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SIMPLE PREPARATIVE LIQUID CHROMATOGRAPHY SYSTEM AND ITS APPLICATION TO THE SEPARATION OF *ALTERNARIA* METABOLITES

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SUMMARY

A simplified device for large-scale preparative liquid chromatography is described. Employing modifications of standard liquid chromatography apparatus, the device uses the laboratory compressed-air supply to achieve elution rates of 150–300 ml/min without the need for an expensive pumping system. Its use in the preparation of gram quantities of toxic *Alternaria* metabolites is described, and illustrates its potential as a convenient and economical alternative to commercial preparative liquid chromatographs.

INTRODUCTION

In the course of studies on the chemistry and toxicology of secondary fungal metabolites, there is often a need for large quantities of purified preparations of these mycotoxins. Investigations in our laboratory and others on new methods for the isolation of gram quantities of mycotoxins have led to the use of a Waters PrepLC System 500 preparative liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), employing PrepPAK 500 silica cartridges, for the preparation of ochratoxin A[1], deoxynivalenol[2], and the major dibenzopyrone metabolites produced by *Alternaria*[3].

Although the speed and capacity of the Waters device greatly facilitates the preparation of purified toxins, the system involves considerable expense, both for initial instrumentation and for the PrepPAK cartridges, which in our case at least, were not re-usable. In the present study, we have designed a liquid chromatography system with comparable flow-rates and sample capacity which allowed rapid and convenient preparation of the mycotoxins of interest in our studies. Details of the construction of this apparatus, and its application to the isolation of gram quantities of *Alternaria* metabolites, are reported in this paper.

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EXPERIMENTAL

Construction of apparatus

The liquid chromatography system is shown in Fig. 1. In place of an expensive high-volume pump, the laboratory air supply, regulated to a pressure of 25–30 p.s.i.g., was used, and provided flow-rates of 150–300 ml/min. For use as a solvent reservoir, we selected a used household pressure cooker (Mirro A 406 M; Aluminum Goods Mfg. Co., Manitowoc. WI, U.S.A.), 5 l capacity, as a convenient and economical pressure vessel. The gauge and safety-valve ports in the lid were employed for the solvent and air-lines, respectively, using fittings with appropriate threading. All solvent lines were of PTFE, either 3.2 mm O.D. × 3.1 mm I.D. (Waters), used as the outlet line from the reservoir, or 3.0 mm O.D. × 1.5 mm I.D. (Anspec, Ann Arbor, MI, U.S.A.), used in all other applications. Air lines were either butyl rubber, 12.8 mm O.D. × 6.4 mm I.D. (Fisher Scientific, Itasca, IL, U.S.A.), or Tygon, 16 mm O.D. × 6 mm I.D. (Scientific Products, McGaw Park, IL, U.S.A.). Valves 1 and 2 were Hamilton miniature valves (Scientific Products), valves 3 and 4 were standard Pyrex 3-way stopcocks (Scientific Products). Air lines were secured with standard ring-style hose clamps; solvent line fittings were Cheminert 1/4 in. screw fittings, 1/8 in. bore (Anspec), except for that in the lid of the solvent reservoir, which was a Pharmacia column outlet fitting (Pharmacia Fine Chemicals, Uppsala, Sweden).

The columns themselves were modifications of Michel-Miller columns (ACE

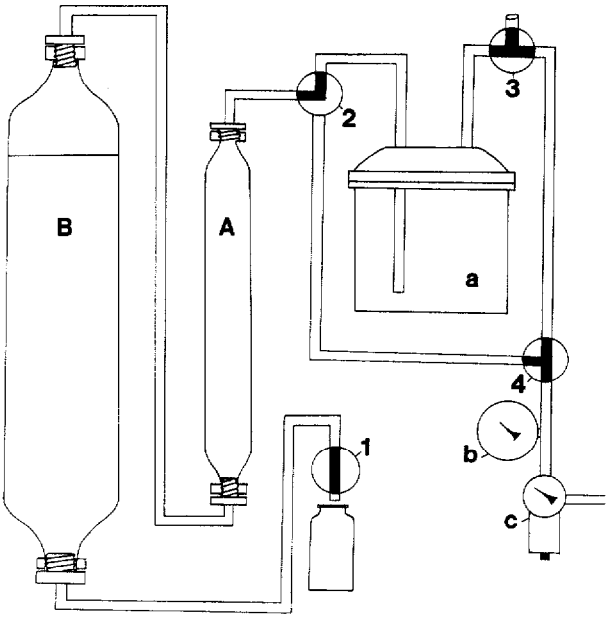


Fig. 1. Preparative-scale liquid chromatograph. See text for complete description. Column A: "sight glass" to monitor solvent level. Column B: oversized column containing gel bed. Actual size: 66 cm (overall) × 8.1 cm O.D. Capacity: 2.3 l (approx. 1.1 kg silica gel when packed in hexane; 900 g used routinely). Valves 1 and 2: solvent control valves. Valves 3 and 4: air control valves. Third outlet in 3 is vent to atmosphere. Other components: (a) solvent reservoir (pressure cooker); (b) air pressure gauge; (c) pressure regulator on laboratory air supply.

