High-performance liquid chromatography comparison of supercritical-fluid extraction and solvent extraction of microbial fermentation products

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Abstract

The use of supercritical fluids for the extraction of biologically active compounds from the biomass of microbial fermentations has been compared with extraction using the organic solvents methanol and dichloromethane. Compounds representing a range of structural types were selected for investigation. All the extracts obtained were examined using reversed-phase high-performance liquid chromatography. The extractability of metabolites using unmodified and methanol-modified supercritical-fluid carbon dioxide was examined in particular detail for six microbial metabolites: chaetoglobosin A, mycolutein, luteoreticulin, 7,8-dihydro-7,8-epoxy-1-hydroxy-3-hydroxy-methylxanthone-8-carboxylic acid methyl ester, sydowinin B and elaiophylin. The extraction strength of super-critical-fluid carbon dioxide alone appeared to be lower than that of dichloromethane. All the components of interest that were extractable with dichloromethane and methanol were also extractable with methanol-modified carbon dioxide.

1. Introduction

A number of natural products and natural product derived compounds have proved to be extremely useful as drugs for the treatment of a range of medical conditions [1]. Screening programmes directed at the discovery of low-molecular-mass compounds with useful biological properties from natural sources continue to constitute an important and expanding interest throughout the pharmaceutical industry [1–3]. New naturally-produced compounds with interesting biological activities are reported regularly and these often provide the basis for pharmaceutical or agrochemical development projects.

The most widely explored sources of natural products to date have been plants, marine organisms and micro-organisms, in particular actinomycetes and fungi. Large numbers of cell (biomass) associated microbial fermentation products are routinely screened in a variety of assays after extraction using a sequence of organic solvents of increasing polarity, to produce several extracts from each organism. The method of preparation of these samples is critical to the efficiency and success of the screening process [3,4] and there is a constant search for new extraction techniques and sample separation procedures. Once samples containing potentially

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useful substances are detected, the active components are isolated chromatographically.

It has been suggested that supercritical fluids can be used as an alternative to organic solvents for the extraction of natural products [5–8], improving the purification process by achieving a selective extraction [9,10] of the desired sample components through careful control of the extraction conditions. The parameters which can be used to vary the extraction conditions are temperature, pressure, modifier or mobile phase [11]. Supercritical-fluid extraction (SFE) can thus reduce the overall time required for the separation and characterisation of particular metabolites from within complex mixtures [12–14].

Reports in the literature on the SFE of microbial natural products from various samples are relatively sparse. Most studies have described the extraction of plant products such as taxanes [15], parthenolide [16] or the constituents of Chinese herbal medicines [17]. The extraction of lipids from lyophilised microalgae has also been investigated [18]. While studies have been performed on the extraction of compounds from microbial sources such as carboxylic acids [19], and ergosterol [20] from mushrooms and aflatoxin B_1 from infected corn [21], there have been very few reports concerning microbial fermentation samples. A detailed account of the extraction of cyclosporin from the fungus *Beauveria nivea* is one of the more thoroughly investigated examples [22]. The use of SFE to remove a sterol from dry mouldy bran produced by solid-state fermentation with *Gibberella fujikuroi*, without extracting any gibberellic acid, has also been described [23].

The aim of this study is to investigate the structural types of compounds that are extractable from microbial fermentation samples using SFE. A subsequent publication will examine the quantitative extraction of a range of microbial fermentation products. We report here a comparative study of the use of supercritical-fluid carbon dioxide, with and without methanol modification, with that of the organic solvents dichloromethane (DCM) and methanol for the extraction of known metabolites from biomass samples obtained by microbial fermentation.

This paper will concentrate on the extraction of chaetoglobosin A, 1; from *Penicillium expansum*; mycolutein, 2; and luteoreticulin, 3; from an unidentified actinomycete; 7,8-dihydro-7,8-epoxy-1-hydroxy-3-hydroxymethylxanthone-8-carboxylic acid methyl ester, 4; and sydowinin B, 5, from *Aspergillus fumigatus*; and elaiophylin, 6, from a *Streptomyces sp.* The structures of these compounds are shown in Fig. 1. Chaetoglobosin A is a member of the cytochalasin group of fungal metabolites and has previously been reported as a product of *Chaetomium globosum* [24]. The γ-pyrone derivative mycolutein and its α-pyrone analogue luteoreticulin are *Streptomyces* sp. metabolites [25,26]. Compound 4 has recently been isolated by us [27] and also reported separately as an *Aspergillus* sp. metabolite [28]. Sydowinin B is co-produced with 4 [27,28]. Elaiophylin is a glycosylated macrodiolide reported to be produced by *Streptomyces* sp. [29]. The extracts obtained by SFE and with organic solvents were compared by reversed-phase high-performance liquid chromatography (HPLC) analysis.

