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Review Article

Evolution of Stationary Phase Design in Chromatography and Electrophoresis: Materials, Mechanisms, and Emerging Trends

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Abstract:

Remarkable transformation has been observed in separation science with the evolution in the design of stationary phases, observing a transition from conventional silica-based columns to monoliths, engineered polymers, metal-organic frameworks (MOFs), and many others. Many of these stationary phases have seen routine or specialized application in industrial and research settings. In this review article, a critical examination of the evolution in stationary phases employed in separation methods is conducted, emphasizing the surface chemistry, analyte retention mechanisms, and emerging sustainability considerations. A comparison of columns shows an evolution in analytes from just molecules and proteins to a wider range of analytes, including cells. While these evolutions have brought significant advances in research, advances in stationary phases have yet to come to a halt, with research being performed in MOFs and more.

Keywords: Chromatography, Electrophoresis, Stationary Phase Chemistry, Sample Isolation, Stationary Phase Design.

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Introduction

Separation science involves methods that separate compounds from a mixture on the basis of their physicochemical properties such as polarity, reactivity, and stereochemistry. Among many methodologies, the first reported method was demonstrated by Mikhail Tsvet for the separation of plant pigments. Since its emergence, separation science has undergone significant changes over the course of the 20th century. In separation methods, the principal components are the analyte, the mobile phase, and the stationary phase. As the name implies, the stationary phase is the non-moving part in the separation method, while the mobile phase is the moving part which transports the analyte through the stationary phase. Both phases are selected such that they interact with the analyte to a varying degree on the basis of a selected physicochemical property. This interaction leads to the retention of the analyte on the column to a certain degree, which is commonly different for different analytes. Thus, when a sample mixture is run through a column, due to the varying degrees of retention, analytes are separated and further detected through appropriate means. This is the primary aim and purpose of separation methods. The stationary phase has observed significant evolution to ensure the efficient and economical separation of analytes. In preparing this review, methods are selected based on their novelty arising specifically from advances in the design of the stationary phase. Methods whose novelty emerged due to advances in other features—like mobile phase composition, methods of detection, or due to integration with auxiliary technologies like mass spectrometry—are not included, as their novelty is not associated with changes in the stationary phase.

Evolution of Chromatographic Separation Techniques and Stationary Phase Chemistry

In 1903, Mikhail Tsvet demonstrated the separation of plant pigments in a column filled with calcium carbonate as the stationary phase, the first reported liquid chromatography method, followed by his formally coining the term "chromatography" in 1906. This laid the foundation of separation science. Soon, the separation of α-amylase using starch as the stationary phase was demonstrated by Emil Starkenstein—the earliest example of affinity chromatography, although the term had not yet been coined [1]. In 1923, J. C. Whitehorn demonstrated the separation of amines through a permutit-filled column, the earliest example of ion-exchange chromatography (IEC) [2]. A major breakthrough happened when Arne Tiselius demonstrated electrophoresis in 1930, establishing an electromigration-based method for the

separation of analytes [3]. N.A. Izmailov and M.S. Shraiber demonstrated the first planar chromatographic method in the form of thin-layer chromatography (TLC) in 1938, using aluminium oxide, calcium oxide, and magnesium oxide as the stationary phase [4], meanwhile, G. M. Henderson and H. G. Rule investigated chiral separation in columns during the same period [5]. Paper chromatography emerged in 1943 and became a widely adopted method for biomolecule analysis. It was first reported by Archer John Porter Martin and Richard Laurence Millington Synge [6,7]. Paper electrophoresis was developed in the 1930s and 1940s and became a foundational method for innovations in biomolecular separation [8].

During the 1950s, chromatographic science expanded into multiple modes. Gas chromatography (GC) was reported in 1952 by Anthony T. James and Martin [9], while starch was also used as a support medium in electrophoresis by Kunkel and Slater [10]. Size-exclusion chromatography (SEC) involving use of starch-packed column was first demonstrated by Lindqvist and Storgårds in 1955 [11]. Protein purification columns based on hydroxyapatite were subsequently developed in 1956 by Tiselius, Stellan Hjertén and Örjan Levin [12]. Electrophoretic methodologies also advanced with free-flow electrophoresis (1958) introduced by Barriollier et al. [13], isoelectric focusing (1961) introduced by Svenssen-Rilbe [14,15], and discontinuous electrophoresis (1964) introduced by Baruch J. Davis and Leonard Ornstein [16,17]. The modern era of high-resolution separations began in 1966 when Yoichiro Ito described the coil planet centrifuge, which led to the development of counter-current chromatography in 1970 [18,19], and Csaba Horváth developed the first high-performance liquid chromatography (HPLC) instrument in 1966 [20] and commercial availability followed in 1967 with the ALC-100 system launched by Waters Corporation. In the same period, Pohl et al. demonstrated the separation of cells using dielectrophoresis [21,22]. Field-flow fractionation (FFF), first developed by J. C. Giddings in 1966, is a separation technique based on the application of an external field perpendicular to the direction of flow [23,24]. The earliest documented instance of gel-based affinity electrophoresis could not be identified; however, the concept of affinity electrophoresis was first introduced by Bøg-Hansen in 1973 and formally termed in 1977 [25,26]. Capillary electrochromatography (CEC), introduced by Pretorius in 1974, is a type of analytical method that merges the concepts of electrophoresis and liquid chromatography [27]. Additional advancements came in the form of flash chromatography (1978) (Still et al.) [28], the official introduction of high-performance thin-layer chromatography (HPTLC) in 1977 (Albert Zlatkis & Rudolf E. Kaiser) [29], and capillary electrophoresis (CE, 1981) (by Jorgenson and Lukacs) [30,31]. Denaturing gradient gel electrophoresis (DGGE) was introduced by Fischer & Lerman in 1983 32, thereby adding yet another methodology to nucleic acid studies. Micellar electrokinetic chromatography (MEKC), introduced by Terabe in 1984 allowed for the separation of neutral compounds using an electric field [33,34]. In addition, the concept of monolithic columns was introduced by Hjertén in 1988 [35] revolutionizing the principles of flow dynamics and stationary phase permeation. Additionally, the field of acoustic manipulation was explored via ultrasonic standing waves for cell positioning developed by Coakley et al. in 1989 [36]. Finally, ultra-high-performance liquid chromatography (UPLC) technology became commercially available in 2004 via the ACQUITY UPLC system developed by Waters Corporation.

All the evolutions in separation techniques resulting from advances in stationary phases are shown in Figure 1.

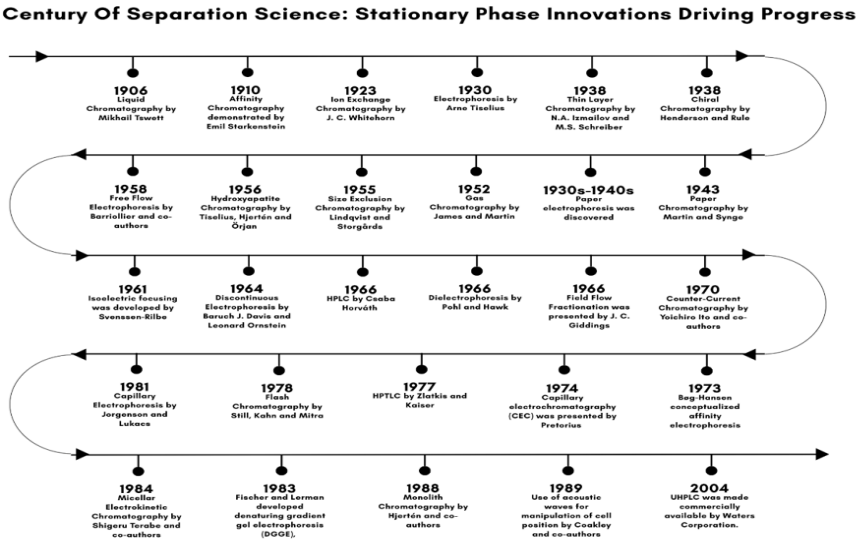

Figure 1: Evolution of Separation Techniques Driven by Advancements in Stationary Phases

In LC, the column employed by Mikhail Tsvet was packed with calcium carbonate for the separation of pigments. The first documented use of silica in a chromatographic column was in partition chromatography by A.J.P. Martin and R.L.M. Synge in 1941; however, in that work, silica served as a support for water rather than as the primary stationary phase [37,38].

