Effect of Extraction Methods on Lipid Yield and Fatty Acid Composition of Lipid Classes Containing γ-Linolenic Acid Extracted from Fungi

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ABSTRACT: The effect of extraction procedures on the lipid yield and fatty acid composition of total lipid and main lipid structures (phospholipids, diacylglycerols, triacylglycerols, free fatty acids, and sterol esters) of fungal biomass (*Mucor mucedo* CCF-1384) containing γ-linolenic acid (GLA) was investigated. Seventeen extraction methods, divided into three groups, were tested: six with chloroform/methanol, five with hexane/alcohols, and six with common solvents or mixtures. The chloroform/methanol procedure (2:1) was selected as standard, where lipid yield (TL/DCW, total lipid per dry cell weight) was 17.8%, considered to be 100% of lipids present. All chloroform/methanol extractions yielded more than 83% recovery of lipids. Use of hexane/isopropanol solvent systems led to a maximum of 75% recovery. The best lipid yield was achieved by a two-step extraction with ethanol and hexane (120%). Extraction efficiency of the other solvent systems reached a maximum of 73%. Triacylglycerols were the main structures of lipid isolated; only methanol-extracted lipid contained 58.5% phospholipids. The fatty acid content of total recovered lipid was variable and depended on both the lipid class composition and the solvent system. GLA concentrations in total lipids isolated by hexane/alcohol procedures (7.3–10.7%) are comparable with classical chloroform/methanol systems (6.5–10.0%). The maximal GLA yield was obtained with chloroform/methanol/*n*-butanol/water/0.1 M ethylenediaminetetraacetic acid (EDTA) (2:1:1:1:0.1, by vol) and after two-step extraction with ethanol and hexane (14.3 and 13.7 g GLA/kg DCW, respectively). The highest GLA content was analyzed in the phospholipid fraction (16.1%) after using chloroform/methanol/*n*-butanol/water/0.1 M EDTA (2:1:1:1:0.1, by vol). Remarkably low concentrations of polyunsaturated fatty acids were determined in the free fatty acid fraction.

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Considerable interest is now focused to the role of polyunsaturated fatty acids (PUFA) in human metabolism. Endogenous γ-linolenic acid (6,9,12,*cis*,*cis*,*cis*-octadecatrienoic acid; GLA) formation, the rate-limiting Δ^6-desaturase metabolite of linoleic acid, is low or impaired in a variety of diseases, including atopic eczema, diabetes, premenstrual syndrome, and so on (1). From this point of view, GLA has a number of possible therapeutic applications and it is an important compound in the medical, pharmaceutical, and food fields. Until recently, GLA has been available in small commercial quantities from certain plant seeds (evening primrose, blackcurrant, borage) (2). Because GLA has long been known to occur in fungi (3), biotechnology would seem to be the obvious route to achieve its large-scale production.

Microbial production of lipids that contain unusual fatty acids with potential commercial applications has mostly been aimed at organism selection and optimization of cultural conditions. Rapid and reliable methods of extraction and purification of PUFA from microbial biomass are required for further development in this area of microbial biotechnology. At the same time, satisfactory treatment must be used to minimize autoxidative degradation and the presence of artifacts. On the other hand, if PUFA are to be used in pharmacological, medical, and food applications, solvents should be selected that are acceptable in terms of toxicity, handling, safety, and cost.

Lipid extraction from biomass is important in the control of microbial lipid preparation. Until now, little attention has been focused on this problem, where most methods applied were originally described for lipid extraction from animal tissues or plant materials. Microbial tissues differ from these materials by composition and cell structure, and problems can arise when applying these classical methods for lipid isolation from such biomass. The choice of a reliable extraction method depends on both the chemical nature of the sample and the type of extract desired. Extraction of membrane-associated lipids, however, requires solvent mixtures that contain alcohols to disrupt the hydrogen bonding and ionic forces between the lipids and proteins.

No single aspect of lipid biochemistry seems to create so much diversity of experimental method as the procedure used for lipid extraction. A major problem that causes much trouble and distortion of the results is lipolysis during lipid extraction and processing. Most lipids are removed by a combination of chloroform and methanol. Bligh and Dyer (4) describe a common procedure in which moist microbial cells,

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directly after harvesting, are extracted by methanol/chloroform (2:1, vol/vol). The other commonly used procedure is to extract dry cells with chloroform/methanol (2:1, vol/vol) (5). After extraction, the solvent system containing lipid is purified with 1% NaCl or KCl, which transfers any nonlipid material that is co-extracted with cell lipids into the aqueous phase. It may, however, also remove some polar and amphiphilic lipids.

For selective removal of particular lipid groups, other solvents may be advocated; for example, hexane/2-propanol (3:2, vol/vol) has been reported as being useful for extraction of phospholipids from animal tissues (6), dimethyl sulfoxide has been applied to extract sterol esters from yeasts (7), and ethyl acetate has been tested for isolation of dihydroxy fatty acids generated enzymatically from arachidonic acid (8). Supercritical CO_2 has been suggested as a useful means for the extraction of lipids from plant seeds (9), and with further work it may be possible to extend technology to the extraction of lipids from microbial sources.

The present study was undertaken to investigate the effect of selected extraction solvent systems and methods on the lipid yield and fatty acid composition of total lipid and of the main lipid classes (phospholipids, diacylglycerols, triacylglycerols, free fatty acids, and sterol esters) isolated from dry fungal biomass. Because similar studies on the lower filamentous fungi have been rather limited, our experiments were done with the aim to compare solvents used in the laboratory with solvents suitable for industrial and human purposes. *Mucor mucedo* CCF-1384, the producer of biologically active GLA (10), was selected for these investigations.

