A NEW COLORIMETRIC METHOD TO QUANTIFY β-1,3-1,6-GLUCANS IN COMPARISON WITH TOTAL β-1,3-GLUCANS AND A METHOD TO QUANTIFY CHITIN IN EDIBLE MUSHROOMS

HELGA MÖLLEKEN, JÖRG NITSCHKE, HENDRIK MODICK, TIM MALOLEPSZY, HANS-JOSEF ALTENBACH *
Department Management of Chemical Processes, *Department Organic Chemistry,
University of Wuppertal, Gaußstr. 20 , D-42097 Wuppertal,
Germany
hmoelle@uni-wuppertal.de

ABSTRACT

Mushrooms contain high amounts of substances like chitin and glucans, which can be used for medical purposes and nutrient matter. Therefore, an easy quantification method for chitin and for glucans, with high precision, is a matter of particular importance.
For this purpose, we developed an easy and unique photometric method for the determination of β-1,3-1,6 glucans in mushrooms. This method is specific for β-glucans with a triple-helix tertiary structure. The used dye, congo red, forms a complex with the triple-helix, which causes a specific bathochromic shift of the absorption maximum from 483 nm to 523 nm.
For the specific determination of total β-1,3 glucans a fluorimetric method was adapted, too, which easily can be performed. By the combination of both methods, it was possible to compare the amount of β-1,3-1,6 glucans with the total β-glucan content.
Furthermore, we developed a new colorimetric method for the quantification of chitin and chitosan, which is based on the specific reaction of polyiodide anions and chitosan. After deacetylation, chitin can also be quantified by this method. With this new spot assay, the chitin content of mycelia and fruiting bodies from several basidiomycetes and an ascomycete was analysed. The presented method can be used for determination in other samples, too.

Keywords: chitin, chitosan, β-1,3 glucans, β-1,3-1,6 glucans

INTRODUCTION

Mushrooms of different species are well-known for their therapeutic effects in traditional chinese medicine since centuries. Their cell-wall β-glucans are ingredients with biological activity, thus these biological response modifiers [1-3] mainly activate the immune system, with the possibility to even having effects as an anticarcinogen [4, 5]. β-Glucans such as Lentinan and Grifolan are known as anticarcinogenic agents since the early 1970s [1, 6, 7]. The structure varies with the linkage degree: β-glucans with none or little β-1,6-linkages mainly have a single helix structure. β-Glucans with higher degrees of β-1,6-glycosidic bonds form a triple helix as their tertiary structure. Recent work describes that the triple helix structure, together with the molecular mass, affects the biological activity of the β-glucans [4-7].

A number of existing methods determine the total amount of β-1,3-glucans [8-13,] but do not make any difference between their important tertiary structure. Up to this day, a direct, fast and quantitative method to determinate the important β-1,3-1,6-glucans with a triple helical structure does not exist. Therefore, a precise method would be of great interest. The aim of our

research was to establish a new method to quantify β-1,3-1,6-glucans in mushrooms and to use it for comparison with the total β-1,3-glucan content in various fungi. Our research can survey which mushroom is more useful for glucan preparation i.e. for functional purposes. Therefore, we would like to present a new colorimetric method for β-1,3-1,6-glucan quantification using congo red dye.

An incorporation of congo red into the triple helix leads to a bathocromic shift. The absorption maximum is moved from 493 to 523 nm [14]. Therefore, congo red can be used for characterisation of glucan tertiary structures, only interacting with the triple helix of β-1,3-1,6-glucans [14,15] and not reacting with other polysaccharides [16]. β-Glucans of selected basidomycetes and an ascomycete were isolated by a sequence of alkali and acid treatment and analysed with the new method and the fluorimetric method. In this work a wide variety of different mushrooms are compared among themselves and their mycelia are compared with some selected fruiting bodies. Therefore, the received data can be used to make comparisons between different and same genus, family and division and between mycelia and fruiting bodies.

