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RESEARCH NOTE

Characterization of mutagenic compounds in heated orange juice by UV and mass spectra

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Gel filtration and high-performance liquid chromatography were used to obtain mutagenic fractions from heated orange juice (93°C, 2 min). Each fraction was submitted to the authors' modified *Salmonella* mutagenicity assay (pH adjustment to 7·4 and 4 h preincubation at 37°C) and examined for UV and mass spectra. Using fast atom bombardment-mass spectrometry and mass analyzed ion kinetic energy techniques, an attempt was made to elucidate the molecular weight and chemical structure of the mutagenic compounds. They consisted of several compounds with molecular weight as follows: 162, 180, 254, 288, 342, 360 and 540 daltons.

INTRODUCTION

Partial characterization of mutagenic compound(s) in heated orange juice has been reported in a previous paper (Ekasari *et al.*, 1990). They are polar, non-volatile, carry no charge, and have molecular weights <700 daltons. The objective of the present study was to characterize further the mutagenic compound(s). This is important in order to be able to isolate the mutagenic compound(s). The chemistry of these compound(s), once known, may become the basis of a more accurate and simpler test to measure heat load of orange juice.

MATERIALS AND METHODS

Samples

Laboratory-prepared orange juice was used which had been heated at 93°C for 2 min as described by Ekasari *et al.* (1986).

Gel filtration and HPLC

Samples were submitted to gel filtration chromatography according to Ekasari *et al.* (1990). The mutagenic fraction was further separated by high-performance liquid chromatography (HPLC). Fractions were collected on the basis of retention time (0·2 ml size/20 s). Fractionation was performed in a Spectra Physics HPLC system (SP 8770) equipped with a Kratos 773 variable wavelength detector. The column used was Aminex HPx-42H (300 × 7·8 mm i.d.; Bio-Rad, Richmond, CA) equipped with precolumn AG 50W-X4 (50 mm × 4·6 mm i.d.). Elution was performed at 30°C with 0·01N H_2SO_4. Sulfate ions in fractions were precipitated by addition of saturated $Ba(OH)_2$ solution. After centrifugation and washing of the pellets, the collected supernatants were brought to pH 7·4 with diluted NaOH solution and subsequently freeze-dried. They were screened for mutagenicity.

Mutagenicity assay

Prior to the assay, freeze-dried fractions were dissolved in 0·5M phosphate buffer pH 7·4 (5× concentrated) and filter-sterilized (0·2 mM, Millipore). The mutagenicity test was performed according to Ekasari *et al.* (1986). This was a modified *Salmonella* mutagenicity assay (4 h preincubation at pH 7·4 and 37°C) using *Salmonella typhimurium* TA100 without S9-mix . Each test was carried out at least in triplicate. Revertants induced by the positive control NQO (4-nitroquinoline-*N*-oxide) were ≥10 000 revertant colonies/μg. A fraction

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was designated mutagenic if the number of induced revertants obtained was at least twice the number of spontaneous revertants (93 colonies per plate). The mutagenic fraction was examined for UV and mass spectra.

UV spectrophotometry

Freeze-dried sample was dissolved in phosphate buffer as described for the mutagenicity assay. The absorbance spectrum, in the range 200–500 nm, was obtained with a Cary-Varian 118 recording spectrophotometer.

Fast atom bombardment-mass spectrometry (FAB-MS)

This was carried out by using a V.G. Micromass ZAB-2HF mass spectrometer, an instrument with a reverse geometry, fitted with a high-field magnet and coupled to a V.G. 11/250 data system. The sample (freeze-dried fraction) was loaded in a thioglycerol solution on to a stainless steel probe and bombarded with xenon atoms. Mass analyzed ion kinetic energy (MIKE) spectra were obtained by varying the electric sector voltage, the main beam being 8 keV. FAB/MIKE spectra record the fragment ions of spontaneously decomposing $(M+H)^+$ ions, while FAB/MIKE/CA (collisional activation) mass spectra record the fragment ions of $(M+H)^+$ ions following collision with helium as the target gas. The helium pressure was such that the intensity of the main beam was reduced by 50%.

