Peroxynitrite oxidises catechols to *o*-quinones

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Abstract Nitration of phenolic compounds is a well-established mechanism on interaction with peroxynitrite. However, while nitration is the predominant reaction for monophenolic hydroxycinnamates, this does not take place with the catechol-containing hydroxycinnamate, caffeic acid. The aim of the present study was to investigate the mechanism of the chemical interaction of caffeic acid with peroxynitrite and to characterise the products formed. A novel compound was detected and characterised as the *o*-quinone of caffeic acid based on its reaction with nucleophilic thiol compounds, glutathione and L-cysteine. The same novel product was identified following the oxidation of caffeic acid in alkaline solutions confirming the identity of this species as a caffeic acid oxidation product.

Key words: Caffeic acid; Oxidation; Antioxidant; *o*-Quinone; Peroxynitrite; Glutathione

1. Introduction

The hydroxycinnamates caffeic, ferulic and *p*-coumaric acids are among the major phenolic components of dietary phytochemicals, usually occurring in various conjugated forms, which result from enzymatic hydroxylation, *O*-methylation, *O*-glycosylation or esterification of *p*-coumaric acid [1]. Chlorogenic acid (the quinic acid ester of caffeic acid), caffeic acid and related compounds are widely distributed in fruit, peas, peaches, plums, cherries and apricots [2] as well as teas, coffee, apple juice, etc. [3–5]. Many studies have demonstrated the antioxidant properties of chlorogenic acid, caffeic acid, of its monohydroxylated precursor, *p*-coumaric and of ferulic acid, the 3-methoxy,4-hydroxycinnamic derivative (Fig. 1) as scavengers of oxygen radicals [6,7], reactive nitrogen species [8] and inhibitors of the *N*-nitrosation reactions [9].

Very little is known about the products formed on interaction of the catechol-containing phenolics with reactive nitrogen species. Our recent studies [8] have shown that hydroxycinnamates can inhibit peroxynitrite-mediated tyrosine nitration, caffeic and chlorogenic acids (the *o*-dihydroxy catechol structures) being more effective than the monohydroxy-containing *p*-coumaric and ferulic acids. Analysis of the products of the direct interaction of peroxynitrite with the hydroxycinnamates demonstrated a mechanism of nitration for monophenolates but, for the catecholates, caffeic and chlorogenic acids, nitration was not detected.

The purpose of this study is to investigate the chemical interaction between caffeic acid and peroxynitrite, the predicted mechanism being that of oxidation, and to establish the chemical nature of the products formed.

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2. Materials and methods

2.1. Chemicals

The hydroxycinnamates, tyrosine, 3-nitrotyrosine, 4-hydroxy-3-nitrobenzoic acid, hydrogen peroxide and sodium nitrite were obtained from Sigma (Poole, Dorset, UK). Sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate, potassium hydroxide and hydrochloric acid were obtained from BDH (Poole, Dorset, UK). HPLC grade acetonitrile was purchased from Rathburn (Walkerburn, Scotland, UK). All other reagents used were of analytical grade. All the reagents were prepared using deionised water (18.2 MΩ Maxima Ultrapure water).

2.2. Peroxynitrite synthesis

Peroxynitrite synthesis was carried out by a modification of the method described by Beckman et al. [10]. Acidified hydrogen peroxide (1 M in 0.7 M HCl, 20 ml) and sodium nitrite (0.2 M, 20 ml) solutions were drawn into two separate syringes, analogous to a stopped flow set up. Simultaneous injection of the contents of both syringes into an ice-cooled beaker containing 1.5 M potassium hydroxide (40 ml) through a 'Y'-shaped junction led to rapid mixing; this results in the formation of peroxynitrous acid followed by stabilisation of the resulting peroxynitrite anion. Manganese dioxide was added to the solution to remove excess hydrogen peroxide. The solution was filtered and the concentration of peroxynitrite was determined by measuring the absorbance at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$). The typical yield of freshly prepared peroxynitrite was 40 mM. The peroxynitrite was diluted in 0.1 M NaOH.

