Journal of Chromatography, 206 (1981) 186–192
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,389

Note

Isolation and purification of Amadori compounds by semi-preparative reversed-phase high-performance liquid chromatography

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(Received October 3rd, 1980)

During heat processing or storage of foods and beverages the non-enzymatic Maillard browning reaction occurs between the carbonyl group of a reducing sugar and the amino group of an amine or amino acid. In the first steps of the reaction, Amadori compounds (1-amino-1-deoxy-2-ketoses) are formed. These compounds have been separated from several biological materials and are considered as precursors of the colour, aroma and flavour of processed foods[1–5]. The bound amino acid in an Amadori compound is not available as a source of amino acid which results in a decrease in the nutritional value of the foods[6–8]. Furthermore, the Maillard reaction has been shown to be responsible for some physiological and toxicological effects[9–11]. There is thus a growing interest in these compounds.

For their analysis in natural products, high-performance liquid chromatography (HPLC) on a cation-exchange resin[12] or in the reversed-phase mode[13] has been proved to be very suitable. However, the Amadori compounds have been investigated to date in relatively few biological products. This fact is due to the tedious procedures for their isolation and purification. The methods generally described[14–18] comprise multiple ion-exchange chromatographic separations which are time-consuming and result in poor yields.

In the first step of our work on the separation of Amadori compounds from biological materials we tried to isolate and purify them in a short time and in a sufficient amount for further systematic studies. We first confirmed the results described in the literature[13] on the separation of Amadori compounds from D-glucose and the aromatic amino acids by ionic suppression and ion-pairing reversed-phase HPLC. These procedures were suitable for the investigation of a number of Maillard reactions starting from aliphatic amino acids. In some cases, the separation of the Amadori compound on a C_{18} reversed-phase analytical column, using water as eluent, has proved to be efficient. An application of these results to the analysis on a C_{18} reversed-phase semi-preparative column of crude Maillard reactions between valine and glucose and between proline and maltose allowed the direct isolation and purification of the Amadori compounds as free products. In our preliminary work, we found it more convenient to use as a model system a mixture of proline (PRO) and its Amadori compound proline–fructose (PRO–FRU) previously obtained by crystallization[1,19].

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Reagents

Amino acids and sugars were from Fluka (Buchs, Switzerland). Water as eluent for the HPLC analysis was very high purity grade. Samples and water for HPLC were passed through a 0.45-μm Sartorius filter.

Thin-layer plates were coated with Polygram SIL G silica gel (Machery, Nagel & Co, Düren, G.F.R.).

Preparation of model Maillard browning systems

L-Valine (0.01 mole) and (+)-D-glucose monohydrate (0.03 mole) were dissolved in methanol (200 ml) and refluxed for 1 h. After cooling, the solution was evaporated to dryness under reduced pressure at 15°C. The residue was extracted with 150 ml anhydrous methanol and refluxed for another 3 h, removing water as its benzene azeotrope. After cooling, the mixture was evaporated to dryness and dissolved in a small amount of water for the HPLC analyses. The crude samples were injected directly into the liquid chromatograph.

L-Proline (0.010 mole) and (+)-D-maltose monohydrate (0.011 mole) were refluxed for 1 h in 200 ml methanol. After cooling, the solution was evaporated to dryness and the residue dissolved in 150 ml anhydrous methanol. Refluxing was continued, removing water as its benzene azeotrope, for another 8 h. The solution was then evaporated to dryness and the residue dissolved in a small amount of water for the HPLC analyses. The reactions were followed by thin-layer chromatography (TLC) on silica gel (eluent: methanol).

HPLC apparatus and procedures

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) included a Model 6000 A pump, a Model U6K injector and a Model R-401 differential refractometer. The analytical column (300 × 3.9 mm I.D.) was packed with Nucleosil 10 C_{18} (Machery, Nagel & Co.). The semi-preparative column (600 × 8 mm I.D.) was packed with 30-μm RSIL C_{18} HL (R.S.L., Eke, Belgium). The precolumn (25 × 3.9 mm I.D.) was packed with 30–38 μm CO:PELL ODS (Chrompack, Middelburg, The Netherlands).

On the analytical column, aliquots (25–30 μl) of the Maillard reactions were separated at a water flow-rate of 0.3–1 ml/min. On the semi-preparative column, aliquots (500–700 μl) were separated at a water flow-rate of 3–7 ml/min. In both these separations, the mobile phase flow-rate was increased after the elution of the Amadori compounds.

