CHROM. 25 255

Buffer conditions affecting the separation of Maillard reaction products by capillary electrophoresis

Andy J. Tomlinson* and James P. Landers

Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905 (USA)

Ivor A.S. Lewis

Department of Chemistry, King's College, London, University of London, London WC2R 2CS (UK)

Stephen Naylor

Department of Pharmacology and Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905 (USA)

ABSTRACT

Capillary electrophoresis was applied to effect the separation of Maillard reaction products generated by the reaction of 5-hydroxymethylfurfural (5-HMF) with glycine. Since many components of this reaction mixture have not been previously identified an empirical approach was taken to develop separation conditions. Buffer composition, ionic strength and pH were all investigated. The effects of these parameters on the separation of a 5-HMF-glycine model Maillard reaction mixture are reported.

INTRODUCTION

Reactions involving amino acids, peptides, or proteins with reducing sugars (Maillard chemistry) are believed to be of great importance in food chemistry. It has been suggested that such reactions are central to the formation of flavours [1–4], colours [5–9], mutagens [10], and antioxidants [11] as foods are cooked or processed. Furthermore, it has been reported that these interactions can occur in dried foods (*e.g.*, milk powders) as they are stored, causing discolouration [12], production of off-flavours [13], and a possible loss of nutritional value [14,15]. In addition, Maillard chemistry has recently been shown to be involved in cross-linking of proteins [16] and nucleic acids [17], thereby influencing the metabolic fate of these biomolecules. Such reactions are also thought to occur *in vivo*, and their role in ageing has been postulated [18,19].

Investigation of the components formed during the reaction of amino compounds with sugars is often aided by analysis of model reaction systems. Typically a pure amino compound (usually an amino acid) is reacted, under a variety of conditions (*e.g.* changes in temperature, reactant concentrations, etc.), with a reducing sugar. These studies have shown that even model systems lead to extremely complex product reaction mixtures that are difficult to analyse [see, for example, refs. 5–9]. This is particularly evident in the investigations of coloured components (the melanoidins) that result from this

---

* Corresponding author.

chemistry [20]. As a result, information regarding these reaction products is rather limited, and complete structural analyses have not been successful. Indeed, to date, despite many attempts, effective means of isolation of the melanoidins have not been established, and HPLC techniques have proved to be of limited use in their investigation [20].

Based on the observation that melanoidins possess amphoteric character [21], there have been attempts to separate them using classical electrophoretic techniques, such as gel and paper electrophoresis [21,22]. Both techniques enabled the separation of model reaction mixtures into a series of coloured bands, but in both instances, the resolution of individual components was reported to be poor. Other electrophoretic techniques applied to the attempted separation of melanoidins include those of electrofocusing and preparative electrofocusing [22]. These latter techniques are reported to enable the separation of amphoteric species that exhibit isoelectric points that differ by 0.005 pH units [23]. However, when applied to the separation of melanoidins, it was found that analysis times were lengthy, often taking up to 14 h to complete [22]. Also, as separations occurred at the gel surface, it was difficult to determine whether the observed coloured bands were real, or artifacts caused by oxidation of the labile melanoidins. Modification of these techniques is required to enable the separation of coloured components of Maillard reaction mixtures in a rapid manner under benign conditions.

A technique that is currently gaining widespread acclaim for the separation of a variety of compound mixtures is capillary electrophoresis (CE) [see refs. 24 and 25 for reviews]. In this form of free solution zone electrophoresis separations are performed in thin-walled, narrow bore (typically 50 μm internal diameter) fused-silica capillaries, that are filled with an aqueous buffer solution [25]. Application of a high voltage across the capillary affords very rapid, highly efficient separations, that are a function of the mass and charge of separated species. Capillary efficiencies in excess of $1 \cdot 10^6$ theoretical plates per meter are not uncommon. In addition, additives/buffer modifiers can be added to the buffers to change separation mechanisms, e.g.,

the addition of surfactants to buffers (as in micellar electrokinetic capillary chromatography; MECC) can enhance separation by introducing a chromatography element to electrokinetic separation mechanisms [25].

