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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).

Separation of Methyl Esters of Fatty Acids and Unsaponifiables by GLC

The methyl esters of the fatty acids, unsaponifiables and authentic compounds were analyzed with a GCV Pye Unicam gas chromatograph equipped with dual flame ionization detectors and dual channel recorder. Separation of fatty acid methyl esters was done using a coiled glass column (1.5 m x 4 mm) packed with Diatomite C (100-120 mesh). The column was operated isothermally at 170 C with nitrogen at 30 ml/min. Detector and injection temperatures were 220 and 200 C, respectively. The unsaponifiables methyl esters were also separated on a coiled glass column (1.5 m x 4 mm) packed with Diatomite C (100-120 mesh) and coated with 1% OV-17. The column was maintained at 270 C and the flow rate of nitrogen was 45 ml/min. Detector and injection temperatures were 300 and 320 C, respectively.

Identification and Determination of Fatty Acids and Unsaponifiables

Peak identification was performed by comparing the relative retention times of each with those of standard materials. The relative retention times for methyl stearate and β-methyl sitosterol are given a value of 1.00. The linear relationship between log retention times of the standard fatty acids or hydrocarbons and the number of carbon atoms of these compounds was used to characterize the odd-carbon fatty acids or the unavailable authentic hydrocarbons (C_{21}, C_{23}, C_{24}, C_{25}, C_{26}, C_{27}, C_{28}, C_{29} and C_{31}). The peak area was measured by triangulation and the percentage of the fatty acids and unsaponifiables were calculated as (area of each peak/area of all peaks) x 100.

RESULTS AND DISCUSSION

Table I shows the fatty acid composition of the lipids of various fungi. Myristic acid was present in all fungi except *A. melleus*. Palmitic acid, stearic acid and arachidic acid occurred in all fungi. Docosanoic acid was present in only two fungi, *A. melleus* and *A. nidulans*. The odd-carbon fatty acids (11:0, 15:0 and 17:0) were found in some fungi. Even-carbon unsaturated fatty acids (16:1, 18:1 and 18:2) were found in all fungi studied. Oleic acid was found as a major fatty acid in all fungi. Similarly, linoleic acid is present as a major fatty acid in all fungi except in *P. oxalicum* in which it occurs as a minor constituent. The odd-carbon unsaturated fatty acid (15:1) was found as a trace component only in *A. nidulans* and *P. oxalicum*.

These results indicate that the variability in fatty acid composition of various fungi largely depends on the fungal

TABLE I

Fatty Acid Composition (%) of Lipids of Various Fungi

Fatty acid	*A. flavus*	*A. melleus*	*A. nidulans*	*A. niger*	*P. oxalicum*	*F. moniliforme*
10:0	---	0.26	0.20	---	0.4	---
11:0	---	---	---	---	---	2.90
14:0	0.50	---	0.15	0.60	0.40	1.90
15:0	0.05	---	0.12	0.30	0.20	1.40
15:1	---	---	0.70	---	0.80	---
16:0	15.80	13.90	19.80	20.50	36.50	0.90
16:1	2.00	3.80	3.30	3.40	4.20	8.20
17:0	0.40	---	0.20	0.50	0.20	---
18:0	17.60	4.80	11.70	15.10	14.40	5.20
18:1	25.10	23.90	39.40	34.50	33.70	30.80
18:2	15.62	19.90	12.60	18.40	5.60	35.10
20:0	22.93	30.80	9.40	6.70	3.60	13.60
22:0	---	2.64	2.43	---	---	---

