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Outstanding Paper

Non-enzymatic glycation of melamine with sugars and sugar like compounds

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ABSTRACT

Melamine (1,3,5-triazine-2,4,6-triamine) is employed in the manufacture of plastics, laminates and glues, yet, it has been found sometimes added illegally to dairy products to artificially inflate foods' protein content. In 2008, dairy products adulterated with melamine were blamed for the death of several infants in China, a situation that forced Beijing to introduce stricter food safety measures. The objectives of this study were threefold: (1) to investigate the susceptibility of the amine groups of melamine to glycation with D-galactose, D-glucose and lactose, sugars commonly found in milk, (2) to study the rate and extent of melamine's glycation with methylglyoxal, glyoxal and DL-glyceraldehyde, three highly reactive metabolites of D-galactose, D-glucose and lactose, and (3) to characterize, using mass spectrometry, the Advanced Glycation Endproducts (AGEs) of melamine with sugars found commonly in milk and their metabolites. Incubation of D-galactose, D-glucose and lactose with melamine revealed that D-galactose was the most potent glycator of melamine, followed by D-glucose, then lactose. Methylglyoxal, glyoxal, and DL-glyceraldehyde glycated melamine more extensively than D-galactose, with each yielding a broader range of AGEs. The non-enzymatic modification of melamine by sugars and sugar-like compounds warrants further investigation, as this process may influence melamine's toxicity *in vivo*.

1. Introduction

In 2008, several infants died in China from milk formulas adulterated with melamine [1–5]. Melamine is an organic base with a 1,3,5-triazine backbone, a toxic chemical that when added to dairy products can artificially inflate foods' protein content. The purpose of this study was to investigate the susceptibility of the amine groups of melamine to glycation (Fig. 1), especially since milk is rich in reducing sugars.

Glycation is a non-enzymatic process that involves a series of steps with reactants, typically including the carbonyl groups in reducing sugars and the free amino groups in proteins. In the initial phase of glycation, the free amino groups on proteins condense with the carbonyl groups of reducing sugars to generate reversible glycosylamines. Once formed, these glycosylamines then convert to the more stable Amadori products that, with time, undergo dehydration, cyclization, oxidation, and rearrangement to form a polymorphic group of compounds referred to as Advanced Glycation Endproducts (AGEs) [6–9].

In this report, we will demonstrate using a variety of analytical techniques: (1) the non-enzymatic reactivity of melamine with sugars commonly found in milk and their metabolites, and (2) the formation of AGEs of melamine under different incubation conditions of time, temperature and pH. Focus will be placed also on the HPLC elution profiles of some the AGEs of melamine and their structures as determined by mass spectrometry.

2. Materials and methods

2.1. Chemicals and supplies

Analytical grade D-galactose, D-glucose, lactose, glyoxal, methylglyoxal, DL-glyceraldehyde and melamine were purchased from Sigma Chemical Co. (St. Louis, MO). Disposable UV-transparent cuvettes (12.5 mm × 12.5 mm × 36 mm) and HPLC analytical grade solvents were obtained from Thermo Fisher Scientific (Rockford, IL). Reverse phase HPLC columns containing C_8 silica resins were obtained from Agilent Technologies (Chelmsford, MA) with all other HPLC supplies from Phenomenex (Torrance, CA).

2.2. Preparation of reaction mixtures

2.2.1. Reaction of lactose, D-glucose and D-galactose (milk sugars) with melamine

Stock solutions of melamine (5 mM) and sugar (300 mM) were prepared in 0.1 M phosphate buffer, pH 6.7. Final incubation

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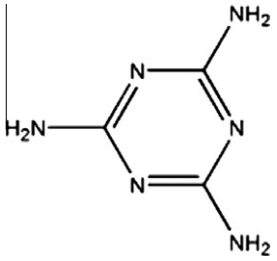

Fig. 1. Chemical structure of melamine (1,3,5-triazine-2,4,6-triamine).

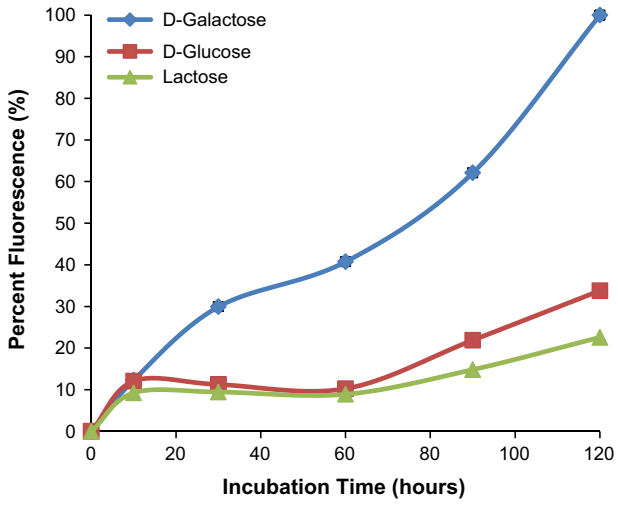

Fig. 2. Time course fluorescence spectral profiles of reaction mixtures containing D-galactose, D-glucose, and lactose with fixed concentrations of melamine (1 mM) at 80 °C for 2 h. Blank solutions were incubated under identical conditions as above and included either sugar (150 mM) alone, or melamine alone (1 mM). All points represent the average of triplicate measurements. Every reading was compared with the highest reading set at 100%. Excitation and emission wavelengths were set at 260 nm and 380 nm, respectively.

mixtures included variable amounts of melamine (0.2, 1, or 2.5 mM) with a set concentration of D-glucose, D-galactose or lactose (150 mM). Blanks included melamine alone (0.2, 1 or 2.5 mM) or sugar alone (lactose, D-glucose or D-galactose) each at a final concentration of 150 mM. Freshly mixed sugars with melamine served as the control solutions. All reaction mixtures and blanks were incubated in the dark in a shaking water bath for 2 h at variable temperatures (40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C). After incubation, samples were placed at −20 °C until analyzed.

