Effective HPLC Method for the Determination of Aromatic Amadori Compounds

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An effective HPLC method was developed to separate and determine the phenylalanine, tryptophan, and tyrosine Amadori compounds in Maillard reaction mixtures. Using ammonium hydroxide as a major eluent component, the Maillard reaction mixtures of aromatic amino acid and glucose were separated in high resolution on an anion exchange column CarboPac PA-1. Aromatic amino acids and their Amadori compounds were determined by a UV detector at 260 nm. Glucose can be determined by a pulsed amperometric detector with the potentials and durations of $E_1 = 0.05$ V ($t = 360$ ms) and $E_2 = 0.60$ V ($t = 120$ ms). The effective gradient program was to increase the content of 0.5 mol/L sodium acetate from 0 to 100% in the 0.1 mol/L ammonium hydroxide solution. The separation was based on the acidity differences between the amino acid residues of amino acid and its Amadori compound. The method was simple and sensitive and was demonstrated to be applicable for the kinetic study of the Maillard reactions in aromatic amino acid and carbohydrate systems.

Keywords: *Maillard reaction; aromatic Amadori compounds; HPLC; ammonium hydroxide*

INTRODUCTION

Amadori compounds are the initial products of the Maillard reaction and also the precursors of many advanced nonenzymatic browning reaction products. The kinetics study of the formation and degradation of Amadori compounds is of great importance to both the understanding of the reaction mechanism and the control of the Maillard reaction. For any kinetics study, it is necessary to have a quick and sensitive method to monitor the compositional changes in the reaction mixture.

Much effort has been made to determine Amadori compounds in either a model or a food system (Yaylayan and Huyghues-Despointes, 1994). Borsook et al. (1955) developed a method to detect Amadori compounds on the basis of their reductivity to potassium ferricyanide in 0.1 mol/L sodium hydroxide solution. Aldachi (1958) modified this method by using phosphate buffer (pH 6.6) to eliminate the possible interference from reducing sugars and used it to determine Amadori compounds in soy sauce. However, some amino acids, such as cysteine, and proteins can interfere with the determination. Moll and Gross (1981) and Moll et al. (1982) separated the Maillard reaction mixtures of valine−glucose and proline−maltose on an RSIL C_{18} HL and an NH_2-bonded silica gel column, and the Amadori compounds were detected by a differential refractometer detector. Some other Amadori compounds were detected by HPLC after proper derivation, such as precolumn treatment with phenylthiocarbanylation as described by Walton and McPherson (1987) and postcolumn derivation as described by Reutter and Eichner (1989). For kinetics study of Amadori compound formation and decomposition, the compositional analysis method must be simple and sensitive. However, there is still no method that is sensitive and quick enough to be applicable for the kinetics study of the Maillard reaction, especially for the direct determination of the Amadori compounds and their parent compounds at the same time.

The Amadori compounds of phenylalanine, tryptophan, and tyrosine have aromatic residues that can be monitored by UV absorption. Using HPLC with a UV detector and a pulsed amperometric detector (PAD), these amino acids and their Amadori compounds can be detected by a UV detector, and glucose can be detected by PAD simultaneously.

The objective of our investigation was to develop an HPLC method to determine Amadori compounds of phenylalanine, tryptophan, and tyrosine and their parent compounds simultaneously in the Maillard reaction mixture.

MATERIALS AND METHODS

Materials and Reagents. Phenylalanine was a SigmaUltra reagent. Glucose, ammonium hydroxide, potassium ferricyanide, sodium metabisulfite, sodium acetate, Amberlite I-6766, Dowex 50W, 50X4-400, and ninhydrin reagent solution were purchased from Sigma Chemical Co., St. Louis, MO. Amberlite CG 120 (200−400 mesh) was from Mallinckrodt Chemical Works, St. Louis, MO. Tryptophan and tyrosine were from the United States Biochemical Corp., Cleveland, OH. All other chemicals used were ACS reagents.

