Chapter 8

Isolation of Natural Products by Ion-Exchange Methods

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Abstract

The primary goal of many natural products chemists is to extract, isolate, and characterize specific analytes from complex plant, animal, microbial, and food matrices. To achieve this goal, they rely considerably on highly sophisticated and highly hyphenated modern instrumentation. Yet, the vast majority of modern instrumentation typically found in the laboratories of natural products chemists is founded on the simple principles of intermolecular forces to achieve separation. Ion-exchange chromatography (IEC) is, at heart, the most fundamental, and strongest, of these interactions and is considered a relatively inexpensive and effective medium in which to "clean-up" a sample. Additionally, IEC offers high recoveries of key analytes and offers the ability to modify the stationary and mobile phases in order to selectively "catch and release" compounds of interest.

Key words: Ion-exchange chromatography, Strong anion exchange, Weak anion exchange, Weak cation exchange, Strong cation exchange, pH gradient elution, Solid phase extraction, Natural products isolation, Organic acid, Alkaloid, Phenol, Glycoside

1. Introduction

Owing to the fundamental principles involved in IEC, this amended chapter draws upon information from previous editions (1, 2) in regard to theory, materials, and medium preparation. Nevertheless, recent instances of the use of IEC as it is applied to natural products as well as current IEC resins (see Note 1) being used are offered in this revised chapter. One of the first recorded examples of IEC being applied to the "recovery of natural products" was a report in 1945 that described the use of a cation exchange column to separate thiamine from riboflavin (3). For more history regarding IEC and nonnatural products applications, there are several detailed reviews, including the early beginnings of phenol–formaldehyde polymers, ca. 1909 (4, 5). IEC is also used extensively for separations

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of biological macromolecules, such as proteins, nucleic acids, and polysaccharides; these applications are covered at length in other book chapters and reviews.

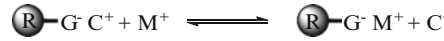
As the chapters of this book exemplify, there are a multitude of methods for the extraction, separation, isolation, and identification of natural products from various origins. Similar to the "teamwork" approach, scientists (coaches) must be versant in several methods and techniques in addition to being able to coordinate the strengths of each. Successful teamwork of these individual methods is critical in order to achieve the overall goal of isolating components, which are frequently present only in trace amounts and from intricate and often enigmatic sources. Fortunately, IEC offers a reliable and relatively inexpensive alternative for the removal, retention, and/or isolation of ionizable components.

In addition to classic uses of IEC as the crude extract "clean-up" medium, there have been innovative approaches and uses for IEC, such as recent reports of "explorative solid-phase extraction" for microbial natural products (6), and rapid separation of neutral, acidic, and basic components via "phase-trafficking" (7). IEC chapters from previous editions of "Natural Products Isolation" have provided elegant examples of the isolation of natural products with marine and microbial origins. Building upon these examples, this chapter edition seeks to provide further examples of IEC use for the extraction or isolation of food or agriculturally related natural products. Additionally, because of the heightened need to reliably extract toxic components from food stuff examples of some common metabolites are included. Other components of interest with microbial or plant origins are also highlighted.

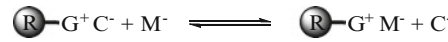
1.1. Theory of Ion-Exchange

As the name succinctly describes, the theory of ion-exchange is simply that—the exchange of ions. In the case of IEC, the equilibrium is between the counterion (C) of a charged functional group (G) attached to an insoluble resin matrix (R), and that of a charged molecule (M) with the same charge as that of the counterion. For natural products, the charged organic molecules are classically represented by protonated bases, such as alkaloids, or deprotonated acids such as fatty acids or amino acid derivatives. Because natural product chemists (or biologists, entomologists, or nutritionists exploring/utilizing separations for that matter) are primarily dealing with organic acids and bases, one can perceive the vital roles pH and pK_a play in the equilibriums and the ability of the resin to "capture" or hold the analyte of interest. Additionally, the ability to alter the charge and the associated charge strengths on the resin allows researchers to selectively remove a charged molecule from complex plant, animal, or food matrices. The equations below illustrate nicely the theory of IEC, the associated equilibriums, and the two principal types of IEC.

Cation exchange (anionic resin for retention of cation analytes)



Anion exchange (cationic resin for retention of anion analytes)



Taking into account that these exchanges are typically in equilibrium, careful management of the mobile and stationary phase conditions allow for predictive adsorption and elution cycles (goodnaturedly coined "catch and release") as well as the ability to regenerate the adsorbent resin.

The large majority of natural products undergoing separation via IEC possess a functional group capable of ionization, e.g., alkaloid, carboxylic acid, or phenolic proton. One critical requirement is that the polarity of the molecule can be altered by simple manipulation of the pH; essentially, the same principles needed for liquid/liquid partitioning, but without the concern of emulsions. Because IEC is at its core simply acid/base chemistry the premises of pH and pK_a play important roles and thus a more mathematical perspective to the ionization process can be applied. The Henderson–Hasselbach equation indicates that most carboxylic acid derivatives will be fully ionized to form the conjugate bases at two pH units above the pK_a value of the protonated form. The same can be said of alkaloids that when protonated provide ammonium derivatives up to two pH units below the pK_a value of their conjugate acid. This useful information provides the guideline that most carboxylic acid derivatives will be ionized at pH values above 6.0, thus be retained on an anion exchange resin, and that amines will exist at their ammonium analogues at pH values below 8.0 and thus be retained on a cation exchange resin. It should be noted that these are not strict rules, but rather guidelines to set initial conditions, and are dependent upon other factors that are discussed presently in-depth.

Now that we have our natural product as an ionized molecule, it must displace the counterion that was initially associated with the resin, and the natural product be retained while the other matrix components or contaminants are eluted from the column. Expressing this equilibrium mathematically, the interactions of the ions may be described in terms of a partition constant (Kp), which is the ratio of the dissociation constants for the counterion (Kc) and the charged molecule (Km) interacting (binding) to the resin-bound charged functional group. This relationship is expressed in the following equations and is regardless of charge.

$$\mathrm{Kp} = \frac{\mathrm{Kc}}{\mathrm{Km}} = \frac{[\mathrm{RGM}][\mathrm{C}]}{[\mathrm{RGC}][\mathrm{M}]}$$

where $\mathrm{Kc} = \dfrac{[\mathrm{RG}][\mathrm{C}]}{[\mathrm{RGC}]}$ and $\mathrm{Km} = \dfrac{[\mathrm{RG}][\mathrm{M}]}{[\mathrm{RGM}]}$.

How effectively the ions undergo exchange between the solid phase, i.e., the counterion of the resin (C), and the mobile phase, i.e., charged molecule (M), is also dependent upon the affinity of the charged resin functional group (RG) toward the ions (expressed as Kc and Km in the equation directly above) as well as the relative concentrations of the counterion (C) and the charged molecule (M). In general, ions that are more polarizable will have a higher affinity to the exchange resin. Additionally, polyvalent ions will typically have a higher affinity for a charged resin functional group than their monovalent counterparts due to the polyvalent ion's ability to bind more than one charged functional group on the resin.

