JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Influence of Phenolic Compounds on the Mechanisms of Pyrazinium Radical Generation in the Maillard Reaction

Qing Bin,[†,§,⊥] Devin G. Peterson,[§] and Ryan J. Elias*[,†]

[†]Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

[§]Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108, United States

ABSTRACT: The generation of pyrazinium radical cations during the early stages of the Maillard reaction has been previously demonstrated. In this study, the effect of food phenolic compounds [4-methylcatechol (4-MeC), (+)-catechin (CAT), and (−)-epigallocatechin-3-gallate (EGCG)] on the fate of these intermediates in Maillard model systems was investigated. Aqueous solutions containing either glyoxal + alanine (GO-A) or glycolaldehyde + alanine (GA-A) were treated with a concentration gradient of each phenolic compound, and quantitative analysis of the resulting pyrazinium radicals in these models was performed using electron paramagnetic resonance (EPR) spectroscopy. CAT and EGCG were observed to affect pyrazinium radical generation rates, in some cases either enhancing or suppressing formation depending on concentration, whereas the simple catechol (4-MeC) had no such effect. A mechanistic study was carried out by LC-MS, which suggested that under some conditions, CAT and EGCG react with imine intermediates via their A-rings, thus influencing the formation of the enaminol radical precursor and, ultimately, pyrazinium radicals. To the authors' knowledge, this is the first study demonstrating imine trapping by phenolic compounds under Maillard conditions and how such phenolic quenching reactions can alter pyrazinium radical formation.

KEYWORDS: *Maillard reaction, pyrazinium radical, glyoxal, glycolaldehyde, glycation, reactive imine trapping, catechins, electron paramagnetic resonance*

INTRODUCTION

The Maillard reaction, often referred to as nonenzymatic browning, refers to the classical reaction between a reducing sugar (carbonyl) and an amino acid (amine) and is an important reaction mechanism in food and regulatory biology.[1] Under food conditions, this reaction is well-known to alter color and flavor, as well as yielding biologically active compounds. The generation of Maillard products in food may be desirable (e.g., the aroma of bakery products, coffee) or undesirable (e.g., discoloration, off-flavor development). Because of its importance to food quality, this reaction has been the subject of intense study for decades; however, given the complexity of this reaction and the difficulty associated with characterizing many Maillard products, in particular, high molecular weight products, many mechanistic aspects of this reaction are unknown. In biological systems, the Maillard reaction has also been studied in relation to pathology associated with aging and diabetes.[2]

In recent decades, the presence of radical intermediate species has been confirmed in the early stages of the Maillard reaction.[3] These species were identified as pyrazinium radical cations using EPR, which appear to be key precursors in subsequent Maillard pathways. Namiki and Hayashi proposed a mechanism (Figure 1) by which this radical is formed and offered evidence that it involved the generation of a glycolaldehyde imine in equilibrium with its enaminol tautomer, which is dimerized and oxidized to a resonance-stabilized 1,4-dialkylpyrazinium radical cation. The authors suggested that the glycolaldehyde imine was formed either from the fragmentation of a Schiff base or by glycolaldehyde−amino acid condensation. Glycolaldehyde (GA) was reported to be the most effective reactant in yielding pyrazinium radicals. More recently, Hofmann and co-workers conducted a series of time course studies that investigated color changes accompanying pyrazinium radical formation during the early stage of the Maillard reaction.[4] It was demonstrated that glyoxal (GO) is generated in abundance in advance of radical formation. Furthermore, GA concentration was seen to increase as a function of reductone concentration, leading to increases in observed radical yields, which was presumably due to enhanced reduction of GO to GA. In the same study, the role of the pyrazinium radical cations in the context of color formation was investigated through the use of a 1,4-diethylpyrazinium diquaternary salt. The authors proposed a mechanism involving transformation of the pyrazinium radical cations into the hydroxylated 1,4-dialkyl-1,4-dihydropyrazines via those diquat intermediates, leading to nonenzymatic browning.[4]

Despite the important role of pyrazinium radical cations as browning precursors, the factors affecting their generation and fate in foods are largely unknown. In particular, little attention has been given to the effect that other food components (e.g., phenolic compounds) have on pyrazinium radical generation. Recent studies by Peterson and Totlani demonstrated that Maillard pathways can be affected by (−)-epicatechin.[5] The generation of several volatile compounds (e.g., pyrazines, furans) in both model systems and bakery products was inhibited in the presence of this compound. It was proposed

Received: March 12, 2012
Revised: May 9, 2012
Accepted: May 9, 2012
Published: May 9, 2012

dx.doi.org/10.1021/jf3010838 | J. Agric. Food Chem. 2012, 60, 5482−5490

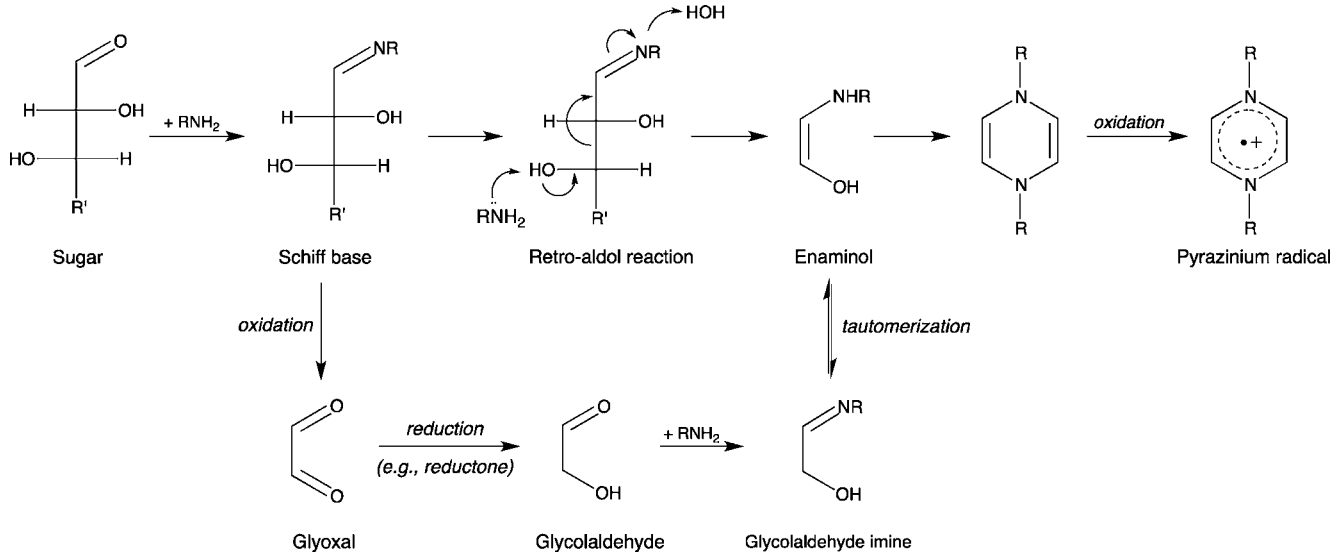

Figure 1. Mechanism of pyrazinium radical cation formation in the Maillard reaction.[3,4]

