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Isotope Labeling Studies on the Formation of Multiple Addition Products of Alanine in the Pyrolysis Residue of Glucose/Alanine Mixtures by High-Resolution ESI-TOF-MS

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ABSTRACT: Pyrolysis was used as a microscale sample preparation tool to generate glucose/alanine reaction products to minimize the use of expensive labeled precursors in isotope labeling studies. The residue remaining after the pyrolysis at 250 °C was analyzed by electrospray time-of-flight mass spectrometry (ESI-TOF-MS). It was observed that a peak at m/z 199.1445 in the ESI-TOF-MS spectrum appeared only when the model system contained at least 2-fold excess alanine. The accurate mass determination indeed indicated the presence of two nitrogen atoms in the molecular formula ($C_{10}H_{18}N_2O_2$). To verify the origin of the carbon atoms in this unknown compound, model studies with [$^{13}U_6$]glucose, [^{13}C-1]alanine, [^{13}C-2]alanine, [^{13}C-3]alanine, and [^{15}N]alanine were also performed. Glucose furnished six carbon atoms, and alanine provides four carbon (2 × C-2 and 2 × C-3) and two nitrogen atoms. When commercially available fructosylalanine (N-attached to C-1) was reacted with only 1 mol of alanine, a peak at m/z 199.1445 was once again observed. In addition, when 3-deoxyglucosone (3-DG) was reacted with a 2-fold excess of alanine, a peak at m/z 199.1433 was also generated, confirming the points of attachment of the two amino acids at C-1 and C-2 atoms of 3-DG. These studies have indicated that amino acids can undergo multiple addition reactions with 1,2-dicarbonyl compounds such as 3-deoxyglucosone and eventually form a tetrahydropyrazine moiety.

KEYWORDS: Maillard reaction, fructosylalanine, pyrolysis, 3-DG, tetrahydropyrazine, ESI-TOF-MS, mass spectrometry, isotope labeling

INTRODUCTION

Isotope labeling can be considered an important tool that allows the elucidation of the mechanism of complex chemical reactions and at the same time permits the assignment of structures to mass spectral ions generated under various ionization conditions. Although such studies can be considered costly and time-consuming, the use of pyrolysis probe as a sample preparation tool can greatly reduce the quantities of labeled precursors needed to perform such analyses. A wide range of temperatures (50−900 °C) can be employed to generate reaction products in which specifically labeled atoms are incorporated from the starting materials into the various products of pyrolysis. In addition, integrating the generation of the reaction mixtures with the process of separation and identification through the use of a pyrolysis−gas chromatography−mass spectrometry (Py-GC-MS) system can generate a wealth of information regarding the molecular mechanism of formation of various volatile and semivolatile compounds formed in different mixtures such as in sugar/amino acids known to initiate the Maillard reaction.[1−3] The analysis of such complex mixtures poses great challenges due to the need for the identification of chemically diverse components. Performing such reactions at a microscale level using pyrolysis has allowed the introduction of ^{13}C- and ^{15}N-labeled atoms into the reaction products, through carrying out the reactions with specifically labeled glucose (independently labeled at C-1, C-2, etc.) and amino acids (N, C-1, C-2, etc.) using Py-GC-MS as an integrated reaction, separation, and identification system.[4,5] This technique requires only submilligram quantities of relatively expensive labeled precursors, thus reducing drastically the cost of such experiments. To test the hypothesis that the residue remaining after such pyrolysis experiments also contains useful chemical information in the form of labeled nonvolatile products, we have analyzed the pyrolysis residue of a glucose/alanine model system for such compounds using high-resolution electrospray ionization−time-of-flight mass spectrometry (ESI-TOF-MS) and triple-quadrupole tandem mass spectrometry (MS/MS) in conjunction with the isotope labeling technique.

