

**Study on the 3-hydroxy-3-methyl-glutaryl CoA reductase inhibitory
properties of *Agaricus bisporus* and extraction of bioactive fractions using
pressurized solvent technologies (ASE and SFE)**

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Abstract

BACKGROUND: *Agaricus bisporus* mushrooms were able to lower cholesterol levels in hypercholesterolemic rats and it was suggested that dietary fibers might inhibit cholesterol absorption. However, *A. bisporus* extracts were also able to inhibit the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR, the key enzyme in the cholesterol biosynthetic pathway) and this might also contribute to the observed lowering of cholesterol levels in serum.

RESULTS: The methanol:water extracts obtained from *A. bisporus* were able to inhibit up to 60% the HMGCR activity using an *in vitro* assay. The HMGCR inhibitory capacities depended on cultivation conditions, strains, etc. The potential inhibitors were not statins, they might be β -glucans able to scavenge the substrate and impair the enzymatic reaction. They were present during all mushroom developmental stages and similarly distributed through all the tissues including the parts discarded as a by-product. Accelerated solvent extractions using 1:1 ethanol:water as pressurized solvent (10.7 MPa, 25°C, 5 cycles of 5 min) were more effective in the extraction of the HMGCR inhibitor/s than supercritical fluid extractions (9 MPa, 40°C) using CO₂ with 10% ethanol.

CONCLUSION: A mushroom cultivation and two extraction procedures were optimized to obtain fractions from *A. bisporus* with high HMGCR inhibitory activities to design novel ingredients for hypocholesterolemic functional foodstuffs.

1 INTRODUCTION

2 High cholesterol or LDL (low-density lipoprotein) levels in serum increases the risk of
3 cardiovascular diseases (CVDs). In public health terms, achieving a reduction in
4 cholesterol by dietary advice (increase consumption of vegetable and fruits) is of limited
5 effectiveness.¹ Many researchers have been exploring the possibility of increasing
6 components in foodstuff which have hypocholesterolemic effects such as *i.e.* β -glucans or
7 phytosterols.^{2,3} These novel functionalized foods reduce serum cholesterol (10 – 20%)
8 because they impair the absorption of exogenous cholesterol during digestion.^{2,4} However,
9 depending on genetic variations, many consumers cannot reduce their cholesterol levels
10 because by avoiding cholesterol intake their 3-hydroxy-3-methyl-glutaryl CoA reductase
11 (HMGCR) activity is stimulated enhancing the biosynthesis of endogenous cholesterol.⁵
12 Thus, another approach to design novel hypocholesterolemic functional foods might be their
13 supplementation with compounds able to inhibit the HMGCR activity.

14 Edible mushrooms were able to lower cholesterol levels *in vivo* (*Pleurotus* spp., *Lentinula*
15 *edodes*, *Ganoderma lucidum*, *Agaricus bisporus* etc.)⁶⁻⁸ and were also able to inhibit the
16 HMGCR *in vitro*. Some reports pointed lovastatin as the compound responsible for the enzyme
17 inhibition.⁹⁻¹¹ Lovastatin (mevinolin) and other statins are drugs usually prescribed to
18 hypercholesterolemic patients because they act as competitive inhibitors of the HMGCR
19 showing approx 200 folds more affinity for them than their real substrate.¹² Moreover, selenium
20 supplementation combined with statins therapy was proved to be significantly beneficial to lipid
21 therapy¹³ and mushrooms can be Se-fortified by addition of sodium selenite to their cultivation
22 substrates to modify their biological properties.¹⁴ Thus, if there are statin-producer mushroom
23 strains, they might also be Se-fortified to enhance their hypocholesterolemic effect.

24 However, other authors found no detectable lovastatin levels but still high HMGCR

inhibition activities.^{6,15} Other compounds such as lanosteroids, ganoderols etc., were described in *G.lucidum* and apparently they were also able to inhibit the enzyme but through an indirect mechanism.⁶

A. bisporus basidiomata were able to lower blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats and it was suggested that dietary fibers might be involved, but via a complex process.⁸ The mushroom dietary fiber fraction includes mainly chitins and in higher amounts β -glucans. Fungal β -glucans are polysaccharides of D-glucose monomers linked by β -glycosidic bonds showing preference for the β -(1,3/1,6) bonds instead of the β -(1,3/1,4), the characteristic pattern of cereals β -glucans.¹⁶ In this study, the HMGCR inhibitory properties of *A. bisporus* extracts are reported as another pathway which might also contribute to the observed lowering of cholesterol levels in serum. The distribution of the potential inhibitor/s depending on the cultivation parameters, different strains (including Se-fortified strains), developmental stages etc., was also monitored.

Supercritical fluid extraction (SFE) with CO₂ as well as accelerated solvent extraction (ASE) are ecologically and economically sustainable processes that are increasingly being developed as a response to recent reports concerning the environmental status of our planet.¹⁷ Moreover, both methods satisfy the demand of environmentally clean extraction processes to produce new extracts or compounds with a potential use as functional ingredients or nutraceuticals. The use of supercritical carbon dioxide, with properties such as low viscosity, high solvent strength and zero surface tension has been encouraged as a substitutive of organic solvents. It has been previously used in the extraction of different compounds from mushroom strains.¹⁸⁻²³ On the other hand, when water is used at higher temperatures and pressures (as occurring in ASE) it can also replace organic solvents. Water

can also be used in combination with ethanol, a GRAS (*i.e.*, generally recognized as safe) solvent used in the food industry, to tailor the extract composition and therefore its beneficial properties. Accelerated solvent extraction using water has also been used to extract polysaccharides and compounds with different biological activities from *L. edodes*, *P. ostreatus* and *G. lucidum*.^{15,24-26} However, to our knowledge there are no studies on the screening of the HMGCR inhibitory properties of *A. bisporus* extracts produced by either SFE or ASE technologies.

