Addition of carbon-centered radicals to aromatic antioxidants: mechanistic aspects

Thomas Nauser\textsuperscript{a,b,a} and Janusz M. Gebicki\textsuperscript{b}

Several recent studies have shown that the rates of formation of adduct radicals between carbon-centred radicals and aromatic molecules are virtually diffusion-controlled and reversible. This contrasts with \textquoteleft radical addition\textquoteright, the well-known multistep reaction in preparative organic chemistry where the rate-determining initial formation of radical adducts is perceived to be several orders of magnitude slower and virtually irreversible. Using pulse radiolysis and spectroscopic analysis, we have now re-examined parts of this complex mechanism. The results have significant implications for biological systems: electron-rich, aromatic structures may act like buffers for radicals, moderating their reactivity resulting in a much slower reaction determining the overall rate of oxidation. In vivo, an organism would gain time for an appropriate antioxidant reaction.

Introduction

Oxidative stress is a common consequence of modern industrialized life, widely held to be responsible for the initiation or aggravation of many diseases and other deleterious conditions in humans.\textsuperscript{1} It is defined as a condition in which the challenge by free radicals and other oxidants exceeds the defensive capacity of the organism. While humans and other aerobic organisms are equipped with a range of endogenous antioxidant systems selected for defense against deleterious oxidative processes, these defenses are clearly inadequate under oxidative stress. Since oxidative stress is strongly associated with deleterious health conditions, there is a well-recognized need for supplementation of the endogenous defenses with additional antioxidants. They should be widely available, effective, readily administered and inexpensive. To fill this need, there is a vast choice of commercially available antioxidant supplements, many of which claim to provide defense against free radicals, frequently without scientific justification.

In fact, there is a large group of natural products with significant beneficial effects on health demonstrated in many careful studies, which were for many years partly explained by their antioxidant properties.\textsuperscript{2} Flavonoids and other plant-derived polyphenols form a group of over 8000 identified compounds, with over 500 forming part of human diet.\textsuperscript{3} While their ability to act as antioxidants by donating electrons to free radicals and other oxidizing agents is well known, later quantitative work identified two principal reasons why they were unlikely to compete with the physiological endogenous antioxidants such as ascorbate or GSH \textit{in vivo}. First, the concentrations of ingested polyphenols in tissues apparently only reach at best micromolar levels and, second, their reactions with radicals are relatively slow.

There are now significant doubts about the validity of both these conclusions. On the concentrations \textit{in vivo}, the accuracy of the commonly used spectrophotometric and LC-MS techniques has been questioned, with poor agreement in laboratory estimates of even standard single polyphenols and their metabolites. Measurements of these compounds and their metabolites in tissues are likely to be even more subject to error, with the general caution that the obtained values are likely to be significantly underestimated.\textsuperscript{3-5} The second reservation, slowness of the reactions of polyphenols with radicals, is at least partly due to differences in the understanding of the term antioxidant. Oxidation is defined by IUPAC as \textquoteleft the complete, net removal of one or more electrons from a molecular entity\textquoteright.\textsuperscript{6} Accordingly, most chemists would think of antioxidant action exclusively in terms of electron-transfer. The requirement of complete electron transfer leads to low reaction rates of the flavonoids \((\text{Fl(OH)}_3)\) and polyphenols (PPH) generally because it involves a large entropic barrier imposed by the need for exchange of protons:\textsuperscript{7}

\begin{align}
\text{Fl(OH)}_3 + \text{R}^\cdot & \rightarrow \text{Fl(OH)}O^\cdot + \text{RH} \\
\text{Fl(OH)}O^\cdot + \text{H}_2\text{O} & \rightarrow \text{Fl(O\text{"}\text{O}^\cdot)}^\cdot + \text{H}_3\text{O}^+ 
\end{align}

As an example of typical reaction kinetics, the rate constants of reactions of 12 flavonoids with superoxide radicals \((\text{O}_2^\cdot /\text{HO}_2^\cdot)\) lie in the range of only \(\sim 3 \times 10^2\) to \(4.1 \times 10^5\ \text{M}^{-1}\ \text{s}^{-1}\) in spite of a very large redox potential difference.\textsuperscript{8,9} In contrast to the...
chemical definition, for biologists an antioxidant is defined by its ability to inhibit deleterious oxidative processes partially or completely, regardless of the mechanism. The practical advantage of the biological approach lies in that it measures the quality of an antioxidant solely by its effectiveness in lowering the activity of damage-inducing oxidants; this may well be sufficient to eliminate the consequences of oxidative stress under physiological conditions. It will be shown that both the chemical and biological definitions of antioxidant action are illustrated by the reactions of polyphenols with C-centered radicals.