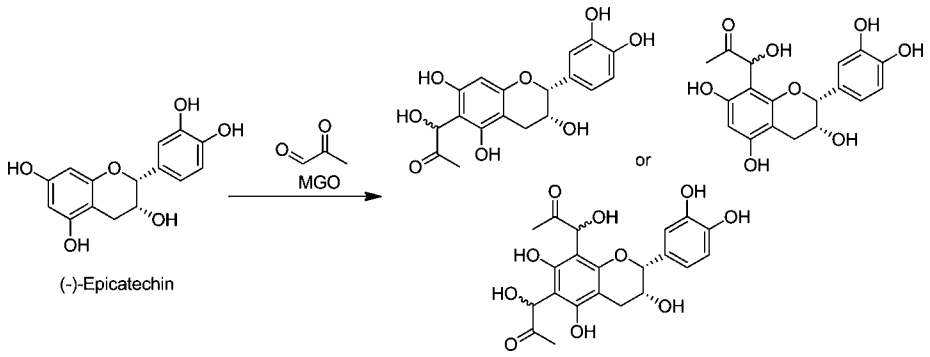

Figure 2. (−)-Epicatechin−carbonyl-trapping reaction and formation of EC−MGO adducts.[7]

that (−)-epicatechin behaves as a trapping agent of carbonyl-containing sugar fragments under such conditions. Isotopic (C^{13}) labeling studies provided evidence of the trapping of C_2 (e.g., glyoxal), C_3 (e.g., methylglyoxal, glyceraldehyde), and C_4 (e.g., erythrose) sugar fragments by (−)-epicatechin, and the formation of these EC−sugar fragment adducts was reported.[6] The structure of the EC−methylglyoxal (MGO) adduct was elucidated by 2D nuclear magnetic resonance (NMR), revealing that the mechanism of trapping was a classical electrophilic aromatic substitution reaction occurring at the highly activated A ring (Figure 2).[7] Trapping of reactive dicarbonyls by catechins has also been studied under physiological conditions. In a subsequent study, Sang and co-workers reported trapping of MGO and GC by EGCG under model physiological conditions.[8] Furthermore, trapping of aldehydes (e.g., glyoxylic acid, acetaldehyde, benzylaldehyde) by (−)-epicatechin in wine has also been reported.[9,10]

It was hypothesized that carbonyl-trapping reactions by food phenolic compounds affect pyrazinium radical formation in the early stages of the Maillard reaction. GO and GA are thought to be the key C_2 fragments involved in pyrazinium radical formation (Figure 1) and, as such, have been studied extensively as important carbonyls connecting many pathways of the Maillard reaction. In the present study, aqueous GO-A and GA-A Maillard models were used to investigate how phenolics affect the mechanism of pyrazinium radical formation.

MATERIALS AND METHODS

Chemicals. Glyoxal (40 wt % solution in water), glycolaldehyde dimer, D-glucose, L-alanine, (−)-epigallocatechin, (+)-catechin hydrate, 4-methylcatechol, and (−)-epigallocatechin gallate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and solvents were of reagent or HPLC grade. Water was purified through a Barnstead Nanopure Diamond water purification system (Thermo Scientific, Dubuque, IA, USA).

Detection of Pyrazinium Radical Cations in Maillard Models with EPR. Solutions consisting of glyoxal (0.2 M) and L-alanine (0.5 M phosphate) or glycolaldehyde (0.1 M) and L-alanine (0.5 M) in phosphate buffer (0.1 M, pH 7.0) were treated with various concentrations of 4-MeC, CAT, or EGCG. Solutions were deoxygenated with nitrogen gas in their respective reaction vessels, and sample aliquots (50 μL) were transferred to, and incubated (25 °C) in, sealed glass micropipets (Brand GmbH, Wertheim, Germany). The EPR spectra of pyrazinium radicals were recorded at regular intervals over the course of 200 h on a Bruker eScan X-band spectrometer (Bruker BioSpin, Billerica, MA, USA). The experimental parameters were as follows: modulation amplitude, 0.69 G; sweep time, 2.62 s; frequency, 9.78 GHz; gain, 1×10^3. EPR calibration was performed using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (5 μM). All EPR experiments were performed in duplicate.

Sample Preparation for LC-MS Analysis. Glyoxal (0.2 M) and L-alanine (0.5 M) or glycolaldehyde (0.1 M) and L-alanine (0.5 M) solutions were prepared in deoxygenated phosphate buffer (0.1 M, pH 7.0) and treated with EGCG (10 or 100 mM). After 1.5 h of incubation (at 25 °C), an aliquot (0.5 mL) of the reaction mixture was loaded on a 500 mg C18 cartridge (Supelco, Bellefonte, PA, USA),

dx.doi.org/10.1021/jf3010838 | J. Agric. Food Chem. 2012, 60, 5482−5490

which was preconditioned with 5 mL of methanol and then 5 mL of water. The cartridge was washed with 2 mL of water and then eluted with 2 mL of methanol. The isolate was subsequently filtered through a 0.20 μm nylon syringe filter (Millex, Billerica, MA, USA) using a 1 mL syringe (Millipore, Bedford, MA, USA) prior to LC-MS analysis.

LC-MS Analysis. Sample analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) interfaced with a Micromass Q-TOF Micro mass spectrometer (Waters, Milford, MA, USA). The HPLC system consisted of a binary pumping system (G1312A), a degasser (G1322A), an autosampler (G1313A), a column compartment (G1316A), and a diode array UV−vis detector (G1315A). An injection volume of 10 μL was used, and samples were separated on an Ascentis Express C18 column (2.1 mm × 150 mm, 2.7 μm) (Supelco) at 35 °C. The mobile phase was maintained at a flow rate of 250 μL/min using a binary solvent system of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The elution gradient started at 10% B (0−2 min), linearly increased to 50% B (2−23 min), linearly increased to 100% B (23−30 min), was maintained at 100% B (30−32 min), decreased to 10% B (32−35 min), and was finally maintained at 10% B (35−50 min). Analytes were detected using an inline Q-TOF Micro mass spectrometer equipped with a LockSpray dual exact mass ionization source inlet system, consisting of two electrospray probes for the co-introduction of analyte and lock mass reference compound. The source was operated at ES^- mode with the following conditions: source temperature, 100 °C; desolvation temperature, 300 °C; capillary voltage, 3.0 kV; cone voltage, 30 V. An internal reference, reserpine (100 mg/L delivered at 50 μL/min), was used to obtain exact mass measurements. The reference spray was sampled at an interval of 10 s. Accurate mass measurement was conducted in MS mode (ranging from 50 to 1100 Da) with a scan time of 1.0 s. Structural information of analytes was obtained in MS/MS mode with the collision energy set between 15 and 20 V.

