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# FLAVONOIDS AND OTHER PHENOLIC COMPOUNDS: CHARACTERIZATION AND INTERACTIONS WITH LEPIDOPTERAN AND SAWFLY LARVAE

Matti Vihakas

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# ABSTRACT

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VIHAKAS, MATTI: Flavonoids and other phenolic compounds: characterization and interactions with lepidopteran and sawfly larvae

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This thesis focuses on flavonoids, a subgroup of phenolic compounds produced by plants, and how they affect the herbivorous larvae of lepidopterans and sawflies. The first part of the literature review examines different techniques to analyze the chemical structures of flavonoids and their concentrations in biological samples. These techniques include, for example, ultraviolet-visible spectroscopy, mass spectrometry, and nuclear magnetic resonance spectroscopy. The second part of the literature review studies how phenolic compounds function in the metabolism of larvae. The harmful oxidation reactions of phenolic compounds in insect guts are also emphasized. In addition to the negative effects, many insect species have evolved the use of phenolic compounds for their own benefit.

In the experimental part of the thesis, high concentrations of complex flavonoid oligoglycosides were found in the hemolymph (the circulatory fluid of insects) of birch and pine sawflies. The larvae produced these compounds from simple flavonoid precursors present in the birch leaves and pine needles. Flavonoid glycosides were also found in the cocoon walls of sawflies, which suggested that flavonoids were used in the construction of cocoons. The second part of the experimental work studied the modifications of phenolic compounds in conditions that mimicked the alkaline guts of lepidopteran larvae. It was found that the 24 plant species studied and their individual phenolic compounds had variable capacities to function as oxidative defenses in alkaline conditions. The excrements of lepidopteran and sawfly species were studied to see how different types of phenolics were processed by the larvae. These results suggested that phenolic compounds were oxidized, hydrolyzed, or modified in other ways during their passage through the digestive tract of the larvae.

**Key words**: flavonoids, larvae, phenolic compounds, secondary compounds

# TIIVISTELMÄ

TURUN YLIOPISTO
Kemian laitos/Matemaattis-luonnontieteellinen tiedekunta

VIHAKAS, MATTI: Flavonoidit ja muut fenoliset yhdisteet: karakterisointi ja vuorovaikutukset perhos- ja sahapistiäistoukkien kanssa

Väitöskirja, 135 s.
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Marraskuu 2014

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Tämä väitöskirja keskittyy flavonoideihin (kasvien tuottamien fenolisten yhdisteiden alaluokka) ja siihen, miten ne vaikuttavat kasveja syöviin perhos- ja sahapistiäistoukkiin. Kirjallisuuskatsauksen ensimmäinen osa tarkastelee erilaisia tekniikoita, joiden avulla flavonoidien kemiallisia rakenteita ja pitoisuuksia määritetään. Näihin tekniikoihin kuuluvat esimerkiksi ultravioletti-näkyvän valon spektroskopia, massaspektrometria ja ydinmagneettinen resonanssispektroskopia. Kirjallisuuskatsauksen toinen osa keskittyy fenolisten yhdisteiden vaikutuksiin toukkien ruoansulatuksessa. Yhtenä pääpainona ovat fenolisten yhdisteiden haitalliset hapettumisreaktiot toukkien mahassa. Monet hyönteislajit ovat myös kehittyneet hyödyntämään kasvien fenolisia yhdisteitä omiin tarpeisiinsa.

Väitöskirjan kokeellisessa osassa löydettiin koivun ja männyn sahapistiäistoukkien hemolymfasta (toukkien ruumiinneste) korkeita pitoisuuksia monimutkaisia flavonoidien sokeriyhdisteitä. Toukat olivat muodostaneet kyseiset yhdisteet yksinkertaisemmista flavonoideista, jotka olivat peräisin koivunlehdistä ja männynneulasista. Flavonoideja havaittiin myös sahapistiäisten kotelokopissa, mikä viittasi siihen, että toukat olivat käyttäneet flavonoideja koteloiden rakentamiseen. Kokeellisen työn toinen osa käsitteli fenolisten yhdisteiden hapettumista oloissa, jotka jäljittelivät perhostoukkien mahan emäksisiä olosuhteita. Tutkituilla 24 kasvilajilla ja niiden sisältämillä yksittäisillä fenolisilla yhdisteillä havaittiin olevan erilaisia taipumuksia hapettua emäksisissä oloissa. Lisäksi perhos- ja sahapistiäistoukkien jätöksiä tutkimalla selvitettiin, miten fenoliset yhdisteet muokkautuivat toukkien ruoansulatuksessa. Tulosten perusteella fenoliset yhdisteet olivat mahdollisesti hapettuneet, hydrolysoituneet tai muokkautuneet muulla tavalla kulkeutuessaan toukkien ruoansulatuksen läpi.

**Asiasanat**: flavonoidit, fenoliset yhdisteet, sekundääriyhdisteet, toukat

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Turku, October 2014

Matti Virkkunen

# 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** Vihakas, M. A., Kapari, L., Salminen, J.-P. 2010. New types of flavonol oligoglycosides accumulate in the hemolymph of birch-feeding sawfly larvae. *J. Chem. Ecol.* 36:864–872.

**II** Vihakas, M., Tähtinen, P., Ossipov, V., Salminen, J.-P. 2012. Flavonoid metabolites in the hemolymph of European pine sawfly (*Neodiprion sertifer*) larvae. *J. Chem. Ecol.* 38:538–546.

**III** Vihakas, M., Pälijärvi, M., Karonen, M., Roininen, H., Salminen, J.-P. 2014. Rapid estimation of the oxidative activities of individual phenolics in crude plant extracts. *Phytochemistry* 103:76–84.

**IV** Vihakas, M., Gómez Avila, I., Karonen, M., Tähtinen, P., Sääksjärvi, I., Salminen, J.-P. Phenolic compounds and their fates in tropical lepidopteran larvae: modifications in alkaline conditions. *Submitted manuscript*

# ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| CE | Capillary electrophoresis |
| CID | Collision induced dissociation |
| COSY | Correlation spectroscopy |
| DAD | Diode array detector |
| ECD | Electronic circular dichroism |
| ESI | Electrospray ionization |
| FAB | Fast atom bombardment |
| FT–ICR | Fourier transform ion cyclotron resonance |
| GC | Gas chromatography |
| HHDP | Hexahydroxydiphenoyl |
| HMBC | Heteronuclear multiple bond correlation |
| HPLC | High-performance liquid chromatography |
| HSQC | Heteronuclear single quantum correlation |
| ICP–MS | Inductively coupled plasma mass spectrometry |
| IT | Ion trap |
| LC | Liquid chromatography |
| MALDI | Matrix-assisted laser desorption/ionization |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| *m/z* | Mass-to-charge ratio |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear overhauser enhancement spectroscopy |
| QTOF | Quadrupole time-of-flight |
| ROESY | Rotating frame overhauser effect spectroscopy |
| ROS | Reactive oxygen species |
| SRM | Selected/single reaction monitoring |
| SPE | Solid phase extraction |
| TOCSY | Total correlation spectroscopy |
| TQ | Triple quadrupole |
| UHPLC | Ultra-high performance liquid chromatography |
| UV | Ultraviolet |
| vis | Visible |

# 1 INTRODUCTION

This thesis focuses on the chemical interactions between plants and plant-feeding organisms, i.e., herbivores. Plants are immobile and cannot hide or run away when they are threatened by herbivores. During the course of evolution, several strategies have evolved in plants for defense against different types of herbivores, such as many insect and mammal species. These defenses include morphological features, such as thorns, trichome hairs, resins, and waxes (Fürstenberg-Hägg et al., 2013). They function as physical barriers against herbivores even though some of them (resins and waxes) can also contain active chemical compounds. Another important defense mechanism of plants is to produce chemical substances that are aimed at harming herbivores (Wink, 2003). Herbivores can perceive the negative effects of plant defensive compounds by their repulsive taste or by their toxic effects. Plant chemicals may also negatively affect the growth of herbivores, so that the overall consumption of plant material is reduced. The above-mentioned physical and chemical defenses are considered as direct defenses, while indirect defenses of plants include, for example, volatile compounds that attract predators and parasitoids of herbivorous insects (Dicke, 2003).

Many insect species have evolved a toleration to the defensive compounds of their host plant, which has enabled the insect species to become specialized in a particular plant species (Ehrlich and Raven, 1964; Després et al., 2007). Some initially noxious plant chemicals have even become attractants that induce the feeding of insect larvae on those plant species. Specialization to specific host plant species can be beneficial for the insect, as the new host can provide a rather unlimited food source that is free of competitors that may still be repelled by the plant's defensive compounds (Harborne and Grayer, 1994). This exemplifies the coevolution of plants and herbivores, where they both impose selective pressures on each other, which slowly drives the evolution of both the plant and herbivore species. It has been proposed that coevolution between plant defense compounds and butterfly species has been a major factor that has provided the impetus for the evolution of these organisms (Ehrlich and Raven, 1964). If insect species adapt to certain plant-derived chemical compounds, this could induce plants to produce even more powerful defenses, to which insect species could respond with new counter-defenses. The ongoing chemical competition between plant and insect species has been illustrated as an arms race (Berenbaum and Feeny, 1981).

Herbivorous insects harm plants mainly during their feeding period as larvae. Interestingly, adult insects are often important pollinators of flowers. Plants reward pollinating insects with nectars containing sugars, amino acids, and other compounds that are used

by the insects as energy, in the construction of proteins, and in other biochemical processes (Kevan and Baker, 1983).

This thesis focused on phenolic compounds produced by plants. The initial purpose of phenolics was probably to defend plants against their natural enemies. However, some insect herbivores have evolved to utilize phenolics for their own benefit. For example, gall-inducing sawfly larvae feeding on willow leaves seem to be able to control, to some extent, the production of phenolic compounds in their host plant (Nyman and Julkunen-Tiitto, 2000). The main objective of the thesis was to study the different mechanisms by which phenolic compounds affect two types of herbivores, the larvae of butterflies and moths (Lepidoptera), and sawflies (Hymenoptera, **Figure 1**). Phenolic compounds are prone to chemical alterations as they move through the digestive tract of larvae. For example, the gut conditions of many lepidopteran species are highly alkaline, and these conditions can promote the oxidation of phenolic compounds, which in turn would cause oxidative stress to the larvae (Appel, 1993; Barbehenn and Constabel, 2011; Salminen and Karonen, 2011). In addition to oxidation reactions, several other metabolic fates are possible for phenolics in herbivorous larvae (Lahtinen et al., 2005; Ferreres et al., 2008). Plant-derived compounds can be further transferred into the larval hemolymph, i.e., the circulatory fluid of insects, where these compounds can be used in the defense of the larvae (Müller and Brakefield, 2003; Prieto et al., 2007).

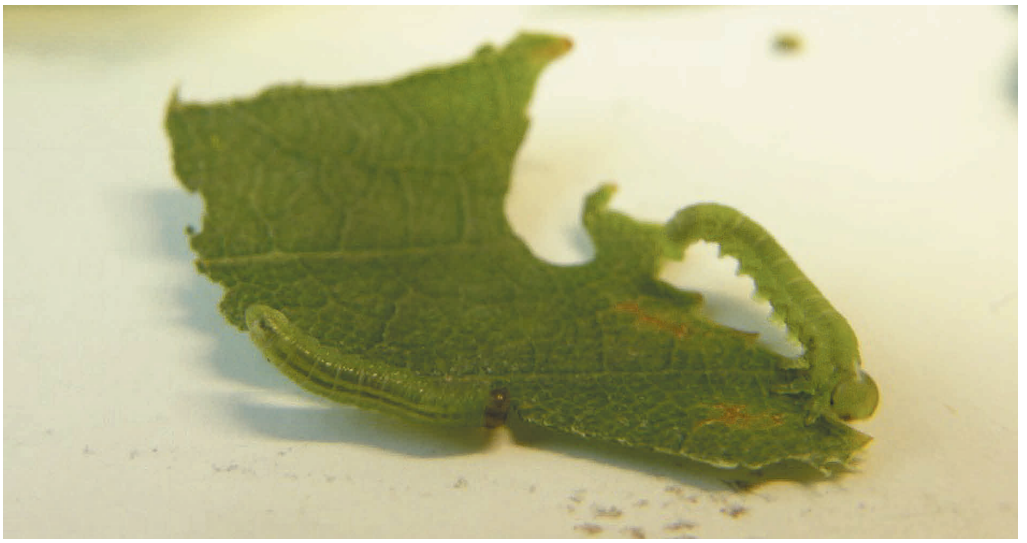


**Figure 1.** Sawfly larvae feeding on birch leaves.

# 2 LITERATURE REVIEW

## 2.1 Phenolic compounds

Plant metabolites can roughly be divided into two categories: primary and secondary metabolites. The primary metabolites participate in the nutrition and essential metabolic processes such as growth, development, or reproduction, and these compounds include, for example, carbohydrates, lipids, and proteins. Secondary metabolites often function in important ecological roles between a plant and its environment, such as being pigments in plant leaves and flowers, or as defensive compounds against herbivores and pathogens (Bernards, 2010). The secondary plant compounds can be divided into three main categories: alkaloids, terpenoids, and phenolics (Bernards, 2010). Phenolic compounds are further divided into subgroups, such as flavonoids, tannins, and phenolic acids. Many phenolics contain several phenolic rings with multiple hydroxyl groups, and thus the term polyphenolic is often used. Quideau et al. (2011) proposed a revised definition of a polyphenolic compound that also explains their biosynthetic origin: "*The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression*". This definition excludes terpenoids, alkaloids, and compounds with only one benzene ring (e.g., vanillin and salicylic acid) outside of polyphenolic compounds. Most of the phenolic compounds in this thesis are polyphenolics.

### 2.1.1 Flavonoids

Flavonoids are based on a C6–C3–C6 backbone (**Figure 2**). Their biosynthesis in plants combines two metabolic pathways: a shikimate-derived phenylpropanoid pathway (producing a C6–C3 unit) and an acetate/malonate-derived polyketide pathway (producing a C6 unit; Waterman and Mole, 1994; Quideau et al., 2011). Flavonoids are divided into three main classes: flavonoids, isoflavonoids, and neoflavonoids (**Figure 2**; Waterman and Mole, 1994). The class of flavonoids is further divided in several subclasses that are distinguished from each other by the chemical differences in their ring C (**Figure 3**; Iwashina, 2000). For example, different subclasses can have a double bond between C2–C3, a hydroxyl group at C3, or a carbonyl group at C4 (**Figure 3**).

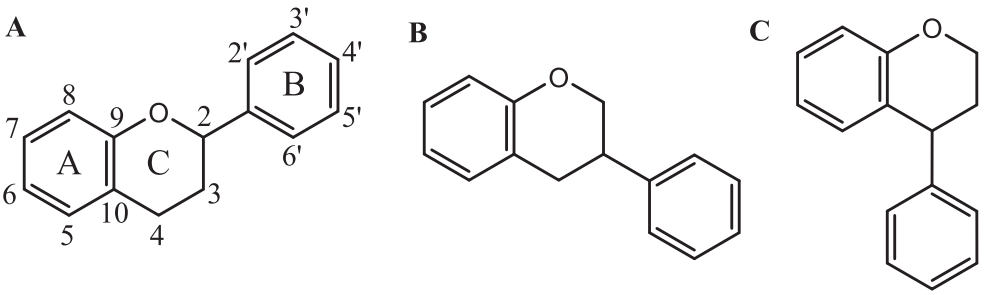



**Figure 2.** The main classes of flavonoids: flavonoids (**A**), isoflavonoids (**B**), and neoflavonoids (**C**). The labeling of flavonoid rings, and the numbering of atoms is presented.

In plants, flavonoids are often found as glycosides (Iwashina, 2000), where one or several glycosyls are attached to the flavonoid aglycone or to other glycosyls (**Figure 4**A). The glycosyls are mostly bound via ether bonds to the flavonoid (*O*-glycosidic flavonoids) but in the less common *C*-glycosyl substituted flavones, the glycosyls are attached to aglycones via C–C bonds. The glycosyls can be acylated with different acids, such as malonic and coumaric acids (Cuyckens and Claeys, 2004). Variable amounts of substituents, such as hydroxyl and methoxy groups, glucuronic acid, and isoprenoid groups can be attached to the flavonoid rings, which increase the diversity of possible flavonoid structures (**Figure 4**B and C). Two or several flavonoid units can be oxidatively coupled to produce complex bi-, tri-, tetra-, penta- and even hexaflavonoids (**Figure 4**D; Ferreira et al., 2006). They are composed of flavonoid units containing a carbonyl group at C4 (e.g., flavones, flavonols, aurones, chalcones), but the presence of a carbonyl group may not always be evident from the structure. These compounds should not be confused with proanthocyanidins that are composed of flavan-3-ol units (see Tannin section below).

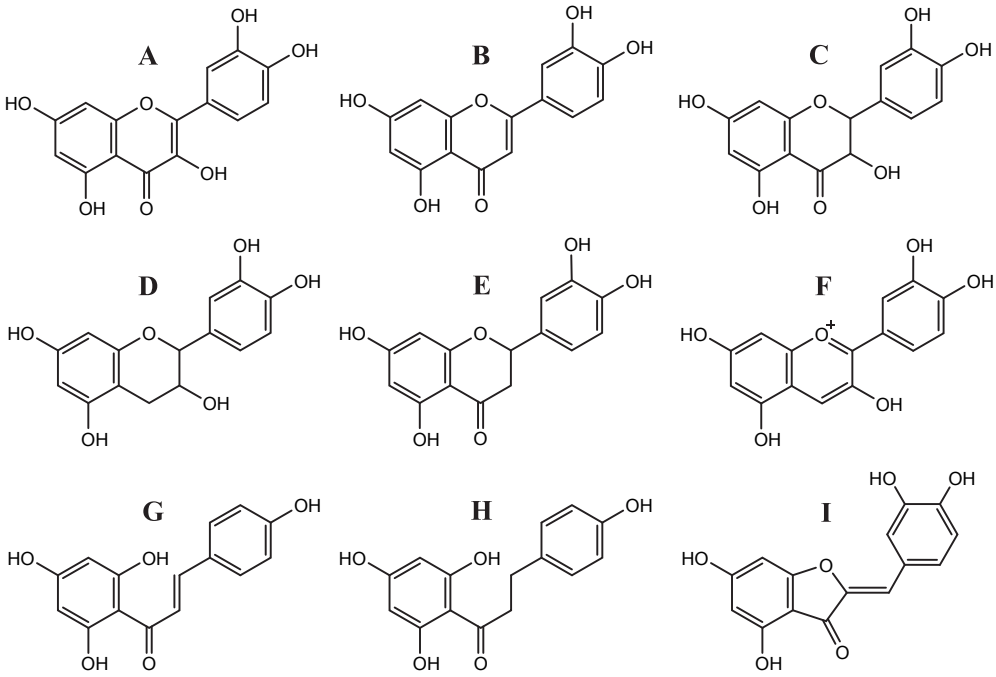



**Figure 3.** Examples of different subclasses of flavonoids: a flavonol (**A**), a flavone (**B**), a dihydroflavonol (**C**), a flavan-3-ol (**D**), a flavanone (**E**), an anthocyanidin (**F**), a chalcone (**G**), a dihydrochalcone (**H**), an aurone (**I**).

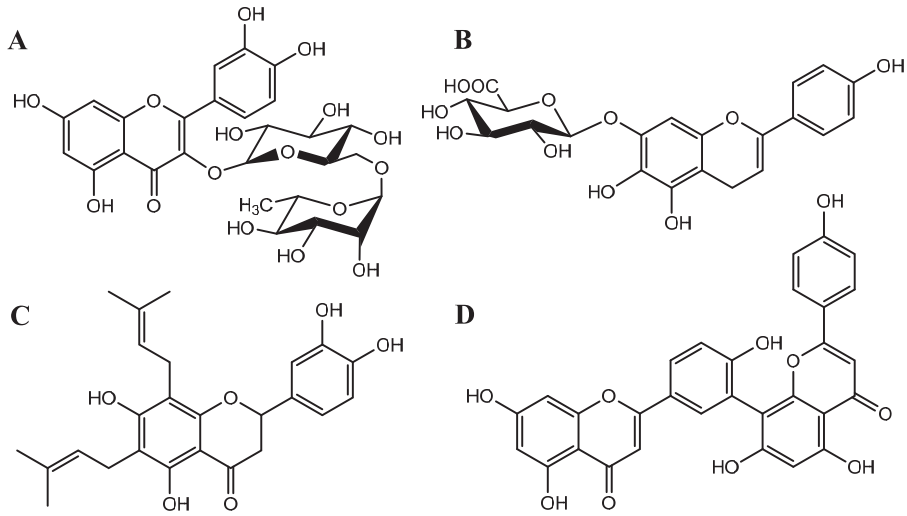



**Figure 4.** Example structures of flavonoids: a flavonol diglycoside (**A**), a flavone glucuronide (**B**), an isoprenylated flavanone (**C**), a biflavone (**D**).

### 2.1.2 Tannins

Tannins are water-soluble phenolic compounds that are traditionally connected with their ability to precipitate proteins and alkaloids (Swain and Bate-Smith, 1962). Tannins are divided in three classes, i.e., proanthocyanidins (condensed tannins), hydrolysable tannins, and phlorotannins.

