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Chemistry and genotoxicity of caramelized sucrose

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Caramelization of a 1% sucrose solution at 180°C accompanied characteristic changes in pH, M_r, UV-absorbance, and fluorescence values as well as increased reducing power activity after 40–60 min. Similar changes occurred to sucrose heated at 150°C, after 150–240 min. Bioactivity of caramelized sucrose samples was tested for mutagenic activity, using *Salmonella typhimurium* strains TA-98 and TA-100, respectively, as well as the *Saccharomyces* D7 yeast strain for mitotic recombination and Chinese hamster ovary cells (CHO) to assess clastogenicity. Caramelized sucrose expressed no mutagenicity in the TA-98 strain, but gave positive ($p < 0.05$) results with the TA-100, base-pair substitution strain. Similarly, mitotic recombination in the *Saccharomyces* D7 yeast strain and clastogenic activity in CHO cells were induced when exposed to caramelized sucrose. In the all cases, preincubation with S-9 reduced ($p < 0.05$) the mutagenic activities of caramelized sucrose. Fractionation of the caramelized sucrose into volatile and nonvolatile compounds was performed and tested for clastogenicity using CHO cells. Volatile components contributed approximately 10% to total clastogenicity, which was enhanced by the presence of S-9. Nonvolatile components recovered, consisting of relatively lower M_r, gave highest ($p < 0.05$) clastogenic activity, denoting that higher M_r caramel colors are relatively free of this property.

Keywords: Caramel / Fractionation / Genotoxicity / Sucrose hydrolysis

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1 Introduction

Caramelization and Maillard reactions represent two distinct sources of nonenzymatic browning that occur in food products which have undergone a thermal process. For example, roasting of green coffee beans that contain 5–10% sucrose, dry weight, will result in the almost complete destruction of sucrose to products that interact with amino acids and result in generation of Maillard reaction products (MRP) [1]. Early MRPs represent compounds that contribute to the odor (*e.g.*, aldehydes, furans, pyrroles, quinolines, and indoles), whereas extended heat treatment leads to aldol condensation and polymerization to advance browning, characterized by the formation of high M_r pigments (*e.g.*, melanoidins) [2]. Caramelization of sucrose can occur simultaneously along with the Maillard reaction and contribute to nonenzymatic browning with the production of high UV-absorbing compounds also derived at intermediate stages of the reaction. Sucrose decomposition products that result from the Maillard reaction have been shown to contribute to the mutagenicity of the brown crust of bread [3] and of coffee [4] as well as numerous model systems [5, 6]. These products have been shown to also exhibit antioxidant [7–9] and chemoprotective [10, 11] activities in a number of cell-free and cell-based models. It is of interest that caramelization of sugars contributes markedly to the production of brown pigments and therefore may contribute to an overestimation of the Maillard reaction and its associated properties in foods [12].

When crystalline sucrose is heated at about 160°C, or above, and in the absence of amino acids or peptides, brown pigments collectively referred to as caramel are formed [13]. These brown pigments result from a series of chemical reactions that include hydrolysis, dehydration, and polymerization, collectively referred to as caramelization. Sucrose, held at 160°C as a melt, will hydrolyze to glucose and fructose anhydride [14, 15]. The production of water and organic acids such as acetic, formic, and pyruvic during sucrose caramelization will enhance the hydrolysis [16]. Hydrolytic products, glucose and fructose, are reactants in the formation of caramel and volatile flavor compounds [17]. Heating sucrose to 300°C in a stream of nitrogen produces volatile furan compounds; namely 2-methyl furan, furan, 2-hydroxyacetyl furan, and other volatile reaction

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Abbreviation: CHO, Chinese hamster ovary

products (methanol, acetone, acrolein, propanol, and acetaldehyde) [18, 19].

Stich *et al.* [20] reported that caramelized sucrose, without the activation by a liver microsomal enzyme extract (*e.g.*, S-9), could induce a relatively high frequency of chromosomal breaks and exchanges in cultured Chinese hamster ovary (CHO) cells. In a parallel study, these workers [21] also reported that furfural, furfuryl alcohol, 5–methyl furfural, 2-methyl furan, 2,5-dimethyl furan, and 2-furyl methyl ketone products were effective at inducing a relatively high frequency of chromatid breaks and chromatic exchanges in the absence of S-9 pretreatment. In more recent studies, imidazole derivative products (2-acetyl-4(5)-(1,2,3,4-terahydroxybutyl)-imidazole) in caramel color III were shown to be immunotoxic [22]. Other studies reported no clastogenicity in CHO cells, nor any mutagenicity in *Salmonella* strains and *Saccharomyces cerevisiae* gene assay following treatment with commercial caramel color III, in both S-9 pretreated or deleted protocols [23, 24].

The purpose of the present study was to determine the changes in the mutagenic and clastogenic activities of caramelized sucrose after heat treatment at both 150°C (below melting point of sucrose) and at 180°C (above melting point of sucrose) over various heating times. Sucrose crystals were dissolved in water to simulate a hydrated food system prior to heating. The caramelized sucrose samples were characterized by recording absorbance readings taken in the visible and UV wavelength regions, measuring relative reducing power and by determining fluorescence intensity. Mutagenicity and genotoxicity assessments were made on these products in the presence and absence of microsomal S-9 mixture to obtain an assessment of toxicity, both before and after liver P_{450} enzyme metabolism. Moreover, volatile compounds residing in the caramelized sucrose were further identified by a GC-MS, whereas fractions of nonvolatile compounds were obtained by Sephadex G-25 gel filtration chromatography. Both components were assessed for clastogenic activity.

