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ABTS radical-driven oxidation of polyphenols: Isolation and structural elucidation of covalent adducts

A.M. Osman *, K.K.Y. Wong, A. Fernyhough

Scion, Private Bag 3020, Rotorua, New Zealand

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Abstract

The formation of covalent adducts obtained from the reaction of the polyphenols, *trans*-3,3′,4′,5,7-pentahydroxyflavan (catechin) and 1,3,5-trihydroxybenzene (phloroglucinol), with ABTS radicals is reported. Two adducts derived from (+)-catechin and three adducts from phloroglucinol were isolated and identified using reversed-phase high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). The molecular masses of the (+)-catechin-derived adducts (I_c and II_c) were found to be 802 and 559 Da, respectively, whereas the masses of phloroglucinol-derived adducts (I_p, II_p, and III_p) were 638, 395, and 381 Da, respectively. The initially formed adducts (I_c, I_p) were unstable and degraded to secondary adducts (II_c, II_p, and III_p) releasing part of the ABTS molecule. The structures of these adducts were elucidated by interpreting the results of MS/MS analysis of prominent ions generated by both positive and negative ion ESI-MS. The adducts were found to scavenge ABTS radicals, an observation that could explain the complex kinetic behaviour manifested by the reactions of ABTS radicals with polyphenols. A mechanism, which accounts for both the formation of the adducts and the degradation products of ABTS radicals, is proposed.

Keywords: ABTS radicals; Polyphenols; Covalent adducts; Degradation products; Antioxidant; Kinetic behaviour

One of the most commonly used organic radicals for the evaluation of antioxidant efficiency of pure compounds and complex mixtures is the radical cation derived from 2,2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) [1–3]. These radical cations could be generated by enzymatic, chemical, and electrochemical means [1–6]. Polyphenols have recently attracted considerable interest because of the beneficial health effects as antioxidants and their abundance in some fresh fruits, vegetable extracts, and beverages [7]. The extent and the rapidity with which polyphenols quench the ABTS radical cation chromophores are the criteria used to assess their relative antioxidant capacity as compared with a standard antioxidant, Trolox [8]. In these $ABTS^{\cdot+}$-based methods it is assumed that the antioxidants simply reduce the radicals back to the parent substrate, ABTS. However, several studies have shown that the kinetics of the reaction between the ABTS radical cations and polyphenols are rather complex and the lack of a relationship between the rate law and stoichiometric factors has been reported [9,10]. In a recent communication, we have reported that, in the presence of the polyphenols, (+)-catechin, (−)-epicatechin, and phloroglucinol, ABTS radicals undergo degradation reactions [11]. The degradation products were purified and identified as 3-ethyl-6-sulfonate benzothiazolinone imine and the corresponding sulfoxide, 3-ethyl-6-sulfonate benzothiazolone [11].

The purpose of this study was to investigate the nature of the ABTS radical-mediated polyphenol oxidation products and to gain an insight into the mechanism of formation of the ABTS-derived degradation products. Catechin, which is the simplest model of condensed tannins [12], and phloroglucinol, a constituent of the catechin structure, were examined in this work (Fig. 1). The results show that ABTS radical-driven oxidation products of these

* Corresponding author. Fax: +6473435507.
E-mail address: Ahmed.Osman@Scionresearch.com (A.M. Osman).

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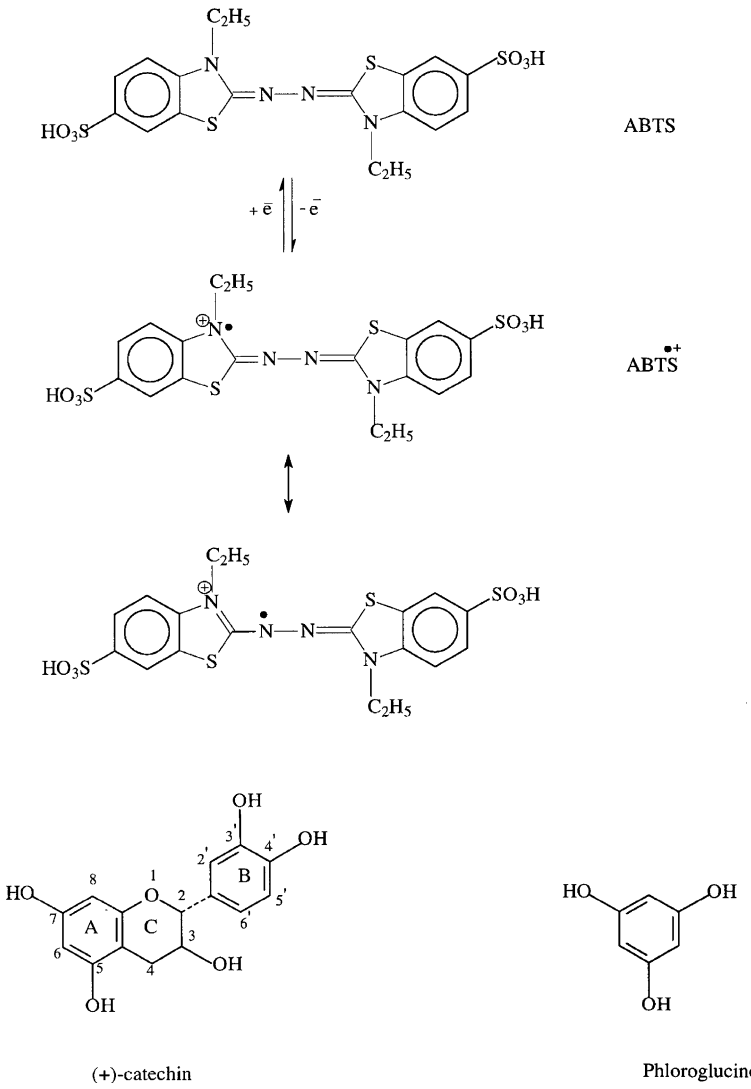

Fig. 1. Structures of ABTS, ABTS radical cation and the polyphenols examined in this work.

polyphenols form unstable primary covalent adducts with the ABTS radicals, which in turn are further converted to secondary adducts, releasing part of the ABTS molecule.

