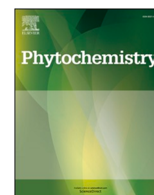
Phytochemistry 179 (2020) 112501

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Distribution of enzymatic and alkaline oxidative activities of phenolic compounds in plants

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ARTICLE INFO

Keywords:
Phenolic profiles of plants
Enzymatic activity
Oxidative activity
Hydrolysable tannins
Flavonoids
Cinnamic acid derivatives

ABSTRACT

In this study, we screened 287 plant tissue samples from 175 plant species for their phenolic profiles. The samples were oxidized enzymatically *in planta* or at high pH *in vitro* to determine how these two oxidative conditions would alter the initial polyphenol profiles of the plant. Compounds that contained a pyrogallol or dihydroxyphenethyl group were highly active at pH 10. Enzymatic oxidation favored compounds that contained a catechol group, whereas compounds containing a pyrogallol group or monohydroxysubstituted phenolic moieties at most were oxidized less frequently. This study gives a broad overview of the distribution and alkaline oxidative activities of water-soluble phenolic compounds in plants as well as the enzymatic oxidative activities of various plant tissues.

1. Introduction

Plants produce phenolic compounds for a number of different purposes. These functions include acting as signaling molecules or coloring agents, and helping the plants to withstand external stress factors such as UV radiation and attacks by pathogens and herbivores (Appel, 1993; Cheynier et al., 2013; Matthews et al., 1997). Phenolic compounds are structurally diverse, ranging from simple phenolic acids to large and complex polyphenols. They can be divided to several classes, e.g. hydrolysable tannins (HTs), proanthocyanidins (PAs; syn. condensed tannins), flavonoids and hydroxycinnamic acid derivatives.

HTs consist of a polyol core with one or more native or modified gallic acid substituents. This group includes simple gallic acid derivatives such as galloyl glucoses, gallotannins and ellagitannins. Gallotannins contain at least one digalloyl group, consisting of two galloyl moieties linked together with a (usually *meta*-)depside bond. In ellagitannins, two galloyl moieties are linked with a C–C bond, forming a hexahydroxydiphenoyl (HHDP) group. The HHDP group can be further modified: for example, geraniin contains a dehydroxy-HHDP group, whereas in vescalagin – an ET with an acyclic glucose core – the HHDP group is linked with a third galloyl moiety, forming a nonahydroxytriphenoyl (NHTP) group. Several oligomeric ellagitannins are known, with the largest oligomer known to date consisting of eleven tellimagrandin I monomer units (Salminen et al., 2011) (Figure S1a).

PAs consists of two or more flavan-3-ol monomeric units. The monomers are linked together via C4→C8 or C4→C6 bond in the B-type PAs, whereas less common A-type PAs are characterized by an additional C2→O→C7 or C2→O→C5 bond. The most common PA class is the procyanidins (PCs), followed by prodelphinidins (PDs). PCs consist of (epi)catechin subunits and PDs of (epi)gallocatechin subunits, but may additionally contain one or more (epi)catechin subunits (Figure S1b).

Flavonoids are built around a $C_6C_3C_6$ skeleton, and can be divided to several subclasses based on the substitution of the heterocyclic C ring, which remains open in chalcones. The most common flavonoid aglycones are the flavonols quercetin and kaempferol, containing a 3′,4′-dihydroxy- and 4′-monohydroxysubstituted B ring, respectively. Flavonolignans consist of a flavonoid and lignan, whereas complex tannins (not detected in thus study) are composed of a flavonoid and hydrolysable tannin. Protoflavones, mainly found in ferns, are characterized by their non-aromatic B-ring and a 1′-OH group (Hunyadi et al., 2014). Structural diversity within flavonoid subclasses arises from alkoxy groups as well as the type, possible substitution and linkage of the glycoside moiety (hexose or pentose sugar, acetate or malonate substitution, *O*- or *C*-glycoside). With few exceptions, flavonoids are stored in plant cells as glycosides (Figure S1b).

Hydroxycinnamic acid derivatives are, like flavonoids, structurally diverse and ubiquitous in the plant kingdom. An archetypal example is 5-*O*-caffeoylquinic acid (5-CQA), also known as chlorogenic acid.

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https://doi.org/10.1016/j.phytochem.2020.112501
Received 25 March 2020; Received in revised form 11 August 2020; Accepted 18 August 2020
Available online 30 August 2020
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Hydroxycinnamic acid derivatives can be divided to several subclasses depending on the substitution of their cinnamic acid moiety or moieties (e.g. caffeic, *p*-coumaric and ferulic acids), their polyol core (e.g. quinic and tartaric acid derivatives), or other substituents or moieties in the compounds (e.g. phenylethanoid derivatives) (Figure S1c).

Other phenolic compound classes detected in this study are phenylethanoids and arylbutanoids, containing a phenolic substituent in a carbon chain, and diarylheptanoids that contain two. The skeleton structure of xanthonoids is $C_6C_1C_6$, while that of stilbenoids is $C_6C_2C_6$. Furthermore, compound classes that are not inherently phenolic may nevertheless include phenolic compounds. For example, sinalbin is a phenolic glucosinolate, and oleuropein is a phenolic secoiridoid (Figure S1d).

Because of their ecological significance, numerous studies covering the occurrence of phenolics in plants have been conducted throughout the decades. Most of the studies focus on a limited number of plant species, a single plant family, or only on certain classes of phenolic compounds, such as ellagitannins or flavonoids. Large-scale systematic screening studies would help to understand the distribution and ecology of phenolic compounds in the plant kingdom (Marsh et al., 2017; Moilanen et al., 2015).

One of the proposed modes of action of phenolics regarding insect herbivores is oxidation, which turns phenolic compounds into highly reactive quinones, which in turn may react with proteins present in the plant material, compromising its nutritive quality, or damage the epithelial cells in the herbivore's gut. Upon consumption, plant phenolics are subjected to at least two types of oxidizing factors: 1) oxidizing enzymes present in the plant material itself, which the phenolics can come into contact with as the plant cell structures are ruptured, and 2) the alkaline environment in the midgut of certain insect herbivore species, such as Lepidoptera, which promotes autoxidation of the phenolics (Appel, 1993).

Plants may contain a variety of enzymes capable of oxidizing phenolic compounds, such as intracellular polyphenol oxidases (PPOs) and extracellular peroxidases (PODs) and laccases (Yoruk and Marshall, 2003). PPOs can be divided to two groups: monophenolase-active enzymes and *o*-diphenolase-active enzymes. While both types can oxidize *o*-diphenols into *o*-quinones in presence of molecular oxygen, only monophenolase-active enzymes can oxidize monophenols into *o*-diphenols before proceeding to oxidize them into quinones (Yoruk and Marshall, 2003). Enzymatic oxidative activity of phenolics, i.e. the propensity of phenolics extracted from plants to be enzymatically oxidized, has been widely studied using commercial PPO, which is usually monophenolase-active tyrosinase extracted from mushrooms. However, monophenolase activity is not as common trait in plants as diphenolase activity, and it is usually also 10–40 times weaker when present (Yoruk and Marshall, 2003). Despite producing monohydroxysubstituted phenolic compounds, such as coumaric acid derivatives and kaempferol glycosides, the monophenolase activity of the plant may be very low. Thus, in most cases the commercial tyrosinase likely overestimates the plant's enzymatic oxidation potential. Alternatively, the enzymatic oxidation potential of a plant sample is estimated by extracting the enzymes and introducing them to commercial standard, typically 5-CQA, which may not accurately represent the phenolic content of the plant.

The enzymatic oxidative activity of a plant is governed by several factors. The amount of PPO and other oxidizing enzymes depends on the species, tissue, stage of development, health and sustained damage of the plant. In a healthy plant cell, some or all of PPO may be stored in a latent state, only activating once the plant has reached a certain developmental stage or been damaged. This is further complicated by the pH and temperature optima as well as substrate specificity of different PPOs produced by different plant species. (Aniszewski et al., 2008; Kim et al., 2018; Yoruk and Marshall, 2003)

Oxidation of various types of phenolic compounds at pH 10 has been studied extensively (e.g. Barbehenn et al., 2006; Kim et al., 2018; Vihakas et al., 2015, 2014). CQAs and phenolics that contain a pyrogallol substructure, e.g. gallic acid derivatives, ellagitannins, (epi)gallocatechin subunits of proanthocyanidins (PAs) and myricetin-type flavonoids, are known to easily oxidize at pH 10, but the activity also varies considerably within the subgroups (e.g. Moilanen and Salminen, 2008). Quercetin, kaempferol, apigenin and coumaroyl derivatives and (epi)catechin subunits of PAs are unaffected or weakly affected by the alkaline conditions (Vihakas et al., 2014). Most likely, the enzymatic and alkaline oxidation of flavonoids is primarily governed by the substitution of their B ring, as the A ring is typically *m*-dihydroxysubstituted and therefore cannot turn into a quinone.

