Supercritical Fluid Extraction of Carboxylic and Fatty Acids from *Agaricus* Spp. Mushrooms†

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Material obtained by the CO_2 supercritical fluid extraction (SFE) of the wild and cultivated mushrooms *Agaricus silvicola* and *Agaricus bisporus*, respectively, was analyzed by gas chromatography (GC)–mass spectrometry and GC–Fourier transform infrared spectrometry. SFE gave results comparable with classical extraction techniques for free fatty acids and esters but with fewer manipulative steps and also resulted in the characterization of acids not previously reported from these sources. The organic acids identified included all of the C_5–C_{26} saturated fatty acids, with palmitic and stearic acid predominating, the C_{15}–C_{22} fatty acid methyl esters, and the C_4–C_{11} α,ω-dicarboxylic acids. Benzoic acid was a major constituent in the *A. silvicola* extract but was only a minor constituent in that from *A. bisporus*. Only the C_8–C_{20} monocarboxylic saturated fatty acids had been previously identified from these species. Unsaturated fatty acids were virtually absent, with only minor amounts of hexadecenoic and octadecenoic acids observed.

INTRODUCTION

The constituents of lipids in the cultivated mushroom *Agaricus bisporus* (Lange) Sing. have been investigated quite extensively (Weete, 1980). The acids include C_{12}–C_{20} even-numbered fatty acids (Holtz and Schisler, 1971; Prostenik et al., 1978; Weete et al., 1985) and C_{16}–C_{24} hydroxy fatty acids (Prostenik et al., 1978), with oleic, linoleic, and palmitic predominating. These acids may exist in their free form or be conjugated to other lipid constituents. Byrne and Brennan (1975) reported on levels of palmitic, stearic, and oleic acids in the free form, and Stancher et al. (1992) expanded the observed range of free and bound fatty acids to include C_8 and C_{13}–C_{17} odd-numbered acids. Isolation of fatty acids typically requires extraction, release (e.g., by saponification), and purification (e.g., by thin-layer chromatography). Analysis involves preparation of the fatty acid methyl esters followed by gas chromatography (GC).

Supercritical fluid extraction (SFE) has emerged as a valuable technique (Lee and Markides, 1990; Hawthorne, 1990; Chester et al., 1992; Tehrani, 1993) for the rapid and efficient isolation of solutes from solid samples. SFE of mushrooms with supercritical carbon dioxide was recently shown to be efficient and rapid for the analysis of the fungal metabolite ergosterol (Young and Games, 1993). This study was conducted to investigate further the application of SFE to other constituents in mushrooms, such as free organic acids.

MATERIALS AND METHODS

Reagents. All reagents and solvents were of analytical reagent grade. Instrument grade carbon dioxide was supplied in cylinders with a dip tube (BOC, London, U.K.) and used without further purification.

Samples. Samples of cultivated mushrooms (*A. bisporus*) were obtained from a local grocery store (Swansea, U.K.) and of wild mushrooms [*Agaricus silvicola* (Vitt.) Pk.] were collected from near Usk, Gwent, U.K. The caps were separated from stems, and each was cut into 2–3-mm-sized pieces, freeze-dried, and ground into powders.

Supercritical Fluid Extraction. Samples of mushroom caps, typically 120 mg in a 7-mL stainless steel extraction thimble, were placed into a Hewlett-Packard 7680A SFE module (Hewlett-Packard, Avondale, PA). Operating conditions were as follows: extraction chamber temperature, 50 °C; extraction conducted with supercritical CO_2 at a density of 0.90 g/mL (pressure, 281 bar) for 11.4 min at a flow rate of 3.3 mL/min (equivalent to 5.5 thimble volumes); analytes from the extraction chamber were trapped on an octadecylsilyl (ODS) column at 40 °C; the trap was then heated to 50 °C and eluted with 1.5 mL of methanol.

Derivatization of Extracts. Extract aliquots were evaporated to dryness, dissolved in about 100 μL of methanol, and treated dropwise with a solution of ethereal diazomethane until a yellow color persisted. The reaction mixture was evaporated to dryness under a stream of nitrogen and the residue redissolved in methanol prior to analysis.