2.2.2. Reaction of methylglyoxal, glyoxal and DL-glyceraldehyde with melamine

Unless otherwise indicated, all reactions were conducted in 0.2 M phosphate buffer, pH 7.2, containing 0.02% sodium azide. Stock solutions of melamine (5 mM), methyglyoxal (40 mM), glyoxal (40 mM) and DL-glyceraldehyde (40 mM) were prepared by separately dissolving each in the 0.2 M phosphate buffer. Final incubation mixtures included variable amounts of methylglyoxal, glyoxal or DL-glyceraldehyde (5 or 20 mM) with different concentrations of melamine (0.2, 1, or 2.5 mM). Blank solutions included methylglyoxal, glyoxal or glyceraldehyde alone (5 mM or 20 mM), or melamine alone (0.2 mM, 1 mM or 2.5 mM). Controls included non-incubated freshly mixed solutions of each aldehyde (methylglyoxal, glyoxal or DL-glyceraldehyde) with melamine. All reaction mixtures and blank solutions were incubated in the dark at 37 °C in a shaking water bath for 30 days. After incubation, samples were placed at −20 °C until analyzed.

2.3. UV and fluorescence spectroscopy

UV readings were obtained at a wavelength of 240 nm with an UltroSpec 2100 instrument (Biochrom Ltd., Cambridge, UK). Fluorescence measurements were made at respective excitation and emission wavelengths of 260 nm and 380 nm using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA). All readings were obtained in thermostatically controlled cuvettes that were maintained at 25 ± 1 °C. The above excitation and emission wavelengths were determined optimal for detecting melamine AGEs.

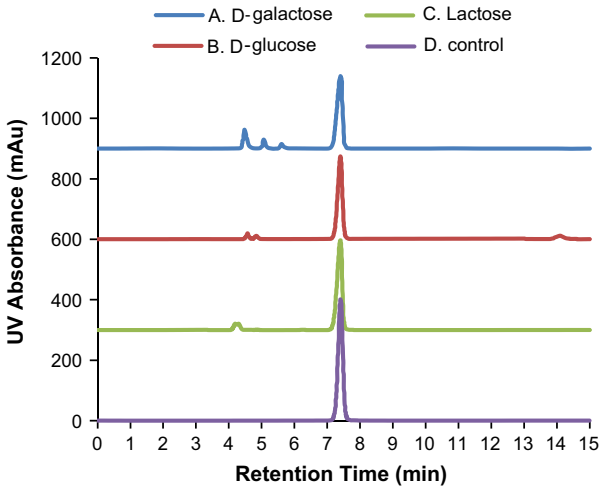

Fig. 3. HPLC elution profile of melamine (1 mM) after 1 h of incubation at 80 °C with 150 mM D-galactose (A), D-glucose (B) and lactose (C). Control reaction mixtures contained freshly mixed solutions of sugar with melamine (D). Repeat chromatographic studies by HPLC revealed no significant difference in the elution profiles and the retention times of the different AGEs.

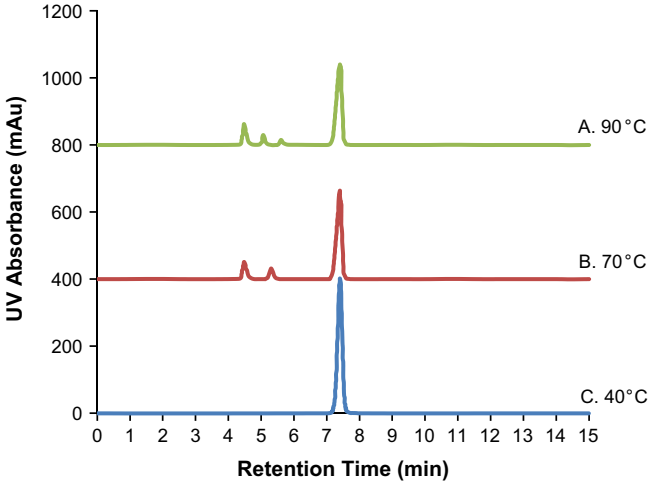

Fig. 4. HPLC elution profile of melamine (1 mM) with D-galactose (150 mM) in phosphate buffer, pH 6.7 after the incubation of the two reactants for 1 h at 90 °C (A), 70 °C (B), and 40 °C (C). Repeat chromatographic studies by HPLC revealed no significant differences in the elution profiles and the retention times of the different AGEs in the above reaction mixtures.

Table 1
UV readings of incubation mixtures containing melamine (2.5 mM) and different aldehydes (5 mM and 20 mM).

Methylglyoxal	5 mM		20 mM	
	Actual UV reading	% Relative reading	Actual UV reading	% Relative reading
0 d	0.000 ± 0.000	0.000	0.000 ± 0.000	0.000
1 d	0.075 ± 0.002	0.336	0.271 ± 0.003	0.321
2 d	0.080 ± 0.001	0.359	0.290 ± 0.003	0.344
30 d	**0.223 ± 0.002**[a]	**1.000**	**0.844 ± 0.002**[a]	**1.000**
Glyoxal				
0 d	0.000 ± 0.000	0.000	0.000 ± 0.000	0.000
1 d	0.046 ± 0.001	0.206	0.173 ± 0.003	0.205
2 d	0.051 ± 0.003	0.229	0.184 ± 0.003	0.218
30 d	0.116 ± 0.001	0.520	0.366 ± 0.004	0.434
DL-glyceraldehyde				
Od	0.000 ± 0.000	0.000	0.000 ± 0.000	0.000
1 d	0.022 ± 0.002	0.099	0.025 ± 0.004	0.030
2 d	0.026 ± 0.001	0.117	0.031 ± 0.002	0.037
30 d	0.080 ± 0.002	0.359	0.302 ± 0.003	0.342

Reaction mixtures included methylglyoxal, glyoxal and DL-glyceraldehyde (5 mM or 20 mM) with melamine (2.5 mM) in phosphate buffer, pH 7.2 at 37 °C for 30 days. Blank solutions were incubated as above with either individual aldehydes alone, or melamine alone. The absorbance of the blank solution was subtracted from all readings. % Relative readings were calculated based on the highest UV values obtained within each set of reaction mixtures (5 mM or 20 mM aldehyde) evaluated.
[a] Denotes the highest UV readings in each set of incubation mixtures.