2. Experimental

2.1. Chemicals

The carbon dioxide was industrial grade (99.98%) from British Oxygen Company (Brentford, UK) and methanol and dichloromethane were HPLC grade from FSA Scientific Apparatus (Loughborough, UK).

2.2. Instrumentation for supercritical-fluid extraction

Supercritical fluid extractions were performed using a Jasco SFE system, (Hachiojo City, Tokyo, Japan), consisting of two Model 980 pumps, one for delivery of carbon dioxide and the other for addition of modifier. The pump head used for carbon dioxide delivery was cooled with an ethylene glycol–water mixture at $-15°C$. Extraction temperature was controlled in a Jasco 860-CO oven and the extraction was monitored
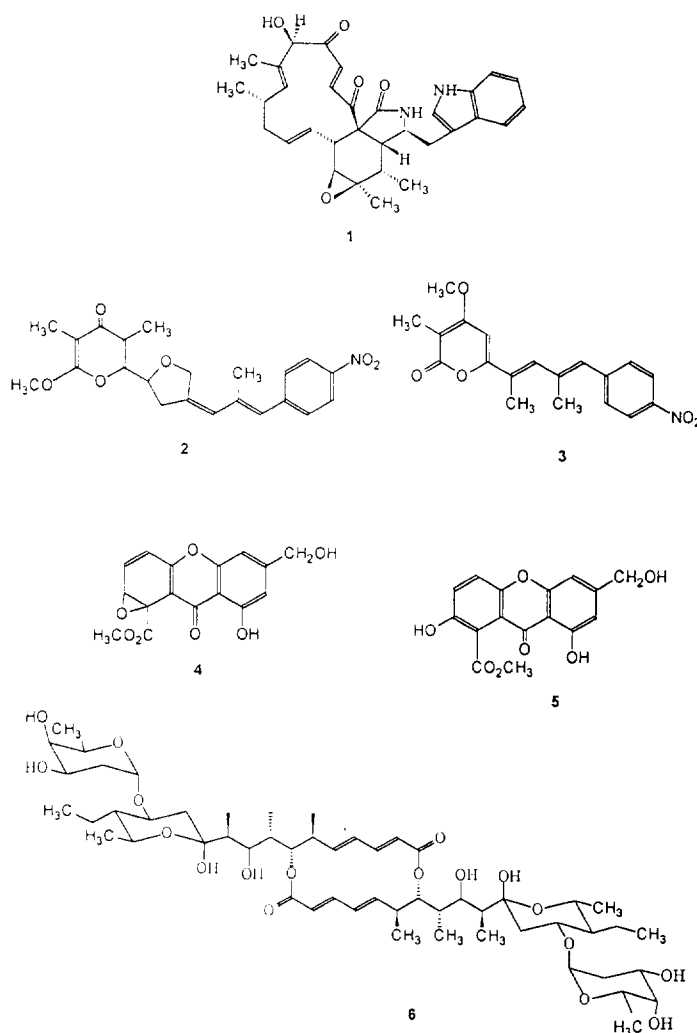
Fig. 1. The structures of: **1**, chaetoglobosin A, $C_{23}H_{36}N_2O_5$, molecular mass (M_r) 528; **2**, mycolutein, $C_{22}H_{23}NO_6$, M_r 397; **3** luteoreticulin, $C_{19}H_{19}NO_5$, M_r 341; **4**, 7,8-dihydro-7,8-epoxy-1-hydroxy-3-hydroxymethylxanthone-8-carboxylic acid methyl ester, $C_{16}H_{12}O_7$, M_r 316; **5**, sydowinin B, $C_{16}H_{12}O_7$, M_r 316; **6**, elaiophylin, $C_{54}H_{88}O_{18}$, M_r 1025.

on a Jasco UV975 variable-wavelength detector. Pressure regulation was performed using a Jasco 880-81 back pressure regulator. Data were captured using Borwin (Mettler-Toledo, Leicester, UK) software on an Elonex 486 computer, (Elonex London, UK).

