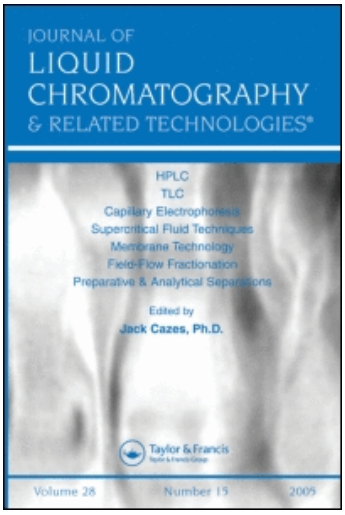

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TLC and GC-MS Probes into the Fatty Acid Composition of some *Lycoperdaceae* Mushrooms

Diana Nedelcheva[a]; Daniela Antonova[a]; Sabina Tsvetkova[a]; Ilko Marekov[a]; Svetlana Momchilova[a]; Boryana Nikolova-Damyanova[a]; Melania Gyosheva[b]

[a] Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria [b] Institute of Botany, Bulgarian Academy of Sciences, Sofia, Bulgaria

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TLC and GC-MS Probes into the Fatty Acid Composition of some *Lycoperdaceae* Mushrooms

Diana Nedelcheva, Daniela Antonova, Sabina Tsvetkova, Ilko Marekov, Svetlana Momchilova, and Boryana Nikolova-Damyanova
Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

Melania Gyosheva
Institute of Botany, Bulgarian Academy of Sciences, Sofia, Bulgaria

Abstract: The efficiency of the successive application of TLC, GC-MS of fatty acid methyl esters and GC-MS of fatty acid 4,4-dimethyloxazoline derivatives in examination of the complex fatty acid mixture in mushrooms is demonstrated on examples of four species of *Lycoperdaceae* family. Twenty seven fatty acids were unambiguously identified in *Bovista plumbea*, *Calvatia utriformis*, *Lycoperdon perlatum*, and *Lycoperdon pyriforme*, grown in Bulgaria. Linoleic (37–65%), oleic (7–24%), palmitic (12–18%), and stearic (2–6%) acids are the major components. The mushrooms contain a characteristic group of three isomeric hexadecenoic fatty acids (double bond in positions 6-, 9-, and 11-), which are resolved and determined separately for the first time. The presence of 9-icosenoic-, 9,12-icosadienoic-, tricosanoic-, pentacosanoic-, hexacosanoic-, and 11-hexacosenoic acids is reported for the first time in Basidiomycetes.

Keywords: Gas chromatography-mass spectrometry, Fatty acids, Dimethyloxazoline derivatives, Puffball mushrooms, *Lycoperdaceae*

Address correspondence to Assoc. Prof. Dr. Boryana Nikolova-Damyanova, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria. E-mail: bmd@orgchm.bas.bg

INTRODUCTION

Mushrooms are a popular part of the human diet not only because of the delicious flavor but also because of the high nutrition value. They are low in calories and fats and high in proteins, vitamins, and minerals.[1,2] In addition, beneficial effects like antitumor, immunomodulating, cardiovascular, and anti-hypercholesterolemic, antiviral, antibacterial, antiparasitic, hepatoprotective, and antidiabetic effects have been described.[3] Mushrooms are reported to have preventing effect in diseases such as hypertension, hypercholesterolemia, atherosclerosis, and cancer, due to their specific chemical composition.[1] However, our knowledge on this composition is far from being complete.

