Bioorganic Marine Chemistry 2

Paul J. Scheuer (Ed.)

R. J. Quinn
Chemistry of Aqueous Marine Extracts: Isolation Techniques

V. A. Stonik and G. B. Elyakov
Secondary Metabolites from Echinoderms as Chemotaxonomic Markers

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The Chemical Ecology of Alcyonarian Corals (Coelenterata: Octocorallia)

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Chemical Defense in Fishes

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With Contributions by
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Preface

Burgeoning research into marine natural products during the past two decades has in no small measure been due to an heightened and world-wide interest in the ocean, to the development of new sophisticated computer-driven instrumentation, and to major advances in separation science. Organic chemists have been fully aware that processes in living systems occur in an aqueous medium. Nevertheless, the chemists who have specialized in the study of small molecules have found it expedient to use organic rather than aqueous solvents for the isolation and manipulation of secondary metabolites. The emergence of new chromatographic techniques, the promise of rewarding results, not to mention the relevance of polar molecules to life itself, have contributed to a new awareness of the importance of organic chemistry in an aqueous medium.

The first chapter in Volume 2 of Bioorganic Marine Chemistry reflects the growing interest and concern with water-soluble compounds. Quinn, who pioneered the separation of such molecules, has contributed a review which closely links techniques with results and is based on practical experience. The second chapter, by Stonik and Elyakov, examines the vast chemical literature of the phylum Echinodermata – over one fourth of it in difficulty accessible Russian language publications. The Soviet authors evaluate the data for their suitability as chemotaxonomic markers.

Two ecological chapters round out Volume 2. Sammarco and Coll offer a comprehensive discussion of octocorals, their coral reef environment, including food, reproduction, predation, competition for space. The final chapter by Tachibana takes up chemical defense in fishes. Although the biology of fishes has a long and distinguished natural history, our knowledge of the molecular basis of some ecological phenomena involving fishes is extremely limited for the obvious reason that this research is difficult, involving as it does highly polar molecules and intricate bioassays.

I should like to thank all contributors for their timely cooperation and, as always, I would like to hear from colleagues who have suggestions or comments for this or future topics in "Bioorganic Marine Chemistry".

February 1988 Paul J. Scheuer

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Chemistry of Aqueous Marine Extracts: Isolation Techniques

Ronald J. Quinn[1]

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Abstract

Various techniques for isolating compounds from aqueous extracts of marine organisms are reviewed. The major isolation techniques involve separation by differences in molecular size, charge or adsorption properties. Successful strategies employed to isolate bioactive constituents from extracts which displayed activity in a variety of biological screens are discussed. The recent marine natural product literature is reviewed and the isolation procedures for compounds isolated from aqueous extracts are summarized.

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Bioorganic Marine Chemistry, Volume 2

1 Introduction

Compounds obtained from aqueous extracts of marine organisms have contributed very little to the increasing number of new organic molecules isolated from marine organisms. We have over a number of years achieved the isolation of the bioactive constituents from aqueous extracts which displayed activity in a variety of biological screens [1]. In many other investigations, where an initial aqueous extraction was performed, subsequent investigations were limited to those compounds which partitioned into an organic phase. The isolation of polar, low molecular weight natural products presents a serious challenge to the organic chemist, particularly from the marine environment with the presence of sodium chloride and other salts in all aqueous extracts. Notwithstanding the difficulties, polar water-soluble constituents can be isolated and purified by a number of different techniques which in combination provide a powerful separating capacity. The recent technique of introducing functional groups onto a number of chromatographic phases has added a new level of versatility and appears to allow the natural product chemist to make many more advances in the chemistry of polar natural products.

It is possible to use several properties of molecules to effect separation; the most useful properties are molecular size, net or potential charge and adsorption capacity. Macromolecules are easily separated from the salts and other low molecular weight contaminants, and further purification of these large molecules can be pursued by standard biochemical methods. The low molecular weight compounds, which include a large number of potentially biologically active constituents, are more interesting to the organic chemist, but have been largely avoided because of the difficulties of separation.