RESULTS AND DISCUSSION

Effect of 4-MeC, CAT, and EGCG on Pyrazinium Radical Formation in a GO-A Model. The effect of the

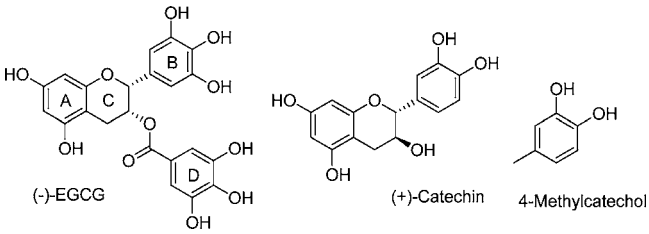

Figure 3. Structures of (−)-EGCG, (+)-catechin, and 4-methylcatechol.

three phenolic compounds 4-MeC, CAT, and EGCG (Figure 3) on the generation rate of pyrazinium radicals in a GO-A model system was studied by EPR. GO is a simple dicarbonyl that is known to be generated during the early stages of the Maillard reaction and is a known precursor to advanced browning products.[11] The influence of EGCG on radical formation in the GO-A model is shown in Figure 4a. In the absence of EGCG, an EPR spectrum characteristic of the pyrazinium radical cation was observed almost immediately, with observed hyperfine coupling constants, in agreement with previous studies.[3] Overall, radical intensity remained low throughout the course of the experiment (200 h), which was anticipated due to the fact that GO was previously observed not to yield high levels of pyrazinium radicals;[3] however, high radical yields were observed once GO was converted to GA by reductones.[4] Radical intensity was observed to increase as a function of EGCG concentration (5−20 mM), with maximum

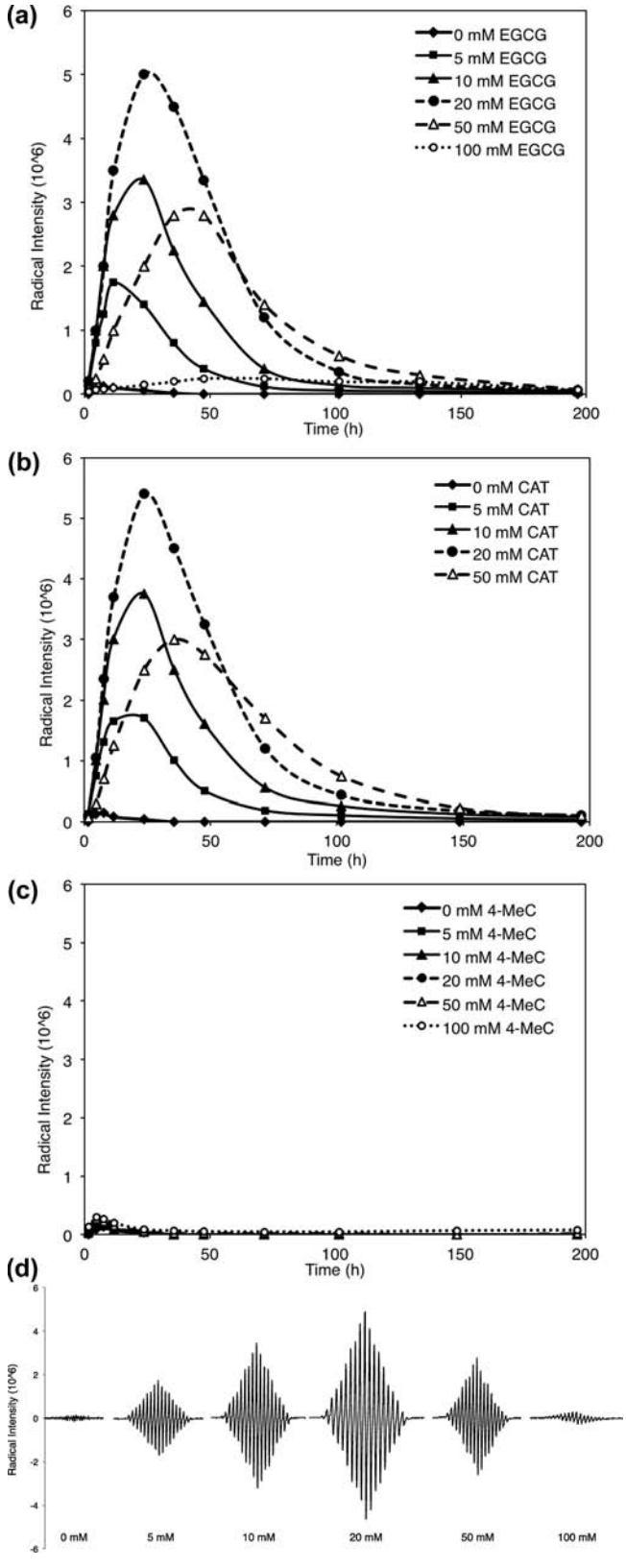

Figure 4. Effects of (a) EGCG, (b) CAT, and (c) 4-MeC on the generation of pyrazinium radical in the GO-A aqueous model at 25 °C; (d) EPR spectra showing the effect of EGCG on radical formation in the GO-A model.

radical yield observed at 20 mM ([GO]:[EGCG] = 10:1). However, the intensity of the pyrazinium radical signal began to

