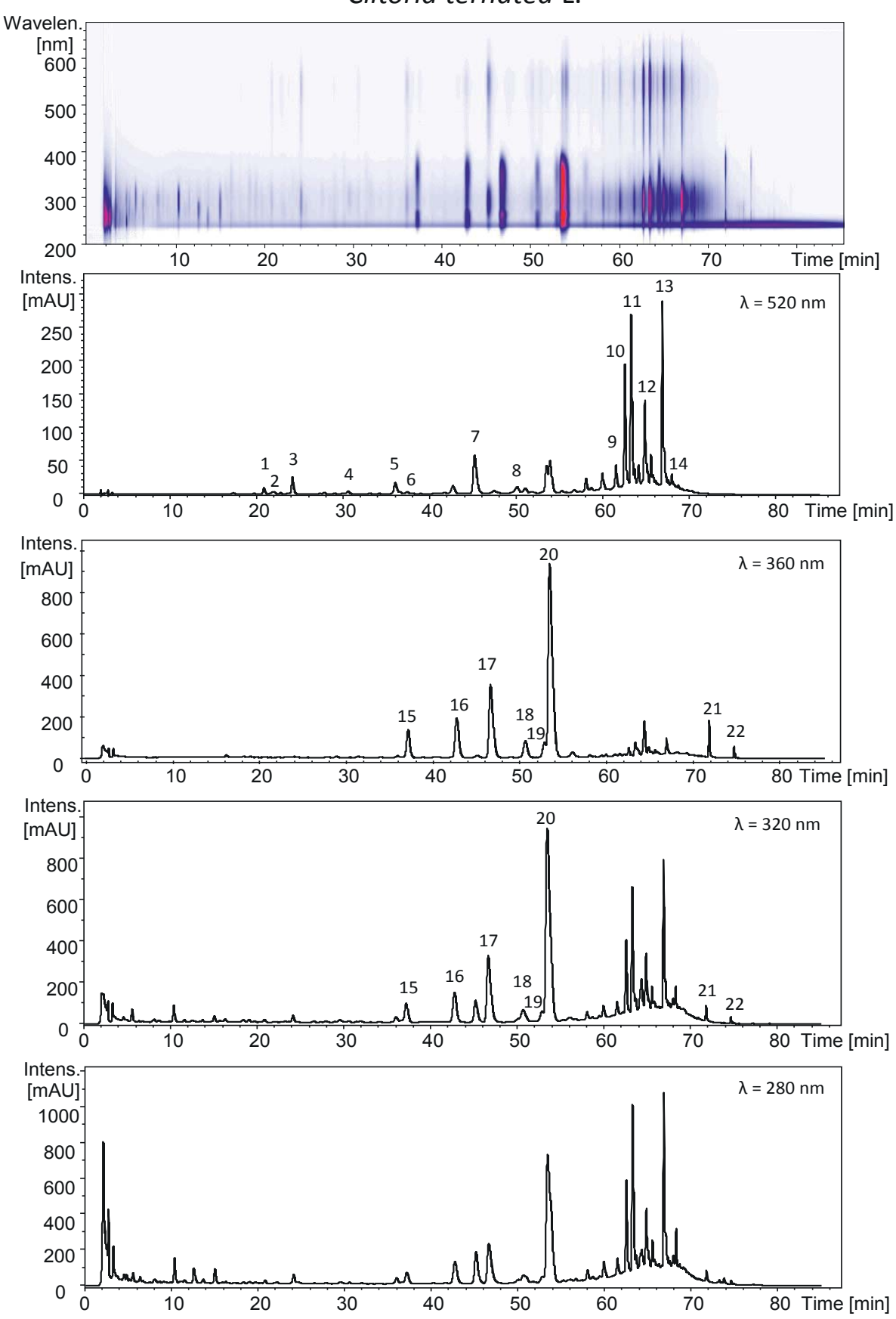
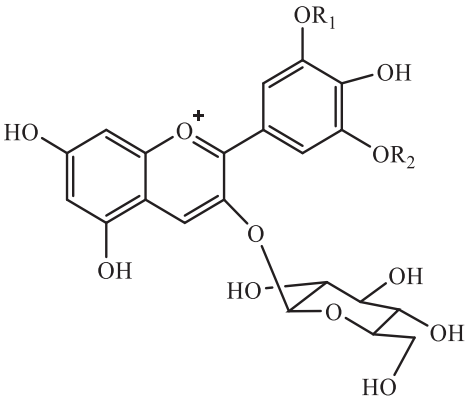


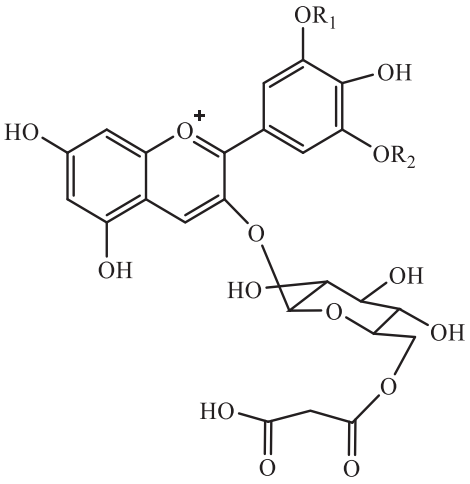
Fig. 3.50. HPLC DAD plot chromatogram from 200 to 660 nm and Base Peak chromatograms of *Clitoria ternatea* L. at 520 nm, 360 nm, 320 nm and 280 nm. For peak numbers see Tables 3.23 and 3.24.

Table 3.23. Anthocyanins in *Clitoria ternatea* L.

Preternatin



Ternatin



Peak No.	t_R (min)	Compound	R_1	R_2	$[M]^+$ (*m/z*)	Fragment ions (*m/z*)
*	17.4	Preternatin C4	GCG	G	1097	935, 773, 611, 465, 303
1	21.0	Preternatin A3	GCG	GCG	1405	1243, 1081, 935, 773, 611, 465
2	22.2	Ternatin C4	GCG	G	1183	935, 773, 611, 465, 303
3	24.5	Ternatin A3	GCG	GCG	1491	1447, 1329, 1243, 1081, 919, 773, 611
4	30.9	Preternatin B4	GCG	GC	1243	1081, 919, 773, 611, 465, 303
5	36.3	Ternatin B4 or Ternatin C1	GCG/ GCGC	GC/ G	1329	1243, 1168, 1081, 1021, 773, 611, 465
6	36.6	Ternatin C3	GC	G	1021	859, 773, 611, 465, 303
7	45.5	Delphinidin-derivative			1167	909, 919, 773, 611, 551, 465, 303
8	50.2	Ternatin D3	GC	GC	1167	919, 859, 773, 611, 465, 303
*	61.2	Del-3-(6"-malonyl)glc			551	303
9	61.8	Preternatin B2	GCGC	GCG	1551	1389, 1227, 919, 773, 611, 465
10	62.7	Ternatin B2	GCGC	GCG	1637	1389, 1227, 1082, 919, 773, 611, 465
11	63.3	Delphinidin-derivative			993	971, 869, 715, 479, 303
12	65.0	Ternatin D2	GCGC	GC	1475	1227, 919, 611, 465
13	67.0	Ternatin D1	GCGC	GCGC	1783	1698, 1535, 1390, 1227, 919, 773, 611
14	67.3	Preternatin D1	GCGC	GCGC	1698	1535, 1227, 919, 773, 611

G: D-glucose; C: *p*-coumaric acid; *traces

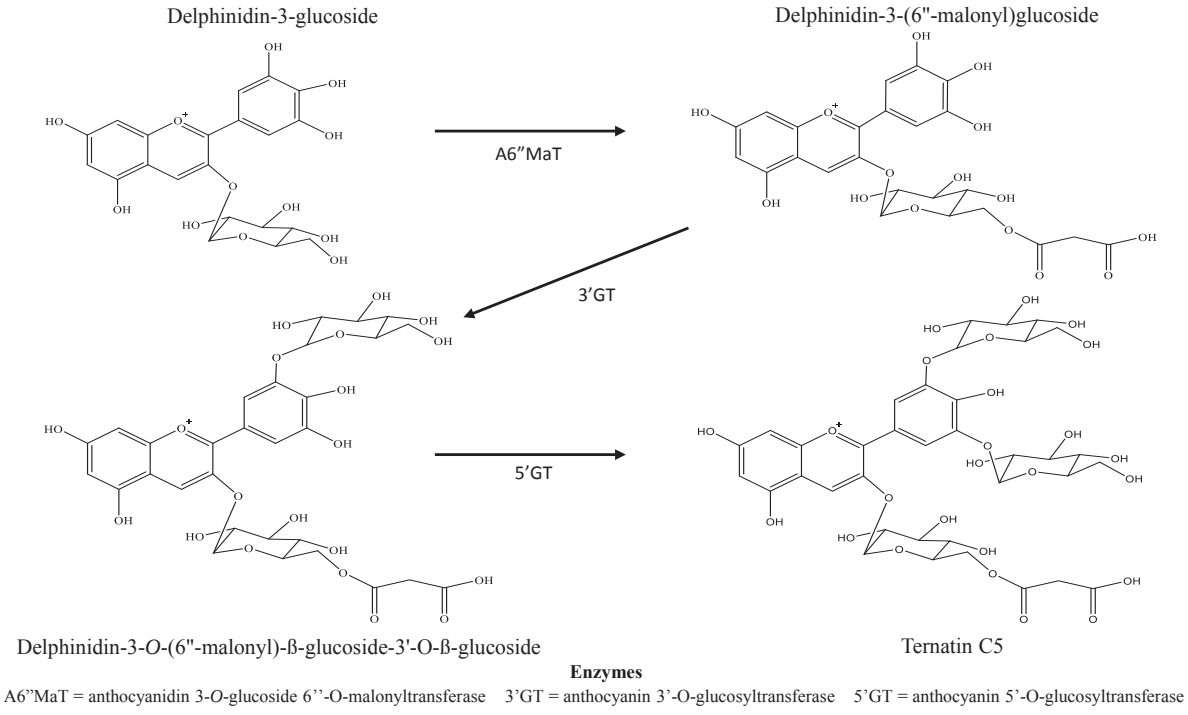


Fig. 3.51. Biosynthesis of Ternatin C5 (Kogawa et al., 2007).

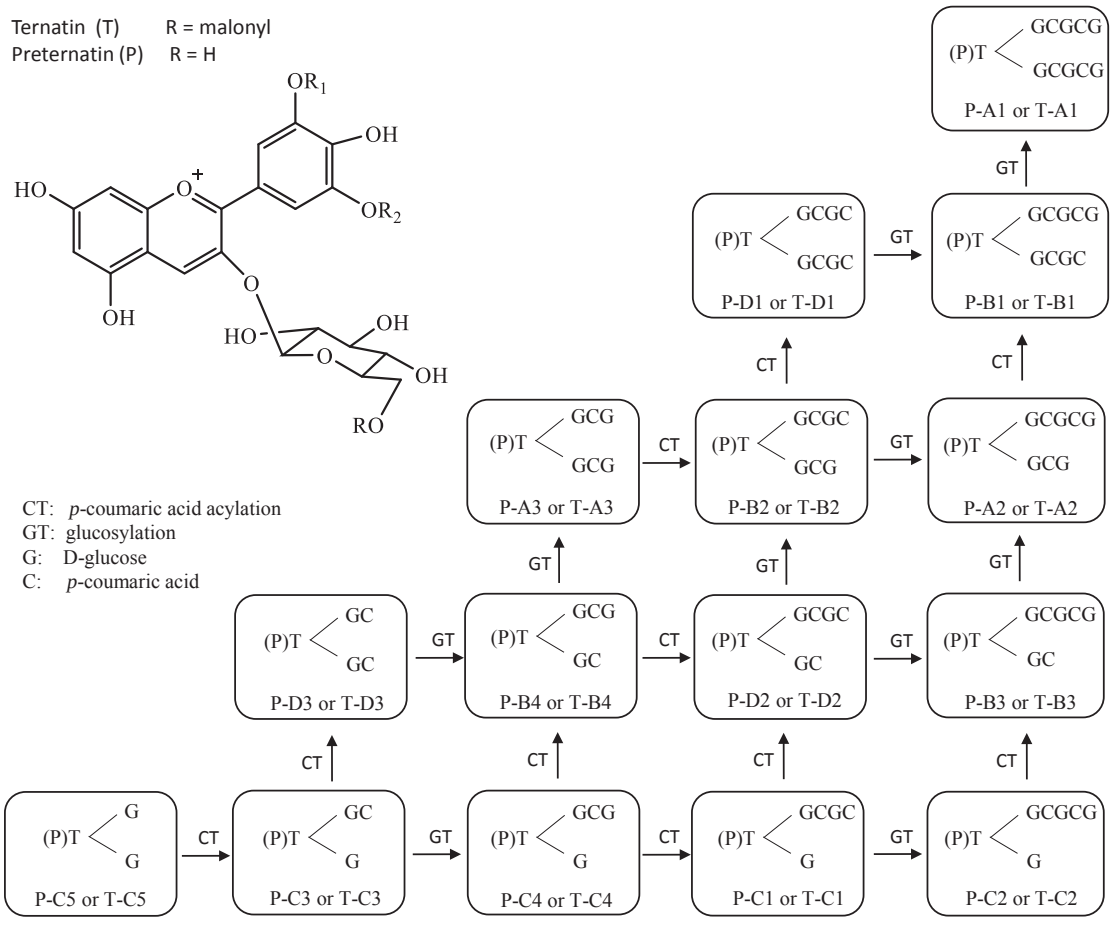


Fig. 3.52. Possible biosynthetic pathways of ternatins and preternatins (Terahara et al., 1998).

