This is to certify that the

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BIOCHEMICAL CHANGES IN THE ONCOM FERMENTATION OF PEANUT PRESS CAKE

presented by

Dedi Fardiaz

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Periles Markakis

Major professor

Date Oct. 27, 1980

# BIOCHEMICAL CHANGES IN THE ONCOM FERMENTATION OF PEANUT PRESS CAKE

By

Dedi Fardiaz

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ABSTRACT

BIOCHEMICAL CHANGES IN THE ONCOM FERMENTATION OF PEANUT PRESS CAKE

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The objective of this investigation was to study some of the biochemical changes that take place in peanut press cake during fermentation by Rhizopus oligosporus and/or Neurospora sitophila.

Peanut press cake was prepared by pressing peanut seeds under a hydraulic Carver press. The cake was soaked overnight in acidified water (pH 4.5) in a refrigerator, and then washed several times. Afterwards, 1% of tapioca flour was added to the cake mass and the mixture was autoclaved at 121°C for 30 minutes. The hot mass was cooled to room temperature, drained, and inoculated with the following mold cultures: (1) N. sitophila ATCC 14151, (2) R. oligosporus ATCC 22959, (3) Neurospora sp. isolated from Indonesian oncom, (4) A mixed culture of (1) and (2), and (5) A mixed culture of (2) and (3). The cake was incubated at 30°C for 72 hours. At 6 hour intervals samples were drawn, freeze dried, pulverized, and analyzed for pH, free

fatty acids, oligosaccharides, soluble protein, electrophoretic pattern of soluble protein, phytic acid, PER, digestibility, and carotenoids.

After 36 hours of fermentation mycelia of R. oligosporus completely covered the peanut press cake into a compact semisolid product, while it took 48 hours for N. sitophila to produce the same product. The pH gradually increased from about 5.1 to 7.2 in 72 hours. Approximately 40% of the peanut oil was hydrolyzed by R. oligosporus, while only 10% by N. sitophila after 72 hours of fermentation. During fermentation with either R. oligosporus or N. sitophila, the sucrose, raffinose, and stachyose contents of peanut press cake decreased. Soluble protein of oncom increased after fermentation, and electrophoresis showed that the protein was hydrolyzed to smaller molecular weight components. At 72 hours of fermentation, about 95% of the phytic acid of the peanut press cake was hydrolyzed by R. oligosporus, while only about 50% by N. sitophila. Only about 60-65% of the phytic acid was hydrolyzed completely to inorganic phosphorus and free inositol, while the remaining phytic acid was hydrolyzed partially to other inositol phosphate forms.

Fermentation did not change the protein content, apparent digestibility, and protein quality of peanut press cake. However, incorporation of 10% of sesame protein raised the PER of peanut press cake from 1.51 to 2.11. The

mycelia and conidia of N. sitophila contained phytofluene, neurosporene, β-zeacarotene, γ-carotene, and β-carotene. However, the concentration of these carotenoids in oncom was too small to increase the vitamin A value of oncom. In general, Neurospora sp. isolated from Indonesian oncom was not very different from N. sitophila ATCC 14151 in its effect in the oncom fermentation. Using a mixed culture of N. sitophila and R. oligosporus resulted in oncom which was low in both oligosaccharides and phytic acid.

To my wife, ANDI, and
my daughter, MIRI

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# INTRODUCTION

Fermentation is one of the oldest methods of preparation and preservation of foods. Throughout the centuries, it has been and continues to be one of the most important methods for preparing and preserving foods. In many parts of the world, especially Southeast Asia, foods prepared by fermentation are important components of diets.

In general, fermented foods have some characteristic flavor, aroma, appearance, or consistency which make them more attractive to the consumer than the raw ingredients. Whitaker (1978) mentioned that some of the advantages of fermented foods were: preservation, improvement of texture, color, flavor and aroma, solubilization, digestibility, nutritional improvement, less cooking, and removal of toxic substances. Among these advantages, flavor improvement may be one of the most important contributions of the fermentation. This is especially true for the Southeast Asian whose diet is rather flat as it mainly consists of rice and vegetables. Besides imparting flavor, in many cases fermented foods make important contributions to the diet as sources of protein, calories, and some vitamins.

Like in all other countries of Southeast Asia, fermented foods are very important in Indonesia. Some of the fermented foods produced can be catagorized as protein-rich meat substitutes. Steinkraus (1978) classified the meat analogues of Indonesia into four general types: tempeh kedele, oncom, tempeh bongkrek, and tempeh gembus. These products are eaten in several forms. They may be sliced, dipped in a salt brine, and fried in vegetable oil to yield a golden brown, crisp product. They may be added to soups, and they may be eaten with soy sauce.

Tempeh kedele is made by fermenting dehulled partially cooked soybeans with molds, chiefly Rhizopus oligosporus (Hesseltine and Wang, 1967; van Veen and Steinkraus, 1970). Oncom is made by fermenting partially cooked peanut press cake with either Neurospora sitophila (orange or red oncom) or R. oligosporus (white oncom) (van Veen et al., 1968). Tempeh bongkrek is similar to oncom, except that the raw material is coconut press cake (van Veen and Steinkraus, 1970). Tempeh gembus is similar to tempeh kedele, except that the raw material is the soybean residue remaining from the manufacture of soybean milk or soybean curd (Steinkraus, 1978).

Traditional food fermentations are characterized by their simplicity and rapidity. Since the fermentation process is usually labor intensive, and does not require a high and expensive technology level, it is entirely appropriate for developing countries like Indonesia. In order

to produce highly acceptable nutritious fermented foods at low prices, the process should be modified or improved using an intermediate technology. This goal can only be achieved if the biochemistry of the fermentation process is fully understood. Among the Indonesian fermented foods, oncom received little scientific attention. This is the reason why oncom was chosen in this study.

# LITERATURE REVIEW

Oncom (pronounced ontsom) is fermented peanut press cake. It is very popular in Western Java (van Veen et al., 1968) where it has been prepared and consumed for centuries. Oncom can be used in soups or fried in vegetable oil for high-protein snacks.

Oncom is prepared from peanut press cake called bungkil (Hesseltine and Wang, 1967). Two types of cake may be used, the commercial press cake which contains small amount of oil, and the village product which contains considerable amount of oil. In oncom preparation, the peanut press cake is first broken up by hand or with a knife, soaked in water overnight, washed, and pressed to remove excess water and oil. The cake mass is steamed and pressed into the form of flat cakes. The flat cakes are placed in a bamboo tray, inoculated with dry oncom from an earlier preparation, and covered with banana leaves. After two or three days the molds have grown and the oncom is ready for consumption. The finished oncom contains approximately 70% moisture, 3-9% oil, 20-30% crude protein, about 4% carbohydrate, 1% ash, and 2% fiber (van Veen et al., 1968).

## Molds in Oncom Preparation

Two different molds are involved in oncom preparation. N. sitophila is used to prepare an orange or red oncom, while R. oligosporus is used to prepare a white oncom. Traditionally, the people in Indonesian villages use small dry pieces of oncom from a previous fermentation as inoculum. Sometimes inoculation is not necessary, since the atmosphere in the fermentation room has been already contaminated by the mold spores. This traditional practice can lead to contamination by undesirable microorganisms; therefore, pure culture fermentation should be introduced to oncom producers.

N. sitophila, one of several known species of the genus Neurospora, is referred to commonly as the red bread mold as a result of its prolific formation of orange to red conidia and mycelia. It is also known as the bakery mold, because it frequently infests bakeries and causes considerable damage (Alexopoulos, 1962). The genus Neurospora can be distinguished from other genera in the family of Moniliaceae by the presence of budding conidia which form tree-like heads as shown in Figure 1. Furthermore, distinguishing characteristics of N. sitophila are: (1) septate mycelium which later may break up into cells, (2) loose network of aerial, long stranded mycelium, (3) aerial hyphae bearing many ovate, pink to orange-red budding conidia, which form branched chains, and are found near the top of the

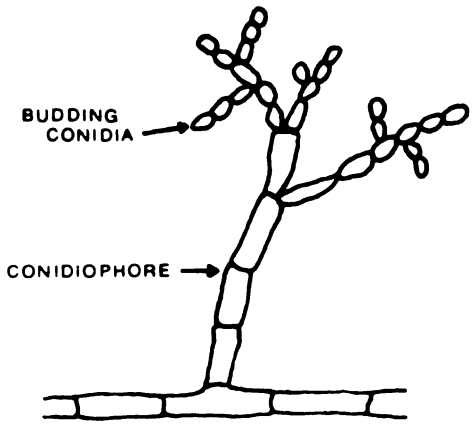


Figure 1. Genus Neurospora.

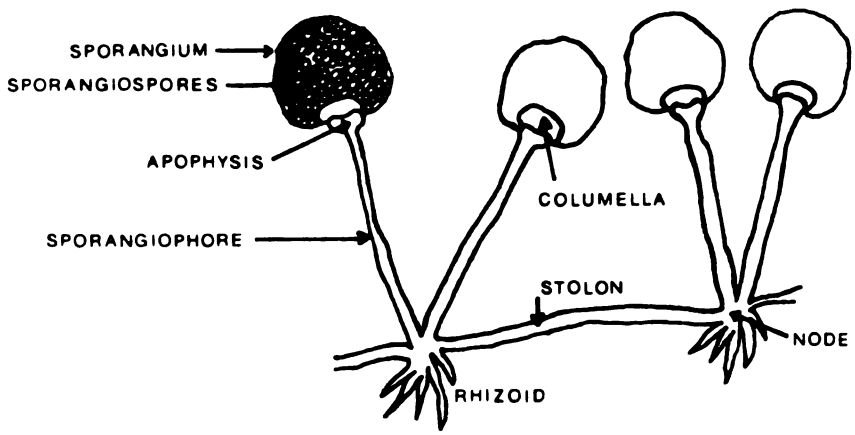


Figure 2. Genus Rhizopus.

plant (Frazier and Westhoff, 1978).

R. oligosporus is one species of the genus Rhizopus (Figure 2). Frazier and Westhoff (1978) summarized that the distinguishing characteristics of Rhizopus are: (1) nonseptate, (2) stolons and rhizoids, often darkening with age, (3) sporangiospores arise at the nodes, where

rhizoids also are formed, (4) sporangia are large and usually black, (5) hemispherical columella and cup-shaped apophysis (base to the sporangium), (6) abundant cottony mycelium which may fill the container, e.g. a petri dish, and (7) no sporangioles.

## Biochemical Changes During Fermentation of Foods

In food fermentations, food acts generally as a satisfactory medium for the growth of a variety of microorganisms. The biochemical changes taking place during fermentation are usually due to the activity of enzymes produced by microorganisms or enzymes inherent to the food (Pederson, 1971). However, microbial enzymes are more important than the inherent enzymes especially in fermented foods that undergo heat treatment prior to the fermentation process. These enzymes act upon plant constituents and presumably enhance the digestibility of raw materials. It has been shown that soluble solids increase during tempeh fermentation (Steinkraus et al., 1960, 1965; van Buren et al., 1972).

### Changes in Lipids

Numerous species of molds have been reported to produce lipolytic enzymes. R. oligosporus is one of them that produces a very strong lipase activity (Wagenknecht et al., 1961; Alford et al., 1964), while N. sitophila

produces lipase of weak activity (Beuchat and Worthington, 1974).

Generally speaking, peanuts are considered a rich source of oil (44-56%), while peanut press cake from the village in Western Java, Indonesia contains 6-20% oil (van Veen et al., 1968). The oil is composed of mixed glycerides of approximately 20% saturated and 80% unsaturated fatty acids (Cobb and Johnson, 1971). The major saturated fatty acids are palmitic (8.4-14.0%), stearic (1.8-3.2%), arachidic (1.0-1.7%), behenic (1.7-3.8%), and lignoceric (0.5-2.6%); whereas, the major unsaturated fatty acids are oleic (33.3-61.3%), linoleic (18.5-47.5%), and 11-eicosenoic (0.7-2.3%).

It is likely that if grown in peanut press cake, both R. oligosporus and N. sitophila will alter lipid components of the peanut. Perhaps, the most notable changes in lipids during fermentation is accumulation of free fatty acids resulted from hydrolysis of triglycerides by lipase. It was demonstrated by Sudarmadji and Markakis (1978) that the total free fatty acid content of tempeh increased from 0.04% to 10.68% after 90 hours of fermentation at 32$^{o}$C by R. oligosporus.

## Changes in Carbohydrates

In fermentation, carbohydrate splitting enzymes are important in providing substrates for the growth of microorganisms (Whitaker, 1978). Addition of 1% tapioca to

peanut press cake was shown to be beneficial for mold growth. Without it, the mold growth was slow and flavor development was poor (van Veen et al., 1968). Peanut press cake contains about 14-20% carbohydrates which are comprised largely of cellulose and simple oligosaccharides. The sugar content of peanut cultivars is highly variable. Glucose, fructose, and galactose are present in small quantities, whereas sucrose is the most abundant sugar and varies from 2.9 to 6.4% depending upon genotype (Newel et al., 1967). The raffinose and stachyose contents of peanuts were reported to range from less than 0.1 to 0.3% and less than 0.1 to 0.5%, respectively (Hymowitz et al., 1972). These two oligosaccharides are considered primarily responsible for flatulence, as they are not hydrolyzed in the small intestine, but they are subject to microbial decomposition, with production of gas, in the large intestine (Rackis et al., 1967; Murphy, 1969).

Shallenberger et al. (1967) reported a marked decrease in stachyose content during tempeh fermentation, while Sugimoto and van Buren (1970) showed that treatment of soy milk with an enzyme preparation from Aspergillus saitoi completely decomposed all the oligosaccharides to their constitutive monosaccharides. Likewise, Mital and Steinkraus (1975) demonstrated that the lactic fermentation reduced the raffinose and stachyose contents of soy milk. It is likely that microorganisms used in food fermentation which are capable of producing invertase and $\alpha$-galactosidase

will hydrolyze raffinose and stachyose. Therefore, the fermentation process may help to reduce the flatulence characteristics of certain foods.

## Changes in Proteins

In most fermented high protein foods, proteolysis is a major factor in the changes in texture and flavor (Whitaker, 1978). The presence of proteolytic enzyme system in the tempeh fermentation was demonstrated by Wang and Hesseltine (1966). In general, most amino acids either declined slightly or were unchanged as tempeh fermentation progressed (Smith et al., 1964; Stillings and Hackler, 1965; Wang et al., 1968). However, free amino acids increased markedly in the fermented product (Stillings and Hackler, 1965). Since in general, fermentation of oilseeds showed little change in protein content except for solubility (Beuchat, 1976), it is unlikely that fermentation would improve the protein quality of peanut press cake.

Besides being rich in oil, peanuts are also rich in protein, and peanut press cake contains 38-51% protein (van Veen et al., 1968). However, the quality of peanut protein is poor because it is deficient in several essential amino acids as shown in Table 1.

Table 1. Essential amino acids in reference and peanut proteins, expressed as g per 16 g N

| Amino acids | Reference[a] | Peanut[b] |
|---|---|---|
| Isoleucine | 4.0 | 3.4 |
| Leucine | 7.0 | 6.4 |
| Lysine | 5.5 | 3.5 |
| Methionine + cysteine | 3.5 | 2.4 |
| Phenylalanine + tyrosine | 6.0 | 8.9 |
| Threonine | 4.0 | 2.6 |
| Tryptophan | 1.0 | 1.0 |
| Valine | 5.0 | 4.2 |

[a] FAO/WHO, 1973
[b] FAO, 1970

Changes in Other Components

Phytic acid or inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) is nutritionally important because of its ability to form insoluble complexes with di- and trivalent minerals, thereby, reducing their availability for absorption in the intestinal tract. Defatted peanut meal was reported to contain approximately 1.5% of phytic acid (Erdman, 1979).

The presence of phytase, an enzyme capable of hydrolyzing phytate, has been reported in germinating seeds (Chang, 1967; Mandal et al., 1972; Lolas and Markakis, 1977). Reinhold (1971) suggested that yeast might contribute in decreasing the phytic acid content of leavened

bread. Recently, it was reported that R. oligosporus produced phytase which reduced the phytic acid content of soybean during tempeh fermentation (Sudarmadji and Markakis, 1977; Wang et al., 1980). No reports have been found regarding phytase production by N. sitophila.

Vitamins, especially some B-vitamins, may change during fermentation. It was reported that the thiamine content of soybean decreased during tempeh fermentation (Roelofsen and Talens, 1964; van Veen and Steinkraus, 1970). However, Quinn et al. (1975) showed that thiamine increased significantly in peanut flour fermented with N. sitophila, R. oligosporus, A. oryzae, and A. elegans. Riboflavin and niacin were shown to increase, while pantothenate was unchanged or decreased slightly, in both tempeh and oncom fermentation (Roelofsen and Talens, 1964; van Veen and Steinkraus, 1970; Quinn et al., 1975).

## Safety of Fermented Foods

R. oligosporus and N. sitophila do not produce mycotoxins (van Veen et al., 1968). The presence of aflatoxin in oncom, if any, may originate from peanut press cake which had been contaminated with aflatoxin. Van Veen et al. (1968) found that N.sitophila could destroy approximately 50% of the aflatoxin $B_1$ present in the Indonesian peanut press cake, while R. oligosporus reduced it by about 70%. However, it is not known whether the metabolic products from degraded aflatoxin are harmless.

Some fermented foods may be contaminated by toxin-producing microorganisms. A good example is tempeh bongkrek (fermented coconut press cake) which is susceptible to development of bongkrek poison. Bongkrek poisoning is caused by the growth of Pseudomonas cocovenenans under conditions unfavorable to the molds (Nugteren and Berends, 1957). Recently, the Indonesian government has banned the production of tempeh bongkrek because of the danger of bongkrek poisoning.

# MATERIALS AND METHODS

## Preparation of Oncom

Peanut press cake was prepared by pressing certain amount of shelled peanut (MSU Foodstore) under the Carver press. The peanut was first broken down into small pieces and pressed at 12,000 psi for 30 minutes. The resulting cake was soaked overnight in water which had been acidified with citric acid to pH 4.5 at refrigeration temperatures. The peanut mass was then washed several times with acidified water to remove the remaining oil that rose to the surface. Afterwards, 1% of tapioca flour was added to the mass and autoclaved at 121°C for 30 minutes. The hot mass was cooled to room temperature, drained, and inoculated with the mold culture.

Mold cultures used in this experiment were: (1) N. sitophila ATCC 14151, (2) R. oligosporus ATCC 22959, (3) Neurospora sp. isolated from Indonesian oncom, (4) mixed cultures of (1) and (2), and (5) mixed cultures of (2) and (3). Mold cultures were grown and stored on potato dextrose agar slants. After one week, the mycelia and spores of each slant were harvested with 4 ml of sterilized distilled water. Ten ml of spore suspension containing

2 x $10^8$ spores per ml were used to inoculate 1500 g of peanut press cake. The well mixed inoculated cake was packed tightly into disposable petri dishes (100 x 15 mm), and incubated at 30°C for 72 hours. At 6 hour intervals samples were drawn, freeze dried, pulverized, and stored in a freezer.

## pH Determination

A 20 g sample of oncom was homogenized with 80 ml of distilled water in a small Waring blender for 5 minutes. The pH determination was made directly on the homogenate using a Corning pH meter, Model 10 (E.H. Sargent and Co., Chicago, IL.).

## Proximate Analysis

Samples used for protein efficiency ratio (PER) evaluation were subjected to proximate analysis. Moisture, crude protein, crude fat, crude fiber, and ash were determined according to the methods of AOAC (1975).

## Analysis of Free Fatty Acids

### Extraction

The method of Mattick and Lee (1959) was used to extract free fatty acids from the sample. Oil was first extracted from the sample with diethyl ether using a Goldfisch extractor. One g of oil and 8 mg of n-heptadecanoic

acid (internal standard) were transferred into a 60 ml separatory funnel. Thirty five ml of a mixture of diethyl ether and petroleum ether (1:1) were added to dissolve the oil, then, 6.5 ml of 95% ethanol and 12.5 ml of 1% $Na_2CO_3$ were added. The mixture was shaked several times and the aqueous layer containing the sodium salts of free fatty acids was separated into another 60 ml separatory funnel.

Extraction of free fatty acids from the ether layer was repeated three times; first, with 1.5 ml of 95% ethanol and 7.5 ml of 1% $Na_2CO_3$; second, with 1.5 ml of 95% ethanol and 5.0 ml of 1% $Na_2CO_3$, and finally with 6.5 ml of distilled water. All the aqueous phases were collected and combined, whereas, the ether layer containing the glycerides was discarded.

To a separatory funnel containing the aqueous layer 1.5 ml of 10% $H_2SO_4$ was added in order to free the fatty acids. The free fatty acids were then extracted with 12.5 ml of the solvent mixture mentioned above. The ether layer was separated and transferred through Whatman # 1 filter paper containing several g of anhydrous $Na_2SO_4$ into a 5 ml screw-cap vial. The solvent was evaporated to dryness by passing a stream of nitrogen gas through the vial. The extraction was repeated three times with fresh solvent.