Proanthocyanidins are oligo- or polymeric structures that are formed from flavan-3-ol monomer units (**Figure 5**, Haslam, 1998a). Flavan-3-ols are derived from flavonoid biosynthesis, which structurally connects proanthocyanidins to flavonoids. Different types of flavan-3-ol monomers exist, such as catechins (hydroxyl groups in C3ˈ and C4ˈ; **Figure 5**A), gallocatechins (hydroxyl groups in C3ˈ, C4ˈand C5ˈ), and afzelechins (a hydroxyl group in C4ˈ). Proanthocyanidins that are formed purely from (epi)catechin units are called procyanidins, while (epi)gallocatechins form prodelphinidins and (epi)afzelechins form propelargonidins. Proanthocyanidins are found in nature in multiple structural variations and isomeric forms, which is caused by several factors. Firstly, flavan-3-ol units contain two stereogenic carbons at C2 and C3, which enables four possible isomers. Secondly, catechin units combine with other types of flavan-3-ols units to produce mixed proanthocyanidins, for example, a catechin–gallocatechin dimer. Thirdly, the flavan-3-ol units can bind to each other in several ways. For example, in B-type proanthocyanidins two flavan-3-ol units are linked via a single C–C bond (mostly from C4 to C8, or from C4 to C6, **Figure 5**B), while in A-type proanthocyanidins two flavan-3-ol units are bound together with a C–C and a C–O–C bond (Ferreira et al., 2006).

Hydrolysable tannins are esters of gallic acid and a central polyol (most usually *β*-D-glucose). Their biosynthesis originates in the formation of gallic acid in the shikimate pathway of plants (Werner et al., 1997; Ossipov et al., 2003; Niemetz and Gross, 2005). Hydrolysable tannins are divided in three subclasses: simple gallic acid derivatives, gallotannins, and ellagitannins (**Figure 5**, Niemetz and Gross, 2005; Salminen and Karonen, 2011). In simple gallic acid derivatives, 1–5 gallic acid units can be esterified to a glucose thus forming mono- to pentagalloyl glucoses, while galloylquinic acids contain quinic acid as the central polyol (**Figure 5**C and D). Gallotannins are modifications of pentagalloyl glucose and they contain six or more galloyl groups (**Figure 5**E). Ellagitannins are characterized by a hexahydroxydiphenoyl (HHDP) group that is formed in the oxidative coupling of two adjacent galloyl groups (**Figure 5**F), but also other structural modifications of the HHDP group exist (Quideau and Feldman, 1996). The central glucose in ellagitannins can be in both cyclic and acyclic forms, and ellagitannins also form oligomers. The third and most uncommon class of tannins, phlorotannins, are composed of phloroglucinol units that are attached to each other by C–C or C–O bonds (**Figure 5**G; Haslam, 1998a). The occurrence of phlorotannins in nature is limited to brown and red algae.

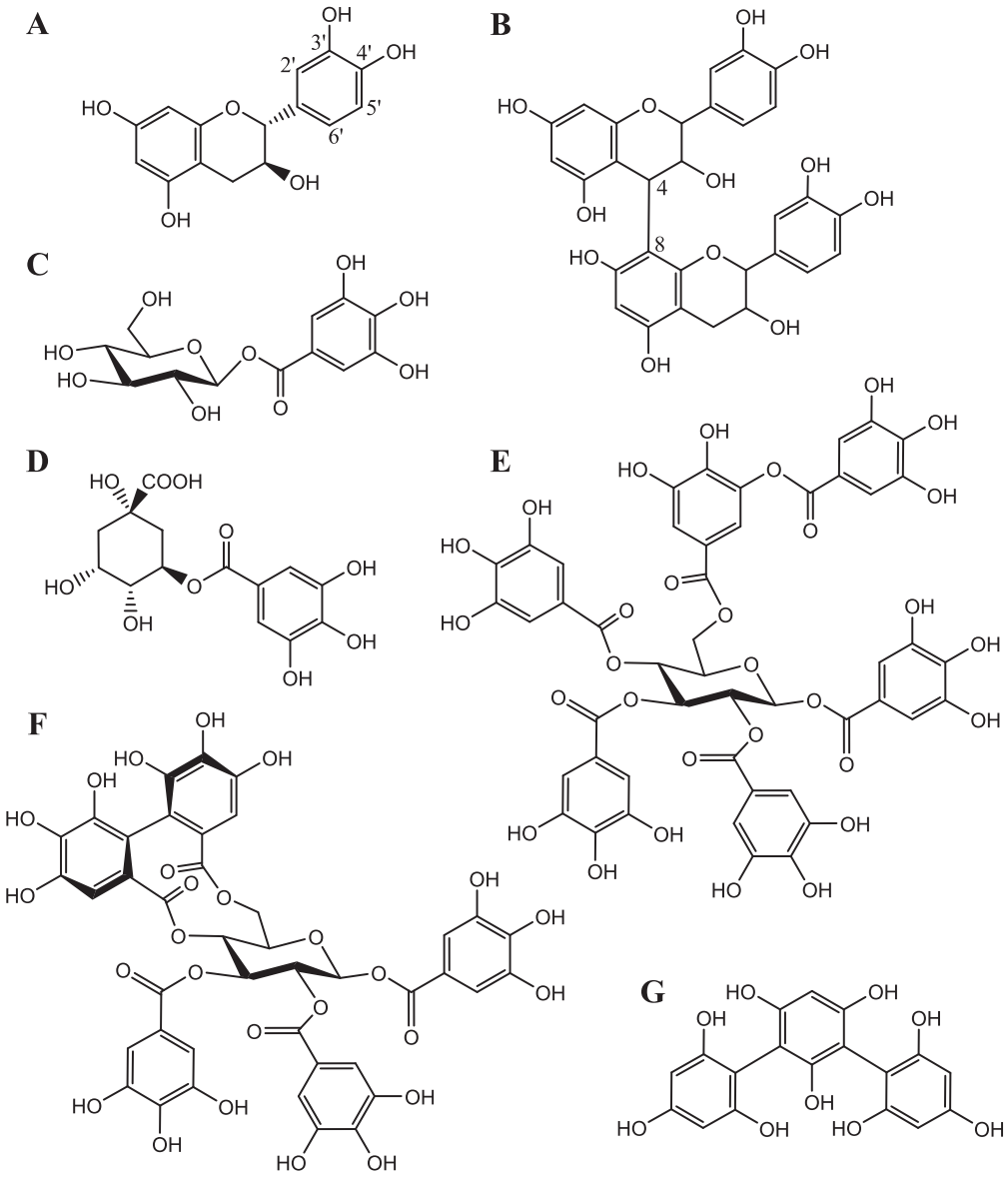



**Figure 5.** Example structures of tannins: a flavan-3-ol ((+)-catechin) (**A**), a dimeric B-type proanthocyanidin (**B**), a monogalloyl glucose (**C**), a galloylquinic acid (**D**), a gallotannin (hexagalloyl glucose) (**E**), an ellagitannin (tellimagrandin II) (**F**), a trimeric phlorotannin (**G**).

Tannins are traditionally thought to function as digestion inhibitors of herbivores. This inhibition is based on the ability of tannins to bind to proteins, which would decrease the dietary value of plant material (Feeny, 1968; Haslam, 1998b). Later studies have pointed out that many tannins and other phenolic compounds would gain their biological activities through oxidative activation in herbivore guts (Appel, 1993), and that this would be especially true for ellagitannins (Barbehenn et al., 2006a,b).

### 2.1.3 Hydroxycinnamic acids

Hydroxycinnamic acids are a subclass of phenolic acids that are based on a C6–C3 backbone (**Figure 6**). They are biosynthesized in plants in the same phenylpropanoid pathway as the C6–C3 building blocks of flavonoids (Waterman and Mole, 1994). Hydroxycinnamic acids are found in plants, for example, as esters with quinic acid and as derivatives with flavonoid glycosides (**Figure 6**; Clifford, 2003). Similarly to other phenolic compounds, also hydroxycinnamic acid derivatives are sometimes connected with possible anti-herbivore activities (e.g. Felton et al., 1989).

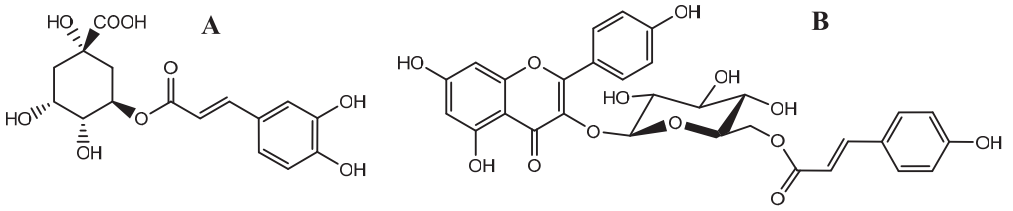



**Figure 6.** Examples of hydroxycinnamic acid derivatives: a 3-*O*-caffeoylquinic acid (chlorogenic acid) (**A**), an acylated flavonol glycoside (kaempferol 3-*O*-[6-*O*-*p*-coumaroyl]-glucopyranoside) (**B**).

## 2.2 Methods to study flavonoids

Flavonoids and other phenolics are usually present in plants in complex mixtures. To facilitate their analysis, a chromatographic step is often needed to separate individual compounds from each other. The separation techniques of flavonoids include, e.g., capillary electrophoresis (CE), gas chromatography (GC), and liquid chromatography (LC; Marston and Hostettmann, 2006). High-performance liquid chromatography (HPLC) has been the most common separation technique for phenolic compounds (Marston and Hostettmann, 2006; Kalili and de Viliers, 2011). In HPLC, a sample mixture in solution is eluted through a column with the aid of eluent flow (isocratic or gradient elution). Different compounds in the sample mixture are retained with variable efficiencies inside the column, which causes their chromatographic separation. Different packing materials of columns are used but octadecylsilyl bonded phases (i.e. $C_{18}$) have been common in the separation of flavonoids (Marston and Hostettmann, 2006). During recent years, HPLC has been followed by ultra-high performance liquid chromatography (UHPLC). Particle sizes in UHPLC columns are smaller (less than 2 μm) than in traditional HPLC columns (approx. 3–5 μm), which are required for higher pressures (approx. 500–1400 bars) in UHPLC columns than in HPLC columns (up to 400 bars; Kalili and de Viliers, 2011; Unger and Weng, 2013). In comparison to HPLC, the use of UHPLC has several advantages, including shorter analysis times and better sensitivity and resolution (Churchwell et al., 2005; Ortega et al., 2010).

The eluent flow from an LC column is directed into a detector that detects flavonoids separated by the column. Several types of detectors are used in combination with LC. Fluorescence detection is sometimes used for the detection of flavonoids, but this method is limited because only certain flavonoids exhibit native fluorescence (de Rijke et al., 2006). Flavonoids can, however, be derivatized with metal cations to produce fluorescent complexes. In an electrochemical detector (coulometric electrode array), flavonoids are gradually oxidized as they move through an array of electrodes that are placed in the order of increasing potential (Brenes et al., 2000; Guo et al., 1997). Ultraviolet (UV) – visible (vis) spectroscopy is a common detection method of flavonoids in LC (Marston and Hostettmann, 2006). The use of UV–vis and mass spectrometry (MS) for detecting flavonoids is covered in the following sections.

Gas chromatography (GC) coupled with mass spectrometry has low detection limits and high resolution to analyze flavonoids (de Rijke et al., 2006). However, sample pretreatment with derivatization agents is needed, so that flavonoids become more volatile. Despite this, flavonoid glycosides can be difficult to analyze with GC–MS, because derivatization may form several derivatives from a single flavonoid (Fossen and Andersen, 2006; de Rijke et al., 2006), and flavonoid glycosides can be thermally unstable.

Electronic circular dichroism (ECD) measurements are used to determine the absolute configuration and conformation of isolated flavonoids (Slade et al., 2005). It is based on the variable abilities of chiral molecules to absorb circularly (left and right) polarized light. For example, flavan-3-ols have stereogenic carbon atoms at positions 2 and 3 of the flavonoid ring C (**Figure 3**D), and thus there are four possible diastereomers that can be distinguished from each other by their different ECD spectra.

### 2.2.1 Ultraviolet-visible spectroscopy

All phenolic compounds contain aromatic conjugated systems that absorb light in the UV–vis spectral region enabling their detection with UV–vis detectors. A traditional UV–vis detector monitors only one or several wavelengths at the same time, whereas a diode array detector (DAD) simultaneously measures a range of wavelengths (e.g., 200–500 nm), which enables the measurement of UV–vis spectra of phenolic compounds.

The UV–vis spectra of flavonoids are comprised of absorptions arising from the benzene rings A and B and their possible conjugations to ring C. The UV–vis spectra of different subclasses of flavonoids give tentative information about the structures of flavonoid aglycones (de Rijke et al., 2006; **Figure 7**). All flavonoids have an absorption maximum at around 240–290 nm (Band II), which is mostly affected by the conjugation of ring A and its substitution pattern (Santos–Buelga et al., 2003). Some flavonoids have another absorbance maximum at around 300–550 nm (Band I), which is detected in flavonoids where rings B and C are conjugated (via a double bond between

carbons C–2 and C–3 in ring C). This absorption maximum is at around 460–560 nm for anthocyanins and at 310–370 nm for flavones and flavonols (Santos–Buelga et al., 2003). The Band I maximum is generally at a longer wavelength in flavonols than in flavones, which can help to differentiate between these two types of flavonoids (**Figure 7**A and B). In addition, chalcones and aurones have an elongated conjugation system from their ring B (see structures in **Figure 3**) and, thus, these compounds show a second absorption maximum, which is at around 345–390 nm for chalcones and at 390–430 nm for aurones (Santos–Buelga et al., 2003).

Flavan-3-ols, proanthocyanidins and dihydrochalcones show mainly one absorption maximum at around 270–290 nm (Band II), while flavanones and dihydroflavonols also have a small shoulder (Band I) at around 320 nm (**Figure 7**C and D; Santos–Buelga et al., 2003; Marston and Hostettman, 2006). The absence or low absorption maximum of Band I is probably caused by the lack of conjugation between ring B and the rest of the molecule (no double bond beside the ring B). For most flavonoids, except for anthocyanins and some aurones, the above-mentioned Band I and II absorption maxima lie in the range of UV radiation (i.e. below 400 nm), so the term UV spectrum of flavonoids is often used instead of the term UV–vis spectrum.

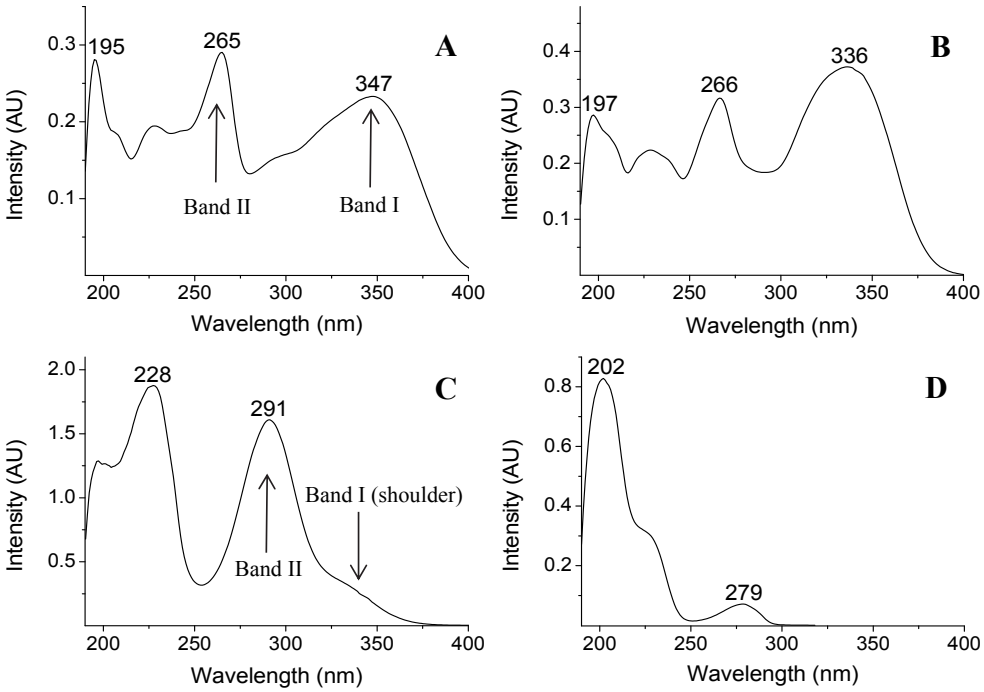



**Figure 7.** Example UV spectra of flavonoids (acquired from UHPLC–DAD chromatograms with 0.1 % formic acid and acetone in the solvent mixture): a flavonol (kaempferol monohexoside) (**A**), a flavone (apigenin glucuronide) (**B**), a dihydroflavonol (dihydromyricetin) (**C**), a flavan-3-ol ((+)-catechin) (**D**).

The locations of Band I and II maxima are affected by the number of hydroxyl groups in rings A and B as well as by the glycosylation of the flavonoid. For example, the glycosylation of a hydroxyl group can cause a decrease in the Band I absorbance maximum (Santos–Buelga et al., 2003). However, the actual sugar molecule has no effect on the UV spectrum of flavonoid glycosides. Acyl substituents in glycosyls cause an additional maximum in the UV spectrum of flavonoid glycosides. For example, a coumaroyl substituent (a conjugated system) produced an extra absorption maximum at around 310–320 nm in the UV spectrum of kaempferol and quercetin glycosides (Soliman et al., 2000; Mihara et al, 2004). When interpreting UV spectra from LC–DAD data, it should be noted that the UV absorption maxima of flavonoids might be slightly shifted depending on the solvent composition by which they were eluted from the LC column (Fossen and Andersen, 2006).

In addition to structure elucidation, UV–vis detectors are used in the quantitative analyses of flavonoids and other phenolics. The sensitivity of detection is improved by choosing a detection wavelength or wavelengths where the phenolics in question show maximum absorption (Marston and Hostettman, 2006). UV–vis quantitation has good reproducibility, because the absorbance of a particular compound stays virtually unchanged between different analysis times. For the best accuracy, phenolic compounds should be quantified with standards that have the same or similar chemical structures as the quantified compounds. The use of unsuitable standards produces quantitation errors (Marston and Hostettman, 2006) because these standards have different UV–vis absorption properties than the quantified compounds. Detection limits of phenolic compounds are often higher with UV–vis than with MS techniques, and the co-elution of phenolics hampers the quantitation of individual compounds (Islam et al., 2011; Verdu et al., 2013).

### 2.2.2 Mass spectrometry

Similarly to UV–vis detectors, mass spectrometers have a crucial role in detecting and analyzing flavonoids and other phenolic compounds. While UV–vis detectors only detect compounds with a UV–vis absorbing chromophore, mass spectrometers detect virtually all kinds of chemical compounds that are ionized in the used analytical conditions. These two instruments are often coupled in line, so that the eluent flow from LC first passes through an UV–vis detector, after which the eluent is directed to MS (LC–DAD–MS).

In MS, analyte molecules are first ionized, after which the ions are analyzed according to their mass-to-charge ratios (*m/z*, Watson and Sparkman, 2007). Mass spectrometers are divided into different groups according to their ionization techniques and mass analyzers. Ionization techniques used in flavonoid analyses include, for example, fast

atom bombardment (FAB) and matrix assisted laser desorption/ionization (MALDI). In FAB, the flavonoid sample is dissolved in a liquid matrix that is bombarded with a beam consisting of fast atoms (e.g. Ar or Xe), which causes the desorption of ions from the sample matrix (Fossen and Andersen, 2006). In MALDI, the sample is dissolved in a matrix that absorbs energy from laser pulses, which causes the formation of plasma that produces ions from the analyte molecules (Fossen and Andersen, 2006). In addition to different ionization techniques, also several mass analyzers exist, such as, ion trap (IT) and Fourier transform ion cyclotron resonance (FT–ICR) analyzers. This chapter concentrates on the electrospray ionization (ESI) technique, and how it is used in combination with two different mass analyzers: triple quadrupole (TQ) and quadrupole time-of-flight (QTOF) analyzers.

ESI functions as an interface between LC and a mass spectrometer. In ESI, the eluent and analyte molecules from LC are sprayed through a needle that is kept at a high potential or there is a potential difference between the needle and the inlet (nozzle) of the mass spectrometer. The applied potential causes the formation of charged droplets that move towards the inlet of the mass spectrometer (Westman-Brinkmalm and Brinkmalm, 2009A). The charged droplets shrink as the assisting drying gas flow helps to evaporate the solvent and the repulsion of positive or negative charges breaks down the droplets until ions are formed from the analyte molecules. The ionization process in ESI source functions at atmospheric pressure, while the inner parts of the mass spectrometer are kept at strong vacuum. Only gas-phase ions move inside of the mass spectrometer, while solvent molecules evaporate in the ESI source, or they are prevented from entering the mass spectrometer by a gas flow (e.g., a curtain gas) at the nozzle of the mass spectrometer. Inside the mass spectrometer, the ions are transferred to a mass analyzer, where they are analyzed according to their *m/z* ratios.

In TQ analyzers, the ion spray from the ion source is guided through quadrupoles that are comprised of four rods that are placed in the corners of a square (**Figure 8**A). The diagonally opposite rods are electrically connected to radio frequency and direct current sources, which causes an electric field between the rods (Watson and Sparkman, 2007). An oscillating electric field is maintained between the rods by changing the radio frequency amplitude and direct current potential, which puts ions in a spiral path through the quadrupole (Watson and Sparkman, 2007; van Breemen and Martinez, 2013). The electric field is adjusted so that only ions with a particular *m/z* ratio pass through the quadrupole at a certain time, while other ions collide with the rods. A selected range of *m/z* values can be scanned with the quadrupoles by gradually changing the parameters of the electric field. After the quadrupoles, the ions reach a detector, which converts the ion signals into electric current signals that are further converted into a mass spectrum by a computer system.