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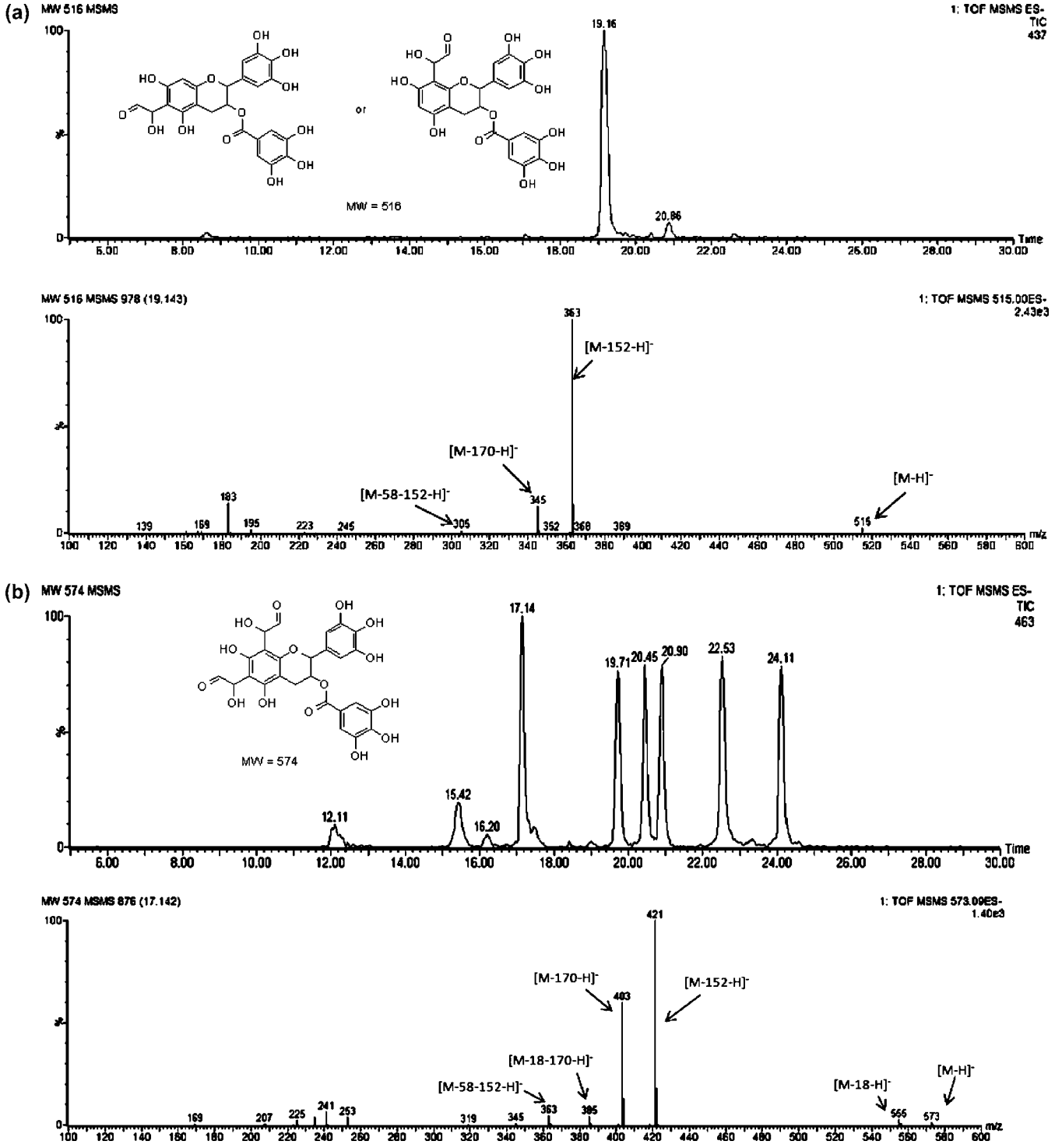

Figure 5. LC-MS/MS chromatogram and spectrum of (a) monosubstituted and (b) disubstituted EGCG−GO adducts generated from the GO-A−EGCG model.

decrease in the presence of EGCG at concentrations equal to, and exceeding, 50 mM. Figure 4d depicts the EPR spectrum of the pyrazinium radical observed in the system described above and related dose response. Substituting CAT for EGCG resulted in a similar trend (Figure 4b), with radical intensity increasing with CAT concentrations up to, and including, 20 mM CAT, and signal suppression observed at 50 mM (100 mM CAT was not included in the present study due to solubility restrictions in the model system). However, this trend was not seen for 4-MeC (Figure 4c), suggesting that catechol or gallate groups were not responsible for this effect. Furthermore, given the similarity between the CAT and EGCG treatments with respect to observed radical intensities, it was hypothesized that the A-rings of these two compounds were the reactive sites for carbonyl trapping, as previously reported by Noda and Peterson.[12]

Identification of Key Intermediates in the GO-A−EGCG Model. To investigate the effect of phenolic compounds

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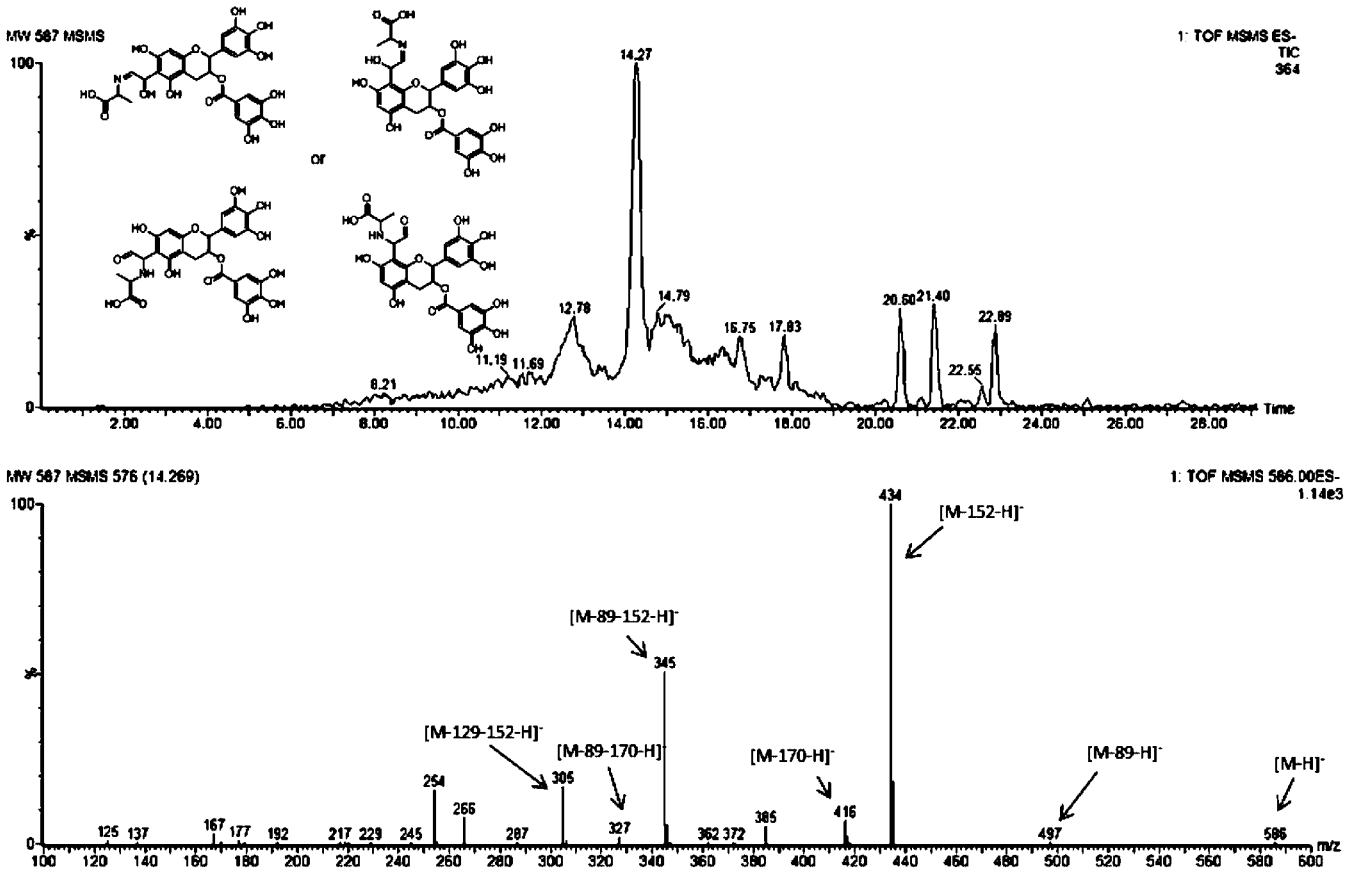

