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# Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications

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ABSTRACT

Mixed-mode chromatography (MMC) is a fast growing area in recent years, thanks to the new generation of mixed-mode stationary phases and better understanding of multimode interactions. MMC has superior applications in the separation of compounds that are not retained or not well resolved by typical reversed-phase LC methods, especially for polar and charged molecules. Due to the multiple retention modes that a single MMC column can offer, often MMC provides additional dimension to a separation method by adjusting the mobile phase conditions. Mixed-mode media is also an effective way to clean up complex sample matrices for purification purposes or for sensitive detection of trace amounts of analytes. In this article, we discuss mixed-mode stationary phases and separation mechanisms and review recent advances in pharmaceutical and biopharmaceutical applications including the analysis and/or purification of counterions, small molecule drugs, impurities, formulation excipients, peptides and proteins.

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## 1. Introduction

Mixed-mode chromatography (MMC) or multimode chromatography is becoming increasingly popular in pharmaceutical and biopharmaceutical applications due to its unique selectivity and retention of a variety of compounds, especially polar and charged molecules [1–8]. MMC is a chromatographic method in which solutes interact with stationary phase through more than one interaction mode or mechanism. MMC has been used as an alternative or complementary tool to traditional reversed-phased (RP), ion exchange (IEX) and normal phase chromatography (NP). Unlike RP, NP and IEX chromatography, in which hydrophobic interaction, hydrophilic interaction and ionic interaction respectively are the dominant interaction modes, mixed-mode chromatography employs a combination of two or more of these interaction modes.

Mixed-mode phenomena in the past were considered "secondary interactions". Most stationary phases are based on rigid support matrices such as silica gel or polymers, to which specific functional groups (e.g. alkyl chain C18, diol, etc) are bonded. Often sample solutes interact differently with the matrices and the functional groups, generating "secondary interaction" characteristics [9]. Mixed-mode IEX and RP interactions can even be observed on classical silica-based RP columns without intentionally introducing an ion-exchanger. Free silanol groups on silica gel matrix are considered as sites of secondary interactions in RP chromatography. Similarly, hydrophobic interactions exist in IEX separation, and ionic interactions exist in SEC separation. While in some cases, the secondary interactions are considered beneficial for selectivity [10,11], most of the time it is considered detrimental to a separation. For example, the free silanol group on silica often contributes to peak tailing, a phenomenon that is minimized by end-capping or by optimizing mobile phase conditions.

MMC can retain and separate small, polar drugs and related substances that are not retained by typical RP HPLC. It has been used as an alternative method to traditional ion chromatography for counterion analysis [1,12,13]. MMC has been used for the purification of biological samples and allowing direct sample injection [14–17]. MMC can retain acidic and basic compounds at mild mobile phase conditions compatible with MS detection. For a given mixed-mode column, the predominant separation mechanism depends on the properties of the sample as well as the mobile phase conditions. The mixed-mode stationary phases introduced in recent years provide desirable and repeatable "secondary interaction" or "tertiary interaction" with the use of carefully designed functional groups of different retention modes and well controlled manufacturing

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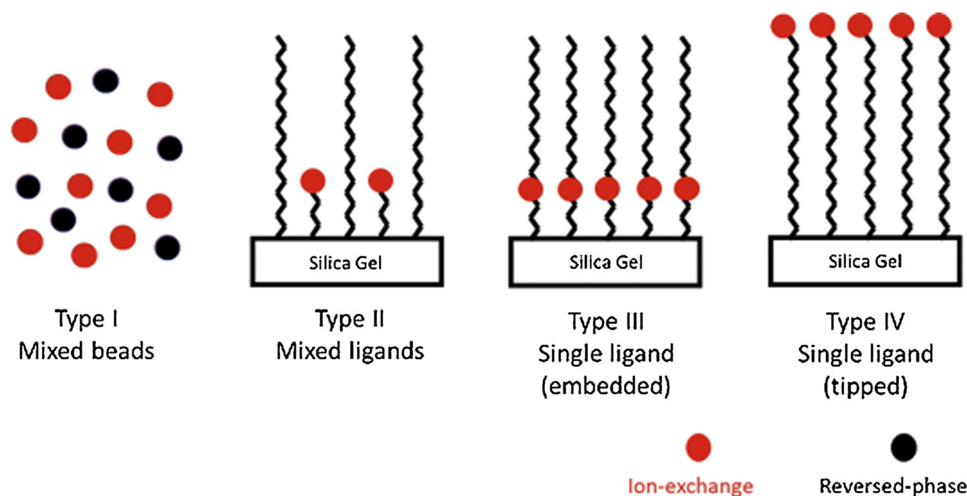



**Fig. 1.** Types of RP/IEX bimodal mixed-mode columns classified by the arrangement of functional groups.
Adapted from Ref. [47].

process. The recent commercialization of mixed-mode columns significantly advanced the utility of MMC in pharmaceutical and biopharmaceutical applications [18–23].

Because MMC is complementary to RP and other separation modes, mixed-mode columns are also used in two-dimensional liquid chromatography (2D-LC) [2,24,25]. Furthermore, the multi-mode retention mechanisms can add dimensionality to a single mixed-mode column by adjusting mobile phase conditions. Therefore, mixed-mode columns are frequently used as an alternative technique for 2D-LC while using a single column and conventional HPLC setup [5,26,27].

## 2. Stationary phase and separation mechanism

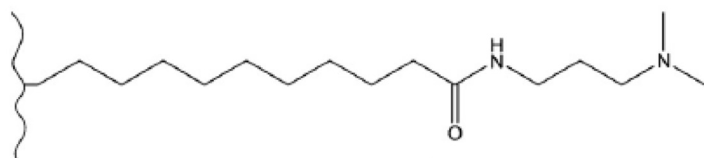
### 2.1. Mixed-mode LC column media

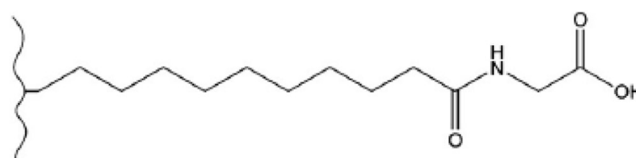
The occurrence of mixed-mode chromatography, including both RP and IEX mechanisms, has been known for decades [9]. The use of mixed-mode chromatography for HPLC separations has been widely reported [18,28–45]. Mixed-mode columns can be divided into RP/anion exchange (AEX), RP/cation-exchange (CEX), hydrophilic interaction liquid chromatography (HILIC)/AEX and HILIC/CEX bimodal phases, as well as RP/AEX/CEX and HILIC/AEX/CEX trimodal materials. According to their chemistry design, bimodal media can usually be classified into four categories (Fig. 1). Type I media are achieved by mixing different types of particulate separation media, each with a single chemistry, such as RP or IEC, and packing the mixture into a column [28,29]. Type II media consist of substrates modified at the surface with a mixture of ligands having different functionalities, RP/IEX, HILIC/IEX, or RP/HILIC [30,32,33]. More sophisticated stationary phases can be prepared using ligands that contain ion exchange functionality as a part of the hydrophobic ligand. Depending on the position of the ionizable functionality with respect to the pore surface, these phases can be "embedded" (Type III), i.e. the functionality is close to the surface and the hydrophobic chain extends in a mobile phase environment [31,34,37], or "tipped"(Type IV) with the functionality at the free end of the hydrophobic chain [34,46]. These stationary phases are advantageous in reproducibility since the chemistry is defined by the attached ligands, not by the preparation process.

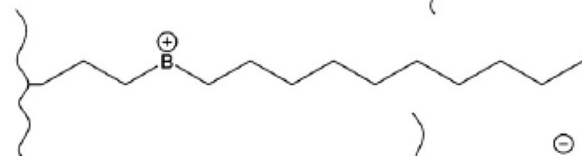
In recent years, mixed-mode stationary phases have received considerable attention by both academia and industrial research organizations. Several RP/weak anion-exchange (WAX) materials consisting of a selector immobilized onto thiol-modified silica gel have been reported [38,40–43,46]. In these phases, the WAX site is located on the outer surface of the lipophilic layer and is linked to the hydrophilic silica support via a lipophilic spacer with polar embedded amide and sulfide groups. Currently, Type I and Type II bimodal columns are not commonly used due to the performance limitations. Columns using Types III and IV media have been commercialized and positioned as both general-purpose LC columns (as an alternative to C18) and application-specific products. An example of commercial Type III columns is the Primesep® column family that each column has a dual chemistry stationary phase with a hydrophobic long alkyl chain and an ionizable cationic or anionic embedded group [35–37,48,49]. When the polar group bears a charge, it effectively shields any other less polar groups of the stationary phase. As a result, the activity of silanol groups, which cause unwanted interaction in many reversed-phase columns, is completely undetectable and does not affect the peak shape or selectivity. Commercial Type IV bimodal columns are also available, such as Acclaim® Mixed-Mode WAX-1 [45], Acclaim Mixed-Mode WCX-1 [18] and Acclaim Surfactant [44]. Structures of some RP/IEX stationary phases with distinctive chemistry designs are illustrated in Fig. 2.

