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Modifications of Tryptophan Oxidation by Phenolic-Rich Plant Materials

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The role of plant phenolics as possible antioxidants was studied toward oxidation of tryptophan. Sources of plant phenolics included byproducts of deoiling processes, such as camelina, rapeseed, and soy meals and Scots pine bark drink, as well as berry phenolics from raspberry, black currant, and rowanberry. The oxidation of tryptophan and its individual oxidation products with and without added phenolics were analyzed by using a validated high-performance liquid chromatography method with diode array and fluorescence detection. Tryptophan in the presence of hexanal and iron ($FeCl_2$) was degraded by 77% after 6 days of oxidation, resulting in oxidation products, such as 3a-hydroxypyrroloindole-2-carboxylic acid B, dioxindolylalanine, 5-hydroxy-tryptophan, kynurenine, *N*-formylkynurenine, and β-oxindolylalanine. The tryptophan modifications formed upon hexanal and iron treatment were efficiently inhibited by camelina meal followed by rapeseed meal and soy meal. In contrast, phenolics from raspberry, black currant, and rowanberry acted as weak pro-oxidants.

KEYWORDS: Tryptophan; protein oxidation; hexanal; HPLC; antioxidants; phenolic compounds; oilseed byproducts; berries

INTRODUCTION

Proteins, peptides, and amino acids in food, feed, and pharmaceuticals are prone to oxidation reactions during processing and storage. Free radicals derived from lipid oxidation reactions are easily transferred to proteins, and the generation of highly reactive protein radicals are further oxidized to secondary compounds. Oxidation of proteins can involve cleavage of the polypeptide chain, modification of amino acid side chains, and conversion of the protein to derivatives that are highly sensitive to proteolytic degradation (*1*), as well as protein damage via cross-linking (*2*). Therefore, lipid oxidation products, such as aldehydes, are among the most important compounds to contribute to food deterioration and modification of food structure (*2*). Changes in food structure cause changes in protein functionality and may ultimately lead to toxic substances (*3*).

Plant materials rich in phenolic compounds exhibit a wide range of activities, such as antimicrobial, antimutagenic, anti-inflammatory, as well as antioxidant activities (*4, 5*). Phenolic compounds act as antioxidants by donating electrons and terminating radical chain reactions (*6*) and as metal chelators by binding metals (*7*). The role of phenolic antioxidants on protein oxidation has only been evaluated in a few studies with phenolics from berries and oilseed byproducts in oxidation models, such as oil-in-water emulsions (*8*), meat (*9, 10*), liposomes (*11*), and low-density lipoproteins (*12*). However, these studies have focused on measuring the overall effect of phenolics on protein oxidation (loss of tryptophan fluorescence and formation of carbonyl derivatives) and do not address what individual oxidation products are actually formed. Thus, it is unclear what compounds are the targets for the phenolic antioxidants.

Because the functional groups of proteins can be modified by oxidative attack from the end products of lipid oxidation during processing and storage, secondary lipid oxidation products, such as aldehydes, are also added to food products as flavoring agents (e.g., cinnamic aldehyde, benzaldehyde, anisaldehyde, and *trans*-2-hexenal) (*13*). Amino acid tryptophan has been shown to react with naturally and food-occurring aromatic and phenolic aldehydes, such as benzaldehyde, anisaldehyde, salicylaldehyde, syringaldehyde, vanillin, acetaldehyde, and formaldehyde, yielding tetrahydro-β-carbolines, for example, in fermented alcoholic and non-alcoholic beverages, such as wines, beers, distillates, as well as in grape and orange juices (*14*). While studies have emphasized the poorer reactivity of the saturated aldehydes such as hexanal, a major contributor to oxidation reactions and commonly used as a marker of food quality, in contrast to unsaturated ones (*2, 15*), the research on elucidating the effects of hexanal on protein, peptide, and amino acid oxidation is still limited. A study by Meynier et al. (*16*) revealed that hexanal and *trans*-2-hexenal caused a rapid and substantial decrease in tryptophan fluorescence as well as in lysine and histidine residues of whey proteins and sodium caseinate. It has also been reported that binding of hexanal to soy glycinin and β-conglycinin induced structural modifications,

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such as an increase in surface hydrophobicity and turbidity as well as a loss of lysine (*17*).

Although research has been performed on the antioxidative properties of plant phenolics, little is known about their role in preventing proteins, peptides, and amino acids from oxidation. In our earlier study (*18*), the role of plant phenolics in inhibiting the H_2O_2 catalyzed oxidation of tryptophan was investigated. However, the antioxidant mechanisms remain incompletely revealed. To further determine the antioxidant action of phenolic compounds, precise information is needed concerning the nature of protein oxidation products formed. The objective of this study was to investigate oxidation of the amino acid tryptophan in the presence of plant phenolics in a model system containing hexanal and $FeCl_2$. The detection of tryptophan oxidation products was analyzed with a fast high-performance liquid chromatography (HPLC) method developed and validated in this study. Modifications of tryptophan oxidation was studied using phenolic-rich plant materials, including rapeseed (*Brassica rapa* L.), camelina (*Camelina sativa*) and soy (*Glycine max* L.) meals, as well as pine bark drink (*Pinus sylvestris*) and berry phenolics from raspberry (*Rubus idaeus*), black currant (*Ribes nigrum*), and rowanberry (*Sorbus aucuparia*), all found antioxidatively active in our previous studies (*4, 9, 10, 19, 20*).

MATERIALS AND METHODS

Materials. The rapeseed (*B. rapa* L.) meal used was the byproduct of the rapeseed deoiling process, in which the oil was expelled by pressing the seeds at an elevated temperature by Mildola Ltd. (Finland). Camelina (*C. sativa*) meal was the byproduct of cold pressed camelina oil obtained from Raisio Ltd. (Finland). Soy meal (*G. max* L.) was obtained from Risetti Ltd. (Finland). Protein, fatty acid, and tocopherol compositions, as well as isoflavone and lignan contents of the oilseed processing byproducts, have been reported in a previous study by Salminen et al. (*9*). Scots pine (*P. sylvestris* L.) bark drink was obtained by extraction with water, so that it contained 30% pine bark and phloem (Ravintorengas Ltd., Siikainen, Finland). All berries, raspberry (*R. idaeus*), black currant (*R. nigrum*), and rowanberry (*S. aucuparia*), were purchased as fresh from a market place. The berry samples were packed immediately into a vacuum and stored in a freezer at −20 °C until use.

Chlorogenic acid, cyanidin-3-glucoside, ellagic acid, genistein, procyanidin B1, sinapic acid, and taxifolin were from Extrasynthèse (Genay, France). Catechin, quercetin, L-tryptophan, 3-hydroxy-tryptophan, 5-hydroxy-L-tryptophan, L-kynurenine, tryptamine, and kynurenic acid were obtained from Sigma. Sodium tetraborate decahydrate, acetic acid, and ironchloride tetrahydrate ($FeCl_2$) were from Riedel-de Haën (Germany). Trifluoroacetic acid was from Sigma. Hexanal was from Aldrich (Germany). All solvents were HPLC-grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). A MilliQ water purification system was used (Millipore, Bedford, MA). Chemicals used were of analytical purity.

Isolation of Plant Phenolics. The extraction of camelina, soy, and rapeseed meal were performed according to methods of Salminen and Heinonen (*9*) and Vuorela et al. (*10*), with some modifications. Plant material (0.8 g) and 20 mL of 80% methanol (70% ethanol for rapeseed) were put in a centrifuge tube that was shaken in a water bath (75 °C) for 60 min. The clear phenolic extract was collected after centrifugation (6000 rpm, 15 min).

