Analysis of the Methanol-Extractable Nonvolatile Maillard Reaction Products of a Model Extrusion-Cooked Cereal Product

Jennifer M. Ames,*,† Anna Arnoldi,‡ Lisa Bates,†,§ and Monica Negroni‡

Department of Food Science and Technology, The University of Reading, Whiteknights, Reading RG6 6AP, U.K., and Dipartimento di Scienze Molecolari Agroalimentari, Sezione di Chimica, Università di Milano, via Celoria 2, I-20133 Milan, Italy

Methanol extracts were prepared from starch−glucose−lysine model food systems prepared by extrusion cooking (pH before cooking 3.4, 5.0, 7.7). Components of the methanol extracts were separated by isoelectric focusing (IEF), capillary zone electrophoresis (CZE), ion-exchange HPLC (IE-HPLC), and reverse-phase HPLC (RP-HPLC). IEF was useful for giving an initial separation of the complex mixtures and for indicating which other separation techniques might be appropriate. CZE gave separations superior to those achieved by both types of HPLC. Both RP-HPLC (with diode array detection) and CZE (with scanning wavelength detection) established differences between the profiles of components of the extracts prepared from extrudates of different initial pH. Semipreparative RP-HPLC was used to isolate selected reaction products of each extrudate. 5-(Hydroxymethyl)furfural (HMF) was identified in each sample at levels that decreased with increasing feedstock pH. Using mainly NMR, 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone was conclusively identified as a component of the methyl extract prepared from the pH 7.7 extrudate. Preliminary spectral data were obtained for three additional Maillard reaction products.

Keywords: *Maillard reaction; colored compounds; extrusion cooking; glucose; lysine; pH; HPLC; CZE; IEF; separation; diode array HPLC; NMR; heteronuclear two-dimensional NMR*

INTRODUCTION

The Maillard reaction is of prime importance in food chemistry, and yet the majority of the colored products formed remain uncharacterized. The colored compounds formed from the Maillard reaction may be classified into two groups, the low molecular weight structures and the large polymeric melanoidins (Ames and Nursten, 1989). Previous Maillard color chemistry studies have concentrated on the low molecular weight structures in aqueous systems (Ledl and Schleicher, 1990; Ames et al., 1993) while only a few have considered intermediate or low moisture systems (Tomlinson et al., 1993a, 1994).

Techniques that have been used to separate low molecular weight Maillard color compounds include reverse-phase HPLC (RP-HPLC) (e.g., Nursten and O'Reilly, 1986; Tomlinson et al., 1993a, 1994; Bailey et al., 1996), ion-exchange chromatography (IE-HPLC) (Hashiba, 1978; Ingles and Gallimore, 1985), isoelectric focusing (IEF) (O'Reilly, 1983), and capillary zone electrophoresis (CZE) (Deyl, 1990; Tomlinson et al., 1993b, 1994). HPLC of melanoidins is considered to be unsatisfactory, since the pigments are chemically ill-defined and consist of a complex mixture of compounds (Lea, 1988). A previous study comparing CZE to RP-HPLC showed CZE to give improved separation of the melanoidins from a 5-(hydroxymethyl)furfural (HMF)−glycine aqueous reaction mixture (Tomlinson et al., 1994).

This study had two aims. The first was to compare RP-HPLC, IE-HPLC, IEF, and CZE for the separation of colored compounds extracted from an extrusion-cooked cereal system, prepared at different pH values. The second aim was to obtain spectral data for selected components isolated from the extrudates.

EXPERIMENTAL PROCEDURES

Materials. Wheat starch type A was obtained from ABR Foods, Corby, U.K. D-(+)-Glucose, L-lysine monohydrochloride, citric acid, and sodium bicarbonate were obtained from BDH, Poole, U.K. The lysine was of biochemical grade, and the other chemicals were of analytical grade. Methanol for the extraction of nonvolatile compounds was AnalaR grade (Hayman Ltd., Witham, U.K.).

For IEF, Ultrodex granulated gel and ampholytes (pH range 1−10) were used (Pharmacia, Uppsala, Sweden). Phosphoric acid (1 M) and 1 M sodium hydroxide solution (AnalaR grade, BDH, Poole, U.K.) were used to soak electrode strips.

The buffers used for CZE were 30 mM borate buffer and 20 mM citrate buffer. Borate buffer at pH 9 was prepared from disodium tetraborate (AnalaR grade, BDH) and water (HPLC grade, Fisons, Loughborough, U.K.). Citrate buffer at pH 2.5 was prepared from 0.1 M citric acid (Sigma, Poole, U.K.) solution and 0.2 M disodium hydrogen orthophosphate (GPR grade, BDH) solution, in the ratio 7:1 diluted with HPLC grade water to give a concentration of 20 mM with respect to citrate.

The methanol and acetonitrile used for HPLC were of HPLC grade (Rathburn, Walkerburn, U.K., or Baker, Deventer, The Netherlands). Water for HPLC was purified using either a reverse osmosis plus polishing system (Purite Ltd., Thame, U.K.) or a Milli-Q water purification system (Millipore, Bedford, MA). All HPLC solvents were filtered through nylon 0.45 μm filters and degassed before use. For IE-HPLC, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride (all AnalaR grade), and concentrated phosphoric acid (to adjust the buffers to pH 2.8) were obtained from BDH.

The silylating reagents *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BTSFA), hexamethyldisilazane, and trimethylchlorosilane were obtained from Fluka (Buchs, Switzerland).

* To whom correspondence should be addressed (tel, +44 1734 318730; fax, +44 1734 310080; e-mail, j.m.ames@afnovell.reading.ac.uk).

† The University of Reading.

‡ Università di Milano.

§ Current address: United Biscuits (UK) Ltd, Group Research and Development, Lane End Road, Sands, High Wycombe, Bucks HP12 4JX, U.K.

S0021-8561(96)00631-0 CCC: $14.00 © 1997 American Chemical Society

Deuterated methanol (methyl-d_3 alcohol-*d*) from Aldrich (Gillingham, U.K.) and dimethyl-d_6 sulfoxide (DMSO, Isotech, Miamisburg, OH) were used for nuclear magnetic resonance spectroscopy (NMR).