The various molecular properties that can be used to achieve separation will be discussed. Several examples will be given, including a series of marine extracts which displayed biological activity and which were investigated by a variety of techniques in order to determine the most appropriate isolation strategy.

Following discussion of the molecular properties, isolation of the biologically active constituents of the sponge *Tedania digitata* [2,3], the red alga *Gracilaria edulis (lichenoides)* [4], the holothurian *Pentacter crassa* [5], the soft coral *Nephthea* sp. [6], the sponges *Haliclona* and *Chalinopsilla* spp. [7], and the octopus *Hapalochlaena maculosa* [8], will be used to illustrate successful strategies for the isolation of bioactive constituents from aqueous marine extracts. This will be followed by an evaluation of the marine natural products literature to highlight constituents isolated from aqueous marine extracts and a discussion of the role of reversed phase adsorbents.

There is little doubt that any biological screening program which screens both organic and aqueous extracts will discover many active aqueous extracts. The broadly based pharmacological screening program carried out on crude extracts at the former Roche Research Institute of Marine Pharmacology in Australia revealed that approximately half of the observed pharmacological activities occurred in the organic extracts and half in the aqueous extracts. While the full details of the pharmacological screening have not been published, a review of the pharmacological and microbiological screening of 159 algae [9] and the results of

the antimicrobial screening of algae have been published [10]. This chapter attempts to provide information that will facilitate the chemical evaluation of aqueous marine extracts.

The isolation of polar low molecular weight constituents should be pursued by mild methods which will not degrade the constituents. Aqueous extracts are a nutrient-rich medium in which bacterial and fungal growth may occur and suitable precautions must be taken.

2 Useful Properties Allowing Separations

2.1 Size

Diafiltration can be used to separate compounds by their effective size. Ultrafilters consist of thin semipermeable membranes bound to a porous sustrate. They are manufactured with a variety of pore sizes and are characterized by nominal molecular weight limits above which most species are retained. It is possible to use ultrafilters in series in order to obtain a fractionation based on the size of the molecules.

Diafiltration in which a constant volume is maintained in the sample solution by continuous addition of wash solution results in the retention of large species in their initial concentration, while the permeable solutes are diluted in the ultrafiltrate. Table 1 gives the results for diafiltration of crude extracts using an ultrafilter of 10 000 dalton cut-off in series with an ultrafilter which is rated at 500 dalton.

Diafiltration offers a coarse fractionation by molecular size, whereas gel permeation chromatography offers the ability to obtain greater resolution. Molecules are retarded as they pass through the porous gel medium depending on the time spent within the pores and are eluted in order of decreasing size, molecules larger than the pore size are excluded and elute first. It is possible to use gel permeation columns connected in series in a similar fashion to ultrafilters in series. If a gel with a lower exclusion volume is connected in tandem to the eluant of an initial gel column, material excluded by the second gel will retain its relative position as eluted from the first gel, while material that enters the pores of the second gel will be further separated. Figure 1 shows the separation achieved on a Pharmacia Sephadex G-25 column connected in series with a Pharmacia Sephadex G-10 column. A crude aqueous ethanolic extract of a sponge of the family Verongidae displayed antimicrobial activity against both *Staphylococcus aureus* and *Escherichia coli* [10]. The active constituent was shown to be the known compound 2,6-dibromo-4-acetamido-4-hydroxycyclohexa-2,5-dienone (3,5-dibromoverangiaquinol) (**1**) [11], which constituted 1.3% of the crude extract or 0.2% of the dry sponge.