Berry samples were freeze-dried prior to analysis and stored at −20 °C until use. Extraction and isolation of berry anthocyanins and raspberry ellagitannins were carried out as described by Kähkönen et al. (*19*), and that of rowanberry phenolics were carried out as described by Kylli et al. (unpublished results). The berry phenolic fractions were further purified by preparative HPLC, and the interfering sugars were removed by solid-phase extraction (SPE) as described by Kähkönen et al. (*19*). Berry isolates were freeze-dried and stored in −20 °C.

Total Phenolics and Phenolic Profiles. The amount of total polyphenols was measured colorimetrically according to the Folin−Ciocalteau procedure (*21*). Phenolic extract (0.2 mL) was evaporated to dryness. After that, 0.2 mL of methanol/water (1:2), 1 mL of Folin−Ciocalteau reagent (1:10), and 0.8 mL of disodium carbonate solution (7.5%) were added. After 30 min, the total phenolic content was measured at 765 nm by a Perkin-Elmer λ25 UV−vis spectrophotometer (Norwalk, CT). Gallic acid was used as a standard compound. The results are expressed as gallic acid equivalents (GAE), μg/g of plant material. The HPLC analysis of phenolics was performed according to the method outlined by Koski et al. (*22*) for phenolic acids and their derivatives and by Kähkonen et al. (*20*) for other phenolic compounds. Catechin, chlorogenic acid, cyanidin-3-glucoside, ellagic acid, gallic acid, procyanidin B1, rutin, and sinapic acid were used as standard compounds. The phenolic profiles of pine bark drink, raspberry, and black currant anthocyanins, as well as raspberry ellagitannins, used in this study, were reported in our previous study (*18*).

HPLC−DAD/FLD Analysis. Determination of tryptophan and its oxidation products was carried out using HPLC combined with diode array detection and fluorescence detection (DAD/FLD). The separation was performed on a 100 × 4.6 mm i.d., 5 μm, 300 Å, C8 Discovery BioWide Pore column (Supelco, Bellefonte, PA), equipped with a 20 × 40 mm i.d., 5 μm, C8 Discovery BioWide Pore guard column (Supelco, Bellefonte, PA). The HPLC system (Waters, Milford, MA) consisted of a 717 plus autosampler, 515 and 510 pumps with a pump control module, a column oven with a temperature control module, a PDA 996 diode array detector, and a 2475 Multi λ fluorescence detector. The best resolution between tryptophan oxidation products was achieved by using a gradient of 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), programmed as follows: 0−30 min from 98 to 70% A, with postrun 10 min programmed as follows: 30−30.1 min from 70 to 50% A, 30.1−32 min from 50 to 75% A, and 32−40 min from 75 to 98% A. The column oven temperature was set at 25 °C. The total flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 10 μL. The diode array detector wavelength of 260 nm was used to monitor oxidation products. The fluorescence program was as follows: 0−7 min, excitation (Ex) at 230 nm and emission (Em) at 340 nm; 7−11 min, Ex at 230 nm and Em at 435 nm; and 11−40 min, Ex at 270 and Em at 350, with gain set at 10 (0−9 min) and at 1 (9−40 min), bandwidth set at 18, and attenuation set at 128. Commercially available tryptophan oxidation product references, such as 3-hydroxy-tryptophan, 5-hydroxy-tryptophan, kynurenine, kynurenic acid, tryptamine, and tryptophan, were used. Tryptophan oxidation products were confirmed by spectroscopic identification and retention times. The quantification of tryptophan and its oxidation products were made using tryptophan, kynurenine, and 5-hydroxy-tryptophan as external standards. The quantifications for 3a-hydroxy-pyrroloindole-2-carboxylic acid, dioxindolylalanine A/B, *N*-formylkynurenine, and β-oxindolylalanine were calculated as equivalents to kynurenine as an external standard because these compounds were not commercially available.

Validation of the HPLC Method. The tryptophan oxidation product references were dissolved in 0.1 M borate (pH 6.3) to a concentration of approximately 0.6 mg/25 mL, and tryptophan was dissolved to approximately 6 mg/25 mL (stock solutions). Limit of detection (LOD) was defined as the concentration of analyte that results in a peak height 3 times the noise. The limit of quantification (LOQ) was defined as the concentration of analyte that results in a peak height 3 times the LOD. Samples (tryptophan, kynurenine, and 5-hydroxy-tryptophan) of three different concentrations (1:1, 1:4, and 1:40 from stock solutions) were analyzed at the beginning and end of every HPLC run with a calibration curve to calculate the precision. The precision is denoted by the intra- and interday coefficient of variation (CV). System suitability parameters, such as capacity factor (k'), relative retention (α), resolution (R_s), and theoretical plate number (N), were tested.

Oxidation of Tryptophan with Hexanal and $FeCl_2$. The 2 mM tryptophan model solution using 0.1 M borate (pH 6.3) was oxidized with 50 mM hexanal and 0.1 mM $FeCl_2$ at 37 °C in a shaking water bath in the dark in the presence of selected extracts of phenolic compounds (at concentrations of 50 and 100 μM) for 8 days (data points collected at days 0.1, 2, 6, and 8). The addition of hexanal and iron into tryptophan solution resulted in a final pH of 5.5. Iron was added to the model because it is ubiquitous in food ingredients and biological

tissues and an important pro-oxidant (*23*). Solutions of phenolics were pipetted into 10 mL screw-top vials, and the solvent was evaporated with nitrogen. In the control sample, no antioxidant was added. Subsequently, tryptophan solution (5 mL) followed by hexanal (31 μL) and $FeCl_2$ (25 μL) was added to the vials. Samples were filtered [0.45 μm Acrodisc syringe filter with hydrophilic polypropylene membrane (GHP), Pall Life Sciences, Ann Arbor, MI] prior to HPLC analysis.

The concentrations (50 and 100 μM) of added phenolic compounds were based on the total phenolic content best reflecting the wide range of phenolics in the cases of soy meal and pine bark drink and the total hydroxycinnamic acid derivatives in the cases of rapeseed and camelina meals because hydroxycinnamates were among their principal phenolic compounds. On the basis of the total phenolic content, rapeseed and camelina meal concentrations at 50 and 100 μM corresponded to concentrations of 42 and 84 μM and 115 and 230 μM, respectively. The concentrations of raspberry and black currant anthocyanin isolates, raspberry ellagitannin isolate, and rowanberry extract were calculated as cyanidin-3-glucoside, ellagic acid, and chlorogenic acid equivalents, respectively, because they presented the main phenolic groups present in the isolates. On the basis of the total phenolic content, rowanberry extract concentrations at 50 and 100 μM corresponded to concentrations of 94 and 188 μM, respectively. All of the calculations of the molar concentrations were made to be consistent with our previous study (*18*). All tested berry isolates and other plant materials were dissolved in methanol, ethanol (rapeseed), or water (pine bark).

The percent inhibition of plant materials against the formation of tryptophan oxidation was calculated: $[(C_0 - C_t) - (S_0 - S_t)/(C_0 - C_t)] \times 100$, where C_0 is the concentration of tryptophan in the control sample at day 0, C_t is the concentration of tryptophan at day 6, S_0 is tryptophan with antioxidant at day 0, and S_t is tryptophan with antioxidant at day 6. The tryptophan concentration of the control sample (without added phenolics) before oxidation (day 0) was 408 μg/mL. The percent inhibition of plant materials against the formation of tryptophan oxidation products was calculated for day 6: $(C_0 - C_1)/C_0 \times 100$, where C_0 is the concentration of the tryptophan oxidation product in the control sample and C_1 is the tryptophan oxidation product in the antioxidant sample. The inhibitions were expressed as percentages.