Methods. *(a) Preparation of Extruded Model System.* Wheat starch, glucose, and lysine were mixed (0.96:0.03:0.01) to give a homogeneous extruder feed. Either the pH of the mix was unadjusted (pH 5.0) or it was adjusted by addition of citric acid (pH 3.4) or sodium hydrogen carbonate (pH 7.7). The feed was cooked in a twin screw corotating extruder, model MPF50 (APV Baker Ltd, Peterborough, U.K.) to give an expanded product. Starch (initial pH 5.0) was also extruded. The moisture content of all the feeds was 18% (m/m), except for the pH 3.4 feed, which had a moisture content of 15% (m/m). The temperatures in the final barrel zone and at the die were 150 ± 3 and 150 ± 7 °C, respectively. The residence time was 32 s. Full experimental details have been reported previously (Bates, 1996; Bates et al., 1994).

(b) Preparation of Colored Extracts. Expanded samples (90 g) were ground and refluxed with methanol (1100 mL) for 3 h using a Soxhlet extraction apparatus at a temperature of 60 °C. The methanol was removed by rotary evaporation, with the water bath temperature below 40 °C, to give a concentrated colored extract. Samples were stored in borosilicate glass vials under nitrogen at −20 °C prior to analysis. Samples were redissolved to 1 mL using buffer (for CZE) or water (for HPLC).

(c) IEF. Three colored extracts of an extrudate prepared at pH 7.7 were combined and concentrated. The sample was added to a suspension of Ultradex gel and ampholytes (pH range 1−10) and poured into a flat bed plate. Isoelectric focusing was conducted using a LKB 2117 multiphore cooling plate with a LKB 2197 constant power supply and a LKB 2209-001 MultiTemp thermostatic controller (LKB Produkter, AB, Bromma, Sweden). The voltage was set at 1500 V (voltage attained was 970 V) for 18 h, and the temperature was maintained at 10 °C. An electrode strip soaked in 1 M phosphoric acid was placed at the anodic end, and another strip soaked in 1 M sodium hydroxide was placed at the cathodic end.

The positions of separated color bands were assessed by viewing under daylight and a UV lamp. The pHs at 30 points on the gel surface were determined with a surface pH electrode (Whatman, Maidstone, U.K.), in order to record the p*I* of each separated band.

(d) CZE. A SpectraPhoresis 1000 (SpectraPhysics, Hemel Hempstead, U.K.) capillary electrophoresis instrument was used, fitted with an uncoated open bore 64 cm × 50 μm capillary column (56 cm to the detector). A voltage of 20 kV was applied to the capillary to enable the electrokinetic separation of components; this gave rise to a current of 37 μA. The capillary temperature was maintained at 30 °C. Injections were made in the hydrodynamic mode, loading for 2 s. Samples were filtered through a 2 μm syringe filter before analysis. Components were detected by a scanning wavelength detector in the UV region (200−300 nm) or in the visible region (370−500 nm). The running buffer used was either 30 mM sodium borate at pH 9 or 20 mM sodium citrate at pH 2.5.

(e) HPLC. All HPLC separations were performed using a Hewlett-Packard (Bracknell, U.K.) 1050 pump and diode array detector. Samples were filtered through a 2 μm syringe filter before injection. Data analysis was carried out using Hewlett-Packard ChemStation software. Chromatograms were obtained at 280 and 420 nm, and raw data were collected from 190 to 600 nm.

IE-HPLC: Samples were separated on a Spherisorb S5SCX cation exchange column (250 mm × 4.6 mm, 5 μm particle size, PhaseSep, Deeside, U.K.) fitted with a guard column packed with the same stationary phase. The optimized gradient for separation of the colored extracts and the flow rates are given in Table 1. The injection volume was 20 μL.

RP-HPLC: Some analytical separations were performed on an Ultracarb ODS30 high carbon loaded reverse-phase column (150 mm × 4.6 mm, 5 μm particle size, Phenomenex, Macclesfield, U.K.) fitted with a guard column packed with the same stationary phase. The gradient and the flow rates used are given in Table 2. Other analytical separations were carried out using a LiChrosorb RP-18 column (250 × 4 mm, 5 μm particle size, Merck, Darmstadt, Germany) fitted with a 4 × 4 mm precolumn packed with the same stationary phase. The gradient went from 5% methanol in water to 50% methanol in water at a rate of 2.25 mL/min and then to 100% methanol at a rate of 3.33 mL/min. The flow rate was 1 mL/min. The injection volume for all analytical separations was 20 μL. In order to separate fractions for further characterization, semipreparative columns were used. When an ODS30 column (150 mm × 10 mm) was used, the separation conditions were those given in Table 2 and the injection volume was 50 μL. Other semipreparative separations were carried out using a Supelcosil SPLC-18 column (250 × 10 mm, 5 μm particle size, Supelco, Bellefonte, PA) and the same gradient as for the Lichrosorb RP-18 analytical column but with a flow rate of 3 mL/min and an injection volume of 200 μL. Organic solvent was removed from collected fractions using a slow stream of nitrogen. Samples were then freeze-dried for 24 h before being dissolved in 1 mL of methanol and stored in borosilicate glass vials under nitrogen, prior to further analysis.

Table 1. Gradient for IE-HPLC Separation of Colored Extracts

time (min)	% A[a]	% B[a]	% C[a]	flow rate (mL/min)
0	100	0	0	0.5
35	100	0	0	0.5
35.5	100	0	0	1.0
47	0	100	0	1.0
60	0	100	0	1.0
100	0	0	100	1.0
120	0	0	100	1.0

[a] Solvent A = 20 mM sodium dihydrogen phosphate in water (pH 2.8). Solvent B = 20 mM disodium hydrogen phosphate in water (pH 7.7). Solvent C = 40% solvent B/60% methanol.

(f) Mass Spectrometry (MS). High-resolution analyses were performed using one of two instruments. The first was a VG70SE magnetic sector instrument (VG Organic, Manchester, U.K.), operated in the electron impact (EI) mode. Significant operating conditions were as follows: electron energy, 70 eV; accelerating voltage, 8 kV; source temperature, 200 °C; probe temperature, ambient initially, gradually increased to the source temperature. The second instrument was a Finnigan-MAT TSQ70 machine equipped with an ICIS data system. It was operated in either the EI or the chemical ionization (CI) mode. Significant operating conditions were as follows: electron energy, 70 eV; source temperature, 170 °C; probe temperature, ambient initally, gradually increased to the source temperature. CI spectra were obtained using isobutane as the reagent gas (pressure 5.5 × 10^{-5} Torr. Samples were introduced by means of a direct introduction (DIS) probe.

