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IMPURITIES OF AMARANTH, A FOOD DYE; THEIR TOXICOLOGICAL IMPLICATIONS

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ABSTRACT: *Ethyl acetate extracts of 6 commercial amaranths produced in 1985 and 1986 were analyzed with a gas chromatograph. The α-naphthylamine ranging from 142 ppb to 4216 ppb was detected, but the β-naphthylamine was not detected. The mutagenicity of the ethyl acetate extract was tested using Salmonella typhimurium TA100 in the presence of the S-9 fraction. Significant mutagenic activity was seen in samples containing high levels of α-naphthylamine. It is suggested that the potential hazard of amaranth to the general public should be reconsidered from the point that the impurities contained in amaranth preparations are the main sources of mutagenicity or carcinogenicity.*

Keywords: *Amaranth, α-naphthylamine (α-NA), β-naphthylamine (β-NA), Salmonella typhymurium TA100, α-nitronaphthalene (α-NN), β-nitronaphthalene (β-NN)*

INTRODUCTION

Amaranth (FD & C Red No. 2; CI16185), A food color additive, has been used worldwide (International Life Science Institute, 1986). In Korea, this dye is now used for coloring confectionery products, beverages, dairy products, etc. (Registration Law on Food Additives, National Institute of Health, 1985).

This dye has been the subject of international controversy because of its well known toxicological test results. Fetotoxicity and carcinogenicity of amaranth was reported in Russia (Baigusheva, 1968; Andrianova, 1970). The U.S.FDA banned the use of amaranth in foods, drugs and cosmetics in 1976, based solely on its own long term animal tests.

It has been tested for both the *in vitro* and *in vivo* mutagenic activities. Some investigators reported negative results with *Salmonella typhymurium, Escherichia coli* mutant and yeast (Maxwell and Newell, 1974; Gardner and Nutman, 1977; Kada et al., 1972) and others had positive data using chinese hamster ovary cell (CHO), human lymphocytes and mouse micronucleus tests with unspecified samples (Ishidate et al, 1984; Vaidya and Godbole, 1978; Combes and Haveland-Smith, 1982).

These discrepancies have prompted an assumption that the amaranth used in these toxicity tests might have been contaminated with potent mutagenic and/or carcinogenic agents. The presence of α-NA and β-NA in amaranth was discovered finally by Stavric et al (1979) using gas chromatography.

The above assumption gives a probable interpretation why the old animal toxicity tests, when the quality of amaranth was relatively poor, showed positive carcinogenicity results and recent ones, when the quality control of the product is improved, are showing negative results.

Results of Stoltz et al (1979), tested with Salmonella / microsome, supported the possibility of negative results due to the suppression effect of amaranth itself even when the mutagenic contaminants were present.

This led the authors to the idea that the commercial amaranth used in Korea might contain certain levels of carcinogenic or mutagenic contaminants which are not under the present regulations, but which may pose potential hazards to the general public. Moreover, it would be of interest to apply a simple biological test to assess the possible hazard without resorting to laborious chemical analysis which have limitations in detecting a few known suspected chemicals. Therefore, an attempt was made in this study to correlate the contaminant level in commercial amaranth with the Ames mutagenicity test (Maron and Ames, 1977; McCann and Ames, 1977).

MATERIALS AND METHODS

Materials

Six certified food grade amaranths (B and O Co., Korea) were collected from markets of Seoul and Taejeon in Korea. They were produced in 1985 and 1986. Ethyl acetate used in extractions was obtained from Shinyo Pure Chemical Co. and was the guaranteed grade. The α-NA from Merck, β-NA from Wako Pure Chemical Co. and 1-nitronaphthalene and 2-nitronaphthalene from Aldrich were also of guaranteed grade. Dimethylsulfoxide (DMSO) was obtained from Merck and Glucose-6-phosphate, NADP, histidine and biotin were purchased from Sigma Chemical Ltd.. All other chemicals used were laboratory reagent grade.

Gas Chromatography

Capillary gas chromatography was carried out on a Varian 3700 equipped with a thermionic specific detector (TSD) and a 12 m × 0.33 mm id. fused silica capillary column (BP-1, 0.25 μm film thickness). An isothermal column temperature of 190°C was used, other operating conditions included the following: injector, 250°C; detector, 250°C; nitrogen carrier gas at 1.9 ml/min; split ratio, 18:1.

JEOL(Japan Electronics Optic Laboratory) JMS-BX-300 Gas Chromatograph / Mass Spectrometer was used for the identification of α-NA and the separation of a major unknown component.