In electrophoresis, in 1807 Reuss at Moscow University noticed that dispersed clay particles showed migration in water under the effect of constant electric field [39]. The experiment performed by A. Tiselius involved the use of a U-shaped tube containing a buffer of lower density and a sample solution of higher density. Within the tube, the denser sample solution remained at the bottom, while the lighter buffer occupied both arms of the tube above the sample layer. Electrodes were placed at both ends of the tube and submerged in the buffer. Upon application of an electric field, analyte ions underwent partial separation and became enriched at the interface of the buffer, resulting in their separation. This technique is also referred as carrier-free electrophoresis [40]. Starch gel was first used as stationary phase by Kunkel and Slater in 1952 [10] and Polyacrylamide gel (PAGE) was first used as stationary phase by Samuel Raymond and Lewis Weintraub in 1959 [41]. This technique is also referred as carrier electrophoresis

Chiral separation of compounds was first shown by Louis Pasteur but analytes were separated manually [42]. First column-based separation was shown by Henderson and Rule in 1938 but were not widely adopted as they were specific for the analytes used by them. π-complex chiral selectors by William H. Pirkle and amylose and cellulose columns by Okamoto were earliest example of chromatographic columns that can separate wide range of analytes but they emerged out several decades later [5].

In Ion exchange chromatography, concept of ion exchange developed due to observation by Thompson and Way, who observed that soils and clays were able to absorb ammonia [43]. The earliest reported ion exchange chromatography was performed by J. C. Whitehorn for separation of amines in 1923 [2]. The earliest reported synthesis of ions exchange resins date back to 1935 and it was synthesized by Adams and Holmes [44].

Earlier columns used in hydroxyapatite chromatography exhibited limited stability; these were subsequently replaced by more stable, spherical ceramic hydroxyapatite-based columns [45]. Affinity based method was first demonstrated by Emil Starkenstein in 1910 when he used starch as stationary phase and also as support for purifying α-amylase 1. Immunoaffinity chromatography by Alessandro et al. in 1935 is the earliest example of immunoaffinity chromatography [46]. Cyanogen bromide was first used as affinity binding agent in 1968 by Cuatrecasas, Anfinsen, and Wilchek. Several more binding agents have also appeared like lipids, dye ligands, aptamers, metals, etc [1].

In 1943, Gordon A. H., Martin A. J., and Synge R. L. attempted to immobilize silica on a glass plate as a stationary phase for amine separation but were unsuccessful. However, in the course of that investigation, they observed that cellulose paper could effectively separate amines, leading to the emergence of paper chromatography [6,7]. In the same period, R. E. Liesegang also developed a related method in which a spot of sample was adsorbed onto filter strips placed within a solvent-saturated chamber for the separation of analytes, an approach conceptually similar to paper chromatography [47–49]. Although the theoretical

basis for size-based separation was first outlined by Tiselius and Synge, the first experimental demonstration using a starch-packed column was reported by Lindqvist and Storgårds in 1955 [11].

The landscape of acoustic manipulation further evolved from particles to cells in 1989 when Coakley et al. first reported the use of ultrasonic standing waves to manipulate cell positions. Since by design this method was using liquid bed, it's application remained confined to manufacturing than the analytical applications [36]. It was not until the work of Hawkes et al. (2004), demonstrating cell manipulation between laminar flow streams [50], and Petersson et al. (2007), who reported the first microchip-based separation of human blood cells [51], that the technology transitioned into a high-precision analytical tool.

Affinity electrophoresis is a special electrophoresis technology, which separates biomolecules through molecular binding interaction. The technique was invented in 1973 by Bøg-Hansen and showed a lot of potential but suffered a slow but steady decline in popularity among researchers due to the advent of newer, higher resolution analytical techniques. Nevertheless, affinity electrophoresis has experienced a considerable resurgence of interest during the last decade, aided by several advances and applications of this technique. This has been especially true in regard to the rapid evolution of 1D and 2D metal affinity electrophoresis and high throughput immuno-affinity electrophoresis systems on microfluidic cards. [52].

Capillary electrochromatography (CEC) was first introduced by Pretorius in 1974 and was developed into a powerful technique by Jorgenson and Lukacs in 1981. This transition was an important step from the theoretical concept to experimental work that laid the groundwork for the emergence of CEC as a new approach, combining the benefits of the two established separation techniques, capillary electrophoresis and chromatography. [27].

Overview of Separation techniques in Analytical Chemistry

Chromatographic Separation Techniques

Molecules in chromatography are mostly separated because of their different interactions with the stationary phase. The molecules interact with the stationary phase according to the separation principle employed, either polarity, ion exchange, molecular weight, or another separation principle, and multiple separation principles can apply at once.

Liquid Chromatography (LC) and its improvisations: Liquid chromatography (LC) is one of the earliest separation methods developed and was followed by numerous variations, such as flash chromatography, high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC). All of these procedures employ a column that is filled with silica or other packing material; the major variation relates to the size of the particles of the stationary phase. Generally, these particles are relatively large (200-150 μm) for LC, smaller (63-40 μm) for flash chromatography, fine (5-3 μm) for HPLC and ultra-fine (less than 2 μm) for UPLC. Separation is based on adsorption: Non-polar compounds (Reverse-Phase) are better retained on non-polar (Reverse-Phase) columns, and polar compounds are better retained on polar (Silica) columns.

Thin Layer Chromatography (TLC) and its improvisation: Thin-layer chromatography (TLC) is a method in which a thin layer of silica gel is coated on a solid planar support forming a flat surface used to separate compounds. With this design, analysis can be conducted quickly, utilizing minimal concentrations of analyte. Consequently, TLC is commonly employed in research and industry in order to rapidly screen samples qualitatively. HPTLC is an advanced form of TLC which is characterized by very small dimensions of silica and very thin layers. The benefits of the refinements include better separation efficiency, better resolution and reduced analyte use and mobile phase consumption [40].

Affinity chromatography: Affinity chromatography is characterized by the special chemistry of the stationary phase material, which contains the binding agents for retaining the analytes as they are separated based on certain chemically related characteristics. The stationary phase usually is one of the column fillings, to which these binding agents are attached. Many agents such as enzymes, lectins, metals, and cell membranes have been used successfully. Techniques that rely on individual binding agents, such as lectin affinity chromatography or cell membrane chromatography, may be regarded as distinct methods. However, for clarity and conciseness, they are collectively categorized under affinity chromatography [1].