2.3. High-performance liquid chromatography

HPLC separations were performed using a Waters (Watford, UK) system comprising a Waters 600E multisolvent delivery system, a Waters 996 photodiode array detector and a Waters 717 WISP sample injection unit. Peak detection and integration were obtained using a Waters Millennium 2010 Chromatography Manager. All chromatographic separations were performed in a temperature controlled room at 21°C.

The column used for the chromatographic analysis was a Waters Nova-Pak C_{18} Radial Pak cartridge (100 mm × 8 mm I.D.) contained in a

Waters radial compression module. All the extracts produced were analysed using the same linear water–acetonitrile gradient, increasing from 0 to 100% acetonitrile over a period of 15 min, after an initial delay of 2 min, at a flow-rate of 2 ml/min. Analysis of each sample was repeated, in some cases up to four times. The compounds of interest were identified by their retention times and comparison of their UV–visible spectra with those in Xenova's in-house spectral library on a Waters 996 photodiode array detector. Each compound had previously been isolated from its producing organism and its structure determined by a combination of spectroscopic techniques including mass spectrometry and nuclear magnetic resonance.

2.4. Microbial biomass sample production and preparation for extraction

The microbial biomass samples were harvested by centrifugation from fermentations produced by inoculation of each organism into 300 ml of an appropriate growth medium contained in a two litre conical flask and incubated with shaking at an appropriate temperature for seven days. The cells were then lyophilised and placed in sealed containers and stored at −20°C until required. Prior to extraction each sample was milled to a fine powder and separated into a minimum of five aliquots in the weight range 0.2–0.5 g, depending on the amount of biomass produced in the fermentation. The metabolites of interest were identified by their UV–visible spectra and HPLC retention times.

2.5. Solvent extraction

One of the pre-weighed aliquots was taken and sequentially extracted with dichloromethane (2 × 50 ml) and then methanol (2 × 50 ml). Extraction involved ultrasonication for 30 min. The extracts for each solvent were combined and filtered before being reduced to dryness on a rotary evaporator. The extracted material was redissolved in a known amount of methanol and transferred to a vial for HPLC analysis. All the

extracts from one organism were dissolved in the same volume of the appropriate organic solvent.

2.6. Supercritical-fluid extraction procedure

An aliquot of the biomass sample was placed in a 3 ml extraction vessel which was placed into the SFE system. All the extractions were performed using a flow-rate of 2 ml/min at 40°C and 300 kg cm^{-2}.

Each sample was extracted for 30 min with carbon dioxide and then for 30 min with 20% (v/v) methanol-modified carbon dioxide. The unmodified and modified extracts were collected separately. In the case of the unmodified fraction the sample was dissolved in methanol. For the modified fraction, the methanol collected during the extraction was first removed under a stream of nitrogen. The dry extracts were redissolved in a known amount of methanol. Both fractions were then analysed by HPLC.

The SFE system was washed with methanol after each individual extraction and dried using carbon dioxide to ensure that no residue was left within the system which could carry over into subsequent extractions.

After each supercritical-fluid extraction the extraction vessel was switched out of line and removed from the system. The biomass was then removed from the vessel and sequentially extracted using the same procedure as detailed in the Solvent extraction section, to determine the quantities of the metabolites of interest not extracted using the SFE procedure. All supercritical fluid and residual extracts from one organism were dissolved in the same volume of methanol as the solvent extracts for that organism.

3. Results and discussion

The aim of the study was to establish a SFE protocol to fractionate and purify components from microbial fermentation biomass samples. The experiments detailed here are a qualitative comparison of organic solvent extraction versus SFE of microbial fermentation biomass samples.

Sequential solvent extraction using dichloro-

methane and methanol was first performed on a biomass sample from each fermentation. A fresh sample of the biomass was then taken and extracted using SFE. The SFE extraction conditions were not optimised for a particular sample but were chosen to maximise the extraction potential of the technique utilising a low extraction temperature with a high solvent density. A total time constraint of one hour was also placed upon the SFE extractions in order to facilitate a high number of extractions per day. Each sample was extracted sequentially with supercritical-fluid carbon dioxide and methanol-modified carbon dioxide. The residual biomass was then extracted with dichloromethane and methanol in order to give an indication of the completeness of the SFE extraction. Direct comparisons of the SFE and residual organic solvent extracts with the standard method of organic solvent extraction were made using reversed-phase HPLC. A detailed examination of six microbial metabolites is described. Each of these extractions was repeated at least once. The extracts obtained in each case were equivalent.