Despite their low content, lipids are primary metabolites and the knowledge of their composition is an important part of the studies on mushrooms. At present, studies on fatty acid composition attract attention. Fatty acids are the main structural element of lipids (see[1,2,4–6] and the references cited therein), and the knowledge of their identity and quantitative proportions provides important characteristics of lipid components present. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) of fatty acid methyl esters (FAME) have been utilized so far.[1,4–6] These useful approaches, however, limit the information to the determination of the chain-length and the overall unsaturation of the component fatty acids. The localization of double bonds remains ambiguous[1,4–6] and, therefore, complete structural analysis of fatty acids is not achieved. More informative is the approach that uses GC-MS analysis of fatty acid derivatives other than methyl esters.[7] Among the several possibilities,[7] conversion of fatty acids in 4,4-dimethyloxazoline (DMOX) derivatives was employed in the present work. While this paper was in preparation, Pedneault et al.[2] reported on the fatty acids of some Boletaceae mushrooms by converting the fatty acids to their picolinyl derivatives prior to GC-MS, and this is the most informative paper on the fatty acid composition of mushrooms published so far.

We present, here, an approach that uses thin-layer chromatography (TLC) to characterize the lipid classes, GC and GC-MS of methyl esters and GC-MS of DMOX derivatives of fatty acids, with the aim to examine in details the fatty acid composition of four edible *Lycoperdaceae* mushroom species (puffballs): *Bovista plumbea* Pers.: Pers.; *Calvatia utriformis* (Bull.: Pers.) Jaap, *Lycoperdon perlatum* Pers.: Pers. and *Lycoperdon pyriforme* Schaeff.: Pers., widespread in Bulgaria. To the best of our knowledge, the fatty acid composition of these species has not been reported as yet.

EXPERIMENTAL

All reagents and solvents were of analytical grade. Hexane was left for 24 h over potassium hydroxide and then distilled; diethyl ether was peroxide-free.

The mushrooms were collected during the summer and autumn of 2005 and 2006 at the foot of the Rila Mountain in the Southwest of Bulgaria. The apparent properties of the fruit bodies of the samples were recorded as were the habitat and the morphological characteristics. Fungal materials were placed in glass containers filled with hexane. The containers were transported in the same day to the laboratory where immediate isolation of lipids was carried out.

A reference mixture of lipid classes was prepared by mixing equal aliquots of 100 mg/mL solutions of docosane, cholesteryl oleate, methyl oleate, oleyl alcohol, cholesterol, 1,3-diolein, 1-monoolein-rac-glycerol, L-dioleylphosphatidyl-choline (all purchased from Sigma-Aldrich Chemie GmbH, Germany), and a purified (preparative silica gel thin-layer chromatography, see bellow) triacylglycerol (TAG) fraction from sunflower oil in dichloroethane.

Isolation of the Lipid Fraction

The procedure described by Christie[7] was applied. When delivered in the lab, the hexane layer was decanted; the fruit body and stem of puffballs were cut into small pieces, briefly blended with methanol (10 mL/g tissue), and stirred for 20 min. Then, a double volume of chloroform was added and the mixture was stirred for additional 4 hours. The combined organic layers were evaporated, the crude extract was taken up in about 24 mL of fresh chloroform-methanol, 2:1 (by volume) and subjected to a "Folch" wash (with 1/4 of the total volume, e.g., 6 mL of 0.88% potassium chloride in water) in order to eliminate non-lipid contaminants. The aqueous (upper) layer, containing non-lipid contaminants, was removed. The organic layer, which contained the purified lipids, was washed with the same volume (as the upper layer) of saline-water, 1:1 (by volume). The bottom layer was transferred to a round bottom flask and the major part of the solvent was evaporated on a rotary evaporator. The rest was quantitatively transferred to a preweighed glass vial using chloroform-methanol, 2:1 (by volume). The solvent was evaporated under a gentle stream of nitrogen to a constant weight in order to determine the lipid content. Lipids were dissolved in hexane to give a 5% stock solution and kept at -20°C until analyzed.

Identification of Lipid Classes by TLC

To determine the main lipid classes, a suitable aliquot of the 5 mg/mL lipid solution (sample size of about 50–100 μg) in hexane was applied on a 19 cm × 4 cm laboratory made glass plate (ca. 0.2 mm thick silica gel G layer). The lipid classes were identified by comparison with the reference lipid mixture (20 μL of 10 mg/mL solution in dichloroethane), which was placed

alongside. The plate was developed once with *ca.* 4 mL hexane-acetone, 100:8 (by volume). The lipid zones were detected by spraying with 50% ethanolic sulphuric acid and heating at 200°C on a temperature controlled hot plate.