Paper Chromatography: Paper chromatography is a method that uses liquids as stationary and mobile phase both and the paper is what holds everything together. Water sticks to the paper due to cellulose and acts as stationary phase. When sample is run, as mobile phase runs along the paper, analytes partition between mobile phase and stationary phase continuously while also moving along with the mobile phase. This movement of analytes varies depending on solubility where polar analytes are retained and non-polar analytes move ahead. The paper used is made from cellulose, which is very common, in nature [53].

Counter Current Chromatography (CCC): In counter current chromatography, liquids are used in both the phases, where one is kept stationary and the other is flowing in a column. Counter current chromatography technique is only applicable when two immiscible liquids phases are used with one liquid kept as stationary phase. Separation of analytes are obtained via the principle of partitioning where, as analytes run along the column, their solubility in both the phases determine their elution [54]. Several variations in counter current chromatography are available like:

- Droplet counter current chromatography.
- Hydrostatic counter current chromatography.
- Hydrodynamic counter current chromatography.

Ion-Exchange Chromatography (IEC): Stationary phase in IEC involve use of resins that can perform ion-exchange, either cationic or anionic in nature. This property helps in separating analytes of opposite charge to that of resins, which can be controlled by altering mobile phase parameters like pH, temperature, and salt concentration. Cationic resins are generally made of sulfonic acid ($–SO_3H$) or carboxylic acid (–COOH) functional group, whereas quaternary ammonium ($–NR_3^+$) or amino ($–NH_3^+$) functional groups are commonly found in anionic resins. These functional groups are commonly immobilized on polymers like polyacrylates, polymethacrylates and more and used in column [40].

Size Exclusion Chromatography (SEC): SEC involves separating analytes based on their physicochemical property of molecular size. The stationary phase in this method is composed of beads formed by crosslinked polymers of dextran, agarose, polyacrylamide, styrene-divinylbenzene, sephadex and more. Due to arrangements of polymers into beads, porous structure is formed which primarily drives separation of analytes. During separation, as sample constituents move along the column, smaller molecules are able to enter the pores and are thus more likely to be retained in column and larger molecules elute out earlier and this leads to size based separation of analytes [40].

Hydroxyapatite Chromatography: This chromatographic method uses hydroxyapatite as the stationary phase. Hydroxyapatite is a calcium-phosphate mineral of natural origin whose chromatography column provides a powerful and versatile separation platform by enabling analyte resolution based on two properties: charge and affinity. These principle are able to act simultaneously due to the inherent cation-exchange and metal-affinity characteristics of hydroxyapatite, making this technique particularly suitable for mixed-mode chromatography [12].

Gas Chromatography: Gas chromatography is employed only for the analysis of volatile or semi-volatile compounds, with either a liquid or solid stationary phase. Liquid stationary phases are commonly synthesized from synthetic polysiloxanes, such as polydimethylsiloxane or polyethylene glycol, whereas solid stationary phases are made of materials such as silica or alumina. Columns can either be fully packed or designed as capillaries with a liquid immobilized on the inner walls of capillary. Solid columns are generally limited in length due to dense packing, but capillary columns, being hollow by design, can extend from 1 to 100 meters. The mobile phase generally includes inert carrier gas that does not contribute to separation via interaction due to physicochemical property; rather, separation is entirely dependent on interactions between analytes and the stationary phase [40].

Chiral Chromatography: Chiral chromatography is a specialized technique used specifically for resolution of physical mixture of chiral molecules. The stationary phase are typically prepared with semi-synthetic cyclodextrins, natural polysaccharides, pirkle-type (brush-type) columns, cellulose, and so on. Cyclodextrins are the semi-synthetic oligosaccharides resulting from the α-1,4-glycosidic bond between glucose units, which permit the formation of a toroidal (cone-shaped) structure, with a hydrophobic cavity and a hydrophilic outer surface. This unique structural chemistry allows the binding of multiple molecules of analytes through inclusion complex as a kind of powerful chiral selectors, and this is further divided into subtypes: α-cyclodextrin and β-cyclodextrin. The primary separation principle of chiral chromatography is based on different interactions between the stationary phase and each enantiomer, influenced by factors such as dipole moments and steric hindrance, resulting in different retention times of each enantiomers leading to effective resolution of chiral molecules [5,55].

Monolith Chromatography: Monolith chromatography uses stationary phase made of a single, continuous block of stationary phase material, with the structure referred to as a monolith. This monolithic structure is by design porous and contains countless interconnected channels, providing a noticeable advantage over traditional packed-bed columns like low backpressure and allowing high flow rates. The material used in monolith can be either inorganic, such as silica, or organic, such as polymers. Organic monoliths show pH stability and customizable properties but are generally mechanically less stable than inorganic monolith, whereas inorganic monoliths exhibit high mechanical stability and efficient separation performance [56].

Micellar Electrokinetic Capillary Chromatography (MEKC or MECC): MEKC is a variation of capillary electrophoresis that uses a capillary filled with surfactant containing buffer for the separation of analytes. The novelty of this method arises from the incorporation of surfactants like dodecyl sulfate, into the buffer at concentrations above the critical micellar concentration, leading to formation of micelles. When electric field is applied, these micelles migrate in the capillary, and analytes are separated based on their partitioning between the aqueous buffer and the micellar phase. This leads to separation of neutral molecules that are generally not resolved by conventional gel or capillary electrophoresis. Though micelles are integral components of the buffer, they are not considered a true stationary phase, but due to the partition based separation taking place due to micelles, they are described as a pseudo-stationary phase. Furthermore, though it's operation is similar to electrophoresis, the primary separation mechanism in MEKC is partitioning; therefore, this technique is classified under chromatographic methods [57].

Capillary Electro-Chromatography: Capillary electrochromatography (CEC) is a hybrid separation technique that integrates the high efficiency of capillary electrochromatography with the range of capillary column stationary phases available in liquid chromatography, such as packed beds and monolithic columns, to yield an integrated separation principle on a single platform. In this method, electroosmotic flow serves as the primary factor for the movement of mobile phase, allowing uniform, plug-like flow profiles and keeping band broadening at minimum, leading to overall enhanced separation performance.

Notably, CEC supports a broad spectrum of stationary phase columns, such as reversed-phase, ion-exchange, hydrophilic interaction, chiral separations, and ligand affinity columns. This versatility, in combination with its high efficiency and selectivity, underscores its potential as a powerful analytical tool for the separation and characterization of complex analytes in advanced analytical applications [58].

Electrophoresis Separation Techniques

In electrophoresis, molecules are separated on the basis of different migration rate through a medium with an applied electric field as the driving factor for movement of analyte. Molecules of different size, charge, or shape show different migration rates, and this variation in electrophoretic mobility drives their separation. A limitation of electrophoresis is that the technique can only displaces charged species; neutral molecules are not affected by the electric field and can only be separated through other mechanisms, such as the micellar partitioning seen in MEKC. The inherent property of a charged analyte to migrate under the influence of an applied electric field is labelled as its electrophoretic mobility.

Gel Electrophoresis: In gel electrophoresis, gelling agents such as agarose and polyacrylamide are used for preparation of gel slab that serves as the stationary phase. The analytes are separated due to the application of an electric field, which induces the movement of charged species. The matrix in gel slab contains a network of pores that influences the migration of analytes. Since the applied electric field can only govern the movement of charged analytes, only charged molecules are separated using this technique. Neutral molecules do not respond to the electric field and, therefore, are generally not resolved due to electrophoresis [40].