2 Materials and methods

2.1 Chemistry methods

2.1.1 Sucrose caramelization

Twelve grams of sucrose were dissolved in 20 mL of distilled water in an evaporation dish. The dish was placed in a forced air circulating oven at either 150 (40 min) or 180°C (20 min) to completely evaporate the free water in the sucrose solution. The final heating time included the evaporation time.

2.1.2 Visible and UV spectral analyses

Caramelized sucrose solutions at concentrations of 24% of original sucrose were prepared. The relative levels of caramel pigment in heat processed sucrose samples were assessed by measuring the absorbance of suitably diluted solutions and calculating the apparent extinction coefficients on the basis of 1% of original sucrose. Spectral analysis was conducted using a Unicam SP 800 spectrophotometer at 200, 224, 284, and 450 nm.

2.1.3 Fluorescence measurement

Relative fluorescence intensity of caramelized sucrose solutions was made at an excitation wavelength of 360 nm and emission wavelength of 420 nm using a slit width of 3 mm in an Amenco-Bowman #4-8202 spectrofluorometer. One microgram of quinine sulfate in 1 mL of 0.1 N H_2SO_4 had a fluorescence intensity of 16 when the meter multiplier was set at 1. Fluorescence values were reported on the basis of 1% of the original sucrose solution.

2.1.4 Recovery of volatile compounds

Volatile compounds in caramelized sucrose heated at 180°C for 90 min were extracted by 50 mL HPLC-grade ethyl ether [25]. A 10% caramelized sucrose solution (one liter) was adjusted to pH 3.5 and heated at 100°C for 1.5 h to volatilize the acidic compounds for capturing in the ether. Thereafter, the caramelized sucrose solution was adjusted to pH values of about 7.5 and 9.5, respectively, prior to two further distillation/extraction periods of 1.5 h each. This was done to recover the volatile neutral and basic compounds in the same ether extraction. These fractions were further dehydrated with anhydrous sodium sulfate and magnesium sulfate prior to ether removal in a Kuderna-Danish concentrator to a working volume of 2–3 mL. The concentrated ether extract was used for GC-MS analysis.

2.1.5 Recovery of nonvolatile compounds

Gel filtration of nonvolatile compounds present in a caramelized sucrose system heated at 180°C for 90 min was performed on Sephadex G-25 (fine) using a 2.5×80 cm column. Five milliliters of a 5% caramelized sucrose solution was applied, and distilled water was introduced as the eluent. Fractions with volumes of 5.5 mL were collected using an LKB fraction collector equipped with a drop-counting system. The absorbance of the eluent was measured continuously at 280 nm. Fractions with similar absorption were pooled and lypholyzed. Nonvolatile fractions recovered by Sephadex G-25 gel filtration were again processed further by HPLC using a gel permeation column (Brownlee labs, Santa Clara, CA, USA) Standard reference proteins were injected into the column for M_r estimates. The M_rs of different fractions were estimated by comparing the retention time of the fractions and the standard proteins.

None of the fractions showed a single symmetric peak; fractions 1 and 2 both exhibited shouldering with lower M_r compounds also present.

2.1.6 Reducing power measurement

Reducing powers of both crude caramelized sucrose samples and component fractions collected by gel filtration were performed using 2,6-dichloroindophenol by colorimetry [26]. Absorbance measurements were taken at 540 nm, with ascorbic acid used as the standard reducing agent for reference equivalent (*e.g.*, milligrams of ascorbic acid *per* 100 g of the original sucrose).

2.1.7 GC-MS

A Varian MAT gas chromatograph-mass spectrometer, was used. Data were recorded on a Varian 620-computer. A column of 5% carbowax 20 M on chromosorb WHP (80/100 mesh, 20 ft × 2 mm id) was used. The column temperatures were programmed at 60–200°C at a rate of 4°C/min. Helium flow rate was 20 mL/min. Injector, separator, and line temperature were 200, and 250°C respectively. The ionization voltage for normal mass spectral fragmentation patterns was 70 eV, and scans were taken from m/e 15 to m/e 750 in 5 s intervals.

2.2 Toxicity testing

Caramelized sucrose extracts recovered in a 50 mL volume of ether were first condensed to about 1 mL and then shaken vigorously with 8 mL of distilled water. The ether from the extract was removed from the aqueous solution reduced by a constant flow of nitrogen gas which reduced the final volume to 10 mL. Caramelized sucrose extracts were tested for mutagenicity and clastogenicity by preincubation with an activated hepatic microsomal enzyme (*e.g.*, +S-9) mixture, that consisted of liver cytosol derived from Aroclor 1254–treated rats. Controls for these samples were not pretreated with activated liver enzyme homogenate (*e.g.*, –S-9).

2.2.1 Mutagenicity activity assays

Salmonella typhimurium (TA-98 and TA-100 strains) and *S. cerevisiae* bioassays were used to assess the mutagenicity [5, 6] of caramelized sucrose, processed by heating the sucrose solution at 180°C for 90 min. The *Salmonella* strain TA-100, which is susceptible to base-pair substitution, and strain TA-98, a frame-shift mutant, were used. Mutagenic activity was measured by both the standard plate method [27] and the modified preincubation method [24]. For the *Saccharomyces* gene conversion assay, the diploid yeast strain D7 was used to assess the mitotic recombination and other genotoxic activities [28]. In addition to recording toxicity of samples for yeast survival, results of this assay are presented as both convertants *per* 10^1 of the plate and convertants *per* 10^5 survivors.

