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Mutagenicity and Possible Occurrence of Flavonol Aglycones in Heated Orange Juice

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ABSTRACT

Orange juices were heated at 93°C for 1–30 min and hydrolysed with 1M HCl for 1 h at 100°C. The resulting heat-treated orange juice and acid hydrolysate were analysed for flavonol aglycones (kaempherol and quercetin) and mutagenic activity towards Salmonella typhimurium *TA 100 without S9-mix. Results show that mild heat treatments normally applied in the processing of orange juices are insufficient to liberate mutagenic flavonol aglycones from their glycosides. These findings support the hypothesis that heating produces Maillard intermediary products (MIP) which, after neutralisation to pH 7·4 and under specific preincubation conditions (4 h at 37°C and pH 7·4), give rise to mutagenicity and cytotoxicity.*

INTRODUCTION

Working on the development of a method to measure heat load in orange juice, we have demonstrated that heated orange juices have mutagenic and cytotoxic effects towards *Salmonella typhimurium* TA 100 under specific test conditions. It is proposed that these effects are due to Maillard intermediary products (MIP; Ekasari *et al.*, 1986*a,b*, 1988). Mazaki *et al.* (1982) reported

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that hydrolysates of citrus juices obtained with acid and enzymes were mutagenic by the Ames test. They suggested that the mutagenicity observed in the ether extract was due to flavonol aglycones such as kaempherol and quercetin detected in the hydrolysates.

In citrus fruit, flavonols are present as glycosides, which are not mutagenic but which may be converted to aglycones, for example, by acid hydrolysis (Rouseff, 1980; Brown, 1980; Uyeta *et al.*, 1981). The pure aglycones quercetin from quercitrin (quercetin-3-rhamnoside) and rutin (quercetin-3-rutinoside), and kaempherol from robinin (kaempherol-7-rhamnoside-3-galactorhamnoside) were reported to be mutagenic in the Ames test (Bjeldanes & Chang, 1977; Hardigree & Epler, 1978; Brown, 1980).

The present study was carried out to elucidate the possibility of involvement of mutagenic flavonols in our test system. Heat-treated orange juice (93°C, 1–30 min) and an acid hydrolysate were examined for the presence of kaempherol and quercetin and mutagenic activity towards *Salmonella typhimurium* TA 100 without S9-mix.

EXPERIMENTAL

Preparation of samples

Samples of laboratory-prepared orange juices (see Ekasari *et al.*, 1986*a*) were divided into three portions and treated according to Fig. 1.

Treatment A resulted in a serum as usually prepared for our test system (Ekasari *et al.*, 1986*a*, *b*). Treatment B resulted in an ether extract. Treatment C was an acid hydrolysis according to Mazaki *et al.* (1982) and resulted in an ether extract.

Mutagenicity assays

The mutagenicity tests were performed according to our modified assay as previously described (Ekasari *et al.*, 1986*a*) and the standard test which was an Ames test with 20 min preincubation at 37°C as described by Maron & Ames (1983). All mutagenicity tests were performed with *Salmonella typhimurium* TA 100 without S9-mix. Each test was carried out at least in triplicate. The numbers of spontaneous revertant colonies (114–152 colonies/plate) were subtracted from the numbers of induced revertant colonies. Revertants induced by the positive control (4-nitroquinolin-*N*-oxide) were $\geq 10\,000$ revertant colonies/μg. An extract was designated mutagenic if the

