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Supercritical CO₂ extraction of solid-state cultivation fungus producing azaphilone polyketides.

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Abstract:

Supercritical Fluid Extraction (SFE) methods dedicated to microorganisms specialized metabolites are very scarce in the literature and limited to liquid cultivation. We proposed here a new sample preparation method to achieve SFE of specialized metabolites from solid-state cultivation. SFE parameters, including CO₂ pressure, temperature of extraction cell and percentage of co-solvent, were optimized in the case of solid-state cultures of *Penicillium sclerotiorum* SNB-CN111, a filamentous fungus producing azaphilone pigments. The metabolic composition of the extracts was then analyzed by reverse-phase liquid chromatography coupled to electrospray ionization and tandem mass spectrometry in data dependent acquisition mode. The resulting molecular networks generated by MetGem software allowed the annotation of the extracted metabolites in the different conditions, confirming the enrichment of fractions according to the polarity of azaphilone subfamilies. First, the 100% CO₂ fraction a yield ten times higher than hexane maceration. The optimization of SFE method led to an extraction yield twice as high as ethyl acetate maceration when mixing CO₂ with ethanol and, indicating that CO₂/ethanol SFE is more environmentally-friendly and efficient than standard maceration methods for the extraction of azaphilones from solid-state fermentation.

Keywords: Supercritical Fluid Extraction, microorganisms, fungus, *Penicillium sclerotiorum*, azaphilones, specialized metabolites

1. Introduction

Dyes and pigments are used in many industries (textile, food) and represent a global market of USD 38.2 billion in 2022 [1]. Synthetic pigments, such as azo dye, are known to persist and accumulate in aquatic environments [2,3]. Therefore, these pigments have adverse effects on aquatic life and food chain due to their UV absorption properties which disrupt photosynthetic activities [4]. Adverse effects on human health have also been reported, such as hepatocarcinogenic effects [5]. To promote more environmentally friendly processes, the exploitation of pigments of natural origin, i.e. mineral, animal, vegetable origin or produced by microorganisms, is an attractive alternative [6,7, 8]. Among the sources of natural pigments, microbial fermentation is the most promising one, as it is renewable, requires less space, produced lower greenhouse gas emission, and is no subject to seasonality [9] Large-scale production of pigments from microorganisms is already explored for specific molecular families such as carotenoids, astaxanthin and lutein [10]. Among other pigments produced by microorganisms, azaphilones, a family of yellow, orange, red and violet metabolites, have been identified as biosourced pigments [11].

Azaphilones are compounds produced by filamentous fungi used for thousands of years in fermentation processes, especially several *Monascus* sp. in Asia [12,13]. However, citrinin, a nephrotoxic metabolite, is co-produced during *Monascus* sp.. fermentation highly limiting their commercialization in Europe and USA [6,14]. Thus, alternative strains to *Monascus* sp., such as *Penicillium* or *Talaromyces* sp. producing azaphilones, are now being explored for pigment production [15]. However, one of the drawbacks of pigment production by microorganisms is the low yield compared to synthetic methods [16].

Supercritical fluid extraction (SFE) based on supercritical CO₂ is a low-temperature extraction method, fulfilling several green chemistry principles, i.e. less toxic solvent, possibly from renewable feedstocks, and easy scalability for industrial purposes [17]. This extraction method is also widely employed for plant material in the field of natural products [18,19]. However, excluding microalgae [20] and cyanobacteria [21–24], examples of SFE of microorganisms, such as bacteria and fungi, are very scarce in the literature. The first supercritical fluid extraction of natural products, published in 1995, tested various microorganisms grown in liquid medium, such as *Penicillium expansum*, *Aspergillus fumigatus* or *Streptomyces* sp. and demonstrated extraction yields equivalent to those achieved by dichloromethane maceration [25]. A supercritical CO₂ extraction of a natural immunosuppressant, mycophenolic acid, produced by solid-state fermentation of *Penicillium brevicompactum* (DSM 2215) was described in 2018 [26]. The last study using SFE to extract metabolites produced by fermentation was published in 2020 and demonstrated advantages of SFE for several specialized metabolites from *Myxococcus xanthus* in liquid fermentation over conventional extraction methods [27]. Thus, despite the extensive use of SFE for the extraction of plant specialized metabolites, the use of SFE on microorganisms and their specialized metabolites remains largely unexplored and unused.

In this study, we propose a SFE method to extract and fractionate specialized metabolites produced during solid-state cultivation. Using *Penicillium sclerotiorum*, a fungus producing azaphilone pigments, we demonstrate that SFE outperforms organic media maceration in both extraction yield and the diversity of extracted metabolites.

2. Materials and Methods

2.1 Fungus material

2.1.1 *Penicillium sclerotiorum* SNB-CN111 cultivation

The strain SNB-CN111 was isolated from *Nasutitermes similis* termite aerial nest sampled in Piste de Saint-Elie in French Guiana. The strain SNB-CN111 from the strain library collection at ICSN was identified as *Penicillium sclerotiorum*. A sample submitted for amplification and nuclear ribosomal internal transcribed spacer region ITS4 sequencing allowed for strain identification by NCBI sequence comparison. The sequence has been registered under registry number KJ023726. Three squares (approximately 0.2 × 0.2 cm) of fungus from an older plate of five days were inoculated into a fresh plate and were cultivated on 10 cm Petri dishes (80 cm²) at 28 °C for 15 days for accumulation of specialized metabolites on potato dextrose agar (PDA) medium (Dominique Dutscher SAS, Brumath, France). At the end of the 15 days, the fungi reached full confluence on the Petri dishes.

2.1.2 Extraction of *Penicillium sclerotiorum* SNB-CN111

For liquid-liquid extraction (LLE), the content of one Petri dish was transferred into a large container and macerated with 25 mL of ethyl acetate or hexane for 24 h. Insoluble residues were removed via filtration and the organic phase was washed three times with 25 mL of water (H₂O) using a separatory funnel, dried with anhydrous solid Na₂SO₄ then evaporated using a rotary evaporator under reduced pressure and at a temperature of 30°C.

For SFE the content of one Petri dish was snap frozen in liquid nitrogen and milled using mortar and pestle until a fine powder was obtained. The powder was thus resuspended in 50 mL of Milli-Q® water and freeze-dried for 16 h. The dry powder (500 mg) was thus inserted in the extraction cell 100 x 4.6 mm, 0.5 mL (Jasco, France).

