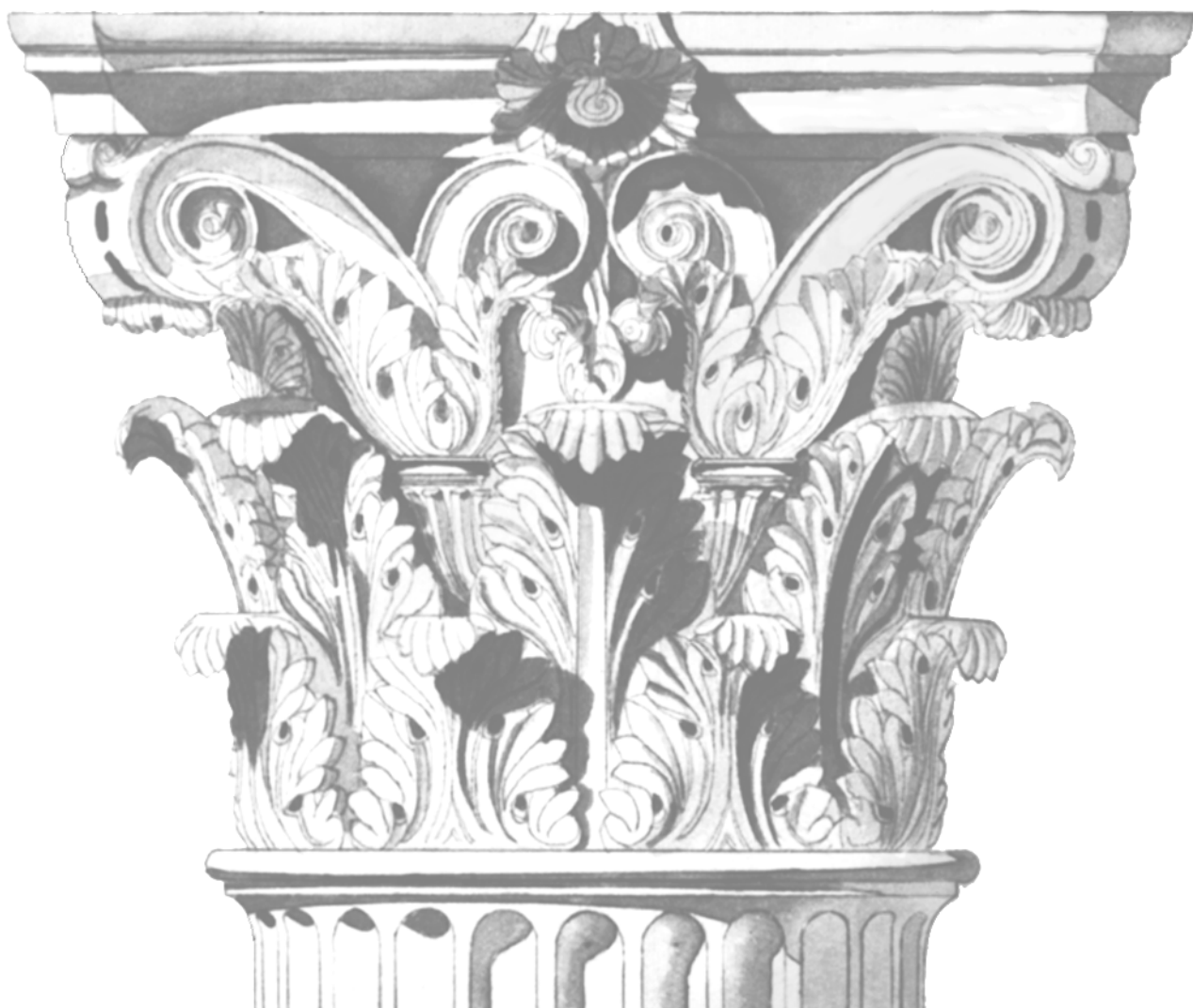


**ANALYSIS OF SECONDARY
METABOLITES IN PLANT AND
CELL CULTURE TISSUE OF
HYPERICUM PERFORATUM L
AND *RHODIOLA ROSEA* L.**

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TOLONEN**

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University of Oulu

OULU 2003



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Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Raahensali (Auditorium L10), Linnanmaa, on November 22nd, 2003, at 12 noon.

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Tolonen, Ari, Analysis of secondary metabolites in plant and cell culture tissue of *Hypericum perforatum* L and *Rhodiola rosea* L.

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2003

Abstract

Sensitive chromatographic methods were developed for the quantitative analysis of secondary metabolites in *Hypericum perforatum* (St. John's wort) and *Rhodiola rosea* (Golden root, rose root) extracts. Sample preparation methods were developed for plant, cell culture and biotransformation suspension matrixes. High performance liquid chromatography (HPLC) was used for the separation of analytes, and chromatographic data was acquired using photodiode array (PDA) detection or atmospheric pressure ionization mass spectrometry (API-MS). Ionization efficiencies with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were compared under different conditions. Specific mass spectrometric detection methods such as multiple reaction monitoring (MRM) and selective ion monitoring (SIM) were utilized. For identification of known and new secondary metabolites in plant tissues, mass spectrometric methods with triple quadrupole and time-of-flight mass spectrometers were used together with one- and two-dimensional nuclear magnetic resonance spectroscopy (NMR).

Keywords: LC/MS, liquid chromatography, mass spectrometry, NMR spectroscopy

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Oulu, September 2003 Ari Tolonen

Abbreviations

ADP	adenosine diphosphate
API	atmospheric pressure ionization
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
ATP	adenosine triphosphate
B ₀	static magnetic field
B ₁	radio-frequency field
BBI	broadband tunable x-channel equipped probe head optimized for inverse detection (i.e. proton detection)
CoA	co-enzyme A
CI	chemical ionization
COSY	correlation spectroscopy
DEPT	distortionless enhancement through polarization transfer
DMAPP	dimethylallyl diphosphate
DQF	double quantum filtered
ESI	electrospray ionization
EI	electron impact (ionization)
FAB	fast atom bombardment (ionization)
GDP	guanosine diphosphate
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation
HMQC	heteronuclear multiple quantum coherence
HMBC	heteronuclear multiple bond correlation
IPP	isopentenyl pyrophosphate
LC/MS	liquid chromatography/mass spectrometry
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
MRM	multiple reaction monitoring
m/z	mass to charge-ratio
NMR	nuclear magnetic resonance spectroscopy
PDA	photo-diode array detector

PFG	pulsed field gradient
RF	radio-frequency
SIM	selective (/single) ion monitoring
S/N	signal to noise
TFA	trifluoro acetic acid
TOCSY	total correlation spectroscopy
TOF	time of flight
TPPI	time proportional phase increment
UDP	uridine diphosphate
UV	ultraviolet
XIC	extracted ion chromatogram
μ	nuclear magnetic moment (in NMR context)
γ	gyromagnetic ratio of nucleus (in NMR context)
ν	frequency of RF-field (in NMR context)
I	nuclear spin (in NMR context)
m	magnetic quantum number (in NMR context)

List of original papers

The present thesis consists of the following original publications that are referred to in the text by Roman numbers:

- I Ari Tolonen, Jouko Uusitalo, Anja Hohtola and Jorma Jalonen, Determination of naphthodianthrones and phloroglucinols from *Hypericum perforatum* extracts by LC/MS/MS, *Rapid Communications in Mass Spectrometry* 16 (2002) 396-402.
- II Ari Tolonen, Anja Hohtola and Jorma Jalonen, Fast HPLC/PDA analysis of naphthodianthrones and phloroglucinols from *Hypericum perforatum* extracts, *Phytochemical Analysis*, 14 (2003) 306-309.
- III Ari Tolonen, Minna Pakonen, Anja Hohtola and Jorma Jalonen, Phenylpropanoid glycosides from *Rhodiola rosea*, *Chemical & Pharmaceutical Bulletin*, 51 (2003) 467-470.
- IV Ari Tolonen, Anja Hohtola and Jorma Jalonen, Liquid chromatographic analysis of phenylpropanoids from *Rhodiola rosea* extracts, *Chromatographia*, 57 (2003) 577-580.
- V Ari Tolonen, Anja Hohtola and Jorma Jalonen, Comparison of electrospray ionization and atmospheric pressure chemical ionization techniques in analysis of main constituents from *Rhodiola rosea* extracts by HPLC/MS. *Journal of Mass Spectrometry*, 38 (2003) 845-853.

Some unpublished results are also briefly presented in this thesis.

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1 Introduction

A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. In addition, in the form of natural products or as functional foods, medicinal plants and their extracts offer an alternative to specifically targeted drugs in the treatment and prevention of many diseases. Both *Hypericum perforatum* (St. John's wort) and *Rhodiola rosea* (Golden root, rose root) are well known plants, which have been used medicinally for a decades, but study of their pharmacological effects and the compounds responsible for these still continues. At the moment, the interest in these plants is focused on the naphthodianthrones and phloroglucinols of *H. perforatum* and the glycosidic phenylpropanoids of *R. rosea*. When attempts are made to generate populations producing maximum concentrations of these bioactive compounds, either *in vivo* or *in vitro*, or when novel structurally related compounds are sought in the plants or their cell cultures, the methods of analytical chemistry become of critical importance.

This thesis describes analytical methods for quantitation of the main constituents in the plant or callus culture tissue of *H. perforatum* and *R. rosea*. The methods utilize high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection (sections 4.2.1 and 4.4.1) and with more specific mass spectrometric (MS) detection (sections 4.2.2 / 4.4.2 / 4.4.3) in selective ion monitoring (SIM) or multiple reaction monitoring (MRM) modes.

In addition to the development of analytical methods, search was made for novel possibly bioactive compounds in the plant extracts with use of modern methods of structural elucidation. Alongside the known main metabolites in *R. rosea* plant material, some new compounds were isolated and identified by mass spectrometry and one- and two dimensional nuclear magnetic resonance spectroscopy (NMR) (section 4.3).

2 Secondary metabolism in plants

2.1 Metabolism and its significance

Metabolism in living matter may be described as a large group of enzyme-controlled and regulated chemical reactions that produce energy in the form of ATP, produce substances needed for growth and the development of tissues, and help the organism to survive in different circumstances. Depending on the biosynthetic origin, general occurrence and biochemical role of the compounds produced in metabolism, they are called either primary or secondary metabolites.

Primary metabolic routes produce primary metabolites, which are present almost everywhere in nature and are essential for all life forms. These compounds include the common carbohydrates, fats, proteins and nucleic acids that are needed to create and maintain life. Apart from fats, the compounds are polymeric and usually chemically large molecules. Typically they are involved in the energy regulation of organisms and with growth and development of tissues; in short, they are the building blocks of organisms.

The secondary metabolites are of more limited occurrence. While some do not have a proven function in the organism, others apparently play a vital role. Some secondary metabolites of plants are found only in certain species of families, showing the individuality of species. Their production may also vary with conditions: the environment of the organism or the available nutrition. They may have a role in providing defense against pest and pathogens, providing protection against UV radiation and stress, or acting as attractive volatile odor compounds or pigments [1-3]. Reactions involving secondary metabolites produce an enormous selection of compounds, some of them substances that may be pharmacologically active in humans and useful as medicines or food additives. Some famous examples (Fig 1) of secondary plant metabolites with pharmacological activities are the analgesic and antipyretic compound salicin (**1**) isolated from willow (*Salix* species) and used as a template for the synthesis of acetylsalicylic acid (aspirin), the anticancer drug taxol (paclitaxel, **2**) isolated from Pacific yew (*Taxus brevifolia*) and the strongly analgesic, narcotic, and addictive compound morphine (**3**) isolated from opium (*Papaver somniferum*).

Cultivation of plant tissue in synthetic media offers an alternative as a way of producing metabolites of interest to the traditional cultivation in fields or greenhouses [3-6]. Traditionally, most of this work has been concerned with undifferentiated cells, but also differentiated cells, such as in hairy root tissue, have been cultivated. In successful cases, cell suspension cultures can offer a repeatable method to produce secondary metabolites from elite mother plants with easily controlled conditions and with a continuous supply of material. An increasing number of compounds are being isolated from cell cultures, including at the moment compounds from at least ten groups of phenylpropanoids, ten groups of alkaloids, five groups of terpenoids, and three groups of quinones [5-6]. Some of these compounds are formed in cell cultures through biotransformation reactions, in which the functional groups of secondary metabolites are transformed into some other functionality via esterification, oxidation, reduction, hydroxylation, or glycosylation. *In vivo*, all these reactions are regio- and stereoselective, which is of great advantage [5-6].

To date, only a few plant metabolites have been produced via cell culture production in industrial scale. There are many problems, including insufficient knowledge of biosynthetic routes and enzymology, problems with bioreactor operation, such as instability of the cell lines and scaling up, and lower concentrations of the target secondary metabolites in cell cultures than in naturally growing plants [3-5]. The reason for the lower concentrations is not fully understood, but it may be that the development of secondary metabolites in plants is connected with the development process of organs (roots, stem, leaves) and the concentrations in cultures of undifferentiated cells (callus culture, suspension) are therefore low [2,4]. In a few cases, however, cell cultures have been found to produce higher levels of secondary metabolites than the differentiated mother plant itself [4-6]. Examples are the production of anti-inflammatory naphthoquinone shikonin from *Lithospermum erythrorhizon* and antiseptic alkaloid berberine from *Coptis Japonica*. If the level of metabolites is to be increased, culture conditions need to be optimized and accurate information is required about even low concentrations the metabolites present. This, in turn, demands much more sensitive and accurate analytical methods than those employed in measuring the larger concentrations in naturally grown plants. The development of such methods was one of the aims of this work.

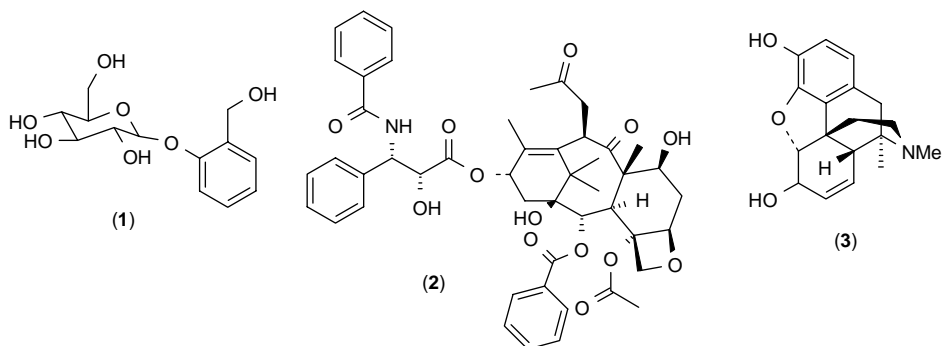


Fig. 1. Famous examples of secondary metabolites with pharmacological activity: (1) salicin, (2) taxol (paclitaxel), (3) morphine.

2.2 Major metabolic pathways

The pathways for modifying and synthesizing carbohydrates, proteins, fats and nucleic acids are the same in all organisms, apart from minor variations. Also, the biosynthesis of most secondary metabolites begins from a relatively small group of compounds, which are modified into an unlimited number of compounds through various synthesis pathways. The most important of these metabolic pathways are those with acetyl coenzyme A (acetyl-CoA), shikimic acid, or mevalonic acid intermediates, and known as the acetate, shikimate, and mevalonate pathways, respectively. These key intermediates are formed from products of the glycolytic pathway or their intermediates (Fig 2). These major metabolic pathways are briefly described below [1,2,7]. In addition to these key intermediates, a wide variety of antibiotics, peptides, proteins, and alkaloids are formed from amino acids derived from acetyl-CoA via citric acid (Krebs) cycle [1, 8], or via the shikimate pathway.

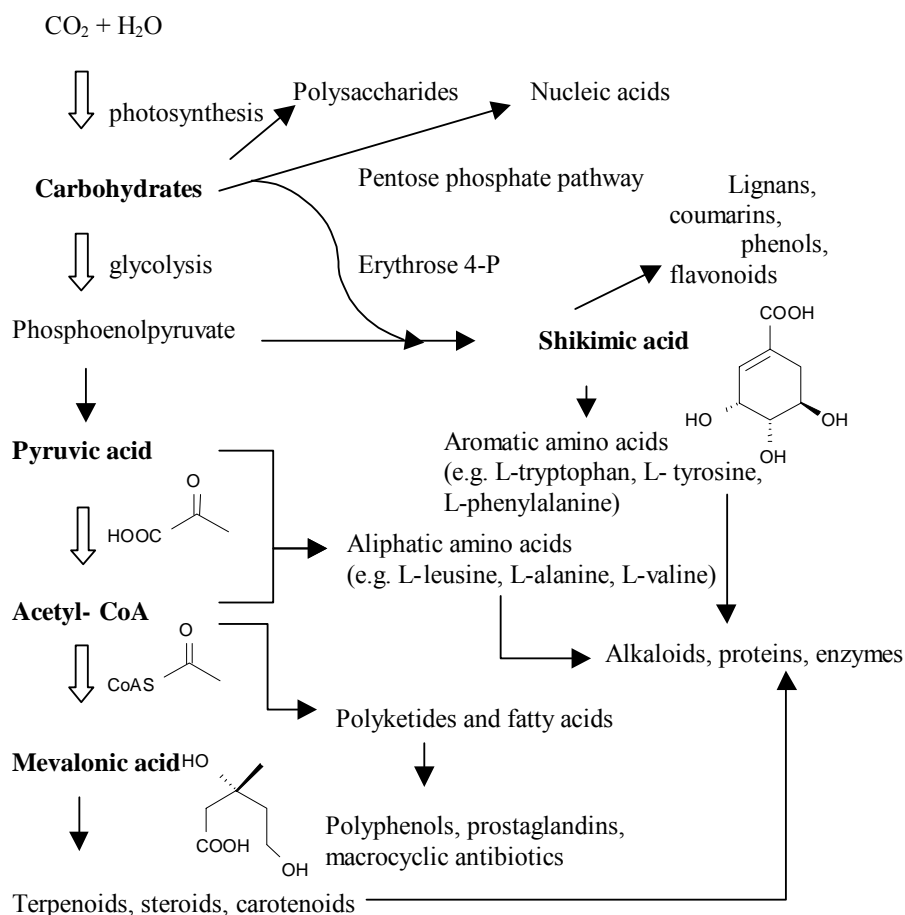


Fig. 2. Common biosynthetic pathways to secondary metabolites via acetyl-CoA, shikimic acid, mevalonic acid and amino acids.

2.2.1 Carbohydrates

Carbohydrates are products of photosynthesis, the most fundamental process in metabolism. In photosynthesis, inorganic carbon in atmospheric carbon dioxide is converted to organic carbohydrates by photosynthetic organisms and light in a very complicated process that can be summarized as in Equation 2.1. Water usually acts as an electron donor in this process, though some photosynthesizing bacteria also use hydrogen sulfide, thiosulfate, hydrogen, lactic acid, or isopropyl alcohol instead, producing other by-products than oxygen.



Carbohydrates are the most common compounds in living organisms and are used in nutrition and energy storage in the form of ATP and as structural components for cell walls. They are present in nature in the form of mono-, oligo- and polysaccharides, aminoglycosides, and an unlimited number of glycosides of molecules from different metabolic pathways.

