

Real Time Monitoring the Maillard Reaction Intermediates by HPLC- FTIR

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Abstract

Maillard reaction products in the food-, nutrition- and pharmaceutical related processes are of great interest in cases where the substances involved are chemically reactive or unstable. Detailed data using vibrational FTIR spectroscopy of the Maillard-type reaction products are limited and needs more experimental evidences with explicit mechanisms of the reaction to demonstrate how the reducing sugars be avoided in formulating the amine-containing substances. The combination of high performance liquid chromatography (HPLC) with Fourier transform infrared (FTIR) spectrometry is a powerful instrumental separation-structure sensitive technique that allows characterization in real time of the separated chemical species. In this short review we demonstrate the benefits of the HPLC-FTIR coupling technique in studying the Maillard reaction model Fructose/Asparagine system.

Keywords: Maillard reaction; Asparagine; Fructose; HPLC- ATR-FTIR

Introduction

The Maillard reaction involves three different stages in which complex reactions take place between reducing sugars and free amino groups of amino acids or peptides. The initial stage involves the formation of the Schiff base and the formation of the Amadori products without the formation of browning compounds. The second stage involves the dehydration of sugar and amino acid degradation. In the final stage Melanoidines are the final heterocyclic nitrogen compounds which are actually nitrogen containing polymeric substances which are difficult to decompose (Figure 1). The formation of acrylamide follows a number of possible routes in the frame of Maillard reactions in food products, in which the asparagine route is the major one. Acrylamide has been shown to originate from the Maillard reaction of the amino acid asparagine with reducing sugars as well as carbonylic compounds deriving from either the Maillard reaction or lipid oxidation processes [1-5] during heating. However, it was demonstrated that the asparagine pathway is the most predominant for the formation of acrylamide through isotope-labelling experiments [1]. This was further supported by model studies showing that the N-glycosyl of asparagine generated twenty times more acrylamide, compared to α -dicarbonyls and the Amadori compound of asparagine concluding that α -hydroxy carbonyl compounds, such as fructose and glucose are more effective than other carbonylic compounds in generating acrylamide [3]. Asparagine alone may form acrylamide through decarboxylation and deamination but the reaction is inefficient giving extremely low yields. Nevertheless, asparagine in the presence of the hydroxycarbonyl moiety of reducing sugars can produce acrylamide in the range of 1 mol percent in model systems [3].

There are only few studies utilizing the monitoring ability of the FTIR technique to elucidate the reaction network in the acrylamide formation pathway [6,7]. The decarboxylated Schiff base (azomethine ylide) and decarboxylated Amadori product are both key intermediates contributing to the formation of acrylamide (Figure 1). Their relative importance was determined when both of the intermediates were synthesized and their relative abilities to generate acrylamide under dry and wet heating conditions were investigated [6]. One of these intermediates is oxazolidin-5-on which was recently monitored by FTIR in a dry model reaction system [7]. Imines formed subsequent to carbonyl-amine reactions were characterized by FTIR spectroscopy. Recently, a DSC-FTIR microspectroscopy method was applied to detect

continuous pathways in the solid state asparagine/glucose reaction [8]. In this work, we demonstrate the feasibility of applying the coupled HPLC-FTIR separation-structure sensitive technique to Maillard-type reaction of the fructose-Asparagine products in real time.

Experimental

Chemicals

Acrylamide (>99.5%) was purchased from Panreac Quimica SA (Barcelona, Spain). L-Asparagine monohydrate (>99%) and fructose (>99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was milli-Q grade water (Millipore, Ireland). Acrylamide stock solutions and calibration standards were prepared in water and kept at -20°C until use. All samples were filtered with disposable syringe filters (45- μ m pore size, PTFE, Millex, Millipore, Ireland) prior to analysis.

Sample preparation

Equimolar solutions of fructose and asparagine (0.2 M) were prepared in phosphate buffer (0.1 M) and the pH was adjusted to the desired value (pH 8). Samples (10 ml) were heated in closed screw-capped tubes at 100, 120, and 140°C in a heating oven (Mettler, Germany). At predetermined heating times (30, 60, 90 and 120 mins), samples were taken and immediately cooled on ice and stored at -20°C prior to analysis.

