JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
REVIEW

J. Agric. Food Chem. 2010, 58, 1371–1382 1371
DOI:10.1021/jf9024294

Oxidative Phenols in Forage Crops Containing Polyphenol Oxidase Enzymes

IFAT PARVEEN,*[,†] MICHAEL D. THREADGILL,[‡] JON M. MOORBY,[†] AND ANA WINTERS[†]

[†]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan, Aberystwyth SY23 3EB, U.K., and [‡]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

Polyphenol oxidases (PPOs) are copper-containing enzymes that catalyze oxidation of endogenous monophenols to *ortho*-dihydroxyaryl compounds and of *ortho*-dihydroxyaryl compounds to *ortho*-quinones. Subsequent nucleophilic addition reactions of phenols, amino acids, and proteins with the electrophilic *ortho*-quinones form brown-, black-, or red-colored secondary products associated with the undesired discolouration of fruit and vegetables. Several important forage plants also exhibit significant PPO activity, and a link with improved efficiency of ruminant production has been established. In ruminant animals, extensive degradation of forage proteins, following consumption, can result in high rates of excretion of nitrogen, which contributes to point-source and diffuse pollution. Reaction of quinones with forage proteins leads to the formation of protein−phenol complexes that are resistant to proteolytic activity during ensilage and during rumen fermentation. Thus, PPO in red clover (*Trifolium pratense*) has been shown to improve protein utilization by ruminants. While PPO activity has been demonstrated in a number of forage crops, little work has been carried out to identify substrates of PPO, knowledge of which would be beneficial for characterizing this trait in these forages. In general, a wide range of 1,2-dihydroxyarenes can serve as PPO substrates because these are readily oxidized because of the ortho positioning of the hydroxy groups. Naturally occurring phenols isolated from forage crops with PPO activity are reviewed. A large number of phenols, which may be directly or indirectly oxidized as a consequence of PPO activity, have been identified in several forage grass, legume, cereal, and brassica species; these include hydroxybenzoic acids, hydroxycinnamates, and flavonoids. In conclusion, a number of compounds are known or postulated to enable PPO activity in important PPO-expressing forage crops. Targeting the matching of these compounds with PPO activity would be a useful plant breeding approach to improve the utilization of feed nitrogen by ruminant livestock and help reduce the environmental impact of livestock agriculture in temperate countries.

KEYWORDS: Polyphenol oxidase; PPO substrates; browning reactions; quinones; forage plants; ruminants

INTRODUCTION

Polyphenol oxidases (PPOs) are a group of enzymes that are widely distributed in forage crops and fruit and vegetable plants (*1−4*). Numerous studies have been devoted to PPOs because they are responsible for browning of fresh fruit and vegetable products, following bruising, cutting, or other treatments causing cell damage (*1*, *5*). Moreover, enzymatic (PPO) and nonenzymatic oxidation of phenolic compounds contribute to quality loss in foods and beverages due to postharvest deterioration reactions which result in shortened shelf life and consequent commercial losses (*5−7*). The importance of PPO activity in forage crops for ruminant animal consumption has also received attention because it has been associated with reductions in proteolysis and lipolysis in forage (e.g., *Trifolium pratense* L.; red clover) when consumed fresh (*8*) or when ensiled (*9*). Reduced proteolysis of forage protein in the silo and in the rumen would lead to a slower uptake of nonprotein nitrogen (NPN) from the rumen, much of which is excreted and is ultimately lost as a potential pollutant on livestock farms.

*Corresponding author. Tel: +44 (0) 1970 823207. Fax: +44 (0) 1970 823245. E-mail: ifp@aber.ac.uk.

POLYPHENOL OXIDASE ACTIVITY AND RUMINANT NUTRITION

PPOs (E.C.1.10.3.1), also termed catechol oxidases, catecholases, diphenol oxidases, *ortho*-diphenolases, phenolases, and tyrosinases, are enzymes containing a dinuclear copper center that inserts molecular oxygen ortho to a hydroxy group in an aromatic ring (monophenol oxidase activity) (*2*, *4−7*, *10*, *11*). This reaction is followed by the two-electron oxidation of the *ortho*-dihydroxyphenol compounds to *ortho*-quinones with a concomitant two-electron reduction of oxygen yielding one molecule of water (*10*). Quinones are powerful oxidants and strong electrophiles that undergo different reaction pathways,

© 2010 American Chemical Society

resulting in the formation of intensely colored secondary products. Secondary reactions involving quinones include (i) oxidation of substrates with lower redox potential, which can be converted into other quinonoids by coupled oxidation, (ii) nucleophilic addition reactions with amino acids and proteins, and (iii) condensation and polymerization (*1*, *7*). The formation of brown-, black- or red-colored polymers is associated with the undesired discolouration of fruit and vegetables (*5*). Several factors determine the rate of enzymatic browning, including the concentrations of active PPO and endogenous phenolic compounds, pH, temperature, and the availability of molecular oxygen (*5*, *13*, *14*). In ruminants, extensive degradation of proteins in the rumen can result in increased absorption of ammonia by the animal, which is largely excreted in the urine (*15*), particularly in the absence of an adequate energy supply for incorporation into microbial protein. This can lead to poor retention of nitrogen and to increased risk of pollution. Reaction of quinones with protein inhibits degradation of the protein, and protein−phenol adducts are formed (*3*, *9*, *16*). PPO in red clover has been shown to improve utilization of proteins by ruminants (*17*). Following harvesting, red clover tends to brown almost immediately (*18*, *19*). In agricultural forages, the loss of protein by proteolysis during ensilage of forage legumes is a common and major problem (*12*, *17*−*19*). It has been speculated that the rate of proteolysis is greater in forage legumes than in forage grasses (*19*). Previous studies have shown that, when ensiled, red clover experiences significantly less degradation of protein to NPN in comparison to that of forages such as alfalfa (*Medicago sativa* L.) (*19*). More recent studies have demonstrated lower levels of breakdown of protein during ensilage in red clover and, moreover, high levels of the enzyme PPO were thought to be responsible for this diminution (*12*, *17*). In a recent study, PPO was shown to decrease the hydrolysis of peptides in crude plant extracts (*17*). The authors demonstrated a 5-fold increase in the extent of proteolysis in leaves of red clover when native *ortho*-diphenols were removed, which returned to initial levels when an exogenous *ortho*-diphenol was added. This suggested that the action of PPO enzyme on *ortho*-diphenol substrates was crucial for postharvest inhibition of proteolysis (*17*). Furthermore, it was shown that the endogenous PPO *ortho*-diphenol substrates disappeared, concordant with the inhibition of proteolysis.

CHEMICAL PROPERTIES AND REACTIONS OF PLANT PHENOLS

Plant phenols are secondary metabolites that are synthesized via complex nonreversible biosynthetic pathways (i.e., shikimate and phenylpropanoid pathways) that are not present in animals and often offer the plant protection against herbivory, microbial pathogen invasion, invertebrate pests, and environmental stresses. It is therefore not unexpected that such compounds may confer antinutritional properties. Plant phenols have multifunctional properties, for example, they are reducing agents, hydrogen-donating antioxidants, and quenchers of singlet oxygen. It is these properties that account for the role of phenols in browning reactions. The presence of the phenolic hydroxy group imparts an acidic nature to phenol molecules and increases the aqueous solubility of these compounds (*20*).

The reactive nature of the hydroxy-substituted cinnamic acids is due to the presence of the acrylic acid group conjugated with the aromatic ring which facilitates two-electron oxidation of the ring hydroxy groups to the corresponding *ortho*-quinone. Interactions occur between the π-electron ring and unpaired electrons on the sp^2-hybridized oxygen atom (*20*). This mesomeric effect results in a decrease of electron density on the oxygen atom which in turn pulls electrons from the hydroxy hydrogen atom with subsequent loss of the hydrogen, leaving a negative charge on the oxygen (*20*). This interaction gives this class of molecules special properties, most notably the ability to generate free radicals, which can be stabilized by ring delocalization, and subsequently modifies radical-mediated oxidation processes (*7*). The oxidation potential of the monocyclic phenolics is highest in the 2,4,5,-trihydroxy compounds and least among the monophenols. Typically, a wide range of 1,2-dihydroxyarenes (*ortho*-dihydroxyphenols) can potentially serve as PPO substrates; these are readily oxidized because of the *ortho* positioning of the hydroxy groups (*5*). The nature of the side chain, number of hydroxy groups, and their position on the benzene ring have a major effect on their oxidation by PPOs (*12*). In addition, these properties will determine the extent to which these compounds maybe indirectly oxidized as a consequence of reactions with PPO products. PPO substrate specificity is dependent on plant species and isoform, and is generally much higher for diphenols than monophenols. Potential PPO substrates must accumulate to sufficient levels to be physiologically significant.

FORAGE PLANT PHENOLS

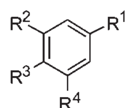
Here, we consider compounds which may be directly or indirectly oxidized as a result of PPO activity. The most probable PPO substrates are the *ortho*-dihydroxyaryl compounds without a high steric environment. Monohydroxyaryl compounds may also be oxidized to *ortho*-dihydroxyaryl compounds; however, as noted above this activity has not been demonstrated with all plant PPOs characterized to date. Enzymes from different plant species generally exhibit distinct substrate specificities. Many PPOs are capable of oxidizing smaller compounds, including hydroxybenzoic acids, hydroxycinnamic acids, and their derivatives. A number of flavonoids have also been identified as the preferred substrate in plant species. Phenols with greater antioxidant properties may also have an important role in browning of forage plant material via secondary reactions with PPO generated quinones.

Hydroxybenzoic Acids. The family of hydroxybenzoic acids or hydroxybenzoates are characterized by the core structure C_6-C_1 (*21*−*23*) (**Figure 1**). In plants, they are formed primarily by the shikimate pathway but can also be formed via the phenylpropanoid pathway (*23*) and are found in high abundance in forage plants. A large number of hydroxybenzoic acids, for example, gallic, *para*-hydroxybenzoic, *para*-hydroxyphenylacetic, and protocatechuic acids, have been identified in kale, sorghum, millet, and oat forages (*24*−*26*). In forage plants, these compounds are also widely distributed as hydroxybenzoic acid glycosides. Naturally occurring PPO substrates include gallic acid and catechol (*11*).