Extraction by supercritical CO₂ was performed on a 1260 Infinity Analytical SFC system (Agilent Technologies, Waldbronn, Germany) consisting of an Aurora module and an “LC-like” system. The chromatographic column was simply exchanged with the extraction cell. A 15 minutes step gradient consisting of supercritical CO₂/ethanol (v:v, 100:0, 99:1, 98:2, 95:5, 90:10, 80:20, 50:50, flow rate 2 mL.min⁻¹) was used to generate 7 fractions. Ethanol with a flow rate of 0.2 mL.min⁻¹ was used as make-up solvent to efficiently transport fractions into collection vials. Oven temperature was set at 50°C and back pressure regulator at 100 bar for a 100% CO₂ fluid density of 384 kg.m⁻³. For condition optimization, 6 densities were chosen, *i.e.* 384, 628, 780, 834, 910 and 975 kg.m⁻³ corresponding to 50°C, 100 bar, 40°C, 100 bar, 40°C, 150 bar, 50°C, 250 bar, 40°C, 300 bar, 25°C, 320 bar using on-line calculator http://www.peacesoftware.de/einigewerte/co2_e.html.

2.2 LC-MS/MS analysis

Crude extracts and fractions of *Penicillium sclerotiorum* SNB-CN111 strain were prepared at 1 mg.mL⁻¹ in methanol and filtered on 0.45 µm PTFE membrane. RPLC-ESI(+)-MS/MS experiments were performed with a 1260 Prime HPLC (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent 6540 Q-ToF (Agilent Technologies, Waldbronn, Germany) mass spectrometer. LC separation was achieved with an Accucore™ RP-MS column (100 × 2.1 mm, 2.6 µm, Thermo Scientific, Les Ulis, France) with a mobile phase consisting of H₂O/formic acid (99.9/0.1) (A)—acetonitrile/formic acid (99.9/0.1) (B). The column oven was set at 45°C. Compounds were eluted at a flow rate of 0.4 mL.min⁻¹ with a gradient from 5% B to 100% B in 20 min and then 100% B for 3 min. Injection volume was fixed at 5 µL for all analyses. For electrospray ionization source, mass spectra were recorded in positive ion mode with the following parameters: gas temperature 325 °C, drying gas flow rate 10 L.min⁻¹, nebulizer pressure 30 psi, sheath gas temperature 350 °C, sheath gas flow rate 10 L.min⁻¹, capillary voltage 3500 V, nozzle voltage 500 V, fragmentor voltage 130 V, skimmer voltage 45 V, Octopole 1 RF Voltage 750 V. For ESI, internal calibration was achieved with two calibrants purine and hexakis (1h,1h,3h-tetrafluoropropoxy) phosphazene (*m/z* 121.0509 and *m/z* 922.0098) providing a high mass accuracy better than 3 ppm. The data-dependent MS/MS events were acquired for the five most intense

ions detected by full-scan MS, from 200-1000 m/z range, above an absolute threshold of 1000 counts. Selected precursor ions were fragmented at a fixed collision energy of 30 eV and with an isolation window of 1.3 amu. The mass range of the precursor and fragment ions was set as m/z 200–1000.

2.3 Data processing and analysis

The data files were converted from the .d standard data format (Agilent Technologies) to .mzXML format using the MSConvert software, part of the ProteoWizard package 3.0 [28]. All .mzxml were processed using MZmine2v51 as previously described [29]. The mass detection was realized with MS1 noise level at 1000 and MS/MS noise level at 0. The ADAP chromatogram builder was employed with a minimum group size of scans of 3, a group intensity threshold of 1000, a minimum highest intensity of 1000, and m/z tolerance of 0.008 (or 20 ppm). Deconvolution was performed with the ADAP wavelets algorithm according to the following settings: S/N threshold=10, minimum features height=1000, coefficient/area threshold=10, peak duration range 0.01-1.5min, t_R wavelet range 0.00-0.04min. MS/MS scans were paired using an m/z tolerance range of 0.05 Da and t_R tolerance range of 0.5 min. Isotopologues were grouped using the isotopic peak grouper algorithm with an m/z tolerance of 0.008 (or 20 ppm) and a t_R tolerance of 0.2 min. Peaks were filtered using Feature list row filter, keeping only peaks with MS/MS scans (GNPS). Adduct identification, *i.e.* sodium- or potassium-cationized species, was performed on the peak list with a retention time tolerance of 0.1 min, an m/z tolerance of 0.008 or 20 ppm, and a maximum relative peak height of 150%. Complex search, such as dimers, was performed with a retention time tolerance of 0.1 min, an m/z tolerance of 0.008 or 20 ppm, and a maximum relative peak height of 150%. Peak alignment was performed using the join aligner with an m/z tolerance of 0.008 (or 20 ppm), a weight for m/z at 20, a retention time tolerance of 0.2 min, and weight for t_R at 50. The MGF file and the metadata were generated using the export/submit to GNPS option.

Molecular networks were calculated and visualized using MetGem 1.34 software [30]. MS/MS spectra were window-filtered by choosing only the top 6 peaks in the ± 50 Da window throughout the spectrum. The data were filtered by removing all peaks in the ± 17 Da range around the precursor m/z . The m/z tolerance windows used to find the matching peaks was set to 0.02 Da, and cosine scores were kept in consideration for spectra sharing 2 matching peaks at least. The number of iterations, perplexity, learning-rate, and early exaggeration parameters were set to 5000, 25, 200, and 12 for t-SNE view.

MASST [31] was queried using https://github.com/robinschmid/microbe_masst script [32] with the following matching parameters: precursor m/z tol = 0.05, m/z tol = 0.05, minimum cosine similarity = 0.7, minimum matched signals = 3, parallel queries = 10

Figures were generated using R and related packages (ggplot2, Rcolorbrewer, and gridextra, metaboanalyst), MetGem export function, and ChemDraw Professional 16.0 (PerkinElmer).

3. Results

3.1 Extraction parameters optimization

To perform supercritical CO₂ extraction of specialized metabolites from *P. sclerotiorum* SNB-CN111, the sample was snap-frozen in liquid nitrogen, then ground using cryogenic mortar and pestle. The resulting powder was resuspended in Milli-Q® water and then freeze-dried for 16 hours. The resulting product was a dry electrostatic powder that was convenient to handle and compatible with supercritical fluid extraction. From one Petri dish containing 25 g of culture medium, approximately 1 g of dry powder was obtained.

