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Investigation of Maillard reaction products using ^{15}N isotope studies and analysis by electrospray ionization–mass spectrometry*

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Structural characterization of reaction products of Maillard Chemistry has proved to be rather challenging. This can be directly attributed to the extreme complexity of product reaction mixtures that are known to range from small organic molecules to extremely large polymers. In addition, contemporary analytical techniques have been significantly challenged by the hydrophilic nature of advanced glycation endproducts (AGEs). Recently, methods such as on-line capillary electrophoresis-electrospray ionization-mass spectrometry (CE-MS) have emerged to provide a means of structurally characterizing molecules of extreme hydrophilicity. However, while this technique provides a means to identify such molecules, it is often difficult to differentiate products of ion/molecule reactions that can occur in the interface from authentic Maillard reaction products (MRPs). These artifacts are misleading, and lead to erroneous conclusions regarding solution composition. In the present study, we have prepared MRPs by reaction of 5-hydroxymethylfurfural (5-HMF) separately with ^{14}N-glycine and ^{15}N-glycine. CE-MS analysis of a 50:50 (v/v) mixture of these solutions readily identifies product of ion/molecule reactions by a multiplicity of isotopes that fit a simple binomial expansion statistical output. By contrast, the mass spectrum of a MRP present in a preformed mixture of isotopically pure reaction products is much simpler. Such spectra display two responses (one for each isotope) that are separated by a mass number that directly corresponds to the number of nitrogen atoms present in the MRP. In addition, tandem mass spectrometric analysis of both of the isotopically pure MRPs aids the structural characterization of molecules of interest. Each fragment ion increases in mass by the number of nitrogen atoms it contains in the spectrum of the ^{15}N labeled MRP. This strategy is demonstrated by the detection and structural characterization of 2-formyl-5-(hydroxymethyl)pyrrole-1-acetic acid.

While Maillard reaction products (MRPs) have known significant implications in food chemistry and biomedicine, the chemical structure of many remain unknown (Yaylayan, 1997). Several factors are attributed to the partial understanding of reaction pathways of Maillard Chemistry, with perhaps none more important than the complex nature of product reaction mixtures. Reactions of relatively simple reagent mixtures, such as amino acids, peptides, or proteins with reducing sugars or other compounds with carbonyl functionality, result in a complex mixture of products that are known to range from small molecules to extremely large polymers (Finot *et al.*, 1990). The chemical diversity of these molecules, and the extreme hydrophilicity of the advanced glycosylation endproducts (AGEs), has significantly challenged contemporary analytical chemistry used for MRP characterization.

Previously, volatile and semi-volatile MRPs have been identified by the routine technique of on-line gas chromatography-mass spectrometry (GC-MS), and by pyrolysis GC-MS (Keyhani and Yaylayan, 1996*a*). Chemical processes involved in the formation of volatile flavors are relatively well understood. However, analysis

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and identification of non-volatile MRPs, such as AGEs or the colored melanoidins, has been more problematic, due to the apparent lack of a suitable analytical approach for the structural characterization of a complex mixture of such hydrophilic molecules. Indeed, even reversed-phase HPLC coupled on-line to a mass spectrometer via a thermospray interface (LC-TSP-MS) was found to be poorly suited to this application (Tomlinson, 1991). While reversed-phase HPLC yielded inadequate MRP resolution, thermospray MS was insensitive and routine use was problematic. Subsequently, electrospray ionization has become a standard MS approach for the analysis of hydrophilic molecules in the biological sciences (Smith *et al.*, 1990). The technique of capillary electrophoresis (CE) is also becoming mature, often affording analyte resolution that is far superior to that observed by more contemporary methods such as reversed-phase HPLC (Kuhr and Monnig, 1992). Recently, CE separation of MRPs has been reported (Deyl *et al.*, 1990; Tomlinson, 1991, 1993, 1994). In addition, on-line capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) has been developed (Smith *et al.*, 1988; Lee *et al.*, 1988), and applied in studies of MRPs (Benson *et al.*, 1993; Lapolla *et al.*, 1995). However, structural characterization of MRPs remains difficult. Indeed, electrospray ionization-mass spectrometry (ESI-MS) further complicates MRP data interpretation by its preponderance to form analyte clusters during ionization and desolvation processes. These artifactual responses are detected at higher m/z values than expected and provide misleading MRP characterization (Benson *et al.*, 1993).

