Journal of Chromatography, 516 (1990) 287–298
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 523

Separation and partial characterization of Maillard reaction products by capillary zone electrophoresis

ZDENEK DEYL*, IVAN MIKSIK and RUDOLF STRUZINSKY
Institute of Physiology, Czechoslovak Academy of Sciences, Videnska 1083, 142 00 Prague 4 (Czechoslovakia)

ABSTRACT

Capillary zone electrophoresis proved useful for separating small amounts of both charged and uncharged solutes that are otherwise difficult to analyse. A typical complex mixture that had previously resisted all analyticl approaches, including reversed-phase separations, is the products arising from the reaction of free amino acids with aldehydic sugars (Maillard reaction products). By using capillary zone electrophoresis [untreated capillary 50 cm × 75 μm I.D., 18 kV, 0.02 mol/l phosphate buffer (pH 7.5)], a number of products resulting from the reaction of glucose or ribose with glycine, alanine and isoleucine were separated and partially characterized. They were separated (1) without derivatization (and profiles of compounds absorbing at 220 nm were obtained), (2) as phenylthiocarbamyl derivatives in a search for reactive amino groups and (3) after derivatization with 2,4-dinitrophenylhydrazine in a search for a method for compounds with a free aldehydic group. Phenylthiocarbamyl derivatives were separated in 0.005 mol/l borate buffer (pH 9.6) at 20 kV and 25 μA. Separation of 2,4-dinitrophenylhydrazones was effected by electrokinetic micellar chromatography in the same apparatus using a 50 cm × 75 μm I.D. capillary at 10 kV in 0.01 mol/l Na_2HPO_4–0.006 mol/l tetraborate, 0.050 mol/l with respect to sodium dodecyl sulphate. The results are compared with those given by high-performance liquid and thin-layer chromatography.

INTRODUCTION

In a previous study[1] we attempted to separate reactive 2,4-dinitrophenylhydrazine compounds arising during the Maillard reaction. It was emphasized that Maillard reaction products represent complex mixtures which are very difficult to analyse. On the other hand, their importance is considerable: they represent a traditional topic in food chemistry[2] and more recently they have been shown to be involved in cross-linking of proteins[3] and even nucleic acids[4], influencing the metabolic turnover of these biopolymers. Biologically they are undesirable side-products of metabolism and their role in ageing is a matter of discussion[5]. Briefly, in the reaction of D-glucose and L-glycine after an Amadori rearrangement D-fruc-

0021-9673/90/$03.50

toseglycine was found which, in the next step, may react to give di-D- fructoseglycine. At weakly acidic pH and at elevated temperature the compound is rapidly split into 3-deoxy-D-*erythro*hexosulose and unsaturated side-products. Dicarbonyl intermediates are prone to reactions with available free amino groups with the formation of brownish and/or polymeric products[6].

Within the Maillard products obtained some individual compounds were identified and a rough estimate of over 150 compounds present was reported[7]. Some of the *in vivo* occurring compounds that were identified were later shown to be preparative artifacts[8].

From the analytical point of view, it is evident that new approaches have to be applied because, *e.g.*, high-performance liquid chromatographic (HPLC) procedures did not give satisfactory results in spite of testing a wide variety of separation conditions. Moreover, a profiling technique would help considerably in obtaining orientation information on the reaction products as it has to be kept in mind that not only are the products numerous but also their nature is likely to depend on the nature of the sugar and amino acid involved.

In this work we have tried to exploit the high separating power of capillary zone electrophoresis (CZE); the profiles were obtained on the basis of UV (220 nm) absorbance and additional information was gained by reacting the products with 2,4-dinitrophenylhydrazine and phenyl isothiocyanate using simple amino acid–sugar mixtures as model systems.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical-reagent grade, *i.e.*, glycine (Reanal, Budapest, Hungary), β-alanine (Loba, Wien-Fischamend, Austria), isoleucine (Calbiochem, San Diego, CA, U.S.A.), glucose monohydrate, ribose and 2,4-dinitrophenylhydrazine (Lachema, Brno, Czechoslovakia). Solvents for the Maillard products preparation and derivatization were used without further purification. Methanol for chromatography (Merck, Darmstadt, F.R.G.), acetonitrile of spectrometric grade (Janssen, Beerse, Belgium) and doubly distilled water were used in chromatographic separations. For sterilization filters of 0.2-μm pore size (Sigma, St. Louis, MO, U.S.A.) were used. All chemicals used in the preparation of electrophoretic buffers were of analytical-reagent grade and were purchased from Lachema.

Preparation of Maillard products

Glucose–glycine Maillard products were prepared by the following procedure[9]: 0.018 mol of glycine (1.35 g) were reacted with 0.0045 mol of glucose monohydrate (0.9 g) in 60 ml of ethanol with 20 drops of glacial acetic acid under reflux. After boiling for 24 h the reaction mixture was filtered and the filtrate was dried and reconstituted in doubly distilled water (*ca.* 10 mg/ml). Maillard reaction products with other amino acids were prepared in a similar way (alternatively 2.36 g of isoleucine or 1.50 g of alanine were used in the reaction).

