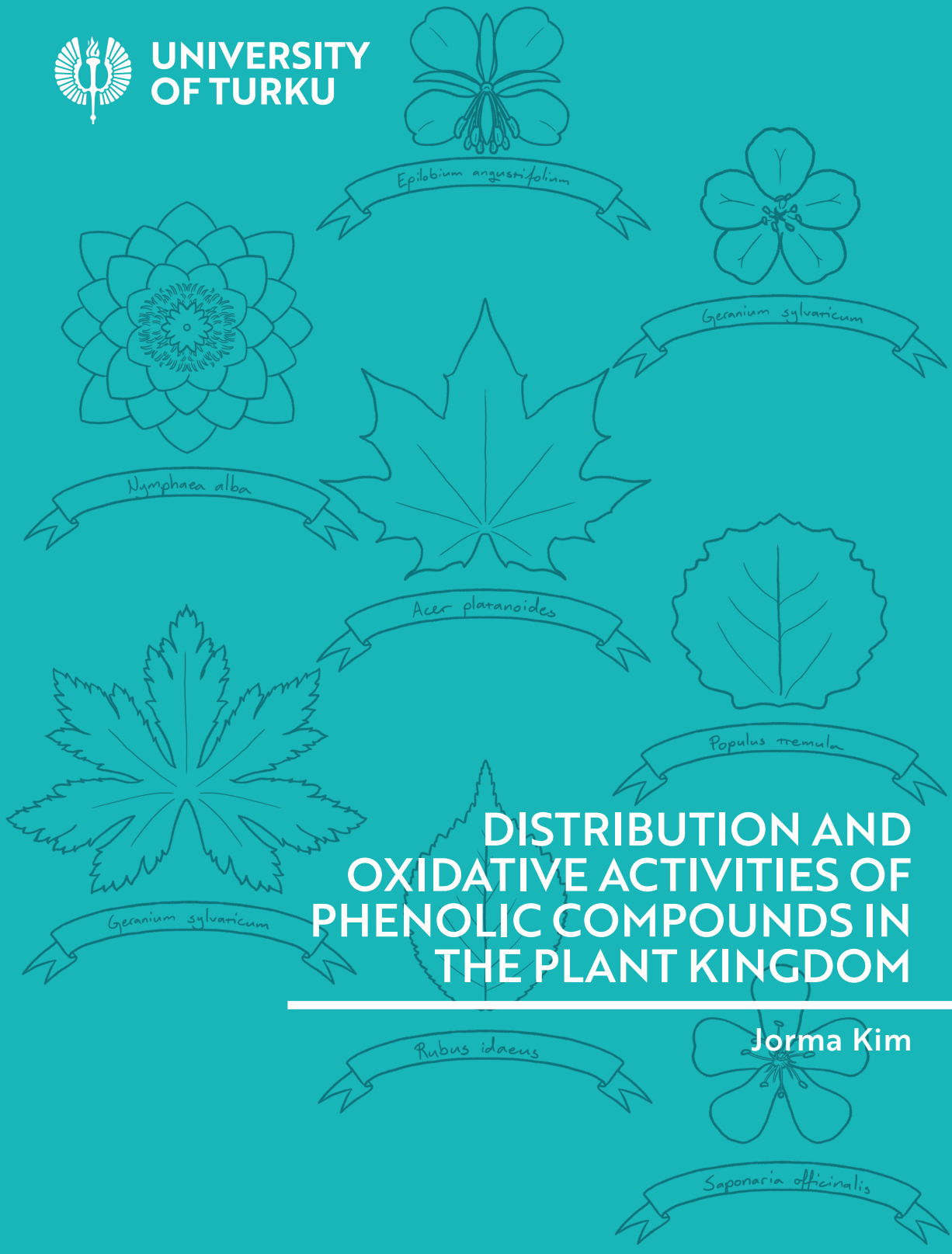
UNIVERSITY OF TURKU



DISTRIBUTION AND OXIDATIVE ACTIVITIES OF PHENOLIC COMPOUNDS IN THE PLANT KINGDOM

Jorma Kim

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 638 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2020

UNIVERSITY OF TURKU

DISTRIBUTION AND OXIDATIVE ACTIVITIES OF PHENOLIC COMPOUNDS IN THE PLANT KINGDOM

Jorma Kim

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 638 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2020

University of Turku

Faculty of Science and Engineering
Department of Chemistry
Natural Compound Chemistry
Doctoral Programme in Physical and Chemical Sciences

Supervised by

Professor Juha-Pekka Salminen
Department of Chemistry
University of Turku
Turku, Finland

Adjunct Professor Maarit Karonen
Department of Chemistry
University of Turku
Turku, Finland

Reviewed by

Professor Heidi Appel
Department of Environmental Sciences
University of Toledo
Toledo, OH, USA

Professor Elisabeth Gross
CNRS, LIEC
Université de Lorraine
Metz, France

Opponent

Professor Anna Kiss
Department of Pharmacognosy and Molecular Basis of Phytotherapy
Medical University of Warsaw
Warsaw, Poland

Cover image by the author.

ISBN 978-951-29-8281-3 (PRINT)
ISBN 978-951-29-8282-0 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)
Painosalama, Turku, Finland 2020

UNIVERSITY OF TURKU
Faculty of Science and Engineering
Department of Chemistry
Natural Compound Chemistry
JORMA KIM: Distribution and oxidative activities of phenolic compounds in the plant kingdom
Doctoral Dissertation, 211 pp.
Doctoral Programme in Physical and Chemical Sciences
September 2020

ABSTRACT

Plant phenolics have been studied for decades because of their role as chemo-defensive compounds against herbivores and pathogens, and their assumed health benefits to humans. One of their proposed modes of actions is oxidation, which can happen enzymatically or spontaneously at alkaline conditions. An underlying issue is that the majority of studies only focus on one or few plant species or families, so gaining a comprehensive overview of the distribution of phenolics across the plant kingdom requires compiling data from several studies which may utilize fundamentally different extraction and analytical methods and have different research foci.

This thesis has two parts. First, an existing analytical method was improved with an addition of a simple *in planta* enzymatic oxidation step. Thereby a single analytical procedure results in three types of data: the phenolic profile of the oxidized and non-oxidized plant samples as well as the alkaline and enzymatic oxidative activity of the individual compounds as well as the raw extract. Analyzing hundreds of samples revealed that at alkaline conditions, the most active compounds either contained a pyrogallol moiety or a catechol moiety at the end of an alkane chain. While enzymatic oxidation tended to favor compounds with a catechol moiety, the activity varied between plant species and even between tissues within the same species. In addition, a method for estimating the prevailing pH in larval gut was developed by studying the isomerization of caffeoylquinic acids at alkaline pH and comparing the results to the existing data of the isomer ratios in larval frass.

Second, a research gap in the field of composition and ecology of the phenolics of tropical plants was covered in a large-scale screening study. Several predictions inspired by theories and hypotheses on plant apparency as well as resource availability and allocation were confirmed. For example, there was a strong positive correlation between seed mass and polyphenol content. The majority of seeds containing phenolics were rich in either various hydrolysable tannins or proantho-cyanidins.

KEYWORDS: alkaline oxidative activity, enzymatic oxidative activity, phenolic compounds, plant kingdom, specialized metabolites

TURUN YLIOPISTO
Luonnontieteiden ja tekniikan tiedekunta
Kemian laitos
Luonnonyhdisteiden kemia
JORMA KIM: Kasvikunnan fenolisten yhdisteiden esiintyvyys ja hapettumisaktiivisuus
Väitöskirja, 211 s.
Fysikaalisten ja kemiallisten tieteiden tohtoriohjelma
Syyskuu 2020

TIIVISTELMÄ

Kasvien fenolisia yhdisteitä on tutkittu vuosikymmeniä, koska ne toimivat kemiallisena puolustuskeinona kasvinsyöjiä ja taudinaiheuttajia vastaan, minkä lisäksi niiden uskotaan olevan hyödyllisiä ihmisten terveydelle. Entsyymien katalysoiman sekä emäksisissä olosuhteissa tapahtuvan spontaanin hapettumisen on esitetty olevan eräs niiden toimintamuodoista. Valtaosa tutkimuksista keskittyy vain yhteen tai korkeintaan muutamaan kasvilajiin tai -heimoon, joten kattavan kokonaiskuvan saaminen edellyttää useiden tutkimustulosten koostamista siitäkin huolimatta, että mahdollisesti eri tavoitteisiin tähtäävissä tutkimuksissa on voitu käyttää oleellisesti erilaisia uutto- ja analyysimenetelmiä.

Tämä väitöskirjatyö on kaksiosainen. Ensimmäisessä osassa olemassa olevaan analyysimenetelmään lisättiin yksinkertainen vaihe, jossa fenoliset yhdisteet hapetetaan itse kasvinäytteessä. Yhdellä analyysikerralla saadaan näin kerättyä kolmenlaista dataa: hapetettujen ja hapettamattoman näytteen sisältämät fenoliset yhdisteet sekä yksittäisten yhdisteiden ja raakauutteen emäksinen ja entsymaattinen hapettumisaktiivisuus. Satojen kasvinäytteiden analysointi osoitti, että emäksisissä olosuhteissa aktiivisimmat yhdisteet sisältävät joko pyrogallooliryhmän tai tyydyttyneen hiilivetyketjun päähän sitoutuneen katekoliryhmän. Katekoliryhmän sisältävät yhdisteet olivat niin ikään altteimpia hapettumaan entsymaattisesti, mutta hapettumisaktiivisuus vaihteli eri kasvilajien ja jopa saman lajin kasvinosien välillä. Lisäksi tässä osassa kehitettiin menetelmä toukkien suolen vallitsevan pH:n arvioimiseksi tutkimalla kahveoyylikviinihappojen isomeroitumista toukkien suolen emäksisiä olosuhteita imitoivissa pH:issa ja vertaamalla tuloksia aiemmin kerättyyn tietoon kahveoyylikviinihappoisomeerien määrällisistä suhteista toukkien ulosteessa.

Työn toisessa osassa tutkittiin trooppisten kasvilajien siemeniä. Trooppisten kasvien sisältämiä fenolisia yhdisteitä ja niiden ekologiaa ei ole aiemmin juurikaan tutkittu, varsinkaan tässä mittakaavassa. Monet kasvien näkyvyyteen sekä ravinteiden saatavuuteen ja kohdentamiseen perustuvista ennusteista saivat kokeista vahvistusta – esimerkiksi siementen painon ja polyfenolikoostumuksen välillä havaittiin vahva korrelaatio. Fenolisia yhdisteitä sisältävistä lajeista valtaosa sisälsi joko erityyppisiä hydrolysoituvia tanniineja tai proantosyanidiineja.

ASIASANAT: emäksinen hapettumisaktiivisuus, entsymaattinen hapettumisaktiivisuus, erikoistuneet metaboliitit, fenoliset yhdisteet, kasvikunta

Table of Contents

Abbreviations

BCI	Barro Colorado Island, Panama
CA	Cinnamic acid derivative
CAF	Caffeic acid derivative
CQA	Caffeoylquinic acid
COU	Coumaric acid derivative
CoQA	Coumaroylquinic acid
DiCQA	Dicaffeoylquinic acid
EC	Enzyme Commission number
ET	Ellagitannin
FC	Folin-Ciocalteu
FL	Flavonoid
GA	Gallic acid, gallic acid derivative
GQA	Galloylquinic acid
GT	Gallotannin
HHDP	Hexahydroxydiphenoyl group
HT	Hydrolysable tannin
KA	Kaempferol
MY	Myricetin
PA	Proanthocyanidin
PC	Procyanidin
PCoA	Principal coordinates analysis
PD	Prodelphinidin
PGLS	Phylogenetic generalized least squares analysis
PPC	Protein precipitation capacity
PPO	Polyphenol oxidase
QU	Quercetin
UHPLC-DAD-QqQ-MS	Ultrahigh-performance liquid chromatography coupled with diode array detector and triple quadrupole mass spectrometer

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kim J, Pälijärvi M, Karonen M, Salminen J-P (2018) Oxidatively active plant phenolics detected by UHPLC-DAD-MS after enzymatic and alkaline oxidation. *J Chem Ecol* 44: 483–496. doi: 10.1007/s10886-018-0949-x.