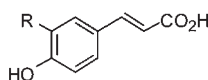
Hydroxycinnamic Acids and Derivatives. The class of hydroxycinnamic acids, otherwise known as phenylpropanoids, is the most widely distributed group of naturally occurring compounds (*21*, *23*), and a large number are recognized PPO substrates. These compounds are characterized by the carbon skeleton C_6-C_3 (**Figures 1** and **2**) (*21*, *23*). In higher plants, they are found in high concentrations and consist of the basic acids (in nature, the phenolic cinnamic acids usually occur in the trans form), known as coumaric and caffeic acids (*21*−*23*, *27*, *28*). The majority of these compounds in forage plants are in the form of esters, the most common being 5-caffeoylquinic acid (5-CQA), also known as chlorogenic acid (**Figure 2**). Where caffeoyl esters are present in relatively high abundance, they may be

considered the most likely endogenous PPO substrates in the forage. Recently, a large number of cinnamoyl conjugates (**Figure 3**) have been reported in cocksfoot (*Dactylis glomerata* L.) (*29*). Cinnamic acids can also undergo esterification with glycerol, saturated long chain alcohols, fatty diols, and fatty hydroxy acids (*26*, *30*−*34*) (**Figure 4**).

Besides forming hydroxycinnamate esters, plants are also able to synthesize, albeit to a lesser degree, cinnamic acid amides (cinnamamides). For example, *para*-coumaroylagmatine, in red clover, is a common type of hydroxycinnamic acid amide, while amides with aromatic amino acids, such as caffeoyl hydroxyphenylalanine (caffeoyl DOPA) (also referred to as clovamide) and *para*-coumaroyl DOPA (also found in red clover) are less widely distributed in the plant kingdom (*35*, *36*) (**Figure 5**). Another subset of hydroxycinnamic acid amides is avenanthramides (also termed *N*-cinnamoylanthranilates), which consist of an anthranilic acid or hydroxyanthranilic acid connected to a hydroxycinnamic acid through the amide bond (*26*) (**Figure 6**). Caffeoyl DOPA is a recognized substrate of red clover PPO (*12*).

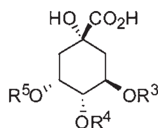


1: gallic acid $R^1 = CO_2H$; $R^2 = R^3 = R^4 = OH$
2: *para*-hydroxybenzoic acid $R^1 = CO_2H$; $R^2 = R^4 = H$; $R^3 = OH$
3: *para*-hydroxyphenylacetic acid $R^1 = CH_2CO_2H$; $R^2 = R^4 = H$; $R^3 = OH$
4: protocatechuic acid $R^1 = CO_2H$; $R^2 = R^3 = OH$; $R^4 = H$



5: coumaric acid R =H
6: caffeic acid R = OH

Figure 1. Structures of hydroxybenzoic and hydroxycinnamic acids found in forage crops.



7: 3-*O*-caffeoylquinic acid $R^4 = R^5 = H$; $R^3 =$
8: 4-*O*-caffeoylquinic acid $R^3 = R^5 = H$; $R^4 =$
9: 5-*O*-caffeoylquinic acid $R^3 = R^4 = H$; $R^5 =$
10: 5-*O*-para-coumaroylquinic acid $R^3 = R^4 = H$; $R^5 =$

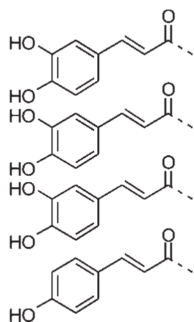


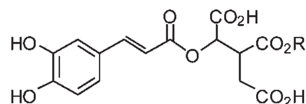
Figure 2. Structures of hydroxycinnamate quinic esters found in forage crops.

Flavonoids. The flavonoids are based on the C_6-C_3-C_6 skeleton and consist of six major subgroups, flavones, flavonols, flavonones, isoflavones, flavan-3-ols, and anthocyanidins (*22*, *23*, *37*), depending on oxidation level and mode of cyclization. A number of these compounds have been identified as PPO substrates. The family of flavonoids is well documented, and the subgroups will only be discussed briefly in this review. **Figure 7** represents the principal structures from the flavonoid group distributed in forage plants (*22*, *23*, *28*). Flavonoid compounds are characterized by two phenyl cycles, designated A- and B-rings, linked by a heterocycle, C ring (**Figure 7**). The B-ring in contrast with the A-ring has been established as the most important site for H-transfer. The oxidation potential of flavonoid compounds is largely determined by the hydroxylation pattern on the B-ring, with a catechol moiety (preferably at the 3′- and 4′-positions) conferring a high degree of stability to the flavonoid phenoxy radical. This is increased by a C2-C3 double bond and a hydroxyl at position C3 (*38*, *39*).

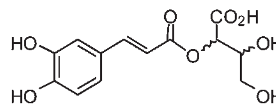
The family of flavonols or 3-hydroxyflavans is widely distributed in the plant kingdom (*22*, *23*). The most common flavonols in forage plants are myricetin, quercetin, and kaempferol; of these, quercetin is reported to be the most widely distributed (*22*, *23*, *37*) and has a wide range of biological activities in mammals (*40*) (**Figure 8**). Generally, the flavonols are found as *O*-glycosides, formed through substitution of a hydroxy group at the 3-position of the C-ring, with substitutions also possible at the 5-, 7-, 4′-, 3′-, and 5′-positions (*23*). The common glycosylated structures include the 3-glucosides, 3-galactosides, 3-rhamnosides, and 3-glucuronides. It must be considered that linked glycosides may cause steric hindrance to the PPO enzyme and prevent enzyme−substrate binding.

Flavones are structurally related to flavonols, with the exception of the hydroxy group at position 3 of the C-ring (*22*, *23*). These compounds are reported to be widely dispersed in angiosperms (*23*). Apigenin and luteolin are the most common flavones in the plant kingdom (*22*, *23*) (**Figure 9**). Flavones can undergo many substitution reactions including hydroxylation, methylation, O- and C-alkylation, and glycosylation (*23*).

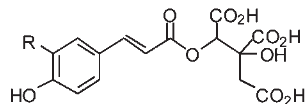
Flavanones are characterized by the presence of an asymmetric chiral carbon at C2 (resulting in two possible optical isomers) and the absence of a double bond in the C-ring (*23*, *41*). In naturally occurring flavanones, the C-ring is attached to the B-ring at C-2 in



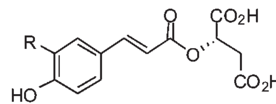
11: 2-*O*-caffeoylisocitric acid R = H
12: 2-*O*-caffeoylisocitric acid 6-methyl ester R = Me



13,**14**: 2-*O*-caffeoylthreonic acid stereoisomers



15: 2-*O*-caffeoylhydroxycitric acid R = OH
16: 2-*O*-coumaroylhydroxycitric acid R = H



17: 2-*O*-coumaroyl-L-malic acid R = H
18: 2-*O*-caffeoyl-L-malic acid R = OH

Figure 3. Structures of hydroxycinnamate esters found in forage crops.

the α-configuration (*23*). Flavanones are formed from flavones by reduction of the double bond in the central C-ring. These types of compounds are highly reactive and readily undergo hydroxylation, glycosylation, and O-methylation reactions. These compounds are less widely dispersed, in comparison to that of the flavones and flavonols. The main flavanone aglycones are naringenin and eriodictyol; these are structurally related to apigenin and luteolin (*23*) (**Figure 9**).

Isoflavones are isomers of the flavones that are generally characterized by attachment of the B-ring at the C-3 position of the central heterocycle (*22*, *23*). In higher plants, a large number of isoflavones have been described, and many have been found exclusively in the leguminous forages red clover and subterranean clover (*Trifolium subterraneum*) (*23*, *42−44*) (**Figure 10**). The most common isoflavone compounds are daidzein, genistein, formononetin, and biochanin A. These compounds are reported to be weakly estrogenic (*45*) and hence are termed phytoestrogens.

Flavan-3-ols or catechins are possibly the most complex subclass of the flavonoids, with structures ranging from the simple monomers (+)-catechin and its optical isomer (−)-epicatechin, to the oligomeric and polymeric proanthocyanidins, also termed condensed tannins (*22*, *23*) (**Figure 11**). Several compounds from this class are recognized PPO substrates in a number of plant species. In the plant kingdom, these compounds are usually distributed as aglycones and not the glycosylated forms (*20*, *22*, *23*). The flavan-3-ols, proanthocyanidins, and flavonones consist of a saturated C-ring system, which make these compounds nonpolar (*23*). In flavan-3-ols, the two asymmetric carbon centers at C-2 and C-3 produce four optical isomers of which (+)-catechin and (−)-epicatechin are most widely distributed in nature. The differences in the configuration of these compounds are thought to influence the compounds' binding properties, which may be important in enzyme−substrate interactions (*23*). There are two types of proanthocyanidins (type-A and type-B), both of which are formed from (+)-catechin and (−)-epicatechin units. In type B proanthocyanidins, oxidative coupling occurs between the C-4 of the heterocycle and the C-6 or C-8 positions of an adjacent unit to produce oligomers and polymers, while type-A proanthocyanidins contain an additional ether bond between C-2 and C-7 (*23*). Both catechin and epicatechin have been demonstrated to be PPO substrates in banana and litchi (or lychee) fruit, respectively, (*46−48*) while procyanidin A2 has been identified as a substrate of PPO in pericarp tissues of litchi fruit (*49*).

Anthocyanidin compounds are highly abundant in the plant kingdom; in fruit and flower tissues, they contribute to red, blue, and purple colors (*20*, *23*, *41*). These compounds protect plants by absorbing light and are susceptible to oxidation because of their oxidizable hydroxy groups in ring B (*20*, *23*). Anthocyanidins have the core structure 2-phenylbenzopyrilium (with the C in the form of a pyrilium cation) or flavium and vary in extent of hydroxylation and methoxylation of the B-ring (*23*). In nature, the most common anthocyanidins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, with cyanidin being the most widely distributed (*20*, *23*). In plant tissues, these compounds do not occur as aglycones; they are usually dispersed as glycosides, which are known as anthocyanins (**Figure 12**). With the exception of apigeninidin and luteolinidin, the anthocyanins carry the sugar residue in the 3-position. It is proposed that part of

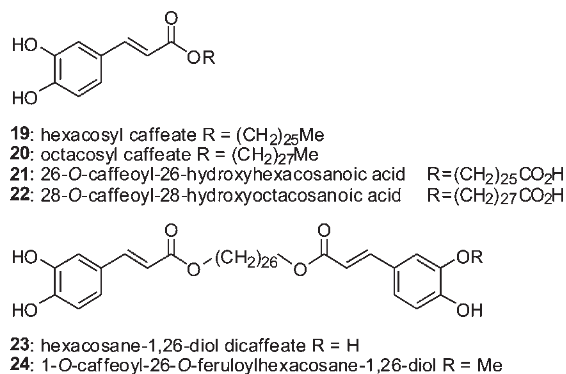


Figure 4. Structures of hydroxycinnamate/caffeoyl fatty acid conjugates (inlcuding saturated long chain alcohols, fatty diols, and fatty dihydroxy acids) found in forage crops.