EXPERIMENTAL PROCEDURES

Microorganism. The culture *M. mucedo* CCF-1384 was obtained from the Culture Collection of Fungi, Department of Botany, Charles University (Prague, Czech Republic). It was maintained on Sabouraud agar slants at 4°C.

Fermentation. The inoculum medium for fermentation contained glucose (30 g/L, separately sterilized) and corn steep (15 g/L). The pH was adjusted by addition of 1 M NaOH to a value of 6.0. The strain was cultivated in 500-mL cultivation flasks with 150 mL liquid medium. All flasks were inoculated with a spore suspension in an isotonic solution at a final concentration of $1–2 \times 10^5$ spores per mL and incubated on a rotary shaker (3 Hz frequency) at 28°C for 3 d. The tank reactor experiment was performed in an LH-20 fermentor (L.H. Fermentation, Ltd., Reading, United Kingdom) with a working volume of 12 L medium (the same composition as the inoculum medium) and was inoculated by 750 mL of inoculum (5 flasks). Fermentation conditions were: temperature, 24°C; stirring, 550 rpm; aeration rate, 1.0 vol/vol; mechanical foam removing time, 96 h. The mycelium after fermentation was harvested by filtration, washed with water, and gently dried at 65°C for 15 h.

Lipid isolation. Dry biomass was disrupted and homogenized by addition of sea sand (2 g sand/1 g dry fungal mass) for 30 min. Dried mycelium (2 g) was taken for lipid isolation, and lipid was extracted by the following methods (triplicate standards of dry mycelium were prepared to assess reproducibility; the reproducibility of individual methods was in the range 91–96%): (i) Fungal biomass was extracted two times by 100 mL chloroform/methanol (2:1, vol/vol) for 3 h at laboratory temperature with occasional stirring. After extraction, the mixture was filtered to remove mycelium, and the extracts were collected. KCl 0.9% (1.2-fold of total extract volume) was added, then the mixture was stirred vigorously for 1 min and centrifuged to effect phase separation. The chloroform/lipid-containing layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. (ii) Method is the same as (i), but chloroform/methanol (1:1, vol/vol) was used. (iii) The same as (i), but chloroform/methanol (1:2, vol/vol) was used. (iv) Method is the same as (i), but chloroform/methanol/*n*-butanol/water/0.5 M ethylenediaminetetraccetic acid (EDTA) (2:1:1:1:0.1, by vol) was used and continuously stirred.(v) Method is the same as (i), but chloroform was used as a sole solvent. (vi) Fungal biomass was extracted two times by 100 mL methanol for 3 h at laboratory temperature with occasional stirring. After extraction, the mixture was filtered to remove mycelium, extracts were collected and extracted twice with hexane (ratio 1:1, vol/vol). The hexane/lipid-containing fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. (vii) Fungal biomass was extracted two times by 100 mL hexane/isopropanol (3:2, vol/vol) for 3 h at laboratory temperature with occasional stirring. After extraction, the mixture was filtered to remove mycelium, and extracts were collected. KCl (0.9%) (1.2-fold total extract volume) was added, stirred vigorously for 1 min, and the mixture was centrifuged to effect phase separation. The hexane lipid-containing layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. (viii) Method is the same as (vii), but hexane/isopropanol (4:1, vol/vol) was used. (ix) Method is the same as (vii), but hexane as a sole solvent was used. (x) Method is the same as (vi), but isopropanol was used as a sole solvent. (xi) Fungal biomass was extracted by 100 mL ethanol for 3 h with occasional stirring. After filtration, the biomass was again extracted with 100 mL hexane for 3 h with occasional stirring and then filtered. The ethanol fraction was evaporated, 100 mL hexane and 120 mL 0.9% KCl were added, strongly mixed for 1 min, and the hexane/lipid containing fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. The hexane fraction after the second extraction was mixed with 0.9% KCl (1.2-fold), stirred vigorously for 1 min, and the hexane/lipid-containing fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. (xii) Method is the same as (vi), but acetone was used as a sole solvent. (xiii) Method is the same as (vi), but acetone/benzene/isopropanol (1:1:1, vol/vol/vol) was used. (xiv) Method is the same as (vi), but benzene/methanol (1:1, vol/vol) was used. (xv) Method is the same as (vi), but ethyl acetate was used as a sole solvent. (xvi) Method is the same as (vi), but diethyl ether was used as a sole solvent. (xvii) Method is the same as (vi), but acetonitrile was used as

a sole solvent. The dielectric constants (ε) of individual solvents (at 20°C) are (11): hexane, 1.89; benzene, 2.284; diethyl ether, 4.335; chloroform, 4.806; ethyl acetate, 6.02; *n*-butanol, 17.80; isopropanol, 18.3 (25°C); acetone, 20.7 (25°C); ethanol, 24.3 (25°C); methanol, 32.63 (25°C); acetonitrile, 37.5; and water, 78.54.

