ISOLATION AND IDENTIFICATION OF NON-VOLATILE WATER SOLUBLE MAILLARD REACTION PRODUCTS

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Short Title:

Water soluble polymers from Maillard Reaction.

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ABSTRACT

A water-methanol solution of glycine and D-glucose was refluxed for seven hr. The solvent was evaporated under vacuum at room temperature and the residue was dialyzed against distilled water. After dialysis, the solvent was evaporated under vacuum at room temperature. The non-dialyzable fraction was further fractionated by gel filtration. The process yielded three polymeric materials ($10,000 < M < 20,000$) whose purity was verified by HPLC. The isolated polymers were further analyzed by UV-VIS and FTIR spectroscopy and by pyrolysis/GC/MS. Elemental analysis indicated that polymer A has the following empirical formula $C_6H_{11}N_1O_4$ and polymers B1 and B2 have the same empirical formula as glucose $C_1H_2O_1$. The origin of nitrogen containing polymer A was assigned to Amadori intermediate or to some of its derivatives and the origin of polymers B1 and B2 was assigned to Glucosone and to 3- or 1-deoxyglucosones; common non-nitrogen containing reactive intermediates during Maillard reaction. Plausible mechanisms were proposed for the formation of polymers.

RÉSUMÉ

Une solution d'eau-méthanol de glycine et de D-glucose a été sous reflux pendant sept heures. Le solvant a été évaporé sous vide à température ambiante et le résidu a été dialysé contre l'eau distillée. Après dialyse, le solvant a été évaporé sous vide à température ambiante de nouveau. La fraction non dialysée a été par la suite fractionnée par filtration de gel. Ce procédé a produit trois matériaux polymériques ($10,000 < M < 20,000$) dont la pureté a été vérifiée par CLHP. Les polymères isolés ont été analysés par la suite par spectroscopie UV-VIS et spectroscopie infrarouge transformée de Fourier et par pyrolyse/CG/SM. L'analyse élémentaire indiquait que le polymère A avait la formule empirique suivante: $C_6H_{11}N_1O_4$ et les polymères B1 et B2 avaient la même formule empirique que le glucose: $C_1H_2O_1$. L'origine du polymère A contenant de l'azote a été attribuée à l'intermédiaire Amadori ou à ses dérivés et celle des polymères B1 et B2 est associée au glucosone ainsi qu'au 3- ou 1- deoxyglucosones; ces derniers sont des intermédiaires de réaction, qui ne contiennent pas d'azote, lors de la réaction de Maillard. Des mécanismes plausibles ont été proposés pour la formation des polymères.

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1. INTRODUCTION

Scientific studies covering flavor and browning phenomena was initiated by Louis-Camile Maillard, 1912. In a misguided attempt to determine the biological synthesis of proteins, he heated a concentrated solution of D-glucose and amino acid mixture then observed a gradual darkening in color, the development of odors reminiscent of baking bread or the roasting of food products. This work attracted sufficient attention to persuade many others to continue the study of what came to be called the Maillard reaction. Maillard reaction can be involved in the processing of food in at least three different ways (Danehy and Wolnak, 1983). First, there is the unconscious role played in the development of flavor in such traditional processes as the roasting of coffee and cacao beans, the baking of breads and cakes, and the cooking of meats. Second, there is a deliberate use of Maillard technology in the production of artificial flavors and/or engineered foods. Third, there are the efforts to inhibit undesirable results of Maillard reaction in food processing. Reduction of the nutritional value of processed food may also occur from the loss of essential amino acids (e.g. lysine) and other nutrients such as ascorbic acid. In addition, there is the possibility of toxic components (e. g. imidazoles) being formed. Most research is directed towards isolation and identification of volatile aromatic compounds. Very little is known about non volatile polymeric compounds generated during Maillard reaction. The objective of this research addresses the lack of information on chemical structures of the Maillard polymers by proposing to isolate and identify such components.

1.1. OBJECTIVES

The objectives of this research are to:

1) Produce polymers (melanoidins) using glucose-glycine model system

2) Isolate pure Maillard polymers through dialysis, gel filtration chromatography and HPLC

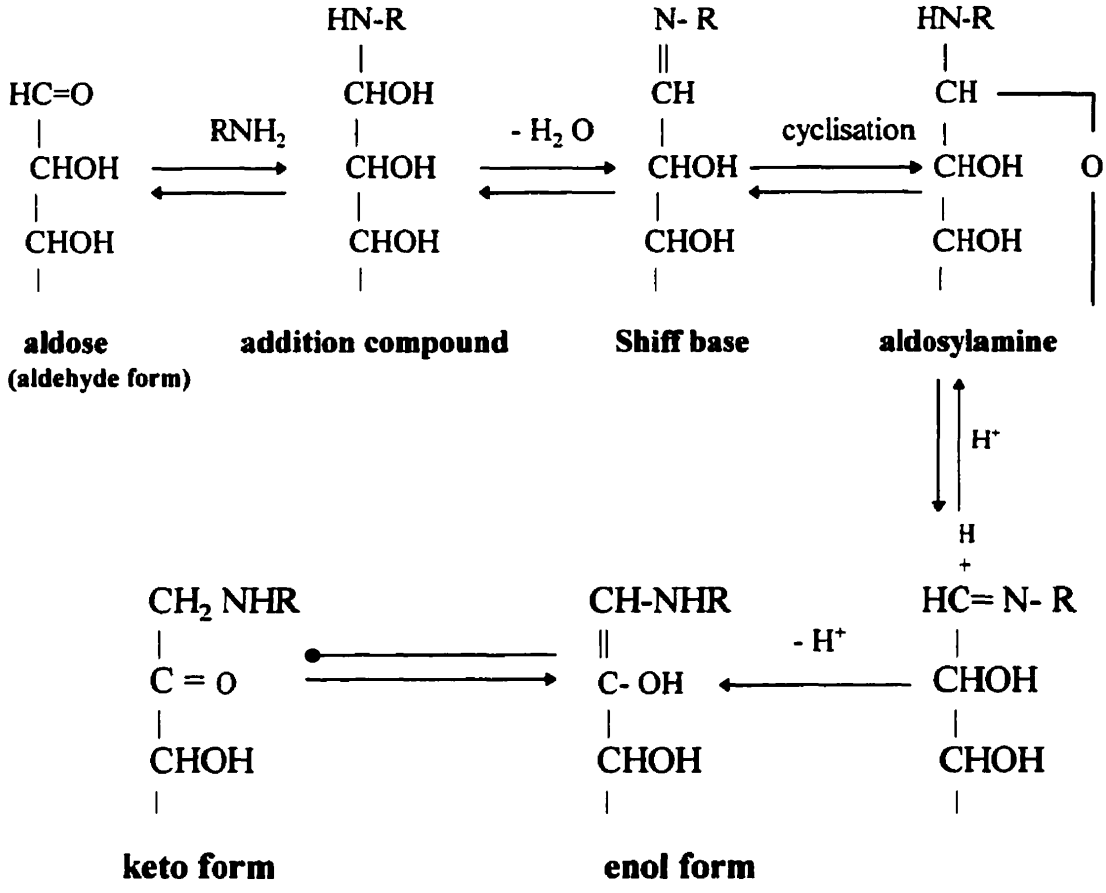
3) Characterize and propose hypothetical partial structures for each polymer.

2. LITERATURE REVIEW

2.1. CHEMISTRY OF BROWNING IN MODEL SYSTEM

The formation of brown colors is a common occurrence in organic chemistry, and particularly in carbohydrate chemistry. Deterioration as a result of the chemical reaction in nonenzymatic browning is especially serious in dried and concentrated foods (Lithebody and Fevold, 1948; Ross, 1948). Undesirable changes in color and flavor together with lose of nutritive value of food can cause considerable waste. However, desirable browning reactions effects, such as the production of flavors and colors characteristic of roasted foods, have an immense effect on the palatability and consequent choice of foodstuffs (Reynolds, 1959).

The first step of the browning reaction is the formation of a N-aldosylamine (Ellis and Honeyman, 1955). Hodge's classical mechanism (Scheme 2.1) proposes that this involves an addition reaction between the carbonyl group of the open chain form of an aldose and the amino group of an amino acid, peptide, or other compound with a primary amino group (Hodge, 1955). It is convenient to view the reaction through the open chain form of the sugar which is the most reactive form. Although some authors suggest that the cyclic pyranose or furanose conformation of the sugar is more likely to be involved since it is the most abundant form of sugars in aqueous solution (Potman and Van Wijk, 1989). The subsequent elimination of water and molecular rearrangement gives a 1-amino -1- deoxy-2-ketose (Amadori compound).



N-substituted
1-amino-1-deoxy-2- ketose
Amadori Compound

Scheme 2.1 Formation of Amadori compound in the initial stages of the Maillard Reaction .

Amadori intermediates themselves do not have any characteristic flavor, however, they are important precursors of flavor compounds. They are thermally unstable and undergo dehydratation and deamination reactions to give furfurals, reductones, pyran derivatives, cyclopentane derivatives, and unique compounds (Fargeson, 1969). Some of these

compounds are further intermediates, while others are end products that contribute to the aroma, flavor as well as the color in thermally processed foods (Figure 2.1).

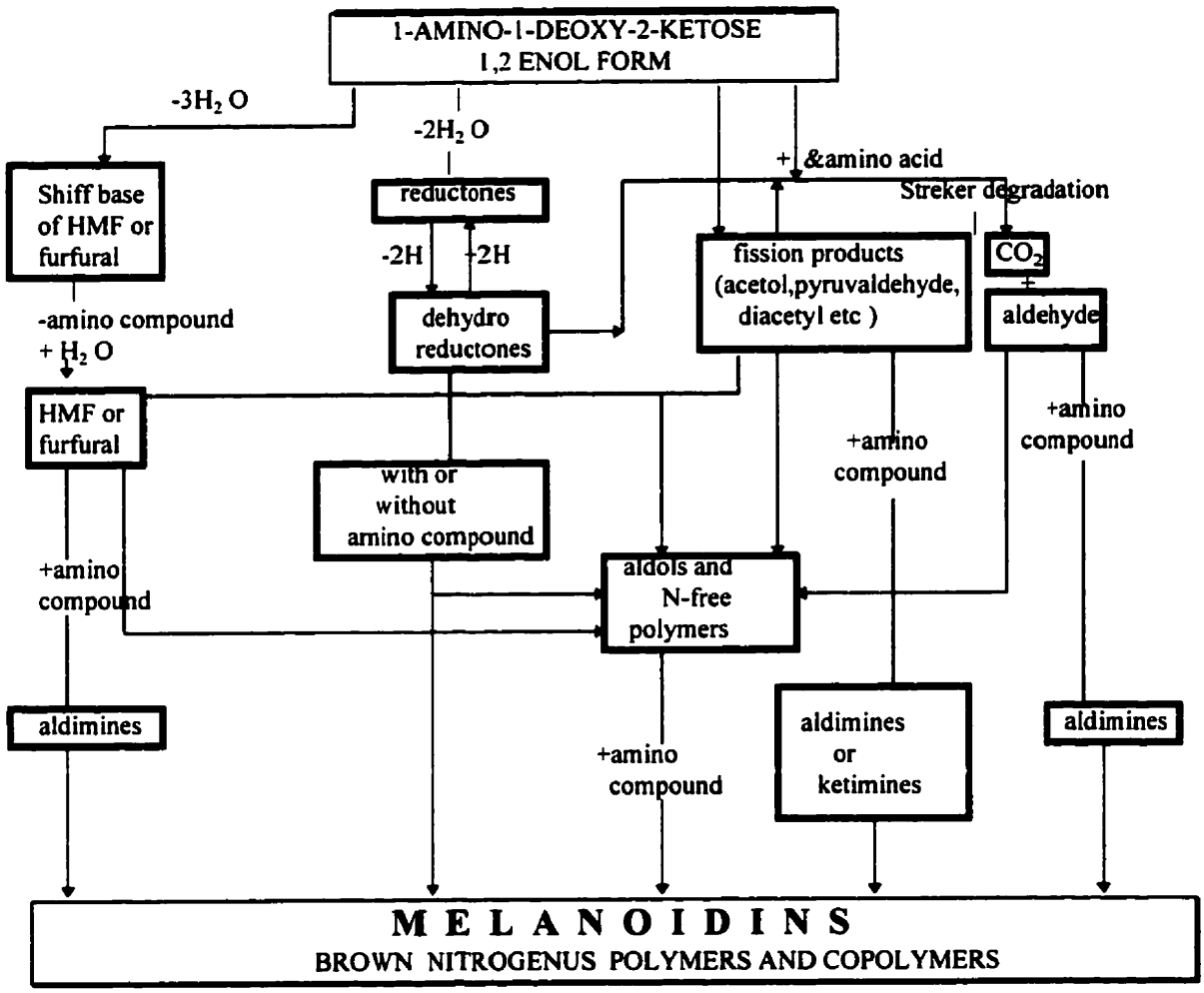


Figure 2.1 Hodge's view of the pathways by which browning products are formed .

(Hodge, 1953)

In the final stage of browning, the intermediates polymerize to form unsaturated, fluorescent and colored polymers. The chief reactions involved are thought to be aldol condensation, aldehyde-amine polymerization, and the formation of heterocyclic nitrogen compounds, such as pyrroles, imidazoles, pyridines, and pyrazines (Hodge, 1953).

2.2. HIGH MOLECULAR WEIGHT BROWNING COMPOUNDS (*melanoidins*)

One of the least understood aspects of the Maillard reaction is the formation of brown polymers characteristic of heated foods. The nature and the mechanism of the formation of these polymers are still unknown (Yayaylan and Huyghues-Despointes, 1994). Spectroscopic studies of the brown compounds in the visible and ultraviolet regions have shown that addition and substitution reactions play a more important role than condensation reactions (Ledl and Schleicher, 1990).

Previous studies have already clarified a number of intermediates involved in the mechanism for the production of melanoidin during the amino-carbonyl reaction (Reynolds, 1963). However, little is known about the chemical characteristic or chemical structure of the melanoidins. In general, melanoidins are similar to humic substances and melanins in their chemical properties (Shalygin, 1941). These polymerized substances are random in structure, highly dispersive in molecular weight and moderate in absorption spectrum (Gomyo et al., 1972). Hence, there are great difficulties in the investigation of the structural features of these substances in detail. Some reports are available with respect to the chemical structure of melanoidins prepared from model

systems consisting of amino compounds and hexoses or pentoses (Kato and Tsuchida, 1981). However, these are limited to descriptions of elemental composition, some functional groups and general properties of melanoidins.

2.2.1. Chemical studies and characterization of melanoidins.

Many investigators have observed the formation of strongly reducing substances, *reductones*, in the Maillard reaction (Figure 2.1). Reductones are known to be the most reactive intermediates during polymer formation in model systems. Kato and Nogushi (1968) prepared eight melanoidin samples by heating an aqueous or a methanolic solution of D-glucose or D-xylose with glycine, ammonium hydroxide, or n-butylamine, followed by dialysis and freeze-drying. They found that the reductone content of the reaction increased along with heating time. This fact indicates that the greater part of the reductones and melanoidins would be formed in the later stage of the Maillard reaction as final products. It could be considered then, that some parts of the reductone are incorporated into the melanoidin structure. Melanoidin pigment, as a nondiffusable fraction from this reaction, is a highly reactive substance, and is especially unstable on heating in aqueous media (Gomyo et al., 1972). They concluded that both colorization and decolorization occur simultaneously during the browning reaction and in the preparation of melanoidins in model systems. It is evident from his work that melanoidins have ambivalent reactivity, and that depolymerization occurs on heat treatment. The factors influencing those changes are summarized in Figure 2.2

DEPOLYMERIZATION

(Discolorization)

O_2 (essential)

Higher pH

Higher temperature

POLYMERIZATION

(Colorization)

Absence of O_2

Lower pH

Higher temperature

Presence of transitional metals

Figure 2.2 The ambivalent reactivity of melanoidin and favorable factors (Adapted from Gomoyo et al., 1972)

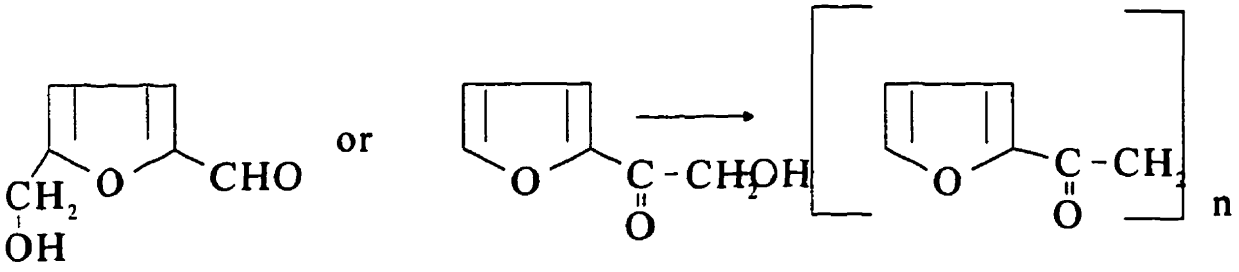
Hayase et al., (1984) treated melanoidin with hydrogen peroxide to investigate the decolorization and subsequent decomposition reactions. Melanoidins were prepared by refluxing in an oil bath (95° C for 7 hours) mixtures of D-glucose (1 mol) and glycine (1 mol) in deionized water. The degree of decolorization was predominantly influenced by the temperature increase at neutral pH. However, the differences were smaller under alkaline conditions if the temperature was increased. The decolorization degree was slightly changed by addition of hydrogen peroxide over 6.72 %. Accordingly, the optimal concentration of hydrogen peroxide was determined to be 6.72%. After all, the most important factor influencing the decolorization was found to be the pH range in which alkaline side was the leader (decolorization reached 94 % at pH 10).