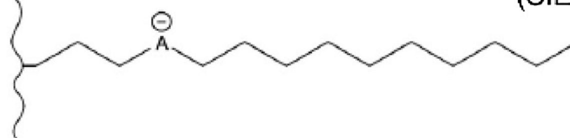
In addition to silica-based mixed-mode columns discussed above, polymer-based mixed-mode HPLC columns are also available [50,51,52–54]. The multimode separation mechanism of the OmniPac PAX-500 is achieved by coating a macroporous, hydrophobic polymer core for RP retention with an anion-exchange latex bead layer for anion-exchange retention. The macroporous structure provides an accessible hydrophobic core where reversed-phase retention occurs. The anion-exchange selectivity is provided by anion-exchange MicroBead™ latex that coats the outer layer of the hydrophobic core. Similarly, the multimode separation mechanism of OmniPac PCX-500 is achieved by coating a macroporous hydrophobic polymer core for RP retention with a CEX latex bead layer for cation-exchange retention.

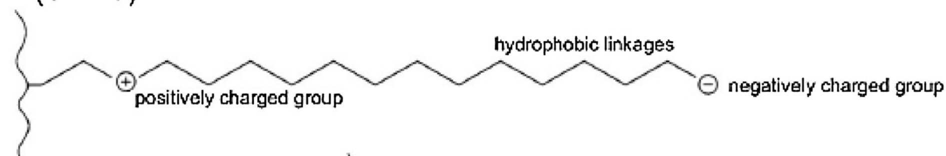
Due to the complexity and variety of analytes in hydrophilicity and ionization, it is highly challenging but desirable to separate anionic, cationic and neutral molecules within a single HPLC analysis. This separation necessitates trimodal stationary phases that can provide CEX, AEX and RP (or hydrophilic) interactions simultaneously. Fig. 3A shows that the Scherzo stationary phase by Imtakt [23] is constructed by mixing two types of bonded silica particles: one modified with C18 and CEX functionalities, and the other one with C18 and AEX functionalities. These columns are positioned as general-purpose columns for a broad range of HPLC applications

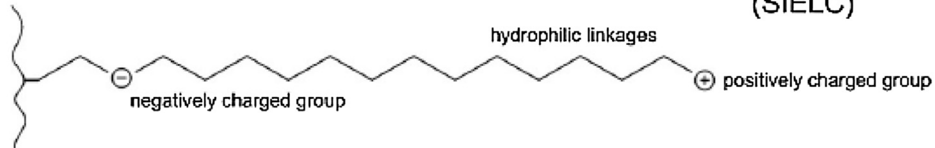


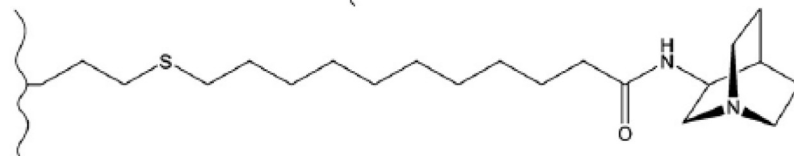



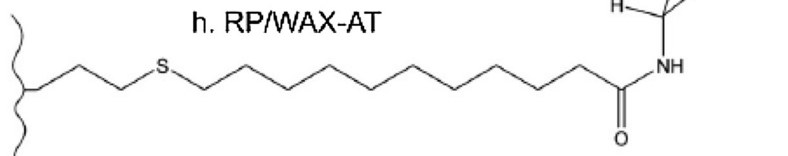



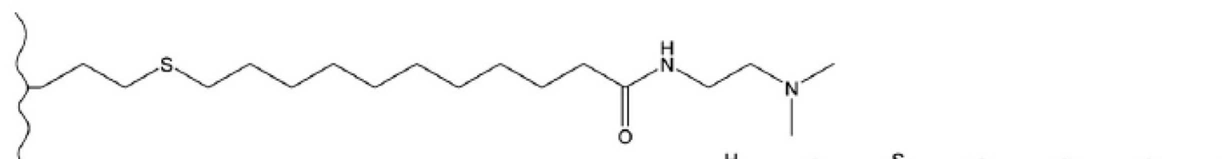



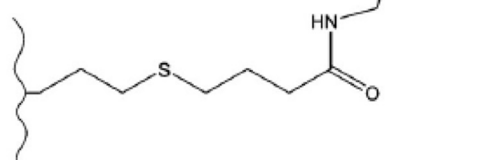




**Fig. 2.** Structures of some RP/IEX mixed-mode stationary phases.

Adapted from Ref. [20].

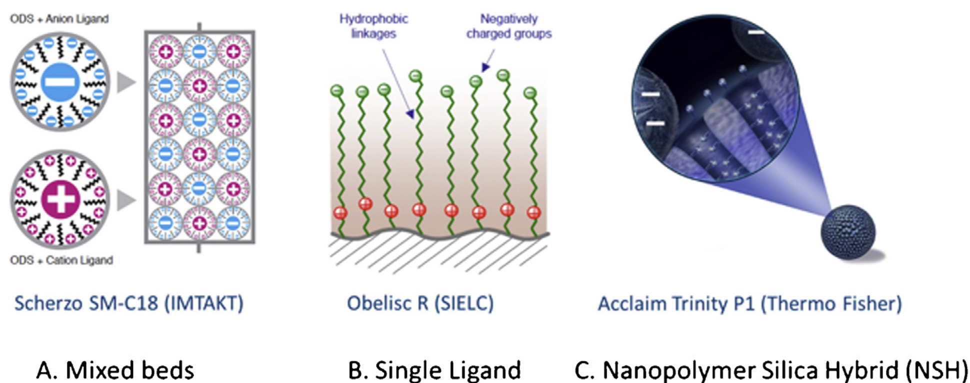



**Fig. 3.** RP/AEX/CEX trimodal mixed-mode phases. (A) Mixed-beds. Adapted from Ref. [23]. (B) Single ligand. Adapted from Ref. [21]. (C) Nanopolymer silica hybrid. Adapted from Ref. [55].

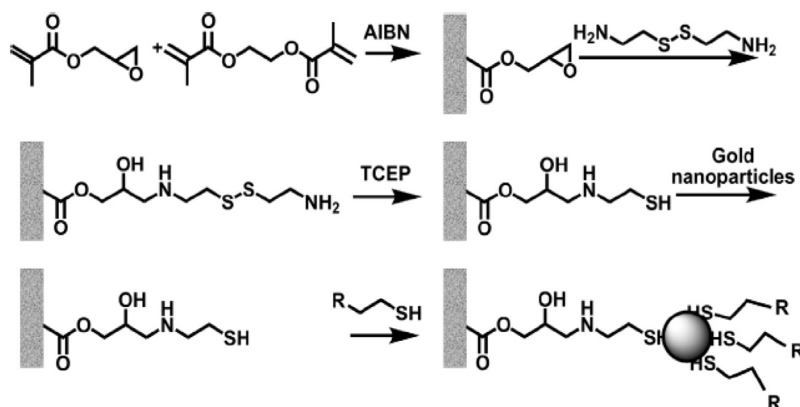



**Fig. 4.** Preparation of poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith and its modifications with cystamine, reduction with tris(2-carboxylethyl)phosphine, attachment of gold nanoparticles, and coating with 1-octanethiol.
Adapted from Ref. [65].

[56–58]. SIELC Obelisc R and Obelisc N differ in the type and proximity of their charged groups and the hydrophobicity of their long chains (Fig. 3B) [58,59]. Obelisc R has cationic groups close to the silica surface separated from anionic groups by a hydrophobic chain. Obelisc N has anions close to the surface separated from cationic groups by a hydrophilic chain. While Obelisc R is a reversed-phase analytical column and can be used in traditional, reversed-phase type applications, Obelisc N is a column which has very polar characteristics and works well for polar and charged analytes [21].

The Acclaim Trinity P1 is prepared by Nanopolymer Silica Hybrid (NSH$^{TM}$) technology through an electrostatically-driven self-assembly process. It consists of high-purity porous spherical silica particles whose inner-pore area is covalently modified with silyl ligands containing both RP and WAX moieties while the outer surface is coated with fully sulfonated nano-polymer beads by electrostatic interactions (Fig. 3C) [55]. The synthetic process was described previously [12,58]. First, spherical porous silica particles are covalently modified with a silane containing both a hydrophobic alkyl chain (for hydrophobic retention) and a terminal tertiary amine (for weak anion-exchange retention) in the same ligand to obtain the surface modified silica particles. Then nanometer-sized fully-sulfonated polystyrene-divinylbenzene polymer beads mixed with the modified silica particles under conditions in which both components are ionized, ensuring an electrostatically driven self-assembly process. As the size of nano-polymer beads is in the range of 1000–3000 Å, much larger than the pore size of silica particles (300 Å), the charged nanometer-sized polymer beads are selectively and permanently attached to the outer surface area by electrostatic attraction, but are excluded from the inner-pore area due to steric hindrance. This chemistry design creates a spatial separation of the AEX and CEX regions, preventing each charged moiety from masking the other, and allowing simultaneous RP, CEX and AEX retention. The Acclaim Trinity P2 column stationary phase is also based on NSH technology [60], but its inner-pore area is modified with a covalently bonded hydrophilic layer that also provides CEX retention while the outer surface is modified with AEX nanopolymer beads. Acclaim Trinity P2 column provides CEX, AEX and HILIC retentions simultaneously.