2.4. High-performance liquid chromatography

Each HPLC run was performed in triplicate using a Hitachi system (San Jose, CA, USA) equipped with a low-pressure gradient pump (L-2130), a four-channel degasser, a sequential auto sampler (L-2200), and a high sensitivity diode-array detector (190–800 nm) (L-2455). AGE species were separated on a C_8 reverse phase HPLC column (5 μm × 4.6 mm × 150 mm) and monitored at 240 nm. Mobile phase consisted of a 92% solution of 10 mM citrate heptane sulfonate buffer, pH 3.0 and 8% acetonitrile. An isocratic condition was applied for 15 min at a constant flow rate of 1.00 ml min^{-1}. Prior to HPLC analysis, all samples were filtered with a 0.45 μm membrane (Millipore, Billerica, MA, USA), degassed for 15 min, and centrifuged.

2.5. Mass spectrometry

Mass spectrometry studies were performed on a ThermoFinnigan LCQ mass spectrometer (ThermoScientific, Waltham, MA) equipped with an electrospray ionization source and a quadrupole ion trap mass analyzer. Samples were diluted into a solvent consisting of 50/50 (*v*/*v*) 0.1% acetic acid in water/acetonitrile and directly infused into the electrospray source using a capillary syringe pump at a flow rate of 3 μL min^{-1}. Nitrogen was used as the sheath gas (setting at 60), and ultrapure helium was used as the collision gas. The ion spray voltage was set as 4.5 kV and the capillary temperature was 210 °C. The mass spectrometer was set to function in the positive ion mode with parameters optimized during direct infusion of caffeine standards with solvent. Samples were analyzed by MS and tandem mass spectrometry (CID fragmentation with He) favoring isolation and fragmentation of singly charged ions. The ESI/MS system was operated with the Xcalibur software (version 2.0, ThermoFinnigan), with the same software used also for data analysis.

3. Results

Fig. 2 shows the time course of melamine's glycation with D-glucose, D-galactose and D-lactose under temperature and pH conditions employed in the manufacture of powdered milk products. The amounts of D-glucose, D-galactose and lactose were adjusted to actual concentrations of the sugars in unprocessed milk and lactose free milk formulas [10]. At pH 6.7 and 80 °C for 120 min, D-glucose and D-lactose were found to be poor glycators

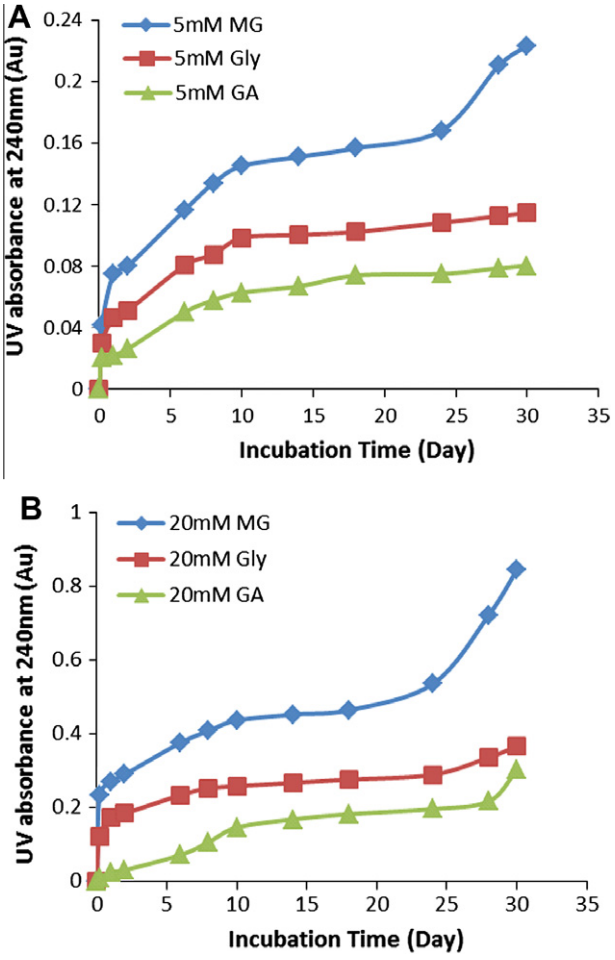

Fig. 5. A and B show the UV absorbance profiles of incubation mixtures containing varied amounts of methylglyoxal, glyoxal and DL-glyceraldehyde with set concentrations of melamine (2.5 mM) in phosphate buffer, pH 7.2 at 37 °C for 30 days. Blank solutions were incubated as above with either aldehyde alone (5 mM and 20 mM), or melamine alone (2.5 mM). All UV readings were subtracted from the blank readings.

Table 2
Peak retention times and percentages of AGE band areas resulting from the separation of incubation mixtures containing methylglyoxal, glyoxal and DL-glyceraldehyde with melamine by HPLC.

Incubation time (day)	[a]% Band area of nonglycated melamine (Rt = 7.4 min)	HPLC Peak Retention Times of Melamine AGEs % Band Areas of Melamine AGEs Relative to Total Band Areas									
		Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10
Methylglyoxal											
0	100.0%										
2	74.9%	8.4 min 12.0%	8.8 min 13.1%								
10	54.4%	6.2 min 6.5%	6.7 min 6.1%	8.3 min 3.4%	8.7 min 16.0%	10.6 min 8.6%	12.1 min 4.9%				
30	39.9%	2.9 min 2.3%	5.2 min 1.1%	5.6 min 9.8%	6.0 min 17.4%	6.5 min 1.5%	6.8 min 1.2%	7.9 min 1.1%	8.5 min 2.5%	11.0 min 3.8%	12.8 min 19.3%
Glyoxal											
0	100.0%										
2	61.6%	6.0 min 38.4%									
10	29.3%	4.4 min 4.0%	5.4 min 3.6%	6.1 min 63.1%							
30	3.5%	2.9 min 1.1%	4.4 min 11.7%	4.7 min 9.5%	5.4 min 12.8%	5.8 min 15.4%	6.0 min 46.0%				
DL Glyceraldehyde											
0	100.0%										
2	91.9%	3.8 min 3.6%	5.0 min 4.5%								
10	75.9%	2.1 min 3.9%	3.8 min 4.7%	4.8 min 13.1%	5.0 min 2.3%						
30	54.5%	2.9 min 1.9%	3.8 min 11.9%	4.9 min 16.3%	5.0 min 14.1%						

[a] Denotes the fractions of nonglycated melamine eluted at retention time of 7.4 min.

