# Technical

## Lupin Protein Concentrates by Extraction with Aqueous Alcohols

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### ABSTRACT

In order to remove the toxic quinolizidine alkaloids and other nonprotein constituents, hexane-defatted flakes of lupin (*Lupinus mutabilis*) were extracted under various conditions with ethanol, methanol or their aqueous solutions. Lupin protein concentrates containing more than 70% protein and 0.1-0.2% alkaloids were obtained in high yields by consecutive extractions, countercurrent extraction or semicountercurrent extraction of the defatted lupin flakes with either 80% ethanol or 80% methanol.


### INTRODUCTION

The lupin has great potential as a valuable oilseed crop in regions of temperate climate (1). Several species of lupin, such as *Lupinus albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis*, are being cultivated in many countries, not only for the oil but also for the protein in the seed (2). *L. mutabilis*, e.g., is a major crop in the Andean regions of Bolivia, Chile and Peru, which is currently being exploited for the production of edible oil (3).

The lupin protein, for which composition and nutritional properties are well documented, has so far found limited use in foods for humans and livestock, mainly due to the occurrence of some toxic quinolizidine alkaloids in the lupin seeds (1). The major alkaloids of the lupin are lupanine, isolupanine, hydroxylupanine and sparteine. A minor part of these alkaloids is recovered together with the oil during conventional extraction with hexane and, subsequently, the alkaloids are removed from the oil by refining. A major part of the alkaloids is retained in the defatted lupin flakes, which, due to their toxicity, find very restricted use in foods and feed formulations. Breeding and cultivation of suitable varieties of "sweet lupin" with low alkaloid content are still at an experimental stage (4). Therefore, a process currently is being sought for the removal of alkaloids from hexane-defatted flakes of common "bitter lupin."

We were given the task by the Gesellschaft für Technische Zusammenarbeit GmbH (GTZ), Eschborn, Germany, to explore the technique of solvent extraction using ethanol, methanol and their aqueous solutions for the removal of alkaloids and other nonprotein constituents from defatted lupin flakes with the aim of obtaining nontoxic protein concentrates.

### EXPERIMENTAL PROCEDURES

#### Materials

All reagents were of analytical grade. For extraction, technical grade solvents, purified by distillation, were used throughout.

Seeds of *L. mutabilis*, grown in Chile, were provided by R. Gross, Deutsche Gesellschaft für Technische Zusammenarbeit, GTZ, Instituto de Nutrición, Lima, Peru. The lupin seeds were processed as follows in the pilot plants of Holtz & Willemsen GmbH, Krefeld, Germany. The seeds were coarsely disintegrated by a single passage through cracking rolls and the ground seeds were treated with live steam at 0.3 atm for 15 min. This material was then subjected to toasting and dehulling in fluidized bed equipment (5). The resulting meal was adjusted to 18% moisture content by treatment with live steam and made into flakes (max. 1 mm thick) by a single passage through stone rolls. The lupin flakes were then defatted with hexane at 50-60 C in large Soxhlet extractors; the solvent adhering to the flakes was removed at 40 C in an oven with forced air circulation. The defatted lupin flakes, for which composition is given in Table I, were used in all extraction studies.

#### Analytical Methods

Official methods of the AOCS were used for determination of moisture (6), total nitrogen (7) and lipids (8). Protein content was calculated as (total nitrogen - alkaloid nitrogen) x 6.25.

Total quinolizidine alkaloids in the lupin flakes were determined by the following modification of a rapid method that has been described recently (9). For a fast quantitative extraction of the alkaloids, 1 g of the ground sample was sonicated with 25 ml chloroform for 10 sec at 60 W using the micro tip of a Branson Sonifier (Branson Sonic Power Co.). After the addition of 1 ml 15% potassium hydroxide solution, the mixture was sonicated again for 10 sec. Three g of basic aluminium oxide (activity grade super I, Woelm Pharma GmbH & Co., Eschwege, Germany) were added to the mixture, which was sonicated once again for 10 sec. The mixture was then filtered through porous glass (G-3) filter, washed with chloroform and the extract concentrated to a volume of 2-3 ml. The total alkaloids in the extract were determined by titration with a solution of *p*-toluenesulfonic acid in chloroform using the potassium salt of tetrabromophenolphthalein ethylester as indicator.

