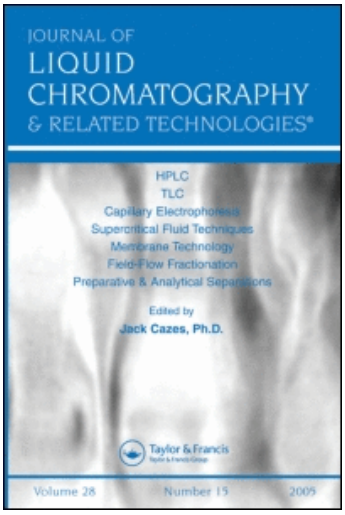

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THE SEPARATION OF A PENICILLIUM FUNGAL EXTRACT BY THIN LAYER CHROMATOGRAPHY AND HPLC: A COMPARATIVE STUDY

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ABSTRACT

The separation of a Penicillium fungal extract by two dimensional-multimodal thin layer chromatography and gradient high performance liquid chromatography is reported. The fungal extract was resolved on a cyano derivatized silica gel TLC plate using two dimensional development; first dimension in methylene chloride: hexane:acetic acid (9:10:1), taken out, dried, turned 90°, and developed in the second dimension in acetonitrile:methanol: water (40:37:32). Over 20 different spots were observed under UV radiation. Three different mobile phase gradients, acetonitrile, methanol, and tetrahydrofuran as primary organic solvents were used.

Different selectivities resulted in each gradient. The best result was obtained using the acetonitrile gradient, 25% to 100% acetonitrile in 30 minutes. Also, it was found that using photo diode array detection in natural products research is a must when quantitation is required, since the mixture contains compounds of differ-

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ent absorptivities. The results show that changing the wave length of detection resulted in different peak heights (areas).

INTRODUCTION

Natural products chemistry deals with the organic molecules, frequently the secondary metabolites, found in nature: in plants (leaves, needles, bark, flowers, seeds and roots), in marine organisms (plants, animals and microbes), in the microbial fauna, and in the soil. In the past, pharmaceutical research has relied heavily on natural products, if not as a source of drugs, then as a source of novel bioactive chemotypes that can be developed into drugs. Successes have been natural and semi-synthetic penicillins, the cephalosporins, taxol, a derivative of camptothecin, and many more, all natural product drugs currently used in the clinic.

The National Cancer Institute (NCI) drug discovery program includes a search for new lead compounds from natural products extracts as anticancer, antiviral, and antimicrobial agents. In a library of more than 150,000 crude natural product extracts are about 19,000 extracts of fungal origin, and among these 1,987 extracts from cultures identified as from the genus Penicillium. The genus Penicillium has about 150 described species, and these various organisms are well known as producers of a variety of bioactive and toxic compounds, such as natural penicillins, penicillic acid, wortmannin, gliotoxin, cyclopiazonic acid, etc., that will be detected by many different sorts of bioactivity screens.

The Berdy Antibiotic Database lists 602 bioactive compounds with Penicillium as the producing organism. But even when taxonomy has determined that two fungal extracts are identical to the species level, they do not necessarily produce the same secondary metabolites. Possible genetic differences within the species, variable culture conditions, and composition of the growth media all affect the mixture of metabolites produced by the fungus. Thus, the natural products chemist in a drug discovery program will be repeatedly presented with bioactive extracts from Penicillium cultures, and be faced with the challenge of determining whether the active substance is a known molecule or a new chemical entity.

Natural product extracts typically are a complex mixture which require high resolving chromatographic and electrophoretic methods to fully investigate. Previously, UV profiles of bioactive substances,[1] HPLC retention tables,[2] and chromatographic plus bioactivity methods[3,4] have been used for the identification of compounds found in fungal extracts. We have previously reported the use of gradient HPLC,[5] two dimensional thin layer chromatography,[6] and capillary electrophoresis[7] for the separation of taxol and congeners from the exceedingly complex needle and bark extracts of *Taxus brevifolia L. (Taxaceae)*.

The fungal extract chosen for this study, was selected because a toxic substance had been detected in a complicated region of an HPLC chromatogram, so improved resolution of components was required if the toxic metabolite was to be isolated and identified. We utilized gradient HPLC with photo diode array UV detection and multimodal-two dimensional TLC to develop methods for resolution of the components of crude extracts from two different Penicillium isolates. Photo diode array UV detection allows the detection and quantification of the resolved compounds at their optimum absorption wavelengths.

