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Study on the Decomposition of the Amadori Compound *N*-(1-Deoxy-D-fructos-1-yl)-glycine in Model Systems: Quantification by Fast Atom Bombardment Tandem Mass Spectrometry

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A new method for the quantification of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) was developed based on isotope dilution fast atom bombardment tandem mass spectrometry using ^{13}C-labelled DFG as an internal standard. This method, which requires neither derivatization nor clean-up of the samples, was used to study the degradation of DFG under different conditions (time and pH). It was found that the decomposition of DFG at 90 °C was favoured at pH 7 compared to pH 6. The higher stability of DFG at pH 6 was due to 1,2-enolization leading to the relatively stable 3-deoxglucosone.

INTRODUCTION

Amadori compounds are *N*-(1-deoxyketos-1-yl)-amine derivatives formed in the course of the non-enzymatic browning (Maillard) reaction.[1] They are key intermediates of the Maillard reaction and responsible for changes in colour, flavour and nutritive value of foods.[2,3] They have been found, for example, in malt and brewed products,[4,5] dried vegetables,[6] soya sauce[7] and cocoa.[8]

The degradation pathways of Amadori compounds have been widely discussed.[9] They decompose (Scheme 1)[1] *via* the enediol or the aminoenol intermediate and form, by 2,3-enolization and 1,2-enolization, the reaction products 1-deoxy-2,3-hexodiulose (1-deoxyosone) and 3-deoxy-2-hexosulose (3-deoxyosone), respectively.[10] These reactive α-dicarbonyls show a tendency to further enolization, dehydration and carbon fission and are thus capable of forming polymeric colourants and volatile compounds.

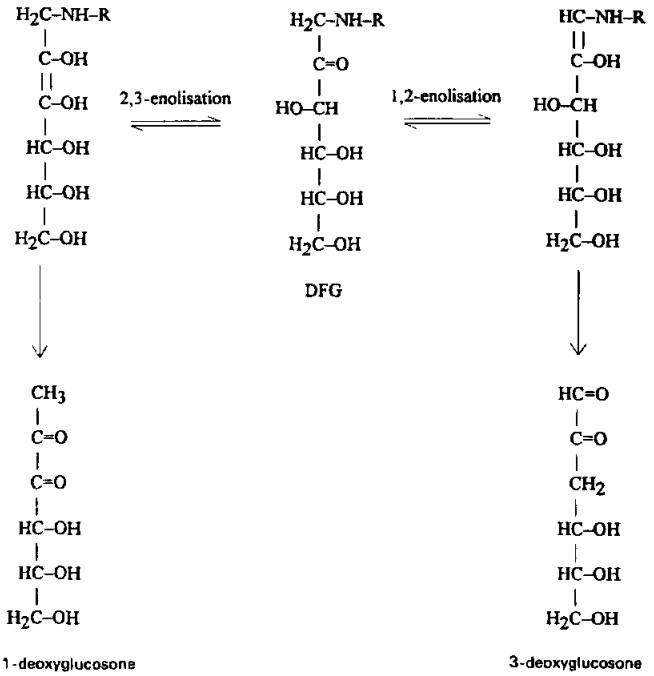
Several methods have been reported for the quantitative analysis of Amadori compounds,[11] including high-performance liquid chromatography (HPLC)[6] and capillary gas chromatography/mass spectrometry (GC/MS).[4] The latter involves oximation of the isolated Amadori compounds followed by silylation, thus theoretically resulting in two peaks (*syn* and *anti* isomers) for each Amadori compound. In order to simplify the isolation procedure, to avoid any derivatization step and to maintain high selectivity and sensitivity of the mass spectrometer, we have developed a new, accurate method based on isotope dilution fast atom bombardment tandem mass spectrometry (FAB MS/MS). Isotope dilution with FAB mass spectrometry provided a high dynamic range.[12] MS/MS gave a compromise between improved selectivity and decreased sensitivity.

The aim of our work was to study the decomposition of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) in model systems. The newly developed quantification method for DFG based on isotope dilution FAB MS/MS is reported here in detail, as is the synthesis of the ^{13}C-labelled DFG used as internal standard.

EXPERIMENTAL

Sythesis of *N*-(1-deoxy-D-fructos-1-yl)-(1-^{13}C)glycine

The target compound was synthesized according to the literature.[13,14] A mixture of 50 g (278 mmol) of D-(+)-



Scheme 1. Decomposition pathway of the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) according to Ledl and Schleicher.[1] R = CH_2COOH.

