SUPERCRITICAL CO_2 AS SOLVENT FOR EXTRACTION OF FUNGAL METABOLITES

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ABSTRACT

Fungi produce a diverse array of metabolites – small molecules that are not necessary for normal growth or development. Some are exploited for their antibiotic and pharmaceutical activities; others are involved in disease interactions with plants or animals and are better known as mycotoxins. Supercritical fluid extraction, especially with supercritical CO_2 as an environmentally safe extraction medium, received a lot of attention in the 1990s. In our study, supercritical CO_2 was used to obtain metabolites from *Wallemia sebi*, better known as the food spoilage fungus. The results of our study revealed that SC CO_2 can be successfully applied as solvent for extraction of fungal metabolites. Due to the simplicity of the method, SC CO_2 seems to be suitable solvent for the extraction of high quantities of biologically active metabolite from *W. sebi* for further identification.

Key words: supercritical fluid extraction, supercritical CO_2, fungal secondary metabolites, mycotoxins

INTRODUCTION

The potential of supercritical fluid extraction (SFE) as an alternative to conventional extraction procedures has been demonstrated in a wide variety of samples **[1]**. Commonly used conventional extraction and purification procedures require time-consuming solvent extraction with high consumption of toxic organic solvents. In comparison to conventional techniques for extraction and sample purification, where a great amount of potentially health-hazardous solvents are employed, supercritical CO_2 (SC CO_2) is non-toxic, non-flammable and chemically inert solvent. Further, one of the greatest advantages of SFE over other extraction techniques is that it can be automated. This makes it highly suitable for rapid, routine analysis as it provides quantitative transfer of extracted analytes to the analytical instrument resulting in a reduced contamination of analytes. Generally the quantitative analysis after extraction of analytes from matrix involves laborious purification procedures **[2]**.

Supercritical fluids generally have low viscosities (e.g. 10^{-3}–10^{-4} g/cm/s), high diffusivities (e.g. 10^{-3}–10^{-4} cm^2/s) and moderate densities (e.g. 0.3–0.8 g/cm) which lead to more rapid and efficient extractions because of the combination of liquid-like solvating and gas-like mass transfer properties. Hence, SFE has been shown to be a quantitative and rapid method for extraction of nonpolar components from different sample matrices. The SC CO_2 as extraction solvent has a strong lipophilic selectivity and unwanted polar substances such as organic acids, salts, sugars and glycosides that are fully insoluble can be easily extractable from matrix**.** Furthermore, modifications of the extraction selectivity can be simply achieved by changing the temperature or pressure during extraction. This offers the advantage of obtaining an extract that is virtually free of other substances **[3].**

Mycotoxins are defined as secondary metabolites produced by fungal species, growing on agricultural products during cultivation, harvest, transport and storage. Their occurrence in food has been recognized as potential human health-hazard either caused by direct contamination of grains and fruits and their products or by accumulation of mycotoxins and their metabolites in animal tissues **[4]**.

Ambrosino et al. **[5]** described the development and the optimization of an SFE protocol for extraction of beauvericin, a cyclic hexadepsipeptide capable of inducing programmed cell death in mammals produced by fungi such as *Beauveria bassiana* and *Paecilomyces fumosoroseus*, on spiked and natural contaminated maize samples. The main recorded advantages of SFE used for isolation and analysis of beauvericin were: a) acquisition of a cleaner extract (more HPLC-friendly) and b) low consumption of organic solvent (more environment-friendly).

Wallemia sebi (*W. sebi*) is a xerophilic mould able to grow in a wide range of water activities (a_w) **[6]**. Wheeler et al. **[7]** consider it ubiquitous, although records of its occurrence in foods are scarce. This is probably due to the use of inadequate techniques of isolation, since *W. sebi* does not grow well on common high a_w laboratory media. Considered as one of the main spoiling fungi of salted and dehydrated fish, it has been isolated from prunes, syrups, grape raisins, jams, jellies, dry peppers, bread, condensed milk, dates and grains such as rice, corn, wheat, peanut and soybeans. Furthermore, *W. sebi* is the only species of *Wallemia* that has been involved in human

infections. Unfortunately, due to the rarity of *W. sebi* infections, there is hardly any data on their clinical features and treatment **[8].** Additionally, only few reports deal with secondary metabolites of *W. sebi* revealing the fungus as a producer of biologically active metabolites, some of which having a minimum inhibitory dose in the bioassays comparable with such mycotoxins as penicillic acid and citrinin **[9, 10, 11]**. Some of its metabolites are yet unidentified but recorded to have antifungal and cytotoxic activity **[11]**.