The main compounds in *Clitoria ternatea* L. are flavonol glycosides as shown in the HPLC chromatogram at 360 nm (Fig. 3.50). Fifteen flavonols glycosides were found and presented mass fragments in positive mode at 287, 303 and 319, which are characteristic of kaempferol, quercetin and myricetin aglycones, respectively. In addition, they showed also mass fragments in negative mode at 285, 301 and 317 corresponding to flavonols. The main copigment was peak 20 that was identified as kaempferol 3-neohesperidoside (*m/z* 595). Subsequently, quercetin-3-2^{G}-rhamnosylrutinoside (peak 15), quercetin-3-neohesperidoside (peak 16), kaempferol-3-2^{G}-rhamnosylrutinoside (peak 17), quercetin-3-rutinoside (peak 18), quercetin-3-glucoside (peak 19) and quercetin (peak 21) were detected. Furthermore, traces of myricetin-3-neohesperidoside, myricetin-3-2^{G}-rhamnosylrutinoside, myricetin-3-glucoside, myricetin-3-rutinoside, quercetin-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside, kaempferol-3-rutinoside, kaempferol-3-glucoside and kaempferol-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside were found. An overview on the identification of flavonols from *Clitoria ternatea* L. is given in Table 3.24. These results were in concordance with previous studies (Kazuma et al., 2003; Mukherjee et al., 2008).

Table 3.24. Flavonols of *Clitoria ternatea* L. flowers.

Peak No.	t_R (min)	Compound	Concentration (mg/g)**	$[M+H]^+$	Fragment ion (*m/z*)
15	37.5	Quercetin-3-2^{G}-rhamnosylrutinoside	30.04	757	611, 465, 303
16	43.0	Quercetin-3-neohesperidoside	82.23	611	465, 303
17	46.9	Kaempferol-3-2^{G}-rhamnosylrutinoside	121.00	741	595, 449, 287
18	50.7	Quercetin-3-rutinoside	62.58	611	465, 303
19	52.5	Quercetin-3-glucoside	15.26	465	303
20	53.6	Kaempferol-3-neohesperidoside	304.72	595	449, 287
21	72.1	Quercetin	14.15	303	
22	75.0	Unknown	-	985	675
*	29.1	Myricetin-3-2^{G}-rhamnosylrutinoside	-	773	627, 465, 319
*	31.5	Myricetin-3-neohesperidoside	-	627	481, 319
*	31.6	Myricetin-3-glucoside	-	481	319
*	36.3	Myricetin-3-rutinoside	-	627	481, 319
*	56.2	Quercetin-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside	-	697	535, 449, 287
*	62.1	Kaempferol-3-rutinoside	-	595	449, 287
*	63.8	Kaempferol-3-glucoside	-	449	287
*	64.4	Kaempferol-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside	-	681	535, 287

* traces; **calculated as kaempferol-3-neohesperidoside equivalents at 320 nm.

3.8.2 Preparative separations

3.8.2.1 Membrane chromatography

Polyphenols of dried *Clitoria ternatea* L. flowers (50 g) were extracted using methode 3 (see section 4.3), thus obtaining about 20 g of raw extract. In this case, 4 g of raw extract of *Clitoria ternatea* L. were separated by membrane chromatography (see section 4.6.5). After separation, 1.3 g of anthocyanin fraction and 2.5 g of copigment fraction were obtained. A comparison of HPLC-DAD analysis of raw extract, anthocyanin fraction and copigment fraction (Fig. 3.53) demonstrated that polyphenols of raw extract were divided in two groups. Anthocyanins, which absorb at 280 nm and 520 nm, were not present in the copigment fraction and this is supported by HPLC chromatograms at 520 nm (Fig. 3.54).

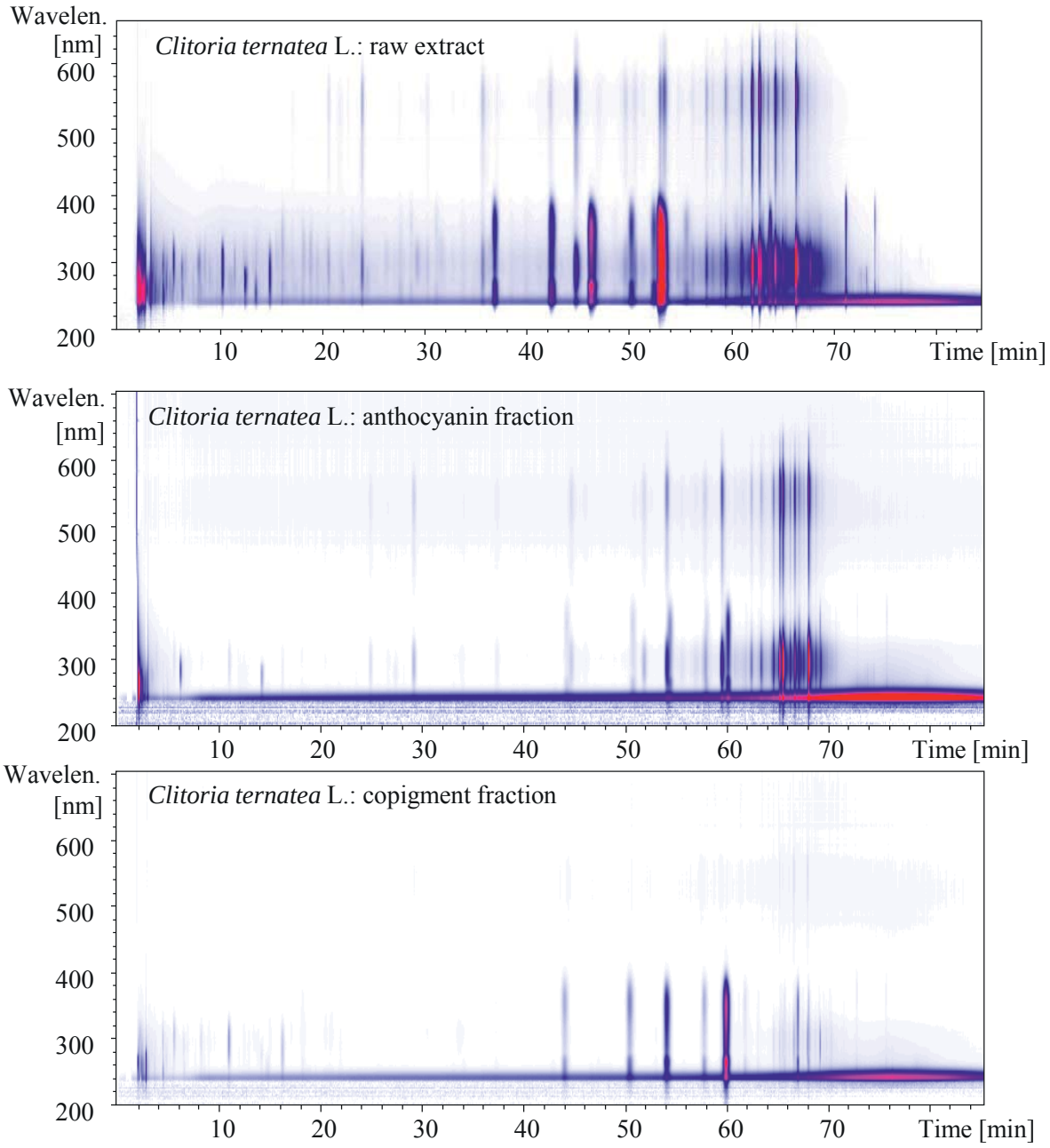


Fig. 3.53. *Clitoria ternatea* L.: HPLC-DAD analysis of raw extract (top), anthocyanin fraction (middle) and copigment fraction (bottom).

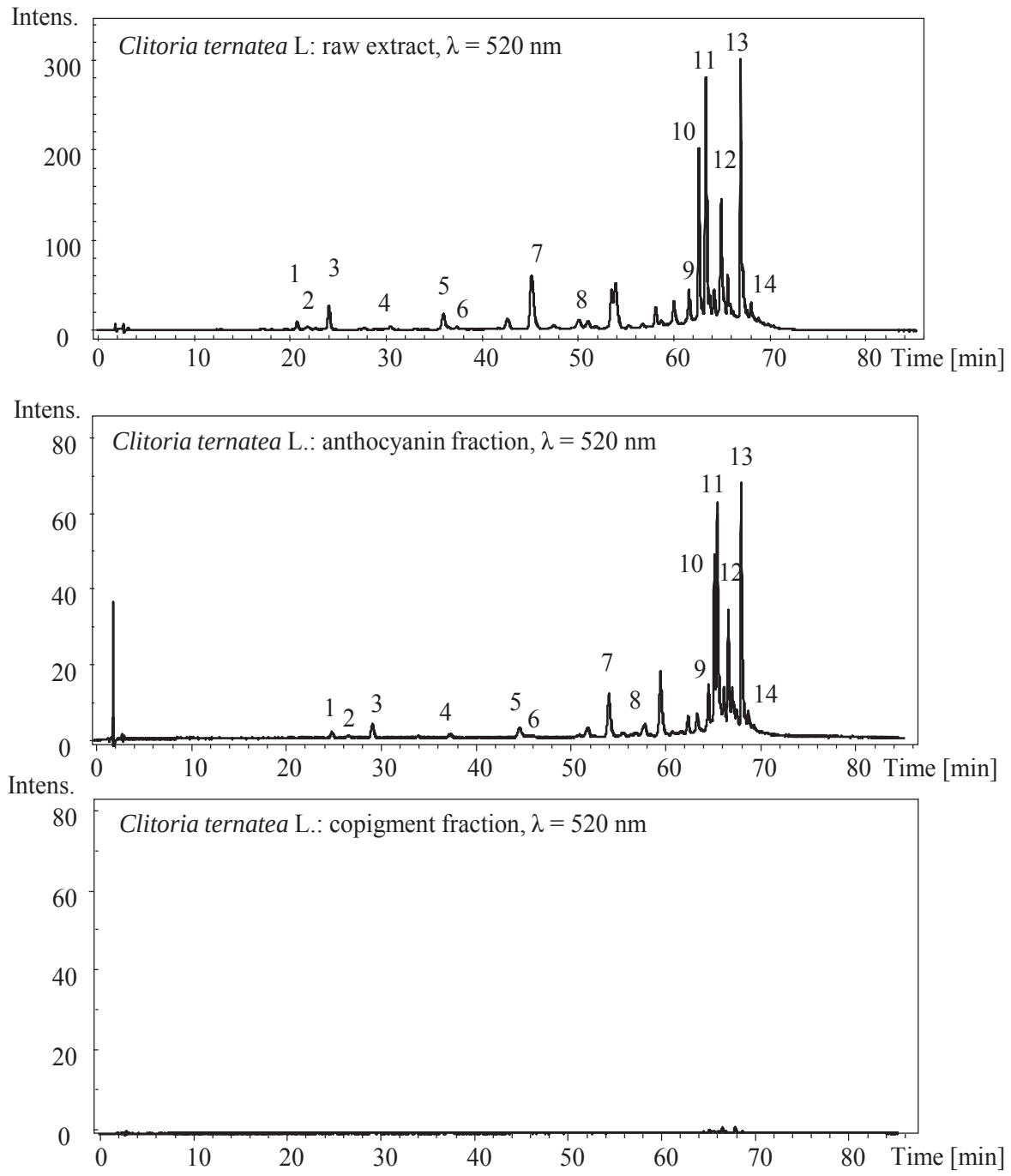


Fig. 3.54. HPLC Chromatogram at 520 nm of raw extract, anthocyanin fraction and copigment fraction of *Clitoria ternatea* L.