#### Extraction Procedures

Single extractions were done as follows. Portions of 20 g defatted lupin flakes were taken in 200-ml glass-stoppered Erlenmeyer flasks. Definite volumes of ethanol, methanol or their aqueous solutions of varying concentration were added and the mixture was shaken for different periods in a temperature-controlled water bath using a gyratory shaker

TABLE I

**Analysis (Dry Weight Basis) of Lupin Seed and Defatted Flakes**

| Constituent (%) | Whole seed | Defatted flakes |
|---|---|---|
| Protein[a] | 42.5 | 53.4 |
| Lipids | 19.8 | 1.1 |
| Alkaloids | 4.2 | 3.2 |

[a](Total N − alkaloid N) x 6.25.

(Type WTR-1, Infors AG, Basel, Switzerland) at 250 rpm. At the end of the extraction, the extract was rapidly separated from the flakes by suction on a heated Buchner funnel. The total extract or an aliquot thereof was evaporated to dryness in a rotary evaporator and weighed, in order to determine the dry matter extracted. The extracted flakes were dried at 40-50 C in an oven provided with forced air circulation and analyzed for alkaloid and nitrogen contents.

Three consecutive extractions of 20-g portions of defatted lupin flakes were done in an identical manner as the single extractions. After each extraction, the residual flakes, without prior drying, were transferred to the Erlenmeyer flask and reextracted with fresh solvent.

Three-stage countercurrent extractions of 60-g portions of defatted lupin flakes, divided into batches of 20 g each, were done according to the scheme outlined elsewhere (10). The same procedure was followed as for single extractions. At each stage, the first of the three batches of flakes was extracted with the fresh solvent and the resulting extract was used for extraction of the subsequent batch, and so on. In each of the following stages, the first batch from the previous stage was placed at the end of the series.

Batches of 2 kg defatted lupin flakes were extracted with aqueous ethanol and methanol in a large-scale extractor, shown schematically in Figure 1. The extractor, a cylindrical stainless steel vessel with a capacity of 20 ℓ, was provided with a heating mantle, a manually operated agitator and inlet as well as outlet for the solvent or extract. In each extraction, the required amount of solvent was added to the extractor and the defatted lupin flakes were loosely stacked into the solvent bed on top of a sieve plate (1 mm × 1 mm holes) that was located at the bottom of the extractor. Care was taken to prevent the entrainment of air bubbles at the flake-solvent interface. The lid was closed and the extractor was rapidly heated to a temperature of 60 C with occasional agitation. The solvent pump was started in order to circulate the solvent downward through the bed of the flakes at a flow rate of 300 ℓ/hr. The extraction was stopped after 1 hr and the extract separated from the flakes. After cooling, the flakes were removed, dried in an oven at 50 C with forced air circulation and weighed.

Three consecutive extractions of 2-kg batches of the defatted lupin flakes were done essentially as just described. After each extraction, the extract was separated and the residual flakes were reextracted with fresh solvent.

Three-stage and five-stage countercurrent extractions of 2-kg batches of the defatted lupin flakes were done in the same extractor according to a scheme outlined elsewhere (10). The extraction procedure was similar to that already described. Several tanks were used for storage of the intermediate extracts while the batches were being transferred.

Three-stage semicountercurrent extractions of 2-kg batches of the defatted lupin flakes were done, as already described, according to a scheme given in Figure 2. After the processing of seven batches, the residual flakes were combined, weighed and analyzed.

## RESULTS AND DISCUSSION

Processing of the whole lupin seeds in a pilot plant yielded defatted flakes, for which composition, in comparison to that of the whole seed, is given in Table I. It is evident from the figures on alkaloid content that some of the alkaloids of the lupin seeds are removed by extraction with hexane.

In order to find a suitable process for the removal of alkaloids from defatted lupin flakes and enrichment of the proteins contained therein, 20-g portions of the defatted

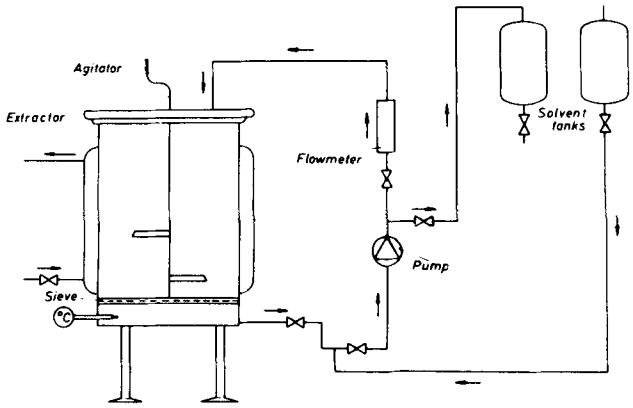


**FIG. 1. Schematic diagram of the large-scale batch extractor.**

flakes were extracted with ethanol, methanol and their aqueous solutions under various conditions.

