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Quenching of α,β-unsaturated aldehydes by green tea polyphenols: HPLC–ESI–MS/MS studies

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ABSTRACT

The aim of this work was to investigate *in vitro* the quenching activity of green tea polyphenols against α,β-unsaturated aldehyde, using 4-hydroxy-nonenal (HNE) as prototype and HPLC–ESI–MS/MS techniques. HNE is the most abundant and genotoxic product of oxidation of dietary polyunsaturated fatty acids, and is believed to be involved in the early stage of colorectal carcinogenesis on account of its genotoxic potential.

Both epigallocatechin gallate (EGCG, 1.0–3.5 mM), the main constituent of green tea polyphenols, and a green tea aqueous extract are able to quench HNE (50 μM) in colorectal physiomimetic conditions (10 mM phosphate buffer, pH 8.0, 37 °C), giving rise to the formation of six diastereomeric covalent adducts at the ring A of EGCG, as indicated by their ESI–MS/MS fragmentation pathways. The specificity of the adduction positions was explained by ^{1}H NMR experiments. HNE quenching is pH-dependent and maximum at pH 8.0. ESI–MS analysis showed no formation of 4-hydroxy-2,3-epoxy-nonanal, or adduction of the epoxide to EGCG. This implies that too little hydrogen peroxide (1 mM, 24 h incubation, FOX-2 method) develops from auto-oxidation of EGCG in our aerobic experimental conditions to oxidize HNE to its corresponding epoxide, so this mechanism is not responsible for the compound's disappearance. EGCG and green tea extract also quenched acrolein, another genotoxic α,β-unsaturated aldehyde, giving one predominant adduct and minor isobaric species, probably due the adduction of acrolein at different positions of the EGCG ring A.

These results suggest that EGCG and green tea extract, beside the proposed mechanisms of chemoprevention that target multiple cell-signaling pathways that control cell proliferation and apoptosis in cancer cells, can also prevent protein carbonylation in the tumor tissue environment, depending on the pH of the medium surrounding the tissue, the type of tumor, the stage of dysregulation of lipid peroxidation and, finally, the stage of carcinoma development.

1. Introduction

Tea drinking is an ancient tradition dating back 5000 years in China and India. It has been long regarded in those cultures as an aid to good health, and current studies are focusing on the possible use of tea in the prevention and treatment of some cancers [1].

All varieties of tea come from the leaves of *Camellia sinensis*. The leaves are picked, rolled, dried, and heated. To make black tea the leaves are then allowed to ferment and oxidize. Since it is less processed, green tea contains higher levels of antioxidants than black tea [2].

There is compelling evidence in literature that dietary intake of green tea and purified green tea components are inversely associated with the risk of colorectal cancer [3–5]. This has been strongly supported by several studies in animal models, and recently by the milestone epidemiological study by Yuan et al. [6], who found that urinary levels of EGC and 4′-MeEGC (EGCG metabolites) were related with a significantly lower risk of colon cancer in males.

Reactive oxygen species (ROS) increase in chronic diseases of the gastrointestinal tract, and are involved in the onset and progression of precancerous lesions [7]. Green tea and its components might exert their beneficial chemopreventive action against colorectal cancer by reducing ROS production associated with inflammation, through a specific antioxidant mechanism [7]. Several other mechanisms have been proposed for the beneficial effects of green tea polyphenols, including inhibition of (1) telomerase activity, (2) over-expression of tumor-associated fatty acid synthase, FAS [8],

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and (3) mitogen-activated protein kinase cascade signaling [9], or effects on multiple signal transduction pathways that control the unwanted proliferation of cells [10], aberrant arachidonic acid metabolism [11], induction of the development of pro-apoptotic hydrogen peroxide (H_2O_2) [12], and of phase-I and phase-II detoxifying enzymes [13].

Surprisingly, it has never been considered whether, with its antioxidant action, green tea and its components also have anticarbonyl activity by entrapping genotoxic α,β-unsaturated aldehydes such as 4-hydroxy-nonenal (HNE) and acrolein (ACR) [14], which arise from oxidative degradation of dietary PUFAs and whose presence can trigger the inflammatory stage preceding the onset and progression of colorectal cancer [7].