2.2.2 Clastogenic activity assay

Chromosome aberration analysis was conducted on caramelized sucrose products and associated fractions using CHO cells (*e.g.*, 140 000 cells on a 22-mm coverslip, positioned in a 3.5 cm Falcon plastics petri dish) [6]. Culture media was Eagle's minimal essential media (MEM), supplemented with 10% fetal calf serum, 1 mg/mL sodium bicarbonate, and antibiotic mixture (*e.g.*, streptomycin sulfate (29.6 μg/mL), penicillin (125 μg/mL), kanamycin (100 μg/mL), and fungizone (2.5 μg/mL). Toxic and bioactive concentrations were predetermined. Experiments were initiated after cells reached 50–60% confluence. Colchicine (0.1%) dissolved in MEM was added to the incubated samples at 16 h postexposure to caramelized samples and associated fractions to determine chromosomal aberration frequency. Ethanol/acetic acid (3:1) was used to fix cells on glass slides, and cells were stained with 2% orcein in 50% acetic acid and water. Each analysis was conducted in duplicate with 200 metaphase plates analyzed for chromosomal exchanges and chromatid breaks [5, 6].

2.3 Statistical analysis

Data are expressed as mean ± SD. Student's *t*-test ($p < 0.05$; $p < 0.01$) was used to compare means treated with (+S-9) and without (– S-9) pretreatment.

3 Results and discussion

3.1 Chemical and physical properties of crude caramelized sucrose preparation

The effects of heating sucrose at temperatures of 150 and 180°C, respectively, over different time durations produced substantial changes on a number of physical and chemical properties of caramelized sucrose samples (Table 1). Generally, it would not be expected that sucrose, a nonreducing sugar, would undergo caramelization when heated in solution or used as a melt below 160°C. Indeed, brown pigments were observed to gradually form in concentrated sucrose solutions and sucrose melts over relatively short periods of heating. This can be explained by the fact that sucrose can be hydrolyzed and a melt occurs at 169°C [14]. A series of chemical reactions are required to reach the polymeric form of higher M_r pigments, which begins with the destruction of simple sugars such as glucose and fructose, at elevated temperatures. For example, the caramelization of sucrose heated at 200°C can be described in three stages. Melting sucrose crystals correspond to a loss of water by dehydra-

tion; thus the first stage involves a weight loss (*e.g.*, 4.5%), which corresponds to one molecule of water *per* molecule of sucrose and the formation of anhydrides. In the second stage, about 9% loss of weight will occur with the reaction and the formation of a brown pigment, called caramelan. Caramelen, (molecular formula $C_{36}H_{50}O_{25}$) is a water-soluble brown pigment formed in the third stage of the reaction. With further heating at 200°C, the dark brown pigment, caramelin (average molecular formula for caramelin $C_{125}H_{188}O_{80}$) is formed.

In this study, heating sucrose solutions at temperatures of 150 and 180°C resulted in a gradual increase in sucrose concentration through water evaporation to an extent that no free water remained. The temperature of the sucrose system increased progressively toward ambient temperature of the oven ensuring a uniform heat treatment of the sucrose. Concentrated sucrose solutions (37.5/100 g) heated in an oven at 150 and 180°C, respectively, from 20 min to 2 h, produced caramelized products that dissolved readily in water. The one exception of this was for sucrose samples that were heated at 180°C for 2 h. In this specific case, partial formation of very high M_r polymers likely occurred which reduced the solubility character of the products. The caramelization reaction was characterized by a color change from colorless to pale yellow, to amber, to an orange brown, and then finally to a dark brown color. Corresponding decreases in pH also occurred from neutral to weak acid (pH 4–5), and then to a stronger acidity (pH 2–3) with increased duration of heating. This reaction also featured the formation of substances which showed a strong absorbance at UV 284 nm as well as the generation of other substances that had a unique fluorescence character and reducing power (Table 1).

Caramelized sucrose solution samples prepared at a final concentration of 1% w/w contained constituents that had maximum absorption at UV ranges of 284, 224, and 200 nm, respectively (Table 1). In general, UV absorbance values increased with the degree of caramelization but leveled off after a certain level of caramel was produced. The reducing power capacity of caramelized sucrose samples also increased with the degree of caramelization, thus higher temperatures and longer heating times produced products with stronger reducing power. This response can be attributed in part to the early production of reductones that consist of conjugated enediol and carbonyl fractions and are produced by carbohydrate dehydration and fission [29]. Moreover, reductones are also generally acidic due to the dissociation of the terminal proton, which explains in part the decrease in pH of the reaction medium [26].

Fluorescence intensities of caramelized sucrose samples were also measured, following necessary dilution of samples to reduce the quenching effect of the color of the solution (Table 1). Fluorescence intensity was proportional to the concentration of the caramelized sucrose solution, and increased as the heating temperature and heating time increased. When the sucrose was caramelized at 180°C, the development of fluorescent products formed quickly after 40 min of heating. Maximum relative fluorescence intensity was reached after 60 min of heating, before reaching a plateau. In contrast, caramelized sucrose samples heated at 150°C exhibited a relative fluorescence intensity pattern that reached maximum values when samples were heated for longer durations of 150–240 min. The importance of temperature in eliciting these changes is best shown by comparing the results obtained when sucrose was caramelized at 150°C *versus* 180°C. At 150°C, maximal changes in these reaction parameters were found to take much longer and often required at least 4 h of heating.