Monolithic stationary phases can also be used for mixed-mode chromatography separation. The ion exchange functionality is easily introduced into the polymer monolith by copolymerizing the functional monomers with the cross linkers. Methacrylic acid has been copolymerized with 2-hydroxyethyl methacrylate and ethylene dimethacrylate to produce a monolith with hydrophilic interaction and weak cation exchange (WCX) capabilities [61]. Strong anion exchange (SAX) can be introduced by copolymerizing 2-(methacryloxy)ethyltrimethylammonium methyl sulfate with different crosslinkers to generate mixed-mode RP/SAX [62] or HILIC/SAX [63] monoliths. Alternately, the IEX functionality can be introduced to the monolith by a surface modification approach. Silica-based mixed-mode monoliths are usually obtained by modifying the monolith surface with desired functionalities. For instance, amine groups have been attached on monolithic silica to create a RP/WAX mixed-mode phase [39,64]. A new approach was recently reported for the preparation of monolithic mixed-mode stationary phases for protein separation [65]. As shown in Fig. 4, the surface of monolithic poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) capillary columns was functionalized with thiols

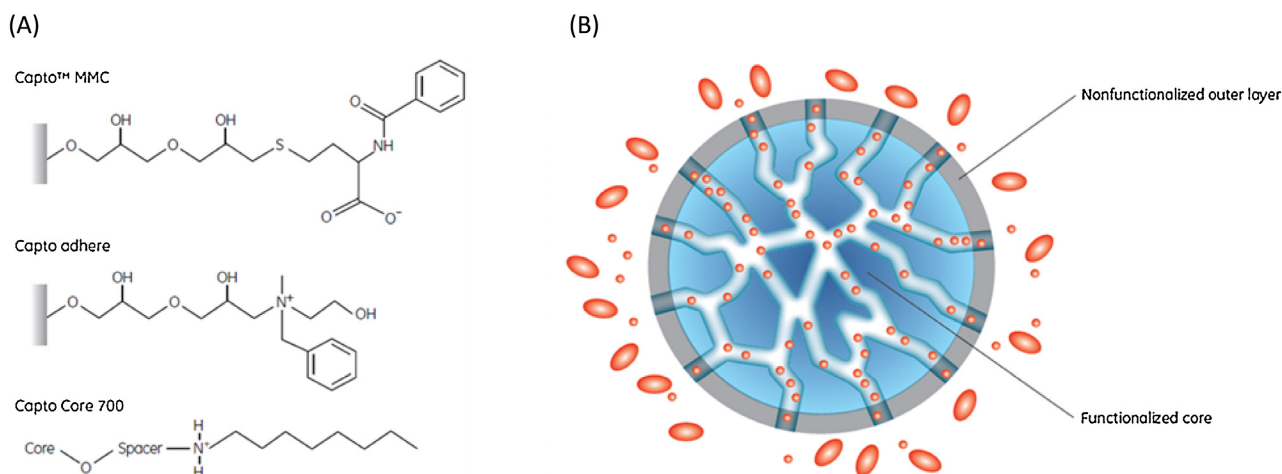



**Fig. 5.** GE Capto staionary phases. (A) Structures of Capto MMC, Capto adhere and Capto Core 700. (B) Schematic cross-sectional view of a Capto Core 700 particle. Adapted from Ref. [19].

and coated with gold nanoparticles. The final mixed-mode surface chemistry was formed by attaching, in a single step, alkanethiols, mercaptoalkanoic acids, and their mixtures on the free surface of attached gold nanoparticles. Use of these mixtures allowed fine tuning of the hydrophobic/hydrophilic balance.

The MMC technology for antibody purification was developed in the late 1950s with hydroxyapatite [66,67]. The following generations of mixed-mode media were developed after 1970 and were used in many applications [68–71]. In the 1980s, these resins were widely used for the purification of nucleic acids rather than the protein purification field. The pioneering work of Burton and Harding started the era of using "Hydrophobic Charge Induction Chromatography" or mixed-mode chromatography to purify proteins [72]. They tested numerous ligands having heterocycles known for their hydrophobicity and demonstrated that the combination of hydrophobic and ionic interactions offered new selectivity. Since then, MMC has been used to evaluate the performance of for mAb purification that does not involve protein A [73–78].

MMC using multimodal functional ligands can adsorb target proteins with the combination of ionic interactions, hydrogen bonds and hydrophobic interactions [79–81]. Mixed-mode resins can directly capture target proteins at relatively high salt concentration without dilution or other additives due to their multiple binding interactions. Currently, commercially available mixed-mode resins include Capto MMC, Capto adhere and Capto Core 700 from GE Healthcare, PPA Hypercel, HEA Hypercel and MEP Hypercel from Pall Corporation, Eshmuno HCX from Merck Millipore, Toyopearl MX-Trp-650 M from TOSOH Bioscience, and Nuvia cPrime, CHT Ceramic Hydroxyapatite, and CFT Ceramic Fluoroapatite from Bio-Rad. Varying properties of these resins such as static and dynamic binding properties [82,83], adsorption kinetics [84], adsorption selectivity [85], ligand design and molecular simulation [80,86–88] and applications [89–92] have been reported in literature.

The column chemistry of Capto MMC, Capto adhere and Capto Core 700 from GE Healthcare is shown in Fig. 5A. Capto MMC is a multimodal cation exchanger with WCX interaction, hydrophobic interaction, hydrogen bonding, and thiophilic interaction [93]. The combined effects of these interaction modes give the media novel selectivity and make it tolerant to high salt. Capto adhere is a SAX mixed-mode medium provided by *N*-benzyl-*N*-methyl ethanolamine functionality. It provides several interactions with proteins including electrostatic interaction, hydrogen bonding, and hydrophobic interaction [6,93]. Capto Core 700 ligand is found only in the core of the beads. The Capto Core concept shown in Fig. 5B is based on a bead with a nonfunctionalized outer layer (without ligand) and a functionalized core with an attached ligand. This design combines properties of gel filtration and adsorption chromatography. The bead's pores in the outer layer, with an approximate exclusion limit of 700 kD, have been specifically designed to exclude large molecular entities such as viruses, DNA, large protein, or protein complexes from entering the internal space, while small protein impurities can enter the interior of the matrix particle and bind to the ligand, thereby enabling an efficient flow-through purification step. The core of each bead is functionalized with octyl amine that is both hydrophobic and positively charged (at $pH < 10$), resulting in a highly efficient multimodal binding of various impurities over a wide range of pH and salt concentrations. This novel core bead technology gives Capto Core 700 a dual functionality with restricted access, combining size separation and multimodal binding [19].

Other forms of MMC have also been used for the separations of bio-molecules. High salt concentrations are often used to promote hydrophobic interactions between proteins and mildly hydrophobic surfaces. Studies have been done using SEC columns with a neutral hydroxyl functionality (e.g. diol) to separate monoclonal antibodies, the aggregates, or bispecific hybrids not only based on size, but also based on hydrophobic interactions resulting from the addition of salt to the mobile phase [3,17]. Zirconia based RP/IEX mixed-mode column [94] and ionic liquid HILIC/AEX MMC [95] have also been reported.

### 2.2. Mixed-mode SPE media

Mixed-mode chromatography has also been used for solid-phase extraction (SPE) sample preparation [96,97]. Most mixed-mode SPE media are polymer-based sorbents modified with ion-exchange groups, which typically enables RP and IEX interaction mechanisms. The introduction of IEX interaction allows the selective extraction of target species or the elimination of interference by adjusting the charge state of analytes and/or sorbents with mobile phase pH change. Mixed mode SPE is mainly used to clean-up samples, pre-concentrate targets, reduce matrix effects, and selectively extract acidic, neutral and basic analytes from complicated samples, including environmental wastewater, food, and biological fluids.