The aim of the application of SC CO_2 as a solvent for extraction of metabolites from *W. sebi* biomass in the present study was: **a)** to avoid the use of aggressive organic solvents for extraction and purification of metabolites **b)** to obtain the target metabolite that in preliminary studies showed to have biological activity for further identification.

MATERIALS AND METHODS

Microorganism and growth conditions

W. sebi EXF-958 (isolate from EXF collection, Ljubljana, Slovenia) was grown in yeast nitrogen based (YNB) broth supplemented with 20% NaCl till stationary phase at 28°C. After filtration, the collected biomass was lyophilized and stored at +4°C.

Preparation of biomass

Prior to extraction, the lyophilized fungal biomass was grounded using a mechanical grinder. The arithmetic mean diameter was used as measure for the average particle size and was measured using a laser granulometer (FRITSCH analysette 22 compact, Germany).

Apparatus

The SFE experiments using SC CO_2 were performed on a semi-continuous flow apparatus (**Figure 1**) build in the workshop of the university. The apparatus was constructed for a maximum pressure of 500 bar and a temperature of 100°C. Approximately 0.5 g of grounded fungal biomass was charged into the extractor. The temperature of the water bath was regulated and maintained at a constant level (±0.5 °C, Lauda dr. R. Wobser GmbH & Co. KG, Lauda Konigshofen, Germany). The apparatus was first purged with the gas used for extraction. In the next step, liquefied gas (CO_2) was continuously pumped with a high pressure pump (ISCO syringe pump, model 200DX, Lincoln, Nebraska, *P*max = 450 bar) through the preheating coil and over the sample bed in the extractor. The solvent flow rate was measured with a flow meter (ELSTER HANDEL GmbH, Mainz, Germany).

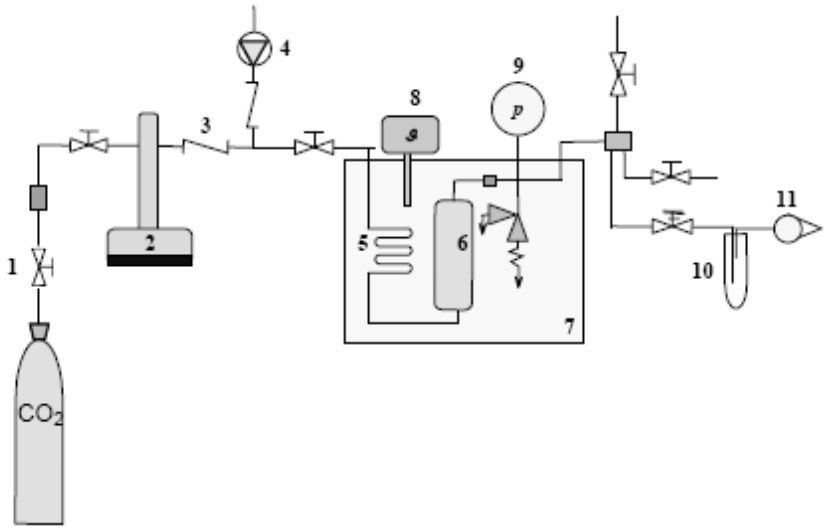

Figure 1: Semi-continuous flow apparatus for SFE **[12]**: **1)** CO_2 supply, **2)** high-pressure pump, **3)** valve, **4)** pump for co-solvent, **5)** pre-heater coil, **6)** extractor, **7)** water bath, **8)** heater, **9)** manometer, **10)** collection vial, **11)** rotameter.

In the study, two SFE flow modes for extraction of metabolites from *W. sebi* biomass were used: **a)** a continuous flow mode and **b)** a pause flow mode. To prevent formation of ice and to maintain the SC CO_2 dynamic flow at 1 mL min^{-1}, a valve before the collection vial was heated. The extract was collected in a glass vial, and from the weight of the extract the yield was calculated. The acquired extract was dissolved in methanol (Merck, Germany), analysed using a UV-VIS spectrophotometer and fractionated on a silica gel plate with thin layer chromatography.

UV-VIS measurement

Absorption spectra of metabolites present in CO_2 extract were determined by making the absorption scan in range from 200 nm to 800 nm using a Varian UV-VIS spectrophotometer (Germany). Peaks were recorded at peak threshold of 0.01.

Thin layer chromatography (TLC)

CO_2 extract was fractionated using thin layer chromatography (TLC) on a 3 x 8 cm silica gel plate (60 F_{254}, 0.2 mm, Merck). The TLC plates were developed using a toluene : ethyl acetate : formic acid (5:4:1) solvent system. Afterwards, the TLC plates were air-dried at 100°C and visualized under the UV-light (366 nm). Retention factor (R_f) for each observed metabolite was calculated as the travel distance of the metabolite divided by the travel distance of the solvent.

