Simultaneous Quantitative Analysis of Maillard Reaction Precursors and Products by High-Performance Anion Exchange Chromatography

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A new analytical setup allowing the simultaneous analysis of precursors and products of the Maillard reaction is described. It is based on high-performance anion exchange chromatography with electrochemical (ECD) and diode array detectors (DAD) coupled in series. Chromatography and detection were optimized to permit simultaneous monitoring of compounds relevant to the Maillard reaction, such as the sugar, the amino acid, and the corresponding Amadori compound as well as the cyclic intermediates 5-(hydroxymethyl)-2-furaldehyde, maltol, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one. Separation was achieved on a CarboPac PA-1 column using a gradient of sodium acetate in aqueous sodium hydroxide. The Amadori compound, glucose, and glycine were monitored by an ECD operating in the integrated amperometry mode. The number of analyzed compounds was further increased by coupling the ECD with a DAD for the analysis of ultraviolet-active constituents. This method was successfully applied to model Maillard reaction mixtures based on glucose and glycine.

KEYWORDS: Maillard reaction; Amadori compounds; quantitative analysis; anion exchange chromatography; integrated amperometry; simultaneous analysis

The Maillard reaction, also called nonenzymatic browning, is a complex network of reactions involving carbonyl and amino compounds, such as reducing sugars and amino acids (1). It is the main reaction responsible for the transformation of precursors into colorants and flavor compounds during food processing. In general, similar mechanisms also occur under physiological conditions, and a considerable part of Maillard research has been devoted to medical chemistry focusing on health and disease (2, 3). In the course of the Maillard reaction, the sugar molecule is broken down into reactive intermediates that enter further reactions, thus increasing not only the complexity but also the diversity of the reaction mixture.

The initial phase of the Maillard reaction leads to the formation of 1-amino-1-deoxyketoses of amino acids, known as Amadori compounds. For example, glucose and glycine give rise to N-(1-deoxy-D-fructos-1-yl)glycine (1) (Figure 1) that undergoes further reactions, basically following two main decomposition pathways: 1,2-enolization (A) and 2,3-enolization (B). 5-(Hydroxymethyl)-2-furaldehyde (4) is formed from hexoses via 3-deoxyhexosones (2, pathway A), whereas 1-deoxy-2,3-hexosuloses (3) generate 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (5) as a typical marker of pathway B. As the early stage of the Maillard reaction influences the course of the whole reaction, understanding of these phenomena is crucial. This requires quantitative data of compounds representing the various reaction stages, preferably obtained using methods allowing simultaneous analysis of various types of Maillard compounds. Even though this is usually a challenging task for the analytical chemist, such quantitative data are the basis for optimization of flavor formation, control of browning reaction, or reaction modeling. This approach reduces significantly the number of analytical runs required for data collection.

Separation and quantitative determination of individual classes of Maillard compounds have been achieved using various chromatographic techniques (Table 1). In general, high-performance liquid chromatography (HPLC) offers neither sufficient resolution nor satisfactory sensitivity for simultaneous analysis of Maillard compounds. An efficient HPLC method for analyzing Amadori compounds, which offers both good separation and sensitivity, has been reported by Eichner and co-workers (8, 9). However, whereas parent sugars and Amadori compounds can be analyzed in the same run, parent amino acids cannot. Although GC shows better separation efficiency as compared to HPLC, the Amadori compounds need to be converted into volatile compounds prior to analysis. The necessity to derivatize and the ability of gas chromatography...
(GC) to separate tautomeric forms of Amadori compounds are the major drawbacks of this method (10).