The possibility of a potential role of polyphenols as effective in vivo antioxidants in reactions not involving transfer of electrons was suggested by results reported in 2014 by Nauser and Carreras, who described a fast reversible addition reaction of carbon-centered radicals to histidine.\(^{10}\) Reaction kinetics were investigated by pulse radiolysis because formation of the adducts was virtually diffusion-controlled, with rate constants 2–3 orders of magnitude higher than in processes involving complete electron transfer. Importantly also, formation of the adducts resulted in significant lowering of the reactivity of the radicals.

The chemistry of polyphenols and their oxidation by radicals have been extensively studied. Most of the results were obtained in alkaline solutions, where the compounds are more soluble.\(^{11}\) This implies deprotonation of at least one phenolic group, which would accelerate the transfer of electrons.\(^{7}\) Kinetics measurements at high pH would, therefore, yield an upper limit for the respective electron transfer reactions at neutral pH. However, results of measurements in alkaline solutions cannot be simply extrapolated to physiological conditions, reducing their relevance to biological systems; it will be shown that the mechanism of the radical–polyphenol reaction appears to change significantly around pH 8. In order to test the antioxidant potential of polyphenols under conditions approaching the physiological, we reported recently on radical addition of carbon-centered amino acid radicals to several polyphenols at neutral pH.\(^{12}\) We confirmed the results of the earlier studies with histidine by finding a close to diffusion-controlled rates of formation of the adducts with a range of polyphenols, resulting in drastic lowering of the reactivity of amino acid radicals. The reactions were reversible, with equilibrium constant of \(5 \times 10^5 \text{ M}^{-1}\) in the case of morin. Adduct formation did not involve electron-transfer or the breaking of bonds.\(^{13}\) The process leading finally to the repaired radical and formation of a phenoxy radical (PPO*) can be represented by reaction (3), with the marked stages corresponding to the biological and chemical definitions of antioxidant action (see above):

\[
PPOH + R^* \rightarrow \text{(adduct)*} \rightarrow \text{PPO* + RH} \tag{3}
\]

Observation of the reaction rate difference of several orders of magnitude between formation of the adducts (biological definition) and the final products (chemical definition) required the availability of fast and sensitive pulse radiolysis technology and was missed in earlier studies. At present there is no information on the intermediate steps following the formation of the complex.

Addition of radicals to aromatic centers is long known\(^ {14}\) and “nucleophilic radical addition”, where carbon-centered radicals are used to add aliphatic side chains to aromatic centers, has been used in organic chemical synthesis since the pioneering work of Minisci in the 1970s.\(^ {15–18}\) Minisci interpreted his experimental product yields and distribution in terms of a mechanism in which formation of a radical adduct was the rate-determining step, followed by a very fast oxidation process, for example by a transition metal ion, resulting in in the final product.\(^ {12}\) The studies of aromatic radical adduct by Nauser et al. revealed a significant complexity of the mechanism of these reactions, particularly at pH values relevant to living organisms.\(^ {10,12,13,19}\)

In an effort to deepen our understanding of the details of the reaction mechanism and to provide a critical test of our initial data, we have now investigated by pulse radiolysis the response of the adducts of the flavonoid morin and amino acid radicals to varying conditions, such as concentration and pH. We also extended the study to additional amino acids and polyphenols. Interpretation of the data was challenging, but the difficulties were not unique: when Minisci investigated the addition of electron-rich radicals to electron-poor aromatic structures, many of the findings had to be rationalized by an educated guess. However, as we were observing the initial fast processes and loss of radical reactivity instead of formation of the products, our findings contribute to the overall mechanistic picture of radical addition reactions, adding some new pieces to the puzzle.

**Experimental**

**Pulse radiolysis**

Experiments were carried out in the pulse-radiolysis facility at the ETH described previously.\(^ {20}\) Briefly, samples were irradiated with 50 ns/2 MeV electron pulses delivered by the electron accelerator. As light-source a 75 W Xe-arc lamp was used. For wavelength selection appropriate order-sorting filters and a 300 mm Czerny–Turner monochromator with 150 l mm\(^{-1}\) gratings (blazed either at 300 nm or at 500 nm) and a slit width of 1 mm were chosen. Prepared samples were taken up in gas-tight syringes and were introduced into the measurement cell through a PEEK capillary. The quartz measurement cell had an optical pathlength of 6 cm.

