STEP GRADIENT ELUTION IN GEL PERMEATION CHROMATOGRAPHY. A NEW APPROACH TO NATURAL PRODUCTS SEPARATIONS

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ABSTRACT.—A step-gradient elution sequence in gel permeation chromatography has been shown to be a mild, rapid and efficient technique for initial fractionation of crude extracts of plants and invertebrates. The procedure is described, and two examples of its application are presented.

The separation and purification of individual constituents from crude extracts is all too frequently the most difficult segment of the process of isolating and identifying natural products. While the advent of hplc has afforded dramatic improvements in the final stages of separations, adsorption chromatography on silica gel, alumina, or Florisil remains the most common technique for initial fractionations.

Adsorption chromatography, despite its broad applications, is fraught with disadvantages: consumption of relatively large quantities of eluants, the length of time required for one chromatographic separation, and, perhaps most important, the potential for rearrangement or decomposition of many naturally occurring compounds during chromatography. This report presents a viable alternative to traditional adsorption chromatography for first fractionations of crude plant or invertebrate extracts, gel permeation chromatography modified by the use of eluant combinations of increasing polarity.

Initial attempts to isolate a toxic, dermatitis-producing agent from the blue-green alga *Lyngbya majuscula* were hampered by apparent decomposition of the toxin on silica gel and inability to separate the toxin from a complex matrix of compounds with similar polarity (1). To counter the decomposition problem, gel permeation of the crude dichloromethane soluble extracts through Sephadex LH–20 with methanol-chloroform (1:1) was attempted, but no significant gains were made in the separation of components. The gel permeation was repeated, this time with hexane-dichloromethane (1:4) until no further band movement was observed on the column. The elution solvent was then changed to dichloromethane-acetone (3:2) and elution continued until, again, movement of the material on the column ceased. Finally, elution with dichloromethane-acetone (1:4) removed the remaining material from the column. Fractionation was based solely on the migration of distinct colored bands! The results of this chromatography are summarized in table 1. Shrinkage of the gel bed was approximately 10% over the entire run.[1] A rapid flow rate, ~3 mL/min, was established at the onset of the chromatography, but gradually slowed to ~2mL/min because of changes of solvent composition and concomitant contraction of the gel bed.

The use of a non-polar solvent combination, followed by substantial increments in eluant polarity, enabled this single chromatography step to combine the normal-size separations expected in gel permeation with enhanced partition (2) and adsorption (3) effects exhibited by Sephadex LH–20 toward aromatic and polar, hydrogen-bonding compounds. In the case of the *Lyngbya majuscula* extracts, the results were startling. Four novel entities, malyngamide A, **1** (4), majusculamides A and B, **2a** and **2b** (5), malyngolide, **3** (6), and lyngbyatoxin, **4** (7), were concentrated in separate fractions.

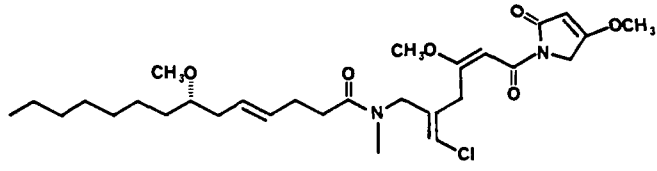
Examination of these structures suggested that the elution of **1–3**, all effected with hexane-dichloromethane (1:4), depended on both molecular size and polarity.

[1]Experiments employing methanol rather than acetone resulted in considerable shrinking of the gel bed, accompanied by cracking and channeling.

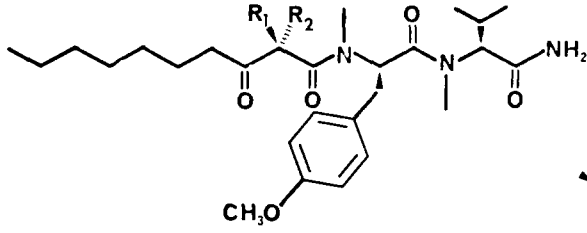
TABLE 1. Gel permeation of 1.52 g *Lyngbya majuscula* extract[a] (10).

