Evaluation of Radical Products from β-Alanine/Sugar Mixtures by Use of GC−MS with the Galvinoxyl Radical

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We have quantified the radical production of Maillard systems with β-alanine and different sugars by a method that uses the free radical scavenger capacity of galvinoxyl (2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadiene-1-ylidene)-*p*-tolyloxy) and a GC−MS separation and quantification. We have compared the results with the spectrophotometrical conclusions and highlight the importance of the radical production in the Maillard systems. The method is precise (CV < 5%), accurate (>97%), sensitive (limit of detection 10 ng/mL galvinoxyl), and may be used for the detection and quantification of radical activity in the chemical and biological systems.

Keywords: *Maillard reaction; free radical; gas chromatography; mass spectrometry; galvinoxyl*

INTRODUCTION

The reaction of amino acids, peptides, and proteins with sugars or with other carbonyl substances leads to the deterioration of foods being stocked for industrial and home use. The reaction results in the destruction of some essential nutritive components and the diminution of digestibility. In addition to this, the products of reaction are sometimes toxic, cancerous, or may be mutagen agents. Namiki and Hayashi (1975, 1983) describes a new mechanism of the Maillard reaction which involves cleavage of the sugar molecule with generation of a highly reactive two-carbon fragment at an early stage of the reaction with the development of radical products. The radical can be detected by electron spin resonance (ESR) but failure to observe the radical in the ESR spectrum does not prove that radicals are not involved, since the concentration may be too low for direct observation. Another technique is the chemically induced dynamic nuclear polarization (CIDNP), when protons in a reacting molecule become dynamically coupled to an unpaired electron while traversing the path from reactants to products. In this paper, the quantification of radical Maillard production from the reaction of an aqueous system consisting of β-alanine with added sugars was investigated. Analysis was made by the galvinoxyl GC−MS method and compared with spectrophotometrical conclusions on the reactivity of sugars.

MATERIALS AND METHODS

Materials and Reagents. β-Alanine and sugars were high-grade commercial materials. All of the reagents were purchased from Fluka Sigma-Aldrich Chimie (L′Isle d′Abeau Chesnes) and Laboratories Merck-Clévenot S. A. (Nogent-sur-Marne).

Sample Preparation. The reaction mixtures consisted of 1 M β-alanine and 0.5 M sugars (sucrose, cellobiose, maltose, *N*-acetylglucosamine, mannose, sorbose, galactose, rhamnose, glucose, xylose, ribose, arabinose), dissolved in sodium phosphate buffer (pH 7.35; 0.5 M in PO_4). The components were placed in test tubes; galvinoxyl (1 mL, 10.15 mg of galvinoxyl in 20 mL of ethanol) and *o*-phenylphenol as an internal standard (100 μL, 49.57 mg of biphenyl in 20 mL of ethanol) were added to make up a 20-mL reaction mixture at three points: from the outset (A), after 25 h (B), and after 50 h (C). Solutions were bubbled with helium to saturate with inert gas and to remove dissolved oxygen. The parallel samples were maintained at 45 °C for 100 h. Spectrophotometrical determinations were realized at 25, 50, and 100 h.

Liquid−Liquid Extraction. A sample (2 mL) was pipetted into a glass centrifuge tube. Hexane (2 mL) was added, and the mixture was vortex mixed for approximately 30 s. The two phases were separated by high-speed centrifugation for 15 min. The organic phase (top layer) was transferred to a glass tube with a Pasteur pipet (operation repeated three times) and dried under a stream of nitrogen at 45 °C in a Büchi 461 water bath. The residue was reconstituted with ethanol (50 μL).

