

# Enzymatic Browning in Sugar Beet Leaves (*Beta vulgaris* L.): Influence of Caffeic Acid Derivatives, Oxidative Coupling, and Coupled Oxidation

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## Supporting Information

**ABSTRACT:** Sugar beet (*Beta vulgaris* L.) leaves of 8 month (8<sub>m</sub>) plants showed more enzymatic browning than those of 3 month (3<sub>m</sub>). Total phenolic content increased from 4.6 to 9.4 mg/g FW in 3<sub>m</sub> and 8<sub>m</sub>, respectively, quantitated by reverse-phase-ultrahigh-performance liquid chromatography–ultraviolet-mass spectrometry (RP-UHPLC–UV-MS). The PPO activity was 6.7 times higher in extracts from 8<sub>m</sub> than from 3<sub>m</sub> leaves. Substrate content increased from 0.53 to 2.45 mg/g FW in 3<sub>m</sub> and 8<sub>m</sub>, respectively, of which caffeic acid glycosyl esters were most important, increasing 10-fold with age. Caffeic acid glycosides and vitexin derivatives were no substrates. In 3<sub>m</sub> and 8<sub>m</sub>, nonsubstrate-to-substrate ratios were 8:1 and 3:1, respectively. A model system showed browning at 3:1 ratio due to formation of products with extensive conjugated systems through oxidative coupling and coupled oxidation. The 8:1 ratio did not turn brown as oxidative coupling occurred without much coupled oxidation. We postulate that differences in nonsubstrate-to-substrate ratio and therewith extent of coupled oxidation explain browning.

**KEYWORDS:** *Beta vulgaris*, phenolic acids, flavonoids, PPO activity, oxidative coupling

## INTRODUCTION

For future protein demand, sugar beet leaves (*Beta vulgaris* L.) seem an attractive new protein source.<sup>1</sup> For the extraction of proteins present, cell rupture is needed.<sup>2</sup> Cell rupture brings polyphenol oxidases (PPOs) stored within chloroplasts and phenolics stored within vacuoles together in the presence of oxygen.<sup>2</sup> In such situations, PPOs oxidize phenolics into highly reactive quinones, which engage in browning reactions. Also, these quinones are prone to nucleophilic attacks by e.g., cysteine and lysine residues, resulting in covalent binding of phenolics to proteins.<sup>3</sup> These reactions affect the final techno-functional properties of the proteins extracted.<sup>4</sup> Often, PPO activity varies with plant age, environmental influences, and activation of latent enzymes in plants.<sup>5,6</sup> Currently, there is limited information on the PPO activity in *B. vulgaris* species.<sup>1</sup>

*B. vulgaris* belongs to the family of Amaranthaceae. Phenolics found in this family can be divided into phenolic acids and flavonoids. Phenolic acids (e.g., hydroxybenzoic and hydroxycinnamic acids), together with the flavonoid subclasses flavonols (e.g., quercetins) and flavones (e.g., vitexins) have been reported in *B. vulgaris* beets and leaves.<sup>7,8</sup> Similar to PPO activity, the content of total phenolics in leaves has been reported to vary with plant age. In lettuce, the total phenolic content decreased by 88% from 28 to 59 days after planting, followed by an increase to the starting content 73 days after planting. The increase in total phenolics content coincided with increases in PPO activity and browning.<sup>5</sup> No investigations on the relation between the phenolic composition and extent of brown coloring have been performed.

Plants contain reducing agents that can, in principle, delay enzymatic browning. Of these, ascorbic acid (AA) and glutathione (GSH) are the most abundant.<sup>9</sup> Ascorbic acid can

reduce the enzymatically formed quinones back into *o*-diphenolics, thereby generating dehydro-ascorbic acid (DHA). Ascorbic acid can be regenerated by glutathione-dependent DHA reductase, upon which DHA and GSH are converted into AA and glutathione disulfide (GSSG), respectively.<sup>10</sup> During plant growth and development, changes in AA and GSH contents have been reported.<sup>5,11</sup> The content of AA and GSH may affect the extent of enzymatic browning.

Earlier research in our laboratory<sup>1</sup> showed that aqueous extracts made from leaves of 3-month old sugar beet plants had a yellow color, whereas similar aqueous extracts made from leaves of 8-month old plants were brown. The observations did not depend on year of harvest or growing condition (field or greenhouse). Neither the 2-fold increase in PPO activity, measured as cresolase (hydroxylation of monophenolics into *ortho*-diphenolics) and catecholase (oxidation of *o*-diphenolics into *o*-quinones) activities, nor the 2-fold increase in total phenolic content could satisfactorily explain the differences in color between the two plant ages.<sup>1</sup> Hence, the main aim of this research was to find explanations for the difference in color formation upon cell rupture in sugar beet (*B. vulgaris* L.) leaves harvested after 3 and 8 months. It was hypothesized that the composition of individual phenolics changes over time, which might lead to increased quantities of mono- and *o*-diphenolic substrates in leaves of older plants.

In this research, the composition of substrate phenolics was determined in leaves from 3-month and 8-month old plants.

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Subsequently, the oxidative enzyme activity toward various classes of endogenous phenolic compounds was measured. In addition, the reaction products were analyzed.

## MATERIALS AND METHODS

**Chemicals.** UHPLC-grade water and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). Vitexin was obtained from Rotichrom TLC (Karlsruhe, Germany). All other chemicals and extraction solvents used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO).

**Sugar Beet Leaves Harvesting and Storage.** The leaves of sugar beets (*B. vulgaris* L. var. "Arrival") harvested in 2014, 3 and 8 months after sowing the seeds and used in our previous research,<sup>1</sup> were used. After collecting the leaves, one part was stored in vacuum sealed bags at  $-20\text{ }^{\circ}\text{C}$ . The other part of the leaves was freeze-dried and ground into powder. The freeze-dried powders were stored at  $-20\text{ }^{\circ}\text{C}$ . Dry matter contents of the leaves were determined gravimetrically after drying overnight at  $105\text{ }^{\circ}\text{C}$ . Dry matter contents were  $10.6 \pm 0.4\%$  and  $15.8 \pm 0.2\%$  w/w fresh weight for 3<sub>m</sub> and 8<sub>m</sub>, respectively.

**Preparation of the Phenolics Extracts.** Phenolics were extracted (1:10 w/v) from freeze-dried leaves with 50% (v/v) methanol containing 0.5% (v/v) acetic acid using four consecutive extractions. Preliminary extractions using larger proportions of methanol did not result in higher extraction yields of phenolics but rather in larger amounts of chlorophyll and saponin contaminants. During each extraction, samples were first sonicated for 5 min and then stirred for 30 min at  $4\text{ }^{\circ}\text{C}$  in darkness. After centrifugation ( $4\text{ }^{\circ}\text{C}$ , 5 min, 5000g), fresh solvent was added to the pellet, after which the procedure was repeated. The four extracts were combined and methanol was removed by evaporation under reduced pressure at  $40\text{ }^{\circ}\text{C}$ . The aqueous residue was freeze-dried and used for further analyses. For each plant age, samples were extracted in triplicate and analyzed separately.