The simplest form of carbohydrates, monosaccharides of general formula $\text{C}_n\text{H}_{2n}\text{O}_n$, are often simply called sugars, of which the most common is glucose. These small units exist in nature primarily in the form of cyclic hemiacetals. The cyclic forms are furanose or pyranose compounds, depending on whether they consist of five- or six-member rings, (Fig 3).

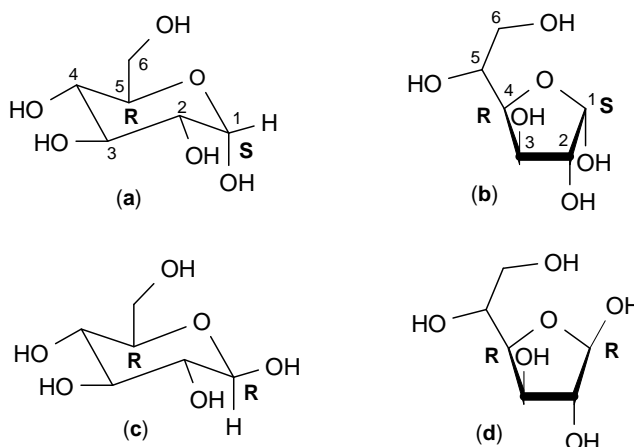


Fig. 3. (a) α -D-glucopyranose, (b) α -D-glucofuranose, (c) β -D-glucopyranose, (d) β -D-glucofuranose.

2.2.2 Acetate pathway

The acetate pathway produces a very large number of acetyl-CoA based compounds, sometimes classified as polyketides. Included as phenols such as phloroglucinols and anthraquinones, fatty acids, prostaglandins, thromboxanes, leukotrienes, and macrolide antibiotics. Polyketide structures are derived from poly- β -ketoester chains that are formed from acetyl-CoA units via malonyl-CoA in condensation reactions, as shown in Figure 4. The polymerization of polyketide chains then either continues further to produce long and highly reactive poly- β -keto chains that form a wide variety of aromatic compounds, or alternatively the carbonyl groups are reduced before reaction with the next malonyl group, and reaction leads to fatty acids and macrolide antibiotics. This

biosynthesis pathway via polyketide formation leads to the usually easily recognizable meta-substitution pattern in phenols and aromatic compounds.

2.2.3 Shikimate pathway

The shikimate pathway begins with a combination of the glycolytic pathway intermediate phosphoenolpyruvate (PEP) and the pentose phosphate pathway product erythrose-4-phosphate, which leads, after a few reaction steps, to 3-dehydroquinic acid and then through various reaction routes to shikimic acid and eventually to a number of different metabolites, including phenols, cinnamic acid derivatives, flavonoids, coumarins, lignans, and alkaloids (Fig 5). In contrast to products of the acetate pathway, the substitution pattern found in phenols of the shikimate pathway is characteristically ortho or para. The C₆C₃ phenylpropane units present in many metabolites originate from phenylalanine and tyrosine structures. Flavonoids are produced through a combination of products of the shikimate and the acetate pathways, where three malonyl-CoA units are added to a shikimate pathway intermediate such as cinnamoyl-CoA to form a polyketide (as in the acetate pathway). The polyketide is then folded to form a chalcone structure that is transformed further to flavonoid.

2.2.4 Mevalonate pathway

Mevalonic acid is formed from three molecules of acetyl Co-A, but it leads the metabolism to different compounds from those of the acetate pathway. The mevalonic acid is transformed into five-carbon-containing C₅ isoprene units, which in the form of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) combine to form a large number of terpenoid and steroid structures (Fig 6).

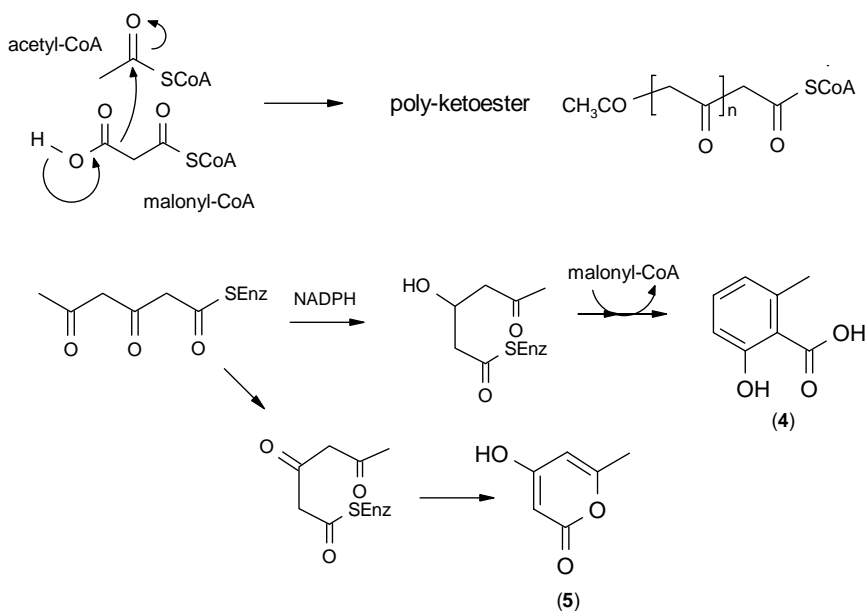


Fig. 4. Formation of polyketides from acetyl-CoA. (4) 6-methylsalicylic acid, (5) triacetic acid lactone.

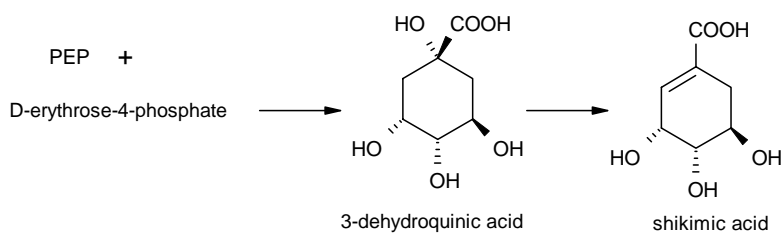


Fig. 5. Origin of the shikimate pathway.

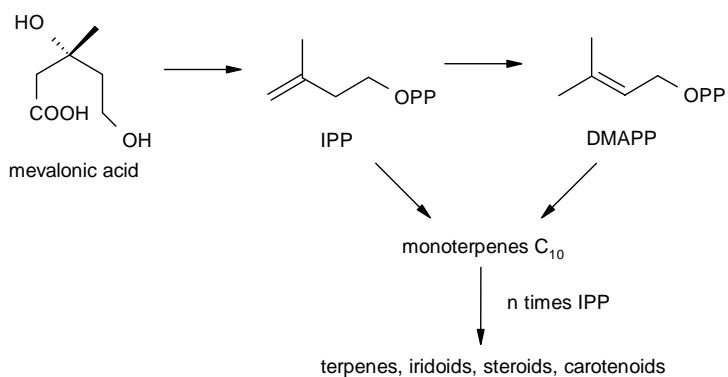


Fig. 6. Formation of terpenoids from mevalonic acid.

2.3 *Hypericum perforatum* L.

St John's Wort (*Hypericum perforatum* L.) is a plant belonging to the Hypericaceae family and is widely distributed over Europe, Northern Africa and Asia. It has long been used in traditional medicine as an anti-inflammatory and healing agent [9]. In modern medicinal applications it is mainly utilized for its anti-depressive activities [10-12], though it also has antiviral effects [13]. The origin of the anti-depressive activities of its extracts is still not entirely clear, but the photosensitizing naphthodianthrone derivatives protopseudohypericin (**6**), pseudohypericin (**7**), protohypericin (**8**) and hypericin (**9**), and the phloroglucinol hyperforin (**10**) and its derivative adhyperforin (**11**) are considered to be the main bioactive constituents of the plant [14-23]. Anti-depressive activities have also been linked to some flavonoid compounds in the extracts [24]. Besides their anti-depressive properties, the naphthodianthrone **7** and **9** have been found to be capable of destroying surface structures of viruses [25-27] and **9** has shown potential in cancer treatment [28]. Compound **10** has been shown to possess anti-inflammatory and anti-allergenic [29], antibacterial [30-31] and anti-tumor activities through inducing apoptosis [32]. Quite recently, reports of drug-herb interactions involving *H. perforatum* extracts have been published, indicating some potential hazards when the herb is co-administered with certain drugs [33-37].

In addition to the naphthodianthrone **6** – **9** and the phloroglucinols **10** – **11**, which are produced via polyketides in the acetate pathway metabolism, *H. perforatum* is known to contain a number of flavonoids produced by combining elements from the shikimate and acetate pathways. These include quercetin (**12**), luteolin (**13**), and catechin (**14**) and their glycosides quercitrin (**15**), isoquercitrin (**16**), hyperoside (**17**), rutin (**18**), luteolin 5-O- β -D-glucopyranoside (**19**), and luteolin-3'-O- β -D-glucopyranoside (**20**), the biflavone derivatives amentoflavon (**21**) and I3-II8-biabinigenin (**22**), and procyanidins formed from catechin and epicatechin, such as procyanidin A2 (**23**), B1 (**24**), B5 (**25**), and C1 (**26**) [38-40].

The physicochemical properties of compounds **6** – **11** add their own challenge to the analysis, since exposure to light converts compounds **6** and **8** to **7** and **9**, respectively, and leads to the degradation of phloroglucinols **10** and **11**. Compounds **10** and **11** are also extremely sensitive to oxidation and unstable in solution [41-44]. In view of this sensitivity, extraction and sample preparation for analysis of compounds **6** – **11** has to be performed in the absence of light.

The concentrations of the naphthodianthrone and phloroglucinols mentioned are usually quite high in *H. perforatum* plant extracts, up to 0.23% and 4.5% in dry weight of the plant, respectively [45]. In cell cultures, only much lower concentrations have been measured, however [46,47].

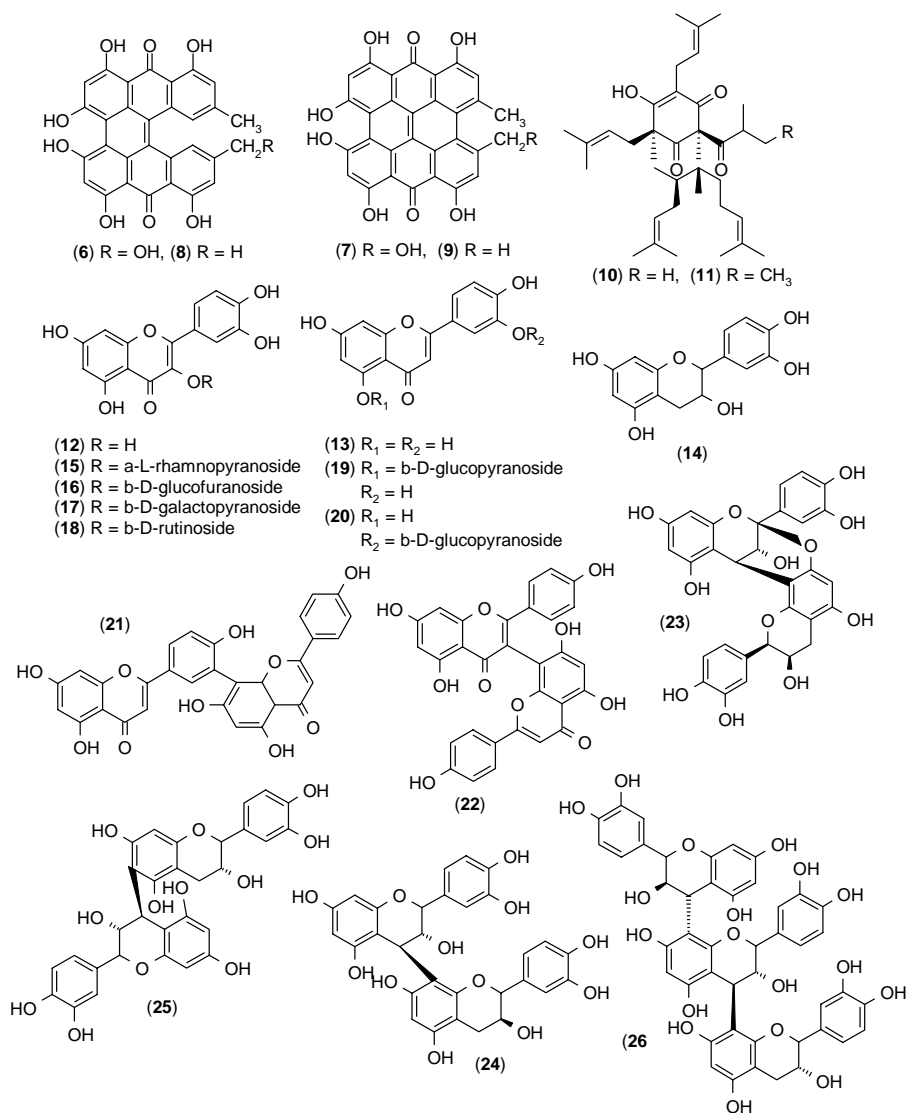


Fig. 7. Structures of some *H. perforatum* constituents. (6) Protopseudohypericin, (7) pseudohypericin, (8) protohypericin, (9) hypericin, (10) hyperforin, (11) adhyperforin, (12) quercetin, (13) luteolin, (14) catechin, (15) quercitrin (quercetin 3-O-α-L-rhamnopyranoside), (16) isoquercitrin (quercetin 3-O-β-D-glucofuranoside), (17) hyperoside (quercetin 3-O-β-D-galactopyranoside), (18) rutin (quercetin 3-O-β-D-rutinoside), (19) luteolin 5-O-β-D-glucopyranoside, (20) luteolin-3'-O-β-D-glucopyranoside (21) amentoflavon (=I3'-II8-biabigenin), (22) I3-II8-biabigenin, (23) procyanidin A2, (24) procyanidin B1, (25) procyanidin B5, (26) procyanidin C1.

2.4 *Rhodiola rosea* L.

Golden root (Roseroot, *Rhodiola rosea* L., *Crassulaceae*) has long been known as a medicinal plant and has been used in Scandinavian, Russian and Chinese traditional medicine to enhance the body's resistance against fatigue and to extend human life [48]. The plant is distributed globally in arctic regions, including Northern Asia, Alaska, and northern parts of Europe.

The plant extract has been shown to possess a number of biological activities, such as effects on prolyl endopeptidase inhibition [49], antiallergic effects [50], antimicrobial effects [51], effect on memory and learning [52], antidepressant and anti-inflammatory effects [53,54], effects in cancer therapy [55-58], and prophylactic effects for ischemic cerebral circulation disorders [59]. Its main active components are believed to be its shikimate pathway metabolites derived via C₆C₃ structure, i.e. hydroxyphenylethyl tyrosol (**27**) and its glycoside salidroside (**28**) [60], and the phenylpropanoids rosin (**29**), rosarin (**30**), and rosavin (**31**) [61], which are reported to be pharmacologically active as antioxidants and neurostimulants [62,63]. Some cinnamyl acid derivatives such as 4-hydroxy-cinnamyl-O- β -D-glucopyranoside (sachaliside 1, triandrin) (**32**) and 4-methoxy-cinnamyl-O- β -D-glucopyranoside (vimalin) (**33**) have been found in cell cultures of the plant [64]. Monoterpenes rosiridol (**34**) and rosiridin (**35**) [65] synthesized by the mevalonate pathway have been studied for their stimulant properties [66]. The plant is also known to contain the flavonoids herbacetin (**36**), gossypetin (**37**), and kaempferol (**38**) and their glycosides rhodionin (**39**), rhodionidin (**40**), rhodiogin (**41**), rhodiogidin (**42**), rhodalin (**43**), rhodalidin (**44**) rhodiosin (**45**), and kaempferol-7-O- α -L-rhamnopyranoside (**46**) [67-70]. In addition, sterols β -sitosterol (**47**) and daucosterol (**48**) [65] and a large number of small volatile compounds [71] have been detected in the plant.

The concentrations of the main metabolites in plant rhizomes and roots are reported to be as high as 3% of dry weight for rosavin and 1% for salidroside after five years of cultivation [72]. Salidroside and rosavin are produced effectively in cell suspensions when the suspensions are fed with precursor compounds, such as tyrosol, tyrosine, phenylalanine, and cinnamyl alcohol. Up to 95% of the added precursors is reported to be biotransformed into other constituents, mainly to salidroside and rosavin [72-75].

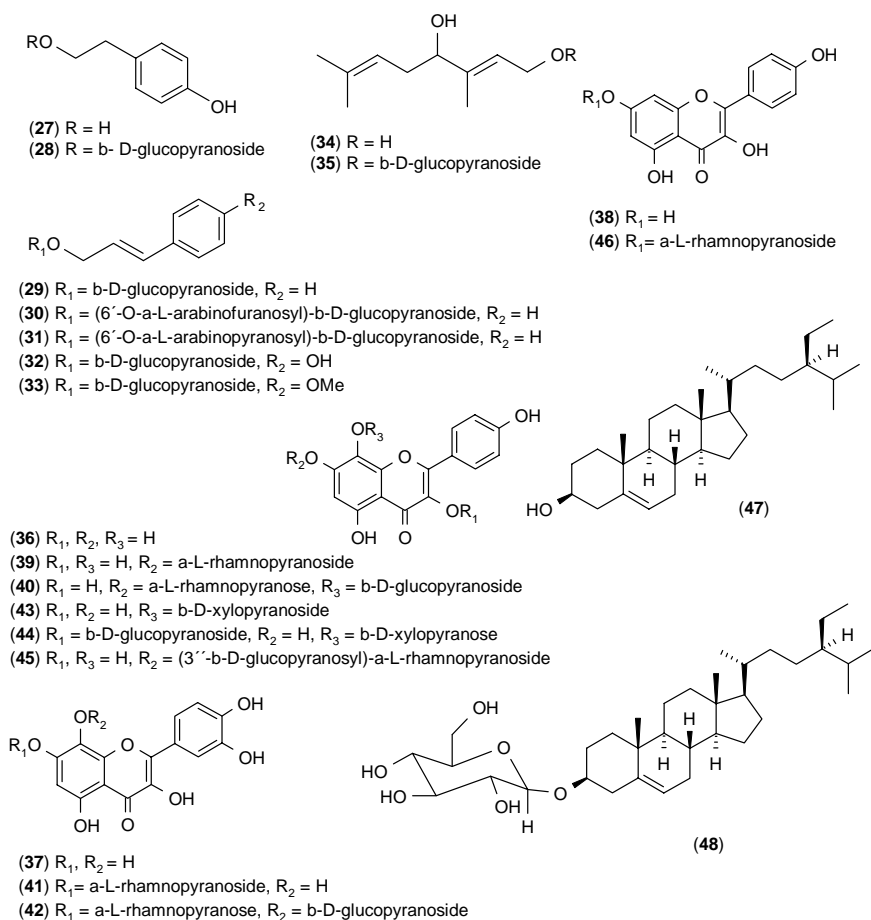


Fig. 8. Structures of some common constituents of *Rhodiola rosea* L. (27) Tyrosol, (28) salidoside (p-hydroxyphenylethyl O- β -D-glucopyranoside), (29) rosin (cinnamyl O- β -D-glucopyranoside), (30) rosarin (cinnamyl-(6'-O- α -L-arabinofuranosyl)-O- β -D-glucopyranoside), (31) rosavin (cinnamyl-(6'-O- α -L-arabinopyranosyl)-O- β -D-glucopyranoside), (32) 4-hydroxycinnamyl-O- β -D-glucopyranoside (sachaliside 1, triandrin), (33) 4-methoxy-cinnamyl-O- β -D-glucopyranoside (vimalin) (34) rosiridol, (35) rosiridin, (36) herbacetin, (37) gossypetin, (38) kaempferol, (39) rhodionin (herbacetin-7-O- α -L-rhamnopyranoside), (40) rhodionidin (herbacetin-7-O- α -L-rhamnopyranose-8-O- β -D-glucopyranoside), (41) rhodioglin (gossypetin-7-O- α -L-rhamnopyranoside), (42) rhodioglidin (gossypetin-7-O- α -L-rhamnopyranose-8-O- β -D-glucopyranoside), (43) rhodalin (herbacetin-8-O- β -D-xylopyranoside), (44) rhodalidin (herbacetin-8-O- β -D-xylopyranose-3-O- β -D-glucopyranoside), (45) rhodiosin (herbacetin-(3''-O- β -D-glucopyranosyl)-7-O- α -L-rhamnopyranoside)), (46) kaempferol-7-O- α -L-rhamnopyranoside, (47) β -sitosterol, (48) daucosterol (β -sitosterol-3-O- β -D-glucopyranoside).