EXPERIMENTAL

Materials

Acetonitrile, methanol, tetrahydrofuran (THF), hexane, dichloro-methane, and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). All solvents used were HPLC grade. Distilled, deionized water was used. All solvents for HPLC analysis were degassed and filtered through a 0.2 μm Nylon membrane filter. The Penicillium extracts were obtained from the NCI Chemical Repository. Cyano-, amino-, and C-18 derivatized silica high performance TLC plates, 0.2 mm thick layer, 10 x 10 cm, were obtained from EM Science (from Alltech Associates, Deerfield, IL). A reversed phase C-18 HPLC column, packed with 5 μm spherical particles, 4.6 x 250 mm, was purchased from Vydac (Hesperia,CA).

Methods

Viable fungal cultures were obtained from the NCI Repository in Frederick, MD, and were grown in several different media, and under different culture conditions, designed to increase the chemical diversity of the secondary metabolites which are produced, examples of which, have previously been published.[8] The cultures investigated here were primary isolates identified as genus Penicillium, species unknown. The entire fungal ferment is extracted by first adding 10%v/v methanol, disruption of the fungal cells by high shear homogenization, and extraction by partitioning against dichloromethane. Solvent was removed by rotary evaporation, and the residue was dried under high vacuum and weighed prior to biological evaluation, followed by chromatographic analysis.

Thin Layer Chromatography

Two μL of the extract, dissolved in methylene chloride, were spotted at the corner of the cyano TLC plate, about 1 cm from the bottom and the left side of

the plate. After drying, the plate was developed in the first dimension in methylene chloride:hexane:acetic acid (9:10:1), taken out, dried, turned 90º, and developed in the second dimension in acetonitrile:methanol:water (40:37:32). After drying, the plate was viewed in a UV box under short and long UV radiation.

HPLC

Five μL of the extract, dissolved in methanol, were injected onto the C-18 column and eluted off the column using gradient elution. The gradient elution program for acetonitrile, methanol, and THF was as follows: For acetonitrile, 25% to 100% acetonitrile in 30 minutes. For methanol, 40% to 70% methanol from 0 to 15 min., then 70% to 100% methanol from 15 to 25 min. For THF, 10 to 25% THF from 0 to 10 min., 25% to 35%THF from 10 to 20 min., 35% to 65% THF from 20 to 30 min., and 65% to 100% THF from 30 to 40 min., all at a flow rate of 1 mL/min. The effluent was monitored using a photo diode array detector set at 225, 254, 280, and 300 nm.

Apparatus

The HPLC instrument used is a Hewlett Packard model 1090 equipped with an automatic injector, photo diode array (PDA) detector, auto sampler, and data station. Chromatograms were recorded on a x-y recorder.

RESULTS AND DISCUSSION

Thin Layer Chromatography

Three different TLC plates coated with cyano-, amino-, and C-18 derivatized silica were tested in this study. The optimum separation of the extract was achieved on the cyano coated plate in a two dimensional format employing normal phase (adsorption) solvent system, dichloromethane:hexane:acetic acid (9:10:1) in the first dimension, and reversed phase (partition) solvent system, acetonitrile:methanol:water (40:37:32) in the second dimension. This is done based on previous experience with resolving natural product extracts.[9] Such extracts contain compounds of different chemical properties and solubilities, therefore, using a two dimensional-multimodal TLC system insures maximum separation of the compounds in the extract. Figure 1 is a reproduction of the TLC chromatogram of the Penicillium fungal extract. Note that more than 20 compounds have been resolved using this procedure.

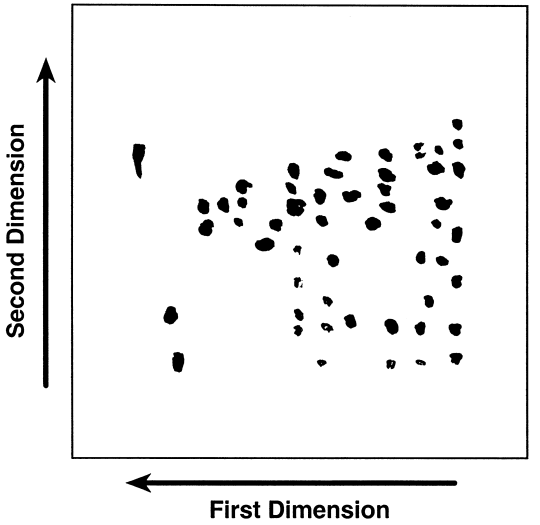

Figure 1. TLC chromatogram of the Penicillium fungal extract. Experimental details as in text.