The formation of fluorescent products in heated reducing sugar-amino acid MRP systems has been well described [9,

Table 1. Physical and chemical properties of caramelized sucrose samples

Temperature (°C)	Heating time (min)	pH[a] (24% solution)	Variable wavelength extinction coefficients[b]				Reducing affinity[c] (mg of ascorbic acid)	Fluorescence value[d] (relative value)
			450 nm	284 nm	224 nm	200 nm		
150	40	3.97	0.002	0.07	0.05	0.05	57.3	0.50
150	90	3.23	0.04	1.19	0.77	0.59	72.9	10.4
150	150	3.05	0.10	3.13	1.51	1.41	104.2	20.8
150	240	2.82	0.55	5.10	2.50	2.34	145.8	250.0
180	20	3.33	0.02	0.76	0.43	0.42	52.1	6.25
180	40	2.85	0.58	8.39	4.32	3.93	145.8	66.7
180	60	2.69	1.96	8.60	5.02	3.65	192.7	333.4
180	90	2.64	2.83	8.21	7.60	–	224.0	301.7
180	120	2.62	3.50	7.40	5.31	4.79	244.8	333.4

a) pH values of 24% (w/w) sucrose solution after heat treatment.
b) Extinction coefficients (nanometers).
c) Reducing power (mg ascorbic acid/100 g sucrose).
d) Fluorescence values represent a relative value measured from 1% w/w caramelized sucrose solution.

Table 2. Volatile compounds of caramelized sucrose (180°C), 90 min, as identified by GC-MS

Elution order	Compound	Reference[a)]
1	Ether (solvent)	
2	Propanal	[35, 40]
3	Ethyl formate	
4	Ethyl acetate	[39, 40, 44]
5	Unknown	
6	2,3 Pentandione	[35, 44]
7	3–Methyl furan	[35]
8	Unknown	
9	2-Methyltetrahydro-3-furanone	[35]
10	Unknown	
11	5-Methyl-3-hydrofuran-3-one	[3, 36–38]
12	Acetic acid	[16, 41]
13	2–Furfural	[35, 41, 44]
14	2-Furylmethyl-ketone	[3, 36–38, 44]
15	2-Furylpropyl-ketone	
16	5-Methyl-2-furfural	[41]
17	7-Methyl-benzo(b)furan	
18	Furfuryl alcohol	[16]
19	4-Methyl-2-butenoic acid-lactone	[39, 40]
20	Same as 2-furylpropylketone	
21	4-Methyl-2-butenoic acid	[35]
22	Unknown	

a) Confirmation, as reported by previous studies.

30, 31]. Fluorescence character generated in an unheated glucose-glycine Maillard reaction solution at pH 5.0–5.2 is very low and will increase five-fold within 10 min when heated at 100°C [31]. This result is attributed in part to the chromophoric group of Schiff base in conjugation with an electron donating group. Water-soluble conjugated unsaturated carbonyl compounds collected from heated model glucose-glycine MRPs [9, 32], products of Strecker degradation of amines [33], and glycation end-products [34] have all in common a fluorescence character. In contrast, less is known about the fluorescent products of caramelized sugars. Aromatic volatiles such as ethylbenzene and trimethylbenzene, potential contributors to sucrose caramelization, have been reported in heat-treated glucose solutions [35]. Similarly, non volatile aromatic compounds with fluorescent properties can also be produced in the caramelization of sucrose.

3.2 Recovery and composition of caramel volatile fraction

Reactions involved in the caramelization of sucrose include mutarotation, enolization and isomerization, dehydration and fragmentation, anhydride formation and polymerization. The extent to which the reaction occurs depends upon pH, temperature, and heating time. In the present study, 2-furfural was identified as a major compound present in sucrose caramelization. Ethyl acetate, 2-furyl methyl ketone, and 5-methyl-2-furyl methyl ketone were minor compounds also present in the volatile fraction (Table 2). Volatile compounds formed during pyrolysis of sugars are furans for the most part, and have been shown elsewhere to occur in response to heating sucrose under a nitrogen atmosphere at 300°C. The generation of 2-methyl furan was a major decomposition product [35] and of 56 volatile compounds recovered, 23 were furan related compounds.

In our study, volatile compounds generated from heating sucrose in an open evaporation dish produced thermal degradation products that included 2-furyl methyl ketone, 2-furyl propyl ketone, methyl-benzo(b)furan, and 5-methyl-3-hydro-furan-2-one. Similar compounds have been recovered in food systems such as milk products [36], licorice [37], roasted peanuts [38]. Ethyl formate, ethyl acetate, 4-methyl-2-butenoic acid, and 4-methyl-2-butenoic acid lactone have also been isolated from the volatile constituents of white bread crust [39, 40].

3.3 Recovery and composition of nonvolatile fraction

After the residue was bubbled with nitrogen overnight to drive off the remaining volatiles, a Sephadex G-25 column was used to further fractionate the residue. Chromatograms were obtained based on the absorbance values taken at 284 nm. Three fractions, namely fraction 1 (tubes 18–26), fraction 2 (tubes 27–45), and fraction 3 (tubes 46–60), corresponded to a yield ratio of 9:12:1:1.8, respectively (Table 3). These fractions contained dark color components eluted at the void volume and lighter colored components eluted from tube 60 onward, until virtually no further absorbance could be detected. The presence of carbonyl compounds were expected in fractions that absorbed at 284 nm [8]. The results of the fluorescence extinction coefficient value, along with the estimated M_r and the yield of each fraction, are also presented in Table 3. The lower M_r fractions exhibited higher fluorescence values. Due to the small quantity of materials collected from gel filtration, especially fractions 3–5, further analysis was not possible.