Materials and methods

Chemicals. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt [ABTS $(NH_4)_2$], (+)-catechin, and potassium peroxodisulfate were obtained from Sigma. Phloroglucinol was purchased from Ajax chemicals LTD (Sydney, Australia). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany) and MilliQ nanopure water was used for the preparation of all aqueous solutions.

Generation of ABTS radical cations ($ABTS^{\bullet+}$). ABTS radical cations were chemically generated by incubating ABTS with potassium peroxodisulfate (PDS) as described by Henriquez et al. [13]. The radical cations were usually prepared by incubating 2 mL of 10 mM solution of PDS with 2 mL of 20 mM ABTS (both dissolved in 100 mM acetate buffer, pH 5) at room temperature for 16 h. The ABTS radical cation concentration was determined at 734 nm ($\varepsilon_{734\,nm} = 0.015\ \mu M^{-1}\,cm^{-1}$) [13,14], or at 420 nm ($\varepsilon_{420\,nm} = 36{,}000\ M^{-1}\,cm^{-1}$) [15]. Values for the ABTS radical cation concentrations determined at the two wavelengths were comparable.

Sample preparations for HPLC analyses. For the analytical HPLC measurements, 400 μL of the radical cation solutions (121.8 μM) obtained as described above was incubated either with 40 μL of catechin (1 mM) or 20 μL of phloroglucinol (1 mM) for ca. 2 h. Then, 20–100 μL of the incubation mixture was loaded onto a C_{18} analytical column.

Isolation of the polyphenol-derived adducts. For the isolation of catechin-derived adduct II_c, 2.5 mL ABTS (20 mM) dissolved in 100 mM acetate buffer, pH 5, was incubated with 1.5 mL of 10 mM PDS (dissolved in the same acetate buffer) at room temperature for 16 h. The radical concentration was estimated to be 2.52 mM. A volume of 900 μL of catechin solution (10 mM) was added to 3.75 mL of the radical solution and incubated at room temperature for 2 h. The final concentration of catechin was 1.94 mM and the concentration of the radical cation was estimated at ca. 2.03 mM.

For the preparation of phloroglucinol-derived adducts, radical solution prepared in the same way as described above was used. The concentration of this radical solution was 3 mM. To this was added 500 μL of phloroglucinol solution (dissolved in buffer). The final concentration of phloroglucinol was 21.8 mM and that of the radical cation was ca. 2.64 mM. Incubation was performed at room temperature for 2 h. A volume of 200–400 μL of these solutions was loaded onto a preparative C_{18} column. To isolate the catechin-derived adduct I_c, the analytical column was used since this product co-eluted with the ABTS substrate under the preparative experimental conditions.

Analytical high-performance liquid chromatography. Separations were achieved using the analytical reversed-phase column Econosphere C_{18}

(150 × 4.6 mm, 5 μm). A volume of 20–100 μL of the reaction mixture was loaded onto this column. Eluent A was 5% acetic acid in water and eluent B acetonitrile. The following gradient was applied with a flow rate of 0.8 mL/min: 100% A for 3 min, then 3% of B in A (3–4 min), followed by isocratic 3% B in A (4–15 min), then a linear gradient 3–40% B in A (15–25 min), isocratic 40% B in A (25–30 min), 40–60% B in A linear (30–33 min), isocratic 60% B in A (33–42 min), 60–0% B in A (42–43 min), and 100% A (43–50 min).

Preparative HPLC. Econosphere C_{18} (250 × 10 mm, 10 μm) was used. Solvent A was 5% acetic acid in water and solvent B acetonitrile. The following conditions were used: isocratic 3% B was applied for 15 min, then a linear gradient 3–10% B (15–25 min), isocratic 10% B (25–60 min), 10–45% B linear (60–62), isocratic 45% B (62–75 min), 45–0% B (75–77 min), 100% A (77–096 min), 0–60% B linear (96–100 min), isocratic 60% B (100–120 min), and 60–0% B (120–124 min), isocratic 100% A (124–135 min). This latter increase of solvent B to 60% was performed to ascertain that all products were eluted from the column. The flow rate was 0.6 mL/min. UV detection and UV–visible absorption spectra of the eluted compounds were performed with a Hewlett–Packard HP 1050 multiple wavelength detector.

ESI-MS analysis. A Finnigan LCQ DECA XP mass spectrometer (CA, USA) was used. The isolated adducts were directly injected into the mass spectrometer operating in both positive and negative modes at a flow rate of 10 μL/min. When the positive ion mode was applied, the following ESI source parameters were used: source voltage +4.8 kV, capillary voltage −30 V, capillary temperature 225 °C, and sheath gas (N_2) flow 30 (arbitrary units). When the negative ion mode was used, the samples were dissolved in ammonium bicarbonate (0.1 M). The conditions were: source voltage −4.8 kV, capillary voltage +35 V, capillary temperature 230 °C, and sheath gas (N_2) 30 (arbitrary units). For full scan MS analysis the spectra were acquired in the range of m/z 50–2000. The prominent ions were selected for CID (collision-induced dissociations) fragmentations and the abundant ions were further subjected to MS^n analyses. The collision energy ranged from 0 to 50%.