We have presented a simple method to study the effects of both the enzymatic and alkaline oxidation on the phenolic profile of any plant sample (Kim et al., 2018). In the enzymatic oxidation step, collected and frozen plant tissue samples are simply incubated at 30 °C before the phenolics are extracted and analyzed. Phenolic compounds and the enzymes produced in the very same plant tissue can come into contact during the incubation, resulting in enzymatic oxidation. The method is physiologically more relevant than the ones utilizing commercial enzymes or phenolic standards. In the alkaline oxidation step, a portion of the non-oxidized plant extract is incubated in a buffer of desired pH – which is 10 in our case – and the oxidation is stopped by neutralizing the solution. Enzymatic and alkaline oxidative activities of the plant sample can be obtained by comparing the quantified total phenolics of the oxidized samples with the non-oxidized sample, whereas the effects of oxidation on individual compounds can be inspected by analyzing the oxidized and non-oxidized samples using chromatography (Fig. 1) (Kim et al., 2018).

In this follow-up experiment, we used our enzymatic and alkaline oxidation methods to analyze the phenolic contents and oxidative activities of 287 plant tissue samples across the plant phylogeny (Table 1), collected in Turku area, Finland. We aimed to collect at least leaf tissues from each species (where applicable), but also collected flowers and other plant parts if they were available. Our analyses included any water-soluble compounds, some of which have never been studied with these oxidative activity methods before.

Based on our previous works on oxidative activities of plants

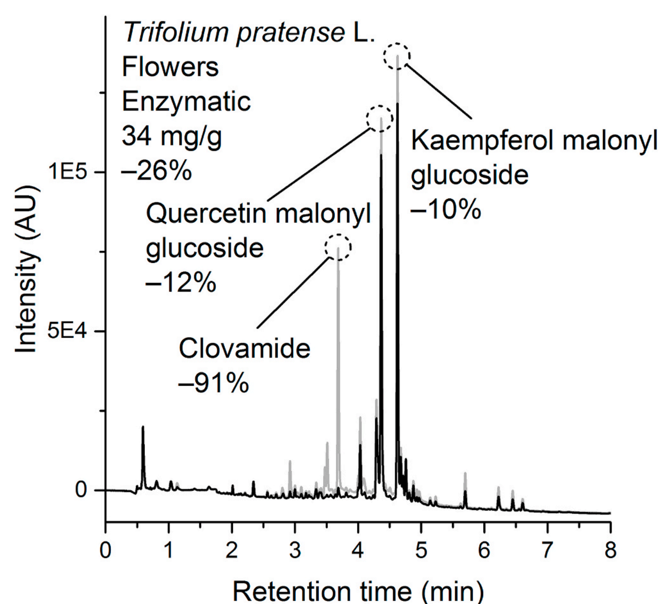


Fig. 1. The content of phenolic compounds, recorded at 280 nm, in non-oxidized (grey) and enzymatically oxidized (black) *Trifolium pratense* flowers. The peak area of clovamide has decreased by 91%, likely due to oxidation, whereas flavonols only have lost ca. 11% of their peak areas. A moderate, 26% decrease in total phenolics suggests that the sample retains most of its phenolic compounds, supporting these observations.

Table 1
List of studied plant orders and families and description of their phenolic composition and activities. Brackets following the family refer to the number of genera and species in the family. (–) No activity: the phenolic content of the samples is low overall (total phenolics concentration 10 mg/g or less) with no major peaks present, or the compounds are not affected by the oxidative conditions, the peak area change staying within ±10%. (*) Weak activity: the areas of most peaks have reduced by ca. 10–30%. (**) Moderate activity: the areas of most peaks have reduced by 30–50%, or the area of the main peak has reduced by ca. 50–70%. (***) High activity: the areas of most peaks have reduced by >50%, or the area of the main peak has reduced by >70%. Further details are given in the General description column. Literature used to confirm the phenolic composition is listed in the References column.

Clade / Order	Family (genera, species)	Activity: Enzymatic	Activity: pH 10	General description of main compounds and activity	References
Equisetopsida					
Equisetales	Equisetaceae (1, 2)	***	**	5-CQA, chicoric acid, KA glycosides and protoapigenin glucoside. High di- and monophenolase activity. 5-CQA completely and chicoric acid partially oxidized at pH 10, whereas flavonoids unaffected.	Veit et al. (1995)
Pinopsida					
Pinales	Cupressaceae (1, 1)	*	**	CoQA, catechin, PC, and QU glycoside. No enzymatic activity. Catechin oxidized at pH 10.	
	Pinaceae (3, 3)	–	*	Various compounds, such as catechin, astringin, KA glycoside, taxifolin 3′-*O*-glucoside, and other flavonoids. Weak enzymatic and alkaline oxidative activity.	Karonen et al. (2004); Slimestad and Hostettmann (1996)
Basal angiosperms					
Nymphaeales	Nymphaeaceae (1, 1)	**	***	Geraniin. Moderate enzymatic activity in *Nymphaea alba*, but in leaves only.	Moilanen et al. (2015)
Monocots					
Alismatales	Araceae (1, 1)	**	–	KA glycosides and a flavone. KAs partially oxidized by enzymes.	
Liliales	Liliaceae (2, 2)	***	**	Anthocyanins (*Fritillaria meleagris*), caffeyl and coumaroyl glucosides, QU diglycoside (*Lilium martagon*). Compounds partially oxidized by enzymes. Anthocyanins unaffected by pH 10, cinnamic acid derivatives completely oxidized. QU only partially oxidized.	
	Melanthiaceae (1, 1)	*	–	KA triglycoside.	
Poales	Poaceae (5, 5)	–	–	Flavonoids and 5-CQA. Low overall content of phenolics.	Jay and Viricel (1980); Nawwar et al. (1980)
Asparagales	Iridaceae (1, 2)	–	*	*C*-glycosidic flavones, MY, mangiferin and 5-CQA. 5-CQA and MY partially and completely oxidized at pH 10.	Hirose et al. (1981); Williams et al. (1997)
	Amaryllidaceae (1, 1)	–	*	KA glycosides.	Lachowicz et al. (2017)
	Asparagaceae (3, 3)	**	*	3-CQA, CoQA and other caffeic and coumaric acid derivatives, QU and KA glycosides. Moderate enzymatic and alkaline oxidative activity observed on *Convallaria majalis* extracts.	
Basal eudicots					
Ranunculales	Ranunculaceae (5, 6)	**	*	Caffeic acid derivatives, such as caffeoylglucoside, fukinolic acid and chicoric acid, and also flavones and KA and QU glycosides. An unidentified peak emerges at pH 10 in *Ranunculus ficaria* and *Anermone nemorosa*. High enzymatic oxidative activity in caffeic acid-rich species, unlike in the flavonoid-rich *Ranunculus ficaria*. Caffeoyl glucoside and fukinolic acid completely oxidized at pH 10. Flavonoids and chicoric acid unaffected by the alkaline conditions.	Kruse et al. (1999)
	Papaveraceae (2, 2)	**	*	Caffeoylmalic acid and other caffeic acid derivatives and QU glycosides. High enzymatic oxidative activity on *Chelidonium majus* leaves. Otherwise the species had a moderate or low enzymatic and alkaline oxidative activity.	Hahn and Nahrstedt (1993)
Saxifragales					
Saxifragales	Paeoniaceae (1, 1)	*	***	1GG, 5GG, gallotannins and KA glycosides. Gallic acid derivatives oxidized completely at pH 10. Total phenolics unaffected by the alkaline oxidation.	
	Crassulaceae (1, 2)	–	**	GA derivative, PD hump, caffeoylmalic acid and KA glycosides. GA derivative and PDs completely oxidized at pH 10.	Li et al. (2016); Nishizawa et al. (1980)
	Grossulariaceae (1, 2)	–	*	MY, QU and KA glycosides, 3-CQA, PD hump. MY glycosides and PD completely oxidized at pH 10, QU and KA glycosides moderately oxidized. Weak to no enzymatic oxidative activity.	Vihakas et al. (2014)
Malvids					
Geraniales	Geraniaceae (1, 3)	*	***	Geraniin, carpinusin, sylvatiins and GQA.	Tuominen et al. (2015)
Myrtales	Onagraceae (1, 3)	**	***	Oenotheins B and A.	Baert et al. (2015)
Sapindales	Sapindaceae (2, 2)	*	**	*Acer platanoides*: gallotannins, *Aesculus hippocastanum*: QU and KA glycosides, catechin, PC oligo- and polymers. Gallotannins oxidized at pH 10. Moderate enzymatic oxidative activity on *A. hippocastanum* leaves, weak in other tissue samples.	Oszmiański et al. (2014)
Malvales	Tiliaceae (1, 1)	*	*	CoQA and an unknown coumaric acid derivative. The only instance where this coumaric acid derivative appears to isomerize at pH 10.	
Brassicales	Brassicaceae (7, 8)	*	–	*C*-glycosidic flavones, QU and KA glycosides. Non-phenolic glucobrassicin in *Cardamine pratensis* vanished during incubation at 30 °C. In *Thlaspi caerulescens*, KA-Glc-MaGlc transformed into KA-DiGlc, possibly because of enzymatic activity.	Bennett et al. (2004); Haribal and Renwick (1998); Pang et al. (2013)
Fabids					
Malpighiales	Hypericaceae (1, 1)	**	–	5- and 3-CQA, epicatechin, PC hump, QU glycoside and dimethylmangiferin. CQAs, epicatechin and PCs oxidized in larger quantity than QU glycoside and dimethylmangiferin. CQAs and epicatechin partially and completely oxidized at pH 10.	Tusevski et al. (2018)