**Sample preparation**

To prepare stock solutions, the polyphenols were dissolved under argon. Polyphenols with slow dissolution kinetics were dissolved at high pH and then brought back to neutral pH within one minute by addition of HCl (diluted from 37%, \textit{pro analyti}, source: Merck, Zug, Switzerland). Morin (95%) was purchased from Adipogen (Liestal, Switzerland), rutin (98%) from ABCR (Karlsruhe, Germany), gallic acid from
Cayman Chemical Company (Ann Arbor, USA), catechin (>98%) from Sigma (Zug, Switzerland), and epigallocatechin gallate was a kind gift from Dr J. Vinson. For the chemical structures of the (poly-)phenols used, see Table 1. Stock solutions of the polyphenols were used within 2–3 h after preparation. Stock solutions of the amino-acid derivatives (Bachem, Bubendorf, Switzerland) were prepared from the pure chemicals and MilliQ water (Millipore, Switzerland) and kept under argon. Phosphate buffer was prepared with NaH2PO4 and Na2HPO4 (puriss. pro analysi) from Fluka (Buchs SG, Switzerland) according to the Henderson–Hasselbach calculation. Because the pH of phosphate buffer around neutral is concentration dependent, slight corrections were applied by addition of NaOH (made from pellets, Ph. Eur. II, source: Siegfried, Zofingen, Switzerland) or HCl.

Before use, glassware, syringes and tubing were first rinsed with dilute HNO3 (to mobilise transition-metal ions) and then with MilliQ water. We chose not to immerse any pH-electrode into our samples before the experiments. Pulse-radiolysis experiments can be very sensitive to even nanomolar traces of contaminations, especially transition metal ions. Working in an inorganic chemistry lab, we could not exclude presence of such contaminations on and in our electrodes. We controlled approximately correct pH with the leftover solution that could not be taken up into the syringe. These drops were put on a pH-paper for control. Appropriate pH-papers were used (depending on expected pH) from Riedel-de-Haen (Reinach, Switzerland), Merck, Fluka, and Macherey-Nagel (Oensingen, Switzerland).

Solutions that were not used up completely were measured with a pH meter after the experiments.

With the stock solutions, 10 ml batches were prepared in Schlenk tubes where they were deaerated and saturated with the required gas. Deaeration was carried out by repeated evacuation to 20 mbar to prevent boiling at ambient temperature, stirring and refilling with argon or N2O. After at least 6 cycles, the sample was equilibrated with the required gas or gas mixture at atmospheric pressure.

Evaluation of results

Absorbed energy doses are given in J kg⁻¹ ≡ Gy (gray). The electron pulses decomposed water in the irradiated solutions, generating 2.7 × 10⁻⁷ M Gy⁻¹ of reducing electrons (eaq⁻), 2.8 × 10⁻⁷ M Gy⁻¹ oxidizing hydroxyl radicals (HO*), and 0.6 × 10⁻⁷ M Gy⁻¹ of hydrogen atoms (H*).21 In solutions saturated with 24 mM N2O, the eaq⁻ were converted to HO*, approximately doubling their yield.

Results

The reaction of amino acid alkyl radicals with morin

1. Near-neutral pH: kinetics and spectra. The results shown in Fig. 1 were generated by pulse irradiating N2O saturated solution of 10 mM AcAlaNH2, 50 μM morin, pH 7 with doses of approximately 8 Gy. Approximately 5 μM of AcAla*NH₂ radicals then reacted with the morin. The 200 fold concentration excess of the amino acid ensured that 98% of the primary radicals generated reacted with the amino acid, leaving the morin intact.

Time vs. absorption trace at 575 nm showed three phases in the first millisecond after the pulse (Fig. 1, upper panel, blue trace), in agreement with an earlier report: Phase A, a fast absorption increase (0–5 μs), followed by a brief decay in Phase B (10–100 μs) and a slow increase in Phase C.11 Phase A was attributed to a fast equilibrium reaction of AcAla*NH₂ radicals forming an adduct with the morin. The kinetics of Phase B were second order (see below). Associated with the reaction in Phase C, there was an absorption increase at 700 nm (Fig. 1, upper panel) which was slower than at 575 nm. During the first two phases, the absorption at 700 nm was small. The build-up at 700 nm may be associated with a reaction consecutive to the 3rd phase observed at 550 nm. Importantly, concomitant
In the presence of 20% oxygen (0.2 mM) and 80% N₂O (20 mM) or 10% oxygen (0.1 mM) and 90% N₂O (22 mM), Phase C was suppressed: under such conditions an absorption at 550 nm was observed and reached maximum after approximately 20 μs, with \( k_{\text{obs}} = 2 \times 10^5 \text{s}^{-1} \). The calculated extinction coefficient was identical to the one derived for N₂O-saturated solution of 50 μM morin in 10 mM phosphate buffer at pH 7.4 irradiated in the absence AcAlaNH₂, providing a correction was applied for the appropriate radiochemical yield of HO• for the respective solutions (Fig. 1, lower panel).