TABLE I

LIQUID CHROMATOGRAPHY COMPONENTS USED IN THIS APPARATUS

Other components used have been described in the text. The mention of manufacturers, suppliers, and part numbers is merely to facilitate identification of the various parts of the system and is not intended to deter substitution of other appropriate components.

Item	*Figure reference**	*Manufacturer/supplier*	*Supplier part No.*
"Sight glass" column	A	ACE	5795
Threaded fittings, glass	(B)**	ACE	7644
End fittings, PTFE	(A and B)	ACE	5801
Safety shield	(A)	ACE	5798
Solvent line fittings	(A, B, 1, 2)	Cheminert/Anspec	43670
Solvent valves	1 and 2	Hamilton/Scientific Products	C5360
Reservoir outlet fitting	(a)	Pharmacia	90-035
Solvent tubing	(a and 2)	Waters	26809
	(A, B, 1, 2)	Anspec	43580

* See Fig. 1. Letters and numbers in parentheses refer to locations of components not specifically identified in Fig. 1.

** Fittings used in the custom fabrication of the oversized column.

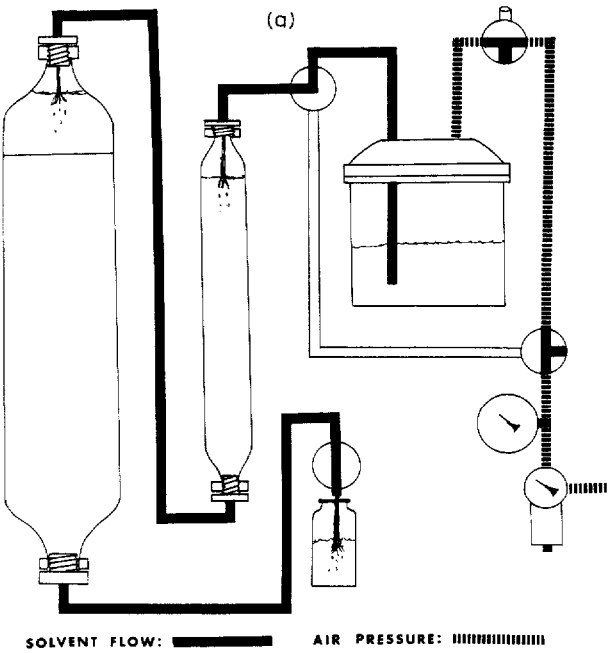
Glass, Vineland, NJ, U.S.A.), equipped with PTFE fittings. The oversized column (B), 66 cm (overall) × 8.1 cm O.D., was fabricated by a local glass shop using ACE threaded fittings. The bores of the PTFE inlet and outlet fittings, originally 1/16 in., were drilled out to 1/8 in. to provide increased flow capacity. The smaller column (A) is a standard ACE catalogue item (see Table I for manufacturers and part numbers of chromatography components used in this apparatus).

The large column was routinely packed with 900 g of silica gel 60, 0.06–0.2 mm particle size (E. Merck, Darmstadt, F.R.G.). This provided a head-space in the column above the bed of approx. 10 cm, allowing large-volume or viscous samples to be loaded directly onto the gel bed.

General operation of apparatus (Fig. 2)

After the column is packed in an appropriate solvent and sample is loaded, 5 l of the first eluting solvent is placed in the reservoir, the valves are placed in the positions in Fig. 2a, and the reservoir is pressurized to 25–30 p.s.i.g. Solvent flows from the reservoir into the empty column (A) and thence into the packed column (B). Eluate flows from the column through valve 1 into the receiving container. As the elution proceeds, column A will slowly fill up with solvent as the entrapped air dissolves into the solvent stream. This column functions as a "sight glass", allowing one to monitor the solvent flow and note when the reservoir empties.

The reservoir can then be refilled without interrupting column elution, as shown in Fig. 2b. In this valve configuration, the pressurized air is applied directly to the "sight glass" column, where the contained volume of solvent (approximately 500 ml in our system) allows continued elution during solvent replenishment. The reservoir is isolated from the pressure line and vented to the atmosphere through valve 3, permitting opening and solvent replenishment. Valves 3 and 4 are returned to their original configuration, re-pressurizing the reservoir; valve 2 is then turned, restoring normal solvent flow with no pressure flux on the column bed.



