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Short communication

Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures

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Abstract

A simple and rapid standardized micro-scale extraction procedure has been developed to prepare extracts from fungal cultures for high-performance liquid chromatographic (HPLC) analysis. The method is based on ultrasonic extraction of three 6-mm plugs cut from a culture using 0.5 ml of solvent followed by a simple solvent change, filtration and injection. Approximately 5 min of work is involved in the extraction and work-up process and the extract can prepared for HPLC analysis within 60–70 min. The method has been used for determination of chromatographic metabolite profiles from 395 fungal isolates, including all terverticillate *Penicillium* species, cultivated on both Czapek Yeast Autolysate agar and Yeast Extract Sucrose agar. The concentration of the extracts proved to be sufficient to determine all secondary metabolites reported to be produced by these species using HPLC with diode array detection. These findings were confirmed by analyses of 132 pure metabolite standards.

Keywords: *Penicillium* species; Fungal metabolites; Sample handling

1. Introduction

Growth of the filamentous fungi is associated with the production of a wide range of secondary metabolites of which many, the mycotoxins, are hazardous to other organisms including humans [1,2]. Today, the usage of the secondary metabolite profiles forms an important part of fungal classification and identification [3–6]. Studies of chemotaxonomy and fungal ecology will, however, often involve screening a large number of cultures. Selective extraction procedures will in some cases be necessary to obtain a full profile of secondary metabolites, and in ecology even in a small part of a colony such as an interaction zone. In both cases a simple and rapid screening method will be necessary. Whereas a large number of HPLC methods have been described for

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screening method will be necessary. Whereas a large number of HPLC methods have been described for specific determination of metabolites [7], only a few methods are suited for general screening of cultures.

A general standardized method for detection of mycotoxins and other secondary metabolites in fungal cultures has previously been presented by Frisvad and Thrane [4,8,9]. Their method is based on a combined extraction of the entire content from 8–10 petri dishes including 3–5 different substrates into one extract and a subsequent HPLC analysis with diode array detection (DAD). The method is, however, laborious and involves the use of large amounts of organic solvents, thus only a limited number of samples are included in most studies. The method offers the possibility to study the production of secondary metabolites on selected substrates, but not in different parts of the culture or in the interaction zone between different colonies growing on the same petri dish.

An alternative to the standardized HPLC method is the TLC agar plug method [10–12], which is one of simplest methods for determination of secondary metabolite profiles directly from fungal cultures. The TLC plug method is based on wetting an agar plug cut from the culture with a drop of organic solvent on the mycelium side and placing it with the mycelium side down on a TLC plate while still wet. After a few seconds the plug is removed and the TLC plate eluted using standard procedures. Although a very broad range of secondary metabolites can be determined as coloured spots using optimized solvent systems and selective spraying reagents, the TLC agar plug methods lack the general selectivity and sensitivity of HPLC based methods. The TLC agar plug method allows rapid screening of cultures and part-colonies and is widely used in fungal taxonomy [13,14].

To combine the simplicity of the TLC method [10–12] with the standardized HPLC method described by Frisvad and Thrane [8,9] a new extraction procedure has been developed. The method was tested on all terverticillate *Penicillium* species as they include the most often encountered species in food spoilage and as a broad range of different secondary metabolites have been reported from these species [7,15–17]. Furthermore, considerations were given to reduce the solvent usage in order to improve work safety and to protect the environment.

2. Experimental

2.1. Cultivation

The isolates used in this study were selected from the IBT culture collection at the Department of Biotechnology (IBT), Technical University of Denmark, to include all terverticillate *Penicillium* taxa isolated from different habitats. Inoculations were done in three point cultures on Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES) (inoculation procedure and formulation of the substrates can be found in Ref. [13], both media used with addition of the described trace element solution) and grown for seven days at 25°C in the dark.