2. Near-neutral pH: stoichiometry. When a sample of 100 μM morin and 20 mM AcAlaNH₂ in 10 mM phosphate buffer at pH 7.5 was repeatedly irradiated with ~13 Gy pulses, the build-up of Phase C became progressively weaker and its rate was lowered with each pulse (Fig. 2, upper panel). Also, the time-dependent absorbance changes at 700 nm disappeared gradually. Something was consumed. More and more, the decay of Phase B dominated the kinetics trace. Phases B and C could be separated because the kinetics of Phase B were of second order and

with the build-up in Phase C, there was also a shift of the absorption band from 575 nm to 550 nm (Fig. 1, middle panel).

![Fig. 1](image1.png)

**Fig. 1** Upper panel: Irradiation of N₂O saturated solution of 10 mM AcAlaNH₂, 50 μM morin, pH 7, with doses of approximately 8 Gy. Kinetics traces at 575 nm (blue) and 700 nm (red). Middle panel: Corresponding transient spectra for 10 μs (black), 50 μs (blue), and 500 μs (green) after irradiation. Lower panel: Kinetics trace at 550 nm 50 μM morin, pH approximately 7.4, irradiation dose 10 Gy, oxidized by HO• radicals (N₂O saturated solution), red trace, or oxidized by peroxyl radicals (in presence of 10 mM AcAlaNH₂, 80% N₂O, 20% O₂), blue trace. In the latter case, the total radical yield is approximately 15% higher – the trace has been scaled accordingly.

![Fig. 2](image2.png)

**Fig. 2** Response of samples of 100 μM morin and 20 mM AcAlaNH₂ in 10 mM phosphate buffer at pH 7.5 to repeated irradiation with 13 Gy pulses. Upper panel: Kinetics traces detected at 550 nm: number of pulses before measurement is shown above the x-axis (0 represents the fresh solution), with the corresponding kinetic traces from 0 on the top to 8 on the bottom. Lower panel: The corresponding apparent rate constants of Phase C absorbance growth.
practically unaffected by repeated pulsing. Therefore, the background signal, i.e. Phase B, is best fitted in the first 400 μs, after repeated pulsing. This background could then be subtracted from the other kinetics traces, and the build-up rate constants of Phase C derived (Fig. 2, lower panel) from the corresponding traces. As long as there was “enough” morin left intact, we observed a linear drop in reaction rate. At very low remaining concentration of still intact morin, its consumption by the irradiation leveled off (Fig. 2, lower panel).

In the experimental results shown in Fig. 2, approximately 7–8 μM of radicals were produced per pulse. The results show that 73% of the reactivity of 100 μM morin in Phase C could be quenched by 5 successive irradiations. Apparently, the stoichiometry of Phase C was not 1 : 1 for the radical : morin ratio. This was corroborated by experiments in which 15 mM AcLysNH₂ and 75 μM morin were pulse irradiated with doses that produced 3–90 μM AcLys∗NH₂. The data showed unambiguously that the relative yield of product in Phase C strongly decreased with increasing dose, i.e. with the starting concentration of radicals (Fig. 3, upper panel). A decrease in the relative yield can be rationalized qualitatively by the influence of competing radical recombination reactions whose occurrence increased with the radiation dose. It is, however, surprising that the absolute yield of absorbance dropped sharply when the concentration of amino acid radicals was larger than that of morin (Fig. 3, upper panel). A decrease in the relative yield indicates a change of the mechanism of the morin–radical reaction. Notably, this is the only trace that cannot reflect a smaller stoichiometry than 1 : 1 of [radicals] : [morin] ratio, with morin not in excess. The initial absorbance in Phase A, 3 μs after pulse, was strictly linear with the amount of radicals produced, with one exception, where [AcLys∗NH₂] > [morin] (Fig. 3, lower panel).

3. Near-neutral pH: thermodynamics. For a more detailed investigation of Phase B, we chose to pre-condition the samples by repeated pulsing, in order to suppress Phase C. Both the first and second Phases of the kinetics were unaffected by repeated irradiation of the solution, even if the total amount of radicals exceeded the initial morin concentration by a wide margin, with [radical]_{tot} > 10[morin]_{initial}. If the morin concentration was varied, given a fixed ratio [morin] : [AcAla] of 1 : 200, the rate of this decay was inversely proportional to the initial morin concentration (Fig. 4, upper panel). In other words, a non-consumed substructure of morin inhibited the radical decay in Phase B. The kinetics corresponded to a recombination of radicals engaged in a fast equilibrium reaction with morin with an equilibrium constant of (4 ± 3) × 10^5 M⁻¹ (for details see ref. 10, ESI). That is almost one order of magnitude higher than the value derived from the build-up kinetics. It should be noted that in our current determination the K_{eq} value was calculated by dividing the slope of the measurement by the offset and that since the latter was small, the result carried a large relative error. Note also that the pH-determination in our experiments had an accuracy of ±0.2 (for implications see below).