The TQ analyzers enable analyses with tandem mass spectrometry (MS/MS). In these studies, the first quadrupole is used to select or scan ions with the desired *m/z* ratios. The selected precursor ions move to the second quadrupole, i.e., the collision cell, where the ions are fragmented with the aid of a collision gas (e.g., argon, **Figure 8**A). The fragment ions, i.e., the product ions, are formed by collision induced dissociation (CID) reactions, where part of the kinetic energy formed in the collision of precursor ions and collision gas is converted to internal energy of the precursor ions (Westman-Brinkmalm and Brinkmalm, 2009B). The excess internal energy breaks down the chemical bonds in the precursor ions and consequently the product ions are formed. The product ions are transferred to the third quadrupole where they are analyzed according to their *m/z* values. Several different types of MS/MS analyses can be performed with TQ analyzers. For example, in product ion analysis a precursor ion is first selected and fragmented, after which all the formed product ions are scanned at the third quadrupole. In selected/single reaction monitoring (SRM), a precursor ion or ions are selected at the first quadrupole and their specific product ions are scanned at the third quadrupole. If multiple product ions from one or more precursor ions are studied, a term multiple reaction monitoring (MRM) can be used (Murray et al., 2013).

Another way of conducting fragmentation in some instruments is by the so called in-source CID fragmentation (Watson and Sparkman, 2007). This means that the newly formed ions are fragmented in the ESI source before they enter the high vacuum region inside the mass spectrometer. The kinetic energy of ions can be adjusted, for example, by the cone voltage of the system. The accelerated ions collide mostly with nitrogen gas that is present in the residual air of ionization chamber, or with the nebulizer or drying gas. Contrary to MS/MS, the use of in-source CID is unselective, because basically all ions present are susceptible to possible fragmentations.

TQ analyzers are sensitive and they are especially suitable for quantitative analyses because they have wide linear response ranges and rapid duty cycles (10–50 ms), which enable the detection of several data points for each chromatographic peak (Watson and Sparkman, 2007; Westman-Brinkmalm and Brinkmalm, 2009B). TQ analyzers produce low-resolution mass spectra, and the maximal *m/z* range is limited to 4000 or less (Watson and Sparkman, 2007; van Breemen and Martinez, 2013).

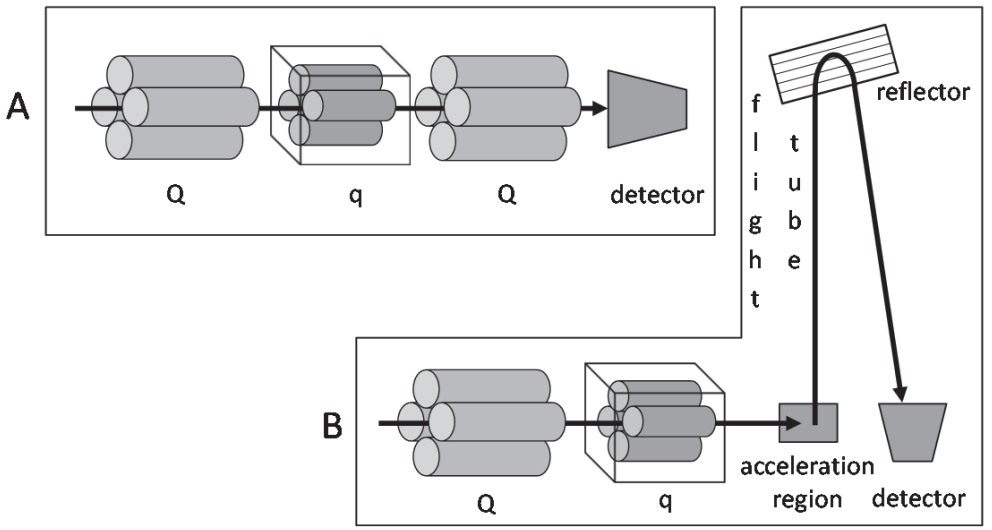



**Figure 8.** A schematic cross-section of a triple quadrupole (**A**) and a quadrupole time-of-flight mass analyzer (**B**). Q denotes a quadrupole and q a collision cell.

A QTOF analyzer is a hybrid technique that combines a quadrupole analyzer (Q) with a time-of-flight analyzer (TOF, **Figure 8**B). The ions first pass through the quadrupole, which resembles the first quadrupole in TQ. Next to the quadrupole is a collision cell that functions in MS/MS measurements in a similar way as in TQ. Sometimes, QTOF is denoted as QqTOF which takes into account the collision cell (Westman-Brinkmalm and Brinkmalm, 2009B). Finally, the ions move to the flight tube that is placed orthogonally to the quadrupole (**Figure 8**B). The ions are accelerated with high-voltage pulses at the beginning of the flight tube after which the ions fly towards the detector (often via a reflector or several reflectors). All ions with a similar charge have a similar kinetic energy at the beginning of the flight, but their velocities are dependent on their masses (Watson and Sparkman, 2007). This causes ions with similar charges but different masses to reach the detector at slightly different times, which is used to determine the mass spectrum.

The linear dynamic range of QTOF analyzers for quantitative analysis is lower than in TQ analyzers (Westman-Brinkmalm and Brinkmalm, 2009B). However, QTOF analyzers produce high-resolution mass spectra and, thus, can be used to measure the accurate masses of analyte ions and their fragments. The accurate masses are used to estimate the molecular composition of ions, which is important when the structures of unknown compounds are determined (van Breemen and Martinez, 2013). The high mass accuracy of QTOF is a combination of several factors (Watson and Sparkman, 2007). First, the flight tube is placed orthogonally to the ion beam coming from the quadrupole region (**Figure 8**). This causes the diminution of longitudinal dispersion of ions (forward and backward) when the ions are accelerated in the TOF tube, which

increases the resolution of ions. Secondly, the reflector equalizes the flight times of ions with the same *m/z* values but slightly different kinetic energies. This means that ions with higher kinetic energies will penetrate deeper into the electric field of the reflector, while similar ions with less kinetic energy will penetrate less in the reflector. Thus, ions with higher kinetic energy will have a slightly longer flying path than ions with lower kinetic energy, so that ions with the same mass reach the detector simultaneously, thereby enhancing mass accuracy. The reflector also increases the flying path of ions enhancing the resolution of ions with different masses.

Both positive and negative ionization modes can be used in the analysis of flavonoids with ESI. The negative ionization often gives better sensitivity for flavonoids, probably because their ionization is enhanced by the deprotonation of the acidic hydroxyl groups (de Rijke et al., 2006; Fossen and Andersen, 2006). However, the positive ionization mode can sometimes give more structural and fragmentation information about the flavonoids (Cuyckens and Claeys, 2004). MS is used, first of all, to elucidate the molecular masses of flavonoids. This can be done by detecting the deprotonated or protonated molecular ions ($[M–H^-]$ or $[M+H]^+$) of the analytes. The ESI conditions may produce adducts both in the positive and negative ionization modes (e.g., a $Na^+$ adduct $[M+23]^+$ or a formic acid adduct $[M+46–H]^-$, de Rijke et al., 2003; Fossen and Andersen, 2006). In addition to molecular ions, also cluster ions (e.g., $[2M–H^-]$) can be formed (Cuyckens and Claeys, 2004; Tian et al., 2002), and they can be used in identifying the molecular masses of unknown compounds. Fragmentation of flavonoids in their first-order mass spectra gives further details about their structures. For example, the cleavage of *O*-glycosidic bonds between the flavonoid and the sugars can be used to elucidate the sugar residues (**Figure 9**). The cleavage of a hexose sugar is detected as an ion that has a 162 Da smaller *m/z* value than the molecular ion, whereas the cleavage of a pentose represents a corresponding ion with a decrease of 132 Da (Tian et al., 2002; Cuyckens and Claeys, 2004).

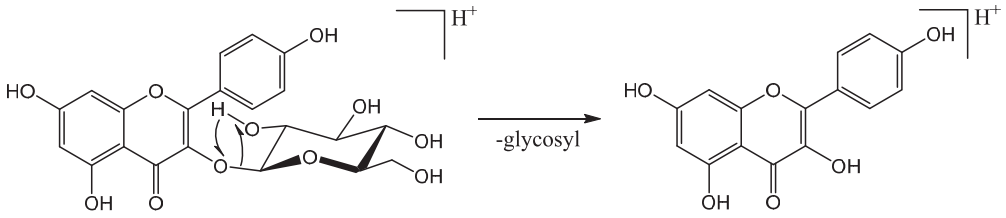



**Figure 9.** A rearrangement reaction cleaves a glycosidic residue from a flavonoid glycoside during its fragmentation in the mass spectrometer. Any of the hydroxyl groups of the sugar can participate in the presented rearrangement reaction (Cuyckens and Claeys, 2004).

The first order mass spectrum of a flavonoid normally reveals only the molecular mass of the flavonoid aglycone and possible acidic or glycosidic residues. More structural

information can be gained with MS/MS analyses of flavonoids (Ma et al., 1997). In a fragmentation reaction, two C–C bonds in the ring C can be cleaved, which cuts the flavonoid aglycone into two fragments: the first fragment includes atoms on the side of ring A ($^{i,j}A^+$ or $^{i,j}A^-$ ion), and the other ion represents atoms on the side of ring B ($^{i,j}B^+$ or $^{i,j}B^-$, **Figure 10**). The masses of the fragments can be used to estimate how many hydroxyl groups or other substituents are attached to rings A and B (Ma et al., 1997; Cuyckens and Claeys, 2004; Marston and Hostettmann, 2006). Ions $^{1,3}A^+$ and $^{1,3}A^-$ are especially significant MS/MS fragments of flavonoids (**Figure 10**; Cuyckens and Claeys, 2004). Further fragments deriving from the loss of small molecules or radicals (e.g., water, carbon monoxide, or methyl group) are also useful in structural elucidation of flavonoids (Ma et al., 1997). MS/MS may also be used to determine the structures of acylated flavonoid glycosides, and the losses of acidic moieties are observed as neutral losses, such as –206 Da (sinapic acid) and –176 Da (ferulic acid; Ferreres et al., 2005; Harbaum et al., 2007).

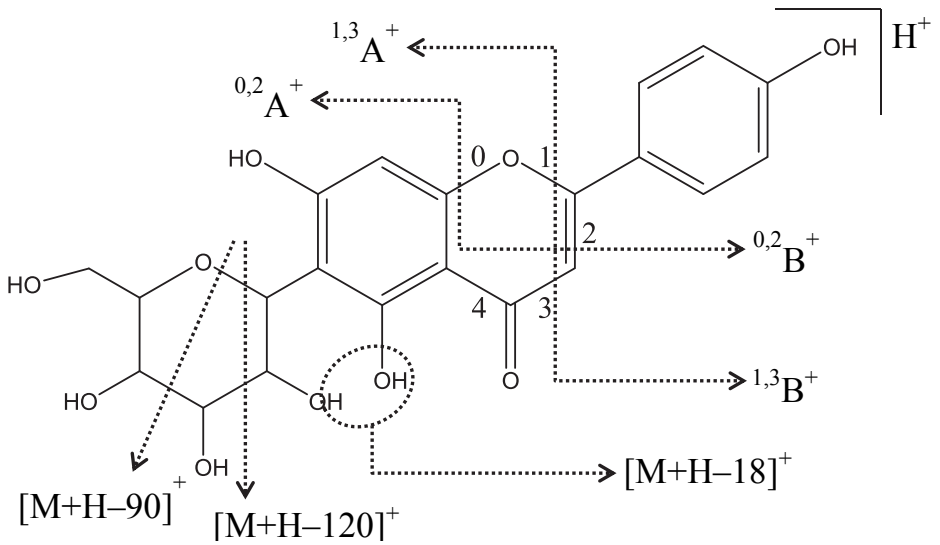



**Figure 10.** Examples of possible MS/MS fragmentation pathways of a flavonoid with a *C*-glycosyl unit (Ma et al., 1997, Cuyckens and Claeys, 2004).

Flavonoids with *C*-glycosyls show only molecular ions without any further fragments in their first-order mass spectra. More structural information about flavonoids with *C*-glycosyls can be achieved with MS/MS. In addition to the $^{i,j}A$ and $^{i,j}B$ ions, fragment ions resulting from the loss of water molecules and the cross-ring cleavage of glycosyl units can be observed (**Figure 10**; Cuyckens and Claeys, 2004; Guo et al, 2013).

### 2.2.3 NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has an important role in the structural elucidation of flavonoids and other phenolic compounds. In NMR spectroscopy, a sample is placed in a homogenous magnetic field after which radio frequency pulses are directed to the sample. Different NMR active nuclei have characteristic frequencies at which they absorb energy from these pulses, which is used to measure an NMR spectrum. NMR spectra provide chemical shifts and coupling constants of NMR active nuclei (most often $^{1}H$ and $^{13}C$). Chemical shifts give information about the chemical environment and neighboring groups and atoms of the NMR active nuclei, while coupling constants show how these nuclei are coupled to each other. Thus, NMR spectra provide information on how individual atoms are spatially situated in the molecule, which can be used to elucidate the chemical structures of unknown compounds. There are a variety of NMR experiments that all give different kinds of structural information. Standard $^{1}H$ NMR experiment provides the relative number of hydrogen atoms (protons), their chemical shifts and spin-spin couplings to neighboring hydrogens (Fossen and Andersen, 2006). Similarly to $^{1}H$ NMR, a $^{13}C$ NMR spectrum provides the chemical shifts of carbon atoms and the relative number of carbon atoms in the molecule.

In the case of flavonoid glycosides, $^{1}H$ NMR spectra also show the signal (or signals) of an anomeric sugar proton whose coupling constant to the neighboring sugar proton determines whether the glycosyl is in $\alpha$- or $\beta$-anomeric configuration (Markham and Geiger, 1994). Further, the chemical shifts and coupling constants of anomeric and other protons help to determine the structure of the glycosyl unit. This is important, since the MS analyses of flavonoid glycosides are unable to differentiate between two different glycosyls that have same molecular masses (e.g. glucose and galactose). The position of an acyl group attached to a sugar unit can sometimes be deduced from the $^{1}H$ NMR spectrum, as the chemical shift of the sugar proton next to the acyl group often shows a marked downfield shift (Markham and Geiger, 1994).

Two dimensional NMR experiments show how different atoms are bonded or spatially situated in relation to each other. For example, correlation spectroscopy (COSY) is used to determine which hydrogen atoms are coupled to each other. Heteronuclear single quantum correlation (HSQC) experiment determines which two nuclei of different atoms (most often $^{1}H$ and $^{13}C$) are directly bonded to each other. Heteronuclear multiple bond correlation (HMBC) spectra show correlations between two different atoms (for example $^{1}H$ and $^{13}C$) that usually are separated by 2–4 bonds. In flavonoid research, HMBC is especially useful in elucidating the attachment sites of glycosyl and acyl groups, which cannot be determined by the first-order mass spectra of flavonoids (Veitch et al., 2005, 2011; Fossen and Andersen, 2006; Hirayama et al., 2008). Total correlation spectroscopy (TOCSY) experiments are useful in the assignment of glyco-

syl protons, especially in flavonoid oligoglycosides where the proton signals of different glycosyls are often overlapping (Fossen and Andersen, 2006). The positions of glycosyl units and other structural details of flavonoids can also be determined with $^{1}H–^{1}H$ experiments with nuclear overhauser enhancement spectroscopy (NOESY) and rotating frame overhauser effect spectroscopy (ROESY) that show protons that are spatially close to each other in the molecule (Tamura, 2002; Veitch et al., 2005; Fossen and Andersen, 2006).

An advantage of NMR is that it keeps analyte compounds intact for their possible further use, whereas in MS the analyte compounds are degraded. However, a rather high amount of an analyte (often a few milligrams) is needed for NMR experiments, and the NMR spectra of mixtures can be difficult to interpret (Fossen and Andersen, 2006). Eluent flow from LC can be coupled to NMR (LC–NMR). This technique could be used to analyze mixtures of phenolic compounds without the time-consuming purification of individual compounds, but the instrumentation and the use of deuterated solvents can be too expensive for routine use (Marston and Hostettman, 2006; Karagianis and Waterman, 2008). As noted above, the need for rather large sample sizes in NMR is another limiting factor for the use of LC–NMR as compared to LC–MS.

## 2.3 The roles of flavonoids in plants

Flavonoids are ubiquitously present in different plant species, where they are often present as glycosides in the vacuoles of cells (Iwashina, 2000). Several roles have been attributed to flavonoids in plants. These include, for example, working as signaling molecules and protecting tissues against UV radiation (Treutter, 2000, and references cited therein). Flavonoids are found as color pigments in many flowers in addition to other pigments, such as carotenoids and chlorophylls. The purpose of flower pigments is to attract pollinating insects. Flavonoid pigments include, for example, anthocyanins that cause cyanic or bluish colors, flavone and flavonol glycosides causing white or cream colors, and chalcones, aurones, and some flavonols contributing to yellow colors (Harborne and Grayer, 1994). Flavonoids can also be used as ultraviolet pigments that are visible to some pollinating insects but are invisible to humans (Harborne and Grayer, 1994).

Flavonoids have many and complex roles in plant–insect interactions (Harborne, 2001; Simmonds, 2001). Depending on the insect species and the individual compound, flavonoids can have both negative and stimulating effects on larval herbivores (Harborne and Grayer, 1994; Renwick, et al., 2001; Simmonds, 2001). Quercetin and its glycosides reduced the growth of gypsy moth (*Lymantria dispar* L.) larvae feeding on different pine species (Beninger and Abou-Zaid, 1997), and flavonoid aglycones in birch leaves possibly acted as defensive compounds against the autumnal moth (*Epirrita*

*autumnata*, Lahtinen et al., 2004). However, in general, flavonoids seem to have only limited defensive effects against insects (Harborne, 2001).

## 2.4 The larvae of Hymenoptera and Lepidoptera

The insect order of Lepidoptera is divided into butterflies (Rhopalocera) and moths (Heterocera). Lepidopterans are mostly phytophagous, which means that their caterpillars consume plant material (mostly leaves, but also sometimes wood and fruits; Holland, 1968). The order of Hymenoptera contains, for example, bees, wasps, and ants, but also the suborder Symphyta of sawflies. The larvae of many sawfly species feed on trees, mostly chewing externally the leaves or needles of their host plant (Haack and Mattson, 1993). Other feeding guilds of sawfly species include, e.g., gall-producing sawflies and borers of shoots and fruits (Haack and Mattson, 1993). Both lepidopterans and sawflies have economic importance since many of their species are pests as regards agriculture and forestry (Haack and Mattson, 1993; Usmani and Knowles, 2001). The larvae of some species have periodic outbreaks, including the pine-feeding sawfly *Neodiprion sertifer* (Larsson and Tenow, 1984) and the birch-feeding moth *Epirrita autumnata* (Kallio and Lehtonen, 1973).

Both lepidopterans and sawflies are found from subarctic to tropical regions (Koponen, 1983; Kouki et al., 1994; Pinto-Tomás et al., 2011; Smith et al., 2013). It has been speculated that tropical lepidopteran larvae could be more specialized to certain host plant species, genera, or families than lepidopteran species in temperate regions (Dyer et al., 2007). One explanation is that tropical plant species possibly contain more efficient and versatile chemical defenses than temperate plants (Coley and Barone, 1996; Dyer et al., 2007). In contrast to a general trend where the highest species richness is often found in tropical regions, most sawfly species are concentrated in northern latitudes (Kouki et al., 1994).

## 2.5 Flavonoids and other phenolic compounds in larval metabolism

The digestive tract of lepidopteran and sawfly larvae is broadly divided into three sections: foregut, midgut, and hindgut (Terra et al., 1996; **Figure 11**). Secondary plant compounds are subject to different chemical environments and metabolic processes as they move through the digestive tract of larvae.

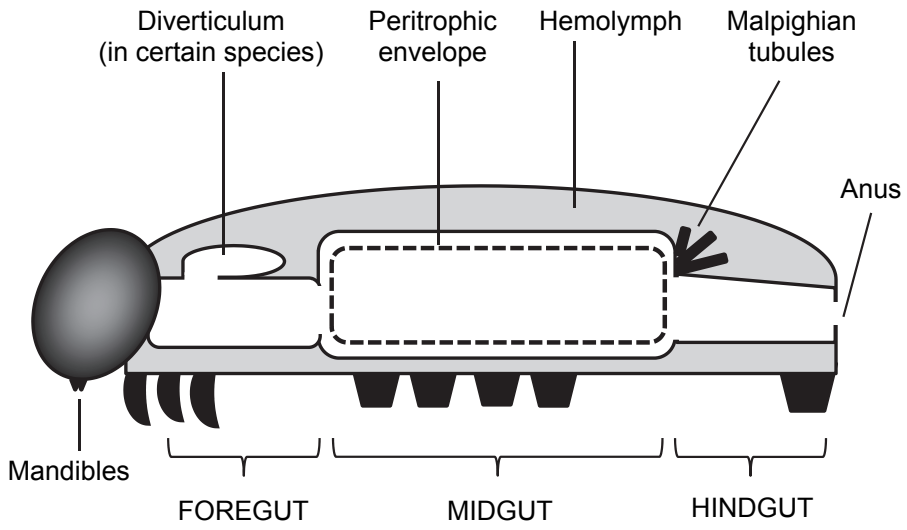



**Figure 11.** A simplified presentation of larval anatomy (Chapman, 1985; Terra et al., 1996).

### 2.5.1 Feeding and foregut

Leaf-chewing insects use their mouthparts (e.g., mandibles) for cutting and grinding the tissues of their host plant (Smith, 1985; **Figure 11**). After feeding, the ground plant material first reaches the foregut. The foreguts of lepidopteran larvae are reported to be mostly slightly acidic to neutral (Appel and Martin, 1990; Appel and Maines, 1995; Barbehenn and Martin, 1994), but the conditions may be alkaline in some species (Appel and Martin, 1990). The acidic or neutral conditions promote the formation of protein-tannin complexes, which possibly decreases the nutritive value of plant material for the larvae (Salminen and Karonen, 2011; Schultz and Lechowich, 1986). The rather neutral conditions in the foregut do not seem to favor the autoxidation of phenolic compounds in a way that is expected in the alkaline midguts of the larvae. The oxidation of phenolics is covered in detail in the Midgut-section.

