Journal of Chemical Ecology (2018) 44:483–496
https://doi.org/10.1007/s10886-018-0949-x

Oxidatively Active Plant Phenolics Detected by UHPLC-DAD-MS after Enzymatic and Alkaline Oxidation

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Received: 2 October 2017 / Revised: 14 February 2018 / Accepted: 15 March 2018 / Published online: 11 April 2018
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Abstract

We developed a combination of methods to estimate the alkaline oxidative conditions of the midgut of insect larvae and to reveal the alkaline and enzymatic oxidative activities for individual phenolic compounds present in the larval host plants. First, we monitored the in vitro isomerization of 5-*O*-caffeoylquinic acid (5-CQA) into 3-CQA, 4-CQA and 5-CQA at pH 9.0–11.0. Then we calculated the isomer ratios of 3-CQA, 4-CQA and 5-CQA from the frass of eight species of insect herbivores fed on foliage containing 5-CQA. The isomer ratios suggested that the midgut pH of these larvae ranged from 9.4 to around 10.1. Second, we developed an in situ enzymatic oxidation method that enabled oxidation of phenolics in a frozen plant sample at 30 °C by species- and tissue-specific enzymes. Then we measured the alkaline and enzymatic oxidative activities of the individual phenolics in 20 plant species by quantifying the proportion of the compound concentration lost due to the auto-oxidation of a plant extract at pH 10 and due to the enzymatic oxidation of the frozen plant sample at 30 °C. Our results showed that both of the oxidative activity types depended primarily on the type of phenolic compound, but the enzymatic oxidative activity depended also on the plant species and tissue type. This combination of methods offers an approach to characterize a wide array of phenolics that are susceptible to oxidation by the plant enzymes and/or by the alkaline conditions estimated to prevail in the insect midgut. We propose that these kinds of compound-specific results could guide future studies on specific plant-herbivore interactions to focus on the phenolics that are likely to be active rather than inactive plant phenolics.

Keywords Antiherbivore defense · Autoxidation · Enzymatic activity · Foliar oxidases · Phenolic oxidation · Polyphenol oxidase

Introduction

Plants produce hundreds of different polyphenols (compounds containing more than one phenolic group) and simple phenolic compounds (containing a single phenolic group) that may function in defense against herbivores, pathogens and abiotic stress factors, such as UV radiation. Insect herbivores are important consumers of the green tissue of plants, and the diversity of the abundant and wide-spread Lepidoptera alone extends well beyond 150,000 species (van Nieukerken et al. 2011). Oxidation of plant phenolics is considered to be one of the plants' defense mechanisms against herbivores (Appel 1993; Salminen 2014). As phenolic compounds oxidize, they produce reactive quinones, which in turn produce colored melanins by binding covalently to proteins, phenolics, or other quinones (Yoruk and Marshall 2003). Binding to proteins reduces the nutritional quality of the ingested plant material (Felton et al. 1992). Furthermore, quinones and melanins can cause oxidative stress to the herbivore via free radicals produced in redox recycling (Thiboldeaux et al. 1998). Damage to midgut tissue caused by e.g. juglone (5-hydroxy-1,4-naphtoquinone) has been demonstrated in vitro (Thiboldeaux et al. 1998).

The oxidative activity of plant phenolics can be measured in multiple ways. One can measure the accumulation and decay rates of the primary products of the phenolic one-electron oxidation, i.e. semi-quinone radicals by EPR spectroscopy (Barbehenn et al. 2006a), or the accumulation of the two-electron oxidation products, i.e. yellowish to brownish quinones by UV spectroscopy (Barbehenn et al. 2006a; Moilanen and Salminen 2008). Another approach is to measure the decrease of phenolic concentrations due to the oxidative reactions (Salminen and Karonen 2011; Vihakas et al.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-018-0949-x) contains supplementary material, which is available to authorized users.

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2014). In all these cases the oxidative activity of phenolics can be defined as their tendency to participate in redox reactions in such a way that potentially harmful oxidation products such as quinones or reactive oxygen species are formed. Here we define two types of oxidative activities for phenolics that may be relevant when insect herbivores feed on their host plants: (1) enzymatic oxidative activity triggered by the oxidizing enzymes of the plant itself—such as polyphenol oxidase (PPO), peroxidase (POD) and laccase—and (2) alkaline oxidative activity triggered by the autoxidation of phenolics at alkaline pH in the midgut of the insect herbivore (Appel 1993; Yoruk and Marshall 2003).

Earlier studies have established that especially C-glycosidic ellagitannins (ETs) are prone to rapid autoxidation at alkaline pH, whereas gallotannins (GTs)—i.e. galloyl glucoses containing more than five galloyl moieties—and flavonoid (FL) glycosides in general are considered less susceptible to rapid autoxidation (Salminen and Karonen 2011). However, the tendency of individual ETs, GTs or FLs to be oxidized by plant species-specific enzymes has not been studied. The enzymatic oxidation of one of the most common plant phenolics, 5-*O*-caffeoylquinic acid (5-CQA, or chlorogenic acid) has been examined (Clifford 2000). 5-CQA can be a substrate for PPOs and is oxidatively active at alkaline pH. In addition, it was shown that 5-CQA isomerizes into a mixture of 3-CQA, 4-CQA and 5-CQA in alkaline conditions and that different insect species produce various isomer ratios of CQAs in the frass after feeding on 5-CQA-rich foliage (Salminen et al. 2004; Lahtinen et al. 2005). If the observed isomer ratios reflect the pH differences of the midguts of the species, studies on 5-CQA isomerization could be used to estimate the alkaline oxidative conditions of the larval midgut. If the enzymatic content of the host plant tissue was known as well, then both the alkaline and enzymatic oxidative activities could be measured at the characteristic pH for the herbivore species and an appropriate enzyme content for the plant species.

PPO is a common, diverse and widely studied enzyme family in the plant kingdom. PPOs contain copper in the active site and vary e.g. in size, the level of glycosylation and substrate specificity (Yoruk and Marshall 2003; Aniszewski et al. 2008). All PPOs can oxidize *o*-dihydroxysubstituted phenolics such as CQAs to *o*-quinones (diphenolase activity; EC 1.10.3.1), and some PPOs can also oxidize monohydroxysubstituted phenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1) immediately followed by oxidation to *o*-quinones (Lerch 1995; Yoruk and Marshall 2003; Fig. 1). Laccases (EC 1.10.3.2) are distinguished by their ability to oxidize *o*-, *m*- and *p*-dihydroxysubstituted phenolics, trihydroxysubstituted phenolics and a variety of other compounds, such as ascorbic acid, but not monohydroxysubstituted phenolics (Yoruk and Marshall 2003; Aniszewski et al. 2008). The content and composition of oxidizing enzymes in plants depends on the species, the maturity of the plant and stress factors in the surroundings, and different enzymes vary in their pH and temperature optima (Yoruk and Marshall 2003).

In a healthy plant cell, phenolic compounds are mainly stored in vacuoles and thus are separated from PPOs that are stored in thylakoid membranes of chloroplasts and from extracellular PODs and laccases (Mayer and Harel 1979; Yoruk and Marshall 2003). As an herbivore feeds on a plant, the cell walls and intracellular membranes of the plant are ruptured, so that plant phenolics and oxidative enzymes come into contact. The phenolics can thus be oxidized to quinones, and the consequent reactions alter the nutritive value of the plant or cause oxidative stress as stated above. The phenolics that escape enzymatic oxidation may be oxidized in the alkaline conditions present in the midgut of many lepidopteran insect larvae (Appel 1993). Either enzymatic or nonenzymatic oxidation could result in oxidative stress to the herbivore.

A simple oxidation method developed by Salminen and Karonen (2011) utilizes a microplate reader to compare the total phenolic content of a crude plant extract before and after oxidation at alkaline pH to estimate what proportion of the phenolics present in the sample are oxidized in these conditions. Vihakas et al. (2014) combined the assay with subsequent analysis by ultrahigh-performance liquid chromatography coupled with diode array and mass spectrometric detection (UHPLC-DAD-MS) to reveal individual phenolic compounds that contributed to the alkaline oxidative activity of the plant extract or remained intact during the oxidation at alkaline conditions. This method does not reveal the individual phenolic compounds that could be oxidized by enzymes present in the same plant individual. Furthermore, the earlier methods did not provide guidance for selecting the proper pH for the autoxidative step, although it is apparent that herbivore species have variable midgut pH values (Harrison 2001).

