CHARLES UNIVERSITY

PHARMACEUTICAL FACULTY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACEUTICAL BOTANY

DIPLOMA THESIS

PURIFICATION OF PHLORIZIN FROM *MALUS DOMESTICA* BORKH. BY SOLID-PHASE EXTRACTIONAND SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.

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Hradec Králové, May 2018   Eva Prachařová

DECLARATION

I declare that this thesis is my copyrighted work. All literature and other resources I used while processing are listed in bibliography and properly cited. The thesis was not misused for obtaining the same or different academic degree.

In Hradec Králové, June 2018   Eva Prachařová

Acknowledgement

ABSTRAKT

Diplomová práce

Purifikace florizinu pomocí extrakce na tuhé fázi a semipreparativní vysokoúčinné kapalinové chromatografie

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Cílem této diplomové práce bylo najít co nejlepší podmínky pro čištění flavonoidního extraktu z listů *Malus domestica* Borkh. a získání co nejčistčí frakce florizinu. Florizin by mohl mít v budoucnosti uplatnění při léčbě diabetu melitu typu 2, má schopnost snižovat glykemii pomocí snížení absorpce glukózy v tenkém střevu a zvýšením vylučování glukosy močí.

Prvním krokem bylo nalézt SPE kartridž s vhodným sorbentem a vhodný eluent pro extrakci na tuhé fázi. Jako nejvhodnější se ukázala kartridž DPA-6S a jako eluent 100% MeOH. Dalším krokem bylo najít co nejlepší podmínky pro semipreparativní HPLC při použití kolny ACE 5 C18 (5 μm, C18, 150 × 10mm i.d., délka 150 mm).Jako nejvhodnějšímobilní soustava pro semipreparativní HPLC se jevila směs 1% (v/v) kyseliny octové ve vodě (solvent A) aEtOH 100% (v/v) (solvent B), chromatografická seperace probíhala lineární gradientovou elucí 10–100% B, 0–60 min, s průtokem 1ml/min.

Touto metodou bylo dosaženo čistoty 91,05% florizinu

Klíčová slova: *florizin, SPE, semipreparativní HPLC, purifikace.*

ABSTRACT

DIPLOMA THESIS

Purification of phlorizin from *Malus domestica* Borkh.by solid-phase extraction and semi-preparative high-performance liquid chromatography

Eva Prachařová

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Department of Pharmaceutical Botany

The aim of this diploma thesis was to find the best conditions for purification of a flavonoid extract from leaves of *Malus domestica* Borkh., and obtaining the purest fraction of phlorizin. Phlorizin may be used in the treatment of diabetes mellitus type 2 in the future, it has the ability to reduce glycemia by reducing the absorption of glucose in the small intestine and by increasing urinary glucose excretion.

The first step was to find an SPE cartridge with a suitable sorbent and a suitable eluent for solid phase extraction. The DPA-6S cartridge and 100% methanol as an eluent were found to be the most suitable for SPE. The next step was to find the best possible conditions for semi-preparative HPLC using an ACE 5 C18 column (5 μm, C18, 150 x 10 mm i.d., 150 mm length). The mobile phase consisted of 1% (v/v) acetic acid in water (solvent A) and ethanol 100% (v/v) (solvent B), and a linear gradient elution was used (10–100% B), 0–60 min, the flow: 1mL/min. This method resulted in the 91.05% purity of phlorizin.

Keywords*: phlorizin, SPE, semi-preparative HPLC, purification*.

List of abbreviations

2D	two-dimensional
ACN	acetonitrile
CE	capillary electrophoresis
CoA	coenzyme A
ESI	electronspray ionisation
EtOH	ethanol
GC	gass chromatography
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LDL	low density lipoprotein
LLE	liquid-liquid extraction
MAE	microwave assisted extraction
MeOH	methanol
NVP	*N*-vinyl-2-pyrrolidone
PDA	photodiode array
PLE	pressuried liquid extraction
PTFE	polytetrafluorethylene
ROS	reactive oxygen species
RP	reverse phase
RE	rutin equivalent
SFE	supercritical fluid extraction
SGLT1	sodium-glucose linked transporter 1
SGLT2	sodium-glucose linked transporter 2
SLE	solid-liquid extraction

SPE	solid-phase extraction
TLC	thin-layer chromatography
UAE	ultrasound assisted extraction
UV/VIS	ultraviolet/visible

LIST OF FIGURES

CONTENT

12

1   **Introduction**

Flavonoids are biologically active secondary plant metabolites and represent the most frequent occurring group of polyphenolic compounds, about 4000 compounds have been isolated. These compounds are mainly known for their antioxidant activity, in addition also acts in plants reproduction and have antimicrobial and anti-inflammatory properties. Flavonoids are divided into several structuraltypes includinge.g.,flavanols, flavones and dihydrochalcones.Phlorizin is a representative of dihydrochalcones and has a rolein plant reproduction, a resistence to plant diseases, a plant growth and itsregulation. It has an antioxidant effects. Previously the flavonoid was used for treatment of malaria. Nowadays, phlorizin is being studiedfor its ability to reduce glycemia independetly of insulin by reducing glucose absorption in the small intestine and increasing glucosuria via affecting sodium-glucose linked transporter (SGLT). These properties make it a promising substance useful in the treatment of diabetes mellitus type 2.

This diploma thesis deals with the purification of phlorizin using SPE (solid-phase extraction) and semi-preparative HPLC (high-performance liquid chromatography).SPEis currently a very popular extraction method and it is a widely used method of a sample preparation. Its advantage is lower consumption of solvents, also it is reasonably a fast method.Substances are distributed on the basis of their physical and chemical properties between a solid (sorbent) and a liquid (gas) phase. HPLC works on the same principle and the analysis is both qualitative (retention time) and quantitative (area of the peak).

The aim of this thesis was to find a method for obtaining pure phlorizin from the flavonoid extract of the lyophilized *Malus domestica* Borkh leaves. Phlorizin should be free of toxic residues to be used for biological tests.

2   The aim of the work

1. Preparation of an extract from lypohilized*Malus domestica* Borkh. leaves,

2. Find the most appropriate SPE cartridge for the extract purification and its eluent composition,

3. Find the most suitable chromatographic conditions for purification of phlorizin by semi-preparative HPLC.

3 Theoretical part

3.1 Phenolic compounds

Phenolic compunds are secondary plant metabolites having one or more hydroxyl groups attached to benzene ring. They occur in both simple and polymerized form.Phenols can be founded as aglycones, but mostly existingin glycosylated form. Phenolic compounds playan important role in the growth and reproductionof plants, providing protection against pathogens and predators as wel contributing to the color and sensory characteristics of fruits and vegetables. There is a correlation between polyphenol-rich foodsand the decreased risk of cardio-vascular diseases. There are four groups of polyphenols: flavonoids, phenolic acids, stilbens and lignans[2].

3.1.1 Flavonoids

Flavonoids are widly distributed phenolic compound, about 4000 compounds have been isolated. They are synthetized from phenylpropanoid precursors produced in shikimate pathway and malonyl CoA produced in acetate/malonate pathway[3].
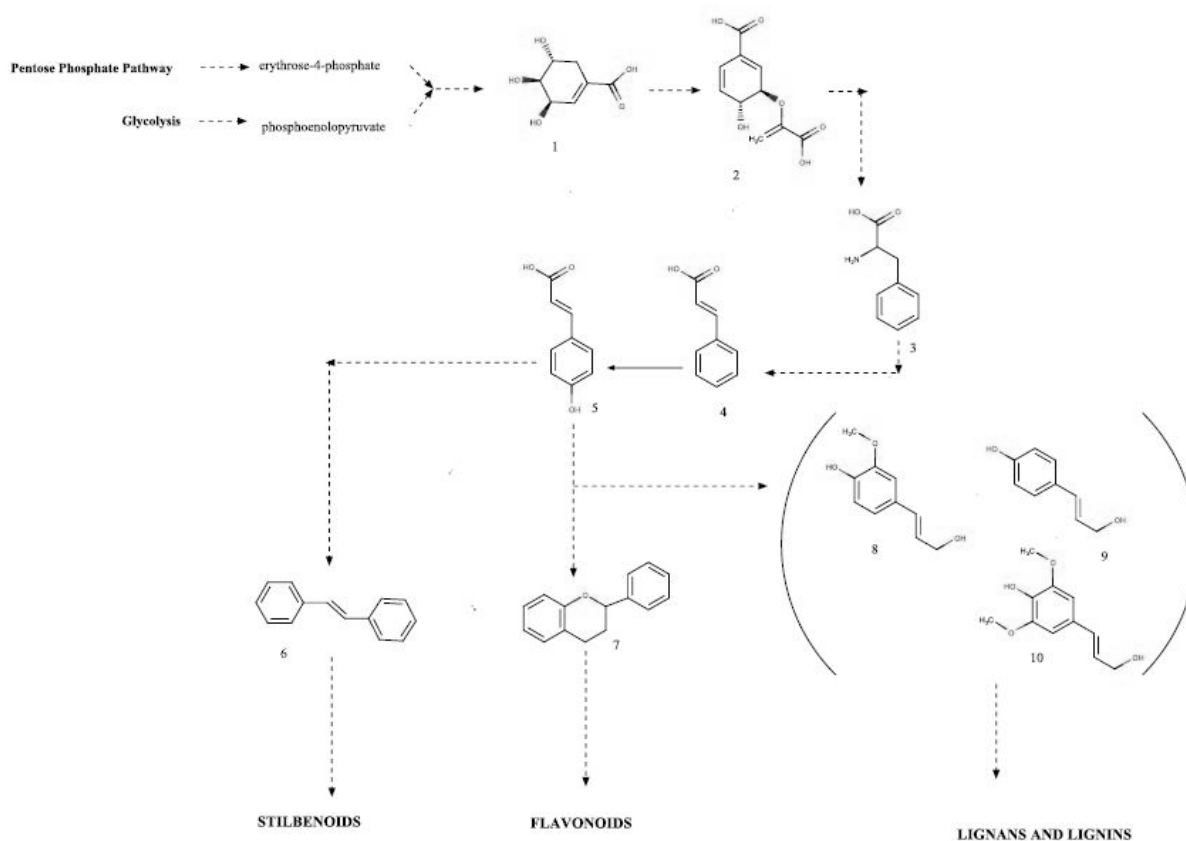
Figure 1 Synthesis of flavonoids, 1 shikimate, 2 chorismate, 3 phenylalanine, 4 trans-cinnamate, 5 *p*-coumarate, 6 stilbene backbone, 7 flavan backbone, 8 coniferyl alcohol, 9 *p*-coumaryl alcohol, 10 sinapyl alcohol[4].

The basic structure of flavonoids is adiphenylpropaneskeleton, which consists of two benzen rings (ring A, ring B) linked by a three-carbon chain. They may be described as C_6-C_3-C_6compounds (Figure 2). Based on its variation (hydroxylation, alkylation, *O*-glycosylation, *C*-glycosylation, different saturation of rings)flavonoids are classifiedintostructural subgroups. Most of flavanoids have third ring containing oxygen (ring C). The B ring is attached tothe position 2 (flavones, flavonols, flavanones, flavanonols, flavanols or catechins and anthocyanins) (Figure3), to the position 3 (prefix iso-) or the position 4 (prefix neo-) position of C ring[4].
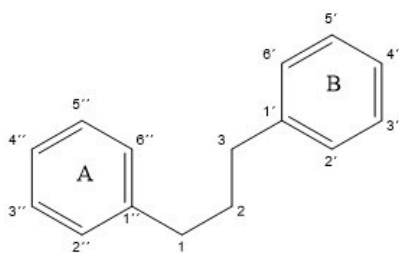
Figure 2 Diphenylpropane
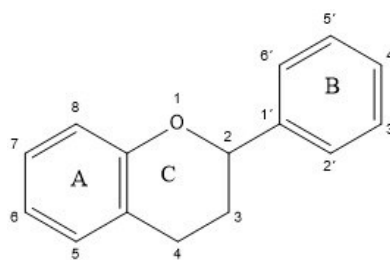
Figure 3 Basic structure of flavonoids

3.1.1.1 Flavanones

Flavanones (Figure 4) have a ketone in the position 4 of C ring, they may be substituted with a number of hydroxyl groups, which may be glycosylated and/or methylated. They represent a major flavonoid group, in which about 350 aglycones and 100 glycosides were identified. Flavanones are widely distributed, especially in plant families Asteraceae, Fabaceae and Rutaceae. For example, naringin and hesperidin belong to these compounds[3,5].
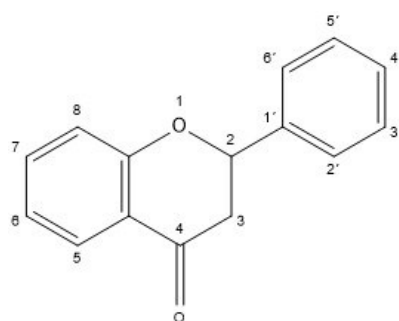
Figure 4 Flavanones