3 Analytical methods

3.1 High performance liquid chromatography

In high-performance liquid chromatography, analyte molecules are injected into a column filled with particles capable of retaining them (stationary phase), and they are eluted out of the column with solvents (mobile phase) so that the least strongly retained analyte is eluted first into detector [76,77]. The speed and efficiency of the separation of analytes can be adjusted through modification of mobile phase composition, flow rate, and pH, temperature of the column, and other column properties such as dimensions and stationary phase material.

At the moment, the most widely used columns in natural product research are reversed-phase columns where the porous silica particles are coated with non-polar material and the usual solvents for elution are polar compounds such as water, methanol, acetonitrile, isopropanol, or tetrahydrofuran. Additives such as formic and acetic acids, trifluoro acetic acid (TFA), ammonium acetate, or phosphate buffers may be added to the mobile phase for pH adjustment and optimization of the separation [76]. Typically, modern analytical columns are 1.0 – 4.6 mm in diameter and 30 – 250 mm in length, while the particle size varies in the range 2 – 7 μm . For preparative columns designed specially for the isolation of compounds the column diameter may be as much as 50 mm.

The particles in reverse-phase columns are usually coated with hydrocarbon chains, four to eighteen carbons ($\text{C}_4 - \text{C}_{18}$) in length, but a large number of different coating materials have been introduced by manufacturers, including moieties containing phenol, amino, amide, nitro and cyano groups, polyethylene glycols, and various fluorinated alkyls [76,78]. The wide variety of modifiers for the hydrocarbon chains and the many different ways of binding the chains into particles have resulted in the availability of stationary phase materials offering a wealth of polarities and capabilities, including different pH ranges and tolerances against temperature and water content in the mobile phase. In addition to traditional particle-type stationary phases, monolithic columns where the particle material in the column is replaced by a single porous rod, are becoming increasingly popular [76,78,79]. This structure leads to decreased pressure in the column and enables the use of higher eluent flow rates than in traditional columns.

Detection of chromatograms in natural product analysis is most often achieved with ultraviolet/visible (UV/Vis, PDA) detectors or mass spectrometry. The aromatic or double bond chromophores of most natural compounds make them easy to detect with a UV detector. However, with the development of atmospheric pressure ionization (API) techniques such as electrospray (ESI) [80-83] and atmospheric pressure chemical ionization (APCI) [84-87], the potential of coupling HPLC with mass spectrometer (LC/MS) has increased enormously during the last decade [88,89].

A very wide range of natural products have been analyzed by HPLC and LC/MS, among them flavonoids and other polyphenols [88-93], terpenes and steroids [88,90,94,95], iridoids [96,97], alkaloids [88, 89, 98, 99], vitamins [88, 100, 101], saccharides [88, 102, 103], and amino acids [104 -106].

Several HPLC methods have been published for analysis of the naphthodiantrone, phloroglucinol, and flavonoid constituents of *Hypericum perforatum* extracts, with either UV/fluorescence [9, 41-43, 107-114] or mass spectrometric [38, 115-117] detection. Most of these methods are not, however, designed for simultaneous analysis of all the main bioactive naphthodianthrone and phloroglucinols of the plant, or alternatively they require very long runs.

A few papers describe HPLC methods for the analysis of the glycosidic phenylpropanoids in *Rhodiola rosea* extracts [72, 118-121]. All these methods rely on UV detection, and, as in the case of *H. perforatum*, they allow the simultaneous analysis of only a few of the compounds present in the plant.

3.2 Mass spectrometry

3.2.1 Mass analysis

In mass spectrometry ionized molecules and their fragment ions are separated according their mass-to-charge ratios m/z and the relative detector responses at each m/z -ratio are plotted in mass spectrum. Depending on the ionization technique and the method used for mass analysis, the spectrum may show a large number of peaks from fragment ions, providing structural data of the analyzed compound, or it may contain a peak solely for the molecular ion, providing information only about the size of the molecule or about the elemental composition as in the case of an accurate mass analysis of the molecule.

In quadrupole instruments [88, 122,123], the mass analysis is performed by a quadrupole consisting of four parallel rods. Two diagonally opposite pairs of rods are held at opposite direct current voltages, while the radio-frequency field of one pair is phase-shifted 180° relative to that of the second pair. When these fields are correctly chosen, only ions with a certain m/z ratio pass through the quadrupole to the detector; ions with other m/z ratios have too large oscillation to pass through. The mass spectrum is obtained by scanning the RF field.

When the quadrupole units are connected linearly with a collision cell (RF-only quadrupole or hexapole) placed between them, the resulting instrument (i.e. triple

quadrupole instrument) is capable of tandem mass spectrometry (MS/MS) [88, 122]. The first analyzer works as a mass filter and chooses the ions of interest for the collision cell where they collide with gas (usually argon or nitrogen) and dissociate to fragment ions that are analyzed with the second mass analyzer (i.e. product ion scanning). The procedure can also be designed to work the other way round, so that the first analyzer scans the precursor ions for a certain fragment ion determined by the second analyzer (precursor-ion scanning), or then both analyzers scan for ions for which a loss of certain mass corresponding to a certain fragment is occurs in collision cell (neutral loss scanning). The main disadvantage of this kind of mass analyzer is that it is not capable of high resolution and is not very suitable for accurate mass measurements.

A common mass analyzer capable of higher resolution is the time-of-flight (TOF) instrument, in which the ions are accelerated to the detector through a flight tube [88, 124]. Because all ions are accelerated with the same amount of energy, ions with different m/z ratios arrive at the detector at different times. Flight tubes of modern TOF instruments are equipped with a reflectron (electrical mirror) that equalizes the flight times of ions with the same m/z but different kinetic energy, thereby increasing the resolution and mass accuracy of the instrument to the level that accurate mass measurements can be performed.

3.2.2 Atmospheric pressure ionization (API)

As mentioned above, molecules to be analyzed must be ionized before entering the mass analyzer. Traditionally this has been performed by electron impact ionization (EI) [88, 125] or chemical ionization (CI) [88, 125,126] in direct inlet under high vacuum conditions. For larger biomolecules, other ionization techniques, such as fast atom bombardment (FAB) [88, 122, 127, 128] and matrix assisted laser desorption ionization (MALDI) [88, 122, 129] have been developed. These techniques are poorly suitable for LC/MS, however, owing to the large amount of solvents used in HPLC, and alternative ionization methods have been developed for interfacing HPLC and MS during last decades. Atmospheric pressure ionization (API) techniques, which are highly suitable for the high eluent flow rates in HPLC, are nowadays very much used in LC/MS. The most common API methods at the moment are electrospray (ESI) [80-83, 88] and atmospheric pressure chemical ionization (APCI) [84-88], and atmospheric pressure photo-ionization (APPI)[130] has recently been introduced as the newest addition. Thermospray ionization [88, 131] is usually considered as the common forefather of these API methods, and has similar fields of applications.

In ESI the sample is injected (or fed through HPLC) into a warmed capillary with 2 – 5 kV voltage. The voltage may be positive or negative, and the oppositely charged ions formed in solution are gathered on to the inner surface of the capillary. When the solution exits the capillary, the solution with the help of a suitably directed nebulizing gas flow expands rapidly and forms a spray with charged droplets where an excess of ionized molecules are present on the surface of the droplets. What happens in the next stage is still unclear, but according to current theories the solvent is vaporized and the size of the

droplets decreases until the repulsive interaction of charges exceeds the surface tension and forces the ions to eject from the droplets (ion evaporation model) [132-134], or alternatively the droplets break into smaller droplets many times until finally single ions are formed (charge residue model)[135,136]. Because of the need for initial ionization in the solvent, the electrospray is highly dependent on the solution chemistry and surface behavior of the analyte ions. For this reason it is not very suitable for non-polar compounds, but instead favors the polar compounds. For the same reason, additives for eluents are commonly used to make the pH of the solution favorable for ionization or to steer the ionization process in a desired direction. This ionization process is referred to as “soft” ionization because it typically produces only a few fragment ions and an intensive protonated molecule (deprotonated in negative ion mode), or in many cases adducts with alkali metal cations (Na^+ or K^+).

In APCI, traditional chemical ionization is carried out at atmospheric pressure. The same API interface as for ESI is suitable for APCI and also the ionization probe is closely similar. The main difference is the use of a corona discharge needle in APCI to produce the electrons that initiate a gas phase ionization reaction in which the analytes are ionized with the evaporated solvents as ionization gas. Thus, in contrast to ESI, APCI is dependent on gas phase chemistry, i.e. gas phase acidity and basicity [126]. Ionization in solution is not necessary, and therefore APCI is more suitable for non-polar analytes than ESI. APCI also works better than ESI at higher HPLC flow rates. In APCI, as in ESI, eluents can be modified with additives to increase ionization reactions, and APCI is thought to be less vulnerable than ESI to some harmful HPLC additives and background suppression of the signals [88].

ESI and APCI have been applied to the analysis of a very wide range of compounds from a large number of plants [88-90, 137-140]. ESI has been applied with LC/MS in the analysis of *H. perforatum* extracts in negative ion mode owing to the poor ionization of naphthodianthrone in positive ion mode [38, 115-117]. Also, intensive tandem mass spectrometric studies have been performed with negative ion ESI for the naphthodianthrone of *H. perforatum* [115] and positive ion ESI for phloroglucinols [44].

R. rosea extracts have been studied by mass spectrometry in only two cases. One was a GC/MS study of the volatile compounds of the plant [71], and in the other negative ion mode APCI was used in LC/MS for identification of two phenolic compounds in the plant extract [121].

3.3 NMR spectroscopy

Nuclear magnetic resonance spectroscopy is probably the most versatile technique in modern analytical and structural elucidation chemistry. With different one- and multi-dimensional NMR methods, an abundance of information can be obtained on the structure of a molecule, almost regardless of its size or elemental composition. Not surprisingly, NMR is widely used in analytical chemistry and the biosciences. The most useful NMR methods in natural product research and the structural elucidation of novel compounds from plants and cell matrices are one- and two-dimensional homo- and

heteronuclear correlation methods utilizing the ^1H and ^{13}C nuclei [141, 142]. The fundamentals of NMR are briefly presented in the following [143, 144].

The nucleus of most atoms possesses a nuclear magnetic moment (μ), which is directly proportional to its nuclear spin.

$$\mu = \gamma\hbar/2\pi \quad (3.1)$$

The value of the spin I , which is given by the magnetic quantum number m , can have values $m = I, I-1 \dots -I$, so there are $2I + 1$ different values of m and as many possible orientations of the spin in the magnetic field. Nuclei with spin $I = 0$ have no nuclear magnetic moment, and cannot be observed by NMR spectroscopy. The gyromagnetic ratio γ is a constant for each isotope of each element and it can be either positive or negative. The detection sensitivity of NMR experiment is proportional to the value of γ , so that nuclei with large value of γ are more sensitive and easier to observe than those with small value of γ . Typical NMR nuclei ^1H and ^{13}C have spin $I = \frac{1}{2}$.

The components of the magnetic moment along the B_0 field with direction z can be presented as:

$$\mu_z = -m\gamma\hbar/2\pi \quad (3.2)$$

The energy of a magnetic dipole in a magnetic field B_0 is:

$$E = -\mu_z B_0 \quad (3.3)$$

For a nucleus with $2I + 1$ possible orientations in a magnetic field there are $2I + 1$ energy states, which are obtained from Equations 3.2 and 3.3:

$$E = -(m\gamma\hbar/2\pi)B_0 \quad (3.4)$$

Since all individual nuclei in a molecule experience the magnetic field differently due to their different environments, the individual energy levels can be written as:

$$E = -(m\gamma\hbar/2\pi)B_{\text{eff}} \quad (3.5)$$

B_{eff} is the magnetic field experienced by a nucleus and is affected by the external field B_0 and the internal components B_{int} , that arise from the internal interactions of the nucleus with its surrounding environments.

$$B_{\text{eff}} = B_0 + B_{\text{int}} \quad (3.6)$$

In NMR experiment, irradiation of nuclei with a perpendicular RF field (B_1) with suitable frequency ν induces transitions between different energy states. Energy differences between the magnetic spin states are determined by measuring the frequency at which transitions between the states occur. At resonance the following condition holds:

$$\omega = \gamma B_{\text{eff}} = \gamma(B_0 + B_{\text{int}}) \quad (3.7)$$

In Equation 3.7, $\omega = 2\pi\nu$ and ν is the frequency of the electromagnetic radiation at which absorption occurs.

Because the internal fields B_{int} are closely related to the structure of the molecule, the spectrum obtained in the experiment provides a diversity of information about the environment of the nucleus and thus about the structure of the molecule. B_{int} arises from different interaction mechanisms: magnetic shielding, magnetic dipole-dipole coupling, scalar coupling, interaction of the spins with a fluctuating magnetic component of the radio-frequency field, Zeeman interaction, and, for nuclei with spin quantum numbers larger than $1/2$, nuclear electric quadrupole coupling. If the molecular motion is rapid in the NMR timescale (as it is in liquids), the orientation of molecules disappears and interactions are averaged, so that chemical shift interaction and scalar coupling obtain their average values, but dipolar and quadrupolar interactions average to zero and they cannot be observed in the liquid state NMR experiment.

4 Applications

4.1 Experimental

4.1.1 Liquid chromatography

All HPLC experiments were performed with a Waters 2690 Alliance HPLC system (Waters Corp., Milford, USA). UV-detector used was a Waters 996 PDA detector (Waters Corp., Milford, USA). The columns, other additional instruments and experimental parameters are described in more detailed manner in the discussion of the experiments or in the original papers [I–V].

4.1.2 Mass spectrometry

All collision induced fragmentation mass spectra were recorded and multiple reaction monitoring and selective ion monitoring LC/MS experiments were performed with a Micromass Quattro II triple quadrupole mass spectrometer (Altrincham, UK). All exact mass measurements were carried out with a Micromass LCT time-of-flight mass spectrometer (Wythenshawe, UK). Both instruments were used in LC/MS experiments with an ESI or APCI source. Both positive and negative ion acquisition modes were employed. In all experiments, nitrogen was used as both drying and nebulizing gas and argon as a collision gas. In accurate mass measurements the analyte compounds were injected through the HPLC instrument and lock mass compounds were introduced to the flow through a T-mode flow splitter with syringe pump at a flow rate of 10 – 30 $\mu\text{l}/\text{min}$, so that the number of ions per scan was between 300 and 500 for analyte ions and between 500 and 1000 for lock mass ions. Typically a protonated molecule of leusine enkefaline ($\text{C}_{28}\text{H}_{38}\text{N}_5\text{O}_7$, 556.2771 Da) or a sodium adduct of raffinose ($\text{C}_{18}\text{H}_{32}\text{O}_{16}\text{Na}$,

527.1588 Da) was used as reference mass. Other experimental parameters are described in the original papers [I–V].

4.1.3 NMR spectroscopy

All NMR spectra were acquired at 11.7 T on a Bruker DRX 500 spectrometer (Bruker, Fällanden, Switzerland) with a BBI probe head equipped with z-axis gradient coils. Samples were dissolved in D₂O and 70–100 µl sample volumes were used in Shigemi NMR tubes (susceptibility matched to water). A special shimming technique [145] producing a map of B₀ field strength along the sample volume was applied. Typically 0.4 – 0.8 s acquisition times with 2 s relaxation delays were used for ¹H experiments. For TOCSY and (DQF)COSY experiments, typically 0.4 – 0.6 s acquisition times were used together with 128 or 256 time increments and a varying number of experiments. Mixing times for TOCSY were 100 – 160 ms. Two-dimensional spectra were acquired either in N-type magnitude mode or as phase-sensitive with TPPI or States-TPPI mode quadrature detection. In HSQC–DEPT and HMBC experiments, 128 increments and a varying number of experiments were acquired, optimizing J_{CH} couplings for 130 Hz and 4 – 10 Hz, respectively. Suppression of the water signal was achieved using either presaturation or the excitation sculpting method described by Shaka & Hwang [146]. ¹H NMR spectra were iterated by using PERCH spectral analysis software [147].

4.2 Analysis of naphthodianthrones and phloroglucinols in *H.*

perforatum

A number of HPLC methods have been published for the analysis of the main active compounds in *H. perforatum*. The methods rely on either UV/fluorescence [9, 4-43, 107, 109-113] or mass spectrometric [38, 115-117] detection. However, many of these methods require very long HPLC runs, up to 60 minutes, or are designed to analyze only a few of the main bioactive compounds in the extracts. Furthermore, the sensitivities of the methods are not usually high enough to detect the very low amounts of metabolites present in cell cultures and their extracts.

4.2.1 Analysis of plant metabolites with PDA detection

With the HPLC method with photodiode array (PDA) detection developed here, the analysis of compounds **6** – **11** was performed in 12 minutes per injection [I]. Solvent consumption in the method is low due to the small column, and only filtration and dilution of the extract are needed for sample preparation before injection to the HPLC.

The samples were prepared from the plants as described in [I] and compounds **6** – **11** were identified by HPLC/ESI–MS and with their distinctive UV-spectra [43] obtained with the PDA detector, as described in [II]. The liquid chromatogram obtained from the extract with PDA detection is shown in Figure 9. The UV trace at 590 nm shows the clear separation obtained for naphthodianthrones **6** – **9** and the UV trace at 270 nm the separation obtained for phloroglucinols **10** and **11**. Retention times were 1.9 min for **6**, 2.4 min for **7**, 3.1 min for **10**, 3.5 min for **11**, 3.6 min for **8**, and 4.2 min for **9**. Experimental details are given in the figure caption.