Statistical Analysis. Statistical analysis was performed using Duncan's multiple range tests by SAS 9.1 (SAS Institute, Inc., Cary, NC). A *t* test was performed to determine differences between the means of intra- and interday precisions. All of the results are given as the mean values of triplicate analyses unless otherwise stated.

RESULTS AND DISCUSSION

Separation and Validation of HPLC Method. The best separation between tryptophan, as well as tryptophan oxidation products of dioxindolylalanine A/B, 3a-hydroxypyrroloindole-2-carboxylic acid B, 5-hydroxy-tryptophan, kynurenine, *N*-formylkynurenine, and β-oxindolylalanine A/B, was achieved by using a gradient of 0.1% trifluoroacetic acid and acetonitrile (**Figure 1**). Dioxindolylalanine gave two peaks, and β-oxindolylalanine gave a double peak, because of their diastereomers. Tryptophan and its oxidation products were identified by their relative retention times (Rt) and their spectroscopic properties and by checking peak purity of the compounds by a photodiode array (PDA) detector. Kynurenic acid (Rt at 3.7 min), 3-hydroxy-kynurenine (Rt at 7.5 min), and tryptamine (Rt 13.0 min) were not detected among tryptophan oxidation products during oxidation with hexanal and iron. Separation of oxidation products was achieved within 13 min, and retention times were reproducible between chromatographic runs. The chromatographic separation was generally good; the resolution value $R_s \geq 1.5$ and capacity factor $k' \geq 1.0$ were obtained for all compounds, including also the standard compounds. The relative retention (α) was between 1.1 and 1.5 for all compounds: it was best for dioxindolylalanine B and kynurenic acid (1.5), as well as for tryptophan (1.4), and the most critical for *N*-formylkynurenine, tryptamine, and 3a-hydroxypyrroloindole-2-carboxylic acid B (1.1). The number of theoretical plates (N) > 2000 was obtained for all compounds: 3900 (3-hydroxy-kynurenine), 4100 (dioxindolylalanine A), 4300 (kynurenic acid), 5800 (dioxindolylalanine B), 7400 (3a-hydroxypyrroloindole-2-carboxylic acid B), 11 000 (kynurenine), 17 000 (*N*-formylkynurenine and β-oxindolylalanine), 17 400 (5-hydroxy-tryptophan), 44 000 (tryptamine), and 46 000 (tryptophan). LOD and LOQ, as well as the linearity of the calibration curves, are available as Supporting Information. Repeatability (intraday precision) of the method was good for tryptophan (CV 6.4%, $n = 17$), kynurenine (CV 5.7%, $n = 17$), and 5-hydroxy-tryptophan (CV 9.8%, $n = 16$). In addition, interday precision was excellent for tryptophan (CV 6.3%, $n = 23$) and kynurenine (CV 6.9%, $n = 23$) and the most critical for 5-hydroxy-tryptophan (14.5%, $n = 21$). There were no significant differences ($p < 0.05$) between interday precision and repeatability for each reference compound (tryptophan, kynurenine, and 5-hydroxy-tryptophan). Values of precision were below 15% of the coefficient of variation (CV). Thus, the HPLC method is selective and rapid for tryptophan and its oxidation products.

Oxidation of Tryptophan. Tryptophan was degraded by 17, 51, 77, and 78% after 0.1, 2, 6, and 8 days of oxidation, respectively, in the presence of hexanal and iron. In previous studies, tryptophan losses after 6 h of oxidation with H_2O_2 were reported to be 50 (*18*) and 18−68% (*24*) depending upon the conditions used. The greatest loss of tryptophan (by 68%) was induced by hourly added H_2O_2 at pH 8.3 and 40 °C (*24*). During the oxidation, tryptophan-derived oxidation products, such as 3a-hydroxypyrroloindole-2-carboxylic acid B, dioxindolylalanine A/B, 5-hydroxy-tryptophan, kynurenine, *N*-formylkynurenine, and β-oxindolylalanine, were formed (**Figure 2**). Some peaks remained unidentified. The formation of tryptamine was not observed with tryptophan oxidized by hexanal and iron (**Figure 1**) compared to tryptophan oxidized with H_2O_2 (*18*). The formation of 3a-hydroxypyrroloindole-2-carboxylic acid A in tryptophan oxidized by hexanal and iron was found to be under the limit of detection. β-Oxindolylalanine and *N*-formylkynurenine were shown to be the main oxidation products of tryptophan, followed by dioxindolylalanine B, 5-hydroxy-tryptophan, kynurenine, dioxindolylalanine A, and 3a-hydroxy-pyrroloindole-2-carboxylic acid B at day 2 (**Figure 2**). **Figure 3** shows the degradation of tryptophan, leading into various oxidation compounds with *N*-formylkynurenine and kynurenine as major end-products. In addition, the indole ring of tryptophan is susceptible to irreversible oxidation, producing 3a-hydroxy-pyrroloindole-2-carboxylic acid, β-oxindolylalanine, and dioxindolylalanine, that can be further transformed to kynurenine and *N*-formylkynurenine, dioxindolylalanine, and kynurenine, and kynurenine, respectively (*24, 25*).

The amount of primary oxidation product, 3a-hydroxypyrroloindole-2-carboxylic acid B, reached a plateau after 2 days of oxidation, whereas the amount of dioxindolylalanine A/B, 5-hydroxy-tryptophan, kynurenine, and *N*-formylkynurenine increased during the oxidation period (**Figure 2**). However, the amount of β-oxindolylalanine began to decrease after the second day of oxidation and continued decreasing over the entire study period. The formation of individual tryptophan oxidation products by hexanal and iron followed a similar pattern as in earlier studies when tryptophan was oxidized with H_2O_2 (*24*). However, the concentrations of oxidation products formed from tryptophan oxidized with hexanal and iron were higher (**Figure 2**) than in tryptophan oxizided with H_2O_2, with similar tryptophan loss (*18*). It has been observed that heated α-lactalbumin and β-lactalbumin oxidation of tryptophan yielded hydroxy-

