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Gradient elution of ionic compounds

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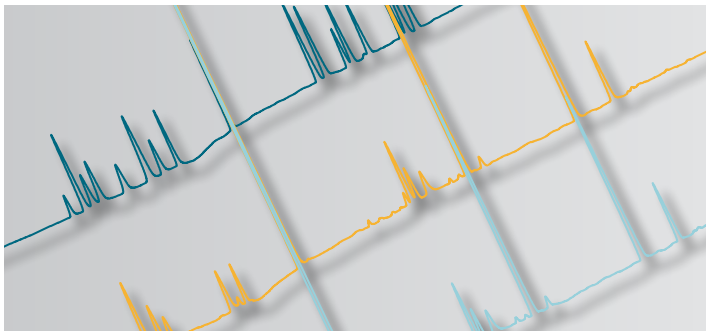


Abstract

In conventional liquid chromatography, the gradient elution of compounds is a well-established technique with a vast number of applications. For example, RPLC in combination with variable detection methods allows the separation of compounds with widely different retention behavior in a single run by changing the amount of organic solvent in the mobile phase. Until the late 1980s, gradient elution techniques had found only limited application in the field of ion chromatography with conductivity detection, primarily because the most important inorganic anions and cations can be separated and determined under isocratic conditions. Conversely, a variation of the ionic strength in the mobile phase during the chromatographic run is inevitable if inorganic and organic anions or cations or ions with different valencies are to be analyzed in a single run. With the introduction of modern high-capacity membrane suppressors the problem of significant changes in background conductivity could be solved. In addition, the application of gradient techniques in anion-exchange chromatography is hampered by a second problem caused by carbonate impurities in the hydroxide eluents, which may severely interfere with the analysis due to baseline drifts and strongly varying retention times of early eluting anions. Utilizing electrolytic eluent generation, the problems of gradient elution in ion-exchange chromatography could finally be solved, so that these techniques are as common in modern ion chromatography as in the field of conventional HPLC. Even though concentration gradients in the chromatography of ionic components are most widely used nowadays, there are numerous other examples for composition, capacity, and pH gradients. Capacity gradients on cryptand-modified stationary phases allow the elution of nonpolarizable *and* polarizable anions in the same run, which is extremely difficult, if not impossible, using conventional anion exchangers. pH gradients are very important for the analysis of recombinant therapeutic proteins such as monoclonal antibodies. Cation-exchange chromatography is currently one of the most popular methods for charge variant analysis. While conventional cation-exchange methods use an eluent of increasing ionic strength to elute the protein from the separator column, the use of a pH gradient to assess charge heterogeneity of monoclonal antibodies offers the key advantage that a single pH method can be applied to a series of monoclonal antibodies (mAbs) having a wide range of isoelectric points. A fundamental problem of ion-exchange chromatography is the simultaneous analysis of anions and cations, which cannot be carried out using conventional anion or cation exchangers. Silica-based mixed-mode phases solve this problem, supporting hydrophilic or hydrophobic and electrostatic interactions at the same time. Mixed-mode phases are especially suitable for applications, which are not possible with either ion-exchange or reversed-phase chromatography.

Due to the special morphology of mixed-mode phases the selectivity of these columns is complementary in comparison with conventional ion exchangers and reversed-phase columns. Mixed-mode columns are suitable, for instance, for the simultaneous separation of active pharmaceutical ingredients (APIs) and their counter ions. Gradient techniques based on volatile buffers with acetonitrile as the organic modifier will be shown that have been developed for this application.

Introduction

Gradient elution is a well-established technique in conventional reversed-phase liquid chromatography for method development as well as for quantitative analysis of compounds with a widely different retention behavior by increasing the strength of the mobile phase during the chromatographic run. We differentiate between continuous and discontinuous gradients, the latter one being most commonly referred to as stepwise elution or step gradient. The two most important advantages of gradient elution are the increase of peak capacity (the number of peaks separated to baseline in a given amount of time) and peak compression (focusing effect). The latter is the result of the permanently increasing elution strength of the eluent during the gradient run, compensating the band broadening effect to some extent, so that late eluting peaks are almost as narrow as early eluting ones. The great potential of gradient elution techniques for the analysis of complex mixtures has already been recognized in the early 1950s by pioneers such as Hagdahl *et al.*[1] and Donaldson *et al.*[2]. Over many decades, this technique has matured extremely well, resulting in a large number of applications as outlined in the book by Snyder and Dolan[3].

The concept of ion chromatography (IC) introduced by Small *et al.*[4] in 1975 only described isocratic separations of inorganic anions and cations, because the separation of the most important inorganic anions such as fluoride, chloride, nitrite, bromide, nitrate, orthophosphate, and sulfate (referred to as standard anions) did not require gradient elution. The same was true for the analysis of alkali and alkaline-earth metals, which were also separated at that time under isocratic conditions, respectively. However, separations of complex samples by gradient elution were not possible at that time for one reason: the packed-bed suppressor columns used by Small *et al.* to lower background conductance by converting the eluent into a less conductive form did not provide sufficient suppression capacity. In addition, the use of gradient elution in anion-exchange chromatography was hampered by a second problem as the commonly used carbonate/bicarbonate eluent is converted to carbonic acid in the suppressor device. Since carbonic acid is in equilibrium with bicarbonate, an increasing carbonate/bicarbonate eluent concentration results in a severe baseline drift when using a conductivity detector, a problem for which no solution existed in the early days of ion chromatography. Rocklin *et al.*[5] were the first to introduce a high-capacity membrane-based suppressor that paved the way for using hydroxide mobile phases in anion-exchange chromatography, which is a more suitable eluent for gradient elution because it is converted to water independent of its concentration. For this reason, Hamish Small originally experimented with hydroxide eluents for anion-exchange chromatography but could not solve the problem of carbonate impurities in manually prepared hydroxide mobile phases. Since carbonate accumulates at the stationary phase, it interferes with the analysis, because carbonate has a higher eluting strength than the hydroxide eluent. This effect results in strongly varying retention times, especially of weakly retained anions.

Only with the introduction of electrolytically generated mobile phases by Liu *et al.*[6] in 1998, outlined below in more detail, gradient elution techniques in ion-exchange chromatography became stable and thus practical, and are as easy to perform as isocratic separations. Although concentration gradients are the most dominating type of gradients in IC, other types of gradients such as composition, capacity, and pH gradients are also applied today.

Gradient types in ion chromatography

As mentioned above, the most common type of gradient in ion-exchange chromatography is a concentration gradient, in which the eluent ion concentration is increased during the chromatographic run, very often interrupted by isocratic steps to better separate critical pairs of analytes. Depending on the application, the gradient profile can be linear or curved (convex or concave). Convex gradient profiles, for instance, are applied for the separation of polyphosphates as the separation of higher oligomers is only successful with a very small increase in eluent ion concentration, while the first members of this homologous series, orthophosphate and pyrophosphate, have a widely different retention behavior, which can be addressed by a rapid increase in eluent ion concentration at the beginning of the run. Conversely, concave gradient profiles are typically used to more rapidly elute very strongly retained ions which otherwise would elute much later than, for instance, a group of earlier eluting analytes exhibiting a similar retention behavior.

In contrast to concentration gradients, composition gradients are less common in ion-exchange chromatography, because they present a problem in practical application due to the change of the type of eluent ion during the chromatographic run. Furthermore, the affinity of the two different eluent ions must differ significantly to cover the entire retention room between weakly and strongly retained analyte ions.

Thus, column re-equilibration after the gradient run is time-consuming as the eluent ions with a high affinity toward the stationary phase have to be replaced by eluent ions with a low affinity. When applying a concentration gradient, this problem is not observed, because the ionic form of the resin (type of counter ions present on the functional groups of the resin material) does not change.

A very unique type of gradient utilized in modern ion chromatography is a capacity gradient[7] performed on cryptand-modified stationary phases. Cryptands are bicyclic polyethers bearing a three-dimensional cavity, which has approximately the same diameter than that of a potassium ion. When using a potassium-containing mobile phase, potassium ions are diffusing into the cavity forming a cryptand-potassium complex that can serve as an anion-exchange site. However, an anion such as hydroxide has to be associated with the positively charged cryptand-potassium complex to maintain electrical neutrality. Woodruff *et al.*[7] were the first who developed a poly(styrene-*co*-divinylbenzene) substrate with covalently bonded cryptand 2.2.2 moieties. Since potassium ions have a much higher binding constant in comparison with sodium or lithium, a potassium hydroxide eluent is typically used for high-capacity applications. Under isocratic conditions, however, multivalent anions such as sulfate and orthophosphate are very strongly retained. However, retention times can be shortened significantly by applying a capacity gradient. For this purpose, the cationic counter ion in the hydroxide eluent is changed for sodium at the time of sample injection. Over time, the potassium ions are more and more replaced by the less binding sodium ions, resulting in a much lower ion-exchange capacity. When switching from a potassium (or sodium) hydroxide eluent to lithium hydroxide, column capacity is almost zero, so that strongly retained anions are eluted much faster. With this approach, polarizable and nonpolarizable anions can be analyzed in the same chromatographic run, which is extremely difficult even with modern conventional anion exchangers.

Occasionally, pH gradients are applied for the analysis of low-molecular weight ions (<500 Da). A typical example is the addition of a strong base to a weak acid, which might also be considered as a concentration gradient as the concentration of the dissociated acid increases during the run by increasing pH. Nowadays, pH gradients are much more important for the chromatographic characterization of therapeutic proteins such as monoclonal antibodies (mAbs). Charge variant analysis, for instance, is almost exclusively carried out by cation-exchange chromatography as stated in the review article by Vlasak and Ionescu[8]. In the past, eluents of an increasing ionic strength were predominantly used to elute the protein from the cation exchanger, until Farnan and Moreno[9] found out that pH gradients exhibit a better peak capacity and thus a higher resolving power. In addition, a single pH gradient method for assessing charge heterogeneity can be applied to a number of mAbs differing in their isoelectric points.

Choice of eluents

For the successful application of concentration gradients in combination with conductivity or mass spectrometric detection, the use of a suppressor system is mandatory to reduce background conductivity to a very low level when increasing eluent concentration during the gradient run. Nonsuppressed conductivity detection cannot be applied, because the resulting background drift is by far too strong. Eluent suppression can be carried out chemically utilizing micromembrane suppressors or electrolytically with self-regenerating suppressors[10], both of which provide the high suppression capacity required for gradient elution techniques. The most suitable eluents for concentration gradients in anion-exchange chromatography are based on hydroxide, which are converted to water in the suppressor independent of the initial concentration. The resulting background conductance is typically around 0.5 µS/cm. Very important in this context, however, is the quality of the deionized water for preparing mobile phases; the specification for its resistivity of 18.2 MΩ·cm is a minimum requirement to achieve such low background conductance of the suppressed eluent.

Carbonate/bicarbonate eluents are totally unsuitable for concentration gradients in anion-exchange chromatography, because the suppression product is carbonic acid, which partly dissociates into bicarbonate according to Eq. (1):

$$H_2CO_3 + H_2O \rightleftharpoons H_3O^+ + HCO_3^- \quad (1)$$

Thus, when increasing the carbonate/bicarbonate eluent concentration, background conductance rapidly increases due to the rising bicarbonate concentration in the suppressor effluent. Moreover, in comparison with hydroxide, even bicarbonate is to strong an eluent to separate highly polar, low-molecular weight organic acids such as formic, acetic, lactic, and glycolic acids.

Because carbonate ions have a much higher elution power than hydroxide ions, even traces of carbonate as an impurity in a hydroxide eluent results in inconsistent retention times, especially at the beginning of a gradient run, affecting the separation and quantitation of weakly retained anions. Hydroxide eluents, therefore, have to be carbonate-free! Hence, carbonate-free hydroxide eluents cannot be prepared manually due to the presence of carbon dioxide in the atmosphere, which immediately diffuses into alkaline solutions where it is converted to carbonate. This problem could not be solved until the late 1990s, when Liu *et al.*[6] described for the first time the preparation of carbonate-free hydroxide eluents by means of electrolysis in a closed system that was finally commercialized under the trade name Eluent Generator™. A detailed description and a schematic illustration of this device can be found in the Handbook of Ion Chromatography[10].

The electrolytic generation of a KOH eluent has a number of advantages. The most important one is that KOH is generated free of any carbonate, because it is generated in a closed system, i.e., carbon dioxide in the atmosphere cannot diffuse into the electrolysis chamber of the eluent generator. This is extremely important when low concentrations of KOH (c < 50 mmol/L) are used, omitting time-consuming postchromatographic rinsing procedures to remove carbonate that has accumulated at the stationary phase. The second advantage is the control of the KOH concentration via the current being applied between the perforated anode in the electrolyte reservoir and the cathode being placed in the electrolysis chamber: the higher the applied current, the higher the resulting KOH concentration. The applied current can be kept constant for isocratic elution of analytes or programmed over time for concentration gradients with minimal delay (gradient delay volume: 15 µL). Thus, linear or curved gradients are realized by electrical current gradients. Last but not least, only a constant flow of high-purity deionized water– provided by an isocratic pump – is needed as a carrier, which saves cost as no proportioning pump is required. The maximum KOH concentration that can be generated with an eluent generator depends on the eluent flow rate.