Fructosylphenylalanine was prepared according to a modification of Hashiba's (1976) procedure developed in our laboratory. We modified the method by placing 10 g of phenylalanine, 100 g of glucose, 10 g of sodium metabisufite, and 100 mL of water into a 250 mL flask. After the pH was adjusted to 8 with 2 mol/L sodium hydroxide solution, the mixture was heated in a boiling water bath for 8 h. The mixture was then diluted with 1000 mL of water and subjected to absorption on the Amberlite CG 120 (H^+ form, 200−400 mesh, 4 × 40 cm) ion exchange resin column. After a wash with 1000 mL of water to remove the unabsorbed compounds, the column was eluted with 0.1 mol/L ammonium hydroxide solution. The fraction that was positive to ferricyanide test (Aldachi, 1958) was collected (about 500 mL) and subjected to the Dowex 50W ion exchange resin column (H^+ form, 200−400 mesh, 4 × 120 cm) to remove the unreacted phenylalanine. The Dowex 50W column was eluted with 0.1 mol/L citric buffer (pH 3.25). The fraction that was positive to ninhydrin test was collected

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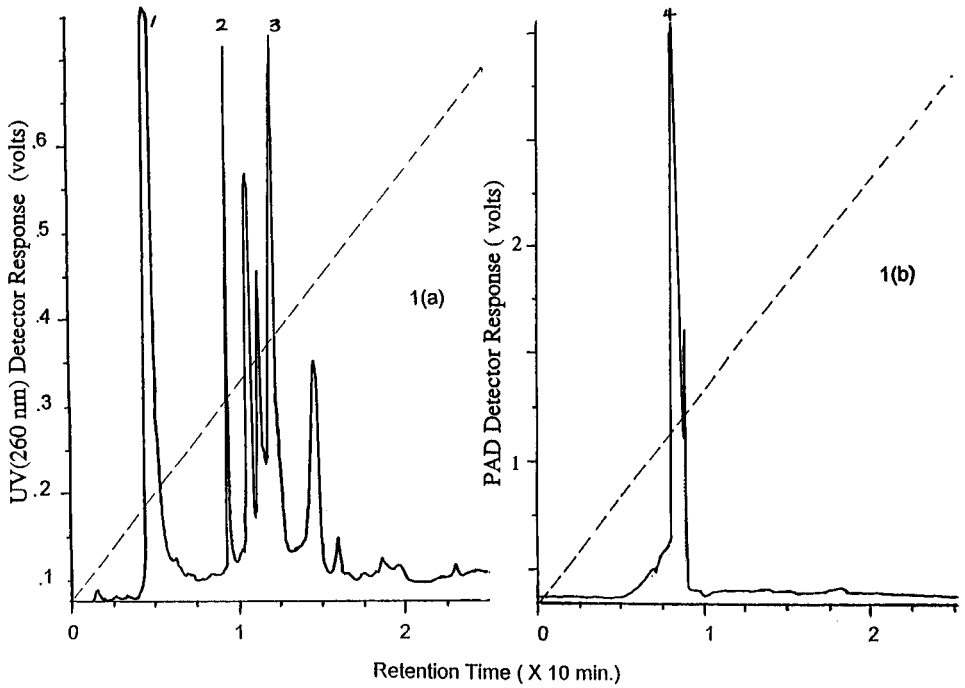

Figure 1. High-performance liquid chromatogram of glucose−phenylalanine reaction mixture. The reaction conditions were 10 g of phenylalanine, 10 g of sodium metabisufite, and 100 g of glucose in 100 mL of water, pH 8.0 at 100 °C for 8 h. (a) Chromatogram from UV detector. Peak 1, Rt 5 min, is unidentified brown compound or compounds. Peak 2, Rt 9.8 min, is phenylalanine. Peak 3, Rt 11.22 min, is phenylalanine Amadori compound. (b) Chromatogram from PAD detector. Peak 4, Rt 8.23 min, is glucose. Other compounds were not detectable under the pulse potential and duration conditions.