(g) Preparation of Silylated Derivatives and Analysis by Gas Chromatography−MS (GC−MS). Small amounts of purified compounds were silylated with BTSFA and pyridine, or hexamethyldisilazane, or trimethylchlorosilane and pyridine. Analyses were performed using the TSQ70 mass spectrometer fitted with a 30 m × 0.25 mm fused silica column coated with a 0.25 μm film of DB5 stationary phase (J&W Scientific, Folsom, CA). The column was temperature programmed from 100 to 200 °C at a ramp rate of 8 °C.

(h) Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AMX-600 spectrometer at 600 and 150.9 MHz, respectively. Methanol-d_4 and DMSO-d_6 were the solvents, and tetramethylsilane was the internal standard. Chemical shifts were expressed in parts per million (δ). Heteronuclear two-dimensional ^{1}H−^{13}C one-bond correlations (heteronuclear multiple quantum correlation, HMQC) (Bax and Morris, 1981) and multiple bond correlations (heteronuclear multiple bond correlation, HMBC) (Bax and Summers, 1986) were carried out in the ^{1}H-detected mode with broad-band decoupling in the ^{13}C domain.

Table 2. Gradients for RP-HPLC Separation of Colored Extracts (Used with the ODS30 Analytical and Semipreparative Columns)

analytical column				semipreparative column			
time (min)	% HPLC water	% acetonitrile	flow rate (mL/min)	time (min)	% HPLC water	% acetonitrile	flow rate (mL/min)
0	92	8	0.5	0	98	2	2.4
5	92	8	0.5	15	98	2	2.4
10	83	17	0.5	22	80	20	2.4
15	76	24	0.8	35	75	25	3.5
25	64	36	0.8	40	64	36	3.5
30	0	100	0.8	45	0	100	3.5
40	0	100	0.8	60	0	100	3.5

Table 3. Bands Separated by IEF of the Extract of the pH 7.7 Extrudate

p*I* range	color
1.7−2.1	pale yellow
2.7−3.1	brown/gray
3.5−4.3	pale yellow
5.1−6.5	dark orange/brown
5.6−6.3	fluorescent white
7.2−8.3	orange/brown
8.8−9.0	yellow
10.1	dark orange/brown

RESULTS AND DISCUSSION

Color measurement data for the ground extrudates, obtained using a color spectrophotometer, showed that, as the pH of the feed mix increased, the L^* values of the extrudates decreased significantly (indicating increased darkness), the a^* value increased significantly (indicating increased redness), and the hue angle decreased significantly (indicating a shift in color from yellow-brown to orange-brown) (Bates et al., 1994). The variation in color of the extrudates with pH was not reflected in the colors of the methanol extracts, which were about the same, regardless of feed pH. Overall, it appeared that only a small proportion of the total color was extracted by the methanol, but no more color could be removed by prolonging the extraction time. Analysis of methanol extracts of extrudates produced at the same pH, but at 15 or 18% moisture, showed that the effect of moisture was very small, especially when compared to the effect of pH. Therefore, it was reasonable to ignore the effect of moisture (over the range 15−18%) when comparing the separation patterns of methanol extracts of the extrudates of different feed pH (Bates, 1996).

IEF. The extract from the pH 7.7 extrudate was analyzed. Seven colored bands and one band that fluoresced white under UV radiation were separated (see Table 3). The bands separated on the basis of both charge and isoelectric point and had p*I* values ranging from 1.7 to 10.1. The bands with the most intense color all had p*I* values above 5 (at 5.1−6.5, 7.2−8.3, and 10.1), suggesting that a large proportion of the colored material was cationic in nature. This contrasts with work on the colored material prepared from glycine model systems. O'Reilly (1983) reported the presence of anionic compounds only in her xylose−glycine system, while Tomlinson (1993b) showed that the major reaction products of a HMF−glycine mixture possessed at least partial anionic character. The cationic nature of much of the colored material in the current study may be attributed to the use of lysine (a dibasic amino acid). It has been shown that the p*I* of total melanoidin formed in aqueous glucose−amino acid systems varies with the amino acid with values of 2.5 and 8.0 being recorded for glycine and arginine, respectively (Gomyo, 1976).

CZE. Preliminary work showed that although the citrate buffer gave better resolution than the borate buffer, migration times were greatly increased and the total run time was unacceptably long (more than 60 min). Therefore, most work was performed using the borate buffer. Figure 1 shows electrophoretograms of extracts of samples extruded at pH 3.4 and 7.7, at 280 and 420 nm. Repeat CZE runs demonstrated that the electrophoretograms were reproducible. Good resolution was observed at both 280 and 420 nm, and clear differences can be seen between the two samples. The electrophoretograms differed with extrudate pH at migration times of 6−7 and 10−13 min. The pH 3.4 extract revealed peaks at *ca.* 11 and 12.5 min on the 420 nm electrophoretogram which were not observed for the pH 7.7 sample. The broad band of migrating material seen on the electrophoretograms for both samples, particularly at 420 nm between 6 and 12 min, may be due to unresolved melanoidin (Tomlinson, 1994). The peaks at 6.6 and 12.3 min on the 420 nm trace of the pH 3.4 sample had λ_{max} values (between 340 and 500 nm) at 400 and 468 nm and 418 nm, respectively, while the two largest peaks on the 420 nm electrophoretogram of the pH 7.7 sample, at 6.3 and 6.7 min, had λ_{max} values (between 340 and 500 nm) at 410 and 475 nm and 405 nm, respectively. This indicates that the major resolved colored compounds in the extracts of the extrudates of different pH were different.

HPLC. The first stage was to optimize the HPLC methods (Bates, 1996). For IE-HPLC, separation of the colored components took more than 2 h. Clear differences were observed for extracts of extrudates of different pH values, and the broad bands (presumably due to polymeric material) observed on the 420 nm chromatograms were more prominent for the extract prepared from the pH 7.7 sample. One aim of the study was to isolate components of the extracts by semipreparative HPLC, prior to analysis by MS and NMR, and this requires removal of buffer salts from the collected fractions. This was a potential difficulty. Therefore, attention was turned to RP-HPLC.

Initially, RP-HPLC separations were performed using an ODS2 column. Although some separation of components was achieved, much of the material was unretained by the column. Ion-pair chromatography was also attempted, but little improvement was observed. Therefore, a high carbon loading stationary phase, ODS30, with 31% available carbon, was employed. This phase is designed for use with polar compounds and gave improved resolution of components. The chromatograms obtained are shown in Figure 2. The chromatograms obtained for the extract of starch extruded alone showed only eight very small peaks, which were not observed at the detector setting used for the extracts of the starch−glucose−lysine extrudates.

