# Aflatoxins and Fermented Foods in Japan

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## Introduction

While 'Miso', 'Shoyu' and 'Sake' are Japanese traditional fermented foods and are all brewed by the use of Koji-molds, it was found in 1960 that *Aspergillus flavus*, a mold closely related to the Koji-mold, produces aflatoxins which are substances very strong in carcinogenic effect.[1] This discovery started from the poisoning of poultry fed with peanuts imported from Brazil to England. Since then we are often questioned by some people both at home and abroad who know little of the differences between brewing Koji-molds and the aflatoxin-producing ones as to whether Japanese industrial Koji-molds produce aflatoxins or not.

In consequence, whether Japanese industrial Koji-mold strains produce aflatoxins or not has become an important question to be settled.

On the other hand, the problems of aflatoxin were taken up by the Toxic Microorganisms Panels in the Joint U.S.-Japan Cooperation on Development and Utilization of Natural Resources (UJNR). Experts of the Japanese and U.S. Government offices, such as Ministry of Agriculture and Forestry, Ministry of Health and Welfare, U.S. Department of Agriculture and U.S. Department of Health, Education and Welfare, cooperated in the investigations, and a symposium and a joint conference on the toxic microorganism were held in October, 1968 in Hawaii.

In the Food Research Institute, Ministry of Agriculture and Forestry of Japan, studies were made in the first place to establish a new method of analysis for aflatoxins and then thorough investigations were made on the possibility of the presence of aflatoxins in Japanese fermented foods and their raw materials, including industrial Koji-mold strains. But no aflatoxin was detected in them as reported by many Japanese and foreign researchers, as Masuda *et al.*, Hesseltine *et al.*, Aibara *et al.*, Yokotsuka *et al.* and Murakami *et al.*

## Fermented foods in Japan

*1) Miso*

'Miso' is one of the most important foods in Japan. It is made from soybeans, rice and others and contains 10–18% of protein and 5–12% of salt. There are at present about 3,000 factories producing 530,000 metric tons of 'Miso' per year in Japan. In addition to this what is called home-made 'Miso' is also popular in agricultural districts. A Japanese consumes about 22 g. of 'Miso' per day mainly in the form of 'Miso-soup'. And just as there are varieties of cheese in Europe, so also we have many kinds of 'Miso' in Japan, depending upon the type of raw materials used, their mixing ratio and the method of processing. The manufacturing method is outlined in Fig. 1.

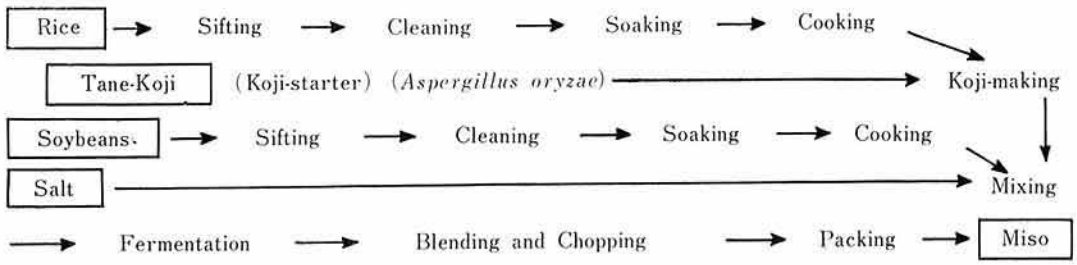



Fig. 1. Flow Sheet of Miso Manufacture.

*2) Shoyu*

'Shoyu' is a traditional seasoning indispensable to Japanese people. It is made from soybeans and wheat, and contains about 18% of salt. There are about 4,500 factories producing 'Shoyu' at present in Japan and the yearly output amounts to about 1,080,000 kl. A Japanese is said to take about 1 *l* of 'Shoyu' per month. Since Japanese foods were generally composed of vegetable sources, some seasoning materials besides ordinary salt, sugar and other spices were necessary for giving delicacies to them. And for this purpose, 'Shoyu' has been known to be the most suitable from very early times. The use of 'Shoyu' is now popular also in many countries besides Japan.

