Analysis of Amadori Compounds by High-Performance Cation Exchange Chromatography Coupled to Tandem Mass Spectrometry

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High-performance cation exchange chromatography coupled to tandem mass spectrometry or electrochemical detection was found to be an efficient tool for analyzing Amadori compounds derived from hexose and pentose sugars. The method allows rapid separation and identification of Amadori compounds, while benefiting from the well-known advantages of mass spectrometry, such as specificity and sensitivity. Glucose- and xylose-derived Amadori compounds of several amino acids, such as glycine, alanine, valine, leucine/isoleucine, methionine, proline, phenylalanine, and glutamic acid, were separated or discriminated using this new method. The method is suitable for the analysis of both model reaction mixtures and food products. Fructosylglutamate was found to be the major Amadori compound in dried tomatoes (~1.5 g/100 g) and fructosylproline in dried apricots (~0.2 g/100 g). Reaction of xylose and glycine at 90 °C (pH 6) for 2 h showed rapid formation of xyluloseglycine (~12 mol %, 15 min) followed by slow decrease over time. Analysis of pentose-derived Amadori compounds is shown for the first time, which represents a major breakthrough in studying occurrence, formation, and decomposition of these labile Maillard intermediates.

Amadori compounds are 1-amino-1-deoxyketoses representing an important class of Maillard intermediates.1 They are formed in the initial phase of the Maillard reaction by the so-called Amadori rearrangement of the corresponding N-glycosylamines,2 the latter obtained by condensation of amino acids and aldose sugars. Amadori compounds play a central role in the early phase of the Maillard reaction and can decompose to α-dicarbonyls of various chain length, such as 3-deoxyhexos-2-ulose, 1-deoxy-2,3-hexodiolulose, 2-oxopropanal, butane-2,3-dione, glyoxal, and others.3,4 Amadori compounds and the very reactive α-dicarbonyls initiate a cascade of further reactions that finally result in the formation of a complex mixture containing volatile compounds and brown pigments, the latter also referred to as melanoidins.3,5 These reactions take place during food processing and storage and also under physiological conditions.6,7

The analysis of Amadori compounds is a challenging task, particularly in complex systems such as foods or biological fluids. Earlier work focused on automatic amino acid analyzers to separate Amadori compounds using postcolumn reaction with ninhydrin and measurement of the developed color by UV/visible detection.8-10 The methods based on amino acid analysis mainly suffer from insufficient separation efficiency, as amino acids may interfere with Amadori compounds. On the other hand, they are quite sensitive and rapid, as no derivatization is necessary prior to analysis.

Gas chromatography (GC) has rarely been used, mainly because derivatization of Amadori compounds is required prior to analysis, which in addition results in tautomeric forms.11,12 The complication arising from the formation of tautomers can be reduced by oximation of Amadori compounds prior to trimethylsilylation. In this case, each Amadori compound, however, forms two peaks, corresponding to syn- and anti-isomers of the resulting oximes. This method was successfully used to separate up to 11 Amadori compounds in several food samples, such as malt and beers11 and cocoa beans.13

The most frequently used methods are based on high-performance liquid chromatography (HPLC) using various types of stationary phases and detectors. However, HPLC techniques require time-consuming cleanup procedures to isolate the Amadori compounds from complex matrices prior to chromatography. Furthermore, the majority of the methods developed offer neither sufficient resolution nor satisfactory sensitivity. The most efficient HPLC method was developed by Eichner et al. separating up to 16 Amadori compounds on a DEAE-Si column and monitoring them by UV detection at 480 nm after postcolumn derivatization with triphenyltetrazolium chloride. This method offers both good separation and sensitivity.19-20

Recently, a method based on high-performance anion exchange chromatography (HPAEC) coupled with an electrochemical or diode array detector has been reported as a powerful analytical technique for the detection and monitoring of known Amadori compounds.20-21 The method permits sensitive and relatively selective detection of hexose-derived Amadori compounds, but also simultaneous analysis of their precursors and some degradation products. However, unequivocal identification of Amadori compounds can hardly be achieved by this method. In addition, the chromatographic conditions for the analysis of hexose-derived Amadori compounds are not suitable for the more labile pentose-derived analogues due to their rapid off-column decomposition.