Lipid fractionation. Lipid was dissolved in chloroform/methanol (1:1, vol/vol) as a 5% solution and applied to thin-layer chromatography (TLC) plates (Silica Gel 60, 20 × 20 cm, 0.5 mm layer; Merck, Darmstadt, Germany). The plates were developed with the solvent system hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol), and spots were visualized by iodine vapor (12). The lipids separated by TLC were identified by comparison of their R_f values with known standards. Phospholipids, diacylglycerols, free fatty acids, triacylglycerols, and sterol esters were scraped off (iodine vapor and water traces were removed under reduced pressure) and extracted with 50 mL chloroform/methanol (1:1, vol/vol). Silica gel was removed by filtration and washed with 50 mL chloroform/methanol (1:1, vol/vol). The organic phases were pooled, and the solvent was removed by rotary evaporation. The amount of each fraction was determined gravimetrically. Recoveries were usually between 85–90%. The sum of individual lipid classes investigated was considered as 100%.

Fatty acid determination. Fatty acids from total lipids and from lipid structures were released by alkaline hydrolysis (refluxing with 1 M KOH in 95% ethanol for 1 h) and converted into their methyl esters by the method of Morrison and Smith (13). Fatty acid methyl esters were analyzed by gas chromatography (GC CHROM 5; Laboratórni Přistroje, Praha, Czech Republic) on a packed column (1.8 m × 2 mm) with diethyleneglycol succinate (15% w/w + 3% w/w H_3PO_4 on Chromaton N AW DMCS; Supelco, Bellefonte, PA) according to our previous work (12). Identification of the fatty acid methyl ester peaks was performed by authentic standards (Supelco). Methyl heptadecanoate was used as an internal standard to quantitate fatty acid content in the lipids. The degree of fatty acid unsaturation, IU (Δ/mole), was calculated by the formula:

$$IU = [1(\% \text{ monoene}) + (\% \text{ diene}) + (\% \text{ triene})]/100 \quad [1]$$

RESULTS AND DISCUSSION

Extraction of total lipids. Lipids play an important role in the cells, where they occur in the membranes and cytosol. They are heterogeneous compounds with different structures and properties. Due to these attributes, it is difficult to obtain their complete isolation. Pure single lipid classes are soluble in a wide variety of organic solvents, but many of these solvents are not suitable for lipid extraction from tissues or cells. The ideal solvent or solvent system for extracting lipids from cells should be sufficiently polar to remove all lipids from their association with cell membranes or with lipoproteins, but should not react chemically with these lipids. At the same time, the solvent should not be so polar that nonpolar lipids do not dissolve. Generally, nonpolar solvents are usually needed for lipid extraction, where most lipids are adequately dissolved. Nevertheless, their combinations with polar solvents are advantageous, mainly because of dehydration, protein denaturation, and degradation of hydrogen bonds between the lipid complex and proteins. The extractants may also have a function in preventing enzymatic hydrolysis. Increasingly, attention is being given to the potential toxicity of solvents. Finally, the extractability of cells or tissues is variable and depends both on the nature of the cells and of the lipids.

Most extraction solvents used for lipid isolation from cells are based on various mixtures of chloroform and methanol that are similar to those of Bligh and Dyer (4) or Folch *et al.* (5). The method by Folch *et al.* (5) (chloroform/methanol, 2:1, vol/vol) is usually the accepted standard for lipid extraction from biological tissues because of high lipid yields. Therefore, we selected this extraction solvent system as standard, and the other solvent systems used were compared with it. Our experiments showed that 17.8% of total lipid [g lipid/100 g dry cell weight (DCW)] was obtained with chloroform/methanol (2:1), and this value was considered as 100%. Figure 1 clearly shows that chloroform/methanol solvent systems [method (i)–(iv)] recovered more than 83% of lipids. The lipid yield with the extraction mixture of methanol/*n*-butanol/chloroform/0.5 M EDTA [1:1:2:0.1, (iv)] was almost the same as with the standard method (98.9%). Butanol is accepted for increasing lysophospholipids yields, and EDTA is needed for phosphatidylserine extraction (14). Chloroform alone [(v)] extracted 84.8% total lipid. Because chloroform is a good solvent for nonpolar compounds, we can state that the *M. mucedo* strain contains higher amounts of nonpolar lipid structures than polar lipids. Analysis of the total lipid com-

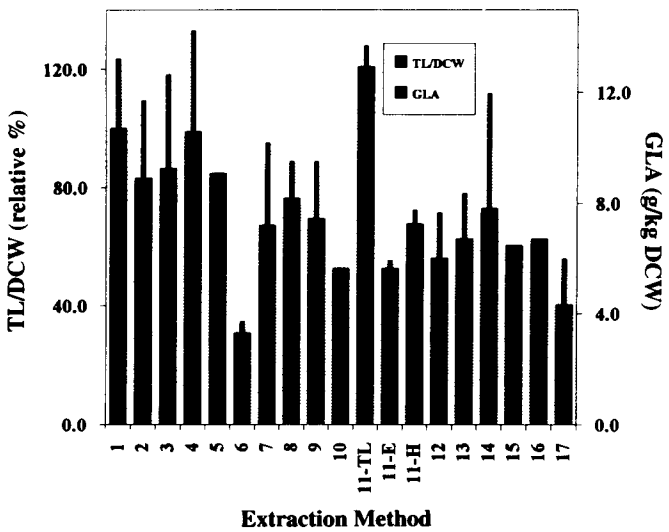

FIG. 1. Effect of various solvents and solvent systems on the lipid and γ-linolenic acid (GLA) yield from dry *Mucor mucedo* mycelium. The chloroform/methanol system [2:1, vol/vol, (i)] was selected as standard, where 17.8% total lipid per dry cell weight (TL/DCW) was considered as 100%. For details, see the Experimental Procedures section (11-TL, total lipid; 11-E, ethanol fraction; 11-H, hexane fraction).

