Food Chemistry 171 (2015) 280–286

Contents lists available at ScienceDirect

Food Chemistry

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

Determination of free diferulic, disinapic and dicoumaric acids in plants and foods

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form 25 August 2014
Accepted 30 August 2014
Available online 8 September 2014

Keywords:
Hydroxycinnamic and dicinnamic acids
Phenylpropanoids
Oxidative coupling
Food
Plant
Liquid chromatography
Mass spectrometry

ABSTRACT

Hydroxycinnamates are common phenolic compounds of plants and plant foods, often found in substantial quantities. Due to their high *in vitro* antioxidant activity they can easily be oxidized under oxidative conditions. In this study, we found that *in vitro* oxidation of coumaric, ferulic and sinapic acids resulted mainly in dimeric compounds. We hypothesized that these dimers are present in plants and plant foods not only in their bound form but also as free acids that can be extracted from non-hydrolyzed samples. By applying sensitive UHPLC–MS/MS method, we were able to identify and quantify four free hydroxycinnamic acid dimers for the first time, namely 8-8′-disinapic, 8-5′-diferulic, 8-*O*-4′-diferulic and 8-3′-dicoumaric acids, in wheat sprouts, Chinese cabbage, millet sprouts, light beer and parsley. Concentrations of dicinnamates in plant tissues ranged from 0.05 to 2.8 μg g^{-1} DW and the monomer:dimer ratio ranged from 2 to 850.

1. Introduction

Undesirable oxidation and browning of plant food diminishes its quality and could also affect its safety (Friedman, 1996). The oxidation of phenolic compounds is poorly understood at the molecular level and the effects of oxidized phenolic compounds on human health are unknown. Hydroxycinnamic acids, the early metabolites of the phenylpropanoid pathway, are present in substantial quantities in all land plants (Emiliani, Fondi, Fani, & Gribaldo, 2009). Their high *in vitro* antioxidant activity suggests that they are easily oxidized under oxidative conditions. Besides direct oxidation by oxidants and oxidizing radicals, phenolics are commonly oxidized by a few enzymes, including polyphenol oxidase (PPO), peroxidase and laccase (Weng & Chapple, 2010; Yoruk & Marshall, 2003). Whereas PPO oxidizes phenols by adding molecular oxygen to their molecule, class III peroxidases and laccases mediate the one electron oxidation of phenolics, resulting in the formation of radical cations or quinones that are subsequently coupled with other molecules. The products have been suggested to be insoluble polymers (e.g., lignin) or soluble low molecular weight compounds, such as oligomeric proanthocyanidins, lignans and some dimeric alkaloids (Costa et al., 2008). The *in vitro* oxidation and subsequent oligomerization of other highly abundant phenolics, such as quercetin, resveratrol, hydroxycinnamic and hydroxybenzoic acids, have been studied several times, but the oxidation products have not yet been unambiguously identified in plants or have only been identified after release from their insoluble form (Bunzel, 2010; Chervyakovsky et al., 2008; Cichewicz, Kouzi, & Hamann, 2000; Liu, Wan, Huang, & Kong, 2007; Mouterde, Flourat, Cannet, Ducrot, & Allais, 2013; Rouau et al., 2003).

Hydroxycinnamic acids, the products of PAL enzyme, have been demonstrated to protect plant cell against free radicals and oxidative damage (Tamagnone et al., 1998; Yamasaki, Sakihama, & Ikehara, 1997). During the elimination of oxidants, hydroxycinnamic acids as electron donors are oxidized and the majority is irreversibly modified although they can partially be regenerated via the ascorbate and glutathione cycles (Sgherri, Cosi, & Navari-Izzo, 2003). In general, hydroxycinnamic acids are found

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http://dx.doi.org/10.1016/j.foodchem.2014.08.131
0308-8146/

both attached to cell wall and as soluble compounds in food plants. However their oligomers have only been determined after saponification, acid hydrolysis or cinnamoyl esterase treatment (Bunzel, 2010; Faulds, Sancho, & Bartolome, 2002). In the present study, we report that diferulic, disinapic and dicoumaric acids are low molecular weight oxidation products present in common plants and foods as free compounds.