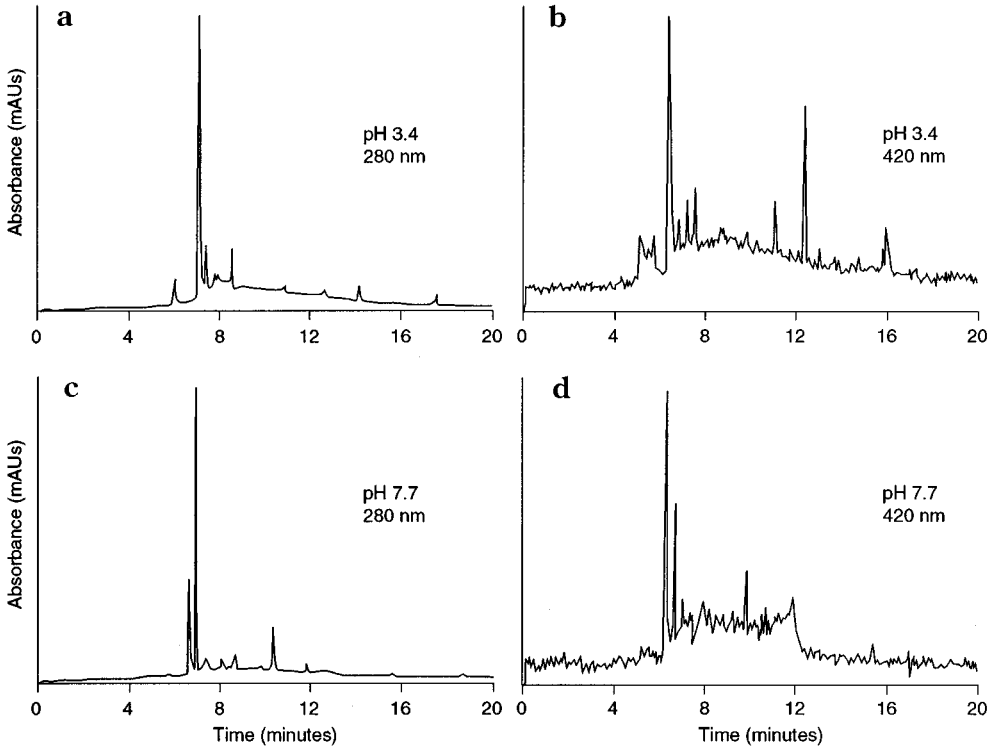

Figure 1. Electrophoretograms of the methanol extracts of the pH 3.4 and 7.7 extrudates: (a) pH 3.4, 280 nm; (b) pH 3.4, 420 nm; (c) pH 7.7, 280 nm; (d) pH 7.7, 420 nm.

As for IE-HPLC, differences were observed between samples of different initial pH, when analyzed by RP-HPLC. Considering the 280 nm traces, the peak at 12.40 min dominated the pH 3.4 extract and accounted for 84.3% of the total peak area. It decreased at pH 5 and was a minor peak at pH 7.7 (1.4% of the total peak area). At pH 7.7, the largest peak was that at 8.28 min, which accounted for 69.6% of the total peak area on the 280 nm chromatogram. A resolved peak (at 15.44 min) was observed on the 420 nm chromatogram for the pH 3.4 sample but was not present at the higher pH values. At pH 7.7, a broad band dominated the 420 nm chromatogram. The λ_{max} values of the largest resolved RP-HPLC peaks obtained for the extracts of the pH 3.4 and 7.7 extrudates are given in Table 4. The UV spectra of the 5.53, 12.40, and 21.84 min peaks from the pH 3.4 extrudate and the 12.46 min peak from the pH 7.7 extrudate are indicative of furan derivatives (Bailey et al., 1996).

CZE gave superior resolution of the extract components compared to RP-HPLC (Figures 1 and 2), and this agrees with Tomlinson et al. (1994). However, semipreparative CZE is not a routine procedure. Since the next step was to isolate components prior to structural analysis, this was performed using RP-HPLC. High-purity samples are crucial for obtaining clean NMR and mass spectra that can be readily interpreted; thus, optimum resolution of components by RP-HPLC was vital. Further attempts to improve the resolution of components of the extract from the pH 7.7 extrudate resulted in the best separations being achieved using the LiChrosorb RP-18 column and a water−methanol gradient. The 280 nm chromatogram is shown in Figure 3.

Structural Analysis. *(a) pH 3.4 Extrudate.* Only the peak at 12.40 min, on the 280 nm ODS30 chromatogram (Figure 2a), was analyzed. The mean retention time from 12 runs was 12.40 ± 0.2 min on the ODS30 analytical column under the separation conditions in Table 2. The HPLC−DAD spectrum showed a λ_{max} at 280 nm with a subsidiary maximum at 230 nm. The isolated compound was colorless and dissolved readily in methanol. The mass spectrum was 126 (85, M), 97 (98, M −29), 69 (79, M − 57), 41 (100, M − 85), and it matched that for HMF given by the MS data system library. The HPLC−DAD data matched exactly those obtained for the authentic compound. The presence of HMF in this extrudate was expected. It is formed in substantial amounts in low-pH systems via 1,2-enolization of the Amadori rearrangement product (ARP) (Ames, 1988). Lower amounts were present in the pH 5.0 and 7.7 samples (see below).

Table 4. λ_{max} Values of the Largest Peaks Observed in the RP-HPLC Chromatograms of the Extracts of the pH 3.4 and 7.7 Extrudates

pH 3.4 extrudate		pH 7.7 extrudate	
retention time (min)	λ_{max} (nm)	retention time (min)	λ_{max} (nm)
4.71[a]	300, 345 (sh)	3.41	300, 265 (sh)
5.53	280, 225	4.79	335
7.70	305, 350 (sh)	8.28[b]	300, 355 (sh)
12.40[c]	280, 230	12.46[c]	285, 235
15.44[a]	385		
21.84	290, 225		

[a] Detected only on the 420 nm chromatogram. [b] Detected on both the 280 and 420 nm chromatograms. All other peaks were detected only at 280 nm. [c] HMF.

