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The Production of Aroma by *Aspergillus oryzae* During the Preparation of Soy Sauce *Koji*

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ABSTRACT

The rôle of koji *in the preparation of soy sauce has been a subject of controversy. A study of the volatiles produced during* koji *preparation and a comparison with uninoculated bean-flour mixture shows that the growth and sporulation of* Aspergillus oryzae *is related to the production of volatiles. The volatiles present at the mycelial stage are different from the volatiles of sporulated* koji. *The ether extracts of the acidic homogenates were different from those of the neutral and alkaline homogenates.*

INTRODUCTION

The manufacture of soy sauce generally involves two stages of fermentation—the *koji* stage and the *moromi* stage.

Koji is a Japanese word describing the preparation of mould growth on cooked cereals and/or soy beans. It serves as an enzyme source for the conversion of natural plant constituents to simpler compounds. *Koji* for Chinese soy sauce is made from a mixture of wheat flour and steamed soy beans with strains of *Aspergillus oryzae* or *Aspergillus soyae* as the *koji* starter. On the other hand, roasted wheat and steamed defatted soy bean meal are often used by the Japanese when they prepare *koji*.

Soy sauce *koji*, with its dark-green appearance, pleasant aroma and sweet taste with a slightly bitter note, contains high protease and amylase

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activity and is generally regarded as of superior quality (Wang & Hesseltine, 1979). During the *koji* stage of fermentation, proteins are broken down to peptides and amino acids by proteolytic enzymes, especially by neutral and alkaline proteases. Polysaccharides are hydrolysed to oligosaccharides, disaccharides and monosaccharides, mainly by α-amylase secreted by the mould, although some invertase activity has also been detected (Yong, 1971). Lipids are also acted upon by the lipase present in the *koji*.

It is generally believed that the *moromi* stage of fermentation is mainly responsible for the flavour of soy sauce. Yokotsuka (1960), however, suggested that a good culture mould must give rise to the characteristic flavour of soy sauce. The metabolic processes involved in the *koji* stage of fermentation, which may have a rôle in the development of the final flavour of soy sayce, have not beeen investigated. Nevertheless, it should be noted that soy sauce manufacturers generally believe that a well-sporulated *koji* is necessary for the production of good quality soy sauce.

The present study seeks to establish whether the *koji* stage of fermentation contributes to the development of volatiles crucial to the characteristic aroma of soy sauce. The study reported in this paper was carried out with samples of *koji* at various intervals from the time of inoculation to sporulation of the mould.

MATERIALS AND METHODS

Purity and source of chemicals

All inorganic chemicals were of Merck 'AnalaR' grade. Soy beans and plain wheat flour were obtained commercially. The diethyl ether for extraction was of British Drug House (BDH) 'AnalaR' grade.

Mould

Aspergillus oryzae NRRL-1989.

Media and culture methods

The mould was maintained on potato dextrose agar (Oxoid), 3·9 g in 100 ml of distilled water. Slopes of this medium were inoculated with

mould spores and incubated at 30 °C for 9 days, by which time profuse sporulation had taken place. Fresh cultures were used for the preparation of the *koji*.

pH measurements

All pH measurements were made on a Corning pH meter model 7.

Preparation of *koji*

The *koji* was prepared according to the method of Yong & Wood (1976), with slight modifications. Instead of a 1:1 ratio of soy beans to flour, a 1:0·8 ratio was used as it was found to produce a drier, and less starchy, *koji*.

A control was set up in which no spores were added. Both the test and control were maintained under aseptic conditions.

Extraction of volatiles from *koji*

Three 50-g samples of *koji* at 0, 24, 48 and 72 h were separately homogenized with 100 ml of sodium phosphate buffer, 0·1 M, pH 7·0. The three homogenates were centrifuged at 3000 rpm and the supernatants retained.

One of the homogenates with unadjusted pH was the neutral fraction. The acidic fraction was obtained by adjusting another of the homogenates to pH 4·5 with 1 M HCl. The third supernatant was made alkaline with 10% NaOH to pH 8·0.

Each fraction was then extracted with a known volume (200 μl) of diethyl ether using a J & W Scientific liquid–liquid extractor. The ether extracts were then analysed by capillary gas–liquid chromatography (GLC).

The control, consisting of uninoculated bean-flour mixture, was homogenized and adjusted to pH 4·5. The homogenate was then extracted in the same way and analysed by capillary GLC.