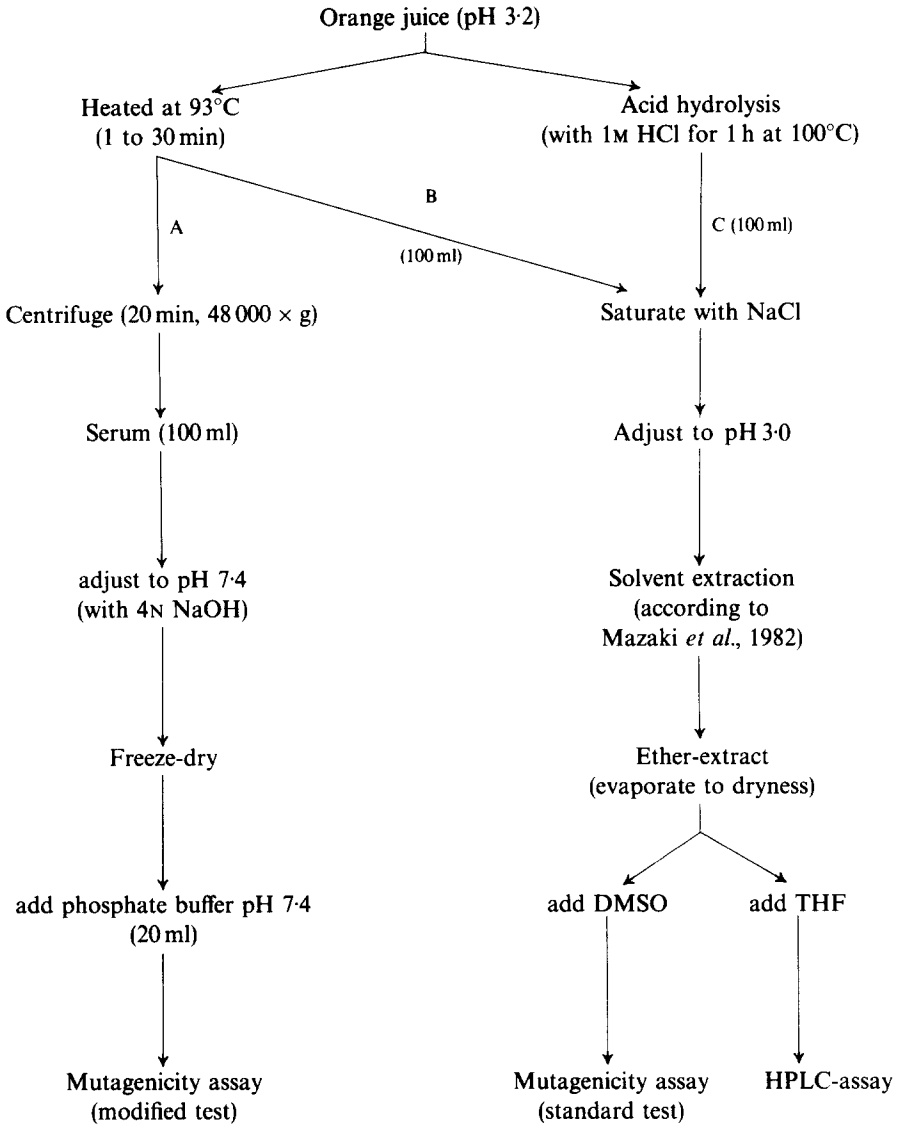

Fig. 1. Sample preparation flow chart.

number of induced revertants obtained was at least twice the number of spontaneous revertants (solvent control).

HPLC analysis of quercetin and kaempherol

A Spectra Physics liquid chromatograph was used, equipped with an SP 8700 XR pump and a Kratos Spectroflow 773 variable wavelength detector (365 nm at 0·05 AUFS range).

TABLE 1
Gradient Program: (A) 4% Acetic Acid in Methanol, and (B) 4% Acetic Acid in Water

Time (min)	*A (%)*	*B (%)*
0	10	90
9	10	90
24	50	50
30	60	40
35	60	40
36	100	0
42	100	0

A Merck reversed phase column (250 × 4·6 mm I.D.) self-packed with Lichrosorb 10 RP 18 equipped with a Chrompack precolumn (100 × 2·1 mm I.D.) packed with Copell ODS 30–38 μm was used.

A gradient program (Table 1) was used. Quercetin and kaempherol were eluted in 29·15 min and 32·32 min, respectively; the flow-rate was 1·5 ml/min.

RESULTS AND DISCUSSION

Table 2 presents the kaempherol and quercetin content in the ether extract of orange juice sample (heated at 93°C for 1–30 min) and its acid hydrolysate obtained from acid hydrolysis according to Mazaki *et al.* (1982). These analytical results were confirmed by complete recovery from spiked samples (data not shown). There was no detectable amount of either kaempherol or

TABLE 2
Flavonol Aglycones in Ether Extract of Laboratory Prepared Orange Juice

Treatment[a]	*Kaempherol*	*Quercetin*	*Total*
	(μg/ml single strength juice)		
B (unheated)	ND	ND	ND
B (93°C, 1 min)	ND	ND	ND
B (93°C, 2 min)	ND	ND	ND
B (93°C, 30 min)	0·16	0·11	0·27
C (acid hydrolysed)	1·8	5·5	7·3

[a] See Fig. 1.
ND: not detected.

quercetin in orange juice samples heated at 93°C for various times and only a very small amount was observed after 30 min heating. The acid hydrolysate showed a higher amount of quercetin and kaempherol.

Furthermore, in agreement with the results of a previous study (Ekasari *et al.*, 1986*b*), it was shown that heated orange juices under their natural acid condition were not mutagenic. Neither the serum (treatment A) nor the ether extract (treatment B) of heated orange juices (93°C, 1–30 min) showed any mutagenic activity in the Ames standard mutagenicity test (Table 3). These findings confirm that heat treatments normally applied in the processing of orange juices are insufficient to liberate either kaempherol or quercetin from their sugar conjugates of the corresponding glycosides. The magnitude of the quercetin and kaempherol levels in the acid hydrolysate implicates that the concentration tested in the mutagenicity assays do not exceed 1·1 μg/plate and 0·4 μg/plate, respectively. According to Bjeldanes & Chang (1977) and Hardigree & Epler (1978) quercetin is a direct-acting mutagen. However, the available amounts observed in treated orange juices (Table 2) would not be sufficient to cause mutagenic effects. Kaempherol requires metabolic activation by mammalian liver S-9 to become mutagenic. The cytotoxic effects observed in the acid hydrolysed sample (Table 3) probably

TABLE 3
Mutagenicity of Heated Orange Juice and Acid Hydrolysate

Treatment[a]	*Test system*	*Dose (ml/plate)*	*Revertants/plate*
A (93°C, 1 min)	Modified	0·1	346[b]
		0·2	268[b]
A (93°C, 2 min)	Modified	0·1	225[b]
		0·2	145[b]
A (93°C, 2 min)	Standard	0·1	9
		0·2	27
B (93°C, 1 min)	Standard	0·05	8
		0·1	9
B (93°C, 2 min)	Standard	0·05	1
		0·1	10
B (93°C, 30 min)	Standard	0·05	8
		0·1	3
C (acid hydrolysed)	Standard	0·05	toxic
		0·1	severely toxic

[a] See Fig. 1.
[b] Indicates mutagenic.

are due to other compounds formed during the extreme acid treatment which resulted in a viscous dark-brown hydrolysate. We can conclude that the mutagenic flavonols i.e. quercetin and kaempherol are not involved in our test system.

This supports our theory that heating produces Maillard intermediary products (MIP) which, after neutralisation to pH 7·4 and under the specific preincubation conditions described, give rise to mutagenicity and cytotoxicity (Ekasari *et al.*, 1986*a*,*b*).

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