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Analytical Methods Section

A multidetector HPLC system for the analysis of Amadori and other Maillard reaction intermediates

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Most of the HPLC- and GLC-based analytical procedures described in the literature and used to analyze Maillard reaction mixtures attempt to separate and identify Amadori products; however, many of the other important intermediates that form during this reaction are fluorescent species or contain redox moieties that do not absorb in the visible or ultraviolet regions. To detect simultaneously (and on-line) a wide variety of such products and to follow their kinetics, an HPLC with diode array detector was coupled in parallel to fluorescence and electrochemical detectors. A fraction collector was interfaced to the system and connected to the effluent, exiting the fluorescence detector to collect fractions for further analysis. The potential of such a system to analyze complex Maillard reaction mixtures and to detect different types of Amadori products (aromatic and non-aromatic) in the presence of starting sugars and amino acids was demonstrated.

INTRODUCTION

The non-enzymatic interaction of reducing sugars with amino acids, proteins and DNA, which is known as the Maillard reaction, results in the formation of important intermediates both *in vivo* and in food systems (Finot *et al.*, 1990). Identification of the intermediates formed under physiological conditions can contribute to the development of diagnostic techniques to assess the damage to proteins and DNA and aid in the detection of the early onset of this reaction in diabetic patients. Knowledge of the composition of Maillard reaction mixtures is also important in heated mixtures of reducing sugars and amino acids due to their contribution to the sensory properties of thermally processed foods. Such mixtures are also known to contain carcinogens and antioxidants (Finot *et al.*, 1990).

One of the main difficulties associated with the analysis of Maillard reaction mixtures, is the formation of a multitude of products with diverse physical and chemical properties (hydrophobic, hydrophilic, volatile, non-volatile, etc.); some chromophores are UV active some are colored; others are fluorescent or redox-active. The initial phase of this reaction with glucose produces the first stable intermediate, 1-amino acid-1-deoxy-D-fructose, known as an Amadori product. Detection and separation of this important intermediate from the starting sugar and amino acid mixture is of crucial importance in studying the early kinetics of the Maillard reaction.

Many systems have been developed for the separation and identification of important Maillard products. These techniques are based mainly on HPLC using single detectors. Gas chromatography, on the other hand, has had only limited success in analyzing the early Maillard reaction intermediates and their immediate degradation products due to their low volatility and instability. However, GC/MS has been extensively used to analyze volatile aroma components of different Maillard model systems. The main advantage of HPLC over GC in the Maillard reaction analysis context is that non-volatile water-soluble compounds can be analyzed directly without the need for prior derivatization. This is important for the early phase kinetic analysis of Maillard reactions. Van den Ouweland *et al.* (1978) used an HPLC system with refractive index detector to separate different Amadori products on an Aminex A-9 cationic column (H^+ form), whereas Takeoka *et al.* (1979) were able to separate *p*-nitrobenzyloxime derivatives of Amadori products on a μBondapak/carbohydrate column, using UV detection at 254 nm. In addition, they separated aromatic amino acids from their corresponding Amadori products on a μBondapak/NH_2 column or μBondapak C_{18} reversed phase column, again using UV detection. Moll & Gross (1981), developed a semi-preparative reversed phase HPLC procedure to separate valine and proline Amadori products from crude extracts of Maillard reactions,

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using refractive index detection and water as the eluent. A more general procedure that can separate five Amadori products involved an NH_2-bonded silica gel column and methanol:water (80:20) as eluent again using refractive index detection (Moll *et al.*, 1982). However, the most efficient separation of Amadori products was reported by Eichner *et al.* (1990). They demonstrated the separation of 16 Amadori products from glucose and fructose, using *N,N*-diethylaminoethyl modified silica gel (DEAE-Si 100, 3 mm, Serva) by a post-column derivatization technique based on the reduction of triphenyl tetrazolium chloride by Amadori products and other reducing sugars to produce red 1,3,5-triphenyl formazane which absorbs at 480 nm. Kawakishi *et al.* (1991) separated alanine Amadori product from both D-*arabino*-hexosulose and the amino acid using a Develosil NH_2-5 column with UV monitoring at 210 nm. A similar separation of proline Amadori product from glucose and proline was achieved by Debrauwer *et al.* (1991) using two reversed phase C_{18} columns connected in series with refractive index detection.

Due to extensive requirement for derivatization and the ability of GLC columns to detect the multiple tautomeric forms of reducing sugars that can complicate the analysis, there are relatively fewer applications of GLC-based techniques to the analysis of early Maillard reaction intermediates. Takeoka *et al.* (1979) showed that injection of a pertrimethylsilylated derivative of a *p*-toluidine Amadori product onto a WCOT column produces five peaks corresponding to five known tautomeric forms of the compound. Complications arising from tautomeric forms can be eliminated by chemically reducing the Amadori products and derivatizing the corresponding alditols. Alternatively, acyclic volatile derivatives can be prepared by oximation followed by trimethylsilylation (Eichner *et al.*, 1990). Although this method eliminates the formation of cyclic tautomeric forms, it introduces the problem of *syn* and *anti* isomers of the oximes that can be separated and detected on capillary columns, thus complicating the analysis.