The use of CE to separate Maillard reaction products has received scant attention [26,27]. In the first study, reaction products resulting from reactions of glucose or ribose with glycine, alanine and isoleucine were separated, before and after the formation of phenylthiocarbonyl or 2,4-dinitrophenylhydrazine derivatives [26]. We have recently reported the separation of reaction products (in particular the melanoidins) resulting from glucose–glycine and 5-hydroxymethylfurfural (5-HMF)–glycine model systems by reversed-phase HPLC (RP-HPLC) and CE [27].

The objective of the present study was to investigate CE conditions that effect the separation of reaction products formed by a 5-HMF–glycine model Maillard reaction mixture. The effects of buffer composition, ionic strength and pH on the separation of components of this reaction mixture are reported.

EXPERIMENTAL

Chemicals

5-HMF and glycine were analytical reagent grade obtained from Aldrich (Poole, UK). Buffer tablets were obtained from FSA (Loughborough, UK). Dichloromethane was HPLC grade from Rathburn (Walkerburn, UK). Gold-grade ammonium acetate was obtained from Aldrich (Milwaukee, WI, USA) and analytical reagent-grade potassium phosphate, sodium acetate and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA). Ammonium hydroxide was from Baxter (Minneapolis, MN, USA). High-purity water was prepared in-house using a Sybron Banstead water purifier system (ex-Millipore) applied by VWR (Minneapolis, MN, USA).

Preparation of a 5-HMF–glycine model Maillard reaction mixture

5-HMF (1.5 g; 0.012 mol) was reacted for 8 h, under reflux conditions, with glycine (0.9 g; 0.012 mol) in aqueous solution (40 ml) buffered

to pH 7.0, with commercially available buffer tablets. After reaction, the mixture was cooled and extracted with dichloromethane (3 × 50 ml) to remove most of the reacted 5-HMF. The resultant aqueous fraction was then freeze-dried to yield a dark brown powder. This powder was reconstituted in deionised water, to produce a solution of 1 mg/ml, prior to separation by CE.

CE separation conditions

All CE separations were performed using a Beckman P/ACE model 2100 CE (Fullerton, CA, USA) coupled to an IBM PS2/76 PC with control and data capture by System Gold Software. An uncoated capillary 57 cm × 75 μm, 50 cm before detector) was used throughout this study. Prior to using the capillary and between each analysis, the capillary was rinsed with a 0.1 M solution of sodium hydroxide followed by water and conditioning with running buffer (5 column volumes of each). The following buffers were used during these investigations: 5 mM potassium phosphate (mono basic) + 5 mM sodium phosphate (dibasic) pH 7.0; 20 mM sodium acetate pH 7.0; 20 mM ammonium acetate pH 7.0; 10 mM potassium phosphate (monobasic) + 10 mM sodium phosphate (dibasic) pH 7.0; 40 mM sodium acetate pH 7.0; 40 mM ammonium acetate pH 7.0; 20 and 40 mM ammonium acetate adjusted to pH 4.6 with trifluoroacetic acid; 20 and 40 mM potassium phosphate (monobasic) adjusted to pH 4.6 with trifluoroacetic acid; and 20 and 40 mM ammonium acetate adjusted to pH 9.2 with ammonium hydroxide.

Samples were applied to the anodic end of the capillary using pressure injection (1 s) and separated by application of a voltage of 10 kV across the capillary. The capillary temperature was maintained at 25°C throughout separations. Components were detected spectrophotometrically at a wavelength of 214 nm.

RESULTS AND DISCUSSION

The complexity of model Maillard reaction mixtures has frequently been demonstrated [10]. In addition, the lack of suitable analytical techniques that affect the separation of components of such reaction mixtures has considerably detracted from the identification of, in particular, advanced Maillard reaction products (MRPs), such as the coloured melanoidins. While several semi-volatile reaction products have been recently identified [28], no advance MRPs have been structurally characterised. Development of separation techniques to effect the resolution of these compounds must therefore be conducted via an empirical approach. For CE, buffer composition, its ionic strength, and pH are parameters that are of primary importance in determining suitable separation conditions. However, it is recognised that capillary dimensions, applied voltage, and sample injection technique are also factors to be considered. In the present study, these latter parameters were fixed; hence, whilst it is understood that increasing capillary length can result in better resolution of components, the effects of buffers for separating MRPs produced by reaction of 5-HMF with glycine were of initial interest.
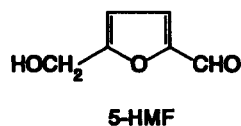
5-HMF

In all samples analysed, unreacted 5-HMF was detected, and under the pH conditions used, was present as the neutral species. Hence, it was utilised to determine electroosmotic flow (EOF). Therefore, since in forward polarity experiments (sample applied at anode and components detected at cathodic end of capillary), cations are detected before neutral compounds whereas anions are detected later, the ionic character of analytes in aqueous solutions of various pH can be determined. In the present study, we have used the migration times of reaction products, relative to 5-HMF, in electropherograms to gain an understanding of the ionic character of MRPs produced by the reaction of 5-HMF with glycine.