Analytical methods

Capillary GLC analysis

Each ether extract was qualitatively analysed using a HP 5880A gas

chromatograph equipped with a flame-ionisation detector. The column used was an HP Ultra No. 2, 25 mm × 0·20 mm inside diameter fused silica with polyethylene glycol, molecular weight 20 000 (Carbowax 20M). The carrier gas was nitrogen set at 10 psi ($\mu = 40$ cm/s). The injector temperature was 200 °C, and the detector temperature, 250 °C, and the column was temperature programmed from 70 to 170 °C at 2 °C/min and with a final time of 80 min. Manual injection of 2 μl was carried out and the split ratio set at 10:1.

RESULTS

Preparation of *koji*

The *koji* was prepared together with a control. The conditions used for the control were similar to those of the test but to spores were added. The objective of the control was to determine the rôle of the mould in the contribution of volatiles present in the *koji*. Both the inoculated and uninoculated mixtures were kept under aseptic conditions.

Samples were taken each day and evaluated for appearance and smell. They were homogenized and extracted for volatiles and the extracts analysed by capillary GLC. The results of the evaluation of *koji* at various stages of preparation are shown in Table 1.

Immediately after inoculation (0 h), the test and control looked and smelled the same. Both had a beany, maltol-like smell. The flour coating the beans was dry. Both the test and the control had a temperature of 30 °C—the ambient temperature.

By 24 h after inoculation, the test had lost its bean aroma. Instead, there was a mouldy smell and the temperature of the mixture had risen to 37 °C. Some of the beans were covered with white mycelia. The appearance and smell of the control were both unchanged from 0 h.

At 48 h, there was no change in the control. In the test there was now a white carpet of mycelia covering the beans. The mixture smelled mouldy and the temperature had fallen slightly to 35 °C. The walls of the perspex container in which the *koji* was incubated were covered with moisture due to condensation. The soy beans had also shrunk in size.

By 72 h profuse sporulation of the fungus had taken place. The beans were now covered with a thick, dark-green coating of fungus with an abundance of fungal spores. There was a mouldy smell with some

TABLE 1
Evaluation of the Appearance and Aroma of *Koji* by Sniffing

Time of incubation (h)	*Description of appearance*	*Aroma*	*GLC profile*
0	Beans covered with yellow–brown flour. Temperature, 30 °C.	Beany, roasted flour smell like that of maltol	Fig. 1. Few peaks initially with distinct and large peaks after 50 min
24	Beans covered with some white mycelia. Temperature, 37 °C	Mouldy	Fig. 2. Peaks towards the end of the run reduced in height; new peaks before 40 min (peaks 'a' and 'b')
48	Whole of the bean-flour mixture coated with white mycelia. Temperature, 35 °C	Mouldy	Fig. 3. Peak 'a' large; peak 12 reduced in height and peak 'b' one-third of peak 'a'
72	Mould sporulated; dark-green spores. Temperature, 35 °C	Mouldy but slightly sweet	Fig. 4. Distinct peaks throughout run

sweetness and a slightly bitter note. The mixture was dry and the temperature had stabilised at 35 °C. The control remained the same as at the time of inoculation.

Capillary GLC analysis

The results of the capillary GLC analyses are shown in the accompanying Figures.

Acidic extract of *koji*

The ether extract of *koji* at 0 time (Fig. 1) showed few significant peaks in the initial part of the chromatogram. Major peaks appeared, however, after 40 min. These are shown in Fig. 1, numbered 14 to 24.

In Fig. 2, the 24-h extract looks different. The major peaks, 14 to 24, have decreased in height and those before 40 min are more distinct. New