Preparation of FAME

The procedure described by Christie[7] was applied. In brief, an aliquot of the stock solution of lipids (ca. 20 mg) was taken and hexane was added to give a total volume of 1 mL. Sulphuric acid in methanol (2 mL, 1% solution) was then added in a stoppered vial and the mixture was left overnight in a block heater at 50°C. Water (5 mL, containing 5% sodium chloride) and hexane (5 mL) were added to the cooled sample and the mixture was vigorously shaken. The aqueous layer was discarded by using a Pasteur pipette and the hexane layer was again washed with 5 mL 2% aqueous potassium bicarbonate. The completeness of the reaction was checked by analytical silica gel TLC with mobile phase hexane-acetone, 100:8 (by volume). FAME zone was visualized by spraying the plate with 50% ethanolic sulphuric acid and heating at 200°C on a temperature controlled hot plate. If needed, the FAME were purified under the same conditions on a laboratory made glass plate (dimensions and layer thickness as above). The edges of the plate were sprayed with 2′,7′-dichlorofluorescein. The zone was scraped, the material was transferred to a small glass column (Pasteur pipette), and FAME were eluted with diethyl ether in a preweighed glass vial. The solvent was evaporated under a gentle stream of nitrogen and the residue was weighed and dissolved in hexane to give a 2% solution.

Preparation of DMOX Derivatives

The procedure described by Christie[7] was followed with small modifications. Initially, the lipid sample (up to 10 mg) was hydrolyzed to free fatty acids by heating for 3 h at 50°C with freshly prepared 0.1 M potassium hydroxide in 90% aqueous ethanol (0.25 mL per mg sample). Then, 2 M hydrochloric acid (0.05 mL per mg sample), 3 mL hexane, 3 mL diethyl ether, and 2 mL water were added and the mixture was vigorously shaken. The upper organic layer was taken with a Pasteur pipette and was passed through a short column with anhydrous sodium sulfate. The solvents were evaporated under a gentle stream of nitrogen and the free fatty acids (up to 2 mg) were immediately subjected to reaction with 2-amino-2-methyl-1-propanol (0.25 g) at 190ºC for 4 hours in a closed sample vial, flushed with nitrogen to eliminate moisture and minimize autoxidation. After cooling, 5 mL *n*-hexane-diethyl ether, 1:1 (by volume) were added, followed by 5 mL of water. The organic layer was transferred to a test tube and was dried over anhydrous sodium sulfate for 1 h. The solution was then taken with a Pasteur pipette and passed

through a small column of anhydrous sodium sulfate into a preweighed vial. The solvent was evaporated (stream of nitrogen), and the residue was dissolved in *n*-hexane to give a 2% solution of DMOX derivatives. Thorough drying of the sample gives a more stable product. The completeness of the reaction was checked by silica gel TLC with a mobile phase of 3 mL chloroform-methanol, 100:3 (by volume) to a front of 10 cm. DMOX derivatives ($R_f = 0.4$) were detected by treating the plate successively with bromine and sulphuryl chloride vapors and heating at 180°C on a temperature controlled hot plate.

Analysis of FAME and Fatty Acid DMOX Derivatives by GC/MS

The same instrumentation, namely an Agilent 6890 Plus System (Agilent Technologies, Santa Clara CA, USA) equipped with a 5793 mass selective detector (Agilent Technologies, Santa Clara CA, USA) and a 30 m × 0.25 mm × 0.25 μm SP 2380 capillary column (Supelco, Bellefonte PA, USA), were used to examine both the FAME and the respective DMOX derivatives. The temperature gradient started from 150°C with 3°C/min to 230°C and held at this temperature for 15 min; solvent delay was 2.2 min, T_{inj} was 260°C, and T_{aux}−280°C. Helium was the carrier gas at 0.8 mL/min. The mass detector operated at T_{quad} 150°C and T_{source} at 230°C. Injection volume was 1.5 μL; split 20:1.

