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NMR lipid profile of *Agaricus bisporus*

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Abstract

Lipids extracted from freeze dried and powdered cultivated *Agaricus bisporus* by the Bligh and Dwyer method, were subjected to 1D-proton and 2D-COSY NMR analysis. The diacylglycerophospholipids, mono-, di- and tri-glycerides, ether lipids, sphingolipids and steroidal lipids were studied qualitatively and quantitatively. Our findings suggested that (a) ethanolamines and cholines were the predominant diacylphospholipids, (b) sterols, mainly ergosterol, were present in relatively large quantities and (c) the phospholipid fatty acid composition consisted almost exclusively of linoleic acid. This type of detailed data on lipid composition was accurately and rapidly obtained in one step, without chemical modification of the sample. Additional information on four classes of lipid, including their fatty acid composition was obtained after separating the total lipid extract by NH_2-aminopropyl Certify II Bond Elut solid phase chromatography and analysing the NMR spectra of each class of lipids. The results demonstrated the potential of the method for the study of plant metabolism, development and taxonomy. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Agaricus bisporus*; Basidiomycetes; Mushroom; NMR; Lipids

1. Introduction

The lipid content of mushrooms, such as *Agaricus bisporus* (Lange) Sing, has been the object of many investigations in relation to studies in subjects as varied as metabolism, nutrition and medicine. As far back as 1962, a study reported by Hugues (1962) identified 10 fatty acids among which linoleic acid predominated. It was shown later that fatty acids with 16 and 18 carbons are most abundant and that linoleic and oleic acids are the principal unsaturated fatty acids, palmitic acid being the major saturated one (Holtz & Schisler, 1971; Byrne & Brennan, 1975; Weete, Furter, & Hander, 1985; Senatore, 1988; Solberg, 1989; Mau, Beelman, Ziegler, & Royse, 1991; Stancher, Procida, & Calabrese, 1992). Determination of the sterol composition of mushrooms was tremendously eased by the advances in mass spectrometry techniques. Ergosterol was confirmed as the predominant sterol in Basidiomycetes and closely related species as well as novel sterols were also identified by this technique (De Simone, Senatore, Sica, & Zollo, 1979; Weete, 1980; Yokokawa & Mitsuhashi, 1981; Senatore, 1992; Kobata, Wada, Hayashi, & Shibata, 1994). Acylglycerols, sterols and the fatty acid contents of mushrooms are thus well established. However, little has been reported on the phospholipid content of mushrooms.

To understand better the importance of technique and experimental process in obtaining comprehensive information on lipid mixtures, it is necessary to review the steps involved in a conventional analytical procedure. The isolation of the lipids is carried out via a typical Bligh and Dyer (1959) or Folch, Lees, and Stanley (1957) extraction of the total lipids, or supercritical fluid extraction of the carboxylic and fatty acids (Weete et al., 1985). The purification and/or analysis is then proceeded to by one or a combination of the following techniques: TLC (Abdullah, Young, & Games, 1994), GC after derivatisation of the fatty acids components into methyl esters (Christie, 1987a), HPLC (Prostenik et al., 1983; Christie, 1987b), ion exchange chromatography (Sparling, Zidovetski, Muller, & Chan, 1989) or GC–MS. Lipid content determinations have thus always been a time consuming several steps process, until one and two dimensional high resolution proton NMR spectroscopy was used to obtain comprehensive information on total lipid extracts without chromatographic separation or any chemical modification (Higgins, 1987). It has indeed been successfully

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applied to determine qualitatively and quantitatively the lipids extracted from cells, tissues and body fluids (Casu, Anderson, Choi, & Gibbons, 1991; Casu et al., 1992; Choi, Casu, & Gibbons, 1993; Casti et al., 1993; Adosraku, Choi, Constantinou-Kokotos, Anderson, & Gibbons, 1994; Nicholson, Foxall, Spraul, Farrant, & Lindon, 1995; Yeboah, Adosraku, Nicolaou, & Gibbons, 1995), parasites and organisms (Adosraku et al., 1993; Adosraku, Smith, Nicolaou, & Gibbons, 1996).

In this study, we report for the first time, the analysis of both intact and purified lipid extracts from *Agaricus bisporus*, using 1D and 2D COSY proton NMR experiments. Different types of phospholipids and neutral lipids were identified from intact lipid mixtures and quantified from their NMR spectra. Information on the fatty acid composition and the average levels of unsaturation were obtained from the same spectra. In order to obtain a more detailed analysis of the sample, the mixtures were separated, before NMR analysis was performed, on solid phase, by ion exchange chromatography, into 4 fractions corresponding to (a) neutral lipids, (b) free fatty acids, (c) neutral phospholipids and (d) acidic phospholipids (Kates, Burdon, & Van Kippenberg, 1988). The spectroscopic method was thereafter used to confirm the identity of the lipids belonging to the different classes and their fatty acids composition (Choi et al., 1993).

2. Results and discussion

NMR spectroscopy has been exploited in many laboratories for the study of lipids in cells, tissues and in human and animal body metabolism (Casu et al., 1991, 1992; Adosraku et al., 1993, 1994, 1996; Casti et al., 1993; Choi et al., 1993; Nicholson et al., 1995; Yeboah et al., 1995). Membranes of animal and plant cells generally possess 50–100 different lipids belonging to separate classes which are related by common structural motifs (Adosraku et al., 1996). It is not surprising therefore, that the individual NMR spectra of the lipids have not been distinguished in these complex plant mixtures. Moreover, the study of lipids in plants, by NMR, is relatively new and progress has been limited by the lack of fully comprehensive maps of membrane lipids.

Here we report, both qualitative and quantitative NMR analysis of lipid extracts from *Agaricus bisporus*. Although the use of total lipid extracts implied overlaps of certain individual lipid spectra, even the simple NMR procedures described in this paper, rapidly (in less than half an hour) and quantitatively yielded information on many lipids. The lipid profiles obtained by TLC, HPLC and GC should thus be contrasted by this NMR procedure. In addition, complementary extraction procedures and/or prior separation by Bond Elut columns can compensate for some of the drawbacks to this simple one step analysis (Choi et al., 1993). These drawbacks include incomplete extraction of some lipids such as highly phosphorylated inositol or glycolipids, very low concentrations of some lipidic vitamins and cofactors, and overlaps of spectra.

2.1. Diacylglycerophospholipids (DAGP)

The corresponding designation of the different glycerophospholipids is as follows: phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), x times phosphorylated phosphatidylinositol (PIPx), phosphatidic acid (PA), phosphatidylglycerol (PG) and cardiolipin (DiPtdGly).