Exposure of human colon tumor cells to butyrate, one of the major products of fermentation of dietary fibers by the gut microflora, increases expression of the primary HNE-metabolizing glutathione transferase (GST) isoform [15] (hGST-A4), and consequently the detoxification of HNE. Thus the present study investigated whether EGCG, which is the major constituent of green tea polyphenols, and an *in toto* aqueous infusion of commercial green tea, was able to eliminate HNE formation by a quenching mechanism. The study employed different analytical techniques: HPLC–DAD–MS/MS–^{1}H NMR, working in physiomimetic conditions and using concentrations of EGCG comparable to those found in a cup of green tea.

2. Material and methods

2.1. Chemicals

All chemicals and reagents were of analytical grade and purchased from Sigma–Fluka–Aldrich Chemical Co. (Milan, Italy); HPLC-grade and analytical-grade organic solvents were from the same supplier. HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). (−)-*R,R*-Epigallocatechin gallate (EGCG), catechin (C), epicatechin (EC) and catalase were purchased from Sigma–Fluka–Aldrich (Milan, Italy). 4-Hydroxynon-2-enal diethyl acetal (HNE-DEA) was synthesized according to the literature [16]. HNE was prepared from HNE-DEA by 1 mM HCl hydrolysis (1 h at room temperature) and quantified by UV spectroscopy (λ_{max} = 224 nm, ε = 13750 M^{-1} cm^{-1}). 4-Hydroxy-2,3-epoxy-nonenal (epoHNE) was synthesized by reacting 33 μmol of HNE with 16 mM of H_2O_2 in 1 mL of 10 mM phosphate buffer solution, and purifying it on a RP-SPE cartridge (3 g silica-bonded C-18 resin, Discovery® DSC-18, Supelco, Bellefonte, PA, USA) following the manufacturer's instructions. Purity was checked by ^{1}H NMR. Commercial green tea was purchased in a local store and an aqueous extract was obtained by infusing 1.5 g of dry leaves in 100 mL of water at 90 °C for 5 min. Deuterium oxide was from Sigma–Fluka–Aldrich (Milan, Italy).

2.2. Consumption of HNE by catechins and green tea

HNE (final concentration 50 μM in 1 mM phosphate buffer, pH 8.0) was incubated with C, EC, EGCG (10 mM phosphate buffer, pH 8.0) or with the green tea aqueous extract for different periods (up to 24 h) at 37 °C. Samples for each different incubation time were directly analyzed by HPLC [H_2O/CH_3CN/H_3PO_4 50/50/0.1 (v/v/v)] to measure HNE consumption (UV detection, 224 nm).

2.3. H_2O_2 (FOX-2)

H_2O_2 was measured according to Nourooz-Zadeh [17].

2.4. HPLC–DAD–ESI–MS analyses

HNE was incubated with EGCG (or with the aqueous extract of green tea) at a 1:1 molar ratio in 10 mM phosphate buffer (pH 8.0) for 24 h at 37 °C. Samples were diluted 1:5 with a water/acetonitrile (70:30) mixture and 20 μL injected into the HPLC–MS spectrometer.

HPLC–DAD experiments were carried out on a Thermoquest Surveyor System (Thermoquest, Milan, Italy), equipped with a quaternary pump, a Surveyor Model 6000 LP UV/VIS diode array programmable detector operating at 280 nm, a Surveyor AS autosampler, a vacuum degasser and Xcalibur Software. Components were separated with a Phenomenex Synergy RP80 A column (150 mm × 2 mm i.d., particle size 4 μm) protected with a Max-RP guard column (4 mm × 2 mm i.d. particle size 4 μm). Gradient elution: 100% solvent A [H_2O, 0.1% HCOOH] to 60% B [CH_3CN, 0.1% HCOOH] in 60 minutes, followed by re-equilibration. Flow rate 0.2 mL/min.

HPLC–ESI–MS analysis was done with a Thermo Finnigan LCQ Advantage ion trap mass spectrometer (Thermoquest, Milan, Italy). The ESI/MS source was set as follows: capillary temperature 220 °C; spray voltage 4.5 kV; capillary voltage 10 V (positive ion mode) or −3 (negative ion mode); sheath gas flow rate 2 L/min; auxiliary gas flow rate 5 L/min. Spectra were detected in positive and negative ion mode (m/z 100–1000, 0.5 scan/s).