Gas Chromatography–Mass Spectrometric (GC–MS) Analysis. GC–MS analyses were conducted on underivatized and derivatized extracts on a Finnigan Model 4500 quadrupole mass spectrometer in the electron ionization (EI) mode at 70 eV and the chemical ionization (CI) mode (methane). A J&W Scientific DB-1701 30 m × 0.32 mm i.d. column with 1-μm film (J&W Scientific, Folsom, CA) was used with on-column injection, and the column was temperature programmed from 100 to 300 °C at 15 °C min^{-1}.

Gas Chromatography–Fourier Transform Infrared (FTIR) Spectrometric Analysis. GC–FTIR analyses were conducted on underivatized extracts on a Hewlett-Packard 5890A GC coupled to a 5965B IRD. A J&W DB-5 30-m column with 0.26-μm film was used in the splitless mode with a purge delay of 30 s, injector 250 °C, and the column was temperature programmed from 100 to 280 °C at 10 °C min^{-1}. Transfer lines were at 280 °C.

RESULTS AND DISCUSSION

SFE on small samples (120 mg) of powdered mushroom proceeded quickly to provide extracts, which were immediately ready for analysis by GC–MS. It should be noted that the use of small amounts of material requires that special attention be paid to ensuring that samples be homogeneous and representative of the whole. Figure 1

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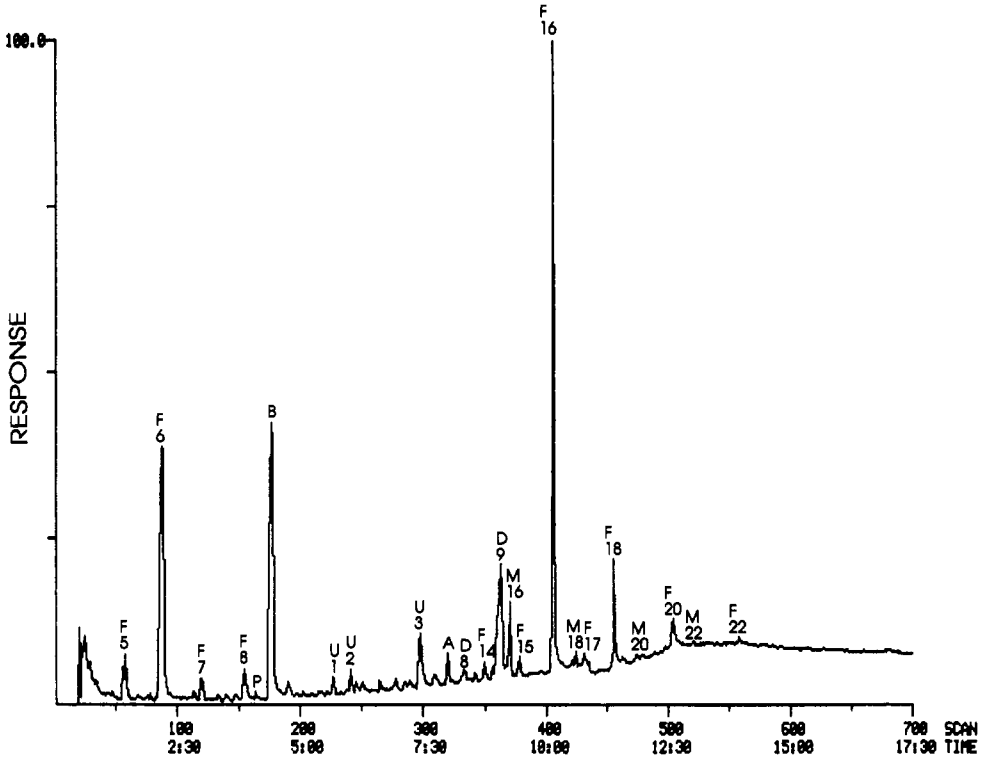

Figure 1. Gas chromatogram (GC) of a CO_2 supercritical fluid extract of the mushroom *A. silvicola*. Separation was done by GC on a 30 m × 0.32 mm i.d. DB-1701 column temperature programmed from 100 to 300 °C at 15 °C min^{-1}. Detection was by electron ionization mass spectrometry. A, monomethyl azelaic acid; B, benzoic acid; D8-D9, α,ω-dicarboxylic acids of 8- and 9-carbon chain length; F5-F22, straight-chain saturated fatty acids of 5–22 carbons; P, pentyl hexanoate; U, unidentified.

