Antioxidant Flavonoid Glycosides in *Viola Tricolor* L.

Ph.D. Thesis

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Abstract

In this thesis the chemical composition of heartsease (*Viola tricolor* L.) was studied to support the evidence-based determination of its biological activities.

As the traditional internal administration of heartsease herb is as a tea, we primarily aimed at the analyses of components, which are supposed to be present in this aqueous extract. Henceforth, a proper sample preparation method was developed, yielding a fraction rich on polar constituents, which was further separated by conventional Sephadex LH-20 column chromatography. HPLC-UV analysis of the fractions suggested that the two main flavonoid components had been successfully isolated. They were definitly identified by LC-MS^n and NMR studies as violanthin (6-*C*-glucosyl-8-*C*-rhamnosyl apigenin) and rutin (3-*O*-rhamnoglucosyl quercetin). Furthermore, sixteen of the minor flavonoid components (*C*-glycosides, *O*-glycosides and *C*,*O*-glycosides) were tentatively identified by nanoLC-MS^n. Although violanthin could not be quantified in the lack of a commercially available reference molecule, rutin was quantitatively determined by HPLC with UV detection ((0.42$\pm$ 0.01) %). The antioxidant capacities (electron-donor and hydrogen donor activities) of the fractions were determined by the TEAC and DPPH assays, respectively. In respect to their antioxidant properties, the highest electron-donor capacity was measured with rutin, and a fraction enriched on flavonoids exhibited the highest hydrogen donor activity.

Garden pansies (*V. x wittrockiana* Gams.) are plants of complex hybrid origin. In this thesis, beside a comparative HPLC study, the anthocyanidin and flavonoid contents as well as the antioxidant capacities of garden pansies of different petal color and heartsease were compared.The anthocyanidin and flavonoid contents of the samples were quantified by spectroscopic methods registered in the European Pharmacopoeia 5.0. While the highest anthocyanidin content was measured in the violet flower sample, the white and yellow pansy samples showed the highest flavonoid content. The antioxidant capacity of the samples was determined by the TEAC assay. The heartsease and pansy samples were observed to be as good antioxidants as the well-known ginkgo leaf. In addition, significant correlation was found between the flavonoid content and the antioxidant capacity of the samples.

Összefoglaló

A vadárvácska (*Viola tricolor* L.) európaszerte ismert tradícionális gyógynövény. Bár eredményesen alkalmazzák felsőlégúti megbetegedések és bőrbetegségek kezelésére, fitokémiai és hatást igazoló vizsgálata egyaránt hiányos.

A tradícionális gyógyszerformával nyert bíztató eredmények alapján célzott extrakcióval, majd Sephadex LH-20 oszlopkromatográfiával eltérő polaritású frakciókat állítottunk elő. A két fő flavonoid komponenst sikeresen izoláltuk, szerkezetüket LC-MS^n és NMR módszerekkel violantinként (6-*C*-glükozil-8-*C*-ramnozil apigenin) és rutinként (3-*O*-ramnoglükozil kvercetin) azonosítottuk. Ez utóbbi komponenst mintánkban mennyiségileg is meghatároztuk ((0,42± 0,01) %). A minor flavonoid komponensek közül tizennégy szerkezetét (*O*-glikozidok, *C*-glikozidok és *C*,*O*-glikozidok) LC-MS^n vizsgálatokkal jellemeztük. A frakciók antioxidáns kapacitását (elektron-donor és hidrogén-donor aktivitását) a TEAC és DPPH *in vitro* tesztrendszerekben határoztuk meg. A legmagasabb elektron-donor kapacitást a rutin esetében mértük, míg a legmagasabb hidrogén donor kapacitást a flavonoidokban dúsított frakció esetében tapasztaltuk.

Bár a kertészeti árvácska fajokat (*Viola x wittrockiana* Gams.) a *V. tricolor* és más *Viola* fajok keresztezésével nemesítették, tartalmi anyagaik összetételéről, esetleges farmakológiai hatásaikról ez idáig nincs irodalmi adat. Kísérleteinkben tehát meghatároztul egy kereskedelmi vadárvácska és négy különböző színű kertészeti árvácska minta flavonoid- és antociántartalmát a Ph. Eur. 5.0 módszerével. A minták flavonoid összetételét VRK és HPLC módszerekkel vizsgáltuk. Az antioxidáns tulajdonság jellemzésére a TEAC antioxidáns rendszert használtuk. A kertészeti árvácska mintákban a vadárvácska mintával megegyező komponensek vannak jelen, csupán azok aránya változik. Egyértelmű összefüggés figyelhető meg a minták morfológiai sajátságai, antociántartalma, valamint flavonoidtartalma és -összetétele között. A használt tesztrendszerben az árvácska minták az ismert antioxidáns ginkgóhoz hasonlóan jó antioxidáns kapacitással jellemezhetők. A minták flavonoidtartalma és antioxidáns kapacitása között szignifikáns korreláció volt megfigyelhető.

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Abbreviations

APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
ATP	Adenosine triphosphate
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
cGMP	Cyclic guanosine monophosphate
COSY	Correlation spectroscopy
CVD	Cardivascular disease
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI	Electrospray inozation
FT-ICR	Fourier-transform ion-cyclotron resonance
GC	Gas chromatography
HAT	Hydrogen atom transfer
HMBC	Heteronuclear multi-bond correlation
HSP	Heat shock protein
HSQC	Heteronuclear single quantum coherence
i.p.	Intra peritoneal administration
IT	Ion trap mass analyzer
LC	Liquid chromatogarphy
LLE	Liquid-liquid extraction
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multi-stage mass spectrometry
NOESY	Nuclear Overhauser-effect spectroscopy
NOS	Nitric oxid synthase (e = endogenous, i = inducible, n = neuronal)
OD	Optical density
PGC-1	Peroxisome proliferator-activated receptorγ coactivator
p.o.	Per oral administration
Q	Quadruple filter mass analyzer
QQQ	Triple-quadruple mass analyzer
r	Square of the sample correlation coefficient

R	Recovery in the fortified sample recovery test
R^2	Pearson's cefficient of regression
RSD	Relative standard deviation
SE	Solvent extraction
SET	Single electron transfer
SPE	Solid-phase extraction
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Thermospray ionization
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight mass analyzer
TSP	Thermospray ionization

CHAPTER 1

Preliminaries and aims of the studies

The use of medicinal plants was always an important part of the medical systems of the world. Herbal medicinal products are useful therapeutic options often providing a safer form of therapy, and in many instances, specific remedies have been shown to be clinically effective. Their use is extensive and increasing. The evidence-based analyses of the traditional medicinal plants' chemical composition and biological effect is inevitable in the development of new and effective phytomedicines.

Although heartsease (*Viola tricolor* L.) has been extensively used in the traditional medicine for the treatment of skin disorders and upper respiratory tract problems for centuries, the biological activity of its main secondary metabolites has hardly been studied. Recent (yet unpublished) results of Kery et al. [1] suggest, however, the beneficial effects of heartsease infusion on the mitochondria. A part of those interesting data are represented and discussed in Tab. 1.1 and Tab. 1.2.

Mitochondria are indispensable energy-producing organelles of the eukaryotic cells. By the process of cellular respiration they generate ATP, which is used as a source of chemical energy. Besides, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth. Apparently, several diseases were found to be brought about by the impairment of mitochondrial functions e.g. Parkinson disease, Morbus Alzheimer's and diabetes [2, 3].

It was demonstrated that a specific mutation of the mitochondrial DNA results in β-cell dependent type 1 diabetes [4], and a decline in mitochondrial

Table 1.1: Effects of a 4-day long administration of heartsease water extract (V.t.e.) and/or glucose solution (g.s.) on protein levels in different cell cultures. n.d. = not detected

		Relative OD unit (sample/control)			
Cell culture	Administration	PGC-1	eNOS	HSP90	HSP72
primer pig endothel	8 μg/ml V.t.e	n.d.	1.6/1.0	1.1/1.0	2.3/1.0
HaCaT keratocyte	50 mM g.s. + 4 μg/ml V.t.e.	8.0/0.0	n.d.	6.0/0.0	6.0/0.0
HaCaT keratocyte	50 mM g.s. + 8 μg/ml V.t.e.	6.0/0.0	n.d.	5.0/0.0	4.0/0.0
HaCaT keratocyte	50 mM g.s. + 16 μg/ml V.t.e.	5.0/0.0	n.d.	4.7.0/0.0	4.2/0.0

PGC-1: Key enzime in mitochondria regulation. Its decreased level was reported to be in connection with insulin resistance.

eNOS: Increases PGC-1 expression. Its decreased level was reported to be in connection with diabetes gastropathy, high blood pressure, and other CVDs.

HSP90,72: Play a key role in the stabilization of the active eNOS complex and in the transport of important mitochondrial proteins.

Table 1.2: Glucose tolerance test of NMRI mice after five days i.p. administration of freeze-dried heartsease water extract.

	2 g/kg glucose i.p.		
time elapsed after glucose administration	Control	+ V. tricolor extract 5x30 mg/kg	+ V. tricolor extract 5x100 mg/kg
min	mM glucose		
0	12	11.2	12.6
30	22	13.9	14.9
60	20.4	18	12.7
90	12.8	16	12.5

oxidative and phosphorylation activity was associated with insulin resistance, the major factor in the pathogenesis of type 2 diabetes [5, 6]. Moreover, reduced level of the PGC-1 transcription factor, was observed in diabetes patients, and the potential role of its genetic variations in diabetes was also reviewed [7–10]. The PGC-1 was reported to play key role in the regulation of mitochondrial biogenesis and function [11]. Nitric oxide was also found to trigger mitochondrial biogenesis. This effect was mediated by the cGMP dependent induction of PGC-1 [12]. The specific action of nitric oxide depends on its enzymatic sources, namely neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), each having distinct tissue localization. nNOS was found mostly in the brain's nonadrenergic, noncholinergic autonomic neurons and in the muscles. Studies on nNOS knockout animals revealed clinical pictures caused by decreased nNOS expression [13]. nNOS-deficient mice develop gastric dilation and stasis [13], as NO regulates gastrointestinal tract motility and the muscle tone of the sphincter in the lower esophagus, pylorus, sphincter of Oddi, and anus [14]. Symptoms of diabetes gastropathy such as postprandial nausea, vomiting, abdominal discomfort and pain, delayed gastric emptying, fullness, and bloating decrease the quality of life of every second diabetes patient [15]. Increase of NOS expression or NO supplementation with NO donors were reported to improve diabetes gastropathy [16]. This was in correlation with the fact that decreased nNOS expression was observed in type 1 and type 2 diabetes patients. Nitric oxide released from the endothelium by eNOS plays an important role in regulation of vascular tone, inhibition of both platelet and leukocyte aggregation and adhesion, hypertension, and inhibition of cell proliferation [17]. These properties suggest that the level of NO production by the endothelium may play a pivotal role in the regulation of cardiovascular diseases (CVDs) [18], and CVDs were reported to be the major causes of mortality and disability in people with diabetes. The macrovascular manifestations include atherosclerosis and medial calcification. The microvascular consequences, retinopathy and nephropathy, are major causes of blindness and end-stage renal failure [19].

In conclusion, increased mitochondrial biogenesis and functions as well as increased NOS expression were supposed to decrease insulin resistance, improve diabetes gastropathy, retinopathy and nephropathy, and contribute to the prevention of CVDs. Consequently, they can lengthen the life-span and

improve quality of life of diabetes patients.

The above demonstrated promising results piqued our interest in the analysis of chemical composition of heartsease's infusion. Studies were undertaken to

1. develop a proper extraction method to replace the water extract of heartsease, and obtain an extract, which was microbiologically more stable and easier to work with.

2. develop a suitable fractionation protocol for the separation of this polar extract by conventional column chromatography.

3. analyze the fraction's chemical composition by chromatographic and spectroscopic methodologies, such as HPLC, LC-MS, and NMR.

4. to characterize the antioxidant properties of the fractions in different *in vitro* test systems.

5. to compare the chemical composition and antioxidant activity of heartsease with other Viola species.

CHAPTER 2

Introduction

2.1 The genus *Viola*

Heartsease (*Viola tricolor* L.), also known as wild pansy, belongs to the genus *Viola*. The later comprises flowering plants in the *Violaceae* family with about 400-500 species distributed around the world. Most species are found in the temperate Northern Hemisphere, however *Viola* species are also found in widely divergent areas such as Hawaii, Australia, or South America. Most *Viola* species are small perennial or annual plants, and a few are small shrubs. They typically have heart-shaped, scalloped leaves, though palmate leaves or other types have also been described. The flowers are zygomorphic with bilateral symmetry and formed of five petals: four are upswept or fan-shaped with two per side, and there is one broad, lobed lower petal pointing downward (Fig. 2.1). Solitary flowers are produced on long stalks, persistent after blooming. The flowers have five free stamens with the lower two having nectary spurs that are inserted on the lowest petal into the spur or pouch. The flower styles are thickened near the top and the stigmas are head-like, narrowed or often beaked. After flowering, fruit capsules are produced that split open by way of three valves. *Viola* flowers are most often spring blooming with well developed petals pollinated by insects. Many species also produce self-pollinating flowers in summer and autumn that do not open and lack petals. The nutlike seeds are often spread by ants. Flower colors vary in the genus, ranging from violet, as their common name suggests, through various shades of blue, yellow, white, and cream, whilst some types are multicolored. Many cultivars and hybrids have been bred in a greater spectrum of colours. Flowering is often profuse, and may last for much of the spring and summer [20–22].

Table 2.1: Structures of flavonoid glycosides discussed in this thesis. In the lack of available literature data, stereochemistry has not always been indicated

Trivial name	MW (Da)	Structure
isoorientin	448	luteolin-6-*C*-glucoside
isovitexin	432	apigenin-6-*C*-glucoside
isoschaftoside	564	apigenin-6-*C*-arabinoside-*8*-C-glucoside
orientin	448	luteolin-6-*C*-glucoside
rutin	610	quercetin-3-*O*-rhamnosyl(1→6)glucoside
saponarin	594	apigenin-6-*C*-glucoside-7-*O*-glucosdie
schaftoside	564	apigenin-6-*C*-glucoside-8-*C*-arabinoside
scoparin	462	chrysoeriol-8-β -D-*C*-glucoside
swertiajaponin	462	7-methoxy-luteolin-6-*C*-glucoside
vicenin-2	594	apigenin-6,8-di-*C*-glucoside
violanin		delphinidin-3-*O*-(p-coumaroyl-rhamnosylglucoside)
violanthin	578	apigenin-6-*C*-β-D-glucoside-8-*C*-α-D-rhamnoside
violarvensin	578	apigenin-6-*C*-β-D-glucoside-8-*C*-β-D-rhamnoside
vitexin	432	apigenin-8-*C*-glucoside

(a)

(b)

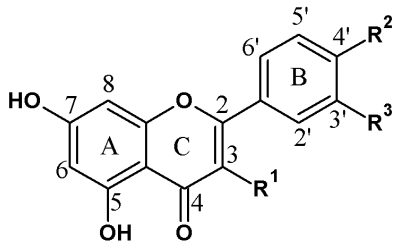
(c)

Figure 2.1: (a) flowering part of heartsease (*Viola tricolor* L.) (b) habitus of garden pansy (*Viola x wittrockiana* Gams.) (c) habitus of *Viola tricolor* L. (left) and *Viola arvensis* Murray. (right)

A number of *Viola* species are grown for their ornamental flowers, but several members of the genus have been widely used in traditional phytomedicine. Leaves and roots of *V. odorata* have been reported to possess expectorant, sudatory, and metabolisms enhancing abilities, whilst its flowers exhibited expectorant, tranquillizer, and antihypertensive effects. Infusions of *V. tricolor* and *V. arvensis* were also described as expectorants and metabolisms enhancers [23]. Other papers review the heartsease herb's expectorant, diuretic, astringent, and anti-inflammatory effects and its indication in skin disorders and upper respiratory tract problems [22, 24]. On the other hand *V. odorata* were utilized in cough mixtures for chronic bronchitis, whooping chough (pertussis), and asthma bronchiale, against migraine, or as a sedative [22]. In addition, heartsease herb also increases mitochondria formation and its application provides prophylaxis and treatment for illnesses brought about by impaired mitochondrial activity or by decreased functioning of the constitutive nitric oxide synthase enzyme (unpublished results of Kery et al.).

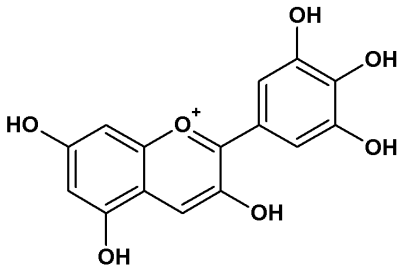
Although the discussed *Viola* species were considered as remarkable herbal remedies, only scarce information was reported on their phytochemical analysis. One of the most significant group of heartsease's active compounds, the flavonoids have been analyzed only by outworn, sometimes even unreliable methodologies. Accordingly, papers from the 1980's report on the presence of *O*-glycosyl (luteolin-7-*O*-glucoside and rutin) and *C*-glycosyl flavonoids (isoorientin, isovitexin, orientin, scoparin, vicenin-2, and vitexin) [22, 25, 26]. From the C,*O*-glycoside group only saponarin was detected [22]. For their chemical structures see Tab. 2.1 and Fig. 2.2a. Anthocyanins are also classified as flavonoid glycosides [27]. In heartsease, the presence of violanin, platyconin, and violanin-chloride (all delphinidin glycosides Fig. 2.2b) was described [28]. On the other hand, from *V. arvensis* a flavone-di-*C*-glycoside was isolated, and identified as violarvensin by NMR spectroscopy [29]. Besides, the presence of ursolic acid based, galactose or galacturonic acid containing triterpene saponins was not reliably confimed in *V. tricolor* [22], and other authors claim a peptide component: violapeptide-1 responsible for heartsease's hemolytic activity [30]. Carotenoids such as violaxantin, violeoxantin, lutein, luteinepoxid, and neoxantin were also identified in heartsease [31–34]. Besides phenolic acids such as p-coumaric, gentisinic, p-hydroxy benzoic, p-hydroxyl-phenylacetic, caffeic, protocatechuic, vanillic, and salicylic acids as well as their derivatives, polysac-

(a)



	MW	R^1	R^2	R^3
apigenin	270	H	OH	H
luteolin	286	H	OH	OH
kaempferol	286	OH	OH	H
chrysoeriol	300	H	OH	OCH_3
diosmetin	300	H	OCH_3	OH
quercetin	302	OH	OH	OH
isorhamnetin	316	OH	OH	OCH_3

(b)



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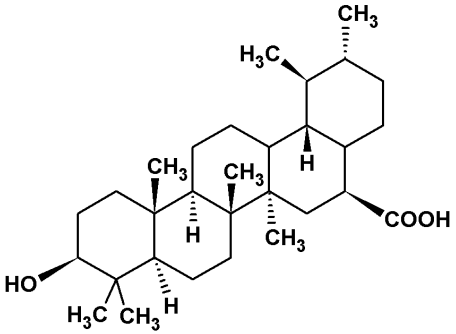

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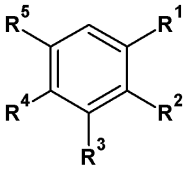


	R^1	R^2	R^3	R^4	R^5
p-hydroxy-benzoic acid	COOH			OH	
salicylic acid	COOH	OH			
protocatechuic acid	COOH		OH	OH	
gentisinic acid	COOH	OH			OH
vanillic aid	COOH		OCH_3	OH	
p-hydroxy-phenylacetic acid	CH_3–COOH			OH	
p-coumaric acid	CH_2=CH_2–COOH			OH	
caffeic acid	CH_2=CH_2–COOH			OH	OH

Figure 2.2: Structures of (a) flavonoid aglycones discussed in this thesis, (b) delphinidin (anthocyanidin), (c) ursolic acid, and (d) phenolic acid derivatives.

charides, vitamin E and C were also reported in *V. tricolor* [22].

2.2 The flavonoids

2.2.1 Chemical structure (Biosynthesis)

All flavonoids possess a three-ring diphenylpropane ($C_6C_3C_6$) core structure and their main subclasses are depicted in Fig. 2.3.

Flavonoids are of mixed biosynthesis, consisting of units derived from both shikimic acid and polyketide pathways (Fig. 2.4) [35]. The polyketide fragment is generated by three molecules of malonyl-CoA, which combine with the C_6–C_3 unit (as a CoA thioester) to form a triketide starter unit. The triketide starter unit undergoes cyclization by the enzyme chalcone synthase. In addition, cyclization can occur to give a pyranone ring containing flavanone nucleus, which can either have the 2,3 bond (Fig. 2.3) oxidized to give the flavones or be hydroxylated at position 3 to give the flavonol group. Flavonols may then be further oxidized to yield anthocyanins.

The basic structure is usually modified by means of hydroxylation and/or methylation at positions C-3, C-5, C-7, C-3', C-4' and/or C-5'. Occasionally, aromatic or aliphatic acids, sulphate, prenyl, or isoprenyl groups are attached to the flavonoid backbone [36, 37]. In the plants, flavonoids are mostly present as glycosides. The purpose of glycosylation is to render a molecule less reactive and more water soluble, thus glycosylation in plants can be regarded as a form of protection to prevent cytoplasmic damage [38]. *O*-glycosides have sugar substituents bound to a hydroxyl group of the aglycone, whereas in the case of *C*-glycosides, the sugar is connected to the aglycone through a carbon-carbon bond. Theoretically, the glycan residue can be attached to any of the aglycone's hydroxyl groups, but certain positions are apparently favored, such as the 7-hydroxyl group for flavones, flavanones, flavonols and isoflavones and positions C-3 for flavonols and anthocyanidins Fig. 2.3 [38–40]. 5-*O*-glycosides are rare for compounds with a carbonyl group at C-4, since the 5-hydroxyl group participates in hydrogen bonding with the adjacent carbonyl [41]. Up until today, *C*-glycosylation has almost exclusively been found at positions 6 or 8, and only in two cases at position 3 [42]. The most frequently found monosaccharides in glycosidic combinations are glucose and rhamnose, and less frequently

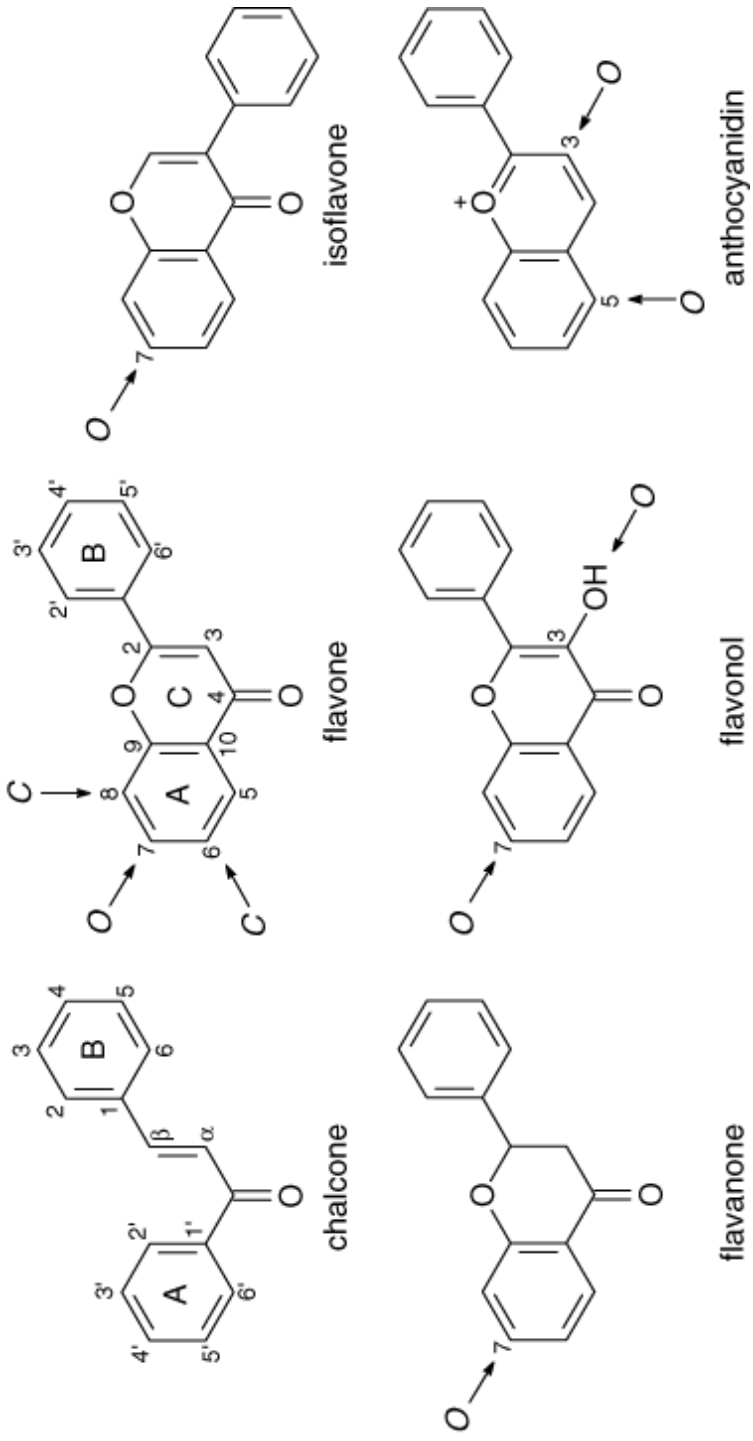


Figure 2.3: Structures of the main flavonoid classes. Common *O*- and *C*-glycosylation positions are indicated by arrows [38].

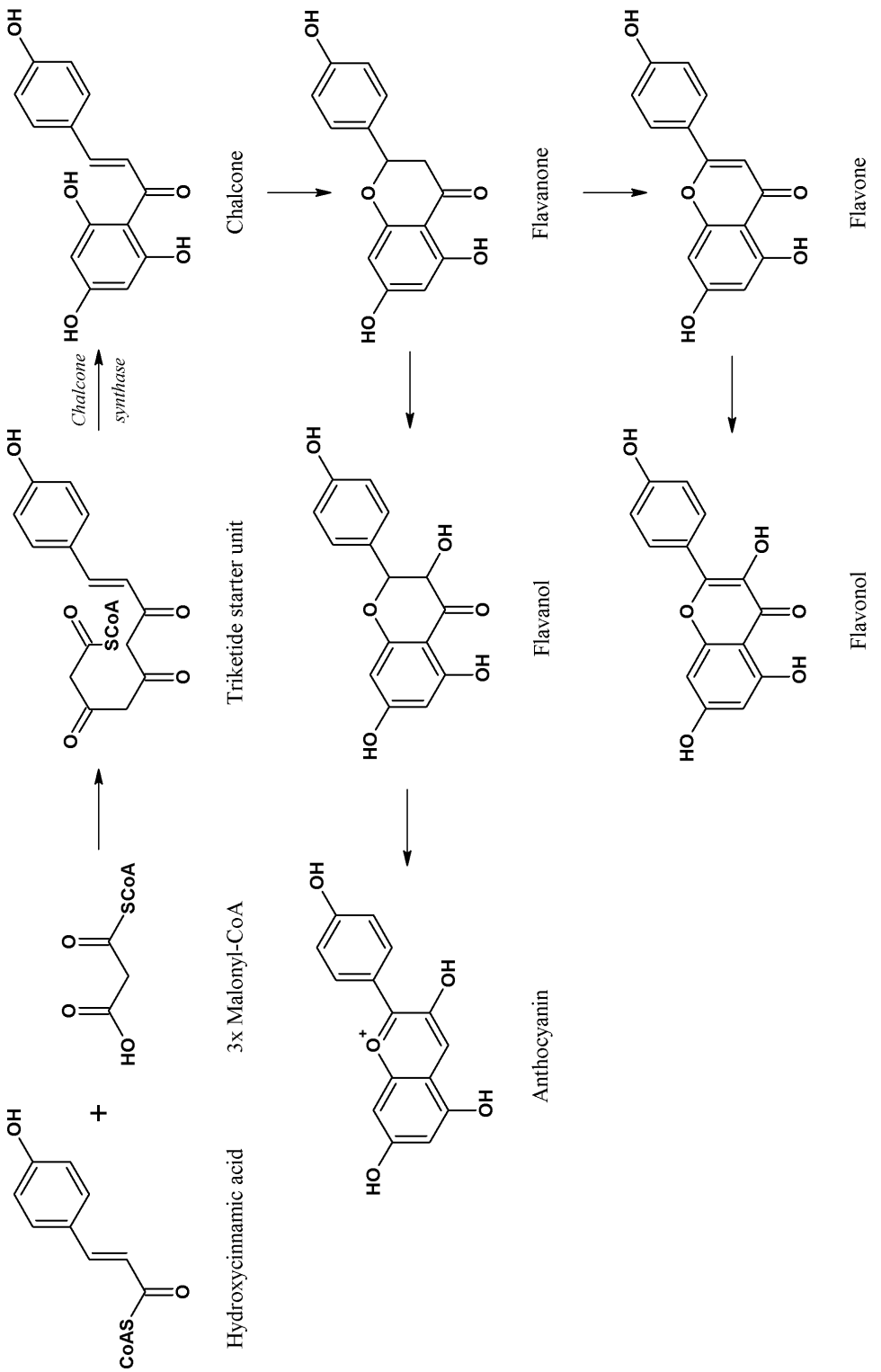


Figure 2.4: Biosynthesis of the major flavonoid classes.

arabinose, xylose, and glucuronic acid [40, 42, 43]. In addition, one or more of the sugar hydroxyls were reported to be esterified with aromatic or aliphatic acids [44–46].

2.2.2 Health effects

Up until today, flavonoids have been found only in plants. Being not only colored pigments, but also enzyme inhibitors and stimulants, metal chelators and reducing agents, they are important for the plant's normal growth, development and defense [27, 47]. In addition, several papers including epidemiological studies and meta-analyses report on their beneficial effects on human health. As the most important examples, flavonoids reportedly reduce the risk of cancer [48–51] and cardiovascular diseases [52–55] as they e.g. hinder the invasion of metastases [56], slow the division of tumor cells [57, 58], reduce hypertension [59, 60], as well as protect and strengthen vascular walls [61, 62]. In addition, flavonoids showed prospective benefits in the treatment and/or ease of symptoms of several serious illnesses such as AIDS [63–65], Morbus Alzheimer's [66, 67], rheumatic diseases [68–70], diabetes [71–73], asthma bronchiale [74], and gastro-intestinal ulcers [75, 76] due to their immun-modulatory, antimicrobial, antioxidant, anti-inflammatory, pain-killing, and smooth muscle relaxant effects [74, 77–80]. Flavonoids also render valuable help in minor problems e.g. wounds, bites, burns, or common cold [74, 81].

2.3 General considerations on qualitative flavonoid analytics

2.3.1 Analyte isolation

Over the years many sample pre-treatment methods have been developed to determine flavonoids in plants. For analyte isolation solvent extraction (SE) – which may be followed by solid-phase extraction (SPE) – is still the most widely used technique, mainly because of its ease of use and wide-ranging applicability. Soxhlet extraction is used less frequently to isolate flavonoids from solid samples. From liquid samples analytes are isolated using liquid-liquid extraction (LLE) or SPE. As regards SE and Soxhlet, in most cases aqueous methanol

or acetonitrile is used as solvent. In the case of LLE the extraction solvent usually is ethyl acetate or diethyl ether containing a small amount of acid. LLE is usually directed at the isolation of aglycones, while the other methods can have the isolation of both aglycones and conjugates as their goal. In flavonoid analysis LC-based methods are by far the most important ones. Less common procedures involve GC, capillary electrophoresis (CE) or thin-layer chromatography (TLC) [82–84].

2.3.2 Separation and detection

LC of flavonoids is usually carried out in the reversed-phase mode, on C_8- or C_{18}-bonded silica columns. However, also other phases, such as silica, Sephadex and polyamide are used. Gradient elution is generally performed with binary solvent systems, e.g. with water containing acetate or formate buffer, and methanol or acetonitrile as organic modifier. LC is usually performed at room temperature, but temperatures up to 40 °Care sometimes recommended to reduce the time of analysis and because thermostated columns give more repeatable elution times [82].

All flavonoid aglycones contain at least one aromatic ring and, consequently, efficiently absorb UV light. The first maximum, which is found in the 242-285 nm range, is due to the A-ring and the second maximum, which is in the 300-550 nm range, to the substitution pattern and conjugation of the C-ring. Simple substituents such as methyl, methoxy and non-dissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima. Already several decades ago, UV spectrophotometry was, therefore, a popular technique to detect and quantify flavonoid aglycones. More recently, UV detection became the preferred tool in LC-based analyses and, even today, LC with multiple-wavelength or diode-array UV detection is a fully satisfactory tool in studies dealing with, e.g. screening, quantification of the main aglycones and/or a provisional sub-group classification [85].

In flavonoid analysis, fluorescence detection is used only occasionally, because the number of flavonoids that exhibit native fluorescence is limited. For these compounds, the limits of detection in LC and CE are typically about an order of magnitude lower than with UV detection. Moreover, their fluorescence facilitates selective detection in complex mixtures [86]. Classes of flavonoids that show native fluorescence include the isoflavones, flavonoids with an OH

group in the 3-position. Since most flavonoids are electroactive due to the presence of phenolic groups, electrochemical detection can also be used [82, 87].

2.3.3 Identification and structural characterization

Today, LC-MS/MS is the most important technique for the identification of target flavonoids and the structural characterization of unknown members of this class of compounds. As regards target analysis, tandem-MS detection has largely replaced single-stage MS operation because of the much better selectivity and the wider-ranging information that can be obtained. Depending on the nature of the application, additional information is derived from LC retention behaviour, and UV absorbance and, occasionally, FLU or ED characteristics, due comparison being made with standard injections and/or tabulated reference data. In studies on the characterization of unknowns, a wide variety of LC-MS/MS techniques is usually applied next to LC-DAD UV for rapid class identification. In addition, LC-NMR often turns out to be an indispensable tool to arrive at an unambiguous structural characterization.

2.4 LC-MS in the characterization of flavonoids

Structural analysis of flavonoids is essential in the search for new biologically active compounds and in the development and quality control of natural products. Determination of the absolute structure of flavonoids is a complicated task, which mostly requires the combination of advanced techniques, e.g., ^{1}H- and ^{13}C-NMR-spectrometry, ^{1}H-^{1}H-correlated spectroscopy, mass spectrometry, and/or X-ray cristallography requiring large amounts of adequately purified sample [87]. Flavonoids, however, are usually present in a complex matrix of plant extracts, thus generally difficult to isolate in higher quantities. To alleviate this drawback, hyphenated techniques, such as LC-MS and LC-NMR can be applied. Both techniques have advantages and disadvantages [82, 88–90]. LC-MS represents a rapid and easy-to-access methodology with high sensitivity and low sample demand. LC-NMR provides more structural information (even about the stereochemistry of glycosides), but only a few laboratories are equipped with such high end instrumentation. Besides, due to its low sensitivity, for an LC-NMR experiment more sample is required.

2.4.1 Instrumentation

Thermospray ionization (TMS), the first soft ionization method applicable to the combination of liquid chromatography with mass spectromerty was introduced in the early 80's [91] for flavonoid analysis. This technique has been in recent years gradually phased out by atmospheric pressure ionization (API), including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The application of matrix-assisted laser desorption/ionization (MALDI) in flavonoid MS has also been reported [92, 93]. Besides the ionization sources, MS instrumentations are usually classified according to the applied mass analyzers: quadruple filters (Q), ion traps (IT), as well as time of flight (TOF) and Fourier-transform ion-cyclotron resonance (FT-ICR) devices [87].

For some years, single-stage MS operations have been replaced by tandem mass spectrometry because of the much better selectivity and wider-ranging structural information it provides. Tandem mass spectrometry is a term which covers a number of techniques, in which one stage of mass spectrometry is used to select the ion of interest and the second stage is then used to analyze it. In multi-stage MS these steps can be repeated consecutively. MS/MS experiments can be achieved on e.g. Q-TOF or triple-quadrupole (QQQ) instruments, the application of IT analyzers, however, allows also MS^n analyses.

2.4.2 Nomenclature and basic fragmentation

Fragment ions yielded by mass spectrometric analysis of flavonoids are usually designated according to a widely accepted nomenclature system for aglycones developed by Mabry [85], improved by Ma [94], and elaborated for glycoconjugates by Domon and Costello [95]. The applied labels are depicted in Fig. 2.5.

For free aglycones labels ${}^{i,j}A_0$ and ${}^{i,j}B_0$ are used to refer to fragments containing intact A- and B-rings, respectively, where superscripts i and j indicate the broken C-ring bonds. Glycoside fragments with retained charges on the carbohydrate portion are designated as A_i B_i and C_i, where i represents the number of broken bond, counted from the terminal sugar unit. On the other hand, ions containing the aglycone are labeled as X_j Y_j and Z_j, where j is the number of the cleaved interglycosidic bond, counted from the aglycone. The glycosidic bond linking to the aglycone is numbered as 0. In the case of *C*,*O*-glycosides, *O*-glycosylated on the *C*-glycosyl moiety, the first glycosidic bond does not con-

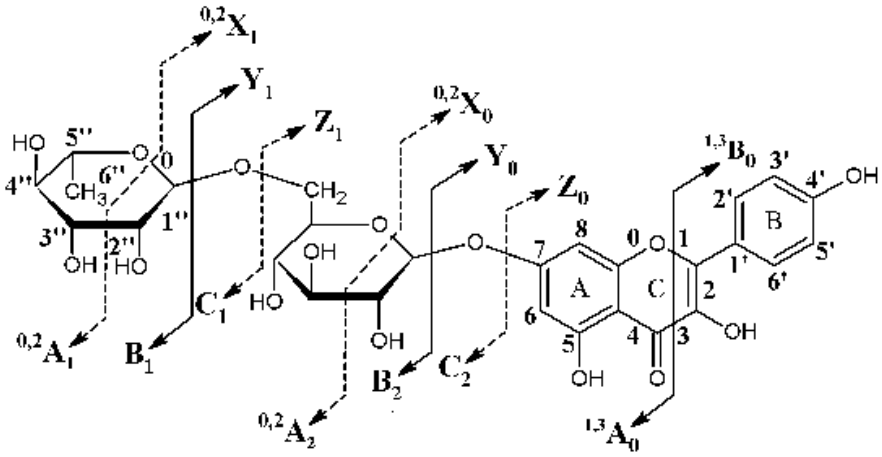


Figure 2.5: Fragment nomenclature commonly applied for for flavonoid glycosides and aglycones.

nect to the aglycone, therefore the numbering starts at the *C*-glycosyl moiety [96]. In addition, for *C*,*O*-glycosides, the Y_0 or Z_0 ions represent fragments, resulting from the loss of the *C*-glycosyl moiety.

Fragments B_i and Y_i are observed both in positive and negative ion spectra, and are generated through the cleavage of the glycosidic bond with retention of the glycosidic oxygene atom by the fragments containing the aglycone. In positive ion mode, B_i and Y_j result from the protonation and subsequent cleavage of the glycosidic bond or alternatively from the cleavage of the glycosidic bond accompanied by a protontransfer (Fig. 2.6a) [95]. In the negative ion mode, this fragmentation follows a more complex pathway. There the deprotonated molecular ion undergoes epoxide formation accompanied by the opening of the sugar ring, the cleavage of the glycosidic bond, and a competitive protontransfer (Fig. 2.6b) [97]. In addition, the glycosidic bond can also fragment with the retention of the glycosidic oxygene by the carbohydrate fragment without aglycone yielding the C_i and Z_j ions. In the negative ion mode the deprotonated sugar ring undergoes epoxide formation resulting in the cleavage of the glycosidic bond. This process is also accompanied by protontransfer reactions (Fig. 2.6c). The formation of $Z_j$in the positive ion mode, however, is a two step process involving a loss of water molecule from the corresponding Y_j ion (Fig. 2.6d) [98].

Product ions generated through the cleavages of the sugar ring's C–C bonds are labeled with ${}^{k,l}A_i$ and ${}^{k,l}X_j$, where k and l indicate the broken sugar ring

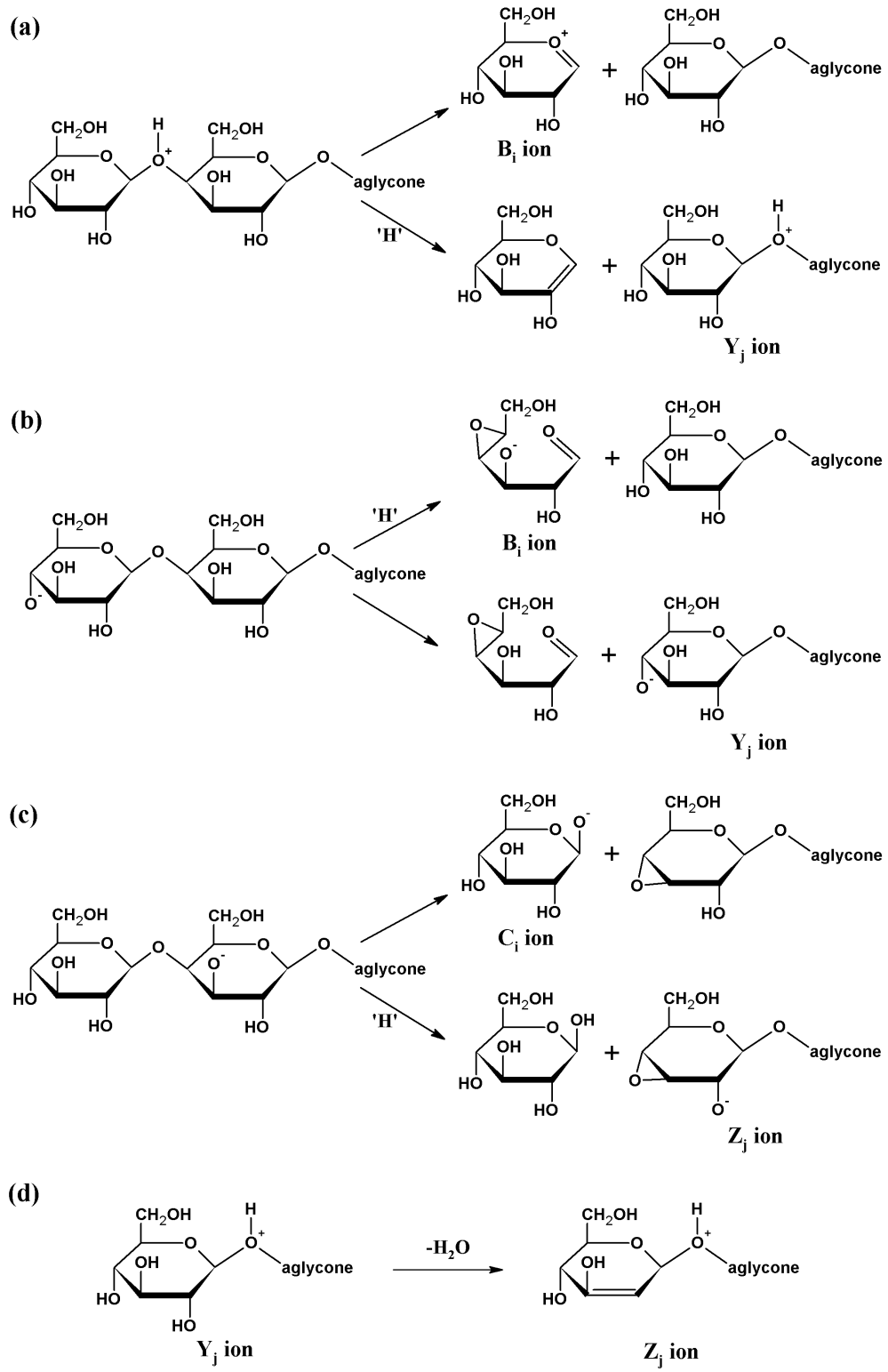


Figure 2.6: Genesis of (a) B_i and Y_j in the positive ion mode, (b) B_i and Y_j in the negative ion mode, (c) C_i and Z_j in the negative ion mode, and (d) Z_j in the positive ion mode.

bonds. Note that ${}^{k,l}A_i$ is easily confused with the aglycone fragment (${}^{i,j}A_0$), therefore to avoid uncertainty the subscript 0 was added to the latter [94] (for sugar fragments $i > 0$). For simplicity, ions formed by the direct loss of radicals or small neutral molecules are usually specified by reference to the parent ion (e.g. ${}^{0,2}X^+$–H_2O). In some cases, subscripts H, D, or P was added to the labels, referring to 'hexose', 'deoxyhexose', or 'pentose', respectively. Label 'E_i' designates the loss of water molecules. In some instances, a number in parenthesis was added to the label to indicate the position where the sugar unit was attached to the aglycone.