Extraction Procedure

10 g of amaranth was dissolved in 150 ml of distilled water and transferred to a 500 ml separatory funnel. Subsequently, 100 ml of ethyl acetate was added and shaken for 2 min, let stand, and the aqueous phase was collected into the same beaker. The ethyl acetate layer was collected through a funnel containing 70 g anhydrous sodium sulfate into a 500 ml round bottom flask. This extraction procedure was repeated twice with 50 ml ethyl acetate. The extract was concentrated with a rotary evaporator (Büchi R110) at 40°C under vacuum to about 1 ml. The concentrate was transferred to a 20 ml vial and washed five times with a total of 15 ml ethyl acetate. The extract was dried under nitrogen at 35°C and 1 ml of ethyl acetate was added. 2 μl of the solution was injected into a gas chromatograph for analysis and GC / MS for the identification of α-NA and the major unknown component.

Salmonella typhimurium TA100 Mutagenicity Test

The ethyl acetate extract equivalent to 10g amaranth was evaporated under nitrogen to dryness and was dissolved in 100 μl of DMSO for plate incorporation. The procedures used were basically those of Ames et al (1977).

RESULTS

The typical chromatogram of ethyl acetate extract of amaranth is shown in Figure 1. The retention time of α-NA and β-NA was 2.45 min and 2.60 min, respectively. Table 1 shows the concentration of naphthylamines in the samples of amaranth. Detection limit of the present method was 50 ppb for both α-NA and β-NA. Recoveries with 500 ppb and 2500 ppb spiking levels of α-NA were greater than 90% and therefore no futher recovery corrections were made for each sample analysis. However, the recoveries of β-NA at both concentrations were approximately 60%. As no samples showed any β-NA, further improvement on the recovery method was not made in this experiment.

As shown in Table 1, all samples contained high levels of α-NA while β-NA was not detected. The content of α-NA varied from 142 ppb to 4216 ppb regardless of production date or manufacturing batch.

Samonella typhimurium TA100 mutagenicity test with S9 was carried out for the ethyl acetate extracts as shown in Table 2. The mutagenic activity was increased with the increasing concentrations of α-NA isolated from amaranth (Fig. 2). Even with the limited number of 4

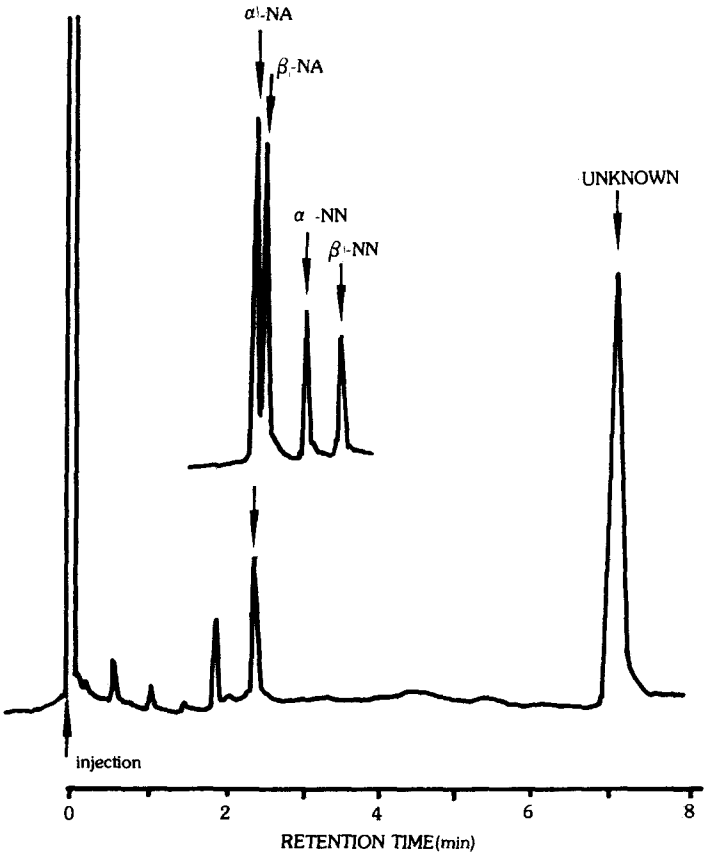

Fig. 1. Typical Chromatogram of ethyl acetate soluble impurities of amaranth sample produced in June, 1986

samples, the numbers of revertants were increased with the increase of α-NA concentration in amaranth at a correlation coefficient of 0.901.

Table 1. Concentration of naphthylamines (ppb) in amaranth

Date of production	α-naphthylamine*	β-naphthylamine
Control**	N.D.***	N.D.
85, 4	142.3 ± 8.13	N.D.
85, 7	2095.0 ± 790.53	N.D.
85,12	813.2 ± 93.86	N.D.
86, 1	290.0 ± 62.22	N.D.
86, 6	437.5 ± 41.46	N.D.
86, 8	4216.3 ± 187.77	N.D.