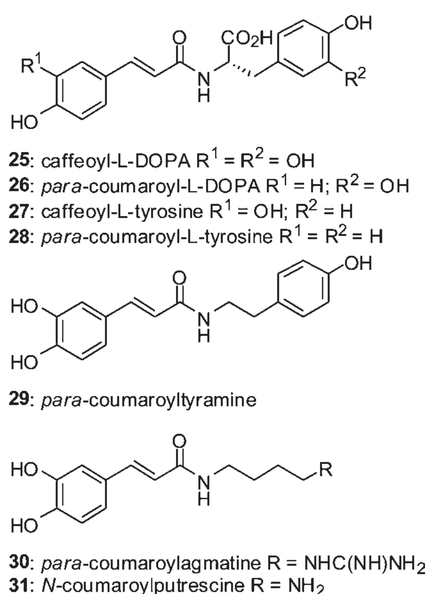


Figure 5. Structures of hydroxycinnamate amides found in forage crops.

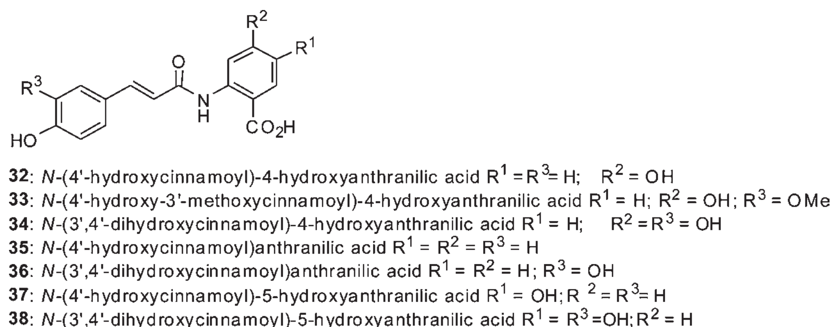


Figure 6. Structures of avenanthramides found in forage crops.

J. Agric. Food Chem., Vol. 58, No. 3, 2010

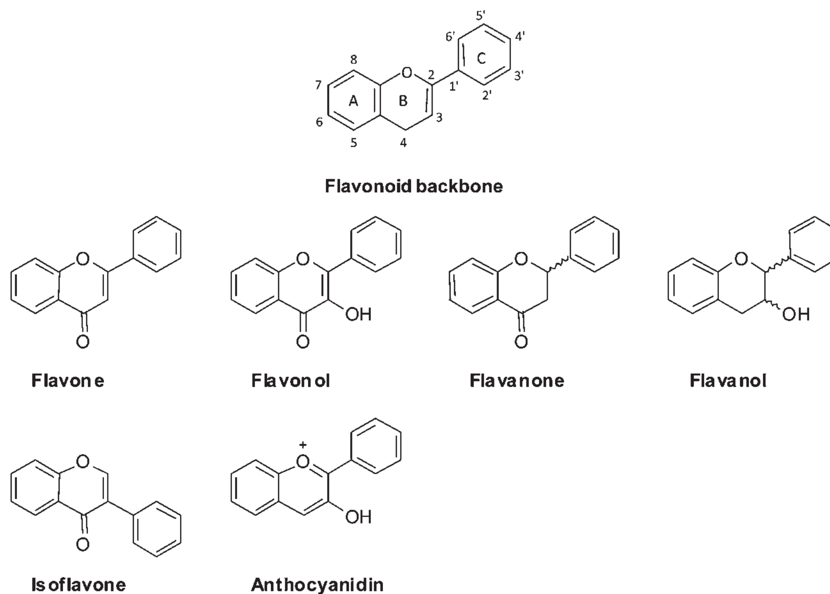


Figure 7. General structures of six major flavonoid subgroups: flavone, flavonol, flavanone, isoflavone, flavanol, and anthocyanidin.

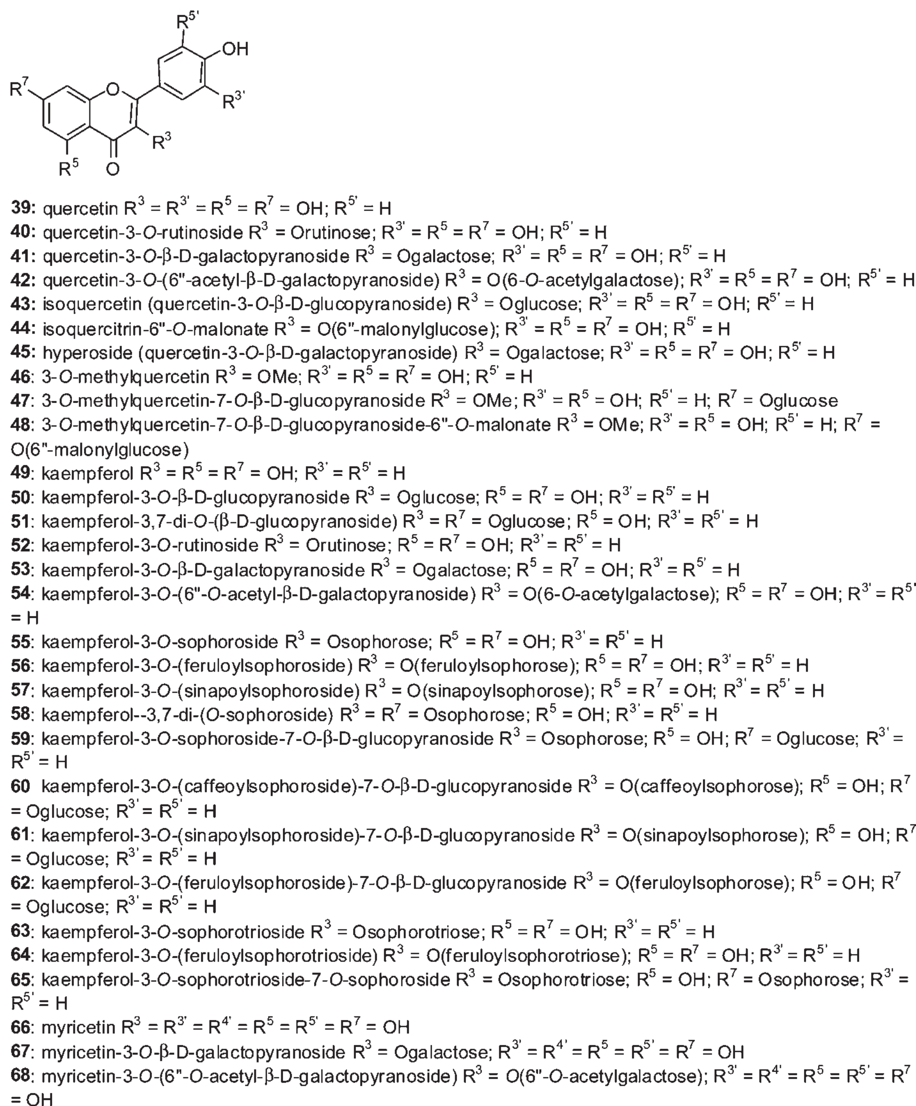


Figure 8. Structures of flavonols found in forage crops.

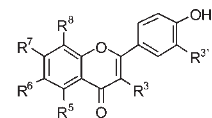
the browning process in litchi fruit involves PPO-catalyzed oxidation of anthocyanidin (*46, 47*).

DISTRIBUTION AND CONCENTRATION OF PHENOLS IN FORAGE PLANTS WITH PPO

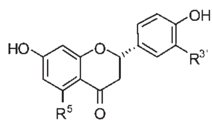
The compositions of the polyphenolic compounds in forage crops vary depending on the species, cultivar and genetics, maturity, climate, and agricultural practices (*7, 20*). Furthermore, factors such as stability, biosynthesis, and degradation will influence the level of these compounds in forage plants. Therefore, the levels and ranges of these compounds may vary considerably. Although the range of natural phenols in forage crops is vast, only those that are potential PPO substrates will be discussed in this review. Of the various classes, the C_6-C_1 (benzoic acids), C_6-C_3-C_6 (flavonoids, isoflavonoids, flavonoid glycosides, and anthocyanins), and $(C_6$-C_3-$C_6)_n$ (condensed tannins or proanthocyanidins) are of major significance in forage plants.

Forage plants reported to have PPO enzymes include grasses, tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratense*), timothy (*Phleum pratense*), perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*), hybrid ryegrass (*Lolium perenne* × *multiflorum*), and cocksfoot (or orchard grass); legumes, red clover and white clover (*Trifolium repens*); cereal grasses, pearl millet (*Pennisetum glaucum*sorghum (*Sorghum bicolour*), wheat (*Triticum spp.*), barley (*Hordeum vulgare*), corn (*Zea mays*), and oat (*Avena sativa*); and brassica forages, kale (*Brassica oleraceae*) and turnip (*Brassica rapa*) (*3, 8, 17, 50−58*).