(b) pH 5.0 Extrudate. Peaks A and B (Figure 2b) eluted at 25.4 and 28.0 min, respectively, using the semipreparative ODS30 column and the separation conditions in Table 4. Both were isolated. Peak A had a mean retention time of 25.4 ± 0.7 min. It had a λ_{max} at 275 nm and a second band of absorption at 380 nm. It was dark yellow and dissolved readily in methanol. The MS data showed the peak to be a mixture of components, including HMF. It contained relatively large amounts of a component with a probable molecular mass of 340 amu. Its accurate mass was 340.2396 amu,

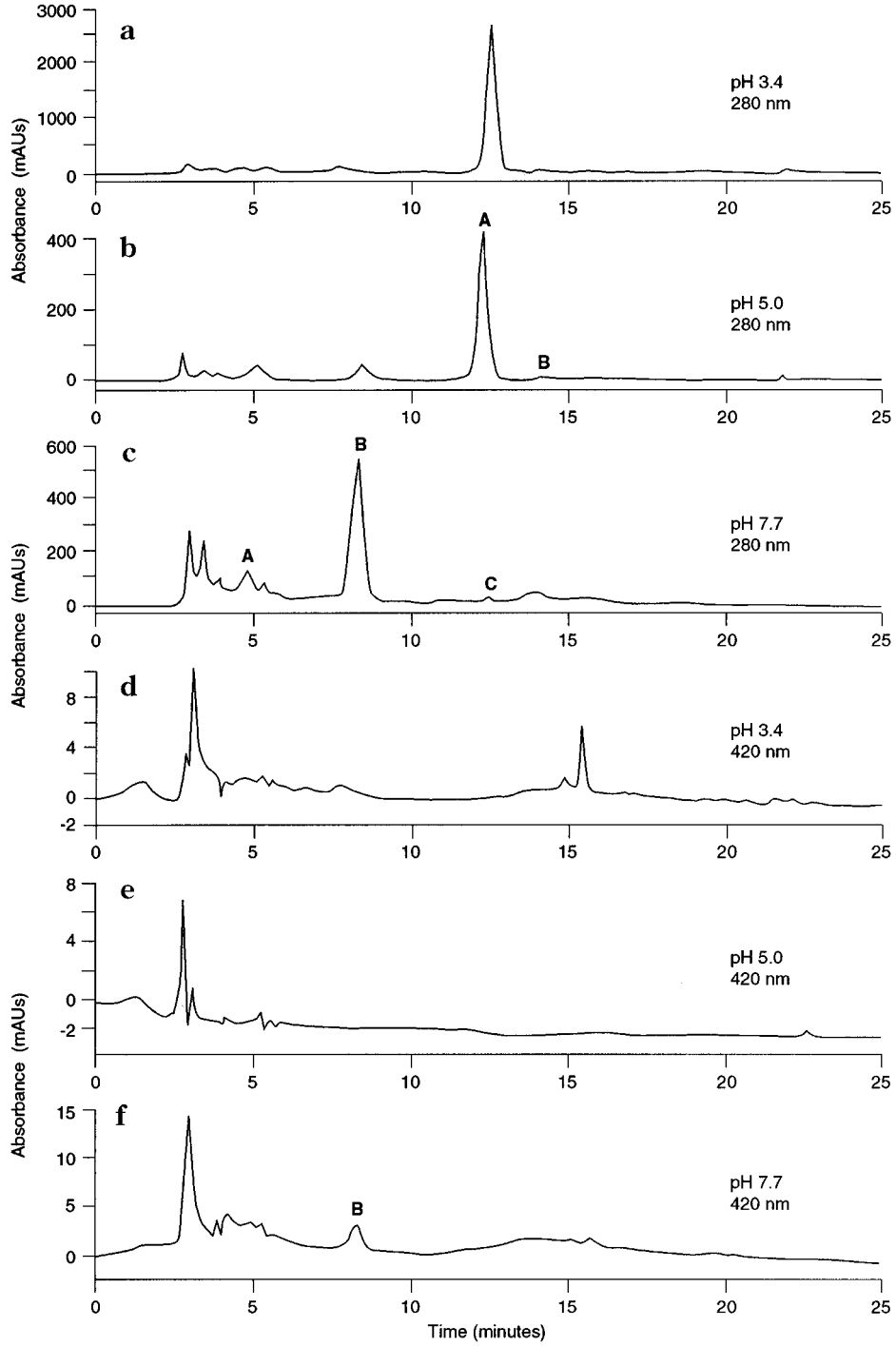

Figure 2. Analytical ODS30 chromatograms of the methanol extracts of the pH 3.4, 5.0, and 7.7 extrudates: (a) pH 3.4, 280 nm; (b) pH 5.0, 280 nm; (c) pH 7.7, 280 nm; (d) pH 3.4, 420 nm; (e) pH 5.0, 420 nm; (f) pH 7.7, 420 nm.

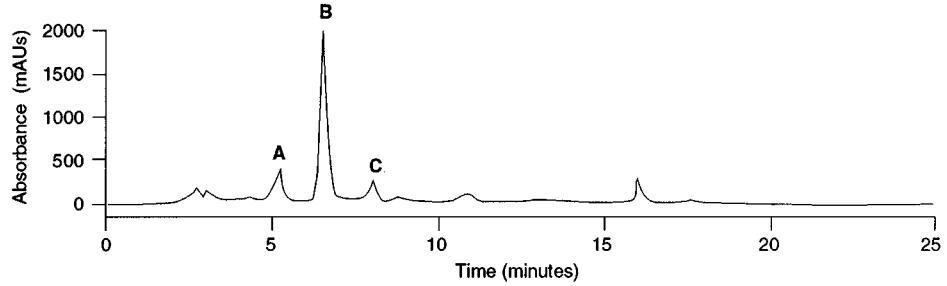

Figure 3. Analytical LiChrosorb RP-18 chromatogram of the methanol extract of the pH 7.7 extrudate at 280 nm.

and it was present at a relative intensity of 12% of the base peak. The most probable empirical formula was $C_{18}H_{32}O_4N_2$ (deviation −3.4 mmu). MS data for the most important fragment ions were as follows: 278.2000(8) $C_{16}H_{26}O_2N_2$; 254.1330(11) $C_{15}H_{14}O_2N_2$; 225.0729(20) $C_{13}H_9O_2N_2$; 189.0726(25) $C_{11}H_{11}O_2N$;