MATERIALS AND METHODS

Materials. L-Alanine, β-alanine, D-glucose, and formic acid (>98%) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). L-[^{13}C-1]Alanine, L-[^{13}C-2]alanine, L-[^{13}C-3]alanine, and L- [^{15}N]alanine were all >98% enriched and purchased from Cambridge Isotope Laboratories (Andover, MA). Fructosylalanine and 3-deoxyglucosone were purchased from Toronto Research Chemicals (North York, ON, Canada). Methanol was of HPLC grade purchased from EMD Chemical (Gibbstown, NJ). Nanopure water was provided by a Millipore Synergy ultrapure water purification system (Billerica, MA).

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Table 1. Model Systems Used To Generate Pyrolytic Residues and ESI-TOF-MS Data of the Target Ions

model system[a]	m/z $(M + H)^+$	molecular formula[b]	MS/MS fragments[c]
glucose + 2 × ala	199.1445	$C_{10}H_{18}N_2O_2 + H^+$	
3-deoxyglucosone + 2 × ala	199.1433	$C_{10}H_{18}N_2O_2 + H^+$	181, 163[d]
3-deoxyglucosone + 2 × ala	243.1335	$C_{11}H_{18}N_2O_4 + H^+$	
fructosylalanine	199.1432	$C_{10}H_{18}N_2O_2 + H^+$	
fructosylalanine +1 × ala	199.1432	$C_{10}H_{18}N_2O_2 + H^+$	
fructosylalanine + 1 × ala	234.0965	$C_9H_{15}NO_6 + H^+$	216, 188, 170, 126[e]
fructosylalanine + 1 × ala	243.1345	$C_{11}H_{18}N_2O_4 + H^+$	
fructosylalanine + 1 × β-ala	243.1341	$C_{11}H_{18}N_2O_4 + H^+$	
labeled model systems	**m/z 199**	**molecular formula**	**no. of label incorporation**
[$^{13}U_6$]glucose + 2 × ala	205.1644	$^{13}C_6C_4H_{18}N_2O_2 + H^+$	6 carbon atoms
glucose + 2 × [^{13}C-1]ala	199.1445	$C_{10}H_{18}N_2O_2 + H^+$	0 carbon atoms
glucose + 2 × [^{13}C-2]ala	201.1507	$^{13}C_2C_8H_{18}N_2O_2 + H^+$	2 carbon atoms
glucose + 2 × [^{13}C-3]ala	201.1507	$^{13}C_2C_8H_{18}N_2O_2 + H^+$	2 carbon atoms
glucose + 2 × [^{15}N]ala	201.1377	$C_{10}H_{18}{}^{15}N_2O_2 + H^+$	2 nitrogen atoms

[a] ala, alanine. [b] Accuracy < 5 ppm. [c] Collision energy = 20 V from QqQ. [d] See Figure 4A. [e] See Figure 4B.

Sample Preparation. Pyrolysis residues were generated using a CDS Analytical Pyroprobe 2000 system (Oxford, PA). About 1 mg of sample mixtures (see Table 1) was packed inside quartz tubes (0.3 mm thickness), which were plugged with quartz wool and inserted inside the coil probe and pyrolyzed for 20 s at a temperature of 250 °C. The quartz tube was then placed inside a 1.5 mL Eppendorf microcentrifuge tube (Mississauga, ON, Canada), and 1 mL of nanopure water was added to dissolve the nonvolatile residues. The samples were vortexed and then sonicated in a Branson model 2510 ultrasonic bath (Danbury, CT) for 5 min followed by a 20-fold dilution in 50% methanol and 0.1% formic acid.

ESI-TOF-MS Analysis. Accurate mass data were obtained using an Agilent 1200 series high-performance liquid chromatography (HPLC) system equipped with an Agilent 6210 time-of-flight (ESI-TOF) mass spectrometer (Santa Clara, CA). An injection volume of 2 μL was introduced by direct (loop) injection without HPLC separation using a mobile phase consisting of 50% methanol and 0.1% formic acid at a flow rate of 0.4 mL/min. The dual ESI source was operated in positive mode, and data were acquired over a mass range of m/z 100−1000 with internal calibration using the reference masses m/z 121.050873 and 922.009798 (Agilent ESI tuning mix), sprayed continuously through the reference electrospray needle. The source was operated with the following parameters: gas temperature, 350 °C; capillary voltage, 4000 V; flow, 12 L/min (ultrapure nitrogen); nebulizer gas pressure, 35 psi; fragmentor and skimmer voltages, 100 and 60 V, respectively.