Besides β-glucans, chitin is an important component of the fungal cell wall. The polymer is characterized by β-(1,4)-branched N-acetylglucosamine units. Partial deacetylation of this biopolymer yields chitosan. The wide variety of possible applications of chitosan in food industry includes, for example, functional foods, use as packaging material and filtration devices. Thus, the cell wall of mushrooms could be an important source for chitosan and glucan production. Basidiomycetes like *Lentinula edodes* or *Grifola frondosa* are well known for their application in various medical domains. β-Glucans, in particular, have been proposed as active immunostimulating agents. After isolation of the glucans, the remaining fibres contain mainly chitin and can be used for chitosan preparation as well. As chitin is insoluble in most solvents, a direct detection is difficult, but it is possible to quantify it as chitosan or N-acetylglucosamine indirectly. Therefore, we developed a modification of a method by Tsuji *et al.* [17] that relies on the formation of a coloured complex of chitosan with Lugol's solution [18]. Generally, Lugol's solution is used in microscopy as a specific dye for chitin in cell walls. Thus, chitin was converted to chitosan with concentrated potassium hydroxide and the hydrolyzed chitosan reacts to glucosamine. With this simple method, the chitin content of a number of mushrooms was determined. As the complex of Lugol's solution and chitosan is insoluble, a spot assay was developed. After pipetting the samples on plates, the optical density of the chitin–polyiodide complex was measured by a colorimetric technique which is commonly used for quantitative analysis of TLC plates as well.

MATERIAL AND METHODS

Chemicals. Chitin and chitosan with a purity of 99 % were obtained from Sigma–Aldrich (Seelze, Germany) for the calibration standard and model reactions. Schizophyllan (β-1,3-1,6-glucan) from S*chizophyllum communue* was used for the photometric quantification method as a calibration standard (Selco Wirkstoffe Vertriebs GmbH, Wald-Michelbach, Germany). Curdlan was used as the calibration standard for the fluorimetric determination of β-1,3-glucans, congo red and anilin-blue diammonium salt were products of Sigma-Aldrich (Seelze, Germany).

Mushroom Samples. The fruiting bodies of the analysed mushrooms were kindly provided by a local breeder. The following mushroom species were chosen for further analysis and mycelia cultivation on a malt-yeast extract media:

Lentinula edodes (Shiitake), *Pleurotus ostreatus* (Oyster Mushroom), *Pleurotus eryngii* (King Trumpet Mushroom), *Hypsizygus tessulatus* (Shimeji Mushroom), *Flammulina velutipes* (Enokitake), *Agaricus bisporus* (Button Mushroom), *Grifola frondosa* (Maitake), *Pleurotus*

pulmonarius (Lung Oyster Mushroom), *Trametes versicolor* (Turkey Tail), *Morchella esculenta* (True Morel).

Cell Preparation. Cell preparation and the preparing of dry cell material was accomplished according to [19, 20].

Determination of Chitin and Chitosan. A chitosan standard was used for calibration in a concentration range of 0.5–5 mg/mL of chitosan. The standard solution or the sample solution (2 lL) was pipetted onto the desired TLC plates or papers. The spots were detected with 1% Lugol's solution, which was sprayed on the plates evenly. Afterwards the optical density of the spots was analysed with a Bio-Rad Geldoc2000 with the contour tool of the Bio-Rad Quantity One quantitation software v.4.2. All results are reported in optical density (OD) units per mm^2. All analyses were performed in triplicate [19, 20].

Colorimetric Determination of β-1,3-1,6-Glucans with Congo Red. A α-Helios photometer was used at a wavelength of 523 nm for the photometric determinations for the standard solution with schizophyllan and the tested samples. A direct measurement of the bathochromic shift is used. Because of the light brownish colour of some fractions a measurement of the background absorbtion at 523 nm is necessary in the range of 50 – 150 µg/ml. All samples were also analysed in triplicate [19, 20].

Fluorimetric Determination of the Total-β-1,3-Glucans with Aniline Blue. The total β-1,3-glucan content was measured according to the method described by Ko and Lin [21]. All fluorimetric measurements were carried out on a Kontron Instrument SFM 25. The excitation wavelength was 392 nm, the emission wavelength was 502 nm and the spectral bandwidth was 20 nm. All samples were analysed in triplicate again.