3.1.1.2 *Flavones*

Flavones (Figure 5), unlike flavanones have a double bond between positions 2 and 3. Otherwise, thestructure is the same as in the case of flavanones. Flavanones are converted by enzymes flavanone 2-hydroxylase and flavanone 1-hydroxylase to flavones. They are widely distributed in various families. They can be found in all parts of plant. For instance, apigenin and luteolin are representatives of this group[4,6].
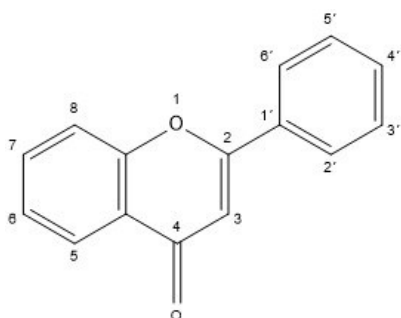
Figure 5 Flavones

3.1.1.3 *Flavonols and dihydroflavonols*

Flavonols (Figure 6) and flavanonols (Figure 7) are hydroxylated in the position 3 and have a ketone in the position 4. The difference between these two structural types of flavonoids is in the presence of the double bond, flavonols have the double bond between the position 2 and 3. Flavonols are the most common and largest subgroup of flavonoids in vegetables and fruits. Members are e.g., quercetin and kaempferol[7].
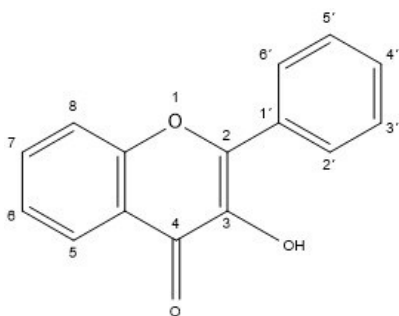
Figure 6 Flavonols
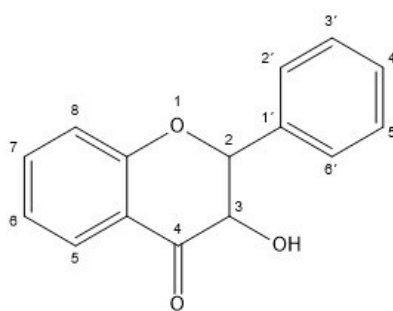
Figure 7Flavanonols

3.1.1.4 *Flavanols*

Flavanols (Figure 8), also called as flavan-3-ols or catechinas (monomers), proanthocyanidins (oligomers), are substitued with hydroxyl in the position 3. There are two chiral centers in the structure, in the position 2 and 3. For example, catechin and epicatechin belong to flavanols[6].
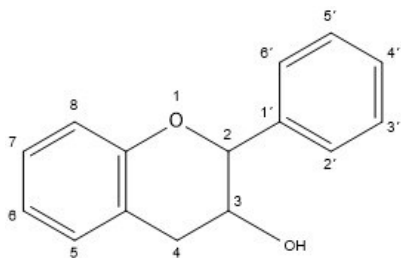
Figure 8 Flavanols

3.1.1.5 *Isoflavonoids*

A structure of isoflavonoids (Figure 9) is somewhat simillar to estrogens, namely 17β-estradiol (E2). They can have estrogenic effects in animals, therefore they are also called phytoestrogens. They are abundant in subfamilyFaboideae. Aglycones (e.g.,genistin, daidzin, glycitin) representa biologically active form of this structural subtype[6].
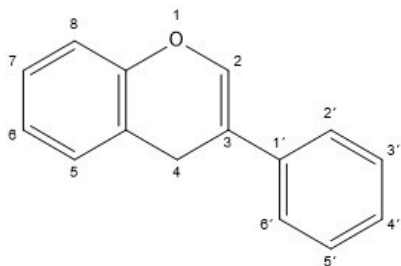
Figure 9 Isoflavonoids

3.1.1.6 *Anthocyanidins*

Anthocyanidins (Figure 10) are cations, most commonly occurring in the form of chloride salts. As the only flavonoids which are colorful and their color depends on the surrounding pH. In the position 3, they are linked by glycosidic bonding to a sugar moiety to form anthocyanin. Among anthocyanidins belong e.g., cyanidin, pelargonidin, petunidin[8].
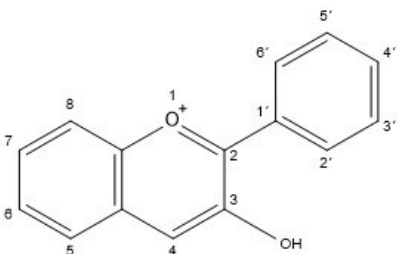
Figure 10 Anthocyanidins

3.1.1.7 *Chalcones and dihydrochalcones*

Flavanoids with open C ring are chalcones and dihydrochalcones(Figure 11). They belong toflavanoidsdue to the same biosynthesis as other flavonoids[9].
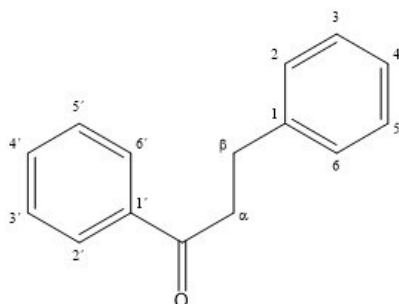
Figure 11 Chalcones and dihydrochalcones

3.1.1.8 *Function in plants*

Flavonoids are common and widely abundant secondary plant metabolites. They protect plants from UV light and an oxidation stress as antioxidants. Another indispensable role is in reproduction, where they act as attractors for pollinators. They can affect insects either positively, as oviposition stimulants and feeding attractans, or negatively, as feeding deterrents. They also participate in a process called aleopathy, where they negatively affect a growth of other organisms nearby. They represent a protective function, some of them also have antimicrobial effects[2,10].

3.1.1.9 *Activity in animals and human*

Their use results from their properties. It is important to highlight their antioxidant effects which are additive with other antioxidants such as ascorbic acid and tocopherol[3]. They themselves have even more pronounced antioxidant effects than above mentioned vitamins. There are several mechanisms involved in antioxidation:

- direct scavengingof reactive oxygen species (ROS),
- activation of antioxidant enzymes, metal chelating activity,
- reduction of α-tocopheryl radicals,
- inhibition of oxidases,
- mitigation of oxidative stress caused by nitric oxide,
- increase in uric acid levels,
- increase in antioxidant properties of low molecular antioxidants[11,12,13].

In the structure of flavonoids, the number and position of hydroxyl groups, which are donors of hydrogen, are important. Wherein the hydroxyls on ring B are more importance and contribute to the antioxidant activity more than those on the A ring. Methylation, the presence of conjugated double bonds and polymerization is also significant. Aglycons have a greater antioxidant effect compared to corresponding glycosides[2].

Some of flavonoidsact hepatoprotectively, for example silymarin, therefore it is usedin diseases where the liver may be affected[2].

Due to their antimicrobial activity, they could be used in the future for some bacterial diseases instead of classic antibiotics[5,14].

They act against viruses via influence of enzymes important for the life cycle of the virus. There are some flavonoids, which act against HIV virus, therefore they could be potential important for the development of new drugs[14].

Another use of flavonoids is in theanti-inflammatory treatment, where they interfere with a synthesis of inflammatory mediators such as eicosanoids, cytokines, adhesion molecules and C-reactive protein. Inflammatory mediators are associated with long-term illnesses and the development of cardiovascular diseases[10,14].

3.2 Phlorizin

Phlorizin (phloretin-2'-β-D-glucopyranoside) (Figure 12) is a representative of the subgroup of dihydrochalcones which possess open C ring and their skeleton consists of C_6-C_3-C_6 (two benzen ringsconnected with propane). It is a glucoside of phloretin[1].
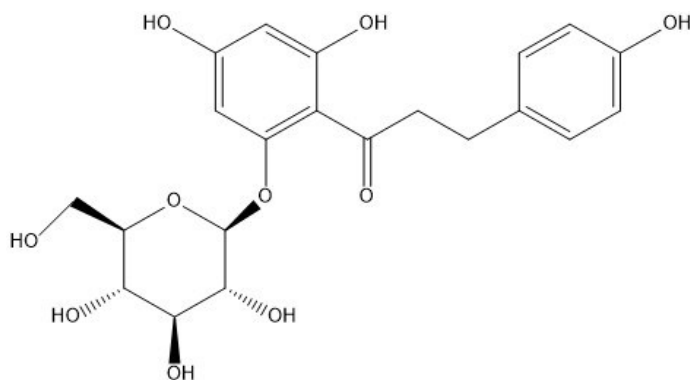
Figure 12 Structure of phlorizin

The compound occurs in apple trees, specifically in the bark of roots, leaves and sprouts.Phlorizinwas isolated in 1835 by L. G. De Koninck for the first time. Its highest concentration occursin apple trees, small amount was found in *Lithocarpus polystachyus* Rehd., *Rosa canina* L., *Fragaria× ananassa* Duch.or *Vaccinium macrocarpon* Aiton. Apple

trees are quite unique, because closely related trees are unable to synthesize phlorizin or phloretin[15]. Phlorizin is stable during storage and drying[16].

3.2.1 Function in plants

Phlorizin plays a role in a resistence to plant diseases, especially the aglycon phloretin. Another its role is in a plant growth and development regulation. It has antioxidant effects, prevents fat oxidation and reduces the risk of developing cardiovascular diseases[1].

3.2.2 Activity in human and animals

Phlorizin was previously used to treat malaria and reduce a fever. Today we are talking about phlorizin in relation to diabetes mellitus type 2 and obesity. Phlorizin has ability to bind on glucose transporters SGLT1 and SGLT2. This bond is specific and inhibition is competitive. SGLT1 is located primarily in the small intestine cells andmay be located in a renal tubular epithelium, SGLT2 in proximal convoluted renal tubule. The compound causes polyuria, reduces glycemia and adsorbtion of sugars in the small intestine. This helps to reduce a caloric intake and a progression of secondary damages caused by hyperglycemia in diabetes. However, an increased level of glucose in the urine can cause more frequent infections of the urinary and genital system[1,17].

3.2.3 In vitro tests

In vitro tests have found that phlorizin has a positive effect on the differentiation of osteoblasts[18]. Another experiment, where the small intestine of the hamster was used, showed that phlorizin is a competitive inhibitor of an energy independent active sugar transponter[17]. The effect of phlorizine and phloretin was investigated on lipolysis and inhibition of macrophage activity inmouse 3T3-LI cells. It was found that activity of phlorizin was lower in comparison to phloretin[19].

3.2.4 In vivo tests on animals

Assays performed on diabetic rats showed that phlorizin is ableto normalize meal tolerance and insulin sensitivity[20]. Another study on diabetic rats demonstrated a protective effect of phlorizin against diabetic nephropathy[21]. A study focused on lactating cows demonstrated effects of phlorizin on a reduction of glycemia and an increased lipolysis as well[22].

3.2.5 In vivo tests on humans

An assay performed on healthy male volunteers exhibited an effect of an apple flavonoid extract (a phlorizin content was 448 mg in each2.8 g tablet) on glycemia, insulinemia

and glycosuria. Glucose and insulin levels were lower after administration of the apple extract, caused by inhibition of a glucose transport in the small intestine. Conversely, after ingestion of the extractthe level of glucose in urine was 4.9 higher than administration of glucose alone[20].

A preliminary study with healthy volunteers proved an effect of phlorizin together with pectin on postprandial glycemia (a content of phlorizin was 325 mg in a dose corresponding to approximately 0.004 g× kg^{-1})[23].

3.3 Phytochemical andanalytic methods offlavonoids

3.3.1 Flavonoid extraction

Extractionis the most important step in a sample preparation for recovering and isolation of active substances from a plant material. Several different methods are usedto extract flavonoids from a plant material.

3.3.1.1 *Conventional methods*

Liquid-liquid (LLE) and solid-liquid extraction (SLE) are basic types of extraction, they utilize a solvent into which an extracted substance passes on the basis of its physical and chemical properties. The solvent is selected based on a polarity of an extracted flavonoid. Diethyl ether, and ethyl acetate can be used for extraction of lesspolar flavanoids (isoflavones, flavanones, methylated flavones and flavonoles), alcohols (methanol, ethanol) are used for flavonoid glycosides extraction. Two mutually immiscible liquids are used for the LLE. After agitation and after phase separation, two layers are visible and the compound of interest should be in the extraction liquid. The main problems of LLE are incomplete phase separations, less than quantitative recoveries, use of expensive, fragile glass ware, and disposal of large quantities of organic solvents and environmental pollution[24,25].