(*continued on next page*)

Table 1 (*continued*)

Clade Order	Family (genera, species)	Activity		General description of main compounds and activity	References
		Enzymatic	pH 10		
	Violaceae (1, 4)	*	*	QU and KA glycosides, flavones, catechin, CoQA and unidentified coumaric acid derivative. Weak overall enzymatic and alkaline oxidative activity, with the exception of catechin which oxidized completely at pH 10.	Sugahara et al. (2019)
	Salicaceae (2, 6)	**	**	5- and 3-CQA, CoQA, KA and QU glycosides, catechin, PC, PD, MY-type flavanonol. Moderate enzymatic activity, strong diphenolase activity in some samples.	Häikiö et al. (2009); Lavola et al. (2018)
Fabales	Fabaceae (6, 10)	*	*	Flavone and flavonol glycosides (QU, KA, and MY), isoflavonoids, caffeoylmalic acid and clovamide. High enzymatic activity in *Trifolium medium* leaves and high mono- and diphenolase activity of *T. pratense* leaves. Low to moderate enzymatic activity in other samples.	Klejdus et al. (2001); Nicholls and Bohm (1982); Polasek et al. (2007); Suzuki et al. (2008)
Fagales	Betulaceae (2, 4)	**	**	3- and 5-CQA, QU glycosides, oregonin and rubranoside A (*Alnus*), rhododendrin (*Betula pendula*) or 5-CQA, 1GG and QU and KA glycosides (*B. pubescens*). Moderate to strong enzymatic and alkaline activity except for rhododendrin in *B. pendula*.	Novaković et al. (2013); Sati et al. (2011); Sunnerheim et al. (1988); Telysheva et al. (2011)
	Fagaceae (1, 1)	***	***	Ellagitannins, such as vescalagin and castalagin.	Moilanen et al. (2015)
Rosales	Rhamnaceae (1, 1)	**	*	Unknown flavonoid and KA glycoside. KA glycoside oxidized enzymatically.	
	Rosaceae – Rosoideae (8, 16)	**	***	Ellagitannins, e.g. agrimoniin, rugosin D, geraniin, tellimagrandin II, gemin A, lambertianin C and sanguiin H-6, as well as QU glycosides. The level of enzymatic oxidative activity varies from low to high.	Moilanen et al. (2015); Okuda et al. (1992)
	Rosaceae – Spiraeoideae (5, 7)	**	*	5- and 3-CQA, QU glycosides and other flavonoids. Moderate to high enzymatic oxidative activity and moderate alkaline oxidative activity.	
	Ulmaceae (1, 1)	***	**	5- and 3-CQA and QU glycosides.	
	Urticaceae (1, 1)	***	**	5-CQA and caffeoylmalic acid. Caffeoylmalic acid only partially oxidized at pH 10.	Pinelli et al. (2008)
Caryophyllales					
Caryophyllales	Caryophyllaceae (5, 5)	*	–	Monohydroxysubstituted flavonoid glycosides (apigenin, saponarin and KA), unidentified flavonoids, caffeic acid derivatives. Caffeic acid derivatives oxidized by enzymes.	Budan et al. (2014); Dubois et al. (1985)
	Polygonaceae (1, 3)	*	*	QU and KA glycosides, other flavonoids, catechin and PC.	
Cornales					
Cornales	Cornaceae (1, 1)	**	***	Galloyl glucoses, ellagitannins and caffeic acid derivatives. Hydrolysable tannins completely oxidized at pH 10.	Hatano et al. (1989), Lee et al. (2000)
Ericales					
Ericales	Ericaceae (3, 6)	*	*	5-CQA, coumaric acid derivatives, QU glycosides, other flavonoids and simple phenolic acids. Varying enzymatic activity: caffeic acid derivatives oxidized completely, partially or not at all.	Hokkanen et al. (2009); Ieri et al. (2013); Jalal et al. (1982)
	Primulaceae (3, 5)	*	*	5-CQA, MY, QU and KA glycosides and other flavonoids, PD. High enzymatic activity on *Lysimachia thyrsiflora* leaves.	Apel et al. (2017)
Campanulids					
Asterales	Campanulaceae (1, 3)	*	**	5- and 3-CQA, caffeoylmalic acid, CoQA, KA and QU glycosides. Weak to high diphenolase activity.	
	Asteraceae (17, 20)	***	**	Caffeic acid derivatives (5-, 3-, di- and triCQA, chicoric acid and others), flavonoids (QU, KA, AP, LU glycosides and others). High diphenolase activity and low to moderate monophenolase activity.	Adesso et al. (2016); Flamini et al. (2001); Milutinović et al. (2018); Schütz et al. (2005)
	Menyanthaceae (1, 1)	***	***	5-CQA, diCQA, QU and KA glycoside. High diphenolase activity.	
Apiales	Apiaceae (4, 4)	***	*	5- and diCQA and unknown caffeic acid derivative, KA and QU glycosides. High di- and monophenolase activity.	
Dipsacales	Adoxaceae (1, 1)	***	**	5-CQA and QU glycoside.	
	Caprifoliaceae (1, 2)	**	**	5-CQA, diCQA, flavanones and QU glycoside.	Liu et al. (2016)
Lamiids					
Gentianales	Rubiaceae (1, 2)	***	**	5-CQA and QU glycoside.	
	Apocynaceae (1, 1)	*	*	5-CQA, QU and KA glycosides.	
Solanales	Convolvulaceae (1, 1)	***	**	5- and diCQA, KA glycoside. High diphenolase activity.	
	Solanaceae (1, 1)	**	***	5-CQA and QU glycoside. Moderate enzymatic activity.	
Boraginales	Boraginaceae (3, 3)	***	***	Rosmarinic acid, 5- and 3-CQA. Rosmarinic acid completely oxidized by enzymes and at pH 10.	Petersen et al. (2009)
Lamiales	Oleaceae (1, 1)	**	***	*Syringa vulgaris* leaves contain QU and LU glycoside, acteoside, ligstroside derivatives and oleuropein. Flowers contain echinacoside and acteoside. High enzymatic and alkaline activity on flowers. No enzymatic activity and moderate alkaline activity on leaves.	Tóth et al. (2016)
	Plantaginaceae (2, 3)	**	**	Plantamajoside (*Plantago major*), a variety of flavone and other flavonoid glucosides and caffeic acid derivatives (*Veronica*). Plantamajoside completely oxidized by enzymes and at pH 10. High enzymatic activity on *V. chamaedrys*, moderate on *V*. longifolia. Tricetin completely oxidized at pH 10.	Barreira et al. (2014); Ravn et al. (2015); Ž ivković et al. (2017)
	Lamiaceae (4, 6)	***	**	Caffeic acid derivatives (5- and 3-CQA, acteoside, caffeoylmalic acid, teupolioside, forsythoside B) and flavonoids (KA, QU, AP and IS glycosides). High enzymatic activity, low to high activity at pH 10.	Czerwińska et al. (2017); Karioti et al. (2010); Leporini et al. (2015)
	Orobanchaceae (1, 2)	*	–	AP and chrysoeriol glycosides and other flavonoids.	Marczak et al. (2010)