2. Materials and methods

2.1. Chemical synthesis

Sinapic, ferulic and 4-coumaric acids were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). 8-*O*-4′-dehydrodiferulic acid **12b** was isolated earlier (Bunzel, Funk, & Steinhart, 2004). 8,5′-dehydrodiferulic **10b** and 8,3′-dehydrodi-*p*-coumaric **10a** acids were prepared according to published protocols (Ralph, Quideau, Grabber, & Hatfield, 1994; Torres & Rosazza, 2001). Proton and carbon chemical shifts (s) are reported in ppm downfield from internal reference of residual (CHD_2)-$CO(CD_3)$ peak in $(CD_3)_2CO$ (for ^{1}H-NMR; calibrated to 2.05 ppm) and the carbonyl peak in $(CD_3)_2CO$ (for ^{13}C-NMR; calibrated to 206.26 ppm). To synthesize bis-lactone **16c**, a solution of sinapic acid **1c** (1.0 g, 4.46 mmol, 1.0 equiv) in EtOH (5 mL) was added to a solution of dry $FeCl_3$ (1.59 g, 9.81 mmol, 2.2 equiv) in EtOH (12 mL) at RT under vigorous stirring. The reaction was stirred for 2 h, followed by removal of the organic solvents under reduced pressure. The residue was suspended in water (20 mL) and extracted with EtOAc (3 × 50 mL). Combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and the solvents were removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 50:1–>20:1), yielding 665 mg (67%) of slightly yellow crystals. Mp = 232–234 °C; ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.79 (s, 12H), 4.07 (d, J = 0.5 Hz, 2H), 5.71 (s, 2H), 6.69 (s, 4H), 7.47 (broad s, 2H); ^{13}C NMR (125 MHz, acetone-d_6) δ [ppm] = 176.7, 149.7, 138.2, 130.5, 104.9, 84.0, 57.4, 49.7. To synthesize lactone (*E*)-**17c**, a solution of dilactone **16c** (150 mg, 0.33 mmol, 1.0 equiv) in THF (1 mL) was cooled to 0 °C and 1.0 M aq. sol. of NaOH (10 mL) was added. The reaction mixture was stirred at 0 °C for 15 min and then acidified to pH 4 (2.0 M aq. HCl). The mixture was extracted with EtOAc (3 × 25 mL) and the resulting organic layers were combined, dried over Na_2SO_4, filtered and the solvents removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 20:1–>10:1), yielding 69 mg (46%) of yellow crystals. Mp = decomp; ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.75 (s, 6H), 3.81 (s, 6H), 4.28 (m, 1H), 5.69 (d, J = 2.4 Hz, 1H), 6.63 (s, 2H), 7.02 (s, 2H), 7.33 (broad s, 1H), 7.56 (d, J = 2.0 Hz, 1H), 7.86 (broad s, 1H), 11.2–13.5 (broad s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ [ppm] = 174.5, 171.4, 147.4, 147.2, 141.9, 137.7, 135.2, 130.2, 124.4, 118.1, 107.7, 101.9, 80.5, 56.4, 56.3, 53.5. To synthesize lactone (*Z*)-**17c**, a solution of (*E*)-**17c** (~1.5 mg) in acetone-d_6 (550 μL) was placed in close proximity to a tungsten light and irradiated for 3 days. ^{1}H NMR spectra indicated >98% conversion of (*E*)-**17c** to (*Z*)-**17c**. ^{1}H NMR (500 MHz, acetone-d_6) δ = 3.83 (s, 6H), 3.86 (s, 6H), 4.11 (dd, J = 1.9, 5.0 Hz, 1H), 5.75 (d, J = 5.1 Hz, 1H), 6.77 (s, 2H), 7.19 (d, J = 2.0 Hz, 1H), 7.43 (broad s, 1H), 7.61 (s, 2H), 7.96 (broad s, 1H), 11.2–13.5 (broad s, 1H). To synthesize diacid **14c**, a solution of dilactone **16c** (11 mg, 0.02 mmol, 1.0 equiv) in DMSO (1 mL) was added to 0.1 M phosphate buffer (9 mL, pH 7.4) and the resulting mixture was stirred at RT for 3 days. The water and remaining DMSO were removed by sublimation under high vacuum and the residue was suspended in EtOAc (50 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvents were removed under reduced pressure, yielding the diacid **14c** (46%). ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.76 (s, 12H), 7.01 (s, 4H), 7.63 (broad s, 2H), 7.85 (s, 2H), 11.5–13.3 (broad s, 2H); 13C NMR (125 MHz, acetone-d6) δ [ppm] = 56.5, 108.8, 126.4, 126.6, 138.8, 142.6, 148.5, 168.6. To synthesize thomasidioic acid **15c**, a solution of sinapic acid **1c** (500 mg, 2.23 mmol) in water (110 mL, 0.02 M) was gently stirred in an open flask at RT. The pH of the solution was adjusted to 7.5–8.0 using 0.5 M aq. NaOH. Air was bubbled through the solution at RT and the resulting mixture was stirred in the dark for 72 h. The whole mixture was then extracted with CH_2Cl_2 (3 × 150 mL). Combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered and the solvents removed under reduced pressure to yield 378 mg (76%) of thomasidioic acid **15c**. 1H NMR (500 MHz, acetone-d6) δ [ppm] = 3.40 (d, J = 1.5 Hz, 1H), 3.61 (s, 3H), 3.69 (s, 6H), 3.89 (s, 1H), 5.05 (broad s, 1H), 6.35 (s, 2H), 6.91 (s, 1H), 7.68 (s, 1H), 8.01 (broad s, 1H), 8.07 (broad s, 1H), 11.5–13.3 (broad s, 2H); 13C NMR (125 MHz, acetone-d6) δ [ppm] = 40.3, 47.3, 56.55, 56.62, 60.4, 106.2, 108.8, 124.07, 124.11, 125.0, 134.7, 1357, 138.3, 142.6, 146.4, 148.45, 148.51, 168.6, 173.4. Spectral data of the isolated acid **15c** were in agreement with those previously reported (Ahmed, Lehrer, & Stevenso, 1973).