(Moore, 1968). The ninhydrin test positive fraction was passed through an Amberlite I-6766 anion exchange resin column (Cl^- form, 100−200 mesh, 4 × 40 cm) and washed with water to remove citric anion. The ninhydrin test positive fraction is fructosylphenylalanine. Fructosylphenylalanine was identified according to the method described by Hashiba (1976). The purity of the fructosylphenylalanine was verified by the HPLC method as described in this paper. The structure was confirmed by mass spectrogram with a VG ZAB-ET mass spectrometer (Mass Spectrum Facility, Center for Advanced Food Technology, Cook College, Rutgers University, New Brunswick, NJ).

Fructosyltryptophan was prepared according to the method of Lee et al. (1979) and fructosyltyrosine according to the method of Hashiba (1976).

HPLC System. The HPLC system (Dionex Corp., Sunnyvale, CA) consisted of an advance gradient pump (Model GPM-2, Dionex), a 25 μL sample loop (Model LCM, Dionex), a variable-wavelength UV detector (Model VDA-2, Dionex), and a pulsed amperometric detector (Model PAD-2, Dionex). Dionex eluent degas module (EDM-2) was used to sparge and pressurize the eluents with helium. A CarboPac PA-1 anion exchange resin column (4.6 × 250 mm, Dionex) and a CarboPac PA guard column (3 × 25 mm, Dionex) were used for the experiment. Detection was made by a PAD with a gold working electrode and dipulse amperometry and a UV detector at 260 nm. The following pulse potentials and durations were used for detection of the aldehyde group: $E_1 = 0.05$ V ($t =$ 360 ms), $E_2 = 0.60$ V ($t = 120$ ms). Chromatographic data were collected and plotted with an 820 work station (Dynamic Solutions, Division of Millipore, Ventura, CA).

Preparation of the Maillard Reaction Mixtures. The phenylalanine−glucose reaction mixture was prepared by mixing 10 g of phenylalanine, 10 g of sodium metabisulfite, and 100 g of glucose and put it into a 250 mL flask. The mixture was solubilized in 100 mL of water. After the pH was adjusted to 8 with 2 mol/L sodium hydroxide solution, the mixture was heated in a boiling water bath for 8 h. This reaction mixture contains phenylalanine, fructosylphenylalanine, glucose, and some advanced nonenzymatic browning products. It was used as a model mixture for developing the HPLC method.

The tyrosine−glucose reaction mixture and tryptophan−glucose reaction mixture were prepared by heating the mixtures of 0.5 mol/L tyrosine or tryptophan in 1 mol/L glucose solution at 95 °C for 10 h.

RESULTS AND DISCUSSION

The CarboPac PA-1 was an anion exchange resin column. The functional group of the ion exchange resin was alkyl quaternary amine. This column was originally developed by Dionex for the separation and determination of complex mixtures of mono- and disaccharides. The eluent for this purpose was sodium hydroxide solution with the concentration varied from 0.016 to 0.1 mol/L for different kinds of saccharide samples.

The Amadori compounds of amino acids and glucose have residues of both fructose and amino acid. The Amadori compound structure is similar to that of saccharides as it can be seen as a derivative of the saccharides. The structural similarity of the saccharides and Amadori compound make it possible to have an effective partition on CarboPac PA1 column. Furthermore, the structure difference between the saccharide and Amadori compound is also great enough to have an effective separation of them on this column if the proper eluent can be found. However, the phenylalanine−glucose reaction mixture could not be separated on the column by the sodium hydroxide solution eluent used for saccharide separation.

We found that the phenylalanine−glucose reaction mixture could be directly separated on the column in good resolution by using ammonium hydroxide solution as the eluent. The chromatogram is shown in Figure 1. The phenylalanine, fructosylphenylalanine, and some advanced browning products were detected by a UV absorbency at 260 nm. Glucose content was determined with a PAD. The PAD detected the glucose by electrochemical oxidation of its aldehyde group on the