II Kim J, Pälijärvi M, Karonen M, Salminen J-P (2020) Distribution of enzymatic and alkaline oxidative activities of phenolic compounds in plants. *Phytochemistry* 179: 112501. doi: 10.1016/j.phytochem.2020.112501

III Gripenberg S, Rota J, Kim J, Wright SJ, Garwood NC, Fricke EC, Zalamea P-C, Salminen J-P (2018) Seed polyphenols in a diverse tropical plant community. *J Ecol* 106: 87–100. doi: 10.1111/1365-2745.12814

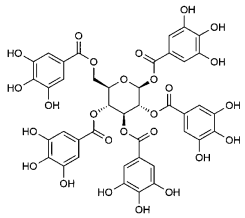
IV Kim J, Gripenberg S, Karonen M, Salminen J-P (2020) Seed tannin composition of tropical plants. Submitted to *Phytochemistry*.

1 Introduction

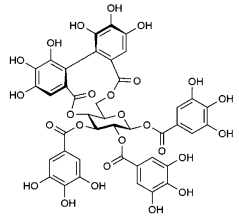
Plant cells produce a variety of specialized metabolites to increase the survival and reproduction of the organism as a whole. These metabolites include alkaloids and terpenoids, as well as polyphenols and simple phenolic acids, which are collectively known as phenolics. Phenolics are common and ubiquitous in the plant kingdom; the vast majority of plant species allocate some of the resources they could spend on growth and development to produce phenolic compounds to act as pigments and as a stress response against UV radiation, pathogens and herbivores, among other things (Appel 1993; Cheynier et al. 2013). Phenolics can be divided into several, partially overlapping classes, some of which will be briefly introduced below. Exemplar structures are presented in Figure 1.

Simple phenolic acids only contain one phenolic moiety, while polyphenols contain two or more. Hydrolysable tannins (HTs) contain a native or modified galloyl group centered around a polyol core. Galloyl glucoses (GGs; **1**), gallotannins (GTs) and ellagitannins (ETs; **2**–**4**) belong to HTs. The respective characteristic structures of the latter two classes are a chained galloyl group and hexahydroxydiphenoyl (HHDP) group, where two gallic acid (GA) moieties are linked via a C–C bond. Structural variety of ETs arises from modifications of the HHDP group and the cyclic or acyclic nature of the central glucose core, and the type of linkage between monomer units in oligo- and polymeric ETs. Complex tannins consist of a HT unit and a flavonoid or proanthocyanidin (PA) unit (Nonaka 1989).

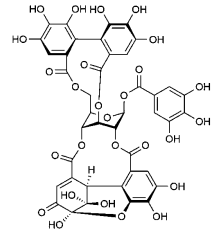
Proanthocyanidins (**5**–**7**) typically consist of two or more flavan-3-ol subunits, but some rare classes may also contain flavan subunits. The two most common PA classes are procyanidins (PCs) and prodelphinidins (PDs), with characteristic subunits being (epi)catechins and (epi)gallocatechins, respectively (in this work, these are referred to as PC and PD subunits at times). In B-type PAs, the monomeric units are linked via a C4→C8 or C4→C6 bond, whereas A-type PAs additionally contain a C2→O→C7 or C2→O→C5 bond. Structural diversity of PAs arises from the stereochemistry of the carbons C2, C3 and the extension units' C4, the possible galloylation of the 3-OH, and the composition on subunit level of each compound, since PA subclasses other than PCs may contain (epi)catechin in addition to their characteristic subunits.



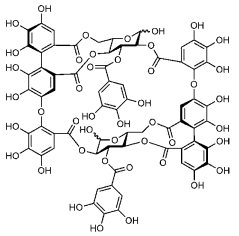
1 Pentagalloyl glucose



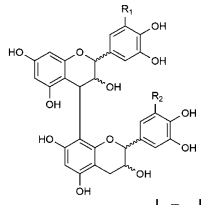
2 Tellimagrandin II



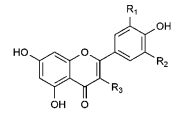
3 Geraniin



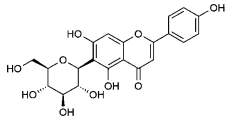
4 Oenothein B



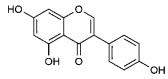
	R_1	R_2
5 Dimeric procyanidin	H	H
6 Dimeric prodelphinidin	OH	H
7 Dimeric prodelphinidin	OH	OH



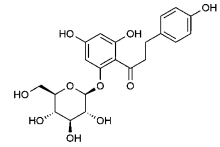
	R_1	R_2	R_3
8 Kaempferol	H	H	OH
9 Quercetin	OH	H	OH
10 Myricetin	OH	OH	OH
11 Apigenin	H	H	H
12 Luteolin	OH	H	H
13 Tricetin	OH	OH	H



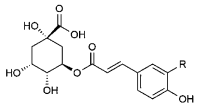
14 Isovitexin



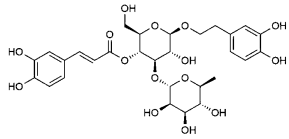
15 Genistein



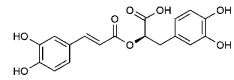
16 Phloridzin



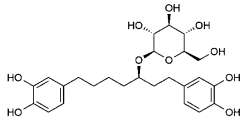
17 R = OH: 5-Caffeoylquinic acid
18 R = H: 5-Coumaroylquinic acid



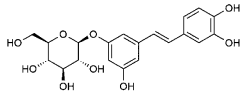
19 Acteoside



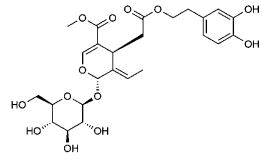
20 Rosmarinic acid



21 Rubranoside A



22 Astringin



23 Oleuropein

Figure 1 Several types of phenolics detected in this study. Compounds **1–4** are hydrolysable tannins: **1** is a galloyl glucose, while **2–3** are different ellagitannins (**2** simple hexahydroxydiphenoyl [HHDP] ester, **3** dehydro-HHDP ester, **4** oligomeric and macrocyclic). Compounds **5–7** are dimeric proanthocyanidins with different subunit compositions. Compounds **8–16** are various flavonoids: **8–10** are flavonols and **11–13** are flavones, usually present in plants as glycosides. **14** is a *C*-glycosidic flavone, **15** is an isoflavone, and **16** with an acyclic C_3 structure is a dihydrochalcone. Compounds **17–20** are hydroxycinnamic acid derivatives: **17–18** are quinic acid esters, **19** is also a phenylethanoid derivative, and **20** is a 3-(3,4-dihydroxyphenyl)-lactic acid ester. **21** can be considered to be a phenylpropanoid derivative. Compound **22** is a dihydroxysubstituted stilbenoid and **23** is a secoiridoid containing a phenylethanoid group.

All flavonoids share the common $C_6C_3C_6$ skeleton. They are divided into subclasses based on the functional groups of the C_3 structure. The most common flavonoids in the plant kingdom are flavonols (i.a. quercetin and kaempferol glycosides; **8**–**10**) and flavones (i.a. apigenin glycosides; **11**–**13**). Flavonoids are usually *O*- or *C*-glycosylated (**14**) and sometimes methoxylated. In isoflavonoids, the B ring is in the position 3 (**15**), while in neoflavonoids (not detected in this study) it is in the position 4. In chalcones and dihydrochalcones, the typically heterocyclic C_3 structure remains open (**16**).

Hydroxycinnamic acid derivatives include caffeic and coumaric acid derivatives (**17**, **18**). A typical example is 5-*O*-caffeoylquinic acid (5-CQA, also known as chlorogenic acid), which is ubiquitous in the plant kingdom. Hydroxycinnamic acids can be centered around a polyol core (e.g. 5-CQA, chicoric acid, fukinolic acid, acteoside; **19**), or esterified with other phenolic acids (e.g. rosmarinic acid; **20**).

Phenolics also include a large variety of other compounds, such as xanthonoids (e.g. mangiferin), phenylethanoid and phenylpropanoid derivatives (e.g. rubranoside A; **21**) and stilbenes (e.g. astringin; **22**). Compound classes that are inherently not phenolic may include compounds that contain phenolic moieties. Such examples are secoiridoids (a type of terpenoid, e.g. oleuropein; **23**) and glucosinolates (e.g. sinalbin).

Two major modes of action against herbivores have been proposed for phenolic compounds: oxidation and protein precipitation. As a herbivore consumes the plant material, the proteins and phenolics in the digested material can come into contact with each other. Certain phenolics are able to bind to the surfaces of the proteins with non-covalent interactions, decreasing the solubility of the protein to the point of precipitation, reducing the digestibility of the protein and consequently, the nutritive quality of the consumed plant material (Feeny 1969). This mode of action is prevalent in mammalian herbivores. Oxidation can occur in two ways: by the oxidizing enzymes present in the plant itself (Yoruk and Marshall 2003), or in the alkaline environment of the midgut of lepidopteran caterpillars (Appel 1993). Both oxidation mechanisms require molecular oxygen, which is available throughout the digestive tract of insect larvae in small quantities. Phenolics can oxidize into highly reactive quinones, which can cause oxidative stress by damaging the epithelial cells in the herbivore gut (Thiboldeaux et al. 1998), or affect the nutritive quality of the ingested material with covalent cross-linkage with proteins (Yoruk and Marshall 2003).

At least four types of enzymes that are capable of oxidizing phenolics can be present in plants (Yoruk and Marshall 2003): *o*-diphenolase-active polyphenol oxidases (PPO; Enzyme Commission number, or EC, 1.10.3.1), monophenolase-active PPO (EC 1.14.18.1), peroxidases (EC 1.11.1.7) and laccases (EC 1.10.3.2). Diphenolase-active PPOs are the most common phenol-oxidizing enzyme in

the plant kingdom, capable of oxidizing *o*-dihydroxysubstituted[1] phenolics into *o*-quinones (Figure 2). Of these four enzymes, monophenolase-active PPO is the only one capable of oxidizing monohydroxysubstituted phenolics, by first turning them into *o*-diphenolics and then into *o*-quinones (Yoruk and Marshall 2003; Aniszewski et al. 2008). Instead of using molecular oxygen as an oxidizing agent, peroxidases use hydrogen peroxide (H_2O_2) which can become available in the digestive tract as a byproduct of PPO-catalyzed oxidation or autoxidation of phenolics (Yoruk and Marshall 2003; Barbehenn et al. 2010). Laccases are capable of oxidizing trihydroxysubstituted and *p*-dihydroxysubstituted phenolics into quinones (Mayer and Harel 1979; Yoruk and Marshall 2003). In healthy plant cells, phenolics are usually stored in vacuoles away from PPOs present in chloroplast lumens and laccases that are extracellular. They can come into contact once the plant cell structures are damaged, for instance, by a herbivore chewing on the plant material (Yoruk and Marshall 2003; Aniszewski et al. 2008).