3.4 Clastogenic and mutagenic properties of caramelized sucrose

Caramelized sucrose prepared at different temperatures and at various heating durations was analyzed for clastogenicity, using CHO cells (Table 4). Caramels prepared at 150 and 180°C both exhibited clastogenic activity, with maximum activity found when heating durations were extended to 150 min for 150°C and 90 min for 180°C, respectively. Prolonged heating time reduced the percent chromosome meta-

Table 3. M_r and other physical properties of the nonvolatile fractions[a)]

Sample	Color	Yield (g/100 g)	Estimated M_r	Extinction coefficient at 284 nm (1% solution)	Fluorescent value (1% solution)
Fraction 1	Dark brown	36.5	6000–12 000	4.08	12.5
Fraction 2	Light brown	57.3	3000–5000	5.92	4.2
Fraction 3	Yellow	0.46	<2500	1146	5000
Fraction 4	Yellow	0.23	<1000	1083	4500
Fraction 5	Yellow	0.10	<1000	126.7	1250

a) Caramelized sucrose extract separation by gel filtration on Sephadex G-25 and monitored at 284 nm.

Table 4. Effect of heating temperature and heating duration on clastogenic activity of caramelized sucrose[a)]

Caramelization process		% Methaphase plates with chromosome aberrations; sample concentration (mg/mL)				
Temp (°C)	Time (min)	250	125	62	31	16
150	40	T	47.1 (1.00)	2.7 (0.01)	0.8 (0.00)	0.00 (0.00)
150	90	T	T	62.3 (l.18)	11.8 (0.06)	2.7 (0.00)
150	150	T	T	18.8 (0.14)	15.5 (0.09)	0.9 (0.00)
150	240	MI	53.6 (0.61)	3.6 (0.00)	0.0 (0.00)	0.0 (0.00)
180	20	T	T	T	20.9 (0.21)	0.9 (0.01)
180	40	T	T	MI	60.0 (1.03)	1.8 (0.00)
180	60	T	T	T	57.1 (0.69)	9.1 (0.05)
180	90	T	T	T	MI	3.09 (0.38)
180	120	T	T	T	50.9 (0.72)	4.5 (0.02)

a) Controls for this experiment had no metaphase plates with chromosome aberrations.
Figures in parentheses indicate average number of exchanges *per* metaphase plate.
T = toxic; MI = mitotic inhibition.

phase plate exchanges. A similar trend was observed when quantitative analysis consisted of recording the average number of exchanges *per* metaphase plate. The addition of S-9 liver microsomal preparation to the same samples significantly ($p < 0.05$) suppressed the clastogenicity of samples that were treated for the longest heating duration at 150°C as well as those samples treated for both 20 and 120 min heating at 180°C (Table 5).

The genotoxic effect of caramelized sucrose may be attributed to furan derivatives, which include furan, furfural, furfuryl alcohol, 5-methyl furfural, 2-methyl furan, 2,5-dimethylfuran, and 2-furymethyl ketone [21]. It was also reported that a relatively high frequency of chromatid breaks and exchanges in cultured CHO cells occurred without S-9 treatment. We extend this finding by reporting that the clastogenicity of caramelized sucrose can be reduced when exposed to hepatic microsome metabolism, especially in samples that were heat treated for prolonged time periods at both 150 and 180°C.

The Salmonella strain TA-100 produced a positive mutagenic response to caramelized sucrose, and unlike the TA-98 strain, this mutagenicity occurred using both the standard plate and preincubation methods (Fig. 1). Positive results obtained with the TA-100 strain refer to the capacity of caramelized sucrose constituents to induce base-paired substation. The preincubation method showed much higher mutagenicity sensitivity than the standard plate method. In all the cases, the addition of S-9 significantly suppressed ($p < 0.05$) the mutagenic activity (Fig 2B). The Ames test has been used to demonstrate mutagenic activity toward different fractions of Maillard reaction products, previously [5, 6, 42] showing positive response to triose reductones [43], furans, ketones, and a number of acids, esters, and alcohols [44]. Similarly, studies that have reported TA-100 and TA-98 tester strains to show relative mutagenicity for caramelized sucrose [44, 45] are in conflict with other workers who have not observed similar mutagenicity over a wide range of concentrations for commercial caramels, both with and without S-9 preincubation [23, 46].

Genotoxicity of caramelized sucrose was also observed in the present study using *Saccharomyces* strain D7 as evidenced by the concentration dependent increases in mitotic recombination and mutation occurring with caramelized sucrose (Fig 2B). The addition of S-9 mixture reduced both the toxicity (Fig. 2A) and suppressed the genotoxic affect of caramelized sucrose ($p < 0.05$). Very similar results have been reported previously with furfural alcohols in both the