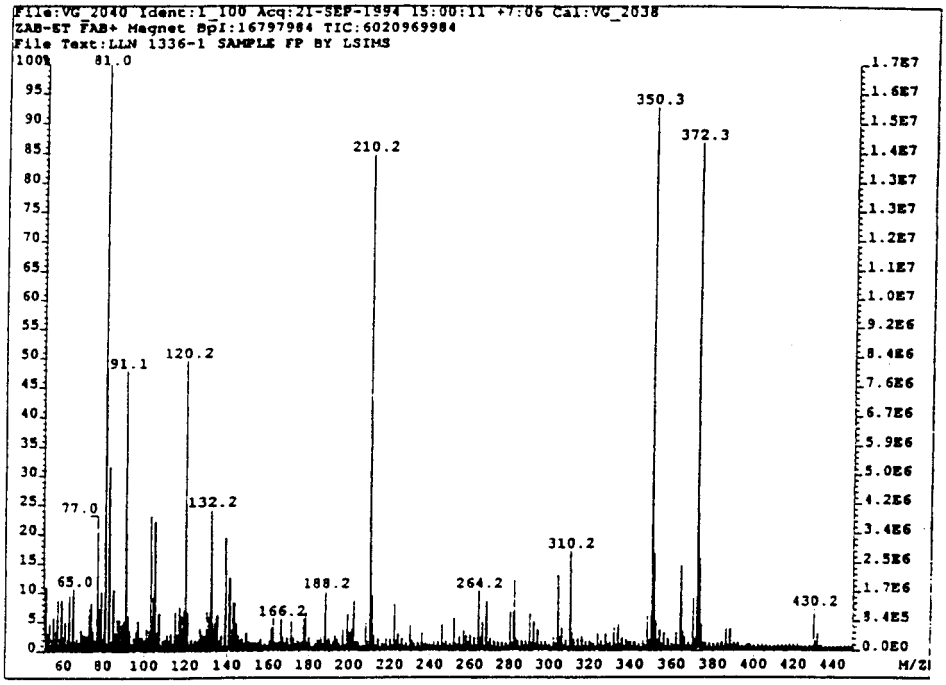

Figure 2. Mass spectrum of the prepared fructosylphenylalanine.

pulse potential and duration conditions as described under Materials and Methods. The Amadori compounds and their amino acids were also detectable using PAD by electrochemical oxidation of the amine group after adjustment of pulse potential and duration conditions. However, interference from ammonium hydroxide in the eluent was too high to determine the Amadori compound and amino acid quantitatively. The effective gradient was to increase the sodium acetate (0.5 mol/L) content in 0.1 mol/L ammonium hydroxide solution linearly from 0 to 100% in a 25 min gradient program. Peaks 2, 3, and 4 with retention times (Rt) of 9.8, 11, and 8.2 min were identified as phenylalanine, fructosylphenylalanine, and glucose, respectively.

The CarboPac PA-1 column was packed with a peculiar anion exchange resin developed by Dionex. The separation of phenylalanine and fructosylphenylalanine should be based on the anion difference between the amino acid and its Amadori compound because of the anion exchange nature of the column. Substitution of hydrogen with glucose in the amino group increases the alkalinity of the carboxyl group of the amino residue, making the anion more stable than that of phenylalanine. Therefore, it is possible to separate the fructosylphenylalanine from phenylalanine. This explanation was further supported by mass spectrum analysis, as shown in Figure 2, of the fructosylphenylalanine. The *m/e* values of molecular peaks were 350.3 and 372.3, corresponding to fructosylphenylalanine and sodium fructosylphenylalanine, respectively. This indicated that sodium fructosylphenylalanine was stable and existed in the prepared phenylalanine Amadori compound.

The high resolution of separation, we believe, was contributed by the use of ammonium hydroxide as the major component of the eluting solution. Because of its gaseous nature under alkali conditions, ammonium hydroxide was seldom used in high-performance liquid chromatography. We think it is the mild alkalinity of ammonium hydroxide that made it possible to separate the phenylalanine from its Amadori compound, because the alkalinity difference between phenylalanine and its Amadori compound is very small.

We have tried to replace the ammonium hydroxide solution with other buffer systems, such as borate buffer and carbonate buffer, and to separate the reaction mixture in the same pH range as the ammonium hydroxide solution. It was possible to separate the phenylalanine with its Amadori compound in the borate buffer system, but quantitative analysis with the borate buffer system was impossible. The borate ion can form a complex with the Amadori compound, making it undetectable. For the carbonate buffer system, the resolution of separation was not as good as with the ammonia hydroxide solution.