We proposed that a method that uses the enzymes present in a plant organ to oxidize the phenolic compounds present in the same organ would complement our earlier method that relies on oxidation by alkaline pH. In addition, we introduced a method to estimate the pH of the insect midgut by measuring the isomer ratios of 3-CQA, 4-CQA and 5-CQA in the frass after the insect had digested 5-CQA rich foliage. If any phenolic compounds are oxidized by enzymes and/or the estimated pH of the midgut, they are considered to be oxidatively active compounds, whereas if both of the treatments result in low responses, the plant organ is considered oxidatively inactive. This type of activity-based selection of compounds could significantly improve the future studies aiming to reveal the role of phenolic oxidation in plant defense, by focusing attention on the analysis of phenolics that appear to be oxidatively active. Moreover, when the autoxidative step (Salminen and Karonen 2011) is done in a pH that is physiologically relevant to the given herbivore species, we could focus on results that suggest a role for alkaline oxidative activity only with species that truly have alkaline gut conditions.

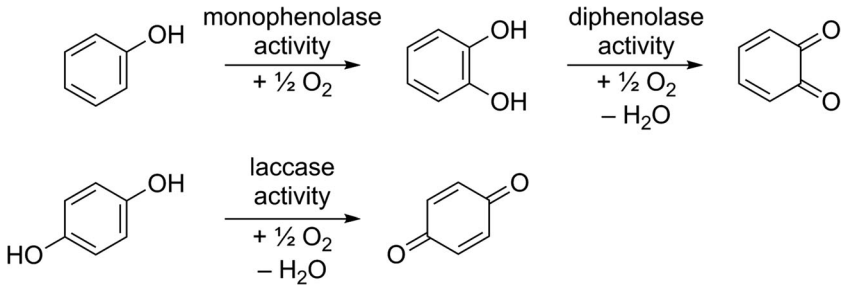

Fig. 1 Different routes of enzymatic oxidation of phenolics. All polyphenol oxidase enzymes are capable of forming *o*-quinones from *o*-diphenols, but only some can form *o*-diphenols from monophenols. Laccase enzymes are versatile, and are able to oxidize e.g. *o*-, *m*- and *p*-diphenols and triphenols to quinones

In this paper, we report new and important improvements to our method to detect and tentatively characterize the plant phenolics with oxidative activity (Salminen and Karonen 2011; Vihakas et al. 2014). The new approach can reveal individual phenolics that are susceptible to in vitro oxidation or other types of modifications either by the foliar enzymes of the plant sample itself or by the pH estimated to prevail in the insect gut. The enzymatic oxidation method, tested with 20 plant species, takes advantage of the loss of plant cell integrity followed by enzymatic oxidation of phenolics when a previously frozen plant sample is incubated at 30 °C. The change in the concentration of total phenolics due to the enzymes present in the plant and the pH of the insect midgut can be estimated by both the UV detection with microplate reader and UV and MS detection with UHPLC-DAD-MS. This combination of both simple and sophisticated applications allows the rapid screening of hundreds of plant samples for most oxidatively active plant species and organs, or individuals within a given species, and the identification of the types of phenolics contributing to the observed activity. This should facilitate closing the current gaps in our knowledge of the oxidatively active and inactive phenolics in plants of interest. This approach may help investigators focus on identification and quantitation of the potentially active compounds instead of the diverse pool of all phenolic compounds produced by the plant cell.

Methods and Materials

Chemicals and Reagents

5-CQA was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). 4-CQA, 3-CQA and LC-MS-grade acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). LC-MS- and analysis-grade formic acid and analysis-grade sodium hydrogen carbonate were from VWR (Helsinki, Finland). Analysis-grade sodium carbonate was from FF-Chemicals Ab (Yli-Ii, Finland). Analysis-grade acetone and 50 mM sodium carbonate/sodium hydrogen carbonate buffer (pH 10, Buffer Concentrate 4797) were from J.T. Baker (Deventer, the Netherlands). Folin-Ciocalteu phenol reagent and gallic acid were from Sigma Chemical Co. (St. Louis, MO, USA). Water was filtered through an Elgastat UHQ-PS purification system (Elga, Kaarst, Germany).

In Vitro Isomerization of Caffeoylquinic Acids

Isomerization of 3-, 4- and 5-CQA was studied first at pH 9, 10 and 11 using UHPLC-DAD-MS. Standard solutions (10 mg/ml) of each isomer were prepared by dissolving the commercial standard in water. A 200 μl aliquot of each standard solution was transferred to an UHPLC vial and mixed with 1800 μl of carbonate buffers at pH 9, 10 or 11 (oxidized samples) or water (nonoxidized samples). The vial was sealed and UHPLC analysis was immediately initiated, using a 6 min separation protocol (Supplementary Information) to enable 24 injections from the same vial in 144 min. The kinetics were studied using three replicate vials for each pH, preparing each oxidation reaction immediately before the UHPLC analysis commenced. The results were plotted using Origin 8.0 software. In the more detailed pH experiment 5-CQA was oxidized for 180 mins at 11 pH values over the pH range of 9.0–11.0 in 0.2 pH unit increments, again using three replicates per condition.

Isomerization of Caffeoylquinic Acids in Herbivorous Insects

We reanalyzed the frass data we had earlier obtained for eight species of insect herbivores (Salminen and Lempa 2002; Salminen et al. 2004; Lahtinen et al. 2005; Salminen 2018) fed on birch leaf diet that contained 5-CQA, but very little 3-CQA and no 4-CQA. These studies included two species of lepidopteran larvae (*Epirrita autumnata* and *Agriopis aurantiaria*) together with larvae of six sawfly species (*Amauronematus amplus*, *Nematus brevivalvis*, *Pristiphora alpestris*, *Priophorus pallipes*, *Arge* sp. and *Nematus viridis*). All leaf and frass samples were freeze-dried, ground into fine

powder, extracted and analyzed by HPLC-DAD as reported in Salminen and Lempa (2002), Salminen et al. (2004), Lahtinen et al. (2005) and Salminen (2018).

Development of the Enzymatic Oxidation Method

The enzymatic oxidation method was optimized with leaves of two model plant species: *Betula pubescens* Ehrh. (white birch) and *Betula pendula* Roth (silver birch), growing in the Botanical Garden of the University of Turku, Finland. The subspecies of the former, *B. pubescens* spp. *czerepanovii* (mountain birch) is known to have a relatively high PPO activity (Ruuhola and Yang 2006).

The enzymatic oxidation was carried out at 30 °C. This temperature is plausible considering the herbivory occurring in nature. Furthermore, this temperature is generally suitable for most reported PPO activities, and is low enough to avoid thermal degradation of PPO that can occur at around 50 °C (Yoruk and Marshall 2003).

The time for enzymatic oxidation was optimized by comparing incubation for 1, 2 and 3 h. Five leaves were collected from single *B. pubescens* and *B. pendula* trees in May 2011 for the control sample and each incubation time sample. Upon collection, the leaves were wrapped in aluminum foil, placed in an insulated box filled with ice and transferred to a freezer (−20 °C) where they were kept a minimum of 18 h. Once frozen, one set of foil-wrapped leaves was placed in an oven at 30 °C and was incubated for the desired time. After incubation, the samples were refrozen, lyophilized and ground to powder using a MM 200 mixer mill (Retsch GmbH, Haan, Germany).

Plant Material

Plant species and genera were selected based on the knowledge of their phenolic or PPO content and activity, as well as their availability in the Turku area. The collected species and plant tissue types are listed in Table 1.

The species rich in ETs were *Epilobium angustifolium* (great willowherb; also known as *Chamerion angustifolium* (L.) Holub), *Epilobium hirsutum* L. (hairy willowherb; Baert et al. 2015), *Geranium sylvaticum* L. (woodland geranium) and *Geranium pratense* L. (meadow geranium; Moilanen et al. 2015). To study the effect of oxidation on GTs, we collected *Acer platanoides* (Norway maple) and *Paeonia* sp. (peony; Haddock et al. 1982). The species rich in FLs were *Pinus sylvestris* L. (Scots pine) containing e.g. taxifolin glucoside (Karonen et al. 2004), *Alliaria petiolata* (M.Bieb.) Cavara & Grande (garlic mustard) containing kaempferol glycosides (Haribal and Renwick 2001), and *Malus* sp. (apple) containing phloridzin (Hunter and Hull 1993).