Since the applied current is inversely proportional to the flow rate, low flow rates enable the use of higher eluent concentration. For analytical ion chromatography at a flow rate of 1 mL/min, the maximum eluent concentration is 0.1 mol/L. Figure 1 compares an isocratic separation of standard inorganic anions on the Thermo Scientific™ Dionex™ IonPac™ AS11 anion exchanger with the respective gradient elution. As can be seen from the bottom chromatogram, the baseline remains stable over the entire gradient run. Since the electrolytically generated KOH eluent is not contaminated by carbonate, baseline drifts are typically in the nanoSiemens/centimeter range. The two chromatograms in Figure 1 also reveal the general advantages of gradient elution over isocratic elution that were already mentioned: the higher peak capacity and peak compression. Due to the focusing effect, the peak of the late-eluting orthophosphate is much narrower in comparison with isocratic elution (top chromatogram).

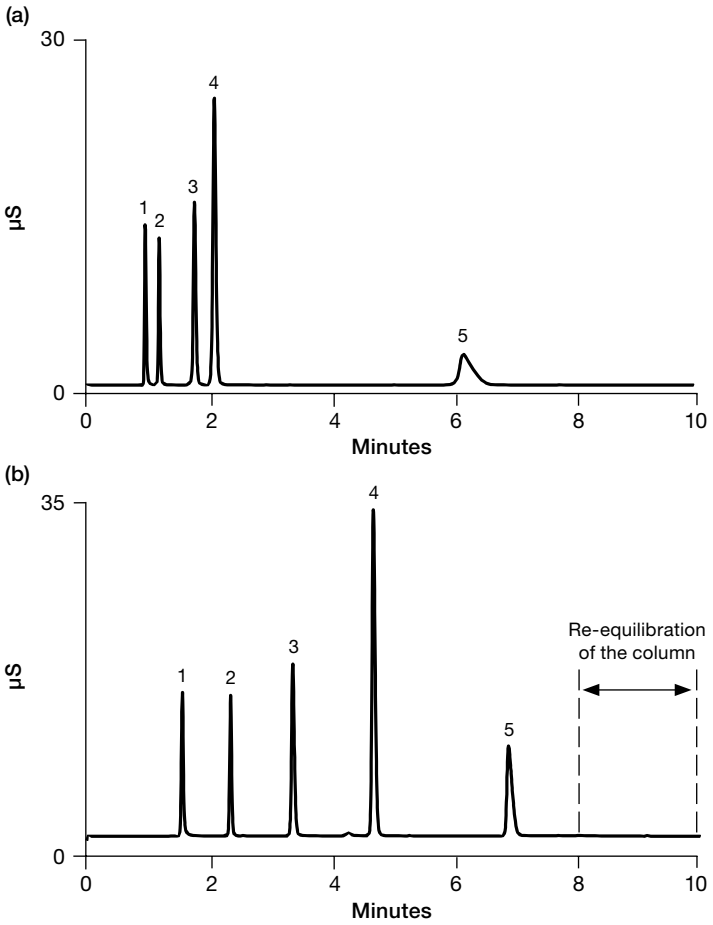


Figure 1. Separation of inorganic anions with an electrolytically generated KOH eluent. Separator column: Dionex IonPac AS11; column format: 250 mm × 4 mm i.d.; eluent: (a) 15.5 mmol/L KOH, (b) 0.5–25 mmol/L KOH in 8 min; flow rate: 2 mL/min; detection: suppressed conductivity; injection volume: 25 µL; peaks: 2 mg/L fluoride (1), 3 mg/L chloride (2), 10 mg/L nitrate (3), 15 mg/L sulfate (4), and 15 mg/L orthophosphate (5).

In addition to hydroxide eluents, salts of other weak acids may also be employed as eluents for gradient elution in anion-exchange chromatography. Weakly retained organic acids, for instance, can be separated together with standard inorganic anions utilizing a tetraborate gradient that has a slightly lower elution strength than hydroxide. However, due to the low solubility of tetraborate in water, the maximum concentration is not high enough to also elute polyvalent anions in the same run. At the end of the 1980s, promising experiments with zwitterionic compounds were carried out by Irgum[11], following the idea that these compounds exist as anions in an alkaline environment and thus can function as an eluent. Product of the suppressor reaction, however, is the zwitterionic form with no intrinsic conductance. Even though this concept is very intriguing, it was never accepted in the market as compounds such as Taurine (2-aminoethanesulfonic acid) or CAPS [3-(*N*-cyclohexylamino)-1-propanesulfonic acid] used by Irgum are not commercially available in the required purity.

In very rare cases, gradient separations have to be optimized by adding organic solvents to the hydroxide mobile phase. Since a long time, it is known that the selectivity of an ion exchanger can be adapted by the type and the concentration of an organic solvent, because the solvation power and hydrophobicity of a solvent are influencing the separation mechanism. Investigations of the influence of various protic and aprotic solvents on the retention behavior of standard inorganic anions were carried out by Stillian and Pohl[12]. Based on their results, the separation of inorganic anions and organic acids in fruit juices and alcoholic beverages by gradient elution could be improved by adding small amounts of methanol to the KOH eluent. In analytical ion chromatography using 4 mm or 2 mm separator columns, the addition of an organic solvent to a hydroxide eluent – either as a constant amount or in form of a solvent gradient – requires the use of a low-pressure proportioning pump. The use of an eluent generator is problematic due to the enhanced noise level when an organic solvent is added to the deionized water pumped into the electrolysis chamber. When employing a gradient pump, however, the hydroxide eluent has to be prepared manually, resulting in significant baseline drifts due to unavoidable carbonate impurities. One way out of this dilemma is to add the organic solvent to the electrolytically generated hydroxide eluent via a manifold prior to entering the injection valve[13].

Gradient elution techniques in cation-exchange chromatography are less common than in anion-exchange chromatography, because the most important inorganic cations such as alkali and alkaline-earth metals can be eluted simultaneously under isocratic conditions since the late 1980s. A gradient elution technique by increasing the acid concentration over time would not really help in this case to shorten analysis time as the time for column re-equilibration has to be taken into account. In case the sample to be analyzed contains a variety of organic amines in addition to inorganic cations, a higher peak capacity is often required which can be provided by an acid concentration gradient. If this is combined with suppressed conductivity detection, methanesulfonic acid (MSA) is the eluent of choice. Since purity problems are not encountered with MSA, the use of an eluent generator would not be imperative but is still recommended for ease-of-use, reproducibility of the gradient steering, and consistency of the generated analytical data. The instrumental setup for electrolytical acid generation is totally analogous to the generation of bases as described by Weiss[10]. Combined acid and organic solvent gradients are only feasible if amines with a high degree of surface-activity toward the stationary-phase surface such as longer-chain aliphatic or aromatic amines are to be separated together with standard inorganic cations.

The only application in cation analysis that requires a gradient elution technique is the analysis of lanthanides, which are strongly hydrated in aqueous solution with only minor differences in physicochemical properties determining their separation. Thus, isocratic cation-exchange chromatography does not lead to satisfactory separations. Based on the differing complexing behavior of the various lanthanides, fast and efficient separations are possible utilizing gradient elution on a bifunctional cation exchanger such as Thermo Scientific™ Dionex™ IonPac™ CS5A bearing defined anion- and cation-exchange capacities. Such separation is another example for a composition gradient in ion chromatography as the oxalic acid eluent at the start of the gradient is partly replaced by diglycolic acid during the run[14].

Examples for gradient elution of anions

As mentioned above, the analysis of standard inorganic acids in aqueous samples is usually performed under isocratic conditions using a carbonate/bicarbonate mobile phase. However, if closely eluting analytes are present in very disparate concentration ratios, gradient elution is the method of choice to obtain a higher peak capacity and shorter run times for more strongly retained anions. A typical example is the separation of oxyhalides together with standard inorganic anions in tap and bottled water that has been disinfected with ozone or a combination of ozone and chlorine dioxide, respectively. This process leads to the formation of chlorite and/or bromate present in the respective samples at concentration levels down to the single-digit microgram/Liter range. The separation of these anions from much larger concentrations of chloride, sulfate, and bicarbonate according to U.S. EPA Method 300.1[15] thus requires the use of a separator column with an optimized selectivity. Figure 2 illustrates the selectivity of such a separator column, the Thermo Scientific™ Dionex™ IonPac™ AS19-4µm column. As can be seen from the overlaid gradient profile, the initial concentration of the electrolytically generated KOH is kept constant for a few minutes to obtain maximal resolution of the early eluting anions, followed by a linear concentration gradient. The small baseline drift of less than 100 nS/cm thereby is remarkable.

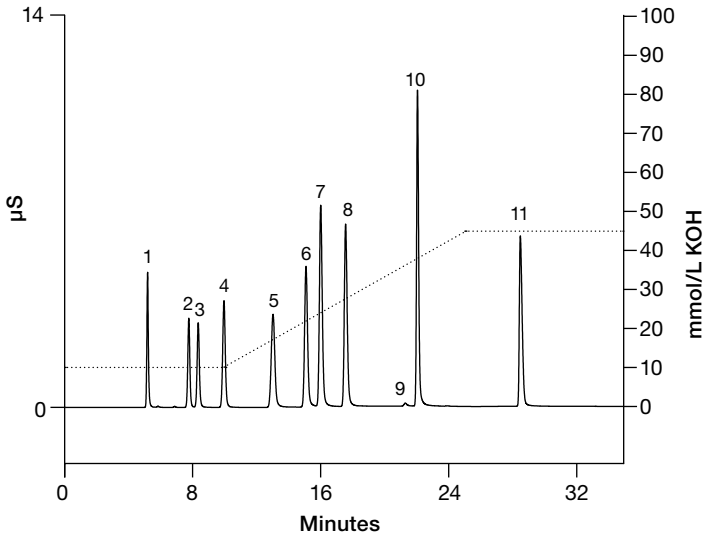


Figure 2. Separation of standard inorganic anions and oxyhalides on Dionex IonPac AS19-4µm. Column format: 250 mm × 2 mm i.d.; column temperature: 30 °C; eluent: KOH (EG); gradient: 10 mmol/L from 0 to 10 min, then 10–45 mmol/L in 15 min; flow rate: 0.25 mL/min; injection volume: 2.5 µL; detection: suppressed conductivity; peaks: 3 mg/L fluoride (1), 10 mg/L chlorite (2), 20 mg/L bromate (3), 6 mg/L chloride (4), 15 mg/L nitrite (5), 25 mg/L each of chlorate (6), bromide (7), and nitrate (8), carbonate (9), 25 mg/L sulfate (10), and 40 mg/L orthophosphate (11).

The advantage of electrolytic hydroxide generation is also evident for the analysis of anions in high-purity water at trace level. Due to a low baseline drift and a very low background conductance, detection limits in the sub-microgram/Liter range can be obtained when applying large-volume injections (up to 2,000 µL). Since high-purity water contains noticeable amounts of dissolved bicarbonate, a significant carbonate peak is typically observed in the respective chromatograms due to the conversion of bicarbonate to carbonate in the mobile phase at high pH. Depending on the concentration ratio with neighboring peaks, optimization of gradient conditions might not be sufficient to achieve adequate separation; in these cases, the use of a carbonate removal device (CRD-200)[10] is recommended, which removes more than 90% of the carbonate introduced by the sample.

Very often, a linear gradient is not sufficient to achieve an adequate separation. In this case, a multistep gradient with isocratic steps in between may solve more complex separation problems, especially in screening analyses. As an example, Figure 3 shows the separation of a wide variety of inorganic and organic anions on the Dionex IonPac AS11-4µm anion exchanger, which has been developed for such screening analyses. Starting with a very low initial KOH concentration to separate weakly retained fluoride and small molecular-weight organic acids, eluent concentration is ramped up to also elute strongly retained trivalent anions such as orthophosphate and citrate. Taking into account that three different isocratic separations would be necessary to analyze this set of analytes, the huge advantage of gradient ion-exchange chromatography becomes apparent[16].