Esterification

Free fatty acids were converted to their methyl esters prior to GLC analysis because these derivatives are

more volatile than the acids. Boron trifluoride in methanol (14%,w/v) was used as esterifying reagent according to the method of Supelco, Inc. (1975).

Into the vial containing dry free fatty acids, 2 ml of benzene was added to dissolve the acids. Two ml of $BF_3$-methanol was further added into the vial and mixed well. The vial was placed in a small beaker with water and boiled for 3 minutes on a steam bath. To stop the reaction, 1 ml of distilled water was added to the reaction mixture, which was then separated into two layers. The top layer contained the methyl esters dissolved in benzene, while the bottom layer was a mixture of methanol, water, and acid catalyst. To separate the two layers, the vial was centrifuged, and the benzene layer was transferred with a syringe into another vial. Two µl of the benzene containing methyl esters was injected into the gas chromatograph using a 10 µl Hamilton syringe # 701 (Hamilton Co., Reno. NEV.).

Gas Chromatography Conditions

All gas chromatographic separations were carried out using a Perkin-Elmer 900 Gas Chromatograph equipped with a Servo/Riter II Flushmount Recorder and a Flame Ionization Detector (Perkin-Elmer Corp., Norwalk, CONN.).

A 3 ft. x 0.125 in. o.d. stainless steel column was packed with 10% DEGS-PS on 80/100 mesh supelcoport (Supelco, Inc., Bellefonte, PA.).

The chromatographic conditions for methyl-ester derivative separations were accomplished with helium as the carrier gas at an inlet pressure of 40 psi and the flow rate of 18 ml per minute. The flame ionization detector was operated at 265°C with hydrogen pressure of 20 psi and air pressure of 40 psi. The injection port temperature was 235°C, attenuation was X64 with attenuation range X100, and chart speed was 15 in. per hour. Temperature of the column was programmed for 130 to 190°C at 10°C per minute and the column was held at 190°C for 22 minutes.

Identification of the Chromatogram Peaks

The chromatogram peaks were identified with two methods: (1) by running the sample directly into a Gas Chromatograph/Mass Spectrometer (GC/MS), and (2) by using a standard mixture RM-3 (Supelco, Inc., Bellefonte, PA.) containing methyl esters of myristate ($C_{14:0}$), palmitate ($C_{16:0}$), stearate ($C_{18:0}$), oleate ($C_{18:1}$), linoleate ($C_{18:2}$), linolenate ($C_{18:3}$), arachidate ($C_{20:0}$), behenate ($C_{22:0}$), erucate ($C_{22:1}$), and lignocerate ($C_{24:0}$).

The GC/MS used was a Hewlett Packard 5840A Gas chromatograph/HP 5985 Mass Spectrometer (Hewlett Packard Corp., Avondale, PA.). The column was a 6 ft. x 0.250 in. o. d. glass column packed with 3% DEGS on 80/100 mesh chromosorb. The helium flow rate was 25 ml per minute, and the temperature was programmed from 130 to 190°C at 10°C per minute. The ion source and analyzer temperatures of the mass

spectrometer were maintained at 200°C. The accelerating voltage was 2000 V, ionizing potential 70 eV, repetitive scan 266.7 a.m.v. per second, and scan time 1.4 seconds.

## Analysis of Oligosaccharides

### Extraction

The oligosaccharides were extracted from the defatted sample with 80% ethanol (Conrad and Palmer, 1976). Two g of the defatted sample was diluted in 100 ml of 80% ethanol and a small amount of $CaCO_3$ was added to it to neutralize the acidity. The mixture was then refluxed at 70°C for 4 hours. The extract was centrifuged for 30 minutes, and the supernatant was collected. The precipitate was extracted with 50 ml of 80% ethanol, and the two supernatants were combined.

The supernatant was decolorized by filtering through activated charcoal Darco G-6 (Matheson, Coleman & Bell, Co.). The charcoal was washed with 20 ml of water to insure that all the sugars had passed through it. One half g of $Ba(OH)_2$ was added to the filtrate and stirred using a magnetic stirrer, followed by the addition of 0.5 g of $ZnSO_4$. This step was done to remove contaminating proteins (Delente and Ladenburg, 1972; Bau et al., 1978). The mixture was centrifuged for 30 minutes, the supernatant was desalted by passing it through Dowex 50W x 8 ($H^+$ form) and Dowex 2 x 8 ($Cl^-$ form which had been converted to $OH^-$ form).

The desalted solution containing mainly sugars was concentrated using a flash evaporator (Buchler Instruments, Fort Lee, N.J.) at 38°C. The concentrated solution was passed through a Sep-pak (Waters Associates Inc., Milford, MA.) to remove lipid materials, and freeze dried.

Just before analysis, the freeze dried sample was dissolved in 0.5 ml of deionized water and filtered through a 0.22 μm pore-diameter membrane filter (Gelman Instrument Co., Ann Arbor, MI.) utilizing a Swinney syringe filter (Millipore Corp.). The sample was subjected to analysis by circular thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

Qualitative Analysis by Circular TLC

Precoated 20 x 20 cm silica gel G-1500 plates (Schleicher & Schuell, Keene, NH.) were soaked in 0.3 M $KH_2PO_4$ solution for 2 minutes, and then dried at room temperature for several hours. The plates were dried at 60°C for 1 hour prior to the application of the samples.

In the center of a TLC plate, a circle 2 cm in diameter was drawn carefully with a compass. Seven samples and 1 standard mixture consisting of glucose, fructose, sucrose, melibiose, raffinose, and stachyose were applied around the perimeter of the circle, each containing approximately 100 μg sugars. The sample spots were thoroughly dried using a hair drier, then the plate was placed in the "SelectaSol" (Schleicher & Schuell, Keene, NH.) holder

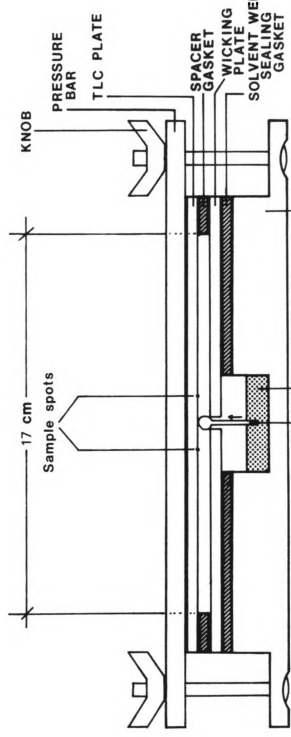


Figure 3. Diagram of circular TLC.

which consisted mainly of a solvent well, wick, and gasket (Figure 3).

Five ml of developing solvent consisting of n-butanol/dioxane/$H_2O$, 4/5/1 by volume (Ghebregzabher et al., 1976) was placed in the center well of the "SelectaSol". A spacer gasket 17 cm in diameter was used along with W-2 polyethylene wick. The solvent was developed until the solvent front reached the edge of the spacer gasket for approximately 3 hours. The development was done twice to insure that the oligosaccharides having low Rf values had been separated. The plate was dried, sprayed, and heated at 60°C for 20 minutes. The detection reagent was a mixture of 0.1 g of orsinol, 0.1 g of resorsinol, 4.4 ml of conc. $H_2SO_4$, and 35.6 ml of 95% ethanol (McLaren and Won, 1979). Identification of sample sugars was based on a comparison of Rfs with those obtained with a standard mixture.

Quantitative Analysis by HPLC

The high pressure liquid chromatograph consisted of a Model M6000 Pump, a Model U6K Injector, and a Model R401 Differential Refractometer (Waters Associates, Inc., Milford, MA.). The response was recorded on a Model 281 Recorder (Linear Instruments Corp., Costa Mesa, CA.).

Oligosaccharides, glycerol, and myo-inositol were separated on a 4.2 mm i.d. x 30 cm long μBondapak/Carbohydrate column (Waters Associates Inc., Milford, MA.). A degassed mixture of acetonitrile and water (80:20, v/v) was

used as eluant at a flow rate of 2.3 ml per minute. Detector attenuation was 8X, and recorder chart speed was 0.25 in. per minute. Two to 10 µl of sample aliquot were injected into the chromatograph using a 25 µl syringe (Precision Sampling Corp., Baton Rouge, LA.). Identification of sample sugars was based on a comparison of retention times with those obtained with a standard mixture containing sucrose, melibiose, raffinose, stachyose, glycerol, and myo-inositol (1% solution of each).

## Analysis of Soluble Protein

### Soluble Protein Determination

Two and half g of finely ground defatted sample was blended with 57.5 ml of sodium phosphate buffer (pH 7.9, $\mu = 0.01$) in a small Waring blender for 5 minutes, and centrifuged for 30 minutes. The supernatant was collected and analyzed for protein content using the method of Lowry et al. (1951). Reagents prepared for the analysis were as follows.

Reagent A: a solution of 10% $Na_2CO_3$ in 0.5 N NaOH.

Reagent B: a solution of 1% $CuSO_4.5H_2O$.

Reagent C: a solution of 2% potassium tartrate.

Folin-phenol reagent: 5 ml of 2 N Folin-phenol solution was diluted to 50 ml with distilled water.

Fifteen ml of reagent A, 0.75 ml of reagent B, and 0.75 ml of reagent C were mixed in a 50 ml erlenmeyer

flask. One ml of this mixture was added to 1 ml of soluble protein sample in a 16 x 150 mm test tube, mixed thoroughly and incubated at room temperature for 15 minutes. Three ml of Folin-phenol reagent was added to the tube, mixed, and incubated at room temperature for 45 minutes. The absorbance was determined at 540 nm with a Beckman DU Model 2400 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA.).

A standard curve was previously prepared using a series of standard solution containing 0 to 300 µg bovine serum albumin per ml (Figure 4). The following linear relationship was obtained:

$$A_{540} = 0.0024\ C + 0.0170$$
$$(r = 0.999)$$

where C was the concentration of protein in µg per ml.

Electrophoretic Pattern of Soluble Protein

The supernatant used for soluble protein determination was freeze dried and subjected to separation by SDS electrophoresis according to the method of Weber and Osborn (1969) as described by Cooper (1977). Materials used for SDS electrophoresis were as follows.

Gel buffer solution (pH 7.2) prepared by dissolving 7.8 g $NaH_2PO_4.H_2O$ and 18.6 g $Na_2HPO_4$ in 1 L of water.

Acrylamide solution prepared by dissolving 22.2 g acrylamide and 0.6 g N,N'-methylene-bis (acrylamide) in water to yield a final solution volume of 100 ml.

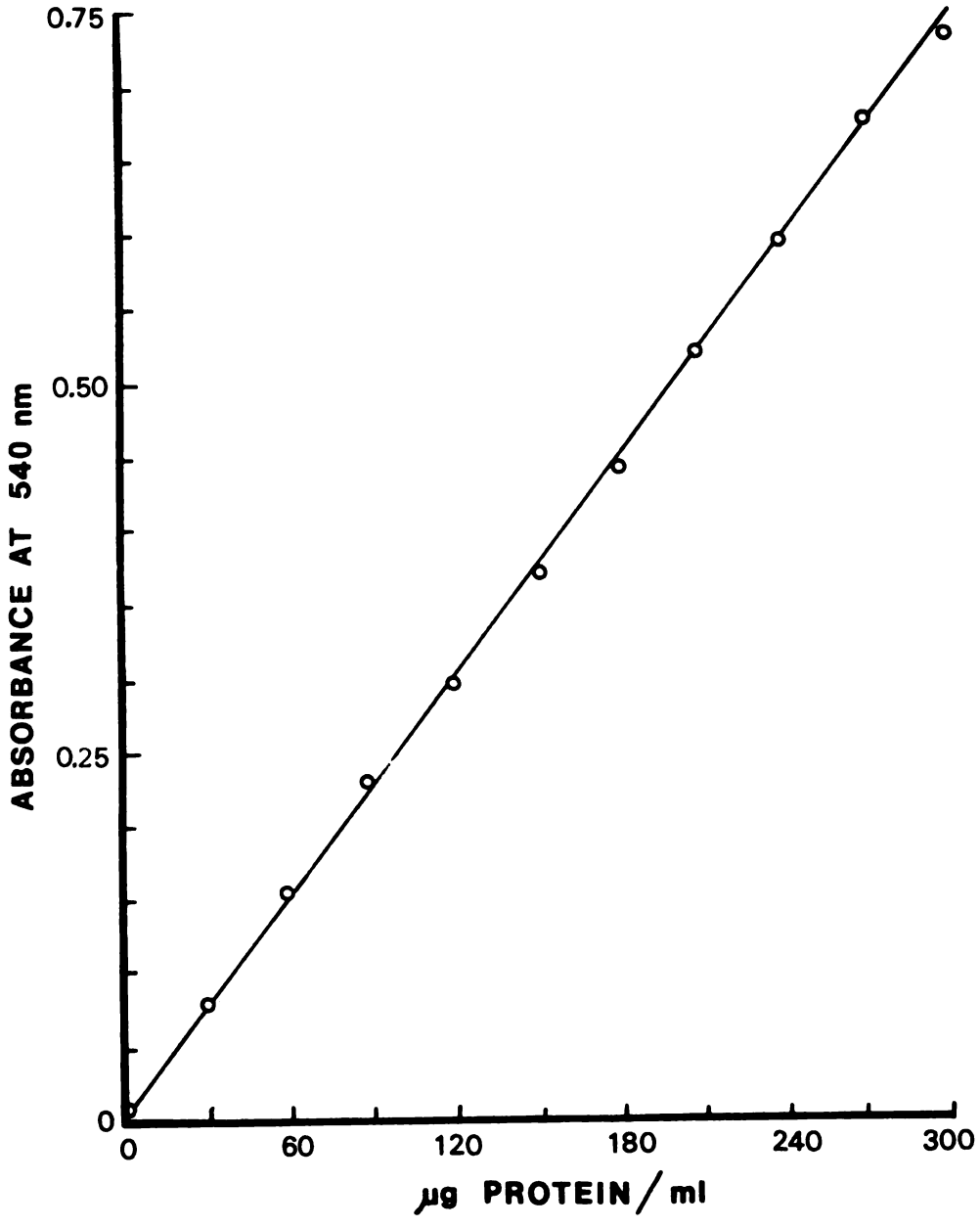


Figure 4. Standard curve for soluble protein determination.

Ammonium persulfate solution prepared by dissolving 35 mg ammonium persulfate in 10 ml of water.

Sample buffer solution prepared by mixing 5 ml of gel buffer solution, 2 ml of 2-mercaptoethanol, 2 g of sodium dodecyl sulfate (SDS), and 93 ml of water.

Ten % gel was prepared by mixing 15 ml of gel buffer solution, 13.5 ml of acrylamide solution, 50 µl of N,N,N,N'-tetramethylethylene diamine (TEMED), and 1.5 ml of ammonium persulfate solution. Forty µl of 0.2% soluble protein samples were applied on the gel and operated at a current of approximately 8 mA per tube. Bromophenol blue was used as a marker dye. Gels were stained with Coomassie brilliant blue G-250 in 3.5% perchloric acid solution.

## Phytic Acid Determination

The extraction and precipitation of phytic acid were performed according to the method of Wheeler and Ferrel (1971). To achieve better recovery, the method had been slightly modified.

One g of finely ground dried sample was extracted with 50 ml of 3% trichloroacetic acid (TCA) using a mechanical shaker for 90 minutes. The suspension was centrifuged and 10 ml of the supernatant were transferred into a 40 ml conical centrifuge tube. Four ml of $FeCl_3$ solution (2 mg $Fe^{3+}$ per ml of 3% TCA) were added to the aliquot by blowing rapidly from the pipet. Ferric phytate ($Fe_4Phy$) was precipitated by heating the tube and its contents in a boiling-

water bath for 45-60 minutes. The tube was centrifuged for 15 minutes and the clear supernatant was decanted carefully. The precipitate was washed by dispersing it well in 25 ml of 3% TCA, heating it in a boiling-water bath for 10 minutes, and centrifuging the mixture for 15 minutes. This step was repeated once more using water instead of 3% TCA.

The $Fe_4Phy$ was converted to sodium phytate and $Fe(OH)_3$ by dispersing the precipitate in a few ml of water, and 3 ml of 1.5 N NaOH. The volume was adjusted to approximately 30 ml with water and heated in a boiling-water bath for 30 minutes. The tube was centrifuged for 15 minutes and the clear supernatant was decanted carefully. The precipitate, consisting of $Fe(OH)_3$, was washed with water, recentrifuged and redecanted. The $Fe(OH)_3$ was dissolved with 40 ml of hot 3.2 N $HNO_3$ and transferred to a 100 ml volumetric flask. The tube was washed with hot water, collecting the washings in the same flask. The flask was cooled to room temperature and the volume was adjusted to mark with water. The iron content was determined according to AOAC (1975). Reagents used for iron determination were as follows.

Orthophenanthroline solution: a 0.1% solution.

Hydroxylamine hydrochloride solution: a 10% $H_2NOH.HCl$ solution.

Acetate buffer solution prepared by dissolving 8.3 g anhydrous sodium acetate and 12 ml of acetic acid which was then diluted to 100 ml with distilled water.

Ten ml of aliquot containing Fe (previous step) was transferred into a 25 ml volumetric flask, and the following solutions were added in order: 1 ml of hydroxylamine hydrochloride, 5 ml of acetate buffer, and 1 ml of orthophenanthroline solution. The content was adjusted to volume, and the absorbance was measured at 510 nm with Beckman DU Model 2400 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

A standard curve was previously prepared using a series of standard solution containing 0 to 2.4 µg Fe per ml (Figure 5). The following linear relationship was obtained:

$$A_{510} = 0.2302\ C - 0.0020$$

$$(r = 0.999)$$

where C was the concentration of Fe in µg per ml.

The phytate phosphorus content was calculated from the Fe results assuming a 4:6 iron:phosphorus molecular ratio. To evaluate the efficiency of the extraction and analysis, 10 and 20 mg standard sodium phytate (NaPhy) were added to the control samples, and recoveries of phytic acid were calculated. Standard NaPhy (Sigma Co., purity 98%, $H_2O$ 43%, Na 2 atoms/mole) was calculated to contain 52.37% of phytic acid.

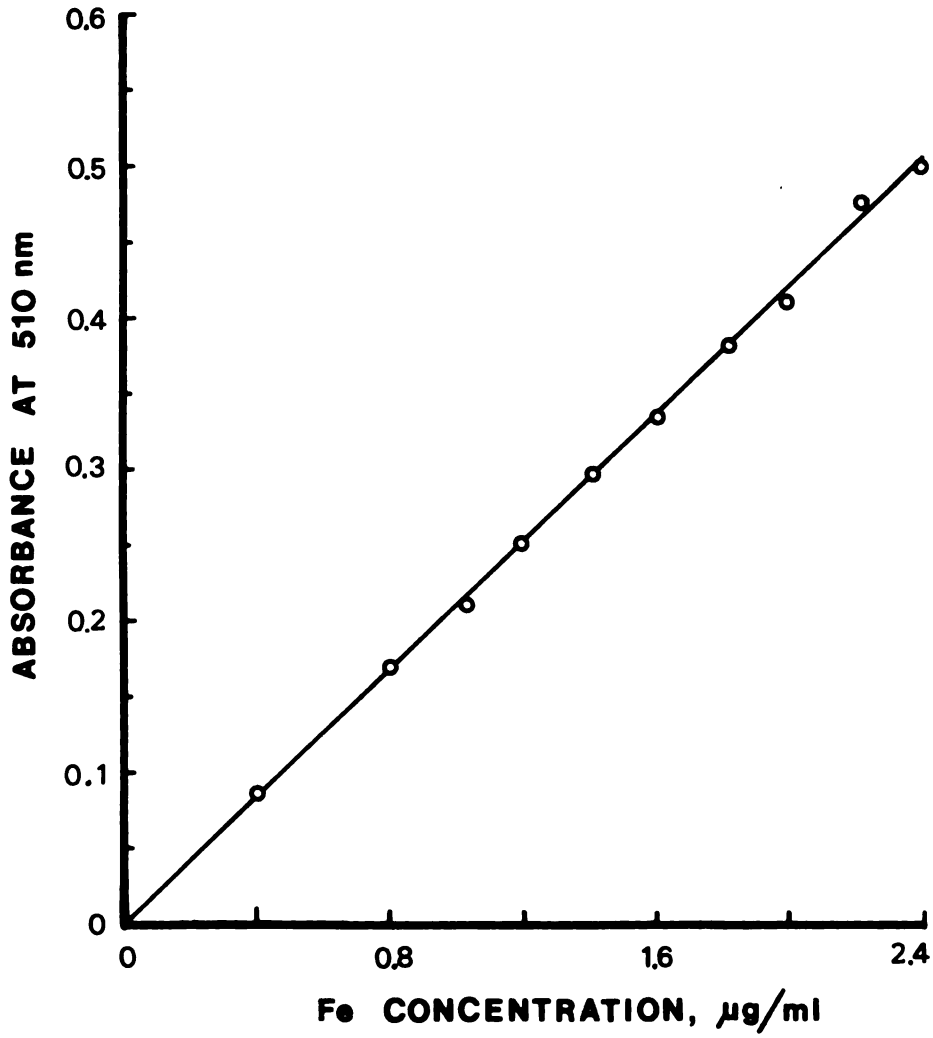


Figure 5. Standard curve for iron determination.