Chromatographic analysis of sugars can be substantially improved by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). This technique takes advantage of the affinity between the ionized group of sugars at alkaline pH and a pellicular quaternary amine stationary phase (15), thus offering excellent resolution, and also the highly selective and sensitive detection of nonderivatized sugars at picomole levels with minimal sample cleanup. HPAEC should also offer good resolution for Amadori compounds as they contain the sugar moiety (Table 1). Indeed, phenylalanine, glucose, and the corresponding Amadori compound were analyzed by HPAEC using a CarboPac PA-1 column with a PAD to determine the sugar and an ultraviolet (UV) detector to monitor the amino acid and Amadori product (12). However, this method was applicable only to aromatic amino acids and their Amadori compounds. Amino acids and Amadori compounds were determined quantitatively by PAD using a gradient of acetonitrile and phosphate buffer on an aminopropyl column (11).

The aims of this work were (i) to define both chromatographic and electrochemical conditions for achieving separation and determination of Amadori compounds and the parent precursors and (ii) to extend the method to UV-active cyclic Maillard products.

Figure 1. Simplified scheme showing parts of the Maillard reaction (for explanation see text): 1, N-(1-deoxy-α-fructos-1-yl)glycine (R = H); 2, 3-deoxyhexosone; 3, 1-deoxy-2,3-hexosulose; 4, 5-(hydroxymethyl)-2-furaldehyde; 5, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: glycine, d-mannose, d-fructose, sodium acetate (NaOAc), diethyl ether, 2-propanol, acetic acid, and sodium chloride (Merck, Darmstadt, Germany); d-glucose, 5-(hydroxymethyl)-2-furaldehyde (4), maltol, disodium hydrogenphosphate, and deuterium oxide (D2O, Sigma-Aldrich, Steinheim, Germany); piperidine and ethanol (Fluka, Buchs, Switzerland); sodium hydroxide (NaOH) 46/48% solution (Fisher Scientific, Pittsburgh, PA); and sodium hydrogen carbonate (Prolabo, Paris, France). The solutions and eluents were prepared using ultrapure deionized water (specific resistivity > 18.2 MΩ·cm) from a Milli-Q-system (Millipore, Bedford, MA). NaOH solutions used as eluents were prepared by diluting a carbonate-free 46/48% (w/w) NaOH solution in water previously degassed with helium gas. The poly(vinylidene fluoride) (PVDF) filter (0.22 μm/25 mm) was from Supelco (Bellefonte, PA). The Amadori compound N-(1-deoxy-β-fructos-1-yl)glycine (1) was prepared from d-glucose and glycine as reported by Staempfli and co-workers (16).

Synthesis. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (5) was prepared following the procedure described by Kim and Baltès (17) using some modifications. After glucose (0.2 mol) and piperidine (0.2 mol) had been refluxed in ethanol (150 mL) for 90 min, acetic acid (0.2 mol in 30 mL ethanol) was added, and the mixture was further refluxed for 22 h. Ethanol was evaporated under reduced pressure to one-third of its original volume, then filtered, and washed with 2-propanol (30 mL). After evaporation of the solvent, water (200 mL) and sodium chloride (60 g) were added. The pH was adjusted to 4.0 with HCl (0.1 mol/L), and the neutral compounds were extracted.
overnight with diethyl ether (150 mL) at 45 °C. The residue was filtered, and the solvent was evaporated under reduced pressure.

Distillation of the residue at 0.03 mbar and 170 °C gave a yellow oil containing the target compound as evidenced by GC. Diethyl ether (1 mL) was added to the distillate leading to the target compound by stirring at 90 °C for a defined period of time. Alternatively, a solution of glucose (150 mmol) and glycine (118.5 mg); solution C contained compound 3 (64 mg), and maltol (63 mg). The solutions were distributed in small tubes (5 mL) and stored at -5 °C for a defined period of time.

The concentration of the eluents was 300 mM/L for both sodium acetate (NaOAc) and sodium hydroxide (NaOH). The flow rate was kept constant at 1 mL/min throughout the program.