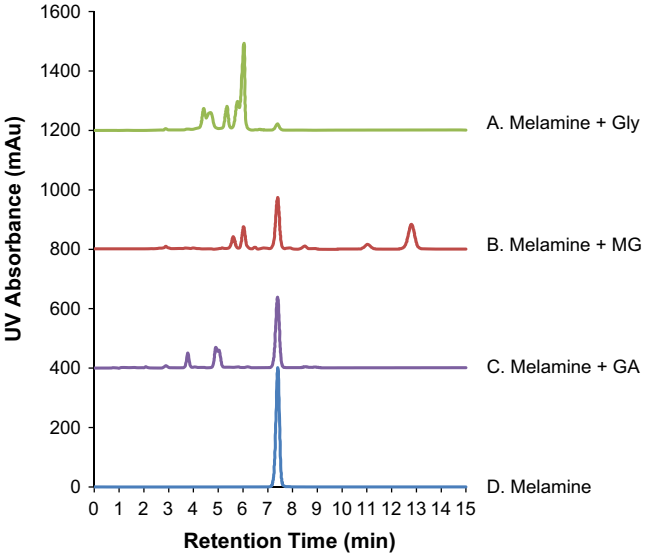

Fig. 6. HPLC elution profiles of melamine (2.5 mM) with methylglyoxal (20 mM) (A), glyoxal (20 mM) (B) and DL-glyceraldehyde (20 mM) (C) after 30 days incubation at 37 °C. Figure (D) shows HPLC elution profile of melamine alone (2.5 mM) or melamine freshly incubated with aldehydes (20 mM each). All separations were performed on C_8 reverse phase HPLC column (5 μm × 4.6 mm × 150 mm) as described previously. Repeat chromatographic studies by HPLC revealed no significant differences in the elution profiles and the retention times of the AGE peaks.

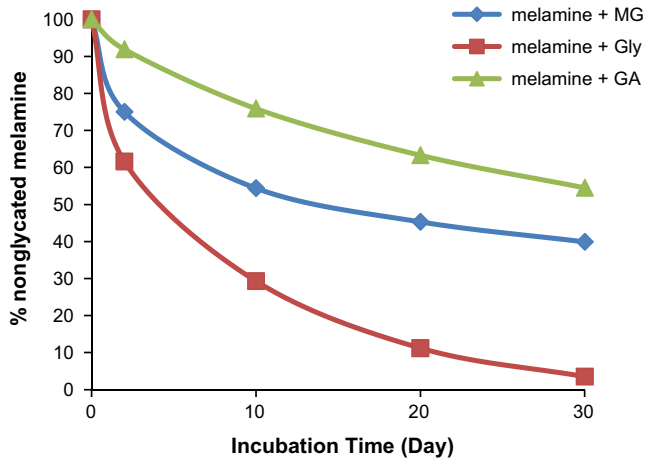

Fig. 7. The order of reactivity of methylglyoxal, glyoxal and DL-glyceraldehyde (20 mM each) with 2.5 mM melamine. Glyoxal was the most reactive glycator of melamine followed by methylglyoxal then DL-glyceraldehyde. The percentages of nonglycated melamine were calculated on the basis of the ratio of HPLC band areas of nonglycated melamine to total band areas in each chromatograph. All values in the figure are the average of triplicate measurements. The variation of each set of triplicate readings is less than 1%.

of melamine yielding fluorescence spectral profiles that were nearly superimposable. Under equivalent incubation conditions, mixtures of D-galactose and melamine yielded higher fluorescence readings demonstrating that relative to D-glucose and D-lactose, D-galactose was a more effective glycator of melamine. As expected, the blank solutions and controls yielded no changes in their fluorescence, confirming that for glycation to occur, both sugar and melamine had to be in the same reaction mixture and incubated over time.

The pronounced reactivity of D-galactose relative to D-glucose may be explained by the enhanced general instability of the hemiacetal ring of D-galactose, which in solution renders the molecule more prone to an open-chain configuration [11,12]. Thus, with a higher percentage of the sugar existing in an open ring structure than D-glucose, D-galactose becomes more vulnerable to nucleophilic attack by the amino groups on melamine [11,12]. This phenomenon may also explain galactose's higher reactivity than D-lactose since as a disaccharide, lactose contains D-glucose at it reducing end.

Fig. 3A–C display the respective HPLC elution profiles of mixtures of melamine incubated with D-galactose, D-glucose and lactose at 80 °C for 1 h. Fig. 3D shows the HPLC elution profiles of solutions containing melamine alone (blank), and melamine freshly added to D-galactose, D-glucose or D-lactose (controls). Melamine alone eluted as one prominent peak with a retention time (R_t) of 7.40 min. Each of the control mixtures yielded an elution

profile identical to the blank, showing one peak coincident to where melamine eluted (R_t 7.40 min).

The HPLC chromatograms in Fig. 3A–D revealed two important things: (1) that glycation was a time dependent process requiring the presence of both melamine and sugar, and (2) that D-galactose was a more potent glycator of melamine than D-glucose and D-lactose, a finding consistent with the fluorescence data in Fig. 2. Incubation mixtures of D-galactose with melamine yielded three distinct AGE products by HPLC. Of the bands corresponding to the AGE products, one eluted with a retention time of 3.85 min, one at 4.49 min and one at 5.08 min (Fig. 3A). A fourth band appearing at a retention time of 7.40 min corresponded to the fraction of melamine that did not condense with D-galactose. Integration of the band areas revealed that with D-galactose as the glycating sugar 32.1% of melamine was non-enzymatically modified; i.e., in contrast to 12.3% with D-glucose and 6.3% with D-lactose. Other results revealed that reducing the incubation temperatures, reduced the extent glycation of melamine and the number of AGEs of melamine in the reaction mixtures. Fig. 4A–C exemplify this behavior with D-galactose as the model glycating agent.