Despite the high resolving power of modern anion exchangers in the 4 µm format, monovalent carboxylic acids such as acetic and lactic acids are only partially separated under the chromatographic conditions in Figure 3. The same is true for divalent pairs of ions such as succinic and malic acids, malonic and tartaric acids as well as sulfate and fumaric acid. Yet those compounds play an important role in the analysis of nonalcoholic and alcoholic beverages due to their impact on organoleptic properties. The organic acid composition of fruit juices, for instance, is analyzed to control the freshness and authenticity of the products as well as to identify potential adulterations[17]. However, a separation of the most important organic acids in fruit juices can only be accomplished by adding methanol to the KOH gradient[18], which improves the separation of divalent species, but unfortunately impairs the separation of weakly retained organic acids[19]. At the moment, the best separation is achieved when overlaying a KOH gradient with a rising methanol gradient declining toward the end of the run[20]. Such separation is illustrated in Figure 4 with the separation of organic acids in beer. The center chromatogram in Figure 4 represents the beer sample spiked with butyrate which is an important component indicating bacterial contaminations; it can be separated from other sample components without any problems under these chromatographic conditions.

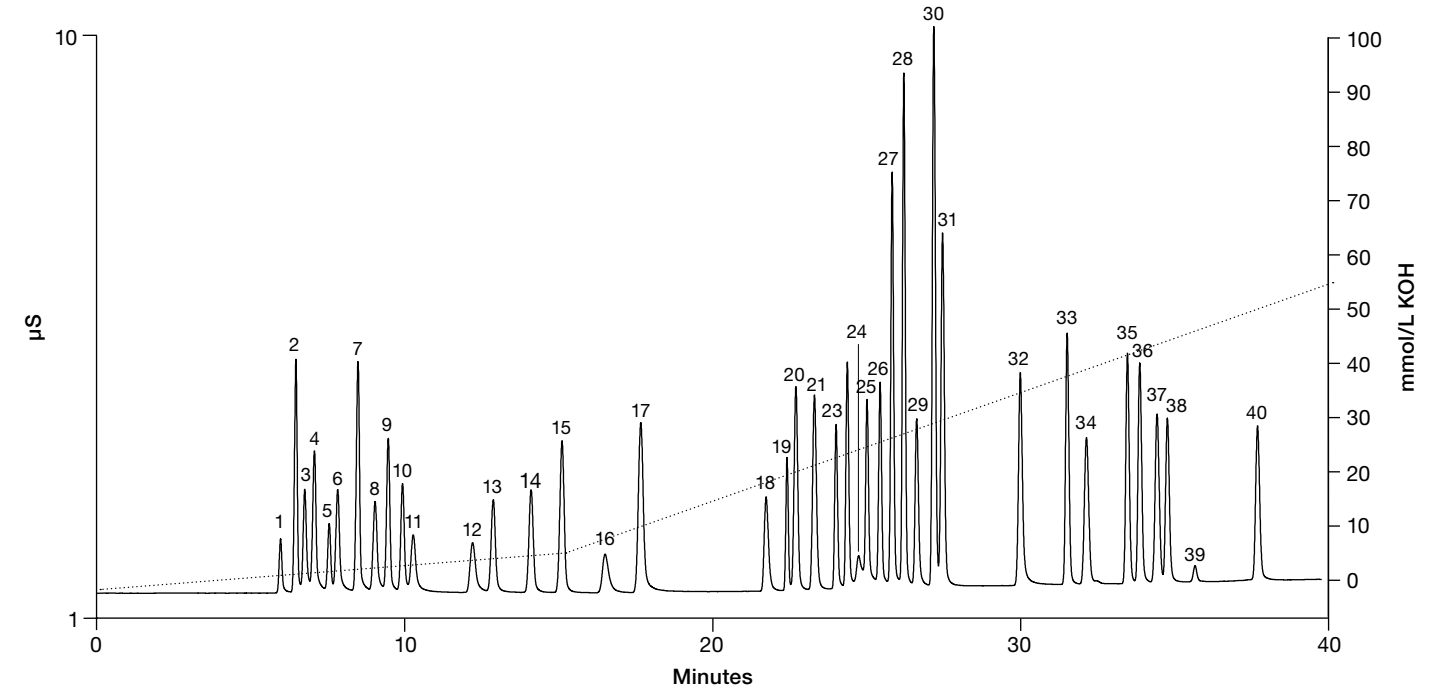


Figure 3. High-resolution separation of inorganic and organic anions utilizing a multistep gradient. Column: Dionex IonPac AS11-HC-4µm. Column format: 250 mm × 2 mm i.d.; column temperature: 30 °C; eluent: KOH (EG); gradient: 1 mmol/L for 0.01 min, 1–5 mmol/L in 15 min; 5–55 mmol/L in 25 min; flow rate: 0.38 mL/min; injection volume: 2.5 µL; detection: suppressed conductivity; peaks: 5 mg/L quinate (1), 1.5 mg/L fluoride (2), 5 mg/L each of lactate (3), acetate (4), 2-hydroxybutyrate (5), propionate (6), formate (7), butyrate (8), methyl sulfonate (9), pyruvate (10), isovalerate (11), valerate (12), monochloroacetate (13), and bromate (14), 2.5 mg/L chloride (15), 5 mg/L each of 2-oxovalerate (16), nitrite (17), ethyl phosphate (18), trifluoroacetate (19), bromide (20), and nitrate (21), 7.5 mg/L each of citramalate (22), malate (23), carbonate (24), malonate (25), citraconitate (26), maleate (27), sulfate (28), α-ketoglutarate (29), oxalate (30), and fumarate (31), 10 mg/L each of tungstate (32), orthophosphate (33), phthalate (34), arsenate (35), citrate (36), chromate (37), isocitrate (38), *cis*-aconitate (39), and *trans*-aconitate (40).

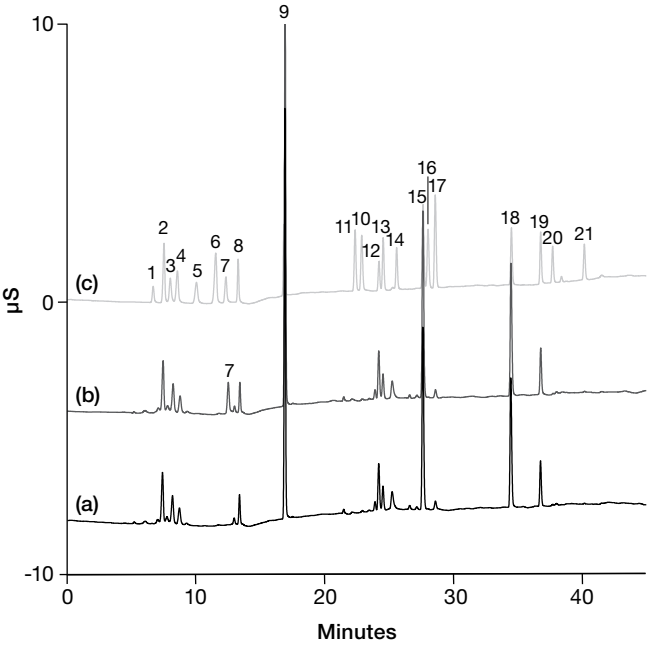


Figure 4. Gradient elution of inorganic and organic acids in beer. Separator column: Dionex IonPac AS11-HC-4µm, column format: 250 mm × 2 mm i.d.; column temperature: 30 °C; eluent: KOH (EG)/MeOH; gradient: 1 mmol/L with 2% MeOH (v/v) for 8 min, 2–10% MeOH (v/v) at 8.1 min, 1–15 mmol/L in 10 min with 10% MeOH (v/v), 15–30 mmol/L in 10 min with 10% MeOH (v/v), 30–60 mmol/L in 10 min with 10% MeOH (v/v); flow rate: 0.38 mL/min; injection volume: 2.5 µL; detection: suppressed conductivity; samples: (a) beer, 1 : 5 diluted with DI water, (b) beer, 1 : 5 diluted with DI water and spiked with 10 mg/L butyrate, (c) standard with 5 mg/L quinate (1), 3 mg/L fluoride (2), 5 mg/L each of lactate (3), acetate (4), propionate (5), formate (6), and butyrate (7), 10 mg/L pyruvate (8), 5 mg/L each of chloride (9), bromide (10), and nitrate (11), 10 mg/L each of succinate (12), malate (13), tartrate (14), sulfate (15), fumarate (16), and oxalate (17), 15 mg/L each of orthophosphate (18), citrate (19), isocitrate (20), *cis*-aconitate (21), and *trans*-aconitate (22).

A low-pressure gradient pump is required, whenever a hydroxide concentration gradient is overlaid with sodium acetate as a second eluent component. A typical example for such a gradient elution technique is the separation of proteinaceous amino acids or their phosphorylated derivatives by anion-exchange chromatography utilizing integrated pulsed amperometric detection (IPAD)[21]. The major advantage of this detection technique is the direct (underivatized) detection of amino acids and their derivatives down to the single-digit picomole level. Since IPAD requires a pH value > 11, anion-exchange chromatography with hydroxide-based eluents is a perfect match. The only difference to classical cation-exchange chromatography is the reversed elution order of amino acids; the analysis time is approximately the same.

Due to the strongly different retention behavior of aliphatic and aromatic amino acids, a simultaneous analysis of all proteinaceous amino acids requires a hydroxide concentration gradient, to which sodium acetate is added over the course of the gradient to elute aromatic and phosphorylated species. A typical profile of hydrolysate amino acids separated on the Thermo Scientific™ Dionex™ AminoPac™ PA10 anion exchanger is shown in Figure 5. As can be seen from this chromatogram, anion-exchange chromatography allows the separation of phenylalanine and tyrosine, which is not baseline-resolved on any commercially available cation exchangers.

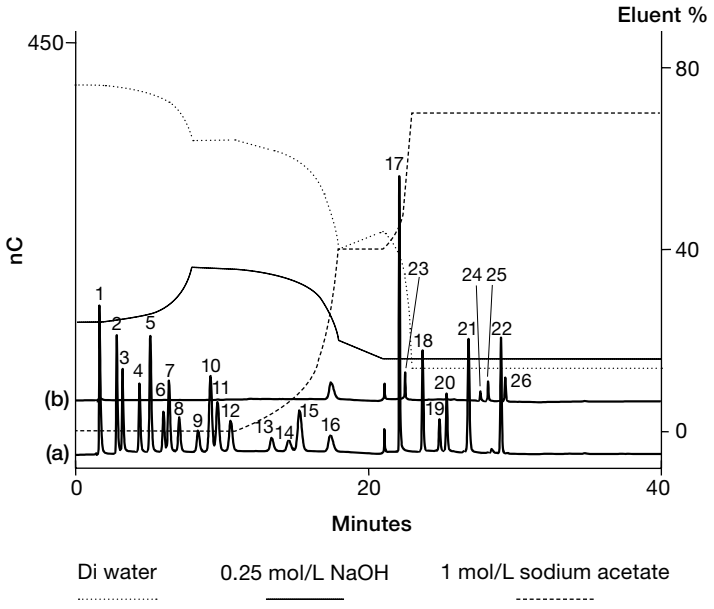
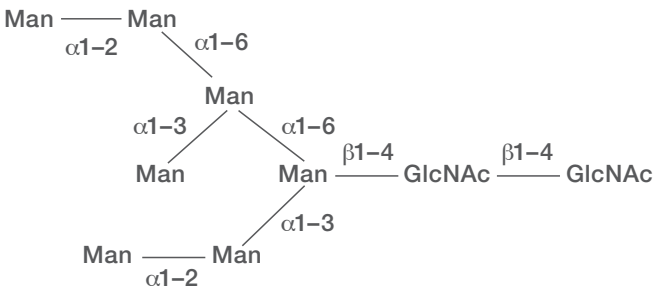


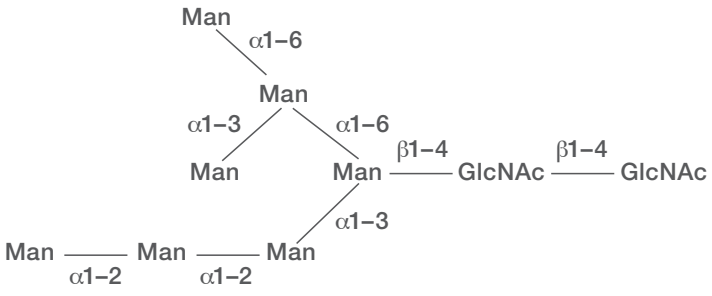
Figure 5. Gradient elution of hydrolysate amino acids and *O*-phosphorylated amino acids. Separator column: Dionex AminoPac PA10; column format: 250 mm × 2 mm i.d.; eluent: NaOH/NaOAc; gradient: graphically highlighted; flow rate: 0.25 mL/min; detection: integrated pulsed amperometry on a gold working electrode; chromatogram (a): 200 pmol each of arginine (1), hydroxylysine (2), lysine (3), glutamine (4), asparagine (5), alanine (6), threonine (7), glycine (8), valine (9), hydroxyproline (10), serine (11), proline (12), isoleucine (13), leucine (14), methionine (15), norleucine (16), histidine (17), phenylalanine (18), aspartate (19), glutamate (20), cystine (21), and tyrosine (22), chromatogram (b): 50 pmol each of P-arginine (23), P-serine (24), P-threonine (25), and P-tyrosine (26).

