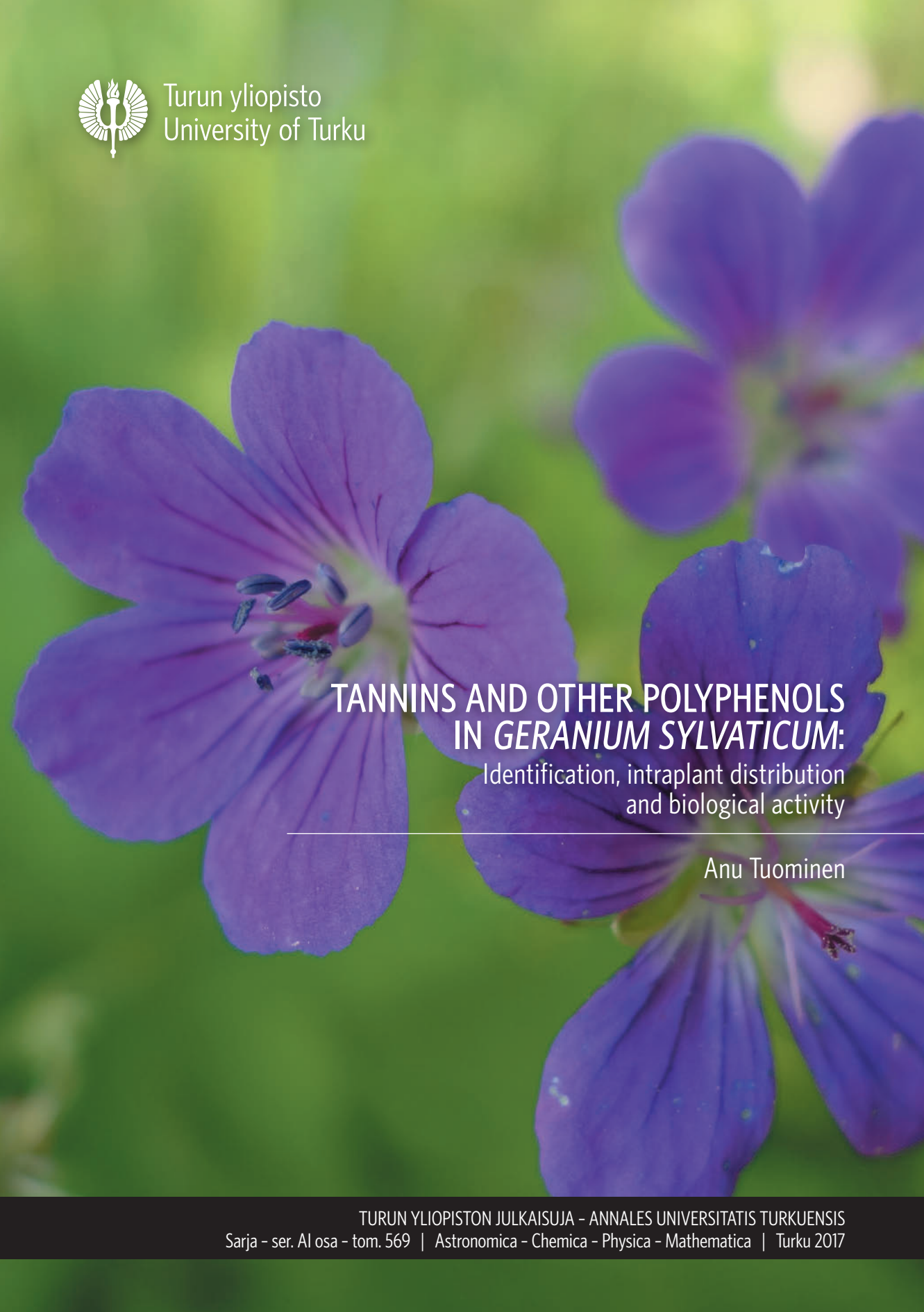

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Identification, intraplant distribution and biological activity

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ABSTRACT

UNIVERSITY OF TURKU
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TUOMINEN, ANU: Tannins and other polyphenols in *Geranium sylvaticum*: Identification, intraplant distribution and biological activity

Doctoral thesis, 241 p.
Laboratory of Organic Chemistry and Chemical Biology/
Natural Compound Chemistry
December 2017

This thesis studies the phenolic compounds of *Geranium sylvaticum* L. It focuses especially on geraniin and related ellagitannins. The literature review covers the phytochemistry of *Geranium* species, the biological activity of tannins and methods for the analysis and identification of tannins.

In the experimental work, methods were developed for the extraction and high-pressure liquid chromatographic analysis of polyphenols in *G. sylvaticum* organs and their degradation products in the alkaline pH. Using a high-resolution mass spectrometry and diode array detection, water-soluble polyphenols in the organs of *G. sylvaticum* were tentatively identified and studied for their seasonal variation. Moreover, the main compounds were isolated and structures further elucidated. The biological activity of organ extracts and fractions was measured with antioxidant activity and pro-oxidant activity assays.

Results showed that the plant organs of *G. sylvaticum* have distinctive intraplant variation in their phenolic profiles. Geraniin and other ellagitannins dominated in all the green photosynthetic organs. While the pistils accumulated gallotannins, the stamens kaempferol glycosides, and seeds and roots accumulated oligomeric proanthocyanidins. Of these, galloylglucoses, gallotannins and minor ellagitannins in particular showed seasonal and ontogenic variation. The petals contained unique acetylglucosylated hydrolysable tannins. These compounds showed capacity to act as copigments for anthocyanins. Due to the high content of ellagitannins and galloyl quinic acids, the leaf, pistil and hairy root extracts showed high antioxidant and pro-oxidant activity. Hydrolyzable tannins degraded fast in the alkaline conditions used in the pro-oxidant test and produced hydrolysis, deprotonation and oxidation products.

The observed intraplant distribution of the polyphenol groups, seasonal and ontogenic variation, and the various biological activities observed may reflect the different functions and importance of various types of tannins in the plants' interaction with pollinators, herbivores and/or pathogens.

Keywords: *Geranium sylvaticum*, tannins, biological activity

TIIVISTELMÄ

TURUN YLIOPISTO
Kemian laitos/Matemaattis-luonnontieteellinen tiedekunta

TUOMINEN, ANU: Metsäkurjenpolven (*Geranium sylvaticum*) tanniinit ja muut polyfenolit: tunnistaminen, kasvinsisäinen jakaantuminen ja biologinen aktiivisuus

Väitöskirja, 241 s.
Orgaanisen kemian ja kemiallisen biologian laboratorio/
Luonnonyhdisteiden kemia
Joulukuu 2017

Tutkimustyö käsittelee metsäkurjenpolven (*Geranium sylvaticum* L.) fenolisia yhdisteitä keskittyen erityisesti geraniiniin ja muihin ellagitanniineihin. Kirjallisuusosiossa käydään läpi kurjenpolvikasvien kemiaa, tanniinien biologista aktiivisuutta ja niiden analysointimenetelmiä.

Kokeellisessa osiossa kehitettiin nestekromatografisia analyysimenetelmiä, joilla tutkittiin metsäkurjenpolven eri kasvinosien polyfenoleja sekä niiden hajoamistuotteita emäksisissä olosuhteissa. Vesiliukoiset polyfenolit tunnistettiin ja niiden kasvukauden aikainen vaihtelu metsäkurjenpolven eri kasvinosissa tutkittiin käyttäen massaspektrometriaa. Lisäksi pääyhdisteet eristettiin tarkempaa rakenteen määrittämistä varten. Kasvinosauutteiden ja fraktioiden biologista aktiivisuutta mitattiin antioksidantti- ja pro-oksidanttiaktiivisuustesteillä.

Tuloksista nähtiin, että metsäkurjenpolven kasvinosien polyfenolikoostumukset erosivat merkittävästi. Geraniinia ja muita ellagitanniineja esiintyi etenkin vihreissä yhteyttävissä osissa. Emeistä löytyi korkeita pitoisuuksia gallotanniineja, heteistä kemferoliglykosideja, siemenistä ja juurista oligomeerisia proantosyanidiineja. Näistä etenkin galloyyliglukooseilla, gallotanniineilla ja ellagitanniineilla havaittiin kasvukaudenaikaista ja kasvuvaiheesta riippuvaa vaihtelua. Terälehdistä tunnistettiin aivan uusi asetyyliglykosyloituneiden hydrolysoituvien tanniinien ryhmä, joiden havaittiin toimivan antosyaanien väriä tehostavina kopigmentteinä. Korkean ellagitanniini- ja galloyylikviinihappopitoisuuden takia etenkin lehti-, emi- ja hiusjuuriuutteet osoittivat voimakasta antioksidantti- ja pro-oksidanttiaktiivisuutta. Hydrolysoituvat tanniinit hajosivat nopeasti pro-oksidanttitestissä käytetyissä emäksisissä olosuhteissa ja reaktiossa syntyi hydrolyysi-, deprotonaatio- ja hapettumistuotteita.

Havaittu kasvinsisäinen jakaantuminen, kasvukauden ja kasvuvaiheen aikainen vaihtelu ja biologinen aktiivisuus kuvastavat kuinka erityyppisillä tanniineilla voi olla erilaisia tehtäviä ja merkitys kasvin ja sen pölyttäjien, kasvinsyöjien sekä patogeenien välisissä vuorovaikutuksissa.

Avainsanat: *Geranium sylvaticum*, metsäkurjenpolvi, tanniinit, biologinen aktiivisuus

TABLE OF CONTENTS

PREFACE AND ACKNOWLEDGEMENTS

This work was carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku since September 2007. I gratefully acknowledge the Emil Aaltonen Foundation, my main funding source for almost four years. I equally acknowledge other sponsors: the Turku University Foundation and the Department of Chemistry. Travel grants from the Turku University Foundation, the Palomaa-Erikoski Scholarship Fund, Finnish Mass Spectrometry Society, the Graduate School of Organic Chemistry and Chemical Biology, and the Doctoral Programme in Physical and Chemical Sciences enabled my participation in international conferences. I am also grateful to Bayer Oy and especially Dr. Piia Hara and Dr. Pirjo Kortesuo for facilitating the completion of this thesis.

First of all, I want to express my deepest gratitude to my supervisor, Professor Juha-Pekka Salminen for giving me the opportunity to this thesis project. I admire your passion, enthusiasm and ambition for science, especially for ellagitannins. I started this project during a time when the future of Natural Chemistry Research Group was still taking shape after the retirement of Professor Kalevi Pihlaja. Juha-Pekka has worked hard to build a strong research group, provided us with up-to-date high-quality instruments and maintained an enthusiastic spirit, which all has been invaluable for me. I am especially grateful that you gave me freedom and trust to do this Ph.D project independently and follow my own ideas. I am also thankful that you gave me the opportunity to the Teaching Lab period. It was a nice experience.

After maternity leave in autumn 2011, I received two other supervisors, Dr. Maarit Karonen and Dr. Jari Sinkkonen. Maarit is a true superwoman who has helped me a lot, especially with the proanthocyanidin and mass spectrometry issues, and her careful proof-reading and positive support has been crucial. I am also grateful about that she offered me the opportunity to act as the secretary of the Chemist Club of Turku for four years. Although this post may have postponed my graduation, it has been rewarding and taught me a lot of useful skills. Jari guided me through his expertise areas of NMR spectroscopy, structural elucidation and multivariate analysis. I needed a push to produce a more structure-oriented sylvatiin paper, and without you, it wouldn't have been possible.

I thank Professor Anurag Agrawal and Professor Herbert Kolodziej for reviewing my thesis and giving me valuable comments to improve it. Likewise, I thank Professor Kristiina Wähälä for accepting the role of acting as my opponent.

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The most important decision for women in science is the selection of a spouse. Jukka has done all the housekeeping work so that I can focus on science. He has given me the most important things in my life, our lovely daughters Minttu and Lilli—and two bonus daughters, Emma and Salla. You have all brought me lots of joy and happiness. You are always ready for adventures and to hit the road, whether it is driving around Finland to collect plants or to drive across Europe to attend a conference. Thank you a thousand times for your patience and love.

Turku, December 2017

Anu Thomin

Live as if you were to die tomorrow. Learn as if you were to live forever.

- Mahatma Gandhi-

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and some unpublished results. The publications are referred to in the text by their Roman numerals.

I Tuominen, A., Toivonen, E., Mutikainen, P., Salminen, J.-P., 2013. Defensive strategies in *Geranium sylvaticum*, Part 1: Organ-specific distribution of water-soluble tannins, flavonoids and phenolic acids. *Phytochemistry* 95, 394–407.

II Tuominen, A., 2013. Defensive strategies in *Geranium sylvaticum*, Part 2: Roles of water-soluble tannins, flavonoids and phenolic acids against natural enemies. *Phytochemistry* 95, 408–420.

III Tuominen, A., Sinkkonen, J., Karonen, M., Salminen, J.-P., 2015. Sylvatiins, acetylglucosylated hydrolysable tannins from the petals of *Geranium sylvaticum* show copigment effect. *Phytochemistry* 115, 239–251.

IV Tuominen, A., Sundman, T., 2013. Stability and oxidation products of hydrolysable tannins in basic conditions detected by HPLC/DAD-ESI/QTOF/MS. *Phytochem. Anal.* 24, 424–435.

V Tuominen, A., Karonen, M., 2017. Off-line 2-dimensional HPLC analysis of proanthocyanidins in *Geranium sylvaticum.* Manuscript submitted to *Phytochemistry*.

VI Tuominen, A., Salminen, J.-P., 2017. Hydrolyzable tannins, flavonol glycosides, and phenolic acids show seasonal and ontogenic variation in *Geranium sylvaticum*. *J. Agric. Food Chem.* 65(31), 6387–6403.

ABBREVIATIONS

AA	ascorbic acid
AO	antioxidant
CA	chlorogenic acid
CD	circular dichroism
CE	Cotton effect
COSY	correlation spectroscopy
DAD	diode array detector
DHHDP	dehydrohexahydroxydiphenoyl
DP	degree of polymerisation
DPPH	1,1-diphenyl-2-picrylhydrazyl
DQF-COSY	double quantum filtered COSY
DW	dry weight
EA	ellagic acid
ESI	electrospray ionisation
ET	ellagitannin
FLA	flavonoids
FW	fresh weight
G	galloyl
GA	gallic acid
GG	galloylglucose
Glu	D-glucose
GT	gallotannin
HHDP	hexahydroxydiphenoyl
HILIC	hydrophilic interaction liquid chromatography
HMBC	heteronuclear multiple bond coherence
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum correlation
HT	hydrolysable tannin
ID	internal diameter
PLSR	partial least squares regression
MALDI	matrix-assisted laser desorption ionization
m/z	mass-to-charge ratio
MRM	multiple reaction monitoring
MS	mass spectrometry
MW	molecular weigth
NHTP	nonahydroxytriphenoyl
NMR	nuclear magnetic resonance

NOESY	nuclear Overhauser effect spectroscopy
NP	normal phase
PA	proanthocyanidin
PC	procyanidin
PD	prodelphinidin
PGG	pentagalloyl glucose
PPC	protein precipitating capacity
PTFE	polytetrafluoroethylene
ROS	reactive oxygen species
RP	reversed phase
RT	retention time
SA	shikimic acid
SD	standard deviation
TOCSY	total correlation spectroscopy
TOF	time-of-flight
UHPLC/UPLC	ultra high-performance liquid chromatography
UV	ultraviolet
VIS	visible
Q	quadrupole
QA	quinic acid

1 INTRODUCTION

Phytochemistry combines chemistry and botany and studies secondary metabolites of plants. In contrast to primary metabolites, secondary metabolites exhibit special characteristics such as broad structural diversity, restricted taxonomic distribution, and specific biological activity.[1] They accumulate in particular organs and their chemical specialization occurs during normal plant growth.[1] Polyphenols are the largest and the most widespread group of the secondary metabolites of plants. They have a great variety of structures from simple compounds with few aromatic rings, such as phenolic acids and flavonoids, to highly polymeric substances, such as tannins and lignin. The newest definition specifies that polyphenols are compounds having more than one phenolic ring, do not contain nitrogen-based functional groups and are produced by the shikimate-derived phenylpropanoid and/or the polyketide pathways.[2] This thesis focuses on tannins in higher plants, which can be classified into proanthocyanidins (PAs) and hydrolysable tannins (HTs); the latter includes both gallotannins (GTs) and ellagitannins (ETs) (Fig. 1).

Chemical ecology tries to discover why secondary metabolites appear in plants, why there are so many different kinds of secondary metabolites and what is their role. Already in 1959, Fraenkel suggested that the purpose of secondary metabolites is to act as repellents or attractants to insect herbivores.[3] Most probably, plant polyphenols play multiple roles: reproduction, nutrition and growth, through interactions with insects, symbiotic fungi and bacteria above and below ground, as allelopathic signaling compounds, and the protection of plants from photodamage.[2,4-5] For example, it is well known that colorful flavonoids and anthocyanins act as pigments to attract pollinators and play a role in UV protection.[6] However, for tannins, the main hypothesis still is that they are produced for defensive chemicals against herbivores and pathogens.[7-11] Tannins have two features distinguishing them from other polyphenols: their tendency to bind to proteins, pigments, other large molecular compounds and metal ions, and high antioxidant activity.[12] These features are behind the health benefits for humans and the wide use of tannin-containing plant extracts in herbal medicine.[13]

The challenge in phytochemical studies is the complexity of plant extracts that can contain hundreds of secondary metabolites. In the past, tannins have been studied with spectrophotometric “total methods” that measured the amount of tannins as a bulk, actually counting the amount of hydroxy groups present in the tannins without acknowledging the real difference between different tannin groups.[14] Nowadays, precise HPLC-DAD-MS methods are used to identify and quantify tens of compounds from the sample during one analysis. Still, the presence of several isomers, oligomers and polymers demands specific knowledge and methods.

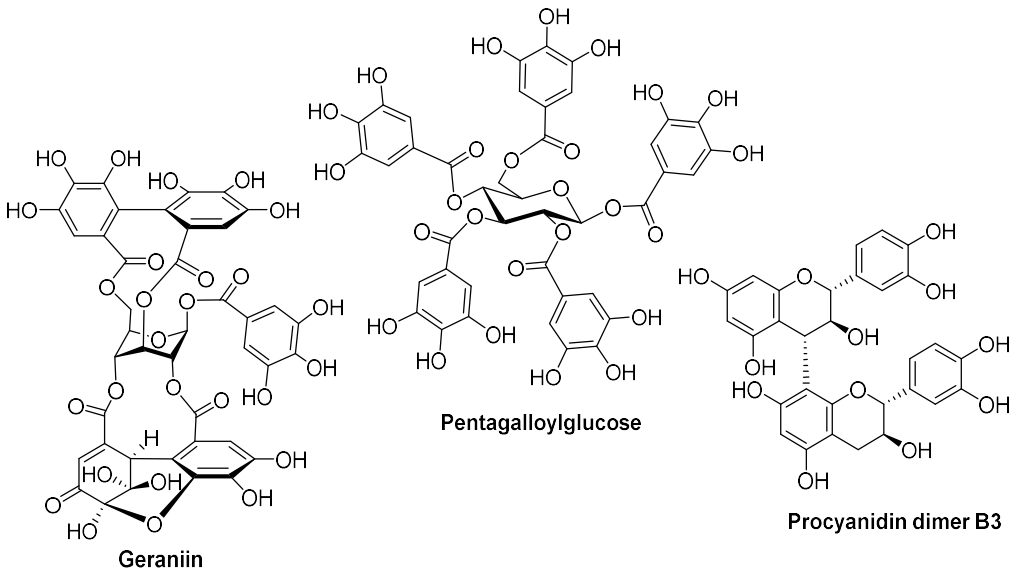


Figure 1. Typical tannin groups in *Geranium* species; pentagalloylglucose (PGG) belongs to galloylglucoses (GGs) and is the key compound of hydrolysable tannins; geraniin is an oxidatively transformed ellagitannin and B3 is a procyanidin dimer that belongs to proanthocyanidins (PAs).

Geranium sylvaticum was selected for the subject of this Ph.D study because of three reasons. First, *G sylvaticum* is known to be rich in geraniin and other tannins. However, the chemistry of this species has not been studied in detail before. Secondly, *G. sylvaticum* is interesting from an ecological point of view because of its gynodieous breeding system. Female plants need to compensate for the lack of male function to survive in populations. It has not been studied how the phenolic content differs between sexual morphs and whether that plays a role in the female's maintenance, for example, due to defense against herbivores or pollination chemistry. Third, *G. sylvaticum* is a common perennial plant in Finland and throughout Europe. Therefore, plant material can be easily cultured or harvested, which makes it a good tannin source for compound isolation or a potential source of natural products. However, the seasonal and regional variation in its tannin content needs to be studied before planning harvesting for commercial use.

In this thesis, water-soluble tannins and phenolic compounds were tentatively identified from eight organs of *G. sylvaticum* using LC-DAD-MS and isolated pure compounds were characterized using a MS/MS, CD and NMR spectroscopy. The qualitative and quantitative differences were studied along several levels: intraplant variation between the organs, seasonal and onthogenic variation, variation between two sexual morphs, populations and flower color polymorphs. Furthermore, the antioxidant and pro-oxidant activity of extracts and fractions, and the protein precipitation and copigmentation capacity of selected compounds were studied to see if these features explain the observed variations and reflect the different functions of compounds or biotic or abiotic pressures that *G. sylvaticum* faces.

2 LITERATURE REVIEW

2.1 *Geranium* species

2.1.1 Classification and abundance

Geraniaceae family consists of approximately 800 species of annual or perennial herbs and shrublets that are widely distributed all over the world. *Geranium* (cranesbills) is the largest genus of the family and contains about 400 species. The name of the genus comes from the Greek word "géranos" that means "crane" because the appearance of the fruit resembles the bill of a crane (Fig. 2). Other genera in this family are *Pelargonium*, *Erodium*, *Monsinia* and *Sarcocaulon* (Fig. 3).

Often *Pelargoniums* are mistakenly called and sold as geraniums or scented geraniums. *Geraniums* and *Pelargoniums* are sometimes confused even in scientific literature ever since Linnaeus (1753) classified them into the same genera.[15] These two genera have distinctive characteristics. *Geraniums* are called hardy geraniums, wild-growing geraniums or true geraniums because in general, they are stress-tolerant perennials that are able to survive outdoors over winter and grow in the northern hemisphere. On the other hand, *Pelargoniums* are frost-tender odorous perennials, which grow mainly in Africa and the southern hemisphere, and are not able to survive the weather in Europe. The most distinctive feature is the structure of the flowers. *Geranium* flowers have five same-size symmetrical petals and ten fertile stamens. *Pelargoniums* have nectar spurs at the base of the flowers and flowers are bilaterally symmetrical with mostly two different smaller/larger petals above and three uniform petals below and seven fertile stamens, while *Erodiums* have only five stamens.[16–18]

Figure 2. *G. sylvaticum* fruit after the seed ejection.

In Finland, 14 *Geranium* species can be found growing wild in nature.[19] The most common is *G. sylvaticum* L. (Wood cranesbill) that grows to some extent all over Finland. Another abundant species is *G. pratense* (Meadow cranesbill) that has larger, bluer, droopier flowers than *G. sylvaticum*. It is not endemic but occurs near old farms in southern and middle Finland. Other rarer species, that grow only in harsher environments in southwestern Finland, in the Åland Islands and the archipelago, include: dark-red flowered *G. sanguineum* (Bloody cranesbill); sticky and smelly *G. robertianum* (Herb Robert); greyish, hairy, small, pink-flowered *G. molle* (Dove's foot cranesbill), *G. pusillum* (Small-flowered cranesbill) with small bluish red flowers and less hair and *G. lucidum* (Shining cranesbill) with smaller, shiny leaves and red flowers.[19–21]

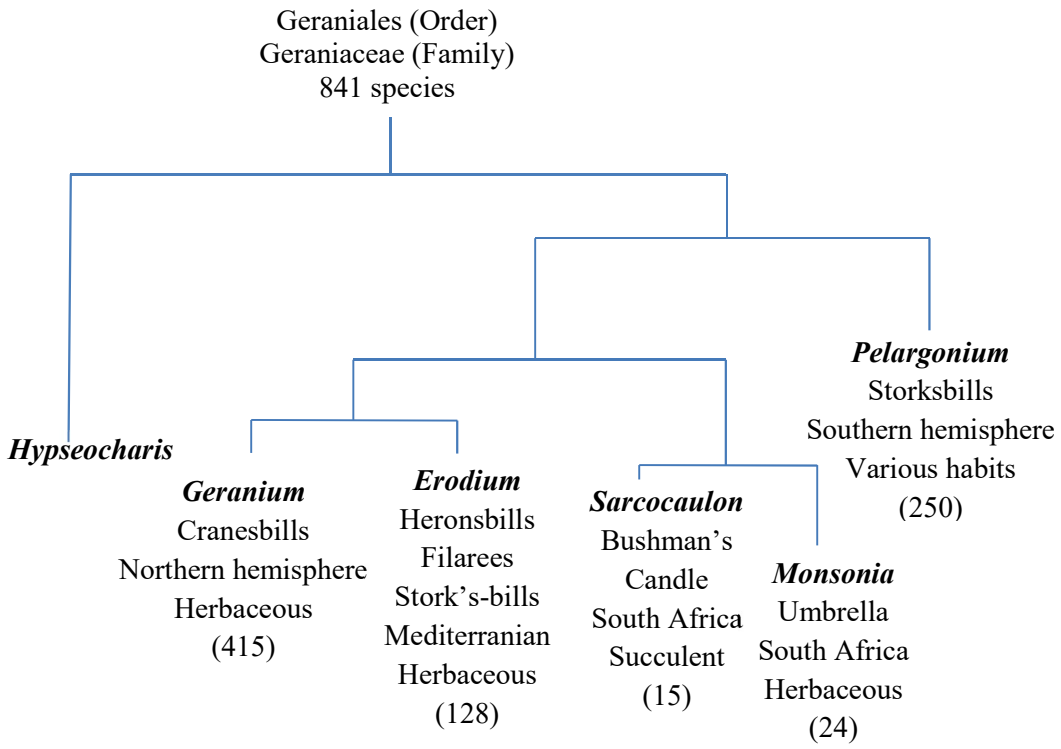


Figure 3. Phylogenetic relationship of Geraniales.[16–18, 22] Number of accepted species names in brackets was adopted from The Plant List 2013.[22]

2.1.2 Commercial use in herbal medicine and gardening

Herbal medicine

Many *Geranium* plants have been used for medicinal purposes all over the world since the late sixteenth century and are still used today in Japan, the USA and in Eastern Europe.[15] The Geranium oil derived from *Pelargonium* species has been used in perfumery, cosmetics and aromatherapy products.[15] The *Geranium* plants are mainly used as hydrophilic extracts in traditional medicines against infectious and other human diseases because of their broad antiviral and antimicrobial activities.[15, 23-24] The antimicrobial properties and other bioactivity are derived from hydrolysable tannins.[9-11] In particular, geraniin has been extensively studied for its various bioactive properties such as antioxidant activity, cancer prevention and hypoglycemic activity.[25] Its production with micropropagation by the tissue cultures of *Geraniums* has been successful.[26]

G. thunbergii is one of the most important medicinal plants in Japan, known as "gen-noshouko" and used for diarrhea and other intestinal, liver and haematological

disorders.[26] Its tannin profile has been well studied and its hydrolysable tannins are considered the main bioactive constituents in *G. thunbergii*.[27-28]

In Europe, *G. robertianum* (Herb Robert) and *G. maculatum* (American cranesbill) have been used as herbal medicines.[15, 21] Probably the root of *G. maculatum* is the most commonly used *Geranium* product worldwide; it has been used as a styptic and astringent for internal bleeding, diarrhea or inflammations.[15] In addition, this dried mainly GT-containing herb is used in herbal medicine.[29] *G. robertianum* has been used for similar symptom: as a decoction or tea to treat diarrhea, excessive bleeding and slowly healing wounds, as a coolant and as a pain reliever. This strongly-smelling plant is also mentioned in the Finnish Flora Fennica that describes how, in old times, the *G. robertianum* decoction was used to wash wounds, as a tea to help breast milk flow in women after parturition, and the walls were brushed with the plant to keep bugs away.[30] Nowadays it is used as a mouthwash for the inflammation of the mouth and pharynx; topically for burns and wounds, and internally for haemorrhage, kidney and bladder stones, liver and gall bladder problems.[29]

The most cultivated and economically important species in Bulgaria and other Eastern European countries are *G. macrorrhizum* and *G. sanguineum*.[31] *G. macrorrhizum* (bigrooted Geranium) is an important plant in Bulgarian Folk tradition where its name "Zdravetz" means healthy.[23, 31] Phytotherapy in Balkan countries uses both herbage and rhizomes of *G. macrorrhizum* as water-soluble ethanolic and methanolic extracts or as compresses for their hypotensive, sedative, astringent and cardiotonic effects.[31] *G. macrorrhizum* is also used for the production of the Bulgarian essential oil called Zdravetz oil that is used in aromatherapy. *G. sanguineum* (Bloody cranesbill) was considered a blood-strengthener in folk medicine because of the red color of its rhizomes. The water-soluble extracts of rhizome have astringent, vasodolatant, anti-inflammatory, hypotensive, cardiotonic and slightly sedative effects.[23, 31]

G. sylvaticum is seldom mentioned as an herbal medicine. In Iceland, it has been traditionally used for inflammation, gastrointestinal sores and arthritis, and is studied today for its synergistic effects with *Angelica archangelica* extract to treat memory sicknesses.[32-33] Studies have shown that the 70% aqueous ethanol extract of *G. sylvaticum* above-ground parts exhibit moderate antibacteriostatic activity against gram-negative bacteria.[24]

Decorative gardening and other use of Geraniums

Pelargoniums are popular floriculture plants, but many *Geraniums* are used as decorative plants in gardens as well.[31] For example, *G. ibericum*, *G. renardii* and *G. sanguineum* are important for garden and landscape uses as good hardy flowers and rock garden ornamentals. *Geraniums* are also cultivated for harvesting purposes and for the feeding of bees.[31] Beekeepers in Finland consider *G. sylvaticum* as a potential nectar plant in mire honeys.[34] The positive side is that hardy *Geraniums* are relatively resistant to pests and plant diseases. Plant extracts of *Geraniums* can even be used as insect or plant disease

control agents; for example, *G. viscosissimum* is active against tobacco budworms and *G. pratense* works against common scab of potato.[35–36] In addition, *G. pratense* have been used to produce dye and its roots have been used in the tanning of leather.[21]

Previously, tannin-containing pasture plants were considered toxic to livestock, because of some incidents that involved extensive foraging of one specific plant that has high amount of tannins (about 20 mg/g DW) usually GTs and ETs.[37–38] However, climate change has driven researchers to find new solutions to animal nutrition, and suitable concentrations of tannins in feeds can be used to achieve positive effects.[37] *G. sylvaticum* is a common plant found in grasslands where ruminants might feed. In *in vitro* screening assay, *G. sylvaticum* was a promising bioactive grass plant, which reduced methane by 30% and ammonia emissions of ruminants by 80% and still had good organic matter digestibility.[39] Plant material could be used as feed or as feed additives for animals that do not forage on grasslands.

2.1.3 *Geranium sylvaticum*, its herbivores and gynodiecy

Geranium sylvaticum is one of Finland's best-known forest plants. It has wide ecological amplitude as it grows wild in most parts of Europe to Turkey.[21] Its common name, wood cranesbill (eng. wood crane's-bill, am. woodland geranium), aptly describes its most abundant growing habitats: rich-type forests, young forest heaths, stream banks, damp and mountain meadows. The species is more common than ever, growing in logging clearings and next to forest roads and road-sides. In the north, it also grows in rich swamps and bogs.[19–21, 40]

G. sylvaticum is a perennial herb, with a height up to 25–80 cm (Fig. 4). The stem is cylindrical and erect-branched. There are three kinds of leaves: long-stalked basal rosette leaves that can be over 20 cm wide, divided into broad 5-7 deeply cut large-toothed lobes with a round blade and palmate venation; stem leaves are more short-stalked or stalkless; and stipulate leaves are even smaller.

Flowering lasts 3–4 weeks from mid-June to mid-August in Finland.[41–42] The whole plant has glandular hairs; especially buds and flowering parts (personal obs.). Flowers usually grow in pairs in axillary or terminating erect flower-stalks. Each plant has one to several flowering shoots per ramet and on average each plant produces 12 flowers.[43–44] The color and size of the *G. sylvaticum* flowers vary more than any other flower of Finnish forest plants.[19] Flowers are regular, with five purplish-blue to white petals 1.5–3.5 cm across, with a white center. Petal tips are round or slightly notched.[45] The strongest purplish colors are found in the southern part of Finland, while pale violet and white flowers are more common in the northern populations, although a variety of colors can be found among neighboring plants.[19, 45] Flowers are usually hermaphrodic, but sometimes *G. sylvaticum* produces female plants, which have clearly smaller flowers and start flowering a couple of days earlier than hermaphroditic plants.[42, 45–46]

Flowers contain ten stamens that are in two whorls of five.[45] The pistil has five stigmatic lobes that are closely joined to each other in a male phase, and then curve out and unfold when the female phase starts and stigmas are receptive to pollen.[47] To prevent self-pollination, *G. sylvaticum* is protandrous, which means that it presents pollen before the stigmas become receptive. Five sepals are relatively small and hairy, clearly shorter than petals, and have membranous edges and a sharp point.[21]

Figure 4. *Geranium sylvaticum* and some examples of natural threats: A) *Zacladus geranii* is a spezialized granivore, B) butterfly larvae are herbivores that damage leaves and C) leaf rust *Uromyces geranii* is a common pathogen.

After pollination, ca. 80% of the flowers develop into glossy, hairy fruit that contain seeds.[48] Fruit changes from green to brown just before the seeds mature for the ejection.[40–41] Each flower contains ten ovules, but typically the fruit contains one to five seeds.[40–41] The fruit breaks apart using the seed ejection. As the fruit splits, the seed is thrown upwards when the five awns curve backwards and remain attached to the central

axis (Fig. 2). Mature seeds are brown and have individual masses between 4 and 6 mg.[40, 46, 49] Seeds are dispersed by cattle, birds and ants.[16] *G. sylvaticum* seeds can survive in the soil seed bank less than 1 year and can be germinated throughout the season.[43]

The rhizome is short (3–10 cm) and branched or unbranched with a strong, horizontal mainroot covered with brown scales and petiole bases.[16, 19, 41, 43] Symbiotic arbuscular mycorrhizal fungi have been found in almost all the rhizomes of *G. sylvaticum* in natural populations; approximately 40–80% of root length has been colonized.[44, 48, 49] Other abundant colonizers are dark septate fungi and other unicellular fungi.[48–49] The plant receives nutrients in return for the carbon that symbiotic fungi consume.[48] The sexual reproduction predominates in *G. sylvaticum*, but meristems of rhizome can be activated and produce asexual clonal offspring.[16, 43] One individual can consist of one or several ramets belonging to the same rhizome.[40]

Herbivores that use G. sylvaticum

The flowers of *G. sylvaticum* are pollinated by a variety of generalist insects, of which bumblebees can be considered the most important pollinators due to their larger, hairy bodies.[41–42, 44] On the other hand, the flowers of *G. sylvaticum* are susceptible to herbivore damage. The petals are heavily consumed by a specialized weevil, *Zacladus geranii* (Fig. 4A).[42, 46] Approximately 27% of the plants have herbivore damage in their flowers, based on a field study of Finnish populations.[42] The flower petals are the main food source for the adults of oliphagous *Z. geranii* and they oviposit their eggs in the ovaries or in the style on the flowers.[42, 46, 50–51] Seeds of *G. sylvaticum* are consumed by the hatched larvae of the *Z. geranii.* Larvae either bore a small hole into the developing seed or eat a part of the seed; ca. 20% of the seeds are predated based on a study of Finnish populations.[42, 46] Usually, only one mature larva is found in each fruit, which emerges and pupates in the ground.[50]

Butterfly larvae and beetles are also found in the *Geranium* flowers as herbivores and adult butterflies use the nectar of plants (Fig. 4B).[19, 40, 52] For example, Geranium Argus (*Pleibeius eumedon*) is a butterfly species that has specialized in *G. sylvaticum*. This species lay their eggs against the pistil or on the leaves, and the larvae feed on the same plant.[52] Other blue butterflies that use *G. sylvaticum* as a foodplant in Finland are *Plebeius Artaxerxes* and *Plebeius nicias* and hymenoptera such as *Corynis obscura*.[52]

Larger animals, such as reindeer, graze the leaves of *G. sylvaticum* intensively in the continental areas of the northern Europe.[48, 53] It is one of the most common forbs of the sheep diet in Norway.[54] Sheep can graze approximately 7% of the plants; however, usually only 1-2 leaves of each plant is grazed and the grazing does not reduce plants' survival.[43] It has been previously observed that the total phenolic content in *G. sylvaticum* leaves increases with a simulated herbivory treatment.[54] Although the phenolic content in *G. sylvaticum* is high, voles and sheep readily consume it and thus small increases in content

probably won't affect their diet choice.[54] On the contrary, rabbits are known to avoid *G. sylvaticum* perhaps because of the high tannin content.[55]

It is not known how these tannins taste to other vertebrates, but it has been studied that to humans, geraniin and ascorgeraniin do not taste astringent as other tannins do.[13] The human diet contains lots of tannin-rich products such as various fruits, berries, nuts, chocolate, wine and tea that can be consumed daily (about 1 g/d polyphenols) without any harmful effects.[56–57] In herbal medicine, the usage of high-tannin content has caused only a few side effects, such as nausea, in sensitive patients.[29]

Plant diseases in G. sylvaticum

Hardy and perennial *Geraniums* are quite resistant to plant diseases. However, in nature *G. sylvaticum* is a principal host plant for several pathogenic fungi. Leaf rusts, caused by *Uromyces geranii*, *Puccinia leveillei* and *Puccinia morthieri* can be observed as yellow to red spots on petioles, on the vascular tissue or entire upper sides of leaves (Fig. 4C).[58] Some fungi cause an intense reddish-violet color on leaves.[58] White-mold *Plasmopara geranii-sylvatici* fungi and downy mildew *Peronospora conglomerata* fungi occur under the leaf surface and makes it crinkled and yellowish.[59–60] The bacterial leaf blight caused by *Xanthomonas campestris* pv. *pelargonii* is the most serious disease of the *Pelargonium* species.[61] However, the seedlings of *G. sylvaticum* and other *Geranium* species are more resistant to the bacterial leaf blight than *Pelargoniums*; brown spots were observed but seedlings did not die.[61]

The high level of resistance may be due to the high tannin content that inhibits microbial decomposers and pathogens. For example, geraniin has been identified as the active compound of the aqueous root extracts of *G. pratense* and *G. neparense* against a wide range of soil-borne plant diseases.[36, 62] There are no studies about plant diseases in the rhizome of *G. sylvaticum* although the beneficial symbiotic fungi is well studied.[48–49] The same cosmopolitan soil pathogens *Penicillium* or *Fusarium* might colonize *Geranium* roots and seeds that cause root rot for *Pelargoniums*.[63–65]

G. sylvaticum has a gynodioecious breeding system

Gynodioecy is a genetic polymorphism that occurs in 7% of plants.[66–67] In gynodioecious populations, female plants that do not produce pollen coexist with hermaphrodites that produce both pollen and seeds. At least 12 species of *Geranium* and *Erodium* are described as gynodioecious and, in addition to *G. sylvaticum,* distinct polymorphism has been observed in *G. pratense*, *G. palustre*, *G. maculatum* and *G. richardsonii*.[45, 68–70] The clearest difference between the sexual morphs of *G. sylvaticum* is the smaller flower size of females (Fig. 5).[42, 45] Actually, three kinds of flowers can be observed: 1) hermaphrodite flowers that have all 10 normally developed stamens; 2) intermediate flowers in which 1–

9 of the stamens have no anthers or anthers are small, dry, orange-colored or white or contain sterile pollen; 3) female flowers that have small or no stamens and have dry, pale anthers and sterile pollen (Fig. 5).[42, 45, 47] Some other differences between the sexual morphs of *G. sylvaticum* are summarized in Table 1.

Figure 5. Female and hermaphrodite flower

The female plant frequency in the Finnish populations of *G. sylvaticum* is approximately 12% and varies from 0.4 to 27.2%.[40, 45] Female plants are more frequent in northern populations where growing conditions are harsher.[40] The female frequency is similar in Sweden; however, no females have been found in smaller nearby Russian populations when plants have been studied at the bud stage.[70–71] The intermediate plants are found in half of the studied Finnish populations and their frequency is low, ca. 1%.[40]

The maintenance of genetically determined polymorphism is one of the key questions in evolutionary biology.[72] Asikainen (2004) suggests that the sex determination of *G. sylvaticum* is under nucleocytoplasmic control.[67] Female plants need to compensate for the lack of male functions to coexist with hermaphrodites, otherwise they transmit their genes half as much.[73] This can be achieved by allocating resources saved from smaller flowers and not producing pollen to female functions, such as to higher fruit and seed production and/or production of higher quality seeds or offspring.[68–69, 73] Furthermore, it has been suggested that herbivores and pathogens may contribute to the maintenance of gynodioecy by preferring one of the gender morphs.[74–75]

In a study of Finnish *G. sylvaticum* populations, females produced more fruits and seeds per flower than hermaphrodites (Table 1). There is contradicting data about the size of seeds; older studies observed that hermaphrodite seeds are significantly larger than female seeds, but newer studies that measured the biomass weight found they are equal.[40, 45, 76] This difference can be due to the variation of seed size among populations, years and during growing season.[40, 46] For example, those *G. sylvaticum* plants that grow in sunny meadows start flowering one week earlier and produce 35% more flowers and four times more seeds with 30% less leaf biomass than the plants growing in shady forests.[41, 49] Also, early flowering plants produce 17% more seeds per fruit than the later ones and the seed mass declines towards the end of the season.[46]

Table 1. Differences of female and hermaphrodite plants of *G. sylvaticum*.

Flowers		**Reference**
Flower size (petal length)	~50% H > F	42, 44, 45
Number of flowers per shoot	44 %[1] H > F	40, 45
Number of flower shoots/flowers	No difference	44, 46, 48
Flower nectar production (sugar content)	H > F	47, 80
Pistil biomass	No difference	76
Pollen size	20% H > F	45
Floral herbivores	8% H > F	42
Pollinator preference	13-50 % H > F	42, 44
Flower color	No difference	45
Number of barren flowers in shoots	No difference	45
Flower and stigma lifespan	50% F>H	81
Seeds		
Individual seed mass	No difference	40, 46, 76
Seed size (length)	6 % H > F	45
Seed P content	No difference	48
Seed predators	No difference	42, 46
Seeds per flower	20% F > H	40, 76, 78
Fruit set (number of fruits/number of flowers)	~40 % F > H	40, 44, 48
Total seed production	~65 % F > H	48
Germination rate of seed	No difference	40, 45, 78
Response to light during maturation	No difference	79
Other		
Seedling survival	No difference	40, 45, 78
Shoot development	H > F	45
Vegetative characters (such as hairiness, plant height, number of rosette leaves, root mass etc.)	No difference	45, 48, 77
Foliar P	No difference	48, 77
Foliar P after defoliation	F > H	48
Colonization of AM fungi	No difference	44, 77
Effect of AM fungi to flowering, flower size and flower visitors	H > F	44, 77
Higher P aqcuisition due to AM fungi	F > H	77
Decrease in fitness after increased reproduction	17% F > H	79
Regional differences in distribution	No difference	45
Chromosome number	No difference	45
Effect of simulated herbivory to reproduction	No difference	48

F = female plant, H = hermaphrodite plant, AM = symbiotic arbuscular mycorrhizae

Studies on *G. maculatum* and *G. richardsonii* have showed similar results.[68–70] *G. maculatum* clearly allocate resources from the pollen production to the seed production and quality; females produced 60% more and larger seeds than hermaphrodites, and these seeds had more frequent germination, larger above- and belowground biomass of seedlings and more frequent flowering.[69–70] The two to four times lesser seed production of hermaphrodites can be explained by inbreeding depression.[70] This risk of inbreeding depression is lower in *G. sylvaticum* because its flowers are protandrous.[76]

Despite the extensive studies, no other significant sex-specific differences in *G. sylvaticum* have yet been found, which could indicate that females can allocate saved resources to other functions than better seed production (Table 1).[48] An interesting factor in the female maintenance is the role of herbivores. Preference for other sex may be explained by the different nutrients or defensive compound content of the gender morphs.[42, 48, 82] In the natural populations of *G. sylvaticum* in Finland, pollinators and floral herbivores prefer bigger hermaphrodites' flowers slightly more often than females' because hermaphrodite flowers provide bigger pollen and better nectar resources.[42, 44, 47] Nevertheless, females are able to produce more seeds. Pollinators' preference for hermaphrodite flowers does not benefit the female maintenance in populations, whereas herbivores' preference for hermaphrodites might.[42] Interestingly, Varga and Kytöviita (2010) observed differences in pollinator preferences, which might be important in the maintenance of gynodioecy: the most important pollinators, bumblebees, did not prefer either sex; whereas syrphid flies, that consume only pollen, visited hermaphrodites more often.[44] It is advantageous to pollen and nectar feeders to recognize hermaphrodites; in contrast, those insects that oviposit to flowers should prefer female plants because there are more seeds for larvae to eat. However, studies have not shown that seed predators prefer either sex.[42, 46] So far there are no studies about the differences in defensive compounds between the sexes of *G. sylvaticum*.

2.2 Phytochemistry of *Geranium* species

The genus *Geranium* is known to contain high amounts of tannins in the complex mixtures of PAs, GTs and ETs.[83-84] The phytochemistry of this genus was reviewed by Harborne and Williams in 2002.[55] Flavonoids are universally present in *Geraniums*. The roots contain high amounts of PAs while the bluish-purple flowers contain several anthocyanins.[55] However, the most prevalent compound in the whole genus is the ET geraniin.[13, 84] The leaves of *G. sylvaticum* have been observed to contain geraniin, flavonoids, ellagic, gallic and hydrocinnamic acids, and seed PAs using colorimetric total methods.[83–86] Since then, only three detailed studies about the structures of compounds from *G. sylvaticum* species have been published concerning anthocyanins, flavonoids and essential oils. The next chapters describe the structures of secondary compounds in *Geraniums* and their intraplant and seasonal variation.