Capillary Gas Chromatography (GC)/Mass Spectrometry (MS). A Hewlett-Packard 5890 Series II gas chromatograph coupled with a HP G 1800A mass spectrometer with a ChemStation data system was used for mass spectral identification and quantification. Chromatographic separation was achieved on a HP-35 capillary column (cross-linked 35% PH ME siloxane, length 30 m, column i.d. 0.25 mm, film thickness 0.25 μm, phase ratio 250). The GC operating conditions were as follows. The injector was used in the splitless mode at 250 °C, the transfer line and the detector temperature was 300 °C. The oven temperature was 60 °C for 1 min, 60−200 °C at 15 °C/min, 200−300 °C at 20 °C/min, and isothermal hold at 300 °C for 10 min. Helium was used as the carrier gas at a flow rate of 0.8 mL/min; ionizing voltage, 70 eV; injections, 2 μL; internal standard, *o*-phenylphenol. Acquisition was realized in the SIM mode to enhance sensitivity. Three ions were selected for each compound *o*-phenylphenol (**170**, 70, 141) and galvinoxyl (**57**, 365, 407) (one for quantitation and two for confirmation, quantitation ions of each compound are shown in bold). Undestroyed galvinoxyl was identified on the basis of comparison of retention time and ion ratio. Ion ratios were calculated by dividing the area of the confirming ion by the area of the quantitative ion. Quantification of galvinoxyl was based upon the ratio of the integrated ion area to the corresponding *o*-phenylphenol internal standard.

Method Validation. Statistical study (Caporal-Gauthier, 1992). Calibration curve, prepared in blank buffer with pure galvinoxyl and *o*-phenylphenol, consisted of seven concentrations in the range of 0, 5, 10, 20, 40, 80, and 100 ng/mL

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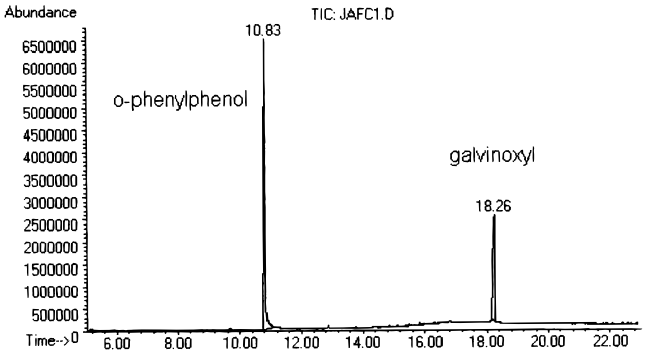

Figure 1. Gas chromatogram used for etalon curve after extraction with hexane (*o*-phenylphenol 0.5 ng/mL, galvinoxyl 40 ng/mL).

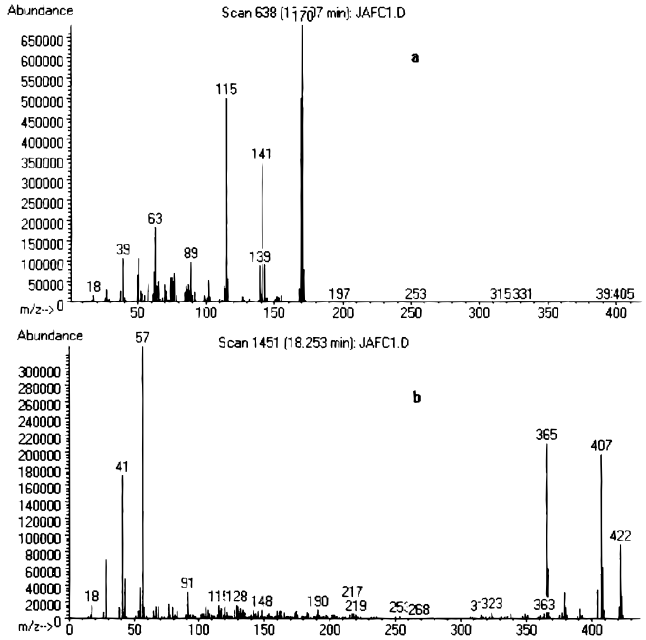

Figure 2. Mass spectra for *o*-phenylphenol (a) and galvinoxyl (b).

galvinoxyl and 0.5 ng/mL *o*-phenylphenol for each batch, including four control samples. Linearity: $r^2 > 0.96$; $t < t_{\text{theoretic}}$ (Student); $C < C_{\text{theoretic}}$ (Cochran), $F < F_{\text{theoretic}}$ (Fischer). Accuracy: confidence interval $F < F_{\text{Fischer}}$.

