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Reactivity of Epicatechin in Aqueous Glycine and Glucose Maillard Reaction Models: Quenching of C_2, C_3, and C_4 Sugar Fragments

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Mechanisms of how epicatechin alters the pathways of the Maillard reaction were investigated. Carbon-13 and nitrogen-15 labeling studies were utilized to define the reactivity of epicatechin with glucose, glycine, and/or reaction products in an aqueous model (pH 7, 125 °C for 30 min) via GC, GC/MS and HPLC/MS analysis. Quantification of the volatile reaction product isotopomers by GC/MS from a 1:1 labeled to unlabeled glucose (carbohydrate module labeling technique) plus glycine model system indicated the formation of 2,3-butanedione and acetol were primarily formed via intact C_4 and C_3 sugar fragments, whereas pyrazine, methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and cyclotene were primarily formed via intact C_2/C_2, C_2/C_3, C_3/C_3, C_3/C_3, and C_3/C_3 sugar fragment pairs, respectively. The formation of these seven compounds was also reported by GC analysis to be dramatically inhibited when epicatechin was added to the glucose/glycine model system (observed 9−113-fold reduction). HPLC/MS analysis of both the glucose-labeled and glycine-labeled model systems with and without epicatechin indicated that epicatechin reacted directly with C_2, C_3, and C_4 sugar fragments, while epicatechin did not report any direct reactivity with glycine. In conclusion, the quenching of sugar fragmentation products via epicatechin was correlated with the observed inhibition on volatile compound formation when epicatechin was added to a glucose/glycine aqueous reaction model system.

KEYWORDS: Maillard reaction; nonenzymatic browning; inhibit; epicatechin; polyphenolic; sugar fragments; reactivity

INTRODUCTION

The Maillard reaction is a ubiquitous food reaction that occurs during storage, cooking, and heat processing. The complex array of reactions that follow the initial condensation of amino-carbonyl groups result in the formation of Maillard reaction products that are broadly classified as lower molecular weight compounds (carbonyl and heterocyclic) and macromolecules such as melanoidin-brown nitrogenous polymers (*1*−*4*). Ultimately, the formation of these compounds is responsible for several changes in the color, flavor, aroma, nutritional value stability, shelf life, and mutagenic properties of foods, and therefore, control of the Maillard reaction is of critical importance to the food industry as it has a direct impact on the overall quality of processed food (*5*). The classical Hodge sequence (*3*) has been traditionally accepted as the primary mechanism for the formation of Maillard reaction products (*1*). The initial stage of the reaction involves the addition of an amino group to the reactive carbonyl, resulting in the formation of an unstable imine (or glycosylamine) which is subsequently transformed to an Amadori rearrangement product (1-amino-1-deoxy-2-ketose). Amadori products further undergo several reactions including deamination (to form deoxyosones), retroaldol and aldol reactions, dehydration, enolization, and Strecker degradation to form a large number of compounds (*2*); only a small portion of these reactions are responsible for imparting characteristic flavor notes to foods. During the final stages of the reaction, high molecular weight pigments melanoidins (color development) are formed by polymerization of advanced Maillard reaction products.

The mechanisms responsible for the generation of taste and aroma compounds via the Maillard reaction along with color formation have been extensively studied in food and model reaction (sugar−amino acid) systems. Historically, kinetic aspects of the Maillard reaction have focused on time−temperature conditions or matrix parameters such as water activity, pH, sugar type, and nitrogen source (*6*−*9*).

More recently researchers have also focused attention toward the influence of natural antioxidants on the formation of Maillard reaction products. Wang et al. (*10*) reported the presence of phenolic compounds such as caffeic, ferulic, and chlorogenic acids inhibited the formation of aroma compounds such as pyrazines, pyridines, furan derivatives, and Strecker degradation products in coffee model systems consisting of L-leucine,

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L-lysine, and L-cysteine with D-glucose. In cocoa liquors, Counet et al. (*11*) reported a negative correlation between procyanidin contents and aroma development generated during roasting (i.e., pyrazine derivatives). They suggested that, during cocoa fermentation, flavor precursors are developed (i.e., sugar and amino acids) while procyanidins are leached from the beans and therefore the procyanidin content is an indirect indicator of the bean fermentation time or the flavor precursor concentration and ultimately flavor yield during roasting. Losses of up to 50% of the bean procyanidin content can occur as a result of leaching during the fermentation step of cocoa production (*12*). Zak and Keeney (*13*) also reported a negative correlation between roast cocoa bean flavor potential and phenolic content but alternatively suggested that phenolic compounds may bind up protein, rendering them unavailable for enzymatic digestion (reducing key Maillard precursors).