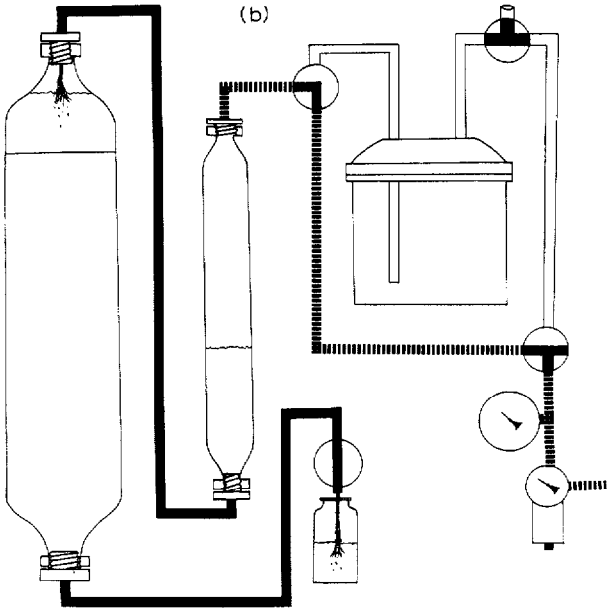


Fig. 2. Operation of chromatograph: (a) valve configuration and paths of air and solvent flow during normal operation; (b) valve configuration and paths of flow during solvent replenishment. See text for complete description.

The system can be easily shut down overnight (valuable during a lengthy column run) by closing valve 1 and setting the other three valves in the configuration shown in Fig. 2b. This maintains pressure in the column but allows the reservoir to be depressurized and opened when not in use. (We found the gasket in the lid of the pressure cooker deformed from the solvent vapors when the reservoir was left closed and pressurized overnight.)

Once pressurized, the column should not be depressurized until the run is completed. As much air dissolves into the solvent due to the nature of the system, venting the column to the atmosphere would result in massive cracking of the column bed as the solvent within the gel degasses.

Preparation of Alternaria metabolites

Alternaria alternata strain RL 671-2 was used to produce the metabolites prepared with this system. A spore suspension of the mold grown on Potato-Dextrose Agar (Difco Labs., Detroit, MI, U.S.A.) was used to inoculate a rice medium, composed of 300 g long-grained rice + 150 ml tap water, in 2.8-l Fernbach flasks. Cultures were incubated 21 days at 27°C in darkness, and shaken once per day by hand to break up the mycelial mat. Cultures were soaked overnight in 500 ml ethyl acetate and extracted three times with the same solvent in a Waring Blender. The extracts were recovered by filtration, pooled, and concentrated.

The column was packed in *n*-hexane (900 g silica gel 60). Approximately 80 g crude extract in 150 ml ethyl acetate was loaded on the column bed using a funnel, Valve 1 was opened, and the sample allowed to enter the bed by gravity. Approximately 200 ml hexane was then added to the column, the upper fitting was attached, 4 l hexane was added to the solvent reservoir, and the system was pressurized to 25 p.s.i.g. Eluate from the hexane wash was collected in a single fraction. Thereafter, 400-ml fractions were collected. Elution solvents were as follows: 20 l ethyl acetate–hexane (15:85); 20 l ethyl acetate–hexane (30:70); 10 l ethyl acetate–hexane (45:55); 10 l ethyl acetate–hexane (60:40); 10 l ethyl acetate; 3 l methanol. Each fraction was spotted on silica-gel thin-layer plates and developed in toluene–ethyl acetate–formic acid (5:4:1). The metabolites, which are fluorescent, were visualized under long-wave UV light. Fractions containing pure, or like mixtures, of toxins were pooled into 13 large fractions, designated A through M, and concentrated using a Büchi Rotary Evaporator R (Brinkmann, Westbury, NY, U.S.A.).

RESULTS AND DISCUSSION

The liquid chromatography system described here has been used routinely in our laboratory during the past year for preparation of large quantities of purified mycotoxins. Results for a typical run for isolation of large quantities of *Alternaria* metabolites are presented here.