2.3. Interaction of hydroxycinnamates with peroxynitrite

Stock solutions of hydroxycinnamates (50 μM) were prepared in 50 mM phosphate buffer, pH 7. Hydroxycinnamates were reacted with either equimolar or excess concentrations of peroxynitrite and the formation of nitration and/or oxidation products was characterised by spectroscopy and HPLC. The reaction between peroxynitrite and hydroxycinnamate was initiated by the addition of a 1 ml stock solution of *p*-coumaric, ferulic or caffeic acid to 20-μl aliquots of varying concentrations of peroxynitrite in alkali. The final concentrations of peroxynitrite in the reaction mixture were between 50 and 400 μM. The samples before and after reaction with peroxynitrite were analysed by spectrophotometry on a Hewlett-Packard 8453 UV-Visible spectrophotometer using Chemstation software (Hewlett-Packard, Waldbronn, Germany). Peroxynitrite allowed to degrade for 10 min in phosphate buffer (pH 7) at room temperature was used as the blank.

To characterise the products formed following the reaction of hydroxycinnamates with peroxynitrite, HPLC analysis was performed. Solutions containing 50 μM hydroxycinnamate in phosphate buffer (50 mM, pH 7) were added to a tube containing a 20-μl aliquot of varying concentrations of peroxynitrite (final concentrations 50–2000 μM peroxynitrite) in a final volume of 1 ml. Samples were analysed by HPLC with UV detection at 280 nm and the spectra of the resolved peaks were obtained between 200–600 nm with photodiode array detection (Waters 996 Photodiode Array Detector). Caffeic acid and reaction products were separated using a Novapak C_{18} 4-μm column (250×4.6 mm, Waters, Watford, UK) and a mobile phase consisting of 50 mM phosphate buffer, pH 7, at a flow rate of 0.5 ml/min (Waters 626 Pump). The injection volume was 50 μl (Waters 717 plus Autosampler) and data acquisition was performed with Waters Millennium 2010 Chromatography Manager software (Waters, Milford, MA, USA). For the analysis of *p*-coumaric acid and ferulic acid, the composition of the mobile phase was modified to an isocratic system consisting of 95% 50 mM phosphate buffer, pH 7, and 5% acetonitrile. The retention times for caffeic, ferulic and *p*-coumaric acids were 11.2 min, 9.5 min and 7.6 min, respectively.

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The loss of substrate following the reaction of caffeic acid with peroxynitrite was quantified using a calibration curve of caffeic acid (μM) vs. peak area ratio using 4-hydroxy-3-nitrobenzoic acid as the internal standard. The formation of the main product with a retention time of approximately 6.3 min was also quantified and expressed as caffeic acid equivalents.

2.4. Interaction of thiol compounds with products formed by the reaction of caffeic acid with peroxynitrite

The reaction between thiol compounds and putative *o*-quinone oxidation products generated from the reaction of caffeic acid with peroxynitrite was investigated. Caffeic acid (50 μM) and peroxynitrite (400 μM) were reacted for 15 min prior to the addition of glutathione, L-cysteine or L-methionine (final concentrations 25–200 μM). The concentration of products remaining in the sample before and after the addition of thiols was quantified by HPLC. Control samples, without the addition of thiol compounds, were analysed throughout the experiment to ensure stability of the products during the analytical procedure.

2.5. Alkaline oxidation

The UV/visible spectra and HPLC profiles of the products arising from caffeic acid in alkaline solution were investigated. The spectra of caffeic acid (50 μM) were obtained immediately, and after prolonged incubation (1 h) with 50 mM phosphate buffer at pH 7 or pH 12. In order to investigate whether changes in spectrum were reversible, the alkaline caffeic acid solution was adjusted back to pH 7 with an appropriate volume of concentrated HCl.

3. Results and discussion

Spectroscopic studies of the interaction of caffeic acid with peroxynitrite at pH 7 show the decline in the major hydroxycinnamate peaks at 290–310 nm but a resolved peak at 430 nm characteristic of nitrated phenolics as previously shown for ferulic and *p*-coumaric acids [8], was not detected (Fig. 2A). The interaction of caffeic acid (50 μM) with increasing concentrations of peroxynitrite, from equimolar up to 40-fold molar excess (50–2000 μM), was examined and the subsequent products were analysed by HPLC. On reaction with peroxynitrite two new peaks are observed at shorter retention times than the native caffeic acid (Fig. 3A), one absorbing in the far UV region (Product A, R_t 5.3 min) and a later eluting peak with a shoulder at 240 nm, a maximum absorbance at 264 nm, a shoulder at 315 nm and a broad smaller band between 400–500 nm (Product B, R_t 6.3 min). Fig. 4 shows the influence of increasing peroxynitrite concentration on formation of Product B, expressed in terms of caffeic acid equivalents. On increasing the concentration of peroxynitrite, both Product A and B increase in area, as the peak area for caffeic acid concomitantly decreases. At 400 μM concentration of peroxynitrite, the formation of Product B is maximal with a concentration of 12.7 μM caffeic acid equivalents (approximately 25% of the starting concentration of caffeic acid). In addition, caffeic acid is 73% and 92% consumed in the presence of 400 and 800 μM peroxynitrite, respectively, indicating that the reaction is approaching completion with these concentrations of peroxynitrite. Increasing the concentration of peroxynitrite above 800 μM does not increase the formation of Product B, rather the peak area progressively decreases. These results could suggest that secondary reactions, perhaps dimerisation or polymerisation of the primary oxidation product are occurring, although we have no conclusive HPLC evidence of a secondary reaction product.

