Food Chemistry **15** (1984) 165–173

Antimicrobial Activities of Heated Glucose and Fructose Solutions and Their Elucidation by High Performance Liquid Chromatography

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(Received: 12 May, 1984)

ABSTRACT

The heating of glucose and fructose in phosphate buffers (pH 5·0 and 7·0) at 100, 110 and 120 °C for 30, 60 and 120 min led to increasing inhibitory activities against the growth of Escherichia coli. *The inhibitory effects of both heated sugars were rather similar, which was in agreement with the similar compositions of solutions after heating. The antimicrobial activity of solutions was separated into seven different fractions by high performance liquid chromatography (HPLC). Furfural or 5-hydroxymethylfurfural was not responsible for this antimicrobial activity.*

INTRODUCTION

The heating of carbohydrates results in the formation of many different compounds (Pigman & Anet, 1972; Feather & Harris, 1973; Houminer, 1973). The fact that some of these compounds have growth-inhibiting effects on bacteria has been known for a long time (Lewis, 1930; Ingram *et al.* 1955; Finkelstein & Lankford, 1957; Mälkki, 1963, 1965; Kato & Shibasaki, 1974), but the exact nature of the compounds has not been ascertained because of the difficulties encountered in their isolation.

Only after prolonged heating (8 h at 120 °C) was the concentration of 5-hydroxymethylfurfural inhibitory, according to Ingram *et al.* (1955). The only study clearly connecting these antimicrobial effects to certain compounds is by Kato & Shibasaki (1974), who separated catechol, 3-methylcatechol, 4-methylcatechol and methylhydroquinone from a

Food Chemistry 0308-8146/84/$03·00

heated xylose solution (120 °C for 20 min at pH 10·6). These compounds were shown to be partially responsible for the antimicrobial activity of the solution. Hitherto, the compounds responsible for the antimicrobial activity developing at neutral and slightly acidic pH values have not been determined. In order to investigate this phenomenon glucose and fructose solutions were heated for various times and at various temperatures. Their antimicrobial activity and sugar composition were assayed. The solutions were fractionated by HPLC and the fractions were tested for antimicrobial activity.

METHODS

Heat treatments

Solutions (50 g litre^{-1}) of D-glucose and D-fructose in 0·2 M $NaOH/H_3PO_4$ buffer (pH 5·0 and 7·0) were sealed in 20 ml glass ampoules under vacuum and heated in an oil bath for 30, 60 and 120 min at 100 °C, 110 °C and 120 °C. After heating they were cooled to room temperature and stored at this temperature for less than 3 h unopened until tested for antimicrobial activity or subjected to HPLC analysis.

Microbiological tests

Half-millilitre samples were pipetted into test tubes. The tubes were transferred into a water bath at 57 °C and 180 μl of Bacto Nutrient Agar (Difco, USA) (92 g litre^{-1} distilled water) were added. The tubes were immediately transferred to another water bath at 44·5 °C and allowed to equilibrate for 10 min. They were then inoculated with 50 μl of a suspension containing about 1000 cells of *Escherichia coli* ATCC 1172-2. After mixing, ten droplet cultures of the infusion were made on a petri dish and incubated at 37 °C for 48 h and counted.

Sugar analysis with HPLC

The instrument consisted of two M-6000A pumps, M-660 gradient programme, U6K injector, μ-Bondapak/carbohydrate column, M-440 dual channel UV-detector monitoring at 254 nm and 280 nm and M-401 refractive index detector (all from Waters Assc, USA). The eluent was acetonitrile:water (80:20) at a flow-rate of 1 ml min^{-1}. The sugars were

identified on the basis of their retention times and quantified by peak height measurements from the trace of the refractive index detector.

HPLC of heated sugar solutions

Two-millilitre samples of glucose and fructose solutions (heated for 120 min at 120 °C and at pH 7·0) were injected into the HPLC instrument equipped with a μ-Bondapak C_{18} column (4 × 300 mm). A linear gradient was run from 0 to 100% methanol in water for 90 min at a flow-rate of 0·7 ml min^{-1}. Fractions of 2·1 ml were collected and released from methanol under reduced pressure at 20 °C, after which they were reconstituted to 2 ml volumes and tested microbiologically.