2.2. Extraction

Culture extracts were prepared by cutting three plugs of 6 mm diameter (approx. 85 mm^2 of surface) from one or several colonies using a cork drill as follows: one in the centre of the colony, one at the rim of a colony as far away from other colonies as possible and one at the rim of a colony as near to another colony as possible to represent the variability in a colony. The plugs were transferred to a 1.5-ml disposable autosampler screw-cap vial and 500 μl of the solvent mixture methanol–dichloromethane–ethyl acetate (1:2:3) containing 1% (v/v) formic acid were added. The plugs were extracted ultrasonically for 60 min. The extraction solvent mixture is similar to the extraction solvent used in the standardized HPLC method [9]. The extract was transferred to a clean vial using a Pasteur pipette and the organic phase was evaporated to dryness under a gentle stream of nitrogen. The residues were re-dissolved ultrasonically for 10 min in 400 μl methanol containing 0.6% (v/v) formic acid, 0.02% (v/v) hydrochloric acid and 2.5% (v/v) water (see note Section 5). Evaporation of the extraction solvent was necessary to make the sample compatible for injection in the HPLC mobile phase used, i.e. to remove the

ethylacetate. All samples were filtered through 0.45-μm Minisart RC4 filters (Sartorius, Germany) into clean vials before analysis.

2.3. HPLC analysis

The HPLC analyses were performed on a HP1090M HPLC (Hewlett-Packard, Germany) using 10-μl injections and DAD with a 6-mm flow-cell collecting approx. 2 UV spectra per second from 200 nm to 600 nm with a bandwidth of 4 nm. Separations were done on a 100×4 mm HP Hypersil BDS-C_{18} Cartridge column (Hewlett-Packard, USA) packed with 3 μm particles and including a 4×4 mm guard column. The column was maintained at 40°C. A linear gradient starting from 85% water (A) and 15% acetonitrile (B) going to 100% acetonitrile in 40 min, then maintaining 100% acetonitrile for 3 min, was used at a flow-rate of 1 ml/min. Both eluents contained 0.005% (v/v) trifluoroacetic acid (TFA). All chemicals used were Merck analytical grade and double distilled water.

3. Results and discussion

The analyses of 790 plug extracts from the 395 isolates studied showed that all the metabolites reported to be produced by these species could be identified either by comparison with analysis of standards or literature reports using retention times and UV spectra [7,15,16]. Several unknown metabolites were furthermore seen in all chromatographic metabolite profiles in agreement with previous observations [16].

Fig. 1 shows an example of HPLC analysis of plug extracts from *P. tricolor* (IBT 11663) cultivated on CYA as the top layer and YES as the bottom layer. The superimposed 210 nm and 280 nm traces show considerable differences. The most obvious difference is the early eluting broad peak which can be identified as terrestric acid and other members from the same biosynthetic pathway. These metabolites are only found when *P. tricolor* is cultivated on YES. Several other differences can be seen, both in the peak profile and in the quantitative occurrence. All metabolites known to be produced by *P. tricolor* [18] can be identified in the two profiles by comparing retention times and UV spectra with analysis of standards (see legend of Fig. 1). Fig. 1 illustrates the generally accepted importance of cultivation substrate on metabolite production; thus, screening metabolite production on different substrates is important in establishing the full metabolic potential of an organism.

P. polonicum is very common in stored cereals and a species from which a wide range of secondary metabolites have been reported. Chromatographic metabolite profiles (shown as 210 nm traces) from 4 isolates of *P. polonicum* collected from different habitats are shown in Fig. 2. Ergosterol (a commonly used marker for fungal biomass production) is found at nearly equal peak heights in the four chromatographic profiles, thus approximately the same amount of biomass has been extracted. The amount of secondary metabolites produced in the cultures can therefore be compared directly using peak heights. Comparing the chromatographic profiles from these isolates of *P. polonicum*, chromatograms (A) and (D) are quantitatively the most similar, which might represent a close relationship between the isolates. This assumption has later been strengthened using electrospray mass spectrometric (ES-MS) profiling [19,20]. The following metabolites can be identified based on analyses of standards (retention times in brackets): orsellinic acid (1.2 min), penicillic acid (3.3 min), cyclopenol (4.7 min), leucyl-tryptophanyl-diketopiperazine (6.3 min), rugulosuvine (7.7 min), cyclopenin (8.4 min), cyclopeptin (9.6 min), asterric acid (12.3 min), viridicatin (12.85 min), 3-methoxy-viridicatin (12.93 min), verrucofortine (15.3 min), puberuline (16.0 min), ergosterol (42.5 min). Viridicatol, aurantioclavine, dehydro- cyclopeptin and verrucosidine can be identified using literature reports [7,17].

