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# Rapid estimation of the oxidative activities of individual phenolics in crude plant extracts

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ABSTRACT

Previous studies of purified phenolic compounds have revealed that some phenolics, especially ellagitannins, can autoxidise under alkaline conditions, which predominate in the midgut of lepidopteran larvae. To facilitate screening for the pro-oxidant activities of all types of phenolic compounds from crude plant extracts, we developed a method that combined our recent spectrophotometric bioactivity method with an additional chromatographic step via UPLC–DAD–MS. This method allowed us to estimate the total pro-oxidant capacities of crude extracts from 12 plant species and to identify the individual phenolic compounds that were responsible for the detected activities. It was found that the pro-oxidant capacities of the plant species (i.e., the concentrations of the easily-oxidised phenolics) varied from 0 to 57 mg/g dry wt, representing from 0% to 46% of the total phenolics from different species. UPLC–DAD–MS analysis revealed that most flavonol and flavone glycosides were only slightly affected by alkaline conditions, thus indicating their low pro-oxidant activity. Interestingly, myricetin-type compounds differed from the other flavonoids, as their concentrations decreased strongly due to alkaline incubation. The same effect was detected for hydrolysable tannins and prodelphinidins, suggesting that a pyrogallol sub-structure could be a key structural component that partially explains their easy oxidation at high pH. Other types of phenolic compounds, such as hydroxycinnamic acids, were relatively active, as well. These findings demonstrate that this method displays the potential to identify most of the active and inactive pro-oxidant phenolic compounds in various plant species.



## 1. Introduction

Plants produce a wide variety of secondary compounds in their various tissues. One of the many functions of these secondary compounds is defence against herbivores. In some cases, the original secondary compound may require chemical activation or modification to perform its intended activity against herbivores. These compounds include cyanogenic glucosides (Morant et al., 2008; Zagrobelny et al., 2004), glucosinolates (Morant et al., 2008), iridoid glycosides (Pankoke et al., 2012), and some phenolic compounds (Klocke et al., 1986; Salminen and Karonen, 2011; Summers and Felton, 1994). Phenolic compounds are a diverse group of secondary plant compounds, some of which may be oxidatively activated to function against herbivores (Appel, 1993). Phenolics can be oxidised enzymatically or by autoxidation to produce quinones and other oxidation products that can harm the growth of herbivores (Appel, 1993; Felton et al., 1989; Summers and Felton, 1994). Autoxidation has been reported for many classes of phenolic compounds, such as flavonoids (Cao et al., 1997) and caffeic acids (Cilliers and Singleton, 1989, 1991). The autoxidation of phenolics is promoted by many factors, such as the alkaline conditions prevalent in the gut of many insect herbivores (Appel, 1993). For example, the midgut of lepidopteran larvae can be highly basic, with a pH as high as 10–12 (Dow, 1984; Gringorten et al., 1993).

Previous studies have examined the autoxidation of phenolic compounds by measuring semiquinone radicals that are generated from phenolics in the gut of lepidopteran larvae (Barbehenn et al., 2003, 2005, 2008). The oxidation of phenolics was also studied via *in vitro* experiments that mimicked the alkaline conditions prevalent in the midgut of lepidopterans (Barbehenn et al., 2006; Moilanen and Salminen, 2008; Tuominen, 2013; Tuominen and Sundman, 2013). The studies described above demonstrated that different classes of phenolics or individual phenolic compounds have variable abilities to function as pro-oxidants in the caterpillar gut. Salminen and Karonen (2011) presented a new method that enabled the comparison of pro-oxidant capacities of different plant

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samples. Their method is based on several analytical steps. First, the total phenolics in the plant samples are measured via a modified Folin–Ciocalteu assay. Next, the samples are incubated in an alkaline buffer at pH 10, which oxidises some of the phenolics in the plant samples. The oxidised samples are then measured via the modified Folin–Ciocalteu assay, and the results are compared to those of the non-oxidised samples. The results reveal the quantity of the total phenolics that was oxidised during the incubation. Their method estimates the pro-oxidant capacity of a given plant sample, and it can be used to compare the pro-oxidant capacities of different plant species. This method, however, is limited in that it does not identify which specific phenolic compounds are responsible for the detected oxidative capacities of the plant species. For instance, different samples could produce similar pro-oxidant capacities, even though the samples contain distinct compositions of phenolic compounds.

Based on these limitations, we further developed the pro-oxidant method of Salminen and Karonen (2011). We combined the conventional spectrophotometric methods with ultra-high performance liquid chromatography coupled to diode array detection and mass spectrometry (UPLC–DAD–MS) to identify the individual phenolic compounds that are oxidised under alkaline conditions. To do so, we analysed both the initial and oxidised plant extracts from 12 selected plant species via UPLC–DAD–MS. These analyses revealed which types of phenolics were responsible for the low or high pro-oxidant activities of each tested plant species. Our results emphasise the capacity of the improved methodology to elucidate the pro-oxidant defence of various plant species. Moreover, these analyses were performed directly from crude plant extracts, which means that the time-consuming purification of secondary compounds performed in earlier studies (Barbehenn et al., 2006; Moilanen and Salminen, 2008; Tuominen and Sundman, 2013) is unnecessary. Our new method could facilitate the detection of the pro-oxidant capacities of multiple plant species and the identification of the types of phenolic compounds responsible for the detected activities. Importantly, this method will help us to understand the types of phenolics that appear to perform anti-herbivore activities in different plant species.