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glucose (Merck, Switzerland) and 8 g (42 mmol) of sodium pyrosulphite (Siegfried, Switzerland) in 7.5 ml of water was heated to 95 °C. After stabilization of the temperature 5 g (66 mmol) of (1-^{13}C)-glycine (Tracer Tech. Inc., Sommerville, Massachusetts) were added step by step. The temperature was then held for 1 h at 95 °C before cooling. The orange-brown reaction mixture was diluted with aqueous ethanol to a final volume of 360 ml containing 61% ethanol. The whole mixture was then transferred to a cationic ion-exchange column (Amberlite IR 120 H^+, Rohm & Haas). The remaining glucose was washed out with ethanol–water (7:3, v/v) and water, then the labelled DFG was eluted with 0.2 N ammonia (15 ml l^{-1} 25% ammonia, Merck, Switerland). The elution was monitored by thin-layer chromatography (SiO_2 HPTLC, Merck) using butanol–water acetic acid (2:1:1, v/v) as mobile phase. The solvent was evaporated and the residue recrystallized in ethanol–water (7:3 , v/v). The yield was 2.3 g (9.7 mmol; 15% theor.) of white *N*-(1-deoxy-D-fructos-1-yl)-(1-^{13}C)-glycine.

Non-labelled DFG was synthesized using the same reaction scheme.

The purity of the labelled and the non-labelled DFG was checked by nuclear magnetic resonance (NMR) and elementary analysis: for (^{13}C)DFG, C 40.76% (40.76%), H 6.33% (6.35%), N 5.84% (5.88%); for non-labelled DFG, C 40.30% (40.51%), H 6.34% (6.37%), N 5.94% (5.94%).

Stock solutions of the labelled and the non-labelled DFG were prepared in water prior to use.

Sample preparation for kinetic studies

The decomposition of DFG was studied at pH 6 and pH 7. For each series of experiments, 1 M solutions of DFG were prepared by dissolving 1.19 g of DFG in 5 ml of distilled water. The pH of the solution was 6. To perform the reaction under neutral conditions, the pH was adjusted with 6 N NaOH prior to heating. The samples were then heated for 15, 30, 60 or 120 min at 90 °C. The kinetics were performed at constant pH using a pH-stat device (Metrohm, Switzerland). About 0–1 ml 6 N NaOH was automatically added dropwise to the mixture during the experiments to maintain the pH at its initial value. The reaction mixture was immediately cooled down to room temperature and stored at −20 °C before further treatment.

Sample preparation for isotope dilution FAB MS/MS

The reaction vials were vigorously shaken on a Vortex and centrifuged to separate possible precipitated material. Then, 50 µl of the sample were added to 1 ml water. Fifty microlitres of the diluted solution were added to 50 µl of (^{13}C)DFG stock solution (2 mg ml^{-1}) and 1 µl of it was mixed directly on the probe tip with approximately 1 µl of a thioglycerol-glycerol (1:1) matrix.

The calibration curve was prepared by mixing 50 µl of various concentrations of (^{12}C)DFG (ranging from 0.02 mg ml^{-1} to 8 mg ml^{-1}) with 50 µl of (^{13}C)DFG stock solution. Aliquots (1 µl) were directly placed on the probe tip and mixed with approxiamtely 1 µl of the matrix.

Mass spectrometry

All experiments were performed on a Finnigan TSQ700 triple-quadrupole mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an Ion Tech saddle-field atom gun operated at 10 kV and 0.2 mA using xenon as gas. The mass spectrometer was run under full data control (ICIS II).

For the isotope dilution experiments the triple quadrupole was set to fragment ion scan mode. With the first quadrupole, the protonated molecular ion of either DFG (*m/z* 238) or labelled (^{13}C)DFG (*m/z* 239) was selected. At the same time the third quadrupole was set to scan the masses 220 Da and 221 Da respectively, representing the loss of one water molecule from the $[M + H]^+$ ion. The offset of the collision cell was set to 10 eV (laboratory reference). Argon was the collision gas at a pressure of 1.6 mTorr. The two mass-analysing quadrupoles scanned alternatively for 0.1 s the two sets of corresponding masses. Both quadrupoles were tuned for unit mass resolution.

RESULTS AND DISCUSSION

Fast atom bombardment tandem mass spectrometry of DFG

FAB mass spectrometry produces from DFG the commonly observed protonated molecular ion with little fragmentation. The protonated molecular ion for DFG is 238 Da and for ^{13}C-labelled DFG is 239 Da.

