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ORIGINAL ARTICLE

Formation of radicals during heating lysine and glucose in solution with an intermediate water activity

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Abstract

Heating glucose with lysine under alkaline conditions (pH 7.0–10.0) was found to take place with consumption of oxygen together with formation of brown-colored compounds. Highly reactive intermediary radicals were detected when lysine and glucose were heated at intermediate water activity at pH 7.0 and 8.0. The detection was based on initial trapping of highly reactive radicals by ethanol followed by spin trapping of 1-hydroxyethylradicals with α-(4-pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN) and Electron Spin Resonance (ESR) spectroscopy. The generation of reactive intermediary radicals from the Maillard reactions was favored by enhancing alkaline conditions (pH 8.0) and stimulated by presence of the transition metal ion Fe^{2+}. The stability of the nitrone spin traps, *N*-*tert*-butyl-α-phenylnitrone and POBN was examined in buffered aqueous solutions within the pH range 1–12, and found to be less temperature dependent at acidic pH compared to alkaline conditions. A low rate (k_{obs}) of hydrolysis of POBN was found at the used experimental conditions of 70°C and pH 7.0 and 8.0, which made this spin trap method suitable for the detection of radicals in the Maillard reaction system.

Keywords: Maillard reactions, intermediary radicals, ESR, spin trapping, oxidation, stability of spin traps

Introduction

The Maillard reactions (MR) generate aroma, flavor compounds, and brown-colored polymers responsible for the characteristic taste and appearance of heat-processed foods. However, compounds known to induce a risk to human health are also formed concurrently. This includes so-called Advanced Glycation End products (AGEs), which are also formed at slower rates and at lower temperatures in the body. The AGEs formed either endogenously or derived from the diet have been associated with development of diabetes and cardiovascular diseases [1,2]. Rate of formation and final content of AGEs in food are influenced by many factors such as pH, heating temperature, time, water activity (a_w), as well as availability of precursors, which most often are reducing sugars, free amino acids, or proteins.

The pathways of the MR in food have been described and studied extensively since its discovery more than 100 years ago by the French chemist Luis Camille Maillard [3]. However, many mechanistic details remain unresolved due to analytical difficulties related to the complex combinations of intermediates and products, and the involvement of high temperatures used during heat processing of foods. At low pH (pH 5.0) the MR is mainly taking place by polar reactions (ionic pathways), while at alkaline conditions oxidative conditions are increasingly favored thereby promoting formation of intermediary radicals [4]. Stable intermediary radicals have been detected by electron spin resonance (ESR) spectroscopy formed as part of the MR. The radicals were found to be generated prior to the Amadori rearrangement by the so-called Namiki pathway probably originating from degradation of the Schiff base [5,6]. Formation of these dialkylpyrazinium radical cations is pH dependent with low levels formed at neutral pH and with increasing levels up to pH 11 [7]. However, other highly reactive intermediary radicals derived from oxidative pathways involving metal-catalyzed degradation of central intermediates also seem to be involved in prolongation of MR [8,9]. Less detailed information exists about their nature and formation, due to difficulties related with detection of short-lived and highly reactive radicals in complex systems. The spin trapping technique can be used to study short-lived radicals. It is a technique, where short-lived radicals are trapped and converted to stable spin adducts with sufficient lifetime for detection by ESR [10]. The majority of spin traps are nitrones such as α-(4-pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN) and α-(4-pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (PBN), which can react with a broad range of radicals, that is, C-, N-, O-, and S-centered radicals, and thereby produce long-lived nitroxyl radicals as spin adducts. Most studies involving the spin trapping technique have been performed under physiological conditions, that is, pH 7.0–7.4, and in the temperature range 20–40°C [11]. However, detection of radicals as intermediates during oxidative degradations or MR in

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heat-processed foods and beverages is becoming relevant, and the high temperatures together with the fact that food systems have pH values ranging from 2.0 to 8.0 makes it necessary to test the stability of spin traps under these conditions. Hydrolysis of nitrone spin traps is likely to be enhanced under such conditions leading to the formation of an aldehyde and a hydroxylamine (reaction 1).

$$ArCH = N(O)R + H_2O \rightarrow ArCHO + HN(OH)R \quad (1)$$

The aim of this study was to examine formation of unstable intermediary radicals during the reactions of glucose with lysine at an elevated temperature [12]. This was used as a model of a low molecular weight (LMW) food-related MR system, in order to explore the effects of pH, water activity, a_w, and presence of transition metals like iron on radical pathways of the MR. Furthermore, the thermal hydrolysis of the spin traps PBN and POBN at both acidic and alkaline conditions were characterized with the aim of establishing their stabilities under the used reaction conditions.