2.4.3 Structure elucidation

The characterization of flavonoid glycosides by mass spectrometry comprises the determination of (i) glycosylation types (*O*-, *C*-, or mixed glycosides), (ii) types of the sugar units (hexoses, deoxyhexoses or pentoses), (iii) distribution of the sugar residues, (iv) order of the glycan sequence, (v) interglycosidic linkages, (vi) glycosylation position, and (vii) nature of the aglycone. Besides summarizing papers, which reporte on the determination of the above characteristic markers, this subsection focuses on the identification options of unknown flavonoid glycosides in complex samples (e.g. plant extracts) with the emphases on the differentiation of isomeric compounds.

Although MS fragmentation patterns may theoretically vary with the instrumentation used, several authors reported that the main fragmentation paths of flavonoids are independent of the actual ionization mode (ESI, APCI, or MALDI) or the types of analyzers applied (QQQ, IT, or QTOF) [92, 99–101]. On the contrary, significant differences could be observed regarding the relative abundances of fragment ions by using different instrumentation [92, 99, 100]. Therefore, methods based on the presence or absence of distinctive fragment ions are preferred to techniques, which rely only on relative intensity changes observed for isomeric compounds.

Similarly, approaches not requiring special sample preparation methods are also favored. Nonetheless, methods including derivatization or complex formation, are also discussed if they provide complementary information on structural elucidation (Sec. 2.4.3.5).

As the majority of the cited references report only on the study of flavones, flavonols, flavanones, and isoflavones, mass spectroscopic analysis of antho-

cyanidins will be summarized in a separate part (Sec. 2.4.3.6). On the other hand, since their unique structure allows their easy differentiation from other flavonoids, chalcones will not be discussed (for further references see [102, 103]). In addition, (i) if no particular comments are given, considerations apply to all major flavonoid classes (except of anthocyanidins and chalcones); (ii) if their labels do not indicate charges, fragments were observed in both positive and negative ionization modes; (iii) the discussed flavonoid glycosides were named referring to their aglycones (Fig. 2.2a).

2.4.3.1 Characterization of glycosylation type

O-, *C*- and *C*,*O*-glycosides can be distinguished by their positive or negative ionization spectra. As reported by many authors, for *O*-glycosides, the application of low or medium fragmentation energy results in heterolytic cleavage of their hemi-acetal O–C bonds, yielding distinctive Y_i fragments [98, 104–110]. For *C*-glycosides, low fragmentation energy does not provide adequate fragmentation. At medium fragmentation energy, however, characteristic ${}^{i,j}X$ and E_i fragments result from intraglycosidic cleavages and from losses of water molecules, respectively [98, 100, 109, 111]. On the other hand, the application of higher fragmentation energy leads to intraglycosidic cleavages of the sugar units of *O*-glycosides and produces Y_i fragments for *C*-glycosides, rendering the spectral interpretation difficult, sometimes misleading. In the case of *C*,*O*-glycosides, both ${}^{i,j}X$ and Y_i ions were observed [96, 98]. The characteristic positive and negative ion fragmentation of *O*- and *C*-glycosides are illustrated in Fig. 2.7 for rutin (quercetin-3-*O*-rhamnosylglucoside) and vitexin (apigenin-6-*C*-glucoside).

2.4.3.2 Nature of the glycan part

As the majority of relevant papers report on the characterization of diglycosides, this subject is discussed primarily. Concerning the characterization of glycosides with three or more sugar residues, for the time being no sufficient information is available to draw general conclusions.

Type of the sugar unit Generally, by LC-MS^n analyses no information can be obtained about the stereochemistry of the flavonoid glycoside's glycan part. The sugar type can be, however, easily determined, since the A_i, B_i, and C_i

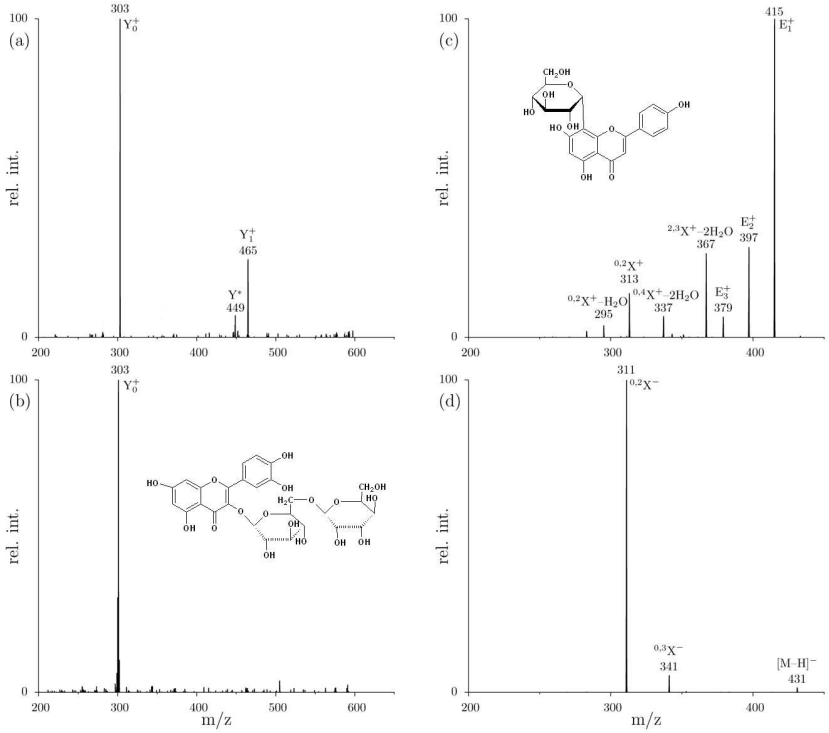


Figure 2.7: Sample spectra for *O*-, and *C*-glycosides. Product ion spectra of (a) protonated and (b) deprotonated rutin, as well as (c) protonated and (d) deprotonated vitexin. The spectra were recorded at medium fragmentation energy (35%). Other parameters were as published in [112].

Table 2.2: Mass losses characteristic of hexose, deoxyhexse, and pentose units

	Hexose	Deoxyhexose	Pentose
C-glycosides			
$^{0,1}X$	150 [b,c]	134 [b]	120 [b]
$^{0,2}X$	120 [a,b]	104 [b]	90 [a,b]
$^{0,3}X$	90 [a,c]	74	60 [a]
$^{0,4}X$	60	44	30
$^{1,5}X$	134 [b]	120 [b]	104 [b]
$^{2,3}X–2H_2O$	66 [c]	66	
$^{0,4}X–2H_2O$	96 [b,c]	80 [b]	66 [b]
$^{0,2}X–H_2O$	138 [a,b]	122 [b]	108 [b]
$^{0,2}X–2H_2O$	156 [b]	140 [b]	126 [b]
O-glycosides			
Y_i	162 [a]	146 [b]	132 [b]
Z_i	180 [a]	164 [a]	150 [a]

[a] Ref. [96] [b] Ref. [98] [c] Ref. [100]

fragments appear at different *m/z* values in the case of hexoses, deoxyhexoses and pentoses. Although these ions are usually not present in the actual spectra, their masses can be inferred from the difference of the parent ion's mass and the masses of the corresponding X_i, Y_i, and Z_i fragments, respectively [96, 98, 100, 111]. These characteristic mass losses are summarized in Tab. 2.2. For instance, if the product ion spectrum of a flavonoid *O*-glycoside comprises three characteristic fragments, which result from the losses of –146, –162, and –308 Da units, this fact indicates that the glycoside contains a deoxyhexose and a hexose unit. Similarly, if in the product ion spectrum of a *C*-glycoside the characteristic fragments were generated by the losses of –120 and –122 Da units, this indicates the presence of a hexose and a deoxyhexose unit, respectively.

Distribution of the sugar residues Flavonoid *O*-glycosides most often contain one or two sugar units, but tri- and tetraglycosides are also not uncommon [40]. By definition, two sugars can be attached to the flavonoid aglycone either at two different positions (di-*O*-glycosides, di-*C*,*O*-glycosides) or at the same position (*O*-diglycosides, *C*,*O*-diglycosides) forming a disaccharide.

To differentiate between the isomers, two basic methods have been described. On one hand, they can be distinguished by their product ion spectra, through the analysis of the Y_1, Y_0, and Z_1 ions. The presence of the Z_1 fragment implies that one sugar unit is linked to another sugar and not directly to the aglycone, therefore indicates an *O*- or *C*,*O*-diglycosyl structure [96, 98, 106]. In addition, differences were observed in the relative abundances of the Y_1^- and Y_0^- ions for the two *O*-glycosyl isomer forms. For di-*O*-glycosides, the Y_1^- ion was reported to be the base peak (100% relative abundance), and the Y_0^- of medium intensity (about 30% relative abundance), whilst in the spectra of *O*-diglycosides the Y_0^- ion exhibited the highest abundance (Fig. 2.8) [106]. Analogously, for di-*C*,*O*-glycosides, the Y_0^- observably exhibited the highest abundance, whereas in the case of *C*,*O*-diglycosides, the Y_0^- showed less than 10 % relative intensity, and the Z_1^- fragment was found to be the base peak [96]. The other method is based on the presence of characteristic radical ions. The product ion spectra of di-*O*-glycosides contained $[Y_1–H]^{-\bullet}$radical ions and a $[Y_0–2H]^-$ fragment. In the spectra of *O*-diglycosides only the $[Y_0–H]^{-\bullet}$radical ion was observed, the $[Y_1–H]^{-\bullet}$ions were missing, as it was demonstrated for flavonol glycosides (Fig. 2.9a and b) [104]. Similarly, the $[Y_1–H]^{-\bullet}$ion was typically observed upon cleavage of the glycosidic bond between the aglycone part and the *O*-linked glycan, thus only for the di-*C*,*O*-glycosyl flavone isomer [113].

Apparently, the determination of the sugar unit distribution would be easier in the case of glycosides with different sugar units. As a first approximation, the product ion spectra of di-*O*-glycosides would contain two Y_1^+ ions at different *m/z* values, whereas in the spectra of *O*-diglycosides only one Y_1^+ fragment would be observed, corresponding to the loss of the external sugar unit. In the product ion spectra of *O*-diglycosides, however, a second Y_1^+ ion was also reported – usually labeled as Y^* –, originating from the loss of the internal sugar residue making spectra interpretation misleading [108].

Glycan sequence This structural characteristic is only relevant for glycosides with different sugar units and determination of the glycan sequence seems self-explanatory [98]. For example, in the product ion spectra of *O*-diglycosides, the $Y_{1(external sugar)}$ and Y_0 fragments were expected. However, as it was demonstrated on the example of rutin (quercetin-3-*O*-rhamnosyl(1→6)glucoside), the negative ion MS/MS spectra of (1→6) diglycosides often did not show signif-

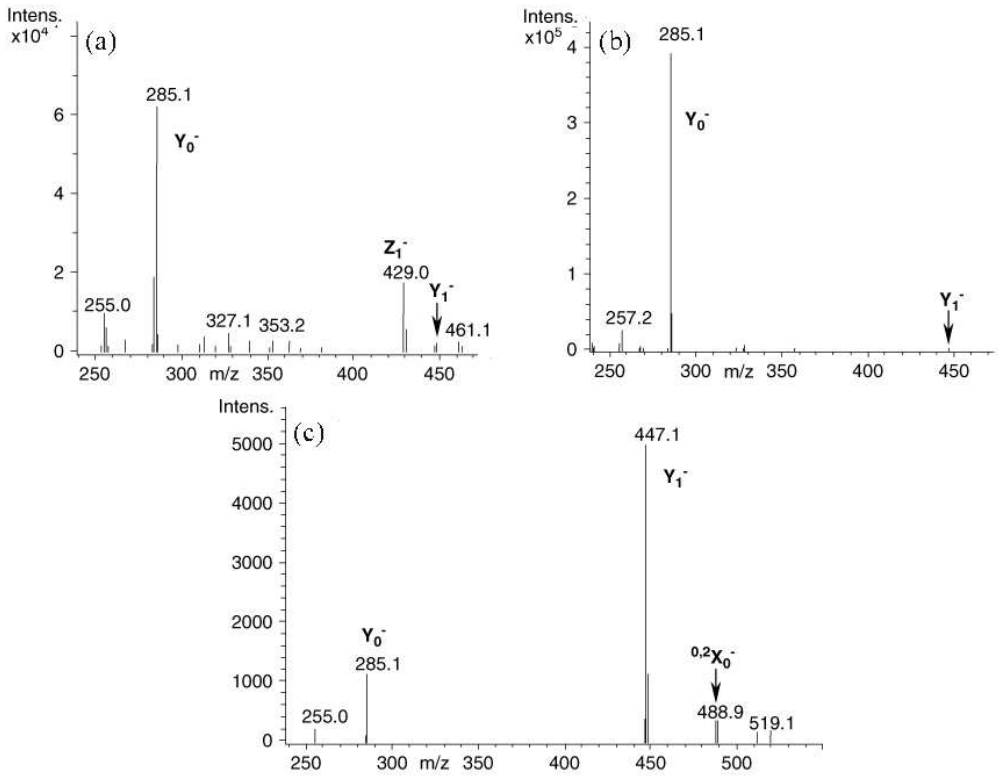


Figure 2.8: Differentiation of flavonoid disaccharides. Product ion spectra of deprotonated (a) kaempferol-3-*O*-glucosyl(1→2)glucoside, (b) kaempferol-3-*O*-glucosyl(1→6) glucoside, and (c) kaempferol-3,7-diglucoside [106].

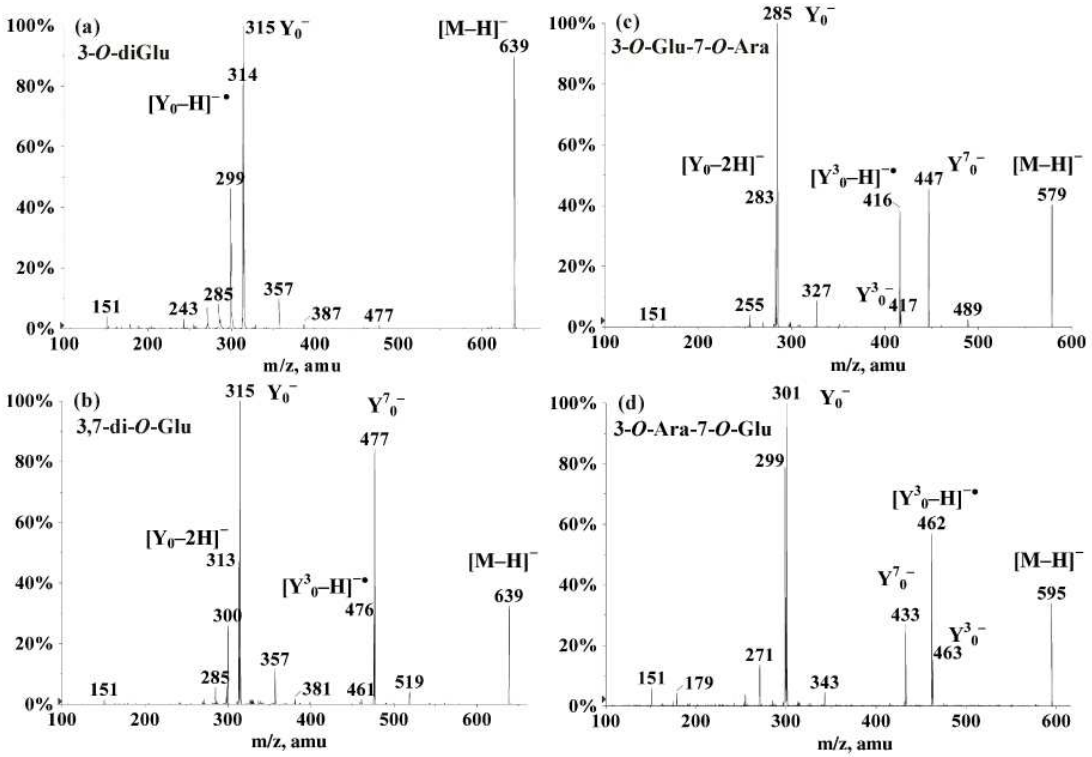


Figure 2.9: Differentiation of di-*O*-glycosides and *O*-diglycosides, as well as sugar distribution determination in case of 3,7-di-*O*-glycosides. Product ion spectra of deprotonated (a) isorhamnetin-3-*O*-glucosyl(1→6)glucoside, (b) isorhamnetin-3,7-di-*O*-glucoside, (c) kaempferol-3-*O*-glucoside-7-*O*-arabinoside, and (d) quercetin-3-*O*-arabinoside-7-*O*-glucoside [104].

icant Y_1^- fragments, while the positive product ion spectra of *O*-diglycosides exhibited both $Y_1{}^+{}_{(deoxyhexose)}$ and Y^* ions, rendering the characterization of the glycan sequence impossible (Fig. 2.7a and b). Although, up to date, no general mass spectrometry based analysis has been reported for flavonoid glycan sequencing, the necessary information can be deduced logically in special cases. For example, the 1→6 linkage between two sugar units is only possible if the deoxyhexose is the external sugar and the hexose the internal, since deoxyhexoses possess a methyl group instead of a hydroxymethyl at position 5″ [112].

Interglycosidic linkage The sugar units in flavonoid oligosaccharides connect through interglycosidic linkages. Although the glycosidic hydroxyl group (at position 1) can produce glycosidic bonds theoretically with four hydroxyl groups (attached to C-2, C-3, C-4, and C-6) of the other sugar unit, the 1→2 and 1→6 linkages are preferred [27, 107].

Regarding the determination of linkage type for *O*-diglycosides, it has been reported that the relative abundances of the $Y_1{}^+$ and $Y_0{}^+$ ions could be used as indicator. Ma et al. [107, 108] found that $Y_0{}^+ > Y_1{}^+$ was indicative of 1→2 linkages, while $Y_0{}^+ < Y_1{}^+$ suggested 1→6 connections. However, others reported contradictory data possibly suggesting the influence of the actual experimental conditions [105]. Nonetheless, the $Y_0{}^+/Y_1{}^+$ ratio was always observably higher for 1→2 linked diglycosides than of in 1→6 linked isomers [105, 107, 108]. On the other hand, the differences observed in negative ion fragmentation of connectional isomers can serve as the bases for their differentiation. In the negative ion mode, the Y_0^- fragment was always the base peak, and the Y_1^- ion exhibited higher abundance for 1→2 linkages than in the case of 1→6 connected glycosides [104–106]. In addition, the presence of the Z_1^- ion [106] and more pronounced glycan fragmentation [105] was reported to be characteristic for the 1→2 linked isomers only.

For 1→2 linked *C*,*O*-diglycosides the internal cleavage of the *C*-glycosyl moiety yields ${}^{0,2}X^-$ fragments, while in all the other cases (1→3, 1→4 or 1→6 linkages), it results in the loss of the *O*-glycosyl sugar unit and a part of the *C*-glycosyl moiety, yielding $[Y_1–{}^{0,2}X]^-$ ions. However, the ${}^{0,2}X_0{}^-$ ion is easily confused with the ${}^{0,2}X_1{}^-$ fragment, which results from the internal cleavage of the *O*-glycosyl moiety and may also be present in the spectra. Therefore, the MS^3 spectrum of the ${}^{0,2}X_0{}^-$ ion has to be analyzed as well. The loss of the

O-glycosyl unit (the presence of Y_0^- confirms the 1→2 interglycosidic linkage [96].

2.4.3.3 Position of glycosylation

Theoretically, the glycan residue can be attached to any of the aglycone's hydroxyl groups, but certain positions are apparently favored, such as the 7-hydroxyl group for flavones, flavonols, flavanones, and isoflavones and positions C-3 flavonols (Fig. 2.3) [38–40].

Differentiation of positional flavonol glycoside isomers can be based on the analysis of distinctive fragment ions in their negative product ion spectra [104]. The $[Y_0–CO]^-$ ion was observed only for 7-*O*-monoglycosyl flavonols, while the $[Y_0\text{-}2H\text{-}CO]^-$ fragment was described to be distinctive for the 3-*O*-monoglycosyl isomers. In the case of flavonol-3,7-di-*O*-glycosides with different sugar units, the glycosylation position can easily be determined, since only the loss of the 3-*O*-glycosyl moiety yields the $[Y_1–H]^{-\bullet}$radical ion (Fig. 2.9c and d). In another approach, significant differences were reported in the relative intensities of the Y_1^+ ions, i.e., $Y_{1(3)}^+$ ions were always more intensive than the $Y_{1(7)}^+$ ions [114]. Moreover, unambiguous information could be obtained about the glycosylation position from the high-energy $[M+Na]^+$ spectra of the compounds of interest (Fig. 2.10) [113]. The presence of a B-ring fragment containing the sugar residue clearly indicates 4'-*O*-glycosylation. The subsequent loss of the B-ring part from the aglycone product ion pointed to 3-*O*-glycosylation, while the occurrence of a B-ring loss from both the aglycone and the $[M+Na]^+$ ions is characteristic of 7-*O*-glycosylation.

Up until today, *C*-glycosylation has almost exclusively been found at positions 6 or 8, and only in two cases at position 3 [42]. Waridel et al. [100] described that the positive ion MS/MS spectra of mono-*C*-glycosyl isomer pairs – such as apigenin-8/6-*C*-glucoside (vitexin/isovitexin) and luteolin-8/6-*C*-glucoside (orientin/isoorientin) – varied only in the relative intensities of the water losses (E_i^+ ions) (Fig. 2.11a and b). Consequently, for the 8-*C*-glycosyl isomers, the E_1^+ ion was observed as base peak, while in the product ion spectra of the 6-*C*-glycosyl isomers, the $^{2,3}X^+–2H_2O$ fragment exhibited the highest intensity. In addition, the same authors reported that in the negative ion MS/MS spectra the E_i^- fragments were observed only for the 6-*C*-glycosyl positional isomer [100]. On the other hand, in their FAB experiments Becchi and Fraisse [111] de-

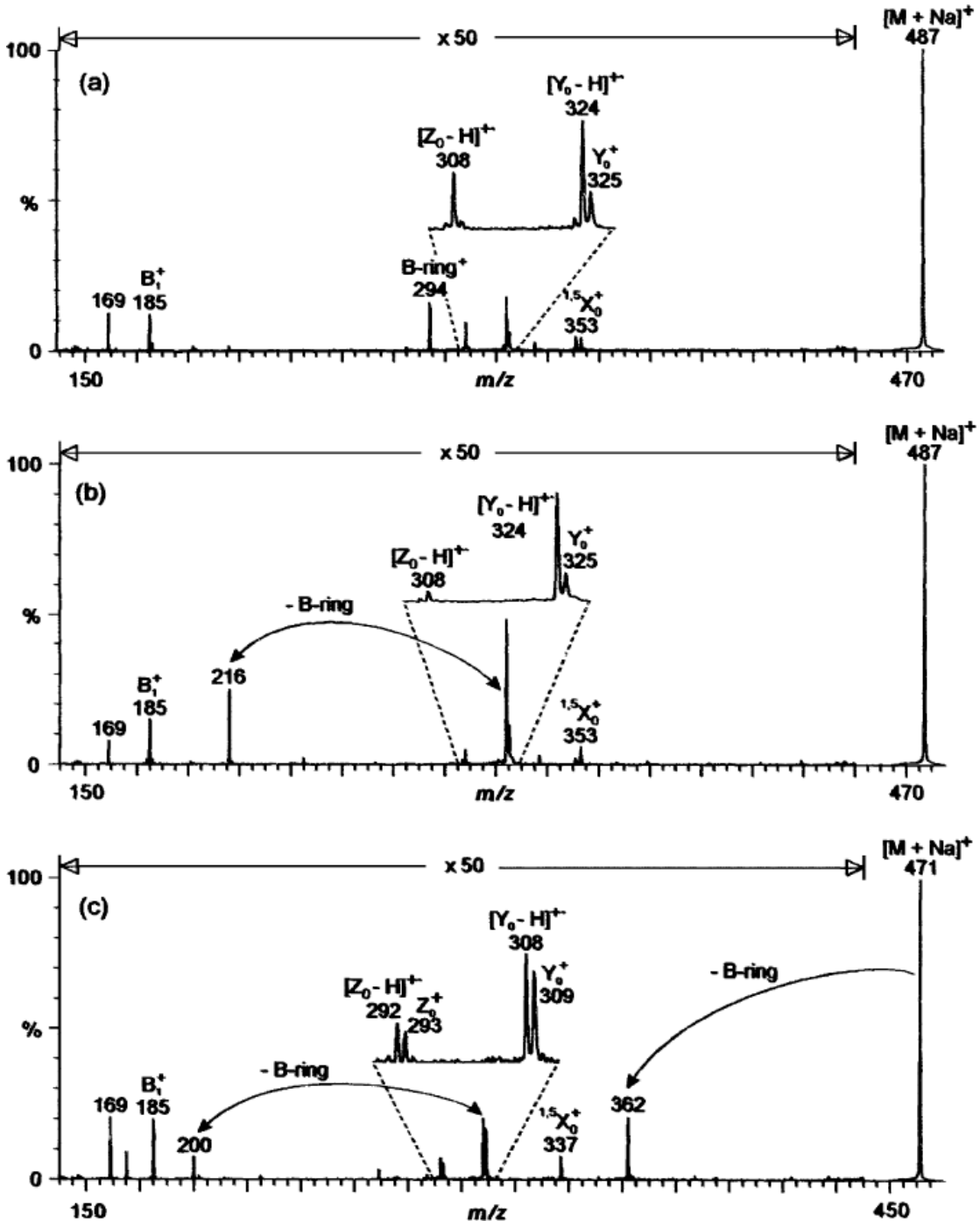


Figure 2.10: Differentiation of positional isomers. (a) quercetin-4′-*O*-glucoside, (b) quercetin-3-*O*-glucoside and (c) luteolin-7-*O*-glucoside. All product ions contained sodium (not indicated in the spectra) [113].

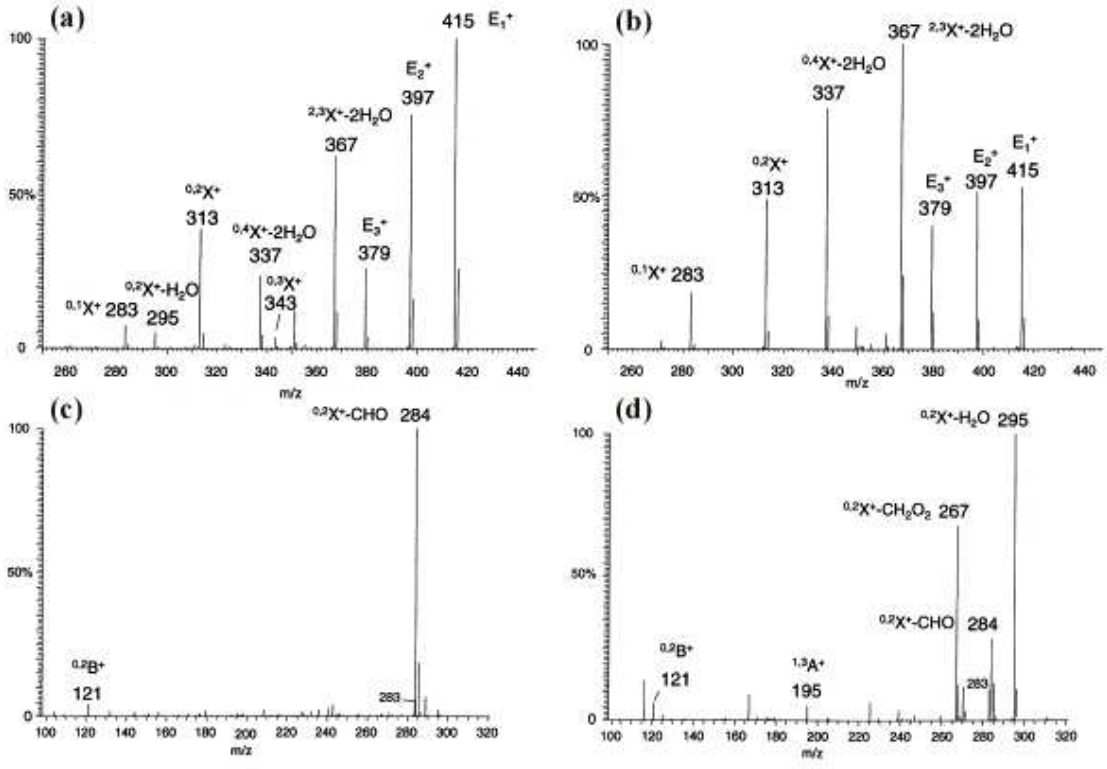


Figure 2.11: Differentiation of 6-*C*- and 8-*C*-glycosides. Product ion spectra of protonated (a) vitexin and (b) isovitexin, as well as the MS^3 spectra of the $^{0,2}X^+$ fragment for (c) vitexin and (d) isovitexin [100].

scribed the E_1^- ion as being characteristic also for the 8-C isomer vitexin. Similarly, the same authors reported higher abundance of the $^{0,3}X^-$ ion for the 6-C isomers than for the 8-*C*-glycosides [111]. Moreover, different small molecule loss patterns were observed in the positive ion MS^3 spectra of the $^{0,2}X^+$ fragments for 6-*C*- and 8-*C*-glycosyl isomers. Using the same fragmentation energy, more pronounced fragmentation occurred in case of C-6 glycosides, resulting in $^{0,2}X^+$–H_2O, $^{0,2}X^+$–CHO, $^{0,2}X^+$–H_2O–CO fragments, whereas in the spectra of the 8-*C*-glycosyl isomers only the $^{0,2}X^+$–CHO fragment was present (Fig. 2.11c and d). This observation was also confirmed by Vukics et al. [112]. In addition, the $^{1,3}A^+$and $^{1,3}A^+$–CO fragments were exclusively present in the positive MS^3 spectra of the $^{0,2}X^+$ ion for the 6-C isomers, such as swertiajaponin (7-methoxy-luteolin-6-*C*-glucoside), isoorientin and isovitexin [115, 116].

On the other hand, in the product ion spectra of hexose and pentose1 containing asymmetrical di-*C*-glycosides, such as schaftoside (apigenin-6-*C*-glucoside-8-*C*-arabinoside) and isoschaftoside (apigenin-6-*C*-arabinoside-8-*C*-glucos-

ide) more extensive fragmentation was found for the C-6 sugar moiety. Consequently, fragments $^{0,2}X^{+}_{P}$, $^{1,5}X^{+}_{P}$, $^{0,2}X^{+}_{H}$–H_2O and $^{0,2}X^{+}_{H}$–$2H_2O$ appeared only for the C-6 pentose isomer, while $^{0,4}X^{+}_{H}$–$2H_2O$, $^{0,2}X^{+}_{P}$–H_2O, and $^{0,1}X^{+}_{H}$ were observed only for the C-8 pentose variant [98, 112]. Please note that the product ion spectra of hexose and deoxyhexose containing di-*C*-glycosides did not exhibit the same differences [112].

On the subject of the differentiation of positional *C*,*O*-glycosyl isomers, only scarce information is available. Examining 53 flavonoid glycoside isomers, Ferreres et al. [96] observed that in the negative ion MS^n spectra a certain fragment ratio was always higher for the C-6 isomers. These fragments were very similar in structure to flavonoid aglycones (Ag), namely, $[Ag+41]^-$ and $[Ag+71]^-$ for mono-*C*-glycosides and $[Ag+83]^-$ and $[Ag+113]^-$ for di-*C*-glycosides.

2.4.3.4 Nature of the aglycone

Identification of the aglycone part of flavonoid *O*-glycosides can be achieved by thorough analyses of the MS^3 spectra obtained for the Y_0 fragment, or by their comparison (i) with the corresponding spectra of reference molecules, (ii) or with literature data [92, 94, 99, 101, 117–121]. In case of *C*-glycosides, the lack of an abundant Y_0 fragment renders the direct comparison of spectra impossible. Still, a thorough analysis of the MS^3 spectra of $^{i,j}X$ or $^{i,j}X$–H_2O fragments, which are similar in structure to the aglycone, can provide sufficient information for the identification. If the instrumentation does not allow the registration of MS^3 spectra, application of higher fragmentation energy is recommended, as then the fragment ions characteristic of the aglycone may appear in the MS/MS spectra [109].

The basic structure of the aglycone part, including the number and nature of substituents, can be assumed from the molecular mass corresponding to the aglycone. For example, for apigenin-7-*O*-glucoside, the molecular mass of MW = 270 can be calculated from the *m/z* value for the Y_0 fragment either in the positive or in the negative ion mode. This fact suggests that it possesses a 2,3 double bond and three hydroxyl substituents connected to a flavone, flavonol or isoflavone skeleton, as no other relevant combination of basic skeletons and substituents adds up the same molecular weight. The structures of flavone and isoflavone isomers differ only in the connection points of the B-ring, they have the same molecular mass. However, as far as their differentiation is concerned,

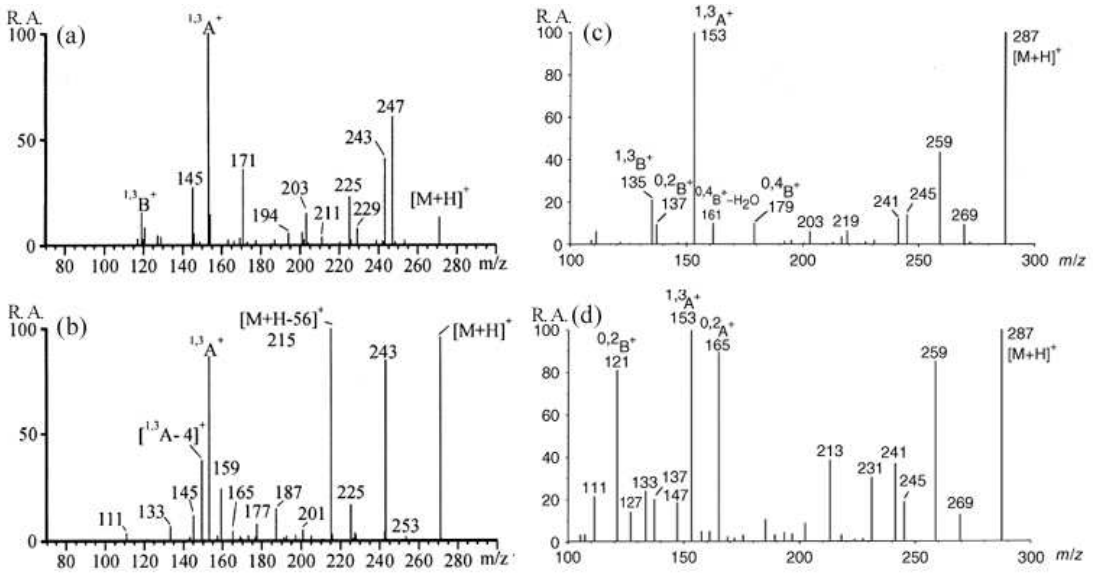


Figure 2.12: Differentiation of flavone, isoflavone and flavonol isomers. Product ion spectra of the protonated (a) flavone apigenin, (b) flavonol genistein, (c) flavone luteolin, and (d) flavonol kaempferol [94, 122].

only contradictory information have been published in the literature. De Rijke et al. [99] compared the fragmentation of isoflavones and flavones, and found that the MS/MS spectra recorded on IT and QQQ instruments showed the same fragmentation behavior for flavones and isoflavones. On the other hand, Prasain at el. [41] and Hughes et al. [118] presented mass spectra of the isoflavone genistein and the flavone apigenin, isomers of the same molecular mass, and reported ways of differentiation. According to their results, in the negative ion MS/MS spectra of apigenin, characteristic fragment ions could be observed at *m/z* 117 and 151, while for genistein a distinctive fragment at *m/z* 133 was detected. Based on the analysis of seven flavone-isoflavone isomer pairs, Kuhn et al. [122] could not confirm these findings, and proposed the loss of a 56 Da unit to be characteristic for isoflavones (Fig. 2.12a and b). Note that as it will be discussed later in this section, this loss was also observed for flavonols and prenyl flavonoids.

The fact that the aglycone fragmentation pathways vary with the substitution pattern of the molecules allows differentiation of flavone and flavonol isomers of the same molecular mass. For example, the presence of a hydroxyl group at position 3 of flavonols results in different fragmentation compared with flavones [94, 101, 117]. Albeit the $^{1,3}A^+$ and $^{0,2}B^+$ions show high abun-

dance for both compound groups, the corresponding $^{1,3}B^+$ion was detected only for flavones, whilst for flavonols the $^{1,3}B^+$–2H ion was observed. Besides, Ma et al. [94] reported the $^{0,4}B^+$ and $^{0,4}B^+$–H_2O ions distinctive for flavones and the $^{0,2}A^+$ fragment characteristic for flavonols. Similarly, according to the study of Fabre et al. [117] the negative ion mode fragmentation of flavonols and flavones significantly differ. While for flavonols they observed $^{1,2}B^-$and $^{1,2}A^-$ fragments, for flavones $^{1,3}B^-$ and $^{1,3}A^-$ ions were found to be characteristic. As the example of the flavone luteolin and the flavonol kaempferol demonstrates (Fig. 2.12c and d), flavones and flavonols of the same molecular mass can easily be distinguished with the help of the above described $^{i,j}A$ and $^{i,j}B$ ions.

The same conclusions could be drawn for *C*-glycosides from the analysis of the MS^3 spectra of their $^{i,j}X$ or $^{i,j}X$–H_2O fragments. Albeit the *m/z* values for the $^{i,j}B$ ions are the same as for the *O*-glycosides, the molecular masses of the $^{i,j}A$ fragments have to be recalculated in each cases, since part of the sugar moiety remains attached to the aglycone (Fig. 2.13).

Besides of the major fragmentation pathways, which yield the $^{i,j}A$ and $^{i,j}B$ ions, small molecule and/or radical losses were reported for flavonoid aglycones both in the positive and negative ion modes [92, 94, 117, 119, 120]. Some of them, such as the losses of H_2O (18 Da), CO (28 Da), C_2H_2O (42 Da), CO_2 (44 Da) are less characteristic, and their significance to differentiate flavonoid classes is not reliably verified. For example, the loss of a 56 Da unit (two CO groups) has been suggested to be distinctive for isoflavones [121, 123], but the same fragment has been observed for flavonols [94, 118], and the loss of 56 Da (a C_4H_8 group) could point also to the presence of a prenyl substituent [124]. On the other hand, the loss of a 15 Da unit ($CH_3^\bullet$) is an unambiguous indicator of methoxy groups, which is generally accompanied by the losses of CO (28 Da) and $HCO^\bullet$(29 Da) [119, 120, 125]. Albeit there is no general method to determine the exact location of the methoxy substituent, one can define if the methoxy group is connected to the A- or the B-ring based on the masses of the A-ring fragments [120].