2.2.1 Non-phenolic lipophilic compounds produced by glandular trichomes

Glandular trichomes are typically hair-like micro-organs on the surface of the leaves, stems, flowers, and fruits that exude lipophilic defensive compounds.[55, 87] *Geranium* species typically have glandular hairs on the upper leaf surface and stems.[55, 87] The trichome exudates of strong-smelling *Geraniums* have especially been studied, because these species are used in the production of essential oils. There are two types of trichomes, which abundance can vary during the development of the leaves. Non-glandular trichomes form the physical layer of hairs that make the plant less palatable to herbivores or can protect them from low humidity levels, high wind or the UV-B light of the sun. Glandular trichomes secrete compounds, which may have protective functions as antifeedants, antifungals, antibiotics or in UV protection.[87] The head of glandular trichomes is often pear-shaped, whereas non-glandular hairs are straight and sharp.[88] The type of glandular trichomes vary in different organs of *G. robertianum*. In leaves, trichomes are short (up to 60 μm), procumbent or erect and have pear-shaped apical cells that secrete essential oils containing terpenoids, resinic acid and phenolic compounds, while in flower structures, trichomes are much longer (up to 600 μm), accumulate anthocyanins in the red apical cells and secrete flavonoids.[89]

As already mentioned, the commercially exploited geranium oil is actually derived from scented *Pelargoniums* in which the volatile essential oil components are stored in leaf trichomes.[88] Geranium oil is mainly used in perfumery (rose-like fragrance with a minty note), and to a lesser extent as a flavor in the food industry and as a potential antimicrobial agent.[90] Two essential oil components are named after this genus: germacrone and geraniol (Fig. 6). These compounds can be found in aerial parts of well-studied *G. macrorrhizum*, but it is not yet known if these are found in other *Geranium* species.[55] Commercial geranium oil derived from *Pelargoniums* contains mainly citronellol (35–58%) and geraniol, which differs from the composition of true geranium

oils of *G. robertianum*, *G. maccrorhizum* and *G. sylvaticum*.[89–91] The essential oil of *G. sylvaticum* (0.08% of the fresh weight of the leaves) was studied using gas chromatography; it contains 39.8% sesquiterpenes, 23.5% aldehydes and 18.2% hydrocarbons: major components were γ-muurolene, (*E*)-caryophyllene and linalool (Fig. 6).[92]

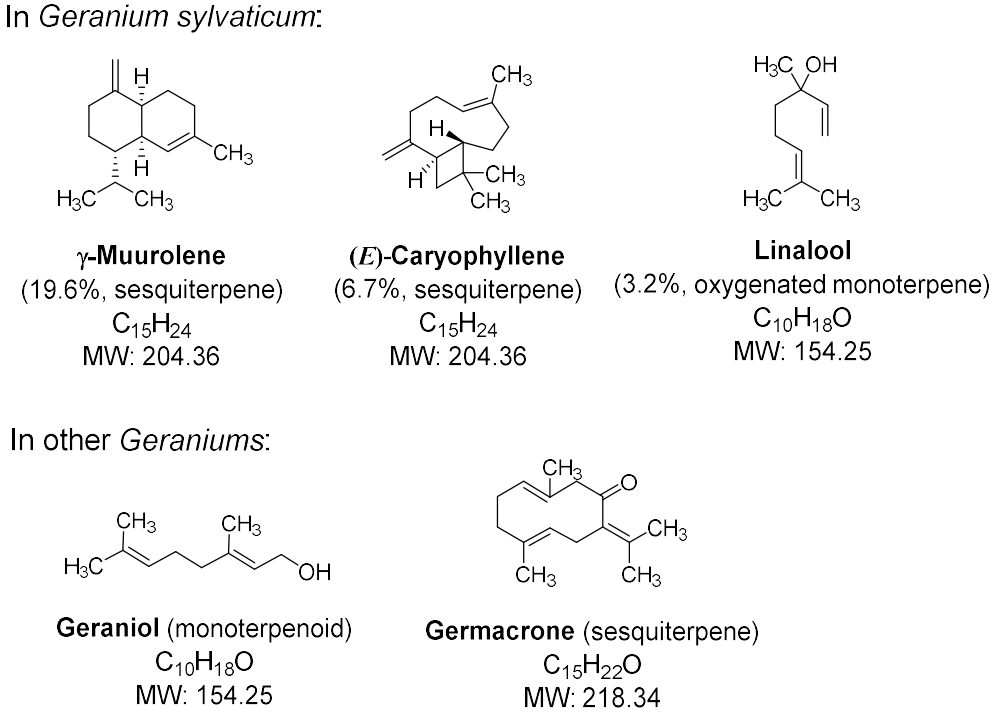


Figure 6. Main volatile essential oil components of *G. sylvaticum* and other *Geraniums*.[55, 92]

Non-volatile secondary metabolites of the glandular trichome exudates of the *Geranium* species are less studied.[87] The lipophilic methyl ethers of kaempferol, quercetin and myricetin have been detected in the leaf surfaces of all 11 studied *Geranium* species.[93] Quercetin 3,7,3',4'-tetramethyl ether (Fig. 7) is a major lipophilic constituent of *G. macrorrhizum* (0.4% of the essential oil).[94] Unique *n*-octyl disaccharides can be isolated from *G. carolinianum;* caroliniaside A is a major constituent (65%) of the carolianiaside fraction (Fig. 7).[87]

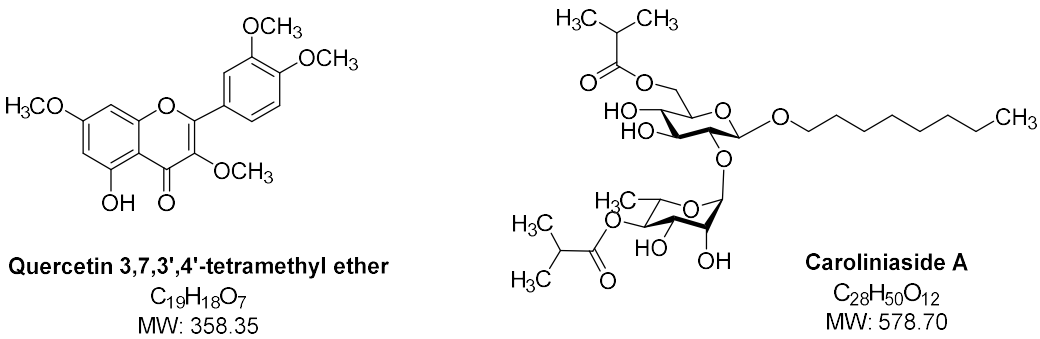


Figure 7. Non-volatile compounds found from the trichome exudates of *Geranium* species.

2.2.2 Water-soluble phenolic acids and flavonoids

Phenolic acids

The function of phenolic acids in plants is poorly understood. Phenolic acids can have diverse functions as antioxidants or signaling molecules.[95–96] Almost all *Geranium* plants contain common phenolic acids such as caffeic acid, gallic acid, ellagic acid, *p*-coumaric acid and ferulic acid (not in *G. sylvaticum*) (Fig. 8).[85–86] Mono- and diesters of hydroxycinnamic acids, such as chlorogenic acid and its isomers, are almost ubiquitous in higher plants and have been identified in *G. sanguineum*.[97–99] Tartaric acid, commonly found in related *Pelargonium* species, is absent in *Geranium* and *Erodium* species.[16, 100] However, caffeic acid (2.41 mg/g DW) and caftaric acid (1.30 mg/g DW) have been found in relatively high concentrations in *G. sanguineum* (Fig. 8).[101] In a study of Romanium *Geraniums* species, the caftaric acid content in *G. pyrenaicum*, *G. palustre* and *G. columbinum* varied between 0.2 and 0.8 mg/g DW whereas the ellagic acid content varied between 9 and 72 mg/g.[102–103] *G. phaeum* has been reported to contain low amounts of sinapic (0.61 mg/g) and cichoric acids (0.17 mg/g).[102]

In contrast to hydroxybenzoic and hydroxycinnamic acid, gallic acid occurs in large esters as it is the precursor of hydrolysable tannins found in many *Geranium* leaves and roots.[83, 86] Furthermore, other compounds related to hydrolysable tannins, such as *m*-digallate, brevifolin carboxylic acid and galloyl quinic acids have been found in *G. thunbergii*.[104–105] Instead of galloyl quinic acids, the presence of galloyl shikimic acid has been found in related species *Erodium cicutarium* and *E. moschatum*; that is a suggested chemosystematic difference between *Geranium* and *Erodium* genera.[106–107] *Geraniums* do not contain alkaloids, however, nitrogen containing amino acid tryptophan has been detected in *G. pratense*.[86, 108]

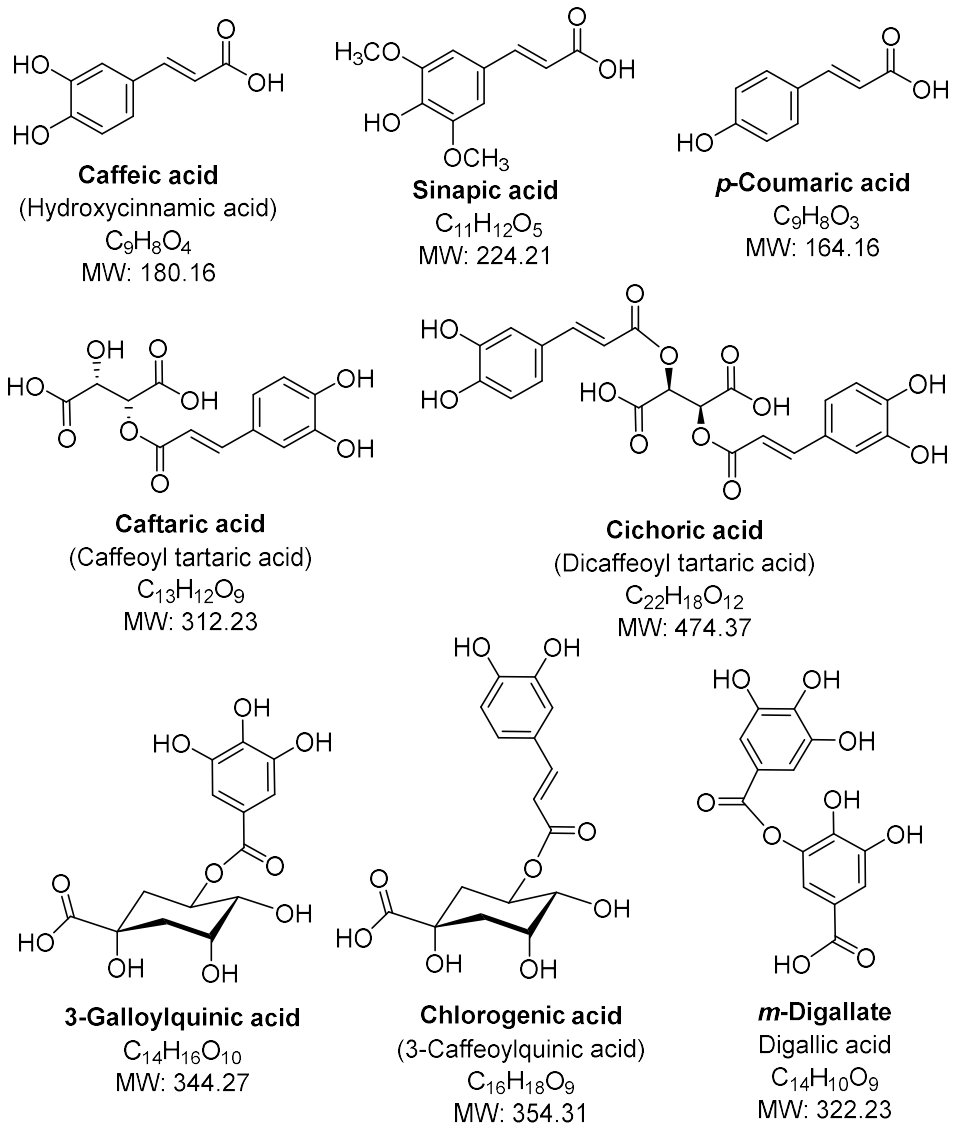


Figure 8. Structures of some common phenolic acids in *Geraniums.*

Flavonoids

Flavonoids are found in nearly all plants, therefore they are one of the most studied secondary metabolite group.[109] The main role of yellow and colorless flavonoids is thought to be UV protection. Other proposed functions include protection against high temperatures, heavy metals, oxidation, acting as pigments or copigments, pollen germination and signaling.[6] Flavonoids can be divided into six subgroups: flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanidins. Flavonols are the dominating flavonoid type in *Geranium* leaves.[55] Quercetin is the most common flavonoid and is typically found in all *Geranium* species as aclygone and derivatives (Table 2).[55, 83, 85, 93, 110] Kaempferol is found in 93% of the *Geranium* species, myricetin in 13% of *Geranium* species, and luteolin is detected in some species.[55, 83, 93, 110] Typical flavonoids in *Geranium* species and *G. sylvaticum* are listed in Table 2. *Geraniums* contain both glucosides and galactosides. Quercetin-3-galactoside is the main flavonol glycoside in most species of *Geranium.* Its content varies between 0.03 and 1.6% of the DW of leaves, whereas some species contain more quercetin-3-glucoside.[111] Interestingly, the petals of blue *Geranium* cultivars contain more kaempferol and myricetin glycosides than quercetin, which is the dominating compound in leaves.[112] Three flavanol aglycones, five glycosides (Table 2) and luteolin 7-glucoside have been characterized from *G. sylvaticum* by thin-layer chromatography.[93]

Other interesting flavonoid structures have been found in *Geraniums*. The glandular trichome exudate, obtained from the upper leaf surface, contains lipophilic kaempferol, quercetin and myricetin methyl ethers that may have a defensive function (Table 2).[93] Moreover, the presence of galloylated flavonols seems to be typical for *Geraniums*: quercetin 3-(2''-galloyl)-galactopyranoside and -glucopyranoside has been reported for the first time in *G. pratense* and later other 3-(2''-galloyls) and 3-(6''galloyls) in *G. tuberosum* and *G. stepporum*.[108, 113–114] A high content of quercetin 3- (6''-galloyl)-glucoside has been found in a few other species that also have a high level of HTs, such as *Tellima grandiflora*, and *Euphorbia*, *Acacia* and *Bergenia* genera.[115] These galloylated flavonoid glucosides are extremely alkali labile, but are resistant to β-glucosidase.[115] New flavonol derivatives having an oxide derivative of rhamnose unit attached to kaempferol and quercetin, named as geranioside A and B, respectively, have been isolated from *G. purpureum*.[116] Several studies have used flavonoids in the classification of species, e.g. Saleh et al. (1983) suggested that *Geranium* species lack 4'-glucosides, which are characteristic for *Erodium* and *Monsonia* species.[110]

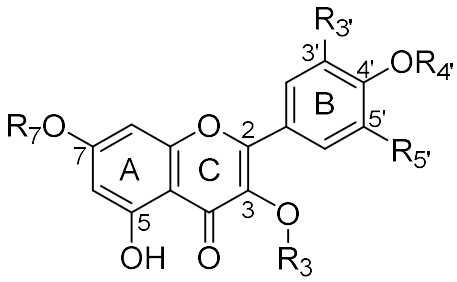


Figure 9. Flavonol core structure. R_3–R_7 refer to Table 2.

Table 2. Some common flavonoids in *Geranium* species (see Fig. 9 for structures).[55, 93, 110, 112, 117]

Aglycone	Glycoside	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_3	R_7	MW	UV-vis max. (nm)		Found in *G. sylvaticum*
								Band II	Band I	
Kaempferol		H	H	H	H	H	286			x
	Astragalin	H	H	H	glu	H	448	266	346	
	Trifolin	H	H	H	gal	H	448			
		H	glu	H	rut	H	756			
	Afzelin, kaempferin	H	H	H	rha	H	432	264	344	xx
	Kaempferitrin	H	H	H	rha	rha	578			
		H	H	H	sop	H	610			
		H	Me	H	H	H	300			
	Kumatakenin	H	H	H	Me	Me	314			x
Quercetin		OH	H	H	H	H	302	256	372	x
	Isoquercetin, isoquercitin	OH	H	H	glu	H	464	258, 264sh	352	xx
	Hyperin, hyperoside	OH	H	H	gal	H	464	257, 263sh	351	
	Quercitrin, thujin	OH	H	H	rha	H	448	257, 264sh	351	xx
	Guajaverin	OH	H	H	arab	H	434	257, 264sh	351	
	Rutin, rutoside, sophorin	OH	H	H	rut	H	610			
		OH	H	H	glu	glu	626			
		Me	H	H	Me	Me	344			
	Retusin	Me	Me	H	Me	Me	358			
Myricetin		OH	H	OH	H	H	318			
	Isomyricitrin	OH	H	OH	glu	H	480	264	354	
		OH	H	OH	sop	H	642			
		OH	H	OH	rut	H	626			
		OH	H	OH	H	glu	480			
	Myricitrin	OH	H	OH	rha	H	464	262	350	x

glu = glucose, gal = galactose, rha = rhamnose, rut = rutinose, sop = sophorose, arab = arabinose
x = low amount, xx = high amount

One of the major flavonoid groups is anthocyanins, water-soluble vacuolar pigments, which give pink, red, violet and blue colors on flowers, fruits and leaves.[118] Anthocyanins act as a visible cue for animals and protect chloroplast from photodamage.[6] Often young leaves are redder and contain relatively more anthocyanins; therefore, it has been suggested that anthocyanins may also act as antiherbivore and antifungal protection in young leaves, although there is not much evidence to support this.[6] The name of the group

comes from the Greek words *anthos* and *kyanos* that mean "flower" and "blue". The color of anthocyanin is dependent on its structure and conditions. The anthocyanin composition of *Pelargoniums* (colors ranging from white to orange-red and purple) is more studied than the composition of purplish-blue *Geraniums*, because of their popularity as potted plants.

Anthocyanidins in *Pelargoniums* and *Geraniums,* as 92% of all identified anthocyanins in the world, are based on the six most common anthocyanidins (Table 3).[119–120] Most anthocyanidins are unstable and thus 97% of anthocyanidins found in plants are glycosylated, typically on the 3 position of the C ring that stabilizes the structure.[118–119, 121] Typically, there are one (49%), two (45%) or three (5%) monosaccharide units that are connected to the anthocyanidins through *O*-linkages. The most typical monosaccharide in plants is glucose (90%). Other common ones are rhamnose (20%), galactose (10%) and xylose (10%); arabinose, and glucuronic acid are much rarer.[118] The three most common disaccharides are 2-glucosylglucose (sophorose), 6-rhamnosylglucose (rutinose) and 2-xylosylglucose (sambubiose).

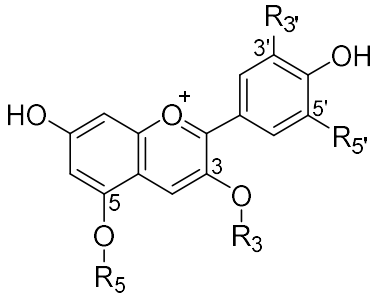


Figure 10. Anthocyanidin core structure. R_3–R_5 refer to Table 3.

Table 3. Common anthocyanins in plants.[118, 122–124]

Aglycone	Anthocyanin	$R_{3'}$	$R_{5'}$	R_3	R_5	MW	Vis. max. (nm)	Color	Occurrence of aglycone in plants %	Relative proportion in *G. sylvaticum* petals %
Pelargonidin		H	H	H	H	271	520	orange-red	18	
	Callistephin	H	H	glu	H	433				
	Pelargonin	H	H	glu	glu	595	495			
Cyanidin		OH	H	H	H	287	535	blue-red	30	
	Chrysanthemin	OH	H	glu	H	449	516*			10.5**
	Cyanin	OH	H	glu	glu	611	512*			1.5
Delphinidin		OH	OH	H	H	303	546	purple-blue	22	
	Myrtillin	OH	OH	glu	H	465	524*			3.4
Petunidin		OMe	H	H	H	317	543	purple-blue		
Peonidin		OMe	H	H	H	301	532	blue-red	20 together	
	Peonin	OMe	H	glu	glu	625	512			
Malvidin		OMe	OMe	H	H	331	542	purple		
	Oenin	OMe	OMe	glu	H	493				
	Malvin	OMe	OMe	glu	glu	655	523*			10.5**
		OMe	OMe	aceglu	glu	697	526*			84.6

glu = glucose, aceglu = acetylglucose

*on-line HPLC in HCOOH/H_2O/MeOH mobile phase (others mainly in 0.01% HCl/MeOH)

** 10.5 together

More than 65% of the identified anthocyanins are acylated with hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids), hydroxybenzoic acids (*p*-hydroxybenzoic and gallic acids) and aliphatic acids (malonic, acetic, malic, oxalic, succinic, and tartaric acids).[118] *Geraniums* and *Pelargoniums* contain anthocyanins acylated with acetic acid; these type of anthocyanins are quite rare, altogether 36 have been found only in angiosperm families.[118] These acetyl groups are typically linked to glucosyl 6-position as in malvidin 3-(6-acetylglucoside)-5-glucoside that is the main anthocyanin in the flowers of *G. sylvaticum*.[118, 122] Other minor anthocyanins in *G. sylvaticum* are the 3,5-diglucosides of malvidin and cyanidin and the 3-glucosides of cyanidin and delphinidin (Fig. 10, Table 3).[122] The acetyl can be attached to both the 3-glucose and 5-glucose, of which the latter is the most probable position.[112] Anthocyanin structures in flowers are typically more complicated than the structures in other plant parts.[119]

2.2.3 Hydrolysable tannins and proanthocyanidins

Hydrolysable tannins

As mentioned earlier, gallic acid derivatives are often found in *Geraniums* in ester form as hydrolysable tannins (HTs) having mostly molecular weights less than 5000 Da.[97] HTs can be divided into three groups: galloylglucoses (GGs) that are galloyl esters of glucose; gallotannins (GTs) that are galloylglucoses where additional galloyl groups are attached with depside bonds; and ellagitannins (ETs) where two galloyl groups are attached to form hexahydroxydiphenoyl (HHDP) or further oxidized groups such as dehydrohexahydroxydiphenoyl (DHHDP) group. Other core polyols can be quinic acid (tara tannins), glucitol (acertannins) or hamamelose (hamamelitannins).[12, 97] The name of the HT group comes from their tendency to easily hydrolyze in the presence of acid or during hot water extraction to smaller compounds. The diagnostic hydrolysis product for GGs is gallic acid and, for ETs, ellagic acid. Other acids can be formed depending on the original structure of ET, for example geraniin produces brevifolin carboxylic acid (Fig. 11). The hydrolysis products of geraniin are shown in Fig. 11, these are fairly stable, especially corilagin.[125]

1,2,3,4,6-pentagalloyl-*β*-D-glucose (PGG) is considered the biosynthetic precursor to all ETs and therefore it is relatively widespread.[38, 97] Central polyol in PGG is typically β-glucose although α-glucosides are also rarely found in some ETs.[97, 126] PGG may have many positional isomers when some galloyl groups are attached with *meta*-depside bonds.[126] PGG is found in most of the *Geraniums*, and di- and tetraGGs have been reported from *G. thunbergii*.[97, 104]

The further metabolism of PGG leads to GTs via additional galloyl group attached by *meta*- or *para*-depside bonds. Usually GTs contain 1–2 depside galloyls and their preferred positions vary depending on the plant.[127] However, GTs up to dodecamers have

been encountered, and the galloyl glucose core can also vary.[128–129] Only a few higher plant families accumulate significant amounts of GTs, such as *Paeonia* spp. and *Arctostaphylos uva-ursi*.[35, 97, 130–131] However, GTs (often erroneously called tannic acid) are produced in high amounts in the galls that are caused by various parasites although leaves of the plant do not synthetize those. Well-known sources of GTs are galls to the leaves of *Rhus semialata* (Chinese gallotannin) and *Quercus infectoria* (Turkish gallotannin).[97, 127] GTs are found in *Pelargoniums* but more rarely in the leaf and stems of *Geranium* species.[97] An exception is the *G. maculatum* herb, which contains mainly GTs.[29]

The oxidative coupling of galloyl groups of PGG leads to ETs. All *Geraniums* are rich in geraniin (1-galloyl-3,6-(*R*)-HHDP-2,4-(*R*)-DHHDP-β-D-glucopyranose, ca 10% DW of the leaves), an ellagitannin that has been first isolated from this genus (Fig. 11).[84, 111, 132–134] Typically geraniin is the main compound in extracts and co-occurs with an isomer named *iso*-geraniin.[97] In addition, several other ETs have been isolated and identified from aboveground parts of *G. thunbergii,* such as corilagin, dehydrogeraniin and geraniinic acids (Fig. 11).[27–28, 55, 104–105, 132, 135] Of these, furosin is presumed to be a degradation product formed during the drying process.[28]

The DHHDP group is very reactive and its further oxidation yields ETs such as chebulinic and chebulagic acid, phyllanthusiins A, B, C and repandusidic acid A.[12] The DHHDP group easily forms condensates with ascorbic acid (moderate aqueous acidic conditions at room temperature; Fig. 12), acetone (refluxing with a small amount of trifluoroacetic acid), and with *o*-phenylenediamine (aqueous acetic acid at room temperature).[12] The condensation product of geraniin with ascorbic acid, i.e., ascorgeraniin, is also naturally present in *Geraniums*.[135] This facile and highly water-soluble ascorgeraniin might be an intermediate in the biosynthesis of geraniin and play a role in the enzymatic oxidation of a HHDP group to a DHHDP group.[104]

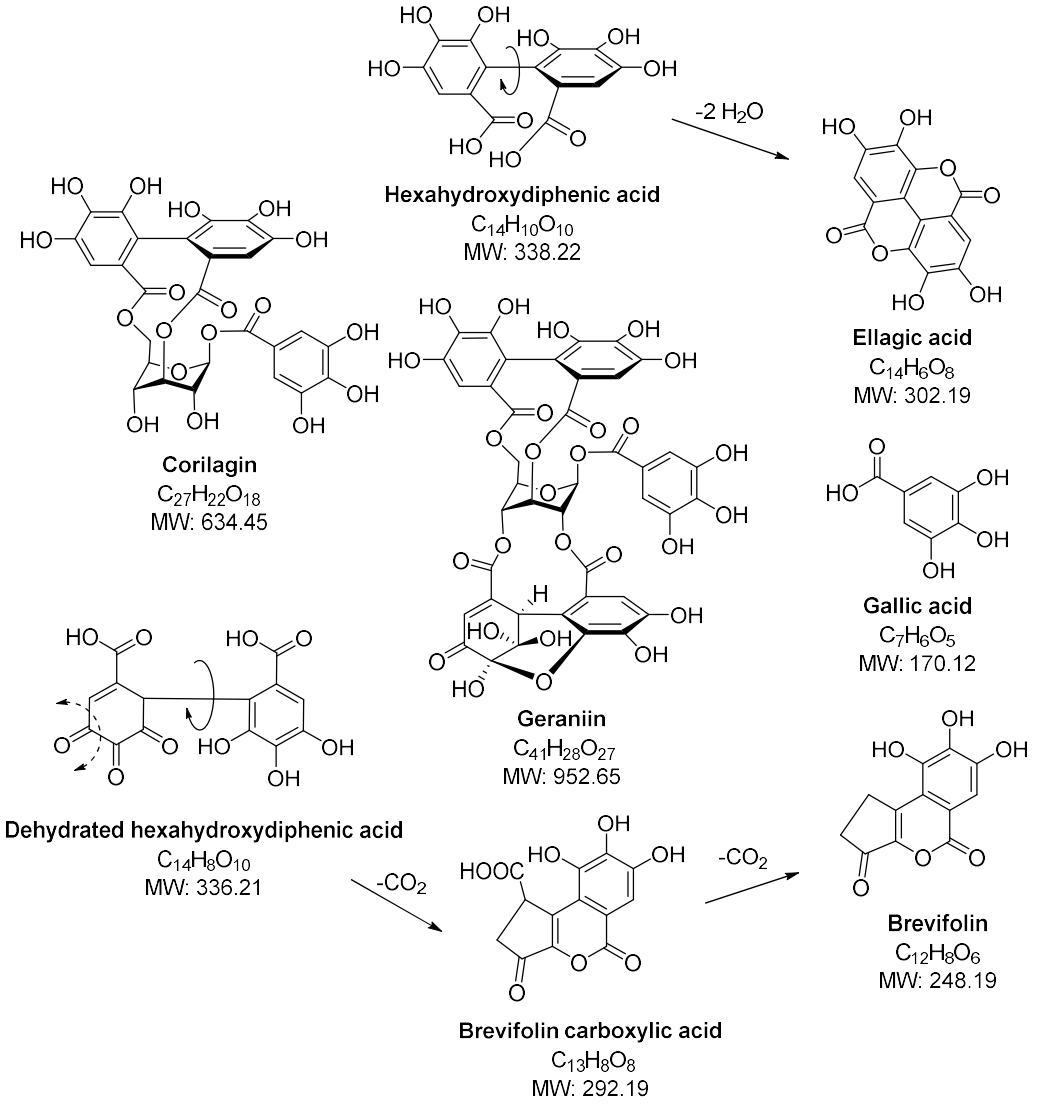
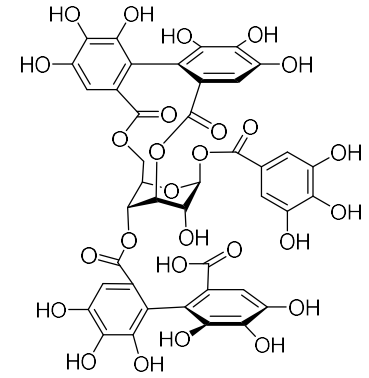
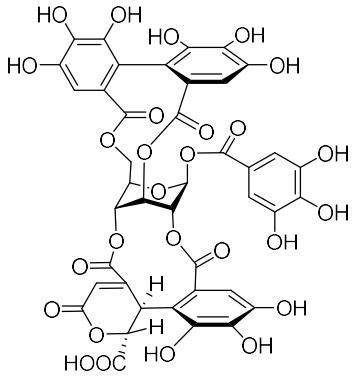


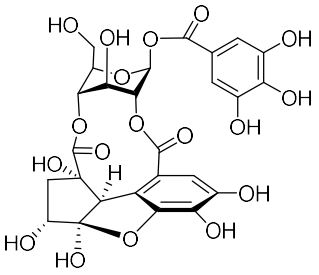
Figure 11. The hydrolysis products of geraniin.[86, 105]



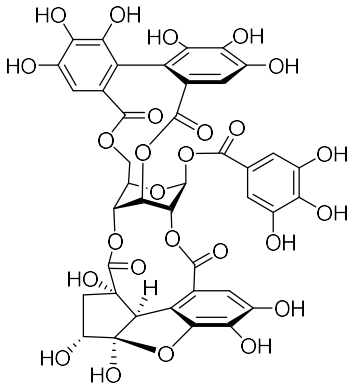
Geraniinic acid A
$C_{41}H_{30}O_{27}$
MW: 954.66



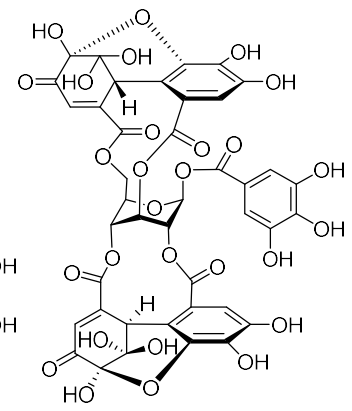
Geraniinic acid B/C
$C_{41}H_{28}O_{27}$
MW: 952.65



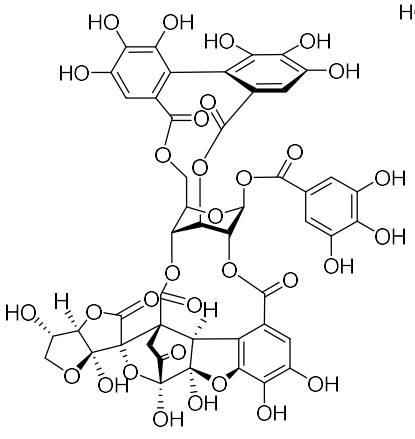
Phyllanthusiin F
$C_{26}H_{24}O_{18}$
MW: 624.46



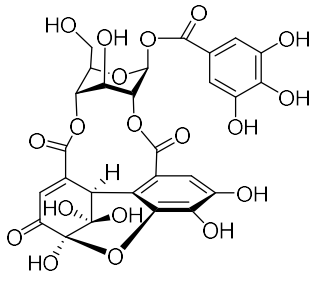
Phyllanthusiin B/C
$C_{40}H_{30}O_{26}$
MW: 926.65



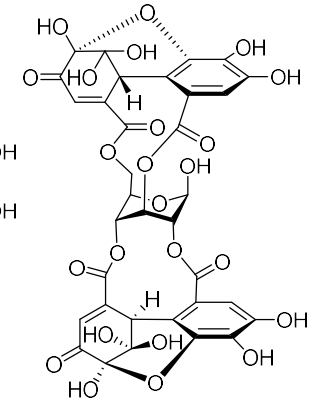
Dehydrogeraniin
$C_{41}H_{28}O_{28}$
MW: 968.65



Ascorgeraniin
Elaeocarpusin
$C_{47}H_{34}O_{32}$
MW: 1110.76



Furosin
$C_{27}H_{22}O_{19}$
MW: 650.45



Furosinin
$C_{34}H_{24}O_{24}$
MW: 816.54

Figure 12. Ellagitannins found from *G. thunbergii*.[27, 28, 135]

HTs typically have D-glucose in $^4C_1$ conformation, whereas in geraniin, glucose is in energetically less favourable $^1C_4$ chair or intermediate skew-boat conformation.[111, 136] In

$^1C_4$ conformation, the oxidative coupling usually occurs between 1,6; 3,6 and 2,4 galloyls.[97] *P. reniforme* is the first member of Geraniaceae where the co-occurrence of ETs with both $^4C_1$ and $^1C_4$ conformations has been found; a few other plants have since been shown to contain both forms.[137] Further possibilities for isomers come from the presence of glucosides and galactosides that have both been found from the flavonoid structures of *Geraniums* (see Section 2.2.2). An ET with D-galactose core has been isolated from *G. pusillum*.[138] This isomer of corilagin is named as pusilagin and it has also been found in *G. purpureum*.[116, 138]

The occurrence of different types of ETs is typically restricted to certain plant families. DHHDP-ETs are a relatively rare group of compounds found in Geraniaceae; moreover, geraniin has been found, although in lesser amounts, in the plants of Hippomaneae, Acalypheae, Aceraceae, Euphorbiaceae, Sapindaceae, Nymphaeaceae and Elaeocarpaceae.[12, 25] Several species belonging to Euphorbiaceae contain geraniin and its dimers and oligomers, such as euphorbins A, B and I and mallotusinic acid.[11, 125, 139–141] This is an interesting difference between these two genera, because dimers or higher oligomers and mallotusinic acid have not been found in *Geraniums*.[11, 125] The closely related *Erodium* species seems to have similar phenolic profiles to *Geraniums*. *Pelargoniums* do not contain geraniin, although these species contain ETs that are oxidized metabolites of geraniin.[107, 125, 137]

Proanthocyanidins

Proanthocyanidins (PAs) are also called condensed tannins because they are composed of flavan-3-ols through condensation reactions. These are large molecules of which molecular weights can be up to 20 000 Da.[142] The name of PAs comes from the reaction where the upper extension units of oligomeric and polymeric PAs yield colored anthocyanidins on acid hydrolysis.[143] PAs are widespread throughout the plant kingdom, unlike ETs that have been found only in dicotyledoneous angiosperms.[11] PAs can be found in fruits, bark, leaves and seeds.[144] Their typical location in the bark and seed coat suggests that their role for plants is to protect them against microbes and herbivores.

PAs can be divided into subgroups based on the flavan-3-ol unit or linkage type. The most common PAs are B-type oligomeric and polymeric tannins that are composed of flavan-3-ols attached through interflavanoid linkages between C4→C8 or more uncommonly between C4→C6 positions.[143] A-type PAs have an additional ether bond at C2→C7 position. Moreover, interflavanoid bonds can be α or β based on two possible configurations at C4. Two common PA groups are procyanidins (PCs), composed of catechin and epicatechin units, and prodelphinidins (PDs), composed of gallocatechin and epigallocatechin units; flavan-3-ol structures with less hydroxylation are rarer. Most commonly PAs have 2*R* absolute configuration; therefore, the rarer 2*S* epimers have the *ent*-prefix. Even polymers with up to hundreds of flavan-3-ol units have been encountered.

This structural diversity is characteristic for PAs. Already in dimers, two diastereomers form four isomeric combinations as seen in Table 4 and even more isomers are found with oligomers (Fig. 13). Galloyl and sugar groups can be attached to the flavan-3-ol unit, most commonly to the 3-hydroxy group in C-ring, and oligomers can be composed of heterogeneous flavan-3-ol units.[144] The diversity increases further with conjugates. PAs with sulphate and amino groups have been found in the roots of *Pelargonium sidoides* and considered an active constituent in herbal medicine Umckaloabo® (EPs® 7630).[145] Because of the complexity and diversity of PAs, typically only the mean degree of polymerization or the mean molecular weight is expressed for plant species.[143]

Table 4. Number of possible isomers as an example in B-type PAs with C4→C8 interflavanoid bond.

Monomers	# Dimers	# Trimers	# Tetramers
Catechin	4	8	16
Gallocatechin	4	8	16
Heterogenous	16	64	256

The PAs in *Geranium* species have been less studied than HTs; only a few detailed studies have been carried out.[55] The roots of *G. sanguineum* and *G. pratense* species, which are used in medicinal purposes and have an intense red color, are known to contain high amount of PAs.[84, 86] A high amount of PAs (16–20% of DW) have been reported for *G. sylvaticum* roots as well.[86] In addition, the seeds of *G. ibericum*, *G. pratense* and *G. sylvaticum* contain PCs.[84, 86] In contrast, leaves of most of the *Geranium* species contain only trace amounts of PCs; only four of 70 species contain PDs when studied with spectrophotometric methods.[84] The type of PAs and the degree of polymerization in *Geraniums* have not been comprehensively studied. Some individual compounds have been isolated, such as epicatechin and PC dimer from the cell suspension cultures, epigallocatechin from *G. thunbergii* leaves and more unusual glycosylated mixed PD and PC tri- and tetramers from the roots of *G. sanguineum*.[104, 146–147] In addition, A-type PAs based on mainly afzelechin units have been isolated from the roots of Mexican medicinal plant *G. niveum* (Fig. 13).[148]

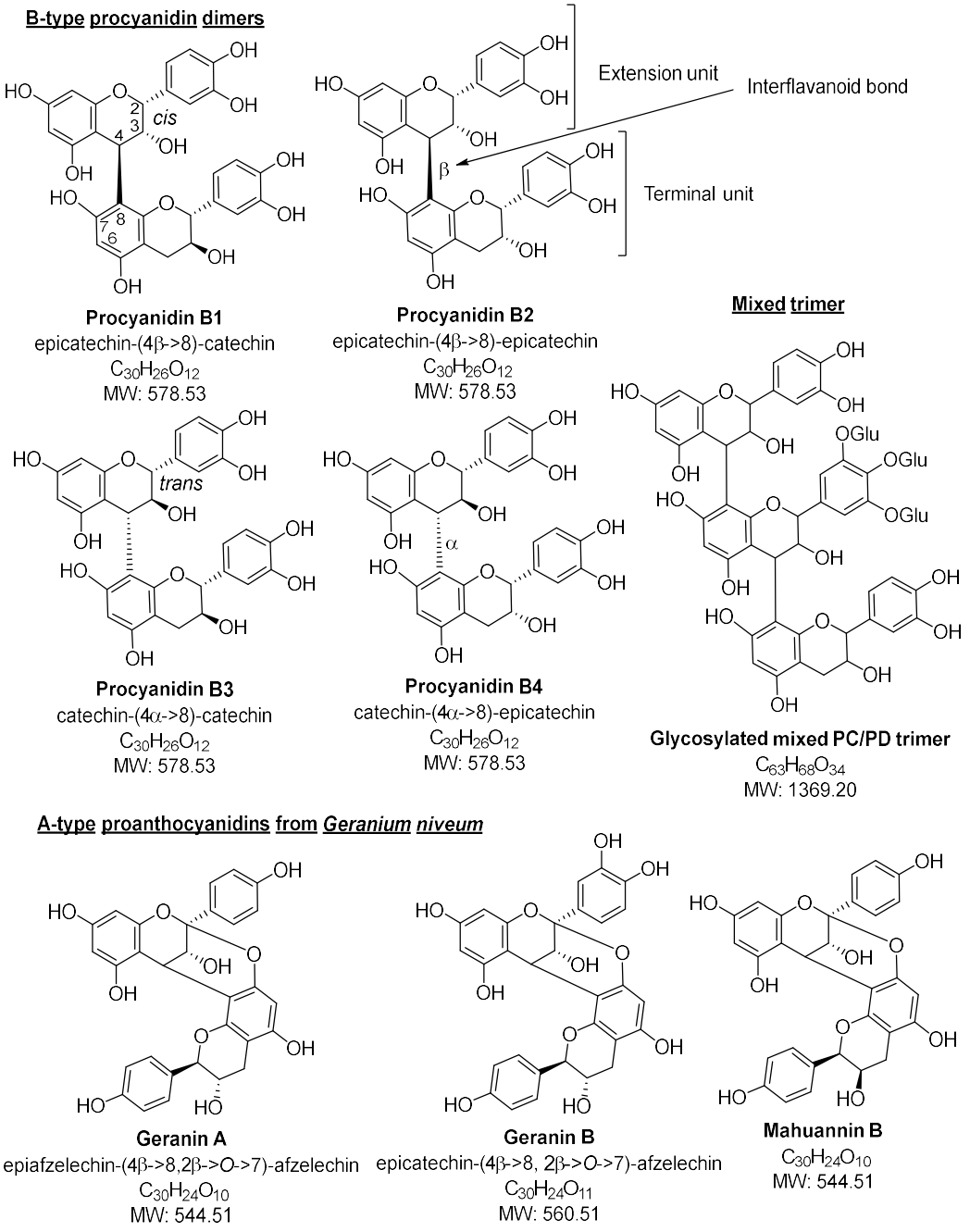


Figure 13. Nomenclature for PAs. Four B-type procyanidin dimers, proposed structure for the mixed trimer of *G. sanguineum* and three A-type PAs found from *G. niveum*.[147–148]

2.2.4 Intraplant and seasonal variation

Most existing studies about the distribution of the phenolic constituents in higher plants have focused on leaves, to ease classification. However, the absence of certain tannin groups in leaves does not mean that other plant organs are not able to produce those.[97] One of the most popular theories that tries to explain the intraplant variation of secondary metabolites is the optimal plant defense theory. It assumes that herbivores and pathogens are the major selective force for the production of secondary metabolites.[149] According to this theory, the allocation of anti-herbivore defenses among plant tissues is based on the probability of herbivore attack on the particular tissue, the value of the plant part to plant fitness, and the costs of defense.[149–150] Therefore, reproductive organs and seeds, which are of high value to the fitness and survival of the plant, should be more protected than the leaves and roots.[150–151] However, the floral tissues are not obviously more protected because they are so short-lived compared with the leaves.[152] Other popular theories that try to explain the type, the distribution and the abundance of secondary metabolites are the plant apparency theory; the resource availability theory states that the level of defensives depends on the available resources; and the carbon-nutrient balance hypothesis predicts that plants construct defensive compounds from the available nutrients and thus depend on the growth environment.[149, 153–156]

There are few references about the intraplant variation of polyphenols in *Geraniums.* Bate-Smith (1972) observed that the tannin content in some *Geranium* species varies between inflorescence and basal leaves.[83] In leaves of *G. thunbergii*, the geraniin content is 12% DW, whereas in stems it is only 1–2%.[125] In contrast, the stems of *G. macrorrhizum* contain more tannins than the foliage, but tannin types were not separated in the study.[23] Several studies have reported that aboveground parts contain mainly HTs, and underground parts have more PAs (Table 5). In *G. sanguineum,* the total phenol content is quite high in both leaves (35% DW) and in rhizome (29% DW).[31, 99] Similar high-tannin content in rhizome have been reported for *G. macrorrhizum*, 16%, and for *G. pratense,* 12–32%.[31, 83, 86] *G. sylvaticum* rhizome contains catechins 42.8% and galloyls 57.2% FW, whereas the same ratio is 12.4% and 81.4% for *G. pratense*.[86]

Table 5. Intraplant variation of phenolics for some well-studied *Geraniums* used in herbal medicine, *G. sylvaticum* and closely related *G. pratense*.

	HERBAGE / content of DW						RHIZOME / content of DW						Reference
	HTs	ETs	EA	PA	FLA	CA	HTs	ETs	EA	PA	FLA	CA	
G. macrorrhizum													
Perennial, southern Europe	12.5%	10%	+	-	quercitrin, kaempferol rhamnoside	+		10%			+	+	23, 31, 83-84, 93
G. sanguineum													
Deciduous perennial, Europe and Turkey	11−20% (GTs)	6% ETs		>1% catechins, epicatechins	>>1% hyperoside, isoquercetin	>1% caffeic acid, caftaric acid	16%	20%, geraniin		3-29% catechin, gallocatechin, mixed oligomers	>1%		23, 31, 83-84, 93, 99, 101, 147
G. robertianum													
Annual, Europe to China, North America	18% PGG	5-8%, 10% geraniin, isogeraniin	+	-	rutin, hyperoside, quercitrin, kaempferol rhamnoside	caffeic acid, ferulic acid		5%					26, 83-84, 86, 93, 125
G. thunbergii													
Perennial, Nothern China and Japan	GGs	12% geraniin, corilagin, dehydrogeraniin, geraniinic acids		epicatechin, epigallocatechin	quercetin (no hyperoside), kaempferitrin, kaempferol rhamnosides	mono- and digalloyl quinic acids, digallate							86, 104, 125, 132
G. pratense													
Deciduous perennial, Europe and western China	19%	9−13% geraniin, isogeraniin	7%	-	>1% hyperoside, isoquercetin, quercetin-galloylglycosides		1.5−4.0%	9-15% geraniin, GGs		9.8−26%		GA, chebula acid	36, 83-84, 86, 97, 103, 108
G. sylvaticum													
Perennial, Europe to Turkey		13% geraniin	+		kaempferin, hyperoside, quercitrin	caffeoyl acids, GA	23% GGs	13%	+	16−20% catechins			83-86, 93

HTs = hydrolysable tannins, ETs = ellagitannins, GGs= galloylglucoses, GER = geraniin, EA = ellagic acid, FLA = flavonoids, CA = caffeic acids and other small phenolic acids, GA = gallic acid

The structural differences between PAs and HTs and their allocation between the roots and leaves of *Geraniums*, suggest that these two tannin classes provide protection against different targets: highly polymerized PAs could protect against the degradative exo-cellular enzymes of soil microbes, nematodes and fungi; and ETs function against the various digesting enzymes of insects or mammalian herbivores present aboveground.[157–158] However, both HTs and PAs have shown only limited antiherbivore activity.[159–162] PAs are usually allocated in the long-lived plant parts and seeds and located in outer plant structures. They are relatively persistent because their C-C bonds are less susceptible to microbial degradation than ester bonds of HTs.[158, 163–165] PA polymers might have a lignin-like structural role, which may function as a deterrent against insect herbivores or as a drought-resistance mechanism.[142, 166] PAs might decrease the decay of roots and seeds in soil by inhibiting degradative microbial action, as it is well-known that catechins and PAs exhibit antiviral and antimicrobial properties.[63] Several mechanisms of action have been proposed, such as enzyme inhibition and iron depletion against bacterial targets, and the lysis of fungal cell membranes.[63] However, geraniin in *G. pratense* root extract also shows antimicrobial effects against soil-borne diseases; it has been suggested that a plant might release geraniin as a root exudate to control pathogens around it.[62]

Seasonal variation

The seasonal variation in the polyphenol content and structures should be studied whenever a large scale harvesting and a use of certain plant is considered. It is also interesting from an ecological point of view because the nutritive value of plant tissues has seasonal and ontogenetic variations that can affect herbivores.