Table II shows the effect of concentration of ethanol and methanol on the extraction of alkaloids, dry matter and protein from the defatted lupin flakes. It can be seen that distinctly higher proportions of alkaloids and dry matter are extracted with methanol and its aqueous solutions than with ethanol at the corresponding concentrations. A slightly higher proportion of proteins is extracted with 100% ethanol than with 100% methanol, however, at concentrations of both 90 and 80%, markedly higher proportions of proteins are extracted with methanol than with ethanol. With both solvents, a decrease in concentration results in an increase in the proportion of alkaloids and dry matter extracted. An increase in the proportion of proteins extracted with decreasing concentration of the solvent is observed with methanol and also with ethanol, at least when the concentration is lowered from 90 to 80%. On the basis of these data, a concentration of 80% ethanol or 80% methanol is considered to be suitable for the extraction of alkaloids and dry matter with a moderate loss of protein.

Table III shows the effect of the ratio of solvent to

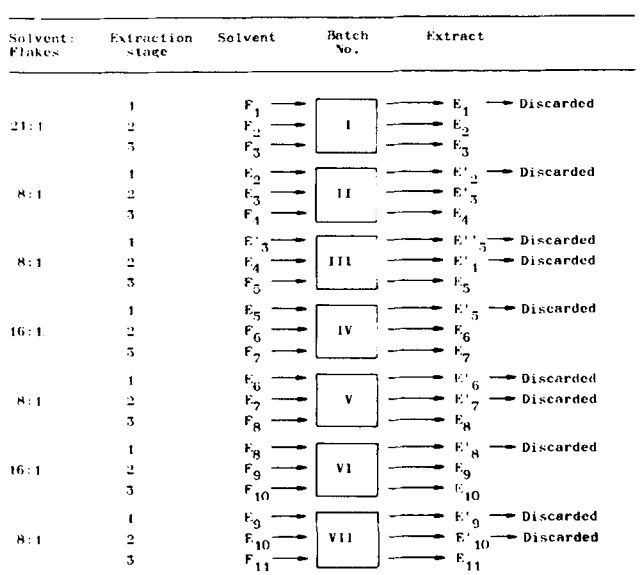


**FIG. 2. Scheme of three-stage semicountercurrent extraction of 2-kg batches of lupin flakes with 80% aqueous methanol. $F_n$ = Fresh solvent, $E_n$ = first extract, $E'_n$ = extract reused once, $E''_n$ = extract reused twice, n = 1, 2, 3 . . . .**

**TABLE II**

**Single Extraction[a] of Defatted Lupin Flakes with Ethanol and Methanol at Different Dilutions with Water**

| Item (%) | Ethanol (%) | | | Methanol (%) | | |
|---|---|---|---|---|---|---|
| | 100 | 90 | 80 | 100 | 90 | 80 |
| Alkaloids extracted[b] | 5.1 | 52.5 | 62.7 | 57.6 | 71.2 | 72.9 |
| Dry matter extracted[b] | 6.1 | 10.9 | 16.6 | 16.9 | 24.0 | 25.7 |
| Protein extracted[b] | 4.6 | 0.9 | 4.1 | 3.9 | 7.0 | 9.3 |
| Alkaloid content of extracted flakes[c] | 3.2 | 1.7 | 1.4 | 1.6 | 1.2 | 1.1 |
| Protein content of extracted flakes[c] | 54.2 | 59.4 | 61.4 | 61.7 | 65.3 | 65.2 |

[a]Conditions of extraction: 20 g lupin flakes; solvent:flakes, 8:1 (v:w); temperature 60 C; time 20 min.

[b]Percentage of alkaloids, dry matter and protein, respectively, in the defatted flakes.

[c]On a dry weight basis.

flakes on the extraction of alkaloids, dry matter and protein from defatted lupin flakes using, as an example, 80% ethanol as solvent. It is evident that the extent of alkaloids and dry matter extracted is distinctly increased by increasing the ratio of solvent to flakes, whereas the ratio of solvent to flakes has only a minor effect on the proportion of proteins extracted.