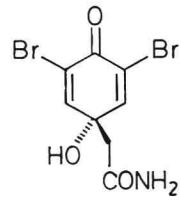

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Table 1. Diafiltration of a series of crude marine extracts: Distribution by size

Species	Extract	Biological activity	Amicon UM10 retentate[a] wt%	Amicon UM05 retentate[b] wt%	Dia-filtrate[c] wt%
Sponge *Ircinia* sp	Water	Atrial stimulant anti-inflam-matory	14.5	11.0	73.5
	Methanol	Atrial stimulant	4.3	8.1	75.9
Soft coral *Sinularia flexibilis*	Methanol	Hypotensive	14.6	13.5	53.2
Gorgonian *Rumphella* sp	Water	Anti-yeast[i]	12.7	2.1	52.3
Sponge	Water	Hypotensive	9.8	12.8	59.5
Sponge	Water	Atrial stimulant	4.4	7.8	70.5
Chondrilla sp	Methanol	Hypotensive	16.7	9.5	70.2
Brown alga *Acrocarpia paniculata*	Water	Anti-bacterial[d]	10.3	3.8	63.5
Red alga *Laurencia elata*	Water	Anti-bacterial[g]	13.4	9.7	67.1
Gorgonian *Briacium* sp	Water	Hypotensive	5.0	10.2	90.1
Soft coral *Sinularia* sp	Water	Hypotensive atrial stimulant	2.3	9.8	84.2
Sponge *Pericharax* sp	Water	Anti-yeast[h]	8.9	19.4	70.1
Soft coral *Sinularia* sp	Water		11.2	8.6	81.0
Sponge	Water	Anti-amphetamine barbiturate potentiation	22.6	6.2	
Brown alga *Scytothaminas australis*	Water	Hypotensive	16.9	6.9	65.9
Sponge *Dysidea fragilis*	Water	Atrial stimulant	1.0	20.4	78.1
Sponge *Pachychalina* sp	Water	Hypertensive anti-fungal[j]	1.2	25.5	60.0
Soft coral *Sinularia* sp	Water	Hypotensive anti-bacterial[e]	5.2	25.4	48.3
Sponge *Ancorina* sp	Water	Anti-bacterial[f, g]	4.7	23.2	49.6
Sea pen *Scytalium sersii*	Water	Hypotensive	10.0	7.9	80.5
Brown alga *Hormosira banksii*	Water	Anti-fungal[k]	25.7	6.7	57.6
Holothurian *Thelenota ananas*	Water	Bronchodilator anti-fungal[j, k]	5.1	11.3	56.6
Soft coral *Sarcophyton* sp	Water	Nicotine blocker	2.6	12.5	80.7

Table 1 (continued)

Species	Extract	Biological activity	Amicon UM10 retentate[a] wt%	Amicon UM05 retentate[b] wt%	Dia-filtrate[c] wt%
Soft coral *Sinularia* sp	Water	Bronchodilator	21.2	36.5	33.5
Sponge *Ianthella filiformis*	Water	EMG	9.8	4.0	51.5
Brown alga	Water	Anti-convulsant	29.4	10.4	63.3
Xiphophora chondrophylla	Methanol	Anti-convulsant	18.7	10.8	70.3

[a] Approximate M.W. > 10,000 dalton.
[b] Approximate M.W. 10,000–500 dalton. UM05 retentate refers to material which passed through the UM10 ultrafilter and was retained by the UM05 ultrafilter.
[c] Approximate M.W. < 500 dalton.
[d] Gram-positive bacteria *Sa Staphylococcus aureus.*
[e] Gram-positive bacteria *Spy Streptococcus pyogenes.*
[f] Gram-negative bacteria *Ec Esherichia coli.*
[g] Gram-negative bacteria *Pm Proteus mirabilis.*
[h] Yeast *Ca Candida albicans.*
[i] Yeast *Cn Cryptococcus neoformans.*
[j] Fungi *Tm Trichophyton mentagrophytes.*
[k] Fungi *Ma Microsporum audouini.*