In the seminal studies of Feeny (1968, 1976), the total tannin content of oak leaves increased from 0.5% to 5.0% DW towards the end of the growing season due to the increasing level of PAs, whereas the HT content stayed more constant throughout the growing season.[153, 167] These extensive studies with birch in Turku have shown that the suitability of birch leaves for insects declines during leaf growth, when the leaves undergo changes in the concentrations of compounds.[168] In young leaves, the content of HTs is high; after that, their content decreases and the content of soluble PAs increases during the leaf maturation.[169–173] Similar trends have been observed in the leaves of oaks and other birch species: the content of HTs, *p*-coumaroylquinic acid derivatives and flavonoid glycosides are at the highest in early spring and decrease towards the end of the growing season (Fig. 14).[171–173] These seasonal changes may reflect that young ET-rich leaves and mature PA-rich leaves in mountain birch are attacked by different herbivorous species, or that tannins might have several functions other than defence.[170–171] Zucker (1983) postulated, based on Feeny's results, that HTs follow the lifecycle of herbivorous insects and are therefore degraded when the insect pressure declines, whereas PAs are formed slowly according to the lifecycles of microbes.[158]

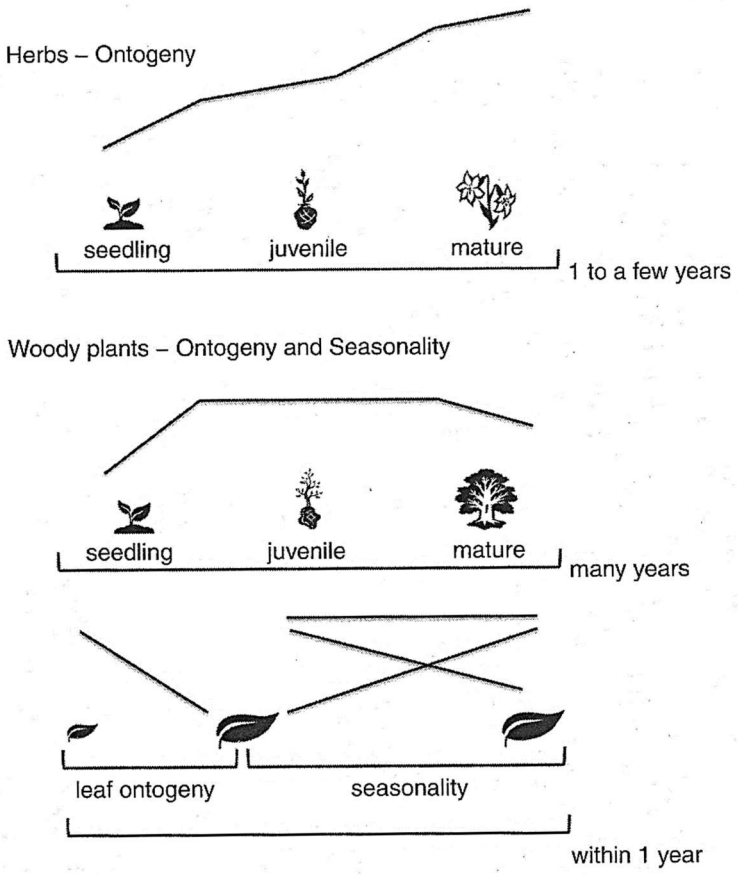


Figure 14. General overall patterns of temporal changes in secondary metabolites.[174]

The approximate 10% DW geraniin content in *Geraniums* is surprisingly high for a secondary metabolite, meaning that it must have a special role for the plant.[125, 175] In contrast to woody plants, studies of *Geranium* species show a pattern where the tannin content is at the highest during flowering. In leaves of *G. lucidum,* the ET content increases from young seedlings at 2.5% to 4.5% DW in the mature ones, whereas the tannin content in aerial parts of *G. sanguineum* diminished during the growing period. However, different tannin groups were not separated in this study.[83] Okuda et al. (1980) observed that geraniin content in the fresh above-ground tissue of *G. thunbergii* growing in Japan is at the lowest in May (0.6%) and at the highest during flowering in August (1.8%) and then decreases slightly to the end of the season in October (1.2%).[125] Similarly, the HT content was at the highest in *G. macrorrhizum* during the flower budding state in aboveground parts and during the seed formation in the rhizomes.[23]

In herbaceous species, the different herbivore pressures, depending on the ontogenic stage, can affect the polyphenolic content; pollinators, pathogens and competing plants might play a role as well (Fig. 14).[174] However, Okuda and Ito (2011) have stated

that tannin structures stay basically constant in herbaceous plants until the leaves decay, in contrast to some woody plants that transform their structures at the beginning of the season.[12] The seasonal variation in herbaceous plants is less studied; there is particularly scant information on non-economic species, because much of the literature deals with agricultural crops. As Feeny (1976) pointed out in his plant apparency theory, woody and herbal plants might have crucial differences regarding their chemical defenses.[153] Long-living plants such as trees are apparent; they are easily found by insects. They adopt a defense such as tannins, which are synthetized in bulk and which are generally repellent to most feeders. In contrast, herbaceous plants are unapparent because they are relatively short-lived and lack the resistant structural materials found in the woody plants. Thus, the whole plant is potentially susceptible to insect herbivory. Their chemical defense is thus more likely to involve the production of specialized toxic chemicals, which are produced in small quantity.[153]

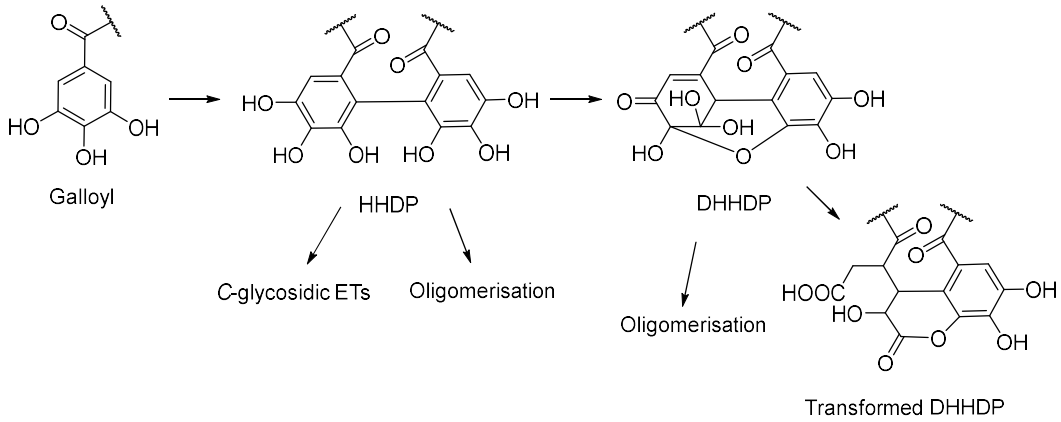


Figure 15. Biogenetic oxidative transformations from the galloyl and HHDP groups to more complex ETs.[140]

Apart from the tannin content, the structures of tannins can show seasonality. During the growing season, ETs in birch trees transform from simple monogalloylglucose and other GG precursors to more complex oxidized compounds, which contain more galloyl and HHDP groups.[172–173] Similar results have been obtained about ETs in leaves of *Liquidambar formosana*.[176] Transformations follow the biogenetic oxidative pathway that Okuda et al. (2000) have outlined (Fig. 15).[140] The DP of PAs is species-specific and seems to be quite uniform throughout the growing season and tissue maturity.[177]

2.3 Biological activity of tannins

The biological activity of polyphenols arises from the several phenolic hydroxy groups that are bonded into an aromatic ring (Fig. 16). One important property of the phenolic hydroxy group is its moderate acidity ($pK_a \approx 8–12$), which means that it easily donates hydrogen and forms negatively-charged phenolate ions.[2, 178] Phenolate anions are nucleophiles that can form ionic bonds with metals, and further loss of an electron leads to a radical. Electrons can delocalize into the aromatic ring and thus cause reactive centers to carbon atoms. Two delocalization-stabilized radicals can undergo oxidative coupling to form carbon-carbon or carbon-oxygen bonds between, for example, two galloyl groups. This forms a basis for the conversion of simple polyphenols to more complex and oligomeric structures.[2, 178]

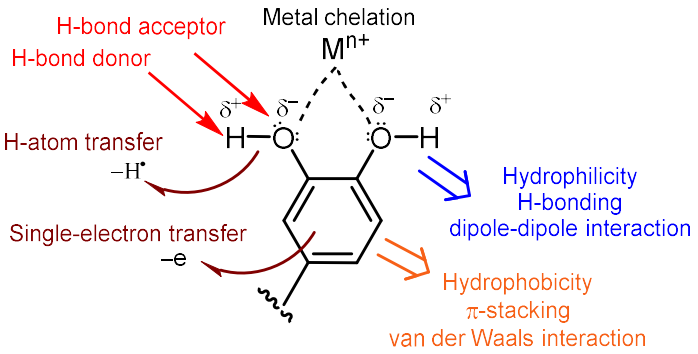


Figure 16. Summary of the physicochemical properties of the phenol functional group.[179]

Other important property of polyphenols is the ability of positively-charged phenolic hydrogen to form hydrogen bonds with negatively-charged centers in other molecules (Fig. 16).[178] In contrast to the hydrophilic character of hydroxy substituents, the planar aromatic rings have a hydrophobic character.[2] These two characters lie behind the ability of HTs to precipitate proteins and form complexes with heavy metals. However, these hydrophobic interactions (<4 kJ/mol) and hydrogen bonds (10–40 kJ/mol) are relatively weak compared with ionic and covalent bonds (100–1000 kJ/mol).[180] The metal complexation is, for example, the mode of action in the hemostatic activity and use of ET-containing plant extracts, such as treatment for extreme bleeding.[35] In the following sections, some biological activities of tannins are described more thoroughly; they focus on those activities that were studied in the experimental part of this study.

2.3.1 Antioxidant activity

Antioxidant (AO) activity is a capability of compound to scavenge free radicals and activated oxygen species, and thus protect other compounds from damaging oxidation.

This phenomenon is called oxidative stress, and it occurs in plants when there is serious imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses.[56, 181–182] Typical ROS are singlet oxygen ($^1O_2$), superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2). ROS are produced during the normal metabolic reactions of photosynthesis and respiration, but under stressful conditions (drought, high temperature, extreme light and salt), their concentration increases to damaging levels.[181] In humans, the oxidative stress has been associated with many age-related diseases such as diabetes, osteoporosis, cardiovascular illnesses, cancer, and neurodegenerative disorders.[57] Plants have both enzymatic antioxidants, such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and non-enzymatic antioxidants such as ascorbic acid, α-tocopherols, glutathione, carotenoids and phenolic compounds.[181] In general, the more hydroxy substituents a phenolic compound has, the stronger are its antioxidant and pro-oxidant activities.[183] Several studies have shown that three main factors affect the antioxidant capacity of polyphenols: 1) the number of phenolic hydroxy groups per molecule; 2) the planar structure that determines the degree of conjugation and resonance effects; and 3) the presence of additional functional groups.[184–186] All phenolic hydroxy groups are not equal in the radical scavenging activity. The *ortho*-dihydroxy (catechol) group is considered the most important radical target site of both tannins and flavonoids, although trihydroxy (pyrogallol) groups have higher redox activities than corresponding dihydroxy groups.[183, 187–190]

In computational studies with simple phenolic monomers, the main antioxidant mechanism is the hydrogen atom transfer, which means the capacity of the phenolic functional group to donate a hydrogen atom to a free radical.[191] In this process, polyphenol antioxidant itself becomes a free radical, and if it is relatively stable and does not continue the radical chain reaction, it is called a chain-breaking antioxidant.[2] In this mechanism, the efficiency of antioxidants is mainly dependent on the bond dissociation energy, i.e., the strength of the O–H bond and the stability of the formed phenoxyl radical. Two structural factors—the number and position of additional hydroxy groups that enables hydrogen bonding, and a possibility to the delocalization of the unpaired electron through conjugation—increase the stability of formed phenoxyl radicals.[2, 184]

Another possible reaction is the single-electron transfer where polyphenol receives an electron from the free radical and forms a stable radical cation.[191] The ionization potential is an important factor in this antioxidant capacity mechanism.[2] The third possible antioxidant mechanism is through a metal chelation by removing the metals or altering their redox potential, thus making them inactive.[2, 184] Metal ions such as iron (II)/copper (I) and iron (III)/copper (II) are involved in the conversion of O_2^- and H_2O_2 into highly reactive $HO^\bullet$ radicals through Haber-Weiss/Fenton-type reactions.[2]

Catechol and pyrogallol groups are particularly good hydrogen donors because of the stabilizing intramolecular hydrogen bonds, whereas, kaempferol-type flavanols might be more prone to act via the single-electron transfer mechanism because they have lower

ionization potential values through the electronic delocalization enhanced by the resonance effects and structural planarity.[2, 192] Other structural features besides the hydroxy groups contribute to antioxidant activity. The antioxidant activity of tannins increases when the number of galloyl groups and the molecular weight increases until the insolubility becomes a limiting factor.[187–188, 190] The stability of the free radical generated by the HHDP group is higher than that of gallates.[190, 193] But a transformation to DHHDP group and further oxidative transformations do not increase the radical scavenging activity.[56, 190] Among flavonoids, the optimal antioxidant activity requires the presence of a dihydroxy group in the B-ring, a 2,3-double bond in the C-ring, and 3- and 5-OH groups adjacent to the 4-keto structure, as in quercetin (see flavonoid core in Fig. 9).[96, 187] In general, the glycosylation and methylation of hydroxy groups reduce the activity of flavonoids.[183, 188] However, the glycosylation of the 3-OH group in quercetin is an interesting exception; in some studies, it increased the radical scavenging activity.[194] Tannins have far more potential as antioxidants than the smaller-size flavonoids, α-tocopherol and ascorbic acid.[187–188, 195–196] A reason for the higher antioxidant activity of tannins might be their preference for the oxidative coupling, which can break the radical chain reactions. These slow polymerization reactions can reproduce the –OH moieties.[187–188, 197–199] As a result, polyphenols have more electrons for the oxidation than can be expected based on their initial structures.[198–199]

One commonly used antioxidant activity assay is the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay that has also been used to study *Geraniums*.[188, 196, 200–202] The DPPH assay basically measures the number of hydroxy groups in the compound.[187] The radical scavenging effects vary depending on the radical used in the assays; DPPH is quite large when compared with, for example, HO· radical used in other assays.[196–197] Other factors that affect the antiradical properties of antioxidants are steric reasons, hydrophilicity or lipophilicity of the antioxidant and its hydrogen-bonding characteristics.[2, 192, 203] The solvent used in the assays can also affect the assay results. In polar solvents, the most acidic OH groups are deprotonated and phenolic compounds react faster.[203]

The methanol extract of the aerial parts of *G. sylvaticum* has shown good antioxidant activity (IC_{50} 31 μg/ml). However, it is not as active as the other *Geranium* species tested in the same study.[202] The free radical scavenging activity of geraniin in the DPPH assay is similar to the activity of epigallocatechin gallate (EGCG), a strong antioxidant found in green tea.[204–205] Several studies have shown that the activity of *Geranium* extracts can be higher than the activity of its constituents, because of the synergistic effects.[113, 201] The reason for synergistic effects might be that the water-solubility of poorly-soluble compounds is enhanced in the presence of other highly water-soluble compounds, such as glycosides.[131]

2.3.2 Pro-oxidant activity

The dual character of reactivity makes the biological activity of polyphenols an interesting subject. Polyphenols can act as protective antioxidants in the reduced form, or damaging pro-oxidants (PRO-OX) in the oxidized form, depending on the environment and free radical source.[2, 183, 206] Oxidative stress is an imbalance between antioxidant and pro-oxidant effects. Active tannin antioxidants might become pro-oxidants through auto-oxidation under special conditions, such as high concentrations, the presence of redox-active metals, copper (II), iron (III) or at high pH.[2, 181, 204] Oxidative stress is not always bad; it can destroy invasive pathogens during inflammation and might be the reaction mechanism behind the anticancer activity of polyphenols.[182, 207]

The redox system phenol ↔ semiquinone ↔ quinone is based on the ability of phenolics to readily donate electrons and protons, and constitutes the molecular basis of the chemical and biological effects of phenolics (Fig. 17).[24] Superoxide anion radicals are produced when the reactive semiquinone donates an electron to molecular oxygen; after that, the superoxide radical can further react and produce other ROS (Fig. 17).[180] The ROS and phenoxyl radicals are behind the actual harmful oxidative reactions that can damage DNA, lipids and other biological molecules.[180, 206] Iron is an oxidant that can be found in insect guts and polyphenols can take part in the Fenton reaction where Fe^{2+} reacts with H_2O_2 and is oxidized to Fe^{3+}; this reaction produces hydroxyl radicals that are the most reactive and harmful of all ROS species.[7, 204, 208] Copper is even a more potent oxidant than iron because of its lower standard reduction potential.[2]

The pro-oxidant action may be a cause of the potential toxicity and harmful effects of polyphenols against herbivores and pathogens that feed on tannin-rich plants.[180, 183, 189, 208–210] The protein precipitation capacity has been considered the main defensive function of tannins against vertebrate herbivores through the digestibility-reducing action. However, it seems that many insect herbivores are able to tolerate high amounts of tannins because they have evolved adaptations to the digestive systems that reduce the harmful action of tannins: hydrolysis by enzymes, adsorption on the peritrophic membrane, surfactants, high pH in the gut and other gut conditions, such as the oxygen level and redox conditions.[161, 180, 211–215] The alkaline digestive tract of insects might have benefits, such as enhancement of the solubility of leaf proteins, stronger activity of the digestive proteinases or the reduction of non-covalent binding between proteins and plant tannins.[216]

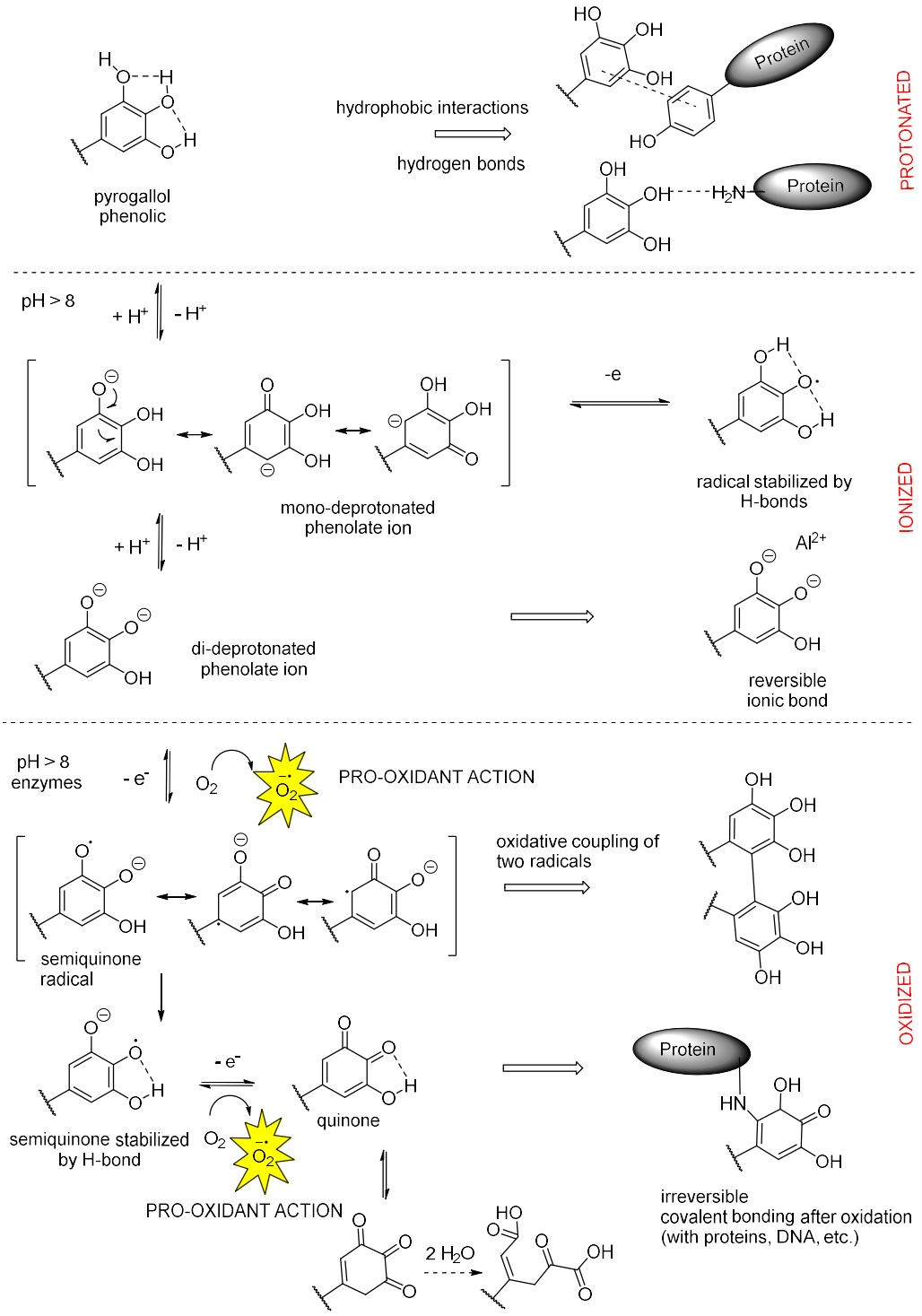


Figure 17. Sequential two-electron dehydrogenative oxidation process of pyrogallol-type phenolics and summary how the oxidation state determines the mode of action; protonated, ionized and oxidized species form different bonds.[2, 180, 191, 219]

In contrast, the gut conditions can transform tannins and make them more harmful.[180] The review of Appel (1993) summarizes this newer theory about how plant tannins function against insect herbivores after activation through oxidation.[180] In particular, many Lepidoptera larvae have highly basic gut conditions (pH 9–12), which favor the auto-oxidation of tannins.[8, 161, 180, 213] Also, enzymes such as peroxidase, polyphenol oxidase and catalase can oxidize tannins in insect guts.[7, 180, 217] Tannin oxidation produces quinones that are generally more toxic substances than polyphenols per se. Semiquinone and quinone-type compounds are highly reactive electrophilic molecules that can take part in a variety of chemical reactions. For example, they can bind covalently with plant proteins, can complex digestive enzymes of herbivores or damage essential nutrients and produce reactive oxygen species that cause oxidative stress on the tissues of herbivores that feed on tannin-rich plants.[24, 180, 209] However, it seems that the balance between antioxidant and pro-oxidant compounds produces a quite high threshold limit for the HT content (circa 15% DW), after which a substantial amount of semiquinone radicals is produced in caterpillars.[159] Furthermore, recent studies have not shown any negative impact of semiquinones on caterpillars' growth rate or essential amino acid utilization.[159, 218]

Important characteristics that determine the pro-oxidant activity of phenolics are their metal-reducing property, chelating behavior, O_2-reducing capacity, the ease of oxidation and lipophilicity.[183, 207, 220] All *o*-dihydroxylated phenolics, such as quercetin flavonoids and chlorogenic acid, and gallic acid derivatives can produce ROS during their oxidation.[208–209, 221] The polyphenols with catechol and pyrogallol moieties are especially prone to oxidation.[2] More stable semiquinone radicals have a higher O_2 reducing potential, and it seems that the resonance delocalization to the side chain that contains double bond or ester bonds diminishes the radical stability.[220]

Among tannins, ETs, which are distinguished by the presence of oxidatively-coupled galloyl groups, can form higher concentrations of semiquinone radicals than GGs or PAs.[210] The free radical generated by the HHDP group also has higher stability than that of gallates.[190, 193] Among ETs, the time needed for the accumulation of maximum semiquinone concentration varies: ETs containing valoneoyl groups reach the maximum in 11 mins and ETs with HHDP groups reach maximum in 30 secs when studied with EPR spectrometry, thus indicating that semiquinones of valoneoyl groups were more stable.[210] Results from the browing assay at pH 10 verified this by showing that ETs, which contain further-oxidized valoneoyl (especially with free COOH group) and nonahydroxytriphenoyl (NHTP) groups, have higher oxidative activity than the HHDP groups.[222] These oxidatively coupled galloyl groups are more rigid, which limits the resonance delocalization; the radical is more likely to reduce surrounding O_2 molecules.[210] It seems that DHHDP-type modifications do not increase the pro-oxidant activity; geraniin and carpinusin have showed relatively low oxidative activity at pH 10, although their pro-oxidant activity at neutral pH was good.[223] In another study, geraniin also showed much

lower reducing ability of Fe^{3+} than ascorbic acid, suggesting that instead of being an effective pro-oxidant, it is a good antioxidant.[204] Among PAs, PDs are more easily oxidized than PCs.[210]

The lower oxidative activity of PAs and GGs may be due to the higher potential that phenoxyl radicals end up with coupling reactions, as shown in Figs 17 and 18.[210] This coupling can reproduce –OH moieties in polymeric products.[198–199] The fate of highly reactive quinonoid species is often overlooked, although these are also formed in the "protective" antioxidant processes.[2] The subsequent reactions of quinones depend on the surrounding conditions, such as the solvent or oxygen content. The oxidized pyrogallol group can further react via hydrolytic ring opening to produce carboxylic acid functions (Fig. 17).[219] These reactions resemble the oxidative transformations observed for DHHDP-ETs (Fig. 15).[140] The DHHDP group of geraniin converts quickly under basic aqueous conditions to dehydrochebuloyl and brevifolin carboxyl groups.[175]

The high pro-oxidant activity of flavonoids has been proved *in vitro* in the presence of transition metals, such as Cu^{2+}, lactic peroxidase and H_2O_2, which are also potentially available *in vivo*.[183, 225] For example, lactic peroxidase catalyzed quercetin semiquinone is stable over 15 min.[225] The oxidation pattern of flavonoids having catechol or pyrogallol units in the B-ring is very similar to tannins, except that in flavonoids, a radical can be delocalized over the C-ring. Furthermore, similar coupling reactions can occur between B-rings (Fig. 18).[224] The substituent at C-3 plays a crucial role: if there is no hydroxy group or it is glycosylated, formation of a keto function is not possible in that position.[96, 203, 224] For example, the oxidation of flavonoids using enzyme or DPPH radicals yielded stable quinones and dimeric compounds under aprotic conditions (acetonitrile), and solvent addition products under protic conditions (MeOH and EtOH) (Fig. 18).[192, 224]

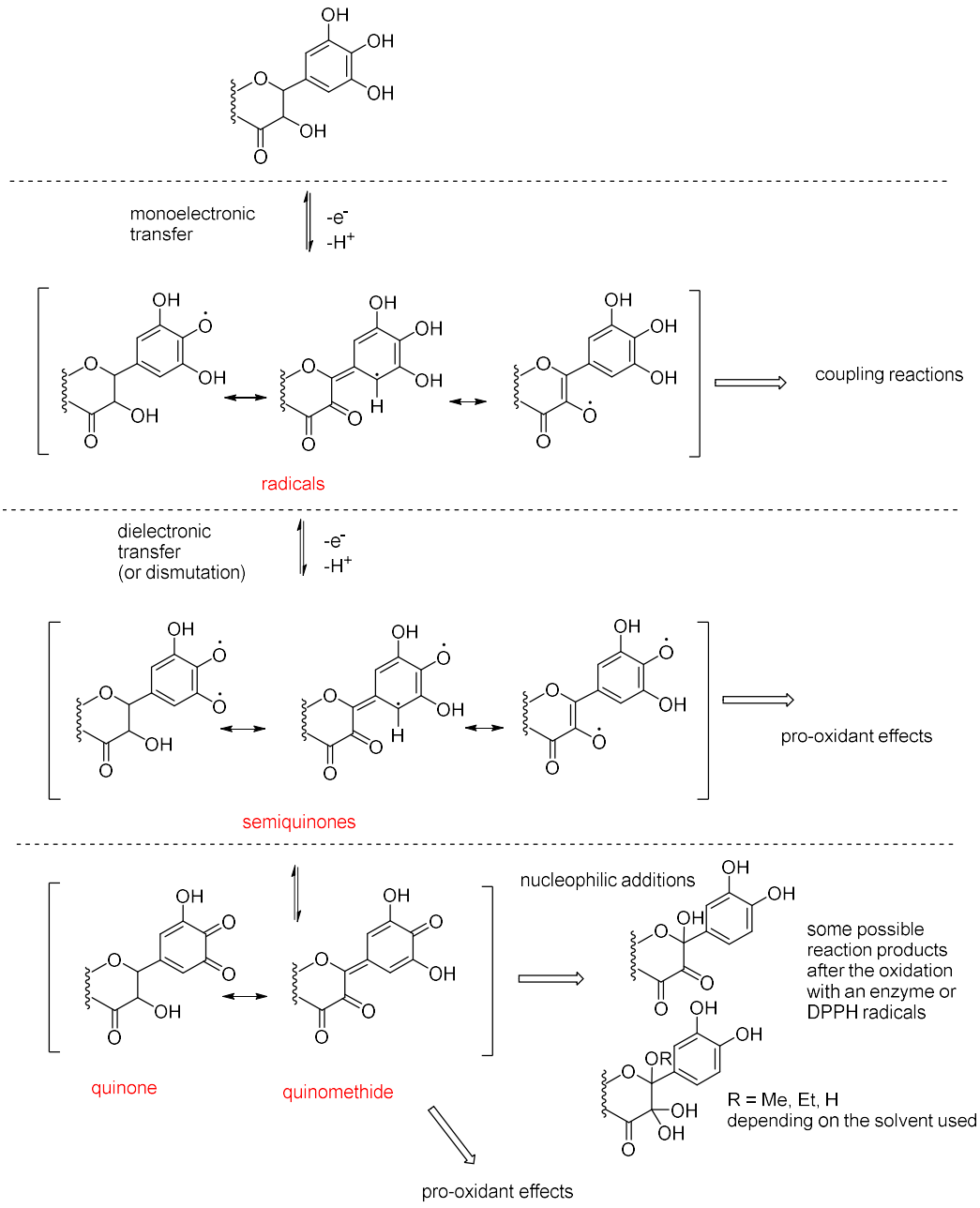


Figure 18. General oxidation pathway for flavonols.[192, 207, 224]

2.3.3 Activation via hydrolysis

One factor affecting the activity of tannins is their metabolism and bioavailability in the particular organism. In the feeding test with insects, the amount of initial compounds that remains unchanged, oxidizes or hydrolyzes, depends on the organism and the tannins used in the test.[161–162, 226] Hydrolysis can be one detoxification mechanism of herbivores against tannins, or the hydrolysis products can be more harmful to herbivores because they are more easily absorbed into insect tissues than the initial tannins.[7] The hydrolysis products of geraniin, ellagic acid (EA) and gallic acid (GA), in *G. viscosissimum* extract inhibited the growth of the larvae of the polyphagous insect pest, *Heliothis virescens*.[35] Based on that, Klocke et al. (1986) have hypothesized that geraniin acts as a "protoxin" by releasing insect growth inhibitors, such as EA, after the hydrolysis in the *in vivo* ingestion.[35] However, geraniin itself is almost twice more an efficient growth inhibitor than its hydrolysis products EA and GA, and the activity of the third hydrolysis product, brevifolin, was not studied (Fig. 11).[35] Similarly, in the antioxidant activity assay, all hydrolysis products of geraniin except corilagin showed a lower radical scavenging activity than geraniin.[227] Niehaus and Gross (1997) came to the same conclusion after they found the tannin acyl hydrolase, a specific enzyme from oak leaves that degrades HTs.[228] Thus HTs might act in two ways in the defensive system of plants: 1) by direct astringency-dependent deterrence and 2) indirectly through their hydrolysis products, which are formed after herbivore attack.[228] Similar defense strategies, where toxic substances are released after the hydrolysis, occur in other secondary metabolites, such as cyanogenic glucosides and glucosinolates.[228]

ETs are fairly labile, especially when compared with PAs under extreme conditions such as high temperature, and in the presence of degrading enzymes, acids or alkaline conditions.[35] The partial degradation of HTs can be conducted with the treatment of tannase, hot water or weak acid.[229] The covalent bonds of PAs are stronger than ester bonds in HTs. In the insect guts, for example, PAs are relatively stable whereas 12–82% of the HTs degrade, depending on the insect species.[7] In the *in vitro* assay of the Eucalyptus plant, the hydrolysis of ETs took 0.1–24 hours in hot water and 5 min to 24 hours in H_2SO_4 depending on the structure.[230] The most sensitive compound to hydrolysis was corilagin, which has the same sugar configuration as geraniin.[230] The highly reactive $^1C_4$ conformation of geraniin might assist the production of hydrolysis products. In spite of this, geraniin is relatively stable among ETs because the highly reactive hydroxyl-*o*-benzoquinone structure of crystalline geraniin is stabilized by intramolecular hemiacetal formation in water. When compared with an HHDP group ($t_{½}$ ca. ½h, H_2O, 90°C), hydrolysis of DHHDP group is much slower in hot water ($t_{½}$ ca. 24h, H_2O, 90°C).[136] In another study, geraniin degraded to corilagin after a half an hour of boiling the extract in water.[231] During hydrolysis in hot water, the reaction rate and formed products are pH-dependent.[229] On the other hand, in enzymatic hydrolysis, the reaction rate is affected by steric factors and the hydrolysis order of typical ET groups is 1-galloyl > 2-galloyl > 3-

galloyl > 6- and 4-galloyl and 4,6-HHDP > 2,3-HHDP.[229] In addition, enzymatic hydrolysis is more effective against smaller molecular weight phenolics than PGG and GTs and methoxy groups are also preferred over hydroxy groups.[228]

Typically, smaller hydrolysis products are more water-soluble and can more easily permeate the peritrophic envelope of insects.[7] The hydrolysis products of HTs, gallic acid and smaller GGs, have been detected in the feces of insects after the ingestion of HTs (Fig. 11).[162, 232–233] The quantitation of only gallic acid in feces underestimates hydrolysis, because the hydrolysis products can be further processed.[162] For example, urolithins, i.e., transformed hydrolysis products, have been detected in the plasma and feces of rats, sheep and humans after the ingestion of geraniin and ellagic acid and other HTs.[227, 234] These intestinal microflora metabolites are ellagic acid-like compounds but with less ester bonds and less phenolic hydroxy groups, and some of the groups are methylated.[227, 234] Urolithins may be behind the positive health effects of ET-rich food because the antioxidant capacity of urolithins is higher than those of original tannins.[227] Corilagin was also detected in rat urine, which means that HTs smaller than 600 Da can be absorbed and excreted as such.[227] Urinary metabolites of PAs in humans have contained glucuronic acid and sulfate conjugates.[37]

2.3.4 Protein precipitation capacity

Protein precipitation capacity (PPC) is a characteristic of tannins, which distinguishes them from other polyphenols.[235] The term "tannin" comes from the French word "tan" that means oak extracts used for the making of leather. In the tanning process, the collagen molecules of raw animal hides are cross-linked with polyphenols via hydrogen bonds.[2] This phenomenon is also behind the taste and astringency properties of tannins.

The size and polarity (described as the octanol-water partition coefficient, K_{OW}) of the polyphenol seem to be the most important characteristics in the protein binding.[235] Other important factors are the size, the type and the solubility of the protein and existing conditions, such as pH, temperature, ionic strength, concentrations and the ratios of protein and tannin, and coexisting compounds that can affect solubility.[131, 235–236] The precipitation process has two stages: first, polyphenol binds to the protein to form a complex, which then further aggregates to form the precipitate.[1, 236] The smallest polyphenol able to effectively bind proteins is epigallocatechin gallate (MW 458 Da) and the upper limit is in highly polymeric PAs (10–15 kDa).[235]

Quideau et al. (2011) have generalized that GGs and GTs are rather hydrophobic, flat but flexible; ETs are more hydrophilic, spherical and rigid; and PAs are hydrophilic, threadlike and flexible—characteristics that most likely determine the PPC of different these tannin groups.[2] Among GGs, the higher number of galloyl groups increases PPC up to pentaGG, which is the most hydrophobic and effective protein binder.[237] The 1-galloyl

group in the anomeric position has the strongest hydrophobic interaction of the galloyl groups.[131] The transformation of GGs to ETs increases the water-solubility and decreases PPC; however, again some ETs that have free a 1-galloyl group can have equal or even higher PPC (affinity to methylene blue) than pentaGG, such as chebulagic acid and chebulinic acid.[238] It seems that the molecular flexibility is more important in hydrophobic interactions than the number of hydroxy groups.[175] Interestingly, the crystalline geraniin shows no astringency on the human tongue, although it precipitates hemoglobin, which makes its use in herbal medicine more favorable.[133] PAs are significantly better protein binders than ETs, and the capacity increases when the degree of polymerization increases.[239]

The defensive properties of tannins against herbivores and pathogens are generally attributed to their ability to bind proteins. Both HTs and PAs can interact with proteins to form soluble or insoluble complexes.[240] Tannins may complex proteins in the guts of herbivores in two ways: non-covalently by forming hydrogen bonds between their hydroxy groups and the electronegative sites on the protein or by hydrophobic interactions; or covalently after the oxidation of tannins.[235, 240] Once proteins are complexed with tannins, they can be difficult to digest and the inactivation of digestive enzymes by tannins may impact the nutrient uptake. Detergents and high pH can inhibit the hydrogen-bonding ability, but high pH can cause oxidation that enables covalent bonds with the nucleophilic parts of proteins.[240] Protein binding can affect the diet choice and the digestions of mammalian herbivores. For example, in sheep, high PA concentrations (6–12% DW) can hinder digestion, whereas moderate concentrations (2–4%) can be beneficial through the protein binding that assists amino acid absorption and reduces ammonia emissions.[241]

2.3.5 Copigmentation

The pollinator-attractive purple color of *G. sylvaticum* petals is caused by anthocyanin pigments. Copigmentation is a phenomenon in which the color of anthocyanins is enhanced by the presense of other colorless compounds. The five main factors that influence the purple and blue flower colours caused by anthocyanins are:

1) the co-existence of several anthocyanin structures
2) cellular concentrations
3) the pH of the cell
4) intra- or intermolecular copigmentation
5) association with metals.[124]

The chromophore of anthocyanidin gives rise to an absorption maximum around 520 nm, which changes according to the fine structure. The most meaningful difference to the

absorption maximum of anthocyanins comes from the number of substituents in the B-ring. More hydroxy groups in the B ring (band I) increase the colour (moves to larger wavelengths) (Table 3). Methylation further causes a small hypsochromic shift of 1–4 nm. Other aromatic acylation causes a bathochromic shift and extra stability, possibly through intra- and intermolecular copigmentation.[121] Glycosylation shifts the spectra maxima of anthocyanins 6–10 nm lower and increases the stability and the water-solubility.[109, 119] Complex anthocyanins have several glycosyl and acyl groups, which leads to extra stability in the solution.[121]

The cellular concentrations of anthocyanins affect the flower color; in high pigment concentrations, anthocyanins self-associate, which increases the color intensity more than can be expected from the concentration (Fig. 28).[119, 123] Other factors that contribute to the observed color of anthocyanins are temperature, the presence of salts, matrix and solvent.[119, 242–243] The vertical stacking of aromatic rings in the self-association leads to peak-broadening in the spectrum (but not to the bathochromic shift) as interaction between two same chromophores leads to exciton-type Cotton effect (CE) (Fig. 26) depending on whether the vertical stacking is clockwise or anti-clockwise; cyanin and pelargonin typically form right-handed aggregates (positive CE) and peonin, delphin and malvin form left-handed ones (negative CE).[244–245]

Willstätter and Zollinger suggested as early as 1913 that the color of anthocyanin is dependent on pH. This unique feature of anthocyanins is due to the existence of four equilibrium forms in aqueous buffer solutions depending on pH; all forms have their own color and stability properties (Fig. 19).[119] The most stable form, reddish flavylium cation (AH^+) dominates at highly acidic conditions in which it is stable for several weeks at room temperature.[119] In mildly acidic pH conditions, there is kinetic and thermodynamic competition between the hydration reaction on position 2 that yields a colorless hemiketal form (B) and the deprotonation reactions of the hydroxy groups that yields quinonoidal bases (A, A^-) (Fig. 19).[119] Hemiketals are also called carbinol pseudo-bases, and they can further degrade through the opening of C-ring to yellow-colored chalcones (C) which ionize in strongly basic solutions as well.[243] When quinonoidal bases further deprotonate, anionic quinonoidal bases, which are resonance stabilized and blue-colored, are formed (Fig. 19).[119] However, without the presence of any copigments, these unstable A and A^- forms degrade, and no more than a few percent of them are present in the equilibrium state.[243] Anthocyanidin aglycones are rarely found in plants; their presence in the sample is probably a result of the degradation during sample preparation through the hydrolysis of glycose moieties of anthocyanins. Also, the aliphatic acyl groups attached to sugars are labile towards mineral acid.[119, 122]

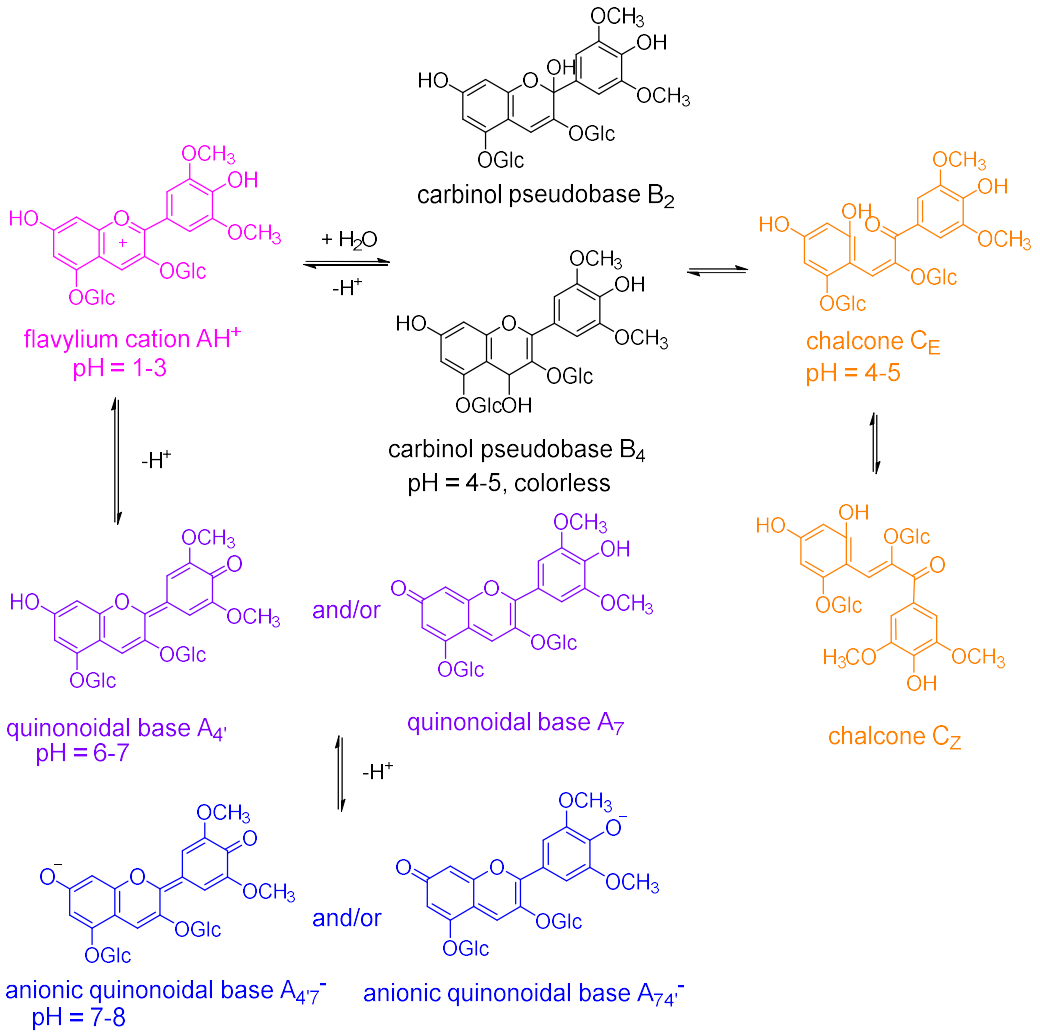


Figure 19. Malvidin diglycoside equilibrium forms, their pH-dependent color changes and the pH with highest concentration of corresponding anthocyanin species.[119, 243, 246]

The pH of the plant cell vacuoles is such that anthocyanins are practically colorless. The vacuolar pH of petal cells is typically 4.5–5.5, but some purple flowers, such as blue geraniums can have pH over 6.[112, 123, 247] Therefore, other organic and phenolic compounds are needed as copigments to produce the blue and purple colors of flowers.[123, 244] This copigmentation theory was first formulated by Willstatter and Zollinger (1916) and Robinson and Robinson (1931).[248–249] The stabilization of the colors of anthocyanins at higher pHs can be achieved with a non-covalent complexation of the flavylium cation and quinonoid bases by other polyphenols and/or metals. The planarity, the potential surface area for π-π-stacking and electron-donating capacity are factors that contribute to the ability of the phenolic compound to act as copigment.[124] Colorless natural compounds such

as hydroxylated benzoic and cinnamic acids, hydroxyflavones and tannins are able to act as copigments of anthocyanins. Flavonol glycosides and chlorogenic acid are the most common copigments.[123, 124, 250–251] Kaempferol, myricetin and quercetin glycosides are well-known flavonoid copigments in some blue-colored geraniums.[112]

Only a few studies have measured the copigmentation effect of tannins.[251–252] In these, HTs and especially GGs have shown to be better copigments than PAs. Tested ETs were poorer copigments than GGs because of their inflexibility and hydrophilic character. This was unexpected, as there are significant amounts of ETs in many flowers such as roses.[124, 251, 253] The studies with galloyl esters of flavan-3-ols suggest that a galloyl group is mainly responsible for the copigment effect.[124, 251] The copigmentation has only been reported to happen with anthocyanins, but it could also happen with other classes of flavonoids.[250]

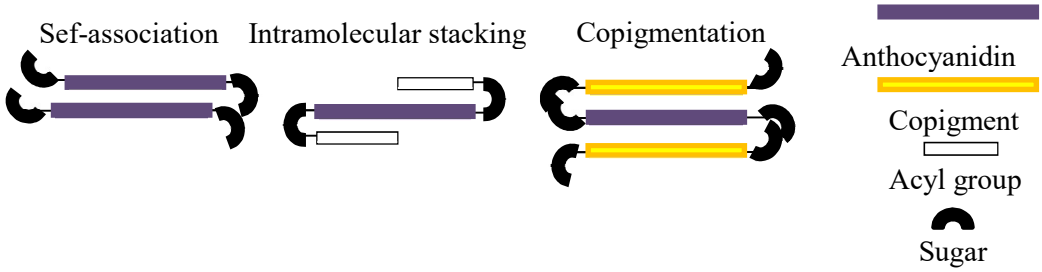


Figure 20. Molecular stacking types of anthocyanins.[254]

The section above described copigmentation where the intermolecular stabilization is caused by other molecules. The intramolecular copigmentation can happen when attached aromatic acyl groups stack sandwich-like to the aromatic rings of the anthocyanin, and stacking is further promoted by intramolecular bonding (Fig. 20).[121, 255] The exact mechanism of copigmentation is not totally revealed, but it is similar to self-association and similar factors affect it. Goto et al. (1990) have suggested the molecular stacking theory in which the hydrophobic interactions in aqueous solutions between the aromatic nuclei of copigments and the anthocyanins with different λ_{max} lead to charge-transfer between them; that causes the bathochromic shift and bluing effect.[256] Other research groups have come to the same conclusion: that the π-π complex and charge transfer from the electron-rich flavonoid or phenolic ester to the electron-deficient flavylium cation or quinonoidal anhydro-base protect the anthocyanin from the water addition and transformation to colorless forms.[121, 124, 250] This hydrophobic chiral stacking can be further stabilized by surrounding the complex with hydrophilic sugar moieties that can form hydrogen bonds.[255–256] The addition of solvents other than water reduces the copigment effect because it interferes with the hydrogen-bond network.[243] Similarly, a higher temperature decreases the copigment effect.[243, 257]

The copigmentation causes a bluing effect, as it enhances the absorption intensity of pigment (hyperchromism) and shifts the absorption maximum to higher wavelengths (bathochromism).[124, 243] The formation of non-covalent copigment complex can be observed as a new, broad, intense spectral band in the UV/Vis spectrum.[124] For example, the copigmentation increased the absorbance of malvin 121% with PGG and 173% with quercetin-3-galactoside (at 520 nm, 22°C).[251] Malvin is most stable at pH 3.65 in time and with temperature, and therefore this pH is optimal to study the copigmentation process.[258]

The copigmentation can also occur with metal ions such as Mg (II), Al (III) or Fe (III) ions.[244, 255] The metal complex theory was first introduced by Shibata 1919.[244] These kinds of metalloanthocyanins are rare and are mostly found in blue-colored flowers. One good example is commelinin, a supramolecule that consists of six anthocyanin molecules and six flavone molecules in a complex with two magnesium atoms.[259] This ratio is typical for metalloanthocyanins.[244] Metal complexes are relatively unstable and break down during the isolation.[119] The association with metals requires an *orto*-hydroxy group, so it not possible with pelargonidin and malvidin aglycones.[244]

2.4 Analysis and identification methods of tannins

For almost a hundred years, the precipitation of proteins, colorimetric reactions with phenolic rings and depolymerisation methods have been used to quantify total tannins or tannin groups.[14, 240] For example, the detection of ETs is based on the detection of its hydrolysis product EA. The disadvantage of this method is that the amount of EA heavily depends on the sample preparation conditions, such as high temperature and prolonged treatment, which increase the hydrolysis of EA.[125] Other disadvantages for spectrophotometric methods are the low sensitivity and the interference of other common constituents present in the samples, such as ascorbic acid, ferrous ions and sugars.[14] Total methods are still useful in the screening of large number of samples or quantifying compound groups that have a poor resolution in the HPLC such as PAs. However, since the 1980s, the HPLC-UV-MS has been the preferred method, as it permits the detection of tannins in complex mixtures.[125] HPLC methods, especially in combination with electrospray ionization mass spectrometry (ESI-MS) and diode array detection (DAD), have been successfully used in the characterizations of various HTs and other polyphenolics from plant tissues.[173, 260–265]

Extraction

Prior to the chromatographic analysis, polyphenols have to be extracted from the plant material. The extraction is most reliably done from vacuum- or freeze-dried samples, and freeze-drying is also recommended for long-term storage of plant material.[266] Typical

solvents for the extraction of water-soluble phenolics are aqueous methanol and aqueous acetone.[14, 266] When the plant contains GTs, the use of methanol and acids should be avoided because these can hydrolyze the depside linkages already at room temperature at neutral pH.[267–268] Acetone is more useful because it inhibits the action of enzymes. The best solvent for the extraction of ETs of birch leaves was 70% acetone with 0.1% ascorbic acid, but the suitability can vary among plant species.[266] Antioxidants can be used to prevent losses due to oxidation, but they might interfere in the activity assays later on. Ascorbic acid should not be used with DHHDP-ellagitannins because it easily forms adducts (see Fig. 12).[14, 135, 269] Grounding, vortexing and sonication make the extraction more effective.