Although there are variations in the method of 'Shoyu' making, the principal processes of natural brewing are as shown in Fig. 2. Defatted soybeans and wheat bran can be used as materials instead of intact soybeans and wheat.

## Aflatoxins[2), 3)]

There are 8 kinds of aflatoxins whose chemical structures are known at present, and $B_1$, and $B_2$ and their derivatives, $M_1$, $M_2$ and $B_{2a}$ are blue~bluish purple and $G_1$, $G_2$ and their derivative $G_{2a}$, are green~yellowish green in fluorescence under ultraviolet light (Fig. 3). $B_1$ is the most toxic among them according to Butler *et al.*, its carcinogenic effect on the rat is about 900 times as high as that of butter yellow.

It is generally accepted that specific strains of *Aspergillus flavus* and *Asp. parasiticus* are the only molds which produce aflatoxins. And

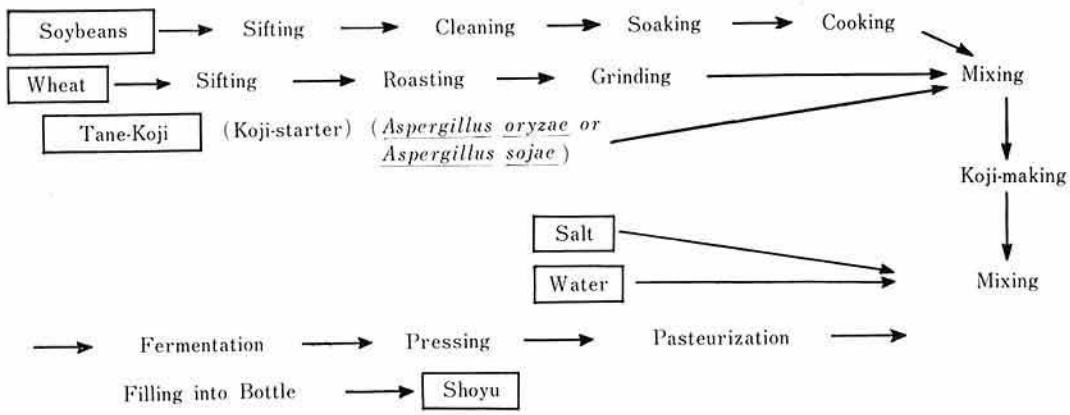



Fig. 2. Flow Sheet of Shoyu Manufacture.

Murakami takes up the position that aflatoxin producing *Asp. flavus* ATCC 15517 is a mold belonging to *Asp. parasiticus*. According to the

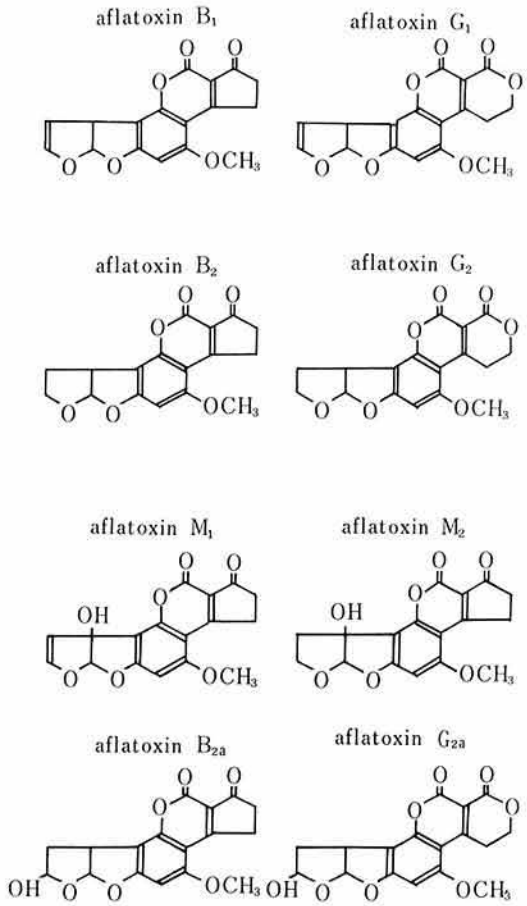



Fig. 3. Chemical structure of aflatoxins.