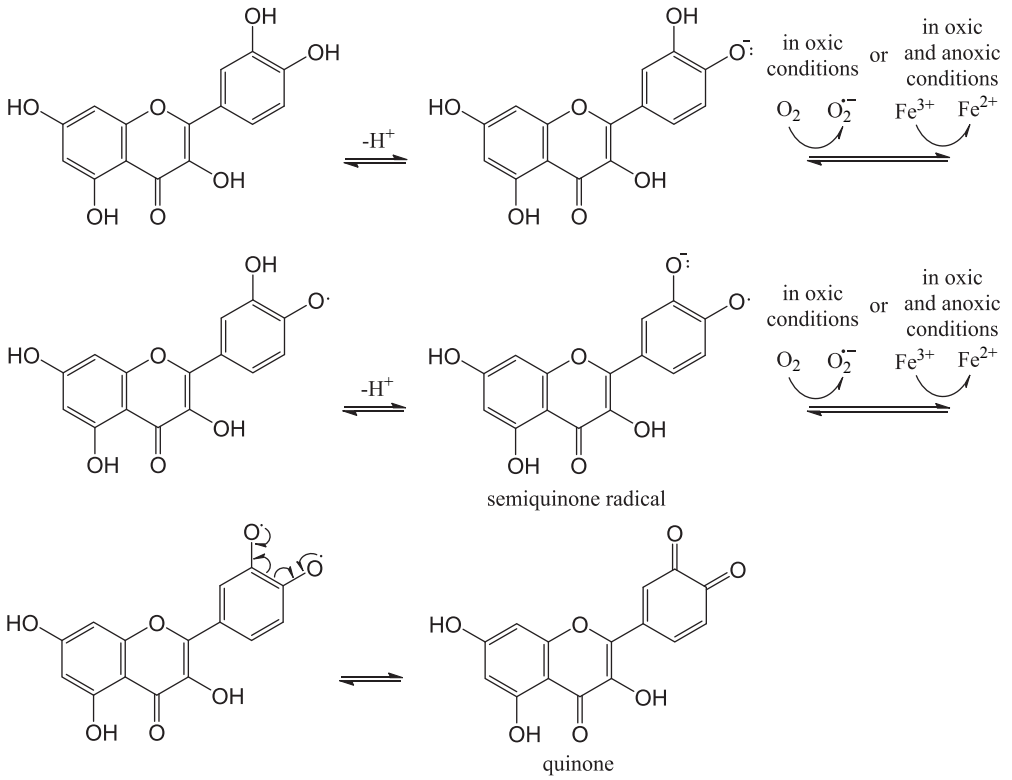
Some sawfly species concentrate plant derived secondary compounds in special pouches (diverticulum) of their foregut (**Figure 11**). For example, sawfly larvae in the subfamily Pergidae concentrate oils (containing terpenoids) from *Eucalyptus* trees into the pouches (Carne, 1962; Morrow et al., 1976), and the larvae of *Neodiprion sertifer* sequester terpenoid resins from the needles of *Pinus sylvestris* (Eisner et al., 1974). When approached, the larvae regurgitate a foul-tasting mixture from the pouches, which repels predators. It has been found that even some lepidopteran species have diverticulum pouches in their foregut, where the larvae store terpenoid-containing oils form their host plant (Common and Bellas, 1977). Moreover, in this case the oily regurgitate seems to have a defensive function.

### 2.5.2 Midgut

After the foregut, the food material is transferred to the midgut, where it is processed by larval digestive enzymes, and most of the food components are absorbed (Chapman, 1985; **Figure 11**). The midguts of lepidopterans probably also contain symbiotic bacteria (Pinto-Tomás et al., 2011), but their possible interactions with phenolic compounds are rather unknown at present.

It has been hypothesized that phenolic compounds would have especially high biological activities in insect midguts, where the phenolics would act as pro-oxidant substances (Appel, 1993). The oxidation of phenolics would be a part of a complex chain of redox reactions that liberate reactive oxygen species (ROS), which in turn causes oxidative stress to the larvae (Appel, 1993; Summers and Felton, 1994; Barbehenn et al., 2001). According to a suggested oxidation scheme, a semiquinone radical is first formed from a phenolic compound, and the semiquinone is quickly transformed to a quinone (**Figure 12**; Appel, 1993). Electrons are liberated during the oxidation of phenolic compounds, and these electrons can be accepted by an oxygen molecule or a metal cation, which leads to their reduction (**Figure 12**). The reduced oxygen molecule forms a superoxide radical ($O_2^{-\bullet}$) that can further produce several other ROS, such as hydroxyl radical, hydrogen peroxide, and other peroxides (Reaction 1 in **Figure 12**; Appel, 1993; Summers and Felton, 1994). Metal cations catalyze the formation of ROS. For example, $Fe^{3+}$ can be reduced to $Fe^{2+}$, which, in the presence of hydrogen peroxide, participates in the formation of detrimental hydroxyl radical ($OH^{\cdot}$) in a Fenton reaction (Reaction 2 in **Figure 12**; Summers and Felton, 1994; Barbehenn et al., 2005).

The quinones formed can covalently bind to plant proteins and amino acids, which may decrease their nutritional value to larvae (Felton et al., 1989). Reactive oxygen species (e.g. hydroxyl radicals) may cause lipid peroxidation (Summers and Felton, 1994) and oxidize proteins, which generates protein carbonyls in larvae (Barbehenn et al., 2005). Steinly and Berenbaum (1985) fed larvae of two species of *Papilio* butterflies with leaves that were supplemented with tannins (chemical structures not specified). They found large numbers of midgut lesions in the butterfly species whose larvae do not naturally consume tannin-containing plant species. The lesions were smaller and less frequent in the species that was specialized to consuming plants with tannins in nature. Similar midgut lesions were detected in the lepidopteran *Spodoptera eridania* when the diet was supplemented with two phenolic glycosides (salicortin and tremulacin, Lindroth and Peterson, 1988). The actual cause of the lesions could not be solved, but it seems possible that they were generated by the oxidative stress imposed by phenolic compounds.




(1) $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

(2) $H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^- + Fe^{3+}$ (Fenton reaction)

**Figure 12.** The oxidation of quercetin and the concurrent formation of hydroxyl radical in alkaline conditions. (Summers and Felton, 1994; Barbehenn et al., 2005).

Different factors influence the oxidation of phenolics in insects. Leaves contain oxidative enzymes (polyphenol oxidases and peroxidases) that are used in defense against herbivores (Barbehenn et al., 2010; Bhonwong, et al. 2009; Felton et al., 1989). The oxidative enzymes are separated from their phenolic substrates in plant cells. When an herbivore ruptures the plant tissue, the oxidative enzymes come into contact with the phenolics, and the resulting quinones can cause oxidative stress to the larvae (Felton et al., 1989). Phenolic compounds are sometimes prone to autoxidation (or autoxidation), which means their spontaneous and non-enzymatic oxidation. The rate of autoxidation of phenolics is accelerated with increasing pH and is especially high in alkaline conditions (Hodnick et al., 1986; Cilliers and Singleton, 1989; Tuominen and Sundman, 2013). The midguts of different species of lepidopteran larvae show variable pH conditions, and values as high as pH 11–12 have been measured in some species (Berenbaum, 1980; Dow, 1984), which would favor autoxidation reactions (Appel, 1993). Many oxidation reactions need atmospheric oxygen to proceed and, thus,

the rates of oxidation are reduced, or the reactions are even stopped, in anoxic conditions (Cilliers and Singleton, 1989). The centers of midguts in lepidopteran larvae are generally nearly anoxic (Gross et al., 2008; Johnson and Barbehenn, 2000), which probably slows down the rates of oxidation reactions. However, oxygen may penetrate into the outer region of the midguts, which may allow oxidation reactions in this part of the midgut (Gross et al., 2008).

Different types of phenolic compounds oxidize with variable efficiencies in alkaline conditions (Barbehenn et al., 2006a; Moilanen and Salminen, 2008; Tuominen, 2013). For example, ellagitannins, a subclass of hydrolysable tannins, have been shown to have higher *in vitro* oxidative activities at high pH than galloyl glucoses or proanthocyanidins (Barbehenn et al., 2006a). Barbehenn et al. (2008) fed larvae of the lepidopteran *Orgyia leucostigma* with leaves from tree species that contained different amounts and compositions of tannins and other phenolic compounds. The authors found high amounts of semiquinone radicals, precursors of phenolic oxidation, in the midguts of larvae that had consumed leaves with high amounts of ellagitannins or galloyl rhamnoses. The leaves dominated by proanthocyanidins produced only little semiquinone radicals in the midguts.

Some lepidopteran species seem to tolerate oxidative stress caused by phenolics compounds. For example, elevated levels of reactive oxygen species did not have a significant effect on the composition of essential amino acids in the midguts of *Lymantria dispar* (Barbehenn et al., 2012). Lepidopteran midguts contain antioxidants (e.g., ascorbic acid and glutathione) and antioxidant enzymes (e.g., catalase, ascorbate peroxidase, and superoxide dismutase; Ahmad and Pardini, 1990; Barbehenn et al., 2001; Felton and Duffey, 1991a; Mathews et al., 1997) that are aimed at diminishing the negative effects of reactive oxygen species. Flavonoids are known to perform as anti- or pro-oxidants depending on their chemical environment (Cao et al, 1997). In some circumstances, phenolic compounds (e.g., catechin and quercetin) showed antioxidant activities in the midguts of lepidopteran larvae (Johnson and Felton, 2001; Johnson, 2005). Moreover, proanthocyanidins decreased the formation of semiquinone radicals from hydrolysable tannins when these two types of phenolics were incubated together at pH 10 (Barbehenn et al., 2006b).

In spite of the above-mentioned negative effects, the larvae should also benefit from maintaining the alkaline conditions in their midguts. For example, alkaline conditions can help to extract proteins and amino acids from plant tissues (Appel and Maines, 1995; Felton and Duffey, 1991b). Additionally, the hydroxyl groups of phenolic compounds are deprotonated in alkaline conditions, which prevents the formation of hydrogen bonds between plant proteins and tannins (Appel, 1993), thus preventing the formation of tannin-protein complexes, or enabling the dissociation of already formed complexes (Feeny, 1969; Berenbaum, 1980). In addition to this, it is possible that the oxidation products of phenolic compounds may disturb midgut pathogens, as was sug-

gested in the interaction between quinones and baculoviruses in the larvae of *Helicoverpa zea* (Felton and Duffey, 1990).

### 2.5.3 Peritrophic envelope

Food material in an insect's midgut is situated inside a peritrophic envelope (or membrane or matrix) that is mainly constructed of chitin, proteins, and proteoglycans (Tellam, 1996; **Figure 11**). The envelope constitutes a semipermeable membrane that allows the movement of dietary compounds and their absorption. For example, some larger molecules (e.g., polymeric macromolecules), viruses, bacteria, or parasites may be kept inside the peritrophic cavity, whereas smaller molecules (e.g., some enzymes or degradation products of dietary compounds) can permeate through the envelope (Tellam, 1996). Compounds permeating through the envelope then reach the epithelial cells of the midgut, where they are absorbed. The peritrophic envelope seems to function as an antioxidant layer that protects the midgut epithelium from oxidative stress (Barbehenn and Stannard, 2004). It has been suggested that the peritrophic envelope of the lepidopteran *H. zea* scavenges hydroxyl radicals and inhibits the formation of reactive oxygen species (Summers and Felton, 1996). Some phenolic compounds seem to have low permeability across the peritrophic envelope of lepidopteran larvae. For example, tannic acid (a commercial and chemically variable mixture of gallotannins; Salminen and Karonen, 2011) did not permeate through the peritrophic matrix of several lepidopteran species (Barbehenn, 2001). Isman and Duffey (1983) found that only 2−5% of the ingested chlorogenic acid and rutin (quercetin diglycoside) were present in the hemolymph of *H. zea* suggesting that only small amounts of these phenolics had passed through the gut wall.

There are several theories as to why the peritrophic envelope is able to permeate or retain some substances, such as phenolic compounds. Phenolic compounds are in an anionic form in alkaline midguts, and according to one theory these polyphenolate anions might not diffuse through the negatively charged sites in the peritrophic envelope (Barbehenn and Martin, 1994). In another study, it was found that tannic acid forms colloidal aggregates when mixed with the midgut fluid of the caterpillar *Manduca sexta*, and these aggregates might explain the ability of the peritrophic envelope to retain tannin-type compounds (Barbehenn and Martin, 1998). The same authors further suggested that these colloids could contain complexes that are formed between phenolic anions and metal cations, or other molecules in the insect gut.

### 2.5.4 Hemolymph

After ingestion by the midgut epithelium, some of the dietary compounds and their metabolic products are moved into the hemolymph of the larvae (**Figure 11**). Hemolymph serves in many important functions, such as transporting nutrients and storing compounds (Mullins, 1985). It also participates in excretion and thermoregulation, and has defensive functions against diseases and parasites. The pH of the hemolymph of lepidopteran and sawfly larvae is slightly acidic to neutral (pH 6.4–6.9; Dow, 1984; Gringorten et al., 1994, Heimpel, 1955). Certain phenolic compounds have been found to transfer from the larval diet into the hemolymph. By using liquid scintillation counting, Isman and Duffey (1983) found that the lepidopteran *H. zea* absorbed approximately 2–5% of the ingested $^{3}$H-labeled chlorogenic acid or rutin into its hemolymph. In another study with *H. zea* and *Spodoptera exigua*, 4–8% of the $^{3}$H-labeled chlorogenic acid was found in the larval hemolymph (Felton et al., 1989). Quercetin mono- and diglucosides were found in the hemolymph of the lepidopteran *Bombyx mori*, and the larvae had probably produced these compounds from dietary quercetin (Hirayama et al., 2008).

Phenolic compounds may increase the antioxidant capacity of larval hemolymph. Johnson and Felton (2001) studied genetically modified tobacco plants that produced elevated levels of phenolic compounds. When these phenolic-rich leaves were fed to the larvae of the lepidopteran *Heliothis virescens*, the antioxidant capacity of the hemolymph was higher than in larvae that had consumed leaves with lower amounts of phenolics. When catechin and quercetin were incubated in the midgut and hemolymph fluids of the lepidopteran *Manduca sexta*, the antioxidant capacity of these fluids was increased, but chlorogenic and caffeic acids sometimes showed slightly pro-oxidant activities in the same conditions (Johnson, 2005). On the contrary, Hirayama et al. (2008) found that the antioxidant capacity of hemolymph of *B. mori* did not increase when the larval diet was supplemented with quercetin.

The larvae of many sawfly species are able to sequester secondary compounds from their host plant and to store them in their hemolymph. This ability has been reported with sawfly species consuming multiple host families, and the variety of sequestered compounds ranges from furostanol saponins to glucosinolates, iridoid glucosides and steroid alkaloids (Müller et al., 2001; Opitz et al., 2010; Prieto et al., 2007; Schaffner et al., 1994). Studies have implied that the sequestered secondary compounds act as defense compounds against the predators of larvae, such as ants (Müller and Brakefield, 2003; Opitz et al., 2010; Prieto et al., 2007). Many sawfly species are able to push out a small droplet of their hemolymph through their integument (“easy bleeding”), and this foul tasting droplet can deter enemies of the larvae (Boevé and Schaffner, 2003; Boevé and Müller, 2005). The sawfly species *Tenthredo zonula* feeding on *Hypericum* plants sequestered flavonoid glycosides into the body of the larvae

(Crockett and Boevé, 2011). Nevertheless, there seems to be no reports where sawfly or lepidopteran larvae have sequestered phenolic compounds in their hemolymph for defensive purposes.

### 2.5.5 Excretion

The malpighian tubules lie in the intersection of the midgut and hindgut (**Figure 11**). Their roles include filtering the hemolymph, removing toxic and waste products, and producing the primary urine of insects (Bradley, 1985). Malpighian tubules also excrete uric acid, which is the main nitrogen-containing waste product of many insects (Cochran, 1985). The last section of the alimentary canal, the hindgut, mainly absorbs water and salts from urine and feces (Chapman, 1985) before the undigested food material is excreted through the rectum and anus. The hindgut pH of several lepidopteran species is reported to be 7.1–7.6 (Appel and Martin, 1990).

Many types of phenolic compounds have been found in the frass of lepidopteran and sawfly larvae (Burghardt, 1997; Lahtinen et al., 2005; Ruuhola et al., 2001; Salminen and Lempa, 2002). It can be estimated how well individual phenolic compounds are degraded or utilized by the larvae, if the concentrations of phenolics in the larval diet and frass are known, and the amount of food consumed by the larvae is measured. This approach was used in the study of Ruuhola et al. (2001), where the generalist moth *Operophtera brumata* was fed with the leaves of different willow species. By analyzing the leaf and frass samples with HPLC–DAD, they estimated that generally more than 50 % of the digested flavonoids were degraded in *O. brumata*. In the freshwater moth *Acentria ephemerella*, a major dietary ellagitannin (tellimagrandin II) was not detected in the larval frass indicating that this compound had been degraded (Gross et al., 2008). Frass analyses have revealed variable metabolic fates for different phenolics. For example, galloyl glucoses were prone to hydrolysis (Salminen and Lempa, 2002; Lahtinen et al., 2005), and chlorogenic acid formed isomers in larval guts (Salminen et al., 2004; Lahtinen et al., 2005). Depending on compound and insect species, flavonoids can be sulfated, glycosylated, deglycosylated, and deacylated in larvae (Ferreres et al., 2008; Salminen et al., 2004; Lahtinen et al., 2005).

## 2.6 Flavonoids and other phenolic compounds in insect life cycles

The lifecycles of lepidopterans and sawflies have many similar features, and they both undergo holometabolism or complete metamorphosis (**Figure 13**). First, an adult female insect lays its eggs on a suitable host plant. After a sufficient time, larvae hatch from the eggs and start consuming their host plant. The fully developed larvae pupate, after which an adult insect (imago) emerges, and the development cycle begins again

from the start. The roles of phenolic compounds in pupal, adult, and egg stages of lepidopterans and sawflies are discussed in the following section.

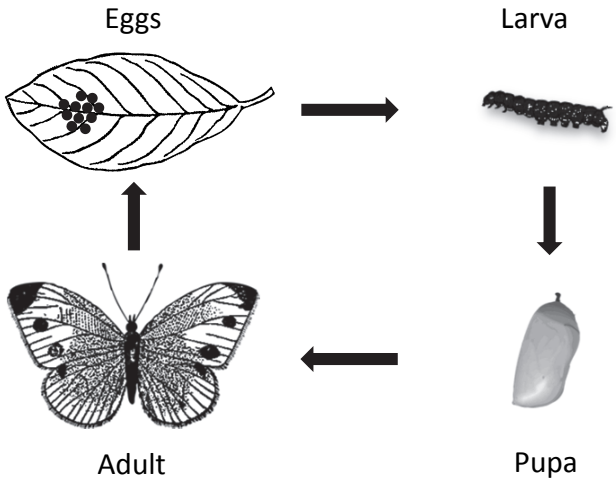



**Figure 13.** Different stages of complete metamorphosis (Holland, 1968).

### 2.6.1 Pupae and cocoons

Lepidopterans and sawflies move to the pupal stage after their larval period. During the pupal stage, the larval structures of the insect are slowly broken down, while the adult structures (such as wings) are formed. Different types of flavone and flavonol glycosides have been detected in the pupae of butterflies (Geuder et al., 1997; Schittko et al., 1999), and these compounds are later transferred to the wings of adult butterflies (see below).

Pupae are inactive, which makes them rather vulnerable to predation. Many lepidopteran and sawfly larvae produce a cocoon to protect the pupae. The cocoons are formed from fibrous protein, i.e., silk, that is produced in the labial glands in the head section of the larvae (Sehnal and Sutherland, 2008). Some larvae are able to sequester plant-derived secondary compounds in their cocoons. For example, the pyralid moth *Uresiphita reversalis* sequesters quinolizidine alkaloids from its host plant in the cocoon silk (Wink et al., 2001). In addition, dietary flavonoids of lepidopteran larvae may be transferred to the cocoons. The cocoons of the silkworm (*Bombyx mori*) feeding on mulberry (*Morus alba*) contain flavonoids (Hayashiya et al., 1959). These flavonoids were identified according to their mass, NMR, and UV–Vis spectral properties as derivatives of kaempferol and quercetin (aglycones and mono–triglycosides; Tamura et al., 2002; Hirayama et al., 2008). Even quercetin with amino acid (proline)

substituents has been found in the cocoons of *B. mori* (Hirayama et al., 2006). The cocoons of *Rondotia menciana*, a relative of *B. mori* also feeding on *M. alba* contained kaempferol and quercetin galactosides (Hirayama et al., 2013). Flavonol glycosides found in the cocoons of these two species were not present in mulberry leaves implying that the larvae had probably modified these compounds from dietary flavonols (Tamura et al., 2002; Hirayama et al., 2013). Flavonoids can possibly shield cocoons against UV–B radiation (Daimon et al., 2010), or increase their anti-oxidant capacity (Kurioka and Yamazaki, 2002). The cocoon extracts of *B. mori* have shown antimicrobial activity that is probably due to antimicrobial proteins (Pandiarajan et al., 2011), but the possible role of flavonoids in this aspect seems to be unknown.