Furthermore, the fact that the fragmentation of the glycan part can vary with the nature of the aglycone may yield additional/supporting information. Reportedly, the irregular Y^* ion, which results from the internal sugar unit loss of *O*-diglycosides, is more pronounced for flavanones, show low abundance for flavonols, and has not been observed for flavones [105, 107, 108]. On the other

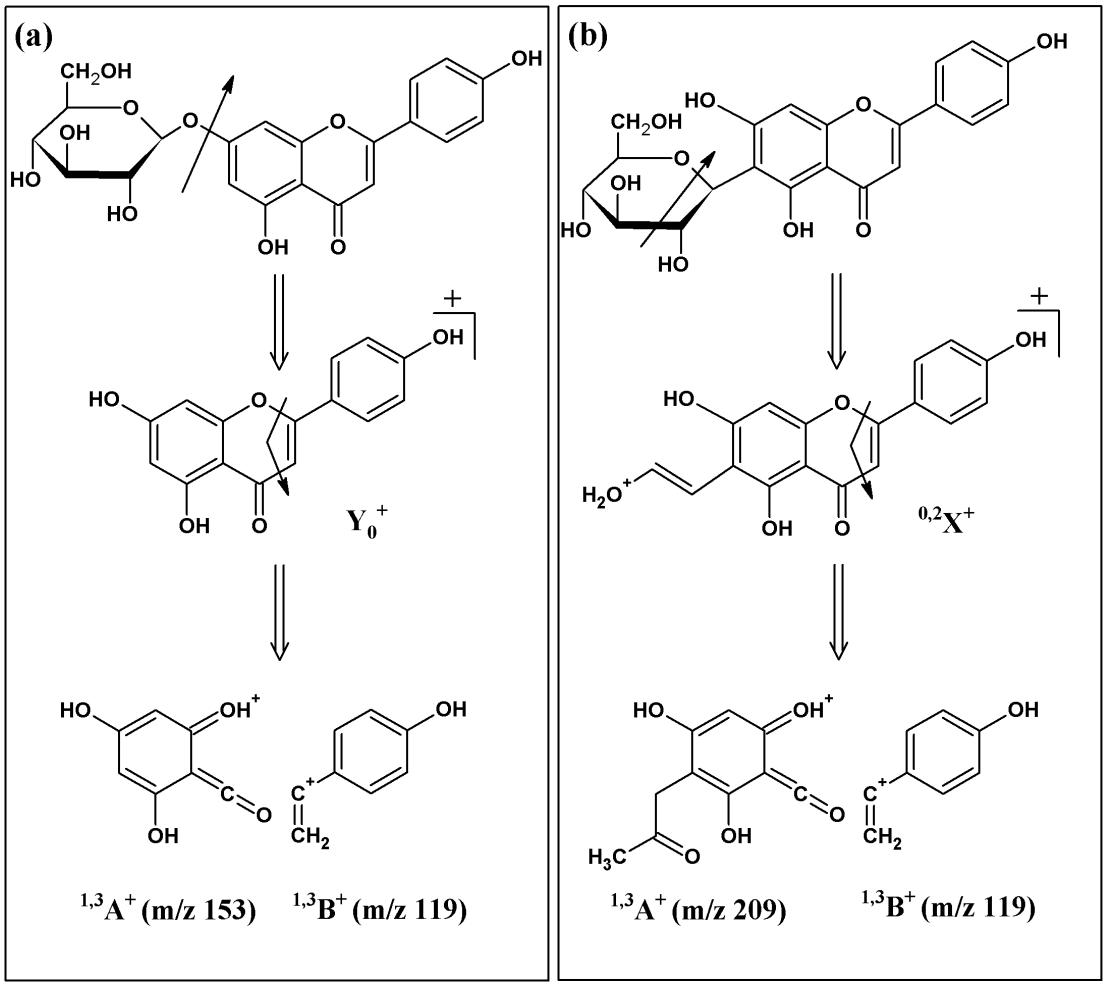


Figure 2.13: The structure and origin of the $^{1,3}A^+$ fragment in case of *O*- and *C*-glycosides. (a) apigenin-7-*O*-glucoside; (b) isovitexin (apigenin-6-*C*-glucoside). The $^{1,3}A^+$ fragment appears at different *m/z* values for *O*- and *C*-glycosides, since in the case of *C*-glycosides it contains a part of the sugar moiety.

hand, contradictory conclusions were drawn about the role of the $(Y_0\text{-H})^{-\bullet}$ion in the differentiation of flavonoid classes. As the 2,3-double bond adjacent to the carbonyl group in position 4 was considered to be essential for its stabilization, the presence of the $(Y_i\text{-H})^{-\bullet}$ion was thought to be opposing to the flavanone structure [126]. However, this ion was later observed in the product ion spectra of the flavanones hesperetin and naringenin [113].

2.4.3.5 Sample derivatization and complex formation methods

At the early days of MS based analysis of flavonoids, derivatization (such as methylation, trimethylsilylation, or acetylation) was inevitable, since electron impact and chemical ionization needed the analyte to be in the gas phase. In addition, only limited structural information could be obtained about the derivatized flavonoid glycosides [127–129], and this methods often lead to difficulties with respect to interpretation of the fragmentation patterns, and required a time-consuming sample preparation step. Another disadvantage of the method was the problem of side reactions [130]. With the introduction of more modern ionization techniques, the analysis of flavonoid glycosides became possible without derivatization, and now it is applied only rarely to obtain additional or complementary information.

Acetylation, for example, can still be useful in the structural characterization of glycoside residues and reportedly allowed differentiation between terminal glucose, galactose and mannose residues of naturally occurring glycosides [131]. Following the same approach, Cuyckens et al. [132] developed a methodology to differentiate and characterize terminal monosaccharide residues of flavonoid *O*-glycosides. According to their FAB and ESI data, a thorough study of some characteristic fragment intensities provided means of differentiation for hexose and pentose isomers. On the other hand, it was demonstrated that the fragmentation behavior of permethylated flavonoid glycosides (the experiments did not include flavanones) depended on the glycosidic linkage between the sugars units [133]. Contrary to non-derivatized glycosides, the Y^* ion was observed for both flavones and flavonols, but the rearrangement yielding the Y^* fragment occurred only for 1→6 linked disaccharides.

Complexation also proved to provide complementary information to structure elucidation. Studies have suggested that certain structural features are required for metal chelation of flavonoids. Proposed chelation sites include the

4-keto and 5-OH groups, the 4-keto and 3-OH groups, and the 3'-OH and 4'-OH groups. Positional flavonoid glycoside isomers, which possess the above chelation sites could be differentiated based on the different fragmentation of their Ca(II) or Mg(II) complexes [134]. Reportedly, the C-7 glycosylated isomer exhibited the most pronounced fragmentation and $^{0,2}X^{+}$ as distinctive fragment. The C-4' isomer implied characteristic loss of the aglycone portion, while the C-3 isomer showed only the loss of a sugar moiety. This loss, however, was typical for the other isomers as well. Based on this approach 6- and 8-*C*-glycosides could also be distinguished. Moreover, the formation of Co(II) complexes with the use of 4,7-diphenyl-1,10-phenanthroline as a new auxiliary chelating ligand, provided means of determination for the glycosidic linkage type, since the loss of the aglycone plus rhamnose unit was observed only for the 1→2 isomer rhoifolin (apigenin-3-*O*-rhamnosyl(1→2)glucoside) [135]. Similarly, additional diagnostic fragmentation paths were reported for the 1→2 isomers in the case of aluminum complexes [136].

2.4.3.6 MS analysis of anthocyanins

According to the few mass spectrometric analysis reports in the literature, anthocyanidins (the aglycones) have similar fragmentation characteristics as the above discussed flavonoid classes. Their positive ion MS/MS spectra show fragments resulted from small molecule losses and C-ring cleavages reported by Oliveira et al. [137]. This paper clearly explains, how the number and nature of substituents, connected to the basic anthocyanidin skeleton, can be determined from the molecular masses of the characteristic $^{0,2}B^{+}$, $^{0,2}B^{+}$–CO, $^{0,3}A^{+}$, $^{0,2}A^{+\bullet}$fragments. Analysis of the anthocyanins (the glycosides) demonstrated that they also produced the above discussed $^{i,j}X^{+}$ and $Y_i{}^{+}$ fragments [137–141]. In addition, the loss of a 15 Da unit ($CH_3^{\bullet}$) was reported to be distinctive for methoxy group containing anthocyanidins [137, 140].

CHAPTER 3

Material and methods

3.1 Chemicals

Isorhamnetin, isovitexin, quercetin, rutin, vitexin, and all solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol was from Fluka (Buchs, Switzerland) and luteolin from Extrasynthese (Genay, France). Apigenin was from Bionorica Ltd. (Neumarkt, Germany).

3.2 Plant materials

For the scouting analyses heartsease herb (*Viola tricolor* L., Violaceae) was obtained from three different sources: (i) collected and identified in Transilvania by Prof. Kalman Csedo (Babes-Bolyai University), (ii) Fitopharma Ltd. (Budapest, Hungary) (SN=28-56-05-VI/24), (iii) MDR 2000 Ltd. (Godollo, Hungary). In the further analyses, the sample from Fitopharma Ltd. was used.

Garden pansies (*Viola* x *wittrockiana* Gams., Violaceae) were cultivated in Nagyrecse (Hungary). They were selected by petal color: violet, violet-white, white and yellow, and collected as herbs, flowers and leaves.

Ginkgo folium (*Ginkgo biloba* L.) was collected in the botanical garden of the Roland Eotvos University, Budapest and identified together with the pansy cultivars in the Department of Pharmacognosy.

3.3 Extraction methods

Method 1 5.0 g dried and freshly powdered heartsease herb was subject of consecutive Soxhlet extraction with (i) n-hexane, (ii) dichloromethane, (iii) ethyl

acetate, and (iv) 70% methanol. The extracts were evaporated to dryness under reduced pressure, and re-dissolved in chloroform or 100% methanol.

Method 2 5.0 g dried and freshly powdered heartsease herb was sonicated two times with 50 mL chloroform for 25 minutes in an ultrasonic bath at 30 °C. The chloroform extract, referred to as *Fraction A* (Fig. 4.1) was evaporated to dryness under reduced pressure at 30 °C. The plant residue was dried at room temperature and re-extracted two times with 40 mL methanol for 15 minutes in an ultrasonic bath at 30 °C. The methanol extract referred to as *Fraction B* (Fig. 4.1) was evaporated to dryness under reduced pressure at 40 °C.

Method 3 1.25 g dried and freshly powdered heartsease plant material was subject of continuous Soxhlet extraction with 100 mL methanol for 9 h (exhaustive extraction). The extract was evaporated to dryness under reduced pressure at 55 °C, weighed and re-dissolved in 25 mL methanol (stock solution). 1.5 mL 0.085% phosphoric acid was added to 3.5 mL of the extract (diluted stock solution) and filtered on a MINIsart RC-15, 0.20μm membrane syringe filter (Sigma-Aldrich). The filtered solution was ready to be analyzed.

Method 4 0.50 g air-dried plant material was sonicated with 20 mL 70% methanol for 20 minutes in an ultrasonic bath at room temperature. The filtered extract was evaporated to dryness in vacuum at 60 °C. The dry residue was re-dissolved in 1.5 mL 70% methanol and separated by solid-phase extraction (Sec. 3.4).

3.4 Solid-phase extraction

Samples for SPE were prepared so as to be equivalent of 20% methanol. The SPE cartridge (Supelclean LC-18, 500 mg / 3 mL, Sigma-Aldrich) was activated with 3 mL methanol followed by 3 mL 2% acetic acid. After sample introduction, the cartridge was washed with 1.5 mL of 70% methanol. The loading and washing solvents were collected and combined for downstream analysis.

3.5 Conventional open column chromatography

Column 1a and b Separation of *Fraction B* (Fig. 4.1) by Sephadex LH-20 column chromatography. The stationary phase bed was prepared by equilibrating Sephadex LH-20 beads for at least 24 hours in 50% methanol. After transferring the slurry to the column (glass, homemade, 35 x 1.5 cm) the bed (final geometry: 28 cm times 1.5 cm) was allowed to settle. 0.2 g dry *Fraction B* was dissolved in 2 mL 50% methanol and introduced to the column. Elution was carried out at a flow rate of 1.0 mL/min with 21 mL 50% methanol then 10 mL 70% methanol and 100% methanol until no more component were detectable.

In case of Column 1a the eluate was collected as follows: Fraction 1 = 8 mL, Fraction 2 = 7 mL, Fraction 3 = until the column became clear. In the following sections, Fraction 2 will be referred to as *Fraction C* and the Fraction 3 as *Fraction D*.

In case of Column 1b the eluate was collected as follows: Fraction 1 = 8 mL, Fraction 2 = 7 mL, Fraction 3 = 3 mL, Fraction 4 = 8 mL, Fraction 5 = 14 mL, Fraction 6 = 2 mL. Again, as above, Fraction 2 will be referred to as *Fraction C*, Fraction 4 as *Fraction E* and Fraction 6 as *Fraction F*. Fractions 3 and 7 were combined and referred to as *Fraction G*.

Column 2 Separation of *Fraction B* by polyamide column chromatography. For the stationary phase we made a suspension of polyamide (Sigma-Aldrich, particle size = 50-160 μm) in water. After transferring the slurry to a homemade glass column (35 x 1.5 cm) the bed (final geometry: 31 cm times 1.5 cm) was allowed to settle. 0.2 g dry *Fraction B* was dissolved in 2 mL 50% methanol and introduced to the column. Elution was carried out at a flow rate of 1.25 mL/min with 40 mL water, followed by 80 mL 10% methanol, 45 mL 20% methanol, 40 mL 30% methanol, 20 mL 40% methanol, 20 mL 50% methanol, 20 mL 60% methanol, 10 mL 70% methanol, 10 mL 80% methanol, 10 mL 90% methanol and finally by 100% methanol until no more component were eluted.

The eluate was collected as follows: Fraction 0 = 76 mL, Fraction 1 = 20 mL, Fraction 2 = 24 mL, Fraction 3 = 24 mL, Fraction 4 = 8 mL, Fraction 5 = 32 mL, Fraction 6 = 20 mL, Fraction 7 = 16 mL, Fraction 8 = 32 mL, Fraction 9 = 32 mL, Fraction 10 = 56 mL. Fraction 3 will be referred to as *Fraction H*, Fraction 8 as *Fraction I* and Fraction 10 as *Fraction J*.

3.6 Thin layer chromatography

Sample solutions were spotted on 0,2 mm Kieselgel 60 F_{254} fluorescent silica-gel plates. As listed below, in each method different developing mixtures and color reagents were used. Plates were read attended and/or unattended in visible and/or UV (λ = 254 and 365 nm) light.

TLC Method 1 EtAc(100)/cc. CH_3COOH(11)/H_2O(26)/HCOOH(11); Naturstoff reagent

TLC Method 2 $CHCl_3$(150)/acetone(33)/HCOOH(17); Naturstoff reagent

TLC Method 3 n-hexane(2)/EtAc(1); $CeSO_4$, 100 °C, 10 min

3.7 High performance liquid chromatography

The ABLE-E & Jasco HPLC (Tokyo, Japan) apparatus consisted of an ERC-3113 degasser, an LG-980-02 solvent mixer, a PU-980 pump and a 20 μL Rheodyne 7725 injector. The instrument was equipped with a type UV-975 UV-VIS detector. UV spectra were recorded during the HPLC separation by manually setting the recording time.

System 1 For the separation, gradient elution from 13% to 18% ACN in 20 minutes (A = 0.5% H_3PO_4) was performed at a flow rate of 1.5 mL/min on a Hypersil ODS (250 x 4.6 mm, 5μm (Sigma-Aldrich) column. Before injection, each sample was filtered on an Acrodisc PVDF 0.20 μm membrane Sartorius syringe filter (Sigma-Aldrich). The eluate was monitored at 340 nm.

System 2 A Supelcosil TM LC-18 (250 x 4.6 mm, 5 μm, Sigma-Aldrich) column was applied during the experiments. Isocratic elution with 20% ACN and 80% phosphoric acid (0.085%, pH=2.2) was performed at a flow rate of 0.8 mL/min. The eluate was monitored at 340 nm.

System 3 For the separation, gradient elution from 13% to 18% acetonitrile in 20 minutes (A = 2.5% acetic acid) was performed at a flow rate of 1.0 mL/min

on a Hypersil ODS (250 x 4.6memm, 5μm, Sigma-Aldrich) column. Before injection, each sample was purified by SPE. The eluate was monitored at 340 nm.

3.8 Liquid chromatography mass spectrometry

Instrumentation 1 Experiments were performed on an Agilent Technologies (Waldbronn, Germany) 1100 HPLC/MSD SL system which consisted of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the LC separation, gradient elution from 10% to 40% ACN in 30 minutes (A = 2.5% CH_3COOH) was performed at a flow rate of 0.5memL/min on a Hypersil ODS (250 x 4.6 mm, 5 μm Sigma-Aldrich) column. The eluate was monitored at UV 340 nm and by the mass selective detector. Scanning was performed from m/z 100 to 1000 in 0.2 minute intervals. The mass selective detector was equipped with a normal-flow electrospray ionization (ESI) source. The electrospray conditions were as follows: drying gas flow: 13 L/min, drying gas temperature: 350 °C, nebulizer pressure: 35 psi, capillary voltage: 3000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

Instrumentation 2 All analyses were performed on a μLC system (LC Packings, Amsterdam, The Netherlands) coupled to linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific Inc., Waltham, MA). The LC device consisted of the Ultimate μ HPLC pump and column oven, the Switchos μ column-switching device with loading pump and two 10-port valves and the FAMOS μ-Autosampler (LC Packings, Amsterdam, Netherlands). A monolithic poly(p-methylstyrene-co-1,2-bis(p-vinylphenyl)ethane) capillary column was used for separation (260 x 0.2memm) [142]. μ-HPLC of flavonoids was carried out employing reversed phase conditions using 0.1% formic acid in water and 0.1% formic acid in acetonitril as solvents A and B, respectively, at a flow rate of 1 μL/min and 35 °C. Separation was performed with a linear gradient (in 50 min from 0% B to 10% B). Hyphenation to the mass spectrometer was carried out by a nanoflow electrospray ionization source from Proxeon (Odense,Denmark) with Pico Tips (FS360-20-10) from New Objective (Woburn MA, USA). Mass spectrometric data were obtained on the linear ion trap LTQ from Thermo Fisher (Thermo Fisher). Measurements in the positive ion mode were performed as

follows: source voltage 1.7 kV, capillary temperature 220 °C, capillary voltage 41 V, tube lens 115 V; in the negative mode: source voltage 1.3 kV, capillary temperature 220 °C, capillary voltage -50 V, tube lens -152 V . Data acquisition and interpretation was done with the Xcalibur software from Thermo Fisher.

3.9 NMR spectroscopy

18 mg *Fraction E* was dissolved in 1 ml DMSO-d6 and characterized on a two-channel Varian Inova 600 MHz (Palo Alto, CA) NMR spectrometer equipped with a waveform generator, a pulsed field gradient (PFG) unit and a dual inverse broad-band probe-head. In addition to registering the ^{1}H-NMR and ^{13}C-NMR spectra standard 2D experiments (COSY, TOCSY, NOESY, HSQC, HMBC) and selective 1D TOCSY-TOCSY and TOCSY-NOESY experiments were applied for assignment. All spectra were calibrated to internal TMS (tetra-methylsilane).

3.10 Anthocyanidin and flavonoid content

The antocyanidin and flavonoid content of the dried samples were determined by applying the methods of the European Pharmacopoeia 5.0, paragraph 'Bilberry fruit, fresh' (*Myrtilli fructus recens*) and 'Wild pansy' (*Violae tricoloris herba*) [143].

3.11 In vitro antioxidant assays

TEAC assay ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in water in 7 mM concentration. Its radical monocation was produced by reacting the ABTS solution with 2.45 mM (final concentration) potassium persulfate and letting the mixture stand in dark at room temperature for at least 12 h before use. The $ABTS^{\bullet+}$ stock solution was diluted with spectroscopic grade ethanol to 0.9 absorbance unit at 734 nm. At least four different volumes of the diluted sample were added to 2.5 ml ABTS solution resulting in different final concentrations and producing inhibition of the blank solvent between 20% - 80%. Absorbance values were measured at 734 nm after 0, 0.5; 0.66; 0.83;

1; 1.5; 2; 2.5; 3; 4; 5 and 6 minutes. For the determination of the so-called inhibition percentage, we extrapolated the final absorbance by numerically solving the simplest possible reaction kinetics model. The inhibition percentage produced by a given sample concentration was calculated as (100-A_t/A_0)*100 (A_t: the extrapolated final absorbance, A_0: the absorbance of the blank solvent). The antioxidant activity was characterized by plotting the inhibition percentage of the samples as a function of sample concentration followed by linear regression (data not shown). The concentration resulting in 50% inhibition is referred to as IC_{50} value.

DPPH assay DPPH$^\bullet$was dissolved in HPLC grade methanol in 0.25 g/L concentration. The DPPH$^\bullet$stock solution was diluted with HPLC grade methanol to 0.9 absorbance unit at 515 nm. At least four different volumes of the diluted sample were added to 2.5 ml DPPH$^\bullet$solution resulting in different final concentrations and producing inhibition of the blank solvent between 20% - 80%. Absorbance values were measured at 515 nm after 0, 0.5, 0.66, 0.83, 1, 1.5, 2, 2.5, 3, 4, 5, 6 minutes. The inhibition percentage was calculated as for the TEAC assay.

3.12 Fortified sample recovery test

1.2 mL of diluted stock solution (prepared according to Method 3) was further diluted with 0.8 mL 70 % methanol. This aliquot served as a blank solution in the fortified sample recovery test. Another 1.2 mL aliquot of the stock solution was diluted with 0.8 mL rutin solution (267 μg/mL, 70% MeOH), corresponding to the addition of 213.6 μg rutin. The recovery (R) was calculated as R = 100(m_{found}-$m_{initial}$)/m_{added}; (m_{found}: rutin content in the fortified sample; $m_{initial}$: rutin content in the blank sample; m_{added}: added rutin amount).

CHAPTER 4

Results and discussion

4.1 Scouting analyses

4.1.1 Basic extraction method

As the traditional internal administration of heartsease herb is as a tea, i.e., by preparing an infusion, in this thesis we primarily aimed at the thorough analyses of this aqueous extract. Aqueous solutions, however, are microbiologically instable and difficult to handle (e.g. to evaporate). Theoretically, the application of methanolic solutions could alleviate these drawbacks. The samness of the heartsease methanolic and aqueous extracts' compositions was demonstrated by HPLC analyses (data not included). In addition, the effectiveness of methanol as extraction solvent was also verified by TLC expreiments. Samples obtained by consecutive extraction with n-hexane, dichloromethane, ethyl acetate, and 70% methanol (Method 1) were compared. The compositions of extracts were characterized by thin layer chromatography. TLC plates were developed in systems of different polarities (TLC Methods 1-3) suitable for the analysis of highly apolar compounds as well as flavonoid aglycones and glycosides, and attended with special color reagents such as Naturstoff. Results clearly demonstrated that the apolar components of heartsease were extracted by the most apolar solvent, n-hexane. In addition, the methanolic extract was enriched in components of similar polarity of flavonoid glycosides and aglycones, which are supposed to be present also in the aqueous extract. Henceforth, in most sample preparation methods after a pre-extraction with chloroform, producing *Fraction A*, heartsease herb was extracted with methanol, yielding *Fraction B* (Method 2) (Fig. 4.1).

4.1.2 Plant materials

As a part of our preliminary examinations, plant materials from three different sources were compared (Sec. 3). The first sample was collected and identified in Transilvania by Prof. Kalman Csedo (Babes-Bolyai University). The second and third samples were purchased from Fitopharma Ltd. and MDR 2000 Ltd., respectively. Our HPLC fingerprint analyses revealed that the compositions of the collected and purchased samples exhibited only slight differences. Thus, for further –especially quantitative– analyses the sample easisiest to obtain (from Fitopharma Ltd.) was used. The similarity of the compositions of collected and purchased plant samples was supported by later MS experiments (data not included).

4.2 Preparative separation of *Fraction B*

For the preparative separation of crude plant extracts, conventional column chromatography methods are widely used. To partition polar components, such as flavonoids or other phenoloids, stationary phases of polyamide, cellulose, silica gel and Sephadex LH-20 were mostly reported [144], most often using aqueous alcoholic solutions (methanol-water or ethanol-water) for their elution. Due to the good light absorption of flavonoids and the fluorescence properties of their derivatives [85], their chromatographic separation can easily be detected. The exact composition of the fractions are usually determined by analytical tools of HPLC, LC-MS, LC-NMR, CE and CEC [82, 145]. However, these methods often require the aid of reference standard molecules. If needed, a second separation step of column chromatography, preparative TLC or even preparative HPLC can be utilized.

The preparative separation of the methanol extract of heartsease (*Fraction B*) was carried out first by Sephadex LH-20 column chromatography. In the preliminary experiments, fractions of identical volumes were collected and the composition of the fractions was screened by TLC (TLC Method 1). Based on the TLC results a fractionation protocol was developed described in Material and methods (Sec. 3) and delineated in Fig. 4.1. This fractionation method provided *Fractions C-G*, which together with *Fraction B* were analyzed by HPLC (Fig. 4.2a-f). The chromatograms were achieved as a result of gradient elution from a RP-C18 column detected at UV 340 nm, a distinctive wavelength for

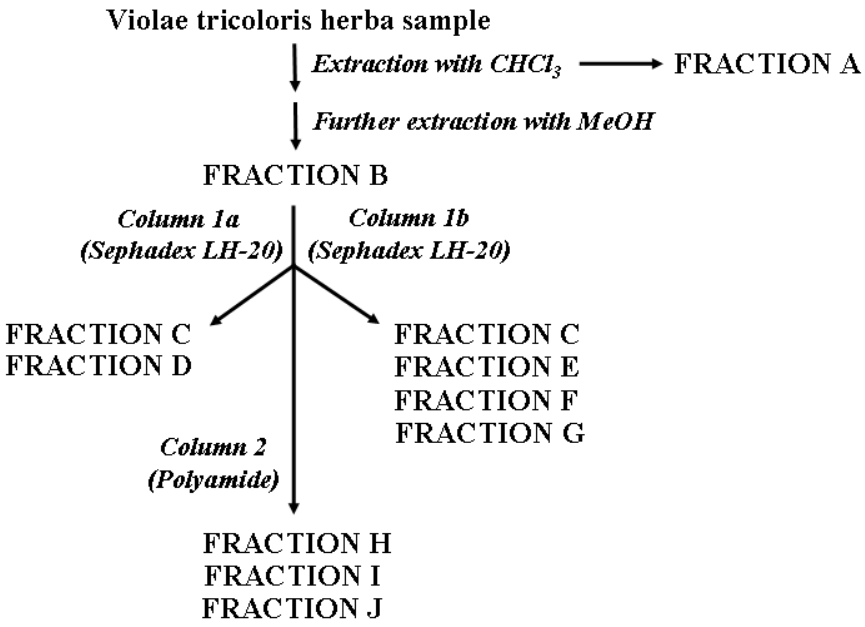


Figure 4.1: Fractionation protocol developed for the isolation of the two main flavonoids (*Fraction E* and *F*) and for the separation of minor flavonoid components.

flavonoid analysis (System 1) [85]. The UV spectra of the characteristic components were also recorded.

The HPLC chromatogram of *Fraction B* (Fig. 4.2a) shows the composition of the methanol extract. *Fraction C* (chromatogram and UV spectra in Fig. 4.2b) comprises the fast-eluting components of *Fraction B*. Specific for Sephadex gels, size exclusion, adsorption and partition mechanisms apply [144], thus, *Fraction C* assumed to contain large molecules and/or highly polar molecules. The retention times and the UV spectra of components **1** and **2** (from Fig. 4.2b) clearly suggest that these components do not belong to the flavonoid family. The UV spectra of flavonoids in polar organic solvents (such as methanol or acetonitrile) exhibit two major absorption maxima in the region of 240 - 400 nm [85], whereas components **1** and **2** have absorption maxima only at 260 nm. In the case of Column 1a, the flavonoid components of the methanol extract were collected together as *Fraction D* (Fig. 4.2c). In the case of the use of Column 1b these flavonoids were separated. *Fraction E* (chromatogram and UV spectrum in Fig. 4.2d) consists mainly of the main flavonoid component of the extract (component **3**). In *Fraction F* (chromatogram and UV spectrum in Fig. 4.2e) the

second main flavonoid (component **4**) was isolated. The UV spectra of components **3** and **4** present typical flavonoid absorption traces with maxima at 274 and 336 nm, as well as at 266 and 361 nm, respectively.

In *Fraction G* (chromatogram and UV spectrum Fig. 4.2f) components **3** and **4** were present at lower quantities. This fraction, however, could not be separated by Sephadex LH-20 column chromatography, not even by systematically changing the elution protocol. Thus, achieving their proper separation was tried by changing the chromatographic stationary phase. Polyamide column chromatography of *Fraction B* (Column 2) resulted among others *Fractions H-J*. These fractions were analyzed by HPLC (System 1) and LC-MS (Instrumentation 1) and their compositions were compared with the methanol extract (Fig. 4.3). *Fractions H-J* (chromatograms in Fig. 4.3b-d) contained five components of *Fraction B* in three combinations: components **5** and **7**, component **8** and components **6**, **8** and **9**, respectively. As a first approximation, their UV spectra suggests that components **5-9** are flavonoids.

The LC-MS analysis of components **5-9** in Instrumentation 1 did not provide sufficient data for structure elucidation (Fig. 4.4). However, molecular mass information of components **5-9** (MW = 594, 448, 578, 611, 662, respectively), and some information about their glycosylation types were obtained. Their molecular masses were higher than 400 Da and the lack of the characteristic fragment ions of sugar losses (146 Da and 162 Da for deoxyhexose and hexose units, respectively) suggested that components **5** and **6** are flavonoid C-glycosides [38], in spite of the fact that in their mass spectra no intensive C-glycoside type fragments (water losses, cleavages of the glycan part [100]) were observed. In Instrumentation 1, where the soft ionization method ESI was used, component **7** showed no fragmentation at all, also suggesting a C-glycosidic structure [38]. Flavonoid compounds of the same molecular masses of vicenin-2 (MW = 595), orientin and isoorientin (MW = 448), violanthin (MW = 578) [22] (see structures in Tab. 2.1) have already been reported in heartsease in early reports of the late 1960's and early 1970's [22]. Their matching with components **5-7**, however, could not been proved. The mass spectra of component **8** displayed characteristic fragments at *m/z* 465 and 303, which could be assigned as aglycone residues after losses of their rhamnosyl and rhamnoglucosyl moieties, respectively. These results suggested a quercetin-*O*-diglycosidic structure, which in correlation with literature data, suggested rutin (quercetin-

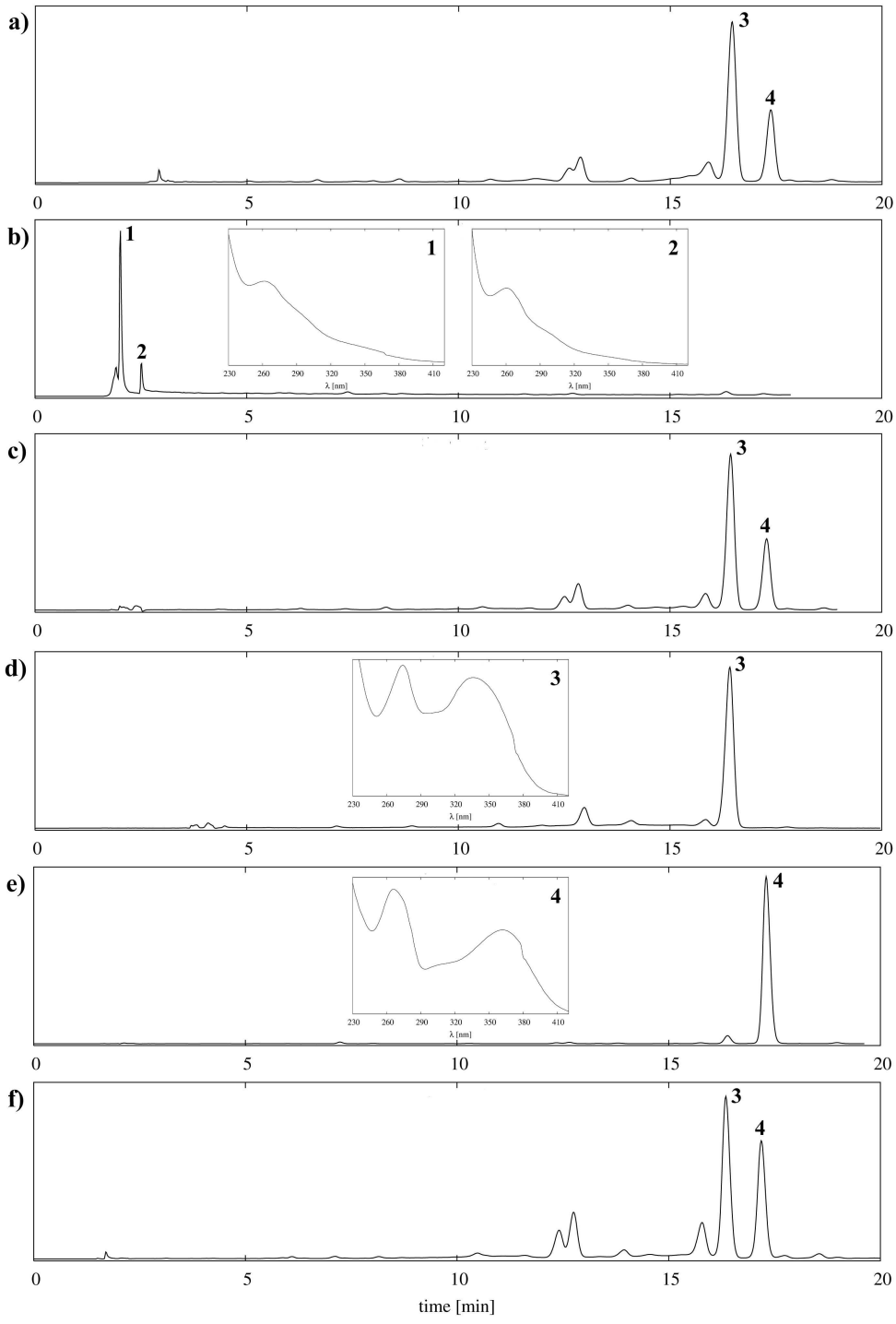


Figure 4.2: HPLC chromatograms of the fractions obtained by Sephadex LH-20 column chromatography (Column 1a and b) and UV spectra of the characteristic components. (a) *Fraction B*, (b) *Fraction C*, (c) *Fraction D*, (d) *Fraction E*, (e) *Fraction F*, (f) *Fraction G*. The LC separation was achieved in System 1.

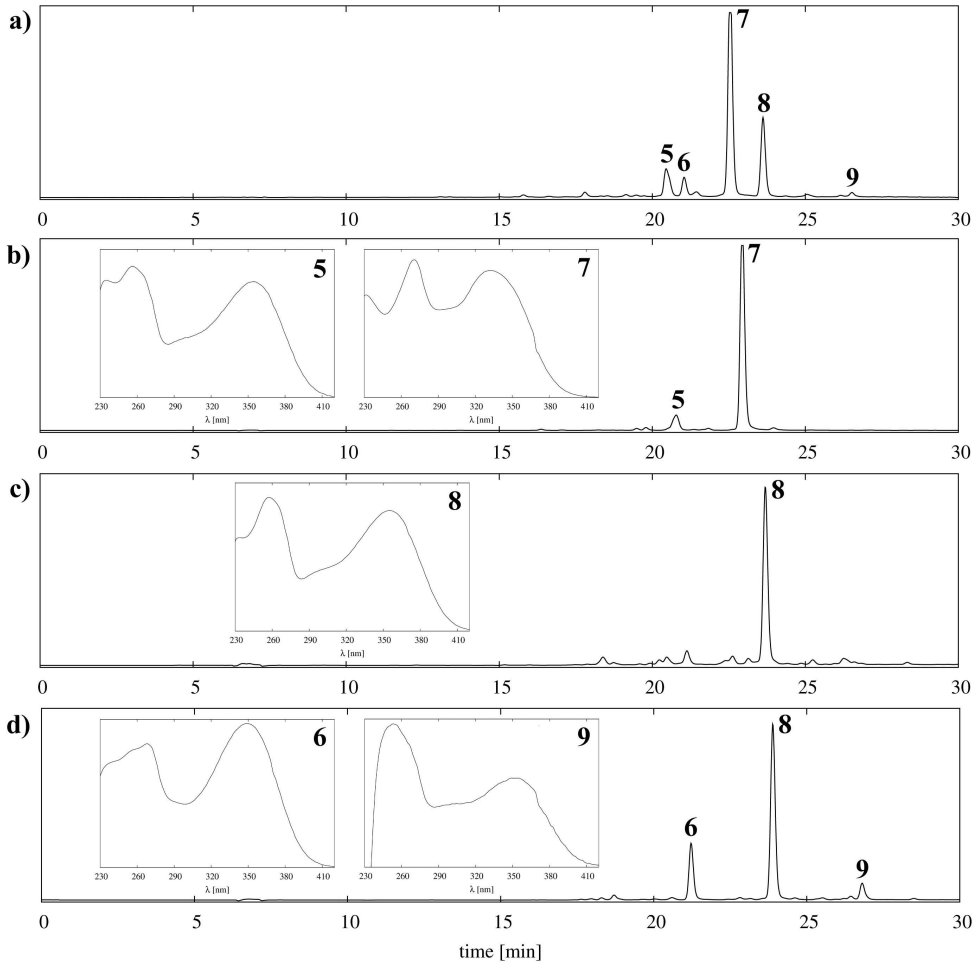


Figure 4.3: HPLC chromatograms of the fractions obtained by polyamide column chromatography (Column 2) and UV spectra of the characteristic components. (a) *Fraction B*, (b) *Fraction H*, (c) *Fraction I*, (d) *Fraction J*. Samples were studied in Instrumentation 1.

3-*O*-rhamnoglucoside) to be present in heartsease in high quantities. The comparison of retention times, UV spectra, molecular masses and MS fragmentation patterns with standard reference molecules also indicated that component **8** should be rutin. In addition, the comparison of their UV spectra and the peak distributions respectively suggest components **4** and **8** to be identical. Consequently, *Fraction F* supposed to consist of rutin.

4.3 Analysis of *Fraction E* by NMR spectroscopy

The structural elucidation of the main flavonoid component of heartsease (*Fraction E*) was achieved by NMR spectroscopy.

The ^{1}H-NMR spectrum of *Fraction E* confirmed the presence of a flavone skeleton characterized by a singlet signal at δ_H 6.78 assigned to H-3 and two doublet signals at δ_H 6.95 and 7.89 assigned to H-3', H-5' and H-2', H-6', respectively. These doublets – with coupling constants (J = 8.4 Hz) distinctive of hydrogenes at ortho position [27] – also suggested that there was only one substitution on the B-ring, namely at position 4'. The absence of signals for H-5 and H-7 implied substituents at positions 5 and 7. The lack of substituent-specific signals in the range of δ 1-2 indicated these substituents to be hydroxyl groups [27]. In addition, the chemical shift of δ_C 182.0 confirmed that a carbonyl group is found at position 4. In conclusion, the the aglycone is proposed to be apigenin Fig. 2.2.

The absence of signals for H-6 and H-8 and the correlations observed in the HMBC spectrum between H-1'' and C-5, and C-6 as well as between H-1''' and C-8 further indicated that the two sugar moieties were attached to positions 6 and 8 (Fig. 4.6). *C*-glycosylation was confirmed by chemical shift values of the anomeric sugar protons (δ_H 4.62 and 5.23) [27] and carbons (δ_C 73.6 and 63.6) [87].

The ^{1}H-NMR and ^{13}C chemical shift (δ_H 1.26 and δ_C 18.1) of the 6''' carbon suggested a methoxy substituent at position 5''' [87], therefore the sugar moiety at position 8 was assumed to be a rhamnose. This assumption was supported by the fact that the ^{1}H-NMR chemical shift of the H-1''' anomeric proton is higher than five (δ_H 5.23) [27]. For the hexose residue, the H-1'' anomeric proton at δ_H 4.62 was coupled to a proton at δ_H 4.10 (J = 9.8 Hz), corresponding to H-2''. This correlation appeared also in the COSY spectrum (Fig. 4.7).

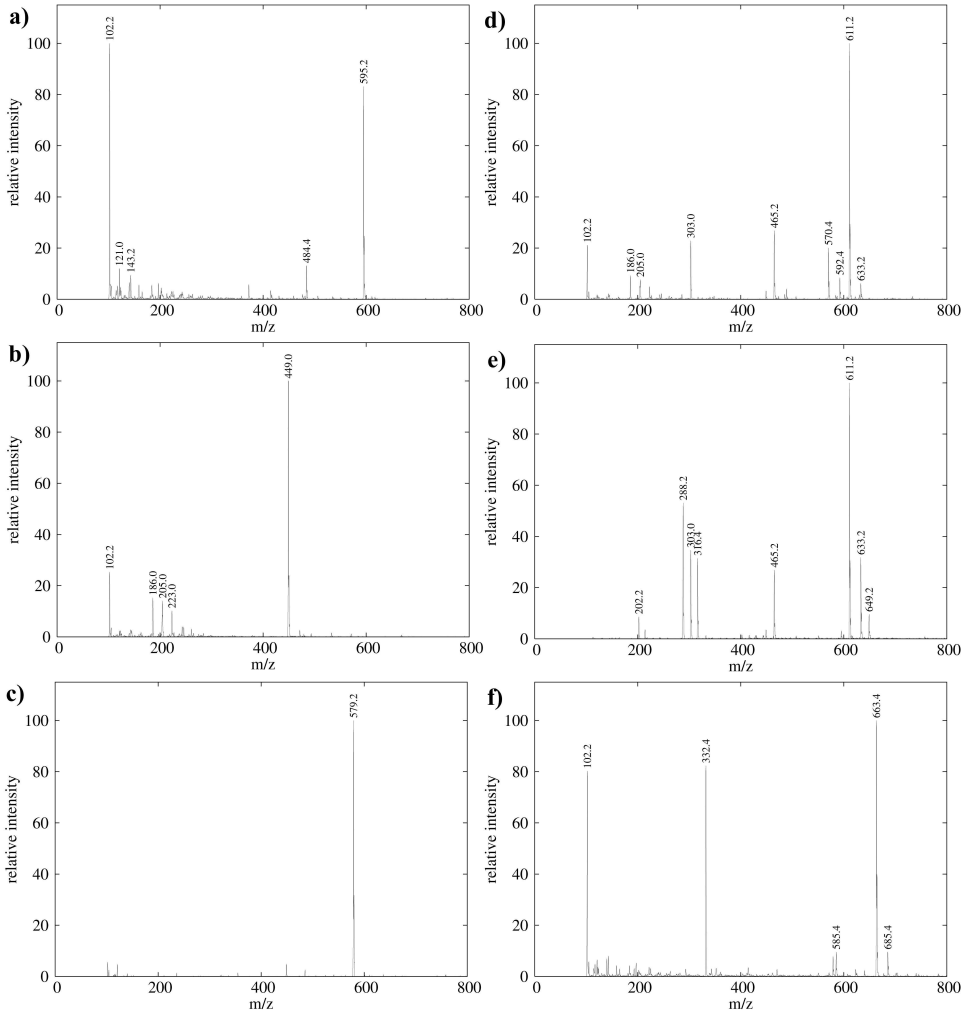
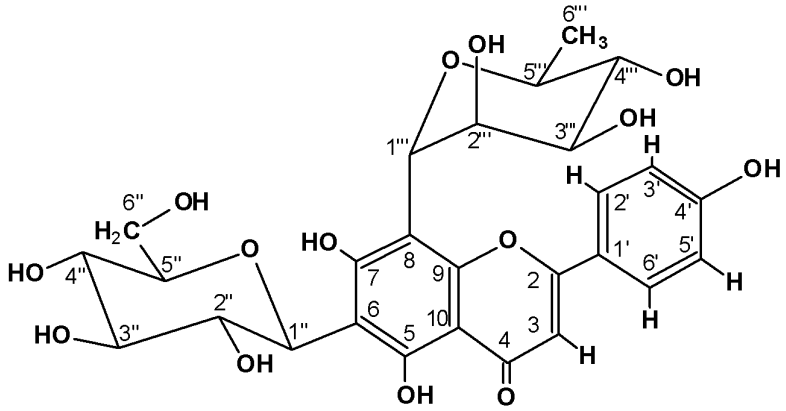


Figure 4.4: Mass spectra of components **5-9** in *Fractions H* and *J*, as well as of rutin reference molecule. (a) component **5**, (b) component **6**, (c) component **7**, (d) component **8**, (e) rutin standard, (f) component **9**. Experiments were performed as described in Instrumentation 1.