RESULTS

Chitin Determination: Calibration. Commercial chitin was used to test whether chitin can be completely converted to chitosan. Therefore, we heated the chitin for 3 h with concentrated potassium hydroxide. The residue was then washed several times with distilled water and dissolved in 10% acetic acid. Then the resulting chitosan content was determined with chitosan standards and applicated in the spot test with Lugol´s solution (Fig. 1). The detection limit according to DIN 32 645 was 107 µg/mL. Mandel linearity test showed that a linear calibration in the concentration range from 0.5 to 5 mg/mL can be arranged (Fig. 2). The recovery rate was also determined. We used commercial chitin and chitosan samples and samples of mushroom chitin preparations together to determine the recovery rate. Repetitions (20) with three different mushroom samples were used for the recovery rate. It was 101 ± 8%. This proves the applicability of this method for mushroom samples.

Determination of chitin and chitosan in edible mushrooms. The chitin content of various mushroom mycelia is summarized in Table 1. It ranges from 0.8 wt % (*P. ostreatus)* to 9.6 wt % (*A. bisporus)* and the average chitin content of the mushroom mycelia is 2.5%.

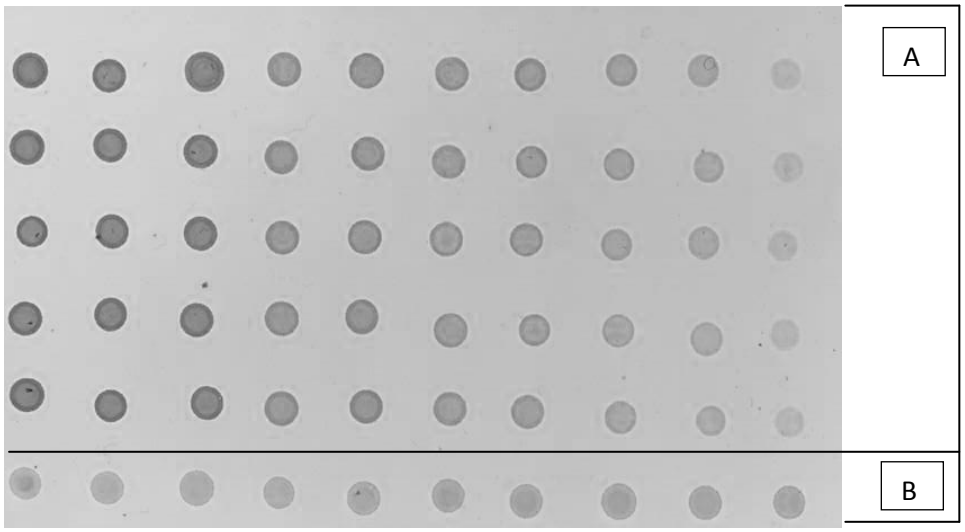

Figure 1: Picture of the spot assay: (A) chitosan standards in a concentration rangeof 5–0.5 mg/mL of chitosan (from left to right); (B) determination of the chitosan from *Agaricus bisporus* mycelia.

