Solid-State ^{13}C CP MAS NMR Spectroscopy of Mushrooms Gives Directly the Ratio between Proteins and Polysaccharides

Laura Pizzoferrato,*[†] Pamela Manzi,[†] Fabio Bertocchi,[‡] Corrado Fanelli,[§] Giuseppe Rotilio,[†,⊥] and Maurizio Paci[‡]

National Institute for Food and Nutrition Research, via Ardeatina 546, 00178 Rome, Italy; INFM and Department of Chemical Science and Technology, Tor Vergata University, via Ricerca Scientifica 1, 00133 Rome, Italy; Department of Plant Biology, La Sapienza University, Largo Cristina di Svezia 24, 00165 Rome, Italy; and Department of Biology, Tor Vergata University, via Ricerca Scientifica 1, 00133 Rome, Italy

The solid-state ^{13}C CP MAS NMR technique has the potential of monitoring the chemical composition in the solid state of an intact food sample. This property has been utilized to study mushrooms of different species (*Pleurotus ostreatus*, *Pleurotus eryngii*, *Pleurotus pulmunarius*, and *Lentinula edodes*), already characterized by chemical analyses for protein and dietary fiber components. Solid-state ^{13}C CP MAS NMR spectroscopy reveals a large difference in the ratio between the glucidic and the proteic resonances probably depending on the mushroom species. An accurate inspection by model compounds and suitable mixtures of proteins and saccharides gives a methodology to interpret these experimental data. A good correlation ($R^2 = 0.93$; $R^2 = 0.81$) has been obtained by comparing the NMR data with the results of the chemical analyses. The results suggest the possibility to perform a taxonomic study and/or a nutritional study on the basis of the ratio between protein and polysaccharide levels determined by NMR or chemical methodologies.

Keywords: *Mushrooms; proteins; polysaccharides; ^{13}C CP MAS NMR*

INTRODUCTION

Edible mushrooms represent an interesting food item that can contribute, in spite of the great variability observed among species, to the formulation of a well-balanced diet.

They are healthy foods, low in calories and in fat, and rich in vegetable proteins of good quality, chitin, vitamins, and minerals and represent an increasing share of the Italian diet (Manzi et al., 1999a). Furthermore, of the nearly 700 known edible species of mushrooms, about 200 may have medicinal value and can be considered functional foods (Chang, 1996). In particular *Lentinula, Pleurotus*, *Auricularia*, *Flammulina*, *Tremella*, *Hericium*, and *Grifola* mushrooms have been reported to show various degrees of immunomodulatory, lipid lowering, and antitumor effects without any significant toxicity (Chang, 1996). One of the most active functional components is the dietary fiber fraction, and in particular, chitosan (deacetylated chitin) and β-glucans have been shown to have cholesterol-lowering activity (Sugano et al., 1988; Bobek et al., 1996; Bajaj et al., 1997), in some cases dose-dependent (Bobek et al., 1997).

The increasing interest in functional ingredients suggests the opportunity to undertake new studies on this traditional food item, with the specific aim to extend knowledge on the dietary fiber fraction, utilizing novel but rapid methodologies.

This study was carried out by solid-state ^{13}C CP MAS NMR, which can perform NMR spectroscopy at high resolution in the solid state, showing easily recognizable bands due to individual carbons of polysaccharide and protein components. This NMR technique has been utilized in model systems (protein and polysaccharide reference compounds and their mixtures) with the aim to make the quantitative evaluation of the protein/saccharide ratio reliable. Finally, the value of the protein/polysaccharide ratio, obtained by this NMR procedure in mushrooms, has been correlated with the relevant results obtained by chemical analysis. The research demonstrates that the CP MAS NMR solid-state technique is able to directly and reliably quantify this ratio in edible mushrooms.

MATERIALS AND METHODS

Samples. The mushroom strains used were *Pleurotus sajor-caju* (Fr.) Singer (SMR 124), *Pleurotus ostreatus* (Jacq: Fr) P. Kumm (SMR 127, SMR 128, SMR 129), *Pleurotus ostreatus florida* (SMR 131, SMR 132), *Pleurotus sapidus* (SMR 130), *Pleurotus eryngii* (DC:Fr.) Quél (SMR 172, SMR 173), and *Lentinula edodes* (Berk.) Singer (SMR 90), belonging to the Mushroom Collection of the National Research Council (Montelibretti, Roma-I), and cultivated in an Italian farm (Italmiko, Senise-Potenza, Italy). The *Pleurotus* spp. and *Lentinula* mushroom analyzed were grown on the same culture medium for the production of the fruiting bodies. The culture medium was prepared by using wheat straw (85% w/w) and sugar beet wastes (15% w/w). The growing techniques applied were previously reported by Quimio et al. (1990).

