Analysis of medicinal plant phenoloids by coupled tandem mass spectrometry

Ph.D. Thesis

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Contents

List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo ionization
BPC	Base to peak chromatogram
BPH	Benign prostate hyperplasia
cAMP	Cyclic adenosine monophosphate
CID	Collision induced decomposition
COSY	Correlation spectroscopy
COX	Cyclo oxygenase enzym
cps	counts per second
DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
EI	Electron ionization
EIC	Extract ion chromatogram
ESI	Electrospray inozation
FAB	Fast atom bombardment
FD	Field desorption
FWHM	Full width half maximum
GC-MS	Gas chromatography-mass spectrometry
HMBC	Heteronuclear multi-bond correlation
HPLC-MS	High performance liquid chromatography-mass spectrometry
HPTLC	High performance thin layer chromatography
HSQC	Heteronuclear single quantum coherence
IT	Ion trap mass analyzer
LC	Liquid chromatogarphy
LOD	Limit of detection
LLOQ	Lower limit of quantitation
LOQ	Limit of quantitation
MRM	Multiple reaction monitoring

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multi-stage mass spectrometry
MW	Molecular weight
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser-effect spectroscopy
RDA	Retro Diels-Alder
RI	Refractive index
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Single reaction monitoring
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoro acetic acid
THF	Tetra hydrofurane
TIC	Total ion chromatogram
TLC	Thin layer chromatography
QQQ	Triple-quadruple mass analyzer
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight mass analyzer
TSP	Thermospray ionization
VIS	Visible light
UV	Ultra violet light

1. Introduction

1.1. Preliminaries

In the last 20 years the way of thinking about human health has changed a lot. Words like „prevention", „synthetic" and „natural" became absolute key words of the modern medical-pharmaceutical approach, and as a result, herbal therapy is living its renaissaince nowadays. Nature, and natural compounds have an unexceptional influence on pharma R & D as they provide an uncountable number of invaluable lead molecules. Phytochemical researches of nowadays focus on bio-assay guided revealing of the therapeutic profile and synergism of medicinal herbs and their constituents. Assesing the clinical and biological potential and determining the pharmacokinetics of herbal constituents is also a hot area. Efficacy of several herbal and plant derived remedies have been shown by clinical trials recently.

Identification and determination of the active substances (either minor or major) is a crucial precondition for the development of modern evidence-based phytomedicines according to the regulations of the EMEA and FDA. Medicinal plant extracts and herbal preparations are complex mixtures of active- and ballast substances which may contain not infrequently up to several hundreds of different constituents with not exactly defined structures. Hence chromatography is undoubtly fundamental to overcome the challenges of phytoanalytics. The usual high performance liquid chromatography (HPLC) associated detectors, such as ultra violet (UV), fluorescent, electrochemical and refractive index (RI), are not as selective and sensitive as a modern detector of today should, and required to be. Mass spectrometry (MS) offers great selectivity and sensitivity and by coupling to high performance liquid chromatography (LC-MS) it enables effective analysis of complex matrices. LC-MS represents a well-established, rapid, powerful and robust technique, available with a wide range of ionization modes for the analysis of non volatile polar, semi-polar and -in some extent- apolar compounds. For the analysis of volatile, rather apolar molecules gas chromatography-mass spectrometry (GC-MS) is the hyphenated method of choice. Since typical and potential drug molecules are rather polar and

water soluble LC-MS has a greater significance than GC-MS. By tandem mass spectrometry (MS/MS) a full structural analysis of a mixture can be accomplished by a few runs involving no time-consuming isolation process. Triple quadrupole MS/MS systems ensure excellent selectivity and sensitivity for quantitative aims. Nuclear magnetic resonance spectroscopy (NMR) serves as a complement analytical tool for LC-MS systems in unambiguous structure elucidation. Today LC-NMR-MS is perhaps the most promising hyphenation technique, but it still needs a few years time to be put in routine, not to talk about its stratospherical price, while LC-MS rapidly becomes a routine technique for the fast and powerful analysis of almost any complex matrix.

2. Aims of studies

Two distinct, but strongly related aims governed our work. First, we aimed to revaluate the phytotherapeutical potential of selected, traditionally well known, but chemically less characterized medicinal plants by the qualitative and quantitative analyses of their active substances. Modern LC-MS methods were applied to investigate the selected traditional herbs of various phenoloid content posessing significant medicinal effects.

Our second aim was to provide modern analytical solutions to replace older, less selective methods of phytoanalytics by studying the versatility and potential of coupled mass spectrometry in phytochemical applications, and draw general conclusion based upon our particular analyses.

Particular model plants were selected with the aim to represent the wide structural and therapeutical variety of phenolics from simple phenolic acids to macrocyclic polyphenols. Phenoloids own highly significant biological and pharmacological action, besides, if not present as active substance, they frequently occur as ballast substances, causing challenging complications in analytical work. Either active substance or ballast, phenolics are nearly always an issue in quality control and quality assurance of herbal products.

With the above mentioned motivation our investigations aimed to identify or characterize the active phenolic substances and determine the acteoside content of the traditional herbal eye-remedy, *Euphrasia rostkoviana* HAYNE (Eyebright) by the help of oriented antioxidant bio-assays.

We aimed to expand the scarce quantitative information on rosmarinic acid, the main antioxidant phenolic compound of *Satureja hortensis* (Savory).

Filipendula ulmaria L. (Meadowsweet) is a poorly characterized herb of serious antypiretic and anti-inflammatory salicylate content, therefore we aimed to analyse its phenolic constituents, characterize and quantify the salicylate components.

The flavonoid content of *Sempervivum tectorum* (Houseleek) is known partly and only at aglycon level. Since glycosilation status affects bioavalibility we aimed the characterization of the *Sempervivum* flavonoids on the glycoside level.

For providing detailed mass spectral information for the quality assurance of *Epilobium parviflorum* Schreb. (Willowherb) we aimed to characterize and interpret the fragmentation of oenothein B, a special macrocyclic phenolic of the herb.

3. Bibliography review

3.1. Mass spectrometry in phenoloid analytics

A crude plant extract may contain up to hundreds of different secondary metabolites of considerably differing chemical nature and spectroscopic parameters [1]. Therefore chromatographic, purification or isolation steps of separation are crucial prior to detection, identification and quantification. Fingerprint chromatograms permits merely comparison of different samples, eg.: search for differences in chemotaxonomy or declaration of adulteration. Confirmation of identity when a conventional LC detector (UV, RI, evaporative light scattering, fluorescence) is in use can only be achieved by corresponding retention time and/or with reference standards. None of the conventional LC detectors are able to detect, within the same analysis, all metabolites encountered in a herbal extract. Characterization and identification of unknown constituents requires a more informative, selective and sensitive analytical tool.

Phenolics, encountered as active substances or ballast compounds, show a great structural variety, but contrary confusingly similar UV absorbtions which are just minimally affected by glycosilation. As phenolics, just like every molecule, posesses a given molecular weight, mass spectrometry can be considered a well fitting detection technique, however it is much more than a simple detector. MS has the potential to yield information on molecular weight as well as on structure. Since the coupling of LC and MS became such an established technique as it is today, the time-consuming isolation and purification procedures being obligatory previously, are mostly redundant today in phytochemical analysis.

3.1.1. Phenoloids

Phenolics is a vast group of plant substances which can hardly be defined simply. They range from simple structures with one aromatic ring to highly complex polymeric substances such as tannins. Fundamental structural elements to

characterize the approx. 8000 naturally occuring phenolics are at least one aromatic ring substituted by at least one hydroxyl group, free or engaged in another function: ether, ester or glycoside. If merely these chemical criteria were considered numerous other structures would be included (e.g. alkaloids and terpenes), this is why a biosynthetic criterion is necessary as well as to exactly define the boundaries of the group. Plant phenolics arise from two main aromatization pathways [2-5]:

- the most common, the shikimic acid pathway (Fig 3.1.) leading from monosacharides to aromatic amino acids (tyrosine and phenylalanine), then by deamination of the first, to cinnamic acids and their derivatives, like benzoic acids, lignanes and coumarins.

- other pathway starts with acetate and leads to poly-β-ketoesters which, by cyclization enables polycyclic products, including depsides, xanthones and quinones. (Fig 3.2.).

Flavonoids are derived from a C_6-C_3 (phenylpropane) unit, from shikimic acid (via phenylalanine) and a C_6 unit that is originated from the polyketide pathway (via malonyl-Co-A) (Fig 3.2.), flavonoids are therefore of mixed biosynthesis, consisting of units derived from both shikimic acid and polyketide pathways.
Structural diversity of phenolics is due to the frequent combination of this two biosynthetic origin. Compounds of mixed origins are flavonoids, stilbenes and pyrones.

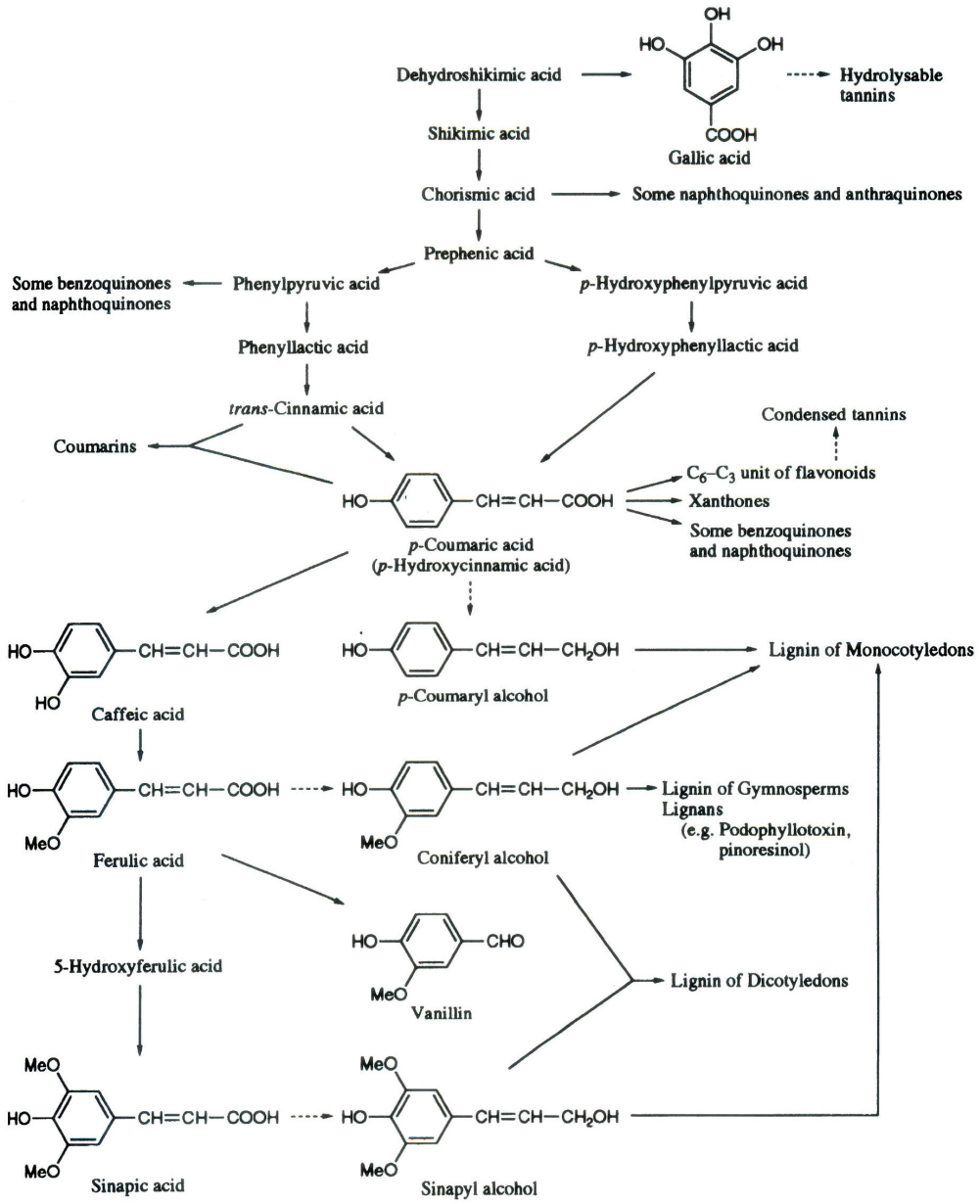


Fig 3.1.: Main steps of the shikimic acid pathway leading to cinnamic acids and their derivatives [4].

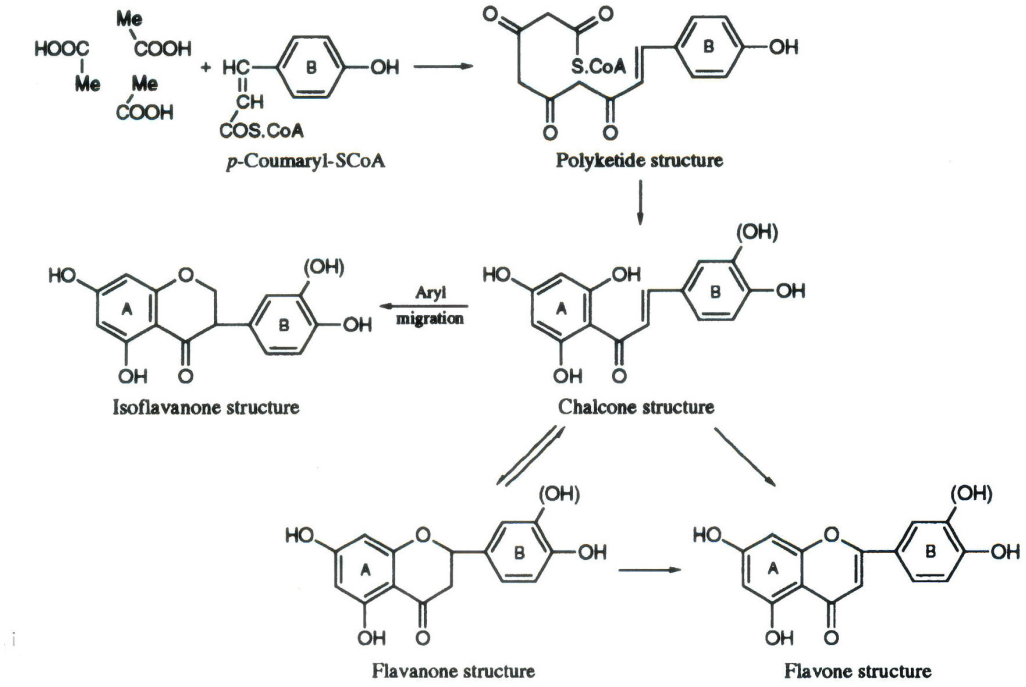


Fig 3.2.: Main steps of the flavonoid biosynthesis [4].

Phenolic classes of pharmaceutical interest are (1) simple phenolic compounds, (2) hydrolysable and non-hydrolysable tannins, (3) coumarins and their glycosides, (4) anthraquinones and their glycosides, (5) naphtoquinones, (6) flavonoids and related glycosides, (7) anthocyanidins and anthocyanins, (8) lignans and lignin [2, 4].

A key feature of the chemical properties of phenolics is their high reactivity due to oxidation of the phenolate ion. This ease of oxidation results in color reactions and directly visible colour. Phenolate ion stabilization by resonance explains the acidity of these molecules, and their good solubility in alkalic solutions.

Pharmacological application and uses of phenolic conatining herbs are closely related to their biosynthetic origin and can hardly be discussed in general, though their antioxidant activity and complex formation ability with macromolecules (proteins) is self-explanatory based upon the chemical attributes [2-5]. Table 3.1. gives an overview of the most common effects and uses of different phenolics classes.

Table 3.1.: Main classes and activities of phenolics.

Classes of phenolics	Activity and therapeutic uses
Phenols, simple phenolic acids	Antiseptic, anti-inflammatory, antipyretic, enzyme inhibitor (cAMP phosphodiesterase, aldose reductase etc.) antibacterial, antifungal, antioxidant [2, 6, 7-10]
Coumarines	venous tonic, vascular protective, anti-psoriatic [2, 4, 11]
Lignans and neolignans	antibacterial, antifungal, enzyme inhibitor [2, 12]
Flavonoids	antioxidant, anti-inflammatory, hepatoprotective, anticancer, decrease capillary fragility and permeability, enzyme inhibitor (cAMP phosphodiesterase, histidine decarboxylase, protein-kinase etc.), estrogenic [6, 9, 10, 13-17]
Anthocyanins	decrease capillary fragility and permeability, antioxidant, anti-bacterial, vision improver [13, 18, 19]
Tannins	complexation with macromolecules and proteins, astringent, antioxidant, enzymatic inhibition (5-lipoxygenase) [4, 6, 20]
Quinones	antibacterial, antifungal, laxative [2, 4, 21]

Phenolics, in general, bear a wide array of health-promoting benefits, such as antioxidant [6], anti-inflammatory [7, 15], antimutagenic and anticarcinogenic activities [22, 23, 14], of which their antioxidant potential is the most frequently investigated. Oxidative stress may underly as a key feature of inflammation process, and polyphenolic compounds as potent natural antioxidant agents, may play an important preventive role in several serious diseases by protecting the body tissues from reactive oxygen species [24, 25]. For all these benefits plant phenolics are in the focus of current pharmacognosy researches, with the hope to understand more their biology, quality, quantitiy in plants, and pharmacological mechanismway, degradation and kinetics in humans.

3.1.2. Extraction and sample preparation

For the extraction of phenolics methanol, ethanol, aqueous ethanol, aqueous methanol, ethyl acetate and aceton suits the best according to polarity [3]. Apolar solvents (hexane, chloroform) are also used for the pre-extraction and removal of

nonpolar constituents. Soxhlet extractor is the most commonly used tool for extracting solid herbal samples. Other methods include ultrasonic bath extraction, microwave assisted extraction, and infrequently supercritical fluid extractor as well [3]. Acidic hydrolysis of glycosides is a common procedure which results free aglycons for further investigation.

For conventional column chromatographic pre-separation, isolation or rough fractionation of the crude extract polyamide, silica gel and Sephadex LH 20 are the generally accepted stationary phases [26, 27]. Flash chromatography or counter current chromatography might be used for standardization or repeatability improvement of fractionation [28]. Chemical separation, concentration or clean up of the samples can be achieved by solid phase extraction (SPE) using organic eluents (methanol, acetonitril) and aqueous solvents preferably the same as of the aqueous HPLC eluent.

3.1.3. Conditions and instrumentation

In the past 20 years the on-line combination of HPLC and MS has become a routinely applicable, extremely powerful and user-friendly analytical tool. The inability of gas chromatography-mass spectrometry (GC-MS) to analyze non-volatile and/or thermolabile molecules of high polarity, such as plant phenolics, and the introduction of atmospheric pressure ionization (API) tecniques, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) gave a massive contribution to LC-MS in gaining its position of today. The rapidly increasing number of papers convincingly proves that LC-MS is a unique technique capable of analysing complex mixtures, like plant extracts, regardless to co-elution or other separation fault, which was a frequent problem when only UV or other usually LC associated detectors were used [29, 30]. This is well supported by the work of Mauri and Pietta in which the ESI characterization of eight selected medicinal plant extracts was accomplished in positive ion mode without chromatographic separation [31]. However, an informative fingerprint can be obtained by this way, chromatography should not be undervalued, since ion suppression and other matrix effects still may result in

inaccurate or false MS respond, not to mention the challenging issue of identifying co-eluting isomers [22, 33].

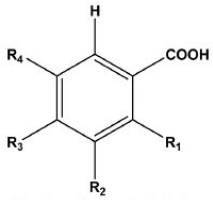
Several reviews are available on HPLC separation of phenolics and there is a great conformity in stationary phase choice. The use of reversed phase (RP) C-18 columns is almost exclusive, ranging from 50 to 250 mm in length, with particle sizes of 1.7-5 µm, mostly endcapped, or polar-embedded to ensure best peak symmetry [3, 34, 35]. An extensive work of Rehová et al. gives an overview and comparison of eleven columns packed with chemically different stationary phases of different diameters used for the separation of flavone aglycons and glycosides and phenolics [36]. Either isocratic or gradient conditions are applied, the most common organic modifiers are acetonitrile and methanol. Contrary to this, there is a great alteration observed among the acidic modifiers of the aqueous phase. The recommended pH range for the analysis of phenolics is between 2 and 4 (most phenolics have a pK_a value around 4, except the higher pK_a values of flavonoids). With an eye kept on MS compatibility, phosphoric acid is unsuitable, due to its non-volatility. Trifluoroacetic acid (TFA) is a popular acidic modifier and however compatible with MS, it is not advisable due to its extensive ionization. The analytes of interest are less or not detectable when TFA is applied. Therefore in majority of the LC-MS investigations acetic acid or formic acid is used in concentrations ranging from 0.1% to 3%. Non-volatile buffers are also banned to use with LC-MS, only formate and acetate buffers are compatible [3, 35, 37]. The column effluent flow rate can influence the sensitivity of mass detection. Optimal flowrates for the LC-MS applications are in the range of 10-50 µL min^{-1}. Though most mass spectrometers of nowadays can operate at flow rates up to 2 mL min^{-1} for ESI and 3 mL min^{-1} for APCI, lower flow rates are strongly advisable for reaching greater sensitivity, for easing instruments maintenance and for economic solvent consumption considerations [35, 38].

Beside the selection of soft ionization (ESI, APCI or APPI), choosing the appropriate mass analyzer is crucial and strictly depends on the analytical aim [35]. For structure elucidation a tandem or multistage mass analyzer is a necessity which is well reflected in the trend of the last 5-6 years: single-stage analyzers have been displaced by tandem or multi-stage instruments. In tandem mass spectrometry only the analyte of interest is transmitted by the first analyzer which is then analysed by the second

one. Multi-stage analyzers can repeat these steps consecutively. An iontrap (IT) or orbitrap analyzer allows fragmentation experiments up to MS^7, but are not the best choice of quantitaive tools [38]. Hybrid constructions, like quadrupole-time-of-flight (Q-TOF), time-of-flight - time-of-flight (TOF-TOF) and classic tandem mass spectrometers, like the triple-quadrupole (QQQ), used in our investigations, especially offers an effective combination of qualitative and quantitative applications. The collision induced decomposition (CID) provides structural information on a selected ion, while the selected ion monitoring (SIM) mode enables high selectivity and with the multiple reaction monitoring (MRM) mode specificity is accomplished in quantitation. Neutral loss scan and precursor ion scan modes offers perfect complement to product ion scan mode [38, 39]. The relative lability of phenoloids, especially glycosides, require the fragmentor voltage (declustering potential) to be kept low (90-120 V). If fragmentor voltage is increased, in-source fragmentation may occur, thus MS^3 can be reached with the QQQ. Fragmentation in this case is not under the operator's control, nevertheless it can be useful [40].

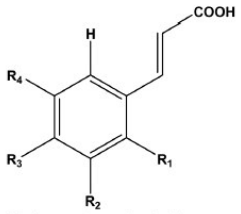
3.1.4. Mass spectrometry of phenolic acids, salicylates and derivatives

The two distinctive carbon frameworks of naturally occuring simple phenolic acids are hydroxicinnamic and hydroxibenzoic structures (Fig 3.3.). The skeleton remaining the same, the variety of these constituents depend on the number, position and conjugation of the hydroxyl groups [3]. Caffeic, coumaric, vanillic, ferulic, protocatechuic acids and their derivatives are common representatives of this group.
The pioneering work on MS analysis of phenolics was accomplished by Alborn and Stenhagen, who described the analysis of caffeic, *o*-coumaric and sinapic acid reference compounds by electron impact ionization (EI) [41]. The EI spectra consisted of low intensity molecular ions but rather diagnostic fragment ions were observable. The effect of ionsource temperature on the fragmentation pattern was studied. The EI fragmentation was too extensive, hence the molecular ion remained undetected in many cases.



Hydroxybenzoic Acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H



Hydroxycinnamic Acids

Name	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Fig 3.3.: Structures and names of the most common simple phenolic acids [3].

The thermospray ionsource (TSP) was a good compromise in detecting the molecular ion, but ESI soon exceeded the potentials of TSP. A pioneering work of ESI interface technology in the mass spectrometry of phenolics was performed by Gioacchini et al., who developed a selective, sensitive method for the determination of phenolic acids and aldehydes [33]. Analytes were identified and determined in negative ion mode, as deprotonated molecules $[M-H]^-$. The research group of Gioacchini concluded that negative ESI provided lower limits of detection of 1 to 2 orders of magnitude as compared to that of other previous ionization methods including TSP in positive mode [33]. This is somewhat straightforward in view of the easy deprotonation of either the hydroxyl or carboxyl group. Although positive and negative modes can be complementary, the latter is much advantageous overall, as it was confirmed by several following studies, and negative mode became the popular choice of ionization mode in the mass spectrometry of phenolics [42-44].

Simple phenolic compounds ans salicylates easily decarboxylate under high temperature giving $[M-H-44]^-$ as a characteristic ion. From salicylic acid to dicaffeoylquinic acids a vast number of molecules produce the loss of -44 amu (CO_2) as can bee seen in Fig 3.4. (*m/z* 163 → *m/z* 119) [45]. Also frequently occurs the formation of $[M-CH_3]^{\cdot-}$ in the case of methoxylated compounds and dehydration of certain fragment ions [46]. An abundant product ion at *m/z* 191 typically refers to quinic acid esters (Fig 3.4.).