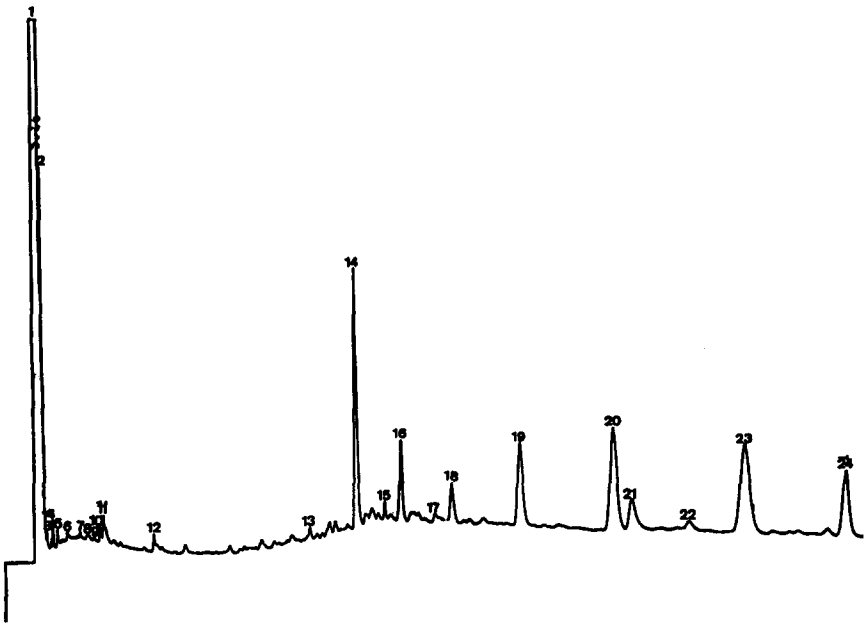

Fig. 1. Chromatogram of *koji* at 0 h, extracted at pH 4·5.

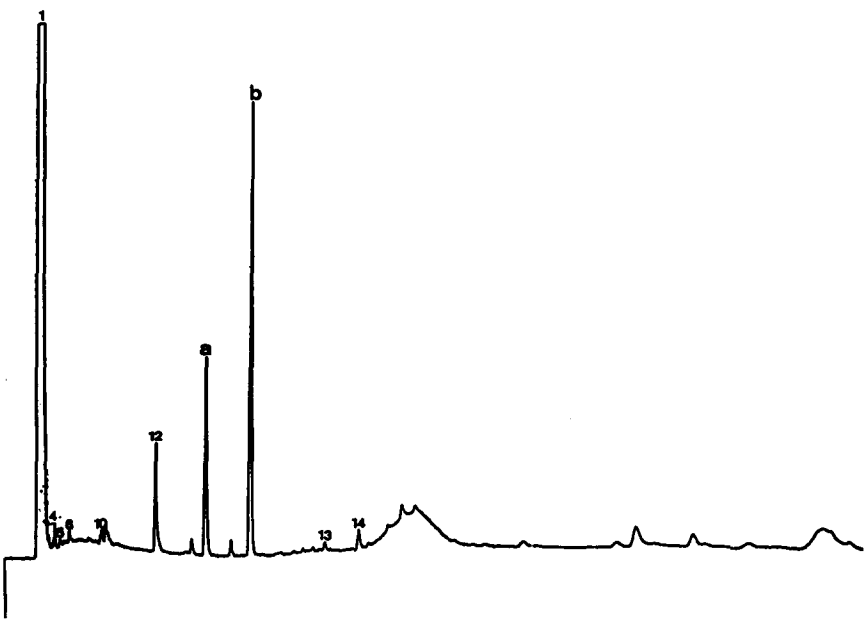

Fig. 2. Chromatogram of *koji* after 24 hours' of incubation, extracted at pH 4·5.

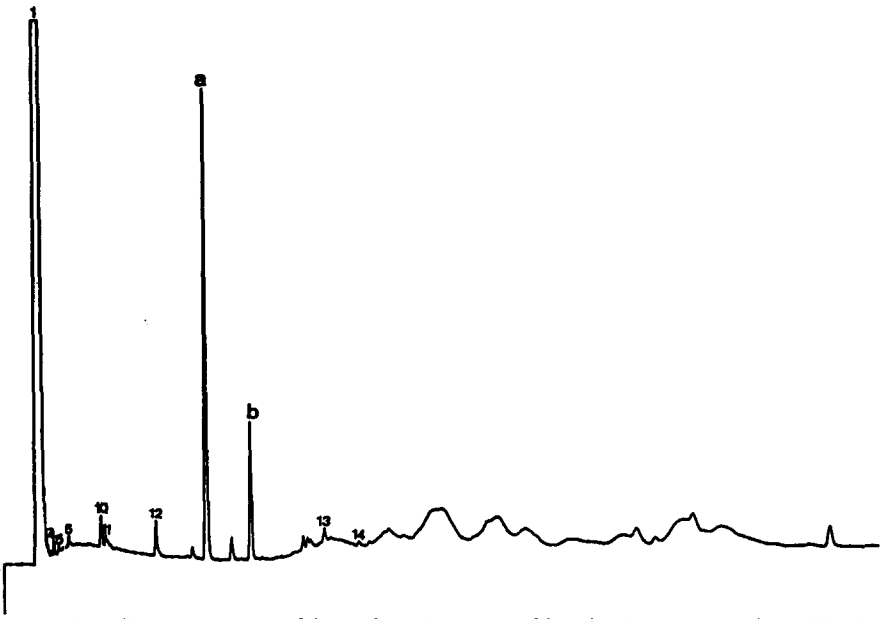

Fig. 3. Chromatogram of *koji* after 48 hours' of incubation, extracted at pH 4·5.