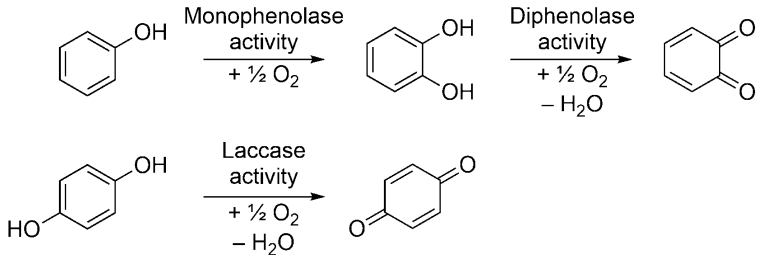


Figure 2 Oxidation of mono- and dihydroxysubstituted phenolics by various enzymes. The monophenolase-active polyphenol oxidase catalyzes the whole reaction on top, while diphenolase-active only can catalyze the latter stage. Thus, monohydroxy-substituted phenolics are unaffected if only diphenolase-active enzyme is present, which is also true for phenolics that are *m*- and *p*-dihydroxysubstituted. Laccases can catalyze the oxidation of *o*- and *p*-dihydroxysubstituted phenolics. The resulting quinones form melanins in a series of unspecific reactions with other quinones, phenolics and biomolecules.

Because of their significance in ecology and potential health benefits to humans, plant phenolics from numerous plant sources have been studied for decades, with

[1] In this thesis, any discussion about the hydroxysubstitution of phenolic moieties refers to the *o*-substitution unless stated otherwise. That is, a compound that is "dihydroxy-substituted" contains a catechol group, where the two OH groups are vicinal, whereas a "trihydroxysubstituted" compound contains a pyrogallol group, where all three OH groups are vicinal. In most flavonoids, the A ring is *m*-dihydroxysubstituted, and this type of structure does not readily form a quinone via oxidation. Thus, the key element in the oxidation of flavonoids is considered to be the substitution of their B ring, and the substitution of the A ring is disregarded.

a wide variety of methods having been developed for the purpose. Nowadays, a typical method involves chromatographic separation, followed by detection and identification using either a diode array detector (DAD), a mass spectrometer (MS), or both. The exact structure is determined using nuclear magnetic resonance (NMR) spectroscopy. Colorimetric "total" methods, e.g. total phenolics and total flavonoids, are still utilized frequently today. These methods reveal the total concentration of phenolic compounds or selected subclasses in plant extracts, but not their exact phenolic composition. Therefore, the information obtained from bioactivity assays can be limited if the phenolics are only quantified using total methods as the relationships between compounds and observed activities of plant extracts cannot be established for certain (Moilanen and Salminen 2008; Salminen and Karonen 2011).

Typically, studies on the phenolic composition of plants focus only on a few plant species or genera, or only focus on a certain class of phenolics, such as ellagitannins or flavonoids. A comprehensive overview of the distribution of plant phenolics across the plant kingdom can be obtained by reviewing the existing literature, but the differences in the methodology of different publications can make the comparison difficult. Large-scale screening studies using consistent methodology throughout overcome this caveat (Moilanen et al. 2015; Marsh et al. 2017), but naturally are more time-consuming to conduct. Furthermore, the study of phenolic composition usually focuses on plants that grow in temperate zones or that are important in the food industry, or both (e.g. Clifford 2000; Häkkinen et al. 2000). In contrast, tropical plants are typically studied for their essential oil composition or compound classes other than phenolics, such as alkaloids or terpenoids (e.g. Scalvenzi et al. 2017; Marini et al. 2018; a literature overview on selected species is available in the Supplementary of Article IV). Oftentimes, the phenolics in tropical plants have been determined using total methods, so details on their exact phenolic composition are scarce.

Enzymatic oxidation potential of plant samples is usually studied by extracting the phenolic compounds from plant material and oxidizing them using the commercially available tyrosinase enzyme extracted from mushrooms (Luo and Xie 2013). However, tyrosinase is monophenolase-active enzyme, and monophenolase activity is relatively uncommon in plants (Yoruk and Marshall 2003; Aniszewski et al. 2008). A plant species may produce monohydroxysubstituted phenolic compounds but no monophenolase-active enzymes. Therefore, the traditional methods may grossly overestimate the enzymatic oxidation potential of plants. An alternative way is to extract the enzymes from a plant source and introduce them to a purified substrate, such as a simple catechol or 5-CQA (Murata et al. 1992; Orenes-Piñero et al. 2006; Schmitz et al. 2008). However, despite being very common in the plant kingdom and a suitable substrate for PPOs, 5-CQA alone may not accurately represent the phenolic composition of the plant in a qualitative or quantitative sense. From

a physiological standpoint, extracting plant material and introducing the extract to an external, commercial material simply is not an appropriate approach.

In alkaline conditions, CQAs can undergo a base-catalyzed isomerization reaction, where the caffeic acid moiety is assumed to migrate within the molecule between the positions 3, 4 and 5 (Xie et al. 2011). This occurs both *in vitro* (Vihakas et al. 2014) and *in vivo* (Salminen et al. 2004; Lahtinen et al. 2005). The observed ratio of these three isomers depends on incubation time and pH. This phenomenon offers a promising alternative for estimating the prevailing gut pH of insect herbivores, which is usually done by using microelectrodes (Gross et al. 2008).

This PhD project covered the aforementioned aspects in the analysis of phenolic compounds. The project had the following aims:

1. Study the distribution of phenolics in the plant kingdom by analyzing hundreds of species across the plant phylogeny, including tropical species (Articles II and IV) and using consistent, high throughput methodologies to achieve comparable results.
2. Develop a physiologically relevant method to study the effects of enzymatic oxidation on phenolics on compound level (Articles I and II).
3. Study the correlation between the phenolic content and non-chemical plant traits of tropical plants (Article III).
4. Study the isomerization rates of CQAs using buffers with various alkaline pH values and compare the observed ratios with the existing data collected from different insect herbivore species (Article I).

Ultrahigh-performance liquid chromatography coupled with a DAD and triple-quadrupole MS (UHPLC-DAD-QqQ-MS) was used to determine the phenolic profiles of each plant sample. The total phenolics and oxidative activities of the raw extracts were determined using the modified Folin-Ciocalteu (FC) assay. In Articles III and IV, the protein precipitation capacity was determined using radial diffusion assay.

A simple *in planta* oxidation method was developed in Articles I and II to study the effects of enzymatic oxidation on compound level using UHPLC-DAD-MS. Frozen plant samples were incubated at 30 °C to allow the phenolics present in the plant sample to be oxidized by the enzymes present in the same plant tissue. In articles III and IV, we analyzed the phenolic content of the seeds of trees, shrubs and lianas from Barro Colorado Island, Panama (BCI), focusing on their significance in the ecology of maternal plants (Article III) and the composition of HTs and PAs (Article IV).

2 Materials and Methods

2.1 Plant material

2.1.1 Plant samples collected in Finland

A collection of 287 plant samples was gathered in the Turku region, South-Western Finland, from May to August 2011 for Articles I and II. The samples were collected in duplicates, A and B. Five plant individuals were selected at the collection site for each sample. At least three undamaged tissue samples, such as flowers, leaves or needles were collected from each specimen and pooled together for the sample A. The same was repeated for the sample B, with the tissue samples growing as closely as possible to the ones collected for the sample A to maximize the similarity of the phenolic content prior to sample processing. Samples were wrapped in aluminum foil and put in an ice box (0 °C) for the remainder of the sample collection and transferred to a freezer (−20 °C) within 3 hours, where they were kept for a minimum of 18 hours before further processing.

Samples B were oxidized enzymatically *in planta* using the method developed in Article I. The incubation temperature was 30 °C, well below the thermal degradation temperature of polyphenol oxidases (around 50 °C), and plausible considering herbivory in nature (Yoruk and Marshall 2003). The incubation time of 1, 2 and 3 hours was tested using five leaves from single *Betula pubescens* and *B. pendula* specimens for each incubation time, with the other five leaves as control samples. After analyzing the phenolic content of the leaves, the optimal incubation time for our purposes was determined to be 2 hours.

Accordingly, the samples B were transferred to an oven set to 30 °C and kept there for 2 hours. As the plant cell structures had been ruptured by the freezing process, this enabled the oxidizing enzymes and phenolics to come into contact with each other, leading to the enzymatic oxidation of phenolics. The samples B were then transferred back to the freezer for another minimum of 18 hours. Samples A and B were freeze-dried and ground into powder.

2.1.2 Plant samples collected in Panama

A collection of 572 seed samples was gathered along the forest trails of BCI from July 2010 to December 2013 for Articles III and IV. This does not include the 62 seed samples that proved to be too hard to grind and had to be excluded from the study. Most of the collected material in a single sample likely shared the same maternal plant individual. The seeds were removed from the fruit pulp and freeze-dried in paper envelopes for a minimum of 72 hours. Each sample of a given species consisted of whole seeds (embryo, endosperm and coat or testa), collected in a given location on the same day. The samples were stored in plastic ziplock bags in a freezer until being exported to Finland under the permit from the *Autoridad Nacional del Ambiente* (ANAM). In Finland, the samples were stored in a freezer before further processing. The seeds were ground into powder. The 178 samples that resulted in thick oily liquid or coarse, sticky granules instead of fine, dry powder were analyzed normally and their data was included in the results. Quantitative difference between dry and oily samples was not evaluated, because the number of species containing more than one dry and oily sample each was very low, making this type of comparison unreasonable.

2.2 Chemical analyses

2.2.1 Sample extraction

Ground samples were weighed (20 mg) in 2 mL Eppendorf tubes and extracted twice with acetone-water (3 h, 1400 µL, 7:3 v/v in Articles I and II, and 8:2, v/v in Articles III and IV to ensure the better extraction of large PAs). The extracts were combined and the acetone was evaporated at room temperature using an Eppendorf concentrator. The remaining aqueous phase was frozen and lyophilized, and the material was then dissolved in 1 mL of ultra-pure water and filtered using a syringe filter (13 mm, 0.2 µm PTFE, VWR International LLC, Radnor, PA, USA), yielding the raw extract of water-soluble phenolics.

2.2.2 UHPLC-DAD-QqQ-MS analyses

All samples were analyzed using UHPLC-DAD-QqQ-MS for their phenolic profiles. In Articles I and II, raw extracts were analyzed either undiluted (comparison between oxidized and non-oxidized samples) or diluted to one-fifth concentration (analyses that included MRM quantitation) by dissolving 40 µL of raw extract in 160 µL of ultra-pure water. In Articles III and IV, the raw extracts were diluted to one-fifth concentration with ultra-pure water prior to analyses.

The UHPLC-DAD-QqQ-MS was an Acquity UPLC® series, consisting of a sample manager, a binary solvent manager, and a photodiode array detector, whereas the triple quadrupole mass spectrometer was a Xevo TQ (Waters Corporation, Milford, MA, USA; Figure 3). The column was a 100 mm × 2.1 mm id, 1.7 µm Acquity UPLC® BEH Phenyl column (Waters Corporation, Wexford, Ireland). The flow rate of the eluent was 0.5 mL/min and the injection volume was 5 µL. The binary gradient elution used acetonitrile (A) and 0.1% aq. HCOOH (B) as solvents. The mass spectrometer was used in a negative ionization mode. The desolvation and source temperatures were 650 and 150 °C, respectively, and the flow rates of the desolvation and cone gas (N_2) were 1000 L/h and 100 L/h, respectively. The collision gas was argon. The photodiode array detector was operated at 190–500 nm, and the phenolic compounds were monitored at 280 nm unless stated otherwise. Elution profiles and MS settings are described below.

The short gradient elution was (reported as % A in B): 0.0–0.5 min, 0.1%; 0.5–5.0 min, 0.1–30%; 5.0–6.0 min, 30–35%; 6.0–9.5 min, column wash and stabilization. UV and MS data were collected from 0 to 6 min, and the ions were scanned at *m*/*z* range 150–1200 on MS. The capillary voltage was set to 2.4 kV (Article II) or 3.4 kV (Articles III and IV). In Articles II–IV, this gradient was used together with the group-specific mass spectrometric multiple reaction monitoring (MRM) methods to quantify galloyl groups (indirectly quantifying gallic acid derivatives), HHDP groups (ellagitannins), (epi)catechin groups (procyanidins), (epi)gallocatechin groups (prodelphinidins), kaempferols, quercetins, myricetins (respective glycosides) and quinic acid derivatives (e.g. CQAs) (Engström et al. 2014, 2015). The short gradient was used to analyze the first 140 samples.