Material and methods

Chemicals

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (99.5%), 2-(*N*-morpholino) ethanesulfonic acid (MES) (99.5%), POBN (95%), PBN (purity 95%), L-lysine (98%), and D-(+)-glucose (99.5%) was from Sigma-Aldrich (St. Louis, MO, USA). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide] (DEPMPO) (99%) was from Alexis Biochemicals (Lausen, Switzerland). Ethanol (96%) was from Kemetyl A/S (Køge, Denmark). 3-Deoxyglucosone (CAS 4084-27-9) (95%) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and hydrogen peroxide (30%) were from Merck (Darmstadt, Germany). Water was purified through a MilliQ purification train (Millipore, Bedford, MA, USA). The concentration of hydrogen peroxide was determined spectrophotometrically ($\varepsilon_{240} = 39.4\ M^{-1} \cdot cm^{-1}$). MES buffer (5 mM) and HEPES buffer (200 mM) adjusted to ionic strength = 0.16 were prepared from the corresponding analytical grade chemicals using MilliQ water and adjusted to pH 7.0 and 8.0.

Kinetic studies of spin trap hydrolysis

Kinetic experiments of thermal and acid/alkaline hydrolysis of POBN and PBN were performed by dissolving 40–50 μM of the spin traps in buffers (concentration 0.10–0.60 M) made from either phosphoric acid, dichloroacetic acid, formic acid, citric acid, and acetic acid for acidic conditions, and sodium hydroxide and potassium hydroxide, for alkaline conditions. The ionic strength of all buffers was adjusted to 1.0 with potassium chloride. The hydrolysis of POBN and PBN were followed at the pH ranging from 1.0 to 12.4 at 40°C. The acidic hydrolysis (pH 1.0–5.0) was also studied at 20 and 60°C. Whereas the alkaline degradation of POBN also was followed at pH 10.0–12.0 at two temperatures: 60 and 70°C. Experiments running for less than 24 h were started by adding 15 μL of stock solutions of POBN or PBN (6.0 mM in ethanol) to 2.25 mL preheated buffer in a cuvette placed in a thermostated cell holder in a Hewlett-Packard HP 8453 diode array spectrophotometer (Palo Alto, CA, USA). Experiments running for more than 24 h were carried out by adding 200 μL of stock solutions of POBN or PBN (6.0 mM in ethanol) to 25 mL preheated buffer in capped 25 ml blue-cap bottles placed in a thermostated water bath. Samples (2.5 mL) were taken at varying intervals and the UV-spectra were recorded. The decay of PBN was followed by measuring the absorbance at 287 nm, while formation of benzaldehyde was followed at 250 nm. Similarly, the decay of POBN was followed by measuring the absorbance at 330 nm, while formation of the aldehyde was followed at 262 nm.

Development of a model system with Fenton reaction

A model system appropriate for detection of radicals formed during heating at intermediary a_w conditions was developed by testing different solvents. MES buffer (5 mM) adjusted to pH 7.0 or 8.0 was mixed with glycerol (30% and 60%), ethylene glycol (30% and 60%) or ethanol (5%, 30%, and 60%). Radicals were generated in the model systems by the Fenton reaction after addition of $FeSO_4 \cdot 7H_2O$ (0.1 mM) and H_2O_2 (0.3 mM) and detected by ESR as spin adducts after reaction with POBN, which was added to a final concentration of 10 mM. Samples (100 μL) were transferred into a 2 mL Eppendorf tube and heated in a glycerol bath at 70 ± 1.0°C for 10, 20, 30, 40, and 50 min prior to analysis.

Preparation of LMW MR model system

LMW MR model systems were prepared by dissolving glucose (0.1 M), lysine (0.1 M), or the mixture of glucose (0.1 M) with lysine (0.1 M) in HEPES buffer adjusted to pH 7.0 and pH 8.0 with 30% ethanol ($a_w = 0.88$) together with POBN (10 mM). The model systems with 3-deoxyglucosone (0.04 M) were prepared by dissolving in HEPES buffer adjusted to pH 7.0 and 8.0 with 30% ethanol ($a_w = 0.88$) together with POBN (10 mM). Iron was added as $FeSO_4 \cdot 7H_2O$ (0.1 mM) to the model systems. Samples (100 μL) were transferred into a 2 mL Eppendorf tube and placed in a glycerol bath at 70 ± 1.0°C for 10, 20, 30, 40, and 50 min prior to analysis.

Water activity (a_w) measurements

The a_w was measured at room temperature by Aqua Lab from ADAB Analytical Devices Ab (Stockholm, Sweden) or calculated by the Raoult's Law [13] for the solutions with 30% and 60% ethanol: $a_w = n/(n + n')$, where n = moles of water and n′ = mole of solute ethanol. All measurements were performed in duplicates.

pH measurements

pH was measured in the samples with a Metrohm 6.0234.100 combination glass electrode from Hamilton Bonaduz AG (Bonaduz, Switzerland) connected to a Metrohm 713 pH-meter from Metrohm (Herisau, Switzerland), which was calibrated at the relevant temperatures with standard buffers of pH 4.01 and 7.00. All measurements were performed in duplicates.