Tandem Mass Spectrometry. An Agilent 1200 series HPLC system equipped with an Agilent 6410 triple-quadrupole (QqQ) mass spectrometer was used for the MS/MS experiments. The mobile phase and flow rate were identical to those for the ESI-TOF analysis, and the injection volume was 5 μL. MS/MS spectra (unit resolution) were obtained by positive electrospray ionization (ESI) using a multimode (MMI) source with the following parameters: gas temperature, 300 °C; vaporizer, 150 °C; gas flow, 5 mL/min; nebulizer pressure, 60 psi; capillary voltage, 3000 V; fragmentor, 135 V; and scan time, 500 ms. Ions at m/z 199.1 and 234.1 were selected as precursor ions with an MS^2 scan range of m/z 30−240 and a collision-induced dissociation (CID) voltage of 20 V using ultrapure nitrogen as collision gas. Raw ESI-TOF and MS/MS data were analyzed by MassHunter Qualitative Analysis software version B.02.00 (Agilent Technologies). Tentative identifications were made on the basis of accurate mass data, isotope labels, MS/MS, and molecular formula assignments.

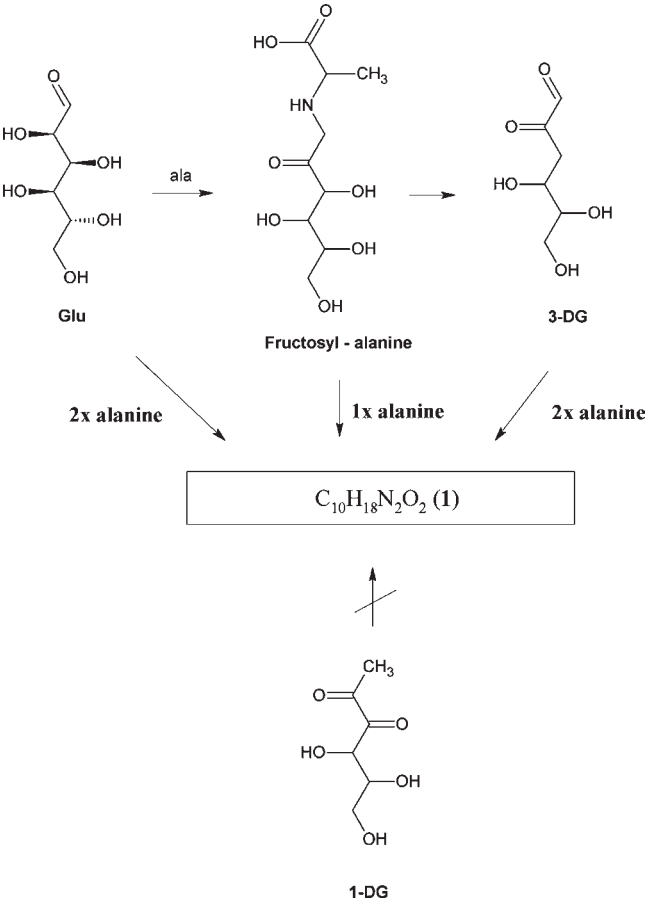

Figure 1. Precursors able to generate compound **1** ($C_{10}H_{18}N_2O_2$). Glu, glucose; 3-DG, 3-deoxyglucosone; 1-DG, 1-deoxyglucosone.