Forage Grasses. Studies conducted at the Institute of Biological, Environmental and Rural Sciences (IBERS) at Aberystwyth have shown 5-CQA to be the major component in several livestock forage grasses including timothy, meadow fescue, Italian, and perennial and hybrid ryegrasses (*50*), and to be a minor component of cocksfoot (*29*) (**Table 1**). Esterification of caffeic acid can also occur to hydroxy groups at positions 1, 3, and 4 on

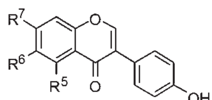


69: luteolin $R^{3'} = R^5 = R^7 = OH$; $R^3 = R^6 = R^8 = H$
70: luteolin-7-*O*-β-D-glucoside $R^{3'} = R^5 = OH$; $R^3 = R^6 = R^8 = H$; R^7 = Oglucose
71: luteolin-7-*O*-rutinoside $R^{3'} = R^5 = OH$; $R^3 = R^6 = R^8 = H$; R^7 = Orutinose
72: apigenin $R^5 = R^7 = OH$; $R^{3'} = R^3 = R^6 = R^8 = H$
73: apigenin-7-*O*-β-D-glucoside $R^5 = OH$; $R^3 = R^{3'} = R^6 = R^8 = H$; R^7 = Oglucose
74: vitexin-2"-rhamnoside $R^5 = R^7 = OH$; $R^3 = R^{3'} = R^6 = H$; R^8 = (2"-*O*-β-L-rhamnosyl)glucose
75: isoswertisin-2"-rhamnoside $R^5 = OH$; $R^7 = OMe$; $R^3 = R^{3'} = R^6 = H$; R^8 = (2"-O-β-L-rhamnosyl)glucose
76: isovitexin-2"-arabinoside $R^5 = R^7 = OH$; $R^3 = R^{3'} = R^8 = H$; R^6 = (2"-*O*-arabinosyl)glucose
77: isoorientin-2"-arabinoside $R^{3'} = R^5 = R^7 = OH$; $R^3 = R^8 = H$; R^6 = (2"-*O*-arabinosyl)glucose



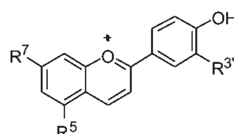
78: naringenin $R^{3'} = H$; $R^5 = OH$
79: eriodictyol $R^{3'} = R^5 = OH$
80: eriodictyol-5-*O*-*b*-D-glucoside $R^{3'} = OH$ R^5 = Oglucose

Figure 9. Structures of flavones and flavanones found in forage grasses.



81: daidzein $R^7 = OH$; $R^5 = R^6 = H$
82: daidzin (daidzein 7-*O*-β-D-glucoside) R^7 = Oglucose; $R^5 = R^6 = H$
83: genistein $R^5 = R^7 = OH$; $R^6 = H$
84: genistein 7-*O*-β-D-glucoside $R^5 = OH$; R^7 = Oglucose; $R^6 = H$
85: genistein 7-*O*-(6"-*O*-malonyl-β-D-glucoside) $R^5 = OH$; R^7 = O(6"-malonylglucose); $R^6 = H$
86: prunetin $R^5 = OH$; $R^7 = OMe$; $R^6 = H$
87: glycitein $R^7 = OH$; $R^6 = OMe$; $R^5 = H$
88: glycitin (glycitein 7-*O*-β-D-glucoside) $R^6 = OMe$; R^7 = Oglucose; $R^5 = H$
89: irilone $R^6,R^7 = OCH_2O$; $R^5 = OH$

Figure 10. Structures of isoflavones found in forage grasses.



96 : apigenindin $R^{3'} = H$; $R^5 = R^7 = OH$
97 : apigenindin 5-*O*-β-D-glucoside $R^{3'} = H$; R^5 = Oglucose; $R^7 = OH$
98 : 5-methoxyapigenindin $R^{3'} = H$; $R^5 = OMe$; $R^7 = OH$
99 : 7-methoxyapigenindin $R^{3'} = H$; $R^5 = OH$; $R^7 = OMe$
100: 7-methoxyapigenindin 5-*O*-β-D-glucoside $R^{3'} = H$; R^5 = Oglucose; $R^7 = OMe$
101: luteolinidin $R^{3'} = R^5 = R^7 = OH$
102: luteolinidin 5-*O*-β-D-glucoside $R^{3'} = R^7 = OH$; R^5 = Oglucose
103: 5-methoxyluteolinidin $R^{3'} = R^7 = OH$; $R^5 = OMe$
104: 5-methoxyluteolinidin 7-*O*-β-D-glucoside $R^{3'} = OH$; $R^5 = OMe$; R^7 = Oglucose
105: 7-methoxyluteolinidin $R^{3'} = R^5 = OH$; $R^7 = OMe$

Figure 12. Structures of anthocyanidins and anthocyanins found in forage crops.

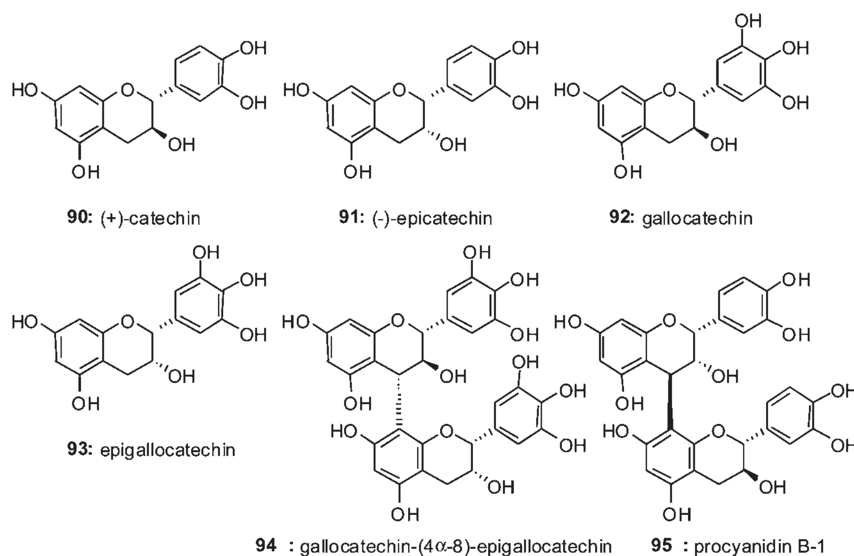


Figure 11. Structures of flavan-3-ols found in forage grasses.

Table 1. Sources of PPO Substrates in Forage Plants

source	PPO substrates
	Forage Grasses
tall fescue (*Festuca arundinacea*)	*hydroxycinnamates*: 5-caffeoylquinic acid
meadow fescue (*Festuca pratense*)	*hydroxycinnamates*: 3-caffeoylquinic acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid
timothy	*hydroxycinnamates*: 4-caffeoylquinic acid; 5-caffeoylquinic acid
perennial ryegrass (*Lolium perenne*)	*hydroxycinnamates*: 3-caffeoylquinic acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid
Italian ryegrass (*Lolium multiflorum*)	*hydroxycinnamates*: 3-caffeoylquinic acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid
hybrid ryegrass (*Lolium perenne x Lolium multiflorum*)	*hydroxycinnamates*: 5-caffeoylquinic acid
cocksfoot (*Dactylis glomerata*)	*hydroxycinnamates*: 3-caffeoylquinic acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid; 2-*O*-caffeoylisocitric acid; 2-*O*-caffeoylisocitric acid 6-methyl ester; 2-*O*-caffeoylthreonic acid; 2-*O*-caffeoylhydroxycitric acid
	Forage Legumes
red clover (*Trifolium pratense*)	*hydroxycinnamates*: 2-*O*-caffeoylmalic acid; caffeoyl DOPA; *para*-coumaroyl DOPA; caffeoyltyrosine; *para*-coumaroyltyrosine; *flavonols*: kaempferol; quercetin; quercetin-3-*O*-β-D-glucoside; quercetin-3-*O*-β-D-glucoside-6′′-*O*-malonate; quercetin-3-*O*-β-D-galactoside; 3-methylquercetin; 3-methylquercetin-7-*O*-β-D-glucoside; 3-methoxyquercetin-7-*O*-β-D-glucoside-6′′-*O*-malonate; *flavones*: apigenin; apigenin-7-*O*-β-D-glucoside; luteolin; luteolin-7-*O*-β-D-glucoside *isoflavones*: daidzein; daidzein-7-*O*-β-D-glucoside; genistein; genistein-7-*O*-β-D-glucoside; genistein-7-*O*-β-D-glucoside-6′′-*O*-malonate; glycitein;glycitein-7-*O*-β-D-glucoside, prunetin
white clover (*Trifolium repens*)	*flavonols*: kaempferol; kaempferol-3-*O*-β-D-galactopyranoside; kaempferol-3-*O*-(6′′-acetyl)-β-D-galactopyranoside; myricetin-3-*O*-β-D-galactopyranoside; myricetin-3-*O*-(6′′-acetyl)-β-D-galactopyranoside; quercetin; quercetin-3-*O*-glucoside; quercetin-3-*O*-β-D-galactopyranoside; quercetin-3-*O*-(6′′-acetyl)-β-D-galactopyranoside *isoflavones*: daidzein; genistein *tannins*: epigallocatechin; gallocatechin; gallocatechin-(4α-8)-epigallocatechin
	Cereal Grasses
pear millet (*Penninsetum glaucum*)	*hydroxycinnamic acids*: cinnamic acid; *para*-coumaric acid; *para*-hydroxybenzoic acid; protocatechuic acid
foxtail millet (*Setaria italica*)	*flavones*: lucenin-1; glucosylorientin; isoorientin; orientin; saponarin; violanthin; glucosylvitexin; isovitexin; vitexin
finger millet (*Eleusine coracana*)	
proso millet (*Panicum miliaceum*)	
sorghum (*Sorghum bicolour*)	*hydroxybenzoic acids*: *para*-hydroxybenzoic acid; protocatechuic acid
Johnson grass (*Sorghum halepense*)	*hydroxycinnamic acids*: caffeic acid; *para*-coumaric acid
jowar (*Sorghum vulgare*)	*flavonols*: kaempferol-3-rutinoside-7-glucuronide; taxifolin *flavones*: apigenin *flavonones*: eriodictyol; luteolin; naringenin *flavanals*: apiforol; luteoforol *anthocyanidins*: apigeninidin; apigeninidin-5-glucoside; 7-methoxylapigeninidin-5-glucoside; luteolidin; luteolidin-5-glucoside; 5-methoxyluteolinidin-7-glucoside; 7-methoxyluteolinidin
wheat (*Triticum* spp.)	*hydroxybenzoic acids*: *para*-hydroxybenzoic acid *hydroxycinnamic acids*: caffeic acid *flavones*: apigenin *anthocyanins*: cyanidin-3-galactoside; cyanidin-3-glucoside; pelargornidin-3-glucoside
barley (*Hordeum vulgare*)	*hydroxycinnamates*: *N*-4-coumaroylputrescine; *para*-coumaroylagmatine
maize (*Zea mays*)	*hydroxycinnamates*: 2-*O*-caffeoylhydroxycitric acid; 2-*O*-coumaroylhydroxycitric acid; *para*-coumaroyltyramine;
oat (*Avena sativa*)	*hydroxybenzoic acids*: gallic acid; *para*-hydroxybenzoic acid; *para*-hydroxyphenylacetic acid; protocatechuic acid *hydroxycinnamic acids*: caffeic acid; *para*-coumaric acid *hydroxycinnamates*: *n*-hexacosylcaffeate; *n*-octacosylcaffeate; 26-*O*-caffeoyl-26-hydroxyhexacosanoic acid; 28-*O*-caffeoyl-28-hydroxyoctacosanoic acid; hexacosane-1,26-diol dicaffeate; 1-*O*-caffeoyl-26-feruloylhexacosane diol *avenanthramides*: *N*-(4′-hydroxycinnamoyl)-5-hydroxyanthranilic acid; *N*-(4′-hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid; *N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid; *N*-(4′-hydroxycinnamoyl)-anthranilic acid; *N*-(3′,4′-dihydroxycinnamoyl)-anthranilic acid; *N*-(4′-hydroxycinnamoyl)-4-hydroxyanthranilic acid; *N*-(3′,4′-dihydroxycinnamoyl)-4-hydroxyanthranilic acid *avenalumic acids*: avenalumic acid; 3-hydroxyavenalumic acid; 3-methoxyavenalumic acid *flavonols*: kaempferol; kaempferol-3-*O*-rutinoside; quercetin; quercetin-3-*O*-rutinoside *flavones*: apigenin; apigenin-6-*C*-glucoside; apigenin-8-*C*-glucoside; luteolin; isoorientin-2′′-arabinoside; isoswertisin-2′′-rhamnoside; isovitexin-2′′-arabinoside; vitexin-2′′-rhamnoside
	Brassica Forages
kale (*Brassica oleraceae* L. var. *acephala* DC.)	*hydroxybenzoic acids*: gallic acid; *para*-hydroxybenzoic acid; protocatechuic acid *hydroxycinnamic acids*: caffeic acid; *para*-coumaric acid *hydroxycinnamates*: *para*-coumaroylquinic acid