The long gradient elution was (reported as % A in B): 0.0–0.5 min, 0.1%; 0.5–5.0 min, 0.1–30%; 5.0–8.0 min, 30–45%; 8.0–11.5 min, column wash and stabilization. UV and MS data were collected from 0 to 8 min, and the ions were scanned at the *m*/*z* range 250–1400 (Articles I and II) or 150–1200 (Articles III and IV) on MS. The capillary voltage was set to 3.4 kV. In Articles I and II, this gradient was used to compare the phenolic profiles of oxidized and non-oxidized samples (Section 2.2.3). In Articles III and IV, this gradient was used together with the MRM methods to analyze the remaining 432 samples to ensure the complete elution of PA humps.

A quick gradient was used to study the isomerization of caffeoylquinic acids in Article I (reported as % A in B): 0.0–0.5 min, 0.1%; 0.5–3.5 min, 0.1–20%; 3.5–3.6 min, 20–0.1%; 3.6–5.0 min, 0.1%. The photodiode array detector was operated at 190–500 nm, and the CQAs were monitored and quantified at their UV maximum of 325 nm. Each isomer (3-, 4- and 5-CQA) was dissolved in ultra-pure water (10 mg/mL). Each standard solution was mixed with a carbonate buffer in a UHPLC vial (1:9, v/v). The UHPLC analysis was then started immediately, and

24 injections were repeated once every 6 min. Each isomer was analyzed at pH 9, 10 and 11, and each analysis was repeated three times. For control, the standard solutions were mixed with ultra-pure water instead of carbonate buffer and analyzed in three replicates. Finally, 5-CQA was incubated at pH 9.0–11.0 in 0.2 pH unit increments, the ratio of isomers after 180 min was determined, and the analysis was repeated three times.

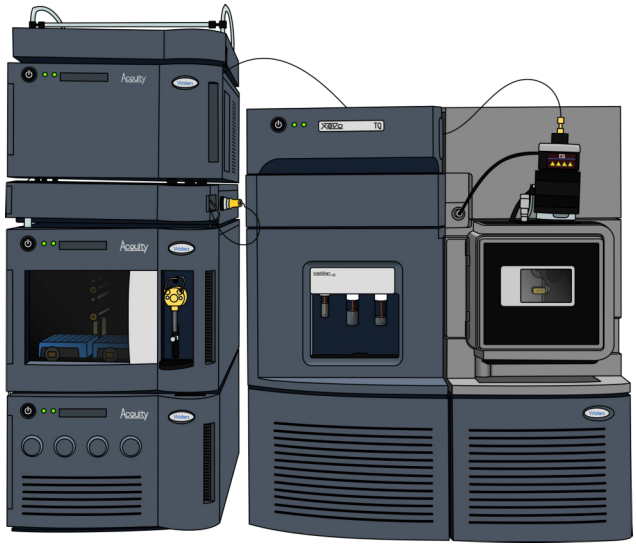


Figure 3 An ultrahigh-performance liquid chromatograph coupled with a diode array detector and triple quadrupole mass spectrometer. Traced from a photograph by the author.

2.2.3 Total phenolics assay

Total phenolics in each sample before and after alkaline oxidation was determined using a modified FC assay to complement the chromatographic analysis and mass spectrometric quantification (Salminen and Karonen 2011). Additionally, the total phenolics were analyzed in the enzymatically oxidized samples in Articles I and II. The analysis was conducted the same way in every case, using a microplate reader (Thermo Multiskan Ascent, Themo Electron Corporation, Shanghai, China; Figure 4), 96-well plates and five solutions: A (pH 10 sodium carbonate buffer), B (0.6% aq. HCOOH), C (a combination of solutions A and B, 9:5, v/v, pH 6), D (1 M FC total phenol reagent), and E (20% Na_2CO_3, m/v).

For alkaline oxidation, 20 µL of undiluted raw extract was transferred to a well-plate, and 180 µL of solution A was added to the same well to initiate the oxidation. The plate was shaken for 10 s every minute for 60 min, after which the oxidation was stopped by adding 100 µL of solution B, lowering the pH of the solution to 6.

The non-oxidized control sample was diluted in the same proportion to obtain a total phenolics value readily comparable to that of the oxidized sample. A 20 µL portion of undiluted raw extract was dissolved in 280 µL of solution C, and the plate was shaken for 10 s every minute for 60 min. Samples B in Articles I and II were given the same treatment, again to obtain comparable total phenolics values.

After this, 50 µL of solution was transferred to a new well, and 50 µL of solution D was added, and the plate was shaken for 60 s. Finally, 100 µL of solution E was added to activate the FC reagent, and the plate was shaken for 10 s every minute, and the absorbance at 742 nm was read after 30 min. All samples were measured in triplicates, and the average values, reported as gallic acid equivalents (mg/g), were compared together to obtain the ratio of oxidized total phenolics.

In Articles I and II, the remaining triplicates of 250 µL were combined and filtered using a syringe filter (4 mm, 0.2 µm PTFE, Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed using UHPLC-DAD-QqQ-MS. This way, the effect of alkaline and enzymatic oxidation could be observed on compound level.

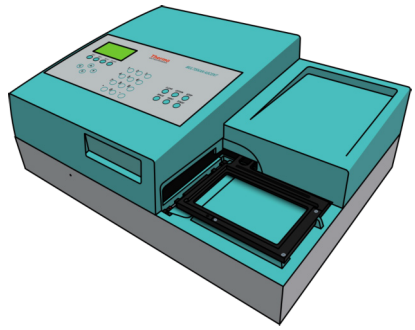


Figure 4 The microplate reader used in the total phenolics assay. Traced from a photograph by the author.

2.2.4 Radial diffusion assay

In Articles III and IV, the protein precipitation capacity of the raw seed extracts was determined using the radial diffusion assay (Hagerman 1987). The concentration of the filtered extract was doubled by freeze-drying 200 µL of extract and redissolving it in 100 µL of ultra-pure water. The model protein was BSA (0.1%, w/v), dissolved in 1% agarose gel (w/v) consisting of 60 µM ascorbic acid and 50 mM acetic acid and buffered to pH 5.0 using 2 M NaOH. A 10 mL portion of gel was dispensed into plastic Petri dishes, nine 4-mm holes were punched in the gel, and 24 µL of concentrated extract was applied into each of three holes. Once the extract had dissolved in the gel, the dishes were sealed with parafilm and incubated upside down at 30 °C for 72 h. The dishes were photographed and the area

of precipitation rings was measured using ImageJ (Abràmoff et al. 2004) and transformed into pentagalloyl glucose equivalents (mg/g). To make these values comparable between samples with varying levels of phenolics, the PPC was reported as the portion of total phenolics.

2.3 Non-chemical traits of tropical plants

The correlation between chemical and several non-chemical traits of woody plants was investigated in Article III (Table 1) based on theories on plant apparency (Feeny 1976) and resource availability and allocation (Coley et al. 1985; Agrawal 2011; Asner and Martin 2012). Non-chemical traits were extracted from existing data or measured using the methods described in the literature (Wright et al. 2010; Fricke and Wright 2016; Gripenberg et al. 2017).

Table 1 Non-chemical plant traits and their assumed correlation with the occurrence and quantity of phenolics in tropical plants.

Non-chemical plant trait	Hypothetical correlation with polyphenol content	Reasoning
Maximum tree height	Positive	Pre-dispersal seed predators can easily find the plentiful crop produced by the large tree.
Length of seed dormancy	Positive	The longer the time span between seed production and seed germination, the more time the pre-dispersal seed predators have time to feed.
Relative growth rate of the plant	Negative	The more the plant invests in chemical defenses, the less resources it has available for growth.
Seed size	Positive	The plant produces few large, but well-protected seeds.
Mechanical seed defenses	Negative	The plant invests only on chemical or mechanical defenses of the seeds.
Defenses expressed by adult-stage plant tissues	Positive	Plants invest in defenses throughout their life cycle.
Trees vs. lianas	More polyphenols in trees	Trees grow slowly, investing in chemical defenses. Lianas focus on fast growth at the expense of chemical defenses.

Statistical analyses were conducted using *R* 3.2.2 (R Core Team 2015). The degree of intraspecific variation in the concentration of polyphenol subgroups

was determined, and the association between chemical and non-chemical plant traits was assessed by the incidence and concentration of phenolics (total and subgroups) in the species. Incidence of phenolics, a binary variable, was tested using phylogenetic logistic regression analysis (Ives and Garland 2010) whereas their concentration, a continuous variable, was tested with phylogenetic generalized least squares (PGLS) analysis. Structures of the correlations were assessed with principal coordinates analysis (PCoA; Gower 1971).

3 Results

3.1 Distribution of phenolic compounds in the plant phylogeny

The complete plant phylogeny of this study is presented in Figure 5. Altogether, 364 plant species from 92 families were represented by 859 plant samples (287 collected in Finland and 572 in BCI). Detected compound classes in each family are summarized in Table 2. Several interesting observations are given below. For a comprehensive presentation, refer to the Supplementary materials of Articles II and IV. Some samples in the family Rosaceae contained HT, while others contained caffeic acid derivatives and PAs; these two sample types fell within the subfamilies Rosoideae and Spiraeoideae, respectively (Potter et al. 2007), and the results of their analyses are examined separately on occasions.

Typically, species that had a high content of total phenolics were also rich in HTs. Families with a concentration of total phenolics of 100 mg/g or more were Cornaceae (155 mg/g; contains galloyl glucoses and ET), Lythraceae (174 mg/g; contains punicalagin and PC), Nymphaeaceae (129 mg/g; contains geraniin), Onagraceae (130 mg/g; contains oenotheins A and B) and Paeoniaceae (130 mg/g; contains GT). Additionally, the average total phenolics concentration of the ET-rich subfamily Rosoideae of the Rosaceae family was 106 mg/g.

Based on the MRM quantitation, PAs, flavonoids and quinic acid derivatives were detected in more species than ETs, but in lower quantities (Table 3). Myricetin glycosides in particular were detected rarely: their average concentration was 1 mg/g or more in only 16 species as opposed to 116 and 113 species for quercetin and kaempferol, respectively. (Epi)catechin subunits were more common than (epi)-gallocatechin subunits based on the MRM.

The family Myrtaceae had a high content of ETs. The unresolved hump of ETs observed in the seeds of *Myrcia splendens* likely consisted of ET oligo- and polymers with simple HHDP ester monomers based on the UV and mass spectra (Figure 6). The seeds of *Doliocarpus multiflorus* contained large amounts of mono- and trigalloyl glucose, but very little digalloyl glucose.

(Continues on page 26.)

