Supercritical CO_2 Extraction of secondary metabolites from *Agaricus blazei*: experiments and modelling.

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The mycelium and young fruiting bodies of *Agaricus blazei* were submitted to supercritical CO_2 extraction, in a modified commercial flow apparatus, at temperatures from 40 to 80 ºC, pressures up to 600 bar and CO_2 flow-rates from 2.0 to 9.0 $g.min^{-1}$.

The best extraction conditions of secondary metabolites, whereby the degree of solubilization (g extract/100 g of fungi) is the highest, was obtained with pure CO_2 at 400 bar, 70 ºC and a CO_2 flow rate of 5.7$g.min^{-1}$. The extract in that conditions were analysed by GC-Ms.

In order to increase the extraction yield of secondary metabolites, which are mostly present in glycolipid fractions, a polar compound (ethanol) was used as co-solvent in the proportions of 5 and 10 % (mol/mol). The presence of ethanol increased the yield when compared with the extraction with pure CO_2. Moreover, a simple model was applied to the supercritical CO_2 extraction of secondary metabolites from *Agaricus blazei*.

INTRODUCTION

The fungi *Agaricus blazei* is a basidiomycete native from Brazil which has been used as nutraceutical due its medicinal properties. This *Agaricus* is an option fit for human consumption, with a somewhat sweet taste and fragrance of almonds. The almond flavor is due to the presence of benzaldehyde, benzyl alcohol, benzonitrile, and methyl benzoat [1]. This mushroom is also well known for its purported medicinal properties. *Agaricus blazei* mushroom in particular assists in the production of interferon and interleukin, which are potent in fighting off cancer cell metastasis. It also reduces blood glucose, blood pressure, cholesterol levels and the effects of arteriosclerosis. The antitumor properties of the fungi were reference in several works [2, 3, 4].

Extracts obtain with hexane dichloromethane and methanol has been study recently [5]. The isolation and characterization of different components from Agaricus *blazei* were also obtain [6].

Supercritical fluid extraction is a separation technique where it is possible to obtain valuable lipids. Carbon dioxide is the most used supercritical solvent, because the obtained extracts are toxic solvent free and the degradation of thermal labile components is avoided due to the moderate temperature used in the process [7, 8, 9].

The objectives of this work are to carry out the supercritical CO_2 extraction of lipids, from *Agaricus blazei*, and to assess the influence of several parameters, namely pressure, temperature and flow rate.

MATERIALS AND METHODS

The supercritical fluid extraction experiments were performed in a flow apparatus (Figure 1). This equipment allows carrying out studies at a temperature up to 120ºC and a pressure up to 600 bar.

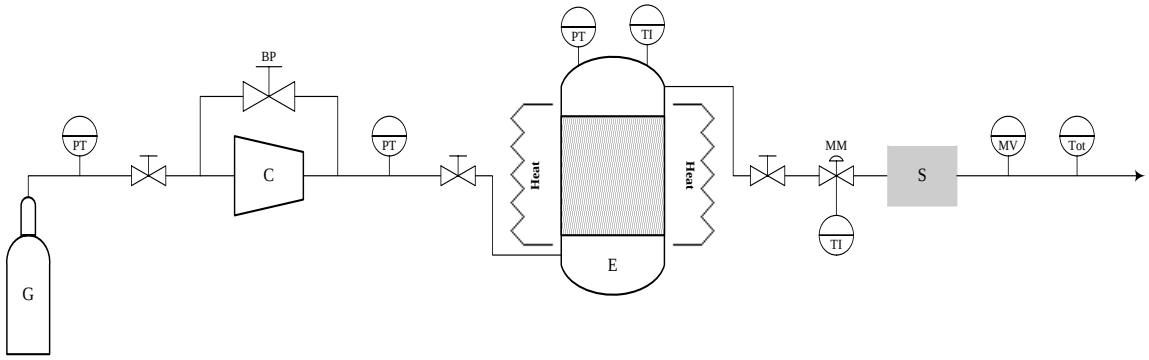

Figure 1: Diagram of the supercritical fluid extraction apparatus.G, CO_2; C, compressor; E, extractor; S, separator; BP, back-pressure regulator; MM, micrometer valve; MV, flow meter; Tot, totalizer; TI, temperature indicator, PT, pressure indicator.