2.2. Oxidation by HRP

Sinapic, ferulic and 4-coumaric acids (1 mM) were oxidized in 35% acetone (in distilled water) by adding hydrogen peroxide (final concentration of 0.5%) and horseradish peroxidases Type II and IV (5–250 μg mL^{-1}; Sigma–Aldrich Fine Chemicals, St. Louis, MO, USA). The reaction was monitored by UPLC–ESI-MS (full scan mode) at various time points (5, 15, 30, 60 and 240 min) after diluting 5 μL of the reaction mixture in 145 μL 10% MeOH.

2.3. Plant and food material

Wheat (*Triticum aestivum*) and millet (*Panicum miliaceum*) sprouts were grown in vermiculite at room temperature and shoots were collected after 4 days. Parsley, Chinese cabbage, radish, cranberries, rice, rice bread, carrot, light beer (lager) and white wine were purchased from a grocery store (Olomouc, Czech Republic).

Table 1
Free dicinnamic acids quantified in plant-derived samples. Concentrations are given as mean ± SD (n = 3).

Compound	MRM	Plant material	Concentration (μg g^{-1} DW)	Monomer/dimer ratio
8-8′-Disinapic acid (**14c**)	401.0 > 357.0	Wheat sprouts	2.30 ± 0.10	2.1
		Chinese cabbage	1.94 ± 0.21	9.9
8-5′-Diferulic acid (**10b**)	385.1 > 341.2	Millet sprouts	0.05 ± 0.01	80.0
8-*O*-4′-diferulic acid (**12b**)	385.1 > 193.1	Light beer	2.78 ± 0.26[a]	852.6
8-3′-Dicoumaric acid (**10a**)	325.1 > 281.2	Parsley	0.15 ± 0.01	133.0

[a] ng mL^{-1}.