**Fractionation of Phenolics.** One gram of freeze-dried phenolics extract was fractionated using a Flash chromatography system (Grace, Deerfield, IL) equipped with a 40 g Reveleris C18 Flash cartridge, 45 mL column volume (CV), particle size  $40\text{ }\mu\text{m}$  (Grace, Deerfield, IL). Elution of phenolic compounds (30 mL/min) was performed using Milli-Q water containing 1% (v/v) acetic acid (eluent A) and with acetonitrile containing 1% (v/v) acetic acid (eluent B). The cartridge was equilibrated with 3 CVs eluent B, followed by 3 CVs eluent A. Elution conditions were as follows: 0–2 min, 0% B; 2–10 min, 0–60% B; 10–11 min, 60–100% B; 11–13 min, 100% B. Fractions (10 mL) were collected and pooled into 4 pools based on ELS and/or UV<sub>280 nm</sub> responses. Pools 1–4 were taken between 0 and 3.5 min, 3.5–5.5 min, 5.5–8 min, and 8–13 min, respectively. Pools 2 and 3 were used for further analyses. Acetonitrile was evaporated from the pools under reduced pressure at  $40\text{ }^{\circ}\text{C}$ . The remaining aqueous phase was freeze-dried and stored at  $-20\text{ }^{\circ}\text{C}$  prior to further analyses. For both 3<sub>m</sub> and 8<sub>m</sub>, two fractionation runs were performed.

**Annotation and Quantitation of Phenolics.** The phenolic fractions were analyzed using UHPLC–UV–MS on an Accela system (Thermo Scientific, San Jose, CA) equipped with a pump, degasser, autosampler (set at  $15\text{ }^{\circ}\text{C}$ ), and photodiode array detector. Samples (10  $\mu\text{L}$ , 1 mg/mL in 50% (v/v) aqueous methanol) were injected onto a Hypersil Gold aQ column, 150 mm  $\times$  2.1 mm i.d.; particle size 1.9  $\mu\text{m}$  (Thermo Scientific, San Jose, CA). The column oven temperature was set at  $30\text{ }^{\circ}\text{C}$ . Elution of phenolic compounds (400  $\mu\text{L}/\text{min}$ ) was performed using water containing 1% (v/v) acetic acid (eluent A) and acetonitrile containing 1% (v/v) acetic acid (eluent B). The elution profile for the pool enriched in phenolic acids (pool 2) was as follows: 0–4 min, 1% B; 4–16 min, 1–22% B; 16–18 min, 22–100% B; 18–20 min, 100% B; 20–21 min, 100–1% B; 21–24 min, 1% B. The elution profile for the pool enriched in flavonoids (pool 3) was as follows: 0–4 min, 1% B; 4–8 min, 1–17% B; 8–25 min, 17–30% B; 25–27 min, 30–100% B; 27–29 min, 100% B; 29–30 min, 100–1% B; 30–32 min, 1% B. For both pools, throughout elution, PDA spectra (200–600 nm) were recorded.

Mass spectrometric ( $\text{MS}^n$ ) data were obtained by analyzing samples on a LTQ-XL Velos mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a heated electrospray ionization probe coupled to the UHPLC system. The instrument was tuned with caffeic acid for pool 2 and with apigenin for pool 3, with capillary temperature at  $350\text{ }^{\circ}\text{C}$ , source heater temperature at  $230\text{ }^{\circ}\text{C}$ , and source voltage at 3.5 kV. A full mass spectrum was recorded over the  $m/z$  range of 150–1500. The annotation of compounds was performed based on the parent molecular ion,  $\text{MS}^n$  data and UV/vis spectra. Data acquisition and reprocessing were performed with Xcalibur 2.07 software (Thermo Scientific, San Jose, CA). For quantitation of phenolics, external calibration curves were used. Quantities of phenolic acids were expressed as caffeic acid equivalents as 42% w/w of the phenolic acid derivatives found contained a caffeic acid backbone. Quantities of flavonoids were expressed as vitexin equivalents as 78% w/w of the flavonoids found were vitexin derivatives. For both compounds, seven concentrations were taken for calibration, based on full MS intensity and measured in duplicate. For caffeic acid: 1.15–115.0  $\mu\text{g}/\text{mL}$ ,  $R^2$  0.91, limit of quantitation (LOQ) was 0.3  $\mu\text{g}/\text{mL}$ . For vitexin: 0.1–13.5  $\mu\text{g}/\text{mL}$ ,  $R^2$  0.97, LOQ was 0.05  $\mu\text{g}/\text{mL}$ . Individual compounds were quantitated based on full MS signal. To account for MW difference between the calibrant and the detected compound, correction factors were calculated by dividing the molecular weight of the analyte by that of the calibrant (Table 1). The MS response was multiplied by the correction factor to obtain the amounts present.<sup>12</sup> Amino acid derivatives were quantitated using caffeic acid as the standard. The phenolic contents in the leaves were expressed in mg/g fresh weight (FW).

**Phenolics Identification Strategy.** Phenolic backbone structures (Figure 1) were identified based on UV/vis spectra and mass spectra of authentic standards (tryptophan, caffeic acid, ferulic acid, sinapic acid, apigenin, vitexin, and quercetin (1 g/L in 50% (v/v) aqueous methanol)). The use of these standards allowed determination of the diagnostic ions for hydroxycinnamic acids<sup>13,14</sup> and flavonoids.<sup>7,15</sup> Phenolic acids show two absorbance maxima, one around 280 nm and one in the range of 320–355 nm.<sup>16</sup> Flavonoids show absorbance around both 265 and 350 nm.<sup>16</sup> The presence of phenolic derivatives was determined by the presence of characteristic neutral losses upon  $\text{MS}^2$  and  $\text{MS}^3$  fragmentation. Phenolic acid *O*-glycosides<sup>17</sup> (2, Figure 2) and flavonoid *O*-glycosides were annotated by a neutral loss of 162 (*O*-hexose). Glycosylation in phenolic acid glycosyl esters (6, Figure 2) was annotated by a neutral loss of 162 (*O*-hexose)<sup>14,18</sup> or 132 (*O*-pentose)<sup>18</sup> and additional polyol fragment ions ( $-30$ ,  $-60$ ,  $-90$ )<sup>13,17</sup> in  $\text{MS}^2$ , originating from ring fission fragmentation within the glycosyl residue. *C*-glycosylation could be distinguished from *O*-glycosylation by a neutral loss of 120 (*C*-hexose).<sup>7,18,19</sup> Acetylation and malonylation were annotated by neutral losses of 42 and 86, respectively.<sup>18</sup>

**Preparation of Enzyme Extracts.** For enzyme extraction, 50 g of frozen leaves from the 3 and 8 months old plants were used, and extraction was performed as described previously.<sup>1</sup> Dialyzed enzyme extracts were used immediately for enzyme assays.

**PPO Activity Assay and Quantitation of Reacting Phenolics.** PPO activities in sugar beet lead extracts were determined by oxygen consumption. The oxygen consumption analyses were performed using an Oxytherm System (Hansatech, Kings Lynn, United Kingdom). Aliquots (700  $\mu\text{L}$ ) of dialyzed enzyme extract were incubated with 200  $\mu\text{L}$  of 5 g/L Flash-fractionated phenolics, 1.2 g/L catechin, or 0.9 g/L vitexin. Substrates were dissolved in 150 mM sodium phosphate buffer pH 6.7. Oxygen consumption was monitored over 30 min. The rate of oxygen consumption during the first 10 s was taken as measure for PPO activity, expressed as nmol of  $\text{O}_2$  per second per gram fresh weight (nmol  $\text{O}_2/(\text{s g})$  FW).