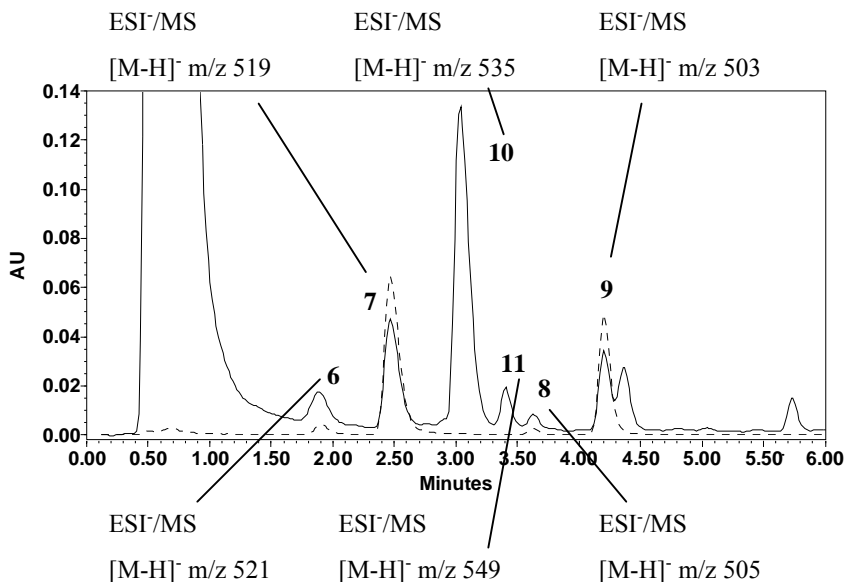


Fig. 9. Liquid chromatogram of *H. perforatum* L. plant extract with PDA detection at UV wavelengths 590 nm (dotted line) and 270 nm (solid line). (**6**) Protoseudohypericin, (**7**) pseudohypericin, (**10**) hyperforin, (**11**) adhyperforin, (**8**) protohypericin, and (**9**) hypericin. Concentrations of compounds in the sample extract are 0.8 – 16 $\mu\text{g/ml}$ for naphthodianthrones **6** – **9** and 3 – 30 $\mu\text{g/ml}$ for phloroglucinols **10** – **11**. The HPLC separation was performed with a Waters XTerra RP18 2.1×50 mm column with $3.5 \mu\text{m}$ particle size at 44°C . Injection volume was 5 μl . The HPLC eluents were aqueous 5 mM ammonium acetate (A) and acetonitrile (B). The initial gradient condition was 50% A and 50% B, changing linearly to 100% B in five minutes. The eluent flow rate was 0.5 ml/min.

The validation of the method was performed with seven concentration levels of standards, each injected in triplicate. For **9**, a correlation coefficient 0.999 was obtained for the range 0.02 $\mu\text{g/ml}$ – 100 $\mu\text{g/ml}$. The regression equation for the calibration curve was $y = 3.62 \times 10^4 x - 2.73 \times 10^4$, where y is the response of the analyte relative to the internal standard and x is the concentration of the analyte ($\mu\text{g/ml}$). The accuracy of quantitation obtained for the standard samples from the generated calibration curve was

acceptable in the range 0.5 – 100 µg/ml, varying from –10% to +5.7%; the precision for the same samples varied from 0.3% to 3.6% (standard deviation).

For **10**, good linearity of response with a correlation 0.999 was obtained for the range 0.1 – 200 µg/ml. The regression equation for the calibration curve was $y = 3.62 \times 10^4 x - 2.73 \times 10^4$. The accuracy and precision for **10** were acceptable in the range 2 – 200 µg/ml, deviations varying from –3.6% to +17% and from 0.2% to 0.8%, respectively. The results of the validation at each concentration level are shown in Table 1 in [I]. It need to be noted that the validation was done with standard solution samples, which means that the background effects present in real samples are not affecting the results. Here, the background compounds at most affect the peak purity and thereby the intensity of the peak. For naphthodianthrones **6 - 9** detected with specific wavelength, the background effects are small, and the real detection limits can be considered close to those given above. For phloroglucinols, the lowest detectable concentrations in real samples may be a little higher than the 0.1 µg/ml given above, and therefore in low concentration samples the peak purity and identification need to be verified by PDA-detected UV spectrum for the correct results. This is usually not a problem, however, because the amounts of phloroglucinols in plant samples are typically at least 20 times the detection limit given above.

The method was used for analyzing batches of over 160 samples of *H. perforatum* extracts within a few days and was found to work well and reliably [148]. In the samples studied the analyte concentrations in extracts typically ranged from 0.5 to 10 µg/ml for the naphthodianthrones and from 10 to 100 µg/ml for the phloroglucinols. Thus, concentrations of the analytes in samples were in very reliable ranges of the calibration curves, and the possible sources of inaccuracy at the low end of the curve, as noted above, do not affect the results.

4.2.2 Analysis of cell tissue metabolites with MRM detection

The concentrations of the naphthodianthrones and phloroglucinols **6 – 11** are usually quite high in *H. perforatum* plant extracts, but only very low (ppm level) concentrations have been found in cell cultures [46, 47]. With the LC/MS/MS method described below, analysis time was 14 minutes per injection and sensitivity was very much improved relative to the method with PDA detection described above [II].

The detection of only compound-specific product ions after collision induced dissociation of molecular ions (multiple reaction monitoring, MRM) reduces the chemical noise level in ion chromatograms, so that the method is even more sensitive than usual LC/MS approaches and allows the use of very fast chromatographic methods. MS/MS data were acquired by selecting the deprotonated molecules $[M-H]^-$ via the first quadrupole mass filter (Q1), activating them in the collision cell (Q2) and monitoring the specific product ions with the last (Q3) quadrupole analyzer with MRM mode.

The sample preparation was performed as in [II] and negative ion mode electrospray detection was used. The product ion mass spectra produced for deprotonated molecules of analyte compounds **6 – 11** and the internal standard reserpine (**49**) are shown in Figure

10. The transitions used in MRM quantitation of analyte compounds and the corresponding most intense fragment ions are shown in Figures 11 and 12, together with proposed structures for some of the fragment ions. An example of the results obtained for a solution containing the internal standard reserpine and all six analyte compounds is shown in Figure 13. Retention times were 1.4 min for internal standard **49**, 1.8 min for **6**, 2.4 min for **7**, 2.9 min for **10**, 3.3 min for **11**, 3.5 min for **8**, and 4.1 min for **9**.

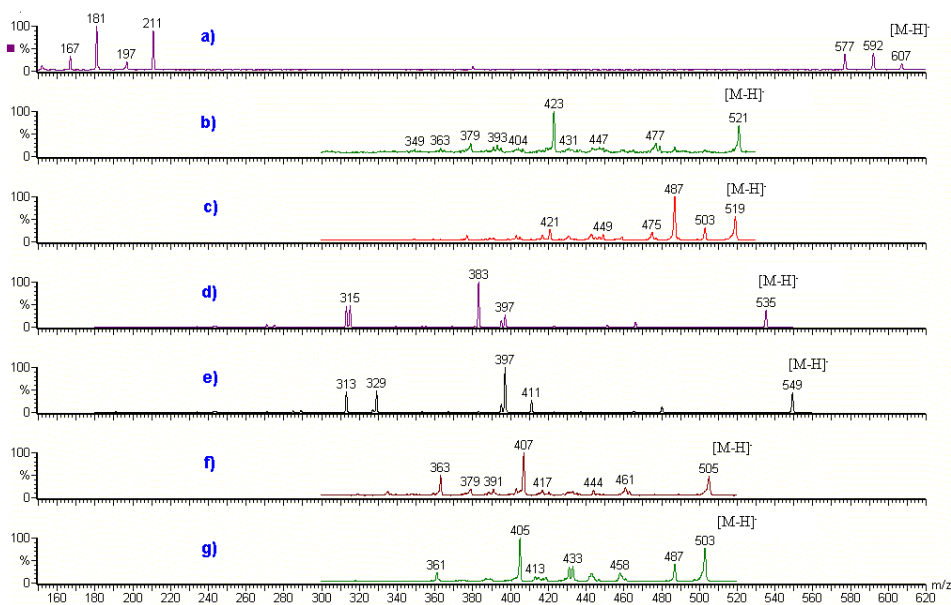


Fig. 10. Negative-ion electrospray LC/MS/MS product ion spectra for analyte compounds and internal standard. a) Reserpine (49) b) protopseudohypericin (6) c) pseudohypericin (7) d) hyperforin (10) e) adhyperforin (11) f) protohypericin (8) g) hypericin (9).

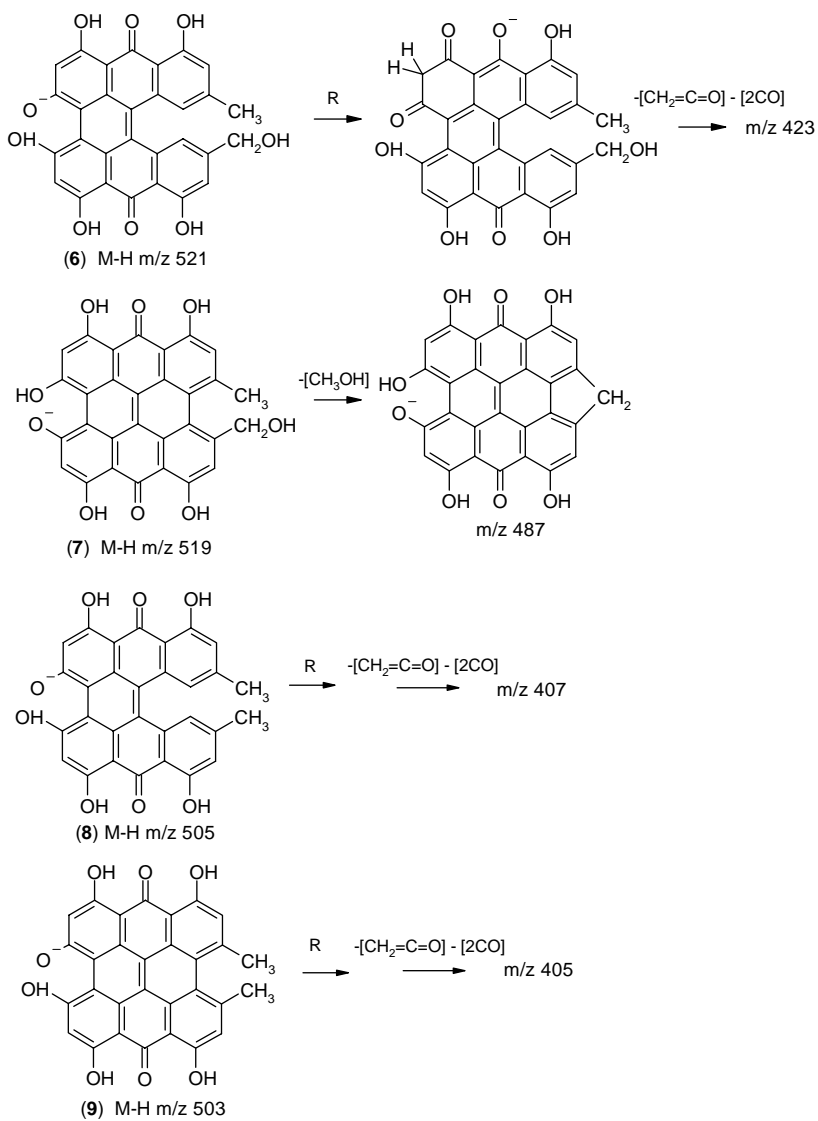


Fig. 11. Monitored fragmentation reactions of 6–9 [115]. The rearrangements (R) for 6, 8, and 9 are identical.

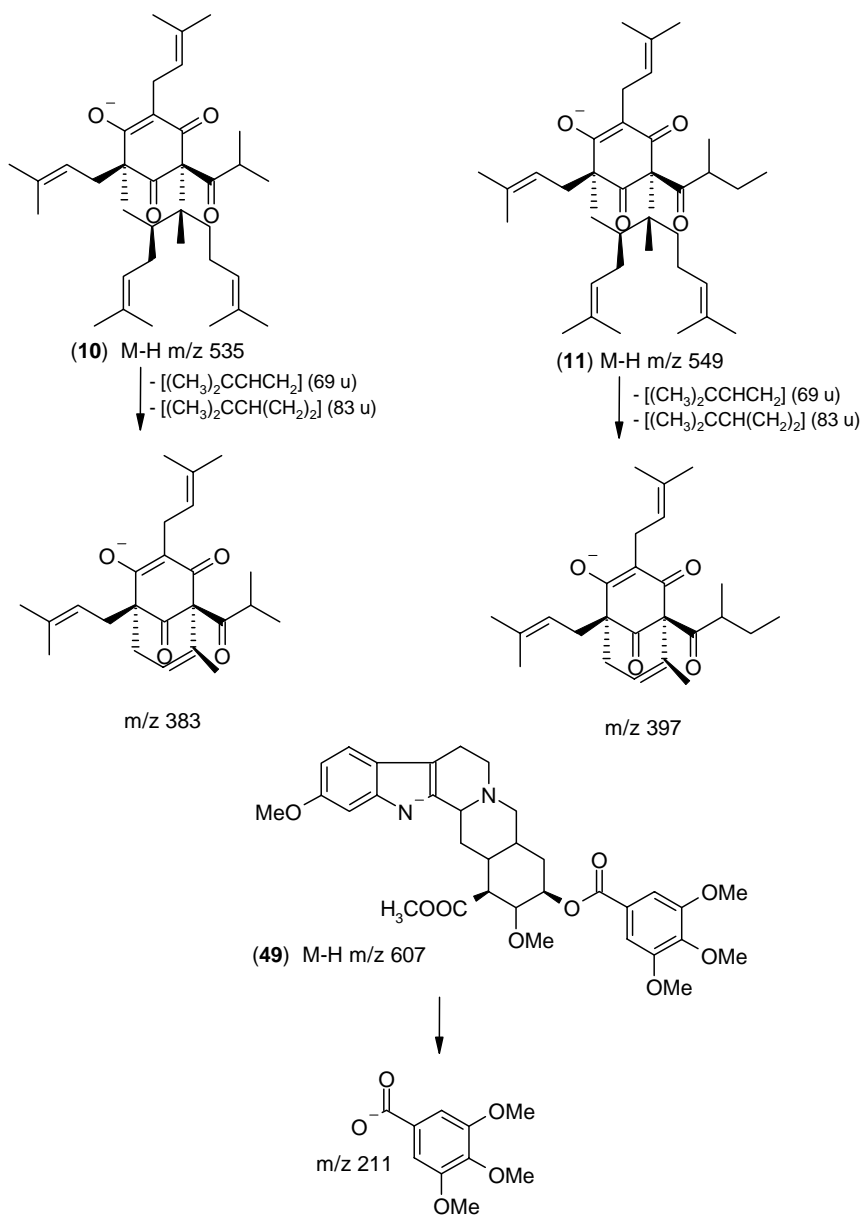


Fig. 12. Monitored fragmentation reactions of 10–11 and 49. Sequential expulsions of side chains occur for 10 and 11. Loss of 69 u is followed by loss of 83 amu [116].

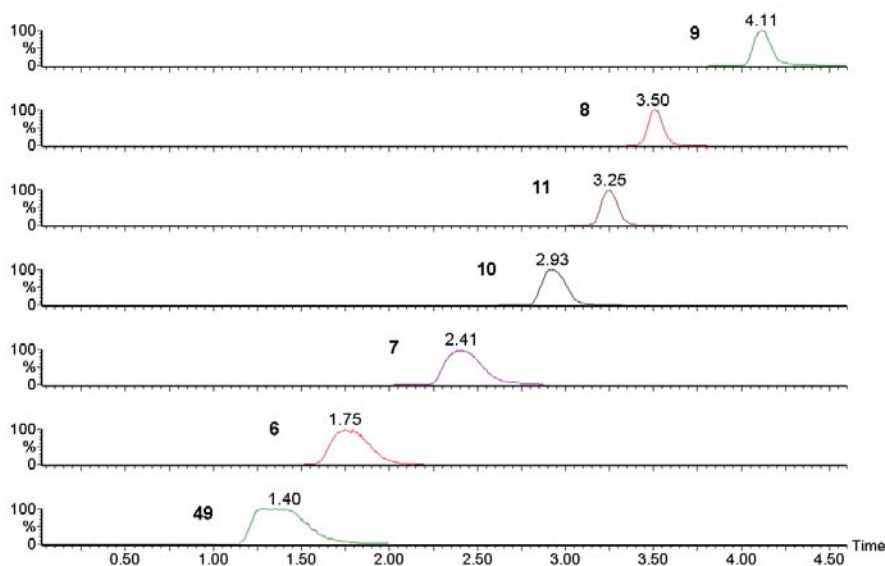


Fig 13. LC/MS/MS chromatogram recorded from *H. perforatum* extract with MRM mode. The experiment was performed with concentrated solution where concentrations of analytes are 0.1–5 $\mu\text{g}/\text{ml}$. Separation was performed with a Waters Xterra RP18 2.1×50 mm column with $3.5 \mu\text{m}$ particle size at 35°C . Injection volume was $20 \mu\text{l}$. The HPLC eluents were aqueous 20 mM ammonium acetate (A) and acetonitrile (B). The initial gradient condition was 50% A and 50% B, changing linearly to 100% B in five minutes. The eluent flow rate was 0.5 ml/min. Negative ion mode electrospray ionization (ESI⁻) was used with MRM detection mode. The time windows and reactions monitored were 1.0 - 2.0 min for internal standard 49 (m/z 607 \rightarrow 211), 1.5 - 2.2 min for 6 (m/z 521 \rightarrow 423), 2.0 - 2.9 min for 7 (m/z 519 \rightarrow 487), 2.6 - 3.3 min for 10 (m/z 535 \rightarrow 383), 3.0 - 3.6 min for 11 (m/z 549 \rightarrow 397), 3.3 - 3.8 min for 8 (m/z 505 \rightarrow 407) and 3.8 - 4.6 min for 9 (m/z 503 \rightarrow 405). More detailed MS parameters are found in [II].

Seven concentration levels of standards containing 8 $\mu\text{g}/\text{ml}$ internal standard 49 were prepared for the testing of linearity and validation of the method. Each concentration level was injected in triplicate. For 9, a correlation coefficient of 0.996 was obtained for the range 2 - 500 ng/ml. The accuracy of quantitation obtained for the same standard samples from the curve ranged from -7% to +19% and the precision for different concentrations was between 5% and 9% (standard deviation). The lower level of quantitation was 2 ng/ml. For 10 a good linearity of response with a correlation coefficient 0.999 was obtained for the range 0.1 - 1000 ng/ml. The accuracy for 10 determined with these same standard solutions varied within the linear range from -8% to +12%, and the precision for different concentrations was between 2% and 15% (standard deviation). The lower level of quantitation was 0.5 ng/ml. The results of validation for 9 and 10 at each concentration level are shown in Table 1 in [II]. As can be seen by comparing these results (quantitation limits) with those obtained with PDA

detection, the gain in sensitivity with MS/MS detection is about 250-fold for hypericin and as much as 4000-fold for hyperforin.