Table 1. Continued

source	PPO substrates
	flavonols: kaempferol-3-*O*-glucoside; kaempferol-3,7-*O*-diglucoside; kaempferol-3-*O*-tetraglucoside-7-*O*-sophoroside; kaempferol-3-*O*-sophoroside-7-*O*-glucoside; kaempferol-3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; kaempferol-3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; kaempferol-3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; kaempferol-3-*O*-sophoroside; kaempferol-3-*O*-(feruloyl)-sophoroside; kaempferol-3-*O*-(sinapoyl)-sophoroside; kaempferol-3-*O*-sophoroside-7-*O*-sophoroside; kaempferol-3-*O*-sophotrioside; kaempferol-3-*O*-sophotrioside-7-*O*-glucoside; kaempferol-3-*O*-sophotrioside-7-*O*-sophoroside
turnip (*Brassica rapa* var. *rapa* L.)	*hydroxycinnamic acids*: caffeic acid *hydroxycinnamates*: *para*-coumaroylquinic acid; caffeoyl malate; coumaroyl malate *flavonols*: kaempferol; kaempferol-3-*O*-glucoside; kaempferol-3,7-*O*-diglucoside; kaempferol-3-*O*-glucoside-7-*O*-sophoroside; kaempferol-3-*O*-(caffeoyl)-sophotrioside-7-*O*-sophoroside; kaempferol-3-*O*-(methoxycaffeoyl)-sophotrioside-7-*O*-glucoside; kaempferol-3-*O*-(coumaroyl)-sophotrioside-7-*O*-sophoroside; kaempferol-3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; kaempferol-3-*O*-(feruloyl)-sophoroside; kaempferol-3-*O*-(feruloyl)-sophotrioside-7-*O*-sophoroside; kaempferol-3-*O*-(sinapoyl)-sophotrioside-7-*O*-glucoside; kaempferol-3-*O*-sophoroside; kaempferol-3-*O*-sophoroside-7-*O*-sophoroside; quercetin; quercetin-3-*O*-glucoside; quercetin-3-*O*-sophoroside; quercetin-3-p-(coumaroyl)-sophoroside-7-*O*-glucoside; quercetin-3-*O*-(diferuloyl)-sophoroside-7-*O*-glucosidequercetin-3-*O*-(feruloyl)-sophotrioside-7-*O*-glucoside; quercetin-3-*O*-(sinapoyl)-sophotrioside-7-*O*-glucoside

quinic acid to yield 1-caffeoyl (1-CQA), 3-caffeoyl- (3-CQA), and 4-caffeoylquinic acid (4-CQA). To date, 1-CQA has not been identified in forage plants. Small quantities of 3-CQA and 4-CQA were detected in meadow fescue and cocksfoot, while 4-CQA was found as the minor component in timothy. In Italian ryegrass, 3-CQA was present in relatively high concentrations (50% abundance relative to 5-CQA acid), but only small quantities were detected in perennial ryegrass. In both of these ryegrasses, 4-CQA was present in only trace quantities. Not surprisingly, PPO activity was reported in temperate grass species, with particularly high activities of the enzyme found in cocksfoot and Italian, perennial and hybrid ryegrasses, while low levels were detected in tall fescue and timothy (*3*).

Recently in our laboratory, we identified a major hydroxycinnamate ester present in cocksfoot leaf tissue, 2-*O*-caffeoylisocitric acid, and its 6-methyl ester (*29*) (**Table 1**). Furthermore, 2-*O*-caffeoylthreonic acid (and diastereoisomer) and 2-*O*-caffeoylhydroxycitric acid were also detected in cocksfoot grass. Typical relative abundance of cinnamate esters in crude grass extracts on a molar basis were 3-CQA (2%), 4-CQA (0.7%), 5-CQA (1.1%), 2-*O*-caffeoylisocitric acid (39.5%), 2-*O*-caffeoylisocitric acid 6-methyl ester (38.6%), 2-*O*-caffeoylthreonic acid (6.3%), its diasteroisomer (1.7%), and 2-*O*-caffeoylhydroxycitric (9.6%). 2-*O*-Caffeoylisocitric acid 6-methyl ester was also found in trace quantities in timothy grass. PPO enzyme isolated from cocksfoot grass showed a high affinity for 2-*O*-caffeoylisocitric acid and the methyl ester, and a high rate of activity with chlorogenic acid. We have also shown that 2-*O*-caffeoylthreonic acid and 2-*O*-caffeoylhydroxycitric acid are substrates of PPO (*29*).

Very few flavonoids have been detected in forage grasses; nevertheless, in leaf extracts of subtropical cocksfoot species, mono-C- and di-C-glycosides of luteolin and di-C-glycosides of apigenin are reported to be abundant (*59*).

Forage Legumes. Red clover (also known as meadow clover, purple clover, and cow clover) is an important forage legume that is grown throughout the world as feed for sheep and cattle (*60*). Red clover is native to Europe, western Asia, and northwest Africa and has been planted and naturalized in many regions of the world, including the Americas and Australasia. Several studies have found 2-*O*-caffeoylmalic acid (also referred to as phaselic acid) and *trans*-clovamide to be the major PPO substrates in the leaves and stems of red clover (*12*, *17*, *61*) (**Table 1**). The abundance of phaselic acid in red clover leaves is reported to be equivalent to 5−20 μmol of caffeic acid g^{-1} FW (*12*, *17*). *Trans*-clovamide is observed at much lower concentrations, with values reported to be equivalent to 1−4 μmol of caffeic acid g^{-1} FW.

Diverse stimuli, such as wounding and elicitor treatments (chitooligosaccharides and jasmonic acid (JA)), have been shown to induce accumulation of hydroxycinnamic acid amides in red clover (*36*). Treatment with JA induced several of these compounds including clovamide, *para*-coumaroyl DOPA, caffeoyltyrosine, and *para*-coumaroyltyrosine. Clovamide was present in the highest concentrations, while the remaining three compounds were present in only minor quantities (*36*).

Recently, Lin et al. (*42*) detected a large number of flavonoids and their glycoside malonates from the flowers and leaves of red clover by liquid chromatography−electrospray ionization mass spectrometry (ESI-MS) (**Table 1**). Concentrations of daidzein and genistein were reported to be the highest in the leaves of red clover, while lower levels were detected in the stems and petioles (*42*−*45*). These compounds have also been identified in subterranean clover (*43*). At IBERS, we have shown that daidzein and genistein are not substrates of the PPO enzyme isolated from red clover (unpublished data); however, this does not necessarily imply that these compounds are not substrates of other PPO isoenzymes.

In New Zealand, the most common pasture for ruminant (cattle and sheep) grazing consists of a mixture of both perennial ryegrass and white clover (*62*). White clover is a highly nutritive legume native to Europe, North Africa, West Asia, and North America (*60*). In contrast to red clover, white clover contains low levels of PPO, which is in the latent form and is not activated during ensilage or grazing (*63*). Several studies have detected condensed tannins and proanthocyanidins in the flowers of white clover and in trace amounts in leaf trichomes (*62*, *64*−*67*) (**Table 1**). Recently, Foo et al. (*67*) detected a number of flavonoid glycoside derivatives in the flower extracts. Quercetin 3-*O*-(6″-acetyl)-β-D-galactopyranoside was found to be the most abundant in the flower extract, while kaempferol 3-*O*-(6″-acetyl)-β-D-galactopyranoside was present in smaller concentrations (*67*). Furthermore, the condensed tannins, gallocatechin, epigallocatechin, gallocatechin-(4α-8)-epigallocatechin, and the corresponding prodelphinidin polymers were also detected. Other compounds isolated from white clover include members of the flavonol and isoflavone classes (*65*, *66*). High levels of soluble hydroxycinnamates have been observed in white clover leaf extracts in our laboratory (unpublished work); however, these compounds have not been identified. These compounds are the most likely substrates of white clover PPO.

Forage Cereals. Forage cereals provide a valuable source of ruminant feed, particularly for housed animals when the forage is conserved and fed out as a whole-crop silage. Whole-crop silages of corn, wheat, barley, triticale, oats, sorghum, and millet are commonly produced in different parts of the world. The occurrence of PPOs in wheat, sorghum, and millet has previously been reported (*51−53*). Hydroxybenzoic acid was relatively abundant in wheat bran extracts (*68*). Caffeic acid was detected in wheat ears, after exposure to fungal infection (*69*). Seven phenolic acids were identified in the free form in extracts of sorghum kernels (*70*). *para*-Coumaric acid was the major phenolic acid observed in millet (*25*). Lesser amounts of protocatechuic acid, *para*-hydroxybenzoic acid, and caffeic acid were also reported.