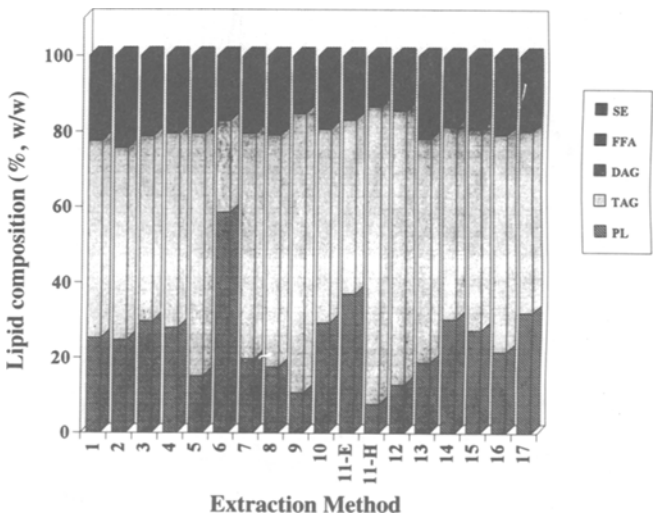

FIG. 2. Composition of individual lipid structures of total lipid recovered after extraction with various solvents and solvent systems from dry *Mucor mucedo* mycelium. The sum of individual lipid classes investigated was considered as 100%. For details, see the Experimental Procedures section (PL, phospholipids; TAG, triacylglycerols; DAG, diacylglycerols; FFA, free fatty acids; SE, sterol esters; 11-E, ethanol fraction; 11-H, hexane fraction).

position showed that triacylglycerols are the main structures of the lipid isolated from *M. mucedo* (Fig. 2). Diacylglycerols, free fatty acids, and sterol esters are minor compounds of neutral lipids. Figure 2 also shows that lipid recovered with chloroform/methanol solvent systems contains more than 50% triacylglycerols and more than 25% polar lipids. Methanol [(vi)], whose polarity is higher than chloroform, extracted only 30.9% lipid. This low lipid yield was due to the variable capability of methanol to extract polar and nonpolar lipid classes. Lipids isolated by methanol consist mainly of polar lipids (58.5%), followed by triacylglycerols (24.4%) and diacylglycerols (12.9%) (Fig. 2). From this point of view, methanol is suitable predominantly for polar compounds and can be accepted for selective extraction of phospholipids from microbial biomass. A similar application of methanol was used by Ramesh *et al.* (15) for animal cells.

The other extractions with hexane/isopropanol solvent systems [methods (vii)–(x)] were tested. These methods are alternatives for chloroform/methanol extractions, and because of their lower toxicity, they have more applications in industrial lipid isolation for human purposes. Hexane/isopropanol mixtures are effective systems for lipid extraction from biological tissues (6,16). However, in contrast to chloroform/methanol experiments, lipid yields from fungal biomass were lower [hexane/isopropanol, 3:2, (vii), 67.4%; hexane/isopropanol, 4:1, (viii), 76.4%], and it seems that a higher ratio of hexane to isopropanol is advantageous. Radin (14) noted that lower lipid yields with hexane/isopropanol solvent systems are caused mainly by reduced isolation of less polar structures. In our experiments with hexane/isopropanol mixtures, we also observed diminished content of phospholipids in total lipid (less than 20%) compared with chloroform/methanol systems (Fig. 2). Hexane alone [(ix)] extracted 69.7% lipids and isopropanol [(x)] isolated only half of the lipid amount (52.8%) compared with the standard method. Figure 2 shows that lipid obtained by hexane consists of 89.3% neutral lipids (74.1% triacylglycerols) and 10.7% polar lipid classes. In contrast to hexane, extraction with isopropanol resulted into a higher concentration of polar structures (29.4%). This was expected due to the different polarities of these two solvents (see dielectric constants in the Experimental Procedures section).

Despite the reduced extraction ability, hexane/isopropanol methods have several advantages compared with chloroform/methanol solvent systems: extracts can be separated from cells by centrifugation (low mixture density); low content of nonlipid structures in the extracts can be used for direct determination on the chromatographic columns without risk of column debasement; extracts contain less pigments (advantage for pigment cell production); and hexane and isopropanol have a long history of safe use in food industry. On the other hand, a higher degree of lipid contamination was observed with hexane/isopropanol. Some enzymes (lipases predominantly) were not inactivated, which caused undesirable reactions (17).

Another hexane/alcohol method was originally described for lower filamentous fungi (genus *Mortierella*) by Suzuki and Yokochi (18). This method is based on the two-step extraction, where in the first step the biomass is extracted by ethanol, and then cells are extracted by hexane (see Experimental Procedures section). Our experiment showed [Fig.1; (xi)] that lipid yield was 20% higher, compared with the standard chloroform/methanol (2:1) method. This is interesting because, after almost 53% of lipid yield from biomass by ethanol, hexane still extracted 68% lipids. However, there were differences in the lipid composition between the first (ethanol) and second (hexane) fractions. Although the ethanol fraction contains larger concentrations of polar lipids (37.1%) compared with hexane extract (7.7%), neutral lipids isolated by ethanol consist of 46% triacylglycerols compared with hexane fraction, where the concentration of triacylglycerols reached a maximum of 78.9% (Fig. 2). It seems that ethanol and hexane can be used for extraction of fungal lipids in the industry. Moreover, due to their low toxicity, they can be applied in the various human fields, such as pharmaceutical, medical, and food areas. Similar good results with these solvents, although on the yeast model, were described by Alexander *et al.* (19) and Davies (20).