## Separation of Inositol Phosphates

### Column Chromatography

Separation of inositol phosphate was done according to the method of Saio (1964). The inositol phosphates were extracted from 1 g of oncom sample with 10 ml of 3% TCA. After centrifugation, 2 ml of the extract was chromatographed on a Dowex 1 x 8 (200-400 mesh, $Cl^-$ form, 1.1 x 10 cm) column. The extract was eluted with 600 ml 0 - 1.0 N HCl linear gradient at a flow rate of 2 ml per minute. Eluant was collected in 120 tubes (5 ml per tube) using a fraction collector (Rinco Instruments Co., Greenville, ILL.).

### Phosphorus Determination of Chromatographic Fractions

The solution in each fraction was evaporated to dryness at 45°C by blowing air to the surface of the solution through a manifold (Figure 6).

To each fraction containing dry residue, 1 ml of 70% perchloric acid was added. The tubes were heated for 1 hour to release the phosphorus from the inositol phosphates. The phosphorus content of each fraction was then determined colorimetrically according to Allen's method (1940) which had been slightly modified. The method was based on the formation of phosphomolybdic acid which was reduced to an intense blue complex. Reagents used in phosphorus determination were as follows.

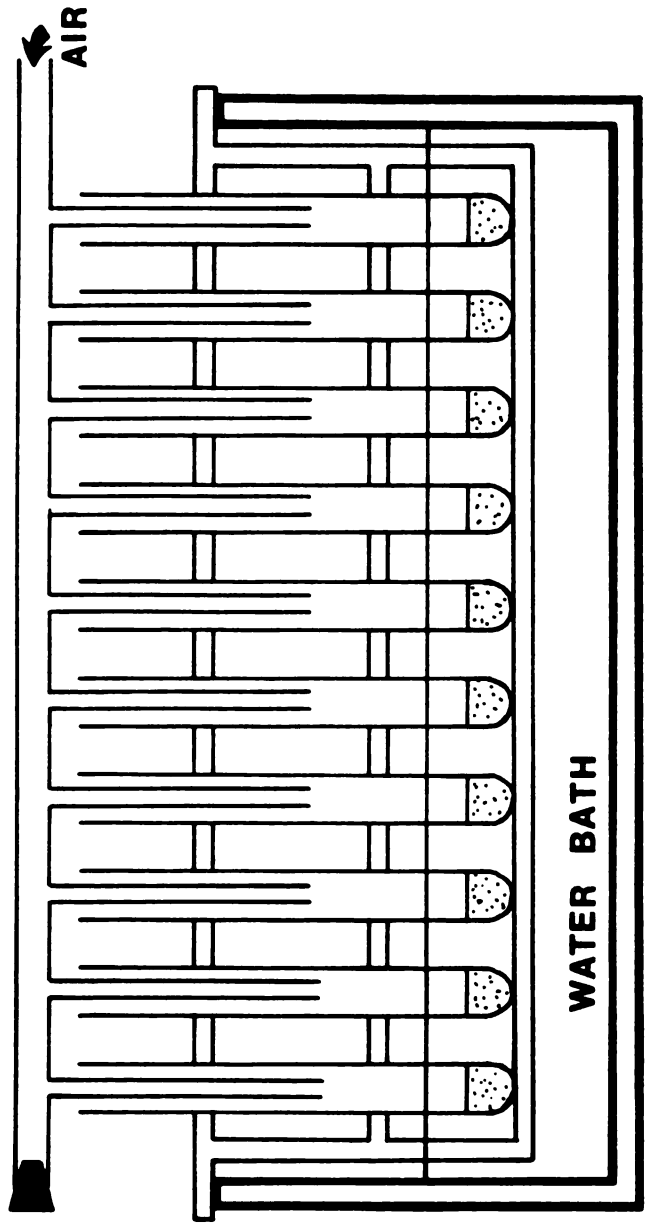


Figure 6. Apparatus used for evaporating liquid in tubes.

Perchloric acid: a 70% solution

Amidol reagent prepared by dissolving 2.5 g 2,4-diaminophenol dihydrochloride and 50 g sodium bisulfite in distilled water and diluted to 250 ml. The solution was kept in a brown bottle and discarded after 1 week.

Ammonium molybdate solution: an 8.3% solution.

Standard phosphorus solution: a 50 μg P per ml solution was prepared by dissolving 0.2197 g $KH_2PO_4$ (dried at 105°C) in distilled water and diluted to 1 L.

After hydrolysis with perchloric acid, 1 ml of amidol reagent and 1 ml of ammonium molybdate solution were added to each fraction, and the volume was adjusted to 10 ml with water. The solution was mixed using a Vortex mixer, and after 5-30 minutes the absorbance was measured at 675 nm with Beckman DU Model 2400 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA.).

A standard curve was previously prepared using a series of standard solution containing 0 to 70 μg P per 10 ml (Figure 7). The following linear relationship was obtained:

$$A_{675} = 0.0125\ C + 0.0021$$

$$(r = 1.000)$$

where C was the concentration of P in μg per 10 ml.

The amount of phosphorus per tube was plotted against tube number to illustrate the separation of inositol phosphate. Identification of the chromatogram was done by combining new fractions of each separated peak. These

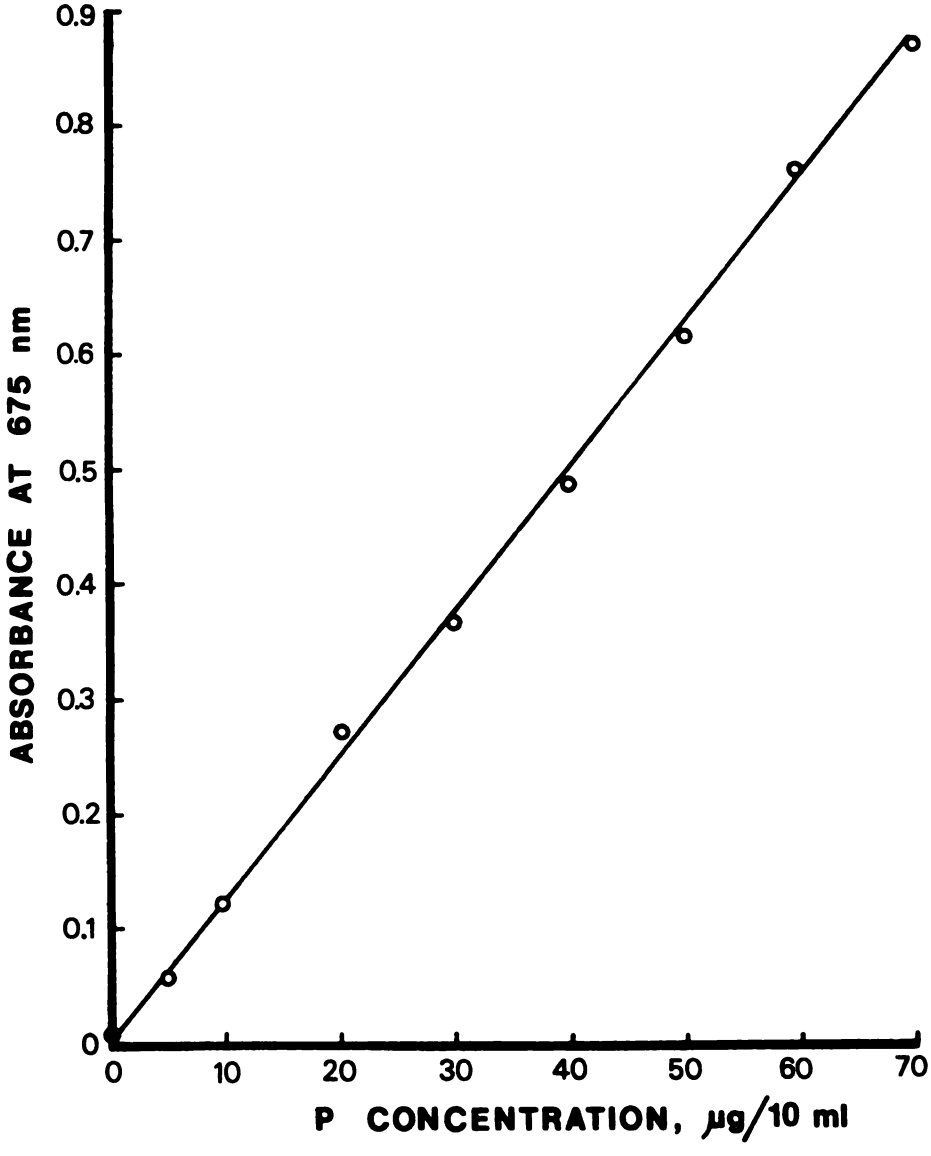


Figure 7. Standard curve for phosphorus determination.

solutions were dried, and hydrolyzed with 6 N HCl in ampules at 110°C for 48 hours. This step was done to liberate phosphorus from inositol phosphate without destroying the inositol moiety. The solution containing free inositol and inorganic phosphorus was subjected to inositol determination (Agranoff et al., 1958) as described by Saio (1964) and to phosphorus determination (Allen, 1940).

In the inositol determination, 2 ml sample containing 0.01 to 0.5 μmole of inositol, 2 ml of 1 M acetate buffer (pH 4.7), and 0.4 ml of 0.01 M sodium periodate were mixed, and the absorbance at 260 nm was immediately read. The absorbance was read again after 30 minutes at room temperature and after 16 hours in water bath at 45°C. The difference in the absorbance before and after heating at 45°C was due to the oxidation of inositol.

A standard curve was previously prepared using a series of standard solution of 0 to 250 μM (Figure 8). The following relationship was obtained:

$$-\Delta A_{260} = 0.0024\ C + 0.0066$$

$$(r = 1.000)$$

where C was the concentration of inositol in μM.

## Biological Evaluation of Protein Quality

Five diets were prepared based on the following sources of protein: (1) casein, (2) uninoculated peanut press cake, (3) fermented peanut press cake, (4) fermented mixture of peanut press cake and sesame flour (9:1), and

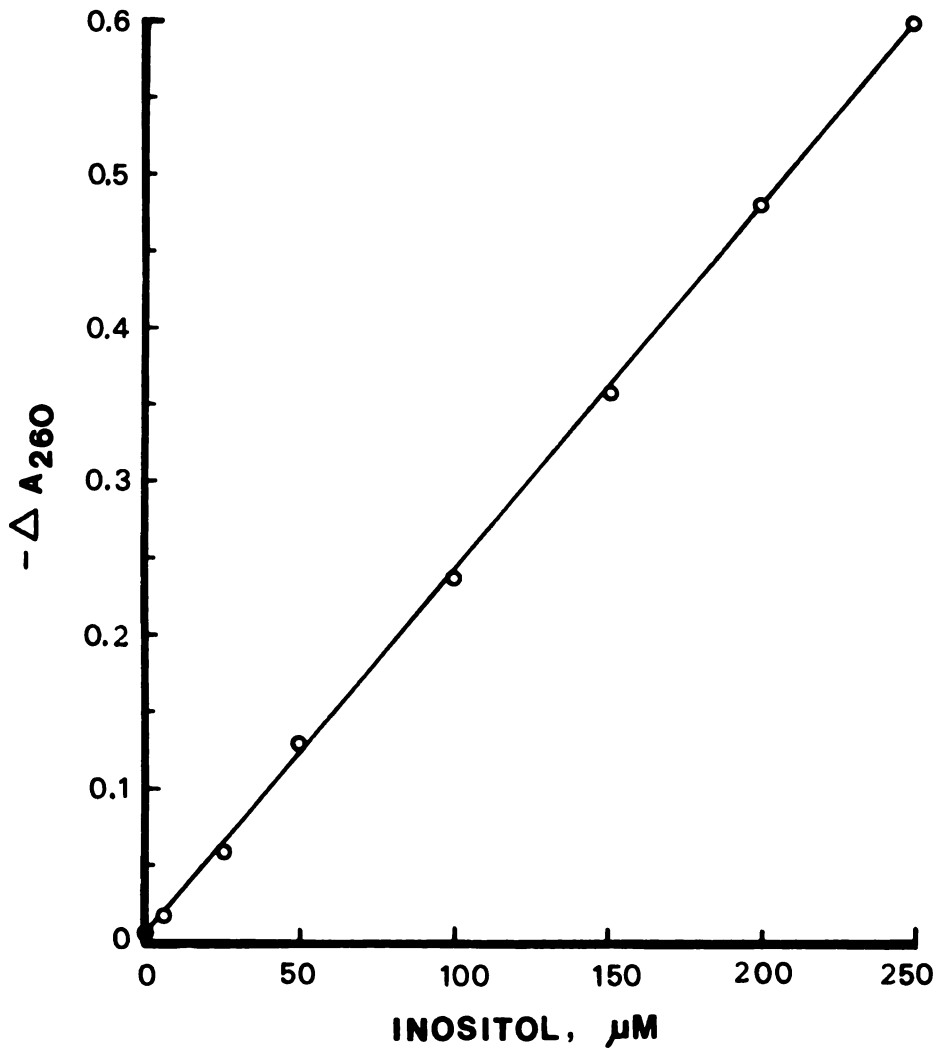


Figure 8. Standard curve for inositol determination.

(5) fermented mixture of peanut press cake and sesame flour (8:2). These ratios refer to protein rather than the entire commodity. A mixed culture of R. oligosporus ATCC 22959 and N. sitophila ATCC 14151 was used to prepare the fermented diets (diet 3, 4, and 5). The fermentation was carried out at 30°C for 48 hours. Each protein source to be evaluated was incorporated into a basal diet to provide a 10% level of protein (Table 2). On the basis of proximate analysis, the diets were equalized with respect to moisture, fat, ash, and crude fiber according to AOAC (1975).

Fifty 21-day old male weanling rats of the Sprague-Dawley strain were used in the evaluation of protein quality. The animals were housed individually in cages with a metal-screen bottom. After 3 days of acclimatization during which the animals were fed a commercial rat diet, the animals were divided into five groups, corresponding to the five experimental diets. Water and diet were offered ad libitum for 28 days. Animal weights and diet intakes were measured weekly for each animal.

## Protein Efficiency Ratio (PER)

The PER value was calculated as the ratio of the weight gained by the animals, to the weight of protein consumed over the 28-day period.

Table 2. Protein quality evaluation diet (AOAC, 1975)

| | g/100 g diet |
|---|---|
| Casein[a] or sample | S to provide 10 g of protein |
| Corn oil | $8 - \frac{S \times \% \text{ ether extract}}{100}$ |
| Salt mixture[b] | $5 - \frac{S \times \% \text{ ash}}{100}$ |
| Vitamin mixture[c] | 1 |
| Cellulose | $1 - \frac{S \times \% \text{ crude fiber}}{100}$ |
| Water | $5 - \frac{S \times \% \text{ moisture}}{100}$ |
| Sucrose and corn starch (1:1) to make 100 g | |

[a] Teklad Test Diets, Madison, WIS. (87% protein).

[b] USP XVIII (ICN Nutritional Biochemicals, Cleveland, OH.). Composition (%): sodium chloride (NaCl), 13.93; potassium iodide (KI), 0.079; potassium phosphate monobasic ($KH_2PO_4$), 38.90; magnesium sulfate ($MgSO_4$), 5.73; calsium carbonate ($CaCO_3$), 38.14; ferrous sulfate ($FeSO_4.7H_2O$), 2.7; manganese sulfate ($MnSO_4.H_2O$), 0.548; cupric sulfate ($CuSO_4.5H_2O$), 0.0477; cobalt chloride ($CoCl_2.6H_2O$), 0.0023.

[c] ICN Nutritional Biochemicals, Cleveland, OH. Composition (mg/100 g diet): vitamin A, 2000 (IU); vitamin D, 200 (IU); vitamin E, 10 (IU); menadione, 0.5; choline, 200; p-aminobenzoic acid, 10; inositol, 10; niacin, 4; Ca-D-pantothenate, 4; riboflavin, 0.8; thiamine.HCl, 0.5; pyridoxine.HCl, 0.5; folic acid, 0.2; biotin, 0.04; vitamin $B_{12}$, 0.003.

## Apparent Diet Digestibility

After 14 days of experimental feeding, carmine red was added to the diet as a dye marker. The red faeces were

collected over a 7-day period. At the end of the seventh day, the marked diet was replaced by the dye-free diet, and the PER experiment was continued. The apparent diet digestibility was calculated as the ratio of diet consumed, to the weight of the faecal output (dry basis) times 100.

Apparent Nitrogen Digestibility

The apparent nitrogen digestibility was calculated according to the following formula:

$$\text{Apparent nitrogen digestibility} = \frac{\text{nitrogen intake (g)} - \text{faecal nitrogen (g)}}{\text{nitrogen intake (g)}} \times 100$$

## Analysis of Carotenoids

Extraction

The carotenoids of N. sitophila and oncom were extracted according to the method of Liu and Luh (1977). N. sitophila ATCC 14151 was grown on potato dextrose agar in ten Roux culture bottles. The culture was incubated at 30°C for 7 days. At the end of the incubation period, the mycelia and conidia were harvested with a small amount of water and blended with a hexane/acetone mixture (1:2, v/v) for 5 minutes. The slurry was filtered through glass wool into a 500 ml separatory funnel. The remaining mycelial residue was washed with the solvent mixture until colorless. The separatory funnel was shaken and the aqueous layer was discarded, while the organic (hexane) layer was washed with

25 ml of 20% KOH in 85% methanol to saponify fatty materials, and the aqueous layer was discarded. The hexane layer was washed first with 25 ml of 85% methanol and then with distilled water several times. The final extract was passed through anhydrous sodium sulfate in a funnel containing glass wool. The filtrate was concentrated to 10 ml in a flash evaporator (Buchler Instruments, Fort Lee, N.J.), and transferred onto the chromatographic column.

In determining the carotenoids of oncom fermented by N. sitophila ATCC 14151 for 72 hours, a 50 g sample was blended with 100 ml of water, a small amount of $CaCO_3$, and 200 ml of the solvent used in the mold extraction. The addition of $CaCO_3$ was done to neutralize the acidity. The extraction was completed as described in the case of the mold.

Separation and Identification

The adsorbent used for the separation of the carotenoids was prepared by mixing MgO and Hyflo supercel (1:1). The mixture was suspended in hexane and wet-packed in a glass column-plugged with glass wool. A 1-cm layer of anhydrous sodium sulfate was placed on the top of 20 x 1.1 cm column of packed adsorbent. Carotenoids were separated by changing the polarity of the elution solvent according to the following order: hexane; 2%, 3%, 5%, 7%, and 10% acetone in hexane; and 8%, and 15% ethanol in hexane. Visible carotenoids were separated easily by watching the color

bands moving through the column, while the movement of phytofluene was traced using a UV light.

The separated carotenoids fractions were dried in a flash evaporator (Buchler Instruments, Fort Lee, N.J.), and redissolved in hexane. The absorbance was scanned and recorded with a Beckman DB-24 recording spectrophotometer (Beckman Instruments Inc., Fullerton, CA.). Carotenoid concentration was calculated based on $E^{1\%}_{1cm}$ at a maximum absorption peak of each component (Foppen, 1971).

## RESULTS AND DISCUSSION

### Growth of Molds and Changes in pH During Oncom Fermentation

During the first 12 hours, the growth of both N. sitophila and R. oligosporus progressed slowly. After 18 hours, a rapid growth of R. oligosporus became obvious where the mycelial growth penetrated deeply into the peanut press cake mass, forming a compact semisolid product. After 36 hours, the cake was completely covered with white mycelia with small amount of black spores produced at the side of petri dish, indicating that the white oncom was ready for consumption.

Unlike R. oligosporus, the growth of N. sitophila was slow. It took 24 hours for this mold to develop obvious mycelial growth, and 40-48 hours to cover completely the peanut press cake mass with mycelia (Figure 9).

It was noticed by touch that during fermentation by either mold, the temperature of the fermenting cake mass gradually increased above that of the incubator. Previous investigators (Steinkraus et al., 1960, 1965; Sudarmadji and Markakis, 1978) showed that during the tempeh fermentation by R. oligosporus the temperature rose to a peak of 40-45$^{o}$C.


Figure 9. Oncom.