The one dimensional spectrum (Fig. 1) and the 2D COSY spectrum (Fig. 2) obtained at 600 MHz revealed the presence of many lipids. However, although there was considerable overlap of resonances from different lipid components, most of the lipids had a structure-specific resonance or set of resonances which enabled their identification and quantification.

All diacylglycerophospholipids contributed to the backbone glycerol Sn2 proton multiplet at δ 5.21. The magnetically inequivalent glycerol Sn1 methylene protons resonated at δ 4.41 (downfield) and δ 4.15 (upfield), while both glycerol Sn3 methylene proton resonances overlapped at δ 3.98. Coupling between these glycerol backbone protons was confirmed by cross peaks in the 2D COSY spectrum that unequivocally gave their assignments. Although these glycerol moiety proton resonances yielded the total number of such lipids, in order to distinguish between diverse diacylglycerophospholipids, the resonances of protons from the head groups were necessary.

Choline lipids were identified by their characteristic $-N^+(CH_3)_3$ proton singlet at δ 3.22. The two choline head group methylene protons ($-OCH_2CH_2N^+(CH_3)_3$) resonated at δ 3.60 ($-CH_2N^+(CH_3)_3$) and δ 4.23 ($-OCH_2-$) and were confirmed by their cross peaks in the 2D COSY spectrum of the total lipids (Fig. 2), and thus represented all choline lipids including diacyl phosphocholines.

Ethanolamine lipids were identified by their characteristic head group $-CH_2NH^+{}_3$ methylene proton resonance at about δ 3.14. The 2D COSY spectrum showed the cross peak (δ 3.14; δ 4.02) which identified the $-OCH_2-$ head group methylene protons at δ 4.02.

Some difficulties were encountered in the determination of acidic diacylglycerol phospholipids such as PS, PI and PG. In particular, their head group resonances largely overlapped with the corresponding glycerol and head group signals from non-acidic phospholipids. It was therefore, quite difficult to identify and quantify all the acidic lipids. However, the 2D COSY spectrum was again of great help and permitted us to gain further information on the presence of these lipids. The presence of some of the cross peaks (δ 3.27 and 3.69, and δ 3.39 and 3.66) corresponding to the six inositol ring protons of PI (C1′

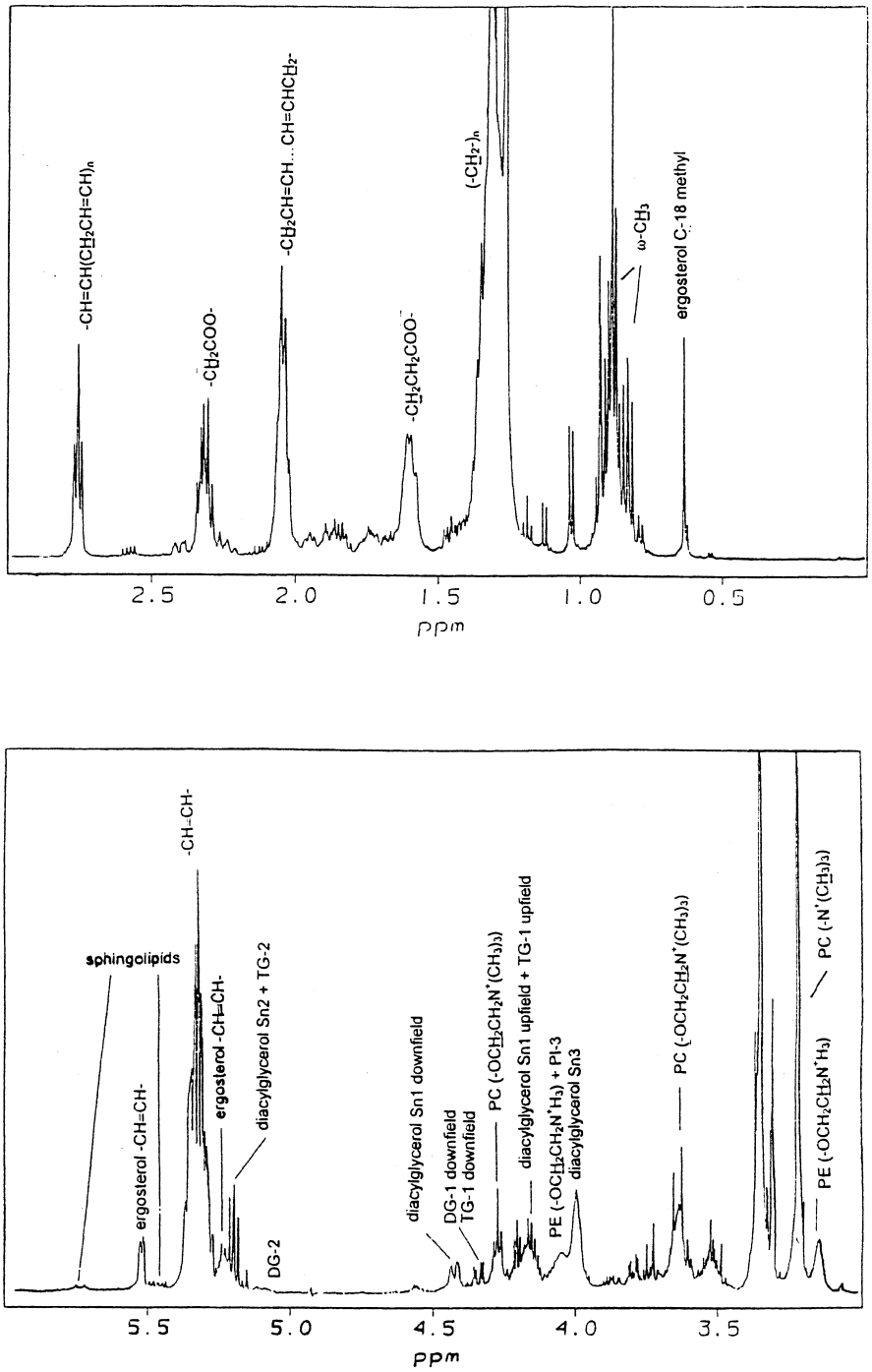


Fig. 1. 1D proton NMR spectrum of lipids extracted from common mushrooms lipids. 128 scans were obtained for Fourier transformation and the residual methanol peak at δ 3.31 was used as the reference chemical shift.