The cocoons of the sawfly *Gilpinia hercyniae* feeding on spruce needles (*Picea abies*) contain catechin (Schopf et al., 1982). It was suggested that catechin from spruce needles was first glycosylated by the larvae and then stored in the silk glands. During cocoon spinning, the catechin glycoside would have been hydrolyzed and used in the sclerotization (tanning) of the cocoon. In sclerotization, catechin was eventually covalently bound to proteins in the cocoon, and the unbound portion of catechin was detected in the study. The sawfly *Perga affinis affinis* sequesters terpenoid oils from eucalyptus leaves in the pouches in its foregut, and the larvae later use this liquid to construct their cocoons (Carne, 1962). On the contrary, no resin acids were found in the cocoon shells of pine sawflies (Björkman and Gref, 1993), even though these compounds are important constituents of the defensive secretions of these larvae (Eisner et al., 1974). The cocoons of the lepidopterans *Myrascia* sp. contained sacs filled with oily terpenoids (Common and Bellas, 1977). These sacs originate from the larval phase, as the larvae sequester oils from their host plant. The oily sacs are possibly aimed at deterring predators of the cocoons.

### 2.6.2 Adult insects

As mentioned above, adults of some butterfly species contain flavonols or flavones that are are especially found in their wings (Morris and Thomson, 1963; Wilson, 1985; Wiesen et al., 1994; Geuder et al., 1997), while a smaller portion of the flavonoids is maintained in other body parts (Geuder et al., 1997). This phenomenon has been detected in approximately 10 % of butterfly species, but it is rare or absent in moths (Harborne, 1985). Larvae seem to selectively sequester certain flavonoids of their host plant and to neglect other compounds (Wiesen et al., 1994; Burghardt et al., 1997; Geuder et al., 1997). For example, kaempferol-3-*O*-rhamnoside was detected as the main flavonol glycoside in the adults of *Polyommatus icarus*, when quercetin-3-*O*-rhamnoside was present in a higher concentration in the host plant (Burghardt et al., 1997). Myricetin glycosides were missing from the adults of *P. icarus* feeding on different hosts (Burghardt et al., 1997; Schittko et al., 1999), and their selective excretion

by the larvae may reflect their possible toxicity to *P. icarus* (Schittko et al., 1999). In some cases, lepidopterans also modify some of the sequestered flavonoids (Geuder et al., 1997; Wiesen, 1994). Flavonoids are assumed to function as UV active pigments of butterfly wings, and they can help with mate recognition between females and males (Burghardt et al., 2000; Geuder et al., 1997). The use of flavonoids for defensive purposes in lepidopteran wings is improbable (Harborne, 2001).

Uric acid (a major nitrogenous waste product of insects) and its metabolites have been detected in the wings and other organs of adult lepidopterans, where they could functions as pigmentation (Lafont and Pennetier, 1975; Tojo and Yushima, 1972). There seem to be no studies that focus on the role of phenolic compounds in adult sawflies.

### 2.6.3 Oviposition and eggs

Female lepidopterans and sawflies search for appropriate plants to lay their eggs (oviposition). In some lepidopteran species, oviposition is stimulated by chlorogenic acid and different flavonoid glycosides (Feeny et al., 1988; Haribal and Renwick, 1996; Nishida, 1994). Some phenolics are inactive alone, but they stimulate oviposition when present in an appropriate combination with other compounds (Feeny et al., 1988; Nishida, 1994). In addition to this, the oviposition of a gall-inducing sawfly was found to be stimulated by tremulacin (a phenolic glucoside) in willow leaves (Roininen et al., 1999).

Flavonoids have even been found in the eggs of the butterflies *Melanargia galathea* and *Pseudozizeeria maha*, and these compounds are probably transferred to eggs via the adult female butterflies (Wilson, 1985; Mizokami and Yoshitama, 2009). The purpose of flavonoids in eggs is unknown. Some lepidopteran species are also known to transfer other types of secondary compounds to their eggs. For example, the eggs of the moth *Cosmosoma myrodora* contain alkaloids with possible anti-predator activities (Conner et al., 2000). Adult Ithomiini butterflies sequester pyrrolizidine alkaloids from nectars in the flowers of their host plants. These compounds are later transferred into the eggs to protect them against predators (Brown, 1987).

# 3 AIMS OF THE STUDY

The main aim of this thesis was to study flavonoids and other phenolic compounds, and to examine their chemical interactions with herbivorous larvae. The first part of the thesis focuses on flavonoids found in the hemolymph of sawfly larvae (Articles **I** and **II**), while the second part concentrates on the modifications of phenolic compounds in the guts of lepidopteran larvae (Articles **III** and **IV**). The main themes of the original publications were:

1. The determination of flavonoids in the hemolymph of birch- and pine-feeding sawfly larvae (Article **I** and **II**).
2. The characterization of phenolic compounds found in the hemolymph of sawfly larvae (Article **II**), and in the host tree leaves and larval frass of lepidopteran larvae (Article **IV**) by MS and NMR techniques.
3. The modification and excretion of phenolic compounds were studied with sawfly and lepidopteran larvae (Articles **II** and **IV**).
4. The *in vitro* estimation of pro-oxidant capacities of plant crude extracts, and the identification of individual phenolic compounds responsible for the detected pro-oxidant capacities (Articles **III** and **IV**).
5. The comparison of the phenolic profiles of leaf extracts incubated *in vitro* and larval frass to determine whether, the *in vitro* results could be used to predict the outcome of phenolic compounds in the metabolism of lepidopteran larvae (Article **IV**).

# 4 MATERIALS AND METHODS

## 4.1 Plant and insect samples

**Table 1.** The plant and insect species studied in articles **I**–**IV**.

| **Study I** | | **Study III** | **Study IV** | | |
|---|---|---|---|---|---|
| **Plant** | **Insect**[a] | **Plant** | **Plant** | | **Insect**[b] |
| *Betula nana* | *Amauronematus amplus* | *Achillea ptarmica* | *Alchornea castaneifolia* | → | Saturniidae* |
| *Betula pendula* | *Arge* sp. | *Aesculus hippocastanum* | *Caryodendron orinocense* | → | *Panacea prola* |
| *Betula pubescens* subsp. *czerepanovii* | *Dineura pullior* | *Betula pubescens* | *Copaifera paupera* | → | *Hyerchiria nausica* |
| | *Nematus brevivalvis* | *Celtis durandii* | *Ormosia macrocalyx* | → | *Acharia* sp. 1 |
| | *Nematus pravus* | *Fragaria moschata* | *Psidium guajava* | → | *Acharia* sp. 2 |
| | *Nematus viridescens* | *Iris* sp. | *Senna reticulata* | → | *Automeris liberia* |
| | *Nematus viridis* | *Oxyanthus stenocarpus* | *Tabebuia ochracea* | → | *Isognathus* sp. |
| | *Pristiphora alpestris* | *Picea abies* | *Terminalia catappa* | → | *Pyrrhopyge amyclas denticulata* |
| | *Trichiosoma scalesii* | *Prunus africana* | *Tetrathylacium macrophyllum* | → | Pyralidae* |
| **Study II** | | *Quercus robur* | *Theobroma bicolor* | → | Saturniidae* |
| **Plant** | **Insect**[a] | *Ribes alpinum* | *Vernonia patens* | → | *Dysschema eurocilia staudingeri* |
| *Pinus sylvestris* → | *Neodiprion sertifer* | *Salix phylicifolia* | *Vochysia braceliniae* | → | Sericochroa sp. |

Abbreviations: [a] sawfly species, [b] lepidopteran species, * identification only on a family level. All nine larval species in **I** were fed with all three Betula species. Larvae in **II** and **IV** consumed only one tree species (marked with an arrow).

The plant and insect species studied in articles **I**–**IV** are presented in **Table 1**, and example pictures of the insect species are shown in **Figure 14**. Larvae for **I** were collected near the Kevo Subarctic Research Station in Utsjoki, northern Finland in 2004. Needles of *P. sylvestris* and the larvae of European pine sawfly (*Neodiprion sertifer*) for **II** originated from Taivassalo, southern Finland in 2009. Plant material for **III** was collected from Finland and Uganda. The Finnish samples (*Achillea ptarmica*, *Aesculus hippocastanum*, *Betula pubescens*, *Fragaria moschata*, *Iris* sp., *Picea abies*, *Quercus robur*, *Ribes alpinum*, and *Salix phylicifolia*) were collected from the Botanical Garden

of the University of Turku and the nearby forests in Turku between 2011 and 2013. The Ugandan samples (*Celtis durandii*, *Oxyanthus stenocarpus*, and *Prunus africana*) were collected near to the vicinity of the Makerere University Biological Station (MUBFS) in Kibale National Park, western Uganda in 2011. Samples for **IV** were collected from the Amazonian National Reserve Allpahuayo–Mishana (NRAM) in the region of Loreto, Peru during 2011.

In addition to the above-mentioned insect species, the hemolymphs of other sawfly species were analyzed for their possible phenolic compounds. Only one hemolymph sample per each of these sawfly species was studied. These species were the pine-feeding *Diprion pini* (common pine sawfly), *Acantholyda posticalis* (great web-spinning pine-sawfly), and three unidentified sawfly species feeding on the leaves of redcurrant (*Ribes rubrum*) and gooseberry (*Ribes uva-crispa*).

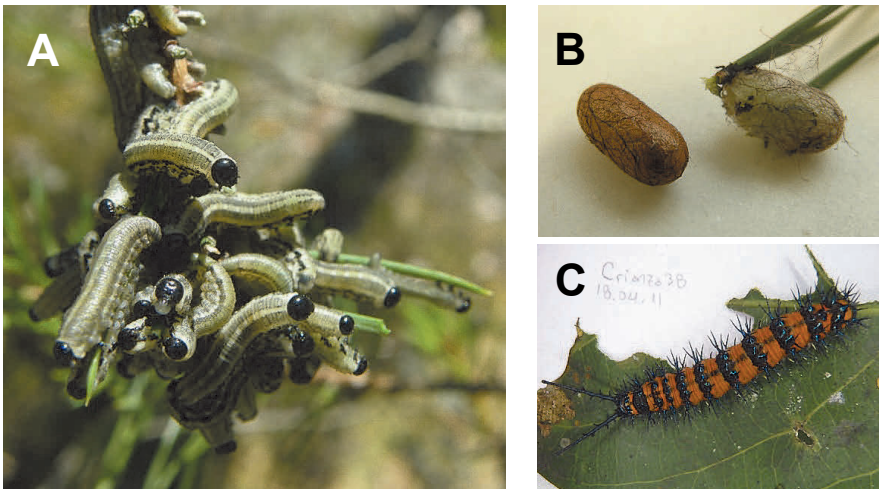



**Figure 14.** Larvae of *Neodiprion sertifer* feeding on *Pinus sylvestris* (**A**), cocoons of *Diprion pini* (**B**), and a larva of *Panacea prola* feeding on *Caryodendron orinocense* (**C**, photo Isrrael Gómez Avila).

## 4.2 Larval rearings

Studies **I**, **II** and **IV** included rearing periods of hymenopteran and lepidopteran larvae. Study **I** used a field-grown "laboratory stock" of sawflies that had been reared from eggs to 2nd-3rd instars in mesh bags on mountain birches (*B. pubescens* subsp. *czerepanovii*). Studies **II** and **IV** used larvae that were collected from the field. The rearings were done rather similarly in each study, but with some modifications (see individual articles for details). To summarize, the larvae were taken to laboratory, where they were reared for several days with the leaves of their natural host trees. In **I**,

the larvae of each sawfly species were divided into three groups that were fed with the leaves of one of the three birch species (**Table 1**). In **II** and **IV**, the larvae were fed with the leaves or needles of a single tree species. After the feeding period of variable length in each study, frass samples were collected from the larvae in studies **II** and **IV**, and hemolymph samples were collected from the sawfly larvae in studies **I** and **II**. The hemolymphs of lepidopteran species in **IV** were tentatively analyzed for phenolic compounds for this thesis (unpublished results).

## 4.3 Preparation of leaf, frass, hemolymph, and cocoon samples

For the chemical analyses of leaf and frass samples in articles **II**, **III** and **IV**, the samples were lyophilized, ground, and extracted with organic solvents. The extraction protocol differed between studies (see more details from individual articles). In brief, the ground leaf or frass samples were first repeatedly extracted with aqueous 70 or 80% acetone, after which the extracts were combined, and acetone was evaporated from the extracts. The extracts were then lyophilized, and finally dissolved in water to give a solution that contained water-soluble phenolic compounds for LC–DAD–MS analyses. For some samples in **III** and **IV**, the samples were further extracted with dichloromethane–methanol (1:1, v:v), after which the combined acetone–water and dichloromethane–methanol extracts were liquid–liquid extracted with hexane and water. The water phase of the liquid–liquid extraction contained water soluble phenolic compounds that were analyzed with LC–DAD–MS.

Freshly collected hemolymph samples (5 or 20 µl) of birch-feeding sawfly larvae were analyzed in **I** with HPLC–DAD and HPLC–ESI–MS without pretreating or filtering the samples. This gradually deteriorated the column and decreased its resolving power. To avoid this, the hemolymph samples in **II** were first fractioned with a solid phase extraction (SPE) cartridge (Sep-Pak® Plus Short tC18 Cartridge, Waters). The cartridge filtered the water-soluble impurities of hemolymph, while lipophilic compounds were retained in the cartridge. Hemolymph samples of 10–20 µl were applied to a SPE cartridge (preconditioned with water), after which the hemolymph was fractioned in the following 2 ml fractions: $H_2O$, 40% aqueous MeOH, and 100% MeOH. The organic solvent was evaporated from the fractions after which the samples were lyophilized. The lyophilized samples were further dissolved in water prior to analysis with LC–DAD–MS. The SPE method was also used for the fractionation of hemolymph samples of the pine-feeding sawflies *D. pini* and *A. posticalis* and the two unidentified sawfly species feeding on *Ribes* leaves (unpublished results).

Hemolymph samples of tropical lepidopteran larvae studied in **IV** were preliminarily studied for their phenolic compounds. Two hemolymph samples from two individual larvae were analyzed from each of the twelve lepidopteran species. The hemolymph

samples had been collected in ethanol, which produced white precipitants in the sample tubes (probably denaturized proteins). The samples were centrifuged, and 100 µl of the supernatant was diluted in 400 µl of water. The samples were analyzed with UHPLC–DAD–MS by detection at 280 nm and with the same SRM/MRM method that were used for the detection of phenolic compounds in leaf and frass samples in **IV** (see next section).

During the rearing of sawfly larvae, a few larvae of the pine-feeding *N. sertifer* and *D. pini* and one birch-feeding larva of *Nematus* sp. constructed cocoons. The cocoons were cut open with small scissors and the pupae were removed. The cocoon shells were repeatedly shaken in water for 5 min (3 × 500 µl) to wash away possible traces of hemolymph from the cocoon. The cocoons were then extracted 2 × 1 hr with 700 µl of 70 % aqueous acetone. The extracts were combined, after which the acetone was evaporated, and the extracts were lyophilized. The extracts were then dissolved in water and analyzed with HPLC–DAD–MS.

## 4.4 Liquid chromatographic and mass spectrometric analyses

Different liquid chromatographic and mass spectrometric equipment and methods were used throughout the thesis. Phenolic compounds were identified by comparing their UV and mass spectra with literature data, and with the aid of commercial standards. An HPLC–DAD system (Merck–Hitachi, Tokyo, Japan) was used to analyze hemolymph samples of birch-feeding sawfly larvae in **I**. The flavonol oligoglycosides were quantitated from the UV chromatograms of hemolymph samples using quercetin as an external standard. In addition to this, hemolymph samples in **I** were qualitatively analyzed with an HPLC system that was attached to a triple quadrupole mass spectrometer (Perkin–Elmer Sciex API 365, Sciex, Toronto, Canada). In **II**, qualitative analyses of samples and the accurate masses of phenolic compounds were acquired with an HPLC–DAD system (Agilent Tecnologies 1200 series, Agilent Technologies, Walbronn, Germany) that was combined with a QTOF mass spectrometer (Bruker micrOTOF$_Q$, Bruker Daltonics GmbH, Bremen, Germany). The chromatographic separation and quantitation of phenolics in **II**–**IV** were obtained with a UHPLC–DAD–MS system (Acquity UPLC®, Waters Corporation, Milford, MA, USA) attached to a triple quadrupole mass spectrometer (Xevo® TQ, Waters Corporation, Milford, MA, USA). MS/MS experiments were used to elucidate the structures of some phenolics in **III** and **IV**.

The quantitation of phenolic compounds in **II** and **IV** was performed with SRM/MRM methods; the MS conditions used are listed in **Table 2**. The changes in the operational factors of the mass spectrometer (e.g. ionization efficiency) between different analysis times were taken into account by using a solution of (+)-catechin as an external stand-

ard. In **II**, flavonoids were quantified with external calibration curves using corresponding flavonoids isolated from the larvae of *N. sertifer* (see next section)**.** The SRM/MRM method in **IV** analyzed the total amounts of phenolics belonging to different subgroups of phenolic compounds (Engström et al., 2014 and a manuscript). The external standards used in **IV** were: kaempferol-3-*O*-glucoside for kaempferol derivatives, quercetin-3-*O*-glucoside for quercetin derivatives, myricetin-3-*O*-rhamnoside for myricetin derivatives, chlorogenic acid for quinic acid derivatives, a dimeric procyanidin for procyanidins, a dimeric prodelphinidin for prodelphinidins, pentagalloyl glucose for galloyl derivatives, and oenothein B for ellagitannins.

**Table 2.** Selected or multiple reaction monitoring methods for detecting phenolic compounds in articles **II** and **IV**.

| **Article II** | | | | |
|---|---|---|---|---|
| **Compound** | **Precursor ion** | **Product ion** | **Cone voltage (V)** | **Collision energy (eV)** |
| (+)-Catechin | 289 | 245 | 34 | 14 |
| Isorhamnetin-3-*O*-glucoside | 477 | 314 | 46 | 28 |
| Kaempferol-3-*O*-glucoside | 447 | 284 | 40 | 26 |
| Quercetin-3-*O*-glucoside | 463 | 300 | 38 | 28 |
| (+)-Catechin 7-*O*-*β*-glucoside | 451 | 289 | 34 | 18 |
| Quercetin 3,7,4′-tri-*O*-*β*-glucoside | 787 | 625 | 42 | 24 |
| Kaempferol 3,7,4′-tri-*O*-*β*-glucoside | 609 | 447 | 64 | 22 |
| Isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside | 639 | 477 | 68 | 24 |
| **Article IV** | | | | |
| Procyanidins | 287 | 125 | 85 | 15 |
| | 289 | 245 | 75 | 15 |
| Prodelphinidins | 305 | 125 | 80 | 20 |
| Quinic acid derivatives | 191 | 127 | 50 | 15 |
| Ellagitannins | 301 | 145 | 95 | 35 |
| | 301 | 200 | 95 | 40 |
| Galloyl derivatives | 169 | 125 | 55 | 15 |
| Kaempferol derivatives | 284 | 255 | 95 | 25 |
| | 285 | 257 | 70 | 25 |
| Myricetin derivatives | 317 | 179 | 45 | 20 |
| Quercetin derivatives | 300 | 271 | 85 | 25 |
| | 301 | 151 | 70 | 20 |

## 4.5 Isolation and identification of phenolic compounds

Three unknown flavonol triglycosides and a catechin monoglycoside present in the hemolymph of *N. sertifer* were isolated from larval extracts (**II**). Several unknown phenolic compounds were isolated from the leaf and frass extracts in **IV**. These compounds included an unknown compound from the leaves of *Caryodendron orinocense*, two compounds from the leaves of *Copaifera paupera*, and one compound from the

frass of the lepidopteran *Dysschema eurocilia staudingeri* feeding on *Vernonia patens*. The obtained extracts were purified with a semi-preparative HPLC system with a DAD detector and a fraction collector (Waters Corporation, USA) to give fractions containing the desired compounds.

NMR spectroscopy was used to elucidate the structures of the isolated compounds. Several 1D and 2D NMR experiments were used for their identification, such as $^{1}$H NMR, $^{13}$C NMR, 1D-TOCSY, HSQC and HMBC. Glycosidic bonds in flavonoid glycosides in **II** were hydrolyzed with hydrochloric acid, and the formed hydrolysis products were derivatized prior to their analysis with GC–MS (GC Autosystem XL with TurboMass Gold mass spectrometer, Perkin Elmer, Norwalk, USA). The absolute configuration of the aglycone part of catechin monoglycoside in **II** was examined with Chirascan™ circular dichroism spectrometer (Applied PhotoPhysics, Leatherhead, UK) by comparing the spectrum of catechin monoglycoside to the spectrum of (+)-catechin. The compound isolated from the frass of *D. eurocilia staudingeri* was further analyzed with inductively coupled plasma mass spectrometry (ICP–MS, Perkin Elmer 6100 DRC Plus, Perkin Elmer Sciex, Toronto, Canada) because the compound was suggested containing manganese (**IV**).