Mixed-mode SPE media are constructed by modifying conventional polymer sorbents, such as polystyrene (PS)–divinylbenzene (DVB) or high-surface-area DVB-based particles, with different IEX functionalities, including quaternary ammonium, sulfonic acid,

amino, and carboxylate groups, for example of Thermo Scientific Dionex SolEx SPE cartridates. To achieve efficient extraction of polar analytes, first the apolar PS-DVB particles are modified with polar groups such as hydroxyls, and then are modified with ionic functionalities, for example of Biotage EVOLUTE® EXPRESS SPE columns [98]. The polar groups counter the hydrophobicity of PS-DVB, imparting a balance of hydrophilicity and lipophilicity, and the ionic functionalities impart IEX characteristics. Strata-X is based on pyrrolidone-modified PS-DVB which is further modified with ionic groups. Strata-X-C contains a sulfonate group, Strata-X-CW contains a carboxylate group, and Strata-X-AW contains a diamine group [99]. Another class of the commonly-used mixed-mode SPE products is based on the copolymerization of lipophilic DVB and hydrophilic N-vinylpyrrolidone followed by modifying the surface with IEX functionalities to produce Oasis WCX (carboxylic acid), Oasis WAX (piperazine group), Oasis SCX (sulfonate group) and Oasis SAX (quaternary amine) [100–110]. The ionic groups can also be introduced by copolymerizing a functional monomer with cross linkers. The imidazole group in N-vinylimidazole (NVIm) −DVB copolymer not only improves the sorbent hydrophilicity, but also introduces an adjustable ion-exchange functionality as it can be protonated by decreasing the pH [111]. 2-Acrylamido-methylpropane sulfonic acid (AMPSA) can be copolymerized with 2-hydroxyethyl methacrylate (HEMA) and pentaerythritol triacrylate (PETRA) to introduce SCX groups onto the polymer sorbent [112].

Mixed-mode SPE products can also be achieved by packing a mixed bed of a special, non-polar C8 sorbent and a strong cation exchanger (e.g. Agilent Bond Elut Certify), or of a non-polar C8 and strong anion exchange sorbent (e.g. Agilent Bond Elut Certify II), or by packing strong cation exchange (SCX) and a strong anion exchange (SAX) sorbent packed into one bed (e.g. Agilent Accu-CAT) [99,113]. Graphite carbon/strong cation exchange (CARB/SCX) mixed-mode SPE has also been explored for simultaneous determination of cyanuric acid (CYA) and melamine (MEL) in dairy products [114].

Solid-phase microextraction (SPME), is a solid phase extraction sampling technique in which a fiber coated with a solid sorbent or polymeric liquid extracting phase is used to sorb a variety of volatile and non-volatile analytes from liquid or gas phase samples. Mixed-mode SPME fibers coated with C18/propylsulfonic functionalities have been used for extraction of freely dissolved basic drugs (amitriptyline and amphetamine) [115,116].

### 2.3. Separation mechanism

The retention of IEX/RP based mixed-mode stationary phases is the result of combination of RP, IEX and ion-exclusion interactions. The relative contribution of each mechanism depends on the hydrophobicity and charge state of solutes as well as chromatographic conditions such as mobile phase ionic strength, pH and organic solvent composition. A simple empirical stoichiometric displacement model can be used for discussion [117–119]. According to this model, plots of log $k$ versus log counter-ion concentration ($C$) are linear, according to the following equation [117]:

$$\log k = \log K_Z - Z \log C$$

wherein $k$ is retention factor, $K_Z$ is a constant related to the ion-exchange equilibrium constant and the ion-exchange capacity and $Z$ is the ratio of the valencies of solute ion ($s$) and counter-ion ($c$) ($Z = s/c$). The empirical linear relationship between log $k$ and log $C$ indicates the degree of ion exchange activity in a separation. The slope $Z$ indicates the number of charges involved in the ion-exchange process, whereby for a monovalent counter-ion the slope is representative for the charge on the solute. In general, all mechanisms function independently, and can be modified as needed by adjusting mobile phase ionic strength, salt type, pH, and organic solvent content [40–43,46].

Mixed-mode stationary phases can be operated in a variety of chromatographic modes, including RP mode (e.g. for neutral molecules), IEX mode (for solutes bearing the opposite charge), ion exclusion chromatography mode (for solutes having the same charge), and HILIC mode (polar neutral, basic, amphoteric, and acidic compounds), depending on the chromatographic conditions and the characteristics of the analytes. While a number of studies on retention behavior and applications in RP/IEX modes have been reported, investigations of RP/IEX mixed-mode columns in HILIC mode have been scarce. Recently, retention behaviors of RP/IEX mixed-mode columns in mobile phases with high acetonitrile content have been the subject of research of academia and industrial research [40,42,43,46,58]. To test the existence of HILIC mechanism in mixed-mode columns, methanol was used as the mobile phase organic solvent as opposed to acetonitrile. Methanol is a strong solvent in HILIC, so comparing its retention vs. solvent content behavior for a group of anlytes to that generated from acetonitrile will provide useful information for better understanding the retention mechanism of mixed-mode columns in high organic solvent conditions. Liu and Pohl investigated the retention behavior of a commercial trimodal mixed-mode column – Acclaim Trinity P1, using three model probes – uracil (neutral), sodium ion (cationic) and nitrate (anionic), in both acetonitrile/ammonium acetate and methanol/ammonium acetate buffer systems [12]. At higher solvent levels (>80%), acetonitrile exhibited significantly higher retention for uracil and sodium ion compared to methanol. For nitrate, a similar observation was noted at a 90% solvent level. Because no HILIC behavior is expected in a methanol/aqueous mobile phase, the increase in retention with increasing levels of acetonitrile is a clear indication of the HILIC effect. At 90% acetonitrile, retention decreased for charged analytes such as sodium and nitrate, indicating the presence of both ion-exchange and HILIC processes existed with the HILIC mechanism.

Due to the presence of polar IEX functionality, modern RP/IEX mixed-mode columns are capable of operating in high organic solvent HILIC mode conditions. Although more studies are needed to thoroughly understand the retention mechanism of RP/IEX mixed-mode columns under HILIC conditions, the existing evidence suggests a retention mechanism consisting of an electrostatic interaction superimposed on a HILIC interaction for highly hydrophilic ionic analytes, and a solely HILIC interaction for highly hydrophilic neutral molecules. Owing to the presence of a hydrophobic moiety, the polarity of the RP/IEX surface imparts intermediate RP interaction strength, but substantial retention for charged analytes can be realized via IEX interaction. As a result, retention of charged analytes can be tuned by adjusting mobile phase pH and ionic strength while the retention of neutral analytes is governed solely by the mobile phase solvent content [1].

## 3. Applications in pharmaceutics and biopharmaceutics

### 3.1. Counterions

Mixed-mode chromatography has gained great popularity in pharmaceutical counterion analysis in recent years [1,120,121]. Among active pharmaceutical ingredients approved by FDA, half of them are in the salt forms [122]. This is due to improved solubility, purity and polymorphism in the salt forms and the resulting gains in drug pharmacodynamics, pharmacokinetics and stability. Counter ion analysis is a key task in pharmaceutical research and quality control. Ion analysis is one of the most popular applications of mixed-mode chromatography. One big attraction of using mixed-

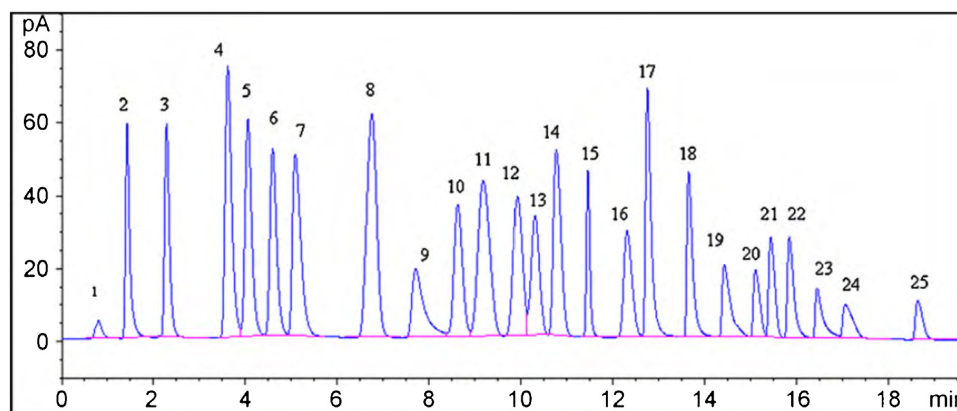



**Fig. 6.** Chromatogram of the 25 common pharmaceutical ions separated by mixed-mode chromatography with charged aerosol detector. Peak identification: 1 = lactate, 2 = procaine, 3 = choline, 4 = tromethamine, 5 = sodium, 6 = potassium, 7 = meglumine, 8 = mesylate, 9 = gluconate, 10 = maleate, 11 = nitrate, 12 = chloride, 13 = bromide, 14 = besylate, 15 = succinate, 16 = tosylate, 17 = phosphate, 18 = malate, 19 = zinc, 20 = magnesium, 21 = fumarate, 22 = tartrate, 23 = citrate, 24 = calcium, 25 = sulfate.
Adapted from Ref. [1].

mode chromatography for ion analysis is that conventional HPLC can be used, with need to purchase and maintain a dedicated ion chromatography (IC) system, typically requiring specialized mobile phase, cation or anion IEX column, ion suppressor, and a conductivity detector. Due to the low UV-absorption of most pharmaceutical counter ions, CAD and ELSD detectors are typically coupled with the mixed-mode LC for their analysis. When using an appropriate MMC column, there is no need to have separate methods for cations and anions, which calls for different ion exchange columns and instrument configuration by IC.

