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Application of capillary electrophoresis to the separation of coloured products of Maillard reactions

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Recent developments in capillary electrophoresis (CE) have demonstrated its usefulness for the separation of trace amounts of materials that are otherwise difficult to analyse. A class of compounds that fall into this category are the melanoidins, the coloured products of Maillard Chemistry. CE techniques were applied to the separation of components produced by the reaction of glucose or 5-hydroxymethyl furfural (5-HMF) with glycine. During these studies, the relative merits of CE were assessed to effect the difficult separation of the melanoidins that were produced by these reaction mixtures. Furthermore a comparative study was undertaken between CE and reversed phase HPLC (rpHPLC). The advantages of CE as a powerful tool for the separation of Maillard Reaction Products (MRPs) were clearly demonstrated.

INTRODUCTION

Non-enzymic browning reactions of sugars with amino acids, peptides and proteins (Maillard Chemistry) have a known importance in food chemistry (Namiki, 1988). Furthermore, their influence on the metabolic fate of proteins and nucleic acids, by the promotion of cross-linking of these biopolymers, has been demonstrated (Bucala *et al.*, 1984; Brownlee *et al.*, 1988), and the role of this chemistry in ageing has been postulated (Harding *et al.*, 1989; Monnier *et al.*, 1990).

Despite the importance and extensive study of Maillard Chemistry many reaction products remain uncharacterised. In particular, the structures of advanced Maillard reaction products (MRPs), such as the coloured melanoidins, are unknown. This can be attributed to the complexity of model reaction mixtures and a lack of analytical techniques that effects the separation of these extremely hydrophilic compounds. Indeed, to date, although many techniques, including rpHPLC (O'Reilly, 1982; Miksik *et al.*, 1990; Tomlinson, 1991), ion exchange chromatography (Ingles & Gallimore, 1985) and electrophoretic methods (Hashiba, 1978; O'Reilly, 1982) have been applied in attempted separations of the melanoidins, there remains no demonstrated means of isolation of these advanced MRPs.

Capillary electrophoresis (CE) is a technique that is currently gaining widespread acclaim for the separation of complex mixtures that have otherwise proved difficult to analyse (Landers *et al.*, 1993). In this form of free solution electrophoresis separations are performed in buffer filled, thin-walled, narrow bore (typically 50μm ID) fused silica capillaries. The application of a high voltage (up to 30kV) across the capillary affords rapid, highly efficient separations, that are a function of the mass and charge of the resolved species (Kuhr, 1990; Kuhr & Monnig, 1992). Capillary efficiencies in excess of 1000000 theoretical plates per metre are not uncommon (Kuhr, 1990). Furthermore additives and buffer modifiers can be incorporated into run buffers to modify separation mechanisms, e.g. detergents may be added to a run buffer (as in Micellar Electrokinetic Capillary Chromatography; MECC) to enhance the separation of neutral compounds, by introducing a chromatography element to the electrokinetic separation mechanisms (Kuhr, 1990; Kuhr & Monnig, 1992).

Recently the potential of capillary electrophoresis for effecting the separation of MRPs was demonstrated (Deyl *et al.*, 1990). In this study MRPs resulting from the reaction of glucose or ribose with glycine, alanine or isoleucine were partially separated before and after the formation of phenylisothiocarbonyl or 2, 4-dinitro-

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phenyl hydrazine derivatives. A comparison of the merits of CE and rpHPLC for the separation of MRPs was also made.

The objectives of the present study were to investigate the potential of CE to specifically effect the separation of melanoidins produced in both spray-dried glucose–glycine and 5-hydroxymethylfurfural (5-HMF)–glycine model Maillard reaction mixtures. We also compare our CE results with those we have obtained from rpHPLC studies of these reaction mixtures.