Therefore the other aim of this work was to compare these two extraction technologies in order to optimize methods to obtain fractions from *A. bisporus* with high HMGCR inhibitory activities to design novel ingredients for hypocholesterolemic functional foodstuffs.

MATERIALS AND METHODS

Biological Material

Agaricus bisporus L. (Imbach) mushrooms were grown in cultivation rooms with automatic control of cultivation parameters (temperature, r.h., CO₂) at CTICH (Centro Tecnológico de Investigación del Champiñón de La Rioja, Autol, Spain) or at the cultivation facilities of some mushroom growers belonging to the La Rioja's mushroom association using as substrate the commonly utilized indoor compost phase II. A more detailed explanation of the experimental trials, utilized substrates, casing layers and spawns and Se supplementations can be found elsewhere.²⁷ Basidiomata were harvested at stage 2-3 according to Hammond *et al.*²⁸ except in those experiments when the effect of the developmental stage was studied. The recorded parameters are described in table 1.

Basidiomata or their separate tissues (manually separated with the help of a knife) were

dehydrated (lyophilized) and ground into fine powder as described by Ramírez-Anguiano *et al.*²⁹ Dried mushroom powders were stored at -20°C until further use.

A minimum of three sporophores were harvested from the same cultivation bed with identical conditions for each different type of sample.

Determination of selenium

The digestion procedure and hydride generation atomic absorption spectrometry (HG-AAS) method was optimized at CTICH based on previous reports.^{30,31} Briefly, mushroom powders (0.4 g) were introduced into a Teflon microwave digestion vessel and HNO₃ 7.7 F was added to an 8 mL final volume. The vessel was closed and fastened into the rotor. The rotor with 6 loaded vessels was placed into the microwave oven (Milestone Ethos Touch control). The applied microwave digestion program was as follows: 0-100°C (3min), 100-150°C (7min), 150-180°C (6 min) and 180°C (15min). The digested samples were adjusted to 20 mL with HCl 4F and heated at 95°C for 20 min to ensure reduction of Se (VI) to Se (IV). Once mixture cooled down, the mixture was diluted with 50 mL HCl 4F. Selenium was determined by HG-AAS with optimized parameters. Detection was performed by an HG-AAS system (Solaar M6 MK2 Dualz, Cambridge, UK). The peak areas of the absorbance were used for calculation of selenium content. HG-AAS technique required a selenium hollow cathode lamp to operate at a wavelength of 196.0 nm with a slit width set to 0.5 nm and an electrodeless discharge lamp set at 10 mA current without background corrector. Aspiration time was 45 s, measuring time 5 s, the height signal evaluation and argon flow 314 mL min⁻¹. Atomizing environment was silicous cell heated to 900 °C, floated solution HCl (10 mol L⁻¹) and reducers were 0.3% NaBH₄ and 0.4%

NaOH. Selenium determination was performed in duplicate and quantified according to a Se calibration curve.

Accelerated solvent extractions (ASE)

Mushroom powder (1g) were mixed with washed sea sand (4 g) (Panreac, Spain) and submitted to pressurized solvent extraction using an Accelerated Solvent Extractor (Dionex Corporation, ASE 350, USA). The sea sand was selected as an inert material to hold the sample inside the extraction cell and to improve efficiency avoiding formation of preferential flow paths. Extraction procedure (per cycle) was carried out at 10.68 MPa (1500 psi) as follows: firstly, the sample was loaded into 11 ml extraction cell, then, the cell was filled with ethanol, heated-up and static extraction was carried out during the selected minutes with all system valves closed. When a cycle was finished, the cell was rinsed, the solvent was purged out of the cell with N₂ gas and the cell remained depressurized. Then, fresh solvent was again added to the extraction cell to carry out another extraction cycle until the programmed number of cycles was finished. The fractions collected after the selected cycles were pooled together in a vial as a single extract.

Several parameters such as solvent, static extraction time or cycles number and temperature were changed in order to optimize the extraction method to obtain fractions with high HMGCR inhibitory activity.

After collection, fractions were immediately concentrated with a rotary evaporator (40°C), frozen and lyophilized. Dried samples were stored at -18°C until further analysis. Extracted dry matter content was measured to calculate the extraction yields. Extractions were carried out in duplicate.

Supercritical fluid extractions (SFE)

Supercritical fluid extractions (pilot-plant scale) with CO₂ (Air-Liquid España, S.A., Madrid, Spain) were carried out in a plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. The extraction vessel has a height/diameter ratio of 5.5 (0.42 m height, 0.076 m internal diameter). A detail explanation of the experimental device can be found elsewhere.³² For each experiment, the extraction cell was filled with 80 g of mushroom powder and 900 g of washed sea sand (Panreac, Spain). In order to optimize the extraction method to obtain fractions with high HMGCR inhibitory activity, parameters such as the use of a co-solvent (ethanol, 10% w/w) and extraction pressure (30, 18 and 9 MPa) were tested. Extraction temperature as well as temperature of separators 1 and 2 was set to 40°C for all the experimental assays. Pressure of separator 1 and separator 2 using ethanol as co-solvent was maintained at 6 and 0.1 MPa respectively, however in CO₂ extraction both separators were kept at a pressure of 6 MPa. The CO₂ flow was set to 2.4 kg h⁻¹ and the total extraction time was of 3 h. For each experiment, extracts collected in separator 1 and 2 were mixed together concentrated to dryness with a rotary evaporator and stored at -18°C until further analysis. Extracted dry matter content was measured to calculate the extraction yields. All the experiments were carried out in duplicate.