3.1. Chaetoglobosin A

The reversed-phase HPLC separations of the dichloromethane and methanol extracts of the biomass of *Penicillium expansum* are shown in Fig. 2. Most of the chaetoglobosin A was extracted by dichloromethane with a small amount
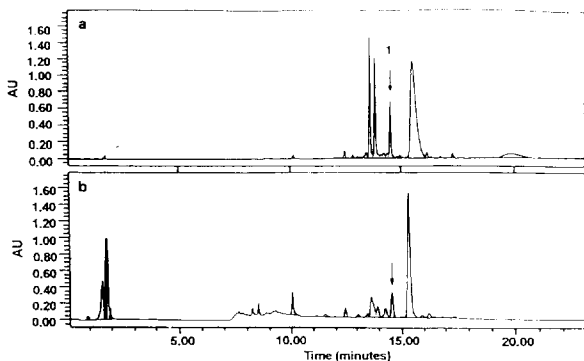
of residual chaetoglobosin A observed in the methanol extract. Generally the methanol extract contained more polar components than the dichloromethane extract although there was no clear distinction. The chromatograms obtained by reversed-phase HPLC analysis of the SFE extracts of the biomass of this fungus are shown in Fig. 3. Chaetoglobosin A was found predominantly in the modified CO_2 extract (Fig. 3b) with small amounts present in the unmodified extract (Fig. 3a). Trace amounts of the compound remained after SFE and were observed in the dichloromethane extract (Fig. 3c). The subsequent methanol extract (Fig. 3d) contained very few metabolites. Chaetoglobosin A was extracted successfully using SFE with the majority of the metabolite of interest being extracted with methanol-modified carbon dioxide. A more selective, preparative extraction could perhaps
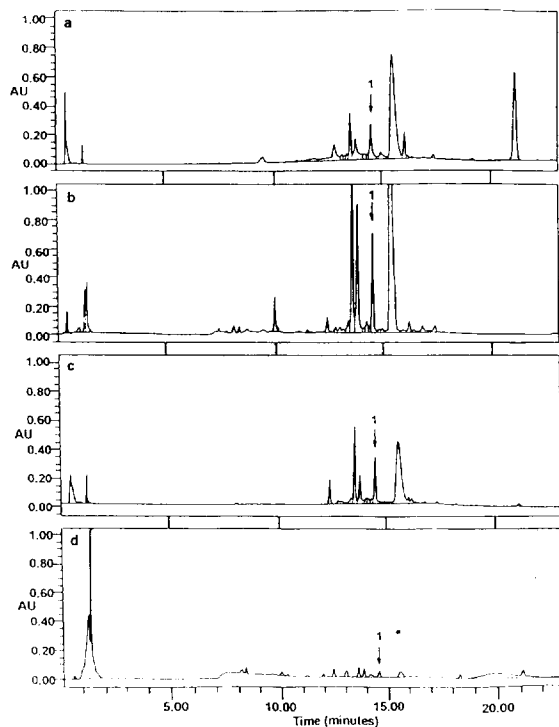
Fig. 3. Chromatograms of SFE and residual extracts of *Penicillium expansum*: (a) unmodified extract (180 μl); (b) methanol-modified extract (50 μl); (c) residual dichloromethane extract (180 μl) and (d) residual methanol extract (180 μl).

Fig. 2. Chromatograms of (a) dichloromethane and (b) methanol extracts of *Penicillium expansum*. The injection volumes were (a) 30 μl and (b) 50 μl.

have been achieved by varying the concentration of methanol modifier and/or increasing the length of the extraction.