GC of FAME was performed on a Hewlett Packard model 5890 (Hewlett Packard GmbH, Austria) gas chromatograph, using the same column and temperature gradient. Injector and detector temperatures were 260°C. Nitrogen was the carrier gas at flow 0.8 mL/min; split 80:1.

RESULTS AND DISCUSSION

Identification of Lipid Classes by Silica Gel TLC

The lipid content was rather low as seen from Table 1 and was in close agreement with the values reported for other Basidiomycetes.[2,4]

Silica gel TLC was used to elucidate the composition of the lipids isolated from the four *Lycoperdaceae* species. The advantages of this technique is that both the reference lipid mixture and the examined sample are applied on the same plate and developed under the same chromatographic conditions, thus, allowing identification of the lipid classes with relatively high probability. If necessary, further hydrolysis and methylation of the sample could distinguish the non-lipid contaminants, which keep the same R_f values as before, while all *O*-acyl lipids and free fatty acids produce FAME.

The following lipid classes were identified in all four *Lycoperdaceae* species: polar lipids (presumably phospholipids, $R_f = 0$), traces of free fatty acids ($R_f = 0.03$), sterols ($R_f = 0.24$), triacylglycerols ($R_f = 0.66$), traces of

Table 1. Lipid content in *Lycoperdaceae* species

Species	Lipid content (%)
Bovista plumbea	3.1 ± 0.7
Calvatia utriformis	1.8 ± 0.2
Lycoperdon pyriforme	4.8 ± 0.7
Lycoperdon perlatum	3.4 ± 0.5

fatty acid esters ($R_f = 0.81$). Mono- and diacylglycerols were not detected, therefore, it was believed that free fatty acids were not artifacts but were originally present in fungal tissues. Triacylglycerols and phospholipids were the main lipid classes.

Fatty Acid Composition

GC is the method of choice when determining the fatty acid composition of a lipid sample and it works perfectly well with simple mixtures. Reference fatty acids are available in most cases that allow for correct identification of the components. Literature data about other mushroom genera[1,2,4–6] and preliminary GC analysis of *Lycoperdaceae* species, reveal that the fatty acids present a mixture of high complexity. Indeed, besides the few major components, a series of minor components has been observed in GC, suspected to be unsaturated isomers, odd chain components, and long chain saturated and monoenoic fatty acids. Complete structural analysis of the component fatty acids was therefore required. We applied a sequence of ordinary GC and GC/MS of methyl esters.

On the other hand, special fatty acid derivatives are designed to provide reliable structural information by GC/MS under the conditions of electron impact ionization. Pyrrolidides, picolinyl esters, and DMOX derivatives have been recommended for the purpose,[7] and of these, the last two have gained wider popularity. DMOX derivatives were chosen in the present work because: (i) they are easy to prepare; (ii) they give unique and readily interpretable mass spectra, and (iii) they are only slightly less volatile than are FAME.[8] There are indeed some disadvantages, of which the main is the need to keep dry the reaction mixture and the products, since traces of moisture can cause ring opening.[7] With careful and rapid work and by immediate injection, the danger of appearance of later eluting intermediates has been successfully minimized. As is evident from Figure 1, the total ion chromatograms (TIC) of FAME and DMOX derivatives of *Lycoperdon pyriforme* lipids are practically identical. DMOX derivatives are eluted slightly later and are better resolved, as is clearly seen from the separation of the two isomers 9-18:1 and 11-18:1 (Figure 1) and of the three 16:1 isomers (Figure 2).