Soxhlet extractor is used for extraction from solid samples. In most cases, aqueous methanol (MeOH) or acetonitrile (ACN) are used as a solvent[26].

3.3.1.2 *Solid phase extraction*

Solid-phase extraction (SPE) is currently a very popular extraction method. SPE is a widely used method of a sample preparation. Its advantage is lower consumption of solvents, also it is reasonably a fast method. This chromatographic method is based on distribution of molecules between a solid phase and a liquid phase, it depends on polarity of a compound of interest and a solvent strength. There are three types of solid phase sorbents – a normal

phase, a RP and an ion-exchange phase. There are plenty of sorbent types and still more are being developed. SPE is suitable for liquid samples, for solid samples, pre-extraction is needed. It has many advantages over the LLE which are incomplete phase separations, less-than-quantitative recoveries, use of expensive, fragile glassware, and disposal of large quantities of organic solvents and environmental pollution[26,27].

In SPE, substances are distributed on the basis of their physical and chemical properties between a solid and a liquid (gas) phase. SPE is frequently used in a sample preparation, which may include concentration or purification of a required compound or compounds from ballast substances. The origin of the sample may be different– biological fluids, environmental samples, pharmaceuticals etc[26,28].

Similarly, this method has some disadvantages. Some skills to managethe process are needed. It is important to maintain the same flow rate due to adsorption of molecules at a sorbent, and the sorbent should not be dried before the sample is applied. SPE can be costly due to consumption of cartridges. Another problem can be also pH of the sample. Silica-based sorbents are stable in a range of 2 to 7.5. If the pH is different, a bounded phase can be hydrolyzed, therefore, polymer sorbents are more often used[27,28].

Nowadays, there are attempts to automate the whole process and connect it with other chromatographic methods. Another development is focused on a format of stationary phases and sorbents, for example mixed-mode sorbents which contain both non-polarand ion-exchange functional groups[26,27,28].

3.3.1.2.1 Liquid and solid phases

A liquid phase containing molecules of interest is applied to SPE columns (cartridges). A solid phase is a stacionary phase. A liquid phase passes through a stacionary phase and molecules are divided according to their chemical and physical properties between both phases. The stationary phase is insertedin a cartridge (Figure 13), which can be made from polypropylene or glass. There are frits made from polyethylene, Teflon or stainless steel that prevent particle release[26,27,28].
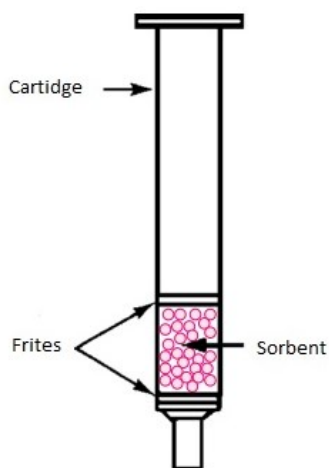
Figure 13 SPE cartridge[29]

3.3.1.2.2 Extraction procedures

A first step of extraction is a conditioning step. A sorbent must be activated to functional groups become accessible. MeOH or ACN followed by water are commonly used for activating hydrophobic sorbents, while hexane or dichloromethane activate hydrophilic sorbents. A small amount of fluid is left over the sorbent for better sample application, then sample can be loaded. A volume of the loaded sample can be ranged from microliters to liters. Some compounds of the sample absorb to the sorbent, some of them pass through the stationary phase. This step is followed with washing and elution steps[26,27,28].

One of the three schemes is used to extract our required compound. Selective extraction, selective washing and selective elution.

Selective extraction means that on the sorbent the selected compounds are binded. It can be a compound of interest or an impurity. Subsequently, the required fraction is collected. If the compoud of interest is binded on the sorbent, the elution is used. If the impurity is binded, we collect the effluent[26,28,29].

In selective washing both a compound of interest and an impurity are bound to a sorbent. To remove the impurity, the enough strong solution to remove the impurity is used, but enough weak to remove the compound of interest[27,28,29].

By selective elution, compound of interest is eluted, while a strongly binded impurity remains bounded[27-29].

Moreover, a sorbent with a bounded material can also be dried and stored for further analysis[27,28,29].
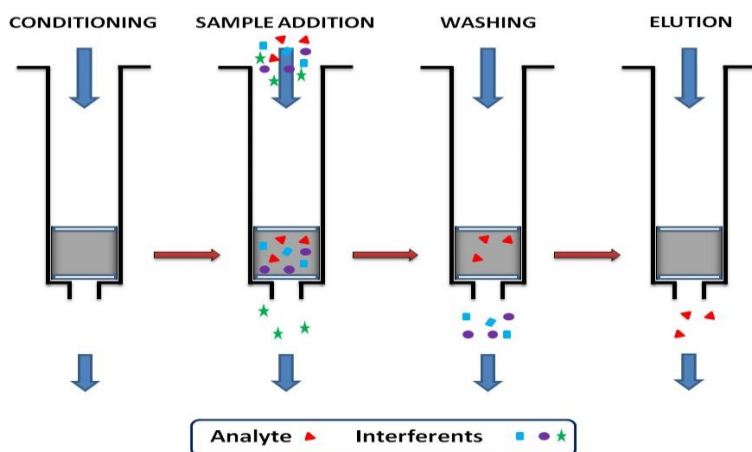
Figure 14 Process of SPE[30]

3.3.1.2.3 Types ofsorbents

Sorbents in SPE can be divided based on an origin of a material into classes such as inorganic porous materials, inorganic porous materials with an organic bound surface, organic synthetic polymers, organic natural polymers, and other materials. Another classification is based on a function related to an analyt. There are conventional SPE, microextraction SPE, head-space SPE utilization, and others[26,27,28].

Materials used as SPE sorbents are often similar to a stationary phase in other chromatographic methods. Sorbents are porous silica (a normal phase) or silica with bounded functional groups (a reverse phase)[26,27,28].

a) Inorganic materials

As sorbents are used hydrated silica, magnesium silicate (Florisil), hydroxyapatite, and hydrated metal oxides such as aluminum oxide, magnesium oxide, and zirkonium oxide. These materials are very porous and polar, so they are used for polar compounds. Silica is often modified by addition of functional groups. Natural silica materials are called diatomites, and they are used for gases retention[28,31,32].

Molecular sieves are also includedin this group. They are synthetic and natural zeolites or aluminiumsilicates. They also contain aluminiumoxidesand silicon, and are tetrahedrally coordinate with a shared oxygen. Cations and water can be contained inpores. Their formula is $Me_{x/n}[(AlO_2)_x\ (SiO_2)_y].mH_2O$, where Me is a cation, x,y and n are numbers of molecules and m is a number of molecules of crystalline water[28,31,32,33].

Finally, carbon in various modifications (graphite) and porosities belong to this group as well. Their use is based on hydrophobic properties[26,28].

b) Inorganic materials with an organic bound surface

This type of a sorbent is based on modified silica. Silanol functional groups are modified by several chemical reactions. Different functional groups may be attached, for a nonpolar phase a linear alkyl like C8 or C18, cycloalkyl, phenyl, etc., and for polar ligans such as $(CH_2)_3$-NH_2, -$(CH_2)_3$ -CN, -$(CH_2)_3$-NO_2, and -CH_2-$CH(OH)$-$CH_2(OH)$[26,31].

c) Organic synthetic polymers

Polymers are used in SPE more often than in HPLC, because there is an emphasis on a mechanical resistence due to higher pressures and the polymers hardly meet it[26].

There are different types of polymerization. For example, polymerization with an inert medium that is removed after the process. A polymer of the desired porosity is obtained in this method. Conditions under which the polymerization takes place, are very important, and include the amount of monomer precursors, the amount of initiator, the temperature, the type of porogenic solvent as well as copolymerization dynamics and cross-linker properties. Because of conditions, there may be problems with getting the material with the same quality[27,31,32,34].

The most common polymer used as a sorbent is poly (styrene-co-1,4-divinlbenzene). It consists of macroporous particles. It retains hydrophobic compounds which contain hydrophylic functionalities, especially aromatics[26,27].

Styrene polymerizations with alkyl methacrylates, *N*-vinylpyrrolidone (NVP), and vinylpyridine produce polymers with large specific surface areas, a range pore diameters and a large total pore volume. Polymerization of 1,3-divinylbenzene and NVP results in a copolymer which combines lipophilic and hydrophilic properties. It can serve to separate compounds with a wide range of polarities. Moreover, functional groups can be attached to the benzene ring, and they modify properties of the polymer[26,28].

Moleculary imprinted polymers belong to a significant group of organic synthetic polymers. These polymers have a molecular-specific binding site for an analyte (or analytes). A template molecule, a monomer (e.g., acrylamide, methacrylamide, methacrylic acid, 2- or 4-vinylpyridne), and a crosslinker (e.g.,, ethyleneglycol dimethacrylate, *N,N*-ethylene-bis-acrylamide) are required for their synthesis. The polymerization takes place around

the template molecule to create a specific binding site with a steric (a size and a shape) and chemical (spatial arrangement of complementary functional groups) memory of the template molecul. This synthesis is placed in a porogenic solvent (e.g., tetrahydrofuran, dichloromethane, chloroform, or ACN). Furthermore, an initiator (e.g., 2,20-azo-bisisobutyronitrile) is added. After the polymerisation the template molecule is extracted and the specific binding site is established. For this step various organic solvents are used[26,28,35].

Limited applicable materials are used for analyzing low-molecular-weight compounds such as drugs or metabolites from biological fluids, and nowadays also from waters containig high level of humic substances. The system is composed from a hydrophobic core (polystyrene) and a hydrophylic envelope. A task of the envelope is to reject hydrophilic molecules such as proteins. Similarly, the pore size plays a role here, only small molecules of drugsor metabolites can penetrated into the core[26,28,30,31].

d) Organic natural polymers

In SPE also organic natural polymers are used as a sorbent. Common used polymers are cellulose, starch, and a macromolecular structure. This material can be modified to be used as an ion-exchange sorbent or an immuno sorbent[25,26].

e) Other materials used as sorbents in SPE

As sorbents are used magnetical particles coated with an organic surface as well. This particles can be manipulated with a strong magnet. As a core of these particles can be used hydrated Fe_3O_4[26].

Other used types are monolithic sorbents which form entire columns made up of one piece. In this case, there is better particle transfer due to elimination of a bedspace. They are designed for small sample analyses[34].
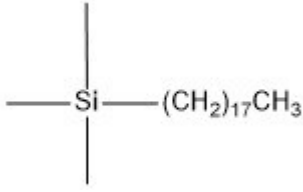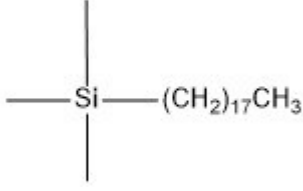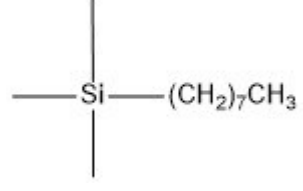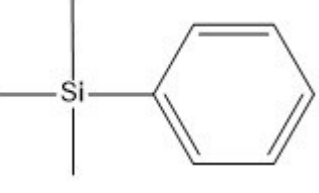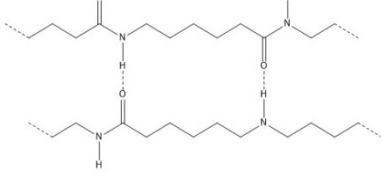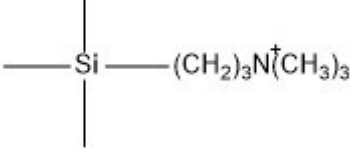
Type of sorbent	Phase	Chemical formula	Description	Application
C-18	Reversed phase	$-Si-(CH_2)_{17}CH_3$	Polymerically bound octadecyl, high percentage of carbon (18% C)	Most organic compounds
C-18Lt		$-Si-(CH_2)_{17}CH_3$	Monomerically bound octadecyl, low percentage of carbon (11% C)	Large hydrophobic molecules which are strongly retained on C-18
C-8		$-Si-(CH_2)_7CH_3$	Monomerically bound octyl	Large gydrophobic molecules which are strongly retained on C-18 and C-18Lt
-Ph		$-Si-$	Monomerically bound phenyl	Weaker retention than C-8, more suitable for aromatic compounds
PA-6S			Polyamide resin	Polar compounds containing multiple -OH and -COOH groups (tannins, flavonoids,..)
-SAX	Ion-exchange and Mixed-Mode	$-Si-(CH_2)_3N^+(CH_3)_3$	Polymerically bound (trimethylaminopropyl) silane, strong anion exchange	Weaker anions (carboxylic acids)
-WCX		$-Si-(CH_2)_3N^+(CH_2COOK)CH_2CH_2N(CH_2COOK)_2$	Polymerically bound carboxylic acid, weak cation exchange	Strong cationic compounds (high pK_a)