Since the early 1980s, concentration gradients based on NaOH/NaOAc in combination with anion-exchange chromatography and pulsed amperometric detection (HPAE-PAD) have also been used for the analysis of sialic acids and oligosaccharides[22]. Since carbohydrates are very weak acids, they dissociate into anions at pH values > 11 and can thus be separated by anion-exchange chromatography. However, sialic acids and oligosaccharides are strongly retained on anion exchangers and, therefore, cannot be eluted with a pure hydroxide eluent. Nevertheless, high alkalinity (pH 13) is required for pulsed amperometric detection; the elution of sialic acids or oligosaccharides is then carried out with a sodium acetate gradient. In contrast to other liquid chromatographic techniques such as reversed-phase or mixed-mode liquid chromatography, carbohydrates and their derivatives can be analyzed in their native state via HPAE-PAD, i.e., a derivatization is not necessary. By means of anion-exchange chromatography, carbohydrates are separated according to size, charge, composition, and linkage isomerism, which explains the success of this analytical method over the years.

A very important application area for HPAE-PAD is the characterization of sialic acids and oligosaccharides derived from glycoproteins. The carbohydrate moieties are enzymatically coupled to the protein during or after the translation (co- or post-translational) and are responsible for a certain biological or therapeutic activity. The empirical correlation between oligosaccharide structures and their retention behavior on anion exchangers has been studied and documented by Rohrer[23]. A typical example for a high-resolution separation of two oligosaccharide linkage isomers is shown in Figure 6. The two main peaks in the chromatogram are mannose-7 isomers of the following structures:



D1 Isomer



D3 Isomer

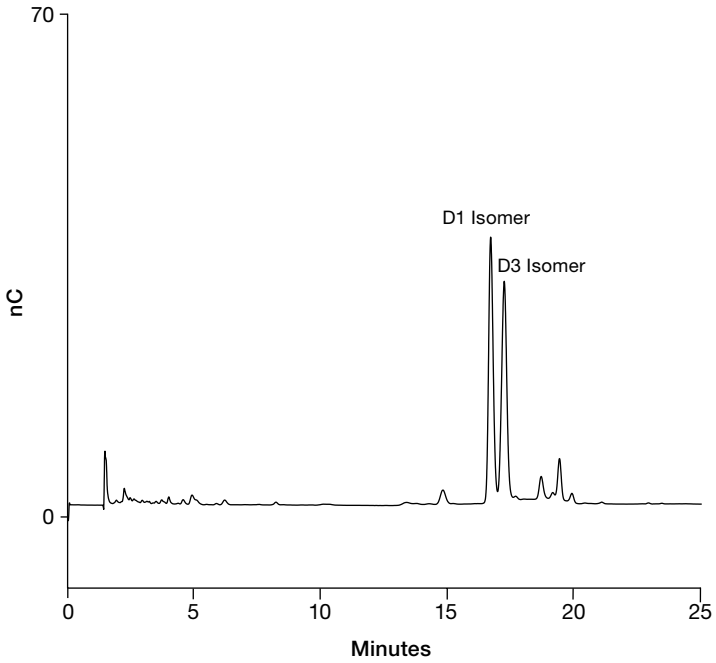


Figure 6. Gradient elution of mannose-7 linkage isomers. Separator column: Dionex CarboPac PA200; column format: 250 mm × 3 mm i.d.; eluent: NaOH/NaOAc; gradient: 0–200 mmol/L NaOAc in 100 mmol/L NaOH in 110 min; flow rate: 0.5 mL/min; detection: integrated pulsed amperometry on a gold working electrode; sample: mannose-7 linkage isomers (Dextra Labs, Reading, UK).

The separation was carried out on the Thermo Scientific™ Dionex™ CarboPac™ PA200, a 5.5 µm nanobead-agglomerated anion exchanger that was specifically designed for high-resolution separations of native oligosaccharides. Alternatively, a mixed-mode stationary phase supporting hydrophobic and electrostatic interactions can be used for such separations, which exhibits a similar resolving power but requires labelling and cannot be operated in conventional ion chromatographs due to the small particle diameter (1.9 µm) of its silica substrate.

Oligosaccharides also occur in many food and beverage products. Using HPAE-PAD, polymers up to DP70 may be analyzed. As an alternative to the classical gradient elution technique based on the eluent combination of sodium hydroxide and sodium acetate, oligosaccharides can also be analyzed utilizing Dual Eluent Generation. In this mode of operation, a methanesulfonic acid and a potassium hydroxide cartridge are used in series to electrolytically generate potassium hydroxide/potassium methansesulfonate eluents, which can replace the cumbersome manual preparation of sodium hydroxide/sodium acetate and eliminate the variation inherent to carbon dioxide intrusion. This operating mode is applicable to the micro (1 mm i.d.) column format. The chromatographic performance of Dual Eluent Generation in comparison to manual preparation of NaOH/NaOAc eluents is illustrated in Figure 7, exemplified through the separation of inulin, a polyfructan that is often found as a component of functional foods. Inulin contains about 30 D-fructose residues and a terminal sucrose unit. Both chromatograms in Figure 7 reveal an excellent separation of the chain length distribution. Due to the different elution strength of the two eluent mixtures, the respective gradient programs are not identical but result in comparable analysis times. Smaller molecular-weight fractions of inulin (DP3–20), also known as fructooligosaccharides (FOS) and used as alternative sweeteners, can also be determined with this method according to AOAC Method 997.08[24]. In addition, Durgnat and Martinez[25] developed a HPAE-PAD method for the simultaneous separation of glucose, fructose, sucrose, lactose, maltose to maltoheptaose, and FOS (DP3–9) and used this method to identify and quantify FOS in a variety of food products.

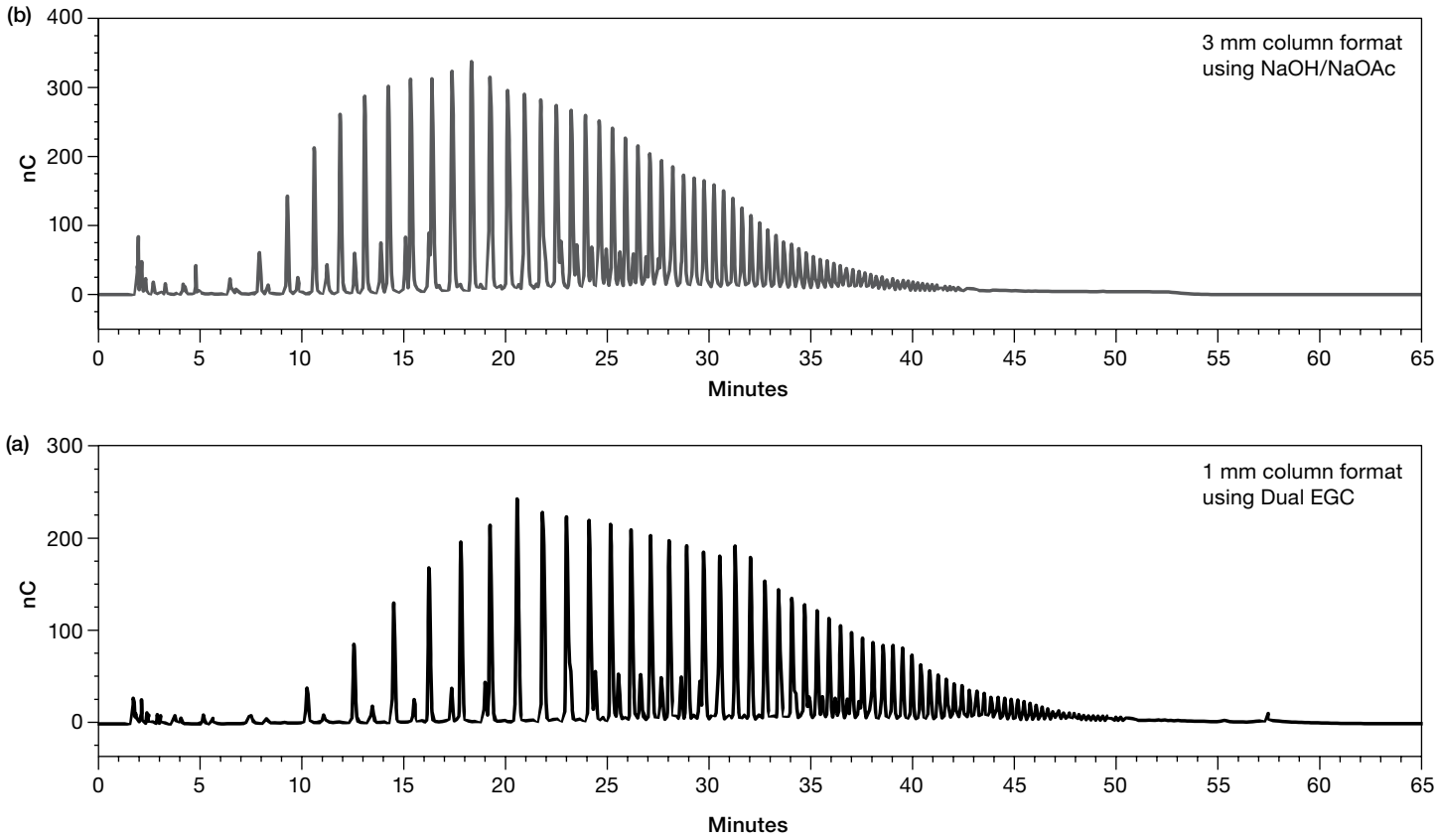


Figure 7. HPAE-PAD analysis of inulin utilizing Dual Eluent Generation in comparison with manually prepared NaOH/NaOAc eluents. Separator column: Dionex CarboPac PA200; column format: (a) 250 mm × 1 mm i.d., (b) 250 mm × 3 mm i.d.; eluent: (a) KOH/KMSA, (b) NaOH/NaOAc; gradient: (a) 0 to 45 min: 40 mmol/L KMSA/60 mmol/L KOH to 156 mmol/L KMSA/22 mmol/L KOH, 45 to 50 min: 156 mmol/L KMSA/22 mmol/L KOH, 50 to 65 min: 40 mmol/L KMSA/60 mmol/L KOH, (b) 100–430 mmol/L NaOAc in 100 mmol/L NaOH in 45 min; flow rate: (a) 0.063 mL/min, (b) 0.5 mL/min; detection: pulsed amperometry on a gold working electrode; sample: 5 mg/mL inulin from chicorée (Sigma Aldrich).

The analysis of oligonucleotides also requires concentration gradients based on buffer/salt mixtures. Oligonucleotides are polyvalent anions, differing in their net charge depending on chain length, so that they can be separated by anion exchangers that satisfy the demand on resolution (*N*, *N*-1 over a wide range of oligomer lengths of more than 50 bases) and speed of analysis (< 30 min). For this purpose, the Thermo Scientific™ Dionex™ DNAPac™ PA200 anion exchanger has been developed, which can be used for a variety of DNA samples such as ssDNA and RNA[26]. The elution of oligonucleotides is typically carried out with NaCl or $NaClO_4$ at pH 8. At this pH, all oligonucleotides of the same length will essentially have the same net charge. Increasing pH will result in a significant increase of retention of mixed-base oligonucleotides due to the ionization of the tautomeric oxygen on guanine and thymine. However, this effect is not observed with oligonucleotides containing only adenine and cytosine. As an example, for the high resolving power of anion-exchange chromatography for oligonucleotides, Figure 8 shows the gradient elution of $d(AC)_{10–11}$ 20–22-mers in less than 15 min.

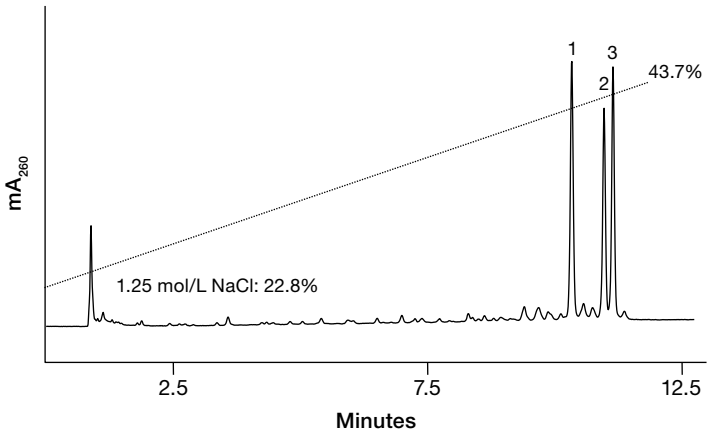


Figure 8. Gradient elution of an oligonucleotide mixture such as d(AC)10–11 20–22-mers. Separator column: Dionex DNAPac PA200; column format: 250 mm × 4 mm i.d.; column temperature: 25 °C; eluent: (A) 20 mmol/L Tris (pH 8), (B) 20 mmol/L Tris + 1.25 mol/l NaCl (pH 8); gradient: linear from 22.8% B to 43.7% B in 12 min; flow rate: 1.2 mL/min; detection: UV (268 nm); injection volume: 8 µL; peaks: (1) 20-mer, (2) 21-mer, and (3) 22-mer.