Quantitation of phenolics before and after reaction with leaf dialyzed enzyme extracts was performed using RP-UHPLC–UV–MS, as described above. An aliquot (200  $\mu\text{L}$ ) of dialyzed enzyme extract was incubated with 100  $\mu\text{L}$  of Flash-fractionated phenolics (5 g/L), dissolved in 150 mM sodium phosphate buffer pH 6.7. After 20 h the reaction was stopped by addition of 300  $\mu\text{L}$  of methanol. A sample at  $t_0$  was prepared in the same way to correct for trace amounts of

Table 1. Overview of Phenolics Annotated and Quantitated in the Aqueous Methanolic Extracts from Sugar Beet Leaves

compd no.	UV max	$[M - H]^-$ m/z	MS <sup>2</sup> m/z	MS <sup>3</sup> m/z	identification	MWCF	3 <sub>m</sub> (mg/g FW)	8 <sub>m</sub> (mg/g FW)
<b>Caffeic Acid Derivatives</b>								
1 <sup>a</sup>	355 <sup>b</sup>	215	<u>179</u> <sup>c</sup> , <u>161</u>	<u>161</u> , <u>89</u>	caffeic acid derivative	1.20	0.08	0.17
2	267, 379	341	<u>179</u> , <u>135</u>	<u>143</u> , <u>135</u>	caffeic acid <i>O</i> - $\beta$ -hexoside <sup>13,17</sup>	1.91	0.94	2.12
4	280, 320	661	<u>661</u> , <u>306</u>	<u>306</u> , <u>593</u> , <u>272</u> , <u>481</u> , <u>179</u> , <u>161</u>	caffeoyl-spermine-hexosyl ester <sup>14</sup>	4.32	0.04	0.02
6	279, 339	383	<u>341</u> , <u>343</u> , <u>179</u>	<u>179</u> , <u>161</u> , <u>119</u>	caffeic acid hexosyl-acetyl ester	2.14	n.q. <sup>d</sup>	0.18
7	- <sup>b</sup>	297	<u>179</u> , <u>161</u> , <u>237</u>	<u>161</u> , <u>89</u> , <u>135</u>	<i>O</i> -caffeoyl threonic acid ester <sup>30</sup>	1.66	0.01	0.02
11	279, 339	261	<u>181</u> , <u>199</u> , <u>215</u>	<u>163</u> , <u>135</u> , <u>119</u>	dihydrocaffeic acid sulfate ester	1.46	n.q.	0.03
18	288, 309	487	<u>307</u> , <u>427</u> , <u>325</u> , <u>161</u> , <u>193</u>		caffeoyl-feruoyltartaric acid <sup>31</sup>	2.72	n.q.	1.47
<b>Amino Derivatives</b>								
3	280	330	<u>222</u> , <u>240</u> , <u>252</u>	<u>150</u> , <u>135</u> , <u>109</u>	dehydro-dopamine derivative	1.84	n.q.	n.q.
5	280	314	<u>224</u> , <u>278</u> , <u>194</u>	<u>152</u>	dopamine-hexosyl ester	1.75	0.12	0.17
8	261	344	<u>150</u> , <u>133</u>	<u>133</u> , <u>107</u>	dehydrodopamine derivative	1.92	0.02	0.06
9	277	282	<u>150</u>	<u>133</u>	dehydrodopamine <i>O</i> -pentoside	1.58	n.q.	n.q.
10	279	192	<u>192</u> , <u>150</u>	<u>150</u> , <u>121</u>	acetyl-dehydrodopamine	1.07	n.q.	n.q.
13	280	203	<u>159</u> , <u>131</u> , <u>203</u>	<u>131</u> , <u>148</u>	tryptophan <sup>23</sup>	1.13	n.q.	n.q.
16	284	354	<u>192</u> , <u>264</u> , <u>294</u>	<u>150</u> , <u>174</u> , <u>192</u> , <u>232</u>	dehydrodopamine-hexosyl-acetyl ester	1.98	n.q.	n.q.
<b>Sinapic Acid Derivatives</b>								
12	281, 310	439	<u>241</u>	<u>97</u> , <u>132</u> , <u>223</u>	unidentified sinapic acid ester	2.45	n.q.	n.q.
21	281, 320	651	<u>223</u> , <u>489</u>	<u>208</u> , <u>179</u>	sinapic acid <i>O</i> -hexosyl derivative	3.64	0.01	0.70
24	280, 322	445 <sup>e</sup>	<u>385</u>	<u>223</u> , <u>295</u> , <u>265</u> , <u>153</u> , <u>161</u> , <u>179</u>	sinapic acid <i>O</i> -hexosyl ester <sup>14</sup>	2.49	0.16	0.34
29	325	489	<u>223</u> , <u>205</u>	<u>164</u> , <u>179</u>	sinapic acid derivative	2.73	0.24	0.21
<b>5-OH Ferulic Acid Derivatives</b>								
14	309	355	<u>191</u> , <u>209</u> , <u>147</u>	<u>85</u> , <u>147</u>	5-OH ferulic acid <i>O</i> -deoxyhexoside	1.98	n.q.	n.q.
15	311	355	<u>191</u> , <u>265</u> , <u>209</u> , <u>111</u>	<u>85</u> , <u>147</u>	5-OH ferulic acid <i>O</i> -deoxyhexoside ester	1.98	n.q.	n.q.
<b>Coumaric Acid Derivatives</b>								
17	288	305	<u>225</u> , <u>165</u> , <u>97</u>	<u>165</u> , <u>181</u> , <u>147</u>	dihydro- <i>p</i> -coumaric acid derivative	1.70	n.q.	0.01
22	284, 309	337	<u>191</u> , <u>289</u> , <u>163</u>	<u>85</u> , <u>147</u> , <u>173</u>	5- <i>p</i> -coumaroylquinic acid <sup>21</sup>	1.88	0.2 <sup>f</sup>	0.28
25	284, 309	337	<u>173</u> , <u>191</u> , <u>163</u> , <u>130</u>	<u>85</u> , <u>57</u> , <u>111</u>	4- <i>p</i> -coumaroylquinic acid	1.88		
<b>Ferulic Acid Derivatives</b>								
20	325 <sup>b</sup>	621	<u>193</u> , <u>459</u> , <u>445</u> , <u>265</u>	<u>149</u> , <u>178</u> , <u>134</u>	Ferulic acid <i>O</i> -hexosyl ester derivative	3.47	0.04	0.63
23	292, 314	487	<u>193</u> , <u>355</u> , <u>149</u>	<u>149</u> , <u>178</u> , <u>193</u> , <u>134</u>	ferulic acid <i>O</i> -pentosyl-hexoside	2.72	n.q.	n.q.
26	283, 309	445	<u>283</u> , <u>343</u> , <u>301</u> , <u>401</u>	<u>138</u> , <u>165</u> , <u>193</u> , <u>286</u>	ferulic acid <i>O</i> -hexosyl-derivative	2.49	n.q.	0.26
27	280, 325	355	<u>193</u> , <u>265</u> , <u>235</u> , <u>175</u> , <u>217</u> , <u>134</u>	<u>134</u> , <u>149</u> , <u>178</u>	ferulic acid-hexosyl ester	1.98	0.32	0.46
28	287, 323	517	<u>193</u> , <u>355</u> , <u>337</u>	<u>134</u> , <u>149</u> , <u>178</u>	ferulic acid <i>O</i> -dihexosyl ester	2.89	0.4	0.13
30	285, 323	443	<u>267</u> , <u>193</u> , <u>134</u>	<u>249</u> , <u>113</u> , <u>175</u>	ferulic acid derivative <sup>14</sup>	2.47	0.29	0.12
31	282, 326	473	<u>267</u> , <u>193</u>	<u>249</u> , <u>113</u> , <u>175</u>	ferulic acid derivative	2.64	0.39	0.53