The lipophilic chemical constituents of the leaf surface can be examined separately from the internal leaf components by briefly (circa 10 s) rinsing the leaf in a solvent such as acetone, diethyl ether or 95% ethanol.[55, 87, 270] Some part of the tannins may stay unextracted, especially those that have a high molecular weight or that are bound to fiber. The amount of bound EA is usually approximately 1–7% of that extracted with boiled 50% methanol.[83] Previously, extraction with boiling water was used, but it is not recommended anymore because it causes the degradation of compounds through hydrolysis; geraniin is particularly susceptible to hydrolysis (Fig. 11).[56] For the same reason, air-drying, especially at elevated temperature, is not recommended.[14]

2.4.1 Chromatography

The main challenge in studying polyphenols and tannins from crude plant extracts is the presence of hundreds of plant metabolites and their structural complexity. Therefore, an effective separation method is needed for their analysis. Chromatography is a separation method where compounds elute through column at different rates depending on the interaction of stationary and mobile phases. As tannins are highly polar, thermolabile and non-volatile, liquid chromatography (LC) is more suitable to study their structures from mixtures than gas chromatography. HPLC, combined at least with the UV detector or the DAD—and nowadays also with mass spectrometer—is used routinely in phytochemical analysis for screening purposes, to purify and follow the isolation of compounds, and study the purity of isolated compounds. These hyphenated systems have a high resolution and good reproducibility and enable on-line quantification and identification at the same time.

Ten years ago, UHPLC (ultra high-performance LC) systems became available from Waters Corporation (UPLC trademark). These systems operate at higher pressures (20,000 psi), have more rapid gradients and an equal or better resolution in separations in much shorter times compared with HPLC. This is achieved with smaller sub 2 μm size particles. Shorter analysis time in the use of UPLC decreases solvent consumption remarkably. Other important advances that improve resolving power are a high-temperature liquid chromatography and multidimensional separations.[271]

New column materials are becoming available all the time. Reversed phase (RP) C18 stationary phases are typically used in routine phenolic analysis. In RP-HPLC, the stationary is less polar than the mobile phase, and thus the more polar compounds elute first. The acidic mobile phase is used to decrease the ionization of the phenolic hydroxy groups that causes peak broadening.[109] The mobile phase under pH 2 is especially recommended to ease the analysis of anthocyanins to ensure that only the flavylium cation forms are present.[109] Newer column materials include monolithic columns, which have much less flow resistance because of their porosity. This allows the use of higher flow rates and thus shorter analysis, and superficially porous particles that provide excellent kinetic power.[271]

The PA oligomers higher than tetramers, and especially polymers have a poor resolution and cause a non-separated chromatographic hump in the RP conditions that raise the baseline.[109, 272] Better resolution for PA polymers, and for HT polymers and GTs, can be achieved with alternative column materials such as normal phase (NP) and hydrophobic interaction (HILIC) that separate the analytes according to their molecular size.[272–274] An especially important new column material in the PA tannin analysis is HILIC, which has replaced the NP columns because of the more convenient mobile phases.[273, 275] The number of separated analytes from complex samples can be further increased by using two-dimensional LC, for example connecting the RPLC method to HILIC.[275–279]

The retention time in HPLC is informative in the identification of compounds. In RP, polar compounds elute first. The polarity of compound increases as the number of hydroxy groups and sugar moieties increase whereas the substitution of hydroxy group, for example with methyl or acyl groups, decreases polarity.[109] Thus, for example, trihydroxylated PDs elute earlier than the corresponding dihydroxylated PCs. Typically, flavanols with 2*R*, 3*S* stereochemistry (catechin) elute earlier than the equivalent 2*R*, 3*R* flavanols (epicatechin).[109] In PA oligomers, the lower unit has a stronger effect on the retention order and A-type PAs elute later than corresponding B-type PAs.[109] GGs and GTs elute in the order of the increasing degree of galloylation.[261] The oxidative coupling of two galloyl groups to form the HHDP group and then the NHTP group or acyclic ET increases water-solubility and shortens the retention time.[261, 264]

A couple of structural features complicates the HPLC peaks of ETs, but can be useful in the identification. Free hydroxy groups at C-1 position of ET produces mixture of α- and β-anomers that can be seen as two peaks in the ratio of ca. 1:1.[56] The α-isomers elute before the β-isomers of corilagin in the RP-18 material.[137] The appearance of two anomeric peaks can be avoided using treatment with $NaBH_4$.[56] Another example is the DHHDP group, which exists as an equilibrium mixture of 5- and 6-membered hemiketal ring tautomers, which causes a broad peak in the HPLC chromatograms.[56, 280] Therefore dehydrogeraniin, which contains two DHHDP groups, exists as a mixture of four isomers, and furosinin with free OH at C-1 has even eight isomeric structures.[12] The retention order

of stereoisomers of geraniin in RP is geraniin (*R*,*R*), helioscopinin A (*S*,*S*) and carpinusin (*S*,*R*).[280]

Fractionation and purification

Crude extracts can be fractionated to ease the analysis and identification. Low pressure chromatography with preparative and semi-preparative columns are used in the fractionation and purification. Although, SiO_2 is used in HPLC columns with acidic buffers, it is not preferred for preparative use because of the strong adsorbtion that most tannins have on silica gel.[56] In the preparative separation of HTs and PAs, hydroxypropylated dextran gels, such as Sephadex LH-20 with aqueous alcohol and acetone eluents, are commonly used.[56, 186, 222, 272, 281] The separation mechanism in Sephadex LH-20 is a size exclusion chromatography and the exclusion limit of Sephadex LH-20 is around 4000–5000 Da. Its disadvantage is its lability and that polyphenols, such as polymeric PAs and ellagic acid, can be adsorbed permanently to the gel.[56] Also, vinyl polymer gels (resins), such as Toyopearl HW-40 and Diaion HP-20 have been used for HTs and PAs.[56, 281] Typically, semi-preparative HPLC step with RP-18 columns is needed to achieve pure tannins.[186, 222, 281] In addition, centrifugal counter-current chromatography (CCC) has been found useful, particularly for oligomeric HTs.[56, 274]

The reason why geraniin, chebulinic acid and chebulagic acid have dominated ET chemistry since the beginning of the 19th century is that they are easily crystallizable compounds.[136] Geraniin can be further purified from the aqueous MeOH as yellow crystals.[133] Crystalline structure has enabled X-ray crystallographic analysis, which confirmed the presence of seven water molecules through hydrogen-bonding ($C_{41}H_{28}O_{27}\cdot 7H_2O$).[282] Isolated geraniin is almost insoluble in water, although it is solved in crude extracts—possibly due to the interaction with other compounds present in the plant extract, such as some amino acids and ascorbic acid.[135] HTs can be isolated from plants without significant degradation of original structures, and they are relatively stable compounds in room temperature.[12] However, purified polymeric PAs are known to slowly alter during long storage.[14]

2.4.2 UV-vis spectroscopy

UV-vis spectroscopy is a suitable detection method for polyphenols because their aromatic rings and other conjugated double bonds are strong chromophores (Fig. 21). The electronic spectra are caused by transitions that occur when the π-electrons or the non-bonding electrons of molecules are excited to higher energy levels due to the absorption of ultraviolet (UV, λ 100–400 nm) or visible light (vis, λ 400–800 nm).

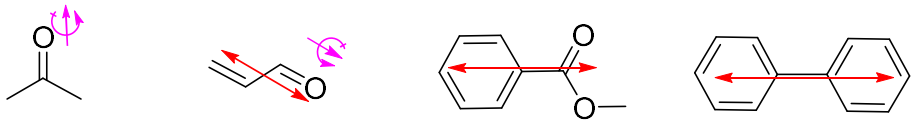


Figure 21. Some common chromophores and their electric transition dipoles in red and magnetic transitions in purple.[283–284]

UV-vis spectroscopy is used in the structure identification of pure compounds, as the absorption spectrum gives information about the chromophore and the color of the compound. It is a popular detection method in the HPLC analysis for screening plant extracts or checking the purity of compounds. The use of DAD allows that, in addition to retention time information achieved from chromatograms, the UV-vis spectra can be recorded to each chromatographic peak and compared with literature values. The use of DAD also enables the extraction of chromatograms with whatever selective wavelength inside the used range. A challenge in the HPLC-DAD analysis is that only preliminary information is achieved, as several compounds may coelute and the shape of observed UV spectra strongly depends on the concentration and HPLC eluent used (Fig. 22).[109]

DAD is especially useful in classifying analytes to different polyphenol classes based on their characteristic UV spectra. The absorption bands of polyphenols arise from the phenolic ring that has two bands over 200 nm, primary band (also called *K* or *E2*) at 210.5 nm with ε 6200 and secondary band (also called *B*) at 270 nm with ε 1450).[2, 284–285] An additional hydroxy group or another electron-withdrawing group shifts the B band further to 280–320 nm, as can be seen if the λ_{max} of gallic acid at 272 nm is compared to acids with one additional double bond attached to phenoyl ring such as *p*-coumaric acid λ_{max} at 305–310 nm or caffeic acids λ_{max} at 325 nm.[2, 109] In the UV spectrum of GGs, there are two maxima at 218 and 280 nm, and the intensity of B band depends on how many galloyl groups the compound contains.[261] For GTs, an auxochromic shift is observed with a characteristic shoulder around 300 nm due to the conjugation of two galloyl chromophores via meta-depsidic bond.[267] The π-electrons of two aryl rings in HHDP group are not conjugated because of the hindered rotation of C-C bond between aryl rings.[197] Therefore, the fine structure of UV spectra is almost totally lost in ETs containing only HHDP and NHTP groups.[264] If the ET contains also galloyl groups, the UV spectra is more similar to GGs; however, the valley between the two wavelength maxima is less steep and maxima are slightly closer to each other.[264] The DHHDP group has a similar absorbance than the galloyl group, in contrast to ETs having gallagyl groups, where the EA-type planar part of compound has a strong characteristic effect on the UV spectrum.[264]

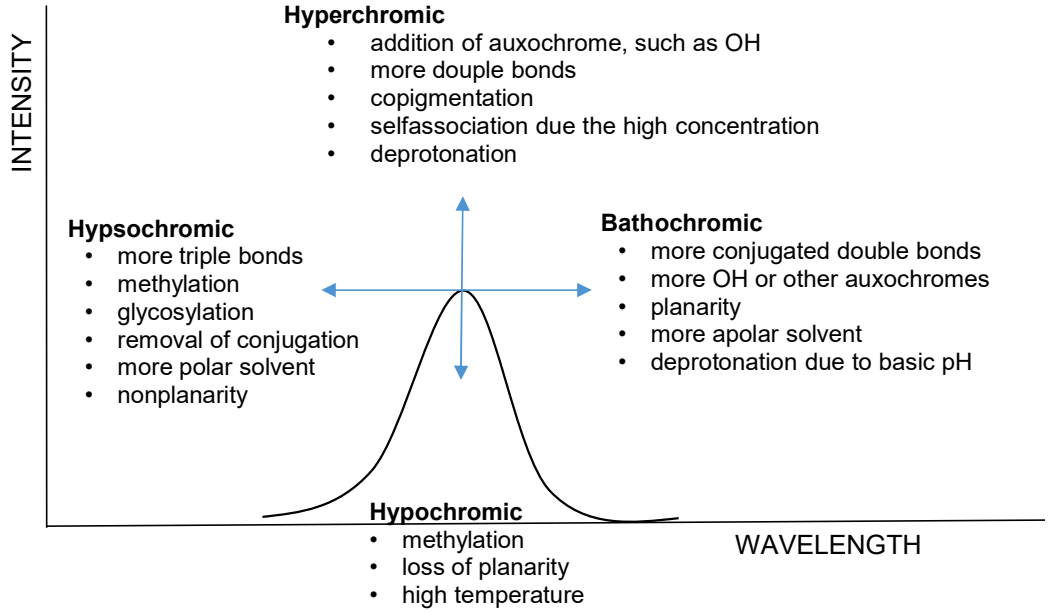


Figure 22. Summary of factors causing different UV spectral shifts.[109, 285]

The UV spectrum of flavonoids has two bands: band II (A-ring benzoyl system) near 240-280 nm and band I (B-ring cinnamoyl system) near 300–380 nm.[109, 286] Some examples of absorption maxima for common flavonoids are presented in Table 2, and for anthocyanins in Table 3. The band I is more informative, as its wavelength maximum and intensity depend on the presence of a double bond in C-ring.[109] The band I is intensive for flavonols with substituted 3-OH (328–357 nm) and for anthocyanins (520–545 nm) (Figs. 9 and 10), whereas PAs that are composed of flavan-3-ols lack it.[109, 286] The PDs show λ_{max} at 270 nm and PCs at 280 nm and mixed PAs something in between.[109] The lower intensity of PAs at 270–290 nm in comparison to other phenolics complicates their detection in the chromatograms.[109] Methylation and glycosylation of free hydroxy groups cause small shifts in the UV spectra of flavonoids, and the acyl groups can be observed according to their maxima.[109] For example, a galloyl group attached to a flavonoid glucoside can be seen as a shoulder in the UV spectra around 265–275 nm.[115]

The pH and thus the protonation status of OH groups strongly affect the UV-vis spectra of phenolics by changing the band shapes and intensities.[203] This has been utilized particularly in the characterization of flavonoids using shift reagents; even weakly alkaline conditions deprotonate the more acidic phenolic groups, such as hydroxy groups at positions 3, 7 and 4' in the flavonoid structure, which moves band I near to 400 nm.[117, 286–287]

2.4.3 Mass spectrometry

The weaknesses of UV detection can be overcome with the use of a mass detector and hyphenated techniques. The molecules in the HPLC eluent flow are transferred into the gas phase and ionized in the ion source of the mass spectrometer. A soft ionization method such as electrospray (ESI) is preferred for large, non-volatile and fragile tannins and because it can be easily connected with LC.[288] ESI-MS is sensitive and has a large mass ratio 100 Da to even 100 MDa due to its capability to generate multiply-charged ions.[289] Another previously used ion source for molecular mass measurements of tannins is fast atom bombardment, but it has a lower mass limit (10 kDa) and an intense background due to the matrix effects.[38, 56] ESI and MALDI (matrix-assisted laser desorption ionization) ion sources are preferred nowadays. The mass ratio of 600 Da to 1 MDa is possible for MALDI, but this ion source cannot be connected to HPLC.

The achieved mass ratio is highly dependent on the analyzer that separates the ions according to their masses. A time-of-flight (TOF) analyzer has a high mass accuracy (< 5 ppm) and resolution (>10 000 FWHM) and is therefore suitable for identification.[290] With a soft ionization method such as ESI and high-resolution (HR) MS equipment such as TOF, a very accurate mass measurement is achieved. The elemental composition can be calculated to predict the unknown structure also for large compounds with a mass range of 50–40 000 Da.[290] More information for the structural analysis, in addition to molecular mass, can be achieved through the fragmentation. In practice, TOF systems often include one quadrupole to enable the MS/MS studies via collision-induced dissociation (CID) for pure samples. Nowadays MS/MS analysis is an invaluable tool and is often required when reporting the characterization of new compounds.

Compared with TOF systems, a quadrupole (Q) mass filter has a lower resolution but a higher dynamic range and good reproducibility, and is therefore more suitable for the quantification of already-identified compounds from samples. In particular, the use of tandem MS increases the sensitivity; these kind of MS systems are now commonly used for the routine analysis of complex or dilute samples. The use of triple quadrupole enables MS/MS studies from the mixtures. TripleQ system has a very good sensitivity for quantification when transition pairs are used.[291] In this method, the first quadrupole is set to filter selected precursor ions. These can be singly- or multiply-charged ions and fragment ions from the in-source fragmentation. Then precursor ions are fragmented by collision-induced dissociation with collison gas flow in the second quadrupole that is really a collision chamber. The third quadrupole can be used to scan the whole spectrum or monitor the selected product ion (selected reaction monitoring, SRM) or even better, several product ions (multiple reaction monitoring, MRM). Tandem-in-time MS^n analysis can be done with Fourier transform ion cyclotron resonance, the orbitrap or quadrupole ion trap mass spectrometer where all these three steps (ion accumulation, precursor-ion selection, precursor-ion dissociation, and product-ion analysis) are done in the same space,

but at different times.[291] The use of the newest analyzer, orbitrap, greatly enhances the mass accuracy and resolution.[289]

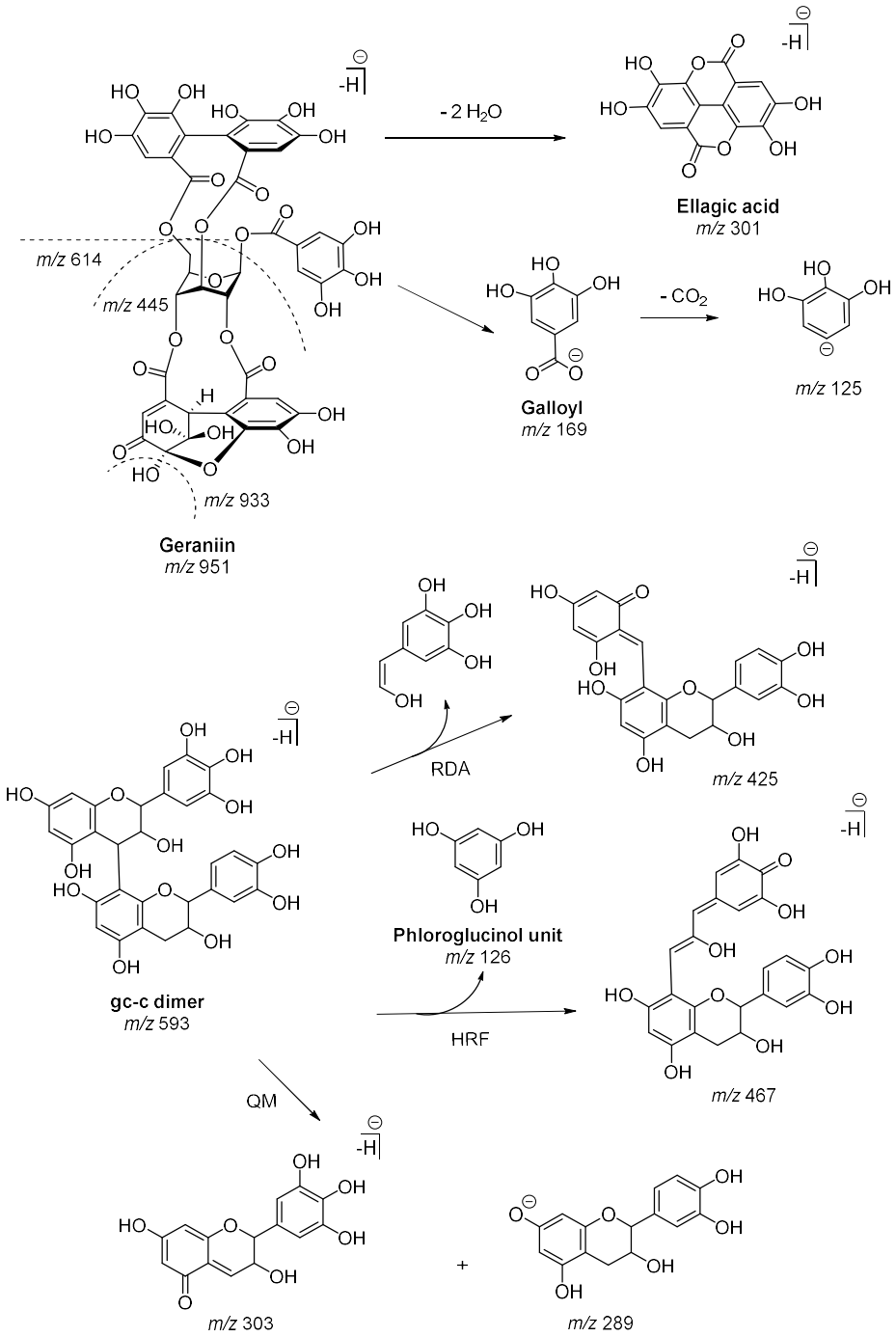


Figure 23. Main fragmentation pathways of geraniin and dimeric PA in the negative ion mode.[204, 292–294] RDA, retro-Diels–Alder fission; QM, quinone methide cleavage and HRF, heterocyclic ring fission.

Mass spectrometers have become a very powerful detector that displaces other detectors and analysing methods. It can be said that a new era began in tannin studies with these advanced mass spectrometers. For example, a new method that utilizes MRM in the study of the DP of PAs has been recently developed, which can replace the conventional degradation methods.[295] Karonen et al. (2010) detected, for the first time in the plant kingdom, ETs up to heptamers in *Oenothera biennis* using HR-HPLC-DAD-ESI-MS, which enabled the use of isotopic patterns in accurate mass calculation for multiply-charged ions.[260] The information about the structures of HTs, previously gained by partial hydrolysis with acid or enzyme, can now be achieved using MS/MS—even from complex mixtures.[296]

In addition to molecular ions, ESI enables the detection of characteristic fragments that can be useful in the structure elucidation. A diagnostic mass fragment for GGs is the loss of gallic acid 170 Da; for GTs it is also the loss of one galloyl group 152 Da.[261] The presence of HHDP group in the structure can be identified from the ion of deprotonated ellagic acid at *m/z* 301 Da.[297] A characteristic fragmentation for geraniin and DHHDP group is the loss of water molecule 18 Da.[204, 264] In MS/MS of geraniin in the negative ion mode, *m/z* values at 933, 715, 613, 445 and 301 have been detected.[204, 292] A diagnostic ion for chebuloyl group is at *m/z* 337 and further peaks at *m/z* 319, 293 and 275 corresponding with the neutral loss of H_2O or CO_2.[186] The loss of common mass fragments, carbon dioxide (–44 Da) and water (–18 Da), can be observed for other ETs that contain free carboxylic acid functions.[264]

The fragment ions observed for flavan-2-ols are reported in Valls et al. (2009).[274] In ESI, the main characteristic fragmentation pathway of PA dimers is the retro-Diels–Alder fission, which generally leads to the loss of ring B from the extension unit (Fig. 23).[293] The ion at *m/z* 441 is observed for PD dimers, the ion at *m/z* 425 for PC dimers (Table 5).[293] These fragments can be used in the determination of the sequence found in mixed PD dimers that are composed of both catechin and gallocatechin units (Table 5).[293] Typically, resolution in RP-chromatography is such that oligomers up to tetramers are separated as defined peaks. After that, the number of isomers causes an unresolved hump (Table 4).[109, 273] However, the use of high-resolution ESI-TOF-MS with a HILIC column has enabled the detection of PC polymers up to the DP of 22.[273] The MS fragmentation pathways for flavonoids and anthocyanins are simpler; the elimination of a sugar unit such as 162 Da (glucose/galactose), 146 Da (rhamnose), 132 Da (xylose or arabinose) and 176 Da (glucuronic acid) is typical; afterwards, the ion of aglycone can be detected when all the sugar units are eliminated.[298]

Table 5. Molecular and fragment ions of dimeric PAs.[293]

$[M-H]^-$	RDA product	HRF product	Extension unit	Terminal unit	Sequence
577	425	451	287	289	c-c
593	425	467	303	289	gc-c
593	441	467	287	305	c-gc
609	441	483	303	305	gc-gc

RDA, retro-Diels–Alder fission; HRF, heterocyclic ring fission; c, catechin; gc, gallocatechin

The negative ionization mode is preferred in the polyphenol analysis because the deprotonation is easier for weakly acidic polyphenols than the protonation, and due its greater sensitivity.[293] Disadvantages of MS when studying complex mixtures, such as plant extracts, include the complex pattern of multiple-charged ions that can complicate the identification; moreover, the matric can seriously affect the ionization of analytes and thus may lead to inaccurate quantification.

2.4.4 NMR spectroscopy

Plant extracts can contain several isomers that have the same molecular mass and mass spectral fragmentation patterns; their structures cannot be fully identified without the isolation of compound and the use of nuclear magnetic resonance (NMR) spectroscopy. In NMR, the atomic nuclei (typically ^{1}H and ^{13}C) of a sample are excited with radio frequency pulses in a static magnetic field. Depending on the nucleus and its chemical environment, it releases different resonances at different frequencies that are detected and transformed to spectra. The resonance frequencies of nuclei are compared with the resonance frequencies of the solvent or added standard, such as tetramethylsilane (TMS), to achieve a comparable value called a chemical shift (δ/ppm). The chemical shift provides information about the chemical environment of the nuclei. The integral of resonance gives the relative number of nuclei that contribute to the ^{1}H signal. The proton signals split if there are chemically different protons present, which affect the spin resonances. This multiplicity reveals how many different types of protons are nearby, and the distance of signals can be used to calculate spin coupling constants (*J*/Hz), which give information about the spatial relationships with neighbouring protons. The complete structural elucidation of an unknown compound requires the use of two dimensional methods, of which the following are typically needed: heteronuclear single quantum coherence (HSQC) that shows the proton-carbon one-bond correlation; heteronuclear multiple bond correlation (HMBC) that shows the proton-carbon correlation across two or three bonds; double quantum filtered correlation spectroscopy (DQF-COSY) that shows the correlations of *J*-coupled protons; total correlation spectroscopy (TOCSY) that shows the proton correlations in the same spin-system; and nuclear Overhauser effect spectroscopy (NOESY) that shows the spatial correlations of protons.

The NMR spectrum provides information about the nature and number of polyphenol groups in HTs. In ^{1}H NMR spectra, a galloyl group gives a 2H singlet around 6.9 to 7.2 ppm and an HHDP group gives two 1H singlets around 6.7 and 7.0.[133–134] The a DHHDP group gives three 1H singlets ca. δ 5.2, δ 6.4 and δ 7.1, in addition to a couple of doublets for a benzylic methine signal at δ 4.8–5.01 (J = 1.5–2) and an olefinic proton signal at δ 6.1–6.27 (J = 1.5–2 Hz), which are characteristic for the two tautomeric forms of DHHDP group (Table 8; Fig. 24).[56, 299] There are some small differences in the sequence of resonances between geraniin and its isomers in the proton spectrum, such as observed for geraniin and granatin B (Table 7).[134] ETs with the same molecular mass but a different oxidatively transformed DHHDP group, such as geraniinic acid B or C, can be easily identified based on their characteristic signals. The main difference in ^{1}H NMR between geraniin and geraniinic acid is that the latter shows the presence of an extra broad singlet of H-6'; it does not show the duplication of signals due to the lack of tautomeric forms.[27]

The thermodynamically less-favoured $^{1}C_4$ conformation of glucose in geraniin is easily distinguished from the more common $^{4}C_1$ conformation by the small J coupling constants for the equatorial-axial coupling of the H-1 and H-2 protons (Fig. 24).[136] The full assignment of glucose protons is more difficult for $^{1}C_4$ type tannins because of the small vicinal couplings of H-1–H-4 and the presence of long-range couplings due to W-arrangements between H-1–H-3 and H-3–H-5. However, these couplings are absent in the NOESY spectrum, which aids the assignment of signals.[56, 300] The chair conformation is suggested for both 6- and 5-membered rings because the chemical shifts and coupling constants are so similar; it seems that only the chemical environment of C-4 changes between these two ring forms of geraniin (Table 7).[136] However, the conformation of glucose core in $^{1}C_4$ tannins can slightly change in different solvents.[137, 300]

The value of chemical shift reveals the nature of a carbon. It is affected by electron withdrawing atoms in the neighbouring atoms or substituents attached to carbon. The carbonyl carbons of galloyl groups are near 165 ppm whereas four other aromatic signals of galloyl groups of HTs are between 110 and 146 ppm. The formation of an HHDP group disturbs the symmetry of galloyl group and yields a fifth different aromatic carbon signal. The oxidative transformation of an HHDP group to a DHHDP group changes these signals totally. In GTs, the addition of galloyl group through a metadepside bond causes an upfield shift of ca. 0.6 ppm to carbonyl carbon signals that are attached to polyol.[128] The typical carbon signals of the B-ring of geraniin are the ketonic carbon at C-4' δ 191.7, geminal diol at C-6' δ 92.4 and hemiasetal at C-5' δ 96.1 ppm, which all are duplicate signals due to two tautomers (Table 8).[27, 111, 133] During the mutarotation of a DHHDP group from 6-ring to 5-ring, an ether linkage is formed, which shifts the C-6' carbon resonances to clearly higher ppm values. However, these values are quite similar for the isomers and therefore are not very useful in the identification (Table 8).[111] Okuda et al. (1989) have reported full assingments of ^{13}C resonances of glucose to aid the determination of the location of acyl groups on the center glucose of HTs.[56] The C-1 signal has the highest ppm

value and the C-6 signal the lowest; the sequence of other signals is affected by the attached groups and their positions.[56, 300] The galloylation of the hydroxy groups in the $^4C_1$ glucose moiety shifts the signals compared with the corresponding carbon resonances of the free glucose: α-effect is between +0.2 and 1.2 ppm and β-effect on neighboroughing carbon nuclei is between –1.4 and –2.8 ppm.[56] For the HHDP group, the corresponding values are for α-carbons between +1.3 and 1.7 ppm and for β-carbons ca. 3 ppm.[56] Therefore, the positional isomers should be distinguished according to ^{13}C resonances; however, values for the DHHDP group were not reported and the literature data of the isomers of geraniin do not provide the full assignments of the carbon resonances of the glucose core, as seen in Table 8.

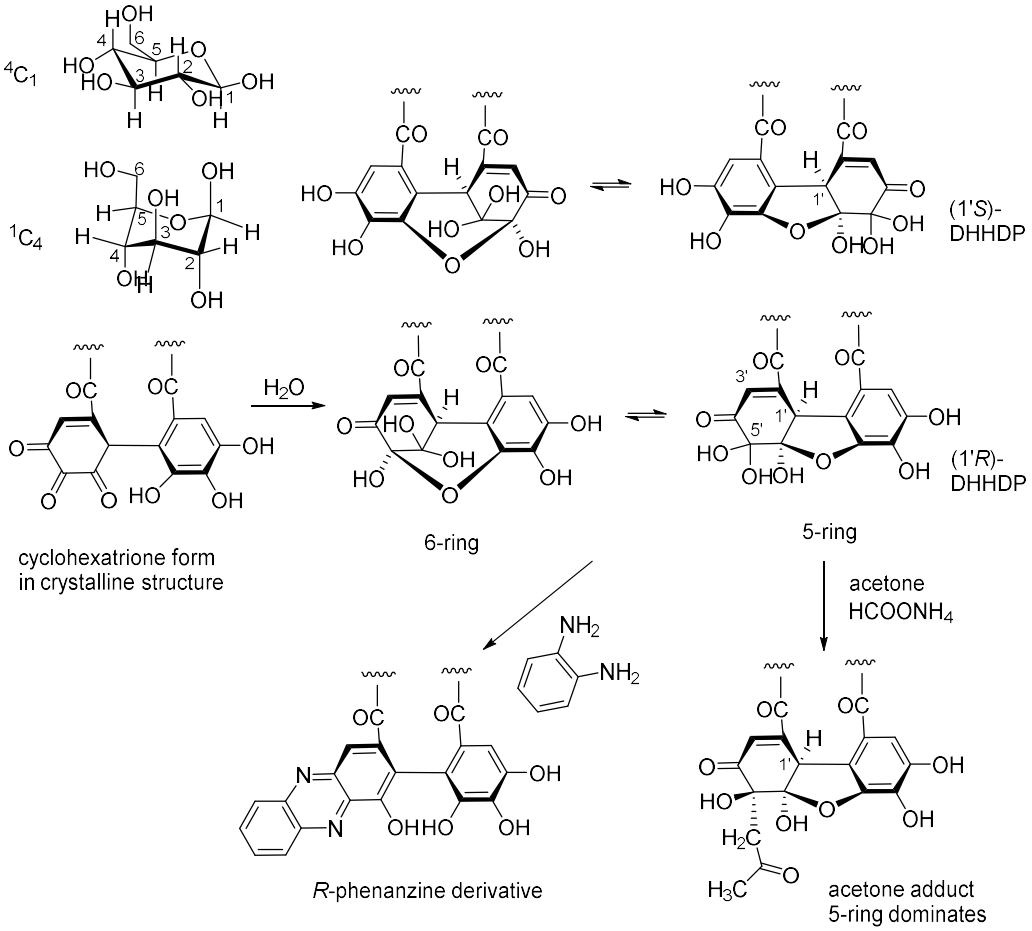


Figure 24. Tautomers and adducts of DHHDP group.

The formation of the equilibrium mixture between 6- and 5-membered rings for hydrated hemiacetal structures in the presence of water, is a characterictic of DHHDP group and duplicates the signals in the NMR spectra (Fig. 24).[27, 111, 301] The equilibration

happens also in other solvents than water, such as acetone and DMSO; the complete equilibration of geraniin in acetone requires 12 hours before the NMR measurement.[133, 302] The water content and solvent especially affect the ^{13}C NMR shifts and patterns.[301] Moreover, the acetal hydroxy groups can be substituted with methyl and ethyl groups in alcoholic solutions, which can make the mixture even more complex.[175] Tautomeric forms are easier to differentiate in the proton spectra if their ratio differs from 1:1, as in carpinusin, where the ratio of 6- and 5-membered ring forms is 7:3.[296] The adduct formation can be used to simplicate the spectra. For example, condensation with *O*-phenylenediamine yields a phenazine derivative attached to C-4 and C-5; condensation with acetone, in the presence of ammonium salt catalyst, yields highly regio- and stereospecifically one stable acetone condensate at the C-5 position (Fig. 24).[280] The acetone adducts of geraniin (MW is 992 Da) and other DHHDP-ETs can be formed during extraction with the unknown compound of plant material acting as a catalyst.[280]

Other features that complicate the NMR spectra of HTs are anomeric forms and oligomerisation. The α- and β-anomers give different signals: the C-1, C-2, C-3 and C-5 signals of β-anomer have 2-5 ppm higher values than corresponding α-anomer.[56] Anomers can be distinguished from each other, as the β-isomers exhibit a doublet for H-1, whereas α-isomers have a broad singlet indicating the different equatorial-axial arrangement of H-1 and H-2.[137] The NMR spectra of dimeric and oligomeric HTs are more complicated; however, the published diagnostic values for different HTs and the comparison of signals to the assigned spectra of monomers can allow assignment of the locations of acyl groups without the use of degradation methods.[56] For example, dimeric tannins, composed of geraniin and PGG (or other DHHDP compounds isolated from *Euphorbia* species), have been identified using the simplified spectra of phenazine derivatives.[139, 141]

Other challenge in NMR is that a quite high amount of pure compound is needed to achieve a good carbon spectrum. Typically, 10 mg is a good amount for a complex molecule. However, more modern and sensitive probes are becoming available, such as cryoprobes where a quarter of the previously-needed sample amount is enough for the analysis. With more sensitive probes, the connection of NMR with HPLC systems might come bemore useful. In addition to the amount of sample, the purity of sample (80%) has to be enough high so that the identification is possible. A high amount of one impurity can disturb the spectral interpretation more than many small impurities. The solvent choice is also important. The solvent can significantly change the conformation of phenolics and thereby the signals observed and solvent signals can hide some important signals.[303] Acetone typically gives clear spectra for HTs and is often used; the addition of water can broaden signals and complicate the calculation of coupling constants (Table 7).

Table 7. ^{1}H shifts and ^{1}H,^{1}H *J*-coupling constants for geraniin, its enantiomer granatin B and for two positional isomers carpinusin and helioscopin that are enantiomers of each other, measured in acetone-d_6 or acetone-d_6/D_2O mixture. See Fig. 24 for positions.

	GLUCOSE							DHHDP		
Position	**1**	**2**	**3**	**4**	**5**	**6a**	**6b**	**1' methine**	**3' olefinic**	**Reference**
Geraniin: 1-G-3,6-(*R*)-HHDP-2,4-(*R*)-DHHDP-glucose										
6-ring	6.60 (*s* br)	5.60 (*s* br)	5.50 (*s* br)	5.56 (*s* br)	4.81 (*m*)	4.93 (*t*; 11)	4.33 (*dd*; 8, 11)	5.16 (*s*)	6.56 (*s*)	296
6-ring	6.59 (1.0, 1.0, 0.9)	5.58 (2.5, 1.0)	5.51 (3.5, 1.2)	5.55 (1.5)	4.82 (8, 11)	4.96 (8, 11)	4.35 (8, 11, 11)	5.12 (*s*)	6.47 (*s*)	136
6-ring	6.17 (*dd*; 1.2, 1.6)	5.57 (*dt*; 1.2, 2.4)	5.48 (*ddd*; 1.6, 2.4, 4)	5.53 (*dd* br; 1.2, 4)	4.80 (*ddd* br; 1.2, 8, 11)	4.93 (*t*; 11)	4.32 (*dd*; 8, 11)	5.18 (*s*)	6.53 (*s*)	132
5-ring	6.60 (*s* br)	5.60 (*s* br)	5.60 (*s* br)	5.46 (*s* br)	4.81 (*m*)	4.78 (*m*)	4.45 (*dd*; 6, 9)	4.72 (*d*; 1.5)	6.26 (*d*; 1.5)	296
5-ring	6.58 (1.0)	5.59 (2.5)	5.50 (3.5)	5.43 (1.5)	4.80 (8, 11)	4.79 (8, 11)	4.45 (8, 11, 11)	4.88 (*d*; 1.3)	6.20 (*d*; 1.3)	136
Acetonyl geraniin										
5-ring	6.58 (*s*)	5.57 (*s* br)	5.57 (*s* br)	5.43 (*s* br)	*4.70-4.94 (*m*)	*4.70-4.94 (*m*)	4.39 (*dd*; 12, 14)	4.92 (*d*; 1)	6.32 (*d*; 1)	280
Granatin B: 1-G-3,6 (*R*)-HHDP-2,4-(*S*)-DHHDP-glucose										
6-ring	6.58 (1.2, 1.2, 1.0)	5.21 (3.3, 1.2)	5.51 (3.2, 1.2)	5.94 (1.3)	4.85 (9.5, 10)	5.28 (9.5, 10, 11)	4.26 (9.5, 10, 11)	5.08 (*s*)	6.60 (*s*)	136
5-ring	6.57 (1.2)	5.12 (3.3)	5.60 (3.2)	6.23 (1.3)	4.85 (9.5, 10)	5.30 (9.5, 10, 11)	4.22 (9.5, 10, 11)	4.97 (*d*; 1.3)	6.30 (*d*; 1.3)	136
Acetonyl granatin B										
5-ring	6.59 (*s*)	5.55 (*s* br)	6.22 (*d*; 3)	5.10 (*s* br)	4.85 (*dd*; 8, 11)	5.31 (*t*; 11)	4.23 (*dd*; 8, 11)	4.92 (*d*; 1)	6.39 (*d*; 1)	280
Helioscopinin A: 3-G-1,6 (*S*)-HHDP-2,4-(*S*)-DHHDP-glucose										
6-ring		*5.08-5.30	5.80	*5.08-5.30	4.70	*5.08-5.30	4.20	5.24 (*s*)	6.59 (*s*)	305
5-ring			5.92					4.99 (*d*; 2)	6.27 (*d*; 2)	305
Acetonyl helioscopinin A										
5-ring	6.21 (*s*)	5.32 (*s* br)	6.00 (*s* br)	5.08 (*s* br)	4.79 (*dd*; 5, 12)	5.40 (*t*; 12)	4.24 (*dd*; 5, 12)	4.95 (*d*; 1)	6.34 (*d*; 1)	280
Carpinusin: 3-G-1,6-(*S*)-HHDP-2,4-(*R*)-DHHDP-glucose										
6-ring 7	6.19 (*s* br)	5.13 (*d* br; 2.5)	5.83	5.39	4.66 (*dd*; 5.5, 13)	5.37 (*dd*; 12, 13)	4.24 (5.5, 12)	5.20 (*s*)	6.55 (*s*)*	304
5-ring 3	6.25	5.04 (*d* br; 3)	6.03	5.45	4.68 (5.5, 12)	5.40 (*t*, 12)	4.16 (5.5, 12)	4.96 (*d*; 1.5)	6.24 (*d*; 1.5)	304
6-ring 1								5.24 (*s*)	6.57 (*s*)	299
5-ring 5	6.26	*5.30-5.60 (*m*)	6.08 (*m*)	*5.30-5.60 (*m*)	4.50-4.80	5.00-5.15 (*m*)	4.06-4.12 (*d*, 12)	5.01 (d; 1.5)	6.27 (*d*; 1.5)	299
Acetonyl carpinusin										
5-ring	6.27 (*s*)	5.46 (*s* br)	6.04 (*s* br)	5.05 (*d* br; 3)	4.71 (*dd*; 5, 12)	5.55 (*t*; 12)	4.12 (*dd*; 5, 12)	4.95 (*d*; 1)	6.34 (*d*; 1)	280
Euphorscopin: 1,3-(*S*)-DHHDP-2-G-4,6-(*S*)-HHDP-glucose										
	6.54 (*d*; 1)	5.84 (*dd*; 1, 4)	5.30 (*dd*; 1, 4)	5.50 (*d*; 8)	4.90 (*t*; 8)	5.06 (*dd*; 8, 11)	3.92 (*dd*; 8, 11)	4.87 (*s*)	6.61 (*s*)	139

*reported as a total for several signals or exact place not determined

G = galloyl, HHDP = hexahydroxydiphenoyl, DHHDP = dehydrohexahydroxydiphenoyl

Table 8. ^{13}C NMR shifts for geraniin, its enantiomer granatin B and for two positional isomers carpinusin and helioscopin. See Fig. 24 for positions.

	Geraniin 1-galloyl 3,6-(*R*)-HHDP 2,4-(*R*)-DHHDP		**Acetonyl geraniin**	**Granatin B** 1-galloyl 3,6-(*R*)-HHDP 2,4-(*S*)-DHHDP	**Acetonyl granatin B**	**Helioscopinin A** 3-galloyl 1,6-(*S*)-HHDP 2,4-(*S*)-DHHDP		**Acetonyl helioscopinin A**	**Carpinusin** 3-galloyl 1,6-(*S*)-HHDP 2,4-(*R*)-DHHDP		**Acetonyl carpinusin**	**Euphorscopin**	
Position	6-ring	5-ring	5-ring	6-ring	5-ring	6-ring	5-ring	5-ring	6-ring	5-ring	5-ring	6-ring	5-ring
1	90.8	91.8	91.7	91.0	91.5			89.9	96.2-60.2 total		90.4	93.3	
2	69.9	70.4	70.1	72.6	69.6*			68.1*			68*	64.6*	
3	63.3	62.3	62.2	64.0	61.1*			60.2*			60.2*	65.5*	
4	66.0	66.8	66.5	69.9	63.8*			66.9*			68*	69.1*	
5	72.6	73.1	73	61.3	72.8*			70.9*			72*	69.1*	
6	63.6	63.8	63.7	64.0	63.7*			63.9*			63.8*	71.3*	
DHHDP-1'	46.21	52.02	51.8		51.7	45.9	51.7	51.7	46.2*	51.8*	51.8	45.9	51.8
DHHDP-2'	154.55	149.21	145.4*		145.9*	154.2	148.9	145.9*			146.0*	148.5	153.6
3' −CH=C−	128.63	124.99	126.9		128.1	129.1	125.7	127.3	128.8*	125.5*	127.3	125.6	129.7
4' C=O	191.69	194.99	197.6		198	192.2	194.9	197.5	191.8*	194.6*	197.7	192.1	195
5'	96.22	92.35	80.8		81.2	96.2	92.6	80.9			80.9	92.4	96.2
6'	92.47	92.35	109.7		109.4	108.8	92.4	109.6			109.8	96.2	109.9
Reference	304	304	280	134	280	305	305	280	299	299	280	139	139

* position not assigned

HHDP = hexahydroxydiphenoyl, DHHDP = dehydrohexahydroxydiphenoyl

2.4.5 CD spectroscopy

The absolute configuration of the compound is difficult to determine with NMR, although some new methods such as optishift agents and chiral solvents are becoming available. Therefore, electronic circular dichroism (or simply CD) spectroscopy can be used in the structural analysis to give information about the stereochemistry using linearly polarized light. Measured compounds need to be optically active and absorb light, i.e., contain chiral chromophores. The ideal functional group for CD is a carbonyl that has an absorption band with a small extinction co-efficient (Fig. 21).

Sugars are the typical chiral centers in tannins. In addition, ETs have an axial chirality, i.e., *R* or *S* atropisomers because of the biaryl structure that has a hindered rotation along the C-C bond between the aromatic rings of HHDP. The steric barrier to rotation is so high that these isomers can be isolated. The determination of the chirality of the HHDP group and related biphenyl groups is one of the main problems in the structural analysis of ETs.[56] The axial stereochemistry of an HHDP group can be accomplished through the use of a Newman projection along the axis of hindered rotation. The priorities of *ortho* substituents are assigned based on Cahn–Ingold–Prelog priority rules. As in Fig. 25, the phenolic OH group has a priority over the carbonyl group. When looking at the HHDP group along the C-C bond, the shortest path from the substituent of the highest priority in the closest ring to the substituent of the highest priority in the other ring determines if the absolute configuration is assigned *S* for clockwise or *R* for counterclockwise (Fig. 25).[306] Ester bonds are known to be *s*-trans and the ester carbonyl is *syn* with respect to the methane hydrogens (Fig. 26).[307]

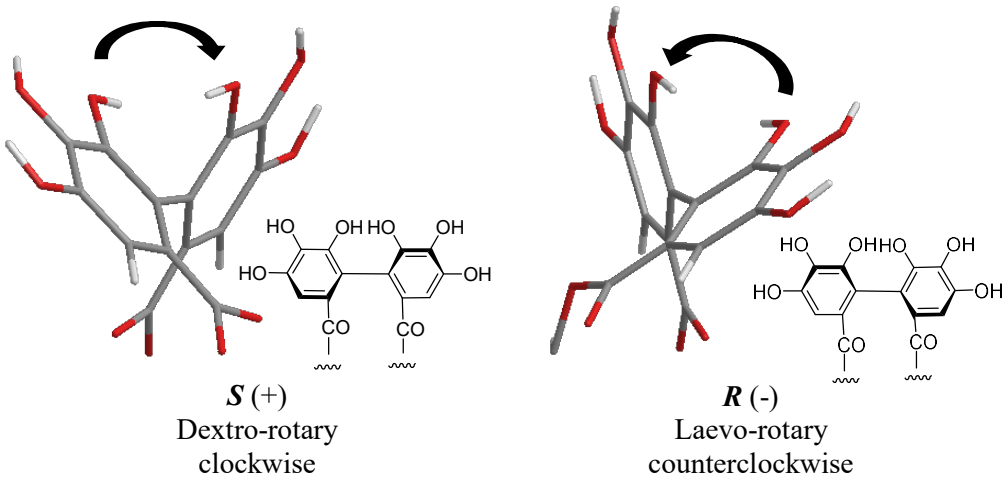


Figure 25. Atropisomers of an HHDP group.