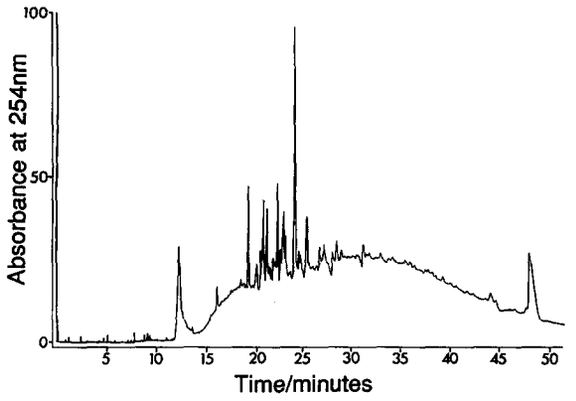

Fig. 1. Electrophoretogram (254 nm) arising from the CE separation of an aqueous solution of a spray-dried glucose–glycine reaction mixture.

EXPERIMENTAL

Chemicals

The chemicals used to prepare the model Maillard reaction mixtures and CE buffers were analytical reagent grade, obtained from Aldrich Chemical Company (Poole, UK). Buffer tablets and ammonium acetate (AR grade) were obtained from FSA (Loughborough, UK). The solvents methanol and dichloromethane were HPLC grade from Rathburns Chemicals (Walkerburn, UK).

Preparation of a spray-dried glucose–glycine model Maillard reaction mixture

Equimolar quantities of glucose and glycine were dissolved in deionised water to give a final concentration of 1M of each reactant. This mixture was spray dried, according to the method of Baines *et al.* (1989), using a Niro Mobile Minor Spray drier (Cornwell Products Ltd, UK). The drier inlet and outlet temperatures were 235 and 170°C, respectively. The flow rate of the aqueous feed stock was approximately 20 ml/min. The resultant brown powder was redissolved in deionised water to produce a solution concentration of approximately 1 mg/ml, prior to separation by CE or rpHPLC.

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

5-HMF (1.5 g; 0.012 moles) was reacted for a period of 8 h, under reflux conditions, with glycine (0.9 g; 0.012 moles) 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 unreacted 5-HMF. The resultant aqueous fraction was 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 or rpHPLC.

CE separation conditions

All CE separations were performed using an Applied Biosystems model 270A CE. An uncoated capillary (122 cm × 50 μm) was used throughout this study. To facilitate UV detection, a window was made by removal of the polymide coating of the capillary; the effective capillary length was 72 cm. Prior to using the capillary and between each analysis, the capillary was conditioned by washing with a solution of sodium hydroxide (1M concentration) followed by conditioning with running buffer (5 capillary volumes of each). The running buffer was prepared by dissolving 30 mM potassium dihydrogen phosphate plus 20 mM disodium hydrogen phosphate in deionised water (100 ml) at pH 6.5.

Samples were applied to the capillary using the instruments vacuum loading technique (3–10 s). A running voltage of 30 kV was applied to the capillary to facilitate the electrokinetic separation of components. This voltage gave rise to a current of 47 μA. The capillary compartment temperature was maintained at 30°C throughout the separation. Components were detected spectrophotometrically at wavelengths of 254 or 430 nm.

Reverse phase HPLC separations

All reverse phase HPLC separations were performed using a Hewlett Packard 1090L liquid chromatograph connected to a Hewlett Packard 3393A integrator. An

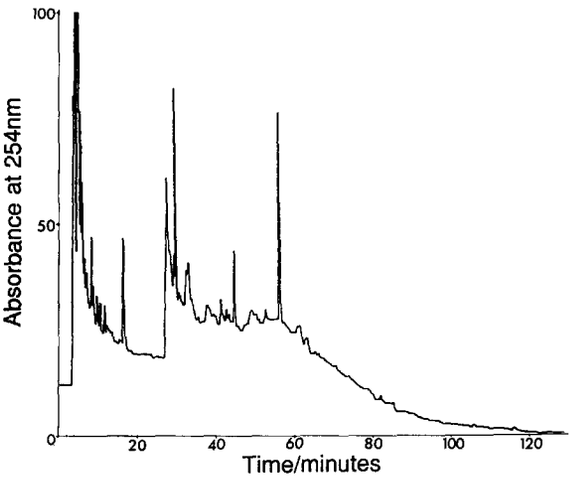

Fig. 2. Reverse phase HPLC chromatogram (254 nm) arising from the analysis of an aqueous solution of a spray-dried glucose–glycine reaction mixture.