Capillary Electrophoresis (CE): CE is an analytical approach that utilizes a narrow capillary filled with a polymer matrix, gel or a buffer solution for the separation of analytes. In this method, both ends of the capillary are placed in the buffer reservoirs present in separate vessels, which are connected through electrodes to complete the circuit and establish an electric field across the capillary. Typical length of capillary ranges from 10 to 100 cm, which allows for efficient separation within a confined pathway. Due to the influence of the electric field, migration of analytes through the capillary takes place with each analyte migrating at different velocities based on their charge-to-size ratio, resulting in efficient separation of analytes. This technique is characterized by high separation efficiency, shorter analysis time, and low sample size and reagent waste generation, making it widely applicable in analytical and research settings [40].

Discontinuous Electrophoresis: The novelty of this separation technique lies in the discontinuous nature of the stationary phase, which is not uniform throughout the system. This discontinuity is mainly attributed to variations in four key parameters: the composition of the gel slab, buffer pH, the ionic strength of the buffer, and the properties of ions present in both the gel and the electrode buffer. The buffer, alongwith other components, contains glycine, which plays a critical role in the separation process. As per the Ornstein and Davis method, the stationary phase is divided into two separate regions: the stacking gel and the resolving gel. The stacking gel is attributed by a larger pore size and a pH of 6.8. The larger pore size leads to reduced resistance to protein movement, allowing the formation of concentrated protein bands based on their mobility. The gel also contains Cl^- ions, which exhibit high mobility under the influence of applied electric field, whereas glycine remains relatively immobile due to its zwitterionic form at this pH. In contrast, the resolving gel is designed to have a smaller pore size and a pH of 8.8. The reduced pore size leads to decrease in protein

mobility, leading to effective band separation. At this higher pH, mobility of glycine is increased and, along with Cl^- ions, migrates ahead of the proteins. Furthermore, the pKa of the amino group in glycine contributes to an increase in pH to approximately 9.5, leading to enhanced protein mobility and accelerating the separation process [59].

Free Flow Electrophoresis: Free-flow electrophoresis (FFE) is a technique in which no solid stationary phase is used, and the sample is continually added into a thin flowing liquid film between two parallel plates. Lateral deflection of analytes according to their electrophoretic mobility is produced by application of an electric field in the direction perpendicular to the flow. This leads to the continual separation in space and analytes can be collected in separate outlet fractions. [43].

Dielectrophoresis: Dielectrophoresis is used to separate without a stationary solid phase. The method involves placing the analytes in a fluid medium between two electrodes producing a non-uniform electric field. The polarizability of particles to the medium causes the dielectrophoretic force that pushes particles towards high or low fields. This allows separation without the need to use a label, which relates to the difference in size and/or dielectric properties. [44].

Isoelectric Focusing: Isoelectric focusing (IEF) is a specialized variant of gel electrophoresis that utilizes a stable pH gradient parallel to electrophoresis within a gel matrix to achieve separation of biomolecules. Amino acids and proteins possess ionisable functional groups, such as amine and carboxyl groups, that give it a net positive or negative charge in the surrounding environment. Adjusting the pH to a point where the opposing charges are equal brings the net charge to zero, and is called the isoelectric point (pI). The main purpose of isoelectric focusing is to separate proteins according to their isoelectric points. The pI of the molecules also affects the migration in electrophoresis: molecules that are more acidic than the pH of the gel will be negatively charged and move toward the anode, while molecules that are more basic than the pH of the gel will be positively charged and move toward the cathode. This process continues until the net charge on each analyte is zero when it reaches the point in the gel where the pH matches its isoelectric point and it no longer moves; the molecules are now then concentrated into separate bands and are separated. [60].

Denaturing Gradient Gel Electrophoresis (DGGE): Denaturing Gradient Gel Electrophoresis (DGGE) is based on the principle that partial denaturation of the DNA occurs during the electrophoretic separation in a gradient of chemical denaturants. Conceptually, the gel slab design in DGGE is similar to isoelectric focusing (IEF) in which a parameter is increased continuously in the direction of electrophoresis. The gradient in IEF is based on pH, and in DGGE the concentration of the denaturing agents in the gel (urea or formamide) is varied. Biomolecules such as proteins and nucleic acids partially denature at a certain concentration of the denaturant during electrophoretic migration. This process results in the formation of discrete bands unique to each fragment. DGGE separates medium-to-short fragments of DNA according to their melting behavior and is sufficiently resolving to detect single-nucleotide polymorphisms without DNA sequencing. [61].

Gel Affinity Electrophoresis: Affinity electrophoresis is an analytical technique which is specific, and involves the use of affinity binding agents in electrophoretic environments to study biomolecular interactions. A variety of methodological variations have been reported such as gel affinity electrophoresis, capillary affinity electrophoresis and affinity-trap polyacrylamide gel electrophoresis. Of these, only gel affinity electrophoresis is discussed in detail herein, since the other methods do not primarily rely on the modification of the stationary phase as a novel aspect of their method and thus do not fit the purpose of this article. The principles of gel affinity electrophoresis are analogous to those of gel electrophoresis, but the unique characteristic of this technique is the modulation of analyte mobility by binding to a specific affinity ligand. These ligands attached to the gel and thus are able to separate analytes according to their binding abilities. This methodological refinement acts as a powerful means for resolving complex mixtures with high specificity for elucidation of molecular interaction, thus expanding the applicability of the method in biochemical research [52].

Paper Electrophoresis: Paper electrophoresis is a separation method using an electric field to separate charged molecules by differential migration through the stationary phase, a sheet of paper. The rate at which the components of the sample move is affected by the charge and molecular size on which they move, allowing them to be separated and analyzed. Although useful at the time, this technique has been replaced by newer techniques that offer higher resolution, accuracy and reproducibility and are more appropriate for the modern scientific needs; the most commonly used of these techniques is gel electrophoresis. However, the resurgence of interest in paper-based electrophoresis has come in the last few decades, when the paper-based platform was used to develop miniaturized analytical devices by implementing microfluidic technologies that are inexpensive and portable [8].

Miscellaneous Separation Techniques

These methods do not fall under the category of chromatography or electrophoresis but are able to separate analytes on the basis of their separation principle.

Field Flow Fractionation (FFF): FFF is a separation methods in which the stationary phase is not used, and the fractionation of analytes is accomplished mainly by size. This is a key difference between FFF and traditional chromatographic and electrophoretic techniques. In FFF, analytes move in a thin open channel of fluid that is located between two parallel plates, with the analytes carried by the liquid stream. The channel is usually 30–100 cm long and 1–3 cm wide, much larger than the thickness of the channel, which is typically 0.05–0.25 mm. This gives a high aspect ratio geometry for the channel. The separation in FFF is based on the combination of the parabolic flow profile and application of an external force field applied transverse to the flow. The channel has a high aspect ratio, which guarantees laminar flow conditions, and a parabolic velocity distribution with fluid velocity being lowest near the walls and highest at the centerline of the channel. A perpendicular external force field is applied at the same time, such as thermal field, sedimentation field (centrifugal), cross-flow field, electrical field, magnetic field or other force field, which pushes analytes to accumulate on the accumulation wall. This force experienced by analyte is typically proportional to it's size, and in some cases, more than one field can be used at the same time. A balance is created between the external force and the counteracting diffusive flux due to Brownian motion. This means smaller analytes, which have greater diffusion coefficients, will be farther from the accumulation wall and will be moving along faster streamlines towards the center of the channel. Larger analytes, however, stay in slower moving areas close to the wall. This spatial separation leads to a velocity difference for the migration and hence good separation. Due to these properties, FFF has been attracting interest for its use in biopharmaceutical and nanotechnology applications; however it has not been extensively adopted for routine use in normal laboratory practice [40].