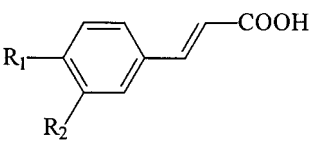

Fig. 1. Structure of hydroxycinnamates: *p*-coumaric acid: $R_1 = OH$, $R_2 = H$; caffeic acid: $R_1 = OH$, $R_2 = OH$; and ferulic acid: $R_1 = OH$, $R_2 = OCH_3$.

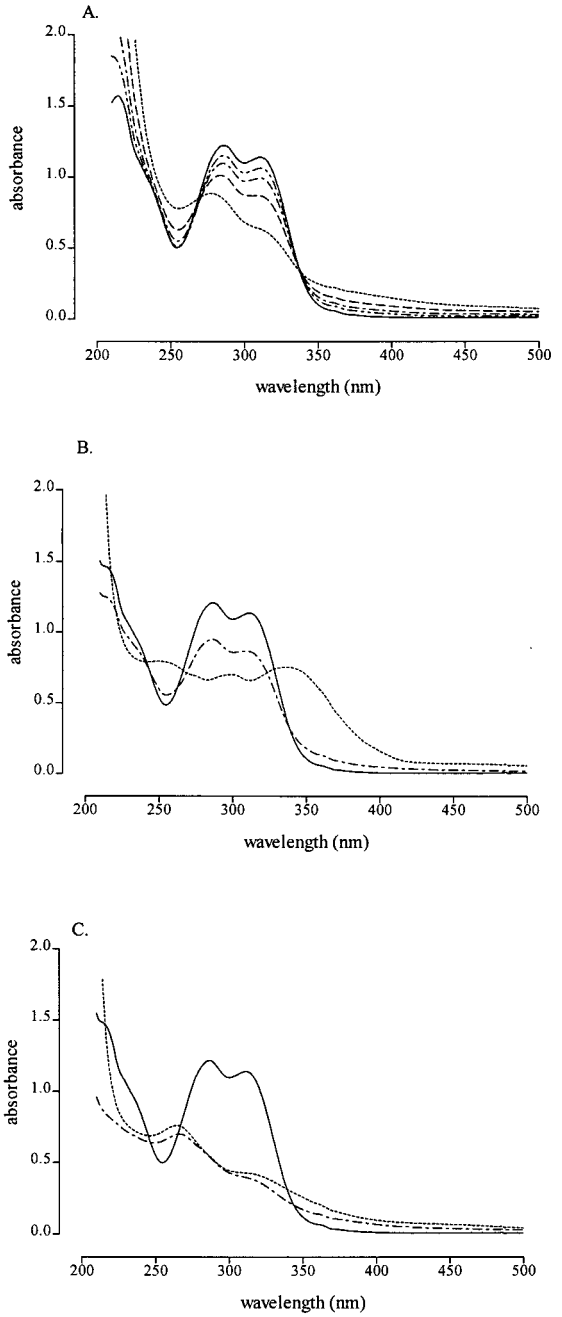

Fig. 2. The UV/visible spectra of caffeic acid (50 μM) in the presence of (A) peroxynitrite (——, 0 μM; – - - –, 50 μM; – - –, 100 μM; – – –, 200 μM; and - - -, 400 μM peroxynitrite) and (B) phosphate buffer, pH 7 (——), pH 12 (- - -) and adjusted from pH 12 to pH 7 (– - –), scanned immediately and (C) after 1 h incubation.