2.5. ESI–MS/MS

For the single ion monitoring (SIM) acquisition, the gradient was modified as follows: 100% solvent A to 60% B in 40 min, followed by re-equilibration.

For ESI–MS/MS experiments the relative collision energy was set at 30% (optimized using the LCQ-Xcalibur software), the isolation width at m/z 1, with and helium as collision gas.

2.6. NMR analysis

NMR spectra were obtained on a Varian Mercury VX 300 spectrometer (Varian, Milan, Italy). Ten milligrams of EGCG were dissolved in 600 μL of D_2O (pH 8, 10 mM phosphate buffer). Spectra were acquired at t = 0, 30 and 60 min using the pulse standard sequence and parameters of the Varian pulse programs library.

2.7. Statistical analysis

All data were expressed as mean ± S.D. of four independent experiments. Prism software (GraphPad Inc., San Diego, CA, USA) was used for Student's t-test and the differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. HNE consumption (HPLC)

Under the physiological conditions of the lower intestinal tract (pH 8, 37 °C), EGCG (1.0–3.5 mM) dose- and time-dependently quenched 50 μM HNE (Fig. 1), a concentration of the carcinogen close to that reached during the progression of the inflammatory burst that precedes the development of cancerous colorectal lesions [7]. Both C and EC were 70% less active (data not shown). Similarly, an aqueous infusion extract of commercial green tea (dry leaves, total catechins 30%, w/w), equivalent to 3.5 mM EGCG, efficiently removed HNE, with quenching values similar to those obtained with the same concentration of EGCG standard, at all observation times.

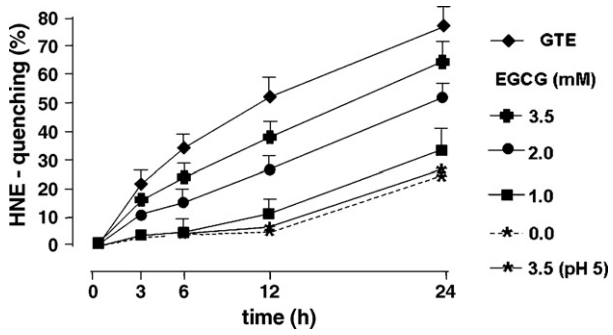

Fig. 1. Trapping of HNE (50 μM) by: (i) EGCG at different concentrations (pH 8), and by (ii) an aqueous extract of commercial green tea (GTE, 1.5 g/100 mL, 95 °C, 5 min) and of (iii) EGCG 3.5 mM at pH 5.

The HNE-trapping activity of EGCG was strictly pH-dependent: at acidic pH there was no quenching of the aldehyde (Fig. 1), indicating that HNE removal requires the formation of the anion and/or the radical-anion form of EGCG.

3.2. Role of hydrogen peroxide (H_2O_2)

With neutral or slightly basic pH, EGCG is oxidized by molecular O_2 to form the EGCG radical (EGCG) and superoxide: the O_2^- can react with another EGCG molecule to form EGCG and H_2O_2 [18]. H_2O_2 can react with HNE to form a cytotoxic epoxide derivative (4-hydroxy-2,3-epoxi-nonanal, epo-HNE) [19]. We therefore investigated whether this could be the primary mechanism involved in HNE disappearance in the aerobic conditions used. However, the addition of catalase (10 U.A.) or superoxide dismutase (20 U.A.) to the incubation mixture at pH 7.4 and 8, at different incubation times, did not affect the extent of HNE quenching by EGCG or by the green tea aqueous extract (data not shown), indicating that H_2O_2 is not the causative agent in HNE removal.

This was confirmed by ESI–MS (direct infusion) which specifically targets epoHNE formation. The reaction mixture of HNE and EGCG after 24 h incubation (1:1, 1 mM, pH 8, 37 °C) did not show either epoHNE or the formation of an adduction product between EGCG and epo-HNE, but only a dehydrated adduct between EGCG and HNE. Very likely, in our conditions and in agreement with Chen and Chung's findings [19], the amount of H_2O_2 formed is too small to oxidize HNE to the corresponding epoHNE, and the quenching is due to its covalent conjugation to EGCG.