Electropherograms obtained from CE analysis of the 5-HMF–glycine model Maillard reaction mixture using three different buffer solutions of the same ionic strength and pH (7.0) are shown in Fig. 1. Comparison of the migration time of 5-HMF (peak 1) in each of these profiles indicates that EOF was fastest in the phosphate buffer (Fig. 1A) and slowest in the ammonium acetate experiment (Fig. 1C). The EOF of the sodium acetate system (Fig. 1B) was closest to
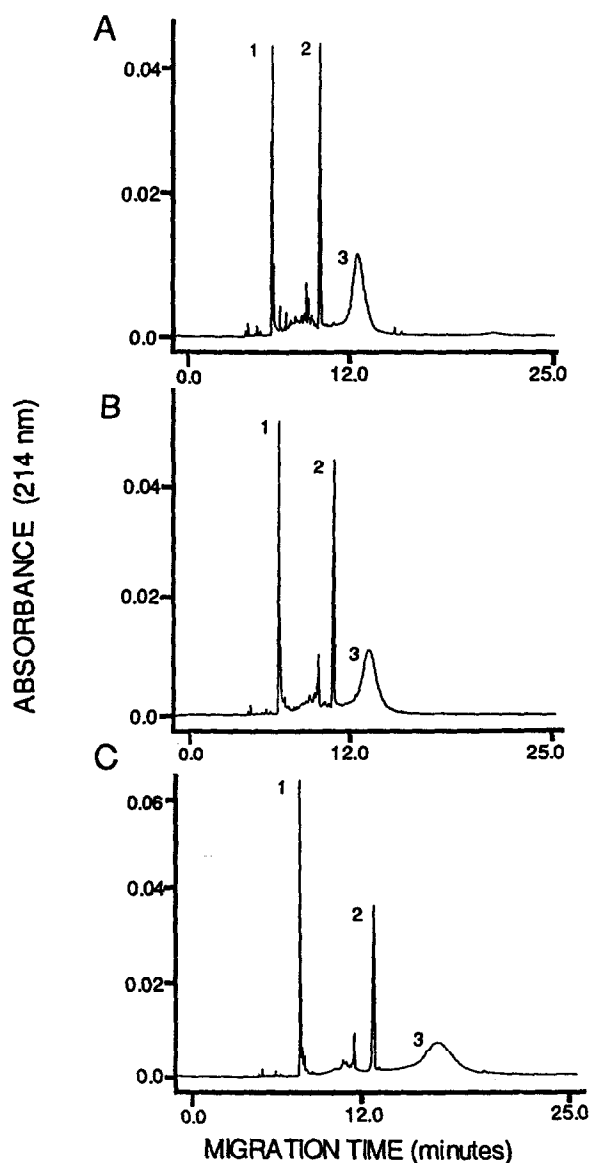
Fig. 1. Electropherograms recorded for a 5-HMF–glycine model Maillard reaction mixture at pH 7.0. (A) 5 mM KH$_2$PO$_4$ + 5 mM Na$_2$HPO$_4$ buffer; (B) 20 mM sodium acetate buffer; (C) 20 mM ammonium acetate buffer. Capillary 57 cm × 75 μm (50 cm prior to detector). Voltage 10 kV. Detection 214 nm. Peaks: 1 = 5-HMF, EOF; 2 = coloured melanoidin product; 3 = complex mixture of MRPs.

