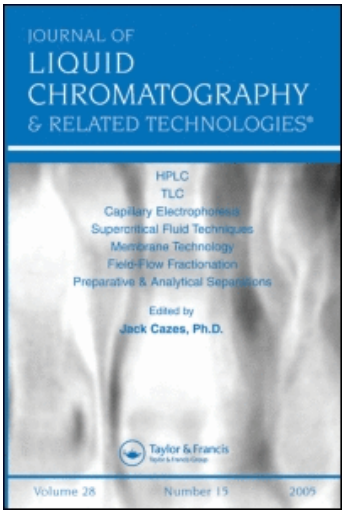

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Use of Millipore Norganic Resin for the Extraction of Proctolin and Other Pharmacologically Active Constituents from Cockroach Tissue Homogenates

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To cite this Article Starratt, Alvin N. and Steele, Robert W.(1986) 'Use of Millipore Norganic Resin for the Extraction of Proctolin and Other Pharmacologically Active Constituents from Cockroach Tissue Homogenates', Journal of Liquid Chromatography & Related Technologies, 9: 10, 2291 — 2303
To link to this Article: DOI: 10.1080/01483918608074149
URL: http://dx.doi.org/10.1080/01483918608074149

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(10), 2291-2303 (1986)

USE OF MILLIPORE NORGANIC RESIN FOR THE EXTRACTION OF PROCTOLIN AND OTHER PHARMACOLOGICALLY ACTIVE CONSTITUENTS FROM COCKROACH TISSUE HOMOGENATES

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ABSTRACT

Millipore Norganic resin, a proprietary product marketed for the preparation of HPLC-grade water, can serve as an alternative to Sep-Pak C_{18} cartridges for the solid phase extraction of proctolin and other physiologically active components from cockroach tissue homogenates. For large scale isolations of such substances, this resin is most useful for first stage purification. Retained substances can be further purified by application to Sep-Pak C_{18} cartridges which are less hydrophobic than the Norganic resin. Results were evaluated by reversed-phase HPLC of extracts and bioassay of fractions. Typical recoveries of proctolin were in the range of 75-90%.

INTRODUCTION

There is growing awareness of the diversity and potent bioactivities of neuropeptides from both vertebrate and invertebrate sources (1). In contrast to the vertebrate situation, progress to-

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wards the identification of these substances from tissues of insect origin has been slow due in large part to difficulties of isolation and purification (2). Recent studies have demonstrated that proctolin, H-Arg-Tyr-Leu-Pro-Thr-OH, the first insect neuropeptide to be identified, and other pharmacologically active peptides can be simply and efficiently isolated from homogenates of insect tissues by Sep-Pak C_{18} cartridge extraction (3-10). In this paper we report that Millipore Norganic resin, a proprietary product marketed for the preparation of HPLC-grade water, can also be employed in the isolation of insect neuropeptides.

MATERIALS AND METHODS

Chromatography and Radioanalysis

Sep-Pak C_{18} cartridges from Waters Scientific Ltd. and Norganic cartridges from Millipore Ltd. were used. Reversed-phase high-performance liquid chromatography (HPLC) was performed on 30 cm x 7.8 mm i.d. Techsil 10 C_{18} (HPLC Technology Ltd.) and 15 cm x 19 mm i.d. μBondapak C_{18} (Waters Scientific Ltd.) columns, using a Waters liquid chromatography system equipped with two model 6000A pumps, a model 660 solvent flow programmer, a model U6K sample injector and a Schoeffel model SF-770 variable-wavelength detector. HPLC water, prepared by passing distilled water through a Norganic cartridge, acetonitrile from Caledon Laboratories Ltd., and trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) from Eastman Kodak Co. were used for the mobile phases.

A Beckman LS-9000 liquid scintillation spectrometer was used for quantitating ^3H-radioactivity which has been expressed as disintegrations per min (dpm). Aliquots from extracts or chromatographic fractions were added to the scintillation cocktail (Omnifluor; New England Nuclear) and counted.

Bioassay

Fractions were dried and residues redissolved in water for bioassay on the isolated cockroach proctodeum (11, 12). To insure

that samples were not subjected to temperature degradation, lyophilization was used for final drying after most of the solvent was removed using a Speed Vac Concentrator (Savant Instruments). Excitatory myotropic activities were quantified as ng proctolin equivalents from the amplitudes of induced contractions, while inhibitory activities were quantified from the diminutions in induced contractions resulting from proctolin standards (1-4 ng) introduced to the bathing chamber 15 sec after sample application.