## 4.6 Pro-oxidant activities of phenolic compounds

Based on a method presented in Salminen and Karonen (2011), plant extracts were incubated in alkaline solutions that mimicked the midgut conditions of lepidopteran larvae (**III** and **IV**). To summarize, plant extracts (20 µl) were first pipetted in wells of a 96-well microplate reader (Thermo Multiskan Ascent, Thermo Electron Corporation, Shanghai, China). Then, 180 µl of a carbonate buffer at pH 10 (50 mM; sodium carbonate/sodium hydrogen carbonate) was added to the wells, which initiated the oxidation of phenolic compounds. The samples were neutralized after 1 hr with 100 µl of 0.6% formic acid, which stopped the oxidation reactions. The amounts of total phenolic compounds in both initial and incubated plant extracts were estimated by a modified Folin–Ciocalteu assay. The amounts of total phenolics in the initial and incubated extracts were compared, which showed how large proportion of the total phenolics disappeared (oxidized) during incubations at pH 10. This proportion was denoted as the pro-oxidant capacity of the plant species, and it was measured for 24 plant species (**Table 1**).

Preliminary analyses showed highly variable pro-oxidant capacities for different plant species. The phenolic compounds causing these differences in oxidative capacities were not known and, thus, an additional analytical step was added to the method of Salminen and Karonen (2011). This UHPLC–DAD–MS step was used to analyze both the initial and incubated plant extracts in **III** and **IV**. In addition to this, the chromato-

graphic profiles of incubated plant extracts were compared with the chromatograms of frass extracts of lepidopteran larvae that had been consuming the same plant species (**IV**).

## 4.7 Feeding test with ants

To test whether sawfly larvae use the flavonoid glycosides of their hemolymph in defensive functions, field tests were performed in Turku, Finland, during the summer of 2011 (unpublished results). The field tests comprised of two choice tests with *Formica polyctena* ants. In choice test 1 (10th June 2011), *F. polyctena* were offered two types of dead larvae of *Epirrita autumnata*: the other larva had been dipped in a water solution of quercetin 3,7,4'-tri-*O*-*β*-glucoside (10 mg/ml, the compound was isolated from *N. sertifer*) and the other larva had been dipped in water (control). The dipped larvae were allowed to dry for few minutes before the choice test. The control and flavonoid-dipped larvae were placed 2 cm from each other on a trail of *F. polyctena* (3–5 m from the ant nest). The two larvae were placed on the ant trail simultaneously, after which it was measured how quickly each larvae was picked up by the ants (**Figure 15**).

Choice test 2 (14th July 2011) was similar to test 1 but in this test dead larvae of *N. sertifer* were dipped in the hemolymph of *N. sertifer* or in water (control). The hemolymph had been collected from multiple larvae. The combined hemolymph was centrifuged and the supernatant was used in the test. Both choice tests were repeated five times in one ant nest, and both tests were made in ten different ant nests, making in total 50 replicates for both tests. Different ant nests were used in tests 1 and 2. Results were statistically analysed with a mixed model analysis of variance (ANOVA) using nest as a random factor.

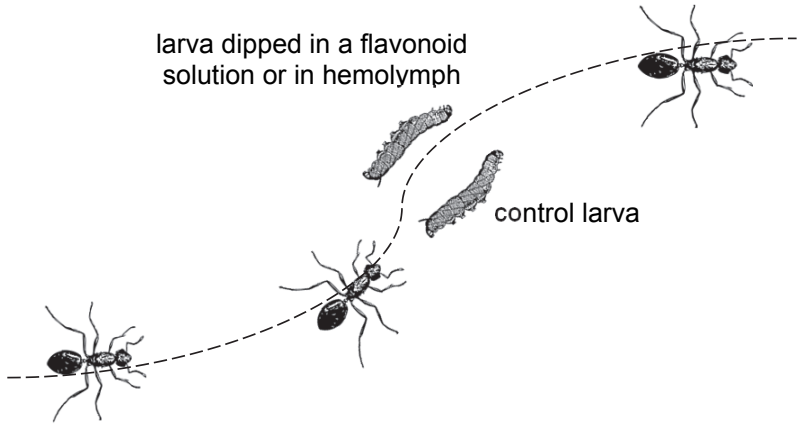



**Figure 15.** Choice tests with *Formica polyctena* ants.

# 5 RESULTS AND DISCUSSION

## 5.1 Considerations of the analytical methods

Flavonoids and other phenolic compounds and their roles in the chemical ecology of lepidopteran and sawfly larvae were studied with the aid of several analytical techniques. The liquid chromatographic and mass spectrometric techniques used became gradually more complex during the course of the work. The first chromatographic analyses in **I** were made with HPLC–DAD, but later studies used modern UHPLC techniques to separate compounds (**II**–**IV**). The use of UHPLC shortened the analysis times from 47 min in **I** to 8.5–11.5 min in **II**–**IV**, produced sharper peaks, and enhanced their separation (**Figure 16**).

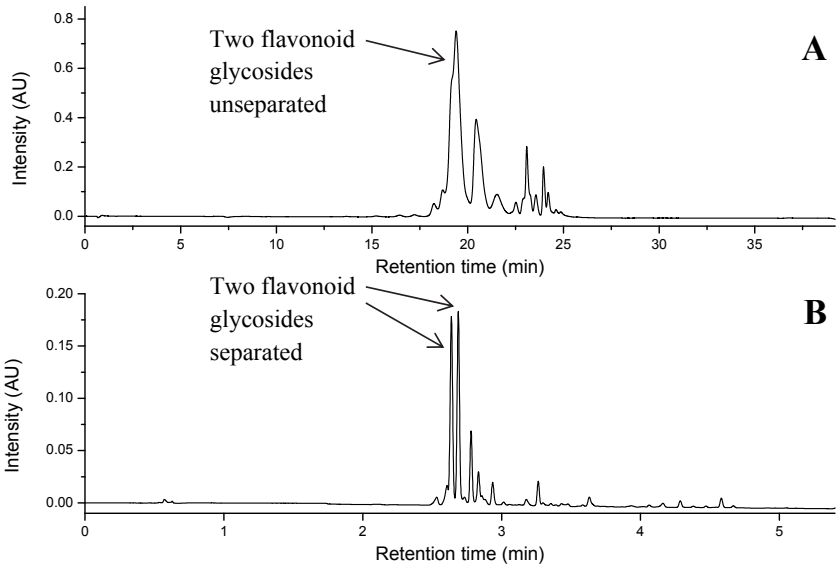



**Figure 16.** The HPLC–DAD (**A**) and UHPLC–DAD (**B**) chromatograms of hemolymph samples of *Neodiprion sertifer* (measured at 349 nm). Different samples were used in the separate LC runs.

The quantitation methods of phenolic compounds developed during the work. Flavonoid oligoglycosides in **I** were quantified with UV detection, but in **II** and **IV**, phenolic compounds were quantified with MS. UV detection is useful for the quantitation of phenolics if individual peaks are chromatographically well separated and they do not co-elute. In **I**, flavonoid oligoglycosides produced a rather unresolved hump in the

HPLC–DAD chromatogram (similar to **Figure 16**A), and flavonoids were thus quantified as "total flavonoids" instead of quantifying individual compounds separately.

Detection limits are lower in MS than in UV detection, and co-eluting compounds can be quantified separately. The operational factors of mass spectrometers (e.g., ionization efficiency) fluctuate with time, and this should be taken into account in quantitative MS analyses. To monitor these changes, runs with an external standard solution of (+)-catechin were measured between analytical samples and different work days. Regardless of whether UV or MS quantitation is used, the most reliable results are obtained when the quantitation standards are identical or chemically close to the analytes. In **II**, flavonoid glycosides in the hemolymph of *N. sertifer* (three flavonol triglycosides and a catechin glycoside) were quantified against standards that were isolated from the same larvae, which increased the accuracy of the quantitation. Phenolic compounds in **IV** were quantified with a group-specific SRM/MRM method that estimated the total amounts of phenolics belonging to different phenolic subgroups (Engström et al., 2014). The SRM/MRM method detected product ions that are typically formed from different subgroups of phenolics during the ionization in ESI–MS. For example, the amounts of quercetin derivatives were estimated by detecting the formation of product ions from precursor ions at *m/z* 300 and 301. Different quercetin derivatives will probably fragment at different efficiencies in MS/MS, which means that the concentrations of individual quercetin derivatives will be either under- or overestimated if only one quercetin standard is used for the quantitation. More accurate quantitation results would be acquired by the use of several quercetin standards that chemically resemble the quantified quercetin compounds.

## 5.2 Phenolic compounds in hemolymph

The hemolymph samples of sawfly larvae were studied with HPLC– or UHPLC–DAD–MS in **I** and **II**. The hemolymph samples in **I** were analyzed without any purification steps, but the hemolymph samples in **II** were first fractioned with SPE. Flavonoids were found in the fraction obtained with 40% aqueous methanol but not in other fractions. The HPLC– and UHPLC–DAD chromatograms of hemolymph samples in **I** and **II** were dominated by peaks whose UV spectra resembled those of flavonols. Band I absorption maxima of flavonol derivatives (close to 344 nm) showed a hypsochromic shift when compared to the flavonol aglycone or monoglycosides (**Figure 17**). Also, the elution times of hemolymph compounds were shorter than would have been expected for simple flavonol monoglycosides. This implied that the flavonols in hemolymph probably contained more than one glycosyl units because more hydrophilic compounds are known to elute earlier in reverse-phase conditions than more lipophilic compounds.

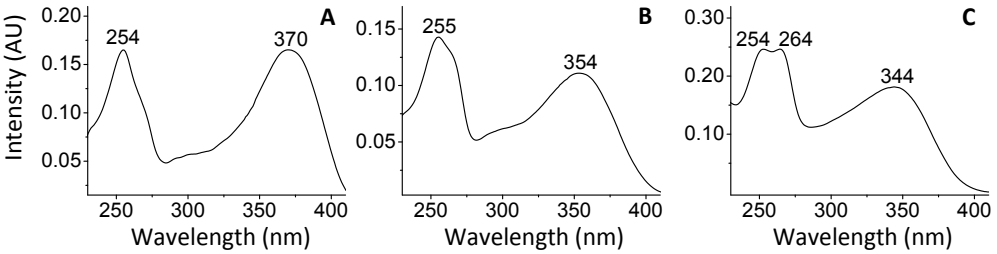



**Figure 17.** The UV spectra of quercetin derivatives from UHPLC–DAD analyses with 0.1 % formic acid and acetonitrile in the solvent mixture: quercetin (**A**), quercetin-3-*O*-glucoside (**B**), and quercetin-3,7,4′-tri-*O*-glucoside (**C**).

The mass spectra of hemolymph flavonoids showed a distinct fragmentation pattern in the positive ionization mode, where the neighboring ions were separated by 132 or 162 Da from each other (Figure 3 in **I**). This fragmentation pattern indicated a stepwise cleavage of glycosyl units from the flavonoid (–132 Da represents a pentosyl unit and –162 Da a hexosyl unit). The hemolymph of birch-feeding sawfly larvae was thus shown to contain flavonol oligoglycosides, i.e., tri-, tetra-, penta-, and hexaglycosides of kaempferol and quercetin (**I**). Examples of flavonol oligoglycosides detected are presented in **Table 3**. The hemolymph samples of birch-feeding sawfly larvae were first analyzed with low resolution HPLC–MS, but the samples were later re-analyzed with high-resolution HPLC–DAD–QTOF, which showed that the hemolymph of several birch-feeding sawfly species also contained small amounts of myricetin oligoglycosides (**Table 3**).

The exact chemical structures of the flavonol oligoglycosides in birch-feeding sawfly larvae could not be solved because of the limited amount of sample material (**I**). Similar flavonol oligoglycosides were tentatively found in the hemolymph of the pine-feeding sawfly *N. sertifer* (**Figure 16**). The larvae of this species sometimes occur as large outbreak populations, which enabled the collection of several hundred larvae, and thus the isolation of four flavonoid oligoglycosides from the larval extracts (**II**). The GC–MS analyses of the isolated compounds proved that the glycosyl units in flavonoid glycosides were glucoses, while ECD data showed that the absolute configuration of the isolated catechin glycoside was *2R,3S*, i.e., (+)-catechin. Finally, based on the ECD, GC–MS, LC–DAD–MS and NMR data, the isolated compounds were identified as a (+)-catechin 7-*O*-*β*-glucoside, isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside, kaempferol 3,7,4′-tri-*O*-*β*-glucoside, and quercetin 3,7,4′-tri-*O*-*β*-glucoside (**Figure 18**, **Table 3**). The NMR data of the isolated compounds is presented in **II**.

**Table 3.** MS and UV spectral data of selected phenolic compounds found in the hemolymph of sawfly and lepidopteran species.

| **Compound** | **UV spectrum $\lambda_{max}$ (nm)** | **$[M+H]^+$** | **$[2M+H]^+$** | ***m/z* values of other ions[a]** |
|---|---|---|---|---|
| Sawfly *Arge* sp. feeding on *Betula pendula* | | | | |
| Quercetin hexaglycoside | 203, 254, 265, 346 | 1275 | – | 1113, 951, 789, 627, 465, 303 |
| Kaempferol pentaglycoside | 267, 344 | 1097 | – | 935, 773, 611, 449, 287 |
| Sawfly *Nematus pravus* feeding on *Betula nana* | | | | |
| Quercetin tetraglycoside | 202, 266, 338 | 951 | 1901 | 789, 627, 465, 303 |
| Myricetin triglycoside | 206, 254, 265, 350 | 805 | 1609 | 643, 481, 319 |
| Sawfly *Neodiprion sertifer* feeding on *Pinus sylvestris* | | | | |
| (+)-Catechin 7-*O*-*β*-glucoside | 203, 227, 278 | 453 | 905 | 291 |
| Isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside | 202, 251, 265, 344 | 803 | 1605 | 641, 479, 317 |
| Kaempferol 3,7,4′-tri-*O*-*β*-glucoside | 195, 265, 319, 338 | 773 | 1545 | 611, 449, 287 |
| Quercetin 3,7,4′-tri-*O*-*β*-glucoside | 202, 253, 264, 344 | 789 | 1577 | 627, 465, 303 |
| Sawfly *Diprion pini* feeding on *Pinus sylvestris* | | | | |
| Catechin monoglycoside | 202, 229, 278 | 453 | 905 | 291 |
| Coumaroylquinic acid | 226, 293 (sh), 311 | 339 | – | 147 ($[M+H–192]^+$) |
| Feruloylquinic acid | 293 (sh), 324 | 369 | – | 177 ($[M+H–192]^+$) |
| Quercetin tetraglycoside | 199, 267, 336 | 951 | 1901 | 789, 627, 465, 303 |
| Quercetin triglycoside | 202, 254, 265, 344 | 789 | 1577 | 627, 465, 303 |
| Kaempferol triglycoside | 195, 267, 320, 339 | 773 | 1545 | 611, 449, 287 |
| Isorhamnetin triglycoside | 202, 252, 266, 345 | 803 | 1605 | 641, 479, 317 |
| Sawfly species 1 feeding on *Ribes uva-crispa* | | | | |
| Quercetin triglycoside | 255, 355 | 757 | 1513 | 611, 465, 303 |
| Kaempferol triglycoside | 265, 348 | 741 | 1481 | 595, 449, 287 |
| Sawfly species 2 feeding on *Ribes uva-crispa* | | | | |
| Coumaroylquinic acid | 229, 292 (sh), 311 | 339 | – | 147 ($[M+H–192]^+$) |
| Quercetin tetraglycoside | 254, 264, 344 | 951 | – | 789, 627, 465, 303 |
| Lepidopteran species of Saturniidae feeding on *Theobroma bicolor* | | | | |
| Catechin monoglycoside | 280 | 451[b] | – | 289 ($[M–H–162]^-$) |
| Lepidopteran *Pyrrhopyge amyclas denticulata* feeding on *Terminalia catappa* | | | | |
| Kaempferol monoglycoside | – | 447[b] | – | – |
| Quercetin monoglycoside | 256, 353 | 463[b] | – | – |

sh = shoulder, [a] The ions of flavonoid glycosides show consecutive cleavages of glycosyls from the flavonoid glycoside (mostly –162 Da, a hexosyl unit), [b] Analyzed with negative ionization mode, ions represent $[M–H]^-$.

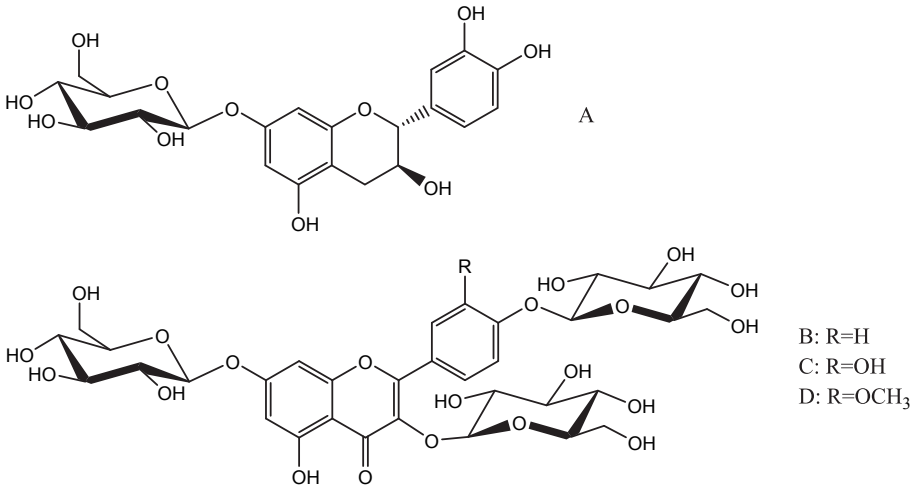



**Figure 18.** The structures of flavonoid glycosides present in the hemolymph of *Neodiprion sertifer*: (+)-catechin 7-*O*-*β*-glucoside (**A**), kaempferol 3,7,4′-tri-*O*-*β*-glucoside (**B**), quercetin 3,7,4′-tri-*O*-*β*-glucoside (**C**), and isorhamnetin 3,7,4′-tri-*O*-*β*-glucoside (**D**).

Hemolymph samples of other sawfly species were fractionated with SPE and analyzed with HPLC– or UHPLC–DAD–MS (unpublished results). The SPE fraction obtained with 40 % aqueous methanol from the pine-feeding *D. pini* showed a complex UHPLC–DAD chromatogram containing phenolic compounds (**Figure 19**; **Table 3**). The main flavonol triglycosides and the catechin monoglycoside in the hemolymph of *D. pini* are probably the same compounds that were characterized in **II** from *N. sertifer* (as suggested by their similar retention times and UV and mass spectral properties, **Table 3**). The hemolymph of another pine-feeding sawfly, *A. posticalis*, did not show clear peaks of flavonol oligoglycosides or other phenolic compounds (chromatogram not shown). The hemolymphs of two unidentified sawfly species feeding on *R. uva-crispa* contained flavonol oligoglycosides and a coumaroylquinic acid (**Table 3**). The hemolymph of a third, unknown, sawfly species feeding on *R. rubrum* and *R. uva-crispa* likewise contained small peaks of flavonol oligoglycosides (data not shown).

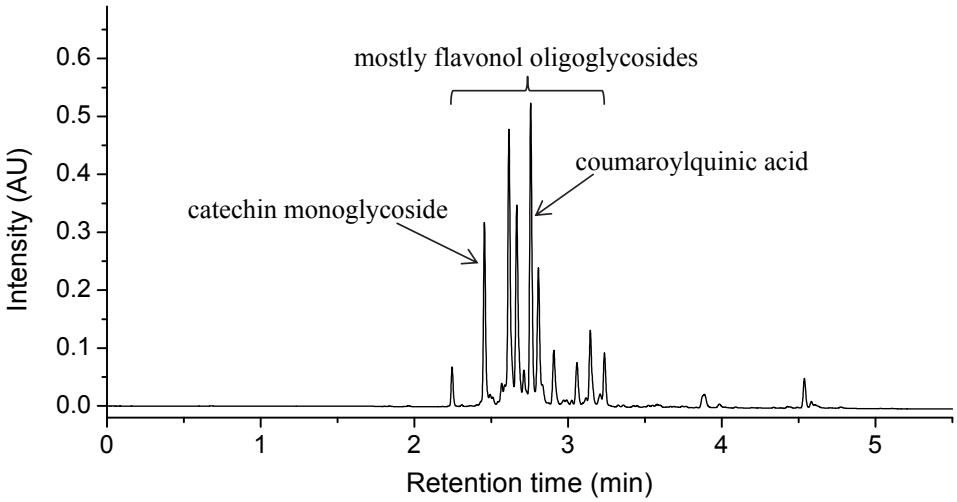



**Figure 19.** The UHPLC–DAD chromatogram of the hemolymph of *Diprion pini* at 349 nm.

None of the flavonoid oligoglycosides present in the hemolymph of birch- or pine-feeding sawfly larvae was found in the leaf or needle diet of the larvae implying that these compounds were probably produced by the larvae from simpler flavonoids present in their diet. It was originally hypothesized that birch-feeding sawflies use flavonoid aglycones in birch leaves as the source of flavonoid oligoglycosides (**I**). To test this hypothesis, birch-feeding sawfly species were fed with the leaves of three birch species that were known to contain different compositions of epidermal flavonoid aglycones (Valkama et al., 2003, 2004). However, these epidermal flavonoids (e.g., methylated flavonoids and the flavones apigenin and acacetin) were missing from the hemolymph samples, except for the flavonol kaempferol in some species. In addition, the hemolymph of larvae feeding on different birch species produced relatively similar HPLC–DAD and chemical profiles regardless of the birch diet that the larvae had consumed (**Figure 20**). This suggested that the larvae had probably not used flavonol aglycones as the source of flavonol oligoglycosides in their hemolymph. Instead, flavonol monoglycosides or (+)-catechin present in birch leaves and pine needles seem to be more likely precursors for the flavonoids in hemolymph. The hemolymph of different birch-feeding sawfly species contained approximately 0.6–12.3 mg/ml of flavonoid glycosides (**I**), whereas the concentration in the hemolymph of the pine-feeding *N. sertifer* was at least 3.7 mg/ml (**II**).