Most of the HPLC-based analytical procedures described in the literature attempt to separate and identify Amadori products in Maillard reaction mixtures; however, many of the other important intermediates that form during this reaction are fluorescent species or contain redox moieties that do not absorb in the visible or ultraviolet regions. To detect simultaneously, a wide variety of such products and to follow their kinetics, an HPLC with diode array detector was coupled in parallel to a fluorescence and an electrochemical detector. A fraction collector was interfaced to the system and connected to the effluent exiting the fluorescence detector to collect fractions for further analysis. In this paper the potential of such a system to analyze complex Maillard mixtures is presented.

MATERIALS AND METHODS

Reagents and chemicals

Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, NJ). Water was distilled and deionized (Milli-Q plus, from Waters). All reagents were purchased from Aldrich Chemical Company

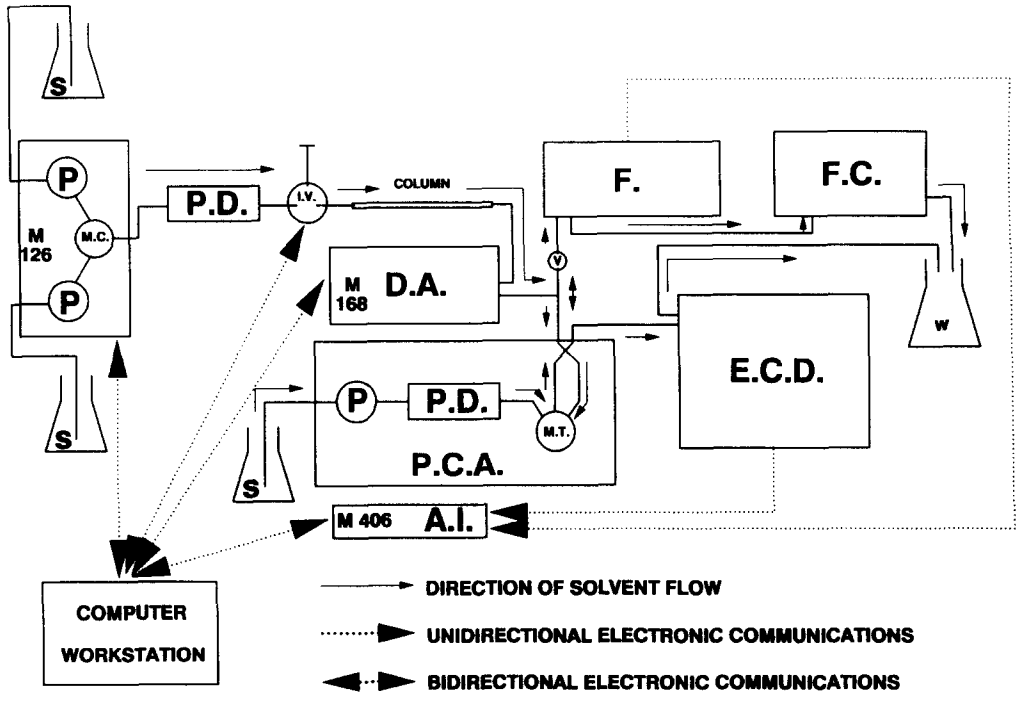

Fig. 1. HPLC system set-up. P, pump; S, solvent; W, waste; M.C., mixing chamber; P.D., pulse dampener, I.V., injection valve; V, splitting valve; A.I., analog digital interface; D.A., diode array detector; P.C.A., post-column addition system; F, fluorescence detector; F. C., fraction collector; E.C.D. electrochemical detector; M. T., mixing T.

(Milwaukee, WI). NaOH was of semiconductor grade (99·99%). HPLC solvents were filtered and degassed (ultrasound/vacuum) prior to use. Amadori compounds of tryptophan (Yaylayan & Forage, 1991) and of proline (Debrauwer *et al.*, 1991) were synthesized using glucose, according to literature methods. The Amadori compound of morpholine was purchased from Sigma Chemical Company (St. Louis, MO).

Apparatus

The HPLC system consisted of a programmable solvent module (Beckman, system GOLD, module 126), a diode array detector (Beckman, system GOLD, module 168), an analog interface (Beckman, system GOLD, module 406), a scanning fluorescence monitor (Shimadzu, model RF-551), a programmable electrochemical detector (Hewlett Packard, model 1049A), and a fraction collector (Gilson, model FC 203). The whole assembly is described in Fig. 1. The pumps, the diode array detector and the analog interface were controlled by an IBM 486-based compatible computer using the system software. The fluorescence and electrochemical detectors were controlled manually; however, the analog signals coming out of these two detectors were sent to the analog interface which digitized the signal and sent it for capture and analysis by the system Gold software on the computer. The diode array detector signal was sent directly to the computer as a digitized output to be captured and analyzed by the software. The carbohydrate dedicated column of Waters (10 μm; 4·6 mm × 30 cm) with an amino propyl guard column, 5 μm × 3 cm Brownlee cartridge system (Amino spheri-5), was used. The solvent systems used consisted of a phosphate buffer (0·001M, pH 2·6 or 0·004M, pH 6·5) and acetonitrile. Their relative compositions are mentioned with each application.