In the absence of zero level samples with real background matrix as with PDA detection, the validation results were determined with standard solution samples,. Thus the discussion at the end of the preceding section, concerning the reliability of the validation results for real samples, equally applies here. In contrast to PDA detection where the background compounds mainly affect the peak purity, in mass spectrometric detection the peak purity is not a problem. Instead, the co-eluting components of background may lower the signal intensity of the analytes by suppressing their ionization. Since the chromatographic conditions used here are very close to those used in the PDA method, however, the analytes are separated from the matrix components very well and the signal suppression due to co-eluting compounds can be assumed to be minor.

The amounts of naphthodianthrones reported for callus samples would roughly correspond to levels of 20 – 2000 ng/ml if the extracts had been prepared as presented here [46,47]. Thus, the method described allows the analysis of much lower concentrations than those reported earlier. In highly concentrated samples, with levels more than 500 ng/ml, an extra dilution step should be included in the sample preparation when this method is used as described in [I].

4.3 Isolation and identification of phenylpropanoid glycosides from *R. rosea*

Different solvent compositions with water, methanol, and acetonitrile and various pH conditions were tested in the extraction of dried and powdered *Rhodiola Rosea* plant material. The most efficient composition for extraction of the bioactive phenylpropanoids was 60% aqueous methanol without pH modification. For more detailed screening of this extract, the twelve compounds were isolated for structure determination on semi-preparative reverse-phased HPLC columns and identified on the basis of their high-resolution mass spectra, fragment ion mass spectra obtained by triple quadrupole instrument (CID), and one- and two-dimensional NMR experiments (TOCSY, (DQF)COSY, HSQC-DEPT or HMQC, and HMBC) [III]. The proton–proton coupling constants obtained from the ¹H spectrum and phase-sensitive COSY spectrum were refined with PERCH spectral analysis software [147].

Six of the isolated compounds were confirmed as known bioactive metabolites of the plant: i.e. tyrosol (**27**), salidroside (**28**), rosin (**29**), rosarin (**30**), rosavin (**31**), and rosiridin (**35**). Two were identified as 4-hydroxy-cinnamyl-O-β-D-glucopyranoside (sachalide 1) (**32**) and 4-methoxy-cinnamyl-O-β-D-glucopyranoside (vimalin) (**33**), which were isolated earlier from the callus culture of the plant but not from the plant itself (Fig 14). Two of the compounds were identified as picein ((4-O-β-D-glucopyranosyl)-acetophenone) (**50**) and benzyl-O-β-D-glucopyranoside (**51**), which have not been found in *R. rosea* before. The last two compounds isolated were, similarly to the known secondary metabolites of the plant, also derived from the shikimate pathway metabolism. These novel compounds, which have not been reported from anywhere else, were

identified as cinnamyl-(6'-O- β -xylopyranosyl)-O- β -glucopyranoside (**52**) and 4-methoxy-cinnamyl-(6'-O- α -arabinopyranosyl)-O- β -glucopyranoside (**53**).

In addition to these identified compounds, also cinnamyl alcohol (**54**) was identified in the extract by comparing its retention times obtained with two different HPLC columns, UV spectrum, and LC/MS spectrum with those of an authentic commercial standard. The retention times obtained for the analyte and the standard were exactly the same, since when standard was added to extract, no new peaks were observed and the intensity of the analyte peak was increased. The UV spectra and positive ion mode ESI spectra for the analyte and the standard were also exactly similar, giving UV absorption maxims at 250 nm and in mass spectra peaks only at m/z 117 for $[M+H-H_2O]^+$.

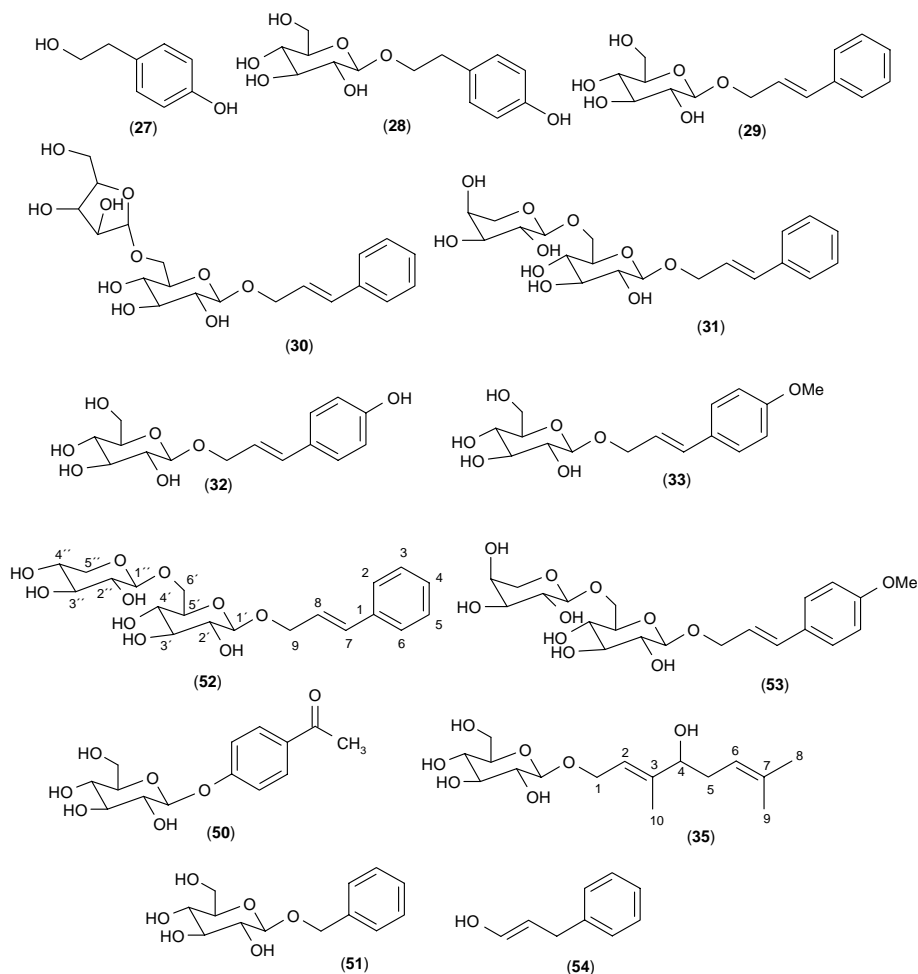


Fig. 14. Compounds isolated from *R. Rosea* aqueous 60% methanol extract. Isolation was performed with HPLC and a fraction collector from 100 HPLC runs using a 100 μ l injection volume, as described in [III].

In mass spectrometric experiments the information obtained from the time-of-flight instrument is the exact molecular weight giving the elemental composition of the compound, while the information obtained from the collision induced dissociation (CID) mass spectra of glycosidic compounds is the fragmentations of the sugar moieties showing the size of the sugar units and aglycone.

Typical fragmentations in the CID spectra of glycosides are dissociations of the glycosidic bonds, leaving the charge either to the sugar unit(s) or to the aglycone of the molecule. In the compounds studied, both the loss of sugar units and the loss of aglycone were seen in positive ion mode, whereas only the loss of aglycone or the loss of aglycone together with one sugar unit (in the compounds with two sugar units) was seen in negative ion mode. As an example, CID spectra measured in positive and negative ion mode for compound **52**, together with the proposed interpretation of the fragment ions, are shown in Figure 15.

Fragmentations of glycosides may also occur on either side of the bonding oxygen atom in the glycosidic bond between the sugar and the aglycone, increasing the number of fragment ions seen in the spectra. Sometimes dissociation of the bonds within sugar rings occurs too, increasing further the number of peaks in the spectrum. These fragmentations can usually be interpreted according to the fragmentation rules of O-linked sugar residues [149].

In positive ion mode electrospray source, many glycosidic compounds have a tendency to ionize via formation of adducts with alkali metal cations (i.e. mainly sodium). Such ionization was observed in this study. These adducts are highly stable and resistant against dissociation, and in some cases, even in collision cell activated reactions, the level of fragmentation and intensity of the fragment ions giving structural information is very low [150, 151]. This poor fragmentation may significantly limit the information to be gained from tandem mass spectrometric experiments. Here, the problem was encountered for the phenylpropanoid compounds with one sugar ring (**29**, **32**, **33**), for which only very low intensity fragment ions were detected. The problem was satisfactorily overcome, however, by steering the ionization process toward the formation of the more easily dissociating NH_4^+ -adduct of the molecule through addition of ammonium acetate to the sample solution (at 10–20 mM level)[III].

The characteristic features of the NMR spectra of simple phenylpropanoids are the region from 5 to 8 ppm showing the nature of aglycone in the compounds, i.e. the aromatic and double bond regions of the phenylpropanoids; the region from 2 to 5 ppm with the chemical shifts and vicinal $^3J_{\text{HH}}$ coupling constants of sugar moiety protons, showing the stereochemistry of these protons and so identifying the sugar; and the 4 to 5.5 ppm region showing the anomeric protons of the sugar rings and their vicinal coupling constant to ring protons, indicating whether the sugar ring is in α - or β -conformation. The chemical shifts of the overlapping sugar signals are mainly obtained from TOCSY spectra, and the coupling constants from phase-sensitive COSY spectra and the iteration of the ^1H spectra with spectral analysis software. The 1D ^1H NMR spectrum and 2D TOCSY and COSY spectra for compound **52** are shown as an example in Figures 16 and 17. The complete UV, MS and NMR spectrometric data used in identification of the isolated compounds is presented in appendix 1, and an explanation of the main points in the elucidation of structures is presented in [III].

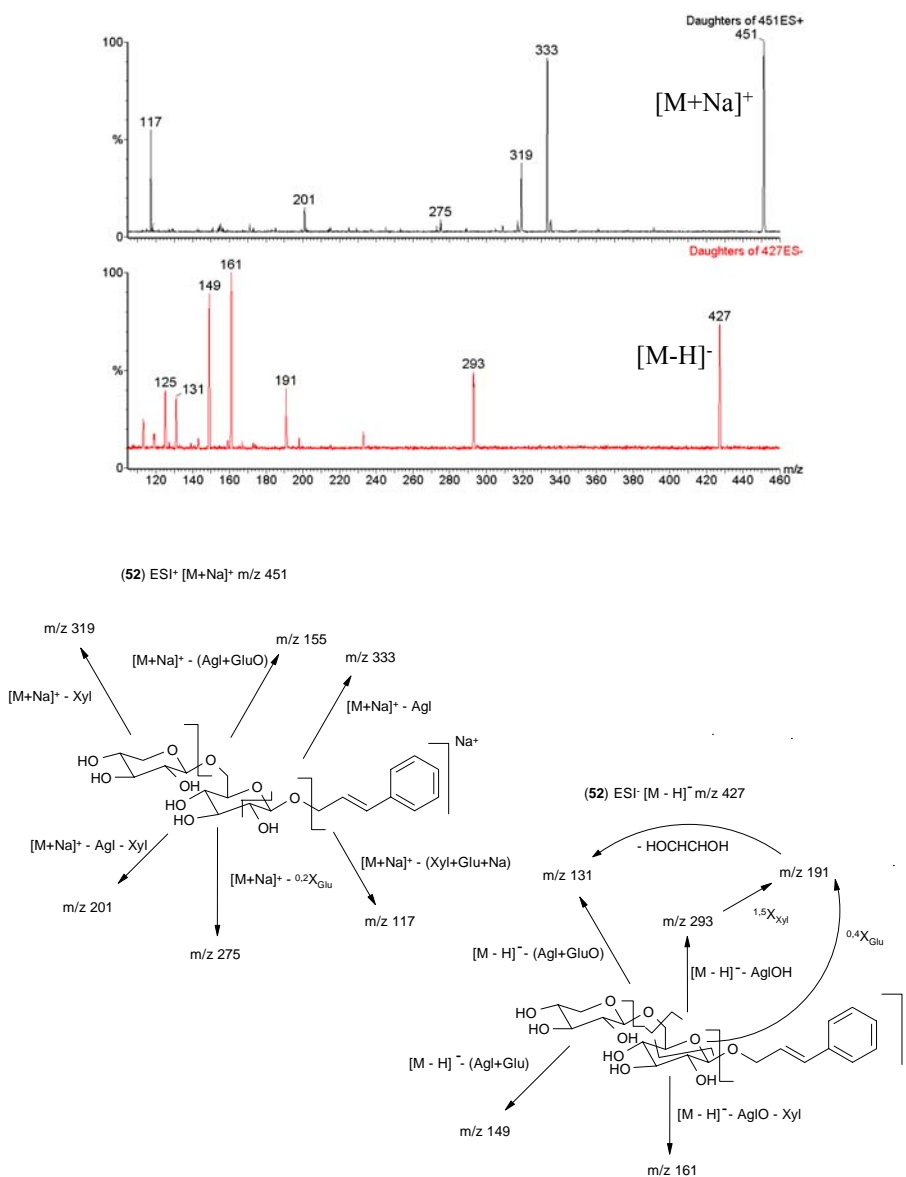


Fig. 15. Collision induced dissociation MS/MS spectra of compound 52 with positive (above) and negative ion mode (below) and suggested interpretations for fragment ions. The parent ion collided in positive ion mode was $[M+Na]^+$ at m/z 451 and that in negative ion mode was $[M-H]^-$ at m/z 427. Experimental parameters are described in [III].

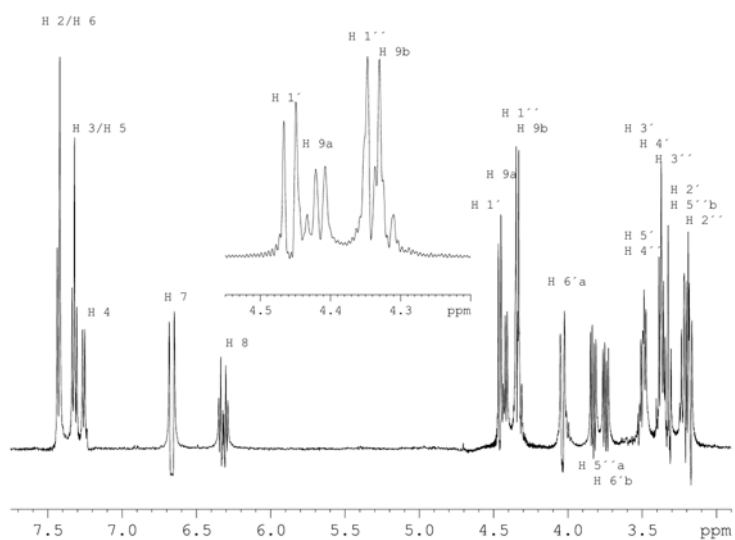


Fig. 16. ^1H NMR spectrum of compound 52 in D_2O . Suppression of water signal was achieved with excitation sculpting [146]. Detailed experimental parameters are given in [III].

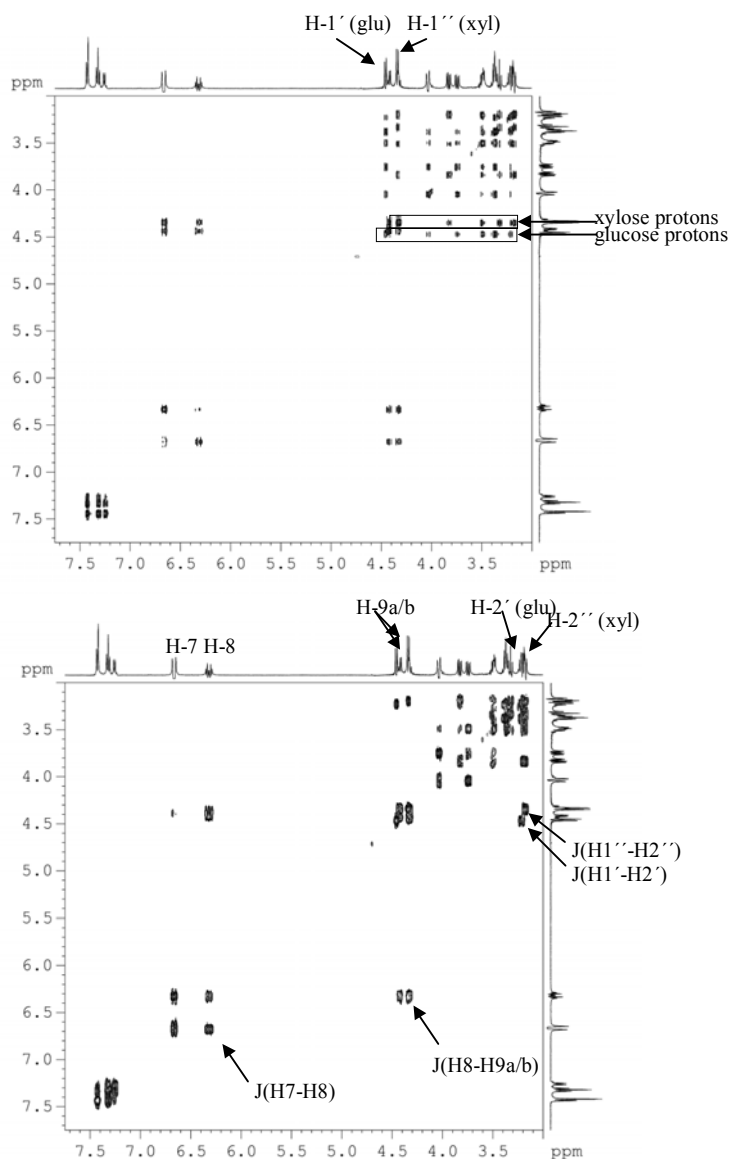


Fig. 17. 2D Gradient selected TOCSY (above) and COSY (below) spectra of compound 52 in D₂O. Mixing time used in the TOCSY experiment was 160 ms. Experimental parameters are as described in [III]. The signals originating from different sugar units are clearly shown in TOCSY (anomeric protons H-1' and H-1'' marked) and are easily identified when the data is used together with COSY spectrum. Complete interpretations of 1D spectra are shown in Fig. 16.

4.4 Analysis of main constituents in *R. rosea*

Earlier methods reported for the analysis of the main bioactive compounds in *R. rosea* plant extracts by HPLC with UV [72, 118-120] or APCI/MS [121] detection are intended for the analysis of just the main components or else they require lengthy analysis times (from half an hour up to 45 minutes). Lengthy analysis times require intolerably long instrument time when large sample sets (e.g. hundreds of samples) are to be analyzed. Furthermore, the sensitivity of these methods is not adequate for the analysis of the very low levels of compounds in cell culture extracts.

4.4.1 Analysis of phenylpropanoids with PDA detection

A new HPLC method relying on a with diode-array detector was developed for determination of the newly identified compounds **52** – **53**, along with the known compounds **28** – **33** and **54** in *R. rosea* plant extract [IV]. The chromatogram for a sample prepared as described in [IV] and detected at UV wavelengths 254 and 276 nm is shown in Figure 18, while the retention times of the analytes are collected in Table 1.