Besides the indirect effects previously suggested for the noted negative correlation between procyanidin content and Maillard-type flavor developed during thermal treatment, Peterson and Totlani (*14*) suggested that the chemical properties of polyphenolic compounds (i.e., epicatechin) may directly influence the mechanisms of the Maillard reaction (function as a key reactant). Using a model aqueous Maillard reaction system (four amino acids and two reducing sugars) as well as two model food systems (cocoa and granola bar), they reported that the addition of flavonoids (i.e., epicatechin and epigallocatechin gallate) altered (inhibited) the formation of Maillard-type aroma compounds (consisting of numerous chemical classes). In a further study, Colahan-Sederstrom and Peterson (*15*) demonstrated that addition of epicatechin to UHT milk prior to processing could be applied to dramatically reduce the thermal generation of key aroma compounds such as methional, furfural, 2-acetyl-1-pyrroline, and 2-acetyl-2-thiazoline (defined by aroma extract dilution analysis). They reported that addition of epicatechin at levels as low as 0.01% to the raw milk significantly decreased the cooked flavor development during ultra-high-temperature processing perceived by a trained sensory panel, while, at 0.2% addition, the UHT flavor intensity was found equivalent to the cooked flavor intensity of a pasteurized milk sample.

Due to the complex nature of mechanistic pathways of the Maillard reaction, extensive use of labeled reactants such as sugar and amino acids (*16*−*19*) has been applied for this study. Carbohydrate module labeling (CAMOLA) is a new approach developed to elucidate the formation pathways of Maillard reaction products (*19*, *20*). This technique utilizes a combination of $^{13}C_6$-labeled and unlabeled glucose in a 1:1 ratio to understand the extent of fragmentation of the sugar skeleton and subsequent formation of key "transient" intermediates involved in the formation of flavor molecules. Sugar fragmentation results in the formation of reactive C_2, C_3, C_4, and C_5 intermediate dicarbonyls that are essential for generation of flavor and aroma molecules (*16*).

On the basis of the observed inhibitory effect epicatechin had on generation of Maillard-type volatile compounds in model systems, it was hypothesized that epicatechin was reactive in the initial stages of the reaction, possibly by quenching key Maillard precursors or intermediate products (i.e., sugar fragments). The purpose of this study was to use labeling experiments to indicate if epicatechin directly reacted with glucose, glycine, and/or resultant reaction products in an aqueous Maillard model system.

MATERIALS AND METHODS

Chemicals. D-Glucose, L-glycine, *n*-dodecane, 2,3-butanedione, pyrazine, methylpyrazine, acetol, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), D-[$^{13}C_6$]-glucose (99% enrichment), D-[1,2-$^{13}C_2$]glucose (99% enrichment), [$^{13}C_2$]glycine (99% enrichment), [^{15}N]glycine (98% enrichment), and [$^{13}C_2$,^{15}N]glycine (99% and 98% enrichment, respectively) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Potassium phosphate, disodium phosphate, ammonium acetate, HPLC-grade methanol, and anhydrous sodium sulfate were obtained from EMD Chemicals (Gibbstown, NJ). (−)-Epicatechin (≥98% purity) was obtained from Zhejiang Yixin Pharmaceutical Co. (Zhejiang, China). High-purity diethyl ether was obtained from Burdick & Jackson (Muskegon, MI).

Table 1. Aqueous Maillard Model Reactions[a]

	reactant concn (mM)							
reactant	model A	model B	model C	model D	model E	model F	model G	model H
glucose	10	10	5	5		10	10	10
glycine	10	10	10	10	10			5
epicatechin		10		10	10	10	10	10
[$^{13}C_6$]glucose			5	5				
[1,2-$^{13}C_2$]glucose					10			
[$^{13}C_2$]glycine						10		
[$^{13}C_2$,^{15}N]glycine							10	
[^{15}N]glycine								5

[a] All reactants in 150 mL of phosphate buffer (0.1 M, pH 7.0) and deionized water at 125 °C for 30 min (does not include reactor heating time to 125 °C).