Linearly polarized light is a sum of right-circularly polarized and left-circularly polarized components. When a linearly polarized light beam passes through an optically active solution, its two circularly polarized rays travel at different speeds and display two chiroptical properties: different optical rotation (different refractive indexies) and unequal absorption (different absorption coefficients $\varepsilon^L \neq \varepsilon^R$). CD measures the difference in the absorption of the right- and left-circularly polarized components of a beam of linearly polarized light as a function of wavelength.[307–308] The resulting dichroic peak is called the Cotton effect (CE) for the French physicist Aimé Cotton who first discovered this phenomenon in 1895. To differentiate it from the UV absorption, in CD spectroscopy the maximum is called a peak and the minimum is called a through.[307] CE is called positive when the peak occurs at longer wavelengths than the through, and negative when it is the opposite. Actually, the CD spectrum is a sum of all CEs of different chromophores in the compound and can thus exhibit several peaks and throughs, with shoulders and inflections. Two enantiomers of a chiral molecule yield mirror images in the CD spectra. CD can be transformed into a concentration-independent unit $\Delta\varepsilon$ by dividing it with concentration (c, mol/L) and pathlength (l, cm). For historical reasons, the output of CD spectrometer can be also given as ellipticity θ (in mdeg) that is 33000 CD.[283]

When the chiral system has two or more chromophores located nearby in space, their electric transition moments interact spatially so that the energy level of the excited states splits (Fig. 26). This so-called exciton coupling of dipoles causes a bisignate or split CD, which means two oppositely-signed CEs, typically where λ_0 is centered on the UV-vis absorption maximum.[307, 309] The distance between opposite CD curves is called *Davydov split* (Fig. 26). In the UV-vis spectra, the coupling typically causes broadened maxima with double intensity—or, if the energy difference is substantial, two different maxima can occur. The exciton coupling is the strongest when chromophores are strong and identical and the projection angle is around 70°.[307] Two chromophores do not have to be in the same molecule; molecular interactions that affect the chirality can be detected with CD, such as clustering and stacking. For example, an intermolecular exciton coupling, which causes characteristic red-shifted exciton-split CD bands, has been observed in the molecular stacking of anthocyanins.[255]

Tannins, which can contain several chromophores and chiral centres, are often too complex to study with theoretical rules; thus empirically determined rules are used for them. The absolute configuration of the HHDP group was first determined by measuring the specific rotation of dimethyl hexamethoxydiphenate obtained from the methanolysis of methylated tannins (Table 9).[56] After that, an empirical rule for determining the absolute structures of ETs was achieved by measuring CD data of a series of tannins and related compounds. TriGGs exhibit the split CE-centered near 273 nm, which corresponds to the intramolecular charge-transfer transition (277 nm) of the galloyl group in the UV spectra (Table 9).[310] The sign of CE around 285 nm shows the chirality between the galloyl groups. The CE of PGG is smaller than that of triGGs because it is the sum of the chiralities of

1,2- and 2,3-galloyl groups that are opposite to each other.[310] Empirically, it has been observed that there is an interaction between adjacent galloyls and HHDP groups, except that the 3-galloyl and β-1-galloyl group do not interact with the 4,6-HHDP group in $^4C_1$ conformation.[310]

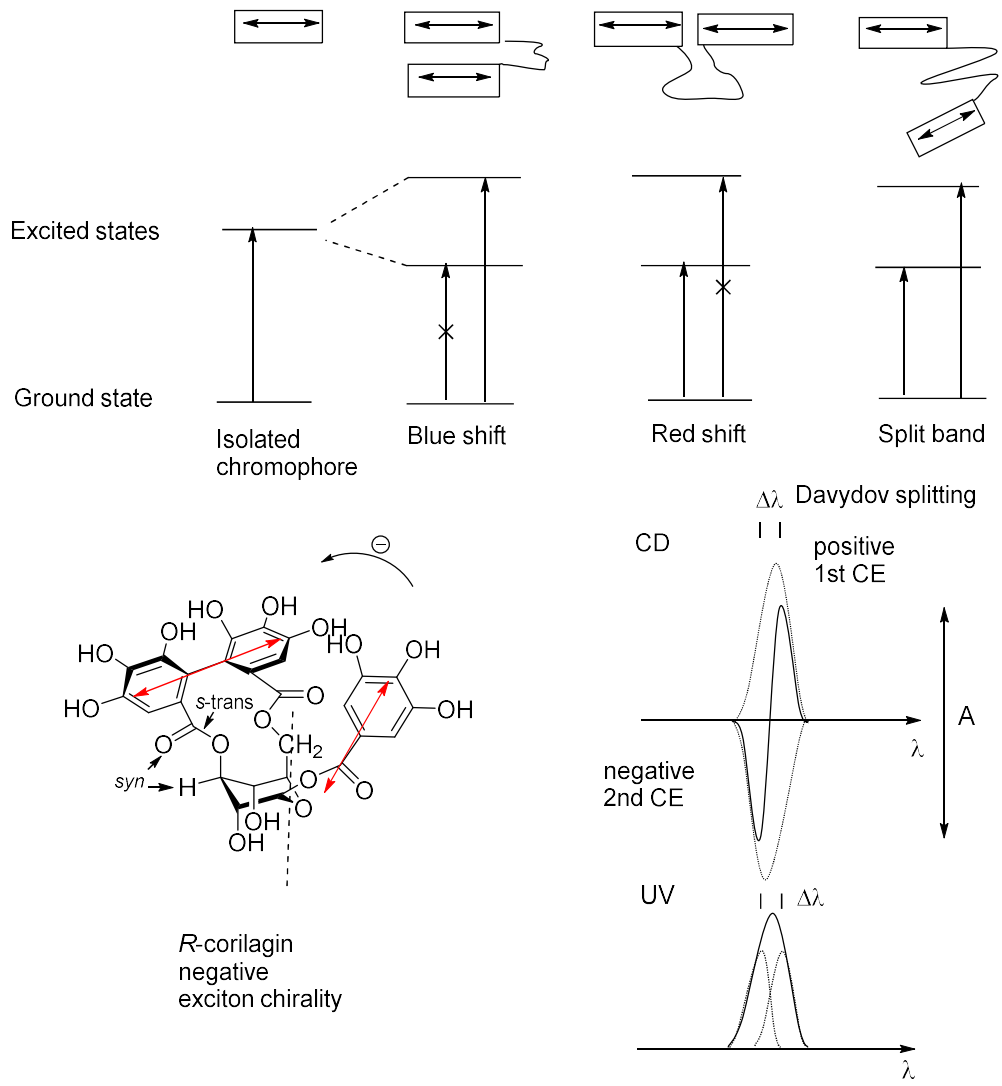


Figure 26. Exciton coupling of two identical chromophores and the coupling of galloyl and HHDP groups in corilagin molecule.[307, 309–310] CE, Cotton effect; CD, circular dichroism; UV, ultraviolet; A, amplitude

The absolute configuration of the HHDP group was confirmed using the *R*-dimethyl hexamethoxydiphenate that exhibited two strong CEs at 225 nm (negative) and 250 nm (positive) that are associated with biphenyl conjugation bands in the UV spectra (Table 9). The *S* configuration has opposite signs. The different twist angle in the HHDP

group attached to a glucose causes a bathochromic shift to longer wavelengths to CEs and thus the positive CE at around 265 nm and negative at 235 nm, are characteristic for the *R* configuration of the HHDP group in ETs.[56, 310] Especially, the CE at 235 nm is a diagnostic for a HHDP group because the CEs of GGs do not overlap.[56] The amplitude of the peak is about twofold if there are two HHDP groups, and higher for oligomers. The smaller CE around 285 nm is regarded as one of the split-type CE centered near 275 nm, arising from the intramolecular charge transfer transition between adjacent galloyl groups and/or HHDP group(s). The sign of the first CE around 285 nm is consistent with the sum of the chiralities among the galloyl groups.

Table 9. CD spectral data of hydrolysable tannins and related compounds in MeOH.[56, 310–311]

Compound		Cotton effect, $[\theta] \times 10^{-4}$ (nm)			K-band	(a,b-unsaturated ketone)
Related compounds		**n→π*(B)**	**Split-type**	**π→π*(B)**	**π→π* (E2, stronger)**	**π→π***
Dimethyl hexamethoxydiphenate	*R*			+4.8 (250)	-5.5 (225)	
Dimethyl hexamethoxydiphenate	*S*			-5.6 (250)	+6.7 (225)	
Galloyl glucoses						
1,2,3-triG-glu			+1.1 (280)	-0.1 (257)		
1,2,6-triG-glu			-3.5 (284)	+2.2 (261)	3.3 (215)	
PentaG-glu			+1.5 (270)	+0.2 (240)	+1.2 (225)	
HHDP-Ellagitannins						
2,3-[(*S*)-HHDP]-glu	*S*		-0.1 (287)	-2.7 (262)	+10.2 (235)	
4,6-[(*S*)-HHDP]-glu	*S*		+0.2 (286)	-2.2 (264)	+8.9 (237)	
3,6-[(*R*)-HHDP]-glu	*R*		-0.4 (290)	+2.4 (256)	-8.5 (236)	
Corilagin (1-G-3,6-(*R*)-HHDP-glu)	*R*		-6.6 (284)	+1.7 (262)	-10.3 (237)	
Strictinin (1-G-4,6-HHDP-glu)	*S*		-0.2 (288)	-3.2 (265)	+12.2 (235)	
Tellimagrandin II (1,2,3-triG-4,6-HHDP-glu)	*S*		+0.8 (285)	-1.0 (264)	+8.1 (235)	
Casuarictin (1-G-2,3; 4,6-di-HHDP-glu)	*S*		+0.7 (281)	-5.3 (261)	+20.2 (234)	
DHHDP-ellagitannins	**C-1'**					
4,6-[(1'*S*)-DHHDP]-glu	*S*	+0.9 (375)			+2.6 (237)	-7.1 (206)
Furosin (1-G-2,4-DHHDP-glu)	*R*	-1.0 (350)	-2.9 (290)	+1.7 (252)	-2.0 (231)	+5.1 (202)
Geraniin	*R*	-1.0 (361)	-6.2 (291)	+4.5 (261)	-7.1 (236)	+10.3 (196)
Granatin B	*S*	+1.1 (360)	-4.6 (285)	-0.8 (263)	-9.0 (243)	-7.1 (211)
Terchebin (1,3,6-triG-2,4-DHHDP-glu)	*R*	-0.8 (350)	+1.1 (290)	+1.4 (251)	-1.3 (233)	+5.2 (213)
Isoterchebin (1,3,6-triG-2,4-DHHDP-glu)	*S*	+0.8 (372)			+4.6 (228)	-7.4 (210)
Furosinin (2,4; 3,6-di-DHHDP-glu)	*R*	-1.9 (356)		1.8 (250)	-2.6 (231)	+10.0 (210)

glu = β-D-glucose; G = galloyl, HHDP = hexahydroxydiphenoyl, DHHDP = dehydrohexahydroxydiphenoyl

The DHHDP group has another chiral center at allylic carbon C-1' of the DHHDP group of which absolute configuration has been empirically determined using chemical degradation methods and phenazine derivatives (see Fig. 24).[311] The strong positive CE around 200 nm and the weak negative peak near 350 nm are characteristic for the *R* configuration at C-1' of the DHHDP group (Table 9). The *S* configuration exhibits

opposite CEs. Other peaks (230 nm) in the CD spectra of geraniin are overlapping with the peaks of HHDP and galloyl groups. Haddock et al. (1982b) substracted the CD spectra of hydrolysis product corilagin from the spectra of geraniin and its enantiomer to resolve the configuration of a DHHDP group.[136]

The CD measurement is useful for colorful compounds but less applicable for compounds that have absorption maxima below 220 nm in the far UV region, because the strong absorption of light by oxygen disturbs the measurement. CD spectroscopy is a very sensitive method; therefore, the total absorbance should be less than 1.5 absorbance units and the temperature should be kept constant during the measurement.[283] Acetonitrile is a good solvent because of its transparency and good solubilizing properties.[307] Methanol is also commonly used, although it can cause problems due to hydrogen bonding. Weaknesses of CD spectroscopy are the low signal resolution and the difficulty of assigning signals without the use of computational chemistry calculations, such as *ab initio* methods.[283]

3 AIMS OF THE STUDY

This study originated from co-operation with the ecologists of the University of Turku who studied the preference of pollinators and herbivores between the female and gynodioecious plants of *G. sylvaticum*. A question arose: What kind of defensive compounds do different plant organs contain, and what is the role of these compounds in these ecological interactions? The other motivation was the previous screening of 100 Finnish plants, which showed that the leaf extract of *G. sylvaticum* was rich in ellagitannins and exhibited high pro-oxidant activity.

The aim was to characterize tannins in *G. sylvaticum* and study their biological activity and variation of tannin content and tannin type throughout the growing season, between sexual morphs and populations. The hypothesis was that intraplant, regional, seasonal and dimorphic differences in the tannin content reflect the different herbivore pressures that plants face.

More detailed goals were:

1. Development of extraction protocol suitable for all eight organs (**I**, unpublished)
2. Development of HPLC method directly suitable for MS and pH 10 measurements (**I**, unpublished, **IV**)
3. Identification of polyphenols and especially tannins in the organs (**I**, **II**, **III**, **V**)
4. Anti- and pro-oxidant activity measurements of extracts and fractions (**II**)
5. Isolation of main/active compounds and their structure elucidation (**I**, **II**, **III**, **IV**)
6. Study the fate of HTs in basic conditions (**IV**)
7. Study the seasonal variation of tannins in *G. sylvaticum* organs (**VI**)
8. Study differences in the phenolic profiles of sexual morphs (unpublished)

4 MATERIALS AND METHODS

4.1 Plant materials

The plant materials for large-scale preparative extraction (**I**, **II, III, IV** and **V**) were collected from natural *Geranium sylvaticum* populations around Finland during the summer of 2007. Aboveground plant parts were collected at the prime time of blooming in June and July. The pistils, stamens, petals, sepals, stems, and leaves were separated from each other and air-dried. The seed materials came from previous ecological studies where seeds were collected after maturation with mesh bags.[40, 42] The roots were collected after the growing season in August and September and washed with water in order to remove soil material, and hairy roots were separated. Plant material was freeze-dried and homogenized into fine powder, except for seeds that were not freeze-dried before homogenization to prevent cold-induced metabolic changes. Different-colored petal samples used in **III** came partly from the summer of 2007 sampling, and more samples were collected during the summer of 2014 from the Turku populations.

Plant samples used in the seasonal variation study (**VI)** were collected during summer 2008. The voucher specimens of these are deposited in the herbarium of the University of Turku (TUR 597241–597244). Four populations from the Turku area were selected to represent different growing conditions: 1) Oriketo 1 is a shadowed forest near a footpath and waste-burning plant; 2) Oriketo 2 is a semi-shadowed forest near Cristian school woods; 3) Oriketo 3 is a high-light and dry roadside near the snow dumping place; and 4) Katariina is a meadow next to the Katariinanlaakso conservation area (Fig. 27). All populations were divided into three smaller plots. Four samples were collected from each plot of the population, and pooled together. Leaf sampling was done fifteen times during growing season in approximately five-day intervals from late May to the end of August. Each time, samples were collected from the small-, medium- and large-sized leaves. Once a month, whole plants were collected from each population to study the roots, stems, and flowers. Samples were stored in the freezer (–20 °C) before freeze-drying.

The leaf, seed, petal and root samples of *G. sylvaticum* in the sexual variation study (unpublished), were collected from both the female and hermaphrodite individuals, which were grown in the greenhouse of the Botanical Garden of the University of Turku. The plants were germinated from seeds that were harvested from the natural populations of *G. sylvaticum* in southern Finland during ecological studies.[312] Samples were freeze-dried and homogenized into fine powder.

Other plant materials used for the isolation of HTs (**IV**) were commercial green tea, *Acer rubrum* leaves and *Betula pubenscens* leaves. Chebulinic acid (**III**) was isolated from fractionated *Terminalia chebula* fruit extract produced in the previous study.[313]

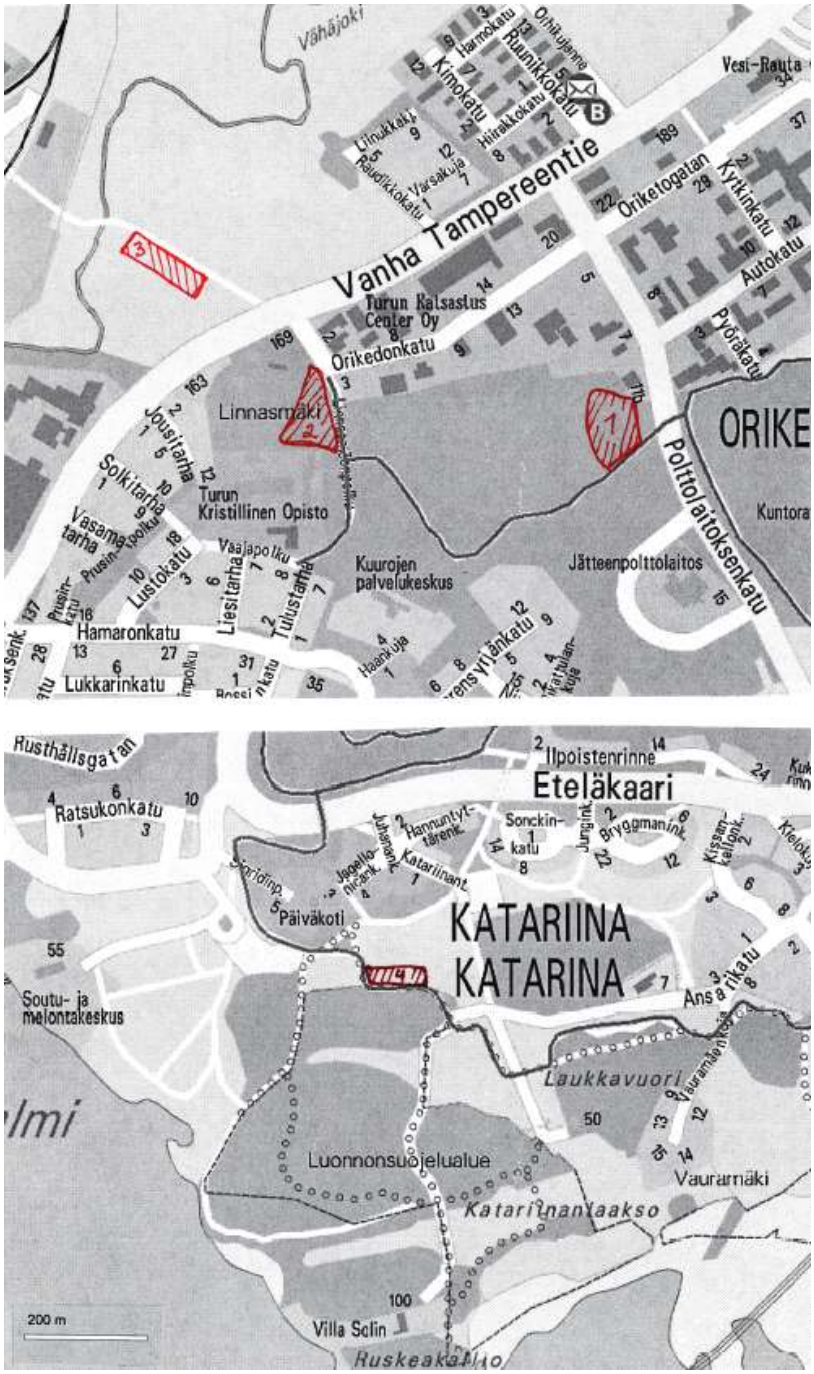


Figure 27. The four *Geranium sylvaticum* populations in Turku from which the samples of the seasonal variation study were collected.

4.2 Extraction and fractionation

Scheme 1 presents the overall workflow used in this study that leads from the plant sample to purified compounds. It includes chromatographic steps and analysis methods used in the process. Aqueous acetone 7:3 (v/v) was selected for extraction solvent based on earlier results and used in all the studies (**I**, **II**, **III**, **IV** and **VI**).[266]

A test to minimize the oxidation of compounds during extraction

It was demanding to collect tens of grams of small flower parts, such as stamens and pistils needed for the large-scale extraction. Therefore, the use of antioxidants or anoxic conditions in the extraction were considered in minimizing the losses of tannins via oxidation during the extraction. Six different treatments were tested in an Eppendorf scale before preparative extractions: (1) control; (2) 0.1% ascorbic acid; (3) 0.2% ascorbic acid; (4) 0.1% $Na_2S_2O_5$; (5) reduced oxygen atmosphere achieved by the sonication of extraction solvent and filling the air space with nitrogen; and (6) a combination of treatments 2 and 5. Five replicates were done for each treatment using leaf, root and petal samples. Ten milligrams of fine powder were extracted two times with 500 µl. The freeze-dried petal and root extracts were dissolved in 1 ml; the leaf extracts were dissolved in 1.2 ml of water and filtered through a 0.45 µm PTFE filter before the HPLC analysis.

Preparative extraction

In the large-scale preparative extraction, samples from different populations and sampling times were pooled to achieve a representative extract of each organ type. Different amounts of fine plant powder (3–50 g depending on the organ size) were separately extracted with varying amounts of extraction solvent, using a planary shaker. The oxidation of tannins during the extraction was minimized by reducing the amount of molecular oxygen from the solvents using sonication, and by keeping the extracts under a nitrogen atmosphere. The extraction efficiency was monitored using HPLC-DAD analysis and the extraction procedures were repeated until the most of the compounds were extracted. After evaporation by rotary evaporation at <40 °C, the aqueous phase was filtered to remove the water-insoluble compounds (mainly chlorophylls), and the filtrate was freeze-dried. Ten milligrams of the freeze-dried crude extracts were dissolved in water to the final concentration of 2 mg/ml and filtered through 0.45 µm PTFE before the HPLC analysis (**I**, **II**).

Fractionation with Sephadex LH-20

The preparative organ extracts were fractionated by Sephadex LH-20 column chromatography as described in **II**. Different amounts of freeze-dried extract, depending on the total amount of crude extract, were weighed and dissolved into 10–20 ml of water

for the column chromatography (see Table 11 for masses). After centrifuging or filtering, the clear sample solution was applied to a Sephadex LH-20 column (40 × 4.0 cm, Pharmacia) and a minimum of 12 fractions (500 ml) per extract were collected by elution with the mixtures of water, MeOH and acetone (see Table 11 or **II** for details). Several fractions were collected with the same eluent when the effluent was still visually coloured at the end of the 500 ml fraction volume. In all, 138 obtained fractions were concentrated into the aqueous phase and freeze-dried.

PLANT SAMPLE	**Leaves (m/g)**	**Petals (m/g)**
Collection		
Organ separation		
Freeze-drying		
Homogenizing into powder	150	8.5
ORGAN POWDER		
Extraction with 7:3 acetone/water	50	6.1
Evaporation of acetone		
Filtration		
Freeze-drying of aqueous phase		
EXTRACT		
Weighting	26	3.6
Dissolvation to water		
HPLC-DAD-MS		
Activity measurements		
Sephadex LH-20 column chromatography	10	3.1
FRACTION		
Evaporation of acetone and/or methanol		
Freeze-drying of aqueous phase		
Weighting (total of all fractions)	8.5	2.5
HPLC-DAD-MS		
Activity measurements		
MAIN or ACTIVE FRACTION	2.8	0.3
Semipreparative HPLC	1.02	0.2
Evaporation of acetonitrile or methanol		
Freeze-drying of aqueous phase		
PURIFIED COMPOUND	**Geraniin**	**Sylvatiin D**
Weighting	0.52	0.02
HPLC-DAD-MS purity		
MS/MS		
NMR	0.01	0.01
CD		
Activity measurements	0.002	0.002

Scheme 1. Overall study protocol and two examples about masses and yields used for the purification of leaf and petal compounds in every step of the protocol.

Analytical extraction

The quantitative scale extraction (**III**, **VI** and unpublished sexual variation study) followed the same protocol as the preparative extraction, but in the smaller Eppendorf scale. An exception was the leaf and petal samples in the sexual variation study, which were extracted before the observations made in this Ph.D work about the use of ascorbic acid in the extraction. Therefore, the freeze-dried and ground leaf (20 mg) and petal (<10 mg) samples of the sexual variation study were extracted with 2 × 750 µl acetone/water (7/3, v/v) containing 0.1% ascorbic acid as an antioxidant using planary shaker for 45 min. The aqueous phase of the extracts was freeze-dried. Leaf extracts were re-dissolved in 1 ml and injection volume was 10 µl. Petal extracts were re-dissolved in 0.6 ml and injection volume 20–60 µl. The seeds and roots of same plants were also studied later. An aliquot of the seeds (40 mg) and roots (20 mg) samples were extracted with 4 × 700 µl acetone/water (7/3, v/v) without ascorbic acid.

In paper **VI**, flower organ samples (0.3–60 mg) were ground as such in the Eppendorf tubes. Larger samples (> 60 mg) were ground on a larger scale and an aliquot of 10 mg of homogenized sample was weighted for the extraction. Samples were extracted in 4 × 700 ml using vortex 30 min. Samples were centrifuged for 10 min, decanted, and extracts were combined into another Eppendorf tube for rotary evaporation and freeze-drying. Extracts were weighted and re-dissolved into the varying amounts of water depending on their mass, and filtered through 0.2 µm PTFE before the UPLC analysis.

4.3 Total quantification methods

In addition to the HPLC quantification, total phenolic content (TP) and total PA content (TPA) were measured for all Sephadex LH-20 fractions and organ extracts in the study **II**, and for the seed and root samples of the sexual variation study (unpublished).

Folin-Ciocalteu for total phenolics

The TP content was analyzed with the Folin-Ciocalteu assay, which is a modification of the traditional method.[314] Samples were dissolved in water and mixed with 1N Folin-Ciocalteu's phenol reagent and 20% sodium carbonate. The absorbance was measured at 730 nm using a well plate reader. The kinetic reaction was followed for one hour and the maximum absorbance of the well was reported. Three replicates were prepared for each sample. Gallic acid was used as a standard. See more details in **II**.

BuOH-HCl for total proanthocyanidins

The TPA content were measured with the BuOH-HCl assay, down-scaled from Ossipova et al. (2001) for the multiplate reader (**II**).[169] The extract, re-dissolved in water, was mixed with the 1-BuOH-HCl solution and heated at 95 °C for 2 h. An aliquot of the sample mixture was pipetted into the well plate, and absorbance was measured at 550 nm by a 96-well plate reader. Three replicates were measured for each sample. In this study, it was observed that many samples that contained anthocyanins were already red before heating (**II**). Therefore, the absorbance of samples before heating was measured separately and subtracted from the reading after heating to avoid false-positive results. The TPA content were quantified against suitable PA standards, which were the Sephadex LH-20 fractions of the *G. sylvaticum* main root extract (fraction 11) and seed extract (fraction 11), which contained mainly pure oligomeric and polymeric PAs. The composition of PAs in the other organs is similar to their composition in the roots; therefore, the PA standard from the roots was used for the quantification of PAs in all other *G. sylvaticum* organs, except for seeds, which contained only PCs (**I**, **V**).

4.4 Biological activity methods

Two different physicochemical properties were measured for the organs of *G. sylvaticum* and 138 Sephadex LH-20 fractions: antioxidant activity (AO) and pro-oxidant activity (PRO-OX) (**II**). In addition, the biological activity of petal compounds was measured for the protein precipitation and copigmentation capacity (**III**).

Antioxidant activity

Antioxidant activity was measured with the modification of DPPH radical scavenging assay, where the stable and purple-colored free DPPH· radical accepts an electron from the antioxidant and changes into a yellow-colored stable compound. The ethanolic DPPH radical solution was mixed with the sample solution, and the decay of the radical was followed by measuring absorbance at 520 nm with a 96-well plate reader. The reaction was followed for 1.5 hours and the maximum absorbance of the well was reported as a result. Four replicates were measured for each sample. Pentagalloylglucose was used as a positive control. Results were expressed as the concentration needed for the 50% inhibition of the radicals (IC_{50}). See more details in **II**.

Pro-oxidant activity

The PRO-OX activity of fractions (**II**) was measured using a method of Barbehenn et al. (2006).[210] The browning observed in the feces of caterpillars is thought to be a result of

quinones formed from tannins under highly alkaline conditions.[7] The incubation of phenolic extracts and compounds at pH 10 has been used as an *in vitro* model for insect gut conditions to study which compounds are potentially relevant to interactions between plants and herbivorous insects.[189, 210] The formation of quinones and other oxidation products was measured as an increase in absorbance at 415 nm with a 96-well plate reader.[210, 222]

Earlier, the results achieved with pure compounds were expressed as a maximum browning rate of the sample (PRO-OX_{max}) during the first 100 seconds.[222] However, more values were needed to express results achieved with plant fractions and extracts. Thus two more rate values were calculated for browning slopes: the average rate of oxidation (PRO-OX_{ave}) during the whole 8-minute measuring period, and the absorbance difference between sample background value and the first absorbance value after the buffer addition (ΔA) (**II**). ΔA measured the fast color change immediately after the addition of buffer, which occurred before the browning measurements began. Also background subtraction was done; it was important especially for those fractions that contained PAs, because those solutions were spontaneously brownish-red or yellow.

Furthermore, the reversibility of color change was tested after oxidation (**II**). Samples were divided into two wells. Water or a neutralizing 0.1% formic acid solution was added, and the color change in wells were compared visually. In addition, oxidation products were analyzed from some selected active fractions using HPLC-DAD after 5 min, 1- and 2-hour incubation in the pH 10 buffer. See more details in article **II**.

Incubation of HTs in buffers

In article **IV**, the fate and degradation products of ten pure HTs were studied with HPLC-DAD-MS. Five commercial buffers at pH 3 and pH 8–11 were used in the incubation. The standard solutions of HTs were prepared in the water and ethanol mixture. In addition, incubation at the reduced oxygen level was studied with two compounds, in order to better cover the full scale of herbivory gut conditions. The oxygen level of buffers was decreased by sparking with nitrogen, and the air space was replaced with nitrogen before the sealing of the well plate with adhesive tape. The effectiveness of oxygen removal was measured with the Winkler method.[315]

The sample treatment was developed to be as fast as possible with the following: 1) buffers and samples were filtered before mixing them together, 2) basic resistant column enabled the HPLC analysis without neutralization step, and 3) incubations were done in the sealed 96-well plate and injected straight from the sealed well plate without transfer step. The incubations of HTs were followed for at least four hours to a maximum of six hours. See more details in paper **IV**.

Protein precipitation activity

Protein precipitation capacity (**III**) was measured with the radial diffusion assay, with some small changes to the previously described method.[316] Compounds were diluted in 40% aqueous MeOH and placed in a well in the bovine serum albumin-containing agarose gel that was coagulated into a Petri dish. Three replicates were prepared for each sample. After the incubation of 72 h at 30 °C, the diameter of visible rings, which were developed when compound diffuses into the gel and complexes with protein, were measured. The diameter of the ring is dependent on the amount of tannin in the sample, and the tannin type and its precipitation capacity. PentaGG was used as a standard. See **III** for more details.

Copigmentation capacity

Copigmentation effect was first studied using petal fraction 1B and pentaGG and sylvatiin A and varying the pH adding NaOH or HCl. These preliminary results encouraged us to repeat the test with commercial pigment and buffers.

Water, methanol or buffers have been used in the dilution of pigments in the previously published copigmentation studies.[243, 250–252] Of these, 30% methanol was used in this study to ensure that less water-soluble compounds would quantitatively solute, yet there is enough water for the copigmentation effect. The new method, suitable for the well-plate reader-scale, was developed based on the previously published methods.[243, 251–252] During the method development, six conditions were tested: pigment:copigment ratio of 1:1; pigment: copigment ratio of 1:2; stability after one hour and the addition of water. Pigment to copigment ratio of 1:2 was chosen for further study based on the concentrations observed in the petal extracts and in the pressed juice.

The anthocyanin-rich Sephadex LH-20 fraction of *G. sylvaticum* petals and commercial malvin chloride were used as pigments and dissolved to (3:7, v:v) MeOH:water in 1 mM concentration. The copigment samples from purified sylvatiins and corresponding HTs were prepared using the same solvent in 2 mM concentration. Commercial buffer solutions were used at pH 4–8 and pure water was used as a reference.

In the final method, solutions were mixed in a well plate in the ratio of: 80 µl of pigment, 90 µl of copigment, 20 µl of buffer, and 80 µl of water. Well plates were shaken and the absorbance values at wavelengths 520 and 550 nm were measured with a 96-well plate reader. The color change in the well plates was also photographed, and the UV spectra of solutions were measured separately using a spectrophotometer. See **III** for more details.

4.5 Chromatographic and mass spectrometric systems

Altogether, three different chromatography systems were used in this Ph.D project. The first chromatographic system, used in the extraction method development test and partly in articles **I**, **II** and **IV**, was an HPLC-DAD system Ultimate 3000 Series (Dionex Corporation, Sunnyvale, CA, USA); see details in **I** or **IV**.

The existing LC method, used in the comparison of antioxidants in extraction and for the leaf and petal samples of sexual variation study, was as follows: The column was a LiChroCART Superspher (75 mm × 4 mm ID, 4 μm) from Merck (Darmstadt, Germany). Two solvents were used (A) 0.05 M phosphoric acid and (B) acetonitrile. The gradient program was as follows: 0–3 min, 2% B in A (isocratic); 3–22 min, 2–20% B in A (linear gradient); 22–30 min, 20–30% B in A (linear gradient); 30–35 min, 30–45% B in A (linear gradient); 35–37 min, 45–70% B in A (linear gradient). The flow rate of the mobile phase was 1 ml/min. The injection volume of sample solution was mainly 20 μl and samples were filtered through 0.45 μm PTFE filters before analysis. Chromatograms were recorded at 280 nm and compounds were quantified as gallic acid equivalents. This previously-published HPLC method has been used in the characterizations of various HTs and other polyphenolics from tree leaves with ESI-MS and DAD detectors.[172–173, 261]

The existing HPLC method used a C18 column with 4 μm particles. The mobile phase consisted of 0.05 M phosphoric acid and acetonitrile with a DAD detector, and 0.4% formic acid instead of phosphoric acid with MS detector, because phosphoric acid is not suitable for the MS. A new single HPLC method was developed, which was suitable for analyzing a high variety of phenolics from all the eight organs of *Geranium sylvaticum*. In the new method, protocol was simplified by replacing the previously-used buffers with 0.1% formic acid that is suitable for the hyphenation of HPLC with high-resolution MicrOTOF-Q mass spectrometer. Three new columns with large pH range stability were compared so that the method was suitable fro use in the pH 10 studies.

From the tested columns, the XBridge™ C18 column C18 (100 mm × 2.1 mm ID, 3.5 mm) from Waters (Milford, USA), which had a large pH range of 1–12, was further used in studies in papers **I**–**V** and for the root and seed samples of the sexual variation study. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile. The linear gradient program was as follows: 0–28 min, 0–20% B in A; 28–34 min, 20–70% B in A. The flow rate of the mobile phase was maintained at 0.3 ml/min. The injection volume of sample solution was 5 μl. The detection wavelength was mainly 280 nm, which is suitable especially for galloylderivatives. Additional fixed wavelengths were recorded at 200 nm (for proanthocyanidins), 349 nm (for flavonoids), 315 nm (for chlorogenic and coumaroyl acids) and 526 nm (for anthocyanins). The UV spectra of compounds were recorded in the wavelength range of 195–600 nm.

Accurate mass analyses

The second system used in the accurate mass HPLC-ESI-MS analyses was an Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to a Bruker micrOTOF-$_Q$ ESI hybrid quadrupole with a time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). The HPLC method used in RP was as described above; more details in **I**, **III**, **IV**. This HPLC-ESI-MS system was also used in the HILIC study (**V**). Phenomenex Luna HILIC column (4.6 × 250 mm, cross-linked diol, 5 µm) was used with SecurityGuard at 30 °C, see more details in **V**.[273] The LC flow was split prior the MS analyses and ca. one ml fractions of the HILIC separations were manually collected from the top of the peaks in the UV chromatogram followed at 280 nm to study with the second dimension using the XBridge™ C18 column.

Seasonal variation study and tripleQ

The third system, used for tannin quantification in the seasonal variation study (**VI**), was an ultra-high-performance liquid chromatographic system (UPLC, Acquity UPLC®, Waters Corporation, Milford, MA, USA) combined with a triple quadrupole mass spectrometer (Xevo® TQ, Waters Corporation, Milford, MA, USA) and a diode array detector. An Acquity UPLC® BEH Phenyl (2.1 × 100 mm ID, 1.7 µm, Waters Corporation, Wexford, Ireland) column was used.

Compound-specific multiple reaction monitoring methods were optimized to detect and quantify the desired compounds (**VI**). At first, the cone voltage (5–100 V) was optimized for the accumulation of precursor ions, and a suitable precursor ion was selected. Then a suitable product ion was sought and the collision energy (0–80V) for the accumulation of this product ion was optimized. The MRM method optimizations were done with the direct infusion of purified compounds or suitable fractions, or by using a gradient UPLC run with methods containing multiple value combinations. Those optimal cone voltage and collision energy combinations were selected that produced the highest intensity for the desired compound (**III**, **VI**). Also, adequate retention time windows were selected. Some of the used MRM methods were previously published.[262, 295, 317] Fluctuations in the ionization efficiency of mass spectrometer were controlled with an external catechin standard solution. See more details in papers **III** and **VI**.

4.6 Isolation of compounds and structure elucidation

The Sephadex LH-20 fractions were further utilized for the isolation of petal compounds and main compounds from the other organs of *G. sylvaticum*. The purification of individual compounds was mainly performed by Waters (USA) preparative HPLC, consisting of a

2998 photodiode array detector, 600 controller and Delta 600 pump using a semipreparative Phenomenex Gemini RP-18 (150 × 21.20 mm i.d., 10 μm; flow rate 8 ml/min) column. Elutions were done with varying gradients with methanol/water (0:100 → 80:20, v/v) and fractions were collected with a Waters fraction collector. The obtained fractions were analyzed with the HPLC-DAD or UPLC-DAD-MS systems and similar fractions were combined. Combined fractions were concentrated into the aqueous phase by rotary evaporation and freeze-dried.

The characterizations were confirmed with MS/MS, UV, CD and NMR spectroscopy. The MS/MS analyses were measured using an HPLC system connected to a TOF mass spectrometer (**I**, **III**) and operated in a negative ion mode. The samples in the MS/MS analysis were introduced by direct infusion. The collision energy was changed from 0 to 100 eV with steps of 5 eV. See more details in **III**.

The NMR spectra were measured with a Bruker Avance 500 spectrometer (Fällanden, Switzerland). Purified compounds were dissolved mainly in acetone-d_6. The techniques used in the structure elucidations were ^{1}H NMR, ^{13}C NMR, DQF-COSY, CH_2-edited HSQC, HMBC, NOESY and 1D-TOCSY.

The UV spectra of isolated compounds were recorded with a Lambda 25 UV/VIS spectrometer (PerkinElmer, Norwalk, USA). The data were handled by UV WinLab 6.0 software. The samples were dissolved in MeOH of HPLC grade.

The CD spectra were recorded with a Chirascan™ circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK) and controlled by Chirascan Pro-Data software. The samples were dissolved in MeOH. The spectra were scanned over the range of 200–400 nm at 22 °C. The data were handled by Applied Photophysics Pro-data Viewer. See more details in **III**.

4.7 Data analysis

Multivariate data analysis in paper **II** was performed for the activity data by LatentiX version 2.00 (Latent5 Aps, Frederiksberg, Denmark). The Partial Least Squares Regression (PLSR) analysis was used to study the relationship between the quantified chemical data (*X*-matrix) and the biological activity data (*Y*-matrix) of 138 fractions in terms of the prediction of *Y*-variables from *X*-variables. The PLSR models were statistically evaluated by the coefficient of correlation (r^2) and the root mean square of the standard error of full cross-validation (RMSECV). Extrapolated values were given to those fractions that were too small for TPA assay or inactive in the activity measurements in order to include those fractions in the models. Statistical analysis in paper **VI** and for the sexual variation results were done with SAS software using mainly ANOVA and *t*-tests.

5 RESULTS AND DISCUSSION

5.1 Extraction and fractionation

Antioxidant test in the extraction

The oxidation of compounds can occur during extraction and decrease the extractable yield of tannins. This can be minimized using antioxidants or reduced oxygen atmosphere. Six different treatments were compared and analyzed with HPLC-DAD (Table 10, unpublished data). Results of these tests were surprising: instead of increasing, antioxidants significantly decreased the content of the main compound, geraniin, in the extracts. Simultaneously, the content of two compounds increased during extraction with ascorbic acid and sodium metabisulfite (Table 10). The effect of treatments was estimated from the content of four compounds: two galloylderivatives and two ellagitannins, and two formed adducts (Table 10). In the root extract, the content of geraniin decreased by (8%) with 0.1% ascorbic acid and twice more (21%) with 0.2% ascorbic acid than with solvents without any antioxidants. Furthermore, the decrease of geraniin was higher (44%) with 0.1% sodium metabisulfite. Corresponding values for the leaf sample were 26%, 40% or 39% lower, respectively.

Further MS studies showed that product formed in the presence of ascorbic acid was ascorgeraniin, a condensate of geraniin and ascorbic acid.[135] However, a small amount of ascorgeraniin was present in the *G. sylvaticum* extracts naturally, as Table 10 shows. Ascorbic acid reacted with the DHHDP group of geraniin, and it seemed that this group reacted in a similar way with $Na_2S_2O_5$ and generated a whole new non-natural ellagitannin with a SO_3H-adduct having the molecular mass of 1034 Da, $C_{41}H_{30}O_{30}S$ (unpublished LC-MS data). The formation of sulfophenolics was observed earlier with sodium hydrogen sulfite and caffeoylquinic acids.[318]

There was no significant difference between treatments in the contents of other galloyl derivatives (Table 10). However, treatment with 0.1% $Na_2S_2O_5$ decreased the content of all compounds. There was small, but not significant, increase in the content of examined compounds with extraction in the reduced oxygen atmosphere. This effect was visible also in the treatment with sonication and 0.1% AA, where content were higher than in the treatment, which contained 0.1% of ascorbic acid (Table 10).

Apparently the DHHDP group of geraniin and other DHHDP-ellagitannins that *G. sylvaticum* contains tend to form adducts or transform in the presence of ascorbic acid or bases. Therefore, to study naturally-occurring tannins and not non-natural artefacts, it is better to avoid the use of antioxidants in the extraction of *G. sylvaticum* plant parts. Instead, the oxidation of tannins during the preparative extraction was minimized by excluding

molecular oxygen by the sonication of the solvents and by keeping the extracts under a nitrogen atmosphere.

Table 10. Contents of selected polyphenols in the root and leaf extracts of *G. sylvaticum* extracted with different antioxidant treatments (unpublished data).

		Content mg/g ± SD (n = 5)					
	Compound	**No treatment**	**0.1% AA**	**0.2% AA**	**0.1% $NA_2S_2O_5$**	**Reduced oxygen**	**Reduced oxygen + 0.1% AA**
Roots	G quinic acid	26.5 ± 0.5	25.5 ± 0.5	26.1 ± 0.5	22.5 ± 2.2	27.8 ± 0.8	26.9 ± 0.8
	Adduct	nd	nd	nd	25.7 ± 3.8	nd	nd
	Geraniin	75.4 ± 1.3	69.3 ± 1.3	59.4 ± 1.2	42.6 ± 5.8	79.2 ± 1.8	75.2 ± 2.4
	Ascorgeraniin	2.1 ± 0.1	5.0 ± 0.2	14.8 ± 0.3	3.3 ± 0.5	2.0 ± 0.1	3.3 ± 0.2
	GG	24.0 ± 2.2	23.0 ± 2.0	20.9 ± 0.5	17.0 ± 2.2	27.8 ± 0.7	25.4 ± 1.5
	Carpinusin	18.6 ± 0.3	17.4 ± 1.0	15.1 ± 0.3	5.7 ± 0.8	19.0 ± 0.6	18.0 ± 0.5
Leaves	Adduct	nd	nd	nd	99.1 ± 1.2	nd	nd
	Geraniin	144.4 ± 1.9	107.0 ± 3.8	86.9 ± 3.6	75.5 ± 0.9	147.2 ± 4.7	105.7 ± 3.3
	Ascorgeraniin	7.7 ± 0.2	52.6 ± 5.7	81.4 ± 4.7	11.8 ± 0.3	7.4 ± 0.2	62.2 ± 3.6

nd = not detected, AA = ascorbic acid, GG = galloylglucose

Extraction and Sephadex LH-20 fractionation

The eluents and collected fractions in the Sephadex LH-20 fractionation are presented in Table 11. The extraction yields showed that the organs of *G. sylvaticum* contained extractable polyphenols approximately half of their dry weight: the leaves and petals were over 50% of DW, whereas the harder tissues such as stems, seeds and roots were ca. 30% of DW (Table 11). Extraction was more efficient in the smaller Eppendorf scale used in the quantitative studies. The first Sephadex fractions, which contained mainly sugars and highly water-soluble small phenolic acids, had the highest relative fraction masses with all organs: in the seeds, stamens and petals, the water fractions accounted for over 50% of the total weight of all fractions (Table 11). Seeds contained a very low amount of extractable compounds; they contained lots of oily, not water-soluble constituents, which were beyond the scope of this study.

However, these Sephadex LH-20 fractions were further used in the isolation of pure compounds and in the biological activity measurements in paper **II**. The HPLC analysis of Sephadex LH-20 fractions revealed the presence of many more compounds in *G. sylvaticum* than could be predicted by analysing the crude extracts only. For example, fractionation revealed that other organs apart from seeds contained a small amount of tryptophan that co-eluted with chlorogenic acid in the HPLC chromatograms.

Table 11. Extraction yield of organs and Sephadex LH-20 fractionation scheme, flavonoids and ellagitannins are expressed as molecular weights, see identifications in the Tables 13–18, 20 and 22.

								Leaves	Sepals	Petals	Stamens	Pistils	Stems	Hairy roots	Main Roots	Seeds
			Yield in large-scale extraction %					51.4	33.4	59.8	48.8	43.7	24.8	42.5	37.8	15.2
			Yield in small-large extraction %					51.2	43.1	67.4	59.0	65.3	30.3	-	39.4	34.6
			Mass used in fractionation (g)					10.0	1.7	3.1	1.5	1.2	10.0	10.0	4.5	3.5
			Yield in fractionation %					85.0	72.6	80.6	74.1	66.7	71.8	84.7	86.2	68.2
Sephadex LH-20 fraction	**Eluent H_20: MeOH: Me_2CO**	**Phenolic acids**	**FLAs**	**ETs**	**DG GGs**	**DP PC**	**DP PD**	**Sephadex LH-20 fraction m/mg**								
1A	100:0:0	GQA, tryptophan	sugars					2500	564	1578	890	248	4536	1745	1844	1985
1B	100:0:0	GQAs, CA, brevifolin	anthocyanins		1			249	-	79	8	16	164	379	39	8
2	90:10:0	GQAs, CA, EA	anthocyanins	970, 374	1-2			173	37	15	5	4	54	109	20	4
3	80:20:0	GSA, GQAs, CA	466	1178, 970, 633				47	17	12	5	1	53	30	24	2
4	70:30:0	GSAs, diGQA	610, 626, 452	970, 633, 952				90	3	12	2	1	51	49	18	1
5A	60:40:0	GSAs, diGQA	626, 610, 482	1364, 908				221	9	52	4	4	91	66	34	3
5B	60:40:0		626	634	2			-	-	-	-	-	63	-	-	-
6A	50:50:0	diGQA, brevifolin	626, 464	633, 970, 952, 1160, 1364	2		1-3	88	23	21	6	10	74	88	50	5
6B	50:50:0	diGQA,	464, 448	633	2		1-2	-	-	-	-	-	186	-	-	-
6C	50:50:0		464	634, 952			oligo	-	-	-	-	-	83	-	-	-
7A	40:50:10	diGQA, triGQA	464, 448, 616	952b, 970, 633	2	1-4	oligo	372	34	66	30	26	169	431	158	19
7B	40:50:10	triGQA	616, 600	952b, 952a, 633, 786, 1110			oligo	2814	175	242	-	22	919	1181	223	-
7C	40:50:10	triGQA		952a, 952b, 1110, 786			oligo	755	195	-	-	-	250	1686	250	-
7D	40:50:10			952, 1104, 954, 786	4		oligo	286	43	-	-	-	-	680	-	-
8A	30:55:15		600	954, 938, 952b, 952acid, 1160, 1144	3-5	2-7	oligo	367	22	313	40	286	85	776	309	74
8B	30:55:15			1890, 954, 1104, 952a, 938	4-5		poly	224	-	-	-	77	93	461	283	-
8C	30:55:15				5		poly	-	-	-	-	-	-	181	161	-
9	70:0:30				4-6	2-8	poly	124	63	65	56	21	25	94	80	109
10A	50:0:50			1890, 938, 952	4-6	3-9	poly	140	5	23	34	33	111	168	192	117
10B	50:0:50			1890, 952	4-7		poly	53	-	-	-	-	85	248	147	-
11	30:0:70				5-9	4-15	poly	3	11	15	29	72	37	78	32	55
12A	20:0:80	WASH	WASH	WASH		poly	poly	12	-	3	3	1	28	15	9	2
12B	20:0:80	WASH	WASH	WASH				4	-	-	-	-	-	4	7	2

GQA, galloyl quinic acid; CA, chlorogenic acid; EA, ellagic acid; GSA, galloyl shikimic acid, FLA, flavonoids, ET, ellagitannins; DG GGs , degree of galloylation of galloylglucoses

DP PC, degree of polymerization of procyanidins; DP PD, degree of polymerization of prodelphinidins

5.2 MRM method optimization

MRM methods were used for the quantification of phenolics in the seasonal variation study (**VI**). These methods enabled the selective and sensitive quantification of compounds that were difficult to quantify using the UV detection because of the large amounts coeluting of compounds, which is typical for plant extracts. An example is a challenging part of leaf-extract UV chromatogram (Fig. 28A), where carpinusin, tetraGG and quercetin galloylglucoside are not well separated. However, with MRM methods, these compounds can be detected and quantified more reliably (Figs. 28B–E). We stated in **I** that the leaves of *G. sylvaticum* contain only traces of carpinusin, because the high content and the intensive absorption of quercetin galloylglucoside masked its presence in the UV chromatogram (Fig. 28). However, the use of MRM methods showed that carpinusin can be found in all parts of *G. sylvaticum* (**VI**).