Abbreviations: 1GG: monogalloyl glucose, CoQA: coumaroylquinic acid, CQA: caffeoylquinic acid, GQA: galloylquinic acid, KA: kaempferol, MY: myricetin, PC: procyanidins, PD: prodelphinidins, QU: quercetin.

phenolics (Kim et al., 2018; Vihakas et al., 2014), we present two hypotheses. First, the plant species rich in ellagitannins, myricetin-type flavonoids and other pyrogallol-containing phenolics would be highly active in alkaline conditions. Second, the enzymatic oxidation of phenolics would highly depend on the plant species and tissue type, but generally, compounds with a catechol moiety would be oxidized more often and to a higher degree than tri- and monohydroxysubstituted compounds. The diphenolase-active PPO is widely distributed in the plant kingdom, whereas the latter types of compounds require less common enzymes to oxidize. With an increased number of species and tissue types, we expected to find large variation in these two types oxidative activities, hopefully with new structure-activity patterns arising.

This study helps to assess the oxidative defense potential based on phenolics in any given plant tissue, as we can directly observe which compounds can readily be oxidized by the enzymes of the plant and by the elevated pH simulating the midgut environment of lepidopteran larvae. We can therefore tell if different plants invest in different phenolics-based oxidative defense strategies – i.e. mainly enzymatic, mainly alkaline, neither or both – or if the same plant uses different strategies between tissues. The coverage of the plant phylogeny and compound classes makes this the most comprehensive study of enzymatic and alkaline oxidation activities on compound level to date.

2. Results and discussion

In total, we collected 287 plant tissue samples, covering 157 identified and 18 unidentified species from 124 genera and 51 families (Fig. 2, according to the Angiosperm Phylogeny Group, 2016); this includes the 24 plant tissue samples of our previous study (Kim et al., 2018). 89 plant species were represented by two tissue types (usually leaves and flowers), 84 species by one tissue type (usually leaves) and 2 species were represented by three tissue types.

2.1. Distribution of polyphenols in plant phylogeny

During the course of plant evolution, some genetic alterations are reflected by synthases and other enzymes, which in turn can have an effect on the metabolic pathways of the plant. This leads to closely related species likely having similar chemistry, i.e. a connection between phytochemistry and taxonomy (Fairbrothers et al., 1975; Hegnauer, 1986). While the distribution of phenolics in the plant kingdom can be studied by compiling existing research, the strength of a single, large-scale study is the comparable, quantitative results made possible by the consistency in methodology.

The family-level phylogeny of the studied plants is presented in Fig. 2 together with their average content of total phenolics determined by the modified Folin-Ciocalteu assay (Salminen and Karonen, 2011) and the average content of hydrolysable tannins, proanthocyanidins, flavonols and quinic acid derivatives determined using group-specific multiple reaction monitoring (MRM) methods introduced by Engström et al. (2015, 2014) (Fig. 3; the method is described briefly in the Experimental section). Families with the highest average concentration of total phenolics were Cornaceae (155 mg/g), Onagraceae (130 mg/g), Paeoniaceae (130 mg/g), Nymphaeaceae (129 mg/g) and Hypericaceae (98 mg/g), and families with the lowest average concentration were Rhamnaceae (15 mg/g), Asparagaceae (14 mg/g), Oleaceae (11 mg/g), Amaryllidaceae (10 mg/g) and Poaceae (6 mg/g). The monocots clade is distinguished by its general lack of any phenolic groups and oxidative activities. In addition, monocots and equisetopsida had the lowest average content of total phenolics (20 mg/g). On species level, the total phenolics varied greatly, ranging from 1 mg/g in *Avena sativa* (Poaceae) to 224 mg/g in *Alchemilla* sp. (Rosaceae; Figures S2.17 and S2.127). Typically, families with a high content of total phenolics were also rich in hydrolysable tannins.

Hydrolysable tannins and proanthocyanidins were detected in 27 plant families by MRM methods, but only 11 families contained both types of tannins. Interestingly, most families containing HTs also contained PAs, but this pattern was not evident at the species level, since of the 24 species containing HTs as their major compounds, only *Sedum telephium* (Crassulaceae) additionally contained PAs (Figures S2.53–55).

HTs were detected in relatively few families using the MRM methods, most of them in the superrosid clade (8 families out of 13), and being completely absent from the families belonging to the clades equisetopsida, pinopsida, monocots, basal eudicots and euasterids. A notable characteristic of HTs is that, when present, their quantity is very high compared to other classes of phenolic compounds. Families richest in HTs were Nymphaeaceae (143 mg/g), Cornaceae (109 mg/g), Geraniaceae (103 mg/g), Fagaceae (67 mg/g) and the Rosoidea subfamily of Rosaceae (62 mg/g; see the following paragraph). In general, the plant families containing HTs had more HHDPs (55 mg/g) than gallic acids (17 mg/g), suggesting that ETs in these families are more common than simple gallic acid derivatives or GTs, or that the HTs contained more HHDP groups than gallic acid moieties.

Two distinct sample subgroups can be seen in the family Rosaceae, as some species contain ellagitannins and other do not. Several ways to classify the species to subfamilies within the family have been proposed, and according to the latest classification by Potter et al. (2007), the species in our sample set represent the subfamilies Rosoideae (16 species covered by 25 samples) and Spiraeoideae (7 species covered by 11 samples). Rosoideae had a high content of HTs (62 mg/g) and low content of quinic acid derivatives (e.g. CQAs; 1.9 mg/g), whereas the Spiroideae contained no HTs (0.4 mg/g) but did have a comparatively high content of quinic acid derivatives (11 mg/g). This is consistent with previous reports noting that only the Rosoideae subfamily contains HTs (e.g. Moilanen et al., 2015; Okuda et al., 1992).