at δ 3.88; C2′ at δ 4.20; C3′ at δ 3.39; C4′ at δ 3.68; C5′ at δ 3.22 and C6′ at δ 3.77) demonstrated a participation of PI above noise level. The lack of detectable PIP, PIP_2, etc., could be attributed to their low concentrations or relative insolubility in the extraction process. The absence of PS or its participation in small quantities to the spectra was assessed by the 2D COSY spectrum (no cross peak at δ 3.98 and 4.28). The glycerol head group (CH_2CHCH_2) of PG is known for a diagnostic set of signal at δ 3.88 (C1′), 3.74 (C3′) and 3.58 (C2′). The two cross peaks at δ 3.60, 3.74 and δ 3.80, 3.60 were thus assigned to PG but it was not possible to confirm the presence of PG using the 1D spectrum, since its features overlapped with other moieties peak. It was more difficult to identify DiPtdGly and PA. The cross peaks that DiPtdGly should have between the $-CH_2CHCH_2-$ pro-

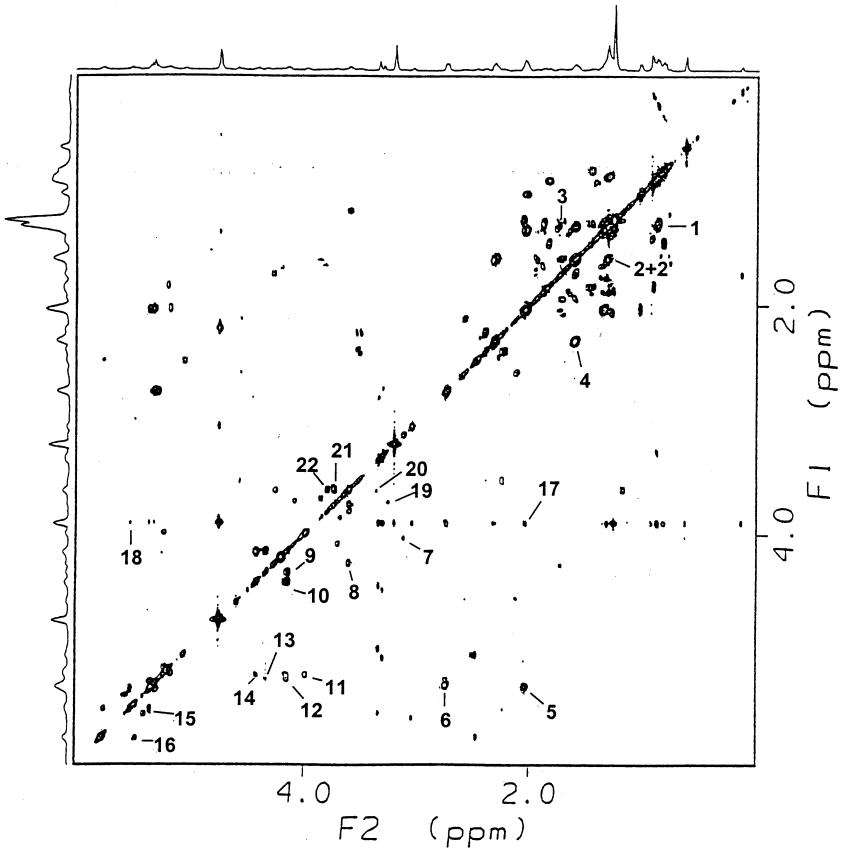
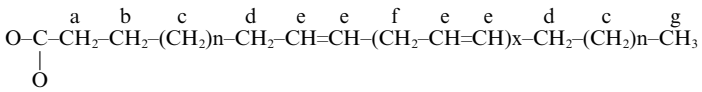


Fig. 2. 2D COSY proton NMR spectrum of lipids extracted from common mushrooms. The 2D spectrum consisted of 4 K data points obtained from 256 FIDs of 16 scans with zero filling in the F1 dimension. 1 (δ 0.87, 1.30): c/g; 2 (δ 1.30, 1.60): b/c; 2′ (δ 1.30, 1.68): b/c for arachidonic acid (–OOC–$(CH_2)_3$–CH=); 3 (δ 1.30, 1.87): d/c; 4 (δ 1.60, 2.32): a/b; 5 (δ 2.05, 5.35): e/d; 6 (δ 2.75; 5.35): e/f; 7 (δ 3.14, 4.02): PE –O$CH_2CH_2NH_3$; 8 (δ 3.62, 4.26): PC –O$CH_2CH_2NH(CH_3)_3$; 9 (δ 4.18, 4.35): TG + DG PL3; 10 (δ 4.18, 4.43): PL3; 11 (δ 3.98, 5.25): PL4; 12 (δ 4.18, 5.25): PL2; 13 (δ 4.35, 5.25): TG PL1; 14 (δ 4.45, 5.25): PL1; 15 (δ 5.35, 5.45): ERG C6/C7; 16 (δ 5.46, 5.72): SL vinyl protons; 17 (δ 3.95, 2.02): SL –CH=CH–CH_2–CH_2–R; 18 (δ 5.72, 3.95): –CH=C*H*–CH_2–CH_2–R; 19 (δ 3.27, 3.69): PI (H4; H5); 20 (δ 3.39, 3.66): PI (H3; H4); 21 (δ 3.60, 3.74): PG head group glycerol PL4; 22 (δ 3.80, 3.60): PG head group glycerol PL1 and PL2. The letters a, b, c, etc., were used to designate fatty acyl chain protons as shown in:



e/d means the cross peak between downfield protons e and upfield proton d of a fatty acid. PC: phosphodiacylglycerocholine; PE: phosphodiacylglyceroethanolamine; SL: sphingolipids; TG: triglycerides; DG: diglycerides; ERG: ergosterol; PL1: cross peak between glycerol Sn1 downfield and Sn2 protons; PL2: cross peak between glycerol Sn1 upfield and Sn2 protons; PL3: cross peak between glycerol Sn1 upfield and downfield protons; PL4: cross peak between glycerol Sn2 and Sn3 protons.

tons of its diacylglycerol moieties (at δ 3.98, 4.17, 4.42 and 5.21) were difficult to distinguish from the corresponding diacylglycerol moiety cross peaks of PE, PC, PG and PI. However, the diagnostic cross peak (δ 3.84, 3.98) corresponding to the degenerate Sn1 and Sn3 proton signals from the central glycerol backbone moiety, was not observed on the 2D COSY spectrum, implying that DiPtdGly was, if present, at a concentration below noise level. PA could not be easily distinguished from the 2D COSY cross peaks corresponding to its diacylglycerol moiety protons (at δ 3.97, 4.46, 4.17 and 5.27) and therefore no quantitative analysis could be carried out on this entity at this stage.