These separations were scaled up first by using 10 ml injections and then by pumping 400 ml of heated sugar solution through four columns in series (8 × 300 mm) packed with Porasil B/C_{18} and eluting the antimicrobial compounds from them with methanol. Methanol was released from the collected fraction under reduced pressure at 20 °C.

The resulting dark brown syrup was injected into a μ-Bondapak C_{18} column (8 × 300 mm) and a gradient was run under the same conditions as above at a flow-rate of 2·8 ml min^{-1}. Fractions of 2·8 ml were collected and treated as above.

GLC/MS and MS

GLC/MS runs were made from samples dissolved in methanol or in freshly distilled diethyl ether with a Hewlett-Packard 5992 A instrument equipped with a FFAP glass capillary column (0·3 mm × 30 m). The operating conditions were as follows: linear temperature programming from 50 °C to 250 °C at 6 °C min^{-1}. Mass spectra were also run with a JEOL JMS-D100 instrument with direct insertion using electron impact ionization at 70 eV.

All the solvents used were of HPLC quality and it was shown with blank runs that none of the antimicrobial activities formed originated from solvents, buffers or unheated sugars.

RESULTS AND DISCUSSION

The results of microbiological tests are presented in Table 1. They demonstrate, as expected, that increases in both heating time and

TABLE 1

Growth Inhibiting Activity of 5% Glucose and Fructose Solutions Heated for Various Times and at Various Temperatures in 0·2 M $NaOH/H_3PO_4$ Buffers and Tested with *E. coli*. Controls Were the Same Sugar Solutions Sterilized by Filtration (0 = no growth, 100 = no antimicrobial activity (as control))

Temperature and dilution	*Antimicrobial activity*					
	Glucose solution			*Fructose solution*		
	30 min	*60 min*	*120 min*	*30 min*	*60 min*	*120 min*
Ph 7·0 100 °C						
1:1	100	100	100	100	100	0
1:5	100	100	100	100	100	100
110 °C						
1:1	100	0	0	50	0	0
1:5	100	100	30	100	50	0
1:10	100	100	100	100	100	20
120 °C						
1:1	5	0	0	0	0	0
1:5	100	40	0	100	0	0
1:10	100	100	30	100	100	0
1:20	100	100	100	100	100	5
pH 5·0 100 °C						
1:1	100	100	100	100	100	50
1:5	100	100	100	100	100	100
110 °C						
1:1	100	100	100	70	50	5
1:5	100	100	100	100	100	100
120 °C						
1:1	100	100	50	10	0	0
1:5	100	100	100	100	100	20
1:10	100	100	100	100	100	100

temperature caused rapid increases in the antimicrobial activities of aqueous glucose and fructose solutions. This result excludes the possible role of osmotic effects and proves that the antimicrobial activity was caused by compounds formed during heating. The rise of pH from 5 to 7 also drastically increased the formation of antimicrobial activity. This result is not surprising as most carbohydrates are known to be thermally

TABLE 2

Results of Sugar Analyses of 50 g litre^{-1} Glucose and Fructose Solutions after Heating for Various Times at Various Temperatures (xyl, xylose; ara, arabinose; glu. glucose; fru, fructose)

Temperature and time	*Glucose solution*				*Fructose solution*			
	xyl	*ara*	*fru*	*glu*	*xyl*	*ara*	*fru*	*glu*
	($g\ litre^{-1}$)				($g\ litre^{-1}$)			
pH 7·0 100 °C								
30 min			1	48	2		48	
60 min			5	45	4	trace	41	4
120 min			8	41	6	1	38	5
110 °C								
30 min	1		7	42	5	1	36	6
60 min	1		9	36	5	2	32	8
120 min	3		13	27	6	2	32	11
120 °C								
30 min	2		10	31	6	1	35	5
60 min	4	1	11	26	5	2	32	6
120 min	5	3	13	17	5	6	11	6
pH 5·0 100 °C	heating had no detectable effect							
110 °C	heating had no detectable effect							
120 °C								
30 min				50	trace		50	
60 min			trace	50	trace		48	
120 min			2	48	1		44	