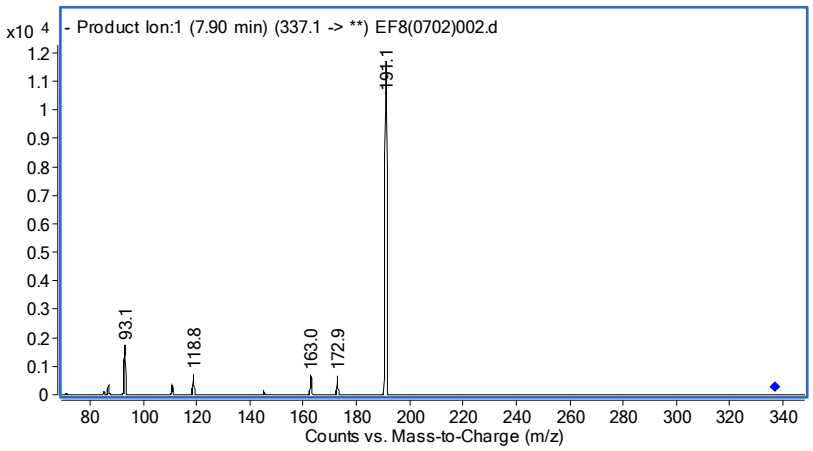
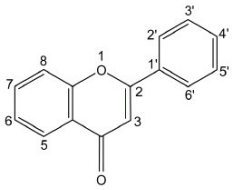


Fig 3.4.: Example for the negative ion CID spectrum of a typical phenolic compound, coumaroyl-quinic acid (collision energy: 30 eV) (self acquired spectrum).

The dehydration product of quinic acid may also emerge in the spectra at *m/z* 173. CID spectra of caffeic acid derivatives usually contain the production *m/z* 179 (caffeic acid unit), whilst further fragments by losses of H_2O (*m/z* 191 → *m/z* 173), CO and CO_2 are also common observations (Fig 3.4.). A neutral loss of -162 amu may point to the loss of a caffeoyl unit, and can also refer to the loss of a hexose (glucose or galactose) moiety which complicates structure elucidation [47]. Another frequent product ion, *m/z* 163 (coumaric acid unit), may point to a coumaroyl ester molecule. The simultanous presence of ion *m/z* 119 (coumaric acid-CO_2) beside *m/z* 163 confirms a coumaroyl type hypothesis [45]. Also, losses of small neutrals, like CO_2, H_2O, CO and product ions at *m/z* 137 (salicylic acid), *m/z* 122 (salicylaldehyde) and *m/z* 124 (salicylalcohol) are common CID fragments of the salicylates and derivatives [48]. In many cases both the $[M-H]^-$ and the ion corresponding to cleavage of the sugar moiety $[M-H-Gly]^-$ are present together in the scan mass spectra, giving two representative ions easing identification. A number of papers, dealing with the mass spectrometric identification of simple phenolics, phenylethanoids and their derivatives, are available in the literature [47, 49-51].

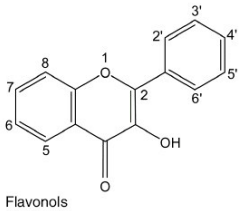
3.1.5. Mass spectrometry of flavonoid aglycons

Flavonoids are one of the largest groups (>6500 known structures) of natural compounds characterized by a chromane type skeleton with a phenyl substituent in the C-2 or C-3 position (C_6-C_3-C_6) (Fig 3.5.). Flavonoids, occuring mainly as *O*-, and *C*-glycosides (hexoses, deoxyhexoses and pentoses), less frequently as aglycons, can be hydroxylated in 3, 5, 7, 3', 4' and/or 5' position. Hydroxyl substituents might be methylated, acetylated, prenylated or sulphated [52, 53]. Mass fragmentation pathways of flavonoid aglycons induced by EI ionization are well recognized, but only a very limited structural information can be obtained regarding glycosides by EI [54, 55]. Due to the high polarity, non-volatility and thermal lability of glycosides, there was no chance of analysis without elaborative derivatization (methylation, trimethylsilylation and acetylation) until „soft" ionization techniques were introduced. After field desorption (FD) ionization was developed in 1975, analysis of underivatized glycosides became possible, which was followed by a number of other „soft" ionization techniques capable of the same [56]. Positive ion CID spectra is more frequently applied than negative ion CID in the investigation of flavonoid fragmentation. This is perhaps because of the more difficult interpretation of the negative spectra, though ionization in negative mode provides greater sensitivity [57].



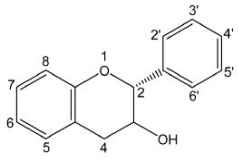
Flavones

Position Compound	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	–	–



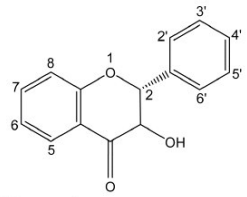
Flavonols

Position Compound	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	–
Kaempferol	OH	OH	–	OH	–
Galangin	OH	OH	–	–	–
Fisetin	–	OH	OH	OH	–
Myricetin	OH	OH	OH	OH	OH



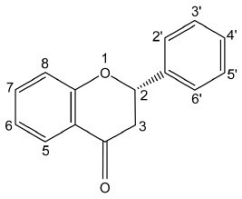
Flavan-3-ols

Position Compound	3	5	7	3′	4′	5′
(+)-Catechin	βOH	OH	OH	OH	OH	–
(−)-Epicatechin	αOH	OH	OH	OH	OH	-
(−)-Epigallocatechin	αOH	OH	OH	OH	OH	OH



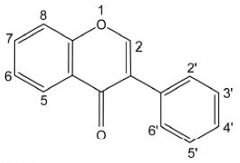
Flavanonol

Position Compound	5	7	3′	4′
Taxifolin	OH	OH	OH	OH



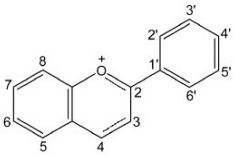
Flavanones

Position Compound	5	7	3′	4′
Naringenin	OH	OH	-	OH
Naringin	OH	0-Rha-Glu	–	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	0-Rha-Glu	OH	OCH_3



Isoflavones

Position Compound	5	7	4′
Genistein	OH	OH	OH
Genistin	OH	0-Glu	OH
Daidzein	–	OH	OH
Daidzin	–	0-Glu	OH
Ononin	OH	0-Glu	CH_3



Anthocyanidins

Position Compound	3	5	7	3′	4′	5′
Cyanidin	OH	OH	OH	OH	OH	–
Cyanin	O-Glu	OH	OH	OH	OH	–
Peonidin	OH	OH	OH	OCH_3	OH	–
Delphinidin	–	OH	OH	OH	–	OH
Pelargonidin	OH	OH	OH	–	OH	–
Malvidin	OH	OH	OH	OCH_3	OH	OCH_3

Fig 3.5.: Structures and names of the most common flavonoids of different types [3].

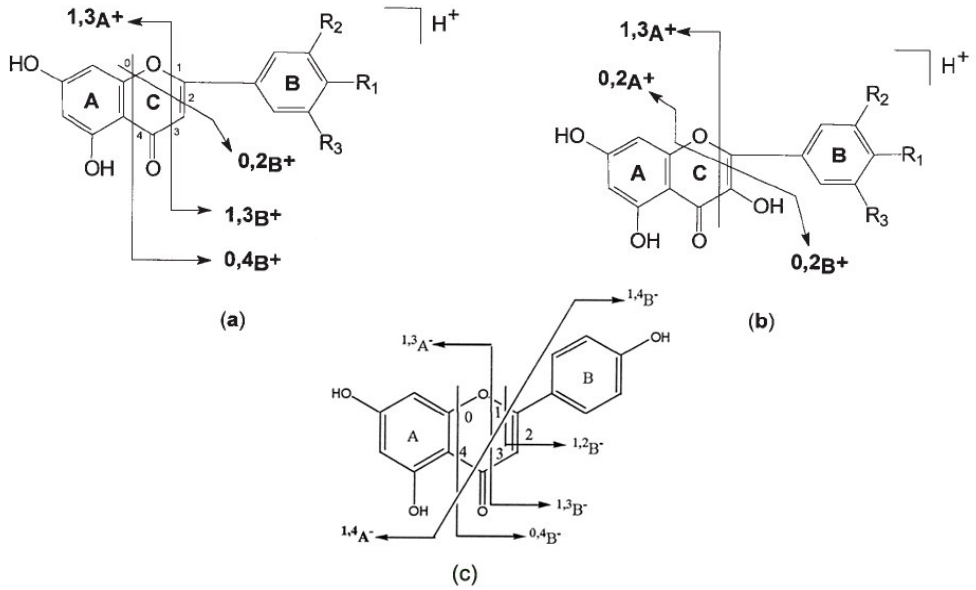


Fig 3.6.: Mass fragmentation pathways of the C ring of flavonoids in positive (a, b) and negative (c) ion CID [58].

The today widespread and accepted nomenclature of flavonoid mass fragmentation pathways were established in principles by Mabry [52] and improved by Ma et al. [58] (Fig 3.6.). Complementing the pathway theory for the glycoconjugates was done by Dommon and Costello [59] (Fig 3.7.). Referring to aglycons, the $^{i,j}A^{+/-}$ and $^{i,j}B^{+/-}$ signs are applied to label main fragments containing intact A or B ring, where superscripts „i" and „j" indicate the numbering of the C-C bond in the C ring (1/3, 0/2, 0/3, 0/4 and 2/4) where fragmentation occured (Fig 3.6.). Glycoside fragments with retained charges on the carbohydrate part are labeled as A_i B_i and C_i, where „i" represents the number of the broken bond, counted from the terminal sugar unit. 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 [60] (Fig 3.7.).

1/3 and 0/4 cleavage of the C ring corresponds to Retro Diels-Alder (RDA) reaction, which, as a key feature of flavonoid aglycon fragmentation, allows one to infer the substitution and oxygenation pattern of rings A and B.

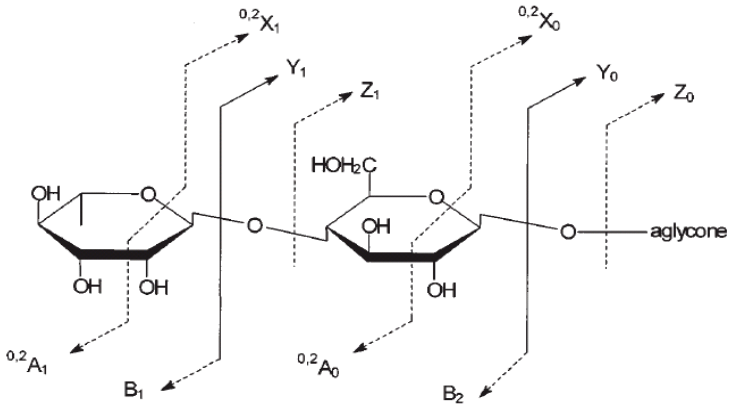


Fig 3.7.: Nomenclature of fragments and the fragmentation paths of the flavonoid glycosides [60].

Superscript +/- provides information on the ionization mode used. The first and most obvious way to characterize the aglycone is to check its molar mass, which reduces at once the number of possibilities, though still several isobars are left (eg. kaempferol and luteolin are both 286 g/mol). Certain fragments resulted by the RDA dissociation are of high significance in further charaterization. The $^{1,3}A^+$ ion (usually *m/z* 153, 137) is observed for all flavonoid groups with characteristic, high abundance in positive ion CID [34, 57] (Fig 3.8.).

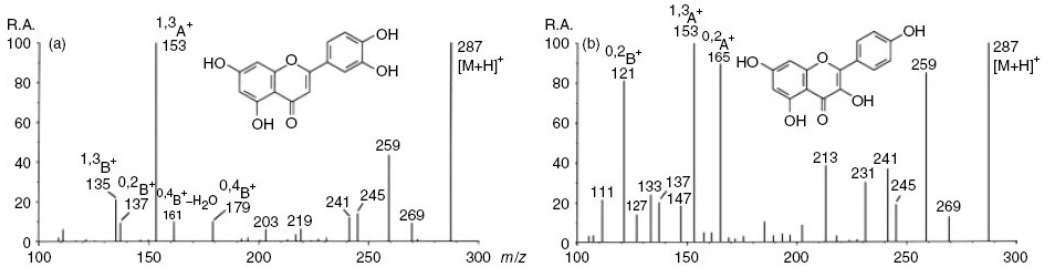


Fig 3.8.: RDA dissociation products of diagnostic *m/z* value in positive ion mode. Figure shows the CID of luteolin (left) and kaempferol (right) [57].

The $^{1,3}B^+$ fragment is distinctive for flavones and isoflavones and can not be found in the MS/MS spectra of flavonols. Observed only under soft ionization conditions, $^{0,4}B^+$ as a product of an alternative RDA fragmentation is also typical for flavones. Ion $^{0,2}A^+$ (*m/z* 165, for compounds with 5,7-di-OH) is likely to emerge at low or medium collision energy CID (≤ 30 eV) being characteristic only for flavonols, while $^{0,2}B^+$ appears in the CID spectra of both flavones and flavonols [34, 54, 58]. $^{1,3}A^+$,

$^{0,2}A^+$, $^{0,2}B^+$ ions all appear as strong peaks at collision energies around 30 eV, as a fingerprint of a typical flavonol CID pattern (Fig 3.6.). The relative abundance of these three main fragments show correlation with collision energy changings [58]. In negative ion CID, similarly to positive ionization, $^{1,3}A^-$ ion is often the major fragment (Fig 3.9.), whereas $^{0,3}B^-$ is characteristic for isoflavones [61].

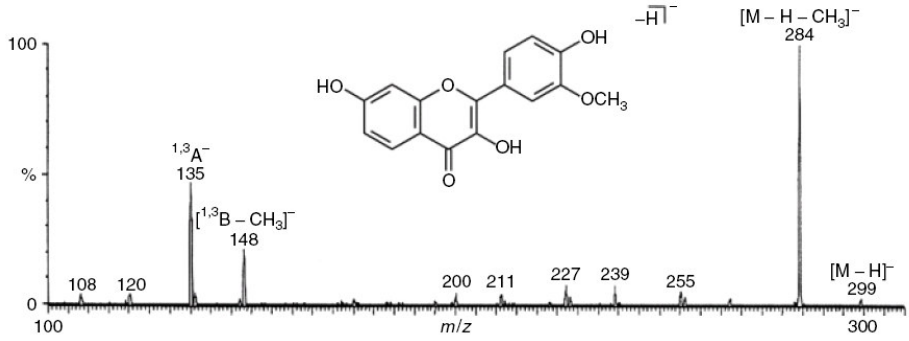


Fig 3.9.: Characteristic $^{1,3}A^-$ fragment in the negative CID of kaempferid [57].

Ion $^{1,3}B^-$ is much more typical of flavones' and flavanones' CID, than that of flavonols. Fragments $^{0,3}A^-$ and $^{0,3}B^-$ are rarely discovered, but $^{0,4}A^-$ frequently appears in all groups of flavonoids in the negative CID. In some cases the direct cleavage of the B ring [M-B ring]$^-$ may also be observed. In general, as compared to the positive CID, no such firmly distinctive rules can be applied for the negative ion fragmentation of flavonoids [34, 57]. As reported by several author, primer fragment ions $^{i,j}A^{+/-}$ and $^{i,j}B^{+/}$ undergo further dissociation by successively loosing small neutrals, like H_2O (-18 amu), CO (-28 amu), CO_2 (-44 amu) C_2H_2O (-42 amu) or combinations of these, labeled as eg.: ($^{1,3}A^-$ - H_2O) [34, 57, 58, 61, 62] (Fig 3.10.).

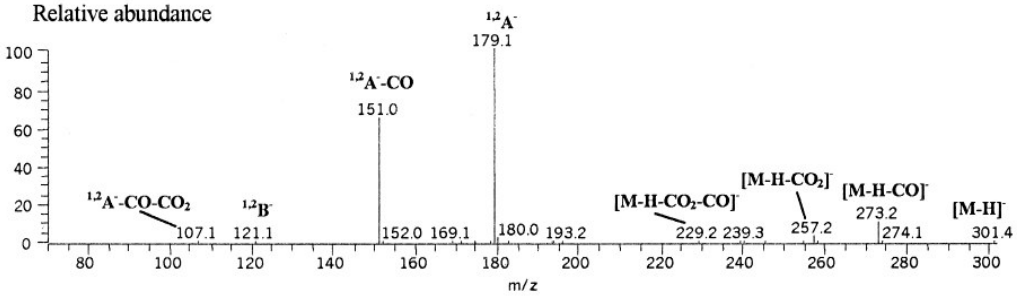


Fig 3.10.: Examples of flavonoid CID products formed by successive losses of simple neutrals, like H_2O, CO_2 and CO. Figure shows the negative CID of quercetin [61].

These losses seems to be less characteristic and provides no absolute evidence for the determination of the aglycone type. Methylated flavonoids are characterized by the loss of -15 amu, resulting in $[M\text{-}H\text{-}CH_3]^{\cdot}$ radical fragment ion, which generally constitutes the base peak. [57]. Fragmentation pathways of flavonoids was reported to be independent of the analyzer type (IT or QQQ) by Rijke et al. [63], which was, however partly disclaimed by other authors [37, 64].

3.1.6. Mass spectrometry of flavonoid glycosides

The characterization of flavonoid glycosides by means of mass spectrometry may cover the determination of glycosylation types (*O-, C*-bonded or both), types of the sugar unit(s) (hexoses, deoxyhexoses or pentoses), distribution of the sugar residues, the glycan sequence, interglycosidic linkages, glycosylation position, and type of the aglycone itself [62, 65]. Differentiation between *O*-, *C*- and *O*-*C*-diglycosides can be reached by examining the low-energy CID spectra of the analyte(s). The protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) *O*-glycosides and *O*-diglycosides give rise to X_j and Y_j ions, respectively. In the case of *O*-*C*-diglycosides only Y_j ions can be observed, formed by fragmentation at the interglycosidic linkage, whereas in the case of *C*-glycosides only $[M+H]^+$ / $[M-H]^-$ ions are detected [57, 65]. High-energy CID spectra shows a more complicated view; product ions of intraglycosidic cleavages of *O*-glycosides emerge, and Z fragments of *C*-glycosides as well [60].

The type of the sugar units of *O*-glycosides can easily be distinguished based upon the neutral losses of -132, -146 and -162 amu (B_i fragments), which correspond to a pentose, a deoxyhexose and a hexose unit, respectively. Glucuronides provide a loss of -176 amu during decomposition. For *C*-glycosides losses of -120 (hexose) and -122 amu (deoxyhexose) are characteristic. There are a number of other mass losses characteristic of sugars from *C*-glycosides which are not discussed here, but can be found in details in recent works [60, 65, 66]. Information on the sugar moieties are limited by a general property of mass spectrometry: stereochemistry can not be determined. Neutral loss scan mode offers a good solution for the fast screeing of flavonoid glycosides concerning the sugar types [61].

Sugars may be attached to the flavonoid aglycons at different positions (eg: di-*O*-glycoside), or at the same position arranged in a row (eg: *O*-diglycoside). Observation of the presence, absence and/or the relative intensities of characteristic product ions (Y_1, Y_0, Z_1) in the CID spectra can be a key in determining the sugar distribution. Presence of Z_1 fragment reveals that one sugar unit is attached to another and not directly to the aglycone, besides, differences in the relative intensity of fragment ions Y_1 and Y_0 may also indicate glycosilation positions. In case of di-*O*-glycosides the Y_1 fragment is two or three times more abundant than the Y_0 fragment, while, for *O*-diglycosides the ratio is inversed [60, 65, 67] (Fig 3.11.).

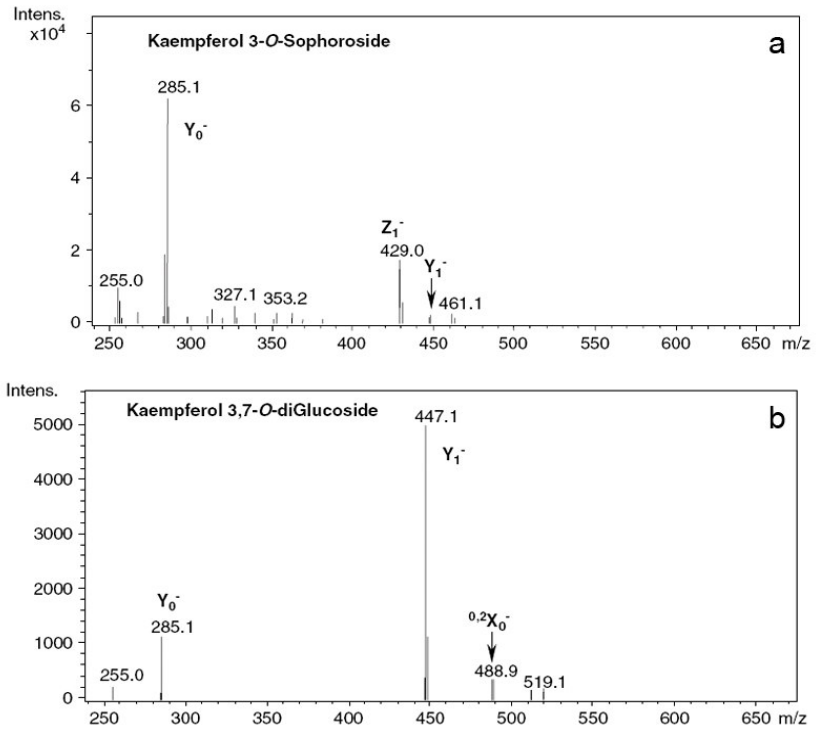


Fig 3.11.: Negative product ion spectra of a kaempferol-*O*-diglycoside (a) and kaempferol-di-*O*-glycoside (b) showing the diagnostic difference regarding intensity of the Y_0^- and Y_1^- fragments [67].

Another way to reveal the sugar distribution is based upon the observation of the collision-induced product ions resulted by homolytic and heterolytic cleavages of the *O*-glycosidic bond. Homolytic cleavage yields a radical aglycone fragment ion $(Y_0\text{-}H)^{-\cdot}$, whilst heterolytic cleavage renders a $(Y_0)^-$ ion corresponding to the aglycone anion (Fig 3.12.). This is however not typical, but was reported by several authors

concerning flavonoid glycosides [40, 57, 68]. Apparently only the CID spectra of a di-*O*-glycoside can contain a fragment ion including the aglycon and one remaining sugar unit (after the loss of the first sugar) $(Y_1\text{-H})^{\cdot\cdot}$. The CID spectra of *O*-diglycosides contain only the $(Y_0\text{-H})^{\cdot\cdot}$ ion [69].

Most preferred glycosilation sites of the aglycon are the next: C-7 hydroxyl in flavones, flavanones, and isoflavones; C-3 and C-7 hydroxyl groups in flavonols, flavanols; and C-3 and C-5 hydroxyls in anthocyanidins. In case of *C*-glycosides positions 3, 6 and/or 8 are mentined by the literature [53, 57].

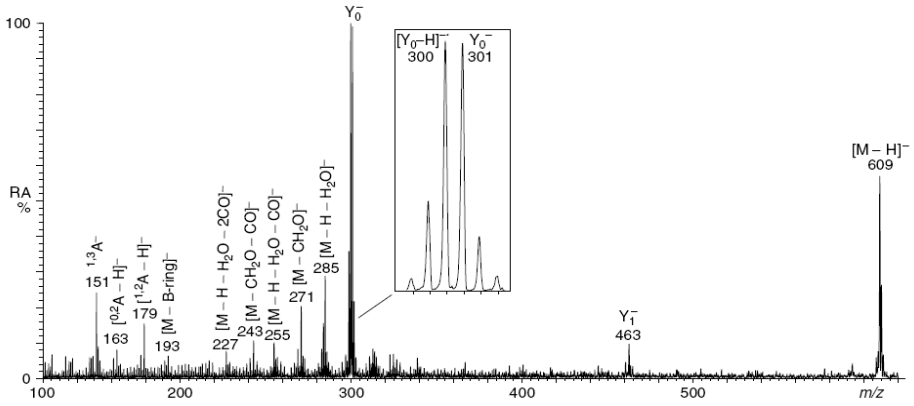


Fig 3.12.: Negative ion CID spectrum of quercetine-rutinoside (rutin). The homolytic cleavage of the *O*-glycosidic bond results an aglycon ion $(Y_0)^-$ and simultaneously a radical aglycone fragment ion $(Y_0\text{-H})^{-\cdot}$ at an *m/z* value of 1 Da differrence as well [57].

Differentiation of glycosilation sites can be based on the presence or lack of distinctive fragments in the negative CID spectra. The $[Y_0\text{–CO}]^-$ ion was observed only for 7-*O*-monoglycosyl flavonols, while the $[Y_0\text{-2H-CO}]^-$ fragment was described exclusively in the MS/MS spectra of 3-*O*-monoglycosyls [69]. The $(Y_0\text{-H})^{-\cdot}$ radical aglycon to $(Y_0)^-$ aglycon anion intensity ratio offers another diagnostic mark, since their relative abundance is in correlation with the position of glycosylation [40, 69]. Relative abundance of the radical aglycone $(Y_0\text{-H})^{\cdot\cdot}$ is higher in case of 3-*O*-glycosides but it is much lower if 7-*O*-glycosides are analysed -as concluded in general by Hvattum and Ekeberg [40] (Fig 3.13.).