Figure 4.5: Assigned NMR chemical shifts and coupling constants (in Hz in parentheses) for violanthin in DMSO-d_6 at 25 °C.



no.	^{1}H	^{13}C
2		163.1
3	6.78 s	102.5
4		182.0
5		160.1
6		109.1
7		163.4
8		104.0
1′		121.5
2′,6′	7.89 d (8.4)	128.4
3′,5′	6.95 d (8.4)	116.1
4′		161.5

no.	^{1}H	^{13}C
1″	4.62 d (9.8)	73.4
2″	4.10 t (9.8)	
3″	3.20 t (9.0)	
4″	3.14 t (8.9)	
5″	3.15 m	82.4
6″	3.69 d (12.0)	61.9
	3.41 dd (12.0, 6.0)	
1‴	5.23 brs	
2‴	3.90 m	
3‴	3.59 d (8.9, 2.5)	
4‴	3.39 t (8.9)	71.9
5‴	3.46 m?	77.2
6‴	1.26 d (6.0)	18.1

s singlet, *d* doublet, *t* triplet, *dd* doublet-doublet, *m* multiplet, *br s* broad singlet

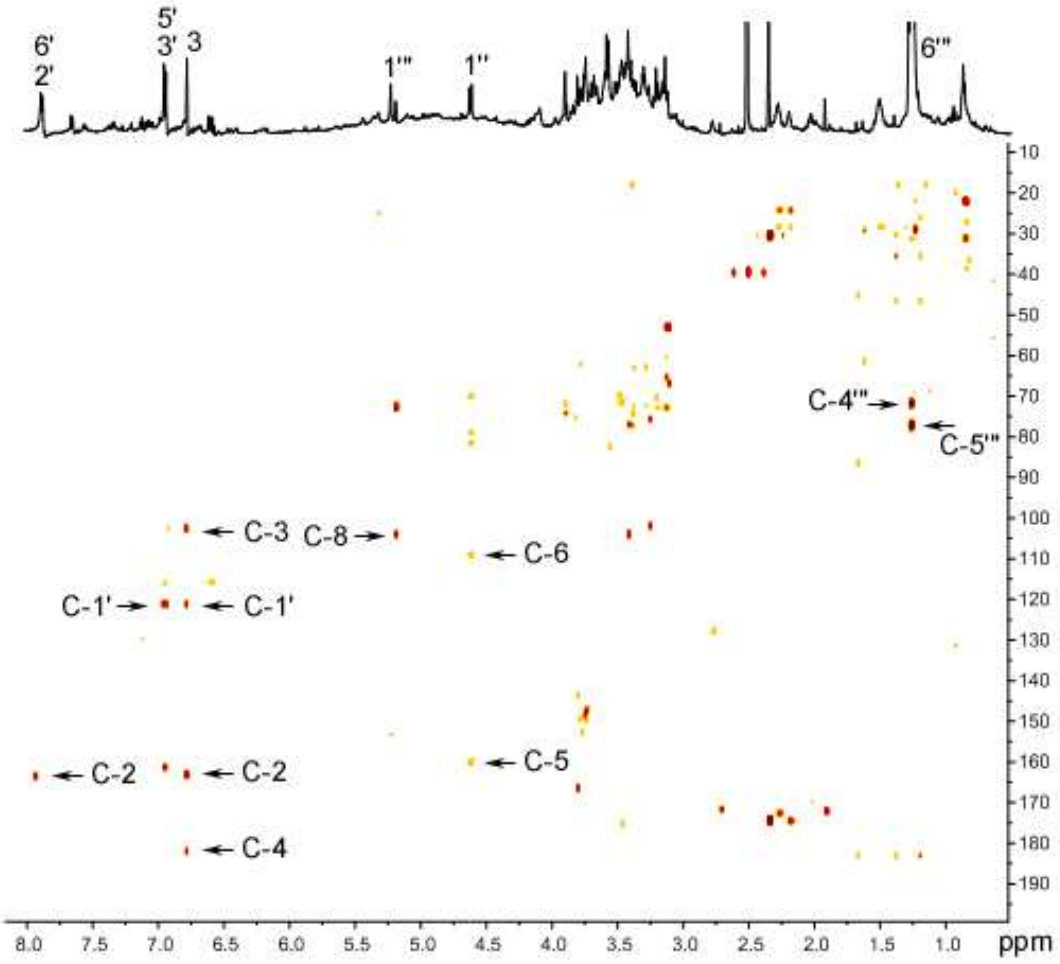


Figure 4.6: HMBC spectrum of *Fraction E*, showing one-bond $_1$H-^{13}C correlation crosspeaks. The 1D ^{1}H NMR spectrum is included as a projecion in the proton dimension.

On the basis of the large coupling constant between H-1‴ and H-2‴, the two protons were established as axial and the configuration of the sugar as β. In addition, assignments of sugar protons in crowded spectral regions by selective 1D TOCSY-TOCSY and TOCSY-NOESY experiments identified this hexose residue as glucose [146].

In conclusion, we consider the major flavonoid component of heartsease (component **3** in *Fraction E*) as violanthin.

4.4 Analysis of *Fraction B* by LC-MS^n

In HPLC based separation of flavonoids generally RP-C18 columns are applied, however, in our case better separation was obtained with a monolithic poly(*p*-methylstyrene-co-1,2-bis(*p*-vinylphenyl)ethane) microcolumn under reversed phase conditions. Separation was performed with a 50 min linear gradient from 0.1% formic acid to 0.1% formic acid containing 10% acetonitrile. Hyphenation to the mass spectrometer was carried out by a nanoflow electrospray ionization source and scouting runs were carried out with medium fragmentation energy setting. However, it was found that for the analysis of *C*-glycosides higher energy was needed in order to obtain adequate fragmentation. On the other hand, for *O*-glycosides, low energy was preferred to prevent the cleavage of the sugar units and provide easily interpretable spectra. One of the most important issues during the MS analysis was how to differentiate compounds, which were characteristic of the sample from the electrospray byproducts, as e.g., in-source fragmentation of some glycosides may result in loss of sugar residues. In other words, in some instances artifacts might appear in the total ion chromatograms, which were not present in the original sample. In the case of rutin (quercetin-3-*O*-rhamnosylhexoside) the aglycone quercetin and isoquercitrin (quercetin-3-*O*-hexoside) were both detected at the same retention time. Their different polarity and binding affinity to the monolithic column, would however suggest different retention behavior. Therefore, in all instances, careful analysis of the full MS spectra was necessary to define the representative sample components. Based on these considerations, sixteen peaks were selected (Tab. 4.1) and their MS/MS and MS^3 spectra thoroughly studied. These sixteen components were classified as *O*-, *C*-, or *C*,*O*-glycosides. Interpretation of their mass spectra and their hypothetical structures are dis-

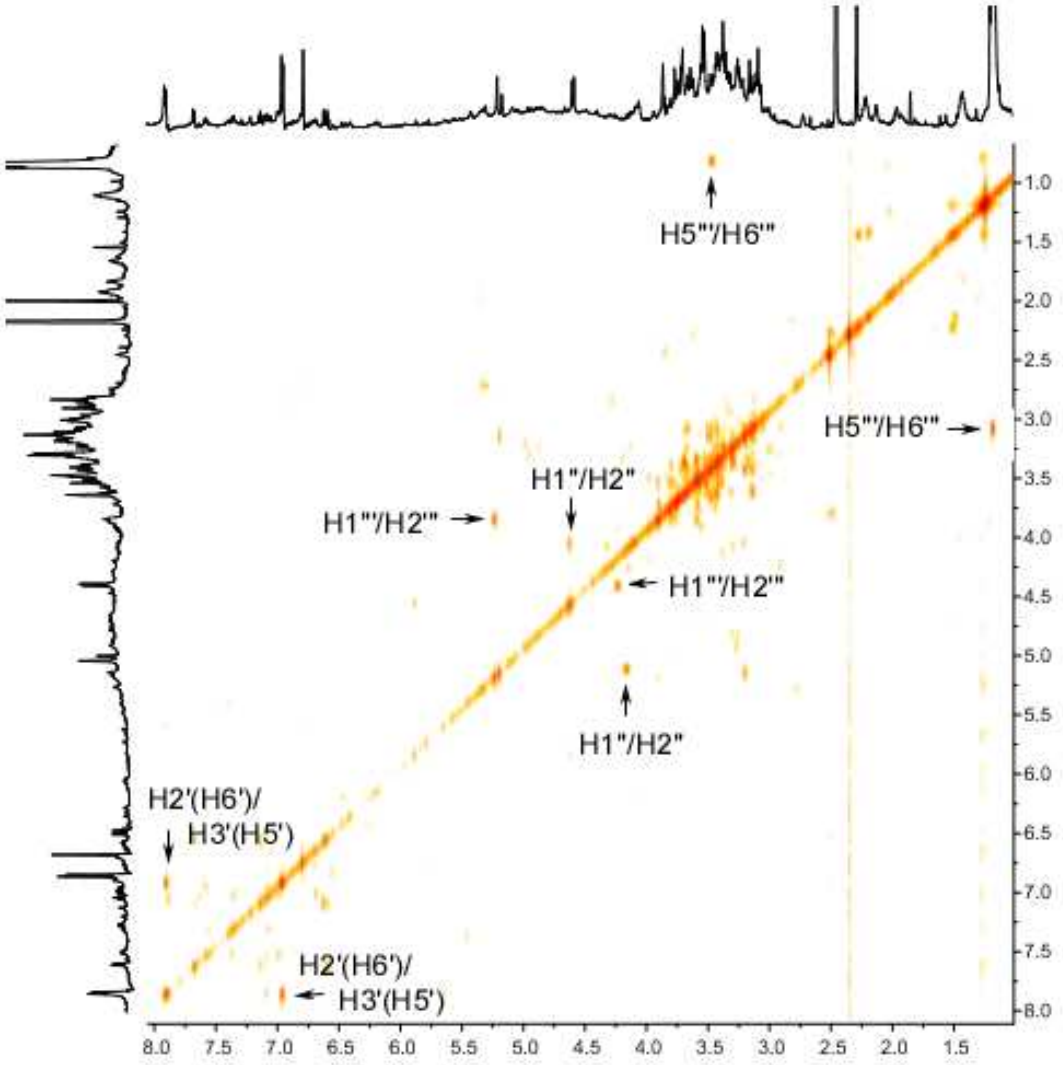


Figure 4.7: COSY spectrum of *Fraction E*, showing one-bond $_1$H-^{1}H correlation crosspeaks. The 1D ^{1}H NMR spectrum is included as a projecion in both dimensions.

Table 4.1: Flavonoid glycoside components in heartsease methanol extract characterized by LC-MS^n

	MW (Da)	Rt	Structure
O-glycosides			
10	594	42.7	kaempferol-3-*O*-deoxyhexosyl(1→6)hexoside
11	610	36.5	quercetin-3-*O*-deoxyhexosyl(1→6)hexoside
12	624	46.7	isorhamnetin-3-*O*-deoxyhexosyl(1→6)hexoside
13	756	34.0	quercetin-3-*O*-deoxyhexosylhexoside-7-*O*-deoxyhexoside
C-glycosides			
14	448	33.0	luteolin-6-*C*-hexoside
15	462	39.5	chrysoeriol-6-*C*-hexoside
16	564	33.0	apigenin-6-*C*-pentoside-8-*C*-hexoside
17	564	35.5	apigenin-6-*C*-hexoside-8-*C*-pentoside
18	578	35.0	apigenin-6-*C*-deoxyhexoside-8-*C*-hexoside
19	578	37.5	apigenin-6-*C*-hexoside-8-*C*-deoxyhexoside
20	594	31.6	apigenin-6,8-di-*C*-hexoside
21	594	33.0	luteolin-6-*C*-deoxyhexoside-8-*C*-hexoside
22	594	35.0	luteolin-6-*C*-hexoside-8-*C*-deoxyhexoside
C,*O*-glycosides			
23	740	30.6	apigenin-*X*-*O*-hexoside-*Y*-*C*-deoxyhexoside-*Z*-*C*-hexoside
24	740	34.3	apigenin-6-*C*-deoxyhexoside-(6″-*O*-hexosyl-8-*C*-hexoside)
25	740	39.7	apigenin-(6″-*O*-hexosyl-6-*C*-hexoside)- 8-*C*-deoxyhexoside

cussed in the following sections.

4.4.1 Characterization of *O*-glycosides

The flavonoid *O*-diglycosides identified in heartsease methanol extract (components **10**-**12**, Tab. 4.1) showed molecular masses of 594, 610, and 624. In their positive and negative ion MS/MS spectra, high abundant Y_0 fragments were observed (for nomenclature see Fig. 2.5). By definition, these fragments originate from the losses of all sugar units and represent the aglycones of the components. Consequently, the molecular masses of 286, 302, and 316, are rep-

resentative for the aglycones of components **10-12**, respectively. In addition, the comparison of the positive and negative ion MS^3 spectra of the Y_0 fragments with literature data [94, 117, 120] as well as with the MS/MS spectra of reference molecules (Tab. 4.2) suggested component **10** to be kaempferol-, component **11** quercetin- and component **12** isorhamnetin-glycoside (Fig. 2.2a).

The positive ion MS/MS spectra of components **10-12** showed Y_1^+ ions corresponding to the losses of 162 and 146 Da (hexose and deoxyhexose units, respectively) [98]. These sugars can be attached to the flavonoid aglycone either at two different positions (di-*O*-glycosides) or at the same position (*O*-diglycosides) forming a disaccharide. The parallel loss of a hexose and a deoxyhexose unit would suggest the presence of a di-*O*-glycosidic isomer. On the other hand, for *O*-diglycosides, appearance of irregular Y_1^+ ions (generally labeled as Y^*) have been reported, resulting from the losses of the internal sugar units [108]. Thus, the positive ion MS/MS spectra alone seem to be insufficient to differentiate the two isomeric structures. In the negative ion MS/MS spectra of components **10-12**, however, no Y_1^- fragments were observed, only abundant Y_0^- fragments. This fact suggests the presence of *O*-diglycosidic isomers with 1→6 linkages between the sugar moieties [106]. As in the positive ion MS/MS spectra both Y_1^+ fragments (Y_H^+ and Y_D^+) were present in contrast to the negative ion MS/MS spectra where no Y_1^- ions were observed, the order of the sugar units in the disaccharide could not be determined by mass spectral analysis. However, the 1→6 linkage between the sugar units is only possible if the deoxyhexose unit is the external sugar unit, since deoxyhexoses possess a methyl group instead of a hydroxymethyl at position 5". Moreover, to date no diglycoside with a 1→6 linkage has been found with internal deoxyhexose unit [40]. Therefore, we propose that in components **10-12**, deoxyhexosyl(1→6)hexoside units are attached to the flavonol aglycones. Although theoretically this disaccharide can substitute any hydroxyl groups, in the case of flavonols, positions 3 and 7 are reportedly favored [38]. While in the case of 7-*O*-diglycosyl flavonols in the positive ion MS/MS spectra no Y^* fragments were observed, for 3-*O*-diglycosyl flavonols low abundant Y^* fragments have been reported [108]. These facts can be the basis of the differentiation of 3-*O*- and 7-*O*-diglycosylated isomers, and in the case of components **10-12** they may indicate the presence of 3-*O*-diglycosylated flavonols. In conclusion, based on our data, we suggest components **10-12** as kaempferol-, quercetin-,

Table 4.2: Principal ESI-MS/MS product ions obtained from the $[M+H]^+$ and $[M–H]^-$ ions of KA = kaempferol, QU = quercetin, and IR = isorhamnetin reference molecules as well as from the Y_0^+ and Y_0^- fragments of components **10-12**

	Fragmentation [a] *m/z* (% relative abundance)											
	Positive ion mode						Negative ion mode					
	KA	C10	QU	C11	IR	C12	KA	C10	QU	C11	IR	C12
M+H/M–H/Y_0	287 (100) [a]	287 (100)	303 (56)	303 (78)	317 (3)	317 (8)	285 (100)	285 (5)	301 (0)	301 (3)	315 (4)	315 ()
–CH_3					302 (100)	302 (100)					300 (100)	300 (100)
–H_2O	269 (12)	269 (19)	285 (52)	285 (51)	299 (5)	299 (4)	267 (6)	267 (44)	283 (14)	283 (5)		
–CO	259 (12)	259 (11)	275 (9)	275 (17)			257 (11)	257 (100)	273 (13)	273 (18)		
–CHO	258 (22)	258 (26)	274 (13)					256 (9)				
–CH_3OH					285 (44)	285 (45)						
–C_2H_2O	245 (22)	245 (3)			275 (3)	275 (6)	243 (13)	243 (6)				
–CO_2							241 (20)	241 (26)	257 (9)	257 (16)		
–H_2O–CO	241 (62)	241 (76)	257 (100)	257 (61)	271 (5)	271 (8)	239 (7)	238 (19)	255 (8)	255 (4)		
–2CO	231 (18)	231 (24)	247 (24)	247 (17)	261 (11)	261 (7)	229 (16)	229 (41)				
–CH_3OH–CO					257 (10)	257 (10)						
–H_2O–2CO	213 (51)	213 (63)	229 (66)	229 (100)	243 (4)	243 (4)	211 (3)	211 (3)				
	203 (6)	203 (10)	219 (6)	219 (8)			201 (6)					
	199 (3)	199 (4)	215 (4)		229 (3)	229 (6)		197 (20)				
	197 (11)	197 (13)	213 (5)				195 (5)	195 (11)	211 (4)	211 (4)		
	185 (6)	185 (10)	201 (7)	201 (6)								
– B-ring			195 (8)	195 (23)					193 (8)	193 (7)		
$^{1,2}A$									179 (100)	179 (100)		
–2CO–$C_2H_4O_2$					177 (3)	177 (6)						
$^{0,2}A$	165 (85)	165 (66)	165 (58)	165 (47)	165 (8)	165 (8)	163 (6)	163 (20)				
$^{1,3}A$ / $^{1,2}A$–CO	153 (26)	153 (30)	153 (16)	153 (13)	153 (4)	153 (6)	151 (25)	151 (7)	151 (73)	151 (67)		
$^{1,3}B$–2H	133 (12)	133 (16)	149 (7)	149 (4)	163 (2)	163 (3)						
$^{0,2}B$	121 (14)	121 (16)	137 (18)	137 (3)		137 (3)						
$^{0,2}A$–CO	137 (4)	137 (4)	137 (18)	137 (3)				135 (3)				
	105 (3)	105 (3)	121 (5)	121 (3)	139 (13)	139 (11)						
$^{1,3}A$–C_2H_2O	111 (8)	111 (9)	111 (6)	111 (11)								
$^{1,2}A$–CO–CO_2									107 (4)	107 (4)		

and isorhamnetin-3-*O*-deoxyhexosyl(1→6)hexosides, respectively.

As a first approximation, based on its molecular mass, we considered component **13** (MW = 756) a triglycoside. The positive and negative ion MS^3 analysis of the abundant Y_0 ion (MW = 302) suggested its aglycone to be quercetin. The mass losses corresponding to the Y_i fragments implied the attachment of two deoxyhexose and one hexose to the aglycone. Because of the presence of the Y^* fragment (Fig. 4.8a and b), the distribution of the sugar moieties could be deduced only from the complementary analyses of Y_i and $^{k,l}X$ ions in both the positive and negative ion modes (Fig. 4.8a and b). The three sugar residues can theoretically be attached to the aglycone at three, two or one position with different sequences. As an abundant Z_i ion was observed in the negative ion MS/MS spectrum, the tri-*X*,*Y*,*Z*-*O*-glycoside form was ruled out [106, 111]. On the other hand, according to the MS^3 analysis of $[M+H]^+ \rightarrow [Y_D - {}^{0,2}X_H]^+$, the presence of the $Y_D - {}^{0,2}X_H - Y_D$fragment at *m/z* 343 is opposed to the X-*O*-triglycoside structure. In addition, the presence of the *m/z* 609 ($Y^-{}_D$) ion and the absence of the *m/z* 593 ion indicated that when only one sugar unit was lost that should have been deoxyhexose. Therefore we consider the structure as quercetin-X-*O*-deoxyhexoside-Y-*O*-deoxyhexosylhexoside, and *m/z* 595 is designated in the positive ion spectra as $Y^*{}_H$. In the case of component **13**, the preferred attachment points for the monoglycosyl and the diglycosyl units were reportedly positions 3 and 7 [38]. Based on the analogy to components **10-12** and the fact that Y^* ions were observed only for 3-*O*-diglycosides [108], we consider component **13** as quercetin-3-*O*-deoxyhexosylhexoside-7-*O*-hexoside. In regard to the linkage type of the diglycosyl unit ambiguous results were obtained. According to Ferreres et al. [96] the presence of an abundant Z_i fragment indicated 1→2 linkage. However, the analogy to other *O*-glycosides identified in the same extract would suggest a 1→6 connection.

4.4.2 Characterization of *C*-glycosides

In the MS/MS spectra of *C*-glycosides analyzed in heartsease methanol extract (components **14-22**, Tab. 4.1), characteristic fragments resulted both from water losses and sugar cleavages. In the positive ion mode, besides the water losses, $^{2,3}X^+$, $^{0,4}X^+ - 2H_2O$, $^{0,2}X^+$, $^{0,1}X^+$ ions and low abundant (relative intensity $< 1\%$) $Y_i{}^+$ fragments were observed. On the other hand, the negative ion

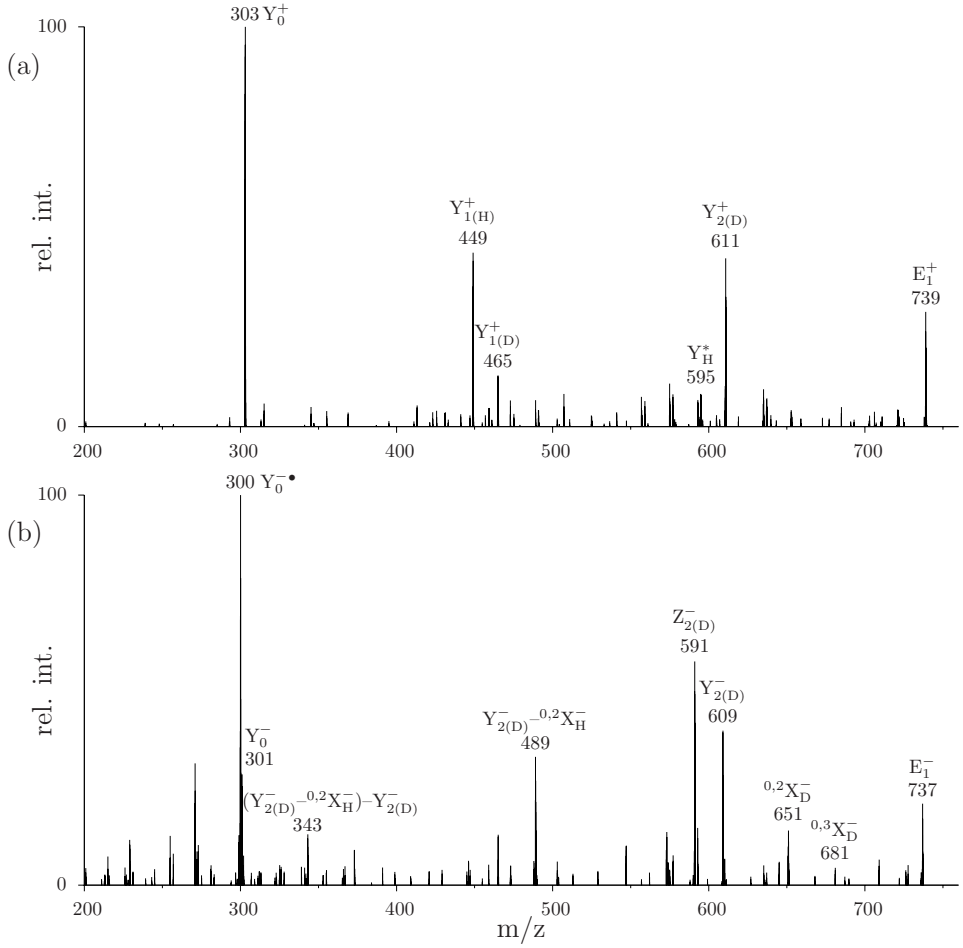


Figure 4.8: (a) Positive and (b) negative ion MS/MS spectra of component **13**

spectra showed abundant $^{0,4}X^-$, $^{0,3}X^-$, $^{0,3}X^-$–$2H_2O$, $^{0,2}X^-$, $^{1,5}X^-$ fragments. In addition, for di-*C*-glycosides, parallel cleavages of both sugar units resulted in $^{i,j}X_1{}^{k,l}X_2$fragments, which at applying the same fragmentation energy, were more abundant in the negative ion mode.

The fragments resulting from sugar unit cleavages corresponded to different mass losses for hexoses, deoxyhexoses, and pentoses, thus, the types of the sugar residues could be determined. Accordingly, we found that the two examined mono-*C*-glycosides (components **14** and **15**, molecular masses of 448 and 462, respectively) contained hexose units. In respect to their attachment points, up until today, *C*-glycosylation has been almost exclusively found at positions 6 or 8 and only in two cases at position 3 [38, 42]. Although no significant differences were observed in the MS/MS spectra of the isomers, the analysis of the $[M+H–^{0,2}X]^+$ ions provided means of differentiation. Waridel et al. [100] observed different small molecule loss patterns in the positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for C-6 and C-8 isomers. Using the same fragmentation energy for C-6 glycosides more fragmentation occurred, resulting in $^{0,2}X^+$–H_2O, $^{0,2}X^+$–CHO, $^{0,2}X^+$–H_2O–CO, $^{1,3}A^+$, and $^{0,2}B^+$ ions, whereas in the spectra of C-8 isomers only the $^{0,2}X^+$–CHO and $^{0,2}B^+$ ions were present. Our results were similar in comparing the positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) reference molecules (Fig. 4.9a and b) The analogy of the MS^3 spectra of components **14** and **15** to the above described fragment patterns (Fig. 4c and d), suggested the presence of C-6 hexosyl isomers. This assumption was further supported by the fact that high abundant $^{0,3}X^-$ fragments were found in the negative ion MS/MS spectra of components **14** and **15** (Fig. 4.9e,g and h) similarly as reported in [100, 111].

In the case of *C*-glycosides, the lack of significant Y_0 ions rendered the characterization of the aglycone part difficult, since no direct comparison with reference molecules was possible. Albeit, a thorough analysis of $^{i,j}A^+$ and $^{k,l}B^+$ ions in the MS^3 spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ provided information about the structure of the flavonoid aglycone. The calculated molecular mass for the aglycone of component **14** (MW = 286) indicated that the flavonoid aglycone contained a 2,3 double bond and four hydroxyl substituents. Ions at *m/z* 177 and 149, designated as $^{1,3}A^+$ and $^{1,3}A^+$–CO, implied the presence of two hydroxyl groups on A-ring. Furthermore, *m/z* 161, 137, and 135, designated as $^{0,4}B^+$–H_2O, $^{0,2}B^+$,

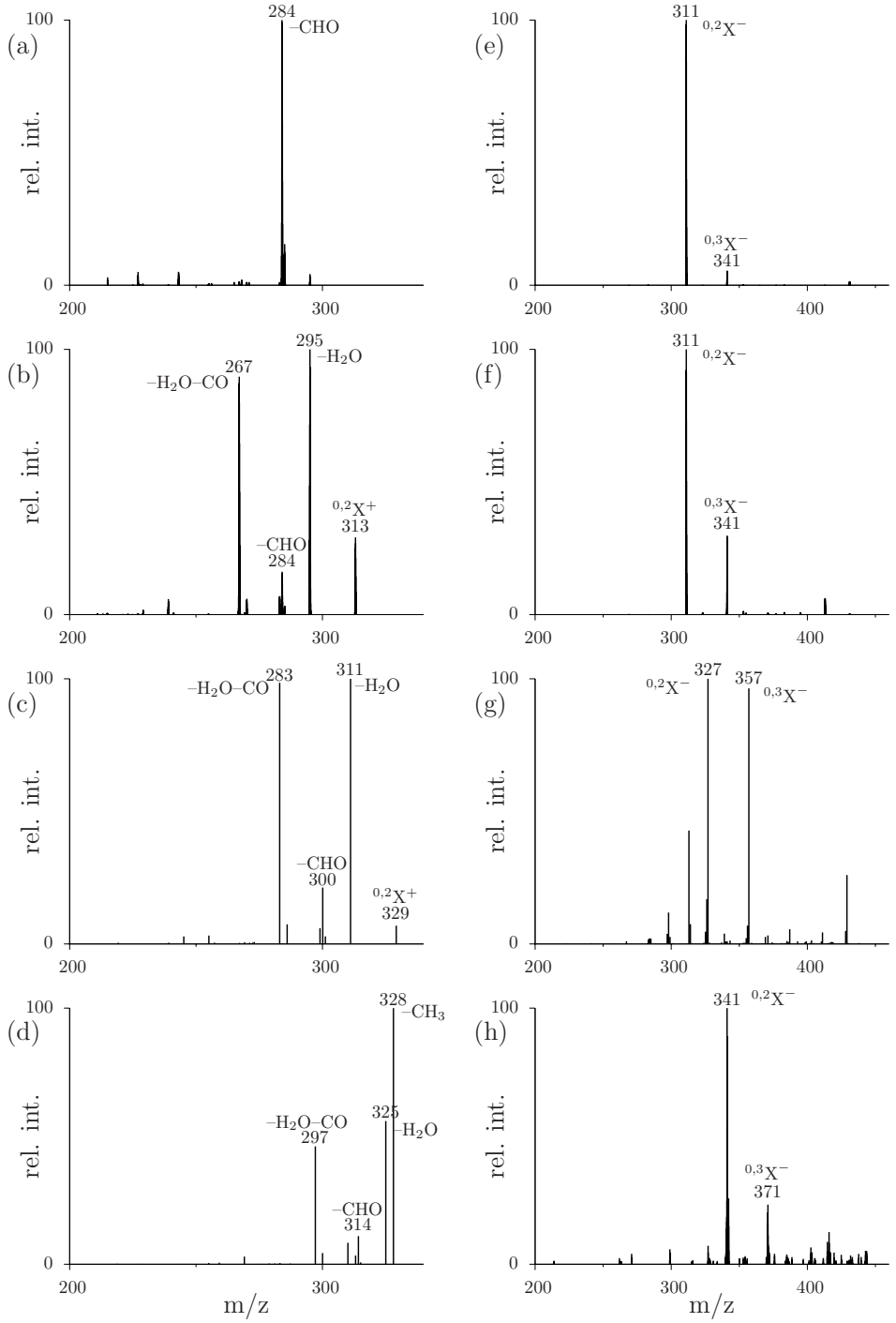


Figure 4.9: Positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for (a) vitexin reference molecule, (b) isovitexin reference molecule, (c) component **14**, and (d) component **15**, as well as negative ion MS/MS spectra of (e) vitexin reference molecule, (f) isovitexin reference molecule, (g) component **14**, and (h) component **15**.

and $^{1,3}B^+$, evidenced that the remaining two hydroxyl groups were connected to B-ring. Consequently, we propose component **14** as a luteolin-6-*C*-hexoside (Tab. 2.2a). On the other hand, in the MS^3 spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ of component **15** a loss of 15 Da was observed, suggesting a methoxy substituent per [120]. Together with this fact, the calculated molecular mass for the aglycone of component **15** (MW = 300) indicated that the flavonoid aglycone contained a 2,3 double bond as well as a methoxy and three hydroxyl substituents. Analogously to component **14**, *m/z* 177 and 149 implied the presence of two hydroxyl groups on A-ring. In addition, *m/z* 151, designated as $^{0,2}B^+$ signaled that a hydroxyl and a methoxy substituent were both attached to B-ring. As for the localization of the methoxy group, positions 3′ and 4′ are the only options as suggested in [42]. Although the exact attachment point of the methoxy group could not be determined at this point, we propose component **15** as chrysoeriol-6-*C*-hexoside rather than diosmetin-6-*C*-hexoside (Tab. 2.2a), based on its structural similarities to other compounds reported in hertsease [22, 25].

The di-*C*-glycosides characterized in the present study (components **16-22**) showed molecular masses of 564, 578, and 594 as depicted in Tab. 4.1. For each of these three mass values we found at least two differently eluting peaks, which vary either in their aglycones and sugar residues or in the attachment points of the sugars to the aglycone. Similarly to the monoglycosides above, the $^{i,j}X$ ions observed in the positive and negative ion MS/MS spectra of components **16-22** (Tab. 4.1) revealed information about the sugar types. Accordingly, we considered components **16** and **17** to contain hexose and pentose residues, while components **18**, **19**, **21** and **22** included hexose and deoxyhexose moieties and component **20** had two hexose substituents. On the other hand, the analysis of the MS^3 spectra of the $[M–H]^- \rightarrow [{}^{0,2}X_i{}^{0,3}X_j]^-$ and $[M–H]^- \rightarrow [{}^{0,2}X_i{}^{0,2}X_j]^-$ ions allowed the characterization of the aglycone part. Comparison of the corresponding spectra indicated that components **16-20** as well as components **21-22** may contain the same aglycones. Their calculated molecular masses (270 and 286) implied the presence of a 2,3 double bond as well as three and four hydroxyl substituents for components **16-20** and components **21-22**, respectively. In addition, the fragment ions $^{1,3}A^-$, $^{1,3}B^-$, and $^{0,4}B^-$ signified that two hydroxyl groups were located on A-ring. The remaining one (for components **16-20**) or two (for components **21-22**) hydroxyl substituents can theoretically be attached either to C-ring or B-ring, yielding flavonol or flavanone struc-

tures, respectively. According to the study of Fabre et al. [117] the negative ion mode fragmentation of flavonols and flavanones significantly differ. While for flavonols they observed $^{1,2}B^-$ and $^{1,2}A^-$ fragments, for flavanones $^{1,3}B^-$ and $^{1,3}A^-$ ions were found to be characteristic. Accordingly, we propose that components **16-20** have apigenin and components **21-22** luteolin aglycones.

Unfortunately, the methods described for monoglycosides were not applicable for the determination of the sugar attachment points for diglycosides. On the other hand, in their FAB experiments Li and Claeys claimed different fragmentation patterns for di-*C*-glycosyl isomers [98]. Among others they presented spectra of schaftoside (apigenin-6-*C*-glucoside-8-*C*-arabinoside) and isoschaftoside (apigenin-6-*C*-arabinoside-8-*C*-glucoside), di-*C*-glycoside isomers containing hexose and pentose moieties with the molecular mass of 564. Based on the analogy to these spectra – fragments $^{0,2}X^+_P$, $^{1,5}X^+_P$, $^{0,2}X^+_H$–H_2O and $^{0,2}X^+_H$–$2H_2O$ appeared only for the C-6 pentose isomer, while $^{0,4}X^+_H$–$2H_2O$, $^{0,2}X^+_P$–H_2O, and $^{0,1}X^+_H$ were observed only for the C-8 pentose variant – we consider component **16** as apigenin-6-*C*-pentoside-8-*C*-hexoside and component **17** as apigenin-6-*C*-hexoside-8-*C*-pentoside.

As described in Sec. 4.3, the main flavonoid component of heartsease methanol extract has been identified by NMR spectroscopy as violanthin (apigenin-6-*C*-glucoside-8-*C*-rhamnoside, MW = 578). Based on this information we differentiated components **18** and **19**. The base peak chromatogram revealed that component **19** is present in much higher concentration than component **18**, thus, we considered component **19** as violanthin, and component **18** as the apigenin-6-*C*-hexoside-8-*C*-deoxyhexoside isomer. Components **21** and **22** could not be differentiated by means of MS analysis. On the other hand, based on the information obtained from their chromatographic behavior (Sec. 4.4.4) we propose component **21** as luteolin-6-*C*-deoxyhexosisde-8-*C*-hexoside and component **22** as luteolin-6-*C*-hexoside-8-*C*-deoxyhexoside.

4.4.3 Characterization of C,O-glycosides

The C,*O*-glycosides in heartsease methanol extract were characterized on the basis of their negative ion MS/MS spectra. The molecular mass of 740 for components **23-25** in Tab. 4.1 indicated that these compounds were most probably triglycosides. Analogously to the *C*-glycosides, the presence of *m/z* 353 and 383 (Fig. 4.10) and their MS^3 spectra suggested apigenin as their aglycone. This fact

Table 4.3: Principal ESI-MS/MS product ions obtained from the $[M+H]^+$ ions of components **16-22**

	C16	C17	C18	C19	C20	C21	C22
MW	564	564	578	578	594	594	594
E_1^+	547 (100) [a]	547 (100)	561 (100)	561 (100)	577 (100)	577 (100)	577 (100)
E_2^+	529 (80)	529 (24)	543 (39)	543 (20)	559 (38)	559 (36)	559 (23)
E_3^+	511(44)	511 (10)	525 (18)	525 (8)	541 (8)	541 (14)	541 (19)
E_4^+			507 (5)	507 (3)	523 (5)	523 (5)	523 (3)
$^{2,3}X^+_H$-$2H_2O$ / $^{0,4}X^+_P$–$2H_2O$	499 (11)	499 (30)	513 (35)	513 (28)	529 (18)	529 (22)	529 (19)
$^{2,3}X^+_H$-$3H_2O$	481 (8)	481 (12)	495 (23)	495 (10)	511(12)	511(18)	511(9)
$^{0,3}X^+_{H/}{}^{0,2}X^+_P$	**475 (6)**						
$^{0,4}X^+_H$–$2H_2O$		**469 (12)**	483 (11)	483 (14)	499 (4)	499 (18)	499 (8)
$^{1,5}X^+_P$ / $^{0,2}X^+_D$	**461 (8)**						
$^{0,2}X^+_P$–H_2O		**457 (18)**					
$^{0,2}X^+_H$/ $^{0,1}X^+_P$	445 (3)	445 (14)	459 (13)	459 (15)	475 (11)	475 (12)	475 (13)
$^{0,2}X^+_D$–H_2O			457 (30)	457 (19)		473 (28)	473 (20)
$^{0,2}X^+_H$–H_2O	**427 (50)**		441 (3)	441 (4)	457 (35)	457 (5)	457 (2)
$^{0,1}X^+_H$		**415 (3)**					
$^{0,2}\mathbf{X}^+_H$**–2H₂O**	**409 (10)**						

[a] *m/z* (relative abundance)

Table 4.4: Principal ESI-MS/MS product ions obtained from the $[M–H]^-$ ions of components **16-22**

	C16	C17	C18	C19	C20	C21	C22
MW	564	564	578	578	594	594	594
E_1^-	545 (20) [a]	545 (46)	559 (17)	559 (34)	575 (14)	575 (18)	575 (20)
E_2^-			541 (4)	541 (4)			
$^{0,3}X^-_P$	503 (10)	503 (38)					
$^{0,3}X^-_D$			503 (23)	503 (18)		519 (9)	519 (23)
$^{0,3}X^-_H$/ $^{0,2}X^-_P$	473 (76)	473 (100)	487 (29)	487 (72)	503 (35)	503 (16)	503 (21)
$^{1,5}X^-_P$ / $^{0,2}X^-_D$			473 (100)	473 (30)	489 (22)	489 (100)	489 (100)
$^{0,2}X^-_P$–H_2O	455 (8)	455 (12)					
$^{0,2}X^-_H$/ $^{0,1}X^-_P$	443 (100)	443 (59)	457 (75)	457 (100)	473 (100)	473 (14)	473 (20)
$^{0,2}X^-_D$–H_2O							
Y^-_P	431 (16)						
$^{0,2}X^-_H$–H_2O					455 (5)		
$[^{0,2}X_i{}^{0,3}X_j]^-$	383 (12)	383 (12)	383 (22)	383 (18)	383 (20)	399 (17)	399 (18)
$[^{0,2}X_i{}^{0,2}X_j]^-$	353 (20)	353 (30)	353 (30)	353 (28)	353 (40)	369 (10)	369 (12)

[a] *m/z* (relative abundance)

together with the molecular mass of 740 implied that components **23-25** most probably contained two hexose and one deoxyhexose units. For component **23**, the presence of a high abundant (relative intensity $> 90\%$) Y_H ion at *m/z* 577 signaled that an *O*-glycosidic hexose unit was attached to the aglycone. The $^{k,l}X$ fragments over *m/z* 600 are characteristic of *C*-glycosyl deoxyhexose and hexose units, directly linked to the aglycone (unconjugated, i.e., no other sugar unit attached to it). The same assumption can be drawn from the parallel cleavages of a hexose and deoxyhexose units after the *O*-glycosidic hexose is lost (mass range of 450-577). In conclusion, we propose component **23** as an apigenin-X-*O*-hexoside-Y-*C*-deoxyhexoside-Z-*C*-hexoside.