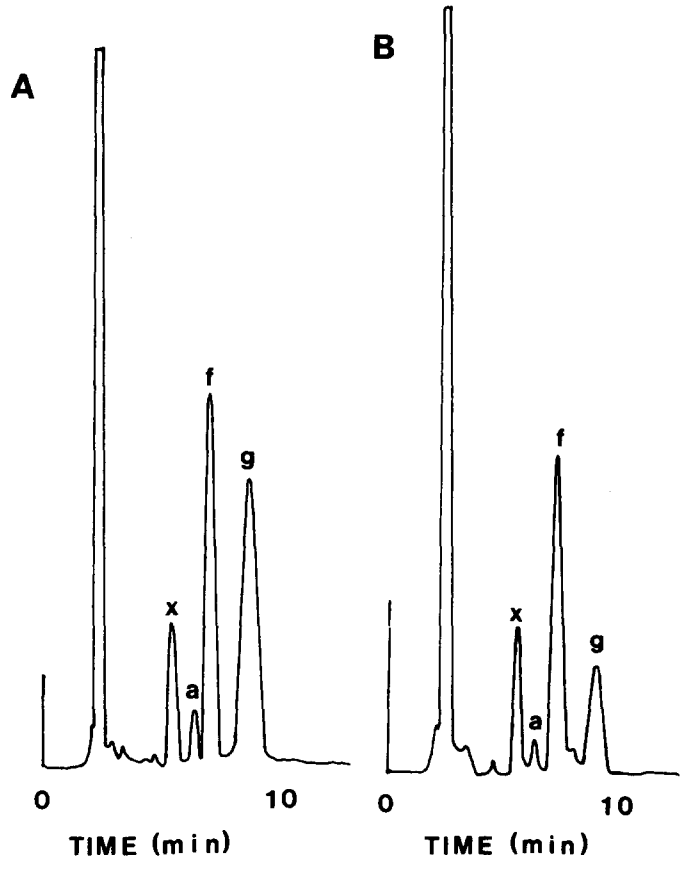

Fig. 1. Chromatography of sugars in heated 50 g litre^{-1} glucose (A) and fructose (B) solutions. Heating was carried out in 0·2 M $NaOH/H_3PO_4$ buffer (pH 7·0) at 120 °C for 120 min. For chromatographic conditions see text. x, xylose; a, arabinose; f, fructose; g, glucose.

most stable in the pH 3–4 range (Kröner & Kothe, 1939; Mathews and Jackson, 1933; McDonald, 1950). Fructose is also known to be thermally less stable than glucose (Houminer & Patai, 1969). This is also reflected in the results presented in Table 2 and in Fig. 1. When either glucose or fructose was heated at pH 7·0 for 120 min the composition of thermal products seemed to be rather similar because of isomerization (Speck, 1958) and a possible common decomposition pathway. Also the antimicrobial fractions from both sugars had the same retention times (Fig. 2).