Figure 15 Table of sorbents sorted by typeof a phase(1/2)[36]
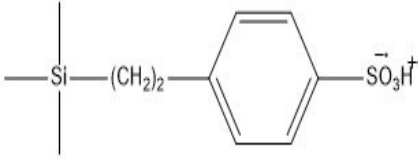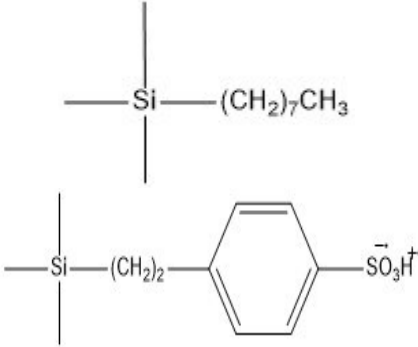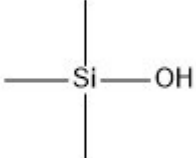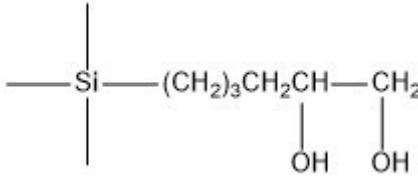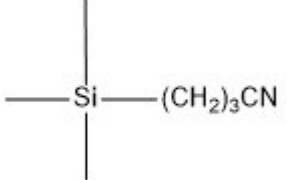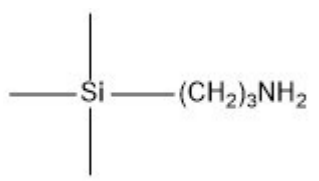
-SCX	**Ion-exchange and Mixed-Mode**		Polymericcaly bound benzensulfonic acid, strong cation exchange	Strong cation exchange for cations, antibiotics, drugs, organic bases, amino acids, etc.
-MCAX			Combination of octyl and benzensulfonic acid, dual retention mechanism (hydrophobic and ion exchange)	Polar basic and zwitterionic compounds
-Si	**Normal phase**		Silicagel without bound phase	Polar compounds, such as alcohols, aldehydes, amines, etc.
-Diol			2,3-dihydroxypropoxypropyl	Polar compounds, structurally similar molecules
-CN			Monomerically bound cyanopropyl, can be used also in reverse and ion-exchange phase	Very polar molecules irreversibly retained on very polar sorbents
-NH₂			Polymerically bonded, aminopropyl, can be used also in reverse and ion-exchange phase	Polar molecules

Figure 16 Table of sorbents sorted by typeof a phase(2/2)[36]

3.3.1.2.4   Solid-phase extraction apparatus

There are two basic formats of a stationary phase – cartridges and discs.

a) Cartridges

Standard SPE columns look as syringes or have a slightly altered shape about various volumes from 100 µL to 60 mL, the most common volume is 3 mL. Furthermore, there are specially shaped cartridges to be inserted into centrifuges to speed up the passage of the sample through the sorbent. Syringes are often made of polypropylene, also they can be made from glass. The sorbent usually fills up one third of the packing volume and is enclosed between two frites (Figure 13). Cartriges usually contain from 10 mg to 10 g of a sorbent is and the amount of an analyte should not be more than 5% of the sorbent weight. Cartridges are produced with various sorbents. Sorbents themselves and empty cartridges are also available for self-filling[29].

Among manufactures belong e.g., Sigma-Aldrich, Waters (Oasis), J. T. Baker, Thermo Fisher Scientific, Macherey-Nagel, Agilent technologies.

b) Disks and pipette tips

The discs are designed to withstand a higher pressure. A small amount of a sorbent, less than 10 mg, is bound to an inert matrix made of polytetrafluoroethylene (PTFE) (90% sorbent, 10% PTFE) or glass fibers. The disk is inserted in a disk cartridge or in a High-flow disk-like cartridge. Commercially available are discs with a diameter from 4 mm to 96 mm. The small length to diameter ratio of the disk enables higher flow rates and faster extractions than can be achieved with cartridges. Producers are e.g., Sigma-Aldrich (Empore), Restek, Avantor 806006 (BAKERBOND Speedisk), Horizon Technology (Atlantic)[26,37].

Based on the discs, a 96-well plate method was developed. This method is designed for rapid extraction of a large number of samples. Each well can be eluted with a small volume of a solvent (100–200 µL)[26,28,37].

Pipette tips with an inserted disk are also used. Pipette tips can contain silica monolith, for example MonoTips (GL science) are manufactured with bound C18, Trypsin or TiO_2. Another manufacturer of chromatographic tips is Thermo Fisher Scientific, which produces HyperSep tips[26,27,28].

3.3.1.2.5 Sample processing

One SPE cartridge can be processed using a single tube processor or with a syringe and an adapter. There are two types of a single tube processor. First one is a with rotate knurled knob for a slow flow. If a fast flowis needed, a piston is used in others. A pressure of air or nitrogen is usedin the syringe with an adapter. Another option is use a vacuum flask for a single cartridge[26,27,28].

Several SPE cartridges can be processed using a centrifuge or a vacuum[26].

3.3.1.3 *Other unconvenctional methods*

In addition to SPE, non-conventional methods also include ultrasound-assisted extraction, microwave-assisted extraction, pressuried liquid extraction and supercritical fluid extraction.

Ultrasound-assisted extraction (UAE) using ultrasonic waves to disturb a surface of a material and facilitate penetration of a solvent. It is suitable for extraction of thermolabile and unstable compounds. UAEis characterized by a short extraction time (often takes less than 30 minutes) and lower solvent consumption[37].

Microwave-assisted extraction (MAE) uses microwaves to heat up a sample. Unlike convenctional methods, where heat is transferd to the sample after a heating vessel, microwaves heat the sample directly. MAE reduces a time required for extraction and increases recovery. A typical extraction procedure takes 15–30 min and uses small solvent volumes in the range of 10–30 mL[38,39,40,41].

Pressurized liquid extraction (PLE) uses for extraction organic solvents which are heated above their boiling point. The sample is extracted at high temperatures and high pressures for a short time. Advantages are a shorter time of extraction and a less volume of solvents[40,42].

Supercritical fluid extraction (SFE) uses fluids in critical conditions. Critical conditions are when the fluid is above its critical temperature and crtitical pressure, then the solvent is between two states, a liquid and a gas. Supercritical carbon dioxide, insted of an organic solvent, is the most widely used for SFE. Carbon dioxide passes through a sample and is trapped in a trapping solvent (e.g.,MeOH), it may be recovered and reused. This method is suitable for thermolabile and rapidly decomposing substances[40,43].

3.3.2 Flavonoid separation

Tha main technique for flavonoid separation is based on liquid chromatography (LC), other methods are gas chromatohraphy (GC), capillary electrophoresis (CE) and thin-layer chromatography (TLC).

3.3.2.1 *Thin layer chromatography*

It is a simple method, used mainly for a rapid sample analysis [33,35]. As a sorbent, the most commonly used silica gel is applied on an aluminum or a glass plate. Another type of a sorbent is alumina. A development occurs in a chromatography chamber filled with a mobile phase. Detection is by UV light at 350–365nmor 250–260nm. The zone of interest may be scraped off, the substance eluted from the sorbent and used for further analysis[36]. Modification of classical TLC is a 2D method, where the sample can be developed with in different mobile phasesin two directions. Thus, 2D-TLCcan provide better separation[44].

3.3.2.2 *Capillary electrophoresis*

A principle of this method is based on a migration of molecules in an electric field. To be able to move of compounds in the electric field, molecules must be ionized. Ionization of Molecules is achieved by addition of buffers having required pH. Neutral moleculs do not move. Advantages of CE are an excellent separation efficiency, a high resolution, a short analysis time, being easy to automate, and a low solvent and a sample consumption. Phosphate and borate buffers are commonly used for flavonoid separation, voltages of 10–30kV and 10–50 nL injection volumes, adetection with UV, fluorescence and mass spectrometry (MS). These parametres are usedin two main types of CE, capillary zone electrophorsis and micellar electrokinetic chromatography[45,46].

3.3.2.3 *Gas chromatography*

As is clear from the title, a mobile phase is a gas. An analyzed sample must be converted to a gaseous state or it is a volatile substance. Since aboiling point of flavonoids is high (above 300 ˚C), they must be derivatized. Hydroxyl groups are derivatized by methylation (e.g., trimethylanilinium hydroxide as an agent) or by converted to trimethylsilylderivatives (e.g., *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamideas an agent)[42,43,46]. Because of the high boiling temperature, it is difficult to analyze glycosides even after their derivatization, the high-temperature-high-resolution GC-MS method can be used. In this case, a column is adapted to temperatures up to 400 ˚C[25]. Low polar capillary columns are used for standard

GCs. For detection, a flame ionization detector (FID) was previously used, nowadays it is replaced by a mass spectrometry detector (MSD)[43].

3.3.2.4 *High-performance liquid chromatography*

It can be used for the separation, identification and quantification of substances. This method is suitable for nonvolatile compounds such as flavonoids.

A stationary phase, a column, may be packing or monolithic. The packing columns are filled with small particles, often with dimensions from 1.5 μm to 5 μm[47,48]. Monolithic columns are made up of one piece of a porous material. There are two kinds of pores, macropores, mainly used to a transport of a mobile phase, and micropores, responsible for separation[34]. The stationary phase can be divided according to a nature of the sorbent into normal, reverse and ion-exchange phases. The reverse phase (RP) columns are most often usedin flavonoid separationas the sorbent C8 orC18. The RP can be used in a wide range of polarities. The normal phase can be also used such as sorbents silica, Spandex and polyamide. Silica is more suitable for compounds after acetylation[48].

A solvent is selected according to a used sorbent and a nature of a analyte. Non-polar slovents such as hexane or chloroform are used in the normal phase. The RP is typical for polar solvents, most MeOH + water and ACN + water. Solvents are pumped into a system, individually or mixed. Eluction is isocratic (no change in a composition of a mobile phase) or gradient (a composition of a mobile phase changes in time). Gradient elution is quite common in flavonoid separation, asystem water + MeOHis preferred. ACN can be used instead of MeOH. For acidification, acetatic or formic acid is added. It is added for better separation and prevention of peak tailing[46,47,48].

Detection is performed by photodiode array, ultraviolet/visible (UV/VIS), UV-fluorescence detectors. Furthermore, electrochemical detection and mass spectral detection are used. Quantification can be done using external or internal standards, a calibration curve (standard addition method) or a method of internal standardization[42].

3.3.3 **Phlorizin identification**

Identification can be done by comparing retention time and spectra, with mass-spectrometry. Standard can be added.

On the ability of molecules to absorb light, UV spectrophotometry is based, each compound has its typical spectrum. Each flavonoid has at least two maxima for UV spectra due to the benzen rings A and C. For phlorizin the first maximum, which is found in the 220–

230 nm range, is due to the A ring, and the second maximum, which is in the 284 nm range, to the substitution pattern and conjugation of the C ring is charasteristic[49].
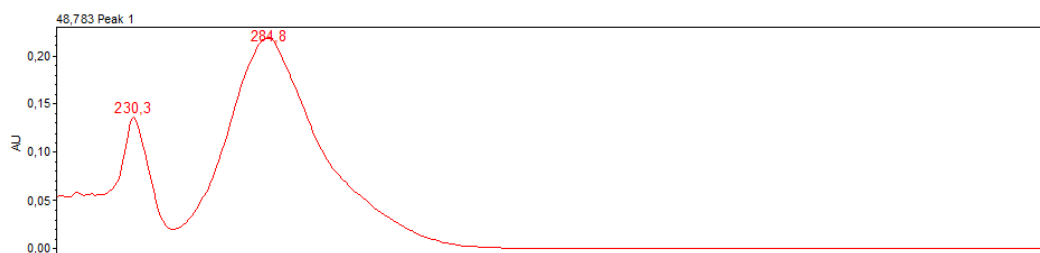
Figure 17 UV/VIS spectrum of phlorizin (measured at the Department of Pharmacognosy, LSMU Kaunas)