Prunus padus (bird cherry) and *Menyanthes trifoliata* L. (bogbean) leaves contain 5-CQA (Martz et al. 2009; Olszewska and Kwapisz 2011). In order to get a more comprehensive view of different cinnamic acid derivatives, the following species were also collected: *Campanula patula* L. (spreading bellflower) rich in CQAs and coumaroylquinic acid (CoQA; Teslov et al. 1983), plantamajoside-rich *Plantago major* L. (major plantain; Ravn and Brimer 1988), 2-*O*-caffeoylmalic acid-rich *Urtica dioica* (stinging nettle; Pinelli et al. 2008), and chicoric acid-rich *Taraxacum* sp. (dandelion; Schütz et al. 2005).

Trifolium pratense L. (red clover) and *Populus tremula* L. (European aspen) were selected on the basis of their rather well-documented PPO activity. Though no experiments have been conducted on *P. tremula*, other *Populus* species have been used in PPO studies (Constabel et al. 2000), with 11 genes coding PPO having been identified in *Populus trichocarpa* (black poplar; Tran et al. 2012). Seven genes coding the synthesis of PPOs have been identified in *T. pratense*, an important source of protein for ruminants (Jakešová et al. 2016). The main compounds of *T. pratense* are FLs and clovamide (*N*-caffeopyl-L-DOPA; Polasek et al. 2007).

Plant samples were collected in duplicates, A and B, in May–July 2011 in Turku area, SW Finland (see Fig. S1 for the complete flow of the experiment). Sample A was the control, and it was also used for oxidation at alkaline conditions, while sample B was used for enzymatic oxidation. At least five individual plants were chosen from a given plant population. At least three pairs of undamaged leaves or flowers were collected from each individual. We tried to maximize the chemical similarity of samples A and B by collecting two closely growing leaves (or flowers) and adding the first to batch A and the second to batch B. Thus both batches A and B were comprised of 15 leaves or flowers. Both batches A and B were immediately wrapped in aluminum foil, placed in an insulated box filled with ice and transferred to a freezer (−20 °C) within 3 h, where they were kept a minimum of 18 h.

Enzymatic Oxidation and Freeze-Drying

For each species, the samples in batch A were taken from the freezer and freeze-dried, without thawing the samples, so that sample A represented the unaltered phenolic content. The B samples were taken from the freezer and each foil packet was incubated for 2 h at 30 °C, so that the tissue was thawed and then exposed to oxidation by the sample-specific enzymes for 2 h. After the 2-h oxidation step, the B samples were refrozen and freeze-dried. The lyophilized samples A and B were ground to fine powder using a MM200 mixer mill.

Table 1 Plant samples used in the study

Species	English name	Parts	Main compound	References
Method optimization				
Betula pendula Roth	Silver birch	Leaves	Betuloside	Sunnerheim et al. 1988
Betula pubescens Ehrh.	White birch	Leaves	5-CQA	Ruuhola and Yang 2006
Ellagitannin-rich species				
Epilobium angustifolium	Great willowherb	Flowers, leaves	Oenothein B	Moilanen et al. 2015
Epilobium hirsutum L.	Hairy willowherb	Flowers, leaves	Oenothein B	Moilanen et al. 2015
Geranium pratense L.	Meadow geranium	Flowers, leaves	Geraniin	Moilanen et al. 2015
Geranium sylvaticum L.	Woodland geranium	Flowers, leaves	Geraniin	Moilanen et al. 2015
Gallotannin-rich species				
Acer platanoides	Norway maple	Leaves	Gallotannins	Haddock et al. 1982
Paeonia sp.	Peony	Flowers, leaves	Gallotannins	Haddock et al. 1982
Flavonoid-rich species				
Alliaria petiolata (M.Bieb.) Cavara & Grande	Garlic mustard	Flowers	Kaempferol triglycoside	Haribal and Renwick 2001
Malus sp.	Apple	Leaves	Phloridzin	Hunter and Hull 1993
Pinus sylvestris L.	Scots pine	Needles	Taxifolin glucoside	Karonen et al. 2004
Cinnamic acid derivative-rich species				
Campanula patula L.	Spreading bellflower	Flowers	CoQA	Teslov et al. 1983
Menyanthes trifoliata L.	Bogbean	Leaves	5-CQA	Martz et al. 2009
Plantago major L.	Major plantain	Leaves	Plantamajoside	Ravn and Brimer 1988
Prunus padus	Bird cherry	Leaves	5-CQA	Olszewska and Kwapisz 2011
Taraxacum sp.	Dandelion	Leaves	Chicoric acid	Schütz et al. 2005
Urtica dioica	Stinging nettle	Leaves	Caffeoylmalic acid	Pinelli et al. 2008
Plants with high enzyme activity				
Populus tremula L.	European aspen	Leaves	3-CQA	Constabel et al. 2000; Tran et al. 2012
Trifolium pratense L.	Red clover	Leaves	Quercetin malonyl glycoside	Polasek et al. 2007; Jakešová et al. 2016

CQA = caffeoylquinic acid, CoQA = coumaroylquinic acid

Sample Extraction and Autoxidation under Alkaline Conditions

For each species, the plant powders (20 mg) from both samples A and B were extracted twice with 1400 μl of acetone–water (7:3, v/v) for 3 h, and the extracts were combined. The acetone was evaporated from each extract with an Eppendorf concentrator at room temperature. The aqueous phases were frozen, lyophilized and dissolved in 1 ml of water, before filtering using a syringe filter (13 mm, 0.2 μm PTFE, VWR International LLC, Radnor, PA, USA). These filtered samples comprised the aqueous extracts (A or B).

A part of the aqueous extract of unaltered sample A was oxidized under alkaline conditions following the protocol of Salminen and Karonen (2011). A 20 μl aliquot of the extract A was mixed with 180 μl of 50 mM sodium carbonate buffer (pH 10) and placed in a microplate reader (Thermo Multiskan Ascent, Thermo Electron Corporation, Shanghai, China) programmed to shake for 10 s every min for 60 min. After 60 min, the oxidation was stopped by adding 100 μl of 0.6% aq. HCOOH (v/v), resulting in pH 6. These steps diluted sample A fifteen-fold and yielded 300 μl of the oxidized extract $A_{ALK\text{-}OX}$.

Total Phenolics Measurement and UHPLC-DAD-MS Analysis

To be able to compare the phenolic contents of both the non-oxidized aqueous extract A and the aqueous extract of the enzymatically oxidized sample B against the autoxidized extract $A_{ALK\text{-}OX}$, the original aqueous extracts A and B needed to be diluted fifteen-fold. Therefore 20 μl of each the aqueous extract (A and B) for each species was diluted in triplicate in a 96-well plate format by adding 280 μl of diluent (pH 10 buffer mixed with 0.1% HCOOH at a ratio of 9:5 v/v, pH 6). These diluted extracts were shaken for 60 min in the well-plate yielding 300 μl extracts of $A_{NON\text{-}OX}$ and $B_{ENZ\text{-}OX}$, parallel to autoxidation step (above) that produced triplicate 300 μl extracts of $A_{ALK\text{-}OX}$.

The total phenolics content of the each sample of A_{NON-OX}, A_{ALK-OX} and B_{ENZ-OX} was measured with the modified Folin-Ciocalteu assay (Salminen and Karonen 2011). Subsamples of 50 μl of all the triplicate extracts were transferred into a new well-plate and mixed with 50 μl of 1 M Folin-Ciocalteu reagent and 100 μl of 20% Na_2CO_3 (m/v). The mixture was shaken in a microplate reader for 10 s every min and the absorbance at 742 nm was read after 30 min. The quantification standard was gallic acid (0, 10, 25 and 100 μg/ml).