Peptides

[3,5-^{3}H-Tyr^{2}]Proctolin (32.8 Ci/mmol) from New England Nuclear, unlabelled proctolin from Sigma Chemical Co. and cockroach myoactive peptides MI and MII from Peninsula Laboratories were used.

Preparation of Cockroach Extracts

Adult Periplaneta americana were taken from stock colonies at 4 to 8 weeks of age and frozen at -70°C until required. Heads were collected in batches of 2400 (approx. 100 g) and homogenized by 3 x 30 sec passes (speed setting 7) at 60 sec intervals in 1200 ml ice-cold methanol-water-acetic acid (90:9:1 by vol) using a PT 10-35 Polytron homogenizer (Brinkmann) equipped with a PT 35 probe generator. Following centrifugation at 20,000 g for 30 min (4°C), the supernatant was separated and the pellets re-extracted twice in the same manner. The supernatants were combined and the solvents removed by rotary evaporation at 25-28°C. The residue was shaken on a wrist-action shaker for 2 h with 400 ml water-ethyl acetate (1:1 by vol). After removing most of the insoluble material by filtration through a pad of glass wool, the organic and aqueous phases were separated and the aqueous phase redried by rotary evaporation. The residue from the aqueous fraction was shaken with 48 ml 10 mM TFA and the resulting solution centrifuged at 27,000 g for 10 min (4°C) to remove a small quantity of insoluble material. In later experiments, the second rotary evaporation step was omitted and traces of ethyl acetate remaining in the aqueous phase were removed by a stream of nitrogen. [^{3}H-Tyr^{2}]Proctolin (up to 40 ng and 4.8 x

10^6 dpm) was added to the extract at this time so that recoveries from the solid phase extraction steps could be monitored.

Similarly, 300 bodies of deheaded adult P. americana (approx. 300 g) were homogenized with a Polytron PT45 homogenizer in 1500 ml ice-cold methanol-water-acetic acid (90:9:1 by vol). The homogenate was centrifuged, re-extracted once, and the combined supernatants taken to dryness by rotary evaporation at 25-28°C. The residue was shaken for 2 h with 400 ml water-ethyl acetate (1:1 by vol) and the aqueous phase separated and subjected to a stream of nitrogen to remove all traces of ethyl acetate. After addition of [^{3}H-Tyr^2]proctolin (approx. 10 ng, 10^6 dpm) this solution was applied directly to a Norganic resin cartridge.

Solid-Phase Extraction Studies

In preliminary studies, Norganic resin was removed from a cartridge and several mini-columns prepared by packing 1.0 or 1.5 g in Pasteur pipettes. These mini-columns and Sep-Pak C_{18} cartridges, which contain about 0.35 g of octadecylsilylsilica, were prewashed with 5 ml acetonitrile-10 mM TFA (1:1 by vol) followed by 20 ml 10 mM TFA before sample application. To determine appropriate solvents for the elution sequence, [^{3}H-Tyr^2]proctolin (5 µg; 1.24×10^5 dpm) in 0.6 ml water was loaded onto these packings and the eluate reapplied twice. Elution was performed by sequentially washing with 20 ml 10 mM TFA and 5 ml portions of 1:9, 3:7 and 1:1 (by vol) acetonitrile-10 mM TFA. Extraction efficiencies were monitored by liquid scintillation quantitation of radioactivity.

The extraction characteristics of the two packings were next compared against a more complex sample matrix, namely aliquots (1.0 ml; 50 head equivalents) from a cockroach head extract. Aliquots spiked with [^{3}H-Tyr^2]proctolin (0.83 ng; 10^5 dpm) were applied to the Norganic mini-columns or Sep-Pak C_{18} cartridges as above. After washing with 20 ml 10 mM TFA, retained substances were eluted with 6 ml acetonitrile-10 mM TFA (1:1 by vol) and dried by rotary evaporation. Material contained in these fractions was redissolved in 100 ul 10 mM TFA and separated by reversed-phase HPLC on the 30

cm x 7.8 mm i.d. Techsil 10 C_{18} column using a 25 min gradient (curve 6) of 15 to 50% (v/v) acetonitrile-water containing 5 mM HFBA at 3 ml/min. ^{3}H-Radioactivity was determined for aliquots of fractions obtained during initial separation steps and following HPLC chromatography.