For mass spectrometry, an analyte is converted to ions and thereafter differentiated by weight/charge ratio (m/z) and their relative intensity is recorded. Ionization is divided according to the amount of a used energy to hard and soft ones. The hard ionizations include electron ionization, which produces a large number of fragments.Soft ionizations include e.g., chemical ionization, electrospray ionization (ESI), atmospheric-pressure chemical ionization and matrix-assisted laser desorption/ionization[50].
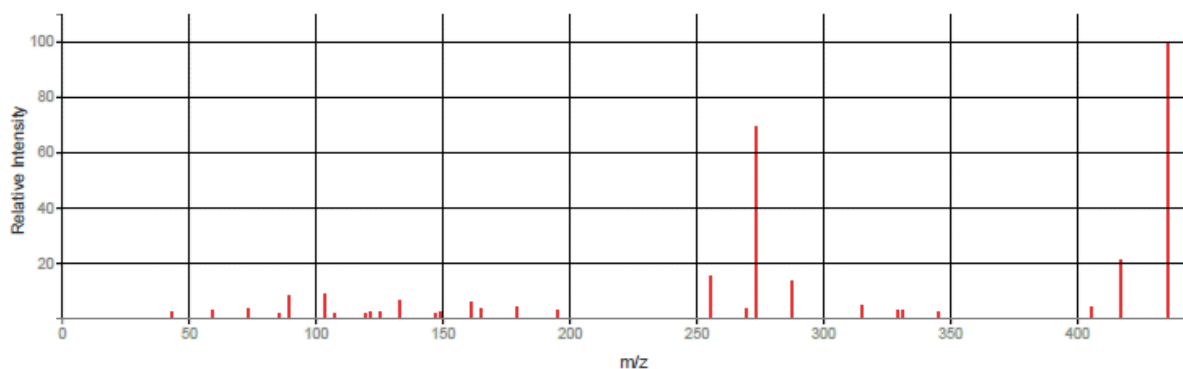
Figure 18 Phlorizin ESI-MS spectrum, ionization mode – negative, colision energy – 10 eV[51]

3.4 *Malus domestica* Borkh. (Rosaceae)

Synonym: *Pyrus malus* L., Malus *communis* var. *sativa* DC. in Lam. et DC., *Malus pumila* var. *domestica* (Borkh.) C. K. Schneider, *Pyrus malus* subsp. *pumila* (Mill.) A. et Gr. B. II. *domestica* A. et Gr., *Malus sylvestris* subsp. *pumila* (Mill.) Domin var. *domestica* (Borkh.) Domin, *Malus communis* subsp. *pumila* (Mill.) Gams var. *domestica* (Borkh.) Gams in Hegi, *Malus sylvestris* subsp. *mitis* (Syme) Mansfeld var. *domestica* (Borkh) Mansfeld[52]

Taxonomic classification

Class: Magnoliopsida

Order: Rosales

Family: Rosaceae

Genus: *Malus*

Species: *Malus domestica* Borkh[53]

3.4.1 Botanical characterisation

Approximately 10 000 cultivars of *Malus domestica* Borkh. are estimated in the world, there are about 1500 in Czech Republic. It is the world's most widly cultivated fruit tree[52]. It belongs to family Rosaceae together with other fruit trees such as *Pyrus* spp. (pear), *Cydonia oblonga* Mill. (quince), *Eriobotrya japonica* Lindl (loquat), and *Mespilus germanica* L.[53]

3.4.2 Morphological description

Trees or shrubs growing up to 6–10 (–14) meters with a grey to dark grey bork. Leaves are elliptical with serrate margins 5–14 cm long and 3.5–8.0 wide. Their upper side is dark green, almost without pubescence, the lower side is felt-like and light grey-green. The inflorescence is a corymb of 4–8 flowers. The flowers are white-pink and consist of five petals. In the ovary there are five cavities, in each are two seeds 8–10 mm long. The apple trees are self-unfruitful, they need a different flowering variety as a pollen donor for pollination. The fruit of the tree is a pome which can be 4–9 cm long and 5–10 cm wide, the shape is spherical, flattened, barrel-shaped or conical[52].

3.4.3   Habitat and distribution

Malus domestica Borkh. was bred from *Malus sieversii* M.Roem., originally grown in Central Asia. Nowadays these trees grow over the world. They are grown on all continents most in a mild climate, fewer in a subtropic climate[52,54].

3.4.4   Traditional use

In particular, fruits are very important and have been used for centuries. Traditional uses include constipation, water retention, fever, scurvy and warts. Fresh fruit or pressed juice is either fresh or fermented (cider) is consumed[55,56].

3.4.5   Terapeutical use

Therapeutic use is not yet available, but results of studies showed that regular consumption of the fruits has an effect on LDL-cholesterol levels[57], inflammation, blood sugar (diabetes melitus), a lower risk of cardiovascular diseases[55,58].

3.4.6   Constituens

An amount of constituents depends on conditions in which a plant grows, acollection period and an age of the plant[59,60].

3.4.6.1   *Phenolic compounds*

The main constituens are phenolic compounds. Two groups, flavonoids and phenolic acids were isolated from *Malus domestica* Borkh. Compounds were isolated from whole plant (Figures 19 and 20)[59,60]. Since this thesis is focused on the extraction of flavonoid phlorizin from leaves, a summary profile of phenolic compounds in the leaves is shown in Figure 21.

	Type	Representative
Flavonoids	flavan-3-ols or flavanols	(+)-catechin
		(−)-epicatechin
	dihydrochalcones	phlorizin
		phloretin
	flavonols	quercetin
		hyperosdie
		rutin
		isoquercitrin
		avicularin
	anthocyanidins	cyanidin
	anthocyanins	cyanidin-3-*O*-galactoside
Phenolic acids	hydroxycinnamic acids	chlorogenic acid
		p-coumaric acid
		caffeic acid

Figure 19 Phenolic compounds detected in *Malus domestica*Borkh.[60,61,62]
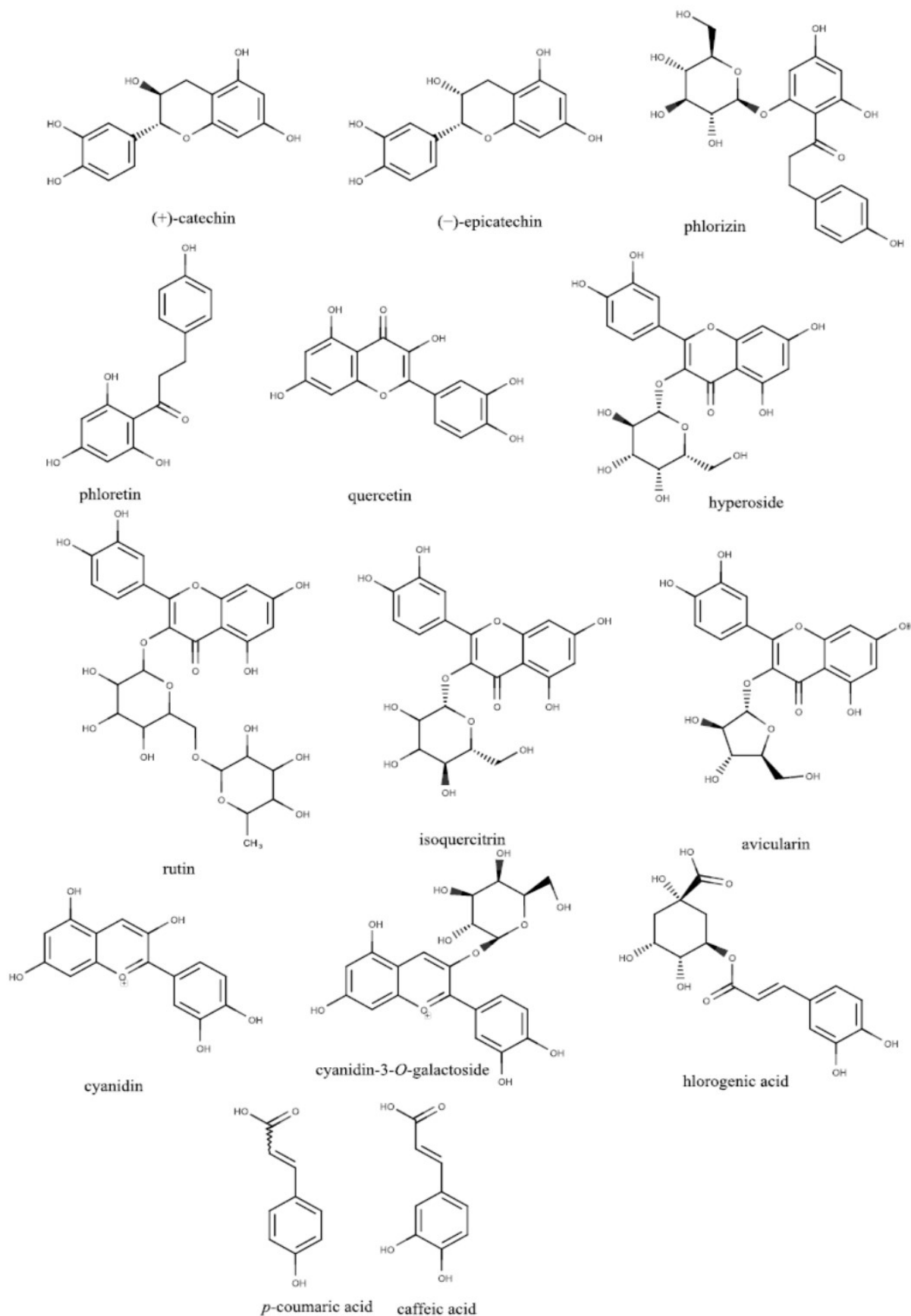
Figure 20 Structures of phenolic compounds isolated from *Malus domestica* Borkh.[60,61,62]
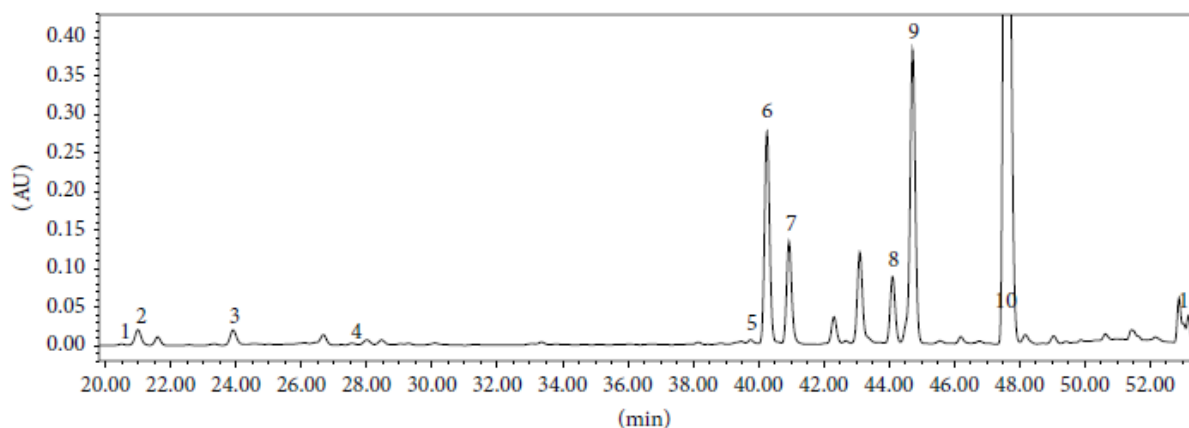
Figure 21 Total content of phenolic compounds in *Malus domestica* Borkh leaves:1 (+)-catechin, 2 chlorogenic acid, 3 caffeic acid, 4 (–)-epicatechin, 5 rutin, 6 hyperoside, 7 isoquercitrin, 8 avicularin, 9 quercitrin, 10 phloridzin, and 11 phloretin[63].

3.4.6.2 *Terpens*

Triterpenes from *Malus domestica* Borkh. were isolated, mainly pentacyclic triterpenes corosolic, betulinic, oleanolic and ursolic acids (Figure 22). All of them were isolated from fruit peels[64,65].

betulinic acid

ursolic acid

corosolic acid

oleanolic acid
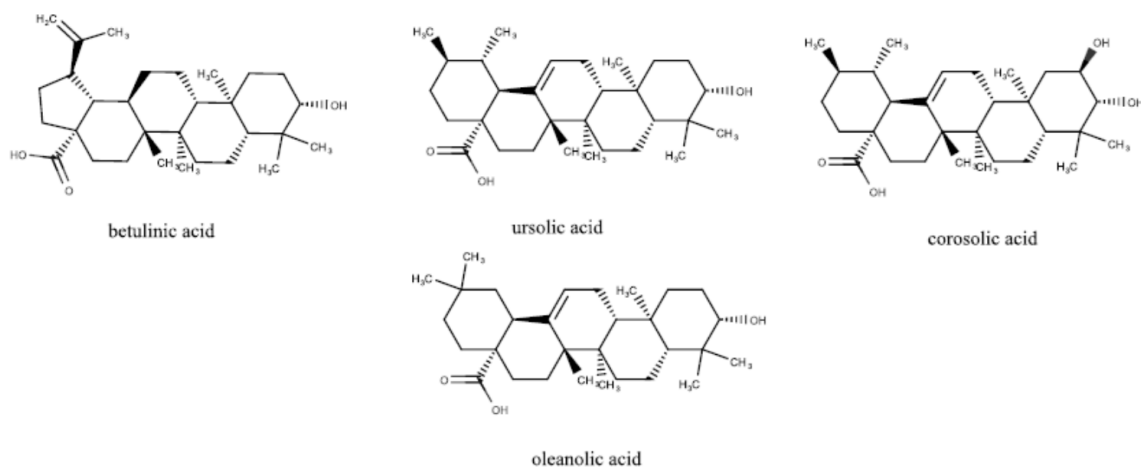
Figure 22 Structures of triterpenoid acids isolated from *Malus domestica* Borkh.[64,65]

4 Experimental part

4.1 Materials

4.1.1 Plant material

Lyophilized apple leaves from the apple cultivar Ligol (winter cv., bred in Poland). The apple trees were grown in the experimental orchard of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Babtai, Lithuania (55°60′N, 23°48′E). Block 2, row 4, trees 21-24. Apple leaves were lyophilized with a ZIRBUS sublimator 3 × 4 × 5/20 (ZIRBUS technology, Bad Grund, Germany) at a pressure of 0.01 mbar (condenser temperature, −85°C). The lyophilized apple leaves were ground to a fine powder by using a Retsch 200 mill (Haan, Germany)[63].