1.2. Role of Counterions

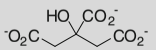
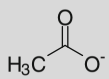
As with most ionic systems the overall charge neutrality of the column and components must be kept in equilibrium. Recalling lessons from general chemistry, the equations described earlier which illustrated the reversibility of the counterion (C) and the charged molecule (M) can be influenced in either direction by simply increasing the concentration of the counterion (Le Châtelier's Principle) or substitution with a counterion of greater affinity (increased value of Kc) to the functional group on the resin. Table 1 highlights the affinity of ions for typical functional groups on most ion-exchange resins, though the functional group make-up of the column will have specific affinities that should be established or verified prior to use. For example, if an anion exchange column was being utilized and a carboxylic acid derivative, represented by the acetate ion, was retained on the resin, the use of chloride ions, which have a higher affinity for the resin, would displace the carboxylic acid derivative and would thus be eluted from the column.

The same would be applicable for a cation exchange column, where the ammonium ion is representative of an alkaloid under acidic conditions. In order to elute the protonated alkaloid, calcium would have to be used since it has a stronger affinity for the resin; whereas sodium would not displace the protonated alkaloid. As will be illustrated presently, instead of using calcium to displace the protonated alkaloid, the pH of the column can simply be altered to deprotonate the alkaloid and effectively elute the analyte from the column.

1.3. Other Interactions

As alluded to earlier there are other discreet but important interactions taking place beyond the obvious ionic interactions; namely, other intermolecular forces, albeit not as strong. Recalling our general chemistry teachings, these other interactions, listed in decreasing strength, are ion-dipole, hydrogen bonding, dipole–dipole, and London dispersion forces. Because there are typically hydrocarbon backbones supporting the charged functional group there are opportunities for these discreet intermolecular interactions between

Table 1
Affinities of some common ions to anion and cation exchange columns

	Affinity to column						
	High						**Low**
Anion Exchange Column			Cl^-				HO^-
	Phosphate	Citrate >	Chloride >	Bicarbonate	Formate >	Acetate >	Hydroxide
Cation Exchange Column	Ca^{++} Calcium >	NH_4^+ Ammonium >		Na^+ Sodium >		H^+ Proton >	Li^+ Lithium

the uncharged portions of the charged organic molecule (analyte) and the functional group hydrocarbon framework. These secondary interactions can help contribute to the overall "catch and release" of the organic molecule and can be influenced with careful manipulation of the organic solvent systems utilized during the separation process. In addition to the above-noted interactions, there is one more type of hydrophobic interaction that can influence the catch and release mechanism—that of pi-stacking between aromatic moieties of the resin functional group backbone (i.e., phenyl group) and the analyte of interest.

1.4. Capacity and Selectivity Rate

As briefly mentioned earlier, there are other important features of IEC—capacity and selectivity. The capacity of a resin can be defined in several ways depending on a user's preferences. As natural products chemists, we (the authors) found the most applicable and easiest to understand definition to be the apparent capacity (effective capacity), which is "the number of exchangeable counterions per specified amount resin" and depends on the experimental conditions, such as pH and concentration of analyte medium (8). The apparent capacity is usually lower than the theoretical capacity. More practically, this definition can be simplified into the number of resin-bound charged functional groups per unit mass of resin. Since the resin may contain multiple binding sites (polyatomic ions) the capacity is typically expressed in milliequivalents of ions adsorbed per gram of resin (meq/g). For example, a capacity for a weakly acidic IEC resin is 4.4 meq/g (dry weight); 5 g of this resin would theoretically be capable of adsorbing 22 mmol of a charged organic molecule (with a single charge). This same amount of the resin would also be capable of adsorbing 11 mmol of a divalent charged molecule.

The selectivity of a resin is its ability to discriminate between the analyte of interest and the other associated components (e.g., contaminants). This involves the idea of a selectivity coefficient, which is "the ability of an ion-exchanger to select one of two ions present in the same solution." This topic is covered in greater detail in the IPUAC Compendium of Chemical Terminology (2nd Edition) or in numerous technical information brochures from suppliers (by simply typing in the keywords ion-exchange and selectivity into your Web browser). Succinctly put, selectivity coefficients are the resin's preference to interact with an ion—the larger the coefficient, the larger the preference for the ion. A visual example of this is found in Table 1.

2. Materials

The stationary phase in IEC consists of a solid support matrix, typically in the form of spherical beads that are functionalized with various ionic groups either throughout or on the surface of the particle.

The nature of the ionic group on the support is what determines the strength and retention properties of the stationary phase. For the isolation of natural products, the most common materials used as supports are polystyrene and silica. The principles of IEC have been exploited for many purposes, from analytical to preparative scales. As a result, there are now numerous commercial stationary phases available in a variety of chemistries and particle sizes: the millimeter range for bulk packings for use in self-packed columns, to the 30–100 μm particle size similar to the examples for solid phase extraction (SPE). There are also smaller particle sizes, 3–10 μm, which are used in high-performance IEC and require specialized equipment for solvent delivery and analyte detection (9). As a general rule, the smaller the particle size, the better the resolution; however, the smaller particle sizes result in higher backpressures.

An intermediate alternative for the isolation of small quantities of charged species is SPE, commonly used for sample cleanup at the analytical scale. Prepacked SPE cartridges are available at a decigram scale, which allows them to be employed for small-scale preparative purposes. Many of the packings used in SPE and analytical IEC are also available as loose bulk materials, making scale-up possible. However, SPE materials are often proprietary and expensive.

The following sections give a cross-section of currently available stationary phases for the isolation of ionizable natural products, provide background information on some of their properties, and attempt to identify current trends of innovation.

2.1. Support Matrices

The support matrices are the insoluble backbone material to which the ionic functional groups are attached, usually by covalent bonds. They may consist of synthetic organic polymers (resins), silica, or carbohydrates. Polymers have the advantage of being stable over a wide range of pH, often from 0 to 14, which is an ideal property for IEC. By contrast, silica-based packings are typically unstable at high pH, and thus applications of silica as a backbone are typically restricted to more acidic conditions. Cross-linked carbohydrate media, which may be agarose- or dextran-based have working ranges from pH 2 to 9 and are less commonly used for natural products, but still find application in biological macromolecules. Because of their ubiquitous use for natural products, polymer and silica support matrices are discussed in more detail.

2.1.1. Polymeric Resins

One of the more common polymeric phases consists of polystyrene which is cross-linked with divinylbenzene (DVB) in different proportions. Two types of polystyrene-DVB matrix are distinguished: microporous gels, which usually contain 2–8% DVB; and, macroporous (also called macroreticular) which have a high surface area due to their porous structure and are made using a higher cross-linking, up to 55% (9). For example, Dowex 50W-X8 contains 8%

cross-linking DVB by weight. Moreover, a microporous bead will swell upon hydration, but a macroporous bead will not due to the higher cross-linking with DVB. Therefore, with microporous gels the column bed expands considerably (see Note 2) with aqueous mobile phase and compacts with high proportions of organic solvent due to dehydration. The spherical beads of bulk materials that can be used in glass columns may be as large as a millimeter, allowing sufficiently high flow rates even with gravity elution. In analytical IEC particle sizes between 3 and 10 μm are currently available with a trend toward the smaller size.

Another polymer currently in use as a backbone for IEC is cross-linked acrylic gel. These exchangers are marketed as being more flexible and resistant to organic fouling during long-term use as compared to polystyrene-DVB. An example is WAX Amberlite-IRA67; however, the differences appear to be minor.