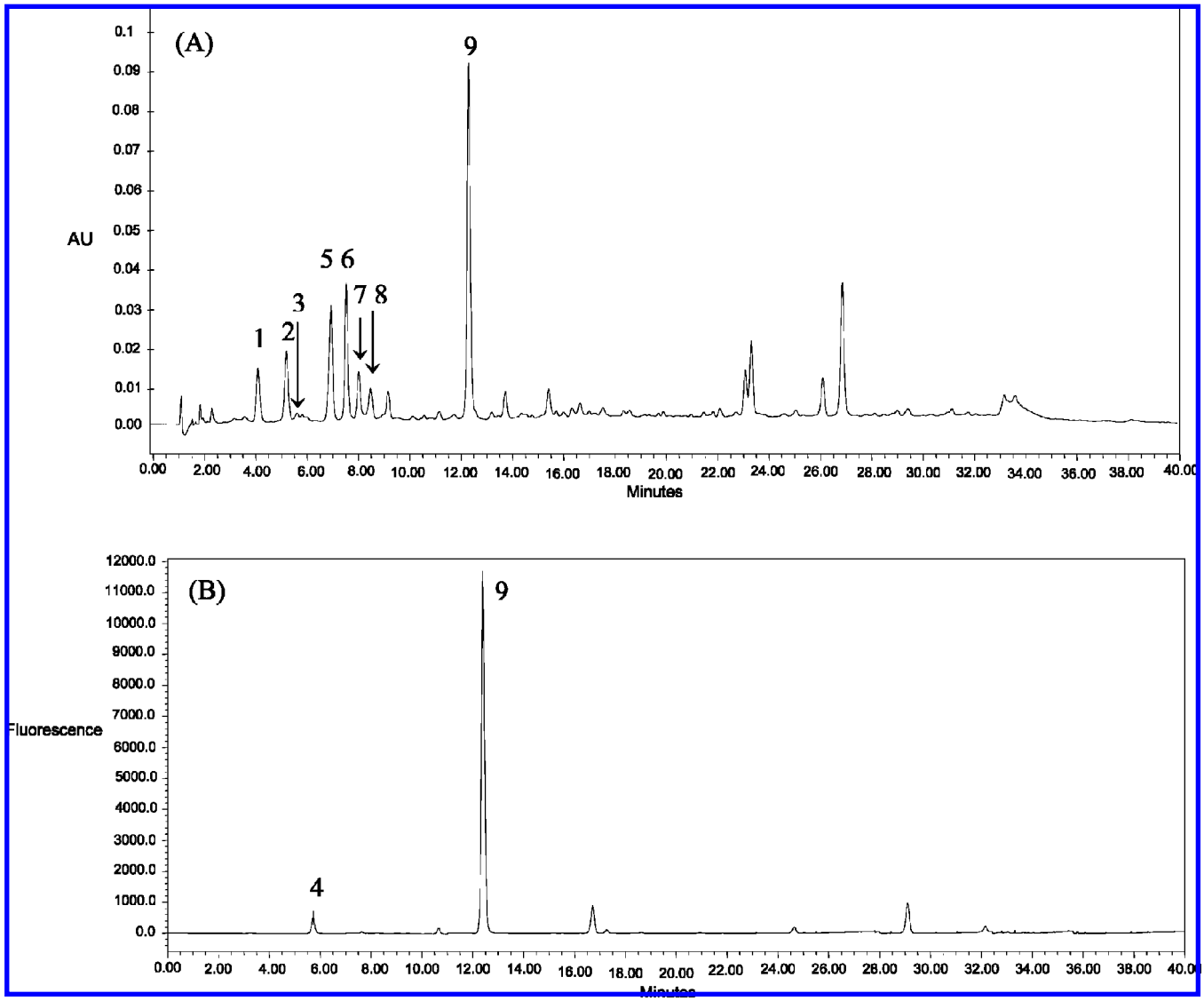

Figure 1. RP-HPLC chromatogram of tryptophan and its oxidation products with (A) UV detection at 260 nm and (B) fluorescence detection: 0−7 min, Ex at 230 nm and Em at 340 nm; 7−11 min, Ex at 230 nm and Em at 435 nm; and 11−40 min, Ex at 270 nm and Em at 350 nm. Experimental conditions are as reported in the Materials and Methods. Identification of peaks: 1, dioxindolylalanine A; 2, dioxindolylalanine B; 3, 3a-hydroxypyrroloindole-2-carboxylic acid B; 4, 5-hydroxy-tryptophan; 5, kynurenine; 6, *N*-formylkynurenine; 7, β-oxindolylalanine A; 8, β-oxindolylalanine B; and 9, tryptophan.

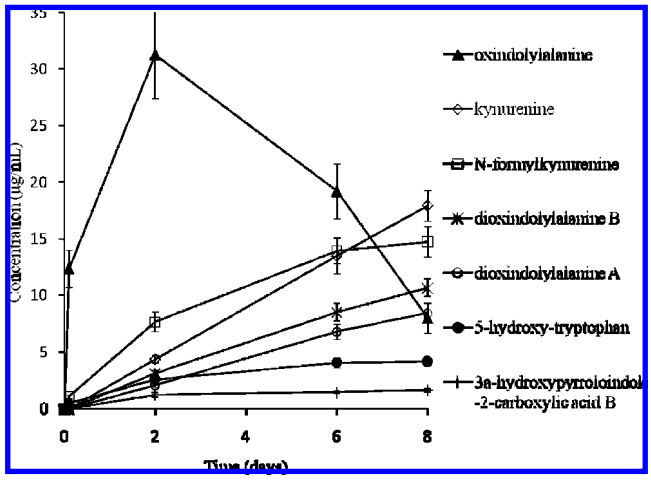

Figure 2. Formation of oxidation products (μg/mL, $n = 18$) in tryptophan solution (2 mM) oxidized with hexanal (50 mM) and $FeCl_2$ (0.1 mM).

tryptophan and hydroxy-tryptophan or *N*-formylkynurenine, respectively (*26*). There were no significant differences between tryptophan losses or the formation of tryptophan oxidation products between days 6 and 8, except for β-oxindolylalanine. Thus, the oxidation rate seems to be stabilized for tryptophan and its oxidation products after day 6. Therefore, all of the results are calculated according to day 6. The results on the inhibition of tryptophan oxidation are calculated according to the level of oxidation at day 6 to be consistent with the calculations of the control. Inhibition values for 3a-hydroxypyrroloindole-2-carboxylic acid B are not included because the amount of 3a-hydroxypyrroloindole-2-carboxylic acid B formed was under the LOQ. In addition, the formation of 3a-hydroxypyrroloindole-2-carboxylic acid A in hexanal- and iron-oxidized tryptophan with all added plant materials were found to be under the LOD.

Approximately 20% of the oxidation products depending upon the tryptophan loss could be elucidated by HPLC determination. This was consistent with previously reported results in H_2O_2-oxidized tryptophan (*18, 24*). The appearance of less polar UV-active and fluorescent compounds was detected after tryptophan was eluted (**Figure 1**). However, the formation of putative tryptophan aggregation was not investigated in this study.