Figure 1 shows the FAB mass spectra of the labelled and the non-labelled DFG. In addition to the molecular ion, the losses of one or two water molecules is observed leading to the ions at 221/220 Da and 203/202 Da, respectively.

The CID spectrum of the protonated molecular ion leads to similar spectra showing enhanced fragment ions $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$, and an additional fragment corresponding to protonated glycine at *m/z* 77 and 76 for the labelled and the unlabelled DFG, respectively. This low-mass fragment ion was of too low intensity to be used for the quantification.

The acquisition of a fragment ion instead of the protonated molecule itself enhances the selectivity and therefore decreases the probability of interference through matrix ions or ions from other compounds in the mixture.

The use of thioglycerol in the matrix and the presence of sodium ions from the stabilization of the pH during the reaction process could form a sodium adduct of two matrix molecules at *m/z* 239 as a possible interfering ion. This ion also loses one water molecule, producing a fragment ion of the mass of 221 Da. However, thioglycerol easily undergoes oxidation to the disulphide,

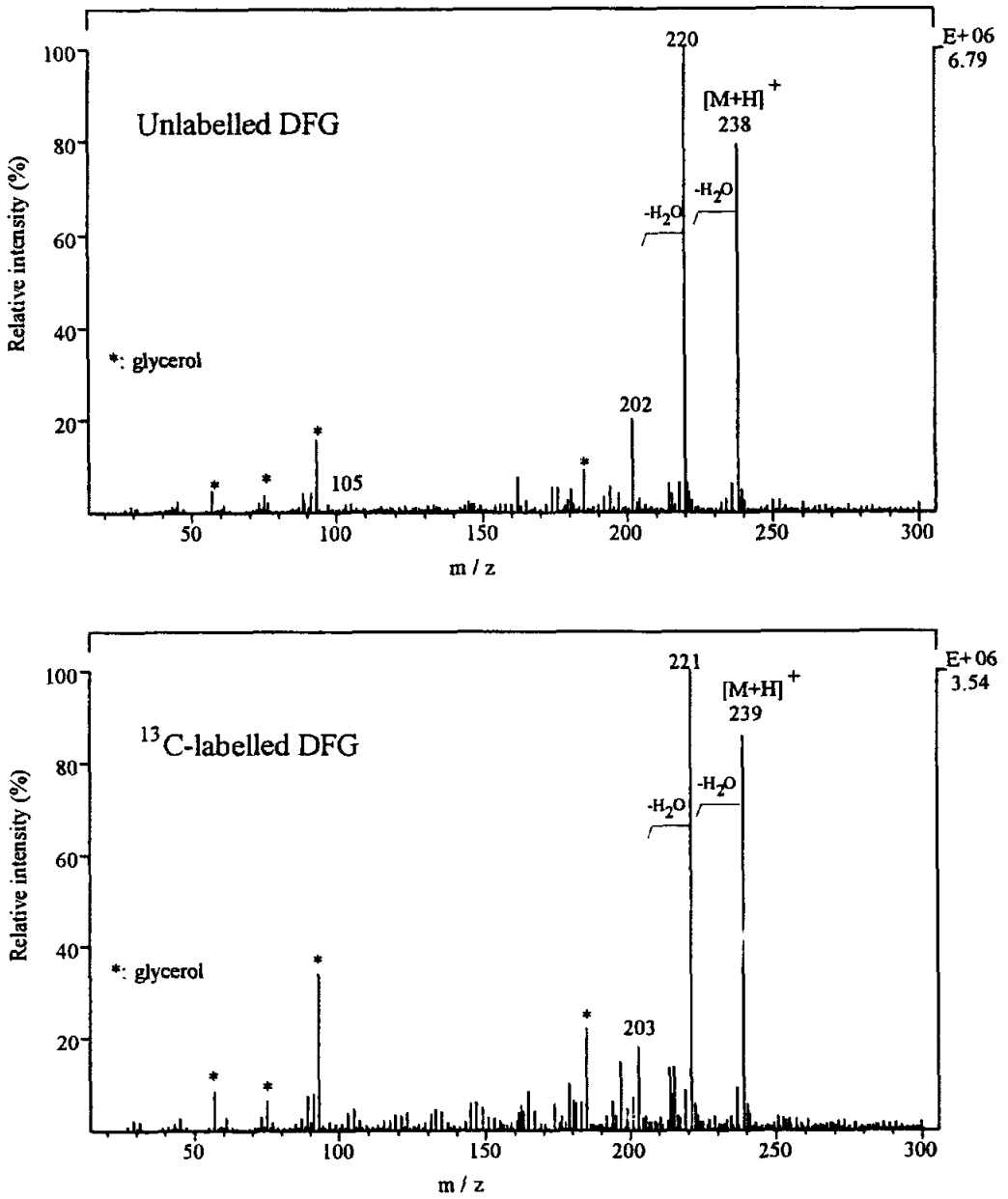

Figure 1. FAB mass spectra of the non-labelled and the ^{13}C-labelled DFG showing the two neutral losses of a water moiety from the protonated molecular ion.

which forms an ion at *m/z* 215. Its sodium adduct appears at *m/z* 237. Therefore no interfering ions were observed.