RESULTS AND DISCUSSION

The Maillard reaction of glucose and alanine proceeds through the formation of Amadori product (Figure 1) with the subsequent generation of 1-deoxy- and 3-deoxyglucosones (1-DG and

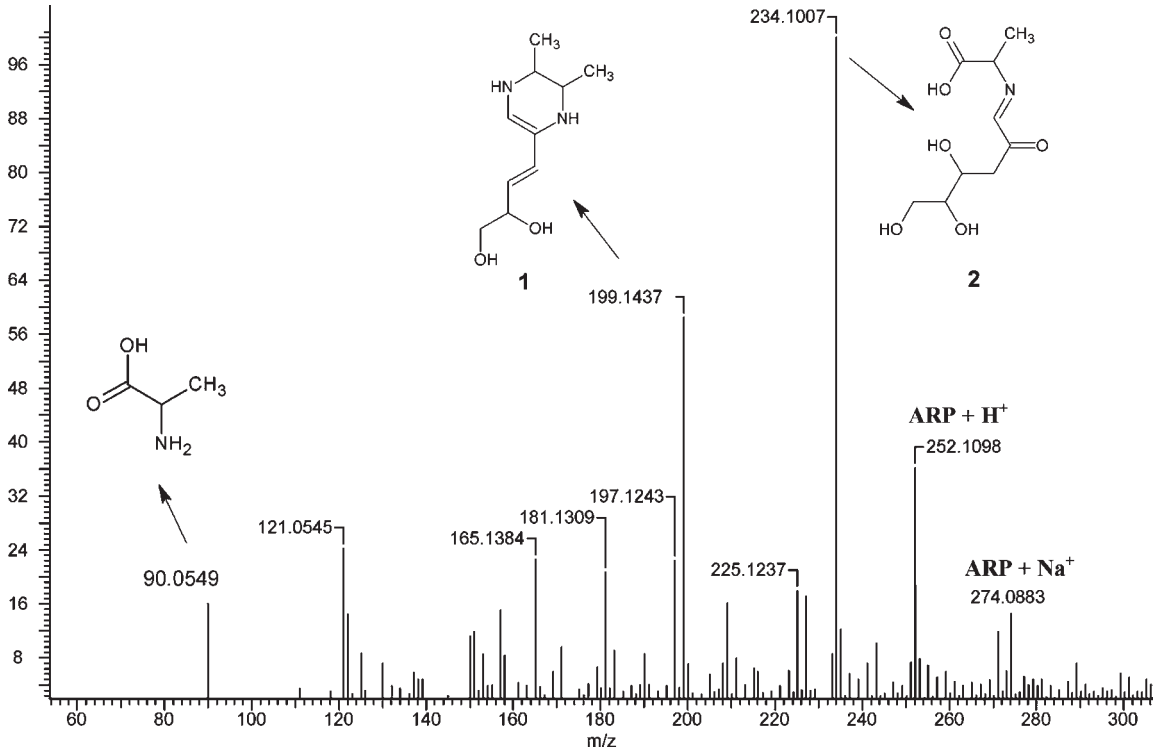

Figure 2. ESI-MS spectrum of the pyrolysis mixure of fructosylalanine/alanine model system showing structures **1** and **2** as neutral molecules. ARP, Amadori rearrangement product.

3-DG). Such 1,2-dicarbonyl intermediates[6] have been identified in various model systems,[4] food products,[7] and physiological fluids,[6] and their transformations in the presence of amino acids are known to generate numerous volatile aroma products in food or cross-linked structures in biological systems. Although 1,2-dicarbonyl intermediates are known to react with 1,2-diaminobenzenes as trapping agents and form quinoxaline derivatives, their ability to react with two amino acids and form double Schiff base adducts has not been documented. Furthermore, such multiple additions of amino acids to 1,2-dicarbonyl compounds may provide further insight into cross-linked structures that may form between two amino acids in processed foods. When a glucose/alanine model was studied using the pyrolysis probe as the sample preparation tool at 250 °C, it was observed that a peak at m/z 199.1445 in the ESI-TOF-MS spectrum appeared only when the model system contained a 2-fold excess of alanine. The accurate mass determination indeed indicated the presence of two nitrogen atoms in the molecular formula of m/z 199.1445 (corresponding to the protonated molecule MH^+ of $C_{10}H_{18}N_2O_2$) as shown in Table 1. To verify the incorporation of two nitrogen atoms and the origin of the carbon atoms in this unknown compound (**1**), model studies with [$^{13}U_6$]glucose, [^{13}C-1]alanine, [^{13}C-2]alanine, [^{13}C-3]alanine, and [^{15}N]alanine were performed. As shown in Table 1, glucose contributed six carbon atoms and alanine provided four carbon atoms (2 × C-2 and 2 × C-3) in addition to two nitrogen atoms. No C-1 atom of alanine was incorporated into the unknown structure **1**, indicating its loss through decarboxylation. Preliminary analysis of the labeling data, however, pointed toward double incorporation of alanine molecule either at the two carbonyl carbons (C-2 and C-3) in 1-DG or at the C-1 and C-2 carbonyl carbons of 3-DG (see Figure 1). When commercially available fructosylalanine (N-attached at C-1) was also pyrolyzed alone or in the presence of 1 mol of alanine, the peak at m/z 199.1445 was again observed (Figure 2). In addition, when commercially available 3-DG was reacted with a 2-fold excess of alanine, the peak at m/z 199.1445 was generated, confirming the points of attachments of the two amino acids as the C-1 and C-2 atoms of 3-DG.