Spectrophotometric Measurements. The optical density at 420 nm was obtained with a Hewlett-Packard 8452 A diode array spectrophotometer for the samples without galvinoxyl and internal standard, buffered at the same pH and diluted until 1:10 with water.

RESULTS

The total chromatogram for the calibration curve is presented in Figure 1. Mass spectra for the galvinoxyl and for the internal standard are given in Figure 2. We used the quantification of the destroyed galvinoxyl in the presence of Maillard radical model systems after 100 h for comparison with the destroyed quantity in the absence of them. We have compared the percentage of destroyed galvinoxyl for each sugar, galvinoxyl being added from outset (set A), after 25 h (set B), and after 50 h (set C) (Figure 3). At the same time we have compared the evolution of optical density at 420 nm for each sugar (Figure 4) in percentage and consider the optical density of the most reactive system after 100 h such as 100%.

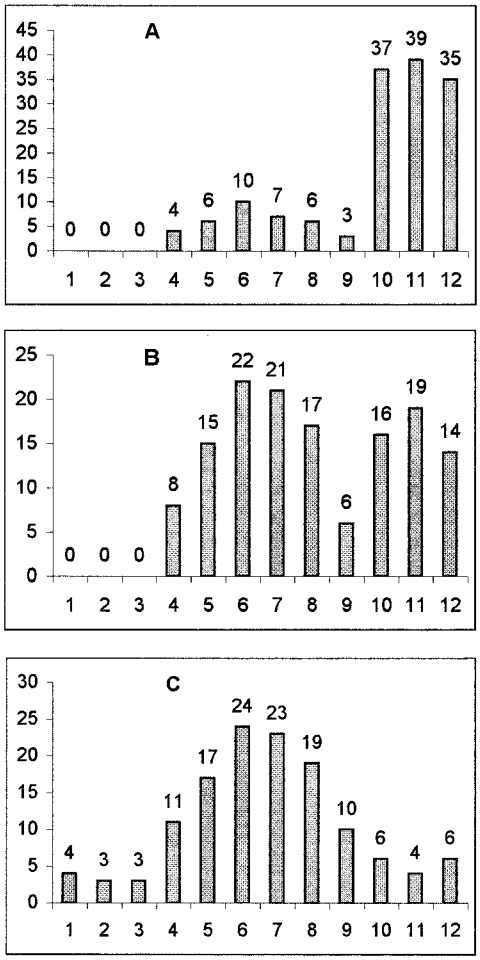

Figure 3. Percentage of destroyed galvinoxyl after 100 h (100% is the quantity of galvinoxyl in the buffer system): set A, galvinoxyl added from the outset; set B, after 25 h; set C, after 50 h. Sugars: 1, sucrose; 2, cellobiose; 3, maltose; 4, *N*-acetylglucosamine; 5, mannose; 6, sorbose; 7, galactose; 8, rhamnose; 9, glucose; 10, xylose; 11, ribose; 12, arabinose.

DISCUSSION

The early stage in the Maillard reaction is the condensation of the free aldehyde group of sugar with the amino group of amino acid (or protein). A single sugar can exist in five forms: the acyclic form, the α- and *β*-pyranose forms, and the α- and *β*-furanose group, with only acyclic forms reacting with amino acids. Their reactivity in the Maillard reaction depends on the proportion of the open form present in solution. Free radicals appear to have no place in Hodge's "classical" scheme but they are undoubtedly formed in Maillard reactions before the Amadori rearrangement.