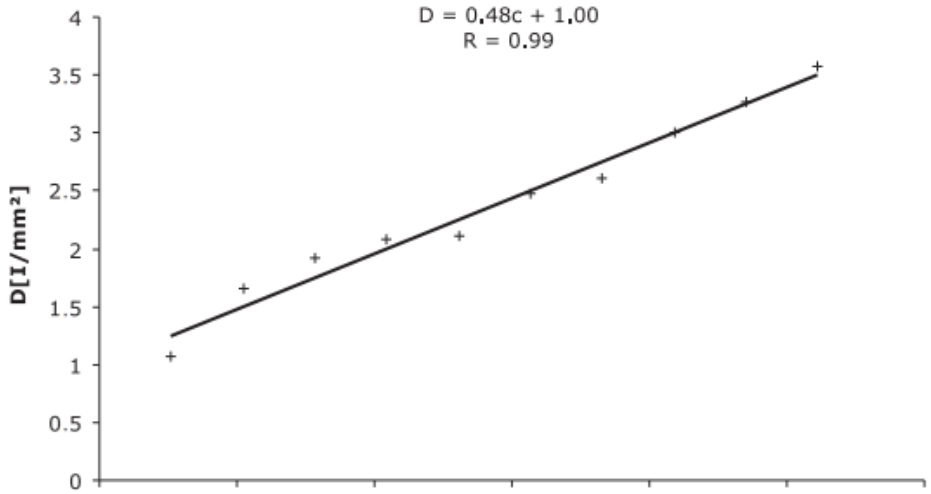

Figure 2: Calibration curve in a concentration range of 0.5–5 mg/mL of chitosan.

Table 1: Chitin content with standard derivations of various mushroom mycelia expressed as g chitosan per 100 g of dry mass (DM)

Mushroom mycelia	Chitin content [g/100 g DM]
Agaricus bisporus	9.60 ± 0.15
Pleurotus eryngii	3.56 ± 0.35
Lentinula edodes	2.49 ± 0.19
Morchella esculenta	1.70 ± 0.40
Grifola frondosa	1.67 ± 0.23
Pleurotus pulmonarius	1.64 ± 0.15
Hypsizygus tessulatus	1.57 ± 0.24
Trametes versicolor	1.35 ± 0.06
Flammulina velutipes	1.21 ± 0.18
Pleurotus ostreatus	0.82 ± 0.08

The chitin content of the fruiting bodies is summarized in Table 2. It varies in a range of 0.8–9.8 g/100 g dry mass. It becomes obvious that *A. bisporus* contains less chitin in its fruiting body in comparison to the mycelia. In contrast, *F. velutipes* has a significantly higher content in the fruiting body than in the mycelia. The other species have comparable amounts in their fruiting bodies and mycelia.

Table 2: Chitin content with standard derivations of various mushroom fruiting bodies expressed as g chitosan per 100 g of dry mass (DM).

Mushroom fruiting bodies	Chitin content [g/100 g DM]
Flammulina velutipes	9.83 ± 0.45
Agaricus bisporus	4.69 ± 0.90
Pleurotus eryngii	3.16 ± 0.40
Lentinula edodes	1.87 ± 0.20
Pleurotus ostreatus	0.76 ± 0.22
Hypsizygus tessulatus	0.39 ± 0.01

Chitosan could not be detected in all samples, an indicator that most of the amino groups of the glucosamine units are acetylated.

Glucan Determination: Calibration. Colorimetric Determination of Triple helical β-1,3-1,6-glucans with Congo Red

The bathochromic shift from the reaction of β-1,3-1,6-glucans with congo Red is effected by the β-1,3-1,6-glucan and dye concentration. For the calibration schizophyllan solutions in a concentration between 50 and 700 µg/ml were used.