Viewing the developed plate under short UV radiation revealed different compounds than those when the plate was viewed under long UV radiation. An amino derivatized silica gel TLC plate was tried, but gave inferior results to those obtained using the cyano derivatized silica gel TLC plate. The separation using a C-18 derivatized silica gel TLC plate in a two dimensional format using methanol:water (80:20) in the first dimension, and acetonitrile:water (70:30) in the second dimension, did not give as good a resolution of the fungal extract as that when the cyano plate was used, results not shown. This is due to the fact that the extract contains compounds of different chemical properties.

High Performance Liquid Chromatography

Gradient HPLC allows the resolution of a complex mixture due to the changes in the mobile phase properties, which will affect the partitioning of the different compounds in the mixture. Also, the use of PDA detection allows the monitoring of compounds of different UV absorption, which would maximize the eluting peak area and results in better quantitative information. In this study, we used three different mobile phase gradients where the primary solvent in each was different. The three primary solvents were acetonitrile, methanol, and tetrahydrofuran. These three solvents resulted in different selec-

tivities, as it is clear from Figure 2, which clearly shows differences in the peak elution order.

The advantages of using PDA detection in natural products research are obvious from Figure 3. This chromatogram, which is recorded at four different absorption wave lengths; 225, 254, 280, and 300 nm, reveals fine structures that would not have been detected at a single UV absorption wave length. Also, the size of the peaks (area and height) at different wave lengths is different. For example, one can clearly see differences in peak sizes in the 10 to 14 minutes elution range. The peak eluting at 14 min appears much smaller than those at 12

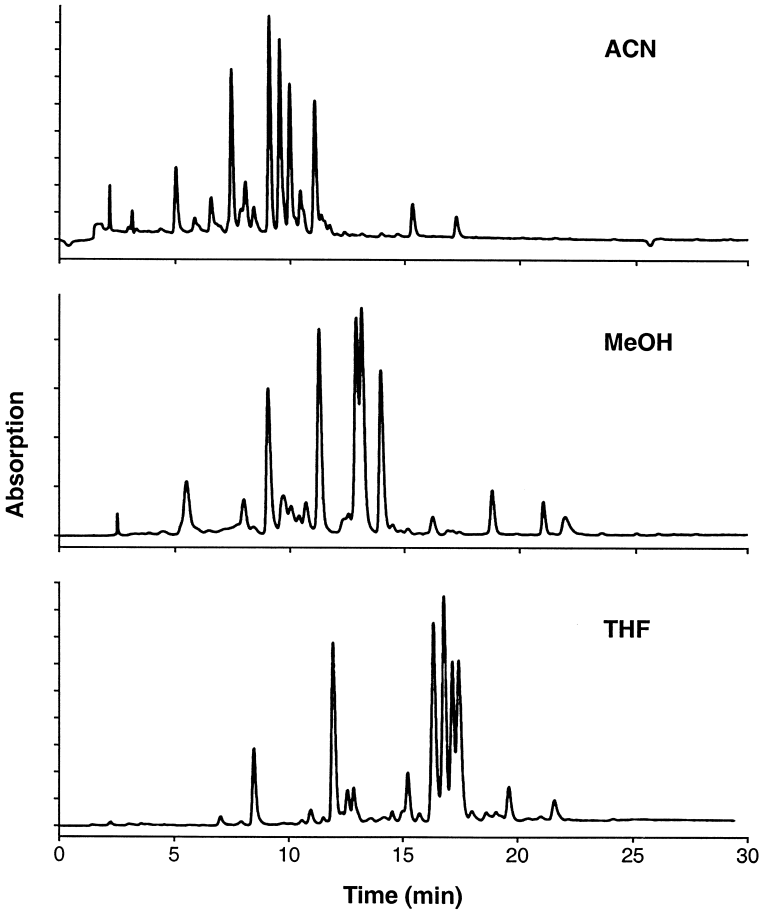

Figure 2. HPLC chromatogram comparing the separation of the Penicillium fungal extract using acetonitrile, methanol, and tetrahydrofuran as the primary solvent gradients.