Hu et al. (*71*) characterized the flavonoid content of dark blue-grained wheat. Cyanidin-3-glucoside was the predominant anthocyanin, and cyanidin-3-galactoside and pelargornidin-3-glucoside were also detected. Diglycosyl apigenins were identified in wheat bran from four varieties of wheat (*68*). Anthocyanidins are the major class of flavonoids identified in sorghum (*25*). These are classified as 3-deoxyanthocyanins because of the absence of a hydroxy group in the 3-position of the C-ring. This feature increases their stability at high pH compared to that of common anthocyanins. Highest levels are observed in sorghums with a black pericarp. Flavan-4-ol compounds such as apiforol have been detected in red pericarp sorghums. The only class of flavonoids reported in millets are flavones (**Table 1**), and several glycosyl flavones have also been identified including glucosylvitexin and glucosylorientin. Ozawa et al. (*72*) isolated cinnamate esters, including 2-*O*-caffeoylhydroxycitric acid and 2-*O*-coumaroylhydroxycitric acid, in the whole corn plant. 2-*O*-Caffeoylhydroxycitric acid has previously been shown to be a substrate of the cocksfoot PPO enzyme (see above).

Oats have a high content of phenolic compounds and are used widely as livestock feed, both as a whole-crop silage and as a grain or groat (*26*, *73*). Hydroxycinnamates (hydroxycinnamic acids and cinnamate esters) are the most abundant antioxidants in oat, while flavonoids are reported to be present in small quantities (*26*,*74−79*). The phenolic acids which may be considered as PPO substrates consisted of hydroxybenzoic acids and hydroxycinnamic acids; the former include *para*-hydroxybenzoic acid, *para*-hydroxyphenylacetic acid, protocatechiuc acid, and gallic acid; while the latter consist mainly of caffeic acid and *para*-coumaric acid (*26*, *75*). In a variety of plant tissues, relatively small concentrations of hydroxycinnamic acids occur in the free form; the large majority exist as covalent conjugates. Trace quantities of free *para*-coumaric acids were detected in aqueous ethanol (70%) extracts of cv. Hinoat (*75*). Moreover, in oat grain meal, the majority of *para*-coumaric and *para*-hydroxybenzoic acids were derived from soluble esters (*75*). Sosulski et al. (*78*) reported that 8.7 mg kg^{-1} of total free phenolic acids were detected in the oat flour extract, while soluble phenolic acid esters totalled 20.6 mg kg^{-1}. *para*-Hydroxyphenylacetic acid and protocatechuic ester were detected in trace amounts in the oat extracts. Emmons et al. (*76*) found caffeic acid (2.4 mg kg^{-1}) as the major component in the oat groats with *para*-coumaric acid (0.9 mg kg^{-1}) present in smaller quantities. In contrast, in oat hulls, *para*-coumaric acid (9.7 mg kg^{-1}) was detected in the highest concentrations, while caffeic acid (0.9 mg kg^{-1}) and gallic acid (0.6 mg kg^{-1}) were minor components.

In plants, phenolic acids have also been shown to exist in the form of esters in conjunction with glycerol, saturated long-chain monoalcohols, fatty diols, and hydroxy acids (*26*, *30−34*) (**Table 1**). These may serve as PPO substrates, provided that the phenolic portion is in a polar environment. Daniels et al. (*32*) isolated and identified several of these compounds in oats extracts, with hexacosyl caffeate and octacosyl caffeate existing in a 3:1 ratio. Similarly, 26-*O*-caffeoyl-26-hydroxyhexacosanoic acid and 28-*O*-caffeoyl-28-hydroxyoctacosanoic acid were found in a 3:1 ratio. Daniels et al. (*32*) also reported the presence of a mixture of diols, hexacosane-1,26-diol and octacosane-1,28-diol (relative ratios 2:1), each of which was esterified by two caffeoyl groups and by one caffeoyl and one feruloyl moiety.

The presence of hydroxy- and/or methoxy-substituted *N*-cinnamoylanthranilates, or avenanthramides in oat groats and hulls has been documented by several researchers (*26*, *79−82*) (**Table 1**). Avenanthramides are synthesized by condensation of substituted anthranilic acids, i.e., 5-hydroxyanthranilic acid and substituted cinnamoyl-CoA such as feruloyl-CoA (*79*). Concentrations of avenanthramides were found to vary among different oat genotypes and their growing environment, with abundance of *N*-(4′-hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid (AV2) ranging from 40 to 132 mg kg^{-1} in 10 oat genotypes (*81*). Quantitative studies conducted by Dimberg et al. (*79*) on avenanthramides *N*-(4′-hydroxycinnamoyl)-5-hydroxyanthranilic acid (AV1), AV2, and *N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid (AV3) in three cultivars (Mustang, Svea, and Kapp) showed concentrations in groats to be in the region 25−47 (AV1), 21−43 (AV2), and 28−62 mg kg^{-1} (AV3). Of the three cultivars, Mustang contained a significantly higher abundance of AV1, AV2, and AV3 when compared to that of the other two cultivars. Emmons et al. (*76*) also identified AV1 and AV2 in oat groats and hulls. The authors reported concentrations of 54, 36, and 52 mg kg^{-1} in oat groats and 25, 17, and 14 mg kg^{-1} in oat hulls.

Small quantities of flavonoids have been reported in cereal grains (*26*). The flavones apigenin and luteolin were identified in oat flour, along with their glycosidic derivatives, apigenin-6-*C*-glucoside and apigenin-8-*C*-glucoside. Furthermore, the flavonols kaempferol and quercetin and their 3-*O*-rutinoside derivatives, i.e., kaempferol-3-*O*-rutinoside and quercetin-3-*O*-rutinoside, were also detected (*26*). Chopin et al. (*83*) identified vitexin 2′′-rhamnoside, isoswertisin 2′′-rhamnoside, isovitexin 2′′-arabinoside, and isoorientin 2′′-arabinoside have been identified in the leaves, stems, and flowers of oats. More recently, vitexin-2-*O*-rhamnoside was detected in leaves, stems, and panicles of oats, with concentrations ranging from 0.04% to 0.18% (*84*).

Forage Brassicas. Kale is also widely used as a forage crop and is cultivated in many regions of the world, particularly in the Mediterranean, Southwestern Europe, and the U.K. (*24*). Kale forages are adapted to a wide range of climate conditions; they are grown usually for summer−autumn−winter grazing in cool temperate conditions and for winter−spring grazing in subtropical climates (*85*). The Brassicaceae family includes a wide range of horticultural crops; of these, turnip (*Brassica rapa* var. *rapa* L.) is also used widely as a forage crop. Turnip grows well in cold climates and can be stored several months after harvest (*86−88*). *Brassica* species are reported to be good sources of natural phenolic antioxidants, containing flavonoids, lignans, and condensed tannins (*86*) (**Table 1**). In kale leaves, potential PPO substrates, including hydroxybenzoic acids (gallic, protocatechuic, and *para*-hydroxybenzoic) and hydroxycinnamic acids (caffeic and *para*-coumaric), were isolated and quantified by HPLC-MS (*24*). Caffeic acid and *para*-hydroxybenzoic acid were found to be among the most abundant free acids and soluble esters in kale leaves, while smaller quantities were detected for *para*-coumaric acid and protocatechuic acid. Gallic acid was present in trace quantities. Of the soluble glycosides, caffeic acid derivatives were present in the highest concentrations, while *para*-coumaric acid derivatives were also detected in relatively abundant levels.

The phenolic acids observed in the leaf extract were also observed in kale seeds. In contrast to kale leaves, *para*-coumaric acid was detected in appreciable levels. Caffeic and *para*-hydroxybenzoic acids were detected in smaller quantities, while protocatechuic and gallic acids were present in trace quantities. The ratios of total hydroxybenzoic acids/total hydroxycinnamic acids in the leaves of kale were 7:93, while in kale seeds, the ratio of abundance was 12:88, respectively (*24*).

In a study conducted by Sousa et al. (*86*), *para*-coumaroylquinic acid (3-*p*-CoQA) and a large number of flavonoids were identified in the flowers of kale (**Table 1**). Turnip contained several phenolic acids and flavonoids distinct from those found in kale (*86*). These compounds were detected in the leaves, stems, and flowers of turnip (*86*, *87*). In kale flowers, kaempferol-3-*O*-sophoroside (2004 mg kg^{-1} dry matter (DM)) was the major component and represented 21% of total phenols, while 3-*para*-CoQA (166 mg kg^{-1} DM) and kaempferol-3-*O*-sophoroside-7-*O*-glucoside (173 mg kg^{-1} DM) were the least abundant, with each contributing to 2% of total phenols (*86*). Fernandes et al. (*87*) also found that kaempferol-3-sophoroside-7-*O*-glucoside, kaempferol-3-*O*-feruloyl/caffeoyl-sophoroside-7-*O*-glucoside, was one of the major components in the flowers of turnip and corresponded to approximately 15−17% of total phenols, while 3-*para*-CoQA was found in minor quantities and represented 1% of the total phenols. A similar profile was observed for the leaves and stems, with the major compounds each accounting for about 13−20% of total phenolics, while the minor components each contributed to less than 0.8%. Following treatment with methyl jasmonate, in turnip leaves, coumaroyl malate, caffeoyl malate, and chlorogenic acid were detected in trace quantities (approximately 0.0004%) (*88*).

Turnip tops (*Brassica rapa* L. subsp. *Sylvestris* L.) also contained a significant number of flavonoids (*89*) (**Table 1**). In contrast to kale and turnip, this species also contained a large number of quercetin flavonoids. Furthermore, the hydroxycinnamic acids coumaric and caffeic were also identified. Quantitative data indicated that the flavonoids were the most abundant ranging from 102 to 139 mg/100 g, while hydroxycinnamic derivatives were found in the range 5.8−52.5 mg/100 g. The most abundant flavonoids among kaempferol and quercetin derivatives were kaempferol-3-*O*-glucoside and quercetin-3-sinapoyl-sophotrisoside-7-glucoside (*89*).

In summary, PPO activity in forage plant species offers a potential mechanism for improving protein utilization by ruminant livestock and limiting nitrogen losses to the environment. It is evident from the literature that a broad range of phenolic compounds accumulate in PPO-containing forage crops. This is relevant to the role of PPO in ruminant nutrition as the extent of browning is determined both by enzyme activity and phenol composition. Oxidation of phenols can occur as a direct result of PPO activity or indirectly as a consequence of secondary reactions with PPO generated quinones. Studies on the effect of total phenol composition on PPO activity in forage crops are limited. This will be affected by both the range and levels of phenol compounds which in turn will be influenced by genetic and environmental factors. Further characterization of the PPO trait in terms of phenol composition will inform breeding targets for improving forages for livestock farming.