The major components in ether-soluble fractions, obtained from melanoidin oxidation by alkaline hydrogen peroxide, were identified as 2-metyl-2,4-pentanediol, N,N-dimethylacetamide, phenol, acetic acid, oxalic acid, methylpropanedioic acid, propanedioic acid, 2-furanocarboxylic acid, butanedioic acid, 2-hydroxypropanoic acid, 2,5-furanodicarboxylic acid, and 5-hydroxymethyl-2-furanocarboxylic acid. Furthermore, glycine was the major degradation product found in the aqueous fraction after hydrogen peroxide treatment of the glycine-glucose melanoidin model system (Hayase et al., 1984). As final result of their investigation, partial chemical structure of the melanoidin can be proposed as follows:

CH_3.CO-R	COMPOUND I
CH_3-C(H or OH)=C(H or OH)-CO-R	COMPOUND II
R-CO-CO-R	COMPOUND III
R-CO-CH(CH_3)-CO-R	COMPOUND IV
R-CO-CH_2-CO-R	COMPOUND V
R-CO-CH_2-CH_2-CO-R	COMPOUND VI
CH_3-CH(OH)-CO-R	COMPOUND VII

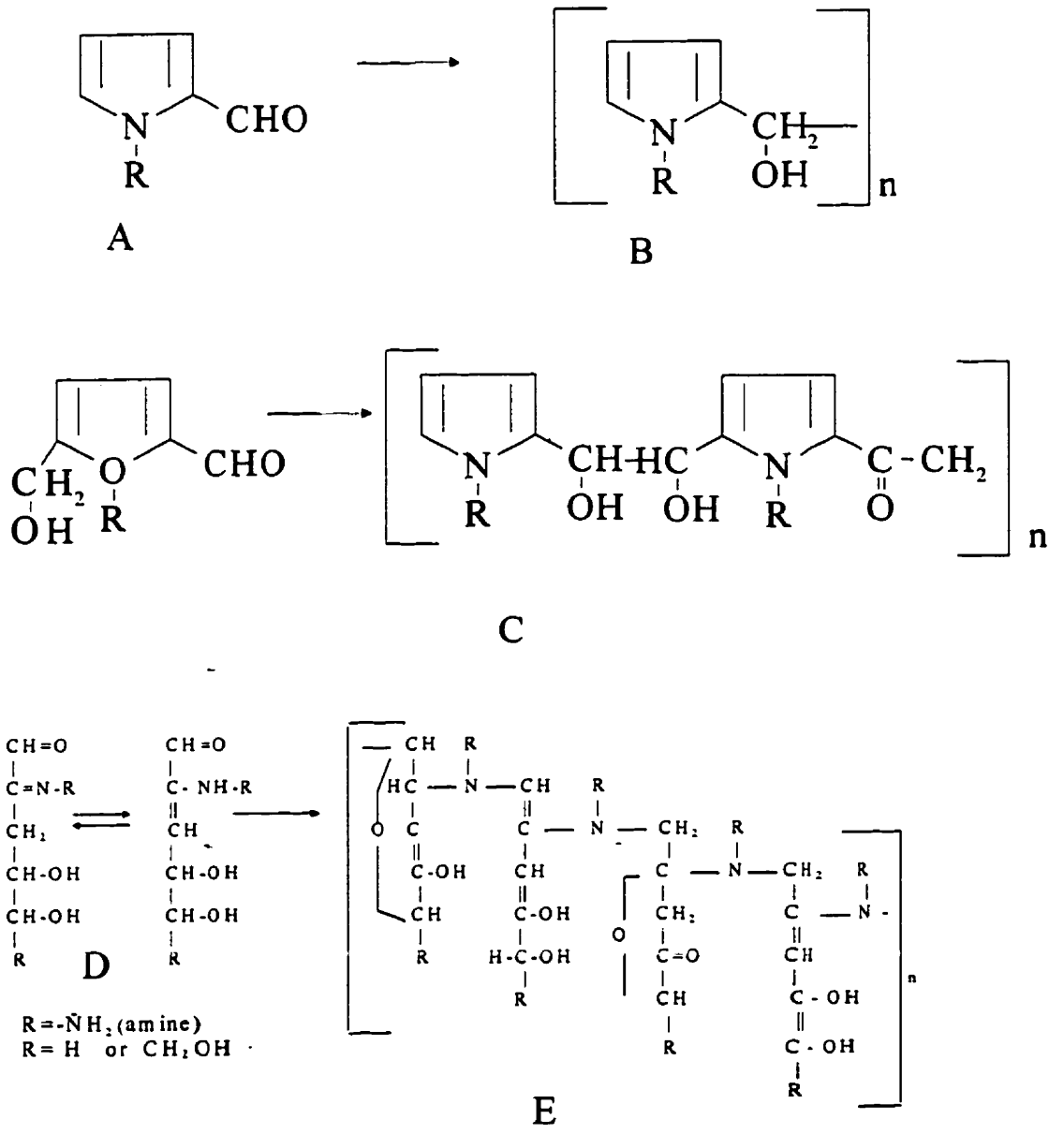
One of the most important observations of this study was the estimation of acetic acid as a major product from the acidic fraction, and compound I as the most common fragment in melanoidin polymers (Hayase et al., 1984).

Furan ring moiety (Scheme 2.2) was postulated as the repeating unit of brown polymers produced from sorbose/glycine under strongly acidic conditions by Heyns and Hauber (1970).



Scheme 2.2 Repeating units of furan rings according to (Heyns and Hauber, 1970)

Other workers carried out experiments using aldose-butylamine under near neutral conditions, and N-substituted pyrrole-2-aldehyde was isolated as one of the major reaction products (Kato, 1967; Kato and Fujimaki, 1970). Results from the other studies of melanoidin polymers (Kato and Tsuchida, 1981) propose Shiff's base or its enamine (D in Scheme 2.3) as one of the repeating units of the latter. They produced polymers from D-glucose, D-xylose with butylamine and ammonia under three different condition (using water or methanolic solution as solvent, acetic acid as catalyst and temperature of 50°C or 100°C). Polymeric products were purified by dialysis followed by ether extraction prior to pyrolysis and oxidative degradation with limited amount of permanganate.



Scheme 2.3. Possible repeating units of polymeric brown products and their precursors (Kato and Tsuchida, 1981).

Although alkylpyrroles (Scheme 2.3 **B** and **C**) were formed by pyrolysis of aldose-butylamine melanoidins, pyrrole carboxylic acids were not formed as major byproducts of permanganate oxidation. These facts suggest that pyrroles are not present in any significant amount in the melanoidin skeleton but are formed during the course of pyrolysis. Shiff's base of 3-deoxyosulose (**D** in Scheme 2.3) or its enamine, which has been suggested as an intermediate of pyrrole-2-aldehyde formation (Kato et al., 1968), is assumed to polymerize to form **E** trough an aldol-type condensation. The monomeric units shown in **E**, are more probable structures than aromatized ring forms. Nevertheless, it is possible that some aromatic rings might be formed under more drastic condition (Kato and Tsuchida, 1981).

Cämmerer and Kroh (1994) found that proposed structures by Kato and Tsuchida (1981) can not have general validity due to the great difficulties involved in structure determination of the polymers. They further suggest that it can not be assumed that melanoidins have a regular composition with repeating units. They proposed a new structure based on the observation of the effect of different reaction condition on preparation of polymers (see Figure 2.3).

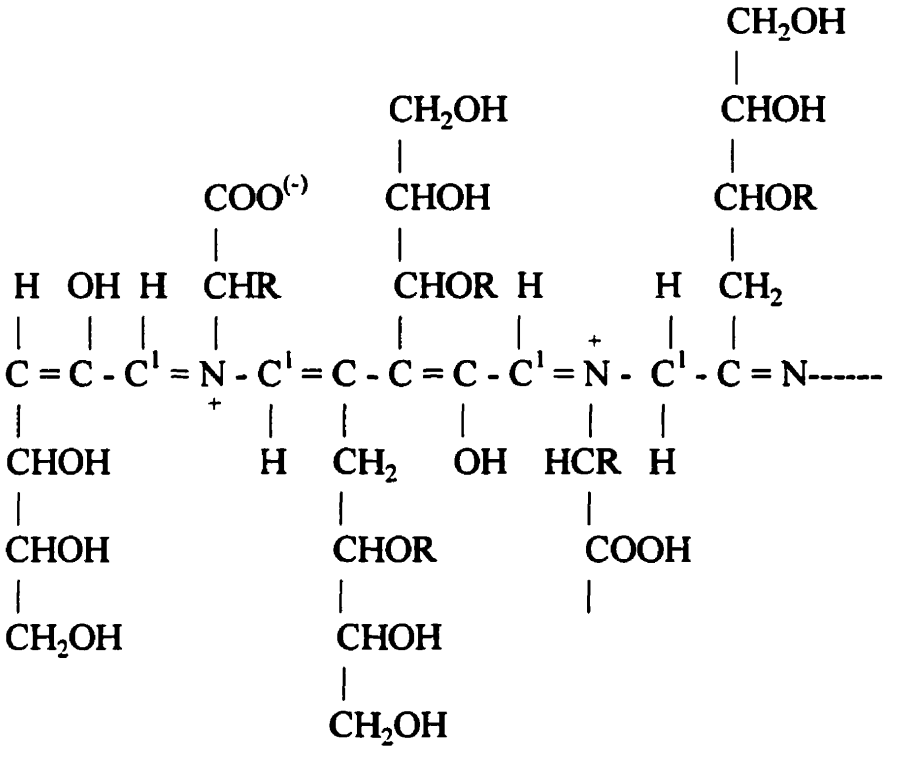
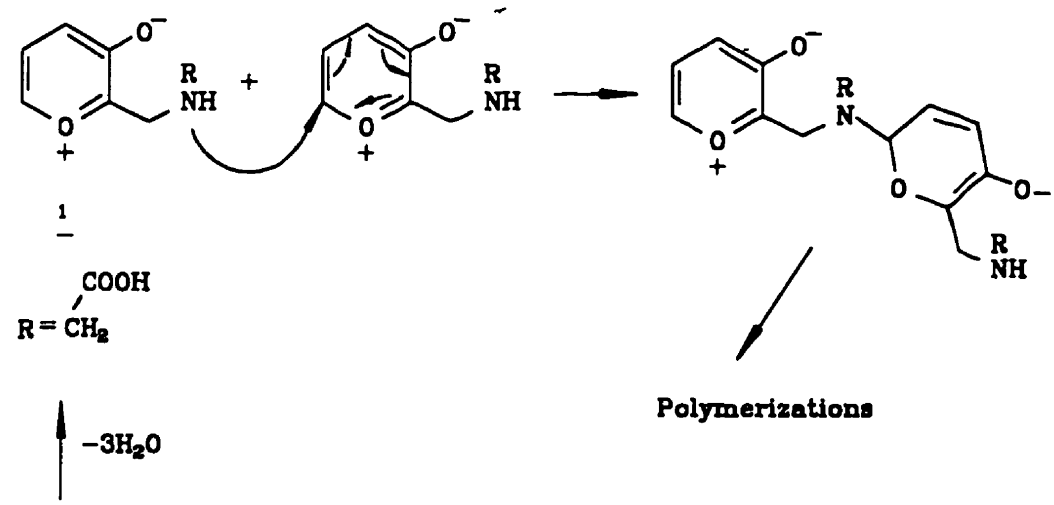


Figure 2.3 Proposed chemical structure of the investigated melanoidins from (glucose/glycine) (Cämmerer and Kroh, 1994)

Evidence was provided for the suggested structure by IR and UV spectral interpretation and by CP-MAS NMR studies. Results suggest that -N=C- or N=C is incorporated into the polymer as the main structural element.

Based on studies of glucose-glycine reaction, Yaylayan and Lachambre (1990) proposed the formation of pyrylium betaines as monomeric units which can polymerize and form brown polymers (Scheme 2.4). Pyrylium betaines are suggested to be formed by loss of three molecules of water from the Amadori intermediates.



Amadori Product

Scheme 2.4 Polymerization of pyrylium betaines (Yaylayan and Lachambre, 1990)

Feather and Nelson (1984) isolated a water soluble polymer from the mixture of glucose-glycine, after refluxing for 8 hours. Elemental analysis has indicated that monomeric units contain one mole of glucose, one mole of glycine, minus three moles of water. Also noteworthy is the fact that the amino acid was incorporated into the polymer, when methaionine was used to produce melanoidin (Feather and Nelson, 1984). By qualitative observation, they reported that the polymers were more soluble in tap water than distilled water, and that they binded metal ions, which could not be removed by dialysis against distilled water.

As we know from references already cited, melanoidins are formed during polymerization of carbohydrate degradation products (e.g. 3-deoxysuloses), or adjuncts of these, with amino compounds. However, the regularity of the polymers with respect to nitrogen-containing or nitrogen-free subunits, and whether the frequency of the subunits is affected by composition of the reaction mixture, is not known. For this reason, Wedzicha and Kaputo, (1992), initiated a systematic investigation of the melanoidin structure with the relationship between composition of reaction mixture and certain properties of the high molecular weight products. They found that melanoidins with Mw > 12 000 consist of at least 60 subunits, and because of their length, may appear homogenous even if the distribution of different types of subunits is random. The results of experiments in which the concentration of glycine far exceeded that of glucose, showed that stoichiometry of the polymer is in the range of four to five molecules of glucose for every four amino acid molecules. The part of the amino acid incorporated into any polymer derived from Strecker degradation product is the nitrogen atom. Therefore, in the polymerization mechanism, two subunits are needed: a carbohydrate degradation product, a glycine unit, and an amino ketone obtained from Strecker reaction (Wedzicha and Kaputo, 1992).

There are few publications cited in the literature describing the process of melanoidin formation in fried foods and whether brown pigment formation is affected by the presence of fat in food mixtures. Malondialdehyde is a natural compound produced in substantial quantities in lipid-rich foods as an end-product of polyunsaturated lipid peroxidation. The latter reacts with amino-sugars, and particularly N-substituted amino

ketoses, giving rise to a variety of heterocyclic compounds (pyrroles, dihydropyridines, and 4-pyridones). These heterocyclic compounds are unstable, and polymerize on standing to give colored, melanoidin-like, polymers (Nursten and O'Reilly 1986).

Obertonov et al., (1993), investigated melanoidin formation in fried meatballs and schnitzel, characterizing spectra from fractions using gel chromatography. This investigation shows convincingly that lipids are active participants in the reaction of nonenzymatic browning. The amount of melanoidins in the fried product increased with the duration of using frying fat. This fact indicates that melanoidin precursors (mainly carbonyls), diffuse from the fat into the product, where nitrogen bases react to form brown polymers. The melanoidins accumulated in fried product were slightly polar and strongly lipophilic. They could be the product of aldol condensation (destruction product of lipid) or (if nitrogen contained) the amino acids which have undergone to a high degree of strecker degradation whose products are nonpolar polymers (Obertonov et al., 1993).