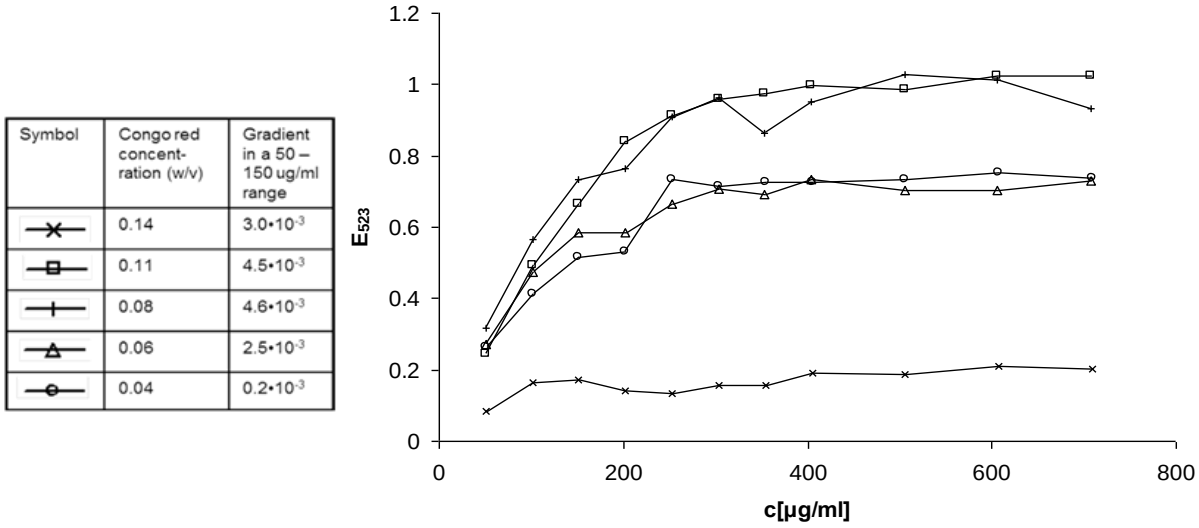

Figure 3: Correlation of different schizophyllan concentrations to the extinction of congo red and the calibration curves in the range of 50 – 150 µg/ml schizophyllan.

Figure 3 shows the dependence of different schizophyllan concentration to the extinction and compares the gradient of the calibration curves in the range of 50 – 150 µg/ml schizophyllan. The results show that a congo red concentration of 0.08 % (w/v) is most sensitive.

Therefore, this concentration is used for further analysis. A calibration curve in the range of 50 – 150 µg/ml schizophyllan is shown in Figure 4.

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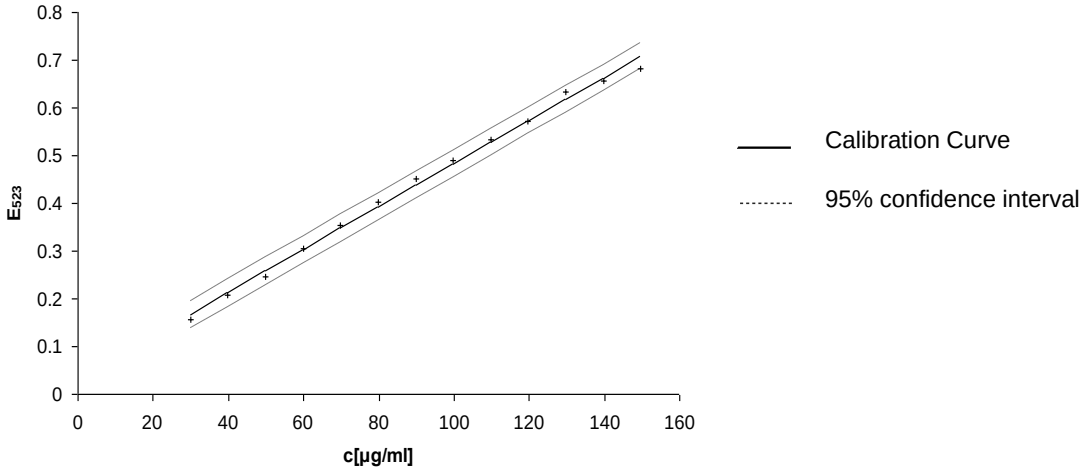

Figure 4: Calibration curve of congo red with schizophyllan with 95% confidence in the range of 50 – 150 µg/ml.

A method validation proved the applicability of the procedure, as the linearity of the calibration curve in the a spired working range of 50 – 150 µg/ml glucan was confirmed by the Mandel linearity test. The limit of detection was determined according to DIN 32 645. Afterwards the matrix influences by using all three glucan fractions of several mushrooms were analysed. The determined average recovery rate of 95.15 % showed that this method can be used for nearly all mushroom extracts. Absorption spectra which show the bathochromic shift are presented in Figure 5.