The eluent exiting the diode array detector was split continuously through a metering valve. The splitting ratio was dependent upon the differential pressure between the two outlets (one after the fluorescence detector, the other after the electrochemical detector). The flow rate was set at 1 ml/min, and once the pressure stabilized (with post-column addition of 0·2N NaOH at 1 ml/min), the flow rate exiting the fluorescence detector was measured and found to be 0·45 ml/min with a mobile phase consisting of 30% phosphate buffer and 70% acetonitrile; the same flow rate was maintained with other solvent systems. The pump head pressure was continuously monitored in order to ensure that the split ratio remained constant. In all cases, unless specifically mentioned, the elution was isocratic.

Pulse amperometric detection

Glucose and fructose were monitored (oxidation) by the electrochemical (EC) detector operating in the pulse amperometric detection (PAD) mode (E_2 = +0·650 V, t_2 = 50 ms; E_3 = −1·050 V, t_3 = 50 ms; E_1 = +0·10 V, t_1 = 200 ms; versus Ag/AgCl reference electrode with internal electrolytes, E_1 being the measuring potential) at a gold electrode. The pulse can be changed (herein only the monitoring voltage E_1 was changed when required) in order to monitor amino compounds, Amadori products and/or glucose.

Fluorescence and diode array detection

The fluorescence detector was operated at excitation/emission wavelengths of 280/360 nm with the low sensitivity setting for tryptophan derivatives and 360/440 nm for other Amadori degradation products at the high-sensitivity setting. The diode array detector was set to monitor the 190–460 nm region. While full advantage of the scanning capability of the system was taken, for simplification purposes only the 192 ± 2 nm and 280 ± 10 nm wavelengths are shown.

Analysis of assay solutions

Standard mixtures

Two standard mixtures were prepared. The first was composed of indole, tryptophan, Amadori-tryptophan, D-glucose, glycine, valine and proline dissolved in 70% methanol solution. The separation was performed with 75% acetonitrile and 25% phosphate buffer (0·001M, pH 2·6 containing 0·01% SDS). The second standard mixture contained D-glucose, D-fructose, Amadori-proline, Amadori-morpholine and Amadori-tryptophan dissolved in 70% acetonitrile solution. The mobile phase consisted of 70% acetonitrile and 30% phosphate buffer (0·004M, pH 6·5).

Assay reaction mixtures

Four aqueous assay reaction mixtures were prepared. The first consisted of Amadori-proline (0·1M) and the second consisted of Amadori-morpholine (0·1M); both heated for 2 h at 100°C. The mobile phase consisted in both cases of 70% acetonitrile and 30% phosphate buffer (0·004M, pH 6·5). The third reaction mixture contained 1mM D-glucose and 1mM L-tryptophan. The mixture was heated in a sealed tube at 100°C for 3 h and subsequently analyzed by GC/MS prior to HPLC analysis. The GC/MS analysis (on DB-1 column; temperature = 70°C with 5°C/min rate of increase until 250°C) indicated the presence of indole, tryptophol, tryptamine and norharman. The mobile phase for the gradient HPLC analysis for the presence of the above components in the reaction mixture consisted of 2·5% hexane in acetonitrile (solution A) and phosphate buffer (0·001M, pH 2·6 containing 0·01% SDS) (solution B). The initial composition was 98% A which was changed after 1·5 min to 70% A during a 15-min interval. The fourth reaction mixture consisted of 1·0mM D-glucose and 1·0mM morpholine in 1 ml of water (basic intrinsic pH). The reaction mixture was heated at 100°C for 3·5 h. A sample was taken every 30 min and injected. In this case the mobile phase consisted of 70% acetonitrile and 30% phosphate buffer (0·004M, pH 6·5).

RESULTS AND DISCUSSION

The inherent limitation of liquid chromatography to analyze complex mixtures is due to its low separation efficiency and lack of universal detectors compared to GLC techniques with capillary columns. The lack of universal detectors entails the use of multidetectors to remedy some of the problems associated with such analyses. Recent developments in capillary zone electrophoresis and in LC/MS interfaces may provide satisfactory solutions to the separation and detection problems in the near future. Most HPLC methods reported in the literature to analyze Maillard reaction mixtures have been focused on the separation of Amadori products either by post-column derivatization techniques to convert the analytes into compounds that absorb in the visible region or by limiting the scope of analysis to Amadori products containing aromatic moieties and subsequent detection by UV. Analyzing Maillard reaction mixtures, however, poses a greater challenge.

Methods have been developed to analyze the three main components present in the initial phase of the Maillard reaction, namely the starting sugar, amino acid and the Amadori product using either refractive index or low UV detection at 190 or 210 nm. Poor sensitivity and selectivity are often limiting for both modes of detection. Gradient incompatibility associated with refractive index (RI) detection also limits the separation potential of such analyses. Separation and detection of a larger number of Maillard reaction products is even more challenging. Deyl *et al.*, (1990) exploited the high separating power of capillary zone electrophoresis with UV detection at 220 nm to profile the products formed in model sugar–amino acid systems and separated 3–7 components from different models. Additional information about these components was gained by reacting them with 2,4-dinitrophenylhydrazine and phenyl isothiocyanate and rechromatographing the mixtures after removal of the derivatized products.