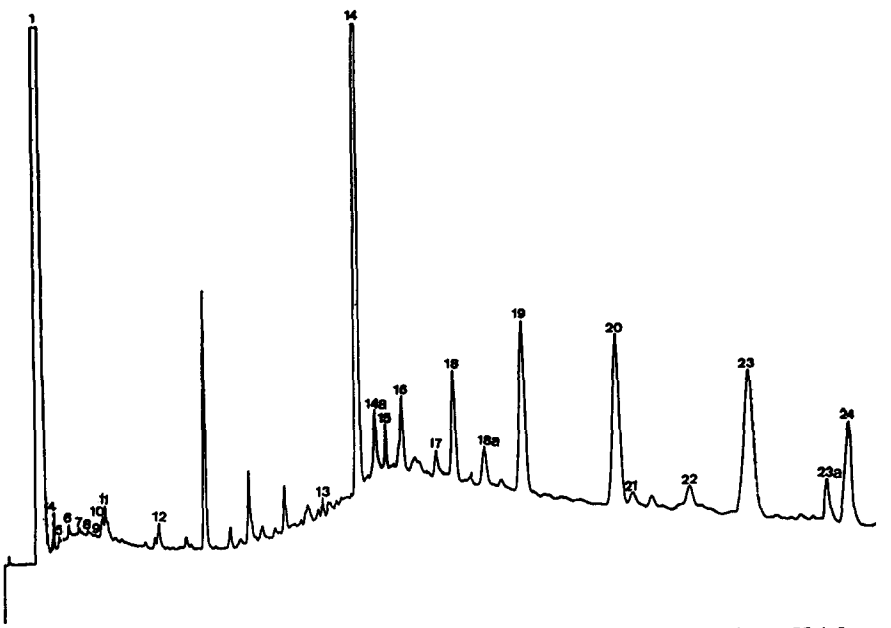

Fig. 4. Chromatogram of *koji* after 72 hours' of incubation, extracted at pH 4·5.

peaks, notably peaks 'a' and 'b', appear. Peak 12, which was shorter than peak 14 in Fig. 1, is now larger.

In the 48-h sample (Fig. 3), peak 'a' remains large. In Fig. 2, peak 12 was half the height of peak 'a'. In the 48-h sample, peak 12 is only 1/13th the height of peak 'a'. Peak 'b', which was twice the height of peak 'a' in Fig. 2, is now one-third its height. In this sample extract, the peaks towards the end of the run are not well resolved.

In the sporulated sample (72 h), shown in Fig. 4, there seems to be the appearance of many peaks throughout the GLC run. On closer examination, some of these peaks fall in the same positions as those shown in Fig. 1. There are new peaks—15, 21, 25 and 28.

The acidic ether extracts had the characteristic aroma of soy sauce.

Control and spore extract

In the control acidic fraction, a different profile is obtained (Fig. 5). There are, on the whole, few significant peaks. The spore extract does show some significant peaks (Fig. 6).

Neutral fraction of *koji*

The ether extract of the neutral fraction of *koji* gave, on inoculation, the profile shown in Fig. 7. There are major peaks, numbered 5 to 27. These do not fall in the same positions as those in Fig. 1. After sporulation of the mould (Fig. 8), there is a central group of peaks followed by three separate peaks—24, 25 and 26. The number of volatiles has therefore increased on sporulation.

Alkaline fraction

The profiles obtained at 0 and 72 h are similar to those extracted under neutral conditions. In fact, when Figs 9 and 7, and Figs 10 and 8, are superimposed upon each other, the peaks are observed to elute at the same retention times, relative to the solvent.

This corresponds to the evaluation of aroma where both the neutral and alkaline fractions and ether extracts smell bitter and 'chemical', unlike soy sauce.