Determination of the stoichiometries (n) of the polyphenols. A typical incubation mixture was performed as follows: to a volume of 550 μL of 100 mM acetate buffer (pH 5) was added 400 μL of radical solution (121 μM) and, after allowing 4 min for stabilization, the reaction was started by adding 50 μL of 0.1 mM polyphenol. The concentration of the radicals was thus 48 μM and the initial concentration of the polyphenol was 5 μM. Different ratios of the radicals and the polyphenol, ranging from 9:1 to 2:1, were used. Kinetic runs of 30–40 min at room temperature were performed. The decay reaction of the radicals was monitored at 734 nm [13,14] using a Cary diode array spectrophotometer (Hewlett–Packard). To investigate whether or not polyphenol-derived adducts consume ABTS radicals. appropriate volumes of phloroglucinol-derived adducts I ($A_{350\,nm} = 0.5$ U) and II ($A_{460\,nm} = 0.495$ U) were also incubated with the 48 μM ABTS radicals.

Results

Formation of covalent adducts from the reaction of phloroglucinol with ABTS radical cations

The incubation of phloroglucinol ($M_W = 126$) with ABTS radical cations ($M_W = 514$ Da) yielded several products, as shown by analytical HPLC (Fig. 2A). The peak with an elution time of ca. 26 min in a typical chromatogram was identified as ABTS, by comparing its retention time and UV–visible absorption spectrum with an authentic standard. Peaks I and II, at 4.8 and 8.7 min, respectively, were previously identified as degradation products from the ABTS radical cation, namely 3-ethyl-6-sulfonate benzothiazolinone imine and 3-ethyl-6-sulfonate benzothiazolone, respectively [11]. For ESI-MS analysis of the peaks at ca. 13 and 24 min, preparative HPLC was carried out (Fig. 2B). These two peaks separated also by preparative HPLC were labelled as adduct I_p and II_p after considering the following results from mass spectrometry.

The base peak detected in adduct I_p had m/z value of 639.6 (+ve ion) (Fig. 3A), which appeared to be an adduct of the oxidation product of phloroglucinol and ABTS radical cation. There were also two minor ions with m/z value of 259 and 381.3. If the analysis was not rapidly performed, an m/z value of 638.9 was obtained for adduct I_p (data not shown), suggesting a deprotonation reaction. Moreover, a decrease of its intensity accompanied by parallel increase of the minor ions was observed. The base peak of adduct II_p had m/z value of 396.1 (Fig. 3B), which was smaller than that of adduct I_p by 243.5. A minor ion of 259.1 was again observed.

Formation of covalent adducts from the reaction of catechin with ABTS radical cations

Similar to the product profile from the reaction with phloroglucinol, analytical HPLC showed that reaction of ABTS radical cations with catechin ($M_W = 290$ Da) yielded two unknown products (with retention times of ca. 19 and 24.5 min, respectively, Fig. 4A), hereafter labelled as adducts I_c and II_c. As preparative HPLC did not resolve adduct II_c from ABTS (peak retention time 62 min, Fig. 4B), multiple analytical HPLC runs were used to isolate sufficient amounts of adduct I_c. The major ion of adduct I_c had m/z value of 803 (Fig. 5A), which appeared to be an adduct of the oxidation product of catechin and the radical cation ($M_W = 802$ Da). The peak labelled as adduct II_c showed a major ion with m/z of 560 (Fig. 5B), which was smaller than that of adduct I_c by 242.9 and thus showed the same mass difference as that found between adducts I_p and II_p.

Fragmentation analysis of the polyphenol-derived adducts

Of the three ions detected in adduct I_p from the reaction of phloroglucinol and ABTS radical cation, MS/MS fragmentation analysis of the major ion (m/z 639.6) yielded the other two ions (Fig. 6). This confirmed that the two smaller ions in adduct I_p were degradation products of the base ion. The m/z value of one of the ions is 259, which is the same as that of a minor ion in adduct II_p and that of product I (previously identified as degradation product from the ABTS radical [11]). Product I therefore appeared to be a degradation product derived from both adducts I_p and II_p.

Fragmentation of adduct II_p ($m/z = 396$) yielded ions with m/z of 244 and 216 (data not shown), corresponding to successive loss of ms fragments 152 and 28, respectively. These fragments are consistent with the proposal that adduct II_p lost a modified phloroglucinol ring (fragment 152) to produce a protonated benzothiazolium ion (m/z 244), which in turn loses an ethylene group (C_2H_4) to give an ion with m/z of 216.