In order to alleviate the problem of detection and identification of complex mixtures by HPLC, an attempt was made to interface an HPLC with a diode array (DA), scanning fluorescence (FL), electrochemical (EC) detector and a fraction collector (see Fig. 1). The signals from the three detectors can be monitored simultaneously through the system software.

Overview of the system capabilities

Pulsed amperometric detection (PAD) is a relatively new technique which uses repeating automated sequences of three applied potentials for specific durations; the first potential (E_1) is to oxidize the analyte; at this potential the current is also measured. A higher potential (E_2) is applied to electrochemically clean the electrode surface (with the concurrent formation of a gold oxide layer) and E_3 (a negative potential) is applied to reduce gold oxide back to gold. For each class of compound these potentials should be optimized. Pulsed amperometry, like other electrochemical techniques, is sensitive to changes in eluant pH; to minimize baseline shifts and to optimize PAD sensitivity a post-

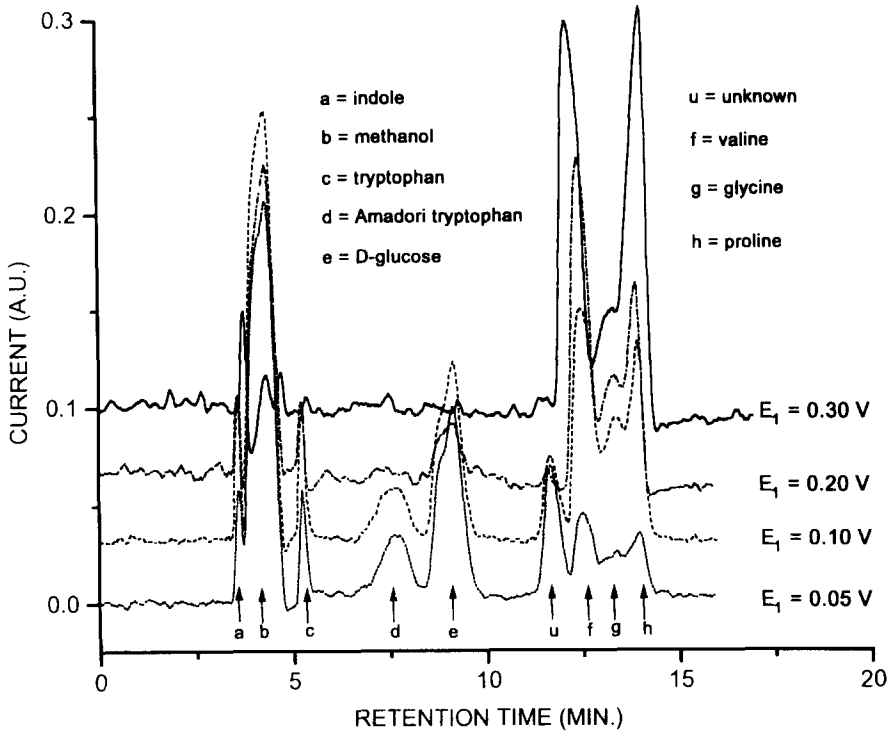

Fig. 2. Chromatograms of the standard mixture containing indole, tryptophan, Amadori-tryptophan, D-glucose, glycine, valine and proline. Using EC/PAD, each at a different measuring voltage (E_1 = 0·05, 0·10, 0·20 or 0·30 V; E_2 = 0·650 V, E_3 = −1·050 V; t_1 = 200 ms, t_2 = 50 ms, t_3 = 50 ms), and a mobile phase consisting of 75% acetonitrile and 25% phosphate buffer (0·001M, pH 2·6). A. U., arbitrary units.

column addition of NaOH is necessary. The use of PAD allows direct analysis of non-derivatized amino acids, Amadori products and especially sugars at low picomole levels with high signal-to-noise ratio. PAD provides the added flexibility of monitoring the three components in question or only one of them by adjusting the applied potentials. Figure 2 shows the separation of indole, valine, glycine, proline, glucose, tryptophan and tryptophan Amadori product using the following applied potentials: E_1 = 0·10 V (t_1 = 200 ms), E_2 = 0·65 V (t_2 = 50 ms) and E_3 = −1·050 V (t_3 = 50 ms). By changing the measuring potential (E_1) it is possible to measure only carbohydrate derivatives such as glucose and Amadori products. Here, even at E_1 = 0·05 V, the signal for the amino acids is still perceivable due to the relatively high concentration of amino acid present (~0·200 mg/ml) relative to glucose (0·004 mg/ml). On the other hand, at higher potentials, only alkyl amino acids are detectable. This is consistent with the relative reducing powers of sugars compared to amino acid.

The relative sensitivity and limit of detection of the PAD under these conditions (E_1 = 0·3 V) for alkyl amino acids is comparable to that of the UV detection at 190 nm. However, in the case of carbohydrates, EC/PAD detection represents approximately 100- to a 1000-fold gain in sensitivity depending on the carbohydrate used (Fig. 3). This type of sensitivity enhancement can be crucial for analyzing biological samples. Figure 3 shows the on-line simultaneous monitoring of the data captured from four channels (UV at 190, 280 nm, fluorescence at 280/360 nm and ECD) during the HPLC analysis of the standard mixture containing glucose, fructose, Amadori-morpholine, Amadori-proline and Amadori-tryptophan. In this particular model system, due to the fluorescence of tryptophan, its Amadori product can also be detected by fluorescence. In this mode the limits of detection can be increased more than 1000-fold over diode array detection. In this multidetector HPLC system, Amadori-tryptophan can serve as the ideal internal standard, as it absorbs in the UV, fluoresces, and can be oxidized electrochemically.