2.2. Neutral acylglycerols

TG (triglyceride) showed its characteristic Sn1,3 downfield and upfield cross peaks at δ 4.35, 4.18 and Sn1,3 downfield and Sn2 δ 4.35, 5.25 in the 2D COSY spectrum but its 1D spectrum features overlapped with other moieties peaks. However, the multiplet at δ 5.15 corresponding to the Sn2 moiety of diglycerides (DG) yielded its quantity in the mixture and the amount of TG could be deduced easily from the integral of the multiplet in the δ 4.35 region, corresponding to DG Sn1 and TG Sn1,3 downfield protons and gave a good approximation to the proportions of DG and TG in the mixture. Accord-

ingly, TG was found to be 1.6 times more abundant than DG. Monoglyceride (MG) resonances (at δ 3.50 and 4.00) overlapped with other features of the spectrum making their determination impossible.

2.3. Ether lipids

These are glycerophospholipids in which a fatty acid chain is attached to the C-1 oxygen of the glycerol moiety by a saturated or vinyl *ether* rather than *ester* linkage. This C-1 o-alkyl chain can be involved in saturated or vinyl ether linkages in which the C–C double bond is contiguous with the ether oxygen. The head groups are usually ethanolamine or choline.

The chemical shifts used to distinguish the saturated ether lipids are those of the $RC^{b}H_2C^{a}H_2OC^{1}H_2C^{2}H$– moiety. The absence of a, b cross peaks at δ 3.44, 1.59 on the 2D COSY spectrum were consistent with the low abundance of this class of saturated ether lipids (Weete et al., 1985). Similarly, the cross peaks for the a, b and c protons of the $RC^{d}H_2C^{c}H_2C^{b}H{=}C^{a}HOC^{1}H_2C^{2}H$– moiety were not observed at δ 5.91, 4.33 (a/b cross peak), δ 5.91 and 2.00 (b/c cross peak), δ 4.33 and 2.00 (a/c cross peak); nor were the glycerol moiety cross peaks at δ 3.92, 5.16 and δ 3.87, 5.16. It was concluded therefore that neither saturated (alkyl–acyl glycerolipids) or unsaturated ether lipids (alkenyl–acyl glycerolipids) were present at concentrations above noise level (0.1%).

2.4. Sphingolipids

These lipids contain the base 4-sphinganine or 4-sphingenine (sphingosine) instead of glycerol although other bases may be present. Other sphingolipids, the cerebrosides and gangliosides, have the phospho head groups replaced by carbohydrate moieties. These latter classes were not present in our extract.

Sphingenine lipids were identified and quantified by their specific vinyl proton resonances of the sphingenine moiety at δ 5.75 (multiplet) and δ 5.48 (quartet). The coupling of these vinyl protons was confirmed by the cross peaks in the 2D COSY. In addition, it was possible, to distinguish if these sphingolipids possessed a choline or ethanolamine head group by checking for an eventual splitting of the choline resonance at δ 3.22. No splitting was observed, therefore, the sphingolipids present in the mixtures were identified as being mainly ceramide phosphoethanolamines.

2.5. Sterols

The only member of this class fully identified here was ergosterol. Its percentage was easily calculated from the high-field C_{18} methyl resonance at δ 0.63 in the 1D spectrum (Casu et al., 1991; Adosraku et al., 1994). This was confirmed with other ergosterol-specific resonances at δ 1.04 (C-21 methyl) and δ 5.46 (C-6 proton). The cross peaks on the 2D spectrum between the C-6 and C-7 protons (δ 5.46, 5.72) agreed with these resonances. Ergosterol proved to be the major lipid in the mushroom extract as already reported by De Simone et al. (1979), Senatore (1988) and Solberg (1989). However, the extensive number of sterol methyl resonances between δ 0.5 and δ 1 on the NMR spectrum (Fig. 1) demonstrates clearly the presence of a large number of sterols in a not negligible concentration, which was estimated at ca 5% of the total lipids. Individual NMR spectra reported by De Simone et al. (1979), Yokokawa and Mitusuhashi (1981) and Kobata et al. (1994), suggest that some of these sterols could be ergost-7-en-3β-ol, ergosta-7,22-dien-3β-ol and ergosta-5,7-dien-3β-ol (peaks at δ 0.54, 0.78 and 0.86). Further sterol analysis could be performed by GC–MS.

Table 1 summarises the calculations and shows that PC and PE were the predominant phospholipids and represented 76% of the glycerophosphatides, in a PC/PE ratio of 1.14. This was in agreement with Holtz and Schisler (1971) and Weete et al. (1985) who found PC and PE equivalent to approximately 70% of the total glycerophosphatides and a PC/PE ratio at 1.16. In agreement with Weete et al. (1985), neither ether phospholipids nor lysophospholipids (δ 4.97) (Yeboah et al., 1995) were detected. Although overlap in the 1D proton NMR spectrum did not permit the precise quantification of the acidic phospholipids, simple subtraction of the PC, PE integrals from the total Sn1 upfield proton one at δ 4.41) that they represented 12% of the total phospholipids.

2.6. Analysis of the fatty acid chains

The number of fatty acid chains was determined from the 1D NMR spectrum using the area of the $-CH_2COOR$ resonances at δ 2.31 as 100% of fatty acid chains. The $-CH_3$ resonances at δ 0.86 can also be a measurement of the total fatty acid chains, but numerous overlaps with peaks from sterol moieties in the δ 0.8–0.9 region made the $-CH_2COOR$ resonances a better choice.

The presence of polyunsaturated fatty chains is normally indicated by the overlapping resonances at ca. δ 2.80. These signals arise from the allylic methylene protons within the series of double bonds in the chain [–CH=CH–CH_2–(CH=CH–CH_2–)$_n$] with $n \geq 1$. For linoleic acid, $n=1$, and its specific methylene gave rise to a triplet at δ 2.75 (Casu et al., 1991; Adosraku et al., 1994). It was thus distinguishable from the overlapping region defined by the other PUFAs methylene resonances. As expected (Holtz & Schisler, 1971; Byrne & Brennan, 1975; Senatore, 1988; Solberg, 1989), the mushroom extract (Table 2) contained almost exclusively linoleic acid and little if any saturated fatty acids or higher PUFAs, since the triplet at δ 2.75 was very well resolved while the region at δ 2.80 showed peaks corresponding

Table 1
Agaricus bisporus lipid composition determined from NMR integrals of characteristic signals

Lipids	Chemical shifts (δ, ppm)	Mol% of total lipids	Mol% of total glycerophosphatides
Total diacylglycero-phospholipids	4.41	53	100
PC	3.22	21	41
PE	3.14	19	36
Total acidic phospholipids	4.41	12	24
PI	3.88	–	–
PS	4.28	–	–
DiPtdGly	3.98	–	–
PA	4.46	–	–
PG	3.74	–	–
TG	4.35	12	NA
DG	5.1	8	NA
MG	4	–	NA
Ether lipids	5.91	<1%	<1%
Sphingolipids	5.48; 5.75	2	NA
Ergosterol	0.63	21	NA
Other sterols	0.54–0.78	5	NA

NA: not applicable.