Due to the absence of anthocyanins in the copigment fraction, the membrane chromatographic separation proved satisfactory results and large amounts of copigments or anthocyanins could be obtained. After membrane chromatography, copigment fraction and anthocyanin fraction are available for the isolation of individual compounds by countercurrent chromatography.

3.8.2.2 Countercurrent Chromatography

HPCCC separation of raw extract of *Clitoria ternatea* L.

Raw extract of *Clitoria ternatea* L. (500 mg) was separated by HPCCC with tert-butyl methyl ether/acetonitrile/water (1:3:1:5, v/v/v/v), acidified with 0.1% TFA. The elution-extrusion mode was run in Head to Tail mode with a 3 mL/min flow rate and operating speed of 1600 rpm. Fractions were collected every minute. In order to detect anthocyanins and copigments in the *Clitoria ternatea* L. extract, compounds were monitored at 280 nm. Nine fractions were obtained as illustrated in Fig. 3.55.

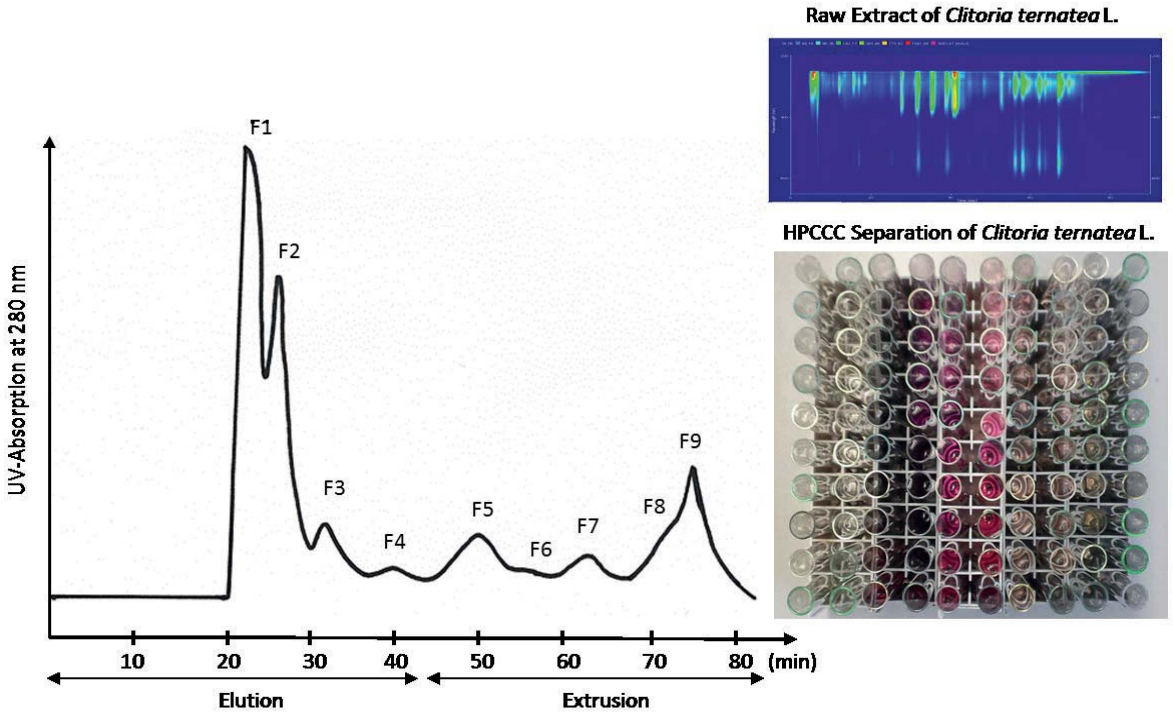


Fig. 3.55. HPCCC chromatogram of raw extract of *Clitoria ternatea* L. at 280 nm.

At the beginning of the HPCCC separation, anthocyanins were eluted in fraction 1 and fraction 2 in which a mixture of ternatin A3, preternatin A3 and ternatin B2 was present. In fraction 3, ternatin B4 or ternatin C1 and ternatin D2 were found. Quercetin-3-2^{G}-rhamnosylrutinoside was detected together with ternatin D1 and D2 in fraction 4. Kaempferol-3-2^{G}-rhamnosylrutinoside was isolated in fraction 5, while quercetin-3-neohesperidoside and quercetin-3-rutinoside were separated in F6 and F7. Kaempferol-3-neohesperidoside, which is the main compound in *Clitoria ternatea* L., was isolated in fraction 8, additionally quercetin-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside was a minor compound in the same fraction. At the end of HPCCC separation, a mixture containing quercetin-3-glucoside, quercetin-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside and quercetin was eluted. The isolation of individual anthocyanins was not possible due the complexity of raw extract from *Clitoria ternatea* L. Consequently, a previous separation of compounds (e.g. by membrane chromatography) is required in order to improve the isolation of copigments and polyacylated anthocyanins.

HPCCC Separation of copigment fraction of *Clitoria ternatea* L.

The semi-preparative separation of flavonols contained in copigment fraction from *Clitoria ternatea* L. was carried out by HPCCC. The solvent system consisted of ethyl acetate/n-butanol/water (5/5/10, v/v/v) acidified with 0.1% acetic acid. The HPCCC run was performed in Head to Tail mode using the elution-extrusion approach. Flow rate was 5 mL/min and rotation velocity was set to 1600 rpm. Fractions of 5 mL were collected and copigments were monitored at 280 nm. About 500 mg of copigment fraction were fractionated obtaining twelve fractions, as shown in Fig. 3.56.

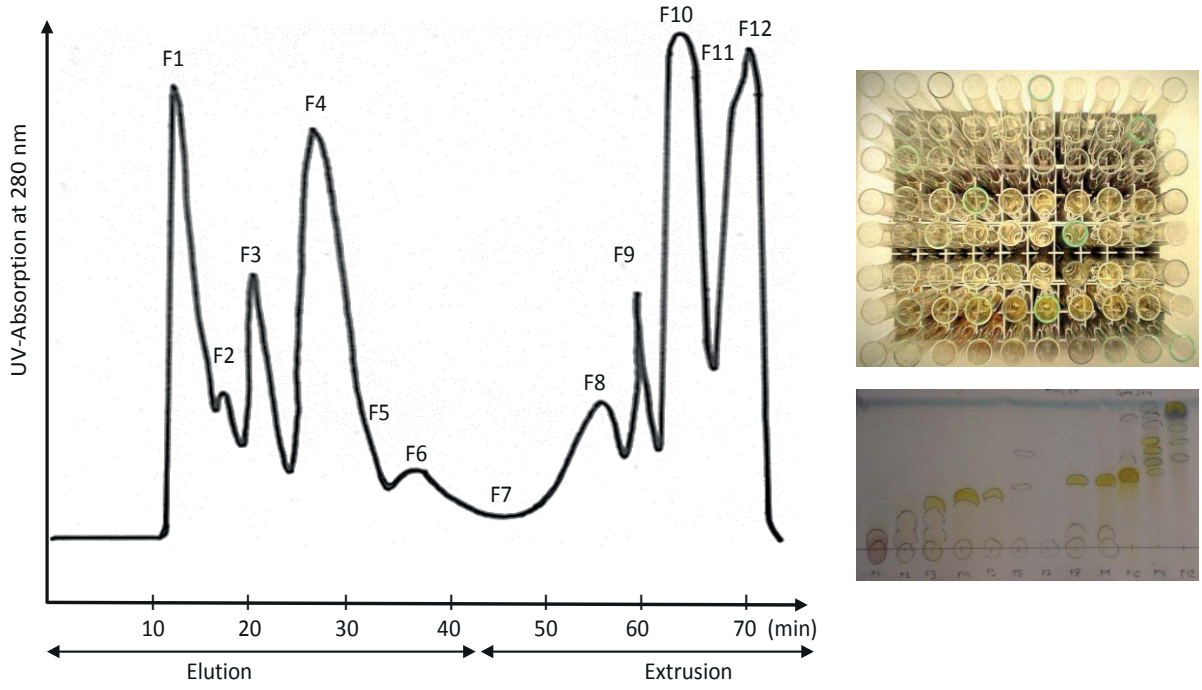


Fig. 3.56. HPCCC chromatogram of copigment fraction of *Clitoria ternatea* L. at 280 nm.

After TLC, HPLC-DAD and HPLC-DAD-ESI-MS^n analysis of every fraction, a polymeric hump was observed in fraction 1 (F1), then myricetin-3-2^G-rhamnosylrutinoside were detected in fraction 2 (17 mg). Quercetin-3-2^G-rhamnosylrutinoside was isolated with a high purity of 98% in fraction 3 (23.3 mg), while kaempferol-3-2^G-rhamnosylrutinoside was found in fraction 4 (54 mg, 98%) and in fraction 5 (5 mg) with a purity of 66%. Two myricetin derivates were found in fraction 6 (8 mg) corresponding to myricetin-3-glucoside and myricetin-3-neohesperidoside. In fraction 8 (22 mg), quercetin-3-neohesperidoside was detected in high purity, while fraction 9 contained quercetin-3-neohesperidoside (78%), quercetin 3-rutinoside (12%) and quercetin-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside (10%). The principal copigment, kaempferol-3-neohesperidoside, was isolated in fraction 10 with a high purity (92%) and in high quantity (131 mg). In fraction 11 (43mg), quercetin-3-glucoside, kaempferol-3-O-(2"-O-α-rhamnosyl-6"-O-malonyl)-β-glucoside, kaempferol-3-glucoside and kaempferol-3-rutinoside were detected. Finally, quercetin was isolated from fraction 12 (19 mg). In addition, the obtained fractions presented a coloration from light yellow to dark yellow.

3.9 Authenticity and Food Adulterations

Due to certain factors such as a limited production, low yield and high prices, food adulteration is a common problem. For example, the addition of cheaper fruit products in the case of fruit juices has been reported (Wrolstad and Durst, 2007). For this reason, the characterization of individual phenolic compounds such as anthocyanins, flavonols and phenolic acids is important for food authenticity assessment. Moreover, polyphenols can be used as markers for routine analysis in order to verify the authenticity of food and its products. The polyphenol content has been used as authentication tool in wines and fruit juices according to cultivars and geographic origin. However, there have been cases of adulterated food over the years. Pomegranate juices have been adulterated with grape, elderberry, raspberry, peach and pear because of limited production (Nuncio-Jáuregui et al., 2014), while the addition of amaranth, mulberries, blackcurrant and elderberry has been published in bilberry adulterations (Penman et al., 2006; Primetta et al., 2013). With a view to detect possible fraudulent cases for anthocyanin-rich food, fingerprints were established in the presented work by spectrophotometric analysis, UV-Vis spectra, HPLC-profiles and mass spectroscometric analysis. One indicator for authenticity is the total phenol content that can be calculated by Folin-Ciocalteu method (see section 4.6.4.1). Consequently, diverse anthocyanin-rich fruit juices were analyzed (Fig. 3.57). The results showed that *Aronia melanocarpa* is a rich source of phenolic compounds, followed by elderberry and beverages based on mixtures of red berries, while cranberry and strawberries presented the lowest phenolic content. In the case of anthocyanins, spectrophotometric methods show an absorption maximum at 520 nm. Nevertheless, this method is limited to determination of total anthocyanin content and cannot identify the individual anthocyanin composition. As a result, chromatographic methods such HPLC and HPLC-ESI-MS^n are required for the analysis in order to identify the individual composition. For instance, authentic *Aronia melanocarpa* juice and commercial aronia extracts (AE1-AE4) were analyzed by HPLC-DAD and HPLC-ESI-MS^n analysis revealing some anomalies in samples AE2, AE3 and AE4, as shown in Fig. 3.58. *Aronia melanocarpa* contains cyanidin-3-*O*-galactoside (peak 1) and cyanidin-3-*O*-arabinoside (peak 3) as main anthocyanins and cyanidin-3-*O*-glucoside (peak 2) and cyanidin-3-*O*-xyloside (peak 4) as minor compounds. Furthermore, *Aronia melanocarpa* has been characterized by the presence of chlorogenic acids, quercetin-derivatives and protocatechuic acid. However, extracts AE2, AE3 and AE4 presented a different anthocyanin profile in which cyanidin-3-*O*-glucoside was the main anthocyanin (80-84%) such as in blackberry (Fan-Chiang and Wrolstad, 2005). In addition, other anthocyanins such as cyanidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside were also detected. On the other hand, AE2 and AE3 had a lack of chlorogenic acids and quercetin derivatives, while AE4 contained only chlorogenic acid, quercetin-3-glucoside and quercetin-3-rutinoside. Because of the absence of characteristic markers of *Aronia melanocarpa*, adulterations by adding other fruits was demonstrated in AE2, AE3 and AE4 extracts. To summarize, adulterations can be detected using simple methods like TLC or spectrometric methods but

the use of chromatographic methods provide the best insight into the chemical composition by generating fingerprints for authenticity control.