Other mushrooms such as *Volvariella volvacea* (Fr.) Singer (VGMG), *Armillariella mellea* (Vahl: Fr) Kummer (AMMG),

* To whom correspondence should be addressed: Phone: 39.06.5032412. Fax: 39.06.5031592. E-mail: pizzof@inn.ingrm.it.

[†] National Institute for Food and Nutrition Research.

[‡] INFM and Department of Chemical Science and Technology, Tor Vergata University.

[§] La Sapienza University.

[⊥] Department of Biology, Tor Vergata University.

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and *Hydnum repandum* L:Fr. (HAMG) were also considered, as a comparison, to complete the NMR study even if the cultured conditions of these fungal samples were not specified by the producer.

Mushrooms were dried immediately after being harvested and then analyzed. Samples for solid-state NMR spectroscopy were prepared by cutting a small cylindrical amount of the carpophore and directly inserting it into the CP MAS rotor.

Methods. Total amino acid analysis was carried out according to the method of Spackman et al. (1958) by ion-exchange chromatography after protein hydrolysis. Methionine and cysteine were preliminarily oxidized by performic acid to convert methionine into methionine sulfone and cysteine into cysteic acid (Schram et al., 1954). Tryptophan determination was carried out by isocratic reversed-phase high-performance liquid chromatography and fluorescence detection after alkaline hydrolysis (Steven and Jorg, 1989).

Dietary fiber was determined according to the enzymatic–gravimetric method of Prosky et al. (1988). For the protein correction of the dietary fiber amount, the factor 4.38 converting nitrogen to protein was utilized (Manzi et al., 1999b).

The chitin content was measured as *N*-glucosamine released after acid hydrolysis (6 N HCl for 24 h) and analyzed during the ion-exchange chromatographic run of amino acids (Manzi et al., 1999b).

All the quantities of lysozyme and cellulose, used as model systems, were calculated as the number of monomer moles. For cellulose the glucose residue (molecular weight 162) was considered. For lysozyme a mean molecular weight of amino acid residues, obtained by dividing the protein molecular weight by the number of residues (i.e., 14300/129 = 110.9), was utilized to calculate the number of amino acid moles in the weighed amount of protein.

Evaluation of the protein/polysaccharide ratio was obtained by integration of resonances including, for carbonyl resonances (170 ppm), the spinning sidebands. The value of the integrals was used without corrections. Other representative resonances were considered: the methyl resonances (20 ppm) for proteins, the CH anomeric carbon (105 ppm) and the CH at about 90 ppm for the resonances from polysaccharides. The correspondence between the ratio and concentration was tested by executing spectroscopy of solid mixtures of a reference protein (lysozyme) and polysaccharide (cellulose). Fixed amounts of lysozyme and cellulose were weighed and finely mixed in the solid state. Different amounts of lysozyme and cellulose were also considered separately utilizing silicon oxide to fill the rotor to the same extent. The FIDs were treated with an exponential multiplication to obtain a line broadening similar to that of the spectra under study.

Simulation of spectra was obtained by summing the Lorentzian line shapes for the chemical shift values of the resonances of the amino acids and saccharides (Saito, 1986) with the intensities proportional to the effective concentration in the sample. A line broadening similar to that obtained in the experimental spectra was applied.

Reagents. All reagents for laboratory determinations from C. Erba (Milan, Italy) were of analytical or HPLC grade as required.

Lysozyme from chicken egg and cellulose used for solid-state calibration were purchased from Sigma Chemical Co. (St. Louis, MO).

Apparatus. ^{13}C CP MAS NMR spectroscopy was performed by a Bruker instrument, AM 400, operating at 100.56 MHz in the magic angle cross-polarization mode. The contact time between proton and carbon magnetization was 1.2 ms, and the number of scans was 1024. The repetition rate was about 3.5 s for every scan. The rotation was typically set at about 4000 Hz.

RESULTS AND DISCUSSION

The shape of resonances of the ^{13}C CP MAS NMR spectra upon changing the source of mushrooms has been examined. In Figure 1 the ^{13}C CP MAS NMR spectra of different samples of the species *P. ostreatus* are reported. In particular the resonances are quite similar, and only slight changes in the relative intensity can be observed. Only in the spectrum of sample SMR 131 (*P. ostreatus florida*) is a slight increase of the resonance at 53 ppm observable.