Table 2
Fatty acid composition of *Agaricus bisporus* as determined from NMR integrals

Fatty chain	Chemical shift (ppm)	% of total fatty chains
Total unsaturated	2.04	91
Total saturated	–	9
Linoleic acid (18:2)	2.75	75
Linolenic acid (18:3)	0.95	–
Arachidonic acid (20:4)	1.65	–
Docosahexaenoic acid (22:6)	2.38	–
Monounsaturated	–	16
Total chains	2.31	100

(C:*n*) denotes fatty acid chains with C carbons and *n* double bonds. Integrals of characteristic resonances were compared to that of the –CH_2COO– resonance at about 2.3 ppm which represented total fatty chains.

to higher PUFAs of less than 1% of the linoleic acid. The crosspeak at δ 5.33 and 2.75 on the 2D COSY spectrum Fig. 2 confirmed coupling between the methylene and vinyl protons of this unsaturated fatty acid.

The diverse PUFAs have diagnostic resonances related to the number and the position of their carbon–carbon double bonds (Casu et al., 1991). Linolenic (18:3) (ω-CH_3 at δ 0.95 characteristic of *n*-3 fatty acids), arachidonic acid (20:4) (–CH═CH–CH_2–CH_2–CH_2–COO– at δ 1.69) and docosahexaenoic acid (22:6) (–CH═CH–CH_2–CH_2–COO– at δ 2.38) could not be accurately determined from the 1D proton spectrum due to their very low concentrations and overlap with sterol resonances. Total unsaturated chains were calculated at about 90.8% of total fatty chains, using the ratio of integral of peak at δ 2.04 to that of the –CH_2COO– signal at δ 2.30. This confirmed the predominance of unsaturated fatty chains in the mushroom extract. To obtain the proportion of monounsaturated fatty acid was thus a matter of simple subtraction of the total polyunsaturated value from the total unsaturated one.

As a typical feature of most higher fungi and in agreement with most papers (Prostenik et al., 1983; Weete et al., 1985; Mau et al., 1991; Stancher et al., 1992; Abdullah et al., 1994), the lipids extracted from *Agaricus bisporus* presented a high degree of unsaturation and the overwhelming constituent fatty acid was linoleic acid. It is interesting to point out the importance of linoleic acid in mushrooms since it is the precursor of the 'mushroom alcohol' (1-octen-3-ol) which together with the associated C_8 ketones (1-octen-3-one, 3-octanone) constitute the main volatiles and are considered the major contributors to the characteristic mushroom flavour (Kates et al., 1988). The identity of the most abundant monounsaturated and saturated fatty chains were confidently attributed to oleic and palmitic acids, respectively (Solberg, 1989).

2.7. Solid phase chromatography and NMR analysis of the various classes of mushroom lipids

Although the NMR approach for analysing lipids has proved versatile and comprehensive, there are several drawbacks still to be overcome: (1) fatty acid analysis was incomplete and only of total lipid, (2) some low abundance lipids, even in 2D NMR spectra were difficult to identify and quantify and (3) acidic and highly acidic

lipids, such as PI, PS, DiPtdGly, PG, PA were not quantitatively analysed.

Solid phase chromatography extraction was the technique of choice to solve these problems since it provides a rapid and effective way of isolating compounds of interest from complex matrices including blood, urine, foods, environmental samples and pharmaceutical formulations (Gurr & Harwood, 1991; Cruz, Noel-Suberville, & Montury, 1997). Bond Elut ion-exchange chromatography has been efficiently used to separate lipids into 4 fractions: neutral lipids especially glycerides and sterols (fraction 1), non-esterified FA (fraction 2), non-acidic phospholipids (fraction 3) and acidic phospholipids (fraction 4) (Table 3). The 1D proton NMR spectrum of total lipid extract is shown expanded in Fig. 1. Figure 3 shows the comparison of this spectrum with those of fractions 1, 3 and 4. Fraction 2 gave rise to resonances characteristic of free FA. The spectrum, of very low resolution due to very low quantities of the equivalent moieties, is not presented here, but it confirmed, as expected, that most FA are tightly bound or associated with the phospholipids. The efficiency of the Bond Elut fractionation was observed by the presence of marker ergosterol at δ 0.63 in the ^{1}H NMR spectrum of fraction 1 (Fig. 3b) but not in fractions 3 (Fig. 3c) and 4 (Fig. 3d). It was quite obvious, by the absence of their characteristic signals from the spectrum in the neutral lipid fraction 1 (Fig. 3b), that no phospholipids were present. The analysis of ergosterol (the main sterol present) and glycerides was therefore made easier by the absence of overlapping fractions 3 and 4 lipid spectra. The results obtained confirmed those found before separation. In addition, the fractionation enabled the presence of MG (resonance at δ 3.50 for the Sn3 protons and δ 4.00 for the Sn2 proton) to be established, previously not possible due to overlaps. Further confirmation of the efficiency of Bond Elut separation of non-acidic phospholipids (Fig. 3c) from acidic phospholipids was discernible by the absence of choline or ethanolamine head group resonance in the spectrum of fraction 4 (Fig. 3d).

Fraction 3, the non-acidic phospholipids were readily analysed by the absence of overlapping resonances from fraction 1 and 4 (Fig. 3c). Unsaturated sphingoid lipids presence was confirmed as well as the absence of ether lipids.