ammonium acetate, the total analysis time is 20 min, whilst in both phosphate and sodium acetate buffers, all components migrated within 15 min. In general, since it is expected that the longer components are on the capillary, the better their separation, it would be predicted that the best resolution of MRPs would be achieved using the ammonium acetate buffer. Indeed, considering the broad peak (peak 3) detected in each electropherogram, the separation of components, whilst poor in all examples, were partially resolved in the ammonium acetate buffer. These components were suspected to be polymeric, since their mass-to-charge ratios (the separation mechanism of free solution CE methods) were apparently very similar. However, for other MRPs of the reaction mixture (earlier migrating species), better resolution was achieved using the phosphate buffer, e.g., a second compound that migrates with a time similar to that of a neutral species has been separated from 5-HMF in this buffer solution (peak 1 in Fig. 1A). This effect is likely to be due to a more effective prevention of analyte-analyte interactions in the phosphate buffer in comparison to the acetate buffers. In all three buffers at this pH (7.0), the major MRPs of this reaction mixture, including peak 2 (a response shown in previous study [27] to be the main melanoidin (coloured) component of the 5-HMF–glycine reaction mixture) migrate with longer times than 5-HMF. These compounds therefore possess anionic character at this pH.

Electropherograms obtained from CE separation of the 5-HMF–glycine model reaction mixture using buffers of twice the ionic strength but the same composition and pH (7.0) as those used to generate the profiles of Fig. 1 are shown in Fig. 2. Comparison of the migration time of 5-HMF (peak 1) in each of these buffers again indicates that EOF was fastest in the phosphate buffer (Fig. 2A) and slowest in the ammonium acetate system (Fig. 2C). However, the EOF of all buffer compositions was slower at increased ionic strength. This can be attributed to a change of zeta potential (the charge on the capillary wall) caused by occupation of the ionised silanol groups by cations, due to the increased concentration of these ions in solution. One effect of
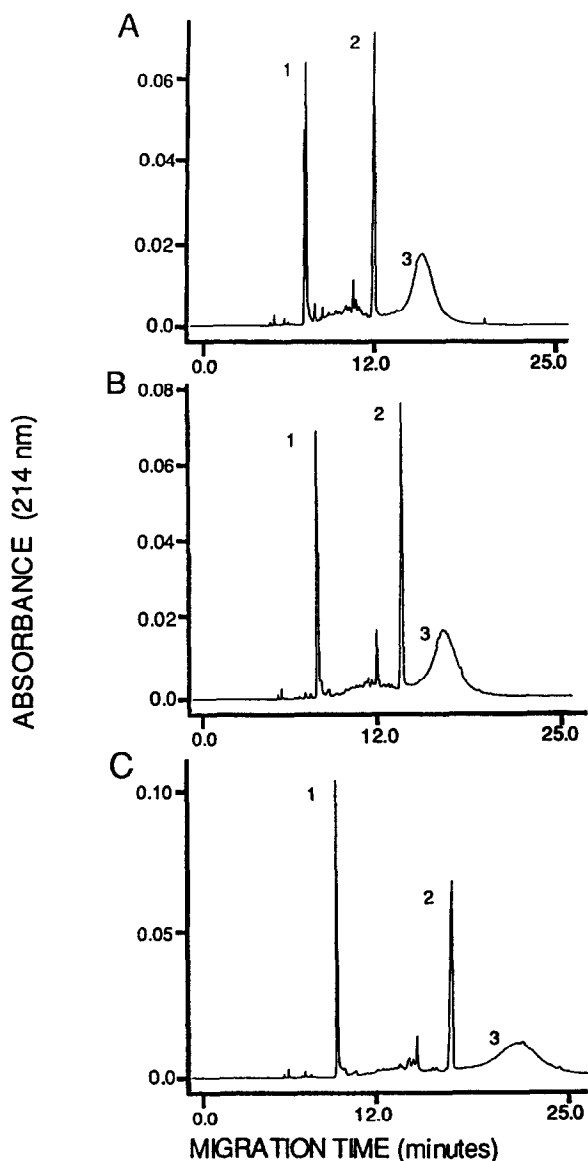
that of the phosphate buffer. The various differences of EOF are mirrored by the length of time MRPs are held on the capillary. Hence, in

Fig. 2. Electropherograms recorded for a 5-HMF–glycine model Maillard reaction mixture at pH 7.0. (A) 10 m*M* KH$_2$PO$_4$ + 10 m*M* Na$_2$HPO$_4$; (B) 40 m*M* sodium acetate; (C) 40 m*M* ammonium acetate. Other parameters and assignments as noted in Fig. 1.

the slower EOF was to hold MRPs on the capillary for a longer time. This increases the resolution achieved in all buffers. Furthermore, since at a high ionic strength there are more buffer ions in solution, analyte-analyte interactions are reduced, the effect of which is also increased resolution of components. However,

as observed with the lower ionic strength buffers, the best separation of the MRPs migrating prior to the major coloured response (peak 2) of the 5-HMF-glycine reaction mixture was achieved in phosphate buffer (Fig. 2A). In these higher ionic strength buffers, since the pH of solution was again 7.0, most MRPs possessed anionic character.