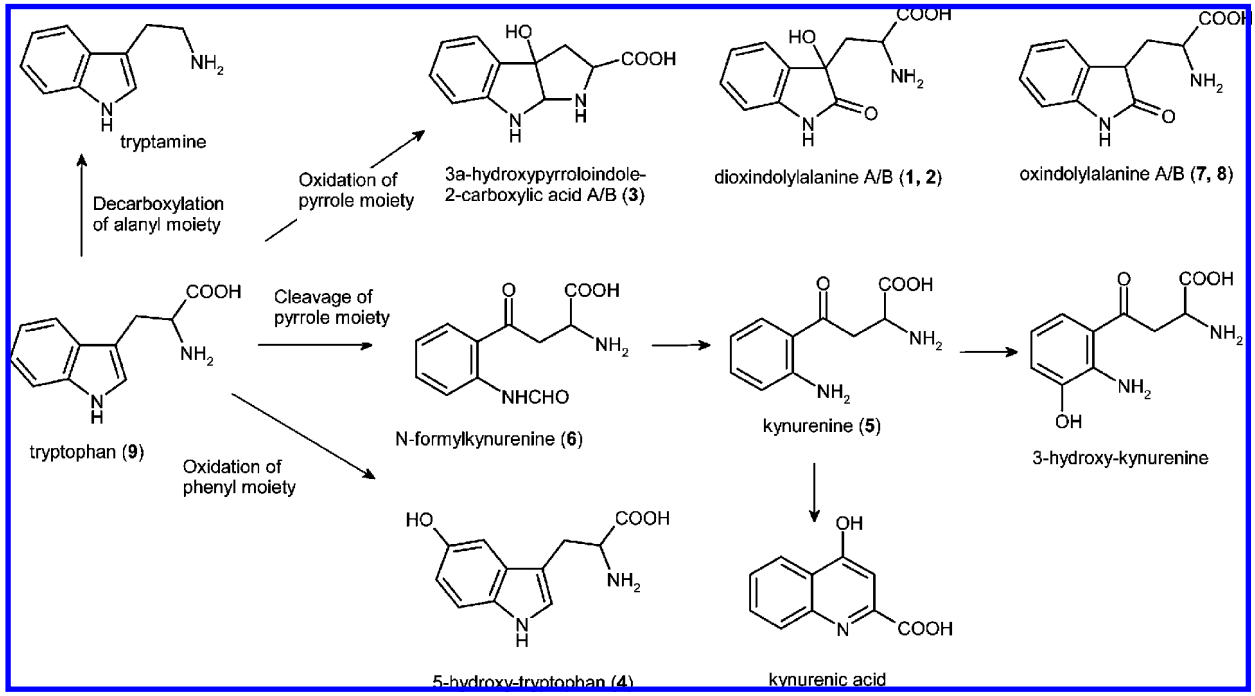

Figure 3. Formation of tryptophan oxidation products. Numbers correspond with peaks in **Figure 1**.

Table 1. Inhibitions of Tryptophan Loss (%) with Phenolics after 6 Days of Oxidation with Hexanal and $FeCl_2$ Compared to Day 0[a]

	inhibitions of tryptophan loss (%) after oxidation (mean ± SD)	
	50 μM	100 μM
	Plant Phenolics	
camelina meal	51.9 ± 4.0 a	49.8 ± 1.0 a
rapeseed meal	24.6 ± 11.4 b	33.6 ± 2.9 b
soy meal	25.7 ± 3.3 b	NA
pine bark	6.7 ± 2.1 c	12.6 ± 2.2 c
black currant anthocyanins	−15.5 ± 1.2 fg	−8.9 ± 0.5 ef
raspberry anthocyanins	−10.7 ± 1.3 ef	−13.1 ± 3.5 f
raspberry ellagitannins	−1.5 ± 0.9 cde	−7.3 ± 0.5 def
rowanberry extract	−23.0 ± 0.5 g	−12.6 ± 1.2 f
	Phenolic Compounds	
cyanidin-3-glucoside	−6.9 ± 4.3 def	−23.7 ± 3.2 g
chlorogenic acid	−1.5 ± 11.5 cde	−9.7 ± 1.3 ef
catechin	−10.5 ± 2.6 ef	10.0 ± 1.3 c
ellagic acid	5.3 ± 8.7 cd	−9.6 ± 8.5 def
genistein	−6.8 ± 5.0 def	6.8 ± 4.7 c
procyanidin B2	−2.4 ± 6.3 cde	−0.9 ± 7.8 d
quercetin	−0.9 ± 1.4 cde	−9.5 ± 1.0 ef
sinapic acid	4.8 ± 1.0 cd	−8.4 ± 7.4 ef
taxifolin	1.1 ± 9.3 cde	−4.7 ± 0.8 de

[a] NA, not analyzed. Values in the same column followed by different letters are significantly different ($p < 0.0001$).

Effect of Plant Phenolics on Tryptophan Oxidation. Different plant phenolics affected the oxidation of tryptophan by hexanal and iron by either acting as antioxidants or as pro-oxidants (**Table 1**). Most of the phenolic reference compounds showed only weak antioxidant or pro-oxidant activities toward tryptophan loss (**Table 1**).

Oilseed Processing Byproducts. Camelina meal phenolics (50 and 100 μM) exhibited the best inhibitions (50−52%) among the plant phenolics against the loss of tryptophan oxidized by hexanal and iron (**Table 1**). However, there was no significant difference in the effect between the concentration levels. In our previous study, camelina meal phenolics were shown to inhibit to a minor extent the loss of tryptophan (by 6−32%) and its oxidation products in a H_2O_2-catalyzed oxidation of tryptophan (*18*). Thus, camelina meal phenolics revealed a more pronounced effect in inhibiting the hexanal- and iron-induced tryptophan oxidation than H_2O_2-induced oxidation of tryptophan. The camelina meal phenolics comprise flavonols, hydroxycinnamic acids, and flavanols (**Table 2**), which presumably have a synergistic effect as the antioxidant activity of their principal reference compounds, such as sinapic acid at 50 μM (5%) and catechin at 100 μM (10%), toward the loss of tryptophan, which was weaker than that of the camelina meal extract. In addition, quercetin and chlorogenic acid showed either no effect or pro-oxidant activity toward tryptophan loss. Camelina meal phenolics showed excellent (≥95%) inhibitions toward dioxindolylalanine A and β-oxindolylalanine and good (≥80%) inhibitions toward *N*-formylkynurenine and kynurenine, as well as moderate (≥50%) inhibitions toward dioxindolylalanine B and 5-hydroxy-tryptophan (**Table 3**). Because tryptophan was less oxidized in the presence of camelina meal phenolics, the formation of primary and secondary oxidation products was delayed and therefore resulted in excellent inhibitions.

Rapeseed meal phenolics at 50 and 100 μM showed 25−34% inhibitions toward the loss of tryptophan (**Table 1**). Similarly to the effect of camelina meal phenolics, the effect of rapeseed meal phenolics in inhibiting tryptophan loss was more pronounced in tryptophan oxidized by hexanal and iron than to that of tryptophan oxidized with H_2O_2 (8−23%) (*18*). Rapeseed meal also provided excellent protection toward oxidation of β-oxindolylalanine (**Table 3**). However, rapeseed phenolics showed moderate (≥50%) protection toward the formation of dioxindolylalanine A and B, 5-hydroxy-tryptophan, and kynurenine compared to camelina meal phenolics. Rapeseed meal phenolics inhibited the oxidation by delaying the formation of primary oxidation products, dioxindolylalanine and β-oxindolylalanine, thereby showing more pronounced protection toward secondary oxidation products, *N*-formylkynurenine and kynurenine. Hydroxycinnamic acid derivatives of principally sinapine and sinapic acid were the most abundant phenolic compounds in the phenolic extract of rapeseed meal (**Table 2**). Among hydroxycinnamic acids, sinapic acid (50 μM) showed weak inhibition (5%) against tryptophan loss, while chlorogenic acid induced oxidation of tryptophan (**Table 1**). In our previous

Table 2. Phenolic Composition of Plant Extracts[a]

compound	rapeseed meal (μg/g)	camelina meal (μg/g)	soy meal (μg/g)	rowanberry (mg/g)
total phenolics[b]	5865 ± 547	9791 ± 993	1761 ± 133	18.7 ± 0.8
	Flavonoids and Phenolic Acids			
flavanols[c]	ND	3233 ± 818	449 ± 127	ND
catechin[c]				52 ± 2
hydroxybenzoic acids[b]	ND	ND	ND	0.1 ± 0.02
hydroxycinnamic acids and derivatives[d]	3773 ± 1063	3590 ± 383	ND	341 ± 23
sinapine[e]	765 ± 134	450 ± 52	ND	NA
sinapic acid[e]	74 ± 9	9 ± 1	ND	NA
flavonols[f]	ND	6029 ± 677	ND	64 ± 2
anthocyanins[g]	ND	ND	ND	9.6 ± 0.3
ellagic acid[h]	ND	ND	ND	0.2 ± 0.04

[a] ND = not detected/concentration under detection limit, and NA = not analyzed. [b] Gallic acid as the standard. [c] Catechin as the standard. [d] Chlorogenic acid as the standard. [e] Sinapic acid as the standard. [f] Rutin as the standard. [g] Cyanidin 3-glucoside as the standard. [h] Ellagic acid as the standard.