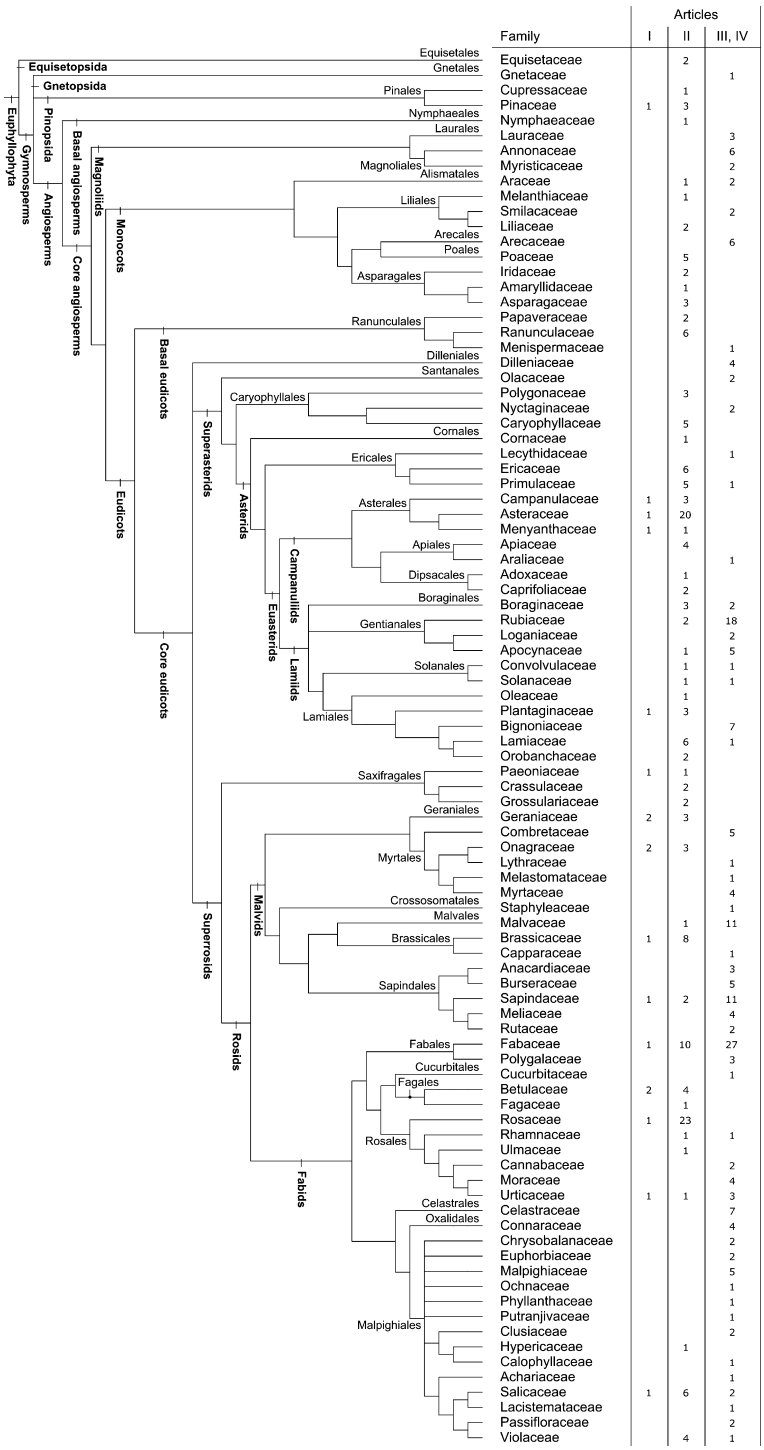


Figure 5 The complete family-level plant phylogeny of this project, together with the number of species from each family in each of the four articles.

Table 2 Detected classes of phenolic compounds in the studied plant families. Abbreviations: CA: cinnamic acid derivatives, CAF: caffeic acid derivatives, COU: coumaric acid derivatives, ET: ellagitannins, FL: flavonoids, GA: gallic acid derivatives, HT: hydrolysable tannins, KA: kaempferol glycosides, MY: myricetin glycosides, PA: proanthocyanidins, PC: procyanidins, PD: prodelphinidins, QU: quercetin glycosides.

Family	HT		PA			FL				CA		
	GA	ET	PC	PD	Other	KA	QU	MY	Other	CAF	COU	Other
Equisetaceae						X			X	X		
Gnetaceae												
Cupressaceae			X				X				X	
Pinaceae			X			X						X
Nymphaeaceae		X										
Lauraceae			X							X		
Annonaceae												
Myristicaceae												
Araceae						X			X			
Melanthiaceae						X						
Smilacaceae												
Liliaceae							X		X	X	X	
Arecaceae			X							X		
Poaceae						X	X		X	X		
Iridaceae								X	X	X		X
Amaryllidaceae						X						
Asparagaceae						X	X			X	X	
Papaveraceae							X			X		
Ranunculaceae						X	X		X	X		
Menispermaceae												
Dilleniaceae	X		X						X			
Olacaceae	X	X										
Polygonaceae			X			X	X		X			
Nyctaginaceae												
Caryophyllaceae						X			X	X		
Cornaceae	X	X								X		
Lecythidaceae												
Ericaceae							X		X	X	X	X
Primulaceae				X		X	X	X	X	X		
Campanulaceae						X	X			X	X	
Asteraceae						X	X		X	X		
Menyanthaceae						X	X			X		
Apiaceae						X	X			X		
Araliaceae												
Adoxaceae							X			X		
Caprifoliaceae							X		X	X		
Boraginaceae										X		
Rubiaceae			X	X	X		X			X		

Family	HT		PA			FL				CA		
	GA	ET	PC	PD	Other	KA	QU	MY	Other	CAF	COU	Other
Loganiaceae												
Apocynaceae						X	X			X		
Convolvulaceae						X				X		
Solanaceae							X			X		
Oleaceae						X			X			X
Plantaginaceae									X	X		
Bignoniaceae										X	X	
Lamiaceae						X	X		X	X		
Orobanchaceae									X			
Paeoniaceae	X					X						
Crassulaceae	X			X		X				X		
Grossulariaceae				X		X	X	X		X		
Geraniaceae	X	X										
Combretaceae		X										
Onagraceae		X										
Lythraceae		X										
Melastomataceae		X	X									
Myrtaceae		X	X	X								
Staphyleaceae												
Malvaceae			X							X	X	
Brassicaceae						X	X		X			X
Capparaceae												
Anacardiaceae	X		X						X			
Burseraceae												
Sapindaceae	X		X	X		X	X					
Meliaceae			X						X	X	X	
Rutaceae												
Fabaceae	X		X	X		X	X	X	X	X		X
Polygalaceae			X	X								
Cucurbitaceae												
Betulaceae	X					X	X			X		X
Fagaceae		X										
Rosaceae		X					X		X	X		
Rhamnaceae						X			X			
Ulmaceae							X			X		
Cannabaceae												
Moraceae			X							X		
Urticaceae			X							X		
Celastraceae			X	X								
Connaraceae			X							X	X	
Chrysobalanaceae				X					X			
Euphorbiaceae	X	X										
Malpighiaceae												
Ochnaceae			X						X		X	

Family	HT		PA			FL				CA		
	GA	ET	PC	PD	Other	KA	QU	MY	Other	CAF	COU	Other
Phyllanthaceae		X										
Putranjivaceae												
Clusiaceae			X						X			
Hypericaceae			X				X			X		X
Calophyllaceae			X									
Achariaceae												
Salicaceae			X	X		X	X		X	X	X	
Lacistemataceae			X	X								
Passifloraceae												
Violaceae			X			X	X		X		X	

The seeds of the genus *Psychotria* contained interesting PAs that could not be identified with the methods utilized in this study. Thus, they were simply referred to as "Psychotria PAs". While fragment ions characteristic of PAs were observed in their mass spectra (Figure 7), assigning the product ions to the correct corresponding precursor ions was not possible due to coelution. Based on the mass spectra, the Psychotria PAs consisted of (epi)catechin with a molecular weight of 290 Da and two other subunits with the molecular weights of 274 and 258 Da. (Epi)afzelechin, (epi)fisetinidol and (epi)guibourtinidol (the subunits of propelargonidins, profisetinidins and proguibourtinidins, respectively) match these molecular weights. However, the high alkaline oxidative activity of the raw *Psychotria* extracts would indicate there are pyrogallol groups present in the samples (see the following section), or that a yet unknown structural factor positively affects the alkaline oxidative activity of phenolic compounds.

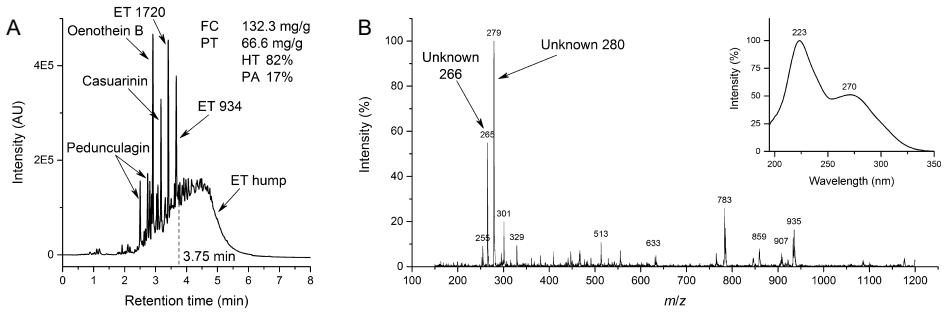


Figure 6 (A) The UV chromatogram of *Myrcia splendens* seeds, recorded at 280 nm. (B) The mass spectrum taken from 3.75–8.0 min. The embedded UV spectrum is taken from 3.75–5.25 min. The ellagitannin hump appears to consist of simple HHDP esters.

Table 3 Multiple reaction monitoring quantitation of phenolic compounds in all species of this study (n = 364). "Average, all" is the average concentration among all species. "Average if present" includes only species with an average concentration of at least 1 mg/g. "Maximum" is the maximum concentration among all species. "c" is the number of species with the given average concentration range. Proanthocyanidins, flavonoids and quinic acid derivatives were present in more species than hydrolysable tannins, but in lower concentrations. Abbreviations: FL: flavonoids, HT: hydrolysable tannins, PA: proanthocyanidins, QA: quinic acid derivatives.

Concentration (mg/g)	HT	PA	FL	QA
Average, all	9	7	4	3
Average if present	15	12	7	7
Maximum	186	81	46	41
1 ≤ *c* < 20	38	117	140	93
20 ≤ *c* < 50	10	36	15	9
50 ≤ *c*	27	8	0	0

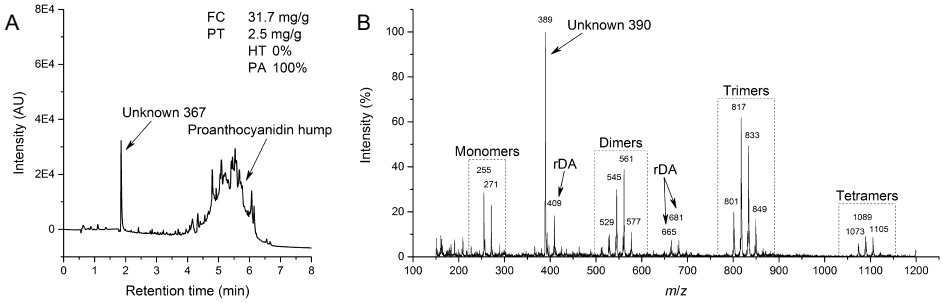


Figure 7 (A) The UV chromatogram of *Psychotria horizontalis* seeds, recorded at 280 nm. (B) The total mass spectrum taken from 0.8–8.0 min. Psychotria proanthocyanidins consist of subunits which contain one or two hydroxyl groups less than catechin (274 Da and 258 Da, respectively). Abbreviations: FC: Folin-Ciocalteu assay (total phenolics), HT: hydrolysable tannins, PA: proanthocyanidin(s), PT: total polyphenols (mass spectrometric quantitation), rDA: retro-Diels-Alder fragmentation.

3.2 Alkaline oxidative activity of phenolics

A modified Folin-Ciocalteu assay was used to determine the alkaline oxidative activity of raw plant extracts by comparing the concentration of total phenolics of non-oxidized and oxidized plant samples. Additionally, the effect of oxidation on compound level was studied on the plant samples collected in Finland, in which case the alkaline oxidative activity of phenolics was determined by comparing the peak areas of the compounds in oxidized and non-oxidized samples at 280 nm on the UV chromatograms. These two methods were used in tandem to observe how the compound-level activities would be reflected in total phenolics and vice versa.