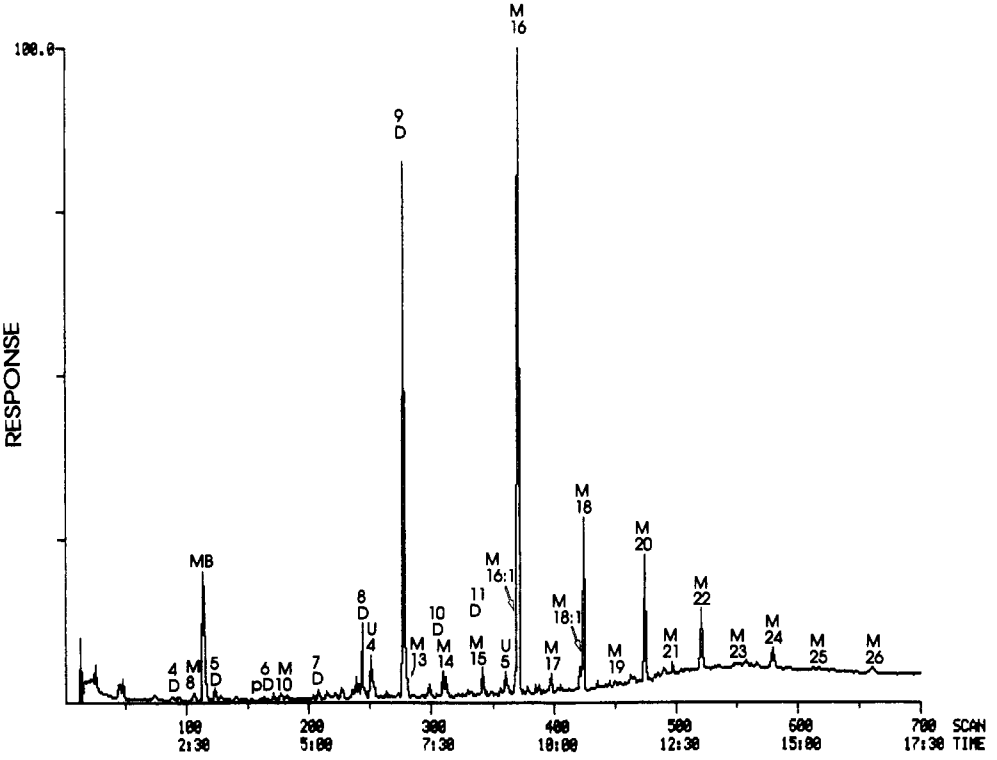

Figure 2. Gas chromatogram of a diazomethane-treated CO_2 supercritical fluid extract of the mushroom *A. silvicola*. Separation was done by GC on a 30 m × 0.32 mm i.d. DB-1701 column temperature programmed from 100 to 300 °C at 15 °C min^{-1}. Detection was by electron ionization mass spectrometry. 4D-11D, dimethyl esters of α,ω-dicarboxylic acid of 4–11-carbon chain length; M6-M26, straight-chain saturated fatty acid methyl esters of 6–26 carbons; M16:1, M18:1,monounsaturated fatty acid methyl esters of 16 and 18 carbons; MB, methyl benzoate; P, pentyl hexanoate; U, unidentified.

shows a total ion chromatogram for such an extract from *A. silvicola*. Initial data suggested that the majority of substances detected were fatty acids, so an aliquot of the extract was methylated with diazomethane and analyzed by GC–MS (see Figure 2). Because molecular ions for some of the analytes were not readily observable in the EI MS, methane CI MS analyses (which provided molecular ion information) of the underivatized and derivatized extracts were also performed.