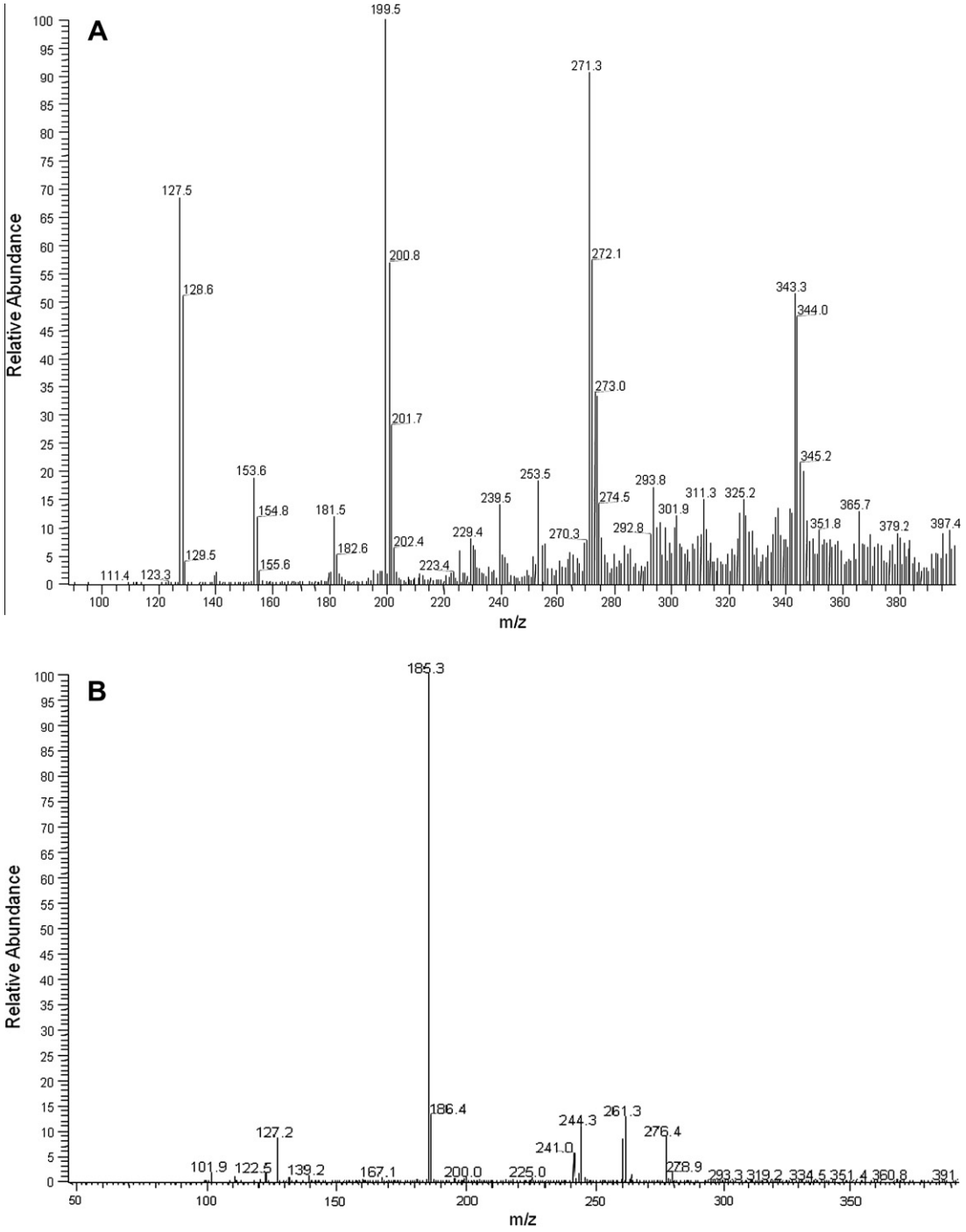

Fig. 8. Mass spectral analysis of solutions of melamine (2.5 mM) incubated with methylglyoxal (20 mM) (A), glyoxal (20 mM) (B) and DL-glyceraldehyde (20 mM) (C) at 37 °C for 30 days, and at 80 °C for 1 h with D-galactose (150 mM) (D).