The low abundance (relative intensity $< 10\%$) of Y_H at *m/z* 577 and the presence of Z_H at *m/z* 559 (Fig. 4.10) together implied that in the case of components **24** and **25**, the *O*-glycosidic hexose unit was connected to another sugar residue. On the other hand, the $^{k,l}X$ fragments over *m/z* 600 indicated that the unconjugated *C*-glycosyl unit was a deoxyhexose, therefore the *O*-glycosidic hexose must have been attached to the other hexose unit. As no significant $^{0,2}X_H$, but high abundant Y_H–$^{0,2}X_H$ fragments were observed, we propose a 1→6 linkage between the hexose units. Regarding to the location of the hexosyl(1→6)hexose moiety, for C-6 isomers higher abundant Z^- ions were observed as suggested by Ferreres at al [96]. Thus, based on the significantly different intensity of *m/z* 559 in the MS/MS spectra of components **24** and **25** (Fig. 4.10), we considered them as 8-*C*- and 6-*C*- diglycosyl isomers, respectively. In conclusion, we suggest component **24** as apigenin-6-*C*-deoxyhexoside-(6''-*O*-hexosyl-8-*C*-hexoside), and component **25** as apigenin-(6''-*O*-hexosyl-6-*C*-hexoside)- 8-*C*-deoxyhexoside.

4.4.4 Chromatographic behavior

Besides the analysis of MS spectra, structural information can also be obtained from the chromatographic behavior of the components. Detailed analysis of the retention times (Tab. 4.1) further supported the proposed structures for the components of interest.

The retention times for *O*-glycosides (components **10-13**) inversely correlated to polarity as the triglycoside (component **13**) preceded the diglycosides (components **10-12**). As components **10-12** assumably possessed the same glycoside substituent, differences in their retention times could be explained by

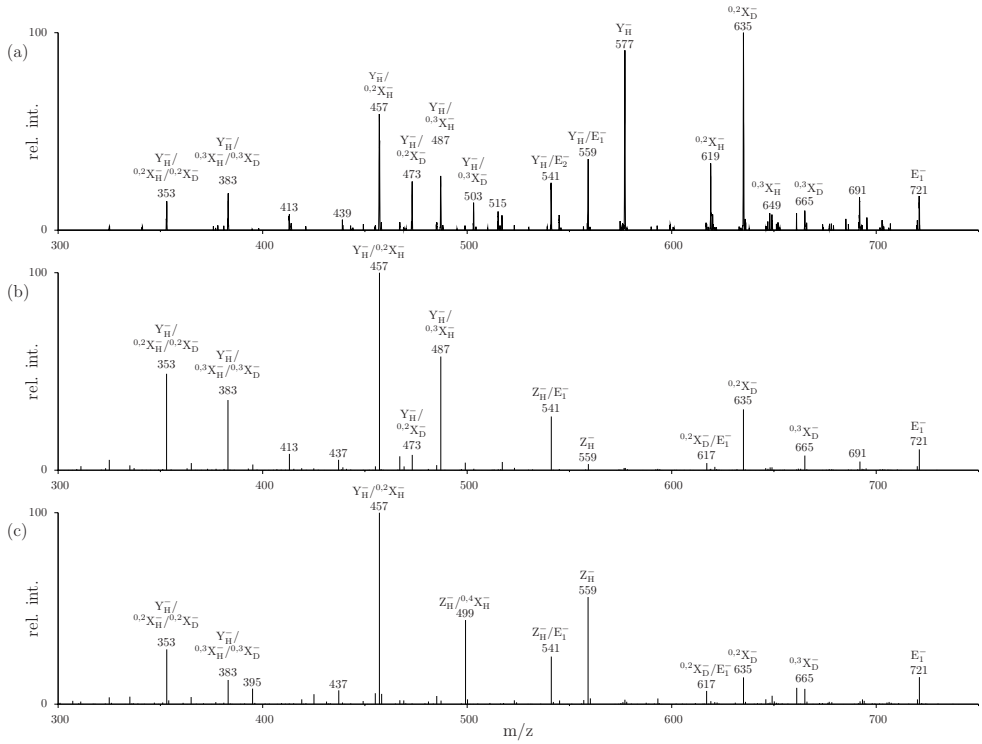


Figure 4.10: Negative ion MS/MS spectra of (a) component **23**, (b) component **24**, and (c) component **25**.

the different polarity of their aglycones. The quercetin-glycoside component **11** (two free hydroxyl groups on the B-ring) eluted first, followed by the kaempferol-glycoside component **10** (one free hydroxyl group on theB-ring) then the isorhamnetin-glycoside component **12** (a free hydroxyl and a metoxyl group on theB-ring).

For *C*-glycosides (components **14**-**22**) the polarity of the molecules were determined by the polarity of the aglycone and the conjugated sugar units. In general, hexosyl glycosides (four free hydroxyl groups in the sugar part) preceded pentosyl glycosides (only three hydroxyl groups), and pentosyl glycosides preceded deoxyhexosyl glycosides (three hydroxyl groups and a methoxyl substituent) in polarity. We observed the same elution tendencies for the di-*C*-glycosides (components **16**-**22**). For components **16**-**20**, which differed only in their sugar residues, the di-hexosyl isomer (component **20**) eluted first, followed by the pentosyl-hexosyl isomers (components **16** and **17**) then the (deoxyhexosyl)-hexosyl isomers (components **18** and **19**). In addition, the luteolin-glycoside components **21** and **22** preceded the apigenin-glycoside components **18** and **19**, in concordance with the fact that luteolin had two hydroxyl groups

on B-ring, while apigenin possessed only one. The examples of components **16-19** demonstrated that 8-*C*-hexosyl isomers eluted earlier than the 6-*C*-hexosyl isomers. Based on this fact, we propose component **21** as luteolin-6-*C*-deoxy-hexosisde-8-*C*-hexoside and component **22** as luteolin-6-*C*-hexoside-8-*C*-deoxy-hexoside.

4.5 Quantitative analyses of rutin

For the quantitative analysis of rutin an isocratic HPLC separation method was developed, reffered to as System 2. This rapid method still featured proper resolution of Peaks A and B (R_s= 1.95), which were identified by peak tracking [147] based on the results of NMR and LC-MS^n experiments as violanthin and rutin, respectively. The characterization of the LC method comprised linearity determination, in addition to accuracy and precision measurements. Quantitative determination of rutin was carried out using the *external standard calibration* technique [147], i.e., standard solution samples in a concentration range of 50-300 μg/mL were injected and measured. Good correlation (y = (30378$\pm$251)x-15422, R^2 = 0.9993) was obtained between the sample concentration (x) and the uncorrected peak area (y). Accuracy was verified by the so called *fortified sample recovery test* (R = 99.36 $\pm$ 0.06 %, RSD = 0.07 %, n = 3) [148]. Precision was tested by HPLC peak area reproducibility. Mean relative standard deviation values for the reference peak areas (n = 10) and the sample peak areas (n = 9) were 0.99 % and 1.58 %, respectively. According to our results, our heartsease herb sample contained 0.42$\pm$0.01 % rutin (RSD = 2.78 %, n = 6).

4.6 The antioxidant activity of heartsease

Antioxidants are molecules capable of quenching harmful free radicals, thus useful in the prophylaxis and treatment of more than eighty types of illnesses including cardiovascular problems, complications of diabetes, inflammations, immune disorders, and liver problems, just to list a few [74]. These illnesses occur if the body's oxidative balance is disturbed. Although natural antioxidants are present in the daily diet in moderate amount, there are cases when additional supplements are necessary to support the body's own antioxidant system. In recent years, considerable attention has been devoted to natural an-

tioxidants in order to replace synthetic ones. Due to its flavonoid and other phenolic compound content, heartsease may represent a promising source for natural antioxidants [39].

The antioxidant capacity of samples can be determined by *in vivo* and/or *in vitro* assays. The *in vitro* techniques are usually classified according to the mechanisms involved. Hydrogen atom transfer (HAT)-based methods and single electron transfer (SET)-based methods are both widely utilized [149]. The trolox equivalent antioxidant capacity (TEAC) [150] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [151] assays are decolorization based methods. Due to their advantages in ease of use and reproducibility they have been applied in many laboratories [149]. In these test systems colorful artificial free radicals ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) are generated and their concentration is determined before and after the introduction of samples. Antioxidant components quench the free radicals whose concentration decreases proportionally to the antioxidant capacity of the sample. The oxidized and reduced forms of the radical absorb at different wavelength maxima, which allows spectroscopic determination of the amount of radicals present. The TEAC and the DPPH assays were reported to characterize the electron-donor [150] and hydrogen donor [152] activity of the samples, respectively.

The antioxidant capacity of *Fractions B-G* were determined both by the TEAC and DPPH assays, whereas *Fraction A* could be studied only by the TEAC assay, since it was obtained by extraction with chloroform, and was apolar in character. The antioxidant capacity of the samples was characterized by calculating the so-called IC_{50} (inhibitory concentration) value; the concentration, which produces 50% quenching of the free radicals. The lower the IC_{50} value, the better antioxidant the sample is. The IC_{50} values for *Fractions A-G* are summarized in Tab. 4.5. Based on the results obtained, we can draw the following conclusions. The polar components of heartsease (*Fraction B*) show better antioxidant activity than the apolar ones (*Fraction A*). If *Fraction B* is cleared from the fast-eluting components (*Fraction C*), which show poor antioxidant activity, a purified flavonoid fraction was obtained (*Fraction D*) with significantly higher antioxidant capacity. The isolated main compound, violanthin (*Fraction E*) showed slightly worse electron-donor properties and approximately the same hydrogen-donor capacity as the unseparated flavonoid fraction, *Fraction D*. The isolated second main component, rutin (*Fraction F*), on the other hand,

Table 4.5: Antioxidant capacity of *Fractions A-G* determined by the TEAC and DPPH *in vitro* assays and characterized by the concentration, which produced 50% quenching of the free radicals

	TEAC assay		DPPH assay	
	$IC_{50}\pm$ SD(μg/mL)	RSD %	$IC_{50}\pm$ SD(μg/mL)	RSD %
Fraction A	6.08 ± 0.25	4.13	n. d.	
Fraction B	3.36 ± 0.16	4.70	13.05 ± 0.41	3.13
Fraction C	17.86 ± 1.28	7.14	52.97 ± 0.95	1.80
Fraction D	1.61 ± 0.05	3.23	7.99 ± 0.17	2.08
Fraction E	2.17 ± 0.14	6.52	7.28 ± 0.05	0.73
Fraction F	0.31 ± 0.01	2.50	11.36 ± 0.39	3.41
Fraction G	0.82 ± 0.03	3.61	4.03 ± 0.13	3.23

n.d. = not detected

exhibited significantly better electron-donor properties, but lower hydrogen-donor capacity. Reduction of the ratio of the two main components in the flavonoid fraction (*Fraction G*) resulted in increase in both the electron-donor and hydrogen-donor activities.

4.7 Comparison of garden pansy and heartsease

Garden pansies (*V. x wittrockiana* Gams.) are plants of complex hybrid origin involving at least three species, *V. tricolor*, *V. altaica* and *V. lutea*. They have several colorful varieties and are widely cultivated as ornamental plants. Albeit, they have been crossbred from *V. tricolor,* a well-know medicinal plant, their secondary metabolite composition has neither been studied nor compared with heartsease. In this thesis, besides a comparative HPLC study, the anthocyanidin and flavonoid contents as well as the antioxidant capacities of garden pansy and heartsease were compared.

Consequently, methanolic extracts were prepared from heartsease as well as herbs, flowers and leaves of several garden pansy varieties of violet, violet-white, white and yellow petal color (Method 4). All samples were analyzed by reversed phase HPLC (System 3) and representative chromatograms are shown

in Fig. 4.11. The chromatograms exhibit very similar peak distribution of all the samples with differences mostly observed in peak ratios. As most panels in Fig. 4.11 exhibit, the two major components were eluted at retention times of 14.0 (*Peak A*) and 15.0 minutes (*Peak B*).

By the different intensity of the individual components in the chromatogram, the samples were classified into the following two major groups of flower and herb/leaf samples. The main component of the flower samples (*Peak B* in Fig. 4.11 d and e) is proposed to be rutin, according to the HPLC and LC-MS experiments described above (Sec. 4.2, Sec. 4.3, and Sec. 4.4). The second group (Fig. 4.11a-c) consisting of the herb and leaf samples, apparently contained much lower quantities of rutin (*Peak B*), but was rich in violanthin (*Peak A*, identified by peak tracking [147]. The similarity between the herb and leaf samples can be associated with the fact that pansy herb samples consisted mostly of leaf (80%, determined by the weight of the fresh species) rather than flowers (20%, determined by the weight of the fresh species). The weight of the stem was negligible. The violet flower sample did not fit into this classification as it only possessed low amounts of rutin and violanthin, as depicted in Fig. 4.11f, thus in the violet flower samples the minor components gained more significance in the HPLC peak pattern.

In addition to their flavonoid content distribution, the actual anthocyanidin and flavonoid contents are distinctive of the samples. Anthocyanidins are natural pigments in plants, consequently, they are present in flowers (especially with colorful petals) in larger quantities than in leaves. The quantity of anthocyanidins and flavonoids were determined by applying the registered methods of the European Pharmacopoeia 5.0 (Tab. 4.6, Fig. 4.12) [143]. The highest anthocyanidin content was measured in violet pansy. Colorless varieties (yellow, white and white with a little violet) possessed only low amounts of anthocyanidins. On the other hand, in regard to the flavonoid content, species with pale petals showed elevated concentration. The highest amount was measured in yellow pansy flower. As Tab. 4.6 and Fig. 4.12 depicts, flower samples had higher flavonoid content than leaf samples, while herb samples were in between.

The antioxidant activity of the samples was determined by the TEAC decolorization assay, The antioxidant capacity was characterized by calculating the IC_{50} value (Tab. 4.6 , Fig. 4.12). The lower the IC_{50} value, the better the

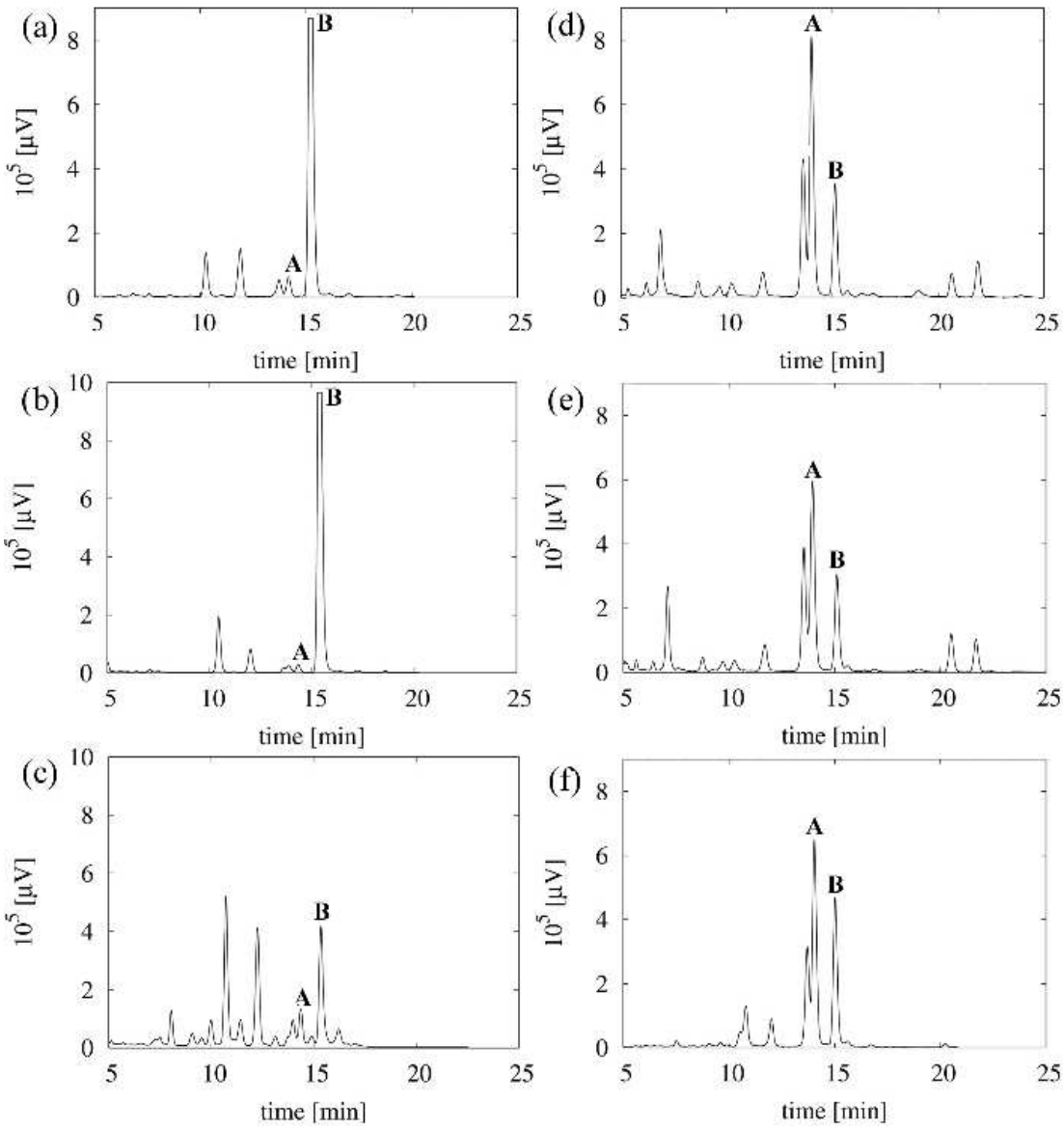


Figure 4.11: HPLC chromatograms of (a) violet-white pansy flower, (b) white pansy flower, (c) violet pansy flower, (d) yellow pansy herb, (e) white pansy leaf, and (f) heartsease herb.

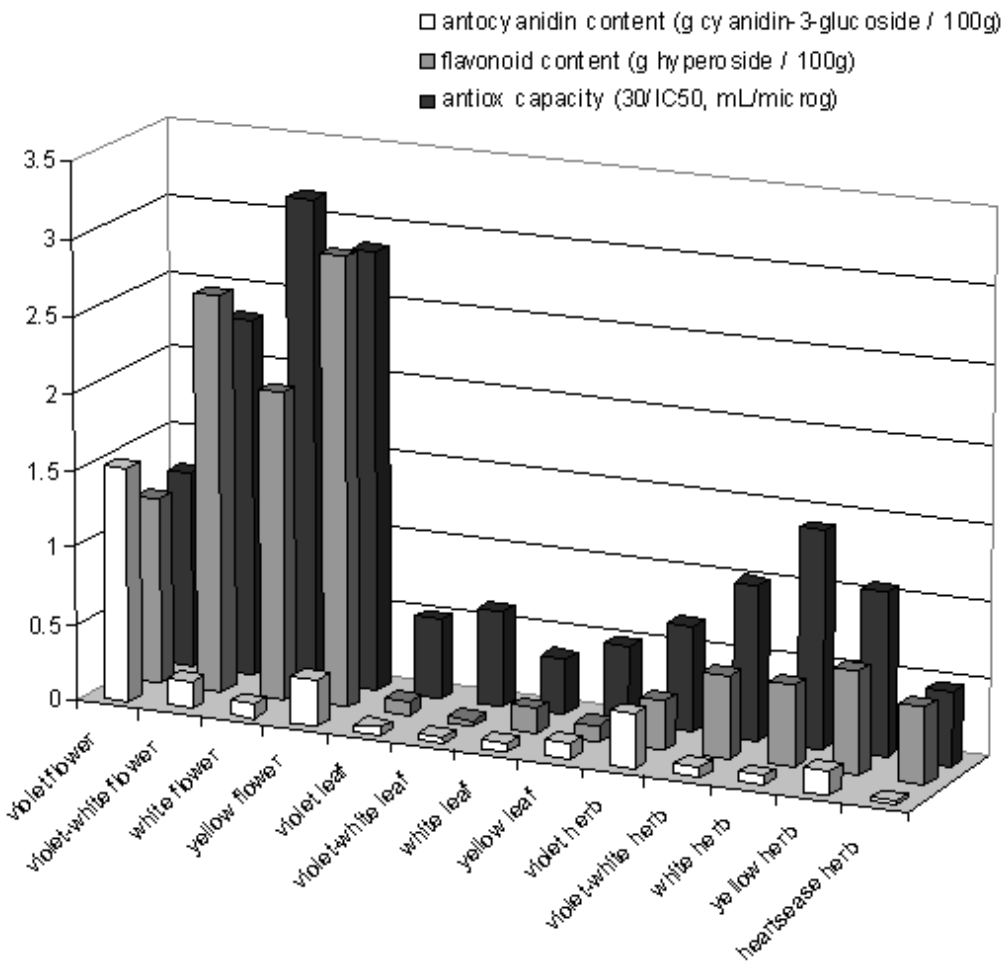


Figure 4.12: The antocyanidin and flavonoid contents, as well as the antioxidant capacity of heartsease and garden pansies of different petal color.

Table 4.6: The antocyanidin and flavonoid contents, as well as the antioxidant capacity of heartsease and garden pansies of different petal color.

		Anthocyanidin content (g cyanidin-3-glucoside/ 100 g sample)	Flavonoid content (g rutin/ 100 g sample)	IC_{50} value (g/ml)
Violet pansy	flower	1.52 ± 0.06	1.21 ± 0.07	(1.57 ± 0.05)x 10^{-5}
	leaf	0.05 ± 0.002	0.10 ± 0.006	(3.86 ± 0.18)x10^{-5}
	herb	0.31 ± 0.01	0.38 ± 0.02	(2.92 ± 0.04)x10^{-5}
Violet-white pansy	flower	0.19 ± 0.01	2.58 ± 0.15	(8.59 ± 0.25)x10^{-6}
	leaf	0.04 ± 0.002	0.03 ± 0.002	(3.18 ± 0.09)x10^{-5}
	herb	0.05 ± 0.004	0.62 ± 0.03	(1.99 ± 0.10)x10^{-5}
White pansy	flower	0.09 ± 0.004	2.01 ± 0.12	(6.35 ± 0.08)x10^{-6}
	leaf	0.06 ± 0.002	0.16 ± 0.01	(5.32 ± 0.12)x10^{-5}
	herb	0.08 ± 0.003	0.59 ± 0.03	(1.42 ± 0.06)x10^{-5}
Yellow pansy	flower	0.31 ± 0.01	2.93 ± 0.18	(6.98 ± 0.28)x10^{-6}
	leaf	0.11 ± 0.004	0.11 ± 0.007	(4.00 ± 0.09)x10^{-5}
	herb	0.11 ± 0.006	0.62 ± 0.04	(1.87 ± 0.05)x10^{-5}
Heartsease	herb	0.02 ± 0.0008	0.50 ± 0.03	(4.17 ± 0.10)x10^{-5}
Rutin				(0.12 ± 0.01)x10^{-5}

antioxidant activity of the sample. Our experiments revealed that in this test system the heartsease and pansy samples both showed good antioxidant activities. Apparently, their antioxidant capacity was at the same level as the well-known antioxidant ginkgo leaf of IC_{50}= (1.82 $\pm$ 0.07) x 10^{-5} mg/mL). In addition, the crossbred garden pansy possessed better antioxidant properties than its mother species, heartsease. Similarly to the flavonoid content, flower samples showed the highest antioxidant activity, whereas herb and leaf samples possessed inferior antioxidant properties according to the IC_{50} values attained. Based on this similarity, we examined the relationship between the antioxidant activity and the flavonoid content of the samples, and found significant correlation between the antioxidant capacity (IC_{50} value) and the flavonoid content (r= 0.6375, p=0.02). On the other hand, no correlation was found between the antioxidant capacity and the anthocyanidin content (r= 0.2798, p=0.35).

CHAPTER 5

Conclusion

Although heartsease (*Viola tricolor* L.) has been extensively used in the traditional medicine for centuries, its biological activities and secondary metabolite composition have hardly been investigated. The chemical composition of heartsease was studied as first in the literature to support the evidence-based determination of its biological activities. Our examinations covered the qualitative and quantitative analyses of heartsease's hydrophyl –potentially bioactive– constituents, the determination of their antioxidant capacities, as well as the comparison of heartsease with an other *Viola* species, namely garden pansy (*V. x wittrockiana* Gams.).

1. As the traditional internal administration of heartsease herb is as a tea, we primarily aimed at the thorough analyses of components, which are supposed to be present in this aqueous extract. Aqueous solutions, however, are microbiologically instable and difficult to handle (e.g. to evaporate). Henceforth, a methanol-applying extraction method was developed, yielding a fraction rich on polar constituents. The samness of heartsease methanolic and aqueous extracts' compositions was verified by TLC and HPLC analyses.

2. A fractionation protocol was developed for the separation of the potentially bioactive hydrophyl components. The methanolic extract enriched on polar components was separated by conventional Sephadex LH-20 or polyamide column chromatography. HPLC-UV analyses of the fractions suggested that the two main flavonoid components had been successfully isolated. In addition, purified flavonoid fractions containing only two-three components were obtained, suitable for the scouting LC-MS analy-

ses.

3. The main flavonoid component –contrary to former literature data– was identified by LC-MS^n and NMR studies as violanthin (6-*C*-glucosyl-8-*C*-rhamnosyl apigenin).

4. By comparing retention times, UV spectra, molecular masses, and fragmentation patterns with reference standard molecules, the second main flavonoid component was suggested to be rutin (3-*O*-rhamnoglucosyl quercetin).

5. In addition, another fourteen flavonoid glycosides were tentatively identified by LC-MS^n. 3 flavonoid *O*-, 7 *C*- and 3 *C,O*-glycosides with kaempferol, quercetin, isorhamnetin, crysoeriol, apigenin and luteolin aglycones were reported for the first time in heartsease. Besides, our results confirmed the presence of rutin, violanthin, isoorientin and vicenin-2.

6. Although violanthin could not be quantified in the lack of a commercially available reference molecule, rutin – the second main flavonoid component – was quantitatively determined by HPLC with UV detection. The examined heartsease herb samples contained 0.42$\pm$ 0.01) % rutin.

7. The antioxidant activity of heartsease herb was characterized as first in the literature. The antioxidant capacities (electron-donor and hydrogen donor activities) of the above described fractions were determined by the trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assays, respectively. In respect to their antioxidant properties, the highest electron-donor capacity was measured with isolated rutin, and a fraction enriched on flavonoid glycosides exhibited the highest hydrogen donor activity.

8. Garden pansies (*Viola x wittrockiana* Gams.) were characterized from a phytochemical point of view as first in the literature. Besides a comparative HPLC study, the anthocyanidin and flavonoid contents as well as the antioxidant capacities of garden pansies of different petal color and heartsease were compared. Apparently, similar major components were found in herb, leaf and flower samples, however, differences were observed in their component ratios. The main component of the flower samples was

proposed to be rutin, as the main component of the herb and leaf samples was suggested as violanthin.

9. The anthocyanidin and flavonoid contents of the samples were quantified by spectroscopic methods registered in the European Pharmacopoeia 5.0. While the highest anthocyanidin content was measured in the violet flower sample, the white and yellow pansy samples showed the highest flavonoid content.

10. The antioxidant capacity of the samples was determined by the TEAC assay. Our data revealed that in this test system the heartsease and pansy samples were as good antioxidants as the well-known antioxidant ginkgo leaf.

11. In addition, significant correlation was found between the flavonoid content and the antioxidant capacity of the samples supporting the assumption that the antioxidant activity of heartsease and of garden pansies are mainly due to their flavonoid coponents. On the other hand, no correlation was observed between the antioxidant capacity and the anthocyanidin content.

12. According to our study garden pansies provide a more valuable flavonoid source than heartsease. This fact –supported by further examinations– may bear also therapeutical significance.

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List of publications

Papers related to the thesis

1. **Viktoria Vukics**, Barbara Hevesi Toth, Thomas Ringer, Krisztina Ludanyi, Agnes Kery, Guenther K. Bonn, and Andras Guttman. Quantitative and qualitative investigation of the main flavonoids in heartsease (*Viola tricolor* L.). *J. Chrom. Sci.*, 46:97–101, 2008.

2. **Viktoria Vukics**, Agnes Kery, Gunther K. Bonn, and Andras Guttman. Major flavonoid components of heartsease (*Viola tricolor* L.) and their antioxidant activities. *Anal. Bioanal. Chem.*, 390:1917–1925, 2008.

3. **Viktoria Vukics**, Thomas Ringer, Agnes Kery, Guenther K. Bonn, and Andras Guttman. Analysis of heartsease (*Viola tricolor* L.) flavonoid glycosides by micro-liquid chromatography coupled to multistage mass spectrometry. *J. Chromatogr. A*, 1206:11–20, 2008.

4. **Viktoria Vukics**, Agnes Kery, and Andras Guttman. Analysis of polar antioxidants in heartsease (*Viola tricolor* L.) and garden pansy (*Viola x wittrockiana* Gams.). *J. Chrom. Sci.*, 46:823–827, 2008.

5. **Viktoria Vukics**, and Andras Guttman. Structural characterization of flavonoid glycosides by multi-stage mass spectrometry. *Mass Spectrom. Rev.*, Published Online: Dec 30 2008. DOI:10.1002/mas.20212

Other papers

1. **Vukics Viktória** és Kéry Ágnes. Metodikai gondok és lehetőségek flavonoid hatóanyagú kivonatok standardizálásánál. *Acta. Pharm. Hung.*, 75:

2. **Viktoria Vukics**, Barbara Hevesi Toth, Adam Fukasz, and Agnes Kery. Impact of flavonoid composition of medicinal plants: Difficulties in selecting an LC method. *Chromatographia Suppl.*, 63:93–100, 2006.

3. Barbara Hevesi Toth, Andrea Balazs, **Viktoria Vukics**, Eva Szoke, and Agnes Kery. Identification of *Epilobium* species and willow-herbs (Onagraceae) by HPLC analysis of flavonoids as chemotaxonomic markers. *Chromatographia Suppl.*, 63:119–123, 2006.

Talks

1. **Vukics Viktória**, Fukász Ádam, Blázovics Anna, Kéry Ágnes. XI. Magyar Gyógynövény Konferencia, Dobogókő, 2005. okt. 13-15: A vadárvácska és izolált fenoloidjainak antioxiáns hatása

2. **Viktoria Vukics** and Agnes Kery. II. PhD. Joint Meeting on Biomedical Sciences, Budapest, 6-7 November 2005: Antioxidant activity of wild pansy (*Viola tricolor*) L. and its phenolic constituents

Posters

1. Agnes Kery, Pal Apati, Andrea Balazs, Ildiko Papp, Eva Nagy, **Viktoria Andrasek**, Eva Szoke, and Anna Blazovics. 3. World Congress on Medicinal and Aromatic Plants, Thailand, Chiong Mai, 3-7 February 2003: Antioxidant Activity of Medicinal Plants in Different Systems. PP04-11

2. **Andrasek Viktória**, Apáti Pál, Balázs Andrea, Blázovics Anna, Papp Ildikó és Kéry Ágnes. Congressus Pharmaceuticus Hungaricus XII., Budapest, 2003. máj. 8-10. : Flavonoid tartalom, összetétel és antioxidáns hatás *Viola* fajokban. P-2

3. Hevesi T. Barbara, Balázs Andrea, **Vukics Viktória**, Szőke Éva és Kéry Ágnes. Semmelweis Egyetem PhD. Tudományos napok, Budapest, 2005. ápr. 14-15. : A kisvirágú füzike flavonoid összetételének és antioxidáns hatásának vizsgálata. PII/2

4. **Viktoria Vukics**, Anna Blazovics, Adam Fukasz, Agnes Kery. 1st BBBB Conference on Pharmaceutical Sciences, Siofok, 25-28 September 2005:

The antioxidant activity of *Viola tricolor* L. measured in different *in vitro* antioxidant systems. P-58

5. Barbara Hevesi Toth, Andrea Balazs, **Viktoria Vukics**, Eva Szoke, and Agnes Kery. 1st BBBB Conference on Pharmaceutical Sciences, Siofok, 25-28 September 2005: Flavonoid composition and antioxidant capacity of willow-herb. P-18

6. Agnes Kery, Bela Simandi, Ildiko Papp, Aniko Gava, **Viktoria Vukics**, Eva Lemberkovics, and Eva Szoke. 1st BBBB Conference on Pharmaceutical Sciences, Siofok, 25-28 September 2005: Quality of medicinal plant products prepared by supercritical fluid extraction. P-23

7. **Vukics Viktória**, Blázovics Anna, Fukász Ádam, Hevesi T. Barbara, Kéry Ágnes. A Magyar Szabadgyök-kutató Társaság III. Konferenciája, Debrecen, 2005. okt. 13-15. : A vadárvácska *Viola tricolor* L. antioxidáns hatásának vizsgálata.

8. **Viktoria Vukics** and Agnes Kery. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak, Budapest, 2005. okt. 19-21. : A vadárvácska *Viola tricolor* L. és néhány kertészeti árvácska faj összehasonlító vizsgálata. page 142

9. **Vukics Viktória**, Tory Kálmán, Kolonics Attila és Kéry Ágnes. Congressus Pharmaceuticus Hungaricus XII., Budapest, 2006. máj. 25-27. : Vadárvácska frakciók összetételének és biológiai hatásának vizsgálata. P-101

10. Alberti Ágnes, **Vukics Viktória**, Hevesi T. Barbara és Kéry Ágnes. Congressus Pharmaceuticus Hungaricus XII., Budapest, 2006. máj. 25-27. : Áfonya fajok fenoloidjainak ósszehasonlító vizsgálata. P-87

11. Gáva Anikó, Simándi Béla, Szarka Szabolcs, **Vukics Viktória**, Szőke Éva és Kéry Ágnes. Congressus Pharmaceuticus Hungaricus XII., Budapest, 2006. máj. 25-27. : Betulin és betulinsav vizsgálata *Alnus*, *Betula* és *Platanus* fajokban. P-89

12. **Viktoria Vukics**, Thomas Ringer, Agnes Kery, Gunther Bonn, and Andras Guttman. 8th Horvath Medal Award Symposium, Innsbruck, Austria,14-15. April 2008. : LC-MS^n analysis of flavonoid glycosides in heartsease (*Viola tricolor* L.) 37B

Quantitative and Qualitative Investigation of the Main Flavonoids in Heartsease (*Viola tricolor* L.)

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Abstract

Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (MS^n) is used for the analysis of flavonoids in heartsease (*Viola tricolor* L.). Our data suggested that the two main flavonoid components were violanthin (6-*C*-glucosyl-8-*C*-rhamnosyl apigenin) and rutin (3-*O*-rutinosyl quercetin). The identification of rutin was confirmed by comparing its retention time, UV spectrum, molecular mass, and fragmentation pattern with the reference standard. In this paper, we also report on the quantitative analysis of rutin by high-performance liquid chromatography. According to our results, heartsease herb contained 420 ± 1.17 µg/g rutin.

Introduction

Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. It has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic (1). Although the use of heartsease as herbal medicine goes back centuries, the biological activity of its secondary metabolites has hardly been studied. Reports deal only with heartsease extract's cyclic peptides' cytotoxicity (2) and its antimicrobial activity (3). Our knowledge about its composition and chemical structure is not complete either. Papers from as early as the 1980's report on compounds such as carotenoids (4–7), antocyanidins (8), and flavonoids (1) being present in heartsease.

Flavonoids represent an important group of secondary plant metabolites. They contain a three-ring C_{15} flavone skeleton and show great structural variety because of the plethora of possible hydroxyl, metoxyl, and glycosyl substituents. The *O*-glycosidic flavonoids have sugar moieties bound to a hydroxyl group, whereas the *C*-glycoside flavonoids have sugar substituents bound to a carbon of the aglycone. The structural characterization of flavonoid glycosides is usually performed by a combination of spectroscopic methods, including UV, nuclear magnetic resonance (NMR), and mass spectrometry (9). In contrast to off-line NMR techniques, which represents one of the most precise methods for chemical structure determination (including stereochemistry) of unknown components, other methods like liquid chromatography–mass spectrometry LC–MS do not require isolation of the target analyte, and thus offer a good choice for the analysis of crude plant extracts (10–12). The usual soft ionization techniques such as electrospray ionization (ESI) and fast atom bombardment (FAB) generate mainly protonated molecules. The molecular mass alone, however, is not sufficient for structural elucidation, therefore fragment information by collision-induced dissociation tandem mass spectrometry (CID-MS–MS) is necessary. A careful study of the fragmentation patterns obtained by CID-MS–MS can be of a particular value in the determination of the nature and site of attachment of important substitution groups such as sugars moieties (13–16).

In this paper, we report on the quantitative and qualitative investigation of the two major flavonoid components of heartsease extract, namely violanthin and rutin. These compounds were identified by regular LC–MS and nanoLC–MS, and rutin was quantitated by high-performance liquid chromatography (HPLC).

Materials and Methods

Chemicals and plant material

Chloroform, HPLC-grade acetic acid, acetonitrile, methanol, and water were purchased from Sigma-Aldrich (St. Louis, MO). Heartsease herb (*Viola tricolor* L.) (SN = 28-56-05-VI/24) was purchased from Fitopharma Ltd. (Budapest, Hungary).

Qualitative analysis of heartsease extract

Sample preparation

Dried and freshly powdered plant material (5.00 g) was sonicated two times with 50 mL chloroform for 25 min in an ultrasonic bath at 30°C. The plant residue was dried at room temperature and re-extracted two times with 40 mL methanol for 15 min in an ultrasonic bath at 30°C. The methanol extract

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was evaporated to dryness under reduced pressure at 55°C. Dry extract (5.3 mg) was re-dissolved in 5.3 mL mixture of 3.7 mL MeOH, and 1.6 mL 2.5% CH_3COOH and was purified by solid-phase extraction (SPE). The SPE cartridge (Supelclean LC-18, 500mg/6mL, Sigma-Aldrich) was activated with 5 mL MeOH, then with 5 mL 2.5% CH_3COOH. After introduction, the sample was washed with a mixture of 3.7 mL MeOH and 1.6 mL 2.5% CH_3COOH. The loading and washing solvents were collected in the same vial, and filtered on an Acrodisc Nylon 0.20-µm membrane Sartorius syringe filter (Sigma-Aldrich).

LC–MS and LC–MS^n conditions

For system 1, experiments were performed on an Agilent Technologies (Waldbronn, Germany) 1100 HPLC/MSD SL system which consisted of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the LC separation, gradient elution from 10% to 40% ACN in 30 min (A = 2.5 % CH_3COOH) was performed at a flow rate of 0.5 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm) (Sigma-Aldrich) column. The eluate was monitored with both the diode array (at 340 nm) and the mass selective detector. Scanning was performed from *m/z* 100 to 1000 in 0.2 min intervals. The mass selective detector was equipped with a normal-flow electrospray ionization (ESI) source. The electrospray conditions were as follows: drying gas flow, 13 L/min; drying gas temperature, 350°C; nebulizer pressure, 35 psi; capillary voltage, 3000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

For system 2, experiments were also performed on a Thermo Fisher LTQ (San Jose, CA) mass spectrometer, equipped with a nanoflow electrospray ionization source (Proxeon, Odense, Denmark). The sample was introduced directly to the ionization source. The electrospray conditions were as follows: spray potential, Ues = 2.02 kV; spray current, Ies = 0.67 µA; capillary temperature, 250°C; capillary voltage, 36.93 V. Scanning was performed from *m/z* 100 to 1000 in intervals. The Excalibur software (Thermo Fisher) was used for data acquisition and evaluation.

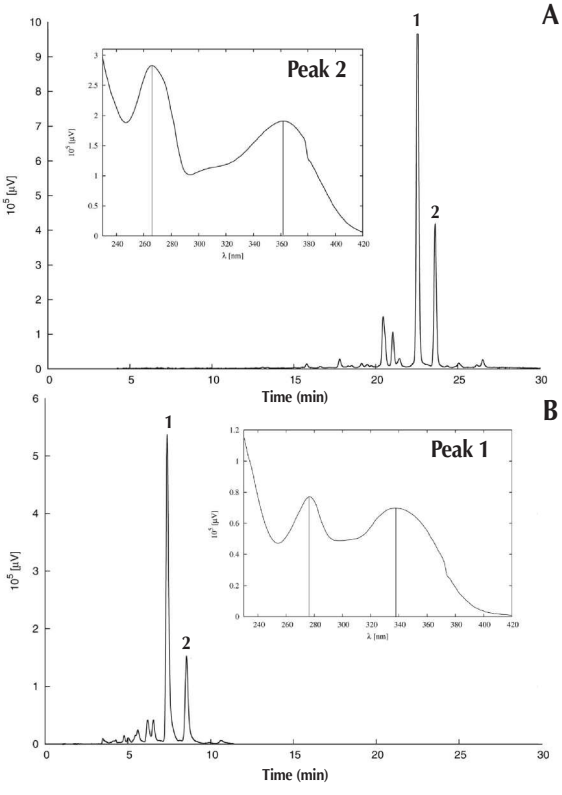

Figure 1. HPLC chromatograms of heartsease extract and the UV spectra of Peak 1 (violanthin) and 2 (rutin). A = System 1 (gradient elution from 10% to 40% ACN in 30 min [A = 2.5 % CH_3COOH) at a flow rate of 0.5 mL/min on a Hypersil ODS (250 x 4.6 mm, 5 µm) column], B = System 3 [isocratic elution with 20% ACN and 80% phosphoric acid (0.085%, pH = 2.2) at a flow rate of 0.8 mL/min on a Supelcosil TM LC-18 (250 x 4.6 mm, 5 µm) column].

Quantitative analysis of rutin

Sample preparation

Dried and freshly powdered heartsease plant material (1.25 g) was subject of continuous Soxhlet extraction (home-made Soxhlet apparatus) with 100 mL methanol for 9 h (exhaustive extraction). The extract was evaporated to dryness under reduced pressure at 55°C, weighed, and re-dissolved in 25 mL methanol (stock solution). Phosphoric acid (1.5 mL 0.085%) was added to 3.5 mL of the extract (diluted stock solution) and filtered on a MINIsart RC-15, 0.20-µm membrane syringe filter (Sigma-Aldrich). The filtered solution was ready to be analyzed.

HPLC conditions (system 3)

The ABLE-E & Jasco HPLC (Tokyo, Japan) apparatus consisted of an ERC-3113 degasser, an LG-980-02 solvent mixer, a PU-980 pump, and a 20 µL Rheodyne 7725 injector. The instrument was equipped with a UV-975 UV–vis detector. UV spectra were recorded during the HPLC separation by manually setting the recording time. A SUPELCOSIL TM LC-18 (250 × 4.6 mm, 5 µm, Sigma-Aldrich) column was applied during the experiments. Isocratic elution with 20% ACN and 80% phosphoric acid (0.085%, pH = 2.2) was performed at a flow rate of 0.8 mL/min. The eluate was monitored at 340 nm.

