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In-situ IR transmission spectroscopic observation and kinetic analyses of initial stage of the Maillard reaction as a simulated formation process of humic substances

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The Maillard reaction is one of the dominant processes forming humic substances in natural waters affecting environmental pollution. In this study, we succeeded in quantitatively following decreases of starting materials (glycine and ribose) of the Maillard reaction by in-situ infrared (IR) transmission spectroscopic observations with the original heatable liquid cell. The obtained kinetic parameters extrapolated to a typical Earth's surface aquatic environments (15 ºC) indicate the half-life of around 2 years for decrease of these components as the first step for the formation of humic substances.

"Humic substance" is a general term of polymerized high molecular weight organic matter, whose structure is not well defined, present in Earth's surface environments. They are formed by partial breakdown and polymerization of organic molecules of living organisms (proteins, carbohydrates, saccharides, etc.), enzymatic reactions, reactions between functional groups, and metabolism of microorganisms. The reactive functional groups of humic substances may influence environmental pollution by adsorbing and transporting pollutants, and promoting the chemical reactions. However, the processes and rates of formation of humic substances are complicated and poorly understood.[1]

In order to examine formation rates of humic substances, we focused on the Maillard reaction, which is a continuous reaction between an amino group in amino acids and a carbonyl group in sugars producing polymerized brown organic compounds, melanoidins[2]. It is considered to be a dominant process in the aquatic environment such as at the bottom of a deep lake where there is few microorganisms.[1] As starting materials of Maillard reaction, glycine and ribose are selected here as representative amino acid and sugar components. Glycine is the simplest amino acid. Ribose is a simple sugar compound and is used in nucleotides forming RNA and DNA.

Kinetics of Maillard reaction has been studied for long years by various researchers, especially in food chemistry. Since browning of food is one of the most important problems in food chemistry, the progress of Maillard reaction is often measured in terms of color formation by visible spectroscopy[3]. However, most of amino acids and sugars as initial materials of simulated Maillard reaction are neither colored nor ultraviolet-visible (UV-Vis) active. Moreover, in-situ IR measurement on aqueous solutions has almost never been performed in the study of Maillard reaction due to strong IR absorptions of water. The aim of this research is to establish a stable in-situ IR transmission spectroscopic measurement method of Maillard reaction as a simulated formation process of humic substances and to evaluate the time scale of the decreases of initial materials. In order to trace intermediates and later products, we are conducting in-situ ultraviolet-visible (UV-Vis) spectroscopic measurement of glycine+ribose mixture solutions. This work will be published later.

We have designed and constructed a new heatable liquid cell (Figure 1). PTFE spacers of 0.05 mm thick were made from commercial PTFE sheets (AS ONE, 7-358-02 and 7-358-07) by piercing them with punches of 12 mm (outside diameter) and 9 mm (inside diameter). 2.0 M glycine and 2.0 M ribose solutions were prepared by dissolving them in pure water (MilliQ). These solutions and pure water were mixed to obtain 0.5 M glycine+ribose mixture solutions, 0.5 M glycine solutions and 0.5 M ribose solutions. About 1 µl of the solution was injected in the sample chamber of the liquid cell (Figure 1).

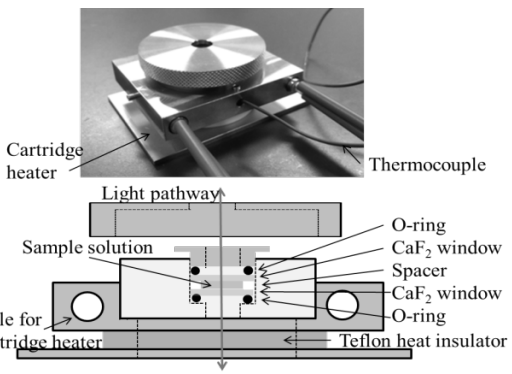

Figure 1. The overview and cross section of the new heatable liquid cell.