3.3. HNE conjugation to EGCG

The formation of a EGCG–HNE covalent adduct was established by HPLC–DAD–ESI–MS. Fig. 2 shows a new sharp peak, after 24 h incubation of EGCG and HNE at a 1:1 molar ratio, eluting at 58 min in the UV chromatogram (Fig. 2 panels a and b). At the same retention time, the mass chromatogram (Fig. 2c and d) contained a set of isobaric peaks with m/z 595 $[EGCG + HNE–H_2O–H]^-$, which could be attributed to: (i) the adduction of HNE at different EGCG positions (very likely positions 6 and 8 as in fact was subsequently demonstrated by LC–ESI–MS/MS and ^1H NMR experiments), with formation of structural isomers, and/or: (ii) a set of different stereoisomers of the dehydrated form of the HNE–EGCG adduct. The presence of these isomers can be explained by the fact that EGCG occurs naturally only in the *R*,*R* absolute configuration. The introduction of a racemic side chain due to (*R*,*S*-HNE) can give rise to the formation of additional stereogenic centers which may generate different diastereoisomers.

Fig. 3a illustrates the most likely explanation for the formation of these six peaks: from the dehydration of the EGCG–HNE adduct two geometric isomers may arise, A with two stereogenic centers (four diastereoisomers) and B with one stereogenic center (two diastereoisomers). This pathway was substantiated by the results relating to the reaction in the same conditions between EGCG and ACR, which does not have any stereogenic center.

The products were analyzed by HPLC–MS in SIM acquisition mode, with a gradient program suitably modified to separate the peaks better. Only one predominant peak, attributable to a 1:1 EGCG/ACR adduction product, was detectable in the HPLC–MS chromatogram (Fig. 3b), as reasonably expected from the introduction of an achiral moiety. This was accompanied by minor isobaric species at m/z 515 $[EGCG + ACR + H]^+$, attributable to the adduction of ACR at different positions of the EGCG ring A or to the higher

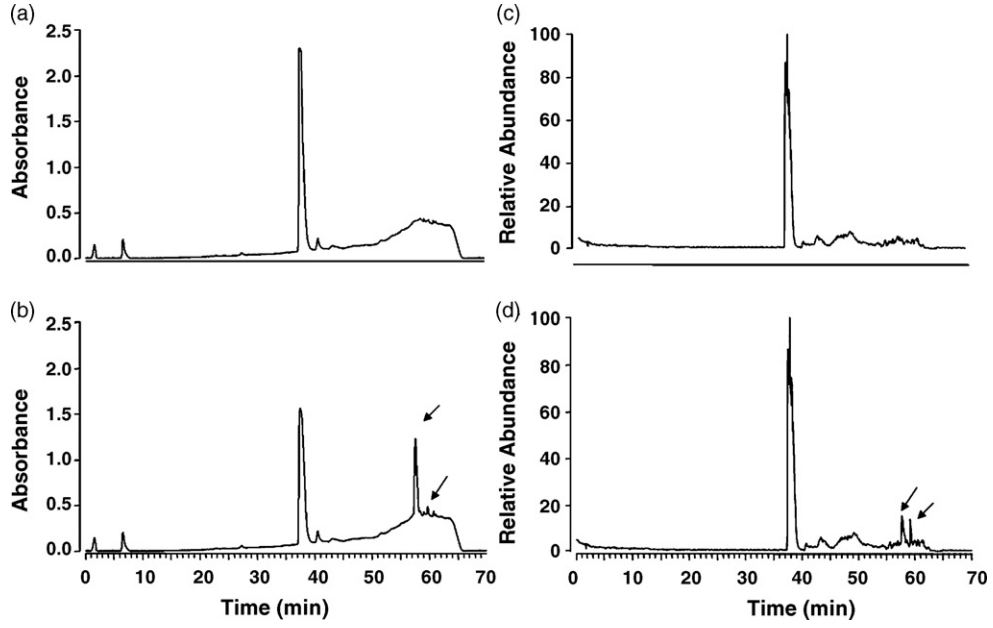

Fig. 2. HPLC–UV–DAD–ESI–MS analysis of EGCG 1 mM ((a) UV and (c) MS chromatograms) and of a 1:1 HNE/EGCG reaction mixture ((b) UV and (d) MS chromatograms) after 24 h incubation. UV, total PDA scan, MS, total ion current.