Applications of the LC/DA/PAD/FL system: Early phase kinetic analysis of Maillard reaction

During the early stages of the Maillard reaction, the analytes of interest are mainly polar water-soluble components comprised of monosaccharides (reducing sugars and Amadori products) and amino acids; however, as the reaction progresses, depending on the pH, temperature and time, different products can be formed, including ketones, aldehydes and heterocyclic compounds; some of them are UV-active, some are colored and others contain redox moieties. In addition, fluorescent species are known to form during the early stages of the reaction but tend to degrade as the solution turns brown. The nature of these fluorescent species is still unknown.

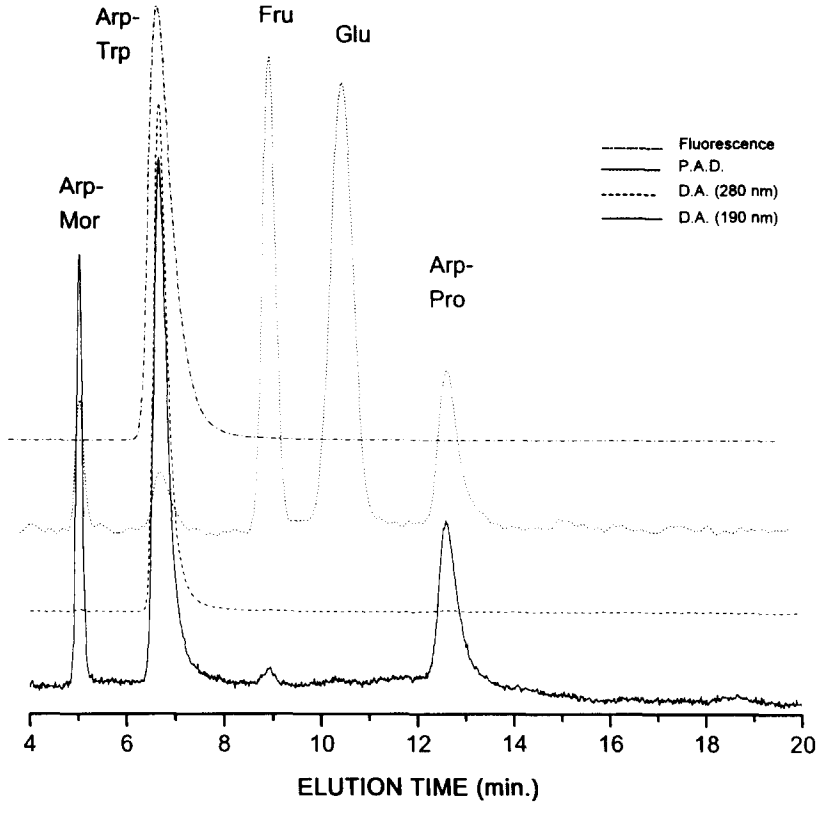

Fig. 3. Simultaneous on-line monitoring of chromatographic data from a single injection of the standard mixture containing D-glucose D-fructose, Amadori-proline, Amadori-morpholine and Amadori-tryptophan. Mobile phase: 70% acetonitrile, 30% phosphate buffer (0·04M, pH 6·5). Arp-Mor, Amadori-morpholine; Arp-Pro, Amadori-proline; Arp-Trp, Amadori-tryptophan; Glu, D-glucose; Fru, D-fructose.