The amount of biomass (surface) extracted into a plug extract is independent of colony diameter (for colonies larger than the cork drill), whereas concentration of extracts from classical "multi-plate" methods, like the standardized method described by Frisvad and Thrane [9], will depend on the colony diameter. The concentration of a plug extract correponds to approx. 212 mm^2 colony surface extracted into 1 ml. Compared to the standardized

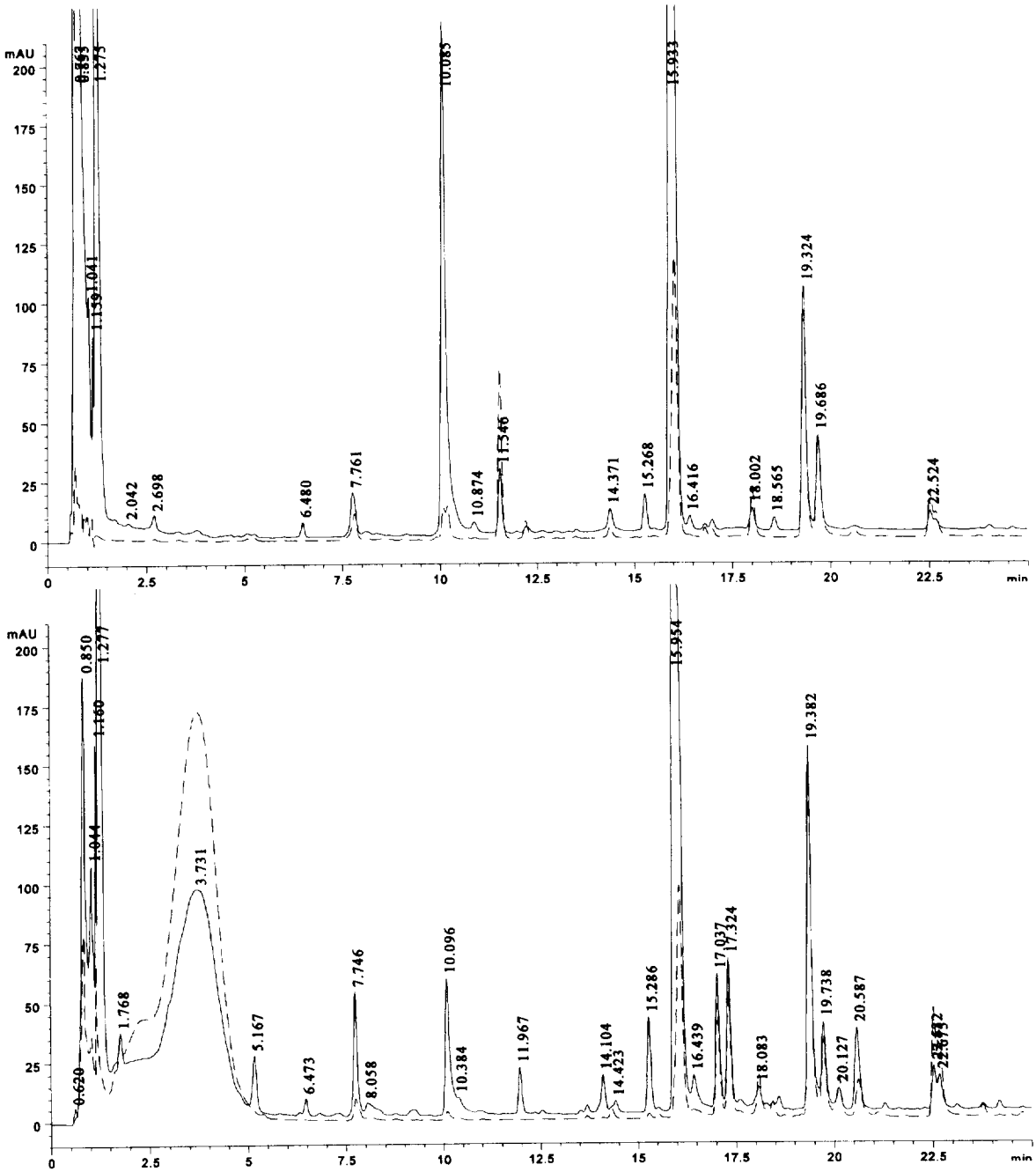

Fig. 1. HPLC analyses of plug extracts from *P. tricolor* (IBT 11663) grown on CYA at the top and on YES at the bottom, both with the 210 nm trace as a full line and the 280 nm trace as a broken line showing significant differences. The following metabolites produced by *P. tricolor* can be identified using retention times and comparing UV spectra from analyses of standards [18]: viridicatic acid at 2.70 min (only CYA), terrestric acid at 3.7 min (only YES), leucyl-tryptophanyl-diketopiperazine at 6.4 min, asteltoxin at 11.6 min (only CYA), verrucofortine at 15.3 min, puberuline at 16.0 min, viomellein at 20.1 min (only YES), xanthomegnin at 22.5 min, vioxanthin at 22.7 min.