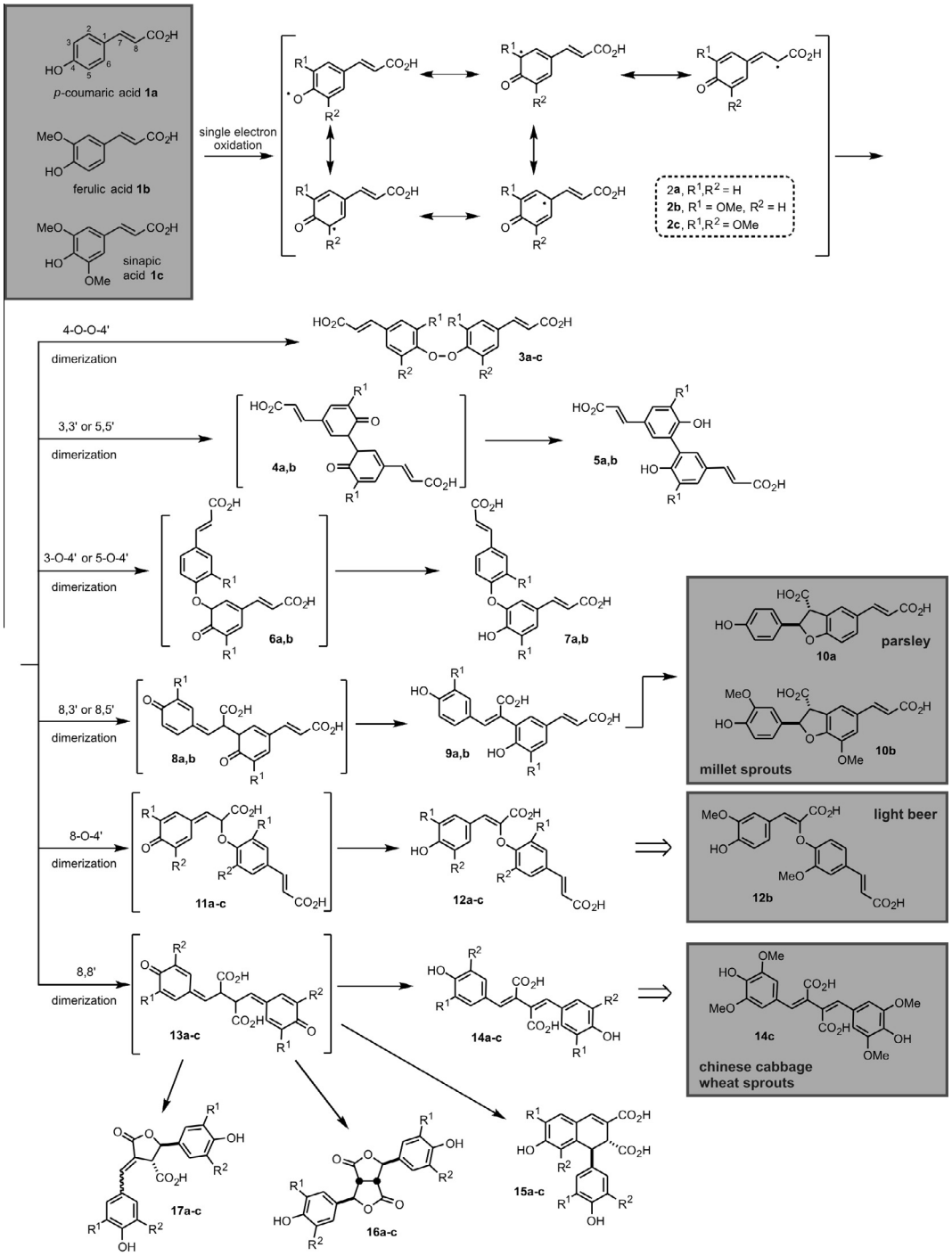

Fig. 1. Overview of possible products formed by single-electron oxidation of hydroxycinnamic acids (**1a-c**). The arrangement of atoms in optically active molecules is shown in a relative configuration.

2.4. Sample preparation

500 mg of lyophilized samples (with the exception of the beer and wine samples, which were directly used for SPE after the addition of the internal standards) were homogenized in 5 mL aqueous solution of the internal standards with or without sodium azide (0.1 mM) using an oscillation ball mill (MM 301, Retsch, Haan, Germany) in 5 mL aqueous solution of internal standards with or without sodium azide (0.1 mM). Deuterated salicylic (3,4,5,6-D4) and 4-hydroxybenzoic (2,3,5,6-D4) acids (0.3 μM in the extraction solvent) were used as internal standards. Test tubes containing finely ground plant material were sonicated for 15 min, vortexed for 30 min and then subjected to centrifugation for 10 min at 20,000*g* and 7 °C (Avanti 30, Beckman, Krefeld, Germany). The supernatant was directly loaded onto an SPE cartridge (Spe-ed C18, 1 g/6 mL, Applied Separations), which was dried with nitrogen and eluted with 100% methanol. The eluent was removed using a vacuum rotary evaporator, re-dissolved in 10% MeOH, filtered through a 0.45 μm Nylon membrane microfilter (Alltech, Breda, Netherlands) and analyzed by UPLC–MS/MS. All samples were analyzed in triplicate.

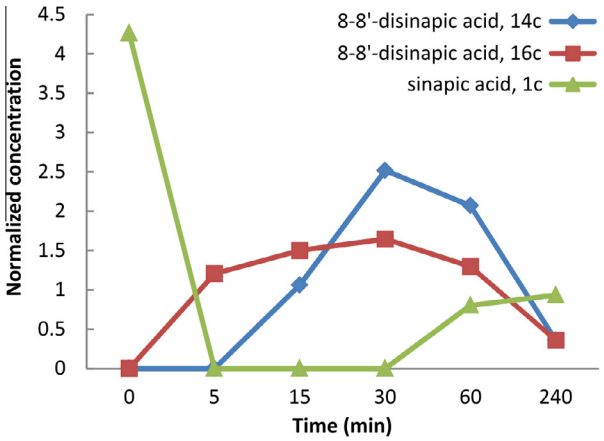

Fig. 2. Time course of the most abundant products (disinapates **14c** and **16c**) detected in a reaction mixture of sinapic acid, hydrogen peroxide and HRP. Normalization of each curve was performed by dividing data points by their mean.

2.5. UPLC–MS/MS

The UPLC–MS/MS instrumentation and conditions were similar to those described earlier (Gruz, Novak, & Strnad, 2008). Briefly, analyses were performed using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) coupled to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro *micro*™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative mode. Samples were injected onto a reversed phase column (BEH C_8, 1.7 μm, 2.1 × 150 mm, Waters, Milford, MA) maintained at 30 °C and eluted by acetonitrile-based mobile phase. The effluent was introduced into a PDA detector (scanning range 210–600 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 100 °C, desolvation temperature 350 °C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was used as collision gas (collision energy 16 eV) and nitrogen as desolvation gas (500 L h^{-1}). MRM transitions used for quantification of dimers