In the present study we demonstrate the usefulness of stable isotopes to aid the characterization of MRPs that were formed by reaction of aqueous solutions of 5-hydroxymethyl-2-furfural (5-HMF) with either ^{14}N glycine or ^{15}N glycine. We show how CE-MS analysis of a 50:50 mixture of independently prepared labeled and unlabeled reaction mixtures overcomes the problem of mischaracterization of MRP molecular weights using ESI-MS. In addition, we demonstrate the usefulness of isotopic labeling for interpretation of MRPs structure from data acquired by tandem MS.

For these studies all solid reagents were obtained from Aldrich Chemical Company (Milwaukee, WI). Polyimide-coated fused silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ) and HPLC grade solvents were obtained from Baxter (Minneapolis, MN). Model reaction mixtures were prepared by dissolving 5-HMF (15 mg) and ^{14}N glycine or ^{15}N glycine (9 mg) in H_2O (400 μl) containing triethylamine (10 μl, pH~10). These mixtures were vortexed and incubated for 5 h at 60°C. The ^{14}N and ^{15}N glycine reaction mixtures were combined 1:1 molar ratios just prior to analysis by CE-MS, which was performed using a Beckman (Fullerton, CA) P/ACE series 2100 CE system interfaced to a Finnigan-MAT 95Q (Bremen, Germany) mass spectrometer of BEQ_1Q_2 configuration (where B is a magnet, E is an electric sector, Q_1 is an octapole and Q_2 is a quadrupole mass filter). A Finnigan-MAT (Bremen, Germany) electrospray ion source was used throughout. CE separations of MRPs were achieved using a capillary (50 μM×86 cm) internally coated with polybrene as previously described (Tomlinson *et al.*, 1995). An aqueous separation buffer of 20 mM ammonium acetate containing acetic acid (6% v/v) and an applied separation voltage of −10 kV was used throughout. A co-axial liquid sheath of methanol:water:acetic acid (75:25:1) was delivered at a rate of 3 μl/min. The electrospray voltage was 3.5 kV referenced against an instrument accelerating voltage of 5 kV. MS data were collected in positive mode over a mass range of 50–600 Da at a scan speed of 2 s/decade. An instrument resolution of ~1000 was used throughout. Ions of interest were subjected to tandem mass spectrometry using argon as the collision gas at a pressure of 1×10^{-5} bar and a collision energy of 24 eV (lab. frame). MS2 was scanned from 60 to 190 Da at a scan speed of 0.3 s/100 u. MS2 resolution was ~3 u at peak base.

As expected, analysis of a ^{14}N glycine-HMF model Maillard reaction mixture by CE-ESI-MS demonstrated many reaction products (results not shown). Data interpretation was also difficult since electrospray ionization yields only prominent protonated molecular ion (MH^+) responses, and identification of MRPs which contain the amino nitrogen of the glycine could not be easily assigned. Using the nitrogen rule, MRPs of odd molecular weight contain an odd number of nitrogen