In further tests, 15 mM solutions of different amino acid derivatives and 75 μM morin at pH (7.4 ± 0.2) were saturated with N₂O and then pulse irradiated. The results were similar to those with AcAla, but with different observed rate constants. Apparent rate constants of Phase B were thus derived for solutions that had been irradiated multiple times (Fig. 2, lower panel), in analogy with results in Fig. 2 (upper panel).

Influence of pH

We found that the kinetics of morin reacting with AcAla∗NH₂ were very sensitive to the conditions of the preparation. It is known that such reactions are pH dependent and that autoxidation of polyphenols during preparation may be an issue, especially if they are dissolved at high pH. Therefore, experiments used for quantitative intercomparison of results have always been carried out using samples prepared from the same stock solutions of polyphenol, buffer and AcAlaNH₂ that have all been kept under argon. In addition, the experiments were carried out with the same concentration ratio of chemicals and within 2–3 hours after preparation of the polyphenol stock solution. This minimized differences in pH and in possible
Impurities in the samples due to degradation. In order to qualitatively survey the response of our experiments to pH changes, we conducted a series of experiments changing the pH between 3 and 9 while keeping the concentration of morin (25 mM) and AcAlaNH$_2$ (5 mM) constant (Fig. 5). Qualitatively, we observed that at 550 nm Phases A and B did become less prominently visible above pH 7 and were obscured almost completely at pH 9 by the strong absorption of Phase C. With increasing pH the associated build-up of Phase C was also accelerated and more prominent and absorption at 700 nm was also more prominent. At pH 9.4 the kinetics trace was clearly sigmoidal and still had a positive slope at 1.5 ms, in clear contrast to the measurement at 550 nm, where at the same time the slope was negative.

The reaction of amino acid alkyl radicals with rutin, gallate, catechin and epigallocatechin gallate

Reactions of the flavone morin were subsequently compared with those of other polyphenols. Qualitative experiments were carried out with rutin, a glycosylated flavone, and with flavonoid epigallocatechin gallate (EGCG) as well as its related substructures catechin and gallate. The pulse-irradiated solutions were saturated with N$_2$O and contained 75 mM polyphenol, 15 mM AcAlaNH$_2$ and 20 mM buffer. No spectra were collected. Except for catechin, kinetics traces between were recorded between 500 nm and 575 nm. For catechin the detection wavelength was 300–350 nm (see below).

Experiments with rutin yielded qualitatively similar traces to morin under the same conditions (Fig. 6). It is unsurprising that the different positions of the hydroxy groups in the B-ring of the flavone (ortho vs. para dihydroxy) as well as glycosylation of the C-ring would have an influence on rate constants and extinction coefficients. For example, the build-up in Phase C appeared to be slower in the case of rutin.

We found that catechin, gallate and EGCG also reacted with AcAla$^+$NH$_2$, as shown by the kinetics traces. With catechin, no distinct traces in the visible range could be observed. However, the decay observed at 310 nm, where the spectrum of AcAla$^+$NH$_2$ has a shoulder, was significantly slowed down (data not shown).
Gallate had a similar effect on the traces measured in the UV, but there was also an absorbance build-up detected in the visible region (Fig. 7, red trace). EGCG showed a response to radicals similar to that of morin and rutin, with 3 different reaction phases visible in the kinetics traces (Fig. 7, blue trace). The slow absorbance build-up of Phase C was faster than with gallate.

**Discussion**

Our previous study of the fast formation of adduct of carbon-centered radicals and polyphenols (PPH) suggested that the initial step was followed by a series of reactions, presumed to reflect stepwise stabilization of the adducts.\(^\text{12}\) We have now carried out additional experiments and analysis as part of a study of the mechanism of this sequence of reactions.

Under the conditions employed, pulse radiolysis generated hydroxyl radicals which were scavenged by an amino acid, producing a C-centered radical AA\(^*\). It seems beyond doubt that reactions (4) and (5) governed the initial phase of our experiments. Subsequently many reactions were possible, but their occurrence cannot be conclusively established with the available data. However, some of our experimental results allow comments on reactions (6)–(14):