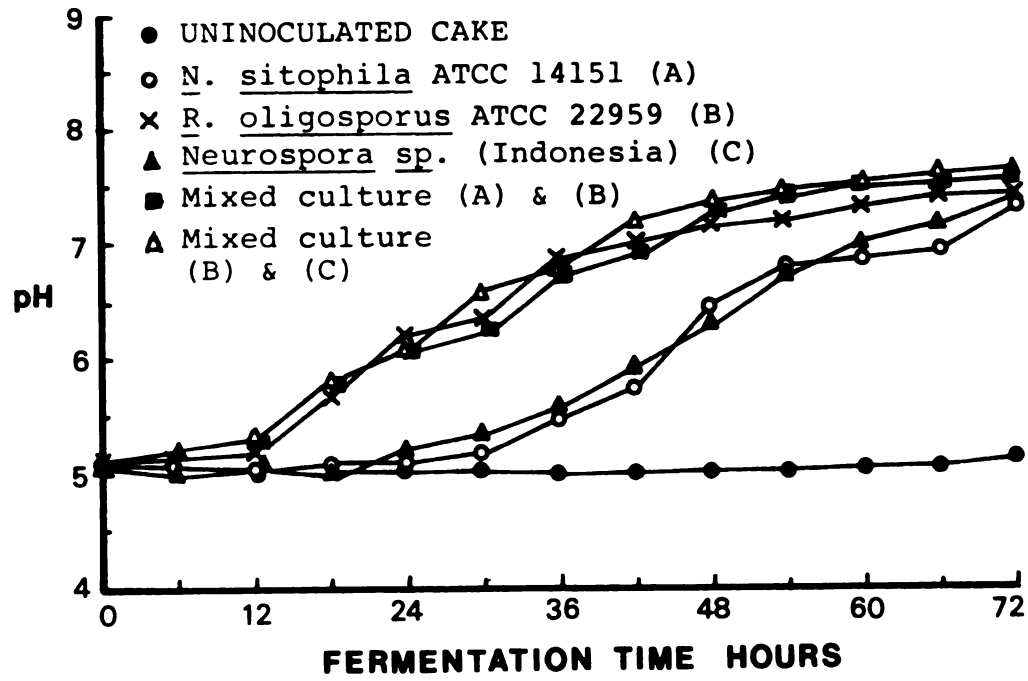


Figure 10. Changes in pH during oncom fermentation.

The development of mycelial growth during fermentation was accompanied by a gradual increase in pH. At the time of inoculation, the pH of peanut press cake was about 4.9-5.2. In oncom fermented by N. sitophila the pH remained rather constant for the first 24 hours and then gradually increased to 7.2 in about 72 hours as shown in Figure 10. In oncom fermented by R. oligosporus, the gradual increase in pH started as early as 12 hours, and it took only 42 hours for this mold to reach pH of 7.2. It was suggested that an increase in pH during fermentation was due to ammonia resulted from deamination of amino acids (van Buren et al., 1972).

## Identification of Free Fatty Acids

Figure 11 illustrates the GLC elution pattern obtained for the methyl esters of free fatty acids extracted from oncom fermented by R. oligosporus after 72 hours of fermentation. Other oncom samples showed similar GLC elution patterns except for their peak heights. Through comparison with the GLC elution pattern of an RM-3 standard mixture (Figure 12), nine peaks of the oncom chromatogram were identified as methyl esters of myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidate, behenate, and lignocerate. Neither the uninoculated cake nor the oncom contained erucic acid.

Figures 13 to 16 show the GC/MS spectra obtained for the methyl esters of free fatty acids extracted from the same oncom sample. All the esters had a base peak at m/e 74 and a large peak at m/e 87 which are usually found in the spectra of methyl esters (Kuksis et al., 1976), with the exception in Figures 14B and 14C. These last two spectra show the mixture of $M^+$ peaks at m/e 294, 296, 297, and 298, indicating the presence of an unseparated mixture probably of methyl esters of stearate, oleate, and linoleate. Instead of 0.125 in. o.d. column used for separation shown in Figures 11 and 12, a larger diameter column (0.250 in. o.d.) was used in GC/MS separation, resulting a decrease in resolution. This is probably the reason why methyl esters of stearate, oleate, and linoleate were not resolved in GC/MS

system.

Traces of methyl pentadecanoate (Figure 13B) were detected in oncom fermented by R. oligosporus only after 72 hours of fermentation. Again, the GC/MS spectra show that neither the uninoculated cake nor oncom contained erucic acid.

For quantitative analysis, calibration curves were prepared by injecting known amounts of pure methyl esters of fatty acids and plotting the amount injected versus the calculated peak areas for each compound. Linear regression equations of the methyl ester calibration curves used for calculation of free fatty acid contents are shown in Appendix 1. The efficiency of free fatty acid extraction was evaluated by spiking internal standard (n-heptadecanoic acid) to peanut oil prior to extraction. The recovery of added internal standard in each extraction ranged from 91 to 96%.

## Fatty Acids Liberated During Fermentation

The peanut press cake had been autoclaved at 121°C for 30 minutes which inactivated the intrinsic enzymes; therefore, any liberation of fatty acids during fermentation should have been caused by the action of lipase of the molds. Table 3 shows the free fatty acids content of oil extracted from oncom during fermentation. Myristic, linolenic, arachidic, behenic, and lignoceric acids were not detected in oncom samples at 0 hour of fermentation, while,

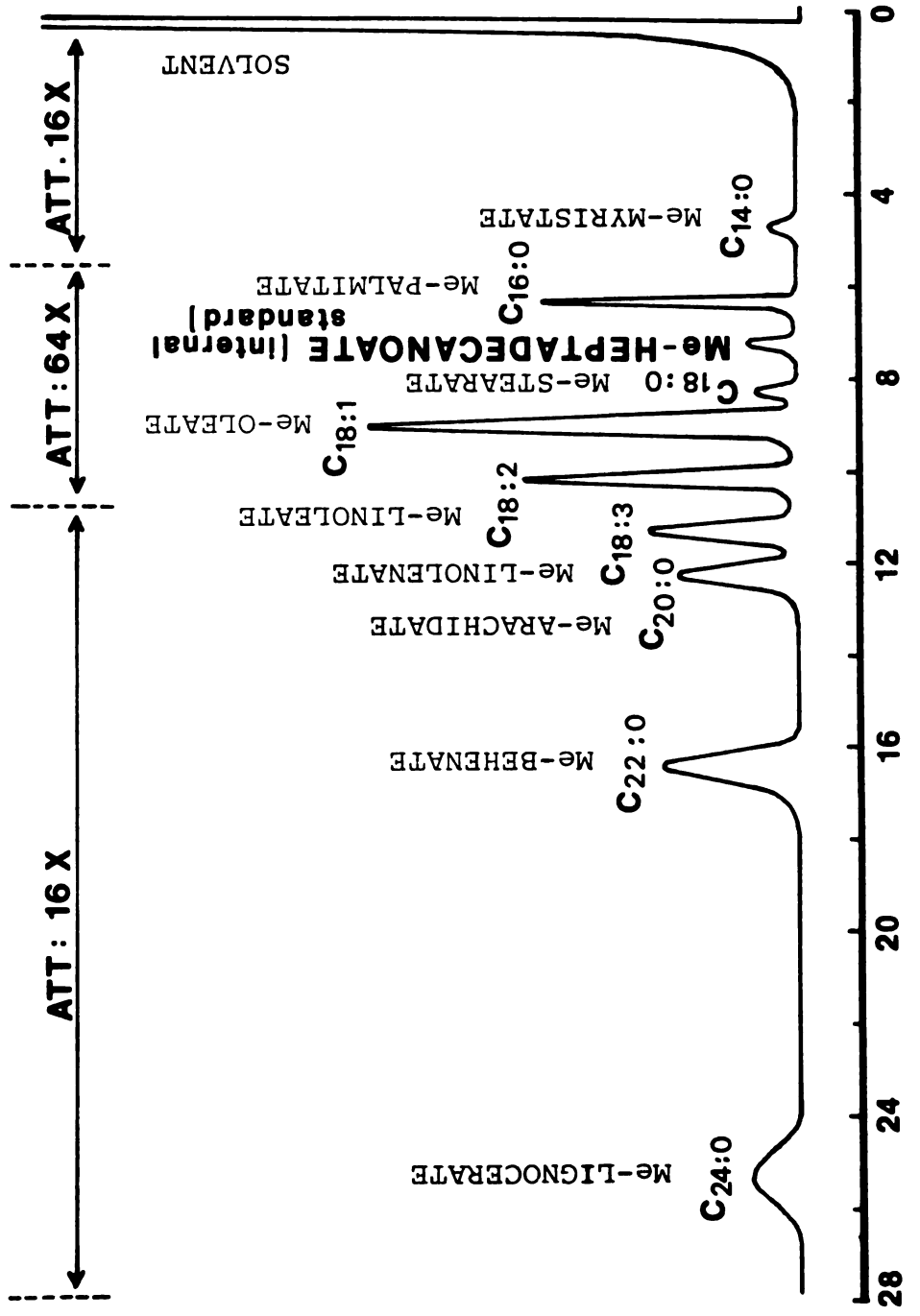


Figure 11. GLC elution pattern of the methyl esters of free fatty acids extracted from oncom fermented for 72 hours by R. oligosporus.

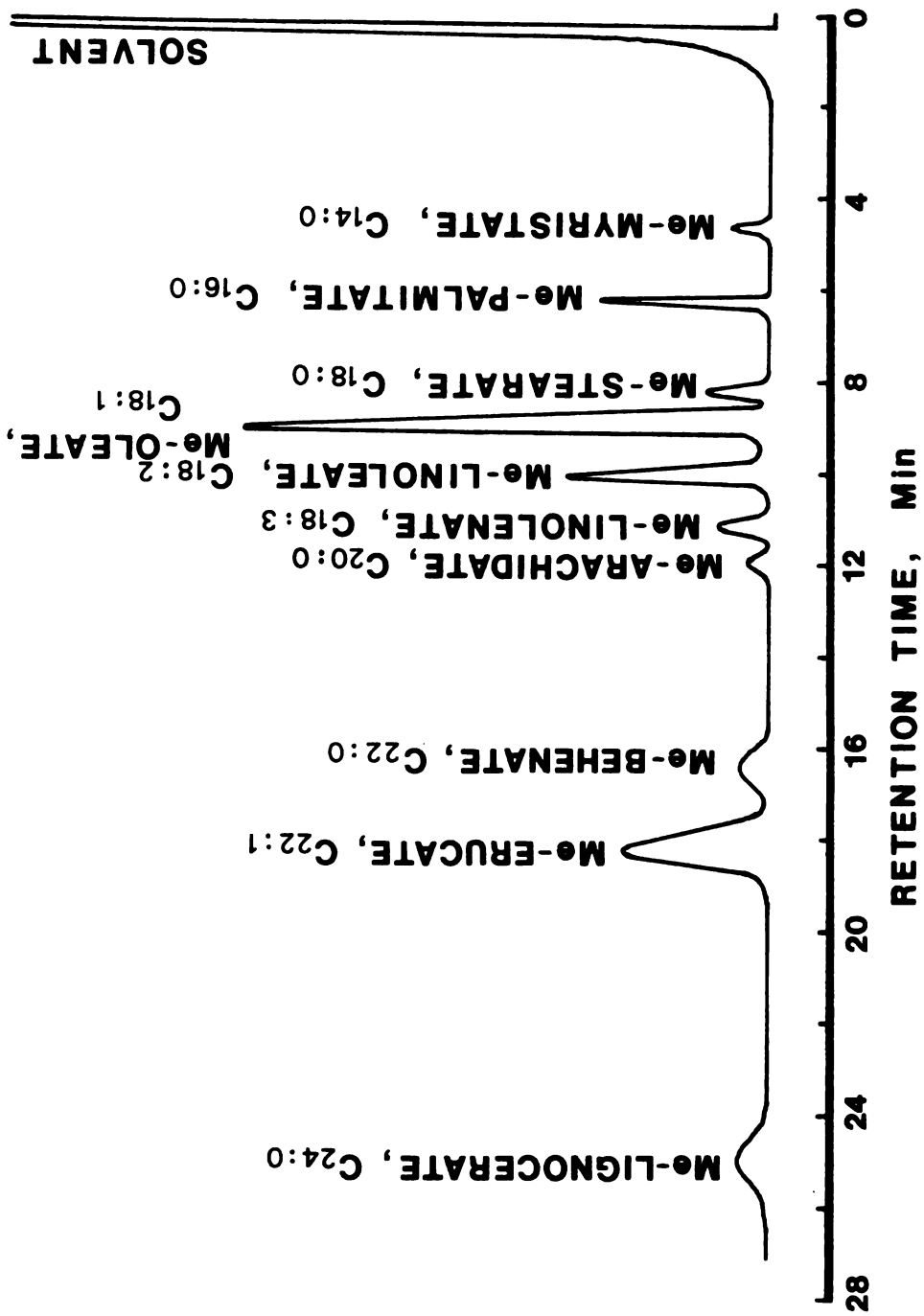


Figure 12. GLC elution pattern of the methyl esters of free fatty acids of a standard mixture.

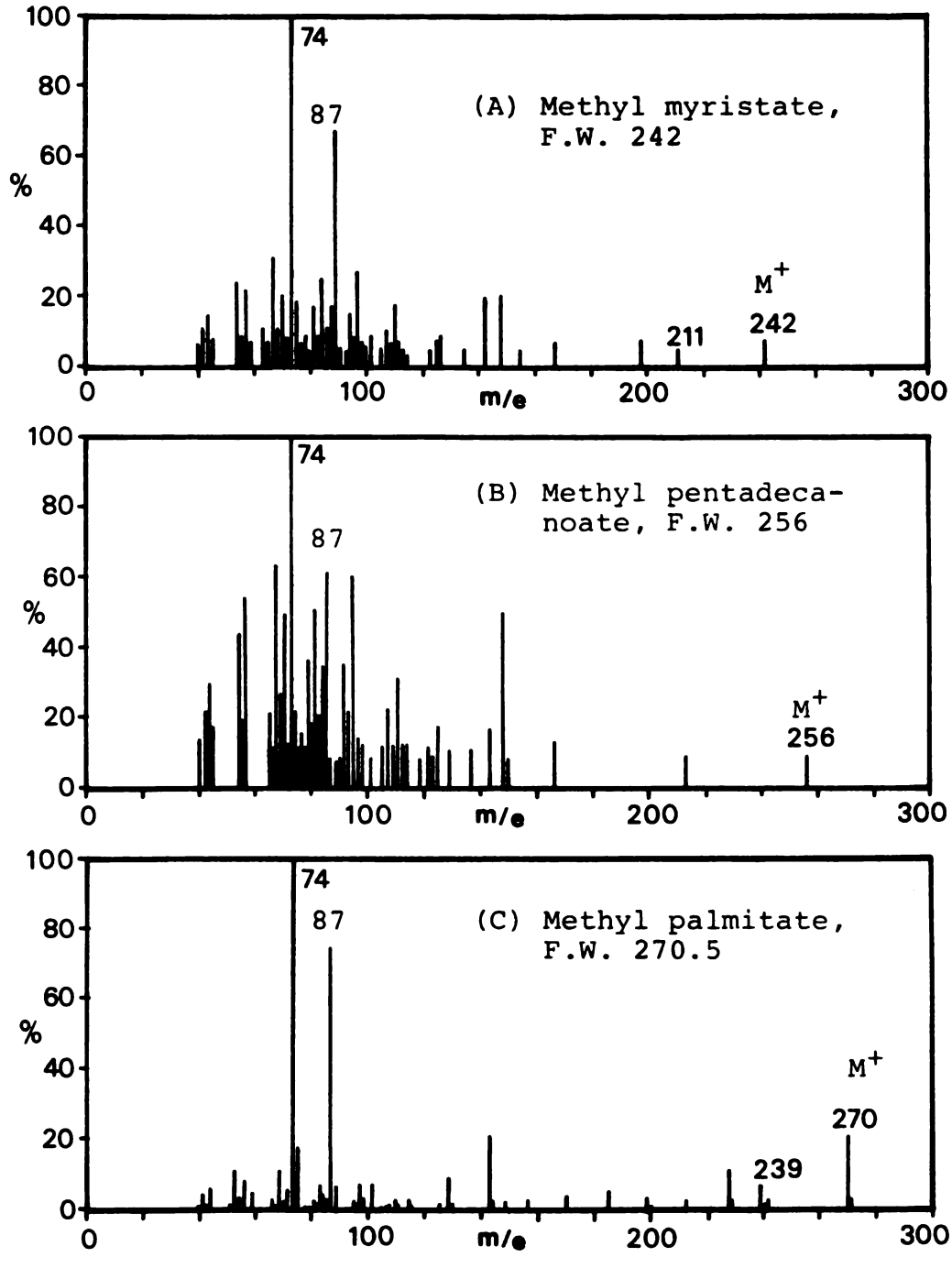


Figure 13. Mass spectra of (A) methyl myristate, (B) methyl pentadecanoate, and (C) methyl palmitate.

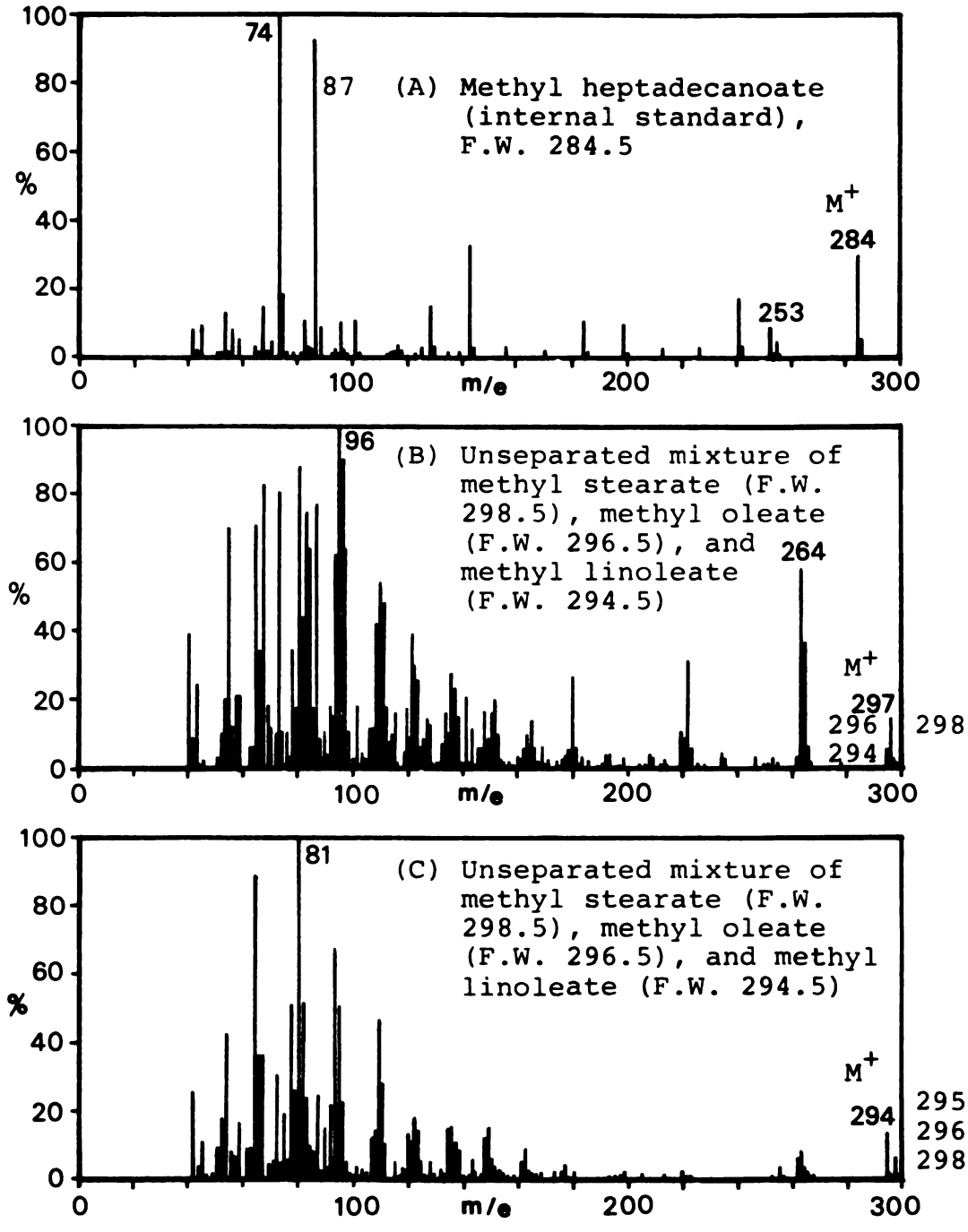


Figure 14. Mass spectra of (A) methyl heptadecanoate, (B) and (C) unseparated mixture of methyl stearate, methyl oleate, and methyl linoleate.