The liquid CO_2 flowing from the cylinder was compressed to the desire pressure (Applied Separations, Spe-ed^{TM} SFE) into the extraction vessel, which is heated. Then CO_2 flows through a bed of glass spheres, propylene wool, sample, propylene wool and bed of glass

spheres. The total volume of CO_2 was determined with a mass flow meter GFM and a totalizer (AALBORG). The CO_2 (99.995% purity) was supplied by Air Liquide (Portugal).

Figure 2 –Global picture of the equipment

Figure 3 – Extraction vessels inside of the furnace (heating system).

The sample, *Agaricus Blazei* mushroom supplement powder, was purchased (MRL – Mycology Research Laboratories, Ltd.) and appears as small spherical beans less than 0.3mm medium diameter, homogeneous and lyophilized.

Supercritical fluid extraction was carried out using 10g of biomass weight in an analytical balance (Sartorius CP224S). Conditions of extraction were: CO_2 flow rates of 2.0 $g.min^{-1}$, 5.7 $g.min^{-1}$ and 9.0 $g.min^{-1}$, pressures up to 600 bar and temperatures up to 80 ºC. The conditions were supervised during all experiments. The extracts were collected in a U tube, at the atmospheric pressure and a temperature controlled with an ice bath. The amount of extract obtained was assessed gravimetrically.

GC-MS-FID analysis

Agaricus extracts were analyzed on a Thermo Trace Ultra Polaris GC-MS with Split/Splitless injector and DPFC. For Identification and quantification analysis, Ion Trap MS and FID detectors were used, respectively.

A RTX-5 (30 m x 0.25 mm x 0.25 μm (Restek)) capillary GC column was used for separation of extract components, programmed from 50 ºC (1 min. hold) to 280 ºC (15 min. hold) with heating rates of 30 ºC min^{-1} to 100 ºC (no hold), 3 ºC min^{-1} to 150 ºC (no hold) and 5 ºC min^{-1}. Split Injector, FID detector, GC-MS transfer line and MS Ion source temperatures were 220, 250, 280 and 220 ºC, respectively. Injection was performed in the split mode with a 1/50 split

ratio. Helium was used as carrier at a constant flow of 1.0 ml/min and Ion Trap damping gas was and 0.3 ml/min, respectively. A 70 eV ionization voltage, a 25 ms max. ion time and a 0.5 s/scan were used in Ion Trap MS analysis. For quantitative analysis the same response factor of 1 was used for all identified compounds. For identification of compounds the NIST mass spectral search program for the NIST/EPA/NIH Mass Spectral Library version 2.0a was used [10].

RESULTS AND CONCLUSIONS

The biomass of *Agaricus blazei* was submitted to supercritical fluid CO_2 extraction at the following conditions: flow rates from 2.0, and 9.0 g.min^{-1} of CO_2, at a pressure of 400 bar and temperature of 70 ºC. Yield in lipids, collected at regular time intervals are shown in Figure 4 as a function of the CO_2 mass.