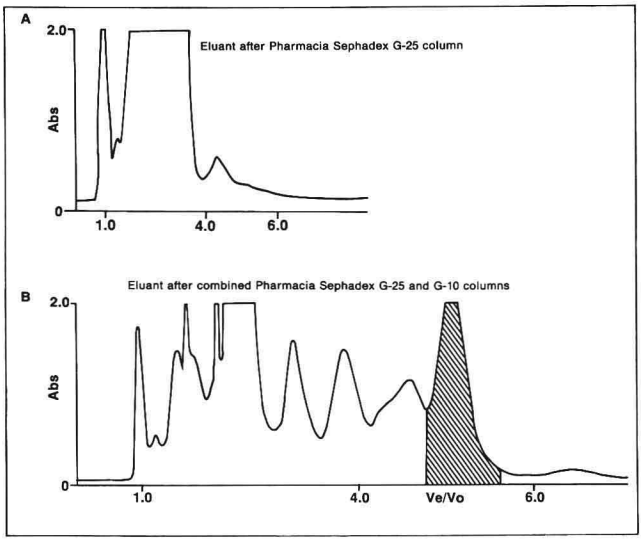


Fig. 1. Chromatography of crude extract (1.2 g) of a sponge of the family *Verongidae* on Pharmacia Sephadex G-25 fine (38.8 × 2.5 cm) in series with Pharmacia Sephadex G-10 (41.4 × 2.5 cm). Eluant: water, UV monitor: 254 nm, 2.0 A full scale

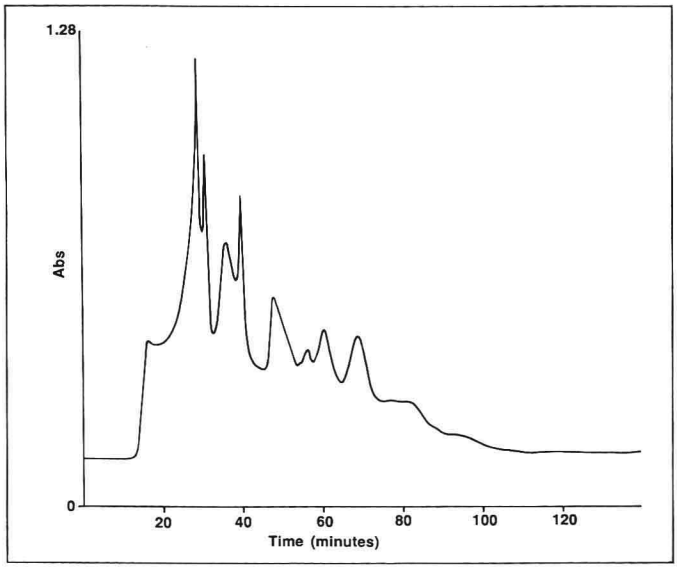


Fig. 2. Chromatography of crude extract (1.0 g) of *Stelleta conulosa* on Merck TSK-HW-40(S). (27 × 3.8 cm). Eluant: water, UV monitor: 254 nm, 1.28 A full scale, flow rate: 5 ml/min, pressure: 100 psi

Gel permeation chromatography with soft compressible gels is a slow process. The example in Fig. 1, where 1.2 g of crude extract yielded 15.7 mg of the biologically active fraction took 20.5 hours. The introduction of more rigid supports for gel permeation chromatography has allowed this process to be speeded up considerably. Figure 2 shows a separation on Merck Fractogel TSK HW-40 (S).

Diafiltration offers a ready answer to the question of molecular size. Stirred cells connected in series can be an effective choice for small scale separations. A pressurized reservoir placed between the nitrogen supply and the stirred cells will automatically maintain the fluid level in the cells and provide diafiltration under conditions of constant volume. The resolving power of diafiltration is not high, as each filter provides only retentate (containing compounds excluded by the membrane) and diafiltrate (containing compounds which pass through the membrane); in addition, there is no distinct cut-off point. This means that the compound of interest, if it is of a molecular size close to the nominal cut-off value, may be distributed over both fractions. Small organic molecules are separated with the fraction that contains the salts; hence the degree of purification is small: however, the technique has decided advantages for purification of excluded larger molecules. Diafiltration is a particularly mild method, as it can be run in a cold room at 4° C. Furthermore, the process can be run unattended if a pressurized reservoir is used; as water is the solvent, the fractions can be directly freeze-dried. Resolution of a mixture is much greater with gel permeation chromatography. Sodium chloride travels as a discrete band and can be separated from many small organic molecules. The ability to use rigid supports in conjunction with HPLC

equipment found in most organic chemistry laboratories may mean that this will be the favored approach for most workers.