Model Maillard Reaction System. The reaction mixtures (shown in **Table 1**) were dissolved in 150 mL of 0.1 M phosphate buffer (pH 7.0) and heated in a 600 mL Parr reactor (model 4563, Parr Instrument Co., Moline, IL) under constant stirring (set to 60% of the maximum speed) at 125 °C for 30 min, subsequently cooled to 20 °C with an internal cooling coil, and immediately prepared for further analysis.

Isolation/Extraction. Each reaction mixture was divided into two analytical categories: nonvolatile and volatile compounds. For the nonvolatile analyses, 70 mL of each reacted mixture was poured (4 mL/min) onto a nonionic polymeric resin (XAD-2, Supelco, Bellefonte, PA; column dimensions 400 mm × 22 mm i.d.; 80 g of resin). The resin was washed once (8 mL/min) first with 100 mL of 10% methanol solution and second with 15% methanol aqueous solution (2 × 200 mL). The final isolate was collected by desorption with 200 mL of 100% methanol (8 mL/min), then concentrated to 5 mL under vacuum (Buchi Rotavapor, model R110, New Castle, DE; 0.1 atm; water bath temperature 30 °C), and filtered prior to HPLC analysis.

For the volatile analyses, 70 mL of each reacted mixture was extracted 3× with 20 mL of diethyl ether (spiked with 0.5 μL of *n*-dodecane/600 mL, internal standard). The ether was pooled, dried with anhydrous sodium sulfate, filtered, and concentrated to 500 μL via spinning band distillation (model 800, B/R Instruments, Easton, MD) for GC analysis.

Liquid Chromatography/Mass Spectrometry. All methanol isolates were analyzed by an HPLC/MS instrument utilizing electrospray ionization (ESI) which consisted of a Shimadzu HPLC system (Shimadzu, Columbia, MD) equipped with a pump (LC-10ATvp), a degasser (DGU-14A), a low-pressure mixture (FCV-10ALVP), an autosampler (SIL-10vp), a Waters column heater (model TCM, Waters, Milford, MA), and a 4.6 × 250 mm, 5 μm packing, RP-18 Allure aqueous column (Restek, Bellefonte, PA) interfaced to a Waters ZMD 2000 mass spectrometer (Waters). HPLC conditions were as follows: injection volume 10 μL, column temperature 35 °C, binary mobile systems A (10 mM ammonium acetate aqueous solution, pH 6.8) and B (100% methanol), column flow rate 1.0 mL/min. A series of linear gradients were used starting with 5% B in A (0−5 min), then increasing to 80% B in A (5−20 min), then increasing to 99% B in A (20−22 min), and then decreasing to 5% B in A (28−30 min). The effluent was split 10:1 postcolumn (zero dead volume T-splitter; Supelco) with one part (100 μL/min) directed to the MS for detection and the other part to waste. The MS conditions were as follows: negative ion mode, capillary voltage 3.0 kV, scan range 100−1000 Da, source temperature 150 °C, probe temperature 250 °C.

Gas Chromatography/Mass Spectrometry. The diethyl ether extracts were analyzed by an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) utilizing an HP5972 mass spectrometer detector (EI mode) equipped with a liquid autosampler (model A200SE, CTC Analytics, Carrboro, NC). All analyses were performed on a DB-Wax capillary column (Agilent Technologies, 30 m × 0.25 mm i.d. with a 0.25 μm film thickness). The analysis parameters were as follows: 2 μL of sample was injected in the split mode (5:1), inlet temperature 200 °C, column flow constant at 0.7 mL/min (H_2), MS temperature 175 °C. The temperature program was 35 °C for 2 min, ramp at 5 °C to 230 °C, and hold for 4 min.

Gas Chromatography. All diethyl ether extracts were analyzed by an Agilent 6890 (Agilent Technologies) utilizing a flame ionization detector equipped with a DB-Wax capillary column (Agilent Technologies, 60 m × 0.25 mm i.d. with a 0.25 μm film thickness) and a Combi-Pal autosampler (CTC Analytics). The GC operation conditions were as follows: 2 μL of sample was injected in the split mode (5:1), inlet temperature 200 °C, detector temperature 250 °C, carrier constant flow rate 1.2 mL/min (H_2). The oven program was 35 °C for 2 min, then increase at 5 °C/min to 230 °C, and hold for 4 min.