ESR spectroscopy

Samples were transferred to 50 µL ESR micropipettes from BLAUBRAND IntraMark (Wertheim, Germany) and placed in the resonator of the Miniscope MS 200 ESR spectrometer from Magnettech GmbH (Berlin, Germany). The following ESR parameters were used: center field, 3357 G; sweep width, 100 G; sweep time, 30 s; microwave power, 10 mW; and modulation amplitude, 1000 mG. The middle duplicate peak-to-peak amplitude of the ESR signal of the spin adducts was measured by the Analysis 2.02 software program (ESR applications, Berlin, Germany). All measurements were performed in duplicates.

Oxygen consumption assay

The oxygen consumption was carried out with lysine (1.0 M), glucose (1.0 M), or glucose (1.0 M) with lysine (1.0 M) dissolved in MilliQ water during heating at 45°C. pH was adjusted with HCl or NaOH to pH 7.0, 8.0, 9.0 or 10.0 using the buffer capacity of lysine itself. A HQ30d electrode connected to HQd meter (HACH LANGE, Germany) was used for oxygen measurements. The electrode was calibrated before use by air-saturated MilliQ water at 25°C. The measurement was started immediately after placing the electrode in 20 mL of sample. Measurement time was adjusted according to pH due to a pH dependent difference in oxygen consumption. All measurements were performed in duplicates.

Measurement of brown polymers

The change of absorbance at 420 nm was used as a measure for development of brown products from lysine (1.0 M), glucose (1.0 M) or glucose (1.0 M) with lysine (1.0 M) dissolved in MilliQ water. pH was adjusted with HCl or NaOH to pH 7.0, 8.0, 9.0, or 10.0 using the buffer capacity of lysine. The samples were heated at 45°C and the absorbance measured in the spectral range 250–500 nm by an HP8453 UV/VIS diode array spectrophotometer from Hewlett-Packard Co. (Palo Alto, CA). All measurements were performed in duplicates.

Results

Reactions of glucose and lysine

The role of oxidative reactions in the Maillard reactions was initially examined by monitoring the oxygen consumption during heating solutions containing either glucose, lysine, or glucose mixed with lysine. No substantial oxygen depletion was observed during heating of glucose alone. Oxygen was consumed during heating of the lysine solution and the rate of oxygen consumption had a maximal rate at pH 8.0 (Figure 1). The rate of oxygen consumption in the mixed glucose and lysine solution increased when enhancing pH from 7 to 10 (Figure 1). During heating of the mixed lysine glucose system brown-colored products were also generated that absorbed light at 420 nm and this formation of brown-colored MR products increased with pH with a maximum at pH 9 (Figure 1). Heating either glucose or lysine alone did not generate colored compounds under the same conditions. The results suggest the MR between glucose and lysine is coupled to oxidation reactions, since the high rates of oxygen consumption also occurred at pH 9 and 10 where the formation of brown compounds was most pronounced.

Stability of spin traps

Studying the possible radical formation at high temperatures in acidic or alkaline solutions requires knowledge about the stability of spin traps under these conditions. The stability of the two spin traps PBN and POBN at varying temperatures and pH was studied by following

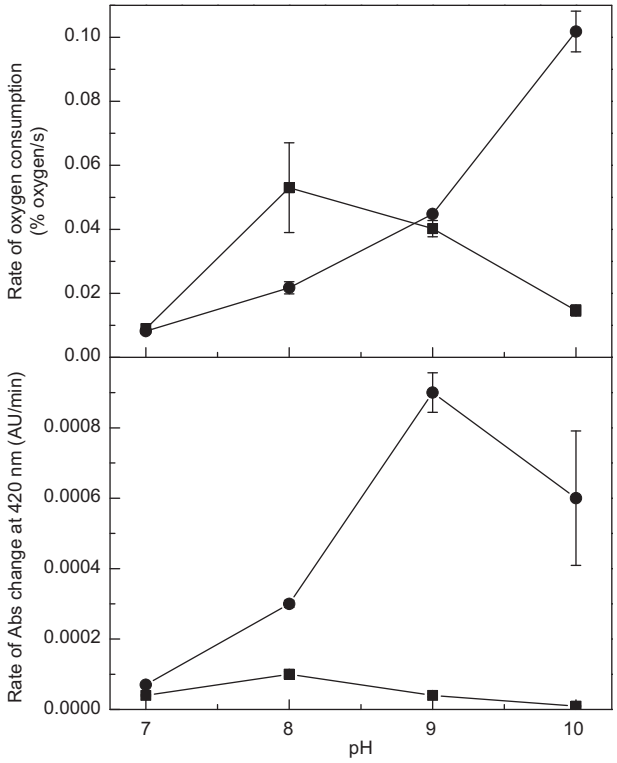

Figure 1. Above: Rate of oxygen consumption during heating (at 45°C) in a closed system with lysine (1.0 M) (■), or glucose (1.0 M) with lysine (1.0 M) (●) dissolved in MilliQ water and pH adjusted with either HCl or NaOH. Bottom: Rate of formation of brown-colored products as determined by absorbance at 420 nm during heating (at 45°C) of lysine (1.0 M), (■) and glucose (1.0 M) with lysine (1.0 M) (●) dissolved in MilliQ water and pH adjusted with HCl or NaOH.