Substances observed in this study were tentatively

identified on the basis of comparison of EI mass spectra with those in the data system library. Subsequent comparisons were made with chromatographic and MS data for those fatty acid methyl esters (FAME) standards that were available. The free and methyl esters of α,ω-dicarboxylic acids were confirmed by comparison of MS with those reported by Viden and Rezanka (1987).

Table 1 shows the various constituents identified by GC–MS and their levels in mushrooms extracted by CO_2 SFE. The previously observed range of fatty acids has been extended to include all saturated fatty acids from C_5 to C_{26}, with palmitic and stearic acids predominating. SFE with unmodified CO_2 is a relatively mild method and likely would extract primarily those fatty acids, such as in the neutral lipids, that are not bound (e.g., as esters). Goncalves et al. (1991) observed that free fatty acids are more soluble (extractable) than triglycerides in supercritical CO_2. The even more polar phospholipids, which are the main constituents of cell walls (Gunstone and Morris, 1983), would likely not be extracted at all. There is usually 2–3 times more bound lipid present in fungal cell walls than unbound, and 70% of the fatty acids in cell walls are unsaturated (Weete, 1980). The virtual absence of unsaturated fatty acids in the SFE is supported by the report (Byrne and Brennan, 1975) that linoleic acid (C18:2) was a minor component of the total neutral lipid fatty acids of *A. bisporus*. When extraction was done under conditions (alcoholic NaOH) that would release bound fatty acids, it is not surprising that Stancher et al. (1993) observed linolenic acid.

A variety of organic acids and derivatives not previously reported in *Agaricus* spp. were observed in the SFE materials. Analysis of the extract prior to derivatization revealed the presence of C_{15}–C_{22} FAME. These compounds have been reported in basidiomycetes other than mushrooms (Laseter et al., 1968). It is possible that FAME were present in extracts isolated in earlier studies of *Agaricus* spp.; they would not have been observed after saponification and would have been otherwise masked by the preparation of methyl esters for the determinative step. The C_4–C_{11} α,ω-dicarboxylic acids were observed as was the monomethyl ester of the C_9 dicarboxylic (azelaic) acid. Although succinic acid has been reported in some mushrooms (Kazuno et al., 1987), the higher dicarboxylic acids have not. Benzoic acid, a significant component in *A. silvicola*, has been reported as a minor constituent of the volatiles from another mushroom (Audouin et al., 1989) and in an extract of an unspecified mushroom (Zolotov et al., 1983). The presence of benzoic acid is somewhat curious since Menon et al. (1990) have reported this substance to have an inhibitory effect on some isoenzymes of *A. bisporus*. There were numerous other minor constituents (<0.1%) that could not be identified because of insufficient material. Other than the levels of benzoic acid, the patterns of acids and esters for the two *Agaricus* spp. were quite similar.

Although the absolute values of the relative percentage levels of the different components were somewhat variable depending upon EI or CI analyses of the underivatized or derivatized samples, they showed similar trends within a given species. Some of the intraspecies differences are attributable to the calculations based on total ion counts without regard to application of response factors for the individual components. Such factors would be applied in a proper quantitative investigation, which was beyond the intended scope of this study. The differences may also due to natural causes; Weete (1980) has noted that there may be considerable variation in lipid content even between different strains of the same species grown under identical conditions.

Table 1. Relative Composition of Acids and Esters Extracted by Supercritical CO_2 from *Agaricus* Spp. Mushrooms