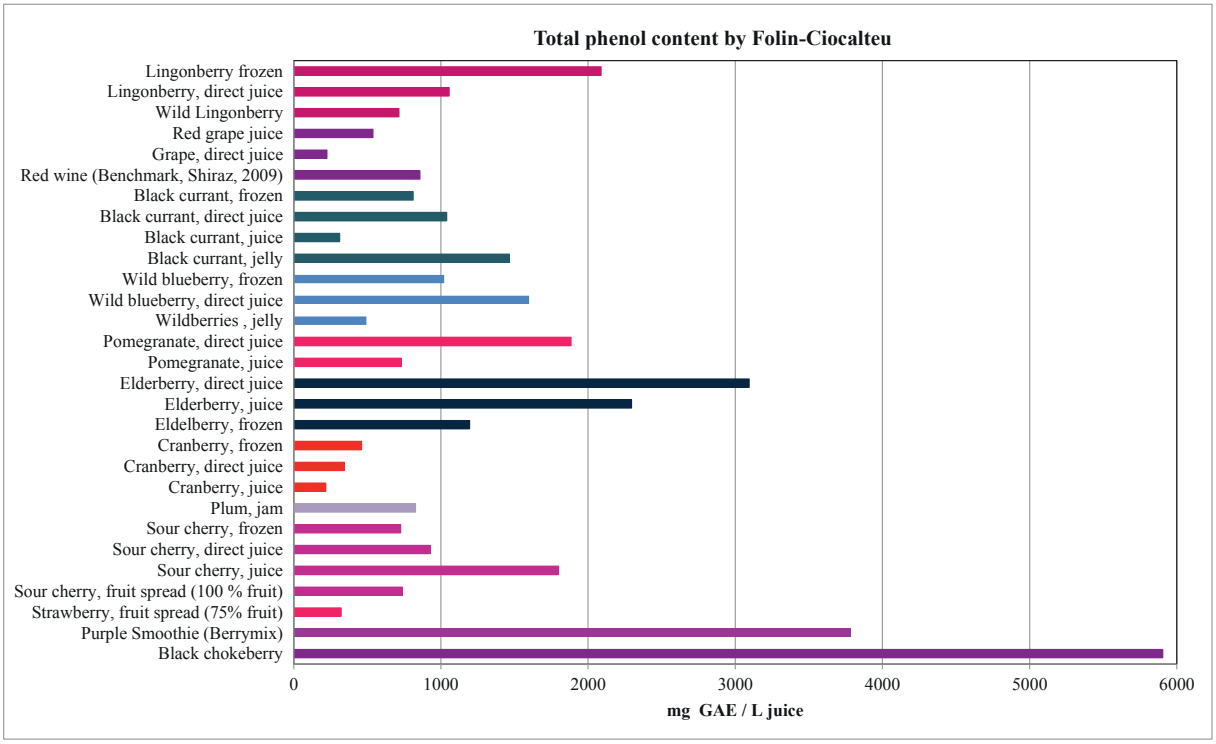


Fig. 3.57. Determination of total phenolic content in red/black fruits.

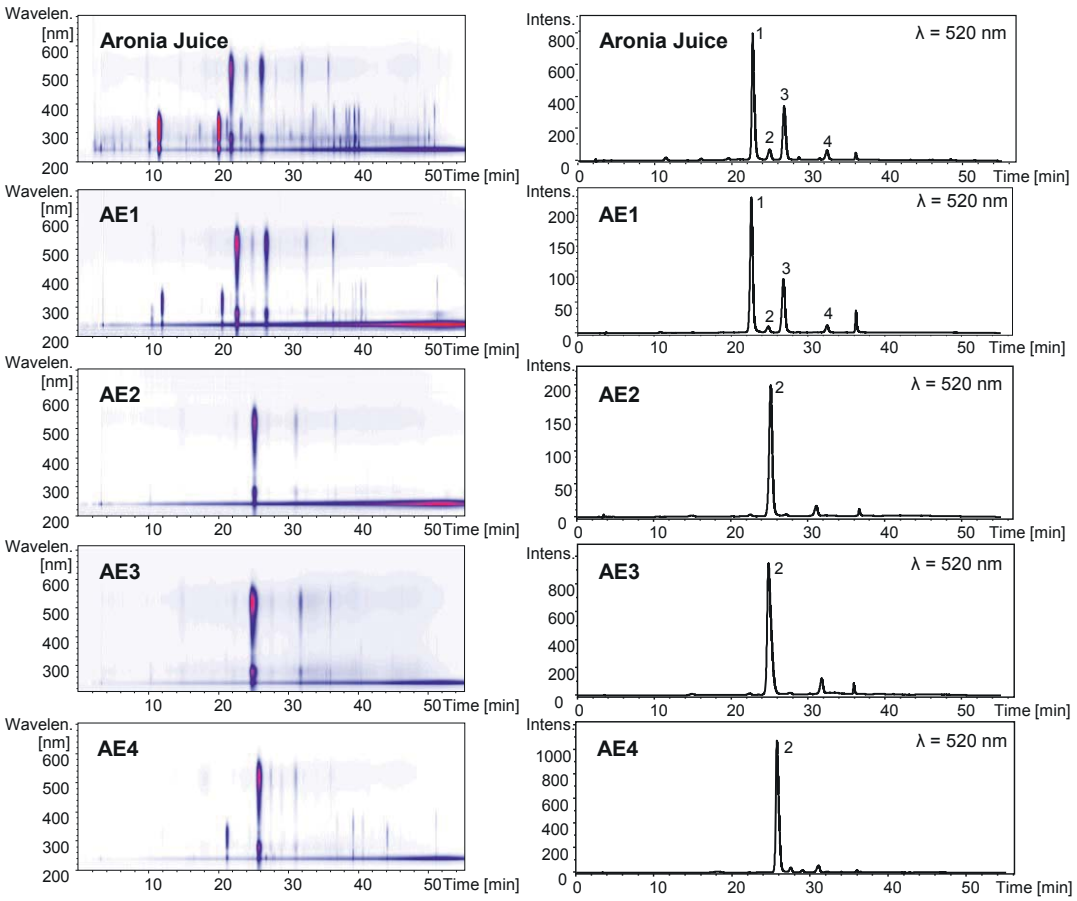


Fig. 3.58. HPLC-DAD Profile of *Aronia melanocarpa* juice and aronia extracts AE1-AE4 (left) and detection of adulteration in AE2-AE4 by UV-chromatogram at 520 nm (right).

4 Material and Methods

4.1 Plant materials

Table 4.1. List of raw material.

Plant material		Manufacturer
Blackberry	direct-cut and frozen	Real
	bio juice	Eden
Black chokeberry	extract 20%	Symrise
	pomace	Symrise
	concentrated juice	Symrise
	concentrated juice	Symrise
	juice	Rabenhorst
	AE1, berry extract	Symrise
	AE2, extract 25%	Symrise
	AE3, extract	Symrise
	AE4, extract	Symrise
Sour cherry	juice	Odenwald
	frozen	Henkel
	bio direct juice	Eden
	juice	Albi
	Fruit spread	Göbber
	Actiplants® Cherry Purevital extract	Symrise
Black carrot	Antonina, root	Provided by the Faculty of Agricultural Sciences and Landscape Architecture of the University of Applied Sciences of Osnabrück, Germany
	Deep Purple, root	
	Purple Sun, root	
	Purple Haze, root	
	Beta Sweet, root	
Purple Sweet Potato	root	Provided from Hefei, China
Roselle	dried calyces	Provided from Veracruz, México
	dried calyces	Provided from Egypt
Butterfly pea	dried flowers	Provided from Thailand

Table 4.1. Continued.

Plant material		Manufacturer
Blackcurrant	bio juice	Eden
	bio direct juice	Rabenhorst
	frozen	Henkel
	frozen	Henkel
	juice	Albi
	direct juice	Rabenhorst
	jelly	Schwartau
Blueberry	bio juice	Eden
	bio direct juice	Rabenhorst
	frozen	Henkel
Elderberry	bio juice	Rabenhorst
	direct juice	Bavaria Waldfrucht
	frozen	Bos Food Lebensmittel
Wild berry	jelly	Schwartau
Cranberry	bio direct Juice	Rabenhorst
	juice	Ocean Spray
	frozen	Bos Food Lebensmittel
Lingonberry	frozen	Henkel
	bio direct juice	Rabenhorst
Wild lingonberry	jelly	Schwartau
	juice	Schwartau
Strawberry	Fruit spread	Zentis
Grapefruit	bio direct juice	Rabenhorst
	juice	Takdaneh Agri
Plum	jam	Schwartau
Red grapes	direct juice, bio	Rabenhorst
	direct juice	Albi
Wein	Benchmark, Shiraz	Grant Burge Wines PYT LTD
Smoothie	Purple (berrymix)	True fruits
	Cherry, apple & cranberry	True fruits

4.2 Chemicals

Table 4.2. List of chemicals.

Chemicals		Manufacturer
Acetonitrile	HPLC grade	Fisher Scientific (Loghborough, UK)
Acetonitrile	LC-MS grade	Honeywell (Seelze, Germany)
Acetic acid	p.a. grade, ≥99.8%	Sigma (Steinheim, Germany)
Acetic acid	HPLC grade, 100%	AppliChem (Damstadt, Germay)S
Amberlite® XAD-7 HP	20-60 mesh	Sigma (Steinheim, Germany)
p-Anisaldehyde	>99%	Fisher Scientific (Loghborough, UK)
(+)-Catechin-Hydrate	≥99.8%	Sigma (Steinheim, Germany)
Chlorogenic acid hemihydrate	≥98%	Sigma (Steinheim, Germany)
Dichloromethane	HPLC grade	Fisher Scientific (Loghborough, UK)
Ethanol	≥99.8 %	Sigma (Steinheim, Germany)
Ethanol	industrial quality	ZCL
Ethyl acetate	p.a. grade, ≥99.5%	Carl Roth (Karlsruhe, Germany)
Ethyl methyl ketone	≥99%	Sigma (Steinheim, Germany)
Folin-Ciocalteu's phenol reagent		Merck (Damstadt, Germay)
Formic acid	p.a. grade, ≥98%	Carl Roth (Karlsruhe, Germany)
Formic acid	LC-MS grade	Fisher Scientific (Loghborough, UK)
Gallic acid monohydrate	98%	Fluka (Buchs, Switzerland)
Hydrochloric acid	37%	VWR Int. S.A.S. (Darmstadt, Germany)
Methanol	gradient grade	VWR Int. S.A.S. (Darmstadt, Germany)
Methanol-d_4	99.5%	Deutero (Kastellaun, Germany)
n-Butanol	HPLC grade, ≥99.8%	Carl Roth (Karlsruhe, Germany)
n-Hexane	industrial quality	ZCL
Quercetin dihydrate	≥95%	Merck (Damstadt, Germay)
Sodium carbonate	≥99%	Fluka (Buchs, Switzerland)
Sodium chloride	≥99%	Carl Roth (Karlsruhe, Germany)
Sodium hydroxide	≥99%	Carl Roth (Karlsruhe, Germany)
Sulfuric acid	p.a. grade, ≥95%	Carl Roth (Karlsruhe, Germany)
tert-Butyl methyl ether	p.a. grade, ≥99.5%	Fluka (Buchs, Switzerland)
tert-Butyl methyl ether	industrial quality	ZCL
Trifluoroacetic acid	99%	Sigma (Steinheim, Germany)
Trifluoroacetic acid-d_1,	99.5%	Deutero (Kastellaun, Germany)
Water	deionized, Nanopure®	Werner (Leverkusen, Germany)

4.3 Extraction of polyphenols

Method 1: Methanolic extraction

Fruits or flowers were cut and blended for 3 min, while black carrots were washed with water, dried, cut into small cubes, around 1cm^3. After that, polyphenols were extracted with a mixture of methanol/acetic acid (19:1, v/v) for 8 hours. Then plant material was removed by filtration (filter paper, MN 615 ¼ Macherey-Nagel, Düren, Germany). Solvents were removed by vacuum evaporation and the extract was freeze-dried.