Experimental setup

HPLC: Maillard reaction product (MPRs) formation was monitored on a Shimadzu Prominence 20A HPLC system consisting of a quaternary pump with online degasser, a temperature-controlled column oven and a UV-SPD detector. A 1 μ l aliquot of sample was injected for LC separation whilst a 100 μ l aliquot of the same sample

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was injected to acquire the FTIR measurements. HPLC analysis was performed on a 4.6×250 mm, $5 \mu\text{m}$ particle size, Zorbax SB-Aq analytical column (Agilent Technologies). Water at a flow rate of 0.5 ml/min was used isocratically as the mobile phase at room temperature. Maillard reaction products (MPRs) and acrylamide were detected at 200 nm.

FTIR: A Bruker Tensor 27 FTIR spectrometer equipped with a triglycine sulfate (TGS) detector was used for spectra acquisition (Figure 1). For controlling the spectrometer the software package OPUS 7.0/IR/3D (Bruker) was used. The OPUS CHROM Version 7 (Bruker) software package was used to acquire time-dependent spectra in the coupled HPLC-FTIR process. The result of the measurement performed with the OPUS CHROM package was a number of spectra taken at defined, constant time intervals. During the measurement, the acquired spectra and generated traces were displayed in real time. A horizontal ATR (HATR) accessory (Pike Technologies, Inc., Madison, USA) was employed fitting a Germanium flow-through liquid ATR cell with ten internal reflections. This enabled the pumping through of solutions over the ATR Germanium surface with minimal dead volume. The Horizontal ATR is designed with a pair of transfer optics to direct the infrared beam of the spectrometer to one end of the IR transmitting ATR crystal. A similar pair of optics directs the beam emitted from the other end of the ATR crystal to the spectrometer detector. The Germanium crystal is trapezoid shape with a thickness of 4 mm (Figure 1). This feature maximizes the signal to noise of the resulting spectra. Spectra were collected in the $900\text{--}1800\text{ cm}^{-1}$ range with 8 cm^{-1} resolution and 16 co-added scans each (apodisation function: Blackman-Harris-3-term). A time resolution of 8 spectra per minute was achieved. The scanner velocity was 20 kHz (HeNe frequency). A background spectrum was collected before each sample measurement with only water flowing through the ATR cell. This was used to calculate the absorption spectra during the CHROM measurement. The single pure

substances were injected and a chromatographic run was performed. These were used as reference spectra for the identification asparagine and sugars. The intensity changes of the spectra as a function of time were stored as traces in a 3D file for all FTIR measurements. Data extraction and processing was carried out using the OPUS 7.0/IR/3D (Bruker) software. The spectra were extracted from the 3D data set of the chromatographic peak maximum and were graphically plotted using Origin Pro v8.0 software.