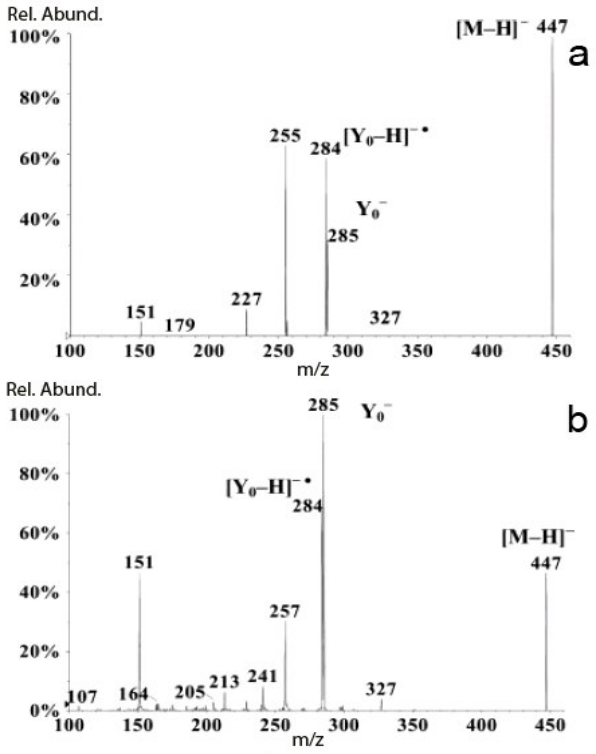


Fig 3.13.: Relative abundance of the radical aglycone ion $(Y_0\text{-}H)^{\cdot-}$ and the aglycone ion $(Y_0)^-$ in case of 3-*O*-glycosides (a) and 7-*O*-glycosides (b). Figure shows the CID spectra of luteolin-3-*O*-glucoside and luteolin-7-*O*-glucoside [40].

Besides it was also reported that the collision-induced homolytic to heterolytic cleavage ratio of the 3-*O*-glycosidic bond of flavonol glycosides increased with the increasing number of hydroxyl groups in ring B. Contrary to this, the ratio was found to be decreasing with the number of hydroxyls of the B ring for 7-*O*-glycosides. Moreover, no radical aglycone fragment was observed in the CID of B ring methoxy substituted 7-*O*-glycosides. The presence of a B ring fragment containing the sugar residue clearly indicates 4'-*O*-glycosylation [40].

3.2. Other methods commonly used for the investigation of phenoloids (HPLC, NMR, *in vitro* assays)

For reasons of simplicity and limited extension this section includes only those methods and instruments which have relevance regarding our particular samples.

By liquid and gas chromatography (HPLC, GC) all phenolics of various structure and polarity can be analysed [3, 34]. The most frequently applied UV detection is appropriate for the reliable detection and pre-differentiation of phenolics, but is inappropriate for accurate distinction since the spectra of phenoloids are very similar in many cases. A smart approach to LC method development is to perform a gradient scout first, and then decide to use an isocratic or gradient run according to results [70]. Only two works have been published about the HPLC of *Euphrasia* phenolics so far, both analysis occured by UV detection. Vukics et al. used acetonitril and phosphoric acid as acidic modifier, while Luczak and Swiatek applied acetic acid in a gradient elution of three mobile phases accomplished with a quaternery LC pump [71, 72]. Among other phenolics Eukovoside A, a phenylethanoid glycoside, is also commonly found in different *Euphrasia* species. Phenylethanoid derivatives, like eukovoside A or acteoside, bear several medicinal effects [73, 74], reports on their HPLC determination by different detectors can be found in the literature [75-77].

Most HPLC methods developed for salicylates apply THF and/or phosphoric acid/phosphate buffers [78, 79]. For reasons mentioned earlier TFA and phosphoric buffers are chemicals that should be avoid in mass spectrometry, therefore these methods were not adoptable. Poblocka-Olech et al. reported a comprehensive coupled NP-RP-HPLC (normal phase - reversed phase - HPLC) method for the separation and analysis of phenolics and salicylates of *Filipendula ulmaria.* Detection occured by UV and evaporative light scattering detectors [80].

Only the research groups of Abram and Stevens developed HPLC methods for the separation of *Sempervivum* flavonoids and for the investigation of their aglycons [81, 82]. However numerous information can be found on the flavonoid aglycons of *Sempervivum tectorum,* the composition of their sugar moieties are hardly known, even though it would be of importance if questions of bioavailability and absorption kinetics is considered [83, 84].

Epilobium parviflorum contains numerous myricetin and quercetin glycosides and several other caffeic acid derivatives. However HPLC, TSP and FAB MS methods are available in the literature for the analysis of *Epilobium* flavonoids and tannins, they lack information on oenothein B and other related macrocyclic polyphenols

which are considered to be the active substances of the *Epilobium* species [26, 85-87].

An excellent review by Silva Elipe covers all the NMR and LC-NMR one and two dimensional methodologies, techniques and key features of NMR spectral interpretation of flavonoids [88]. Other detailed publications are also available on the NMR investigation of flavonoids [34, 88, 89]. Examples for the NMR of phenylpropanes and other phenolics are to be found in the works of Wu et al., Pereira et al, Owen et al., and Lia et al. [90-93]. and perhaps most important biological

The most obvious effect of phenolics is their antioxidant effect which is due to the high reactivity of the aromatic -OH group. This is of importance since anti-inflammatory effect of natural phenolics is strongly linked to their antioxidant activity, which is further in relation with the number and glycosilation-status of -OH groups [94-97]. Several simple, mostly spectrophotometrical, *in vitro* assays are available for the fast testing of the antioxidant capacity of phenolic samples. In spite of their limitations, the simplicity, ease of use and basic instrument demand makes ABTS and DPPH *in vitro* decolorization assays the most widespread methods to evaluate the scavenging activity of natural antioxidants [98-101]. Since the synergistic effects of components enhance their antioxidant activity [102].

3.3. Model plants

For presenting the phytochemical potentials of mass spectrometry phenolic containing model plants of long traditional usage were selected. The medical benefits of these herbs are known since centuries or millenary, and experience about their extraction, effect, application and dosage have been existed without extensive knowledge on their qualitative and quantitative composition. These medicinal plants were already analysed previously in the seventies and eighties by methods which we consider today not modern. Reinvestigation and extension of prevoious results by modern powerful analytical methods is desirable, LC-MS may offer a perfect solution.

3.3.1. *Euphrasia rostkoviana* HAYNE (Scrophulariaceae)

Fig 3.14.: *Euphrasia rostkoviana* HAYNE

Euphrasia rostkoviana Hayne, synonym: *Euphrasia officinalis* L. (Eyebright) is a delicate semiparasitic annual herb with a short, branched 10- 50 cm long leafy stem. The opposite leaves shape ovate to lanceolate, toothed and hairy. The solitary flowers are axillary, white, yellowish or tinted violet and have two pronounced lips. The fruit is capsule. The identification of European *Euphrasia* species, even for specialists, is a difficult task, hence, for convenience, many of them are grouped in an aggregate species, *E. officinalis*. Eyebright is common in damp meadows, pastures and woods throughout whole Europe. The dried flowering herb is used, after cut above ground it is dried in shade and stored in dark [103, 104].

The principal compounds in the aerial part of various *Euphrasia* species are iridoids (aucubin, ixoroside, euphroside), phenolic acids, phenylpropane glycosides (eukovoside A) and derivatives and glycosides and/or aglycons of flavones (luteolin, apigenin) and flavonols (quercetin, kaempferol). Other constituents include bitter principles, tannins, phytosterols (*β*-sitosterol) and carbohydrates [72, 105-109, 110-113]. Former elucidations and identifications in the eighties were performed by thin-layer chromatography (TLC) [110], paper chromatography, differential spectrophotometry [107-109] and ^{13}C- and ^{1}H-NMR spectroscopy [111-113]. Modern liquid chromatographic technique was involved in merely two works. Vukics et al. used eyebright as a model plant among 3 others for LC method development for flavonoid aglycones [71], while Luczak and Swiatek analysed only the simple phenolic acids of Eyebright [72]. No mass spectral analysis occured yet.

According to the traditional use and the well established effects of its main compoundgroups (iridoids and flavonoids) Eyebright has anti-inflammatory, analgetic and antioxidant effect, though there have not been any aimed pharmacological assay to prove this by investigating Eyebright extracts. In 2000 Porchezian et al. showed antihyperglycemic activity of *Euphrasia officinale* leaves

on alloxan-induced diabetic rats [114]. A multinational prospective cohort trial, conducted in 2000 as well, gave evidence based support for the anti-conjunctivitis effect of Eyebright. Stoss et al. found that symptoms of conjunctivitis, including allergic conjunctivitis, totally disappeared in 3-17 days in more than 95% of patients treated with Euphrasia eye drops [115]. As the vernacular name and historical records indicate, eyebright has long been used as a folk medicine primarily for the treatment of various eye-disorders such as cataract, conjunctivitis, red-, inflamed-, irritated- and sore-eyes. The infusion is used externally as an eyebath or applied as compress for styes and blepharitis. Internally a weaker infusion is to be taken 3-4 times daliy for the same ailments, jaundice and abdominal spasms [103-105].

3.3.2. *Satureja hortensis* L. (Lamiaceae)

Fig 3.15.: *Satureja hortensis* L.

Satureja hortensis, summer savory or garden savory, is an aromatic perennial subshrub with ascending or trailing stems and 10-45 cm high, erect branches. The opposite greyish leaves are leathery, lancolate, flowers are light purple or white coloured. Fruit consists of four smooth nutlets. Savory is native to the mediterranean Europe, but it is grown many places elsewhere in the world as a seasoning herb. For medicinal benefits the fresh or dried flowering shoots are used.

The whole plant is aromatic, its essential oil content, mainly carvacrol and cymene, may reach up to 3%. Other constituents are tannins and mucilage [103, 104, 116]. Phenolic constituents include rosmarinic acid and flavonoids. Kemertelidze et al. identified several flavonoid aglycons (apigenin and luteolin) and their various glycosides in the aqueous ethanolic extract of *Satureja hortensis* with paper chromatography and differential spectrophotometry, which are considered anachronistic today [117]. Bros et al. quantified some flavonoids in summer savory by high performance thin layer chromatography (HPTLC) methods [118]. HPLC

methods for phenolic acid derivatives of Savory is available in the publication of Liu et al. [119].

The high carvacrol content and other essential oil components of the herb provide antimicrobial, antitumor, analgesic, antispasmodic, antiparasitic effects [120]. Astringent, stomachic, carminative effects are also known [103, 104]. The phenolic constituents of *Satureja* ensures the plant's notable antioxidant activity [121]. Other recent promising studies have shown that *Satureja* extracts own in vitro protective effect on DNA, and inhibits platelet adhesion [122, 123]. It is used mainly for stomach and intestinal disorders, against intestinal worms, flatulence and to stimulate appetite. The herb's effect makes it a good gargle. The distilled essential oil is used as a flavouring, fresh or dried leaves serves as popular season for meats, fish and beans [103, 104].

3.3.3. *Filipendula ulmaria* L. MAXIM (Rosaceae)

Fig 3.16: *Filipendula ulmaria* L. MAXIM

Filipendula ulmaria L. Maxim (Meadowsweet) is a perennial herb with a short, pink rhizome, usually erect, and stems as high as 50-120 cm. Basal leaves are up to 60 cm long. Stem leaves are ovale shaped, dark green. Small densely arranged flowers are of creamy-white fragrant. Meadowsweet is to be found in damp woods, meadows, in fens and by riversides throughout Europe. Dried flowers, or rarely leaves separately, the whole plant above ground and the rhizomes are used medicinally as drugs [103, 104].

Constituents of *Filipendula ulmaria* include numerous salicylate aglycons and glycosides (eg.: salicylic acid, salicylalcohol, methylsalicylate, salicin), various glycosides of dominantly quercetin (e.g. spiraeosid) and kaempferol, tannins and phenolglycosides [79, 106, 124]. The flavonoid profile of the herb was studied in details by two detailed works [125, 126]. Meier et al. were the only to use modern

analytical tools, such as HPLC-UV, for the analysis of *Filipendula* salicylates [79]. The analysis and determination of volatile salicylates is established by GC [127].

Meadowsweet, such as other salicylate containing herbs own significant antipyretic, analgesic, antirheumatic effect. Beside these, urinal disinfecting and sweat gland inhibiting effects are also mentioned by the literature [106, 124]. Pharmacological investigations have shown the antimicrobial and anti-inflammatory activities of ethanolic and aqueous extracts from Meadowsweet flowers [128, 129]. A heparin-like complex found in *Filipendulae ulmariae flos* showed anticoagulant and fibrinolytic effects [130]. Various extracts of Meadowsweet flowers showed strong immunomodulatory activity towards the classical pathway of complement activation [131]. The flavonoid complex is considered to be responsible for the antiulcerogenic activity of the drug [132, 133]. Meadowsweet is used traditionally to treat rheumatism, fevers and pain in much the same way as Aspirin is used, but it is free of the synthetic drug's side effects. Since the active *Filipendula* substances are to be found as glycosides in the gastrointestinal tract and salicylic acid is formed only in the liver via oxydation, the harmful side effects, such as gastric bleeding are not associated with the use of *Filipendula* extracts. It is one of the most powerful herbal remedy for gastritis and peptic ulcer. Infusion of the flower administered 2-3 times a day is used against cold, influenza and headache. Alone or added to other preparations it is frequently used for the treatment of rheumatic pain and arthritis [103, 104].

3.3.4. *Sempervivum tectorum* L. (Crassulaceae)

Fig 3.17.: *Sempervivum tectorum* L.

Sempervivum tectorum, common houseleek, is a robust perennial herb, with dense basal rosette of fleshy obovate to oblong sharp-tipped bluish-green leaves. The numerous dull-red flowers are arranged in terminal cymes. Hoseleek grows on weathered rocks, roofs and

screes, and is native in central and southern Europe. Traditionally the leaves are used, normally freshly, rarely dried.

Constituents of Houseleek are tannins, bitter compounds, sugars, flavonoids and mucilage [103, 104, 106]. Flavonoid type compounds, on aglycon levels (kaempferol, quercetin, myricetin and herbacetin), were studied with gas chromatography coupled to mass spectrometry (GC-MS) and HPLC-UV [81, 82]. Yet we have no information on their glycosilation status, nor any structural detail on the previously mentioned other substances.

Some recent studies have described antinociceptive, liver-protecting and membrane stabilizing effects related to *Sempervivum tectorum*, which have been attributed partly to the antioxidant activity of its phenolic compounds [134-137]. Traditionally Hoseleek is a good astringent and wound healer. Its external applications are numerous: pressed juice of the leaves is applied on herpetic erruptions of the skin, on suppurating wounds, in inflammations caused by insect bites, and against external inflammation the ears [103, 104, 106].

3.3.5. *Epilobium parviflorum* Schreb. (Onagraceae)

Fig 3.18.: *Epilobium parviflorum* Schreb.

Though several *Epilobium* species (*E. parviflorum* Schreb., *E. angustifolium* L., *Epilobium montanum* L., *Epilobium tetragonum* L., and *Epilobium roseum*) bear therapeutical significance, our investigatations aimed the *Epilobium parviflorum* Schreb. which is the most commonly used species of the genus by the folk medicine. Willowherb, is a perennial herb with a height of 50-70 cm, unbranched thin stem covered by fine trichomes. Leaves are lanceolate, sized 3-5 cm. Light-purple flowers are radial symmetrical. The herb grows throughout Europe in clumps, in woodland clearings, and in waste places. Young shoots and leaves are used medicinally [103, 106].

Constituents, which characterize the whole genus, include polyphenols, tannins, up to 20%, flavonols (glycosides of myricetin and quercetin), hydroxy-cinnamates, phytosterols (*β*-sitosterol), mucilage and sugars [86, 103, 104, 106, 138, 139]. Investigations were carried out by means of TLC [87, 139], HPLC-DAD [87], LC-MS [26, 86]. Several constituents, present in other species of the genus as well, were identified and confirmed by ^{1}H and ^{13}C NMR spectroscopy [26]. Oenothein B, the active component of *E. parviflorum* was first identified by Lesuisse et al. by means of HPLC and NMR techniques [140]. Ducrey and co-workers also investigated the molecule by NMR and FAB-MS for determining the molar mass [141].

Pharmacological investigations have been restricted mostly to two species, *E. angustifolium* L. and *E. parviflorum* Schreb. Notable antioxidant [142, 143] antibacterial (against S. aureus and E. coli) and general antimicrobial [143, 144], anti-inflammatory effect (inhibition of COX-1 and COX-2 enzymes) [143] and antiproliferative effects were demonstrated of the two abovementioned species [145, 146]. Vitalone and co-workers presumably explained the antiproliferative effect with the oenothein B content, which is a macrocylic tannin, widespread in the *Epilobium* genus. This was confirmed by Lesuisse et al., who showed that oenothein B inhibits the 5-*α*-reductase enzym and thus *Epilobium* has beneficial effect in benign prostatic hyperplasia (BPH) [146]. Young shoots and leaves are used traditionally as a decoction or infusion. Efficient against headache and migraine, and being rich in vitamine C, the tea of the herb is recommended as a spring tonic as well. Leaves can also be consumed as vegetable or salad [103, 104].

4. Materials and methods

Chemical substances and instrumentation common in all analyses are described below, in section „General", followed by other data on conditions which are plant specific.

4.1. General

4.1.1. Solvents and chemicals

All solvents of reagent grade and HPLC super gradient grade, and the acidic HPLC modifiers were purchased from Sigma-Aldrich (Budapest, Hungary). Pure water for chromatography was prepared with a Millipore Direct Q5 water purification system (Millipore, Bedford, MA, USA). Aqueous eluents for HPLC and LC-MS were filtered through a 0.45 µm mixed cellulose ester membrane (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath before use. Reference substances: chlorogenic acid, caffeic acid, cinnamic acid, quercetin-rutinoside (rutin), apigenin, quercetin, luteolin, rhamnetin, kaempferol, salicin, salicylic acid and aucubin were purchased from Sigma-Aldrich (Steinheim, Germany). *p*-coumaric acid was supplied by Fluka (Buchs, Switzerland), while spiraeoside was obtained from Carl Roth GmbH (Karlsruhe, Germany).

4.1.2. Solid-phase extraction

In order to pre-separate and purify the analytes of interest samples (except *Filipendula* and *Satureja*) were subjected to an SPE procedure prior to analysis. The Supelclean LC-18 SPE tubes (500 mg/3mL, Supelco, Bellefonte PA, USA) were conditioned with 2 x 2.5 mL methanol and with 2 x 2.5 mL 2.5% acetic acid. Dried extracts (different amount from plant to plant, approx. 10-12 mg) were redissolved in 4-5 mL of a mixture of HPLC super gradient grade methanol and 2.5% acetic acid in

water (ratio 1:5). 4-500 µL of these solutions were added to the tubes. SPE tubes were eluted in three steps, with 3 mL of 25%, 70% and 100% methanol. In cases of *Sempervivum* and *Epilobium* samples the first elution step of 25% was cancelled.

4.1.3. Liquid chromatography-mass spectrometry apparatus

Chromatographic and on-line mass spectral analyses were performed with an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source coupled to an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) (Agilent Technologies, Palo Alto, CA, USA). The Masshunter B.01.03 software was used for data acquisition, and for qualitative and quantitative analysis. Identification of the components was carried out by the comprehensive interpretation of UV, and CID spectral data and by the comparison with those of literature data and authentic standards.

4.2. *Euphrasia rostkoviana* Hayne

4.2.1. Plant material

Euphrasia Rostkoviana Hayne plant samples were collected in meadow-lands of Mures County, Romania, in the stage of blooming in July 2006. Plant samples were authenticated in the Department of Pharmacognosy, Semmelweis University, Budapest, where voucher specimen is deposited (E.r. 008).

4.2.2. Extraction, fractionation and isolation

10 g air-dried, freshly powdered (meshsize 2 mm) plant material (*Euphrasiae herba*) was extracted with methanol in a Soxhlet apparatus according to the instructions of the Ph. Eur. 5.8. After filtrating the extract, methanol was evaporated under reduced

pressure with a rotary evaporator at 50 °C. Sample residue was redissolved in HPLC super gradient garde methanol and subjected to the SPE purification step.

Fractionation of the methanolic extract and isolation of the main phenolic compound was carried out by conventional open column chromatography. An aqueous suspension of 59 g MN polyamide SC-9 was prepared and used as stationary phase filled in a homemade glass column. Before introducing the sample, the suspension was allowed to settle (final dimensions of the bed: 390 mm x 30 mm). 2.4 g methanolic extract was dissolved in 4 mL methanol, adsorbed on a reasonable amount (4-5 g) of polyamide, dried until the solvent evaporated and layered on the top of the settled stationary phase. The column was eluted successively with water (1000 mL, 100 separate fractions) and aqueous-methanol (100 mL 50% methanol, 100 mL 70% methanol and 800 mL 100% methanol, 76 separate fractions and the following amounts were combined). Fractions of 10 mL were collected and those of similar fingerprint were combined and used for further studies.

4.2.3. Content of main groups of compounds

Contents of the leading constituents: flavonoids, polyphenols, tannins, and hydroxycinnamic derivatives were determined from the dried plant sample by applying the refering UV-VIS spectrophotometric methods of Ph. Eur. 5.08. and Ph Hg. VII., respectively.

4.2.4. Antioxidant activity assay

The scavenging activity of the methanolic fractions was determined spectrophotometrically using free radicals of ABTS $^{\bullet+}$ and DPPH $^{\bullet}$ in a decolorization *in vitro* assay according to Re et al. with some modifications, described below. 20 µL of samples in 5 different concentrations, in three parallels were introduced to 2.5 ml of the solution containing the free radical. After shaking for a few seconds, absorbance changes were recorded at 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 300 and 360 seconds. For determining the inhibition %, the final absorbance (at t = 360

sec) was extrapolated by numerically solving the simplest possible reaction kinetics model. Methanolic solutions of ascorbic acid, chlorogenic acid, trolox and kaempferol standards were measured for comparison with both free radicals as well. Inhibition % was calculated as:

$$\text{Inhibition \%} = (A_b - A_f) / A_f \times 100$$

where A_b = the absorbance of blank (t = 0 sec); A_f = extrapolated final absorbance. Ploting the inhibition % vs concentration, IC_{50} (concentration of antioxidant required for a 50% loss in the initial free radical concentration) values were determined for all samples and standards.

Radical cation solutions were prepared as follows. 10 mg ABTS was dissolved in 2.6 ml HPLC grade water and was reacted with 1.72 mg potassium persulfate to generate ABTS radical cation (ABTS $^{\bullet+}$). The solution was diluted with spectroscopic grade ethanol to an absorbance of 0.900 (± 0.05) at 734 nm, immediately before the measurement. 10 mg DPPH was dissolved in 25.0 ml HPLC grade methanol. The solution was diluted with HPLC grade methanol to an absorbance of 0.900 (± 0.05) at 515 nm, immediately before the measurement. Measurements were carried out with a HITACHI U-2000 spectrophotometer. All analyses were run in triplicate and averaged. Data were analyzed at significance level $P<0.05$. The results are expressed as mean values and standard deviation (SD). Students' t-test and F probe were applied to compare and evaluate results.

4.2.5. High performance liquid chromatographic conditions

- Column: Supelco ODS Hypersil (150 mm x 4.6 mm, 5 µm; Supelco, Bellefonte PA, USA), guard-column of the same stationary phase
- Eluents: A: acetic acid in water (2.5%, v/v %) B: methanol
- Gradient program: 0 min: 25 B% (v/v %), 25 min: 52 B% (v/v %), 30 min: 90 B% (v/v %), 33 min: 25 B% (v/v %).
- Flow rate: 1 mL min^{-1}
- Column thermostate: 25 ^{o}C
- Injection: 15 µL
- UV detection: 260 and 340 nm (spectra: 200-400 nm)

4.2.6. Triple quadrupole mass spectrometric conditions

- Ionsource: ESI, negative
- Temperature: 350 ^{o}C
- Nebulizer pressure: 45 psi (N_2)
- Drying gas flow: 9 L min^{-1} (N_2)
- Fragmentor voltage: 130 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50-700 (cycletime: 800 msec)
- Postcolumn splitter: 60%-40% : waste-MS

Before quantitation both fragmentor voltage (from 70 to 140 V, with steps of 10 V) and collision energy (from 5 to 50 eV, with steps of 5 eV) were optimized by parameter ramping. Optimal setting for collision energy was 35 eV, and for fragmentor voltage, 130 V. High purity N_2 was used as collision gas. Quantitation was achieved in MRM mode.

4.2.7. Time-of-flight mass spectrometric conditions

Accurate mass and elemental analysis was achieved by an Agilent 6210 time-of-flight mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) operating with a dual-nebulizer ESI source in the negative ion mode. TOF analyses were accomplished in collaboration at the Research and Training Laboratory for Separation Science, Eötvös Lóránd University. ESI conditions were the same as described above. Two reference masses (*m/z* 112.985587 and *m/z* 1033.988109) were used to recalibrate the mass axis during analysis. Full scan mass spectra were acquired over a range of *m/z* 100-1500. For confirmation the measured mass of the deprotonated molecular ion was compared with the calculated theoretical value. All spectra were acquired at FWHM of the corresponding chromatographic peaks.

4.2.8. NMR spectroscopy, apparatus and conditions

NMR experiments were carried out in collaboration at the Department of Pharmaceutical Chemistry, Semmelweis University, on a 600 MHz Varian VNMRS spectrometer (Palo Alto, CA, USA) equipped with a dual 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences, as ^{1}H, ^{13}C, COSY, TOCSY, NOESY, HSQC, HMBC and processing routines available in VnmrJ 2.2C / Chempack 4.0 were applied. The probe temperature was maintained at 298 K and standard 5 mm NMR tube was used. 14.0 mg of purified solid compound was dissolved in 700 μL methanol-d4 (99.8 atom% D, Sigma-Aldrich). ^{1}H and ^{13}C chemical shifts (δ) are reported in ppm relative to CHD_2OD (δ = 3.30 ppm for ^{1}H) and CD_3OD (δ = 49.0 ppm for ^{13}C).