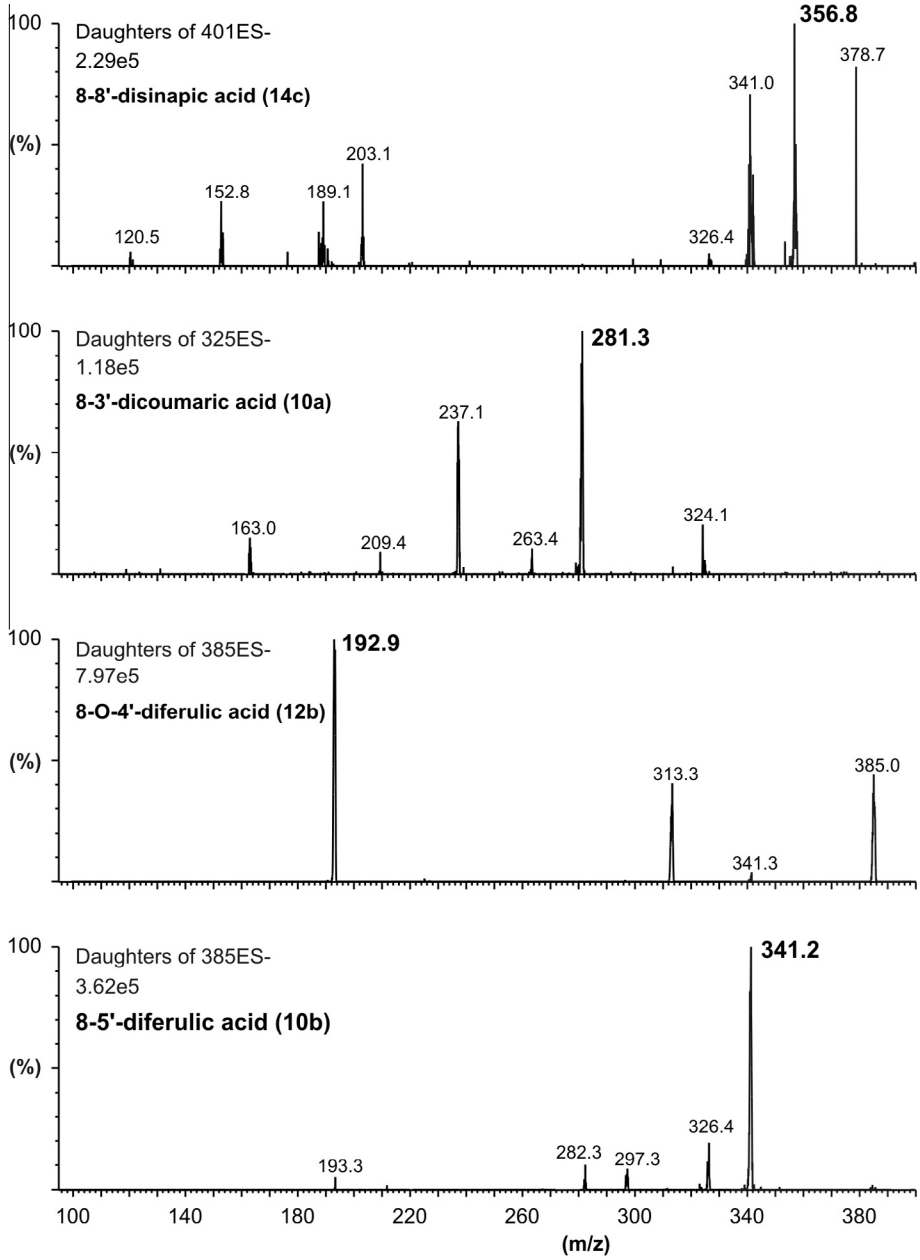

Fig. 3. Product ion spectra of selected dicinnamic acids detected in reaction mixtures with H_2O_2 and horseradish peroxidase (ions given in bold were used in MRM mode).

are listed in Table 1. The quantification of both monomers and dimers was based on deuterium labeled internal standards of 4-hydroxybenzoic and salicylic acids to compensate for losses during the extraction procedure and reduce matrix effects that can suppress the ionization efficiency. Analyte concentrations were calculated by using the ratio of analyte:internal standard.

3. Results and discussion

3.1. Oxidation of phenolic acids by HRP

Hydroxycinnamic acids **1a-c** (Fig. 1) were oxidized *in vitro* by HRP, and the reactions were monitored at several time points by UPLC-PDA–MS operating in the full scan mode. The main products under the described conditions were dimers with molecular weights of ($2 \times MW_{cinnamate} - 2H$). Typically, not only one dimeric compound but various stereo and regioisomers were formed. As minor products, several other oxidation products, such as oxodimers ($2 \times MW_{cinnamte} - 2H + O$) and dehydrodimers ($2 \times MW_{cinnamate} - 4H$), were also detected. Although the cinnamic acids reacted quickly with hydrogen peroxide in the presence of HRP, they were stable for several hours when only hydrogen peroxide was added. An unexpected time course was observed for the reaction of sinapic acid, i.e., its re-appearance in the reaction mixture after 60 min (Fig. 2). By adding synthesized disinapates to the reaction mixture, we found that this increase was not due to their breakdown but more likely due to the transformation of other products undetectable by our system (e.g., hydrolysable oligomers or stable radicals). This phenomenon was not observed at lower concentrations of hydrogen peroxide (<0.1%).