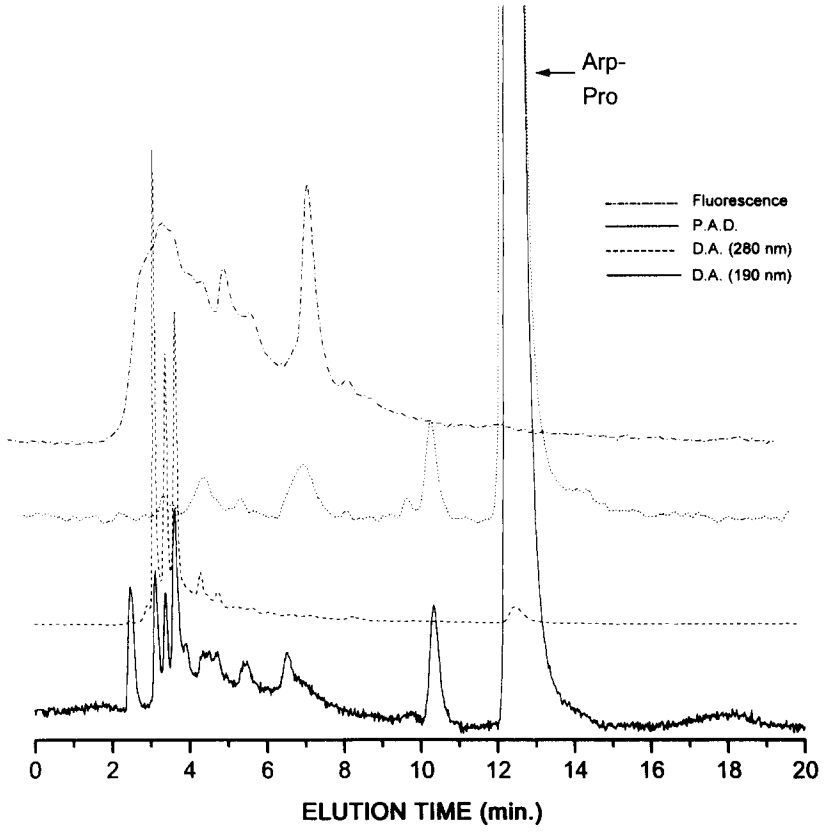

Fig. 4. Simultaneous on-line monitoring of chromatographic data from a single injection of the assay reaction mixture containing Amadori-proline heated at 100°C for 2 h, using 70% acetonitrile and 30% phosphate buffer (0·004M, pH 6·5) as the mobile phase. Arp-Pro, Amadori-proline.

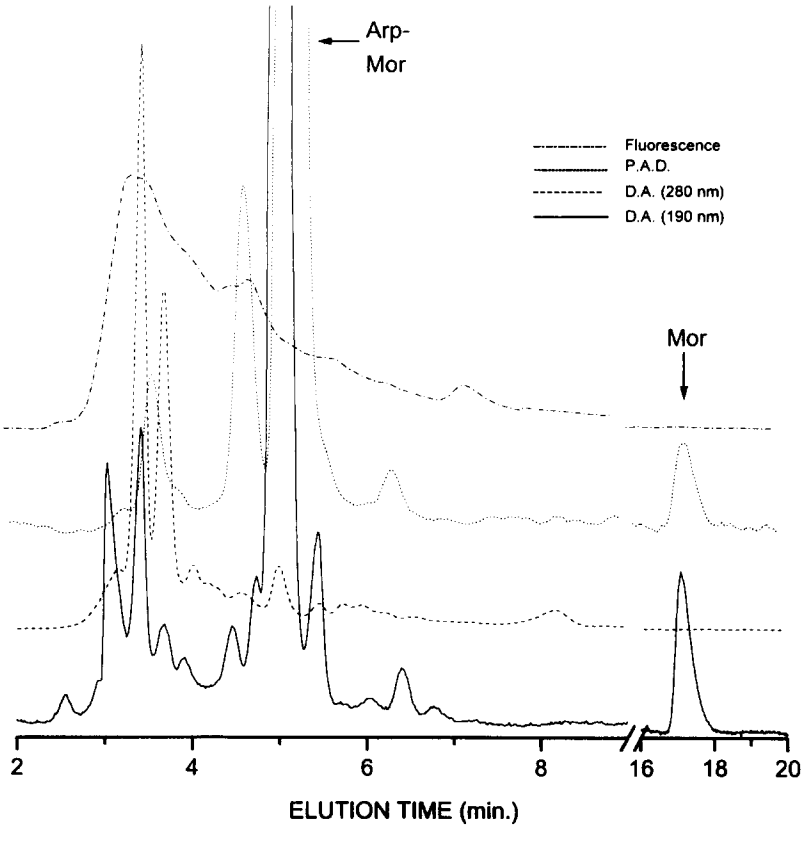

Fig. 5. Simultaneous on-line monitoring of chromatographic data from a single injection of the assay reaction mixture containing Amadori-morpholine solution heated at 100°C for 2 h, using 70% acetonitrile, 30% phosphate buffer (0·004M, pH 6·5) as the mobile phase. Arp-Mor, Amadori-morpholine; Mor, morpholine.