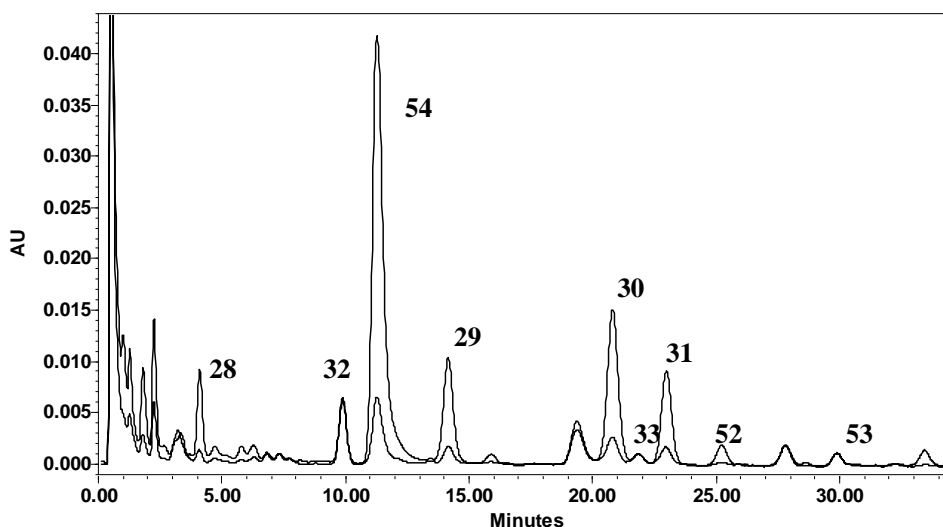


Fig. 18. Liquid chromatogram of *R. Rosea* 60% methanol extract at UV wavelengths 254 nm (solid line) and 276 nm (dotted line). The separation was performed with a Waters Xterra RP18 2.1 × 50 mm column with 3.5 μm particle size at 26 °C. Injection volume was 20 μl. Initial gradient elution conditions were 100% 10 mM aqueous ammonium acetate (A) and 0% acetonitrile (B), which changed to 99% A : 1% B in four minutes, and to 91% A : 9% B in the next 34 minutes. The flow rate was 0.6 ml/min. The identification for peak of the compound **54** is not shown in [IV], since the origin of the peak was identified afterwards.

Table 1. Retention times of the analytes.

Retention time	Analyte
4.2 min	Salidroside (28)
9.9 min	4-Hydroxy-cinnamyl-O- β -glucopyranoside (sachalide 1) (32)
11.2 min	Cinnamyl alcohol (54)
14.2 min	Rosin (29)
20.8 min	Rosarin (30)
21.8 min	4-Methoxy-cinnamyl-O- β -glucopyranoside (33)
23.0 min	Rosavin (31)
25.2 min	Cinnamyl-(6'-O- β -xylopyranosyl)-O- β -glucopyranoside (52)
29.9 min	4-Methoxy-cinnamyl-(6'-O- α -arabinopyranosyl)-O- β -glucopyranoside (53)

The method was calibrated with use of eight concentration levels of salidroside (**28**) and rosavin (**31**) standards, each of which was injected in triplicate. Since the UV spectrum of **28** differs noticeably from the spectra of the other analyte compounds, a specific wavelength, 276 nm, was used to obtain more sensitivity. For the other compounds the wavelength 254 nm was found to be suitable, since their peak maximums in UV spectra were found either at 250 nm or 259 nm and the shapes of the maximum peaks are similar, producing similar responses for similar quantities at 254 nm. The assumption of similar response for compounds **29** – **33** and **52** – **53** at 254 nm was thus found reasonable and compound **31** was used as an external standard for all.

For **28**, good linearity of response with a correlation coefficient 0.999 was obtained for a 0.2 – 50 $\mu\text{g/ml}$ range. The regression function giving the slope and y-axis intercept of the curve was $y = 2.64 \times 10^4 x + 4.45 \times 10^3$. The detection limit obtained was about 0.05 $\mu\text{g/ml}$. The accuracy for the standard solutions determined from the obtained curve (within linear range) varied from – 8.4% to + 2.7%, and the precision for different concentrations ranged between 0.8% and 9.0% (standard deviation). For **31** a correlation coefficient 0.999 was obtained for a range 0.2 – 100 $\mu\text{g/ml}$. The regression function for the curve was $y = 2.19 \times 10^5 x + 1.10 \times 10^4$, while the detection limit obtained was 0.05 $\mu\text{g/ml}$. The accuracy ranged within the quantitation limits from – 15% to + 10% and the precision for different concentrations was between 1.0% and 4.7%.

Since the validation was performed using standard solutions without the background effects of a real matrix, the possible effect of the sample matrix needs to be considered. In fact, the background effects should be low, because a real sample with correct background was used in development of the chromatographic method, and, according to the PDA detector, the peak purities of the separated analyte peaks were good. With extracts prepared as here, the highest amounts of **28** and **31** reported earlier for *R. rosea* would roughly correspond to concentrations of 25 $\mu\text{g/ml}$ for **28** and 70 $\mu\text{g/ml}$ for **31**.

4.4.2 Analysis of phenylpropanoids from plants and cell tissue with MS detection

In the method described above, all analyte compounds were for the first time analyzed simultaneously in a single HPLC run. Owing to the UV detection, however, the

separation required over 40 minutes analysis time and the sensitivity was not good enough for measuring low level concentrations in cell culture extracts. The use of a mass spectrometer as detector for HPLC analysis enables a highly specific detection of the analyte compounds and makes it possible to use a much faster chromatographic method. The sensitivity of the method is also improved drastically relative to the traditional UV-detection method.

A liquid chromatographic method with mass spectrometric detection based on selective ion monitoring (SIM) of sodium adducts of analyte compounds was developed for the simultaneous analysis of salidroside (**28**), rosin (**29**), rosarin (**30**), rosavin (**31**), sachalaside 1 (**32**), 4-methoxy-cinnamyl-O- β -glucopyranoside (**33**), cinnamyl-(6'-O- β -xylopyranosyl)-O- β -glucopyranoside (**52**), 4-methoxy-cinnamyl-(6'-O- α -arabinopyranosyl)-O- β -glucopyranoside (**53**), rosiridin (**35**), and benzyl-O- β -glucopyranoside (**51**) in callus and plant extracts. The total run time was 22 minutes [V].

4.4.2.1 Comparison of ionization methods and conditions

To find the most sensitive ionization method for the compounds studied, electrospray and atmospheric pressure ionization were tested with the same HPLC gradient in both positive and negative ion mode with four different additive compositions for the aqueous mobile phase (Table 2).

Table 2. Aqueous eluent phases used in solvent optimization studies. The percentage of organic solvents (acetonitrile (B) and methanol (C)) was the same in the gradient with all eluent systems. See experimental in [V].

Eluent system	Composition
I	Water
II	0.1% formic acid (v/v)
III	0.02% formic acid+10 mM ammonium acetate
IV	10 mM ammonium acetate

The sample used in these studies was a plant extract to which small amounts of isolated analyte compounds had been spiked to ensure their presence. The pseudo-molecular ions and most intense fragment ions were identified in the LC/MS spectra acquired in full scan mode and ion chromatograms corresponding to the analyte main peaks were extracted. An example of an extracted ion chromatogram (XIC) obtained is presented in Figure 19, with the eluent system I. All peaks detected in spectra, with their interpretations, are collected in Table 2 in [V.] The intensities of the XIC peaks with different eluent systems and ionization methods were compared with signal-to-noise ratios and are presented in Figure 20. Retention times were 2.1 min for internal standard salicin (**1**), 2.6 min for **28**, 4.4 min for **51**, 5.6 min for **32**, 12.7 min for **29**, 13.7 min for **30**, 14.9 min for **31**, 15.7 min for **52**, 15.7 min for **35**, 15.8 min for **53**, and 16.0 min for **33**.

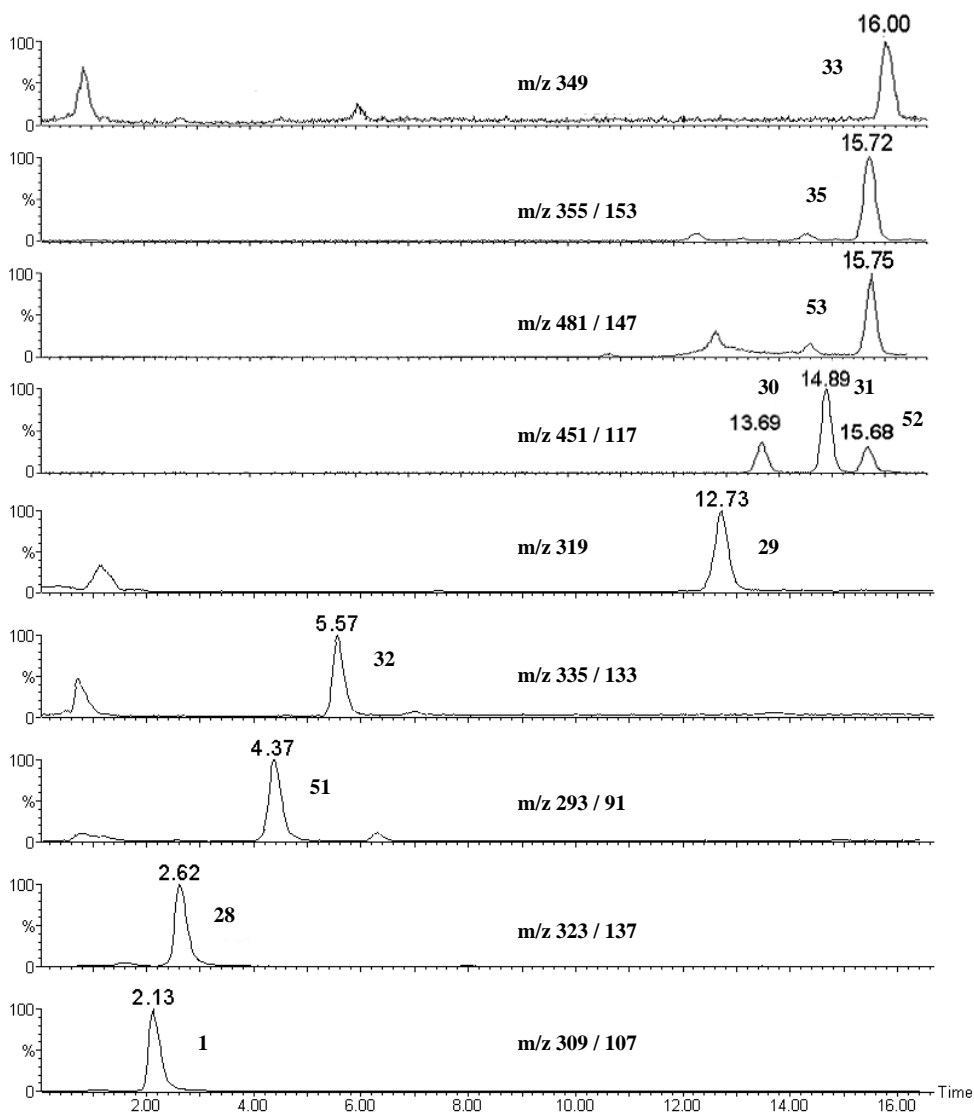


Fig. 19. Extracted ion chromatograms of an LC/MS experiment (full scan mode at range m/z 80-530) with ESI⁺ ionization and eluent system I. Ions used for extraction are marked in the chromatograms ($[M+Na]^+$ / fragment ion). Concentrations of the compounds in the sample used were 200 – 1200 ng/ml. The HPLC separation was performed using a Waters Polarity 2.1 \times 50 mm column with 3 μ m particle size together with a Phenomenex Max-RP 2.0 \times 4.0 mm precolumn. Temperature of the column oven was 35 $^{\circ}$ C and the injection volume was 5 μ l. The eluent flow rate was 0.3 ml/min. Gradient: (A = aqueous phase with different modifiers (see Table 2), B = acetonitrile and C = methanol) 0 min 90% A: 5% B: 5% C, changing linearly during 16 min to 76% A: 12% B: 12% C.

Positive ion mode electrospray ionization with no mobile phase additives was clearly the most sensitive ionization method for the analyte compounds: S/N ratios for XIC peaks were in general two to five times as high as those obtained in negative mode ESI and positive ion mode APCI. (Intensities of the latter were about the same.) Negative ion mode APCI gave S/N ratios ten to 100 times lower than positive ion mode ESI, and was clearly the most unsuitable ionization method for the analytes.

In positive ion mode the compounds showed a strong tendency to ionize by forming a sodium adduct $[M+Na]^+$ at $m/z [M+23]^+$. This was mostly replaced by the formation of ammonium adduct $[M+NH_4]^+$ at $m/z [M+18]^+$ when ammonium acetate was added to the eluent (systems III and IV). Also potassium adduct $[M+K]^+$ at $m/z [M+39]^+$ was detected in some spectra. Ionization via protonation was not observed, but rather, adduct formation was the main ionization process, even in APCI conditions. With eluent system I the sensitivity was best in both ESI and in APCI in positive ion mode; with electrospray this was as expected, since the dominant ionization process is the formation of stable sodium adducts in liquid phase. For hydroxy-substituted analytes **28** and **32** the calculated pK_a values taken from SciFinder [152] are 10.2 ± 0.15 and 9.9 ± 0.15 , and 12.9 ± 0.70 for the other compounds. Since these phenols and their glycosides are acidic compounds, one would expect them to be neutral in solutions under the conditions employed. The weak protonation and effective ionization via adduct formation is as expected therefore.

The addition of mobile phase modifiers decreased the efficiency of sodium adduct formation and, especially with formic acid with eluent system II, greatly increased the background noise, lowering the sensitivity. The suppression of adduct formation with formic acid addition may partly be due to the capability of $[HCOO]^-$ ions to neutralize positive sodium cations. The ammonium adduct formation occurring with eluent systems III and IV replaced the sodium adduct formation, as is typical for glycosides and phenolics [139].

In negative ion mode, ionization mostly occurred through the formation of deprotonated molecule $[M-H]^-$, but with eluent systems II – IV, peaks were also observed for formate adduct $[M+HCOO]^-$ at $m/z [M+45]^-$ or acetate adduct $[M+OAc]^-$ at $m/z [M+59]^-$. In some spectra these peaks were more intense than the peak for $[M-H]^-$. In negative ion ESI, sensitivity with eluent system II was almost as good as with eluent system I, and in negative ion mode APCI the sensitivity was best with eluent system II. In contrast to opposite to positive ion mode, none of the eluent systems led to best sensitivity for all compounds.

The formation of $[HCOO]^-$ adducts as a result of formic acid addition (eluent system II) suggests that the protons present are transferred to the weaker acids water, methanol, and acetonitrile with higher proton affinities, in either liquid or gas phase reaction. The low amount of $[M-H]^-$ ions suggests that no effective proton transfer from analyte compounds to $[HCOO]^-$ occurred at pH 2.6, in contrast to the findings of an earlier study with flavonoids [153].

Owing to their acidity, ionisation of the analyte compounds would be expected to be more effective in negative ion mode under high pH conditions. However, as the pK_a values of the analyte compounds are high, the pH conditions used here were apparently too low to deprotonate the analyte ions effectively in liquid phase. The better ionization efficiency obtained in ESI⁻ with the eluent system I (at highest pH) when than with the other eluent systems was as expected.

The fragmentation was not very intense, the main fragment ions being due to the loss of sugar units with intensities usually lower than those of the molecular ions. In positive ion mode these main fragment ions were seen at m/z $[M-163]^+$ for **28**, at $[M-179]^+$ for **29**, **32**, **33**, **35**, and **51**, and at $[M-(149+162)]^+$ for **30**, **31**, **52**, and **53**. The relative intensities of fragment ions were lowest with eluent systems I and II, being about half of the main peaks, whereas in solvent systems with ammonium acetate the intensity of fragment ions was as high as that of $[M+NH_4]^+$ and $[M+Na]^+$ or higher. This was probably due to the lower stability of $[M+NH_4]^+$ than $[M+Na]^+$ ion.

In negative ion mode the fragment ions were due to the losses of neutral aglycones $[M-H-AglOH]^-$, leading to ions at m/z 161 for glucosides and at m/z 293 for compounds **30**, **31**, **52**, and **53**. Also the fragment ions of one sugar unit retaining the charge were detected, at m/z 179 for glucosides and at m/z 149 for compounds **30**, **31**, **52**, and **53**.

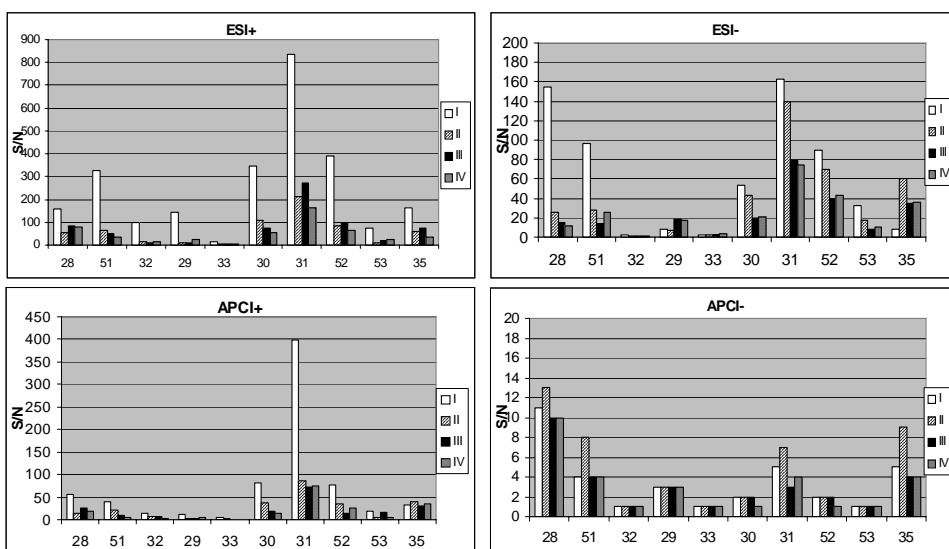


Fig. 20. Signal-to-noise ratios for analytes with eluent systems I-IV. a) ESI^+ b) ESI^- c) $APCI^+$ d) $APCI^-$. See Table 2 for interpretation of columns.

4.4.2.2 LC/MS method validation with SIM detection

Electrospray ionization with eluent system I, which was found to be the most suitable for the compounds studied was used in optimization of the mass spectrometric detection mode for analytical purposes [V]. The usual multiple reaction monitoring (MRM) with fragment ion detection was not suitable with this eluent system because the sodium adducts $[M+Na]^+$ that formed, were highly stable for analyte compounds with only one sugar unit, producing product ions with very low intensities in CID experiments. Interestingly, the adducts formed for compounds with two sugar moieties (**30** – **31** and **52** – **53**) were not as stable, but instead fragment ions $[M+Na-334]^+$ and $[M+Na-Aglycone]^+$

were observed. One reason for this different behavior may be a different coordination site for sodium cations in molecules with one and two sugar units. Instead of MRM, selected ion monitoring (SIM) for sodium adducts of analyte compounds was preferred as a detection method and was also used for validation.