Acoustophoresis: Acoustophoresis is a stationary-phase-free technique for separating particles and cells of different size, density and compressibility. The technique used involves introducing ultrasonic standing waves inside the fluid-filled microchannel, creating pressure nodes and anti-nodes. The particles move toward the acoustical anti-nodes or nodes depending upon their acoustic contrast factor, which depends upon the density and compressibility of the particles as compared to the suspending fluid. This allows for label-free and contactless separation of biological particles with high viability [62].

Analysis of Separation Methods: A summary of the main analytical separation techniques classified according the stationary phase chemistry and separation mechanism is listed in Table 1. Silica based methods (LC, HPLC, UPLC, TLC, HPTLC, GC and flash chromatography) are used to separate compounds, with interactions taking place between the compounds and silica in both the adsorption and partition process, covering a large molecular size range. Ionic analytes are separated in ion-exchange chromatography based on the net charge of the substance, and macromolecules are separated on the basis of their size in size-exclusion chromatography alongwith electrophoretic mobility in electrophoresis.

Specific molecular recognition and stereoselective interactions work in affinity and chiral chromatography, respectively. Specialized separations obtained by the use of liquid–liquid partition methods, HPLC with hydroxyapatite columns, Acoustophoresis, and Micellar electrokinetic chromatography make it possible to analyse compounds that are not easily separated by regularly used HPLC columns.

Table 1: Comparison of Composition of stationary phase, principle and its uses in different separation techniques

Nature of Stationary Phase	Size of molecules separated	Separation Methods	Example	Reference No.
Silica	Small (<1000 Dalton)	HPLC, UPLC, TLC, HPTLC, GC, Flash Chromatography	Phytoconstituents, Fat, Volatile Organic Compounds, Drugs, Polysaccharides	[63–70]
	Large (>1000 Dalton)	HPLC, UPLC, TLC, HPTLC, Flash Chromatography		
Ion exchange	Small (<1000 Dalton)	Ion Exchange Chromatography, Monolith Chromatography	Proteins, Immunoglobulin, Virus, Extracellular Vesicles, Peptides, Capsids	[71–81]
	Large (>1000 Dalton)			
Gel	Large (>1000 Dalton)	SEC, Gel Electrophoresis, CE, Discontinuous Electrophoresis, DGGE, Paper electrophoresis,	Enzymes, Extracellular Vesicles, Proteins, Genetic Material	[82–90]

		Isoelectric focusing,		
Biorecognitive	Small (<1000 Dalton) Large (>1000 Dalton)	Affinity Chromatography using various affinity agent: Lectin, Metal, Cells, Phospholipids, etc. Affinity electrophoresis	Phytoconstituents, Proteins, Antibodies, Drugs, Genetic Material	[1,52,91–96]
Chiral	Small (<1000 Dalton)	Chiral Chromatography using various agents: Polysaccharides, Cyclodextrins, Proteins, etc.	Any Chiral Molecule's Enantiomer Mixture.	[42,97–100]
Liquid bonded	Small (<1000 Dalton)	Paper Chromatography, Counter-Current Chromatography	Fatty Acids, Phytoconstituents	[101]
Hydroxyapatite	Large (>1000 Dalton)	Hydroxyapatite Chromatography	Monoclonal Antibody, Protein,	[102–105]
Micelles	Small (<1000 Dalton)	MEKC	Drugs, polyamines	[106–109]
Polarizable	Large (>1000 Dalton)	Dielectrophoresis, Field-flow fractionation, Free flow electrophoresis	Cells, Liposome	[110–112]
Acoustic Contrast	Large (>1000 Dalton)	Acoustophoresis	Cells	[113–116]
Hybrid Method	Small (<1000 Dalton)	Capillary electrochromatography	Drugs	[117–120]

Abbreviations in table

- HPLC- High-performance liquid chromatography
- UPLC- Ultra-high-performance liquid chromatography
- TLC- Thin-layer chromatography
- HPTLC- High-performance thin-layer chromatography
- GC- Gas chromatography
- SEC- Size exclusion chromatography
- MEKC- Micellar electrokinetic chromatography
- DGGE- Denaturing gradient gel electrophoresis

Comparative Performance Analysis of Stationary Phase Architectures

Choosing a stationary phase architecture for a particular analytical or preparative application has to consider a variety of performance parameters including theoretical plate number (N), resolution (R_s), back pressure, mass transfer characteristics, sample throughput, analyte load capacity and cost/reliability of the instrument.

Table 2 shows a tabular comparison of the representative literature values of these parameters for key stationary phase architectures which would be useful in making rational choices for phase selection.

Table 2: Comparative Performance Parameters of Major Stationary Phase Architectures

Stationary Phase Type	Typical Particle / Feature Size	Efficiency (N/m)	Operating Pressure	Mass Transfer	Load Capacity	Key Advantage	Key Limitation
HPLC (3–5 µm silica)	3–5 µm	~50,000–80,000	Moderate (up to ~400 bar)	Moderate (diffusion-limited)	Moderate	Versatility; broad analyte compatibility; mature technology	Slower analysis vs. UPLC; higher solvent use
UPLC (sub-2 µm silica)	<2 µm	~150,000–200,000	High (>600 bar)	Excellent	Moderate–low	High efficiency; fast analysis; low solvent consumption	High instrument cost; requires ultra-high pressure hardware
Monolith (silica / polymer)	2 µm skeleton / 5 µm macropore	~100,000–200,000	Low (<200 bar)	Excellent (convective flow)	High (esp. for biomolecules)	Fast flow rates; low backpressure; high throughput	Batch-to-batch reproducibility; limited column selection

MOF-based	Sub-micron crystals (0.3–1 μm)	~50,000–120,000 (reported)	Low–moderate	Good (large pore volume)	Variable; often low due to strong binding	Tunable pore size; unique selectivity for shape/size isomers	Limited aqueous stability; poor reproducibility; not commercialized
Polysaccharide chiral (cellulose / amylose)	3–5 μm coated silica	~50,000–100,000	Moderate	Moderate	Moderate	Broad chiral recognition; well established; regulatory acceptance	Solvent restrictions for coated phases; method development can be lengthy
Capillary electrophoresis	25–100 μm i.d. capillary	Up to 10,000,000+	None (voltage-driven)	Excellent (plug flow)	Very low (nl injections)	Extremely high efficiency; minimal reagent use; separates charged and neutral species (MEKC)	Low sensitivity; limited load capacity; precision can be poor

Abbreviations in table

- HPLC- High-performance liquid chromatography
- UPLC- Ultra-high-performance liquid chromatography
- MOF- Metal-organic framework

The relative significance of these several change pathways becomes clear in terms of the several important comparative insights that emerge from Table 2. First of all, UPLC provides the best possible chromatographic efficiency for a particle-packed system because of its small particle size of less than 2 μm; this disadvantage, however, is a fast rise in backpressure, since most conventional HPLC systems can only withstand up to 400 bar [121]. The reduction of the length scale, which includes the particles size, results in better mass transfer, with 1.7 μm particles offering h.e.t.p. values 2-3× lower than 3.5 μm particles to yield comparable separation efficiency [122]. This also gives a significantly increased range of usable flow rates, thus providing an increase in sample throughput without any loss in separation quality [123]. A desirable "solution" in between is obtained with the monolithic columns: The macroporous structure has interconnected pores which enable convective transport of substrates through the column support, thus avoiding diffusional mass transfer limitations, and the velocity of operation may be high [35]. The monolithic type of supports is characterized by a combination of mesopores and macropores, which enables high flow rates at low mass transfer resistance and low backpressure on a large surface area [124]. In high throughput screening of large biomolecules like proteins and viral vectors, monolithic supports prove to be especially appealing for their capability to separate empty capsids from viral capsids containing DNA and to successfully isolate supercoiled plasmid DNA and remove the mRNA therapeutics impurities of double-stranded RNA [125]. Metal-organic framework (MOF) stationary phases are currently being studied in an exploratory way, because MOFs are crystalline porous compounds that consist of metal ions and organic linkers, have tunable pore environment and are structurally versatile allowing the control of the size, shape, hydrophobicity and chemical functionality of the pores, as needed for the separation of molecules that require finely tuned host–guest interactions [126].