Method 2: Blanching extraction

Purple Sweet Potatoes were washed with water, dried, cut into small cubes, around 1cm^3, including their peels. For the extraction of polyphenols, purple sweet potatoes were blanched with hot water at 100 °C for 3 min. Then a solution of water/hydrochloric acid (19:1, v:v) was added and cooled at 0 °C for 3 hours, subsequently stored at room temperature for 8 hours. To remove the plant material, the suspension was filtered, then solvents were removed by vacuum evaporation.

Method 3: Ethanolic extraction

Clitoria ternatea L. flowers were blended for 3 min and then sample was defatted three times with n-hexane. Subsequently, sample was defatted with dichloromethane (3x500 mL), then 400 mL of a mixture of ethanol/water (8:2, v/v) was added and homogenized for 1 min, thereupon addition of 600 mL of a mixture of water:formic acid (98:2, v/v), then stored for 8 hours. The eluate was concentrated after filtration, and freeze-dried.

4.4 Adsorption chromatography on Amberlite XAD-7

The raw extract was applied onto an Amberlite XAD-7 column that had a diameter of 6 cm and a filling level of 60 cm. Afterwards the column was washed with water in order to eliminate salts, proteins and sugars. Polyphenols were eluted with a mixture of methanol/acetic acid (19:1, v/v). Solvents were evaporated and the XAD-7 extract freeze-dried.

4.5 Solvent precipitation

The freeze-dried extract was dissolved in 150 mL of ethanol, then stirred for 1 h. The insoluble residues were removed by filtration. Subsequently, 150 mL of n-hexane were dropped into the solution at a flow rate of 10 mL/min, then the solution was filtered. Solvents of the filtrate were evaporated and the extract freeze-dried.

4.6 Device parameters

4.6.1 HPLC-DAD

4.6.1.1 Analytical HPLC-DAD

Analysis of Anthocyanins:

Pump:	PU-980 Plus, Intelligent HPLC Pump, Jasco (Groß-Umstadt)
Degasser:	DG-980-50, 3-Line-Degaser, Jasco (Groß-Umstadt)
Gradient former:	LG-980-09, Ternary Gradient Unit, Jasco (Groß-Umstadt)
Detector:	MD-910, Multiwavelenghth detector
Injector:	AS-950, Intelligent Sampler, Jasco (Groß-Umstadt)
Software:	Borwin-PDA Version 1.0

Column:	RP-18 Luna 5µ C-18(2) 100 Å, 250 x 4.6 mm, Phenomenex (Aschaffenburg)
Precolumn:	4 mm x 4 mm HPLC guard cartridge system of the same material as column
Wavelength:	280 nm, 320 nm, 360 nm and 520 nm
Flow rate:	0.5 mL/min
Solvent A:	water/acetonitrile/formic acid (87:3:10, v/v/v)
Solvent B:	water/acetonitrile/formic acid (40:50:10, v/v/v)

Gradient 1: for blackberry, black chokeberry, sour cherry, black carrots and roselle

Time (min)	0	20	35	40	45	55	60
A (%)	94	80	60	40	10	94	94
B (%)	6	20	40	60	90	6	6

Gradient 2: for purple sweet potato

Time (min)	0	20	25	40	45	70	80	90
A (%)	98	86	86	85	85	10	98	98
B (%)	2	14	14	18	18	90	2	2

Gradient 3: for butterfly pea

Time (min)	0	2	5	10	20	30	40	45	50	55	60	65	70	75	83	90
A (%)	94	80	89	87	84	82	80	78	75	70	60	30	10	30	94	94
B (%)	6	10	11	13	16	18	20	22	25	30	40	70	80	70	6	6

Analysis of Proanthocyanins:

Pump:	PU-2080 plus, Intelligent HPLC Pump, Jasco (Groß-Umstadt, Germany)
Degasser:	DG-2080-53, 3-Line-Degasser, Jasco (Groß-Umstadt, Germany)
Gradient former:	LG-2080-02, Ternary Gradient Unit, Jasco (Groß-Umstadt, Germany)
Detector:	MD-2010 plus, Multiwavelength Detector, Jasco (Groß-Umstadt, Germany)
Injector:	AS-2057 plus, Intelligent sampler, Jasco (Groß-Umstadt, Germany)
Thermostat:	Jetstream Plus Column Thermostat, Jasco (Groß-Umstadt, Germany)
Software:	ChromPass Chromatography Data System, Version 1.8.6.1

Column:	Aqua 5µ C-18, 125 Å, 250 x 4,6 mm, Phenomenex (Aschaffenburg)
Precolumn:	4 mm x 4 mm HPLC guard cartridge sytems of the same material as column
Wavelength:	280 nm
Temperature oven:	25 °C
Flow rate:	0.8 mL/min
Solvent A:	water/acetic acid (98:2, v/v)
Solvent B:	acetonitrile

Gradient:

Time (min)	0	25	45	50	55	60	70
A (%)	97	90	65	25	75	97	97
B (%)	3	10	35	75	25	3	3

Table 4.3. Calibration range for quantification by HPLC.

Compounds	Standard	Calibration range (mg/L)
Anthocyanins	Cyanidin-3-glucoside	40-480
Ternatins	Delphinidin-3-glucoside	10-1300
Chlorogenic acids	Chlorogenic acid (5-CQA)	22-260
Quercetin-derivatives	Quercetin	13-180
Copigments of *Clitoria ternatea* L.	Kaempferol-3-neohesperidoside	34-670

4.6.1.2 Preparative HPLC-UV/VIS

Pump:	Wellchrom HPLC Pump K-1001, Knauer (Berlin)
Degasser:	Knauer
Gradient former:	Solvent Organizer K-1500, Knauer (Berlin)
Mixing	Dynamic mixing chamber, Knauer (Berlin)
Detector:	Wellchrom UV K-2600, Knauer (Berlin)
Injector:	Knauer 55960 with 500 µL loop
Software:	Eurochrom 2000 for Windows Version 2.05

Column:	RP-18 Luna 5µ C-18(2) 100 Å, 250 x 15.0 mm, Phenomenex (Aschaffenburg)
Wavelength:	280 nm and 520 nm
Flow rate:	3 mL/min
Solvent A:	water/acetonitrile/formic acid (92:3:5, v/v/v)
Solvent B:	water/acetonitrile/formic acid (45:50:5, v/v/v)

Gradient 1			Gradient 2			Gradient 3		
Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
0	94	6	0	95	5	0	94	6
20	80	20	8	94	6	10	70	30
35	60	40	12	92	8	30	60	40
40	40	60	18	90	10	40	40	60
45	10	90	20	88	12	48	10	90
55	94	6	25	84	16	53	94	6
60	94	6	30	73	27	60	94	6
			50	10	90			
			55	94	6			
			60	94	6			

Gradient 1: for neochlorogenic acid, protocatechuic acid and cyanidin 3-arabinoside.

Gradient 2: for cyanidin-3-galactoside and cyanidin-3-glucoside.

Gradient 3: for cyanidin-3-sambubioside and delphinidin-3-sambubioside.

4.6.2 HPLC-DAD-ESI-MS^n

Mass spectrometer:	HCT Ultra PTM Discovery system, Bruker Daltonics (Bremen)
Pump:	Series 1100-Bin Pump G1312A, Agilent Technologies (Waldbronn)
Autosampler:	Series 1100-Autosampler ALS SL G1329B, FC/ALS Therm G1330B, Agilent Technologies (Waldbronn)
Injection:	1-10 µL
Detector:	Series 1100-Autosampler ALS SL G1329B, FC/ALS Therm G1330B, Agilent Technologies (Waldbronn)
Software:	Bruker Hystar 3.2, Bruker ESI-Compass 1.3 for HCT/Esquire, Data Analysis Version 3.0, Bruker Daltonics (Bremen)

Analysis of Anthocyanins:

Ion source:	ESI
Ion Polarity:	positive (Anthocyanins) and negative (Copigments) and alternating mode
Trap Drive:	120
Dry Gas Temp (set):	330 °C
Nebulizer (set):	60 psi
Dry gas (set):	N2, 11 L/min
HV Capillary:	-4500 V
HV End Plate Offset:	-1500 V
Scan range:	100-2200 *m/z*
Max.Accu Time:	200 ms
ICC Target:	100000

Column:	C18 (2) Luna column (Phenomenex, Germany), 150 mm x 2.0 mm, 3 µ.
Solvent A:	water/acetonitrile/formic acid (95:3:2, v/v/v)
Solvent B:	water/acetonitrile/formic acid (48:50:2, v/v/v)
Samples:	were dissolved in mobile phase A and analyzed
Flow rate:	0.2 mL/min
HPLC gradient:	as described above see section 4.6.1.1; Analysis of anthocyanins Gradient 1: for blackberry, black chokeberry, sour cherry, black carrots and roselle Gradient 2: for purple sweet potato Gradient 3: for butterfly pea

Analysis of Proanthocyanins:

Ion source:	ESI
Ion Polarity:	negative and alternating mode
Trap Drive:	50
Dry Gas Temp (set):	325 °C
Nebulizer (set):	40 psi
Dry gas (set):	N2, 10 L/min
HV Capillary:	-70 V
HV End Plate Offset:	-500 V
Scan range:	100-2200 *m/z*
Max.Accu Time:	200 ms
ICC Target:	100000

Column:	Aqua C-18 column, 150 mm x 2.0 mm, 3µ, 125 Å (Phenomenex, Germany)
Solvent A:	water/acetic acid (98:2, v/v)
Solvent B:	acetonitrile
Samples:	were dissolved in mobile phase A and analyzed
Flow rate:	0.2 mL/min
HPLC gradient:	as described above see section 4.6.1.1; Analysis of proanthocyanins

4.6.3 TLC

TLC-sheets:	ALUGRAM® SIL G/UV_{254}, Silica gel 60 with fluorescent indicator UV_{254}, Macherey-Nagel (Düren, Germany)
Solvent 1:	ethyl acetate/butanone/formic acid/water (40:35:10:10, v/v/v/v)
Solvent 2:	dichloromethane/methanol/water (7.5:2.7:0.3, v/v/v)
Detection:	UV light at 254 nm and 366 nm
Spray reagents:	*p*-anisaldehyde-sulfuric acid reagent
After reagent:	heated to 105 °C until visualization of spots.
Lamp:	HP-UVIS, 366 nm and 254 nm, Desaga, Sarstedt

4.6.4 UV Spectrophotometer

Instrument:	UV/VIS/NIR Spectrophotometer V-570 Jasco (Groß-Umstadt, Germany)
Software:	Spectramanager v1.27.02

4.6.4.1 Total phenolic content by Folin Ciocalteu method

The total phenolic content of sample was determined using Folin-Ciocalteu´s method and gallic acid as standard. A 7.5 % Na_2CO_3 solution, 10% Folin-Ciocalteu's reagent and a standard solution of gallic acid (concentration interval from 10 to 60 mg/L) were used. Solution of the samples in a concentration of 1 mg/mL was used in the analysis. 200 µL of sample, water or standard solution of gallic acid were pipetted into a semi-micro cuvette, and then 1 mL of 10% Folin-Ciocalteu's reagent was added. After an interval of time (30 s-8 min), 800 µL of 7.5 % Na_2CO_3 solution was put into the cuvette. Two hours later, the absorbance of blue coloration was measured at $\lambda = 760$ nm against a blank sample. Absorbance of calibration standards, sample and blank value calculated the absorbance difference. The standard curve is in m/L, results were reported in milligrams gallic acid equivalents per liter sample.

4.6.5 Membrane chromatography

Table 4.4. Technical data of Sartobind SIEX 150 mL, filter capsule and pumpe system.