0.5 M glycine+ribose mixture solutions were heated at 80, 75 and 70 ºC for 48 hours (2 days) and at 65 and 60 ºC for 96 hours (4 days) in an IR spectrometer (Jasco, FT-IR4100). IR spectra were measured every 2 minutes with a wavenumber resolution of 4 cm^{-1} and 64 scans in the 400-7800 cm^{-1} spectral range. As reference experiments, IR spectral changes of 0.5 M glycine solution only and 0.5 M ribose solution only were measured for 2 days heating at 80 ºC. The other measurement conditions were the same as those of 0.5 M glycine+ribose mixture solutions.

Representative in-situ IR spectra of 0.5 M glycine+ribose mixture solutions heated at 80 ºC (0-48 hours) in the 400-7800 cm^{-1} spectral range are shown in Figure 2. They show peaks around 1600, 3400, 5200 and 7000 cm^{-1} corresponding to vibrations of water molecules[4]. These band areas were almost constant (5200 cm^{-1} band area with a

baseline of 4500-5500 cm^{-1} was 70~77, and 7000 cm^{-1} band area with a baseline of 6500-7300 cm^{-1} was 20~22.) throughout each heating experiment indicating that there was almost no escape of the sample solutions.

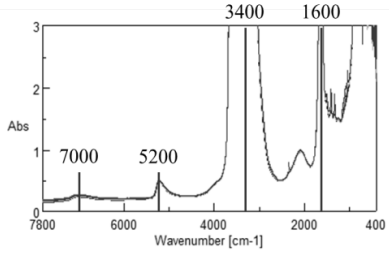

Figure 2. Representative IR spectra of 0.5 M glycine+ribose mixture solutions heated at 80 ºC (0-48 hours) in the 400-7800 cm^{-1} spectral range.

Details of in-situ IR spectra in the 900-1800 cm^{-1} spectral range of 0.5 M glycine+ribose mixture solutions, 0.5 M glycine solutions and 0.5 M ribose solutions heated at 80 ºC (0-48 hours) are presented in Figure 3. They show peaks at 1044, 1088, 1120, 1156, 1220 and 1246 cm^{-1} corresponding to absorptions by ribose[5], and 1330, 1410, 1442 and 1510 cm^{-1} due to absorptions by glycine[6]. These absorptions in the spectra of 0.5 M glycine solutions alone and 0.5 M ribose solutions alone showed no significant changes with heating time. Therefore, no significant changes occurred on IR active functional groups of glycine alone and ribose alone during their heating at 80 ºC for 48 hours. On the other hand, in the spectra of 0.5 M glycine+ribose mixture solutions, these absorptions decreased with heating time. This indicates that their functional groups were broken by the Maillard reaction. It should be noted that no increasing absorption bands have been recognized.

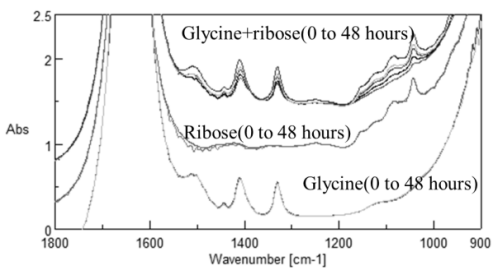

Figure 3. Representative IR spectra of 0.5 M glycine+ribose mixture solutions, 0.5 M glycine solutions and 0.5 M ribose solutions heated at 80 ºC (0-48 hours) in the 900-1800 cm^{-1} spectral range. (They were shifted vertically for clarity.)

Small bands around 1156, 1120, 1088 and 1044 cm^{-1} are corresponding to C-O stretching vibrations of different C-O bonds in ribose[5]. Changes with time in these individual band areas around 1044, 1088, 1120, 1156 cm^{-1} (baseline: 1020-1060, 1075-1105, 1110-1145, 1145-1170 cm^{-1}, respectively) showed similar trends. Therefore, they can be regarded as one global C-O band of ribose and its band area was determined with a baseline of 1020-1170 cm^{-1}, because of its larger precision. This C-O stretching band around 1100 cm^{-1} is selected for further quantitative analyses of ribose. The 1100 cm^{-1} band areas (baseline: 1020-1170 cm^{-1}) were divided by the 1100 cm^{-1} band areas of the 0 second spectrum of 0.5 M glycine+ribose mixture solutions heated at each temperature to normalize differences of the sample solution thickness. These normalized band areas showed exponential decreases at each heating temperature (Figure 4a).