4.1.1.1 *Chemicals*

- Acetic acid (> 99.7% Sigma-Aldrich GmbH, Lithuania),
- Acetonitrile (ACN) (> 99.9%, Sigma-Aldrich GmbH, Lithuania),
- Destilated water (producedby Milli-Q® by Merck),
- Ethanol (EtOH) (>95.00%Vilniaus degtine), Lithuania,
- Methanol (MeOH) (>99.9%, Honeywell, Lithuania)

4.1.1.2 *Equipments*

- Extraction manifold, pos 20 (Waters, USA)
- Vacuum drying chamber VD 23 (BINDER, Germany)
- Analytical HPLC with Waters 2695 chromatograph equipped with a Waters 2998 PDA detector (Waters, USA)
- Acquity LC system (Waters, USA) equipped with a triple quadrupole tandem mass spectrometer (Xevo, Waters, USA)
- AutoPurification system with 2767 Sample Manager, 2545 Binary Gradient Module System, Fluidics Organizer, 2489 UV/Vis Detector (Waters, USA)
- Ultrasonic bath ELMASONIC P (Elma Schmidbauer GmbH, Germany)
- DSC-PH cartridge (1mL tube, 50mg) (Sigma-Aldrich, Germany)
- DSC-NH$_2$ cartridge (1mL tube, 50mg) (Sigma-Aldrich, Germany)
- DSC-18LT cartridge (1mL tube, 50mg), (Sigma-Aldrich, Germany)

- DSC-CN cartridge (1mL tube, 50mg), (Sigma-Aldrich,Germany)
- DPA-6S cartridge (1mL tube, 50mg), (Sigma-Aldrich, Germany)

4.1.2 Methods

4.1.2.1 *Extraction*

Extraction of a sample of lyophilized apple leaves (0.2537 g)was carried out after the addition of 10 mL of 70% (v/v) EtOH in water using ultrasonic bath (ELMASONIC P). The sample was extracted for 40 minutes at 60 ˚C, a frequency was set at 80 kHz and a power at 100 W. The sample was filtered through a paper filter after extraction[63].

4.1.2.2 *Solid phase extraction*

The extract prepared in the previous step was used for solid phase extraction. In first step, five different cartridges were compared, DSC-PH, DSC-NH$_2$, DSC-18LT, DSC-CN and DPA-6S. First, each cartridge was conditioned by washing with 1 mL of 100% MeOH and followed with 1 mL of water. The sample was applied in a volume of 300 μL. After sample application, cartridges were eluated with 1 mL of water followed with 1 mL 60% MeOH, and finally with 1 mLof 100% MeOH. Each fraction was analysed by mass spectrometry and HPLC.

After comparing, the 6S cartridge was selected and used in a subsequent experiment. Five of these 6S cartridges were used. A conditioning procedure remained the same. The sample was applied in a volume of 300 μL in each cartridge. After sample application, cartridges were washed with 1 mLof water and then with eluated 1 mLof 100% MeOH. Fractions from all cartridges eluted with 100% MeOH were colected and subsequently concentrated in a vacuum drying oven to the volume 1 mL.

4.1.2.3 *HPLC quantification*

Qualitative and qunatitative analyses of phlorizin were performed by HPLC method developed by Liaudanskas [67]. A Waters 2695 chromatograph equipped with a Waters 2998 photodiode array (PDA) detector was used for the HPLC analysis. Chromatographic separations were carried out by using a YMC-PackODS-A (5 μm, C18, 250 × 4.6mm i.d.) column equipped with a YMC- Triart (5 μm, C18, 10 × 3.0mm i.d.) precolumn. The column was operated at 25 C˚. The volume of the extract being investigated (sample volume) was 10 μL. The flow rate was set at 1 mL/min, and continual and convex gradient elution was used. The mobile phase consisted of 2% (v/v) acetic acid in water (the solvent A) and 100% (v/v) ACN (the solvent B). The following conditions of elution were applied:

0–30 minutes, 3–15% B; 30–45 minutes, 15–25% B; 45–50 minutes, 25–50% B; and 50–55 minutes, 50–95% B. Identification was done by comparing retention time and spectral characteristics of the reference compounds[67].

4.1.2.4 *Semi-preparative HPLC*

For separation preparative a column ACE 5 C18 (5 µm, C18, 150 × 10mm i.d., the lenght 150 mm) was used. A PDA detector was set at 288 nm. The column was operated at a constant temperature of 25 C°. Four tests were performed. The injection volume of extract was in the first, the second and the fourth test 500 µL, in third it was 1mL. The flow rate was 1 mL/min and gradient elution was used for all tests. In first three test the mobile phase consisted of 1% (v/v) acetic acid in water (solvent A) and 100% (v/v) EtOH (solvent B), in fourth test the mobile phase consisted of 1% (v/v) acetic acid in water (solvent A) and 100% (v/v) ACN (solvent B). The following conditions of elution were applied in the first, the second and the fourth test: 0–60 minutes, 10–100% B. In third test following conditions were applied: 0–37 minutes, 10–65% B; 37–40 minutes, 65% B; 40–42 minutes 65–67% B; 42–45 minutes, 67% B; 45–47 minutes, 67–69% B; 47–50 minutes, 69% B; 50–53 minutes 69–71% B; 53–56 minutes, 71% B; 56–58 minutes, 71–73% B; 58–61 minutes, 73% B; 61–67 minutes, 73–80% B. At the beginning of the test, fractions were collected at a two-minute interval, after reaching the peak, the interval was shortened to one minute.

4.1.2.5 *Liquid chromatography-mass spectrometry (LC-MS)*

Identification of flavonoids was performed by Acquity LC system (Waters, USA) equipped with a triple quadrupole tandem mass spectrometer (Xevo, Waters, USA) with an electrospay ionization source (ESI) to obtain MS/MS data. YMC Triart C18 (100 x 2.0 mm; 1.9 µm) column was used for analysis. The column temperature was maintained at 40°C. Gradient elution was performed with a mobile phase consisting of 0.1% formic acid in water solution (the solvent A) and ACN (the solvent B) with the flow rate set to 0.5 mL/min. A linear gradient profile was applied with following proportions of solvent A: 0–1 minutes, 95%; 1–5 minutes, 70%; 1–7 minutes, 50%; 7.5–8 minutes, 0%; 8.1–10 minutes, 95%. Negative electrospray ionization was applied for the analysis with the following settings: capillary voltage – 2 kV, a source temperature –150°C, a desolvation temperature –400°C, a desolvation gas flow – 700 L/h, a cone gas flow – 20 L/h. Collision energy and cone voltage were optimized for each compound separately.

5   Results

5.1   Extraction

Extraction was performed by a validated method. Under the conditions, the amount of flavonoids in the extract was 26.97 ± 0.63 mg ER/g[63].

5.2   Solid phase extraction

As a first step, a suitable cartridge and an eluent for the extraction of phlorizin was found. For all types of cartridges, it was apparent that the sample was absorbed, but only with the 6S cartridge showed that the extract was bound after washing with water. These results were supported by LC-MS (5.4.) andquantitative HPLC (5.3.).

5.3   HPLC quantification

To calculate the phlorizin content a calibration curve (Figure 23) was used. The calibration curve was determined by measuring five samples of known concentration of phlorizin and their coresponding areas. The curve equation is as follows: $Y = 18500X - 7090$ (Y corresponds to the peak area and the X content of phlorizine in mg/mL).
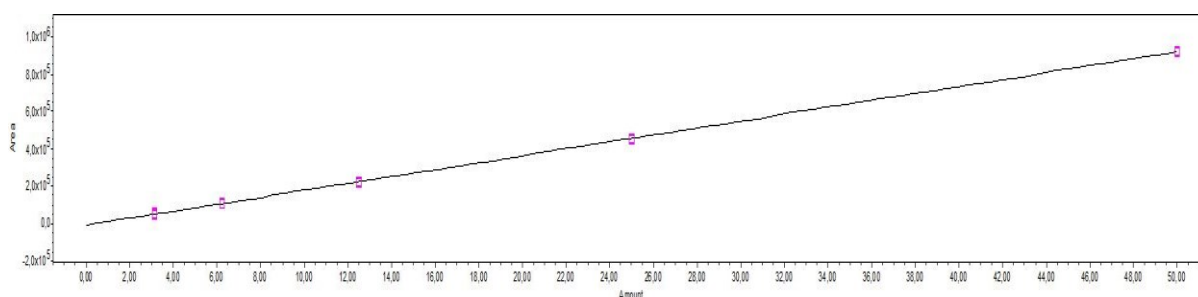
Figure 23 Calibration curve for phlorizin content calculation

By calculating the phlorizin content in mg/mL (Figure 24), the three fractions with the highest content were found. The DSC-NH$_2$ cartridge eluted with water, the DSC-PH cartridge eluted with water, and the DPA-6S cartridge eluted with 100% MeOH.

Cartri-dge	Area Water fraction	Phlorizin [mg/mL]	Area 60% MeOH fraction	Phlorizin [mg/mL]	Area 100% MeOH fraction	Phlorizin [mg/mL]
6S	0	0	169239	9.53	3381699 (Figure 25)	183.18
18LT	2404415	130.35	1168651	63.55	24779	1.72
NH2	4995983 (Figure 27)	270.44	432939	23.79	14377	1.16
PH	3494904 (Figure 29)	189.30	2174544	117.93	23174	1.64
CN	3099048	167.90	2115283	114.72	39336	2.51

Figure 24 Amount of phlorizin in each fraction of different cartridges

By comparing the areas of the peaks in the fractions, obtained by analytical HPLC, the percentage of phlorizin in the fractions was calculated. Highest percentage of phlorizin was found in cartidge DPA-6S, as solvent 100% MeOH was used, its purity was 92.48% (Figure 26). In the other two cartridges with the highest phlorizin the purity was less than 90% (Figure 28 and 30). Content in DSC-PH cartridge with solvent water was 80.65%, in DSC-NH$_2$ with solvent water 89.20% of phlorizin.
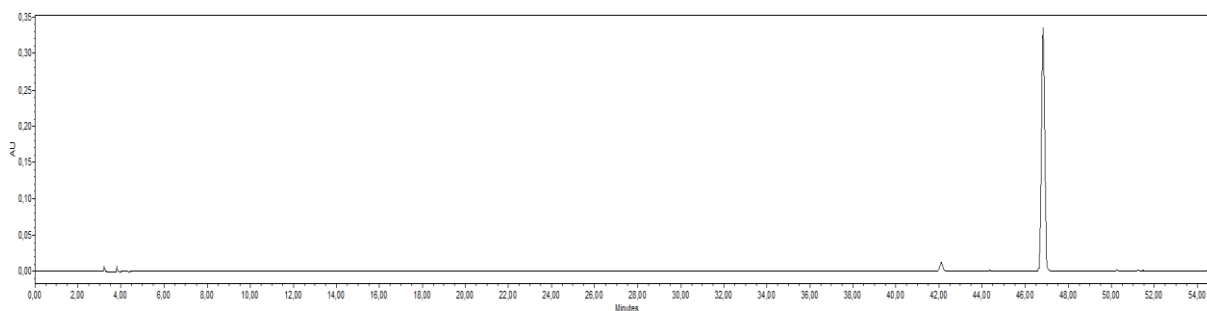
Figure 25 Chromatogram, cartridge DPA 6-S, solvent 100% MeOH