2.2.2. Factors influencing browning

Many food processes, such as baking, frying, and roasting, are based upon the Maillard reaction for flavor and color formation. This can be used when designing and controlling food production in order to obtain the desired product quality and stability. Due to the interactions between the variables involved in Maillard reaction, it is often difficult to apply results from model systems directly to food processes. However, they

are highly important in the investigation of reaction effects, and crucial for the further study of the interaction between various factors influencing the Maillard reaction.

Controlling the main factors, such as temperature, time, water activity, pH, and reactant availability, in the browning process is very important in minimizing the nutritional losses while obtaining optimal color and flavor (Lingnert, 1990). A large number of model studies have been reported in the literature. Results obtained from different investigation vary, due to differences between model systems used. The rate of browning in foods increases rapidly with increasing temperature, it also depends on the water content in the system, and the pH, and varies with different amino acids (Reynolds, 1963). The influence of the molar ratio of the reagent, water content, time, and temperature on the rate of formation of nondialyzable melanoidins have been studied in the model system (Obertonov et al., 1986).

D-glucose-L-cysteine, and D-glucose-L-glutamic acid model systems have been recently studied by Obertonov et al., (1990). The rate constants for molar ratios of 2.5:1, 1:1, 1:2.5 have been established as 2.69×10^{-5} s^{-1} , 1.43×10^{-5} s^{-1} , 0.76×10^{-5} s^{-1} respectively, for the D-glucose-L-cysteine model system, and 2.61×10^{-5} s^{-1} , 2.32×10^{-5} s^{-1} , 4.41×10^{-5} s^{-1} for the D-glucose-L-glutamic acid model system. The estimated activation energies (E_a) for the two model systems is 111.29 Kj/mol and 134.27 Kj/mol respectively (Obertonov et al., 1990). Another study (Motai, 1974) has been presented to prove the correlation between molecular weight and color intensity of melanoidin. Based on this investigation, a linear relationship between the distribution coefficient (in dextrin

gel), molecular weight and color intensity (log $^{1\%}_{1\ cm}$ E_{450}) has been established. Using this correlation, the following equation has been proposed:

$$\mathbf{E = k \times M^{\alpha}}$$

where :

α – distribution coefficient (degree of polymerization)

k – constant value

E - color intensity

M - molecular weight

The equation representing a relationship between color and molecular size, can be applied to most prepared melanoidin by Maillard reaction. Values k and α varied with the type of amino compound. Amino acid-xylose system, showed low α value and high k value compared to those of the melanoidins from peptide-xylose system. Additionally, the α value tends to increase and the k value tends to decrease with increasing molecular size of the amino compound. The α values for glycine-, diglycine-, triglycine-xylose systems were found to be proportional to the degree of polymerization. It was suggested that the variation of the α value was due to the variation in the structure of the polymerizing site and the chromophore of melanoidin with the type of amino compound. However, they were not affected by sugars and slightly influenced by heating (Motai and Inoue, 1974). The α value is also considered to indicate the degree of stability to

oxidative browning. Melanoidin having a low α value requires a high degree of polymerization to form the same amount of color as melanoidin having a high α value.

One very important class of food proteins, which are significantly high in amide content, are wheat proteins. They are known as a gluten proteins due to high glutamin content (about 30% of the overall amino acid content of gluten is glutamine) (Hoseeney and Rogers, 1992). Interestingly, a recent study indicates that Maillard reaction derived aromas and brown color formation, decreased as the amide levels decreased (Izzo and Ho, 1993). The high level of brown color in the less deamidated samples can be attributed to the increased levels of residual amides. Consequently, with volatile generation, these amides were converted to ammonia via deamidation, which subsequently interacted with glucose. Izzo and Ho., (1993) proposed that this interaction led to polymerization of sugars followed by brown pigment formation.

2.2.3. Melanoidins as enzyme inhibitors and active oxygen scavengers.

There are several reports on the formation of antioxidative Maillard reaction products during food processing. It has been widely accepted that nonenzymatic browning reactions produce strongly reducing substances known as *reductones,* which are responsible for antioxidant properties of the reaction products (Hodge, 1953; Evans et al., 1958). However, chemical properties of reductones formed in the browning reaction between reducing sugars and primary amines or amino acids are still obscure. Kirigaya et al., (1968) studied the relation between color intensity and reductones with antioxidant

activity of melanoidins. Using a heated (100°C) mixture of 0.8 M D-xylose and 0.8 M glycine, they showed an inhibitory effect against autoxidation of linoleic acid. The antioxidant activity increased in proportion to the color intensity of the browning reaction solution, where reductones formed during the browning process showed little contribution to the activity. Therefore, it was concluded that melanoidin pigment plays an important part in the antioxidant activity (Kirigaya et al., 1968).

Recent work presented by Hayase et al., (1990), concluded that melanoidins have strong oxygen scavenging activity. Hydroxyl radicals are well known to react rapidly with various organic compounds such as proteins, carbohydrates, and lipids. It has been reported that high-molecular weight melanoidin (prepared from a D-glucose-glycine system) at concentrations of 0.3 % and 0.03 % scavenged 86 % and 47 % of hydroxyl radicals respectively. Furthermore, hydrogen peroxides were also largely scavenged by low-molecular weight and high-molecular weight melanoidins (Hayase et al., 1990).

Previously reported antioxidant properties of different fractions of melanoidins suggest that the brown product of the Maillard reaction may have a potential anticarcinogenic property by inhibiting cytohrome P-450 enzymes (Kitts et al., 1993). Partial structures in the melanoidin molecule can be involved in blocking the active site of trypsin or in interacting with another site, which probably has an allosteric effect on the inhibition. Consequently, melanoidin obtained by the Maillard reaction (D-glucose and glycine) was found to exert a potential inhibitory effect on the activity of trypsin (Hirano et al., 1994).

2.2.4. Mutagenic and antimutagenic activity of melanoidins.

Maillard reaction products could be involved in either cancer induction or protection (Aeschbacher, 1990). Human cancer can be produced by various agents and factors in the environment and its development depends on life-style. It is generally accepted that human carcinogenic processes are composed of multisteps; including initiation step by mutagens, promotion step by tumor promoting agents, progression and formation of fully malignant cancers (Sugimura et al., 1990).

A series of heterocyclic amines have been isolated from cooked fish and meat by adopting a short-term microbiological mutagenic test as a monitoring system. Sugimura et al., (1990) isolated and described the structure of heterocyclic amines which have been involved in carcinogenic mutation of various organs of female and male mice, among them were pyridoindole, dipyridoimidazole and aminocarboxylic compounds some of which were found in cooked food. It has been suggested that Maillard reaction are involved in the formation of the aminoimidazoazaarenes (very genotoxic compound), identified from the crust of fried or broiled meat and fish. Further studies in model systems have shown that with increasing amount of sugar added to the model system, less aminoimidazoazaarenes were produced (Skog et al., 1990).

Apart from the effect of the temperature on the formation of food mutagens, the Maillard reaction, under certain conditions, might be either catalytic or inhibitory. This offers the possibility to control the formation of food mutagens by guiding Maillard reactions in suitable direction. The mode of cooking could play an important part for

mutagens formation. It was found that proline, tryptophan, and other indoles possess specific inhibitory attribute which compete with creatinine in the heterocyclic amines formation (Weisburger and Jones, 1990). Based on the fact that Maillard reaction products (melanoidins) exhibit antioxidant properties, it could be concluded that these antioxidants can function as antimutagenic agents. Preliminary results to support this hypothesis were recently obtained with a melanoidin which exerted a desmutagenic effect *in vitro* trough scavenging oxygen radicals (Aeschbacher, 1990). It was also suggested that the antioxidant activity of food product could be further improved by adding synthesized or fractionated Maillard reaction products or by optimization of food processing (Lingnert, 1990).

2.2.5. Nutrition and antioxidant activity of melanoidins

It has been mentioned earlier that melanoidin prepared from the model system showed antioxidant activity against unsaturated fatty acids. Therefore, it seems to be possible to apply melanoidins in food processing as natural additive (antioxidant).

Considering effective usage of melanoidin as a food component it will be necessary to undertake nutritional study of melanoidins. Recently, Fujimaki et al., (1979) reported growth response of rats fed with a diet containing nondialyzable melanoidin. It was found that nondialyzable melanoidin had no effect on the growth response of rats (Fujimaki et al., 1979). It has been also assumed that melanoidin could have physiological effects similar to dietary fiber because of its indigestible polymeric nature.

The study concluded that dietary melanoidin suppressed the elevation of cholesterol level of plasma and liver in rats and effected intestinal metabolism of cholesterol (Miura and Gomyo, 1990).

Maillard reaction products react generally by the same mechanism as other food components such as vitamin C, α-tocopherole, and several phenolic compounds with antioxidant property (Figure 2.4).

Mechanism of Antioxidant Inhibition

➡ **inhibition of nitrosamine formation**
(competition with electrophilic reaction of nitrite with secondary amines)

➡ **scavenging of reactive molecules**
(trap positively charged electrophilic metabolites or scavenge oxygen radicals)

➡ **modulation of monooxygenase / " radical defense" enzyme system**
(alteration of electrons of **NADPH**

➡ **antipromotion**
(free radicals are involved in tumor promotion)

Figure 2.4 Mechanism of antioxidant inhibition (Adapted from Aeschbacher, 1990).

3. MATERIALS AND METHODS

3.1. MATERIALS

D-Glucose (anhydrous) was purchased from BDH Inc. and glycine from Fisher Scientific. BIO-GEL P-10 (exclusion limit 1,500 - 20,000) and BIO-GEL P-2 (exclusion limit 100 - 1,800) were purchased from Bio-Rad Laboratories (Richmond, CA). Slide-a-lyzer cassettes (3.0 - 15 mL capacity, 10,000 MW cut off) from Pierce. Water was purified using Milli-Q water purification system from Millipore Corp. Infrared spectra were recorded in D_2O on Nicolet 8210 Fourier transform spectrometer. UV/VIS spectra were recorded in water on a Beckman DU-64 spectrophotometer. Hunter tristimuls values were calculated using Color™ add-on application for GRAMS/386 software (Galactic Industries, New Hampshire). Elemental analysis was performed by Guelph Chemical laboratories Ltd. (Ontario, Canada).

3.2. POLYMER PREPARATION TECHNIQUE AND APPARATUS

Glucose (3.1 g ,0.02M) and glycine (1.46 g, 0.02M) were dissolved in 30 mL (2:1 v/v) of methanol water solution and refluxed under continuous heating at 65°C for seven hours. The brown solution was decanted from the precipitated residue which has been formed after cooling the reaction mixture. Clear solution was transferred into glass vials and vacuum dried for 48 hours using Speed Vac Dryer, (SG110A, SAVANT /UVS400). Dark sticky paste was easily dissolved in water and placed in Slide-A-Lyzer Cassette (15

mL sample volume with nominal molecular weight cutoff = 10 000) and dialyzed against deionized distilled water for 24 hours.

The content of the cassette was transferred to the 15 mL glass vials and vacuum dried under room temperature condition for 24 hours in order to evaluate the weight (yield) of the high molecular weight polymers before performing a second separation by Gel Filtration Chromatography. The external solution was freeze dried and gave an average yield of 3.7 g. Both, previously separated fractions by dialysis were dried and restored in deionized water before Gel Filtration Chromatography.

3.2.1. Gel Filtration Chromatography System

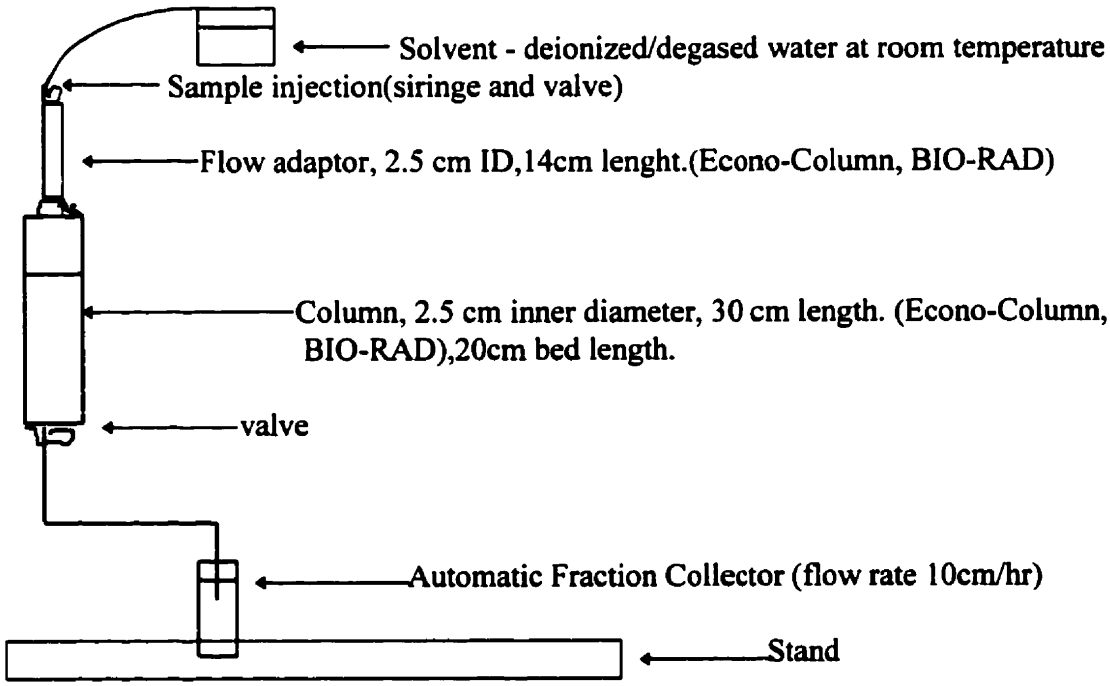


Figure 3.1 Diagram of the Gel Chromatography system

Approximately 5 mL of the concentrated sample was introduced cerfully by flow adaptor into the column with gel (P-2) for fraction MW< 10 000 and (P-10) for fraction MW > 10 000. For each run 40 x 10 mL fractions were collected and analyzed by UV/VIS Spectrophotometer. Fractions exhibiting similar spectra were combined and solvent was evaporated. The purity of the separated polymers was verified by HPLC. The pure samples were anylysed by FTIR and Py/GC/MS and submitted for elemntal analysis.

3.2.2. **HPLC** *(High Performance Liquid Chromatography)*

HPLC Beckman system Gold consisted of a programable solvent delivery module 110B and variable wavelenght UV detector model 166. The UV detector and all data handling system were controlled by an IBM 486-based computer with Gold system software. The pump system and flow rate (1mL/min) were controlled manually, Supelco, Progel-TSK G2500PWXL (30 cmx7.8 mm ID) column was used. Deionized and degassed water was used as a mobile phase.

3.2.3. **FTIR** *(Fourier Transform Infrared Spectroscopy)*

Previously vacuum dried fractions were dissolved in D_2O and placed in CaF_2 cell with 25 μm Teflon spacer. Spectra were collected on a Nicolet 8210 Fourier-Transform or Midac Prospect-IR spectrometer purged with dry air and equipped with DGTS detector. Total 128 scans were acquired at 4 cm^{-1} resolution (Nicolet 8210, spectral analysis by OMNIC) and 68 scans at 4 cm^{-1} resolution (Midac Prospect-IR, spectral analysis by GRAMS).

3.2.4. **Pyrolysis GC/MS** *(Gas Chromatography with Mass Selective detector)*

A Hewelt Packard GC with MS detector (5890 GC/591B MSD) interfaced to a CDS pyroprobe 2000 units was used for the Py/GC/MS analysis. Samples (1-4 mg) were introduced inside the quartz tube (0.3 mm thickness) and pluged with quartz wool and inserted inside the coil probe. The Pyroprobe was set at desired temperature (350 ° C) at the heating rate of 50 ° C/ms with a THT (total heating time) of 20 seconds. The GC column flow rate was 0.8 mL/min. for a split ratio of 92:1 and a septum purge of 3 mL/min. Capileary direct MS interface temperature was 180 ° C, ion source temperature was 280 °C. The ionization voltage was 70 eV, and the electron multiplier was 1682 V. The mass range analyzed was 30-300 amu. The column was fused silica DB-5 (30 m length x0.25 mm ID x 25 μm film thickness; Supelco Inc.). The column initial temperature was -5°C for 3 minutes and was increased to 50 °C at a rate of 30 ° C /min; immediately the temperature was further increased to 270 ° C at a rate of 8°C/min. and kept at 270 ° C for 5 minutes.