Determination of HMGCR inhibitory activity

Mushroom powders or obtained mushroom extracts (50 mg mL⁻¹) were mixed with water (heated at 50°C or room temperature) or with methanol:water (1:1 v/v). Suspensions were shaken in a Vortex for 1 min and centrifuged at 9659 x g for 2 min. Supernatants (20 µL)

were applied into a 96 wells-plate (Corning Incorporated Life Sciences, MA, USA) according to the user's manual of the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) Assay (Sigma, Madrid). Absorbance at 340 nm was monitored at 37°C using a microplate reader (Tecan Group Lt, Switzerland). Determinations were carried out in duplicate.

HPLC-MS/MS analysis of the extracts

Mushroom extracts (3 mg mL⁻¹) showing HMGCR inhibitory capacity were injected (20µL) into an Accela HPLC-MS/MS (Thermo Electron Corporation, San Jose, CA) equipped with an ACE 3 C18-AR column 150 x 4.6 mm, 3 µm particle size (Advanced Chromatography Technologies, Aberdeen, UK). The mobile phase was a mixture of acetonitrile: 0.5% acetic acid in water (60:40 v/v) running isocratic for 30 min. Flow rate was 0.5 mL min⁻¹. Detection was accomplished by using photodiode array detector (200-700 nm) and a mass spectrometer (triple quadrupole) (TSQ-Quantum, Thermo Electron Corporation, San Jose, CA) with an ESI (Electrospray Ionization) interface.

For mass spectrometry analysis spray voltage was set in 3500 V in positive mode, nitrogen was used as sheath gas at 350°C and 35 arbitrary flow/pressure units. Mass analyzer was set simultaneously in full scan and SRM (Single Reaction Monitoring) modes, in this case SRM experiments were done using 1 precursor ion and 1 daughter as recommended by UE regulation (Comision Decission 657/2002 about performance of analytical methods and the interpretation of results). SRM filters were set for a better quantification of statins, precursor and product ions. Moreover, a full scan mode was used within a mass range of 200-800 m/z. Statins including pravastatin, lovastatin, simvastatin and atorvastatin (Cinfa, Spain) were used as standards. Determinations were carried out in duplicate.

Determination of the HMGCR inhibitors chemical nature

Agaricus bisporus dry powder (50 mg mL⁻¹) showing HMGCR inhibitory activity was mixed with methanol:water (1:1) vigorously shaken in a Vortex for 1 min, centrifuged at 9659 x g in a microfuge for 2 min and the obtained supernatant separated. Supernatant was filtrated through a 0.45 µm filter and the filtrate was submitted to several filtrations using centrifugal filters (VWR, Barcelona, Spain) with cut off of 10 and 3 kDa Microcom filters (Millipore, Madrid, Spain) using a microfuge. Fractionated samples were then applied to the HMGCR activity test in order to estimate the molecular weight of the potential HMGCR inhibitors.

The mushroom samples in methanol:water (1:1) were also prepared by mixing them with enzymatic preparations such as: i) a mixture of exo-1,3-β-glucanase (100 U mL⁻¹) and β-glucosidase (20 U mL⁻¹), ii) a mixture of invertase (500 U mL⁻¹) and amyloglucosidase (1630 U mL⁻¹) and iii) pepsin (536 U mL⁻¹). Treated samples were incubated for 1 min and centrifuged (9659 x g 2 min.). Supernatants were submitted to filtration using the 10kDa filters and the microfuge (13147 x g 30 min) in order to separate the enzymes avoiding interferences. Then, the HMGCR inhibitory activity of the separated fractions was determined.

The same methanol:water extracts showing HMGCR inhibitory activity were applied to the HMGCR activity test but altering the application procedure. Usually, in this test solutions are applied following the sequence: first the buffer, then NADPH, substrate, standard inhibitor (pravastatin) as control or sample (potential inhibitory preparation) and at the end the HMGC_oA reductase. Then, the test was performed by mixing the sample and substrate separately and applying the mixture when substrate should be added or mixing the

sample with the enzyme and applied at the end. Moreover, similar mixtures were also prepared and applied but mixing the sample with a diluted solution (1/2) of pravastatin or with the enzyme but in the presence of the diluted pravastatin concentration. All the experiments were carried out in duplicate.

Statistical analysis

One way analysis of variance (ANOVA) was performed using a Statgraphics® Plus 3.1 for Windows software (Statistical Graphics Corporation, Maryland, USA). The mean comparison test used was Fisher's least significant differences procedure (LSD).