\[
\begin{align*}
\text{2AA}^* & \rightarrow \text{products} \quad (4) \\
\text{AA}^* + \text{PPH} & \rightleftharpoons [\text{AA} \cdot \cdot \cdot \text{PPH}]^* \quad (5) \\
\text{AA}^* + \text{PPH} & \rightarrow \text{AAH} + \text{PP}^* \quad (6) \\
[\text{AA} \cdot \cdot \cdot \text{PPH}]^* & \rightarrow \text{AAH} + \text{PP}^* \quad (7) \\
[\text{AA} \cdot \cdot \cdot \text{PPH}]^* + \text{PPH} & \rightleftharpoons [\text{PPH} \cdot \cdot \cdot \text{AA} \cdot \cdot \cdot \text{PPH}]^* \quad (8) \\
\text{AA}^* + \text{O}_2 & \rightarrow \text{AA-O}_2^* \quad (9) \\
\text{AA-O}_2^* + \text{PPH} & \rightarrow \text{AA-O}_2 \cdot \cdot \cdot \text{H} + \text{PP}^* \quad (10) \\
\text{PP}^* + \text{O}_2 & \rightarrow \text{PP-O}_2^* \quad (11) \\
\text{PP-O}_2^* & \rightarrow \text{PP}^*_{\text{ox}} + \text{O}_2^* \quad (12) \\
[\text{AA} \cdot \cdot \cdot \text{PPH}]^* + \text{O}_2 & \rightarrow \text{AA-PPH-O}_2^* \quad (13) \\
\text{AA-PPH-O}_2^* & \rightarrow \text{AA-PP} + \text{HO}_2^* \quad (14)
\end{align*}
\]

The range of possible reactions indicates the complexity of the mechanism of the reactions following the well-documented formation of the complex [AA \cdot \cdot \cdot PPH]\(^*\). Already 20 years ago we were cautioned in personal communications by W. Bors that the mechanism of the kinetics of reaction of radicals with polyphenols would be very complicated and that sample preparation would be difficult, particularly in solutions close to pH 7. After the conclusion of our study, we entirely concur. However, the finding of very high rates of formation of adducts of polyphenol–radical adducts near pH 7, in order to test whether polyphenols are likely to fulfil the role of effective antioxidants in \textit{vivo}.

**Possible role of contaminants**

The possibility that impurities in the supplied chemicals affected the reproducibility of experimental results cannot be excluded, although none had claimed purity of less than 98%. It is also possible that autoxidation during sample preparation and small changes in pH between samples from different stock solutions may be partly responsible. However, we need to emphasize that such variations did at no point change the qualitative or even the semiquantitative results. Other than impurities in the chemicals, oxygen is always a strong candidate as contaminant. One of the major problems in our
new absorption maximum evolving at 700 nm. A maximum absorbance at 550 nm was reported for one-electron oxidation of morin\textsuperscript{12} and, therefore, could indicate phenoxyl radical formation. After 1 ms the absorption at 550 nm band started to bleach while the one at 700 nm was still growing, indicating that the bands stemmed from different species and different processes. That would imply at least one additional process occurring within the first millisecond at pH 7.

There was a stoichiometric consumption of the reactive species or reactive groups involved in Phase C of the reaction cascade (Fig. 2). Like the band at 550 nm, this observation may indicate involvement of a redox process, as suggested in the early literature on radical reactions with polyphenols (reviewed in ref. 25). In contrast to equilibria, oxidation processes require irreversible stoichiometric consumption of reactants. Interestingly, the drop in reactivity of the adducts implies the consumption of two equivalents of morin per radical: fresh solutions showed a rate constant of Phase C of \( k = 7.5 \times 10^3 \) s\(^{-1}\), with the initial six 13 Gy pulses causing a rate constant drop of 1.1 \( \times 10^4 \) s\(^{-1}\) per pulse (Fig. 2, lower panel). While a dose of 13 Gy produced 7–8 \( \mu \)M carbon-centered radicals, the reactivity drop implies a destruction of about 14–15 \( \mu \)M morin. “Strange” reactions often point to the presence of impurities in the solutions. However, the persistent and reproducible lack of the expected stochiometry for all polyphenol samples investigated does not support this hypothesis. In addition, the observation of a reaction stochiometry different than 1:1 for reactions of radicals and aromatic molecules is not unique. A reaction with two aromatic centres per radical is similar to earlier findings with the reaction of alcohol radicals and His, where at elevated His concentrations more than one His participated in the reaction.\textsuperscript{10} There was an obvious mechanism change if [radical] > [morin] with Phase C not observed any more (Fig. 3, upper panel, brown trace). However, for experiments with more than 2 morin molecules per radical, \textit{i.e.} with a dose <50 Gy, the kinetics traces were qualitatively similar and we expect a similar mechanism, in keeping with the notion of a 1:2 [radical] : [morin] stochiometry. These observations, however, do not prove conclusively that a 1:2 stochiometry applied for Phase C. It may also be only effective at later times, \textit{i.e.} at the product formation step (reaction (6)). Importantly, because of the high reactant concentrations needed for these experiments, current data do not allow extrapolation of our observations to possible similar reactions \textit{in vivo}.