On the individual compound level, two structures could be attributed to a high alkaline oxidative activity of phenolics. One of them was the pyrogallol group, which can be found in gallic acid derivatives, myricetin-type flavonoids and prodelphinidins, and has been observed to lead to the high alkaline oxidative activity of phenolics before (Figure 8; Vihakas et al. 2014). Another one was the catechol moiety at the end of an alkane chain. While accurately determining the effects of substitution of the alkane chain would require more dedicated studies, it appears that the structure should be at least the phenylethanoid-type – that is, the carbon immediately following the phenyl group should be saturated and unsubstituted, while the following one may be hydroxysubstituted. Conjugation seems to lower the alkaline oxidative activity of the compound. This is evident when comparing the activities of oleuropein (average peak area decrease: 100%), oregonin (97%) and rubranoside A (96%), all containing a phenylethanoid or phenylpropanoid group, to those of astringin (30%) and mono-CQAs (35%), which contain the catechol group at the end of an unsaturated carbon chain (Figure 9).

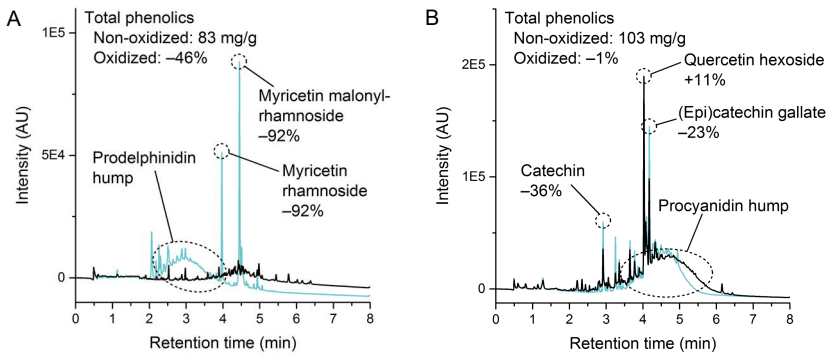


Figure 8 The UV chromatograms of non-oxidized (light blue) and alkalinically oxidized (black) *Ribes alpinum* leaves (A) and *Rumex longifolius* seeds (B), recorded at 280 nm. The trihydroxysubstituted myricetin glycosides and prodelphinidins completely oxidize, whereas the dihydroxysubstituted quercetin glycoside, (epi)catechin and procyanidins oxidize only moderately at best.

Monohydroxysubstituted compounds, such as kaempferol-type flavonoids and coumaric acid derivatives, were generally not active or only slightly active at pH 10. Their peak areas decreased by merely 0–15%.

There was an inconsistency in the alkaline oxidative activity of GTs, detected in *Paeonia* sp. and *Acer platanoides*. On an individual compound level, GTs larger than hexagalloyl glucoses tended to oxidize completely, losing 82–100 % of their peak areas. The observation was not supported by the FC assay, as the quantity of total phenolics only changed by ±15 %. The exact cause of this discrepancy is open to

speculation. For example, the oxidation products may still be able to reduce the FC reagent, but they are not detected with the utilized chromatographic method, e.g., due to a lowered UV absorbance capacity, late elution or removal by pre-analysis filtration.

While caffeic acid derivatives that did not additionally contain a phenylethanoid group could be classified as "moderately active" at pH 10 (average loss of peak area ca. 30%), their total peak area decrease could vary considerably between samples. This deviation could range from ca. 20% decrease of caffeoylmalic acid to even 80% decrease of diCQAs. This was true for other "moderately active" compounds as well, suggesting that the oxidation of these compounds is greatly dependent on factors in the sample matrix that promote or inhibit their oxidation.

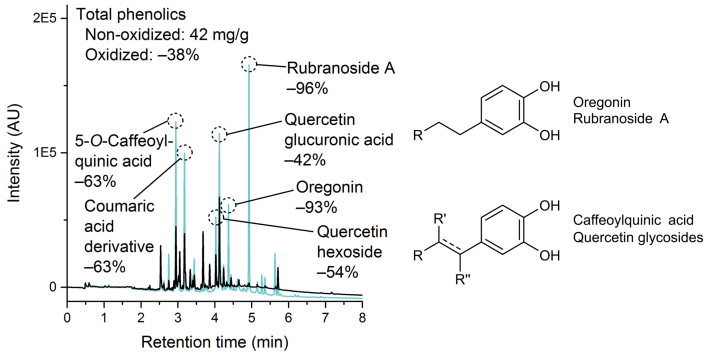


Figure 9 The UV chromatogram of non-oxidized (light blue) and alkalinically oxidized (black) *Alnus incana* leaves, recorded at 280 nm. Compounds with a dihydroxyphenethyl-type structure (e.g. oregonin and rubranoside A) oxidized nearly completely. Compounds with a carbon chain that was unsaturated, branching or both (e.g. caffeic acid derivatives and quercetin glycosides) were not oxidized as much. Note: Taking isomerization into account, the decrease in total peak area of caffeoylquinic acids and coumaric acid derivatives were 24% and 23%, respectively.

On the raw extract level, the changes of 10% or less in total phenolics were considered negligible. A 25% decrease or more in total phenolics could usually be associated with a relatively large portion of moderately or highly active compounds, though the total phenolics rarely decreased by over 50%. The main compounds of the samples with the highest decrease of total phenolics (around 45–55%) were usually myricetin glycosides or PDs. The high content of ETs typically resulted in a 20–35% decrease of total phenolics, whereas with PCs, the decrease was usually 0–20%. Oxidative activities of *Psychotria* samples were high in this regard, 30% on average.

3.3 Enzymatic oxidative activity of phenolics

The enzymatic oxidation of phenolics depended not only on their structure (namely, the mono-, di- or trihydroxysubstitution of their phenolic moieties), but also on the plant species and tissue as the content of oxidizing enzymes varies between different plants species and plant parts. Since diphenolase-active PPO is the most common enzyme capable of oxidizing phenolics in plants, it was not surprising that dihydroxysubstituted phenolics were oxidized more frequently and to a higher extent than the mono- and trihydroxysubstituted ones which would require less common monophenolase-active PPO and laccases to oxidize (Figure 10).

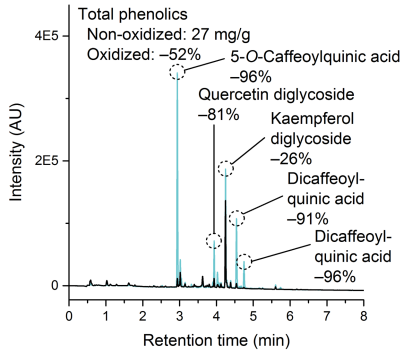


Figure 10 The UV chromatogram of non-oxidized (light blue) and enzymatically oxidized (black) *Trifolium pratense* flowers, recorded at 280 nm, demonstrating the tendency of enzymatic oxidation to favor dihydroxysubstituted phenolic compounds (such as caffeic acid derivatives and quercetin-type flavonoids) over monohydroxysubstituted ones (such as kaempferol-type flavonoids). The 52% decrease in total phenolics confirms that the phenolic content of the sample has substantially decreased.

Strong monophenolase activity was observed i.a. on *Malus* sp. (Figure 11), whereas *Quercus robur*, *Nymphaea alba*, the genus *Epilobium* and the subfamily Rosoideae of the family Rosaceae had strong triphenolase activity. Variation in enzymatic activity between tissue parts was observed e.g. in *N. alba* and the *Epilobium* species as their flowers had no enzymatic activity, while the same ellagitannins were oxidized in their leaves (Figure 12).

In general, if the enzymatic oxidative activity was high on the individual compound level (the decrease in peak area of all compounds was close to 100%), the decrease of total phenolics tended also to be high. In such cases, the total phenolics were often decreased by over 50%, with drops of 75% or more not being uncommon. This is in contrast with the alkaline oxidation, where the level of total phenolics rarely decreased by over 50% even when the oxidation was nearly complete on compound level (Table 4). This suggests that the final products of

enzymatic and alkaline oxidation are different, with alkaline oxidation *in vitro* resulting in compounds that retain some of their phenolic nature or ability to reduce the FC reagent, while enzymatic oxidation results in compounds that have lost all of their phenolic nature, their FC-reducing capacity, or both.

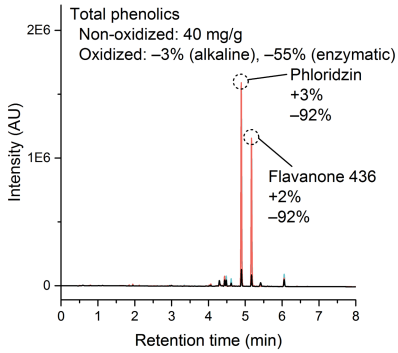


Figure 11 The UV chromatogram of the non-oxidized (light blue, mostly obscured), alkalinically oxidized (red) and enzymatically oxidized (black) *Malus* sp. leaves, recorded at 280 nm, demonstrating that monohydroxysubstituted phenolics (here, phloridzin) can oxidize in suitable conditions. In accordance with what we know about the oxidation of monohydroxysubstituted phenolics, at alkaline conditions, phloridzin does not oxidize at all. However, its complete oxidation *in planta* suggests that the *Malus* leaves contain monophenolase-active PPO.

Table 4 The number of oxidized samples with a given decrease of total phenolics (n = 276). Samples that had a low phenolic content (<10 mg/g) or a non-negative change in total phenolics have been excluded. The concentration of total phenolics could decrease substantially as a result of enzymatic oxidation, while the decrease after the alkaline oxidation tended to be relatively moderate in comparison. Based on the data of Article II.

Oxidation	0–20%	21–40%	41–60%	61–80%	81–100%
pH 10	98	153	15	0	0
Enzymatic	101	59	50	33	14

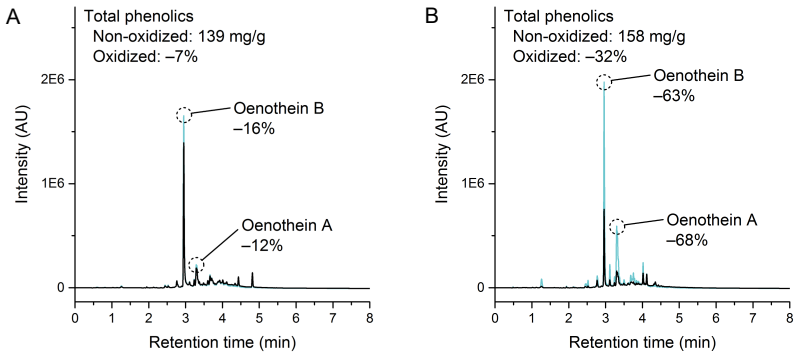


Figure 12 The UV chromatogram of non-oxidized (light blue) and enzymatically oxidized (black) *Epilobium angustifolium* flowers (A) and leaves (B), recorded at 280 nm. The same phenolic compounds – oenotheins A and B – have oxidized to the same extent within the sample, but differently between the two tissues, likely due to different contents of (active) oxidizing enzymes in the tissues. The total phenolics have reduced by a negligible level in flowers and noticeably in leaves, supporting the compound level observations.

3.4 Isomerization of monocaffeoylquinic acids

The total peak area of all CQA isomers decreased faster at the higher pH: during the first 60 min, the total peak areas had decreased by ca. 25, 70 and 90% at pH 9, 10 and 11, respectively, and by ca. 40, 85 and 95% after 120 min, regardless of the original isomer. When 3- or 5-CQA was the original isomer, 4-CQA was the first new isomer to form, and when 4-CQA was the original isomer, the first one to form was 3-CQA. Eventually, the ratio of isomers would reach a steady state, but the total level of isomers kept decreasing. At pH 9, the isomerization occurred so slowly that the steady state was not reached during the analysis time. At pH 10 it took approximately 140 min to reach the steady state, and at pH 11, ca. 30 min. At the steady state, the ratio of 3-, 5- and 4-CQA (in their retention order) was approximately 4:3:3 (Figure 13).