Fortified sample recovery test

1.2 mL of diluted stock solution (see the sample preparation section) was further diluted with 0.8 mL 70% methanol. This aliquot served as a blank solution in the fortified sample recovery test. Another 1.2 mL aliquot of the stock solution was diluted with 0.8 mL rutin solution (267 µg/mL, 70% MeOH), corresponding to the addition of 213.6 µg rutin. The recovery (R) was calculated as $R = 100(m_{found} - m_{initial})/m_{added}$; ($m_{found}$: rutin content in the fortified sample; $m_{initial}$: rutin content in the blank sample; m_{added}: added rutin amount).

Results and Discussion

Qualitative analysis of heartsease extract

The purified flavonoid fraction of heartsease methanol extract was first analyzed by normal-flow LC–MS (System 1). Good separation was obtained by applying gradient as elution shown in Figure 1A. Peaks A and B represent the two main flavonoid com-

ponents. By comparing retention time, UV spectrum, molecular mass and fragmentation pattern with the reference standard molecule (Table I), we suggest Peak B as rutin (Figure 2). The main component (Peak A) could not be identified under these particular MS conditions due to poor fragmentation; however, its molecular mass was determined as M = 578.2. Because the usual molecular mass of flavonoid aglycones ranges from 200 to 350, an apparent molecular mass larger than 500 indicates that Peak A is probably a conjugate.

As higher stage MS analysis provides insights about the chemical structure of the molecules of interest, Peak A was analyzed with MS–MS (System 2). Fragment ions with relative intensity higher than 1% are listed in Table II. For MS^2, *m/z* 579.14 was selected as precursor ion. The characteristic fragments might have derived from water losses and/or the cleavage of sugar units. This latter, together with the absence of Y_G^+,Y_R^+, and $Y_{R,G}^+$ (depicted in Figure 3, *m/z* 411.37, 433.37, and 271.24, respectively,) suggested that Peak A was not a di-*O*- but a di-*C*-glycosylflavonoid. In case of flavonoid-*C*-glycosides, the most usual connection site of the sugar unit is at position 6 or 8 (17) (Figure 2).

As Becchi et al. (15) described, the presence of $^{0,2}X^+$ and the absence of $^{0,3}X^+$ indicates that the component of interest is a di-*C*-hexosylflavonoid. Because in the MS–MS spectrum of Peak A $^{0,2}X^+_{hexose}$ (neutral loss = 120 Da) is a significant fragment, and $^{0,2}X^+_{pentose}$ (neutral loss = 90) is not detectable, we suggest that the component in Peak A is a di-*C*-hexosylflavonoid. As a first approximation, we can propose the presence of glucosyl and rhamnosyl moieties from the neutral losses of $[M+H–120]^+$ and $[M+H–104–H_2O]^+$, respectively. The early work of Li et al. (14) described how isomers whose structure differ only in the binding position of the sugar units (e.g., violanthin and isoviolanthin, Figure 2) can be differentiated with the help of characteristic fragment ions. Following their detailed analysis scheme of similar *C*-glycoside isomers the fragmentation of the 6-*C*-carbohydrate substituent generally results in a set of typical product ions: $^{0,4}X^+$-$2H_2O$, $^{0,2}X^+$, $^{1,5}X^+$, $^{0,1}X^+$, and Y^+. Similarly, the fragmentation of the 8-*C*-carbohydrate substituent gives rise to another group of characteristic product ions: $^{0,2}X^+$-H_2O, $^{0,2}X^+$-$2H_2O$, and $^{0,3}X^+$-$3H_2O$ (Table III). In case of Peak A, the fragment ions distinctive to glucose and rhamnose are $^{0,4}X^+$-$2H_2O$, $^{0,4}X^+$-$3H_2O$, $^{0,2}X^+$, $^{1,5}X^+$-$2H_2O$, $^{0,1}X^+$, and $^{0,2}X^+$-H_2O, respectively. Based on the analogy in the fragmentation patterns, we consider that one glucose unit is connected at position 6, and one rhamnose unit is connected at position 8 to ring C, respectively.

As demonstrated, water losses are explained by the elimination of water molecules from the 2‘’-hydroxyl groups of the sugar moieties and the 5- or 7-hydroxyl groups of the aglycone part (16). The high intensity of the E_1^+, E_2^+, and E_3^+ fragments, therefore, indicate the presence of hydroxyl groups either at position 5 or 7 or both. For further examinations we recorded the MS^3 spectra of *m/z* 337. This fragment ion bears special importance because it has lost both sugar moieties, and is therefore similar to flavonoid aglycones. Ma et al. (13) suggested three main fragmentation pathways for flavone and flavonol aglycones. According to their descriptions, the characteristic fragment ions for flavones and flavonols, which derive from the cleavage of ring C, are $^{1,3}B^+$, $^{0,2}B^+$, $^{0,4}B^+$, and $^{1,3}B^+$-2H, $^{0,2}B^+$, respectively (Figure 4). One can calculate from the molecular mass of $^{0,2}B^+$ that the presence of *m/z* 153, 137, 121, and 105 suggests that ring B has 3, 2, 1, or no hydroxyl substituents, respectively. Similarly, *m/z*

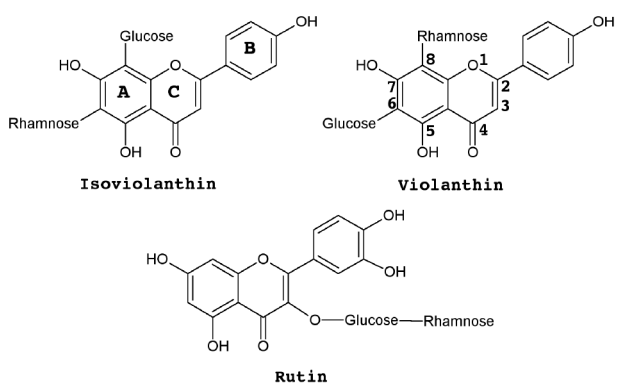

Figure 2. Chemical structures of isoviolanthin, violanthin, and rutin.

Table I. Fragment Ions of Peak A (Violanthin), Peak B (Rutin), and Rutin Reference Molecule (System 1)

Peak A *m/z* (RI* %)	Peak B *m/z* (RI %)	Rutin standard *m/z* (RI %)
581.2 (7)	633.2 (14)	633.2 (21)
580.2 (38)	–	613.2 (23)
579.2 (100)	–	612.2 (31)
449.0 (6)	611.2 (100)	611.2 (100)
121.0 (7)	593.4 (8)	593.4 (4)
–	465.2 (27)	465.2 (29)
–	–	316.4 (24)
–	303.0 (22)	303.0 (37)
–	–	288.2 (39)
–	–	202.2 (7)

* RI = relative intensity.

Table II. Fragment Ions Obtained by MS–MS analysis of *m/z* 579.14 (System 2)

Label	Structure	MS^2 579.14
E_1^+	$[M+H-H_2O]^+$	561.17 (100)
E_2^+	$[M+H-2H_2O]^+$	543.25 (29)
E_3^+	$[M+H-3H_2O]^+$	525.17 (12)
$^{2,3}X_{G,R}^+$-$2H_2O$	$[M+H-30-2H_2O]^+$	513.17 (28)
	$[M+H-2C_2H_2O]^+$	495.17 (12)
$^{0,4}X_G^+$-$2H_2O$	$[M+H-60-2H_2O]^+$	483.25 (11)
$^{0,4}X_G^+$-$3H_2O$	$[M+H-60-3H_2O]^+$	465.2 (5)
$^{0,2}X_G^+$	$[M+H-120]^+$	459.25 (14)
$^{0,2}X_R^+$-H_2O	$[M+H-104-H_2O]^+$	457.17 (24)
$^{0,2}X_G^+$-H_2O	$[M+H-120-H_2O]^+$	441.1 (4)
$^{0,1}X_G^+$	$[M+H-150]^+$	429.0 (1)
$^{1,5}X_G^+$-$2H_2O$	$[M+H-134-2H_2O]^+$	409.1 (1)

151, 135, 119, and 103 ($^{1,3}B^+$) indicates the presence of 3, 2, 1 and no hydroxyl substituents on ring B. The *m/z* 149, 133, and 117 ($^{1,3}B^+$-2H) demonstrates that ring B has 2, 1 or no hydroxyl substituents and that the molecule is a flavonol (3-hydroxyl substituent). In the MS^3 spectrum of *m/z* 337, the fragment ion of *m/z* 121 is significant (relative intensity = 14%), whereas the relative intensity of the fragment ion *m/z* 117 is lower than 1%. Accordingly, we propose that the component in Peak A has only one hydroxyl substituent on ring B and has no hydroxyl substituent on ring C at the position 3. As a conclusion, the component in Peak A is apparently violanthin (Figure 2).

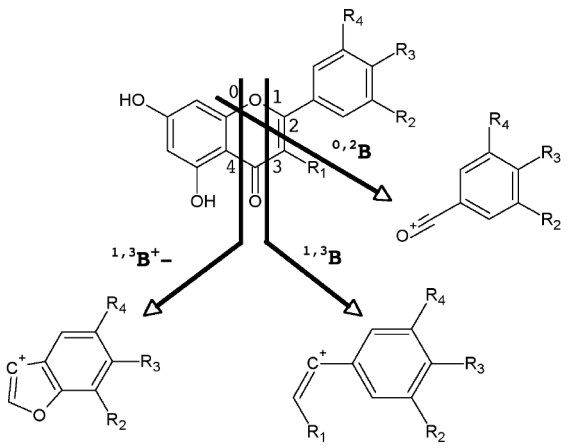

Figure 4. Suggested fragmentation patterns.

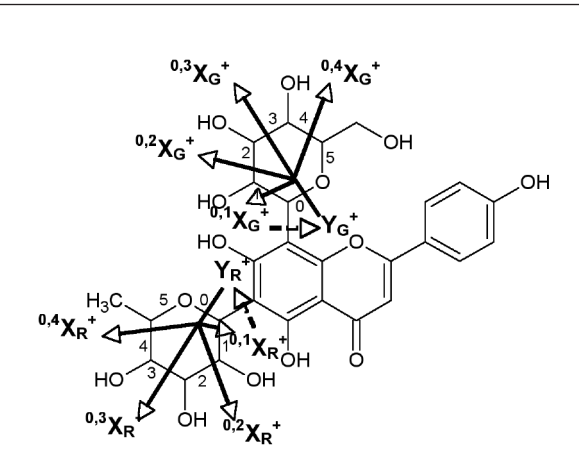

Figure 3. Characteristic carbohydrate cleavage patterns.

Table III. Characteristic Carbohydrate Fragments (System 2)*

A* Fragments characteristic of:		B† Fragments characteristic of:	
6-*C*-carbohydrate substituent	8-*C*-carbohydrate substituent	glucose	rhamnose
$^{0,4}X^+$-$2H_2O$	–	$^{0,4}X^+$-$2H_2O$, $^{0,4}X^+$-$3H_2O$	–
$^{0,2}X^+$	–	$^{0,2}X^+$	–
$^{1,5}X^+$	–	$^{1,5}X^+$-$2H_2O$	–
$^{0,1}X^+$	–	$^{0,1}X^+$	–
Y^+	–	–	–
	$^{0,2}X^+$-H_2O	–	$^{0,2}X^+$-H_2O
	$^{0,2}X^+$-$2H_2O$	–	–
	$^{0,2}X^+$-$3H_2O$	–	–

* A = described in the literature (14).
† B = obtained by the MS–MS analysis of *m/z* 579.14.

Quantitative analysis of rutin

For the quantitative analysis of rutin, an isocratic HPLC separation method was developed, referred to as System 3 (Figure 1B). This rapid method still featured proper resolution of Peaks A and B (R_s = 1.95). The validation process of the method is comprised of linearity determination, in addition to accuracy and precision measurements. Quantitative determination of rutin was carried out using the external standard calibration technique (18), that is, standard solution samples in a concentration range of 50–300 µg/mL were injected and measured. Good correlation [$y = (30378 \pm 251)x - 15422$, $R^2 = 0.9993$] was obtained between the sample concentration (x) and the uncorrected peak area (y). Accuracy was verified by the so called fortified sample recovery test (R = 99.36 ± 0.06 %, RSD = 0.07 %, n = 3) (19). Precision was tested by HPLC peak area reproducibility. Mean relative standard deviation values for the reference peak areas (n = 10) and the sample peak areas (n = 9) were 0.99% and 1.58%, respectively. According to our results, our heartsease herb sample contained 420 ± 1.17 µg/g rutin (RSD = 2.78 %, n = 6).

Conclusion

In this work, the two main flavonoid components of heartsease's crude methanol extract were analyzed by regular LC–MS and nanoLC–MS^n. Multistage mass spectral data analysis suggested one of the main components as violanthin. Identification of the second main flavonoid component as rutin was attempted by direct comparison of its retention time, UV, and multistage mass spectral data with a conventionally available standard. Our quantitative HPLC analysis revealed that the heartsease herb sample contained 420 ± 1.17 µg/g rutin.

Acknowledgment

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ORIGINAL PAPER

Major flavonoid components of heartsease (*Viola tricolor* L.) and their antioxidant activities

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Abstract Sephadex LH-20 column chromatography was used to separate flavonoid components in a heartsease methanol extract. One of the main components was identified by NMR as violanthin (6-*C*-glucosyl-8-*C*-rhamnosylapigenin). As a first approximation, the other main flavonoid component was considered to be rutin (3-*O*-rhamnoglucosylquercetin), based on comprehensive comparison of retention times and UV spectra of reference molecules, as well as molecular mass and fragmentation patterns obtained by mass spectrometry. The minor flavonoids were separated by polyamide column and analyzed by LC-MS. The antioxidant capacity of different flavonoid fractions was determined using both Trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) in vitro antioxidant assays. The highest electron-donor capacity was found for the major flavonoid component (rutin), whereas one minor component-rich flavonoid fraction exhibited the highest hydrogen-donor activity.

Keywords Flavonoid isolation · Column chromatography · NMR · LC-MS · Antioxidants

Abbreviations

COSY	correlation spectroscopy
HMBC	heteronuclear multibond correlation
HSQC	heteronuclear single-quantum coherence
NOESY	nuclear Overhauser effect spectroscopy
TOCSY	total correlation spectroscopy

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Introduction

Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. It has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic [1]. Although the use of heartsease as herbal medicine goes back centuries, the biological activity of its main secondary metabolites has hardly been studied. Reports deal only with the cytotoxicity of its cyclic peptides [2] and its antimicrobial activity [3]. Our knowledge in regards to the chemical structures of its main components is also very limited. Papers from as early as the 1980s report on carotenoids [4–7], anthocyanidins [8], and flavonoids [1] being present in heartsease.

For the preparative separation of crude plant extracts, conventional column chromatography methods are widely used. To partition polar components, such as flavonoids or other phenoloids, stationary phases of polyamide, cellulose, silica gel, and Sephadex LH-20 were mostly reported [9], most often using aqueous alcoholic solutions (methanol/water or ethanol/water) for their elution. Owing to the good light absorption and fluorescence properties of flavonoids [10], their chromatographic separation can easily be detected. The exact compositions of the fractions are usually determined by analytical tools such as HPLC, LC-MS, NMR, LC-NMR, CE, and CEC [11, 12]. However, these methods often require the aid of reference standard molecules. If needed, a second separation step of column chromatography, preparative TLC, or even preparative HPLC can be utilized. In general, there are no well-defined single isolation strategies for the separation of flavonoids and usually one or more steps are necessary for their complete isolation.

The antioxidant capacity of flavonoids can be determined by in vivo and/or in vitro assays. The in vitro techniques are classified according to the mechanisms involved. Hydrogen atom transfer (HAT)-based methods and single electron transfer (SET)-based methods are both widely utilized [13]. The Trolox equivalent antioxidant capacity (TEAC) [14] and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [15] characterize the electron-donor [14] and hydrogen-donor properties [16] of samples, respectively. These assays have been applied in many laboratories owing to their advantages in terms of ease of use and reproducibility [13].

In 1965 Kolosne Pethes et al. isolated rutin from heartsease by paper chromatography [17] and reported it to be its main flavonoid component. However, considering the poor resolution power of paper chromatography it is very likely that other structurally similar flavonoid components will coeluate with rutin. The application of state-of-the-art LC-MS techniques can, however, confirm some uncertainties connected to older separation techniques. In this paper we report on the isolation and identification of the major flavonoid components of heartsease and their antioxidant capacities.

Materials and methods

Chemicals and plant material

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heartsease herb (*Viola tricolor* L.; SN= 28-56-05-VI/24) was purchased from Fitopharma Ltd. (Budapest, Hungary).

Extraction

A 5.0-g batch of dried and freshly powdered plant material was sonicated twice with 50 mL chloroform for 25 min in an ultrasonic bath at 30 °C. The chloroform extract, referred to as fraction A (Fig. 1) was evaporated to dryness under reduced pressure at 30 °C. The plant residue was dried at room temperature and re-extracted twice with 40 mL methanol for 15 min in an ultrasonic bath at 30 °C. The methanol extract referred to as fraction B (Fig. 1) was evaporated to dryness under reduced pressure at 40 °C.

Column chromatography

Separation of fraction B by Sephadex LH-20 column chromatography The stationary phase bed was prepared by equilibrating Sephadex LH-20 beads for at least 24 h in 50% methanol. After transferring the slurry to the column (glass, homemade, 35×1.5 cm) the bed (final geometry 28 cm×1.5 cm) was allowed to settle. A 0.2-g dry fraction B was dissolved in 2 mL 50% methanol and introduced onto the column. Elution was carried out at a flow rate of 1.0 mL min^{-1} with 21 mL 50% methanol then 10 mL 70% methanol and 100% methanol until no more component was detectable.

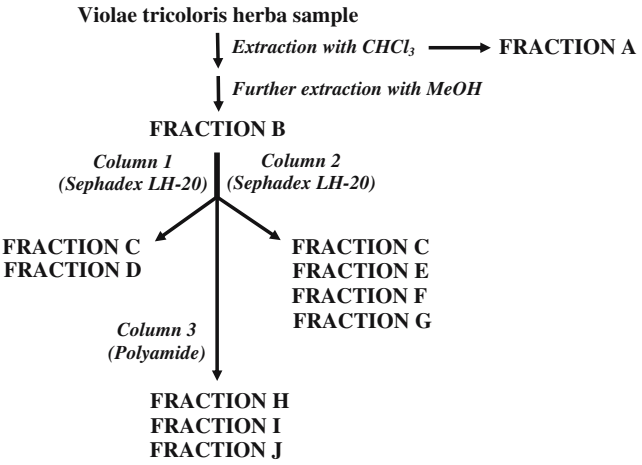

Fig. 1 Fractionation protocol developed for the isolation of the two main flavonoids (*fractions E* and *F*) and for the separation of the minor flavonoid components (*fractions H–J*) from heartsease methanol extract. *Column 1* and *2* homemade glass columns, 35 cm×1.5 cm, Sephadex LH-20 bed (final geometry 28 cm×1.5 cm). Elution with different mixtures of methanol and water. *Column 3* homemade glass column, 35 cm×1.5 cm, polyamide bed (particle size 50–160 μm, final geometry 31 cm×1.5 cm). Elution with different mixtures of methanol and water

In the case of column 1 the eluate was collected as follows: fraction 1=8 mL, fraction 2=7 mL, fraction 3=until the column became clear. In the following sections, fraction 2 will be referred to as fraction C and fraction 3 as fraction D.

In the case of column 2 the eluate was collected as follows: fraction 1=8 mL, fraction 2=7 mL, fraction 3= 3 mL, fraction 4=8 mL, fraction 5=14 mL, fraction 6=2 mL. As above, fraction 2 will be referred to as fraction C, fraction 4 as fraction E, and fraction 6 as fraction F. Fractions 3 and 7 were combined and referred to as fraction G.

Separation of fraction B by polyamide column chromatography (column 3) For the stationary phase we made a suspension of polyamide (Sigma-Aldrich, particle size 50–160 μm) in water. After transferring the slurry to a homemade glass column (35×1.5 cm) the bed (final geometry 31 cm×1.5 cm) was allowed to settle. A 0.2-g dry fraction B was dissolved in 2 mL 50% methanol and introduced onto the column. Elution was carried out at a flow rate of 1.25 mL min^{-1} with 40 mL water, followed by 80 mL 10% methanol, 45 mL 20% methanol, 40 mL 30% methanol, 20 mL 40% methanol, 20 mL 50% methanol, 20 mL 60% methanol, 10 mL 70% methanol, 10 mL 80% methanol, 10 mL 90% methanol, and finally by 100% methanol until no more component was eluted.

The eluate was collected as follows: fraction 0=76 mL, fraction 1=20 mL, fraction 2=24 mL, fraction 3=24 mL, fraction 4=8 mL, fraction 5=32 mL, fraction 6=20 mL, fraction 7=16 mL, fraction 8=32 mL, fraction 9=32 mL, fraction 10=56 mL. Fraction 3 will be referred to as fraction H, fraction 8 as fraction I, and fraction 10 as fraction J.

HPLC separation and LC-MS analysis

System 1 The ABLE-E & Jasco HPLC (Tokyo, Japan) apparatus consisted of an ERC-3113 degasser, an LG-980-02 solvent mixer, a PU-980 pump, and a 20-μL Rheodyne 7725 injector. The instrument was equipped with a UV-975 UV–Vis detector. UV spectra were recorded during the HPLC separation by manually setting the recording time. For the LC separation, gradient elution from 13 to 18% acetonitrile (ACN) in 20 min (A=0.5% H_3PO_4) was performed at a flow rate of 1.5 mL min^{-1} on a Hypersil ODS (250×4.6 mm, 5 μm; Sigma-Aldrich) column. Before injection, each sample was filtered on an Acrodisc PVDF 0.20-μm membrane Sartorius syringe filter (Sigma-Aldrich).

System 2 Experiments were performed on an Agilent Technologies (Waldbronn, Germany) 1100 HPLC/MSD SL system which consisted of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the LC separation, gradient elution from 10 to 40% ACN in 30 min (A=2.5% CH_3COOH) was performed at a flow rate of 0.5 mL min^{-1} on a Hypersil ODS (250×4.6 mm, 5 μm; Sigma-Aldrich) column. The eluate was monitored at 340 nm and by the mass-selective detector. Scanning was performed from *m*/*z* 100 to 1,000 in 0.2-min intervals. The mass-selective detector was equipped with a normal-flow electrospray ionization (ESI) source. The electrospray conditions were as follows: drying gas flow 13 L min^{-1}, drying gas temperature 350 °C, nebulizer pressure 35 psi, capillary voltage 3,000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

NMR analysis

Fraction E (18 mg) was dissolved in 1 mL DMSO-d_6 and characterized on a two-channel Varian Inova 600-MHz (Palo Alto, CA) NMR spectrometer equipped with a waveform generator, a pulsed field gradient (PFG) unit, and a dual inverse broadband probe head. In addition to registering the ^{1}H-NMR and ^{13}C-NMR spectra standard 2D experiments, i.e., correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multibond correlation (HMBC), and selective 1D TOCSY–TOCSY and TOCSY–NOESY experiments were applied for assignment. All spectra were calibrated to internal tetramethylsilane (TMS).

TEAC assay

A 7 mM aqeous solution of 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was prepared by dissolving in water. The radical monocation of ABTS was produced by reacting the ABTS solution with 2.45 mM (final concentration) potassium persulfate and letting the mixture stand in the dark at room temperature for at least 12 h before use. The $ABTS^{\cdot+}$ stock solution was diluted with spectroscopic grade ethanol to 0.9 absorbance units at 734 nm. At least four different volumes of the diluted sample were added to 2.5 mL ABTS solution resulting in different final concentrations and producing inhibition of the blank solvent between 20 and 80%. Absorbance values were measured at 734 nm after 0, 0.5, 0.66, 0.83, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 min. For the determination of the so-called inhibition percentage, we extrapolated the final absorbance by numerically solving the simplest possible reaction kinetics model. The inhibition percentage produced by a given sample concentration was calculated as $A_t/A_0 \times 100$ (A_t is the extrapolated final absorbance at time t, A_0 is the absorbance of the blank solvent). The antioxidant activity was characterized by plotting the inhibition percentage of the samples as a function of sample concentration followed by linear regression (data not shown). The concentration resulting in 50% inhibition is referred to as the IC_{50} value.

DPPH assay

A 0.25 g L^{-1} solution of DPPH was prepared by dissolving in HPLC grade methanol. The DPPH stock solution was diluted with HPLC grade methanol to 0.9 absorbance units at 515 nm. At least four different volumes of the diluted sample were added to 2.5 mL DPPH solution resulting in different final concentrations and producing inhibition of the blank solvent between 20 and 80%. Absorbance values were measured at 515 nm after 0, 0.5, 0.66, 0.83, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 min. The inhibition percentage was calculated as for the TEAC assay.

Results and discussion

Extraction

The traditional internal administration of heartsease herb is as a tea, i.e., by preparing an infusion. This water extract is supposed to contain the polar constituents, e.g., phenolic

acid derivatives, flavonoids, tannins, and peptides. Water solutions, however, are microbiologically unstable and difficult to handle (e.g., due to evaporatation). Hence, after pre-extraction with chloroform (Fig. 1), producing fraction A, a methanol extract was also prepared (fraction B) from heartsease herb. The solubility profiles of the water and methanol extracts were identical, as checked by HPLC (data not shown). Fraction B was further separated by conventional Sephadex and polyamide column chromatography as displayed in Fig. 1.

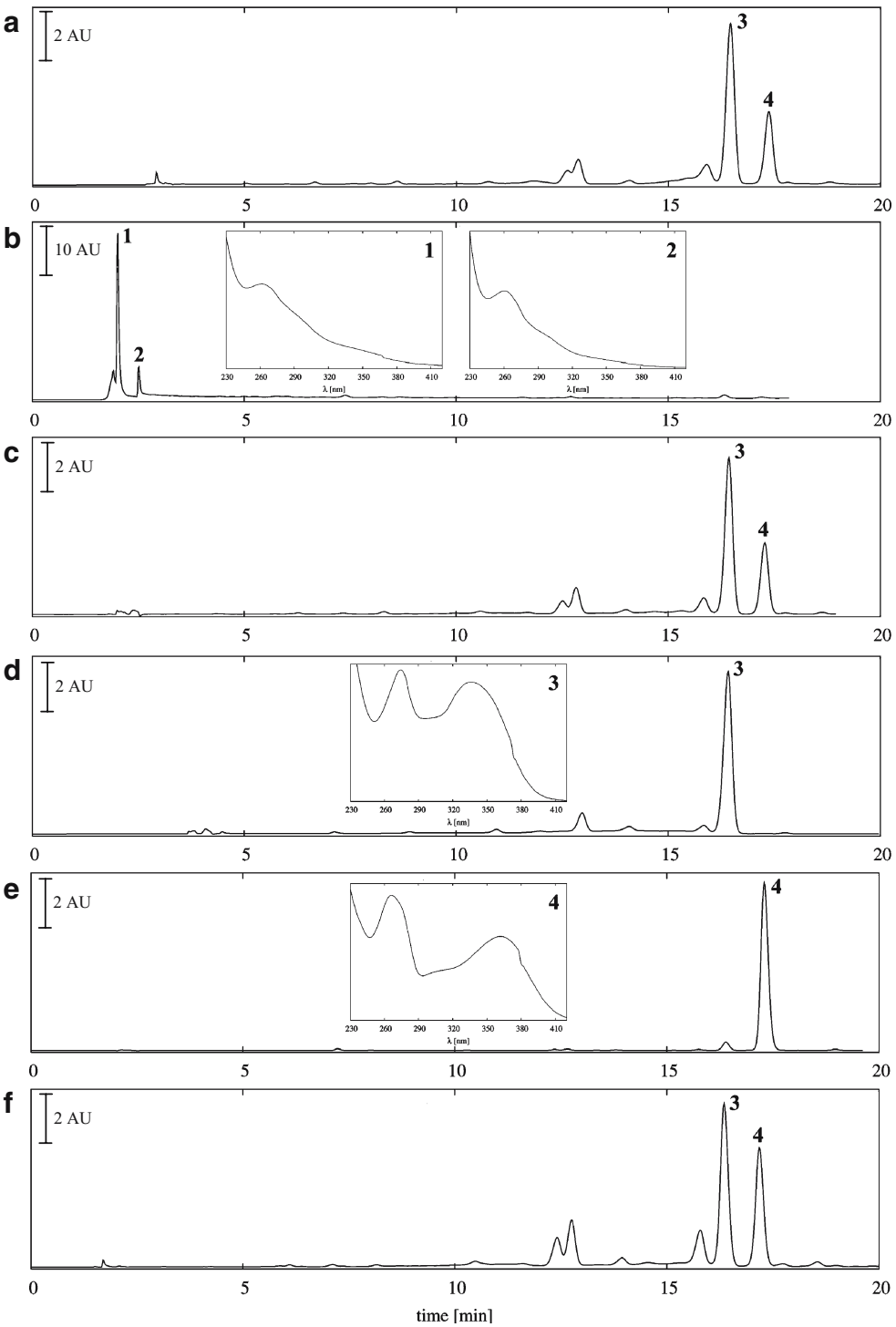

Fig. 2 HPLC chromatograms of the fractions obtained by Sephadex LH-20 column chromatography (columns 1 and 2) and UV spectra of the characteristic components: **a** fraction B, **b** fraction C, **c** fraction D, **d** fraction E, **e** fraction F, **f** fraction G. For the LC separation, gradient elution from 13 to 18% ACN in 20 min (A=0.5% H_3PO_4) was performed at a flow rate of 1.5 mL min^{-1} on a Hypersil ODS (250×4.6 mm, 5 μm) column

Column chromatography and qualitative analysis of the fractions

The preparative separation of the methanol extract of heartsease (Fig. 1, fraction B) was carried out by Sephadex LH-20 column chromatography. In the preliminary experiments, fractions of identical volumes were collected and the composition of the fractions was screened by thin-layer chromatography (TLC). Based on the TLC results we developed the fractionation protocol described under Materials and methods and delineated in Fig. 1. This fractionation method afforded fractions C–F, which together with fraction B were analyzed by HPLC (Fig. 2a–f). The chromatograms (system 1) were achieved as a result of gradient elution from an RP-C18 column detected at 340 nm, a distinctive wavelength for flavonoid analysis [10]. The UV spectra of the characteristic components were also recorded.

The HPLC chromatogram of fraction B (Fig. 2a) shows the composition of the methanol extract. Fraction C (chromatogram and UV spectra in Fig. 2b) comprises the fast-eluting components of fraction B. Because size-exclusion, adsorption, and partition mechanisms apply to specific Sephadex gels [9], fraction C was assumed to contain large molecules and/or highly polar molecules. The retention times and the UV spectra of components **1** and **2** (from Fig. 2) clearly suggest that these components do not belong to the flavonoid family. The UV spectra of flavonoids in polar organic solvents (such as methanol or acetonitrile) exhibit two major absorption maxima in the region 240–400 nm [10], whereas components **1** and **2** have absorption maxima only at 260 nm. With column 1, the flavonoid components of the methanol extract were collected together as fraction D (Fig. 2c). With column 2, however, these flavonoids were separated. Fraction E (chromatogram and UV spectrum in Fig. 2d) consists mainly of the main flavonoid component of the extract (component **3**). In fraction F (chromatogram and UV spectrum in Fig. 2e) the second main flavonoid (component **4**) is isolated. The UV spectra of components **3** and **4** present typical flavonoid

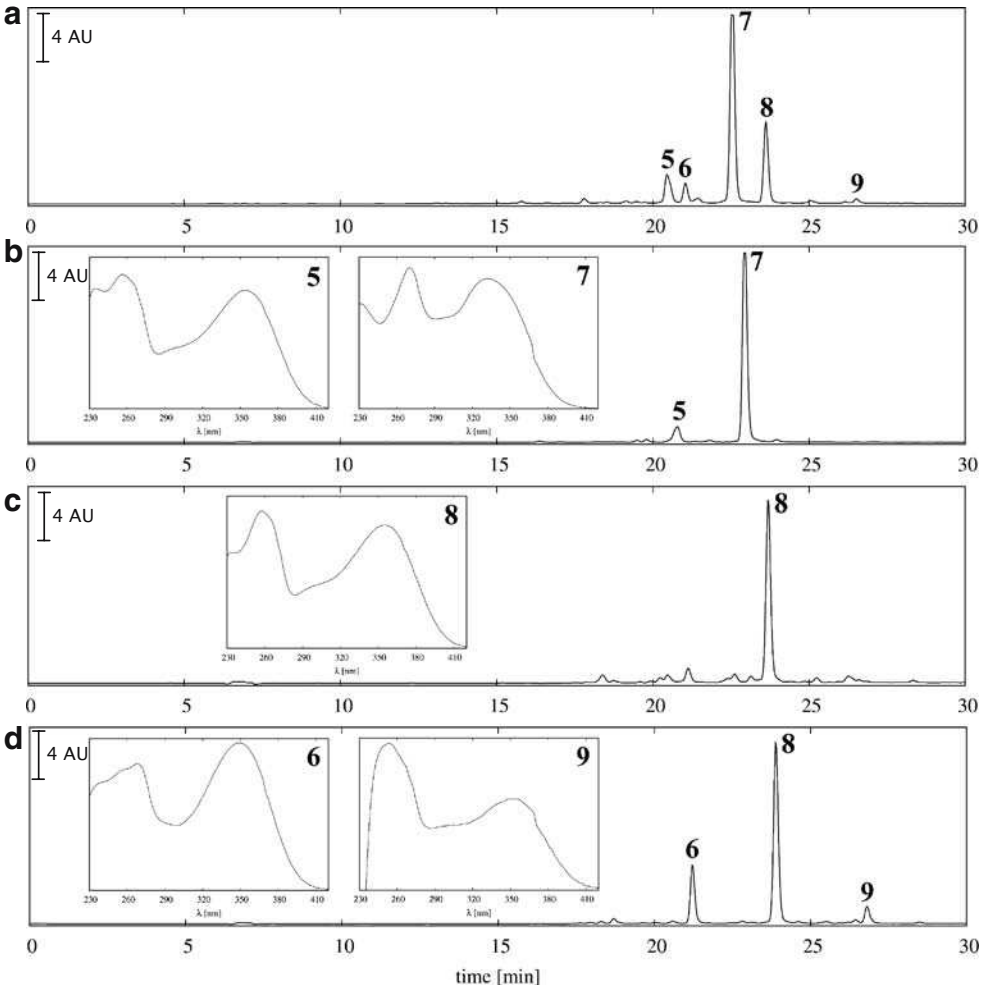

Fig. 3 HPLC chromatograms of the fractions obtained by polyamide column chromatography and UV spectra of the characteristic components of **a** fraction B, **b** fraction H, **c** fraction I, **d** fraction J. For the LC separation, gradient elution from 10 to 40% ACN in 30 min (A=2.5% CH_3COOH) was performed at a flow rate of 0.5 mL min^{-1} on a Hypersil ODS (250×4.6 mm, 5 μm) column

absorption traces with maximums at 274 and 336 nm, as well as at 266 and 361 nm, respectively.

In fraction G (chromatogram and UV spectrum in Fig. 2f) components **3** and **4** were present at lower quantities, representing relatively more concentrated minor flavonoid components. This fraction, however, could not be separated by Sephadex LH-20 column chromatography, not even by systematically changing the elution protocol. Thus, we tried changing the chromatographic stationary phase. Polyamide column chromatography of fraction B resulted, among others, in fractions H–J. These fractions were analyzed by HPLC and LC-MS using system 2, and their compositions were compared with the methanol extract (Fig. 3). Fractions H–J (chromatograms in Fig. 3b–d) contain five components of fraction B in three combinations: components **5** and **7**; component **8**; and components **6**, **8**, and **9**, respectively. As a first approximation, their UV spectra suggests that components **5**–**9** are flavonoids.

The LC-MS analysis of components **5**–**9** using system 2 did not provide sufficient data for structure elucidation (Fig. 4). However, molecular mass information of components **5**–**9** (MW=594, 448, 578, 611, 662, respectively), and some information about their glycosylation types were obtained. Their molecular masses were higher than 400 Da and the lack of the characteristic fragment ions of sugar losses (146 Da and 162 Da for deoxyhexose and hexose units, respectively) suggested that components **5** and **6** are flavonoid *C*-glycosides [18], in spite of the fact that in their mass spectra no intensive *C*-glycoside-type fragments (e.g., water losses, cleavage of the glycan part [19]) were observed. Using system 2, where a soft ionization method (ESI) was used, component **7** showed no fragmentation at all, also suggesting a *C*-glycosidic structure [18]. Although reports from the late 1960s and early 1970s suggest that flavonoid compounds of the same molecular masses as vicenin-2 (MW=595), orientin, isoorientin (MW=448), and violanthin (MW=578) [1] (see structures in Fig. 5.) occur in heartsease, we could not prove that they match components **5**–**7**. The mass spectra of component **8** displays characteristic fragments at *m*/*z* 465 and 303, which could be assigned as aglycone residues after losses of their rhamnosyl and rhamnoglucosyl moieties, respectively. These results suggested a quercetin-*O*-diglycosidic structure,

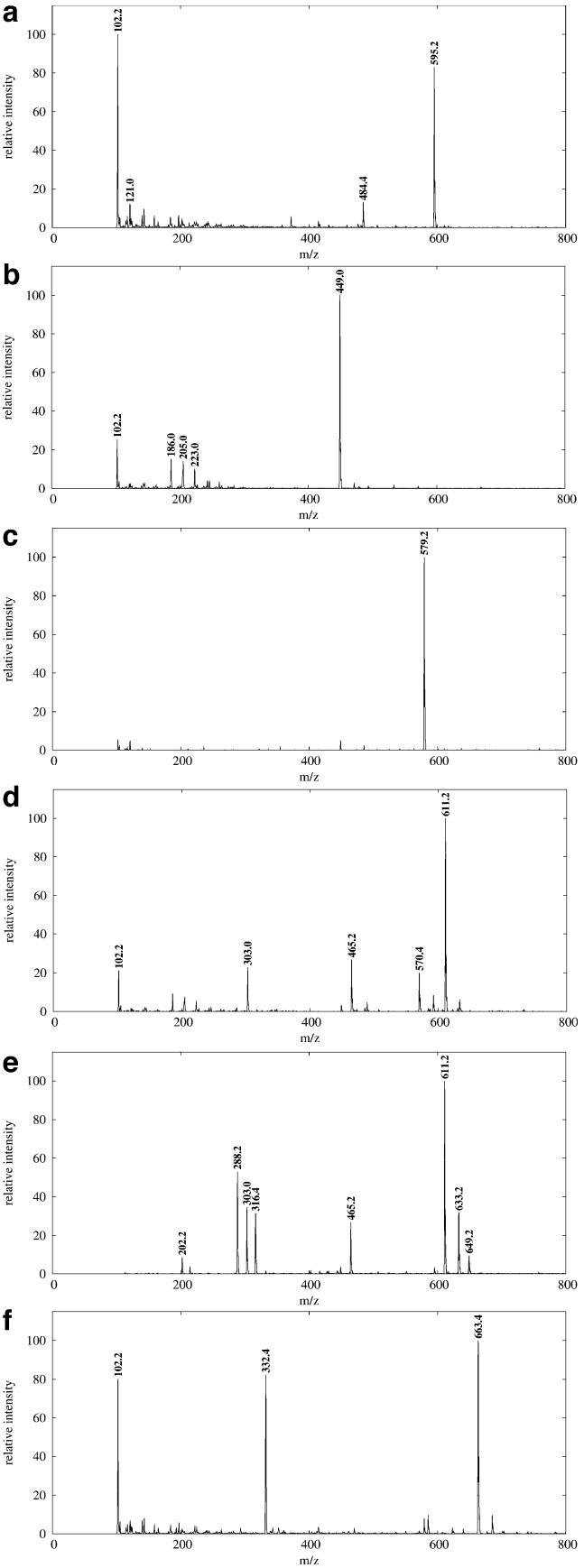

Fig. 4 Mass spectra of components **5**–**9** in fractions H and J, as well as of rutin reference molecule: **a** component **5**, **b** component **6**, **c** component **7**, **d** component **8**, **e** rutin standard, **f** component **9**. Experiments were performed on an Agilent 1100 HPLC/MSD SL system. The mass-selective detector was equipped with a normal-flow electrospray ionization (ESI) source. The electrospray conditions were as follows: drying gas flow 13 L min^{-1}, drying gas temperature 350 °C, nebulizer pressure 35 psi, capillary voltage 3,000 V

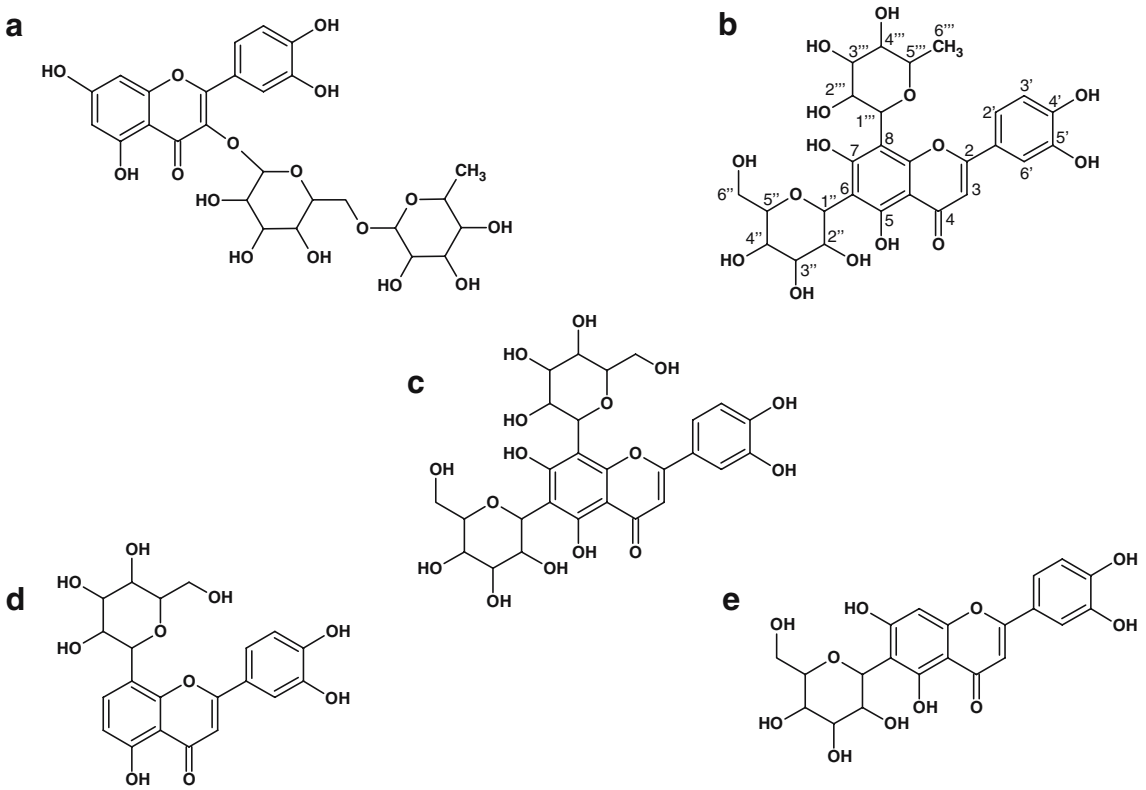

Fig 5 Chemical structures of **a** rutin (3-*O*-rhamnoglucosylquercetin), **b** violanthin (6-*C*-rhamnosyl-8-*C*-glucosylapigenin), **c** vicenin-2 (6-*C*-glucosyl-8-*C*-glucosylapigenin), **d** orientin (8-*C*-glucosylapigenin), and **e** isoorientin (6-*C*-glucosylapigenin)

which in correlation with literature data, suggested rutin (quercetin-3-*O*-rhamnoglucoside, see structure in Fig. 5.) to be present in heartsease in high quantities. The comparison of retention times, UV spectra, molecular masses, and MS fragmentation patterns with standard reference molecules also indicated that component **8** is rutin. In addition, the comparison of their UV spectra and the peak distributions of the methanol extract using systems 1 and 2 respectively suggest that components **3** and **7** as well as **4** and **8** were identical. Consequently, fraction F consists of rutin, and

Table 1 Assigned NMR chemical shifts (ppm) and coupling constants (in Hz in parentheses) for violanthin in DMSO-d_6 at 25 °C

No.[a]	^{1}H	^{13}C	No.[a]	^{1}H	^{13}C
2		163.1	1″	4.62 d (9.8)	73.4
3	6.78 s	102.5	2″	4.10 t (9.8)	
4		182.0	3″	3.20 t (9.0)	
5		160.1	4″	3.14 t (8.9)	
6		109.1	5″	3.15 m	82.4
7		163.4	6″	3.69 d (12.0), 3.41 dd (12.0, 6.0)	61.9
8		104.0	1‴	5.23 br s	
1′		121.5	2‴	3.90 m	
2′,6′	7.89 d (8.4)	128.4	3‴	3.59 d (8.9, 2.5)	
3′,5′	6.95 d (8.4)	116.1	4‴	3.39 t (8.9)	71.9
4′		161.5	5‴	3.46 m	77.2
			6‴	1.26 d (6.0)	18.1

s singlet, *d* doublet, *t* triplet, *dd* double doublet, *m* multiplet, *br* broad
[a] See Fig. 5b

Table 2 Antioxidant capacity of the fractions obtained by Sephadex LH-20 column chromatography

Fraction	TEAC assay		DPPH assay	
	IC_{50}±SD (μg mL^{-1})	RSD (%)	IC_{50}±SD (μg mL^{-1})	RSD (%)
A	6.08±0.25	4.13	nd	
B	3.36±0.16	4.70	13.05±0.41	3.13
C	17.86±1.28	7.14	52.97±0.95	1.80
D	1.61±0.05	3.23	7.99±0.17	2.08
E	2.17±0.14	6.52	7.28±0.05	0.73
F	0.31±0.01	2.50	11.36±0.39	3.41
G	0.82±0.03	3.61	4.03±0.13	3.23
Rutin	0.12±0.01	7.66	10.26±0.47	4.58

nd not detected
Antioxidant capacity was determined by the TEAC and DPPH in vitro assays and characterized by IC_{50} value

fraction E contains the main flavonoid component of heartsease, i.e, violanthin (MW=578).