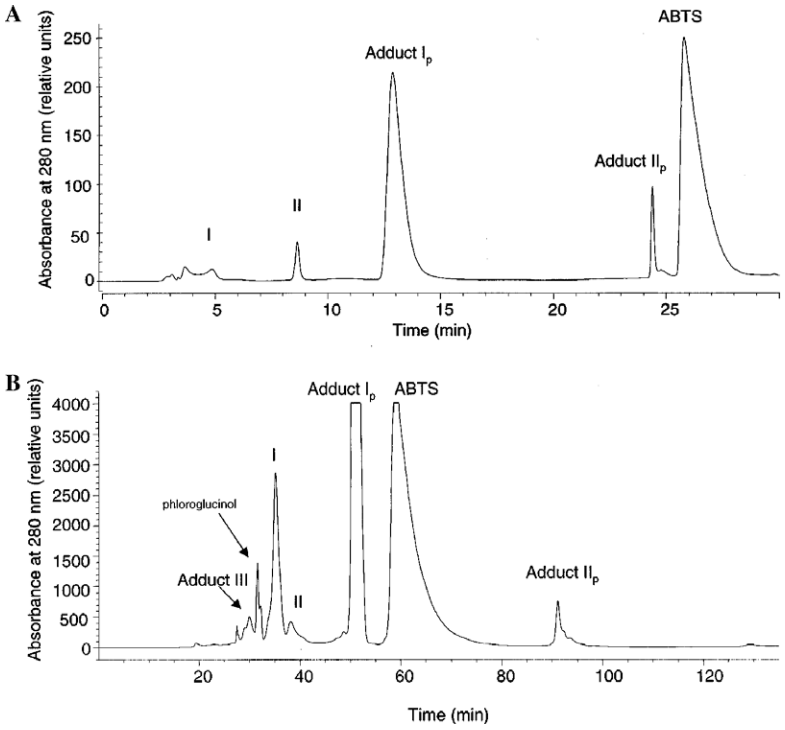

Fig. 2. A typical HPLC chromatogram of the reaction products of phloroglucinol with ABTS radical cations (A) analytical and (B) preparative. The products I and II are ABTS degradation products [11].

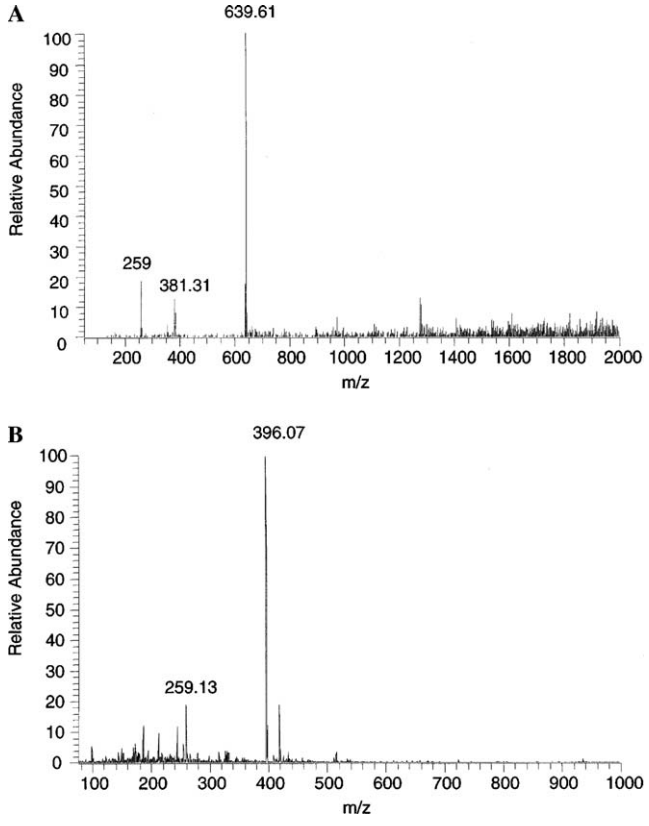

Fig. 3. Full-scan ESI-MS spectrum of (A) adduct I_p and (B) adduct II_p.

The fragmentation of adduct II_c ($m/z = 560$), obtained from the reaction of catechin with ABTS radical cation, produced a prominent ion with m/z of 408,which in turn gave an ion with m/z of value 244 and the latter an ion with m/z 216, corresponding to successive loss of fragments 152, 164, and 28, respectively. Analogous to adduct II_p, the pattern suggested the involvement of a modified phloroglucinol ring (fragment 152), and a protonated benzothiazolium ion ($m/z = 244$) that loses an ethylene group. Since the ion with m/z value of 165 is considered to be diagnostic of the B ring of catechin [16], the fragment 164 originates from the B ring of catechin. The ion with m/z of 408 resulted from the collapse of fragment 164 with a protonated benzothiazolium ion, after elimination of the modified phloroglucinol.

Interestingly, the UV–visible spectra of the I_c and I_p were comparable and had maxima absorbance at 240, 300, and 350 nm (Fig. 7). Likewise, the UV–visible spectra of the second adducts II_c and II_p were also similar and had maxima absorbance at 240 and 455 nm (Fig. 7C and D). This suggests structural similarity of the adducts' chromophores.

Stability of the polyphenol-derived adducts

To assess the stability of the isolated adducts, freeze-dried samples were dissolved in 100 mM acetate buffer, pH 5, and rechromatographed on the analytical column (Fig. 8). These chromatograms indicated that the analytical peaks with retention times of ca. 13, 20.8, 24 and 25 were adducts I_p, I_c, II_p, and II_c, respectively. All four adducts degraded to generate product I, while there was evidence of product II being generated from adducts I_p and II_p (Fig. 8). adducts I_p and I_c appeared to generate small