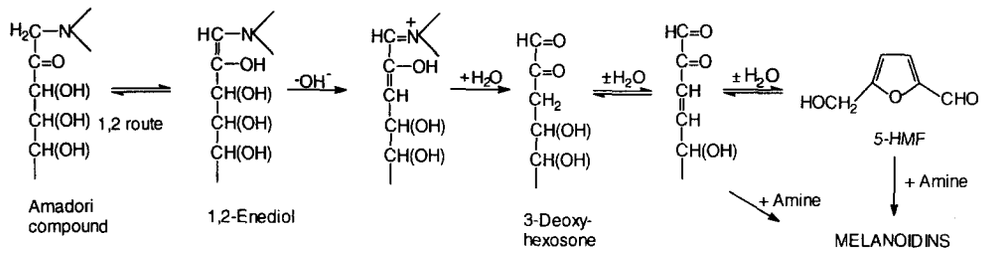

Fig. 3. Reaction scheme of the formation of 5-HMF from a sugar–amine reaction mixture.

ODS-2 column (25 cm × 4.6 mm ID, 5 μm particle size, ex. Chromex, Manchester, UK) was used throughout this study. A mobile phase linear gradient of 100% 0.1 M aqueous ammonium acetate (held for 10 min after injection) to 100% methanol was developed over 100 min after which the column was further eluted with methanol for 10 min. The mobile phase flow rate was 1.0 ml/min. The column eluent was monitored spectrophotometrically at 254 or 430 nm using the HP1090L filter photometric detector.

RESULTS AND DISCUSSION

CE and rpHPLC methods were developed to enable investigation of reaction mixtures prepared under Maillard conditions (results not shown), including the novel spray-dried reaction of glucose and glycine (Baines *et al.*, 1989).

A CE electropherogram recorded, at a wavelength of 254nm, for a spray-dried glucose–glycine reaction mixture is presented in Fig. 1. A comparative profile obtained by analysing this mixture by rpHPLC is shown in Fig. 2. Both profiles (as those recorded at 430nm; not shown) clearly demonstrated the complexity of this product reaction mixture and the difficulty encountered when attempting separation of MRPs. In particular, the coloured compounds (detected at retention times of between 30 and 60 min, Fig. 2) were not resolved by rpHPLC and although some fine detail is observed, in general, the melanoidins were eluted from the column as a broad diffuse hump. In contrast, electrophoretograms recorded for this reaction mixture show that the high efficiency of CE separations enable a better resolution of the MRPs of this model Maillard system. Hence whilst a broad hump of migrating material is observed during CE separations of this product reaction mixture, the resolution of the melanoidins (compounds of migration times, between 13 and 50 min, Fig. 1), is increased dramatically.

It is noteworthy that the increased resolution of the melanoidins of this reaction mixture, afforded by CE, would diminish, if there was inadequate conditioning of the capillary between sample analysis. This would have possibly been due to coating of, in particular the front of the capillary, by components of the analysed reaction mixture. Reconditioning the capillary with 1M sodium hydroxide and running buffer prior to further analyses was found to overcome this problem and became an essential component of our CE method for the analysis of model Maillard reaction mixtures.

An interesting feature of CE is the co-analysis of reaction mixtures with a neutral marker, such as mesityl oxide. This technique enables determination of the electro-osmotic flow (a bulk flow of solution caused by the interaction of ionised surface silanol groups of the capillary and the migration of hydrated cations towards the cathode upon application of a high voltage) of the CE method and permits a prediction of the ionic character of the detected species (Wainright, 1990). For example, in forward polarity CE experiments (sample application at the anodic end of the capillary), species that are detected with shorter migration times than the neutral marker are positively charged, whilst those compounds that are detected with longer migration times than the neutral marker possess anionic character. Components that migrate with the same velocity as the neutral marker are themselves neutral. Such investigations during the study of the components of the spray-dried glucose–glycine reaction mixture, revealed that under the developed CE conditions, most MRPs and especially the melanoidins were detected with longer migration times than the neutral marker. Hence, under these conditions, it is apparent that MRPs and particularly the melanoidins possess anionic character.