Long-term incubation of ribose and glycine was performed by the following procedure: 0.0015 mol of glycine (0.113 g) and 0.003 mol of ribose (0.450 g) were incubated at 37°C in 15 ml of 0.2 mol/l phosphate buffer (pH 7.4). Sterilization was

effected by filtration through a 0.2-μm pore size filter. Samples of 2 ml were taken at 2, 6 and 36 days.

Preparation of 2,4-dinitrophenylhydrazones

The reagent was prepared as a saturated solution of 2,4-dinitrophenylhydrazine in 2 mol/l hydrochloric acid[10]. Reaction was performed by addition of 6 ml of reagent solution to 1 ml of the solution of the Maillard products. After 1 h at room temperature the sediment was filtered off and washed with distilled water on the filter. 2,4-Dinitrophenylhydrazones obtained in this way were dissolved in ethyl acetate (*ca.* 5 mg/ml).

Preparation of phenylthiocarbamyl (PTH) derivatives

Derivatization of Maillard products with phenyl isothiocyanate followed the procedure recommended in the Operator's Manual for the Waters Assoc. PICO-TAG system[11].

Electrophoretic apparatus

Capillary zone electrophoresis was effected in a laboratory-assembled apparatus (described in detail elsewhere[12]) resembling the set-up published by Jorgenson and De Arman-Lukacs[13]. An untreated fused-silica capillary protected on the external surface with a silicone-rubber layer (Institute of Physics, Slovak Academy of Sciences, Bratislava, Czechoslovakia) was used for separation. The capillary was 50 cm long (to the detector) with an additional 10 cm to the cathode and had an inside diameter of 75 μm. Before analysis the capillary was washed with 1 ml each of methanol, chloroform, methanol, 1 mol/l sodium hydroxide solution and 3 mol/l hydrochloric acid. Finally, the capillary was washed with 2 ml of the running buffer, attached to the high-voltage source and left running at 10 kV until the current dropped to 20 μA.

The light beam generated by a deuterium lamp at 220 nm passed a slit and entered the capillary. This was conditioned so that a 1-cm long piece of the plastic covering sheet was removed near the cathodic end and this part was used as a cuvette. The outcoming light was then measured with a UV detector.

The capillary was attached to a variable-voltage source. If the current exceeded 70 μA, the voltage automatically decreased to avoid overheating. The sample was applied electrophoretically (0.05 min, 10 kV).

Electrophoretic operating conditions

Samples for the analysis of underivatized Maillard products were run in 0.02 mol/l phosphate buffer (pH 7.4) at 18 kV and 60 μA. Separation of phenyl isothiocyanate-derivatized Maillard reaction products was effected in 0.005 mol/l borate buffer (pH 9.6) at 20 kV and 25 μA. Electrokinetic chromatography of 2,4-dinitrophenylhydrazones was carried out in 0.01 mol/l Na_2HPO_4–0.006 mol/l tetraborate which was 0.05 mol/l with respect to sodium dodecyl sulphate (SDS). The applied potential was 10 kV. Owing to the higher conductivity, the current limit was set at 80 μA.

Reversed-phase chromatography

All separations except those of phenylthiocarbamyl derivatives were carried out

on a Spectra-Physics (San Jose, CA, U.S.A.) SP 8100 liquid chromatograph connected to a Spectra-Physics SP 4100 computing integrator. The eluent was monitored at 270 nm (underivatized Maillard products) or at 360 nm (2,4-dinitrophenylhydrazones) using a Waters Assoc. (Milford, MA, U.S.A.) 490E programmable multi-wavelength detector. A glass column (150 × 3.3 mm I.D.) packed with Separon SGX C_{18} (7 μm) (Tessek, Prague, Czechoslovakia) was mounted in the instrument. Separation of phenylthiocarbamyl derivatives of Maillard reaction products was done on the PICO-TAG Amino Acid Analysis System (Millipore, Milford, MA, U.S.A.).

Separation of underivatized Maillard reaction products. The column was conditioned with acetonitrile for 15 min before every analysis. The flow-rate was maintained at 1.0 ml/min. At time zero (100% acetonitrile) the sample (10 μl) was applied to the column and was eluted by decreasing the proportion of acetonitrile to give acetonitrile–methanol–water (70:28:2, v/v/v) at 2 min. Then the elution was isocratic with acetonitrile–methanol–water (70:28:2) from 2 to 10 min and acetonitrile–methanol–water (70:20:10) from 10.1 to 20 min. From 20 min the sample was eluted with an increasing proportion of water in the mobile phase, reaching 100% at 30 min. Finally the column was eluted for 10 min with water. The temperature was maintained at 40°C.