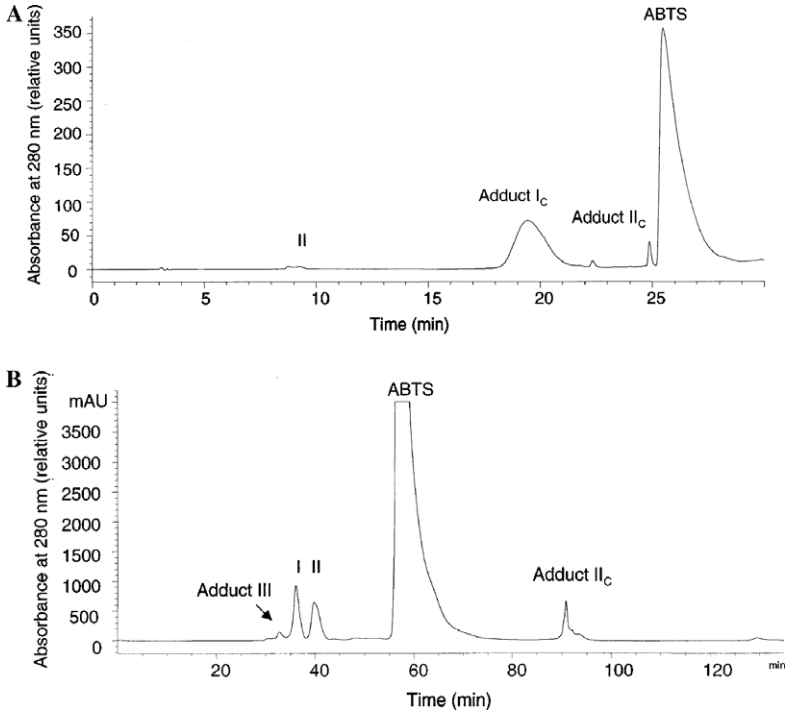

Fig. 4. HPLC chromatogram of the reaction of catechin with ABTS radical cations (A) analytical and (B) preparative.

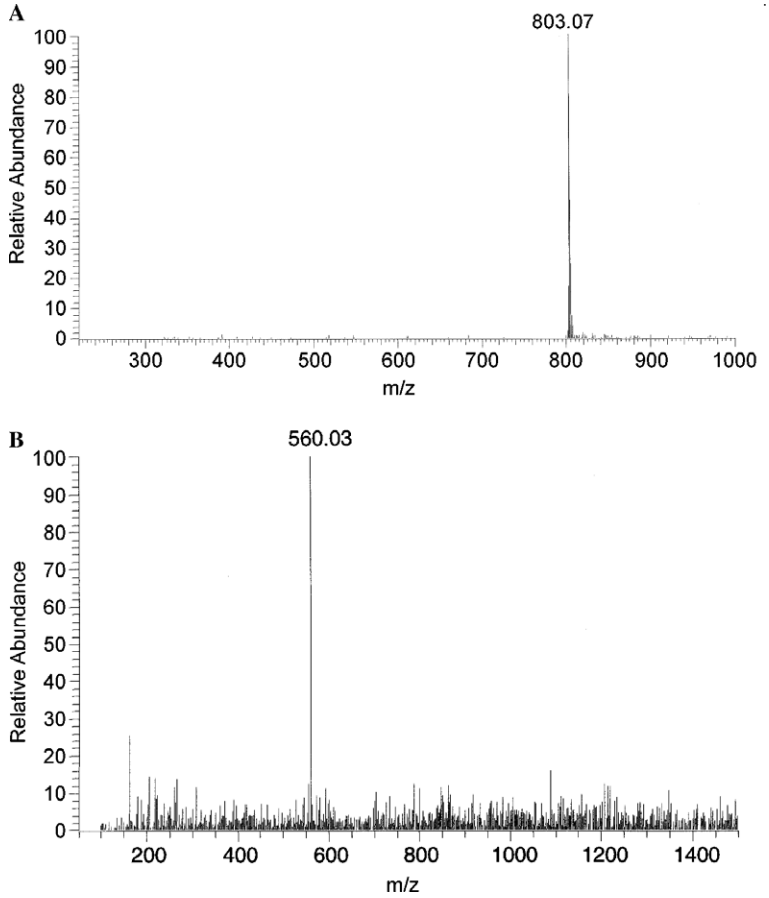

Fig. 5. Full-scan ESI-MS spectrum of (A) adduct I_c and (B) adduct II_c.

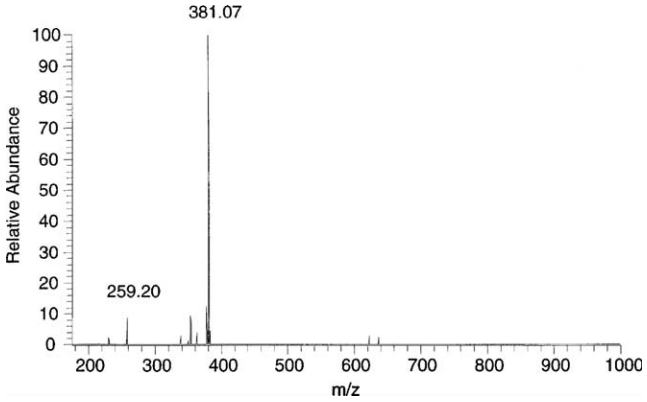

Fig. 6. MS/MS fragmentation of the base peak of adduct I_p.

amounts of adducts II_p and II_c, respectively. In contrast, adducts II_p and II_c generated minor peaks with similar retention times near 25 min on the analytical column. As these minor peaks had similar UV–visible absorption spectra as the adducts, they could be different ionisation states of the same adducts.

In comparison with the analytical HPLC profiles (Figs. 2A and 4A), the preparative HPLC profiles (Figs. 2B and 4B) had additional minor peaks, in addition to the expected peaks of ABTS radical degradation products I and II. One of these minor peaks was identified as phloroglucinol (residual) and the other appeared to be a third adduct in the case of phloroglucinol reaction. This latter product could be ascribed to the base ion with m/z value 381, which represented a loss of 258.6 from adduct I_p (Fig. 3A). The HPLC analysis of the isolated adduct I_p generated a product that eluted before product I (Fig. 8A), that, most likely, is adduct III_p. A product with similar retention time (Fig. 4B) and UV–visible absorption spectrum to adduct III_p was detected on the preparative HPLC analysis of the catechin reaction mixture. However, due to the small