Other solvents were tested in our experiments, but total lipid recoveries were lower compared with chloroform/methanol methods (Fig. 1). Acetone [(xii)] extracted only 56.2% of the lipids extracted by the standard chloroform/methanol procedure. However, acetone is used mainly as a selective solvent for nonpolar lipids (87.1%, Fig. 2), and polar lipid residues can be further isolated with more polar solvents. The mixture acetone/benzene/isopropanol [1:1:1, (xiii)] extracted only 62.9% lipids, although Allen (21) stated that this solvent system isolated more lipids than chloroform/methanol (2:1). The advantage of this method is its possible application for wet

cells, as well as for the determination of enzyme activities in cells after lipid isolation. The other extraction method described for yeasts and for microorganisms with firm cell walls is based on the benzene/methanol (1:1) mixture (22). However, from our results it is clear that this solvent system [(xiv)] isolated 73% of the lipid, and it is not suitable for quantitative lipid determination from fungal biomass. Moreover, application of benzene in industry is impossible due to its carcinogenic attributes. Ethyl acetate [(xv)] has similar properties as chloroform, but its extraction effectiveness is low (60.7%). Ethyl acetate was applied for isolation of the dihydroxy fatty acids generated enzymatically from arachidonic acid (8). Diethyl ether also has weak extraction capability [(62.9%, (xvi)], but as a nondenaturation solvent it can be used for the study of enzyme activities either in the absence of lipids or in the presence of certain lipid structures (14). Acetonitrile was the last investigated solvent [(xvii)], and its lipid yield reached only 40.4% compared with the standard chloroform/methanol mixture. From this point of view, acetonitrile is not a convenient extractant for quantitative lipid isolation from fungal biomass. However, this solvent can be used for the direct analyses of some lipid structures by ultraviolet spectra (14).

Finally, we can state that chloroform/methanol mixtures (mainly ratio 2:1, vol/vol) and two-step extraction with ethanol and hexane are the most suitable methods for lipid extraction from dry *M. mucedo* cells. Chloroform/methanol solvent systems are conceivable for use under laboratory conditions, but extraction with ethanol and hexane can find application in industrial lipid isolation for human purposes.

Fatty acid composition of total lipid and isolated lipid structures. The effect of various extraction solvents and mixtures on the fatty acid composition of the total lipid isolated from *M. mucedo* and of the main lipid structures (phospholipids, diacylglycerols, triacylglycerols, free fatty acids, and sterol esters) was further examined.

Total lipid. The nonpolar hydrocarbon chain of fatty acids is one of the main features of lipids that affect their solubility in organic solvents. Their solubility in nonpolar reagents increases with chainlength but decreases with the number of double bonds. Similarly, shorter chainlengths of the fatty acid residues cause their higher extractability in more polar solvents. The effect of extraction methods on fatty acid composition in total lipid is shown in Table 1. From our results it is evident that oleic acid is the dominant fatty acid in the intracellular lipid of *M. mucedo*, and its content varies from 36.6 to 46.6%. Levels of essential GLA were in the range of 6.5–11.5%. Low concentrations of this fatty acid were detected after extraction with chloroform, ethyl acetate, and diethylether. Conversely, values of GLA above 11% were found after using benzene/methanol (1:1) and acetonitrile. The highest degree of fatty acid unsaturation (IU), which expresses the amount of double bonds per length unit of carboxyl chain, was calculated for the extraction with acetonitrile (1.04). However, chloroform alone caused the lowest IU (0.83). It is interesting that, after chloroform extraction, the ratio C_{18} fatty acids/C_{16} fatty acids (C_{18}/C_{16}) was elevated (3.42). These results are in accordance with the polarity of individual solvents or mixtures tested in these experiments. It was confirmed that more polar extractants are suitable for the isolation of more unsaturated fatty acids, and less polar solvents are appropriate for the extraction of fatty acids with longer carbon chains. We can also state that the fatty acid composition of total lipid recovered is affected predominantly by the ratio of the indi-

TABLE 1
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Total Lipid After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	1.9	19.3	5.3	11.3	41.6	11.7	8.9	0.97	2.99
2	1.6	18.6	4.9	9.9	43.1	12.2	9.7	1.02	3.19
3	1.7	19.8	3.7	12.9	39.7	12.2	10.0	0.98	3.18
4	1.6	19.8	4.1	13.4	39.4	12.0	9.7	0.97	3.12
5	1.4	18.5	3.8	18.9	42.3	8.6	6.5	0.83	3.42
6	1.6	20.0	3.8	13.7	40.3	11.4	9.2	0.95	3.13
7	2.1	19.3	4.0	13.5	38.2	12.2	10.7	0.99	3.20
8	2.4	21.2	3.7	14.5	37.8	11.7	8.7	0.91	2.92
9	1.4	19.6	4.4	10.9	41.7	12.4	9.6	1.00	3.11
10	1.6	18.4	3.9	11.0	45.6	12.2	7.3	0.96	3.41
11-E	2.4	20.7	3.5	16.0	39.2	10.0	8.2	0.87	3.03
11-H	3.0	20.7	4.1	17.0	36.6	10.5	8.1	0.86	2.91
12	1.8	19.5	4.5	12.7	40.4	11.2	9.9	0.97	3.09
13	1.7	20.1	4.2	12.0	41.0	11.5	9.5	0.97	3.05
14	1.6	20.5	4.6	11.0	39.9	10.9	11.5	1.01	2.92
15	1.5	20.8	4.3	13.5	42.7	10.6	6.6	0.88	2.92
16	1.5	19.3	3.6	11.9	46.6	10.5	6.6	0.91	3.30
17	2.4	18.3	4.4	9.9	41.9	12.1	11.0	1.04	3.30

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

vidual lipid classes in the total lipid after extraction with various solvents of different polarities (Fig. 2).