The quantification of the Amadori compound N-(1-deoxy-D-fructos-1-yl)glycine was also achieved by fast atom bombardment (FAB) tandem mass spectrometry (MS). Its particular advantage is the simple sample preparation procedure, as the samples are directly introduced into the ion source. However, simultaneous analysis of several Amadori compounds cannot be achieved by this method.

Over the past decade, FAB has been almost completely replaced by electrospray ionization (ESI). This technique, offering very “soft” ionization and easy coupling to on-line separation tools as particular advantages, has recently been used in combination with hydrophilic interaction liquid chromatography (HILIC-ESI-MS) to identify five glucose-derived and three maltose-derived Amadori compounds in wheat gluten hydrolysates.22 The coupling of tandem mass spectrometry to capillary electrophoresis (CE-MS) has successfully been applied to discriminate six glucose-derived Amadori compounds. However, the analysis of pentose-derived Amadori compounds by the techniques mentioned above has not been achieved so far.

The aim of this work was to develop a high-performance ion exchange chromatography method that would be compatible with MS detection and also suitable for rapid analysis of Amadori compounds derived from both hexose and pentose sugars.

**EXPERIMENTAL**

**Materials.** The following compounds were obtained commercially: D-glucose and sodium dihydrogen phosphate (Merck, Darmstadt, Germany); D-xylene, glycine, L-phenylalanine, L-leucine, L-isoleucine, L-valine, L-proline, L-methionine, L-alanine, and 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (Aldrich/Fluka, Buchs, Switzerland); sodium hydroxide 46/48% solution (Fisher Scientific, Pittsburgh, PA). The glucose-derived Amadori compounds N-(1-deoxy-D-fructos-1-yl)glycine (Fru-Gly), N-(1-deoxy-D-fructos-1-yl)-L-proline (Fru-Pro), and N-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (Fru-Glu) were prepared using already reported procedures.22,25,26 The solutions and eluents were prepared using ultrapure deionized water (specific resistivity ≥18.2 MΩ·cm) from a Milli-Q system (Millipore, Bedford, MA). The poly(vinylidene fluoride) (PVDF) syringe filters (0.45 μm) were from Supelco (Bellefonte, PA). All other reagents were of analytical grade and were used without further purification.

**Synthesis of N-(1-Deoxy-D-xylulos-1-yl)glycine (Xyl-Gly).** The Amadori compound Xyl-Gly was prepared as described in the literature using some modifications.27 Xylose (270 g) and water (45 mL) were placed in a round-bottom flask and heated in a boiling water bath. When the temperature of the solution reached 90 °C, glycine (33.8 g) and sodium disulfite (40.5 g) were added and the reaction mixture was heated at 90 °C for 20 min. After cooling, the reaction mixture was dissolved in an ethanol/water mixture (70 + 30, v/v, 1650 mL) and the solution was passed through a column (5 × 40 cm) filled with a Dowex 50WX4 ion exchange resin (H+ form). The resin was washed with an ethanol/water mixture (70 + 30, v/v, 5 L) and then with water (1.5 L) until the eluent was negative to the TTC test.28 Xyl-Gly was eluted with ammonium hydroxide (0.2 mol/L) collecting 100-mL fractions. Each fraction was tested using the TTC and Elson–Morgan test.29 The fractions positive in both tests were collected, then charcoal was added, and the filtrate was freeze-dried. Xyl-Gly was obtained as an amorphous white powder (19 g, 22% yield) with a purity of 98% by NMR. The product is hygroscopic and must be stored under argon. A ternary mixture of the α, β, and open-chain form was obtained in a ratio of 30/40/30 (Figure 1) based on the integration of H-C1 and H-C3 NMR signals.