RESULTS AND DISCUSSION

Influence of the cultivation systems

According to preliminary results,^{27,33} cultivation parameters influence the HMGCR inhibitory activity found in *A. bisporus* basidiomata because when different growers were cultivating the same mushroom variety, large differences in the percentage of inhibition were observed.³³ However, some of those parameters such as the casing layer utilized to induce fructification did not affect significantly the presence of HMGCR inhibitory compounds and no correlation was neither observed with other parameters such as crop quality or infections (bacterial, fungal and pests).³³ Other reports also indicated that water was the best solvent to extract HMGCR inhibitors from several mushrooms except for *A. bisporus* which showed higher HMGCR inhibitory activity in methanol:water extracts.¹⁵ Thus, *A. bisporus* basidiomata from the same spawn variety (Fungisem H-15) were cultivated at the same cultivation room in three different batches and harvested to investigate the variability from trial to trial and between the first three flushes. The

mushroom powders obtained from the collected basidiomata were mixed with both solvents to compare and results indicated that there were no significant differences between trials. The methanol:water extracts obtained from mushrooms harvested in the first and second flush showed a lower HMGCR inhibitory capacity (respectively 14.4 and 11.2%) than those harvested from the third flush (29.5%). Thus, the potential inhibitor/s might be compounds related to the secondary metabolism (*i.e.* statins) since in the third flush, mushrooms obtain less nutrients from the substrate than during the first and second flushes. On the other hand, it might also suggest that the HMGCR inhibitory activity of the basidiomata might be modulated by the presence/absence of specific compounds. Mushrooms can be Se-fortified by addition of sodium selenite to their cultivation substrates and this supplementation can modify their biological properties.¹⁴ Moreover, selenium supplementation combined with statins was proved to enhance the statin effect on the reduction of cholesterol levels.¹³ Thus, if these *A. bisporus* strains were able to produce statins or related compounds, Se-fortified mushroom strains could perhaps enhance their HMGCR inhibitory activity. Therefore, *A. bisporus* mushrooms (Fungisem H15) were cultivated in substrates supplemented with a low and a high dose of selenite. The selenium absorbed by the Se-fortified basidioma was also measured and the HMGCR inhibitory activity evaluated. However, no correlation was found between the selenium supplementation nor the levels of selenium absorbed by the basidioma and the inhibitory capacity. Selenium content in the basidiomata ranged from an average value of 3.2 ppm in control samples, 10.9 ppm in the lower selenium application up to 31.6 ppm in the highest applied dose and the HMGCR inhibitory activity ranged from 5.7 up to 48.9 % independently of the Se content evaluated. These inhibition values were in the range of those usually found for non Se-fortified mushrooms therefore, no synergistic effect or

enhancing of the HMGCR inhibitory activities was observed by selenium supplementation.

Variability within the *Agaricus bisporus* commercial strains

Spawns from several commercial *A. bisporus* varieties were cultivated and the obtained basidiomata from the first flush harvested. Only the methanol:water (1:1 v/v) extracts obtained from Somycel A-15 showed a significantly lower HMGCR inhibitory activity compared to the others varieties (Figure 1). No differences were found between summer varieties such as Gurelan 60 and the other strains preferentially cultivated in winter. Wise to mention was the fact that water extracts obtained from Fungisem H15 also showed inhibitory capacities opposite to the rest of the varieties suggesting the presence of different inhibitory compounds within the strains.

Developmental and tissue distribution of the HMGCoA-reductase inhibitors

A. bisporus basidiomata (Fungisem H15 from the third flush) from the same cultivation tray were harvested at different developmental stages including from primordia until the veil was broken and the gills were visible (stage 5). However, the HMGCR inhibitory activity did not correlated to the basidioma developmental stage since primordia showed a slightly higher inhibitory activity (37.8%), stages 3 and 4 showed 30.8% inhibition and mature mushrooms (stages 5-6) an insignificant increase (32.7%). Therefore, the HMGCR inhibitory capacity was ever present through the complete sporophore life cycle.

Similarly, the tissue distribution showed no preferential location of the potential inhibitor/s within the basidioma. Epidermis, dermis, gills, veil, stipe and lower part of the stipe were analyzed and only gills extracts appeared to have a slightly lower inhibitory capacity compared to the other tissues (Fig 2). These results were in disagreement⁹ with those of

pointing gills as the tissue with higher lovastatin concentration, indicating that perhaps this compound was not in this case the responsible for the observed HMGCR inhibitory activity. Interestingly, the lower part of the stipe, usually removed during harvesting and discarded, showed 56.9% inhibitory capacity indicating that these mushroom by-products could be valorized and utilized to extract HMGCR inhibitors.

ASE extraction to obtain fractions with high HMGCR inhibitory activity

A. bisporus basidiomata were submitted to extraction with pressurized solvents in order to obtain mushroom fractions with high HMGCR inhibitory activity. Two advanced extraction methodologies were tested, supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE).

According to recent studies¹⁵ the extraction yield of HMGCR inhibitors was highly influenced by the extraction temperature (for other mushroom species such as *Pleurotus ostreatus* and *Lentinula edodes*), while other parameters such as extraction time and cycles did not remarkably improved the extraction of the potential inhibitors. Thus, *A. bisporus* extractions were carried out selecting 5 cycles of 5 min (optimal conditions for the two other species) but at different temperatures and using water (as used for the other mushroom species) and a mixture of ethanol:water (1:1 v/v). The same extraction procedures were applied to two of the mushroom varieties which showed more interesting HMGCR inhibitory activities (Fungisem H15 and Gurelan 60).