RESULTS

The extractability of metabolites from *W. sebi* was studied using SFE technique. Modern extraction technique using SC CO_2 was applied in order to avoid the usage of aggressive organic solvents and to maximize recovery of target metabolite that in preliminary studies (data not shown) demonstrated strong "*in vitro*" biological activity.

To achieve a better contact between fungal biomass and solvent, the biomass was grounded before filling into the extractor. The average size of particles used for extraction was 80.9 µm (arithmetic mean diameter).

In the study, two flow modes were used: **a)** continuous flow mode and **b)** pause flow mode. In the continuous flow mode, CO_2 continuously extracted metabolites from the biomass in the extractor to the collection vial for an entire dynamic time. In the pause flow mode, first the static equilibrium was achieved for a certain time followed by dynamic extraction step. The yield of extracted metabolites was calculated separately for continuous flow mode and static flow mode according to extraction time (**Figure 2**).

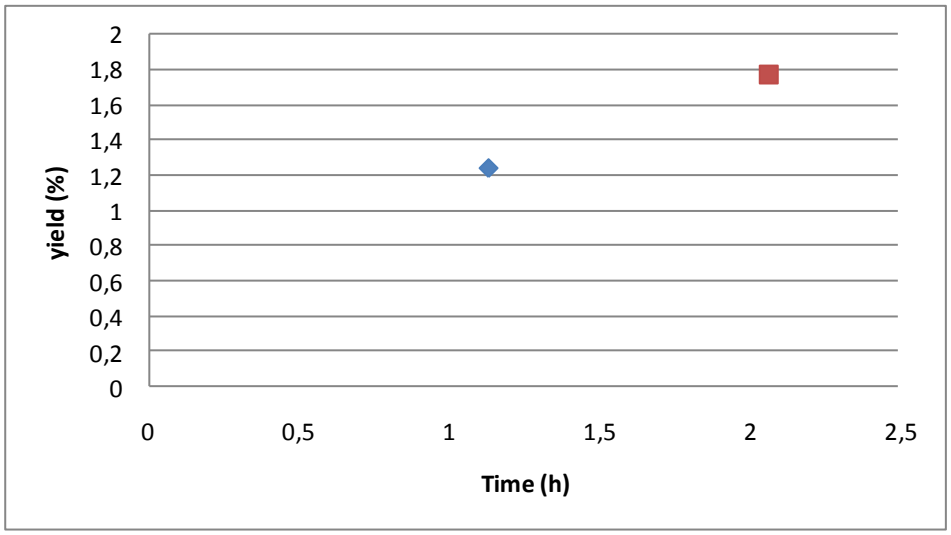

Figure 2. Extraction yield (%) for continuous flow mode (■) and static flow mode (♦) according to extraction time (h).

From Figure 2, it can be observed that continuous flow mode resulted in a better yield, while pause flow mode shortens the extraction time. Optimization of process is currently under the study.

Further, to check the purity of CO_2 extract acquired with continuous flow mode the absorbance spectrum of metabolites dissolved in methanol was recorded. Absorption maxima of metabolites are presented in **Figure 3.**