4. PREPARATION AND ISOLATION OF MAILLARD POLYMERS

4.1. INTRODUCTION

Brown pigments have been isolated from the reaction between aldoses and amino acids by many workers (Maillard, 1916; Ellis, 1959; Hayase et al., 1984) using different model systems. As a result, they obtained polymeric compounds with diversified chemical structures. The rate of browning in foods and model systems increases rapidly with increasing the time and temperature of the reaction (Benzing-Purdie et al., 1986; Danehy and Pigman, 1951). Water activity also plays a very important role in the melanoidin formation, as well as the pH of the solution. Type of amino acid used in the model system is one of the most important factors responsible for the aroma and flavor formation. However, yield of melanoidin seems to be relatively unaffected by the molar ratio of the reactants (Wedzicha et al., 1992). Dialysis is perhaps the most straightforward method of separating high molecular weight components if mainly molecular weight is to be evaluated. This method of separation has been used recently by many researchers (Kato et al., 1967; Gomyo at al., 1972; Motai and Inoue, 1974; Motai, 1974; Kato and Tsuchida, 1981; Feather and Nelson, 1984; Kato et al., 1987; Obertonov at al., 1990 ; Wedzicha and Kaputo, 1992; Kroh, 1994).

However, for the more elaborate isolation of structuraly different fractions, gel filtration chromatography could be used along with dialysis separation. Gel filtration chromatography has been used mainly for polymer weight estimation (Obertonov at al., 1993) and HPLC for separation. However, combination of dialysis with gel filtration

chromatography has not been reeported. Therefore, objectives of this research are; the evaluation of more effective methods (dialysis combined with repeated gel filtration chromatography) for Maillard polymer separation and isolation.

4.2. PREPARATION OF THE POLYMERS

To generate initial polymers, the glycine (0.02 M) and glucose (0.02 M) model system was refluxed under mild condition (methanol/water 2:1 v/v) for seven hours. Eight experiments were performed (Table 4.1), similar fractions from each gel filtration run were combined and subjected to chemical and spectroscopic analysis.

TABLE 4.1. MELANOIDIN PREPARATION

Sample	Glucose(g)	Glycine(g)	Residue(g) (glycine)	MELANOIDIN(g)	
				>10 000	<10 000
1	3.0500	1.249	0.4793	0.1132	3.6510
2	3.0782	1.5420	0.6871	0.1901	3.7804
3	3.0614	1.5667	0.6692	0.1114	3.4687
4	3.0939	1.5705	0.9236	0.1234	3.9821
5	3.1094	1.4945	0.6250	0.1226	3.7251
6	3.0860	1.5420	0.7628	0.1131	3.6543
7	3.1445	1.4715	0.5664	0.1045	3.6821
8	3.1150	1.5281	0.8561	0.1521	3.6987

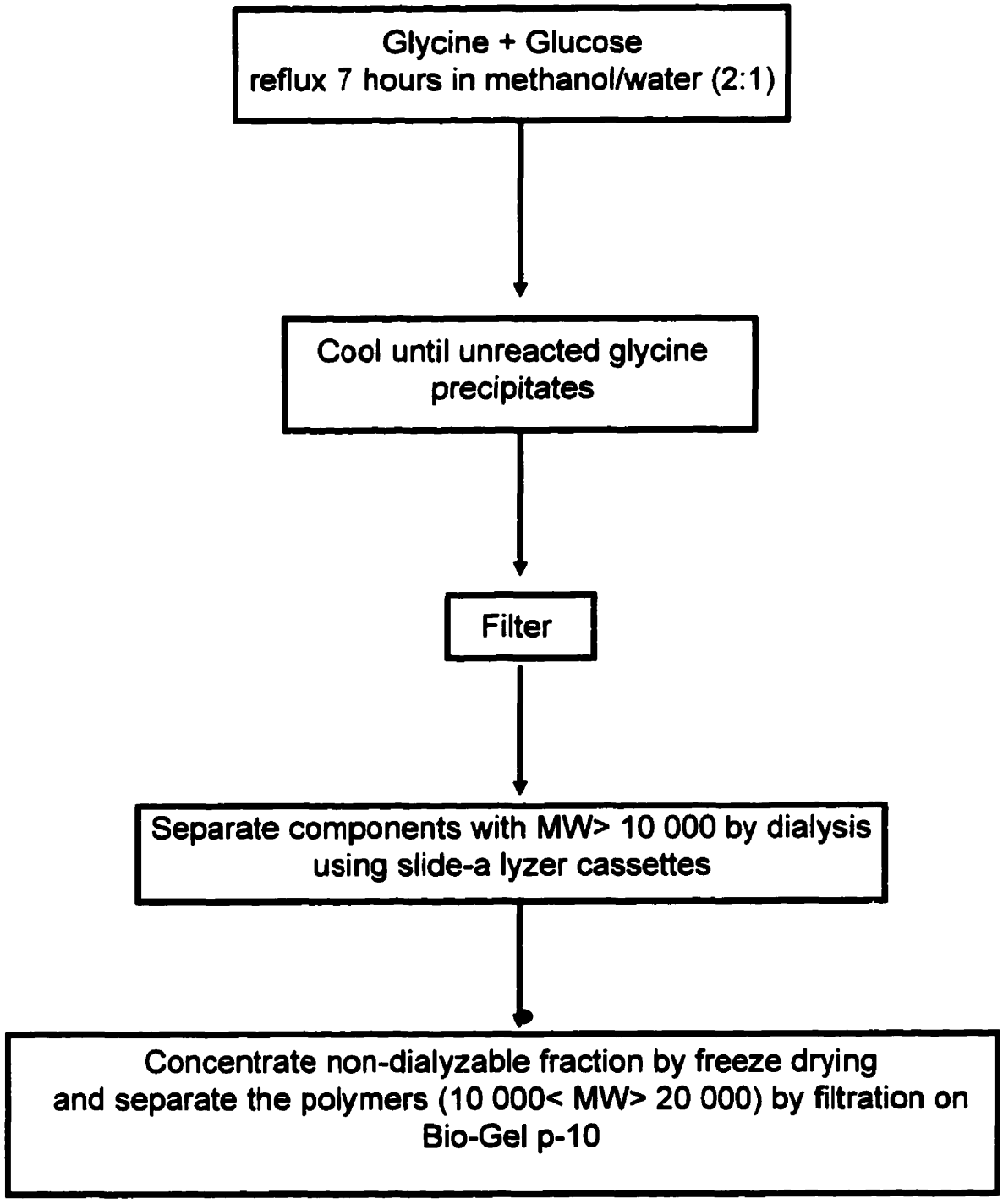
The brown solution obtained after several hours of reflux was decanted to separate the precipitated residue which was formed after cooling the reaction mixture. Weight of the residue varied from 0.9236 g to 0.4793 g. The precipitated solids were recrystalized

and were found to be unreacted glycine (the white powder had the same melting point and same FTIR or GC/MS spectrum as pure glycine). Further separations were carried out after removal of unreacted glycine. Scheme 4.1 summarizes the isolation of polymers from the reaction mixture. The samples were dialyzed, followed by gel filtration to collect the fractions containing polymeric materials.

4.3. ISOLATION OF THE POLYMERS

4.3.1. INITIAL SEPARATION BY DIALYSIS

The reaction mixtures from each experiment were dialyzed against deionized water in order to remove lower than 10 000 MW compounds from the reaction solution. Dialysis process (12 hours without changing water) was always performed just after completing polymer preparation for each run (Scheme 4.1). Content of the dialyzing cassette was transfered into vial (30 mL) and evaporated on Speed Vac. dryer to an average weight of 0.1089g. After removing the dialyzing cassette from the beaker with a deionized water solution (1000 mL) contained compounds having amolecular weight of < 10 000. The dialysis solution (1000 mL) was freeze dried and gave an average yield of 3.6 g for each separation run.



Scheme 4.1 Preparation and isolation of Maillard polymers

4.3.2. ISOLATION BY GEL FILTRATION CHROMATOGRAPHY

All further separations were performed by gel filtration chromatography using UV/VIS spectrophotometer to monitor the composition of each eluting fraction. Content of the dialysis cassette was separated by repeated gel filtration chromatography and approximetly 50 x 10 mL fractions were collected at each separation run. Solution in every vial was examinated by spectrophotometer and those with similar spectra were combined. Vials from 1 to 10 contained only solvent, content of vials 11 - 22 was termed as fraction A, content of vials 23 -35 were mixed A and B and vials from 36 to 50 were named as fraction B. All fractions containing pure A and pure B were combined and concentrated. Gel filtration was repeated three times in order to remove impurites before HPLC analysis.

Fraction which eluted first was labeled as the polymer A and the second eluted fraction was termed as polymer B. Both polymers even after only visual observation seemed to be different by their color intensity and some of their physical properties. Polymer A was much darker than polymer B and more solid-like. The second polymer (B) had a very sticky caramel like consistence and this made it very difficult to dry it completely. Elemental analysis indicated that polymer B was not homogenous. Further gel filtration of the polymer B was conducted on the same column, however, HPLC (instead of UV/VIS spectophotometer) was employed as the verification system for the outcoming fractions. Approximetaly 50 fractions (10 mL vial) were collected. HPLC analysis indicated two distinct fractions with different retention times of 3.5 minutes for

the first fraction named polymer B1 and 5.9 minutes for the second fraction termed polymer B2. The purity of the three polymers (A, B1 and B2) was further verified by HPLC and their chemical structures were determined using different techniques such as FTIR, PY/GC/MS and elemental analysis. Results of the analysis are shown in Tables 5.1-5.7 (Chapter 5).

4.3.3. VERIFICATION OF THE PURITY OF ISOLATED POLYMERS BY HPLC

In this assay progel-TSK G2500PWXL column has been used and the mobile phase consisted of 100% water. Flow rate was set at 1.0 mL/min. Wavelengths were set for each polymer based on UV\VIS spectra. Consequently for polymer A the wavelength was set at 326 λ_{max} (mμ) and polymers (B1 and B2) at 290 λ_{max} (mμ). HPLC was a particularly suitable method for non-volatile water soluble Maillard polymers for its versatility, simplicity and high degree of detection. To test the ability of the column to separate polymers A, B1, and B2 a mixture of three polymers was injected and three separated peaks were observed (Figure 4.1). The wavelength for obtained polymeric mixture was set at 280 λ_{max} (mμ). Pure solutions of the polymer A, polymer B1 and B2 were also analyzed and corresponding chromatograms are shown in Figures 4.2-4.4

4.4. CONCLUSION

Dialysis, along with repeated gel filtration chromatography can be considered as an effective method for separation and isolation of Maillard polymers

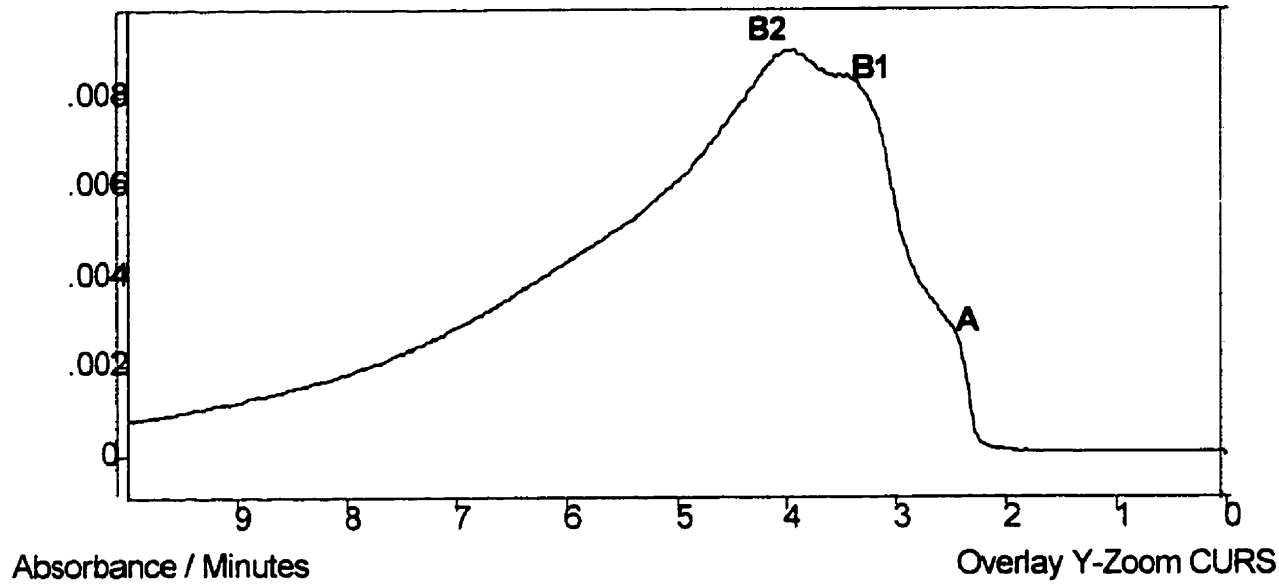


Figure 4.1. HPLC chromatogram of the brown polymeric solution before gel filtration chromatography. (280 λ_{max} (mμ).

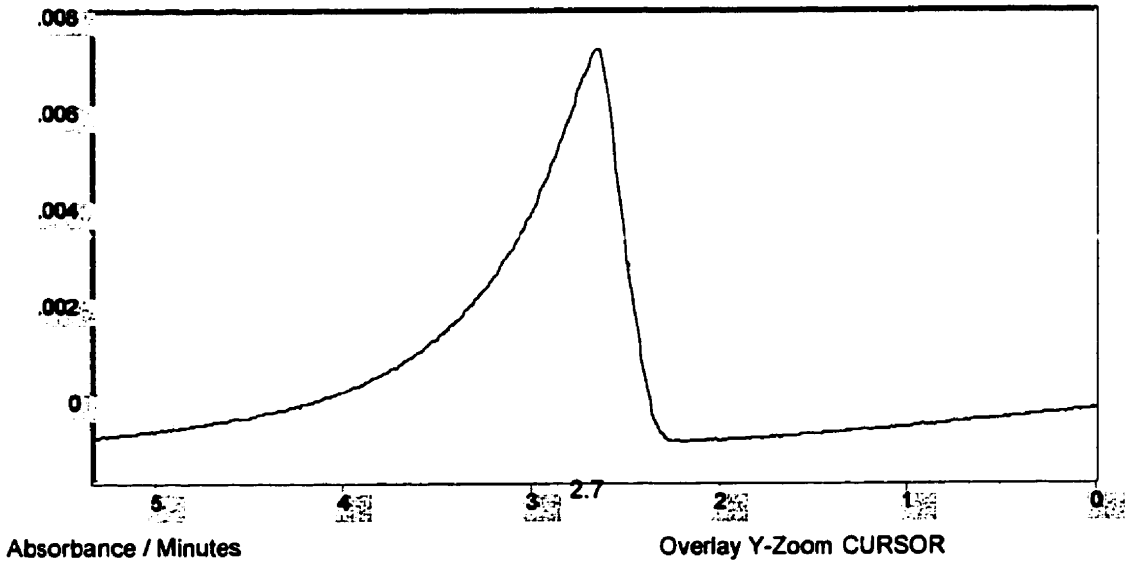


Figure 4.2 HPLC chromatogram for polymer A (326 λ_{max} mμ).