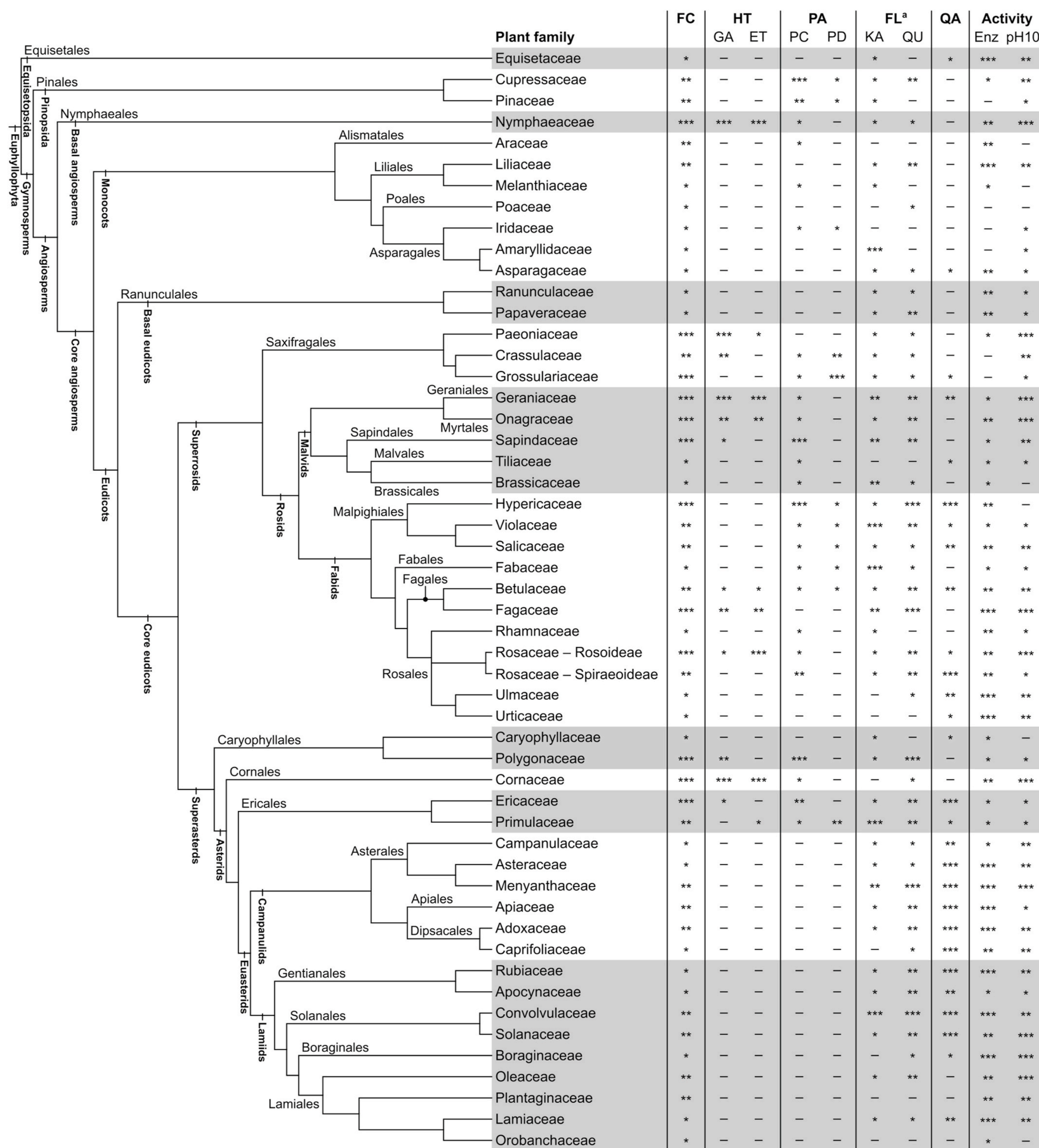
PAs were detected in most of the clades using the MRM methods, with the exception of euasterids, basal eudicots and equisetopsida. The (epi)catechin subunits (13 mg/g) of PAs were more common than the (epi)gallocatechin subunits (6 mg/g). This pattern was opposite only in Grossulariaceae, Crassulaceae and Primulaceae.

Likewise, kaempferol and quercetin glycosides were detected in all clades using the MRM methods, albeit in relatively small quantities when compared to tannins. Quercetin glycosides were slightly more abundant than kaempferol glycosides (6 mg/g vs. 5 mg/g). However, there was huge variation in the ratio of kaempferol to quercetin glycosides between the families, e.g. Amaryllidaceae (kaempferol-rich) and Polygonaceae (quercetin-rich) (Fig. 2). As with flavonols, the quantity of quinic acid derivatives is usually low compared to tannins. Quinic acid derivatives are the most abundant in the superasterids clade, and relatively common in the fabids clade.

Based on these results alone, families with a high content of gallic acid derivatives, ellagitannins or PDs could be predicted to be highly active in alkaline conditions according to the earlier studies (Barbehenn et al., 2006; Kim et al., 2018; Vihakas et al., 2015, 2014). Species rich in PCs or kaempferol and quercetin glycosides are likely not active under alkaline conditions. Families that contain quinic acid derivatives – namely, CQAs – can be expected to be highly active enzymatically, as mono- and trihydroxysubstituted phenolic compounds require less common specialized enzymes to oxidize.

2.2. Oxidative activities of phenolic compounds

We assessed the oxidative activities of the compounds by comparing their peak areas at 280 nm before and after either oxidation step (Fig. 1). As in our previous study, peak area variation within ±10% was considered negligible (Kim et al., 2018). An overview of the plant families, their content of various classes of phenolic compounds, and their general activities are presented in Fig. 2, and a more detailed description of their phenolic content and activities are given in Table 1. The samples we collected consist of various species with very heterogeneous content of phenolic compounds with varying levels of oxidative



Plant family	FC	HT: GA	HT: ET	PA: PC	PA: PD	FL[a]: KA	FL[a]: QU	QA	Activity: Enz	Activity: pH10
Equisetaceae	*	–	–	–	–	*	–	*	***	**
Cupressaceae	**	–	–	***	*	*	**	–	*	**
Pinaceae	**	–	–	**	*	*	–	–	–	*
Nymphaeaceae	***	***	***	*	–	*	*	–	**	***
Araceae	**	–	–	*	–	–	–	–	**	–
Liliaceae	**	–	–	–	–	*	**	–	***	**
Melanthiaceae	*	–	–	*	–	*	–	–	*	–
Poaceae	*	–	–	–	–	–	*	–	–	–
Iridaceae	*	–	–	*	*	–	–	–	–	*
Amaryllidaceae	*	–	–	–	–	***	–	–	–	*
Asparagaceae	*	–	–	–	–	*	*	*	**	*
Ranunculaceae	*	–	–	–	–	*	*	–	**	*
Papaveraceae	*	–	–	–	–	*	**	–	**	*
Paeoniaceae	***	***	*	–	–	*	*	–	*	***
Crassulaceae	**	**	–	*	**	*	*	–	–	**
Grossulariaceae	***	–	–	*	***	*	*	*	–	*
Geraniaceae	***	***	***	*	–	**	**	**	*	***
Onagraceae	***	**	**	*	–	*	**	–	**	***
Sapindaceae	***	*	–	***	–	**	**	–	*	**
Tiliaceae	*	–	–	*	–	–	–	*	*	*
Brassicaceae	*	–	–	*	–	**	*	–	*	–
Hypericaceae	***	–	–	***	*	*	***	***	**	–
Violaceae	**	–	–	*	*	***	**	*	*	*
Salicaceae	**	–	–	*	*	*	*	**	**	**
Fabaceae	*	–	–	*	*	***	*	–	*	*
Betulaceae	**	*	*	*	*	*	**	**	**	**
Fagaceae	***	**	**	–	–	**	***	–	***	***
Rhamnaceae	*	–	–	*	–	*	–	–	**	*
Rosaceae – Rosoideae	***	*	***	*	–	*	**	*	**	***
Rosaceae – Spiraeoideae	**	–	–	**	–	*	**	***	**	*
Ulmaceae	*	–	–	–	–	–	*	**	***	**
Urticaceae	*	–	–	–	–	–	–	*	***	**
Caryophyllaceae	*	–	–	–	–	*	–	*	*	–
Polygonaceae	***	**	–	***	–	*	***	–	*	*
Cornaceae	***	***	***	*	–	–	*	–	**	***
Ericaceae	***	*	–	**	–	*	**	***	*	*
Primulaceae	**	–	*	*	**	***	**	*	*	*
Campanulaceae	*	–	–	–	–	*	*	**	*	**
Asteraceae	*	–	–	–	–	*	*	***	***	**
Menyanthaceae	**	–	–	–	–	**	***	***	***	***
Apiaceae	**	–	–	–	–	*	**	***	***	*
Adoxaceae	**	–	–	–	–	*	**	***	***	**
Caprifoliaceae	*	–	–	–	–	–	*	***	**	**
Rubiaceae	*	–	–	–	–	*	**	***	***	**
Apocynaceae	*	–	–	–	–	*	**	**	*	*
Convolvulaceae	**	–	–	–	–	***	***	***	***	**
Solanaceae	**	–	–	–	–	*	**	***	**	***
Boraginaceae	*	–	–	–	–	–	*	*	***	***
Oleaceae	**	–	–	–	–	*	**	–	**	***
Plantaginaceae	**	–	–	–	–	–	–	–	**	**
Lamiaceae	*	–	–	–	–	*	*	**	***	**
Orobanchaceae	*	–	–	–	–	–	–	–	*	–

Fig. 2. Plant families arranged according to APG IV, and their phenolic compound classes and total phenolic levels. The dash (–) and one, two or three asterisks denote different average quantity levels depending on the compound class as follows: FC and ET: <1, 1–30, 31–60 and >60 mg/g; GA: <1, 1–10, 11–20 and >20 mg/g; PC and PD: <1, 1–15, 16–30 and >30 mg/g; KA, QU and QA: <1, 1–5, 6–10 and >10 mg/g. For the oxidative activity column, dashes and asterisk represent the following: (–) No activity. The phenolic content of the samples is low overall (total phenolics concentration 10 mg/g or less) with no major peaks present, or the compounds are not affected by the oxidative conditions, the peak area variation remaining within ±10%. (*) Weak activity. The area of most major peaks have reduced by ca. 10–30%. (**) Moderate activity. The areas of most major peaks have reduced by ca. 30–60%. (***) High activity. The areas of most major peaks have reduced by >60%. Abbreviations: FC: Folin-Ciocalteu assay (i.e. total phenolics), HT: hydrolysable tannins, GA: gallic acid derivatives, ET: ellagitannins, PA: proanthocyanidins, PC: procyanidins, PD: prodelphinidins, FL: flavonols, KA: kaempferols, QU: quercetins, QA: quinic acid derivatives, Enz: enzymatic oxidative activity, pH10: alkaline oxidative activity at pH 10. [a] Myricetin has been left out because of its low quantity in the sample set. It was detected in 20 families, with a maximum concentration of 7 mg/g.