During this study, it became apparent that the spray-dried glucose–glycine reaction mixture was extremely complex. We therefore investigated ways of producing less complicated model Maillard reaction mixtures. In this regard, we reacted a number of known intermedi-

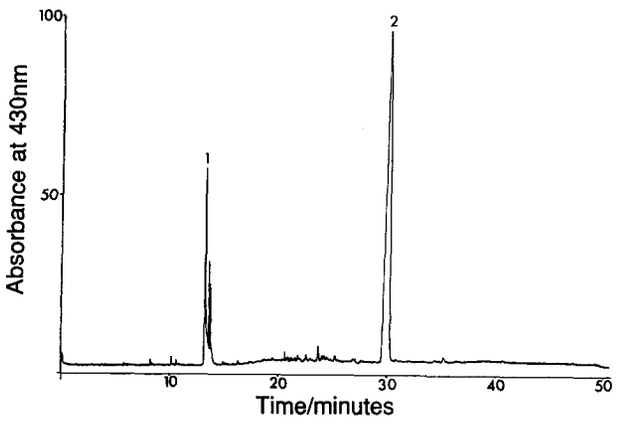

Fig. 4. Electrophoretogram (430 nm) arising from the CE separation of an aqueous solution of a 5-HMF–glycine reaction mixture.

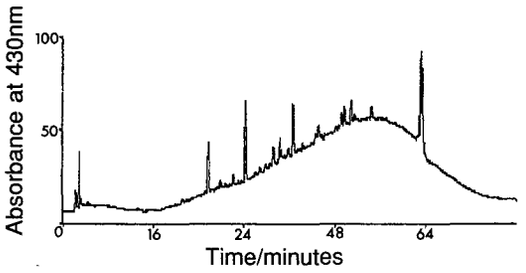

Fig. 5. Reverse phase HPLC chromatogram (430 nm) arising from the analysis of a 5-HMF–glycine reaction mixture.

ates of Maillard chemistry with glycine to determine those compounds that are intermediate to melanoidin formation. One compound that was found to be particularly prone to browning with glycine was 5-HMF. This compound is reported to be formed in Maillard Chemistry via a 1,2-enolisation process of the Amadori intermediate (see for example, Namiki, 1988; also Fig. 3). Furthermore we had detected (by GC-MS techniques, Tomlinson, 1991; Tomlinson *et al.*, 1993) this compound in our spray-dried glucose–glycine model Maillard reaction mixture.

A CE electrophoretogram recorded at a wavelength of 430nm (to specifically show the melaniodins) for a 5-HMF–glycine model reaction mixture is given in Fig. 4; a comparative rpHPLC profile (also recorded at a wavelength of 430nm) is shown in Fig. 5. Inspection of these data shows that in comparison with the glucose–glycine model system, the 5-HMF–glycine reaction mixture was much simpler. The rpHPLC profile of this reaction mixture shows that little separation of components has occurred and a broad hump of eluting material is observed. In contrast, the CE electrophoretogram indicates a much better resolution of components of this reaction mixture. In addition, only two major bands of material (peaks 1 and 2) were detected by CE. The first of these (peak 1; if only in part) was determined as unreacted 5-HMF. The composition of the second band of detected material (peak 2), is at present unknown. This response does, however, represent the major coloured component(s) of our 5-HMF–glycine model reaction mixture and as such the compounds migrating in this region require further investigation, particularly by CE-MS techniques, to enable their characterisation.

The detection of 5-HMF (a neutral compound under these conditions) again indicates that under these CE conditions MRPs, coloured or otherwise, were predominantly of anionic character.

CONCLUSIONS

Through our studies of two model Maillard reaction mixtures, we have demonstrated the power of CE for the separation of MRPs and, particularly, the melanoidins. In addition, we have shown that Maillard reaction mixtures can be simplified by reacting potential intermediates of the melanoidins with glycine. In our experiments, CE separation of a 5-HMF–glycine reaction mixture indicated one major band of coloured material. Whilst the nature of this response is currently unknown, it is predicted that under our conditions of analysis, this material has anionic character. In addition, our investigations tentatively suggest that the majority of MRPs detected during these investigations possessed anionic character when analysed under the CE conditions studied.

In conclusion, this study shows that future investigations of, in particular, the melanoidins should involve CE for the separation of these advanced MRPs.

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