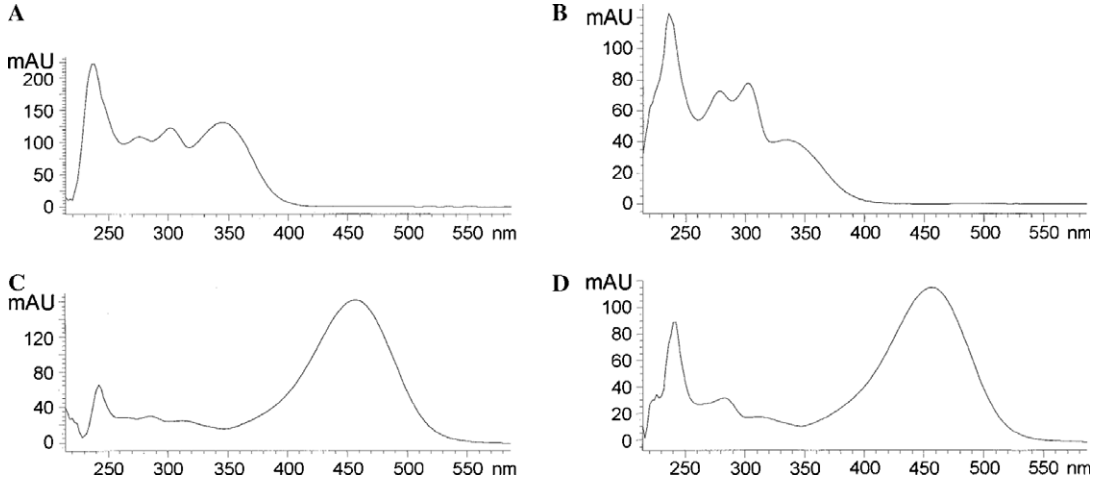

Fig. 7. UV–visible absorption spectra of (A) adduct I_p, (B) adduct I_c, (C) adduct II_p, and (D) adduct II_c.

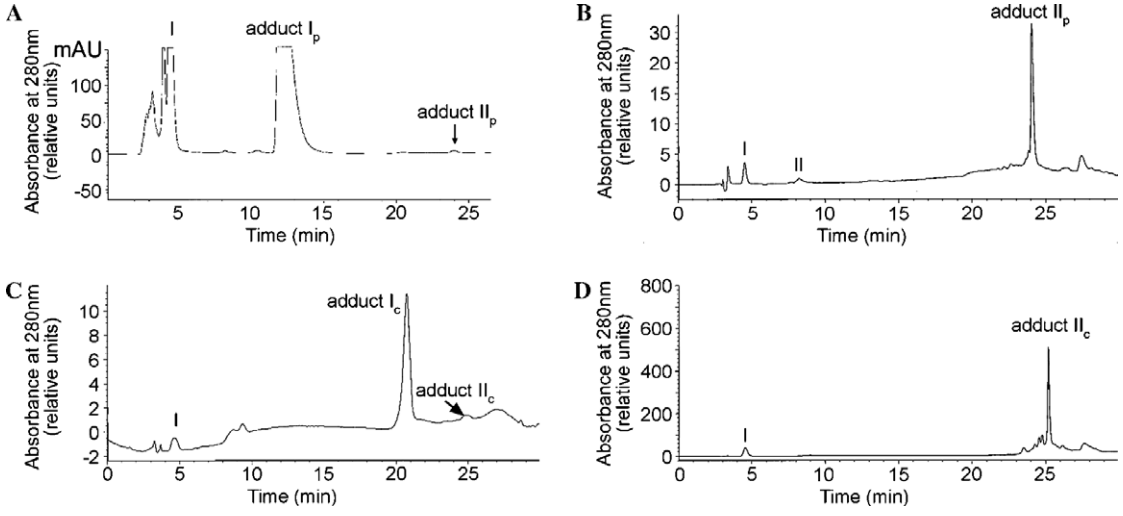

Fig. 8. HPLC profile of isolated adducts rechromatographed to assess stability: (A) adduct I_p, (B) adduct II_p, (C) adduct I_c, and (D) adduct II_c. The products I and II were identified as ABTS degradation products [11].

amount formed, this product was not further characterised, but may be inferred to be adduct III_c.

Reaction kinetics and stoichiometry

Finally, the kinetic profile of the reaction between ABTS radical cations and polyphenols is reported in Fig. 9. As has been previously reported [9,17], the kinetic profile of this reaction displayed two distinct phases (Fig. 9), a fast and slow step. After 4 min pre-incubation and upon addition of the polyphenol, in the initial rapid step ABTS radicals decayed over the first 2 min. The stoichiometry (n) of the reaction could be determined according to the equation: $n = (A_0 - A_F)/\varepsilon C$ [17], where C is the initial concentration of the polyphenol, ε is the molar extinction of ABTS radical cations at 734 nm, and A_0 and A_F are the initial and final (end of fast step) absorbance of the reaction mixture. Using various ratios of radicals to polyphenols, ranging from 2:1 to 9:1, the stoichiometric factor was estimated to be close to 2 (n varied from 1.7 to 2.3) for both catechin and phloroglucinol. The estimated n might not be the true stoichiometric factor because ABTS radicals consumed during the subsequent slow step were not taken into consideration. Because the slow step was found to be more marked in the phloroglucinol reaction (Fig. 9B), the rate of ABTS radical decay was also monitored in the presence of its adducts. The adducts also showed the two-step decay (Fig. 9C and D) observed in the parent phenol.

