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Characterization of Mutagenic Compound(s) in Heated Orange Juice

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ABSTRACT

Heated orange juice (93°C, 2 min) was fractionated using solvent extraction, vacuum distillation, ion-exchange chromatography and gel filtration. The fractions were subjected to our modified Salmonella *mutagenicity assay (pH adjustment to 7·4 and 4 h preincubation at 37°C). Results showed that the mutagenic compound(s) are polar, non-volatile, carry no charge and have molecular weights ≤ 700 daltons. Three fractions, separated by gel filtration, showed a stronger mutagenicity than the serum; one fraction with molecular weight between 200 and 700 daltons possessed the highest mutagenic activity.*

INTRODUCTION

In preceding papers (Ekasari *et al.*, 1986*a,b*; 1988) it was reported that heat-treated orange juices induced mutagenicity towards *Salmonella typhimurium* TA100 under the conditions of the modified *Salmonella* mutagenicity assay. The mutagenic response can be interpreted as heat load and its measurement gives an expression of inner quality of (concentrated) orange juice. On this basis it has been possible to classify various commercial orange juices into four groups. Juices with a higher heat load show a stronger tendency for browning upon storage; a similar effect was also reported by other workers (Saguy *et al.*, 1978; Mannheim & Havkin, 1981; Marshall *et al.*, 1986).

In the present study we attempt to define some properties of the compound(s) which cause mutagenicity. This will eventually lead to their

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identification. We, therefore, conducted fractionation of heated orange juice by vacuum distillation, solvent extraction, ion-exchange chromatography and gel filtration to separate and concentrate the mutagenic compound(s). Since the strongest mutagenic response appeared in laboratory-prepared orange juices heated at 93°C for 2 min, such juices were used for the purpose of this study. Juices heated for longer periods of time at this temperature (93°C) show a strong cytotoxic effect (Ekasari *et al.*, 1988) and therefore were not included in the present study.

MATERIALS AND METHODS

Samples

Laboratory prepared orange juice heated at 93°C for 2 min as described by Ekasari *et al.* (1986*a*).

Preparation of the volatile and non-volatile fractions (vacuum distillation)

Heated orange juice (100 ml) was evaporated under vacuum at 60°C in a rotary evaporator until *c.* 40 ml condensate was obtained. The resulting condensate (volatile fraction) and concentrate (non-volatile fraction) were adjusted to pH 7·4 with $Na_2HPO_4.12H_2O$ and $NaH_2PO_4.H_2O$, filter sterilized (0·2 μm, Millipore) and tested for mutagenicity.

Ion-exchange chromatography

Heated orange juice was centrifuged (48 000 × *g* for 20 min) and the serum, after pH adjustment to 7·4 (with 4N NaOH), was chromatographed on a 9·5 cm × 2·0 cm column of Biogel CM (cation-exchanger, Biorad) and a 12 cm × 1·6 cm column of Biogel DEAE (anion-exchanger, Biorad). The columns were equilibrated with 6 M $(NH_4)_2CO_3$ solution; NH_4^+ and CO_3^{2-} were counter ions for CM and DEAE, respectively. Ten millilitres of serum, after two-fold dilution with distilled water, was loaded onto each column and then eluted in 5 ml fractions with five successive column volumes of 0·0012M $(NH_4)_2CO_3$ and five column volumes of 0·6M $(NH_4)_2CO_3$. The eluates were collected and pooled into three portions called eluate I (fraction Nos 1–10), eluate II (fraction Nos 11–35) and eluate III (fraction Nos 36–70); eluates I and II were obtained with the lower molar (0·0012M) eluent. Each portion was freeze-dried, dissolved in 2 ml of 0·5M phosphate buffer, pH 7·4 (five-fold concentration), filter-sterilized (0·2 μm, Millipore) and tested for mutagenicity.

Solvent extraction

Heated orange juice was centrifuged (48 000 × *g* for 20 min) and the serum after pH adjustment to 7·4 (with 4N NaOH) was freeze-dried in a 100 ml portion. The freeze-dried serum (*c.* 12 g) was extracted in a Soxhlet apparatus, first with 250 ml of petroleum ether for 3 h and then successively with chloroform and methanol in the same manner. Each solvent fraction was evaporated to dryness in a rotary evaporator under vacuum at 35°C. The freeze-dried serum (unextracted), the solvent extracts and the ultimate residue were dissolved in 20 ml 0·5M phosphate buffer, pH 7·4, i.e. five-fold concentration with regard to the 100 ml serum used. The aliquots were filter-sterilized (0·2 μm, Millipore) and tested for mutagenicity.