Results and Discussion

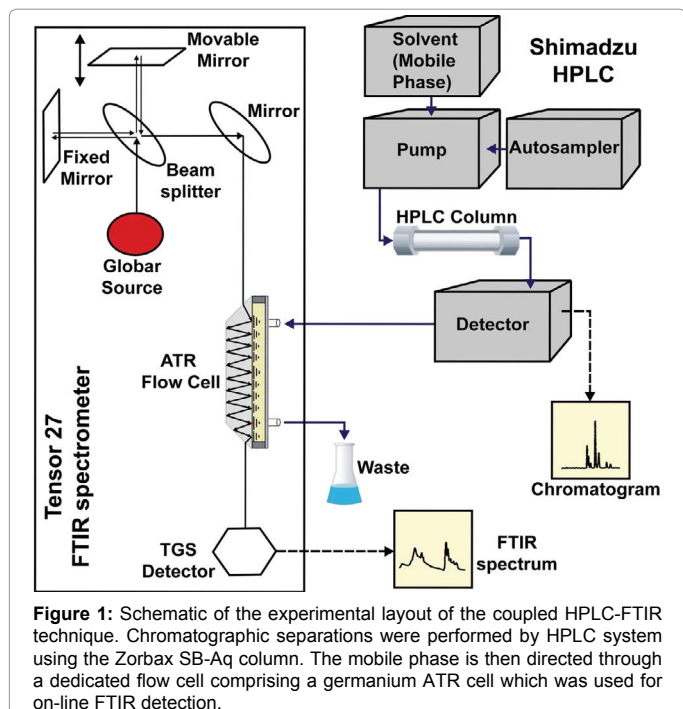
A new analytical setup allowing the concurrent identification of precursors and products of the Maillard reaction is described. It is based on high-performance liquid chromatography (HPLC) with UV-vis detector and a FTIR-ATR flow cell coupled in series. Separation and qualitative determination of individual Maillard species have been achieved using various chromatographic techniques including GC-MS and High-Performance Anion Exchange Chromatography (HPAEC) [9]. The main advantage of HPLC over GC in the MR analysis context is that non-volatile water-soluble compounds can be analyzed directly without the need for prior derivatization. However, high-performance liquid chromatography (HPLC) on its own has limited ability to adequately resolve and give structure sensitive information when analyzing Maillard reaction compounds. The collected fractions were then analyzed by the ATR-FTIR technique.

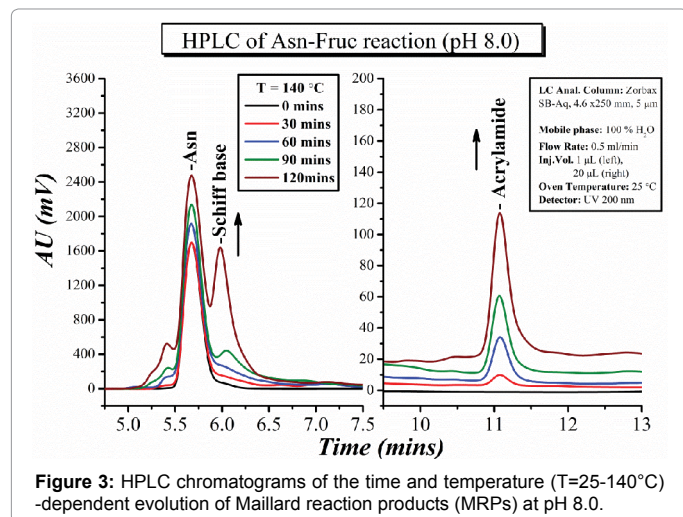
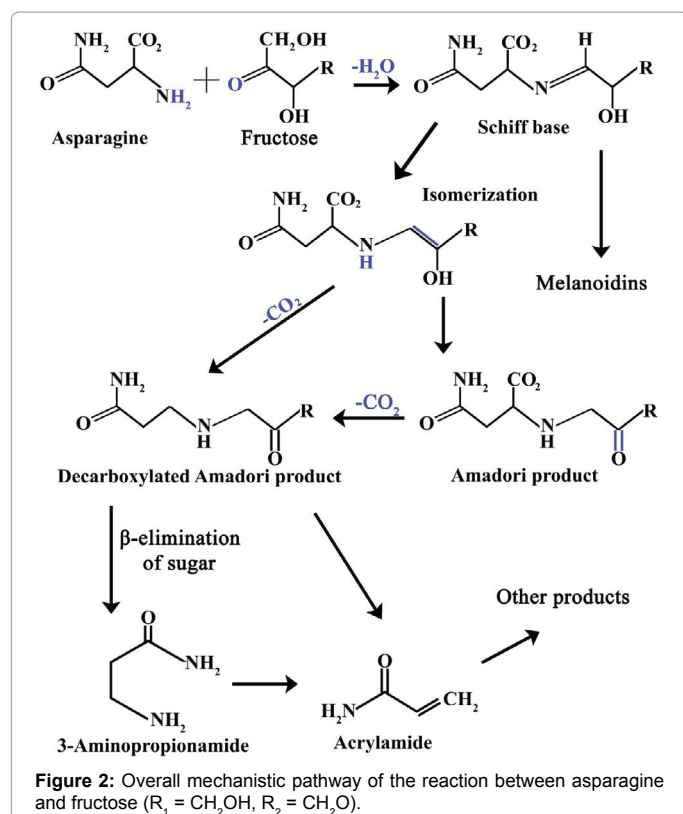
Glycoconjugates, such as N-glycosides and related compounds formed in the early phase of the Maillard reaction have been proposed as key intermediates leading to acrylamide formation [10]. This hypothesis was supported by the work of Yaylayan et al. and Zyzak et al. [1,11]. Both groups provided evidence of the importance of Schiff base of asparagine which is formed after a dehydration step. The Schiff base is formed early in the Maillard reaction as the result of elimination of water from the conjugate of glucose and asparagine [1,3].