Each total supercritical CO₂ extraction (total SFE) was performed once, using a sequence of 15-minute isocratic steps of increasing percentage of ethanol as a co-solvent together with pressure/temperature (T/P) coupled optimization. The objective was a CO₂ density distribution of the initial fraction (0% ethanol) ranging from 384 (100 bars, 50 °C) to 975 kg.m⁻³ (Figure 1a); the 975 kg.m⁻³ condition, the CO₂ was considered as a subcritical fluid. When performing total SFE, the masses of the crude extracts decreased when initial fluid density increased (Figure 1b, Table S1). It was observed that for the 100 bar, 50 °C condition (lowest initial fluid density), the total extraction yield was 36.95 mg for one Petri dish.

This value decreases to 26.28 mg for 320 bars and 25 °C condition (highest initial fluid density). Furthermore, for the 0% ethanol fraction (Figure 1c, black dot, TableS1) the extraction yield does not change with modification of pressure and temperature pairs, indicating that initial fluid density does not affect extraction yield. The same result was observed for 1, 2, 5, and 10% ethanol fractions. However, the yield for the 20 and 50% ethanol fractions drops dramatically from 11.48 mg (100 bar and 50 °C, 50% ethanol) to 5.13 mg (320 bar, 25 °C, 50% ethanol) (Figure 1c, Table S2Erreur ! Source du renvoi introuvable.).

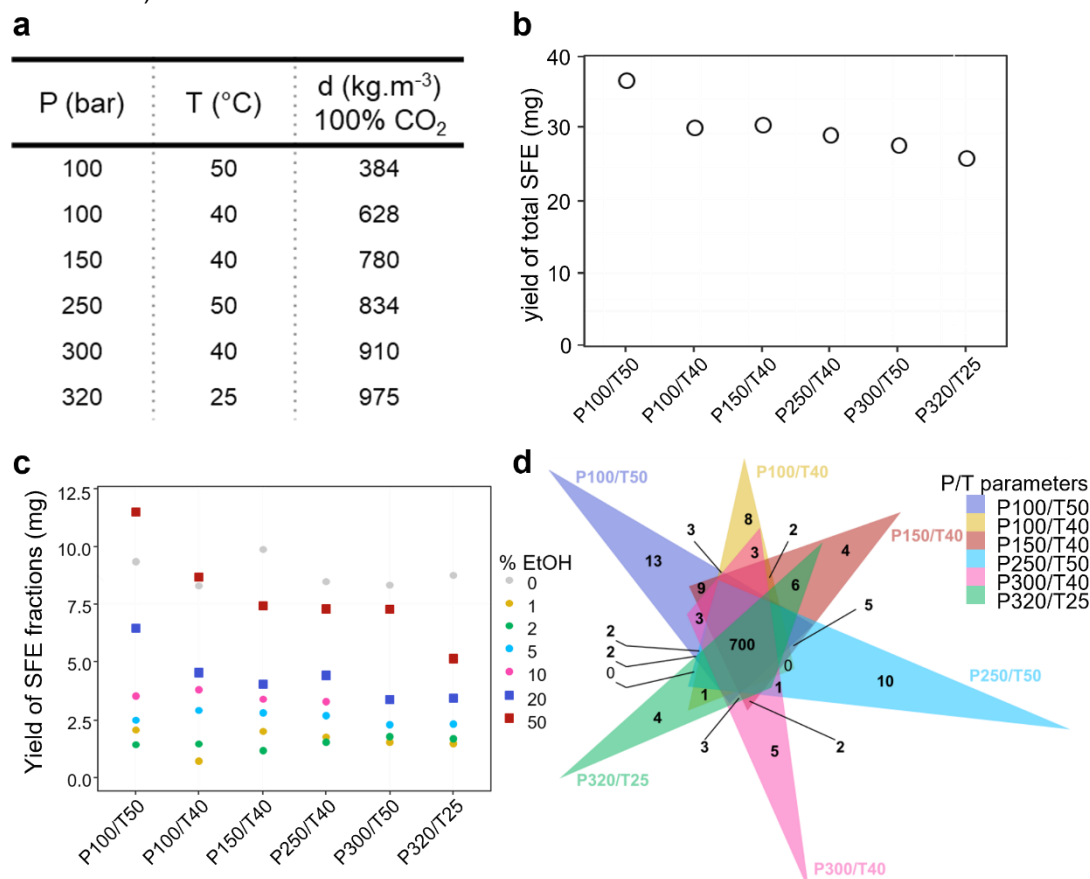


Figure 1: Extraction of *P. sclerotiorum* SNB-CN111 specialized metabolites by supercritical fluid extraction as a function of different pressure and temperature pairs. (a) 100% CO₂ density with the temperature and pressure conditions. (b) Evolution of total SFE yield of *P. sclerotiorum* SNB-CN111. (c) SFE yield of fractions as a function of percentage of ethanol co-solvent. (d) Venn diagram displaying shared and unique metabolite features (intensity > 50,000) extracted from *P. sclerotiorum* SNB-CN111 by total Supercritical Fluid Extraction. P: pressure in bar, T: temperature in °C

In order to select the optimal SFE method, we compared the molecular compositions of extracts from different pressure and temperature conditions. For this purpose, all the extracts were analyzed by reverse-phase liquid chromatography hyphenated to positive electrospray ionization tandem mass spectrometry (RPLC-ESI(+)-MS/MS) in data dependent acquisition (DDA) mode and their features, *i.e.* a pair of *m/z* and retention time with MS/MS spectrum associated, were combined in one data table. The total number of metabolites detected in the analyses ranged from 2051 to 2314 for different pressure and temperature pairs (Table S2). The common features among total SFE in the different (P, T) conditions were examined. One thousand thirty-nine of these features are shared by all samples corresponding to half of them. In contrast, only 131 to 267 features were unique to one particular (P, T) condition, corresponding to 6-12% of features. Regarding the most intense metabolites (> 50,000 counts corresponding to 877-903 features), the proportion of compounds shared by all (P, T) conditions increases to 80% of total features. Furthermore, the number of unique features for an extraction condition drastically decreases to 0-1% of the total features (Figure 1d). If we consider the most intense features, these results indicate that the total SFE obtained from different pressure and temperature pairs extracts contained the same molecular composition.

Beyond a calculation of the number of common features between extraction conditions, a correlation heatmap was calculated based on molecular composition and intensity of features in each fraction from

each (P, T) condition (Figure 2). Three distinct clusters of fractions were highlighted. The molecular profiles of all 0% ethanol fractions were the most similar to each other compared to the rest of the fractions, with similarity scores between 0.75 and 1. Similarly, the 1 and 2% ethanol fractions of each extraction condition clustered together with a similarity greater than 0.9. The 5, 10, 20% and 50% ethanol fractions from the different pressure and temperature couples clustered together with similarities greater than 0.8. Therefore, pressure and temperature conditions did not significantly alter the extraction profiles since both total extracts and fractions were very similar when considering a fixed percent of ethanol as a co-solvent. The extraction condition with 100 bar, 50 °C was selected as it led to the highest extraction yield. It must be noted that increasing the percentage of ethanol at such (P,T) conditions will rapidly leads to subcritical fluids rather than supercritical ones.