Table 1. continued

compd no.	UV max	$[M - H]^-$ m/z	MS <sup>2</sup> m/z	MS <sup>3</sup> m/z	identification	MWCF	3 <sub>m</sub> (mg/g FW)	8 <sub>m</sub> (mg/g FW)
Quercetin Derivatives								
32	271, 301, 395	625	301, 300, 273, 179	179, 257, 151	quercetin O-dihexoside <sup>8,15</sup>	1.45	0.03	0.05
Vitexin Derivatives								
33	269, 331	593	431, 293	293	vitexin-2''-O-hexoside <sup>19,21</sup>	1.38	0.34	0.41
34	269, 332	563	413, 443, 311, 293	293	vitexin-2''-O-pentoside <sup>7,19</sup>	1.31	0.34	0.36
35	269, 330	431	311, 341, 191	283, 311, 191	vitexin	1.00	n.q.	0.02
36	269, 330	635	473, 311	311, 413	vitexin O-hexoside	1.47	0.30	0.20
38	269	679	635, 593	455, 413, 293, 269	malonyl-hexosylvitexin	1.58	n.q.	n.q.
40	269, 330	649	605, 455	455, 311, 473, 563, 269	malonyl-pentosylvitexin	1.51	0.13	0.13
41	268, 331	473	413, 293	293, 161	vitexin acetyl derivative	1.10	0.03	n.q.
iso-Rhamnetin Derivatives								
37	254, 351	639	315, 300, 271, 255	300, 315, 287, 107, 151	iso-rhamnetin O-dihexoside <sup>7,15</sup>	1.48	0.14	0.35
39	267, 341	609	315, 300	300	isorhamnetin derivative <sup>32</sup>	1.41	0.13	0.09

<sup>a</sup>The numbers correspond to the peak numbers in Figure 3. MWCF, molecular weight correction factors used for other unidentified compounds; therefore, absorbance maxima were not or only partially provided. <sup>b</sup>Values underlined represent diagnostic ions for the respective compounds.<sup>14,15,19</sup> n.q. = not quantitated, concentration was below the limit of quantitation. <sup>c</sup>Parent ion for compound 24 is supposed to be an acetic acid adduct ( $M + 60 - H$ ).<sup>7</sup>For quantitation, 22 and 25 were combined.

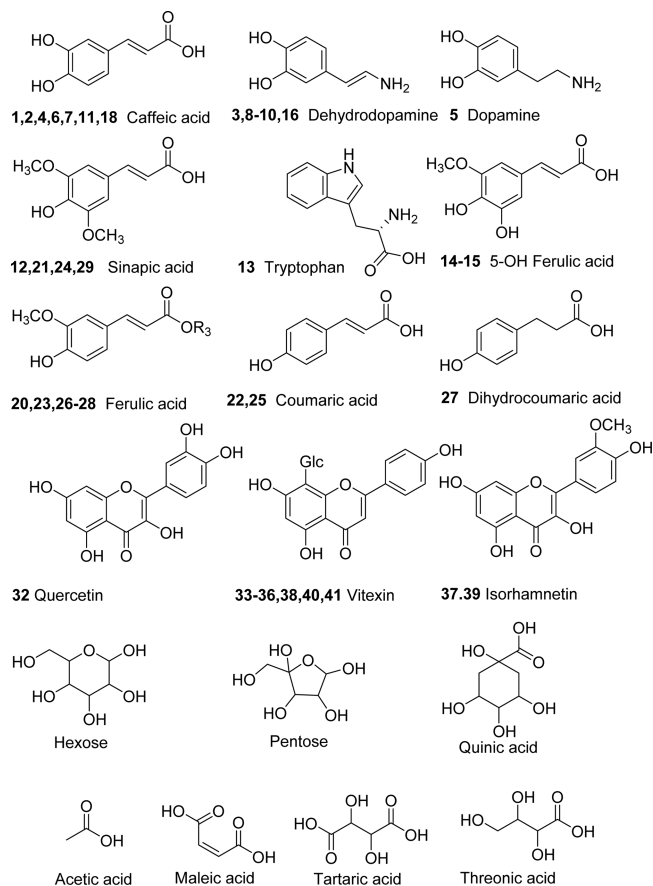


Figure 1. Phenolic backbone structures and potential side groups annotated in leaves of *B. vulgaris*.

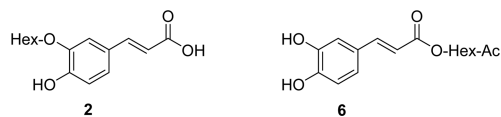


Figure 2. Structures of (2) caffeic acid hexoside and (6) caffeic acid hexosyl-acetyl ester.

phenolics present in the dialyzed enzyme extract. In this sample, methanol was added directly to prevent the enzyme reaction.

Contributions of nonsubstrate phenolics to browning via oxidative coupling was investigated using model systems. Equimolar contents of chlorogenic acid with sinapic acid, chlorogenic acid with both ferulic acid and sinapic acid (1 mM), and different molar ratios of chlorogenic acid with ferulic acid (1:1, 1:3, 1:8) in 10 mM sodium phosphate buffer pH 6.7 were incubated with 15 U<sup>20</sup> mushroom tyrosinase for 30 and 60 min. Reaction products were diluted 10 times in 50% (v/v) methanol. The reaction mixtures with 1:1 molar ratios were analyzed using RP-UHPLC-UV-MS as described above with an altered gradient: 0–1 min, 5% B; 1–9 min, 5–50% B; 9–10 min, 50–100% B; 10–12 min, 100% B; 12–13 min, 100–5% B; 13–15 min, 5% B. The injection volume was 5  $\mu$ L. The UV/vis spectra of the reaction mixtures were recorded using a UV-1800 spectrophotometer and UV Probe 2.00 software (Shimadzu, Kyoto, Japan).

**Ascorbic Acid and GSH Quantitation.** The method to quantitate ascorbic acid (AA) and dehydro-ascorbic acid (DHA) in leaves was adapted from Davey et al.<sup>9</sup> Freeze-dried leaves (50 mg) were ground in liquid nitrogen. Next, 1.5 mL of 3.3% (w/v) metaphosphoric acid was added, and the mixture was sonicated on ice for 10 min. The mixture was centrifuged (4  $^{\circ}$ C, 10 min, 25 000g) and the supernatant was filtered through a 0.45  $\mu$ m cellulose filter (Whatman, Dassel, Germany) prior to AA and GSH analysis. To convert DHA

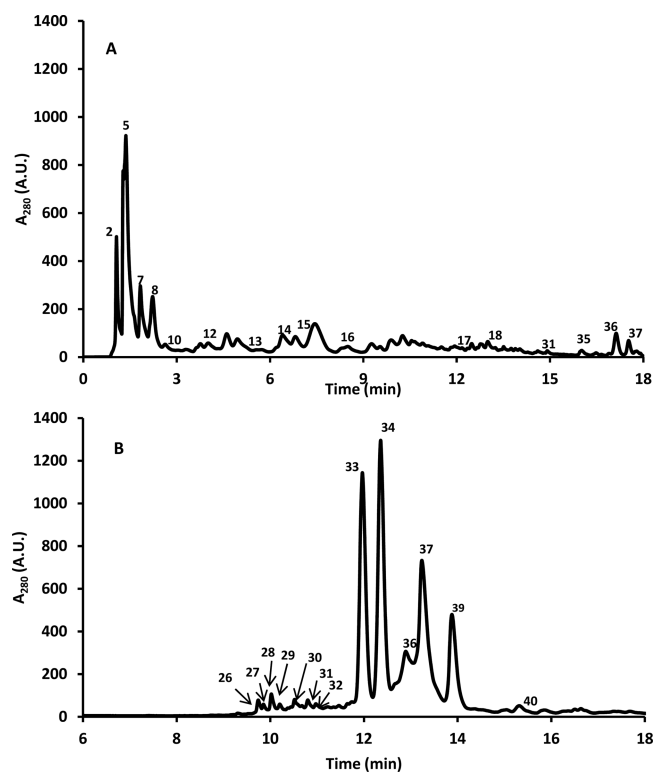
and GSSG into AA and GSH, respectively, 100  $\mu\text{L}$  of the filtered supernatant was mixed with 5 mM DTT in 400 mM Tris base, pH 9.0, and incubated at room temperature in the dark for 15 min. The reaction was stopped by addition of 50  $\mu\text{L}$  of 8.5% (w/v) *o*-phosphoric acid. Four samples of both ages were injected onto a ProntoSIL 120-3 C18 AQ column (Knauer, Berlin, Germany) and eluted isocratically with water containing 0.04% (v/v)  $\text{H}_3\text{PO}_4$ , 0.25% (v/v) methanol, and 0.1 mM EDTA. Flow rate was 0.35 mL/min and elution was followed by a wash step with 30% (v/v) aqueous acetonitrile. AA and GSH were detected at 243 and 197 nm, respectively. For quantitation, external AA and GSH calibration curves were used (0–0.1 g/L in 3% (v/v) *m*-phosphoric acid containing 2.5 mM DTT,  $R^2 = 0.98$  for both AA and GSH). The DHA and GSSG contents were determined by the differences in AA and GSH contents after and before DHA and GSH reduction, respectively.