4.2.9. Method validation

Calibration plot, precision and quality control samples

The standard solutions for calibration were prepared by use of the isolated component. Purity and identity of the isolate was thoroughly checked by means of NMR spectroscopy HPLC-DAD-MS/MS and LC-TOF before using it as a standard compound. The isolate was dried with a rotary evaporator at 50 ^{o}C in an analiticaly pre-weighed flask, which was weighed after that, thus amount of the isolate was known.

Standard solutions were prepared at 40, 100, 300, 500, 800 and 1500 ng/mL with 70% methanol. Each standard solution was prepared in triplicate and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept and correlation coefficient were determined by least squares linear regression analysis. Linearity was checked by residuum curves of the ideal and measured calibration calculated by the accuracy data (expressed as RSD%) of the calibartion points. Quality control samples were prepared in the same manner at concentrations of 100, 300 and 800 ng/mL and used for the determination of the intra-day precision (low, mid and high concentrations of the standard in three parallel

runs on the same day) and inter-day precision (low, mid and high concentrations of the standard in three parallel runs on three successive days). Retention time repeatability was checked with 6 successive runs of the isolated compound. Limit od detection (LOD) and lower limit of quantitation (LLOQ) were determined based upon the signal to noise ratio (S/N) of the analyte.

Accuracy, recovery test

SPE recovery

Accuracy was verified by the recovery test using fortified samples and additionally an SPE recovery test was also performed. Three parallel standard stock solutions (each 1750 µg/mL) were prepared with the mixture of 1 mL methanol and 5 mL 2.5% acetic acid in water and subjected to the SPE procedure described previously. 400 µL of the stock solution was injected on to the SPE tube. The solution, collected during the SPE elution with 25% methanol, was diluted up to a volume of 10.00 mL with methanol. 5 µL of this solution was diluted up to 1 mL with 70% methanol and used for the LC-MS/MS determination. Recovery (*R*) was calculated as R = 100 C_{found} / $C_{applied}$. (C_{found} = measured concentration in sample, $C_{applied}$ = concentration in the standard solution used).

Method recovery, fortified samples

1 g air-dried, freshly powdered plant material alone and in another set after mixed with 8 mg of the isolated compound was extracted in three parallels with methanol in a Soxhlet apparatus according to the instructions of the Ph. Eur. 5.8. After filtration methanol was evaporated under reduced pressure at 50 ^{o}C. Method recovery was tested in a concentration range to match with that of acteoside in the plant sample. 45 mg of the fortified dry extract was redissolved in 1 mL of methanol and 2.5% acetic acid (1:5) solution. 500 µL of this solution was subjected to the SPE procedure previously described. The solution, collected during the SPE elution with 25% methanol, was diluted up to a volume of 10.00 mL with methanol. 5 µL of this solution was diluted up to 1 mL with 70% methanol and used for LC-MS/MS. Recovery was calculated as R = 100 (C_{found} − $C_{initial}$) / C_{added}. (C_{found} = measured

concentration in fortified sample, $C_{initial}$ = initial concentration in sample, C_{added} = concentration in the standard solution used).

4.3. *Satureja hortensis* L.

4.3.1. Plant material

Satureja hortensis plant sample was obtained from commercially available Satureja products.

4.3.2. Extraction and sample preparation

Saturejae herba plant samples were extracted with 50%, 70%, 96% and 100% (v/v%) ethanol in a Soxhlet extractor according to the guidance of Ph. Eur. 5.8. Solvents were then evaporated at 50 ^{o}C under reduced pressure, residues were redissolved in HPLC super gradient grade methanol and filtrated through a 0.2 µm RC syringe membrane filter (Phenomenex, Torrance, CA, USA) prior to analyses.

4.3.3. High performance liquid chromatographic conditions

- Column: ODS Supelcosil (150 mm x 2.1 mm, 5 µm; Supleco, Sigma-Aldrich, Stenheim, Germany)
- Eluents: A: acetic acid in water (2.5%, v/v%) B: acetonitrile
- Gradient program: 0 min: 13 B% (v/v %), 20 min: 46 B% (v/v %), 22 min: 100 B% (v/v %), 23 min: 13 B% (v/v %).
- Flow rate: 0.4 mL min^{-1}
- Column thermostate: 25 ^{o}C
- Injection: 5 µL
- UV detection: 330 nm (spectra: 200-380 nm)

4.3.4. Triple quadrupole mass spectrometric conditions

- Ionsource: ESI, negative
- Temperature: 350 ^{o}C
- Nebulizer pressure: 30 psi (N_2)
- Drying gas flow: 8 L min^{-1} (N_2)
- Fragmentor voltage: 120 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50-700 (cycletime: 800 msec)
- Collision energy: changed between 20 and 45 eV according to differences in molecule structures (high purity nitrogen was used as collision gas)

4.3.5. Testing of method performance

Standard solutions for calibration of rosmarinic acid were prepared at 1, 5, 20, 50, 150 and 300 µg mL^{-1} with 70% methanol. Each dilution of the standard was prepared in triplicates and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept and correlation coefficient were determined by least squares linear regression analysis. Quality control samples were prepared at concentrations of 5, 50 and 150 µg mL^{-1} and were used to determine the intra-day precision and inter-day precision. Retention time repeatability was checked with 6 successive runs of the 70% methanol sample. LOD and LLOQ were determined based upon the signal to noise ratio (S/N) of the analyte.

4.4. Filipendula ulmaria L. MAXIM

4.4.1. Plant material

Filipendula ulmaria L. Maxim plant samples; herb and flower (*Filipendulae herba* and *flos*) were collected around Bükkszentkereszt, Hungary, in June 2007.

Identification was carried out in the Department of Pharmacognosy, Semmelweis University, where the voucher specimen is deposited (F.u. 002).

4.4.2. Extraction and sample preparation

5 g air-dried, freshly powdered (meshsize 2 mm) plant material (*herba* and *flos*) was extracted with methanol in a Soxhlet apparatus according to the instructions of the Ph. Eur. 5.8. After filtrating the extract methanol was evaporated under reduced pressure with a rotary evaporator at 50 ^{o}C. Samples were redissolved in 70 % super gradient grade methanol and filtered through a 0.2 µm RC syringe membrane filter (Phenomenex, Torrance, CA, USA) prior to analyses.

4.4.3. High performance liquid chromatographic conditions

- Column: Zorbax SB-C18 column (150 mm x 3 mm, 3.5 µm; Agilent Technologies, Waldbronn, Germany)
- Eluents: A: formic acid in water (0.5%, v/v%) B: acetonitrile
- Gradient program: 0 min: 10 B% (v/v %), 20 min: 25 B% (v/v %), 24 min: 90 B% (v/v %), 26 min: 100 B% (v/v %), 28 min: 10 B% (v/v%).
- Flow rate: 0.5 mL min^{-1}
- Column thermostate: 35 ^{o}C
- Injection: 5 µL
- UV detection: 230, 270 and 340 nm (spectra: 200-400 nm)

4.4.4. Triple quadrupole mass spectrometric conditions

- Ionsource: ESI, negative
- Temperature: 350 ^{o}C
- Nebulizer pressure: 40 psi (N_2)
- Drying gas flow: 9 L min^{-1} (N_2)
- Fragmentor voltage: 100 V
- Capillary voltage: 4000 V

- Scan range: *m/z* 50-800 (cycletime: 800 msec)
- Collision energy: changed between 10 and 45 eV according to differences in molecule structures (high purity nitrogen was used as collision gas)

Prior to quantitation using formic acid peak area of the standards and all other salicylates vs. different ESI settings were investigated and evaluated. By parameter ramping fragmentor voltage (from 70 to 160 V, with steps of 10 V), capillary voltage (from 3000 to 4000 V, with steps of 500 V) and ESI temperature (from 275 to 350 ^{o}C, with steps of 25 ^{o}C) were investigated. Quantitation was performed in SIM mode (dwell time: 200 msec). For the studying the acetate adduct formation during HPLC method development fragmentor voltage was ramped between 70 and 140 V with steps of 10 V.

4.4.5. Method validation

Calibration plot, precision and quality control samples

Standard solutions of salicin were prepared at 100, 300, 800, 2000 and 5000 ng mL^{-1} and solutions of salicylic acid standard at 150, 300, 600, 1500 and 3000 ng mL^{-1} with 70% methanol. Each standard solution was prepared in triplicates and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept and correlation coefficient were determined by least squares linear regression analysis. Quality control samples were prepared at concentrations of 100, 800 and 5000 ng mL^{-1} for salicin and at 150, 600, 3000 ng mL^{-1} for salicylic acid. These were used to determine the intra-day precision and inter-day precision. Retention time repeatability was checked with 6 successive runs of the herba sample. Blank sample (pure solvent) was analysed to check the occurence of any impurity or co-elution with the same *m/z* as that of the analytes. LOD and LLOQ were determined based upon the signal to noise ratio (S/N) of the analyte.

Method recovery, fortified samples

For the method recovery test 5 g air-dried freshly powdered plant materials (*Filipendulae herba* and *flos*) were extracted in three parallels with methanol as described above, after addition of 0.789-0.789 mg of salicin and 2.368-2.368 mg of salicylic acid standards. After filtration methanol was evaporated under reduced pressure at 50 ^{o}C. Fortified samples were then redissolved in 70% super gradient grade methanol and filtered as described above. Method recovery was tested in a concentration range to match with that of the target analytes (salicin and salicylic acid) in the plant sample. Method recovery was calculated as already mentioned at section 4.2.9. (method recovery).

4.5. *Sempervivum tectorum* L.

4.5.1. Plant material

Sempervivum plant material was cultivated at the Research Station of the Corvinus University of Budapest, in Soroksár. *Sempervivum tectorum* samples and herbarium specimen are deposited at the Semmelweis University, Department of Pharmacognosy, where authentication of the sample was accomplished as well.

4.5.2. Extraction and sample preparation

Polar extract (80% (v/v%) methanol) of lyophylized and powdered *Sempervivum tectorum* leaves has been studied. 3 g of *Sempervivum tectorum* leaves were extracted with 100 mL 80% (v/v%) methanol in a Soxhlet apparatus and the extract was purificated by solid phase extraction. The samples were evaporated to dryness at 50 ^{o}C, under reduced pressure and residues were redissolved in 70% (v/v%) HPLC supergradient grade methanol and were subjected to the SPE purification step.

4.5.3. High performance liquid chromatographic conditions

- Column: Supelcosil C18 column (250 mm x 4.6 mm, 5 µm; Sigma-Aldrich, Stenheim, Germany)
- Eluents: A: acetic acid in water (2.5%, v/v%) B: methanol
- Gradient program: 0 min: 20 B% (v/v%), 20 min: 70 B% (v/v%), 24 min: 20 B% (v/v%).
- Flow rate: 1 mL min^{-1}
- Column thermostate: 25 ^{o}C
- Injection: 15 µL
- UV detection: 340 nm (spectra: 200-400 nm)

4.5.4. Triple quadrupole mass spectrometric conditions

- Ionsource: ESI, negative
- Temperature: 350 ^{o}C
- Nebulizer pressure: 45 psi (N_2)
- Drying gas flow: 9 L min^{-1} (N_2)
- Fragmentor voltage: 120 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50-1000 (cycletime: 1 sec)
- Collision energy: changed between 8 and 45 eV according to differences in molecule structures (high purity nitrogen was used as collision gas)
- Postcolumn splitter: 60%-40% : waste-MS

4.6. *Epilobium parviflorum* Schreb.

4.6.1. Plant material

Epilobium parviflorum Schreb. (cultivated plant material; voucher No.: EPP0607/S), and two commercially available *Epilobium parviflorum* samples were studied. Plant

material was collected in the Budai-hegység, Hungary, in 2006. Macroscopical and microscopical identification was accomplished in the Department of Pharmacognosy, Semmelweis University, Budapest, where the samples and herbarium specimen are deposited.

4.6.2. Extraction and sample preparation

5 g freshly powdered plant material (meshsize: 2 mm) was extracted three times succesively, with 20 mL 80% (v/v%) acetone in an ultrasound sonicator (2 h). Extracts were evaporated to dryness at 50 ^{o}C, under reduced pressure. The residues were redissolved in HPLC supergradient grade methanol and were subjected to the SPE purification step.

4.6.3. High performance liquid chromatographic conditions

- Column: ODS Supelcosil (250 mm x 4.6 mm, 5 µm; Supleco, Sigma-Aldrich, Stenheim, Germany)
- Eluents: A: acetic acid in water (2.5%, v/v%) B: acetonitrile
- Gradient program: 0 min: 11 B% (v/v %), 20 min: 21 B% (v/v %), 25 min: 41 B% (v/v %), 28 min: 100 B% (v/v %) 30 min: 11 B% (v/v%).
- Flow rate: 1 mL min^{-1}
- Column thermostate: 25 ^{o}C
- Injection: 20 µL
- UV detection: 260 and 340 nm (spectra: 200-380 nm)
- Postcolumn splitter: 60%-40% : waste-MS

4.6.4. Triple quadrupole mass spectrometric conditions

- Ionsource: ESI, negative
- Temperature: 350 ^{o}C
- Nebulizer pressure: 45 psi (N_2)

- Drying gas flow: 9 L min^{-1} (N_2)
- Fragmentor voltage: 135 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50-1600 (cycletime: 1 sec)
- Collision energy: changed between 10 and 60 eV (high purity nitrogen was used as collision gas)

5. Results and discussion

Results and discussion are presented in subsections according to model plants. The examples of phytoanalytical MS applications follow a row from simple phenolic acids and salicylates towards more complex structures, like flavonoid aglycons, glycosides and macrocyclic phenolics.

5.1. *Euphrasia rostkoviana* Hayne

In order to gain an overview on the constituents of the *Euphrasiae herba* sample, content of flavonoids, polyphenols, tannins and hydroxycinnamic derivatives were determined according to the instructions of Ph. Eur. 5.08, 5.1-5.5. with the following results: flavonoids: 0.38 ± 0.03 g/100g (hyperoside), polyphenols: 1.47 ± 0.09 g/100g (pirogallol), tannins: 0.56g ± 0.04 /100g (pirogallol) and hydroxycinnamic derivatives: 1.97 ± 0.09 g/100g (rosmarinic acid).

5.1.1. Polyamide fractionation and isolation

Considering that a variety of anti-inflammatory natural components possess antioxidant activity as well [94, 95], the presumtion was logical to attribute eyebrights anti-inflammatory effect to its antioxidant components and investigate the assumed coherence. In order to study the antioxidant effect of the compoundgroups separately, the methanolic total extract was fractionated. For the rough fractionation polyamide was applied, which is a common stationary phase for conventional column chromatography [27]. By aqueous and by a following stepwise change to 100% methanolic elution provided seven separate fractions containing iridoid glycosides, and eight separate fractions of phenolics/flavonoids (fractions I-VIII), respectively. The yellowish VIS absorbtion of flavonoids made detection and fractionation easy [52]. After a fast screen of the methanolic fractions it was concluded, that glycosylated caffeic acid- and phenolic acid derivatives were first to

elute followed by flavonoids with sugar moiety then caffeic acid derivatives without sugar entitites. Column chromatography served as a proper tool also for the preparative isolation of the main compound of the *Euphrasia* extract. Although the main constituent was present throughout methanolic fractions 6-10, after LC-DAD-MS/MS analysis only fraction 8 and 9 (combined and named as fraction I) was found to contain it solely without any additional compound. The isolation process yielded 51 mg pure substance.

5.1.2. Antioxidant effect

All methanolic fractions of *Euphrasia* showed a concentration dependent scavenging activity in both assays. Samples tested in the ABTS assay are usually characterized by the TEAC value (The amount of Trolox (mM) required to produce the same activity as 1 mM of the compound under investigation), however, it was not possible, since the fractions were multicomponent thus no definite molecular weight was to be matched.

Gallic acid was the strongest antioxidant among the investigated standards in both assays. The number of free OH groups, their inductive effect and close position resulting in ability for several possible mesomeric sctructures may give an explanation for its high scavenging acitvity [101]. Results obtained with the flavonoid standards confirmed that the free-radical scavenging activity is in strong relation with the number and glycosilation-status of OH groups [96, 97]. The higher the number of free OH groups, the higher the scavenging activity (see quercetin vs kaempferol and quercetin vs rutin in table 5.1.). Fraction I proved to be the strongest DPPH and ABTS radical cation scavenger with a mean IC_{50} of 11.88 µg/ml and 4.24 µg/ml, respectively, followed by fraction V, IC_{50}: 14.31 µg/ml and 4.63 µg/ml, respectively (table 5.1.). The IC_{50} value of quercetin standard was 4.1 times (DPPH assay) and 3.8 times (ABTS assay) lower than that of the flavonoid fraction with lowest IC_{50} (fraction V.). The IC_{50} of glycosilated quercetin (rutin) standard was much more comparable to that of fraction 5. The identified flavonoids in the *Euphrasia* samples were present as *O*-glycosides, which have obviously lower scavenging activity than flavonoid aglycones as discussed above. Though having the

highest activity, fraction I was 2.3 times (DPPH) and 2.1 times (ABTS) less effective than caffeic acid standard itself. On behalf of the flavonoids, also a 2 fold difference -in average- was experienced between the strongest flavonoid fraction (fraction V) and rutin standard. IC_{50} data of DPPH and ABTS assays showed a relatively good correlation, R^2 was 0.9124 (Fig. 5.1.).

Table 5.1.: Antioxidant activity of the methanolic soxhlet extract and methanolic fractions of *Euphrasia rostkoviana* Hayne and of the investigated standards.

Sample	IC_{50} (µg/ml) ± SD		Standard	IC_{50} (µg/ml) ± SD	
	ABTS	DPPH		ABTS	DPPH
Fr. I	4.24 ± 0.18	11.88 ± 0.39	Ascorbic acid	4.74 ± 0.15	4.78 ± 0.19
Fr. II	6.92 ± 0.42	17.88 ± 0.96	Caffeic acid	1.93 ± 0.12	5.05 ± 0.20
Fr. III	13.15 ± 0.87	37.70 ± 2.54	Chlorogenic acid	4.72 ± 0.17	7.43 ± 0.32
Fr. IV	5.56 ± 0.24	17.93 ± 1.37	Gallic acid	0.89 ± 0.52	2.64 ± 0.16
Fr. V	4.63 ± 0.22	14.31 ± 1.18	Kaempferol	8.37 ± 0.22	18.86 ± 0.67
Fr. VI	6.71 ± 0.40	17.72 ± 1.40	Quercetin	1.21 ± 0.06	3.48 ± 0.19
Fr. VII	10.55 ± 0.94	36.33 ± 1.62	Rutin	3.27 ± 0.11	7.36 ± 0.26
Fr. VIII	11.42 ± 0.82	74.53 ± 4.27	Trolox	2.07 ± 0.09	5.32 ± 0.22
MeOH extract	17.46 ± 0.88	34.78 ± 1.93			

The IC_{50} values resulted by the DPPH method were significantly different as compared to those of the ABTS method, while the precision (standard deviation) of the two methods did not differ significantly. The joint use and comparison of the methods is reasonable. All fractions exhibited in average 2.8 times stronger scavenging activity against ABTS $^{\bullet+}$ free radical than against DPPH $^{\bullet}$ free radical. A number of anti-inflammatory natural compound is a good antioxidant at the same time, therefore the traditionally known anti-inflammatory effect of the multi-component Eyebright extract may be partly attributed to its antioxidant phenolic constituents. Conclusion and interpretation of the applied rapid antioxidant methods is hardly easy or widely comparable, results are in contrast even with each other in many cases [100, 101]. On the other hand, applying at least two different assays, a rough overview of the scavenging activity can be gained and used as guidelines for further studies.

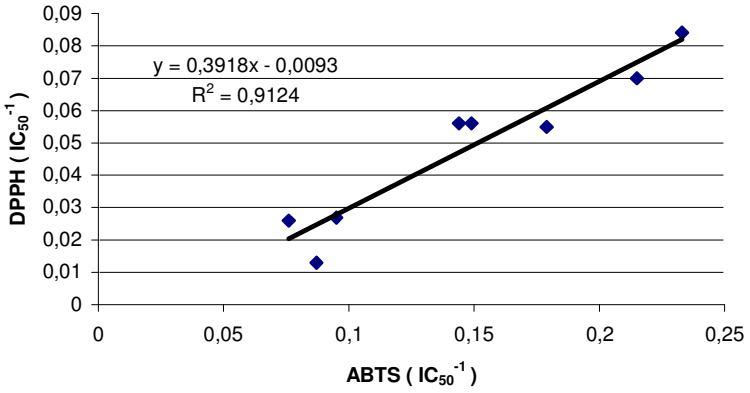


Fig 5.1.: Correlation of IC_{50}^{-1} results obtained with the ABTS and DPPH methods.

5.1.3. Qualitative analysis

With the aim of finding correlation between effect and quality, moreover, a chemical explanation for the effect, fractions were comprehensively analysed by coupled tandem mass spectrometry and nuclear magnetic resonance.

In view of the previous HPLC methods of the literature [71, 72], simplicity and MS compatibility considerations, acetic acid was choosed, tested and found to fit well for our method development. In agreement with the approach of Dolan a gradient scout was first applied using acetonitril as organic modifier [70]. Isocratic runs of 12% and 18% acetonitrile was used (based on the result of gradient scout) but it was soon concluded, that isocratic conditions provide insufficient resolution, and a gradient run was developed. Acetonitril, in contrast of its higher selectivity, was changed to methanol to enhance ionization efficiency in the ESI.

The HPLC-UV fingerprint chromatogram of the methanolic *Euphrasiae herba* extract showed the predominance of phenolic acid derivatives, whilst flavonoids were detectable as minor components (Fig 5.2.), which is interesting in light of the notable flavonoid content of the herb (0.38 ± 0.03 g/100g (hyperoside)).

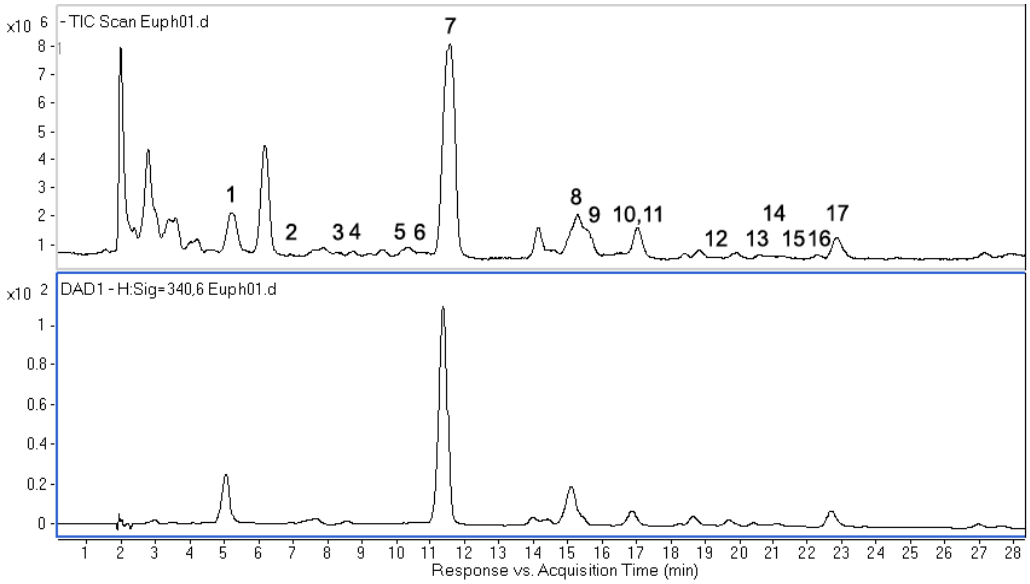


Fig 5.2.: TIC and UV (340 nm) chromatograms of the methanolic soxhlet extract of *Euphrasiae herba.* For details of numbering please see table 5.2.

Preliminary distinction of the flavonoids, phenol carboxylic acids, their derivatives and other plant constituents was accomplished by comparing their retention times and UV spectra. One absorbtion maximum around 330 nm suggested a caffeic acid derivative, while maxima at 260-280 nm and 330-360 nm was a clear mark for flavonoids. Using merely UV detector for the exact differentiation of the closely related structures was insufficient [72]. With on-line mass spectral analysis a higher confidence in identification was reached, particularly if the co-elutions around 15 mins are considered (Fig 5.2.).

For the effect-quality comparability fractions of the total extract were analysed. Based on the UV spectral data the only compound (t_R: 11.68 min) in fraction I was assumed to be a phenylpropane (cinnamic acid) derivative (λ_{max} = 332 nm). This fraction contained the main compound of *Euphrasia rostkoviana* owing high antioxidant activity, and what is more, in high purity. Therefore this fraction is discussed more in details. The molecular ion $[M-H]^-$ was detected at *m/z* 623, neither adducts, nor characteristic isotope pattern was observed. The $^{13}C/^{12}C$ peak intensity ratio suggested an approximate carbon atom number of 30. The molar mass of 624 Da and the high carbon number indicated that the molecule is much more than a simple cinnamic acid derivative.

Table 5.2.: List of identified and/or characterized compounds in *Euphrasiae herba* sample (S = identity was confirmed by standard compound, all aglycons of flavonoid glycosides were identified by standards as well).