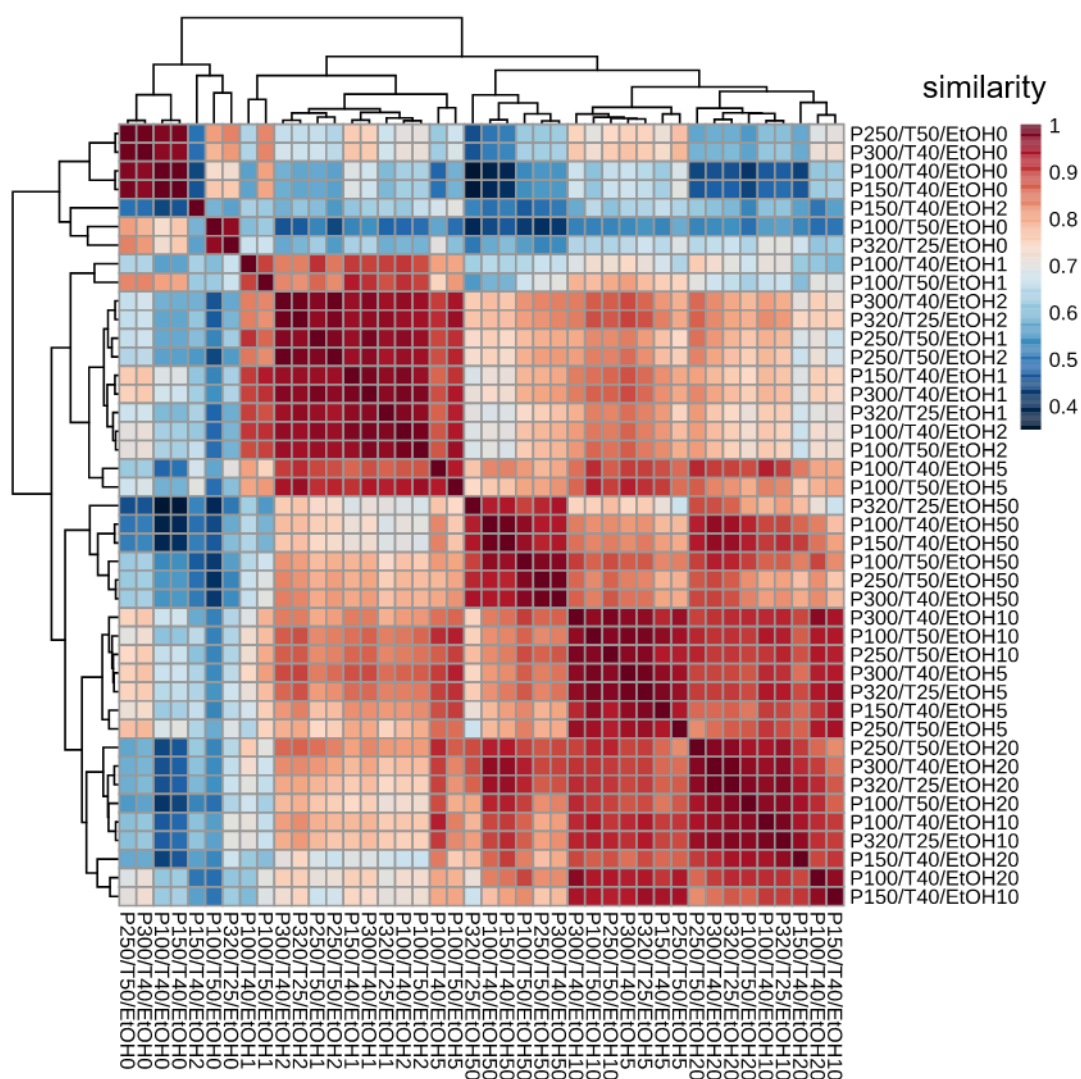


Figure 2: Correlation heatmap of *P. sclerotiorum* SNB-CN11 SFE fractions molecular composition similarities as a function of pressure, temperature, and percentage of ethanol.

3.2 Comparison of 100% CO₂ Supercritical Fluid Extraction of fungus, cultivation medium and hexane maceration

After the selection of the extraction condition leading to the highest yield, the yield extraction was compared to conventional methods. An extraction control (negative control, *i.e.* culture medium, solid PDA, 15 days at 28 °C without microorganisms) was included to assess that supercritical CO₂ with or without ethanol mainly extracts specialized metabolites of *P. sclerotiorum* SNB-CN11. A t-SNE molecular network constructed from RPLC-ESI(+)-MS/MS data highlights the differences of molecular composition between *P. sclerotiorum* total SFE, and PDA medium total SFE. In molecular networks, features cluster together based on their MS/MS spectra similarity, that is used as a proxy for their chemical structure.

Few clusters are shared between SFE of PDA medium and *P. sclerotiorum* SNB-CN111 cultivated on PDA (Figure 3a). Supercritical CO₂ is often compared to hexane in terms of polarity [33]. Thus, we also compared maceration with hexane and SFE 0% ethanol fraction (Figure 3b). A t-SNE visualization indicates that hexane maceration and SFE 0% ethanol fraction from *P. sclerotiorum* indeed share 310 features and some clustered together. Despite those molecular similarities, *P. sclerotiorum* SNB-CN111 SFE 0% ethanol fraction outperformed hexane maceration with a 10.5-fold higher yield (9.39 mg and 0.89 mg respectively). To confirm those qualitative observations, a correlation heatmap was generated from SFE molecular composition of PDA, *P. sclerotiorum* SNB-CN111 cultivated on PDA fractions and hexane maceration. It indicates that SFE fractions from PDA medium have similarities lower than 0.2 compared to extractions from *P. sclerotiorum* SNB-CN111 cultivated on PDA. Moreover, SFE 0% ethanol from *P. sclerotiorum* SNB-CN111 cultivated on PDA is more similar to hexane maceration than other SFE fractions (Figure 3c).