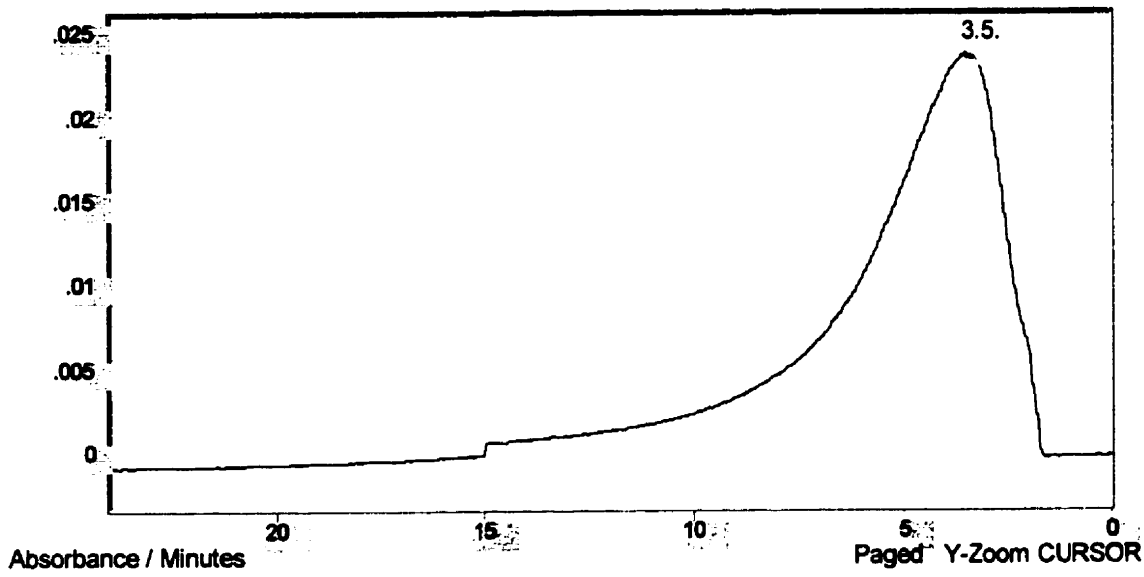


Figure 4.3 HLPC chromatogram of the Polymer B1 (290 λ_{max} mμ).

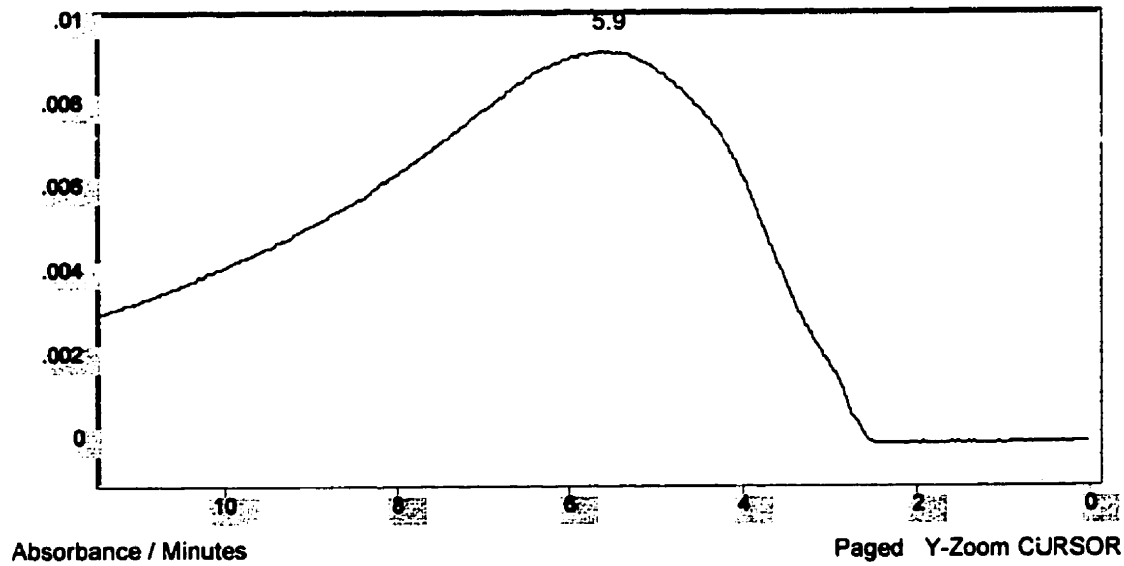


Figure 4.4 HPLC chromatogram of the polymer B2 (290 λ_{max} mμ).

5. SPECTROSCOPIC AND CHEMICAL CHARACTERISATION OF POLYMERS

5.1. SPECTROSCOPIC CHARACTERIZATION

5.1.1. FTIR EXAMINATION

5.1.1.1. Introduction

To evaluate the structure and the chemical properties of melanoidin, FTIR has been used as a preliminary technique to gain information regarding functional groups. This is the chief asset in the utilization of infrared spectroscopy for gaining structural information (Conley, 1974). In determining structural differences in unknown materials, it is necessary to minimize the effect of environment (polar molecules exist in association with each other, and generally the position of the bands are shifted to the lower frequencies when compared to the position of the same bands in nonpolar solvents). Therefore, where possible it is advisable to conduct measurements in nonpolar solvents or even better in solids state which has been found to reflect the most the reality of the molecule. However, polymers A, B1 and B2 were only soluble in water and hence extra caution was necessary in data interpretation. A polymeric structure in its nature is a very complex product to analyze. Evaluation of the latter in a solid state (concentrated sample) was difficult due to very high intensity of the absorbed peaks in IR spectrum.

5.1.1.2. Results and Discussion

Purified samples were analyzed in D_2O as described under experimental section (Chapter 3). Figures 5.1 to 5.3 display the FTIR spectra of polymers A, B1 and B2

respectively and Table 5.1 summarizes their spectroscopic data. Tables 5.2, 5.3 and 5.4 show possible band assignments for polymers A, B1 and B2 in the IR spectra. All three spectra had a typical polymeric band spreading which was difficult to define. However, some characteristic absorptions were observed which helped to assign group frequencies for unknown polymeric structures.

TABLE 5.1 SPECTROSCOPIC DATA OF THE POLYMERS

POLYMER	UV-VIS	IR (cm^{-1})	COLOUR*
Polymer A	219, 326	2937(w), 1607(s), 1465(s) 1379(m)	X = 0.1527 Y = 0.1347 Z = 0.6005
Polymer B 1	466(sh), 347(sh) 295, 209	2936, 2890, 1620, 1458,1410, 1321,1152,1086,1038	X = 0.089 Y = 0.076 Z = 0.356
Polymer B 2	293	2936, 2890, 1620, 1458,1389, 1321, 1336, 1152, 1086, 1038	X = 0.0429 Y = 0.036 Z = 0.1562

w = weak, s = strong, m = medium, sh = shoulder

* Hunter tristimulus values

5.1.1.2.1. FTIR analysis of polymer A

The absorption band at 1607 cm^{-1} is characteristic to conjugated, C=N and CH=CH, or carboxylate ion COO^-. Due to relatively dark colour of the polymer it can be concluded that conjugated double bonds of C=N and CH=CH types are present in higher proportions than carboxylate ion. The greater the number of conjugated double bonds present in the molecule, the further absorption band will be shifted to region of longer

wavelengths and darker the colour will become (increased presence of yellow to brown chromophores). Very strong peak at 1465 cm^{-1} indicates the presence of CH, CH_2, and CH_3 groups which are probably parts of glucose moiety incorporated somehow into the conjugated polymeric structure of the melanoidin A.

TABLE 5.2 BAND ASSIGNMENTS FOR POLYMER A

Band Observed (cm^{-1})	**Possible Assignments**
2937(weak)	**CH_2, CH_3** symmetric stretching
1607(strong)	**CH=CH, conjugated C=N, amide I, C=C ring, NH_3^+ or Carboxylate ion**
1465(very strong)	CH_2,CH_3,
1386 (shoulder)	CH_2,CH_3

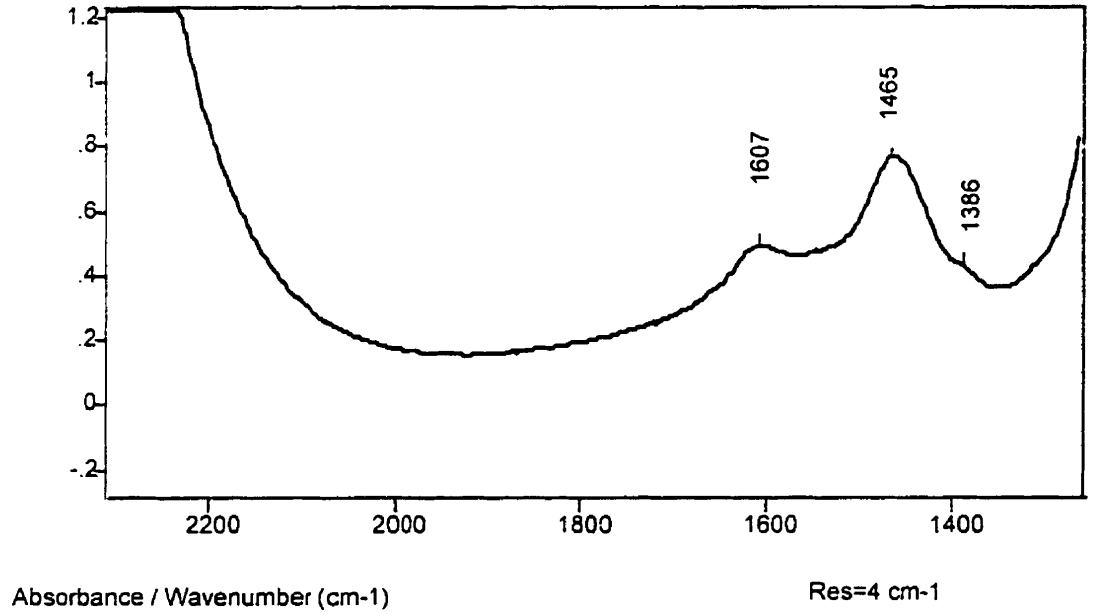


Figure 5.1. FTIR spectrum of the polymer A

5.1.1.3. FTIR analysis for polymer B1 and B2

From the FTIR spectra and its band assignments it can be concluded that polymers A (Figure 5.1) and B1 (Figure 5.2) and B2 (Figure 5.3) have different chemical structures. However, there are more differences between the structure of the polymer A and B (1, 2) than polymers B1 and B2 which suggest that B1 and B2 can be structurally related.

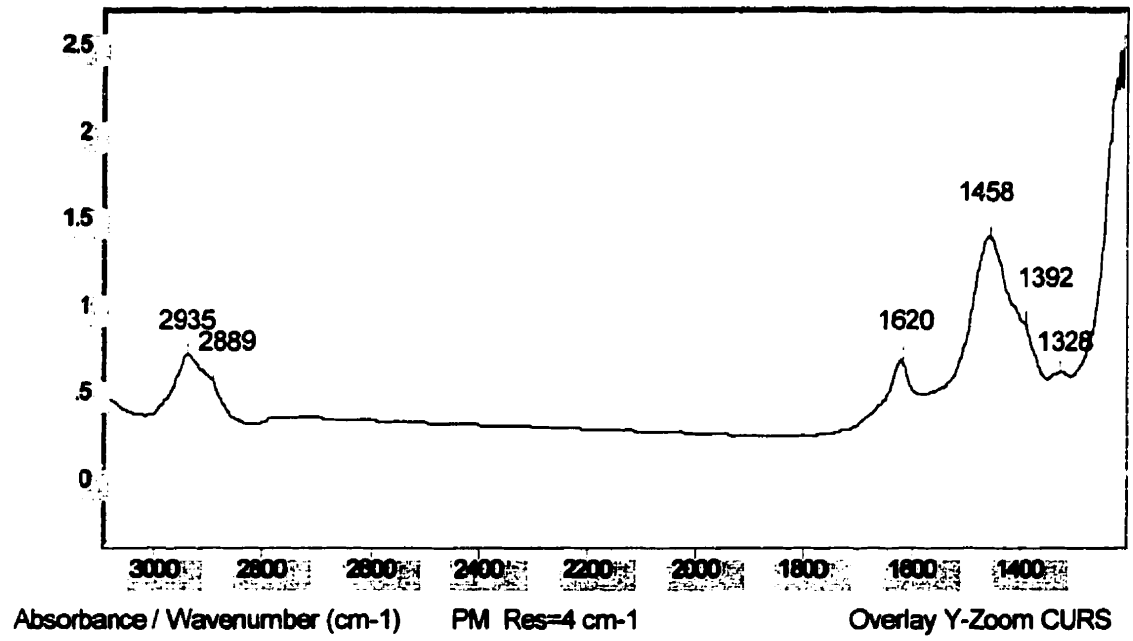


Figure 5.2 Infrared spectrum of polymer B1

TABLE 5.3 FTIR BAND ASSIGNMENT FOR POLYMER B1

Band Observed (cm^{-1})	**Possible assignments**
2935(strong) and **2889**(shoulder)	**CH_2, CH_3** symmetric stretching
1621(medium)	**Carboxylate ion** (symmetric stretching), **C=C** ring
1458(very strong)	**CH_2, CH_3** asymmetric stretching or **Carboxylate ion** (asymmetric stretching),
1392(shoulder)	**COO^-** asymmetric stretching, alkyl CH_3 ,CH_2 asymmetric stretching
1328 (weak)	unknown
1087 (medium)	CH glucose fragment , rocking characteristic for furan ring
1059 (strong)	C=C-C C-H C-OH

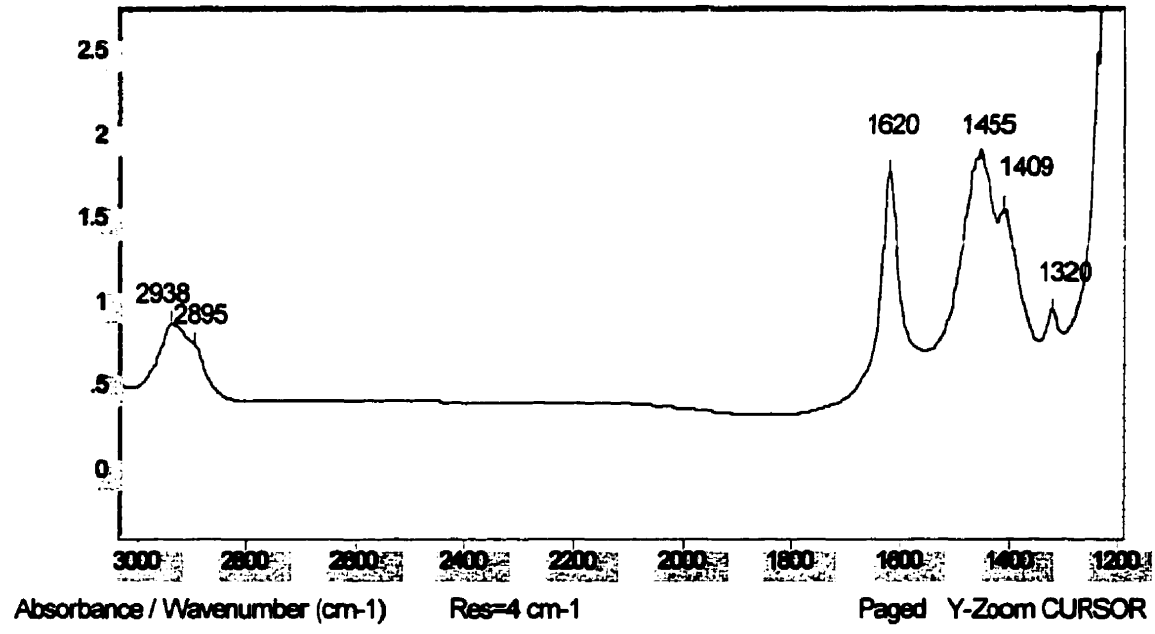


Figure 5.3 Infrared spectrum of polymer B2

Table 5.4 FTIR BAND ASSIGNMENT FOR POLYMER B2

Band Observed (cm^{-1})	Possible assignments
2938(strong) and 2895 (shoulder)	CH_3, CH_2 symmetric stretching
1620(very strong)	Carboxylate ion (symmetric stretching), or C=N ring
1455 (very strong)	alkyl CH_3,CH_2 asymmetric stretching
1409 (broad shoulder)	COO^- asymmetric stretching
1321(medium)	unknown
1087 (strong)	CH glucose fragment , rocking characteristic for furan ring
1059 (extremely strong)	C=C-C ; C-H ; C-OH

Yellow brown colour and thick caramel-like consistency of the polymers B1 and B2 indicate the presence of the aldose parts in the polymeric structures. It was described by Ledl (1990) that yellow colour is formed by condensation of hydroxy-methylfurfural with pyranone. Both of these products can be derived from 3-deoxyaldoketose. Moreover, in an aqueous solution hemicetals of pyranone are not stable and it can be assumed that rearrangement occurs first, followed by a subsequent condensation leading to furanone formation. IR spectra of both polymers indicated strong appearance of furanoid species (1087 cm^{-1}) as well as other glucose structure related moieties (C-H ; C-OH; C-C , 1059 cm^{-1}). The same band appearance at (2935-37) cm^{-1} and (2890-95) cm^{-1} regions for glucose and polymers (B1 and B2) can indicate the presence of alkyl groups in the polymer B1 and B2. IR spectra (Figure 5.2 and 5.3) displayed two very broad

bands one at 1620 cm^{-1} (symmetric stretching) and the second at 1411 cm^{-1} (asymmetric stretching) which are very typical for carboxylate ion structures originating from amino acid (glycine).