For calibration of the LC/MS method (in SIM mode) with electrospray ionization and eluent system I, solutions of eight concentration levels of standards were prepared for recording of calibration curves and each solution was injected in triplicate. In validation for **28**, a good linearity of response with a correlation coefficient 0.994 was obtained in the range 0.5 – 500 ng/ml. The accuracy for **28** from determined with same curve varied within linear range from – 9.1% to +13.2% and the precision for the different concentrations was between 1.0% and 8.9% (standard deviation). A 15% deviation was considered as the limit of acceptance for quantitation, so the –22.2% deviation with 2000 ng/ml standard was not acceptable.

For **31**, a correlation coefficient 0.999 was obtained for range 2.0 – 2000 ng/ml. The accuracy within the quantitation limits ranged from –2.9% to +11.7% and precision for the different concentrations varied between 1.0% and 4.3%. For **51**, a correlation coefficient 0.999 was obtained for the range 2.0 – 500 ng/ml. The accuracy within the quantitation limits ranged from –10.0% to +11.0% and precision for the different concentrations was between 0.8% and 8.2%. Results of the validation in each concentration for all standards are shown in Table 3 in [V]. As with the methods described above, the validation was performed with solutions free of background effects, which means that the accuracy and detection limits in real sample analyses may not be as good due to signal suppression caused by the matrix.

The method was applied to a number of plant and callus samples with good results. However, the concentrations of analyte **28** determined in studied callus samples exceeded the highest point of the linear detection, so an extra dilution step (with 6% methanol containing 400 ng/ml of internal standard, 1:9) was added in the sample preparation. After this, the concentrations detected in the samples were in the range 30 – 90 ng/ml for **28** and 10 – 60 ng/ml for **31**. As these concentrations are much over the lower limits of detection given above, the sensitivity of the method is good enough, even if the matrix effects cause some decrease in intensities.

4.4.3 Analysis of biotransformation in *R. rosea* cell suspensions by LC/MS

In addition to the HPLC methods designed for the analysis of plant and cell culture metabolites of *R. rosea*, need arose for a method capable of analyzing the biosynthetic precursor compounds of these metabolites. In biotransformation studies, the precursor compounds are fed to a cell suspension, where cell enzymes transform them into secondary metabolites [5, 6]. Biotransformation studies on *R. rosea* and *R. sachlinensis* have shown the transformation of cinnamyl alcohol (**54**) precursor into rosavin (**31**) [72] and the transformation of L-tyrosine (**55**) and tyrosol (**27**) precursors into salidroside (**28**) [73-75]. Since also some other compounds, such as phenylalanine, can be expected to

take part into these transformation reactions [74] (Fig 21), an LC/MS method was developed for simultaneous analysis of all these precursor and product compounds. The method was refined to be capable as well of determining the phenylpropanoids rosin (**29**), rosarin (**30**), and cinnamyl- (6'-O- β -xylopyranosyl)-O- β -glucopyranoside (**52**), since the precursors of rosavin (**31**) are expected to transform into these compounds in cell suspensions.

The aim in the analyses was to follow the transformation of precursor compounds added to the suspension into certain products. This required that the sample preparation be developed for both suspension media and the cells themselves. Since a LC/MS method for analyzing compounds **28** – **31** and **52** was already available, as described in the previous section, the HPLC conditions of the method were simply re-optimized to retain the polar amino acids L-tyrosol (**55**) and L-phenylalanine (**56**), while still resolve the compounds **30**, **31**, and **52** of same molecular weight from each other. Figure 22 shows SIM traces of the LC/MS method developed, using positive ion mode electrospray ionization and selective ion monitoring with the quadrupole MS instrument. Retention times were 1.0 min for **55**, 1.6 min for **56**, 4.3 min for internal standard **1**, 5.6 min for **27**, 6.8 min for **28**, 14.6 min for **30**, 14.7 min for **29**, 15.2 min for **31**, 15.6 min for **52**, and 16.8 min for **54**. For amino acids **55** and **56** the ions monitored were the protonated molecules $[M+H]^+$, while for compounds **28** – **31**, and **52** the sodium adducts $[M+Na]^+$ and for compounds **27** and **54** the fragment ions $[M+H-H_2O]^+$ were monitored.

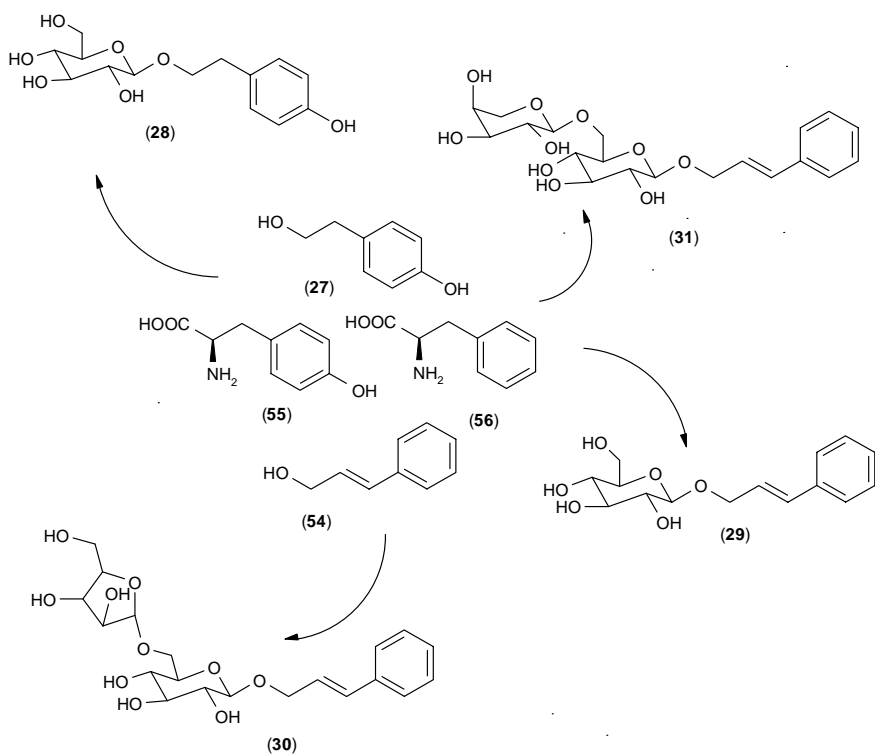


Fig. 21. Assumed biotransformation in *R. rosea* cell suspension.

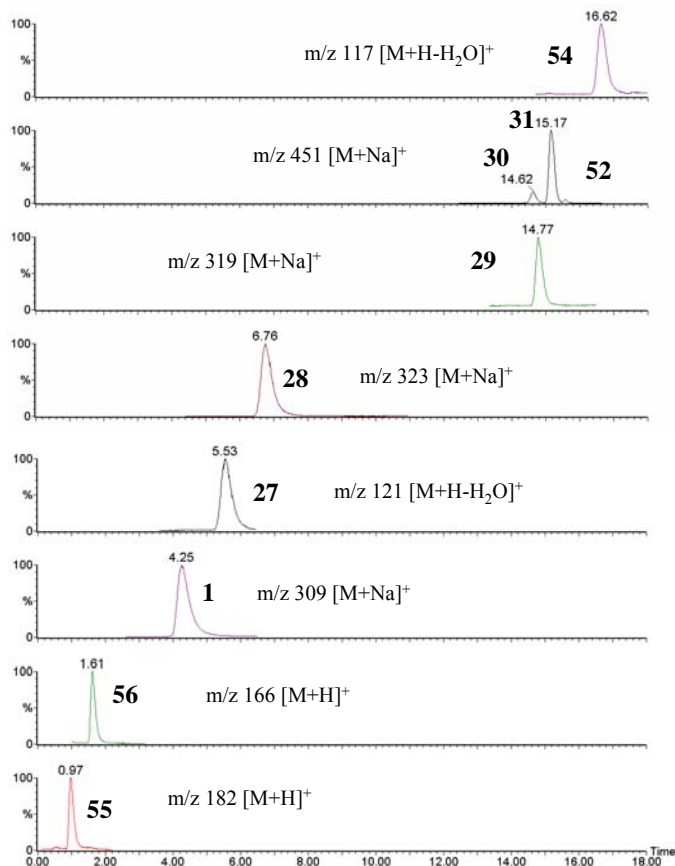


Fig. 22. LC/MS (ESI⁺) SIM chromatograms for precursor and product compounds in *R. rosea* biotransformation studies. The monitored ions are as indicated. Compound 1 is the internal standard salicin. A Waters Polarity 2.1×50 mm column with 3 μm particle size was used together with Phenomenex maxRP 2*5.0 mm precolumn at 35°C. Flow rate was 0.30 ml/min and injection volume 5 μl. Gradient: (A= Water, B=acetonitrile, C=methanol) 0 min 96% A : 2% B : 2% C, 2 min 96% A : 2% B : 2% C, 16 min 68% A : 16% B : 16% C. Nitrogen was used as nebulizer and desolvation gas at 150 °C and 320 °C, respectively. Cone voltages were 24 V for amino acids 55 and 56, 30 V for 1 and 28, 22 V for 29 – 31, and 52 and 32 V for 27 and 54. Capillary voltage was 3.4 kV. The cell samples were freeze-dried and powdered and 30 mg was extracted for 60 min in ultra sound sonication with 1.5 ml 60% methanol containing 400 ng/ml salicin. The extract was filtered and diluted (depending on concentration, 1:999 for cells obtained from 5 mM precursor medium) with 6% methanol containing 400 ng/ml salicin. The suspension medium samples were analyzed with direct injection after filtration and dilution (depending on concentration, 1:999 for 5mM precursor medium) with 6% methanol containing 400 ng/ml salicin (1).

For calibration of the method, eight concentration levels of standards were prepared for **55**, **56**, **27**, **28**, **31**, and **54** and each level was injected in triplicate. Good linearity of detection with correlation coefficients higher than 0.989 was obtained for all compounds for the ranges presented in Table 3. The method has been applied successfully in a number of biotransformation studies relevant to the production of rosin, rosavin, and salidroside in cell culture suspensions and in bioreactors [154].

Table 3. Linear ranges and accuracy and precision results for LC/MS method in SIM mode for biotransformation precursor and product compounds of R. rosea.

Compound	Linear range (ng/ml)	Correlation coefficient	Accuracy (%-dev) in different concentrations	Precision (%-SD) in different concentrations
55	5 – 500	0.999	-2.2 ... +10.1	0.5...4.8
56	5 – 500	0.999	-6.0 ... +14.0	1.6...5.7
27	5 – 2000	0.991	-7.4 ... +12.0	1.7...8.0
28	5 – 500	0.998	-4.7 ... +7.2	1.5...8.0
31	5 – 2000	0.999	-8.9 ... +1.5	0.5...5.7
54	5 – 500	0.989	-10.2 ... +15.0	2.5...8.6

5 Conclusions

The broad capability of HPLC in natural product analysis was exploited in the development of accurate and reliable methods for quantitative analysis of plant secondary metabolites. Naphthodianthrone and phloroglucinol metabolites were measured in *H. perforatum* plant and cell culture samples, and phenylethyl- and phenylpropanoid glycosides in matrixes of *R. rosea*. Liquid chromatography was also used as a semi-preparative isolation method, with compounds of interest collected directly from the column. Atmospheric pressure ionization mass spectrometry provided a highly sensitive and selective detector for HPLC. Relative to UV detection, it produced a marked improvement in sensitivity it and shortened the analysis times by allowing a more straightforward and faster elution of the compounds. The high selectivity was achieved by detection solely of compound specific ions (selective ion monitoring) or of compound specific dissociation reactions in collision cell of a triple quadrupole instrument (multiple reaction monitoring).

Together with one- and two-dimensional NMR spectroscopy, mass spectrometry was also used as a powerful tool in the structural elucidation of novel compounds in plant extracts. Tandem mass spectrometric (MS/MS) experiments were done to collect structural data of the compounds in the form of fragment ions. The informational value of these MS/MS experiments was particularly for compounds with similar aglycone-glycoside-type structures. The elemental composition of compounds was obtained from high resolution accurate mass measurements with a time-of-flight MS instrument. The results underline the importance of the using several analytical methods in a versatile way to obtain the highest possible amount of structural data from unknown compounds and so to elucidate their exact structure. Here, mass spectrometry provided confirmation for the structural data obtained by NMR, and vice versa, and both techniques also offered some unique structural data.

The methods developed in this work make possible the identification and quantitative analysis of a wider variety of secondary metabolites in the extracts of the *R. rosea* and *H. perforatum*, in shorter time and more sensitively than earlier.

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Appendix 1

Tyrosol (**27**): UV (in water-acetonitrile) λ_{\max} nm: 276. ESI⁺ (m/z, rel. Int %) 161 [M+Na]⁺ (100). ESI⁻ (m/z, rel. Int %) 137 [M-H]⁻ (100). ¹H NMR (500 MHz, in D₂O) 7.05 ppm (d, 9.0 Hz, H-2 & H-6), 6.72 ppm (d, 9.0 Hz, H-3 & H-5), 3.66 ppm (t, 7.3 Hz, H-4), 2.65 ppm (t, 7.3 Hz, H-7).

Salidroside (**28**): UV (in water-acetonitrile) λ_{\max} nm: 276. HRMS: 323.1098 Da, (calc for C₁₄H₂₀O₇Na 323.1107 Da). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 318 [M+NH₄]⁺ (10), 265 [M+NH₄-53]⁺ (15), 247 [M+NH₄-71]⁺ (15), 229 [M+NH₄-89]⁺ (15), 205 [M+NH₄-113]⁺ (35), 187 [M+NH₄- 296]⁺ (30), 180 [M+NH₄-131]⁺ (20), 163 [M+NH₄-155]⁺ (100), 145 [M+NH₄-173]⁺ (95), 127 [M+NH₄-191]⁺ (15), 121 [M+NH₄-197]⁺ (95), 85 [M+NH₄-233]⁺ (25). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 299 [M-H]⁻ (10), 179 [M-H-120]⁻ (15), 161 [M-H-138]⁻ (6), 149 [M-H-150]⁻ (8), 119 [M-H-180]⁻ (90), 113 [M-H-186]⁻ (20), 101 [M-H-198]⁻ (20), 89 [M-H-210]⁻ (100). ¹H NMR (500 MHz, in D₂O) 7.09 ppm (d, 8.8 Hz, H-2 & H-6), 6.73 ppm (d, 8.8 Hz, H-3 & H-5), 4.32 ppm (d, 8.3 Hz, H-1'), 3.96 ppm (td, -10.3/7.4 Hz, H-8a & H-8b), 3.76 ppm (dd, -13.6/2.2 Hz, H-6'a), 3.57 ppm (dd, -13.6/6.5 Hz, H-6'b), 3.33 ppm (dd, 10.2/9.4 Hz, H-3'), 3.28 ppm (ddd, 9.8/6.5/2.2 Hz, H-5'), 3.23 ppm (dd, 9.8/9.4 Hz, H-4'), 3.10 ppm (d, 10.2/8.3 Hz, H-2'), 2.75 ppm (t, 7.4 Hz, H-7).

Rosin (**29**): UV (in water-acetonitrile) λ_{\max} nm: 250. HRMS: 319.1170 Da, (calc for C₁₅H₂₀O₆Na 319.1158 Da). ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 319 [M+Na]⁺ (100), 287 [M+Na-32]⁺ (10), 215 [M+Na-104]⁺ (10), 201 [M+Na-218]⁺ (20), 179 [M+Na-140]⁺ (8), 155 [M+Na-164]⁺ (8), 117 [M+Na-202]⁺ (15), 105 [M+Na-214]⁺ (6), CID of [M+NH₄]⁺, 15 eV, 314 [M+NH₄]⁺ (15), 117 [M+NH₄-197]⁺ (100). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 314 [M+NH₄]⁺ (15), 117 [M+NH₄-197]⁺ (100). ¹H NMR (500 MHz, in D₂O) 7.42 ppm (d, 8.2 Hz, H-2 & H-6), 7.32 ppm (d, 8.2 Hz, H-3 & H-5), 7.25 ppm (dd, 7.9/1.4 Hz, H-4), 6.67 ppm (d, 16.4 Hz, H-7), 6.33 ppm (ddd, 16.4/7.3/6.9 Hz, H-8), 4.46 ppm (d, 8.4 Hz, H-1'), 4.44 ppm (dd, -13.2/7.3 Hz, H-9a), 4.32 ppm (dd, -13.2/6.9 Hz, H-9b), 3.82 ppm (dd, -13.9/1.8 Hz, H-6'a), 3.62 ppm (dd, -13.9/7.2 Hz, H-6'b), 3.38 ppm (dd, 10.6/8.9 Hz, H-3'), 3.35 ppm (ddd 8.6/7.2/1.8 Hz, H-5'), 3.29 ppm (dd, 10.6/8.6 Hz, H-4'), 3.24 ppm (d, 8.9/8.4 Hz, H-2').

Rosarin (**30**): UV (in water-acetonitrile) λ_{\max} nm: 250. HRMS: 451.1590 Da, (calc for C₂₀H₂₈O₁₀Na 451.1580 Da). ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 451 [M+Na]⁺ (50), 333 [M+Na-118]⁺ (60), 319 [M+Na-132]⁺ (25), 275 [M+Na-176]⁺ (7), 155 [M+Na- 296]⁺ (20), 117 [M+Na-334]⁺ (100). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 427 [M-H]⁻ (10), 293 [M-H-134]⁻ (25), 161 [M-H-266]⁻ (100), 149 [M-H-278]⁻ (35), 131 [M-H-296]⁻ (30), 125 [M-H-302]⁻ (30), 113 [M-H-314]⁻ (8), 101 [M-H-326]⁻ (10), 89 [M-H-338]⁻ (75). ¹H NMR (500 MHz, in D₂O) 7.69 ppm (dd, 7.6/1.4 Hz, H-2 & H-6), 7.63 ppm (dd, 7.7/7.6 Hz, H-3 & H-5), 7.58 ppm (dd, 7.7/1.4 Hz H-4), 6.97 ppm (d, 17.3 Hz, H-7), 6.63 ppm (ddd, 17.3/6.9/6.0 Hz, H-8), 5.28 ppm (s, H-1'), 4.77 ppm (d, 7.0 Hz, H-1'), 4.9-3.5 ppm (m, H-9a & H9b, H-2'-H6', H2''- H-5').