TABLE II

Composition (%) of the Unsaponifiables of Various Fungi

Component	RRT[a]	*A. flavus*	*A. melleus*	*A. nidulans*	*A. niger*	*F. moniliforme*	*P. oxalicum*
C-21	0.063	---	---	---	6.00	---	---
C-22	0.071	---	33.29	30.30	20.30	40.00	---
C-23	0.107	8.79	---	---	---	0.80	---
C-24	0.116	11.62	2.71	2.27	---	0.80	---
C-25	0.119	---	---	---	1.69	---	---
C-28	0.169	27.63	27.41	22.73	---	5.60	64.22
C-29	0.232	4.71	1.02	1.52	---	0.53	---
C-30	0.250	---	1.36	1.52	0.51	0.53	---
C-31	0.313	2.51	---	---	---	---	5.55
C-32	0.446	3.92	4.41	2.53	1.25	---	7.39
C-33	0.518	1.88	---	---	---	---	3.70
Cholesterol	0.643	6.44	12.20	4.55	---	28.33	3.70
Campesterol	0.830	4.40	8.14	29.53	28.26	---	2.77
Stigmasterol	0.893	1.88	6.10	---	---	3.20	0.55
Unknown	0.946	---	---	---	---	14.09	---
β-Sitosterol	1.000	21.98	3.36	5.05	34.81	3.72	8.32
Unknown	1.214	---	---	---	---	2.40	3.70
Unknown	1.411	4.24	---	---	6.23	---	---
Hydrocarbons (%)		61.06	74.47	60.07	30.14	48.26	80.97
Campesterol: Stigmasterol ratio		1:5	1:0.4	1:0.17	1:1.23	---	1:3

[a]Relative retention time for β-sitosterol (retention time = 7.4 min) taken as 1.00.

species and genera. Hence, the fatty acid composition might be applied as an additional tool together with classical microscopical examination for the identification of fungi. For instance, *Fusarium* had a higher concentration of linoleic acid (35.1%) than other genera, *Penicillium* was characterized by a higher concentration of palmitic acid (36.5%) than others. Moreover, the ratio between the most common fatty acids could be used as an index to identify fungal species belonging to a given genus. For example, the (18:0):(18:1) ratios for the *A. flavus, A. melleus, A. nidulans* and *A. niger* were: 0.7:0.2:0.3:0.44, respectively. Also, the (18:1):(18:2) ratios were 1.61:1.20:3.13:1.88 for the species just listed, respectively. A variation among the species of *Aspergillus* genus has been demonstrated (7). Thus, 16:1 and γ-linoleic acids were present in *A. niger* and not in *A. flavus*. These results and ours support the idea of using the fatty acid composition in the identification of fungi belonging to a particular genus.

The fatty acid composition of various fungi is quite different from that of corn oil extracted from deliberately infected embryos (2). Thus, some short chain fatty acids (8:0 and 10:0) were found in infected corn oil but not in the fungi. Furthermore, the infected corn oil did not contain arachidic acid which was found as a major constituent in every fungus.