Peak	Compound, fraction	TIC t_R (min)	$[M-H]^-$ (*m/z*)	CID product ions (*m/z*)
1 S	chlorogenic acid V., VI.	5.35	353	191, 179, 173, 135, 93
2 S	caffeic acid V., VI.	6.77	179	135, 109
3	coumaroyl-quinic acid VIII.	8.11	337	191, 173, 163, 119, 93
4	unidentified VII., VIII.	8.98	367	191, 173, 160, 134, 111, 93
5	unidentified III.	9.72	161	133, 117, 105, 89, 77
6 S	coumaric acid IV., V., VI.	10.49	163	119, 93
7 S	acteoside I., II., III.	11.68	623	461, 315, 179, 135, 113
8	luteolin-diglucuronide II.,	14.88	637	461, 285, 175, 133
9	luteolin-hexoside V., VI., VII.	15.65	447	285/4, 256, 151, 107
10 S	quercetin-rutinoside (rutin) IV., V., VI.	17.08	609	301/0, 271, 255, 179, 151
11	quercetin-hexoside VI., VII.	17.08	463	301/0, 271, 255, 151
12	apigenin-hexoside IV., V.	19.09	431	269/8, 227, 183, 151, 117
13	kaempferid-glucuronide V., VI.	20.09	475	299, 284, 256, 151, 147
14	luteolin-hexoside V., VI., VII.	20.66	447	285, 255, 227
15	fisetin-deoxyhexosil-hexoside III., IV.	20.94	593	285/4, 255, 234, 227, 151
16	isorhamnetin-3-*O*-hexoside V., VI.	21.15	477	315/4, 299, 285, 271, 243
17	luteolin-glucuronide VI.	22.90	461	285, 243, 217, 199, 175, 151, 133

CID experiments at collision energies of lower level (20-25 eV) resulted two abundant product ions at *m/z* 161 and 461 (Table 5.2.). At higher energies (~ 45 eV) four more product ions appeared with lower intensities at *m/z* 113, 135, 179 and 315. The loss of -162 amu between *m/z* 623 and *m/z* 461 typically referrs to a hexose sugar unit and the 146 amu difference between *m/z* 461 and *m/z* 315 may indicate another sugar moiety (deoxyhexose) (Fig 5.3.).

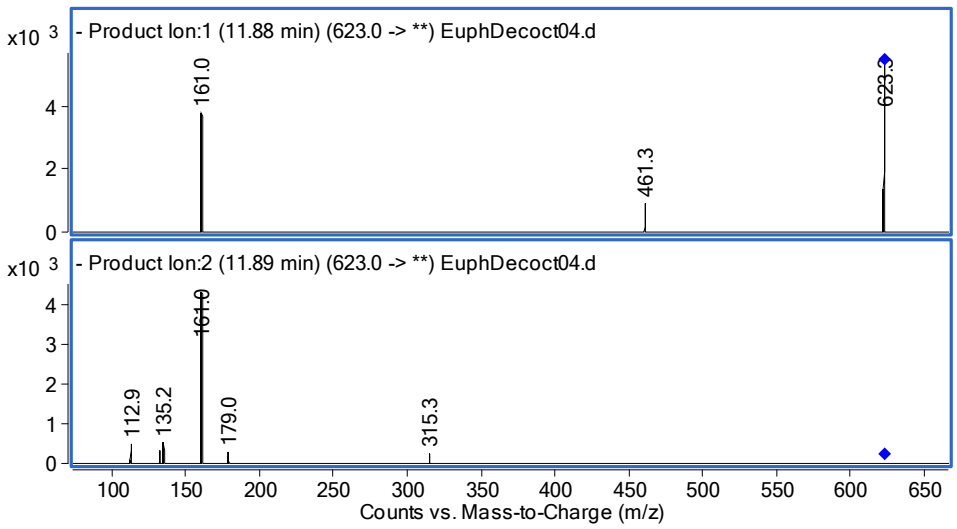


Fig 5.3.: Product ion spectra of acteoside at collision energies of 25 (top) and 45 eV (bottom).

The low-abundant fragment at *m/z* 179, resulting the ion *m/z* 161 after loss of a water molecule, may support the phenylpropane theory, if the molar weight of caffeic acid (MW = 180 g/mol) is considered. The loss of water may point to a phenyl or carboxyl group [33]. Though being aware of several structural information in light of the CID, the identity was still obscure. To gain information about the elemental composition, ESI-TOF in conjunction with molecule formula calculation was utilized. Mass accuracy of TOF analysers may change over a broad ppm range according to the ion intensity of the analyte of interest compared to that of the lock-mass. Therefore fraction I was investigated in three different concentrations, at S/N=3, at S/N=5.7 and at S/N=12.3, where the lock mass abundance were 300 %, 150 % and 40 % of the abundance of the molecular ion, respectively. Since halogens are very rarely found in plants and an absence of typical isotope pattern was observed, the search for the molecular formula was restricted to elements C, H, O and N. There was no restriction in the double bond equivalent. At levels of S/N=3 and S/N=12.3 the formula calculator algorithm ranked the formula $C_{29}H_{36}O_{15}$ as first score (100%) with -1.37 ppm and -0.16 ppm mass error, respectively. At S/N=5.7 the formula $C_{24}H_{36}N_2O_{17}$ was ranked first (100%) with -2.25 ppm mass error, while $C_{29}H_{36}O_{15}$ only as fourth (72.1%), with +4.01 ppm mass error. The formula $C_{24}H_{36}N_2O_{17}$ is unlikely, just like the nitrogen content, especially if the $^{13}C/^{12}C$

intensity ratio is taken into consideration. Based on the UV, MS/MS and TOF experiments we presumably identified the molecule as acteoside, a phenylethanoid glycoside known mainly in the Scrophulariaceae family. For structure and fragmentation details please see Fig 5.4.

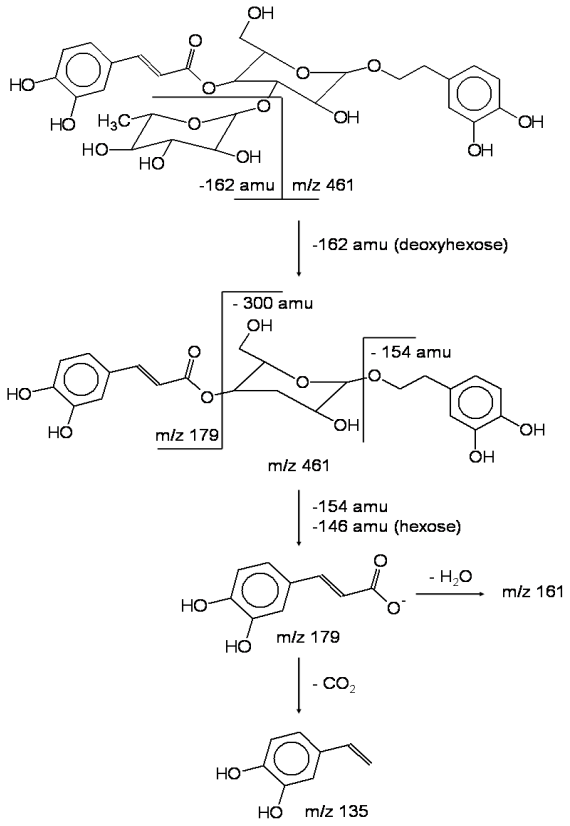


Fig 5.4.: Structure and supposed fragmentation pathway of acteoside (top) and its CID fragments with referring *m/z* data.

Fraction II contained acteoside as well, accompanied by another constituent of high polarity at *m/z* 637. The double neutral loss of -176 amu (*m/z* 637 → *m/z* 461 → *m/z* 285) marked glucuronide sugar moieties (Fig 5.5.d). The aglycon provided few product ions, even at high collision energies, but fragment *m/z* 175 ($[M\text{-}H\text{-}C_3\text{-}O_2\text{-}C_2H_2O]^-$) seemed to be distinctive and the aglycon was determined as luteolin. Acteoside was included in Fraction III also, but at an uncomparably lower concentration. Abundant ions in this fraction were *m/z* 353, 161, 609 and 593 (Table 5.2.). Telling fragments in the CID of *m/z* 353 were at *m/z* 191, 179, 135, 93. Based on typical losses (-44) and the chief fragment at *m/z* 191 this constituent was

identified as chlorogenic acid, which was confirmed with reference standard. Product ions of *m/z* 161 $[M-H]^-$ were detected at *m/z* 133, 117, 105 and 89. Consecutive losses of CO (-28 amu) and O (-16 amu) are obvious. Difference between *m/z* 117 and 105 is 12 amu, between *m/z* 105 and 89 is 16 amu, and between *m/z* 89 and 88 is 12 amu. Loss of 12 may correspond to a carbon atom, but is a very unlikely loss. Despite of the many product ions this compound remained unknown. Typical fragments of ion *m/z* 609 $[M-H]^-$ were *m/z* 301 $(Y_0)^-$, *m/z* 255 $[M-H-CO-H_2O]^-$, *m/z* 229 $[M-H-CO_2-CO]^-$, *m/z* 179 $^{1,2}A^-$ and *m/z* 151 $^{1,3}A^-$ -CO. Based on the unambiguous products, the relative intensity of the $(Y_0-H)^{\cdot-}/(Y_0)^-$ fragments (100-150%) and the loss of -308 amu (deoxyhexose-hexose) this constituent was charaterized as rutin [40]. Identity of rutin was confirmed by reference standard.

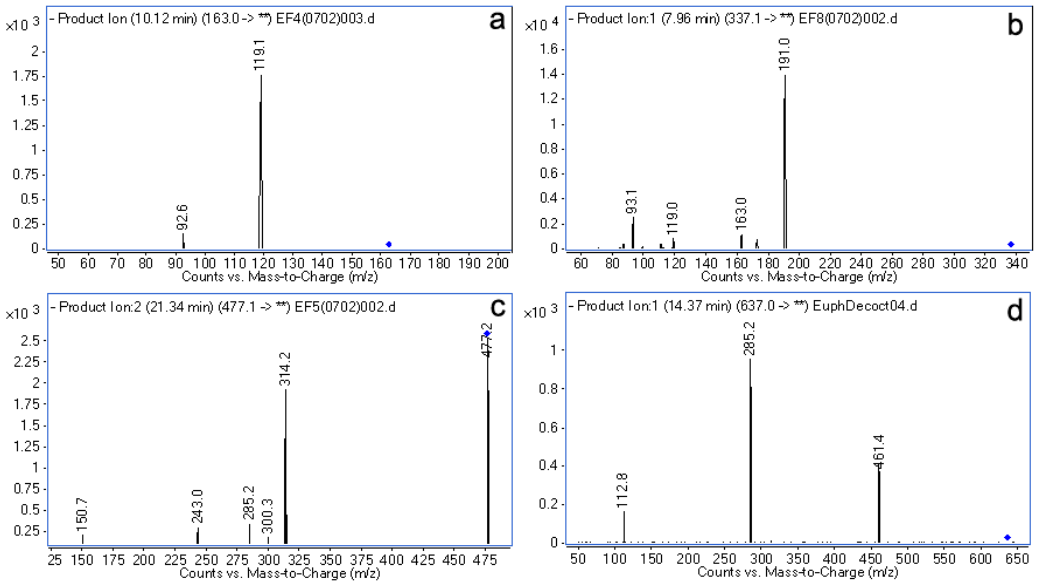


Fig 5.5.: Examples for the fragmentation of phenolic acids and flavonoids in *Euphrasia rostkoviana*. Fig shows the CID spectra of coumaric acid (a), coumaroyl-quinic acid (b), isorhamnetin-3-*O*-hexoside (c) and luteolin-diglucuronide (d).

Ion $[M-H]^-$ observed at *m/z* 593 exhibited abundant fragments at *m/z* 285, 273, 255, 227 and 163. The pronounced product at *m/z* 163 corresponds to $^{1,2}A^-$, a main fragment of the flavonoid skeleton. Thus the aglycone was characterized as fisetin. Other widespread flavonoids of the same molar mass (MW = 286 g/mol), luteolin and kaempferol, does not exhibit $^{1,2}A^-$ product ion in their CID [61]. The loss of -308 amu indicates a deoxyhexose and a hexose sugar part, which are supposed to be in a

chain linked to the -OH group at position 3, based on the radical aglycon ion to aglycon ion ratio (*m/z* 284 : 285 = 120% : 100%). Constituents of fraction IV, dominated by flavonoids, exhibited molecule ions $[M-H]^-$ at *m/z* 163, 609, 593, 431 and 461. The latter two molecules were detected at lower levels by an order of 1,5-2 magnitude than the others. Ion *m/z* 163 provided a meaningful -44 amu loss (CO_2), and a further loss of -26 amu (-CH=CH-), which resulted the product of *m/z* 93. This compound was identified as coumaric acid, as confirmation, 100% match was found with the referring spectral data of the coumaric acid reference standard. Constituents at *m/z* 609 and 593 were exactly those already revealed in fraction III. Dissociation of molecule ion at *m/z* 431 resulted alternative aglycon fragments at *m/z* 269 and 268 (*m/z* 117, $^{1,3}B^-$) pointing to apigenin as aglycon, and the loss of -162 amu marks a hexose sugar moiety. Ion at *m/z* 461 lost a neutral part of 176 amu, suggesting glucuronide sugar part and displayed in the CID typical fragments of luteolin aglycon. Composition of fractions V and VI were the most representative regarding the versatile flavonoid profile of Eyebright, including several flavonoid glycosides accompanied by simple phenolics (Fig 5.6. and 5.7.).

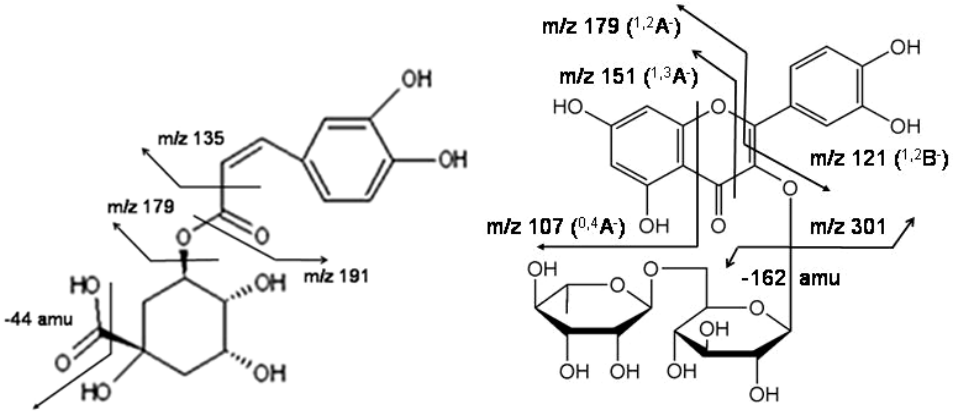


Fig 5.6.: Examples for the fragmentation pathways of typical *Euphrasia* phenolics: chlorogenic acid (left) and rutin (right).

Caffeic acid (*m/z* 179) and coumaric acid (*m/z* 163) were the dominant phenolic acids in fraction V. Flavonoids of this fraction were detected at *m/z* 447 (at two retention times, t_R: 15.65 and 20.66 min), 609, 431, 475 and 477. Presence of abundant fragments at *m/z* 285 $(Y_0)^-$ and 133 ($^{1,3}B^-$) in the CID of $[M-H]^-$ at *m/z* 447 (t_R: 15.65 min) revealed clearly that the aglycone is luteolin, which was supported by the UV

spectral maximums as well [61]. The one at t_R: 20.66 min displayed the same fragments in its CID, indicating the conclusion of a luteolin aglycone also. Such difference in retention properties might be explained by differing glycosilation positions, nevertheless $(Y_0)^-$ and $(Y_0\text{-H})^{\cdot-}$ ion ratios were not informative enough to draw any conclusions. Ions *m/z* 431 and 609 were apigenin-hexoside and rutin, respectively, as discussed earlier, in connection with fraction III and IV. Ion at *m/z* 475 provided pronounced fragments at *m/z* 299, 284 and 147, and strangely no other distinctive fragments were to be observed at lower *m/z* levels. Based on these few products we presume the molecule being kaempferid as loosing a $-CH_3$ group (*m/z* 299 → *m/z* 284) and forming a $[M\text{-H-}CH_3]^{\cdot-}$ radical ion [45, 57]. Ion $[M\text{-H}]^-$ at *m/z* 477 yielded main CID products at *m/z* 314 ($(Y_0\text{-H})^{\cdot-}$), 285, 271, 243 and 151. The great loss of -308 amu referrs to a hexose and a deoxyhexose sugar. The molar weight of the aglycon, and the 15 amu difference between *m/z* 300 and 315 (methyl group) suggests a rhamnetin or an isorhamnetin aglycon.

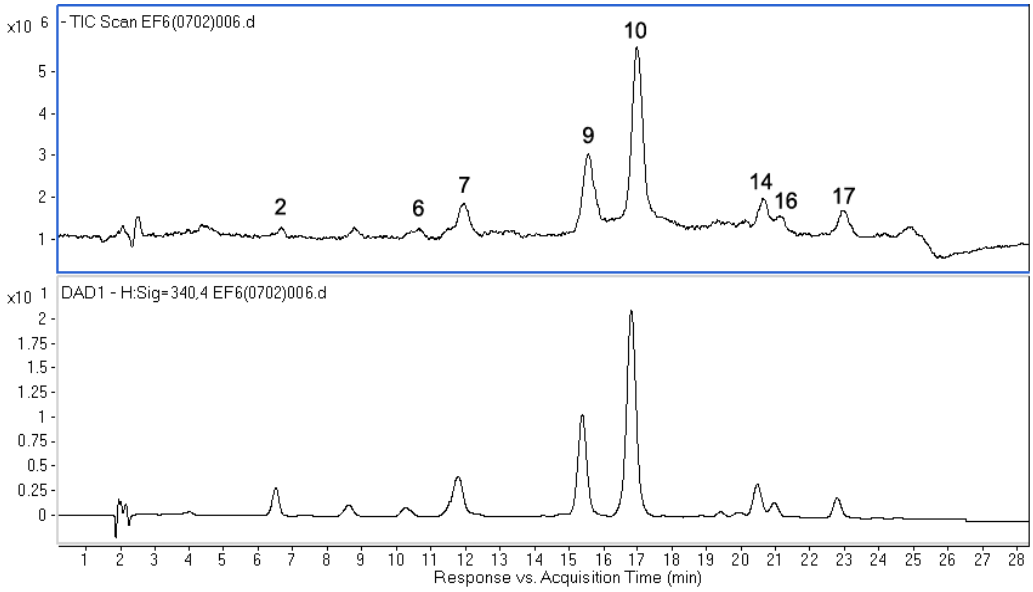


Fig 5.7.: TIC and UV chromatogram of fraction VI of *Euphrasia rostkoviana* sample. For details of numbering please see table 5.2.

The two isomers differ in the position of methoxylation: C7 - OCH_3 for rhamnetin and C3'- OCH_3 for isorhamnetin. The fragment at *m/z* 151 corresponds to a $^{1,3}A^-$ product ion, and the lacking fragment at *m/z* 121 ($^{1,2}B^-$) confirms that the molecule is an isorhamnetin-3-*O*-hexoside. A $[M\text{-H-}CH_3]^{\cdot-}$ radical aglycon was also observed at

m/z 299, which is rendered by the homolthic clevage of the methoxy group [45, 57]. Flavonoid constituents of fraction VI were detected at the same *m/z* data as of fraction V by greater abundance, except *m/z* 475. The only new compound in this fraction was *m/z* 463 eluting close to rutin (t_R: 17.08 min). This $[M-H]^-$ ion resulted telling fragments at *m/z* 301/300 (rel. intensity ratio = 200%:100%) and at *m/z* 151 after a neutral loss of -162 amu (hexose), indicating a quercetin-3-*O*-hexoside. It is presumed to be a product of rutin formed via in-source fragmentation, otherwise, such a 2-fold difference in the number of sugars would result a greater discrepancy in retention [40]. Ionpeaks at *m/z* 447 (both) and 463 also emerged in fraction VII. Composition of fraction VIII were dominated by phenolic acids, observed at *m/z* 337 and 367. CID of the first included pronounced fragments at *m/z* 191 (quinic acid unit), 163 (coumaroyl unit) and 119, by which and by studying the CID pattern of chlorogenic acid (caffeoyl-quinic acid) standard, the molecule was characterized as coumaroyl-quinic acid. Similar products were observed after fragmentation of *m/z* 367: *m/z* 173, 155, 134, 93. Product *m/z* 191 corresponds to a quinic acid part, as suggested by the identical CID pattern of chlorogenic acid and coumaroyl-quinic acid [33, 45]. Fragment *m/z* 135 might match with that fragment of chlorogenic acid at *m/z* 135 (caffeoyl unit-CO_2). The same stands for *m/z* 93. Calculation based on previously succesfully interpreted CID spectra shows that the molar weight of the phenolic part in this particular molecule is 194 g/mol (367-191)+18, which is by 14 Da greater than that of caffeic acid. Therefore a caffeic acid-like structure is suggested where a -CH_2- part is positioned between the carboxyl group and the substituted ring.

As previously mentioned, flavonoid glycosides were present in the methanolic Soxhlet extract as minor components. Some of them turned observable only after the polyamid column chromatography which suggests, that a serious enrichment occured simultaneously with fractionation.

5.1.4. Quantitation of acteoside

The traditional medical application of *Euphrasia* increases the notability of the experienced scavenging capacity exhibited by fraction I which was practically due to acteoside alone. A new point of view is added to the antioxidant effect when the dominance of acteoside is observed in the methanolic total extract as well, not just in fraction I. It is thus likely that acteoside has a characteristic and basic influence on the biological effect of *Euphrasia*, therefore we aimed to determine the acteoside content.

Qualitative studies already showed that fraction I was highly pure regarding acteoside, and the column chromatographic isolation yield was 51 mg. It was straightforward to check wether the purity in question may reach up to a reference standard obtained from analytical suppliers. After thorough chromatographic tests purity of the acteoside isolate was proved to be 98.2 ± 0.82% (DAD, n=3) and 97.1 ± 0.79% (TIC n=3) (Fig 5.8.). Results indicate, that our isolate was equal to that of commercially available phytochemical reference standards regarding purity, and was proper as a standard for quantitation but its unambiguous identity was still to be declared.

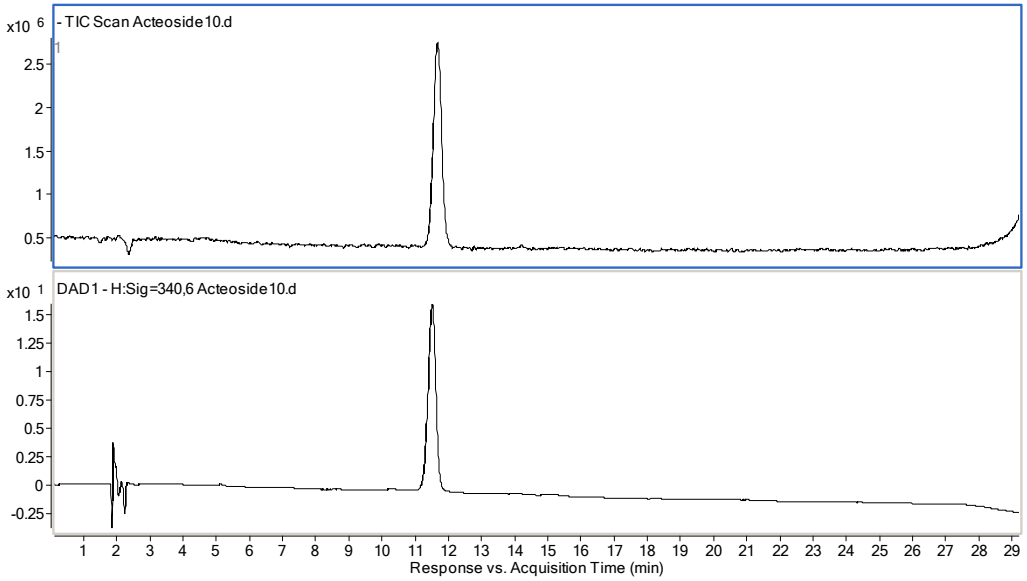


Fig 5.8.: TIC and UV (340 nm) chromatograms of the isolated acteoside (fraction I).

5.1.5. NMR spectroscopy

The NMR analysis confirmed the tentative identification based on MS findings. ^{1}H and ^{13}C assignments of the investigated compound were based on chemical shifts, multiplicity patterns and 2D experiments (Fig 5.9.). Here we discuss only few key parameters for structure elucidation of acteoside. The stereochemistry of the caffeoyl moiety was obvious in the ^{1}H spectrum, since the coupling constant between H-7' and H-8' was 15.9 Hz, which is characteristic of a trans arrangement. Configuration of the β-D-glucose unit was confirmed by trans-diaxial couplings, while the low *J* value between H-1 and H-2 in the rhamnose moiety proved their equatorial-equatorial positions. The HMBC correlation from H-4 of the glucose unit to the C-9' of the caffeic acid established the attachment position of this moiety; consequently the isoacteoside structure (where caffeic acid is attached to the glucose C-6 position) can be excluded. Further evidence to exclude the isomeric structure is the chemical shift values of glucose H-6 protons, these values should be shifted approximately 0.8 ppm downfield in case isoacteoside.

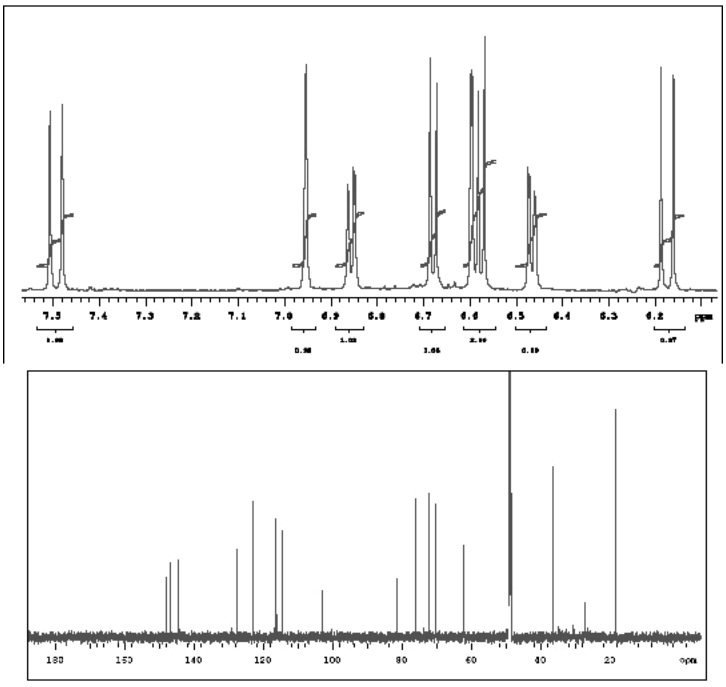


Fig 5.9.: The aromatic region of the ^{1}H NMR spectrum (top) and the ^{13}C NMR spectrum (bottom) of acteoside.