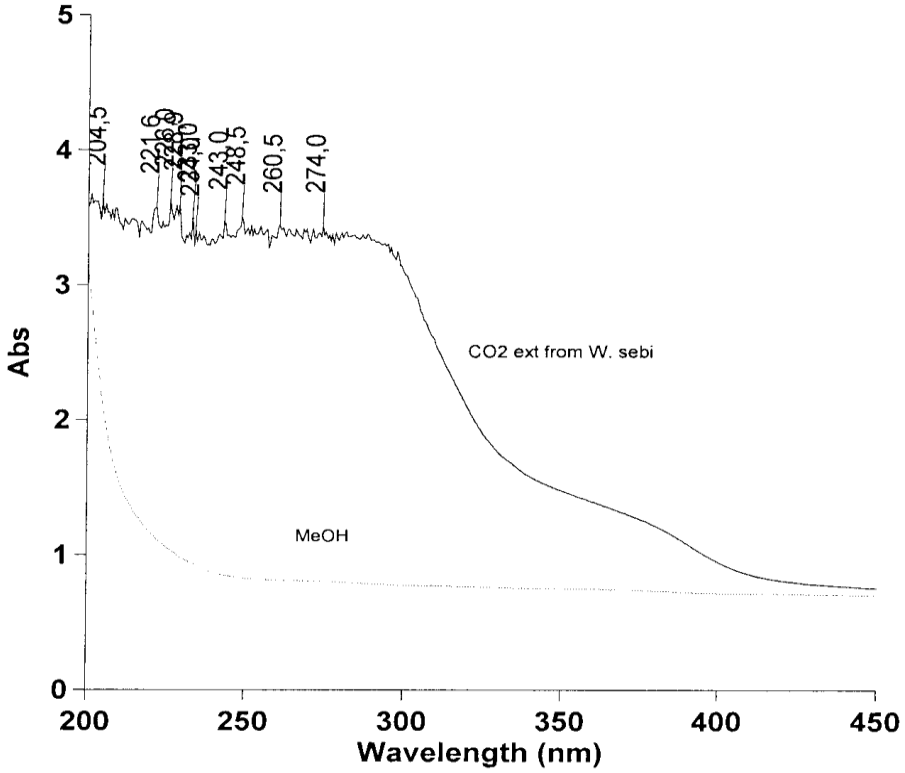

Figure 3. UV-VIS spectrum of metabolites acquired using SC CO_2 from *W. sebi* biomass.

UV-VIS measurement of CO_2 extract dissolved in methanol revealed that metabolites acquired in extract have absorption maxima between 201.0 nm and 297.5 nm range. Peaks recorded in mentioned range reflects also the purity of extract. It can be observed that extract contain numerous metabolites. Extract was further subject of fractionation with TLC.

In order to separate metabolites and compare whether the target metabolite was acquired using both flow modes, collected CO_2 extracts were visualised under UV-light (366nm) after being dissolved in methanol and fractionated on silica gel plate (**Figure 4**).

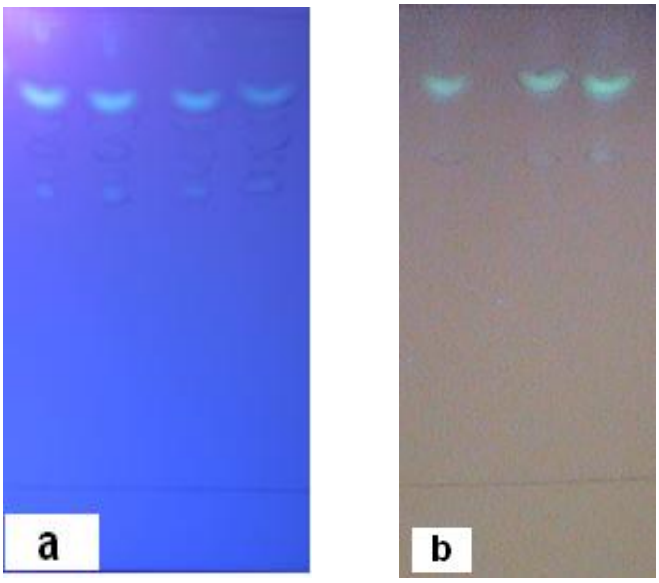

Figure 4: TLC silica gel plate with fractionated metabolites acquired from *W. sebi* using SC CO_2 with **a)** continuous flow mode and **b)** pause flow mode.

Additionally, the R_f values of each fractionated metabolite in the CO_2 extract acquired with continuous flow mode was calculated from the TLC silica gel plate (**Table 1**).

Table 1: Retention factors (R_f) of metabolites in the CO_2 extract acquired from lyophilized fungal biomass of *W. sebi* grown in YNB broth with 20% NaCl, fractionated on silica gel plate using TLC, and visualized under the UV-light (366nm).

Fractionated metabolites from *W. sebi* biomass	
Fraction on silica gel TLC plate	**R_f**
1	0.64
2	0.67
3	0.735
4	0.786
5	**0.83**

Extraction of *W. sebi* fungal biomass with SC CO_2, regardless of the flow mode used, resulted in extraction of non-polar metabolites that travel high along the silica gel plate. Illumination with a 366 nm wavelength caused fluorescence quenching and visualisation of five separated metabolite fractions. Among all the metabolites present in the extract, the one with R_f of 0.83 was acquired

in the highest quantity (**Figure 4**). Preliminary assay showed that mentioned metabolite exhibits strong "*in vitro*" biological activity and is a target metabolite of the reported study acquired using SCF extraction technique.

CONCLUSION

This is the first report on usage of modern extraction techniques for the extraction of metabolites from *W. sebi*. While supercritical fluid extraction (SFE) has been described as efficient method for extraction of other fungal metabolites, the isolation and purification of biologically active metabolites from *W. sebi* has previously only been performed using organic solvent extraction, TLC and HPLC **[8, 9]**.

The results of our study revealed that SC CO_2 can be successfully applied as solvent for extraction of metabolites from *W. sebi* regarding the flow mode used. Furthermore, due to the simplicity of the method SFE seems to be suitable for the acquisition of high quantities of biologically active metabolite from *W. sebi* for further identification.

Additionally, application of modern extraction method, such are SFE, could lead to the identification of new metabolites produced by xerophilic mould used in the study.

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