5.1.2. UV/VIS SPECTROPHOTOMETER

In practice, ultraviolet spectrometry is mostly limited to conjugated systems. However, there is an advantage to the selectivity of ultraviolet absorption. Characteristic groups can be recognized in molecules of widely varying complexities. A large portion of a relatively complex molecule (e.g. polymeric structures) may be transparent in the ultraviolet. In consequence the spectrum of the very complex molecule may be similar to that of much simpler molecule. Major objective of the UV/VIS spectrometry in this study was the initial evaluation of the eluted fractions from gel filtration chromatography and separate them into groups with similar spectra exhibition. However, the correlation between spectra and chemical structure of the polymers can be used for their characterisation. Table 5.1 demonstrate the results of UV/VIS spectroscopy for all three polymers. Polymer A is characterized by absorption bands at 219 λ_{max} (mμ) and 326 λ_{max} (mμ) which can be produced by a wide group of different compounds such as furan species or aldehyde, $-(C=C)_2-$ acyclic, -C=C-C=O-, -C=C-C=C and -C=C-C= N.

Polymer B1 shows absorption bands at 209 λ_{max} (mμ), 295 (347 sh) λ_{max} (mμ) and 466 λ_{max} (mμ) (weak shoulder) which can be characteristic of a group of compounds such as furan species, aldehydes or -C=C-C=O-. Third polymer, B2 was not different from the polymer B1 in the UV/VIS spectrum. The main difference was the appearance of the two

shoulders (347 and 466) λ_{max} (mμ) which can not be used to distinguish between the polymers.

5.1.3. COLOUR MEASUREMENT

Colour formation is the primary characteristic of the Maillard reaction products. One of the principal system in colour measurement is the Hunter system which is based on sensing colour by human eye. This system proposes that the eyes contain three light sensitive receptors (red, green and blue). Food colorimetry can be a useful method in the evaluation of physical properties of polymers. Table 5.1 presents the Hunter tristimulus value of each separated polymer. Polymer A, even from only visual evaluation was found to be the darkest one. Values of the X,Y,Z (Table 5.1) for the polymer A are almost double as for polymer B1 and more than triple for the polymer B2. This observation suggests that polymer A contains more chromophores. As the first eluted peak, polymer A should have a higher molecular weight than the two other polymers and consequently more units in the whole molecule. Furthermore, it could be proposed that the latter is the most complicated (from all three isolated in this study) in its chemical structure.

5.1.4. PY/GC/MS ANALYSIS

5.1.4.1. INTRODUCTION

Pyrolysis connected directly to GC/MS can provide a fast and convenient method of partial structure determination of polymers. Huyghues-Despointes et al., (1994) demonstrated usefulness of the PY/GC/MS to study proline Amadori product. Effect of temperature on the polymers and generation of volatiles produced can be used as an important tool for chemical characterisation. Pyrograms can be used as fingerprints for polymer identification. The latter with other information obtained from different types of analysis will be crucial for determing the mechanism and pathway of formation of polymers.

5.1.4.2. Results and Discussion

In the present study, Py/GC/MS was used to investigate the differences in the fragmentation products of the three isolated polymers. The main product formed from pyrolysis of the polymer A was acetic acid (25.23% of the peak area) which could be formed from either glucose fragments or glycine during pyrolysis. Table 5.5 lists pyrolysis products of the polymer A identified by GC/MS. Pyrogram (Figure 5.8) of the latter indicates significant presence of the pyrolysates typical for glucose-glycine Amadori products (Keyhani and Yaylayan, 1996) such as 5-methyl-1H-pyrrole-2-carboxaldehyde, 1-methyl-1H-pyrrole, pyridine, 4-methyl pyridine, methyl pyrazine, trimethyl pyrazine, 2,3-dihydroxy-2-methyl-4H- pyran-4-one. These preceding data

imply the presence of the Amadori like units incorporated into structure of the polymer A.

TABLE 5.5. PYROLYSIS PRODUCTS OF THE POLYMER A

% AREA	COMPOUND
3.52	Acetic acid, methyl ester
1.47	2,3- Butanedione
2.58	2- Butanone
25.23	Acetic acid
6.04	1-Hydroxy-2-propanone
1.38	Ethanamine, N,N-diethyl
2.77	1-Methyl-1H pyrrole
1.96	Pyridine
0.43	4-Methyl-pyridine
3.43	Methyl-pyrazine
2.02	2-Furancarboxaldehyde
0.49	2-Propanone , 1-(acetyloxy)
0.96	1H-pyrrole-2,4-dimethyl
2.87	Cyclopent-2-en-1,4-dione
0.76	1-(2-Furanyl)-ethanone
3.88	2,6-Dimethyl-pyrazine
0.88	2.3-Dimethyl-pyrazine
1.72	5-Methyl-2-furancarboxaldehyde
2.16	Trimethyl-pyrazine
0.67	1-(1H- pyrrol-2-yl)-ethanone
1.53	3-Hydroxy-2-methyl-4H-pyran-4-one
1.89	5-Methyl-1H- pyrrole-2-carboxaldehyde
2.43	2,3-Dihydro-3,5-dihydroxy-2-methyl-4h-pyran-4-one
1.28	4-Amino-3-methyl-phenol
72.35	

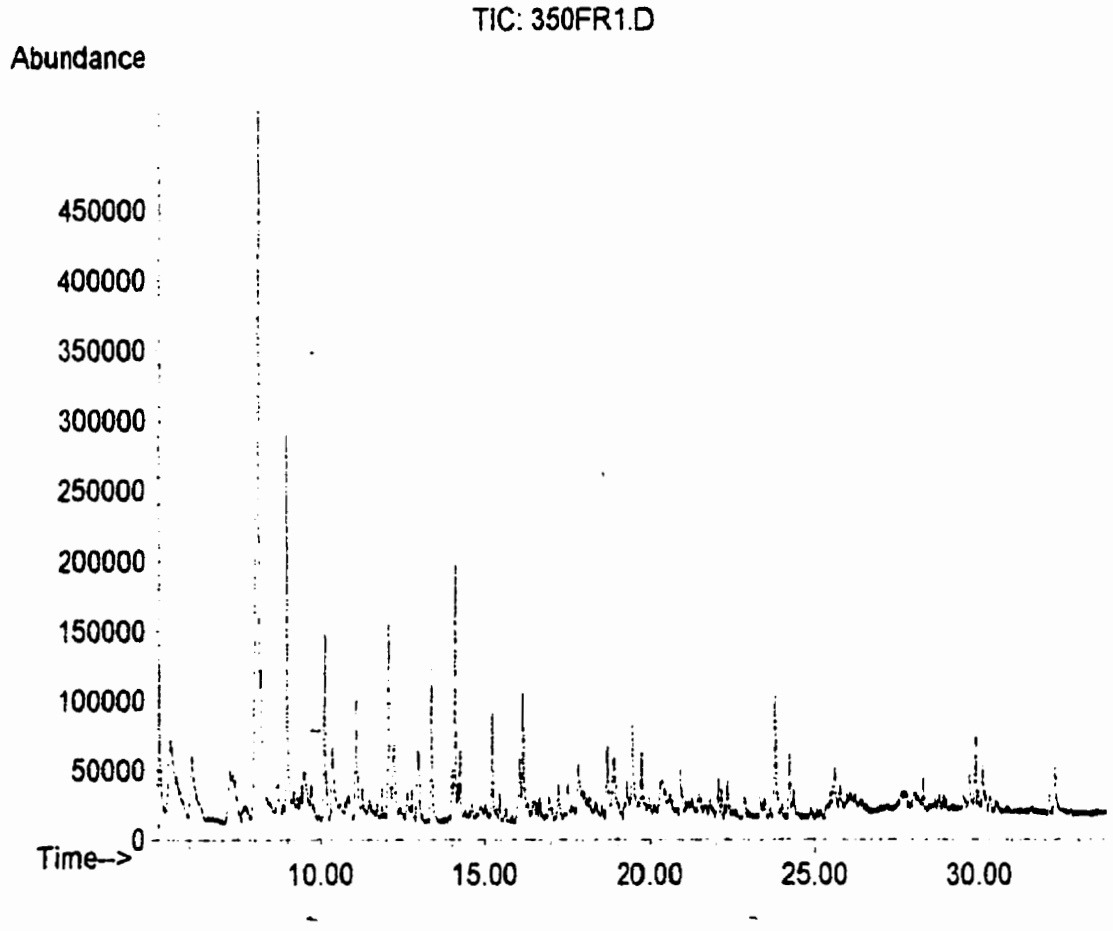


Figure 5.4. Py/GC/MS chromatogram of the polymer A

Products formed from the polymers B1 and B2 are summarized in Table 5.6. The data in this table show the absence of nitrogen species in both polymers. Pyrolysis products from the polymer B1 and B2 are similar in their chemical structures, with only few differences indicated in Table 5.6. Both polymers produced predominantly furanoid species (66 % of total peak area), 5-hydroxymethyl-2-furaldehyde, 2,2 bifuran, 2-furanmethanol, 2-furancarboxyaldehyde, formic and acetic acid. Py/GC/MS analysis indicates that polymers B1 and B2 contain the structures which have the ability of

producing furan upon heating. Furanone has been found as the most important Maillard reaction product involved in color and fruity aroma development (Hodge et al., 1963; Danehy, 1985). It is already known that Amadori product can lose a glycine molecule and form 3-deoxyglucosone which can further dehydrate to 6-hydroxymethyl-2-furaldehyde and to 5-hydroxymethyl-2-furaldehyde. However, little is known about role and importance of the browning reaction intermediates such as 1-deoxyglucosone, 3-deoxyglucosone, HMF in the polymerization reaction that leads to Maillard polymer formation or the role of released amino acid in such a reaction.

The major pyrolysis products of the dialyzable material derived from glucose-glycine model system were 4H-pyran-4-one and acetic acid (Table 5.7). Evidence from the composition of nondialysable and dialysable fractions strongly suggest that all three polymers were formed in the early stage of the Maillard reaction. This process involves Amadori formation followed by its degradation by forming intermediates which can further polymerize with each other as well as with other molecules present in the reaction milieu.

TABLE 5.6 PYROLYSIS PRODUCTS OF POLYMERS B1 AND B2

% AREA		COMPOUND
B1	**B2**	
2.18	**2.36**	Formic acid
7.47	**5.3**	Acetic acid
0.00	**1.82**	2-Methyl-furan
1.60	**4.30**	1-Hydroxy-2-propanone
7.65	**10.21**	2-Furancarboxaldehyde
0.48	**0.73**	2-Furanmethanol
0.36	**0.40**	2(3H)-Furanone-5-methyl
2.67	**1.24**	Cyclopent-2-en-1,4-dione
0.60	**0.70**	1-(2-Furanyl)-ethanone
0.00	**1.49**	1,3-Cyclopentanedione
6.91	**2.35**	5-Methyl-2-furancarboxaldehyde
0.00	**0.17**	2-Ethyl-furan
0.00	**0.13**	**2,2'-Bifuran**
0.00	**0.25**	2-Hydroxy-3-methyl 2-cyclopenten-1-one
2.46	**1.46**	2-Furancarboxylic acid
0.00	**0.84**	3-Furancarboxylic acid, methyl ester
0.00	**0.26**	2H-Pyran-2-one
0.02	**0.06**	3-Hydroxy-2-methyl -4H-pyran-4-one (Maltol)
8.64	**4.32**	2,3-Dihydro-3,5-dihydroxy-2-methyl-4H-pyran-4-one,
1.48	**0.86**	3,5-Dihydroxy-2-methyl-4H-pyran-4-one
34.5	**35.59**	5-(Hydroxymethyl)-2-furancarboxaldehyde
0.07	**0.00**	**[2,2'Bifuran]-3-carboxylic acid**
0.48	**0.00**	**5-[(5-methyl-2-furanylmethyl)]-2-furancarboxaldehyde**
76.52	**74.73**	

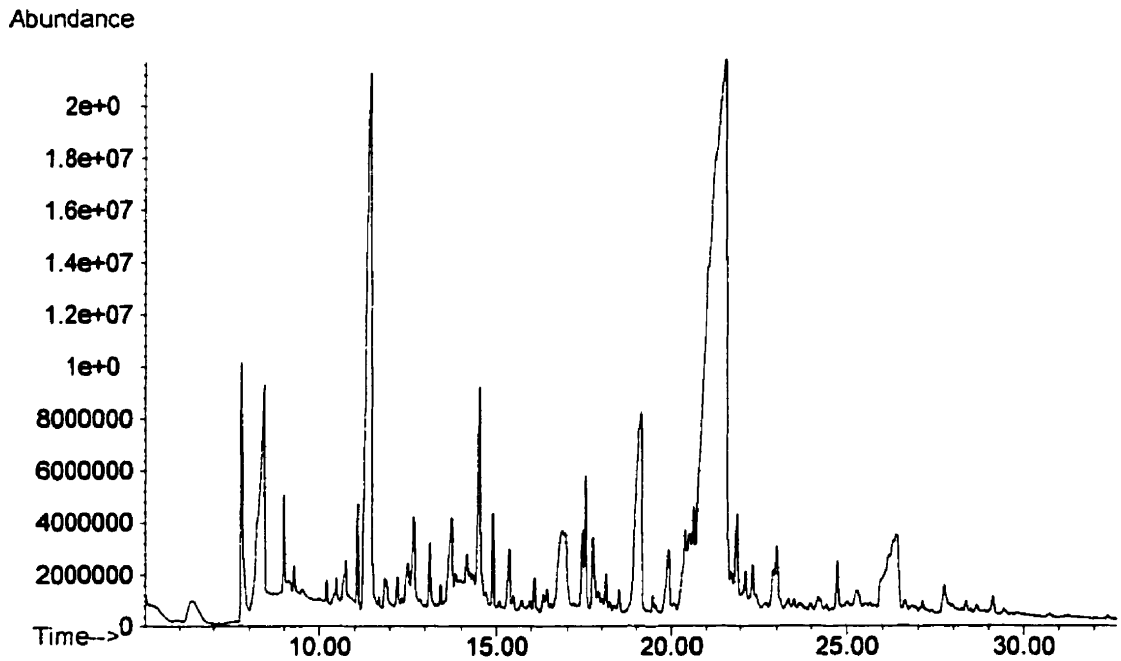


Figure 5.5. Py/GS/MS chromatogram of the polymer B1

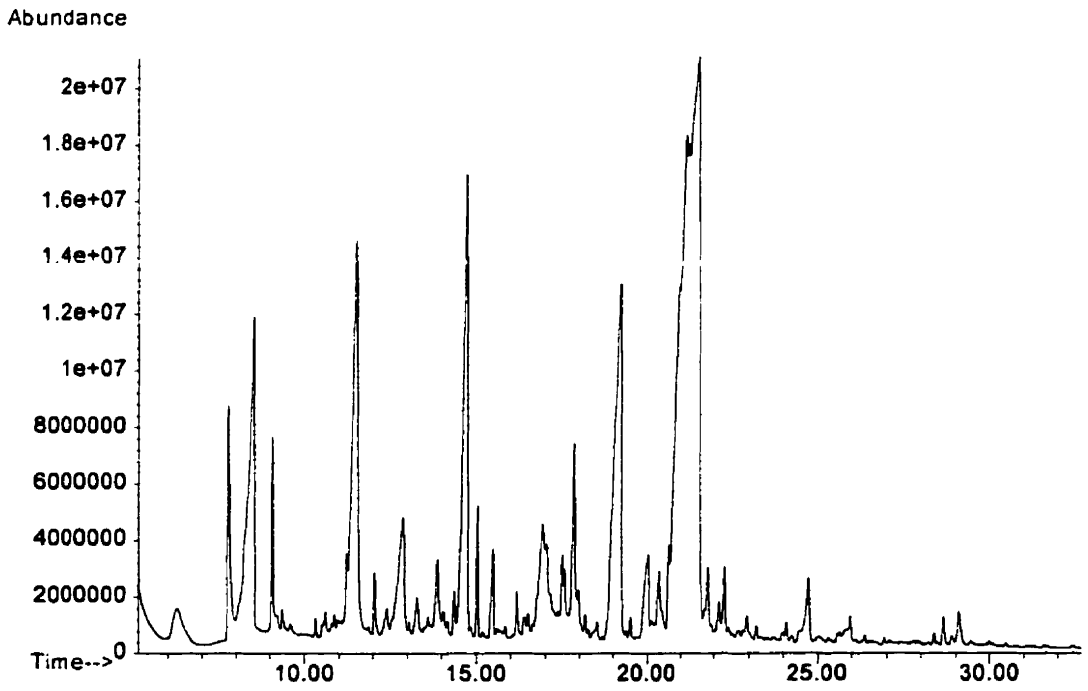
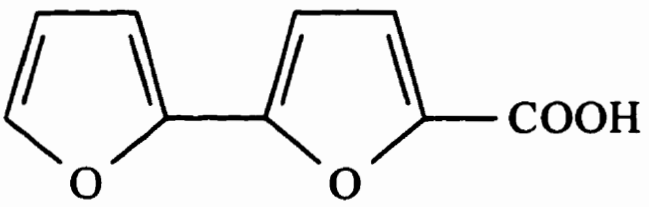
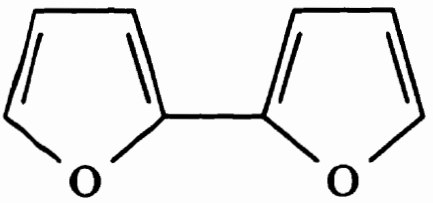