Separation of 2,4-dinitrophenylhydrazine-reactive products. The column was conditioned with methanol–water (30:70, v/v) for 15 min before every analysis. The flow-rate was 1.0 ml/min. The sample was applied to the column in ethyl acetate (*ca.* 5 mg/ml) and elution was started with methanol–water (30:70, v/v) followed by a linear gradient reaching 65% methanol in 15 min after application; isocratic elution with methanol–water (65:35, v/v) followed for another 15 min. The column was washed with methanol for another 10 min. The temperature was maintained at 30°C. Preparative separations were done with the same system and corresponding fractions were pooled.

Separation of phenylthiocarbamyl derivatives. Analysis of these derivatives was done on a PICO-TAG Amino Acid Analysis System[11] with standard derivatization. Briefly, samples were separated by reversed-phase HPLC using two eluents: (A) 19.0 mg/l of sodium acetate trihydrate, 0.5 ml/l of triethylamine, titrated to pH 6.40 with glacial acetic acid and subsequently 60 ml of acetonitrile being added to 940 ml of this buffer and (B) 400 ml of water added to 600 ml of acetonitrile. The column was conditioned with 100% eluent A for 18 min before each analysis at a flow-rate of 1.5 ml/min. During analysis the flow-rate was 1.0 ml/min. Elution was started with 100% eluent A followed by a linear gradient reaching 46% eluent B at 10 min and 100% eluent B at 10.5 min. Between 10.5 to 12.0 min elution was isocratic with 100% eluent B. The temperature was maintained at 38°C and the effluent was monitored at 254 nm.

Affinity chromatography

A Glyco-gel boronate–agarose column (Pierce, Rockford, IL, U.S.A.)., which is cross-linked 6% beaded agarose prepacked with 1.0 ml of immobilized *m*-aminophenylboronic acid, was equilibrated with 10 ml of wash buffer (0.25 mol/l amonium acetate, 0.05 mol/l magnesium chloride, 0.003 mol/l sodium azide, pH 8.5). The sample in wash buffer (0.4 ml) was applied to the column and allowed to soak the gel. Then 5 ml of wash buffer were added to the column and allowed to drain into the gel. This non-bound fraction was collected. The bound fraction was eluted with 4 ml of elution

buffer (0.2 mol/l sorbitol, 0.05 mol/l Na_2EDTA, 0.003 mol/l sodium azide, pH 8.5). The column was regenerated by washing with 5 ml of water and then 10 ml of 0.1 mol/l acetic acid.

High-performance thin-layer chromatography (HPTLC)

Silica gel 60 F_{254} HPTLC plates (10 × 10 cm) for nano-TLC (Merck) were used. Samples were developed twice with carbon tetrachloride–ethanol–*n*-butanol (90:5:5,