Finally, the obtained NMR datasets were compared with those reported in the literature [90-93]. It is worth to mention that the ^{1}H NMR assignment is fully in agreement with the pioneer work of Owen et al. and also with Pareira's and Wu's work, since in Lia's own set of data H-7 and H-8 are inverted. Similarly to that, there are also conflicting ^{13}C data published by Wu et al., where the chemical shift of glucose C-3 was taken for hydroxytyrosol C-8. In the latter constituent of acteoside, C-2 was also confused with C-5. In agreement with MS experiments the target compound was identified as acteoside (Fig 5.4.).

5.1.6. Quantitative result and method validation

The result for acteoside content was 2.56 ± 0.19 g (mean ± SD) / 100 g dry plant material (RSD: 7.57%, n = 3), which is prominently high, and may explain, in part, the beneficial effect of *Euphrasia* concerning inflamed eye-disorders.

SPE method

Though specificity can be reached by MRM mode, it does not solve the problem of ionsupression. To minimize this effect an SPE purification step was employed. The 3 mL 25% methanol eluted practically all amount of acteoside (signal of acteoside was below S/N = 3 in the sample of elution of 70% methanol) added to the SPE sorbent and no acteoside was lost during the sample addition. As a result, optimal elution of acteoside was achieved with high recovery and good precision, SPE recovery was 98.6% (RSD: 1.56%, n =3).

Selectivity, specificity

Great selectivity was ensured by the MRM mode of the triple quadrupole instrument. The *m/z* 161 fragment ion (transition *m/z* 623 → *m/z* 161) was choosen as the quantifier ion, and to avoid the quantitation of an ion providing the same fragment, *m/z* 461 was set as the qualifier ion. Pure solvent (70% methanol) was injected onto the HPLC column and analysed in order to exclude co-elution which may be a possible source of ionsupression. No interfering peak appeared at the retention time of acteoside.

Linearity, precision and accuracy

Five calibration standard samples (40-1500 ng mL^{-1}) were analyzed in triplicate in order to determine the linearity. The assay posessed acceptable linearity (r^2 = 0.997) within the investigated range with good precision and accuracy. Linear regression equation was y = 16.232x - 69.975 (y = ax + b, where „y" is the peak area, „a" is the slope, „x" is the analyte concentration in ng mL^{-1} and „b" is the intercept). Retention time repeatability was satisfactory (RSD: 0.7%, n=6). Intra- and inter-day precision, studied by the quality control samples, were also found to be acceptable (highest RSD: 6.27%) (Table 5.3.). Accuracy was acceptable, referring to the results of the fortified method recovery test, recovery was 91.0% with good standard deviation (RSD: 2.1%, n=3). Our quantitative method provided good linearity, precision, accuracy, and low quantitation limits (Table 2.), which are in accordance with, or better, than those of previous works [75, 76].

Limits of detection and quantification

According to the lowest point of the calibration curve the LOQ was 40 ng mL^{-1} for acteoside. LLOQ (S/N=10) was 15 ng mL^{-1}, while LOD (S/N=3) was found to be 5 ng mL^{-1}.

Table 5.3.: Precision (relative standard deviation) and accuracy of the quantitative method studied with the isolated acteoside.

Nominal concentration (ng mL^{-1})	Measured concentration (ng mL^{-1})	Precision (RSD %)	Accuracy (%)
Intra-day			
100	102.1	1.51	102.1
300	297.9	0.84	99.3
800	811.2	0.87	101.4
Inter-day			
100	98.7	6.27	98.7
300	292.6	6.18	97.5
800	852.1	3.66	106.5

5.1.7. Summary

A total of seventeen phenolic constituent were identified or characterized by LC-DAD-MS/MS in the methanolic extract of *Euphrasiae herba*. Our qualitative results are partly in agreement with those of previous studies, moreover one phenolic acid, one phenylpropane glycoside, one flavonoid aglycone and six flavonoid glycosides were reported for the first time in *Euphrasia rostkoviana*. In contrast to the usually time comsuming and less selective chromatographic and spectroscopic methods of former results on the herb we suggest modern, powerful and fast LC-MS/MS methods as a replacement.

According to our literature search, acteoside has neither been identified, nor quantified in *Euphrasia rostkoviana* before. Product ions of the acquired CID spectra were in agreement with that of Wu et al. [77]. Isolation purity of acteoside was as high (≥ 97.1%), as its calibration standard application was amenable. We achieved the same result as Li et al. regarding isolation purity of acteoside [28]. Our validated, reliable, sensitive and selective SRM method is amenable for wider application including medicinal plant screening, biofluid analysis and quality control of raw plant materials.

Beside its antioxidant property other medicinal effects of acteoside were also shown [73, 74], which underline the significance of the notably high acteoside content in Eyebright, which may give a reasonable explanation, in part, for its anti-inflammative action concerning eye-disorders.

The antioxidant *in vitro* assays served well for the identification and pre-differentiation of the potentailly active compoundgroups. In view of its significant antioxidant effect and high content acteoside was supposed as active component of Eyebright herb.

5.2. *Satureja hortensis* L.

A recent, but yet not published study, conducted at the Budapest University of Technology and Economics by the research group of Simándi showed a notable antioxidant capacity of the polar and semi-polar extracts of *Saturejae herba.* Soxhlet

extracts of 50%, 70%, 96% and 100% (v/v%) ethanol, acetone, ethyl acetate and isopropyl alcohol were included in investigations. The differences in antioxidant efficacy observed among the extracts of different polarity called for analysis of the phenolic content. The significant scavenging effect of rosmarinic acid standard directed our interest to the determination of the rosmarinic acid content of the extracts.

HPLC method development was started with a gradient scouting [70] using methanol as the organic mobile phase. It was soon realised that methanol provides unsatisfactory resolution, broad bands (width of main peak: 0.9 min) and in addition analysis time was rather long (28 min). Changing eluent B to acetonitrile and increasing flowrate resulted in sharper peaks (0.4 min width), better resolution and an analysis time of 23 min (Fig 5.11.). The main compound (t_R: 7.79 min) was detected as $[M-H]^-$ at *m/z* 359. CID experiments resulted fragment ions typical of phenolic acids, such as *m/z* 179 (caffeoyl unit) or *m/z* 135 (caffeoyl unit -CO_2) by which the component was characterized as rosmarinic acid (Fig 5.10.) [45, 119]. By full match with the rosmarinic acid reference standard, regarding retention time, UV and CID spectral data, identitiy of the main compound was confirmed.

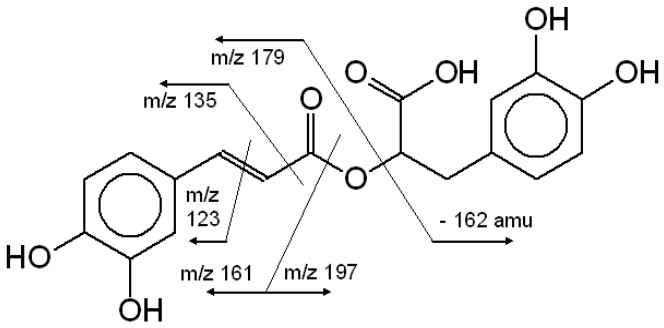


Fig 5.10.: Structure and CID fragmentation pathways of rosmarinic acid.

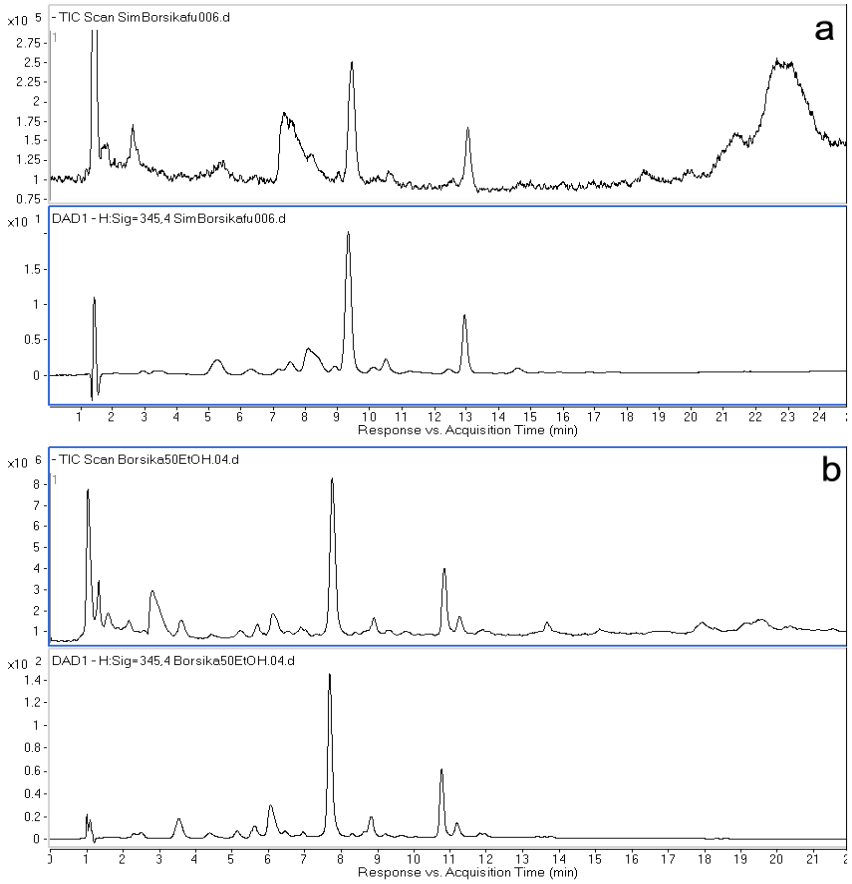


Fig 5.11.: TIC and UV (345 nm) chromatograms of the 50% ethanolic extract of *Satureja hortensis,* acquired with methanol (a) and acetonitrile (b) as B%.

Under the peak of rosmarinic acid, both in the sample and standard solution, another $[M-H]^-$ ion was detected at *m/z* 719 with lower abundance which, after CID investigation, was found to correspond to rosmarinic acid as well. We presume that a $[2M-H]^-$ dimer of rosmarinic acid was formed in the ion-source via gas phase reactions (Fig 5.12.). Dimerization phenomena was already reported about rosmarinic acid and other phenolic acids [119].

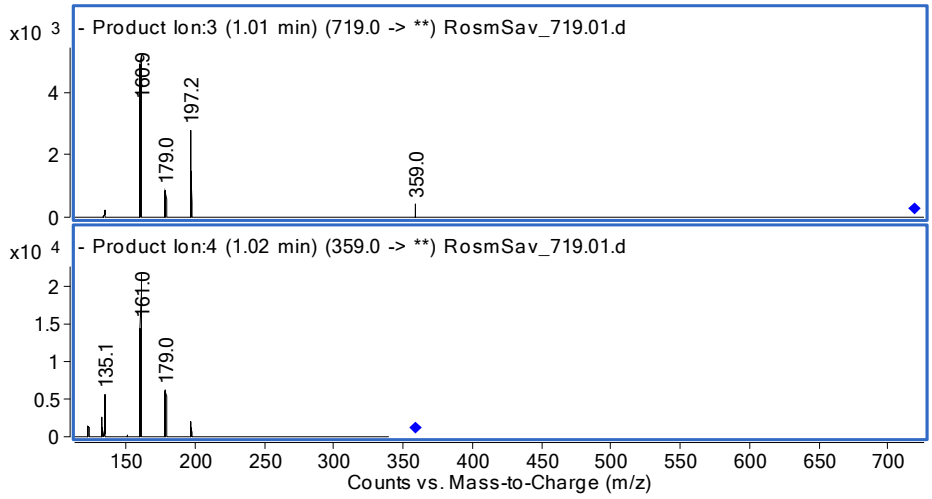


Fig 5.12.: CID spectra of the $[2M-H]^-$ ion (top) and the $[M-H]^-$ (bottom) of rosmarinic acid detected at *m/z* 719 and 359, respectively.

To exclude that our observation is due to chemical heterogenity or questions of quality of the reference standard, rosmarinic acid was analysed in four different concentration (5, 20, 50, 150 µg mL^{-1}). The dimer to monomer ion ratio was increasing with the increasing concentration (5 µg mL^{-1}: 0.2/4.6, 150 µg mL^{-1}: 3.9/3.7), thus the theory of low quality standard, or special chemical structure in the sample has been declined. Since the non quantitative simultaneous presence of the $[2M-H]^-$ dimer and the original $[M-H]^-$ ion makes quantitation impossible either in SIM or MRM mode, we investigated the robustness and conditions of the dimerization process. Fragmentor voltage was changed between 70 and 170 V by parameter ramping and as a result no trend was found in dimerization, both ions appered in the scan spectra at all voltage settings. After all we concluded that determination of the rosmarinic acid content with the available LC-MS system is not feasible in SIM, neither in MRM mode, since the analyte was detected in two different ionic forms, regardless to changes in conditions.

UV detection, though with lower sensitivity and selectivity, still offered a good solution for quantitation. Our LC separation ensured good resolution for rosmarinic acid and peak purity was already investigated by MS, content of the main compound of *Satureja* was determined by UV detection at 330 nm. Results of contents are to be found in table 5.4.

Table 5.4.: Quantitative results of the rosmarinic acid content determined in different ethanolic extracts of *Satureja hortensis*.

Samples (ethanolic extracts)	Rosmarinic acid content (% of extract ± SD)	RSD %
50%	4.69 ± 0.02	0.42
70%	6.69 ± 0.05	0.77
96%	6.47 ± 0.09	1.39
100%	5.59 ± 0.07	1.34

Summary

The leading compound of *Satureja* was identified as rosmarinic acid by LC-MS/MS, and quantified by UV detection. The 70% ethanolic extract exhibited the highest rosmarinic acid content. Content was decreasing if the % volume of ethanol was either lowered than, or increased from 70%. The high rosmarinic acid content supports the antioxidant test results.

5.3. *Filipendula ulmaria* L. MAXIM

5.3.1. Qualitative analysis

As a first step of LC method development the referring literature data was studied. HPLC methods from the litertaure for salicylates were not adoptable to our aims [78, 79]. In spite of its efficacy the coupled NP-RP-HPLC (normal phase - reversed phase - HPLC) method of Poblocka-Olech et al. seemed too complicated, in addition, not as many diverse compounds were expected in *Filipendula* samples as much they separated [80]. After the above considerations a gradient of acetonitrile and 0.5% (v/v%) formic acid in water was applied for achieveing separation [48]. Acteic acid was also tested as an alternate, but formic acid was chosen for analysis, becaue a significant presence of disturbing and only partly formed acetate adducts were detected in the scan mass spectra. Nevertheless, formate adducts were also observable rarely. There was no notable difference between the two acids regarding separation.

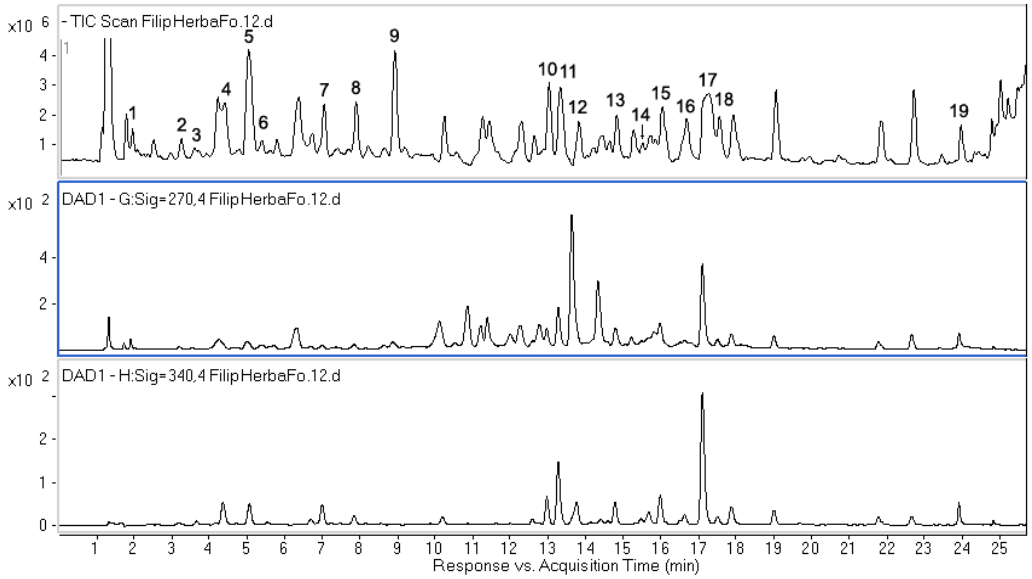


Fig 5.13.: TIC and UV (270 and 340 nm) chromatograms of *Filipendula* herba sample. For details of numbering please see table 5.5.

Table 5.5.: List of identified and/or tentatively characterized compounds in *Filipendula ulmaria* herba and flower sample (S = identity was confirmed by standard compound).

Peak No.	Compound	TIC t_R (min)	*m/z*
1	salicylalcohol derivative	1.96	169 $[M-H]^-$
2 S	salicin (formate adduct)	3.25	331 $[M-H+HCOOH]^-$
3 S	caffeic acid derivative	3.72	297 $[M-H]^-$
4	salicylic acid monohexoside	4.46	299 $[M-H]^-$
5 S	chlorogenic acid	5.07	353 $[M-H]^-$
6	salicylalcohol derivative	5.4	183 $[M-H]^-$
7	caffeic acid derivative	7.06	297 $[M-H]^-$
8	coumaroyl-quinic acid	7.91	337 $[M-H]^-$
9	monotropitin (formate adduct)	8.92	491 $[M-H+HCOOH]^-$
10 S	quercetin-rutinoside (rutin)	13.03	609 $[M-H]^-$
11	quercetin monohexoside	13.33	463 $[M-H]^-$
12	quercetin monohexoside and quercetine monoglucuronide	13.83	463 $[M-H]^-$ 477 $[M-H]^-$
13	quercetin monopentoside	14.82	433 $[M-H]^-$
14	kaempferol monohexoside	15.5	447 $[M-H]^-$
15	quercetine monopentoside	16.06	433 $[M-H]^-$
16	quercetine dezoxihexoside	16.68	447 $[M-H]^-$
17 S S	spiraeoside and salicylic acid	17.25	463 $[M-H]^-$ 137 $[M-H]^-$
18	quercetin derivative	17.57	585 $[M-H]^-$
19 S	kaempferol monohexoside	17.95	447 $[M-H]^-$
20 S	quercetin aglycone	23.96	301 $[M-H]^-$

The applied gradient provided acceptable resolution for almost all components in the chromatogram (Fig 5.13.). A total of six salicylates, four phenylpropanes and ten different glycosides of quercetin and kaempferol and quercetine aglycons were identified and/or structurally characterized in the methanolic extract of *Filipendulae herba* and *flos* (Table 5.5.). Several molecules of salicylate type eluted right after the solvent peak. The peak at 1.96 min exhibited a UV spectra typical of salicylalcohol (λ_{max}: 220, 272 nm), and contrary to this, a mass of 170 g/mol (*m/z* 169). By CID experiments fragments of *m/z* 124/125, 97 and 79 were obtained (Fig 5.14.a). The -44 amu (*m/z* 169 → *m/z* 125) indicates a carboxyl group on the salicylalcohol skeleton [45]. Strangely, the fragment resulted by decarboxylation was detected at *m/z* 124 and 125 as well, which may point to a homolytic cleavage of the carboxyl group giving rise to a radical fragment ion. See our suggestion for tentative structure in Fig 3.15. Salicin, a commonly cited constituent of *Filipendula ulmaria* was detected as a formate adduct $[M-H+formate]^-$ at *m/z* 331 with a retention of 3.25 min (Fig 5.14.b). The glucose moiety cleaved off in all CID experiment together with the formate unit, and informative product ions at *m/z* 123 $[M-H-Glu-formate]^-$ and *m/z* 93 $[M-H-Glu-formate-CHOH]^-$ were formed. UV spectral results were in full agreement with the MS findings. Identity was confirmed by comparison of relevant data with those of salicin standard. The next salicylate was detected at *m/z* 299 $[M-H]^-$ (t_R: 4.46 min). A loss of -162 amu between fragments *m/z* 299 and 137 points to a hexose sugar compound, and the further loss of -44 amu (*m/z* 137 → *m/z* 93) reveals a carboxyl functional group (Fig 5.14.c). As MS/MS results were complemented with the UV data (λ_{max}: 205, 237 and 303 nm) this compound was tentatively identified as salicylic acid hexoside. Another salicylalcohol derivative was characterized at *m/z* 183 $[M-H]^-$ (t_R: 5.4 min). Product ions of the CID were *m/z* 168, 124, 106 and 95. Losses of -15 amu ($^{\cdot}CH_3$), -44 amu (CO_2) and -18 amu (H_2O), respectively, confirms the first idea of identity which was based on UV absorbtion maxima (λ_{max}: 220, 272 nm). Our presumed structure can be seen in Fig 3.15. A salicylate of extra-ordinarly high molar mass was detected in the middle of the chromatogram (t_R: 8.92 min) at 491 *m/z*.

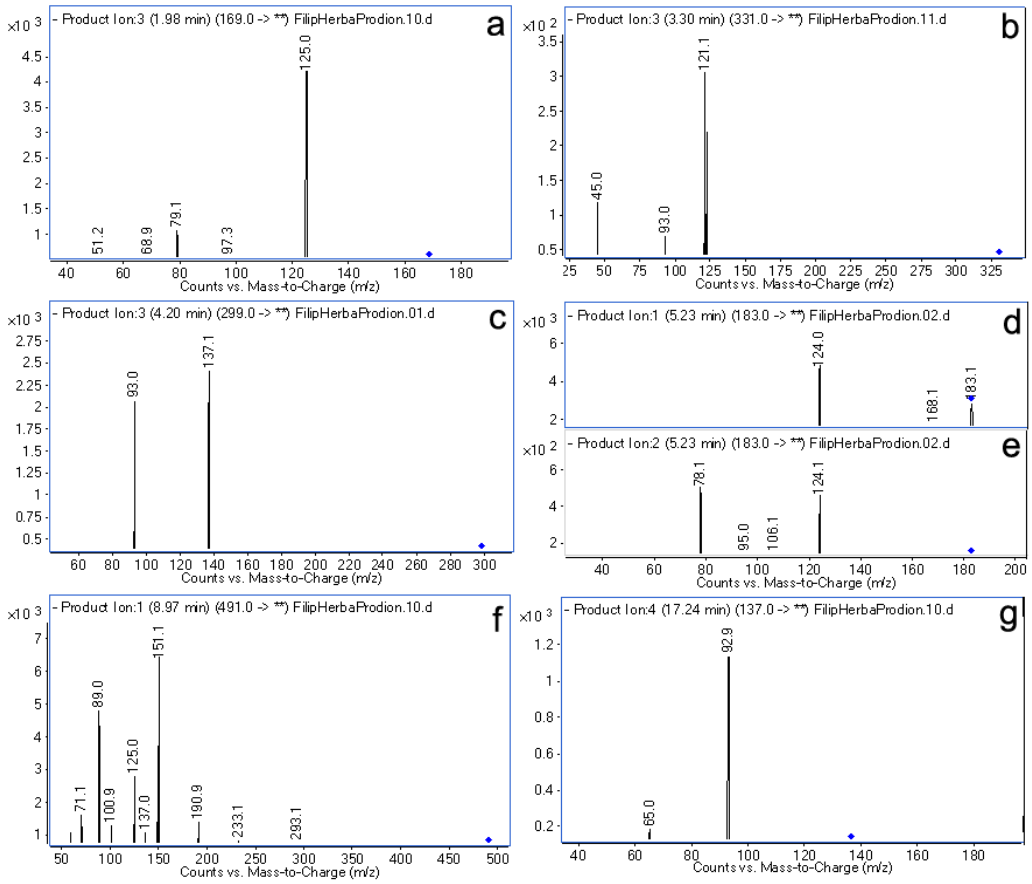


Fig 5.14.: CID spectra of the salicylates identified or characterized in the *Filipendula* sample. salicylalcohol derivative (a), salicin (b), salicylic acid hexoside (c), salicylalcohol derivative (d and e), monotropitin (f), salicylic acid (g).

CID of this constituent showed a loss of -46 amu from the molecule ion $[M\text{-}H]^-$ and a consecutive great loss of -294 amu resulting an ion at 151 *m/z*, which generated further products of *m/z* 137 and 125 (Fig 5.14.f). This molecule was tentatively identified as the formate adduct $[M\text{-}H+formate]^-$ of monotropitin (methylsalicylate-primverose), which was in match with the UV spectral data. Salicylic acid was detected in a peak of co-elution with spiraeoside, another characteristic constituent for *Filipendula*, at t_R: 17.35 min with an *m/z* of 137. Fragments were *m/z* 93 and 65. MS and UV characteristics of identity were compared with that of salicylic acid standard. Neither salicylaldehyde, nor its conjugated form was detectable in the Soxhlet extracts, which is perhaps due to its volatility and/or decomposition. Pseudomolecular ions $[M\text{-}H]^-$ of those compounds detected as formate adducts did

not show up in the scan mass spectra. Beyond the novel two structures our qualitative findings are in agreement with previous works [125, 126].