Dimensions	190 x 77 mm
Beed height	8 mm
Membrane volume /area nano capsule	3 ml /110 cm^2
Membrane volume/ area 150 ml capsule	150 ml /5500cm^2
Binding capacity 10%	3.9 g
Ion exchange ligand Sartobind S	Strong acidic cation exchanger:sulfonic acid ($R\text{-}CH_2\text{-}SO_3^-$)
Housing	Polypropylene
Membrane matrix	Stabilized reinforced cellulose Nominal pore size > 3µm
Ion capacity per cm^2 of membrane	2-5µeq
Maximum pressure at 20 °C	4 bar, 0.4 Mpa, 58 psi
Maximun pressure during venting	0.5 bar, 0.05MPa, 44 psi
Pumpe	Tandem 1082, Sartorius, Göttingen (Germany) Dimensions: width: 14.6 cm; height: 21.6 cm; depth: 27.9 cm
Filter capsule	Sartopore 2300, Sartorius, Göttingen (Germany)

The sample was dissolved in 1 L methanol/acetic acid (19:1, v/v) and was filtered with a filter paper (MN 615 ¼ Macherey-Nagel, Düren, Germany). A Sartopore 2300 filter capsule was connected between the adsorber and the pumping system. The membrane absorber was regenerated with 2.5 L of 1N NaOH by using a peristaltic pump with a flow rate of 100 mL/min. After regeneration, an equilibration step was carried out with 2.5 L of 0.01N HCl and then 1 L methanol/acetic acid (19:1, v/v). Subsequently,

the extract solution was loaded. To remove copigments, the membrane adsorber was flushed with one liter of methanol/acetic acid (19:1, v/v) and this copigment-fraction was collected. Anthocyanins were eluted with 1 L of 1:1 (v:v) mixture of aqueous 1M NaCl solution and methanol. This anthocyanin-fraction was acidified with acetic acid with a final concentration of 1% for stabilization. The solvents of copigment-fraction and anthocyanin-fraction were removed in vacuo and the residue was freeze-dried. During the loading, washing and elution step, every 200 mL, a sample of 2 mL of the solution was collected, dried under nitrogen and analyzed by HPLC-DAD.

Desalination of Anthocyanin-Fraction

The anthocyanin fraction contained sodium chloride. To remove this salt, an Amberlite XAD-7 HP column (Sigma, St. Louis, MO) was used. A glass column (120 cm x 6 cm) filled with polyacrylic resin XAD-7 was washed with 1 L of methanol and then equilibrated with 3 L water/acetic acid (995:5, v/v). Anthocyanin fraction was dissolved in 500 mL of water/acetic acid (995:5, v/v) and applied onto an Amberlite XAD-7 HP column. The column was washed with 3 L water/acetic acid (995:5, v/v). After elimination of salts, anthocyanins were eluted with methanol/acetic acid (19:1, v/v). Anthocyanin-fraction was concentrated in vacuo and freeze-dried.

4.6.6 Countercurrent Chromatography

4.6.6.1 High-Speed Countercurrent Chromatography

Instrument:	High-speed model CCC-1000,
	Pharma-Tech Research Corp. Baltimore, MD (USA)
	Triple coil system, Teflon tube i.D. 2.56 mm
Coil volume:	850 mL
Pump:	Biotronik BT 3020, HPLC Pump Jasco
Fraction collector:	Pharmacia LKB Superfrac
Detector:	UV/Vis Detector k-2500, Knauer (Berlin)
Recorder:	ABBC Goerz Servogor 120, 3cm/h
Loop:	20 mL or 50 mL

4.6.6.2 High Performance Countercurrent Chromatography

Instrument:	Spectrum HPCCC, Dynamic Extractions Ltd (UK)
	equipped with two columns, PFA tubing i.D. 1.6 mm
Coil volume:	125.5 mL (63 mL + 62.5 mL)
Temperature:	30 °C
Thermostat:	RC6 CS, Lauda Dr. R.Wobser, Lauda-Königshofen (Germany)

Pump	WellChrom HPLC-Pump K-501, Knauer (Berlin)
	with a preparative pump head (up to 50 mL/min)
Fraction collector	Pharmacia LKB Super Frac, Pharmacia LKB, Bromma (Sweden)
Detector	WellChrom Spectro-Photometer K-2500 detector, Knauer (Berlin)
Loop	5 mL
Software	Eurochrom 2000 for Windows Version 2.05, Knauer (Berlin)

4.6.6.3 Low-Speed Rotary Countercurrent Chromatography

Instrument:	Prototype Pharma-Tech Research Corp. Baltimore, MD (USA)
	Single coil system, convoluted tubes,
	Teflon tube i.D. 8.2 mm, 107 m long
Coil volume:	5.5 L
Pump:	Biotronik BT 3020, HPLC Pump Jasco
Fraction collector:	Pharmacia LKB Superfrac
Detector:	UV/Vis Detector k-2500, Knauer (Berlin)
Recorder:	ABBC Goerz Servogor 120, 1 cm/h
Sample load:	manuel injection over 3 way tap direct to coil, 300 mL sample extract

Table 4.5. Parameters for CCC separations.

CCC System	Sample	Sample		Solvent system
LSRCCC	Blackberry XAD-7	35.5 g	4 mL/min 60 rpm 12 min/tube	TBME/n-BuOH/ACN/Water (2/2/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Blackberry F5/F6 LSRCCC	700 mg	4 mL/min 900 rpm	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Aronia A20 AF	700 mg	3 mL/min 900 rpm 4 min/tube	TBME/ACN/Water (3/1/3; v/v/v) + 0.1% TFA
HPCCC Semi-preparative	Aronia A20 AF	250 mg	3 mL/min 1600 rpm 1 min/tube	EtOAc/n-BuOH/Water (2/8/10; v/v/v) + 1% formic acid
HSCCC	Aronia A20 CF	700 mg	3 mL/min 900 rpm 4 min/tube	TBME/ACN/Water (2/2/3; v/v/v)
HSCCC	Aronia AP AF	500 mg	3 mL/min 900 rpm 4 min/tube	EtOAc/EtOH/Water (5/2/3; v/v/v) + 0.1% TFA

Table 4.5. Continued.

CCC System	Sample	Sample		Solvent system
HSCCC	Aronia AP CF	1000 mg	3 mL/min 900 rpm 4 min/tube	n-Hexane/EtOAc/n-BuOH/ HCOOH/Water (1/2/2/0.8/5; v/v/v/v/v)
HSCCC	Black carrot Antonina AF	500 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Black carrot Deep Purple AF	500 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Black carrot Purple Sun AF	500 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Black carrot Antonina CF	300 mg	3 mL/min 900 rpm 4 min/tube	EtOAc/EtOH/Water (5/2/3; v/v/v)
HSCCC	Black carrot Deep Purple CF	300 mg	3 mL/min 900 rpm 4 min/tube	EtOAc/EtOH/Water (5/2/3; v/v/v)
HSCCC	Black carrot Purple Sun CF	300 mg	3 mL/min 900 rpm 4 min/tube	EtOAc/EtOH/Water (5/2/3; v/v/v)
HSCCC	Purple sweet potato PSP AF	700 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Purple sweet potato PSP CF	600 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HSCCC	Roselle HSL XAD-7	500 mg	3 mL/min 900 rpm 4 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HPCCC Semi-preparative	*Clitoria ternatea* L. Raw extract	500 mg	3 mL/min 1600 rpm 1 min/tube	TBME/n-BuOH/ACN/Water (1/3/1/5; v/v/v/v) + 0.1% TFA
HPCCC Semi-preparative	*Clitoria ternatea* L. CF	500 mg	5 mL/min 1600 rpm 1 min/tube	EtOAc/n-BuOH/Water (5/5/10; v/v/v) + 0.1% acetic acid

4.6.7 Nuclear Magnetic Resonance Spectroscopy

Instrument 1: AVII-600 Bruker Daltonics (Germany)

Measuring frequency: ^{1}H: 600, 1 MHz, ^{13}C: 150.9 MHz

Instrument 2: AMX-300 Bruker Daltonics (Germany)

Measuring frequency: ^{1}H: 300, 1 MHz, ^{13}C: 75.5 MHz

Physicochemical description of the purified substances

ANTHOCYANINS

Cyanidin-3-O-β-D-glucopyranoside (Cyanidin-3-glucoside)

Origin: Blackberry, Black chokeberry

Molecular formula: $C_{21}H_{21}O_{11}^{+}$

Molecular mass: 449.38 g/mol

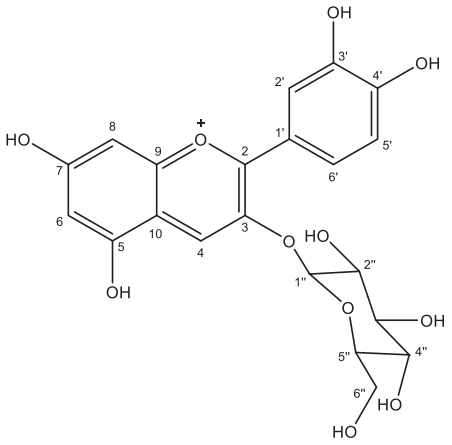
UV (HPLC method, Solvent A):

λ_{max} = 515 nm and 279 nm

ESI-MS (pos)

Pseudo molecular ion *m/z* 449 $[M]^{+}$;

m/z 287 [M-Glucose]$^{+}$



^{1}H-NMR (600 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 3.48 (dd, 1H, J=9.3Hz, J=9.4Hz, H4''), 3.57 (d, 1H, J=9.1Hz, H3''), 5.59 (ddd, 1H, J=2.3, J=6.1Hz, J=8.9Hz, H5''), 3.7 (dd, 1H, J=7.9Hz, J=9.1Hz, H2''), 3.76 (dd, 1H, J=6.1Hz, J=12.1Hz, H6b''), 3.96 (dd, 1H, J=2.2Hz, J=12.1Hz, H6a''), 5.33 (d, 1H, J=7.8Hz, H1''), 6.69 (d, 1H, J=2.0Hz, H6), 6.93 (dd, 1H, J=0.8Hz, J=2.0Hz, H8), 7.05 (d, 1H, J=8.7Hz, H5'), 8.08 (d, 1H, J=2.3Hz, H2'), 8.29 (dd, 1H, J=2.3Hz, J=8.7Hz, H6'), 9.06 (sbr, 1H, H4).

^{13}C-NMR (75.5 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 62.5 (C6''), 71.2 (C4''), 74.9 (C2''), 78.2 (C3''), 78.9 (C5''), 95.4 (C8), 103.5 (C6), 103.9 (C1''), 113.5 (C10), 117.5 (C5'), 118.5 (C2'), 121.3 (C1'), 128.3 (C6'), 137.1 (C4), 145.7 (C3), 147.5 (C3'), 155.9 (C4'), 157.9 (C9), 159.4 (C5), 164.5 (C2), 170.6 (C7).

Cyanidin-3-O-β-D-galactopyranoside (Cyanidin-3-galactoside)

Origin: Black chokeberry

Molecular formula: $C_{21}H_{21}O_{11}^{+}$

Molecular mass: 449.38 g/mol

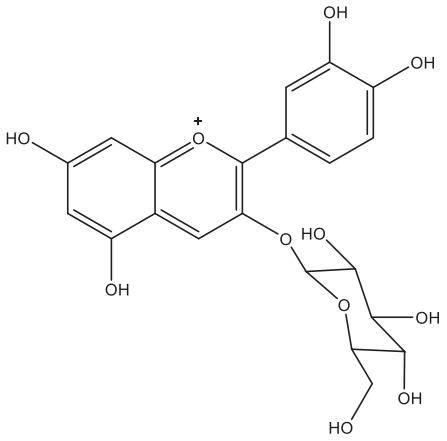
UV (HPLC method, Solvent A):

λmax = 515 nm and 279 nm

ESI-MS (pos)

Pseudo molecular ion *m/z* 449 $[M]^{+}$;

m/z 287 $[M\text{-Galactose}]^{+}$



^{1}H-NMR (300 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 3.72 (dd, 1H, J=3.2Hz, J=9.6Hz, H3''), 3.80-3.85 (m, 3H, H5'', H6a'', H6b''), 4.00 (d, 1H, J=3.1Hz, H4''), 4.04 (dd, 1H, J=8.1Hz, J=8.9Hz, H2''), 5.29 (d, 1H, J=7.5Hz, H1''), 6.68 (d, 1H, J=1.7Hz, H6), 6.92 (d, 1H, J=1.8Hz, H8), 7.04 (d, 1H, J=8.7Hz, H5'), 8.09 (d, 1H, J=2.2Hz, H2'), 8.29 (dd, 1H, J=2.2Hz, J=8.7Hz, H6'), 9.05 (1H, H4).