Table 3. Inhibitions (%) of Tryptophan Oxidation Products after 6 Days of Oxidation by Plant Phenolics in a Tryptophan Model Solution Oxidized with 50 mM Hexanal and 0.1 mM $FeCl_2$[a]

	dioxindolyl-alanine A	dioxindolyl-alanine B	5-OH-tryptophan	kynurenine	*N*-formyl-kynurenine	oxindolyl-alanine
			Camelina Meal			
50 μM	100.0 ± 0.0 a	77.9 ± 4.3 a	74.2 ± 1.1 a	77.9 ± 5.4 a	80.6 ± 3.0 a	100.0 ± 0.0 a
100 μM	100.0 ± 0.0 a	37.1 ± 3.8 b	78.6 ± 0.6 a	83.4 ± 3.0 a	82.4 ± 2.1 a	100.0 ± 0.0 a
			Rapeseed Meal			
50 μM	32.4 ± 9.5 b	37.5 ± 20.0 b	34.8 ± 5.2 b	20.8 ± 5.9 b	18.9 ± 24.1 bc	91.4 ± 7.9 ab
100 μM	53.6 ± 0.9 b	52.6 ± 10.0 a	47.5 ± 3.1 b	53.5 ± 4.7 b	35.1 ± 10.8 c	100.0 ± 0.0 a
			Soy Meal			
50 μM	36.3 ± 10.1 b	32.5 ± 4.6 b	42.2 ± 2.5 b	76.7 ± 9.0 a	80.7 ± 3.4 a	100.0 ± 0.0 a
			Pine Bark			
50 μM	−84.9 ± 21.1 e	−75.3 ± 17.2 e	15.8 ± 12.1 c	0.3 ± 7.4 c	32.0 ± 5.2 b	89.7 ± 2.9 b
100 μM	10.7 ± 3.2 c	11.9 ± 1.9 c	41.8 ± 8.1 b	58.5 ± 5.2 b	68.7 ± 5.1 b	58.8 ± 3.3 c
			Raspberry Anthocyanins			
50 μM	−51.4 ± 7.7 cd	−25.5 ± 6.2 c	−6.0 ± 5.8 d	−42.7 ± 4.3 e	−11.8 ± 3.8 d	59.1 ± 5.2 d
100 μM	−64.6 ± 2.5 d	−68.3 ± 0.8 de	−55.9 ± 2.7 e	−54.3 ± 4.3 d	−13.5 ± 4.7 f	57.9 ± 1.4 c
			Raspberry Ellagitannins			
50 μM	−33.8 ± 3.0 c	−41.7 ± 3.7 cd	−5.3 ± 10.9 d	−35.5 ± 0.2 e	3.0 ± 7.4 cd	39.0 ± 4.0 e
100 μM	−73.8 ± 5.5 d	−77.3 ± 6.4 e	−37.0 ± 6.6 d	−30.5 ± 0.3 c	2.8 ± 4.3 e	44.8 ± 3.3 d
			Black Currant Anthocyanins			
50 μM	−70.1 ± 0.5 de	−63.9 ± 1.9 de	−49.2 ± 5.9 e	−13.4 ± 2.6 d	−11.9 ± 0.9 d	50.5 ± 9.6 d
100 μM	−101.4 ± 25.8 e	−76.7 ± 2.0 e	−15.0 ± 10.6 c	−31.4 ± 5.9 c	16.6 ± 2.9 d	83.3 ± 5.9 b
			Rowanberry			
50 μM	−68.0 ± 22.9 de	−40.8 ± 13.1 cd	3.0 ± 9.1 cd	−42.0 ± 4.3 e	26.7 ± 6.9 b	80.0 ± 1.4 c
100 μM	−98.8 ± 2.1 e	−63.3 ± 5.6 d	−10.9 ± 1.3 c	−61.2 ± 0.7 d	32.7 ± 2.2 c	84.7 ± 4.2 b

[a] Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.0001$).

study, sinapic acid was somewhat more potent (20−27%) in inhibiting tryptophan loss in oxidation induced by H_2O_2 (*18*). It may be possible that the choline ester of sinapic acid, sinapine, is the more effective form. Thus, the diverse structures of hydroxycinnamic acids may be contributing to the oxidative protection of tryptophan in both rapeseed and camelina meals.

Soy meal (50 μM) inhibited tryptophan loss by 26% (**Table 1**). **Figure 4** presents the chromatogram of loss of tryptophan and the formation of tryptophan-derived oxidation products with and without soy meal phenolics. In our earlier study, soy meal phenolics were able to inhibit tryptophan loss in H_2O_2-oxidized tryptophan by 7% (at 50 μM) and 18% (at 100 μM) (*18*). Thus, soy meal phenolics were slightly more potent in inhibiting the oxidation of tryptophan oxidized by hexanal and iron compared to tryptophan oxidized by H_2O_2. Soy meal phenolics were potent in inhibiting the oxidation of β-oxindolylalanine, *N*-formylkynurenine, kynurenine, and to a lesser extent also the oxidation of dioxindolylalanine A/B and 5-hydroxy-tryptophan (**Table 3**). Soy meal phenolics showed more pronounced inhibition toward the formation of tryptophan oxidation products, such as kynurenine and *N*-formylkynurenine, than rapeseed meal phenolics (50 μM), with less tryptophan loss (**Table 3**). The concentrations of oxindolylalanine with oilseed byproducts increased rapidly after oxidation had started, as it did in the control sample, although the concentrations of β-oxindolylalanine with all oilseed phenolics (soy, camelina, and rapeseed) were lower (5−7 μg/mL at day 0.1; individual data not shown) compared to that of the control sample (12 μg/mL at day 0.1; **Figure 2**). However, after 2 days of oxidation, the concentrations of β-oxindolylalanine had decreased significantly with added oilseed byproducts (0−2 μg/mL; individual data not shown) compared to the control (31 μg/mL; **Figure 2**). The concentrations of β-oxindolylalanine with oilseed byproducts remained low (0−2 μg/mL; individual data not shown) during the rest of the oxidation period (days 6 and 8) compared to that of the control (19 and 8 μg/mL; **Figure 2**). This phenomenon is due to the fact that β-oxindolylalanine itself is also susceptible to oxidation, yielding dioxindolylalanine and kynurenine (*24, 25*). This would explain the higher