3.2. Mycolutein and luteoreticulin

The dichloromethane and supercritical-fluid extracts of the biomass of an unidentified actinomycete known to produce mycolutein and luteoreticulin were examined by HPLC (Fig. 4). The compounds were identified as being present in these extracts by their UV–visible spectra. Mycolutein is the more polar of the metabolites with a retention time of 15.2 min whilst luteoreticulin has a retention time of 15.8 min. On organic solvent extraction, these compounds were found predominantly in the dichloromethane extract with lesser amounts of both compounds also present in the methanol extract. On
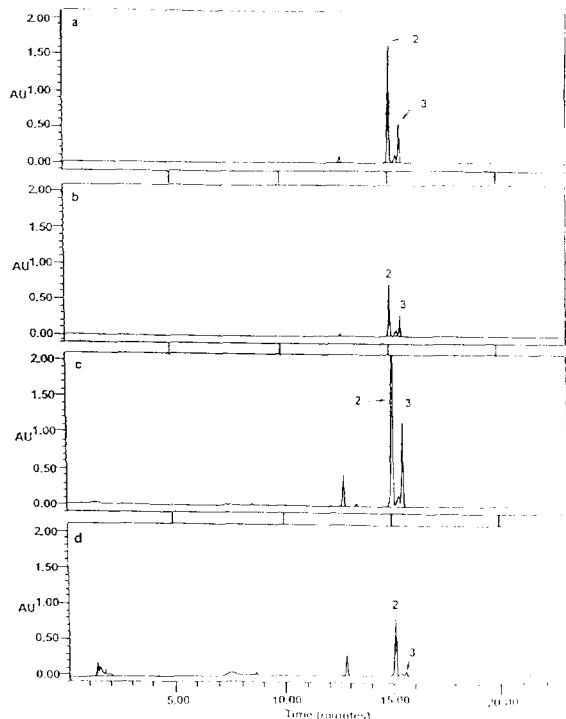
SFE, both compounds were extracted in small quantities with unmodified carbon dioxide (Fig. 4b) and in much larger amounts with methanol-modified carbon dioxide (Fig. 4c). Only trace amounts of both compounds were found in the organic solvent extracts of the biomass sample after SFE (Fig. 4d). Again successful extraction of the compounds of interest has been achieved by SFE in 1 h.

3.3. 7,8-Dihydro-7,8-epoxy-1-hydroxy-3-hydroxymethylxanthone-8-carboxylic acid methyl ester and sydowinin B

The chromatograms obtained by reversed-phase HPLC analysis of the dichloromethane and supercritical-fluid extracts of the biomass of *Aspergillus fumigatus* are shown in Fig. 5. The chromatogram of the dichloromethane extract (Fig. 5a) shows the presence of **4** and sydowinin B (**5**) as peaks with retention times of 11.2 and 11.0 min, respectively. The methanol extract contained some sydowinin B but compound **4** was not present in detectable amounts.

The extract obtained with carbon dioxide alone (Fig. 5b) contained **4** and a small quantity of **5**. The methanol-modified extract (Fig. 5c) contained no detectable amount of **4** and large
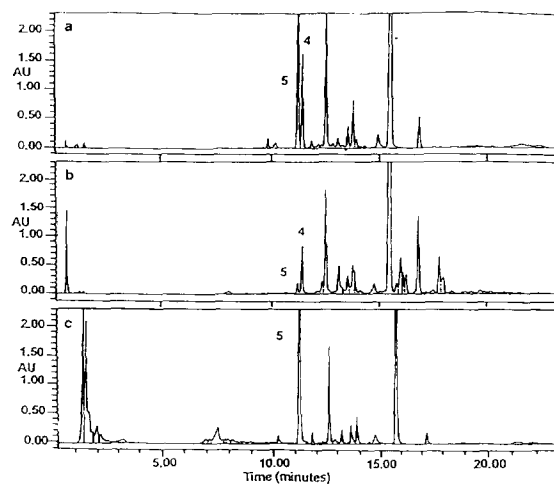
Fig. 4. Chromatograms of extracts containing mycolutein and luteoreticulin: (a) dichloromethane extract (5 μl); (b) unmodified CO₂ extract (5 μl); (c) methanol-modified extract (5 μl of sample diluted 1:9 with methanol) and (d) residual dichloromethane extract (5 μl).

Fig. 5. Chromatograms of the dichloromethane and SFE extracts of *Aspergillus fumigatus*: (a) dichloromethane extract (50 μl); (b) unmodified CO₂ extract (80 μl) and (c) methanol-modified CO₂ extract (30 μl).

peak for **5**, now with a retention time of 11.1 min. The identification of the sydowinin B peak in these extracts was confirmed by HPLC analysis of the extracts after addition of a solution of a sample of sydowinin B, which had been previously purified and characterised spectroscopically, as an internal standard. The organic solvent extracts of the supercritical-fluid extracted biomass did not contain any detectable amounts of the two compounds of interest. The short retention times of **4** and sydowinin B indicate that these compounds are more hydrophilic than the other metabolites described so far. The results show that compound **4** was extracted well by dichloromethane and by unmodified super-critical-fluid carbon dioxide. The supercritical carbon dioxide extract, however, contained a relatively small quantity of sydowinin B compared to the dichloromethane extract. Extraction with supercritical carbon dioxide was thus more selective for compound **4** over sydowinin B than extraction with dichloromethane. Compound **4** was the metabolite of greater pharmaceutical interest in this fermentation [27,28] and these results demonstrate the utility of SFE as a selective preliminary fractionation step. Similar results have been obtained for the extraction of turmeric [30].