classification by Sakaguchi *et al.*, *Asp. oryzae* and *Asp. sojae* which are Japanese industrial Koji-molds can be clearly distinguished from aflatoxin-producing *Asp. parasiticus* and *Asp. flavus*. But the discrimination is not always clear in the classification by Raper and Fennell and gives rise to various questions.

Hesseltine *et al.* reported that aflatoxin-producing molds grew well on various agricultural products, as rice, wheat, corn, sorghum and soybeans, including peanuts, to produce aflatoxins. The production of aflatoxins, however, is the lowest in soybeans among them. The aflatoxin-producing molds are considered to be distributed now in all the world centering around the tropics.

## Investigation on the possibility of the presence of aflatoxins in fermented foods

*1) Establishment of a new method of analysis for aflatoxins*[4]

Thin-layer chromatography (TLC) has been a usual method of chemical detection for aflatoxins. In TLC, silica gel is used generally as adsorbent and methanol-chloroform is a popular developer. But it is difficult to maintain the activity of silica gel thin-layer always uniform, and separation and quantitative analysis of the four kinds of aflatoxin ($B_1$, $B_2$, $G_1$ and $G_2$) often become difficult due to fluctuation in Rf value when the thin-layer is not uniform. In view of this fact, the authors carried out studies on TLC to improve their mutual separation.

As a result, it was found that 5% acetone in chloroform was a favorable developer giving good separation in case Silica gel G (ex Merck) was used as adsorbent and 1–3% methanol in chloroform was suitable to Silica gel HR (ex Merck) (Fig. 4).

However, TLC can not be a satisfactory method for exact detection of aflatoxins, and at

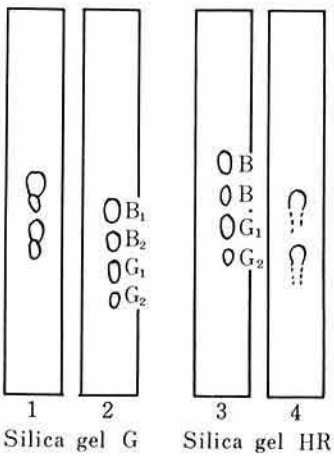



Fig. 4. TLC chromatogram of aflatoxin mix.
1. 3% methanol in chloroform
2. 5% acetone in chloroform
3. 3% methanol in chloroform
4. 5% acetone in chloroform

least examination of ultraviolet absorption is necessary to make up for the deficiency. The authors, therefore, developed a new liquid chromatographic (LC) method for separation of aflatoxins in liquid state to meet the need of larger amount of samples for their examination of ultraviolet absorption.

Among the various kinds of columns, such as alumina, silica gel, Sephadex G-10, G-25 and LH-20, Sephadex G-10 showed good mutual separation of the aflatoxins. When crude aflatoxins dissolved in a minimal volume of methanol were developed on a Sephadex G-10 column and eluted with 1% methanol in water or with distilled water, the fraction eluted first was a mixture of coloring matters, and then fractions of aflatoxins $G_2$, $B_2$, $G_1$ and $B_1$ were obtained in this order successively. And it was proved that this chromatographic method could be used to analyze aflatoxins quantitatively within 10 per cent error when their contents in a sample were 10 $\mu$g or more (Fig. 5 and Table 1).