While polystyrene-DVB sorbents show excellent stability over the whole pH range, one drawback is their hydrophobic nature. This factor may limit the efficacy of a sorbent when the typical water-based sample solution is loaded and the polar analytes do not interact. Other polymeric supports are continuously being explored. For instance, some current SPE supports are made of macroporous *N*-vinylpyrrolidone-DVB copolymer (Table 2 provides examples; also see Note 3). These supports are marketed as combining reversed-phase (RP) characteristics (DVB for example) with a "polar hook" for enhanced capture of polar analytes and good wettability due to the more hydrophilic *N*-vinylpyrrolidone.

2.1.2. Silica Gel

Silica gel has the advantage that it is nonporous, thermally and mechanically stable, and does not swell in aqueous systems. This is exemplified in other chromatographic techniques, such as HPLC, in which silica gel is the preferred material for the stationary phase. However, as a support matrix for IEC silica plays only a secondary role because of its instability at high pH (ca. >8) and loss of functional groups at low pH (<2). Instead, the trend in IEC support matrices is toward organic polymer-based supports owing to their versatility under varying isolation conditions (5). On silica-based phases, the functional groups are usually covalently bonded to the outer surface of the particles through silica–OH groups. This localization to the outer surface results in good mass transfer and chromatographic efficiency since the analytes do not have to diffuse into pores or deeper into the resin matrix. This in turn facilitates a high flow rate with acceptable separation. One method to address the limited pH range of silica-based phases is to coat the silica particle with a polymer, thus combining desirable properties of both materials. For example, in the Waters SAX phase Sep-Pak Accell Plus QMA the silica particle is coated with acrylamide–acrylic acid copolymer.

Table 2
Commercially available mixed-mode ion-exchange (MMX) resins

Stationary phase	Manufacturer/ supplier	Support matrix	Functional group	Counter-ion	pH range	Particle size (μm)	Reference
Oasis Max RP/SAX	W	*N*-Vinyl-pyrrolidone-DVB copolymer	$-CH_2(N(CH_3)_2C_4H_9)^+$	n/p	0–14	30 and 60	(6)
Oasis Wax RP/WAX	W	*N*-Vinyl-pyrrolidone-DVB copolymer	$-CH_2$–piperazine	n/p	0–14	30 and 60	
Strata-X-C Multi-mode, polar, and IEC	Px	n/p	Polymeric	n/p	1–14	33	(6)
Oasis WCX RP/WCX	W	*N*-Vinyl-pyrrolidone-DVB copolymer	Carboxylate	H^+	0–14	30 and 60	
Oasis MCX RP/SAX	W	*N*-Vinyl-pyrrolidone-DVB copolymer	$-SO_3^-$	H^+	0–14	30 and 60	

Px Phenomenex, *W* Waters, *n/p* not provided

2.1.3. Mixed Mode and Other Supports

As mentioned previously, the nature and geometry of the backbone support can exert important secondary effects (intermolecular forces) and thus assist in the separation of analytes with similar charge and polarity characteristics. Interestingly, these secondary effects were originally seen as a detriment; however, today some of these same effects are targeted for desired effects in IEC applications. These backbones, labeled mixed-mode phases with both hydrophilic and hydrophobic (RP) secondary properties, are employed depending on the purpose of the separation and the nature of the sample.

Mixed-mode sorbents have become popular in SPE and for use in isolation schemes. The essential concept with mixed-mode IEC is an RP support interspersed with an ionic-exchange media, e.g., an RP/anion-exchanger (MAX) was utilized for the screening process of fungal metabolites (6). The MAX column was utilized to provide polarity information about the fractions from the crude extract, thus decreasing the amount of time required for separations.

Similarly, monolith columns are increasingly being employed for natural products applications. Monolith columns, which are meant as an alternative to particulate supports, rely on interconnected microscopic channels for surface area and thus create lower backpressures. The resulting advantages over particle-based packings are shorter analyses times and reduced solvent consumption.

The self-styled latex-agglomerated exchangers (10), which are available from Dionex primarily for analytical applications, consist of beads of 9 or 13 μm diameter that are surface-coated with smaller latex particles of 70 or 85 nm diameter. The latex particles are the part of the support matrix bearing the functional groups. The larger beads are made of ethylvinylbenzene (EVB) cross-linked with 55% DVB. The small latex particles consist of functionalized polyvinylbenzylammonium polymer cross-linked with 6% DVB. Like other DVB copolymers these can be microporous (IonPac AS11 column) or macroporous (IonPac AS11-HC column); the latter is marketed as the high-capacity, high-resolution version of the two. The high-capacity version is available as monolithic column.

2.2. Functional Groups

The functional groups are covalently bonded to the support and exert the greatest influence on the selectivity of different ionic analytes. Recall that the functional groups under discussion here are the same charged functional group (G) discussed under theory section in this chapter. The type of charged groups attached to the support matrix define the type of resin: cation or anion, strong or weak. Additionally, the number of functional groups (available charges) present on the support determines the capacity of the resin which is expressed as either meq/volume unit, meq/weight unit, or in analytical high pressure IEC μeq/column. However, the dynamics of the ion-exchange process also depends on the

Table 3
Approximate pK_a values for typical IEC resin functional groups

Resin type	Functional group	Approximate pK_a value	Ionized state, pH	Example of functional group retained
SCX	$-SO_3H$, sulfonic acid	<1	$-SO_3^-$, >1	Anthocyanin, alkaloid
WCX	$-CO_2H$, carboxylic acid	5	$-CO_2^-$, >6	Alkaloid
WAX	$-NR_2$, tertiary amine	8	$-NHR_2^+$, <7	Carboxylic acid
SAX	$-NR_3^+$, quaternary ammonium	>13	$-NR_3^+$, <13	Phenol, carboxylic acid

position of the functional groups. The closer the functional groups are located toward the surface, and the smaller the beads, the better the mass transfer which improves chromatographic efficiency. Conversely, the efficiency will be relatively low if functional groups are located inside the resin beads to which the analyte has to diffuse in and out of, especially if the resin beads are large.

The role of spacer groups, which are typically hydrocarbon backbones that connect the functional group to the support matrix, should also be mentioned. Though these are considered as secondary effects relative to the ionic interaction of the functional group and ion of interest, their make-up as well as chain length have an influence on the complex interactions. For example, the use of phenyl bonded on or within the spacer group may increase pi-stacking interactions. These interactions can be further explored in other books and/or technical notes.

Lastly, the following additional definitions help to summarize the other terminology, and their associated functional groups, used for IEC exchangers: *s*trong *a*nion e*x*changer (SAX), quaternary ammonium; *w*eak *a*nion e*x*changer (WAX), amines; *s*trong *c*ation e*x*changer (SCX), sulfonic acid; *w*eak *c*ation e*x*changer (WCX), carboxylic acid. These, along with some working pH ranges are outlined in Table 3. It should be noted that the weak cation and anion exchangers have the advantage of being more easily regenerated.