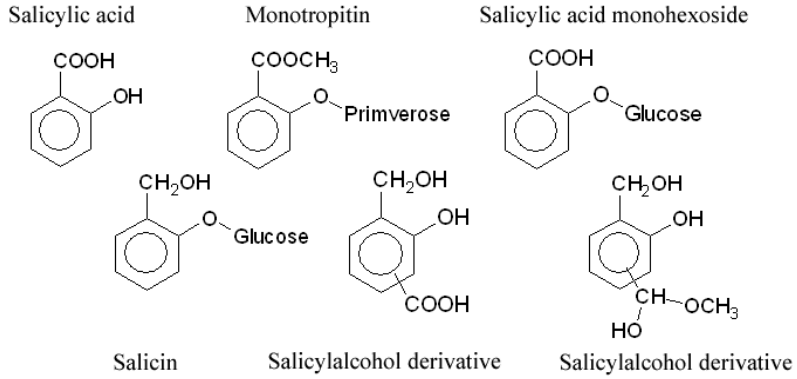


Fig 3.15.: Structures of the identified and/or tentatively characterized salicylates in *Filipendula ulmaria.*

The flavonoid profile of the herb consisted of diverse quercetin and kaempferol glycosides, which were already studied in details by previous works [125, 126]. Guidelines for flavonoid MS identification was already presented in section „Bibliography review", therefore only a few data are provided here. Distinctive fragments such as $^{1,2}A^-$ (*m/z* 179) and rather general fragments of $^{1,3}A^-$ (*m/z* 151) and $^{0,4}A^-$ (*m/z* 107) played key role in the identification of quercetin [34, 58, 61]. Kaempferol was distinguished from luteolin on the basis of product ions $^{0,3}A^-$, $^{1,3}B^-$ and the UV spectral differences [34, 58, 61]. However all flavonoid glycosides in the sample were fragmented with 3 different collision energies, the glycosilation positions were hardly determinable unambiguosly. The $(Y_0)^-$ ion to $(Y_0\text{-H})^{-\cdot}$ ion ratio was decreasing at all flavonoid glycosides with increasing collision energy which gave no clear evidence for position differentiation. The quercetin hexoside at t_R: 13.33 min was supposed to be a quercetin-3-*O*-hexoside (intensity $_{(Yo)^-}$ / intensity $_{(Yo\text{-}H)^{-\cdot}} \leq 0.1$), while the one at t_R: 13.83 min seemed to be a 7-*O*-hexoside (intensity$_{(Yo)^-}$ / intensity$_{(Yo\text{-}H)^{-\cdot}} \geq 5$). The quercetin hexoside at t_R: 17.18 min, exhibiting a more abundant $(Y_0)^-$ ion than $(Y_0\text{-H})^{-\cdot}$ radical ion, was identified as spiraeoside after comparison with reference standard, which is only partly in agreement of the findigs of Hvattum and Ekeberg [40]. The quercetine derivative (glycoside?) at t_R: 17.57 min yielded in its CID neither the $(Y_0\text{-H})^{-\cdot}$ ion, nor the $[Y_0\text{-2H-CO}]^-$ ion, which indicates

perhaps a glycosilation on the B ring [40, 69]. For details of the identified and characterized compounds in *Filipendula* samples please see Table 5.5.

5.3.2. Quantitative analysis

In view of the therapeutical potential of the *Filipendula* salicylates we aimed to determine all the six characterized molecules of the salicylate type from the flower and herb samples. Since only two of the target analytes were commercially available as standards (salicin and salicylic acid), the four remaining were quantified by the use of the two existing standards as well. For this, ESI ionization and sensitivity of the target compounds have had to be assumed identical to that of the standards. In lack of standards it is impossible to prove that sensitivity is identical or similar, but comparison of the ESI ionization behaviours is amenable. Peak areas for all salicylates (standards as well) were investigated and evaluated at different ESI settings. It was concluded in general that fragmentor voltage exhibited far the greatest influence on peak area. For instance peak *m/z* 331 was detected with an area of 344866 counts per second (cps) at 100 V, but at 160 V only a peak of 22933 cps was detectable, meaning a 93.3 % loss. Even smaller differences in the fragmentor voltage could decrease mass signal dramatically, a drop from 130 V to 100 V caused a 54.2 % loss in the intensity of ion *m/z* 137. The capillary setting affected intensity by a much lower influence (maximum deviation was 28.72 %). ESI temperature had even less effect on ionization, greatest deviation between lowest and highest settings was merely 15 %. Trends of area changings at varying ESI parameters were reasonably similar for all salicylates. In order to get a picture about the extent of error rising from determination with calibration of a same type, but different molecule, quantitative results were calculated with both calibration plots for all six salicylate compounds. In view of the above, results of salicylates with no matching standard can only be considered as estimative contents.

When acetic acid in water was used for chromatography, both the pseudomolecular ion $[M\text{-}H]^-$ and the acetate adduct $[M\text{-}H+CH_3COOH]^-$ of salicin were present in the spectra. Attempts to force ionization process to result only the $[M\text{-}H]^-$ ion failed, both the $[M\text{-}H]^-$ and the adduct were present simultaneously at all fragmentor voltages

between 70 and 160 V, hence MS based determination using acetic acid was beyond possibilities. In case of formic acid adduct formation of salicin (*m/z* 331 [M-H+HCOOH]$^-$) was quantitative, peak at *m/z* 285 (salicin, [M-H]$^-$) was not observable in any of the scan mass spectra. Only salicin showed adduct formation, the other five target analytes, including salicylic acid (*m/z* 137 [M-H]$^-$), no. Since fragmentation of an adduct (salicin formate adduct) may significantly differ from the fragmentation of the referring pseudomolecular ion, determination in SRM (single reaction monitoring) mode was not feasible. In fact the target analytes had no CID fragment in common, which is another reason why SRM was found inappropriate. Based on the above considerations quantitation was performed in SIM mode using salicin formate adduct (*m/z* 331 [M-H+HCOOH]$^-$) and salicylic acid (*m/z* 137 [M-H]$^-$) for calibartion. This is of importance since the calibration occured with an adduct, but, was used in part for the estimative determination of compounds which did not form adducts.

The general two-threefold difference between precision (standard deviation) of salicin and salicylic acid calibration may point out the instability and/or low repeatability of salicin adduct formation and calibration, though all RSD% were within an acceptable range (Table 5.6.). Sensitivity (slope of calibration) for salicylic acid was in average 2.48 times higher than for salicin as Fig 5.16. clearly shows.

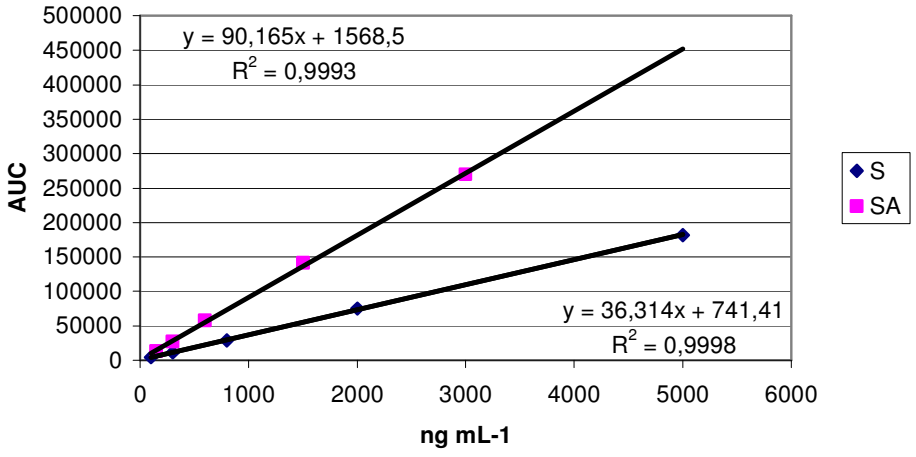


Fig 5.16.: Calibration plots and linear regression equations of salicin (S) and salicylic acid (SA) standards.

There was a difference of two orders of magnitude among the determined quantities in the herb sample and three orders of magnitude deviation in the flower sample (Table 5.6.). The analysed herb (1.94 mg/g calc. in salicylic acid and 4.84 mg/g calc in salicin) and flower (3.7 mg/g calc. in salicylic acid and 9.23 mg/g calc. in salicin) samples were richest in the tentatively characterized salicylalcohol derivative (*m/z* 183) while salicylic acid was present in a lower, but relatively high amount (Table 5.6.). The flower sample was 1.5-2 times richer in some salicylates than the whole aerial part (*m/z* 169, 183 and 299), but not in general extent. Differences between results of salicin and salicylic acid contents calculated vice-verse by their calibrations shows expressively the error what comes out if no matching standard is available for calibration. Quantitative results of salicylates without standards can only be considered as semi-quantitative, nevertheless there is no way to provide more accurate results unless matching standards are accessible.

Table 5.6.: Quantitative results of *Filipendulae herba* and *flos* (standard used for calibration: SA: salicylic acid, S: salicin)

m/z	**Compound**	**Quantity (mg/1g dry plant material, mean ± SD)**	**RSD %**	**Quantity (mg/1g dry plant material, mean ± SD)**	**RSD %**
		Herba		Flos	
169	salicyl alcohol derivative	0.97 ± 0.07 (SA)	7.76	3.34 ± 0.08 (SA)	2.41
		2.30 ± 0.27 (S)	11.78	8.36 ± 0.20 (S)	2.4
331	salicin	0.06 ± 0.002 (SA)	4.34	0.0002 ± 0.0001 (SA)	58.9
		0.16 ± 0.007 (S)	4.19	0.0039 ± 0.0004 (S)	11.19
299	salicylic acid monohexoside	0.15 ± 0.01 (SA)	9.27	0.02 ± 0.001 (SA)	9.37
		0.4 ± 0.03 (S)	8.93	0.1 ± 0.004 (S)	4.2
183	salicyl alcohol derivative	1.94 ± 0.14 (SA)	7.22	3.7 ± 0.08 (SA)	2.15
		4.84 ± 0.34 (S)	7.18	9.23 ± 0.2 (S)	2.16
491	monotropitin	1.1 ± 0.06 (SA)	5.5	1.09 ± 0.06 (SA)	6.06
		2.75 ± 0.15 (S)	5.68	2.78 ± 0.15 (S)	5.65
137	salicylic acid	1.46 ± 0.04 (SA)	3.0	1.37 ± 0.04 (SA)	2.72
		3.65 ± 0.1 (S)	2.95	3.48 ± 0.09 (S)	2.64

5.3.3. Method validation

Specificity was ensured by the SIM mode of the mass spectrometer, no co-elution was detected at the retention times of the analytes when pure solvent was analysed. A good linear response was observed between peak area and concentration for both standards within the investigated range (salicin: y = 36.314x + 741.41; salicylic acid:

y = 90.165x + 1568.5), accuracy of the quality control samples verified linearity. Intra-day, inter-day precision and accuracy were all within acceptable ranges, and precision of salicylic acid was better, as mentioned above (Table 5.7.). Retention time repeatability was good (RSD % = 0.14, n = 6). Recovery for salicylic acid was 89.97 % (RSD % = 9.24, n=3) and for salicin 54.1 % (RSD % = 9.43, n=3). The low, but repeatable recovery of salicin might be explained with the instability of the formate adduct, and/or with an incidental cleavage of the glucose moiety of salicin during Soxhlet extraction. The LOQ (lowest point of calibration) was 150 ng mL^{-1} for salicylic acid, which was the LLOQ (S/N=10) as well. LOQ for salicin was 100 ng mL^{-1} , while the LLOQ was 24 ng mL^{-1}. LOD (S/N=3) for salicylic acid was 50 ng mL^{-1}, for salicin 8 ng mL^{-1}.

Table 5.7.: Precision (relative standard deviation) and accuracy of the quantitative method studied with salicin and salicylic acid standards.

Nominal concentration (ng mL^{-1})	Measured concentration (ng mL^{-1})	Precision (RSD %)	Accuracy (%)
Salicin			
Intra-day			
100	106.8	6.72	106.8
800	773.5	6.11	96.7
5000	4986.9	3.6	99.7
Inter-day			
100	97.1	9.85	97.1
800	752.4	7.31	94.0
5000	4831.6	6.76	96.6
Salicylic acid			
Intra-day			
150	122.1	4.09	81.4
600	603.9	3.03	100.6
3000	2939.9	0.79	97.9
Inter-day			
150	123.5	5.14	82.4
600	580.2	0.93	96.7
3000	2844.1	3.71	94.8

5.3.4. Summary

We performed an extensive qualitative and quantitative study on the active constituents of Meadowsweet, with a particular focus on the salicylate type. Six

salicylates, four phenylpropanes and ten different flavonoids were identified and/or structurally characterized in the methanolic extract of the herb and flower. This work is the first to supply quantitative data on six salicylates in *Filipendula ulmaria*. Salicylates were unobtainable as standards, except salicin and salicylic acid, therefore quantitative results reported here have to be considered partly as semi-quantitaive or approximation.

It was conluded that ESI ionization is out of control in some extent, and depends on the type of analyte. All salicylates were detected as acetate adducts $[M-H+CH_3COOH]^-$ when acetic acid was applied as the acidic modifyer. A switch to formic acid changed ionization dramatically giving rise to pseudomolecular ions $[M-H]^-$. Parameter changing (fragmentor and capillary voltage) did not influence adductformation significantly.

The development of such selective and sensitive methods for the determination of non-volatile salicylates is desirable, in lack of any instruction either in the European or Hungarian Pharmacopoeae to quantify and screen the salicylate content of *Filipendula ulmaria*. This is more urgent if the preventive and mild therapeutic potential of *Filipendula* is taken into account especially contrary to the harmful side-effects of non-steroid anti-inflammatory drugs.

5.4. *Sempervivum tectorum* L.

Research groups of Abram and Stevens described already the flavonoid aglycons of *Sempervivum*, but provided not, if any information on the glycosilation status [81, 82]. However composition of the sugar moieties plays a key role in absorption kinetics [83, 84].

Studying the distinctive spectra in the UV chromatogram of the flavonol compounds in the 80% (v/v%) methanol extract of the *Sempervivum* sample yielded a preliminary structural description. Absorption maximums were characteristic of kaempferol- and quercetin-*O*-glycosides [52, 54]. To overcome the very limited structural information capabilities of the UV detector tandem mass spectrometry was applied. The deprotonated pseudomolecular ions $[M-H]^-$ were examined in product ion scan mode. Beside the univocal *m/z* values of the $[M-H]^-$ ions CID fragments

representative for flavonols were detected, furthermore frequently occuring radical aglycons $(Y_0\text{-}H)^{\bullet}$ were also observable at *m/z* 284 and 300 for kaempferol and quercetin, respectively. (Fig 5.17.).

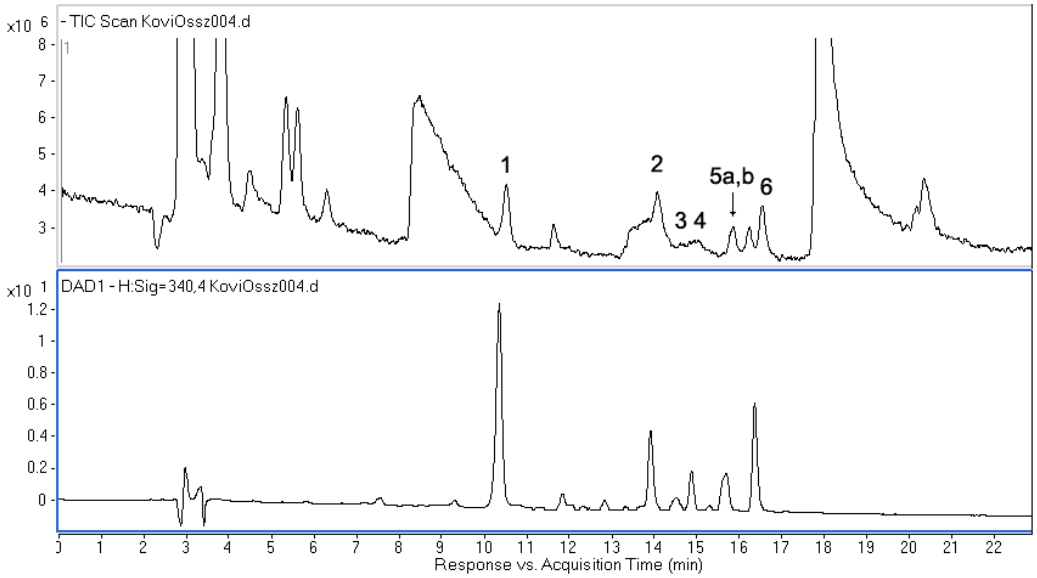


Fig 5.17.: TIC (top) and UV (340 nm) (bottom) chromatograms of the 80% methanolic extract of *Sempervivum tectorum* L. For details of numbering please see table 5.8.

For unambiguous identification the fragmentation patterns and UV data of kaempferol and quercetin were compared with those of standard compounds and literature data. Relative abundance of the $(Y_0\text{-}H)^{\bullet}$ radical ion was by 50-400% higher in mass spectra of compounds 1, 2, 4, 5a and 6 at *m/z* 284 and of compound 3 at *m/z* 300 than that of $(Y_0)^-$ aglycon ion. Therefore these compounds were assumed to be 3-*O*-glycosyls. In the CID of peak 5b the radical aglycon at *m/z* 284 was practically not present. The MS/MS spectra of peaks 1, 2, 4, 5a and 5b were further complicated by the presence of radical aglycon $(Y_0\text{-}2H)^{\bullet}$ indicating di-*O*-glycosidic flavonoids. Further study of the CID spectra revealed radical ions $(Y_1\text{-}H)^{\bullet}$ and $(Y_1\text{-}2H)^{\bullet}$ in peak 1, and $(Y_1\text{-}H)^{\cdot\cdot}$ in peaks 2, 4, 5a and 5b, which is in match with the previous indication of di-glycosides. Formation of $(Y_0\text{-}2H)^{\bullet}$ radical ion is explained by the simultaneous cleavage of two *O*-glycosidic bonds at two different positions (commonly 3-*O* and 7-*O*), resulting a successive elimination of two radical sugar units [67, 69]. Compounds with a $(Y_0\text{-}2H)^{\cdot\cdot}$ radical ion in their CID are thus 3,7-di-

O-glycosides. Table 5.8. shows the most frequent ions which characterize the fragmentation of flavonol *O*-glycosides present in *Sempervivum tectorum* leaves.

Peak 1 (t_R: 10.54 min) at *m/z* 739 was identified as a kaempferol-triglycoside: kaempferol-di-(deoxyhexoside)-hexoside. Fig 5.18. shows the CID spectra of the triglycoside at *m/z* 739, which is representative example for the flavonoid tri- and tetra glycosides in the sample.

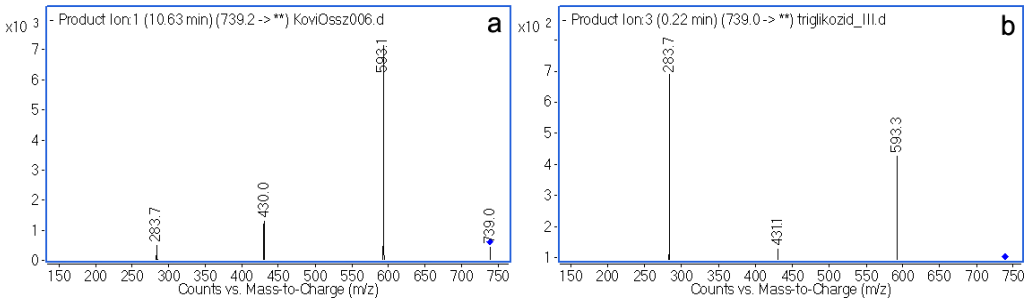


Fig 5.18.: CID spectra of peak 1. ($[M-H]^-$ *m/z* 739) of the *Sempervivum* sample. Diagnostic fragment ions $(Y_1)^-$ and $(Y_1\text{-}H)^-$ are visible at *m/z* 431 (b) and 430 (a), respectively.

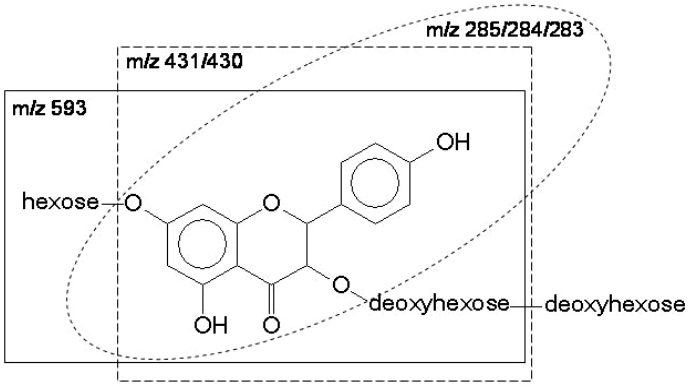


Fig 5.19.: Example for a possible glycosilation profile (kaempferol-7-*O*-hexosyl-3-*O*-dideoxyhexose) and tentative structures of fragments of compound 1. ($[M-H]^-$ *m/z* 739).

Product ions at *m/z* 430, 284 and 283 suggested two homolytic cleavages at different bonds resulting in a radical fragment ion $[M\text{-}H\text{-deoxyhexose-hexose-}H]^{-\bullet}$ and a radical aglycone anion $(Y_0\text{-}H)^-$, respectively. The -146 amu loss between *m/z* 739 and *m/z* 593 corresponds to a deoxyhexose moiety and the loss of -162/163 amu between *m/z* 593 and *m/z* 431/430 suggests a hexose unit, and makes clear that the hexose is directly linked to the aglycon [57]. Possible structures based upon the MS/MS findings for the compound are kaempferol-7-*O*-dideoxyhexosyl-3-*O*-hexose, kaempferol-7-*O*-hexosyl-3-*O*-dideoxyhexose, kaempferol-7-*O*-hexosyl-

deoxyhexosyl-3-*O*-deoxyhexoside, or kaempferol-7-*O*-deoxyhexoside-3-*O*-hexosyl-deoxyhexose (Fig 5.19.). A more accurate determination of the sugar distribution is beyond the capabilities of the mass spectrometer. Presence of this kaempferol-triglycoside in houseleek might be of importance, since flavonoid-triglycosides occur infrequently in the Crassulaceae family. Peak 2 (t_R: 14.09 min) gave a $[M-H]^-$ pseudomolecular ion at *m/z* 593; producing main fragments at *m/z* 430 and 285/284/283, which corresponded to the homolytic cleavage of a hexose and a deoxyhexose moiety, respectively.

Table 5.8.: MS data of characterized flavonoid glycosides in *Sempervivum tectorum* L.

Peak No.	Compound	TIC t_R (min)	$[M-H]^-$ (*m/z*)	CID product ions (*m/z*)
1	K-triglycoside (2 deoxyhexose + 1 hexose)	10.54	739	593, 431/430/429, 285/4/3
2	K-diglycoside (1 deoxyhexose + 1 hexose)	14.09	593	430, 285/4/3, 255
3	Q-rutinoside (rutin)	14.61	609	463, 301/0, 271, 255, 151
4	K-tetraglycoside (2 deoxyhexose + 1 pentose + 1 hexose)	15.05	871	725, 430, 285/4/3
5a	K-triglycoside (2 deoxyhexose + 1 pentose)	15.8	709	430, 285/4/3
5b	K-diglycoside (2 deoxyhexose)	15.8	577	430, 285/3, 255
6	K-hexoside	16.53	447	285/4, 255, 227, 151, 135

K: kaempferol, Q: quercetin

Radical product ion $(Y_0\text{-}2H)^{\cdot-}$ at *m/z* 283 proves that this kaempferol glycoside is a di-*O*-glycoside, not a *O*-diglycoside. Peak 3 (t_R: 14.61 min) was identified as rutin after comparison with the rutin reference standard. Peak 4 (t_R: 15.05 min) was assumed to be a kaempferol-tetraglycoside by its $[M-H]^-$ ion at *m/z* 871 and fragment ions at *m/z* 725, 431/430, 285/284/283. Two losses of -146 amu indicate deoxyhexose units. The lack of the referring radical product ion around *m/z* 725 makes it evident, that on of the deoxyhexose sugars is linked to another sugar as a sugarchain ending. The loss of -294 amu between *m/z* 725 and 431/430 was supposed to be a pentose and a hexose moiety (132+162 = 294). Peak 5 (t_R: 15.8 min) was proved to be a co-elution of compounds characterized by *m/z* data 709 and 577. With the same approach of elucidation as of peak 4, the lacking radical product

around *m/z* 563 indicates that the deoxyhexose is attached to another sugar, and not to the aglycon. The pentose and one deoxyhexose is bonded together to the aglycon at a different position as the other deoxyhexose. The other analyte under peak 5 was (*m/z* 577) was determined as kaempferol-di-*O*-deoxyhexoside [40, 57]. It should be noted, however, that the lack of ion at *m/z* 284 is surprising, but is not in contrast with the tentative identity (Table 5.8.). Peak 6 (t_R: 16.53 min) at *m/z* 447 ($[M-H]^-$) was identified as kaempferol-hexoside, with glycosilation presumably in position 3 [40].

Summary

Glycosilation status of flavonoids might be of importance in view of bioavailability and absorption kinetics. A simple LC method with linear gradient was developed for separation of flavonoids in *Sempervivum tectorum* L. and LC-ESI-MS/MS was used for the characterization of flavonol *O*-glycosides. Glycosilation status of six kaempferol glycosides of different sugar composition and rutin were tentatively characterized. Distinguishing tri- and tetra glycosides of structuraly strongly related isobaric aglycons (luteolin and kaempferol, $C_{15}H_{10}O_6$, MW = 286 g/mol) proved to be a difficult issue, since characteristic aglycon product ions were hardly formed during the CID of glycosides of such high molar weight (700-800 Da). These highly polar flavonoid glycosides may play a significant role in the traditionally experienced effect of *Sempervivum* leaves.