The calibration curve (Fig. 2) was prepared by adding various amounts of DFG to a constant known amount of the ^{13}C-labelled DFG and, thereafter, by measuring the ratio of the signal at *m/z* 220 to the signal at *m/z* 221. The results show that the calibration line represents a second-order curve. This is due to the natural ^{13}C isotope peak of the unlabelled DFG (9.8% at mass 221) which coincides with the acquired mass of the internal standard.[15] This second-order calibration curve was used to quantify DFG in model reactions.

The goodness of fit (accuracy) of the measured values with the theoretical values was checked as follows. The theoretical ^{13}C enrichment of the labelled DFG was calculated for each calibration point, knowing the

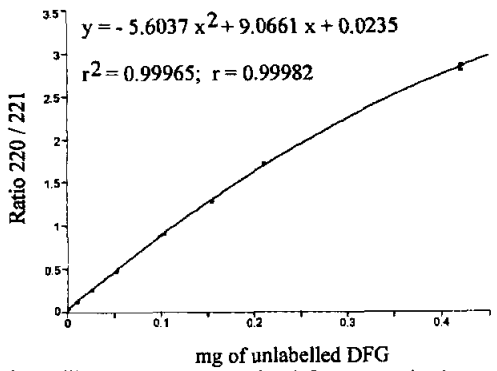

Figure 2. Calibration curve obtained from standard compounds in water. The curve was prepared by adding various amounts of DFG to a constant known amount of the ^{13}C-labelled DFG. Each point represents the mean of three determinations.

amount of labelled and unlabelled DFG in the mixture. The ^{13}C enrichments were expressed in mole per cent excess (MPE) and were calculated according to the formula:

$$[Q_l/(Q_l + Q_u)] \times 100$$

where Q_l = quantity of (^{13}C)DFG in the calibration point; and Q_u = quantity of (^{12}C)DFG in the calibration point.

Furthermore, with the measured ratio of the intensities of the masses 221/220, we calculated the measured ^{13}C enrichment:[15]

$$[(R_s - R_O)/(1 + R_s - R_O)] \times 100$$

where R_s = ratio 221/220 in each calibration point; and R_O = ratio 221/220 measured with pure unlabelled DFG.

Finally, we plotted theoretical ^{13}C enrichment versus measured ^{13}C enrichment (Fig. 3). This curve displays a slope of 0.9727, which slightly deviates from the theoretical value of 1.0000. This difference is due to the isotopic enrichment of the starting (1-^{13}C)glycine (99 at.% excess) used for the preparation of (^{13}C)DFG and due to the chemical purity of the synthesized DFG isotopomers (99% each). The correlation coefficent of the curve was calculated as 0.99977. These experiments demonstrate that the measured results are in good agreement with the theoretical values.

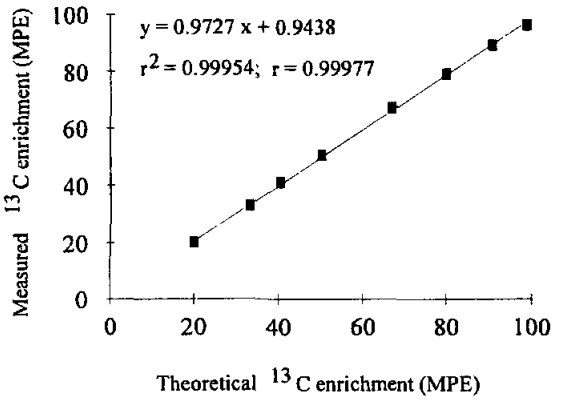

Figure 3. Determination of the accuracy of the measurements. The theoretical ^{13}C enrichments were calculated from the known amounts of (^{13}C)DFG and (^{12}C)DFG mixed together. The measured ^{13}C enrichments were obtained from the ratio of the measured masses 220 to 221. Each point represents the mean of three determinations.