Fraction	Wt (mg)	mL Eluant	Description (Major Constituent)	Ref
1	225	100[b]	chlorophylls, triglycerides	—
2	208	45[b]	malyngamide A	4
3	168	85[b]	majusculamides	5
4	129	90[b]	majusculamides	5
5	160	180[c]	malyngolide	6
6	58	100[c]	lyngbyatoxin	7
7	207	100[c]	saturated fatty acids	—
8	171	250[d]	unsaturated fatty acids	—
9	42	100[d]	unsaturated fatty acids	—
10	111	125[d]	unsaturated fatty acids	—
11	32	200[d]	undetermined	—

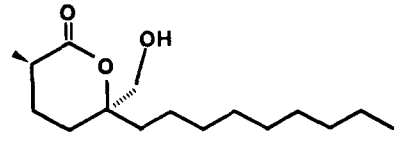
[a]column 2.5×180 cm, void volume 325 ml; flow rates: initial, ~3.0 ml/min; final, ~2.0 ml/min.
[b]hexane-dichloromethane (1:4).
[c]dichloromethane-acetone (3:2).
[d]dichloromethane-acetone (1:4).



1



2 a $R_1 = CH_3$, $R_2 = H$
b $R_1 = H$, $R_2 = CH_3$



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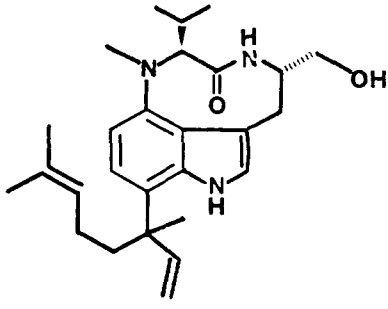

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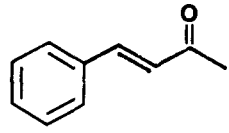

5

Malyngamide A (**1**) is relatively large but possesses no acidic hydrogens available for hydrogen bonding and, therefore, was not retained long. While the majusculamides are comparable in size to **1**, they possess hydrogen bonding functionalities and aromatic rings and were retained longer. Malyngolide (**3**) is both considerably smaller than **1** and **2** and capable of hydrogen bonding; predictably, it was eluted after **1** and **2**. Lyngbyatoxin (**4**) is bulkier than **3** but required a more polar solvent for elution; the aromaticity and multiple hydrogen bonding sites in **4** would seem to be responsible for the increased retention. The carboxylic acid moieties of the fatty acids are so strongly hydrogen-bonded to the gel that the acids are retained long after the much more highly functionalized **1**, **2**, and **4** are eluted.

In an effort to demonstrate the general utility of this method, the crude extracts of the tunicate *Eudistoma olivaceum* were fractionated by this technique. A similar pattern was observed. Triglycerides and other large nonpolar molecules were eluted early, followed by fractions containing mixtures of aromatic constituents. Aliphatic alcohols followed, then fatty acids and, then, small, polar aromatic compounds. Table 2 summarizes the results of this experiment.

TABLE 2. Gel filtration of 1.90 g *Eudistoma olivaceum* extract[a].