The remaining portion (3 × 250 μl) of each of the triplicate extracts A_{NON-OX}, A_{ALK-OX} and B_{ENZ-OX} was used in the UHPLC-DAD-MS analysis. The triplicate extracts were pooled to yield 750 μl and filtered using a syringe filter (4 mm, 0.2 μm PTFE, Thermo Fischer Scientific Inc., Waltham, MA, USA). The extracts A_{NON-OX}, A_{ALK-OX} and B_{ENZ-OX} were then analyzed following the method of Vihakas et al. (2014). A detailed description of the UHPLC method is given in the Supplementary Information. Individual phenolic compounds were identified by comparing their UV and mass spectra to the values reported in the literature and the spectral libraries of our laboratory (Table 2; e.g. Moilanen et al. (2013) and references given above for the plant species used).

Results

Alkaline Oxidation and Isomerization of Caffeoylquinic Acids

We followed the oxidation and isomerization of three CQA isomers (3-CQA, 4-CQA and 5-CQA) separately at three pH values. The rate of decrease of the sum of CQA isomers depended on the pH: the higher the pH, the faster the depletion of CQAs. The overall levels of the three isomers decreased by ca. 25%, 70% and 90% at pH 9, 10 and 11, respectively, during the first 60 min of the incubation period regardless of the original isomer, and by ca. 40%, 85% and 95% after 120 min (data not shown). When 3- or 5-CQA was used as the initial isomer, 4-CQA was the first new isomer to be formed, whereas 3-CQA was the first to be formed from 4-CQA. Thus 5-CQA was the dominant isomer at any pH or incubation time only when it was used as the initial compound. The general trend of isomerization was the same with all CQA isomers: the higher the pH, the faster the isomerization. Late in the reaction, the ratio of isomers did not change although the overall level of CQAs continued to decrease slowly. Figure 2 summarizes the possible combinations of isomeric ratios that could be obtained at these three pH values at any given time point. The incubation of 5-CQA at a series of close pH increments between pH 9.0–11.0 for 180 mins showed how the steady state isomer ratios, reported in their LC elution order of 3-CQA:5-CQA:4-CQA, changed from 1:7:2 to 4:3:3 (Fig. S2).

Isomerization of Caffeoylquinic Acids in Herbivorous Insects

The reanalysis of the frass chemistry data obtained from the previous feeding tests done with 5-CQA-rich *Betula* species (Salminen and Lempa 2002; Salminen et al. 2004; Lahtinen et al. 2005; Salminen 2018) showed that lepidopteran and sawfly larvae differed in the ratios of CQA isomers quantified from the frass. These ratios varied between 1:3:1 (*Pristiphora alpestris*, *Priophorus pallipes*, *Arge* sp. and *Nematus viridis*), 1:2:1 (*Nematus brevivalvis*), 3:4:3 (*Epirrita autumnata* and *Amauronematus amplus*), and 4:3:3 (*Agriopis aurantiaria*). The isomer ratios were highly consistent between insect individuals within a species, even though the recovery rate of CQAs in the frass varied 10–20% between the individuals.

Development of the Enzymatic Oxidation Method

The enzymatic oxidation step was first optimized by incubating *B. pubescens* and *B. pendula* leaves at 30 °C for 0–3 h, with varying levels of activity observed. *B. pubescens* contained e.g. monogalloyl glucose and 5-CQA, which are natural substrates of PPO, containing tri- and dihydroxysubstituted phenolic moieties, respectively. The loss of these compounds, i.e. the decrease of their peak areas at 280 nm, was ca. 80 and 70% after 1 h, ca. 95 and 85% after 2 h, and ca. 100 and 95% after 3 h, respectively (data not shown). In contrast, the main compounds of *B. pendula* leaves were betuloside and CoQA, i.e. monohydroxysubstituted phenolics, and after 2 h of incubation, their peak areas only decreased by ca. 15 and 35%, respectively. In order to avoid fully oxidizing the most easily oxidized phenolic compounds, but to achieve enough oxidation to be easily detected by both UHPLC-DAD-MS and total phenolic assay, we decided that the incubation time of 2 h is the most suitable for an average plant sample.

Enzymatic and Alkaline Oxidation of Phenolics

ETs and simple galloyl glucoses together with other simple galloyl derivatives are relatively easily oxidized at alkaline pH, while gallotannins are much less prone to oxidation (Barbehenn et al. 2006a). Therefore, it was expected that the oxidation of *G. pratense* and *G. sylvaticum* leaf and flower samples under alkaline conditions would result in a consistent pattern between species and tissue types for both geraniin and galloylquinic acids. Figure 3 shows that they were almost fully oxidized at pH 10 and the total phenolic assay showed a decrease of 25–30% of total phenolics due to the oxidation. The enzymatic oxidation, however, resulted in moderate tissue- and species-specific variation in the levels of oxidized phenolics, presumably due to differences in the enzyme activities. Chromatograms in Fig. 3a–c show that geraniin and

Table 2 Identification of compounds based on their retention times, UV spectra and MS data

Compound name	Abbreviation	Compound class	Plant species	Rt (min)	UV maxima (nm)	*m/z* values
Betuloside		Cou	*Betula pendula*	2.75	195, 220, 277	327 $[M-H]^-$
Betuloside isomer		Cou	*Betula pendula*	2.89	195, 220, 277	327 $[M-H]^-$
Biochanin A		FL	*Trifolium pratense*	6.45	260	283 $[M-H]^-$
Caffeoylmalic acid		CF	*Urtica dioica*	3.49	249, 327	295 $[M-H]^-$
3-Caffeoylquinic acid	3-CQA	CF	*Populus tremula* *Campanula patula*	2.52	244, 325	353 $[M-H]^-$
4-Caffeoylquinic acid	4-CQA	CF		3.04	244, 325	353 $[M-H]^-$
5-Caffeoylquinic acid	5-CQA	CF	*Betula pubescens* *Prunus padus* *Menyanthes trifoliata* *Urtica dioica*	2.94	244, 325	353 $[M-H]^-$
Chicoric acid		CF	*Taraxacum* sp.	4.33	252	473 $[M-H]^-$
Clovamide		CF	*Trifolium pratense*	3.69	322	358 $[M-H]^-$
Coumaroylquinic acid	CoQA	Cou	*Betula pendula* *Populus tremula* *Campanula patula*	2.90	310	337 $[M-H]^-$
Dicaffeoylquinic acid	di-CQA	CF	*Menyanthes trifoliata*	4.75	247, 326	515 $[M-H]^-$ 353 $[M-CF-H]^-$
Flavone		FL	*Alliaria petiolata*	3.64	269, 336	593 $[M-H]^-$
Formononetin		FL	*Trifolium pratense*	5.70	256	267 $[M-H]^-$
Galloyl quinic acid	GaQA	GA	*Geranium pratense* *Geranium sylvaticum*	1.94	215, 275	343 $[M-H]^-$
Geraniin		ET	*Geranium pratense* *Geranium sylvaticum*	3.42	223, 276	951 $[M-H]^-$ 933 $[M-H_2O-H]^-$
Heptagalloyl glucose	7GG	GT	*Acer platanoides* *Paeonia* sp.	4.85	221, 278 (sh)	1243 $[M-H]^-$ 1091 $[M-GA-H]^-$ 545 $[M-GA-2H]^{2-}$ 469 $[M-2GA-2H]^{2-}$
Hexagalloyl glucose	6GG	GT	*Acer platanoides* *Paeonia* sp.	4.57 4.70	220, 278 (sh)	1091 $[M-H]^-$ 939 $[M-GA-H]^-$ 469 $[M-GA-2H]^{2-}$
Kaempferol diglycoside 1		FL	*Acer platanoides*	3.99	267, 350	745 $[M-H]^-$
Kaempferol diglycoside 2		FL	*Acer platanoides*	3.77	197, 265, 347	593 $[M-H]^-$
Kaempferol diglycoside 3		FL	*Alliaria petiolata*	3.86	268, 349	609 $[M-H]^-$
Kaempferol triglycoside		FL	*Alliaria petiolata*	2.71	197, 265, 347	771 $[M-H]^-$ 609 $[M-Glu-H]^-$
Monogalloyl glucose	1GG	GG	*Betula pubescens*	1.27	215, 275	331 $[M-H]^-$
Octagalloyl glucose	8GG	GT	*Acer platanoides* *Paeonia* sp.	5.01	220, 276 (sh)	1395 $[M-H]^-$ 1243 $[M-GA-H]^-$ 621 $[M-GA-2H]^{2-}$ 545 $[M-2GA-2H]^{2-}$ 469 $[M-3GA-2H]^{2-}$
Oenothein A		ET	*Epilobium hirsutum* *Epilobium angustifolium*	3.28	222, 265	1175 $[M-2H]^{2-}$
Oenothein B		ET	*Epilobium hirsutum* *Epilobium angustifolium*	2.94	223, 261	783 $[M-2H]^{2-}$
Pentagalloyl glucose	5GG	GG	*Paeonia* sp.	4.37	219, 280	939 $[M-H]^-$ 469 $[M-2H]^{2-}$
Phloridzin		FL	*Malus* sp.	4.89 5.17	196, 225, 284	435 $[M-H]^-$ 273 $[M-Glu-H]^-$
Plantamajoside		CF	*Plantago major*	4.02	197, 220, 328	639 $[M-H]^-$
Quercetin glycoside		FL	*Prunus padus*	3.74	255, 354	595 $[M-H]^-$
Quercetin malonyl glycoside		FL	*Trifolium pratense*	4.36	255, 353	549 $[M-H]^-$ 505 $[M-COOH]^-$
Taxifolin glucoside		FL	*Pinus sylvestris*	3.88	129, 225, 289	465 $[M-H]^-$