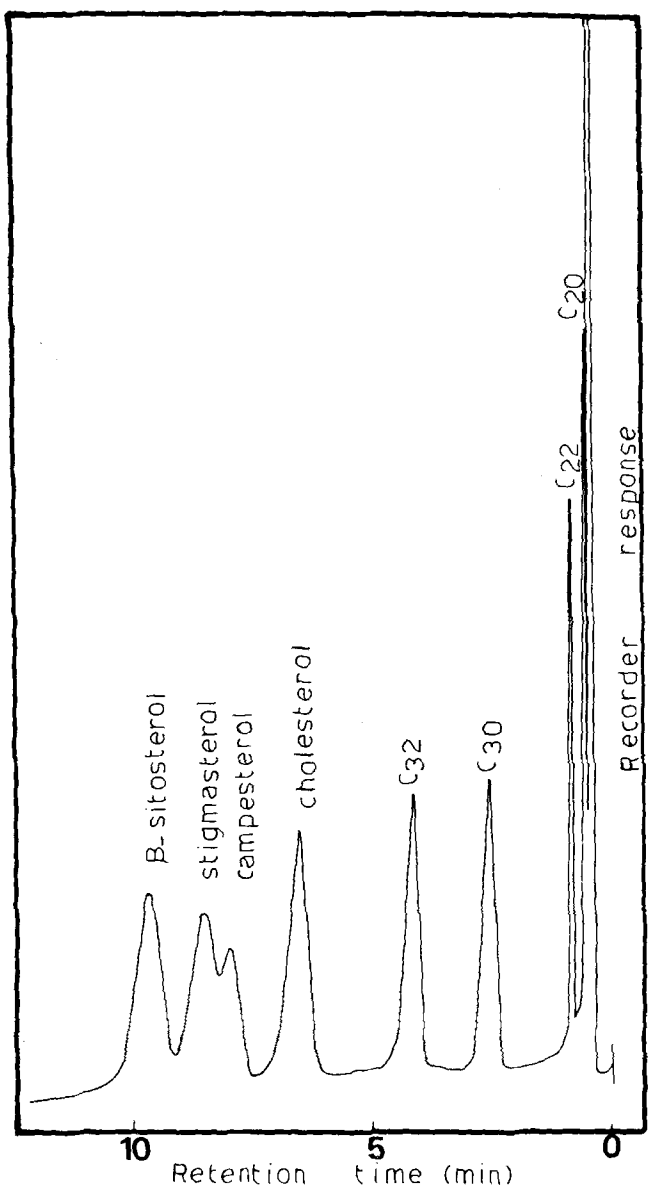

FIG. 1. Gas liquid chromatogram of the standard materials.

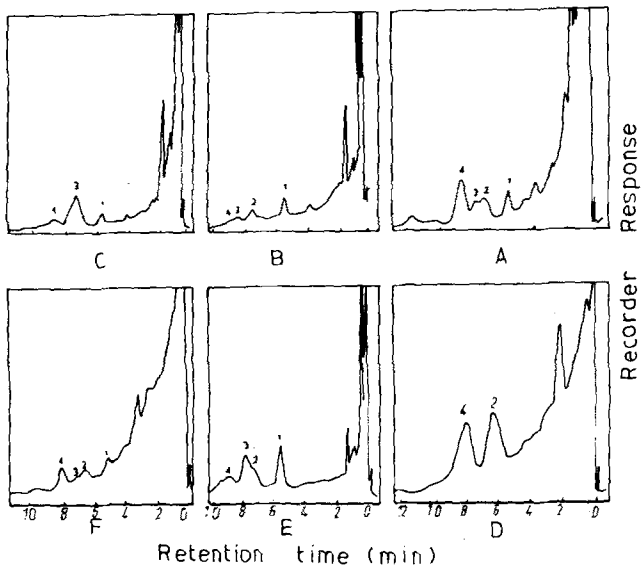

FIG. 2. Chromatograms of the unsaponifiable matter of various fungi. A: *A. flavus*; B: *A. melleus*; C: *A. nidulans*; D: *A. niger*; E: *F. moniliforme*; F: *P. oxalicum*. 1: cholesterol; 2: campesterol; 3: stigmasterol; 4: β-sitosterol.

GLC was also used to study the unsaponifiable matter composition of the fungi used in this investigation, and the results are shown in Table II. Figure 1 shows the standard chromatogram which was used to characterize the unsaponifiable components of the various fungi. Each chromatogram of the fungal unsaponifiables (Fig. 2A, B, C, D, E and F) could be divided into two parts. The first part, the hydrocarbon fraction, constituted 30.14–80.97% of the unsaponifiable matter. The second part of each chromatogram presents the sterol fraction of the fungal unsaponifiables.

The results in Table II show that the distribution of hydrocarbons largely depends on the fungal species. For instance, *n*-eicosane and *n*-pentacosane were present only in *A. niger* whereas *n*-octacosane was detected in all fungi except *A. niger*. Similar conclusions can be made for other fungi.

The sterol fraction of the unsaponifiable part of the fungal lipids was much simpler in composition. Four sterols were found in the fungal unsaponifiables, i.e., cholesterol, campesterol, stigmasterol and β-sitosterol. Also, the data in Table II indicate that sterol composition varies markedly with the type of fungus. In other words, the analysis of the fungal sterols could be a useful method to characterize the various fungi. For instance, the *Fusarium* genus is characterized by a higher concentration of cholesterol (28.33%) than the other genera. Also, the ratio between campesterol and β-sitosterol could be used to differentiate between *Aspergillus* and *Penicillium* genera. The campesterol:β-sitosterol ratio for *P. oxalicum* was 1:3. This ratio was higher than the ratios for *A. niger* (1:1.23), *A. nidulans* (1:0.17) and *A. melleus* (1:0.4) and was lower than the ratio for *A. flavus* (1:5). Also, the campesterol:β-sitosterol ratios for the species of the *Aspergillus* genus were quite different. Hence, sterol composition could be used to characterize various fungi belonging to one genus.