For preparative scale extractions, the post-ethyl acetate aqueous extract of 2400 cockroach heads or 300 cockroach bodies, prepared and spiked with [^{3}H-Tyr^{2}]proctolin as above, was applied to an intact Norganic resin cartridge which had been prewashed with 300 ml acetonitrile-10mM TFA (1:1 by vol) and then with 300 ml 10 mM TFA. A Pharmacia P-3 peristaltic pump was used to apply samples and solvents in this and subsequent steps (flow rate 3 ml/min). After application, the Norganic cartridge was eluted with 10 mM TFA (300 ml) and then with acetonitrile-10 mM TFA (1:1 by vol; usually 800 ml). On some occasions the Norganic cartridge was subsequently eluted with acetonitrile-10 mM TFA (3:1 by vol). Materials eluted from the Norganic cartridge by the acetonitrile-10 mM TFA mixtures were dried, redissolved in 10 mM TFA, and applied sequentially to a series of Sep-Pak C_{18} cartridges. Following application, each Sep-Pak C_{18} cartridge was washed with 10 mM TFA (20 ml) and the eluate tested for ^{3}H-radioactivity. Typically, 4 Sep-Pak C_{18} cartridges were required to retain endogenous proctolin-like materials from 2400 heads and 6-8 for extracts of 300 bodies. Retained substances were obtained by eluting each cartridge with 6 ml acetonitrile-10 mM TFA (1:1 by vol). This Sep-Pak C_{18} retained material from 2400 heads could be further purified by HPLC (3 runs) on a 30 cm x 7.8 mm Techsil 10 C_{18} column using the solvent system described above. Alternatively, all of the corresponding extract from 2400 heads or 300 bodies was separated in a single run on a 15 cm x 19 mm i.d. μBondapak C_{18} column using a 50 min gradient (curve 6) of 0 to 50% (v/v) acetonitrile-water containing 5 mM HFBA at 9 ml/min. For reference purposes, proctolin and the cockroach myoactive peptides MI and MII were chromatographed under the same conditions.

RESULTS AND DISCUSSION

Although earlier studies indicated that a single Sep-Pak C_{18} cartridge could rapidly and efficiently extract proctolin and other myotropic peptides from homogenates of 100 cockroach heads (3), there was a need for a system which could be used for larger samples. Preliminary studies suggested that Millipore Norganic resin, used for scavenging organic substances from water intended for HPLC analyses, might meet this requirement. Small quantities (5 μg) of [^{3}H-Tyr^2]proctolin could be extracted from aqueous solution by either a Sep-Pak C_{18} cartridge or a Norganic resin mini-column. In both cases, good recoveries resulted from elution with mixtures of acetonitrile-10mM TFA with an indication that the Norganic resin was slightly more hydrophobic than Sep-Pak C_{18} (Table 1).

Similar recoveries of radioactivity were observed when aliquots of cockroach head extracts containing added [^{3}H-Tyr^2]proctolin were chromatographed on Sep-Pak C_{18} cartridges and Norganic resin mini-columns (Table 2). However, the acetonitrile-10mM TFA (1:1) eluate from the Norganic resin mini-column yielded approximately four times more residue than the corresponding fraction from Sep-Pak C_{18} cartridges. Chromatographs from HPLC separations of these fractions clearly reflect differences in the binding characteristics of the two packings (Figure 1) but no significant difference was observed in the amount of radioactivity recovered in the proctolin zone fractions (Table 2). With the exception of fraction 5, similar patterns and levels of myotropic activity were observed for fractions from the two preparations (Figure 2). The total quantity of myotropic substances, other than that of fraction 5, estimated as proctolin equivalents was 5.99 $\pm$ 1.79 ng/head (n = 4) using Norganic resin mini-columns, compared to 6.80 $\pm$ 1.65 ng/head (n = 4) using Sep-Pak C_{18} cartridges. It is not known whether the lower amount of inhibitor(s) observed from the Norganic resin preparation (Figure 2B; fractions 20-24) is due to poorer recovery from the column or to decreased resolution from other co-eluting myotropic components, perhaps attributable to the larger amount applied to

TABLE 1

Recovery of [^{3}H-Tyr^2]Proctolin[a] from a Sep-Pak C_{18} Cartridge and a Millipore Norganic Resin Mini-Column[b]

Fraction	^{3}H-Radioactivity (dpm)	
	Sep-Pak C_{18}	Norganic Resin
Non-retained	90	126
10mM TFA wash, 20 ml	0	0
CH_3CN-10mM TFA (1:9), 5 ml	32630	2250
CH_3CN-10mM TFA (3:7), 5 ml	91120	110990
CH_3CN-10mM TFA (1:1), 5 ml	4330	8770
	128170	122136

[a] 5 μg, 123900 dpm applied.
[b] 1.5 g resin packed in a Pasteur pipette.