Identification and Quantification of Volatile Compounds. Positive identifications of unknowns were made by using mass spectra and comparing linear retention indices (LRIs) of authentic compounds under identical analytical conditions. Volatile compounds were quantified by both internal and external standard techniques. The relative response of each volatile compound to the internal standard was determined by GC (same operating conditions as described previously) using a solution consisting of all compounds (2,3-butanedione, pyrazine, methylpyrazine acetol, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, cyclotene) plus the internal standard (*n*-dodecane) each at a concentration of 5 mg/50 mL of diethyl ether. The concentration for the internal standard in each isolate was determined by an external standard (response factor calculated from the internal standard solution).

RESULTS AND DISCUSSION

The volatile compounds identified from Maillard reaction model A (glucose/glycine) and model B (glucose/glycine/epicatechin) as well as the quantities generated are reported in **Table 2**. Overall the formation of Maillard-type volatile compounds in model B was inhibited in comparison to that in model A; the range of inhibition was between 8.5- and 113.4-fold reduction in the generation of acetol and 2,5-dimethylpyrazine, respectively. These results were in agreement with those of Peterson and Totlani (*14*), who reported epicatechin strongly inhibited the formation of Maillard-type volatile compounds using a 4:2 amino acid/reducing sugar aqueous model system.

To investigate how epicatechin inhibited the formation of volatile compounds (blocked key precursors), ^{13}C- and ^{15}N-labeling studies were used. Model C utilized equal mixtures of unlabeled/labeled glucose to elucidate key sugar fragments for the formation of the reported volatile compounds (**Table 2**). The isotopomer analyses of the volatile compounds from model C are shown in **Table 3** and indicate that 2,3-butanedione and acetol were primarily formed via intact C_4 and C_3 sugar fragments, whereas pyrazine, methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and cyclotene were primarily formed via intact C_2/C_2, C_2/C_3, C_3/C_3, C_3/C_3, C_3/C_3 sugar fragments pairs, respectively. Consequently, the respective sugar fragment(s) would be considered key precursors for the formation of each compound. **Figure 1** illustrates the theoretical 1:2:1 proportion of unlabeled to labeled isotopomers in pyrazine (M^+:$M^+ + 2$:$M^+ + 4$) if intact two-carbon glucose fragments were exclusively utilized for compound formation (based on CAMOLA technique). The equivalent 1:2:1 unlabeled to labeled isotopomeric proportion was observed in pyrazine under the specified reaction conditions of model C (**Table 3**), indicating C_2 glucose fragments were key precursors for compound formation. All of the compounds listed were primarily formed via an intact sugar fragment except for 2,3,5-trimethylpyrazine, where C_3/C_3 sugar fragment pairs were the most abundant intact sugar fragment reactants (on the basis of the proportion of isotopomers), and thus, one of the C_3 fragments undergoes a C_3/C_1 recombination (aldol condensation) for compound formation.

The noted inhibitory effect epicatechin had on generation of Maillard-type volatile compounds may have resulted due to the reactivity of epicatechin with the key sugar fragments reported in **Table 3** (quench intermediates). To test this hypothesis, the identification of possible epicatechin−sugar fragment adducts was explored using models B, D (1:1 unlabeled/labeled glucose), and E ([1,2-$^{13}C_2$]glucose) via HPLC/MS analysis (see **Figure 2**). HPLC/MS analytes were reported to consist of a sugar moiety if the following three conditions were observed: (1) the

Table 2. Concentration of GC Analytes in Models A and B[a]

	concn (ng/mL)		
	model A	model B	model A:model B ratio
2,3-butanedione	1067.9	39.6	27
pyrazine	250.5	4.1	61
methylpyrazine	94.1	2.6	36
acetol	2962.8	346.8	9
2,5-dimethylpyrazine	90.3	0.8	113
cyclotene	59.3	nd[b]	>100[c]
2,3,5-trimethylpyrazine	16.6	nd[b]	>50[c]

[a] Quantification via internal and external standard techniques. [b] Not detected. [c] Based on the detection limit (signal-to-noise ratio of 5:1).

Table 3. Proportion of Isotopomers from GC/MS Analysis of Model C

	m/z	% of enriched ^{13}C atoms in molecule[a]							
compound	(M^+)	0	1	2	3	4	5	6	7
2,3-butanedione	86	43	5	7	10	36			
pyrazine	80	25	3	49	1	23			
methylpyrazine	94	20	4	27	22	8	20		
acetol	74	46	4	5	46				
2,5-dimethylpyrazine	108	23	1	5	44	1	3	22	
cyclotene	112	24	5	5	31	3	9	22	
2,3,5-trimethylpyrazine	122	18	6	5	35	13	3	16	5

[a] Corrected for the natural abundance of ^{13}C (1.1%) and for the loss of hydrogen for the M^+ ion in labeled ions by the ratio ($M^+ - 1$):M^+.