Spectrometric apparatus and procedures

The IR spectrophotometer was a Model PE 580 E (Perkin-Elmer). The Cameca NMR spectrometer was operated at 250 MHz. The ^{13}C NMR spectra were conducted in ^2H_2O as solvent with DSS as internal standard (δ TMS = δ DSS*). The Ribermag R 10/10 quadrupole mass spectrometer coupled to the Sidar 111 A data processing system was purchased from Nermag. The analyses were carried out according to the chemical ionization–desorption (CI/D) technique[20]. The reactant gas

* DSS = Sodium trimethylsilyl propionate-d_4.

used was NH_3. In this case the CI/D analyses generally give three ions corresponding to M, M + H^+ (M + 1) and M + NH_4^+ (M + 18). Conditions: desorption, 40–500 mA; speed 0.7 mA/sec; desorption point 378 mA; source temperature 70°C. For the desorption process, 1 μl of a methanolic solution of the sample (5 mg in 5 ml) was deposited on the filament.

RESULTS AND DISCUSSION

The analytical HPLC profiles of the crude Maillard reactions between valine and glucose and between proline and maltose are shown in Figs. 1A and 2A respectively. In the latter case, the presence on the chromatogram of glucose and PRO–FRU is proof of some thermal decomposition of PRO–maltulose during the reflux process.

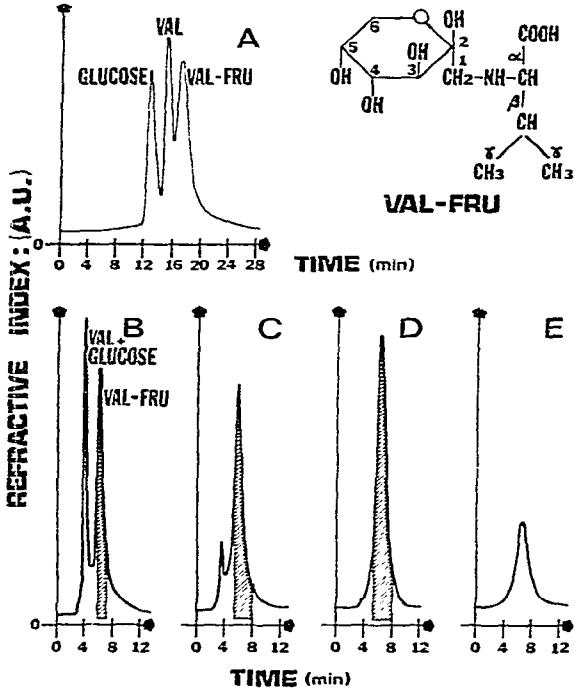

Fig. 1. HPLC profiles of the crude Maillard reaction between valine and glucose on the analytical column (A) and the semi-preparative column (B). Two successive purifications (C and D) of the collected peak of VAL–FRU in B give a pure compound (E). Chromatographic conditions: A, Nucleosil 10 C_{18}, mobile phase (water) flow-rate 0.3–1 ml/min; B, 30-μm RSIL C_{18} HL, mobile phase (water) flow-rate 3–7 ml/min. A.U. = Arbitrary units.

Figs. 1B and 2B show the analysis of the two samples on the semi-preparative column. The peaks are not as well resolved as in A, but the fraction containing the Amadori compound can be recovered (shaded portions on the chromatograms).

After evaporation of the fractions to a small volume a second chromatographic separation was carried out. Three purifications were generally sufficient to obtain

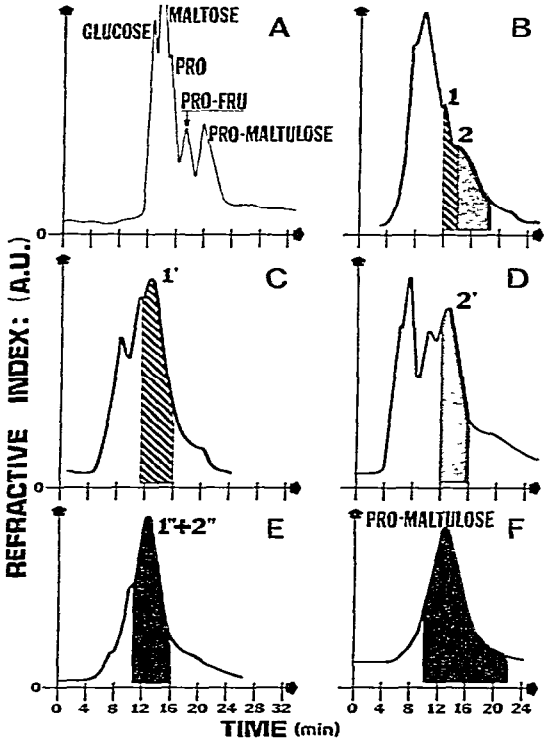

Fig. 2. HPLC profiles of the crude Maillard reaction between protine and maltose on the analytica column (A) and the semi-preparative column (B). Fraction 1 containing PRO–FRU and significant traces of PRO–maltulose is purified in C. Fraction 2 containing PRO–maltoluse is purified in D. Both collected fractions 1′ and 2′ contain PRO–maltulose and are purified together in E and F. Chromatographic conditions as in Fig. 1.

the pure Amadori compound. The residue, obtained after evaporation to dryness of the last purified fractions, was crystallized three times from a small amount of anhydrous methanol. We obtained 900 mg VAL–FRU (yield:30% from valine) and 500 mg PRO-maltulose (yield:10% from proline). Both yields can be optimized. The purity of the Amadori compounds was examined by TLC and HPLC on the analytical column. Their structures were confirmed as follows.