The repeatability of the method was checked with several samples containing the same amount of DFG. Each sample was diluted separately. The results are listed in Table 1, where the ratio 220/221 is the ratio of the observed fragment ion and represents the mean of two measurements.

This rapid method using the labelled DFG as internal standard works well for the quantification of DFG. Other Amadori compounds vary in their hydrophobicity and therefore their presence at the surface of the matrix would change in a non-linear manner. For each target compound the corresponding labelled Amadori compound therefore has to be synthesized and the experiment has to be repeated.

It must be pointed out that this FAB MS/MS method does not tolerate too strong salt concentrations or buffer solutions. Preliminary experiments showed that high concentrations of alkali metals favour the formation of the single- or multi-metalated molecular species which lowers the accuracy of quantification. Buffers, on the other hand, lead to a complete suppression of all the signals, and thus prevent any quantification.

Kinetics of the degradation of DFG

The method was applied to different model reaction mixtures to determine the kinetic properties of the degradation of DFG. Figure 4 represents the decomposition rate of DFG in an unbuffered aqueous solution at 90 °C over a period of 2 h at pH 6 (■) and pH 7 (○), kept constant during the whole reaction.

Continuous decomposition of DFG was observed in all samples analysed. The decomposition rate was

Table 1. Repeatability of the method after measuring twice several samples containing the same amount of DFG

	Ratio of the ions 220/221	Standard deviation	Variation coefficient (%)
Sample A	2.96	0.03	1.00
Sample B	2.84	0.03	0.91
Sample C	2.88	0.01	0.35
Sample D	2.80	0.01	0.52

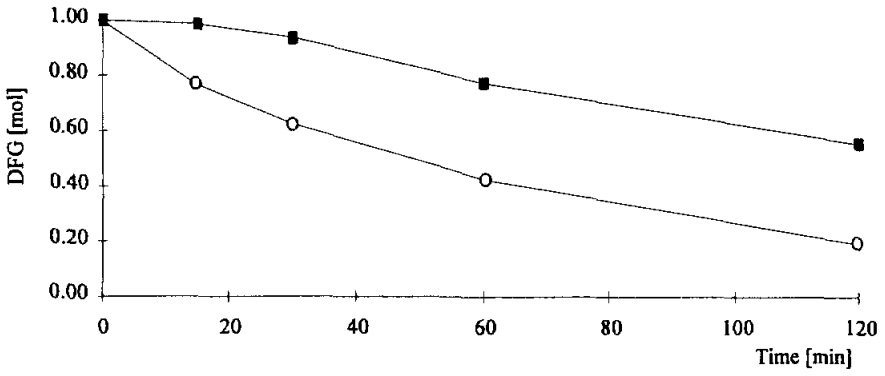

Figure 4. Kinetics of the degradation of DFG at pH 6 (■) and pH 7 (○).

strongly affected by pH. DFG decomposed at pH 6 moderately (■) and more than 50% was still unreacted after 2 h heating. The rate of decomposition was considerably increased at pH 7. Thus, more than 50% was already degraded after 1 h reaction (○). The lower decomposition rate of DFG at pH 6 compared to pH 7 may be explained by the type of enolization favoured and the stability of the resulting deoxyosones. At pH 6, DFG mainly decomposes via 1,2-enolization forming relatively stable 3-deoxyglucosone, whereas at pH 7 the 2,3-enolization pathway dominates, thus leading to the very unstable 1-deoxyglucosone.

According to Anet,[10] the pH of the reaction medium is one important parameter which controls the decomposition pathway of Amadori compounds. 1,2-Enolization is promoted under acidic conditions, resulting in 3-deoxyosones, whereas neutral and alkaline conditions favour 2,3-enolization, forming 1-deoxyosones. This effect has been explained by the degree of protonation of the nitrogen atom.[10]. Ledl and co-workers have recently confirmed the predominance of 1-deoxyosones at pH 7.[16] Significantly higher amounts of 3-deoxyosones were found at pH 5 compared to pH 7. A more rapid fragmentation of these reactive α-dicarbonyls was observed with increasing pH. So far, 1-deoxyosones could only be detected as quinoxaline derivatives,[16,17] which is an indication for their high instability, whereas 3-deoxyosones have long been isolated in pure form.[18]

In conclusion, these experiments show that FAB MS/MS is a straightforward method to study mechanistic aspects of the degradation of DFG in model systems. These results will contribute to our basic knowledge on the Maillard reaction. To continue this work, we will link kinetic experiments with studies on the formation of volatiles which will allow us to evaluate the role of Amadori compounds in generating flavours.

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