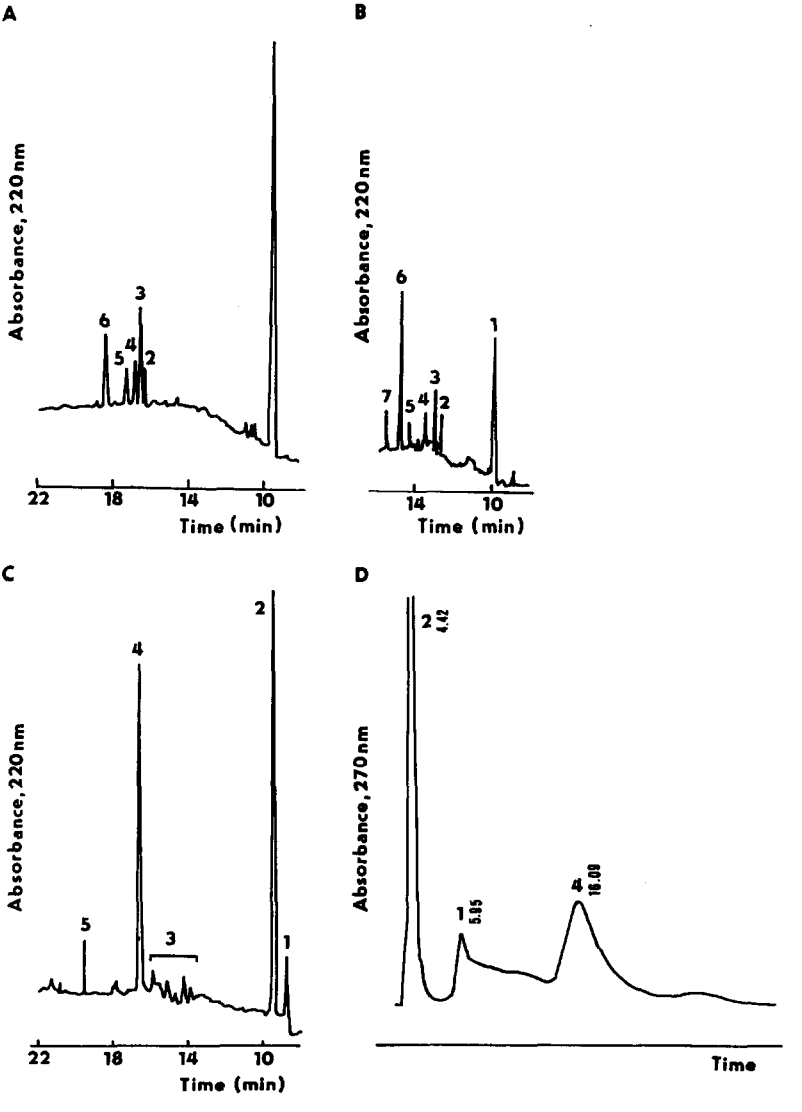

Fig. 1. Electrophoretic profiles of Maillard reaction products during the reaction of different amino acids and glucose. (A) Glycine; (B) isoleucine; (C) alanine. (D) Reversed-phase HPLC separation of products arising during the reaction of glucose and alanine.

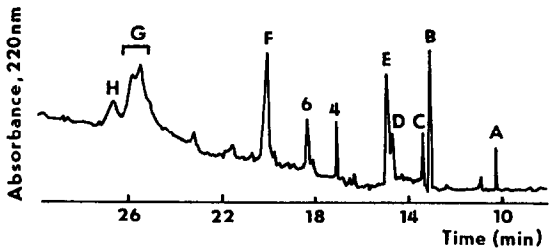

Fig. 2. Electrophoretic patterns of products arising during the reaction of glycine with glucose after hydrolysis.

v/v/v). The compounds were detected as quenching spots under UV light at 254 nm (Min UVIS; Desaga, Heidelberg, F.R.G.).

RESULTS

UV absorbance (220 nm) profiles of Maillard reaction products arising during the reaction of glucose with glycine, alanine and isoleucine by CZE are presented in Fig. 1A–C. Fig. 1D shows a comparative separation obtained by reversed-phase

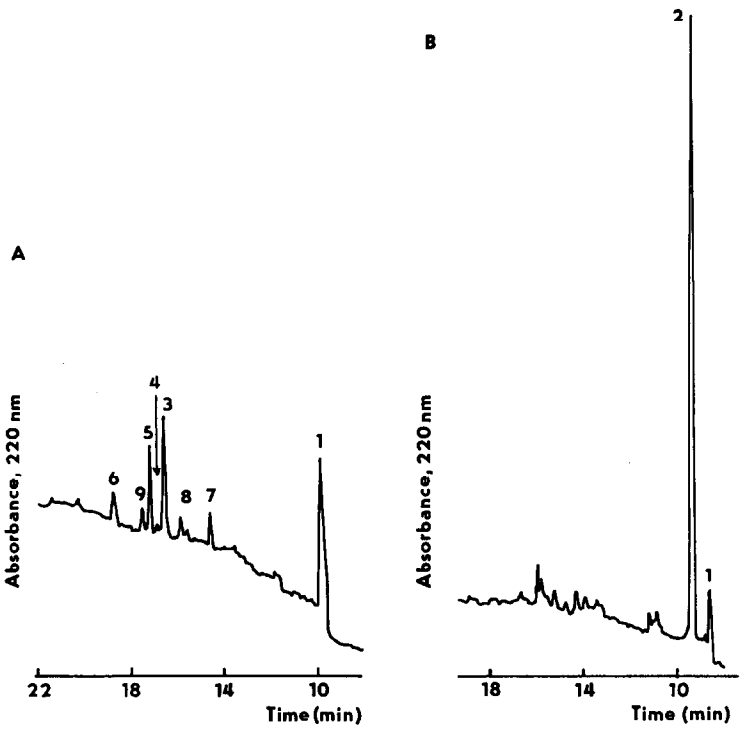

Fig. 3. Electrophoretic patterns of products arising from the reactions of (A) glucose with glycine (B) glucose with alanine after removal of 2,4-dinitrophenylhydrazine-reactive products.

chromatography of the glucose–alanine Maillard products. Five to seven peaks can be distinguished in individual CZE separations. The results clearly show that the composition of the mixture formed differs according to the amino acid component of the starting mixture. For glycine and alanine the profile is typical in containing two prominent peaks present at comparable concentrations, one of which moves much faster than the other in the electric field. Most of the separated peaks are unstable during acid hydrolysis in 6 mol/l hydrochloric acid (Fig. 2); this indicates that the types of compounds present in the unstable peaks are precluded from being detected in naturally occurring samples of glycated (non-enzymatically glycosylated) proteins. Thus, *e.g.*, the main peak of the glucose–glycine profile disappears completely and only peaks 4 and 6 (Fig. 2) withstand routine protein treatment for amino acid analysis.