In order to perform kinetic analyses, the normalized 1100 cm^{-1} band area decreases with time were fitted by the following exponential equation, assuming the first order reaction:

$$y = C\exp(-k_1 t) \quad (1)$$

, where y is the normalized 1100 cm^{-1} band area, t is the heating time (seconds), k_1 is the apparent first order rate constant (/s) and C is the intercept at time zero of the band area axis. Because the reaction might have already proceeded to some extent during the increase of temperature and the time zero is taken when the target temperatures were attained, C is not set to 1.

k_1, C and correlation coefficient r values obtained by fitting of the experimental data by the above equation are listed in Table 1. Obtained C values are close to 1. The obtained apparent first order rate constants k_1 increased with temperature. They can be described by Arrhenius equation:

$$\ln(k_1) = \ln(A) - \frac{E_a}{RT} \quad (2)$$

, where A is the frequency factor (/s), E_a is the activation energy (kJ/mol), R is the gas constant (8.31 J/mol/K) and T is the absolute temperature (K).

Arrhenius plots of k_1 and T (333-350 K) show a quasi-linear trend (Figure 5). The fitting line of these experimental data gives E_a and A for the decreases of 1100 cm^{-1} band area during the heating of 0.5 M glycine+ribose mixture solutions. The obtained E_a and A values are 98 kJ/mol and 4.8×10^9 /sec, respectively.

The same kinetic analyses can be conducted on the bands due to glycine. Since similar changes with time were mostly obtained for these bands, the 1330 cm^{-1} band area was selected here as a representative band because its assignment is known (NH_2 bending+ CH_2 bending of glycine[6]) (Figure 4b). The normalized 1330 cm^{-1} band area decreases with time were fitted by the exponential equation assuming the first order reaction:

$$y = C_1\exp(-k_1 t) + C_2 \quad (3)$$

, where y is the normalized 1330 cm^{-1} band area of glycine, t is the heating time (seconds), k_1 is the apparent first order rate constant (/s), C_1 is the constant corresponding to the intercept at the normalized band area axis and C_2 is the vertical offset corresponding to glycine residues, which is unreacted or dissociated from intermediates (see later considerations). k_1, C_1, C_2 and correlation coefficient r values obtained by fitting of the experimental data by the above equation are also listed in Table 1.

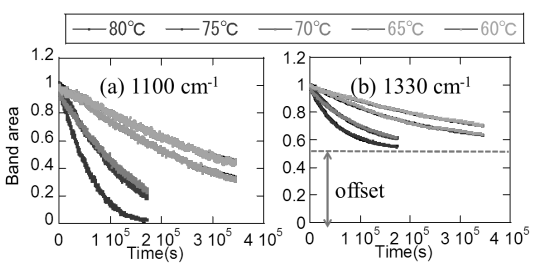

Figure 4. Changes with time of the normalized **(a)** 1100 cm^{-1}

and **(b)** 1330 cm^{-1} band areas during in-situ IR spectroscopy of 0.5 M glycine+ribose mixture solutions heated at 60, 65, 70, 75 and 80 ºC with exponential fitting curves (the first order reaction).

Table 1. k_1 (/s), C and r values of the apparent first order reactions for the normalized band areas of 1100 cm^{-1} and 1330 cm^{-1} in the heating experiments of 0.5 M glycine+ribose mixture solutions at 60, 65, 70, 75 and 80 ºC.

		80 ºC	75 ºC	70 ºC	65 ºC	60 ºC
1100 cm^{-1}	k_1	1.9×10^{-5}	9.2×10^{-6}	7.6×10^{-6}	3.3×10^{-6}	2.4×10^{-6}
	C	1.04	1.05	0.973	1.02	1.01
	r	0.996	0.997	0.996	0.998	0.996
1330 cm^{-1}	k_1	1.8×10^{-5}	1.1×10^{5}	9.2×10^{-6}	4.6×10^{-6}	2.5×10^{-6}
	C_1	0.45	0.46	0.44	0.46	0.50
	C_2	0.54	0.54	0.53	0.54	0.50
	r	0.999	0.999	0.999	0.999	0.999

NH_2 + CH_2 bending of glycine decreased almost simultaneously with C-O bonds of ribose. While C-O bonds of ribose continue to decrease until zero, about halves of NH_2 + CH_2 bonds of glycine decreased and the other halves remained (Figure 4; C_2 ~ 0.5 in Table 1). In previous studies of the Maillard reaction, amino acids were considered to be separated from the reaction intermediates produced by combination of amino and carbonyl groups after Amadori rearrangement, and only sugar moieties were supposed to continue to be dehydrated[2]. Therefore, the above glycine residues observed in our experiments can correspond to these separated amino acids.