2.2.1. Anion Exchangers

Typical strong anion exchangers (SAX, Table 4) contain a quaternary ammonium group ($-NR_3^+$) which remains positively charged over essentially the entire operating range at pH <13. By contrast, with weak anion exchangers (WAX, Table 5) the neutral support matrix is typically functionalized with a secondary or tertiary amine ($-NR_2$), which can be protonated ($-NR_2H^+$) and carry a positive charge at pH <7. Above this value in the basic pH range, it will be deprotonated and therefore be neutral. In general, an SAX can be used

Table 4
Commercially available strong anion exchange (SAX) resins

Stationary phase	Manufacturer/ supplier	Support matrix	Functional group	Counter-ion	pH range	Particle size	Reference
Dowex-1XB	D	Polystyrene-DVB (8%)	Trimethyl benzyl ammonium	Cl^-	0–14	35–75 µm	
Sep-Pak Accell Plus QMA	W	Acrylamide-acrylic acid on diol silica	$–CONH(CH_2)_3NCH_3)_3^+$	Cl^-	2–9	37–55 µm	(11)
QAE Sephadex A25	P	Cross-linked dextran	Quaternary amino ethyl	Cl^-	2–12	40–120 µm	
IonPac AS11	Dn	Latex on EVB-DVB (55%), microporous	Alkanol quaternary ammonium	n/p	0–14	13 µm Latex 85 nm	
IonPac AS11-HC	Dn	Latex on EVB-DVB (55%), macroporous	Alkanol quaternary ammonium	n/p	0–14	9 or 13 µm Latex 70 nm	
TSKgel SAX	T	Polystyrene-DVB	Trimethylamine	Cl^-	1–14	5 µm	

D Dow Chemicals, *Dn* Dionex, *P* Pharmacia, *T* Tosoh, *W* Waters, *n/p* not provided

Table 5
Commercially available weak anion exchange (WAX) resins

Stationary phase	Manufacturer/ supplier	Support matrix	Functional group	Counter-ion	pH range	Particle size (μm)	Reference
Dowex-M-43	D	Polystyrene-DVB macropore	Dimethyl amine	Free base	0–14	n/p	
Dowex-Marathon WBA	D	Polystyrene-DVB macropore	Tertiary amine	Free base	0–7	n/p	(7)
Amberlite IRA-67	R&H	Acrylic gel	Diethyl amino ethyl	Free base	0–7	500–1,000	(12)
Dianion WA 10	M	Polyacrylate gel	Diethyl amino ethyl	Cl^-	0–9	500–1,000	
DEAE A-25	P	Cross-linked dextran	Diethyl amino ethyl	Cl^-	2–9	40–120	

D Dow Chemicals, *R&H* Rohm and Haas (Dow Subsidiary), *M* Mitsubishi, *P* Pharmacia, *n/p* not provided

for isolating weak acids since even under neutral and basic conditions the SAX charge will be available.

For natural products, the most common functional group that can easily exist as an anion is the carboxylic acid, which will dissociate at pH >6 and thus be negatively charged and interact with the positively charged $-NR_3^+$ group of a strong anion exchanger. Any neutral or positively charged compounds would then be eluted with relatively neutral or basic pH of the sample solution. Then, to elute the natural product containing the carboxylic acids from the SAX, the pH would simply be adjusted to pH <4 to protonate the acid and convert the natural product to neutral. Alternatively, by use of a WAX, which typically carries a tertiary amine, the stationary phase itself can be neutralized rather than the analyte, by increasing the pH to >9. The analyte will still be deprotonated, thus charged, and unable to interact with the now neutral column.

In an interesting twist in IEC, newer preparative and analytical columns typically exhibit lower ion-exchange capacity and have replaced some traditional bulk materials with higher capacity. The apparent reason for this is that the charges in the newer materials are concentrated near or on the surface of the bead—this is referred to as pellicular structure—and are now locally at a high density.

2.2.2. Cation Exchangers

Cation exchangers contain immobilized anionic groups to which cationic natural products can reversibly attach. The typical functional group of an SCX (Table 6) is the sulfonic acid group ($-SO_3^-$) which is typically neutralized with H^+, Na^+, or NH_3^+ as counterions. WCX (Table 7) typically have polymeric supports functionalized with the carboxylic acid ($-CO_2^-$) or carboxymethyl ($-CH_2CO_2^-$) residue. As with anion exchangers, the difference between strong and weak cation exchangers is that the sulfonic acid residue remains charged, and is therefore available for ion exchange, over the full pH working range. By contrast, the carboxylic acid functional group of a WCX will only dissociate above a pH of ca. 6 and will be neutral under more acidic conditions. For natural product isolation, this means if a compound or group of compounds is positively charged, such as cations of tertiary amines under acidic conditions with pH <4 will be adsorbed onto the SAX functional groups. Increasing the pH to >9 will deprotonate the amine group of the analyte and elute the analyte.

2.2.3. Zwitterionic Phases

Zwitterionic IEC, which can be used for simultaneous separation of acids, bases, and neutral compounds of the same extract, is becoming popular for analytical applications and have been used for SPE. Zwitterionic phases should not be confused with mixed-bed sorbents, which contain a mixture of anion and cation exchangers in a 1:1 ratio. The two different functional groups with opposite charges reside on separate beads. Mixed-bed columns are rarely used for natural products isolation. Conversely, with zwitterionic

Table 6
Commercially available strong cation exchange (SCX) resins

Stationary phase	Manufacturer/ supplier	Support matrix	Functional group	Counter-ion	pH range	Particle size (μm)	Reference
Dowex 50-X8	D	Styrene-DVB 8% cross-link	$-SO_3^-$	H^+	0–14	38–75	(13–15)
Strata SCX	Px	Silica	Benzyl sulphonate	n/p	n/p	55	(16, 17)
TSK gel SP-3PW(30)	T	Polymethacrylate	$-SO_3^-$	Na^+	2–13	20–40	
AG 50W-X8	B-R	Styrene-DVB	$-SO_3^-$	H^+, Na^+, or $NH4^+$	n/p	106–250	
AG MP 50	B-R	Styrene-DVB macroporous	$-SO_3^-$	H^+, Na^+, or $NH4^+$	n/p	75–150	

B-R Bio-Rad, *D* Dow Chemicals, *Px* Phenomenex, *T* Tosoh, *n/p* not provided

Table 7
Commercially available weak cation exchange (WCX) resins

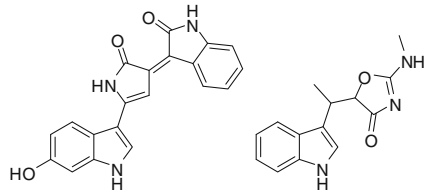
Stationary phase	Manufacturer/ supplier	Support matrix	Functional group	Counter-ion	pH range	Particle size (μm)	Reference
Dowex-MAC-3	D	Acrylic macroreticular	Carboxylic acid	H^+	4–14	16–50	(7)
Amberlite IRC-50	R&H	Acrylic 4% cross-link macroreticular	Carboxylic acid	H^+	5–14	500–1,000	
Dianion WK 10	M	Methacrylic porous	Carboxylic acid	H^+	5–14	500–1,000	
CM-Sephadex C25	P	Cross-linked dextran	Carboxymethyl	Na^+	2–12	40–120	

D Dow Chemicals, *R&H* Rohm and Haas (Dow Subsidiary), *M* Mitsubishi, *P* Pharmacia

supports, the opposite charges reside on the same functional group (ligand). An example of a zwitterionic support is the use of a protonated alkaloid tethered to a compound containing either a sulfonic or carboxylic anion (18). Interestingly, zwitterionic phases, as well as investigations into functionalizing them with chiral selectors, have been recently used for chiral separations.