CF = caffeic acid derivative, Cou = coumaric acid derivative, GA = gallic acid derivative, GG = galloyl glucose, Glu = glucose, GT = gallotannin, ET = ellagitannin, FL = flavonoid, sh = shoulder

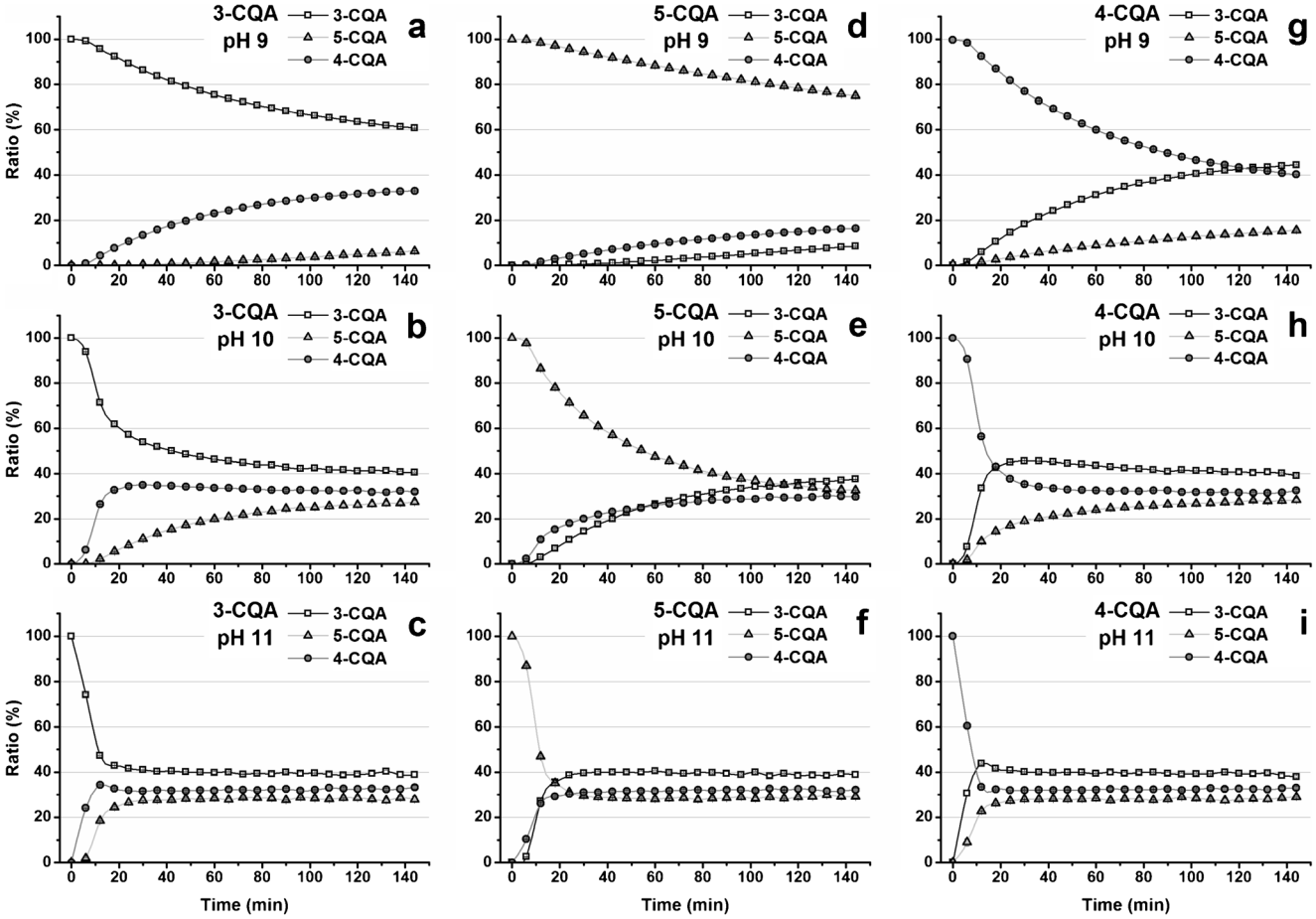

Fig. 2 Isomeric ratios of 3-caffeoylquinic acid (3-CQA), 5-CQA and 4-CQA at pH 9, 10 and 11 measured by UHPLC-DAD at 325 nm

galloylquinic acid remained intact (their peak areas varied only by ±5% at 280 nm), except the flowers of *G. sylvaticum*, where the peak areas of these compounds decreased by 24%. The decrease was supported by the total phenolic assay (−17%).

The species *E. angustifolium* and *E. hirsutum* contain tellimagrandin I-based macrocyclic oligomers, such as oenothein A and B. The oxidation at alkaline conditions produced the expected results: oenothein A and B were almost fully oxidized (over 90% loss as witnessed by UHPLC-DAD), and the level of total phenolics decreased by 25–35% (Fig. S3). Much higher enzymatic oxidative activity was found with *E. angustifolium* leaves and *E. hirsutum* leaves and flowers (30% decrease of total phenolics and 56–64% decrease of oenothein B) than with any of the *Geranium* tissues. However, the enzymatic oxidation of the phenolics in *E. angustifolium* flowers (Fig. S3) did not match this pattern, since only 2% decrease in total phenolics and 18% decrease of oenothein B was observed. This clearly demonstrated that oenothein B can be oxidized enzymatically, but the level of oxidation depends on the enzymatic activity of the plant species and tissue type.

The leaves of *A. platanoides* and the leaves and flowers of *Paeonia* sp. were rich in GTs that are expected to be more stable to oxidation than other galloyl derivatives or ETs (Barbehenn et al. 2006a). However, when the oxidation of these extracts at alkaline conditions continued for 60 min, we witnessed a significant decrease in GT levels by UHPLC-DAD (Fig. S4). In contrast, the total phenolic assay showed relatively stable levels of phenolics in these extracts under alkaline conditions. Our measure of the enzymatic oxidative activity of these species and tissues with both UHPLC-DAD and total phenolic assays detected only minor changes in GT levels with no changes in the levels of total phenolics. This implied that either the samples had a very low enzymatic activity or GTs were not prone to enzymatic oxidation, or both.