At a fixed concentration of 75 \( \mu \)M morin, the measured absorbance in Phase A was linear with the radical concentration, provided [morin] > [radical] (Fig. 3, lower panel). If, however, [radical] > [morin], [morin] was the determining concentration as visualized by the red dot at 75 \( \mu \)M in the plot. This again supports our previous notion that the initial reaction (Phase A) strictly followed a 1:1 stochiometry. Further insight into the mechanism can be gained from Fig. 4, upper panel. The reactive center involved in the formation of the adduct (Phase A) was not consumed,\textsuperscript{13} when morin had been degraded by repeated irradiation so that Phase C was completely suppressed, the apparent rate constant of the decay in Phase B, \( k_{obs,Phase B} \), was inversely
proportional to the initial concentration of morin. This is indicative of a mechanism analogous to adducts of carbon-centered radicals with histidine. Phase B is governed by the recombination reaction (4) and equilibrium (5). Mathematics shows that the observed recombination rate \( k_{\text{obs}, \text{Phase B}} \) then is dependent on \( k_4 \) and \( K_5 \):

\[
k_{4, \text{obs}} = k_4 \varepsilon / l
\]

(15)

\( \varepsilon \) represents the difference of molar absorptivity between reactants and products, \( l \) the optical pathlength. While reaction (4) is an elementary reaction, strictly limited for reactions of AA, Phase B is an overall reaction linked to “all radicals”, i.e. [AA···morin] and AA and

\[
[[\text{AA} \cdot \cdot \cdot \text{morin}^*]] = [\text{AA}^*] \times K_5 \times [\text{morin}]
\]

(16)

\[
[\text{AA}^*]_{\text{tot}} = [\text{AA}^*] + [[\text{AA} \cdot \cdot \cdot \text{morin}^*]] = [\text{AA}^*] \times (1 + K_5 \times [\text{morin}])
\]

(17)

With \( K_5 \) the equilibrium constant of reaction (5), \([\text{AA}^*]_{\text{tot}}\) the amount of “amino-acid related” radicals directly or indirectly involved in Phase B, and \( k_{\text{obs}} \) (eqn (15) and (20)) the slope derived from the linearization of a second-order kinetics trace, 1/\( A_0 \) vs. \( t \). Even though all amino-acid related radicals \([\text{AA}^*]_{\text{tot}}\) will eventually react \( \text{via} \) reaction (4), only a part \([\text{AA}^*]\) is in a reactive form (eqn (17)). Therefore, \( k_{\text{Phase B}} \), the experimentally observed overall rate constant of Phase B, is smaller than \( k_4 \) and decreases with increasing morin concentration. The integration of the second order rate law for reaction (4) and the observed overall reaction, Phase B, yield:

\[
1/[\text{AA}^*] - 1/[\text{AA}^*]_{(t=0)} = k_4 \times t
\]

(18)

\[
1/[\text{AA}^*]_{\text{tot}} - 1/[\text{AA}^*]_{\text{tot}(t=0)} = k_{\text{Phase B}} \times t
\]

(19)

\[
k_{\text{obs}, \text{Phase B}} = k_{4, \text{obs}} / (1 + K_5 \times [\text{morin}])
\]

(20a)

\[
1/k_{\text{obs}, \text{Phase B}} = (1 + K_5 \times [\text{morin}])/k_{4, \text{obs}}
\]

(20b)

Fig. 4, lower panel, shows that the kinetics traces of Phase B decay depended on the nature of the amino acid radical. The morin concentration, the optical pathlength \( l \) and \( k_4 \) were by design the same for all these experiments. As we do not expect the extinction coefficient to change much between adducts, we suspect the equilibrium constant for adduct formation, \( K_5 \), to depend on the radical. This is not surprising; earlier work with His showed the equilibrium constant to depend on the electrophilicity of the radical and we also expect steric factors and charge to play a role.

Some of the reproducibility problems mentioned above can be explained by results of qualitative experiments with morin at varying \( pH \) (Fig. 5): the observed response of the samples was strongly \( pH \)-dependent. The data recorded at 550 nm imply two \( pH \)-equilibria involved in the reactions, one between \( pH \) 5 and 6 and one around \( pH \) 8. All parameters seemed to be affected: kinetics, spectra and perhaps even the mechanisms. Whether the experiments were carried out with different flavonoids (Fig. 6 and 7) or different amino acid radicals (Fig. 1, 3 and 4) at around \( pH \) 7, the qualitative overall observations were the same. We take this as indication that the same types of relevant reaction mechanisms are likely to govern the chemistry at a given \( pH \) region. If true, that would imply that our data would be representative for the family of (iso-)flavonoids at physiological \( pH \). This, in turn, would support a significant antioxidant role for the aromatic metabolome made up of the wide range of endogenous metabolites and ingested aromatic compounds.