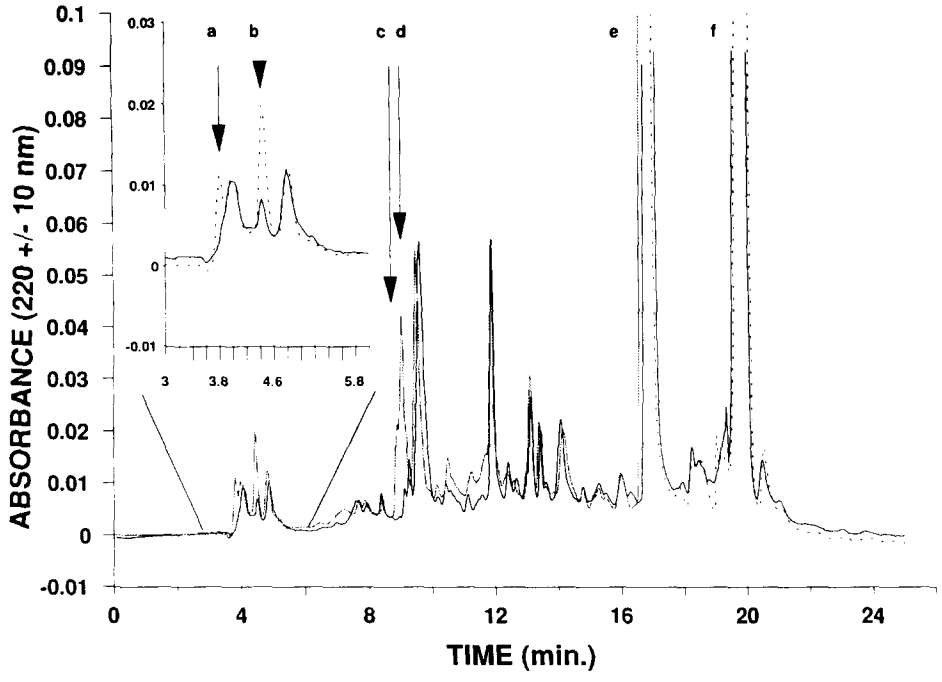

Fig. 6. Chromatogram of the assay reaction mixture containing tryptophan and glucose heated at 100°C for 3 h, using UV detection at 220 nm and a mobile phase consisting of 2·5% hexane- in acetonitrile (solution A) and 0·001M phosphate buffer (pH 2·5) containing 0·01% SDS (solution B). The initial composition was 98% A which was changed after 1·5 min to 70% A during 15-min interval. a, Indole; b, tryptophol; c, tryptamine; d, norharman; e, typtophan; f, tryptophan Amadori product.

Kinetics of degradation

Determination of the rates of sugar and amino acid disappearance and the rate of accumulation of Amadori products is crucial for understanding the Maillard reaction in food and biological systems. Although, there are HPLC methods available based on low-wavelength UV or RI detection, both of these methods suffer from poor sensitivity and selectivity, in addition, gradient incompatibility of RI detectors greatly limits the range of amino acids and sugars that can be separated by isocratic elution. The potential of this system in studying complex Maillard reaction mixtures was demonstrated by analyzing aqueous degradation mixtures of Amadori-proline (Fig. 4) and Amadori-morpholine (Fig. 5) after heating at

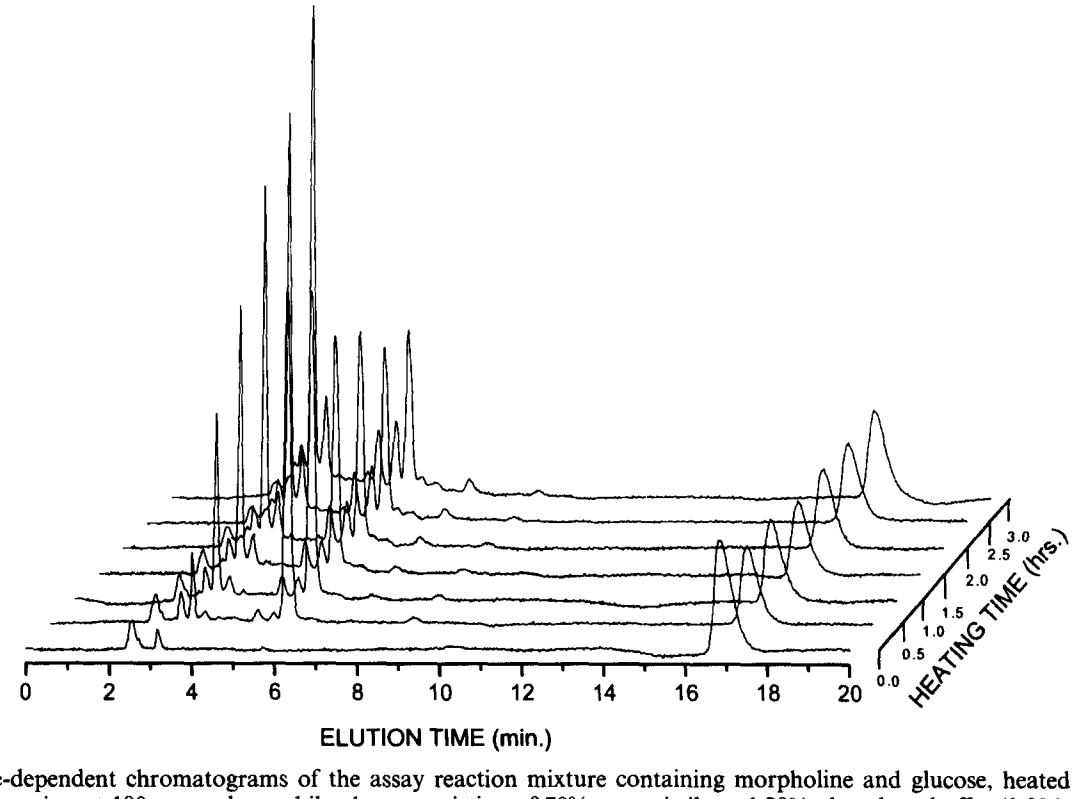

Fig. 7. Time-dependent chromatograms of the assay reaction mixture containing morpholine and glucose, heated for 3 h using UV detection at 190 nm and a mobile phase consisting of 70% acetonitrile and 30% phosphate buffer (0·004, pH 6·5).