To assess the presence of oxidation products following the reaction of caffeic acid with peroxynitrite, reduced glutathione, L-cysteine and, as a negative control, L-methionine were added to the samples. A concentration-dependent decrease in the peak area of Product B (R_t 6.3 min) is observed,

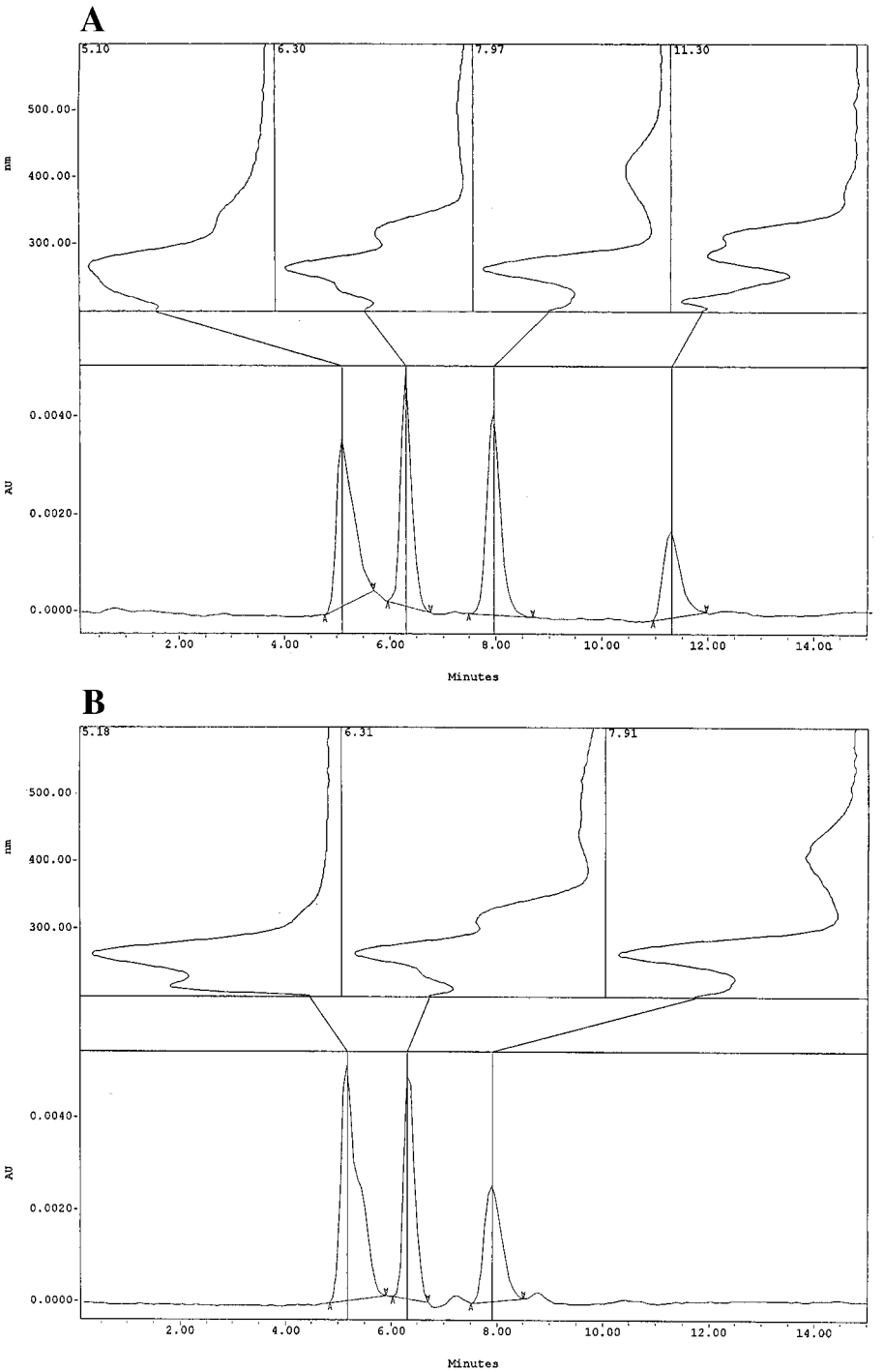

Fig. 3. Chromatogram and photodiode array spectra of Product A (R_t 5.3 min) and Product B (R_t 6.3 min) formed following the reaction of caffeic acid (50 μM) with (A) peroxynitrite (400 μM) and (B) 50 mM phosphate buffer, pH 12. Peaks at R_t 8.0 min and 11.2 min are the internal standard and native caffeic acid, respectively.

as determined by HPLC, after the addition of glutathione and L-cysteine (Fig. 5) indicating that these thiol compounds were reacting with Product B, the tripeptide glutathione being most effective. As predicted, methionine had no effect. The results suggest that the *o*-quinone of caffeic acid is the identity of Product B. Quinones characteristically undergo addition reactions with sulfur nucleophiles, forming thiol conjugates (reviewed in [11]). Previous studies have demonstrated the reaction of thiol compounds with the *o*-quinone compounds formed upon enzymatic oxidation of noradrenaline [12], L-DOPA and dopamine by tyrosinase [13].