Total area	3656812
Area of phlorizin	3381699
Purity of phlorizin	92.48%

Figure 26 Calculation the percentage of phlorizin
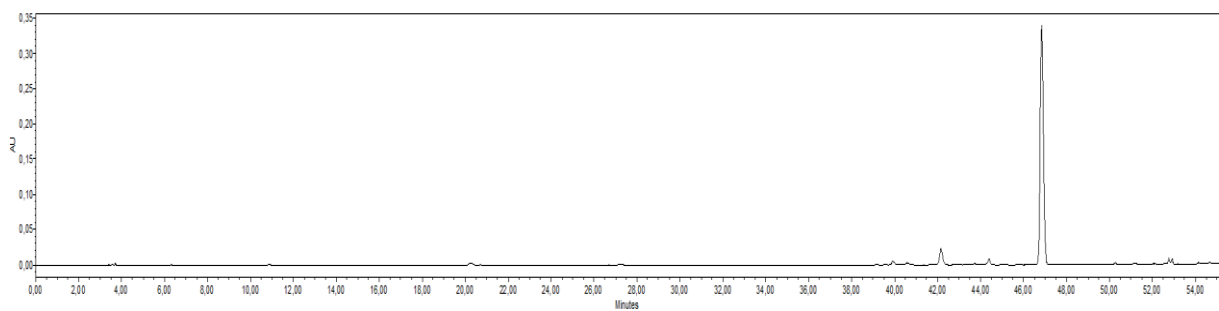
Figure 27 Chromatogram, cartridge DSC-PH, solvent water

Total area	4333326
Area of phlorizin	3494904
Purity of phlorizin	80.65%

Figure 28 Calculation the percentage of phlorizin
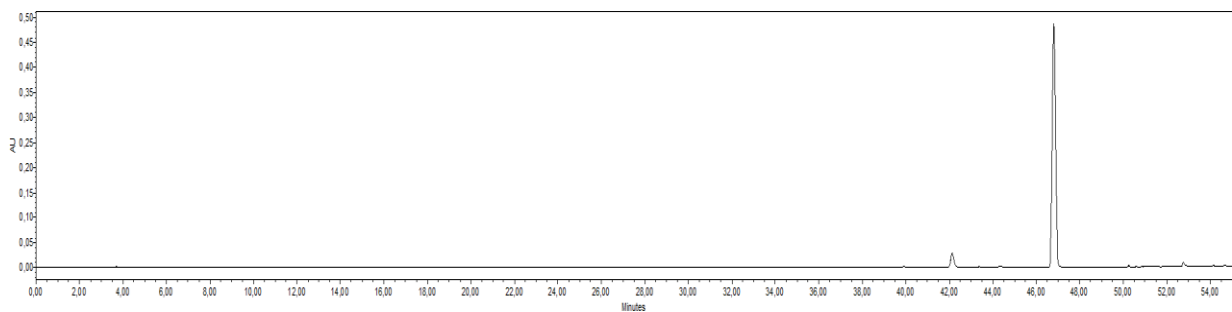
Figure 29 Chromatogram, cartridge DSC-NH₂, solvent water

Total area	5600577
Area of phlorizin	4995983
Purity of phlorizin	89.20%

Figure 30 Calculation the percentage of phlorizin

In the fraction 13 of the second test, which was evaluated as the purest, the amount of phlorizine was 6.84 mg/g (related to the dried weight). By comparing the areas of peaks percentage of phlorizine in this fraction was calculated, which was 91.05% (Figure 32).
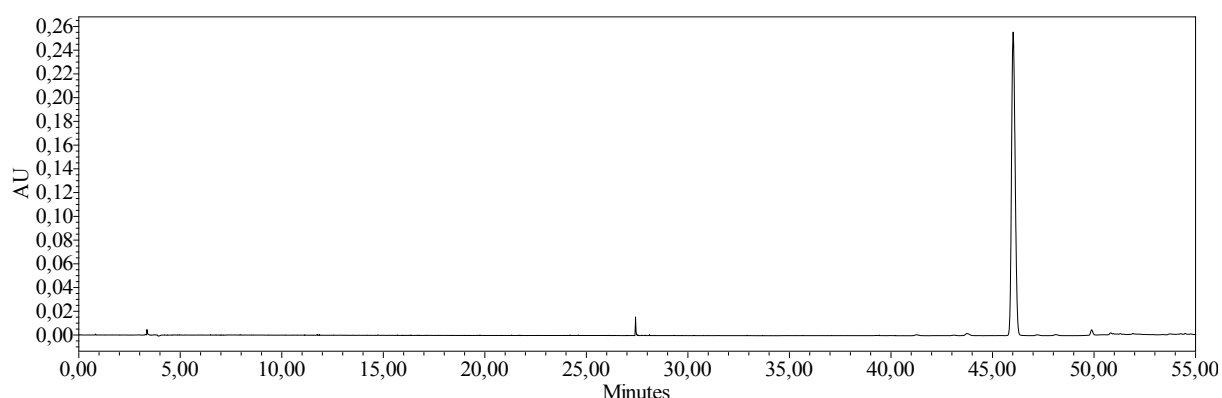
Figure 31 chromatogram based on quantitative HPLC analysis, fraction 13 of the second test (highlighted area in Figure 35)

Total area	3485344
Area of phlorizin	3173394
Purity of phlorizin	91.05%

Figure 32 Calculation the percentage of phlorizin

Using the calibration curve (Figure 23) and its equation Y = 18500X − 7090 (Y corresponds to the peak area and the X content of phlorizine in mg/mL), the amount of phlorizin obtained in fraction 13 was calculated.

$$X = \frac{Y - (-7090)}{18500}$$

$$X = \frac{3485344 - (-7090)}{18500}$$

$$X = 188.78 \; \mu g/mL$$

Conversion to lyophilized leaves weight – 7,53 mg/g (c is concentration [mg/mL], V is total volume of extract, m is weight of lyophlized leaves).

$$X = \frac{c \times V}{m}$$

$$X = \frac{0.18878 \times 10}{0.2537}$$

$$X = 7.44 mg/g$$

46

5.4 Semi-preparative HPLC

5.4.1 Test No. 1

Linear gradient EtOH (solvent B) 10–100%, analysis time 60 min. Injection volume –500 µL.
Theflow rate was 1 mL/min PDA detector was set at 288nm.

Figure 33 Chromatogramof semipreparative HPLC, test no. 1

Fraction	Fraction collection time [min]	EtOHin the mobile phase [%]
1	9.40–11.10	17.1–20.2
2	12.28–14.73	22.4–26.8
3	15.27–17.70	33–36.4
4	20.13–22.15	33.9–43
5	22.15–24.16	43–46.7
6	28.77–30.77	52.4–55.7
7	30.77–32.65	55.7–58.7
8	32.65–34.45	58.7–61.69
9	34.45–37.77	61.69–66.9
10	37.77–40.73	66.9–70.8
11	40.73–42.08	74.2–76.6

Figure 34 Fractions, collection time and composition of the mobile phase, test no. 1

Phlorizin was detected in fractions 8–11.

47

5.4.2   **Test No. 2**

Linear gradient EtOH (the solvent B) 10 –100%, analysis time 60 min. Injection volume –500 µL. The flow rate was 1 mL/min . PDA detector was set at 288nm. Highlighted area represents fraction evaluated as the purest, its quantitative HPLC analysis is shown in Figure 31.
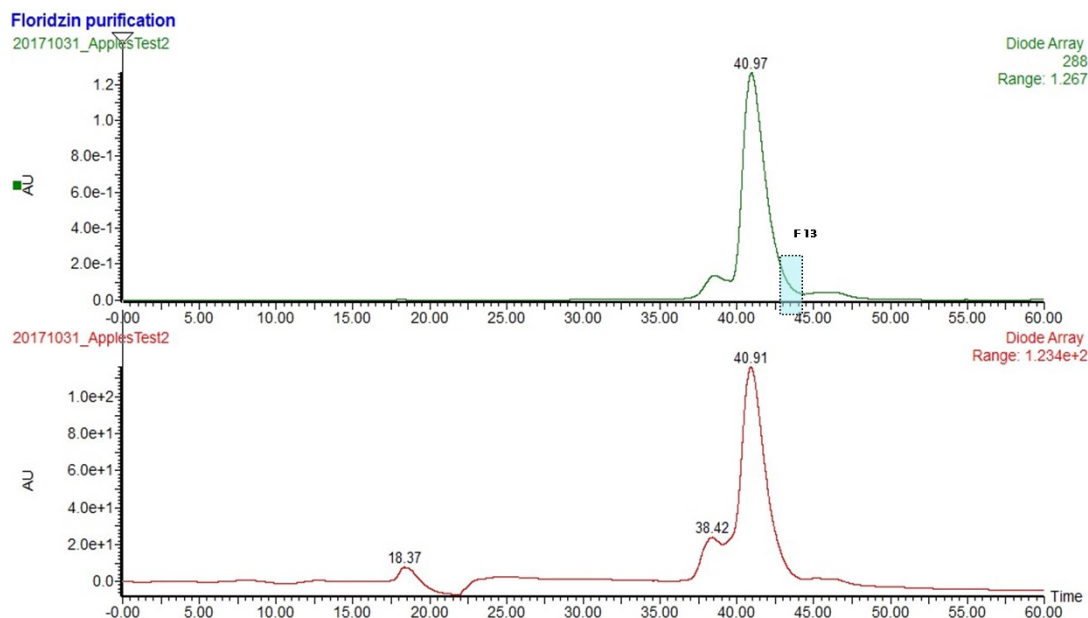
Figure 35 Chromatogram of semipreparative HPLC, test no. 2, highlighted area represents fraction number 13, quantitative HPLC is shown in Figure 31

Fraction	Fraction collection time [min]	EtOHin the mobile phase [%]
1	7.44–9.22	21–23
2	12.18–14.22	28.4–31.2
3	17.43–19.32	35.4–38.4
4	29.15–31.58	53.4–57.3
5	31.58–33.58	57.3–60.2
6	33.58–35.62	60.2–62.8
7	35.62–37.65	62.8–66.4
8	37.65–39.40	66.4–69.3
9	39.40–40.47	69.3–70.4
10	40.47–41.28	70.4–71.4
11	41.28–42.22	71.4–73.2
12	42.22–43.20	73.2–74.5
13	43.20–44.25	74.5–76

Figure 36 Fractions, collection time and composition of the mobile phase, test no. 2

Phlorizin was detected in fractions 10–13.

5.4.3 **Test No. 3**

In this test was an effort to improve separation by changing conditions compared to the previous tests. Gradient and injection volume were changed.

Step gradient EtOH (solvent B) 0–37 minutes, 10–65% B; 37–40 minutes, 65% B; 40–42 minutes 65–67% B; 42–45 minutes, 67% B; 45–47 minutes, 67–69% B; 47–50 minutes, 69% B; 50–53 minutes 69–71% B; 53–56 minutes, 71% B; 56–58 minutes, 71–73% B; 58–61 minutes, 73% B; 61–67 minutes, 73–80% B. Aanalysis time 67 min. Injection volume – 1mL. The flow rate was 1 mL/min. PDA detector was set at 288nm.
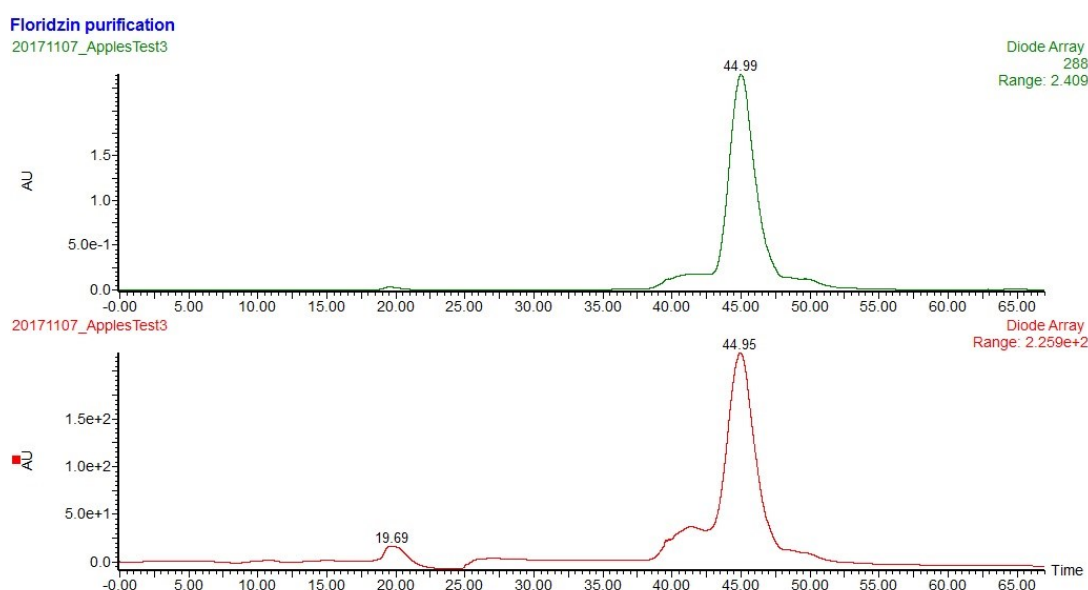
Figure 37 Chromatogramof semipreparative HPLC, test no. 3

Fraction	Fraction collection time [min]	EtOHin the mobile phase [%]
1	38.83–40.35	65
2	40.35–42.48	65.5
3	42.48–43.45	67
4	43.45–44.52	67
5	44.52–45.58	67
6	45.58–46.52	67.6
7	46.52–47.62	68–69

Figure 38 Fractions, collection time and composition of the mobile phase, test no. 3

Phlorizin was detected in fractions 5–7.