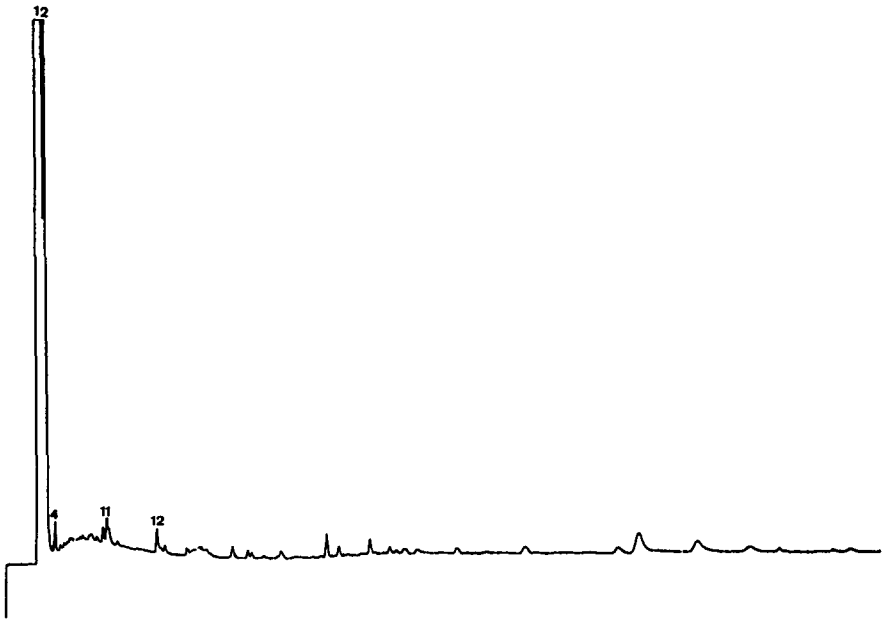

Fig. 5. Chromatogram of control *koji* with no spores added, extracted at pH 4·5.

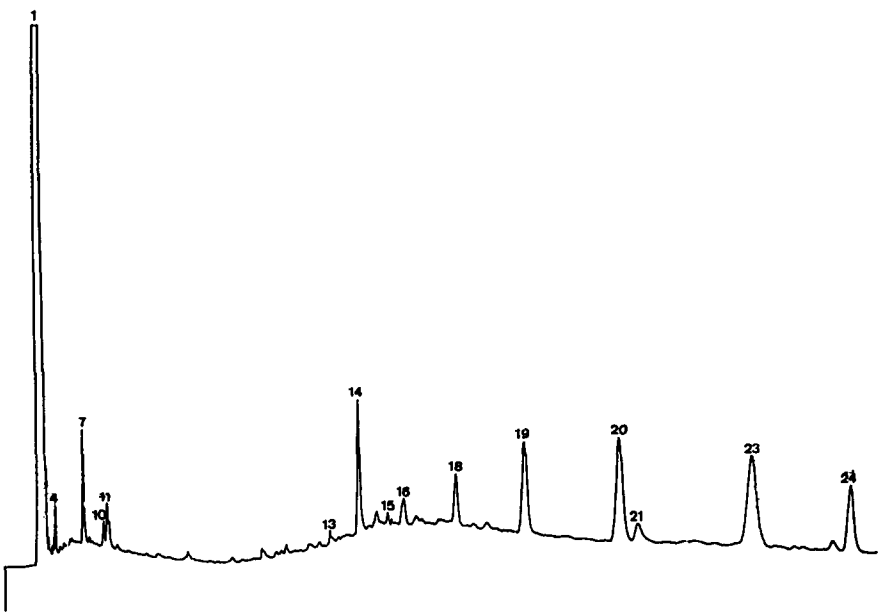

Fig. 6. Chromatogram of ether extract of a suspension of spores of *Aspergillus oryzae*, extracted at pH 4·5.