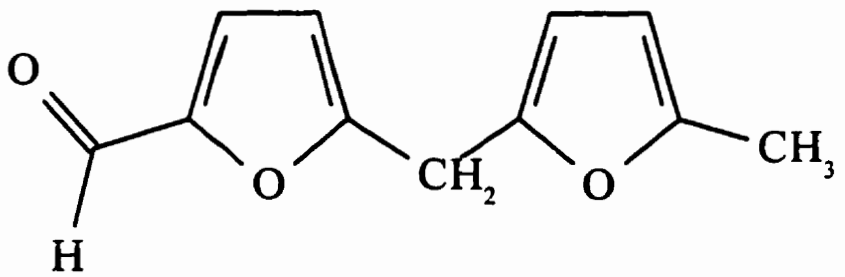
Figure 5.6. Py/GC/MS chromatogram of the polymer B2



2,2'-bifuran-5-carboxylic acid



2,2'-bifuran



2-furancarboxaldehyde-5-[(5'-methyl-2'-furanylmethyl)]

Figure 5.7 Furan derivatives observed Py/GC/MS analysis of the polymer B1 and B2

TABLE 5.7.PYROLYSIS PRODUCTS OF DIALYZABLE FRACTION

% Area	Compound
1.38	2,3-Butanedione
20.93	Acetic acid
1.64	2-Propanone, 1-hydroxy-
0.54	2-Butanone, 3-hydroxy-
0.76	1H-Pyrrole, 1-methyl-
0.47	3-Butene-1,2-diol
2.26	2-Furancarboxaldehyde
0.77	2-Furanmethanol
4.38	Cyclopent-2-ene, 1,4-dione
2.49	Ethanone, 1-(2-furanyl)-
1.21	4-Hydroxybut-2-enoic acid lactone
2.32	2-Furancarboxaldehyde, 5-methyl-
3.45	Pyrazine, trimethyl-
4.2	1,2,3-Propanetriol
0.49	2-Cyclopenten-1-one, 2-hydroxy-3-methyl
0.74	Ethanone, 1-(2-pyridinyl)-
1.06	3-Furanone, 2,3-dihydro-4-hydroxy
8.03	Ethanone, 1-(1H-pyrrol-2-yl)-
0.54	Ethanone, 1-(1-methyl-1H-pyrrol-2-yl)
0.7	Phenol, 4-amino-
21.63	**4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-2-methyl**
1.47	Phenol, 4-amino-3-methyl-
0.57	Dimethylpyrazinone
0.76	Trimethylpyrazinone
0.37	quinoxalinone

5.2. CHEMICAL CHARACTERIZATION

5.2.1. ELEMENTAL ANALYSIS

Elemental analysis indicated that polymer A has the following empirical formula $C_7 H_{11} N_1 O_4$ and polymers B1 and B2 have the same empirical formula as glucose (see Table 5.8). This is consistent with the fact that almost 70% of the Glycine was recovered after the reaction which indicates that under the experimental conditions the amino acid was mainly involved to catalyze the conversion of the sugar onto reactive form which is able to polymerize.

Table 5.8.Microanalysis data* of the purified Maillard poymers

(10 000<MW>20 000) on Bio-Gel P-10.

Sample	%Carbon	%Hydrogen	%Nitrogen	%Oxygen	Emp. formula
Polymer A	46.10 ± 0.11	6.43± 0.00	8.08± 0.00	37.43	$C_7 H_{11} N_1 O_4$
Polymer B1	36.50 ± 0.02	7.50± 0.11	0.00	53.84	$C_1 H_2 O_1$
Polymer B2	38.22 ±0.15	7.28 ±0.09	0.00	51.90	$C_1 H_2 O_1$

* average of duplicate measurements, detection limit 0.01 %

5.2.2. ACID / BASE HYDROLYSIS

5.2.2.1. INTRODUCTION

In the final stage of Maillard reaction polymerisation can proceed through different types of condensation reactions such as glycosylation, aldol condensation, estrification or aldehyde-amine reactions. An important factor in the determination of melanoidin structure is the knowledge of the type of condensation reaction during polymer formation. Hydrolysis can reveal the type of linkage by breaking polymeric units. Acid-catalyzed hydrolysis of ester type condensation results in an acid and alcohol fragments and base-promoted hydrolysis can produce an alcohol and the salt of an acid.

5.2.2.2.HYDROLYSIS REACTION

Polymer A and mixture of polymers B1 and B2 were hydrolyzed both under acid (1N HCL) and base catalysis (1N NaOH) by refluxing for 1 hour. Reaction mixtures were cooled, neutralized to pH 7 and analyzed by FTIR. All changes caused by hydrolysis (acid or base) and observed by IR spectra are summarized in Table 5.9-5.10.

5.2.2.2.1.FTIR analysis of polymer A (after hydrolysis)

There were significant changes in the structure of polymer A after acid and base hydrolysis treatment. In the 1607 cm^{-1} region where possible band assignments include conjugated C=N, C=C, amide I or carboxylate anion, changes in acid hydrolysis can be

explained in different ways. It could be proposed that it was probably decarboxylation and deamination, due to the observance that band at 1607 cm^{-1} region disappeared completely and conjugated C=C-C=C system was probably shifted to 1590 cm^{-1} region. Furthermore, acid and base hydrolysis reactions brought a broad band appearance between 1445-1449 cm^{-1} which indicates strong presence of the alkyl group CH_2, CH_3. These groups originate probably from the glucose fragments incorporated into polymer as well as from decarboxylation of glycine (relatively high temperature under reflux conditions). From the visual observation a decrease in colour was noticed in the reaction of acid hydrolysis and slight increase in basic hydrolysis.

In a basic condition polymer A was hydrolyzed to fragments with fewer conjugated units and the carboxylate ion groups might be shifted to 1590 cm^{-1}. There is also the possibility of sodium salt formation as a result of further reaction between sodium and fragments of the polymer.

Table 5.9. HYDROLYSIS REACTION OF POLYMER A

Band Observed (cm^{-1}) (before reaction)	**Band Observed (cm^{-1}) (after reaction)**	
	HCL	**NaOH**
2937(weak)	present	reduced
1607(strong)	not present	not present
	1590 (strong)	1590 (weak)
1465(very strong)	**1445**(very strong)	**1449 strong**
1379 (shoulder)	not present	not present

5.2.2.2.2. FTIR analysis of polymer B (after hydrolysis)

There are differences and similarities of the basic and acidic hydrolysis performed on fraction B (reactions were performed before further separation of this fraction and were not repeated due to hypothetical conclusion that B1 and B2 are isomers). After hydrolysis reaction two new bands were formed (1650 cm $^{-1}$ and 1592 cm $^{-1}$) and previously assigned to COO^- ion band at 1621 cm^{-1} disappeared completely. Shift of the first band to 1650 cm $^{-1}$ from 1621 cm^{-1} can be explained by the fact that carboxylate ion bands after hydrolysis of the polymer to smaller units were less conjugated. Noteworthy is also the appearance of very strong alkyls bands which are probably due to furan rings. After base hydrolysis alkyl bands were even stronger. Furthermore, all bands related to furanoid species disappeared completely from the IR spectrum after base hydrolysis which indicates NaOH caused polymerization of furanoid species, as evidented by increased browning of the solution during base hydrolysis reaction. One of the interesting observation is the presence of the band at 1592 cm $^{-1}$ in both situations, which can be explained by formation of conjugated aromatic compounds.

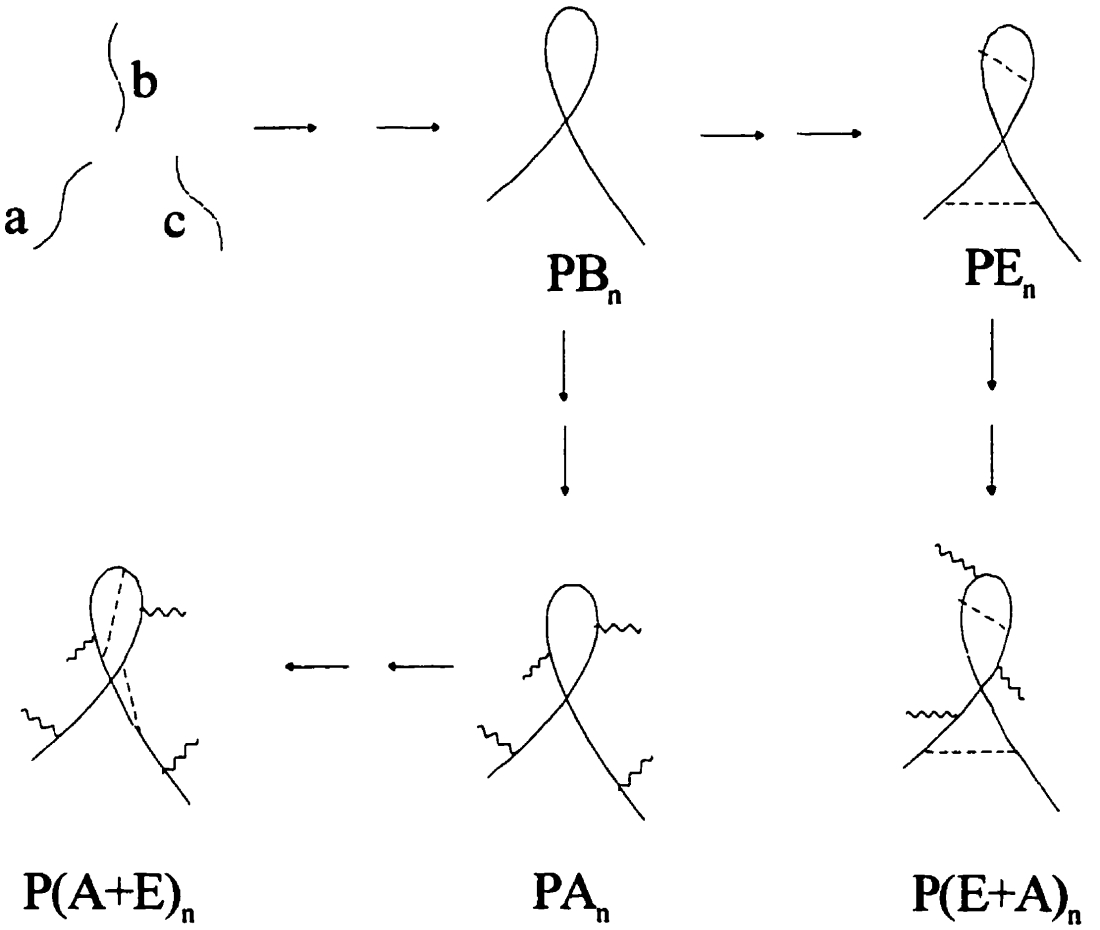
Table 5.10 HYDROLYSIS REACTION FOR POLYMER B1 and B2

Band Observed (cm^{-1}) **(before reaction)**	**HYDROLYSIS REACTION** **NaOH**	**HCL**
2935(strong)and **2889**(shoulder)	reduced	no changes
1621(medium)	not present new peak at **1592**	not present new peaks at **1650 and 1592**
1458(very strong)	very strong broad peak at **1452**	very strong broad peak at **1452**
1392(shoulder)	not present new peak at 1354	not present
1328 (weak)	present	present
1087 (medium)	not present	reduced
1059 (strong)	not present	reduced

6. PROPOSED MECHANISM OF FORMATION OF THE MAILLARD POLYMERS

6.1. INTRODUCTION

The complex process of polymerization during Maillard reaction, produces a variety of polymeric material with different molecular weights, structures and elemental compositions. Some incorporates carbon, nitrogen, oxygen and hydrogen and others only carbon, oxygen, hydrogen and hence could be classified as caramels. The type of polymer produced will depend on the amino acid used, temperature, time, pH control and solvent used. It is simplistic to assume that all polymeric materials from Maillard reaction could have similar structures. However, process of the polymerization reaction during Maillard reaction could be summarized as shown in Scheme 6.1. The initial backbone polymeric materials formed (Pb_n ; n = integer) could undergo elimination type reaction such as (dehydration, decarboxylation, intermolecular substitution followed by elimination, etc.). Alternatively, the reactive sites on the initial polymers can interact with other components in the Maillard mixture to produce a more complex series of derivative polymers designed as PA_n (n = integer). Both series can undergo either addition or elimination process to produce P(A+E)n or P(E+A)n series of polymers as shown in Scheme 6.1.



Scheme 6.1 Process of the polymerization reaction (a, b and c are oligomers).

According to Figure 6.1, the origin of nitrogen containing polymer A is assigned to Amadori intermediates or some of its derivatives. The origin of the polymers B1 and B2 is assigned to glucosones and 1-deoxyglucosone or 3-deoxyglucosones: common non-nitrogen containing reactive intermediates during Maillard reaction. GC\MS analysis of the dialysable fraction (see Table 5.7) indicates formation of the Amadori product during the reaction since Amadori specific products were found in this fraction (Keyhani and Yaylayan, 1996).

6.2. PROPOSED STRUCTURE OF NON-NITROGEN CONTAINING POLYMER

In the early stage of the Maillard reaction the amines rearrange with aldoses to the amino ketoses (Amadori compound) which are more or less stable intermediates. Amadori compounds in certain pH range (4-7) can release amino acid and be degraded to its derivatives such as 1-deoxyglucosone and 3-deoxyglucosone (Figure 6.1). The latter are very reactive α-dicarbonyl compounds which are the precursors of heterocyclic and carbocyclic compounds (Anet, 1959; Kato, 1960).

Elemental composition of the non-nitrogenous polymers B1 and B2, indicates the presence of high oxygen content and a carbon to oxygen ratio similar to glucose as indicated in Table 5.8. Band assignment from FTIR analysis confirm the presence of CH_3, CH_2, C-O-H, C-O-C and COO^- moieties. Pyrolysis of both polymers predominantly produces furanoid species (Table 5.6 and Figure 5.7) indicating the presence of furanose rings or their precursors. To generate polymers with elemental composition similar to glucose could be envisaged as to be formed from the known deoxyglucosones or glucosones intermediates formed during Maillard reaction. As depicted in Scheme 6.1, the monomeric units could be generated from furanose forms of glucosone or 3-deoxyglucosones (structure I in Scheme 6.1) which after oxidation can form carboxylic acid derivatives IIa and IIb. On the other hand 1-deoxyglucosones can exist in furanose form III. Polymerisation through glycosylation of IIa, IIb and III can generate structures that are consistent with experimental data. Polymerization can be affected by 2,6-, 2,3- 2,4-glycosilation reaction, the commonly observed polymerization

process in nature. It was found already by other workers (Igaki et al., 1990) that 3-deoxyglucosone as a major intermediate in Maillard reaction, had a very strong cross-linking activity, accelerated polymerization and increased fluorescence intensity. Accordingly, it could be concluded that this very active carbonyl compound will be glycosidically linked in order to generated the B1 and B2 polymers.