Oxidation results obtained for FL-rich species *A. petiolata*, *P. sylvestris* and *Malus* sp. are shown in Fig. S5. The results agreed with Vihakas et al. (2014), who showed that FLs in general are not susceptible to oxidation at alkaline pH with the exception of myricetin derivatives containing a trihydroxysubstituted B ring. However, although phloridzin in *Malus* sp. remained stable at alkaline pH, it was effectively oxidized by the enzymes present in the leaf tissue: the total phenolic assay showed a 55% decrease

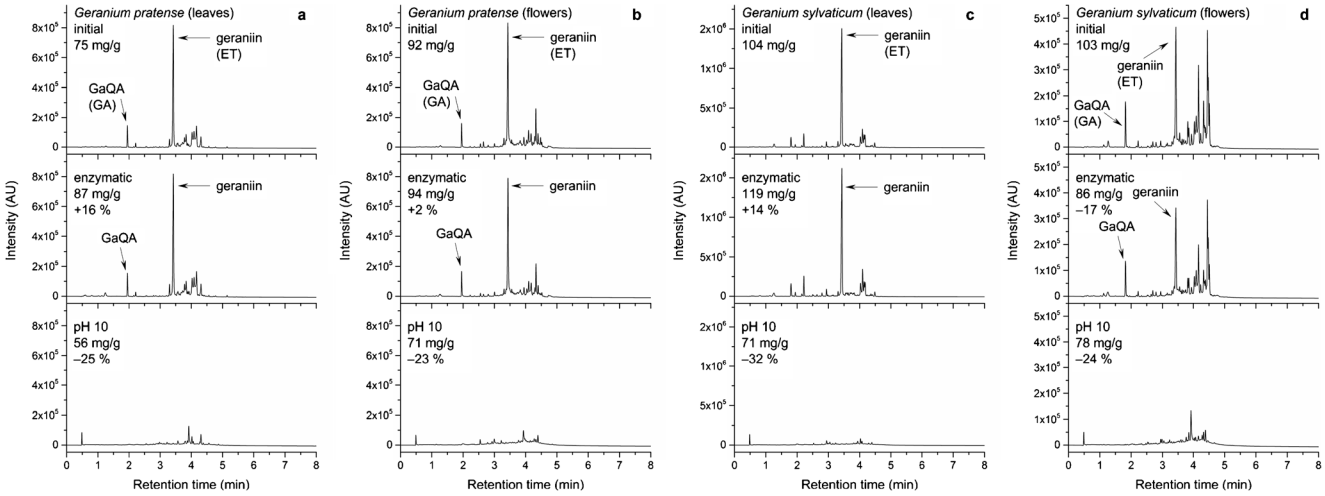

Fig. 3 UHPLC-DAD chromatograms (at 280 nm) of phenolic extracts of *Geranium pratense* leaves (**a**) and flowers (**b**) and *G. sylvaticum* leaves (**c**) and flowers (**d**) rich in dehydrohexahydroxydiphenoyl ester geraniin. Top, middle and bottom chromatograms show initial phenolic profiles, profiles after the enzymatic oxidation and profiles after the oxidation at pH 10, respectively. The mg/g values show the total phenolic content measured by Folin-Ciocalteu assay and the % values show how much the total phenolic content is altered during the oxidation. ET: ellagitannin, GA: gallic acid derivative. For other abbreviations and the UV and MS data of the compounds, see Table 2

in total phenolics and UHPLC-DAD a 92% decrease in the content of phloridzin. The monohydroxysubstitution in the phenolic B ring of phloridzin may explain why it did not undergo autoxidation at alkaline conditions, while its high reactivity under enzymatic oxidation suggested that *Malus* sp. leaves contained monophenolase-active PPO.

Both UHPLC-DAD and total phenolic assays showed that the foliage of *T. pratense* (Fig. S6a), *M. trifoliata* (Fig. S4b), *P. major* (Fig. S7a), *U. dioica* (Fig. S7b) and *Taraxacum* sp. (Fig. S7c) had high enzymatic activity that was able to oxidize most of the phenolics present in the leaves. Similar compounds were also found in *P. tremula* (Fig. S6b), *C. patula* (Fig. S6c) and *P. padus* (Fig. 4a). Among these species, only the enzymes of *P. tremula* were able to efficiently oxidize the CQAs, but apparently foliage of this species did not have much monophenolase activity, since CoQA was only slightly oxidized during the enzymatic incubation. The phenolics in *C. patula* and *P. padus* were largely unaltered by the enzymatic oxidation step, although the species did contain the same phenolic substrates that were oxidized by enzymes present in other species.

The oxidation at alkaline pH of the above eight species revealed variable patterns for the caffeic acid derivatives, which is surprising considering that the structure of caffeic acid should enable it to undergo autoxidation at alkaline conditions. Clovamide was the only individual phenolic compound of *T. pratense* to oxidize completely. Caffeoylmalic acid (Fig. S7b) and chicoric acid (Fig. S7c) were surprisingly stable at alkaline pH, as they were only 30% oxidized according to UHPLC-DAD. In *P. padus* the total level of CQAs decreased only by 12%, whereas in *M. trifoliata* it decreased by 63% (Fig. 4). This suggests that these species contained variable amounts of antioxidants that partially prevented the oxidation of these polyphenols (Barbehenn et al. 2006b).

Discussion

Plant phenolics may be modified in many ways during their passage through the gut of an insect herbivore. If we simplify this relatively complex chain of metabolic events, we may expect that highly stable and inactive compounds are excreted by the insect larvae quantitatively unchanged, while the most active compounds are unstable or modified and thus excreted in lower or non-existing levels. In this study we were interested in developing a combination of methods that would give us new insight into the effects of enzymatic and alkaline oxidation of phenolics on the concentrations of different types of plant phenolics. With the methods, we specifically wanted to be able to estimate the midgut pH of the herbivore species so that the host plant phenolics could be oxidized at this specific pH. Similarly in the quest for the most relevant oxidative conditions, we wanted to oxidize the plant phenolics with the specific enzyme content that co-occurrs in the plant with the phenolics, instead of using a commercially available enzyme preparation that might have little to do with the true enzyme content of the plants. With this combination, we aimed to create a tool to reveal the oxidatively active and inactive phenolics in any given plant-herbivore pair.

We realize that there are several factors affecting the oxidation of plant phenolics in addition to PPOs and other enzymes and alkaline midgut conditions. For example, the presence of Fe^{2+} ions may promote the oxidation of phenolics in the gut. Factors hindering the oxidation are the low concentration of

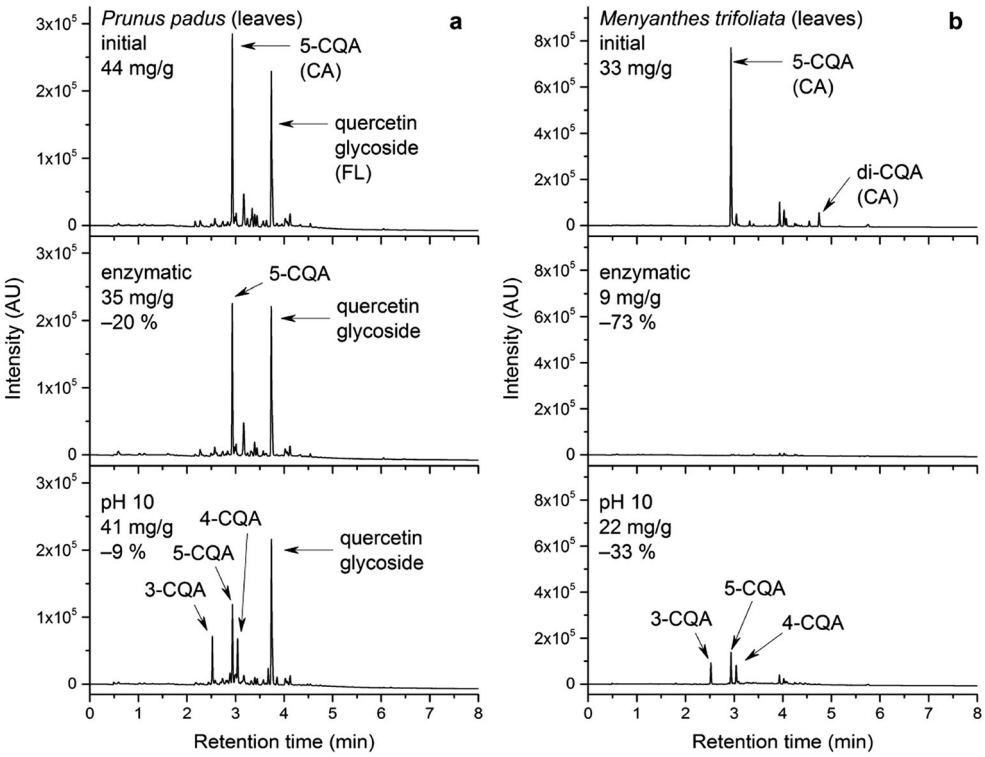

Fig. 4 UHPLC-DAD chromatograms (at 280 nm) of phenolic extracts of *Prunus padus* leaves (**a**) and *Menyanthes trifoliata* leaves (**b**) rich in 5-caffeoylquinic acid. Top, middle and bottom chromatograms show initial phenolic profiles, profiles after the enzymatic oxidation and profiles after the oxidation at pH 10, respectively. The mg/g values show the total phenolic content measured by Folin-Ciocalteu assay and the % values show how much the total phenolic content is altered during the oxidations. CA: cinnamic acid derivative, FL: flavonoid. For other abbreviations and the UV and MS data of the compounds, see Table 2

molecular oxygen especially at the middle and end sections of the midgut, as well as the presence of antioxidants such as ascorbate or glutathione (Johnson and Barbehenn 2000; Barbehenn et al. 2005). The final amount of phenolic compounds that remains intact in the gut and gets excreted is a result of many co-occurring factors that are very difficult to simultaneously mimic in vitro. However, some of the factors that can be easily controlled in vitro can be used to achieve a relatively accurate imitation of the conditions in vivo.