the time-dependent changes of UV-absorption in buffered solutions. Generally, isosbestic points were observed in the UV-spectra during the decay of the spin traps indicating a reaction, where formation of products takes place without accumulation of intermediates (Figure 2A). The decay of spin traps and the formation of products were both found to follow first-order kinetics with identical rate constants at a given pH and temperature (Figure 2B). The UV-spectra observed at the end of the reactions were identical to the spectra of the aldehydes expected to be formed by hydrolysis of the nitrone spin traps, that is, benzaldehyde in the case of PBN and 4-pyridinecarboxaldehyde *N*-oxide in the case of POBN (Figure 3). In both cases *tert*-butylhydroxylamine is also formed, however, it is not expected to absorb light with wavelengths longer than 200 nm and does not contribute to the UV-spectra.

In acidic and alkaline solutions, the decay of the spin traps could be followed for more than two or three half-lifes, which allowed reliable determination of the observed first-order rate constants, k_{obs}. However, in neutral solutions the decays of both spin traps were extremely slow and could only be followed during a time period that was shorter than one half-life. Under these conditions, k_{obs} was determined assuming a first-order decay. The observed first-order rate constants, k_{obs}, for the hydrolysis of POBN and PBN increased under both acidic (pH 1–7) and alkaline (pH 10–12) conditions during heating at 40°C

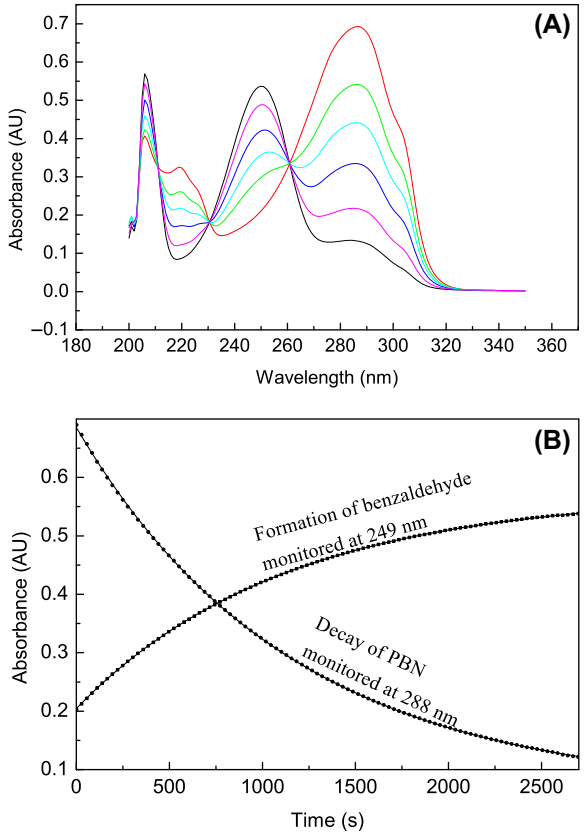

Figure 2. Spectra (A) and time traces (B) recorded during the hydrolysis of PBN (40 μM) in a phosphoric acid buffer (pH = 1.68) at 20°C.

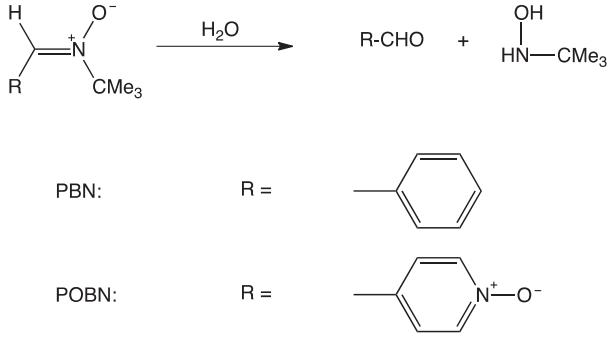

Figure 3. Hydrolysis of spin traps.

indicating specific acid and base catalysis (Figure 4). The hydrolysis of POBN was fastest under basic conditions, while the hydrolysis of PBN was fastest under acidic conditions.