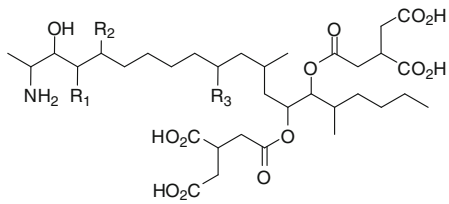
2.3. Column Operation

Having covered the basics of IEC, it is time to prepare for separation of an extract, be it from a plant, animal, microbial, or food matrix. The following subsections provide a rough outline for your isolation scheme, including selection of the IEC resin, resin preparation, column-size selection, sample loading, and elution of your analyte(s). For more detailed schematics please refer to Figs. 1–6 and Table 8 (see Note 4), or the manufacturer's technical notes. It is important to keep in mind the pH of solvents and the pK_a of functional groups both on the resin as well as of the analyte, if known.



1.	Extraction of bacterial broth with 1:1 mixture of Diaion HP20SS and XAD-7. Wash with water (pH 7) for removal of salts, extraction with 50% aqueous MeOH and 100% MeOH.	Extraction of compounds excreted from cells and desalting. No pH adjustment necessary for the XAD-7.
2.	Crude dissolved in MeOH-H_2O (90:10, v/v) and absorbed directly onto Phenomenex Strata-XC. Dried and packed onto Biotage SNAP column with pure resin in the base. Column wetted with H_2O and 1% formic acid.	Dry loading of extract onto SCX. Pure resin packed in bottom of column to absorb potential overloaded material. IEC can successfully be performed on automatic flash system on large scale (Isolera from Biotage).
3.	Neutrals and acids eluted with 100% MeOH with 1% formic acid.	Most of the extract is eluted from this fraction.
4.	Bases (violacein and indolmycin) eluted with 100% MeOH with 2% NH_4OH.	Violacein and indolmycin quantitatively retained.
5.	Basic fraction concentrated, reconstituted in 50:50 v/v MeOH-H_2O and loaded onto Oasis MAX.	Mixed-mode anion exchanger used for the separation of indolmycin and violacein.
6.	Indolmycin is eluted with 100% MeOH.	Non-acidic indolmycin unretained on anion exchanger.
7.	Violacein is eluted with 100% MeOH with 1% formic acid.	Violacein was retained due to acidic phenol.

Fig. 1. The structures of violacein (*left*) and indolmycin (*right*) and their extraction scheme from the bacterial broth of *Pseudoalteromonas luteoviolacea.*



	R_1	R_2	R_3
FB_1	H	OH	OH
FB_2	H	OH	H
FB_4	H	H	H
FB_6	OH	OH	H

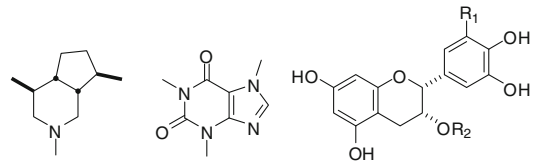
1.	Extraction of fungal mycelium with acidified ethyl acetate.	Extraction of medium to non-polar compounds.
2.	Crude extract dry-loaded onto Phenomenex C_{18} and subjected to flash chromatography on Biotage Isolera using an acetonitrile-H_2O gradient.	Reduction of biomass prior to IEC. Fractions containing fumonisins were pooled prior to further fractionation.
3.	Fraction containing fumonisin was dry-loaded onto a mixed-mode cation-exchanger containing Strata-X-C with pure resin the in the base. Column equilibrated with H_2O, 1% formic acid.	Polymeric mixed-mode cation-exchanger found to have the best recovery and reproducibility.
4.	Neutrals and acids are eluted with 100% MeOH containing 1% formic acid.	High levels of aurasporines, kotanin, and malformins are removed from extract.
5.	Bases (fumonisins B_1, B_2, B_4, and B_6) are eluted with 100% MeOH containing 2% NH_4OH.	Fumonisins are almost quantitatively retained. Only minor impurities remaining in this fraction.
6.	Final purification of fumonisins on a Luna II C_{18} column.	Separation of individual fumonisin analogues.

Fig. 2. The structures of isolated fumonisins and their extraction scheme from the fungal mycelium of *Aspergillus niger.*

2.3.1. Selection of Stationary Phase

The selection of the appropriate exchanger will depend upon the technical level available at the lab for the process itself and desired amount of target compound. For instance, if a specialized high performance IEC setup, such as supplied from Dionex, Inc. is available and only small amounts (mg) of target compound are needed, then prepacked semipreparative columns may be used. Or, if large amounts are desired (g), for instance for biological testing, and large columns are available, then it is advisable to use bulk materials. When small amounts are to be obtained, or utilization of a simple system is desired, the best choice may be an SPE setup as it only requires a vacuum be applied, either through a manifold or through a vacuum flask.

Next, to follow the elution progress, some sort of analytical assay to monitor stability and breakthrough, or a simple bioassay is required. Again, sophisticated equipment in IEC now has amperometric detection (Dionex). Alternatively, a conductivity cell at the outlet, TLC, or analytical HPLC can be used for monitoring the process. If the target has a chromophore, HPLC with photodiode-array detector may be a possibility. Finally, if the target has no chromophore, an evaporative light scattering detector would be a relatively inexpensive alternative compared to mass spectrometry.

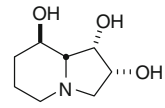


	R_1	R_2
epicatechin	H	H
epigallocatechin	OH	H
epicatechin gallate	H	galloyl
epigallocatechin gallate	OH	galloyl

1. Extract dry ground plant material exhaustively with MeOH and CH_2Cl_2	
2. Remove solvent *in vacuo*.	
3. Suspend 2.5 g residue in 500 mL MeOH-H_2O (1:1, v/v).	
4. Immerse 2 tea bags, one filled with 20 g WAX (Dowex Marathon WBA) and the other filled with 20g WCX (Dowex MAC-3).	Alkaloids bind to theWCX resin, phenolics and acids bind to WAX.
5. Stir solution for 8-10 h.	
6. Rinse tea bags with MeOH ×2.	Neutrals remain in the working solution.
WAX resin	
7. Immerse WAX resin into 500 mL of 2% HCl (v/v in MeOH-H_2O, 1:1), leave overnight under agitation.	
8. Neutralize with NH_4OH to pH 6-7.	
9. Remove MeOH *in vacuo.*	
10. Extract aqueous phase ×3 with EtOAc.	Liquid/liquid extraction.
11. Evaporate organic phase.	Provides phenolics and acids.
WCX resin	
12. Immerse WCX resin into 500 mL of 2% ammoniated MeOH, stir overnight.	
13. Remove solvent *in vacuo*.	Provides alkaloids.
Original working solution	
14. Concentrate solution *in vacuo*.	Provides neutral components.

Fig. 3. The structures of green tea components separated by "phase-trafficking" and the associated extraction scheme.