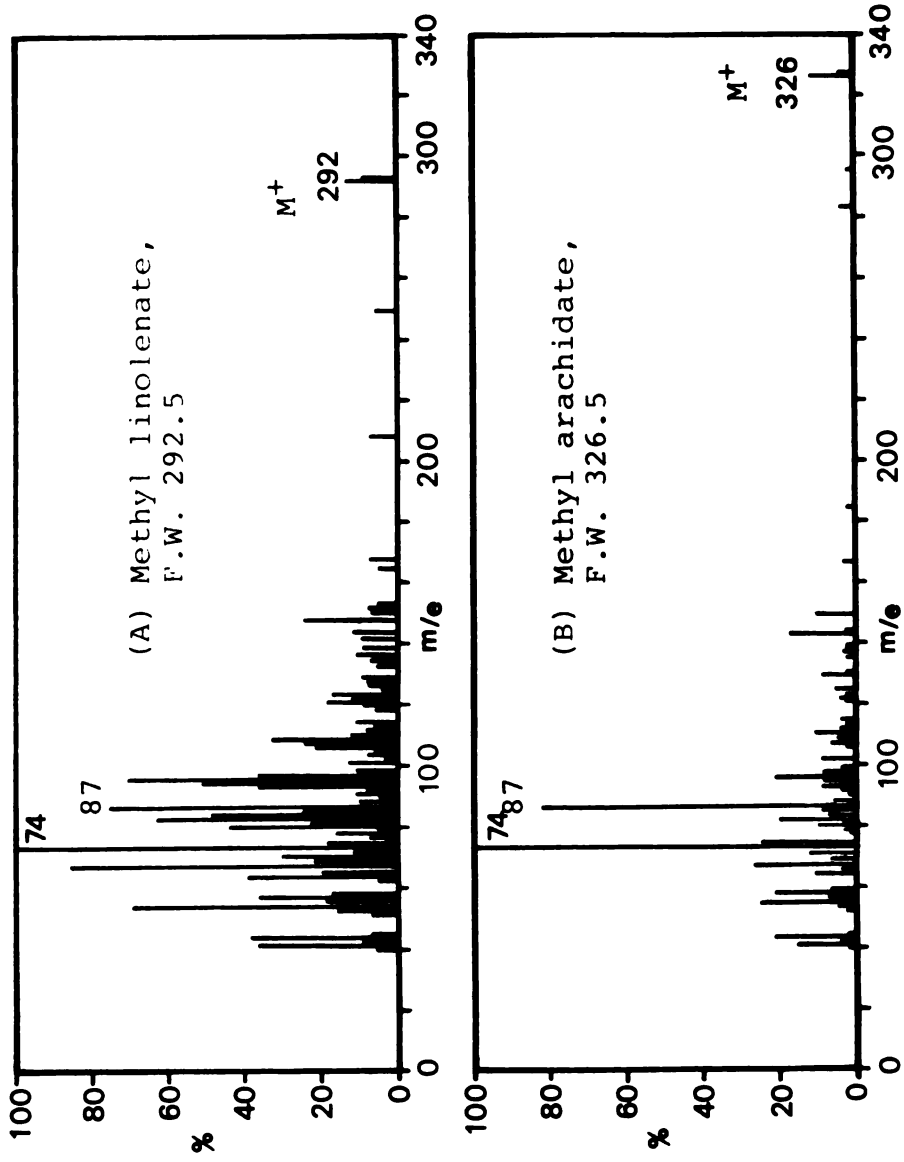


Figure 15. Mass spectra of (A) methyl linolenate, and (B) methyl arachidate.

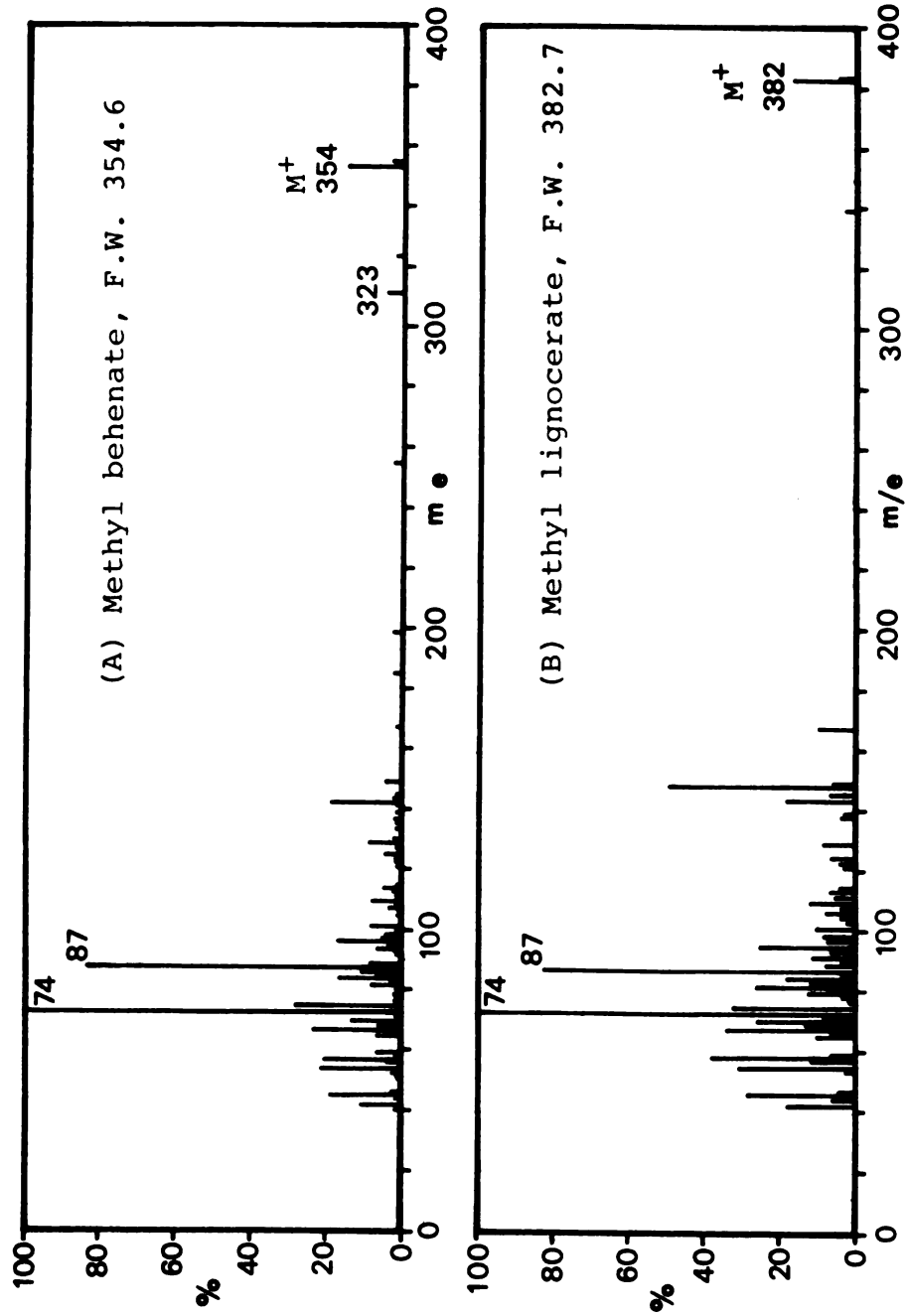


Figure 16. Mass spectra of (A) methyl behenate, and (B) methyl lignocerate.

Table 3. Free fatty acids content of oil extracted from oncom during fermentation (mg/g)

| Time (hr) | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | $C_{20:0}$ | $C_{22:0}$ | $C_{24:0}$ | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| UNINOCULATED CAKE | | | | | | | | | | |
| 0 | ND[a] | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 18 | ND | 1.11 | 0.30 | 1.87 | 3.00 | ND | ND | ND | ND | 6.28 |
| 36 | ND | 1.26 | 0.30 | 2.73 | 4.28 | ND | ND | ND | ND | 8.57 |
| 54 | ND | 1.58 | 0.34 | 3.83 | 4.87 | ND | ND | ND | ND | 10.62 |
| 72 | ND | 1.91 | 0.35 | 5.79 | 6.00 | ND | ND | ND | ND | 14.05 |
| ONCOM FERMENTED BY: | | | | | | | | | | |
| *N. sitophila* ATCC 14151 (A) | | | | | | | | | | |
| 0 | ND | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 6 | ND | 1.18 | 0.25 | 3.61 | 3.17 | TR[b] | ND | ND | ND | 8.21 |
| 12 | ND | 2.00 | 0.46 | 6.73 | 5.28 | TR | ND | ND | ND | 14.47 |
| 18 | ND | 3.58 | 0.49 | 7.29 | 6.29 | TR | ND | ND | ND | 17.65 |
| 24 | ND | 4.03 | 0.50 | 10.89 | 12.83 | TR | TR | 0.02 | ND | 28.27 |
| 30 | ND | 5.68 | 0.98 | 18.68 | 17.59 | 0.09 | 0.90 | 0.59 | ND | 44.51 |
| 36 | TR | 6.09 | 1.40 | 24.32 | 20.62 | 0.34 | 1.39 | 0.49 | ND | 54.65 |
| 42 | TR | 6.10 | 1.40 | 28.91 | 20.68 | 0.38 | 1.41 | 0.59 | TR | 59.47 |
| 48 | TR | 6.26 | 1.42 | 30.20 | 20.90 | 0.39 | 1.49 | 0.69 | 0.48 | 61.83 |
| 54 | TR | 6.59 | 1.59 | 30.51 | 22.32 | 0.45 | 1.57 | 0.85 | 0.59 | 64.47 |
| 60 | TR | 7.09 | 1.60 | 33.82 | 23.29 | 0.60 | 1.69 | 0.98 | 0.59 | 69.66 |
| 66 | 0.03 | 7.85 | 1.81 | 33.91 | 25.00 | 0.65 | 2.01 | 1.29 | 0.63 | 73.18 |
| 72 | 0.08 | 8.15 | 2.01 | 34.87 | 25.63 | 0.66 | 2.04 | 1.32 | 0.68 | 75.44 |
| *R. oligosporus* ATCC 22959 (B) | | | | | | | | | | |
| 0 | ND | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 6 | ND | 5.76 | 0.79 | 17.27 | 11.14 | 0.15 | 0.76 | 0.78 | ND | 36.65 |
| 12 | ND | 10.14 | 2.81 | 29.46 | 30.00 | 0.34 | 3.27 | 1.05 | ND | 77.07 |
| 18 | TR | 19.01 | 4.15 | 61.77 | 62.16 | 0.60 | 3.75 | 1.46 | ND | 152.90 |
| 24 | TR | 19.23 | 4.49 | 72.74 | 73.03 | 0.58 | 3.48 | 3.92 | 0.54 | 178.01 |
| 30 | 0.08 | 19.75 | 5.08 | 94.45 | 90.13 | 0.67 | 4.52 | 5.50 | 2.28 | 222.46 |
| 36 | 0.14 | 21.11 | 6.24 | 109.83 | 105.35 | 0.61 | 5.76 | 5.84 | 4.67 | 259.55 |
| 42 | 0.15 | 23.43 | 6.52 | 120.28 | 117.34 | 0.78 | 4.13 | 6.30 | 5.34 | 284.27 |
| 48 | 0.15 | 25.48 | 7.41 | 130.30 | 127.65 | 1.10 | 4.36 | 7.37 | 5.45 | 309.27 |
| 54 | 0.15 | 27.90 | 7.63 | 140.21 | 129.09 | 0.99 | 5.79 | 8.96 | 7.14 | 327.86 |
| 60 | 0.17 | 29.89 | 9.41 | 150.77 | 140.34 | 1.59 | 7.04 | 9.52 | 7.38 | 356.11 |
| 66 | 0.17 | 31.28 | 9.79 | 162.54 | 145.13 | 1.02 | 8.40 | 10.49 | 7.52 | 376.34 |
| 72 | 0.18 | 35.51 | 11.01 | 162.67 | 149.27 | 1.12 | 8.67 | 10.76 | 7.49 | 386.68 |

[a] Not detected

[b] Trace, less than 0.01 mg/g

Table 3. Free fatty acids content of oil extracted from oncom during fermentation (mg/g) (continued)

| Time (hr) | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | $C_{20:0}$ | $C_{22:0}$ | $C_{24:0}$ | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| *Neurospora sp.* (Isolated from Indonesian oncom) (C) | | | | | | | | | | |
| 0 | ND | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 6 | ND | 1.02 | 0.30 | 2.91 | 2.86 | ND | ND | ND | ND | 7.09 |
| 12 | ND | 2.87 | 0.31 | 3.16 | 2.87 | ND | ND | ND | ND | 9.21 |
| 18 | ND | 2.80 | 0.35 | 5.86 | 5.08 | TR | ND | ND | ND | 14.09 |
| 24 | ND | 3.43 | 0.98 | 10.86 | 9.88 | TR | TR | ND | ND | 25.15 |
| 30 | ND | 3.91 | 0.98 | 12.71 | 10.02 | 0.09 | 0.25 | 0.11 | 0.21 | 28.28 |
| 36 | 0.06 | 4.95 | 1.09 | 17.23 | 9.95 | 0.24 | 0.57 | 0.48 | 0.42 | 34.99 |
| 42 | 0.05 | 5.62 | 1.08 | 18.63 | 15.28 | 0.24 | 0.86 | 0.47 | 0.45 | 42.68 |
| 48 | 0.09 | 5.88 | 1.26 | 20.66 | 16.38 | 0.39 | 1.09 | 0.50 | 0.50 | 46.75 |
| 54 | 0.13 | 5.90 | 1.88 | 24.21 | 16.46 | 0.41 | 1.57 | 0.69 | 0.53 | 51.78 |
| 60 | 0.16 | 8.52 | 2.90 | 29.67 | 27.68 | 0.44 | 1.88 | 0.73 | 0.66 | 72.74 |
| 66 | 0.20 | 9.09 | 3.06 | 35.05 | 30.97 | 0.59 | 2.58 | 0.83 | 0.70 | 83.87 |
| 72 | 0.20 | 9.32 | 3.42 | 49.23 | 33.69 | 0.66 | 2.95 | 0.99 | 0.71 | 101.17 |
| Mixed culture (A) & (B) | | | | | | | | | | |
| 0 | ND | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 6 | TR | 7.82 | 0.52 | 18.38 | 11.94 | 0.25 | 0.26 | 0.29 | ND | 39.46 |
| 12 | TR | 10.60 | 3.37 | 34.38 | 31.50 | 0.65 | 0.64 | 1.45 | ND | 82.59 |
| 18 | TR | 16.15 | 4.44 | 69.33 | 65.34 | 0.74 | 1.04 | 1.38 | ND | 158.42 |
| 24 | 0.07 | 24.13 | 5.38 | 79.51 | 76.05 | 0.99 | 1.33 | 1.53 | ND | 188.99 |
| 30 | 0.09 | 25.05 | 5.55 | 104.84 | 99.45 | 1.04 | 2.15 | 1.55 | 0.41 | 240.13 |
| 36 | 0.14 | 28.93 | 6.77 | 156.00 | 113.34 | 1.12 | 2.50 | 1.58 | 0.86 | 311.24 |
| 42 | 0.15 | 29.64 | 6.93 | 164.84 | 115.35 | 1.13 | 3.42 | 3.26 | 1.49 | 326.21 |
| 48 | 0.18 | 31.66 | 7.50 | 169.15 | 122.95 | 1.04 | 5.15 | 3.84 | 1.92 | 343.39 |
| 54 | 0.19 | 34.54 | 8.15 | 170.04 | 139.28 | 1.00 | 6.28 | 4.04 | 2.00 | 365.52 |
| 60 | 0.20 | 36.37 | 8.49 | 172.64 | 142.80 | 1.28 | 7.03 | 5.79 | 3.15 | 377.75 |
| 66 | 0.27 | 37.28 | 8.90 | 175.03 | 145.11 | 1.22 | 7.98 | 7.85 | 4.42 | 388.06 |
| 72 | 0.24 | 38.43 | 9.17 | 176.80 | 148.43 | 1.42 | 8.46 | 8.51 | 5.38 | 396.84 |
| Mixed culture (B) & (C) | | | | | | | | | | |
| 0 | ND | 0.92 | 0.29 | 1.48 | 2.64 | ND | ND | ND | ND | 5.33 |
| 6 | ND | 8.41 | 0.65 | 18.95 | 10.37 | 0.13 | 0.29 | 0.44 | ND | 39.24 |
| 12 | TR | 14.16 | 2.65 | 36.26 | 33.15 | 0.77 | 0.63 | 1.43 | ND | 89.05 |
| 18 | TR | 18.49 | 3.89 | 70.34 | 62.89 | 0.84 | 1.46 | 2.26 | ND | 160.17 |
| 24 | 0.08 | 22.98 | 4.51 | 100.15 | 93.83 | 0.76 | 2.26 | 4.50 | 0.63 | 229.70 |
| 30 | 0.17 | 34.65 | 6.65 | 140.34 | 95.13 | 1.13 | 4.13 | 5.15 | 2.14 | 284.49 |
| 36 | 0.21 | 35.93 | 7.83 | 151.35 | 98.80 | 1.06 | 5.29 | 6.44 | 3.92 | 310.83 |
| 42 | 0.20 | 36.37 | 7.86 | 165.14 | 110.00 | 0.87 | 5.34 | 6.46 | 3.96 | 336.20 |
| 48 | 0.22 | 42.63 | 8.63 | 172.89 | 119.34 | 1.01 | 5.64 | 6.29 | 3.49 | 360.14 |
| 54 | 0.22 | 42.13 | 9.88 | 174.15 | 126.30 | 1.09 | 6.28 | 6.50 | 3.81 | 370.36 |
| 60 | 0.23 | 44.49 | 9.78 | 180.33 | 140.34 | 1.01 | 6.35 | 6.64 | 4.02 | 393.19 |
| 66 | 0.24 | 45.15 | 10.13 | 187.64 | 150.11 | 1.19 | 6.50 | 6.55 | 4.44 | 411.95 |
| 72 | 0.27 | 45.86 | 10.52 | 195.01 | 151.84 | 1.12 | 6.79 | 6.59 | 3.73 | 421.73 |

palmitic, stearic, oleic, and linoleic acids totally were present at a level less than 0.6%. As the fermentation progressed, more fatty acids were liberated from triglycerides. The order of liberation of major fatty acids in all treatments appeared to be oleic, linoleic, palmitic, and stearic acid. This order is apparently similar to that which occurred in groundnut oil infected by Aspergillus (Tomlins and Townsend, 1968).

The rate of fatty acids liberation in oncom fermented by R. oligosporus was much higher than that in oncom fermented by N. sitophila. Beuchat and Worthington (1974) monitored lipolytic activity of different molds by standard alkali titration of extracted oil and found similar results. In oncom fermented by N. sitophila the total free fatty acid content of oil increased from 0.6 to 10%, while, in oncom fermented by R. oligosporus the increase was from 0.6 to 39% after 72 hours of fermentation.

Substrates and environment conditions (temperature, moisture, humidity) were uniform in all treatments; therefore, the difference in free fatty acid contents was mainly due to different specific ability in producing lipase between two molds. Alford et al. (1964) reported that from 82 microorganisms studied, only 13 were highly lipolytic; one of these was R. oligosporus. It was also reported that many microbial lipases were typical pancreatic-type 1-3 lipases which liberated fatty acids much more rapidly from the 1- and 3- positions of a triglyceride than from the 2-

position, except Geotrichum candidum which had a high degree of specificity for unsaturated fatty acids regardless of their positions. Glycerol was also detected in oncom (Figure 20). This indicates that some triglycerides might have been hydrolyzed completely to glycerol and free fatty acids. It is possible that liberation of fatty acids increases the digestibility of peanut lipids and thereby increases their nutritional value. In addition, free fatty acids may contribute to the development of typical oncom flavor.

## Analysis of Oligosaccharides by Circular TLC and HPLC

Sugars may be separated by TLC on silica gel, but resolution is usually unsatisfactory. However, resolution may be improved by addition of various inorganic salts such as boric acid, tetraborate, bisulfite, and mono- or dibasic phosphates (Ghebregzabher et al., 1976). Recently, McLaren and Won (1979) demonstrated that the separation of oligosaccharides by circular TLC was better than that by a classical linear TLC.

Two factors are known to be involved in enhancing the resolution power of circular TLC (Schleicher and Schuell, 1976). The first of these factors is that in circular TLC the component zones are continuously expanding, so that, in effect, the zones are drawn out into progressively narrower concentric rings. In linear TLC, on the other hand, there is no corresponding expansive effect and

as development proceeds, the component zones become progressively broader and wider due to the effects of diffusion. The second factor is a result of the combination of a progressively expanding solvent front and a limited point source of solvent input. With this arrangement, solvent feed will always be faster at the trailing edge of a component zone, tending to compress it. This factor, along with a progressive drop in the sample loading, significantly supresses the tendency of a component to tail during development.