For hydrolysis of PBN the pH dependence of the observed first-order rate constants, k_{obs}, could be satisfactorily fitted to equation (2) (Figure 4 and Table I). This equation is based on the assumption that the hydrolysis is taking place by three competing pathways, where k_1 is the first-order rate constant for a noncatalyzed hydrolysis, k_H is the second-order rate constant for a specific acid-catalyzed hydrolysis, and k_{OH} is the second-order rate constant for a specific base-catalyzed hydrolysis. K_w is the ionization constant for water.

$$k_{obs} = k_1 + k_H \cdot [H^+] + k_{OH} \cdot K_w/[H^+] \quad (2)$$

For hydrolysis of POBN a truncated version of equation (2), where k_1 was omitted, gave good fits to k_{obs} (Figure 4). The V-shape of the logarithmic plot of k_{obs} as a function of pH suggests that $\log(k_1)$ must be lower than −8.5 for POBN at 40°C. The hydrolysis of POBN and PBN in acidic solutions (pH < 5) was also studied at 20 and 60°C (data not shown). In the acidic solutions, k_H could be determined using the simplified equation, $k_{obs} = k_H \cdot [H^+]$, which adequately described the pH-dependence

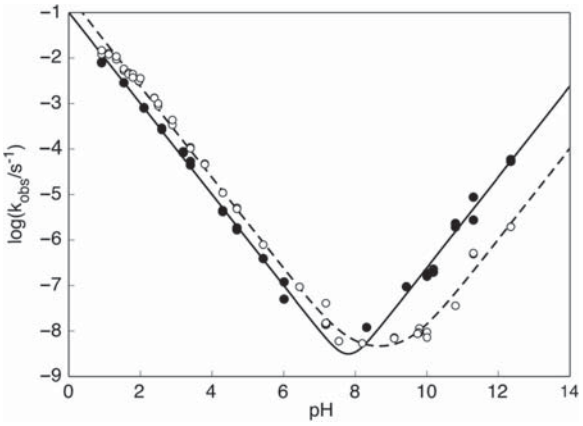

Figure 4. Logarithmic plot of observed first-order rate constants for hydrolysis of PBN (○), and POBN (●) as a function of pH at 40°C and ionic strength 1.0. The dashed line shows the best fit to the expression $k_{obs} = k_1 + k_H \cdot [H^+] + k_{OH} \cdot K_w/[H^+]$ for the PBN data, and the full line shows the best fit of $k_{obs} = k_H \cdot [H^+] + k_{OH} \cdot K_w/[H^+]$ for the POBN data.

Table I. Kinetic data for the hydrolysis of PBN and POBN at 40°C and I = 1.0.

	Spin traps	
Rate constant	PBN	POBN
k_1	3.7×10^{-9} s^{-1}	–
k_H	0.24 M$^{-1}\cdot$s^{-1}	0.10 M$^{-1}\cdot$s^{-1}
$k_{OH}\cdot K_w$	1.0×10^{-18} M$\cdot$s^{-1}	2.4×10^{-17} M$\cdot$s^{-1}

of k_{obs}. Based on an Arrhenius plot of k_H, the activation energies, E_a, for the acid catalyzed hydrolysis were determined to be 16.8 kcal/mol for POBN and 16.7 kcal/mol for PBN.

The observed rate constants, k_{obs}, of thermal alkaline hydrolysis of POBN at 70°C were determined at pH 10, 11, and 12. Based on a linear extrapolation of these rate constants k_{obs} at pH 7.0 and pH 8.0 were estimated to be 1.2×10^{-6} s^{-1} and 1.6×10^{-5} s^{-1}, respectively. Based on these rate constants, it was estimated that more than 95% of POBN will be present after 50 min heating at pH 8.0, which makes it suitable to use POBN as spin trap under the conditions used in the present study of the Maillard reactions.

Model system with intermediate water activity

The Maillard reactions are known to proceed at a high rate in systems with intermediate water activities. A MES-buffered model system with an intermediate a_w was therefore developed to obtain optimal conditions for radical formation during the MR between glucose and lysine. Different solvents glycerol, ethylene glycol, and ethanol were tested according to their ability to decrease a_w (Table II) and the ability to detect highly reactive radicals. The latter was tested by generating radicals by the Fenton reaction by addition of $FeSO_4$ (0.1 mM) and H_2O_2 (0.3 mM) (reactions 3–5), and evaluating the intensity of the ESR signals of spin adducts obtained by trapping radicals with the spin trap POBN in combination with added ethanol (reactions 5 and 6). All systems contained at least 5% ethanol to achieve an improved sensitivity toward trapping reactive radicals such as alkoxyl and hydroxyl radicals [20]. Hydroxyl radicals formed by the Fenton reaction abstract hydrogen atoms from ethanol leading to 1-hydroxyethyl radicals, which after being trapped by POBN is converted into spin adducts that are detectable by ESR [14].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (3)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\cdot}OOH + H^+ \quad (4)$$

$${}^{\cdot}OH + CH_3CH_2OH \rightarrow H_2O + CH_3C\cdot HOH \quad (5)$$

$$CH_3C\cdot HOH + POBN \rightarrow \text{spin adduct} \quad (6)$$

Model systems based on glycerol and ethylene glycol were first tested. Addition of glycerol and ethylene glycol to the MES buffer resulted in a lowering of a_w, however, the amount of detected radicals decreased with increasing concentrations of glycerol or ethylene glycol (data not shown). Radicals were only observed at room temperature in the presence of 30% glycerol, while increasing the concentration of glycerol to 45 and 65% prevented detection of radicals. Glycerol therefore seemed to quench radicals in a concentration dependent manner. No clear pattern was found between the amounts of radicals detected and a_w.