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⁂Estimation of Solid-Liquid Ratios in Bulk Fats and Emulsions by Pulsed Nuclear Magnetic Resonance

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ABSTRACT

Several pulsed nuclear magnetic resonance (NMR) methods were evaluated to estimate the solid fat content of fats and oil-in-water emulsions. The methods were checked with samples of paraffin oil or triolein containing known quantities of crystalline tristearate. A method based on the signal of solid fat (with use of a correction factor, the "*f*-factor") was rejected in this work for general use. Correct results were obtained with methods that used only the signal of the liquid phase. With emulsions, disturbances could arise due to the surfactant present and to possible solubilization of water in the oil phase, presumably by monoglycerides. Without these disturbances, solid fat content in emulsions could be estimated as in bulk fats, after correction of the liquid phase signal for the contribution of protons from the aqueous phase. The signal from fat crystals inside emulsion droplets differed from that of crystals of the same fat in bulk, which may have been due to difference in crystal size but not to difference in crystal modification. Measurements on natural cream showed that disturbances were also possible in this type of emulsion.

INTRODUCTION

Knowledge of the solid fat content of fats and emulsions is important in the food industry as well as in research. Of the methods available for measuring the solid fat content, pulsed nuclear magnetic resonance (NMR) is the most promising (1,2). The method is based on the difference in spin-spin relaxation time T_2 of hydrogen nuclei in the solid and liquid states, on the assumption that differences in proton content of solid and liquid fats are negligible (as is usually the case for natural fats).

The magnetization decay after a 90° pulse allows estimation of the solid fat content directly at one temperature, but a correction factor (the "*f*-factor") is needed since the signal can only be measured some 10 μsec after the pulse. The solid fat content can also be estimated indirectly by taking the signal of the liquid phase into account at a temperature at which the solid fat is dissolved, analogous to the continuous-wave wide-line NMR method (3).

However, the pulsed NMR method can introduce errors if the *f*-factor is used. The use of a mean *f*-factor is justified when it is estimated for a specific group of fats exposed to the same temperature treatment, e.g., margarine fats (1). Care should be taken, however, not to use this mean *f*-factor for other fats or for fats that are exposed to a different temperature treatment. The *f*-factor depends on adjustment of the equipment and on mobility of protons, hence on fat composition, formation of compound crystals, polymorphism, crystal size and temperature (1,3,4).

An attempt has been made (5) to avoid use of the *f*-factor by extrapolation of the free induction decay after a 90° pulse to time zero. Since the exact shape of a signal after a pulse is unknown, this method should be discouraged. In our opinion, use of this erroneous method caused, at least in part, the large discrepancy with other methods (5).

Estimation of the solid fat content in emulsions is hampered by the contribution of protons of the aqueous phase to the NMR signal. Saturation may offer a possibility of distinguishing the aqueous phase from the oil phase (6). To suppress the water signal significantly, however, fat protons will also be saturated somewhat. Another suggestion was addition of paramagnetic ions, which lower the relaxation time of water so that oil and water protons can be distinguished by their relaxation times (7). Addition of ions to an emulsion, however, could cause its instability. A possibility that remains is to correct the signal of the liquid phase by subtracting the contribution of the aqueous phase. This contribution can be calculated from the fat content of the emulsion when the signal of unemulsified aqueous phase has been measured separately. Trumbetas et al. (8) did so, but they used a very short trigger time (i.e., the time between pulses), namely 100 msec, so that fat signals must also have been partly saturated. Oil signals become saturated when the trigger time is below 800 msec.

The aim of this study was to evaluate several pulsed NMR methods for estimation of the content of solid fat in bulk fats as well as in oil-in-water emulsions containing fat crystals in the dispersed phase. The methods were checked with samples containing oil and tristearate crystals; the content of solid fat in these samples was precisely known since the solubility of tristearate in oil is known.

EXPERIMENTAL PROCEDURES

Materials

Paraffin oil (Ph. Ned. VI, density 860 kg m^{-3}, viscosity 68-81 mPa sec) was obtained from Lamers & Indemans, 's-Hertogenbosch, The Netherlands.

Triolein was supplied by K&K Laboratories Inc., USA (no further specifications available).

Tristearate was kindly supplied by P. de Bruyne and D. Waddington of Unilever Research, Vlaardingen, The Netherlands. We found that 99.3% of the fatty acids in this triglyceride was stearic acid, and the content of mono- and diglycerides was less than 0.5%.

Deuterium oxide (D_2O) was obtained from Biorad Chemicals.