Figure 17 illustrates a pattern of oligosaccharides obtained by a circular TLC on $KH_2PO_4$-treated silica gel with two solvent developments. Chromatogram of standard mixture shows that oligosaccharides are well separated with the following Rf values: 0.78 (sucrose), 0.66 (melibiose), 0.52 (raffinose), and 0.31 (stachyose). Although analysis by circular TLC is qualitative, it shows clearly that sucrose disappeared almost completely, while small amount of melibiose appeared after 72 hours of fermentation.

For quantitative analysis purposes, these oligosaccharides were separated by HPLC using a μBondapak/CHO column. Pretrials indicated that acetonitrile/water mixture (80:20, v/v) with a flow rate of 2.3 ml per minute was the best elution solvent for the separation of glycerol, sucrose, myo-inositol, melibiose, raffinose, and stachyose (Figure 18). Retention times of these sugars and alcohols are (in minutes): 1.95 (glycerol), 3.19 (sucrose),

3.69 (myo-inositol), 4.25 (melibiose), 5.00 (raffinose), and 8.44 (stachyose). The second peak (2.60 min) in the chromatogram is the peak of glucose and fructose which could not be separated by HPLC under the conditions of this run. No further attempt was made to resolve monosaccharides since these sugars were present in peanut only in trace amounts.

Within a range of 20 to 100 μg, the peak width of known sugars remained constant; therefore, sugar concentrations were calculated on the peak height basis. The standard curves for oligosaccharides and myo-inositol are shown in Figure 19.

Figure 20 illustrates HPLC chromatograms for oligosaccharides and alcohols in uninoculated cake, and oncom fermented for 72 hours by N. sitophila ATCC 14151; and R. oligosporus ATCC 22959. Concentrations of sucrose, raffinose, and stachyose are listed in Table 4.

After 36 to 72 hours of fermentation, trace amounts of melibiose were detected in all oncom samples. Circular TLC in Figure 17 also showed the presence of melibiose after 72 hours of fermentation. The melibiose which appear during fermentation might be an intermediate product resulting from the hydrolytic breakdown of raffinose and/or stachyose. The appearance of melibiose as an intermediate product was also observed in soymilk treated with an enzyme preparation from Aspergillus saitoi, which completely decomposed all the oligosaccharides to their constitutive

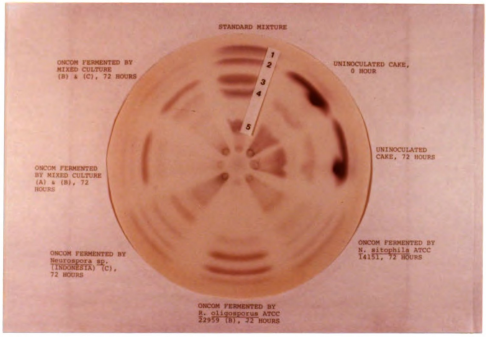


Figure 17. Circular TLC of oligosaccharides extracted from fermented and unfermented peanut press cake. (1) Glucose, Fructose, (2) Sucrose, (3) Melibiose, (4) Raffinose, (5) Stachyose.

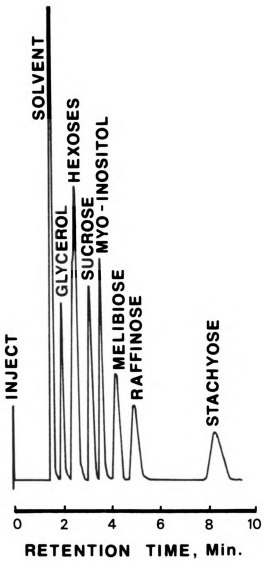


Figure 18. HPLC chromatogram of a standard mixture consisting of oligosaccharides, glycerol, and myo-inositol.

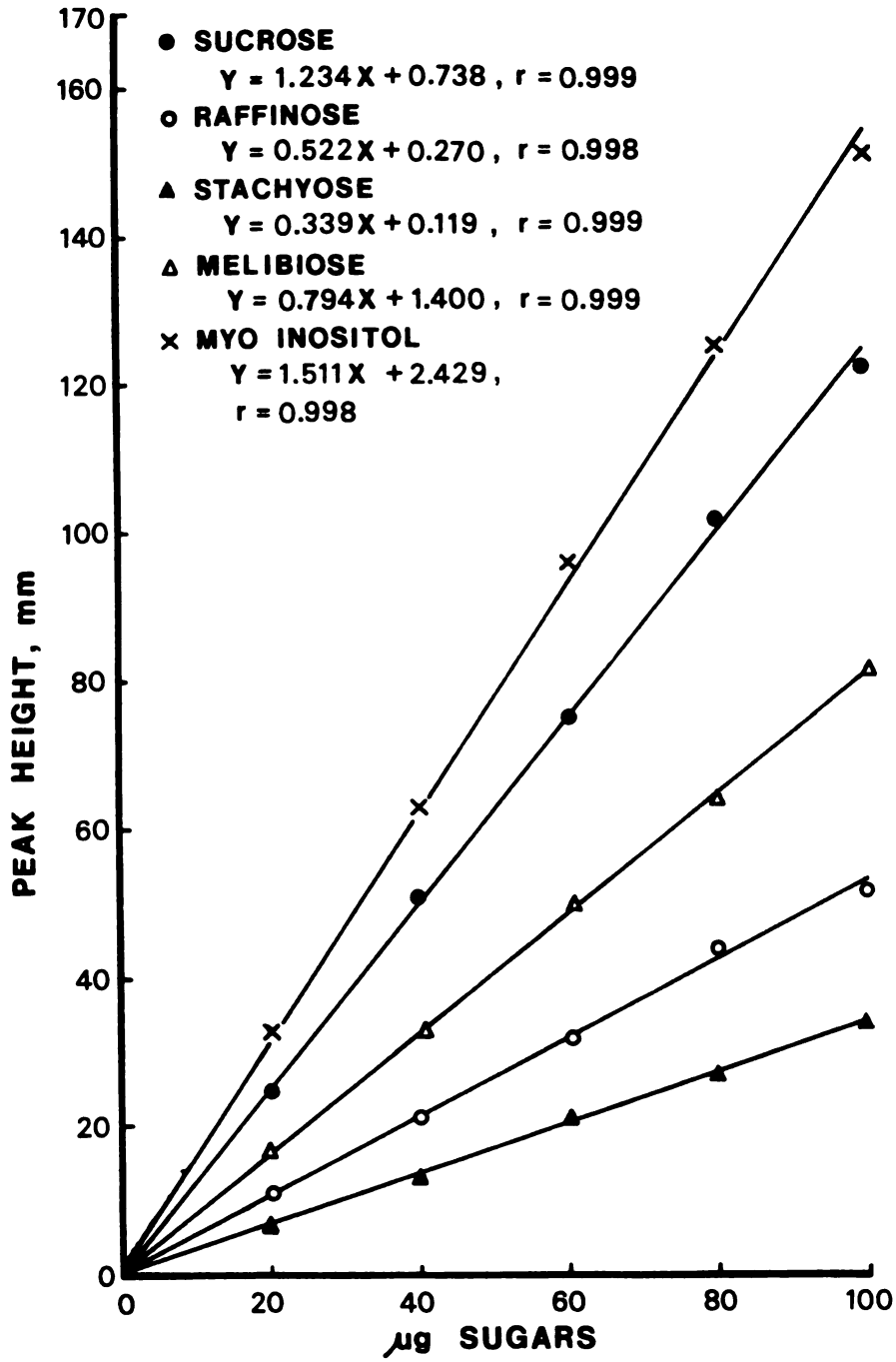


Figure 19. Standard curves for oligosaccharides and myo-inositol.

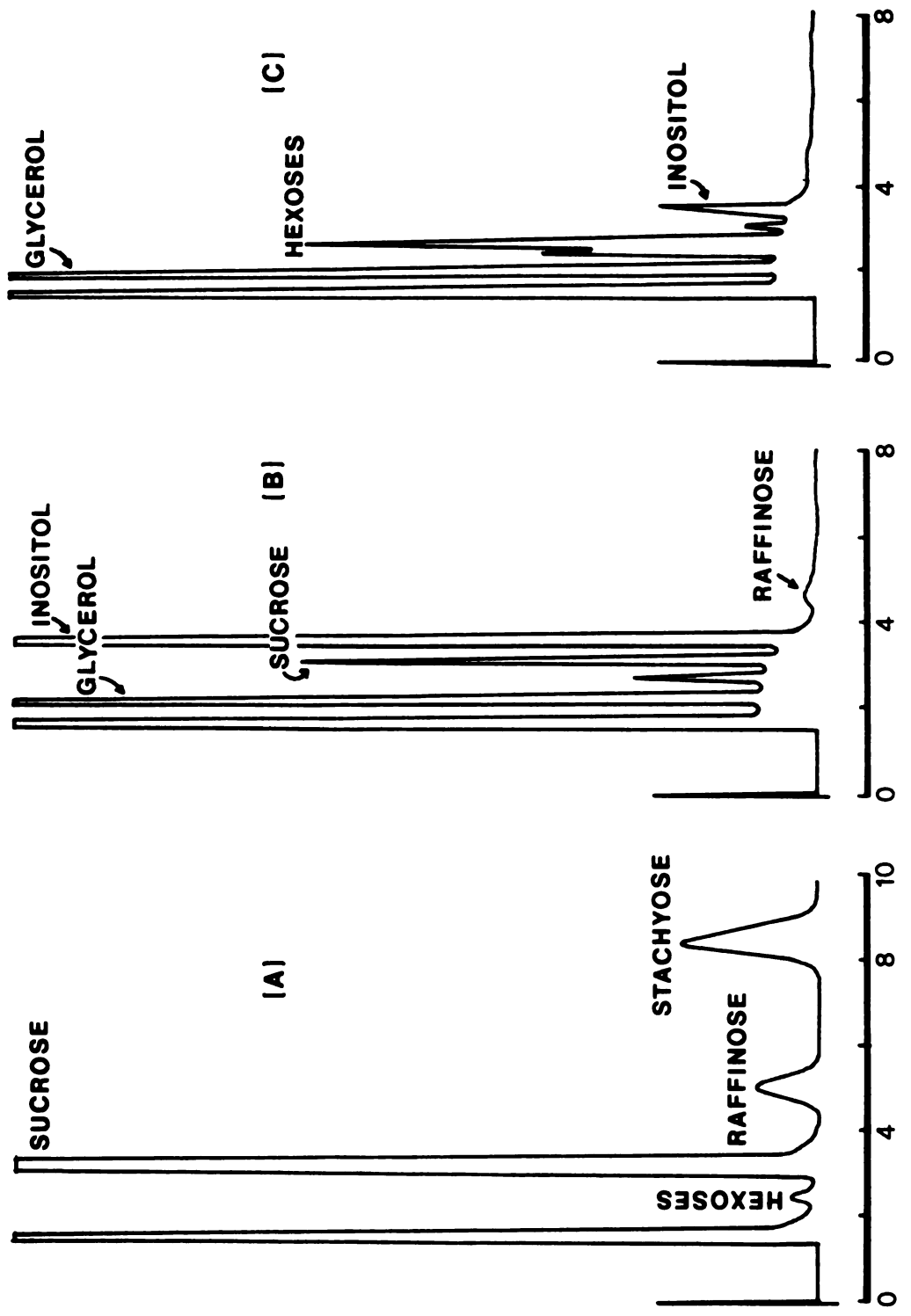


Figure 20. HPLC chromatograms of (A) uninoculated cake, (B) oncom fermented for 72 hours by R. oligosporus, and (C) oncom fermented for 72 hours by N. sitophila.

Table 4. Sucrose, raffinose, and stachyose contents of uninoculated cake and oncom prepared with various mold cultures

| Sample | Ferm. time, hours | Content (%)[a] | | |
|---|---|---|---|---|
| | | Sucrose | Raffinose | Stachyose |
| UNINOCULATED CAKE | 0 | 3.39 | 0.08 | 0.16 |
| | 36 | 3.25 | 0.06 | 0.14 |
| | 72 | 3.30 | 0.09 | 0.15 |
| ONCOM FERMENTED BY: | | | | |
| *N. sitophila* ATCC 14151 (A) | 18 | 0.14 | TR[b] | 0.08 |
| | 36 | TR | ND[c] | 0.04 |
| | 72 | TR | ND | TR |
| *R. oligosporus* ATCC 22959 (B) | 18 | 3.10 | 0.10 | 0.10 |
| | 36 | 1.56 | 0.09 | TR |
| | 72 | 0.89 | 0.06 | ND |
| *Neurospora sp.* (Indonesia) (C) | 18 | 0.09 | 0.03 | 0.07 |
| | 36 | TR | TR | 0.05 |
| | 72 | TR | TR | TR |
| Mixed culture (A) & (B) | 18 | 0.12 | TR | 0.05 |
| | 36 | TR | ND | TR |
| | 72 | TR | ND | ND |
| Mixed culture (B) & (C) | 18 | 0.13 | TR | 0.07 |
| | 36 | TR | ND | TR |
| | 72 | TR | ND | ND |

[a] Average of three determinations.
[b] Trace, less than 0.01%.
[c] Not detected.

monosaccharides (Sugimoto and van Buren, 1970). The reduction of sucrose and stachyose (Table 4) accompanied with the appearance of melibiose strongly indicates that the molds used in oncom fermentation possess an invertase which

hydrolyzes the glucose-fructose bond of the oligosaccharide and an α-galactosidase which hydrolyzes the other bonds of the molecule (Figure 21).

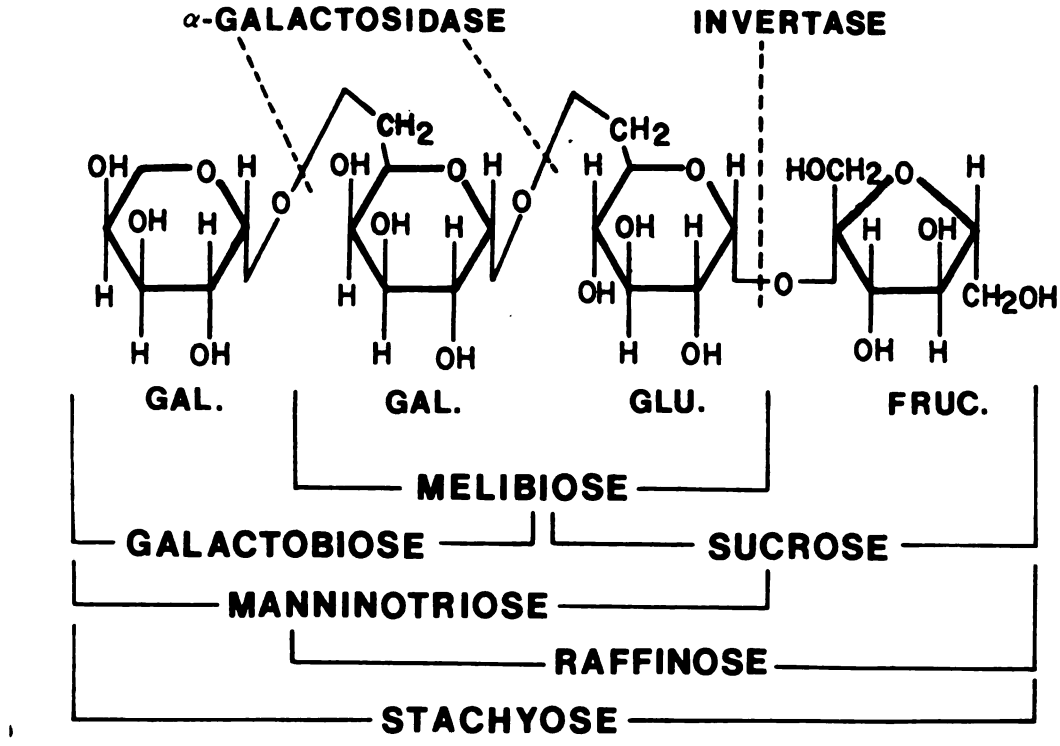


Figure 21. Structure of stachyose.

The activity of invertase produced by N. sitophila seemed to be very strong. It eliminated sucrose and raffinose almost completely in 36 hours of fermentation. While, it took 72 hours for R. oligosporus to hydrolyze 70% of the existing sucrose. Stachyose content decreased during the fermentation; however, the degradation rate was much slower than that in the other oligosaccharides.

The raffinose content of oncom fermented by R. oligosporus did not decrease as much as that of sucrose

and stachyose. Perhaps, this is due to raffinose formed from the partial hydrolysis of stachyose. This finding is in agreement with the report by Shallenberger et al. (1967) in which the stachyose and sucrose contents decreased without apparent change in the raffinose content in the tempeh fermented by a Rhizopus mold over a 72-hour period. On the other hand, Worthington and Beuchat (1974) found that raffinose and sucrose were not utilized by R. oligosporus, while small amounts of stachyose were utilized only after 68 hours of fermentation. When both N. sitophila and R. oligosporus were used as a mixed culture, all oligosaccharides were eliminated almost completely after 36 hours of fermentation. Raffinose and stachyose are thought to be involved in flatulence and their reduction in oncom is a desirable effect.

## The Soluble Protein of Oncom

During 72 hours of fermentation, the soluble protein content of uninoculated cake, as measured by the method of Lowry et al. (1951), remained unchanged and in the range of 70-78 mg per g sample. During the first 18 hours, the soluble protein content of oncom fermented by R. oligosporus increased to 86.4 mg per g, remained unchanged during the second 18 hours, and then increased to 125.9 mg per g at 72 hours of fermentation (Figure 22). Likewise, the soluble protein of oncom fermented by N. sitophila changed during fermentation according to the same trend as in oncom

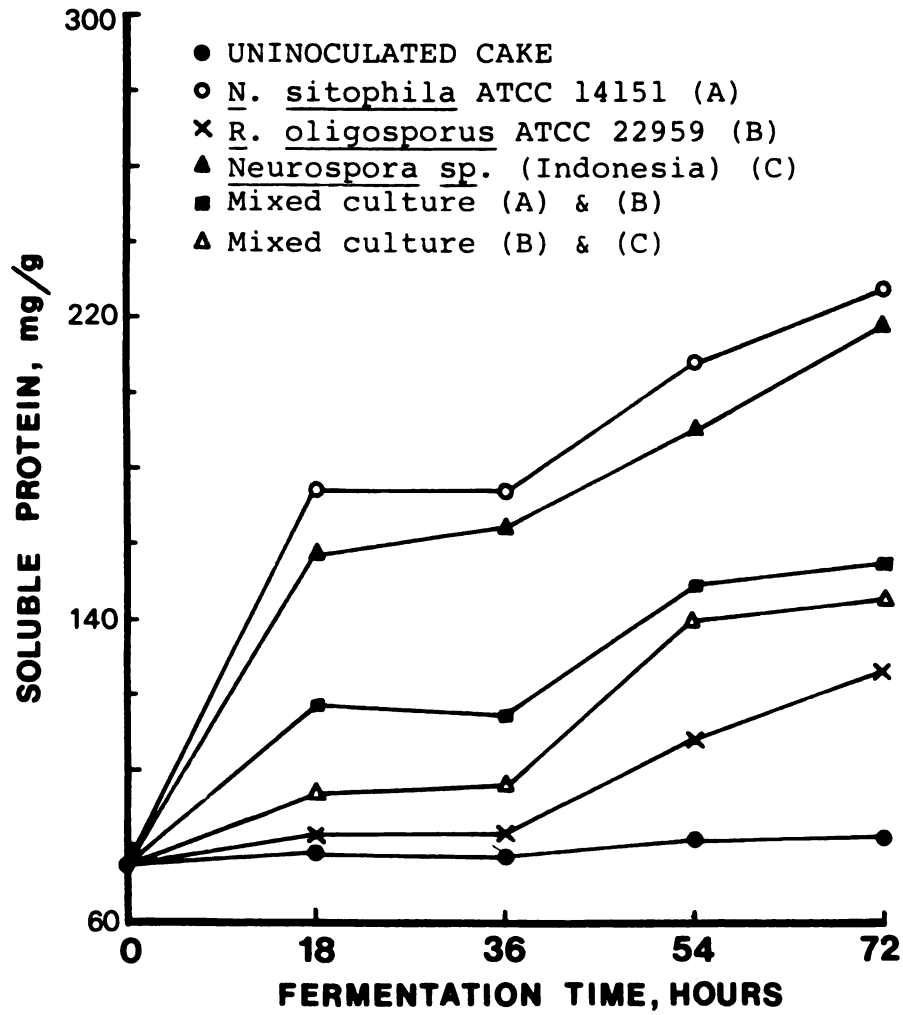


Figure 22. Soluble protein of fermented and unfermented peanut press cake.

fermented by R. oligosporus except that the soluble protein content of oncom fermented by N. sitophila was much higher. Apparently the proteolytic activity in oncom fermented by N. sitophila was stronger than that in oncom fermented by R. oligosporus. Cherry and Beuchat (1976) observed that the free amino acid content was greater in peanuts inoculated with N. sitophila than in those inoculated with R. oligosporus during the infection period. Van Buren et at. (1972) reported that at the end of a 72-hour tempeh fermentation, about one half of the crude protein had become water soluble.