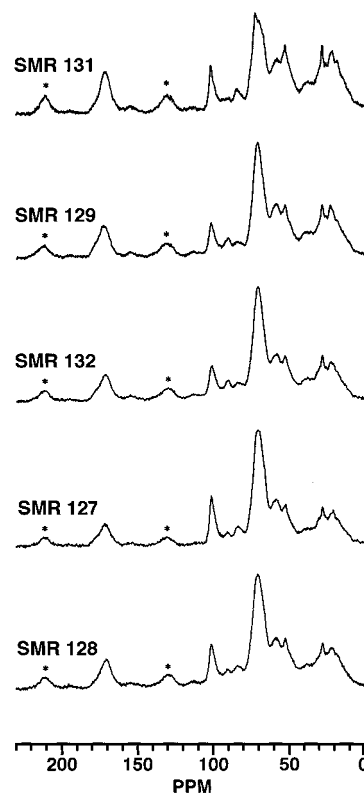

Figure 1. ^{13}C CP MAS NMR spectra of samples of *P. ostreatus* (SMR 131, SMR 129, SMR 132, SMR 127, SMR 128). Spectra have been obtained as reported in the Materials and Methods. Asterisks indicate the spinning sidebands. Assignments of resonances: CO, 170 ppm; aromatics, 120 ppm; C1 of carbohydrates, 105 ppm; CH proteins and carbohydrates, 90–70 ppm; CH_2, 60–40 ppm; CH_3, 30–10 ppm. More details are given in the Materials and Methods where the simulations of the ^{13}C CP MAS NMR spectra are described.

In the spectra the bands due to the carbons of the amino acids of proteins are clearly distinguishable from those due to the polysaccharides where a single resonance at about 105 ppm is attributed to the anomeric C1 of polysaccharidic moieties and the bands around 90 ppm are due to the remaining carbons of the glucidic moiety (Saito, 1986). The resonance at 53 ppm is characteristic of methoxyl groups of lignins (Jung and Himmelsbach, 1989; Hatcher, 1987; Cyr et al., 1988; Gerasimowich, 1984) but is not particularly intense. The assignment of resonances is reported in the legends to the figures also on the basis of the chemical shifts of carbons of amino acids reported in the literature (Saito, 1986).

In Figure 2 two samples of *P. sajor-caju* (SMR 124) and *P. sapidus* (SMR 130) are reported. The spectra are different from those reported in Figure 1; in particular resonances due to protein components (170 ppm) are weaker than the previous ones, and the spectra show that sample SMR 130 has a poorer protein content than sample SMR 124. The relative intensities of the resonances are markedly different, indicating that the protein/polysaccharide ratios are dependent on the

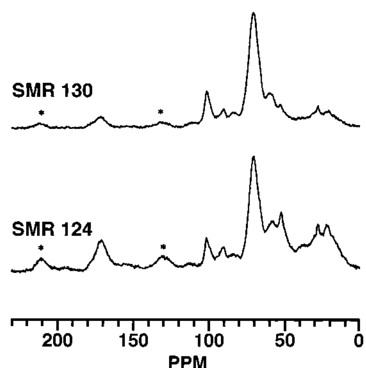

Figure 2. ^{13}C CP MAS NMR spectra of samples of *P. sapidus* (SMR 130) and *P. sajor-caju* (SMR 124). Spectra have been obtained as reported in the Materials and Methods.

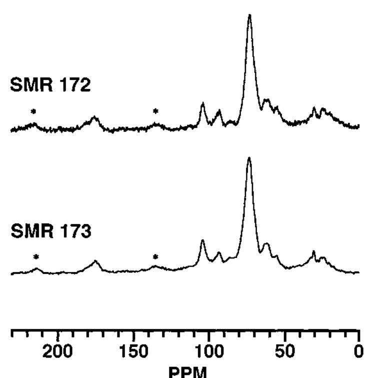

Figure 3. ^{13}C CP MAS NMR spectra of samples of *P. eryngii* (SMR 172, SMR 173). Spectra have been obtained as reported in the Materials and Methods.