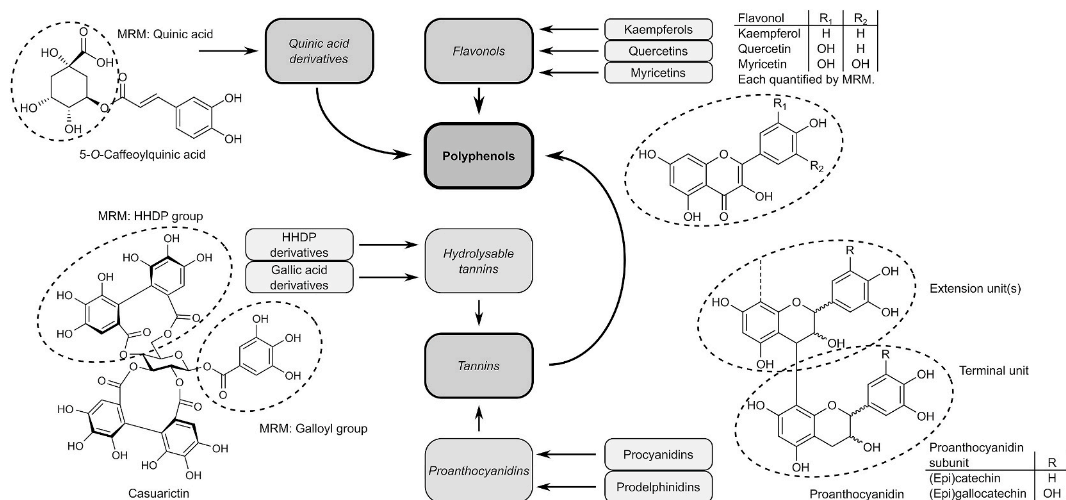


Fig. 3. Phenolic compound structures quantified using MRM methods of Engström et al. (2015, 2014), and how the measurements are related to each other.

activity, and describing these observations in detail is not feasible. Therefore, we have included the results of chromatographic analyses, compound identifications and total phenolic assays in the Supplementary (Figures S2.1 to S2.287, Table S1; various compound structures are presented in Figure S1).

It is worth to note that not all decreases in peak areas can be attributed to oxidation. The clearest example is the isomerization of CQAs at pH 10; for example, the peak area of 5-CQA in the leaves of *Prunus padus* (Figure S2.155) decreases by 58%, but the total peak area of all isomers (3-, 4- and 5-CQA) only decreased by 8% in total; this pattern is a combination of CQA isomerization and oxidation at alkaline pH. In the case of *Thlaspi caerulescens* leaves (Figure S2.89), enzymatic oxidation step resulted in demalonylation of kaempferol hexosyl malonylglucoside, resulting in a subsequent peak area increase of kaempferol dihexoside and the total peak area increase of 5%. However, there were not many these types of examples, but the UHPLC-DAD chromatograms do enable the observation of additional peaks should any emerge from either type of oxidation.

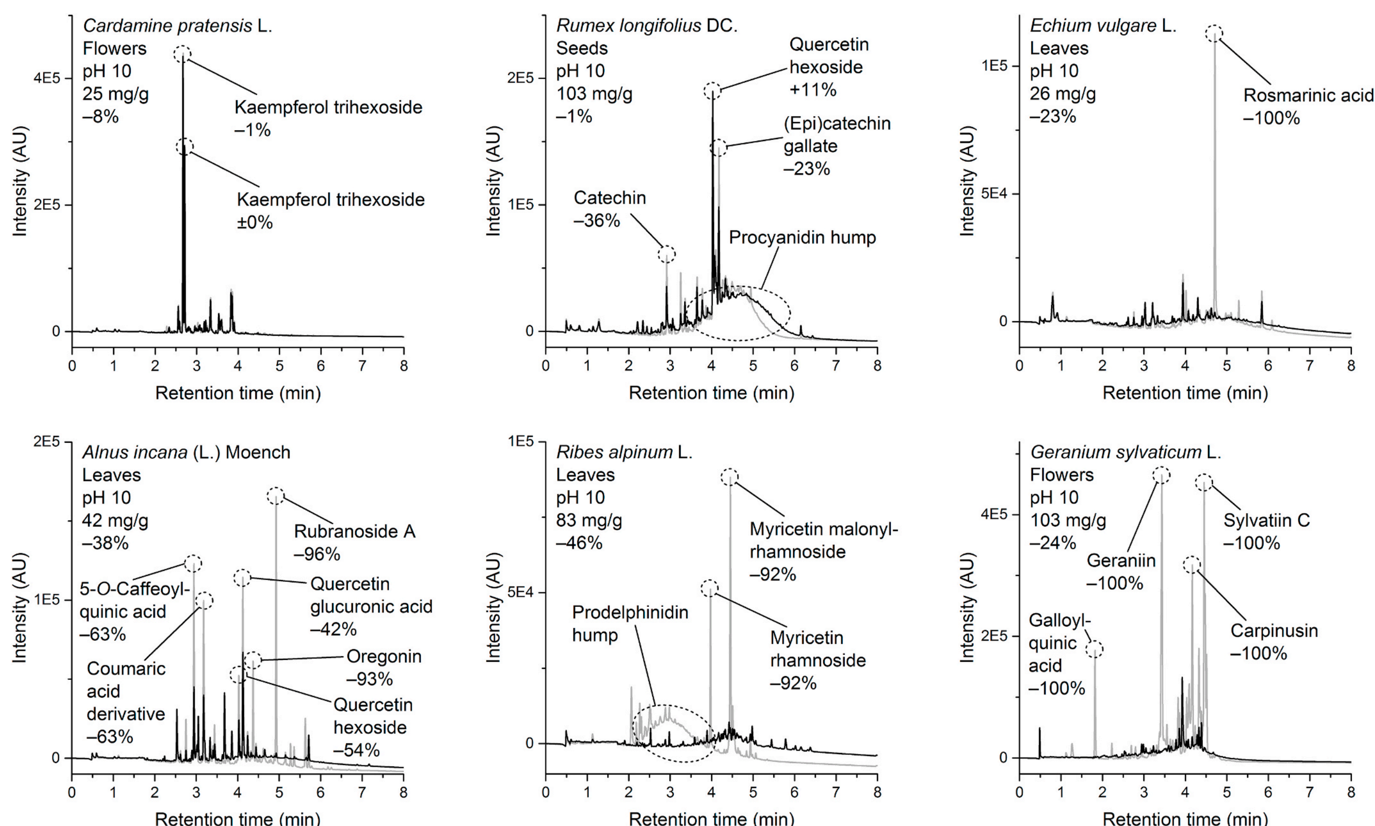


Fig. 4. UV chromatograms (280 nm) of non-oxidized (grey) and oxidized (black) samples of selected species, illustrating alkaline oxidative activities of various types of compounds. Monohydroxysubstituted compounds, such as kaempferol glycosides, are inactive. The same applies for compounds containing a catechol moiety, e.g. catechin, quercetin glycosides and procyanidins. However, if the catechol moiety is at the end of an alkane chain, as is the case with e.g. rosmarinic acid, rubranoside A, and oregonin, the alkaline oxidative activity is greatly increased. Myricetin glycosides, prodelphinidins, gallic acid derivatives and ellagitannins – all containing a pyrogallol moiety – are highly active and oxidize completely.