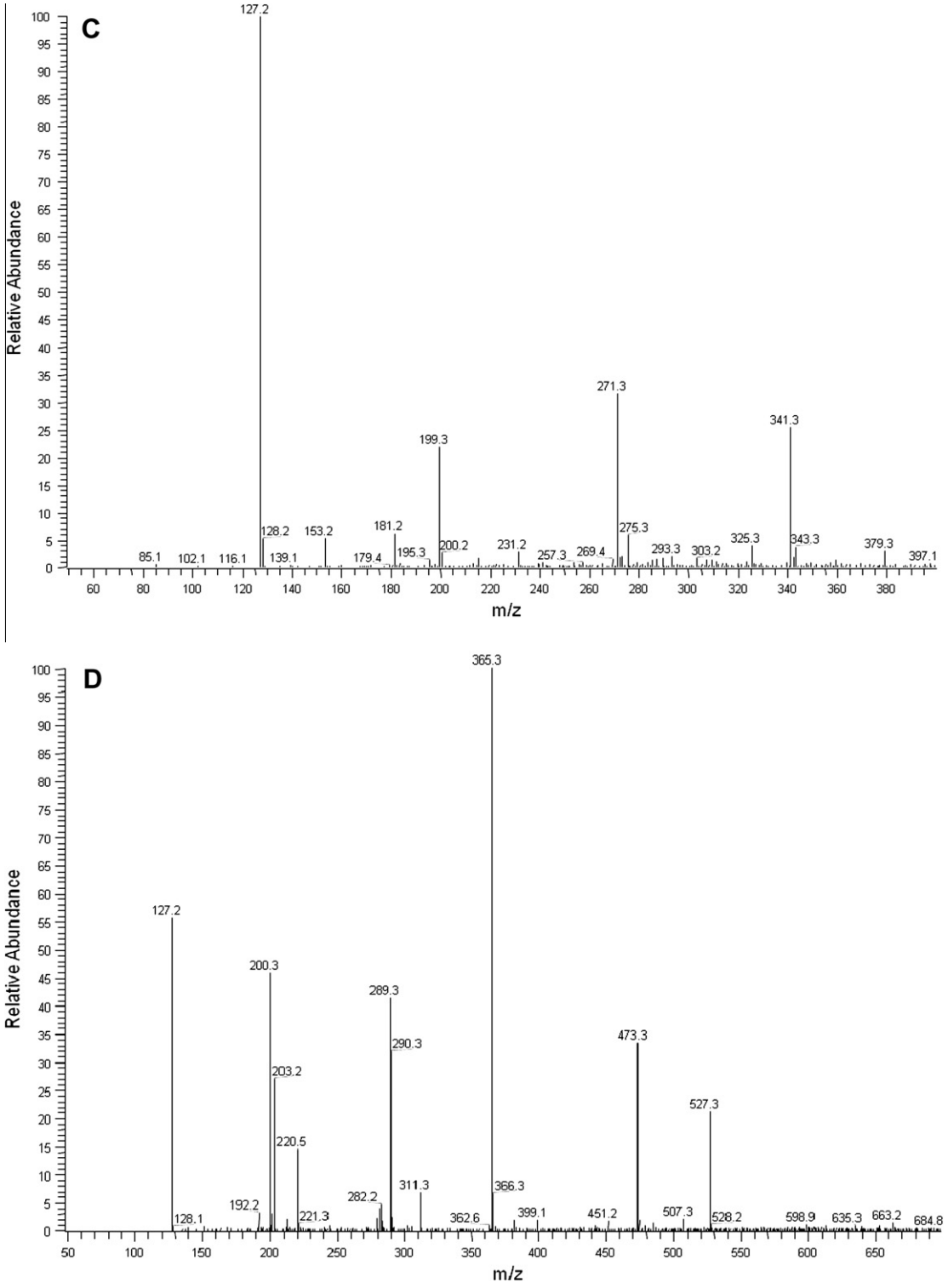

Fig. 8. (*continued*)

Tables 1 displays the changes in UV absorption readings of incubation mixtures of melamine with methylglyoxal, glyoxal and DL-glyceraldehyde (hereon referred to as "aldehydes") at 37 °C covering different time intervals. Increasing incubation time enhanced the AGE formation of melamine with the highest levels of glycation achieved when aldehyde concentrations were adjusted to 20 mM. A close scrutiny of the table confirmed the earlier observations that the extent glycation of melamine was a time dependent process relatable to the chemistry and concentration of the aldehydes in the incubation mixtures. Increases in the aldehyde concentrations prompted AGE formation, with methylglyoxal yielding the highest UV absorbing products followed by glyoxal then DL-glyceraldehyde (Fig. 5A and B). Increases in melamine concentration in the presence of set amounts of the various aldehydes also promoted AGE

formation, demonstrating that the rate of glycation reaction was dependent on both the concentrations of melamine and the various aldehydes (data not shown). Using variable amounts of melamine in the incubation mixtures had no effect on the intensity order of the glycated compounds that were formed with methylglyoxal and DL-glyceraldehyde, once again, producing AGEs with the highest and lowest UV intensities, respectively.

Table 2 and Fig. 6A–C show the HPLC retention times of the various AGEs of melamine with methylglyoxal, glyoxal and DL-glyceraldehyde at 37 °C over 30 days. Included in Table 2 is also the number of AGE products and their percent amount relative to the total AGEs in each incubation mixture over time. The band eluting at retention time 7.4 min was assigned to nonglycated melamine; an observation based on the HPLC elution profiles of both the blank and control solutions (Fig. 6D). Analysis of the data in Table 2 showed that with methylglyoxal there were more AGE products formed than with glyoxal and DL-glyceraldehyde, with DL-glyceraldehyde yielding the lowest number of AGEs. A further evaluation of the results in Table 2 revealed that glyoxal was the most active glycator of melamine followed by methyglyoxal then DL-glyceraldehyde (Fig. 7).

Fig. 8A–D shows the mass spectral profiles of melamine incubated with individual aldehydes at 37 °C for 30 days, and with D-galactose at 90 °C for 2 h. Mass spectral analysis of melamine alone yielded one primary peak located at m/z 127 (data not shown). In reaction tubes containing melamine and methylglyoxal (Fig. 8A), the main product ions were at m/z 199, 271, and 343. The product with m/z 199 corresponded to the addition of one methylglyoxal monomer (Δ m/z 72) to melamine, whereas those with m/z 271 and 343 corresponded to the condensation of two and three monomers.

For the reaction with glyoxal (Fig. 8B), a prominent peak at m/z 185 was observed. This ion corresponded to the addition of one glyoxal monomer (Δ m/z 58) to melamine. In contrast to glyoxal and methylglyoxal, glyceraldehyde condensed with melamine through a Schiff base/Amadori reaction, forming a product ion at m/z 199 (Fig. 8C). The mass spectrometric profile of D-galactose with melamine was more difficult to interpret (Fig. 8D); however, the product ion at m/z 289 suggested the occurrence of Schiff base formation between the anomeric carbon of D-galactose with one of the amino groups of melamine. The additional ions indicated that more than one D-galactose monomer could have condensed with