The acidic lipids (fraction 4) NMR spectrum is presented Fig. 3d. Prior to the fractionation, the analysis of this fraction of lipids was quite difficult due to low concentrations and overlaps of their signal resonances. The presence of PI and PG was confirmed and they could be quantified. Figure 4 presents an expansion of the relevant region from the fraction 4 spectra (Fig. 3d). PS was found to be the predominant acidic phospholipid, in agreement with the findings of Byrne and Brennan (1975). PI and PG were present in lesser quantities. DiPtdGly and PA were still difficult to identify due to overlaps with glycerol backbone resonances and PI head group resonance. Weete et al. (1985) reported PA as the most abundant acidic phospholipid. DiPtdGly represented some 3.5% of *Agaricus bisporus*. These differences

Table 3
Quantification of lipids from common mushrooms determined from the NMR spectra of NH_2-Bond Elut fractions

				A_f			C_f			
Lipids	δ (ppm)	A_i	C_i	F1	F3	F4	F1	F3	F4	R
Total DAGP	4.40	5.2	58	0	3.9	1.1	0	39	12	97
PC	3.22	2.1	24	0	2.1	0	0	21	0	99
PE	3.14	1.9	21	0	1.6	0	0	16	0	87
Total ADAGP	4.41	1.2	14	0	0	1.1	0	0	12	93
PI	3.88	–	–	0	0	0.4	0	0	4	NA
PS	4.28	–	–	0	0	0.7	0	0	7	NA
DiPtdGly	3.98	–	–	0	0	T	0	0	<1%	NA
PA	4.46	–	–	0	0	T	0	0	<1%	NA
PG	3.74	–	–	0	0	0.2	0	0	2	NA
TG	4.35	0.6	7	0.5	0	0	6	0	0	85
DG	5.10	0.4	4	0.3	0	0	3	0	0	74
MG	4.00	–	–	T	0	0	<1%	0	0	NA
Ether lipids	5.9	T	<1%	0	T	0	0	<1%	0	NA
SL	5.75	0.2	3	0	0.2	0	0	2	0	91
Ergosterol	0.63	2	23	1.7	0	0	18	0	0	85
Other sterols	0.54	0.5	5	0.4	0	0	4	0	0	88

δ: chemical shift; A_i: area before separation; C_i: %mol of total lipids, before separation; A_f: Area after separation; C_f: %mol of total lipids, after separation; R: % recovery of each lipid class after separation; F1: fraction 1; F3: fraction 3; F4: fraction 4; total DAGP: total diacylglycerophospholipids; total ADAGP: total acidic diacylglycerophospholipids; SL: sphingolipids; T: traces.

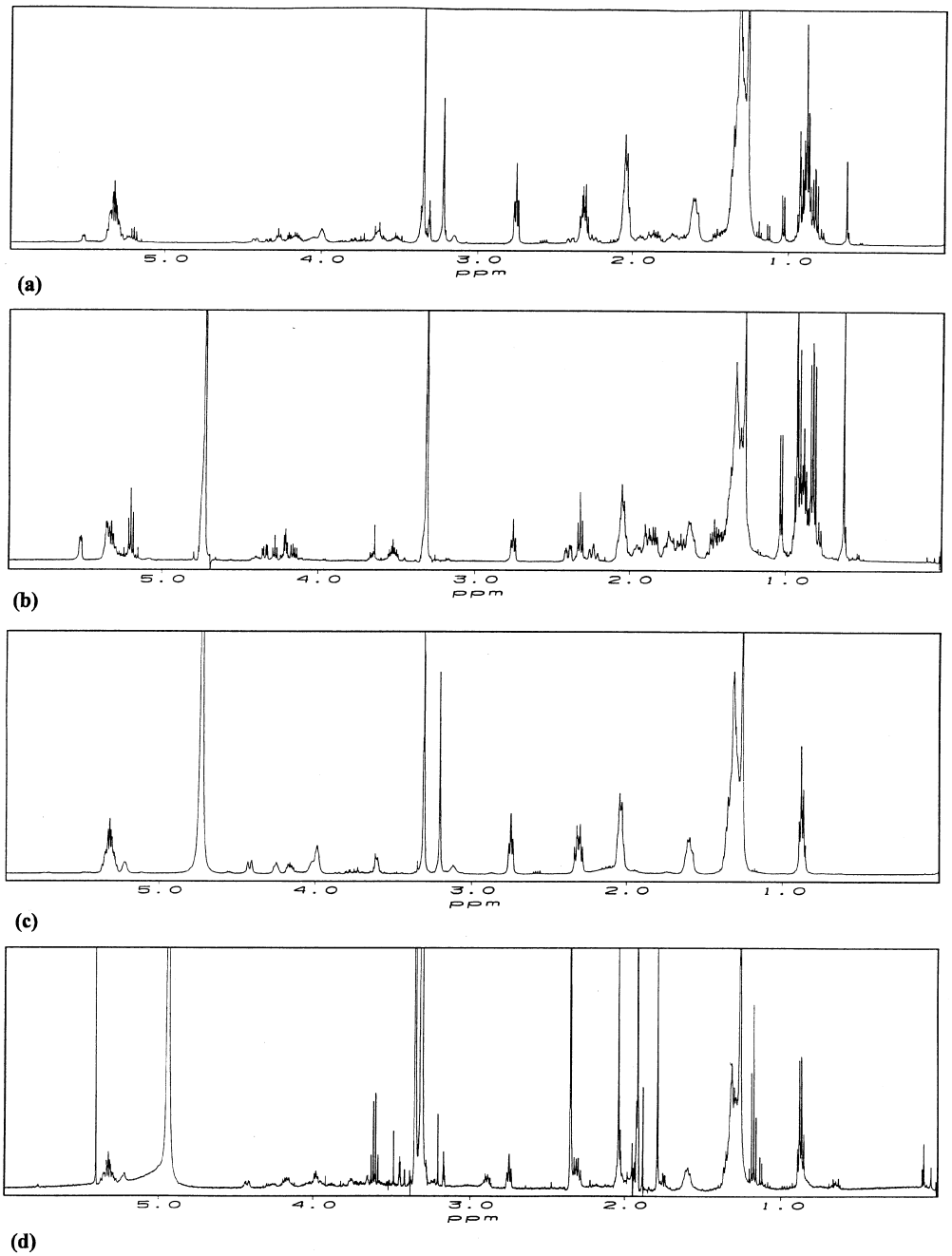


Fig. 3. 1D proton NMR spectra of lipids extracted from common mushrooms. 256 scans were obtained for Fourier transformation and the residual methanol peak at δ 3.31 was used as the reference chemical shift. (a) Total lipid extract, (b) Bond Elut fraction 1: neutral lipids, (c) Bond Elut fraction 3: non-acidic phospholipids and (d) Bond Elut fraction 4: acidic phospholipids.

in the identity rather than the quantity of the acidic phospholipids could be linked to the conditions of growth and/or the genetic differences between the mushrooms used in each case. Weete et al. (1985) cultured the mushrooms as a suspension in a fully synthetic medium, whereas Byrne and Brennan (1975) obtained them directly from commercial mushroom beds.