Huang and coworkers previously [121] reported the separation of inorganic counterions using Sequant ZIC-*p*HILIC columns by two different methods based on the analyte valence. A 150-mm column with pH 7.0 ammonium acetate buffer/acetonitrile (25/75) mobile phase was used in the analysis of monovalent ions such as $NO_3^-$, $Cl^-$, $Br^-$, $Na^+$ and $K^+$. A 50-mm column with pH 3.5 ammonium formate/acetonitrile (30/70) mobile phase was used for multivalent ions such as $Ca^{2+}$, $Mg^{2+}$, $SO_4^{2-}$ and $PO_4^{3-}$. Better accuracy was observed for $Cl^-$ in several drug substances when compared to IC. A SeQuant silica based zwitterionic column ZIC-HILIC operated in the HILIC mode in conjunction with evaporative light scattering detection was also used for the separation of pharmaceutical counterions [120]. A SeQuant polymeric zwitterionic column ZIC-pHILIC was also reported for the simultaneous analysis of cations and anions [120].

Simultaneous separation of pharmaceutical counterions has been significantly improved in recent years, attributed to the recent advance and commercialization of mixed-mode column technologies [47] [12]. As shown in Fig. 6, using the mixed-mode Acclaim Trinity P1 column separation and CAD detection, Zhang and coworkers developed a generic method for simultaneous separation of 25 commonly used pharmaceutical counterions within 20 min, including cation and anion, inorganic and organic ions in the same run [1]. As describe earlier, Trinity P1 is a silica based column with the mixed modes of RP/CEX/AEX interactions. The effects of mobile-phase organic strength, buffer ions, ionic strength, pH and column temperature were investigated to optimize the method as well as to understand the retention and separation mechanisms. The anions studied were chloride, sulfate, bromide, maleate, mesylate, tartrate, citrate, phosphate, fumarate, nitrate, lactate, succinate, besylate, malate, gluconate, tosylate. The cations studies were sodium, calcium, potassium, meglumine, tromethamine (Tris), zinc, magnesium, procaine and choline. Significant physicochemical property differences between counterions and active pharmaceutical ingredients (API) permits complete separation of API and counterions with no interference from API matrix. Fig. 7 shows the separation of counterions and their APIs. The method showed good linearity in the defined range and the accuracy was in the range of 99.0–101.0% with %RSD less than 2.0%. This method was adopted and further validated by the laboratory department of the European Pharmacopoeia (Ph. Eur.) and applied to the counterion identification and quantification in drug substances as well as for the control of inorganic ions as impurities [13]. Method performance was demonstrated by analysis of Ph. Eur. reference standards and pharmaceutical substances (e.g. cloxacillin sodium, somatostatin). This method can easily be coupled to a mass selective detector without any modification for identification of API as well as organic ions.

### 3.2. Active pharmaceutical ingredients and related substances

In the area of small molecule API analysis, RP columns still dominate the applications, but MMC has been used for polar and charged API molecules that typically cannot be handled by RP columns [123–125]. Bisphosphonates are a class of drugs that prevent the loss of bone mass. The analysis of bisphosphonate has been challenging because of these molecules are polar, ionic and most of them do not have a UV chromophore. Pre-column derivatization, indirect UV analysis, ion-pair and capillary electrophoresis (CE) methods have been used to separate and detect these molecules. Etidronate, (1-hydroxyethylidene) bisphosphonate, and its impurities phosphate and phosphite do not have a UV chromophore and are highly ionic, so it is difficult to separate these compounds by conventional HPLC columns and detected by ordinary spectrophotometric methods. Liu et al. developed a stability indicating method for the analysis of etidronate disodium and its related substances by using a mixed-mode column (Primesep SB) and charged aerosol detector [123]. Good sensitivity, accuracy and precision were demonstrated for etidronate and its impurities. Analysis of atovaquone, proguanil and related compounds in the antimalarial combination drug Malarone was accomplished using a mixed-mode column composed of 50% C18 and 50% strong cation exchanger and UV detection [126]. A RPLC/HILIC mixed-mode stationary C18-DTT (dithiothreitol) silica prepared through “thiol-ene” click chemistry was recently reported [127]. The application of the C18-DTT column was demonstrated in the separation of non-steroidal anti-inflammatory drugs, aromatic carboxylic acids, alkaloids, nucleo-analytes and polycyclic aromatic hydrocarbons. The MMC retention behaviors of Waters CSH (charged surface hybrid) stationary phases for ten pharmaceutical com-

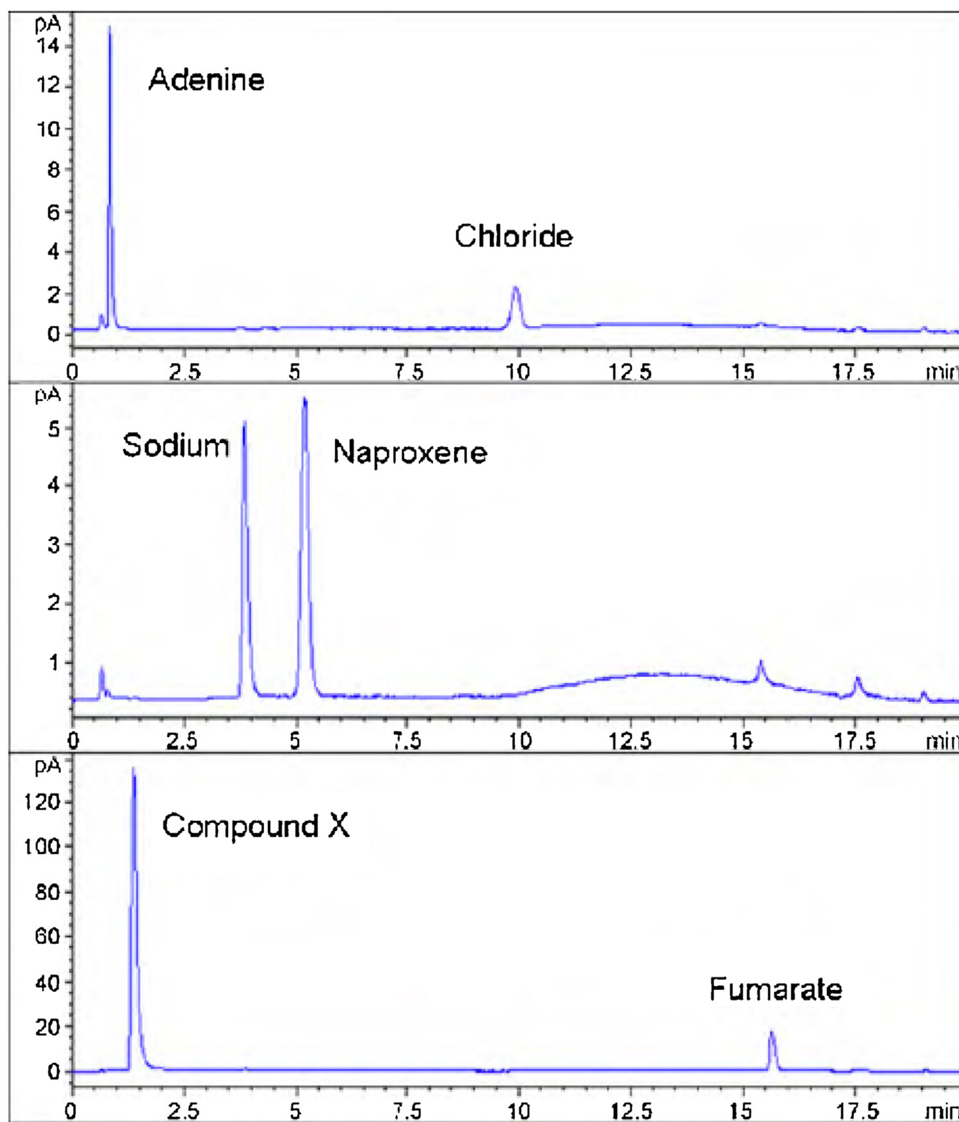



**Fig. 7.** Chromatograms of the separation of active pharmaceutical ingredients and their counterions by mixed-mode chromatography and CAD detector. (a) adenine hydrochloride; (b) naproxen sodium; (c) Pharmaceutical API X and fumarate salt.
Adapted from Ref. [1].

pounds encompassing acids, bases and neutral were reported [128], even though CSH is not marketed as mixed-mode. It was found the CSH stationary phases represent a selectivity tool preferably for separation of basic compounds.

MMC separations have been used in the analysis of challenging analytes in complex biological matrices. Cytarabine (ara-C), an anticancer agent, is highly polar and not retained on the column by typical RP chromatographic conditions. Hsieh et al. developed a mixed-mode HPLC-APCI-MS/MS assay for the determination of cytarabine in mouse plasma using a SIELC Primesep A RP/IEX column (3.2 mm × 50 mm) and a simple sample treatment procedure [124]. MMC C18/SCX was reported for determination of propranolol and furosemide in human plasma [129]. *S*-propargyl-cysteine (SPRC), a cardioprotective agent, is a sulfur-containing amino acid derivative. Like most amino acids, SPRC is highly polar and difficult to extract from biological matrices. It may also co-elute with endogenous, polar substances when use conventional RP chromatography. Zheng et al. applied a MMC method for the determination of SPRC pharmacokinetics in rats [125]. In this method, a CAPCELL PAK CR 1:4 (150 mm × 4.6 mm) mixed-mode RP/CEX column containing C18 bonded silica particles and sulfonic acid CEX particles was used. The assay utilized methanol to achieve a simple and fast deproteinization and the MS quantification was operated in multiple reactions monitoring (MRM) mode.