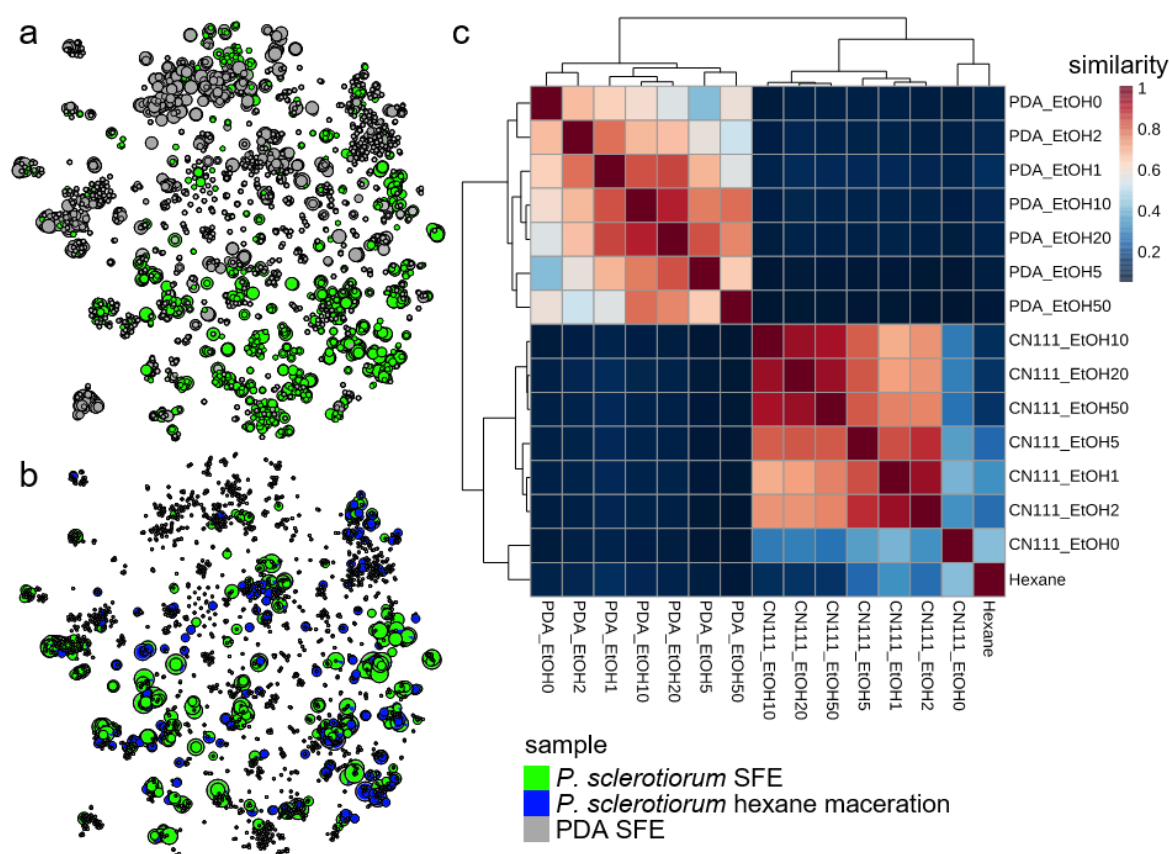


Figure 3: Supercritical Fluid Extraction (100 bar/50°C) of *P. sclerotiorum* SNB-CN111 and PDA medium and *P. sclerotiorum* SNB-CN111 hexane maceration. t-SNE molecular network representations of (a) *P. sclerotiorum* SNB-CN111 and PDA medium total SFE and (b) *P. sclerotiorum* SNB-CN111 SFE 0% ethanol and hexane maceration. t-SNE molecular network representations are constructed on MS/MS data homologies, with the size of node related to their intensity and color to the sample. (c) Correlation heatmap of SFE fractions obtained from *P. sclerotiorum* SNB-CN111 and PDA medium or crude extract from hexane maceration.

3.3 Extraction of azaphilones using Supercritical Fluid Extraction

The *P. sclerotiorum* SNB-CN111 specialized metabolites, especially azaphilones molecules, have been previously characterized from classical ethyl acetate maceration by our research group [34–36]. In order to more deeply explore the metabolome extracted by SFE, each produced extract was submitted to RPLC-ESI(+)-MS/MS experiments in DDA mode (Figure 4). Extracted compounds were annotated using public MS/MS databases and an in-house database [34]. Eight clusters (A-H, Figures S2-S9, Tables S3-S10) related to azaphilones with structural variations were annotated.