LITERATURE CITED

(1) Amiot, M. J.; Fleuriet, A.; Cheynier, V.; Nicolas, J. Phenolic Compounds and Oxidative Mechanism in Fruits and Vegetables. In *Phytochemistry of Fruits and Vegetables*; Thomas-Barberan, F., Ed.; Oxford University Press: New York, 1997; pp 51−85.

(2) Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* (*Elsevier*) **1979**, *18*, 193–215.

(3) Lee, M. R. F.; Colmenero, J. D. O.; Winters, A. L.; Scollan, N. D.; Minchin, F. R. Polyphenol oxidase activity in grass and its effect on plant-mediated lipolysis and proteolysis of *Dactylis glomerata* (orchard) in a simulated rumen environment. *J. Sci. Food Agric.* **2006**, *86*, 1503–1511.

(4) Mayer, A. M. Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochem.* **2006**, *67*, 2318–2331.

(5) Martinez, V. M.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195–200.

(6) Lattanzio, V.; Cardinali, A.; Palmieri, S. The role of phenolics in the postharvest phyiology of fruits and vegetables: browning reactions and fungal diseases. *Ital. J. Food Sci.* **1994**, 3–22.

(7) Lattanzio, V. Bioactive polyphenols: Their role in quality and storability of fruit and vegetables. *J. Appl. Bot.* **2003**, *77*, 128–146.

(8) Lee, M. R. F.; Parfitt, L. J.; Scollan, N. D.; Minchin, F. R. Lipolysis in red clover with different polyphenol oxidase activities in the presence and absence of rumen fluid. *J. Sci. Food Agric.* **2007**, *87*, 1308–1314.

(9) Lee, M. R. F.; Scott, M. B.; Tweed, J. K. S.; Minchin, F. R.; Davies, D. R. Effects of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (*Lactobacillus plantarum* L54). *Anim. Feed Sci. Technol.* **2008**, *144*, 125–136.

(10) Solomon, E. I.; Chen, P.; Metz, M.; Lee, S.; Palmer, A. E. Oxygen binding, activation, and reduction to water by copper proteins. *Angew. Chem., Int. Ed.* **2001**, *40*, 4570–4590.

(11) Yoruk, R; Marshall, M. R. Physicochemical properties and function of plant polyphenol oxidase: A review. *J. Food Biochem.* **2003**, *27*, 361–422.

(12) Winters, A. L.; Minchin, F. R.; Michaelson-Yeates, T. P. T.; Lee, M. R. F.; Morris, P. Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. *J. Agric. Food Chem.* **2008**, *56*, 2817–2824.

(13) Jimenez, M.; Garcia-Carmona, F. The effect of sodium dodecyl sulphate on polyphenol oxidase. *Phytochemistry* (*Elsevier*) **1996**, *42*, 1503–1509.

(14) Gerdemann, C.; Eicken, C.; Galla, H.-J.; Krebs, B. Comparative modelling of the latent form of a plant catechol oxidase using a molluskan hemocyanin structure. *J. Inorg. Biochem.* **2002**, *89*, 155–158.

(15) van der Walt, J. G. Nitrogen metabolism of the ruminant liver. *Aust. J. Agric. Res.* **1993**, *44*, 381–403.

(16) Broderick, G. A.; Walgenbach, R. P.; Maignan, S. Production of lactating dairy cows fed alfalfa or red clover silage at equal dry matter or crude protein contents in the diet. *J. Dairy Sci.* **2001**, *84*, 1728–1737.

(17) Sullivan, M. L.; Hatfield, R. D. Polyphenol oxidase and o-diphenols inhibit postharvest proteolysis in red clover and alfalfa. *Crop Sci.* **2006**, *46*, 662–670.

(18) Jones, B. A. Screening legume forages for soluble phenols, polyphenol oxidase and extract browning. *J. Sci. Food Agric.* **1995**, *67*, 109–112.

(19) Jones, B. A.; Hatfield, R. D.; Muck, R. E. Characterisation of proteolysis in alfalfa and red clover. *Crop Sci.* **1995**, *35*, 537–541.

(20) Ribéreau-Gayon, P. *Plant Phenolics*; Oliver & Boyd, T. and A. Constable Ltd.: Edinburgh, Scotland, 1968.

(21) Clifford, M. N.; Kirkpatrick, J.; Kuhnert, N.; Roozendaal, H.; Salgado, P. R. LC−MS^n analysis of the *cis* isomers of chlorogenic acids. *Food Chem.* **2008**, *106*, 379–385.

(22) Harborne, J. B. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd ed.; Chapman and Hall: London, U.K., 1998.

(23) Crozier, A.; Clifford, M. N.; Ashihara, H. *Plant Secondary Metabolites, Occurrence, Structure and Role in the Human Diet*; Blackwell Publishing Ltd.: Oxford, U.K., 2006.

(24) Ayaz, F. A.; Hayirlioglu-Ayaz, S.; Alpay-Karaoglu, S.; Grúz, Valentová, K.; Ulrichová, J.; Strnad, M. Phenolic acid contents of kale