Examples for gradient elution of cations on cation exchangers

Since the introduction of weak cation exchangers with a carboxylate functionality at the end of the 1980s[27], alkali and alkaline-earth metals can be separated simultaneously under isocratic conditions. The detection method of choice is suppressed conductivity using an electrolytically operated suppressor and a methanesulfonic acid eluent. If high sensitivity is not an issue, nonsuppressed conductivity detection can also be employed, but only in combination with low-capacity separator columns; the most common eluent for this type of analysis is typically dipicolinic acid. However, if the sample contains organic amines in addition to inorganic cations, the situation might be different; it very much depends what kind of amines and how many have to be analyzed, whether or not a gradient elution technique is required. This is exemplified through the separation of inorganic cations and ethylamines in Figure 9a. Using one of the most modern, high-resolution cation exchangers such as the Dionex IonPac CS19-4µm, all analytes are separated under isocratic conditions. However, to resolve the critical pair of analytes – monoethylamine and potassium – a relatively low acid concentration is required. This, in turn, leads to unacceptably long retention times for divalent cations such as magnesium and calcium. In this case, a gradient elution technique can help to shorten analysis times significantly, as shown in Figure 9b. For fair comparison, the time for column re-equilibration at the end of the chromatographic run has to be added, but the decline in total analysis time (injection to injection) is still remarkable.

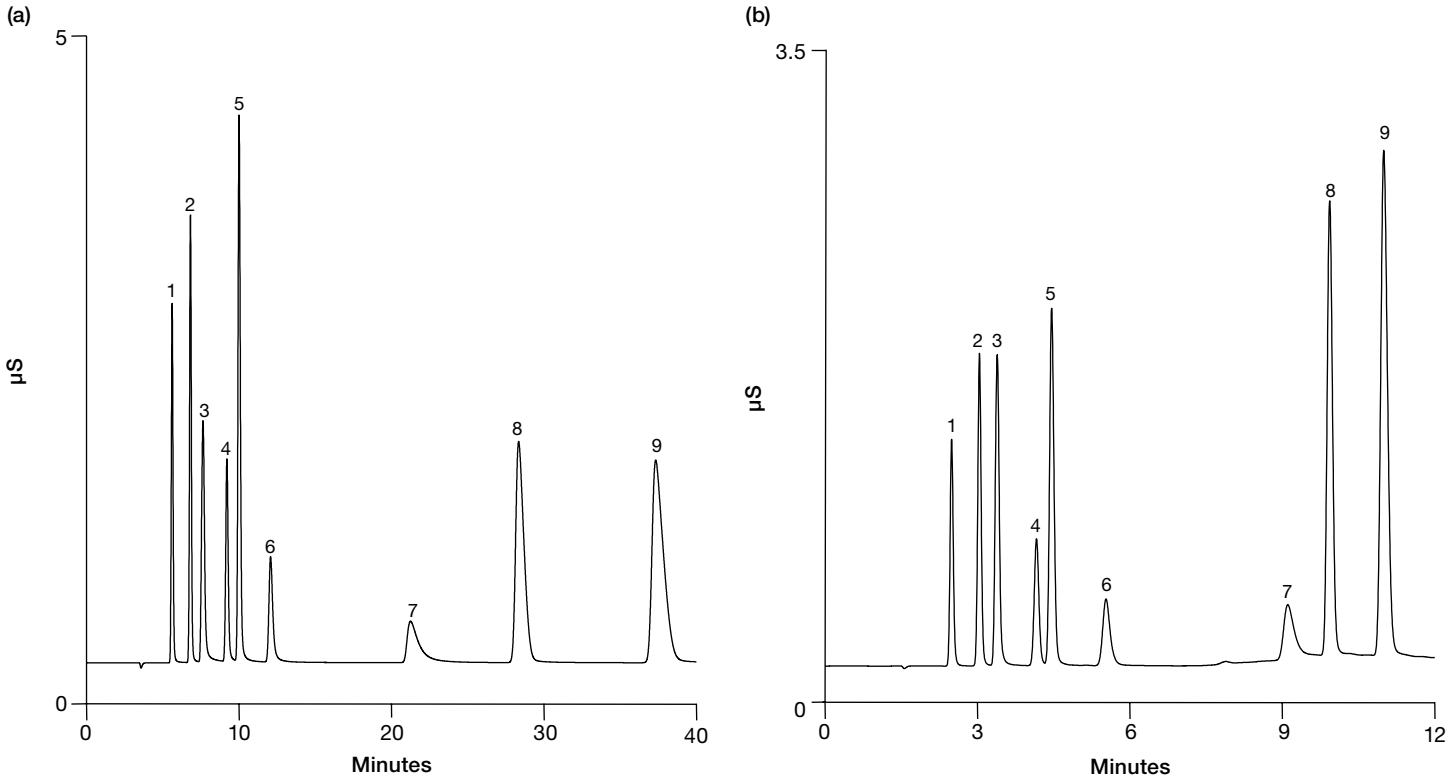


Figure 9. Isocratic elution of inorganic cations and ethylamines in comparison to gradient elution. Separator column: Dionex IonPac CS19-4µm; column format: 250 mm × 0.4 mm i.d.; column temperature: 30 °C; eluent: MSA (EG), (a) 4 mmol/L, (b) 4 mmol/L for 5 min isocratic, then linearly to 8 mmol/L in 7.5 min; flow rates: (a) 10 µL/min, (b) 20 µL/min; detection: suppressed conductivity; injection volume: 0.4 µL; peaks: 0.125 mg/L lithium (1), 0.5 mg/L sodium (2), 0.62 mg/L ammonium (3), 0.7 mg/L monoethylamine (4), 1.25 mg/L potassium (5), 1 mg/L diethylamine (6), 2 mg/L triethylamine (7), 0.62 mg/L magnesium (8), and 1.25 mg/L calcium (9).

In contrast, gradient elution in cation-exchange chromatography cannot be dispensed when amines with a widely different retention behavior are to be separated in the same chromatographic run. A typical example is petrochemically relevant amines, which are less strongly retained than alkaline-earth metals, so that the retention space between mono- and divalent cations has to be spread. As shown in Figure 10, such separation is obtained on a Thermo Scientific™ Dionex™ IonPac™ CS18 cation exchanger using a shallow, multistep concentration gradient at an elevated column temperature of 50 °C. Under these conditions, all three ethanolamines that are important in natural gas and biogas processing can be separated from each other. However, diethanolamine and potassium are co-eluting, but can be separated under different chromatographic conditions.

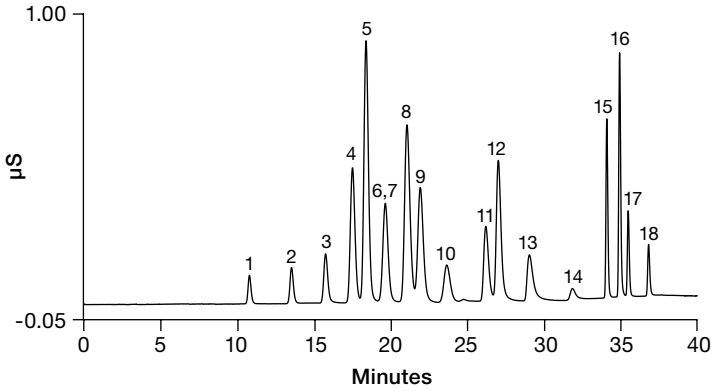


Figure 10. Gradient elution of inorganic cations and petrochemically relevant amines. Separator column: Dionex IonPac CS18; column format: 250 mm × 2 mm i.d.; column temperature: 50 °C; eluent: MSA (EG); gradient: 0.5–1 mmol/L in 20 min, then to 4 mmol/L in 8 min, then to 11 mmol/L in 6 min; flow rate: 0.3 mL/min; detection: suppressed conductivity; injection volume: 5 µL; peaks: 0.05 mg/L lithium (1), 0.2 mg/L sodium (2), 0.25 mg/L ammonium (3), 3 mg/L monoethanolamine (4), 3.6 mg/L monomethylamine (5), 3.6 mg/L diethanolamine (6), 0.5 mg/L potassium (7), 3 mg/L monoethylamine (8), 1.4 mg/L dimethylamine (9), 3 mg/L *N*-methyldiethanolamine (10), 3.2 mg/L morpholine (11), 3.7 mg/L 1-dimethylamino-2-propanol (12), 7.5 mg/L *N*-methylmorpholine (13), 1.5 mg/L *n*-butylamine (14), 0.25 mg/L magnesium (15), 0.5 mg/L each of calcium (16), strontium (17), and barium (18).

Biogenic amines are frequently analyzed in the food and beverage industry. They include aliphatic, aromatic, and heterocyclic compounds, which are biologically active and have a physiological relevance for humans. Biogenic amines are present in food and beverages that contain proteins or free amino acids and can be considered as an indicator for food spoilage through microbial contamination. Since humans show symptoms of sickness, migraines, or heart problems when consuming elevated levels of biogenic amines, the analysis of this class of compounds in various foods and beverages is important. In the past, cation-exchange separations of biogenic amines were not very popular due to the poor peak shapes resulting from hydrophobic interactions with older generation stationary phases. Therefore, RPLC techniques were predominantly used, but they required pre- or postcolumn derivatization. Based on the works of Draisci *et al.*[28] more than 20 years ago, modern weak-acid cation exchangers such as Dionex IonPac CS19-4µm in combination with integrated pulsed amperometric detection provide a welcome alternative for the analysis of biogenic amines, especially when highest sensitivity is required. In general, other detection methods such as suppressed conductivity, UV, and mass spectrometry can also be employed. However, dopamine, serotonin, and tyramine are not ionized after passing the suppressor device, and UV detection is only applicable for aromatic biogenic amines. Thus, integrated pulsed amperometry (IPAD) and MS are the most versatile detection systems for this class of compounds. If inorganic cations are to be analyzed together with biogenic amines, an acid concentration gradient cannot be avoided as biogenic amines are more strongly retained than calcium. Figure 11 shows the fast and baseline-resolved separation of the most important alkali and alkaline-earth metals together with biogenic amines on Dionex IonPac CS19-4µm in less than 10 min with a purely aqueous acid gradient, employing suppressed conductivity detection. Due to the low specific surface area of the supermacroporous substrate of this separator column, organic solvents used by Draisci *et al.* are no longer required in the mobile phase. Using similar gradient conditions, this analytical method can be extended to amines that are much more strongly retained, including those with more than one amino group and quaternary ammonium compounds. Bipyridines such as paraquat and diquat used as herbicides, which until recently could only be eluted with a salt gradient and thus not detected by suppressed conductivity, can now be analyzed under the conditions being used in Figure 11.

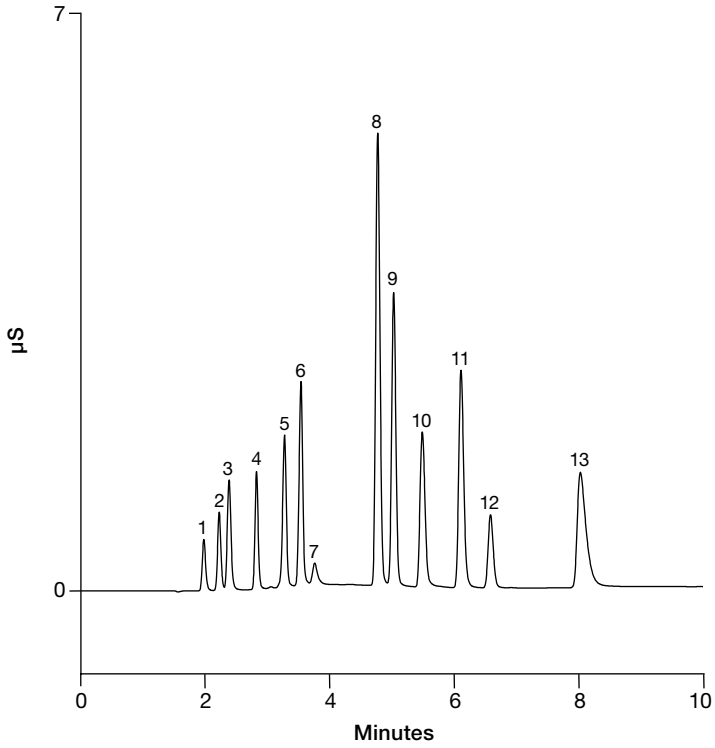


Figure 11. Gradient elution of inorganic cations and biogenic amines. Separator column: Dionex IonPac CS19-4µm; column format: 250 mm × 0.4 mm i.d.; column temperature: 30 °C; eluent: MSA (EG); gradient: 9–70 mmol/L in 7 min; flow rate: 20 µL/min; detection: suppressed conductivity; injection volume: 0.4 µL; peaks: 0.05 mg/L lithium (1), 0.2 mg/L sodium (2), 0.25 mg/L ammonium (3), 0.5 mg/L potassium (4), 0.25 mg/L magnesium (5), 0.5 mg/L calcium (6), impurity (7), 7.5 mg/L putrescine (8), 4.5 mg/L cadaverine (9), 6.5 mg/L histamine (10), 5 mg/L agmatine (11), 3 mg/L spermine (12), and 1.5 mg/L spermidine (13).