2.3. Alkaline oxidative activity of phenolic compounds

Most compound classes followed the alkaline oxidative activity trends found in the previous studies. The most active compounds contained either a catechol moiety at the end of an alkane chain or a pyrogallol group. The former includes oregonin and dihydroxyphenethyl derivatives (e.g. acteoside, rosmarinic acid and oleuropein), and the latter, HTs, (epi)gallocatechin-containing PAs and myricetin-type flavonoids (Fig. 4). These were followed by compounds containing a catechol moiety at the end of a conjugated alkyl chain, and lastly by the generally inactive monohydroxysubstituted compounds.

Ellagitannins, such as geraniin, oenotheins A and B and agrimoniin, as well as myricetin-type flavonoid glycosides typically lost 85–100% of their peak areas (Fig. 4). PD humps (consisting of PAs that contain (epi) gallocatechin subunits in addition to (epi)catechin subunits) disappeared during the oxidation, while PC humps (consisting of PAs containing only (epi)catechin subunits) remained. Gallotannins, i.e. hexa-, hepta-, octa- and nonagalloyl glucoses, lost 100% of their peak areas, as in our previous study (Kim et al., 2018). However, the observations were not supported by the total phenolics measurements which changed only by ca. ±15% (Figures S2.46 to S2.51, S2.71).

Changes in the peak areas of quercetin glycosides ranged from a 22% increase to a 57% decrease (average: 18% decrease) as opposed to peak area changes of kaempferol glycosides ranging from a 34% increase to a 41% decrease (average: 6% decrease). Despite the large variation, the peak areas of quercetin glycosides tended to decrease more than those of kaempferol glycosides.

Caffeoyl phenylethanoids (acteoside, echinacoside, forsythoside B, plantamajoside, and teupolioside) were highly active at pH 10, their peak areas decreasing by 85% on average. The same is true for diarylheptanoids (oregonin and rubranoside A; average: 96% decrease) and rosmarinic acid, a depside of caffeic acid and 3,4-dihydroxyphenyllactic acid (average: 96% decrease). Of the two caffeoyl tartaric acids that were detected, the fukinolic acid was more active at pH 10 than chicoric acid (average: 99 and 27% decreases, respectively). The high activity of these compounds may stem from their catechol moiety at the end of an alkane chain – chicoric acid, which was the least active compound in this group, lacks this kind of a structure.

Oleuropein, a secoiridoid containing a catechol moiety, was highly active (average: 100% decrease), while ligstroside, with a monohydroxysubstituted phenyl, was inactive (4% increase), which follows the patterns established before. However, the activity of demethylligstroside and its aglycone were surprisingly high, considering their monohydroxysubstituted nature (average: 56 and 100% decrease, respectively), though the cause of this discrepancy could not be determined within this study.

Compounds that contained a catechol moiety at the end of a conjugated alkyl chain included caffeic acid derivatives and astringin, a stilbenoid. The activity of most of these compounds varied from weak to moderate. The peak area of caffeoylmalic acid decreased by 8–31% (average: 21%), that of chicoric acid by 11–50% (average: 27%), that of verminoside by 48%, and that of astringin by 30%. Taking isomerization into account, the total peak area of caffeoylarbutin decreased by 36%. The total peak area change of mono-CQAs ranged from a 3% increase to a 63% decrease (average: 35% decrease), and for diCQAs, from a 13% increase to a 77% decrease (average: 59% decrease).

2.4. Enzymatic oxidative activity of phenolic compounds

The enzymatic oxidative activity varied a lot between the identified mono-, di- and trihydroxysubstituted compounds. In general, dihydroxysubstituted phenolic compounds, such as caffeic acid derivatives and quercetin glycosides, oxidized to a higher extent and more frequently than their mono- and trihydroxysubstituted counterparts (e.g. in the leaves of *Populus tremula* and flowers of *Menyanthes trifoliata*, Fig. 5).

The enzymatic oxidation activity of flavonoids is mainly governed by their substitution of B ring, as the typical *m*-OH substitution of the A ring in most flavonoids is not favorable for common PPOs. Thus, quercetin, luteolin and their derivatives can be oxidized by most PPOs, whereas kaempferol, apigenin and their derivatives can only be oxidized by monophenolase-active PPO. HTs and myricetin-type flavonoids with a trihydroxysubstituted B ring can only be oxidized by laccases, a family of extracellular enzymes that is not as common as diphenolase-active PPO. Appropriately, they were not oxidized much during the incubation step in the majority of cases. The enzymatic oxidative activity of tri- and dihydroxysubstituted phenolics did not differ in the few instances where they coexisted in the same sample (e.g. *Trifolium repens*, and *Lysimachia vulgaris*, S2.116 and S2.189). Thus, we believe that in most cases the weak enzymatic oxidative activity of trihydroxysubstituted phenolics can be attributed to the overall lack of specific oxidizing enzymes or at least laccases. An exception to the weak enzymatic oxidative activity of trihydroxysubstituted compounds were ellagitannins in *Quercus robur*, *Nymphaea alba* and the species belonging to the *Epilobium* genus and the Rosoideae of the Rosaceae family. Variation in activity between different tissues is also demonstrated in these samples, as flowers of *Nymphaea alba* and *Epilobium* species are not enzymatically active unlike their leaves. Within-species comparison between leaf and flower tissues did not reveal any major trends on a large scale; both tissues were approximately equally active (data not shown).

An interesting case was the oxidation of isoflavonoids genistein (unmethylated), biochanin A and formononetin (both methylated) in the leaves and flowers of *Trifolium medium* (Figures S2.111 and S2.112). They were completely oxidized by enzymes (loss in peak area 79% or more) despite their monohydroxy- or methoxysubstituted B ring. On the other hand, orobol, an isoflavonoid with a dihydroxysubstituted B ring, lost only ca. 8% of its peak area. This was the only instance where a dihydroxysubstituted compound was significantly less active than its monohydroxysubstituted counterpart.

3. Conclusions

Chemical analysis of a large collection of plant species expanded our previous understanding of alkaline and enzymatic oxidative activities of water-soluble phenolics. Compounds that were the most active at pH 10 contained a pyrogallol group (HTs, myricetin-type flavonoids and PDs) or a catechol group at the end of an alkane chain (e.g. oregonin and dihydroxyphenethyl derivatives). Conjugation between an alkane chain and catechol group lowers the compound's activity at pH 10, as seen with caffeic acid derivatives that did not contain a dihydroxyphenethyl structure (c.f. chicoric acid and fukinolic acid).

Compounds that contained a catechol group were enzymatically the most active, being oxidized in more occasions and to greater extent on average than compounds containing two or three vicinal hydroxyl groups in their aromatic rings. This could be attributed to the abundance of catechol-specific enzymes (e.g. diphenolase-active PPO) and the relative rarity of enzymes capable of oxidizing mono- and trihydroxysubstituted compounds (i.e. monophenolase-active PPO and laccases).

Compounds that did not contain vicinal hydroxyl groups in their structure (i.e. kaempferol-like flavonoids and coumaroylquinic acids) were affected by either type of oxidation less than their more substituted counterparts.