^{13}C-NMR (75.5 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 62.4 (C6''), 70.14 (C4''), 72.1 (C2''), 75.0 (C3''), 77.80 (C5''), 95.2 (C8), 103.4 (C6), 104.5 (C1''), 113.4 (C9), 117.4 (C5'), 118.5 (C2'), 121.3 (C1'), 128.3 (C6'), 137.0 (C4), 145.7 (C3), 147.4 (C3'), 155.8 (C4'), 157.7 (C10), 159.2 (C5), 164.4 (C2), 170.5 (C7).

Cyanidin-3-O-β-D-arabinopyranoside (Cyanidin-3-arabinoside)

Origin: Black chokeberry

Molecular formula: $C_{20}H_{19}O_{10}^{+}$

Molecular mass: 419.36 g/mol

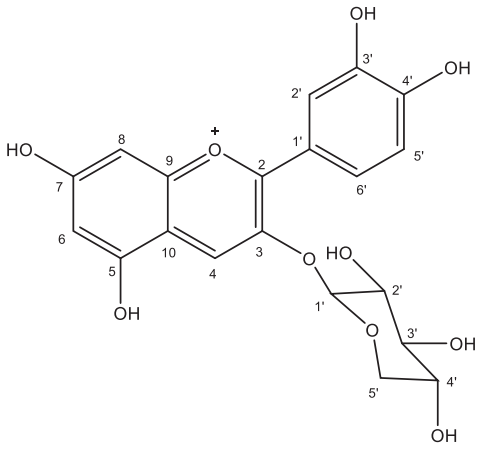
UV (HPLC method, Solvent A):

λmax = 515 nm and 279 nm

ESI-MS (pos)

Pseudo molecular ion *m/z* 419 $[M]^{+}$;

m/z 287 $[M\text{-Arabinose}]^{+}$



^{1}H-NMR (300 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ =3.76 (d, 1H, J= 5.4Hz, H5b''), 3.79 (dd, 1H, J=2.2Hz, J=5.5Hz, H3''), 3.97-3.99 (m, 2H, H2'', H5a''), 4.04 (ddd, 1H, J=1.5Hz, J=6.3Hz, J=7.0Hz, H4''), 5.27 (d, 1H, J=6.1Hz, H1''), 6.64 (d, 1H, J=1.7Hz, H6), 6. 87 (dd, 1H, J=0.7Hz, J=1.7Hz, H8), 6.99 (d, 1H, J=8.8Hz, H5'), 8. 03 (d, 1H, J=2.2Hz, H2'), 8.29 (dd, 1H, J=2.3Hz, J=8.8Hz, H6'), 8.91 (s, 1H, H4).

^{13}C-NMR (75.5 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 66.7 (C5''), 68.7 (C4''), 72.1 (C2''), 73.7 (C3''), 95.2 (C8), 103.5 (C6), 104.1 (C1''), 113.3 (C10), 117.5 (C5'), 117.7 (C2'), 118.5 (C1'), 121.3 (C6'), 128.7 (C4), 136.4 (C3), 145.6 (C3'), 147.5 (C4'), 157.7 (C9), 157.7 (C5), 164.5 (C2), 170.5 (C7).

Cyanidin-3-O-[β-D-xylopyranosyl-(1'''→2'')-β-D-glucopyranoside]
(Cyanidin-3-sambubioside)

Origin: *Hibiscus sabdariffa* L.

Molecular formula: $C_{26}H_{29}O_{15}^+$

Molecular mass: 581.51 g/mol

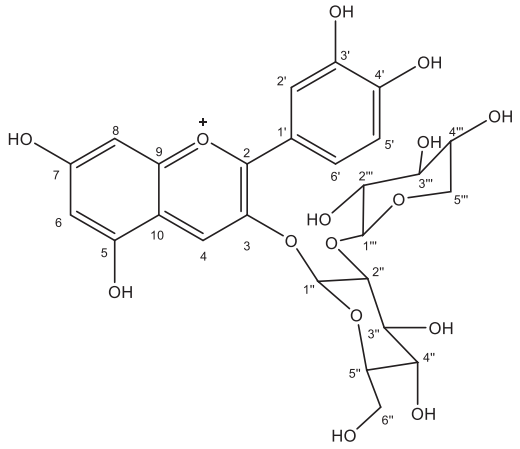
UV (HPLC method, Solvent A):

λmax = 523 nm and 275 nm

ESI-MS (pos)

Pseudo molecular ion *m/z* 581 $[M]^+$;

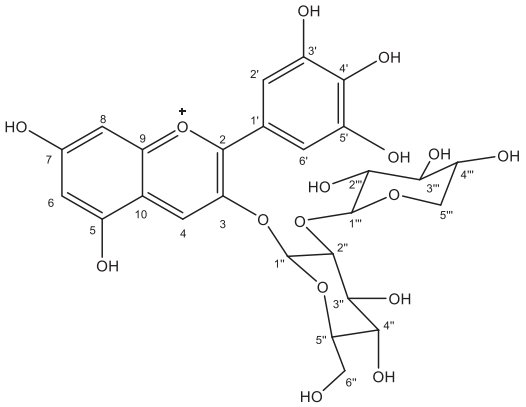
m/z 287 $[M\text{-Glucose-Xylose}]^+$



^{1}H-NMR (300 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ =3.06 (dd, 1H, J=10.5Hz, J=11.5Hz, H5b'''), 3.18 (dd, 1H, J=7.5Hz, J=8.5Hz, H2'''), 3.27-3.35 (m, 1H, H3'''), 3.42 (ddd, 1H, J=10.5Hz, J=8.5Hz, J=5.5Hz, H4'''), 3.51 (dd, 1H, J=9.0Hz, J=9.5Hz, H4''), 3.58 (ddd, 1H, J=2.0Hz, J=5.5Hz, J=9.5Hz H5''), 3.69 (dd, 1H, J=5.5Hz, J=11.5, H5a'''), 3.73 (dd, 1H, J=5.5Hz, J=12.0Hz, H6b''), 3.78 (dd, 1H, J=9.0Hz, J=9.0Hz, H3''), 3.92 (dd, 1H, J=12.0Hz, J=2.0Hz, H6a''), 3.95 (dd, 1H, J=9.0Hz, J=7.5Hz, H2''), 4.76 (d, 1H, J=7.5Hz, H1'''), 5.44 (d, 1H, J=7.5Hz, H1''), 6.65 (d, 1H, J=2.0Hz, H8), 6.88 (d, 1H, J=2.0Hz, H6), 7.01 (d, 1H, J=8.5Hz, H5'), 8.02 (d, 1H, J=2.5Hz, H2'), 8.26 (dd, 1H, J=2.5Hz, J=8.5Hz, H6'), 8.95 (s, 1H, H4).

^{13}C-NMR (75.5 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 62.3 (C6''), 67.2 (C5'''), 70.8 (C4''), 71.0 (C4'''), 75.7 (C2'''), 77.9 (C3'''), 78.2 (C3''), 78.8 (C5''), 81.8 (C2''), 95.0 (C8), 101.7 (C1''), 104.0 (C6), 105.7 (C1'''), 113.2 (C10), 117.3 (C5'), 118.5 (C2'), 121.2 (C1'), 128.6 (C6'), 136.1 (C4), 145.3 (C3), 147.5 (C3'), 156.0 (C4'), 157.5 (C9), 159.2 (C5), 164.1 (C2), 170.4 (C7).

Delphinidin-3-O-[β-D-xylopyranosyl-(1’’’→2’’)-β-D-glucopyranoside]
(Delphinidin-3-sambubioside)



Origin: *Hibiscus sabdariffa* L.

Molecular formula: $C_{26}H_{29}O_{16}^{+}$

Molecular mass: 597.50 g/mol

UV (HPLC method, Solvent A):

λmax = 523 nm and 275 nm

ESI-MS (pos)

Pseudo molecular ion *m/z* 597 $[M]^{+}$;

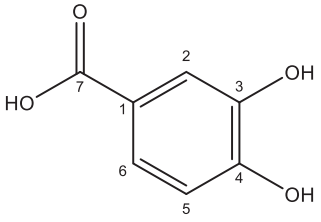
m/z 303 $[M\text{-Glucose-Xylose}]^{+}$

^{1}H-NMR (600 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ =2.98 (dd, 1H, J=11.6Hz, J=10.4Hz, H5a’’’), 3.20 (dd, 1H, J=9.1Hz, J=7.7Hz, H2’’’), 3.29-3.34 (m, 1H, H3’’’), 3.38 (ddd, 1H, J=10.3Hz, J=8.9Hz, J=5.4Hz, H4’’’), 3.54 (dd, 1H, J=9.2Hz, J=9.7Hz, H4’’), 3.60 (dd, 1H, J=5.2Hz, J=11.5Hz, H5’’’), 3.62 (ddd, 1H, J=2.5Hz, J=5.7Hz, J=9.8Hz, H5’’), 3.76 (dd, 1H, J=5.7Hz, J=12.2Hz, H6a’’), 3.80 (t, 1H, J=9.1 H3’’), 3.94 (dd, 1H, J=12.3Hz, J=2.3Hz, H6b’’), 4.01 (dd, 1H, J=9.0Hz, J=7.6Hz, H2’’), 4.71 (d, 1H, J=7.7Hz, H1’’’), 5.48 (d, 1H, J=7.6Hz, H1’’), 6.66 (d, 1H, J=2.0Hz, H6), 6.86 (d, 1H, J=2.0Hz, H8), 7.75 (s, 2H, H2’, H6’), 8.89 (s, 1H, H4).

^{13}C-NMR (150 MHz, Methanol-d_4/TFA (d_1), 19:1, ppm): δ = 62.0 (C6’’), 66.8 (C5’’’), 70.4 (C4’’), 70.6 (C4’’’), 75.7 (C2’’’), 77.6 (C5’’), 77.7 (C3’’), 78.4 (C3’’’), 81.4 (C2’’), 95.0 (C8), 101.3 (C1’’), 103.1 (C6), 105.9 (C1’’’), 112.5 (C10), 112.7 (C2’, C6’), 120.0 (C1’), 135.0 (C4), 144.7 (C4’), 145.5 (C3), 147.6 (C3’,C5’), 157.4 (C9), 158.6 (C5), 164.0 (C2), 170.2 (C7).

COPIGMENTS

3,4-Dihydroxybenzoic acid (Protocatechuic acid)



Origin: Black chokeberry.

Molecular formula: $C_7H_6O_4$

Molecular mass: 154.12 g/mol

UV (HPLC method, Solvent A):

λmax = 523 nm and 275 nm

ESI-MS (neg)

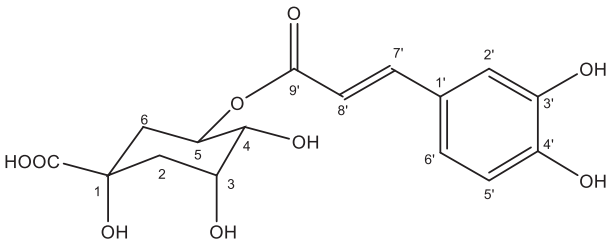
Pseudo molecular ion *m/z* 153 $[M\text{-}H]^{-}$;

m/z 109 $[M\text{-}H\text{-}CO_2]^{-}$

^{1}H-NMR (300 MHz, Methanol-d4, ppm): δ =6.8 (1H, d, J=8.5 Hz, H5), 7.43 (1H, dd, J=8.5, 2.0 Hz, H6), 7.44 (1H, s, H2).

^{13}C-NMR (75.5 MHz, Methanol-d4, ppm): δ =115.8 (C5), 117.8 (C2), 123.3 (C6), 123.9 (C1), 146.1 (C3), 151.5 (C4), 170.2 (C7).

5-O-Caffeoylquinic acid (Chlorogenic acid)



Origin: Black chokeberry

Molecular formula: $C_{16}H_{18}O_9$

Molecular mass: 354.31 g/mol

UV (HPLC method, Solvent A):

λmax = 242 nm and 326 nm

ESI-MS (neg)

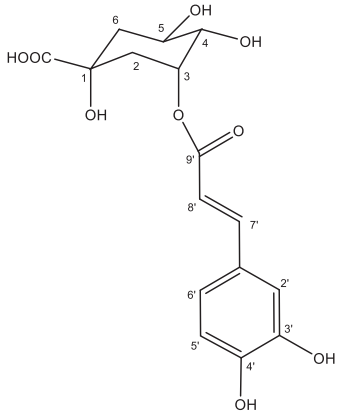
Pseudo molecular ion *m/z* 353 $[M-H]^-$;

m/z 191, 179, 161

^{1}H-NMR (300 MHz, Methanol-d4, ppm): δ = 2.03 (m, 1H, H2eq), 2.07 (m, 1H, H6ax), 2.13 (m, 1H, H2ax), 2.23 (m, 1H, H6eq), 3.72 (dd, 1H, J=3.1Hz, J=8.6Hz, H4), 4.17 (dd, 1H, J=4.0Hz, J=7.0Hz, H3), 5.34 (dt, 1H, J=4.3Hz, J=9.1Hz, H5), 6.26 (d, 1H, J=15.9Hz, H8´), 6.78 (d, 1H, J=8.2Hz, H5´), 6.95 (dd, 1H, J=2.0Hz, J=8.2Hz, H6´), 7.05 (d, 1H, J=2.0Hz, H2´), 7.56 (d, 1H, J=15.9Hz, H7´).