*2) Investigation on the possibility of the presence of aflatoxins*[5), 6)]

Extensive studies were made by the above-mentioned method to investigate whether or not aflatoxins are contained or produced in industrial Koji-mold strains (*Aspergillus oryzae* and *Asp. sojae*), rice and fermented foods in Japan. Liquid or solid cultures of Koji-mold strains, rice, rice 'Koji' in factories, 'Miso' and 'Shoyu' were treated with chloroform to extract aflatoxins, and the extracts obtained were analyzed by the silica gel thin-layer chromatography.

The tests proved no presence of aflatoxins in such samples as a) 238 Koji-mold strains in use for manufacturing of 'Miso' and 'Shoyu' in Japan, b) 46 samples of domestic rice from all prefectures in Japan (produced in 1966 or 1967), c) 11 samples of imported rice (from U.S.A., Spain, Burma, Thailand, Communist China, Taiwan in 1966–1967), d) 28 kinds

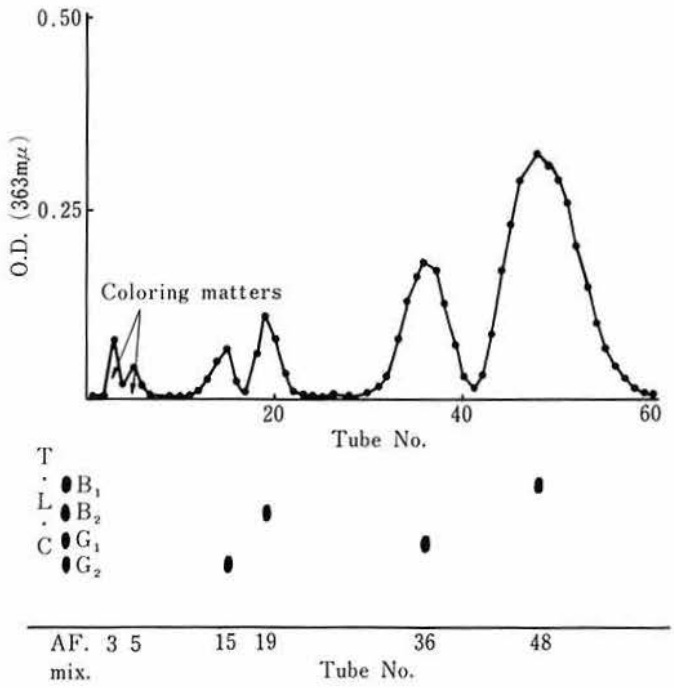



Fig. 5. Above: Fractionation of aflatoxin mix. on a 8 × 500 mm Column of sephadex G-10. Eluant: $H_2O$. Below: Chromatogram of fractionated aflatoxin on TLC.

**Table 1. Separation and refine method of aflatoxin**

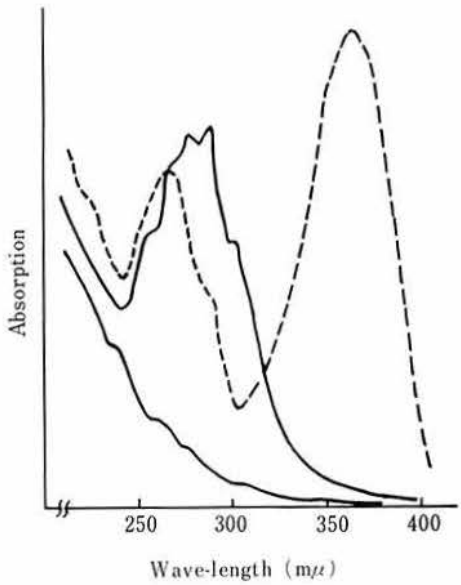
Sample (contaminated by aflatoxin)
↓
Defatting .....hexane
↓
Extraction .....chloroform or methanol
↓
Dehydration .....sodium sulfate
↓
Concentration
↓ ←——hexane
Precipitation
↓
Liquid chromato. Silica gel column, 1% methanol in chloroform
↓
Dry in vacuo.
↓ ←——methanol
Liquid chromato. Sephadex G-10 column $H_2O$ or 1% methanol in $H_2O$
↓
Fractionation ($B_1$, $B_2$, $G_1$ and $G_2$)
↓
Extraction.....chloroform
↓
Shotwell et al:
Dry in vacuo.
↓ ←——chloroform 6.6 ml
Filtering ←——chloroform 2.0 ml
↓ ←——hexane 16.5 ml
at 20°C overnight
↓
Crystallization