The effect of time on the extraction of alkaloids, dry matter and protein from defatted lupin flakes was studied using 80% ethanol as solvent. The results show that increasing the time beyond 20 min does not alter the extent of extraction of alkaloids and dry matter to any appreciable extent. A slight decrease in the extent of proteins extracted with increasing time was observed, which may be attributed to a reduction in solubility due to denaturation.

Consecutive extractions of 20-g portions of defatted lupin flakes with 80% ethanol or 80% methanol were done with the objective of assessing the number of extractions that may be required to obtain products with very low alkaloid and high protein contents. At the same time, the course of extraction of alkaloids, dry matter and proteins during each extraction was studied. The results, given in Table IV, show that three consecutive extractions with 80% ethanol or 80% methanol at a solvent-to-flakes ratio of 8:1 in each extraction yield products containing as little as 0.4 and 0.2% alkaloids and as much as 67.6 and 72.2% of proteins, respectively. Thus, well over 90% of the alkaloids are removed by extraction with either solvent whereas the loss of proteins is only 6.8% with 80% ethanol and 4.0% with 80% methanol. It should be noted, though, that the solvent requirement is fairly high in consecutive extractions. Thus, a solvent-to-flakes ratio of 8:1 in each extraction adds up to a ratio of 24:1 when three consecutive extractions are done.

**TABLE III**

**Single Extraction[a] of Defatted Lupin Flakes with Aqueous Ethanol at Different Ratios of Solvent to Flakes**

| Item (%) | 80% Ethanol:flakes (v:w) | | |
|---|---|---|---|
| | 5:1 | 8:1 | 12:1 |
| Alkaloids extracted[b] | 53.0 | 62.7 | 71.8 |
| Dry matter extracted[b] | 12.8 | 16.6 | 19.3 |
| Protein extracted[b] | 2.4 | 4.1 | 4.1 |
| Alkaloid content of extracted flakes[c] | 1.7 | 1.4 | 1.1 |
| Protein content of extracted flakes[c] | 61.0 | 61.4 | 63.6 |

[a]Conditions of extraction: 20 g lupin flakes; temperature 60 C; time 20 min.

[b]Percentage of alkaloids, dry matter and protein, respectively, in the defatted flakes.

[c]On a dry weight basis.

**TABLE IV**

**Three Consecutive Extractions of Defatted Lupin Flakes with Aqueous Alcohols**

| Item (%) | 80% Ethanol | | | | 80% Methanol | | | |
|---|---|---|---|---|---|---|---|---|
| | Number of extractions | | | | Number of extractions | | | |
| | 1[a] | 2[a] | 3[a] | 3[b] | 1[a] | 2[a] | 3[a] | 3[b] |
| Alkaloids extracted[c] | 57.6 | 81.7 | 91.7 | 95.1 | 63.4 | 84.7 | 95.2 | 98.1 |
| Dry matter extracted[c] | 16.0 | 22.6 | 26.2 | 29.0 | 17.1 | 24.2 | 27.6 | 31.4 |
| Protein extracted[c] | 4.6 | 6.6 | 6.8 | 3.2 | 1.9 | 2.4 | 4.0 | 3.1 |
| Alkaloid content of extracted flakes[d] | 1.6 | 0.8 | 0.4 | 0.2 | 1.4 | 0.6 | 0.2 | 0.1 |
| Protein content of extracted flakes[d] | 60.6 | 64.7 | 67.6 | 72.9 | 64.0 | 69.1 | 72.2 | 72.6 |

[a]Conditions of extraction: 20 g lupin flakes; solvent:flakes, 8:1 (v:w) for each extraction; temperature 60 C; time 10 min for each extraction.

[b]Conditions of extraction: 2 kg lupin flakes; solvent:flakes, 8:1 (v:w) for each extraction; flow rate of recycling solvent 300 ℓ/hr; temperature 60 C; time 1 hr for each extraction.

[c]Percentage of alkaloids, dry matter and protein, respectively, in the defatted flakes.

[d]On a dry weight basis.