When the glucose–glycine reaction mixture is reacted with 2,4-dinitrophenylhydrazine to eliminate components possessing oxo groups, the profile presented in Fig. 3A is obtained, showing a distinct decrease in peaks 1 and 6 and complete disappearance of peaks 2 and 4. The same treatment of the alanine–glucose mixture (Fig. 3B) shows the complete disappearance of peak 4 emerging in the untreated sample at 16.4 min.

If the glucose–glycine reaction products are prepared by long-term incubation at 37°C rather than by boiling, the profile obtained is considerably simplified (Fig. 4). Both main peaks (1 and 2) are virtually eliminated after 2,4-dinitrophenylhydrazine treatment.

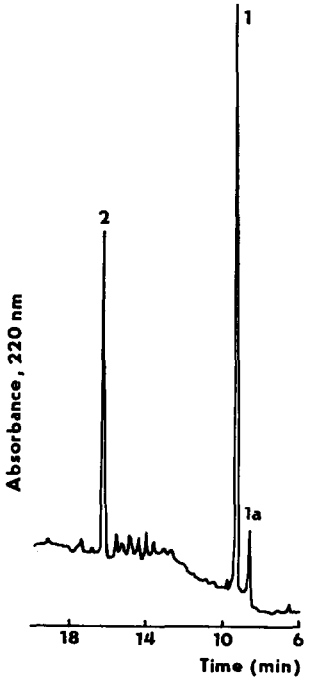

Fig. 4. Electrophoretic profiles of glucose–glycine Maillard reaction products obtained after incubation for 36 days at 37°C.

TABLE I

PROPERTIES OF THE INDIVIDUAL PEAKS OF MAILLARD REACTION PRODUCTS OBTAINED DURING THE GLUCOSE–GLYCINE REACTION

Six peaks can be distinguished after PTH derivatization, indicating the possibility that all the separated peaks may possess a reactive amino group.

Peak No.	*Susceptible to acid hydrolysis (6 mol/l HCl)*	*Susceptible to reaction with 2,4-dinitrophenylhydrazine (oxo-group possessing)*	*Retained during boronate affinity chromatography (possessing vicinal diol)*
1	Yes	Yes (partly)	No
2	Yes	Yes	Yes
3	Yes	No	Yes
4	No	Yes	Yes
5	Yes	No	Yes
6	No	Yes (partly)	Yes

When the glucose–glycine Maillard product mixture obtained at high temperature is subjected to boronate–agarose affinity chromatography, the whole profile is clearly split into two parts: the compound(s) present in the first peak of the electrophoretic profile pass unretained through the column, indicating that it is devoid of vicinal OH groups, whereas the compounds present in the remainder of the electrophoretic profile are bound to the column. A survey of the properties of individual peaks is presented in Table I.

So far the experiments were oriented only to the analysis of products arising by the reaction of glucose. If ribose is used as the sugar component for the preparation of Maillard reaction products a different profile is obtained, indicating that the nature of the products depends on both the carbohydrate and the amino acid component of the reaction mixture. Fig. 5 shows the profiles obtained after incubation of glycine with ribose for 2, 6 and 36 days: the profile consists of a cluster of rapidly emerging peaks (with low electrophoretic mobility) followed by a series of rapidly electrophoresed peaks at the longest incubation time. Within the rapidly emerging cluster of peaks, that designated as 2 is split into 2a and 2b at longer incubation times. Comparative HPLC separations are presented on the right-hand side.

Fig. 6 demonstrates the attempt to separate the 2,4-dinitrophenylhydrazine-reactive products (oxo-containing) arising during the Maillard reaction between glycine and glucose. It is evident that all the three methods used, *i.e.*, CZE (electrokinetic chromatography in the presence of 0.5 mol/l SDS), reversed-phase (RP) HPLC and normal-phase TLC yielded reasonable separations of the main five peaks. Similarly to RP-HPLC, in micellar electrokinetic chromatography the more hydrophobic solutes interact more strongly with the hydrophobic (micellar) phase and, consequently, should be retained longer than hydrophilic compounds. In other words, the order of elution of individual peaks should be similar to that for reversed-phase separation. Further, the order of eluted peaks during normal-phase TLC separation should be the reverse of that in both RP-HPLC and micellar electrokinetic chromatography. It is evident that this is not completely true. On comparing electrokinetic chromatography with RP-HPLC, particularly the retention time of peak