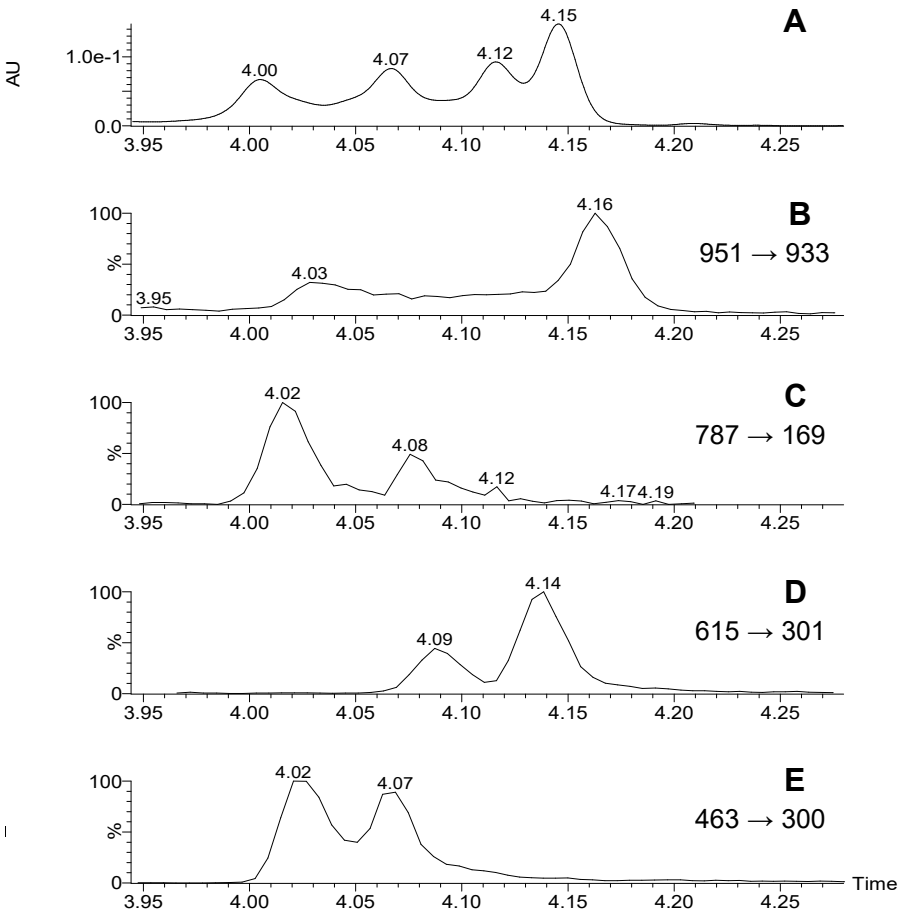


Figure 28. Example of a challenging part of leaf UV chromatogram (A) where many interesting compounds overlap and the ion traces of carpinusin (B), tetraGGs (C), quercetin galloylglycosides (D) and quercetin 3-glycosides (E) detected with MRM methods.

Another challenging sample was the petal extract, where the resolution of sylvatiins A and C was not sufficient for reliable UV quantification (Fig. 29). Also, the quantification of anthocyanins was challenging because of the broad peak detected in the used mobile phase solution and lack of strong absorbance. However, with the MRM method, their quantification was possible (Fig. 29).

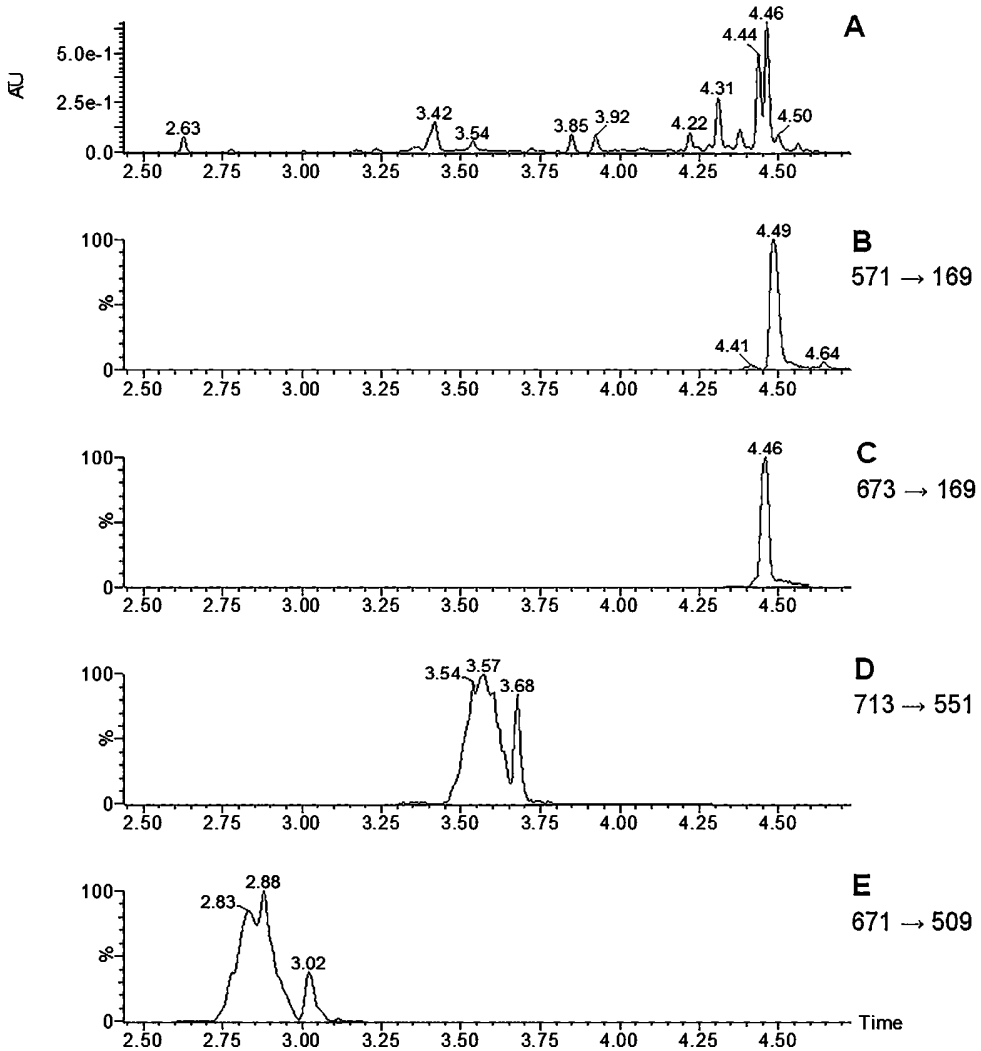


Figure 29. Petal extract UV chromatogram (A), MRM traces for sylvatiin A (B), sylvatiin C (C) and for two anthocyanins, acetylmalvin (D) and malvin (E) of which quantification would be difficult from the UV chromatogram because of the poor separation.

In the development of the compound-specific MRM method using tripleQ, the cone voltage to accumulate the suitable precursor ion, and collision energy to accumulate the selected product ion, were optimized to achieve the highest intensity for the compound. The optimization of cone voltages and collision energies for sylvatiin D is presented as an

example of the process in Fig. 30. The highest intensity for the precursor ion was achieved with the doubly charged molecular ion at 579 Da at cone voltage of 30 V (Fig. 30). This can be the case for compounds that have molecular masses higher than 1000 Da: the doubly charged molecular ion is detected with higher intensity than the molecular ion. However, both ions were used in the selection of product ion and in the optimization of collision energy to study which combination yielded the highest intensities (Fig. 30). As a result, the transition of 579→169 with the collision energy of 25 eV was selected to detect sylvatiin D from the petal samples (**III**, **VI**).

In addition to suitable transitions, it was also important to find a correct retention time window for the compounds, because the MRM method might find another compound that produces the same ions in these kinds of complex plant extracts that contain polymeric structures. For example, the MRM method of sylvatiin B with the molecular mass of 992 Da, which uses transition 495→169, finds also digalloyl quinic acids. Optimized and used MRM methods for *G. sylvaticum* plant organs are listed in papers **III** and **VI**.

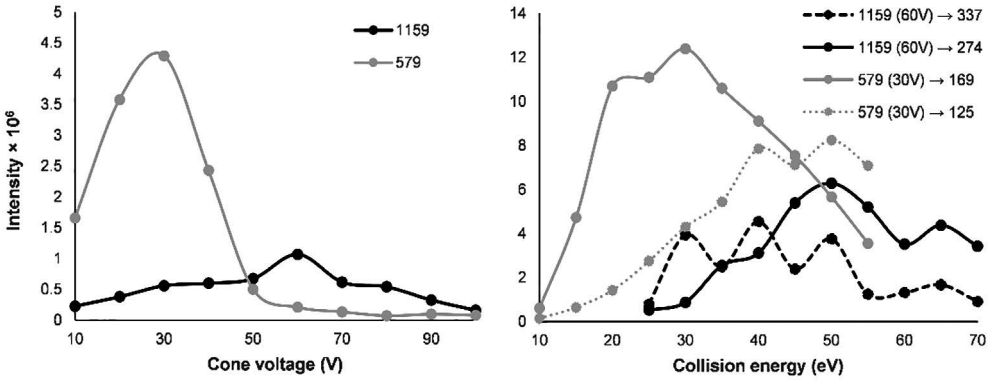


Figure 30. Optimization of cone voltage and collision energy for the MRM method of sylvatiin D.

5.3 Identification and structure elucidation

A systematic procedure was employed for the determination of phenolics from the crude extracts of the organs of *Geranium sylvaticum* as described in **I**. First, the compounds were classified into five polyphenol groups on the basis of their UV spectra analysed with HPLC-DAD: phenolic acids, galloylglucoses, ellagitannins, flavonoids and proanthocyanidins. Secondly, high-resolution mass spectra were analysed to obtain the molecular composition and structural information from the characteristic mass fragments. Previously published literature about *Geranium* species and screening possible structures from the SciFinder database were also used for further identification.

In all, more than 60 compounds were tentatively identified, of which 14 structures were further verified with isolated compounds (Fig. 31). In addition to four sylvatiins, of which structure elucidation was presented in paper **III**, ten other compounds were isolated and structures were confirmed (**I**). These included four ellagitannins: geraniin, 1,2-digalloyl-4,6-HHDP-*β*-D-glucopyranose and carpinusin (3-galloyl-1,6-(*S*)-HHDP-4,2-(*R*)-DHHDP-glucopyranose), and euphorbin B type dimeric ellagitannin (Fig. 33). The other smaller compounds were as follows: 3-galloyl shikimic acid, tryptophan, 4-galloyl quinic acid and 3,4-digalloyl quinic acid. Quercetin 3-(2''-galloyl)-*β*-D-galactopyranoside, and 3-(2''-galloyl)-*β*-D-glucopyranoside mixture was used in the NMR test. See details in paper **I** and in Fig. 31.

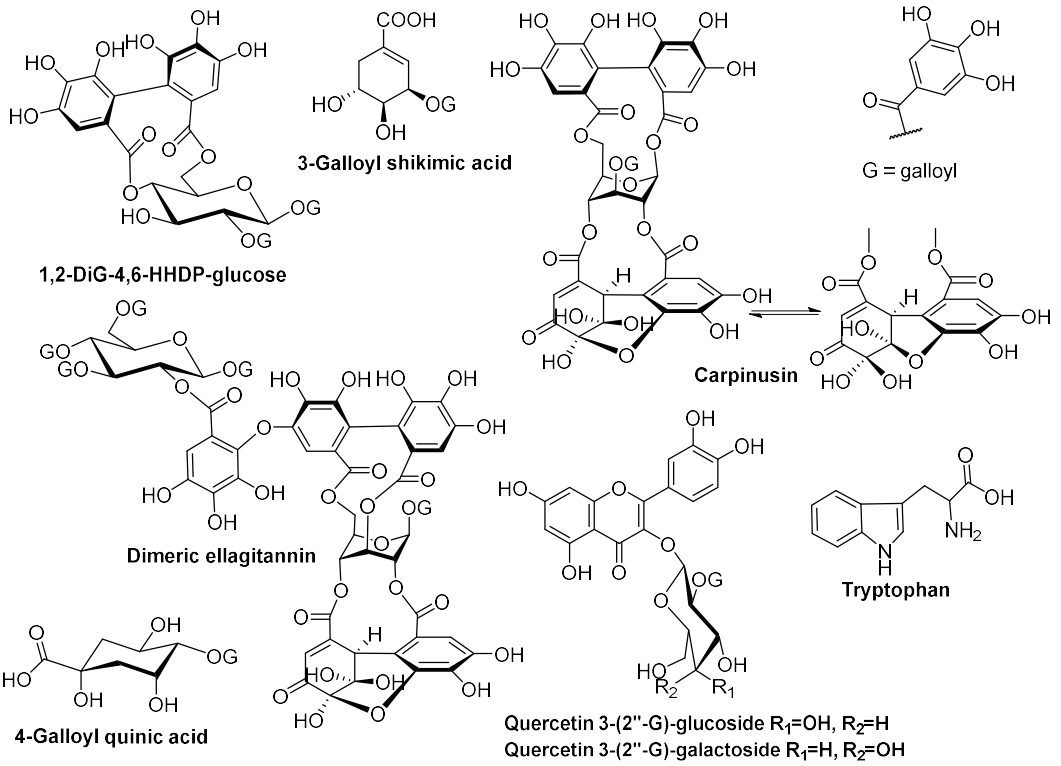


Figure 31. The structures of isolated compounds from *G. sylvaticum.*

UV spectroscopy

UV spectroscopy was used in the classification of *G. sylvaticum* phenolics into different polyphenol classes based on their characteristic UV spectra. The UV spectral properties of polyphenols were discussed in details in the literature section, part 2.4.2, and typical spectra for the polyphenol groups are presented in paper **I**. A challenge regarding the use

of UV spectra in the identification was that the extracts of *G. sylvaticum* contain a complex mixture of compounds. Thus, it cannot be verified which features in the UV spectra are typical for the studied compound or which are caused by some coeluting compounds.

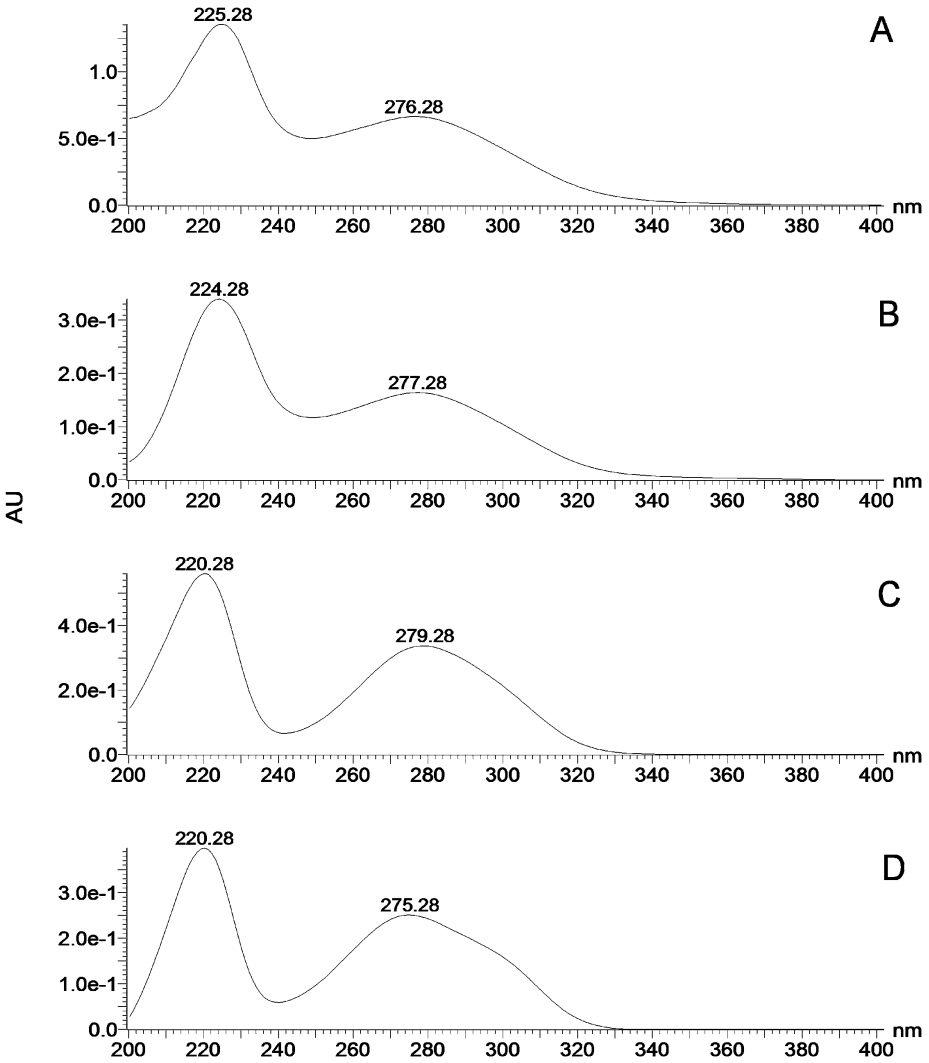


Figure 32. The UV spectra of geraniin (A), carpinusin (B), sylvatiin A (C) and hexaGG (D).

Isomeric compounds, such as geraniin and carpinusin, showed no significant difference in their characteristic UV spectra (Figs. 32A and B). In some cases, the UV spectra can give useful hints for identification. That was the case with the unknown group of compounds of petals, which were assumed to be gallotannins. However, the UV spectra did not show the shoulder at around 300 nm in the second band, which is typical for the galloyl groups conjugated with metadepsidic bonds (Figs. 32C and D). Therefore, some other extra

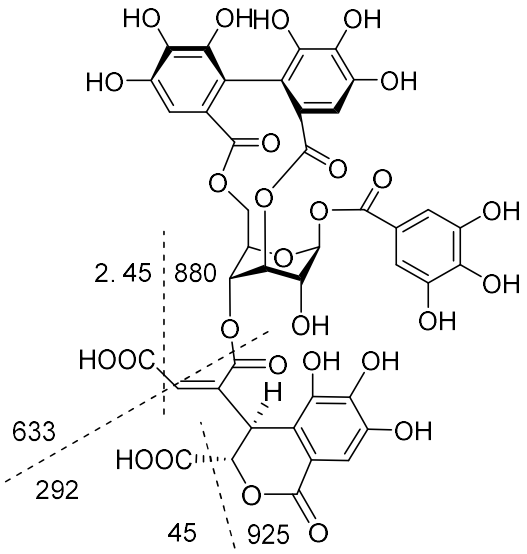
moiety than a galloyl group must be attached to the structures. This moiety was later identified to be an acetylglucose group, which has no effect on the UV spectra of core GG. The UV maxima of identified polyphenols are listed in Tables 13–18, 20 and 22.

Mass spectrometry

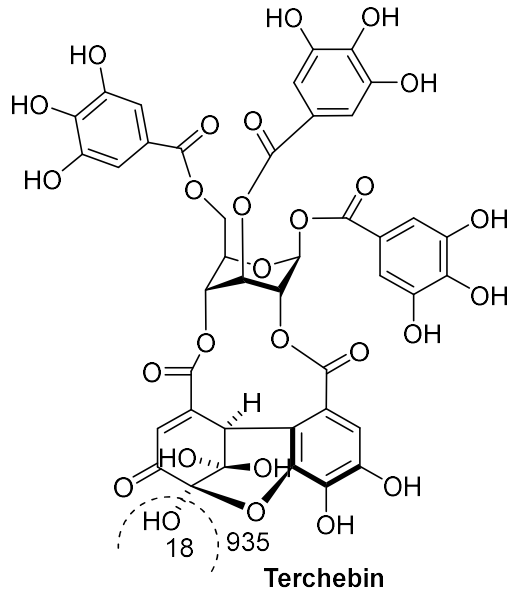
The typical mass spectra of different polyphenol groups are presented in paper **I**. Fragmentation followed the patterns discussed in the literature review. There were differences in the mass spectra produced with the conditions used in the QTOF and tripleQ, although the ESI and negative mode was used for both. Molecular ions, doubly charged ions and clusters were more abundant in the QTOF, whereas in the tripleQ the elimination of water molecules and acid groups were more pronounced. The ability of QTOF to produce multiply charged molecular ions was utilized in paper **V** to determine the degree of polymerization of proanthocyanidins.

The characteristic fragmentation of DHHDP group—the cleavage of water—was detected at *m/z* 933 for geraniin. This ion was clearly seen in the tripleQ MS spectra, although it was not abundant in the QTOF MS spectra. Characteristic fragmentation for the sylvatiins was the loss of acetylglucose moiety (204 Da) and the loss of acetylglucosylated galloyl group (374 Da). The fragmentation patterns of sylvatiins are presented in paper **III** in detail.

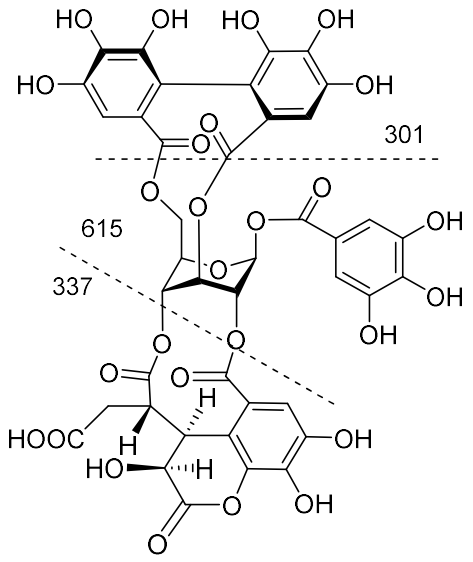
All ETs that have further oxidized DHHDP groups were characterized as G-HHDP-DHHDPmodified-glucoses in paper **I**, but are classified according to their molecular mass and characteristic mass spectral fragmentation to three groups in Tables 13–18, 20 and 22 as follows: geraniinic acid-type compounds having a molecular mass of 952 Da and exhibiting the fragmentation of the one –COOH group; repandusidic acid-type where the cleavage of two acid groups is possible and the ion for the brevifolin at *m/z* 247 Da is detected; and a chebulagic acid-type compound with a molecular mass of 954 Da and not showing the fragmentation of acid groups or water molecule (Fig. 33). The tertiary acid group of geraniinic acid was more easily fragmented than the primary acid group of chebulagic acid in the MS conditions used. Because of the easy cleavage of the acid group, no molecular ion was detected for the geraniinic acids in the tripleQ; instead, ion at *m/z* 907 was strong. Similarly, for repandusidic-type ellagitannins, ions at *m/z* 925 Da were more abundant in the tripleQ than the molecular ions. Figure 35 shows structures for these modified ETs that were tentatively identified based on the MS spectra and their fragmentation patterns. However, compounds have to be isolated to verify these identifications.



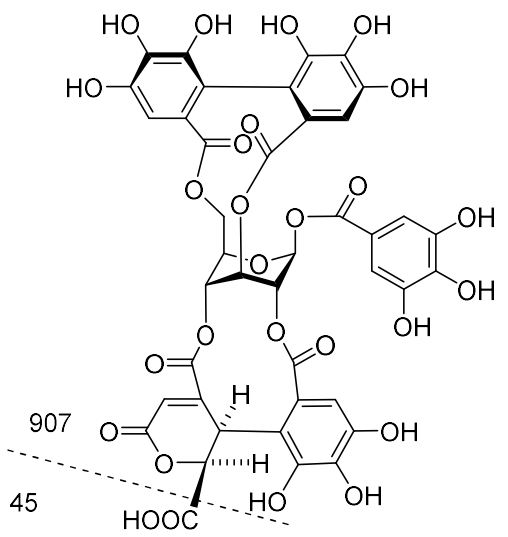
Repandusidic acid
$C_{41}H_{30}O_{28}$
MW: 970.66



Terchebin
$C_{41}H_{30}O_{27}$
MW: 954.66



Chebulagic acid
$C_{41}H_{30}O_{27}$
MW: 954.66



Geraniinic acid B/C
$C_{41}H_{28}O_{27}$
MW: 952.65

Figure 33. Structures of some tentatively identified oxidatively modified ETs in *G. sylvaticum* and their tentative MS fragmentation patterns.

NMR spectroscopy

The ^{1}H NMR spectra of DHHDP ellagitannins are complex, as described in the literature review. Table 12 shows the NMR data of geraniin and carpinusin measured in this work that were compared with the literature values in Tables 7 and 8. The published ^{1}H NMR shifts were more useful in the identification of the purified isomer of geraniin, because the exact positions of carbon signals were not assigned for the tautomers in the literature. The observed signals corresponded the best with the shifts reported for carpinusin: The ^{1}H NMR shift of C-1 was at 6.2 ppm, whereas other isomeric compounds had the corresponding shift near 6.6 ppm, and the shift of C-3 was near 6.0 ppm whereas other isomers had near 5.6 ppm. In addition, the coupling constants of C-6 protons were identical to the values reported in the literature for carpinusin (compare Tables 12 and 7). Observed values also fit best for the carpinusin when ratio of tautomers was considered. The 5-ring tautomer was more visible for carpinusin. This difference was also seen clearly in the LC chromatograms as a broad peak for carpinusin (see Fig. 45).

The assignment of sylvatiins was done by comparing the NMR data with the data of pentaGG. Figure 34 shows ^{1}H NMR spectra of sylvatiin C as an example of the NMR spectra of *G. sylvaticum* compounds. The proton spectra showed the presence of signals from three sugars and galloyl groups. The use of 1D-TOCSY confirmed the presence of three sugars and allowed the assignment of overlapping signals (Fig. 34). NMR spectroscopy was indispensable in the determination of the position of acetylglucose group in the sylvatiin structures. The attachment of acetylglucose to the hydroxy group of galloyl resulted in two aromatic proton signals of the galloyl group being split to two doublets with a coupling constant of 2 Hz due to the asymmetry. Also, the C-2, C-4 and C-6 carbon signals of that galloyl group were circa +2 ppm values compared with the corresponding signals of the galloyl group without acetylglucose, whereas all the other galloyl group shifts remained identical. Signals corresponding with the presence of the acetyl group were observed for carbonyl and methyl. The HMBC long-range correlations confirmed that the acetyl group was attached to the C-6’ of extra glucose and that this glucose was attached via C-1’ to the hydroxy of the galloyl group in C-3. The NMR shifts of sylvatiins are presented in detail in paper **III**.

Table 12. Measured NMR data of the center glucose and the DHHDP group of geraniin and carpinusin.

Compound	Position	$\delta^{13}C$	$\delta^{1}H$	multip.	$J_{H,H}$	HMBC(H->C) correlations	NOESY correlations
Geraniin major tautomer 6-ring	1	90.74	6.55	*t* br	3.0	C-5, C-7galloyl	
	2	69.99	5.56	*m* br	1.0	C-6, C-7'A	
	3	63.34	5.51	*dd* br	1.1, 2.4	C-1	
	4	65.99	5.52	*m* br		C-2 minor	H-5
	5	72.69	4.79	*t* br	9.3	C-6, C-1	H-6e, H-4
	6a	63.75	4.95	*t*	8.2, 10.0	C-5, C-7'HDDP	H-6e
	6e		4.30	*dd*	8.2, 10.0	C-5, C-4	H-5, H-6a
	1" B ring	46.31	5.17	*s*		C-2"B, C-3"B, C-5"B, C-6'B, C-7"B, C-1'A, C-6'A	
	2" B ring	154.67					
	3" B ring	128.68	6.53	*s* br		C-1",C-5", C-7"	
	4" B ring	191.82					
	5" B ring	96.35					
	6" B ring	92.53					
Geraniin minor tautomer 5-ring	1	91.88	6.56	*m* br			
	2	70.57	5.58	*m* br			
	3	62.5	5.58	*m* br		C-1	
	4	66.96	5.42	*d* br	3.7	C-3	
	5	73.37	4.79	*t*	6.4		H-6e
	6a	63.90	4.77	*t*		C-5	
	6e		4.42	*dd*	2.9, 6.4	C-4	H-5
	1" B ring	52.06	4.77	*s*			
	2" B ring	149.29					
	3" B ring	125.04	6.25	*d*	1.4	C-1"B, C-7"B	
	4" B ring	194.57					
	5" B ring	92.45					
	6" B ring	109.23					
Carpinusin major tautomer 6-ring	1	89.70	6.21	*s* br		C-5	
	2	67.66	5.14	*m* br	1.3, 2.0	C-3	
	3	61.37	5.87	*m*	1.2, 2.0	C-4	
	4	67.91	5.40	*dd*	1.1, 1.2	C-3	
	5	71.79	4.67	*dd*	5.3, 13.0	C-1	
	6a	63.62	5.43	*t*	11.7, 13.0	C-5	H-6e, 3'HHDP
	6e		4.25	*dd*	5.3, 11.7		H-6a
	1" B ring	46.37	5.23	*s*		C-6"B ring, C-5"B ring	
	2" B ring	154.59					
	3" B ring	128.81	6.57	*s*			
	4" B ring	191.89					
	5" B ring	96.24				C-3"B ring	
	6" B ring	92.60					
Carpinusin minor tautomer 1 5-ring	1	90.43	6.28	*s* br			
	2	68.15	5.05	*m* br	1.3, 2.6		
	3	61.80	6.08	*m*	< 1		
	4	67.91	5.47	*m* br			
	5	71.89	4.70	*dd*	5.3, 12.9		
	6a	63.73	5.50	*dd*	11.8, 12.9		H-6e
	6e		4.17	*dd*	5.3, 11.8		H-6a
	1" B ring	51.85	5.00	*d*	1.3		
	3" B ring	124.74	6.26	*d*	1.3		
	4" B ring	194.62					
Carpinusin minor tautomer 2	1						
	2		5.17	*m* br			
	3						
	4		5.54	*s* br			
	5		4.76	*dd*	5.4, 13.4		
	6a			*t*	4.7		
	6e		4.22	*dd*	5.2, 11.7		
	1" B ring		4.98	*d*			
	3" B ring						
	4" B ring	194.62					

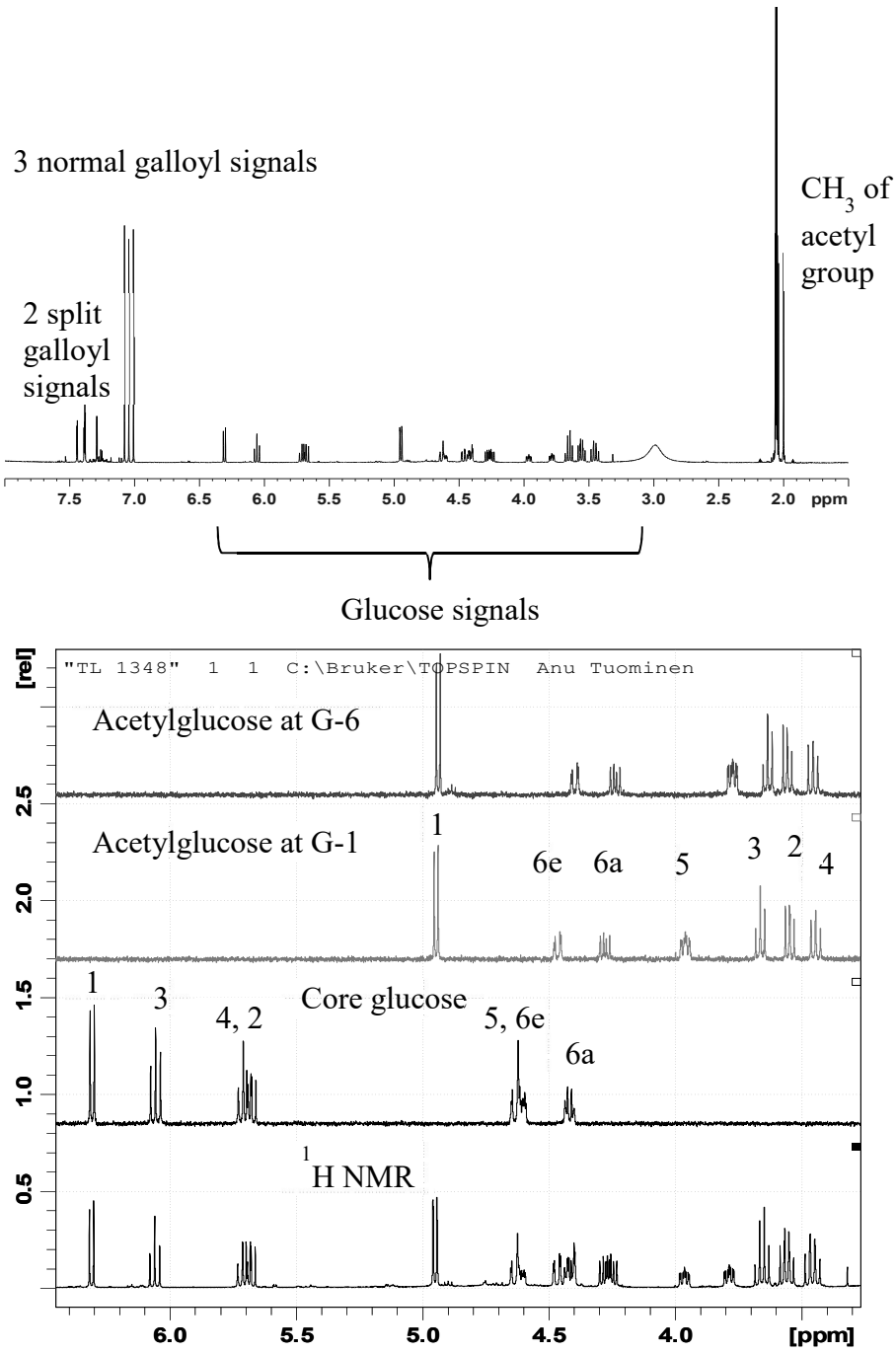


Figure 34. ^{1}H NMR and 1D-TOCSY spectra of sylvatiin C.

CD spectroscopy

The stereochemistry of the geraniin isomer was studied with the CD spectroscopy and compared with the values presented in the literature (Table 9). The DHHDP group of this compound was observed to be in the same configuration as in the geraniin (1-G-3,6-(*R*)-HHDP-2,4-(*R*)-DHHDP-β-D-glucopyranose) structure according to the characteristic CE for *R*-DHHDP: a weak negative CE around 350–360 nm and a strong positive CE near 200–210 nm (Fig. 35B). In contrast, the HHDP group of the isomeric compound was in the *S*-configuration as it showed CEs opposite that of geraniin, characteristic for the *S*-HHDP group: positive CE at 240 nm and negative CE at 265 nm. Results showed that HHDP group does not interact with the free galloyl group, as no strong splitted CE was seen in the spectra (Fig. 35B). Based on the data gained from the NMR and CD spectra, it was suggested that the compound is 3-G-1,6-(*S*)-HHDP-4,2-(*R*)-DHHDP-glucopyranose, which has the common name of carpinusin.

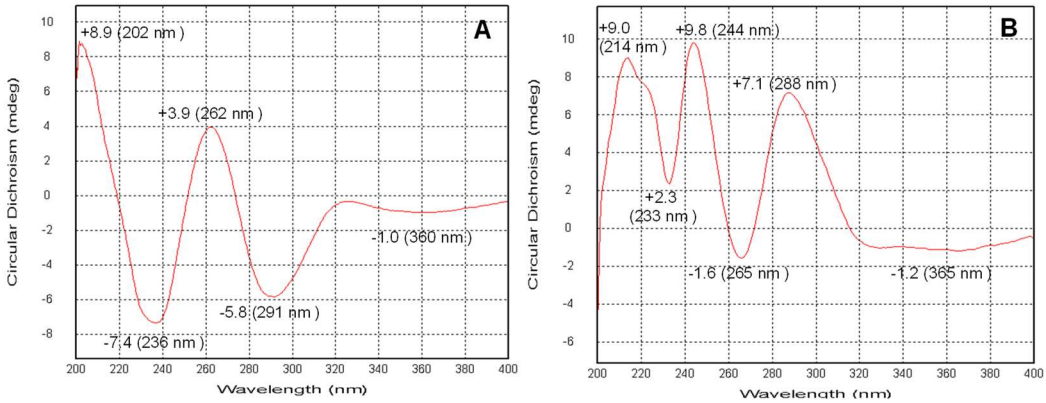


Figure 35. The CD spectra of A) geraniin and B) carpinusin in MeOH.

Similarly, the stereochemistry of sylvatiins was studied with the CD spectroscopy. Figure 39 shows the CD spectra of the measured chebuloyl group containing compounds. Typically, the C-1' of chebuloyl group in the chebulinic acid and chebulagic acid structures remains in the *R* configuration, because these compounds are biogenetically derived from geraniin that has the *R* configuration.[11] The CD spectra of chebulanin and chebulagic acid were similar to that observed for geraniin (compare Figs. 35 and 36). On the other hand, the interaction of galloyl groups and the lack of HHDP group produced different CEs for chebulinic acid. The CD spectrum of sylvatiin D was almost identical to that of chebulinic acid, and it seemed that the acetylglucose moiety only slightly decreased

the intensity of CEs of galloyl groups (**III**). This effect was observed for all sylvatiins (see the CD spectral data of other sylvatiins in paper **III)**.

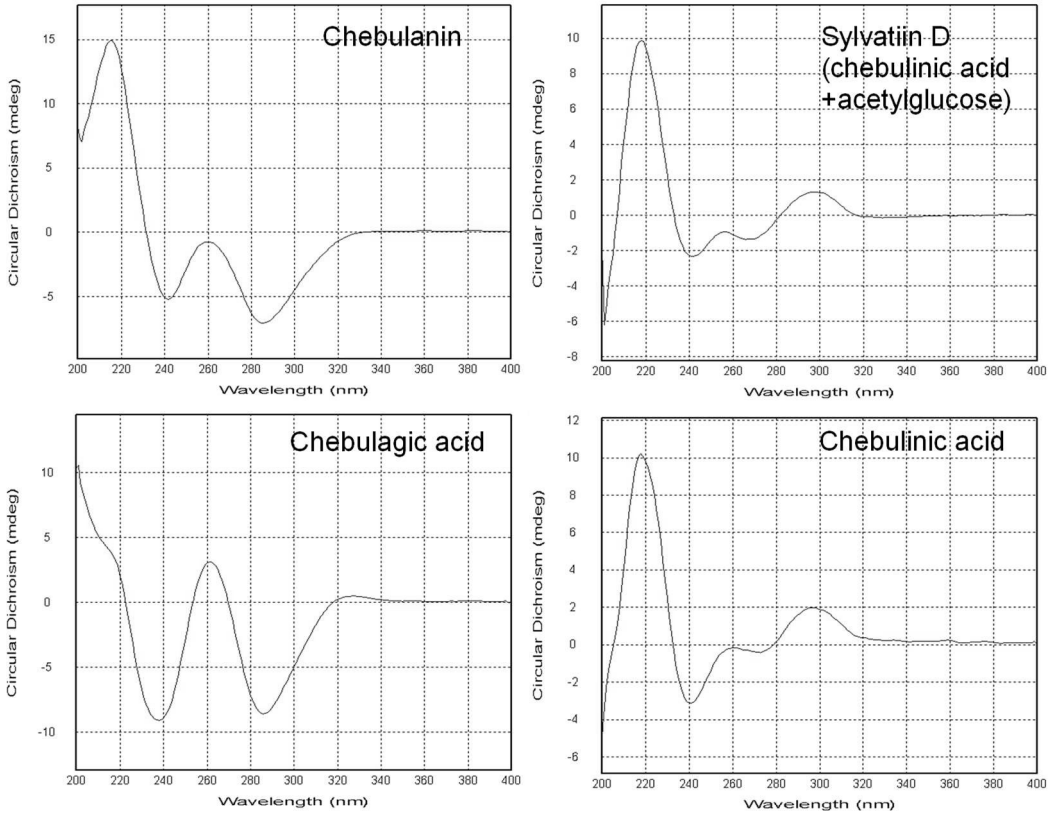


Figure 36. CD spectra of compounds that contain chebuloyl group.

5.4 Intraplant and seasonal variation

The total quantification results achieved in paper **II** corresponded well with the previous results published about *G. sylvaticum*. The average tannin content in the aboveground parts was 12.4% in *G. sylvaticum* (**II**, Fig. 37) while previous studies have reported the ellagitannin content of 13% DW in the leaves.[83–86] Similarly, the average PA content in the roots was 20.2% (**II**) when literature reported ca. 20% of the DW in the roots, composed of catechin and galloyl derivatives, and ellagic acid.[83–86]

This chapter combines HPLC-DAD-MS analysis results from papers **I**–**VI** and describes the phytochemistry of *G. sylvaticum* organs. Molecular masses of identified compounds are presented as nominal masses to save space. Exact masses can be found in article **I**. A clear advantage gained from the present study was that all the organs were studied separately, enabling detailed analyses of the chemical profiles.

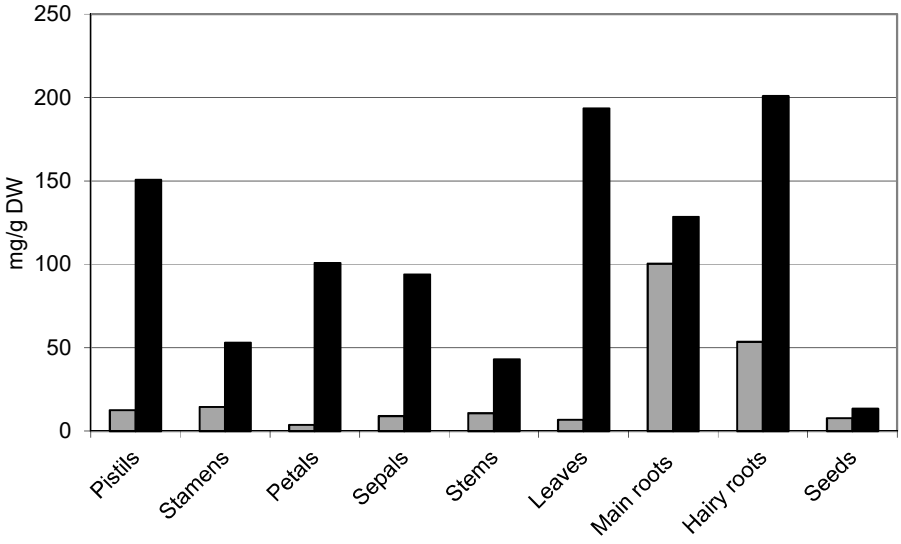


Figure 37. Summary of the total phenols (black) and total proanthocyanidins contents (grey) in *G. sylvaticum* organs.

5.4.1 Pistils synthesize gallotannins more than other organs

Pistil is the female reproductive part of a flower that protects the ovules, and screens out inappropriate pollen. The lifetime of pistils is longer than the other flower parts, because seeds develop inside the ovary until the fruit is ready for the seed ejection, which can take several weeks. The herbivore pressure on the pistils of *G. sylvaticum* is high, as several specialized insects lay their eggs beside the style and hatched larvae consume the developing seeds directly from the ovaries.[42]

The TP content in the pistils of *G. sylvaticum* was 175 mg/g DW (**II**) and the main compounds identified are listed in Table 13. Geraniin was the main compound in the pistils (Fig. 38); the amount of geraniin was the second-highest after the leaves, circa 142 mg/g DW. Pistils showed a distinctive ontogenic variation (**VI**, Fig. 41). Smaller pistils in the male phase contained more geraniin (155 mg/g DW) than pistils in the fruit phase (118 mg/g). In contrast, the amount of carpinusin increased toward the fruit phase from 23 to 59 mg/g DW (**VI**). Also, the amount of galloylated flavonoids increased in the fruit phase; however, the amount of galloylated flavonoids was significantly less in the pistils than in the leaves (**VI**).

The most distinctive feature in the pistils of *G. sylvaticum* was that the pistils accumulated more GGs and GTs than other plant organs: over 70 mg/g DW (**I**, **II,** Fig. 38; Table 13). The detailed MS analysis showed the presence of GTs up to octa- and nonaGGs (**I**). The content of GTs followed a strong ontogenic variation (**VI**). The amount of penta- and hexaGGs multiplied during the pistil growth toward the fruit phase whereas the amount of other GGs remained more constant (**I**, **VI**, Fig. 38). The high content of both ETs and GTs in the pistils was unusual, since the hydrolysable tannin pathway is known to branch after pentaGG to produce either ETs or GTs. Typically, both are not accumulated in the plant tissues simultaneously.[8, 97] The production of both types of HTs suggested that the pistils need a special type of defense against specialized herbivores in order to protect the seeds developing inside the ovary.

The pistils contained a relatively low content of PAs, approximately 33 mg/g DW (**II**). The pistils contained both catechins and gallocatechins, and traces of polymeric PAs up to the DP of 17 were detected (**V**). PC dimer and tryptophan were used as marker compounds of seeds and their content increased toward the fruit phase (**VI**).

The high content of ETs and other phenolics contributed to the high pro-oxidant and antioxidant activity of pistils. Also, the GGs and GTs containing fractions showed high antioxidant activity (**II**). GGs are known to have high protein precipitation capacity, and protein precipitation is one of the assumed defense mechanisms of plants against herbivores, as it hinders the food digestion of herbivores. The protein precipitation capability of the pistils might provide more generalized antiherbivore properties. However, this activity was not measured for the fractions and extracts in this study.

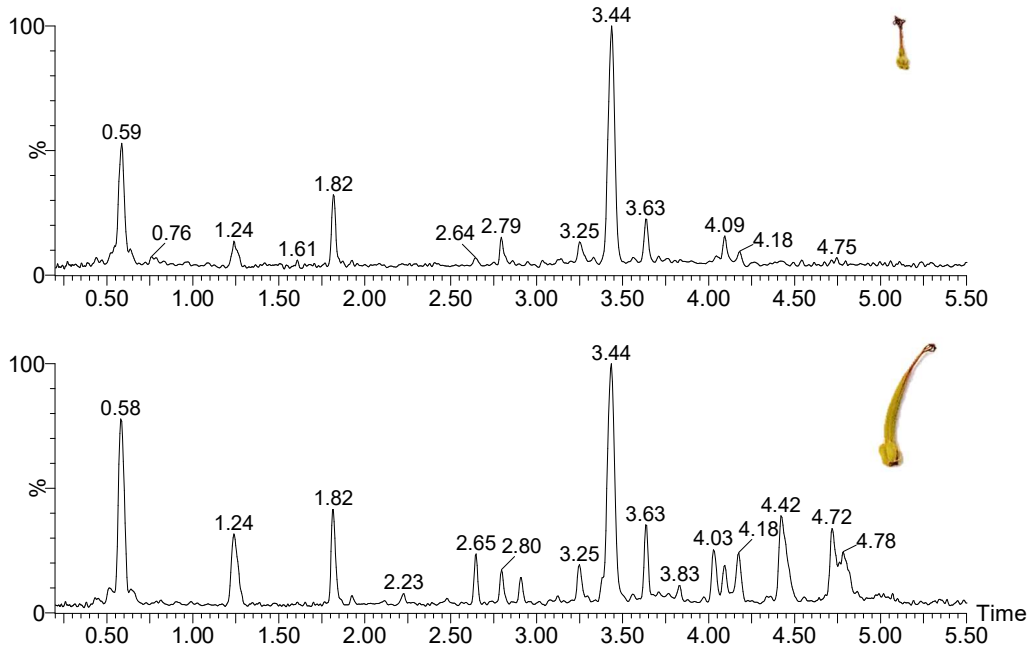


Figure 38. UPLC MS base peak chromatograms of the pistil extracts of *G. sylvaticum* in the male phase (above) and in the fruit phase (below).