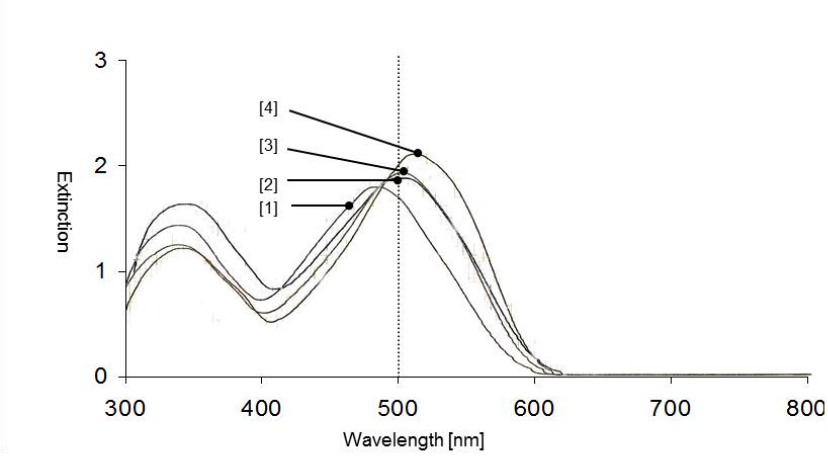

Figure 5: Absorption spectra of: [1] congo red without glucan, [2] with the sodium hydroxide extract of *Pleurotus ostreatus*, [3] 100µg/ml and [4] 200 µg/ml schizophyllan

There are two exceptions when using the KOH-fraction of *H. tessulatus* and *P. eryngii* mycelia, as no bathochromic shift could be observed. This method is appropriable for mushrooms extracts, because of recovery rates >95%. β-Glucans can be detected with good precision and sensibility, as the gradient of the calibration curve shows.

Glucane Determination: β-1,3-1,6-Glucan and β-1,3-Glucan Content of Various Basidomycetes. The total amount of congo red positive β-1,3-1,6-glucans of the mycelia from different basidomycetes, as presented in Table 3, differ from 0.4 – 4,0 g per 100 g for the mycelia and 1.9 – 12.9 g per 100 g dry mass for the fruiting bodies (Table 4)

Table 3: Total-β-glucan and triplehelicale-β-glucan content of mycelia from various mushrooms

Mushroom (Mycelium)	Total-β-Glucan [g/100 g DM]	Triplehelical-β-Glucan (β-1,3-1,6-glucans) [g/100 g DM]
Lentinula edodes	4,2 ±0.1	2,6 ±0.1
Hypsizygus tessulatus	4.2 ±0.1	2.1 ±0.1
Pleurotus pulmonarius	2.5 ±0.1	0.4 ±0.0
Grifola frondosa	4.9 ±0.3	1.8 ±0.1
Pleurotus ostreatus	4.6 ±0.1	3.0 ±0.1
Trametes versicolor	6.8 ±0.2	3.3 ±0.1
Agaricus bisporus	3.8 ±0.2	3.4 ±0.1
Morchella esculenta	4.0 ±0.2	4.0 ±0.2

The maximal difference between the various species is 87.5% for mycelia and 85 % for fruiting bodies. Furthermore, the β-1,3-glucan contents of the mycelium samples, as indicated in Table 3, too, vary between 2.5 g - 4.2 g in mycelia and between 3.8 – 13.5 g per 100 g dry mass for the fruiting bodies (Table 4). *Pleurotus pulmonarius* has the lowest content. In contrast, the Polyporales mushroom *T. versicolor* has the highest content of β-glucans. The contents in fruiting bodies are as mentioned notably higher, however an exception occurs. *Agaricus bisporus* has a lower content than the other analysed fruiting bodies. It is the only mushroom that has a higher content in its mycelia than in its fruiting body. *Agaricus bisporus* is the only secondary decomposer which we analysed. The other mushrooms are primary decomposer. The nutrient uptake may play an important role in cell wall synthesis and therefore in the glucan content and composition. Furthermore, the content of *G. frondosa* is not higher than the contents of *A. bisporus*. *M. esculenta*, the analysed ascomycete, has equal contents as the analysed Basidomycetes. That means that there is no correlation between family, genus or even division and total-β-1,3-glucan content.