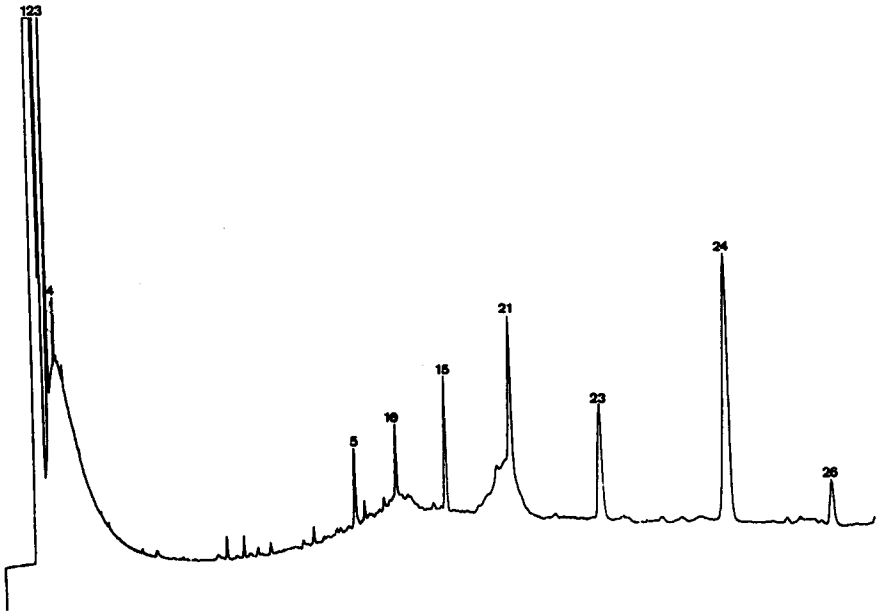

Fig. 7. Chromatogram of *koji* at 0 h, extracted at pH 7·0.

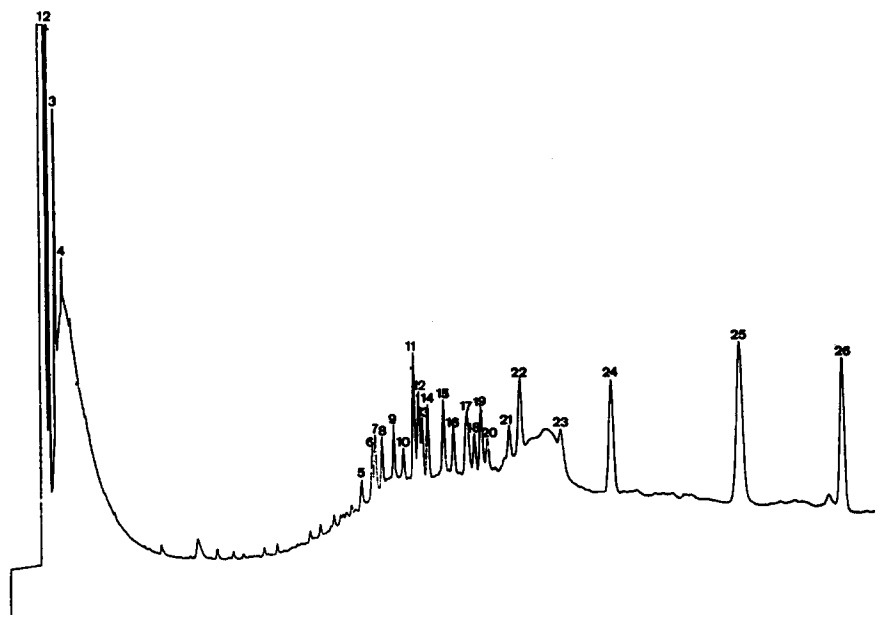

Fig. 8. Chromatogram of *koji* at 72 h, extracted at pH 7·0.

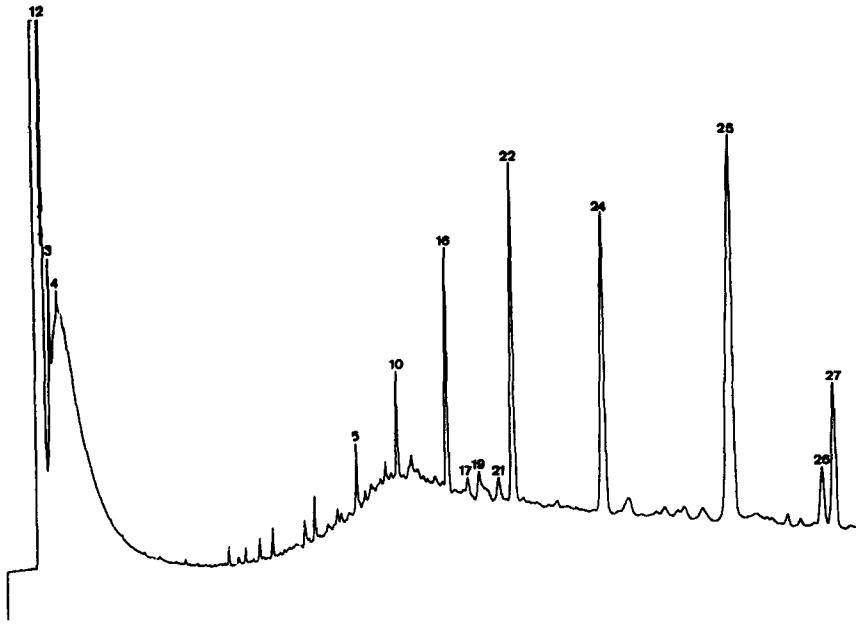

Fig. 9. Chromatogram of *koji* at 0 h, extracted at pH 9·0.