Incubating 5-CQA at various pH buffers for 180 min resulted in isomeric ratios presented in Figure 14. For example, at pH 9.0, the ratio of 3-, 5- and 4-CQA was approximately 1:8:2, at pH 9.6 it was 1:2:1, and at pH 11.0, 4:3:3. Assuming that no major changes in the isomeric ratios occur after the 180-minute incubation time, this knowledge could be used to estimate the gut pH of insect larvae by feeding them 5-CQA and comparing the ratios of the CQA isomers in their frass to Figure 14. For example, the CQA isomeric ratios detected in the frass of *Pristiphora alpestris* and *Agriopis aurantiaria* were ca. 2:6:2 and 4:3:3, respectively, suggesting that the gut pH of these lepidopteran species was close to 9.4 and 10.1.

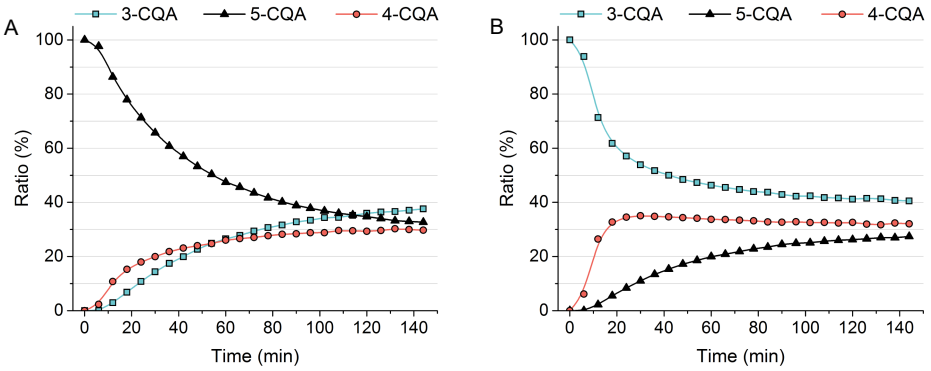


Figure 13 Isomerization of 5-*O*-caffeoylquinic acid (5-CQA, A) and 3-CQA (B) at pH 10. In both cases, 3-CQA becomes the dominant isomer towards the end of the reaction, and at the steady state, the ratio of 3-, 5- and 4-CQA is approximately 4:3:3.

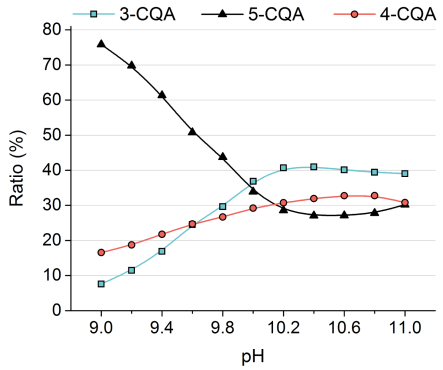


Figure 14 The ratio of caffeoylquinic acid (CQA) isomers resulting from incubating 5-CQA at various pH values for 180 min.

3.5 Protein precipitation capacity of the raw extracts

In general, the PPC of samples with a similar phenolic content could vary considerably. The relative PPC of the species *Combretum fructicosum*, *C. laxum* and *Lafoensia punicifolia*, containing punicalagin in large quantities, was high. The same was true for the genus *Psychotria*, which contained Psychotria PAs, though their concentration of total phenolics was only a fraction of the aforementioned species. The relative PPC of these samples was 92% on average. This far surpasses the other species that had a high relative PPC: *Mabea occidentalis* (72%, contains ETs), *Tontelea richardii* (64%, contains PCs and PDs), *Anacardium excelsum* (58%, contains high quantities of GAs) and *Myrcia splendens* (56%, contains high quantities of ETs).

3.6 Investment in chemical defenses of tropical plants

The observed correlations between chemical and non-chemical plant traits are summarized in Table 5. With few exceptions, the direction of correlations was the same across the bivariate and continuous variables.

Table 5 Non-chemical plant traits and their correlation with the occurrence and quantity of phenolics in tropical plants.

Non-chemical plant trait	Observed correlation with polyphenol content
Maximum tree height	Positive
Length of seed dormancy	Negative
Relative growth rate of the plant	Negative
Seed size	Positive
Mechanical seed defenses	Negative
Defenses expressed by other adult-stage plant tissues	Positive
Trees vs. lianas	No clear difference

4 Discussion

Phenolics were detected throughout the plant phylogeny. Flavonoids, caffeic acid derivatives and PAs were particularly abundant, whereas HTs were detected in relatively few families – most of them from the orders Saxifragales, Geraniales and Myrtales – but in much higher quantities.

The enzymatic oxidation of phenolics depends on the presence of oxidizing enzymes, which varies between different plant species and tissues and is further affected by the life stage of the plant as well as sustained damage – damage by herbivory on one part of the plant can induce the activation of latent-state PPO in other parts of the plant individual. The enzymatic oxidation activity of phenolics not only depends on their suitability to be substrates for enzymes, but also on the life history of the plant specimen being studied. Therefore, when studying the enzymatic oxidation potential of phenolics *in planta* using the method presented in Article I, it is imperative to collect the samples from plant individuals that have not been damaged by herbivores.

While the oxidation of phenolics on compound level was determined by comparing their peak areas in non-oxidized and oxidized samples in the UV chromatogram, it should be noted that peak areas of the compounds may be affected by reactions other than oxidation. A typical example of this is the isomerization of CQAs at alkaline pH, in which case one or more new peaks emerge while the area of the original (or originals) decreases. Care should be taken to identify the instances where the peak areas decrease as a result of isomerization or other non-oxidizing modification of the original compound before drawing conclusions (Figure 9).

The oxidation of "moderately active" compounds may be highly dependent on factors present in the plant extract that inhibit or promote oxidation (such as other phenolics or antioxidants), the nature and quantity of which vary between plant samples. In contrast, compounds containing a pyrogallol group or phenethyl-type group are highly active at alkaline conditions, consistently oxidizing completely.

It is assumed that a trade-off for producing phenolic compounds to defend against herbivores is that the energy and resources spent on their biosynthesis are taken away from other functions of the plant (Herms and Mattson 1992). Furthermore, plants that are likely to be attacked by herbivores would presumably invest in

chemical defenses (Feeny 1976). Consequently, there should be a connection between investment in chemical defenses and, for example, relative growth rate and seed production of the plant. In this regard, the observed correlations between chemical and non-chemical plant traits match the hypothesized ones well (compare Table 1 and Table 5), with two exceptions. There is a negative correlation between the length of seed dormancy and polyphenol content, which may arise from the mechanical protection of the seeds that have a long period of dormancy. Lianas were hypothesized to trade chemical defenses for fast growth as opposed to slow-growing and chemically well-defended trees, but no clear difference was observed between the polyphenol content of trees and lianas.

The results of this project could serve as a starting point for several types of studies. For example, the method for estimating the gut pH of lepidopteran larvae using the isomerization of CQAs as an indicator is based on *in vitro* isomerization data retrospectively compared against the data obtained from larval frass. This method could be verified by comparing the ratio of CQA isomers in the larval frass to the gut pH measured from the same larva individual or species. In some cases, only the compound class could be determined, and studies using NMR or more sophisticated mass spectrometric methods could reveal the structures of these compounds and therefore help understanding their structure–activity relationships. Finally, the benefit of realizing the factors inhibiting the alkaline oxidation of the "moderately active" compounds would be two-fold. If a given plant species was known to contain these constituents, the oxidative activity of its "moderately active" compounds could be predicted to be low. Conversely, a low alkaline oxidative activity of "moderately active" compounds could be used as an indicator for the presence of these constituents in the plant sample.

5 Summary

This thesis gives a comprehensive overview of the phenolic content and the *in vitro* alkaline oxidative activity of a large number of species throughout the plant phylogeny. This includes tropical plants, the phenolic composition of which has not been covered well in the past (Articles II and IV, and their Supplementary material in particular). The identification of the phenolic constituents of the plants, as well as the active compounds, compound types and plant species under two different types of oxidation greatly expands the current knowledge in the field of chemical ecology.

Effects of oxidation by enzymes *in planta* were studied using the simple method developed specifically for this purpose (Articles I and II). Compounds containing a pyrogallol group or a catechol group at the end of a saturated alkane chain were the most active at alkaline conditions, whereas the enzymatic oxidation generally tended to favor compounds that contained a catechol group (Article II).

The investment in phenolics-based chemical defenses in seeds in relation to several non-chemical traits of the seeds or maternal plants was studied (Article III). Most of the hypothesized relationships were supported by the observations: plant types that were assumed to invest in the chemical defenses of their seeds did so. For example, maternal tree height and large seeds were associated with a high content of polyphenols, whereas the opposite was true for seeds that were mechanically well protected.

When subjected to alkaline conditions, CQAs isomerize, reaching a certain time- and pH-dependent ratio of isomers. Assuming that the ratio does not change after enough time has passed, the observed ratio of mono-CQAs could be used to estimate the oxidizing pH – for example, the gut pH of a lepidopteran larva (Article I).

6 Acknowledgements

This work is based on the chemical analyses conducted in Natural Chemistry Research Group at the Department of Chemistry, University of Turku, during 2011–2017. The project was funded by the Kone Foundation and the Academy of Finland. Personal grants from the University of Turku, Emil Aaltonen Foundation, Väisälä Foundation, Finnish Chemical Societies and Palomaa-Erikoski Foundation are gratefully acknowledged. Travel grants from the Doctoral Programme in Physical and Chemical Sciences, Alfred Kordelin Foundation, University of Turku Foundation and Finnish Society of Chromatography enabled me to attend several conferences.

First and foremost I need to thank Professor Juha-Pekka Salminen for introducing me to polyphenol chemistry. Whether it has been about summer jobs, civilian service, analytics, interpretating the data or whatnot, I knew I could always rely on your assistance whenever needed. Your work in integrating the first-year students in their studies have been valuable for the department, and I look forward to a successful ICP2021.

I am also grateful to my second supervisor Maarit Karonen, "the good cop" and the voice of reason during our PhD meetings. Your knowledge in mass spectrometry has been inspiring, and your cheerful nature has ensured a relaxed atmosphere during the coffee breaks.

Professor Elisabeth Gross and Professor Heidi Appel took the task of reviewing this thesis, providing refreshing feedback. Professor Anna Kiss kindly accepted to be my opponent.

The PhD project has been a thoroughly pleasant experience, thanks to the joyful spirit of the Natural Chemistry Research Group. Pre-Christmas parties, coffee breaks, extracurricular activities, you name it, we have had fun together. My colleagues and co-workers throughout the years have been many: "The old school PhD generation" Dr. Jaana Liimatainen, Dr. Johanna Moilanen, Dr. Matti Vihakas, and Dr. Anu Tuominen served as an inspiration to pursue a PhD project of my own. Their work has set the basis for my research and contributed to my ways of presenting data. The methodologies of "the intermediates" Dr. Marica Engström and Dr. Nicolas Baert have been the backbone of my work. The current generation, Jussi Suvanto, Milla Leppä, Juuso Laitila, Suvi Vanhakylä, Marianna Manninen, Valtteri

Virtanen and Niko Luntamo have been my within-group peer support. Vladimir Ossipov supervised my Bachelor's work, and Anne Koivuniemi started to work in the Kone Project when it was launched back in 2011. Knowing that I can trust the accuracy of the data collected by Maija Pälijärvi has been invaluable when preparing and writing the Articles I and II.