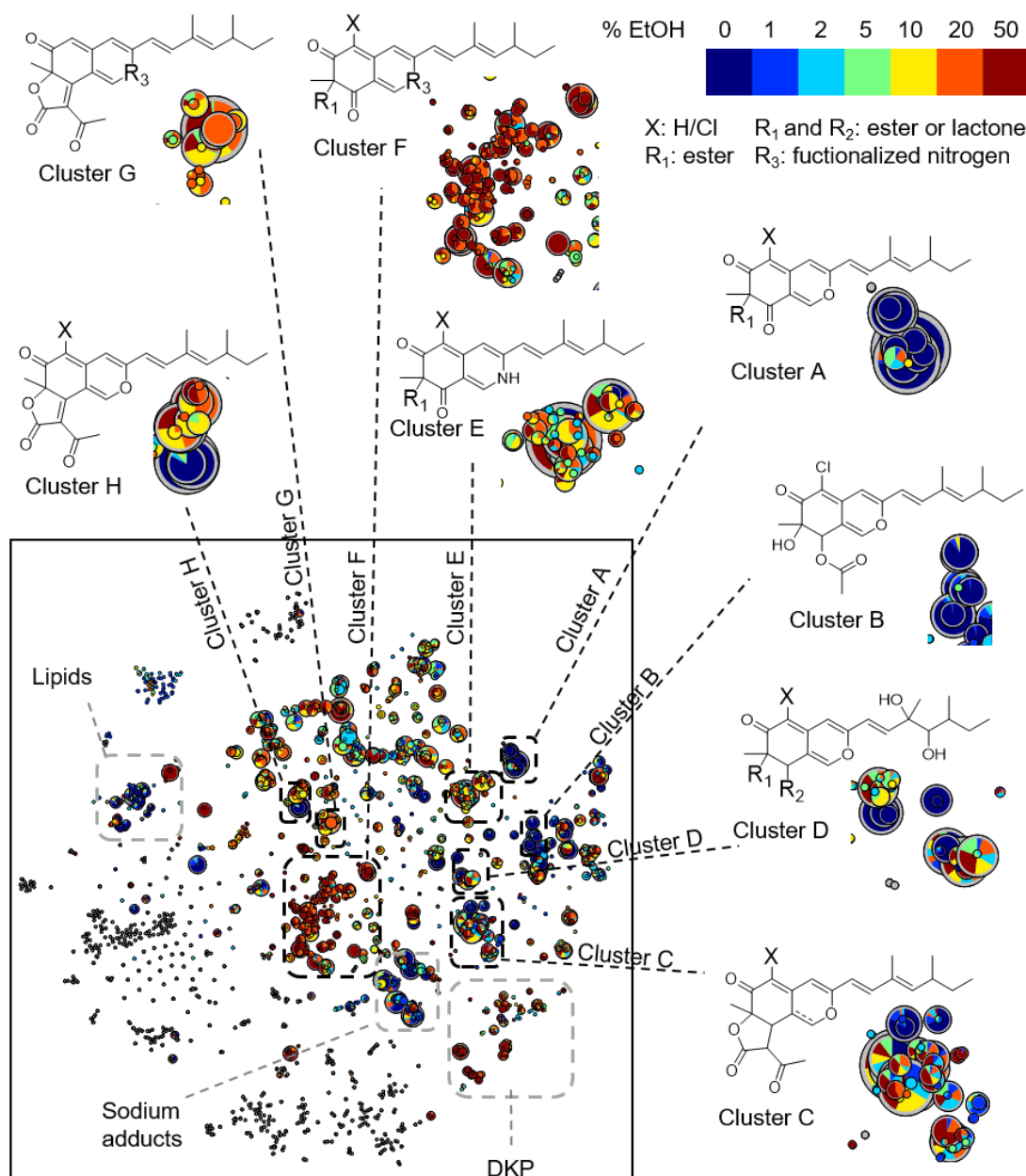


Figure 4: t-SNE molecular network representation constructed on MS/MS data homologies with the size of node related to their intensity and color to the percentage of ethanol used as co-solvent. Annotation was performed by MS/MS standard database query. DKP: diketopiperazines

In cluster A, 12 analogs of sclerotiorin (**1**) were annotated. These molecules were mainly extracted in the 0% ethanol fraction. Cluster B is composed of isochromophilone IV (**2**) and non-annotated analogs also extracted in the 0% ethanol fraction. Cluster C is composed of analogs of ochrephilone (**3**) (6 annotated molecules) and arose from two SFE fractions: at 0% ethanol, azaphilones with an intact angular lactone are extracted while azaphilones with an open lactone ring are extracted at 50% ethanol. Cluster D (8 annotated azaphilones) is related to azaphilones with hydroxyls on their 3-5 dimethyl heptadiene chains mainly extracted at 50% ethanol. Cluster E (6 annotated metabolites) is related to sclerotioramine (**4**) analogs mainly extracted with 5 and 10% ethanol, although **4** is mostly extracted at 50% ethanol. For cluster F (13 annotated molecules), which includes all azaphilones with a functionalized nitrogen and the same scaffold as sclerotioramine (**4**), metabolites are extracted in fractions ranging from 5 to 50% ethanol. The same pattern was obtained for azaphilones with an angular lactone and functionalized nitrogen from cluster G (2 annotated azaphilones). Finally, 5-chloroisotiorin (**5**) and its analogs are extracted at 0% ethanol (cluster H, 2 annotated compounds).

Thus, the full chemical diversity of azaphilones produced by *P. sclerotiorum* SNB-CN111 is successfully extracted by SFE. Fractionation using various percentages of ethanol allows a rather specific extraction of azaphilones based on their polarity.

3.4 Comparison with ethyl acetate maceration.

After selecting the best SFE (P, T) condition and verifying that the metabolic fractions contained azaphilones, we compared SFE with the standard extraction method classically employed in natural product research field, *i.e.* maceration by an organic solvent like ethyl acetate. Hexane maceration is not a common method of extraction but was included prior in this study as a control for SFE 0% ethanol fraction. To control the impact of the grinding step on the extraction yield, the maceration extraction with ethyl acetate was also performed under the same grinding conditions as for the SFE. Yields were calculated as milligrams of crude extract per Petri dish (Figure 5). The yield of total SFE (pool of all fractions) (100 bar, 50°C) was 2.1 times higher than the yield of ethyl acetate extraction, and 41 times higher than the yield of hexane extraction.

Each ethyl acetate, hexane maceration extracts and SFE fractions were therefore analyzed by RPLC-ESI(+)-MS/MS in DDA mode. A heatmap representing the similarity of the metabolic profiles was then calculated (Figure S10). As mentioned before, hexane maceration shared the highest similarity to the 0% ethanol fraction. On the other hand, ethyl acetate extract shares the highest similarity with the 1, 2 and 5% ethanol fractions. In terms of raw numbers, 2237 features were detected from SFE, compared to 986 and 651 from ethyl acetate and hexane maceration respectively (Figure 5b, Table 2). Sixty-five percent of SFE features were unique to this extraction method, that is a raw number of 1459 total unique features out of 2774 total features. Regarding azaphilones, SFE also outperformed Ethyl acetate and hexane maceration (313, 207 and 88 total features respectively) and accounted for the most unique azaphilones, 137 out of 488 from the azaphilone molecular features in cluster A to H (Figure 5c, d, Table 2). For specific scaffolds, azaphilones from clusters A and B were populated by SFE 0% ethanol and hexane extraction. For the rest of the azaphilones clusters, SFE with ethanol and ethyl acetate extractions were the most suitable methods of extraction, and SFE led to the highest yield per compound.

Then, we investigated if SFE offers the opportunity to extend the metabolome characterization to characterize undescribed compounds. In order to reach this objective, we queried the fragmentation spectra of each feature against the whole MASSIVE/GNPS repository using MASST [31] (Table 2). In total, 253 of the detected features were already detected in other LC-MS/MS analyses accounting for 9.1% of the total features. SFE achieved the lowest hit rate on the number of total features with only 9.6% of the total features being detected in MASSIVE repository. However, hexane maceration had the lowest hit rate, 5.2% when it comes to features specific to one extraction method. For the specific class of specialized metabolites produced by *P. sclerotiorum* SNB-CN111, azaphilones, SFE scored the lowest hit rate on the total number of azaphilones and achieved 7.3% hits for features unique to this extraction method, compared to 15.4% for hexane and 20.5% for ethyl acetate.

Table 2: Detected number of features and azaphilones based on the extraction method and their occurrence in the public repository MASSIVE.