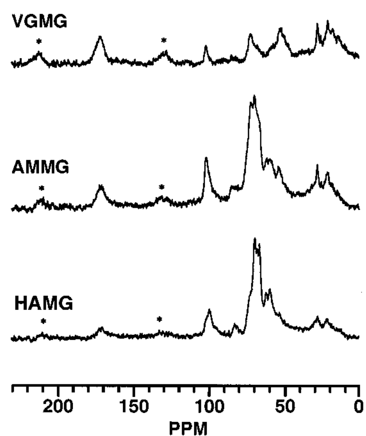

Figure 4. ^{13}C CP MAS NMR spectra of fungi *V. volvacea* (VGMG), *A. mellea* (AMMG), and *H. repandum* (HAMG). Spectra have been obtained as reported in the Materials and Methods.

species of mushrooms studied. This observation was further confirmed by examining the spectrum reported in Figure 3, where two other isolates of *Pleurotus* are reported. The *P. eryngii* samples analyzed show similar shapes, a high content of polysaccharides, and a low content of proteins.

Also in the other fungi analyzed some differences in the spectra appear evident (Figure 4). In fact this varies from the very low content of proteins in HAMG with a very high polysaccharide content, to a more equivalent ratio between the two as in AMMG, to finally a very low polysaccharide content in VGMG. The spectrum of *Lentinula* in Figure 5 also indicates a further difference

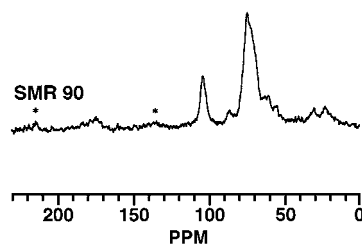

Figure 5. ^{13}C CP MAS NMR spectra of *L. edodes* (SMR 90). Spectra have been obtained as reported in the Materials and Methods.

Table 1. Relative Intensity of Selected Resonances of the ^{13}C CP MAS NMR Spectra of Mushrooms

label	species	170 ppm	105 ppm	90 ppm	20 ppm
SMR 127	*P. ostreatus*	12	7	37	22
SMR 128	*P. ostreatus*	20	8	40	27
SMR 129	*P. ostreatus*	24	7	35	35
SMR 131	*P. ostreatus florida*	23	6	24	33
SMR 132	*P. ostreatus florida*	13	4	22	17
SMR 172	*P. eryngii*	11	5	41	17
SMR 173	*P. eryngii*	10	5	40	10
SMR 90	*L. edodes*	20	18	68	15
SMR 130	*P. sapidus*	12	9	51	15
SMR 124	*P. sajor-caju*	25	6	35	27
VGMG	*V. volvacea*	25	3	11	42
AMMG	*A. mellea*	17	8	34	16
HAMG	*H. repandum*	10	6	27	12

from all the samples above with a very high content of polysaccharides. The relative intensity of the polysaccharidic and proteic components in the different samples has been calculated, and the results are reported in Table 1.

An attempt to obtain the complete fitting of some of the spectra was performed by computer simulation using the chemical shifts of the resonances of carbons of amino acids and those for polysaccharides (Saito, 1986), and the experimental data of amino acid and polysaccharide compositions for the different mushrooms (Manzi et al., 1999a,b). This procedure produced spectra (not shown) that have been compared to those of Figures 1 and 2. The rough similarity obtained indicated that no other macroscopic component is present in the samples, but the multiplicity and dispersion of resonances produces a nonregular line broadening different from one resonance to another, thus giving spectra which are too difficult to fit with a few mathematical operations. As a consequence, this kind of approach was demonstrated to be not feasible without a time-consuming fitting and moreover subject to large errors. For testing the applicability of the NMR techniques to the study of polysaccharide and protein components of mushrooms, a direct approach has been performed using the NMR resonance integrated intensity of resonances.

Calibration of the Protein/Saccharide Ratio. The correspondence of NMR data to the effective molar concentration was checked by using a model for the protein/polysaccharide ratio, able to overcome the different intensities obtained in the solid-state ^{13}C NMR spectra from nonprotonated, singly protonated, and doubly protonated carbons. In fact the cross-polarization, which allows the carbon resonances to become visible in the spectra, is due to the magnetization transfer from protons to carbons and has a very different efficiency in the case of different carbons. This fact can preclude a direct proportionality between integrated resonances and the molar concentrations without direct