A very important application of modern cation-exchange chromatography is the separation of proteins, including monoclonal antibodies. A special challenge in the development and production of these therapeutic proteins is the characterization of the structural variants. Monoclonal antibodies are glycoproteins with a molecular weight of 150 kDa consisting of two identical heavy chains with 430 amino acid residues and two identical light chains with 214 amino acid residues that are linked to each other and to a light chain each by disulfide bonds. A frequent structural variation requiring thorough analysis[29] is the *C*-terminal processing of lysine residues at the heavy chain of monoclonal antibodies. Incomplete protein processing leads to charge heterogeneity due to the absence of *C*-terminal lysine residues, which can be identified by cation-exchange chromatography using salt gradients. Figure 12 shows a separation of these structural variants from the native antibody on the 10 µm Thermo Scientific™ Dionex™ MAbPac™ SCX-10 cation exchanger, which currently provides the highest resolution for this application. The substrate of this separator column is coated with a strongly hydrophilic material to eliminate nonspecific interactions between the protein and the hydrophobic bead core. On this hydrophilic layer, polymer chains with sulfonate groups as the cation-exchange functional groups are attached using an atom transfer radical polymerization (ATRP) grafting approach to tightly control chain length and density of the functional groups. The nature of the two lysine residues in Figure 12 can be verified by treating the sample with carboxypeptidase B, which specifically cleaves *C*-terminal lysine residues. By doing so, the peaks of the two lysine variants disappear accompanied by a corresponding increase in the peak area of the native antibody. The additional peaks eluting before the native antibody and after the lysine variants are more acidic and more basic variants, respectively, which can only be resolved when using a 4-morpholineethanesulfonic acid (MES) buffer in the mobile phase; traditional phosphate buffers do not resolve these peaks.

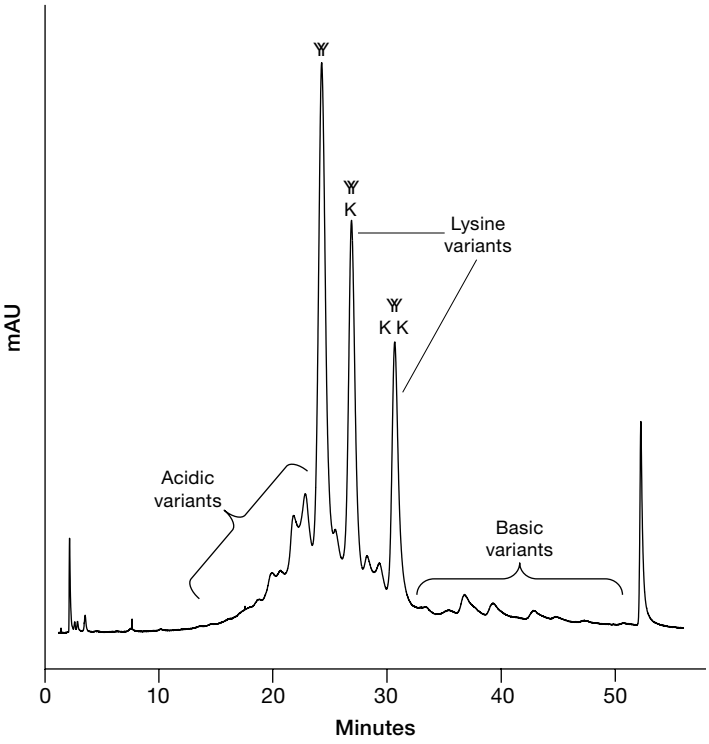
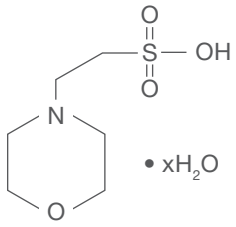


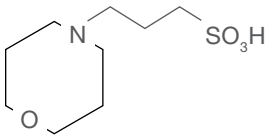
Figure 12. Gradient elution of a monoclonal antibody with a MES/NaCl eluent. Separator column: Dionex MAbPac SCX-10, 10 µm; column format: 250 mm × 4 mm i.d.; column temperature: 30 °C; eluent: A. 20 mmol/L MES + 60 mmol/L NaCl, pH 5.6, B. 20 mmol/L MES + 300 mmol/L NaCl, pH 5.6; gradient: 15–36.44% in 50 min; flow rate: 1 mL/min; detection: UV (280 nm); injection volume: 10 µL; sample: 5 mg/mL mAb.

Faster separations of monoclonal antibodies are demanded by the pharmaceutical industry today to more effectively control the production of these therapeutic proteins, despite the fact that this might compromise resolution. Different strategies can be followed to speed up the separation of mAbs, including compressed gradients, shorter separator columns, and the use of packing materials with smaller particle diameters. Since compressed gradients and shorter columns packed with the same resin material result in a certain loss of resolution, high-resolution separations of monoclonal antibodies with short run times can only be accomplished through the use of shorter columns with 3 or 5 µm particles size resins.

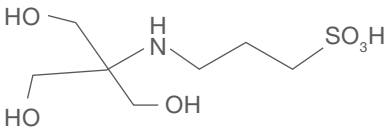
The only disadvantage of cation-exchange chromatography using salt gradients is that those methods are usually product-specific and do not tolerate major changes in chromatographic conditions. Moreover, a lot of parameters such as column selectivity, eluent pH, mobile-phase additives, and gradient profiles have to be optimized for separating a particular mAb. As an alternative, monoclonal antibodies can also be analyzed using pH gradients[9], employing a totally different retention mechanism. In this method, the pH value of the starting buffer has to be maintained at a constant level to ensure that the protein has the opposite charge of the stationary phase and thus binds to it. By changing buffer pH in the way that the protein net charge transitions to zero, proteins are caused to elute from the separator column. The use of pH gradients to assess charge heterogeneity offers a key advantage in that a single method can be applied to a series of mAbs with different isoelectric points. However, it is very difficult to create a buffer system that provides a truly linear pH gradient, partly because of the buffering effect of the stationary phase. Therefore, accurate monitoring of eluent pH is only possible, if an on-line pH meter such as the Thermo Scientific™ Dionex™ PCM-3000 module is placed directly behind the separator column. Since pH is temperature depending, the flow cell of this device is equipped with a temperature sensor, so that the respective chromatography data system reads out the temperature value when calculating pH. Nonlinearities of pH gradients are observed with the piperazine/imidazole/Tris buffer that was originally used by Farnan and Moreno[9] as well as with traditional phosphate buffers. When using the latter ones, positive or negative deviations from the set pH value are observed, which depend on a number of parameters, including the type and the concentration of the buffer, the separator column being used, and the pH value and concentration of the sample. In contrast, truly linear pH gradients over a wide range can be obtained using zwitterionic components in the buffer such as the Good buffers introduced by Thermo Fisher Scientific (Waltham, MA, USA) under the trade name CX-1[30]. With the following four components, a pH gradient covering the range from pH 5.6 to pH 10.2 can be obtained:



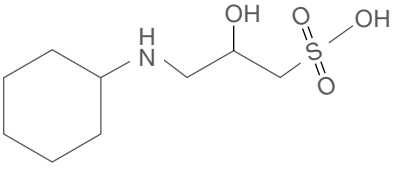
MES p*K* 6.1



MOPS p*K* 7.2



TAPS p*K* 8.4



CAPSO p*K* 9.6

pH gradients can serve as a platform for charge variant analysis of monoclonal antibodies. Considering that the p*I* values of monoclonal antibodies are typically between 6 and 10, separations can be optimized in a very simple way. This is illustrated with the separations of a mAb sample in Figure 13. The chromatogram in Figure 13a results when applying a gradient over the entire range from pH 5.6 to 10.2 with a gradient slope of 0.153 pH unit per minute. Depending on the p*I* value of the antibody, further optimization can simply be achieved by running a shallower pH gradient over a narrower pH range (pH 5.6 to 7.9, 0.078 pH unit per minute). The resulting chromatogram is shown in Figure 13b. The optimal separation in Figure 13c was obtained with an even narrower pH range from pH 6.75 to 7.9 (0.038 pH unit per minute). Even though the gradient slope was significantly reduced in comparison with the initial run, we see linear gradient profiles in all three chromatograms. Thus, the chromatographic behavior of the charge variants remains predictable when running shallower gradients.

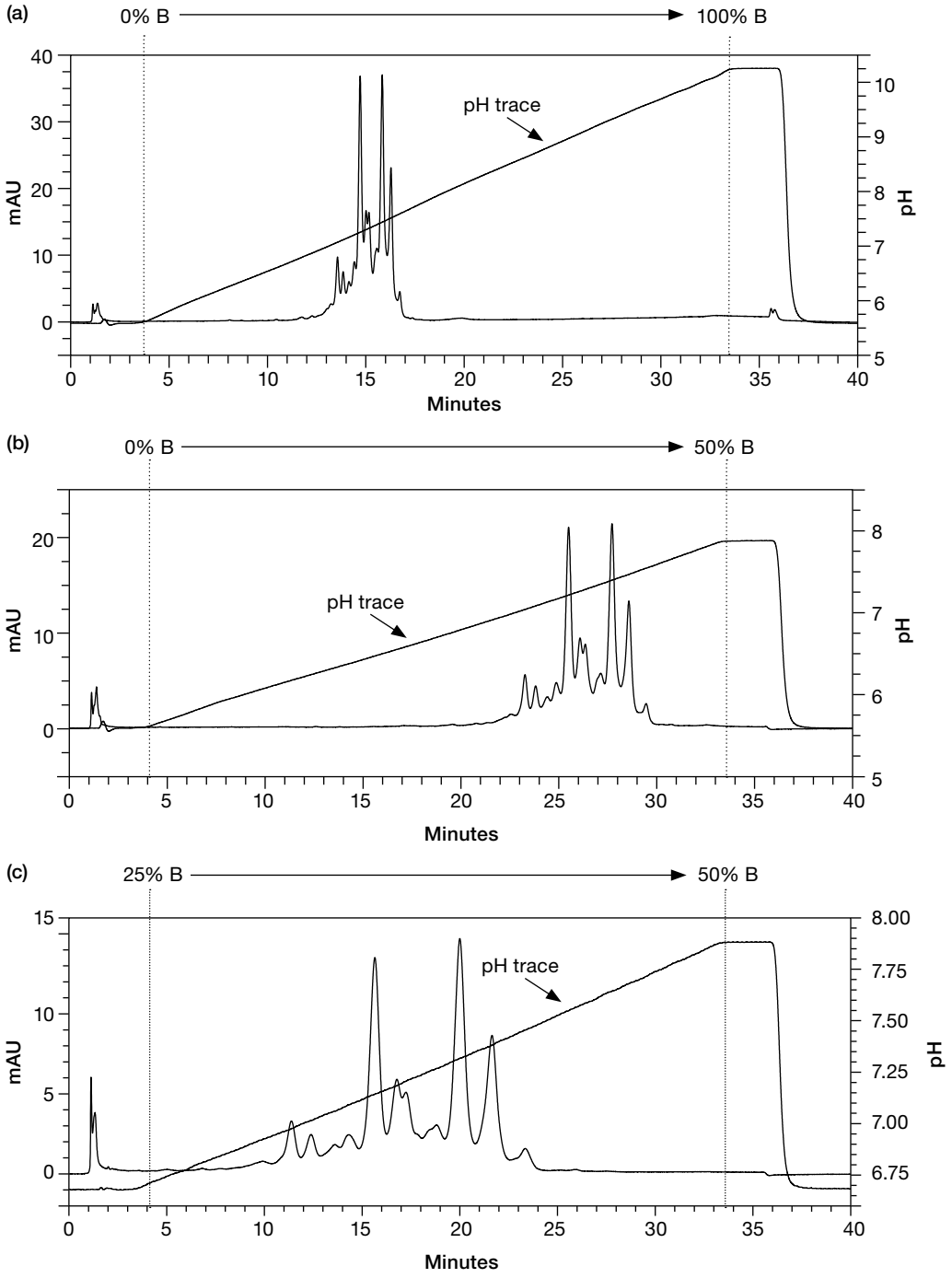


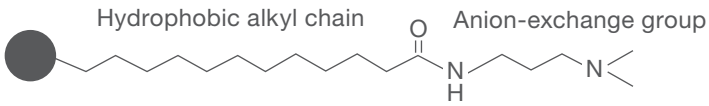
Figure 13. Optimization of the separation of mAb charge variants with a linear pH gradient. Separator column: Dionex MAbPac SCX-10, 10 µm; column format: 250 mm × 4 mm i.d.; eluent: pH gradient based on the CX-1 buffer system; gradient: (a) 0% B (pH 5.6) to 100% B (pH 10.2) in 30 min, (b) 0% B (pH 5.6) to 50% B (pH 7.9), (c) 25% B (pH 6.75) to 50% B (pH 7.9).