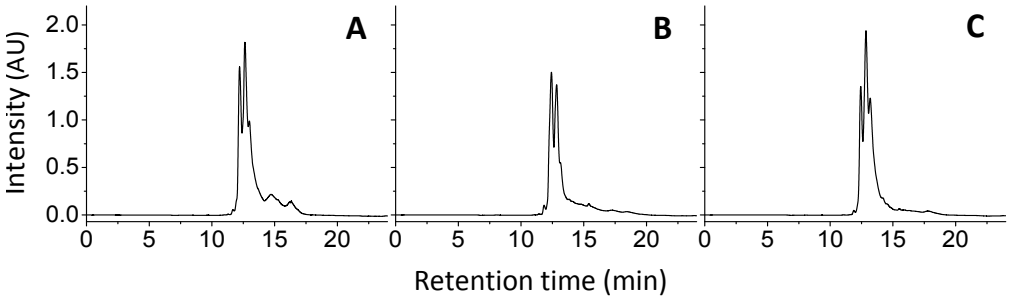



**Figure 20.** The HPLC–DAD chromatograms of hemolymph of *Nematus viridis* at 349 nm after feeding on the leaves of different birch species. Birch species: *Betula pubescens* ssp. *czerepanovii* (**A**), *Betula nana* (**B**), and *Betula pendula* (**C**).

The hemolymph samples of twelve Peruvian lepidopteran species were preliminarily analyzed with UHPLC–DAD–MS (unpublished results). The UHPLC–DAD chromatograms did not show clear peaks of phenolic compounds. Furthermore, the more sensitive mass spectrometric SRM/MRM method did not detect phenolic derivatives in most of the hemolymph samples, but two species showed weak signals of catechin and flavonol derivatives in their SRM chromatograms. The hemolymph of larvae consuming *Theobroma bicolor* contained a catechin monoglycoside, and the hemolymph of larvae feeding on *Terminalia catappa* included monoglycosides of kaempferol and quercetin (**Table 3**). No signs of flavonol oligoglycosides were found in the hemolymph of Peruvian lepidopteran larvae.

## 5.3 Feeding test with hemolymph and ants

The possible deterrent activity of hemolymph of *N. sertifer* or quercetin 3,7,4'-tri-*O*-*β*-glucoside against *F. polyctena* ants was tested in a field trial (**Table 4**, unpublished results). Our hypothesis was that if quercetin 3,7,4'-tri-*O*-*β*-glucoside or the hemolymph of *N. sertifer* have a deterrent effect against *F. polyctena* ants, the flavonoid or hemolymph treated larvae would be picked up more slowly by the ants in comparison to the control larvae. In general, the picking up times varied considerably between replicates, which can be seen as a high variation in the results. The average picking up time for larvae treated with quercetin 3,7,4'-tri-*O*-*β*-glucoside solution was longer than for the control larvae, but the difference was not statistically significant ($F_{1,89} = 2.24$, $P = 0.14$). Moreover, the difference between hemolymph-treated and control larvae was not statistically significant ($F_{1,89} = 0.92$, $P = 0.34$). Our data thus imply that the hemolymph of *N. sertifer* or quercetin 3,7,4'-tri-*O*-*β*-glucoside do not possess considerable deterrent activity against *F. polyctena*. No deterrent effect was found even though the tested concentration of quercetin 3,7,4'-tri-*O*-*β*-glucoside (10 mg/ml) was higher than the quantified concentration in the hemolymph of *N. sertifer* (1.5 mg/ml, **II**). Ants

picked up the larvae of *N. sertifer* faster than the larvae of *E. autumnata*, and this possibly reflects a higher attractiveness of *N. sertifer* as a prey object. Alternatively, the nests of *F. polyctena* may have had lower needs for nutrition during the time of test 1 (in June) than during the test 2 (in July).

**Table 4.** The estimated marginal means of time needed for *Formica polyctena* ants to pick up differently treated larvae.

| | Dipping solution | Estimated marginal means of picking up times (s) | Standard error |
|---|---|---|---|
| **Test 1** (larvae of *E. autumnata*) | Quercetin 3,7,4'-tri-*O*-*β*-glucoside | 126 | 32 |
| | Water (control) | 92 | 32 |
| **Test 2** (larvae of *N. sertifer*) | Hemolymph | 30 | 5 |
| | Water (control) | 29 | 5 |

Some sawfly species are known to sequester plant secondary compounds (e.g., iridoid glycosides and furostanol saponins) in their hemolymph. These compounds can deter ants, as the larvae release droplets of their deterring hemolymph upon predator attack (Opitz et al., 2010; Prieto et al., 2007). Flavonoid oligoglycosides did not deter ants in our tests, but instead these compounds could have bioactivities against other organisms. For example, endoparasitoid eggs develop inside the larvae (Strand and Pech, 1995) and flavonoid oligoglycosides could function against these kinds of organisms. This hypothesis was, however, not tested in this thesis.

## 5.4 Phenolic compounds in cocoons

The HPLC–DAD chromatograms of *N. sertifer* cocoon extracts showed clear peaks of flavonoids (unpublished results; **Figure 21**). These compounds included flavonol monoglycosides and smaller peaks of their corresponding diglycosides and aglycones (**Table 5**). It was assumed that these compounds derive from the flavonoid glycosides in the larval hemolymph, and thus the molecular ions of flavonol triglycosides (ions at *m/z* 773, 789 and 803 in the positive ionization mode) and a catechin monoglycoside (ion at *m/z* 453) were extracted from the total ion chromatograms of cocoon extracts. However, these compounds were not found in the cocoons, except for possible traces of a quercetin triglycoside and a catechin monoglycoside in one cocoon sample. In addition to flavonols and their glycosides, the cocoon extracts also contained flavan-3-ols and dimeric and trimeric procyanidins and several other compounds, whose structures were not identified in this study (**Table 5**).

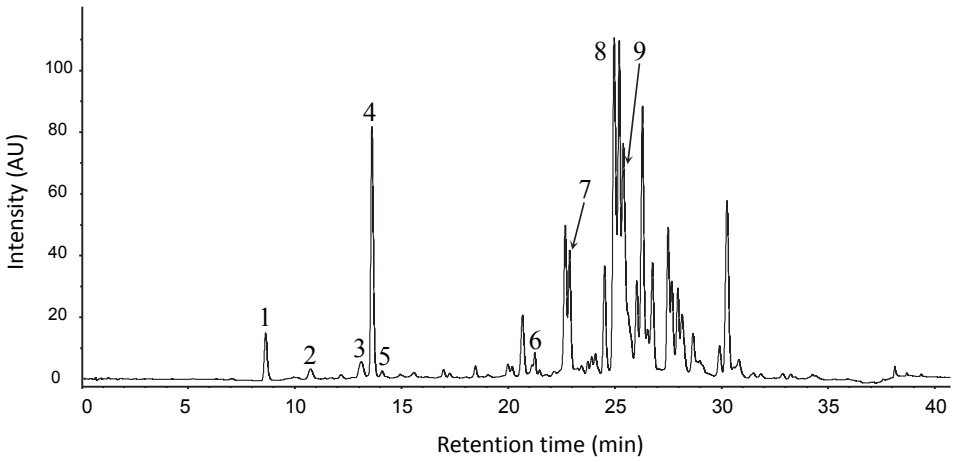



**Figure 21.** The HPLC–DAD chromatogram of a cocoon shell extract of *Neodiprion sertifer* at 280 nm. Compounds 1–9 are listed in **Table 5**.

**Table 5.** Phenolic compounds found in the cocoon shells of *N. sertifer* and *Nematus* sp. Numbers 1–9 refer to peaks in the chromatogram in **Figure 21**.

| No | Compound | UV spectrum $\lambda_{max}$ (nm) | $[M+H]^+$ | *m/z* values of other ions |
|---|---|---|---|---|
| | | | | |
| ***Neodiprion sertifer*** | | | | |
| 1 | Gallocatechin | 206, 272 | 307 | 139 |
| 2 | Proanthocyanidin (PC–PD dimer) | 205, 278 | 595 | 427, 289, 247 |
| 3 | Proanthocyanidin (PC–PC dimer) | 202, 279 | 579 | 427, 289, 247 |
| 4 | Catechin | 202, 279 | 291 | 139 |
| 5 | Proanthocyanidin (PC trimer) | 279 | 867 | 579, 289 |
| 6 | Quercetin diglycoside | 265, 347 | 627 | 465, 303 |
| 7 | Quercetin monoglycoside | 256, 265 (sh), 354 | 465 | 303 |
| 8 | Kaempferol monoglycoside | 265, 348 | 449 | 287 |
| 9 | Isorhamnetin monoglycoside | 256, 266 (sh), 353 | 479 | 317 |
| * | Kaempferol diglycoside | 266, 341 | 611 | 449, 287 |
| * | Isorhamnetin diglycoside | no clear UV spectrum | 641 | 479, 317 |
| * | Kaempferol | 265, 366 | 287 | – |
| * | Isorhamnetin | 254, 369 | 317 | – |
| ***Nematus* sp.** | | | | |
| * | Quercetin diglycoside | 256, 265 (sh), 353 | 627 | 465, 303 |
| * | Quercetin diglycoside | 256, 266 (sh), 353 | 597 | 435, 303 |

Abbreviations: PC = a procyanidin unit/catechin, PD = a prodelphinidin unit/gallocatechin, sh = shoulder, sp = species, * = chromatograms not shown

The cocoon shell of another pine-feeding sawfly, *D. pini,* showed a complex HPLC–DAD chromatogram with several peaks, but no clear flavonoid peaks were found in this sample (chromatogram not shown). The chromatogram of a cocoon of birch-feeding *Nematus* sp. showed several compounds with a UV spectrum similar to flavonols. Two of these compounds were identified as quercetin diglycosides (**Table 5**).

It is unknown why sawfly species transfer plant-derived phenolic compounds to their cocoon shells. These compounds could, for example, function as UV light shielding compounds in a similar way that was suggested for flavonoids found in the cocoons of the lepidopteran silkworm *B. mori* (Daimon et al., 2010). Another possibility is that phenolic compounds are used in the sclerotization (tanning) of the cocoons. In sclerotization, the insect cuticle (outer layer of insects) is made stiffer and more resistant to degradation (Andersen, 1985). Sclerotization starts with the oxidation of *O*-diphenolic compounds, such as *N*-acetyldopamine, after which the formed quinone is bound with the amino group of a protein (**Figure 22**). This complex can further bind to other proteins and quinones, thus forming cross-linked polymeric structures. In addition to *N*-acetyldopamine, also other phenolic substrates can be used in the sclerotization of cocoons. For example, larvae in the lepidopteran family of Saturniidae utilize monoglycosides of 3-hydroxyanthranilic acid and gentisic acid in this process (Brunet and Coles, 1974). The phenolics are stored in larval silk glands as glycosides, but the glycosidic bonds are enzymatically cleaved during the spinning of cocoon. The formed phenolic aglycones are then oxidized, and the formed quinones or free radicals bind covalently to silk proteins producing tanned silk cocoons. The tanned cocoons showed increased mechanical stiffness and higher tolerance against chemical degradation if compared to the untanned cocoons (Brunet and Coles, 1974). Schopf et al. (1982) found catechin in the cocoons of the spruce-feeding sawfly *G. hercyniae*, and the authors proposed that the larvae had used catechin in the tanning process. According to the authors, the sawfly larvae would have evolved to take advantage of the phenolic compounds of their host plants, which would release the larvae from the need to produce similar kinds of phenolics from their own important amino acid sources (e.g., tyrosine and phenylalanine).

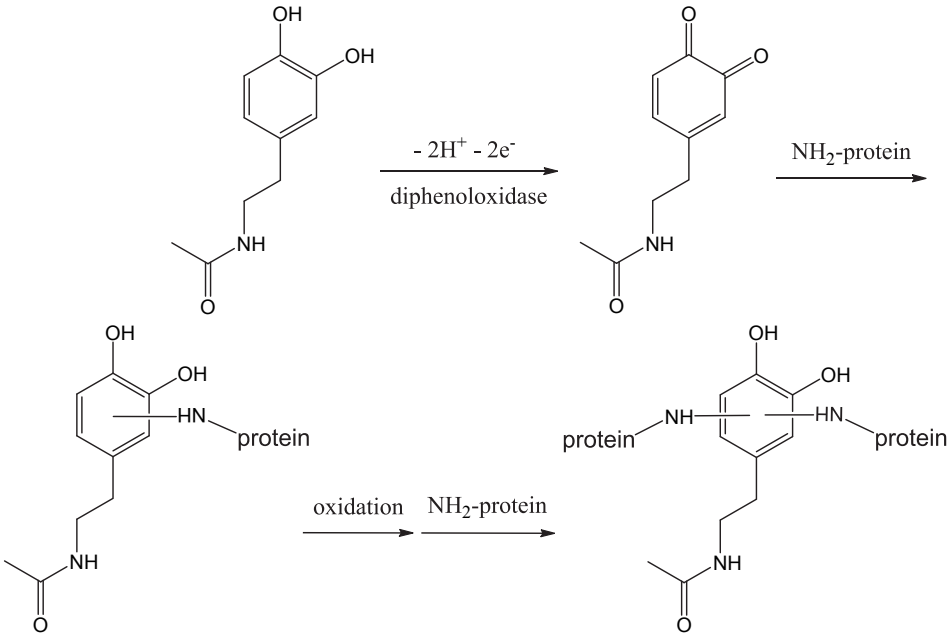



**Figure 22.** A schematic presentation of the sclerotization process of *N*-acetyldopamine with a protein (a nucleophilic addition reaction). The reaction can continue with the re-oxidation of the phenolic ring and with the addition of another protein to the phenolic ring (Andersen, 1985).

Both *G. hercyniae* studied by Schopf et al. (1982) and *N. sertifer* in this thesis belong to the conifer-feeding family Diprionidae. It thus seems possible that similar chemical processes could also occur during the cocoon formation of *N. sertifer*. The findings of Schopf et al. (1982) and Brunet and Coles (1974) could actually link the existence of flavonoid glycosides both in the hemolymph and cocoons of sawflies. A potential explanation is that sawfly larvae glycosylate simple phenolic compounds from their diet and store the formed glycosides in their hemolymph. The formation of oligoglycosidic flavonols (**I** and **II**) and a catechin monoglycoside (**II**) probably transforms these phenolics into a more soluble form for their storage in hemolymph. The phenolic glycosides are later transferred to the silk glands where they are used in the formation of cocoons. Then, flavonoid glycosides would be partially deglycosylated during cocoon formation (Brunet and Coles, 1974; Schopf et al. 1982). Indeed, the cocoons of *N. sertifer* contained mostly flavonol monoglycosides, smaller amounts of their diglycosides, and only traces of flavonol triglycosides, which supports the deglycosylation of flavonol oligoglycosides during cocoon formation.

In addition to being transferred to cocoons, flavonoid (oligo)glycosides in hemolymph could have other functions in the larvae. They could, for example, serve as a carbohydrate storage. The pupating larva does not feed, but it needs energy for constructing the cocoon and for becoming an adult insect. Sawfly larvae could thus store carbohy-

drates in a soluble form as flavonoid (oligo)glycosides, and the carbohydrates could be released during the immobile pupal phase as energy for different metabolic processes.

## 5.5 Pro-oxidant activities of phenolic compounds

Phenolic compounds are suggested to be oxidized in the alkaline midguts of herbivorous larvae. The pro-oxidant capacities of 24 plant species were estimated in **III** and **IV**. The pro-oxidant capacities of the studied plant species varied from almost 0 to 46 % of the total phenolics (**Table 6**). This suggested that the phenolics in different species possess highly variable capacities to function as pro-oxidant defenses against larval herbivores. The comparison of UHPLC–DAD chromatograms of initial and incubated plant extracts showed the individual phenolic compounds that probably caused the measured pro-oxidant capacities (**III** and **IV**). For example, the UHPLC–DAD peaks of ellagitannins always disappeared from plant extracts incubated at pH 10, which suggested that these compounds could cause oxidative stress in herbivore guts (**Table 6**). In addition to probable oxidation reactions, ellagitannins were also hydrolyzed at pH 10, which was seen as the formation of ellagic acid in the incubated samples (**III** and **IV**). Ellagitannins were estimated to possess higher oxidative activities than condensed tannins, galloyl glucoses, or gallotannins at pH 10 (Barbehenn et al., 2006a). Additionally, the midguts of the lepidopteran *Orgyia leucostigma* consuming ellagitannin-rich plant species contained higher amounts of semiquinone radicals than the larvae consuming other types of phenolic compounds (Barbehenn et al., 2008).

Different types of flavan-3-ols and oligomeric proanthocyanidins were detected in **III** and **IV**, including procyanidins (A– and B–types), prodelphinidins and propelargonidins. The intensities of UHPLC–DAD peaks of these compounds at 280 nm were often decreased due to incubations in alkaline conditions implying their partial oxidation *in vitro*. However, in some plant species the peaks of flavan-3-ols or oligomeric procyanidins seemed to be only mildly affected by alkaline conditions (**III**). Some of these compounds seemed to be tolerant to alkaline conditions in one plant species (e.g., *Aesculus hippocastanum*), whereas in other plant species (e.g., *Ormosia macrocalix*) the UHPLC–DAD peaks of similar compounds were efficiently depleted (**Figure 23**). This discrepancy suggested that the susceptibility of individual flavan-3-ols or proanthocyanidins to oxidize *in vitro* could be strongly affected by the presence of other compounds in the solvent mixture. Barbehenn et al. (2006b) found that proanthocyanidins could in some cases act as anti-oxidants, thereby decreasing the oxidation of hydrolysable tannins. It is thus possible that a similar kind of antioxidant effect prevented the oxidation of flavan-3-ols and procyanidin oligomers in *A. hippocastanum* (**Figure 23**A).

**Table 6.** The pro-oxidant capacities of plant species in **III** and **IV** (in order of magnitude). The column "Pro-ox capacity" shows how large a proportion of the initial total phenolics in plant extracts were lost due to incubation at pH 10, and the column "Pro-ox active phenolics" shows the amount of total phenolics lost during the incubation.

| Plant species | Study | Pro-ox capacity | Pro-ox active phenolics (mg/g Dw) | Main phenolics whose concentrations decreased at pH 10 |
|---|---|---|---|---|
| *Quercus robur* | III | 46% | 57 | Ellagitannins, GG |
| *Ribes alpinum* | III | 46% | 38 | Myricetins, prodelphinidins |
| *Betula pubescens* | III | 40% | 25 | Ellagitannins, GG, HCA |
| *Terminalia catappa* | IV | 37% | 151 | Ellagitannins |
| *Salix phylicifolia* | III | 35% | 29 | Myricetins |
| *Alchornea castaneifolia* | IV | 33% | 69 | Ellagitannins |
| *Iris* sp. | III | 32% | 18 | Myricetins |
| *Vernonia patens* | IV | 32% | 46 | HCA |
| *Ormosia macrocalyx* | IV | 27% | 26 | Procyanidins |
| *Fragaria moschata* | III | 23% | 24 | Ellagitannins |
| *Oxyanthus speciosus* | III | 23% | 15 | HCA |
| *Achillea ptarmica* | III | 22% | 6 | HCA |
| *Prunus africana* | III | 22% | 12 | HCA |
| *Psidium guajava* | IV | 21% | 44 | Ellagitannins |
| *Tabebuia ochracea* | IV | 20% | 10 | Scutellarein glucuronide |
| *Tetrathylacium macrophyllum* | IV | 18% | 23 | Phenolic profile mostly unchanged |
| *Picea abies* | III | 13% | 6 | Phenolic profile mostly unchanged |
| *Copaifera paupera* | IV | 12% | 31 | Galloylquinic acids |
| *Vochysia braceliniae* | IV | 8% | 9 | Procyanidins and propelargonidins |
| *Aesculus hippocastanum* | III | 5% | 4 | Phenolic profile mostly unchanged |
| *Theobroma bicolor* | IV | 5% | 6 | Phenolic profile mostly unchanged |
| *Caryodendron orinocense* | IV | ~ 0% | 0 | Phenolic profile mostly unchanged |
| *Celtis durandii* | III | ~ 0% | 0 | Phenolic profile mostly unchanged |
| *Senna reticulata* | IV | ~ 0% | 0 | Phenolic profile mostly unchanged |

Abbreviations: GG = galloyl glucoses, HCA = hydroxycinnamic acids. Compounds were identified based on their UV and mass spectral properties. All values in **IV** and the values of *O. speciosus*, *P. africana* and *C. durandii* in **III** are averages of values obtained from six individual trees. Other values in **III** were obtained from a pooled plant sample.

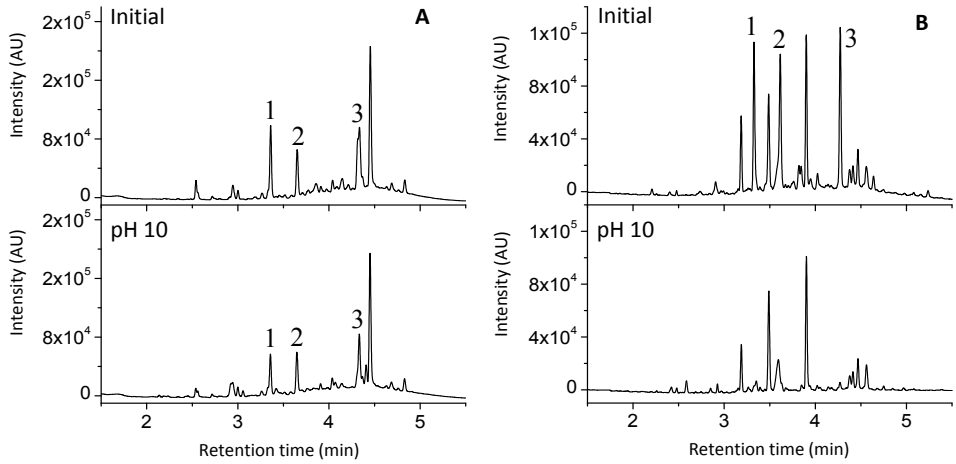



**Figure 23.** The UHPLC–DAD chromatograms of *Aesculus hippocastanum* (**A**) and *Ormosia macrocalix* (**B**) before (Initial) and after (pH 10) the leaf extracts were incubated at pH 10 (detected at 280 nm). Compound 1: (–)-epicatechin, 2: procyanidin trimer (A–type), 3: procyanidin dimer (A–type). The molecular ions of compounds 1–3 (in negative ionization mode at *m/z* 289, 863, 575, respectively) were extracted from the total ion chromatograms of different samples, but these ions were not detected in the chromatograms of incubated extracts of *O. macrocalix*.