I have supervised the students' advanced lab work several times with Heidi Korhonen and Petri Tähtinen, and their experience has come in handy several times. Too bad I won't be seeing what the course will be like in Aurum – don't quote me on that – but I trust you will manage it just fine.

The internship at Kasey Barton's lab at the University of Hawai'i at Manoa was as unique experience as it was out of the blue. Kasey, Kealoha, Amanda, Zoey, Kari, Alanna, Arby and Bruno, my warmest gratitude to you all for your hospitality. Special thanks to Andrea and Tiffany for a chance to join several field trips of theirs at Oahu and Kauai. The hospitality of the family Howe deserves praise as well. Hoping to visit Hawai'i again some day!

Several groups of friends are to be thanked for pastiming and otherwise. First of all, the long-lasting "Kermistit" for the countless get-togethers and other activities throughout the years. Second, the interdisciplinary lunch group "Joko syödään" for the… "interesting scientific discussions." Third, the movie evening group "Cradle of Civilization" for broadening my horizon on the movie spectrum – occasionally more than necessary. Fourth, all the people I keep playing board, role-playing and video games with ("Monday Evening Board Games," "Happy Chaps," "Casualfilter," "Hogwarts," several Dungeons & Dragons groups), and in that respect, I am especially thankful to Tatu for introducing me to Arkham Horror and D&D, the stepping stones for so much more (and financially, for so much less). Fifth, "Party Zone," again for countless get-togethers and movie evenings. And sixth, all those who do not fall to any of the aforementioned groups, i.e. Venla et al. and Krista. It has been fun with you people, and let's keep it that way.

Aleksi, Tytti and Edvin, you may already know that you are awesome, but I'm going to tell you anyway that you are awesome. Finally, my parents have not been holding back their encouragement and support during my studies. Thank you.

Turku, November 2020

Jorma Kim

7 List of References

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics International* 11: 36–42.

Agrawal AA (2011) Current trends in the evolutionary ecology of plant defence. *Functional Ecology* 25: 420–432. doi: 10.1111/j.1365-2435.2010.01796.x.

Aniszewski T, Lieberei R, Culewicz K (2008) Research on catecholases, laccases and cresolases in plants. Recent progress and future needs. *Acta Biologica Cracoviensia* 50: 7–18.

Appel HM (1993) Phenolics in ecological interactions: The importance of oxidation. *Journal of Chemical Ecology* 19: 1521–1552.

Asner GP, Martin RE (2012) Contrasting leaf chemical traits in tropical lianas and trees: Implications for future forest composition. *Ecology Letters* 15: 1001–1007. doi: 10.1111/j.1461-0248.2012.01821.x.

Barbehenn R, Dukatz C, Holt C, Reese A, Martiskainen O, Salminen J-P, Yip L, Tran L, Constabel CP (2010) Feeding on poplar leaves by caterpillars potentiates foliar peroxidase action in their guts and increases plant resistance. *Oecologia* 164: 993–1004. doi: 10.1007/s00442-010-1733-y.

Cheynier V, Comte G, Davies KM, Lattanzio V, Martens S (2013) Plant phenolics: Recent advances on their biosynthesis, genetics, andecophysiology. *Plant Physiology and Biochemistry* 72: 1–20. doi: 10.1016/j.plaphy.2013.05.009.

Clifford MN (2000) Chlorogenic acids and other cinnamates – nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture* 80: 1033–1043. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1033::AID-JSFA595>3.0.CO;2-T.

Coley PD, Bryant JP, Chapin, III FS (1985) Resource availability and plant antiherbivore defense. *Science* 230: 895–899. doi: 10.1126/science.230.4728.895.

Engström MT, Pälijärvi M, Fryganas C, Grabber JH, Mueller-Harvey I, Salminen J-P (2014) Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *Journal of Agricultural and Food Chemistry* 62: 3390–3399. doi: 10.1021/jf500745y.

Engström MT, Pälijärvi M, Salminen J-P (2015) Rapid fingerprint analysis of plant extracts for ellagitannins, gallic acid, and quinic acid derivatives and quercetin-, kaempferol- and myricetin-based flavonol glycosides by UPLC-QqQ-MS/MS. *Journal of Agricultural and Food Chemistry* 63: 4068–4079. doi: 10.1021/acs.jafc.5b00595.

Feeny PP (1969) Inhibitory effect of oak leaf tannins on the hydrolysis of proteins by trypsin. *Phytochemistry* 8: 2119–2126. doi: 10.1016/S0031-9422(00)88169-8.

Feeny P (1976) Plant apparency and chemical defense. In: Wallace JW (ed) *Biochemical Interaction Between Plants and Insects. Recent Advances in Phytochemistry, Vol 10*. Springer, Boston, MA, 1–40. doi: 10.1007/978-1-4684-2646-5_1.

Fricke EC, Wright SJ (2016) The mechanical defence advantage of small seeds. *Ecology Letters* 19: 987–991. doi: 10.1111/ele.12637.

Gower JC (1971) A general coefficient of similiarity and some of its properties. *Biometrics* 27: 857–

871. doi: 10.2307/2528823.

Gripenberg S, Rota J, Kim J, Wright SJ, Garwood NC, Fricke EC, Zalamea PC, Salminen J-P (2017) Data from: Seed polyphenols in a diverse tropical plant community. *Dryad Digital Repository*. doi: 10.5061/dryad.5r083.

Gross EM, Brune A, Walenciak O (2008) Gut pH, redox conditions and oxygen levels in an aquatic caterpillar: Potential effects on the fate of ingested tannins. *Journal of Insect Physiology* 54: 462–471. doi: 10.1016/j.jinsphys.2007.11.005.

Hagerman AE (1987) Radial diffusion method for determining tannin in plant extracts. *Journal of Chemical Ecology* 13: 437–449. doi: 10.1007/BF01880091.

Häkkinen SH, Kärenlampi SO, Mykkänen HM, Heinonen IM, Törrönen AR (2000) Ellagic acid content in berries: Influence of domestic processing and storage. *European Food Research and Technology* 212: 75–80. doi: 10.1007/s002170000184.

Herms DA, Mattson WJ (1992) The dilemma of plants: To grow or defend. *Quarterly Review of Biology* 67: 283–335. doi: 10.1086/417659.

Ives AR, Garland T (2010) Phylogenetic logistic regression for binary dependent variables. *Systematic Biology* 59: 9–26. doi: 10.1093/sysbio/syp074.

Lahtinen M, Kapari L, Ossipov V, Salminen J-P, Haukioja E, Pihlaja K (2005) Biochemical transformation of birch leaf phenolics in larvae of six species of sawflies. *Chemoecology* 15: 153–159. doi: 10.1007/s00049-005-0307-7.

Luo YW, Xie WH (2013) Effects of polyphenol oxidation on in vitro iron availability in faba bean (Vicia faba L.) flour. *International Journal of Food Science & Technology* 48: 701–706. doi: 10.1111/ijfs.12016.

Marini G, Graikou K, Zengin G, Karikas GA, Gupta MP, Chinou I (2018) Phytochemical analysis and biological evaluation of three selected Cordia species from Panama. *Industrial Crops and Products* 120: 84–89. doi: 10.1016/j.indcrop.2018.04.037.

Marsh KJ, Kulheim C, Blomberg SP, Thornhill AH, Miller JT, Wallis IR, Nicolle D, Salminen J-P, Foley WJ (2017) Genus-wide variation in foliar polyphenolics in eucalypts. *Phytochemistry* 144: 197–207. doi: 10.1016/j.phytochem.2017.09.014.

Mayer AM, Harel E (1979) Polyphenol oxidases in plants. *Phytochemistry* 18: 193–215. doi: 10.1016/0031-9422(79)80057-6.

Moilanen J, Koskinen P, Salminen J-P (2015) Distribution and content of ellagitannins in Finnish plant species. *Phytochemistry* 116: 188–197. doi: 10.1016/j.phytochem.2015.03.002.

Moilanen J, Salminen J-P (2008) Ecologically neglected tannins and their biologically relevant activity: chemical structures of plant ellagitannins reveal their in vitro oxidative activity at high pH. *Chemoecology* 18: 73–83. doi: 10.1007/s00049-007-0395-7.

Murata M, Kurokami C, Homma S (1992) Purification and some properties of chlorogenic acid oxidase from apple (Malus pumila). *Bioscience, Biotechnology, and Biochemistry* 56: 1705–1710. doi: 10.1271/bbb.56.1705.

Nonaka G (1989) Isolation and structure elucidation of tannins. *Pure & Applied Chemistry* 61: 357–360. doi: 10.1351/pac198961030357.

Orenes-Piñero E, García-Carmona F, Sánchez-Ferrer A (2006) Latent polyphenol oxidase from quince fruit pulp (Cydonia oblonga): purification, activation and some properties. *Journal of the Science of Food and Agriculture* 86: 2172–2178. doi: 10.1002/jsfa.

Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault M, Dickinson TA, Campbell CS (2007) *Phylogeny and classification of Rosaceae*. doi: 10.1007/s00606-007-0539-9.

R Core Team (2015) R: A language and environment for statistical computing. R Foundation for

Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Salminen J-P, Lahtinen M, Lempa K, Kapari L, Haukioja E, Pihlaja K (2004) Metabolic modifications of birch leaf phenolics by an herbivorous insect: detoxification of flavonoid aglycones via glycosylation. *Zeitschrift für Naturforschung* 59: 437–444.

Salminen J-P, Karonen M (2011) Chemical ecology of tannins and other phenolics: we need a change in approach. *Functional Ecology* 25: 325–338. doi: 10.1111/j.1365-2435.2010.01826.x.

Scalvenzi L, Grandini A, Spagnoletti A, Tacchini M, Neill D, Ballesteros JL, Sacchetti G, Guerrini A (2017) Myrcia splendens (Sw.) DC. (syn. M. fallax (Rich.) DC.) (Myrtaceae) essential oil from Amazonian Ecuador: A chemical characterization and bioactivity profile. *Molecules* 22: 1–12. doi: 10.3390/molecules22071163.

Schmitz GE, Sullivan ML, Hatfield RD (2008) Three polyphenol oxidases from red clover (Trifolium pratense) differ in enzymatic activities and activation properties. *Journal of Agricultural and Food Chemistry* 56: 272–280. doi: 10.1021/jf072488u.

Thiboldeaux RL, Lindroth RL, Tracy JW (1998) Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in Saturniid moth larvae. *Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology* 120: 481–487. doi: 10.1016/S0742-8413(98)10070-1.

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. doi: 10.1016/j.phytochem.2014.02.019.

Wright SJ, Kitajima K, Kraft NJB, Reich PB, Wright IJ, Bunker DE, Condit R, Dalling JW, Davies SJ, Díaz S, Engelbrecht BMJ, Harms KE, Hubbell SP, Marks CO, Ruiz-Jaen MC, Salvador CM, Zanne AE (2010) Functional traits and the growth-mortality trade-off in tropical trees. *Ecology* 91: 3664–3674. doi: 10.1890/09-2335.1.

Xie C, Yu K, Zhong D, Yuan T, Ye F, Jarrell JA, Millar A, Chen X (2011) Investigation of isomeric transformations of chlorogenic acid in buffers and biological matrixes by ultraperformance liquid chromatography coupled with hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight mass spectrometry. *Journal of Agricultural and Food Chemistry* 59: 11078–11087. doi: 10.1021/jf203104k.

Yoruk R, Marshall MR (2003) Physicochemical properties and function of plant polyphenol oxidase: a review. *Journal of Food Biochemistry* 27: 361–422. doi: 10.1111/j.1745-4514.2003.tb00289.x.

UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-8281-3 (PRINT)
ISBN 978-951-29-8282-0 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)