Examples for gradient elution on mixed-mode stationary phases

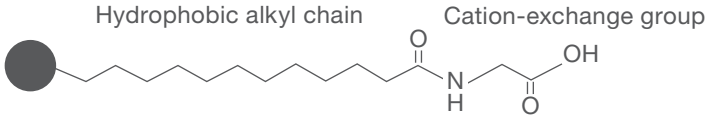
Although ion-exchange chromatography became the most dominating method in ion analysis over the past four decades, there are still some challenges. Baseline-resolved separations of inorganic anions and organic acids, for instance, can only be accomplished to a certain degree, despite the use of gradient elution on modern 4 µm anion exchangers. The same is true for the simultaneous analysis of inorganic cations and organic amines. Another fundamental problem of ion-exchange chromatography is the simultaneous analysis of anions and cations, which cannot be carried out using conventional ion exchangers as ions with the respective opposite charge are not retained. Bifunctional ion exchangers with a defined anion- *and* cation-exchange capacity do exist, but it is basically impossible to find an eluent that elutes both kind of ions from such columns with sufficient resolution. In addition, suppressed conductivity, the most common detection method for ionic species, cannot be employed for such application, because ions of the opposite charge are exchanged for hydronium or hydroxide ions in the suppressor device, respectively. UV detection cannot be employed either as most of the inorganic anions and cations are nonchromophoric.

Mixed-mode liquid chromatography helps to solve these problems, because it utilizes more than one form of interaction between the analyte ions and the stationary phase for their separation. Before mixed-mode liquid chromatography was considered as a chromatographic approach, secondary interactions were believed to be the main cause of peak tailing[31]. Reversed-phase/ion-exchange mixed-mode liquid chromatography has been known for more than 30 years[32]. Columns supporting this mode of liquid chromatography separate analytes by hydrophilic or hydrophobic and electrostatic interactions and provide a number of benefits, including adequate retention of ionic and ionizable analytes and adjustable selectivity[33]. This, in turn results in greater flexibility for selectivity tuning during method development and an expanded application range.

Mixed-mode columns can be classified into bimodal (RP/anion exchange and RP/cation exchange) and trimodal (RP/anion exchange/cation exchange) ones. Under the trade name Thermo Scientific™ Dionex™ Acclaim™ Mixed-Mode WAX-1 and WCX-1[34, 35] Thermo Fisher Scientific has commercialized two silica-based, polar-embedded bimodal columns that carry terminal ion-exchange groups:



Acclaim Mixed-Mode WAX-1



Acclaim Mixed-Mode WCX-1

The selectivity for both stationary phases can be adjusted by changing the ionic strength of the mobile-phase, pH, or the amount of an organic modifier, either independently or concurrently. The variation of ionic strength predominantly impacts the retention of ionic species, while the retention of nonionic species is not affected. Changing mobile-phase pH significantly affects ionization of the terminal functional group, i.e., depending on eluent pH the ion-exchange functional group can be ionized or not. Hydrophobic interactions are markedly affected by organic solvents in the mobile phase. Thus, mixed-mode phases are especially suitable for applications, which are difficult or not possible at all using either ion-exchange or reversed-phase chromatography. Due to the special morphology of bimodal mixed-mode phases the selectivity of these columns is complementary in comparison with conventional ion exchangers and reversed-phase columns.

Combining reversed-phase and anion-exchange characteristics, Acclaim Mixed-Mode WAX-1 provides adequate retention and an ideal selectivity for a variety of anionic compounds. It is effective for even weakly charged anions such as highly polar monocarboxylic acids. Although small molecular-weight organic acids can sometimes be separated by reversed-phase chromatography in the ion-suppression mode at low pH, highly aqueous conditions are required, often resulting in a phase collapse (dewetting effect) with a sudden irreversible loss of retention. Even aqueous-compatible polar-embedded stationary phases fail to separate these acids, primarily because hydrophobic interactions alone are not sufficient to differentiate compounds with similar hydrophobicities. Thus, the separation of the first five monocarboxylic acids in Figure 14 is very difficult if not impossible on any modern reversed-phase or anion-exchange column.

However, on Acclaim Mixed-Mode WAX-1 these acids are not only resolved to baseline but also well separated from the column void. The baseline-resolved separation of ascorbic acid and isoascorbic acid under the isocratic conditions being used is also astonishing. The only disadvantage of this approach is the incompatibility of the phosphate buffer with suppressed conductivity detection. Since all the analytes in Figure 14 are chromophoric, UV detection can be applied instead. Because of its unique column chemistry, Acclaim Mixed-Mode WAX-1 clearly outperforms other commercially available mixed-mode columns, in which the ion-exchange functional groups are embedded in the alkyl ligands, primarily because of secondary interactions between the negatively charged analytes and the positively charged embedded functional groups.

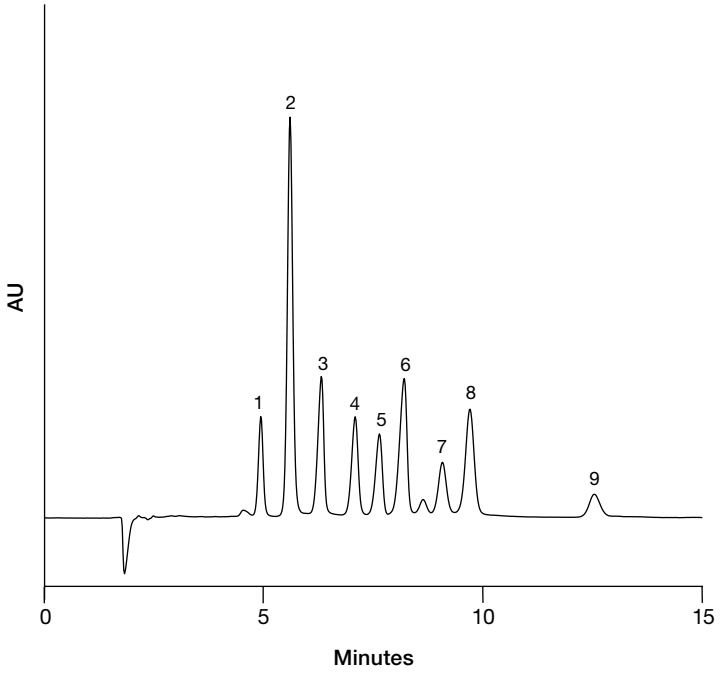


Figure 14. Separation of various monocarboxylic acids of similar hydrophobicities on a mixed-mode stationary phase. Separator column: Dionex Acclaim Mixed-Mode WAX-1, 5 µm; column format: 150 mm × 4.6 mm i.d.; column temperature: 30 ˚C; eluent: 25 mmol/L phosphate buffer, pH 6; flow rate: 0.8 mL/min; detection: UV (210 nm); peaks: (1) quinic acid, (2) shikimic acid, (3) glycolic acid, (4) lactic acid, (5) acetic acid, (6) formic acid, (7) ascorbic acid, (8) isoascorbic acid, and (9) propionic acid.

Bimodal mixed-mode columns are also used for the separation of oligosaccharides derived from glycoproteins. While highest resolution of native glycans is usually accomplished with HPAE-PAD, mixed-mode phases offer the advantage of using volatile buffers in combination with an organic solvent such as acetonitrile as the mobile phase, which allows the use of direct MS detection without a carbohydrate membrane desalter (CMD)[10] that is required for hyphenating anion-exchange chromatography with mass spectrometry. In addition, mixed-mode phases can also be used for LC with fluorescent detection after labeling with 2-aminobenzamide (2AB). However, native glycan profiles are significantly different from fluorescent-labeled ones, especially when analyzing higher sialylated compounds. Figure 15 shows a separation of neutral and acidic *N*-glycans derived from bovine fetuin and labeled with 2-AB on the Thermo Scientifc™ Dionex™ GlycanPac™ AXR-1, a column that supports weakly basic anion-exchange with reversed-phase interactions. Using a volatile buffer such as ammonium formate in combination with acetonitrile as the mobile phase, the column effluent can be directly introduced into the ion source of a mass spectrometer. The elution profile in Figure 15 consists of peaks that are grouped into several clusters. The first one represents a group of neutral oligosaccharides, followed by clusters of sialylated oligosaccharides, eluting according to the degree of sialylation and retained by electrostatic interactions. The equally charged glycans in each cluster are further separated according to branching, size, and linkage isomerism by hydrophobic interactions, which could be confirmed independently by UHPLC-MS^n studies.

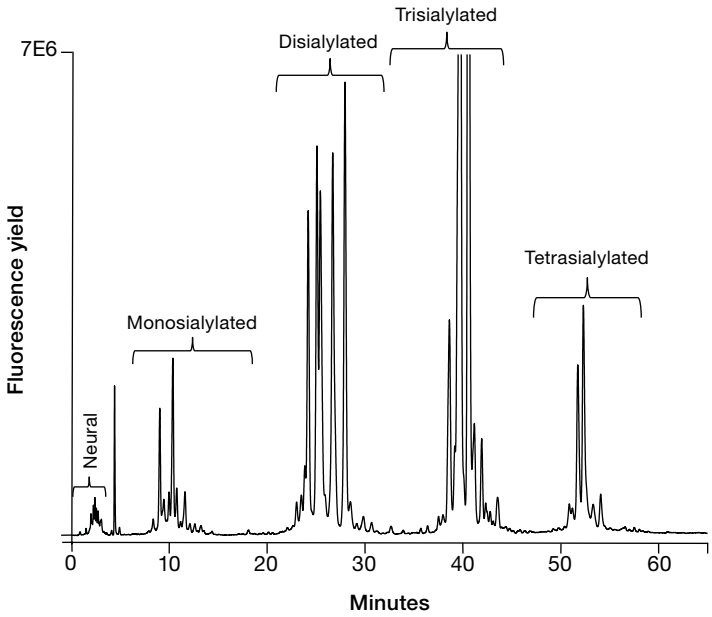


Figure 15. Gradient elution of labeled *N*-glycans derived from bovine fetuin with fluorescence detection. Separator column: Dionex GlycanPac AXR-1, 1.9 µm; column format: 150 mm × 2.1 mm i.d.; column temperature: 40 ˚C; eluent: MeCN/ammonium formate, pH 4.4; gradient: 5 mmol/L for 1 min isocratic, then to 25 mmol/L/MeCN (99 : 1 *v/v*) in 29 min, then to 30 mmol/L/MeCN (80 : 20 *v/v*) in 35 min; flow rate: 0.4 mL/min; detection: fluorescence (320 nm/420 nm); sample: 100 pmol *N*-glycans, labeled with 2-AB.

Acclaim Mixed-Mode WCX-1 is ideally suited for the analysis of basic compounds, which are important in a variety of industrial applications. The analysis of these compounds is often challenging when using silica-based reversed-phase columns. At neutral pH, basic compounds exhibit peak tailing because of the secondary interactions between the analytes and unreacted silanol groups on the stationary-phase surface. Although this difficulty is minimized with polar-embedded stationary phases, which separate a wide variety of basic compounds with optimal peak shape, strongly hydrophilic basic compounds are not adequately retained without the addition of ion-pair reagents to the mobile phase. Acclaim Mixed-Mode WCX-1 not only does retain those compounds, ranging from very hydrophilic to strongly hydrophobic ones, but also elutes them with symmetrical peak shapes and high chromatographic efficiencies. Compounds best analyzed with Acclaim Mixed-Mode WCX-1 also include quaternary ammonium compounds used as bactericides and corrosion inhibitors. In this case, ammonium acetate is used as a buffer, which is volatile enough to allow the use of nebulization detection techniques such as evaporative light scattering (ELS) or charged aerosol detection (CAD), because quaternary ammonium compounds are nonchromophoric.