**Table 2.** Gradient Program for the Simultaneous Analysis of Sugars, Amadori Compounds, Amino Acids, and Cyclic Maillard Intermediates

<table>
<thead>
<tr>
<th>time (min)</th>
<th>modification</th>
<th>gradient (% by vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>initial conditions</td>
<td>96  4  0</td>
</tr>
<tr>
<td>1</td>
<td>increase NaOAc</td>
<td>93  4  3</td>
</tr>
<tr>
<td>29</td>
<td>isocratic conditions</td>
<td>93  4  3</td>
</tr>
<tr>
<td>34</td>
<td>increase NaOAc</td>
<td>16  4  80</td>
</tr>
<tr>
<td>54</td>
<td>cleaning, regeneration</td>
<td>0  100  0</td>
</tr>
<tr>
<td>64</td>
<td>conditioning of column</td>
<td>96  4  0</td>
</tr>
</tbody>
</table>

**Figure 2.** Chemical structure of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(3H)-pyran-4-one (5)

**Table 1.** Analytical Methods for Separation and Quantification of Nonvolatile Maillard Reaction Products

<table>
<thead>
<tr>
<th>method</th>
<th>column</th>
<th>detection</th>
<th>compounds</th>
<th>comments</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>Aminex A-9 (H+)</td>
<td>refractometer</td>
<td>5 Amadori compounds, amino acids</td>
<td>limited detection sensitivity and separation efficiency</td>
<td>4</td>
</tr>
<tr>
<td>HPLC</td>
<td>µBondapak CH</td>
<td>UV (254 nm), as p-nitro-benzoxyoximes</td>
<td>Amadori compounds</td>
<td>separated from aromatic amino acids using µBondapak-NH2</td>
<td>5</td>
</tr>
<tr>
<td>HPLC</td>
<td>µBondapak NH2</td>
<td>refractometer</td>
<td>4 Amadori compounds and parent precursors</td>
<td>semipreparative isolation and purification of Amadori comp.</td>
<td>6</td>
</tr>
<tr>
<td>HPLC</td>
<td>DEAE-Si</td>
<td>bis (480 nm), as triphenyl formazans</td>
<td>16 Amadori compounds</td>
<td>postcolumn derivatization with triphenyl tetrazolium chloride Amadori compound separated from glucose and proline</td>
<td>7, 8</td>
</tr>
<tr>
<td>HPLC</td>
<td>RP-C18</td>
<td>refractometer</td>
<td>1 Amadori compound and parent precursors</td>
<td>double peaks due to oximation (syn and anti isomers separated)</td>
<td>9</td>
</tr>
<tr>
<td>GC</td>
<td>capillary, OV-101</td>
<td>FID, MS, as trimethylsilyl-oxime derivatives</td>
<td>11 Amadori compounds</td>
<td>fluorescence detector for tryptophan and derivatives</td>
<td>10</td>
</tr>
<tr>
<td>HPAEC</td>
<td>aminopropyl</td>
<td>PAD, fluorescence detector, DAD</td>
<td>3 Amadori compounds and parent precursors</td>
<td>only aromatic compounds, interference from NH4OH amino acids not detected</td>
<td>12</td>
</tr>
<tr>
<td>HPAEC</td>
<td>CarboPac PA-1</td>
<td>PAD Amadori, sugar, UV (amino acid)</td>
<td>3 Amadori compounds and parent precursors</td>
<td>applied to kinetic studies in Maillard model systems</td>
<td>13</td>
</tr>
<tr>
<td>HPAEC</td>
<td>CarboPac PA-1</td>
<td>PAD</td>
<td>5 Amadori compounds and glucose</td>
<td>applied to kinetic studies in Maillard model systems</td>
<td>14</td>
</tr>
<tr>
<td>HPAEC</td>
<td>CarboPac PA-1</td>
<td>ECD, integrated amperometry</td>
<td>1 Amadori compound and parent precursors</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a DAD, diode array detector; ECD, electrochemical detector; FID, flame ionization detector; GC, gas chromatography; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometer; PAD, pulse amperometric detector; RP, reversed phase; UV, ultraviolet detector; vis, visual.
Nuclear Magnetic Resonance (NMR) Spectroscopy. Samples were prepared in Wilmad 528-PP 5 mm Pyrex NMR tubes, using deuterated water as solvent. NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrupolar 5 mm probe head, at 360.13 MHz (1H) and 75.56 MHz (13C) under standard conditions (18).