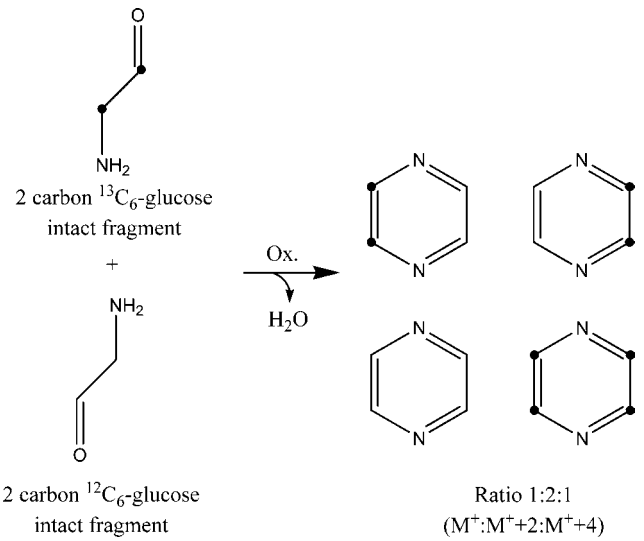

Figure 1. Theoretical isotopomeric distribution in pyrazine from CAMOLA studies if formed from intact two-carbon sugar fragments.