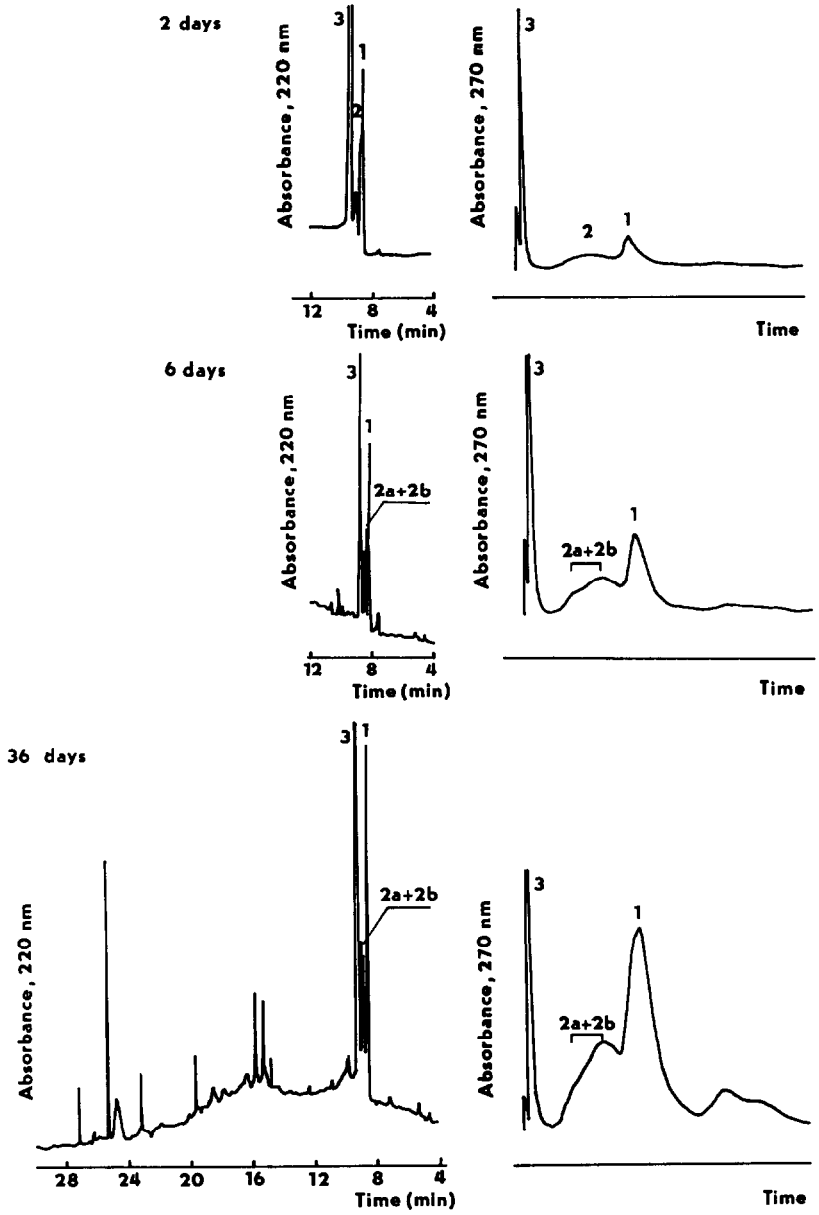

Fig. 5. Profiles of ribose–glycine Maillard reaction products obtained after incubation for 2, 6 and 36 days at 37°C. Capillary zone eletrophoresis runs are on the left and RP-HPLC separations on right.

3 is unexpectedly prolonged in the former instance. The normal-phase TLC separation does not follow any theoretical predictions at all. Peaks 1a and 3 were identified by comparison with standards and by mass spectra as formaldehyde and acetaldehyde, respectively.