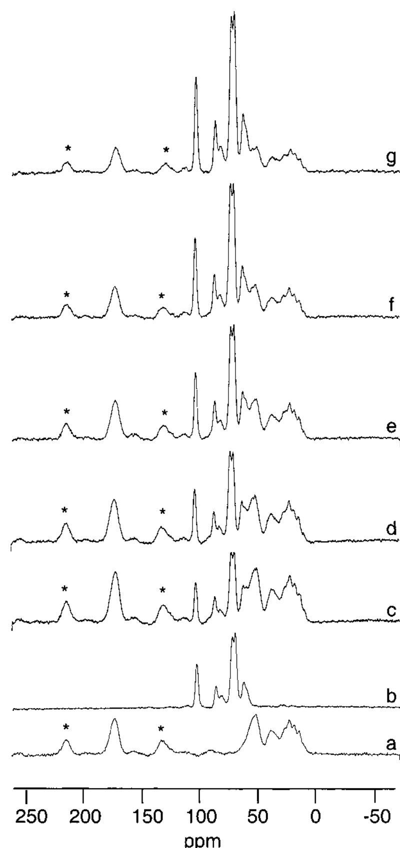

Figure 6. ^{13}C CP MAS NMR spectra of a solid mixture of lysozyme and cellulose at different molar ratios: lysozyme alone (a), cellulose alone (b), mixtures of lysozyme and cellulose at molar ratios of 0.4994 (c), 0.7763 (d), 1.136 (e), 1.623 (f), and 2.319 (g). The ratio 3.394 has not been reported here but is included in the calculations. Bands labeled with asterisks are spinning sidebands. Spectra have been obtained as reported in the Materials and Methods.

evidence that these effects can be overcome and compensated by a calibration curve in a model system.

Furthermore, the study of model compounds has also been undertaken to check the reliability of the protocol of measuring the ratio between protein and saccharides directly by a simple integration of the solid-state spectrum. In Figure 6 the NMR spectra of lysozyme (a), cellulose (b), and their mixtures (c−g) are shown. A linearity test performed between the amount in moles of lysozyme and the relevant resonances (CO at 170 ppm and CH_3 groups at 20 ppm) or the amount in moles of cellulose and the relevant resonances (anomeric CH at 105 ppm, other CH groups at 90 ppm) has shown a very good correlation ($R^2 = 0.97-1.00$) both in separated standards and in their mixtures.

The correlation between the values of the ratio between selected resonances versus the lysozyme/cellulose molar ratio is of similar quality, and also in this case the correlation is excellent ($R^2 = 0.99-1.00$). In particular the resonance ratios measured have been 20 ppm/90 ppm, 170 ppm/90 ppm, 170 ppm/105, and 20 ppm/105 ppm. The best correlation between quantitative molar data and resonance integrals is the ratio between methyl resonances and the CH anomeric carbons (20 ppm/105 ppm) and the ratio between methyl resonances and other polysaccharide carbons (20 ppm/90 ppm) both with a correlation coefficient $R^2 = 1.00$. This observation does not mean that the correlation should be directly proportional with a slope of 1. In fact a linear fit gives

Table 2. Molar Ratio[a] between the Protein and Glucidic Components Chemically Determined in Mushrooms

label	species	protein/dietary fiber	protein/chitin
SMR 127	*P. ostreatus*	0.77	7.24
SMR 128	*P. ostreatus*	1.21	9.68
SMR 129	*P. ostreatus*	1.59	9.24
SMR 131	*P. ostreatus florida*	1.06	9.25
SMR 132	*P. ostreatus florida*	0.68	7.05
SMR 172	*P. eryngii*	0.93	7.00
SMR 173	*P. eryngii*	0.94	5.53
SMR 90	*L. edodes*	0.38	3.45
SMR 130	*P. sapidus*	0.73	6.48
SMR 124	*P. sajor-caju*	0.82	7.66

[a] The molar ratio has been obtained considering the actual number of moles of amino acids for proteins and the number of moles of glucosamine for chitin or the number of moles of glucose for the whole dietary fiber content.

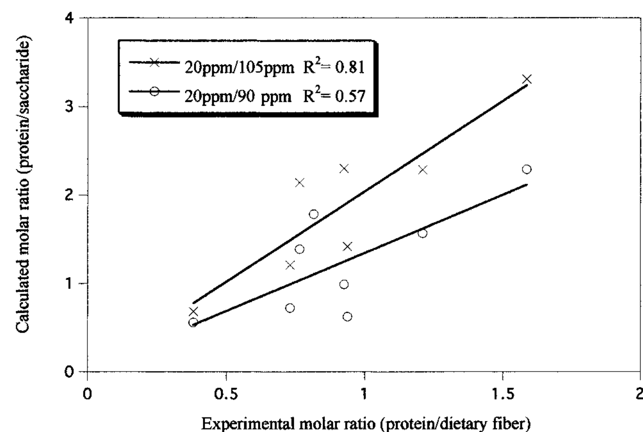

Figure 7. Correlation plot between the experimental molar ratio (protein/dietary fiber) obtained by the chemical analysis data and the molar ratio (protein/saccharide) calculated by the NMR resonance integrals according to eqs (a) and (b).