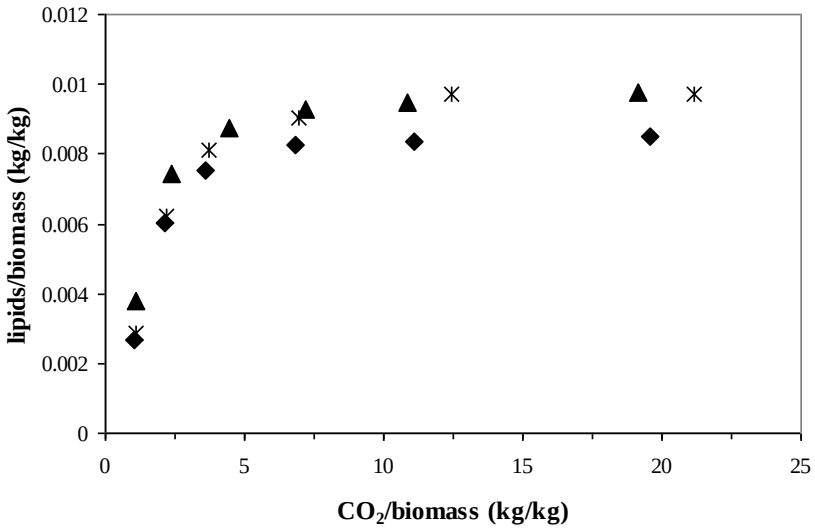

Figure 4: Yield of extracted lipids from *Agaricus blazei* as a function of carbon dioxide mass at 400 bar and 70 ºC, at several flow rates of CO_2: ◆ - 2.0 g.min^{-1}, ▲ – 5.7 g.min^{-1}, ✸ - 9.0 g.min^{-1}.

It can be seemed that the increase of flow rate increases the yield, but after 5.7 g.min^{-1} no significantly evaluation can be detected.

To assess the effect of pressure and temperature, at a flow rate of 5.7 g.min^{-1} experiments were carried out up to 600 bar and temperatures up to 80 ºC. The results are shown in Figure 5 and Figure 6.

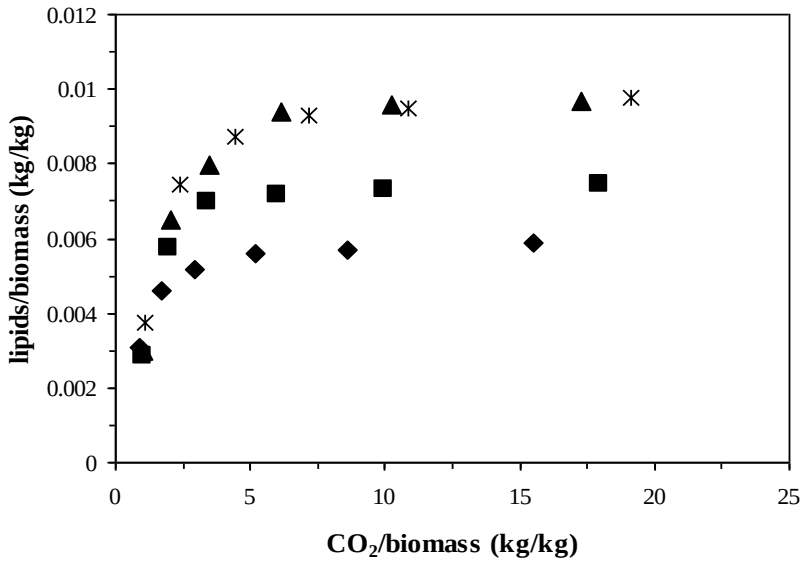

Figure 5: Yield of extracted lipids from *Agaricus blazei* as a function of carbon dioxide mass at 70 ºC and CO_2 flow rates of 5.7 g.min^{-1} for different pressures ◆ - 300 bar, ■ - 350 bar, * - 400 bar and ▲- 600 bar.

At constant temperature and flow rate, the yield increased with the pressure (Figure 5) until 400 bar. The pressure improved the solvent power of the supercritical fluid, due to the increase of its density, but it seems that the higher solubility of the lipids were obtain at 400 bar, since no improved in the yields of extracted lipids were obtained after that.

On the other hand, at constant pressure and flow rate, the yield increased with the temperature until 70 ºC (Figure 6). In fact, the temperature increases the vapor pressure of the lipids, leading also to its higher solubility. From the results that effect, seems to be more important until the temperature of 70 ºC.

With the aim of increasing the extraction yield in lipids from the dried biomass, a polar compound (ethanol) was used as co-solvent in the proportions of 5 and 10 % (mol/mol). The extractions were performed at 400 bar, 70ºC and flow rate of CO_2 of 5.7 g.min^{-1}. The

maximum yields obtain were 0.011 and 0.013 kg of lipids by kg of biomass, respectively. In that case a small increase in the total yield was obtained.