In addition to investigating the effects of increasing ionic strength of buffer solutions, the effect of changing buffer pH was studied. Changes in this parameter cause two major events in CE separations, both of which alter the separation of components. First, since the pK_a of the surface silanol group is *ca.* 2.7, these groups are ionised above a pH of 2.7. Since it is the interaction of the ionised silanol groups with buffer cations that leads to development of EOF, this parameter can be drastically affected by pH changes. Hence, it is observed that at low pH, EOF is slow, whilst at high pH, EOF is much faster. At pH values above 8.5, since all surface silanol groups are ionised, the effect on separation is caused by a change of charge on the analyte species. Hence, changing pH causes both a modification of EOF and a possible change of charge state of analytes.

Results of investigation of the affect of pH on the separation of MRPs of the 5-HMF–glycine reaction mixture are presented in Fig. 3 (ammonium acetate and phosphate buffers at pH 4.6) and Fig. 4 (ammonium acetate buffer at pH 9.2). At low pH, a dramatic change in the appearance of the electropherogram was observed for both buffers (Fig. 3). However, again increasing the ionic strength results, as expected, in increased resolution of components, and in both buffers, the broad response detected (peak 3) is flattened with increased ionic strength (Fig. 3B and D). An unexpected result was at this pH, the MRPs exhibited sizeable anionic character. In fact, this was also found in buffers of pH 1.7 (data not shown). This therefore tends to suggest that the majority of MRPs, and especially the melanoidins, contain a number of functional groups that possess low pK_a values, such that unless extremes of pH are encountered, these molecules exist in solutions as anions.
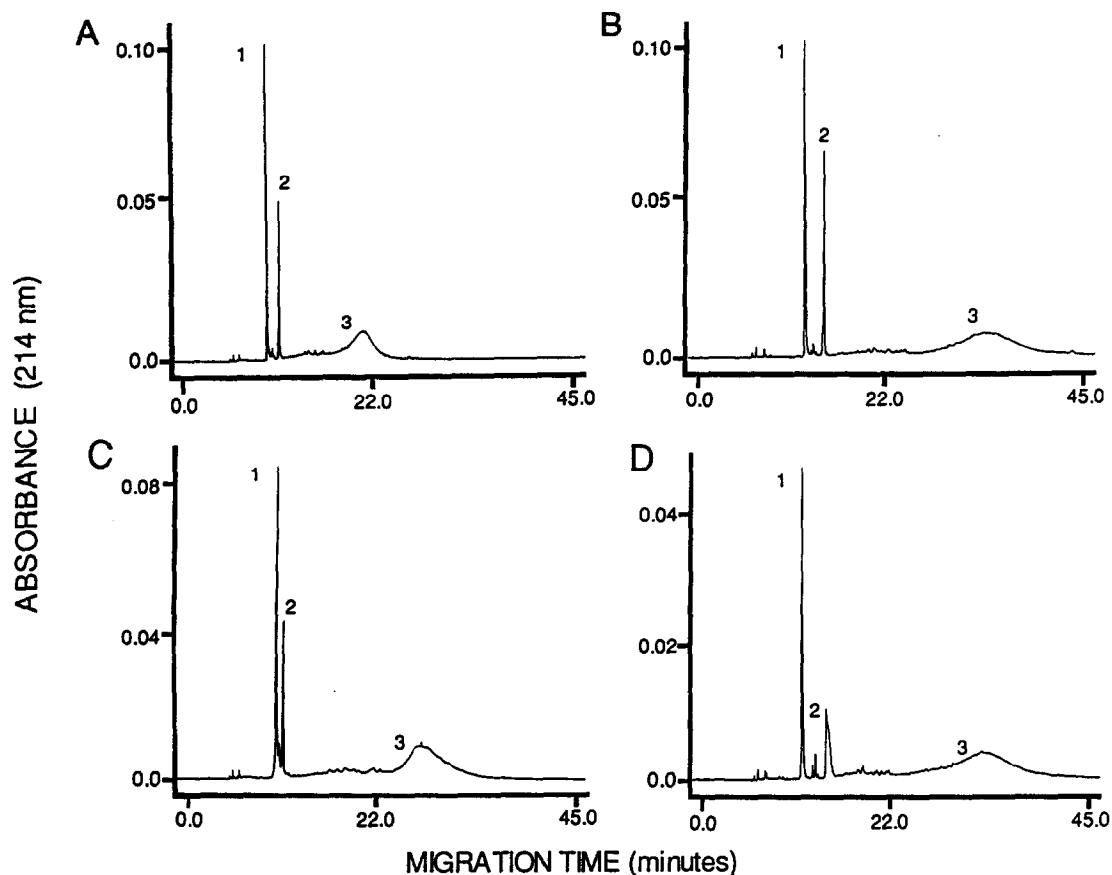
At high pH (Fig. 4A and B), the response due