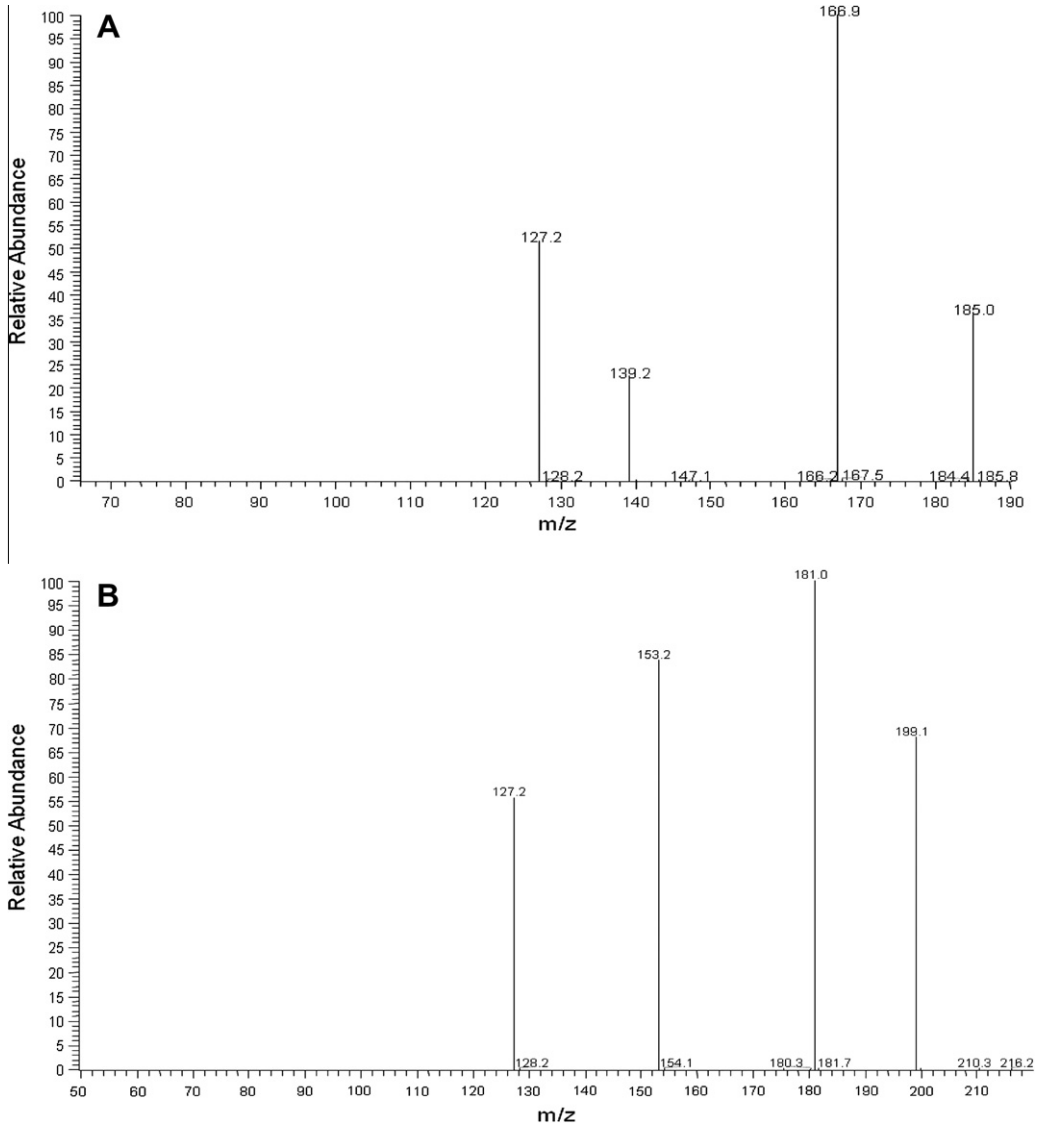

Fig. 9. CID mass spectra of the $(M + H)^+$ ion for (A) glyoxal melamine product at m/z 185 and (B) methylglyoxal melamine product at m/z 199.

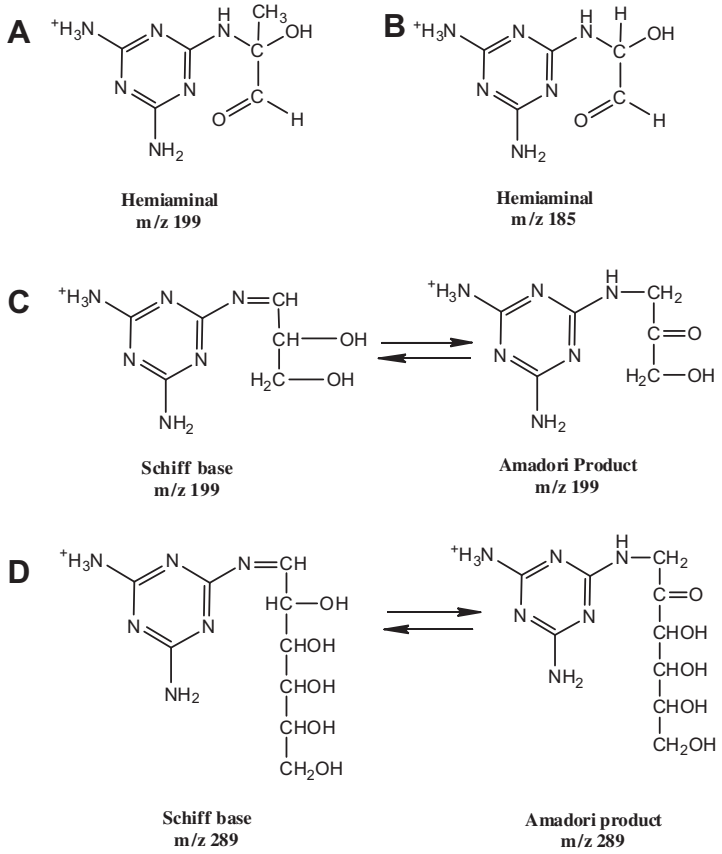

Fig. 10. Elucidation of structures consistent with mass spectrometric data for primary products formed in incubation mixtures containing melamine with methyglyoxal (A), glyoxal (B), DL-glyceraldehyde (C), and D-galactose (D).

melamine with the latter then rearranging to yield multiple products.

Fig. 9A shows the CID mass spectrum for glyoxal's primary product ion at m/z 185 (Fig. 8A) with the main fragmentation pattern revealing a loss in water (−18 Da) and a loss in CO (−28 Da). The absence of a peak at m/z 141 indicated that no carboxylic acid functional group was present. This conclusion was further supported by the absence of a peak at m/z 183 after repeated analysis of the sample in the negative ion mode. The product ion at m/z 185 appears to contain a hemiaminal structure without loss of water forming a Schiff base at pH 7.2.

Fig. 9B shows the CID mass spectrum for methylglyoxal's product ion at m/z 199 with the main fragmentation pattern, again, showing a loss in water (−18 Da), and a loss in CO (−28 Da). This suggests that the reaction of melamine with methylglyoxal was similar to that of glyoxal in forming a hemiaminal structure.