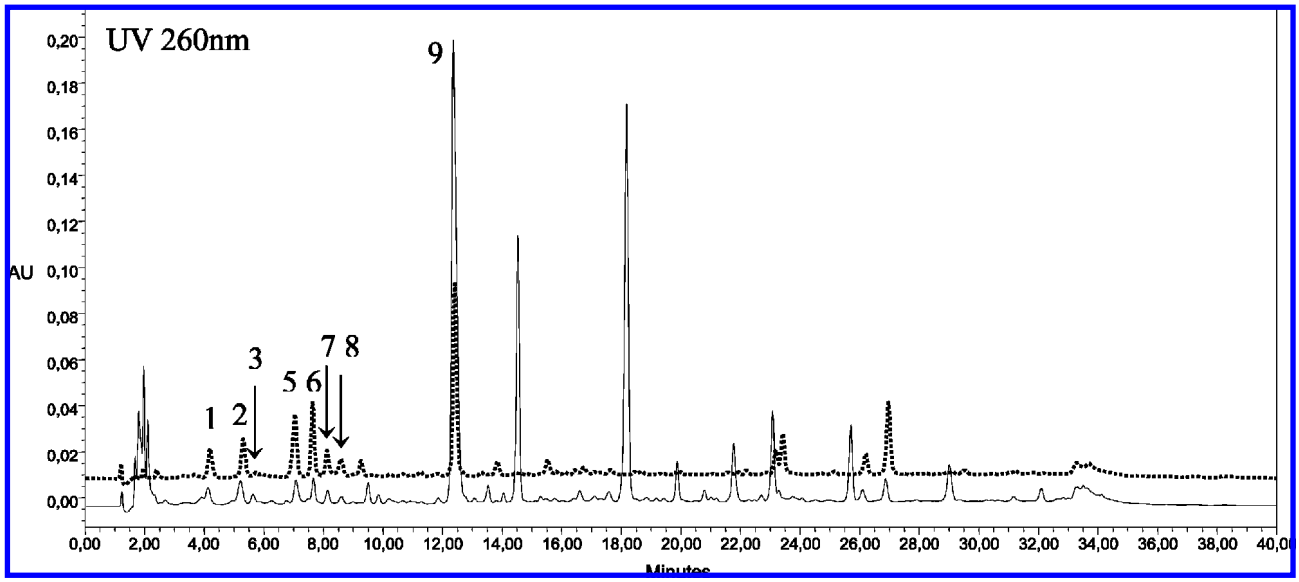

Figure 4. RP-HPLC chromatogram of tryptophan-derived oxidation products of hexanal- (50 mM) and $FeCl_2$- (0.1 mM) oxidized tryptophan (2 mM) (· · ·) with added soy meal phenolics (50 μM) (—) with UV detection after 8 days of oxidation. Peak identifications are as described in **Figure 1**.

Table 4. Inhibitions (%) of Tryptophan Oxidation Products after 6 Days of Oxidation by Phenolic Compounds in a Tryptophan Model Solution Oxidized with 50 mM Hexanal and 0.1 mM $FeCl_2$[a]

	dioxindolyl-alanine A	dioxindolyl-alanine B	5-hydroxy-tryptophan	kynurenine	N-formyl-kynurenine	oxindolyl-alanine
			Cyanidin-3-glucoside			
50 μM	−57.7 ± 2.0 d	−37.9 ± 1.4 c	4.4 ± 13.1 ab	−23.5 ± 1.8 de	20.2 ± 3.8 ab	60.9 ± 4.4 c
100 μM	−27.4 ± 2.4 b	−23.1 ± 3.5 b	16.8 ± 19.2 a	−1.6 ± 3.7 bcd	33.2 ± 4.7 abc	59.3 ± 7.3 bc
			Catechin			
50 μM	−28.7 ± 12.2 ab	−10.0 ± 6.8 ab	−3.7 ± 12.9 abc	−6.9 ± 1.8 bc	13.6 ± 4.7 b	70.3 ± 0.5 abc
100 μM	−10.6 ± 1.2 a	−6.2 ± 0.3 a	7.2 ± 6.1 a	1.6 ± 1.3 bc	44.8 ± 0.1 a	67.1 ± 3.6 ab
			Chlorogenic Acid			
50 μM	−25.7 ± 6.3 ab	−22.6 ± 3.8 b	11.4 ± 7.1 a	−28.2 ± 2.4 e	14.2 ± 14.7 ab	63.3 ± 2.1 bc
100 μM	−61.2 ± 5.6 d	−44.8 ± 0.1 cd	−10.9 ± 2.2 b	−32.9 ± 3.4 e	16.3 ± 1.2 d	66.0 ± 6.2 ab
			Ellagic Acid			
50 μM	−73.0 ± 7.5 e	−77.7 ± 7.8 d	−5.3 ± 1.3 bc	5.2 ± 3.4 a	28.3 ± 7.3 ab	71.7 ± 6.8 ab
100 μM	−125.7 ± 5.4 f	−150.3 ± 2.3 e	−14.2 ± 0.8 b	4.7 ± 1.1 abc	20.6 ± 1.8 cd	79.9 ± 6.3 a
			Procyanidin B2			
50 μM	−46.4 ± 3.7 cd	−22.1 ± 3.0 b	−16.0 ± 1.9 cd	−19.4 ± 0.2 de	10.4 ± 1.0 b	39.7 ± 5.1 d
100 μM	−44.2 ± 2.1 c	−34.2 ± 1.1 bc	−15.1 ± 3.7 b	−10.9 ± 0.1 cd	27.3 ± 3.0 bcd	37.7 ± 3.7 d
			Sinapic Acid			
50 μM	−14.3 ± 3.0 a	−1.4 ± 0.9 a	−26.5 ± 0.8 de	−6.3 ± 3.5 abc	10.5 ± 0.1 b	21.6 ± 3.8 e
100 μM	−51.6 ± 8.4 cd	−34.7 ± 18.4 bc	−50.4 ± 0.5 c	19.9 ± 15.8 a	16.2 ± 4.8 d	46.3 ± 6.6 cd
			Taxifolin			
50 μM	−28.4 ± 4.5 ab	−38.4 ± 10.3 c	−35.5 ± 3.7 e	−17.4 ± 6.8 cde	21.3 ± 7.4 ab	41.3 ± 4.6 d
100 μM	−43.2 ± 2.6 c	−38.3 ± 4.0 bcd	−35.3 ± 3.0 c	−7.6 ± 2.6 cd	35.7 ± 1.8 ab	42.0 ± 3.0 d
			Quercetin			
50 μM	−33.1 ± 8.0 bc	−42.0 ± 10.7 c	−3.7 ± 1.3 abc	−14.4 ± 10.0 cd	33.0 ± 9.3 a	76.0 ± 7.2 a
100 μM	−76.1 ± 11.3 e	−55.0 ± 0.7 d	−17.0 ± 4.9 b	−16.8 ± 2.7 de	28.5 ± 2.5 bcd	81.7 ± 1.5 a
			Genistein			
50 μM	−18.7 ± 3.3 ab	−21.4 ± 0.3 b	−5.6 ± 1.6 bc	0.8 ± 4.6 ab	18.6 ± 8.4 ab	28.9 ± 1.3 e
100 μM	−27.7 ± 2.7 b	−32.9 ± 13.4 bc	16.1 ± 1.1 a	12.1 ± 15.8 ab	32.8 ± 15.5 abc	70.8 ± 22.9 ab

[a] Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.005$).

inhibition toward dioxindolylalanine and kynurenine compared to other plant phenolics (pine bark and berries) because a smaller amount of β-oxindolylalanine was formed with oilseed phenolics. Genistein at 100 μM (7%) was only somewhat more effective than at 50 μM (−7%) in inhibiting tryptophan loss (**Table 1**). In addition, genistein was able to inhibit *N*-formylkynurenine and oxindolylalanine, as well as 5-hydroxy-tryptophan (**Table 4**). In H_2O_2-oxidized tryptophan, genistein and daidzein, isoflavones present in soy, provided 12−17% protection against tryptophan loss (*18*).