With the help of ESR spectroscopy or the CIDNP method it is possible to identify radicals in chemical processes. An alternative and a more simple solution is to detect radical activity by chemical methods which also deliver information about the radical Maillard intermediates. Galvinoxyl possesses the unusual ability to react quantitatively with free radicals. The main mechanism in the reaction with galvinoxyl and Maillard radical products is the coupling to form a molecule similar to that found in the termination reaction in the radical mechanism. The next step is the decomposition of the new molecule and the decomposition of the galvinoxyl (see Figure 5) (Bartlett, 1962; Georgescu, 1997). Using a sensitive GC−MS method of free radical

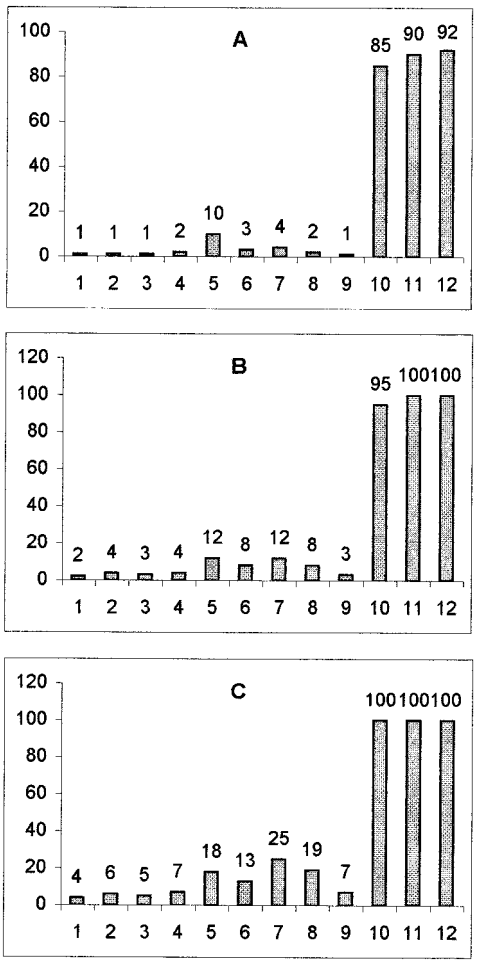

Figure 4. Evolution of $OD_{420\ nm}$ for the Maillard systems (in percentage considering $OD_{arabinose}$ after 100 h as 10%) after 25 (A), 50 (B) and 100 (C) h. Sugars: 1, sucrose; 2, cellobiose; 3, maltose; 4, *N*-acetylglucosamine; 5, mannose; 6, sorbose; 7, galactose; 8, rhamnose; 9, glucose; 10, xylose; 11, ribose; 12, arabinose.

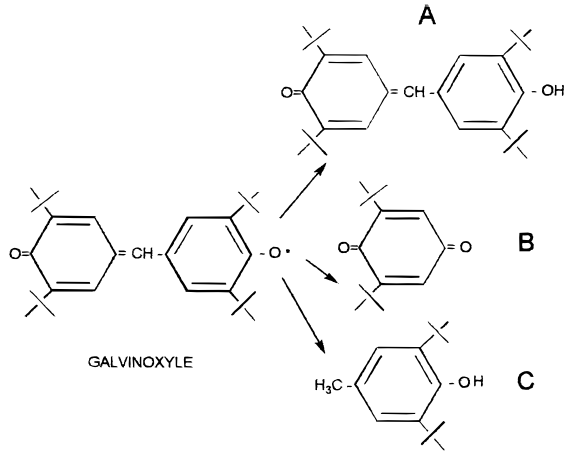

Figure 5. Main products from the decomposition of galvinoxyl: (A) hydrogalvinoxyl; (B) 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione; (C) butylated hydroxytoluene.

detection and quantification based on the degradation of galvinoxyl, we could show the dependence of Maillard radical production as a function of sugar. It is possible to utilize galvinoxyl to answer two questions of Maillard reaction: (a) to what extent are radicals involved in the reaction of sugar and amino acid (determine by examining the effect on the degree of consumption of galvinoxyl)?; and (b) if radicals are involved, to what extent do they enter into browning reactions (determined by examining the effect of different conditions on the rate and the period of disappearance of galvinoxyl)?