α-Xyl-Gly: 1H NMR (360 MHz, 2H2O, δ/ppm) 3.31 (d, 1H, J = 12.9 Hz, C1), 3.38 (d, 1H, J = 12.9 Hz, C3), 3.61–3.66 (m, 2H, C2), 3.90 (m, 1H, C4), 4.13 (d, 1H, J = 5.5 Hz, C5), 4.24 (m, 1H, C3); 4.26 (m, 1H, C4); 13C NMR (90 MHz, 2H2O, δ/ppm) 49.8 or 48.9 or
isocratic with 0.1% (w/w) aqueous formic acid as eluent and a flow rate of 0.25 mL/min. After each run, the column was washed with 0.5% aqueous formic acid for 8 min. The column temperature was maintained at 30 °C with a column heater. The injection volume was 3 μL for model systems and 1 μL for dried fruit samples, corresponding to 1–500 ng of Amadori compounds injected onto the column. The Amadori compounds were detected in positive electrospray ionization (ESI⁺) mode using enhanced monitoring scan (EMS), enhanced product ion (EPI), or multiple reaction monitoring (MRM) experiments. The turbo ion spray (TIS) source was operated at 450 °C with ion spray voltage at 5000 V, nebulizer gas at 241 kPa (35 psi), heater gas (N2) at 414 kPa (60 psi), an entrance potential at 10 V, and a declustering potential of 30 V. For EPI experiments, collision voltage was set to 20 V and nitrogen was used as collision gas. Trap fill time was 20 ms for both EPI and EMS experiments. Scans were acquired at 4000 amu/s over the range 85–350 amu. Data were treated with the manufacturer’s Analyst 1.4 software.

Model Reaction Mixtures. Samples of individual Amadori compounds were prepared by refluxing anhydrous glucose (0.1 mol) or xylose (0.1 mol) and the corresponding amino acid (0.1 mol) in methanol (100 mL) under nitrogen for 4 h. After cooling, the precipitate, containing unreacted amino acids and sugars, was removed by filtration (0.45 μm), and the samples were diluted with water (150–15 000 times) followed by HPCEC analysis. Samples containing mixtures of Amadori compounds were prepared using a similar procedure replacing the single amino acid by a mixture of amino acids (0.1 mol total). The glucose-derived Amadori compounds of the various amino acids are named fructosyl amino acids, i.e., Fru-Gly (for glycine), Fru-Glu (for glutamic acid), Fru-Pro (for proline), Fru-Phe (for phenylalanine), Fru-Met (for methionine), Fru-Ile (for isoleucine), Fru-Val (for valine), Fru-Leu (for leucine), and Fru-Ala (for alanine). Similarly the xylose-derived Amadori compounds are named xylulosyl amino acids, i.e., Xyl-Gly (for glycine), Xyl-Glu (for glutamic acid), Xyl-Pro (for proline), Xyl-Phe (for phenylalanine), Xyl-Met (for methionine), Xyl-Ile (for isoleucine), Xyl-Val (for valine), Xyl-Leu (for leucine), and Xyl-Ala (for alanine).

Quantification of Xyl-Gly. Samples for monitoring the formation of Xyl-Gly were prepared by heating xylose (3.75 g, 25 mmol) and glucose (1.88 g, 25 mmol) in a phosphate buffer (25 mL, 0.2 mol/L, pH 6) at 90 °C for 2 h. Aliquots (1 mL) were taken at defined time intervals and passed through a PVDF syringe filter (0.45 μm). Samples were diluted with water to be in a linear range of the calibration curves and then analyzed by HPCEC-ED. All samples were prepared in duplicate.

Analysis of Amadori Compounds in Dried Fruits. Dried tomatoes (10 g) were placed in a Pyrex bottle and macerated with water (40 mL) for 3 h under shaking. The mixture was then homogenized using a Waring blender for 30 s, diluted with water (50 mL), and shaken for another 30 min. The solid tomato residue was removed with a PVDF syringe filter, and the filtrate was analyzed by HPCEC-MS/MS. Extracts of other dried fruits were prepared using the same procedure.

Selected Amadori compounds (Fru-Glu, Fru-Pro) were quantified in dried tomatoes and apricots on the basis of calibration curves by comparing the peak areas with those of standard solutions containing known amounts of pure compounds. Good
RESULTS AND DISCUSSION

for both Fru-Glu (linearity was obtained in the concentration range up to 300 mg/L and determined). The variability of retention times was significantly shorter retention times for less polar Amadori compounds. On the other hand, the addition of acetonitrile (10%) led to only a very small effect on the retention of Amadori compounds. The retention of Amadori compounds on a cation exchange resin was often used as a cleanup step during the synthesis or prior to analysis. To evaluate whether cleanup was not necessary prior to analysis due to the selectivity of the MRM experiment so that sample preparation was limited to simple dilution in water.