Pine Bark Phenolics. Pine bark phenolics provided 13% inhibition at 100 μM toward oxidation of tryptophan compared to only 7% at 50 μM (**Table 1**). It has been shown that pine bark added to H_2O_2-oxidized tryptophan was able to inhibit tryptophan loss by 20−36% depending upon the concentration level (*18*). Vuorela et al. (*10*) observed that pine bark phenolics showed antioxidant activity toward protein oxidation in cooked pork meat patties. Pine bark (100 μM) was able to inhibit the formation of 5-hydroxy-tryptophan and oxindolylalanine (**Table 3**). In addition, the oxidation of primary oxidation products,

dioxindolylalanine A and B, were slowed down by pine bark phenolics, consequently inhibiting the secondary oxidation products *N*-formylkynurenine and kynurenine. Pine bark phenolics consist mainly of flavanols (~80 μg/g) (*18*), with catechin dominating (*10*). In this study, catechin at 100 μM was able to inhibit tryptophan loss by 10% (**Table 1**), whereas taxifolin, another phenolic compound reported in pine bark, showed either no effect or weak pro-oxidant activity (−5%). In our previous study of tryptophan oxidized by H_2O_2, catechin and taxifolin (at 50 and 100 μM) were able to inhibit the loss of tryptophan by 9−25 and 4−8%, respectively (*18*). Flavonols (e.g., quercetin) and flavanones (e.g., taxifolin) have been shown to have higher metal-initiated pro-oxidant activity (*27*). This may explain why reference compounds quercetin and taxifolin exhibited pro-oxidant or no effect toward the loss of tryptophan.

Berry Phenolics. Raspberry ellagitannins showed pro-oxidant effect on tryptophan oxidation by hexanal and iron as measured by the loss of tryptophan (**Table 1**). Raspberry ellagitannins were reported to have a weak antioxidant effect (12−17%) toward the oxidation of tryptophan induced by H_2O_2 (*18*). It is known that the affinities of tannins for binding, cross-linking, and consequently precipitating proteins are dependent upon the structural flexibility of both the tannin and protein molecule (*28*). The loss of conformational freedom of ellagitannins significantly affects their binding capability (*28*). In addition, it has been shown that metal catalyzes the oxidation and polymerization of the phenolic compounds, therefore reducing their available binding sites (*6*). Therefore, redox scavenging is unlikely to contribute to inhibiting the formation of tryptophan oxidation compounds. Raspberry ellagitannins were less effective in inhibiting the formation of β-oxindolylalanine (by ~40%) compared to the other berry phenolics (58−85%) (**Table 3**). High concentrations of β-oxindolylalanine were generated rapidly after starting the oxidation of tryptophan with hexanal and iron with all of the added berry phenolics, and after 2 days of oxidation, the concentrations of β-oxindolylalanine with all of the berry phenolics were higher (32−40 μg/mL; individual data not shown) than of that of the control (12 μg/mL; **Figure 2**). β-Oxindolylalanine was rapidly decomposed to dioxindolylalanine and kynurenine after the second day of oxidation with all added berry phenolics. In addition, the rapid oxidation of dioxindolylalanine also contributed to an increased formation of kynurenine with all berry phenolics.

Black currant and raspberry anthocyanins and rowanberry phenolics were not able to inhibit the loss of tryptophan because of oxidation (**Table 1**). On the contrary, these sources of plant phenolics also showed pro-oxidant properties. Thus, tryptophan was oxidized more than the control, yielding also more oxidation products. Cyanidin-3-glucoside (50 and 100 μM) as a reference compound showed pro-oxidant activity toward tryptophan loss (**Table 1**). Cyanidin-3-glucoside, however, was able to inhibit the formation of β-oxindolylalanine (by 60%) and *N*-formylkynurenine (by 20−33%) (**Table 4**). In our earlier study with H_2O_2-oxidized tryptophan, black currant anthocyanins at 50 μM were shown to be effective (42%) in inhibiting tryptophan loss (*18*). However, black currant anthocyanins at 100 μM as well as raspberry anthocyanins were not able to inhibit tryptophan loss induced by H_2O_2. Under present conditions, raspberry and black currant anthocyanins did not appear to be correlated to their molecular structures contributing to their antioxidant properties because they exhibited pro-oxidant or weak activities toward tryptophan oxidation. The antioxidant properties of flavonoids are mainly due to the 3′,4′-dihydroxy group located on the B ring and the 3-hydroxy or 5-hydroxy and the 4-carbonyl groups in the C ring (*7*). In addition, the antioxidant activity increases with the number of hydroxyl groups in rings A and B. The inability to protect tryptophan from oxidation may be due to the fact that anthocyanins have a very low oxidation potential (spontaneous oxidation), which renders them into either pro-oxidants by redox cycling or good antioxidants depending upon the conditions, as was described by van Acker et al. (*29*). Moreover, the reaction conditions with a pH of 5.5 may not have been optimal for anthocyanins to exhibit antioxidant properties.

Although the berry phenolics did not protect tryptophan from oxidation by hexanal and iron, they were effective in inhibiting the formation of β-oxindolylalanine, with rowanberry phenolics and black currant anthocyanins showing the best inhibitions (80%) (**Table 3**). The formation of *N*-formylkynurenine was, however, weakly inhibited with black currant anthocyanins (100 μM) and rowanberry extract. Other tryptophan oxidation products were not inhibited by berry phenolics. Catechin (100 μM) was able to inhibit oxindolylalanine (70%) and *N*-formylkynurenine (45%) (**Table 4**). Tryptophan oxidation was faster especially with berry phenolics, which attributed to an increase in primary oxidation products.

In conclusion, this study describes the effects of plant phenolics toward the oxidation of tryptophan with hexanal and $FeCl_2$. The results show that exposure to hexanal and $FeCl_2$ increases the oxidation of tryptophan and the formation of tryptophan-derived oxidation compounds. In this study, the effects of oilseed processing byproducts, such as camelina, rapeseed, and soy meals, as well as pine bark phenolics, were more pronounced on both the inhibitions of tryptophan loss and individual oxidation compounds than berry phenolics, which showed pro-oxidant effects. The indole (pyrrole) moiety of tryptophan is the most likely group to be involved in the reaction with the phenolic compounds because the oxidation of the indole structure yielding *N*-formylkynurenine and kynurenine was effectively inhibited by camelina and soy meals, followed by rapeseed meal and pine bark. This is in accordance with Rawel et al. (*30*), proposing that the semiquinones or quinones of phenolic compounds may react with the heterocyclic N atom of tryptophan. The antioxidant activity of plant phenolics may be due to the ability of flavonoid semiquinones or quinones in binding directly to tryptophan and, thereby, blocking it from further reactions. Further oxidation of this product can lead to the formation of tryptophan dimers or polymers. Another possibility is the reaction between the flavonoid radical and tryptophan radical. However, flavonoid termination reactions do not necessary lead to the termination of radical scavenging because oxidation products (dimers or quinones) and their degradation products may still be reactive (*31*). The network of phytochemicals is essential for their activity, particularly when considering that an antioxidant may become a pro-oxidant if suitable and sufficient co-antioxidants are missing (*32*). Most active antioxidants are likely to be pro-oxidants when they lie beyond the optimum (*29*). This may explain the differences in efficacy for camelina and rapeseed meals compared to rowanberry phenolics, even though they comprise similar phenolics. Therefore, it may be that the concentration levels of the phenolics applied in this study were not optimal for the antioxidant activity, especially when the concentrations of the phenolics (50 and 100 μM) were low compared to the tryptophan concentration (2 mM). More research is needed to optimize the levels of phenolics to be effective and to further explicate their antioxidant effect toward protein oxidation by investigating the unknown compounds formed during the

oxidation. Our results contribute to elucidating the effects of natural phenolic compounds as potential antioxidants to control and prevent protein oxidation reactions in food and pharmaceutical applications.

Supporting Information Available: Characteristics of the HPLC method: limit of detection (LOD) and quantification (LOQ); equation, correlation coefficient (R), and concentration range of the calibration curve. This material is available free of charge via the Internet at http://pubs.acs.org.

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