Discussion

To our knowledge, this is the first report on the formation of covalent adducts between ABTS radical cations and polyphenols. A mechanism is proposed to account for the formation of these adducts (Fig. 10). In this mechanism one molecule of ABTS radical cation abstracts an electron (or hydrogen atom) from the polyphenol and forms a semiquinone radical, regenerating the parent substrate, ABTS. Subsequently, the semiquinone radical reacts with another molecule of ABTS radical cation resulting in the formation of the first polyphenol-derived adducts (I_p and I_c) (Fig. 10). The absence of polymerisation products that might be formed from the coupling of the phenoxyl radicals supports the proposal that the phenoxyl radicals react with the ABTS radical cation.

The initially formed adducts were found to be unstable and degraded to other products (Fig. 10A and B). The results are consistent with further transformation of the initially formed adducts (I_p and I_c) in two different pathways (Fig. 10, pathways 1 and 2). In pathway 1, the polyphenol-derived adducts I_p and I_c undergo a degradation reaction and form adducts II_p and II_c, respectively, releasing a benzothiazolium ion (X). The benzothiazolium ion (X) is known to be unstable, and easily undergoes hydrolysis [18,19] and oxidation to form compound (Y), which was shown to exist predominantly in the keto form [20]. This latter product was identified as 3-ethyl-6-sulfonate-benzothiazolone, one of the previously reported degradation products of ABTS radical cation [11]. The difference

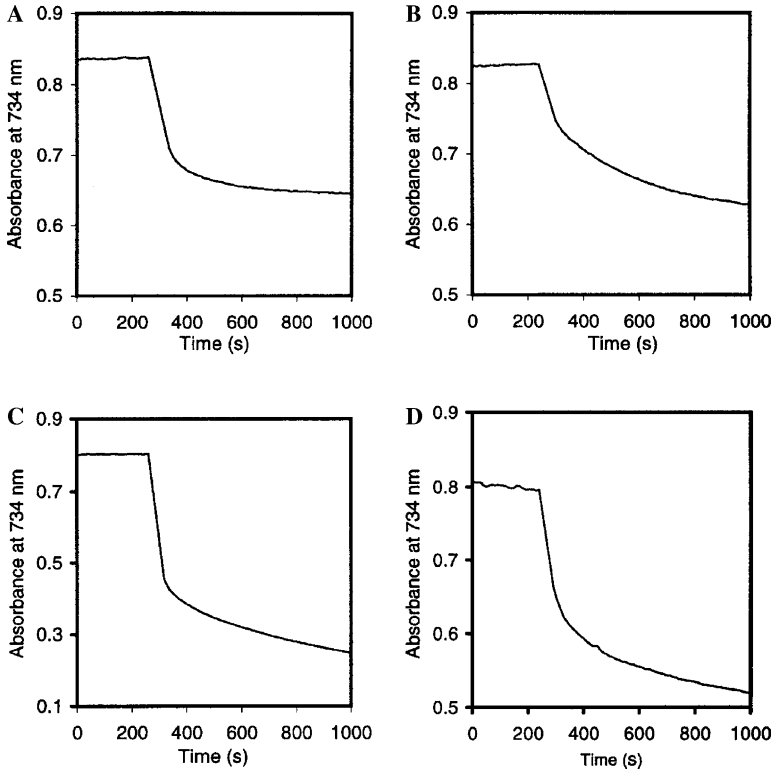

Fig. 9. ABTS radical decay following addition with (A) catechin, (B) phloroglucinol, (C) adduct I_p, and (D) adduct II_p.