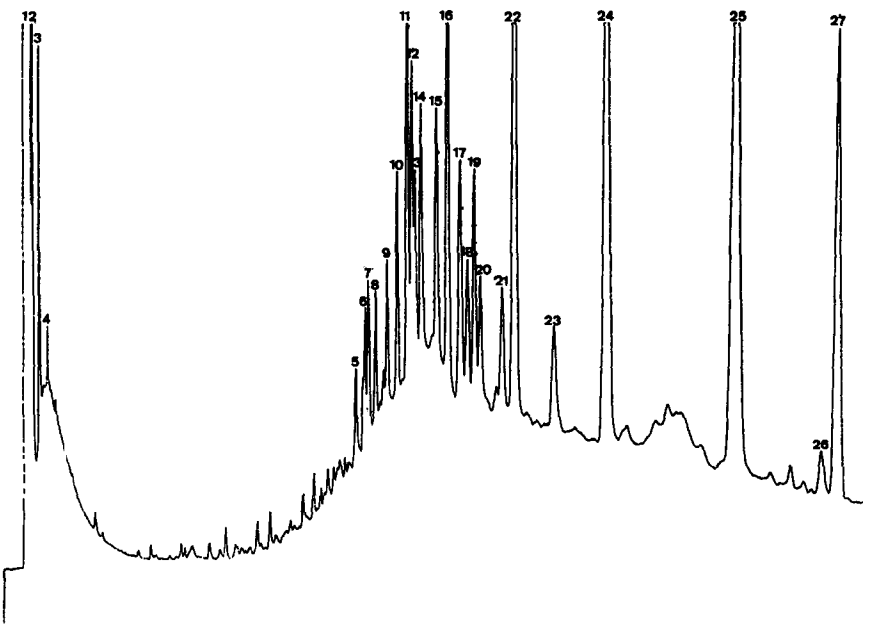

Fig. 10. Chromatogram of *koji* at 72 h, extracted at pH 9·0.

DISCUSSION

The rôle of the fungus in *koji* preparation

The GLC profiles (Figs 1 to 4) show that volatiles are present during all stages of *koji* preparation. Volatiles are not significantly detected in the control (Fig. 5) extracted at acidic pH where no spores were added. Thus, the contribution of volatiles must arise from the fungus *Aspergillus oryzae*.

The change in the aroma of *koji* at various stages of its preparation could be related to the physiological changes undergone by the spores added—that is, germination, vegetative growth and sporulation, as well as the accompanying metabolism. These changes have been discussed by Gottlieb (1976).

Relationship between the GLC profiles and aroma

Table 1 shows that, between inoculation and germination into mycelia, there is a change from a beany, maltol-like aroma of cooked flour to one of a mouldy nature. At 24 and 48 h after inoculation, where the mycelia form a major part of the *koji*, the smell remains mouldy. However, when sporulation of the mould has taken place, the aroma of the *koji* is different. The mouldiness is accompanied by a somewhat bitter–sweet aroma. Accordingly, the GLC profiles of the extracts of acidified *koji* homogenates at various time intervals are also different.

There is also a difference in the neutral and alkaline extracts of *koji* upon inoculation (Fig. 9) and after sporulation (Fig. 10).

Spore germination involves synthetic processes, as well as energy-forming reactions such as the oxidation of carbon compounds, usually sugars or their polyalcohols. Some of these products of metabolism could be volatile and contribute to the observed aroma of the *koji*.

Although the GLC profiles of *koji* at various time intervals are different, the sporulated sample (Fig. 4) resembles the sample at 0 h (Fig. 1). Thus, it was suspected that the similarity was in the spores, as both inoculated and sporulated samples contained spores. An extract of spore suspension, equivalent to the amount used as inoculum, was analysed by GLC. The chromatogram obtained (Fig. 5) was remarkably similar to Figs 1 and 4.

This supports the contention that the spores are responsible for the

pattern of volatiles at acidic pH. The peaks shown in the 72-h sample (Fig. 4) are higher than the 0 h sample (Fig. 1). This is not surprising as the amount of spores in the 72-h sample far exceeds the amount present in the inoculum.

Since a change in the aroma of a sample corresponds to a change in the GLC profile, it suggests that the subjective sensory descriptions of the aroma of *koji* can be correlated with an objective instrumental analysis by capillary GLC, as has been done in this study. A similar approach of investigating the relationship between aroma quality and GLC profiles was carried out by Aishima (1982).