The fatty acid content of the neutral, non-acidic and acidic phospholipids fractions was calculated and the information obtained confirmed and refined the results we had from the interpretation of the spectra of unseparated extracts (Table 4). Linoleic acid was found to be the predominant unsaturated fatty acid present in all 3 fractions. However, each class of lipids appeared to have different degrees of unsaturation. Holtz and Schisler (1971) compared the fatty acid compositions of 'neutral' (including the non-acidic phospholipids) and 'polar' (acidic phospholipids) lipids and found that polar lipids were richer in polyunsaturated fatty acids but poorer in monounsaturated and saturated ones. Their observations

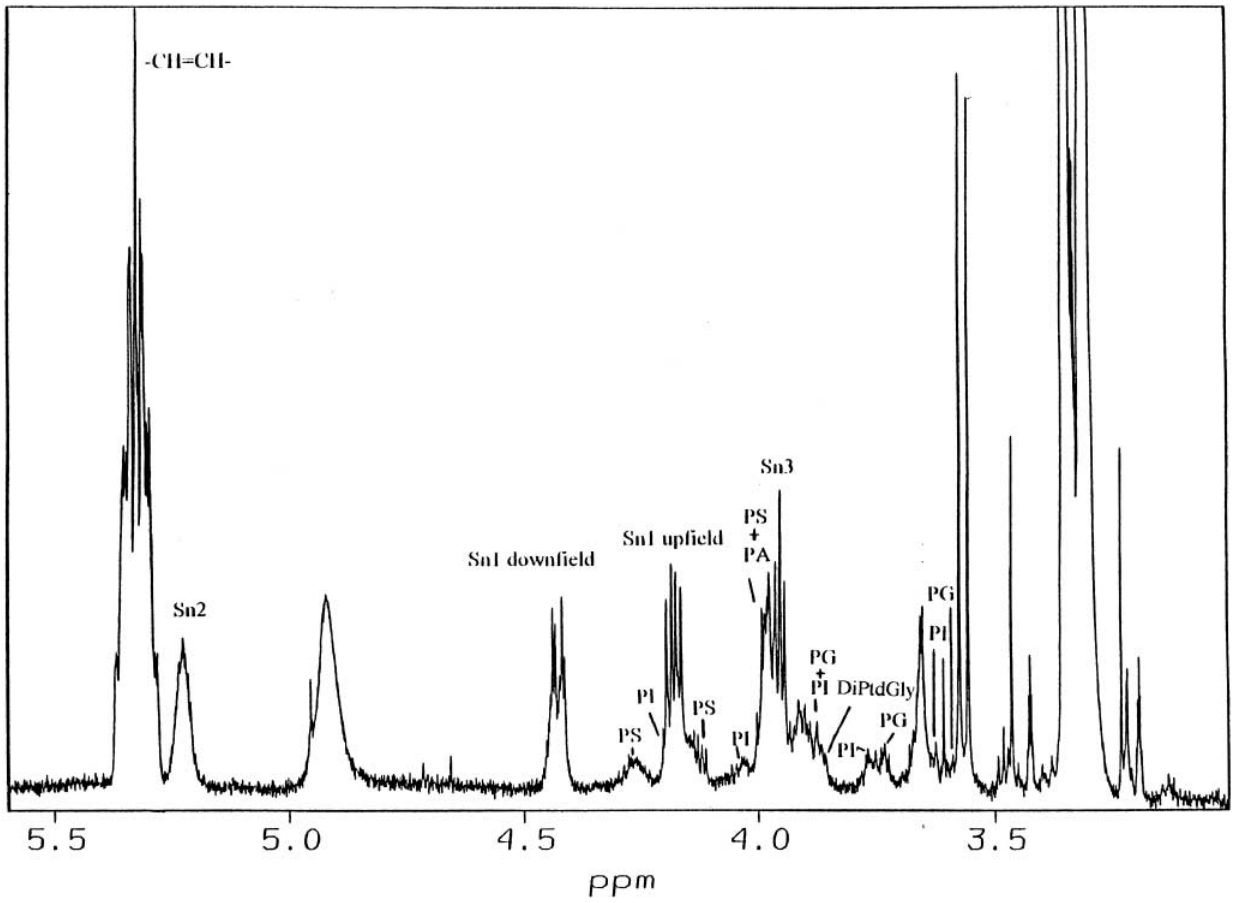


Fig. 4. 1D proton NMR spectra of lipids extracted from common mushrooms: expansion of Bond Elut fraction 4 (acidic phospholipids).

Table 4
Fatty acid analysis of the lipids from common mushrooms determined from the NMR spectra of Bond Elut fractions 1, 3 and 4

				A_f			C_f			
Fatty chains	δ (ppm)	A_i	C_i	F1	F3	F4	F1	F3	F4	R
Total unsaturated	2.04	9.8	91	0.1	7	2.4	88	87.9	93.5	98
Total saturated	–	1	9	0	1	0.2	11.5	12.1	6.5	117
Linoleic acid (18:2)	2.75	8	75	0.1	5.6	2	61.5	70.7	79.7	97
Linolenic acid (18:3)	0.95	–	–	–	T	T	–	<1%	<1%	NA
Arachidonic acid (20:4)	1.65/2.10	–	–	–	T	T	–	<1%	<1%	NA
Docosahexaenoic acid (22:6)	2.38	–	–	–	T	T	–	<1%	<1%	NA
Mono-unsaturated	–	1.8	16	0.1	1.4	0.4	10	26.9	13.8	101
Total chains	2.31	10.8	100	0.2	8	2.6	100	100	100	99

δ: chemical shift; A_i: area before separation; C_i: %mol of total fatty chains, before separation; A_f: area after separation; C_f: %mol of total fatty chains, after separation TR: total % recovery of each fatty chain, after separation in fractions 1, 3 and 4; F1: fraction 1; F3: fraction 3; F4: fraction 4; T: traces; NA: not applicable.

were in agreement with the results obtained for fractions 1 and 3 (respectively, 88 and 88% unsaturated, 12 and 12% saturated; 10 and 27% monounsaturated) as opposed to fraction 4 (94% unsaturated, 7% saturated; 14% monounsaturated). In the non-acidic phospholipid fraction the percentage of saturated fatty acids was 1.9 times higher and there was 1.3 times more mono-unsaturated fatty acids than in the acidic phospholipid fraction.