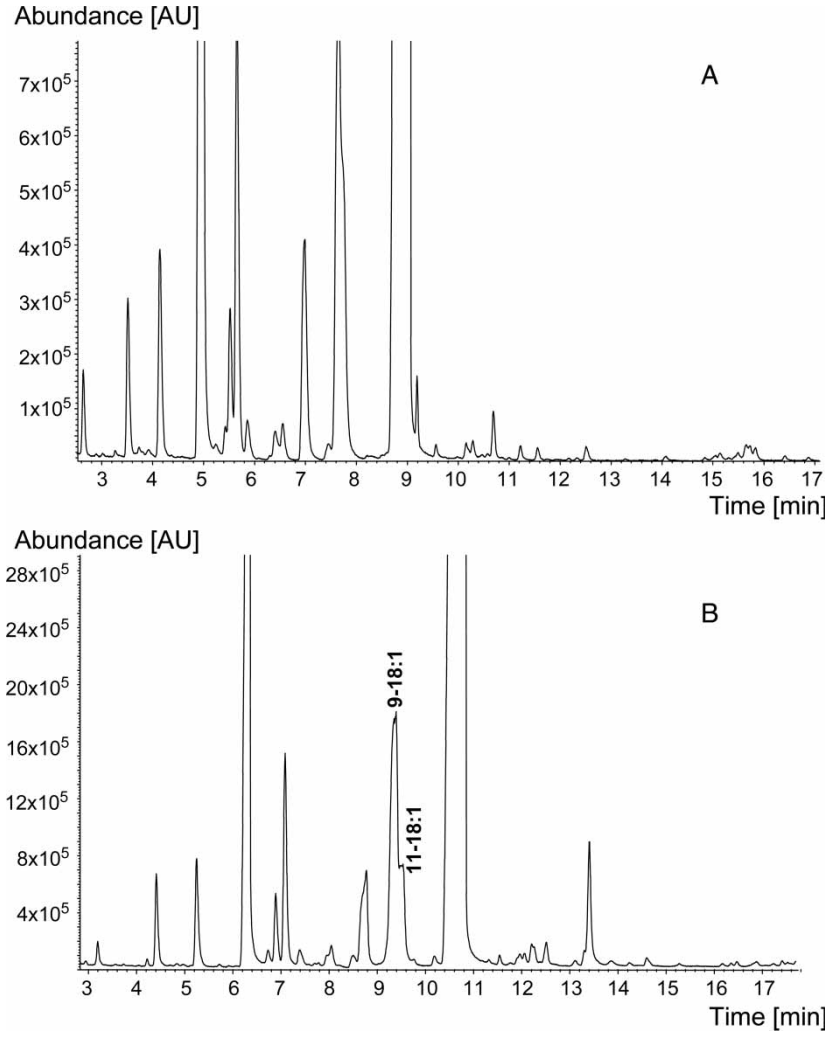

Figure 1. Total ion current (TIC) chromatograms of fatty acids of *Lycoperdon pyriforme*: A. as methyl esters; B. as DMOX derivatives.

Twenty seven fatty acids were identified in total, not all of which were present in all four *Lycoperdaceae* species (Table 2). They were identified by interpreting their mass spectra as methyl esters and as DMOX derivatives according to Christie.[8] Comparison with reference spectra published in the Internet[9] was carried out also. As has been found for other mushroom species belonging to the class Basidiomycetes, four fatty acids are dominating: linoleic acid (9,12-18:2, 37–65%), oleic acid (9-18:1, 7–24%), palmitic acid (16:0, 12–18%), and stearic acid (18:0, 2–6%). In addition, the presence of