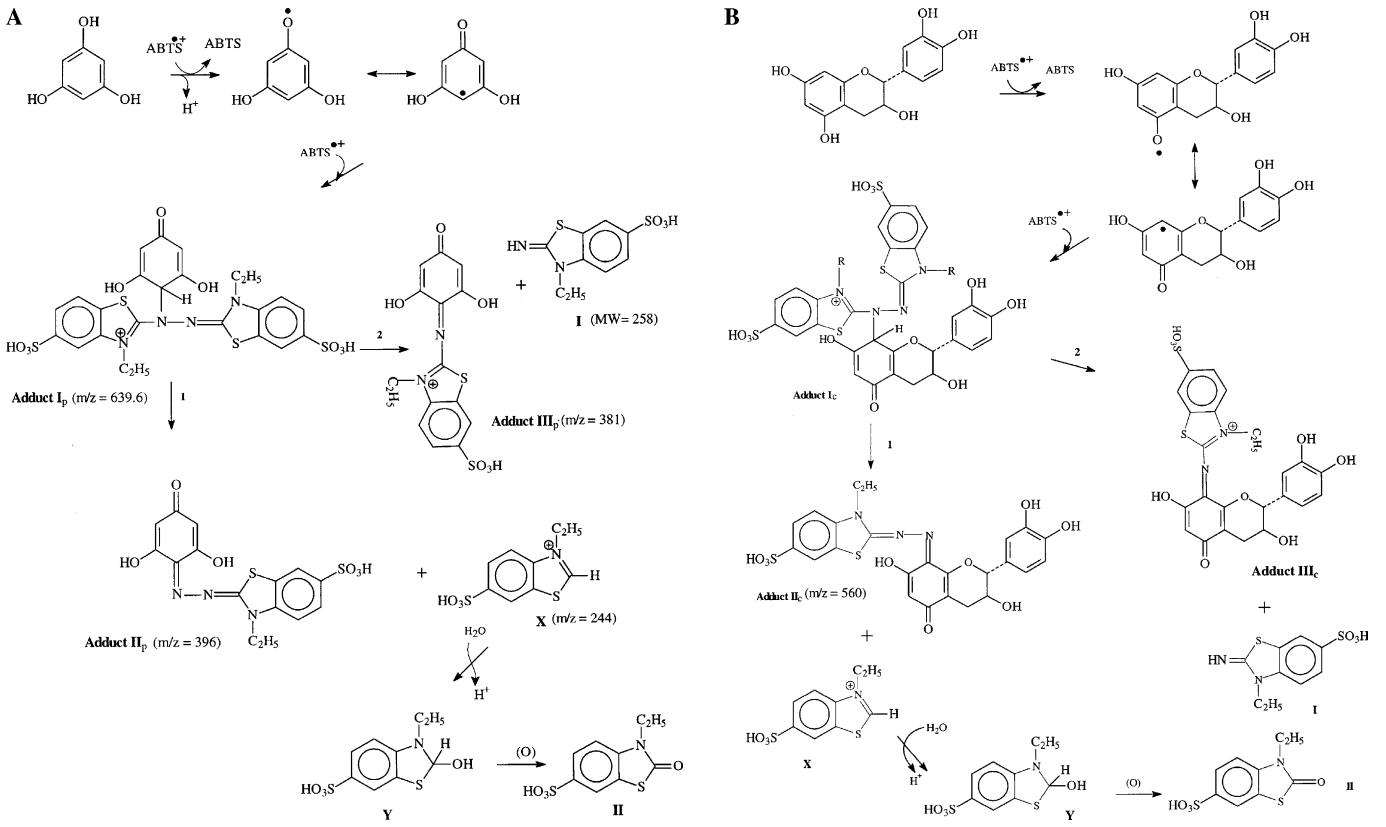

Fig. 10. Proposed mechanism for the formation of the polyphenol-derived adducts with ABTS radical cations and their subsequent degradation pathways. (A) Phloroglucinol-derived adducts and (B) catechin-derived adducts. In pathway 1, adduct I is transformed to adduct II and the benzothiazolium ion (X), and in pathway 2 adduct I is converted to adduct III and the imine (I).

in mass between the polyphenol-derived adduct I and that of adduct II corresponds to a loss of a fragment of 243 Da, which is identical to the mass of the benzothiazolium ion (see the protonated form (X), Fig. 10). Alternatively, adducts I_p and I_c could degrade by a second pathway (Fig. 10, pathway 2) and form third adducts III_p and III_c, releasing 3-ethyl-6-benzothiazolinone imine, the other degradation product of ABTS radical cation [11]. Again, the difference in mass between adducts I and III is consistent with the loss of a fragment of 258 Da, which is identical to the mass of 3-ethyl-6-benzothiazolinone imine (Fig. 10) [11]. We cannot exclude the degradation of adducts II_p and II_c to 3-ethyl-6-sulfo-benzothiazolone hydrazone, which is known to be unstable and to decompose to the imine [19].

The proposed mechanism of ABTS radical-driven oxidation of polyphenols is consistent with the data reported in the literature. It is known that phenols can be oxidized to phenoxyl radicals by radicals less reactive than OH-radicals such as azide and $Br^{\bullet-}$ radicals [21]. In particular, the formation of phloroglucinol-derived radicals was demonstrated [21]. Furthermore, the evidence that adducts are formed between ABTS radical cations and the phenoxyl radicals is in accord with a previous study in which the formation of adducts between ABTS radical cations with alkyl radicals was reported [22]. This mechanism is also in agreement with an earlier suggestion that DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals oxidize butylated hydroxyanisole via its phenoxyl radical and that the latter forms a stable intermediate with DPPH [23].

In addition, the results presented here suggested a plausible explanation for the complex kinetic behaviour observed for the reactions of ABTS radicals with monophenols and polyphenols [9]. The biphasic kinetics, the fast and slow steps, manifested by these reactions could be accounted for by the scavenging potential of the parent phenols and the adducts formed, respectively. The observed lower rate in the second phase could be attributed to the lower steady-state concentrations of the adducts formed before the depletion of ABTS radicals. Moreover, this additional consumption of ABTS radicals by the adducts combined with the ABTS degradation products could account for the lack of relationship between the rate law and stoichiometric factors previously reported for ABTS radical-mediated reactions of different substrates [9,10].

Finally, in contrast to reports that the oxidation of catechin ring by 1,1-diphenyl-2-picrylhydrazyl (DPPH) -derived radicals may occur at the B-ring, forming the corresponding *O*-quinones, or even at the C-2 position of the C-ring [24], our results showed that the oxidation of catechin by ABTS radical cations occurs at the A-ring, which is also the modified part of the molecule (Fig. 10). This is probably due to the fact that the phloroglucinol moiety of the catechin ring might easily form hydrogen bonds with

the radicals. In fact, a previous study showed that flavylium ion interacted non-covalently with the phloroglucinol moiety of the catechin ring prior to the formation of a covalent bond [12]. This would also be in agreement with the recent findings that phloroglucinol forms complexes with diverse reagents including 1,3,5-benzene tricarboxylic acid [25], and substituted pyridines [26]. A possible explanation for the difference in the site of oxidation is probably due to the inaccessibility of the antioxidant to the radical centre, especially when both the antioxidant and the radical are bulky as is the case with DPPH and tannins [27].

In conclusion, we have demonstrated the formation of covalent adducts between ABTS radical cations and the oxidation products of the polyphenols, (+)-catechin and phloroglucinol. These adducts were isolated using HPLC and their structures were elucidated by MS/MS fragmentation analysis. The primary adducts were unstable and formed secondary adducts, releasing part of the ABTS molecule. The latter accounted for the previously reported ABTS degradation products [11]. A mechanism is proposed that explains the formation of these adducts and the ABTS radical degradation products. In the light of the results presented here, we conclude that the assumption that ABTS radicals upon reaction with polyphenols are reduced back to the parent substrate, as the sole mechanism of interaction between the radicals and the polyphenols, is unjustified.

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