Attempts to separate the Maillard reaction mixtures of other aromatic amino acids, tryptophan−glucose and tyrosine−glucose, were also successful. As shown in Figures 3 and 4, these two mixtures were also separated with high resolution. The retention times for fructosylphenylalanine (11.22 min), fructosyltyrosine (11.16 min), and fructosyltryptophan (11.13) were almost the same in value. This further supported the mechanism of separation as described above, because the side chain of the amino acid seemed not to make a significant difference in their retention times. According to the mechanism of separation as described above, it can be expected that other amino acids and their Amadori compounds can be also separated with high resolution by this method. However, for other amino acids and their Amadori compounds, the problem was the lack of the proper method of detection. Therefore, application of this method was still limited to the aromatic amino acids and their Amadori compounds.

This method made it possible to determine both the aromatic Amadori compound and its parent compounds on a single column without precolumn or post column derivation. Thus, it is an ideal method for the kinetics study of the Maillard reaction of aromatic acids and carbohydrates, as both the reactants and products can be determined at the same time. We evaluated this possibility in a phenylalanine−glucose model system.

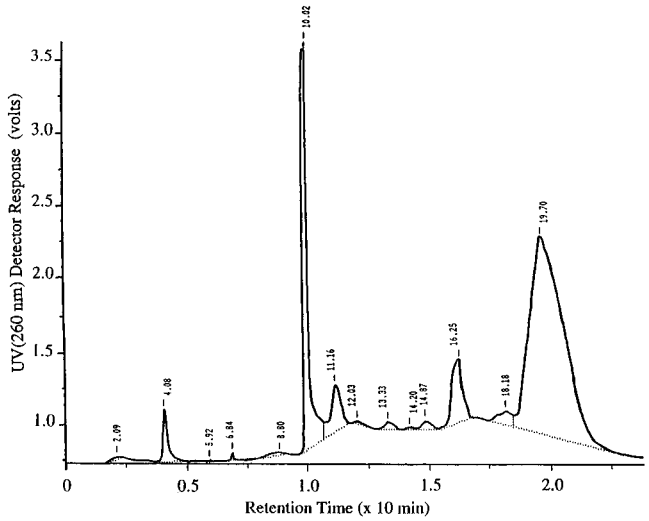

Figure 3. High-performance liquid chromatogram of the tyrosine−glucose reaction mixture. Reaction conditions were 0.5 mol/L tyrosine and 1 mol/L glucose, pH 10.0 at 95 °C for 10 h.

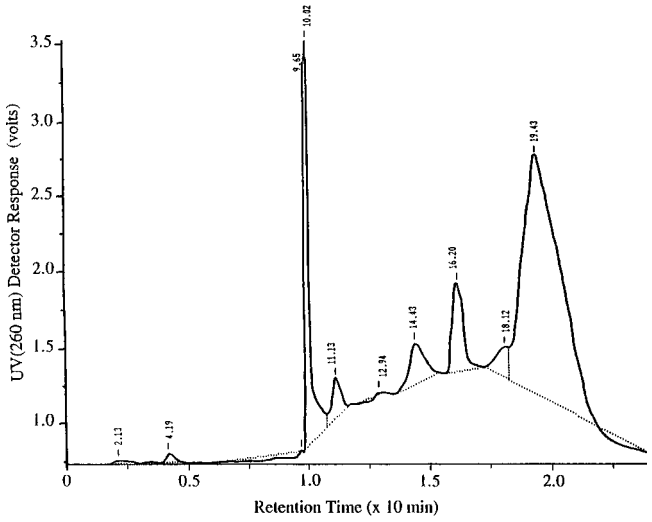

Figure 4. High-performance liquid chromatogram of tryptophan−glucose reaction mixture. Reaction conditions were 0.5 mol/L tryptophan and 1 mol/L glucose, pH 10.0 at 95 °C for 10 h.