By comparing the isomerization of a selected common plant phenolic, i.e. CQA, in vitro at pH 9, 10 and 11, to the isomer ratios found in the frass of eight herbivore species fed on 5-CQA-rich *Betula* leaf diet, we were able to cluster the lepidopteran and sawfly larvae into four groups depending on their frass ratios of the three CQA isomers (reported in their LC elution order 3-CQA:5-CQA:4-CQA). These ratios varied between ca. 1:3:1 (group 1, *Pristiphora alpestris*, *Priophorus pallipes*, *Arge* sp. and *Nematus viridis*), ca. 1:2:1 (group 2, *Nematus brevivalvis*), ca. 3:4:3 (group 3, *Epirrita autumnata* and *Amauronematus amplus*), and ca. 4:3:3 (group 4, *Agriopis aurantiaria*). The ratios were highly consistent between insect individuals of the same species, suggesting that the steady state of isomerization was reached in the gut. Recently Salminen (2018) showed with radiolabelled pentagalloylglucose that it was excreted by individual 5th instar *E. autumnata* so that maximal excretion was achieved between three and five hours after ingestion. This time should be more than sufficient to reach the steady state of the CQA isomer ratios in insect larvae, since a stable isomer ratio was reached in vitro in <30 mins (pH 11), ~140 mins (pH 10) or in slightly over 140 mins (as extrapolated from pH 9 measurements; Fig. 2). Midgut pH 9 did not seem to be relevant for any of the species, since the 144-min in vitro incubation under pH 9 resulted in ca. 1:7:2 isomeric ratios (Fig. 2d), i.e. an excess amount of remaining 5-CQA (when compared to the faecal ratios from 1:3:1 to 4:3:3). The pH 10 experiment was able to produce ca. 1:3:1 ratio (group 1) in 36 min, ca. 1:2:1 ratio (group 2) in 54 min, ca. 3:4:3 ratio (group 3) in 78 min and ca. 4:3:3 ratio (group 4) in 138 min (Fig. 2e). Already this suggested that alkaline oxidative conditions in the midgut increase as follows: group 1 < group 2 < group 3 < group 4. The midgut pH 11 seemed possible only for the insects of group 4, since the CQAs isomerized so rapidly that the steady state ratio of ca. 4:3:3 was reached in <30 min (Fig. 2f). From the oxidation point of view the experiment at pH 11 was problematic, since the CQAs were oxidized by >95%, i.e. it became more difficult to detect the CQA isomers accurately. For this reason we suggest that this isomerization approach with 5-CQA works best at pH values below pH 11.

To reach a better estimation of the midgut pH of the species in groups 1–4, we did a more detailed isomerization experiment over the pH range from pH 9.0 to pH 11.0. This experiment yielded the following pH estimates for the insect midguts: pH 9.4 for group 1, pH 9.6 for group 2, pH 9.8 for group 3 and pH 10.2–11.0 for group 4 (Fig. S2). All the other isomer ratios obtained in vivo matched very well with those obtained at the specific pH in vitro, but for group 4 the 4:3:3 ratio could be obtained quite accurately at the pH 10.2 and 11.0, and less accurately in between these pH's. However, the more detailed

inspection of the isomer ratios in *A. aurantiaria* frass showed it to be 38:33:30 that corresponds best to pH 10.1 in Fig. S2. The pH differences between the species may look small, but the 0.4 pH unit difference (2.5-fold difference in proton concentration) resulted in ca. 48% decrease in the summed concentration of the CQA isomers during the in vitro experiment. Moreover, Tuominen and Sundman (2013) showed that the most dramatic change in the oxidation of hydrolysable tannins takes place between pH 9 and pH 10. Thus even a small change of pH at this area may result into a significant difference in the oxidation of polyphenols in the midgut. Other considerations such as the levels of molecular oxygen and antioxidants present in the gut could also contribute to the reactions possible in an insect species.

These results give two options for those interested in doing the alkaline oxidative activity measurements under as correct oxidative conditions as possible. One can either do the oxidations at the specific pH obtained for the species, or one can do all the oxidations at the same pH, but alter the time used for the oxidation species by species. For instance, the pH 10 oxidation could be conducted for 36 min (group 1), 54 min (group 2), 78 min (group 3) or 138 min (group 4) to obtain the same CQA isomer ratios as was achieved at the steady state of the pH experiments at the pH's 9.4, 9.6, 9.8 and 10.1, respectively. However, we argue that the latter approach may overestimate the oxidative conditions present in the midgut of especially the groups 3 and 4, since such long oxidation times at pH 10 in the presence of molecular oxygen result into more extensive phenolic oxidation than what is witnessed in vivo by the frass chemistry in e.g. Salminen et al. (2004) and Lahtinen et al. (2005). It would be thus better to use the pH estimated by the 5-CQA isomerization test and restrict the oxidation to the maximum of 60 min. However, if the pH and/or the 5-CQA isomerization rate of the target insect species are not known, the original 60 min oxidation method at pH 10 (Salminen and Karonen 2011) offers a good starting point. In these cases with uncertain midgut pH, results interpretation should be done cautiously, since the species could have had significantly less oxidizing gut conditions than assumed. If the alkaline oxidative activity is measured at an unrealistically high pH, then the activity may be significantly overestimated leading to lack of correlation with insect performance (Marsh et al. 2017).

To complement our alkaline oxidative activity methods, we showed that our new method for the enzymatic oxidative activity followed by UHPLC-DAD-MS analysis is able to use a single source tissue to reveal the phenolic compounds that are potentially oxidized by plant in situ enzymes. Incubating the tissue samples for 2 h at 30 °C was a generally suitable incubation condition for the enzymatic oxidation step for differentiating easily oxidized compounds from more stable compounds. If complete enzymatic oxidation is sought, then the incubation time can be increased to three or four hours, but comparing the compound activities in a sample may be less informative if the time is prolonged so that all the phenolics are oxidized during the incubation. With very active samples the incubation time can be shortened to e.g. one hour, since that was enough to oxidize e.g. monogalloyl glucose and 5-CQA in *B. pubescens* leaves by more than 70%. It is very difficult to know the exact time that plant enzymes are active and able to oxidize foliar phenolics once the tissue has been chewed by an herbivore species. In our method, the main point is compound-to-compound or plant tissue-to-plant tissue comparison, not the exact simulation of the biological system. However, our combination of enzymatic and alkaline oxidation methods would allow the simultaneous optimization of the oxidation times for both oxidation types so that the phenolic content remaining after the two oxidation steps would mimic as accurately as possible the phenolic contents measured from the frass. Vihakas et al. (2015) showed that for some herbivore species the pH 10 oxidation method alone is able to mimic the changes in phenolic chemistry that are observed between leaf and frass analyses. It is possible that even better mimic would be achieved if the frozen leaves (samples B) were first oxidized enzymatically for an optimized time X min and then the oxidation continued with the aqueous extract B for an optimized time Y min at the pH Z estimated by the isomerization method with 5-CQA. After these herbivore species-specific X + Y + Z optimizations one could end up with an in vitro method that could reveal quite accurately the combined in vivo oxidative activity for the individual phenolics of the plant sample.