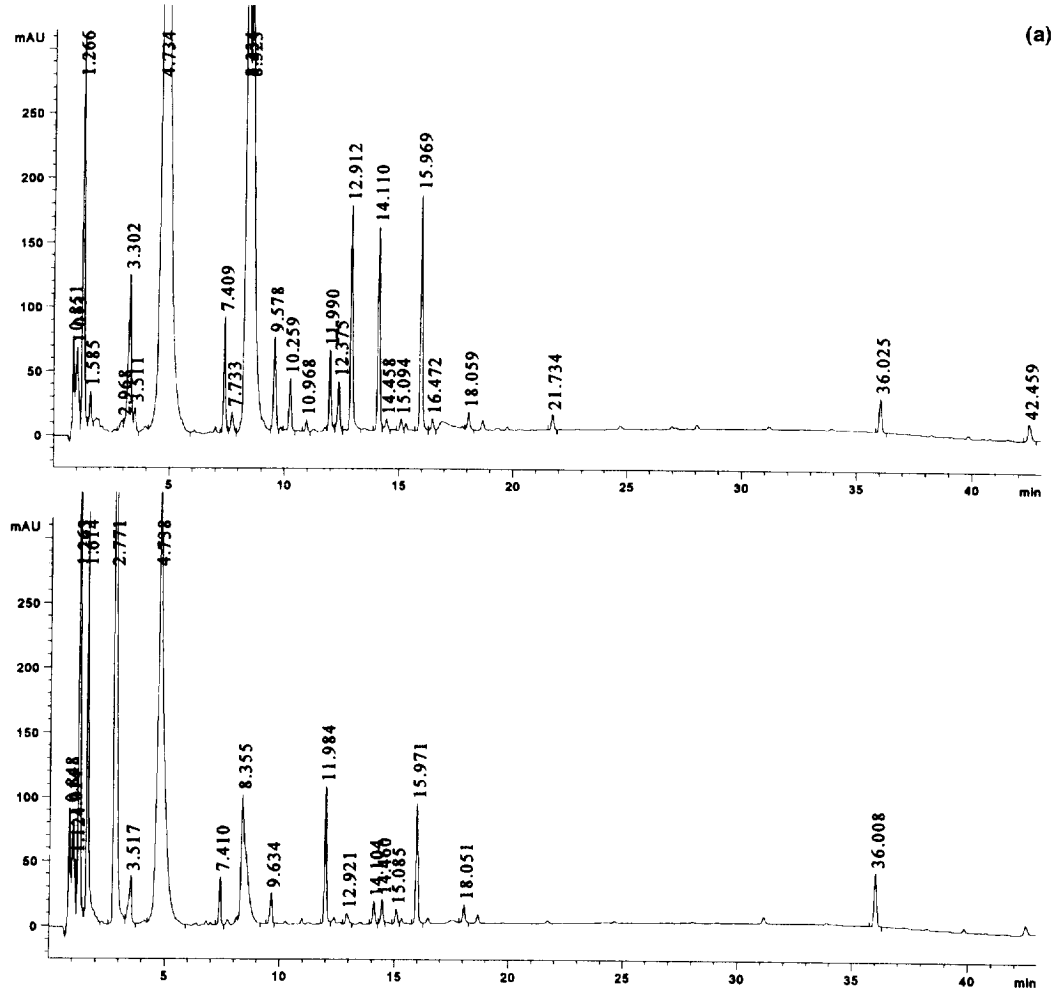

Fig. 2. Four isolates of *P. polonicum* cultivated on YES are shown as the 210 nm chromatographic trace. The isolates are: (A) IBT 14323 (ex ginger), (B) IBT 15771 (ex wheat, Bulgaria), (C) IBT 15780 (ex maize, Bulgaria), (D) IBT 15982 (ex pig feed, Bulgaria).

method [9] (extraction of 8 plates, resulting in 3 ml extract) about the same concentration will be obtained in plug extracts for a colony diameter less than 6 mm, whereas the standardized method will result in approximately 27 times more concentrated extract for a colony diameter of 30 mm. The concentrations of the plug extracts are, however, sufficient for detection of all important secondary metabolites by HPLC–DAD analysis.