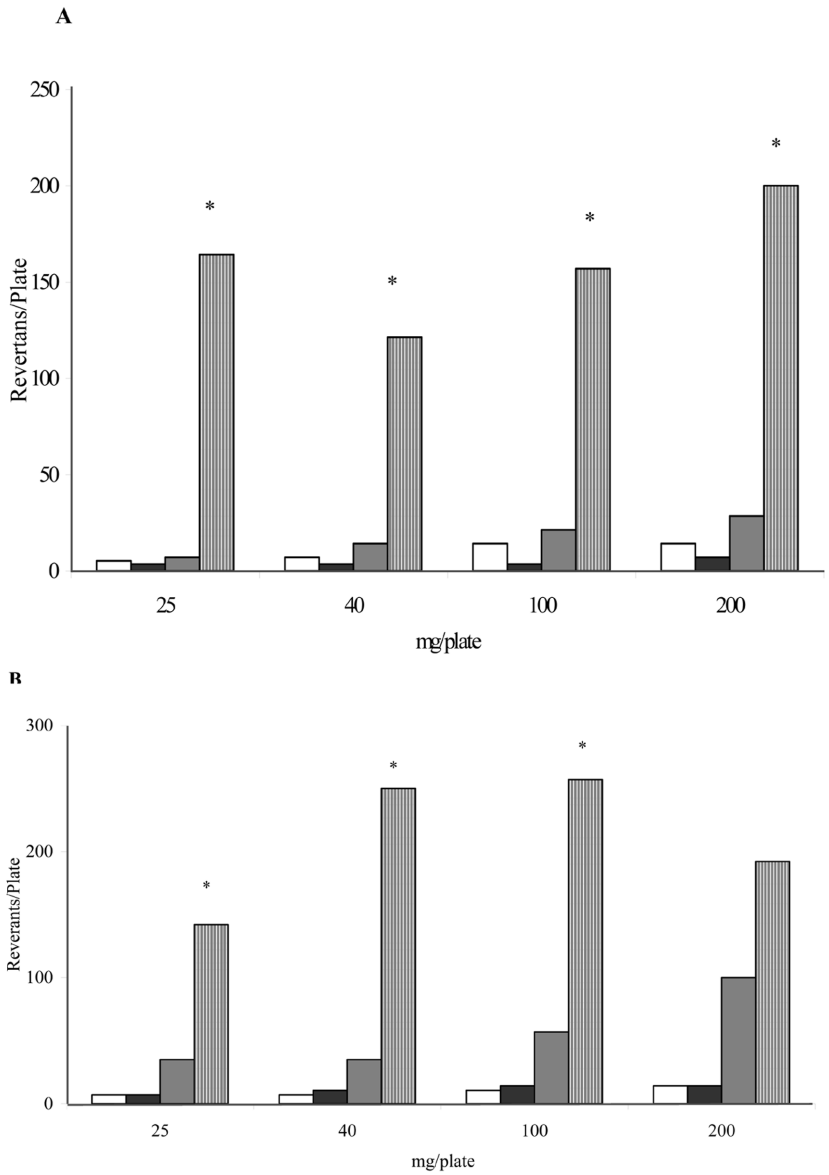

Figure 1. (A) Mutagenicity of caramelized sucrose tested with *Salmonella* strain TA-98 and TA-100 without S-9 premix. Mean values have <5% variance. □: TA-98 strain plate, ■: TA-98 strain preincubated, ▩: TA-100 strain plate, ▥: TA-100 strain preincubated. * denotes significance between TA-100 and TA-98 ($p < 0.05$) for preincubated test. (B) Mutagenicity of caramelized sucrose tested with *Salmonella* strain TA-98 and TA-100 with exposure to S-9 premix. Mean values have <5% variance. □: TA-98 strain plate, ■: TA-98 strain preincubated, ▩: TA-100 strain plate, ▥: TA-100 strain preincubated. * denotes significance between TA-100 and TA-98 ($p < 0.05$) for preincubated test.

presence and absence of S-9 mixture [44]. These findings contrast the negative results obtained for caramel colors using *S. cerevisiae* strain D4, which has led to the conclusion that there is minimal risk associated with caramel color on human health [24].

3.5 Clastogenicity of volatile fraction

The volatile fraction collected from steam distillation and extraction of caramelized sucrose contained strong clastogenic effects on CHO cells over a wide range of concentrations as well as having mitotic inhibition and toxicity at the highest concentrations tested (Table 6). Nearly 50% of the total aberrations *per* metaphase occurred at a concentration of 16 μL/mL. The residue fraction remaining after steam distillation, had greater ($p < 0.05$) clastogenic activity than the caramelized solution without the distillation/extraction process. This was especially true when samples at the highest concentrations were preincubated with the S-9 mixture (Table 6). From the active dose level, it can be calculated that the volatile fraction contributed to approximately 10% of the total clastogenicity of the crude sucrose caramelization reaction. Preincubating fractionated components with the microsomal S-9 liver mixture actually enhanced genotoxicity of the volatiles and suppressed the activity of the residue fraction, while having very little effect on the control sample. These results agree with others, who also reported weak clastogenic activity of caramel color III in CHO cells [24], activity that was lost when samples were

preincubated with a S-9 mixture. Our findings of the volatile fraction when in the presence of S-9 mixture producing an enhanced clastogenic effect, may be the response of components, such as furfural and 5–methyl furfural [21, 44].

3.6 Clastogenicity of the nonvolatile fractions

Fractionation of nonvolatile caramelized sucrose components collected off the Sephadex G-25 gel chromatography column with an absorbance value of 420 nm, displayed