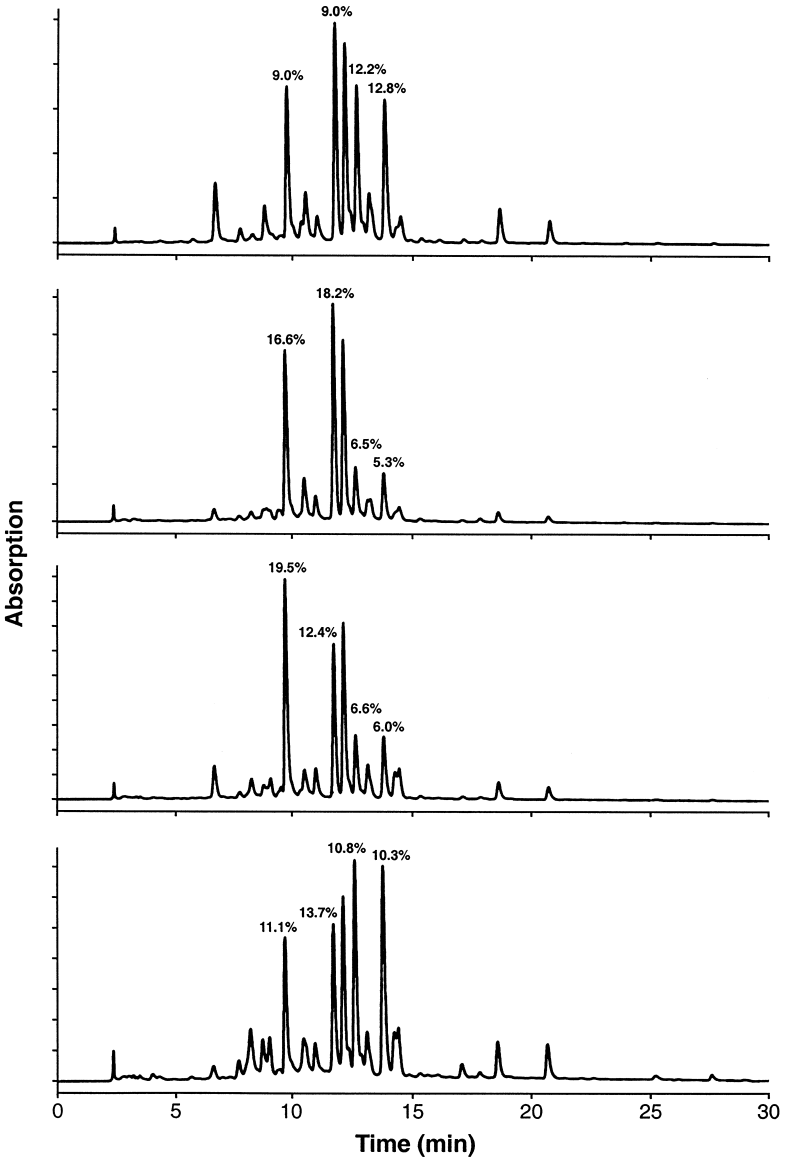

Figure 3. An HPLC chromatogram showing the effect of detection wave length on the peak height (area) of the Penicillium fungal extract.

Table 1. Effect of $\bullet_{max}$ of Absorption (% area) of Different Solutes in the Penicillium Fungal Extract

Peak #	% Area $\bullet_{225}$	% Area $\bullet_{254}$	% Area $\bullet_{280}$	% Area $\bullet_{300}$
1	9.0	16.6	19.5	11.1
2	9.0	18.2	12.4	13.7
3	12.2	6.5	6.0	10.8
4	12.8	5.3	6.0	10.3

min when the detection λ_{max} is 254 or 280 nm, while it appears to be larger than the others when the λ_{max} is 300 nm.

Table 1 is a summary of the % peak areas of 4 different compounds at the 4 selected wavelengths of detection. Such comparison of detection is important in quantitative determination of a solute in a mixture. For example, while the peak at 14 min appeared to be a minor one at 254 and 280 nm, it seems to be a major one at 300 nm. This is extremely important when searching for compounds of medical and pharmaceutical interest. The table also shows that the four compounds can be divided into three groups; compound number 1, compound number 2, and compounds number 3 and 4. Based on their absorption properties (% area), compounds 1 and 2 are different, while compounds 3 and 4 can be assumed to be similar, i.e., possess the same chromophore.

A comparison of the TLC results with the HPLC results shows that two dimensional-multimodal TLC offers higher resolution than gradient HPLC, because two orthogonal modes of separation, adsorption and partitioning, are employed. However, HPLC with PDA detection offers more accurate and simultaneous quantitation of the eluted peaks, and allows detection at predetermined wave lengths, while in TLC that is not possible. HPLC allows the collection of any peak using fraction collection easily, while in TLC spots would have to be lifted off the plate by scraping then extracting with an appropriate solvent, filtering, and evaporation of excess solvent. The advantage of TLC in natural products research is that it can be run in the field with no special requirements.

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