Table 13. Main phenolic compounds identified in the *G. sylvaticum* pistil extracts

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.23	4.3	MonoGG	217, 280	332	331, 633
1.82	5.6	MonoG quinic acid	217, 275	344	343, 191
1.92	7.5	4-MonoG quinic acid	216, 274	344	343, 687
2.23	9.3	3-MonoG shikimic acid	217, 275	326	325, 651, 307
2.48	10.9	Neochlorogenic acid A	220, 280, 330	354	353, 707
2.65		Methylated monoGG	218, 267	346	345, 183
2.79	14.4	3,4-DiG quinic acid	204, 285	496	495, 991
2.80	14.8	DiGG	217, 277	484	483, 967
2.91	16.1	PC dimer	204, 283	578	577, 425
3.11	16.5	G-HHDP-glucose	216, 275	634	633, 316
3.25	18.5	Geraniinic acid	218, 278	952	951, 453, 475
3.44	19.3	Geraniin	222, 277	952	951, 466, 1903
3.40	20.5	Repandusidic acid	220, 274	970	969, 633, 484, 247
3.56	21.1	Ascorgeraniin	221, 281	1110	1109, 554, 467, 635
3.70	21.7	1,2-DiG-4,6-HHDP-glucose	218, 277	786	785, 392, 300
3.63	22.4	TriGG	218, 278	636	635, 317
3.83	23.7	Tellimagrandin II	217, 279	938	937, 468
3.94	24.0	Ellagic acid	255, 366	302	301
4.03; 4.09	25.4	TetraGG	218, 279	788	787, 393
4.18	26.4	Carpinusin	222, 279	952	951, 466
4.14	26.7	Quercetin-G-glycoside	221, 271, 315	616	615, 1231
4.37; 4.42 br	29.2	PentaGG (metadepsidic)	218, 279	940	939, 393, 469
4.49	29.7	Kaempferol-G-glycoside	212, 275, 360	600	599, 1199, 313, 285
4.78 br	31.3	HexaGG	218, 277	1092	1091, 469, 545
5.02 br	32.5	HeptaGG	220, 276	1244	1243, 545, 621

5.4.2 Stamens accumulate chebulagic acid and kaempferol glycosides

Stamens are the male reproductive organs within the flowers. They are composed of anthers, which contains pollen grains, and filaments. The lifetime of stamens is relatively short. The immature anthers are first yellow in the bud phase, and turn blue in the mature stage when the pollen is ready for the presentation.[45]

The total phenolic content in the stamens of *G. sylvaticum* was approximately 60 mg/g DW (**II**) and the main compounds identified from stamens are listed in Table 14. The amount of geraniin was notably low in comparison with the other flower parts; geraniin content was 33 mg/g DW in the bud phase, and decreased to 19 mg/g DW in the open flowers (**VI**, Fig. 39). Filaments contained most of the geraniin, as after the fallout of anthers, the geraniin content was still 28 mg/g DW. Instead of geraniin, stamens contained a distinctively high amount of chebulagic acid; this content decreased during ontogenic phases from buds to the anther fallout (**I**, **VI**, Fig. 39).

Another substantial difference in the chemical profile of stamens compared with the other organs was the high amount of kaempferol glycosides and mono- and digalloyl quinic acids (Fig. 39) (**I**, **II**). Flavonols accumulate into the pollen grains as pollinator attractant pigments, and for other assumed roles.[6, 319–321] However, the amount of flavonol glycosides in the stamens of *G. sylvaticum* was higher in the filaments without anthers and pollen. The only exception was the amount of one kaempferol diglycoside (**IV**). Interestingly, the main pigment anthocyanin in the stamens was malvin, instead of acetylmalvin as observed in the petals (**VI**). Malvin was not detected in the filaments after anthers had fallen off. Therefore, malvin and the other kaempferol diglycoside may be related to the colour change of anthers from yellow to blue, which signals to pollinators that pollen is mature and ready for pick-up.

The sample set in paper **IV** also included some stamens of female plants (unpublished results). Interestingly, there was a clear difference in the level of some compounds in the female stamens, which have undeveloped anthers without pollen or sterile pollen. Female samples contained less chebulagic acid and kaempferol diglycoside and no malvin (unpublished results). Those were exactly the same compounds, which content followed the clear ontogenic trend in the hermaphrodite stamens. This strengthened the suggestion that these compounds were related to the blue color of anthers and pollen, as undeveloped female stamens have a pale or yellow color (see Fig. 5.). Instead, the amounts of geraniin, geraniinic acid and carpinusin were higher in the female stamens.

The PA content in the stamens of *G. sylvaticum* was 34 mg/g DW (**II**). The use of HILIC column, in which the PAs elute according to their DP, revealed that the stamens contained a wide variety of PAs, such as PCs, PDs and mixed PC/PD oligomers, and traces of polymeric mixed PAs up to the DP of 20 were detected (**V**). Stamen extract was rather

inactive in the AO and PO assays, although some stamen fractions that contained high amounts of kaempferol and quercetin glycosides showed high PO activity (**II**).

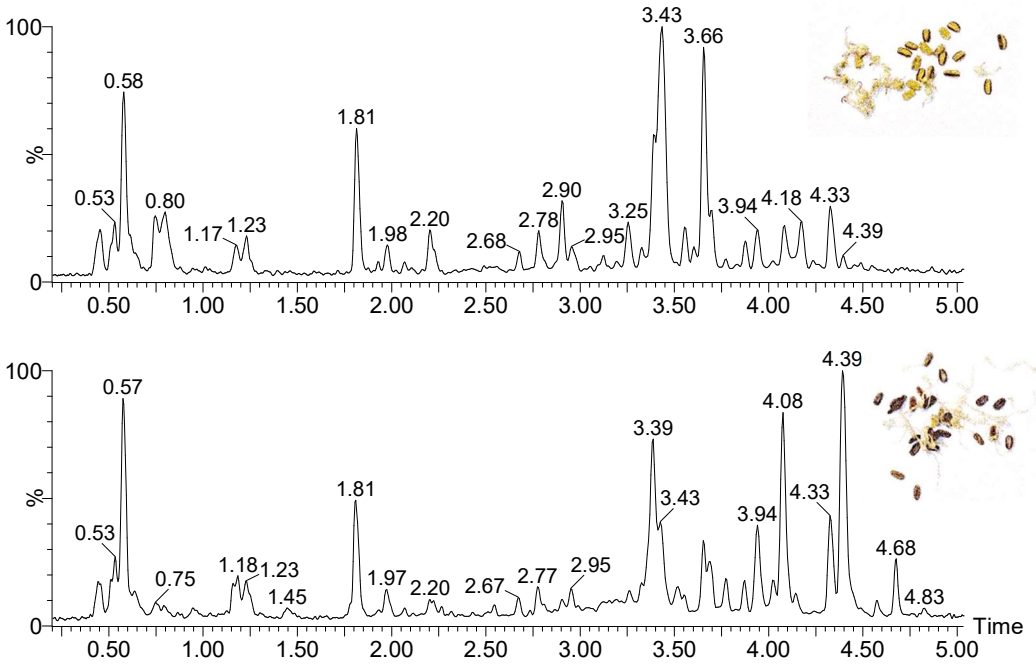


Figure 39. UPLC MS base peak chromatograms of the stamen extracts of *G. sylvaticum* in the immature bud phase (above) and with mature pollen in the open flower phase (below).

Table 14. Main phenolic compounds identified in the *G. sylvaticum* stamen extracts

RT $_{UPLC\ TIC}$ (min)	RT $_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.23	4.4	MonoGG	218, 279	332	331, 663
1.81	5.8	MonoG quinic acid	217, 275	344	343, 687, 191
2.20	10.1	MonoG shikimic acid	217, 275	326	325, 651
2.67	14.0	DiG quinic acid	217, 276	496	495, 343, 991
2.78	15.2	Myricetin glycoside	217, 258+	498	497, 317, 995
2.86	16.3 broad, 18.3	Malvin	230, 276, 535	671	671, 551
2.90	14.5	Catechin	204, 280	290	289
3.25	18.5	Geraniinic acid	218, 278	952	951, 907, 453, 467, 476
3.39	18.2	Leucocyanidin	203, 281	306	305, 225
3.43	19.3	Geraniin	221, 277	952	951, 933, 466, 633, 1903
3.26	19.9	Corilagin	220, 277	634	633, 1267, 301
3.55	21.0	Ascorgeraniin	222, 281	1110	1109, 554, 935, 467
3.69	21.6	1,2-DiG-4,6-HHDP-glucose	216, 271, 361	786	785, 392, 300
3.58	21.9; 22.1	Quercetin diglycoside	206, 269, 350	626	625, 1251
3.66; 3.83	21.9	Kaempferol diglycoside	199, 267, 343	610	609, 415
3.68	22.4	Myricetin glycoside	210, 264, 355	480	479, 317, 959
3.94	24.4	Chebulagic acid	223, 277	954	476, 1430, 301
4.03; 4.08	25.5; 26.2	TetraGG	219, 280	788	787, 393
4.08	25.8	Quercetin 3-glycoside	205, 258, 354	464	463, 927
4.15	26.5; 26.9	Quercetin G-glycoside	212, 271, 358	616	615, 301
4.18	26.6	Carpinusin	222, 280	952	951, 933, 466
4.33	28.1	Quercetin arabinoside	205, 258, 351	434	433, 301, 867
4.39	28.6	Kaempferol 3-glycoside	197, 267, 348	448	447, 895, 285
4.48	29.8	Kaempferol G-glycoside	209, 268, 350	600	599, 417, 1199
4.58, 4.68	31.0	Kaempferol arabinoside	198, 266, 347	418	417, 285, 835

5.4.3 Petals produce sylvatiins that showed copigmentation capacity

Petals provide a visually arresting colorful target for flower pollinators. After pollination, petals drop; thus their lifespan is only a couple of days. Petals of *G. sylvaticum* contain high amount of nutritionally valuable sugars and are heavily consumed by specialized herbivores (**II**). The TP content in the petals of *G. sylvaticum* was 114 mg/g DW (**II**). The petals differed clearly from the other organs of *G. sylvaticum* by being relatively poor in geraniin-type ETs and galloyl quinic acids. Instead, they produced a group of unknown compounds that were not detected in the other organs (**I**, **III**, Table 16 and Fig. 40). The amount of geraniin was higher in the bud phase (41 mg/g DW) than in the open flower phase (10 mg/g DW) (Fig. 40, **VI**). Also, the total PA content in the petals was the lowest of all the studied organs (7.1 mg/g DW). Petals contained mainly PDs; traces of polymeric PAs up to a DP of 14 were detected (**II**, **V**). Like stamens, petals contained high amounts of kaempferol glycosides, instead of quercetin glycosides, which dominated in the other plant parts of *G. sylvaticum* (**VI**). The same feature has been observed within the petals of other blue *Geraniums*, and kaempferol-3-sophoroside (610 Da) has been identified as the main copigment in some cultivars.[112]

Four of the unknown compounds were isolated (**III**). It was discovered that compounds were totally new HTs, GGs and ETs that contained one or two acetylglucose units, which were named sylvatiins A–D (**III**, Table 15, Fig. 41). The same acetylglucose moiety is attached to the main anthocyanin pigment in the petals of *G. sylvaticum*, malvidin 3-(6-acetylglucoside)-5-glucoside.[122] In addition to purified sylvatiins A–D, four other HTs that presumably belong to the sylvatiin family were detected in the petal extract: Two GGs, of which the first had a monoGG core and one attached acetyl group with a molecular mass of 374 Da (G-acetyl-glucose); and second with a triGG core and one acetylglucose, and a molecular mass of 840 Da (sylvatiin E) (Fig. 41). ET with a molecular mass of 1178 Da fitted to a structure where one water molecule is added to the sylvatiin D structure, such as the dehydrochebuloyl group. A second ellagitannin with a molecular mass of 1364 Da fitted to a structure where a second acetylglucose moiety is attached to a sylvatiin D core (Fig. 41).

The petal extract showed only moderate PO and AO activity. The addition of acetylglucose did not increase the activity compared with regular GGs or GTs, when the fractions of pistils that contained GTs and the corresponding petal fractions were compared (**II**). Furthermore, the addition of acetylglucose moiety slightly decreased the PPC activity of sylvatiins when compared with corresponding core compounds (**III**). Therefore, instead of antiherbivore activity, it was tested if these compounds might have a role in the pollinator attraction and petal color, and act as copigments, like flavonoids. The observed ontogenic variation of sylvatiins strengthened the copigmentation theory, as the compounds associated with the color of petals had the same ontogenic trend (**VI**). The amount of acetylmalvin (13 mg/g), kaempferol glycosides (41 mg/g), sylvatiin D (66 mg/g) and C (54 mg/g), was the highest in the fully open phase or remained rather constant

(**VI**). In contrast, the amount of other phenolics was significantly higher in the bud phase (**VI**). When the color polymorphs were studied, it was noticed that the whiter petals had less acetylmalvin, sylvatiin A and sylvatiin C and more flavonol glycosides, as compared with the deep purple petals (**III**).

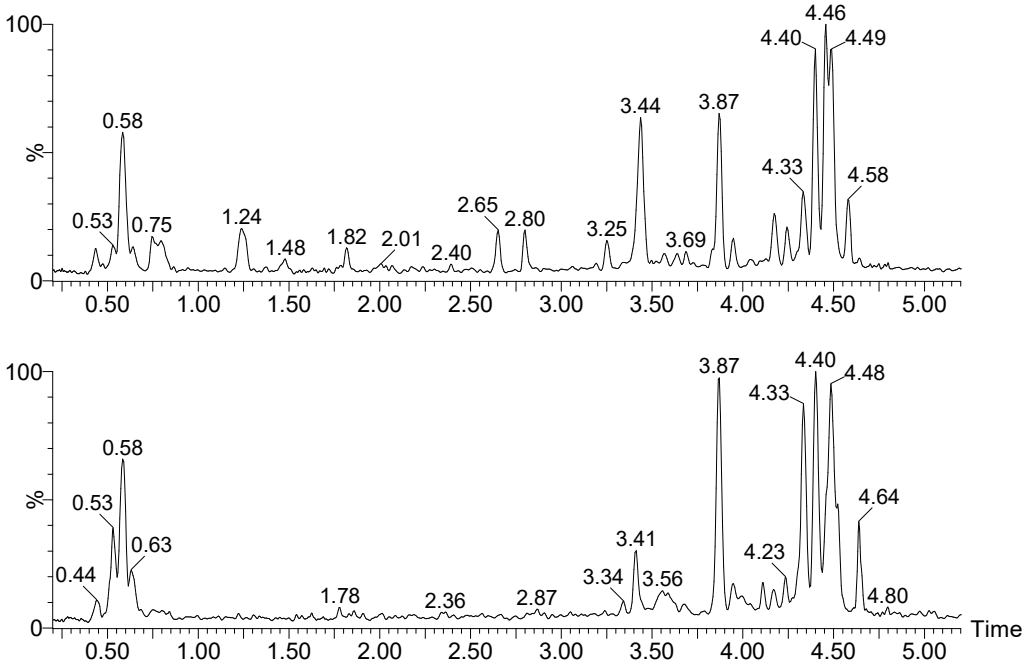


Figure 40. UPLC MS base peak chromatograms of the petal extracts of *G. sylvaticum* in the bud (above) and in the open flower phase (below).

Table 15. Main phenolic compounds identified in the *G. sylvaticum* petal extracts

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.24; 1.48	4.3	MonoGG	218, 280	332	331, 663
1.82	5.6	MonoG quinic acid	217, 275	344	343, 687, 191
2.65	12.7	G-acetyl-glucose	218, 280	374	373, 747
2.80	15.2	DiGG	217, 277	484	483, 967
3.25	18.6	Geraniinic acid	218, 278	952	951, 453
3.41	18.2	Leucocyanidin	203, 281	306	305
3.44	19.3	Geraniin	222, 279	952	951, 466
3.55	21.1	Ascorgeraniin	222, 280	1110	1109, 554, 467
3.56 broad; 3.68	24.4	Acetylmalvin	207, 278, 524	714	713, 551, 1427
3.61	21.9	Quercetin diglycoside	207, 267, 349	626	625, 1251
3.69	22.4	Myricetin glycoside	211, 267, 354	480	479, 959, 317
3.94	21.4; 23.8; 25.6	Sylvatiin type ellagitannin	218, 281	1178	1177, 839, 588, 337
3.86	24.2	Kaempferol diglycoside	197, 267, 347	610	609, 1219
3.87	24.9	Sylvatiin E	218, 277	840	839, 419, 317
4.03	25.4	TetraGG	216, 280	788	787, 393
4.17	26.5	Carpinusin	222, 278	952	951, 466
4.29; 4.40	28.5	Kaempferol 3-glycoside	197, 267, 348	448	447, 895
4.23	27.8	Sylvatiin B	217, 278	992	991, 495
4.33; 4.42	28.4	Sylvatiin D	217, 277	1160	1159, 579
4.40	31.1	Sylvatiin type ellagitannin	223, 273	1364	1363, 681
4.50	29.4	Kaempferol G-glycoside	218, 279	600	599, 1199, 285
4.46	29.7	Sylvatiin A	218, 280	1144	1143, 571
4.48; 4.64	30.4; 31.4	Sylvatiin C	218, 279	1348	673, 1347, 1157
4.58	32.5	Sylvatiin type ellagitannin	222, 274	1140	1139, 569

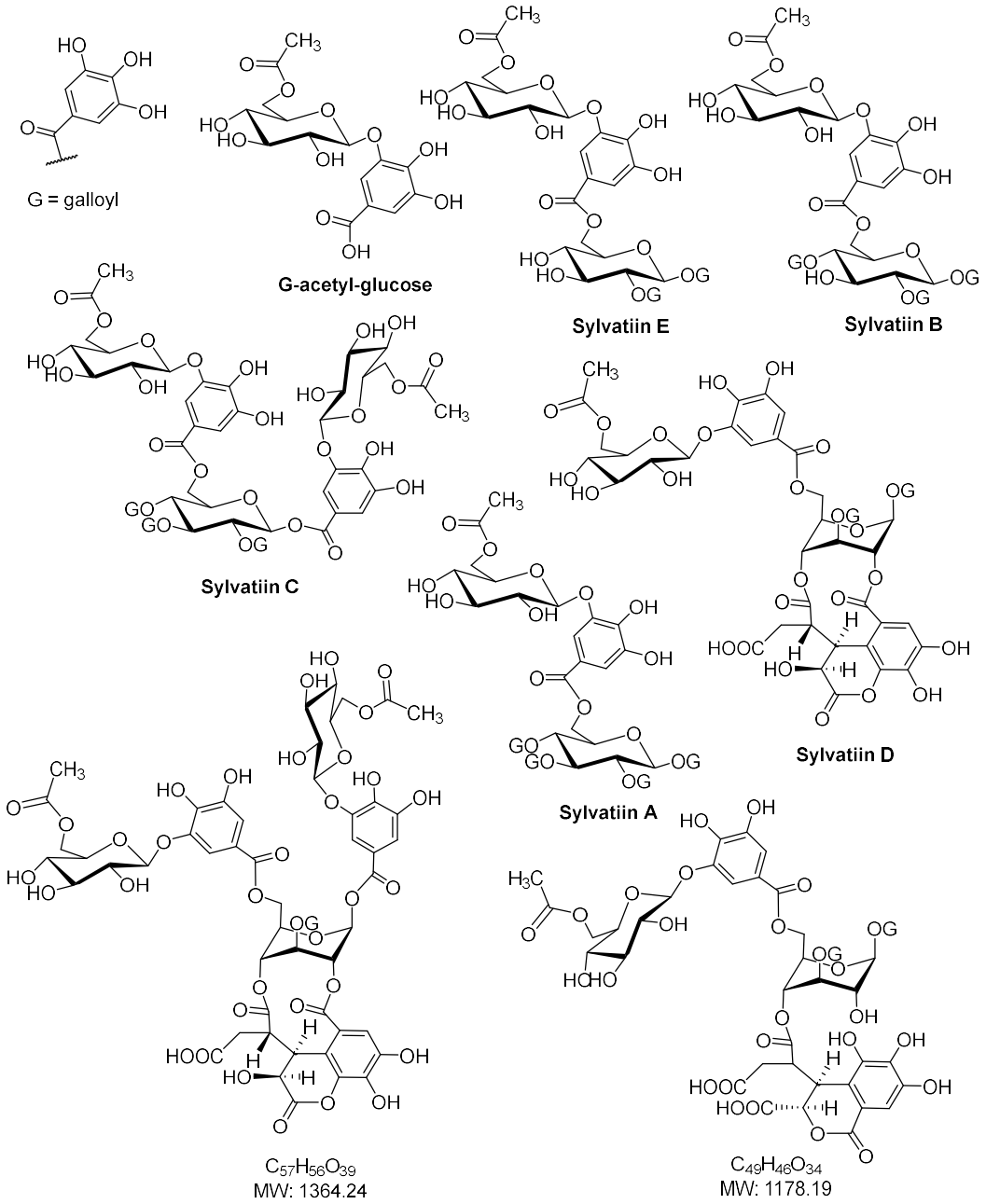


Figure 41. Structures of isolated sylvatiins A–E and the tentative structures of other compounds found in the petals of *G. sylvaticum* and belonging to the sylvatiin family.

A new assay was developed to measure the copigmentation capacity (**III**). The pH of *G. sylvaticum* petals was 4.8 (**III**), and in that pH the anthocyanin fraction and malvin chloride solutions tested were pale pink without the presence of copigments (**III**). Sylvatiin A and D, having one acetylglucose unit, were observed to act as strong copigments and changed the color of pigment:copigment solution to strong purple (**III**, Fig. 42).

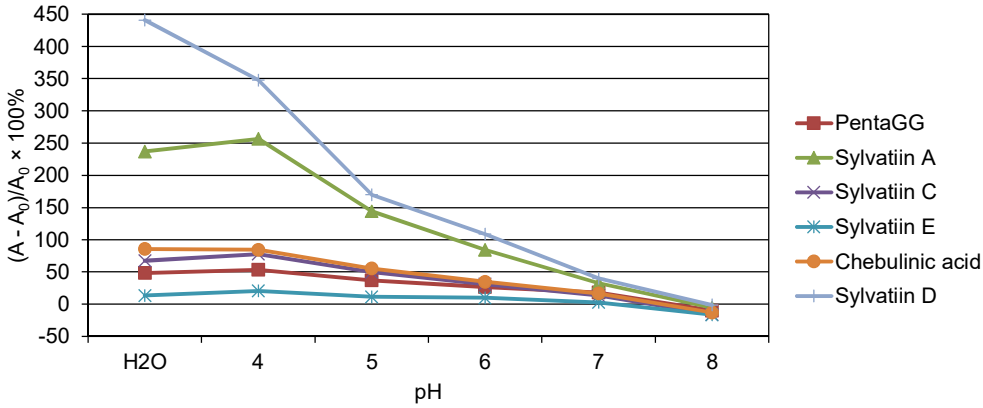


Figure 42. The effect of pH to the copigmentation capacity of sylvatiins and reference compounds measured with petal fraction at 550 nm.

It was suggested that the acetylglycosylated sylvatiins have an optimal structure for copigmentation for several reasons: 1) water is essential for the copigmentation process and the addition of acetylglucose moiety enhances the water-solubility of HTs compared with the core compounds, such as chebulinic acid and pentaGG, 2) at least two free galloyl groups are needed so that it is possible to form a pocket where anthocyanin can intercalate and enable the hydrophobic π-π stacking of aromatic nuclei between malvidin and galloyl groups (Fig. 43), 3) the hydrophilic layer of sugar moieties enables further stabilization expecially in the important 6-galloyl position (Fig. 43) and 4) acetyl moieties bring more hydrogen bonding sites between the hydroxy and carbonyl groups that further enhance the conformation stability. However, more studies are needed, for example, to investigate the role of chebuloyl group in the copigmentation effect. Is it an important factor for the water-solubility or does it also interact with anthocyanin?

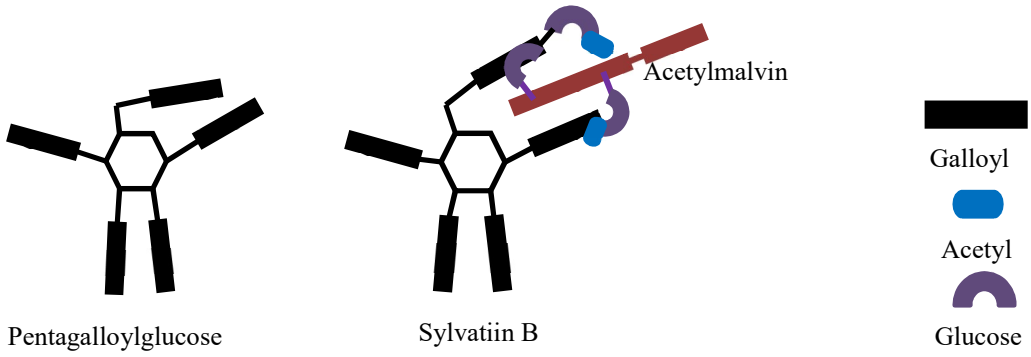


Figure 43. Suggested intermolecular copigmentation mechanism for the sylvatiins and malvidin-3-acetylglucoside-5-glucoside (as an anthocyanin anhydrobase form at petal pH).

5.4.4 Sepals contain highest amount of carpinusin

Sepals are the green leaf-like organs with a primary function of protecting the developing flower. Therefore, sepals are often rich in defensive compounds.[322] The protective role of sepals can be seen in the high amount of glandular trichomes on the outer surface of the sepals of *G. sylvaticum* (Fig. 44). These hairs form a physical barrier against herbivores and often contain defensive compounds.

Figure 44. Glandular trichomes in the outer surface of *G. sylvaticum* sepals.

The chemistry of sepals differed from the other green tissues of *G. sylvaticum*, in that they contained a distinctive amount of carpinusin—even more than geraniin (**I**, **VI**, Fig. 45). The only difference between these ETs is the different location of the free galloyl and HHDP groups in the sugar core. Although these ETs have only a small structural difference (**I**), the fractions that contained carpinusin showed higher pro-oxidant activities than fractions that contained geraniin (**II**). This suggests that carpinusin may be more harmful to herbivores than geraniin. However, studies with purified geraniin and carpinusin have not showed that carpinusin has higher antioxidant, pro-oxidant or anthelmintic activity than geraniin.[223, 323]

The TP content in the sepals of *G. sylvaticum* was 106 mg/g DW (**II**) and the main compounds identified are listed in Table 16. The total PA content in sepals was 30 mg/g (**II**) and only traces of PDs and mixed PC/PD oligomers were detected up to DP of 14 (**V**). The amount of monogalloyl quinic acids and chlorogenic acids were equal to that of leaves. The average amount of carpinusin was 146 mg/g DW and its content was slightly higher in the bud phase; however, the difference was not statistically significant (**VI**). The average amount of geraniin was lower than the carpinusin content—only 63 mg/g DW. However, the sepals contained high amounts of other ETs as well, such as diG-HHDP-glucose (34 mg/g DW) and geraniinic acid (10 mg/g DW) (**VI**). No significant ontogenic variation was observed in the content of ETs. This might be due to the sampling, which did not cover

the true ontogenic phases of sepals as some of the buds might have evolved already before May.

In contrast, population-specific differences were detected in the carpinusin content. The highest amount of carpinusin was found in the Katariina (163 mg/g DW) and Oriketo 1 populations. The carpinusin:geraniin ratio was 2.5:1, whereas in the Oriketo 2 population the amount was lower (ca. 109 mg/g DW) and the carpinusin:geraniin ratio was different, nearly 1:1. Similar differences between Turku populations were observed in the carpinusin contents of other plant organs (**VI**). Differences in the populations can partly explain why carpinusin was not observed in the leaves and stems in papers **I** and **II**, as those samples contained plants also from the Tampere, Orivesi and Lapland populations.

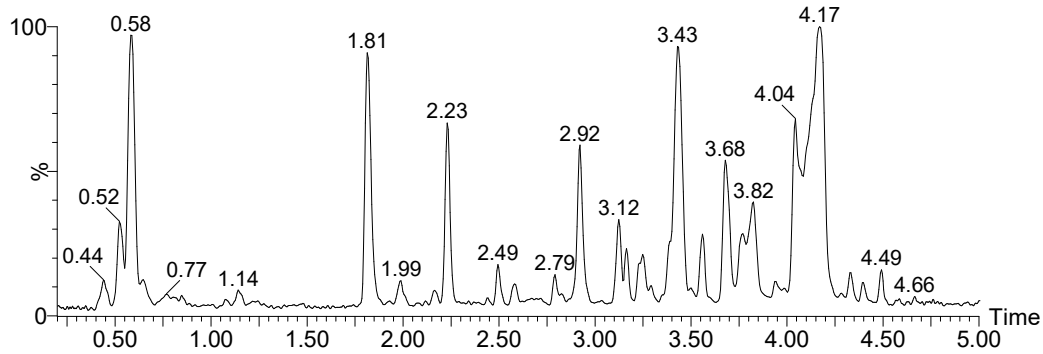


Figure 45. UPLC MS base peak chromatogram of the sepal extract of *G. sylvaticum*.

Table 16. Main phenolic compounds identified in the *G. sylvaticum* sepal extracts

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.23	4.3	MonoGG	219, 278	332	331, 633, 295
1.81; 1.92	5.7; 7.7	MonoG quinic acid	217, 275	344	343, 687, 191, 1031
2.23	9.5	MonoG shikimic acid	216, 276	326	325, 651, 301
2.49	10.9	Neochlorogenic acid A	220+, +325	354	353, 707, 483
2.58	12.2	G-HHDP-glucose	217, 269	634	633, 345, 446, 1267
2.79	13.8; 14.8	DiG quinic acid	217, 275	496	495, 343, 633, 969
2.92	14.5	Chlorogenic acid	219, 240, +326	354	353, 707, 191, 451, 1061
3.12	16.5	G-HHDP-glucose	219, 264	634	633, 316, 484
3.16	17.4	DiG-HHDP-glucose	217, 277	786	785, 392
3.25	18.6	Geraniinic acid	219, 277	952	951, 453, 907, 475
3.43	19.5	Geraniin	221, 276	952	951, 933, 466, 1904
3.56	21.3	Ascorgeraniin	222, 281	1110	1109, 554, 467, 933
3.58	21.8; 22.1	Quercetin diglycoside	206, 264, 350	626	625, 1251
3.68	22.5	Geraniinic acid	225, 281	952	951, 453, 907
3.11, 3.72	20.5	Repandusidic acid	222, 280	970	969, 633, 247
3.74	22.9	DiG-HHDP-DHHDP-glucose	220, 282	1104	1103, 466, 542, 951
3.83	23.9	Tellimagrandin II	217, 279	938	937, 468, 301
3.94	24.6	Chebulagic acid	218, 282	954	953, 476, 301
4.03	25.4; 25.9	Quercetin 3-glycoside	217, 268, 352	464	463, 927, 817, 1415
4.04, 4.17	26.7	Carpinusin	222, 278	952	951, 933, 466, 475, 1903
4.14	26.4; 26.8	Quercetin G-glycoside	215, 271, 358	616	615 ,1231
	27.7	TriGG-DHHDP-glucose	220, 279	954	953, 467, 935, 301
4.39	28.2; 28.7	Kaempferol 3-glycoside	210, 264, 350	448	447, 895
4.36	28.6	PentaGG	217, 281	940	939, 469
4.49; 4.70	29.8	Kaempferol G-glucoside	209, 269, 350	600	599, 285, 1199

5.4.5 Stems contain low amounts of ETs and PAs

Stems carry the branches, foliage and flowers. The nutritive value of stems is often less than leaves; and stems are also less likely to be attacked by herbivores. The stems and leaves of *G. sylvaticum* were qualitatively similar in their phenolic profiles (**I**, **II**, Figs. 46 and 47), but stems contained significantly less TPs, geraniin and FLAs compared with the leaves.

The TP content in the stems was 57 mg/g DW (**II**), and the main compounds identified are presented in Table 17. The average amount of geraniin in the stems was 26 mg/g DW. There were no qualitative differences between leaf stalks and flowering shoots (**VI**). The seasonal trend in the phenolic contents of the leaf stalks was similar to that of the leaves; the lowest amount of geraniin (16 mg/g DW) was detected in the leaf stalks in June during the flowering period, when the biosynthetic resources were allocated to the flower parts. The amount of geraniin in the leaf stalks increased significantly toward the fall to the maximum of 35 mg/g DW. The geraniin content in the flower shoots remained more constant throughout the growing season. Some leaves of *G. sylvaticum* are green the whole year, and this increase in geraniin content might be preparation for the wintertime and against possible plant diseases. Other major ETs in the stems were corilagin (15 mg/g DW) and carpinusin (21 mg/g DW). These amounts were relatively high in comparison with the amount of geraniin in the other plant organs of *G. sylvaticum* (**VI**).

The total PA content in the stems was rather high, at 58 mg/g DW (**II**). The amount of PAs in the stems was actually almost the same as that measured for the seeds. The HILIC study was especially useful for the stems fractions, as the high content of HTs masked the presence of PAs in the UV and MS chromatograms. There were more PDs and mixed PC/PD oligomers than PCs in the stems (**V**). Oligomeric PAs up to DP of 18 were detected, and mDP was 5, as in the other parts (**V**). The maximum content of PCs was detected in July. Also, the amount of prodelphinidins was higher in July, but it increased towards the end of the season (**VI**). Due to a low amount of phenolics, the stem fractions were rather inactive in the bioactivity tests (**II**).

The amount of total flavonoids was about 5 mg/g DW in the stems and remained constant throughout the growing season. There was no significant difference in the flavonoid content between the leaf stalks and flower shoots. The amount of galloylated quinic acids was approximately 4 mg/g DW, and the seasonal trend was similar to the trend observed for ETs. The detailed seasonal study conducted in paper **VI** revealed the presence of methylated mono- and digalloyl glucoses that were not observed in papers **I** and **II**. The amounts of these increased slightly toward the fall and were the highest in the Oriketo 1 population, whereas the Katariina population was clearly different and contained significantly less amounts of methylated GGs.

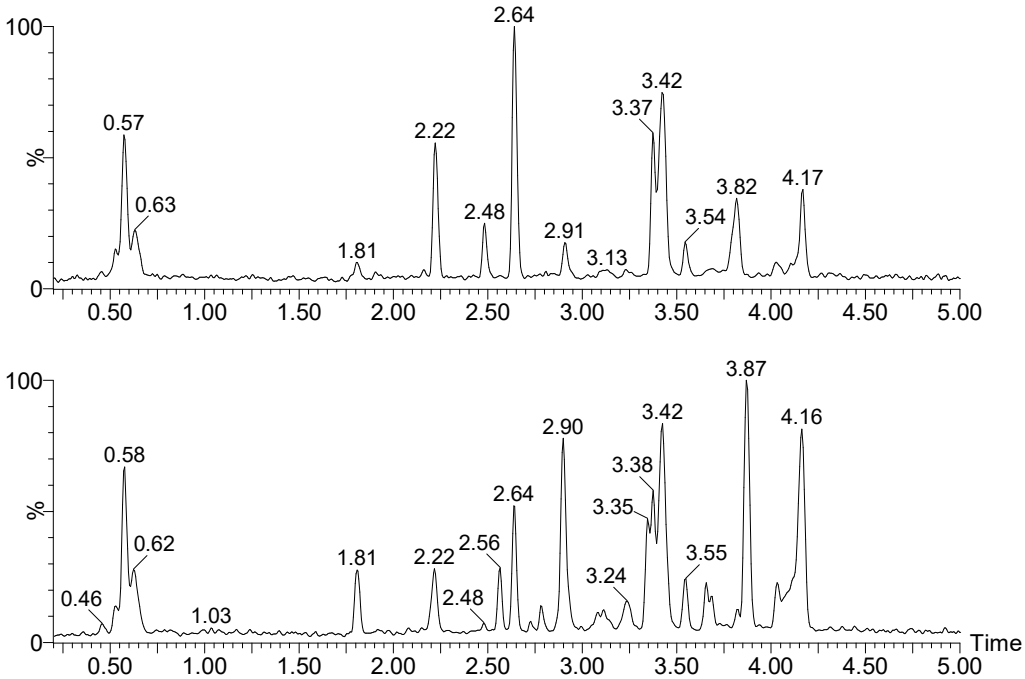


Figure 46. UPLC MS base peak chromatograms of the flower shoot extract of *G. sylvaticum* in June (above) and the leaf stalk extract in August (below).

Table 17. Main phenolic compounds identified in the *G. sylvaticum* stem extracts.

RT $_{UPLC\ TIC}$ (min)	RT $_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.81, 1.92	5.6, 7.6	MonoGG quinic acid	217, 275	344	343, 687, 1031
2.16, 2.22	8.9, 9.5	MonoG shikimic acid	218, 276	326	325, 651
2.48	11.0	Neochlorogenic acid	219+, +325	354	353, 707
2.91	14.6	Chlorogenic acid	203+,+326	354	353, 707, 191
2.64		Methylated MonoGG	218, 266	346	345, 183
2.90	16.1	PC dimer	201, 279	578	577, 289
3.15	16.4	Brevifolin carboxylic acid	278, 354	292	291, 247
2.57	16.7	Corilagin	219, 273	634	633, 316
3.24	18.6	Geraniinic acid	219, 278	952	951, 907, 453, 475
3.37		Methylated DiGG	220, 270	498	497, 345
3.42	19.5	Geraniin	220, 275	952	951, 466, 1904
3.11, 3.72	20.6	Repandusidic acid	219, 274	970	969, 633, 484, 247
3.54	21.3	Ascorgeraniin	222, 281	1110	1109, 554, 467, 933
3.58	21.8	Quercetin diglycoside	213, 268, 352	626	625, 301, 1251
3.74	22.9	DiG-HHDP-DHHDP-glucose	221, 284	1104	1103, 542
3.66		Geraniinic acid	224, 276	952	951, 907, 453
4.04	24.2	Ellagic acid	255, 366	302	301
3.94	24.4	Chebulagic acid	222, 282	954	953, 476, 300
3.87, 4.03	25.2, 25.8	Quercetin 3-glycoside	204, 258, 354	464	463, 927, 1391
3.82		Geraniin II	227, 275	952	951, 933, 475, 466, 301
4.03, 4.09	26.3	TetraGG	219, 282	788	787, 393, 617
4.14	26.5, 26.8	Quercetin G-glycoside	207, 262, 358	616	615, 301, 1231
4.17	26.6	Carpinusin	222, 278	952	951, 933, 475, 466
4.39	28.5	Kaempferol glycoside	210, 255, 350	448	447, 895
4.48	29.8	Kaempferol G-glycoside	216, 271, 350	600	599
4.36	29.2	PentaGG	217, 281	940	939, 469

5.4.6 Leaves contain highest amount of geraniin

The main role of leaves is in photosynthesis and as storage for carbohydrates and water. Due to the high amount of resources allocated to leaf production, leaves represent a valuable organ for plant fitness. Consequently, various strategies have evolved, such as the production of tannins, to protect the leaves from herbivores, pathogens and/or unfavorable abiotic environmental conditions.

The TP content in the leaves of *G. sylvaticum* was 232 mg/g DW (**II**). The content of geraniin in the leaves was the highest among the *G. sylvaticum* organs (**I**, **II**). The leaves of flowering shoots had slightly higher content levels of geraniin (178 mg/g) than the basal leaves (168 mg/g), and the content remained constant throughout the growing season (**VI**). The content of geraniin was unusually high for a secondary metabolite, which indicates that geraniin may play a special role in the *G. sylvaticum* lifecycle. Geraniin-containing extracts and fractions were active in the pro-oxidant and antioxidant tests (**II**). However, studies with purified compounds have shown that geraniin is not very pro-oxidatively or antioxidatively active among other plant ellagitannins.[223]

The main compounds identified in the leaves of *G. sylvaticum* are listed in Table 18. The MRM studies revealed that leaves contained carpinusin circa 50 mg/g DW. The leaves also contained smaller amounts of other ETs, such as chebulagic acid, corilagin, ascorgeraniin and diG-HHDP-glucose. These ET concentrations increased toward the fall (**VI**). The seasonal trend of galloylglucoses can be clearly seen in Fig. 47. The amounts of galloylglucoses, which are precursors of ellagitannins, were highest in the spring in the small-sized leaves, and decreased near to zero toward the fall. The seasonal difference was highest in the monoGG content, which decreased from 27 mg/g in May to less than 4 mg/g in September. The same trend was observed for the contents of tetraGG, triGG and diGG, and in the other plant organs of *G. sylvaticum* as well (**VI**).

Leaves contained more galloylated FLAs than the other organs. For example, quercetin galloylglycoside content was circa 40 mg/g DW, and the content was slightly higher at the beginning of the season, in larger leaves and in the sunnier Oriketo 3 population (**VI**). These galloylated flavonoids have been found in other *Geraniums* as well. It was typical for the flavonoids in the *G. sylvaticum* that both glucosides and galactosides were found. The leaves contained only low amount of PAs (TPA circa 16 mg/g); only traces of PCs and PDs up to the DP of 14 mers were detected (**V**).

All the green photosynthetic parts also contained galloyl shikimic acids (20 mg/g in the spring) and chlorogenic acids (14 mg/g during flowering). Shikimic acid is a precursor of quinic acids, and its content was highest in the small leaves in spring, as was observed for small GGs. Fecka and Cisowski (2005) have suggested that shikimic acids can be used as a chemosystematic marker to distinguish between *Erodiums* and *Geraniums*.[106] However, the presence of shikimic acids in *G. sylvaticum* was now confirmed.

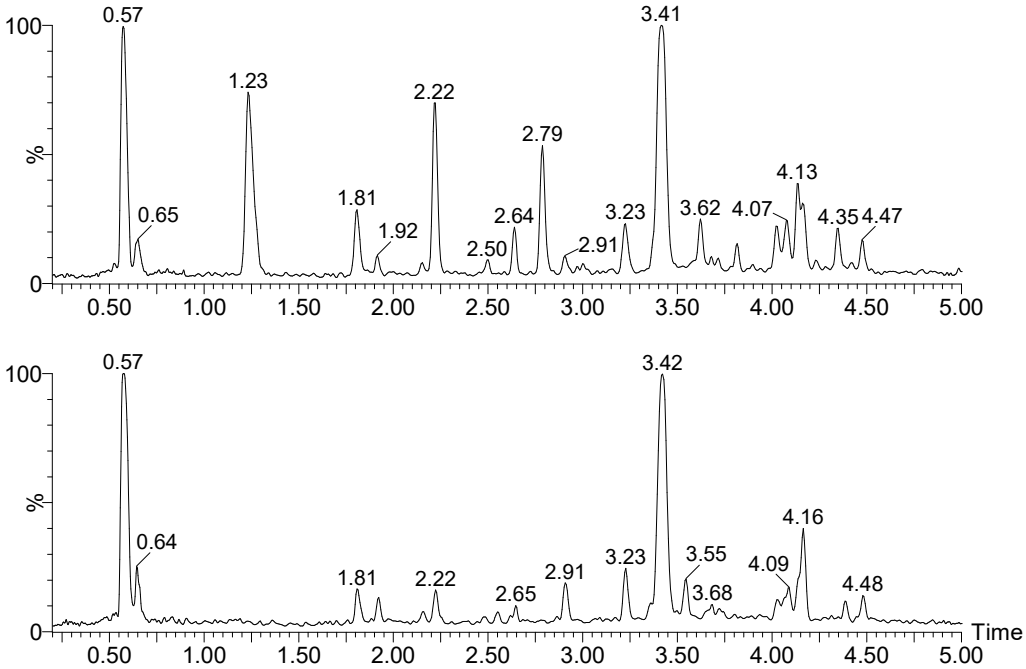


Figure 47. UPLC MS base peak chromatograms of the basal leaf extract of *G. sylvaticum* from the Oriketo 2 population in May (above) and in August (below).

Table 18. Main phenolic compounds identified in the *G. sylvaticum* leaf extracts.

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
0.57	1.2	Disaccharide		342	341, 191, 173, 683, 1025
1.23	4.5	MonoGG	217, 280	332	331, 663
1.81, 1.92	5.9, 8.1	MonoG quinic acid	217, 275	344	343, 687, 191, 1031
2.22	9.3, 9.8	MonoG shikimic acid	218, 275	326	325, 651, 169
2.50	11.2, 14.8	Chlorogenic acid	219, +325	354	353, 707, 191, 1061
2.79	12.8, 16.4	DiGG	216, 276	484	483, 967
2.91	15.4	DiG quinic acid	218, 276	496	495, 343, 991
3.23	18.6	Geraniinic acid	219, 276	952	453, 951, 475, 633, 907
3.41	19.4	Geraniin	222, 277	952	951, 466, 1904, 933
3.11, 3.72	19.9, 20.6	Repandusidic acid	219, 276	970	462, 969, 633, 440, 484, 247
3.55	21.3	Ascorgeraniin	222, 282	1110	467, 554, 1109, 495
3.58	21.8, 22.1	Quercetin diglycoside	207, 259, 353	626	625, 1251
3.62	22.6	TriGG	219, 280	636	635, 317, 1271
3.83		TriGG-HHDP-glucose	217, 279	938	937, 468
3.74	22.8	DiG-HHDP-DHHDP-glucose	221, 281	1104	1103, 466, 542, 1085
4.04	24.2	Ellagic acid	255, 224, 366	302	301
3.94	24.5	Chebulagic acid	222, 273	954	953, 476
4.08	25.1, 25.8	TetraGG	219, 277	788	787, 393
4.02	25.4	Quercetin 3-glycoside	209, 260, 354	464	463, 927
3.95	26.3	Euphorbin dimer	219, 280	1890	944, 1889
4.08, 4.13	26.5, 26.6	Quercetin-G-glycoside	211, 269, 359	616	615, 1231, 301
4.16	26.6	Carpinusin	222, 278	952	951, 466, 933
4.42	27.7	TriG-DHHDP-glucose	220, 279	954	467, 953, 935, 1907
4.39		Kaempferol 3-glycoside	197, 267, 348	448	447
4.35	28.8	PentaGG	217, 281	940	469, 939
4.47	29.3, 30.0	Kaempferol-G-glycoside	216, 271, 350	600	599, 313, 285

5.4.7 Roots accumulated both various PAs and ETs

The roots of *G. sylvaticum* contained high amounts of polyphenols (TP 179 mg/g in the main roots and 225 mg/g in the hairy roots), and the high content of both tannin types made the root fractions highly active in the antioxidant test (**II**). The main roots contained PAs 14% of the DW, whereas the hairy roots contained three times more geraniin than the main roots (**II**). The average amount of geraniin in roots was 32 mg/g DW. The content did not show statistically significant seasonal or between-population variation (**VI**). Also, the amount of other ETs was relatively high compared with the geraniin content: diG-HHDP-glucose content was circa 19 mg/g DW, carpinusin content was circa 23 mg/g, tellimagrandin II content was 12 mg/g DW and corilagin content was 16 mg/g DW (**VI**).

The hearts of the main roots contained more PAs than the bark or hairy roots, whereas the hairy roots contained more total phenolics, and especially geraniin, than the main root part (Table 19). This may imply that the softer hairy roots are more vulnerable to herbivores than the hard main root heart. Roots accumulated a different isomer of galloyl quinic acids, 4-galloyl quinic acid (circa 17 mg/g DW, RT 1.92 min) than the other organs of *G. sylvaticum* (Fig. 48). The content of very water-soluble galloyl quinic acids was higher in the *G. sylvaticum* hairy roots than in the other organs, and hairy roots accumulated three times more galloyl quinic acids than the main roots (**II**). Galloyl quinic acids exhibited high pro-oxidant and antioxidant values (**II**) and thus might be important for the defense of roots, for example, as water leachable exudates.