The next step is the transformation of the Schiff base intermediate to an Amadori product through an Amadori rearrangement. Following this, there is a decarboxylation reaction of the Amadori product to the decarboxylated Amadori product. It is therefore considered that the key mechanistic step is the decarboxylation of the Schiff base leading to other intermediates that can directly release acrylamide (Figure 2). With respect to structure, asparagine alone is in theory capable of being converted thermally to acrylamide through decarboxylation and deamination reactions, however experimentally this was not the case with maleimide being the main reaction product [11].

When asparagine and fructose react at high temperatures it is evident mainly from the HPLC data that there is a mixture of reactants and multiple products in the reaction vessel (Figure 3). Time and temperature-dependent evolution of Maillard reaction products (MPRs), including that of acrylamide were monitored on the Zorbax column which is highly selective for polar molecules in aqueous phases. Despite the fact that there was only partial separation of the two reactants we notice that at 140°C there is degradation of asparagine and fructose as we can observe from the absorption peaks at Reaction time (Rt) 5.4 and Rt 6.0 mins respectively (Figure 3). It is also apparent that the peak at Rt 5.7 mins corresponding to asparagine increases with time at 140°C (Figure 3). In the FTIR spectra, the spectral range between 1200 and 1800 cm^{-1} is shown where the majority of the asparagine FTIR absorptions are found [12-30].

In Figure 4a-c we show the coupled HPLC-FTIR spectra at room temperature (Figure 4a) and $T=140^\circ\text{C}$ (Figure 4b), in the Rt 5.5-6.5