a linear proportionality as follows:

$$\text{protein/saccharide (mol)} = 0.63(\text{NMR resonance integral 20 ppm/105 ppm}) + 0.16 \quad \text{(a)}$$

$$= 2.22(\text{NMR resonance integral 20 ppm/90 ppm}) + 0.07 \quad \text{(b)}$$

The proportionality constant can thus be used for obtaining the protein/saccharide ratio in other samples.

Protein/Saccharide Ratio in Mushrooms. The NMR resonance integrals in the studied samples of mushrooms are reported in Table 1. Utilizing the above-reported eqs (a) and (b), the ratio between the protein and glucidic components has been calculated on the basis of the ratio between the selected signals (20 ppm/105 and 20 ppm/90 ppm).

The calculation for the relevant chemical analysis data (Table 2) was more elaborate. In this case the molar ratio has been obtained by considering the actual number of moles of amino acids for proteins (Manzi et al., 1999a) and the number of moles of glucosamine for chitin or the number of moles of glucose for the whole dietary fiber content (Manzi et al., 1999b). Subsequently a correlation has been performed between the molar ratios of the experimental chemical results (protein/dietary fiber or protein/chitin) and the molar ratio (protein/saccharide) calculated by the NMR resonance integrals. The relevant results are reported in Figures 7 and 8, respectively. In Figure 7, where the molar ratio protein/dietary fiber is shown, the correlation between

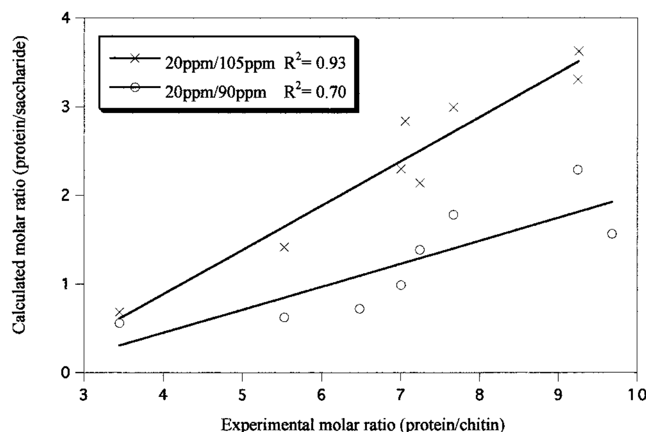

Figure 8. Correlation plot between the experimental molar ratio (protein/chitin) obtained by the chemical analysis data and the molar ratio (protein/saccharide) calculated by the NMR resonance integrals according to eqs (a) and (b).

experimental and calculated values is good if eq (a) is utilized in the calculation ($R^2 = 0.81$), but a broad data dispersion is evident when eq (b) is used ($R^2 = 0.57$). The molar ratio protein/chitin, reported in Figure 8, is more promising, and the linear correlation coefficients are $R^2 = 0.93$ when the ratio 20 ppm/105 ppm is utilized and $R^2 = 0.70$ in the other case. On the whole the correlations performed on the basis of the 20 ppm/105 ppm ratio values are quite good considering the analytical errors associated with each analytical determination and although only some constituents (dietary fiber or chitin) of the polysaccharide fraction have been considered for comparison with the NMR experimental data.

Finally, it is interesting to note that the molar ratio protein/saccharide gives very different values for different species of mushrooms grown on the same cultural medium (*Pleurotus* spp.), and a marked similarity exists between the results obtained with the two methods NMR and analytical analyses. This approach seems particularly interesting because it shows some variabilities among the different genera of the analyzed mushrooms and can be considered a very useful tool to characterize, together with genetic and molecular methods (Urbanelli et al., 1999), not only fungal species but also possible variations among populations of the same species. In fact the simple morphological characterization of mushrooms does not consider that the composition of the media used for fungal growth can induce qualitative−quantitative changes in the organic composition of the fruiting bodies of the mushrooms. Although further research on a larger number of mushrooms is necessary, these results suggest the possibility to perform a taxonomic study of cultivated mushrooms directly on the basis of the ratio between protein and polysaccharide components obtained by either NMR or chemical methodologies.

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