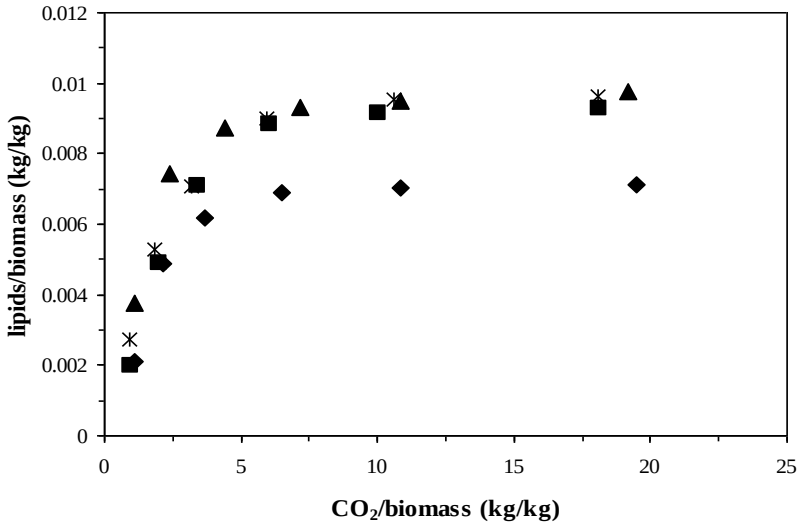

Figure 6: Yield of extracted lipids from *Agaricus blazei* as a function of carbon dioxide mass at 400 bar and CO_2 flow rates of of 5.7 $g.min^{-1}$ for different temperatures ◆ - 40 ºC, ■ - 60ºC, ▲ –70 ºC and * - 80 ºC

The extract obtained in the best conditions (400 bar and 70 ºC) without co-solvent (ethanol) were analyse in the GC-MS-FID system as describe previous. In Table 1 it is presented the (%) percentage composition of the total extract analyse assuming the same response factor of 1 for all identified compounds in the DB-1 column. Mainly all the extracts obtained presents analogous composition.

The main compounds identified in the extract were hexadecanoic acid (49 %) and oleic acid (34 %). The others free fatty acids are presented in lower concentration like stearic (4.15 %) acid, linoleic acid (3.87 %) and arachidic acid (1.35 %). The fatty acids present in *Agaricus blazei* can have antimutagenic as well as desmutagenic activity such as a bio-antimutagenic effect [11]. Its desmutagenic activity involves the capture of mutagenic agents in lipid micelles or an interaction with enzymatic mechanisms that inhibit the activation of promutagens [12].

Table 1 - Composition of the extract obtained by supercritical CO_2 extraction (400 bar, 70 ºC) from *Agaricus blazei.*

Compound	Retention time (min)	Área (%)
Unknown	6.64	0.170
Hexanoic acid	6.80	1.792
Heptanoic acid	8.83	0.179
Octanoic acid	11.43	0.824
Glicerol	11.97	0.074
But-2-enedioic acid	14.20	0.111
Nonanoic acid	14.50	0.247
Decanoic acid	17.85	0.024
3-Hydroxyoctanoic acid	18.69	0.012
Dodecanoic acid	24.03	0.068
Tetradecanoic acid	28.80	0.358
Decanedioic acid, Sebacic acid	29.89	0.059
Pentadecanoic acid	30.88	0.131
Palmitoleic acid	32.33	0.084
Palmitelaidic acid	32.44	0.114
Hexadecanoic acid	32.85	49.00
2-Hydroxysebacic	33.17	t
Heptadecanoic acid	34.64	0.237
Linoleic acid ethyl ester	35.06	2.353
Linoleic acid	35.87	3.870
Oleic acid	35.97	34.07
Elaidic acid	36.06	0.961
Stearic acid	36.38	4.151
Arachidic acid	39.62	1.346
Hydroxystearic acid	39.94	t
Henicosanoic acid	41.15	0.117
Erucic acid	42.28	0.050
Behenic acid	42.62	0.854
Tricosanoic acid	44.04	0.127
1-Monooleoylglycerol	44.48	t
Lignoceric acid	45.41	0.895
Pentacosanoic acid	46.90	t
Hexacosanoic acid	48.64	0.087

Retention index in the DB-1 column, **t = trace** (< 0.05 %).