With this method, the formation sequence of Maillard reaction product can be tracked easily by HPLC analysis of the reaction mixture at different times. In a typical phenylalanine−glucose reaction system, as shown in Figure 5, the Amadori compound (Rt 11.22 min) formed after 30 min of heating in the boiling water bath (a) and the brown compound or compounds (Rt 5 min), identified by the postcolumn collection of eluents, appeared within 2 h of the start of the reaction of phenylalanine and glucose (b). The Amadori compound reached near the highest concentration after the reaction took place for about 8 h. Figure 6 presented the composition change of a phenylalanine−glucose reaction mixture with the progression of reaction time. Calibration of phenylalanine and fructosylphenylalanine was made by standard curves for the quantitative analysis of the composition of reaction mixtures. It can be seen from Figure 6 that this can be used for quantitative determination of phenylalanine, fructosylphenylalanine, and some advanced nonenzymatic browning products in the Maillard reaction mixture. A complete kinetics study of the formation of phenylalanine Amadori compound by using the present method will be reported in a separate paper. These results demonstrated that this method was able to monitor the compositional changes of both the aromatic amino acid and its Amadori compound in the phenylalanine−glucose reaction system.

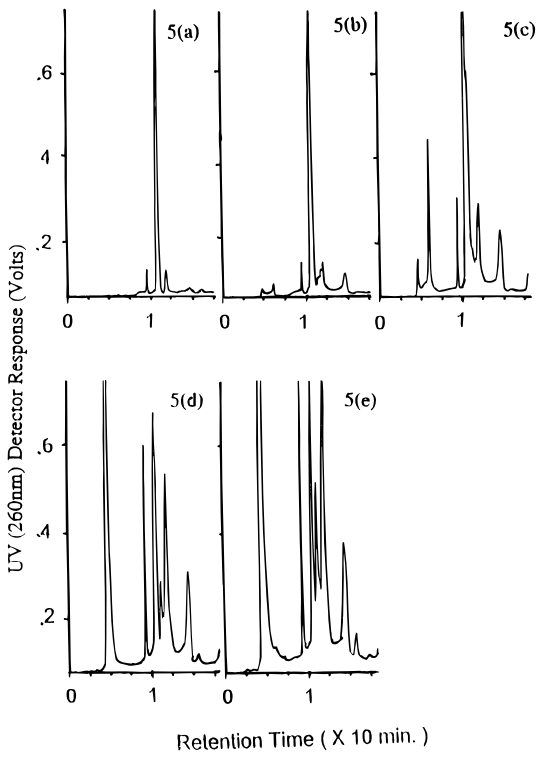

Figure 5. Chromatogram of the phenylalanine−glucose reaction mixture at different time. Reaction conditions were the same as those in Figure 1. (a) 0.5 h; (b) 2 h; (c) 4 h; (d) 7 h; (e) 8 h.

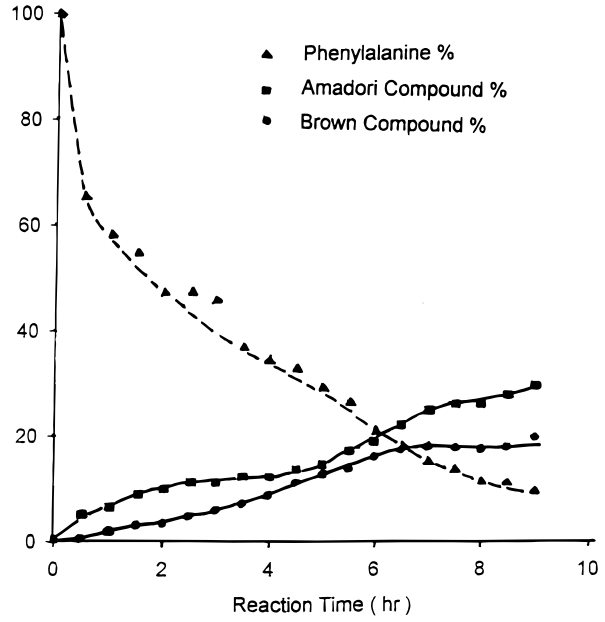

Figure 6. Composition changes in the phenylalanine−glucose reaction mixture. Reaction conditions were the same as those in Figure 1.

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