Phosphorylated carbohydrates are important metabolites in various central metabolic pathways. Chromatographic separation of individual isomeric forms of phosphorylated carbohydrates is critical as these isomers give similar fragmentation patterns and cannot be distinguished by MS/MS detection. However, the chromatographic separation and detection is challenging because of the hydrophilicity of phosphorylated carbohydrates and lack of strong UV-absorbing groups, which is characteristic of most carbohydrates. Hinterwirth et al. [130] reported the separation of isomeric sugar phosphates by RP/WAX mixed-mode column and charged aerosol detector. The stationary phases used were 3-aminoquinuclidine-derived and 3-α-aminotropane-derived RP/WAX mixed-mode columns. Optimal results were obtained when the column was operated under HILIC mode. Acidic conditions led to the complete separation of α- and β-anomers of glucose 6-phosphate at low temperature.

Restricted access columns allow large molecules to pass through the column quickly by hindering their access to the surface with bulky hydrophilic groups, while the small drug molecules are retained by interaction with the bonded hydrophobic group [16,131]. This type of columns is not expected to offer large plate numbers for compound separation, but it provides sufficient chromatographic efficiency for high speed LC–MS analysis. Direct injection of biological samples is desired to eliminate time-consuming cleanup steps and increase analytical accuracy. Normally, direct injection methods utilize dual-column LC systems that need one extraction column for online purification followed by an analytical column for chromatographic separation. By using MMC, a single column can perform all the functions required for direct sample analysis. Hsieh et al. applied a polymer-coated mixed-function (PCMF) column CAPCELL MF C8 for direct and simultaneous LC–MS/MS analysis of two drug candidates in monkey plasma samples [15]. Fleming [16] applied Supelco mixed-mode column Hisep for the quantification of free maytansinoid drug in DM1 antibody drug conjugate (ADC).

### 3.3. Impurities

MMC is very useful in pharmaceutical impurity profiling, especially for complex impurities with diverse physicochemical properties such as hydrophobicity, polarity and charge status. Simultaneous determination of four related impurities in a developmental Drug A was reported using a SIELC mix-mode RP/CEX Primesep 200 column [8]. The four impurities are quite structurally diverse, including a small ionic compound aminoglutarimide hydrochloride, a neutral compound containing aromatic nitro and glutarimide functional groups, and two zwitterion degradants that are geometric isomers containing aromatic amine and glutamine. The charged degrandants and Drug A were retained by both IEX and RP partitioning mechanisms, the small ionic compound was primarily retained by IEX, and the neutral compound was retained through RP partitioning without IEX. Method selectivity, sensitivity and accuracy have been demonstrated to be suitable to determine the related impurities in the capsules of Drug A.

Impurities in drug substance and drug product can also be carried from raw materials, upstream synthesis or other sources of contamination. For example, lithium containing reagents are frequently used in drug synthesis process and can be carried into final drug products. High level of lithium in blood could pose serious even lethal toxicity, so it is very important to accurately quantify residual lithium. Dai et al. recently reported a new approach to determine lithium by using MMC Acclaim Trinity P1 column coupled with CAD [132]. High sensitivity was achieved by systematically studying the retention behaviors of lithium, potential interfering ions and different type of pharmaceutical API matrices under RP, HILIC and CEX/AEX mechanisms, and selecting the chromatographic conditions that effectively reduce the interferences. Samples were directly analyzed without pretreatment such as derivatization and extraction.

By using a single mixed-mode column Acclaim Trinity P1 in conjunction with dual sample injections with different mobile phases in each run, Kazarian and coworkers were able to comprehensively separate hydrophobic and hydrophilic APIs, their counter-ions (organic, inorganic) and excipients and detect them with both UV and refractive index detectors [26]. RP and IEX interaction conditions were applied in the first injection run using a mobile phase consisting of a dual organic modifier/salt gradient to separate API, counter-ions and an unknown degradant. HILIC conditions under high organic solvent mobile phase were applied in the second sample injection run to provide retention of the saccharide excipients. The method was applied to the analysis of two pharmaceutical dry powder inhalers formulations Flixotide® and Spiriva®, which respectively contained fluticasone propionate or tiotropium bromide, and lactose excipient.

In a following study, Kazarian et al. [5] coupled a mixed-bed column and a HILIC column with dual sample injections and used three detectors in series, UV-conductivity- RI to profiling the components of pharmaceutical formulations including Robitussin® cough syrup. A switching valve was used after the pump to switch the mobile phase between two columns. The mixed-bed column Imtakt Scherzo SS C18 provided separation of inorganic anions and cations under isocratic conditions, followed by a dual organic/salt gradient to elute APIs and their respective organic counterions and potential degradants. Then, the mobile phase flow was switched to the HILIC column Shodex Asahipak NH2P-50 4E and the samples were re-injected for the separation of hydrophilic excipients carbohydrates. In this respect, MMC is somewhat like 2D-LC. Additional separation dimension can be added even using the same single column, but changing the mobile phases according to the separation and retention purpose. Wang and coworkers developed a mixed-mode C18/Diol column and applied it to the profiling of the extract of traditional Chinese medicine *Lonicera japonica* [27]. A single column and a conventional HPLC were used with the addition of a six-port, two-position valve controlled sample loop. RP separation mobile phase condition was applied first, and the poorly retained co-eluting hydrophilic components of the extract of *Lonicera japonica* were collected into the sample loop and re-injected online for the second step HILIC separation by conveniently varying the mobile phase components.

Mixed-mode SPE systems are often more advantageous and provide better separation than standard RP or IEX sorbent systems alone. Mixed-mode SPE is a simple but powerful technique to quickly extract and concentrate impurities for MS structure identification of pharmaceutical impurities and degradations, especially for drug forced degradation and excipient compatibility studies. Landis reported the protocols of using mixed-mode CEX SPE Oasis MCX cartridges and AEX SPE Oasis MAX cartridges, and applied them to identify the degradation products and understand the degradation pathways of the hydrolysis of pharmaceutical compounds benzocaine and bezafibrate and oxidation of chlorpromazine and benzocaine [133].

### 3.4. Formulation excipients

Many formulation excipients are highly water soluble and/or charged, so typically they are not retained well on the RP columns and elute at the void volume. Using mixed-mode column to retain and separate these excipients in the presence of high concentration of drug matrix has gained increasing attention in recent years.

Non-ionic surfactants such as polysorbate 20 or 80, in the trade name as Tween 20 or 80, are commonly used in protein drug formulations as excipients or vehicle to increase drug solubility and stability. Due to the strong interference from the high concentrations of proteins and the molecular heterogeneity nature of the polysorbates, the quantitation of polysorbates in protein drug formulations has been challenging and typically requires lengthy sample preparation. Hewitt et al. developed a method using a mixed-mode stationary phase Waters Oasis MAX (mixed-mode anion-exchange and reversed-phase sorbent) and evaporative light scattering detection (ELSD) detector to quantify the total polysorbate in protein formulations [134,135]. Proteins are not retained in the Oasis Max column because of electrostatic repulsions from the quaternary amine in the mixed-mode resin. Hydrophobic polysorbate 20 is retained, eluted with a step gradient and quantified as a single peak. The assay method was qualified using two monoclonal antibodies (mAbs) in terms of accuracy (96–108%), repeatability (2.3% RSD) and linearity ($r2 > 0.999$). A total of 25 unique proteins ranging from 25 to 150 kDa were analyzed with this assay.

Mixed-mode column was used in the first dimension of a multidimensional UHPLC-CAD-MS method to separate the interference from the high concentration of mAbs to further characterize the heterogeneity and stability of polysorbate 20 [24]. A Waters Oasis Max column that has both anion-exchange and reversed-phase properties was used in this study. A two-step elution was employed, with the acidified high aqueous mobile phase used in the first step to wash out the positively charged proteins while retaining the neutral polysorbate, and a high organic mobile phase in the second step to elute all of the polysorbate esters.

He and coworkers used on-line coupling of a size exclusion column with a mixed-mode column for the comprehensive profiling of biopharmaceutical drug products [2]. Proteins and excipients were separated by a SEC column. A switching valve was used after the SEC column to switch the later eluted excipient peak into the Trinity P1 mixed-mode column to separated different excipients including cations $Na^+$ and $K^+$, anion $Cl^-$, nonionic hydrophobic surfactant polysorbate 80 and hydrophilic sucrose. The applications of the method were demonstrated in the analysis of mAb, ADC and vaccine drug product samples.