RESULTS AND DISCUSSION

In this work, HPAEKC was evaluated for the simultaneous determination of glucose, glycine, and the corresponding Amadori compound N-(1-deoxy-d-fructos-1-yl)glycine (1), as well as the cyclic Maillard intermediates 2,3-dihydroxy-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (5), 5-(hydroxymethyl)-2-furaldehyde (4), and maltol using a nondestructive DAD coupled in series with an ECD. These compounds represent some of the major reaction pathways and intermediates of the Maillard reaction (Figure 1).

Analytical Conditions. As Amadori compounds contain both the sugar and amino acid moiety, they possess positive or negative charges depending on the pH of the solution. Therefore, Amadori compounds can be retained not only on an anion exchange resin but also on a cation exchanger. Retention of Amadori compounds on a cation exchange resin is often used as a cleanup step in the synthesis or prior to the analysis of Amadori compounds. However, these columns are not capable of retaining sugars and, thus, are not suitable for simultaneous analysis of sugars and corresponding Amadori compounds. As anion exchange columns can retain not only the Amadori compounds and amino acids but also sugars, the anion exchange column CarboPac PA-1 containing a pellicular quaternary amine stationary phase was selected in this work.

The gradient of sodium acetate in aqueous sodium hydroxide was evaluated to find chromatographic conditions enabling (i) separation of glucose, glycine, and Amadori compound 1 and (ii) quantification of all three compounds by electrochemical detection. No interferences with the ECD were observed under these conditions. Best results were obtained with a gradient composed of water, sodium hydroxide (300 mmol/L), and sodium acetate (300 mmol/L) as shown in Table 2. The gradient starts with a mixture of water and NaOH (4%), followed by a first rapid increase of sodium acetate to 3% (1 min), isocratic for 28 min, and a second increase of sodium acetate to 80% (5 min), then isocratic for 5 min. Each analytical cycle was followed by cleaning and regeneration of the column with NaOH (300 mmol/L) and equilibration of the column using the gradient conditions.

Glucose, glycine, and Amadori compound 1 were detected using an ECD working in integrated amperometry mode. This detection permits direct and simultaneous analysis of all three compounds without any derivatization (Figure 3A). In addition, mannose and fructose can be detected in the same run. Using the conditions described above, three hexose sugars, glycine, and the Amadori compound 1 were separated in one chromatographic run within 40 min.

The spectrum of the compounds of interest was further increased by coupling the ECD with a DAD. The analysis of compound 4 and cyclic enolones is of special interest as these compounds represent markers of specific degradation pathways (1,2-enolization or 2,3-enolization) in the Maillard reaction. Under the chromatographic conditions described above, a DAD was successfully used to analyze compound 4 (λ = 285 nm), maltol (λ = 320 nm), and pyranone 5 (λ = 350 nm) in solutions prepared from reference compounds (Figure 3B–D). Depending on the specific interest, the wavelength can be adapted to increase detection sensitivity. For example, compound 4 is preferably detected at 285 nm (Figure 3B), whereas compound 5 responds best at 350 nm (Figure 3D).

Detection Limits and Linearity. The detection limits, defined as the amount of a compound producing a signal-to-noise ratio of ≥3, were determined using standard compounds (Table 3). They were in the range of 0.01–2.0 μmol/L, depending on the substance. Compound 4 showed the lowest detection limit of 0.01 μmol/L and glycine the highest detection limit of 2.0 μmol/L. The detector response for individual compounds was linear over a broad concentration range and went up to ~100 μmol/L for compounds detected by ECD and to ~500–1000 μmol/L for compounds detected by DAD. Parts A and B of Figure 4 show the calibration curves of Amadori compound 1 and pyranone 5 based on electrochemical and UV detection, respectively.