To search for oxidation products in food sources, we developed a sensitive MRM-based UPLC–MS/MS method based on analyzing collision induced fragmentation spectra of major peaks (ca 25 compounds) present in the reaction mixtures of HRP induced dimerization of hydroxycinnamic acids (Fig. 3). Pseudo-molecular ions $[M-H]^-$ were used as parent ions, with the exception of disinapic acid **14c**, which exhibited an abundant $[M-H-COO]^-$ ion with *m/z* 401 due to in-source fragmentation.

3.2. Chemical synthesis and stability of dimers

Stereo- and regio-isomers of disinapic acid, compounds **14–17c**, were synthesized in mg amounts from bislactone dimer **16c**, which was the primary product of chemical oxidation of sinapic acid **1c**. Depending on the pH of the reaction media and reaction time, the bislactone **16c** was transformed in the absence of oxygen into diacids **14c** and **17c** (Fig. 4). It is interesting to note that we observed the formation of 2–5 different dimers during *in vitro* oxidation, but typically only one isomer was detected in plants. This might be caused by both enzymatic and chemical factors. Plant enzymes are usually more specific than HRP and can work together with other stereospecific proteins, such as dirigent proteins, to form specific stereoisomers (Davin & Lewis, 2000). The presence of only one isoform can also be explained by chemical means. We found that the ratio of the different 8,8′-coupled disinapic acid dimers (diacid **14c**, monolactone **17c** and dilactone **16c**) strongly depended on the pH of the aqueous media, as well as on the presence/absence of oxygen. Since phenolic acids are mostly located in the vacuole and apolast, compartments with generally low pH, dimer formation and stability may be affected by this factor. The experiments with bislactone **16c** demonstrated that under acidic pH conditions, the stability of the open form, diacid **14c**, was markedly improved. Accordingly, **14c** was the only detected disinapate in plant samples. These findings suggested that pH adjustments or usage of buffers during the extraction procedures can accelerate the transformation of natural isoforms of phenolic dimers.

3.3. Determination of oxidation products in foods

The initial screening of food products, including parsley, wheat sprouts, millet sprouts, radish, cranberries, rice, rice bread, carrot, Chinese cabbage, beer and wine, was performed on extracts used later for SPE. As a result, a few tentative identifications were made based on the MS fragmentation, UV–VIS spectra and retention times of the products identified in the *in vitro* reaction mixture with HRP. To further improve the UPLC–MS/MS detection, we performed SPE pre-concentration of all samples to remove interfering compounds and to increase the signal-to-noise ratio. However, we

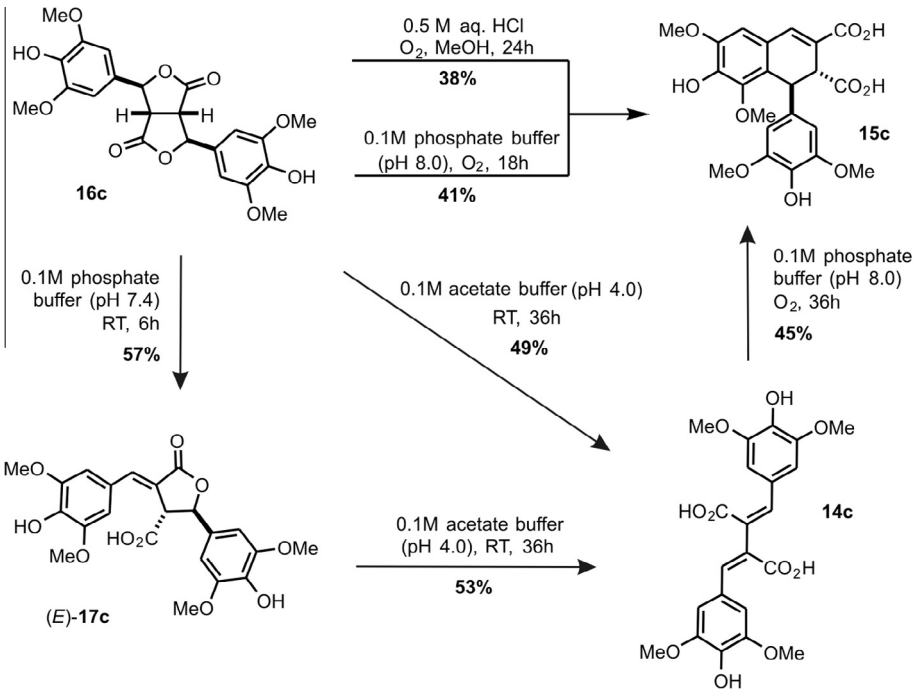

Fig. 4. Conversion of 8-8′-disinapic acid into different stereo- and regio-isomers under specified conditions.