Figure 6. LC-MS/MS chromatogram and spectrum of analyte MW 587 (586 $[M - H]^-$) generated from the GO-A–EGCG model.

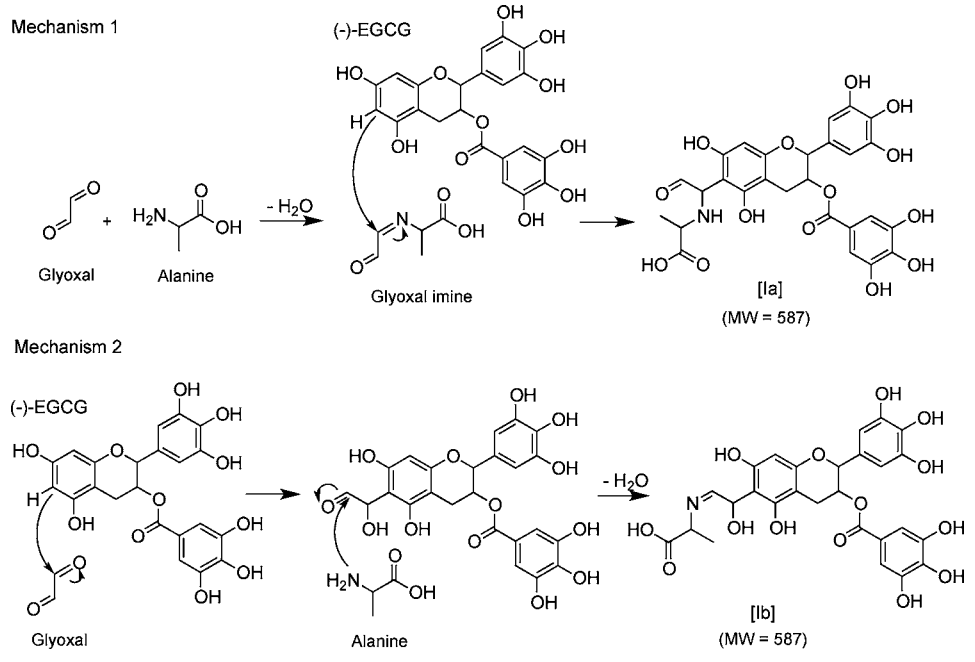

Figure 7. Two possible mechanisms for the formation of analyte MW 587 (**Ia** and **Ib**) in the GO-A–EGCG model. Conjugation is predicted also to occur at position 8 of EGCG (not shown).

in our model systems on pyrazinium radical formation, a link between the precursors of pyrazinium radicals (GO and GA) in our models and the previously reported ability of the A-ring of catechins to covalently trap these sugar fragments[7,8] by electrophilic aromatic substitution reactions was explored. LC-MS analysis of related phenolic–sugar fragment products in the GO-A Maillard model containing low and high concentrations of EGCG (10 and 100 mM) was conducted. When the EGCG concentration was held at 10 mM, several major pseudomolecular ions were observed by LC-MS. On the

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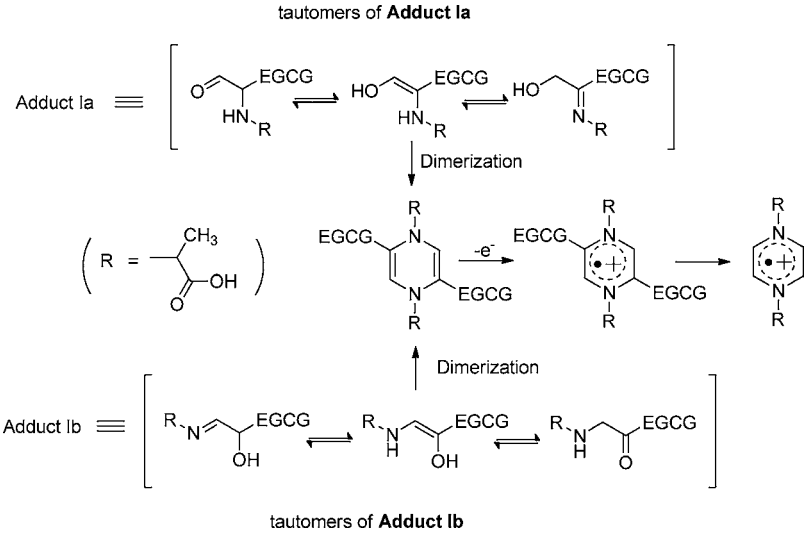

Figure 8. Proposed mechanism for the effect of low EGCG concentration on pyrazinium radical formation in the GO-A model. EGCG conjugation products **Ia** and **Ib** are predicted to dimerize to form pyrazinium radicals given by their sp^2 orbital hybridization.