Analysis of Maillard Samples. Although Maillard reaction samples are known to be very complex, containing a broad range of small and polymeric substances, no sample cleanup was required for the analysis of Maillard reaction mixtures due to the sensitivity and selectivity of the electrochemical detection. Thus, sample preparation is very convenient and rapid, as it consists only in dilution of the sample with water. An example of a typical chromatogram obtained after glucose and glycine had been heated in a phosphate-buffered solution of pH 9 at 90 °C for 3 h is shown in Figure 5. Elution of the compounds was accelerated, compared to Figure 4, by increasing the NaOAc concentration. The data indicate isomerization of glucose to mannose and fructose under alkaline conditions and also the formation of the Amadori compound 1. It should be noted that this type of result can hardly be obtained by any other analytical method in a single run.

Similarly, the degradation of the Amadori compound 1 can be studied using the method described in this work. As shown in Figure 6, electrochemical detection permitted quantification of Amadori compound 1, glycine, glucose, and mannose. In addition, compound 4 and pyranone 5 were quantified in the

Table 3. Detection Limit and Linear Range for the Analysis of Some Hexose Sugars, the Amadori Compound (1), Glycine, 5-Hydroxy-2-furaldehyde (4), Maltol, and 2,3-Dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (5)
same run using UV detection at 285 and 350 nm, respectively. Maltol was not detected as it is formed only in trace quantities from glucose or Amadori compound 1. The simultaneous analysis of these Maillard compounds represents a breakthrough...
The reversibility of the Amadori rearrangement (14) has recently been used to substantiate the hypothesis of the for studying mechanistic aspects of the Maillard reaction and has recently been used to substantiate the hypothesis of the reversibility of the Amadori rearrangement (14). This has become possible as mannose, the epimer of glucose, could be analyzed in the same run along with the Amadori compound, without any isolation step prior to analysis.

In principle, the method described here is also applicable to food and biological samples, provided an appropriate cleanup or concentration step is established. As this depends very much on the sample to be analyzed, optimization would be required to adapt sample cleanup to food and biological systems. For example, in food samples containing high levels of free amino acids, the determination of compound 1 or other glucose-derived Amadori compounds may be complicated by interferences from amino acids. In this case, an ECD can be used in pulsed amperometric mode, instead of integrated amperometry, leading to higher selectivity of the detection due to application of potentials normally used for the determination of sugars ($E_1 = 0.05$ V; $E_2 = 0.75$ V; $E_3 = −0.15$ V). Under these conditions, only sugars and Amadori compounds would be detected (13).

In conclusion, a new analytical method based on HPAEC was developed and demonstrated in this work to be an excellent analytical tool to simultaneously monitor the early and intermediate phases of the Maillard reaction. The method permits one, in a single analytical run, to follow not only the Maillard precursors (e.g., glucose and glycine) but also key intermediates such as Amadori compounds and cyclic intermediates (e.g., compound 4 and pyranone 5), the latter being markers of the 1,2- and 2,3-enolization degradation pathways, respectively. Simple and rapid sample preparation in combination with high sensitivity of detection are the principal advantages of this method as compared to HPLC or GC. It is especially suited to generate kinetic data in a medium/high-throughput manner for multiresponse modeling of the Maillard reaction and to estimate Maillard reaction efficiency as a function of processing conditions. This in turn provides major guidance in the understanding of reaction mechanisms, which is in progress and will be published elsewhere.

**ABBREVIATIONS USED**

DAD, diode array detector; DEAE-Si, N,N-diethylaminoethyl-modified silica gel; ECD, electrochemical detector; EI, electron impact; FID, flame ionization detector; GC, gas chromatography; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detector; PVDF, poly(vinylidene fluoride); RI, retention index; RP, reversed phase; UV, ultraviolet; vis, visual.

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