The type of stationary phase will also naturally be dependent on the targeted class of compounds. If the targeted analyte is known, then the choice of IEC resin is narrowed considerably—if the analyte is acidic (e.g., carboxylic acid such as fumonisins), an anionic exchanger would be appropriate; if the analyte is basic (e.g., an alkaloid), a cationic exchanger would be applicable. As a general guideline, examples of the typical functional groups that are retained on the specific resin types are provided in Table 3. However, this said, one must still consider the pK_a and stability of



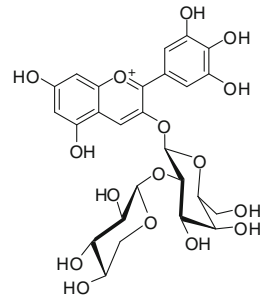
1. Extract dry ground plant material with MeOH using Soxhlet extractor.	16-60 h for typical plant material
2. Remove solvent *in vacuo*.	
3. Dissolve residue with 2% AcOH and extract twice with $CHCl_3$.	Protonated swainsonine remains in aqueous layer.
4. Add aqueous layer to SCX resin (Dowex 50W-8X).	Swainsonine will bind to IEC resin
5. Filter resin from solution and rinse 3× with de-ionized H_2O to a neutral pH.	Need to remove all the acid and neutral components.
6. Rinse resin with 1 M NH_4OH 2-3× to elute swainsonine from resin.	Basic components will elute.
7. Evaporate solution to dryness.	
8. Dissolve residue in 1 M NH_4OH and 1 mL of 10% NaOH and extract with CH_2Cl_2 in a liquid/liquid extractor.	A very basic solution is required to partition the swainsonine into the CH_2Cl_2. The partition coefficient is low, thus a liquid/liquid extractor is best.
9. Remove CH_2Cl_2 solvent *in vacuo*.	
10. Crude swainsonine can be further purified by recrystallization from CH_2Cl_2-MeOH or NH_3 saturated $CHCl_3$.	

Fig. 4. The structure of the polyhydroxylated alkaloid swaisonine and its extraction scheme.

the targeted compound and further contemplate whether a strong/weak IEC resin is suitable. The use of a strong ion exchanger may be suitable when one particular analyte is sought, or if the analyte is known to be stable under harsh pH conditions. If the extraction requires subtle pH manipulation due to labile functional groups or components, the use of a weak ion exchanger may be more appropriate. Avoiding harsh conditions may be a reason why weak anion or cation exchangers are often preferred. These can also be more easily regenerated. As an example, Araya et al. (7) used weak exchangers in their tea experiment.

Often the nature of the compound to be isolated is not known. The isolation procedure will have to be guided by bioassay, or an analytical method will have to be used to acquire information about the nature of the target analyte. First of all, the target should be stable under the conditions used in ion chromatography, but many compounds are unstable under basic or acidic conditions.

Once the stability of the structure and/or activity is confirmed, the solution may have to be desalted. Fermentation broths, hydroponic solutions, marine samples, etc., all may contain high levels of salts that could interfere with the IEC by competing with and displacing the analyte. Desalting can be achieved by adsorbing the analyte onto a nonionic sorbent, such as Amberlite XAD-7 or



1.	Extract homogenized whole kiwifruit in acidified EtOH at 1°C.	Fruit (500 g) in EtOH-H_2O-HCO_2H 1000:500:40 mL for 24 h.
2.	Extract is filtered and remaining solid extracted once more.	EtOH-HCO_2H 1000:40 mL for 24 h.
3.	Combined extracts concentrated *in vacuo*.	
4.	Aqueous layer defatted with hexanes.	Removed lipids, then residual hexanes removed *in vacuo*.
5.	Extract combined with XAD-7 to extract polyphenols, 24 h at 4°C.	Pre-conditioned with HCO_2H, MeOH, and H_2O.
6.	XAD-7 washed with H_2O (5 × 3 L).	Removed sugars.
7.	XAD-7 washed with MeOH (2 × 1.5 L), then MeOH concentrated *in vacuo*, resulting extract dried overnight under high vacuum.	Eluted and extracted all polyphenolics.
8.	Powered polyphenolic extract dissolved in acidic MeOH and loaded onto a column of Dowex 50W-X8.	SAX pre-conditioned with 10% HCl-MeOH and H_2O.
9.	SAX washed with H_2O (500 mL).	Removed non-anthocyanins.
10.	SAX washed 10% HCl-MeOH (1.5 L).	Eluted anthocyanins.

Fig. 5. The structure of an anthocyanin and its extraction scheme from kiwifruit.

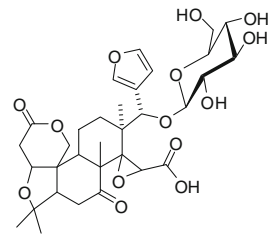
Strata-X which rely on polar and nonpolar interactions, but not ionic interaction with the desired analyte. Once desalted, the sample is now ready for IEC.

One may employ small sample columns with different media and use an isolation scheme similar to that presented in Fig. 1 or SPE supplier Web sites (e.g., method development layout) in order to gain useful information on the nature or the possible pK_a range of the target compounds.

If the plant is to be chemically characterized or is part of an investigation of closely related plants (e.g., same genus) that contain a certain class of compounds, one may start with conditions specifically targeting the analyte. An example of this would be isolation schemes targeting specific alkaloids.

2.3.2. Resin Preparation

Commercial bulk resins usually need preconditioning. The purpose is to remove impurities and residues from the manufacturing process. It is easiest to consult the manufacturer's instructions that come



1. Dilute molasses with distilled H_2O (3:1) for decreased viscosity.	
2. Adjust pH to 3.5 with 6 M HCl.	
3. Centrifuge at 13,000 *g*, 15 min, 5°C.	
4. Filter supernatant through Celite.	
5. Dowex 50W-X4-100 (3 L bed volume).	SCX for decolorization, LG mix passes.
6. Effluent directed to a connected SP70 Sepabeads (3 L bed volume).	Neutral polystyrene-DVB beads absorbs by hydrophobic interaction.
7. Wash with distilled H_2O (10 mL/min) through connected columns overnight.	
8. Elute paritally pure LG mixture from SP70 (80% EtOH, 3 L).	
9. Evaporate to near dryness *in vacuo*.	
10. Adjust to 200 mL with distilled H_2O.	
11. Adjust pH to 6.5 with 0.1 M NaOH and load onto Q-Sepharose column (5 mL/min).	SAX used to adsorb LG (pH for ionized LG, pK_a near 2.8).
12. Run with an aqueous NaCl gradient (0-0.4 M NaCl, 6 L, linear).	Elute and remove other acidic impurities with salt gradient.
13. Combine LG-containing fractions.	
14. Pass LG fractions through Dowex-50 coupled to an SP70 column.	SCX used since LG adsorbed on neutral adsorbent.
15. Rinse overnight with distilled H_2O.	Distilled H_2O rinse to remove salts.
16. Elute LG mixture with 80-100% EtOH.	Evaporate *in vacuo*.
17. Rinse gummy residue repeatedly with EtOH to remove H_2O.	Yields 5 g dry golden-brown powder, 60% recovery from 1 L molasses.

Fig. 6. The structure of limonin glucoside (LG) and its extraction scheme from citrus molasses.

with the product to properly precondition the resin. Usually, washing with methanol or a solvent that simulates the final elution conditions will minimize the likelihood of having impurities in the final product.

Another process during preparation of the resin is swelling, which exposes the functional groups to the mobile phase, hydrating groups, and counterions by opening pores. For carbohydrate polymers, it is important to use dilute HCl and NaOH in addition to water in order to break the hydrogen bonding on the polar backbone and allow complete swelling. The resin can now be neutralized and equilibrated with the initial buffer solution.