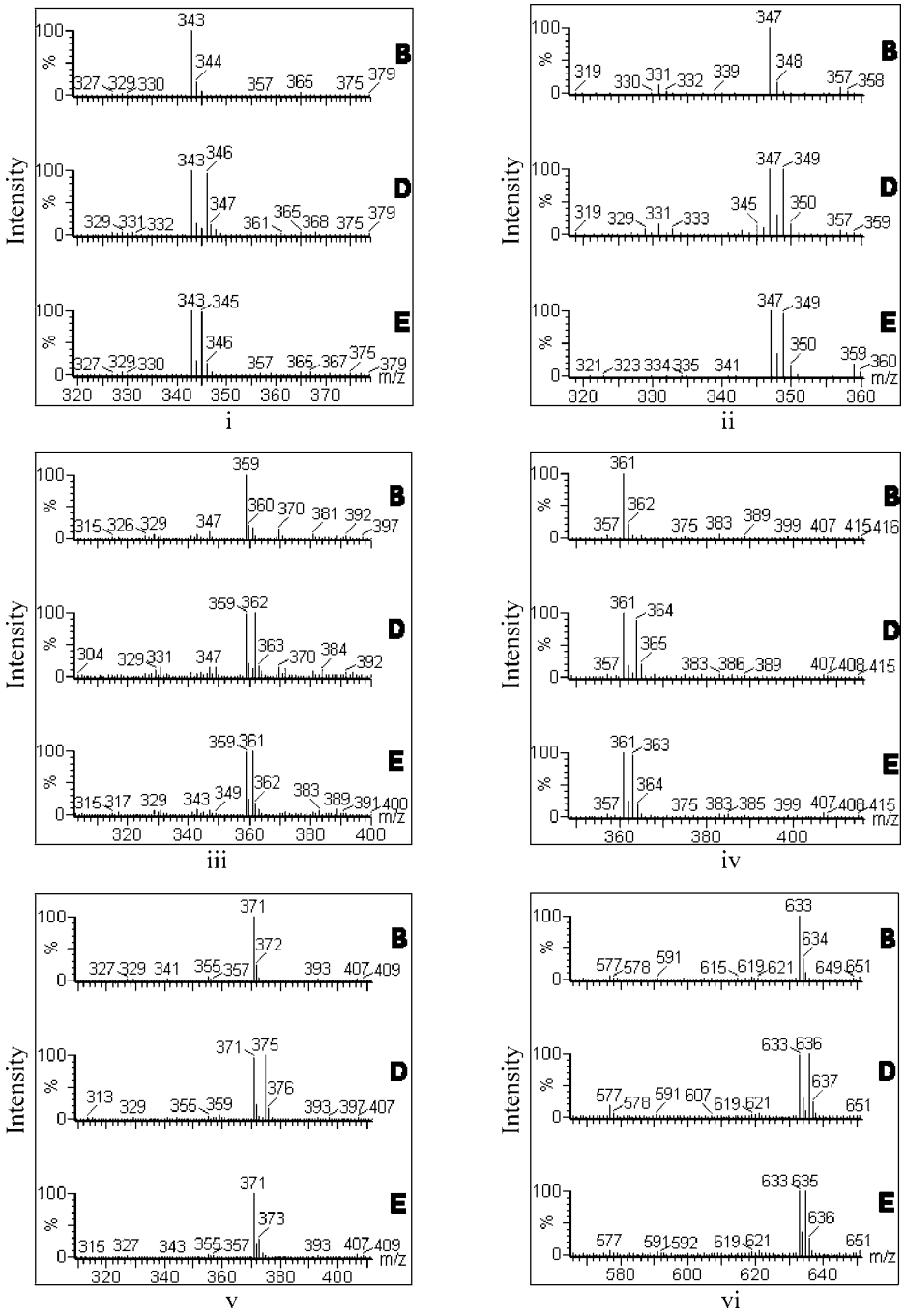

Figure 2. Measured isotopomers of select LC/MS analytes from models B, D, and E.

analytes had a predicted weight greater than that of epicatechin (290) but not equal to that of the flavonoid polymer [i.e., dimer (578), trimer (866)] in model B; (2) the analytes were not detected in model A (contained no epicatechin); and (3) the analytes displayed equivalent ion intensity isotopomers of MW + 1−6 in model D (at a retention time equivalent to that in model B). Six analytes were identified as potential epicatechin−sugar fragmentation products and are illustrated in **Figure 2** (i−vi, top and middle ion spectra). Again, none of these analytes in **Figure 2** were detected in model A. On the basis of isotopomer analysis from model D, the corresponding analytes with *m*/*z* [(M − H)$^-$] values of 343, 347, 359, 361, 371, and 633 were noted to consist of intact C_3, C_2, C_3, C_3, C_4, and C_3 sugar fragments, respectively.

Furthermore, analysis of the isotopomers from model E (**Figure 2**, i−vi, bottom ion spectrum) which utilized [1,2-$^{13}C_2$]-glucose indicated the relative reactivity of different fragments from the intact glucose molecule with each analyte. For the C_3 fragments (**Figure 2**, i, iii, vi), both the labeled (presumable 1−3C) and the unlabeled (presumable 4−6C) fragments were of equal ion intensity and therefore suggested both of these C_3 fragments were equally incorporated in the respective analyte (of similar reactivity). However, for the C_4 fragment (**Figure 2**, v), the primary fragment was from the unlabeled glucose portion (3−6C). For the C_2 fragment (**Figure 2**, ii), the unlabeled and labeled portions were also of equal intensity and therefore suggested that only one C_2 fragment from the unlabeled glucose portion (3−6C) was reactive as well as the labeled (1,2C) position.

The role of glycine and/or intermediate products in contributing to the structure of the analytes illustrated in **Figure 2** (model B) was evaluated using both ^{15}N- and ^{13}C-labeling studies. HPLC/MS analysis of models F ([$^{13}C_2$]glycine), G ([$^{13}C_2$,^{15}N]-glycine), and H (glycine:[^{15}N]glycine = 1:1) indicated no

$$\text{epicatechin} = C_{15}H_{14}O_6$$

$$C_{15}H_{14}O_6 + C_3H_2O \rightarrow MW = 344\ (m/z = 343)$$

$$C_{15}H_{14}O_6 + C_2H_2O_2 \rightarrow MW = 348\ (m/z = 347)$$

$$C_{15}H_{14}O_6 + C_3H_2O_2 \rightarrow MW = 360\ (m/z = 359)$$

$$C_{15}H_{14}O_6 + C_3H_4O_2 \rightarrow MW = 362\ (m/z = 361)$$

$$C_{15}H_{14}O_6 + C_4H_2O_2 \rightarrow MW = 372\ (m/z = 371)$$

$$(C_{15}H_{14}O_6)_2 + C_3H_2O \rightarrow MW = 634\ (m/z = 633)$$

Figure 3. Proposed composition of LC/MS analytes $[M - H]^-$ from model B.

isotopomers (i.e., M + 1−3) were identified for any of the analytes using glycine-labeling experiments, in comparison to analytes in model B (data not shown). This indicated that glycine was not part of the identified analytes as was observed for glucose under these reaction conditions. However, it should be noted the analytes in **Figure 2** (i−vi, top ion spectrum, model B) were similarly identified in models F, G, and H.