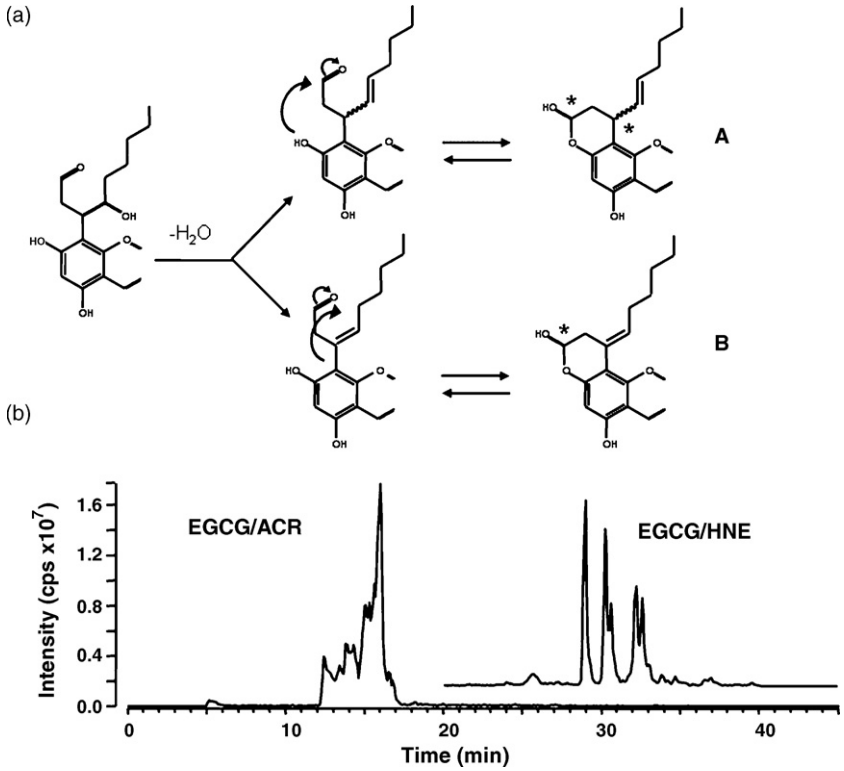

Fig. 3. Formation of the diastereogenic EGCG/HNE adducts A and B (upper panel) and HPLC–ESI–MS analysis (single-ion monitoring mode) of EGCG with (a)) ACR and (b) HNE (lower panel). Molar ratio 1:1, $T = 37\,^{\circ}C$, 24 h incubation, pH 8.

reactivity of ACR at other positions of the molecule (rings C or D).

Besides this information from the ACR experiment we have evidence that EGCG can quench different types of α,β-unsaturated aldehydes.

The same adduct peaks were observed in the UV (Fig. 4a and b) and MS (Fig. 4c and d) chromatograms of the reaction mixture between HNE and the components of an aqueous extract from the commercial green tea sample. This latter was prepared by infusing dry leaves (1.5 g) in 100 mL of distilled water at 90 °C (the common content of a cup of tea). In line with the results of the HPLC study, which showed that EGCG is the most active of the green tea catechins in HNE entrapping, only adducts between HNE and EGCG were detected in the green tea chromatogram, indicating that EGCG plays a primary role in HNE sequestering.

3.4. Structure of the EGCG/HNE adducts, by LC–ESI–MS/MS

To clarify the structures of these main adducts, we used ESI–MS/MS (positive ion mode) to analyze the ions at m/z 597. Fig. 5