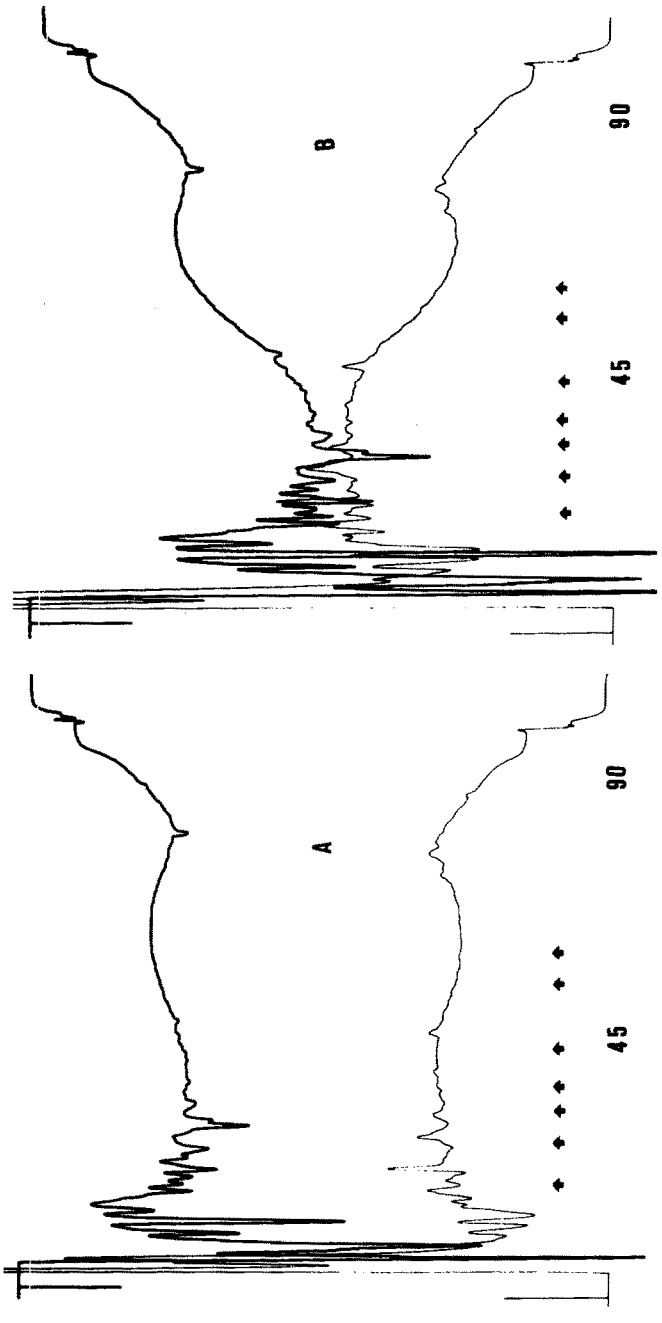

Fig. 2. HPLC chromatograms of heated 50 g litre^{-1} glucose (A) and fructose (B) solutions. Heating was carried out in 0·2 M $NaOH/H_3PO_4$ buffer (pH 7·0) at 120 °C for 120 min. Arrows denote the antimicrobial fractions. Upper line 280 nm and lower line 254 nm both at 0·2 aufs: 20 μl injections. For other details see text.

The microbiologically active fractions were first located using 2 ml and 10 ml injections but isocratic rechromatography led to the loss of most of the antimicrobial activity. Only the rechromatography of antimicrobial fractions from 400 ml samples revealed that the antimicrobial activity results from at least seven different compounds. The loss of activity resulted partly from losses during manipulation of samples and partly from the fact that most of the activities were the result of two or more compounds in one fraction, so that the better resolution during isocratic conditions resulted in loss of activity because the compounds were separated into different fractions after rechromatography. The resulting seven fractions from 400 ml samples were totally inhibitory to the growth of *E. coli* when diluted to 5 ml.

None of the fractions yielded any interpretable mass spectra because of the big number of peaks in the spectra. Furfural and 5-hydroxymethylfurfural were shown not to be responsible for the antimicrobial activity as shown by the microbiological test of the fractions with similar retentions to pure compounds. Although these compounds are slightly active, the amounts formed under the test conditions were too small to be significant.

The active compounds are slightly acidic, which was revealed by their better retention time stability after addition of 0·4% acetic acid to the eluents. Acetic acid had no other effects on the compounds, as was shown by duplicate separation with and without added acetic acid. Under acidic conditions, the active compounds could be partially extracted from water into diethyl ether but because of many co-extracting compounds this did not help in their identification. Storage of the compounds at room temperature rapidly decreased their activities, whereas storage for three months at −20 °C under nitrogen had no measurable effect.

Further attempts are in progress to clarify this phenomenon because of its possible importance both as a beneficial effect in heat sterilization of food and as a harmful effect from the toxicological and nutritional points of view.

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