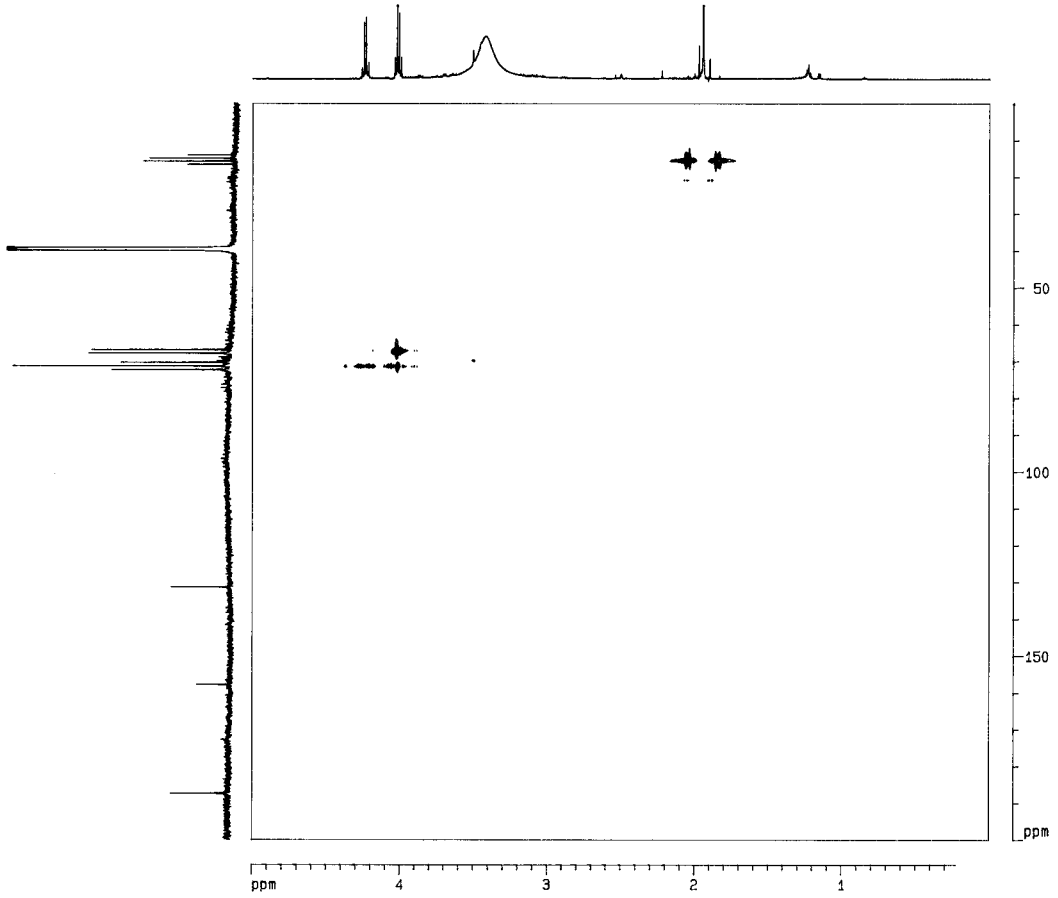

Figure 4. Inverse $^{1}H-^{13}C$ heteronuclear one-bond correlation spectrum in DMSO-d_6 of peak B from the methanol extract of the pH 7.7 extrudate.

177.1290(20) $C_{12}H_{17}O$; 87.0698(30) C_4H_9ON. NMR data are required before the structure of this colored compound can be elucidated.

The retention time of peak B was 28.0 ± 0.8 min. The λ_{max} was at 365 nm with shoulders at 255 and 275 nm and tailing to about 450 nm (into the visible region). The peak was dark yellow and dissolved readily in methanol. HPLC analysis of the isolated peak showed it to be a mixture of two compounds with retention times of 28.2 and 28.7 min. The diode array spectra were almost identical, indicating the presence of two structurally similar compounds, possibly isomers. The MS data revealed the presence of two minor additional components with spectra which were the same as the those of the compound with a molecular mass of 340 amu (the major component of the 25 min peak of the pH 5.0 extrudate separated using the semipreparative ODS30 column) and a compound with a molecular mass of 285 amu. The two major components of this 28 min peak apparently ionized together and gave a probable molecular ion at *m/z* 303. Its accurate mass was 303.2702 amu with a likely empirical formula of $C_{19}H_{33}N_3$ (deviation −2.8 mmu) or $C_{14}H_{33}O_2N_5$ (deviation −6.8 mmu). Abundances of the higher mass ions are as follows: 252.1837(9); 247.2059(13); 240.0958(16); 226.0791(24); 220.1221(21); 211.0574(21); 189.0703(31); 170.0721(28); 165.0703(26); 145.1002(23); 141.0695(23). Again, NMR data are required for elucidation of the structure of this colored compound.

(*c*) *pH 7.7 Extrudate.* Three peaks were isolated, i.e., peaks A, B, and C in Figure 3. They had retention times of 7.76, 9.33, and 10.72 min, respectively, using the Supelcosil SPLC-18 semipreparative column. Peak A, with a retention time of 7.76 ± 0.11 min, gave the following EI-MS data: 144 (100, M^+), 121 (8), 117 (16), 106 (21), 103 (97), 101 (35). The molecular ion was confirmed by CI (145, 100). NMR data are required for structural elucidation.

Peak B had a retention time of 9.33 ± 0.10 min. After purification, the purity of the peak was 98%. It was a white solid with a λ_{max} at 298 nm. The EI-MS data were as follows: 144 (37, M^+), 101 (14, $M - COCH_3$), 72 (14), 55 (16), 43 (100). CI (145, 100) confirmed the molecular mass as 144 amu. Elucidation of the structure was based mostly on NMR experiments in DMSO-d_6. The ^{1}H-NMR data and the suggested assignments were as follows: (600 MHz, DMSO-d_6) δ 2.00 (s, 3, C*H*$_3$), 4.01 (m, 1, C*H*), 4.02 (s, 1, C*H*), 4.23 (m, 1, C*H*), 5.80 (s, 1, O*H*), 7.60 (s, 1, O*H*). A second-order spectrum was obtained, which excluded the direct determination of coupling constants. The observed signals from the ^{13}C-NMR spectrum are as follows (150.9 MHz, DMSO): δ 14.88 (q, primary) 67 (d, ternary), 70.83 (t, secondary), 130.81 (s, quaternary), 157.25 (s, quaternary), 186.4 (s, quaternary). Two-dimensional heteronuclear correlations with inverse detection revealed all the one-bond and multiple-bond ($^{1}H-^{13}C$) correlations (see Figures 4 and 5). Only the disilylated derivative was obtained. It had a retention time of 9.88 min by GC−MS. The molecular mass of 288 amu confirmed the presence of two hydroxyl groups. Taking into account all the analytical data, in particular the NMR information, the compound was identified as 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone.