TABLE 2

Separation of Cockroach Head Extracts on Sep-Pak C_{18} Cartridges and Norganic Resin Minicolumns[a] and Recovery of Added [^{3}H-Tyr^2] Proctolin[b]

Fraction	Sep-Pak C_{18}		Norganic Resin	
	Weight(mg)	^{3}H(dpm)	Weight(mg)	^{3}H(dpm)
1. Non-retained	49.7±5.4	320±700	29.9±5.8	720±860
2. 10mM TFA wash, 20 ml	18.4±2.7	0	14.8±2.0	840±1000
3. CH_3CN-10mM TFA (1:1), 6 ml	5.6±0.5	82800±5160	21.9±1.8	71820±3960
4. Proctolin zone[c] from HPLC separation		78710±1890		81960±8060

[a] 1.0g resin packed in a Pasteur pipette.
[b] Cockroach extract (50 head equivalents) containing 99800 dpm [^{3}H-Tyr^2]proctolin applied. Results ± SD (n = 4).
[c] See Figure 1 for HPLC conditions.

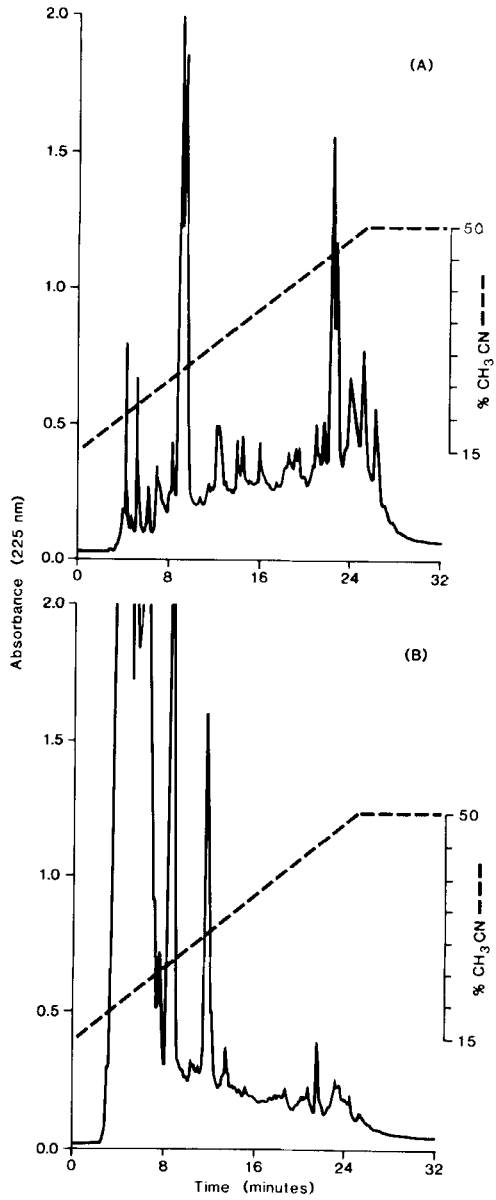

FIGURE 1. HPLC separation of substances extracted by a Sep-Pak C_{18} cartridge (A) and by a Norganic resin (1.0 g) mini-column (B) from identical aliquots (50 head equivalents) of a cockroach head homogenate. Column: Techsil 10 C_{18} (30 cm x 7.8 mm i.d.). Elution: 25 min linear gradient from 15-50% (v/v) acetonitrile-water containing 5 mM HFBA (3 ml/min).