Table II. Water activity (a_w) of model systems.

Ethanol (%)	MES buffer (%)	a_w
Ethanol model systems		
5	95	0.98*
30	70	0.88*
60	40	0.68*

Glycerol (%)	MES buffer (%)	Ethanol (%)	a_w
Glycerol model systems			
30	65	5	0.93 ± 0.01
45	50	5	0.83 ± 0.02
65	30	5	0.66 ± 0.01

Ethylene glycol (%)	MES buffer (%)	Ethanol (%)	a_w
Ethylene glycol system			
65	30	5	0.89 ± 0.02

*Calculated by Raoult's Law.

In model systems with only ethanol, Raoult's Law was used to calculate a_w according to equation, where n is moles of water and n′ is moles of ethanol (Table II) [13].

$$a_w = n/(n + n') \quad (7)$$

The rate of formation of radicals in the MES-buffered ethanol model systems was also found to be concentration dependent, and radicals formed by the Fenton reaction were detected even at room temperature (Figure 5). A significant higher amount of radicals was detected in the model system with 30% ethanol compared to the model systems with 5% and 60% ethanol. The observed ESR spectra (see Figure 6) were triplets of doublets with coupling constants $a_N = 15.2$ gauss and $a_H = 2.5$ gauss, typical for spin adducts of 1-hydroxyethyl radicals with POBN [14]. In the model system with 5% ethanol, the concentration of ethanol seemed to be too low to react with all the radicals formed, leading to fewer radicals detected compared to the model system with 30% ethanol. The same were seen at high ethanol concentration (60%), where less radicals were detected compared to 30% ethanol, probably due to an overload of radicals in the model system leading to reactions between spin adducts and radicals, which reduce concentration of ESR-detectable paramagnetic spin adducts. POBN is known to trap R$^{\cdot}$ and RO$^{\cdot}$ radicals, whereas the spin trap DEPMPO has an affinity to broader range of radicals, such as $^{\cdot}OH$, $O_2^{\cdot -}$, $RO_2^{\cdot}$, R$^{\cdot}$, and sulfur radicals, and it was therefore also tested in the model system. However, detection of spin adducts from DEPMPO decreased with increasing heating time (data not shown), probably due to a shorter lifetime of the spin adducts, and this spin

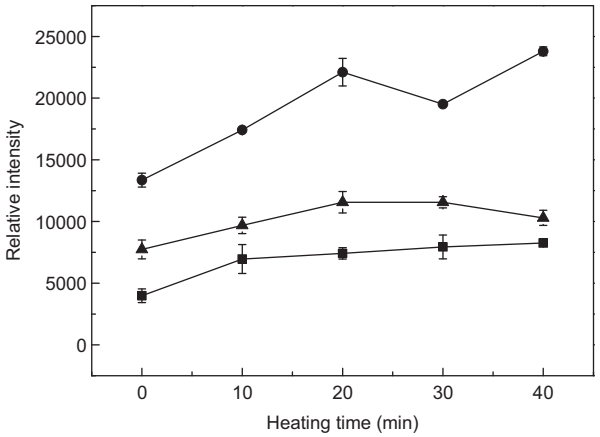

Figure 5. Intensity of spin adducts generated by mixing $FeSO_4$ (0.1 mM) and H_2O_2 (0.3 mM) and trapping radicals with POBN (10 mM) during heating at 70°C in mixtures of MES buffer (pH 7.0) and varying amounts of ethanol: (■) 5% ethanol, (●) 30% ethanol, and (▲) 60% ethanol.

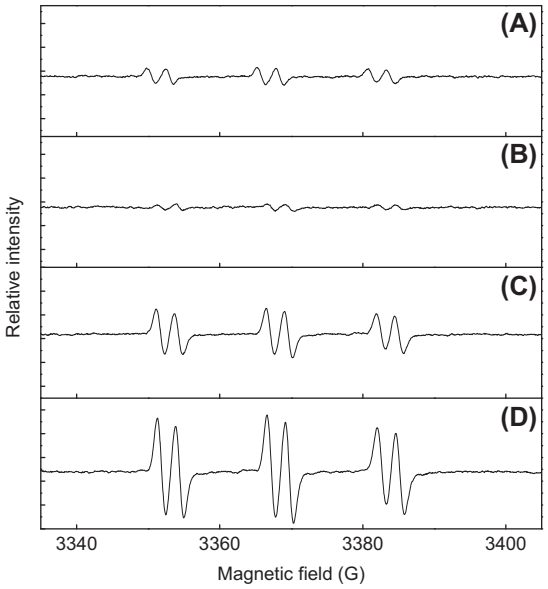

Figure 6. Electron spin resonance (ESR) spectrum of spin adducts generated by trapping radicals with POBN (10 mM) in 30% EtOH/MES buffer (pH 7.0) containing: A) $FeSO_4$ (0.1 mM) and H_2O_2 (0.3 mM) B) Without addition. C) Lysine (0.1 M). D) Glucose (0.1 M) and lysine (0.1 M). All systems were heated at 70°C for 30 min before recording the ESR spectra. The peak-to-peak amplitude of the second doublet peak was used for quantification.

trap was therefore not found suitable for experiments involving heat treatment.