Gel filtration

Heated orange juice was centrifuged (48 000 × *g* for 20 min) and the serum after pH adjustment to 7·4 (with 4N NaOH), was chromatographed at 2°C on a Biogel P2 from Biorad (100–200 mesh; MW cut-off 100–1800 daltons). The gel material was packed into the column, 100 cm × 2·6 cm; for each run 2 ml of sample was loaded onto the column. Elution was carried out with sterilized distilled water at a flow rate of 25 ml/h. Fractions (3 ml) were collected and measured for UV absorbance at 210 nm (Carl Zeiss M4QIII spectrophotometer). The pools were divided into four portions (see Fig. 2) i.e. fractions Nos 40–69, Nos 70–110, Nos 111–130 and Nos 131–170. Each portion was freeze-dried, dissolved in 0·5M phosphate buffer pH 7·4 to original volume of sample (2 ml), filter-sterilized (0·2 μm, Millipore) and tested for mutagenicity. In order to estimate the molecular weight of fractions, the elution curve (Fig. 2) was compared with the one established with a water solution containing galactan (MW >2000), stachyose (MW = 666), raffinose (MW = 504), sucrose (MW = 342) and glucose (MW = 180) where, in each fraction, the phenol–sulfuric acid test (Dubois *et al.*, 1956) was performed.

Mutagenicity assay

Modified *Salmonella* mutagenicity assay (4 h preincubation at pH 7·4 and 37°C) as described by Ekasari *et al.* (1986*a*). *Salmonella typhimurium* TA100 without S9 mix was used. Mutation ratio (number of induced revertant colonies per plate divided by number of spontaneous revertant colonies per plate) ≥2·0 indicates positive mutagenic response. The numbers of spontaneous revertants were in the range of 96–178 colonies/plate. Revertants induced by the positive control (4-nitroquinoline-*N*-oxide) were ≥1000/0·1 μg per plate.

Each experiment was done at least twice and each concentration of the sample was tested at least in triplicate.

RESULTS AND DISCUSSION

Vacuum distillation

Table 1 shows the results of the mutagenicity test on condensate and concentrate. Mutagenic activity was observed only in the concentrate, indicating that the mutagenic compound(s) are non-volatile at pH 7·4. In this context the mutagenicity of commercial concentrated orange juices (Ekasari *et al.*, 1986*a*) should be noted.

Ion-exchange chromatography

As shown in Table 2, mutagenicity was observed only in the first few fractions of the lower molar eluent in both anion- and cation-exchanger. This indicates that the mutagenic compound(s) were not bound to the columns and, therefore, directly washed out from the exchanger beds. Thus, the mutagenic compound(s) appeared to carry no charge.

Solvent extraction

Figure 1 presents the dose–response curves of the mutagenic activity of the unextracted serum, the ultimate residue, and the petroleum ether extract. Results from the chloroform and methanol extract (not shown) were similar to the petroleum extract; in all cases no mutagenic activity was observed. Furthermore, mutagenic effect was observed in the unextracted serum and

TABLE 1
Mutagenicity of Volatile and Non-volatile Fraction from Heated Orange Juice

Fraction[a]	*Mutation ratio*[b]			
dose (ml/plate)	*0·1*	*0·2*	*0·3*	*0·4*
Concentrate (Non-Volatile)	2·6*	3·2*	2·8*	2·5*
Condensate (Volatile)	1·0	0·9	0·9	0·9

[a] See 'Material and Methods' (vacuum distillation).
[b] Asterisk indicates positive mutagenic response.

TABLE 2
Mutagenic Activity of Fractions from Ion-Exchange Chromatography of Heated Orange Juice

Fraction[a]	*Dose (ml/plate)*	*Mutation ratio*[b]	
		Anion-exchanger	*Cation-exchanger*
Eluate I	0·10	2·6*	2·9*
	0·20	2·3*	1·7
	0·45	1·2	0·6 (toxic)
Eluate II	0·10	1·1	1·2
	0·20	1·2	1·1
	0·45	1·3	1·2
Eluate III	0·10	1·1	1·1
	0·20	0·8	0·9

[a] See 'Material and Methods' (ion-exchange chromatography).
[b] Asterisk indicates positive mutagenic response.

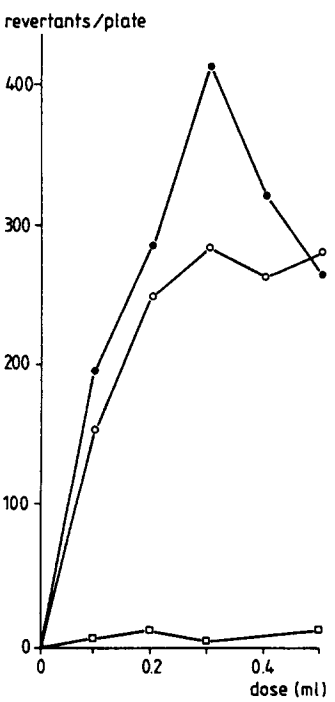

Fig. 1. Dose–response curves of mutagenic activity of (●) unextracted serum 5 × concentrated; (○) ultimate residue (water extract); (□) petroleum ether extract. For details, see 'Material and Methods' (solvent extraction). The numbers of induced revertants/plate are corrected for the number of spontaneous revertants.