Increasing of temperature from 25 up to 200°C resulted in a higher yield (higher percentage of extracted compounds) with the temperature. Similar results and extraction yields were obtained for both Gurelan 60 (ranging from 243 up to 787 mg g⁻¹ mushroom dw) and Fungisem H15 (table 2) varieties being 200°C the best condition to extract approx. 80 % of

1 the material using water as pressurized solvent. When ethanol:water (1:1) was used as
2 extraction solvent a slightly lower yield than with plain water was obtained when the
3 extraction was performed at 25°C. But, increasing of temperatures resulted in increasing
4 extraction yields up to similar percentages than using 100% water. Higher temperatures
5 were not considered because at 150 and 200°C, the presence of the organic solvent induced
6 a remarkable browning in the extracts provoking color interferences when analyzing their
7 HMGCR inhibitory activities. Moreover, the increase of temperature was detrimental for
8 the HMGCR inhibitory capacity of the fractions suggesting that the compounds responsible
9 for the inhibition were thermo labile (Figure 3).

10 Water extracts obtained at room temperature (with or without pressure) did not show
11 interesting HMGCR inhibitory activity. Temperatures higher than room temperature should
12 be used in order to increase the extraction of the potential HMGCR inhibitor/s being the
13 extracts obtained at 50°C the fractions which showed higher inhibitory activities (ASE and
14 standard extractions). However, the extractions carried out with ethanol:water as
15 pressurized solvent showed higher HMGCR inhibitory activities than water extracts and in
16 this case, the use of temperatures higher than 25°C was detrimental. Standard solvent
17 extraction using a mixture of ethanol:water at 50°C could not be carried out because of
18 discolouration. On the contrary than occurring in ASE extractions, the oxygen present in
19 the solution and the organic solvent induces activation of the mushroom polyphenol
20 oxidases³⁴ impairing the proper determination of the HMGCR inhibitory activity.

21 These results indicate that *A. bisporus* might contain compounds able to interfere with the
22 extraction process or that the compounds responsible of the inhibitory capacity of *A.*
23 *bisporus* extracts might be different than those extracted by ASE from *P. ostreatus* and *L.*
24 *edodes* since in the latter mushrooms pressurized water was a more effective solvent to

extract fractions with high HMGCR inhibitory activities.¹⁵

SFE extraction to obtain fractions with high HMGCR inhibitory activity

Results obtained from the ASE experiments showed that the extraction of potential HMGCR inhibitor/s detected in *A. bisporus* basidioma was enhanced when using a mixture of water:organic solvent instead of plain water, indicating certain apolarity in the compounds of interest and suggesting that they might also be susceptible of SFE extraction. Then, *A. bisporus* basidiomata were submitted to extraction with pressurized CO₂ and CO₂ combined with 10% ethanol as modifier. Results showed that the highest extraction yields were obtained when ethanol was added to the CO₂ but higher extraction pressure did not significantly increased the extraction yield (Table 3). In terms of HMGCR inhibition, fractions obtained with the help of ethanol and at 9 MPa showed a slightly higher inhibitory activity than those obtained at lower pressures (Figure 4). However, no significant differences were found in samples extracted without modifier indicating that the fractions obtained with ethanol and low pressure were more selective to isolate the potential HMGCR inhibitor/s. These results can be explained in terms of CO₂ density: at higher pressures the density –and hence the extracting power- of supercritical CO₂ increases at constant temperature, so higher extraction yields were expected when working at 30 MPa. Nevertheless, the higher number of compounds present in the fractions obtained at higher pressures could have caused a dilution effect on the compounds responsible of the HMGCR inhibition leading to better results in fractions recovered under lower extraction pressures (9 MPa).

Since it was previously indicated that the lower part of the *A. bisporus* stipes (which are usually discarded) showed interesting HMGCR inhibitory activities, SFE extractions were

also performed using these by-products. Extractions carried out at 30 MPa with ethanol as co-solvent showed 82.74 % inhibition, 2 fold higher inhibitory activity than the same SFE extract obtained from the basidioma, pointing these by-products as an interesting source of HMGCR inhibitor/s.

However, direct extractions with methanol:water (1:1) from the powdered by-product applied at the same concentration than the SFE extract showed already 56.9% inhibition and 80 g of mushroom powder were necessary to obtain 1.42 g SFE extract with only 1.5 fold higher activity than the mushroom powder thus, SFE was not a method highly selective to obtain fractions with high HMGCR inhibitory activity. On the contrary, ASE extractions at 25°C showed 1.6 fold higher inhibitory activity than direct extraction when the same amount of starting material (1g) was used. Thus, the latter method was more suitable than SFE for potential HMGCR inhibitor/s extraction.

Presence of statins in the *Agaricus bisporus* extracts

A. bisporus extracts with HMGCR inhibitory activity were injected in an LC-MS/MS system to investigate whether the observed inhibition was due to the presence of statins as it was described by other authors^{10,11} since they also observed higher inhibitory capacity in samples obtained with methanol:water rather than with water.⁹ However, no detectable peak at the retention time of pravastatin (3.82 min), atorvastatin (8.06 min), simvastatin (9.83 min) or lovastatin (20.26 min) was observed.

Agaricus bisporus extracts showed three peaks at 4.8, 8.8 and 13.2 min with 567, 459 and 520 m/z different from those of the utilized standard statins (Table 4). The statins SRM transitions were similar to those used previously by other authors,³⁵ therefore, statins were not in this case responsible for the observed HMGCR inhibitory activity.