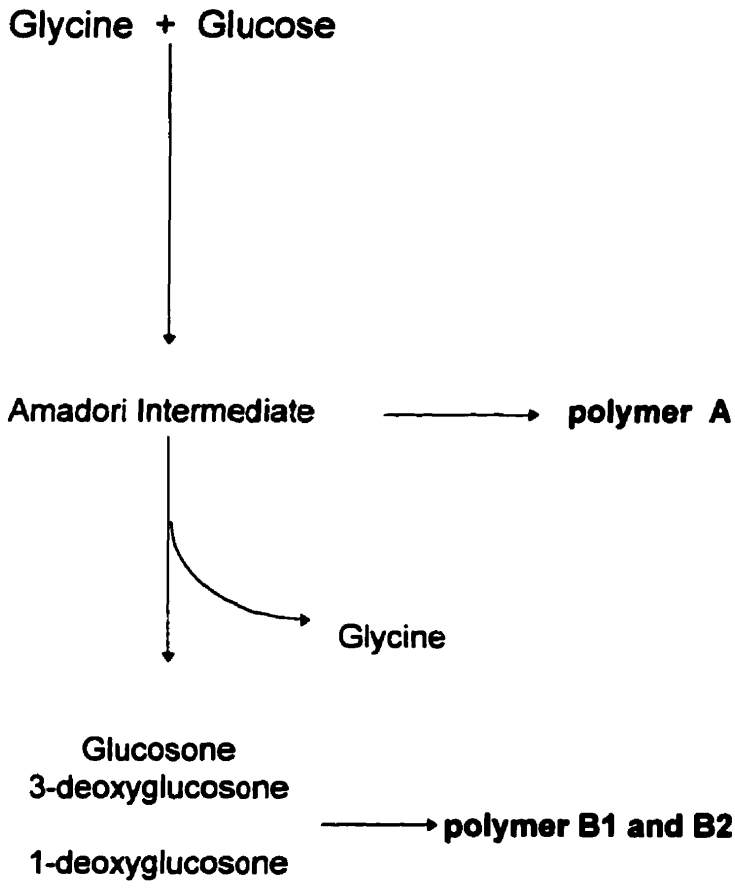
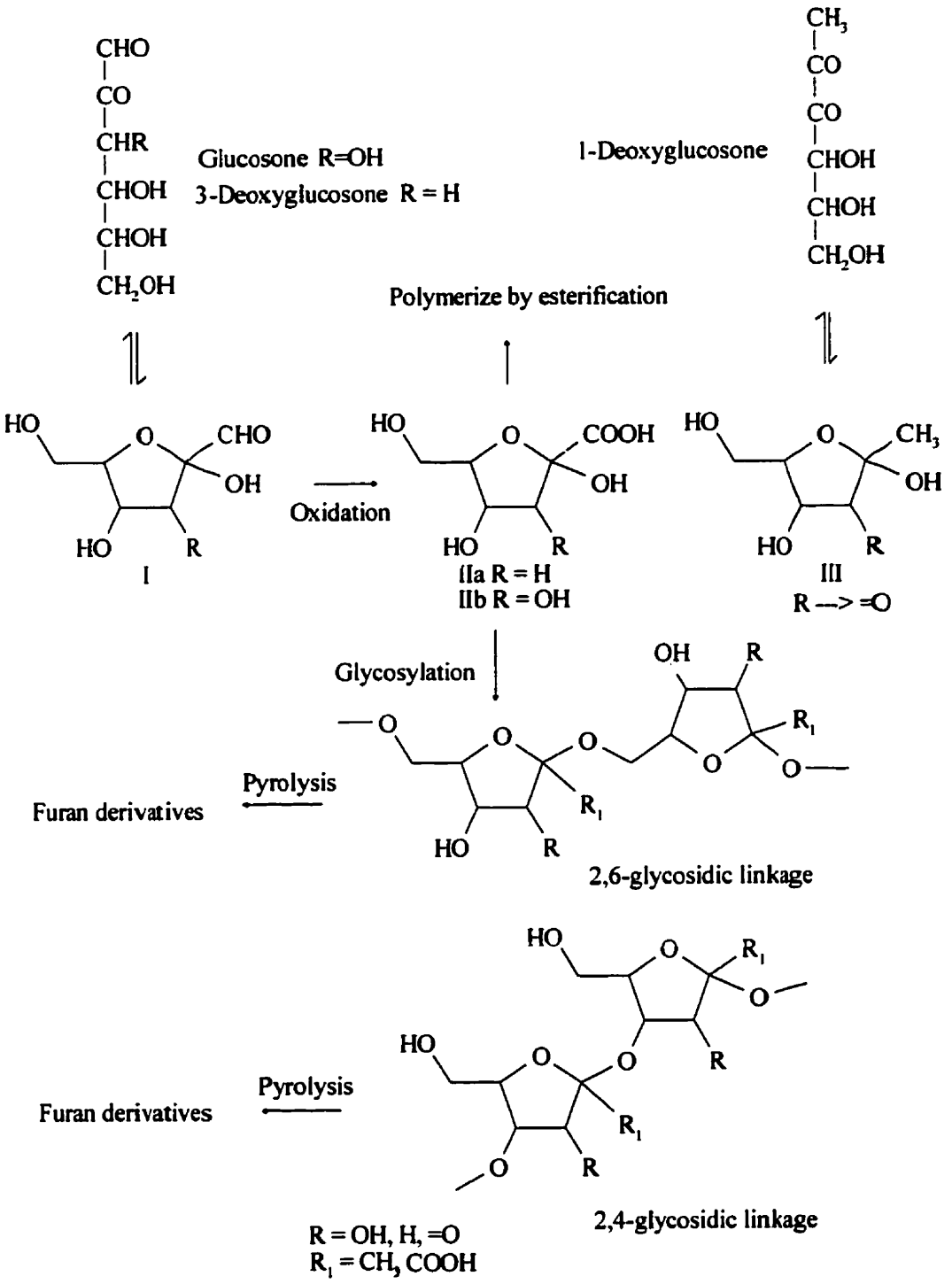


Figure 6.1 Intermediates in polymer formation

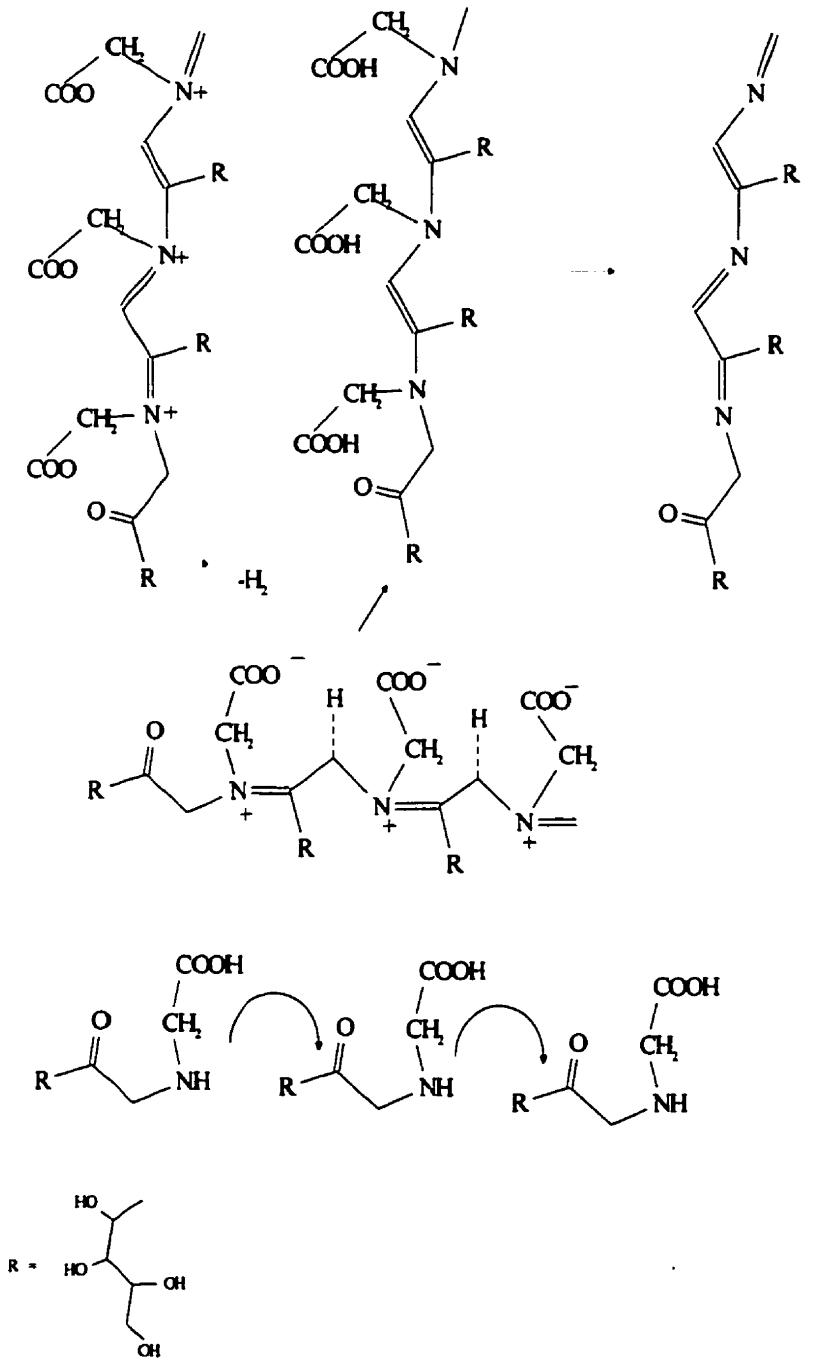


Scheme 6.2 Proposed polymerization reaction (polymer B1 and B2)

6.3. PROPOSED STRUCTURE OF THE NITROGEN CONTAINING POLYMER

Elemental analysis of the polymer A (Table 5.8) indicates that the empirical formula ($C_7H_{11}N_1O_4$) differs from that of glycine Amadori product ($C_8H_{15}N_1O_7$) by a CH_4O_3 unit. This implies a loss of a molecule of CO_2, H_2O and H_2. FTIR analysis confirms the presence of CH_2, C-O-H and COO^- and / or conjugated moieties. Due to the dark brown color of this polymer (Table 5.1), the strong absorption band at 1607 cm^{-1} in the FTIR spectrum is mainly attributed to extensive conjugation and partially to the presence of carboxylate moiety. Pyrolysis of polymer A (Table 5.5) generates components typical to Amadori products such as pyrazines, pyrroles, pyridines, furans, etc. In addition, the presence of 2,3-dihydro-3,5-dihydroxy-2-methyl-4H-pyran-4-one, in the pyrolysis products of polymer A, strongly suggests that the polymer is formed by minimal dehydration at the sugar moiety. Based on the experimental observations, a mechanism is proposed for the formation of polymer A as depicted in Scheme 6.2. According to this scheme, Amadori products can polymerize through nucleophilic addition reactions of amino groups to the carbonyl moieties of a second molecule, followed by dehydration to form the zwitterionic polymer I. The polymer I can either lose a hydrogen molecule to form conjugated zwitterionic polymer IIa or undergo an intramolecular hydrogen transfer to form a neutral derivative IIb which can be converted into the conjugated derivative III, through the loss of a succinic acid moiety as depicted in Scheme 6.2. The structure of the isolated polymer could incorporate in different percentages, the above mentioned polymeric moieties I, IIa, IIb and III. The calculated empirical formula reflects an average of the latter.

II a II b III



Scheme 6.3 Proposed mechanism of the polymer A formation

7. CONCLUSION

Three different polymers have been isolated from a group of high molecular weight fraction in glycine-glucose model system and tentative mechanism of their formation was proposed. Elemental analysis indicated empirical formula for polymer A as $C_7H_{11}N_1O_4$ and for B1 and B2 same as glucose. Polymers B1 and B2 are proposed to be formed through polymerisation of reactive dicarbonyls such as 1-deoxyglucosone and 3-deoxyglucosone. Polymer A is proposed to be formed from Amadori product by the loss of a CH_4O_3 (CO_2, H_2O, H_2). Determination of the structures of all three polymers was made difficult due to their structural complexity and high molecular weight. However, spectroscopic methods of analysis such as UV/VIS, Py/GC/MS, FTIR in conjunction with chemical analysis was quite useful in their structural determination.

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IMAGE EVALUATION
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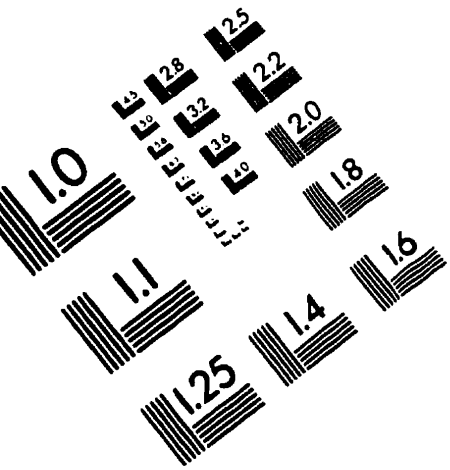

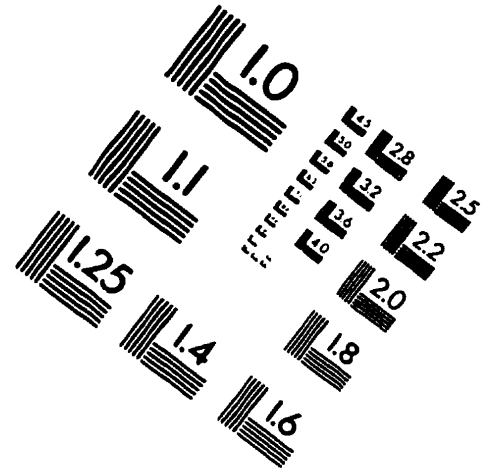

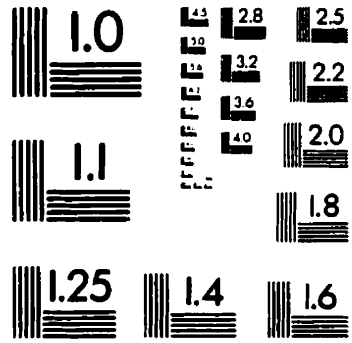

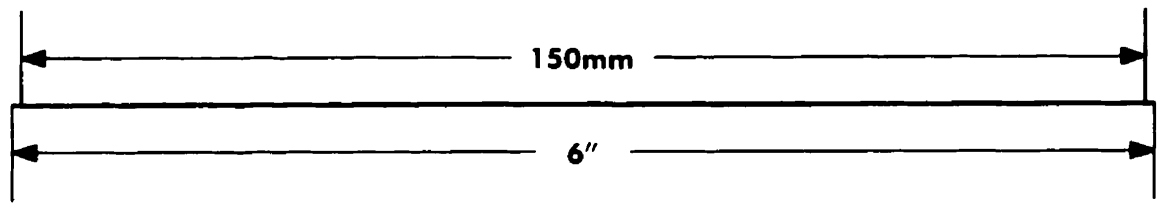

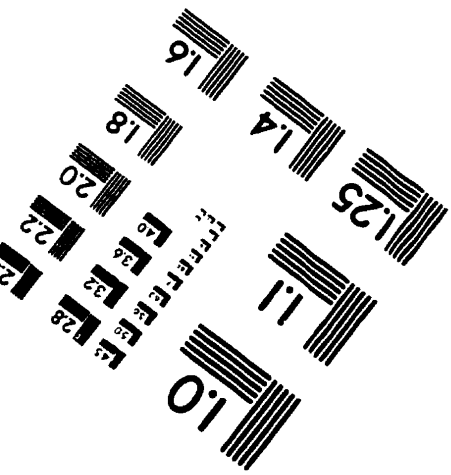

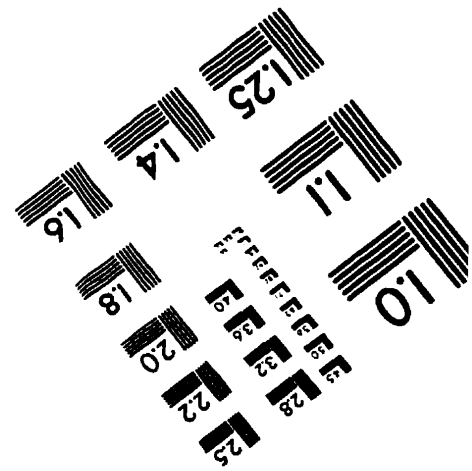

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