Detection of radicals in glucose and lysine reactions

Based on the pre-trials a 30% ethanol MES-buffered model system with POBN as spin trap was selected as an example of a LMW MR model system for studies of formation of intermediary radicals from lysine and glucose during heating. Formation of radicals was detected during heating of the model system containing glucose and lysine and the amounts of spin adducts increased with increasing pH (Figure 7). Spin adducts were only detected in very low levels by heating glucose alone under the given conditions. Autoxidation of monosaccharides has previously been suggested to occur through an enediol tautomer involving enediol oxy radicals, when examined in phosphate model system at pH 7.4 and 37°C using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as spin trap and detection by ESR. The consequence is loss of monosaccharide, oxygen

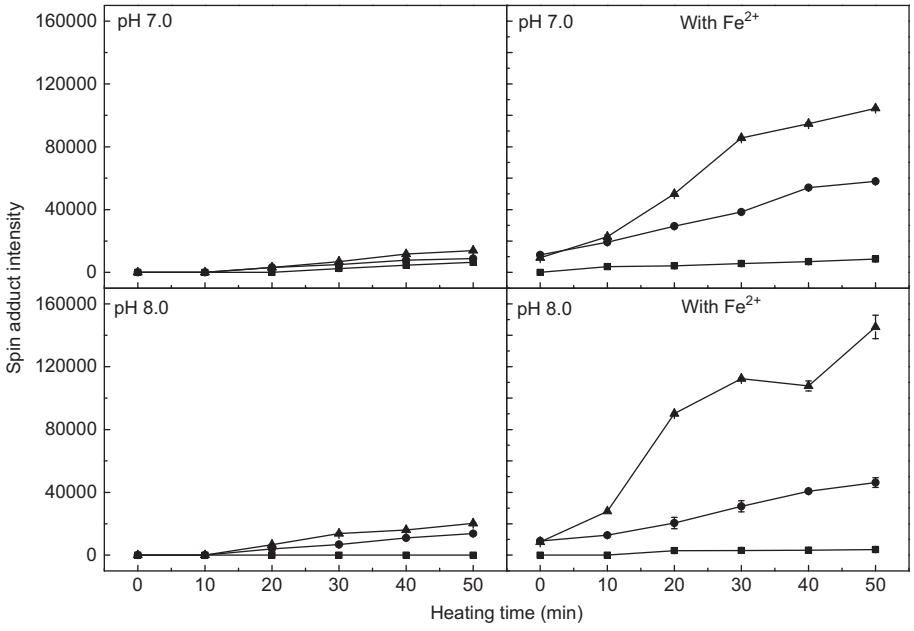

Figure 7. Formation of spin adducts in 30% ethanol/HEPES model system at pH 7.0 (above) or in 30% ethanol/HEPES model system at pH 8.0 (below) with and without addition of $FeSO_4$(0.1 mM). (■) Control with POBN alone. (●) With added lysine (0.1 M). (▲) With added glucose (0.1 M) and lysine (0.1 M). All systems contained POBN (10 mM) and were heated at 70°C for 30 min before recording the ESR spectra.

consumption, and formation of α-oxo-aldehyde, and the formation of hydroxyl and hydroxyalkyl radicals appear also to be involved [11].

Spin adducts derived from 1-hydroxyethyl radicals were detected by heating lysine alone due to heat-induced oxidative degradation of lysine, and similar spin adducts were also formed by heating lysine together with glucose (Figure 6). The levels of spin adducts increased when heating lysine together with glucose as compared to heating lysine alone (Figure 7). More radicals were detected during heating glucose with lysine at pH 7.0 and 8.0 for 50 min at 70°C, as compared to heating lysine alone under similar conditions (68% and 56%, respectively). A catalytic effect of the transition metal Fe^{2+} was observed on formation of the 1-hydroxyethyl radical spin adducts both from lysine alone and lysine mixed with glucose (Figure 7). The presence of transition metals like iron induces the formation of radicals as part of the Maillard reactions as has previously been found for copper induced autoxidation of Amadori products or monosaccharaides [11,15]. Addition of $FeSO_4$ increased the formation of radicals, where 48% and 70% more spin adducts were formed in the glucose/lysine system compared to heating lysine alone at pH 7.0 and pH 8.0. Fe^{2+} catalyzes the oxidation of lysine more efficiently at pH 7.0, whereas Fe^{2+} has a higher catalytic effect on formation of radicals from glucose and lysine at pH 8.0.