Table 8
Brief examples of IEC resins used for extraction and/or isolation of natural products

Target	Extract matrix	Stationary phase	Method summary	Reference
Pyrrolizidine alkaloids and their *N*-oxides	MeOH extract of fresh flowers and leaves of *Echium plantagineum*	Strata SCX; benzyl sulphonate, silica-based cartridges	Analytical scale: fresh plant material; MeOH extract residue with 0.05 M sulfuric acid; apply sample to SCX-SPE cartridges; elute with ammoniated MeOH; remove solvent (N_2 stream); reconstituted in MeOH; reduce with activated indigocarmine resin; C_8 RP-HPLC–ESI/MS	(16)
Pyrrolizidine alkaloids and their *N*-oxides	Honeys from borage (*Echium vulgare*)	Strata SCX; benzyl sulphonate, silica-based cartridges	Analytical scale: dilute honey with 0.05 M sulfuric acid; 40°C for 10 min; cool to rt; centrifuge at 12,000 × *g* for 15 min; 40°C water bath; apply 10 g of honey to SCX-SPE cartridges; elute with ammoniated MeOH; remove solvent (N_2 stream); reconstituted in MeOH; reduce with activated indigocarmine resin; C_{18} RP-HPLC–ESI/MS	(17)
Pyrrolizidine alkaloids and indolizidine alkaloids	MeOH extract of dried leaves, seeds, and bark of *Peripentadenia mearsii*	SCX Dowex 50W-X8-400; 200–400 mesh	Preparative scale: dried plant material; alkaloid screening with Dragendorff's reagent; MeOH extraction; filter through SCX under vacuum; wash sequentially with MeOH and water; elute alkaloids with 1.0 M NaCl; apply to C18 silica gel; wash with water to remove NaCl; elute alkaloids with 1% TFA–MeOH; monitor fraction by (+)ESI/MS; evaporate solvent; preparative C_{18} RP-HPLC separation	(15)

(continued)

Table 8 (continued)

Target	Extract matrix	Stationary phase	Method summary	Reference
Aristolochic acids	Crude drugs, Kampo formulations	Acrylamide-copolymer of SAX (Sep-Pak QMA) coupled to diol silica	Analytical scale: crude drug; extract with MeOH containing 1% ammonia; shake; centrifuge; repeat extraction with pellet; combine supernatants; transfer aliquot onto SPE cartridge; wash with MeOH; elute AAs with acetonitrile–water–phosphoric acid; fill up to volume with MeOH; analyze on TKS gel ODS 80TsQA HPLC, detection at 254 nm	(11)
Ochratoxin A	Red wine	SAX, silica-based	Analytical scale: dilute wine 1:1 *v/v* with aqueous 1% PEG 6000 and 5% $NaHCO_3$; clean-up SAX silica from Bond ElutSAX-columns on top of second flow-through column containing the same type of packing; remove clean-up column, outlet of clean-up column placed into inlet of lower flow-through column; this column used for new gel-based immunochemical assay; compare with membrane-based immunochemical assay and IAC-HPLC	(19)
79 Fungal metabolites, including mycotoxins, ergot alkaloids	Pure compounds	New MMX RP/WAX AQ-RP/WAX and AT-RP/WAX on thiol modified silica	RP/WAX separation; LC–UV–ESI/MS/MS, method comparison, basic compound chiral separation acidic acid/ammonium acetate buffer and organic modifiers acetonitrile, MeOH Luna® NH_2 (5 m, 100 Å, 200 m^2/g RP column)	(25)

Resins may not necessarily come with the counterion form that is suitable for the target compounds. To convert the resin from one counterion to another large volumes of the counterions are added and excess ions are washed off with water; this process is called the resin cycle. The most important conversion for cation exchangers is cycling from the hydrogen ion to the sodium ion, and for anion exchangers cycling from the chloride ion to the acetate ion.

2.3.3. Column-Size

For self-packed columns, it is important to consider the dimensions of the column. If columns are too long, the separation may be compromised by excessive backpressure and band broadening due to diffusion. Columns that are relatively short (10–20 cm) but wide are usually preferred. If the sample size is uncertain, a recommended starting point for the resin amount would be 5 g/100 mL of sample solution (1). This ratio can subsequently be adjusted during upscaling.

2.3.4. Sample Loading

To maintain the pores of the resin free of particulate matter, samples should be clarified, ideally by centrifugation. Filtration by filter paper or other membranes are often difficult and may result in nonspecific adsorption and substantial loss of analyte. Celite is a good initial choice for filtering for samples with a high proportion of fine particulate matter that could pass through or clog filter paper.

There are two methods by which the sample can be loaded—batch or column. With the batch method, the resin and clarified sample are mixed for a sufficient time. The solution is then decanted from the resin or the resin is filtered, the resin is slurry packed into the column, and the analyte eluted. By contrast, when using the column method, the clarified sample is slurry-packed into the column and the sample is applied. A slow flow rate is required (e.g., 5–10 mL/min for a 100 mL column) during sample application to ensure the sample has time to diffuse into the pores. The sample application times for nonporous materials, such as silica may be somewhat less. For SPE applications, follow the manufacturer's recommendations for flow rate.

2.3.5. Elution

The following introduces important elution parameters and strategies, including ionic strength, pH, co-solvents, stepwise and gradient elution. Two related strategies can be used to elute the ionically bound analytes from the column—changing the ionic strength or the pH. In each case, the use of water-miscible organic solvents, such as methanol, ethanol, or acetonitrile, may be effective in the mobile phase. NaCl is often used to displace charged solutes from both anion and cation exchangers since both ions have medium affinities to most resins. By using a change in pH, the elution can be more easily controlled and the process is more selective. At minimum, a two pH unit difference around the pK_a of the analyte between adsorbing and desorbing can be used as a general guideline.

For instance, carboxylic acids (pK_a near 5, depending on the rest of the structure) can be adsorbed at pH 6 and eluted at pH 4.

Natural products bearing lipophilic moieties may interact sufficiently with nonpolar supports, such as polystyrene-DVB resulting in band broadening and poor recovery. Neutralization of their charge by pH adjustment will not only cause them to be much less hydrophilic, but the high concentration may cause them to precipitate. The use of organic co-solvents with the aqueous eluent can reduce the interactions of the analyte with the backbone surface. Using solvent–salt combinations may be the only way to disrupt some of these interactions; thus, the combination of pH adjustment and solvent–salt eluents can lead to a sharp elution profile.

For instance, in order to elute various anions from an anion exchanger, such as Dowex-1, an eluent composition of 3% (ca. 0.5 M) ammonium chloride in 90% methanol/water is usually a good starting point. Alternatively, organic salts, such as the combination acetic acid and pyridine, can be mixed with water in different proportions such that a wide range of pH values can be achieved (Table 9). These organic salts have the advantage of being volatile and can be removed by simple evaporation under reduced pressure.

For preliminary experiments designed to define appropriate versus ineffective eluent compositions, a step gradient with fixed volumes will provide unambiguous answers. For the final elution, a linear gradient elution may be more efficient, and save solvent. For the actual chromatography, a simple gradient generator can be employed, which adds the final solvent (solvent B) to the primary solvent (solvent A) reservoir while stirring it.

It should be kept in mind that in IEC is more of an on/off switch than in typical column chromatography using silica gel. In column chromatography, the preferable narrow band of the mixture travels the column in near equilibrium with the stationary phase while continually interacting with the mobile phase. In IEC, once the desired pH and eluent composition has been determined there will be virtually no interactions of the target analytes with the IEC resin other than the desired strong ionic-bonding column which results in the "catching" of the targeted analyte (the "on"

Table 9
Compositions for pyridine/acetic acid buffers

Composition	pH 3.0	pH 5.0	pH 8.0
Water	970	970	980
Acetic acid	28	14	0.2
Pyridine	2	16	19.8

switch). After elution of the contaminants is achieved, the solvent conditions are changed and the analyte is "released," thus turning "off" the column. In general, a total elution volume of 5–10 times the column volume is appropriate to ensure full recoveries.