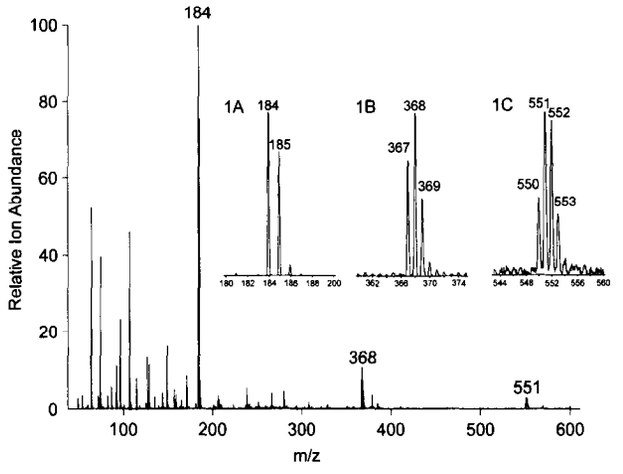

Fig. 1. Mass spectrum from CE-MS analysis of an equimolar mixture of 5-HMF reactions with ^{14}N and ^{15}N-labeled glycine depicting a mononitrogen MRP. CE capillary internally coated with polybrene, separation buffer 2 mM ammonium acetate in 6% (v/v) acetic acid in water. Separation voltage −10 kV, sheath liquid methanol:water:acetic acid (75:25:1 v/v/v), sheath liquid flow rate 3 μl/min. Electrospray voltage was 3.5 kV referenced against an instrument accelerating voltage of 5 kV. Scan range 50–600 Da at a scan speed of 2 s/mass decade. Instrument resolution was ~1000. (A) Expansion of mass scale to show isotope distribution at m/z 184. (B) Expansion of mass scale to show isotope distribution at m/z 367. (C) Expansion of mass scale to show isotope distribution at m/z 550.

atoms. However, this is the limit of the information regarding nitrogen content in detected MRPs. MRPs of even molecular weight may contain zero or an even number of nitrogen atoms. To overcome this limitation we elected to separately prepare two 5-HMF/glycine model reaction mixtures. One reaction mixture was prepared with ^{14}N glycine and the other with ^{15}N labeled glycine. CE-MS analysis of a 50:50 mixture of these model reaction mixtures allowed easy detection of ion/molecule reactions that occur in the electrospray ion source and result in cluster formation. The mass spectrum of a reaction product that is formed by an ion/molecule reaction in the electrospray source manifests itself by showing a multiplicity of isotopes that fit a simple binomial expansion statistical output, and examples will be given later in this manuscript. However, the mass spectrum of a mixture of preformed, isotopically unique reaction products does not show such multiplicity. Instead, the spectrum displays two distinct peaks (one for the labeled and one for the unlabeled MRP) of equal intensity that are separated by a mass number that directly represents the number of nitrogen atoms contained in the molecule of interest. The power of this approach is demonstrated in Fig. 1A. Here, a MRP of molecular weight 183 is characterized by two peaks of almost equal intensity at *m/z* 184 and *m/z* 185. This pattern is indicative of a MRP that possesses one nitrogen atom. The deviation from the expected 1:1 intensity ratio of the peaks at *m/z* 184 and *m/z* 185 in Fig. 1A is likely to be due to differences in the concentration of labeled and unlabeled MRPs. This generally occurs due to inaccuracies induce by mixing two independently prepared reaction mixtures.

The complexity of the mass spectrum shown in Fig. 1 is the result of co-migration of several MRPs using the described CE-MS conditions. Of initial interest were those responses observed at 367 and 550 Da. A wide

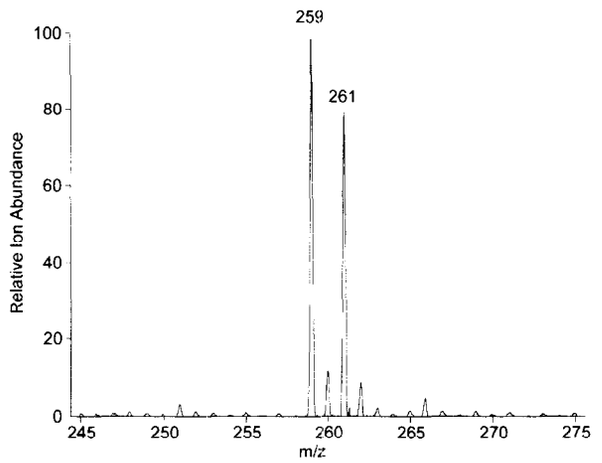

Fig. 2. Expanded mass spectrum (*m/z* 245–275) from CE-MS analysis of an equimolar mixture of 5-HMF reactions with ^{14}N and ^{15}N-labeled glycine showing a dinitrogen MRP. All conditions as described in Fig. 1. Identification of this MRP from CE-MS/MS data is in progress.