5.4.4 Test No. 4

In this test was an effort to improve separation by changing conditions compared to the previous tests. Solvent B was chnaged, linear gradient was retained.

A linear gradient ACN (solvent B) 10–100%, analysis time 60 min. Injection volume −500 µL. Theflow rate was 1 mL/min PDA detector was set at 288nm.
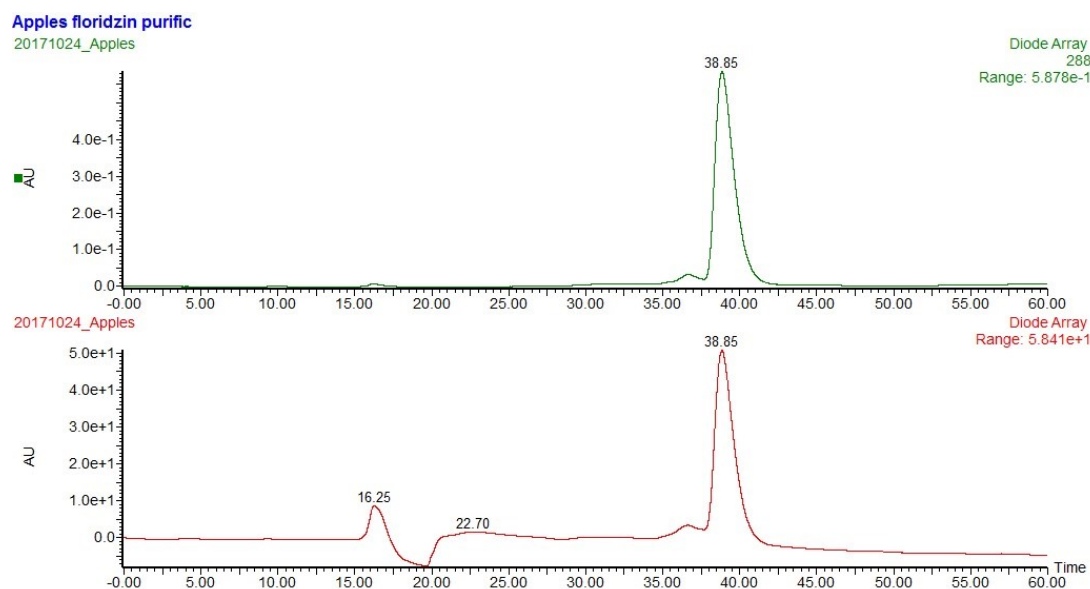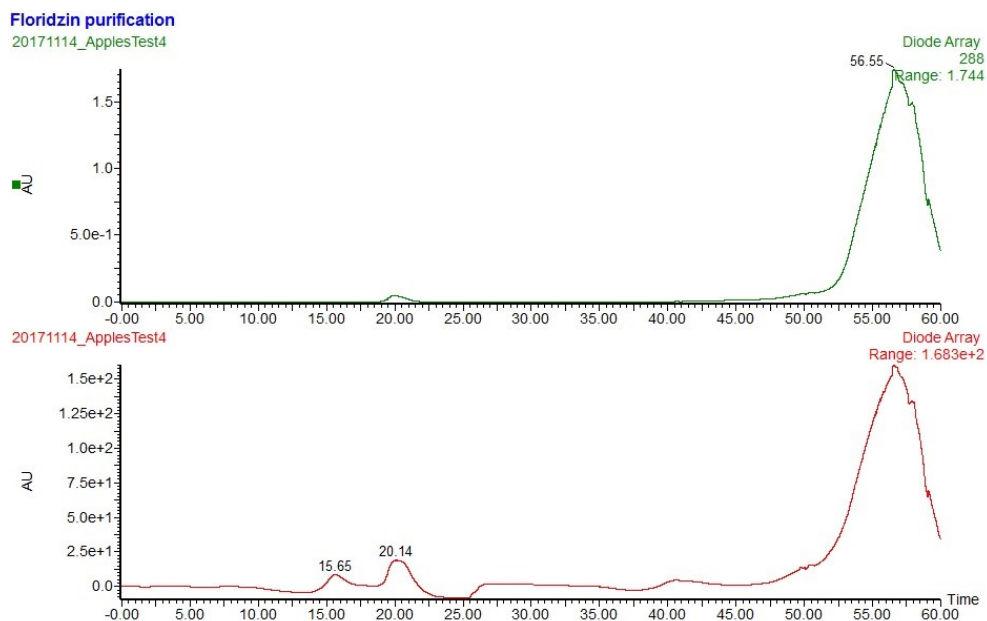

Figure 39 Chromatogram of semipreparative HPLC, test no. 4

As shown in the Figure 39, separation in this test was the worst of all analyses.

Fraction	Fraction collection time [min]	Acetonitrille in the mobile phase [%]
1	16.30–18.12	29.0–30.9
2	18.12–18.93	30.9–32
3	18.93–19.72	32–32.5
4	19.72–20.82	32.5–34
5	20.82–21.75	34–35.3
6	25.67–27.08	39.7–42.4
7	27.08–29.83	42.4–44.8
8	29.83–37.72	44.8–46
9	39.00–41.00	55–57.5
10	41.00–43.00	57.5–60.2
11	43.00–45.05	60.2–62.4
12	45.05–47.07	62.4–64.7
13	47.07–49.10	64.7–67.2
14	49.10–57.18	67.2–69.4
15	51.18–53.14	69.4–71.9
16	53.14–55.15	71.9–74.1
17	55.15–57.17	74.1–76.4
18	57.17–60.00	76.4–80

Figure 40 Fractions, collection time and composition of the mobile phase, test no. 4

Phlorizin was detected in fractions 16–18.

5.5 LC-MS

Identification of phlorizin in fractions was performed with ESI-MS. Negative ion ESI-MS of the phlorizin provided a quasi molecular ion peak [M–H]⁻ at m/z 435 which corresponds to phlorizin, and its fragment of m/z 273 which relates to the phloretin aglycon. Obtained ESI-MS data correlated with those in literature[67].

ESI-MS (*m/z*)[M–H]⁻ 435(100),273(55), 436(23).
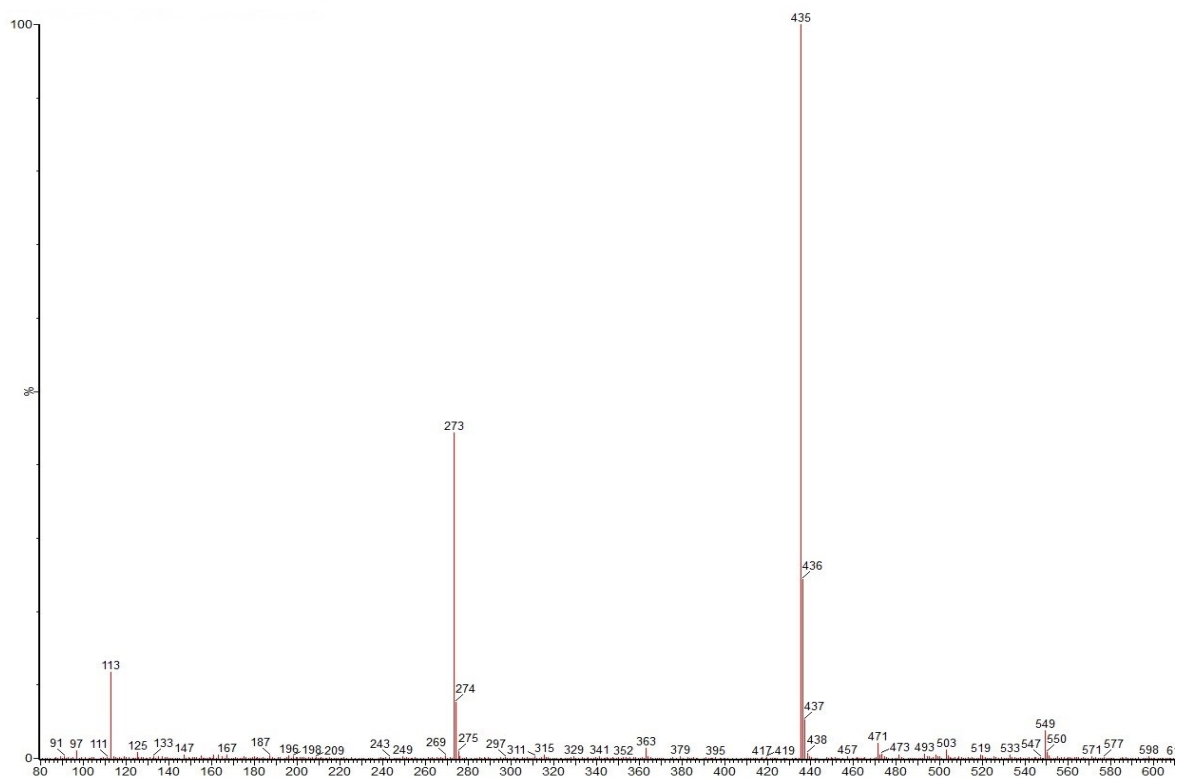
Figure 41 ESI-MS spectra of phlorizin, ionization mode – negative, collision energy – 10 eV

6   Discussion and Conclusion

The aim of this work was to develop a method for obtaining pure phlorizin from an flavonoid extract of the lyophilized *Malus domestica* Borkh leaves. Phlorizin should be free of toxic residues to be used for biological tests.

The first step was to find a suitable cartridge and a solvent for this compound, and to remove ballast compounds. Five cartridges and three solvents were compared. As shown in Figure 24, the three fractions with the highest amount of phlorizin were found, DPA-6S with solvent 100% MeOH, DSC-NH$_2$ with solvent water and DSC-Ph with solvent water. Furthermore, an analytical HPLC analysis was performed with these three fractions. The percentages content of phlorizin were calculated using the areas. Cartidge DPA-6S with solvent 100% MeOH was the most suitable to get the most pure phlorizin and it was used for the next part of the experiment.

The next step was to purify the extract obtained in SPE by semi-preparative HPLC. Four experiments were performed, the first two under the same conditions, another with another mobile phase composition. The attempt was to find the mobile phase without the use of ACN, which could leave the residue after evaporation. If such purified phlorizin were used in biological tests, it could be toxic. ACN was used in test No. 4, because better condicions for the separation were investigated. Howerver, the substitution of ACNfor EtOH did not improve separation. In the first two experiments, separation of the substance on the column was quite good and in second test the 91.05% purity of phlorizin was achieved, but this obtained purity of the compound would not be ideal for biological assays, so the conditions were changed. The amount of phlorizine was calculated using a calibration curve – 188.78 µg/mL, conversion to lyophilized leaves weight – 7.44 mg/g . In the other two experiments no improvement of separation was achieved, subsequent separation was worse.

Phlorizin from *Malus domestica* Borh has been so far purified from the bark and thinned young apples. Purification from the tree bark was performed using High-Speed Counter-Current Chromatography, as a solvent mixture chloroform-methanol-*n*-butanol-water (5:4:0.5:3, v/v) was used, the purity of phlorizin was 99.0%[68]. In another study, acolumn with macroporous resins X-5 was used for purification from thinned young apples. After absorption the column was eluted with 70% EtOH. The obtained purity of phlorizin was 97.52%[69].

This study resulted in relatively pure phlorizin (91.05%). For biological assays, a purity of 95% is required. Compared to the above mentioned experiments to my obtained results, the

difference in the purity of the compound was about 8%. Only the column ACE 5 C18 (5 μm, C18, 150 × 10mm i.d., the lenght 150 mm) was available for this experiment, and the best conditions for the column were investigated. An increased purity of phlorizin could be achieved by selecting columns with different sorbents such as C8, Spandex, polyamide[43] or macroporous resin X5 whichwas used for the purification of phlorizine from thinned young apples[68]. Another approach how to improve the purity of obtained phlorizin could be perfomed by using semi-preparative HPLC separation with another subsequent purification method (e.g., crystallization).

7   **References**

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