The flow rate for adsorption is typically slower than that for elution. A general rule of thumb is 2–5 min contact time between eluent and exchanger, and collected fractions should be small enough to take advantage of the achieved resolution. And finally, collection of 5–10 fractions per column volume is an appropriate starting point; these fractions can always be combined later after evaluation of fraction content via the chosen method. If resources allow, there are fraction collectors available that operate on a timed basis, or can respond to a signal from a detector, such as a conductivity cell.

3. Methods

Beyond biological macromolecules IEC is widely known as a "clean-up" step during the isolation of natural products. However, recent investigations have helped to once again highlight the versatility and importance of IEC during exploratory studies of plants, microbes, and associated extracts. In their research with microbial natural products, Mansson et al. (6) offer some remarkable numbers concerning the chemical composition of microbial extracts. For instance, when the pH range of 2–11 was considered, 44% of the compounds contained an acidic functional group, 17% of the compounds contained a basic functional group, and 9% contained both an acidic and basic functional group. These numbers indicate that ca. 70% of the compounds within a typical microbial extract may be receptive to the use of IEC for their extraction from the complex microbial matrix.

An example of this process is provided in Fig. 1 for the extraction of the alkaloids violacein and indolmycin produced by the marine bacterium *Pseudoalteromonas luteoviolacea* (6). For the alkaloids, the authors noted that these compounds can be efficiently separated by application of an SAX and a mixed-mode anion-exchange, and that desalting prior to IEC is achieved by use of the Diaion HP20SS and XAD-7 resin.

A second example of their IEC process for microbial natural products is provided in Fig. 2 for the extraction and isolation of the four mycotoxins, fumonisins B_1, B_2, B_4, and B_6, produced by the fungus *Aspergillus niger* (20, 24). For the isolation of the structurally complex fumonisins, which contain four carboxylic acids and one amine group, the authors noted that the high levels of small organic acids within the mycelium matrix impairs the traditional use of anion-exchange for purification. However, in this example

the fumonisins, with their amine functionality, can be selectively retained in high purity on a mixed-mode cation-exchange resin. The use of the mixed-mode resin greatly facilitated fumonisin purification on both the analytical and preparative scale.

Another approach recently developed was by Araya et al. (7), in which they coined the idea of "phase-trafficking" for rapid and simple extraction of small sample sizes using "tea bags" filled with WCX resins. Their stated goal for the investigation was "to develop a selective and rapid method for trapping/recovery of acidic and/or basic compounds from plant extracts using solid-phase reagents…" and to apply their methodology toward plant examples. They provided proof of concept via a mixture of phenylacetic acid, *N*,*N*-dimethylbenzylamine, and methyl phenylacetate extracted over a period of 48 h using Dowex Marathon WBA and Amberlite IRC-50. After optimization, they went on to demonstrate the efficacy of the system by extracting skytanthine, caffeine, and several catechins from 2.5 g of green tea (Fig. 3) via the simultaneous use of Dowex Marathon WBA (10 g) and Dowex MAC-3 (20 g) with an overall percent yield of the catechins 10.8% in just 6–8 h. The authors noted the use of sonication minimized oxidation of the phenolics, the use of lower acid and base concentrations decreased side-reactions and decomposition products, and the use of a 1:1 mixture of methanol and acetonitrile effected higher yields.

Some helpful hints regarding phase-trafficking were provided by the authors. It was observed that washing and activating the resins according to the manufacturer's guidelines reduced the time required for trapping compounds, increased the yields, and increased the purity of the recovered compounds as observed by HPLC analysis (UV 254 nm detection). Interestingly, the activation and washing of the resins were achieved by flushing the resins in a packed column, but also could be performed "in bag" by dipping the teabag containing the resin in the appropriate basic or acidic solution (ca. 4–6 h), followed by three water washes (30–60 min). In addition, it was found that the trap-and-recovery process was almost quantitative, despite several washing operations included in their final optimized separation scheme. Also, when working with the "real" plant experiments like ginger extract, solubility problems were encountered with the trapping solution ($MeOH–H_2O$, 1:1); however, it was discovered that basic and acidic compounds will ultimately be trapped into the solid phase by equilibrium shift. Finally, experiments were conducted with recycled resins, observing no change in performance after washing and activating the used resins.

We now turn our attention to the more classical use of IEC and the extraction of the toxic indolizidine alkaloid, swainsonine, from plant material (Fig. 4). Several groups have performed extraction and quantification research on this highly polar alkaloid (21, 22), but

Gardner et al. (13) have provided an elegant use of SPE and IEC in the preparative isolation of swainsonine. In their work, the authors initiate the extraction using conventional acid/base partitioning, but make use of an SCX after acidification to retain the alkaloid and flush away many of the impurities. They then resume the extraction by converting to the free base and eluting the purified alkaloid from the resin. The authors note the low partition coefficient of the free base and the judicious use of a liquid/liquid extractor to achieve a higher yield. For the initial extraction, the authors suggest 16 h of Soxhlet for small (1–5 g) samples, and ca. 60 h for large (1 kg) samples.

Anthocyanins are plant components of particular interest to agricultural scientists due to their content in many fruits. These compounds are inherently polar due to their oxonium ion within the parent structure, but also have acidic phenolic protons, and additionally can exist as glycosides. As an example of the isolation of several anthocyanins from kiwifruit, one of which is shown in Fig. 5 (14), the investigators subjected the homogenized fruit to several extractions with polar solvents and after defatting with hexane utilized SPE to partition the sugars and polyphenolics from the fruit matrix. Next, an SAX resin was used to catch the anthocyanins while rinsing the nonanthocyanin polyphenolics from the resin with water. Finally, the anthocyanins were eluted from the SAX resin using acidic methanol and further purified via preparative HPLC (see Chapter 10 for details on prep-HPLC methods).

As a last example of IEC use for the large-scale extraction of natural products from an agricultural source, limonin glucosides (LGs) (Fig. 6) were isolated in good recovery yield from citrus molasses (23). In their extraction scheme, the authors utilize both SAX and SCX to isolate LGs from a complex food matrix.

4. Notes

1. The term resin for stationary phases in IEC is discouraged by the IUPAC. However, it remains in common usage and therefore our use of it in the text refers to synthetic organic polymers.
2. Safety precaution: to avoid injury when self-packing glass columns with resins it needs to be considered that especially microporous gels with little cross-linking may expand considerably upon hydration. Refer to the specifications for the packing provided by the manufacturer to estimate volume increase.
3. Particle size: the examples given in Tables 2, and 4 through 7 provide only particle sizes for consistency. However, on supplier's Web sites particle size may also be expressed in mesh sieve number, usually as a range. The following table can be used to

convert from diameters to wet mesh (US standard). Source: Bio-Rad Instruction Manual AG 50W.

Diameter (μm)	38	45	53	75	106	150	180	300	425	850	1,180
Mesh (sieve no.)	400	325	270	200	140	100	80	50	40	20	16

4. Because several notations were provided in the methods section, the authors wanted to take one more opportunity to provide the readers with additional recent examples of IEC being incorporated into natural product isolation schemes from a variety of matrices.

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