On the basis of the observed incorporation of glucose fragments and not glycine or a glycine fragment, the analytes from model B (see **Figure 2**) likely consisted of a phenolic−sugar fragment molecular structure. Proposed reactants with the appropriate molecular composition for the formation of the analytes from model B are furthermore illustrated in **Figure 3**. Each analyte with the appropriate MW (based on $M - H^-$) can be formed by combining epicatechin and the respective sugar fragment of varying degrees of oxidation (via redox reactions and dehydration reactions). The quenching of sugar fragments derived from the Maillard reaction directly by epicatechin has not been, to our knowledge, previously reported, although epicatechin has been shown to react with other reactive carbonyl compounds such as dehydroascorbic acid (*21*). Furthermore, catechin has been reported to react with formaldehyde or acetaldehyde to form the dimer with a methylene bridge or a methylmethylene bridge, respectively, in wine and wine model systems (*22*−*24*).

The chromatograms of each of the six analytes with a sugar fragment moiety illustrated in **Figure 2** are furthermore shown in **Figure 4**. Each analyte recorded multiple peaks which likely resulted from the formation of numerous isomers during the reaction, suggesting multiple bonding sites for the analyte formation. On the basis of the chemical properties of epicatechin, ionic and/or radical mechanisms may be involved. For example, C-acylation or O-acylation substitution reactions are possible (ionic), or perhaps, sugar fragments with an enaminol structure (i.e., aminoreductones) which have also been linked to the formation of radical products (*25*, *26*) may be participating in dimerization reactions with a radical epicatechin molecule, followed by a subsequent deamination step.

Additional studies on elucidating the mechanisms involved in the formation of the reported phenolic−sugar fragments and how epicatechin alters or inhibits the Maillard reaction are currently being investigated by our laboratory (i.e., structural characterization of the identified adducts, spin trapping studies, carbonyl reactivity, etc.). Phenolic compounds are ubiquitous in plant-based food materials (i.e., cocoa, cereal grains, coffee beans) and, likewise, may have an important role in the flavor properties of numerous food products besides their well-documented bitter attributes. Furthermore, phenolic−sugar fragment interactions may have positive implication on human health by reducing the so-called "carbonyl stress" directly in vivo or by reducing the carbonyl load ingested from the diet. Ultimately, improving our understanding of how to control this critical food reaction will also provide valuable information that can be used to improve the quality of food products, as it influences the flavor properties, the color development, and the nutritional value.

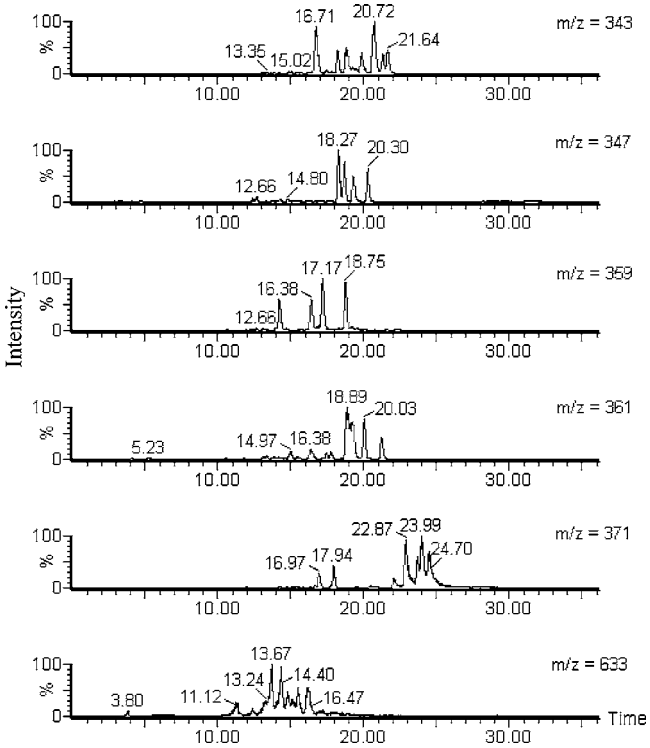

Figure 4. Chromatogram of select LC/MS analytes from model B.

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