Fig. 3 depicts a thin-layer chromatogram of the pooled eluted fractions from chromatography in this system of the crude *Alternaria* culture extract. Alternariol monomethyl ether (AME), the toxin produced in most copious quantities by this strain of *Alternaria,* crystallized in fractions C, D and E upon concentration. The crystallized toxin was recovered by filtration and analyzed for degree of purity, and quantified, by analytical high-performance liquid chromatography, using a Waters

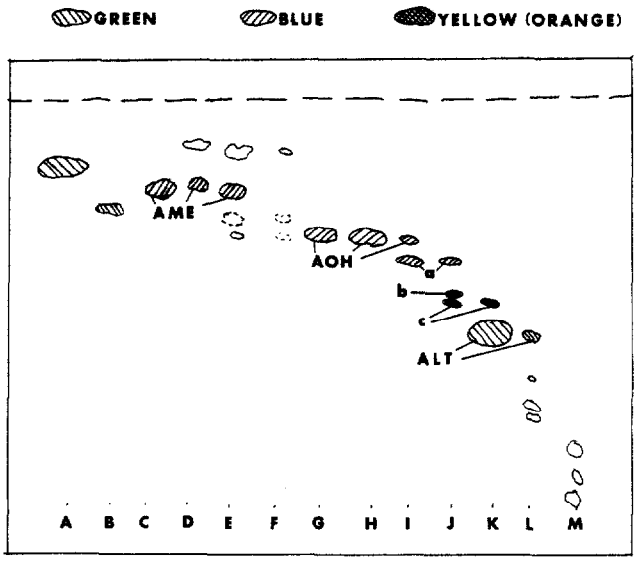


Fig. 3. Thin-layer chromatogram of pooled eluted fractions from column chromatography of crude *Alternaria* culture extract. Conditions of column elution and TLC are described in the text. Visualization is by fluorescence with excitation by UV light (366 nm). Major dibenzopyrone metabolites are identified. AME = alternariol methyl ether; AOH = alternariol; ALT = altenuene. a, b and c indicate the minor toxins altenuisol, altertoxin II, and altertoxin I, respectively.

μBondapak C_{18} column, with detection by UV absorption at 254 nm[4]. Approximately 1100 mg AME were recovered in this way from a single column run, with purity of 90% or greater, suitable for our toxicologic investigations.

To recover alternariol (AOH) and altenuene (ALT), the other major dibenzopyrones produced by this strain of *Alternaria*, as well as the AME remaining in solution, like fractions from several column runs were pooled, concentrated, loaded onto another silica-gel column, and rechromatographed using similar solvent systems to obtain preparations of similar purity (data not shown).

The minor *Alternaria* toxins indicated in Fig. 3 can if desired be purified from these fractions using a Sephadex LH-20 (Pharmacia) gel filtration column as previously described[5].

The run reported in this paper is representative of those we perform at frequent intervals. For chromatography of crude *Alternaria* culture extracts, we believe this apparatus compares favorably with the Waters device used previously[3]. The column satisfactorily resolves the major toxins of interest, and allows chromatography of larger samples of the crude starting material per run, increasing the efficiency and economy of the operation. Using this system, we have also isolated gram quantities of partially purified deoxynivalenol (vomitoxin) from crude culture extracts of *Fusarium* sp. in runs using similar solvents and of similar duration. In addition to the large-scale runs, we have used the system for smaller-scale runs, with a smaller column and lower flow-rates. Even in this mode, the apparatus offers the advantage of obviating the need for a mechanical pumping system, usually the most expensive part of a preparative liquid chromatography apparatus.

The basic design of this apparatus may be adapted to a variety of preparative liquid chromatography applications. The open-bed column style allows the loading of samples too concentrated or viscous to pass through a 17-gauge cannula, the loading method used on the Waters device. If solvent systems are used which are incompatible with the metal of the solvent reservoir (such as aqueous buffers), a glass or plastic vessel of appropriate outside diameter may be cut off to fit inside the reservoir as a suitably inert liner. Although we used the pressure cooker as a conveniently available pressure container, other suitable or more traditional laboratory pressure vessels can be easily substituted. If separation of compounds which may be sensitive to the high oxygen tensions present in this system is desired, the laboratory air supply may be replaced by bottled nitrogen, helium, or other inert gas, as the pressure source. We would recommend in this case, however, that a solvent reservoir of larger capacity be used, to lessen the frequency of refilling, with its accompanying venting of the reservoir and loss of pressurized gas.

CAUTIONARY NOTES

Ethyl acetate and *n*-hexane are highly flammable solvents and their vapor can be toxic if present in sufficient concentration. In this system, due to the large volumes of solvent, and the high flow-rates and pressures used, significant quantities of vapor are generated. In our laboratory this apparatus is permanently installed within a large fume hood, where our culture extractions and all other manipulations involving large quantities of volatile solvents are performed. When this chromatography system is used for other types of separations, the hazards of the materials involved should be assessed, and the device positioned or altered accordingly.

During routine operation of this system safety shields are installed on both glass columns, as recommended by the manufacturer (ACE). The shield on the smaller column (A) is a standard catalogue item listed in Table I. The shield for the custom-made oversized column (B) was fabricated from a polypropylene pipette discard receptacle, cut to fit over the column.

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