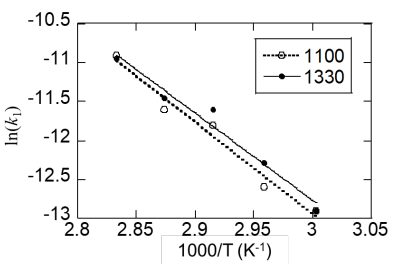

Figure 5. The Arrhenius plots of k_1 for the normalized band areas of 1100 cm^{-1} and 1330 cm^{-1} and the fitting lines.

Arrhenius plots of k_1 and T (333-350 K) for 1100 cm^{-1} band area of ribose and 1330 cm^{-1} band area of glycine show relatively good linear trends (Figure 5). The fitting lines of these experimental data give E_a and A for the decreases of ribose and glycine bands during the heating of 0.5 M glycine+ribose mixture solutions (E_a =98 kJ/mol and $A = 4.8\times10^9$ /s for ribose and E_a = 93 kJ/mol and $A = 1.2\times10^9$ /sec for glycine, respectively). These values are on the same order of magnitudes. These quasi-linear trends suggest the similar reaction mechanisms in this temperature range (60-80 ºC).

In previous works, decreases of initial materials of the Maillard reaction have been fitted by either the first order or the second order reactions[7, 8]. The decrease with time of two starting materials can be usually fitted by the second order reaction. However, the second order fitting of the present experimental data were not good. On the other hand, their fittings by exponential equations were good with correlation coefficient r > 0.996 (Table 1). This indicates the validity of the first order reaction model. The collision of glycine and ribose in this study can be fast and the transformation of formed glycine-ribose composite might be the rate-limiting step following the first order reaction.

Previous kinetic analyses of batch Maillard reactions reported wide ranges of activation energies (16-238 kJ/mol) possibly due to different experimental conditions and kinetic analyses[7, 8]. The present data by in-situ IR measurements with the activation energy around 95 kJ/mol would give constraints on the better understanding of the Maillard reaction.

The obtained apparent first order rate constants k_1 at 60-80 ºC can be extrapolated to lower temperatures by the above Arrhenius equation (eq 2). 15 ºC is selected here as a representative temperature of Earth's aquatic environment. The extrapolated k_1 values at 15 ºC are 9.4×10^{-9} /s for 1100 cm^{-1} and 1.4×10^{-8} /s for 1330 cm^{-1}. In order to better represent time scales of these reaction rates at the earth's surface environments, the half-life $t_{1/2}$ of the decreases of 1100 and 1330 cm^{-1} band areas can be calculated by (ln 2)/ k_1 and the $t_{1/2}$ values at 15 ºC are 2.3 years for 1100 cm^{-1} and 1.5 years for 1330 cm^{-1}.

In natural aquatic environments, amino and carbonyl groups generally exist as portions of complicated structures in proteins, polysaccharides, RNAs and DNAs. These might affect the kinetic parameters of the Maillard reaction. Therefore, further experiments with different starting materials and with different conditions, such as concentrations, pH, etc., are needed to simulate more realistic natural systems.

Although decreases of starting materials could be followed by the present in-situ IR method, no significant increasing absorption bands corresponding intermediates and later products were recognized. Their concentrations or extinction coefficients were possibly too small to be detected in IR range. However, in-situ UV-Vis spectroscopy of glycine+ribose mixture solutions with the same types of heatable liquid cell showed increases of 280 nm absorbance (due possibly to furfural-like intermediates of the Maillard reaction), indicating that intermediate and later products might be produced. We are conducting additional observations and kinetic analyses by in-situ UV-Vis method and this work will be published later.

Moreover, products in batch experiments will be characterized by three-dimensional fluorescence spectroscopy, size exclusion liquid chromatography (SEC) and other methods.

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