This important Maillard reaction product was reported for the first time by Ledl (1979), who formed it from 4-hydroxy-5-methyl-3(2*H*)-furanone and formaldehyde. It was later reported among the reaction

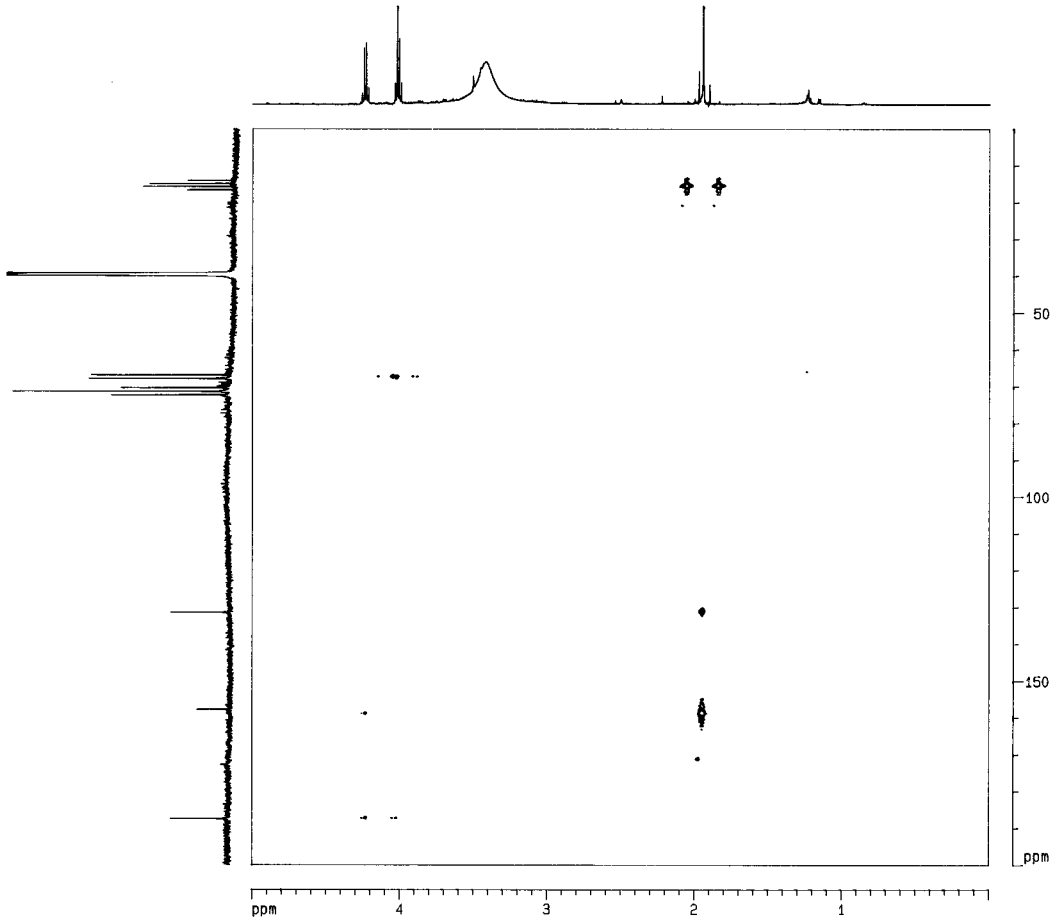

Figure 5. Inverse $^{1}H-^{13}C$ heteronuclear multiple-bond correlation spectrum in DMSO-d_6 of peak B from the methanol extract of the pH 7.7 extrudate.

products of a glucose−glycine model system by Hiebl et al. (1987). Some discrepancies were apparent between the MS and NMR data of those authors and the data reported here. Also, the literature reports the formation of a trisilylated derivative (Hiebl et al., 1987) while we observed a disilylated compound. Therefore, 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone was synthesized from glucose and propylamine according to Knerr and Severin (1993). HPLC−DAD of the synthesized and isolated compounds and GC−MS of both samples after silylation gave identical data, providing conclusive evidence that peak B is 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone.

4-Hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone forms from the ARP via the 1-deoxyosone and is analogous to 4-hydroxy-5-methyl-3(2*H*)-furanone (HMF^{one}) formed from pentoses. It is reported to react with primary amines, such as amino acids, with the formation of pyrrolinones (Hiebl et al., 1987). Although not colored itself, it possesses several reactive groups and it is reasonable to expect it to participate in reactions leading to the formation of structures with extended conjugation. 4-Hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone can form HMF^{one} (10% yield) and formaldehyde on refluxing in phosphate buffer at pH 7 (Hiebl et al., 1987). Therefore, HMF^{one} may be expected to form in our pH 7.7 extrudate and, if so, it should be extracted by methanol. A comparison of the retention time and the diode array spectra of the pH 7.7 extract peaks with those of the authentic compound, run using the same HPLC conditions, failed to detect it. This implies that the extrusion conditions do not favor the formation of HMF^{one} and/or it is consumed by subsequent reactions.

Peak C, with a retention time of 10.72 ± 0.13 min, gave MS and NMR spectra which matched exactly those obtained for authentic HMF.

In conclusion, IEF is a useful technique for the initial separation of a complex mixture of colored Maillard reaction compounds. It also gives an indication of which other separation techniques are appropriate, based on the net charge and p*I* values of the colored bands. CZE has been shown to be a powerful tool for the separation of colored Maillard reaction products, giving both shorter analysis times, compared to HPLC, and superior resolution of components. The disadvantage of this technique is that scaling up to preparative separations is not straightforward. IE-HPLC is able to demonstrate clear differences between samples extruded under different conditions, but the analysis time is very long and the isolation of components can be difficult due to the presence of buffer salts in the eluting solvent. RP-HPLC proved to be the most suitable preparative technique. Separations on a high carbon loaded (ODS 30) column give adequate resolution of components in a relatively short analysis time (40 min) while the LiChrosorb RP-18 column gave better resolution of components of the extract prepared from the pH 7.7 extrudate. Spectral data of selected HPLC peaks have established the presence of HMF in the methanol extracts of the extrudates of all three initial pH values, in amounts that decreased as pH increased. Using mainly NMR, 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone was conclusively identified in the metha-

nol extract prepared from the pH 7.7 extrudate. Preliminary data have been obtained for three additional Maillard reaction products. Additional data are required for their structural elucidation.

This paper has reported data on the components of methanol extracts of model extrudates. Most of the spectroscopic data were obtained off-line, and the necessary steps to isolate and purify each compound were very time-consuming. Over the last 10 years, there have been dramatic developments in the coupled techniques of liquid chromatography−mass spectrometry (LC−MS) and capillary electrophoresis−mass spectrometry (CE−MS), involving the use of mild ionization procedures, such as atmospheric pressure ionization (API) (e.g., Huang et al., 1990; Smith and Udseth, 1993). The application of such powerful on-line techniques for the separation and structural analysis of small amounts of complex mixtures may be expected to offer great advantages for samples of Maillard reaction products, such as those analyzed here. Most of the colored material was not extracted from the extrudate but was retained by the starch matrix. Future work must also address the nature of this material.