2.2 Charge

Ion exchange chromatography can offer a highly selective process. Many polar organic constituents will have ionizable functional groups. Such compounds can be adsorbed to ion-exchange resins bearing a suitable functional group of opposite charge.

Table 2 provides data for the behavior of a series of extracts of marine organisms with strong cation and anion exchangers. The goal of this experiment was to determine if a biologically active constituent could be adsorbed on an ion-exchange resin, for which strong exchangers offer the best choice. For compounds to be adsorbed they must be charged at the pH at which the chromatography is conducted. They can then be eluted by changing the pH to make the adsorbed molecules neutral, or to make the ion exchange resin neutral. Elution of bound constituents from strong ion exchange resins will often require extreme conditions, under which decomposition may occur. In that case a purification scheme must be developed in which a suitable weak ion exchange resin is employed together with a volatile buffer so that the eluants can be directly freeze-dried. Pharmacia SP-Sephadex and BioRad AG 50 are both sulfonic acid cation exchange resins and were used as free sulfonic acids. In one case, extracts were dissolved in 3% acetic acid in order to protonate any basic groups, while in the other the extract was dissolved in water. Pharmacia QAE-Sephadex and Bio-Rad AG 1 are quaternary ammonium anion exchange resins. In one case, extracts were dissolved in 1% ammonia in order to ionize any acids, while in the other the extract was dissolved in water. The recovered weights are often greater than the amount added, as the counter ions are eluted and compounds may be eluted as salts. Regarding the behavior of a biologically active constituent, it must be borne in mind that initial adsorption to the resin that results in loss of biological activity indicates that the biologically active constituent has a potentially ionizable functional group and that isolation by ion exchange chromatography should be explored further. While in many cases biological activity may not be observed in the fractions eluted under the rather harsh conditions of this initial screening, it is possible to modify the conditions of the ion exchange chromatography to allow its use in a separation scheme. These further investigations will take into account the stability of the constituents, the counter-ion selectivity and elution of the constituent of interest by neutralizing the charge on the constituent, by neutralizing the charge on the resin, by increasing the ionic strength of the buffer, or by using a buffer containing highly selective ions.

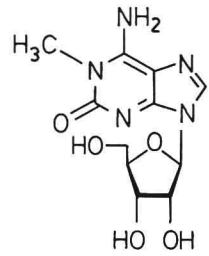
The selectivity of ion-exchange chromatography is well illustrated by the isolation of the pharmacologically active nucleoside 1-methylisoguanosine (**2**) from the sponge *Tedania digitata* [3]. By buffering the extract at pH 3.5, the active constituent could be absorbed on a sulfonic acid cation exchange resin. Elution with a buffer at pH 5.3 resulted in elution of the bioactive material. Rechromatography of this active fraction under the same conditions, followed by recrystalli-

Table 2. Ion exchange of a series of crude extracts

Species	Extract	MB-3	SP-Sephadex		AG-50W-X8 (H form)			QAE-Sephadex		AGI-X8 (acetate form)	
			3% HOAC	1% NH_3	H_2O	2N HCl	5N HCl	1% NH3	3% HOAC	H_2O	2N HCl
Sponge	Water	14.8	71.3	13.7	20.3	74.0	–	84.2	21.1	91.2	11.5
Ircinia sp	Methanol	6.1.	73.1	31.2	9.5	86.4	–	112.1	36.4	90.3	7.8
Soft coral *Sinularia flexibilis*	Methanol	16.9	58	27.2	14.8	64.5	8.5	50.0	44.6	64.6	3.4
Gorgonian *Rumphella*	Water	5.4	75.5	17.9	27.4	74.7	–	75.1	26.2	98.4	15.2
Sponge	Water	1.2	54.5	7.1	16.8	148.9	0.8	84	40	104	1.1
Sponge	Water	11.9	92.3	19.2	14.9	71.7	4.0	111.0	59.0	111.9	17.2
Chondrilla sp	Methanol	5.7	86.6	27.2	21.6	60.8	4.1	78.0	45.9	82.5	4.2
Brown alga *Acrocarpia paniculata*	Water	18.4	66.0	8.4	35.1	62.1	–	91.5	4.8	101.5	4.7
Red alga *Laurencia elata*	Water	22.6	97.0	0.6	41.0	66.7	–	134.5	11.8	111.8	11.0
Gorgonian *Briacium* sp	Water	3.8	86.0	10.2	19.7	81.3	6.1	117.0	23.3	87.1	13.5
Soft coral *Sinularia* sp	Water	3.9	61.3	13.4	13.6	83.5	1.6	85.2	13.5	107.2	11.9