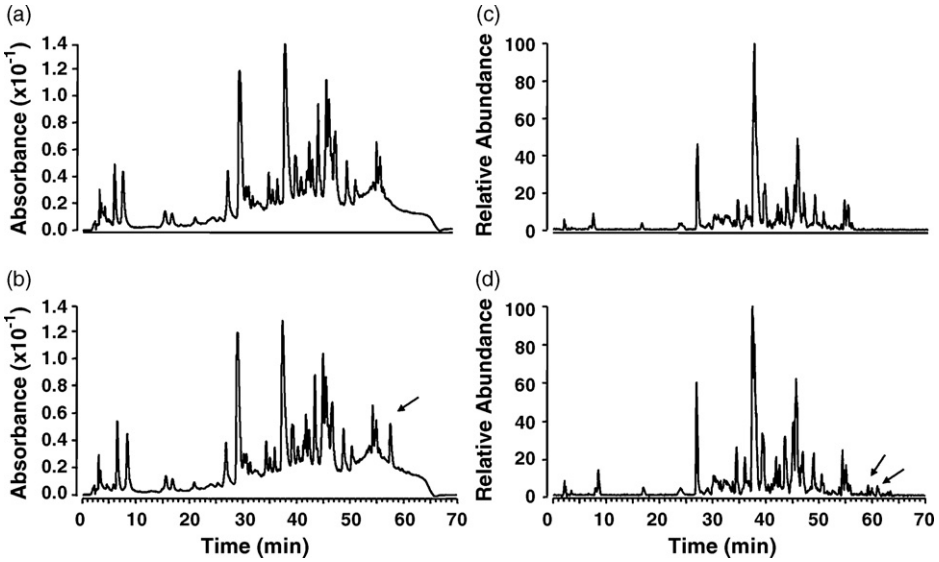

Fig. 4. HPLC–UV–DAD–ESI–MS analysis of GTE 1 mM ((a) UV and (c) MS chromatograms) and of a 1:1 HNE/EGCG reaction mixture ((b) UV and (d) MS chromatograms) after 24 h incubation. UV, total PDA scan, MS, total ion current.

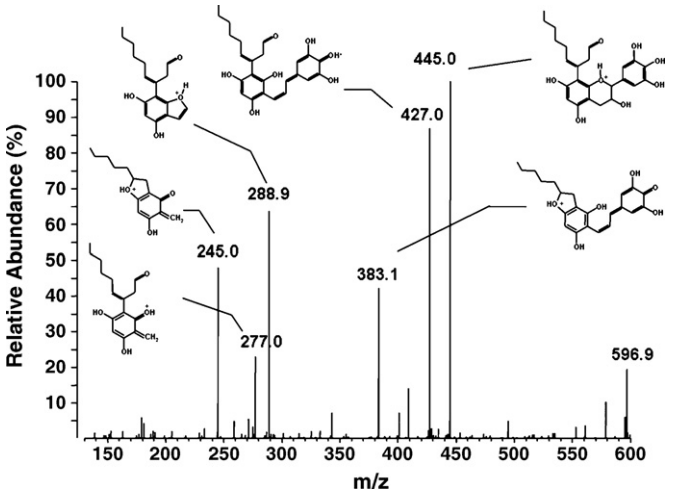

Fig. 5. ESI–MS/MS spectrum of the ion [EGCG + HNE–H_2O + H]$^+$ at *m*/*z* 597 (positive ion mode) and attributions of the main fragment ions.

shows their spectra, with the attribution of the fragment ions. There were several fragment ions: those at *m*/*z* 445, 427 and 383 were due to ions deriving from the loss of the EGCG galloyl moiety followed by the sequential neutral loss of respectively water and acetaldehyde. These ions were diagnostic of a free aldehyde group attached to the side chain of the HNE moiety, suggesting that HNE adduction occurs through a Michael mechanism to the electrophilic C-3 of HNE.

Previous studies on the reaction between HNE and the prototype nucleophilic quencher carnosine showed that the HNE moiety was present in a hemiacetal form, arising from the ring closure between its C-1 carbonyl group and the OH group in C-4 [20]. Surprisingly, in the ESI–MS/MS spectrum of the adduct EGCG–HNE, we did not detect any peak attributable to a retro-Michael fragmentation, strongly suggesting that dehydration of the EGCG–HNE adduct involves the OH group of HNE, thus hampering the hemiacetal formation. These findings highlight EGCG's ability to quench HNE through the formation of a compound with a highly stable C–C bond, a mechanism different from HNE quenching by histidine-containing dipeptides, which involves the C–N bond.

The ions at *m*/*z* 289, 277 and 245, due to the loss of both the galloyl and pyrogallol moieties, indicated that HNE adduction basically involves the ring A of EGCG, and not the less nucleophilic rings C and D. These findings agree with those of Sang et al. who found that EGCG reacted with glyoxal and methylglyoxal at both the C-6 and C-8 positions of ring A [21].

From a physio-pathological view, the ability of green tea components to remove α,β-unsaturated aldehydes arising from lipoxidation, as well as the products generated by glycoxidation (glyoxal, methylglyoxal), extends its chemopreventive window of action, particularly in compartments such as the colon where it can reach higher concentrations even after an acute oral dose (up to 100 μM) [22] than in serum [23].