5.5. *Epilobium parviflorum* Schreb.

The HPLC separation for the analysis of the *Epilobium parviflorum* was developed primarily for revealing the whole phenolic profile of the herb. Numerous polar compounds eluted with very low retention, which were presumed to be macrocyclic ellag- and gallotannins. Oenothein B representing a huge number of free hyroxyl groups and a high molar mass was likely to be found in this region of the chromatogram. Oenothein B is responsible for the 5-α-reductase inhibiting effect of *Epilobium* which establishes its therapeutical effect in BPH. Ducrey and co-workers

investigated already oenothein B by FAB-MS, but their only aim was to determine the molar mass [141].

An abundant compound was detected in our sample at *m/z* 783 ($[M-2H]^{2-}$) throughout the retention range of 3-7 min among several other ions with also high *m/z* values. This finding is in accordance with the molar mass of oenothein B (MW: 1568 g/mol). The single charged pseudomolecule ion $[M-H]^-$ was detected as well, but at an abundance of 1.5 magnitude lower at *m/z* 1567. Considering the molar weight of oenothein B and the presence of the $[M-2H]^{2-}$ ion, the general rule of „one extra charge over every 1000 Da" is justified [38]. CID experiments of the $[M-2H]^{2-}$ at 20, 30 and 40 eV provided product ion spectra rich in fragments (Fig 5.20.).

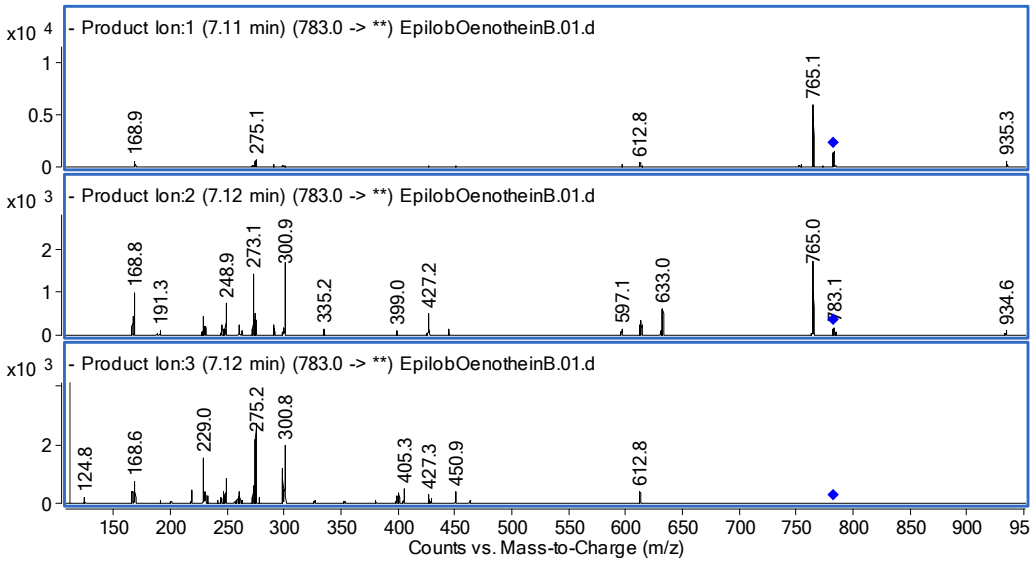


Fig 5.20.: CID spectra of the $[M-2H]^{2-}$ ion of oenothein B detected at *m/z* 783. Collision energies: 20, 30, 40 eV, from top to down, respectively.

Fragmentation of the $[M-H]^-$ ion required greater collision energies (50-60 eV) and resulted less fragment ions (Fig 5.21.). Referring to the retention property and the pseudomolecular ions this compound was identified as oenothein B.

The product ion spectra of the $[M-2H]^{2-}$ exhibited 5 characteristic clusters of fragment ions differing with approx. 150-180 Da from each other, whilst nothing similar was observable in the spectra of the $[M-H]^-$ ion (Fig 5.21.).

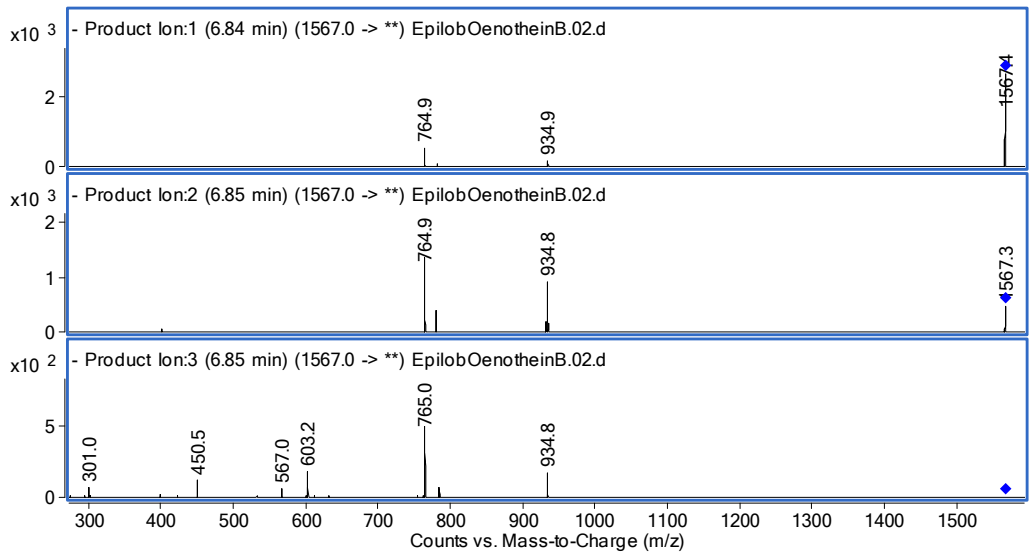


Fig 5.21.: CID spectra of the $[M-H]^-$ ion of oenothein B detected at *m/z* 1567. Collision energies: 50, 55, 60 eV, from top to down, respectively.

The 150-180 Da differences referr to the galloyl monomers of which the molecule is built up (8 gallic acid units and two hexose sugar units). The gallic acid unit fragment was present with notable abundance at *m/z* 169 at all applied collision energies, but surprisingly it was not detected in the MS/MS spectra of the $[M-H]^-$ ion. After loss of CO_2 (-44 amu) a product ion is formed from the gallic moiety, also detectable with low intensity at *m/z* 125 [45]. Fragment *m/z* 169 corresponds to the two galloyl units attached to the sugars exclusively by their carboxyl groups with all three hydroxyls free, marked with letter „G" on Fig 5.22. The intense fragment ion at *m/z* 301, present in both CID, may correspond to two gallic acids attached to each other by C-C bonds released by the cleavage of three ester C-O bonds. At collision energies of 30-40 eV this product ion further looses CO (-28 amu) resulting another product at *m/z* 273. Ion at *m/z* 229 may be due to a simultaneous further loss of oxygen (-16 amu) and CO (-28 amu) from the product of *m/z* 273 (Fig 5.20.) [33]. The next fragment cluster was grouped around the main fragment of *m/z* 427, emerging at all collision enrgies but only characteristic for the CID spectra of the $[M-2H]^{2-}$.

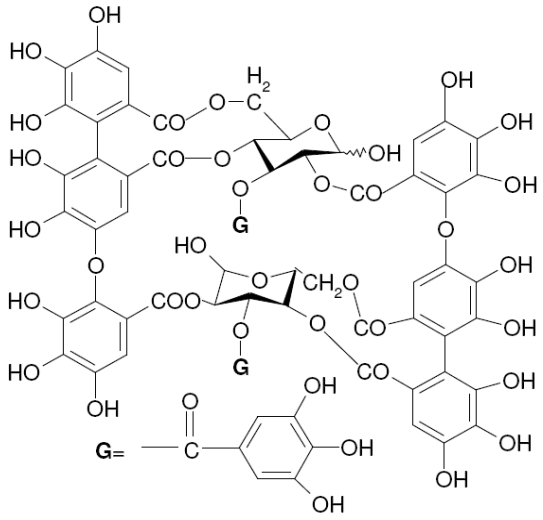


Fig 5.22.: Structure of oenothein B

We presume the ion at *m/z* 427 is built up of three galloyl moiety after the cleavage of two ester C-O and one C-C bonds (Fig 5.23.). Similarly to the previously mentioned, fragment ion *m/z* 427 also looses CO (-28 amu) resulting ion *m/z* 399. If the clevage occurs at the adjacent bond of the ester group keeping both ester oxygen at the trigalloyl structure, a fragment of *m/z* 443 might be formed, which bears a plus oxygen as compared to *m/z* 427. The low abundant fragment at *m/z* 613 is likely to be generated from the fragment *m/z* 765 after the clevage of the intact galloyl unit (-152 amu) from the sugar. Ion *m/z* 597 could be interpreted as the ion *m/z* 613 but less with an oxygen atom, perhaps due to the cleavage occuring at the nearby bond as that of 613. The main fragment (*m/z* 765) at low collision energy (20, 30 eV) is generated by the loss of water (-18 amu) from the $[M\text{-}2H]^{2-}$ molecule ion. Ion *m/z* 765 is the absolute dominant fragment in the CID spectra of the $[M\text{-}H]^-$ ion as well (Fig 5.21.), which is practically the molecule cleaved into half. Fragment at *m/z* 935 in the MS/MS spectra of the $[M\text{-}H]^-$ ion is by 170 Da greater than *m/z* 765 which can only be explained by a gallic acid unit, as we consider. Formation of the product ion at *m/z* 603 is by 162 Da less than the half molecule detected at *m/z* 765, which may correspond to a hexosyl loss. The digalloyl fragment (*m/z* 301) was the smallest detectable structure unit among the products of the $[M\text{-}H]^-$ ion. Our suggestions for tentative structures of the main fragments of oenothein B can be seen in black in Fig 5.23.

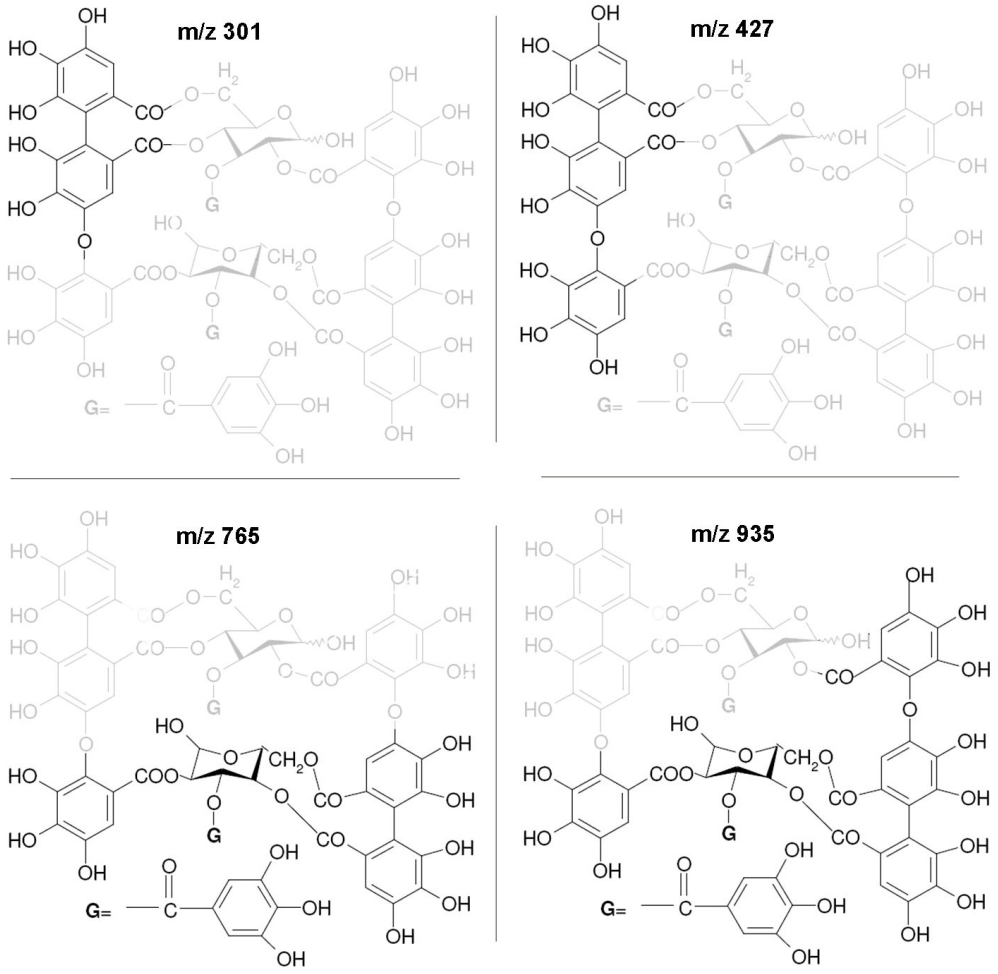


Fig 5.23.: Tentative **s**tructures and *m/z* values of the abundant CID fragments (marked with black) of oenothein B. For details please see text.

Summary

Our tandem mass spectral analysis supplied valuable and detailed structural information about oenothein B which may support the quality control of *Epilbium parviflorum* herbal products or the fast differentiation of oenothein B from other similar macrocyclic polyphenols of the herb. Further NMR investigation is inevitable to confirm our tentative structure. Detection of the $[M-H]^-$ pseudomolecular ion (*m/z* 1567) was almost over the scan range (0-1600 *m/z*) of the quadrupole analyzer in use. Spectra interpretation would have been much more difficult if the $[M-H]^-$ had fallen out of the scan range.

6. Conclusion

We achieved succesfuly the comprehensive qualitative and quantitative analysis of five, yet less characterized medicinal plants. Coupled mass spectrometry served indeed a powerful analytical tool from both qualitative and quantitative point of view for all types of phenolics investigated. All aims were accomplished by the use of LC-MS instrumentation and data processing software (eg.: BPC and EIC of qualitative analysis) with great selectivity and sensitivity at high throughput.

Phytochemistry

A total of seventeen simple phenolic acids and flavonoid glycosides were identified and/or structurally characterized in *Euphrasia rostkoviana* Hayne by LC-MS/MS. Among these one phenolic acid, one phenylpropane glycoside, one flavonoid aglycone and six flavonoid glycosides were reported in Eyebright for the first time. We isolated -at high purity (>97%)- characterized and identified acteoside, a widespread phenylpropane, by UV, MS and NMR spectroscopy for the first time in the species.

A validated, reliable and fast SRM method was developed for the quantitation of acteoside in *Euphrasiae herba*. Our method reached determination at ppb level and yielded an acteoside content of 2.56 ± 0.19 g (mean ± SD) / 100 g dry plant material. We were the first to quantify the acteoside content of *Euphrasia rostkoviana*.

According to the *in vitro* antioxidant bio-assay results the fractions rich in phenylpropanes and rutin displayed the highest free-radical scavenging activity in a concentration dependent manner. It was concluded that acteoside, the main and simultaneously the most potent antioxidant constituent of Eyebright, is to be considered the active substance of the herb.

By studying the ethanolic *Satureja* extracts, it was proved that the MS based determination of rosmarinic acid is amenable neither in SIM, nor in SRM mode, which is due to a supposed non quantitative in-source dimer formation of the analyte. UV detection was thus concluded as the method of choice for the quantification of rosmarinic acid according to the available LC-MS system operated at the mentined settings. The extract of 70% ethanol exhibited the highest rosmarinic acid content (6.69 ± 0.05 (mean ± SD) extract%), which was decreasing if the % volume of ethanol was either lowered or increased from 70%.

Five flavonoid glycosides, three phenylpropane derivatives and three salicylates among the investigated ten flavonoids, four phenylpropanes and six saliclyates were reported in *Filipendula ulmaria* for the first time. This work is the first to determine the content of six salicylates in the herb and flower samples using salicin and salicylic acid standards in SIM mode. Beside salicin and salicylic acid four salicylates lacking appropriate standards were also quantified, therefore their results are to be considered as approxiamtion or semi-quantitative. All contents were calculated and compared based on the calibrations of salicin and salicylic acid, and the methods were validated. Our work provided results for understanding the undeservingly less known phytochemistry of herbal salicylates in view of their therapeutical significancy.

We were the first to accomplish the tentative characterization of the glycosilation profile of seven *Sempervivum* flavonoid di-, tri- and tetra glycosides based upon the relative intensities of fragments $(Y_0\text{-}2H)^{\cdot-}$, $(Y_0\text{-}H)^{\cdot-}$ and $(Y_0)^-$ formed via different CID mechanisms. Information on the type and distribution of sugar units, the glycan sequence and the glycosilation position is of importance considering bioavailability.

Interpretation of the mass spectral fragmentation of the macrocyclic polyphenol, oenothein B, the active substance of *Epilobium parviflorumban*, was reported the first time. The structural elments were matched with the referring main fragments based upon the complementary investigation of the $[M\text{-}H]^-$ and $[M\text{-}2H]^{2-}$ pseudomolecular ions.

Mass spectrometry

The rapidly reachable information density of the mass spectral results should be highlighted first among the numerous advantage, which makes coupled tandem mass spectrometry such a widely applicable exceptionally powerful analytical tool. Even a simple scan mass spectrum supplied with a wealth of information (eg.: acteoside, molar weight, isotopes, N content, C atom number). CID fragmentation provided valuable structural information, SIM and SRM modes ensured highly selective and sensitive determination. Mass spectrometry vs. UV detection served as an uncomparably efficient tool for interpreting co-elutions.

According to our particular studies the very few phytochemical limitations of the technique include the problem of isomeria (eg.: orto, meta or para coumaric acid in *Euphrasia* samples, stereochemistry of sugar moieties), the problematic distinction of structuraly similar isobar flavonoid glycosides of high molar weight (aglycon: kaempferol vs. luteolin, $C_{15}H_{10}O_6$ MW = 286 g/mol) and adduct formation (acetate and formate adducts) which rended difficulties in the analysis of the *Filipendula* samples.

In spite of the experienced limitations liquid chromatography coupled mass spectrometry -in general- and the QQQ analyzer have an immense phytoanalytical potential in the hands of an analyst of high expertise.

Összefoglalás

A gyógynövénykivonatok és növényi gyógyszerek mindig több, nem ritkán többszáz komponensű, kémiailag gyakran nem pontosan definiált szerkezetű ható- és ballasztanyagok keverékei. Mindez a növényi gyógyszer minőségi vizsgálatában és analitikai értékelésében komoly kihívást jelent. A nagyhatékonyságú folyadékkromatográfiával kapcsolt tandem tömegspektrometria (LC-MS/MS) érzékenységének, hatékonyságának és robosztusságának köszönhetően választ jelenthet a kihívásra, hiszen széleskörűen alkalmas komplex folyékony minták vizsgálatára.

Jelen munkánkat kettős célkitűzés vezette. Egyrészt hozzá kívántunk járulni néhány fenoloid tartalmú tradícionális gyógynövény újraértékeléséhez tartalmi/hatóanyagaik feltárásával és mennyiségi vizsgálatával, másrészt további bizonyítékokat, konklúziókat kívántunk szolgáltatni az LC-MS módszerek fitokémiai és fitoanalitikai lehetőségeinek megismeréséhez.

Az *Euphrasia rostkoviana* Hayne, *Filipendula ulmaria* L. MAXIM, *Sempervivum tectorum* L., *Satureja hortensis* L. és az *Epilobium parviflorum* Schreb. metanolos és vizes-metanolos extraktumainak vizsgálatára LC-DAD-MS/MS módszereket adaptáltunk és fejlesztettünk ki. A vizsgálatokat elektroporlasztásos ionforrással (ESI) ellátott hármas kvadrupól tömegspektrométerrel végeztük negatív ionizációs üzemmódban. Az *Euphrasia rostkoviana* potenciális hatóanyagának feltárása céljából bioassay (szabadgyökfogó képesség *in vitro* dekolorizációs módszer) vezetett extrakciót és frakcionálást választottunk.

Vizsgálatainkat nagy érzékenység és jó szelektívitás mellett nagy áteresztőképességgel sikerült elvégeznünk. A kapcsolt tandem tömegspektrometria univerzális kvalitatív és kvantitatív analitikai eszköznek bizonyult a fenoloidok vizsgálatára az egyszerű fenolsavaktól a bonyolultabb flavonoid glikozidokon át a makrociklusos polifenolokig. A single reaction monitoring (SRM) üzemmód jó precízitás és pontosság mellett ppb szinten tette lehetővé az *Euphrasia* vezető antioxidáns hatóanyagának (akteozid) meghatározását. A szemvidítófű kivonatokban összesen tizenhét egyszerű fenolos komponenst és flavonoid glikozidot azonosítottunk és/vagy jellemeztünk. Antioxidáns hatásvizsgálataink alapján

megállapítottuk, hogy a jelentős koncentrációban feldúsuló akteozid a szemvidítófű hatóanyagának tekinthető. Kísérletesen igazoltuk a *Satureja* kivonatok példáján, hogy, egy vélhetően az ionorrásban végbemenő, nem-kvantitatív dimérképződés miatt az antioxidáns rozmaringsav kvantálása tömegspektrometriás detektálással sem selected ion monitoring (SIM), sem SRM módban nem végezhető el. Így meghatározását UV detektálással végeztük. Salicin és szalicilsav standardek segítségével hat szalicilát mennyiségét határoztuk meg SIM üzemmódban, valamint számos flavonoidot azonosítottunk a *Filipendula* mintákban. A fragmens ionok és a gyökös fragmens ionok relatív intenzitásarányai alapján sikerrel térképeztük fel számos *Sempervivum* flavonoid glikozilációs profilját, a cukrok kapcsolódási helyét és sorrendjét. Az egyszeres $[M-H]^-$ és kétszeres $[M-2H]^{2-}$ töltésű molekulaionok szimultán detektálása és fragmentációja alapján elsőként írtuk le az oenothein B makrociklusos fenoloid tömegspektrometriás fragmentációját. A változatos szerkezetű fenoloidok LC-MS/MS adattárának bővítése mellett rámutattunk néhány, a módszer előnyeihez képest nem jelentős hátrányra is (pl: izoméria).

Summary

Medicinal plant extracts and herbal preparations are complex mixtures of active- and ballast substances which may contain numerous, not infrequently up to several hundreds of different constituents with not exactly defined structures. Quality, safety and efficacy of these herbs is thus a great issue and their analysis is challenging. Tandem mass spectrometry coupled to high performance liquid chromatography (LC-MS/MS), as a sensitive, powerful and robust technique, capable of analysing very diverse complex liquid samples, may offer a solution.

The aim of our work was to revaluate traditionally well known, but less characterized herbs of various phenoloid content by the qualitative and quantitative analysis of their active substances, and to study the versatile capabilities of mass spectrometry in phytochemistry, and draw general conclusions regarding phytoanalytical mass spectral applications.

For the analyses of the methanolic and/or aqueous methanolic extracts of *Euphrasia rostkoviana* Hayne, *Satureja hortensis* L., *Filipendula ulmaria* L. MAXIM, *Sempervivum tectorum* L., and *Epilobium parviflorum* Schreb. LC-DAD-MS/MS methods were adopted and developed. Samples were analysed with a triple quadrupole analyzer with electrospray ionsource (ESI) in negative ion mode. For revealing the potential active components of *Euphrasia rostkoviana* a bioassay (*in vitro* antioxidant decolorization assay) guided extraction and fractionation was accomplished.

All measurements were accomplished with great selectivity and sensitivity at high throughput. LC coupled mass spectrometry served indeed as a universal analytical tool for the qualitative and quantitative analysis of phenolics from simple phenolic acids and salicylates towards more complex structures, like flavonoid glycosides and macrocyclic phenolics.

The single reaction monitoring (SRM) mode enabled the quantitation of the leading active substance of the *Euphrasia* sample, acteoside, at ppb level by great precision and accuracy. A total of seventeen different phenolic acids and flavonoid glycosides were identified or characterized in the extract and in its fractions. Quantification of the antioxidant rosmarinic acid in *Satureja hortensis* was not amenable nor in

selected ion monitoring (SIM) nor in SRM mode due to non-quantitative dimer formation, thus UV based quantitation was performed. Contents of six salicylates were determined with salicin and salicylic acid standards in SIM mode in the *Filipendula ulmaria* sample, and several flavonoids were characterized. A comprehensive LC-MS characterization of the glycosilation profile of the *Sempervivum* flavonoids was accomplished based upon rel. intensities of the fragment ions and radical fragments. The simultaneous formation of the single $[M-H]^-$ and double $[M-2H]^{2-}$ charged molecule ion of oenothein B helped the interpretation of its fragmentation in the *Epilobium* sample. However some limitations regarding constitutional isomeria and stereochemistry (sugar moieties) of mass spectrometry in phytochemistry were pointed out which, still, are of almost no significance if compared to the possibilities of the technique.

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List of publications

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B. Blazics, Á. Kéry.
Antioxidant activity of compounds in *Euphrasia officinalis* L. - revaluation of a traditional medicinal plant
Planta Medica (2007) 73: P_266 DOI: 10.1055/s-2007-987047

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Investigation of *Euphrasia rostkoviana* Hayne using GC-MS and LC-MS
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I. Papp, B. Simándi, **B. Blazics**, Á. Alberti, É. Héthelyi, É. Szőke, Á. Kéry.
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XIV. Congressus Pharmaceuticus Hungaricus, Budapest 2009. 11. 13-15.

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