MMC can add dimensionality to a separation by using a single column with stepped mobile phase elution program that promote a certain interaction mode in a certain elution step. In this respect, MCC is an alternative technique for 2D-LC. Kazarian and coworkers used a single Acclaim Trinity P1 column and a dual injection approach to comprehensively separate APIs, their counter-ions, degradation products and excipients by the combined separation modes of RP, IEX and HILIC [26]. A dual buffer and organic solvent gradient was used to elute API, counter-ions and an unknown degradant. A second sample injection was applied in the middle of the chromatographic run when the high organic buffer HILIC condition was established on the column, so the retention of the excipient lactose was achieved. The method was applied to two pharmaceutical formulations Flixotide and Spiriva.

### 3.5. Drug in environmental samples

Mixed-mode solid phase extraction has found a large application in the analysis of drugs in environmental samples. MMC is used as the sample cleaning and concentrating tool to remove matrix interference and increase method sensitivity. Most of the published methods for the determination of illicit drugs in water environmental samples employ SPE as the pre-concentration technique. Oasis MCX [7,22,136] and Oasis MAX [22] [137] are used frequently to extract the drug followed by LC–MS analysis. New mixed-mode SPE material has also been applied [138].

Gonzalez-Marino et al. developed a selective mixed-mode SPE and LC-QTOF-MS method for the simultaneous determination of 24 drugs of abuse and metabolites in urban sewage samples [7]. Analytes were concentrated using mixed-mode Oasis MCX sorbents, improving the selectivity and sensitivity for basic drugs by adopting a fractionated elution strategy, which allowed a significant reduction of matrix effects observed during electrospray ionization of basic drugs.

By using a similar approach, Casado et al. assessed the capability of the OASIS MCX sorbent for the extraction of basic antimycotic drugs from environmental water samples [136]. It was found the mixed-mode SPE improved the selectivity of the concentration process, in comparison with the RP sorbents. The use of a sequential elution SPE protocol allowed the removal of neutral and acidic interferences in the sample which led to a significant reduction of ion suppression and variation during electrospray ionization. The mixed-mode SPE protocol followed by LC-QTOF-MS determination provided LOQs low enough for the selective and unambiguous determination of target compounds in sewage treatment plants, such as fluconazole, clotrimazole, ketoconazole and miconazole, and the non-target antimycotic drug climbazole.

A recent study reported the simultaneous determination of six non-steroidal anti-inflammatory drugs (NSAIDs) in environmental water samples by mixed-mode SPE and LC-QTOF-MS [137]. The method involved an off-line mixed-mode (RP/SAX) SPE for the selective concentration of COXIBs. It was found the use of a strong SAX sorbent (Oasis MAX) led to a significant reduction of matrix effects, during electrospray ionization (ESI), in comparison with results obtained for mixed-mode WAX sorbent (Oasis Wax) and polymeric RP sorbent (Oasis HLB and Strata X). Among the pharmaceuticals investigated, celecoxib and etoricoxib were detected at low levels (ppt) in treated and raw wastewater samples, and two metabolites carboxylated celecoxib and the hydroxylated etoricoxib were also found.

### 3.6. Drug in biological samples

In addition to using MMC as primary analytical columns to analyze small molecule drugs in biological samples [15,125,129] as discussed in Section 3.2, clean up the biological matrix interference using mixed-mode SPE is an effective way to sensitively determine drugs in biological samples. Murkitt et al. earlier reported the use of a mixed mode SPE followed by RPLC-UV to assay GR117289X (an angiotensin II receptor antagonist) in human plasma [139]. The mixed-mode SPE Bond Elut Certify II@ employed is a chemically modified silica gel material supporting three different types of interactions: hydrophobic, polar and anion ion exchange. The extraction procedure has been fully automated by a Zymate XP robot and linked on-line to the HPLC system. The extraction efficiency of the assay was approximately 75%. The assay method was applied to the analysis of GR117289X in plasma of volunteers from a number of clinical studies and has been shown to be robust in sustained use over several months. Determination of dopamine and 3,4-dihydroxyphenylacetic acid in mouse striatum using mixed-mode RP/CEX column CAPCELL PAK CR followed by LC-fluorescence detection was reported [140].

Mixed mode SPE sample treatment can significantly reduce the matrix interference during MS analysis and improve drug recovery. SPE-LC–MS has been applied to the identification and determinations of metoprolol and its metabolites in horse urine and blood [141], nicotinic acid in plasma [142], opioids, cocaines, amphetamines and adulterants in human blood [143], cytarabine in mouse plasma [124], corticosteroids in bovine milk [144], and paroxetine, fluoxetine and norfluoxetine in fish tissues [145]. Gaboxadol is a small, polar compound and a zwitterion. Attempts to develop an applicable method for bioanalysis utilized a time consuming pre-column derivatization. Kall et al. extracted gaboxadol from plasma by mixed mode solid phase Waters Oasis MCX extraction and then analyzed it on an Asahipak NH2P HPLC column under HILIC condition with tandem MS [146]. The validated method was applied to the quantitative determination of gaboxadol in human heparinized plasma.

SPE using Biotage Evolute CX mixed-mode resin cartridges was reported for clean up the blood samples, and combined with LC–MS using QTRAP mass spectrometry in MRM mode and product ion spectra in the linear ion trap mode for opioids, cocaines, amphetamines and adulterants analysis [147]. The cartridge is a mixed-mode resin based cation exchange SPE sorbent with an optimized pore size that minimizes retention of high molecular weight matrix components. a number of 18 drugs were analyzed in human blood, including morphine, codeine, 6-monoacetylmorphine, cocaine, benzoylecgonine, dihydrocodeine, cocaethylene, 3,4-methylenedioxyamphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine,

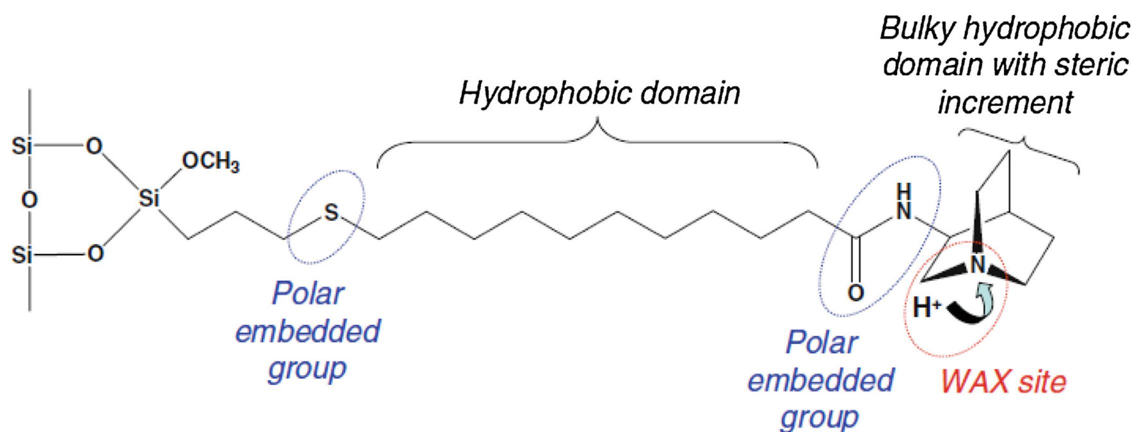



**Fig. 8.** Structure of mixed-mode reversed-phase/weak anion exchange (RP/WAX) stationary phase utilized in Ref. [158].

benzylpiperazine, methamphetamine, amphetamine, methadone, phenethylamine and levamisole.

Recently a mixed-mode C18/SCX SPME coating that combines octadecyl and propylsulfonic acid groups as strong cation exchange sites, was used to measure freely dissolved concentrations of cationic drugs amitriptyline, amphetamine, diazepam and tramadol to different binding matrices, including bovine serum albumin (BSA), human serum albumin (HSA), human plasma and human whole blood [148].

### 3.7. Peptides

Mixed-mode column separation of peptides was recognized by Hancock in 1981 [10] during the development of RP stationary phases, which contains significant concentrations of both free silanol and hydrocarbon groups. Zhu et al. reported the mixed-mode HILIC/SCX separation of peptide when using SCX columns [149,150]. Bell et al. [151,152] explored the ion-exchange characteristic of a pentafluorophenylpropyl-bonded phase (Discovery HS F5) and reported a “U-shape” relationship between retention and organic modifier percentage; the ion-exchange properties were attributed to the ionized silanols of the stationary phase. Gilar [153] reported a silica sorbent with a pentafluorophenyl (PFP) ligand that demonstrated mixed-mode RP/SCX interactions, and it is believed that the silanols in the vicinity of the perfluorinated ligand act as strongly acidic sites. The separations of peptides, phosphopeptides, and sialylated glycopeptides were demonstrated. Alpert [154] reported that when a predominantly organic mobile phase is used, IEX column can retain solutes through hydrophilic interaction even if they have the same charge as the stationary phase, and termed this combination as electrostatic repulsion-hydrophilic interaction chromatography (ERLIC). By using this mixed-mode separation, phosphopeptides can be isolated selectively from a tryptic digest. Yates [155,156] used a biphasic SCX/RP capillary column that was first packed with Zorbax Eclipse XDB C18 and then with Partisphere strong cation exchange (SCX) coupled with MS/MS for proteomic analysis of peptides.