The roots were especially rich in PAs, which causes the characteristic hump on the UV chromatograms (Fig. 48). Roots contained PCs, PDs, mixed PC/PD oligomers and polymers (**I**, **V**). The studies with HILIC column revealed that the hairy roots contained longer polymers than main roots, up to the DP of 20, and a significantly wider variety of PDs and mixed oligomers that were detected in the analysis with the RP column (**V**). In the main roots, the highest polymers detected were with the PD of 13 (**V**). In the seasonal variation study (**VI**), the mDP was estimated to be 6; it did not change during the growing season.

Table 19. Distribution of PAs and total phenolics in the rhizome of *G. sylvaticum* (unpublished results)

	Total PA (mg/g DW)	Total phenolics (mg/g DW)
Main root bark	114	113
Main root heart	186	166
Hairy roots	125	189
Root hairs	88	141

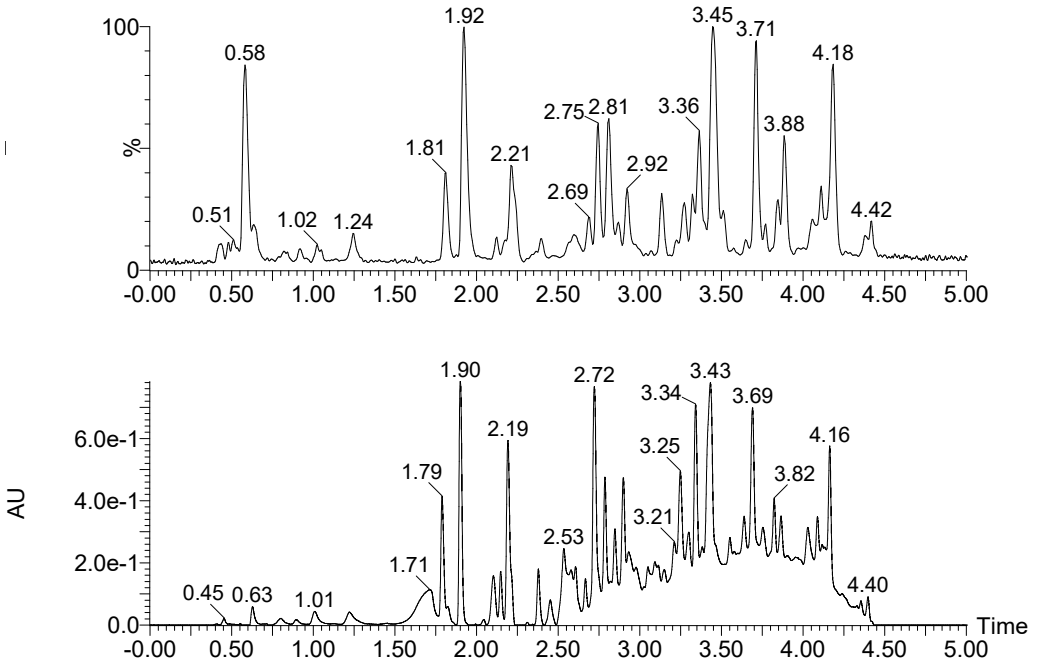


Figure 48. UPLC MS base peak chromatogram (above) and UV chromatogram with wavelength 205 nm (below) of *G. sylvaticum* root extract. Identification of compounds is presented in Table 20.

Table 20. Main phenolic compounds identified in the *G. sylvaticum* root extracts

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.02	2.6	MonoG quinic acid	217, 275	344	343, 687, 191
1.24	4.4	MonoGG	218, 279	332	331
	3.9	PD trimer	216, 272	914	913, 456, 607
1.81	5.7	MonoG quinic acid	216, 275	344	343, 687, 191
1.92	7.7	4-G quinic acid	217, 275	344	343, 687, 1031, 169, 191
1.81, 2.12, 2.17, 2.59	4.7, 6.4, 8.7, 10.8	PD dimer	209, 272	610	609, 441
2.21	8.7	Gallocatechin	207, 272	306	305, 261, 611
2.81	11.0, 13.8, 14.8	DiG quinic acid	219, 275	496	495, 991, 343
2.74	13.7	Epigallocatechin	198, 218, 272	306	305, 611, 917, 1223, 261
2.22, 2.39, 2.63, 2.86	10.6, 14.7	PC/PD dimer	210, 275	594	593, 425
2.92	14.5	Catechin	204, 281	290	289, 245, 579, 869
2.79	15.3	DiG glucose	217, 278	484	483, 967
3.15	16.3	Brevifolin carboxylic acid	278, 218, 355	292	291, 247
3.13	16.7, 18.5	G-HHDP-glucose	218, 271	634	633, 316, 1266
2.80, 2.95, 3.26	13.9, 14.3, 17.0, 18.2	PC dimer	201, 281	578	577, 425, 1155
3.36	18.9	Epicatechin	199, 212, 280	290	289, 245, 579, 869, 1159
3.32	19.4	TriG quinic acid	217, 276	648	647, 1295
3.45	19.5	Geraniin	221, 278	952	951, 933, 466, 1903, 301
3.65	20.1	TriG glucose	218, 278	636	635, 465, 317
	21.3	PC trimer	203, 280	866	865, 432, 1731
3.71	21.9	DiG-HHDP-glucopyranose	218, 277	786	785, 392, 615, 301, 316, 1571
3.85	23.9, 24.6	TriGG-HHDP-glucose	216, 285	938	937, 468, 301
4.02	24.2	Ellagic acid	255, 366	302	301
4.11	25.6, 26.3	TetraG glucose	218, 280	788	787, 393, 617, 317
4.18	26.6	Carpinusin	222, 279	952	951, 933, 466, 475
4.38	28.7	PentaG glucose	217, 281	940	469, 939

5.4.8 Seed coat contains PCs

Seeds are essential in the reproduction of flowering plants. The seeds of *G. sylvaticum* suffer from specialized seed predators that consume the developing seeds directly from the ovaries.[42] In spite of this, the seeds of *G. sylvaticum* contained a low amount of total phenolics (18 mg/g DW) and a particularly low content of geraniin as compared with the other organs (**II**, **V**, Table 21).

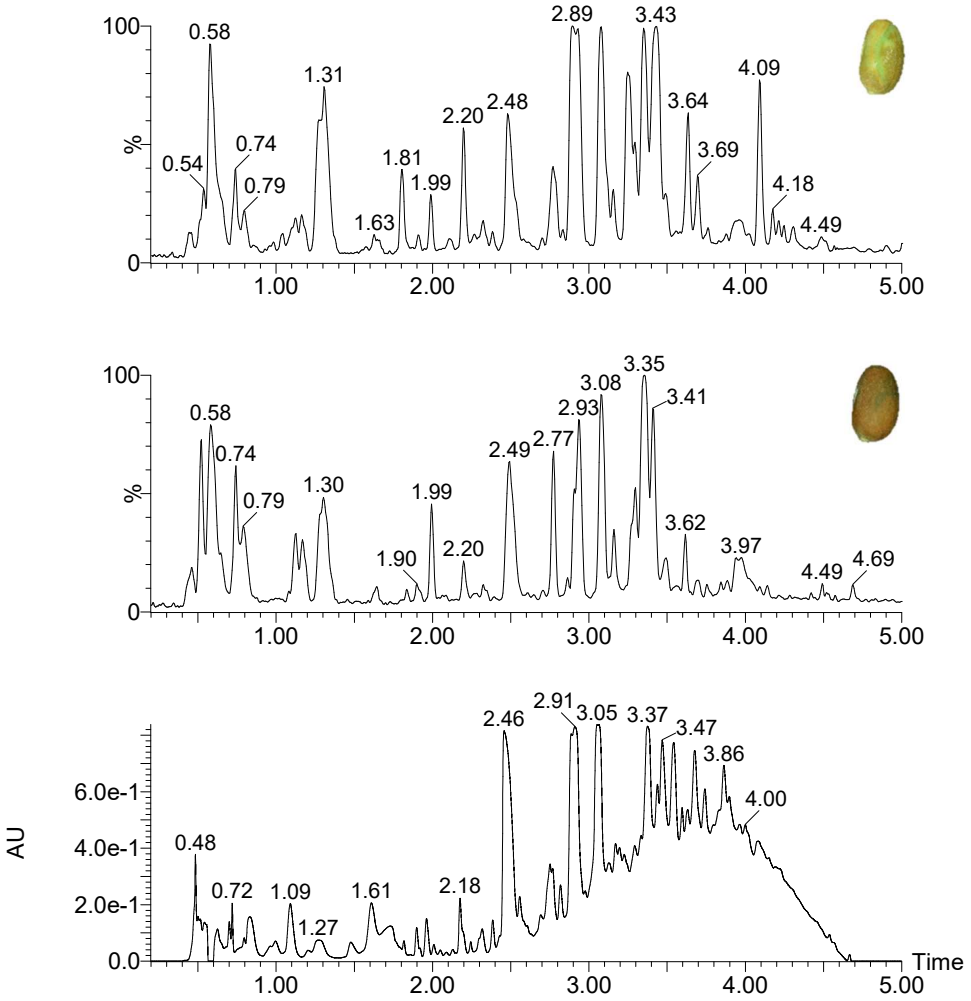


Figure 49. UPLC MS base peak chromatograms of immature green (above), mature brown and UV chromatogram with wavelength 205 nm (below) of *G. sylvaticum* seed extracts. Identification of main compounds is presented in Table 22.

The total PA content in the seeds of *G. sylvaticum* was 70 mg/g DW (**II**). There was only 1 mg/g DW of TP and TPAs located at the heart of the seed, whereas in the seed coat the corresponding contents were 68 mg/g DW and 118 mg/g DW, respectively (unpublished results). The inside part of the seed mainly contained the amino acid tryptophan. In contrast

to other PA-rich organs, seeds mainly contained PCs and only smaller amounts of mixed PC/PDs (**I**, **V**). PC polymers up to the DP of 16 and mixed polymers up to the DP of 20 were detected (**V**).

Although the PAs dominated the phenolic profile of seeds, it already contained some of the HTs that were found in the mature plants. The content of geraniin was ten times higher in the immature seeds than in the brown matured seeds (Table 21, Fig. 49, unpublished results). Immature yellow seeds contained also more galloylglucoses and catechin than mature brown seeds, but the content of oligomeric PAs remained constant (Table 21). PAs in the seed coat may have a protective function against microbial degradation in soil.

Table 21. Ontogenic variation of selected compounds in the seeds of *G. sylvaticum* (unpublished data)

	Yellowish immature	Greenish brown	Brown mature
Tryptophan (mg/g DW)	0.33 ± 0.26	0.72 ± 0.01	0.57 ± 0.16
Geraniin (mg/g DW)	5.64 ± 4.17	2.59 ± 0.76	0.61 ± 0.16
TriGG (mg/g DW)	4.92 ± 4.0	1.75 ± 0.45	1.50 ± 0.89
TetraGG (mg/g DW)	2.95 ± 2.09	1.13 ± 0.18	0.74 ± 0.48
Catechin (mg/g DW)	0.58 ± 0.14	0.52 ± 0.26	0.12 ± 0.05
PC dimer (mg/g DW)	0.82 ±0.27	0.76 ± 0.27	0.49 ± 0.20
PC trimer (μg/g DW)	5.71 ± 1.26	6.32 ± 2.29	4.40 ± 1.61
PC tetramer (μg/g DW)	15.06 ± 1.14	18.12 ± 2.60	15.89 ± 8.14

Table 22. Main phenolic compounds identified in the *G. sylvaticum* seed extracts

$RT_{UPLC\ TIC}$ (min)	$RT_{HPLC\ UV}$ (min)	Compound	UV λ_{max} (nm)	MW	Characteristic *m/z* values
1.31	4.3	Unknown	194	612	611, 303
1.81	5.8	MonoG quinic acid	217, 275	344	343, 191
2.03	7.6	PC dimer, A-type	202, 265, 290	576	575, 879, 423
2.18	8.8	PA dimer mixed	216, 277	594	593, 329, 425
2.21	9.6	PC trimer	201, 280	866	865, 569, 329
	10.2	PC trimer, A-type	204, 280	864	863, 469, 593
2.48	11.0	Tryptophan	198, 220, 280	204	203, 407
2.77, 2.92		PC/PD mixed trimer	204, 280	882	881, 577, 443, 425, 289
2.76	11.6	PC tetramer, A-type	205, 287	1152	865, 1151, 575
2.91	14.8	catechin	204,209, 280	290	289, 865, 579, 719
2.93	13.4	PC dimer	202, 281	578	577, 425, 289
3.08	14.4	PC trimer	201, 280	866	865, 575, 425, 1731, 287
3.12	16.0	Brevifolin carboxylic acid	278, 354	292	291, 247
3.25	18.6	Geraniinic acid	219, 278	952	951, 907, 633, 453
3.34	18.2	Leucocyanidin	203.281	306	305, 225
3.40	17.1, 19.3	PC tetramer	202, 281	1154	1153, 576, 289, 863
3.43	19.6	Geraniin	223, 272	952	951, 933, 466
3.46, 3.50	17.1, 17.7	PC trimer	201, 281	866	865, 575, 1151, 425
3.49	18.2	PC dimer	202, 281	578	577, 425, 1155
3.56	18.6	PC pentamer	202, 281	1442	720, 577, 1441
3.64	20.1	TriGG	218, 278	636	635, 317
3.69	20.2	PC tetramer	202, 282	1154	1153, 576, 865
3.86	21.7	PC pentamer	202, 281	1442	1441, 720, 577
3.96	24.3	Ellagic acid	255, 365	302	301
4.09	26.0	TetraGG	217, 280	788	787, 393, 617, 169
4.18	26.6	Carpinusin	222, 278	952	951, 933, 301

5.5 Biological activity

The aim of the biological activity studies was to measure how the quantity of polyphenols affects the activity of the organ extracts of *G. sylvaticum* and to measure which compounds and polyphenol classes are the most active. Compounds in 138 Sephadex LH-20 fractions were characterized and quantified for individual phenolics using HPLC-DAD/MS, for total PAs by BuOH-HCl assay, and for total phenolics by the Folin-Ciocalteu method. Two different physicochemical properties of biological relevance were measured: antioxidant activity with the DPPH method and pro-oxidant activity with the browning assay under highly alkaline conditions. The exposure of phenolic extracts and compounds at pH 10 has been used as an *in vitro* model for insect gut conditions to study which compounds are potentially the most harmful pro-oxidants in the interactions between plants and herbivorous insects.[189, 210, 222] In this work, the *in vitro* metabolic products of the phenolic compounds of the most active fractions were also analyzed using HPLC-DAD; a separate study was conducted with purified HTs.

5.5.1 Pro-oxidant activity at alkaline pH

Pro-oxidant assay measures the maximum rate of production of quinone-type oxidation products of polyphenols at the pH 10 using a well plate reader and detection at 415 nm.[189, 210, 222–223] In this work, the multivariate models were used to study which polyphenol classes contribute the most to the PO activity of the *G. sylvaticum* organ extracts (**II**). The PRO-OX_{max} values observed for *G. sylvaticum* organ fractions correlated best with the content of flavonoids and galloyl quinic acids; however, the explanatory power of the model was low (**II**).

Therefore, two additional pro-oxidant activity values were used in the data analysis. The color of some fractions changed immediately to yellow after the addition of a pH 10 buffer. This change was calculated with ΔA value. The highest ΔA values were observed for fractions that contained high amounts of kaempferol and quercetin glycosides or other flavonoids and galloyl quinic acids (**II**). In addition, it seemed that the browning of some fractions continued after the 100-s time period that PRO-OX_{max} value covers. The PRO-OX_{ave} value, which follows the 8-min time period, measured better the more slowly forming browning products compared with the PRO-OX_{max} and the ΔA values. The content of galloyl quinic acids and geraniin and total ETs correlated best with PRO-OX_{ave} values (**II**). The difference between the PRO-OX models, the shapes and the slopes of the browning reactions (**II**), suggested that several reactions might occur for polyphenols under alkaline conditions. It was concluded that the spectrophotometric method can be used to estimate the pro-oxidant activity of fractions; however, it is inadequate in clearly showing differences in the reactions of different polyphenol classes.

Therefore, the pro-oxidant method was improved by adding two other steps to the protocol. In order to more closely examine the reactions that happen in the alkaline conditions, the products of the most pro-oxidant active fractions after the incubation at pH 10 were analyzed using HPLC (**II**). Secondly, the reversibility of color change was tested by changing the pH to acidic. These results showed how the *in vitro* reactions at pH 10 (**II**) varied for different phenolic compound groups.

All fractions containing small phenolic acids, such as galloyl quinic acids, galloyl shikimic acids and small GGs, degraded quickly mainly through hydrolysis; the main product was ellagic acid (**II**). These types of fractions yielded both high PRO-OX_{max} and high PRO-OX_{ave} rates in a way similar to gallic acid. Exceptions were fractions that contained chlorogenic acid; instead of hydrolysis, chlorogenic acid isomerized (**II**).

Fractions containing HTs reacted at an intermediate rate and caused browning slopes that were slowly ascending (**II**). The color change of ET and GG containing fractions was not reversible, and the HPLC analysis showed that compounds were degraded (**II**). The main HPLC-detectable products after the one-hour incubation were the hydrolysis products, i.e., smaller GGs, ellagic acid and brevifolin carboxylic acid.

The oxidation reaction of polymeric PAs followed a similar slowly ascending slope to that of GGs, and the browning of PA fractions was irreversible. All pro-oxidant activity rates were small for fractions containing PAs. Some new peaks of degradation or oxidation products appeared. They could be A-type PAs or catechinic acid, which have been produced from B-type PAs and catechin in the alkaline media (**II**, Ferreira et al., 1992).

Flavonoid fractions were highly reactive in the PO assay, as the color sample changed to yellow immediately after the addition of the buffer (**II**). Flavonoid-rich fractions yielded high ΔA values, and this oxidation reaction was too fast for the PRO-OX_{max} calculation. The color change was reversible, which was confirmed using formic acid addition and HPLC. Therefore, it was concluded that the strong yellow color was due to deprotonation that causes a bathochromic shift to the UV spectra of flavonoid at pH 10. This is typical for flavonoids.[203] The oxidation of flavonoids did not continue forward from the deprotonation to the quinone stage or to further reactions, as the HPLC analysis showed that flavonoids were not degraded at pH 10 (**II**). It seems that the conjucated structure of flavonoid core protects dominant quercetin and kaempferol glycosides of *G. sylvaticum* from the degradation that happened to other polyphenol classes. The glycosylation of the 3-OH group observed in most of the flavonoids of *G. sylvaticum* may explain this (**I**) as the 3-OH group has a crucial role in the enzymatic oxidation process of flavonoids.[224] Quercetin and kaempferol glycosides have been rather resistant under alkaline conditions in the other *in vivo* and *in vitro* studies as well.[173, 233, 324–325]

The colorimetric assay for pro-oxidant activity has some limitations because of the inheritable characteristic of phenols to ionize in basic conditions that causes color change, which can mask the formation of quinones and other products. Although the predictive values of three different PRO-OX values varied (**II**), it is suggested that all of

them should be analyzed when evaluating the pro-oxidant activity of fractions, to ensure that very rapidly oxidized flavonoids and slower reacting tannins are both taken into account. When the average value of all these three pro-oxidant values were calculated for organ extracts, the pro-oxidant activity of *G. sylvaticum* organs decreased in the following order: leaves >> pistils > main roots > sepals > petals > hairy roots > stems > stamens >> seeds (**II**). The order of the organs shows clearly how the different mixtures of polyphenol classes are more effective than compounds from only one polyphenol class in the PO activity.

5.5.2 Degradation products and rate at a larger pH scale

In paper **II**, it was observed that ellagic acid was formed at alkaline conditions from various fractions. This observation about EA was verified with the study using 10 isolated compounds (**IV**). Ten structurally distinct hydrolysable tannins were chosen, including three GGs, four other galloyl derivatives with different polyols and three ETs.

The pH 10, used in the pro-oxidant measurement, was originally designed to reflect the pH in lepidopteran larvae. However, other insects that forage *G. sylvaticum* may have different gut conditions. For example, the specialized seed predator of *G. sylvaticum*, the oligophagous weevil *Zacladus geranii*, belongs to Coleopterans, which in general have more acidic or neutral gut conditions.[216, 326] Therefore, the pH scale was expanded to include the pH values 3, 8–11 and reduced oxygen conditions.

The sample treatment was designed to be as fast as possible. Sample solutions were incubated in a well plate and reactions were monitored by HPLC–DAD as such without pre-treatments. The acidic mobile phase neutralised the sample on-line. Reaction products were identified based on the UV spectra and mass spectral fragmentation obtained with the high-resolution HPLC–DAD–ESI/QTOF/MS. The clear advantage of the developed method was that the use of base-resistant HPLC column enabled injections without the sample pre-treatment, and thus the detection of short-lived products.

Hydrolysable tannins were unstable in the basic conditions, and half-lives were mostly less than 10 min at pH 10. Degradation rates were faster at pH 11 but slower at pH 8 and 9 (**IV**, Fig. 50A). Reduced oxygen levels slowed down the degradation reactions significantly, but did not change the end products (**IV**). HPLC analyses revealed that various products were formed, and these were identified as the result of hydrolysis, deprotonation and oxidation reactions. One of the key results was that the main hydrolysis product was ellagic acid; also for those galloyl derivatives that do not contain oxidatively coupled galloyl groups in their initial structures. It has been traditionally thought that galloyl glucoses yield gallic acid as hydrolysis products and ETs yield ellagic acid or other similar products, depending on the initial structure; this has been the basis for their detection and classification for a long time. This is no longer valid, because GGs also yielded EA in the basic conditions after hydrolysis. In fact, pentaGG and other GGs

yielded more EA than ETs, although the formation rate was slower (Fig. 50B; **IV**). Geraniin yielded less EA because it released also brevifolin as a hydrolysis product of the DHHDP group.

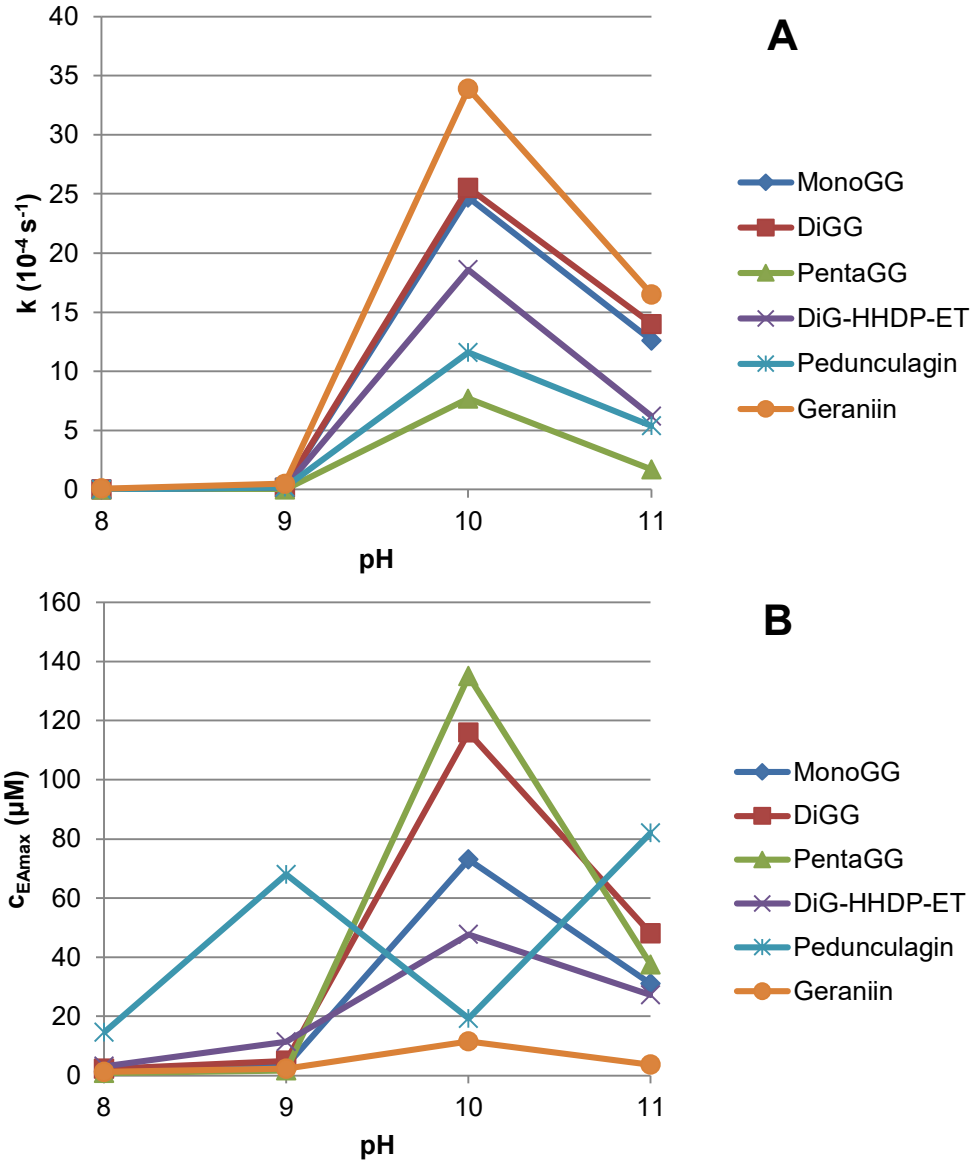


Figure 50. The rate constants for degradation (A) of hydrolysable tannins and maximum ellagic acid formation (B) in various pH conditions.

A clear anomaly in Fig. 50B is that pedunculagin produces less EA at pH 10 than at pH 11. It seems that buffer conditions favored the production of HHDP-glucose (**IV**). Also HHDP group in the geraniin was rather resistant to hydrolysis, as the amount of EA remained low (Fig. 50B; **IV**).

Studies with pure ETs showed that the HHDP and DHHDP groups first oxidize to quinones (**IV**, Fig. 51). The quinone group then oxidized further to transformed ET structures that contained two acid groups. The formation of the diacid group explained the addition of two oxygens to the molecular mass of the product that was detected for pedunculagin (**IV**). The diacid group can further transform to modified ET structures, such as chebuloyl groups in the alkaline conditions, as outlined in Fig. 51. These products exhibit a third UV maximum near 350 nm that yields a yellow color in the alkaline conditions and can contribute to the slowly ascending slopes observed in the PO assay (**II**, **IV,** Fig. 52). However, the oxidation products further degraded to hydrolysis products after two hours (**IV**). The maximum EA content was achieved after one hour, whereas the lifetime of observed possible reddish quinone products was less than 10 minutes (**IV**).

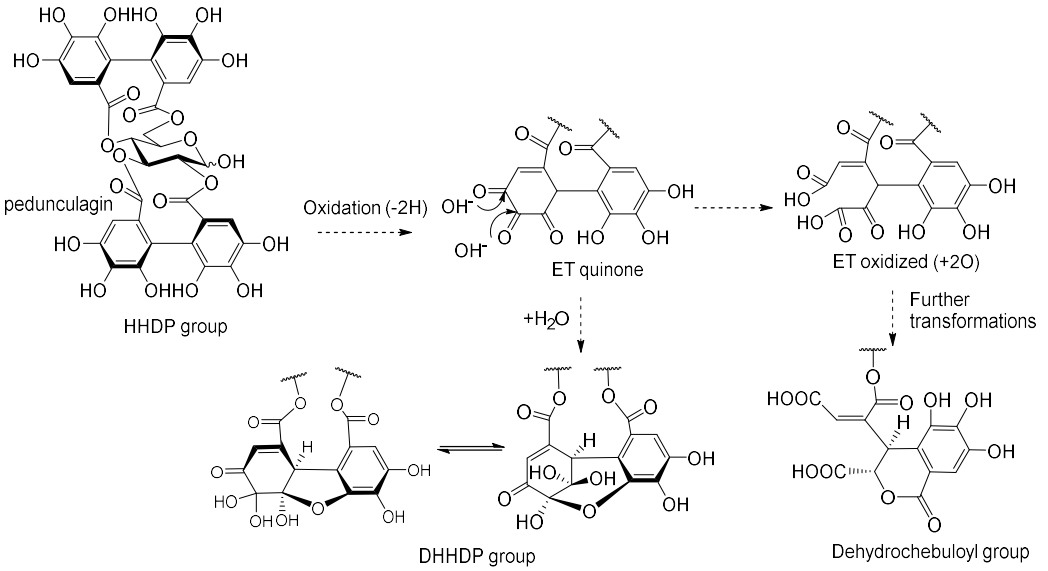


Figure 51. Proposed oxidation pathway for the HHDP group of ellagitannins.

The HPLC analyses of fractions after incubation at pH 10 revealed that the browning observed in the spectrophotometer in the PO assay is a sum of several products produced by hydrolysis, oxidation and deprotonation, not just quinones (Fig. 52). All these products may be meaningful in the plant-herbivore interactions. It is still unknown which of the products are more harmless to insects and which particular compounds are the most susceptible to react when all compounds are mixed together in the herbivore's gut.

The developed method has superior capacity to detect the degradation products of phenolic compounds compared with the PO activity methods, which use only the spectrophotometer to follow colorful products. In addition, many of the products detected in this study are missed with methods that use pre-neutralization or one incubation time-point, such as 1 hour, before the chromatographic analysis.

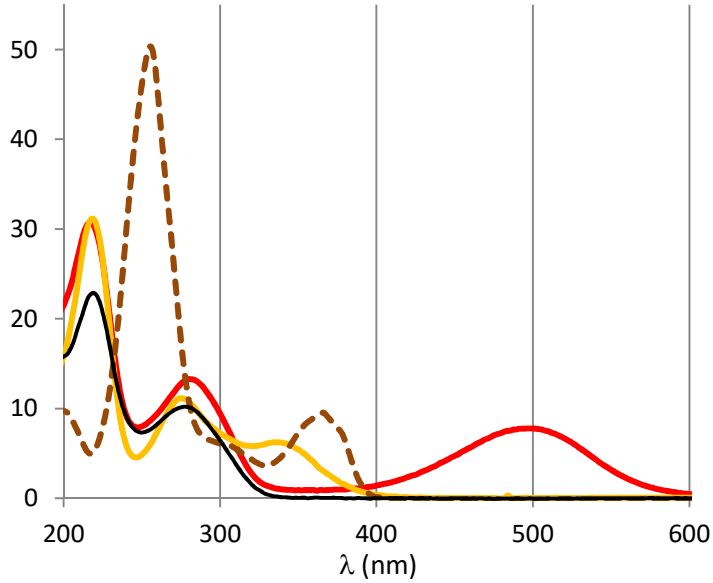


Figure 52. The UV spectra of different products of diG-HHDP-glucose that were formed in the basic conditions. Red line, quinone; yellow line, oxidized product; brown dashed line, ellagic acid; black line, initial compound.

5.5.3 Antioxidant activity of fractions

The antioxidant activity of the extracts and fractions of *G. sylvaticum* organs were measured by DPPH radical scavenging assay (**II**). One-third of the fractions showed high AO activity. The most active fractions were mainly from the pistils, sepals, stems and hairy roots and contained mostly geraniin, other ETs, di- and trigalloylquinic acids, GTs, di-, tri- and tetrameric PAs (**II**). Based on the multivariate models, different polyphenol classes showed AO activity in the *G. sylvaticum* fractions in the decreasing order: ET > GQA > GG > GT > FLA > PA. In particular, fractions that contained geraniin and other ETs with the oxidatively transformed DHHDP groups were highly AO-active (**II**).

Some synergistic effects were also observed. The most antioxidant active crude organ extracts were those of the hairy and main roots that contained a mixture of galloyl quinic acids, ETs and PAs; the same observation was made with fractions (**II**). The AO activity was higher when the fraction contained both type of tannins. For example, the fractions of the hairy roots were more antioxidant-active in comparison with the corresponding main root and leaf fractions, because they contained more hydrolysable tannins in addition to PAs (**II**). The higher DPs of PAs did not seem to increase the AO activity of *G. sylvaticum* fractions, and PCs seemed to be more active than PDs (**II**).

5.6 Variation of phenolics between sexual morphs

Female plants produce only seeds and not pollen. Female plants might compensate for the lack of male function by higher phenolic content than in the hermaphrodites, thus having a better defense against herbivores, pathogens and other unfavourable environment conditions. If they invest more in the constitutive defensive compounds, females might gain an advantage to maintain in the gynodieous populations.[312]

The leaves, seeds, petals and roots of *G. sylvaticum* were selected for the study of sexual differences, because those differed the most in their phytochemistry or measured biological activity (**I**, **II**). Plants were grown in the glasshouse but originated from the following *G. sylvaticum* populations in the Turku area: P3 is a deciduous forest in Paimio (F%, female frequency 16.5%); P4 is a mixed forest in Raisio (F% 10.2); P6 is a meadow in Ruissalo (F% 4.4); P8 is a meadow in Katariinanlaakso (F% 4.6); P9 is a meadow in the middle of the forest on the island of Seili (F% 23.0); P13 is a mixed forest in Oriketo (F% 18.7); P14 is a meadow in Seili (F% 9.2).[40] Samples were collected from both female and hermaphrodite individuals (Table 23, unpublished results). Differences in the contents of polyphenols between sexual morphs were tested with the *t*-test among all populations.

Leaves of female plants had significantly higher TP content ($t = –2{,}88$, $p = 0.0054$) and total ellagitannin ($t = –2.87$, $p = 0.0055$) content than hermaphrodites (Fig. 53, Table 23). However, difference in the geraniin concentrations was not statistically significant ($t = –1.98$, $p = 0.0514$). However, one weakness in this study was that ascorbic acid was used in the extraction and a portion of the geraniin was transformed to ascorgeraniin. The amounts of compounds presented in Table 23 differ from the amounts presented in paper **VI**, because UV detection was used and amounts were calculated as gallic acid equivalents.

Differences in the content of polyphenols in the petals of *G. sylvaticum* between sexual morphs were tested with the *t*-test among all the populations. In petals, the TP content ($t = 0.48$, $p = 0.6323$) was lower in females, but this difference was not statistically significant (Fig. 53, Table 23). Lower TP content was a consequence of the significantly lower content of the main compounds, GTs, in females than in hermaphrodites ($t = 2.59$, $p = 0.0115$) (Table 24). This total GT group includes sylvatiins; however, this study was conducted before the sylvatiins were identified in papers **I** and **III**. Interestingly, also the content of kaempferol 3-glycosides and total flavonoids ($t = 1.46$, $p = 0.1477$) were lower in females, although the difference was not statistically significant. It seems that less co-pigments are produced in the female petals. Unfortunately, the content of anthocyanins were not studied. It is known that female plants of *G. sylvaticum* invest less resources into the pollinator attraction, as the female flowers are smaller and contain less nectar with lower sugar content than hermaphrodites.[42, 45, 80] Still, female plants produce more seeds, which might be a consequence of pollinators' preference for hermaphrodite flowers; this might also protect flowers from the herbivores.[40, 42]

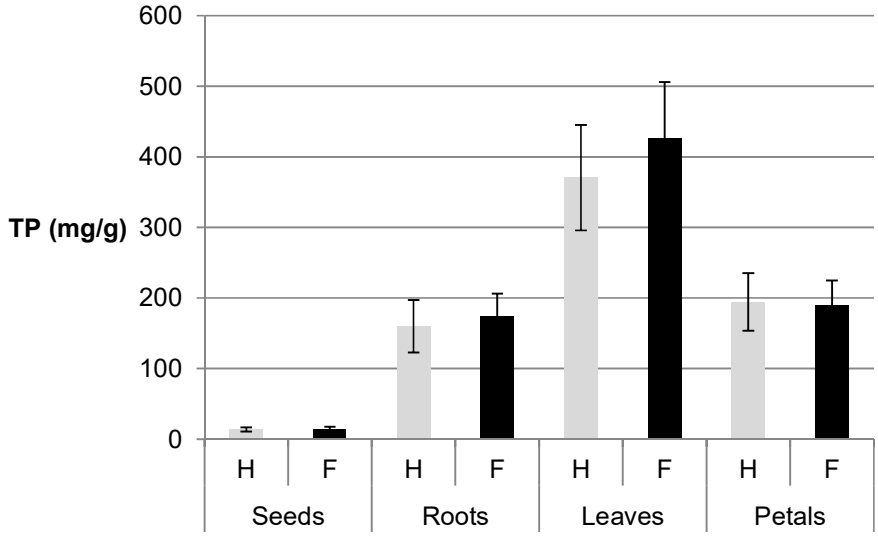


Figure 53. Variation of total phenol (TP) content in the organ extracts of *G. sylvaticum* between two morphs (F = female, H = hermaphrodite) in several populations.

Female plants may increase the seed production or seed quality compared with hermaphrodites by the reallocation of resources from male function to seeds.[68–69] The amount of defensive compounds can be an ecological factor that contributes to the seed fitness. For example, the high polyphenol content might improve seed persistence in soil by inhibiting microbial decomposers. However, no significant differences were observed in the TP or the TPA content of *G. sylvaticum* seeds between sexual morphs, although there were meaningful differences in the TPA content inside populations 8 and 14 (Fig. 53, Table 23). No sex-biased differences were observed in the contents of individual quantified polyphenols, PC dimer, PC trimer, or in the content of amino acid tryptophan (Table 23). This fit well with the previous observation that seed predators did not prefer either sex.[42] However, other compounds that were not investigated in this study might be meaningful in the defensive actions and fitness of seeds.

Some small sex-specific differences were observed in the chemistry of roots. The TP contents of roots were higher in the females in three populations; however, the difference was not statistically significant (Table 23). The combination of ETs and PAs showed high antioxidant capacity in the root fractions of *G. sylvaticum*, but still there were no significant sex-biased differences in the TPA or ET content (Table 23). A similar result was gained with individual quantified polyphenols such as more water-soluble galloyl quinic acid (GQA3) and geraniin (Table 23).

Table 23. Content of total phenols and individual polyphenols in the leaves, seeds, roots and petals of *G. sylvaticum* in different populations and in two sexual morphs. Expressed as average mg/g ± SD and quantified as gallic acid equivalents, except TPA. H, hermaphrodite; F, female

LEAVES							
Population	**Sex**	**n**	**TP**	**Geraniin**	**ET2**	**GQA1**	**Other ETs**
P4	H	8	373.9 ± 55.0	174.0 ± 40.7	27.3 ± 9.5	6.0 ± 3.2	149.5 ± 49.2
	F	6	426.0 ± 105.7	195.0 ± 54.9	43.3 ± 23.4	6.4 ± 4.6	176.4 ± 62.5
P6	H	10	338.6 ± 75.8	160.8 ± 35.5	30.8 ± 21.7	4.6 ± 2.7	132.9 ± 60.3
	F	2	389.6 ± 111.2	173.5 ± 25.9	33.6 ± 4.5	5.5 ± 0.4	168.8 ± 76.7
P8	H	6	342.0 ± 51.6	168.2 ± 17.2	22.8 ± 6.6	4.1 ± 2.1	125.6 ± 40.7
	F	7	451.2 ± 88.8	229.5 ± 40.9	22.5 ± 10.2	5.6 ± 3.3	177.6 ± 52.6
P9	H	2	321.6 ± 146.3	151.8 ± 85.7	29.6 ± 8.5	3.6 ± 0.6	134.4 ± 69.0
	F	3	467.0 ± 34.1	187.8 ± 34.1	49.7 ± 25.3	5.8 ± 2.4	202.6 ± 44.7
P13	H	8	406.4 ± 53.9	197.3 ± 31.8	37.5 ± 18.1	4.1 ± 1.8	158.2 ± 55.7
	F	3	388.9 ± 50.7	189.5 ± 39.4	36.0 ± 4.5	4.4 ± 0.3	152.8 ± 17.4
	H average	43	370.3 ± 74.6	179.3 ± 40.7	29.7 ± 17.3	4.6 ± 2.4	144.8 ± 51.7
	F average	24	426.4 ± 79.6	200.2 ± 42.2	34.7 ± 17.4	5.3 ± 3.1	172.5 ± 48.9
SEEDS							
Population	**Sex**	**n**	**TP**	**TPA**	**Trythophan**	**PC dimer**	**PC trimer**
P3	H	6	11.6 ± 2.5	26.1 ± 5.6	2.1 ± 0.6	1.3 ± 0.4	2.0 ± 0.5
	F	6	14.2 ± 2.9	29.9 ± 3.8	2.1 ± 0.7	1.3 ± 0.3	2.0 ± 0.4
P4	H	6	16.6 ± 4.2	32.0 ± 9.0	3.3 ± 1.7	1.6 ± 0.4	2.3 ± 0.6
	F	6	15.7 ± 4.7	35.0 ± 9.8	1.9 ± 0.3	1.4 ± 0.7	2.4 ± 0.7
P6	H	6	14.0 ± 1.3	32.9 ± 4.7	2.3 ± 0.9	1.5 ± 0.5	2.1 ± 0.3
	F	6	14.9 ± 3.6	27.8 ± 7.4	2.3 ± 0.6	1.7 ± 0.4	2.3 ± 0.4
P8	H	6	13.4 ± 2.8	26.5 ± 6.8	2.4 ± 1.1	1.4 ± 0.4	1.9 ± 0.4
	F	6	13.0 ± 1.3	33.2 ± 2.8	1.7 ± 0.9	1.4 ± 0.5	1.8 ± 0.4
P14	H	6	14.6 ± 1.9	23.6 ± 2.6	2.2 ± 1.7	1.3 ± 0.3	1.8 ± 0.3
	F	6	13.9 ± 2.3	27.2 ± 3.8	1.6 ± 0.6	0.9 ± 0.2	1.5 ± 0.2
	H average	30	14.0 ± 2.9	28.2 ± 6.8	2.5 ± 1.3	1.4 ± 0.3	2.0 ± 0.4
	F average	30	14.3 ± 3.2	30.6 ± 6.5	1.9 ± 0.7	1.4 ± 0.5	2.0 ± 0.5
ROOTS							
Population	**Sex**	**n**	**TP**	**TPA**	**GQA3**	**Geraniin**	**DiG-HHDP-glu**
P4	H	9	174.0 ± 41.3	126.8 ± 34.5	12.6 ± 3.0	40.5 ± 12.7	11.0 ± 4.0
	F	4	182.4 ± 12.7	143.4 ± 32.2	13.0 ± 3.8	40.3 ± 18.8	11.6 ± 6.3
P8	H	10	148.8 ± 23.4	106.2 ± 29.0	10.8 ± 2.8	29.6 ± 8.5	12.1 ± 4.7
	F	7	150.2 ± 36.3	112.6 ± 36.4	9.3 ± 2.1	26.9 ± 9.2	7.6 ± 2.9
P9	H	3	156.0 ± 25.0	125.0 ± 12.8	10.0 ± 3.4	25.6 ± 9.9	9.7 ± 3.6
	F	2	193.0 ± 18.1	114.6 ± 11.3	14.4 ± 1.3	41.9 ± 0.4	16.3 ± 5.1
P13	H	6	177.8 ± 29.9	180.3 ± 35.3	9.6 ± 4.5	25.6 ± 12.4	5.4 ± 3.3
	F	4	172.1 ± 35.8	150.4 ± 75.3	10.6 ± 3.0	32.0 ± 12.4	9.2 ± 2.8
	H average	40	160.0 ± 37.0	129.7 ± 41.9	11.3 ± 3.3	33.0 ± 12.6	10.4 ± 4.8
	F average	22	174.8 ± 31.6	135.1 ± 41.9	11.7 ± 3.2	34.3 ± 12.5	10.7 ± 4.6
PETALS							
Population	**Sex**	**n**	**TP**	**Total GTs**	**Total GGs**	**Kaemp glys**	**Total FLA**
P4	H	10	197.9 ± 29.9	141.9 ± 25.6	45.1 ± 5.8	4.6 ± 1.3	9.6 ± 1.4
	F	6	197.8 ± 26.4	121.0 ± 32.3	65.5 ± 22.0	4.1 ± 1.2	9.0 ± 1.5
P6	H	8	216.0 ± 51.2	159.0 ± 47.2	45.5 ± 18.6	3.6 ± 1.0	10.4 ± 1.6
	F	2	163.0 ± 47.6	102.9 ± 63.8	48.7 ± 18.5	3.4 ± 1.2	10.0 ± 1.8
P8	H	10	168.2 ± 22.4	105.7 ± 19.1	53.0 ± 8.7	3.4 ± 1.1	8.0 ± 2.4
	F	8	175.7 ± 34.8	89.6 ± 29.9	75.6 ± 13.8	3.2 ± 1.4	7.9 ± 3.0
P9	H	3	181.0 ± 41.5	126.0 ± 24.2	44.2 ± 14.6	4.0 ± 1.5	9.2 ± 2.4
	F	3	197.7 ± 25.6	122.0 ± 46.1	64.3 ± 21.7	3.0 ± 0.9	8.2 ± 1.9
P13	H	7	197.7 ± 54.8	133.1 ± 44.6	51.0 ± 10.9	5.2 ± 1.3	12.5 ± 2.2
	F	4	232.5 ± 30.5	145.0 ± 30.4	74.2 ± 12.9	3.3 ± 1.7	11.3 ± 3.8
	H average	48	194.4 ± 40.9	134.1 ± 36.8	48.9 ± 11.3	4.1 ± 1.3	10.0 ± 2.3
	F average	27	189.9 ± 35.0	111.4 ± 35.6	66.9 ± 17.2	3.5 ± 1.2	9.1 ± 2.6

These preliminary results indicate that there might be sex-specific differences in the polyphenol content of *G. sylvaticum*, such as higher content of polyphenols in the leaves, roots and seeds and less copigments in the petals. This test should be repeated with larger amount of samples collected from natural populations and analyzed with the methods developed in this Ph.D work. One reason that can explain why there was no significant difference was that, although original seeds were collected from the plants from different natural populations, actual study plants have been grown in the glasshouse conditions for several years, where growing conditions are more stable and there are less herbivores. The differences in the defensive chemical concentrations between tissues have been smaller under the artificial conditions in the glasshouse compared with the field studies.[327] Therefore, it is possible that differences are more significant if samples are collected from natural populations than the differences observed in this data set.

6 CONCLUSIONS

This work successfully studied the phenolic profiles of the plant organs of *G. sylvaticum* and the variation of their content seasonally, regionally and between sexual morphs. *G. sylvaticum* is a rich source of several polyphenols. A detailed knowledge of the chemistry of plant compounds is needed for utilization of the plant, e.g., in natural products.

More than 60 compounds were identified, of which 14 were confirmed with isolation. *G. sylvaticum* showed significant organ-specific variation in the accumulation of tannins. The amount of many tannins exceeded the 5% of DW, which indicates that these most probably have a special function in the ecological interactions of *G. sylvaticum.* Leaves contained high amounts of geraniin and quercetin galloylglucosides. The sepals accumulated carpinusin and the stamens accumulated chebulagic acid. The high amounts of gallotannins showed distinctive ontogenic variation in the pistils. The new group of hydrolysable tannins were identified from the petals. These sylvatiins showed copigmentation capacity and thus may act in the attraction of pollinators. In particular, seeds and roots accumulated proanthocyanidins, and the type and the degree of polymerization varied among organs.

The hypothesis was that differences in the tannin content might reflect the different herbivore pressures that plant organs face. Therefore, much effort was exerted to study the pro-oxidant activity of hydrolysable tannins and thus to prove their antiherbivore effects. Although ellagitannin-rich extracts and fractions showed high pro-oxidant capacity, they also showed antioxidant activity. The pro- and antioxidant activity of the extracts varied significantly among different plant organs, and the results showed that the tannin content has to be quite high, more than 5%, to show activity. Observations emphasize the dual nature of polyphenols that depends on the surrounding conditions.

There is still lot to explore in the phytochemistry of *G. sylvaticum*. Many more minor compounds can be isolated and elucidated. The study can delver deeper into the smaller parts of organs. It would be interesting to study, using the modern methods developed in this study, the phylogeny and chemical profiles of other *Geranium* species as well. It would also be interesting to study copigment effects of other hydrolysable tannins. Sexual variation should be repeated with samples from natural populations, as there are not many studies that have explored the role of phytochemistry in the female maintenance.

These results provide an example of how biosynthetic pathways can produce delicate differences in the phytochemical profiles among the plant organs of one plant species, even within a single class of compounds. The distribution of active compounds among organs reflects the herbivore pressure and the importance of the particular organ. However, these results showed that it is more likely that tannins may play multiple roles and perform multiple modes of actions in *G. sylvaticum* that are specialized in each organ.

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