Table 4: Total-β-glucan and triplehelical-β-glucan content of fruiting bodies from various mushrooms

Mushrooms (frutiing bodies)	**Total-β-Glucan [g/100 g DM]**	**Triplehelicale-β-Glucan [g/100 g DM]**
Lentinula edodes	9.5 ±0.1	9.5 ±0.1
Flammulina velutipes	9.0 ±0.1	7.8 ±0.1
Pleurotus eryngii	13.5 ±0.1	12.9 ±0.4
Hypsizygus tessulatus	9.1 ±0.3	7.0 ±0.3
Pleurotus ostreatus	9.1 ±0.2	8.3 ±0.3
Agaricus bisporus	3.8 ±0.2	1.9 ±0.0

Table 3 and 4 show that the amount of the important triple helical β-1,3-1,6-glucans on the total-β-1,3-glucan content is different in mycelia and fruiting bodies. The amount of β-1,3-1,6-glucans comports a maximum of 64.0 % and is in average 45 % in mycelia, while in some fruiting bodies like *L. edodes* and *P. eryngii* almost all β-1,3-glucans have a triple helix structure. The other fruiting bodies also have higher amounts of β-1,3-1,6-glucans than the mycelia. The average β-1,3-1,6-glucan amount is 88 %.

DISCUSSION AND CONCLUSION

A reliable specific colorimetric determination for triple helical β-glucans offers the first possibility to analyse β-1,3-1,6-glucans with high precision, fastly and without extensive clean up. Together with an fluorimetric method, both the triple helix β-1,3-1,6-glucans and the total-β-1,3-glucan content of basidomycetes can be compared. The used fluorimetric determination is also fast and our work proves that it is applicable for all mushroom samples. The results are reasonable and can be compared to existing data. The isolation process used by our group is quite similar to the processes described in several publications, so the obtained results can be compared with results of recent research.

Manzi et al [8, 9] quantified β-1,3-glucan contents of various fruiting bodies with an enzymatic hydrolysis of isolated mushroom cell walls and then analysed the released free reduced sugars. With this method 0.58 g for *P. pulmonarius*, 0.38 g for *P. eryngii* and 0.22 g/100 g for *L. edodes* on a dry mass basis were determined. The limitation of this direct enzymatic hydrolysis has been discussed previously [12]. Consequently, other groups first extracted the polysaccharides and then digested the glucans by β-glucanases [11, 13]. Their results show that the fruiting bodies of *Inotus obliquus* contain 8.3 g and of *Agaricus brasiliensis* 10.1 g β-1,3-glucans per 100 g dry mass. Even though these species were not analysed in our present work the contents are comparable with those of our work for related species.

Recent works showed that most of fungal β-glucans are highly branched β-1,3-1,6-glucans with a triplehelical structure [4, 12]. The presented analyses support that a triple helix is the dominant form of β-glucans in most mushrooms.

There is less information on the quantity of glucans in mushrooms that can be compared with our results. With an ELISA method, the content of β-glucans of the fruiting bodies from *L. edodes* and *G. frondosa* were analysed by Zhang [4]. Their results showed that *G. frondos*a contains 2.4 g, *L. edodes* 3.4 g and *F. velutipes* fruiting bodies 6.4 g β-glucans per 100 g dry mass. These values are comparable to our analyses with the direct determination with congo red of these mushrooms.

Several fruiting bodies and mycelia were examined and in summary the results support that the fruiting bodies have the most β-glucans and the highest amount of β-1,3-1,6-glucans, too. The high amount of triplehelical β-glucans in fruiting bodies corresponds to current structure examinations. They show that β-glucans of *P. ostreatus* [11] and *L. edodes* [4] mainly consist of highly branched β-1,3-1,6 -glucans with a triplehelical structure.

The high amounts of β-1,3-1,6 glucans in *L. edodes* and *P. ostreatus* manifest their role as important medical mushrooms. But the analyses support that other edible mushrooms such as *M. esculenta*, *H. tessulatus* and *P. eryngii* have comparable β-glucan quantities. Comparisons between different families, genus and division show that there are no real correlations.