More recently [13] it was recommended that a more in detail biochemical and mutagenic studies of the active principles in the components of these extracts are essential to better establish and distinguish their medicinal properties against more diverse diseases, which is normally recommended

Modelling

A simple empirical model based on a function of the Langmuir gas adsorption isotherm type was used [14] to model the results:

$$Y = \frac{Y_{\infty} t}{B + t} \qquad (1)$$

The model is represented by Equation (1) that has only two parameters of adjustment: $\mathbf{Y}_{\infty}$ and **B**. The value found for Y (yield) is equivalent to the ratio of recovered mass of extract on time t (s) to the initial mass of solute-free feed (kg of extract/ kg of solute-free feed) and $\mathbf{Y}_{\infty}$ is the **Y** value for infinite extraction time or the maximum amount of solute recovered; while the ratio $\mathbf{Y}_{\infty}$/B corresponds to the initial slope of the extraction yield curve versus time. The results to different flow rates the results were presented in Table 2.

Table 2 – Parameters obtained to the different CO_2 flow rates at 400 bar and 70 ºC

Flow rates ($g.min^{-1}$)	Y_{∞}	B
2.0	0.010	1.7432
5.7	0.011	1.4999
9.0	0.011	1.7900

To the different flow rates $\mathbf{Y}_{\infty}$ parameter presented very low values almost without variation, but **B** parameter presented high values with a diminutive variation indicating its dependency on the process conditions. Since this model is based on Langmuir isotherm it should represent well the initial period of the extraction process, which is associated to desorption stage and a convective mechanism. It is important to remember that extraction processes occur in a more complex approach.

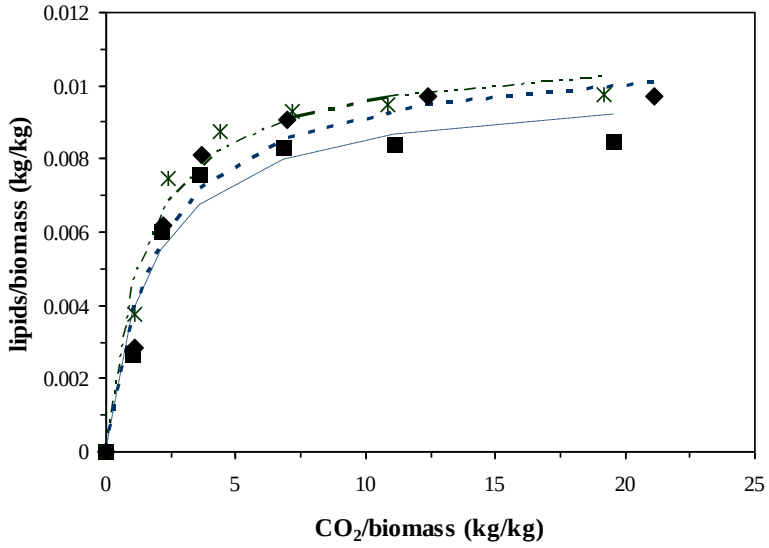

Figure 7: Yield of extracted lipids from *Agaricus blazei* as a function of carbon dioxide mass at 400 bar and 70 ºC, at several CO_2 flow rates : Experimental: ▲ - 2.0 g.min^{-1}, *– 5.7 g.min^{-1}, ◆- 9.0 g.min^{-1}; lines represent the model used.

Figure 7 shows experimental data and curves calculated with model. The empirical model was able to represent the initial stage of the studied extraction process that is controlled by the convection mechanism. The parameters estimated by the model could be used to estimate the necessary time to obtain extracts from *Agaricus blazei* in diverse conditions.

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