^{13}C-NMR (75.5 MHz, Methanol-d4, ppm): δ = 38.3 (C2), 39.0 (C6), 71.5 (C3), 72.1 (C5), 73.7 (C4), 76.3 (C1), 115.3a) (C8´), 115.4a) (C2´), 116.6 (C5´), 123.0 (C6´), 127.9 (C1´), 146.9 (C3´), 147.1 (C7´), 149.6 (C4´), 168.8 (C9´), 177.2 (COOH).
a:shifts are interchangeable

3-O-Caffeoylquinic acid (Neochlorogenic acid)



Origin: Black chokeberry

Molecular formula: $C_{16}H_{18}O_9$

Molecular mass: 354.31 g/mol

UV (HPLC method, Solvent A):

λmax = 242 nm and 326 nm

ESI-MS (neg)

Pseudo molecular ion *m/z* 353 $[M-H]^-$;

m/z 191, 179, 135

^{1}H-NMR (300 MHz, Methanol-d4, ppm): δ = 1.90-2.28 (m, 4H, H2ax, H2eq, H6ax, H6eq), 3.66 (dd, 1H, J=2.5 Hz, J=8.0 Hz, H4), 4.14 (dt, 1H, J=3.2 Hz, J=8.5 Hz, H5), 5.36 (d, 1H, J=2.6 Hz, H3), 6.30 (d, 1H, J=15.9 Hz, H8´), 6.78 (d, 1H, J=8.1 Hz, H5´), 6.94 (dd, 1H, J=1.6 Hz, J=8.2 Hz, H6´), 7.05 (d, 1H, J=1.7 Hz, H2´), 7.59 (d, 1H, J=15.9 Hz, H7´).

^{13}C-NMR (75.5 MHz, Methanol-d4, ppm): δ = 36.9 (C2), 41.4 (C6), 68.6 (C3), 73.0 (C5), 74.8 (C4), n.d. (C1), 115.4 (C8´), 116.0 (C2´), 116.6 (C5´), 122.9 (C6´), 128.1 (C1´), 146.8 (C3´), 146.8 (C7´), 149.5 (C4´), 169.1 (C9´), n.d. (COOH).

5 Summary and Conclusion

Public scandals, awareness of food safety of colorants and health promotion are factors that have marked changes in the legislation of food additives worldwide. Furthermore, the substitution of synthetic food colorants with natural food colorants and the importance of finding sources for natural food colorants have significantly increased.

In the first part of this work, the main aim was to characterize the phenolic composition of anthocyanin-rich food, especially blackberry, black chokeberry, sour cherry, black carrots, purple sweet potatoes, roselle and butterfly pea. Based on glycosylated or acylated anthocyanin classification, all anthocyanin-rich food presented glycosylated anthocyanins. However, black carrots and purple sweet potato contained both classes of anthocyanins, i.e. glycosylated and acylated anthocyanins, while butterfly pea exhibited the presence of polyacylated anthocyanins, called ternatins that are characterized by high molecular weights. Based on anthocyanin type, there is a classification in which foods are assigned as cyanidin, peonidin, delphinidin or multiple type. In this case, blackberry, black chokeberry, sour cherry and black carrots were assigned as cyanidin type, while purple sweet potato was assigned as peonidin type. Butterfly pea contained ternatins that are based on delphinidin aglycone, while roselle was of cyanidin/delphinidin type. In addition, the presence of pyranoanthocyanins was a distinctive feature of sour cherry.
With respect to other phenolic compounds, phenolic acids were found in all these anthocyanin-rich foods. Flavonols were detected in blackberry, black chokeberry, sour cherry, roselle and butterfly pea. Traces of flavanols were found in blackberry, black chokeberry and sour cherry. On the other hand, characteristic for blackberry was the presence of ellagitannins, which are hydrolysable tannins.
After qualitative and quantitative knowledge of the phenolic composition, the identification of their fingerprints were established in order to be used in routine analytic and authenticity controls of blackberry, black chokeberry, sour cherry, black carrots, purple sweet potatoes, roselle, butterfly pea and their products.

In the second part, the preparative isolation of anthocyanins by membrane and countercurrent chromatography was performed. On the one hand, the separations by membrane chromatography were possible in all cases. If samples were loaded in excess, superior to the maximum binding capacity of membrane adsorber, a repetition of separation by membrane chromatography was necessary as in the case of black chokeberry, sour cherry and purple sweet potato. After membrane separations, anthocyanins and copigments have been successfully fractionated in two groups. This membrane chromatographic method allowed the isolation of anthocyanins on a large scale in high purity and quantity, as well as in a short time (around 2 h).

On the other hand, countercurrent chromatographic techniques, especially LSRCCC, HSCCC and HPCCC, provided separation of polyphenols and isolation of individual compounds on a large scale, particularly anthocyanins, chlorogenic acids, quercetin glycosides and kaempferol glycosides.

Nonetheless, this study demonstrated that for determination of chemical composition, chromatographic methods such as HPLC-DAD and HPLC-ESI-MS^n are necessary for the identification of individual compounds. In the cases of anthocyanins, spectrophotometric methods showed only an absorption maximum at 520 nm and this method is limited to determine the total anthocyanin content. However, some food adulterations were found in commercial black chokeberry extracts that presented a different anthocyanin profile and characteristic markers of black chokeberry.

In conclusion, the chemical composition of anthocyanins-rich foods was obtained by TLC, UV spectra, HPLC-DAD, HPLC ESI-MS^n and NMR analysis. In addition, this work provided fingerprints for blackberry, black chokeberry, sour cherry, black carrots, purple sweet potato, roselle and butterfly pea. The application of membrane and countercurrent chromatography enabled the separation and isolation of anthocyanins on a preparative scale. In both chromatographic procedures, high quantities of pure compounds were obtained. Finally, some of the obtained fractions (purple sweet potato and black carrots) are now available for futher biological tests.

6 Zusammenfassung

Öffentliche Lebensmittelskandale, Bewusstsein bezüglich Lebensmittelsicherheit von Farbstoffen und Gesundheitsförderung sind Faktoren, die einen Wechsel in der Gesetzgebung für Lebensmittelzusatzstoffen weltweit bewirkt haben. Darüber hinaus haben der Ersatz von synthetischen Farbstoffen durch natürliche Farbstoffe und die Erschliessung von Quellen für natürliche Lebensmittelfarbstoffe zunehmend an Bedeutung gewonnen.

Im ersten Teil dieser Arbeit war das Hauptziel die Charakterisierung von phenolischen Inhaltsstoffe in Lebensmittel, die reich an Anthocyanen sind, ins besondere Brombeere, Apfelbeere, Sauerkirsche, schwarzen Karotten, violetten Süßkartoffeln, Hibiskus und *Clitoria ternatea* Blüten.
Basierend auf der Klassifizierung als glykosylierte oder acylierte Anthocyane enthielten alle Anthocyan-reichen Lebensmitteln glykosylierte Anthocyane. Allerdings wurden in schwarzen Karotten und violetten Süßkartoffeln beide Klassen von Anthocyanen gefunden, während in *Clitoria ternatea* Blüten polyacylierte Anthocyane nachgewiesen werden konnten, die als Ternatine bezeichnet werden und durch hohe Molekulargewichte charakterisiert sind.
Auf dem Anthocyan-Typ basiert eine Klassifizierung, in der Lebensmittel dem Cyanidin-, Peonidin-, Delphinidin- oder Mehrfach-Typ zugeordnet werden. In diesem Fall wurden Brombeere, Apfelbeere, Sauerkirsche und schwarze Karotten dem Cyanidin-Typ zugerechnet, während violette Süßkartoffeln der Sorte Purple Sweet dem Peonidin-Typ zugewiesen wurden. *Clitoria ternatea* Blüten enthalten Ternatine, die auf einem Delphinidin-Aglykon basieren, während Hibiskus als Cyanidin/ Delphinidin-Typ kategorisiert werden konnte. Außerdem war die Anwesenheit von Pyranoanthocyane eine besondere Eigenschaft von Sauerkirschen.
In Bezug auf andere phenolische Verbindungen wurden Phenolsäuren in alle ausgewählten Anthocyan-reichen Lebensmitteln gefunden. Flavonole konnten in Brombeere, Apfelbeere, Sauerkirsche, Hibiskus und *Clitoria ternatea* Blüten identifiziert werden. Spuren von Flavanolen wurden in Brombeere, Apfelbeere und Sauerkirsche gefunden. Die Anwesenheit von Ellagitanninen, die hydrolysierbare Tannine sind, war andererseits charakteristisch für Brombeere.
Nach qualitativen und quantitativen Bestimmung der phenolischen Zusammensetzung wurde eine Identifizierung über fingerprints (Food Profiling) für die Anwendung in Routineanalysen und Authentizitätskontrolle von Brombeere, Apfelbeere, Sauerkirsche, schwarzen Karotten, violetten Süßkartoffeln, Hibiskus und *Clitoria ternatea* Blüten erstellt.

Im zweiten Teil dieser Arbeit war das Ziel die präparative Isolierung von Anthocyanen mittels Membranchromatographie und Gegenstromverteilungschromatographie. Einerseits waren die Trennungen der Polyphenole mittels Membranchromatographie in alle Fällen möglich. Wenn die

dynamische Bindungskapazität des Adsorbers auf Grund einer hohen Anthocyankonzentration überschritten wurde, war eine Wiederholung der Trennung mittels Membranchromatographie nötig, wie im Fall von Apfelbeere, Sauerkirsche und violetten Süßkartoffeln. Nach der Trennung mittels Membranechromatographie wurden Anthocyane und Copigmente erfolgreich in zwei Gruppen aufgetrennt. Diese membranchromatographische Verfahren erlaubte die Isolierung von Anthocyane in großem Maßstab und in hoher Reinheit, sowie in kurzer Zeit (etwa zwei Stunden). Auf der anderen Seite ermöglichte die Gegenstromverteilungschromatographie, besonders LSRCCC, HSCCC und HPCCC, die Trennung der Polyphenole und die Isolierung einzelner Verbindungen in großen Maßstab, besonders von Anthocyanen, Chlorogensäuren, Quercetin-Glykosiden und Kaempferol-Glykosiden.

Gleichwohl zeigte diese Studie, dass chromatographische Verfahren wie HPLC-DAD und HPLC-ESI-MS^n zur Bestimmung der chemischen Zusammensetzung und zur Identifizierung von individuellen Verbindungen notwendig sind. Im Falle der Anthocyane zeigten die photometrischen Verfahren nur ein Absorptionsmaximum bei 520 nm dieses Verfahren ist auf die Bestimmung des Gesamtanthocyangehaltes begrenzt. Trozdem wurden einige Verfälschungen von Aronia-Extrakten gefunden, die ein anderes Anthocyan-Profil und charakteristische Marker von Apfelbeeren aufweisen.

Zusammengefasst wurde die chemische Zusammensetzung von Anthocyan-reichen Lebensmitteln mittels TLC, UV Spektren, HPLC-DAD, HPLC-ESI-MS^n und NMR Analysen erhalten. Darüber hinaus lieferte diese Arbeit Food Profilings für Brombeere, Apfelbeere, Sauerkirsche, schwarzen Karotten, violetten Süßkartoffeln, Hibiskus und *Clitoria ternatea* Blüten. Außerdem ermöglichte die Anwendung von Membranechromatographie und Gegenstromverteilungschromatographie die Trennung und Isolierung von Anthocyanen im präparativen Maßstab. In beiden chromatographischen Verfahren wurden hohe Mengen an reinen Verbindungen erhalten. Schließlich stehen einigen der erhaltenen Fraktionen aus violetten Süßkartoffeln und schwarzen Karotten für weitere biologische Tests zur Verfügung.

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