Fraction	Wt (mg)	mL Eluant	Description (major component)
1	217	52[b]	triglycerides
2	100	138[b]	aromatic (**5**)
3	163	69[b]	sterols, aromatic
4	375	296[b]	diacetone alcohol[e]
5	68	390[c]	aliphatic alcohols
6	338	124[c]	unsaturated fatty acids
7	196	105[d]	saturated fatty acids
8	92	197[d]	aromatic
9	31	87[d]	aromatic
10	25	50[d]	possible glycosides
11	175	150[d]	undetermined

[a]column 2×190 cm, void volume 260 ml; flow rates: initial, ~3.0 ml/min; final ~2.0 ml/min.
[b]hexane-dichloromethane (1:4).
[c]dichloromethane-acetone (3:2)
[d]dichloromethane-acetone (1:4).
[e]artifact from extraction with aqueous acetone

Fraction 2 has yielded a small nonpolar compound tentatively identified as *E*-1-phenylbut-1-en-3-one, **5**[2]. The aromatic constituents of fraction 3 are larger molecules (MW 300–400) but are nitrogenous heterocycles with at least one hydrogen bonding functionality. Full characterization of these compounds is in progress and will be described in a subsequent report.

Since partition effects and the adsorption of aromatic compounds are most pronounced when methanol or ethanol is used in the eluant (8), compounds like **1**, **2**, and **4**, and the aromatic compounds in the *Eudistoma* fractions could be further purified by a second Sephadex LH-20 gel permeation, this time with chloroform-methanol or dichloromethane-methanol eluant combinations. At this point, the target constituents were, in most cases, amenable to final purification by additional gel permeation chromatography or by hplc on nitrile or octadecylsilyl bonded phase columns.

Although there are published reports on the use of solvent gradients in gel permeation chromatography (9), previous studies employed relatively small changes in the eluant compositions and dealt with far less complicated mixtures. The use of this technique for the isolation of **1** and **4** resulted not only in a sub-

[2] ^{1}H-nmr ($CDCl_3$, 250 MHz): 7.53 (2H, m) 7.50 (1H, d, J=16.5), 7.38 (3H, m), 6.70 (1H, d, J=16.5), 2.37 (3H, s).

stantial reduction in the number of separation steps required (from five to three for **1** and from seven to three for **4**), but in a dramatic improvement in isolated yields as well (30% for **1** and up to 600% for **4**).[3] Moreover, malyngolide, **3**, was eluted with a large fatty ester fraction in silica gel adsorption chromatography experiments and was difficult to detect and purify. Use of this step gradient gel permeation gave a fraction very rich in **3**; this hydroxy lactone was readily purified by one additional gel permeation through Bio-Beads SX-8.

This technique is currently being utilized in several projects in this laboratory and is expected to enjoy broad application in our natural products research.

EXPERIMENTAL

The hexane and dichloromethane were either hplc grade or distilled in glass; the acetone was analytical reagent grade. Eluant combinations were equilibrated for thirty minutes prior to use. Sephadex LH-20 was allowed to swell in hexane-dichloromethane (1:4) for 3–4 hours prior to packing of the column. Several void volumes of eluant were passed through the column prior to use in order to insure complete bed settling.

Used gel could be recycled in the following manner. The gel was stirred in hot (50–60°) methanol for 10 minutes and the solvent removed by suction filtration; this process was repeated once or twice. The partially dried gel was then dried in an oven at 55–60° for twelve to sixteen hours. Thus purified and dried, the gel was ready for reuse.

Lyngbya majuscula was collected at Kahala Beach in Hawaii, freeze-dried, and extracted successively with petroleum ether and dichloromethane. The dichloromethane solubles were used in this study.

Eudistoma olivaceum was collected in Tucker Town Bay, Bermuda, and was extracted successively with acetone and dichloromethane. The extracts were evaporated and the combined residues partitioned between dichloromethane and water. The dichloromethane solubles were used in this study.

ACKNOWLEDGMENTS

Field work in Bermuda was supported by grants from the H. B. Wilkinson Foundation, Continental Corporation and Exxon Corporation. Funds for the work at Montana State University were provided from the Montana extension of NSF grant ISP-8011449 and from the Lilly Research Laboratories.

Received 27 May 1982

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[3]The vinyl ether moiety of **1** is quite unstable in the presence of silica gel, alumina or traces of mineral acid; the amino-indole system in **2** decomposed readily in the presence of acids or oxidizing agents.