Sponge *Pericharax* sp	Water	13.3	71.8	2.6	25.4	74.0	4.4	90.7	18.1	97.2	11.7
Soft coral *Sinularia* sp	Water	11.5	72.8	5.9	18.6	90.5	–	107.1	44.8	105.4	10.0
Sponge *Dysidea fragilis*	Water	14.5	93.1	52.3				84.4	9.7		
Sponge *Pachychalina* sp	Water	1.2	81.2	2.0	20.0	63.6	10.3	112.4	28.0	64.0	19.2
Soft coral *Sinularia* sp	Water	21.8	56.8	12.6	37.1	103.9	10.1	77.2	20.4	76.9	6.0
Sponge *Ancorina* sp	Water	5.5	56.5	19.7	21.5	61.7	10.6	80.4	16.3	79	9.6
Sea pen *Scytalium sersii*	Water	3.0	59.7	19.1	55.7	141.3	1.9	123	15.0	88.2	8.9
Brown alga *Hormosira banksii*	Water	21.8	51.5	28.9	25.1	62.0	1.0	72.3	20.9	91.1	8.5
Holothurian *Thelenata ananas*	Water	4.0	82.9	5.5	14.7	101.6	0.4	93.8	14.8	89.8	5.0
Soft coral *Sarcophyton* sp	Water	9.6	70.5	8.6	86.8	74.5	1.0	77.5	20.8	86.8	7.9
Soft coral *Sinularia* sp	Water	17.2	41.2	50.5	40.1	41.8	1.0	54.0	37.4	81.2	4.1

zation from water yielded 1-methylisoguanosine. This procedure was highly selective since pH was controlled for adsorption and elution. By this procedure 385 g of crude extract yielded 2.75 g (0.71%) of 1-methylisoguanosine.



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The crude extract was processed in two batches on a 40 × 5 cm column and the combined active fraction was rechromatographed on a column of the same size. Ion-exchange adsorbents may have a high capacity dependent on the number of functional groups present on the polymeric matrix. The resin used for this work (Bio Rad AG50W-X8, ammonium form, 200–400 mesh) has a capacity of 1.7 meq/mL. In this case, purification of an extract from 2.41 kg of freeze-dried sponge was achieved by three chromatographies on a regular size laboratory column. This may be compared with the use of gel permeation chromatography, where a column of about the same dimensions (52 × 5 cm) could only separate 4 g of a diafiltrate (500 dalton nominal exclusion limit) from 8 g of the crude extract; if one extrapolates, one finds that it would have taken 48 consecutive chromatographies to achieve the same result.

Another operational point must be noted for the isolation of low molecular weight components. A volatile buffer must be used so that the buffer salts can be removed by lyophilization. Ammonium formate at pH 3.5 and 5.3, which was 0.1 M with respect to formate, was used for the above described separation.

2.3 Adsorption

Adsorption chromatography is the most widely used method for the isolation and purification of small organic molecules. Normal adsorption chromatography has played a key role in the isolation of marine natural products. The strong polarity of water and other aqueous solvent mixtures precludes the use of normal adsorption chromatography for the examination of polar natural products occurring in aqueous extracts of marine organisms. In reversed phase chromatography, where the adsorbent is less polar than the mobile phase, it is possible to adsorb compounds from aqueous solutions and to elute the bound compounds by decreasing the polarity of the eluting solvent. Reversed phase chromatography has found extensive use in analytical applications and has increasingly been used for preparative work. The reversed phase adsorbent must possess low polarity. Two general methods may be used to obtain such adsorbents. Polymers of suitable polarity can

be used directly, or other adsorbents can be modified so that their polar groups are substituted by non-polar groups.