* Average of triplicate determination and one standard deviation
** solvent blank; followed the whole procedure without sample
*** Not detectable; 50 ppb

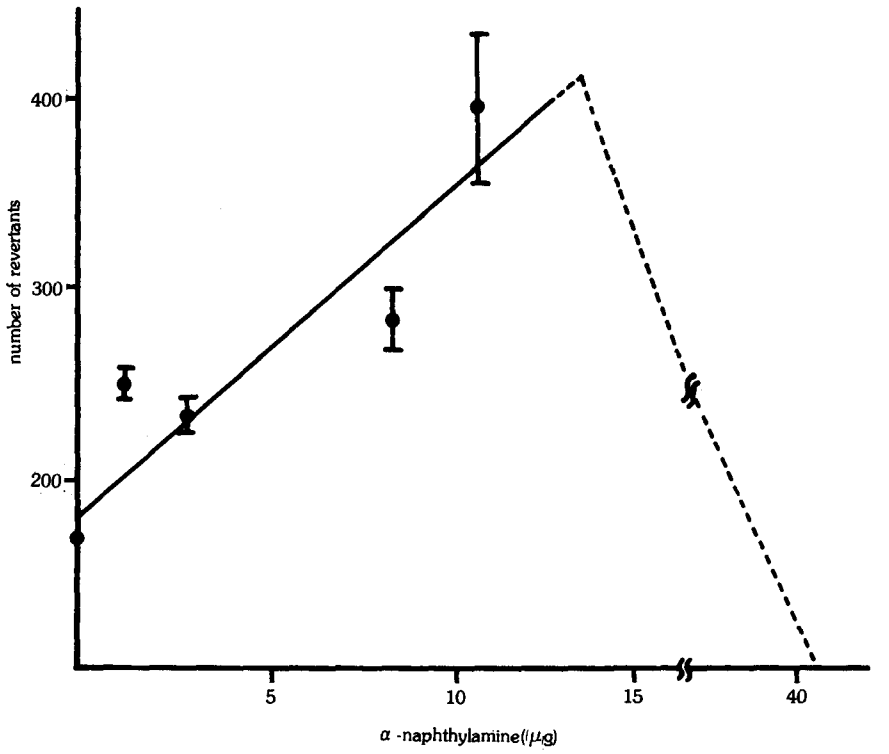

Fig. 2. Revertants with TA100 with S9 vs. the α-NA content in the ethyl acetate extract of amaranth. Correlation coefficient of 4 samples was 0.901.

Table 2. The number of revertants of *Salmonella typhymurium* TA100 with metabolic activation

sample	number of revertants*
solvent control**	167
85, 4	252 ± 8
85, 7***	392 ± 38
85,12	286 ± 15
86, 1	232 ± 11
86, 6	K****
86, 8	K

* Amount of the applied ethyl acetate extract per plate was equivalent to 10g of amaranth

** Solvent control; followed the whole procedure without samples

*** Amount of the applied ethyl acetate extract per plate was equivalent to 5g of amaranth

****K; not countable due to killing

DISCUSSION

The α-NA levels from the samples in this study are far higher than the 7 ppb level of the major amaranth samples from U.S., Europe, Japan, and Mexico analyzed by Stavric et al (1979). Therefore, it is indicated that relatively poor quality amaranth is consumed in Korea and these commodities have a potential of being contaminated by potent β-NA although this study failed to show the presence of β-NA in amaranth.

One interesting finding in this study was that an unknown peak appeared at 7.1 min. It was once tentatively identified as α-or β-nitronaphthalene. The identity of the compound partly came from the major ion peak at 173 m/e with GC/MS. The possibility is also partly supported by the fact that α-nitronaphthalene is an intermediate for the production of amaranth. But the retention times of the standard nitronaphthalenes failed to match with that of the unknown peak. As the amount of the unknown compound is much more than that of α-NA, the presence of that compound could possibly act as an additive or synergistic factor for the toxicological effect of α-NA and/or β-NA.

As shown in Fig. 2, mutagenicity increased with the increase of α-NA content of the extract. This indicates that α-NA could be an index compound for contaminants showing mutagenicity, but the compound alone could not explain their actual mutagenicity. It became evident in the present study that a number of organic impurities other than α-NA and β-NA are present in amaranth. However, as this study is limited to *in vitro* test, it is premature to draw any conclusion about the direct hazard to human being caused by the use of amaranth in food in Korea.

The present result with amaranth gives us a general suggestion which should be emphasized in assessing the safety of chemicals. There is sample possibility that the sample for toxicity test is different from the one the general public consumes. Therefore, a thorough identification of impurities contained in the test material is needed. Additionally, quality control at manufacturing level should be conducted with the aim of not exceeding the specified impurities. Though reasonable, practical implementation could be very difficult. The approach taken in this study, namely, chemical analysis in conjunction with a simple biological test after the isolation of impurities from active ingredient at the state of toxicity test, would ease the process of quality control for potential carcinogens. This approach especially seems applicable to any chemical which uses carcinogenic intermediates in its production.

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