The alkaline oxidative activity of quercetin and kaempferol glycosides and caffeic acid derivatives varied considerably. This inconsistency was not detected in our earlier *in vitro* stability test with pure CQA in various pH buffers (Kim et al., 2018): after 30 min at pH 10, the total peak area of CQA isomers decreased by 50–53% among the three replicates when 5-CQA was the starting isomer, and by 47–51 and 41–45% when the starting isomer was 3-CQA and 4-CQA, respectively. Thus, it can be assumed that the alkaline oxidative activity of these "moderately active" compounds is highly dependent on other factors promoting or inhibiting oxidation present in the plant extract, unlike with ellagitannins,

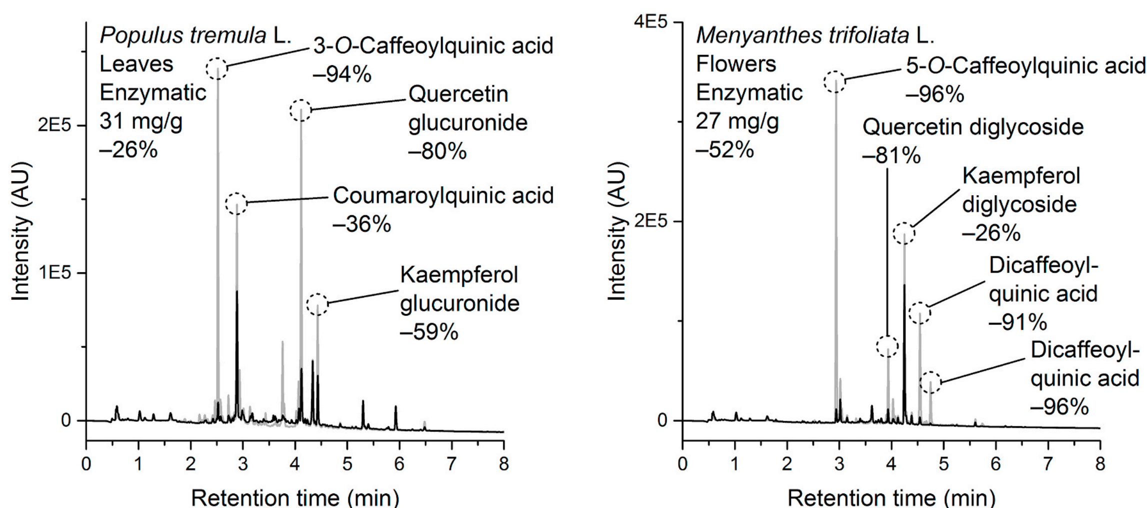


Fig. 5. UV chromatograms (280 nm) of non-oxidized (grey) and oxidized (black) samples of selected species, illustrating enzymatic oxidative activities of various types of compounds. Dihydroxysubstituted compounds, such as quercetin glycosides and caffeic acid derivatives, are oxidized more efficiently than their monohydroxysubstituted counterparts, i.e. kaempferol glycosides and coumaric acid derivatives.

myricetin-type flavonoids and dihydroxyphenethyl-containing structures that are all consistently active at pH 10.

On a general view (Fig. 2), high enzymatic oxidative activity is observed in the clades equisetopsida, campanulids, lamiids and in the orders Fagales and Rosales from the clade fabids. In most cases, the high activity can be attributed to the oxidation of caffeic acid derivatives found in these clades. On the other hand, the high alkaline oxidative activity observed in the clades nymphaeales, cornales and in the families Paeoniaceae from the clade saxifragales and in the families Fagaceae and Rosaceae (Rosoideae) from the clade fabids can be attributed to the oxidation of hydrolysable tannins. In lamiids, the high alkaline oxidative activity arises from the oxidation of phenylethanoids and similar structures where the catechol moiety is at the end of an alkane chain.

It is important to understand the defensive capacities and mechanisms of plants in the field of chemical ecology, e.g. plant-herbivore interactions. When studying the chemical defenses of plants by analyzing plant extracts, "total" methods do not suffice if the active components in the extracts cannot be assessed with certainty. This study has demonstrated the compound types which tend to be the most active at high pH (those that contain a pyrogallol group, or a catechol group at the end of an alkane chain) and the most likely to be oxidized by plant enzymes (those that contain a catechol group). While it is still advisable to test each plant sample to evaluate their enzymatic activity as well as the alkaline oxidative activity of their "moderately active" compounds, this study offers a good starting point for determining the active components in the plant samples that have been analyzed via chromatography and mass spectrometry, especially if the sample only contains few compounds that are known to be of active type.

4. Experimental

4.1. Collection and extraction of the plant material

Sample collection, extraction and analyses were conducted as described comprehensively in Kim et al. (2018). Briefly, the samples were collected in May–August 2011 in Turku area, SW Finland. The species were picked based on their availability on each field trip; we aimed to cover as much of the plant phylogeny as possible by non-discriminately collecting any sort of recognizable plant encountered. Five plant individuals belonging to the same species were selected from a collection site, and at least three undamaged tissue samples (leaves, flowers, needles, etc.) were collected from each specimen to form the sample A. Sample B consisted of a similar set of tissue samples growing as close as possible to the samples collected for the set A. If applicable, the samples A and B came from the same plant individual. Samples were kept in a freezer for a minimum of 18 h. Samples B were enzymatically oxidized by transferring them to an oven (30 °C) and kept there for 2 h, permitting the oxidizing enzymes to come into contact with the phenolic compounds in the plant due to freezing process rupturing the membranes in the plant cells. The sample B was then transferred back to a freezer for another minimum of 18 h. Samples A and B were lyophilized and ground into powder. 20 mg of pulverized sample was weighed in a 2 ml Eppendorf tube and extracted twice with acetone-water (1400 μl, 7:3, v/v, 3 h). An Eppendorf concentrator was used to evaporate the acetone at room temperature. The remaining aqueous phase was frozen, lyophilized, dissolved in 1 ml of water and filtered using a syringe filter (13 mm, 0.2 μm PTFE, VWR International LLC, Radnor, PA, USA), yielding an extract of water-soluble phenolics.

4.2. Chemical analyses

The extracts of oxidized and non-oxidized plant samples were analyzed using a UHPLC-DAD-MS (Acquity UPLC® series, Waters Corporation, Milford, MA, USA) to obtain their phenolic profile at 280 nm before and after enzymatic and alkaline oxidation. In order to obtain comparable chromatograms, the samples were diluted on a 96-well plate according to the alkaline oxidation protocol (Salminen and Karonen, 2011). Triplicates of 20 μl of aqueous extract of sample A and B were diluted 15-fold by adding 280 μl of a mixture of sodium carbonate buffer (pH 10) and 0.6% aq. HCOOH (9:5, v/v, pH 6). Another triplicate of 20 μl of sample A was oxidized at pH 10 by adding 180 μl of sodium carbonate buffer. The plate was shaken for 10 s every min for 60 min. After that the oxidation was stopped by adding 100 μl of 0.6% aq. HCOOH, bringing the pH to 6.

A portion of these samples was used for the modified Folin-Ciocalteu assay (Salminen and Karonen, 2011) to measure the total phenolics before and after the oxidation. 50 μl of each triplicate was transferred to a new 96-well plate, mixed with 50 μl of 1 M Folin-Ciocalteu phenol reagent and 100 μl of 20% Na_2CO_3 (m/v). The plate was shaken for 10 s every min and the absorbance at 742 nm was read after 30 min. Gallic acid was used as the quantitation standard at 0, 10, 25 and 100 μg/ml concentrations.

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 by UHPLC-DAD-MS to obtain UV chromatograms at 280 nm. The UHPLC method is described in detail in

page 1 in the Supplementary. Compounds were identified by comparing their UV and mass spectra to the existing reports in the literature and our research group's spectral library (Table S1). Class of several flavonols was confirmed by the group-specific MRM method. The substituted positions of flavonoids were not determined experimentally. Plant phylogeny was determined using the online tool Phylomatic, version 3 (Webb and Donoghue, 2005) and visualized with Interactive Tree of Life, version 3 (Letunic and Bork, 2016). The chromatograms were processed using Origin software (Version 2016, OriginLab Corporation, Northampton, MA, USA).

Quantity of various subclasses of phenolic compounds were determined by analyzing the non-oxidized extracts of samples A using group-specific MRM methods introduced by Engström et al. (2015, 2014), quantifying their gallic acid derivatives, ellagitannins PCs, PDs, kaempferol-, quercetin- and myricetin glycosides, as well as quinic acid derivatives (Fig. 3). 200 μl of filtered, undiluted extract was analyzed by UHPLC-DAD-MS with the analytical conditions described in the Supplementary. In the MRM methods, precursor ions specific to various polyphenol subgroups (e.g. m/z 169 for gallic acid derivatives) were formed from their parent ions in the ion source by setting optimal voltages to the sample cone. The precursor ion was then fragmented in the collision cell, and the resulting product ion was detected and quantified and transformed to mg/g using external standards. A separate qualitative fragmentation ensured that the detected precursor ion of the quantitative product ion was not a false positive.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The following parties are acknowledged for their help during the project: Anne Koivuniemi and Marica Engström for collecting the plant samples, and Suvi Vanhakylä for species identification. This work was supported by the Kone Foundation and the Academy of Finland [grant number 258992]. Purchase of the UHPLC-DAD-MS system was made possible by a Strategic Research Grant of the University of Turku (Ecological Interaction). We thank Valtteri Virtanen and the Natural Chemistry Research Group in general for scientific discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112501.

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