**TABLE V**

**Countercurrent and Semicountercurrent Extractions of Defatted Lupin Flakes with Aqueous Alcohols**

| Item (%) | Three-stage countercurrent | | | Five-stage countercurrent | Three-stage semicountercurrent |
|---|---|---|---|---|---|
| | 80% Ethanol[a] | 80% Methanol[a] | 80% Ethanol[b] | 80% Ethanol[b] | 80% Methanol[b] |
| Alkaloids extracted[c] | 87.6 | 85.2 | 81.5 | 87.5 | 96.0 |
| Dry matter extracted[c] | 17.5 | 23.9 | 28.5 | 22.4 | 33.0 |
| Protein extracted[c] | 1.2 | 0.8 | 8.2 | 3.5 | 9.8 |
| Alkaloid content of extracted flakes[d] | 0.5 | 0.6 | 0.6 | 0.5 | 0.1 |
| Protein content of extracted flakes[d] | 64.8 | 70.7 | 68.8 | 66.7 | 71.9 |

[a]Conditions of extraction: 60 g lupin flakes; solvent:flakes, 8:1 (v:w) for the entire extraction; temperature 60 C; time 10 min for each extraction.

[b]Conditions of extraction: 2 kg lupin flakes; solvent:flakes, 8:1 (v:w) for the three-stage and five-stage countercurrent extractions and 12:1 (v:w) for the three-stage semicountercurrent extraction; flow rate of recycling solvent 300 ℓ/hr; temperature 60 C; time 1 hr for each extraction.

[c]Percentage of alkaloids, dry matter and protein, respectively, in the defatted flakes.

[d]On a dry weight basis.

Countercurrent extractions of defatted lupin flakes were done with the objective of ensuring maximal removal of alkaloids at a least solvent requirement. The results of three-stage countercurrent extraction of 60-g portions of flakes, divided into three batches of 20 g each, with 80% ethanol and 80% methanol, each at a solvent-to-flakes ratio of 8:1, are summarized in Table V. It is evident that the proportions of alkaloids and dry matter removed by three-stage countercurrent extraction (Table V) approach those attained by three consecutive extractions (Table IV). Surprisingly, the proportion of proteins extracted with both 80% ethanol and 80% methanol is remarkably lower in the countercurrent extraction (Table V) than in three consecutive extractions (Table IV). In the extracted flakes obtained by three-stage countercurrent extraction, the level of alkaloids was slightly higher and the protein content somewhat lower than in those obtained by three consecutive extractions (Tables IV and V).

In order to assess the reproducibility of data obtained from small-scale extractions in the processing of larger quantities, batches of 2-kg defatted lupin flakes were subjected to consecutive extractions, countercurrent extraction and semicountercurrent extraction with aqueous ethanol and methanol. The large-scale extractor (Fig. 1) chosen resembled a stationary batch extractor, which has recently been installed in Peru for the processing of lupin flakes in a pilot plant scale.

The results of three consecutive extractions of 2-kg batches of defatted lupin flakes with 80% ethanol and 80% methanol are included in Table IV. It is evident that with both solvents, the proportions of alkaloids and dry matter extracted is distinctly higher and the proportion of proteins extracted markedly lower in the large-scale extraction than in the extraction of small batches. The flakes obtained by large-scale extraction contain as little as 0.1-0.2% alkaloids and well over 70% proteins.

Three-stage and five-stage countercurrent extractions of 2-kg batches of defatted lupin flakes were done with 80% ethanol only. The results, included in Table V, show that, in comparison to three-stage countercurrent extraction of small batches, somewhat lower proportions of alkaloids are removed and markedly higher proportions of both dry matter and proteins are extracted in the processing of 2-kg batches. Increasing the number of extraction stages to five increases the proportions of alkaloids removed but both the proportions of dry matter and proteins extracted are decreased. Both three-stage and five-stage countercurrent extractions of the 2-kg batches yield flakes with similar alkaloid content as those obtained in extraction of small batches, but the protein content is somewhat higher in the large-scale extractions.

Although from the viewpoint of solvent economy, countercurrent extraction in a continuous manner should be the method of choice in industrial processing, rather sophisticated equipment is required for such a process. Therefore, an attempt was made to optimize a semicountercurrent extraction of defatted lupin flakes, a process which could be readily adapted to plants employing conventional batch extractors.

Three-stage semicountercurrent extraction of 2-kg batches of defatted lupin flakes was done according to the scheme given in Figure 2. Analyses of pooled samples of flakes from seven successive batches of extraction gave the average data summarized in Table V. It is evident that by semicountercurrent extraction with 80% methanol using, on an average, a solvent-to-flakes ratio of 12:1, as much as 96% of the alkaloids can be extracted with a concomitant loss of ca. 10% of the proteins. The extracted flakes contain, on an average, only 0.1% alkaloids and as much as 72% proteins.