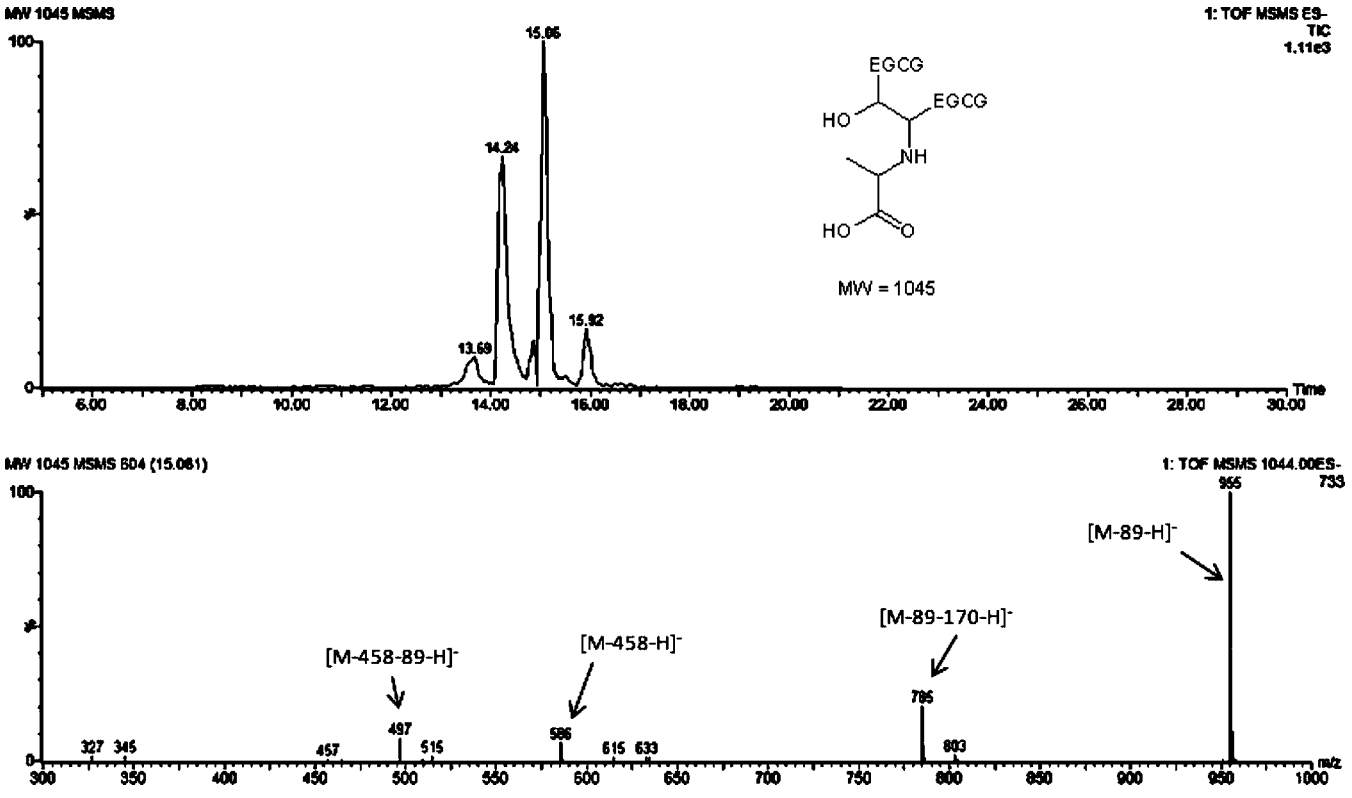

Figure 9. LC-MS/MS chromatogram and spectrum of analyte MW 1045 (1044 $[M - H]^-$) generated from the GO-A–EGCG model.

basis of the observed masses, reaction product structures were predicted, which were further supported by MS/MS fragmentation patterns. Panels a and b of Figure 5 illustrate the pseudomolecular ions 515 $[M - H]^-$ and 573 $[M - H]^-$ detected and supported as mono-GO and di-GO adducts of EGCG, respectively. These reaction products were in agreement with other studies wherein the same products were observed in both food systems[7] and under biological conditions.[8]

In addition to GO adducts, glyoxal imine adducts of EGCG were also reported (Figure 6) and supported by the fragmentation ions of its sibling scan: fragments 434 $[M - 152 - H]^-$ and 416 $[M - 170 - H]^-$ indicate the neutral loss of one galloyl group (MW 152) and one gallic acid group (MW 170) on the gallate ring, respectively; fragment 497 $[M - 89 - H]^-$ indicates the loss of an alanine molecule (MW 89); fragment 305 $[M - 129 - 152 - H]^-$ indicates the loss of a glyoxal imine molecule (MW 129) and a galloyl group (MW 152). The predicted structure of this analyte was further supported by accurate mass measurement with a reported molecular mass of 586.1199 Da and a predicted formula of $[C_{27}H_{24}NO_{14}]^-$ (0.3 ppm). On the basis of the product structure, two possible mechanisms are proposed for the formation of this adduct, illustrated in Figure 7. In mechanism

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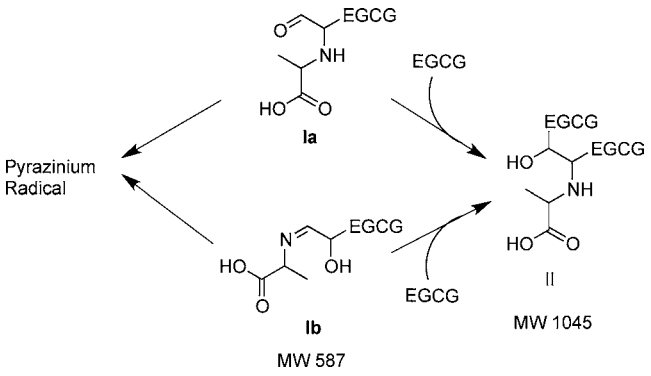

Figure 10. Proposed mechanism of the generation of analyte MW 1045 (**II**) and the effect of high EGCG concentration on pyrazinium radical formation in the GO-A model.

1, this product was generated by the reactive intermediate, glyoxal imine, formed from the initial condensation between glyoxal and alanine. EGCG appears to trap this intermediate through electrophilic aromatic substitution on the A ring to form the MW 587 adduct (**Ia**). In mechanism 2, GO is more reactive toward EGCG and forms the EGCG−GO adduct, which further reacts with the amino group of alanine using the aldehyde group to form adduct **Ib**. Both adducts **Ia** and **Ib** provide a basis to explain the enhancement of pyrazinium radical formation by EGCG in the GO-A model (Figure 8). Both structures have the trigonal planar conformation for the N−C−C moiety (i.e., the moiety forming the pyrazine ring structure) that freely tautomerize and easily dimerize and, subsequently, oxidize to generate the same pyrazinium radical cations consisting of a stable π-system. EGCG is predicted to be liberated from the radical's pyrazine ring and, once liberated, is available to serve as a catalyst for further reactions. The hyperfine structure of the pyrazinium radical from the EPR spectra did not change with EGCG addition, and the enhancement of radical formation increased with EGCG concentration up to 1:10 (EGCG:GO). Other than the MW 587 adduct, a disubstituted EGCG−imine adduct (6- and 8-positions substituted by a glyoxal imine and a GO) was also observed as a minor product.