Proposed Mechanism of the Multiple-Addition Reaction of Alanine. In glucose/alanine reaction mixtures, fructosylalanine, the ARP, is the main precursor that initially forms and subsequently undergoes enolization followed by β-elimination to generate **2** as shown in Figure 3. After hydrolysis, intermediate **2** can generate free alanine and as well as 3-DG.[8] This reactive intermediate can undergo various reactions such as the formation of 5-hydroxymethyl-2-furaldehyde (HMF), or it can react with available amino acids in the reaction medium to regenerate **2**. Structure **2**, whether formed from fructosylalanine or through 3-DG, can undergo decarboxylation through the well-known Strecker reaction[9] and generate **3**. The latter can either undergo hydrolysis to generate the Strecker aldehyde[9] or react with the available free amino acids to form the adduct **4** after dehydration and isomerization of the double bond. Decarboxylation of the amino acid moiety from structure **4** can generate the structure **1** with a molecular formula of $C_{10}H_{18}N_2O_2$. The observed ion at m/z 199.1445 is consistent with the label incorporation data and with the elemental formula derived from the high-resolution ESI-TOF-MS experiments.

Proposed Structure of Multiple Addition Product of Alanine. Initially, it was assumed that the decarboxylation of **4** would generate structure **1′** shown in Figure 3. However, this structure is not consistent with the stability required for samples dissolved in acidic aqueous solutions for ESI-MS analysis due to their ease of hydrolysis. Additional experiments were carried out using the