Rosavin (**31**): UV (in water-acetonitrile) λ_{\max} nm: 250. HRMS: 451.1592 Da, (calc for $C_{20}H_{28}O_{10}Na$ 451.1580 Da). ESI⁺ MS/MS of $[M+Na]^+$ (30 eV, m/z, rel. Int %) 451 $[M+Na]^+$ (25), 333 $[M+Na-118]^+$ (70), 319 $[M+Na-132]^+$ (15), 275 $[M+Na-176]^+$ (8), 201 $[M+Na-250]^+$ (10), 155 $[M+Na-296]^+$ (20), 117 $[M+Na-334]^+$ (100). ESI⁺ MS/MS of $[M+NH_4]^+$ (15 eV, m/z, rel. Int %) 446 $[M+NH_4]^+$ (10) 117 $[M+NH_4-329]^+$ (100). ESI⁻ MS/MS of $[M-H]^-$ (15 eV, m/z, rel. Int %) 427 $[M-H]^-$ (20), 293 $[M-H-134]^-$ (45), 233 $[M-H-194]^-$ (8), 191 $[M-H-236]^-$ (25), 161 $[M-H-266]^-$ (5), 149 $[M-H-278]^-$ (85), 131 $[M-H-296]^-$ (30) 125 $[M-H-302]^-$ (25), 113 $[M-H-314]^-$ (15), 101 $[M-H-326]^-$ (10), 89 $[M-H-338]^-$ (100). ¹H NMR (500 MHz, in D₂O) 7.44 ppm (dd, 7.6/1.4 Hz, H-2 & H-6), 7.33 ppm (dd, 7.7/7.6 Hz, H-3 & H-5), 7.27 ppm (dd, 7.7/1.4 Hz H-4), 6.68 ppm (d, 16.0 Hz, H-7), 6.34 ppm (ddd, 16.0/6.9/6.0 Hz, H-8), 4.48 ppm (d, 8.1 Hz, H-1'), 4.45 ppm (dd, -12.8/6.0 Hz, H-9a), 4.35 ppm (d, -12.8/6.9 Hz, H-9b), 4.30 ppm (d, 7.7 Hz, H-1''), 4.07 ppm (dd, -11.8/1.8 Hz, H-6'a), 3.83 ppm (ddd, 3.4/2.6/0.6 Hz, H-4''), 3.82 ppm (dd, -12.9/2.6 Hz, H-5'ax), 3.75 ppm (dd, -11.8/6.4 Hz, H-6'b), 3.55 ppm (dd, 9.6/3.4 Hz, H-3''), 3.54 ppm (m, -12.9/0.6 Hz, H-5''eq), 3.52 ppm (m, 10.1/6.4/2.1 Hz, H-5'), 3.49 ppm (dd, 9.6/7.7 Hz, H-2''), 3.40 ppm (dd, 9.1/8.8 Hz, H-4'), 3.40 ppm (dd, 9.9/8.8 Hz, H-3'), 3.24 ppm (dd, 9.9/8.1 Hz, H-2'). ¹³C NMR (500 MHz, in D₂O) 137.1 ppm (C-1), 134.3 ppm (C-7), 129.4 ppm (C-3 & C-5), 128.6 ppm (C-4), 127.2 ppm (C-2 & C-6), 125.1 ppm (C-8), 103.9 ppm (C-1''), 101.6 ppm (C-1'), 75.9 ppm (C-3'), 75.2 ppm (C-5'), 73.0 ppm (C-2'), 72.3 ppm (C-3''), 70.8 ppm (C-2''), 70.4 ppm (C-9), 69.1 ppm (C-4'), 68.3 ppm (C-6'), 68.0 ppm (C-4''), 66.0 ppm (C-5').

Sachalicide 1 (Triandrin, **32**): UV (in water-acetonitrile) λ_{\max} nm: 260. HRMS: 335.1110 Da, (calc for $C_{15}H_{20}O_7Na$ 335.1107 Da). ESI⁺ MS/MS of $[M+Na]^+$ (30 eV, m/z, rel. Int %) 335 $[M+Na]^+$ (15), 203 $[M+Na-132]^+$ (20), 155 $[M+Na-180]^+$ (20), 133 $[M+Na-202]^+$ (100), 105 $[M+Na-230]^+$ (10). ESI⁺ MS/MS of $[M+NH_4]^+$ (15 eV, m/z, rel. Int %) 330 $[M+NH_4]^+$ (10), 133 $[M+NH_4-197]^+$ (100). ¹H NMR (500 MHz, in D₂O) 7.30 ppm (d, 8.7 Hz, H-2 & H-6), 6.74 ppm (d, 8.7 Hz, H-3 & H-5), 6.57 ppm (d, 17.3 Hz, H-7), 6.13 ppm (ddd, 17.3/8.4/7.2 Hz, H-8), 4.44 ppm (d, 8.3 Hz, H-1'), 4.40 ppm (dd, -12.9/7.2 Hz, H-9a), 4.27 ppm (dd, -12.9/8.4 Hz, H-9b), 3.80 ppm (dd, -13.6/2.2 Hz, H-6'a), 3.61 ppm (dd, -13.6/6.8 Hz, H-6'b), 3.37 ppm (dd, 10.2/9.3 Hz, H-3'), 3.33 ppm (ddd 9.8/6.8/2.2 Hz, H-5'), 3.28 ppm (dd, 9.8/9.3 Hz, H-4'), 3.19 ppm (d, 10.2/8.3 Hz, H-2'). ¹³C NMR (500 MHz, in D₂O) 157.6 ppm (C-4), 134.5 ppm (C-7), 134.0 ppm (C-1), 128.7 ppm (C-2 & C-6), 122.0 ppm (C-8), 116.4 ppm (C-3 & C-5), 101.3 ppm (C-1'), 76.1 ppm (C-3'), 76.0 ppm (C-5'), 73.0 ppm (C-2'), 70.7 ppm (C-9), 69.5 ppm (C-4'), 60.6 ppm (C-6').

4-methoxy-cinnamyl-O- β -D-glucopyranoside (vimalin, **33**): UV (in water-acetonitrile) λ_{\max} nm: 260. HRMS: 349.1241 Da, (calc for $C_{16}H_{22}O_7Na$ 349.1263 Da). ESI⁺ MS/MS of $[M+Na]^+$ (30 eV, m/z, rel. Int %) 349 $[M+Na]^+$ (100), 331 $[M+Na-18]^+$ (3), 201 $[M+Na-148]^+$ (4), 147 $[M+Na-202]^+$ (70), 91 $[M+Na-258]^+$ (22). ESI⁺ MS/MS of $[M+NH_4]^+$ (15 eV, m/z, rel. Int %) 344 $[M+NH_4]^+$ (8), 147 $[M+NH_4-197]^+$ (100). ¹H NMR (500 MHz, in D₂O) 7.39 ppm (d, 9.2 Hz, H-2 & H-6), 6.91 ppm (d, 9.2 Hz, H-3 & H-5), 6.61 ppm (d, 17.4 Hz, H-7), 6.19 ppm (ddd, 17.4/7.7/6.7 Hz, H-8), 4.45 ppm (d, 8.5 Hz, H-1'), 4.42 ppm (dd, -12.9/6.7 Hz, H-9a), 4.27 ppm (dd, -12.9/7.7 Hz, H-9b), 3.81 ppm (dd, -13.6/2.1 Hz, H-6'a), 3.74 ppm (s, -Ome), 3.62 ppm (dd, -13.6/6.4 Hz, H-6'b), 3.38 ppm (dd, 9.8/9.6 Hz, H-3'), 3.34 ppm (ddd 9.6/6.4/2.1 Hz, H-5'), 3.29 ppm (dd, 9.6/9.6 Hz, H-4'), 3.20 ppm (dd, 9.8/8.5 Hz, H-2').

Cinnamyl-(6'-O- β -xylopyranosyl)-O- β -glucopyranoside (**52**): UV (in water-acetonitrile) $\lambda_{\text{max}}^{\text{nm}}$ 250. HRMS 451.1600 Da (calc for C₂₀H₂₈O₁₀Na 451.1580 Da). ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 451 [M+Na]⁺ (60), 333 [M+Na-118]⁺ (70), 319 [M+Na-132]⁺ (25), 275 [M+Na-176]⁺ (6), 201 [M+Na-250]⁺ (20), 155 [M+Na-296]⁺ (6), 117 [M+Na-334]⁺ (100). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 446 [M+NH₄]⁺ (15), 117 [M+NH₄-329]⁺ (100). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 427 [M-H]⁻ (85), 293 [M-H-134]⁻ (45), 233 [M-H-194]⁻ (7), 191 [M-H-236]⁻ (35), 161 [M-H-266]⁻ (100), 149 [M-H-278]⁻ (85), 131 [M-H-296]⁻ (25), 125 [M-H-302]⁻ (30), 113 [M-H-314]⁻ (15), 101 [M-H-326]⁻ (30), 89 [M-H-338]⁻ (90). ¹H NMR (500 MHz, in D₂O) 7.43 ppm (dd, 8.2/1.0 Hz, H-2 & H-6), 7.32 ppm (dd, 8.2/7.6 Hz, H-3 & H-5), 7.25 ppm (tt, 7.6/1.0 Hz H-4), 6.66 ppm (d, 17.3 Hz, H-7), 6.32 ppm (ddd, 17.3/7.8/6.5 Hz, H-8), 4.46 ppm (d, 8.5 Hz, H-1'), 4.43 ppm (dd, -12.7/6.5 Hz, H-9a), 4.34 ppm (d, 8.7 Hz, H-1''), 4.33 ppm (d, -12.7/7.8 Hz, H-9b), 4.04 ppm (dd, -12.8/1.7 Hz, H-6'a), 3.82 ppm (dd, -12.4/6.0 Hz, H-5''ax), 3.74 ppm (dd, -12.8/6.2 Hz, H-6'b), 3.50 ppm (dd, 9.7/6.0 Hz, H-4''), 3.50 ppm (m, 9.1/6.2/1.7 Hz, H-5'), 3.38 ppm (dd, 9.7/9.6 Hz, H-3'), 3.36 ppm (dd, 10.1/9.3 Hz, H-4'), 3.32 ppm (dd, 9.7/9.6 Hz, H-3''), 3.22 ppm (dd, 9.8/8.5 Hz, H-2'), 3.20 ppm (d, 12.4 Hz, H-5''eq), 3.18 ppm (dd, 9.6/8.7 Hz, H-2''). ¹³C NMR (500 MHz, in D₂O) 137.0 ppm (C-1) 134.2 ppm (C-7), 129.2 ppm (C-3 & C-5), 128.9 ppm (C-4), 126.9 ppm (C-2 & C-6), 125.2 ppm (C-8), 103.9 ppm (C-1'), 101.6 ppm (C-1''), 76.1 ppm (C-3'), 75.9 ppm (C-3''), 75.1 ppm (C-5'), 73.4 ppm (C-2'), 73.2 ppm (C-2''), 70.7 ppm (C-9), 69.7 ppm (C-4'), 69.2 ppm (C-4''), 68.9 ppm (C-6'), 65.5 ppm (C-5'').

4-Methoxy-cinnamyl-(6'-O- α -arabinopyranosyl)-O- β -glucopyranoside (**53**): UV (in water-acetonitrile) $\lambda_{\text{max}}^{\text{nm}}$ 260. HRMS 481.1661 Da (calc for C₂₁H₃₀O₁₁Na 481.1686 Da) ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 481 [M+Na]⁺ (30), 333 [M+Na-148]⁺ (25), 275 [M+Na-206]⁺ (6), 201 [M+Na-280]⁺ (5), 147 [M+Na-334]⁺ (100). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 476 [M+NH₄]⁺ (10), 147 [M+NH₄-329]⁺ (100). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 457 [M-H]⁻ (20), 293 [M-H-164]⁻ (40), 233 [M-H-224]⁻ (15), 191 [M-H-266]⁻ (15), 161 [M-H-296]⁻ (15), 149 [M-H-308]⁻ (60), 131 [M-H-326]⁻ (35), 125 [M-H-332]⁻ (20), 113 [M-H-344]⁻ (10), 101 [M-H-356]⁻ (8), 89 [M-H-368]⁻ (100). ¹H NMR (500 MHz, in D₂O) 7.39 ppm (d, 9.3 Hz, H-2 & H-6), 6.91 ppm (d, 9.3 Hz H-3 & H-5), 6.61 ppm (d, 17.3 Hz, H-7), 6.19 ppm (ddd, 17.3/6.9/6.0 Hz, H-8), 4.46 ppm (d 8.8 Hz, H-1'), 4.41 ppm (dd, -12.7/6.0 Hz, H-9a), 4.30 ppm (d, -12.7/6.9 Hz, H-9b), 4.29 ppm (d 8.4 Hz, H-1''), 4.05 ppm (dd, -12.6/1.8 Hz, H-6'a), 3.82 ppm (dd, 4.5/2.9 Hz, H-4''), 3.80 ppm (dd, -13.1/2.9 Hz, H-5''ax), 3.75 ppm (s, -OMe), 3.72 ppm (dd, -12.6/5.6 Hz, H-6'b), 3.54 ppm (dd, 9.3/4.5 Hz, H-3''), 3.53 ppm (d, -13.1 Hz, H-5''eq), 3.49 ppm (dd, 11.5/5.6 Hz, H-5'), 3.48 ppm (dd, 9.3/8.4 Hz, H-2''), 3.38 ppm (dd, 11.5/8.8 Hz, H-4'), 3.38 ppm (dd, Hz, 8.8/8.7 H-3'), 3.22 ppm (dd, 8.8/8.7 Hz, H-2'). ¹³C NMR (500 MHz, in D₂O) 159.3 ppm (C-4), 133.7 ppm (C-7), 129.9 ppm (C-1), 128.5 ppm (C-2 & C-6) 123.3 ppm (C-8), 114.8 ppm (C-3 & C-5) 104.0 ppm (C-1''), 101.6 ppm (C-1'), 76.0 ppm (C-3'), 75.3 ppm (C-5'), 73.5 ppm (C-2'), 72.6 ppm (C-3''), 70.8 ppm (C-9), 70.1 ppm (C-2''), 69.5 ppm (C-4'), 68.7 ppm (C-6'), 68.5 ppm (C-4''), 66.5 ppm (C-5''), 55.7 ppm (-OMe).

Picein (4-O- β -D-glucopyranosyl)-acetophenone (**50**): UV (in water-acetonitrile) λ_{\max} nm 266. HRMS 321.0943 Da (calc for $C_{14}H_{18}O_7Na$ 321.0950 Da) ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 321 [M+Na]⁺ (100), 303 [M+Na-18]⁺ (15), 257 [M+Na-243]⁺ (75), 158 [M+Na-163]⁺ (15). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 316 [M+NH₄]⁺ (10), 137 [M+NH₄-179]⁺ (100). ¹H NMR (500 MHz, in D₂O) 7.93 ppm (d, 9.2 Hz, H-3 & H-5), 7.11 ppm (d, 9.2 Hz, H-2 & H-6), 5.16 ppm (d, 7.7 Hz, H-1'), 3.84 ppm (dd, -12.9/2.3 Hz, H-6'a), 3.66 ppm (dd, -12.9/6.0 Hz, H-6'b), 3.59 ppm (ddd 9.6/6.4/2.1 Hz, H-5'), 3.53 ppm (dd, 10.8/6.0/2.3 Hz, H-3'), 3.52 ppm (dd, 9.0/7.7 Hz, H-2'), 3.41 ppm (dd, 10.8/9.4 Hz, H-4'), 2.25 ppm (s, H-Me). ¹³C NMR (500 MHz, in D₂O) 196.7 ppm (C-7), 161.2 ppm (C-1), 131.5 ppm (C-3 & C-5), 131.4 ppm (C-4), 116.4 ppm (C-2 & C-6), 99.6 ppm (C-1'), 76.5 ppm (C-5'), 75.8 ppm (C-3'), 73.2 ppm (C-2'), 69.9 ppm (C-4'), 60.9 ppm (C-6'), 33.6 ppm (C-Me).

Benzyl-O- β -D-glucopyranoside (**51**): UV (in water-acetonitrile) λ_{\max} nm 206, 256. HRMS 293.1009 Da (calc for $C_{13}H_{18}O_7Na$ 293.1001 Da) ESI⁺ MS/MS of [M+Na]⁺ (30 eV, m/z, rel. Int %) 293 [M+Na]⁺ (100), 207 [M+Na-86]⁺ (7), 175 [M+Na-118]⁺ (9), 107 [M+Na-186]⁺ (15). ESI⁺ MS/MS of [M+NH₄]⁺ (15 eV, m/z, rel. Int %) 288 [M+NH₄]⁺ (2), 163 [M+NH₄-125]⁺ (20), 145 [M+NH₄-143]⁺ (25), 97 [M+NH₄-191]⁺ (30), 91 [M+NH₄-197]⁺ (100). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 269 [M-H]⁻ (15), 161 [M-H-108]⁻ (50), 113 [M-H-156]⁻ (45), 101 [M-H-168]⁻ (100). ¹H NMR (500 MHz, in D₂O) 7.38 ppm (m, 7.7/1.4 Hz, H-4), 7.37 ppm (m, 7.6/1.4 Hz, H-2 & H-6), 7.34 ppm (m, 7.7/7.6 Hz, H-3 & H-5), 4.83 ppm (d, -11.6 Hz, H-7a), 4.66 ppm (d, -11.6 Hz, H-7b), 4.42 ppm (d, 8.0 Hz, H-1'), 3.82 ppm (dd, -12.3/2.2 Hz, H-6'a), 3.63 ppm (dd, -12.3/6.1 Hz, H-6'b), 3.35 ppm (dd, 9.4/9.2 Hz, H-3'), 3.34 ppm (ddd 10.0/6.1/2.2 Hz, H-5'), 3.29 ppm (dd, 10.0/9.2 Hz, H-4'), 3.19 ppm (dd, 9.4/8.0 Hz, H-2'). ¹³C NMR (500 MHz, in D₂O) 136.7 ppm (C-1) 129.1 ppm (C-4), 128.9 ppm (C-2 & C-6), 128.7 ppm (C-3 & C-5), 101.3 ppm (C-1'), 76.2 ppm (C-5' & C-3'), 73.2 ppm (C-2'), 71.9 ppm (C-7), 69.9 ppm (C-4'), 61.4 ppm (C-6').

Rosiridin (**35**): HRMS 355.1783 Da (calc for $C_{16}H_{28}O_7Na$ 355.1773 Da) ESI⁺ MS (m/z, rel. Int %) 355 [M+Na]⁺ (100). ESI⁻ MS/MS of [M-H]⁻ (15 eV, m/z, rel. Int %) 331 [M-H]⁻ (25), 179 [M-H-152]⁻ (100). ¹H NMR (500 MHz, in D₂O) 5.47 ppm (t, 7.8 Hz, H-2), 5.03 ppm (t, 7.7 Hz, H-6), 4.37 ppm (d 8.4 Hz, H-1'), 4.28 ppm (d, 7.8 Hz, H-1), 4.07 ppm (d, 8.2 Hz, H-4), 3.81 ppm (dd, -13.5/2.2 Hz, H-6'a) 3.62 ppm (dd, -13.5/6.4 Hz, H-6'b), 3.37 (dd, 9.6/9.4 Hz, H-3'), 3.32 ppm (ddd 9.7/6.4/2.2 Hz, H-5'), 3.28 ppm (dd, 9.7/9.4 Hz, H-4'), 3.17 ppm (dd, 9.6/8.4 Hz, H-2'), 2.21 ppm (dd, 8.2/7.7 Hz, H-5), 1.61 ppm (s, H-CH₃ 8/9), 1.57 ppm (d, 14.5 Hz, H-CH₃ 10), 1.53 ppm (s, H-CH₃ 8/9).