In order to substantiate further the oxidation of caffeic acid to the *o*-quinone, experiments were undertaken in which caffeic acid was oxidised in alkaline solutions, a process independent of nitrating species, enzymes and radical intermediates. Altering the pH of caffeic acid solutions results in ionisation of hydroxyl groups with subsequent electron rearrangement to form the corresponding *o*-quinone. Fig. 2B il-

lustrates the spectrum of caffeic acid in phosphate buffer, pH 12. Alkalinisation produces spectral changes in caffeic acid characterised by a decreased absorbance at 290 and 310 nm and increased absorbance, as shown by shoulder formation and a resolved peak, at 250 nm and 345 nm, respectively (Fig. 2B). The broad absorbance band in the visible region (350–400 nm, Fig. 2B) is associated with the yellow chromophore observed following the alkalinisation of caffeic acid. Immediate reversal of the pH of the alkaline solutions back to pH 7 results in decolorisation of the sample and a spectral profile identical to that of caffeic acid, albeit at a decreased intensity. The pH-dependent changes in the spectrum of caffeic acid on exposing to pH 12 and reversing to pH 7 indicate that the product profile is dependent on the duration of alkalinisation. On prolonged exposure to alkaline pH there is a loss of absorbance at 345 nm and increased absorbances at 265 nm and in the visible region (350–450 nm). When the pH of the alkaline sample is returned to 7, the spectral profile remains unchanged suggesting complete, irreversible oxidation of caffeic acid (Fig. 2C).

The spectral profile obtained by prolonged incubation of caffeic acid in alkaline solution is qualitatively similar to that reported by Hapiot et al. [14] following the irradiation of caffeic acid. The observed tailing broad absorption between 350–450 nm can be attributed to polymer formation, which is the basis of the browning reactions in foods and wines. A mechanism concerning a comproportionation reaction between *o*-quinone and the caffeic acid has been proposed to give the semiquinone, which might then dimerise. However, the semiquinone does not readily disproportionate to *o*-quinone in aqueous solution at alkaline pH. The oxidation of caffeic acid by peroxynitrite at neutral pH might involve formation of the *o*-quinone by disproportionation of the *o*-semiquinone [14]. These observations are consistent with those obtained following the exposure of the caffeic acid ester (chlorogenic acid) to polyphenol oxidase which catalyses the oxidation of catechols to corresponding *o*-quinones. Further reaction leads to the formation of brown polymerised pigments [15].

The spectral profile of caffeic acid during the prolonged alkalinisation reaction (1 h) is similar to that observed with high concentrations of peroxynitrite, as seen in Fig. 2A and C. HPLC analysis of the alkalinised caffeic acid sample revealed an identical profile of oxidation products to those obtained

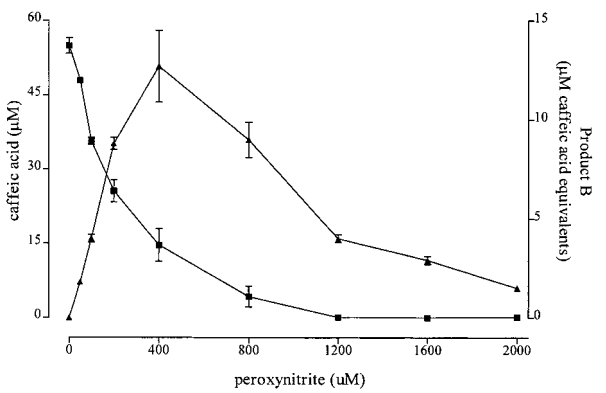

Fig. 4. The loss of substrate and formation of product following the reaction of caffeic acid (50 μM) with peroxynitrite. Caffeic acid (– ■ –) and Product B (– ▲ –) were quantified by HPLC. The values represent mean ± S.D. of $n = 3$ experiments.