The net amounts of GLA, expressed as g GLA obtained from 1 kg of dry mycelium (DCW), were also influenced by the type of extraction solvents and solvent mix-tures. Figure 1 shows that the highest amount of GLA (14.3 g/kg DCW) was isolated with chloroform/methanol/*n*-bu-tanol/water/0.5 M EDTA (2:1:1:1:0.1, by vol) solvent system. The yield of GLA, after employing the other chloro-form/methanol mixtures, decreased in the order chloro-form/methanol (2:1), chloroform/methanol (1:2), and chloroform/methanol (1:1) (13.2, 12.6, and 11.7 g GLA/kg DCW, respectively). Two-step extraction with ethanol and hexane resulted into 13.7 g GLA, which is comparable with the standard chloroform/methanol (2:1) method. Figure 1 also shows that more than 10 g GLA/kg DCW was achieved with the benzene/methanol 1:1 and hexane/isopropanol 3:2 solvent systems (11.9 and 10.2 g GLA, respectively). However, despite the relative high capability of the benzene/methanol (1:1) mixture to extract GLA, possible application of this method is problematic due to the carcinogenic effect of benzene. The lowest GLA yield was obtained with methanol (3.7 g GLA/kg DCW). Finally, from the perspective of human applications of GLA, we can state that a two-step extraction with ethanol and hexane yielded high amounts of GLA. It can be useful for industrial isolation of this essential fatty acid from lower filamentous fungi.

Phospholipids. Phospholipids belong to the polar lipids, and they occur mainly in the cell membranes. Their amount in the intracellular lipid is determined by the polarity of solvent system used. The ratio of phospholipid/total lipid in lipid recovered usually increased with more polar extractants (Fig. 2). Oleic acid is the dominant fatty acid of polar lipid classes (Table 2), although its concentrations were a bit lower than in the total lipids (25.4–40.6%). It is surprising that, after application of methanol, the oleic acid level was lower (25.4%) than stearic acid (37.4%). In contrast to total lipid, the percentage composition of GLA was elevated, but after lipid isolation with diethyl ether and methanol, its concentration dropped (5.8 and 6.1%, respectively). On the other hand, the highest level of this acid (16.1%) was detected with the chloroform/methanol/*n*-butanol/water/0.1 M EDTA (2:1:1:1:0.1) system. Similarly, a high degree of fatty acid unsaturation was observed with the chloroform, isopropanol, acetone/benzene/isopropanol (1:1:1) solvent systems (1.25 for all). Low values of lipid unsaturation analyzed after extraction with methanol (0.56) and diethyl ether (0.75) were due to the small amounts of linoleic acid and GLA. The C_{18}/C_{16} ratio was high after application of acetone (4.01), and the lowest value was calculated for diethyl ether (2.29). From our results it is obvious that the phospholipids contain increased amounts of C_{18} fatty acids when less polar solvents are used. However, it is amazing that the more polar unsaturated fatty acids were better extracted by nonpolar reagents (chloroform, acetone, hexane).

Diacylglycerols. Oleic acid is the main fatty acid in both 1,2- and 1,3-diacylglycerols. In contrast to total lipids, higher levels of palmitic and stearic acids, as well as reduced amounts of di- and triunsaturated fatty acids, were detected (Table 3). The maximum GLA concentration of this fraction was analyzed after lipid isolation with acetonitrile (6.6%), but low values were found with chloroform/methanol (2:1) and ethyl acetate systems (both 1.5%). The degree of lipid unsaturation can be characterized similarly. It is interesting that with chloroform and methanol solvents, which differ greatly

TABLE 2
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Phospholipid Fraction After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	1.7	21.4	4.5	13.6	36.8	12.5	9.5	0.95	2.80
2	2.7	21.4	3.0	13.8	34.1	13.4	11.6	0.99	2.99
3	2.3	20.6	4.0	13.4	34.6	13.3	11.8	1.01	2.97
4	2.4	16.4	3.8	7.2	36.7	17.4	16.1	1.24	3.83
5	1.4	16.3	5.1	4.5	40.2	17.7	14.8	1.25	3.61
6	2.2	22.2	1.1	37.4	25.4	5.6	6.1	0.56	3.20
7	3.8	19.7	3.0	14.9	31.4	13.5	13.7	1.03	3.24
8	4.4	20.1	3.6	13.8	35.3	12.3	10.5	0.95	3.03
9	1.2	18.6	5.2	4.2	40.5	17.3	13.0	1.19	3.15
10	1.5	17.5	4.3	5.0	37.8	18.9	15.0	1.25	3.52
11-E	5.6	18.4	3.0	12.7	32.8	14.2	13.3	1.04	3.41
11-H	6.2	19.3	2.7	19.8	30.7	11.7	9.6	0.86	3.26
12	1.4	15.7	4.0	9.2	37.8	18.5	13.4	1.19	4.01
13	1.4	16.8	5.1	6.1	37.1	18.2	15.3	1.25	3.50
14	1.8	20.3	5.5	8.2	38.2	13.5	12.5	1.08	2.81
15	1.7	20.0	6.8	13.1	35.4	12.4	10.6	0.99	2.67
16	1.9	24.5	5.3	18.3	35.7	8.5	5.8	0.75	2.29
17	1.8	19.6	6.9	16.2	31.7	12.7	13.9	1.06	2.81