	All features					Azaphilones				
	Total	Unique	Common			Total	Unique	Common		
Hexane	651	309	92		178	88	26	20		30
SFE	2237	1459		508		313	137		126	
Ethyl acetate	986	228				207	39			
	Percent of features in MASSIVE					Percent of Azaphilones in MASSIVE				
	Total	Unique	Common			Total	Unique	Common		
Hexane	11.3	5.2	14.1		22.9	26.1	15.4	20.0		43.3
SFE	9.6	7.0		11.8		13.1	7.3		11.1	
Ethyl acetate	12.9	10.1				17.9	20.5			

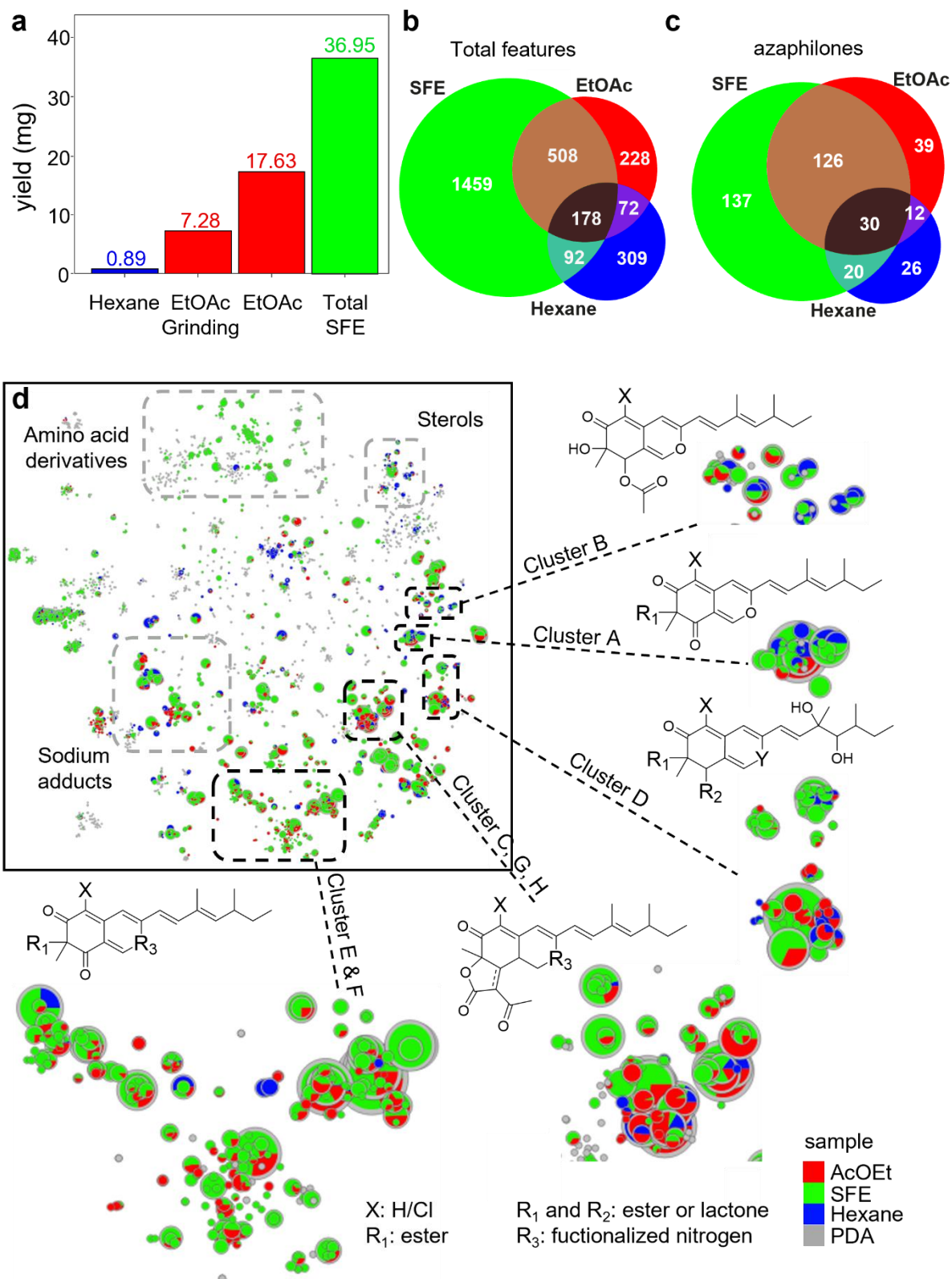


Figure 5: Comparison of extraction methods. (a) Yields of extraction. (b) Qualitative analysis with unique and common features detected for each extraction method. (c) Qualitative analysis with unique and common azaphilones features detected for each extraction method. (d) t-SNE molecular network representation constructed on MS/MS homology with the size of node related to their intensity and color to the extraction method, with dereplicated features from MS/MS standard database query.

4. Discussion

There are few examples in the literature of the microorganism extraction by SFE, which may be due to the fact that the extraction of these biological materials requires more sample preparation steps than plants. Indeed, the extraction by supercritical CO₂ requires samples with a water content lower than 20% [37]. Thus, in order to minimize the amount of water to be eliminated from the samples, the cultures in liquid medium are first centrifuged to recover the biomass which is dried in an oven [38] or freeze-dried [27] then milled. An inert matrix can be added to the sample such as silica sand [39] or resin [27], which facilitates the handling of the sample after freeze-drying in order to fill the extraction cell. As no protocol had ever been reported to perform SFE from solid culture medium, we assumed that the polymerized agar constituting the culture medium (2%) could serve as a solid matrix to fill the extraction cell. Indeed, polysaccharides are described as being insoluble in supercritical CO₂ [40] and our analysis of library hits from the molecular networking data also pinpoints that polymerized agar is not extracted by supercritical CO₂, even with 50% ethanol. Hence, we used a cryo-grinding step to grind the sample before freeze-drying. After this step, a slightly electrostatic powder was obtained allowing for the easy filling of the extraction cell (100 x 4.6 mm, 0.5 mL). The significantly different metabolic profiles obtained from potato dextrose agar or *P. sclerotiorum* SNB-CN111 cultivated on potato dextrose agar (Figure 3) indicates that after 15 days of culture most of the constituents from the PDA medium have been metabolized by the fungus and therefore, most of the compounds extracted are *P. sclerotiorum* metabolites.