3.5. NMR analysis of EGCG in D_2O: role of EGCG tautomerism

The specificity of HNE adduction to ring A of EGCG was explained by the ^{1}H NMR analysis of EGCG dissolved in D_2O at pH 8.0. After only 5 min incubation (Fig. 6a), a fast hydrogen-deuterium (H/D) exchange of its C-6 and C-8 attached hydrogens occurred with almost identical rates of disappearance of the signals (practically complete after 30 min incubation, Fig. 6b). This exchange was much faster than that seen at the same times for the hydrogens of C-2 (ring B), C-2′ and C-3′ (ring C), and C-2″ and C-3″ (ring D), suggesting that

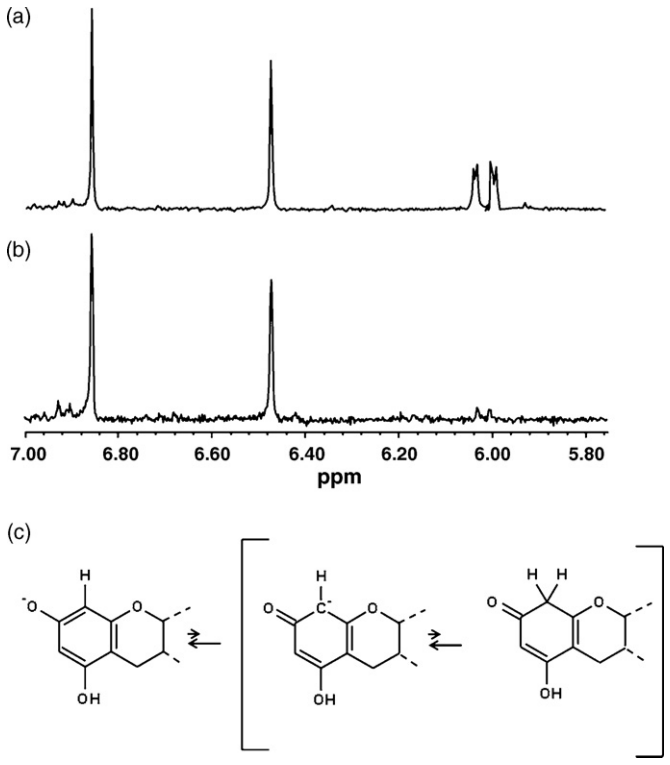

Fig. 6. ^{1}H NMR analysis of EGCG dissolved in D_2O (pH 8). Expanded spectral region at (a) 5 min and (b) 60 min of incubation at 37 °C.

in an aqueous environment EGCG is present as a tautomeric pair, as shown in Fig. 6c. This equilibrium involves the carbanionic form of dissociated EGCG, with the negative charge equally distributed between positions C-6 and C-8. Thus, at physiological pH, the EGCG ring A bears two activated nucleophilic centers which could explain the higher reactivity of EGCG toward HNE at slightly basic pH.

4. Conclusions

These findings show – to our knowledge for the first time – that green tea *in toto* and its main constituent EGCG potently and efficiently remove toxic and mutagenic α,β-unsaturated aldehydes generated from oxidation of diet-derived PUFAs, HNE being one of the most abundant. We do not know to what extent this mechanism plays a role in its chemoprotective action *in vivo*, but the finding is of outstanding interest.

What stems from this study is that a pro-apoptotic effect due to H_2O_2 liberation can be operative under most cell culture conditions, but it can be definitely excluded *in vivo,* considering that, in physiological conditions the partial pressure of oxygen is around 1 or 2 mmHg in the intestinal tract [24], so only trace amounts of H_2O_2 from EGCG auto-oxidation can form. For the same reason we can exclude any cytotoxic action of the metabolite epoHNE against tumor cells. We can also assume that in the colorectal lumen it is the anionic form of EGCG that predominates, and this may be the species that intervenes in quenching ROS and carbonyl species.

Extrapolation of these findings to an *in vivo* situation, with a view to understanding the mechanism of the chemoprotective action of green tea, can be difficult and questionable. However, if we exclude a mechanism based on H_2O_2 liberation *in vivo*, the finding that green tea catechins have strong removing ability for α,β-unsaturated aldehydes helps provide an explanation in addition to those previously proposed for the beneficial effects of green tea in colorectal cancer prevention.

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