In the presence of higher EGCG concentrations, the reverse trend was observed with a decrease in radical intensity observed at concentrations above 1:2 (EGCG:GO) (Figure 4a). We hypothesize that when EGCG concentrations are relatively high, the MW 587 adduct is more likely to form novel reaction products (e.g., the GO-EGCG−EGCG adduct) at the expense of forming pyrazinium radical compounds. To further investigate this possibility, the GO-A model was incubated in the presence of 100 mM of EGCG and analyzed by LC-MS. A comparable amount of molecular ion 1044 $[M - H]^-$ was detected, which was not observed (or observed at only low levels) in the low EGCG system. MS/MS analysis of this 1044 $[M - H]^-$ ion (Figure 9) revealed the major fragmentation ions and structural information as follows: fragment 586 $[M - 458 - H]^-$ indicates the loss of an EGCG molecule (MW 458); fragment 955 $[M - 89 - H]^-$ indicates the loss of an alanine molecule (MW 89); fragment 785 $[M - 89 - 170 - H]^-$ indicates the loss of a gallic acid molecule (MW 170) and an alanine molecule (MW 89); fragment 497 $[M - 458 - 89 - H]^-$ indicates the loss of an EGCG (MW 458) and an alanine molecule (MW 89). This compound was predicted to be a glyoxal imine di-EGCG adduct (**II**), which was further

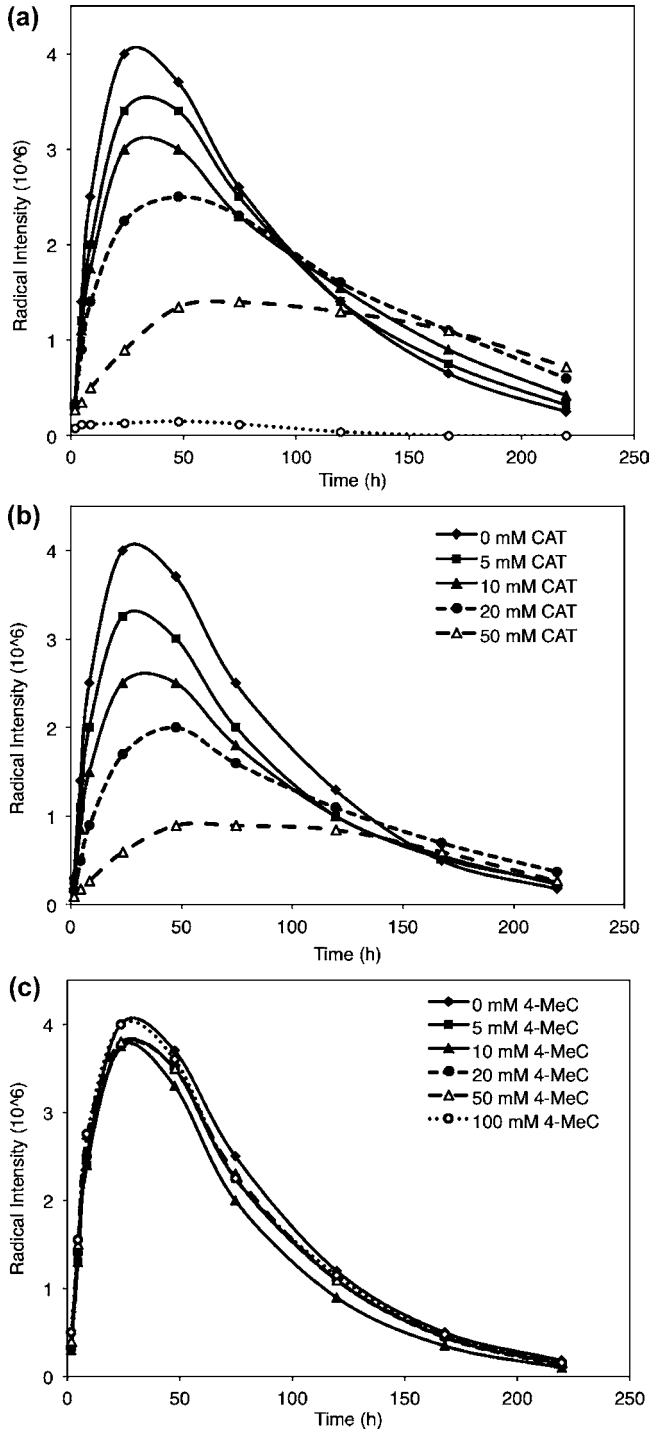

Figure 11. Effects of (a) EGCG, (b) CAT, and (c) 4-MeC on the generation of pyrazinium radical in the GA-A aqueous model at 25 °C.

supported by accurate mass measurement giving a molecular mass of 1044.2094 Da and a predicted formula of $[C_{49}H_{42}NO_{25}]^-$ (4.1 ppm). The proposed mechanism for the formation of adduct **II** is shown in Figure 10. At higher EGCG concentrations, the MW 587 adduct (**Ia** or **Ib**) is predicted to react more readily with other EGCG molecules to form adduct **II**. Once bound to another EGCG molecule, it is proposed that the MW 587 adduct loses its reactivity and its sp^2 planar conformation required to form pyrazinium radicals. Therefore, at high concentrations of EGCG, the phenolic quenches the radical precursors by disarming their chemical reactivity (i.e.,

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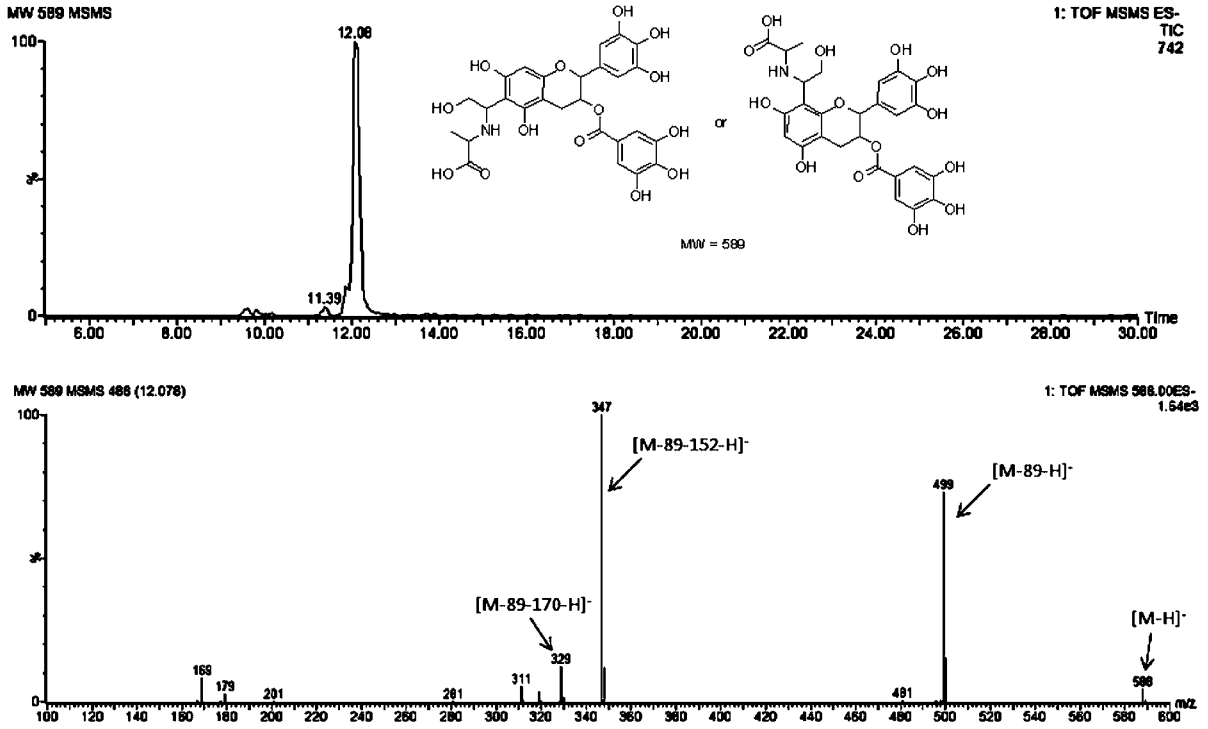