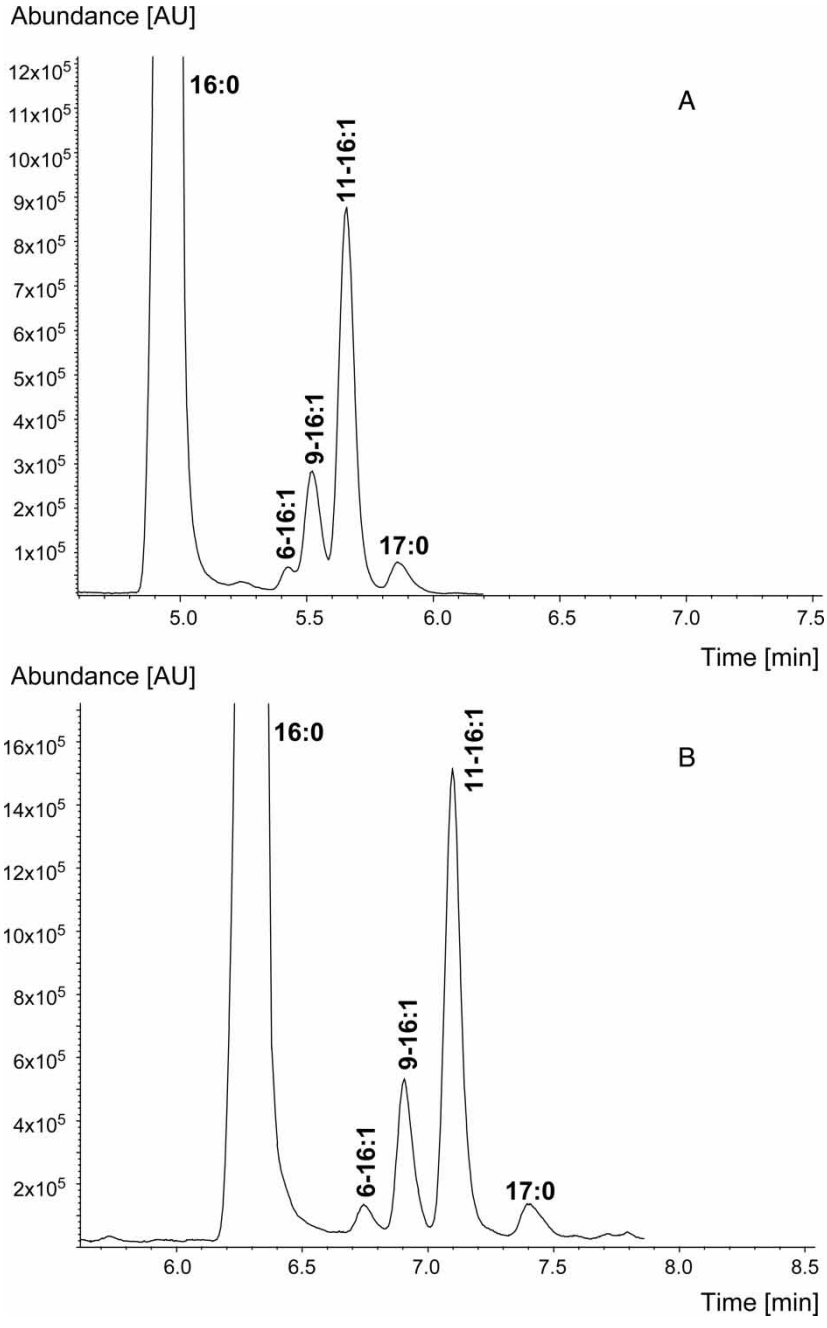

Figure 2. Separation of 16:1 isomers in *Lycoperdon pyriforme*: A. as methyl esters; B. as DMOX derivatives.

Table 2. Fatty acid composition of some Lycoperdaceae species (wt.%)

Fatty acid	*Bovista plumbea*	*Calvatia utriformis*	*Lycoperdon pyriforme*	*Lycoperdon perlatum*
12:0	0.4	0.4	0.4	0.4
14:0	0.6	0.7	0.9	0.8
12-Me-14:0	0.2	0.3	0.1	0.2
15:0	1.0	1.4	1.3	0.9
16:0	14.1	12.2	18.1	14.5
6-16:1	1.0	0.4	0.2	0.6
9-16:1	0.8	0.8	1.0	0.7
11-16:1	1.1	1.7	2.9	0.4
17:0	1.2	0.8	0.3	0.6
16:2	—	—	0.3	—
18:0	3.4	3.6	2.0	6.4
9-18:1	14.0	23.0	6.8	24.1
11-18:1	4.4	5.9	—	0.7
9,12-18:2	51.9	42.4	64.8	37.6
20:0	0.5	0.7	0.1	0.8
9,12,15-18:3	0.4	0.8	0.1	3.9
9-20:1	0.7	0.4	0.1	1.5
11-20:1	—	—	0.3	0.2
9,12-20:2	0.8	0.6	0.1	1.1
22:0	0.5	0.8	0.1	2.4
13-22:1	—	1.2	—	—
23:0	–	0.7	–	0.5
24:0	0.8	1.1	0.1	0.6
15-24:1	0.5	—	—	0.1
25:0	0.7	—	—	0.4
26:0	0.4	—	—	0.1
17-26:1	0.5	—	—	0.4
Sum S	23.8	22.8	23.4	28.6
Sum U	76.2	77.2	76.6	71.4
U/S Ratio	3.2	3.4	3.3	2.5