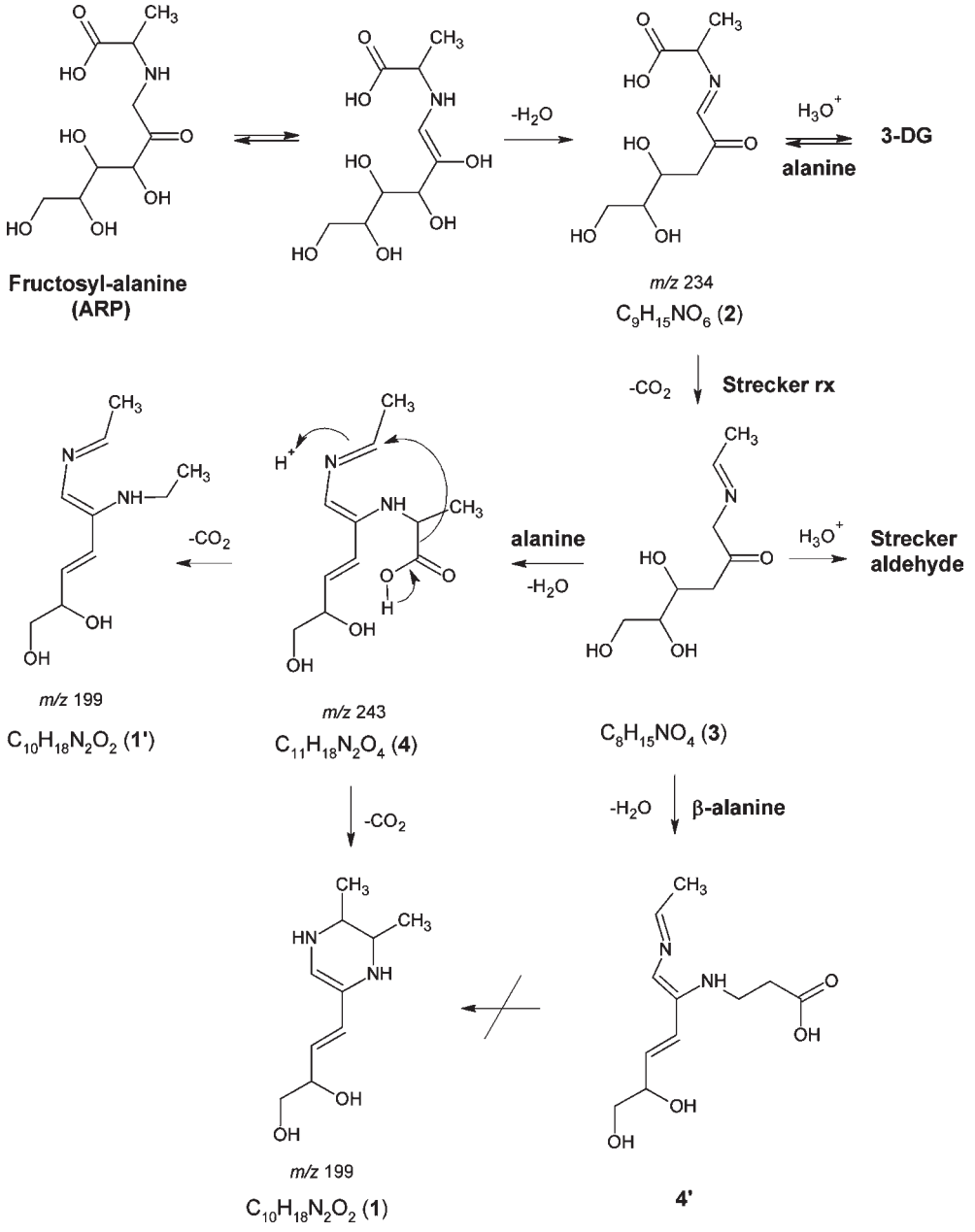

Figure 3. Proposed mechanism of formation of compound **1** (structures are shown as neutral molecules).

reaction mixture of β-alanine/fructosylalanine to confirm or discount the proposed structure. β-Alanine was chosen due to the fact that it is unable to cyclize and generate structure **1** from **4**′, although it can be converted into **1**′ through a simple decarboxylation step similar to **4**. The use of fructosylalanine as the reactant will ensure the attachment of β-alanine mainly at the C-2 position of the glucose moiety. Consequently, if the reaction mixture of β-alanine/fructosylalanine still exhibits a peak at m/z 199, it implies the formation of structure **1**′; otherwise, it confirms the formation of structure **1**. The results of β-alanine/fructosylalanine studies indicated the complete absence of the peak at m/z 199.1445, confirming the assignment of structure **1**.

Evidence in Favor of the Proposed Mechanism of Formation of 1. In addition to the isotope labeling data and precursor studies, the ion at m/z 234.0965 corresponding to the structure of the key proposed intermediates **2** ($C_9H_{15}NO_6$) was also detected as a major peak in the mass spectrum of the fructosylalanine/alanine mixture (Figure 2) and its MS/MS spectrum was found to be consistent with the proposed structure (see Figure 4B). Further evidence supporting structure **1** relative to structure **1**′ comes from its MS/MS spectrum (Figures 2 and 4A), which shows the sequential loss of two water molecules and the formation of ions at m/z 181 and 163, respectively. Such a loss of two water molecules is difficult to rationalize on the basis of structure **1**′, whereas structure **1** can easily lose two water molecules, as shown in Figure 4A.