ABBREVIATIONS USED

UV, ultraviolet; HPLC, high-performance liquid chromatography; HPLC−DAD, high performance liquid chromatography−diode array detection; IE-HPLC, ion-exchange HPLC; RP-HPLC, reverse-phase HPLC; CZE, capillary zone electrophoresis; IEF, isoelectric focusing; MS, mass spectrometry; GC−MS, gas chromatography−mass spectrometry; EI-MS, electron impact mass spectrometry; db + r, double bond plus ring; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple bond correlation; DMSO, dimethyl sulfoxide; HMF, 5-(hydroxymethyl)furfural; HMF^{one}, 4-hydroxy-5-methyl-3(2*H*)-furanone; ARP, Amadori rearrangement product; CE-MS, capillary electrophoresis−mass spectrometry; LC−MS, liquid chromatography−mass spectrometry; API, atmospheric pressure ionization.

ACKNOWLEDGMENT

We thank the following for performing analyses. CZE: Dr. L. Castle and Dr. J. S. Coffey (CSL, Norwich, U.K.). MS: Dr. C. Chapman (Dalgety FTC, Cambridge, U.K.). NMR: Mr. P. Heath (Department of Chemistry, The University of Reading, U.K.).

LITERATURE CITED

Ames, J. M. The Maillard reaction−an update. *Chem. Ind. (London)* **1988**, 558−561.

Ames, J. M.; Nursten, H. E. Recent advances in the chemistry of coloured compounds formed during the Maillard reaction. In *Trends in Food Science*; Lien, W. S., Foo, C. W., Eds.; Singapore Institute of Food Science and Technology: Singapore, 1989; pp 8−14.

Ames, J. M.; Apriyantono, A.; Arnoldi, A. Low molecular weight coloured compounds formed in xylose-lysine model systems. *Food Chem*. **1993**, *46*, 121−127.

Bailey, R. G.; Ames, J. M.; Monti, S. M. An analysis of non-volatile reaction products of aqueous Maillard model systems at pH 5, using reversed-phase HPLC with diode array detection. *J. Sci. Food Agric.* **1996**, *72*, 97−103.

Bates, L. Analysis of the colour of an intermediate moisture Maillard system. Ph.D. Dissertation, The University of Reading, U.K., 1996.

Bates, L.; Ames, J. M.; MacDougall, D. B. The use of a reaction cell to model the development and control of colour in extrusion cooked foods. *Lebens. Wiss. Technol*. **1994**, *27*, 375−379.

Bax, A.; Morris, G. An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. *J. Magn. Reson*. **1981**, *42*, 501.

Bax, A.; Summers, M. F. ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J. Am. Chem. Soc*. **1986**, *108*, 2093−2094.

Deyl, Z.; Miksik, I.; Struzinsky, R. Separation and partial characterization of Maillard reaction products by capillary zone electrophoresis. *J. Chromatogr*. **1990**, *516*, 287−298.

Gomyo, T.; Horikoshi, M. On the interaction of melanoidin with metallic ions. *Agric. Biol. Chem*. **1976**, *40*, 33−40.

Hashiba, H. Isolation and identification of amadori compounds from soy sauce. *Agric. Biol. Chem*. **1978**, *42*, 763−768.

Hiebl, J.; Ledl, F.; Severin, T. Isolation of 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone from sugar amino acid reaction mixtures. *J. Agric. Food Chem*. **1987**, *35*, 990−993.

Huang, E. C.; Wachs, T.; Conboy, J. J.; Henion, J. D. Atmospheric pressure ionization mass spectrometry. *Anal. Chem*. **1990**, *62*, 713A−722A, 724A−725A.

Ingles, D. L.; Gallimore, D. A new method for the isolation of melanoidins from the Maillard reaction of glucose and glycine. *Chem. Ind. (London)* **1985**, March, 194.

Knerr, T.; Severin, T. Investigations of the glucose/propylamine reactions by HPLC. *Z. Lebensm.-Unters. Forsch*. **1993**, *196*, 366−369.

Lea, A. G. H. HPLC of natural pigments in foodstuffs. In *HPLC in Food Analysis;* Macrae, R., Ed.; Academic Press: London, 1988; pp 255−270.

Ledl, F. 4-Hydroxy-2-hydroxymethyl-5-methyl-2,3-dihydrofuran-3-on: ein Zucher Wandlungsprodukt? (4-Hydroxy-2-hydroxymethyl-5-methyl-2,3-dihydrofuran-3-one: a sugar decomposition product?). *Z. Lebensm.-Unters. Forsch*. **1979**, *169*, 176−178.

Ledl, F.; Schleicher, E. New aspects of the Maillard reaction in foods and in the human body. *Angew. Chem., Int. Ed. Engl*. **1990**, *29* (6), 565−594.

Nursten, H. E.; O'Reilly, R. Coloured compounds formed by the interaction of glycine and xylose. *Food Chem*. **1986**, *20*, 45−60.

O'Reilly, R. Application of electrofocusing for the fractionation of coloured products formed during the xylose−glycine Maillard reaction. *Chem. Ind. (London)* **1983**, September, 716−17.

Smith, R. D.; Udseth, H. R. Mass spectrometric detection for capillary electrophoresis. In *Capillary Electrophoresis Technology;* Guzman, N. A., Ed.; Dekker: New York, 1993; pp 525−567.

Tomlinson, A. J.; Mlotkiewicz, J. A.; Lewis, I. A. S. An investigation of the compounds produced by spray-drying an aqueous solution of glucose and glycine. *Food Chem*. **1993a**, *48*, 373−379.

Tomlinson, A. J.; Landers, J. P; Lewis, I. A. S.; Naylor, S. Buffer conditions affecting the separation of Maillard reaction products by capillary electrophoresis. *J. Chromatogr*. **1993b**, *652*, 171−177.

Tomlinson, A. J.; Mlotkiewicz, J. A.; Lewis, I. A. S. Application of capillary electrophoresis to the separation of coloured products of Maillard reactions. *Food Chem*. **1994**, *49*, 219−223.

Received for review August 19, 1996. Revised manuscript received January 7, 1997. Accepted January 12, 1997.[⊗] The BBSRC, RHM, APV-Baker, and the Leatherhead Food RA are thanked for financial support.

JF9606316

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1997.