**Electron transfer**

While our results do not support electron-transfer as the major process in the reaction sequence following formation of the initial adduct (reaction (3)), we also cannot rule it out as minor reaction: the maximum shift from 575 nm to 550 nm at \( pH \) 7.4 in Phase C may well be indicative of an electron transfer. That would parallel our findings with carbon-centred radicals with those of earlier papers on electron transfer with other radicals. However, the absorptivity of the deaerated samples in Phase C at 550 nm was less than 30% of that observed with peroxyl or hydroxyl radicals. If electron transfer did indeed occur, the efficiency of the process would be low. When we repeatedly irradiated the same sample, with a repetition rate of approx. 1 min \(^{-1} \), we observed that absorbance of the solutions did not change permanently. That means that all absorptions seen above 400 nm were transient and bleached fully within some 30 s. We concluded that none of these “Phases” were directly associated with product formation. In agreement with earlier assumptions this is consistent with a complicated multi-step mechanism, with more than 4 reactions.

There is indication that more than one polyphenol was consumed per radical under our experimental conditions. There is, however, no convincing explanation yet on what that would mean on a mechanistic level. There may be some chain reaction involved or annulation reactions might be a reason. We carried out control experiments to detect possible influence of contaminations and failed. However, as long as there is no good hypothesis for a mechanism, we cannot completely rule out contamination as contributor to Phase C.

**The mechanism of radical addition**

Minisci et al. used radical clock experiments to establish the reaction mechanism. As with every kinetics experiment, data analysis depends on the mechanistic assumptions made. With radical clock experiments the standard assumption is of two irreversible reactions with rate constants derived as from a competition reaction by determining the reaction yields. Therefore an undetected, (almost) diffusion-controlled and reversible initial (π-)adduct formation, may skew the interpretation. If the “trapping” reaction of one of the two reactions is reversible, determination of the rate constants of elementary reactions from product yields becomes questionable. As Ashton and Buxton pointed out, product distribution is governed by the conversion of π-adduct to σ-adduct with known directing effects. Our data suggest (vide supra), that for carbon-centered radicals even this step is largely reversible.
The terminology of “nucleophilic radical addition” for the Minisci reaction is questioned by the fast initial adduct formation, as in ref. 12, because the addition step is electrophilic: the stronger the electrophilicity of the incoming radical, the larger the equilibrium constant of the addition reaction.19 Such behavior might have been predicted from Minisci’s statement “the radical must be oxidative in nature”, i.e. a good electron acceptor, which characterizes an electrophile. This statement does not question the vast amount of data on the kinetics of the adduction product formation, the yields of stable products, or the product distribution, which are determined by the electron deficiency at the reaction site. It does, however, change the perception of the mechanism involved. If the equilibrium constants of the adducts increase with the electrophilicity of the incoming radical, one may assume that they would increase with the electron-excess of the aromatic structures. This seems to be the case. Why then would the Minisci reaction require electron-poor aromatic systems?20 While the reaction of a strong electrophile with an electron-rich aromatic structure is thermodynamically favorable, strong electrophiles are usually good oxidants, with electron-rich aromatic structures more easily oxidized than electron-poor ones. There is a good probability of an electron-transfer process, for example by elimination of the reduced radical (see reaction (7) or the reaction of HO• with phenols26), competing with the formation of the desired final product of the synthesis. That may prohibit practical use for preparative purposes. If our hypothesis is true, electron-rich aromatic structures might act like buffers for radicals, moderating their reactivity. Thermodynamics dictate that such spontaneous reaction must result in lowering of the activity of the adduct-forming radicals. In biological systems, adduct formation would therefore constitute an antioxidant reaction. It would be additionally beneficial, after initially buffering of a potentially harmful damaged site, by repairing it in reaction (7) at a later stage. Secondly, more stable intermediates would slow down the possibly rate determining oxidation process, yielding the final product. In vivo, an organism would gain time for an appropriate antioxidant reaction, i.e. to reduce the radical, for example by ascorbate. In a preparative environment, recombination of radicals may outcompete the formation of stable addition products. That said, we do pretend to be able to present a comprehensive alternative to Minisci’s model of radical addition. We do disagree with the first step in his mechanism based on direct observations of initial intermediates, a technique not used in his work. That raises again the question on the nature of the rate-determining step which appeared settled. However, our disagreement leaves the practical use and implications of Minisci’s work untouched.

Conflicts of interest
The authors declare no conflict of interest.

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