Determination of the HMGCR inhibitors chemical nature

Since statins were not present in the *Agaricus bisporus* extracts showing HMGCR inhibitory activity, a preliminary attempt to identify the nature of the inhibitors was carried out. Thus, the methanol:water extracts (showing 47.2% inhibitory activity) were fractionated by molecular weight using specific filtering devices with cut off of 10 and 3 kDa. The obtained fraction including compounds with a molecular weight higher than 10 kDa showed 61.5 % inhibitory activity. However, the fraction with molecular weight lower than 10 kDa also showed high inhibitory capacity (50.4 %). Thus, the latter fraction was further fractionated using a 3kDa filter and the higher inhibitory activity was found in the fraction with a molecular weight between 10 and 3kDa (44.2 %) being lower than 23% in the filtrate corresponding to the low molecular weight fraction. Results indicated that the potential HMGCR inhibitors might be molecules of high molecular weight, higher than 10 kDa. However, the fact that the fractions with molecular weight higher than 3 kDa also showed interesting inhibitory activity could indicate on the one hand, that the possible degradation products obtained from the molecules with molecular weight higher than 10 kDa might still acts as inhibitors or on the other hand, that the inhibitors were not a single compound but molecules with a wide range of molecular weights. In both cases, results suggested that the inhibitor/s were macromolecules, thus, they could be proteins or polysaccharides (or a mixture of both type of structures, proteoglucans etc.). Therefore, in order to define furthermore the chemical nature of the inhibitors, the mushroom extracts showing HMGCR inhibitory activities were treated with specific enzymes mixtures. When pepsin was applied to the mushroom extract only a slight

reduction of the initial inhibitory capacity was observed (from 48 to 41% inhibition). The mushroom extracts mixed with enzymes able to hydrolyze β -linked glucans (*exo*-1,3- β -glucanase and β -glucosidase) showed only a 24.3% of HMGCR inhibition indicating that almost half of the inhibitory capacity was lost in the presence of β -hydrolytic enzymes. When the extract was mixed with amyloglucosidase (hydrolyze α -linked polysaccharides) and invertase still 34.1% inhibitory activity was observed indicating that α -glucans (such as starch or glycogen) might not be so much involved in the inhibitory effect as β -glucans.

Study of the potential in vitro mechanism of action of the HMGCR inhibitors

If, as above suggested, certain β -glucans might be the compounds responsible of the observed inhibition, their mechanism of action cannot be similar to statins. The latter are small molecules acting as competitive inhibitors for which the enzyme showed 1000 folds more affinity ($K_I \approx \text{nM}$) than for its substrate ($K_M \approx \mu\text{M}$) HMGCoA (3-hydroxy-3-methylglutaryl-CoA).¹² However, fungal β -glucans showed gel forming properties able to scavenge small molecules within their complex structures²⁶ thus, perhaps these water/methanol-soluble mushroom β -glucans might be able to inhibit the enzyme by scavenging its substrate. Moreover, these polysaccharides can also bind proteins and if they form linkages close to the catalytic center they might also impair the enzymatic reaction.

Thus, the HMGCR inhibitory test was carried out by mixing firstly the mushroom extract with the substrate or with the enzyme and then performing the standard procedure. As expected, addition of the mushroom extract inhibited the activity however, no significant differences were found if the sample was firstly mixed with the substrate or with the

enzyme. Thus, the same procedure was carried out by mixing the samples first with pravastatin instead of the substrate and with the enzyme in the presence of pravastatin. The selected statin dilution was able to inhibit 87.8% of the HMGCR activity however, if the pravastatin was mixed with the mushroom extract and added to the test 71.5% inhibition was recorded suggesting that pravastatin might have been partially scavenged because of the decrease (approx. 16%) of its inhibitory effect. But, when the sample was firstly mixed with the enzyme also 11% reduction in the pravastatin inhibitory activity was observed suggesting that the sample was also impairing the proper binding of the inhibitor into the catalytic site (since the enzyme has more affinity for pravastatin than for the substrate). Results indicated that both proposed mechanisms could be possible and might take place simultaneously at least in the well of the *in vitro* test since the same mushroom extract was acting as HMGCR inhibitor or as inhibitor of the pravastatin inhibitory action depending on the testing conditions. However, in order to test whether these mechanisms have a real significance *in vivo*, animal models should be utilized. Moreover, it is wise to mention that not all the β -glucans present in *A. bisporus* were able to inhibit the HMGCR activity since for instance the ASE extracts obtained using pressurized water at 150°C (Figure 3) contained large amounts of β -glucans²⁶ but they showed very low HMGCR inhibitory activity.

CONCLUSIONS

Agaricus bisporus extracts able to inhibit the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) activity could be obtained by environmentally friendly advanced technologies such as supercritical fluid extractions (SFE) with CO₂ and 10% ethanol and accelerated

1 solvent extractions (ASE) using methanol:water (1:1 v/v) as pressurized solvent being the
2 latter more effective than SFE or standard extraction methods. Cultivation parameters, flush,
3 strain and tissue type could modulate the higher or lower HMGCR inhibitory activity but
4 not the substrate supplementation with selenium. LC-MS analysis confirmed the absence of
5 statins in the inhibitory fractions obtained instead, certain β -glucans easily extracted with
6 methanol:water at moderated temperatures (lower than 100°C) and with molecular weight
7 higher than 10 kDa., were pointed as the compounds which might be responsible the
8 observed activity. They might scavenge the HMGCR substrate and bind to the enzyme
9 impairing the enzymatic reaction in the *in vitro* HMGCR activity test.

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Table 1. Cultivation parameters recorded and correlated with the HMGCR inhibitory activity values obtained within the analyzed *A. bisporus* samples.