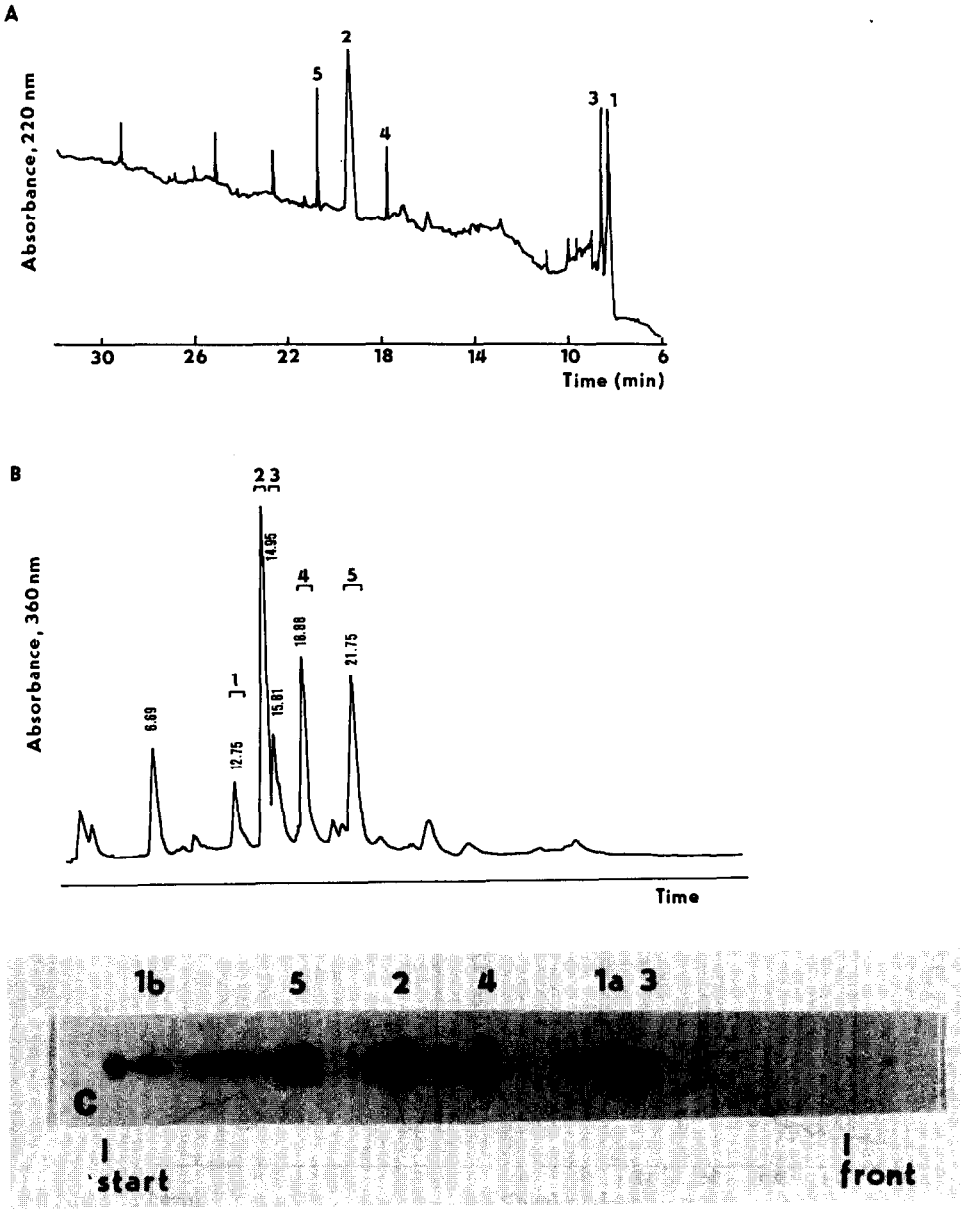

Fig. 6. Comparison of (A) CZE, (B) RP-HPLC and (C) TLC of the separation of 2,4-dinitrophenylhydrazones of the glycine–glucose Maillard reaction products.

DISCUSSION

Capillary zone electrophoresis offers new possibilities for separating complex mixtures that are otherwise difficult to separate. Maillard reaction products represent

a typical example: in liquid column chromatographic separations in both the normal- and reversed-phase modes and under a wide variety of experimental conditions the Maillard products yield broad incompletely resolved peaks. Application of gas chromatographic techniques suffers from problems regarding the low volatility of most of the products; this is particularly true for polymeric Maillard products arising at a later stage of the reaction when coloured polymers are formed. For these reasons we applied CZE to separate Maillard reaction products arising during the reaction of glucose and ribose with glycine, alanine and isoleucine.

As demonstrated, it was possible to separate a number of differently charged peaks in any particular model Maillard system. It was easily possible to follow, *e.g.*, the resistance of individual peaks towards acid hydrolysis, the susceptibility of the products formed towards reaction with 2,4-dinitrophenylhydrazine or phenyl isothiocyanate and the content of vicinal diols. Finally, it was possible to obtain at least general information about the types of compounds formed (Table I).

Separation of the phenylthiocarbamyl derivatives of glycine–glucose Maillard reaction products is shown in Fig. 7. Six main peaks were obtained both by CZE and with the PICO-TAG Amino Acid Analysis System.

If we consider next the general profile of glycine–glucose products, of the six peaks present (Fig. 1a), four contain 2,4-dinitrophenylhydrazine-reactive compounds. At least some of these four peaks are not homogeneous, as revealed by the