The exact structural elucidation of the main flavonoid component of violanthin was achieved by NMR spectroscopy. The ^{1}H-NMR spectrum of fraction E confirmed the presence of a flavone skeleton characterized by a singlet signal at δ 6.78 assigned to H-3 and two doublet signals at δ 6.95 and 7.89 assigned to H-3′, H-5′ and H-2′, H-6′, respectively. The absence of signals for H-6 and H-8, and the presence of two anomeric sugar protons at δ 4.62 and 5.23 indicated a 6,8-di-*C*-glycosylapigenin structure (Table 1, Fig. 5). For further assignments standard 2D experiments (COSY, TOCSY, NOESY, HSQC, HMBC) were used (manuscript in preparation). In addition, the assignment of carbohydrate protons in crowded spectral regions was further confirmed by selective 1D TOCSY–TOCSY and TOCSY–NOESY experiments. The coupling patterns observed in these spectra yielded clear information on the configuration and thus on the identity of the C-6 and C-8 carbohydrate rings (D-glucose and L-rhamnose, respectively). In conclusion, we consider components **3** and **8** as violanthin.

Antioxidant capacity measurements of fractions A–G

The Trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are decolorization-based methods. In these test systems colorful free radicals ($ABTS^{\cdot+}$ and $DPPH^{\cdot}$) are generated and their concentration is determined before and after the introduction of samples. Antioxidant components quench the free radicals whose concentration decreases proportionally to the antioxidant capacity of the sample. The oxidized and reduced forms of the radical absorb at different wavelength maxima, which allows spectroscopic determination of the amount of radicals present. The TEAC assay was reported to characterize both the electron-donor [14] and hydrogen-donor properties [16] of samples, respectively.

The antioxidant capacity of fractions B–G were determined both by the TEAC and DPPH assays, whereas fraction A could be studied only by the TEAC assay, since it was obtained by extraction with chloroform, and was apolar in character. The antioxidant capacity of the samples was characterized by calculating the IC_{50} value, i.e., the concentration which produces 50% quenching of the free radicals (Table 2). The lower the IC_{50} value, the better antioxidant the sample is. The IC_{50} values for fractions A–G are summarized in Table 2. Based on the results obtained, we can draw the following conclusions. The polar components of heartsease (fraction B) show better antioxidant activity than the apolar ones (fraction A). If fraction B is cleared from the fast-eluting components (fraction C), which show poor antioxidant activity, a purified flavonoid fraction is obtained (fraction D) with significantly higher antioxidant capacity. The isolated main compound, violanthin (fraction E), showed slightly worse electron-donor properties and approximately the same hydrogen-donor capacity as the unseparated flavonoid fraction, fraction D. The isolated second main component, rutin (fraction F), on the other hand, exhibited significantly better electron-donor properties, but lower hydrogen-donor capacity. Reduction of the ratio of the two main components in the flavonoid fraction (fraction G) resulted in an increase in both the electron-donor and hydrogen-donor activities.

Conclusion

A proper fractionation protocol have been developed for the preparative separation of the methanol extract of heartsease by Sephadex LH-20 and polyamide column chromatography. The two main flavonoid components of heartsease were isolated by Sephadex LH-20 column chromatography, and qualitatively analyzed by spectroscopic methods. The main flavonoid component was identified as violanthin (6-*C*-glucosyl-8-*C*-rhamnosylapigenin) by a complex NMR study including 2D (COSY, TOCSY, NOESY, HSQC, HMBC) and selective 1D (TOCSY–TOCSY and TOCSY–NOESY) experiments. By comparing retention time, UV spectra, molecular mass, and fragmentation pattern with the reference standard molecules, we suggest the second main flavonoid component to be rutin (3-*O*-rhamnoglucosylquercetin). Purified fractions rich in minor flavonoids were obtained by polyamide open-column chromatography and analyzed by mass spectrometry. The LC-MS experiments further confirmed the identity of rutin. In addition, minor flavonoids (with molecular masses of 448, 595, and 662) were separated; however, their structural elucidation requires further complex spectroscopic studies (MS^{n}, NMR). In respect to their antioxidant properties, the highest electron-donor capacity was measured with rutin (fraction F), and fraction G exhibited the highest hydrogen-donor activity.

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Analysis of heartsease (*Viola tricolor* L.) flavonoid glycosides by micro-liquid chromatography coupled to multistage mass spectrometry

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ABSTRACT

Micro-liquid chromatography (μLC) in conjunction with multistage mass spectrometry (MS^n) was introduced to study several major heartsease flavonoid glycosides. High-resolution μLC separation was achieved by using a monolithic poly(*p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane) column under reversed-phase conditions. The MS/MS and MS^3 analysis of the flavonoid components of interest provided data about their glycosylation type and position, nature of their aglycones, and the structure/linkage information of their glycan moieties. With our μLC–MS^n approach, four flavonol *O*-glycosides, nine flavone-*C*-glycosides, and three flavone *C*,*O*-glycosides were characterized in heartsease methanol extract. All of these glycoconjugates were found to be the derivatives of six aglycones: apigenin, chrysoeriol, isorhamnetin, kaempferol, luteolin, and quercetin.

1. Introduction

Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. It has been utilized to treat various skin disorders, upper-respiratory problems and also used as a diuretic [1]. Most of heartsease's biological activities are attributed to its antioxidant flavonoid compounds. Flavonoids are important secondary plant metabolites and have been proved useful in the prophylaxis and treatment of cardiovascular problems, complications of diabetes, inflammations, immune disorders, and liver problems, just to list a few indications [2].

The term flavonoids comprise a large group of structurally related compounds with a chromane-type skeleton and a phenyl substituent. In plants, the basic three-ring flavonoid structure is usually modified by means of hydroxylation, methylation or glycosylation [3]. Occasionally, aromatic or aliphatic acids, sulphate, prenyl, or isoprenyl groups are attached to the flavonoid aglycone [4,5]. Glycosylated flavonoids commonly occur as flavonoid *O*-glycosides, where one or more hydroxyl groups of the aglycone are bound to a sugar unit trough an acid-labile glycosidic O–C bond. While glucose is the most commonly encountered monosaccharide type, galactose, rhamnose, xylose, and arabinose are not uncommon either [6]. Disaccharides were also found in association with flavonoids: rutinose (rhamnosyl-(α1 → 6)-glucose) and neohesperidose (rhamnosyl-(α1 → 2)-glucose) being the most frequent [7]. Trisaccharides and tetrasaccharides have also been reported [7,8] in flavonoids. In addition, sugars can be attached to the flavonoid aglycone via acid-resistant C–C bonds, referred to as flavonoid *C*-glycosides. Flavonoid *C*-glycosides are classified into mono-*C*-glycosyl flavonoids, di-*C*-glycosyl flavonoids and flavonoid-*O*,*C*-glycosides. In this latter category, a hydrolysable sugar is linked either to a phenolic hydroxyl group or a hydroxyl group of the *C*-glycosyl residue.

For structural characterization of natural compounds in crude plant extracts hyphenated chromatographic techniques such as GC–MS, LS–MS or LC–NMR proved to be very efficient. Direct interfacing of gas chromatography with mass spectrometry is effective for the analysis of free flavonoid aglycones, but is not well suited for the highly polar, thermally labile, and high molecular weight glycosidic conjugates [9]. Liquid chromatography combined with mass spectrometry prevail those applicability limitations. In addition, with LC coupled tandem mass spectrometry information can be obtained on (i) the glycosylation type (*O*-, *C*-, or mixed glycosides) [7], (ii) the aglycone moiety [10,11], (iii) the types of carbohydrates (hexoses, deoxyhexoses or pentoses) [12], (iv) the sequence of the glycan part [8,13], (v) interglycosidic linkages [8,13,14], and (vi) attachment points of the substituents to the aglycone [15–20].

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2. Materials and methods

2.1. Chemicals and plant material

Quercetin (3,3′,5,7-tetrahydroxyflavone), isorhamnetin (3,4′, 5,7-tetrahydroxy-3′-methoxyflavone), vitexin (8-*C*-glucosylapigenin), isovitexin (6-*C*-glucosylapigenin) and all solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Kaempferol (3,4′,5,7-tetrahydroxyflavone) was from Fluka (St. Louis, MO, USA) and luteolin (3′,4′,5,7-tetrahydroxyflavone) from Extrasynthese (Genay, France). Apigenin (4′,5,7-trihydroxyflavone) was from Bionorica (Neumarkt, Germany). Heartsease herb (*Viola tricolor* L.) (SN = 28-56-05-VI/24) was purchased from Fitopharma (Budapest, Hungary).

2.2. Sample preparation

Five grams dried and freshly powdered plant material was sonicated two times with 50 mL chloroform for 25 min in an ultrasonic bath at 30 °C. The plant residue was dried at room temperature and re-extracted two times with 40 mL methanol for 15 min in an ultrasonic bath at 30 °C. The methanol extract was evaporated to dryness under reduced pressure at 55 °C. 10 mg dry extract was re-dissolved in 20 mL 100% methanol and filtered on an Acrodisc nylon 0.20 μm membrane Sartorius syringe filter (Sigma–Aldrich).

Isorhamnetin was dissolved in a mixture of 10% acetone and 90% methanol. Kaempferol, quercetin, luteolin, isovitexin and vitexin were dissolved in 100% methanol. The apigenin was received as solution from Bionorica.

2.3. LC/ESI–MS analysis

All analyses were performed on a μLC system (LC Packings, Amsterdam, The Netherlands) coupled to a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, Waltham, MA, USA). The LC device consisted of the Ultimate μ HPLC pump and column oven, the Switchos μ column-switching device with loading pump and two 10-port valves and the FAMOS μ-Autosampler (LC Packings). A monolithic poly(*p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane) capillary column was used for separation (260 mm × 0.2 mm) [21]. μ-HPLC of flavonoids was carried out employing reversed-phase conditions using 0.1% formic acid in water and 0.1% formic acid in acetonitril as solvents A and B, respectively, at a flow rate of 1 μL/min and 35 °C. Separation was performed with a linear gradient (in 50 min from 0% B to 10% B). Hyphenation to the mass spectrometer was carried out by a nanoflow electrospray ionization (ESI) source from Proxeon (Odense, Denmark) with Pico Tips (FS360-20-10) from New Objective (Woburn, MA, USA). Mass spectrometric data were obtained on the linear ion trap LTQ from Thermo Fisher (Thermo Fisher). Measurements in the positive mode were performed as follows: source voltage 1.7 kV, capillary temperature 220 °C, capillary voltage 41 V, tube lens 115 V; in the negative mode: source voltage 1.3 kV, capillary temperature 220 °C, capillary voltage −50 V, tube lens −152 V. Data acquisition and interpretation was done with Xcalibur from Thermo Fisher.

2.4. Nomenclature

Fragment ions yielded by mass spectrometry were designated according to conventionally used nomenclature system for aglycones developed by Mabry et al. [22] and improved by Ma et al. [11]. The Domon and Costello [23] nomenclature was used for glycoconjugates. In some cases a subscript H, D, or P was added to the labels referring to 'hexose', 'deoxyhexose', or 'pentose', respectively. Label 'E_i' designates the loss of water molecules. For labels see Fig. 1a and b.

3. Results and discussion

3.1. General considerations

In HPLC based separation of flavonoids generally RP-C18 columns are applied, however, in our case better separation was obtained with a monolithic poly(*p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane) microcolumn under reversed-phase conditions. Separation was performed with a 50 min linear gradient from 0.1% formic acid to 0.1% formic acid containing 10% acetonitrile. Hyphenation to the mass spectrometer was realized by nanoflow electrospray ionization and scouting runs were carried out with medium fragmentation energy (35%) setting. However, it was found that for the analysis of *C*-glycosides higher energy (45–55%) was needed in order to obtain adequate fragmentation. On the other hand, for *O*-glycosides, low energy (25%) was preferred to prevent the cleavage of the sugar units and provide easily interpretable spectra. One of the most important issues during the MS analysis was how to differentiate compounds, which were characteristic of the sample from electrospray byproducts, as e.g., in-source fragmentation of some glycosides may result in loss of sugar residues. In other words, in some instances artifacts might appear in the total ion chromatograms, which were not present in the original sample. In the case of e.g. rutin reference molecule (quercetin-3-*O*-rhamnosylhexoside), the aglycone quercetin and isoquercitrin (quercetin-3-*O*-hexoside) were both detected at the same retention time. Their different polarity and binding affinity to the monolithic column, would however suggest different retention behavior. Therefore, in all instances, careful analysis of the full MS spectra was necessary to define the representative sample components. Based on these considerations, 16 peaks were selected (Table 1) and their MS/MS and MS^3 spectra thoroughly studied. These 16 components were classified as *O*-, *C*-, or *C*,*O*-glycosides. Interpretation of their mass spectra and their hypothetical structures are discussed in the following sections.

3.2. Characterization of O-glycosides

The flavonoid *O*-diglycosides identified in heartsease methanol extract (components 1–3, Table 1) showed molecular masses of 594, 610, and 624. In their positive and negative ion MS/MS spectra, high abundant Y_0 fragments were observed (see Fig. 1a and b). By definition, these fragments originate from the losses of all sugar units and represent the aglycones of the components. Consequently, the molecular masses of 286, 302, and 316 are representative for the aglycones of components 1–3, respectively. In addition, the comparison of the positive and negative ion MS^3 spectra of the Y_0 fragments with literature data [10,11,24] as well as with the MS/MS spectra of reference molecules (Table 2) suggested component 1 to be kaempferol-, component 2 quercetin-, and component 3 isorhamnetin-glycoside (Fig. 2).

The positive ion MS/MS spectra of components 1–3 showed Y_1^+ ions corresponding to the losses of 162 and 146 Da (hexose and deoxyhexose units, respectively) [18]. These sugars can be attached to the flavonoid aglycone either at two different positions (di-*O*-glycosides) or at the same position (*O*-diglycosides) forming a disaccharide. The parallel loss of a hexose and a deoxyhexose unit would suggest the presence of a di-*O*-glycosidic isomer. On the other hand, for *O*-diglycosides, appearance of irregular Y_1^+ ions (generally labeled as Y^*) have been reported, resulting from the losses of the internal sugar units [14]. Thus, the positive ion MS/MS

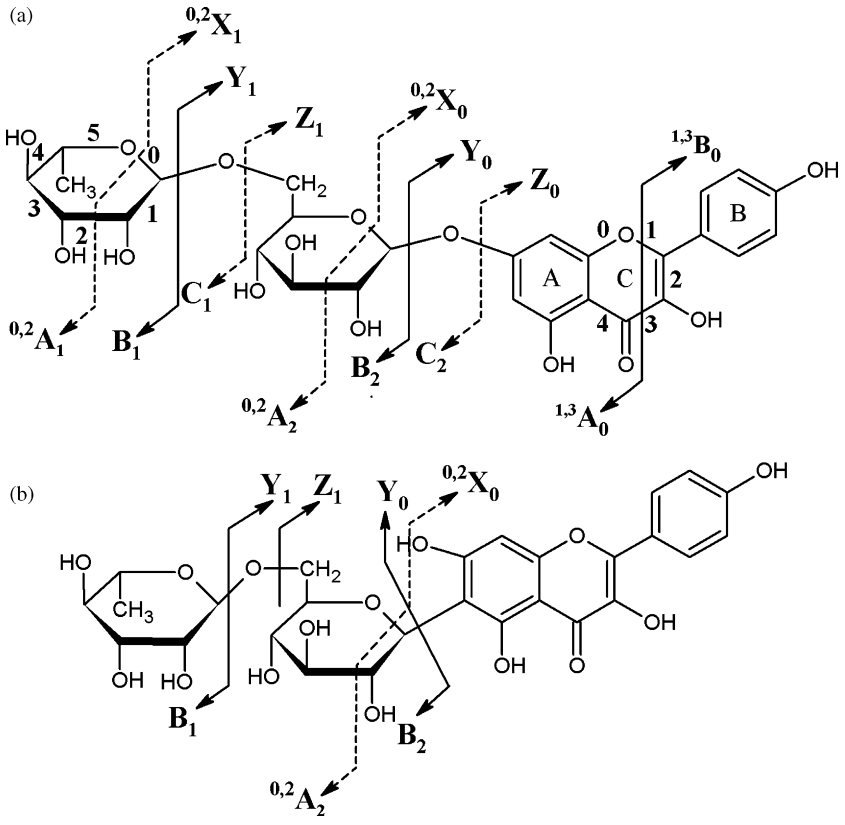


Fig. 1. Fragment nomenclature applied for (a) *O*-glycosides, and (b) *C*,*O*-glycosides and *C*-glycosides.

spectra alone seem to be insufficient to differentiate the two isomeric structures. In the negative ion MS/MS spectra of components 1–3, however, no Y_1^- fragments were observed, only abundant Y_0^- fragments. This fact suggests the presence of *O*-diglycosidic isomers with 1 → 6 linkages between the sugar moieties [8]. As in the positive ion MS/MS spectra both Y_1^+ fragments (Y_H^+ and Y_D^+) were present in contrast to the negative ion MS/MS spectra where no Y_1^- ions were observed, the order of the sugar units in the disaccharide could not be determined by mass spectral analysis. However, the 1 → 6 linkage between the sugar units is only possible if the deoxyhexose unit is the external sugar unit, since deoxyhexoses possess a methyl group instead of a hydroxymethyl at position C-6″. Moreover, to date no diglycoside with a 1 → 6 linkage has been found with internal deoxyhexose unit [6]. Therefore, we propose that in components 1–3, deoxyhexosyl(1 → 6)hexoside units are attached to the flavonol aglycones. Although theoretically this disaccharide can substitute any hydroxyl groups, in the case of flavonols, positions C-3 and C-7 are reportedly favored [7]. While in the case of 7-*O*-diglycosyl flavonols in the positive ion MS/MS spectra no Y^* fragments were observed, for 3-*O*-diglycosyl flavonols low abundant Y^* fragments have been reported [14]. These facts can be the basis of the differentiation of 3-*O*- and 7-*O*-diglycosylated

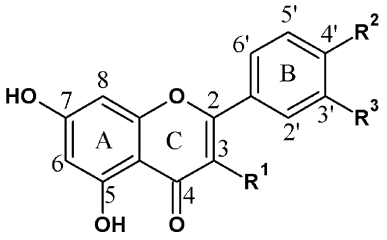
Table 1
Flavonoid glycosides in heartsease methanol extract characterized by LC–MS^n

Component	MW	t_R (min)	Structure
O-Glycosides			
1	594	42.7	Kaempferol-3-*O*-deoxyhexosyl(1 → 6)hexoside
2	610	36.5	Quercetin-3-*O*-deoxyhexosyl(1 → 6)hexoside
3	624	46.7	Isorhamnetin-3-*O*-deoxyhexosyl(1 → 6)hexoside
4	756	34.0	Quercetin-3-*O*-deoxyhexosylhexoside-7-*O*-deoxyhexoside
C-Glycosides			
5	448	33.0	Luteolin-6-*C*-hexoside
6	462	39.5	Chrysoeriol-6-*C*-hexoside
7	564	33.0	Apigenin-6-*C*-pentoside-8-*C*-hexoside
8	564	35.5	Apigenin-6-*C*-hexoside-8-*C*-pentoside
9	578	35.0	Apigenin-6-*C*-deoxyhexoside-8-*C*-hexoside
10	578	37.5	Apigenin-6-*C*-hexoside-8-*C*-deoxyhexoside
11	594	31.6	Apigenin-6,8-di-*C*-hexoside
12	594	33.0	Luteolin-6-*C*-deoxyhexoside-8-*C*-hexoside
13	594	35.0	Luteolin-6-*C*-hexoside-8-*C*-deoxyhexoside
C,*O*-Glycosides			
14	740	30.6	Apigenin-X-*O*-hexoside-Y-*C*-deoxyhexoside-Z-*C*-hexoside
15	740	34.3	Apigenin-6-*C*-deoxyhexoside-(6″-*O*-hexosyl-8-*C*-hexoside)
16	740	39.7	Apigenin-(6″-*O*-hexosyl-6-*C*-hexoside)-8-*C*-deoxyhexoside

Table 2
Principal ESI–MS/MS product ions obtained from the $[M+H]^+$ and $[M-H]^-$ ions of KA = kaempferol, QU = quercetin, and IR = isorhamnetin reference molecules as well as from the Y_0^+ and Y_0^- fragments of components 1–3

	Fragmentation[a] *m/z* (% relative abundance)											
	Positive ion mode						Negative ion mode					
	KA	C1	QU	C2	IR	C3	KA	C1	QU	C2	IR	C3
M+H/M−H/Y_0	287 (100)[a]	287 (100)	303 (56)	303 (78)	317 (3)	317 (8)	285 (100)	285 (5)	301 (0)	301 (3)	315 (4)	315
$-CH_3$					302 (100)	302 (100)					300 (100)	300 (100)
$-H_2O$	269 (12)	269 (19)	285 (52)	285 (51)	299 (5)	299 (4)	267 (6)	267 (44)	283 (14)	283 (5)		
–CO	259 (12)	259 (11)	275 (9)	275 (17)			257 (11)	257 (100)	273 (13)	273 (18)		
–CHO	258 (22)	258 (26)	274 (13)					256 (9)				
$-CH_3OH$					285 (44)	285 (45)						
$-C_2H_2O$	245 (22)	245 (3)			275 (3)	275 (6)	243 (13)	243 (6)				
$-CO_2$							241 (20)	241 (26)	257 (9)	257 (16)		
$-H_2O$–CO	241 (62)	241 (76)	257 (100)	257 (61)	271 (5)	271 (8)	239 (7)	238 (19)	255 (8)	255 (4)		
–2CO	231 (18)	231 (24)	247 (24)	247 (17)	261 (11)	261 (7)	229 (16)	229 (41)				
				233 (10)			215 (6)	215 (3)				
							213 (10)	213 (22)	229 (4)	229 (7)		
$-CH_3OH$–CO					257 (10)	257 (10)						
$-H_2O$–2CO	213 (51)	213 (63)	229 (66)	229 (100)	243 (4)	243 (4)	211 (3)	211 (3)				
	203 (6)	203 (10)	219 (6)	219 (8)			201 (6)					
	199 (3)	199 (4)	215 (4)		229 (3)	229 (6)		197 (20)				
	197 (11)	197 (13)	213 (5)				195 (5)	195 (11)	211 (4)	211 (4)		
	185 (6)	185 (10)	201 (7)	201 (6)								
$-C_2H_2O$–CO								199 (16)				
– ring B			195 (8)	195 (23)					193 (8)	193 (7)		
$^{1,2}A$									179 (100)	179 (100)		
							169 (12)	169 (5)				
$-2CO-C_2H_4O_2$					177 (3)	177 (6)						
$^{0,2}A$	165 (85)	165 (66)	165 (58)	165 (47)	165 (8)	165 (8)	163 (6)	163 (20)				
$^{1,3}A$ / $^{1,2}A$–CO	153 (26)	153 (30)	153 (16)	153 (13)	153 (4)	153 (6)	151 (25)	151 (7)	151 (73)	151 (67)		
$^{1,3}B-2H$	133 (12)	133 (16)	149 (7)	149 (4)	163 (2)	163 (3)						
$^{0,2}B$	121 (14)	121 (16)	137 (18)	137 (3)		137 (3)						
$^{0,2}A$–CO	137 (4)	137 (4)	137 (18)	137 (3)				135 (3)				
	105 (3)	105 (3)	121 (5)	121 (3)	139 (13)	139 (11)						
$^{1,3}A-C_2H_2O$	111 (8)	111 (9)	111 (6)	111 (11)								
$^{1,2}A$–CO–CO_2									107 (4)	107 (4)		

[a] *m/z* (relative abundances).



	MW	R^1	R^2	R^3
apigenin	270	H	OH	H
luteolin	286	H	OH	OH
kaempferol	286	OH	OH	H
chrysoeriol	300	H	OH	OCH_3
diosmetin	300	H	OCH_3	OH
quercetin	302	OH	OH	OH
isorhamnetin	316	OH	OH	OCH_3

Fig. 2. Structures of flavonoid aglycones discussed in this work.

isomers, and in the case of components 1–3 they may indicate the presence of 3-*O*-diglycosylated flavonols. In conclusion, based on our data, we suggest components 1–3 as kaempferol-, quercetin-, and isorhamnetin-3-*O*-deoxyhexosyl(1 → 6)hexosides, respectively.

As a first approximation, based on its molecular mass, we considered component 4 (MW = 756) a triglycoside. The positive and negative ion MS^3 analysis of the abundant Y_0 ion (MW = 302) suggested its aglycone to be quercetin. The mass losses corresponding to the Y_i fragments implied the attachment of two deoxyhexose and one hexose to the aglycone. Because of the presence of the Y^* fragment (Fig. 3a and b), the distribution of the sugar moieties could be deduced only from the complementary analyses of Y_i and $^{k,l}X$ ions in both the positive and negative ion modes (Fig. 3a and b). The three sugar residues can theoretically be attached to the aglycone at three, two or one position with different sequences. As an abundant Z_i ion was observed in the negative ion MS/MS spectrum, the tri-X,Y,Z-*O*-glycoside form was ruled out [8,12]. On the other hand, according to the MS^3 analysis of $[M+H]^+ \rightarrow [Y_D{-}^{0,2}X_H]^+$, the presence of the $Y_D{-}^{0,2}X_H{-}Y_D$ fragment at *m*/*z* 343 is opposed to the X-*O*-triglycoside structure. In addition, the presence of the *m*/*z* 609 (Y_D^-) ion and the absence of the *m*/*z* 593 ion indicated that when only one sugar unit was lost that should have been deoxyhexose. Therefore, we consider the structure as quercetin-X-*O*-deoxyhexoside-Y-*O*-deoxyhexosylhexoside, and *m*/*z* 595 is designated in the positive ion spectra as Y_H^*. In the case of component 4, the preferred attachment points for the monoglycosyl and the diglycosyl units were reportedly C-3 and C-7 [7]. Based on the analogy to components 1–3 and the fact that Y^* ions were observed only for 3-*O*-diglycosides [14], we consider component 4 as quercetin-3-*O*-deoxyhexosylhexoside-7-*O*-hexoside. In regard to the linkage type of the diglycosyl unit ambiguous results were obtained. According to Ferreres et al. [13] the presence of an abundant Z_i fragment indicated 1 → 2 linkage. However, the analogy to other *O*-glycosides identified in the same extract would suggest a 1 → 6 connection.

3.3. Characterization of C-glycosides

In the MS/MS spectra of *C*-glycosides analyzed in heartsease methanol extract (components 5–13, Table 1), characteristic fragments resulted both from water losses and sugar cleavages. In the positive ion mode, besides the water losses, $^{2,3}X^+$, $^{0,4}X^+{-}2H_2O$, $^{0,2}X^+$, $^{0,1}X^+$ ions and low abundant (relative intensity <1%) Y_i^+ fragments were observed. On the other hand, the negative ion spectra showed abundant $^{0,4}X^-$, $^{0,3}X^-$, $^{0,3}X^-{-}2H_2O$, $^{0,2}X^-$, $^{1,5}X^-$ fragments. In addition, for di-*C*-glycosides, parallel cleavages of both sugar units resulted in $^{i,j}X_1\,^{k,l}X_2$ fragments, which at applying the same fragmentation energy, were more abundant in the negative ion mode.

The fragments resulting from sugar unit cleavages corresponded to different mass losses for hexoses, deoxyhexoses, and pentoses, thus, the types of the sugar residues could be determined. Accordingly, we found that the two examined mono-*C*-glycosides (components 5 and 6, molecular masses of 448 and 462, respectively) contained hexose units. In respect to their attachment points, up until today, *C*-glycosylation has been almost exclusively found at positions C-6 or C-8 and only in two cases at position C-3 [7,25]. Although no significant differences were observed in the MS/MS spectra of the isomers, the analysis of the $[M+H{-}^{0,2}X]^+$ ions provided the means of differentiation. Waridel et al. [20] observed different small molecule loss patterns in the positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for C-6 and C-8 isomers. Using the same fragmentation energy for C-6 glycosides more fragmentation occurred, resulting in $^{0,2}X^+{-}H_2O$, $^{0,2}X^+{-}CHO$, $^{0,2}X^+{-}H_2O{-}CO$, $^{1,3}A^+$, and $^{0,2}B^+$ ions, whereas in the spectra of C-8 isomers only the $^{0,2}X^+{-}CHO$ and $^{0,2}B^+$ ions were present. Our results were similar in comparing the positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) reference molecules (Fig. 4a and b). The analogy of the MS^3 spectra of components 5 and 6 to the above described fragment patterns (Fig. 4c and d), suggested the presence of C-6 hexosyl isomers. This assumption was further supported by the fact that high abundant $^{0,3}X^-$ fragments were found in the negative ion MS/MS spectra of components 5 and 6 (Fig. 4e, g and h) similarly as reported in Refs. [12,20].

In the case of *C*-glycosides, the lack of significant Y_0 ions rendered the characterization of the aglycone part difficult, since no direct comparison with reference molecules was possible. Albeit, a thorough analysis of $^{i,j}A^+$ and $^{k,l}B^+$ ions in the MS^3 spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ provided information about the structure of the flavonoid aglycone. The calculated molecular mass for the aglycone of component 5 (MW = 286) indicated that the flavonoid aglycone contained one 2,3 double bond and four hydroxyl substituents. Ions at *m*/*z* 177 and 149 (Fig. 5a), designated as $^{1,3}A^+$ and $^{1,3}A^+{-}CO$, implied the presence of two hydroxyl groups on ring A. Furthermore, *m*/*z* 161, 137, and 135 (Fig. 5a), designated as $^{0,4}B^+{-}H_2O$, $^{0,2}B^+$, and $^{1,3}B^+$, evidenced that the remaining two hydroxyl groups were connected to ring B. Consequently, we propose component 5 as a luteolin-6-*C*-hexoside (Fig. 2). On the other hand, in the MS^3 spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for component 6 a loss of 15 Da was observed, suggesting a methoxy substituent per [24]. Together with this fact, the calculated molecular mass for the aglycone of component 6 (MW = 300) indicated that the flavonoid aglycone contained one 2,3 double bond as well as a methoxy and three hydroxyl substituents. Analogously to component 5, *m*/*z* 177 and 149 (Fig. 5b) implied the presence of two hydroxyl groups on ring A. In addition, *m*/*z* 151, designated as $^{0,2}B^+$ (Fig. 5b) signaled that a hydroxyl and a methoxy substituent were both attached to ring B. As for the localization of the methoxy group, positions C-3′ and C-4′ are the only options as suggested in Ref. [25]. Although the exact attachment point of the methoxy group could not be determined at this point, we propose component 6 as chrysoeriol-6-*C*-hexoside rather than diosmetin-6-*C*-hexoside (Fig. 2), based on its structural similarities to other compounds reported in hertsease [1,26].

The di-*C*-glycosides characterized in the present study (components 7–13) showed molecular masses of 564, 578, and 594 as depicted in Table 1. For each of these three mass values, we found

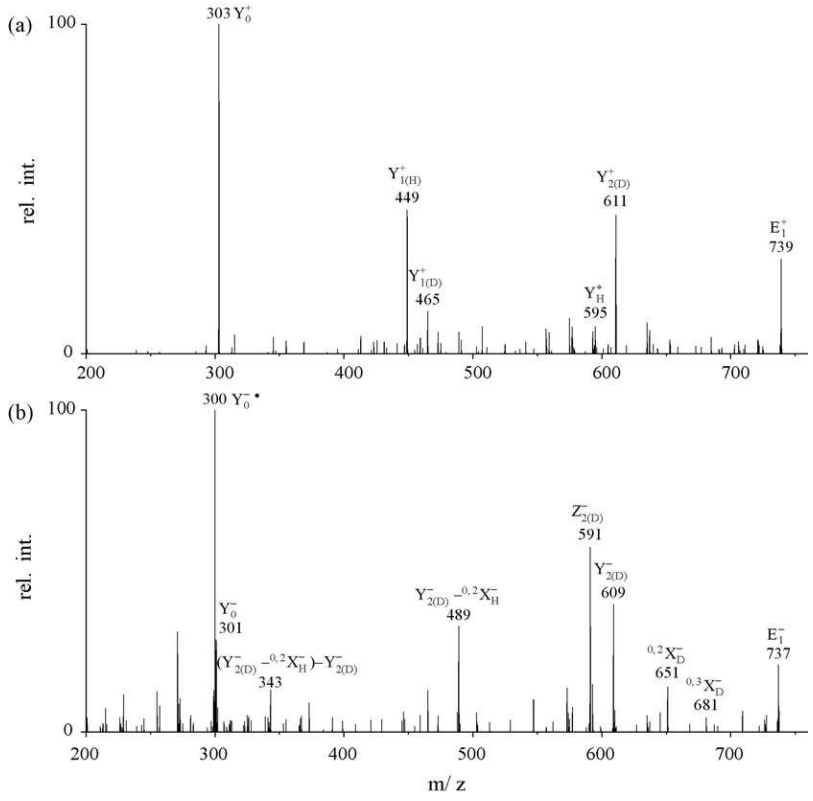


Fig. 3. (a) Positive and (b) negative ion MS/MS spectra of component 4.

Table 3
Principal ESI–MS/MS product ions obtained from the $[M+H]^+$ ions of components 7–13

	C7 (MW = 564)	C8 (MW = 564)	C9 (MW = 578)	C10 (MW = 578)	C11 (MW = 594)	C12 (MW = 594)	C13 (MW = 594)
E_1^+	547 (100)[a]	547 (100)	561 (100)	561 (100)	577 (100)	577 (100)	577 (100)
E_2^+	529 (80)	529 (24)	543 (39)	543 (20)	559 (38)	559 (36)	559 (23)
E_3^+	511(44)	511 (10)	525 (18)	525 (8)	541 (8)	541 (14)	541 (19)
E_4^+			507 (5)	507 (3)	523 (5)	523 (5)	523 (3)
${}^{2,3}X_H^+ - 2H_2O / {}^{0,4}X_P^+ - 2H_2O$	499 (11)	499 (30)	513 (35)	513 (28)	529 (18)	529 (22)	529 (19)
${}^{2,3}X_H^+ - 3H_2O$	481 (8)	481 (12)	495 (23)	495 (10)	511(12)	511(18)	511(9)
${}^{0,3}X_H^+ / {}^{0,2}X_P^+$	**475** (**6**)						
${}^{0,4}X_H^+ - 2H_2O$		**469** (**12**)	483 (11)	483 (14)	499 (4)	499 (18)	499 (8)
${}^{1,5}X_P^+ / {}^{0,2}X_D^+$	**461** (**8**)						
${}^{0,2}X_P^+ - H_2O$		**457** (**18**)					
${}^{0,2}X_H^+ / {}^{0,1}X_P^+$	445 (3)	445 (14)	459 (13)	459 (15)	475 (11)	475 (12)	475 (13)
${}^{0,2}X_D^+ - H_2O$			457 (30)	457 (19)		473 (28)	473 (20)
${}^{0,2}X_H^+ - H_2O$	**427** (**50**)		441 (3)	441 (4)	457 (35)	457 (5)	457 (2)
${}^{0,1}X_H^+$		**415** (**3**)					
${}^{0,2}X_H^+ - 2H_2O$	**409** (**10**)						

Bold values indicate the fragments, which based the differentiation of C7 and C8.
[a] *m*/*z* (relative abundance).

Table 4
Principal ESI–MS/MS product ions obtained from the $[M-H]^-$ ions of components 7–13

	C7 (MW = 564)	C8 (MW = 564)	C9 (MW = 578)	C10 (MW = 578)	C11 (MW = 594)	C12 (MW = 594)	C13 (MW = 594)
E_1^-	545 (20)[a]	545 (46)	559 (17)	559 (34)	575 (14)	575 (18)	575 (20)
E_2^-			541 (4)	541 (4)			
${}^{0,3}X_P^-$	503 (10)	503 (38)					
${}^{0,3}X_D^-$			503 (23)	503 (18)		519 (9)	519 (23)
${}^{0,3}X_H^- / {}^{0,2}X_P^-$	473 (76)	473 (100)	487 (29)	487 (72)	503 (35)	503 (16)	503 (21)
${}^{1,5}X_P^- / {}^{0,2}X_D^-$			473 (100)	473 (30)	489 (22)	489 (100)	489 (100)
${}^{0,2}X_P^- - H_2O$	455 (8)	455 (12)					
${}^{0,2}X_H^- / {}^{0,1}X_P^-$	443 (100)	443 (59)	457 (75)	457 (100)	473 (100)	473 (14)	473 (20)
${}^{0,2}X_D^- - H_2O$							
Y_P^-	431 (16)						
${}^{0,2}X_H^- - H_2O$					455 (5)		
$[{}^{0,2}X_i {}^{0,3}X_j]^-$	383 (12)	383 (12)	383 (22)	383 (18)	383 (20)	399 (17)	399 (18)
$[{}^{0,2}X_i {}^{0,2}X_j]^-$	353 (20)	353 (30)	353 (30)	353 (28)	353 (40)	369 (10)	369 (12)

[a] *m*/*z* (relative abundance).