4. Conclusion

All important secondary metabolites produced by several *Penicillium* species can be determined using the micro-scale extraction, followed by a standardized HPLC–DAD analysis. The chromatographic profiles obtained are analogous to those obtained from the more laborious "multi-plate extraction" HPLC methods [9]. Several extractions, e.g., using different solvents, can for most species be performed on the same colony or even part-colony. The complete preparation of a plug extract involves a few minutes of work and a plug extract can be ready in about 70 min. Thus around 50 extracts can easily be prepared in a working day during the normal examining of 7-day old cultures in the standard identification process [21].

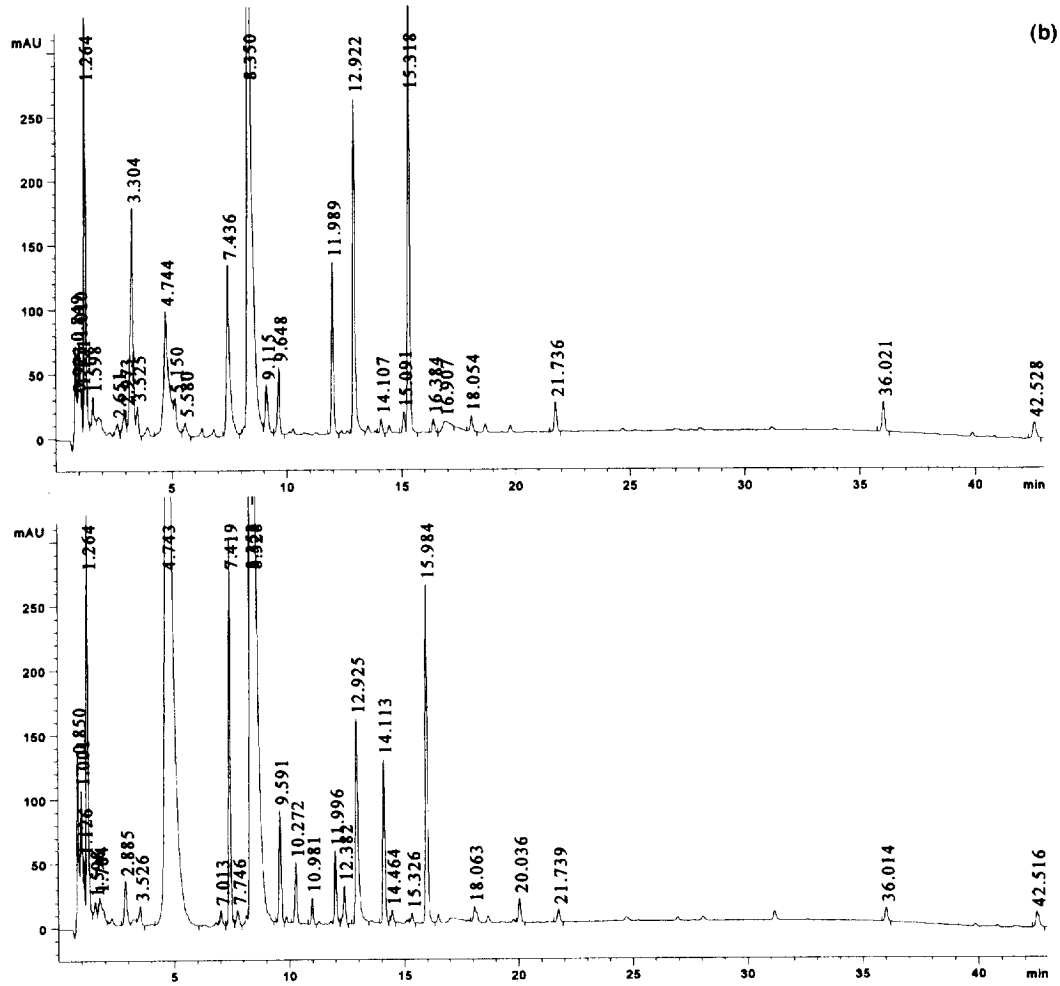

Fig. 2. (*continued*)

An important advantage of the micro extraction method is the reduced amount of solvent in use, less than 1 ml per culture studied, and minimal usage of disposable glassware thereby eliminating the handling and cleaning of large amounts of highly contaminated glassware. This is beneficial for work safety, environment and economy.

5. Note

The solvent used for re-dissolving of the samples was selected to enhance sensitivity of a flow injection ES-MS analysis [19,20,22] performed on the same extracts.

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