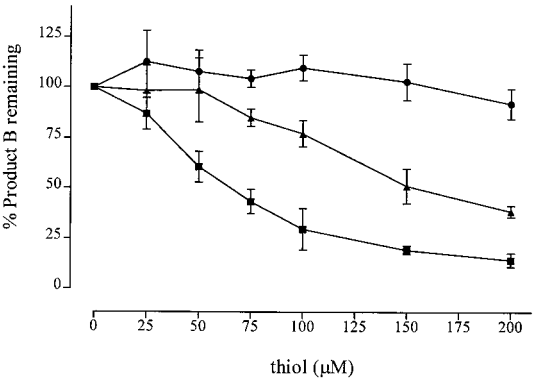

Fig. 5. The effect of glutathione (– ■ –), L-cysteine (– ▲ –) and L-methionine (– ● –) on the amount of Product B formed by the reaction of caffeic acid (50 μM) with peroxynitrite (400 μM) as quantified by HPLC. The values are expressed as % of Product B in the absence of thiol compound. The values represent mean ± S.D. of $n = 3$ experiments.

with peroxynitrite treatment (Fig. 3A and B). In addition the product eluting at 6.3 min reacted with thiol compounds, consistent with its identity as the *o*-quinone.

The identity of Product A following the reaction of caffeic acid with peroxynitrite is unknown (Fig. 3A and B). HPLC analysis revealed that a degradation product of peroxynitrite itself elutes at a comparable retention time to that of Product A. However, this accounts for only a small fraction of the total peak area of Product A obtained following the reaction of caffeic acid with peroxynitrite. Furthermore, this unidentified peak eluting at approximately R_t 5.3 min is also present in alkaline samples containing caffeic acid, indicating that the product is not a nitrated derivative of caffeic acid. The presence and magnitude of Product A are not affected by the presence of thiol compounds, suggesting that it is not an electrophilic quinone. One possibility is that Product A represents polymerised oxidation products following condensation of *o*-quinone [14]. It is also likely that this peak is a sum of at least two co-eluting products since peak resolution and diode array spectra were variable between samples. For these reasons quantification and characterisation of Product A were not performed.

The reaction of monohydroxy substituted hydroxycinnamates, *p*-coumaric and ferulic acid with peroxynitrite was investigated. Ferulic acid yielded two main products following its reaction with peroxynitrite, which have been putatively characterised as a nitrated ferulic compound (R_t 10.5 min) and a possible dimer of ferulic acid (R_t 23.3 min) based on their retention times and characteristic absorption spectra. The peak with R_t 10.5 min displayed a maximum absorbance at 430 nm which is characteristic of a nitrated phenolic species. The peak at R_t 23.3 min has a longer retention time than monomeric ferulic acid with an almost identical photodiode array spectrum, indicative of a ferulic acid dimer. A nitrated compound has also been identified following the reaction of *p*-coumaric acid with peroxynitrite [8]. All the products derived from peroxynitrite-mediated reactions with monohydroxy-substituted hydroxycinnamates were unchanged after the addition of glutathione, consistent with previously published reports indicating the formation of nitrated products [8].

Oxidation of caffeic acid, catechin and other structurally-related catechols to *o*-quinones by enzymatic oxidation, pulse

radiolysis and radical-initiated reactions have been previously described by several authors [14,16–18]. For example, Hapiot et al. [14] have applied electrochemical methods and pulse radiolysis in aqueous and organic solvents to study hydroxycinnamate oxidation. Dimerisation of the monohydroxycinnamates, ferulic acid, and *p*-coumaric acids is shown to occur through radical-radical coupling mechanisms following hydrogen donation, whereas the oxidation of caffeic acid leads to the formation of the corresponding *o*-quinone through a mechanism attributed to disproportionation of the initial semiquinone radical.

The identification of a novel product which reacts with thiol compounds provides experimental evidence of peroxynitrite-mediated oxidation of caffeic acid resulting in *o*-quinone formation. This might be a direct product or arise via a mechanism of semiquinone formation. These findings have implications for the interaction of peroxynitrite with pathologically and physiologically relevant catechol-structures, leading potentially to quinone formation and subsequent polymeric compounds. The findings reported here are consistent with recently published studies on the interaction of caffeic acid with nitrogen dioxide radical generated by pulse radiolysis, demonstrating formation of the corresponding phenoxyl radical, not nitration of the phenolic ring [19].

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