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

TABLE 3
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Diacylglycerol Fraction After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	2.8	31.5	5.9	17.9	35.4	5.0	1.5	0.56	1.60
2	6.1	27.5	4.6	15.5	36.2	7.3	2.8	0.64	1.93
3	4.5	27.9	4.5	20.2	32.7	6.9	3.3	0.61	1.95
4	3.1	25.6	5.1	19.0	33.4	9.2	4.6	0.71	2.16
5	1.9	23.3	4.6	12.7	41.8	10.3	5.4	0.83	2.52
6	1.9	24.4	4.1	21.8	34.7	8.4	4.7	0.70	2.44
7	3.3	25.8	5.1	18.6	32.5	9.1	5.6	0.73	2.13
8	3.2	27.4	4.5	20.9	33.6	6.8	3.6	0.63	2.03
9	2.4	24.4	5.5	11.9	39.8	11.2	4.8	0.82	2.26
10	2.4	24.7	5.4	12.1	39.1	11.1	5.2	0.82	2.24
11-E	3.5	26.9	4.2	22.4	31.6	7.4	4.0	0.63	2.10
11-H	4.6	25.8	3.3	27.4	26.9	7.5	4.5	0.59	2.28
12	2.1	23.1	5.0	16.2	37.1	10.6	5.9	0.81	2.48
13	2.0	25.1	4.5	14.2	39.9	9.6	4.7	0.78	2.31
14	2.2	24.7	4.7	13.3	43.0	8.2	3.9	0.76	2.33
15	2.4	29.2	3.6	15.2	41.9	5.7	1.5	0.61	1.96
16	2.3	29.9	1.2	16.5	40.1	6.9	3.1	0.64	2.14
17	2.2	23.3	5.7	11.6	40.0	10.7	6.6	0.87	2.38

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

in polarity, the C_{18}/C_{16} fatty acid ratio was high (2.52 and 2.44, respectively). This ratio was also elevated for acetone (2.48), but a reduced value was determined after application of the standard chloroform/methanol method (1.60).

Triacylglycerols. Triacylglycerols are major but variable components of fungal lipids. They occur as storage lipids and usually form oil granules within cells. In view of their low polarities, nonpolar solvents are generally required for their extraction (Fig. 2). Our experiments showed that oleic acid was again the dominant fatty acid (Table 4). In contrast to total lipids, triacylglycerols are more saturated, which is due to reduced levels of PUFA. The concentration of GLA varied from 4.7 to 7.5%, with exception of methanol, where 10.7% of GLA was detected. The highest degree of fatty acid unsaturation (1.00) was observed as well for the methanol method. The C_{18}/C_{16} fatty acid ratio also depends on the solvent fea-

TABLE 4
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Diacylglycerol Fraction After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	2.6	23.7	3.8	22.4	39.1	2.5	5.9	0.66	2.54
2	2.5	20.6	4.6	17.0	42.7	7.2	5.4	0.78	2.87
3	2.7	23.9	3.9	18.7	40.4	4.9	5.5	0.71	2.50
4	2.4	20.3	3.4	15.4	41.6	9.8	7.4	0.87	3.13
5	1.9	19.5	3.8	14.6	44.1	10.8	5.3	0.85	3.21
6	1.2	17.2	5.1	14.6	39.5	11.7	10.7	1.00	3.43
7	3.4	21.2	4.2	17.1	37.9	8.9	7.3	0.82	2.80
8	3.0	21.2	4.4	15.9	40.7	8.5	6.3	0.81	2.79
9	2.0	20.3	4.8	10.6	47.9	8.6	5.8	0.87	2.90
10	2.2	22.9	4.4	14.0	43.5	8.3	4.7	0.79	2.58
11-E	3.8	22.3	4.1	17.6	42.1	5.0	5.1	0.72	2.64
11-H	4.3	20.0	3.6	20.5	41.2	4.8	5.6	0.71	3.06
12	1.8	18.4	5.3	14.6	42.0	10.4	7.5	0.91	3.14
13	3.9	21.0	3.7	19.2	40.1	7.4	4.7	0.73	2.89
14	1.9	19.9	3.9	10.9	45.3	10.6	7.5	0.93	3.12
15	1.8	18.1	5.5	13.8	42.4	10.8	7.4	0.92	3.15
16	2.0	23.8	3.4	14.7	45.3	5.1	5.7	0.67	2.60
17	2.3	20.3	5.6	15.3	44.6	6.4	5.5	0.80	2.77