VAL–FRU

IR (KBr):ν (C = 0) at 1620 cm^{-1}. Mass spectrum: parent VAL–FRU, 280 (M + 1); fragments: fructose, 180 (M) and valine, 118 (M + 1). ^{13}C NMR: the chemical shifts with respect to tetramethylsilane (TMS) (Table I) were compared with literature data[21–23]. In ^2H_2O as solvent, VAL–FRU can occur as an equilibrium mixture of the furanose and pyranose structures α and β via the open-chain structure. Based on C-2 signals, the percentages of the different configurations are: β-pyranose, 71%; β-furanose, 23% and α-furanose, 6%. The predominance of the β-pyranose configuration at neutral pH confirms the literature[12] results.

TABLE I

^{13}C NMR ASSIGNMENTS FOR VAL–FRU (FIG. 1)

p = Pyranose; f = furanose.

δ (ppm)/TMS	*Fructose*		*Valine*
	Carbon atom	*Configuration*	*carbon atom*
175.21 175.03			COOH
101.59 98.06 98.00	2	α-p, β-f, β-p	
85.38 85.29	3 4	α-f, α-f, β-f	
80.88 78.73	3 5	β-f α-f, β-f	
72.91 72.11 71.85 71.73 71.67 71.52	3, 4, 5	α-p, β-p	
66.64 63.55	1, 6	α-p, α-f, β-f	
60.22 56.13			C-α
31.80 31.68			C-β
21.24 21.09 19.89 19.74			C-γ

PRO–maltulose

IR (KBr):ν (C = O) at 1627 cm^{-1}. Mass spectrum:no parent peak of PRO–maltulose was found, probably due to the thermal instability of this compound. Fragmentation peaks: proline 116 (M + 1), 133 (M + 18); glucose, fructose 180 (M), 198 (M + 18), 216 (M + 2 × 18); PRO–FRU 278 (M + 1); maltulose 342 (M − H_2O), 378 (M + 2 × 18). ^{13}C NMR:the assignments to the sugar moiety could not be made with precision on account of the complexity of the spectrum due to the equilibrium in solution between the different configurations (Table II). The assignments were compared with the literature data[21–23].

Based on the above results, we consider that the semi-preparative HPLC separations of Amadori compounds from crude Maillard reactions can be conducted rapidly, with good recoveries of the desired product and low solvent cost. The availability of sufficient amounts of the Amadori compounds in a shorter time would permit more systematic studies of them and of their biological roles.

TABLE II

^{13}C NMR ASSIGNMENTS FOR PRO–MALTULOSE (FIG. 3)

δ (ppm)/TMS	*Maltulose*		*Proline*
	Carbon atom	*Configuration*	*carbon atom*
176.59			COOH
176.45			
103.36			
100.65			
98.50	2	α-p, α-f, β-p, β-f	
98.45			
85.20			
82.97	3, 4	α-f, β-f	
80.53			
75.61			
75.50			
75.29			
75.17	2′, 3′, 4′, 5′		
74.52			
74.23			
73.91			
72.41			
72.23	3, 4, 5	α-p, β-p	
72.11			
71.58			
66.58			
63.37	1, 6	α-p, α-f, β-f	
63.23			
60.02			C-α
51.72			C-δ
31.48			C-β
31.04			
26.15			
26.06			C-γ
26.01			

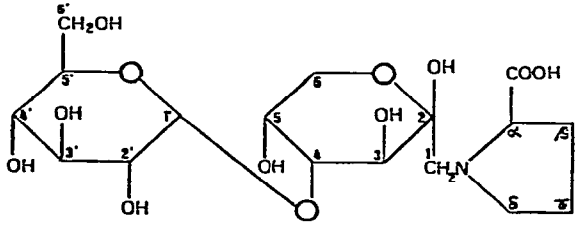

Fig. 3. Structure of PRO-maltulose.

ACKNOWLEDGEMENTS

We wish to thank the D.G.R.S.T. (No. 79.7.1447) for suppor of this work. We also wish to acknowledge the Research Center TEPRAL (B.S.N. Food Branch) for the HPLC packing materials and for recording the mass spectra. We are grateful to Mrs. Eppiger (Centre Regional de Mesures Physiques de l'Academie de Nancy-Metz) for recording the ^{13}C NMR spectra.

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