chain carbon no.	*A. silvicola* underivatized EI[b]	*A. silvicola* underivatized CI[c]	*A. silvicola* derivatized[a] EI	*A. silvicola* derivatized[a] CI	*A. bisporus* underivatized	*A. bisporus* derivatized	*A. bisporus* literature[d]
Fatty Acids[e]							
5	2.5[f]	3.4	–[g]	–	–	–	nr[h]
6	18.4	14.0	2.0	3.1	1.0[i]	–	nr
7	1.3	1.3	0.4	0.2	0.2	–	0.1
8	1.8	2.0	0.4	0.7	0.6	–	1.3
9	–	–	0.3	0.3	–	0.2[i]	nr
10	–	–	0.3	0.4	–	0.4	0.2
11	–	–	0.6	0.3	–	–	nr
12	–	–	0.5	tr[j]	–	–	0.6
13	–	–	2.1	tr	–	–	0.4
14	0.6	1.1	1.0	1.4	2.5	3.3	1.1
15	0.8	3.8	1.4	3.0	4.2	3.1	3.0
16	24.7	24.6	31.1	22.7	51.1	32.1	14.8
16:1	–	–	tr	tr	–	–	0.5
17	1.4	1.2	0.9	2.1	1.7	2.0	1.3
18	4.2	7.4	7.5	10.0	13.2	12.7	4.4
18:1	–	–	1.0	0.8	–	–	1.6
18:2	–	–	–	–	–	–	68.4
19	–	–	0.3	0.5	0.4	0.6	nr
20	2.2	2.7	5.1	7.9	2.7	6.6	0.6
21	–	–	0.5	1.0	–	0.9	nr
22	0.5	3.1	3.1	5.4	0.8	3.4	nr
23	–	–	0.7	0.7	–	0.6	nr
24	–	–	1.2	1.8	–	1.5	nr
25	–	–	0.3	0.6	–	tr	nr
26	–	–	0.7	0.8	–	tr	nr
Fatty Acid Methyl Esters							
6	0.2	0.1			–		nr
8	0.1	0.2			–		nr
15	0.2	1.0			0.3		nr
16	2.4	11.8			1.6		nr
17	tr	0.4			–		nr
18	0.8	1.1			0.5		nr
19	0.2	0.2			–		nr
20	0.1	0.2			0.4		nr
21	tr	tr			–		nr
22	tr	tr			–		nr
Dicarboxylic Acids							
4	–	–	0.3	0.3	–	0.4	nr
5	–	–	0.7	1.1	–	0.7	nr
6	–	–	0.3	0.4	–	0.4	nr
7	–	–	0.4	0.5	–	1.1	nr
8	1.2	0.1	2.7	3.5	2.1	5.4	nr
9	9.7	0.2	20.9	15.7	14.2	22.0	nr
10	–	–	1.1	2.3	–	1.2	nr
11	–	–	tr	–	–	tr	nr
Dicarboxylic Acid Monomethyl Esters							
9	1.2	–			2.3		nr
Other Acids							
benzoic	19.5	10.5	8.3	7.8	0.2	0.2	nr
U1[k]	0.8	3.2			–	–	nr
U2	0.7	0.7			–	–	nr
U3	4.2	5.3			–	–	nr
Other Esters							
P[l]	0.3	0.4	0.2	0.2	–	–	nr
U4			2.4	2.9		0.3	nr
U5			1.3	1.9		0.9	nr

[a] After treatment with diazomethane. [b] Electron ionization mass spectrometry. [c] Chemical ionization (methane) mass spectrometry. [d] Stancher et al. (1992), from lipids extracted by alcoholic NaOH. [e] Saturated fatty acids unless specified otherwise. [f] Percent of total, based on the total ion count as determined by mass spectrometry. [g] Not detected. [h] Not reported. [i] Determined by EI-MS only. [j] Trace (<0.1%). [k] Unidentified. [l] Pentyl hexanoate.

GC–FTIR analysis of an underivatized extract (chromatogram for *A. silvicola* illustrated in Figure 3) served