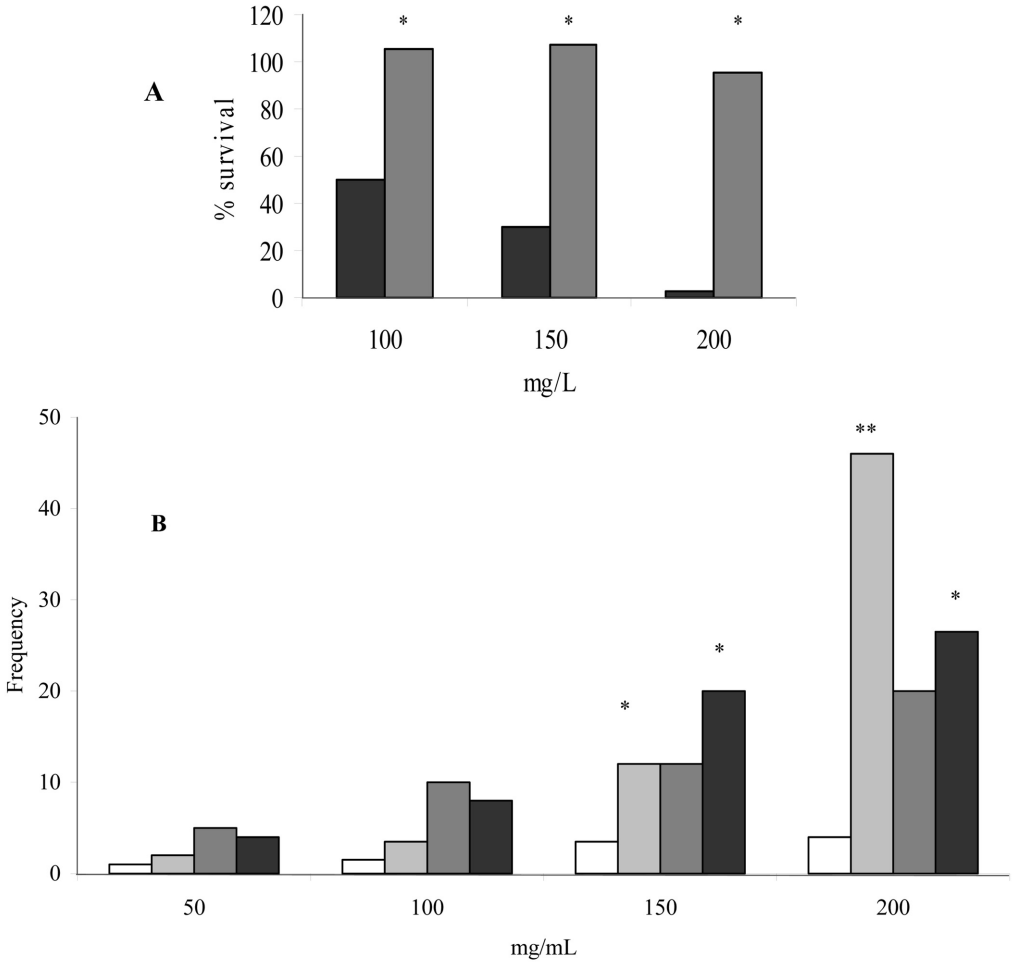

Figure 2. (A) Survival of *S. cerevisiae* treated with caramelized sucrose. ■: −S-9; ■: +S-9. * denotes difference between +S-9 and −S-9 ($p < 0.05$). (B) *S. cerevisiae* treated with caramelized sucrose. □: convertants/10 survival −S9; ■: convertants/10^{-1} survival +S9; ■: convertants/10^5 survival −S9; ■: convertants/10^5 survival+S9. * denotes significance ($p < 0.05$) between +S-9 and −S-9. ** $p < 0.01$ between +S-9 and −S-9.

Table 5. Effect of S-9 liver microsomal preparation on caramelized sucrose induced clastogenic activity[a)]

Caramelization conditions		Concentration (mg/mL)	−S-9			+S-9		
Temp. (°C)	Time (min)		% Metaphases with aberrations	Average no. of exchanges/cell	Average no. of breaks/cell	% Metaphases with aberrations	Average no. of exchanges/cell	Average no. of breaks/cell
150	40	94	26.4	0.30	0.04	22.7	0.24	0.02
150	240	47	65.5	1.58	0.01	17.3	0.24	0.01
180	20	94	18.2	0.22	0.04	9.1	0.08	0.01
180	120	31	36.0	1.16	0.03	13.6	0.13	0.02

a) Concurrent controls for this experiment were: untreated cells 0.7% metaphase plate with aberrations, 0.00 chromatid exchange/cell and 0.01 chromatid breaks/cell; aflatoxin B with activation, 87.5% metaphase plates with aberrations, 2.88 chromatid exchanges/cell, and 0.13 chromatid breaks. −S-9 denotes absence of S-9 premix; +S-9 denotes presence of S-9 premix.

Table 6. Clastogenic effect of volatile and residue fractions collected from caramelized sucrose[a)]

% Chromosome aberration, average chromatid breaks and exchanges/metaphase							
Volatile fraction			Residue concentration (mg/mL)	Caramelized sucrose after distillation/extraction		Caramelized sucrose before distillation/extraction	
(uL/mL)	−S-9	+S-9		−S-9	+S-9	−S-9	+S-9
4	0	24.5 (0.34) 0.054[b)]	6	2.7 (0.02) 0.01[b)]	0	0	0
6	0	MI	8	10.0 (0.13) 0.03[b)]	0	0	0
8	4.55 (0.03) 0.036[b)]	MI	12	56.4 (1.05) 0.1[b)]	0	0	0
12	37.3 (0.57) 0.036[b)]	MI	15.5	MI	0.9(0) 0.02[b)]	2.7 (0.02) 0.01[b)]	2.7 (0.02) 0.01[b)]
16	48.2 (0.85) 0.073[b)]	T	23.5	T	13.6 (0.16) 0.01[b)]	52.5 (1.2) 0.125[b)]	MI
24	MI	T	41.5	T	34.5 (0.47) 0.09[b)]	T	T
31	T	T	47	T	MI	T	T

a) Data refer to percent of chromosome aberration. Values in parentheses indicate average number of exchanges *per* metaphase.
b) Values denote average number of breaks *per* metaphase.
T = toxic. MI = mitotic inhibition; less than 1 metaphase plate among 6000 cells. −S-9 denotes absence of S-9 premix; +S-9 denotes presence of S-9 premix.