Fig. 3. Electropherograms recorded for a 5-HMF–glycine model Maillard reaction mixture at pH 4.6. (A) 20 mM ammonium acetate adjusted to appropriate pH with TFA; (B) 40 mM ammonium acetate adjusted to appropriate pH with TFA; (C) 20 mM KH$_2$PO$_4$; (D) 40 mM KH$_2$PO$_4$. Other parameters and assignments as noted in Fig. 1.

to 5-HMF (peak 1) was reduced, and new responses were detected in electropherograms. There responses also possessed anionic character. Thus, it was apparent that several components that were previously neutral in solution had been ionised. These latter components were therefore weakly acidic. Once again, as expected, resolution of components was increased at a higher ionic strength. The overall resolution of components was, however, not as good as was achieved in either pH 4.6 or 7.0 buffers.

CONCLUSIONS

The complexity of model Maillard reaction mixtures was demonstrated by separation of MRPs by CE produced on reaction of 5-HMF

with glycine. Comparison of buffer composition, ionic strength, and pH for effecting the separation of these compounds indicated that at pH 7.0, a phosphate buffer would be of choice, whilst at pH 4.6, an ammonium acetate buffer gave improvements of component resolution.

Throughout these studies, the major MRPs of the 5-HMF–glycine reaction mixture were detected with longer migration times than 5-HMF (a neutral compound at all pH conditions used in this study). These compounds therefore possess at least partial anionic character in solution over a wide pH range. The amphoteric nature previously reported for such compounds was not detected [21,22]. This leads to suggestions that the MRPs formed in the reaction studied possess a number of acidic groups that exhibit low pK_a
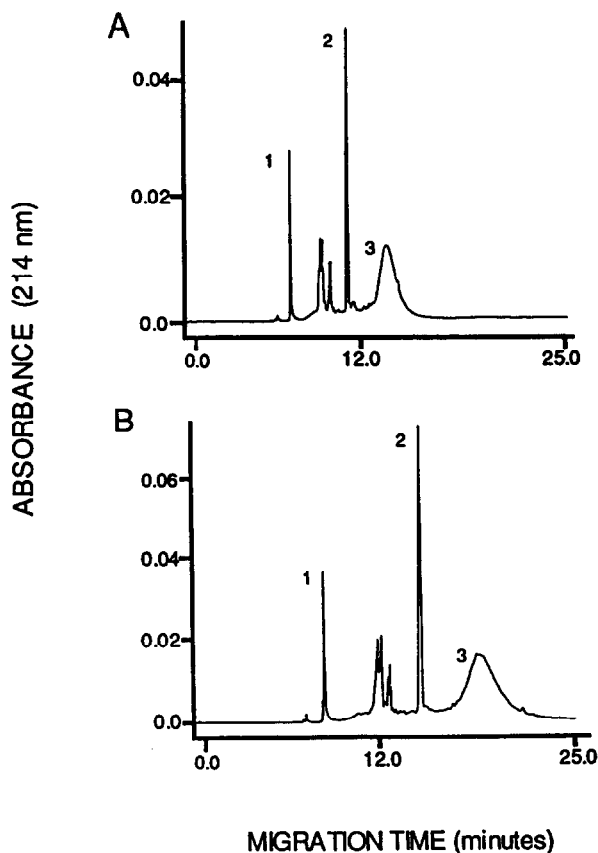
Fig. 4. Electropherograms recorded for a 5-HMF–glycine model Maillard reaction mixture at pH 9.2. (A) 20 mM; (B) 40 mM ammonium acetate (pH of both adjusted with ammonium hydroxide). Other parameters and assignments as noted in Fig. 1.

values. Hence, the formation of cationic species is unfavourable under common pH conditions.