The major findings of the present lipid analysis of *A. bisporus* can be summarised as follows: (1) the glycero-lipids are the major class of lipids in the total extract; PC,

PE and PS constitute the major ones, (2) no ether lipids were found present in the mixture, (3) linoleic acid was the almost exclusive fatty acid component of the glycerolipids extracted and almost no saturated fatty acid chains were present and (4) ergosterol and other sterols were detected in high quantities. They were not analysed extensively due to the absence of sufficient parent sterols spectra for comparison and analysis.

In conclusion, the proportions of all major lipids as well as their fatty acid chain composition were obtained by analysis of 1D and 2D proton NMR spectra of lipid extracts from *A. bisporus*. Without using any chromatographic or chemical modification procedures, the NMR approach provided a quick and effective analytical tool to determine and quantify many lipids in *A. bisporus*. In addition, the combination of Bond Elut chromatography to the above techniques gave superior qualitative and quantitative analyses and enabled in particular to accurately assess the fatty acid composition of the major lipidic class which is the non-acidic phospholipids.

This spectroscopic method could be of importance in the understanding and the discovery of mechanisms involving mushrooms as potent source of medicinal substances (Khanna et al., 1993; Molitoris, 1994). It could also provide a useful tool for the studies of plant lipid profiles, plant diseases and toxicity and the monitoring of metabolic changes (e.g. ripening processes in plants).

3. Experimental

3.1. Samples

Samples of cultivated mushrooms (*A. bisporus*) were obtained from a local grocery store (London, UK). 200 g of fresh caps were cut into 1–2 cm sized pieces, frozen in liquid nitrogen and ground into powder.

3.2. Extraction of total lipids from cultivated mushrooms

The powdered mushrooms were mixed with 2.5 vol. of $CHCl_3$–MeOH (2:1, v/v) in a glass tube, vortexed and sonicated in an ultrasonic bath at 0° for 20 min. The suspension was filtered through cheese cloth to remove particles and centrifuged at 2000 rpm for 10 min at 4°C to separate the aqueous and organic layers. The water layer and the remaining tissue were re-treated with the same vol. of $CHCl_3$–CH_3OH (2:1, v/v) and the extracts obtained from both steps were combined. The organic layer was washed twice with equal vol. of 0.5 M KCl in 50% MeOH, dried over Na_2SO_4 and filtered through glass wool. The volume of the resulting solution was reduced using a rotor evaporator and then concentrated to dryness under a stream of nitrogen. The lipid residue was redissolved in 0.8 ml of CD_3OD–$CDCl_3$ (2:1, v/v) and transferred to 5 mm NMR tubes, under nitrogen. The tubes were kept at −20°C waiting NMR analysis (Bligh & Dyer, 1959).

3.3. Bond Elut (NH_2-aminopropyl) solid phase separation method

200 mg of the extracted lipids were dissolved in 1 ml of $CHCl_3$. A 0.2 ml aliquot of the solution was then applied to each one of 3 Bond Elut Certify II (200 mg, Varian) column preconditioned with 8 ml of HPLC grade dry hexane and another 0.2 ml aliquot was retained as a control for the determination of recovery after separation. According to their different polarities, lipids were separated into four individual fractions (Folch et al., 1957) by passing different eluents through the column in the following order: (1) $CHCl_3$ (eluted non-polar lipids and sterols), (2) Et_2O with 2% HOAc (eluted non-esterified fatty acids), (3) MeOH (eluted non-acidic phospholipids) and (4) 0.05 M ammonium acetate in $CHCl_3$–MeOH (4:1, v/v) and containing 2% (v/v) of 28% NH_4OH soln (eluted acidic phospholipids). A volume of 2×3 ml of mobile phase was used for all elutions. All column elutions were achieved in 5 min under low-speed centrifugation conditions (500*g*). The acidic phospholipids fraction required additional processing to remove ammonium acetate that is detrimental to high resolution NMR analysis. Consequently, this fraction was dried under a stream of nitrogen in order to avoid oxidation and resuspended in $CHCl_3$–MeOH (2:1, v/v, 3 ml) and distilled H_2O (0.6 ml). The contents were vortexed thoroughly for 15 s and left to partition on ice, to limit oxidation. The upper aqueous phase was removed and discarded. $CHCl_3$–MeOH–H_2O (3:48:47, by vol., 1.4 ml) was added to the organic phase and the procedure repeated once. The combined resulting lower organic phase from fraction 4, the 3 eluates from fraction 1 to 3, and the 0.2 ml unseparated lipid control extract were evaporated under a stream of nitrogen. The residues were then resuspended in 0.6 ml of CD_3OD–$CDCl_3$ (2:1, v/v) and transferred to 5 mm NMR tubes.

3.4. ^1H NMR of extracted lipids

Samples were bubbled with nitrogen prior to recording the spectrum in order to avoid oxidation. All NMR spectra were determined using a Bruker AM 600 NMR spectrometer. Chemical shifts were referenced to the residual methanol peak at δ 3.31.

For the 1-dimensional NMR determination, a 45° pulse was applied with solvent presaturation during relaxation to remove excess HOD signal at about δ 4.7. The FID was acquired with 16 K data points in the Fourier transform mode; the temperature was regulated at 298 K.

The 2-dimensional COSY experiment was performed on total lipid extracts in the non-phase sensitive mode.

Connectivity among protons was established with the magnitude COSY pulse sequence with solvent persaturation. 2D COSY experiments were performed at 298 K by using standard pulse sequence, a spectral width of 6714.113 Hz and a relaxation delay of 2.0 s. They were recorded in 4096 data points obtained from 256 FiDs of 16 scan each, with zero filling in the F1 dimension. Water persaturation was performed during relaxation to remove excess HOD signal. The persaturation power was then switched to a minimum level during pulses and evolution to hold the saturation.

3.5. Calculation of lipid proportion

Chemical shifts were identified as described elsewhere (Casu et al., 1991; Adosraku et al., 1994; Yeboah et al., 1995) and from the 2D COSY spectra of the lipid extracts. After baseline correction, characteristic peaks in the 1D NMR spectra were integrated. The integrals directly related to the amounts of each lipid present, correcting for any signal overlap. The number of protons giving rise to the signal was considered in the calculations. In estimating the fatty acid composition of phospholipids, the integral at ca. δ 2.3 was taken as a measure of total fatty chains. The integrals of individual fatty acid were compared to this value.

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