3.4. Elaiophylin

Elaiophylin was the largest compound studied with a molecular mass of 1025. The reversed-phase HPLC chromatograms of the organic solvent extracts of the biomass of a *Streptomyces sp.* known to produce elaiophylin are shown in Fig. 6. The chromatogram of the dichloromethane extract contained elaiophylin as its major component with a retention time of 13.8 min. Several other peaks with retention times in the range 13 to 18 min were also identified by UV–visible spectral matching as analogues of elaiophylin. The methanol extract of the biomass contained similar quantities of the elaiophylin components with the additional presence of many more polar, unrelated metabolites.

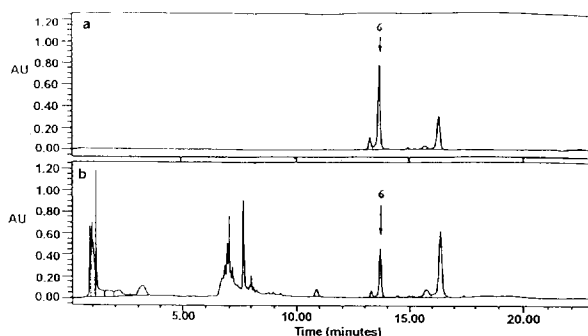None of the elaiophylin components were extracted by SFE using carbon dioxide alone.

Fig. 6. Chromatograms of solvent extracts containing elaiophylins: (a) dichloromethane extract (50 μl) and (b) methanol extract (13 μl).

The chromatogram of the methanol-modified carbon dioxide extract (Fig. 7a) shows significant quantities of elaiophylin and its analogues. The chromatogram of the methanol extract of the biomass after SFE (Fig. 7b) contained lesser amounts of elaiophylin components. Successful but incomplete extraction of the elaiophylins was thus achieved using methanol-modified carbon dioxide.

4. Conclusions

These studies have demonstrated that microbial metabolites representing a range of structural classes and having molecular masses ranging
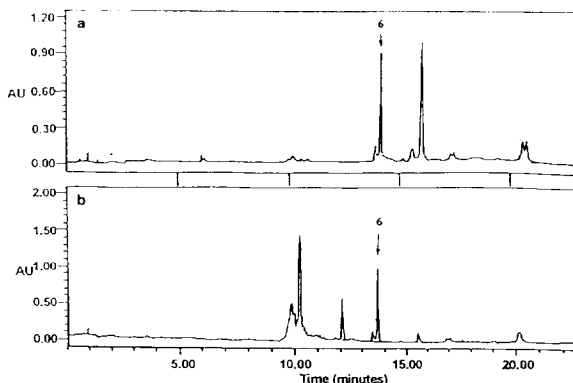

Fig. 7. Chromatograms of samples containing elaiophylins: (a) methanol-modified SFE extract (10 μl) and (b) residual methanol extract (50 μl).

from 316 to 1025 can be efficiently extracted from microbial fermentation biomass samples using supercritical-fluid carbon dioxide. In most cases supercritical-fluid carbon dioxide alone extracted slightly less of the compounds of interest than dichloromethane. Subsequent extraction with supercritical-fluid carbon dioxide modified by the addition of methanol achieved virtually complete extraction of most of these compounds. The efficiency of SFE is thus similar to that of organic solvent extraction for these metabolites. SFE has the advantage, however, that it has the potential to be controlled more finely to obtain a more selective, cleaner extract. The carbon dioxide and methanol modified extracts of 4 and sydowinin B from *Aspergillus fumigatus* also show how unmodified carbon dioxide can be used as a preliminary fractionation step with the more polar material being extracted only when methanol modifier is present. The compounds reported here are all of low to medium polarity and have good solubility in organic solvents.

Acknowledgements

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