Fig. 10A–D displays the proposed structures consistent with the mass spectral data for the primary products formed in incubation mixtures containing melamine with methylglyoxal, glyoxal, DL-glyceraldehyde and D-galactose. A detailed analysis of the predominant product ions generated from the reaction of melamine with methylglyoxal (Fig. 9A) leads to the postulated mechanism pathway for the formation of AGEs seen in Fig. 11. It should be noted that a portion of the signal for ion at m/z 181 in the mass spectrum (Fig. 9A) could be arising from the dehydration of the ion at m/z 199 in the electrospray source.

Other results revealed that increases in pH promoted the formation of melamine AGEs with the sugars commonly found in milk and their metabolites (data not shown).

4. Discussion

Melamine has been employed in many commercial products such as adhesives, laminates, and plastics. Yet lately, it has been used also as an adulterant in many dairy products to artificially elevate food's protein content [13,14]. The adulteration of dairy products with melamine was recently blamed for the death of several infants in China, a situation that resulted in stricter food safety controls not only in China but other countries [2,15,16].

Melamine is rich in nitrogen and contains three amino groups which form part of its hexagonal structure (Fig. 1). These amino groups make melamine a target for glycation as we have shown here in this study. Our work with melamine revealed four important findings. First, it demonstrated that melamine can non-enzymatically react with sugars most abundant in milk and their metabolites. Second, it revealed that glycated products of melamine can with time form AGEs that could be readily resolvable by HPLC. Third, the observation was made that not all sugars and aldoses reacted at the same rate and extent with melamine and that increased temperatures and pH accelerated AGE formation. Lastly, mass spectrometric data revealed that glycation of melamine occurred via the Amadori pathway (Fig. 11) yielding different AGE products with methylglyoxal, glyoxal, DL-glyceraldehyde and D-galactose.

At present, there is no or little information on the uptake and toxicity of glycated melamine products. The high reactivity of melamine with the sugars commonly found in milk and their metabolites warrants further evaluation of melamine's AGEs *in vitro* and *in vivo*.

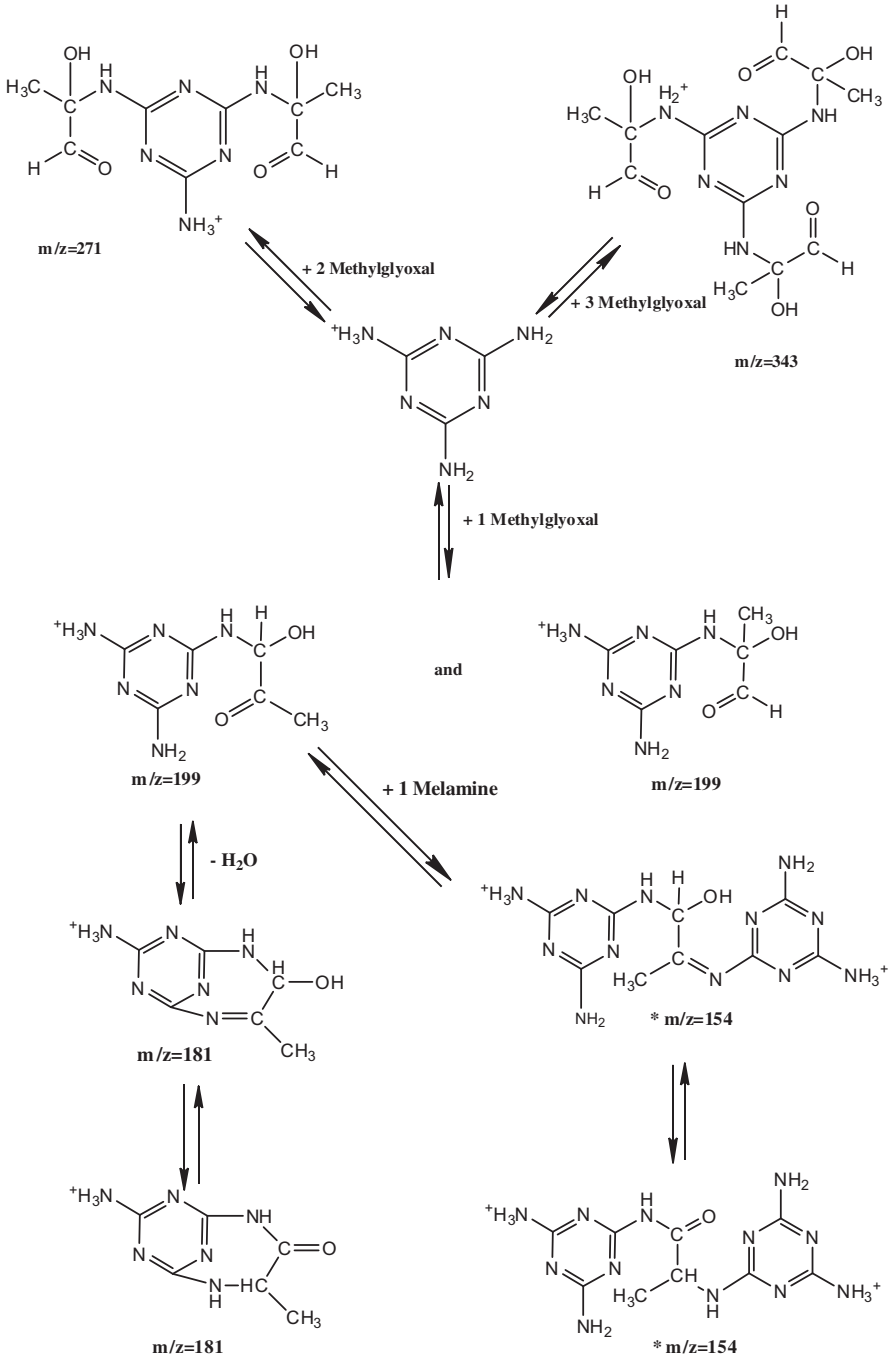

Fig. 11. Postulated pathway for the formation of AGEs of melamine with methylglyoxal. *Denotes $(M + 2H)^{+2}$ ion at m/z 154.

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