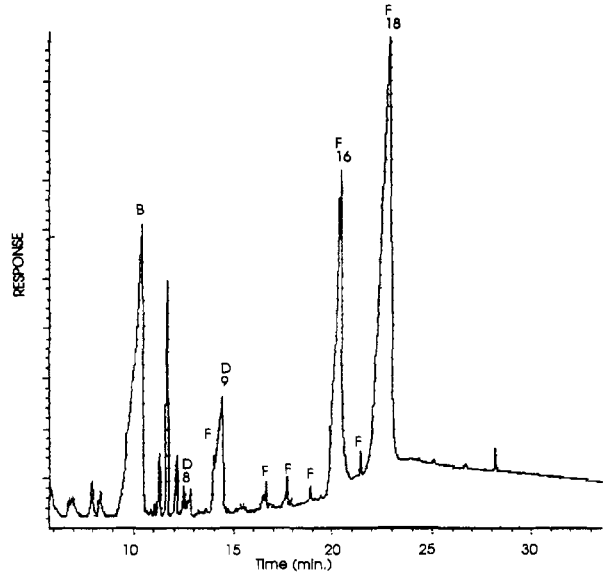

Figure 3. Gas chromatogram of a CO_2 supercritical fluid extract of the mushroom *A. silvicola*. Separation was done by GC on a 30 m × 0.25 mm i.d. DB-5 column temperature programmed from 100 to 280 °C at 10 °C min^{-1}. Detection was by Fourier transform infrared spectroscopy. B, benzoic acid; D, α,ω-dicarboxylic acids; F, straight-chain saturated fatty acids with number of carbons.

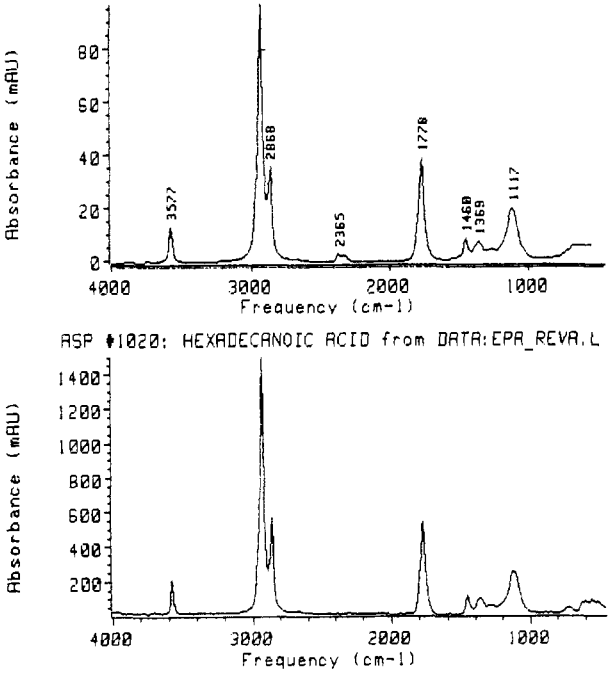

Figure 4. (a, top) FTIR spectrum of hexadecanoic acid extracted by supercritical CO_2 from the mushroom *A. silvicola*. (b, bottom) FTIR spectrum of a hexadecanoic acid standard.

to confirm the presence of free fatty acids, α,ω-dicarboxylic acids, the monomethyl ester of azelaic acid, and benzoic acid. Figure 4a shows the FTIR spectrum of the component identified as F16 and, compared to the best match in the FTIR database library, hexadecanoic acid (Figure 4 b). A search of the total chromatogram for absorbances at 3770–3780 and 1775–1785 cm^{-1} (together characteristic for COOH) revealed the presence of F16, its homologues, and other fatty acids (such as those labeled F).

The GC–MS analyses of extracts also indicated the presence of later eluting sterols; the characterization of these compounds will be reported elsewhere.

CONCLUSIONS

It has been shown that CO_2 supercritical fluid technology can be effectively applied to the extraction of mushrooms. Given that traditional methods for free organic acid constituents in lipids are multistep and somewhat cumbersome, the SFE method employed herein proceeded quickly and simply and provided, in virtually one step, a sample ready for analysis. The results generally were comparable with those based on classical extraction techniques. It also provided a method for analysis of free fatty acids and other substances not previously reported in *A. bisporus* and *A. silvicola*, such as fatty acid methyl esters, α,ω-dicarboxylic acids, and benzoic acid.

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LITERATURE CITED

Audouin, P.; Vidal, J. P.; Richard, H. Volatile compounds from aroma of some edible mushrooms: morel (*Morchella conica*), wood blewitt (*Lepista nuda*), clouded agaric (*Clitocybe nebularis*), and false chanterelle (*Hygrophoropsis aurantiaca*). *Sci. Aliments* **1989**, *9*, 185–193.

Byrne, P. F. S.; Brennan, P. J. Lipids of *Agaricus bisporus*. *J. Gen. Microbiol.* **1975**, *89*, 245–255.