found that evaporation from aqueous solutions of up to 80% organic solvent resulted in substantial losses of some dicinnamates, especially 8-3′-dicoumaric acid **10a**. Therefore, we minimized the sample processing by using water extraction, which allowed direct application onto the SPE column, thus avoiding problematic evaporation of the primary extract. The retention of diacids was always checked in parallel experiments. After loading the primary extract, we dried the SPE cartridge with nitrogen to remove any remaining water and subsequently eluted analytes with 100% MeOH. Due to low solubility of diacids in water, we verified that all of them were quantitatively redissolved in 10% MeOH when the final concentration was $\leqslant$0.5 μM (highest concentration allowed). The pre-concentrated extracts dissolved in 10% MeOH were stable for at least 24 h at 4 °C and for weeks at −80 °C. The signal-to-noise ratio was increased by approximately 6× times and was sufficient to clearly identify dimers of sinapic, ferulic and coumaric acids in wheat sprouts, Chinese cabbage, millet sprouts, light beer and parsley (Fig. 5). In contrast, we were not able to detect any dicinnamates in radish, cranberries, rice, rice bread, carrot and white wine samples.

The oxidation of hydroxycinnamates seems to be mainly mediated by redox enzymes, such as peroxidases, laccases and PPO, which are compartmentalized in the plant cell (Costa et al., 2008; Strack, Pieroth, Scharf, & Sharma, 1985). Compartmentalization can be destroyed during food processing using harsh processing methods (e.g., grinding, steaming, roasting), which cause cell membrane disruption and exposure to oxidative conditions. Homogenizing plant tissues can therefore result in mixing the formerly separated components and subsequent formation of extraction artifacts. To prevent overestimation of hydroxycinnamic acid dimers, we freeze-dried all solid samples to remove hydrogen peroxide, a substrate of class III peroxidases. We also tested how addition of sodium azide, an inhibitor of both laccase and peroxidase activities, affects the concentrations of hydroxycinnamic acid dimers (Johannes & Majcherczyk, 2000; Tuisel, Grover, Lancaster, Bumpus, & Aust, 1991). We found only a minor decrease in the concentration of disinapate (ranging from 20 to 30%) and no significant decrease in the concentrations of dicoumaric and diferulic acids extracted with sodium azide, demonstrating that these dimers are mostly formed in plants before extraction. Besides class III peroxidases and laccases, phenolics are also oxidized by PPO, which is commonly associated with food browning. However, PPO incorporates molecular oxygen into the substrate molecule and is therefore unlikely to be directly involved in the formation of the described dicinnamates.

Mostly unprocessed plant foods, such as wheat sprouts and parsley, where the production of dicinnamates can mainly be attributed to enzymatic activity *in planta*, were investigated in this study. As one exception, we also analyzed light beer, which is a highly processed end-product formulated to be stable for months.