ACKNOWLEDGEMENTS

REFERENCES

1 G. Verin and C. Paranyi, in G. Verin (Editor), *The Chemistry of Heterocyclic Flavouring and Aroma Compounds*, Ellis Horwood, Chichester, 1982, p. 151.

2 J.P. Danehy, *Adv. Food Res.*, 30 (1986) 77.
3 L.L. Buckholz, Jr., *Cereal Foods World*, 33 (1988) 547.
4 J.M. Ames, *Food Manuf.*, (1989) 61.
5 H.E. Nursten, *Food Chem.*, 6 (1981) 263.
6 H.E. Nursten, in D. MacCarthy (Editor), *Concentration and Drying of Foods*, Elsevier, Oxford, 1986, p. 53.
7 J.M. Ames, *Food*, (1987) 67.
8 J.M. Ames, *Chem. Ind., London*, (1987) 558.
9 F. Ledl, in P.A. Finot, H.U. Aeschbacher, R.F. Hurrell and R. Liardon (Editors), *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*, Birkhauser, Basel, 1990, p. 19.
10 M. Namiki, *Adv. Food Res.*, 32 (1988) 115.
11 H. Lingnert and G. Hall, in M. Fujimaki, M. Namiki and H. Kato (Editors), *Amino-Carbonyl Reactions in Food and Biological Systems —Proc. 3rd Int. Symp. on the Maillard Reaction, Susono, Shizuoka, July 1–5, 1985 (Developments in Food Science*, Vol. 13), Elsevier, Amsterdam, 1986, p. 273.
12 K.M.S. Henry, S.K. Kon, C.H. Lea and J.C.D. White, *J. Dairy Sci.*, 15 (1948) 292.
13 A. Ferritti and V.P. Flanagan, *J. Dairy Sci.*, 54 (1971) 1764.
14 J. Mauron, *Prog. Food Nutr. Sci.*, 5 (1981) 5.
15 J. O'Brien and P.A. Morrissey, *Crit. Rev. Food Sci. Nutr.*, 28(3) (1989) 211.
16 M. Brownlee, A. Ceramic and H. Vlassara, *N. Engl. J. Med.*, 318 (1988) 1315.
17 R. Bucala, P. Model and A. Ceramic, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 105.
18 V.M. Monnier, D.R. Sell, M. Satoshi and H.N. Ramanakoppa, in P.A. Finot, H.U. Aeschbacher, R.F. Hurrell and R. Liardon (Editors), *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*, Birkhauser, Basel, 1990, p. 393.
19 J.J. Harding, H.T. Beswick, R. Ajiboye, R. Huby, R. Blakytny and K.C. Rixon, *Mech. Age Dev.*, 50 (1989) 7.
20 A.J. Tomlinson, *Ph.D. Thesis*, University of London, London, 1991.
21 H. Hashiba, *Agric. Biol. Chem.*, 42 (1978) 763.
22 R. O'Reilly, *Ph.D. Thesis*, University of Reading, Reading, 1982.
23 H. Hagland, in J.P. Arbuthnott and J.A. Becley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, p. 3.
24 J.P. Landers, R.P. Oda, T.C. Spelsberg, J.A. Nolan and K.J. Ulfelder, *Biotechniques*, 14(1) (1993) 98.
25 W.A. Kuhr, *Anal. Chem.*, 62 (1990) 403R.
26 Z. Deyl, I. Miksik and R. Struzinski, *J. Chromatogr.*, 516 (1990) 287.
27 A.J. Tomlinson, J.A. Mlotkiewicz and I.A.S. Lewis, *Food Chem.*, in press.
28 A.J. Tomlinson, J.A. Mlotkiewicz and I.A.S. Lewis, *Food Chem.*, in press.