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

TABLE 5
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Free Fatty Acid Fraction After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	2.0	24.9	0.3	46.4	20.4	3.2	2.8	0.36	2.89
2	8.4	20.5	0.3	41.7	24.5	2.5	2.1	0.36	3.40
3	11.5	22.8	0.5	44.8	15.3	2.8	2.3	0.28	2.80
4	6.5	24.3	0.1	45.4	19.6	2.2	1.9	0.30	2.83
5	1.3	23.3	0.5	35.3	33.9	5.0	0.7	0.47	3.15
6	1.9	21.5	1.2	38.8	27.2	5.4	4.0	0.51	3.32
7	10.0	22.5	0.2	42.5	19.3	2.5	3.0	0.34	2.96
8	7.4	25.6	0.3	41.2	22.4	1.5	1.6	0.31	2.58
9	1.1	21.0	1.6	30.0	35.7	2.1	8.5	0.67	3.38
10	1.3	24.7	0.4	29.4	39.7	3.0	1.5	0.51	2.93
11-E	8.4	26.9	0.1	45.6	16.6	1.3	1.1	0.23	2.39
11-H	11.6	25.1	0.2	43.1	15.8	2.6	1.6	0.26	2.49
12	1.6	21.2	1.1	38.1	32.2	2.7	3.1	0.48	3.41
13	1.5	22.1	0.9	36.1	26.9	6.6	5.9	0.59	3.28
14	1.2	23.1	0.3	30.3	40.1	4.0	1.0	0.51	3.22
15	1.1	25.1	0.3	34.5	35.9	2.4	0.5	0.43	2.89
16	1.5	25.9	0.7	35.0	32.2	1.2	3.5	0.46	2.70
17	0.9	21.7	0.1	31.7	41.1	3.8	0.7	0.51	3.55

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

ture. Its maximal level was found after application of methanol (3.43), and low values of C_{18}/C_{16} fatty acid ratios were calculated after extraction with chloroform/methanol 2:1 (2.54) and chloroform/methanol 1:2 (2.50) procedures. Because triacylglycerols are the main lipid components of total lipid isolated from *M. mucedo* (Fig. 2), the choice of a reliable extraction method is important from the point of view of PUFA. We can state that concentrations of GLA in the triacylglycerol fraction achieved by hexane/alcohol solvent systems are comparable with chloroform/methanol methods. This notion can be useful for obtaining and producing GLA-containing preparations for pharmaceutical, medical, and food applications.

Free fatty acids. Free fatty acids are usually minor or negligible compounds of total lipids, but sometimes the hydrolysis of lipid structures by active enzymes (lipases especially)

TABLE 6
Fatty Acid Composition, Degree of Fatty Acid Unsaturation (IU), and Ratio C_{18} Fatty Acids to C_{16} Fatty Acids (C_{18}/C_{16}) of the Sterol Ester Fraction After Extraction with Various Solvents and Solvent Systems from Dry *Mucor mucedo* Mycelium[a]

Extraction	Fatty acids (% w/w)							IU	
no.	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$[b]	(Δ/mole)	C_{18}/C_{16}
1	2.9	25.2	1.3	48.9	17.6	2.3	1.8	0.29	2.66
2	2.7	23.2	2.4	39.8	20.3	5.5	6.1	0.52	2.80
3	3.2	24.6	1.9	43.3	19.8	3.0	4.2	0.40	2.65
4	7.6	25.1	0.4	34.6	24.4	3.1	4.4	0.44	2.61
5	2.9	30.9	0.1	33.3	22.6	4.4	5.8	0.49	2.13
6	1.1	20.3	1.7	32.6	34.5	4.9	4.9	0.61	3.50
7	6.6	23.8	1.4	33.3	22.4	6.2	6.1	0.55	2.70
8	6.4	23.1	1.6	30.1	32.5	2.6	3.8	0.51	2.79
9	2.2	19.5	3.8	12.7	46.1	12.7	3.0	0.84	3.20
10	1.9	22.8	2.8	16.9	44.2	7.7	3.7	0.74	2.83
11-E	8.5	21.4	1.4	40.2	23.7	2.5	2.3	0.37	3.01
11-H	10.6	26.4	0.1	43.9	14.4	1.0	2.2	0.23	2.32
12	0.8	22.5	1.2	33.4	30.1	5.1	6.9	0.62	3.19
13	2.1	28.7	0.4	48.5	15.3	2.3	2.7	0.28	2.36
14	2.6	31.4	0.2	26.0	30.2	8.4	1.2	0.51	2.08
15	2.2	28.6	0.2	40.3	23.1	3.8	1.9	0.37	2.40
16	2.0	28.2	0.4	39.8	24.3	3.6	1.7	0.37	2.43
17	2.3	24.3	0.2	35.9	22.7	6.0	8.6	0.61	2.99

[a]For details, see the Experimental Procedures section (11-E, ethanol fraction; 11-H, hexane fraction).
[b]γ-Linolenic acid.

can occur during extraction. In contrast to previous lipid classes isolated, free fatty acid fractions contain stearic acid as the main fatty acid, and there is a remarkably low content of polyunsaturated fatty acids (Table 5). Concentrations of GLA varied from 0.7 to 5.9%, and only after hexane extraction was its highest level (8.5%) detected. The degree of fatty acid unsaturation is also highest with the same solvent (0.67). The C_{18}/C_{16} ratios are comparable with other lipid structures and vary from 2.39 [ethanol fraction from method (xi)] to 3.55 (acetonitrile).

Sterol esters. Stearic acid, as in the free fatty acid fraction, is also the dominant fatty acid of the sterol ester class (Table 6). Oleic acid was found as the main fatty acid only when the hexane [(ix)] and isopropanol [(x)] solvent systems were used. The degree of lipid unsaturation was low again. Its maximum value was analyzed after extraction with hexane (0.84), which was due to the increased linoleic acid amount (12.7%). Concentration of the essential GLA was reduced after using the mixture of benzene/methanol 1:1 (1.2%), but 8.6% GLA was found after acetonitrile extraction. The highest C_{18}/C_{16} fatty acid ratio was determinated after using methanol (3.50), and its minimal values were found for methanol/benzene 1:1 (2.08) and chloroform (2.13) solvent systems.

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