Analysis of Hexose-Derived Amadori Compounds. Chromatographic Conditions. The column IonPac CS-17 was selected for method development. This column is based on a weak cation exchange resin with reversed-phase properties, and it is fully solvent-compatible with acetonitrile, alcohols, and acetonitrile. To find suitable chromatographic conditions for the separation of Amadori compounds, the column was coupled to an ECD, which required a postcolumn addition of sodium hydroxide to enhance the sensitivity for the detection of Amadori compounds.

Initial experiments indicated that Amadori compounds were retained on the IonPac CS-17 column and eluted using water as a mobile phase. All glucose-derived Amadori compounds investigated in this study were eluted in less than 10 min (Table 1). The addition of formic acid to the mobile phase (5 mmol/L) had only a very small effect on the retention of Amadori compounds. On the other hand, the addition of acetonitrile (10%) led to significantly shorter retention times for less polar Amadori compounds such as Fru-Phe (Table 1). None of the chromatographic conditions tested permitted the separation of all nine Amadori compounds. This implies that only samples containing the Amadori compounds that do not coelute can be analyzed on this column if electrochemical detection is used. However, as mass spectrometry detection was targeted, baseline separation of Amadori compounds was not required.

ESI-MS and Tandem MS. To facilitate protonation of Amadori compounds required for the detection by ESI-MS, water containing 0.1% formic acid was used as eluent. When submitted to low entrance potential and collision voltage (EP = 10, CV = 10 V), the Amadori compound Fru-Gly showed the most abundant peak [M + H − H2O]+ at m/z = 220 corresponding to the loss of water from the [M + H]+ ion (m/z = 238). Apart from these two ions, the fragment [M + H − 2H2O]+ (m/z = 202) and the adduct ions [M + Na]+ (m/z = 260) and [M + K]+ (m/z = 276) were also detected in the mass spectrum. The MS/MS of the [M + H]+ ion acquired at higher CV (20–40 V) contained additional fragments, including those formed by cleavage between the sugar and amino acid moieties. Interpretation of the MS/MS spectrum of Fru-Gly acquired at CV = 20 V is shown in Figure 2.

The fragmentation pattern observed for Fru-Gly was also typical for other Amadori compounds studied. The characteristic fragments of nine hexose-derived Amadori compounds obtained by MS/MS (CV = 20 V) are listed in Table 2. In all mass spectra, the ion [M + H − H2O]+ was the most abundant peak. The loss of formaldehyde [M + H − 3H2O − H2CO]− was found to be characteristic for hexose-derived Amadori compounds.

**Table 1. Effect of the Mobile Phase on the Retention of Glucose-Derived Amadori Compounds on the IonPac CS-17 Column**

<table>
<thead>
<tr>
<th>compound</th>
<th>mobile phase</th>
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<tbody>
<tr>
<td></td>
<td>100% water</td>
</tr>
<tr>
<td>Fru-Gly</td>
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<tr>
<td>Fru-Ala</td>
<td>4.18</td>
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<td>Fru-Glu</td>
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<tr>
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<td>3.77</td>
</tr>
<tr>
<td>Fru-Phe</td>
<td>8.22</td>
</tr>
</tbody>
</table>

* Retention time (in minutes) on IonPac CS17 column (nd, not determined). The variability of retention times was <5% for Fru-Phe and <3% for all other Amadori compounds. Instrument: Dionex DX500 system with electrochemical detection. Dead volume: 2.50 min.

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and Fru-Ala. The concentration of Fru-Glu was 1536 mg/100 g (recovery 83%), which is the same order of magnitude as the concentrations found by Eichner et al. in tomato powder (676 and 3788 mg/100 g).^{12}

The analysis of other food samples, for example, dried apricots, dried plums, or grapes, was also performed (data not shown) without using time-consuming cleanup procedures, making this method very convenient for rapid analysis of Amadori compounds in food products. The concentration of Fru-Pro, which was the major Amadori compound in dried apricots, was 206 mg/100 g (recovery 93%).