The isotope labeling studies have indicated that 3-DG, a common reactive Maillard reaction intermediate, can undergo sequential Schiff base formation with 2 mol of amino acids at the two carbonyl carbons (Figure 3). The initial adduct at m/z 234

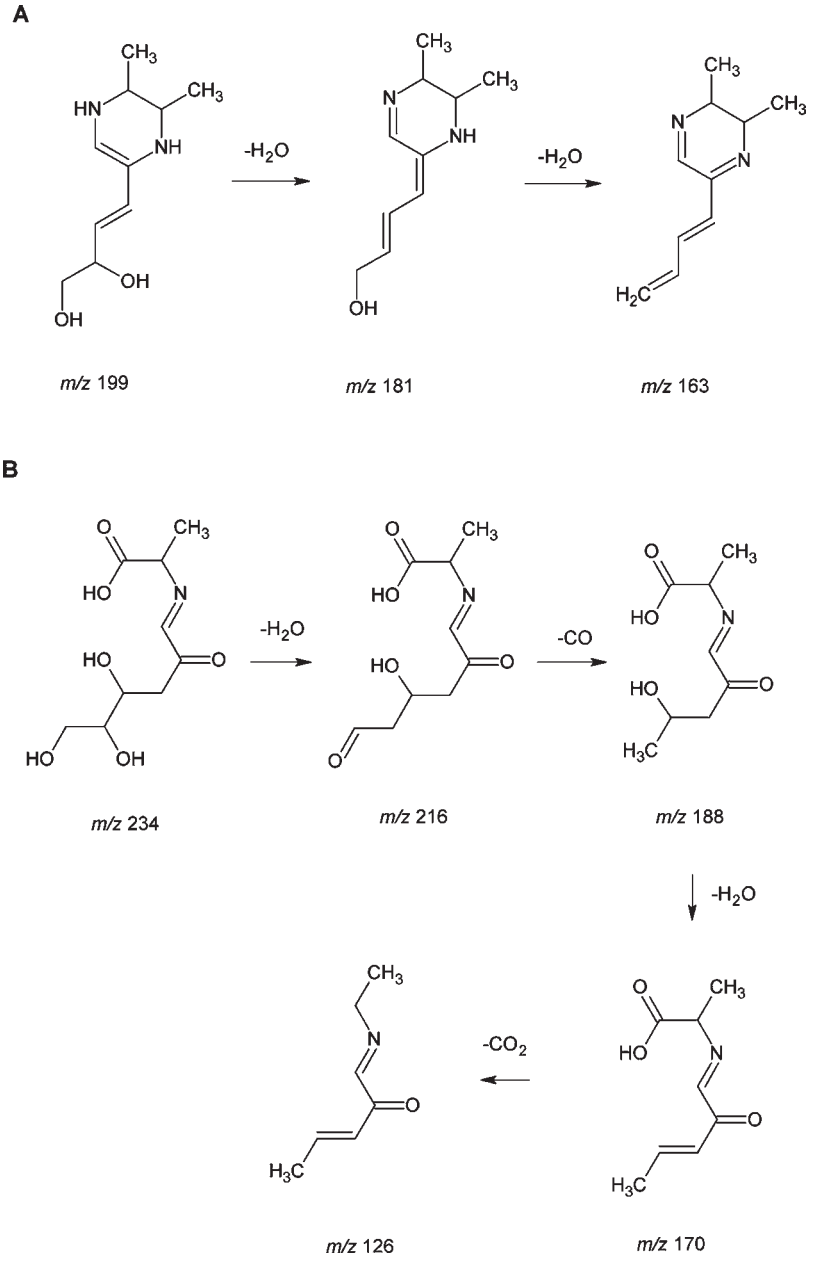

Figure 4. Proposed MS/MS fragmentations of (A) *m/z* 199 and (B) *m/z* 234 obtained at 20 V. Structures are shown as neutral molecules.

(structure **2**) and a critical intermediate at *m/z* 243 (structure **4**) have been observed in the ESI-TOF-MS spectra as listed in Table 1. Such interactions with shorter chain α-dicarbonyl compounds in processed food can allow the formation of aroma-active tetrahydropyrazine-based structures.[10]

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