## RESULTS AND DISCUSSION

**Effect of Plant Age on Phenolics Composition.** The phenolics composition was analyzed in sugar beet leaves from 3 ( $3_m$ ) and 8-month old plants ( $8_m$ ). In order to facilitate annotation of phenolics, the crude extract was fractionated into 4 pools. The majority of the crude methanolic extract consisted of mono- and disaccharides which were collected in pool 1 ( $49.5 \pm 2.9\%$  w/w of the extract). UHPLC–UV–MS analysis showed components eluting in the void of the column, which had no 280 nm absorbance. During MS/MS fragmentation, characteristic neutral losses of  $-162$  for sugars were observed. For  $3_m$  and  $8_m$ , pools 2 and 3 represented  $8.7 \pm 1.7\%$  w/w and  $14.8 \pm 1.5\%$  w/w of the methanolic extract, respectively. Pool 4 contained saponins, which had high column retention and no 280 nm absorbance. Pools 1 and 4 were not included in further analysis as no phenolics were present.

Pool 2 (Figure 3A, Table 1) consisted mainly of *O*-glycosylated derivatives of caffeic- (2, 4, 6), ferulic- (20, 23, 26–28), and sinapic acid (21, 24). Compound 2 was annotated as caffeic acid *O*- $\beta$ -hexoside, with characteristic caffeoyl fragments in MS.<sup>3,17</sup> Glycosylated coumaric acids were not annotated. Trace amounts of dopamine (3, 5, 8–10, 16) and tryptophan (13) derivatives were annotated. Pool 3 (Figure 3B, Table 1) consisted mainly of iso-rhamnetin, quercetin, and vitexin (8-*C*-hexosides of apigenin) derivatives. Vitexins have been previously identified in Swiss chard (*B. vulgaris* subsp. *cycla*),<sup>7</sup> passiflora,<sup>19</sup> and tea leaves.<sup>21</sup> The vitexin derivatives included those which contained an extended *C*-glycosyl residue: vitexin-2'' *O*-hexoside (33), vitexin-2'' *O*-pentoside (34), malonyl-hexosylvitexin (38), and malonyl-hexosylvitexin (40).

On the basis of the phenolic structure, PPO substrates were annotated. Phenolics containing a free hydroxyl (e.g., coumaric acid) or a free *o*-dihydroxyl (e.g., caffeic acid) motif on the benzene ring could be considered as potential substrates. The presence of mono- or *o*-dihydroxyl motifs in a phenolic molecule does not necessarily mean that it is a PPO substrate, as other substituents may be present. Phenolics containing a single hydroxyl- and additional methylated *o*-hydroxy substituents on the benzene ring (e.g., ferulic acid) are not considered to be substrates, because an *o*-diphenol and subsequently an *o*-quinone, cannot be formed. Additionally, phenolic acid glycosides (e.g., 2, Figure 2) and phenolic acid glycosyl esters (e.g., 6, Figure 2) were distinguished. In the former, the glycosyl residue is connected via an acetal linkage to an OH group on the phenolic ring, which thereby hinders quinone formation. In the latter, the glycosyl residue is esterified to the carboxyl group, maintaining the *o*-diphenol function. Caffeic acid *O*- $\beta$ -hexoside (2) and dopamine-hexoside



**Figure 3.** RP-UHPLC–UV chromatograms at 280 nm of (A) pool 2 (phenolic acids) and (B) pool 3 (flavonoids). Numbers represent compounds as listed in Table 1.

(5) were tentatively annotated as glycosides (Table 1). The derivatives of caffeic acid (4, 6), sinapic acid (24), and ferulic acid (27, 28) were tentatively annotated as glycosyl esters. Derivatives of ferulic acid, sinapic acid, and iso-rhamnetin are nonsubstrates for PPOs, as one or more of the *o*-hydroxyl oxygens are methylated. Qualitatively, the phenolics profile obtained (Table 1) correlated with those of *B. vulgaris* species, determined previously.<sup>8,22,23</sup>

To study differences in individual phenolics upon plant aging, all components were quantitated individually (Table 1). Phenolics might be quantitated based on UV-intensity or based on mass signals. As the UV-chromatograms showed high baselines and partial coelution of compounds (Figure 3), quantitation was performed on the basis of full-MS signals, using external calibration curves of caffeic acid and vitexin. The total contents of phenolics quantitated increased from  $4.6 \pm 0.7$  mg/g FW in  $3_m$  to  $9.4 \pm 0.8$  mg/g FW in  $8_m$  (Table 2). Contents were comparable to those found in Swiss chard (*B. vulgaris* subsp. *cycla*).<sup>8</sup> Phenolic acid derivatives represented the major part of the phenolics and increased from 3.1 mg/g FW in  $3_m$  to 7.6 mg/g FW in  $8_m$ . Of the phenolic acids, caffeic acid hexosyl ester content showed the largest increase from 0.2 to 1.9 mg/g FW. The overall content of coumaric acid derivatives was 0.3 mg/g FW for both ages. Sinapic acid derivatives content increased, from 0.4 mg/g FW in  $3_m$  to 1.2 mg/g FW in  $8_m$ . Contents of quercetin- (0.04 mg/g FW), vitexin- (1.1 mg/g FW), and iso-rhamnetin (0.4 mg/g FW) derivatives did not change with age. In both plant ages, glycosylated amino acids (3, 5, 8–10, 16) comprised 2% w/w of the total phenolics.

Overall, these results suggested that the composition of phenolics, rather than the total content of phenolics, is a

**Table 2.** Contents of Phenolic Acids and Flavonoids (mg/g FW) in Leaves of Sugar Beets Harvested 3 and 8 Months after Sowing

phenolic class	3 <sub>m</sub>	8 <sub>m</sub>	substrate for PPO
<b>Amino Acids</b>			
dopamine derivatives	0.1 ± 0.04	0.2 ± 0.05	yes
tryptophan	n.q. <sup>a</sup>	n.q.	no
subtotal	0.1	0.2	
<b>Phenolic Acids</b>			
coumaric acid derivatives	0.2 ± 0.05	0.3 ± 0.1	yes
caffeic acid hexosides	0.9 ± 0.2	2.1 ± 0.2	no
caffeic acid esters	0.2 ± 0.03	1.9 ± 0.1	yes
ferulic acid derivatives	1.4 ± 0.07	2.1 ± 0.1	no
sinapic acid derivatives	0.4 ± 0.03	1.2 ± 0.06	no
subtotal	3.1	7.6	
<b>Flavonoids</b>			
vitexin derivatives	1.1 ± 0.3	1.1 ± 0.1	no
quercetin hexoside	0.03 ± 0.0	0.05 ± 0.0	yes
iso-rhamnetin derivatives	0.3 ± 0.03	0.4 ± 0.1	no
subtotal	1.4	1.6	
total	4.6	9.4	

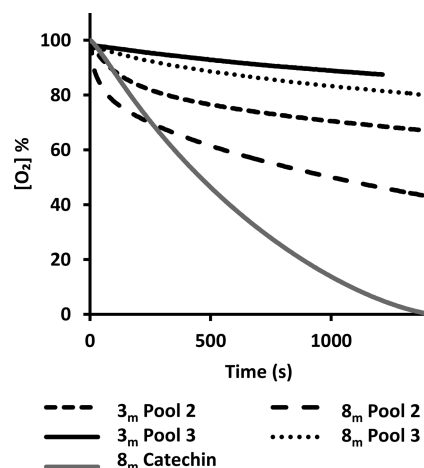
<sup>a</sup>n.q. = not quantitated, concentration below quantitation limit.

determinant factor in explaining the difference in color between the 3<sub>m</sub> and 8<sub>m</sub> leaf extracts.