Our studies show that defatted lupin flakes prepared by conventional processing with hexane can be converted to products having low alkaloid and high protein contents by extraction with aqueous ethanol and methanol. Although the residual alkaloid content of these lupin protein concentrates is slightly higher than that of sweet lupin (11), it should be possible to use such concentrates in admixture with other proteins, such as cereals and legumes. Further work is being directed toward nutritional properties and possible applications of such protein concentrates.

## ACKNOWLEDGMENTS

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# The Lipids of Various Fungi Grown on an Artificial Medium

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## ABSTRACT

The lipids extracted from various fungi belonging to the genera *Aspergillus, Fusarium* and *Penicillium* cultivated on Davis medium were studied. The fatty acids from fungal lipids were fractionated by gas liquid chromatography (GLC) and the main fatty acids were palmitoleic, oleic, stearic, linoleic and arachidic. The results demonstrated that the fatty acid composition of various fungi could be used as criteria for fungal taxonomy. The unsaponifiable matter of the fungi could be divided into two fractions, i.e., hydrocarbons and sterols. The hydrocarbon fraction constitutes an important part because its amount ranged from 30.14 to 80.97% according to the fungal species. The sterol fraction of the unsaponifiable part of fungal lipids was much simpler in composition. Analysis by GLC indicated that sterol composition could be used to differentiate among fungi belonging to different genera as well as among species belonging to one genus.

## INTRODUCTION

The lipid industry in Egypt suffers from a shortage of oils, and, therefore, great attention has been given to finding alternative sources instead of relying on classical oil crops. Fungi can be used as a source of lipids. They need a limited space for cultivation, the time required to reach maximal lipid yield is ca. 2-3 weeks and the expense is much less. Also, some fungi can be used to produce arachidonic acid, one of the essential fatty acids and the precursor of prostaglandins, by culturing them on a hydrocarbon or carbohydrate medium containing fatty acids such as linoleic, linolenic or oleic acids (1). However, further research is needed on the culture conditions, including the temperature, pH and nutrient concentration. Obviously, the selection of fungi is an important factor, because some fungi can produce mycotoxins.

Previous work in our laboratory (2) showed the deleterious effect of some fungal species on the lipids of deliberately infected corn oil. This work was done to determine the lipid composition of such fungi. In addition, the work was extended to demonstrate that the changes in corn oil previously reported were due to fungal effects and not to the inherent lipids of various fungi.

## MATERIALS AND METHODS

### Culture Medium

The fungi were artificially cultured on Davis medium (3) which has the following composition (g/ℓ): 300 g sucrose, 0.5 g $MgSO_4$, 3 g $KNO_3$ and 7 g yeast extract.

### Isolation of Fungi

Fungi were isolated from infected corn kernels (4).

### Preparation of Spore Suspensions, Inoculation and Incubation

Spore suspensions were prepared from 15-day-old pure cultures of *Aspergillus flavus, A. melleus, A. nidulans, A. niger, Fusarium moniliforme* and *Penicillium oxalicum.* One ml spore suspension of each fungus was inoculated into 250-ml, flat-bottomed flasks containing 100 ml Davis medium and incubated at 30 C for 15 days.

### Sources of Standard Materials

A set of standard fatty acids 10:0, 11:0, 12:0, 13:0, 14:0, 16:0, 18:0, 18:1, 18:2, 18:3 and 20:0 with stated purity of 99% by gas liquid chromatography (GLC) was purchased from Nu-Chek-Prep. Pure, saturated hydrocarbons (*n*-eicosane, *n*-docosane, *n*-triacontane and *n*-dotriacontane), cholesterol, campesterol, stigmasterol and β-sitosterol were Sigma grade. The purity of each standard compound was checked by GLC and gave one peak.

### Extraction and Preparation of Fatty Acids and Unsaponifiables

The fungal growth was separated from the medium by filtration, then washed several times with distilled water and dried at 60 C under vacuum. Lipids were extracted by treating the dried fungal growth with a methanol chloroform mixture (1:2, v/v) and blending the mixture in a Waring blender (5). Lipids were saponified with methanolic potassium hydroxide (20%, w/v) overnight at room temperature. The unsaponifiables were extracted three times with petroleum ether (40/60 C). The combined extract was washed several times with distilled water and dried over anhydrous sodium sulfate. The fatty acids were freed from their potassium salts with sulfuric acid solution (5 N), then extracted with petroleum ether. The petroleum ether extract containing the fatty acids was washed three times with distilled water and then dried over anhydrous sodium sulfate. Fatty acids and unsaponifiables were methylated using a diazomethane ethereal solution (6).