1-Hydroxyethyl spin adducts were also found to be formed by heating the central MR intermediate, 3-deoxyglucosone, alone at pH 8.0 (Figure 8). Addition of $FeSO_4$ also accelerated the radical formation. Heat-induced oxidative degradation of 3-deoxyglucosone probably leads to formation of glucosone and carboxylic acids, but may also involve a radical mechanism [16]. Radical containing sugar fragments formed from a C3–C4 cleavage of 3-deoxyglucosone has previously been found to be generated in solution at room temperature in dimethyl sulfoxide [17].

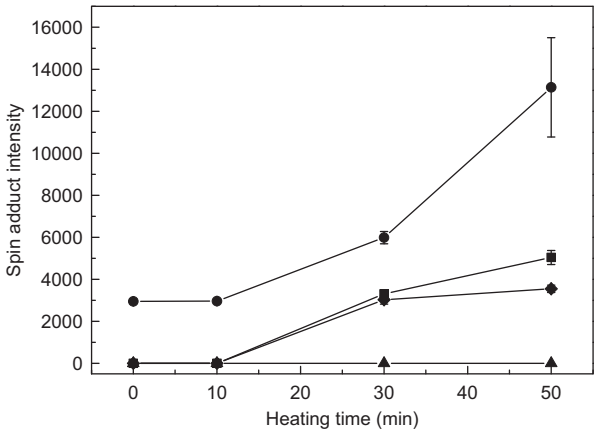

Figure 8. Spin adducts formed by POBN (10 mM) during heating at 70°C in 30% ethanol/70% HEPES model system at pH 8.0 with and without the addition of Fe^{2+}; (▲) control with POBN alone, (◆) with added $FeSO_4$ (0.1 mM), (■) 3-deoxyglucosone (0.04 M), and (●) 3-deoxyglucosone (0.04 M) with $FeSO_4$ (0.1 mM).

The pathways of the Maillard reactions have been attempted explained by Hodge involving the Amadori rearrangement [18], but alternative routes involving the cleavage of sugars with liberation of glycolaldehyde imine have been proposed [19]. Dimerization and oxidation of glycolaldehyde imine lead to formation of 1,4-dialkylpyrazinium radical cations, which are stable enough to be detected directly by ESR, when alanine is heated at 95°C with pentoses or hexoses [20]. Therefore LMW MR model systems without a spin trap were heated under the same conditions to test if 1,4-dialkylpyrazinium radical cations were formed. However, no signal were observed, and the amount of 1,4-dialkylpyrazinium radical cations from reacting glucose with lysine at these conditions of high ethanol content and slightly decreased a_w was below the detection limit of the instrument.

Discussion

Intermediary radicals with high reactivity and short-lived existence are formed as part of the Maillard reactions, but little is known about the mechanism of formation, since their direct detection by ESR is difficult. Spin traps were used in the present study to trap unstable reactive radicals during the heating lysine and glucose leading to formation of stable spin adducts detected directly by ESR. The applied model system contains ethanol, which both reduces the water activity of the system, but also acts as an initial radical trapping agent, thereby enhancing the detection of radical intermediates [14]. The exact identity of the formed radicals is unknown, since they are detected as spin adducts of POBN and 1-hydroxyethyl radicals. However, the radicals formed in the LMW MR model systems were able to abstract hydrogen atoms from ethanol prior to detection, and must therefore be of highly reactive nature such as hydroxyl radicals or alkoxyl radicals. These radicals formed in the LMW MR model systems can possibly be derived from several pathways involving autoxidation of lysine or MR intermediates, which both are catalyzed by iron. Oxidative fragmentation of sugars into C2-, C3-, and C4-sugar fragments involving radical mechanisms is also expected to occur in the LMW MR model system [17] as well as degradation of deoxyxones leading to the formation of H_2O_2 and hydroxyl radicals [9] could lead to highly reactive radicals. Reactive oxygen species (ROS) formed by autoxidation of Maillard reaction products such as Amadori compounds have also previously been suggested [9]. However, the 1,4-dialkylpyrazinium radical cations which have been suggested as initial intermediates in the MR, is not expected be able to abstract hydrogen atoms from ethanol, due to the delocalized nature of the unpaired spins, and they will therefore not give rise to formation of spin adducts in the model systems in the present study [10].

The results obtained using the model systems in this study indicate that radicals will be formed as part of the MR that take place during heat processing of foods and

that heating time and pH conditions will be important factors. However, the presence of other compounds in the food matrix would be important for the involvement of highly reactive radicals in the MR, such as the availability of transition metals like iron, which for example can be found in high concentrations in products like meat. More experiments are needed exploring the full effect of temperature and pH on both formation of highly reactive radicals in food as well as involvement of other compounds with consequences for formation. Knowledge of the participation of radical processes in the MR can be useful in controlling the reaction in relation to formation of beneficial compounds like color and flavor, in contrast to harmful compounds like AGEs.

In conclusion, slightly alkaline conditions were found to increase oxygen consumption and formation of brown-colored products, as well as formation of highly reactive intermediary radicals in the Maillard reactions, when glucose and lysine is heated together at elevated temperatures.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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