**Effect of Plant Age on PPO Activity.** In our previous research,<sup>1</sup> we observed that the cresolase activity increased 6-fold in leaves from 3 to 8 months old, whereas the catecholase activity did not change. The cresolase activity in 8<sub>m</sub> was still 10 times lower than catecholase activity. Activities were determined colorimetrically using pure catechin and tyrosine. Of these compounds, it is unknown whether these are ideal substrates for PPO from sugar beet leaves, as these substrates are normally not present in high amounts. In the present research, pools of endogenous leaf phenolics were used, which were assumed to be better PPO substrates than catechin and tyrosine. The PPO activity was analyzed by incubation of the dialyzed enzyme extracts with catechin as the positive control and with fractionated phenolic pools and monitoring the oxygen consumption (Figure 4). The reaction continued until all oxygen was depleted. For catechin, reaction rates (0.03 ± 0.01 nmol of O<sub>2</sub>/(s g) FW) were similar for 3<sub>m</sub> and 8<sub>m</sub> dialyzed enzyme extracts. Results were comparable to those determined previously.<sup>1</sup> For pool 2, the color changes and reaction rates were high in the beginning, especially for the 8<sub>m</sub> incubation. For this pool, the initial rates were 6.7 ± 0.6 times higher in 8<sub>m</sub> than in 3<sub>m</sub>, i.e., 0.29 nmol of O<sub>2</sub>/(s g) FW and 0.05 nmol of O<sub>2</sub>/(s g) FW, respectively. For both 3<sub>m</sub> and 8<sub>m</sub>, incubations with pool 2 resulted in a red/brown color.

For both plant ages, the dialyzed enzyme extracts had low activity toward pool 3 and rates were similar, 0.02 nmol of O<sub>2</sub>/(s g) FW for 3<sub>m</sub> and 0.04 nmol of O<sub>2</sub>/(s g) FW for 8<sub>m</sub>. During the reaction, the color did not change.

The difference in activity with plant age might be caused by activation of latent enzymes in 8<sub>m</sub> leaves, resulting in higher absolute amounts of PPO. Similar observations have been made for red clover and wheat leaves.<sup>6,24</sup> There, the amounts of PPO, however, were not quantified. The difference in activity might



**Figure 4.** Monitoring of oxygen consumption over time during incubations of dialyzed enzyme extract from sugar beet leaves of 3- and 8-month old plants with pool 2 (1 g/L), pool 3 (1 g/L), and catechin (1.2 g/L) (only the incubations with 8<sub>m</sub> leaf extracts are presented; 3<sub>m</sub> leaf extracts gave similar profiles).

explain part of the differences in browning behavior of sugar beet leaves.

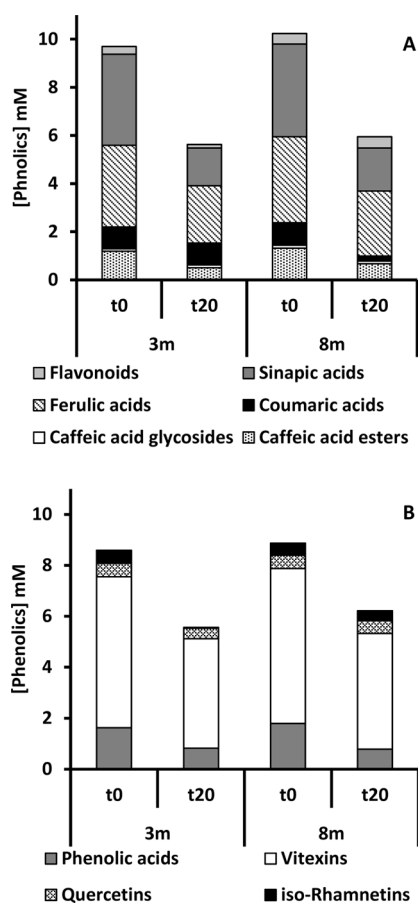
#### Activity of Sugar Beet PPO on Endogenous Phenolics.

As both pools consisted of mono- and *o*-diphenolics, no distinction between cresolase and catecholase activity could be made by oxygen consumption measurements. In order to distinguish these enzyme activities, the concentrations of individual phenolics were determined by UHPLC–UV–MS analysis before and after incubation. Regarding the quantitation of the incubations with pools, only results obtained with pools 2 and 3 from 3<sub>m</sub> plants are shown (Figure 5), as those obtained from 8<sub>m</sub> were comparable. The compositions of residual phenolics after incubation were similar for both plant ages, indicating that the enzymes by themselves had similar substrate specificities.

Prior to incubation, pool 2 from 3<sub>m</sub> contained 8 mol % mono-, 12 mol % *o*-diphenolics, and 80 mol % nonsubstrate phenolics. After incubation of this pool with dialyzed enzyme extract of both plant ages, the total amount of phenolics originally present decreased by 42% (Figure 5A). Amino acid-derivatives were not quantitated as the concentrations found were below the LOQ. No MS peaks indicating the formation of particular oxidative condensation products were annotated. Given the formation of insoluble aggregates during incubation and the increase in UV absorbance >400 nm in the UHPLC–UV chromatograms, it is likely that such condensation products were formed.

The caffeic acid hexosyl esters content in pool 2 was about 12 mol %, and these molecules were the main reactants (Figure 5A). The total content of caffeic acid esters decreased by 60% and 49% in 3<sub>m</sub> and 8<sub>m</sub>, respectively. No changes in compound 2 (caffeic acid hexoside) were found, confirming that it is not a catecholase substrate. The total content of coumaric acid derivatives did not change in 3<sub>m</sub> and decreased by 79% in 8<sub>m</sub>. All three components (17, 22, and 25) reacted. Ferulic- and sinapic acid derivatives decreased for both plant ages, except the ferulic acid *O*-hexoside (27). Overall, sinapic acid diglycosides (23, 28) decreased by 30%, while sinapic and ferulic acid monohexosides (20, 21, 24, 26, and 27) decreased by 60%.

The lack of activity toward pool 3 observed during oxygen consumption measurements (Figure 4) was not expected as



**Figure 5.** Phenolics contents (mM) of (A) pool 2 and (B) pool 3 before ( $t_0$ ) and after ( $t_{20}$ ) incubations with dialyzed enzyme extract from sugar beet leaves of 3- and 8-month old plants.

vitexin was the main flavonoid present (Figure 5B). Potentially, vitexin is a cresolase substrate due to the monohydroxylated benzene ring, which might be converted into an *o*-diphenol. The lack of activity raised the question whether vitexin is indeed a cresolase substrate. This was tested by incubating vitexin with dialyzed enzyme extract and determination of oxygen consumption (data not shown). No activity was observed and the nonreactivity of vitexin is most likely related to the 8-C-hexosyl function, hindering access to the active site.