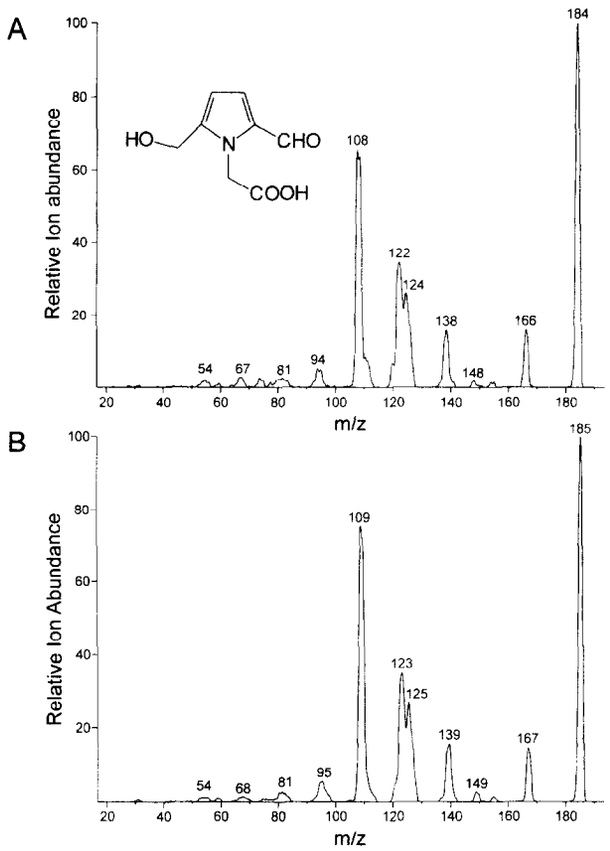

Fig. 3. MS/MS spectra from CE-MS/MS analysis of MRPs. (A) $MH^+ = 184$ MRP (^{14}N-MRP) and (B) $MH^+ = 185$ MRP (^{15}N-MRP). CE and electrospray conditions as Fig. 1. MS1 was used to select precursor ions which were passed in to an octapole collision cell containing argon at a pressure of 1×10^{-5} bar. The collision energy was 24 eV (lab frame), and MS2 was scanned from 60 to 190 Da at a scan speed of 0.3 s/100 u. MS2 resolution $\sim$ 3 u at peak base. Insert of Fig. 3A shows the chemical structure of 2-formyl-5-(hydroxymethyl)pyrrole-1-acetic acid.

variety of MRPs with a large range of molecular weights are known to be produced under Maillard reaction conditions, and detection of species of higher molecular weight in the 5-HMF/glycine reaction was expected. However, examination of the isotopic distribution of the higher *m/z* responses in Fig. 1 highlighted a statistical distribution of isotope responses. Such distribution would have been characteristic of a 'one-pot' reaction mixture of 5-HMF with an equimolar mixture of ^{14}N and ^{15}N-labeled glycine. This explanation is based upon mathematical probability. For example, it is apparent that the ion represented by the peak at *m/z* 367 possesses two nitrogen atoms. In a 'one-pot' reaction scheme in which the glycine is a 50:50 mixture of ^{14}N and ^{15}N-labeled reagents, a readily recognizable statistical distribution of ^{14}N and ^{15}N incorporation into a dinitrogen species is expected. Hence the probability of the dinitrogen reaction products containing $^{14}N_2$ compared to $^{14}N + {}^{15}N$ and $^{15}N_2$ is 1:2:1. Similarly under such 'one-pot' reaction conditions

Table 1. Use of ^{14}N and ^{15}N-labeled responses in the identification of a 5-HMF-glycine MRP from CE-MS/MS spectra