Chester, T. L.; Pinkston, J. D.; Raynie, D. E. Supercritical fluid chromatography and extraction. *Anal. Chem.* **1992**, *64*, 153R–170R.

Games, D. E.; Berry, A. J.; Mylchreest, I. C.; Perkins, J. R.; Pleasance, S. Supercritical fluid chromatography–mass spectrometry. In *Supercritical Fluid Chromatography*; Smith, R. M., Ed.; Royal Society of Chemistry: London, 1988; pp 159–174.

Goncalves, M.; Vasconcelos, A. M. P.; Gomes de Azevedo, E. J. S.; Chaves das Neves, H. J.; Nunes da Ponte, M. On the application of supercritical fluid extraction to the deacidification of olive oils. *J. Am. Oil Chem. Soc.* **1991**, *68*, 474–480.

Gunstone, F. D.; Norris, F. A. *Lipids in Foods Chemistry, Biochemistry and Technology*; Pergamon Press: New York, 1983.

Hawthorne, S. B. Analytical-scale supercritical fluid extraction. *Anal. Chem.* **1990**, *62*, 633A–642A.

Holtz, R. B.; Schisler, L. C. Lipid metabolism of *Agaricus bisporus* (Lange) Sing. I. Analysis of sporophore and mycelial lipids. *Lipids* **1971**, *7*, 176–180.

Kazuno, C.; Nishijima, M.; Amakawa, E.; Sakai, S.; Kamoi, I. Determination of organic acids in edible mushrooms by high performance liquid chromatography. *Nippon Shokuhin Kogyo Gakkaishi* **1987**, *34*, 42–47.

Laseter, J. L.; Weete, J. D.; Weber, D. J. Alkanes, fatty acid methyl esters, and free fatty acids in surface wax of *Ustilago maydis*. *Phytochemistry* **1968**, *7*, 1177–1181.

Lee, M. L.; Markides, K. E. Analytical supercritical fluid extraction. In *Analytical Supercritical Fluid Chromatography and Extraction*; Lee, M. L., Markides, K. E., Eds.; Chromatography Conferences: Provo, UT, 1990; pp 313–352.

Menon, S.; Fleck, R. W.; Yong, G.; Strothkamp, K. G. Benzoic acid inhibition of the alpha, beta, and gamma isoenzymes of *Agaricus bisporus* tyrosinase. *Arch. Biochem. Biophys.* **1990**, *280*, 27–32.

Prostenik, M.; Burcar, I.; Castek, A.; Coscovic, C.; Golem, J.; Jandric, Z.; Kijaic, K.; Ondrusek, V. Lipids of higher fungi. III.

The fatty acids and 2-hydroxy acids in some species of basidiomycetes. *Chem. Phys. Lipids* **1978**, *22*, 97–103.

Stancher, B.; Procida, G.; Calabrese, M. Characteristics of the most common mushrooms cultivated in Italy. IV. Lipids: determination of the content of free and bound fatty acids. *Ind. Aliment.* **1992**, *31*, 744–747, 750.

Tehran, J. Successful supercritical fluid extraction strategies. *Am. Lab.* **1993**, *25* (2), 40HH–40MM.

Viden, I.; Rezanka, T. Capillary gas chromatography–mass spectrometry of aliphatic saturated α,ω-dicarboxylic acid dimethyl esters and direct inlet mass spectrometry of the corresponding free acids. *J. Chromatogr.* **1987**, *408*, 145–155.

Weete, J. D. *Lipid Biochemistry of Fungi and Other Organisms*; Plenum Press: New York, 1980.

Weete, J. D.; Furthe, R.; Haenseler, E.; Rast, D. M. Cellular and chitosomal lipids of *Agaricus bisporus* and *Mucor rouxii*. *Can. J. Microbiol.* **1985**, *31*, 1120–1126.

Young, J. C.; Games, D. E. Supercritical fluid extraction and supercritical fluid chromatography of the fungal metabolite ergosterol. *J. Agric. Food Chem.* **1993**, *41*, 577–581.

Zoltov, Y. A.; Ivanov, A. A.; Shpigun, A. A. Determination of organic acids by ion chromatography. *Zh. Anal. Khim.* **1983**, *38*, 1479–1483.

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