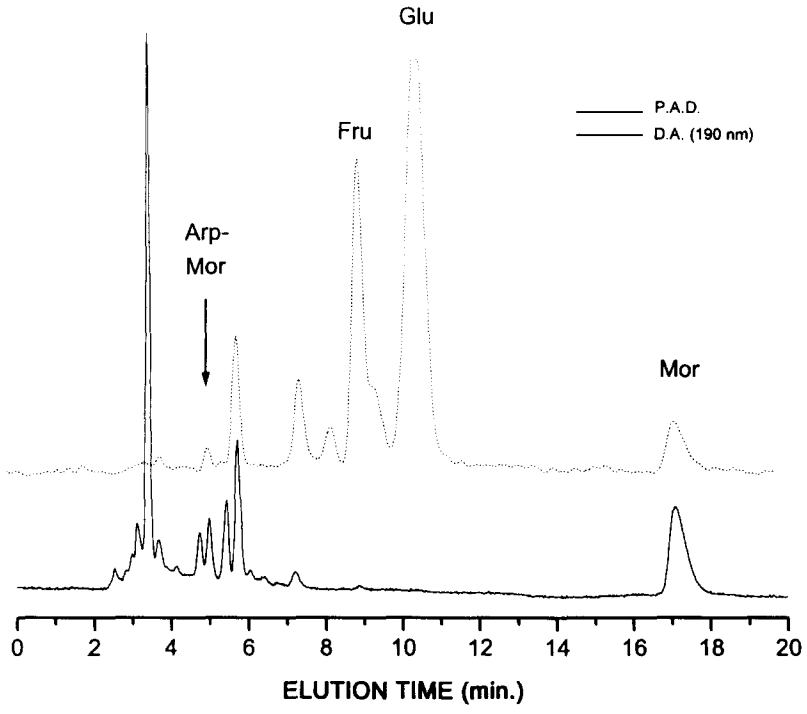

Fig. 8. Simultaneous on-line monitoring of chromatographic data (DA at 190 nm and EC/PAD at E_1 = 0·1 V) from a single injection of the assay reaction mixture containing morpholine and glucose, heated for 3·5 h at 100°C. Arp-Mor, Amadori-morpholine; Mor, morpholine; Glu, D-glucose; Fru, D-fructose. The mobile phase consisted of 70% acetonitrile and 30% phosphate buffer (0·004M, pH 6·5).

100°C for 2 h. Although the solutions turned deep-yellow, most of the Amadori compound remained intact. Still, many compounds were formed and each detector (FI/PAD/DA) proved useful in detecting these degradation products.

Kinetics of formation

To demonstrate the ability of the system to efficiently separate low concentrations of Maillard reaction products in the presence of excess amine and sugar, an aqueous solution of tryptophan and glucose was heated for 3 h at 100°C. At the end of the third hour, the reaction mixture was first analyzed by GC/MS, which indicated the presence of indole, tryptophol, tryptamine and norharman. These compounds were subsequently shown by HPLC to be present in the mixture. Figure 6 shows the HPLC separation of the assay reaction mixture before and after spiking the mixture with the GC/MS-identified compounds and monitored by UV detector set at 220 nm. The identities of the peaks were also confirmed by comparison of their scanned spectra with that of the authentic standards. Similarly, the presence of unreacted tryptophan and its Amadori product in the mixture was also confirmed.

The purities of the peaks were confirmed by real-time purity check algorithm of the resident software. This procedure, for example, indicated the presence of an impurity as a shoulder of the first peak in the chromatogram shown in Fig. 6. The impurity was subsequently shown to be indole (peak a in Fig. 6). Figure 7 shows the time-dependent chromatograms of a morpholine/glucose mixture heated for 3 h; only diode array detection at 190 nm is depicted. This mode of detection indicates a clear build-up over time of a UV-active compound at about 3 min and that another compound (at t_R = 6 min) is formed after 0·5 h of heating, which then starts to degrade. Figure 8 represents an injection of a sample of the same assay reaction mixture taken at 3·5 h as seen by the DA at 190 nm and by the PAD (E_1 = 0·100 V).

It is possible to follow, not only the reduction in time of glucose and morpholine concentrations, but also the formation of the Amadori-morpholine and other intermediates during a single injection. This illustrates that early phase kinetics of the Maillard reaction can be investigated by the present HPLC system in great details. It is interesting to note the formation of fructose in the reaction mixture of morpholine/glucose. Fructose can be formed either from glucose through a Lobry de Bruyn Alberda van Ekenstein transformation or from the Amadori product.

CONCLUSION

HPLC separation and identification of the main components of the Maillard reaction in the early phase and profiling of the components formed during the advanced phase is facilitated by employing a multidetector system. The advantages of such a system include (1) structural identification of the components can be facilitated by categorizing them into UV-, VIS-, FL- and redox-active compounds; (2) the scanning ability of diode array and fluorescence detectors can be used to

compare the UV, VIS and fluorescence spectra to those of known standards for identification purposes; (3) the increased sensitivity for certain compounds in the EC/PAD and fluorescence modes of detection compared to UV or RI detection allows direct analysis of non-derivatized substrates at low concentrations with high signal-to-noise ratios. This is especially important, since many of the products formed in the Maillard systems are in low concentration; (4) alcohols, glycols, reducing and non-reducing carbohydrates, amino acids, amines, sulfur compounds, aldehydes and ketones can be detected by EC/PAD; (5) the compatibility of the system with gradient elution is clear and the flexibility of using different columns other than ion-exchange based columns to separate Maillard reaction mixtures.

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