Amberlite XAD resins are examples of polymeric materials which can be used for reversed phase chromatography. These resins have large specific surface areas and are used as adsorbents for organic materials. Amberlite XAD-2 is a porous polystyrene-divinylbenzene copolymer, while Amberlite XAD-7 is a cross-linked acrylate polyester. The structural differences parallel differences in adsorption properties. Amberlite XAD-2 thus is a nonpolar adsorbent and is used to adsorb nonpolar substances from aqueous solutions, while Amberlite XAD-7 is more polar and is used to adsorb polar compounds from nonpolar solvents. It also finds useful application for aqueous marine extracts, where nonpolar compounds may be adsorbed from polar solvents. Information on the use of Amberlite XAD-2 as a chromatographic phase is available [12–16]; we have reported applications with Amberlite XAD-7 [17]. The following eluotropic series have been established for Amberlite XAD-2 [18] and Amberlite XAD-7 [17], and are detailed in Table 3.

In practice, it is not necessary to proceed past 100% acetone as the eluant, since almost everything that is going to be desorbed by an organic solvent will have been desorbed. Amberlite XAD-2 has a greater affinity for aromatic compounds (benzene, naphthalene, anthracene) than Amberlite XAD-7 [17]. In methanol, the capacity factor [$k' = (Ve - Vo)/Vo$ where Ve is the elution volume and Vo is the void volume] for naphthalene is 3.42 on Amberlite XAD-2 and 0.41 on Amberlite XAD-7 [17]. However, Amberlite XAD-7 has a greater affinity for more polar compounds. For *N,N'*-dimethylthiourea in water, Amberlite XAD-7 had a capacity factor of 2.97, while Amberlite XAD-2 had a capacity factor of 1.72 [17]. Of possibly greater relevance for the isolation of bioactive constituents is the observation that catecholamines are eluted at the void volume with methanol on Amberlite XAD-2, but are measurably retarded by Amberlite XAD-7 [17]. Thus catecholamines can be adsorbed from aqueous solutions by Amberlite XAD-7 and eluted by water/methanol mixtures or methanol depending on the specific compound.

Application to four crude aqueous extracts of marine organisms is shown in Table 4. The samples dissolved in water were applied to columns packed with the

Table 3. Eluotropic series for Amberlite XAD-2 and XAD-7

XAD-2	XAD-7	
	In methanol	In water
Water	Ethyl acetate	Dimethyl sulfoxide
Methanol	Diethyl ether	Methanol
Dimethyl sulfoxide	Acetone	Acetone
Acetone	Dichloromethane	Tetrahydrofuran
Diethyl ether	Tetrahydrofuran	Diethyl ether
Dichloromethane	Benzene	
Ethyl acetate		
Tetrahydrofuran		
Benzene		

Table 4. Behavior of crude aqueous extracts on Amberlite XAD-2 and XAD-7

Organism	Sample in water	Resin	Resin volume	Weight adsorbed and eluted		Approx. % of organics adsorbed and eluted	Approx. % of organics irreversibly bound
				Total (g)	mg/100 ml resin		
Brown alga	5.9 g in 100 ml	XAD-2	400 ml	1.08	270	25	31
Cystophora retorta	5.9 g in 100 ml	XAD-7	400 ml	1.56	390	36	24
Brown alga	5.4 g in 100 ml	XAD-2	400 ml	0.60	150	18	1
Cystophora congesta	5.4 g in 100 ml	XAD-7	400 ml	0.88	220	26	9
Sea anemone	1.7 g in 220 ml	XAD-2	90 ml	0.09	100	7	15
Bunodactis chrysobathis	1.6 g in 210 ml	XAD-7	90 ml	0.099	110	8	20
Sponge	1.3 g in 70 ml	XAD-2	90 ml	0.072	80	10	8
	1.2 g in 70 ml	XAD-7	90 ml	0.09	100	14	15