Parameter	Different type of samples
Selenium-enriched mushrooms	Low (10.9 ppm)/ high (31.6 ppm)/ control (3.2 ppm) Se content
Commercial spawn	Fungisem H15 and H5, Gurelan 60, Mispaj 365 and Somycel A15
Developmental stage	Primordia(stage 1), small(2), medium(3-4), large(5-6)
Flush number	First, second and third flush

Table 2. Extraction yields obtained after submission of *Agaricus bisporus* (Fungisem H15) basidiomata to ASE at different temperatures using two different pressurized solvent mixtures.

ASE temperatures	100% water		Ethanol:water (1:1)	
(°C)	(mg g ⁻¹ dw)	%	(mg g ⁻¹ dw)	%
25	185±94.75 ^a	18.5	62±13.11 ^a	6.2
50	261±127.99 ^a	26.1	181±29.70 ^b	18.1
100	283±70.71 ^a	28.3	333±25.71 ^c	33.3
150	484±44.55 ^b	48.4	NC	-
200	749±156.98 ^c	74.9	NC	-

NC: not considered

^{a,b,c} Denotes statistically significant differences ($P < 0.05$) among values from the same column

Table 3. Extraction yields obtained after submission of *Agaricus bisporus* (Fungisem H15) basidioma to SFE at different pressures with or without ethanol as co-solvent.

SFE pressures (MPa)	With 10% ethanol		Without 10% ethanol	
	Extracted weight (g)	%	Extracted weight (g)	%
9	1.24±0.1 ^a	1.55	0.50±0.13 ^a	0.62
18	1.36±0.22 ^a	1.70	0.60±0.20 ^a	0.75
30	1.50±0.31 ^a	2.08	0.56±0.16 ^a	0.70

^{a,b} Denotes statistically significant differences ($P < 0.05$) among values from the same column

1

2 **Table 4:** SRM transitions and energies used for statins analysis.

3

Compound	Parent ion (m/z)	Product ion (m/z)	Collision Energy (V)
Lovastatin	427.101 M ⁺ Na ⁺	324.921	24
Simvastatin	441.106 M ⁺ Na ⁺	324.812	24
Pravastatin	447.099 M ⁺ Na ⁺	326.924	18
Atorvastatin	559.114 M ⁺ H ⁺	439.998	24

4

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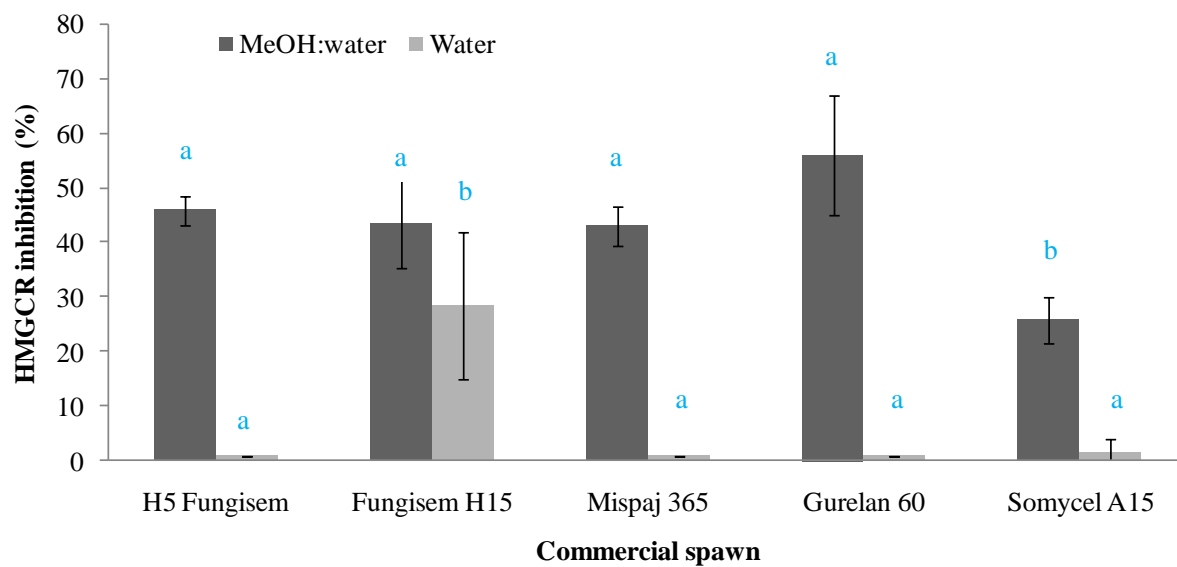


Figure 1. HMGCR inhibitory activity of *A. bisporus* extracts obtained from different commercial varieties.

^{a,b} Denotes statistically significant differences ($P < 0.05$) among the values from the same extraction solvent.