(*Brassica oleraceae* L. var. *acephala* DC.) extracts and their antioxidant and antibacterial activities. *Food Chem.* **2008**, *107*, 19–25.
(25) Dykes, L.; Rooney, L. W. Sorghum and millet phenols and antioxidants. *J. Cereal Sci.* **2006**, *44*, 236–251.
(26) Peterson, D. M. Oat antioxidants. *J. Cereal Sci.* **2001**, *33*, 115–129.
(27) Buren, J. V. *Fruit Phenolics in Biochemistry of Fruits and Their Products*; Hulme, A. C., Ed.; Academic Press: London, U.K., 1970; pp 267−304.
(28) Escarpa, A.; Gonzalez, M. C. An overview of analytical chemistry of phenolic compounds in foods. *Crit. Rev. Anal. Chem.* **2001**, *31*, 57–139.
(29) Parveen, I.; Winters, A.; Threadgill, M. D.; Hauck, B.; Morris, P. Extraction, structural characterisation and evaluation of hydroxycinnamate esters of orchard grass (*Dactylis glomerata*) as substrates for polyphenol oxidase. *Phytochemistry* (*Elsevier*) **2008**, *69*, 2799–2806.
(30) Daniels, D. G. H.; Martin, H. F. Isolation of a new antioxidant from oats. *Nature* **1961**, *191*, 1302.
(31) Daniels, D. G. H.; King, H. G. C.; Martin, H. F. Antioxidants in oats: Esters of phenolic acids. *J. Sci. Food Agric.* **1963**, *14*, 385–390.
(32) Daniels, D. G. H.; Martin, H. F. Antioxidants in oats: Mono-esters of caffeic and ferulic acids. *J. Sci. Food Agric.* **1967**, *18*, 589–595.
(33) Daniels, D. G. H.; Martin, H. F. Antioxidants in oats: Glyceroyl esters of caffeic and ferulic acids. *J. Sci. Food Agric.* **1968**, *19*, 710–712.
(34) Min, D. B.; Smouse, T. H. *Flavor Chemistry of Fats and Oils*; American Chemical Society: Washington, DC, 1986.
(35) Yoshihara, T.; Yoshikawa, H.; Kunimatsu, S.; Sakamura, S.; Sakuma, T. New amino acid derivatives conjugated with caffeic acid and DOPA from red clover (*Trifolium pratense*). *Agric. Biol. Chem.* **1977**, *41*, 1679–1684.
(36) Tebayashi, S.; Ishihara, A.; Tsuda, M.; Iwamura, H. Induction of clovamide by jasmonic acid in red clover. *Phytochemistry* (*Elsevier*) **2000**, *54*, 387–392.
(37) Williams, C. A.; Harborne, J. B. Flavone and Flavonol Glycosides. In *The Flavonoids: Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, U.K., 1993.
(38) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152–159.
(39) van Acker, S. A. B. E.; de Groot, M. J.; van den Berg, D. J.; Tromp, M. N. J. L.; Donné-Op den Kelder, G.; van der Vijgh, W. J. F.; Bast, A. A quantum explanation of the antioxidant activity of flavonoids. *Chem. Res. Toxicol.* **1996**, *9*, 1305–1312.
(40) Cunningham, B. D. M.; Threadgill, M. D.; Groundwater, P. W.; Dale, I. L.; Hickman, J. A. Synthesis and biological evaluation of a series of flavones designed as inhibitors of protein tyrosine kinases. *Anti-Cancer Drug Des.* **1992**, *7*, 365–384.
(41) Salunkhe, D. K.; Chavan, J. K.; Kadam, S. S. *Dietary Tannins: Consequences and Remedies*; CRC Press Inc.: Boca Raton, FL, 1990.
(42) Lin, L.-Z.; He, X.-G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qui, S.-X.; Cordell, G. A. LC-ESI-MS study of the flavonoid glycoside malonates or red clover (*Trifolium pratense*). *J. Agric. Food Chem.* **2000**, *48*, 354–365.
(43) Wong, E. Detection and estimation of oestrogenic constituents in red clover. *J. Sci. Food Agric.* **1962**, *13*, 304–308.
(44) Saviranta, N. M. M.; Anttonen, M. J.; wright, A. V.; Karjalainen, R. O. Red clover (*Trifolium pratense* L.) isoflavones: determination of concentrations by plant stage, flower, colour, plant part and cultivar. *J. Sci. Food Agric.* **2008**, *88*, 125–132.
(45) Booth, N. L.; Overk, C. R.; Yao, P.; Totura, S.; Deng, Y.; Hedayat, A. S.; Bolton, J. L.; Pauli, G. F.; Farnsworth, N. R. Seasonal variation of red clover (*Trifolium pratense* L., fabaceae) isoflavones and estrogenic activity. *J. Agric. Food Chem.* **2006**, *54*, 1277–1282.
(46) Jiang, Y.; Duan, X.; Joyce, D.; Zhang, Z.; Li, J. Advances in understanding of enzymatic browning in harvested litchi fruit. *Food Chem.* **2004**, *88*, 443–446.
(47) Zhang, Z. Q.; Pang, X. Q.; Yang, C.; Ji, Z. L.; Jiang, Y. M. Purification and structural analysis of anthocyanins from litchi pericarp. *Food Chem.* **2004**, *84*, 601–604.
(48) Wuyts, N; De Waele, D; Swennen, R. Extraction and partial characterisation of polyphenol oxidase from banana (*Musa acuminate Grandenaine*) roots. *Plant Physiol. Biochem.* **2006**, *44*, 308–314.
(49) Sun, J.; Jiang, Y.; Wei, X.; Zhao, M.; John, S.; You, Y.; Yi, C. Identification of procyanidin A2 as polyphenol oxidase substrate in pericarp tissues of litchi fruit. *J. Food Biochem.* **2007**, *31*, 300–313.
(50) Winters, A. L.; Robbins, M. P.; Gallagher, J. A.; Lee, M. R. F.; Minchin, F. R.; Parveen, I.; Morris, P. *Biochemistry and Molecular Biology of Polyphenol Oxidases in Grasses. Abstracts, Highlights in the Evolution of Phytochemistry*, Phytochemical Society of Europe, 50th Anniversary Meeting, Cambridge, Apr 11−14, 2007.
(51) Raj, S. N.; Sarosh, B. R.; Shetty, H. S. Induction and accumulation of polyphenol oxidase activities as implicated in development of resistance against pearl millet downy mildew disease. *Funct. Plant. Biol.* **2006**, *33*, 563–571.
(52) Vaughn, K. C.; Duke, S. O. Tissue localization of polyphenol oxidase in *Sorghum*. *Protoplasma* **1981**, *108*, 319–327.
(53) Demeke, T.; Morris, C. F. Molecular characterization of wheat polyphenol oxidase. *Theor. Appl. Genet.* **2002**, *104*, 813–818.
(54) Jennings, P. H.; Dufus, C. M. Effect of gibberellic acid on polyphenol oxidase activity in de-embryonated wheat and barley grains. *New Phytol.* **2006**, *78*, 383–389.
(55) Harpaz, I.; Klein, M. Polyphenol oxidase activity in healthy maize plants and in those infected by the maize rough dwarf virus (MRDV). *Cell. Mol. Life Sci.* **1964**, *20*, 274–275.
(56) Dimberg, L. H; Peterson, D. M. Phenols in spikelets and leaves of field-grown oats (*Avena sativa*) with different inherent resistance to crown rust (*Puccinia coronata* f. sp. *avenae*). *J. Sci. Food Agric.* **2009**, *89*, 1815–1824.
(57) Kim, D.; Song, H; Sangyong, L.; Hyejeon, Y.; Chung, J. Effects of gamma irradiation on the radiation-resistant bacteria and polyphenol oxidase activity in fresh kale juice. *Radiat. Phys. Chem.* **2007**, *76*, 1213–1217.
(58) Stahmann, M. A.; Clare, B. G.; Woodbury, W. Increased disease resistance and enzyme activity induced by ethylene amd ethylene production of black rot infected sweet potato tissue. *Plant Physiol.* **1966**, *41*, 1505–1512.
(59) Jay, M.; Lumaret, R. Variation in the subtropical group of *Dactylis glomerata* L. 2. Evidence from phenolic compound patterns. *Biochem. Syst. Ecol.* **1995**, *23*, 523–531.
(60) Laidlaw, A. S.; Charlton,J. F. L.; Frame, J. *Temperate Forage Legumes*; CAB Intl.: Wallingford, U.K., 1998.
(61) Jones, B. A.; Muck, R. E.; Hatfield, R. D. Red clover extracts inhibit legume proteolysis. *J. Sci. Food Agric.* **1995**, *67*, 329–333.
(62) Jones, W. T.; Broadhust, R. B.; Lyttleton, J. W. The condensed tannins of pasture legume species. *Phytochemistry* (*Elsevier*) **1976**, *15*, 1407–1409.
(63) Theodorou, M. K.; Kingston-Smith, A. H.; Winters, A. L.; Lee, M. R. F.; Minchin, F. R.; Morris, P.; MacRae, J. Polyphenols and their influence on gut function and health in ruminants: a review. *Environ. Chem. Lett.* **2006**, *4*, 121–126.
(64) Burggraat, V.; Waghorn, G.; Woodward, S.; Thom, E. Effects of condensed tannins in white clover flowers on their digestion *in vitro*. *Anim. Feed Sci. Technol.* **2008**, *142*, 44–58.
(65) Carlsen, S. C. K.; Understrup, A.; Fomsgaard, I. S.; Mortensen, A. G.; Ravnskovi, S. Flavonoids in roots of white clover: Interaction of arbuscular mycorrhizal fungi and a pathogenic fungus. *Plant Soil* **2008**, *302*, 33–43.
(66) Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press Inc. London Ltd.: London, U.K., 1967.
(67) Foo, L. Y.; Lu, Y.; Molan, A. L.; Woodfield, D. R.; McNabb, W. C. The phenols and prodelphinidins of white clover flowers. *Phytochemistry* (*Elsevier*) **2000**, *54*, 539–548.
(68) Kim, K. H.; Tsao, R.; Yang, R.; Cui, S. W. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effects of hydrolysis conditions. *Food Chem.* **2006**, *95*, 466–473.
(69) Gogoi, R; Singh, D. V.; Srivastava, K. D. Phenols as a biochemical basis of resistance in wheat against Karnal bunt. *Plant Pathol.* **2001**, *50*, 470–476.
(70) Hahn, D. H.; Faubion, J. M.; Rooney, L. W. Sorghum phenolic acids, their high performance liquid chromatography separation and their relation to fungal resistance. *Cereal Chem.* **1983**, *60*, 255–259.

(71) Hu, C.; Cai, Y.; Li, W.; Corke, H.; Kitts, D. D. Anthocyanin characterization and bioactivity assessment of a dark grained wheat (*Triticum aestivum* L. Cv. Hedong Wumai) extract. *Food Chem.* **2007**, *104*, 955–961.
(72) Ozawa, T.; Nishikiori, T.; Takino, Y. Three new substituted cinnamoyl hydroxycitric acids from corn plant. *Agric. Biol. Chem.* **1977**, *41*, 359–367.
(73) Dimberg, L. H.; Molteberg, E. L.; Solheim, R.; Frohlich, W. Variation in oat groats due to variety, storage and heat treatment. I: Phenolic compounds. *J. Cereal Sci.* **1996**, *24*, 262–272.
(74) Peterson, D. M.; Wesenberg, D. M.; Burrup, D. E.; Erickson, C. A. Relationship among agronomic traits and grain composition in oat genotypes grown in different environments. *Crop Sci.* **2005**, *45*, 1249–1255.
(75) Durkee, A. B.; Thivierge, P. A. Ferulic acid and other phenolics in oat seeds (*Avena sativa* L. var Hinoat). *J. Food Sci.* **1977**, *42*, 551–552.
(76) Emmons, C. L.; Peterson, D. M. Antioxidant activity and phenolic contents of oat groats and hulls. *Cereal Chem.* **1999**, *76*, 902–906.
(77) Xing, Y. M.; White, P. J. Identification and function of antioxidants from oat groats and hulls. *J. Am. Oil Chem. Soc.* **1997**, *74*, 303–307.
(78) Sosulski, F.; Krygier, K.; Hogge, L. Free, esterified and insoluble-bound phenolic acids. 3.Composition of phenolic acids in cereal and potato flours. *J. Agric. Food Chem.* **1982**, *30*, 337–370.
(79) Collins, F. W.; Mullin, W. J. High performance liquid chromatographic determination of avenanthramides, N-aroylanthranilic acid alkaloids from oats. *J. Chromatogr.* **1988**, *445*, 363–370.
(80) Ishihara, A.; Miyagawa, H.; Matsukawa, T.; Ueno, T.; mayama, S.; Iwamura, H. Induction of hydroxyanthranilate hydroxycinnamoyl transferase activity by oligo-N-acetylchitoologosaccharides in oats. *Phytochem.* **1998**, *47*, 969–974.
(81) Dimberg, L. H.; Theander, O.; Lingnert, H. Avenanthramides: A group of phenolic antioxidants in oats. *Cereal Chem.* **1993**, *70*, 637–641.
(82) Collins, F. W. Oat phenolics: avenanthramides, novel substituted N-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* **1989**, *37*, 60–66.
(83) Chopin, J.; Dellamonica, G.; Bouillant, M. L.; Besset, A.; Popovici, G. Weissenböck. C-glycosylflavones from *Avena sativa*. *Phytochemistry* (*Elsevier*) **1977**, *16*, 2041–2043.
(84) Gavrilin, M. V.; Senchenko, S. P.; Gusov, R. M. Quantitative determination of vitexin-2-*O*-rhamnoside in common oats using HPLC and capillary electrophoresis. *Pharm. Chem. J.* **2007**, *41*, 396–398.
(85) Wong, E. Plant Phenolics. In *Chemistry and Biochemistry of Herbage*; Butler, G. W., Ed.; Academic Press: London, U.K., 1973; Vol. 1, pp 265−322.
(86) Sousa, C.; Taveira, M.; Valentão, P.; Fernandes, F.; Pereira, J.; Estevinho, L.; Bento, A.; Ferreres, F.; Seabra, R.; Andrade, P. B. Inflorescences of brassicacea species as source of bioactive compounds: A comparative study. *Food Chem.* **2008**, *110*, 953–961.
(87) Fernandes, F.; Valentão, P.; Sousa, C.; Pereira, J. A.; Seabra, R. M.; Andrade, P. B. Chemical and antioxidative assessment of dietary turnip (*Brassica rapa* var. *rapa* L.). *Food Chem.* **2007**, *105*, 1003–1010.
(88) Liang, Y.-S.; Kim, H. K.; Lefeber, A. W. M.; Erkelens, C.; Choi, Y. H.; Verpoorte, R. Identification of phenylpropanoids in methyl jasmonate treated *Brassica rapa* leaves using two-dimensional nuclear magnetic resonance spectroscopy. *J. Chromatogr., A* **2006**, *1112*, 148–155.
(89) Romani, A.; Vignolini, P.; Isolani, L.; Ieri, F.; Heimler, D. HPLC-DAD/MS characterisation of flavonoids and hydroxycinnamic derivatives in turnip tops (*Brassica rapa* L. subsp. *Sylvestris* L.). *J. Agric. Food Chem.* **2006**, *54*, 1342–1346.

Received for review July 14, 2009. Revised manuscript received November 4, 2009. Accepted November 06, 2009.