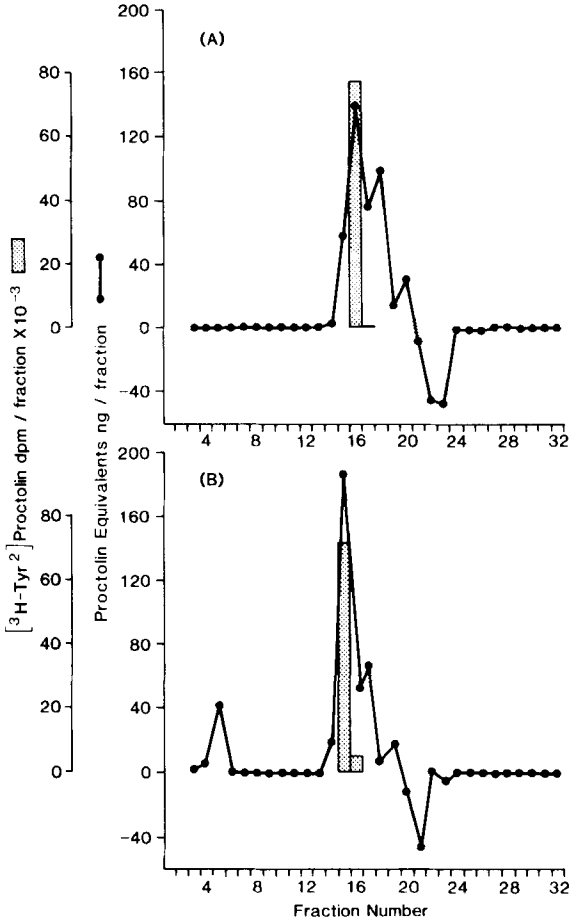

FIGURE 2. Pharmacological activity and ^{3}H-radioactivity of 1 min (3 ml) fractions from HPLC separations shown in Fig. 1. (A) Sep-Pak C_{18} cartridge. (B) Norganic resin mini-column.

the HPLC column. Preparations at this stage are far too impure to relate HPLC peaks to biological activity.

The factor(s) responsible for the myotropic activity exhibited by fraction 5 from the Norganic resin extraction (Figure 2B) has not been previously described. Briefly, this factor(s) induces contractions of the cockroach proctodeum that are far more rapid in onset and also sustained longer than contractions elicited by proctolin. The factor(s) could be isolated from the non-retained and 10 mM TFA wash fractions of Sep-Pak C_{18} cartridge extractions by rechromatography on a Norganic resin mini-column. Elution as before yielded biologically active material in the acetonitrile-10 mM TFA (1:1) eluate and HPLC separation and bioassay showed potent activity in the same zone.

On one occasion, increasing proportions of 10% (4 ml), 50% (10 ml) and 75% (10 ml) acetonitrile in 10 mM TFA were used to elute a Norganic resin mini-column to which 50 cockroach head equivalents of extract had been applied. The fraction eluted with 10% acetonitrile contained 65% by weight of the retained material but only 6% of the ^{3}H-radioactivity. Bioassay of fractions after HPLC separation of the retained material indicated that, apart from the myoactive substance(s) in fraction 5 which occurred mainly in the 10% eluate, most of the myotropic as well as inhibitory material was present in the 50% acetonitrile fraction. The 75% acetonitrile eluate contained no proctolin, as indicated by an absence of radioactivity, yet retained some myotropic material(s).

Similar success in extracting proctolin and other active substances was obtained when homogenates of 2400 cockroach heads were applied to an intact Norganic resin cartridge and eluted with acetonitrile-10 mM TFA (1:1). However, since such preparative runs extract about 850 mg, it was found beneficial to use Sep-Pak C_{18} cartridges to further purify the active substances before HPLC. This reduced the quantity to about 60 mg and, after HPLC, results similar to those shown in Figure 2 were obtained. Recovery of proctolin was 75-90% (n = 6) as judged by [^{3}H-Tyr^2]proctolin measurements. Attempts were not made to optimize recoveries of active