Because of the notable higher amounts of β-glucans in fruiting bodies, a technical isolation of glucans from fruiting bodies seems preferable from this point of view. On the contrary the mycelium is a fast growing raw material, which grows with less effort. For fructification an induction period is necessary, which is expensive and time-consuming, while the mycelium is a renewable resource for β-glucan isolation.

A reliable and specific determination for chitin and chitosan is also achieved. The average chitin content in the analysed mushroom species is about 2.5 g/100 g for the mycelia and 3.5 g/ 100g for the fruiting bodies. Some exceptions occur: the fruiting bodies of *A. bisporus*, *F. velutipes and P. eryngii* have notably higher chitin content and the mycel of *A. bisporus* and *P. eryngii* contain the highest amounts of chitin. As the culture conditions for all mycelia are similar, this seems to be a characteristic for the particular species.

A correlation between chitin content and mushroom systematics like species, family or order could not be observed. The results can be compared with data recently published: Chen [22] has determined high concentrations of chitin in basidiomycetes with the Ride and Drysdale method [23] for quantification and found a content of 21.8 % for the mycelium of *L. edodes.* They did not look at the proportion of other aminohexoses that could additionally be detected with this method. Manzi et al. [8, 9] also used the Ride and Drysdale method [23] to quantify the chitin content as N-acetylglucosamine after hydrolysis with hydrochloric acid. For *A. bisporus* they measured 0.32 % and for *P. ostreatus* 0.43 % on a fresh weight basis. Because of the variation in water content of the mushrooms, comparison with our data is not possible. Vetter *et al.* [24] applied the Smith and Gilkerson method [25] to determine chitin in several wild growing and cultivated mushrooms by measuring the N-acetylglucosamine released by hydrolysis. They quantified chitin in a range from 0.1 to 9.7 g per 100 g of dry mass, including *P. ostreatus* with 2–3 g of chitin. The quantifications by our new method, where the optical density of the insoluble polyiodide–chitosan complex is measured, are comparable with these data.

Vetter later [26] quantified 6–8%, 2–5% and 5–6% chitin for different variations of *A. bisporus*, *P. ostreatus* and *L. edodes* fruiting bodies on a dry-mass basis. The variation in chitin content even in different varieties is remarkable. However, the magnitude of the chitin contents is again comparable to our data. Chen and Johnson [27] measured a chitin content for *Schizophyllum commune* fruiting body of 9.6% on a dry-mass basis. We did not analyse this specific mushrooms, but we measured equal amounts in related mushrooms such as *A. bisporus* or *F. velutipes*. Ofenbeher-Miletic´ *et al.* [28] determined chitin in wild-growing mushrooms. They quantified the N content in purified dietary fibre from fruiting bodies and then calculated the chitin content. Even though our work concentrates mainly on commercially available mushrooms, their measured amounts are comparable with our analyses, with chitin contents in a range of 1.9–13.6% on a dry mass basis. Furthermore, Dikeman *et al*. [29] quantified 7.7% chitin in the dry mass of the fruiting bodies of *F. velutipes* by using HPLC to determine the N-acetylglucosamine after digestion with chitinase. We determined a comparable chitin content of 9.8 % of dry mass.

These comparisons of known methods reveal similarity to our results, but in contrast to the other methods, we have established a method to analyse both chitin and chitosan directly with high precision. Chitosan should be dissolved in acetic acid, after a cleanup with sodium hydroxide, whereas chitin needs deacetylation to chitosan with highly concentrated alkaline solutions. Though the conversion of chitin to chitosan takes up to 3 h, it could be proven that the conversion was complete. We also proved that this assay is specific for cell extracts from mushrooms. Cross-reactions were not observed. We added up to 5 mg/mL of bovine serum albumin to various chitosan standards, but it did not affect the determination. We used an enzymatic starch assay to prove that our samples are starch free. As there are no starch and starch derivates in our mushroom samples, no other colour formation can occur.

The high amount of β-1,3-1,6-glucans could result in a more solid cell wall. This could be necessary for the compact fruiting bodies. On the other hand the high amount of single helix glucans could result in a more flexible cell wall, which is important for the mycelia for nutrient-uptake purposes.

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