Prior to incubation, pool 3 from  $3_m$  contained 16 mol % mono-, 9 mol % *o*-diphenolics, and 75 mol % nonsubstrate phenolics, taking into account that vitexin was a nonsubstrate. The pool contained 69 mol % vitexin derivatives and about 2 mol % caffeic acid esters (Figure 5B). The total decrease (26% for both plant ages) in phenolics quantitated after reaction was mainly caused by a decrease in the content of the vitexin derivatives. Quantitations performed on both pools 2 and 3 indicated that nonsubstrates (ferulic acid, sinapic acid, vitexin derivatives) participated in nonenzymatic continuation reactions.

Of the PPO substrates annotated in the leaves, mainly the caffeic acid esters reacted, independent of the enzyme extract applied (Figure 5A). Monophenolic derivatives decreased three times less than *o*-diphenolic derivatives. Tyrosinase-like enzymes possess both the cresolase and catecholase activity within a single enzyme. The use of *o*-diphenolics is preferred and the hydroxylation step (cresolase activity) is known to be rate limiting.<sup>25</sup> Within the  $8_m$  leaves, the total amount of

diphenolic substrates was 10-fold higher than in  $3_m$  and we propose that this contributed to higher browning potential. There were no changes in total monophenolic substrate content, and this represented on average only 3.5% w/w of the total phenolics pool. Therefore, cresolase activity is expected to have minor influence. Additionally, low monophenolic substrate content might explain the low cresolase activity observed previously as the same leaves were used for this research.<sup>1</sup>

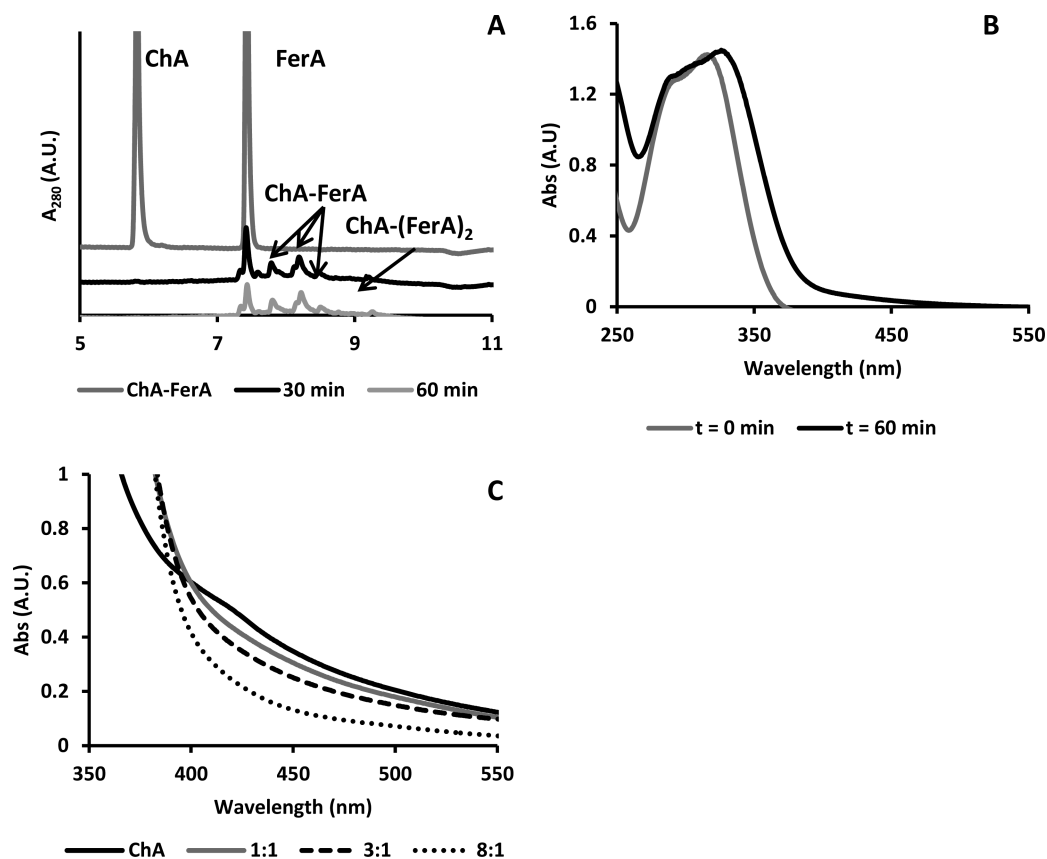
#### Participation of Nonsubstrate Phenolics in Color Formation.

Both PPO activity and nonenzymatic continuation reactions result in the formation of colored oxidative coupling products.<sup>26</sup> In this process, nucleophilic phenolics, which are not necessarily PPO substrates, attack the enzymatically formed quinones and form a covalent bond. To test whether this could indeed explain the observed decrease of nonsubstrates, a model system was used in which chlorogenic acid was incubated with tyrosinase either with or without an equimolar amount of ferulic acid or sinapic acid. After 30 min, a chlorogenic acid dimer ( $m/z$  705) (Figure 6A) was annotated in all samples. In addition, when a mixture of phenolics was incubated, products with  $m/z$  values of 545 (chlorogenic acid-ferulic acid) and 575 (chlorogenic acid-sinapic acid) were annotated. This was indicated by MS<sup>n</sup> fragmentation spectra showing the loss of chlorogenic acid and diagnostic ions for chlorogenic acid, ferulic acid, and sinapic acid. After 60 min of incubation, (ferulic acid)<sub>2</sub>-chlorogenic acid with  $m/z$  737 and (chlorogenic acid)<sub>2</sub>(sinapic acid)<sub>2</sub> with  $m/z$  1153 were tentatively annotated. Sinapic acid was somewhat more reactive than ferulic acid as after 30 min of incubation all sinapic acid and 85% of the initial ferulic acid had reacted. Sinapic acid was also more reactive than ferulic acid when all three compounds were incubated together (data not shown). The final reaction mixtures showed absorbance around 400 nm (Figure 6B), which was expected to relate to formation of coupling products with enlarged conjugated systems. The PDA spectra of the dimers ( $\lambda_{max}$  320–340 nm) themselves had no absorbance in the visible wavelength range. With increasing ferulic acid-to-chlorogenic acid molar ratio, the color formation decreased (Figure 6C).

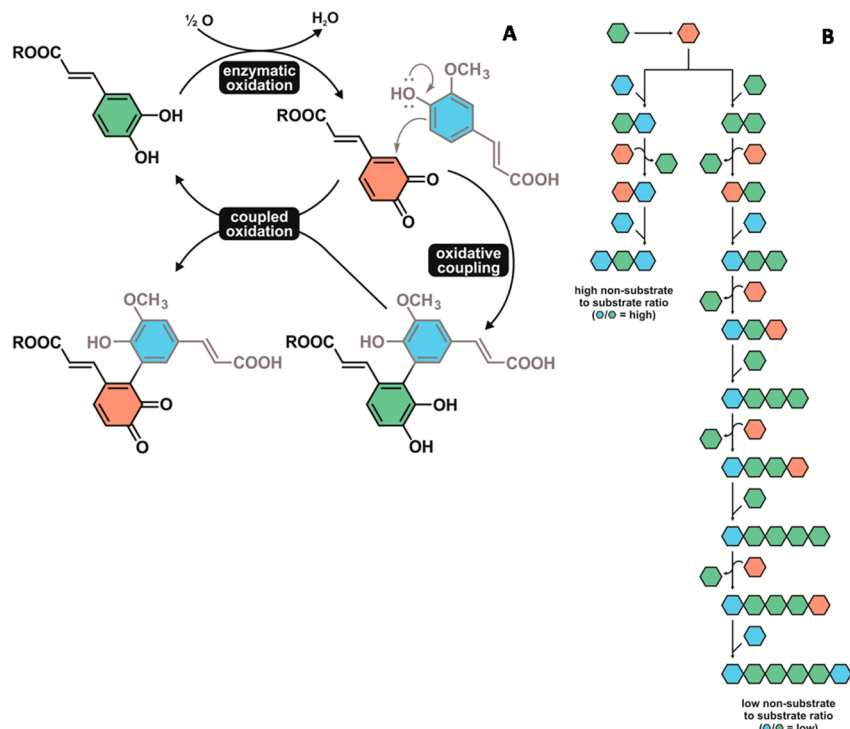
#### Effect of the Nonsubstrate-to-Substrate Ratio on Color Formation.