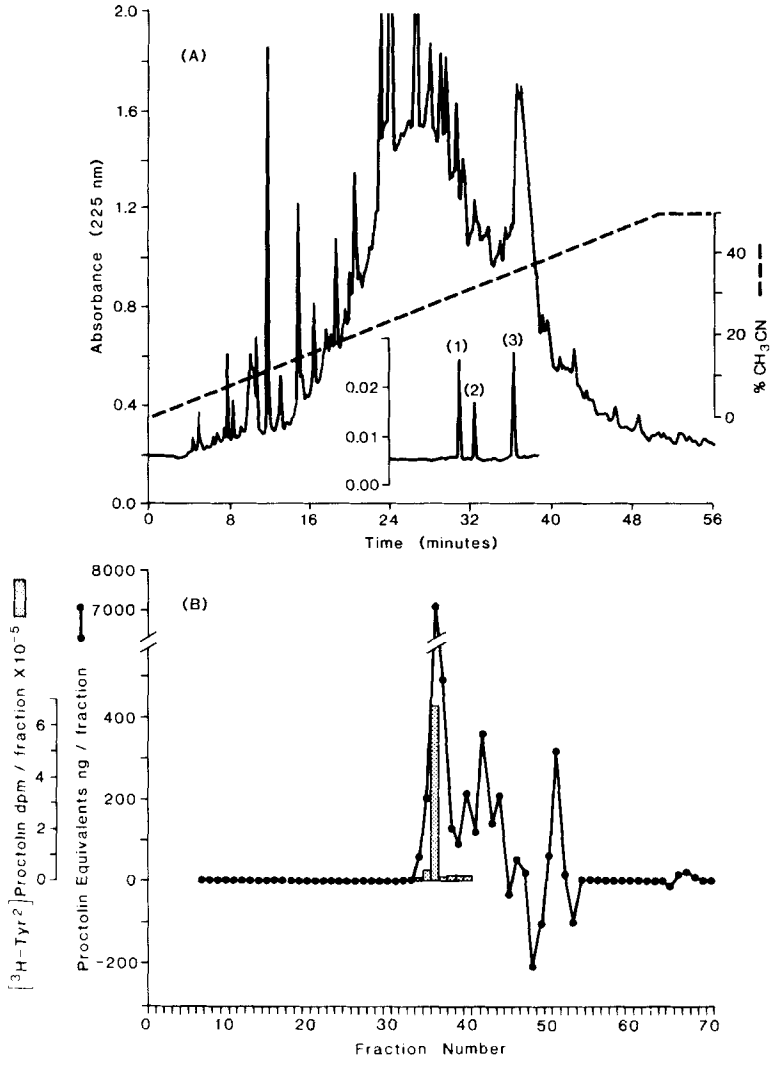

FIGURE 3. (A) HPLC separation of substances extracted from a homogenate of 300 cockroach bodies by a Norganic resin cartridge and further purified on Sep-Pak C_{18} cartridges (see text). Column: μBondapak C_{18} (15 cm x 19 mm i.d.). Elution: 50 min linear gradient from 0-50% (v/v) acetonitrile-water containing 5 mM HFBA (9 ml/min). Inset shows HPLC separation of proctolin (1) and cockroach myoactive peptides MI (2) and MII (3) using the same conditions. (B) Pharmacological activity and ^{3}H-radioactivity of 0.8 min (7.2 ml) fractions from separations shown in (A).

substances other than proctolin but, based on the small scale study described above, it seems possible that some were not quantitatively eluted from the Norganic resin using acetonitrile-10 mM TFA (1:1).

The utility of the method is even more apparent when one is extracting proctolin and other myotropic constituents from cockroach body extracts. These extracts are markedly more intransient to purification than corresponding head extracts. However, experiments with homogenates of cockroach bodies to which [^{3}H-Tyr^{2}]proctolin had been added indicated that a Norganic cartridge could extract all of the proctolin from homogenates of 300 bodies. Figure 3A shows the separation of the total Norganic resin - Sep-Pak C_{18} retained fraction (195 mg) from 300 bodies on a 15 cm x 19 mm i.d. C_{18} column. The elution profile of proctolin and the recently identified (7, 8) myoactive substances MI and MII is also shown (see inset). The distribution of myoactivity observed is presented in Figure 3B. As indicated by radioanalysis, proctolin in the crude preparation eluted faster than in the analytical sample.

These results show that Millipore Norganic resin is a low cost alternative to Sep-Pak C_{18} for the isolation and first stage purification of proctolin and other physiologically active constituents from cockroach tissues. Norganic cartridges have been reused several times with no noticeable loss of performance. Because of its greater retentiveness, the Norganic resin may find application for the isolation of substances lacking sufficient hydrophobicity to be extracted by Sep-Pak C_{18}. It is probable that it will also be generally useful for separating hydrophobic compounds from salts and polar substances.

ACKNOWLEDGMENTS

We wish to thank M.E. Stevens, M. Bosilac, S. Smith and S. Longman for technical assistance and G. Lambert for the illustrations.

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