It is worthwhile to note that the enzymatic oxidation method relies on the cells being damaged by crystallizing water during the freezing step, and thus it may not work well with plant samples that are very fibrous or have a relatively low water content. In these specific cases, the cell walls may remain intact during the freezing period, preventing extracellular enzymes such as laccase from entering the cell and limiting oxidation of phenolics in the sample. In theory, such samples could be frozen with liquid nitrogen prior the oxidation step, but we did not test this approach.

The main aim of the combination of alkaline and enzymatic oxidative activity methods is to find out whether phenolic oxidation is favored by (1) the combined phenolic and enzymatic content of a plant species or organ being studied, and/or (2) the combined phenolic content and the estimated midgut pH of the herbivore. The methods first reveal the individual compounds that exhibit significant changes in their peak areas between non-oxidized and oxidized samples. Small (less than 10%) changes in the peak areas obtained with the enzymatic oxidative activity method are not likely to be meaningful, since our approach did not use exactly the same plant material for sample A (the initial plant sample) and B (the enzymatically oxidized sample). We tried to maximize the homogeneity of the samples A and B by sampling multiple pairs of two close-growing leaves per plant individual, but it is still remains

possible that the two close-growing leaves were chemically slightly different. We did not use an alternative method for creating samples A and B, e.g., by cutting single leaves in two from the middle of the main vein, because cutting the leaf would break the plant cells at the incision line, enabling the enzymatic oxidation of phenolics and thereby altering the phenolic composition and content of the sample. These problems do not interfere with the alkaline oxidative activity method, since it analyses extracts made from the same sample A before and after alkaline oxidation (marked as $A_{NON\text{-}OX}$ and $A_{ALK\text{-}OX}$ in the Materials and Methods section).

Once the phenolic compounds with enzymatic or alkaline oxidative activity have been detected, they can be characterized by UV and mass spectral information (Moilanen et al. 2013), or by the polyphenol-group specific MS/MS tools reported by Engström et al. (2014, 2015). The latter methods show semi-automatically if the active compound belongs to any of the eight main polyphenol groups: gallic acid derivatives, ellagitannins, procyanidins, prodelphinidins, kaempferol derivatives, quercetin derivatives, myricetin derivatives or quinic acid derivatives. The quantitative UHPLC analysis before and after the enzymatic or alkaline oxidation can be used to monitor the differences in the compound-specific enzymatic and alkaline oxidative activities between hundreds of samples. The active compounds are most selectively quantified by compound-specific UHPLC-MS/MS methods (Baert et al. 2015), but if they are chromatographically not overlapped by other compounds, can they be quantified by UHPLC-DAD as well. In fact, we always recommend quantitation by diode array over MS detection, when the compounds of interest are well separated during the LC separation. Unfortunately, the peak purities cannot be known without simultaneous MS analysis, since UV detection cannot reveal the overlapping compounds like MS detection can.

We believe that our enzymatic oxidative activity method offers biologically more relevant information than by separately measuring the enzyme activities of these same samples without knowing if the activities would actually result in the oxidation of any phenolics present in the sample, or by using commercial enzymes to see which of the phenolics in the sample are oxidized without knowing if such enzymes are present in the sample. Plant tissues contain a variety of oxidases, including mono- and diphenolases, and commercial enzyme mixtures fail to accurately represent the enzymatic contents of a studied plant. To our knowledge, the only commercially available PPO is tyrosinase, a monophenolase enzyme purified from mushrooms. The difference between mono- and diphenolase enzymes is that while both can oxidize *o*-diphenols to *o*-quinones, only monophenolase enzymes are capable of oxidizing monophenols to *o*-diphenols and further to *o*-quinones (Lerch 1995; Yoruk and Marshall 2003; Fig. 1). In addition, the ratio of mono- and diphenolase activities varies between plant species: diphenolase activity can be 10 to 40 times higher than monophenolase activity, if monophenolase activity is present at all (Yoruk and Marshall 2003). Thus, commercial tyrosinase may grossly overestimate the enzymatic oxidative activity of a studied plant species if the plant produces relatively large amounts of monophenolic compounds, but contains little to no monophenolase-active PPO. Furthermore, traditional methods for the enzymatic activity do not take the possible presence of antioxidants into account.

It has been suggested that quinic acid esters of caffeic and coumaric acids together with flavonoid glycosides are the most common groups of low molecular weight phenolics present in plants (Herrmann and Nagel 1989). Therefore, CQAs and CoQAs are suitable model compounds for finding out what type of enzymatic activity is found in plant samples: CQA, being an *o*-diphenol, can be oxidized by PPO and laccase enzymes, while CoQA, a monophenol, requires monophenolase-active PPO in order to be oxidized. Assessing the level of oxidation of 5-CQA offers a simple way to establish potential differences in the enzymatic activity of two plant species or tissue types. This was demonstrated with the 5-CQA rich-plants *P. padus* and *M. trifoliata*; the 5-CQA was oxidized to a different extent in each plant because of different enzyme distribution. The choice of PPO-active compound is not limited to CQAs: any suitable compound can be used as long as the compound is found in the plant samples being compared, as seen in *Epilobium* species which are rich in oenothein B.

Laboratories equipped with only a UV spectrophotometer or a microplate reader can use the Folin-Ciocalteu assay to study how the alkaline and enzymatic oxidation affects the total phenolic content of plant extracts. Based on Vihakas et al. (2014) and our study, a decline of over 20% in total phenolics as determined by Folin-Ciocalteu assay is often supported by a very clear decrease in the peak areas in UHPLC-DAD chromatograms. With lower levels of oxidation, the results of the UHPLC measurements seem to deviate from the Folin-Ciocalteu assay. For instance, the total phenolics of *A. petiolata* and *P. sylvestris* declined by 14 and 18% respectively after the enzymatic oxidation, yet their UV chromatograms remained unchanged. This implies that the total phenolic change was due to phenolics or other oxidizing compounds not detectable by UHPLC, or more likely due to the non-phenolic oxidizable compounds witnessed by the less specific Folin-Ciocalteu assay.

It is possible that during the enzymatic or alkaline oxidation, phenolic compounds are modified in other ways than being oxidized. For example, some of the *Geranium* samples showed an increase of 14% and 16% (Fig. 3a, c) of total phenolics due to the enzymatic oxidation. In here the most probable explanation is the natural variation across different samples (in this case, A and B). Another pattern was noted for the GT of *Acer* and *Paeonia* samples, with peaks in the

UHPLC chromatogram significantly decreasing during the alkaline oxidation and no new peaks emerging at 190–500 nm. Meanwhile the results of the total phenolic assay had an insignificant variation from −8% to +15%. Presumably GT oxidation results in the formation of oxidation products or even adducts that either have decreased UV absorbance or are not eluted during the 8-min UHPLC gradient we used, but remain efficient or even better than the original GTs in their ability to reduce the Folin-Ciocalteu reagent. We know that hydrolysable tannins may also be hydrolyzed under alkaline conditions, but the GT oxidations did not reveal the typical hydrolysis products of gallic acid derivatives, i.e. gallic acid and ellagic acid (Tuominen and Sundman 2013; Salminen 2014).

In conclusion, the new enzymatic oxidation method can provide additional data on the ease of oxidation of total and individual phenolic compounds by species-specific enzymes. In addition to finding the most enzymatically active phenolics, the assay may prove useful for finding out how much the foliar enzyme activity relevant for phenolic oxidation varies within a plant species due to different developmental, environmental or stress factors, such as UV radiation or herbivory. When used with the alkaline oxidation method the analyses have the potential to reveal the combined oxidative activities of all plant species and tissue types. Finally, if the alkaline oxidation method is used under oxidative alkaline conditions that mimic the conditions in an insect of interestit is possible to obtain data that provide deeper insight into specific plant-insect interactions.

Acknowledgements Vladimir Ossipov is acknowledged for the valuable scientific discussions about this topic. The anonymous referees and journal editors are acknowledged for their assistance with the earlier versions of the manuscript. Anne Koivuniemi and Marica Engström are acknowledged for collecting the plant samples, and Suvi Vanhakylä for species identification. The study was funded by Kone Foundation (J-P S) and the Academy of Finland (grant 258992 to J-P S). Purchase of the UHPLC-DAD-MS system was made possible by a Strategic Research Grant of the University of Turku (Ecological Interactions).

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