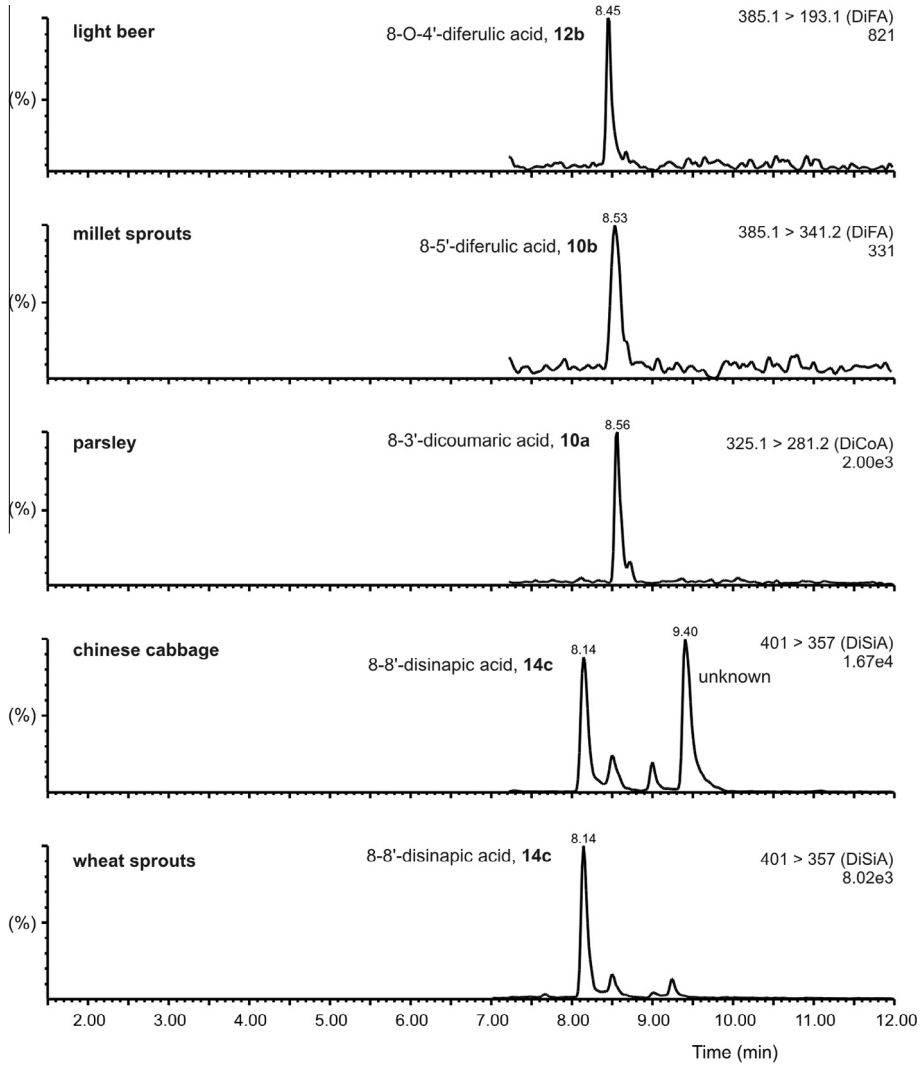

Fig. 5. MRM chromatograms of free dicinnamic acids identified in food and plant samples.

Beer was found to contain 8-*O*-4′-diferulic acid **12b**, possibly originating from either the barley sprouts or formed during processing, such as mashing and wort boiling, where dicinnamates can be formed by enzymatic oxidation, chemical oxidation and/or may be released from their insoluble form (cell wall bound). Whereas 8-*O*-4′-diferulic acid **12b** was present in beer, 8,5′-diferulic **10b** was found in millet sprouts, suggesting different mechanisms (or enzyme specificity) by which these dimers are produced. It is also unlikely that the different structural forms were a consequence of the extraction procedure, providing further evidence of the extraction procedure validity. The concentrations of **12b** and **10b** were more than 1000× times lower than the reported concentrations of bound diferulates released after saponification of barley (0.7 mg g^{-1}) and millet (2.6 mg g^{-1}) samples (Bunzel, Ralph, Marita, Hatfield, & Steinhart, 2001). The most abundant free dicinnamic acids in the studied samples was 8-8′-disinapic acid, which was found in both wheat sprouts and Chinese cabbage. The chromatogram of an extract from Chinese cabbage also showed another peak with the same transition (Fig. 5), but its retention time of 9.40 min did not match with any of the studied disinapate isoforms (**14c**, **15c**, **16c** or **17c**). This might be explained by in-source fragmentation of unknown disinapate derivatives, such as glucosides or esters, resulting in pseudo MS^3 spectra. Unfortunately, we were not able to gather clean full/daughter scan MS spectra due to the low intensity of this unknown compound. Although the concentration of free disinapic acid was relatively high, it was lower than that of bound disinapates released after saponification from rice samples and ranging between 5 and 19 μg g^{-1} (Qiu, Liu, & Beta, 2010).

In general, all dimers were found in lower quantities than their corresponding monomers (between 2 and 850 times). The high ratio of monomer/dimer may be due to several reasons. One is the incorporation of dimers into the cell wall, which contains high quantities of dehydrodiferulic acids attached to arabinoxylans (Allerdings et al., 2005; Bunzel, Allerdings, Ralph, & Steinhart, 2008). In addition, we found that dimers were chemically less stable than simple cinnamates, and thus were degraded faster. It was also demonstrated that dehydrodihydroxycinnamates can be further oxidized to trimers and higher oligomers that are difficult to detect by conventional LC methods, and thus would also remain undetected by our method (Bunzel, Heuermann, Kim, & Ralph, 2008; Mouterde et al., 2013; Rouau et al., 2003).

4. Conclusion

In the present study, we demonstrated that hydroxycinnamic acid dimers are present as free and soluble compounds in common foods and plants. The dimers are products of the oxidation of hydroxycinnamic acids which are mostly considered as health-promoting compounds with antioxidant properties. It is however unclear whether they remain beneficial or become detrimental after fulfilling their antioxidative role, which results in their oxidation and dimer formation. As oxidation products are known to be present in common foods, further work is needed to study their biological activities.

Acknowledgements

We would like to thank Ing. Michaela Šubrtová for excellent technical assistance and Sees-Editing, U.K. for editing the manuscript. This research was supported by project GACR 503/12/P166 and a Czech Ministry of Education grant from the National Program for Sustainability I (LO1204). J.G. and K.D. also acknowledge the support of the Operational Program Education for Competitiveness – European Social Fund (project CZ.1.07/2.3.00/20.0165).

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