Figure 12. LC-MS/MS chromatogram and spectrum of analyte MW 589 (588 $[M - H]^-$) generated from the GA-A–EGCG model.

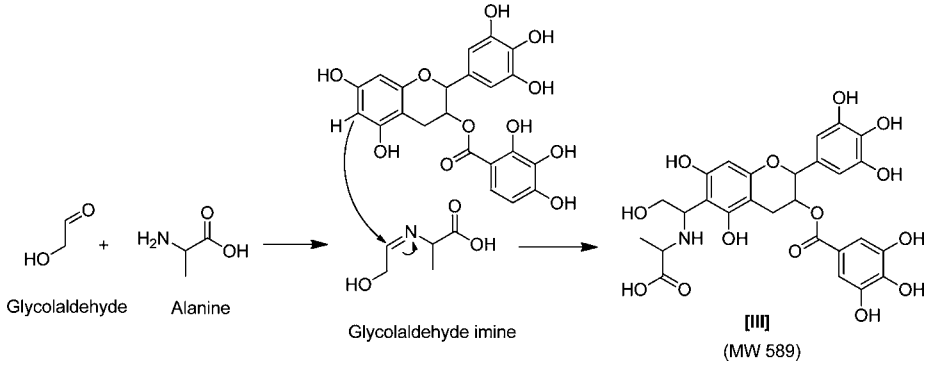

Figure 13. Proposed mechanism of generation of analyte MW 589 (**III**) and the inhibitory effect of EGCG on pyrazinium radical formation in the GA-A model. Conjugation is predicted also to occur at position 8 of EGCG (not shown).

conformation required for the production of pyrazinium radicals). This proposed mechanism explains the suppression of pyrazinium radical intensity observed when concentrations of EGCG/CAT are high relative to GO.

Effect of 4-MeC, CAT, and EGCG on Pyrazinium Radical Formation in a GA-A Model. The effect of the model phenolic compounds 4-MeC, CAT, and EGCG on the generation rate of pyrazinium radicals in a GA-A model was also investigated by EPR. Glycolaldehyde (GA) is the smallest hydroxyl carbonyl that is known to be generated from sugar fragmentation during the early stages of the Maillard reaction[3] and has been reported to efficiently generate pyrazinium radicals via condensation with amino acids, yielding the direct radical precursor, enaminol (Figure 1). The GA-A model was treated with the test phenolic compounds at concentrations ranging from 0 to 100 mM (Figure 11a). It was observed that both EGCG and CAT significantly inhibited pyrazinium radical formation in the GA-A model and that the radical intensity declined as a function of EGCG/CAT concentration. 4-MeC, again, showed no such effect under these conditions.

Identification of Key Intermediates in the GA-A–EGCG Model. LC-MS analysis of the GA-A Maillard model containing EGCG was performed, which revealed a pseudomolecular ion of 588 $[M - H]^-$ (Figure 12). Fragmentation ions and structural information obtained from MS/MS were as follows: fragment 499 $[M - 89 - H]^-$ indicates the loss of an alanine molecule (MW 89); fragment 347 $[M - 89 - 152 - H]^-$ indicates the loss of an alanine (MW 89) and a galloyl group (MW 152). The structure of this compound was predicted to be the glycolaldehyde imine adduct of EGCG (**III**) that was further supported by accurate MS analysis with a reported molecular mass of 588.1371 Da and the predicted formula $[C_{27}H_{26}NO_{14}]^-$ (3.1 ppm). In addition to adduct **III**, the disubstituted EGCG–imine adducts (6- and 8-positions substituted by two glycolaldehyde imine molecules or by imine + GA) were also observed as minor products in the GA-A–EGCG model. Similar to adduct **I**, the formation of adduct **III** (Figure 13) was proposed to occur via the reactive intermediate, glycolaldehyde imine (by condensation between GA and alanine), which is further trapped by EGCG to give

dx.doi.org/10.1021/jf3010838 | J. Agric. Food Chem. 2012, 60, 5482–5490

adduct **III**. Glycolaldehyde imine was reported to be a reactive precursor in the generation of pyrazinium radicals, through tautomerization (to enaminol) and dimerization.[3] Once bound to EGCG, the imine loses its sp^2 orbital hybridization to tautomerize to enaminols and, thus, its ability to form pyrazinium radicals. Hence, as observed in the EPR spectra above, in the presence of EGCG (or CAT), pyrazinium radical formation was suppressed in the GA-A Maillard model.

In summary, catechins were reported to affect the formation rates of pyrazinium radicals in both GO-A and GA-A models, presumably through trapping of electrophilic imine intermediates. This mechanism is proposed to either catalyze the radical generation or function as a quenching agent of the radical precursor enaminol depending on the sugar fragment involved (hydroxycarbonyl vs dicarbonyl sugar fragment). Carbonyl trapping by phenolics was, for the first time, reported as a mechanism to alter reactive imine intermediates linked to pyrazinium radical formation in Maillard systems. As pyrazinium radical formation is linked to nonenzymatic browning, these findings provide key insights into the effect that phenolics have on the Maillard reaction, which is of major importance to food quality and, potentially, human health.

AUTHOR INFORMATION

Corresponding Author

*Fax: (814) 863-6132. E-mail: elias@psu.edu.

Present Address

[⊥]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55455.

Notes

The authors declare no competing financial interest.

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