RESULTS AND DISCUSSION

The most mutagenic fraction was eluted at about 14·5 min. Figure 1 shows the dose–response curve of the mutagenic activity. At high doses a severe cytotoxic effect was observed. Figure 2 shows the UV absorption

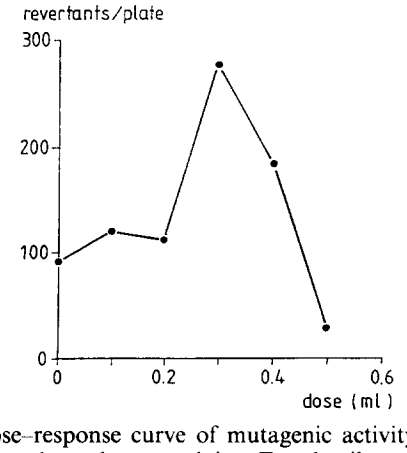

Fig. 1. Dose–response curve of mutagenic activity of HPLC fraction from heated orange juice. For details, see Materials and Methods.

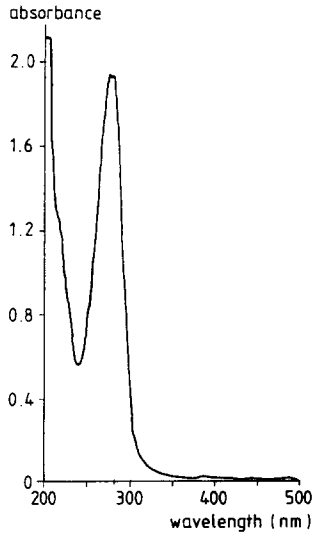

Fig. 2. UV absorption spectrum of HPLC fraction of Fig. 1. For details, see Materials and Methods.

spectrum of this mutagenic fraction. Absorbances around 210 and 280 nm were observed, which resembled the spectrum of α,β unsaturated carbonyl sugars derived from fructose-amino acid systems as reported by Schubert and Sanders (1971) and Zegota and Bachman (1987). These compounds are browning precursors (Namiki, 1988) and have been reported to be mutagenic towards *Salmonella typhimurium* TA100 (Bjeldanes and Chew, 1979) and cytotoxic toward *Salmonella typhimurium* LT_2 and other microorganisms (Stack, 1957; Schubert & Sanders, 1971).

The FAB-MS shows that the fractions consisted of several compounds (data not shown). Each fraction was found to contain the same group of compounds but with different concentrations. The molecular weights of these compounds were determined to be as follows: 162, 180, 254, 288, 342, 360 and 540 daltons. The fragmentation pattern of some of these compounds was studied by the application of MIKE technique. The results are presented in Table 1. The elemental analysis shows that none of these compounds was linked to an amino acid or any other N-compound. For the compounds with molecular weights 180, 288, 360 and 394, respectively, the following elemental compositions have been established: $C_6H_{12}O_6$,

Table 1. Fragmentation pattern of HPLC fractions of Fig. 1 obtained by MIKE technique (for details, see Materials and Methods)

Selected ion $(M+H)^+$	Molecular weight	Fragmentation
395	394	377, 215, 203, 197
361	360	343, 325, 253, 215, 199, 181, 163, 145
289	288	271, 213, 181, 163, 145, 127, 91, 73
181	180	163, 147, 107, 105, 91, 73

$C_8H_{16}O_{11}$, $C_{11}H_{20}O_{13}$ and $C_{11}H_{22}O_{15}$. In all cases a sugar molecule is involved. The remaining R-group is different according to the selected masses. The fact that no N-compounds were detected may be due to any of the following: the small amount of Maillard intermediate product (MIP) released during mild heat treatment of orange juice; further degradation of unstable MIP; or the fractionation. The chemical structure of the unknown compounds responsible for mutagenicity remains to be elucidated, thus preventing the development of a simple chemical assay. In the meantime, the authors' microbiological assay (Ekasari *et al.*, 1988) remains valuable in measuring heat load.

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