More applications have been reported in recently years using stationary phases purposely designed to have multiple functional groups to provide different interaction mechanism. Nogueira et al. [157] reported a mixed-mode RP/WAX stationary phase based on *N*-(10-undecenoyl)-3-aminoquinuclidine selector, which is covalently immobilized on thiol-modified silica particles by radical addition reaction. The stationary phase has hydrophobic RP domains through the alkyl chains and hydrophilic WAX domains through the cationic sites, which also enables repulsive ionic interactions with positively charged functional groups, leading to ion-exclusion phenomena. This mixed-mode phase was applied to the separation and purification.of the N- and C-terminally protected tetrapeptide *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide from its side products. Better selectivity and enhanced sample loading capacity in comparison to RP-HPLC (gradient of ACN containing TFA as ion-pairing agent) was demonstrated. The yield was improved by a factor of about 15 higher compared to the standard gradient elution RP purification protocol.

Lämmerhofer et al. [158] introduced a RP/WAX mixed-mode silica based phase devised for peptide separations. As shown in Fig. 8 the stationary phase contains a hydrophobic alkyl strand with polar embedded groups (thioether and amide functionalities) and a terminal weak anion-exchange-type quinuclidine moiety. It demonstrated that depending on the solute properties and mobile phase conditions, the column can be used in RP (neutral compounds), AEX (acidic compounds), ion-exclusion mode (cationic solutes), HILIC (polar compounds), and HIC (e.g., hydrophobic peptides) modes.

### 3.8. Proteins

In this section, we review the recent advances of the MMC in the therapeutic protein and related area. Readers are encouraged to read earlier reviews for ligands for mixed-mode protein chromatography [14], and its applications to biopolymer [159].

Protein aggregation can happen during biopharmaceutical manufacturing processes, formulation processes, shipping and storage. Aggregation may cause side effects and reduce the efficacy of the products, and have the potential to induce undesirable immunological responses. Aggregation is a critical quality attribute of therapeutic protein products. It is important to effectively remove, monitor and control mAb aggregates. Gao et al. [6] evaluated antibody monomer separation from associated aggregates using three mixed-mode resins, Capto adhere (CA) and two home-made resins with benzylamine and butylamine as the functional ligands (named BA and AB). It was found that the removal efficiency was highly dependent on the mass loading. With the sample load of 50 mg/ml resin both Capto adhere and BA resins can significantly reduce the aggregate level from 20.5% to 2.6% and 2.4%, respectively. The results indicate that both hydrophobic interaction and electrostatic interaction are critical for the aggregate removal and that the cooperation of different molecular interactions is important for the effective aggregates removal with mixed-mode resins.

Misfolding and aggregation frequently occurs during production of recombinant proteins. Misfolding species are made of the same protein and may have similar electrostatic charges and hydrophobicity, and therefore could be difficult to be separated from the

native species. The isoform separation of proteins by mixed-mode chromatography has been reported [160]. Two GE mixed-mode resins, anion-exchange Capto adhere and cation-exchange Capto MMC are applied to two model proteins, etanercept, which has been shown to misfold and bovine serum albumin (BSA) that has been shown to aggregate. The mixed-mode ligands have a chemical structure that provides different interaction modes, primarily electrostatic and hydrophobic/aromatic for protein binding. Effective separation of the misfolded etanercept species and BSA oligomers has been developed in this study using elution conditions that combine pH change and NaCl or arginine at different concentrations.

The purification of the recombinant allergen rBet v 1a was investigated using a family of three mixed-mode sorbents, namely, HEA HyperCel (hexyl amine), PPA HyperCel (phenyl propyl amine) and MEP HyperCel (mercapto ethyl pyridine) [161]. Because it is difficult to predict the protein binding and desorption conditions of mixed-mode sorbents with multi-modal interactions, the screening of sorbents was carried out on a high throughput platform in a 96-well microplate combined with SELDI-MS. The results showed the capture and 9-fold purification of rBet v 1a in a single chromatography step on HEA or PPA HyperCel sorbents without the pre-treatment of the crude E. coli feedstock, saving the cost and time of an extra step often necessary prior to IEX (i.e. dilution or diafiltration) or conventional HIC (addition of lyotropic salts).

Studies also show that proteins of lower stability may exhibit unfolding and aggregation during IEC and MMC separations, as they can with hydrophobic interaction chromatography. This could lead to decreased yield and product degradation. Gospodarek et al. [162] using hydrogen exchange mass spectrometry, investigated the unfolding behavior of the model protein BSA on IEX and MMC (Capto TMMMC and Capto TMAdhere) resin surfaces under different solution conditions at 25 °C. The study suggested that adsorbed BSA unfolds at lower pH values and may show aggregation, and it is the cationic moieties, rather than the hydrophobic ligands, which cause greater surface unfolding at low salt concentrations.

It is important to characterize therapeutic antibody heterogeneity due to the fact that structural heterogeneity often affects the bioactivity and efficacy of a drug. Heterogeneity of mAbs can come from post-translational modifications as well as inherent modifications during manufacturing process and storage conditions. Modifications that result in structural heterogeneity include but are not limited to incomplete disulfide bond formation, glycosylation, isomerization, C-terminal lysine processing, deamidation, and oxidation etc. [163,17]. Measuring oxidative variants can be challenging due to its similarities to the native antibody molecule in size, charge, and hydrophobicity. Wong et al. developed size exclusion – ultra performance liquid chromatography (SE-UPLC), employing a Waters Acquity BEH200 size exclusion column along with a mobile phase consisting of sodium acetate and sodium sulfate that separates IgG into aggregate, monomer, and fragment [17]. The mixed-mode retention behavior of SEC column was observed when use of a moderate salt concentration based on hydrophobicity, resolved a Trp-oxidized IgG monomer pre-peak from the monomer main peak. Method qualification of the mixed mode UPLC method showed good recovery for the spiked monomer pre-peak and Fab fragment. However, the recovery of spiked dimer was low. This method is suitable for the determination of variant monomeric IgG species.

Recently, Yang et al. employed a mixed-mode chromatography using a Sepax Zenix SEC-300 column to separate an IgG4 bispecific hybrid from the parental antibodies [3]. Comparing to other available methods that mostly necessitate labeling or alteration of the model IgG4 molecules, or rely on time-consuming immunoassays and mass spectrometry, the mixed-mode method not only allow the analysis of the bispecific hybrids to study half molecule exchange of in vitro as well as in vivo samples, but also allow the isolation of hybrid antibodies from intact parental antibodies.

Porous polymer monolithic columns with gold nanoparticles for the separation of proteins in RP-IEX mixed mode was recently reported [164]. It was demonstrated the mixed mode character of these monolithic stationary phases in the separations of proteins that could be achieved in the same column using gradient elution conditions typical of reverse phase (using gradient of acetonitrile in water) and ion exchange chromatographic modes (applying gradient of salt in water), respectively. The purification of rabbit polyclonal immunoglobulin G using mixed mode chromatography SepFast™ MM AH-1 was recently reported [165]. A three-step process involved two mixed mode resins (HEA HyperCel and Capto MMC) and an anion exchange membrane polishing was recently reported for the purification of mAb from CHO cell culture supernatant [4].

## 4. Conclusion and perspectives

Mixed-mode chromatography provides unique selectivity especially for polar and charged analytes. The multiple interaction mechanisms allow the users to adjust the mobile phase/eluent conditions to promote certain interactions for certain analytes. MMC has advanced from original “secondary interaction” to purposely designed and controlled multi-mode interactions. The new generation of mixed-mode stationary phases is more robust and diverse. The recent commercialization of mixed-mode stationary phases greatly enhanced the applications of MMC.

MMC has been successfully applied in pharmaceutics for counter ion analysis, polar and/or charged APIs, impurities, formulation excipients, and environmental and biological samples. Mixed-mode sorbents, SPEs have been effectively applied in sample matrices cleanup and increasing LC–MS sensitivity. MMC plays an important role in peptide and protein analysis and purification, antibody aggregation and heterogeneity characterization.

MMC can replace IEX in many applications and allow using MS compatible mobile phases. MMC is superior to RP for some applications, but mostly for the analytes that are not retained and separated well by the traditional RP columns. Although MMC has been applied in impurity profiling of pharmaceutical samples, it is mainly for impurities that have diverse properties. MMC is not used as a primary tool replacing RP for assay and impurity method in pharmaceutical industry yet for general applications. However, it is highly desirable to have such a mixed-mode column that not only separate inorganic ions polar and charged analytes and organic molecules, but also provide the resolution and peak capacity that comparable to RP columns to separate structurally similar compounds.

While MMC provides an increased number of tunable variables that provides more flexibility in method development and more applications to a variety of analytes, it also complicates the method development. Method development of MMC is more time consuming and less straight forward comparing to any of the individual chromatography modes alone. Platform methods and protocols are efficient approaches to apply MMC in pharmaceutical and biopharmaceutical analysis. Further understanding of the separation mechanisms of MMC is necessary to help method development.

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