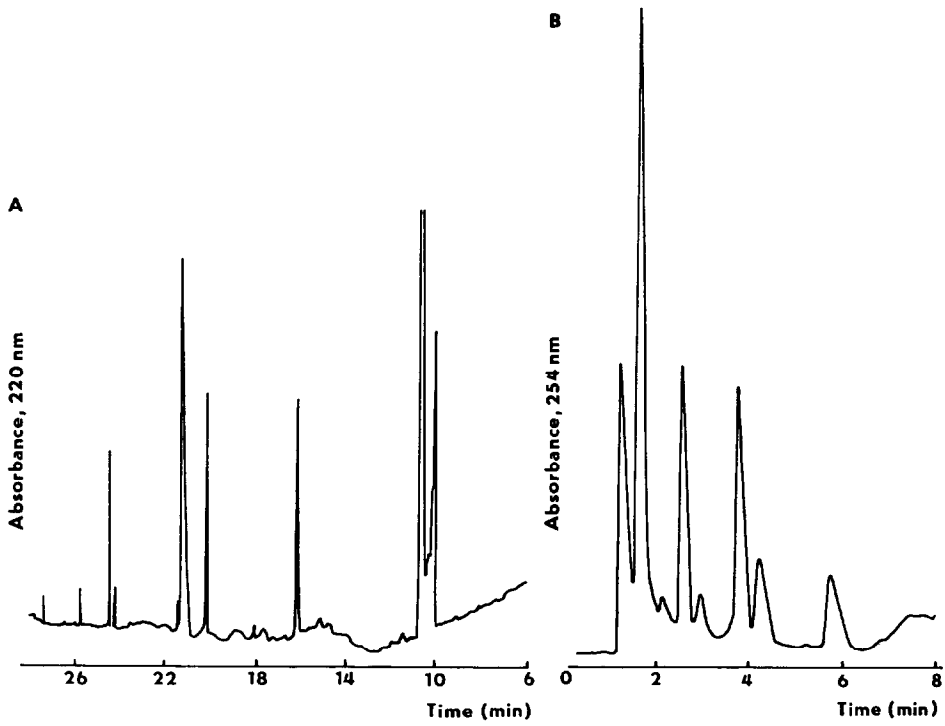

Fig. 7. Capillary zone electrophoresis profile in comparison with the RP-HPLC pattern of glycine–glucose Maillard reaction products after PTH derivatization.

electrokinetic micellar chromatography in the next step. Electrochromatographic separation resulted in five distinct peaks, two of which were identified as formaldehyde and acetaldehyde and one of which can be further separated into two zones by normal-phase TLC. Individual peaks in the electrochromatographic separation of components possessing oxo groups as 2,4-dinitrophenylhydrazones were (except for peak 1) obviously homogeneous and their number was coincident with the number of peaks obtained during RP-HPLC and normal-phase TLC. Two of these peaks were identified, on the basis of their migration in three different separation systems, as acetaldehyde and formaldehyde. By comparing the individual profiles (the original mixture and the mixture of 2,4-dinitrophenylhydrazine-reactive products), at least eight products originate during the Maillard reaction between glucose and glycine.

On reacting the Maillard reaction mixture of glucose and glycine with phenyl isothiocyanate, six peaks can be discerned both in CZE and RP-HPLC, indicating that there are six compounds with a reactive amino group present in the mixture, provided that none of the compounds present yields more than one phenyl isothiocyanate derivative.

The products arising during the glycine–glucose Maillard reaction are neither of clearly polar nor distinctly hydrophobic nature, which causes most of the problems in their separation. These unfavourable properties are preserved even after derivatization, as clearly demonstrated by comparing the different techniques applied to 2,4-dinitrophenylhydrazones (Fig. 6). The separation mechanisms involved are apparently of the multiple-mode type. Consequently, theoretical predictions for the electromigration (and also chromatographic) separations of Maillard products are very difficult indeed.

Regarding the main product(s) arising in all the model systems used at both low and high temperatures, our present knowledge of its properties can be summarized as follows: it is a poorly charged compound (or a category of compounds) at pH 7.4 (or higher), susceptible to acid hydrolysis, which does possess 2,4-dinitrophenylhydrazine-reacting groups and is devoid of vicinal diols.

REFERENCES

1 I. Miksik, R. Struzinsky, K. Macek and Z. Deyl, *J. Chromatogr.*, 500 (1990) 607.
2 M. Namiki, *Adv. Food Res.*, 32 (1988) 115.
3 M. Brownlee, A. Cerami and H. Vlassara, *N. Engl. J. Med.*, 318 (1988) 1315.
4 R. Bucala, P. Model and A. Cerami, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 105.
5 J. J. Harding, H. T. Beswick, R. Ajiboye, R. Huby, R. Blakytny and K. C. Rixon, *Mech. Age Dev.*, 50 (1989) 7.
6 H. Paulsen and K.-W. Pflughaupt, in W. Pigman, D. Horton and J. D. Wander (Editors), *The Carbohydrates, Chemistry and Biochemistry*, Vol. IB, Academic Pres, New York, 1980, p. 881.
7 W. Boltes and L. Mewissen, *Z. Lebesm.-Unters.-Forsch.*, 187 (1988) 209.
8 F. G. Njoroge, A. A. Fernandes and V. M. Monnier, *J. Biol. Chem.*, 263 (1988) 10646.
9 M. Sengl, F. Ledl and T. Severin, *J. Chromatogr.*, 463 (1989) 119.
10 R. H. Brandenberger and H. Brandenberger, in K. Blau and G. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, London, 1978, p. 234.
11 Waters Assoc., *PICO-TAG Amino Acid Analysis System, Operator's Manual*, Millipore, Milford, MA, 1984.
12 V. Rohlicek and Z. Deyl, *J. Chromatogr.*, 494 (1989) 87.
13 J. W. Jorgenson and K. De Arman-Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266.