Fragment Ion in ^{14}N system (m/z)	Fragment Ion in ^{15}N system (m/z)	Inferred Ion composition
54	54	$C_3H_2O^{+\cdot}$
67	68	$C_4H_5N^{+\cdot}$
81	81	$C_5H_5O^+$
94	95	$C_5H_4NO^+$
108	109	$C_6H_6NO^+$
122	123	$C_7H_8NO^+$
124	125	$C_6H_6NO_2^+$
138	139	$C_7H_8NO_2^+$
166	167	$C_8H_8NO_3^+$

the isotopic distribution for the species at m/z 550 (a trinitrogen species) would have a product ratio of 1:2:2:1 ($^{14}N_3$:$^{14}N_2$+^{15}N:^{14}N+$^{15}N_2$:$^{15}N_3$). However, if the reaction mixtures are prepared independently and were mixed minutes prior to analysis a different isotopic distribution is expected. For example, if the MRPs of 367 and 550 Da represented in Fig. 1 were products of the 5-HMF/glycine solution chemistry, the detected isotope distribution would be two peaks of equal intensity separated by a mass number representative of the nitrogen content of the MRP. This is further demonstrated in Fig. 2 for a dinitrogen MRP that has a molecular weight of 258. When the labeled and unlabeled reaction mixtures are prepared independently and mixed just prior to analysis there is no possibility of a statistical distribution of isotopically labeled products. The more complex statistical distribution of peaks observed at m/z 367 and m/z 550 (Fig. 1B,C) are indicative of ion/molecule reaction products that were formed during electrospray ionization and desolvation processes. The heavier ions observed in Fig. 1 were merely clusters of the m/z 184 species, that is also observed in this mass spectrum. The phenomenon of analyte clustering during electrospray processes is well known and particularly prevalent for analytes at relatively high concentrations (Smith *et al.*, 1990).

Further demonstration of the applicability of stable isotopes to aid MRP identification is shown in Fig. 3. Here, CE was coupled on-line with a tandem mass spectrometer (CE-MS/MS), and experiments were conducted to acquire MS/MS data for the responses identified as $MH^+ = 184$ (^{14}N-MRP, Fig. 3A) and $MH^+ = 185$ (^{15}N-MRP, Fig. 3B). Comparison of these data permits easy identification of fragment ions that contain a nitrogen atom. Such ions increase in mass by one unit in the mass spectrum acquired for the ^{15}N-labeled product. In this example, peaks in the ^{14}N MRP spectrum (Fig. 3A) at m/z 67, m/z 94, m/z 108, m/z 122, m/z 124, m/z 136 and m/z 166 are all seen to increase by one mass unit in the corresponding ^{15}N MRP data (Fig. 3B). From this we conclude that all of these fragment ions contain a single nitrogen atom. Clearly, this approach aids the structural characterization of detected MS/MS fragment ions (Table 1), which ultimately yields MRP identification. Here, the detected MRP was a small molecule of molecular weight 183, which we identified as 2-formyl-5-(hydroxymethyl)pyrrole-1-acetic acid (insert of Fig. 3A). This MRP was previously detected, identified and synthesized several years ago by Olsson (Olsson *et al.*, 1978), and was a predictable component of our 5-HMF/glycine model reaction system.

In summary, while the use of labeled reagent to aid the identification of MRPs has been previously described (Tomlinson, 1991; Benson *et al.*, 1993; Tressl *et al.*, 1993; Weenan *et al.*, 1994; Huyghues-Despointes and Yaylayan, 1996; Keyhani and Yaylayan, 1996*b*, 1997), these studies further demonstrate this powerful approach for structurally characterizing products of Maillard Chemistry. In addition, CE-MS analysis of an equal ratio combination of labeled and unlabeled reaction mixtures clearly identifies artifactual responses that can be formed within the electrospray ion source. Finally, the efficacy of this approach for the identification of MRPs produced when 5-HMF was reacted in aqueous solution with glycine was demonstrated. Using the CE-MS and CE-MS/MS approaches described in this report there was no requirement for analyte modification or derivatization prior to analysis of the model reaction mixture.

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