of rice 'Koji' from various 'Miso' factories in eight districts of Japan, e) 108 items of 'Miso' from all prefectures in Japan, f) 21 items of farmers' home-made 'Miso' from various districts of Japan and g) 6 representative brands of 'Shoyu', showing that Japanese fermented foods were non-toxic in every step from raw materials to products so far as aflatoxins are concerned.

Although chloroform-soluble fluorescent compounds were found to be contained in some of the samples tested and showed aflatoxin-like behaviors on the chromatogram, they were proved to be not aflatoxins by the examinations of ultraviolet absorption spectra and others. For example, all Koji-molds tested contained

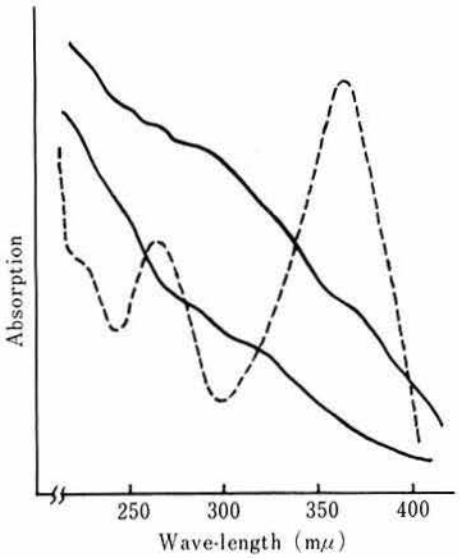
**Table 2. Statistics of the Koji-molds that produce compounds similar to four aflatoxins on Czapek's solution**

| Usage | Aflatoxin-like fluorescent compound | | | Aflatoxin | | Total |
|---|---|---|---|---|---|---|
| | + | ? | − | + | − | |
| Miso | 6 | 7 | 34 | 0 | 47 | 47 |
| Shoyu | 21 | 8 | 36 | 0 | 65 | 65 |
| Sake | 4 | 0 | 25 | 0 | 29 | 29 |
| Miso or Sake | 0 | 0 | 10 | 0 | 10 | 10 |
| Tamari | 3 | 0 | 3 | 0 | 6 | 6 |
| Enzyme | 0 | 0 | 1 | 0 | 1 | 1 |
| Shochu (Spirits) | 1 | 0 | 3 | 0 | 4 | 4 |
| Ama-zake | 1 | 0 | 2 | 0 | 3 | 3 |
| Others | 16 | 10 | 47 | 0 | 73 | 73 |
| Total | 52 | 25 | 161 | 0 | 238 | 238 |
| Per cent | 21.8 | 10.5 | 67.7 | 0 | 100 | 100 |




------- : Authentic aflatoxin $B_1$.
——— : Aflatoxin-like substances.

Fig. 6. Ultra-violet absorption spectra Blue-purple fluorescent substances (Methanol).




------- : Authentic aflatoxin $G_1$.
——— : Aflatoxin-like substances.

Fig. 7. Ultra-violet absorption spectra Green fluorescent substances (Methanol).

no aflatoxin but aflatoxin-like compounds were found in about 30% of them (Table 2). And these compounds were clearly different in ultraviolet absorption spectra from aflatoxins as shown in Fig. 6 and Fig. 7.

The Japanese fermented foods which were feared to be toxic by some people, especially foreigners, with the question of aflatoxins have been thus cleared of the suspicion and assure their positions as traditional foods. It is necessary hereafter to be fully prepared for periodical checks on aflatoxin-producing molds to meet their invasion from abroad.

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