A distinguishing feature is their ability to separate structurally similar substances, like positional isomers, and the ability to do this is believed to be a combination of molecular sieving and adsorption properties however, an important limitation is that because of the irregular shapes of the crystals, a high pressure is required to achieve good column efficiency [127]. While columns filled with MOFs reported at up to 10200 plates/m show good profile, this is significantly lower than the modern chromatographic efficiencies for silica-based columns [128]. Unlike all chromatographic systems, however, eddy diffusion and mass transfer terms do not exist in CE; it is separated in free solution with a relatively steep electroosmotic flow profile, with a number of plates typically much higher than those found in conventional LC separations, to the extent of close to one million [129].

However, the major drawback of its application is that very small amounts of injection can lead to significant decreases in the reliability and accuracy of results, limiting its possible use in trace level analysis [130]. The aforesaid trade-offs show that there is no one best architecture of stationary phases for achieving universal separability; however, choosing the appropriate stationary phase

should account for the physicochemical attributes of the target analyte, the separation scale desired, and the regulatory nature of the application.

Applications of Emerging and Less explored Separation Techniques

Methods that are reliable for scientific work but not in routine or special work are emphasized, and methods which are universally used in research, or which have been superseded by methods of greater sophistication, are not covered. While capillary electrochromatography was satisfying the criteria, it's applications were not considered here, as most studies published between 2020-2025 focused on fabricating new columns for analyte separation, in contrast to other methods that do not involve column fabrication [117–120].

a. Acoustophoresis:

1. The separation of specific cell populations from blood is critical for diagnostic and research applications, and efficient isolation methods are therefore of great importance. Alsved et al. reported a label-free acoustophoretic method for the isolation of peripheral blood mononuclear cells from whole blood [113].
2. During radiolabelling of cells, multiple centrifugation steps are required to separate unbound radiotracers from bound radiotracers, which is necessary to ensure accuracy of the results. Adler et al. presented an acoustophoresis-based method for separating bound and unbound radiotracers [114].
3. In hematopoietic stem cell transplantation for neuroblastoma, the risk of relapse is exacerbated by contamination of the stem cell product with tumour cells. Olm et al. presented an acoustophoretic method for separating tumour cells from the mixture and for enriching the tumour cell fraction, which holds diagnostic and prognostic value [115].
4. While methods for separating complex cell mixtures are well established, their application to limited-volume samples remains challenging, particularly when labelling or affinity-based approaches are required. Urbansky et al. presented an acoustophoretic lab-on-a-chip method that addresses these limitations [116].

b. MEKC:

1. Several anti-breast cancer drugs require therapeutic drug monitoring during treatment. Although liquid chromatography-mass spectrometry (LC-MS) has been applied for this purpose, reports of simultaneous determination of six such drugs are scarce, and existing methods have notable limitations. Mlinarić et al. identified advantages and disadvantages of capillary electrophoresis relative to liquid chromatography, and hyphenated CE with mass spectrometry to address the identified shortcomings. Ultimately, sweeping MEKC-MS/MS, preceded by an appropriate sample extraction step, was proposed as a clinically viable approach for therapeutic drug monitoring [106].
2. Second-generation triazole antifungals are used for the treatment of invasive systemic fungal infections, and therapeutic drug monitoring is required for these agents. Although HPLC-based methods exist for this purpose, Liang et al. developed a sweeping MEKC-based method for the simultaneous determination of three second-generation triazole antifungals [107].
3. Nonsteroidal anti-inflammatory drugs are widely used for therapeutic purposes in both humans and animals. Their widespread use has resulted in environmental contamination, as conventional wastewater treatment methods are generally insufficient for removing these compounds. Several analytical methods — including HPLC and CE — have been developed for nonsteroidal anti-inflammatory drug monitoring, though many are limited by high cost and long analysis times. Alatawi et al. presented an MEKC-based method as a cost-effective and efficient alternative for the analysis of nonsteroidal anti-inflammatory drugs in environmental samples [108].
4. Polyamines are present in both animals and plants, where they play important roles in numerous biological processes. While LC- and CE-based methods exist for polyamine analysis, Olędzka et al. developed an alternative method employing MEKC coupled with laser-induced fluorescence detection for the sensitive analysis of polyamine-containing samples [109].

c. Monolith Chromatography:

1. Research related to extra cellular vesicles are increasing due to it's potential as a novel drug delivery system. Here, Colao et al. used monolith chromatography as a potential bioprocessing method for extra cellular vesicles [71].
2. Attenuated strains of Newcastle Disease virus have demonstrated utility as oncolytic agents in oncolytic virus therapy. A major challenge associated with their use has been the development of robust purification processes. Rogerson et al. reported the use of anion-exchange monolith chromatography for the purification of the virus [72].
3. Analysis of human immunoglobulins is valuable in the study of disease development, and their efficient isolation is a prerequisite for such analyses. Martinović et al. presented an

affinity-based monolithic chromatography column as an alternative approach for the isolation of immunoglobulins [73].

4. Plasmid DNA has established utility in gene therapy and vaccination, and high-purity plasmid DNA is essential for these applications. However, plasmid DNA purification remains technically challenging. Kodermac et al. presented a modified anion-exchange monolith chromatography method for the efficient purification of plasmid DNA [131].

d. Dielectrophoresis and Free Flow Electrophoresis;

1. Age-related macular degeneration is prevalent in older populations, and one therapeutic strategy involves the transplantation of healthy retinal pigment epithelial cells. Yadav et al. proposed a dielectrophoresis-based method for the isolation of pure, healthy retinal pigment epithelial cells suitable for therapeutic application [111].
2. Analysis of circulating tumour cells is of importance in cancer diagnosis, prognosis, and therapy. Nguyen et al. presented a microfluidic device employing dielectrophoresis for the label-free separation of circulating tumour cells from other blood components [132].
3. Charge variants in monoclonal antibodies are routinely monitored in the biopharmaceutical industry, typically by imaged capillary isoelectric focusing. However, this technique has limitations with respect to fractionation and sample recovery. Liu et al. presented a free-flow electrophoresis-based method for the characterization of charge variants in monoclonal antibodies [133].
4. Intratumour heterogeneity in cancer cell populations presents a challenge for their preparative fractionation into distinct subpopulations, particularly when charge-based separation is required. Sohail et al. demonstrated the applicability of free-flow electrophoresis for separating healthy lung cells from cancerous lung cells [112].

e. Field-flow fractionation:

1. A DNA amplification method called loop-mediated isothermal amplication was used by Safenkova et al to produce amplicon of invA gene present in Salmonella enterica. For characterization of amplicons asymmetric flow field-flow fractionation (AF4) was used by author [134].
2. Liposomal doxorubicin formulations are commercially available as alternatives to free doxorubicin, which is associated with systemic toxicity. Both branded and generic liposomal doxorubicin products are marketed in the United States. Ansar et al. conducted a comparative evaluation of generic products against the branded reference standard. Asymmetric flow field-flow fractionation (AF4), LC-MS, and nanoparticle tracking analysis were employed in this assessment [110].
3. Inductively coupled plasma-mass spectrometry (ICP-MS) is used for characterization of samples containing metals in them. Meili-Borovinskaya et al. has used asymmetric flow field-flow fractionation (AF4) coupled with ICP-time-of-flight mass spectrometry (ICP-TOFMS) (AF4-ICP-TOFMS) to show advantage of AF4 coupling with ICP-TOFMS [135].
4. Although AF4 has been applied to the analysis of nanopharmaceuticals, robust, standardized operating procedures suitable for industrial use had not previously been established. Through a collaborative effort involving experts from the European Union Nanomedicine Characterization Laboratory and the National Cancer Institute-Nanotechnology Characterization Laboratory, Caputo et al. developed a quality reference standard for multi-detector AF4 (MD-AF4) [136].

f. Countercurrent Chromatography:

1. For separation of compounds from olive leaves, conventional methods are available but are rendered inefficient in long-term usage which leads to decrease in purity of compound. Sun et al. has developed high performance countercurrent chromatographic method to address this issue. Author achieved better purity of compounds from this method [137]
2. Persistent Organic Pollutants and Halogenated Natural Products are found in marine lifeforms, mainly fish. During analysis of sample, it's lipid matrix can be removed using established techniques such as sulfuric acid treatment or gel permeation chromatography. Here, Schweizer et al. has developed alternative method for removal of lipid matrix which involves Countercurrent chromatographic method which is operated in co-current mode. Author found separation result similar to gel permeation chromatography method [138]
3. Leaves of Eriobotrya japonica contains certain constituents which can inhibit α-glucosidase. Here, Ma et al. has used high-speed countercurrent chromatography for preparative separation and purification of bioactive sesquiterpene glycosides 139
4. Sciadonic acid, a polyunsaturated fatty acid obtained from oil of Torreya grandis is reported to have several health benefits. There are several methods for purification, but it was observed by Xianghe et al. that no methods report it's purification by silver ion combined high speed countercurrent chromatography. Here,

author has developed a method for their purification with above method [140]

g. Denaturing Gradient Gel Electrophoresis:

1. Effluents released by poultry industry are filled with waste of biological origin and thus pose a public health hazard risk. To study and compare the bacterial diversity of effluents, Author (Ouslati et al) used DGGE [141].
2. Petroleum refinery wastewater generated in petrochemical industry are required to be treated before they are deemed fit for release. Here, author Dai et al has developed a biological processing method that can be used for treatment of wastewater. For microbial analysis of process polymerase chain reaction- denaturing gradient gel electrophoresis was used [[142].
3. European foulbrood and American foulbrood is a bacterial disease linked to honeybees. Here, author Masry et al. developed nanoparticles for treatment of disease and simultaneously tried to find any uncommon strain of bacterial entities. For detecting any uncommon strain, polymerase chain reaction- denaturing gradient gel electrophoresis was used [143].
4. Root rot disease is caused due to bacterial entities like R. solani that can significantly damage crops. Here, Elsharkawy et. al. developed a disease decline soil for controlling such diseases. For analysing the soil microbial variety, polymerase chain reaction- denaturing gradient gel electrophoresis was used [88].

Bio-Based Stationary Phase

Bio-based stationary phases are a significant and recent class of separation materials in which the material is natural products, or is a semi-synthetic derivative of a natural product. They are classified as stationary phases, and are part of a larger classification that includes silica based, polymeric, monolithic and Metal-organic frameworks (MOF) based stationary phases, based on their features of biocompatibility, most of the stationary phases, and their accord to green chemistry principles.

Both polysaccharides, such as cellulose, amylose and agarose, and phospholipids and even whole cells or cell membranes represent important bio-based materials.

The greatest advantage of polysaccharide derived phases, in their application as chiral stationary phases in the pharmaceutical field, is the ability to recognize one enantiomer over the other and mechanical stability, particularly in the case of cellulose and amylose coated/immobilized on silica supports. Due to its well-defined pore structure, hydrophilicity and biocompatibility, seaweed-derived agarose gel is the standard matrix used for gel electrophoresis. Bio-based stationary phases are available for use in the methods listed in Table 3.

Table 3: List of Methods that includes bio-based stationary phase

Method	Natural material used in stationary phase
Gel electrophoresis	Agarose gel bed [144]
Hydroxyapatite chromatography	Hydroxyapatite
Immobilized Liposome Chromatography	Phospholipid
Immobilized Artificial Membrane Chromatography	Phospholipid
Cell Membrane Chromatography	Cells
Capillary Electrophoresis	Agarose [145]
Chiral Chromatography	Cellulose, Amylose [42]

Research on biologically derived stationary phases is continuing and signifies the general trend in separation science focusing upon green analytical chemistry.

One of the most sustainable redesign elements that affect the process is the stationary phase itself, as most traditional silica-based supports are energy intensive to produce and operate using halogenated or high-toxicity organic solvents. Substituting conventional stationary phase materials with ones of bio origin is increasingly on the agenda to reduce this, and generally takes place by the following types of stationary phase materials that have been developed to solve the same separation tasks, but take biological materials as an alternative. Table 4 shows some stationary phases made with material derived from nature for modified separation. These bio-derived columns have shown promise of being more cost effective, environmentally friendly columns compared to standard ones, and even separating mixtures of analytes that were not resolved by the use of standard ones in some instances.

The long term stability, scalability of synthesis and column-to-column reproducibility, however, challenges will have to be overcome before it is generally adopted.

Table 4: List of Separation techniques with modified stationary phase using materials of biological origin

Separation Method	Group of Compound	Material Used
GC	Fibres	Palm Fibres [170]
HPLC	Ionic Liquids	Imidazolium ionic liquid of dehydroabietic acid

Separation Method	Group of Compound	Material Used
		(Rosin derived) [171]
	Deep Eutectic Solvents	HCl protonated L-proline: Xylitol [172]

Abbreviations in table

- HPLC- High-performance liquid chromatography
- GC- Gas chromatography

Sustainability and Green Analytical Chemistry in Stationary Phase Design

Environmental issues of analytical chemistry have received more attention as a policy and science issue and separation science is no exception. Traditional analytical workflows typically use large amounts of organic solvents, many of which are toxic, carcinogenic, and long-lasting in the environment, and produce chemical waste streams that pollute the environment where labs operate. The stationary phase is one of the leverage points to enhance the sustainability not only by influencing mobile phase composition but also due to its manufacturing and disposal footprint during the stationary phase. The principles of "Green Analytical Chemistry (GAC)," aimed at the reduction of hazardous reagent use, minimizing waste generation and reducing energy use for analytical performance have started to influence the criteria used for the design and evaluation of new stationary phases. Many tools have been created to evaluate the greenness of analytical approaches, such as Analytical Eco-Scale [146], the Green Analytical Procedure Index (GAPI) [147], and the AGREE (Analytical GREEnness) metric [148]. These frameworks have been developed to assess methods in terms of various aspects: toxicity of the solvent, energy use, waste generation, analyst safety, consumption of sample and reagent, and give a quantitative score so that methods can be compared [149]. The stationary phase can affect these scores in two ways: directly, by its synthesis pathway and waste disposal, and indirectly, by its impact on mobile phase selection. Aqueous-alcoholic mobile phase or supercritical CO_2 program will perform significantly better than a program that would use solvents with a high boiling-point or a chlorinated solvent on eco-scale metrics.