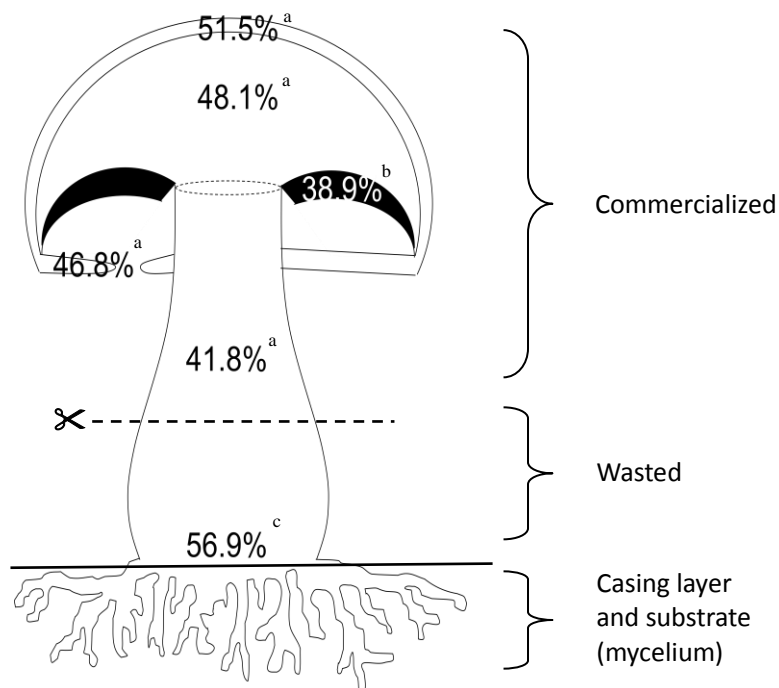


Figure 2. Tissue distribution of the HMGCR inhibitory activity observed within the *A. bisporus* basidioma. The dotted line delimitate the tissues which are usually commercialized (above the line) or discarded during the harvesting (lower part of the stipe and mycelium).
^{a,b,c} Denotes statistically significant differences ($P < 0.05$) among the values.

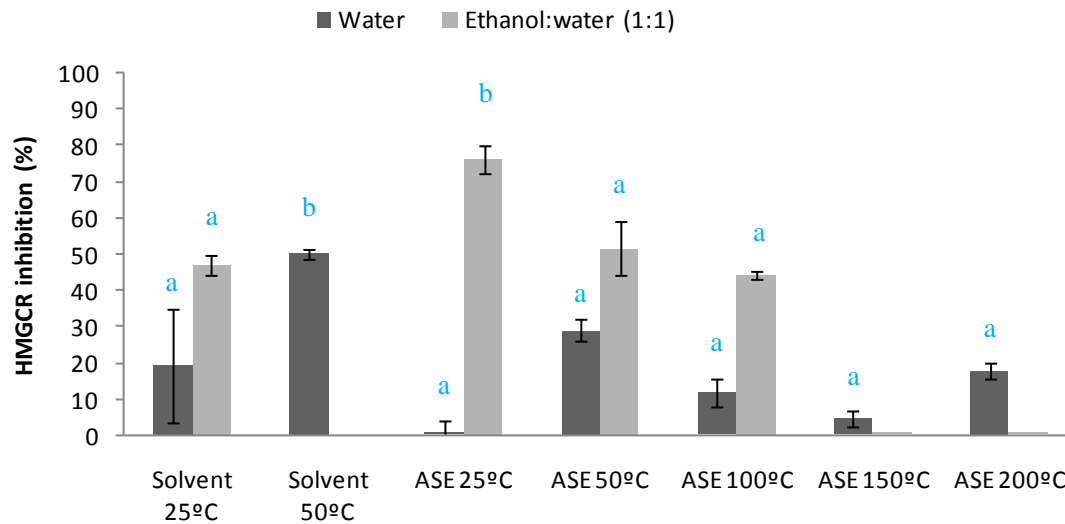


Figure 3. HMGR inhibitory activity of the ASE fractions obtained using two pressurized solvents at different temperatures from *A. bisporus* (Fungisem H15) compared with standardized solid:liquid extractions.

^{a,b} Denotes statistically significant differences ($P < 0.05$) among the values from the same extraction solvent.

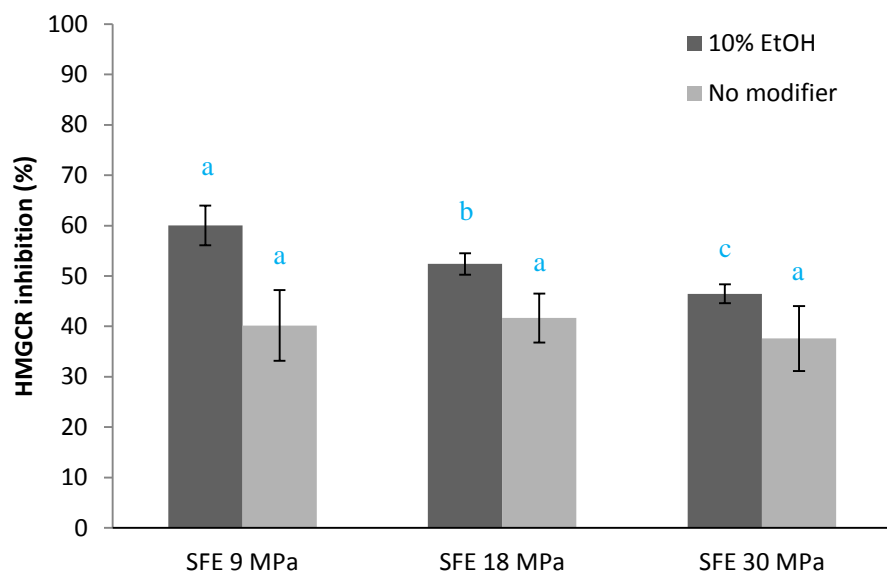


Figure 4. HMGCR inhibitory activity of the SFE fractions obtained using different pressures with or without ethanol as modifier from *A. bisporus* (Fungisem H15).

^{a,b,c} Denotes statistically significant differences ($P < 0.05$) among the values from the same extraction solvent.