## 2. Results

Different plant species contained varying concentrations of total phenolics (from 20 mg/g to 125 mg/g dry wt). The oxidation measurements revealed that approximately 0 to 57 mg/g dry wt of the initial total phenolics were lost at pH 10 (Figs. 1 and 2). In some cases, the oxidised proportions of the total phenolics represented only 0–5% of the initial total phenolics, but in other cases, up to 46% of the initial total phenolics were lost due to oxidation. We separated the 12 examined plant species in three categories according to their pro-oxidant capacities: plants with weak, moderate or high oxidative capacity.

### 2.1. Plants with weak oxidative capacity

In the weak oxidative capacity category, we placed the plant species whose total phenolic concentrations changed only slightly after incubation at pH 10. This result suggested that the composition of the phenolic compounds remained fairly constant during the incubation in alkaline conditions. UPLC–DAD chromatograms of the initial and oxidised samples are presented in Fig. 1A–C. The chromatographic profiles of the initial and oxidised samples were very similar, and the intensities of the individual peaks typically remained at the same level in both samples. A negligible amount of total phenolics in *Celtis durandii* Engl. (hackberries) were oxidised under alkaline conditions, and a similar trend was found in the UPLC data: two predominant compounds in the leaves were unaffected by alkaline conditions (Fig. 1A). These two compounds were classified as apigenin diglycosides with *C*-glycosyls based on their UV spectra and MS/MS fragmentation patterns (Table 1; Ferreres et al., 2007). Similar types of compounds were previously found in the leaves of *Celtis* plants (Kaltenhauser et al., 2010; Spitaler et al., 2009).

Only 5% of the total phenolics in *Aesculus hippocastanum* L. (horse chestnut) were oxidised at pH 10 (Fig. 1B). The chromatographic profiles of the initial and oxidised samples were similar, but the intensities of some individual peaks were lower in the chromatogram of the oxidised sample. A hump in the middle of the chromatogram was interpreted as oligo- to polymeric proanthocyanidins (Karonen et al., 2004), and the calculated mass spectra from the hump area in the total ion chromatogram revealed representative peaks of A-type procyanidin oligomers (Table 1; Morimoto et al., 1987). The procyanidin hump was still present after the incubation in alkaline conditions, and the calculated mass spectra of this area did not change, which suggested that the procyanidins in the sample were resistant to oxidation at pH 10. A similar hump containing procyanidins was detected in the chromatograms of *Picea abies* (L.) Karst (Norway spruce) needles (Fig. 1C), and the chromatograms also contained peaks of catechin and a kaempferol monoglycoside (Table 1; Slimestad and Hostettmann, 1996). The analysis of the total phenolics suggested that 13% of the total phenolics of *P. abies* were lost during the incubation. Alkaline conditions caused a slight decrease in the size of the proanthocyanidin hump, even though the calculated mass spectra of the hump area still contained representative peaks of oligomeric procyanidins.

### 2.2. Plants with moderate oxidative capacity

The moderate oxidative capacity category of plants contained species whose phenolic compounds appeared to be slightly more prone to autoxidation under alkaline conditions than the plant species in the weak category (Fig. 1D–F). For example, 22% of the total phenolics in the leaves of *Prunus africana* (Hook.f.) Kalkman (African cherry) were oxidised at pH 10. The chromatographic profile of the *P. africana* extract changed during the incubation: the height of the peak corresponding to monocaffeoylquinic acid (Nakatani et al., 2000; Ossipov et al., 1996) decreased substantially, and two new peaks appeared on each side of the original peak (Fig. 1D). The new compounds displayed UV and mass spectra similar to those of the original compound (Table 1), which suggested that they were all isomers of monocaffeoylquinic acid. The isomerisation of monocaffeoylquinic acid was also detected in *Achillea ptarmica* L. (sneezewort; Fig. 1E). In addition to this compound, *A. ptarmica* contained a dicaffeoylquinic acid, and the height of the corresponding peak decreased substantially, but it did not form isomers under the alkaline conditions. A flavone (an apigenin glucuronide) in the same species remained unaffected by alkaline conditions.

The leaves of *Oxyanthus speciosus* DC. (whipstick-loquat) displayed a medium level of pro-oxidant capacity based on the total phenolics assay, and the predominant compound based on the UPLC chromatogram was unaffected by the incubation (Fig. 1F). This compound was identified as a cyanogenic glycoside, and these types of compounds have previously been detected in the *Oxyanthus* genus (Rockenbach et al., 1992). The leaves also contained several isomers of mono- and di-caffeoylquinic acids, and one of the isomers of monocaffeoylquinic acid partially co-eluted with the cyanogenic glycoside (as detected on the extracted ion chromatogram of monocaffeoylquinic acid). The heights of the peaks of monocaffeoylquinic acids decreased considerably after the incubation, and the peaks of the dicaffeoylquinic acids disappeared