Table 7. Clastogenicity of nonvolatile caramelized sucrose fractions collected by gel filtration and monitored at 420 nm[a)]

Sample	Concentration (mg/mL)	Activation[b)]	% Metaphases with chromosome-aberrations	Chromatid breaks *per* cell	Chromatid exchanges *per*cell
Fraction I	150	−S-9	1.82	0.018	0
Fraction I	150	+S-9	1.82	0.015	0
Fraction I	200	−S-9	MI	0	0
Fraction I	200	+S-9	20.9	0.336	0.009
Fraction II	15.5	−S-9	15.5	0.41	0
Fraction II	15.5	+S-9	26.4	0.31	0.01
Fraction II	25.0	−S-9	46.9	0.64	0.054
Fraction II	25.0	+S-9	40.0	0.60	0.20
Fraction III	8.2	−S-9	0.91	0	0.009
Fraction III	8.2	+S-9	1.82	0	0.018
Fraction III	16.5	−S-9	21.8	0.26	0.036
Fraction III	16.5	+S-9	17.3	0.18	0.018

a) Concurrent controls for this experiment were: untreated cells 0.7% metaphase plate with aberrations, 0.00 chromatid exchange/cell and 0.01 chromatid breaks/cell; aflatoxin B with activation, 87.5% metaphase plates with aberrations, 2.88 chromatid exchanges/cell, and 0.13 chromatid breaks.
b) −S-9 denotes absence of S-9 premix; +S-9 denotes presence of S-9 premix.

varying degrees of clastogenicity as expressed by the percentage of metaphases with chromosome aberrations, the number of chromatid breaks *per* cell, and to a much lesser extent the number of chromatid exchanges *per* cell (Table 7). The first fraction, which eluted at the void volume, contained constituents which had the highest M_r, but also the lowest ($p < 0.05$) level of genotoxicity without exposure to the S-9 premix. Preincubating this fraction with S-9 significantly ($p < 0.05$) enhanced the clastogenicity as expressed by both the percent metaphases with chromosome aberrations and the chromatid breaks *per* cell. Fraction II contained smaller M_r compounds, but exhibited significantly greater ($p < 0.05$) clastogenic activity, both with and without S-9 treatment. Fraction III showed the relatively lowest clastogenicity compared to Fraction II, which varied between 2 and 10 times lower activity compared to Fraction II.

Fractionation of nonvolatile components absorbing at 280 nm was also assessed for clastogenicity (Table 8).

Table 8. Clastogenicity of nonvolatile caramelized sucrose fractions collected by gel filtration and monitored at 284 nm[a)]

Sample	Concentration (mg/mL)	Activation	% Metaphases with chromosome aberration-	Chromatid breaks *per* cell	Chromatid exchanges *per* cell
Fraction I	37.5	– S-9	3.19	0.12	0
Fraction I	37.5	+S-9	2.70	0	0.03
Fraction I	50	– S-9	MI	0	0
Fraction I	50	+S-9	20.9	0.35	0.05
Fraction II	25	– S-9	25	0.57	0.07
Fraction II	25	+S-9	–	0	0
Fraction II	50	– S-9	MI	0	0
Fraction II	50	+S-9	3.6	0.03	0.01
Fraction III	0.14	– S-9	40.9	1.0	0.11
Fraction III	0.14	+S-9	22	0.26	0.04
Fraction III	0.28	– S-9	MI	0	0
Fraction III	0.28	+S-9	36	0.76	0.46
Fraction V	0.164	– S-9	0	0	0
Fraction V	0.164	+S-9	0	0	
raction IV	1.16	– S-9	26.4	0.30	0.09
Fraction IV	1.16	+S-9	2.7	0.05	0.01

a) – S-9 denotes absence of S-9 premix; +S-9 denotes presence of S-9 premix.

Unlike the three principle fractions recovered with an absorbance of 480 nm, six fractions of the nonvolatile mixture were collected from the Sephadex G-25 column that absorbed at 284 nm. Clastogenicity was greatest in Fraction III, which represented 40.9% chromosome aberration occurring at a concentration as low as 0.14 mg/mL. Fraction IV also showed lower activity at higher concentrations of 1.16 mg/mL, a feature which was further reduced when preincubated with S-9 premix. Fraction V was not active under our experimental conditions, which may be due to the fact that only a very small amount of material was collected and the highest concentration tested was not able to reach the active dosage.

The nonvolatile compounds formed during thermal degradation of sugars have been reported in a former review article [17]. Other workers have described 19 nonvolatile constituents recovered from a heated glucose solution [35, 47, 48] that included furan and benzene derivatives as well as di- or trisaccharides. Our findings of smaller M_r nonvolatile fractions absorbing at 284 nm derived from caramelizing sucrose and expressing the relatively strongest clastogenicity are in agreement with these reports and warrants additional studies to confirm the identify of the active compounds present in these fractions.

4 Concluding remarks

Crystalline sucrose dissolved in water and heated at 150 and 180°C for different times (*e.g.*, 20–240 min) resulted in caramelization, the extent and composition of the reaction being very much dependent on temperature. Changes in chemical properties revealed important differences in caramelized sucrose which were related to bioactive properties involving both mutagenicity and clastogenicity. Caramelized sucrose solutions induced base-pair substitution in *Salmonella* TA-100 strain, gene conversion in *S. cerevisiae* D7, and chromatid breaks and exchanges in CHO cells. The addition of S-9 premix generally reduced the extent of change in mutagenicity and genotoxicity. Fractionation of the caramelized sucrose prepared at 180°C showed that both volatile and nonvolatile components exhibited clastogenic activity. This significant bioactivity was attributed mostly to the lower M_r, nonvolatile, components and not to the high M_r, polymeric pigments. It is concluded that mutagenic and clastogenic activity observed with sucrose degradation and subsequent polymerization reactions is reduced by exposure to metabolic activation enzyme systems. Further studies are encouraged to determine the bioavailability of the bioactive constituents present in caramelized sucrose before absolute estimates of safety are established.

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