The disaccharide's are able to form free radicals and participate in browning probably only after hydrolysis of the glycosidic bond. Thus, the lengths of induction periods for nonenzymatic browning of disaccharides are directly proportional with the trend in activation energies for hydrolysis. In our conditions (initial pH = 7.35, temperature 45 °C), the hydrolysis of disaccharides limits the browning and the radical production and cancels the differences dues to the composition and to the type of glycosidic bond. Consequently, the reactivities of sucrose (1) (glucose + fructose, β-glycosidic bond), cellobiose (2) (glucose + glucose, β-glycosidic bond), and maltose (3) (glucose + glucose, α-glycosidic bond) are close (Figures 3 and 4). If we assume that disaccharides are able to form sugar radicals only after cleavage of the C_2-C_3 bond in the reducing part, with or without previous hydrolysis (Cämmerer and Kroh, 1996), we can explain the much higher rate in radical production for monosaccharides by the fact that they are able to form C2 and C3 fragments easily by the retroaldol reaction.

Most hexoses exist primarily as pyranoses, but a few have substantial amounts of furanose forms. In most cases there is relatively little of the open-chain carbonyl form of the sugar. There are two factors that influence the rate of interaction of monosaccharides with amino acids and respectively the radical production. Aldose sugars, mannose (5), galactose (7), and glucose (9), react more rapidly than the ketose sugars (sorbose, 6) in the acyclic state, because the aldehyde carbonyl groups are relatively more electrophilic than ketone carbonyl groups but ketoses present a greater percentage of open carbonyl forms (for sorbose >100-fold more than glucose, Bunn and Higgins, 1981).

We would expect that pentoses react with a higher rate than hexoses because at this pH (buffer pH = 7.35) the percentage of acyclic forms of pentoses is greater (Marc Loudon, 1989). The radical production and browning for xylose (10), ribose (11), and arabinose (12) are close with a small superiority for ribose in which the furan form is in majority.

The substituted form of glucose, *N*-acetyl-D-glucosamine (4), and the derivatives of mannose, rhamnose (8), have approximately the same radical and browning activity as its sugar of origin. The amino carbonyl condensation in the early stage of the Maillard reaction seems not to be influenced significantly by the substitution of hydroxy groups of sugars.

Whereas, in the pentose/β-alanine models the optical density increases up to 50 h and remains constantly high after that, the radical production decreases rapidly after 25 h. This is probably due to two causes: (1) the decreasing of pH in the Maillard systems (Adrian, 1993; Georgescu, 1997) influencing the decreasing of percentage of acyclic forms of sugars and the diminution of nucleophilic potential of amino acids; and (2) the stabilization of the Maillard reaction in the presence of known radical scavengers such as melanoidins. Scavenging of active oxygens by melanoidins was demonstrated by means of ESR experiments (Aeschbacher, 1990). At the same time, because of steric hindrance, a melanoidin−galvinoxyl reaction is unlikely. As a result, the quantity of destroyed galvinoxyl in the C system is smaller than in the B and A systems. The important

decrease in consumption of galvinoxyl for the systems in which it was added after Amadori rearrangement indicates that scavengeable radicals are being formed before the appearance of browning products.

CONCLUSIONS

The radical production is specific to the first phase of Maillard reaction and is strongly dependent on the sugar structure, especially of the percentage of the acyclic form. The galvinoxyl GC−MS method is able to quantify, with high accuracy, the radical activity. We can specify the better period for an eventual inhibition of radical production (earlier in the presence of pentoses and later in their absence), and we can appreciate the efficacy of scavenger additives, using galvinoxyl like a marker.

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