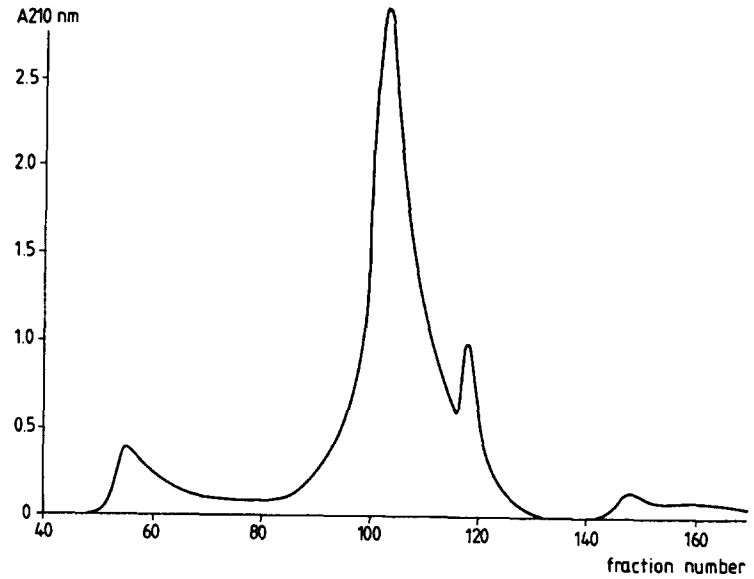

Fig. 2. Gel filtration profile of serum of heated orange juice (93°C, 2 min) on Biogel P2. Column: 100 cm × 2·6 cm; eluent: sterilized distilled water; flow rate: 25 ml/h; fraction size: 3·0 ml.

the ultimate residue (water extract). Together with the results from ion-exchange chromatography (which showed the mutagenic compound(s) carried no charge) this suggests that the mutagenic compound(s) are polar.

Gel filtration

Figure 2 shows the elution profile of serum from heated orange juice obtained with distilled water and measured for absorbance at 210 nm. Figure 3 presents dose–response curves of the mutagenic activity of the pools. Mutagenic activity was observed starting from fraction No. 70 up to 170 (Fig. 2). It should be noted that the pools had a much stronger mutagenic activity than the unfractionated serum, indicating a concentration of the mutagenic compound(s). The pool of fractions Nos 70 up to 110 shows the strongest mutagenic activity. The molecular weight of these mutagenic fractions estimated from the elution profile from the same column with carbohydrates (not shown; see 'Material and Methods') was found to be $200 < MW < 700$.

In conclusion, partial characterization of the mutagenic compound(s) has been achieved and shows them to be polar, non-volatile, carrying no charge and having molecular weights from 200 to 700 daltons. Gel filtration appeared to be a good method to obtain fractions, from heated juice,

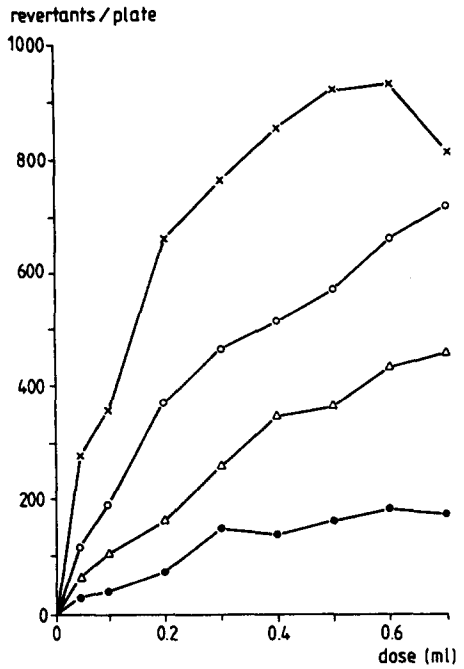

Fig. 3. Dose–response curves of mutagenic activity of (●) unfractionated serum; (△) fraction Nos 131–170 (MW ≤ 200); (○) fraction Nos 111–130 (MW ≤ 200); (×) fraction Nos 70–110 (200 < MW < 700). For details, see Fig. 2 and 'Material and Methods' (gel filtration). The numbers of induced revertants/plate are corrected for the number of spontaneous revertants.

which are strongly mutagenic. This is an important prerequisite for further investigations to isolate mutagenic compound(s) in order to determine their molecular structure.

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