In this context there are several categories of stationary phase development that tie directly to GAC goals. One of the most commercially developed examples of bio-based phases with reliable green credentials are polysaccharide chiral stationary phases, which are based on cellulose and amylose, and are renewable and biodegradable and supercritical fluid chromatography (SFC) which uses supercritical CO2 is green as CO2 vaporises in atmosphere after leaving supercritical fluid state. The most popular stationary phases for chiral SFC are those based on polysaccharides because of their many properties, such as a broad range of potential applications, repeatability, high loadability and the availability of a wide variety of commercially available polysaccharides [150]. Their enantioselective ability has been proven for a large variety of structurally diverse biologically active compounds and for the amylose-based phases especially high rates of baseline enantioseparation have been obtained [151]. In the pharmaceutical industry, SFC with chiral polysaccharide columns has become the preferred approach for enantioseparation, not only due to its high performance as well as requiring minimal solvent consumption and being process-friendly-enantioseparation [150,151]. The naturally occurring hydroxyapatite mineral can be synthesized using biogenic calcium sources like cuttlefish bones, mussel shells and chicken eggshells which are obtained from biological tissues and eliminate many of the energy consuming chemical processes used in the functionalisation of silica-based materials [152].

Bio-renewable materials like deep eutectic solvents (DES) have also gained considerable attention as greener modifier for stationary phases used in HPLC and GC. Deep eutectic solvents are generated through the blending of hydrogen bond donors and acceptors (such as choline chloride and amino acids, or choline chloride and glycerol) that have a range of environmental benefits due to their biodegradability and recyclability, mechanical, chemical, and thermal stability, but are also hindered by their viscous nature and tendency to degrade in aqueous solution [153]. A variety of DES formulations can be readily prepared, are biodegradable, and have reasonable costs associated with them, including amides, carboxylic acids, and alcohols [154]. Designed for use as stationary phase components, DES modified columns have been shown to have utility for polar analytes while also affording a decreased reliance on the use of halogenated solvents as shown in Table 4 from this review [153]. Likewise, HPLC staionary phase materials made from rosin (abietic acid is the major component of rosin, and it has extremely good renewability and biocompatibility) have been tested as HPLC stationary phases, which is also in keeping with the principles of ‘green chemistry' making them strong candidates for sustainable chromatographic applications 155. Ionic liquid stationary phases, such as imidazolium modified silica, have been shown to be selectively tunable over a variety of chromatographic modes [156]. Another major sustainability-elevating lever in separation science is miniaturisation. Mobile

phase can be used in the nanolitre to microlitre per minute range with capillary and nano-LC systems, which require several orders of magnitude less mobile phase than conventional HPLC, which in turn results in fewer waste products and the associated costs of safe solvent disposal [157,158]. UPLC also saves valuable space of both analysis time and solvent consumed per sample injection; switching to a UPLC column format can translate to savings in injection per sample greater than 85% per injection compared to traditional approaches [159]. The absence of particle packing in monolithic columns also results in a lower working pressure, and therefore lower energy use, compared to particle-packed UPLC systems, at the same throughput; further, monolithic and core–shell column geometries with shorter columns and lower internal diameters reduce analysis time, solvent consumption and improve overall method sustainability [160]. They are not revolutionary per se, but their total beneficial effects in all of the many high volume industrial and clinical laboratories can be significant.

Though these innovations have developed, challenges yet exist to scale and integrate stationary phase innovation with GAC principles. The synthetic pathways needed to prepare many novel bio-derived or functionalised phases are multi-step and often involve the use of hazardous reagents, which defeats the end use environmental benefits of such phases. A rapid degradation of the stationary phase and the need to replace it frequently is an important life factor: a short-lived stationary phase produces more overall waste than a traditional silica column with a long service life [160]. An additional barrier is regulatory acceptance of novel stationary phases in the pharmaceutical quality control, where validated, internationally recognised methods are required. This will involve more than just chemical innovations; lifecycle analysis, standardisation, and regulatory engagement will all play significant roles in the path towards truly sustainable stationary phase design.

Future Perspectives

More than a hundred changes have been made in stationary phases over the years, some of which have been brought to commercial use. However, studies in the field are still underway. There are several modifications under active investigation that seek either to enhance the separation efficiency of the currently existing methods, or to develop new sustainable analytical methods. Below are discussed selected examples:

1. Monolith Chromatography:

Monolithic supports have been prepared from silica and various organic polymers like methacrylate, styrene/Divinylbenzene, or acrylamide-based propagates, and they have also been functionalised in their surface with reversed phase, ion-exchange, or affinity ligands. Some commercially available monolithic columns are available such as CIM® disk monoliths (BIA Separations) and Chromolith® silica monoliths (MilliporeSigma) and many other new stationary phases are currently being investigated, especially for biopharmaceutical applications [35,161–164]. Future development of monolithic technologies is envisioned as leading to more complex and stable hybrid materials characterized by superior stability, selectivity, and mass transfer properties. These developments in surface chemistry, nanostructuring and additive manufacturing will allow the development of a wider range of customized and efficient platforms, potentially broadening their application to biopharmaceutical production and analytical separations.

2. Metal-organic framework based stationary phase:

Metal-organic frameworks (MOFs) are porous crystalline materials that are built by linking organic linker molecules with metallic or inorganic nodes. This produces a three dimensional structure with clearly defined pores which permit the mobile phase to flow through, while the metal centres interact with analytes to promote separation. As mentioned before MOFs are tunable and this makes it a topic of interest as active research is still conducted for fabrication of MOF based columns. This can lead to emergence of columns that can have multiple separation factors working simultaneously and more [165–170].

3. Functional groups-based separation of analyte:

Traditional separation methods typically separate analytes according to their polarity, charge, size, or stereochemical attraction to the separation matrix. In 2019, a paper reported a fundamentally different method in which novel, phosphonium ionic liquids have been tested for use as stationary phases for gas chromatography. A standard analyte mixture was chromatographed on an stationary phase consisting of trihexyl(tetradecyl)phosphonium chloride [P66614+][Cl−]: separation was seen as a function of the functional group identity of the analytes and not bulk polarity. To our best knowledge, there is no existing method on the market to selectively separate analytes based on their functional group solely. In-depth evaluation would also offer the potential for a new mode of chromatography, but significant systematic investigation of this mechanism with a large number of analyte classes and ionic liquid structures would need to be completed before it could be confirmed to be general [171].

Conclusion

In the early 20th century the first separation technique was developed and separation science has since grown significantly and is an important component of many industrial processes such as pharmaceuticals, forensics, and academic research. The resolution of an expanding repertoire of analytes, ranging from simple plant pigments to complex gas mixtures, synthetic chemicals, biological fluids and enantiomers, has been the key to this development, thanks to advances in stationary phase design. In most cases, the fundamental chemical structure of the stationary phase material has been preserved, and new separation approaches emerged when its physical and/or physicochemical characteristics were modified. All of these have combined to increase the sensitivity and shorten the time until an analysis is complete. Research on new stationary phase materials has continued without much pause and more improvements are expected over the coming years.

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