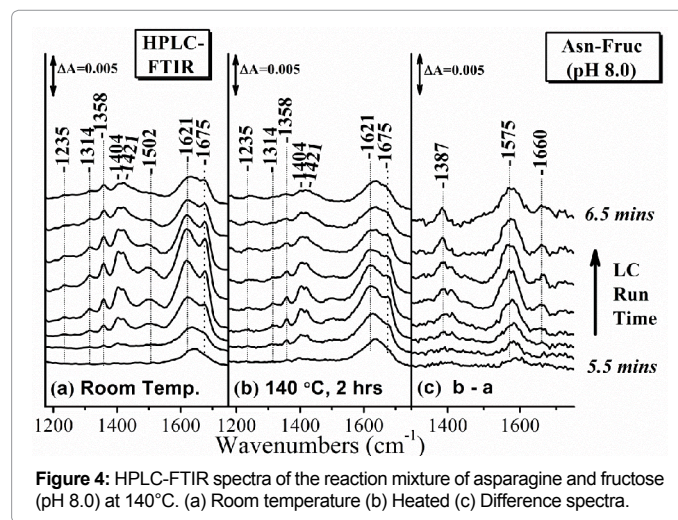




min and 1200-1700 cm^{-1} spectral region. All traces in Figure 4a are the unheated, room temperature reactants. All traces in Figure 4b are those of the reaction products at $T=140^\circ\text{C}$. All traces in Figure 4c are the difference spectra of the heated asparagine-fructose reaction product in the $R_t=5.5\text{-}6.5$ min range at pH 8 and $T=140^\circ\text{C}$ (Figure 4b) minus the unheated spectra shown in Figure 4a. The spectra in Figure 4b reveals intensity changes in the region of the $\text{C}=\text{O}$ band of Asn at 1675 cm^{-1} as well large broadening in the region of the Asn $\delta(\text{NH}_2)$ at 1621 cm^{-1} . However, from the absolute FTIR data due to the overlapping vibrations with the unreacted species and since in the newly formed species the $\nu(\text{C}=\text{O})$ and $\delta(\text{NH}_2)$ are expected to retain similar frequencies with those of the unreacted, it is not possible to assign the vibrations of the new species with certainty. The FTIR difference spectra in Figure

4c reveal features at 1660, 1575, and 1387 cm^{-1} . Based on our HPLC data at $R_t=5.5\text{-}6.5$ we expect that the FTIR spectra have contributions from the formation of the Schiff base Imine group, the enaminol group stretching frequency and/or from the Amadori and decarboxylated Amadori products with distinct $\text{C}=\text{O}$ vibrations in the 1700 cm^{-1} region. For the Schiff base, the carbonyl ($\text{C}=\text{O}$) and NH_3 groups have very similar frequency with that of the unreacted Asn and thus they are spectators in the reaction without any contributions to the bands observed in the difference spectra. Imines under Maillard reactions have been characterized, and as the first step in the carbonyl-amine interaction have been proposed to play a crucial role in the formation of the Schiff base and degradation of the Amadori products towards the formation of the 3-Aminopropionamide and Acrylamide (Figure 1). The main feature in the FTIR spectrum of the imine (Schiff base) species is the vibration of $\nu(\text{-C}=\text{N})$ at 1660 cm^{-1} . Given that the 1660 cm^{-1} mode originates from the Schiff base we assign the other vibrations at 1569 cm^{-1} to HN-C and that at 1388 cm^{-1} to $\nu(\text{C}_{\text{Fru}}\text{-N}=\text{C}_{\text{Asn}})$. We have excluded the possibility that the species we have observed originate from either the Amadori or the decarboxylated Amadori product due the lack of a $\text{C}=\text{O}$ vibration in the 1700 cm^{-1} region. However, we can't exclude the possibility that the species we have observed originate from the isomerization of the Schiff base only, forming the enaminol moiety $\text{C}_{\text{Fru}}\text{-NH}=\text{CH}_{\text{Asn}}$ (1660 cm^{-1}). In this case, the 1589 and 1388 cm^{-1} modes originate from the N-H and C-N stretching vibrations of the enaminol moiety $\text{C}_{\text{Fru}}\text{-NH}=\text{CH}_{\text{Asn}}$, respectively. It should be noted that the concentration of the imine intermediate is increased at elevated temperatures and under alkaline conditions without being converted to an Amadori product. Our data find also support from the results of the model systems where it was shown that imines are converted to Amadori products only under strong acidic conditions and raising temperatures [12]. This suggests that the initial deprotonated amine attack is favored by a protonated form of the imine in order to proceed to the Amadori products and beyond. Another feature that is clearly evident is the loss of the 1502 cm^{-1} asparagine band corresponding to the backbone amino and/or carboxylate group. This further supports the hypothesis that there is loss of the backbone amino group and successive decarboxylation reaction occurring.

The new approach of the HPLC-FTIR application to the Maillard reaction has applications to other similar chemical reaction in food, nutrition and pharmaceutical industries without the need to isolate the species and characterize them separately. This is the major



advantage of this coupling technique. It can be concluded that the real time monitoring of the imine bonds it is reported for the first time in the Fructose-Asparagine system under physiological conditions. In addition, the formation of Acrylamide at T=140°C raises major concerns about the safety in using fructose with asparagine in such temperatures. Although the data in our manuscript only covered qualitative analysis a more detailed quantitative analysis in terms of monitoring the conversion percentage is necessary. These experiments are in progress in our lab [31-41].

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