Electrophoretic patterns of the soluble protein of uninoculated cake, and oncoms fermented for 72 hours by N. sitophila and R. oligosporus on 10% SDS gel are shown in Figure 23. These patterns are indicative of the protein hydrolysis that might have occurred during fermentation.

## Degradation of Phytic Acid in Oncom

Recovery of added phytate ranged from 94-96% (Appendix 2). The phytic acid content of uninoculated cake was 13.60 $\pm$ 0.18 mg per g or 1.36% (3.83 $\pm$ 0.05 mg phytate P per g). It was reported that the phytic acid content of whole oilseeds including peanut was about 1.5% (Erdman, 1979).

It was assumed that there was no destruction of phytic acid during soaking overnight at refrigeration temperature, because pH (4.5) and temperature were low enough

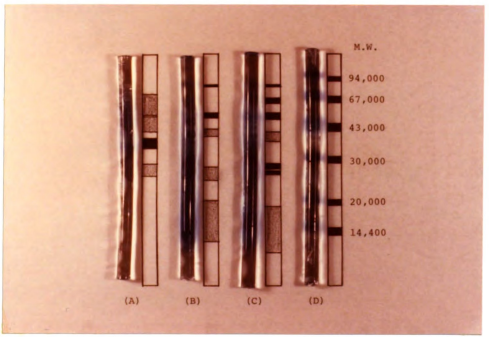


Figure 23. Electrophoretic pattern of soluble protein. (A) Uninoculated cake, (B) Oncom fermented for 72 hours by N. sitophila, (C) Oncom fermented for 72 hours by R. oligosporus, (D) Molecular weight protein standard.

to prevent any phytase activity. Lolas and Markakis (1977) reported that the pH optimum for the Navy bean phytase was 5.3 with an optimum temperature of about 50°C. Others reported that pH optimum of different sources of phytase ranged from 5.0 to 7.5 (Peers, 1953; Chang, 1967; Mandal et al., 1972). Prior to inoculation, peanut press cake was autoclaved at 121°C for 30 minutes. This step might decrease the phytic acid content of the cake. Reddy et al. (1978) reported that autoclaving Black gram beans in excess of water at 116°C for 5 minutes resulted in losses of phytic acid and total P content due to leaching. Likewise, Tabekhia and Luh (1980) showed that cooking dry beans at 100°C for 3 hours had little effect on phytate retention, while canning the dry beans at 115.5°C for 3 hours resulted in a reduction of phytate.

During 72 hours of fermentation, the phytic acid content of uninoculated cake remained constant (Figure 24), while the phytic acid content of oncom decreased cosiderably. The decrease in phytic acid content was accompanied by an increase in inorganic phosphorus content as shown in Figure 25. The decrease in phytic acid and an increase in inorganic phosphorus content were probably due to phytase released by the molds. Autoclaving prior to inoculation should have inactivated the phytase which might be inherent to the peanuts. Therefore, any phytase activity found in oncom must have originated from the molds.

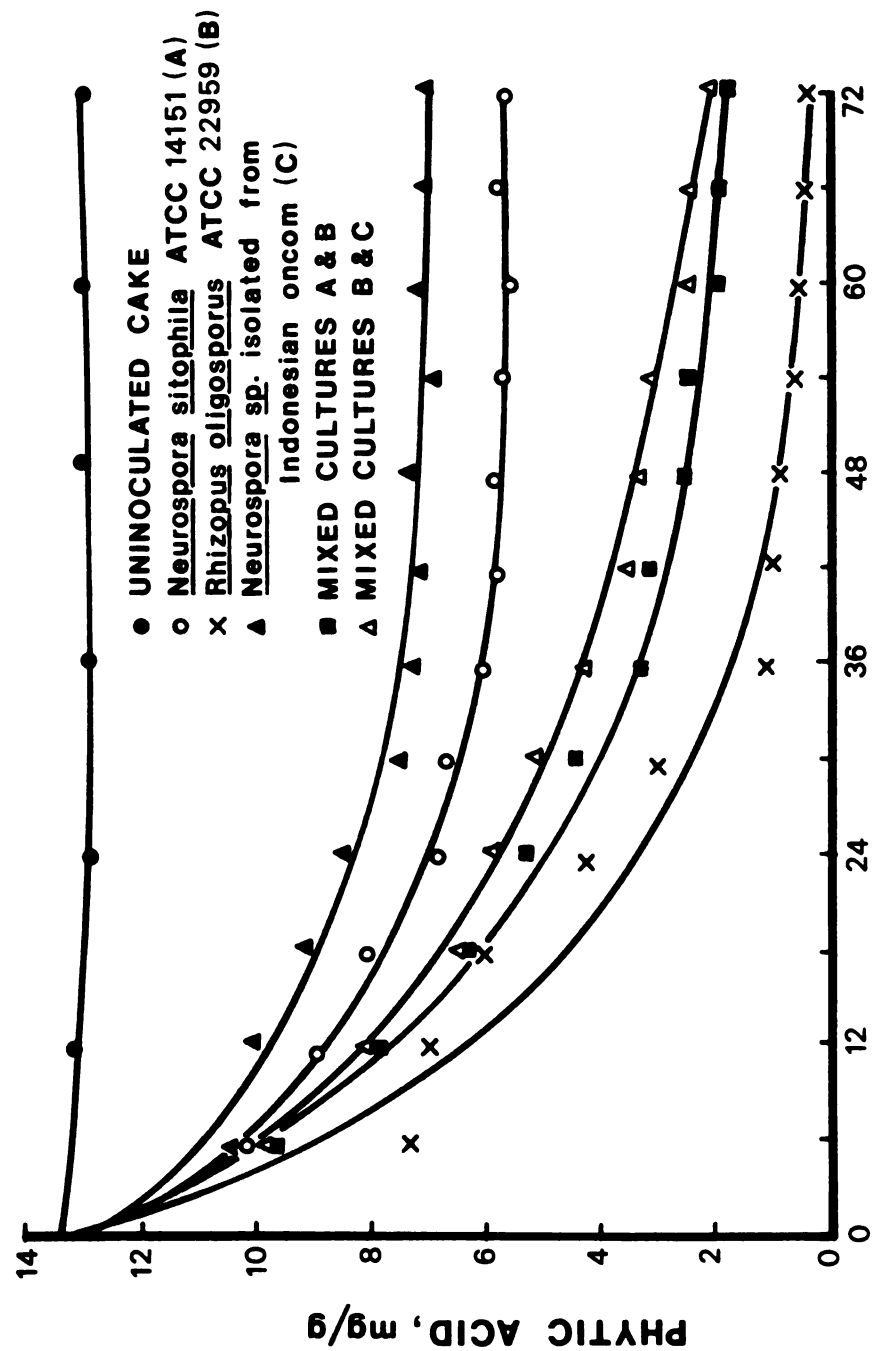


Figure 24. Phytic acid content of fermented and unfermented peanut press cake.

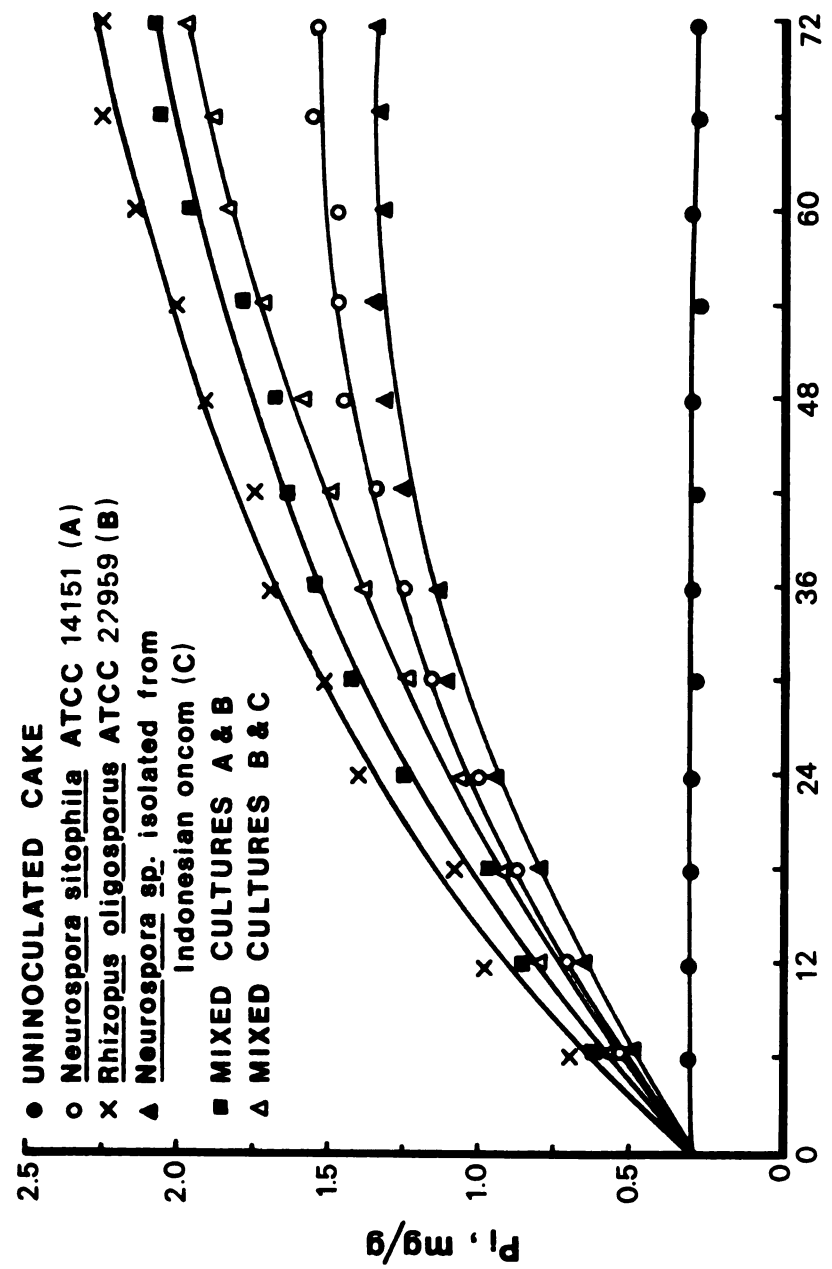


Figure 25. Inorganic phosphorus content of fermented and unfermented peanut press cake.

Judging from the disappearance of phytic acid, it may be deduced that the phytase activity of R. oligosporus is greater than that of N. sitophila. Direct evidence for the presence of phytase in R. oligosporus has been recently reported by Sudarmadji and Markakis (1977) and Wang et al. (1980).

As shown in Figure 20, free inositol was detected in oncom samples, indicating that phytic acid had been hydrolyzed completely by phytase. However, calculations on the free inositol and inorganic phosphorus contents of oncom fermented by R. oligosporus (Table 5) showed that only 60-65% of phytic acid was hydrolyzed completely to free inositol and inorganic phosphorus after 72 hours of fermentation. Some phytic acid was hydrolyzed partially to different forms of inositol phosphates which could be separated on anion exchange resins (Saio, 1964; Cosgrove, 1969; Asada et al., 1969). Inositol phosphates produced in oncom fermented by R. oligosporus were separated on a Dowex 1 x 8 ($Cl^-$ form) with 0 to 1.0 N HCl linear gradient elution. The peaks appearing in the chromatogram were identified by calculating the molecular ratio of phosphorus to inositol (Table 6).

As shown in Figure 26, phytic acid ($IP_6$) appeared as a major peak, while inositol tri- ($IP_3$) and tetraphosphates ($IP_4$) were detected as minor peaks. As the fermentation progressed, phytic acid was dephosphorylated to other inositol phosphate forms (inositol mono-, di-, tri-, tetra-, and pentaphosphate) and inorganic phosphorus.

Table 5. Free inositol and inorganic phosphorus released from phytic acid during fermentation of oncom by R. oligosporus

| Ferm. time, hours | Initial phytic acid (mg/g) | Phytic acid lost (mg/g) | Inositol | | |
|---|---|---|---|---|---|
| | | | Released theoretically (mg/g) | Found (mg/g) | Released (%) |
| 0 | 13.60 | 0 | 0 | 0 | 0 |
| 36 | 0.96 | 12.64 | 3.45 | 1.08 | 31.30 |
| 72 | 0.41 | 13.19 | 3.60 | 2.32 | 64.44 |

| Ferm. time, hours | Initial phytate P (mg/g) | Phytate P lost (mg/g) | Inorganic phosphorus | |
|---|---|---|---|---|
| | | | Found (mg/g) | Released (%) |
| 0 | 3.83 | 0 | 0 | 0 |
| 36 | 0.27 | 3.56 | 1.72 | 48.31 |
| 72 | 0.12 | 3.71 | 2.25 | 60.65 |

A scheme of dephosphorylation of phytic acid by phytase was suggested by Tomlinson and Ballou (1962)(Figure 27). These results demonstrate that fermentation by molds, especially R. oligosporus, improves the nutritive value of peanuts by lowering their phytic acid level. However, it is not known whether these inositol phosphate forms also reduce bioavailability of minerals.

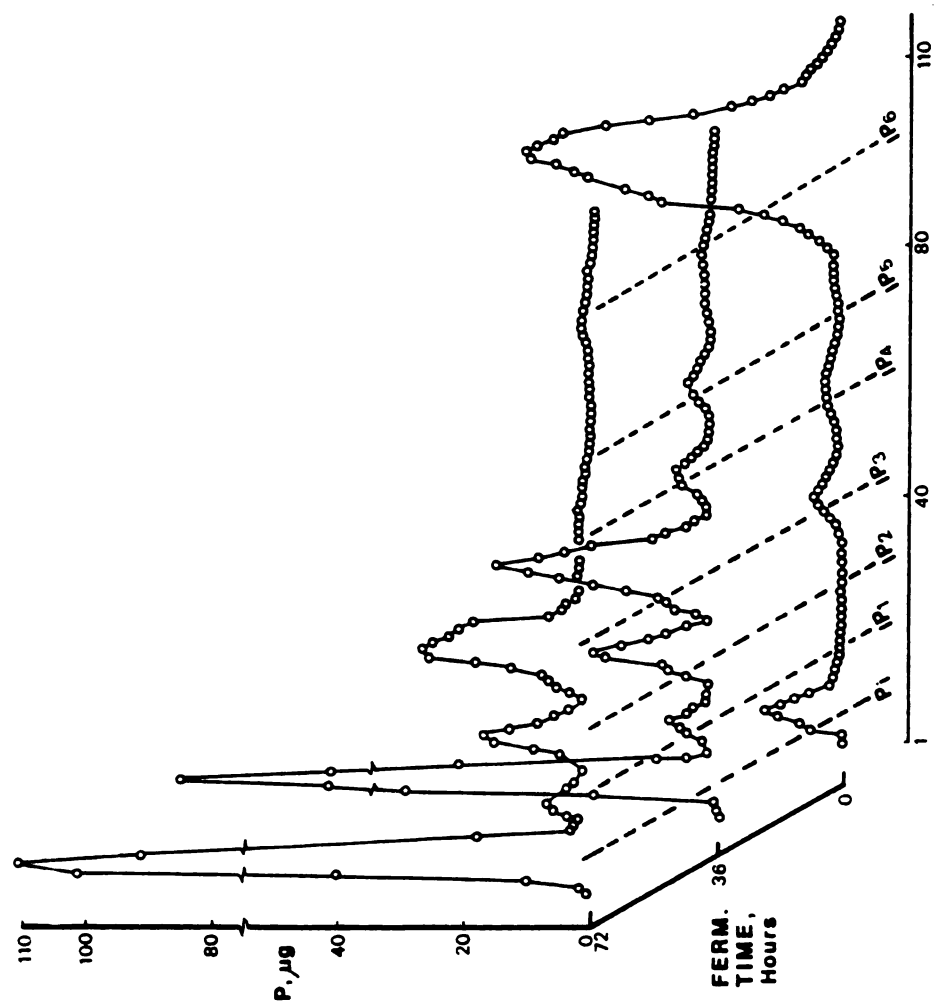


Figure 26. Elution pattern of inositol phosphates on Dowex 1x8 ($Cl^-$) column.

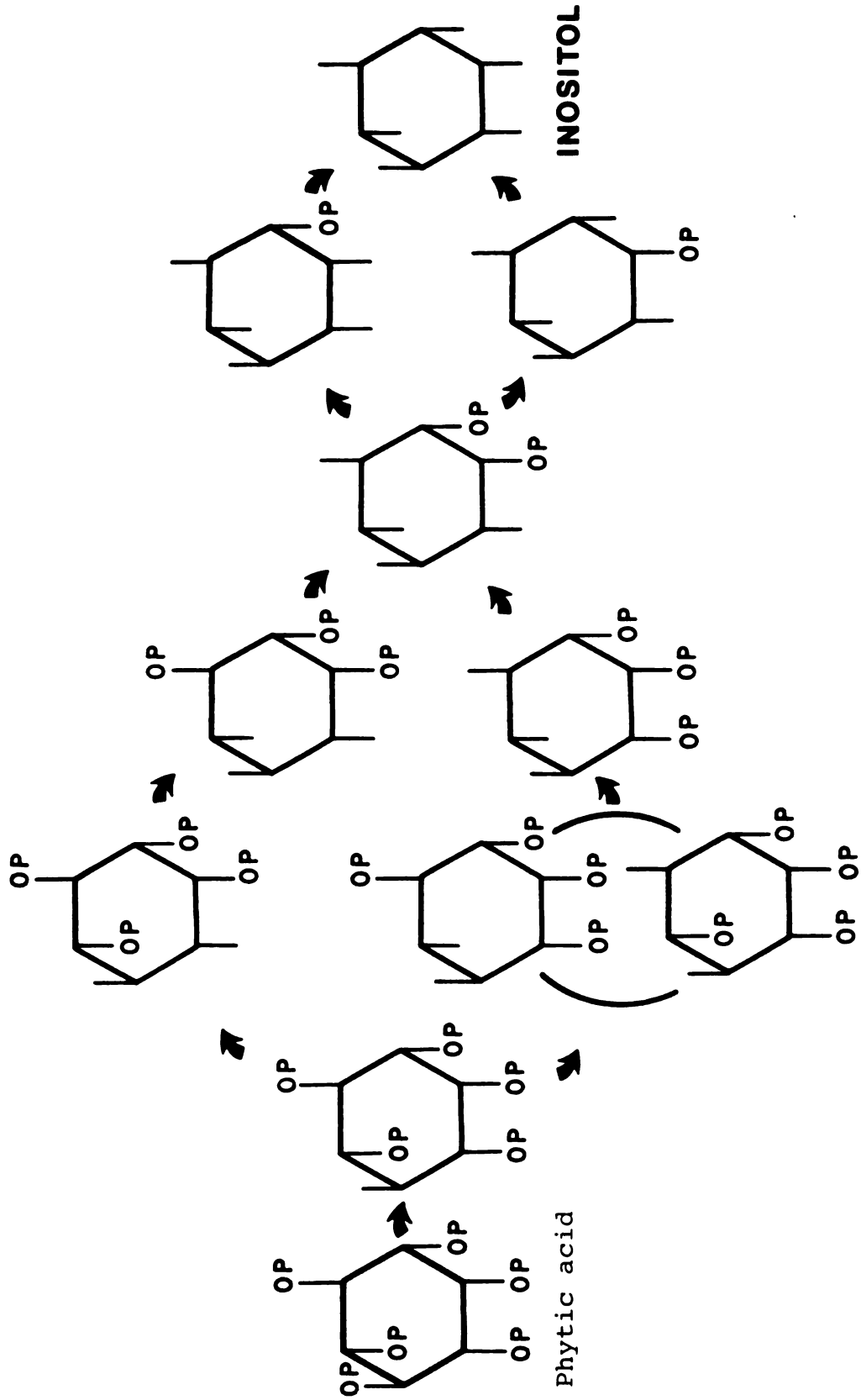


Figure 27. Scheme of dephosphorylation of phytic acid by phytase (Tomlinson and Ballou, 1962).