Mycelia versus spores

The question arises as to whether it is the mycelia or the spores which are responsible for the characteristic aroma of *koji*. This problem was also examined in the case of *Penicillium roqueforti* where there was controversy regarding the source of the methyl ketones which give blue cheese its characteristic flavour (Fan *et al.*, 1976).

From odour evaluation, the mycelial *koji* (24- and 48-h samples) smell differently from the sporulated *koji*. The wet mouldiness of the mycelial *koji* is replaced by a dry, bitter-sweet mouldiness in the sporulated *koji*.

Fan *et al.* (1976) observed that, whilst sporulation was important for the flavour of blue cheese, both the mycelia and resting spores produced methyl ketones by the oxidation of fatty acids. However, while the mycelia, with a larger amount of vegetative cells, produced a larger amount of flavour compounds, they also metabolised methyl ketones faster. On the other hand, resting spores were characterised by their high resistance to the toxic effects of fatty acids, their comparable efficiency in the production and limited metabolism of methyl ketones. A similar occurrence in soy sauce *koji* could explain the distinctiveness in aroma at the mycelial and sporulated stages.

The observation of a difference in aroma of mycelial and sporulated *koji*, as well as different GLC profiles, supports the practice of soy sauce manufacturers in using well-sporulated *koji* for the production of soy sauce. Sugita (1956) studied the relationship between the organoleptic evaluation of cultured *koji* and the quality of soy sauce made from it and recognised a fairly good correlation between them.

It was also noted that the acidic extracts carried the characteristic aroma of soy sauce whilst the neutral and alkaline extracts of *koji* did not.

This was also observed by Yokotsuka (1981). As the neutral and alkaline extracts also contain volatiles, this demonstrates that the GLC profile at a certain pH may not be representative of the total aroma of a sample. GLC has to be used in conjunction with sensory evaluation for a meaningful interpretation of the analysis of aroma.

REFERENCES

Aishima, T. (1982). Discrimination and cluster analysis of soy sauce GLC profiles. *J. Food Sci.*, **47**, 1562–7.

Fan, T. Y., Hwang, D. H. & Kinsella, J. E. (1976). Methyl ketone formation during germination of *Penicillin roqueforti*. *J. Agric. Food Chem.*, **24**(3), 443–8.

Gehring, R. F. & Knight, S. G. (1958). Formation of ketones from fatty acids by spores of *Penicillium roqueforti*. *Nature* (*London*), **182**, 1237.

Gehrig, R. F. & Knight, S. G. (1961). Formation of 2-heptanone from caprylic acid by spores of various filamentous fungi. *Nature* (*London*), **192**, 1185.

Gottlieb, D. (1976). Carbohydrate metabolism and spore germination. In: *The fungal spore* (Weber, D. J. & Hess, W. M. (Eds)). John Wiley & Sons, NY, 141–63.

Sugita, N. (1956). Evaluation of *koji* in soy sauce industry and its relation to the quality of soy sauce. *J. Ferment. Technol.* (*Japan*), **34**, 607–12.

Wang, H. L. & Hesseltine, C. W. (1979). Mold-modified foods. In: *Microbial technology, Vol. II: Fermentation technology* (Pepplar, H. J. & Perlman, D. (Eds)), 95–129.

Wood, B. J. B. & Yong, F. M. (1975). Oriental food fermentations. In: *The filamentous fungi, Vol. I, Industrial mycology* (Smith, J. E. & Berry, D. R. (Eds)). Edward Arnold, London, 265–80.

Yokotsuka, T. (1960). Aroma and flavour of Japanese soy sauce. *Adv. in Food Res.*, **10**, 75–134.

Yokotsuka, T. (1981). Industrial applications of proteinaceous fermented foods. *Proceedings of a Tech. Seminar, Medan, Indonesia.*

Yong, F. M. (1971). *Studies on soy sauce fermentation*. MSc Thesis, University of Strathclyde, Glasgow.

Yong, F. M. & Wood, B. J. B. (1974). Microbiology and biochemistry of soy sauce fermentation. *Advances in applied microbiology*, **17**, 157–93 (Perlman, D. (Ed.)). Academic Press, London, New York.

Yong, F. M. & Wood, B. J. B. (1976). Microbial succession in experimental soy sauce fermentation. *J. Fd Technol.*, **11**, 525–36.

Yong, F. M. & Wood, B. J. B. (1977). Biochemical changes in experimental soy sauce koji. *J. Fd Technol.*, **12**, 163–75.