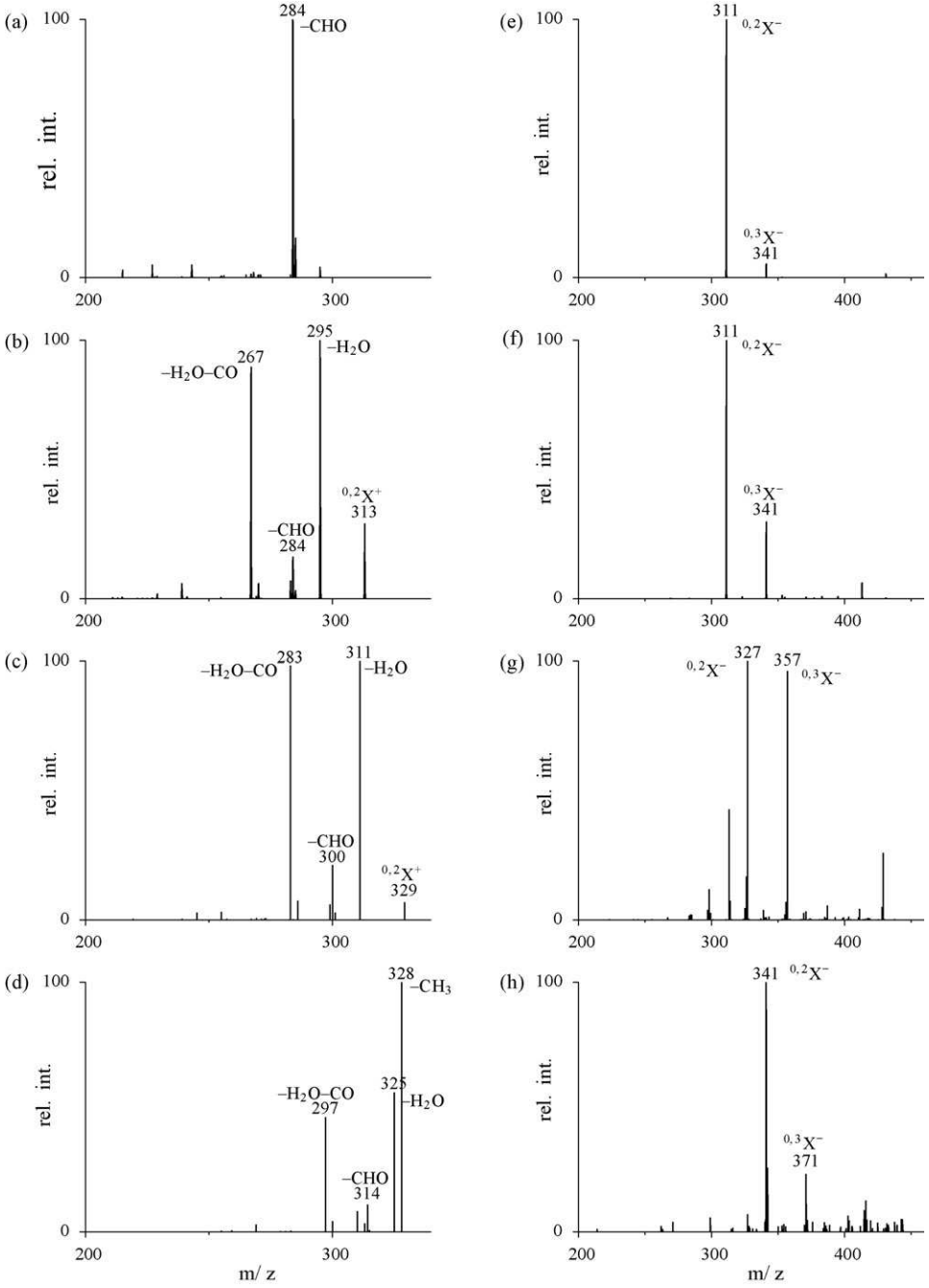


Fig. 4. Positive ion MS^3 spectra of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for (a) vitexin reference molecule, (b) isovitexin reference molecule, (c) component 5, and (d) component 6, as well as negative ion MS/MS spectra of (e) vitexin reference molecule, (f) isovitexin reference molecule, (g) component 5, and (h) component 6.

at least two differently eluting isomers, which vary either in their aglycones and sugar residues or in the attachment points of the sugars to the aglycone. Similarly to the monoglycosides above, the ${}^{i,j}X$ ions observed in the positive and negative ion MS/MS spectra of components 7–13 (Table 1) revealed information about the sugar types. Accordingly, we considered components 7 and 8 to contain hexose and pentose residues, while components 9, 10, 12 and 13 included hexose and deoxyhexose moieties and component 11 had two hexose substituents. On the other hand, the analysis of the MS^3 spectra of the $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,3}X_j]^-$ and $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,2}X_j]^-$ ions allowed the characterization of the aglycone part. Comparison of the corresponding spectra indicated that components 7–11 as well as components 12–13 may contain the same aglycones. Their calculated molecular masses (270 and 286) implied the presence of one 2,3 double bond as well as three and four hydroxyl substituents for components 7–11 and components 12–13, respectively. In addition, the fragment ions ${}^{1,3}A^-$, ${}^{1,3}B^-$, and ${}^{0,4}B^-$ (Fig. 5c–f) signified that two hydroxyl groups were located on ring A. The remaining one (for components 7–11) or two (for components 12–13) hydroxyl substituents can theoretically be attached either to ring C or ring B, yielding flavonol or flavanone structures, respectively. According to the study of Fabre et al. [10] the negative ion mode fragmentation of flavonols and flavanones significantly differ. While for flavonols they observed ${}^{1,2}B^-$ and ${}^{1,2}A^-$ fragments, for flavanones ${}^{1,3}B^-$ and

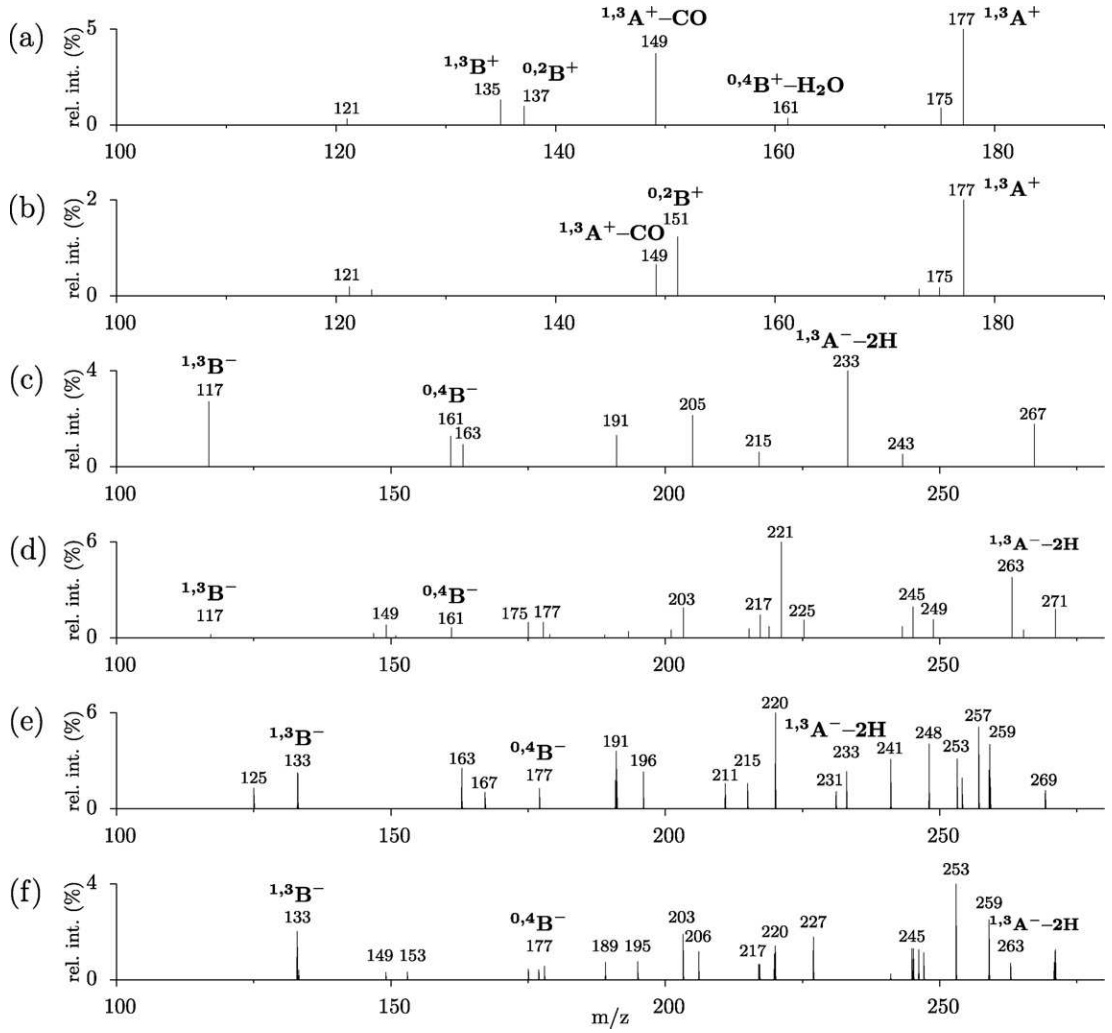


Fig. 5. Lower mass ranges of the MS^3 spectra containing the $^{i,j}A$ and $^{i,j}B$ ions, which were used in the identification of the aglycones of components 7–16. (a) product ion spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for component 5, (b) product ion spectrum of $[M+H]^+ \rightarrow {}^{0,2}X^+$ for component 6, (c) product ion spectrum of $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,2}X_j]^-$ for component 7, (d) product ion spectrum of $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,3}X_j]^-$ for component 9, (e) product ion spectrum of $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,2}X_j]^-$ for component 12, and (f) product ion spectrum of $[M-H]^- \rightarrow [{}^{0,2}X_i{}^{0,3}X_j]^-$ for component 13.

$^{1,3}A^-$ ions were found to be characteristic. Accordingly, we propose that components 7–11 have apigenin and components 12–13 luteolin aglycones (Tables 3 and 4).

Unfortunately, the methods described for monoglycosides were not applicable for the determination of the sugar attachment points for diglycosides. On the other hand, in their fast atom bombardment (FAB) experiments Li and Claeys claimed different fragmentation patterns for di-*C*-glycosyl isomers [18]. Among others they presented FAB spectra of schaftoside (apigenin-6-*C*-glucoside-8-*C*-arabinoside) and isoschaftoside (apigenin-6-*C*-arabinoside-8-*C*-glucoside), di-*C*-glycoside isomers containing hexose and pentose moieties with the molecular mass of 564. Based on the analogy to these spectra – fragments $^{0,2}X_P{}^+$, $^{1,5}X_P{}^+$, $^{0,2}X_H{}^+$–H_2O and $^{0,2}X_H{}^+$–$2H_2O$ appeared only for the C-6 pentose isomer, while $^{0,4}X_H{}^+$–$2H_2O$, $^{0,2}X_P{}^+$–H_2O, and $^{0,1}X_H{}^+$ were observed only for the C-8 pentose variant – we consider component 7 as apigenin-6-*C*-pentoside-8-*C*-hexoside and component 8 as apigenin-6-*C*-hexoside-8-*C*-pentoside.

In a previous study we isolated the main flavonoid component of heartsease methanol extract [27] and identified it by NMR spectroscopy as violanthin (apigenin-6-*C*-glucoside-8-*C*-rhamnoside, MW = 578). Based on this information we differentiated components 9 and 10. The base peak chromatogram (data not shown) revealed that component 10 is present in much higher concentration than component 9, thus, we considered component 10 as violanthin, and component 9 as apigenin-6-*C*-hexoside-8-*C*-deoxyhexoside isomer. Components 12 and 13 could not be differentiated by means of MS analysis. On the other hand, based on the information obtained from their chromatographic behavior (see Section 3.5) we propose component 12 as luteolin-6-*C*-deoxyhexosisde-8-*C*-hexoside and component 13 as luteolin-6-*C*-hexoside-8-*C*-deoxyhexoside.

3.4. Characterization of *C*,*O*-glycosides

The *C*,*O*-glycosides in heartsease methanol extract were characterized on the basis of their negative ion MS/MS spectra. The molecular mass of 740 for components 14–16 (Table 1) indicated that these compounds were most probably triglycosides. Analogously to the *C*-glycosides, the presence of *m*/*z* 353 and 383 (Fig. 6) and their MS^3 spectra (data not shown) suggested apigenin as their aglycone. This fact together with the molecular mass of 740 implied that components 14–17 most probably contained two hexose and one deoxyhexose units. For component 14, the presence

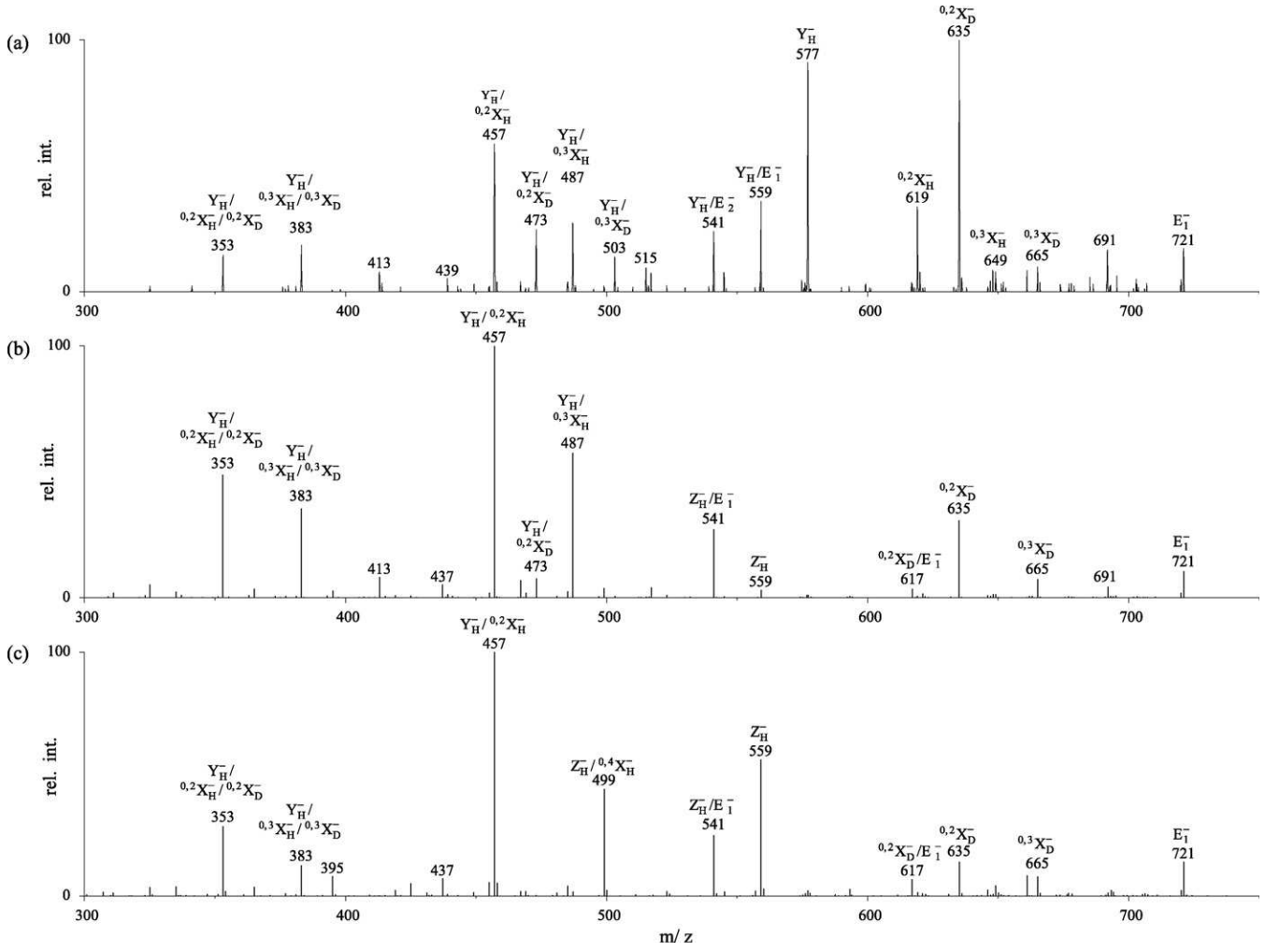


Fig. 6. Negative ion MS/MS spectra of (a) component 14, (b) component 15, and (c) component 16.

of a high abundant (relative intensity >90%) Y_H ion at *m*/*z* 577 (Fig. 6) signaled that an *O*-glycosidic hexose unit was attached to the aglycone. The $^{k,l}X$ fragments over *m*/*z* 600 are characteristic of *C*-glycosyl deoxyhexose and hexose units, directly linked to the aglycone (unconjugated, i.e., no other sugar unit attached to it). The same assumption can be drawn from the parallel cleavages of a hexose and deoxyhexose units after the *O*-glycosidic hexose is lost (mass range of 450–577). In conclusion, we propose component 14 as an apigenin-X-*O*-hexoside-Y-*C*-deoxyhexoside-Z-*C*-hexoside.

The low abundance (relative intensity <10%) of Y_H at *m*/*z* 577 and the presence of Z_H at *m*/*z* 559 (Fig. 6) together implied that in the case of components 15 and 16, the *O*-glycosidic hexose unit was connected to another sugar residue. On the other hand, the $^{k,l}X$ fragments over *m*/*z* 600 indicated that the unconjugated *C*-glycosyl unit was a deoxyhexose, therefore the *O*-glycosidic hexose must have been attached to the other hexose unit. As no significant $^{0,2}X_H$, but high abundant Y_H–$^{0,2}X_H$ fragments were observed, we propose a $1 \rightarrow 6$ linkage between the hexose units. Regarding to the location of the hexosyl($1 \rightarrow 6$)hexose moiety, for C-6 isomers higher abundant Z^- ions were observed as suggested by Ferreres et al. [13]. Thus, based on the significantly different intensity of *m*/*z* 559 in the MS/MS spectra of components 15 and 16 (Fig. 6), we considered them as 8-*C*- and 6-*C*- diglycosyl isomers, respectively. In conclusion, we suggest component 15 as apigenin-6-*C*-deoxyhexoside-(6″-*O*-hexosyl-8-*C*-hexoside), and component 16 as apigenin-(6″-*O*-hexosyl-6-*C*-hexoside)-8-*C*-deoxyhexoside.

3.5. Chromatographic behavior

Besides the analysis of MS spectra, structural information can also be obtained from the chromatographic behavior of the components. Detailed analysis of the retention times (Table 1) further supported the proposed structures for the components of interest (1–16 in Table 1).

The retention times for *O*-glycosides (components 1–4) inversely correlated to polarity as the triglycoside (component 4) preceded the diglycosides (components 1–3). As components 1–3 assumable possessed the same glycoside substituent, differences in their retention times could be explained by the different polarity of their aglycones. The quercetin-glycoside component 2 (two free hydroxyl groups on ring B, Fig. 2) eluted first, followed by the kaempferol-glycoside component 1 (one free hydroxyl group on ring B) then the isorhamnetin-glycoside component 3 (a free hydroxyl and a metoxyl group on ring B).

For *C*-glycosides (components 5–13) the polarity of the molecules were determined by the polarity of the aglycone and the conjugated sugar units. In general, hexosyl glycosides (four free hydroxyl groups in the sugar part) preceded pentosyl glycosides (only three hydroxyl groups), and pentosyl glycosides preceded

deoxyhexosyl glycosides (three hydroxyl groups and a methoxyl substituent) in polarity. We observed the same elution tendencies for the di-*C*-glycosides (components 7–13). For components 7–11, which differed only in their sugar residues, the di-hexosyl isomer (component 11) eluted first, followed by the pentosyl-hexosyl isomers (components 7 and 8) then the deoxyhexosyl-hexosyl isomers (components 9 and 10). In addition, the luteolin-glycoside components 12 and 13 preceded the apigenin-glycoside components 9 and 10, in concordance with the fact that luteolin had two hydroxyl groups on ring B, while apigenin possessed only one. The examples of components 7–10 demonstrated that 8-*C*-hexosyl isomers eluted earlier than the 6-*C*-hexosyl isomers. Based on this fact, we propose component 12 as luteolin-6-*C*-deoxyhexosisde-8-*C*-hexoside and component 13 as luteolin-6-*C*-hexoside-8-*C*-deoxyhexoside.

4. Conclusion

Sixteen flavonoid glycosides have been separated from the methanol extract of heartsease herb by micro-liquid chromatography and characterized by tandem mass spectrometry. The MS^2 and MS^3 analysis of the flavonoid components of interest provided data about their glycosylation type and position, nature of their aglycones, and the structure/linkage information of their glycan moieties. The stereochemical assignment of hexose, deoxyhexose and pentose residues, however, could not be achieved by means of mass spectrometry. On the other hand, in our former studies [27,28] the two main constituents of heartsease components 10 and 2 were isolated and identified as violanthin (apigenin-6-*C*-glucoside-8-*C*-rhamnoside) and rutin quercetin-3-*O*-rhamnosyl(1 → 6)glucoside, respectively, by NMR and mass spectroscopic experiments.

Acknowledgments

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Analysis of Polar Antioxidants in Heartsease (*Viola tricolor* L.) and Garden Pansy (*Viola x wittrockiana* Gams.)

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Abstract

Heartsease (*Viola tricolor* L.) is a well-known medicinal plant. Its biological activities are supposed to be related to its antioxidant capacity. Garden pansies (*Viola x wittrockiana* Gams.) have been crossbred from heartsease and are applied as ornamental plants only. In this study, the mother and the daughter species are compared from a phytochemical point of view. Their flavonoid and anthocyanidin contents are determined by spectroscopic methods recommended by the European Pharmacopoeia 5.0. The compositions of the samples (heartsease and garden pansy varietas of several petal color) are analyzed by high-performance liquid chromatography with UV detection and their antioxidant capacity is determined by trolox equivalent antioxidant capacity assay. Our results suggest that garden pansy, especially its flower, is a promising source of natural antioxidants. In addition, a significant correlation is found between the flavonoid content and antioxidant activity.

Introduction

Antioxidants are molecules capable of quenching harmful free radicals; thus they are useful in the prophylaxis and treatment of more than eighty types of illnesses including cardiovascular problems, complications of diabetes, inflammations, immune disorders, and liver problems, just to list a few (1). These illnesses occur if the body's oxidative balance is disturbed. Although natural antioxidants are present in the daily diet in moderate amount, there are cases when additional supplements are necessary to support the body's own antioxidant system. In recent years, considerable attention has been devoted to natural antioxidants in order to replace synthetic ones. Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. Heartsease herb has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic (2). Due to its flavonoid and other phenolic compound content, heartsease may represent a promising source for natural antioxidants (5). Garden pansies (*V. x wittrockiana* Gams.) are plants of complex hybrid origin involving at least three species: *V. tricolor*, *V. altaica*, and *V. lutea*. They have several colorful varieties and are widely cultivated as ornamental plants. Since garden pansies have been crossbred from *V. tricolor*, they are believed to possess similar good antioxidant activity.

The antioxidant capacity can be determined by means of in vitro and in vivo assays. The in vitro techniques are classified according to the reaction mechanisms involved. Hydrogen atom transfer (HAT) based methods and single electron transfer (SET) based methods are both widely utilized (6). The so-called trolox equivalent antioxidant capacity (TEAC) assay characterizes the electron-donor (7) property of the samples. Due to its advantages in ease of use and reproducibility, this technique is applied in many laboratories (6). Although, due to the complex reaction paths involved, this assay does not always allow exact quantitative evaluation of the antioxidant capacity, it is still a proper method to provide the ranking order of antioxidants (8).

Besides the antioxidant capacity and phenolic content, their chemical compositions are characteristic of the samples of interest from the phytochemical point of view. Because of the unknown composition and complexity of plant extracts, high-performance liquid chromatography (HPLC)-based quantitation of the samples is apparently not an option. For the determination of the approximate anthocyanidin and flavonoid content, spectroscopic methods are being suggested by the European Pharmacopoeia 5.0 (9). The qualitative analysis of the flavonoid composition, however, is usually carried out by reversed-phase HPLC (C8 or C18) (10). The most common detection methods are UV and photodiode array based, although recently mass spectrometry has been rapidly gaining ground (11). In spite of the fact that for the separation of reference molecule mixtures proper methods have been developed (12,13), the qualitative analysis of plant extracts can not be standardized. For each plant sample, therefore, an identical HPLC method must be developed to be able to obtain the so-called fingerprint chromatograms. In phytomedicine, these chromatograms are important indicators of plant material quality. In some cases they reveal the absence of certain components (e.g., pesticide residues) (14,15), or prove that the components responsible for the biological activity are present in appropriate quantities (16–18).

Albeit, garden pansies have been crossbred from *V. tricolor*,

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their secondary metabolite composition has neither been studied nor compared with heartsease. In this paper, we report on a comparative study including HPLC and spectroscopic methods of the polar components of heartsease and garden pansy extracts. These compounds are believed to contribute to the antioxidant properties as well as to the biological activities of heartsease.

Materials and Methods

Chemicals and plant materials

Heartsease herb (*Viola tricolor* L., Violaceae) (SN = 28-56-05-VI/24) was purchased from Fitopharma Ltd. (Budapest, Hungary). Garden pansies (*Viola* x *wittrockiana* Gams., Violaceae) were cultivated in Nagyrécse (Hungary). They were selected by petal color: violet, violet-white, white and yellow, and collected as herbs, flowers and leaves. Ginkgo folium (*Ginkgo biloba* L.) was collected in the botanical garden of the Roland Eotvos University, Budapest and identified together with the pansy varietas in the Department of Pharmacognosy, Semmelweis University (Budapest, Hungary), where voucher specimens were deposited (n = 2).

All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Sample preparation

0.50 g air-dried plant material was sonicated with 20 mL of 70% methanol for 20 minutes in an ultrasonic bath at room temperature. The filtered extract was evaporated to dryness in vacuum at 60°C. The dry residue was re-dissolved in 1.5 mL 70% of methanol and separated by solid-phase extraction. The SPE cartridge (Supelclean LC-18, 500 mg/3 mL, Sigma-Aldrich) was activated with 3 mL methanol followed by 3 mL 2% acetic acid. After sample introduction, the cartridge was washed with 1.5 mL of 70% methanol. The loading and washing solvents were collected and combined for downstream analysis.

HPLC and LC–MS analysis

HPLC separation of the heartsease herb and garden pansy samples were performed by means of an ABLE-E & Jasco (Tokyo, Japan) apparatus consisting of the following components: ERC-3113 degasser, LG-980-02 solvent mixer, PU-980 pump, 20 µL Rheodyne 7725 injector, and UV-975 UV–vis detector. For the separation, gradient elution from 13% to 18% acetonitrile in 20 min (A = 2.5% of acetic acid) was performed at a flow rate of 1.0 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm, Sigma-Aldrich) column.

The LC–MS analysis of the heartsease herb sample was performed on a Model 1100 HPLC–MSD SL system (Agilent Technologies, Waldbronn, Germany), which was comprised of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the chromatographic separation, gradient elution from 10% to 40% ACN in 30 min (A = 2.5% acetic acid) was performed at a flow rate of 0.5 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm) (Sigma-Aldrich) column. The eluate was monitored with both the diode array (at 340 nm) and the mass selective detectors. MSD scanning was performed from *m/z* 100 to 1000 in 0.2 min intervals. The mass selective detector was equipped with a normal-flow electrospray ionization source (ESI). Electrospray conditions were as follows: drying gas flow, 13 L/min; drying gas temperature, 350°C; nebulizer pressure, 35 psi; capillary voltage, 3000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

Determination of the anthocyanidin and flavonoid content and their correlation to the antioxidant activity of the samples

The anthocyanidin and flavonoid content of the dried samples were determined by applying the methods of the European Pharmacopoeia 5.0, paragraph "Bilberry fruit, fresh" *(Myrtilli fructus recens)* and "Goldenrod" *(Solidaginis herba)* (9).

To be able to characterize the samples applied in the TEAC assay, we also determined their flavonoid content. Accordingly,

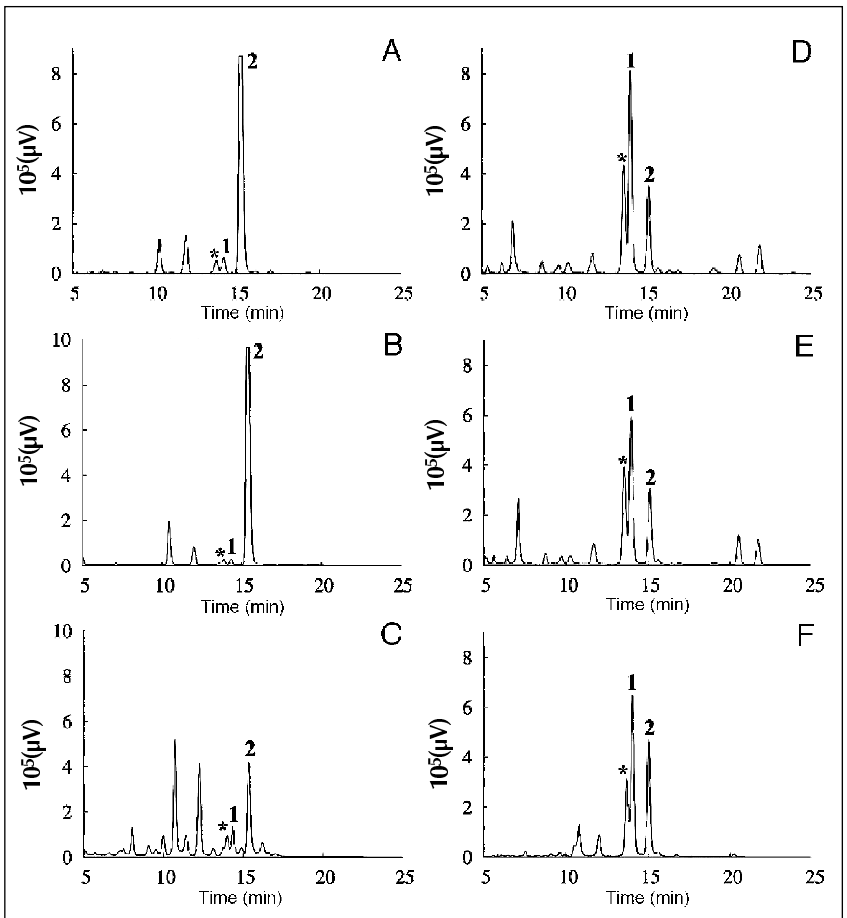

Figure 1. HPLC chromatograms of violet-white pansy flower (A), white pansy flower (B), violet pansy flower (C), yellow pansy herb (D), white pansy leaf (E), and heartsease herb (F). Gradient elution from 13% to 18% acetonitrile in 20 min (A = 2.5% acetic acid) was performed at a flow rate of 1.0 mL/min on a Hypersil ODS (250 x 4.6 mm, 5 µm) column.

0.5 mL of the extracts (see the TEAC assay section) was diluted and treated as described in the original method of the European Pharmacopoeia 5.0. The results were then used for calculating the correlation between the flavonoid content and antioxidant capacity. Data were imported into the Origin 6.0 software (Microcal Inc., Northampton, MA, x = flavonoid content / anthocyanidin content, y = IC_{50} value (g/mL)) and the "Correlation" statistical test was applied. The software calculated the correlation coefficient (r) and the statistical significance (p).

TEAC assay

1.0 g of dried and freshly powdered plant material was sonicated with 20 mL of 70% methanol for 20 min in an ultrasonic bath at room temperature. Five mL water and 5 mL CCl_4 were added to the filtered extract to get rid off the chlorophyll. After centrifugation (9000/min, 10 min) the chlorophyll-free supernatant was evaporated to dryness in vacuum at 60°C. The residue was re-dissolved in methanol for further analysis.

For the TEAC assay, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) was dissolved in water in 7mM concentration. Its radical mono-cation was produced by reacting the ABTS solution with 2.45mM (final concentration) potassium persulfate, and the mixture was stored in the dark at room temperature for at least 12 h before use. The $ABTS^{\bullet+}$ stock solution was diluted with spectroscopic grade ethanol to 0.9 absorbance unit at 734 nm. Five different volumes of the diluted sample were added to 2.5 mL ABTS solution resulting in different final concentrations and producing inhibition of the blank solvent between 20–80%. Absorbance values were measured at 734 nm after 0.0, 0.50, 0.66, 0.83, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00, and 6.00 min. To determine the so-called inhibition percentage, we extrapolated the final absorbance by numerically solving the simplest possible reaction kinetics model. The inhibition percentage produced by a given sample concentration was calculated as $(100 - A_t/A_0) \times 100$ (A_t = extrapolated final absorbance; A_0 = absorbance of the blank solvent). The antioxidant activity was characterized by plotting the inhibition percentage of the samples as a function of concentration and performing linear regression (data not shown). The concentration resulting in 50% inhibition is referred to as IC_{50} value.

Results and Discussion

Qualitative and quantitative analysis of heartsease and garden pansy polar extracts

Because the characteristics of the anticipated antioxidant components of anthocyanidins, flavonoids, and other phenoloids in heartsease and garden pansy are mostly polar, we have chosen to use methanol extracts of the plant materials for our studies. Extracts were prepared from herbs, flowers, and leaves of heartsease and several garden pansy varieties of violet, violet-white, white, and yellow petal color. All samples were analyzed by reversed-phase HPLC and representative chromatograms are shown in Figure 1. The chromatograms exhibit very similar peak distribution of all the samples with differences mostly observed in peak ratios. As most panels in Figure 1 exhibit, the two major components were eluted at retention times of 14.0 (Peak 1) and 15.0 min (Peak 2). Their UV spectra was typical of flavonoids (19), with absorption maxima at 274 and 336 nm, as well as 266 and 361 nm for the two peaks, respectively. Comparing the retention times, UV spectra, molecular masses (MW = 610.2) and fragmentation patterns with reference standard molecules, as a first approximation we consider Peak 2 to be rutin (see structure in Figure 2B). Peak 1 of Figure 1 was identified as violanthin (see structure in Figure 2A) by NMR (data not shown) and mass spectrometry. As this study was focused on rutin and violanthin, this publication did not aim to obtain any structural information about the rest of the peaks in Figure 1. However, according to their chromatographic retention data and absorption spectra profile, these components are most probably flavonoid glycosides. The peak marked by the asterix in Figure 1 with the retention time of 13.7 min (Peak*) might be isoviolanthin, based on UV spectroscopic considerations (absorption maxima at 274 and 335 nm).

By the different intensity of the individual components in the chromatogram, the samples were classified into the following two major groups of flower and herb/leaf samples. The main component of the flower samples (Peak 2 in Figure 1D and 1E) is proposed to be rutin, according to the HPLC and LC–MS experiments described earler. The second group (Figure 1A–1C) comprised the herb and leaf samples, apparently contained much lower quantities of rutin (Peak 2), but was rich in violanthin (Peak 1). The similarity between the herb and leaf samples can be associated with the fact that pansy herb samples consisted mostly of leaf (80%, determined by the

Table I. The Anthocyanidin and Flavonoid Contents, as well as the Antioxidant Capacity of Heartsease and Garden Pansies of Different Petal Color

Sample		Antocyanidin content (g cyanidin-3-glucoside per 100 g sample)	Flavonoid content (g rutin per 100 g sample)	Antioxidant activity IC50 value (g/mL)
Violet pansy	flower	1.52 ± 0.06	1.21 ± 0.07	$(1.57 \pm 0.05) \times 10^{-5}$
	leaf	0.05 ± 0.002	0.10 ± 0.006	$(3.86 \pm 0.18) \times 10^{-5}$
	herb	0.31 ± 0.01	0.38 ± 0.02	$(2.92 \pm 0.04) \times 10^{-5}$
Violet-white pansy	flower	0.19 ± 0.01	2.58 ± 0.15	$(8.59 \pm 0.25) \times 10^{-6}$
	leaf	0.04 ± 0.002	0.03 ± 0.002	$(3.18 \pm 0.09) \times 10^{-5}$
	herb	0.05 ± 0.004	0.62 ± 0.03	$(1.99 \pm 0.10) \times 10^{-5}$
White pansy	flower	0.09 ± 0.004	2.01 ± 0.12	$(6.35 \pm 0.08) \times 10^{-6}$
	leaf	0.06 ± 0.002	0.16 ± 0.01	$(5.32 \pm 0.12) \times 10^{-5}$
	herb	0.08 ± 0.003	0.59 ± 0.03	$(1.42 \pm 0.06) \times 10^{-5}$
Yellow pansy	flower	0.31 ± 0.01	2.93 ± 0.18	$(6.98 \pm 0.28) \times 10^{-6}$
	leaf	0.11 ± 0.004	0.11 ± 0.007	$(4.00 \pm 0.09) \times 10^{-5}$
	herb	0.11 ± 0.006	0.62 ± 0.04	$(1.87 \pm 0.05) \times 10^{-5}$
Heartsease	herb	0.02 ± 0.0008	0.50 ± 0.03	$(4.17 \pm 0.10) \times 10^{-5}$

weight of the fresh species) rather than flowers (20%, determined by the weight of the fresh species). The weight of the stem was negligible. The violet flower sample did not fit into this classification as it only possessed low amounts of rutin and violanthin, as depicted in Figure 1F, thus in the violet flower samples the minor components gained more significance in the HPLC peak pattern.

In addition to their flavonoid content distribution, the actual anthocyanidin and flavonoid contents are distinctive of the samples. Anthocyanidins are natural pigments in plants; consequently, they are present in flowers (especially with colorful petals) in larger quantities than in leaves. The quantity of anthocyanidins and flavonoids were determined by applying the registered methods of the European Pharmacopoeia 5.0 (Table I, Figure 3). The highest anthocyanidin content was measured in violet pansy. Colorless varieties (yellow, white and white with a little violet) possessed only low amounts of anthocyanidins. On the other hand, in regard to the flavonoid content, species with pale petals showed elevated concentration. The highest amount was measured in yellow pansy flower. As Table I and Figure 3 depict, flower samples had higher flavonoid content than leaf samples, while herb samples were in between.

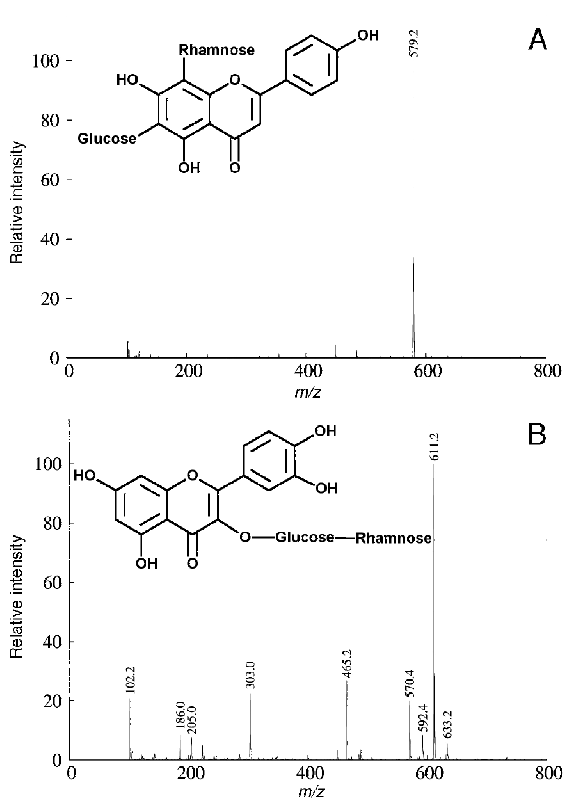

Figure 2. Chemical structures and MS spectra of component 1 (A) and component 2 (B).

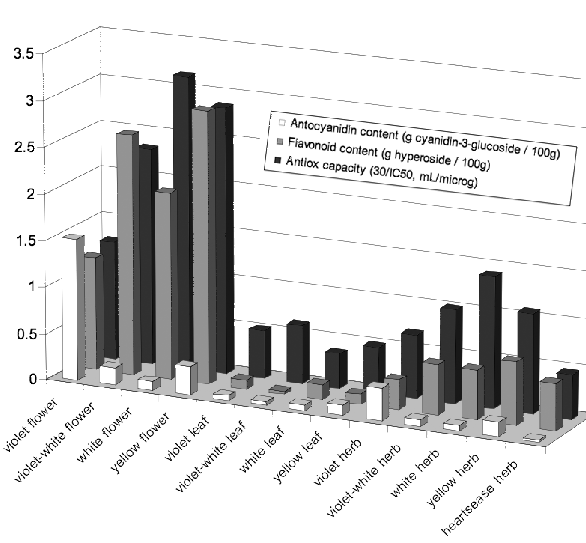

Figure 3. Anthocyanidin and flavonoid contents, as well as the antioxidant capacity of heartsease and garden pansies of different petal color.

Antioxidant activity of the samples

The antioxidant activity of the samples was determined by TEAC decolorization assay, in which colorful artificial free radicals ($ABTS^{\bullet+}$) were generated and their concentration was determined before and after sample introduction. Antioxidant components in the sample quenched the free radicals, whose concentration decreased proportionally to their antioxidant capacity. The oxidized and reduced forms of the radical absorbed at different wavelengths, allowing quantitative spectroscopic determination of the free radical concentration in the samples. The antioxidant capacity was characterized by calculating the IC_{50} value: the concentration, which produces 50% quenching of the free radicals (Table I, Figure 3). The lower the IC_{50} value, the better the antioxidant activity of the sample. Our experiments revealed that in this test system the heartsease and pansy samples both showed good antioxidant activities. Apparently, their antioxidant capacity was at the same level as the well-known antioxidant ginkgo leaf of $IC_{50} = 1.82 (\pm 0.07) \times 10^{-5}$ mg/mL. In addition, the crossbred garden pansy possessed better antioxidant properties than its mother species, heartsease. Similarly to the flavonoid content, flower samples showed the highest antioxidant activity, whereas herb and leaf samples possessed inferior antioxidant properties according to the IC_{50} values attained (Table I, Figure 3). Based on this similarity, we examined the relationship between the antioxidant activity and the flavonoid content of the samples, and found significant correlation between the antioxidant capacity and the flavonoid content ($R = -0.6375$, $p = 0.02$). On the other hand, no correlation was found between the antioxidant capacity and the anthocyanidin content ($R = -0.2798$, $p = 0.35$).

Conclusion

In this paper we report on the study of polar antioxidants of heartsease (*Viola tricolor* L.) and its hybrid, garden pansy (*Viola* x *wittrockiana* Gams.). The flavonoid compositions of the extracts prepared from heartsease herbs, flowers, and leaves of several garden pansy varieties of violet, violet-white, white, and yellow petal color were analyzed by HPLC. Apparently similar major components were found in herb, leaf, and flower samples; however, differences were observed in their component ratios. According to the chromatographic peak distribution, the sam-

ples were classified into two major groups: flower and herb/leaf samples. Comparing the retention times, the UV spectra, the molecular masses, and fragmentation patterns with reference standard molecules, as a first approximation we consider the main component of the flower samples as rutin. The main component of the herb and leaf samples were identified as violanthin using NMR and MS.

The anthocyanidin and flavonoid contents of the samples were quantitated by spectroscopic methods registered in the European Pharmacopoeia 5.0. While the highest anthocyanidin content was measured in the violet flower sample, the white and yellow pansy samples showed the highest flavonoid content.

The antioxidant capacity of the samples was determined by TEAC assay. Our data revealed that in this test system the heartsease and pansy samples were as good antioxidants as the well-known antioxidant ginkgo leaf. In addition, significant correlation was found between the flavonoid content and the antioxidant capacity of the samples, whereas no correlation was observed between the antioxidant capacity and the anthocyanidin content.

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