The SFE conditions, including the choice and percentage of co-solvent, the extraction time, and the flow rate were fixed prior to this study. The temperature and pressure were then optimized to reach the highest yield. As temperature and pressure both influence the density of the fluid, we first focused on these parameters to achieve global extraction of azaphilones. If tabulated data are reported to determine the density of a 100% CO₂ fluid, few experimental data or models are available for CO₂/methanol or CO₂/ethanol mixtures. We found an article referencing CO₂/ethanol fluid density values ranging from 6 to 27% ethanol and for temperature and pressure conditions ranging from 35 to 55 °C and from 100 to 500 bar [41]. This article shows that an increase of the ethanol percentage tends to decrease the density differences between the operating conditions, making the fluid density value plateau at 1000 kg.m⁻³, a value similar to our operating condition of 325 bar, 25°C at 100% CO₂. We then observed an increase in the extraction yield inversely linear to the fluid density. The operating condition that allowed an optimal extraction (100 bar, 50°C, 384 kg.m⁻³) is not commonly encountered in the literature of natural products SFE. In fact, the majority of optimal SFE conditions are distributed between 200-400 bar at 40 °C, *i.e.* 840-960 kg.m⁻³ [19,42]. Our findings are counter-intuitive since increasing the fluid density is described to possess a favorable effect on the extraction of natural products [19,42]. This observation might be related to the ethanol percent because fractions for which the yield decreases while density increases are fractions with 20 and 50% of ethanol as co-solvent. Furthermore, Figure 3 indicates that the main parameter influencing the molecular composition of the fractions is also the ethanol percentage. Thus, the condition at 100 bar and 50°C was determined as optimal, as it allows the best extraction yield while extracting the full chemical diversity of *P. sclerotiorum* SNB-CN111 azaphilones (Figure 5). Nevertheless, all the SFE conditions of *P. sclerotiorum* outperformed maceration in ethyl acetate (Figures 1 and 5, Table S1).

The specialized metabolome of *P. sclerotiorum* SNB-CN111, and in particular the azaphilone family has been already explored recently [34–36]. Thus, after exploring the annotated t-SNE molecular network, we found out the different sub-families of azaphilones were all extracted under the condition of 100 bar and 50°C, using 15-minute isocratic steps with increasing percentage (0, 1, 2, 5, 10, 20 and 50%) of ethanol as a co-solvent. Using the query of databases (including one in-house constituted of metabolites annotated in previous studies [34-36]), it was possible to annotate 49 azaphilones, distributed in 8 clusters. Sclerotiorin (**1**), nitrogenated sclerotiorin analogs, or analogs of (**1**) with an angular lactone or decorated with two hydroxyls on their 3,5-dimethyl-1,3-heptadienyl chain were well extracted by SFE. It must be noted that the rate of annotation of the molecular network is mainly depending on the database completion. We observed that GNPS and related databases are overfed of lipid species whereas some natural families have a small number of reference spectra or even none. Moreover, this extraction method enables a fractionation according to the percent of co-solvent allowing a partial separation of the azaphilone subfamilies. Of interest, fractionation allows separation of yellow/orange (in 0% ethanol

fraction) and red/purple azaphilones (in 20-50% ethanol fractions). Because the modulation of azaphilone color is related to the integration of a functionalized nitrogen into their scaffold, our SFE method could be easily too other azaphilone families produced by other fungi [43]. As azaphilones are well extracted by SFE, this developed method was compared with the most used extraction method in natural product research: maceration with ethyl acetate [44]. Maceration with hexane was also include to compare it with the SFE 0% ethanol, as supercritical CO₂ is often compared to hexane in terms of polarity [33]. Hence, supercritical fluid extraction shows a 2 times higher yield than ethyl acetate maceration, while allowing to combine extraction and fractionation steps. The most similar SFE fractions in terms of azaphilone molecular composition to ethyl acetate extraction are 1, 2 and 5% ethanol fractions (Figure S11). Concerning the 100% CO₂ fraction, it does have a similar azaphilone molecular composition to hexane extraction, but SFE has a 10 times higher yield than maceration in hexane. For the potential of SFE to discover new metabolites, we queried the biggest, to date, repository of LC-MS/MS data (MASSIVE) using MASST query system with cosine similarity of 0.7. If a MS/MS spectrum hasn't been detected to date, it would translate to a compound with higher chances to be an uncharacterized one. Surprisingly, all extraction methods have low hit rates (9.6 to 12.9%) for all the detected features. This might be because of DDA experiments, low abundance features have lower quality MS/MS spectra that can negatively influence the cosine similarity. Still, when focusing only on azaphilones the total of features being detected in MASSIVE increases (13.1% for SFE feature, 17.9% for ethyl acetate features, 26.1% for hexane features). In that regard, SFE outperforms ethyl acetate and hexane maceration as only 7.3% of the features (out of 137 total azaphilone features) have been detected in other samples available at MASSIVE, compared to 15.4% for hexane and 20.5% for ethyl acetate. Thus, due to its high extraction yield and better coverage of azaphilone chemistry, SFE outperformed ethyl acetate maceration.

Conclusion.

This study reports a new extraction process of specialized metabolites produced by microorganism solid-state fermentation. Using pigments of fungal origin, *i.e.* azaphilones molecules, we developed a sample preparation process that is less toxic for the handler while being greener by substituting the maceration steps in organic solvents with an extraction based on supercritical CO₂ with ethanol as cosolvent. This sample preparation goes through a cryogenic grinding step followed by freeze-drying and allows to obtain a powder that can be handled and compatible with SFE without adding an inert matrix in contrast to SFE extractions based on fermentations in liquid medium. After optimization of the extraction conditions, we selected an extraction method at 100 bar, 50 °C, with increments of the ethanol percentage every 15 minutes. This allows to combine the extraction and fractionation steps while ensuring a better extraction coverage of the specialized metabolome. SFE extraction of *P. sclerotiorum* SNB-CN111 allowed the extraction and separation of azaphilones produced by *P. sclerotiorum* with higher extraction yields than those obtained by ethyl acetate maceration. Although not much reported in the literature for microorganisms and their specialized metabolome, SFE appears to be an attractive alternative to maceration for its advantages regarding metabolome coverage, integration in green chemistry procedures and its compatibility with industrial scale-up. In the latter case, the optimization of the culture medium could be optimized to minimize costs, potentially utilizing food waste. However, it is essential to consider that the extraction of undesired metabolites from the substrate may impose limitations on the feasibility of employing such low-cost cultivation media.

Author Contributions

Conceptualization, T.H., V.E., D.T.; methodology, T.H., D.T.; validation, T.H., D.T., V.E.; investigation, T.H., D.T., V.E.; resources, V.E., D.T.; data curation, T.H., V.E., D.T.; writing—original draft preparation, T.H., V.E., D.T.; writing—review and editing, D.T., V.E.; visualization, T.H., V.E.; supervision, V.E., D.T.; project administration, V.E., D.T.; funding acquisition, V.E., D.T.; All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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