**Analysis of Pentose-Derived Amadori Compounds. Chemical Synthesis.** To check whether the IonPac CS-17 column was also suitable for the analysis of pentose-derived Amadori compounds, N-(1-deoxy-o-threo-2-pentulos-1-yl)glycine, the xylulosyl...
derivative of glycine (Xyl-Gly), was prepared in gram amounts by chemical synthesis. The synthesized sample was composed of the α, β, and acyclic forms (Figure 1) in a ratio of 30/40/30, as shown by integration of the H–C1 and H–C3 NMR signals. This distribution was in good agreement with the data published by Feather and Mossine.30 Interestingly, the open-chain and more reactive keto form was well represented in the aqueous solution. In the crystalline state, Xyl-Gly has been reported to occur exclusively as acyclic keto tautomer on the basis of solid-state 13C NMR measurements and crystal structure determination.31

Chromatography and Tandem-MS. Xyl-Gly was analyzed using the same chromatographic conditions as for the hexose-derived analogues. It eluted as a symmetric peak with the same retention time as Fru-Gly. The fragmentation of Xyl-Gly was similar to that of Fru-Gly, as shown in the MS/MS spectrum (Figure 5). Although the m/z values of certain fragments were identical for both compounds (e.g., fragments corresponding to protonated amino acid), the major fragments were shifted by 30 units, which permits discrimination of both compounds by MS.

The characteristic ions and fragments of eight xylose-derived Amadori compounds, obtained by reacting xylose with the corresponding amino acids, are shown in Table 3. Compared to the glucose-derived analogues, the fragment [M + H – 3H2O – H2CO]⁺ did not appear in the spectra of xylose-derived Amadori compounds. Mass chromatograms of the sample are shown in Figure 6. With the exception of Xyl-Leu and Xyl-Ile, all other xylose-derived Amadori compounds could be discriminated by MRM using the ion transition [M + H]⁺ → [M + H – H2O]⁺.

Table 3. MS/MS Spectra of Xylose-Derived Amadori Compounds

<table>
<thead>
<tr>
<th>compd</th>
<th>[M+H]⁺ (m/z)</th>
<th>ions in MS/MS spectra of [M+H]⁺ (m/z)²</th>
</tr>
</thead>
</table>

* AA, protonated amino acid moiety. "AA+CH₄" denotes the amino acid moiety with a residual CH₂ group from the sugar (cf. Figure 4).
Analysis Based on HPCEC-ECD. Although MS detection provides several advantages, such as identification of compounds and very high selectivity, other detectors can also be employed in the analysis of Amadori compounds. The use of an electrochemical detector can be an interesting option for samples that contain one or several Amadori compounds, providing they do not coelute. For illustration, Figure 7 shows a chromatogram of a reaction mixture of xylose heated with alanine and leucine in methanol at 70 °C for 4 h.

The HPCEC-ECD method was applied to study the formation of Xyl-Gly from xylose and glycine at 90 °C in a phosphate buffer (pH 6). A representative chromatogram obtained after 30 min of heating is shown in Figure 8A. Xyl-Gly was rapidly generated at the beginning of the reaction, reaching ~12 mol %, followed by a slow decline as the reaction progressed (Figure 8B). The total analysis time was very short due to the low retention time and limited sample preparation, which basically consisted of dilution with water. Precautions were taken to avoid analytical error due to the degradation of the Xyl-Gly. The samples were either analyzed within 8 h after sample preparation or they were frozen and stored at -20 °C prior to analysis. Solutions for establishing the calibration curves were freshly prepared. Under these conditions, no degradation of Xyl-Gly was observed. The analysis of pentose-derived Amadori compounds represents a major breakthrough in studying occurrence, formation, and decomposition of these labile Maillard intermediates.

In conclusion, a new analytical method based on HPCEC-MS/MS was developed for the analysis of Amadori compounds requiring a minimum sample cleanup. It was demonstrated that the method is an excellent analytical tool that permits monitoring of both hexose- and pentose-derived Amadori compounds in model reaction mixtures and in food products. Compared to HPAEC-ECD, the new method does not permit the simultaneous detection of parent sugars, as they are not retained on cation exchange columns. This represents the major limitation compared to HPAEC, which allows sensitive detection of hexose-derived Amadori compounds, but also simultaneous analysis of their precursors and some degradation products. On the other hand,
the new HPCEC-MS/MS method allows unequivocal identification of Amadori compounds, which is essential for the analysis of complex systems, such as foods or biological fluids. In addition, it is also suitable for the analysis of pentose-derived Amadori compounds, which are known to be very labile and readily decompose on anion exchange columns. The cation exchange column IonPac CS-17 can also be used with electrochemical detection for the analysis of Amadori compounds that do not coelute.

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