The majority of the leaf phenolics were nonsubstrates for PPO. The decreases observed in these derivatives were caused by combinations of oxidative coupling<sup>26</sup> and coupled oxidation<sup>27</sup> reactions, as evidenced with the model substrate chlorogenic acid. The mechanism is proposed in Figure 7A, in which chlorogenic acid is displayed more generically as caffeic acid ester. Initially, monomeric caffeic acid ester is enzymatically oxidized into a quinone. Subsequently, there are two continuation reactions in which this quinone can participate. The first one is oxidative coupling, in which the quinone is attacked by nucleophiles. Nucleophiles present in the model system were caffeic acid ester, ferulic acid, and sinapic acid. The reaction of these phenolics toward quinones is expected to follow their nucleophilic strength; i.e., caffeic acid ester = sinapic acid > ferulic acid.<sup>28</sup> These differences in nucleophilicity can explain the faster decrease of sinapic acid than of ferulic acid in the leaf phenolic extracts upon incubation with the dialyzed enzyme extract (Figure 5A).

The nucleophilic attack on the quinone yields a dimer, such as (caffeic acid ester)<sub>2</sub> or ferulic acid-caffeic acid ester. Thereby, the quinone motif is reduced back into its *o*-diphenol. This *o*-diphenolic dimer has potential to be oxidized. Because of its size, it is unlikely that enzymatic oxidation occurs directly. The



**Figure 6.** RP-UHPLC-PDA traces of (A) chlorogenic acid (ChA) (0.1 mM) and ferulic acid (FerA) (0.1 mM) at several time points after incubation with mushroom tyrosinase and (B) absorbance spectra of the reaction mixtures before and after 60 min incubation. (C) Absorbance spectra of chlorogenic acid after 60 min incubation with mushroom tyrosinase at increasing molar ratios of ferulic acid-to-chlorogenic acid.



**Figure 7.** (A) Proposed mechanism of enzymatic oxidation of a caffeic acid ester, followed by oxidative coupling to ferulic acid, and subsequent coupled oxidation. (B) Schematic representation of coupled oxidation and oxidative coupling reactions at high and low nonsubstrate-to-substrate ratio.



reoxidation of the *o*-diphenolic motif can occur via coupled oxidation.<sup>27</sup> This coupled oxidation is the second continuation reaction for the caffeic acid ester quinone (Figure 7A). In this reaction, the enzymatically formed quinone of monomeric caffeic acid ester can oxidize the dimer and reduce back into a monomeric caffeic acid ester again. For caffeic acid ester-ferulic acid or caffeic acid ester-sinapic acid dimers, only the caffeic acid ester motif will be oxidized. As ferulic acid and sinapic acid do not possess an *o*-diphenolic structure, they are not expected to be oxidized by quinones. These oxidized dimers can participate in further oxidative coupling reactions.

The oxidative coupling and coupled oxidation reactions can comprise various cycles, the number of which depends on the ratio of nonsubstrates-to-substrates present in the mixture. The elongation of phenolics by subsequent cycles of oxidative coupling and coupled oxidation are schematically shown in Figure 7B. At a high ratio (8:1 in the model system), mainly caffeic acid ester-ferulic acid dimers will be formed. Because of the relatively low caffeic acid ester concentration, all caffeic acid ester is consumed after a few reaction cycles and connected to nonsubstrate phenolics. This results in small oligomers without an extensive conjugated system. The dimers found during the model incubations did not show absorbance in the visible wavelength range. The formation of smaller oligomers explains the decreased color formation at increased ferulic acid/caffeic acid ester ratio in the model system (Figure 6C). At low ferulic acid/caffeic acid ester molar ratios (3:1 and 1:1 in the model system), there is increased probability of (caffeic acid ester)<sub>2</sub> formation (Figure 7B). This dimer is prone to further coupled oxidation as there is sufficient caffeic acid ester present, which can be oxidized into quinones by PPO. The monomeric caffeic acid ester quinones oxidize the dimers into quinones. These dimeric quinones are then further elongated by oxidative coupling. During the oxidative coupling, a dimer can be attacked by a monomeric as well as a dimeric nucleophile, resulting in the formation of many different coupling products, which are not necessarily a linear chain as depicted in Figure 7B.

In the same way, the nonsubstrate-to-substrate ratio is suggested to influence the degree of browning in sugar beet leaves. In 3<sub>m</sub> leaves, the molar nonsubstrate-to-phenolic acid-ester ratio was 8:1, whereas in 8<sub>m</sub> leaves this ratio was 3:1. The high ratio in 3<sub>m</sub> leaves inhibits extensive polymerization and browning.

#### Effect of Plant Age on Contents of Reducing Agents.

Besides the phenolics composition and enzyme activity, reducing agents AA and GSH were expected to affect color formation. The freeze-dried 3<sub>m</sub> leaves contained less AA than 8<sub>m</sub> ones, i.e.,  $5.5 \pm 1.5$  mg/100 g FW and  $17.5 \pm 4.1$  mg/100 g FW in 3<sub>m</sub> and 8<sub>m</sub>, respectively. GSH was not detected. After reduction with DTT, the contents of AA and GSH did not increase, indicating that both DHA and GSSG were not initially present. It was hypothesized that the AA content was higher in the younger leaves. The hypothesis had to be rejected as the AA content was 3-fold higher (w/w) in 8<sub>m</sub> than in 3<sub>m</sub>. On a molar basis, the substrate contents were 8 and 3 times higher than AA contents in 3<sub>m</sub> and 8<sub>m</sub> leaves, respectively. Therefore, the activity of AA as reducing agent cannot be a major factor of importance. Plants possess the capacity to reduce DHA back into AA by the expression of dehydroascorbate reductase. This enzyme reduces DHA back to AA by oxidizing GSH into GSSG.<sup>29</sup> This mechanism does not seem to be at play in sugar beet leaves as GSH was not detected.

To conclude, there are several factors influencing the extent of enzymatic browning in plant materials. The increases in enzyme activity and phenolics content have been reported earlier. With the observations in this research, we postulate that besides PPO the ratio between nonsubstrate-to-substrate phenolics drive enzymatic browning, rather than the absolute quantities of phenolics, indicating that the total content of phenolics combined with enzyme activity cannot solely predict the occurrence of enzymatic browning.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01897.

Quantitation of individual phenolics in pools 2 and 3 upon incubations with endogenous leaf oxidases from 3<sub>m</sub> and 8<sub>m</sub> sugar beet leaves (Table S1); mass fragmentation spectrum of caffeic acid *O*- $\beta$ -hexoside (2) (Figure S1); colors of pools 2 and 3 upon incubation with leaf enzyme extract (Figure S2A) and of the model incubations of chlorogenic acid and sinapic acid (Figure S2B); chromatograms and UV/vis spectra of the combined chlorogenic acid and sinapic acid incubated with mushroom tyrosinase (Figure S3); and mass fragmentation spectra of a chlorogenic acid-ferulic acid and chlorogenic acid-sinapic acid oxidation product (Figure S4) (PDF)

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### Notes

The authors declare no competing financial interest.

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