interesting monoenoic isomers was unambiguously established. Of these, 6-16:1, 9-16:1, 11-16:1, and 9-20:1 were identified in all four *Lycoperdaceae* species (Table 2). As is evident from Table 2, the proportions of the saturated, monounsaturated, and polyunsaturated fatty acids differ, depending on the species. Despite that, the U/S ratio (U, unsaturated; S, saturated fatty acids), which is a measure of the total unsaturation degree, is almost equal for *Calvatia utriformis*, *Bovista plumbea* and *Lycoperdon pyriforme*, but is lower for *Lycoperdon perlatum*. However, comments on the fatty acid composition should be made carefully, since it might strongly depend on the stage of development of the fruit bodies. Figure 3 presents the data obtained for young and mature fruit bodies of *Bovista plumbea* collected in the same year and in

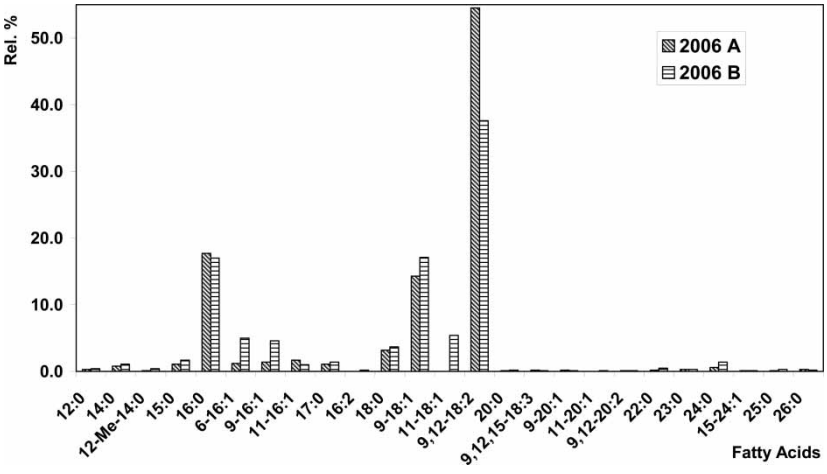

Figure 3. Fatty acid composition of A. young and B. mature fruit bodies of *Bovista plumbea*.

the same location. The significant differences between the fatty acid proportions are clearly seen.

As has been stated above, the fatty acid composition of *Lycoperdaceae* mushrooms is reported here for the first time. Moreover, this is the first report on clearly resolved and separately determined 16:1 isomers in Basidiomycetes. Also, 9-20:1, 9,12-20:2, 23:0, 25:0, 26:0, and 11-26:1 fatty acids are reported in Basidiomycetes for the first time.

This work confirms the previous finding[2] that Basidiomycetes posses a rather complex and characteristic fatty acid composition. Differences between the species and differences due to the development stages of fruit bodies have been established. Some preliminary data show that differences due to the vegetation seasons can also be expected. It is essential to use advanced chromatographic methods for establishing the fatty acid composition and the correct structure of the components. GC/MS of fatty acid derivatives, other than methyl esters is an important step, while TLC helps to determine with rather great certainty the lipid class composition and provides rapid control of the completeness of the derivatization and the purity of the products at each stage of the analysis.

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