Table 6. Ratio of phosphorus to inositol in fractions obtained from Dowex 1x8 ($Cl^-$) chromatography[a]

| Fraction # | Phosphorus (μg) | Inositol (μg) | μmole P/ μmole inositol | | Inositol phosphates identified[b] |
|---|---|---|---|---|---|
| | | | Observed | Theory | |
| 4- 10 | 125.1 | ND[c] | - | - | Inorganic phosphorus |
| 13- 18 | 38.7 | 225.0 | 1.00 | 1 | $IP_1$ |
| 23- 31 | 101.2 | 303.4 | 1.94 | 2 | $IP_2$ |
| 34- 48 | 201.4 | 423.3 | 2.76 | 3 | $IP_3$ |
| 52- 59 | 150.6 | 224.8 | 3.89 | 4 | $IP_4$ |
| 63- 79 | 210.1 | 257.6 | 4.73 | 5 | $IP_5$ |
| 82-107 | 418.1 | 416.2 | 5.83 | 6 | $IP_6$ |

[a] Sample was oncom fermented by R. oligosporus for 12 hours.

[b] Inositol mono- ($IP_1$), di- ($IP_2$), tri- ($IP_3$), tetra - ($IP_4$), penta- ($IP_5$), and hexaphosphate ($IP_6$).

[c] Not detected.

## Biological Evaluation of Protein Quality

The composition of peanut press cake, oncom, and fermented mixtures of peanut press cake and sesame flour is shown in Table 7. On a dry weight basis, the protein contents of peanut press cake and oncom are practically the same, 46.6 and 46.5%, respectively. This indicates that fermentation did not affect the total protein content of the cake.

The PER values and apparent digestibility of the

Table 7. Composition of peanut press cake, oncom, and fermented mixtures of peanut press cake and sesame flour

| | Moisture (%) | Fat (%) | Protein (%) | Fiber (%) | Ash (%) | Carbohydrate (%, by difference) |
|---|---|---|---|---|---|---|
| Peanut press cake (P) | 8.8 | 15.3 | 42.5 | 2.7 | 3.2 | 27.5 |
| Oncom | 9.3 | 15.4 | 42.2 | 2.8 | 3.4 | 26.9 |
| Fermented mixture: | | | | | | |
| P + sesame (9:1) | 8.5 | 13.8 | 43.2 | 3.0 | 3.3 | 28.2 |
| P + sesame (8:2) | 8.3 | 12.6 | 42.8 | 2.8 | 3.5 | 30.0 |

experimental diets are shown in Table 8. The PER and apparent digestibility of oncom did not differ significantly ($P < 0.05$) from those of peanut press cake. This indicates that fermentation did not improve protein quality of raw materials. These results were in agreement with those reported by van Veen et al. (1968).

However, when peanut protein was supplemented by 10% sesame protein, the PER of fermented product was improved significantly by 50%. There was no further improvement when the level of added sesame protein was increased to 20%. The improvement is probably due to the high methionine content of sesame, since this amino acid is

Table 8. Protein efficiency ratio (adjusted to casein PER = 2.50) and digestibility of casein, peanut press cake, oncom, and fermented mixtures of peanut press cake and sesame flour

| | PER ($\bar{x} \pm$ SEM) | Apparent digestibility | |
|---|---|---|---|
| | | Diet ($\bar{x} \pm$ SEM) | Nitrogen ($\bar{x} \pm$ SEM) |
| Casein | 2.50±0.05 | 93.1±0.8 | 89.8±0.8 |
| Peanut press cake (P) | 1.51±0.07[a] | 86.7±1.3[a] | 79.8±0.5[a] |
| Oncom | 1.41±0.08[a] | 85.3±1.2[a] | 80.0±0.6[a] |
| Fermented mixture: | | | |
| P + sesame (9:1) | 2.11±0.06[b] | 84.9±0.6[a] | 79.4±0.4[a] |
| P + sesame (8:2) | 2.13±0.06[b] | 84.6±0.8[a] | 79.1±0.5[a] |

SEM = standard error of means; Means with the same letter are not significantly different at P = 5% (Tukey's test).

limiting nutritional value of peanut protein (Table 9). On the other hand, sesame is low in lysine and this amino acid may become limiting when the proportion of sesame protein reaches a certain level in a mixture of sesame and peanut. The supplementary effect of sesame protein on legume protein has been shown by Boloorforooshan and Markakis (1979) and Akpapunam and Markakis (1980).

There were no changes in the digestibility of either the diet or the protein as a result of the supplementation. Therefore, poor protein quality of oncom and peanut press cake was mainly due to a poor over-all amino acid

Table 9. Essential amino acids in peanut (Arachis hypogaea) and sesame seed (Sesamum indicum) proteins, expressed as g per 16 g N[a]

| Amino acids | Peanut | Sesame seed |
|---|---|---|
| Isoleucine | 3.4 | 3.6 |
| Leucine | 6.4 | 6.7 |
| Lysine | 3.5 | 2.7 |
| Methionine + Cysteine | 2.4 | 4.6 |
| Phenylalanine + tyrosine | 8.9 | 7.6 |
| Threonine | 2.6 | 3.6 |
| Tryptophan | 1.0 | 1.3 |
| Valine | 4.2 | 4.6 |

[a] FAO, 1970

balance. These results suggest that the protein quality of oncom can only be improved by supplementation with other protein sources.

## Carotenoids of N. sitophila

Numerous species of molds are reported to produce carotenoids. Neurospora crassa which produces a complex mixture of carotenoids was often used in studies on carotenoid biosynthesis (Goodwin, 1954). On the other hand, N. sitophila which is also believed to produce carotenoids is rarely mentioned in a literature. This experiment was

done to investigate whether the carotenoids produced by N. sitophila have vitamin A value.

N. sitophila ATCC 14151 was grown on potato dextrose agar in ten Roux culture bottles at 30°C for 7 days. The yield of mycelia and conidia harvested from all culture bottles was 58.2 g wet weight. As shown in Table 10, five carotenoids extracted from mycelia and conidia of N. sitophila were identified as phytofluene, neurosporene, β-zeacarotene, γ-carotene, and β-carotene. The identification of these carotenoids was based on their UV and visible absorption spectra in hexane. Their absorption maxima were the same as those reported by Foppen (1977). The absorption spectra of the N. sitophila carotenoids are presented in Figure 28 and 29. Neurosporene, β-zeacarotene, γ-carotene, and β-carotene as illustrated, absorbed in the visible region, indicating that the red or orange color appeared in conidia of the mold might be due to the presence of these carotenoids.

Phytofluene, neurosporene, β-zeacarotene, and γ-carotene were found as intermediate products in the biosynthesis of β-carotene from mevalonic acid (Davies, 1973). Phytoene and ζ-carotene which were also reported as intermediate products in the biosynthesis of β-carotene were not detected.

The concentration of carotenoids identified above is shown in Table 11. Among the five carotenoids found in

Table 10. Identification of carotenoids in N. sitophila

| Carotenoids identified | Absorption maxima in hexane (nm) | | | |
|---|---|---|---|---|
| Phytofluene | reported[a] | 331 | 347 | 366 |
| | observed | 330 | 347 | 367 |
| Neurosporene | reported | 416 | 440 | 470 |
| | observed | 416 | 439 | 469 |
| β-zeacarotene | reported | 400 | 425 | 450 |
| | observed | 400 | 425 | 450 |
| γ-carotene | reported | 431 | 462 | 494 |
| | observed | 430 | 460 | 492 |
| β-carotene | reported | 425 | 450 | 476 |
| | observed | 425 | 448 | 475 |

[a] Foppen (1971).

N. sitophila, only γ- and β-carotenes were detected in oncom sample. Both of these carotenoids have vitamin A value. Based on calculations, 100 g of oncom only contains 1.92 retinol equivalents, where 0.42 and 1.50 retinol equivalents are contributed by γ- and β-carotenes, respectively. Since the recommended daily allowance for the adult man is 1000 retinol equivalents, the oncom carotenoids have little nutritional significance as provitamin A.

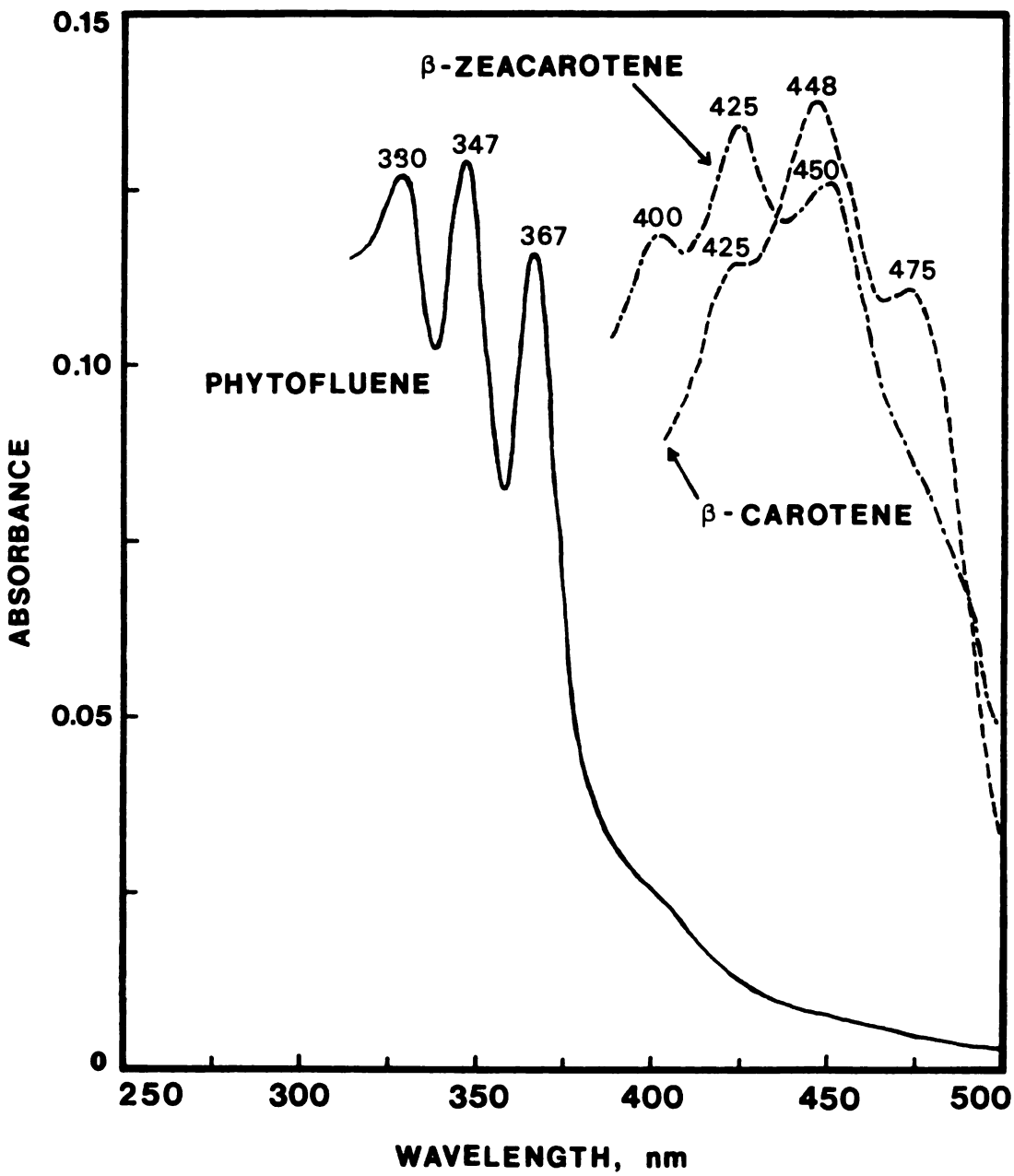


Figure 28. Absorption spectra of phytofluene, β-zeacarotene, and β-carotene extracted from N. sitophila.

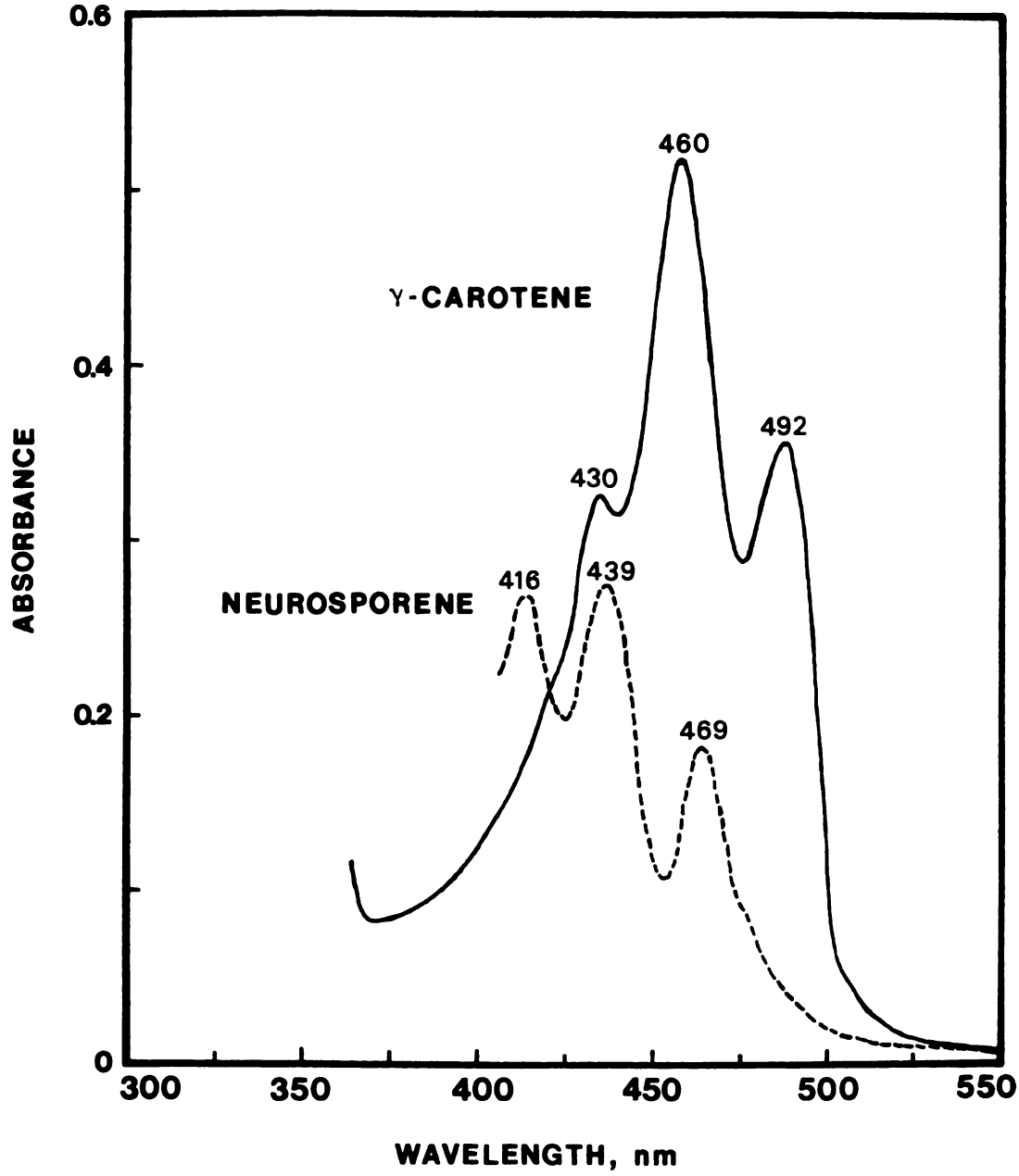


Figure 29. Absorption spectra of neurosporene and γ-carotene extracted from N. sitophila.

Table 11. Concentration of carotenoids found in mycelia and conidia of N. sitophila ATCC 14151 and oncom sample

| Carotenoids | $E^{1\%}_{1cm}$ [a] | N. sitophila ATCC 14151 | | | Oncom sample | | |
|---|---|---|---|---|---|---|---|
| | | Abs.[b] | Vol (ml) | Conc. (µg/ 100g wet wt.) | Abs. | Vol (ml) | Conc. (µg/ 100g wet wt.) |
| Phytofluene | 1100 | 0.32 | 25 | 125 | ND[c] | - | - |
| Neurosporene | 2768 | 0.16 | 25 | 25 | ND | - | - |
| β-zeacarotene | 1940 | 0.48 | 25 | 106 | ND | - | - |
| γ-carotene | 3100 | 1.90 | 25 | 263 | 0.14 | 5 | 5 |
| β-carotene | 2592 | 1.10 | 50 | 365 | 0.25 | 5 | 9 |

[a] Foppen (1971).

[b] Measured at absorption maxima.

[c] Not detected.

# CONCLUSIONS

The pH of the fermenting oncom increased gradually from about 5.1 to 7.2 in 72 hours. Oncom seemed to be ready for consumption when pH was about 6.5-6.8, or after 36 hours fermentation by R. oligosporus or 48 hours fermentation by N. sitophila.

During fermentation, free fatty acids were liberated from triglycerides as a result of lipase released by the molds. Approximately 40% of the peanut oil was hydrolyzed by R. oligosporus, while only 10% by N. sitophila after 72 hours of fermentation. The order of fatty acid liberation was oleic, linoleic, palmitic, and stearic acids followed by minor fatty acids.

During fermentation with either R. oligosporus or N. sitophila the sucrose, raffinose, and stachyose contents of peanut press cake decreased. Raffinose and stachyose are thought to be involved in flatulence and their reduction in oncom is a desirable effect.

The soluble protein content of oncom fermented by either R. oligosporus or N. sitophila gradually increased. Electrophoresis of the soluble protein fraction of oncom showed protein hydrolysis.

During fermentation, about 95% of the phytic acid was hydrolyzed by R. oligosporus, while only about 50% by N. sitophila. Apparently the phytase activity of R. oligosporus is much stronger than that of N. sitophila. About 60-65% of the phytic acid was hydrolyzed completely to inorganic phosphorus and free inositol, while the remaining phytic acid was hydrolyzed partially to other inositol phosphate forms.

Fermentation did not change the protein content, apparent digestibility and protein quality of raw materials. Therefore, the protein quality of oncom can only be improved by supplementation with other protein sources. Incorporation of 10% of sesame protein raised the PER of peanut press cake from 1.51 to 2.11.

The mycelia and conidia of N. sitophila contained phytofluene, neurosporene, β-zeacarotene, γ-carotene, and β-carotene. However, only γ- and β-carotenes were detected in oncom. Both of these carotenoids have vitamin A value, but the concentration of these carotenoids was too small to have nutritional significant as provitamin A.

In general, Neurospora sp. isolated from Indonesian oncom was not very different from N. sitophila ATCC 14151 in its effect on the oncom fermentation. Using a mixed culture of N. sitophila and R. oligosporus in oncom preparation resulted in considerable decrease in both oligosaccharides and phytic acid content.

For future study, it is recommended to include different protein sources as supplements to improve the protein quality of oncom. Typical oncom flavor produced during fermentation has not been analyzed; therefore, the study on flavor of oncom and its relation to sensory evaluation would be interesting. To encourage local oncom producers in the village area, a study on a simple, rapid and hygienic method of oncom preparation would be helpful. Preparation of oncom using a mixed culture of R. oligosporus and N. sitophila should be introduced to produce oncom which is low in both oligosaccharides and phytic acid.

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# APPENDIX

# Appendix 1

Table A1. Linear regression equations of the methyl ester standard curves

| Methyl esters | Conc. range (μg) | Linear regression equations |
|---|---|---|
| Me-myristate | 0- 1.5 | Y = 5.3805 X - 0.4448 (r = 0.9870) |
| Me-palmitate | 0- 6.0 | Y = 6.9830 X - 0.6514 (r = 0.9925) |
| Me-stearate | 0- 4.5 | Y = 5.5158 X + 0.0661 (r = 0.9948) |
| Me-oleate | 0-22.5 | Y = 4.7111 X + 5.2933 (r = 0.9854) |
| Me-linoleate | 0-22.5 | Y = 3.7101 X + 0.4019 (r = 0.9977) |
| Me-arachidate | 0- 4.5 | Y = 5.0738 X - 0.5069 (r = 0.9925) |
| Me-linolenate | 0- 4.5 | Y = 1.9667 X - 0.3000 (r = 0.9923) |
| Me-behenate | 0- 4.5 | Y = 5.1583 X - 0.6375 (r = 0.9922) |
| Me-lignocerate | 0- 4.5 | Y = 3.6190 X - 0.2143 (r = 0.9977) |
| Me-heptadecanoate (internal standard) | 0- 8.5 | Y = 8.5715 X - 0.9027 (r = 0.9991) |

# Appendix 2

Table A2. Recovery of phytic acid added to the control sample[a]

| Sample | PA in control sample (mg) | Added PA[b] (mg) | Total PA (mg) | PA found (mg) | % Recovery |
|---|---|---|---|---|---|
| Control | 12.05 | - | - | - | - |
| Control + 10 mg NaPhy | 12.05 | 5.24 | 17.57 | 16.57 | 95.84 |
| Control + 20 mg NaPhy | 12.05 | 10.47 | 22.52 | 21.37 | 94.89 |

[a] Average of three determinations.

[b] PA = phytic acid = 52.37% of standard NaPhy.