In general, both bimodal mixed-mode columns mentioned above are suitable for the simultaneous analysis of acidic, basic, and neutral compounds. Thereby, the retention of charged species is always enhanced due to electrostatic interactions with the respective functional groups of the separator column, while the retention of compounds with the opposite charge is diminished through electrostatic repulsion. However, retention of oppositely charged species is still possible through hydrophobic interaction, which is also the retention mechanism for neutral compounds.

In the evolution of mixed-mode stationary phases, trimodal columns represent the current state-of-the-art. Trimodal columns such as the Thermo Scientific™ Dionex™ Acclaim™ Trinity P1 and P2 columns support hydrophobic or hydrophilic, anion-exchange, *and* cation-exchange interactions and have an even broader range of applications than bimodal columns[36]. Both separator columns are nanopolymer silica hybrids based on a 3 µm silica substrate. In analogy to the bimodal columns mentioned above, these silica particles are covalently modified with polar-embedded alkyl groups that carry terminal ion-exchange functional groups. The silica particles are then coated with either strong or weak acid polymeric nanobeads. In this way, a distinctive spatial resolution between the oppositely charged ion-exchange functional groups is ensured, which allows both retention mechanisms to function simultaneously and to be controlled independently. In analogy to bimodal columns, the selectivity of such separators can be optimized by adjusting the mobile-phase ionic strength, pH, and organic solvent content.

The most significant advantage of a trimodal column is the possibility to analyze anionic and cationic species in the same chromatographic run, which cannot be performed with traditional anion or cation exchangers. Supporting anion-exchange, cation-exchange, and hydrophobic interactions, the Dionex Acclaim Trinity P1 column provides optimal selectivity for pharmaceutical counter ion analysis. Utilizing a mixture of ammonium acetate and acetonitrile as the mobile phase for such separations, various detection methods can be employed, including nebulization techniques such as ELSD and CAD as well as UV and MS. As an example, Figure 16 shows the separation of 16 anionic and cationic pharmaceutical counter ions in less than 15 min. Due to the morphology of that column, cationic species elute before anionic ones. Since some of the analytes in Figure 16 are nonchromophoric, a nonspecific detection method such as ELS had to be employed in this case. The gradient profile used for this separation can be a starting point for optimizing similar separation problems by adjusting mobile-phase ionic strength, pH, and/or the amount of organic solvent. In addition to pharmaceutical counter ion analysis, trimodal columns are also used to analyze pharmaceutical formulations containing basic and acidic drug components. A typical example is Advil Allergy and Sinus, an over-the-counter drug formulation containing a decongestant (pseudoephedrine), a pain reliever (ibuprofen), and an antihistamine (chlorpheniramine maleate) as active pharmaceutical ingredients (APIs). As can be seen from Figure 17, all four organic components can be separated to baseline without any interferences in a very short time utilizing UV detection. In principle, sodium as the counter ion of ibuprofen could be analyzed in the same chromatographic run, but this would require a nonspecific detector such as a charged aerosol detector.

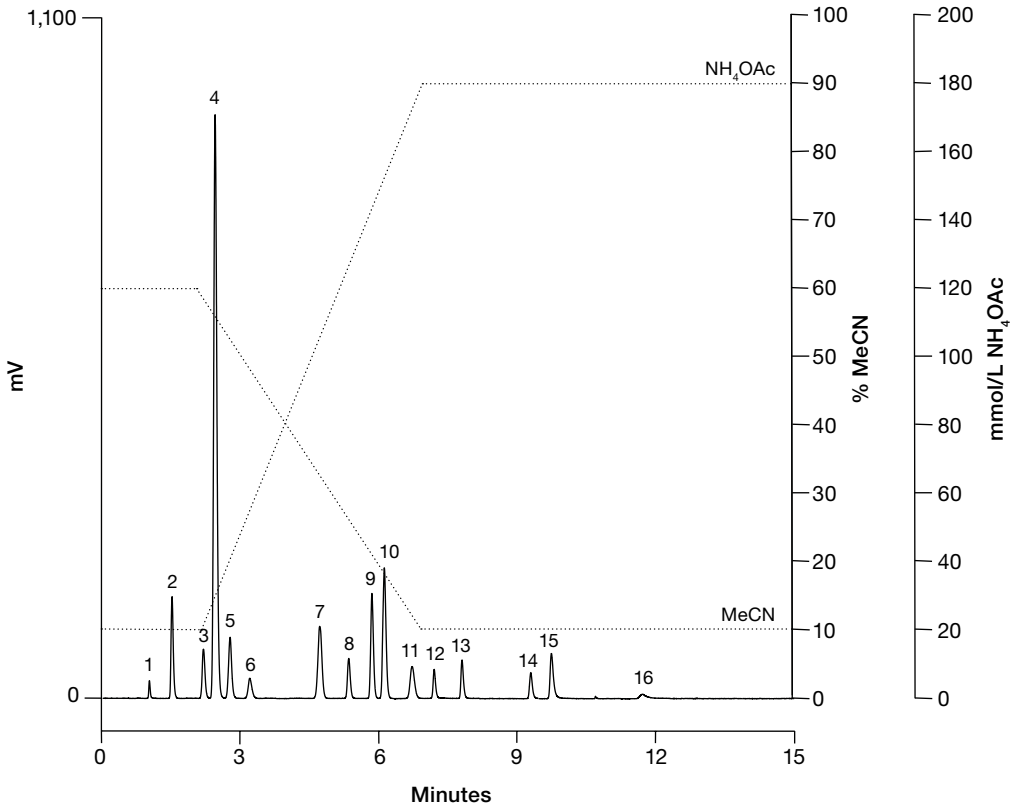


Figure 16. Simultaneous gradient separation of pharmaceutically relevant counter ions. Separator column: Dionex Acclaim Trinity P1, 3 µm; column format: 50 mm × 3 mm i.d.; column temperature: 30 °C; eluent: MeCN/ammonium acetate, pH 4; gradient: MeCN/10 mmol/L ammonium acetate (60 : 40 *v/v*) isocratic for 2 min, then to MeCN/180 mmol/L ammonium acetate (10 : 90 *v/v*) in 5 min; flow rate: 0.5 mL/min; detection: ELS; injection volume: 5 µL; peaks: 50–100 mg/L procaine (1), choline (2), tromethamine (3), sodium (4), potassium (5), meglumine (6), mesylate (7), maleate (8), chloride (9), bromide (10), iodide (11), orthophosphate (12), malate (13), tartrate (14), citrate (15), and oxalate (16).

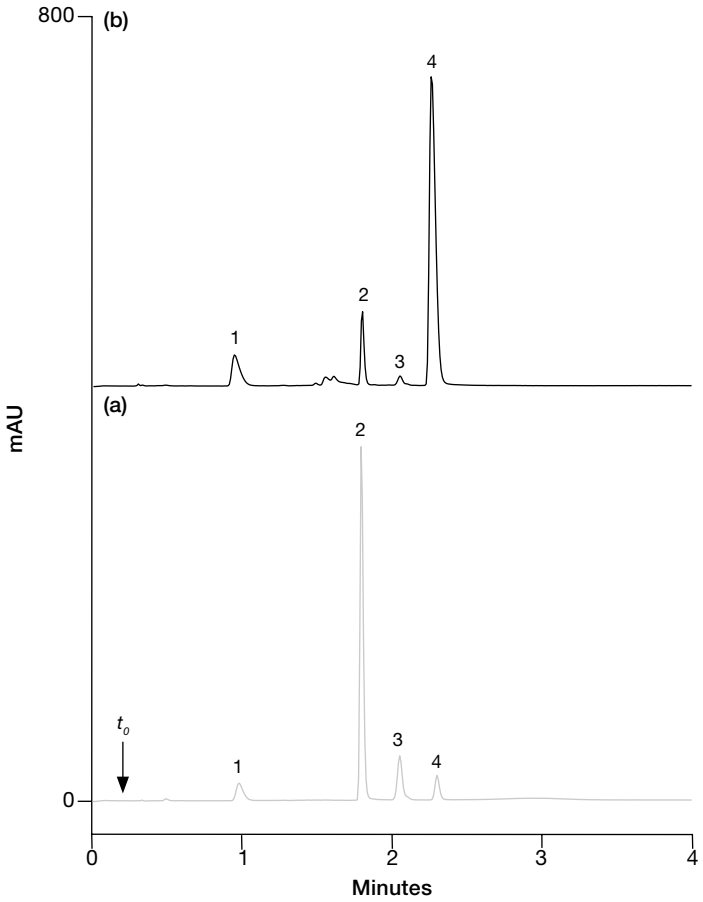


Figure 17. Gradient elution of acidic and basic APIs in a pharmaceutical formulation. Separator column: Dionex Acclaim Trinity P1, 3 µm; column format: 50 mm × 3 mm i.d.; column temperature: 30 °C; eluent: MeCN/ammonium acetate, pH 4.1; gradient: MeCN/20 mmol/L ammonium acetate (25 : 75 *v/v*) to MeCN/40 mmol/L ammonium acetate (80 : 20 *v/v*) in 1 min; flow rate: 1 mL/min; detection: UV (254 nm); injection volume: 2 µL; sample: (a) standard with 0.33 mg/mL API in MeCN/water (1 : 1 *v/v*), (b) Advil Allergy & Sinus (OTC), sample preparation: weigh 20 mg of the powdered tablet in 10 mL MeCN/water (1 : 1 *v/v*) and filtrate (0.2 µm); peaks: (1) pseudoephedrine, (2) chlorpheniramine, (3) maleate, and (4) ibuprofen.

Since the electrostatically agglomerated nanobeads of the Dionex Acclaim Trinity column are sulfonated, divalent anions and cations are highly retained. For this reason, another trimodal column, the Dionex Acclaim Trinity P2, was developed, featuring hydrophilic and cation-exchange interactions through the modification of the silica substrate with an amide. The functional groups of the agglomerated nanobeads are quaternary ammonium groups which support anion-exchange interactions. Thus, Dionex Acclaim Trinity P2 allows the separation of mono- *and* multivalent ions, including divalent ions such as sulfate, magnesium, and calcium by employing an ammonium formate concentration gradient at pH 3.65.

Conclusions

In the original embodiment of ion chromatography introduced in 1975, only isocratic separations of inorganic anions and cations were described. Gradient elution techniques for the analysis of complex samples could not be employed due to the lack of high-capacity suppression devices. In addition, the use of gradient elution in anion-exchange chromatography was hampered by a second problem as the commonly used carbonate/bicarbonate eluent is not suitable for gradient elution. In the end, the introduction of high-capacity membrane-based suppressors paved the way for using hydroxide mobile phases, which is the eluent of choice for gradient elution in anion-exchange chromatography. The problem of carbonate impurities in manually prepared hydroxide eluents could be solved by the concept of electrolytic eluent generation. Together with a large variety of hydroxide-selective stationary phases, gradient elution techniques in anion-exchange chromatography became practical and are as easy to perform as isocratic separations today. The concept of electrolytic eluent generation can also be applied to cation-exchange chromatography for the simultaneous analysis of inorganic cations and organic amines. Although concentration gradients are the most common type of gradients for small molecular-weight anions and cations, composition and capacity gradients are also applied for the analysis of lanthanides and strongly retained anions, respectively.

Over the more than forty years that encompass the development of ion chromatography, the definition of this term became much broader to include ion-exchange separations for biologically relevant classes of compounds such as carbohydrates, amino acids, oligonucleotides, and proteins. High-resolution separations of these compounds are typically performed by gradient elution, proportioning more than one eluent component with low-pressure gradient pumps. Examples are the sodium hydroxide/sodium acetate eluents for the analysis of carbohydrates and amino acids as well as salt gradients for the analysis of oligonucleotides and proteins.

Despite the separation power of gradient elution on modern 4 µm ion exchangers, there are still some challenges such as the baseline-resolved separation of inorganic and organic ions as well as the simultaneous separation of anions and cations. Some of these challenges have been overcome by employing mixed-mode stationary phases supporting more than one retention mechanism. While bimodal columns supporting weak anion-exchange and hydrophobic interactions, for instance, significantly improve the simultaneous separation of inorganic and organic ions, the separation of inorganic and organic anions and cations can be accomplished on trimodal columns that support both anion- and cation-exchange together with hydrophobic interactions. Gradient elution techniques in combination with mixed-mode columns are predominantly used for the analysis of pharmaceutical APIs and their counter ions in one chromatographic run.

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