The possible oxidation of flavan-3-ols and proanthocyanidins could be examined in more detail from the SRM chromatograms of procyanidins and prodelphinidins. The initial, un-incubated leaf extracts often showed a rather unresolved hump in the SRM chromatograms of procyanidins and prodelphinidins (**Figure 24**). In the case of procyanidins, the height of the procyanidin hump usually decreased after the sample had been incubated at pH 10, which implied the partial oxidation of procyanidins (**Figure 24**A and B). For example, the peak intensities of a procyanidin dimer (ion at *m/z* 577 in negative ionization mode) and a trimer (ion at *m/z* 865) in the SRM chromatogram of *Theobroma bicolor* were lowered in the incubated samples indicating their partial oxidation (**Figure 24**A). Prodelphinidins seemed to be oxidized in alkaline conditions (**Figure 24**C and D). For example, a hump containing prodelphinidins and a peak of a prodelphinidin trimer (at *m/z* 913 in negative ionization mode) disappeared from the incubated sample of *Ribes alpinum* (**Figure 24**C), and a similar fate was found for a gallocatechin (ion at *m/z* 305 in negative ionization mode) and a gallocatechin gallate (ions at *m/z* 457, 305) in *Chrysophyllum albidum* (a plant species tested preliminarily in **III**, **Figure 24**D).

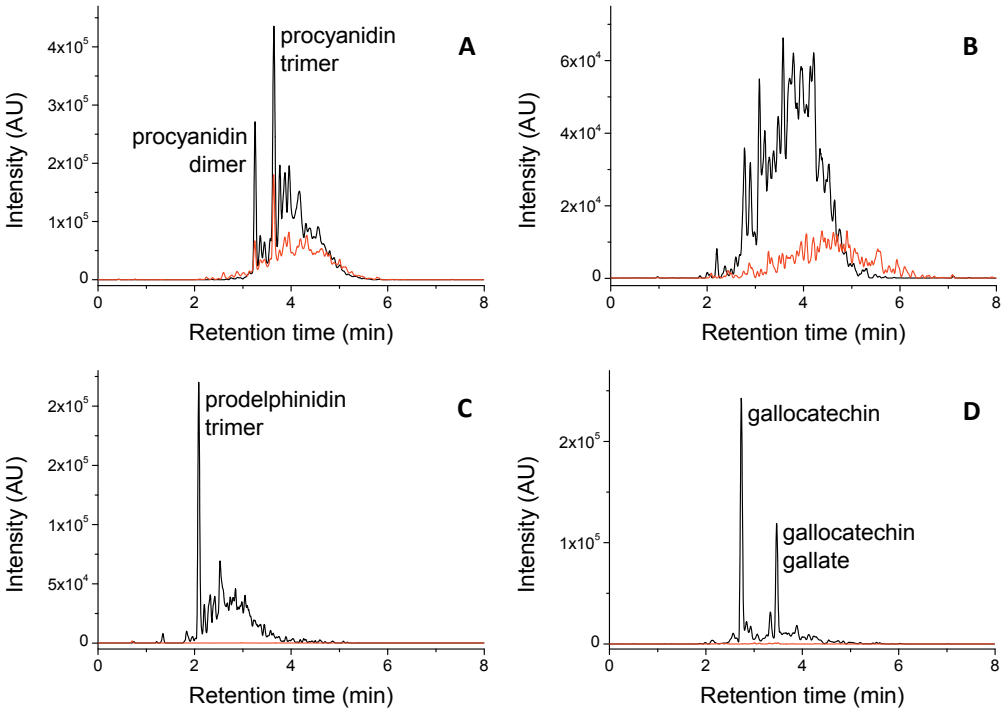



**Figure 24.** The SRM chromatograms of procyanidins and prodelphinidins in leaf extracts before (black line) and after incubations at pH 10 (red line): procyanidins in *Theobroma bicolor* (**A**), procyanidins in *Psidium guajava* (**B**), prodelphinidins in *Ribes alpinum* (**C**), prodelphinidins in *Chrysophyllum albidum* (**D**). For the detected SRM transitions, see Table 2.

The heights of UHPLC–DAD peaks of flavonols and flavones usually slightly decreased during incubations in alkaline conditions, which suggested that these compounds could be, in general, less effectively oxidized than hydrolysable tannins and proanthocyanidins (**III** and **IV**). For example, two plant species with the weakest prooxidant capacities (*Celtis durandii* and *Senna reticulata*, **Table 6**) contained mostly flavonols and flavones, whose UHPLC–DAD peaks at 280 nm were practically unaffected due to incubation at pH 10. These flavonoids included a kaempferol diglycoside, a kaempferol monoglycoside, and an isorhamnetin monoglycoside in *S. reticulata*, and two apigenin diglycosides with *C*-glycosyls in *C. durandii*. However, the quantitative SRM data showed variable oxidative activities for kaempferol and quercetin derivatives in different plant species. In many plant species, only 1–6% of the quantified total kaempferol and quercetin derivatives were lost *in vitro*, but in other plant species up to 40–60% of the quantified flavonol derivatives disappeared (**IV**). When the fates of individual flavonols in alkaline conditions were observed from the SRM chromatograms of flavonol derivatives, it was found that certain flavonoids performed variably in different plant species. For example, the peak area of a quercetin diglyco-

side (ion at *m/z* 609 in negative ionization mode) in the total ion chromatogram of *S. reticulata* stayed almost unchanged due to incubation at pH 10, whereas the peak area of the same compound in *Caryodendron orinocense* decreased on average by 40% during the incubation. This suggested, as noted above for proanthocyanidins, that individual phenolic compounds could show variable tendencies to be oxidized if other phenolic compounds in the solution function as antioxidants.

Myricetin glycosides and dihydromyricetin differed from many other flavonoids because the peaks of these compounds almost completely disappeared from the UHPLC–DAD chromatograms of the incubated plant extracts (**III**). In addition, the leaves of *Tabebuia ochraceae* contained a flavone (scutellarein glucuronide), whose chromatographic peak was similarly affected by alkaline conditions (**IV**). The oxidative activities of these flavonoids are possibly related to the similarities in their chemical structures. Myricetin, dihydromyricetin, and scutellarein contain a pyrogallol substructure (three adjacent hydroxyl groups) in their rings A or B, whereas the seemingly less oxidatively active derivatives of apigenin (a flavone) and kaempferol and quercetin (flavonols) have only one or two hydroxyl groups in their ring B. The autoxidation of myricetin and pyrogallol in alkaline conditions has been earlier reported (Hodnick et al., 1986; Gao et al., 1998). In fact, many other phenolic compounds showing high oxidative activities in alkaline conditions contained at least one pyrogallol group within their structures. These compounds include ellagitannins, galloyl glucoses, and prodelphinidins (**Figure 25**). This implies that a pyrogallol substructure could partly explain the reactivity of several types of phenolics at pH 10. It should be noted that the oxidative activities of individual phenolics vary within these oxidatively active phenolics. For example, different ellagitannins produce variable amounts of semiquinone radicals in alkaline conditions (Barbehenn et al., 2006a), and the oxidative activities of individual ellagitannins at pH 10 have been estimated to vary by a factor of six (Moilanen and Salminen, 2008).

In humans, flavonoids are known for their dual role as both anti- and pro-oxidants. In Cao et al. (1997), flavonoids showed anti-oxidative activities against two types of oxygen radicals, while the same flavonoids acted as pro-oxidants in the presence of $Cu^{2+}$ ions. Interestingly, myricetin was found to be both a better antioxidant and pro-oxidant than kaempferol and quercetin, which the authors related to the higher amount of hydroxyl groups in the ring B of myricetin. The oxidation of myricetin and quercetin has been associated with lipid peroxidation and increased damage to genetic material in humans (Duerte Silva et al., 1996; Sahu and Gray, 1993; Sahu and Washington, 1991), which can promote mutagenesis and carcinogenesis (Sahu and Gray, 1993; Sahu and Washington, 1991). Once again, myricetin induced more severe DNA damage than did quercetin (Sahu and Gray, 1993; Sahu and Washington, 1991).

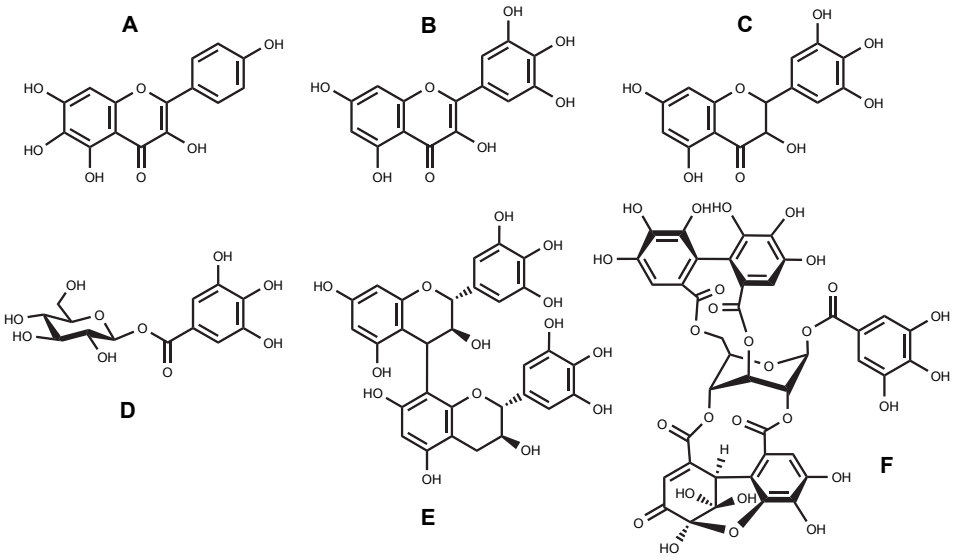



**Figure 25.** The concentrations of phenolics containing a pyrogallol substructure were effectively decreased at pH 10: scutellarein derivative (**A**), myricetin derivative (**B**), dihydromyricetin (**C**), galloyl glucose (**D**), prodelphinidin (**E**), and ellagitannin (**F**).

The role of myricetins in chemical ecology has been rarely studied, which leaves room for indirect scientific speculations. The lepidopteran *Polyommatus icarus* feeding on different plant species was found to selectively excrete myricetin glycosides, while kaempferol or quercetin glycosides were partly transferred to adult butterflies (Burghardt et al., 1997; Schittko et al., 1999). This difference in sequestration of flavonols could partly be due to the toxicity of myricetin to *P. icarus* (Schittko et al., 1999). In another study, lepidopteran larvae consumed willow leaves containing myricetin and dihydromyricetin derivatives and other flavonoids (Ruuhola et al., 2001). However, the larvae seemed to utilize and degrade myricetin and dihydromyricetin derivatives in a similar way as any other flavonoids. The leaves of different birch species in **I** contained myricetin glycosides in addition to kaempferol and quercetin glycosides (Keinänen and Julkunen-Tiitto, 1998; Graglia et al., 2001; Riipi et al., 2002), but birch-feeding sawfly larvae stored mainly kaempferol and quercetin oligoglycosides in their hemolymph. This could possibly reflect the detrimental effect of myricetin-type compounds on these larvae.

Many earlier studies have concentrated on the effects of oxidized phenolic compounds to lepidopteran larvae (e.g. Felton et al., 1989; Felton and Duffey, 1991a and b; Gross et al., 2008; Barbehenn et al., 2001, 2005, 2008, 2012) but there seem to be no studies about the oxidation of phenolics in sawfly larvae. A few scarce reports showed that the pH values in the midguts of sawfly species ranged from close to neutral to mildly alkaline (approximately pH 6.6–8.7), whereas the pH of lepidopteran midguts in the same studies ranged mostly between pH 9–10 (Angus, 1956; Heimpel, 1955). The pH values

as high as 12 of hemolymph have been reported for some lepidopterans (e.g., Dow, 1984). The differing pH values in the midguts of lepidopteran and sawfly larvae suggest that the oxidation of phenolics could be less prominent in sawfly than in lepidopteran larvae. In addition to this, it should be taken into account that many insect herbivores have probably developed to tolerate, perhaps via their antioxidant systems, the deleterious effects of phenolic oxidation (Barbehenn et al., 2001; Felton and Duffey, 1991a; Mathews et al., 1997).

## 5.6 Phenolic compounds in frass

Phenolic compounds were detected in the frass of both lepidopteran and sawfly larvae (**II** and **IV**). The frass of the pine-feeding *N. sertifer* contained the same flavonol monoglycosides (isorhamnetin, kaempferol and quercetin) and (+)-catechin that were detected in the needles of the host plant, *P. sylvestris* (**II**). However, flavonol triglucosides or a (+)-catechin 7-O-*β*-glucoside present in the larval hemolymph were not found in the frass. This implied that these glucosylated flavonoid metabolites were not simple excretory products of the larvae, but that the compounds could have other functions in the larval ecology (see above for the hypothesis about sclerotization in larval cocoons). The frass of birch-feeding sawfly larvae were studied earlier (Lahtinen et al., 2005), and also in this case the authors found only simple flavonoid monoglycosides and other flavonoid metabolites in the frass. However, no flavonoid oligoglycosides were reported.

The frass of 12 lepidopteran species contained several phenolic compounds that were also detected in the leaves of their host plants (**IV**). The UHPLC–DAD chromatograms of frass samples were compared with plant extracts incubated at pH 10 to see, whether individual phenolic compounds would show similar trends in *in vitro* and frass samples. In several cases, phenolic compounds seemed to perform in a comparable way both in alkaline conditions and in the frass samples. For example, the concentrations of several ellagitannins, galloylquinic acid derivatives, and proanthocyanidins decreased during incubation in alkaline conditions (detected at 280 nm), and the concentrations of corresponding phenolics seemed to have decreased in the frass samples in comparison to other compounds. This suggested that these phenolics had been modified in larval metabolism (**Figure 2** in **IV**).

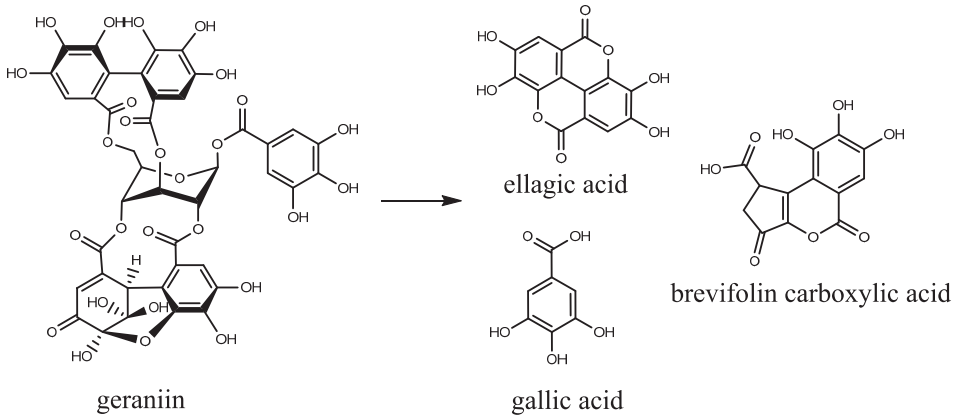



**Figure 26.** An ellagitannin (geraniin) in the leaves of *Alchornea castaneifolia* and its hydrolysis products found in larval frass.

Ellagitannins were hydrolyzed to ellagic acid *in vitro*, and this compound was also detected in the frass of larvae consuming ellagitannins (**Figure 26**). Gallic acid, another hydrolysis product of ellagitannins, was also found in frass samples. Brevifolin carboxylic acid was found in the frass of larvae consuming *Alchornea castaneifolia*, and this compound was probably hydrolyzed from the ellagitannin geraniin (Tuominen and Sundman, 2013).

The peaks of certain flavonols and flavones in the UHPLC–DAD chromatograms of leaf extracts were not strongly influenced by the incubation in alkaline conditions. Accordingly, many of these phenolics were notably present in the UHPLC–DAD chromatograms of frass of larvae consuming these plant species (**IV**). This suggested that lepidopteran larvae may not have modified these flavonoids as effectively as ellagitannins or proanthocyanidins. One atypical flavone was a scutellarein glucuronide in *T. ochraceae*. The intensity of its UHPLC–DAD peak was substantially lower in larval frass when compared with the host plant indicating that this compound had been modified in larvae. Ruuhola et al. (2001) studied the degradation rates of flavonoids in lepidopteran larvae by analyzing the frass of the *Salix*-feeding *Operophtera brumata*. They found that generally more than 60% of the total flavonoids (including flavones and flavonols) had been degraded in the larval metabolism.

In many cases, the *in vitro* incubations of plant extracts and the analyses of larval frass showed rather similar fates for individual phenolic compounds (**IV**). However, the comparisons did not always produce straightforward results. For example, ellagitannins (e.g., punicalagin) were detected as the main phenolics in the frass of larvae consuming *Terminalia catappa*, even though the *in vitro* results suggested that these ellagitannins would be depleted in alkaline conditions (**Figure 2** in **IV**). In another case, a kaempferol glycoside (ion at *m/z* 447 in negative ionization mode) was mostly missing

from the frass of larvae consuming *Senna reticulata* suggesting that this compound was modified by the larvae. However, the same compound remained rather unaffected when the leaf extract was incubated at pH 10 (**Figure 2** in **IV**). The above examples emphasize that, in addition to possible oxidation reactions, many other factors should be taken into account when the fates of phenolic compounds in larval metabolism are studied. These factors include, for example, plant- and insect-derived enzymes, and gut microflora and their effects on phenolic compounds.

Chemical modifications of phenolic compounds were detected in the frass of several lepidopteran species (**IV**). These modified phenolics included kaempferol and quercetin sulfates, and similar types of compounds were earlier detected in the frass of the lepidopteran *Pieris brassicae* (Ferreres et al., 2008). The frass of larvae consuming *Ormosia macrocalyx* contained a compound that was tentatively identified as an oxidized form of catechin (ion at *m/z* 287 in negative ionization mode), which probably derived from flavan-3-ols or proanthocyanidins in the larval diet. The main compound in the frass of larvae feeding on *Vernonia patens* was isolated and purified, and its structure was elucidated with UV and NMR spectroscopy, MS/MS, and ICP–MS (**IV**). The unknown compound was characterized to contain two protocatechuic acid units coordinated to a manganese cation. The protocatechuic acid units probably derived from mono- and dicaffeoylquinic acids present in the larval diet. Protocatechuic acid was earlier found in the frass of the sawfly *Pristiphora alpestris*, and this compound was suggested to derive from hydroxycinnamic acid derivatives in the larval diet (Lahtinen et al., 2005). In addition to phenolic derivatives, several lepidopteran species excreted uric acid, which is a nitrogenous excretory product of many insects.

# 6 CONCLUSIONS

This thesis studied the roles of phenolic compounds in the chemical ecology of lepidopteran and sawfly species. It was found that sawfly larvae transfer flavonols and other flavonoids to their hemolymph, where they are stored mainly as oligoglycosides (**I**, **II**). Flavonoid oligoglycosides were not found in the hemolymph of lepidopteran larvae. The glycosylation of dietary flavonoids seems to be a widespread phenomenon in sawflies because flavonoid oligoglycosides were found in the hemolymph of several species feeding on different host species. The ecological significance of hemolymph flavonoids is unknown, but they seem not to be used in larval defense against ant predators. The possible role of flavonoid oligoglycosides against larval parasitoids should be studied in the future. Flavonoids were also transferred to the cocoon shells of sawflies, which imply that flavonoids could have important roles in the sclerotization process of the insect cocoon.

Flavonoids and other phenolic compounds may produce oxidative stress in the guts of lepidopteran and sawfly larvae. The pro-oxidant capacities of different plant species were shown to be highly variable (**III, IV**). This implies that phenolic compounds in some plant species are able to cause high oxidative stress in some larval species, while other plants contain phenolics that are less effectively oxidized at pH 10. It should be remembered that specialist herbivores have co-evolved with their host plant species and that these larvae may therefore have developed counter-defenses to prevent or reduce the oxidation of phenolic compounds in their guts. Among flavonoids, myricetin derivatives were prone to oxidize in alkaline conditions (**III**). It would be interesting to see whether, myricetin derivatives produce oxidative stress for lepidopteran or sawfly larvae *in vivo* feeding experiments.

The frass of lepidopteran larvae was studied to see whether phenolic compounds are modified as they move through the digestive tract the lepidopteran larvae (**IV**). Leaf extracts incubated at pH 10 and frass samples often showed similar fates for individual phenolic compounds, which suggested that certain phenolic compounds had been oxidized or hydrolyzed in larval metabolism. In addition to these reactions, other types of modifications are also important in affecting the fates of phenolic compounds in lepidopteran larvae.

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