Chapter 8
Characterization Techniques for Chromatography Analysis

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Abbreviations

μPACs	Micropillar array columns
μ-TAS	Micrototal analysis system
ACS	Activated charcoal strip
AEX	Anion-exchange chromatography
ASE	Accelerated solvent extraction
BEH	Bridged ethyl hybrid
CEX	Cation-exchange chromatography
CIC	Chelation ion chromatography
CM	Conditioned medium
CPCs	Cardiomyocyte progenitor cells
DAD	Diode array detection
ECD	Electron capture detector
ErA	Erwinia chrysanthemi l-asparaginase
EVs	Extracellular vesicles
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FID	Flame ionization detector
FS columns	Fused silica columns
GC	Gas chromatography
GC×GC	Two-dimensional gas chromatography
GC×GC–TOFMS	Time-of-flight mass spectrometry
GPC	Gel permeation chromatography
HISD	High-intensity short duration
HPLC	High-performance liquid chromatography
HSDM	Headspace single-drop microextraction
HT-GPC	High temperature-gel permeation chromatography
IC	Ion chromatography

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IC/MS	Ion chromatography-mass spectrometry
IEC	Ion-exclusion chromatography
IEX	Ion-exchange chromatography
IPC	Ion pair chromatography
LC	Liquid chromatography
LILD	Low-intensity long duration
LLE	Liquid–liquid extraction
mAbs	Monoclonal antibodies
MEMS	Microelectromechanical systems
MW	Molecular weight
MWD	Molecular weight distribution
M_n	Number average molecular weight
M_v	Viscosity average molecular weight
M_w	Average molecular weight
M_z	Average molecular weight
M_{z+1}	Higher average molecular weight
NS	Not specific
OAs	Organic acids
PLOT	Porous layer open tubular
PS	Polystyrene
PTM	Post-translational modification
PTV	Programmed temperature vaporizer
TB	Tuberculosis
QC	Quality control
RPLC	Reversed-phase liquid chromatography
SCOT	Support coated open tubular
SEC	Size-exclusion chromatography
SEC-EVs	Extracellular vesicles isolated by size-exclusion chromatography
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
TD	Sorbent thermal desorption
TLC	Thin-layer chromatography
UC	Ultracentrifugation
UC-EVs	Extracellular vesicles isolated by ultracentrifugation
WCOT	Wall-coated open tubular
ZIC	Zwitterionic ion chromatography

8.1 Gas Chromatography for Material Characterization

8.1.1 History of Gas Chromatography

In 1941, Martin and Synge conducted a set of experiments regarding amino acid separation and published an article titled "A new form of chromatogram employing two liquid phases" in which they commented that the mobile phase could be gas instead of liquid. They consequently discovered liquid partition chromatography; however, it was not until 1952 that Martin and Synge made a breakthrough in the field. In 1941, Hesse used absorption chromatography to separate molecules with a molecular mass below 200 Da. This study allowed him to observe that small molecules were poorly adsorbed on the surface of the adsorbent due to the presence of solvents; hence, he suggested that gases could make better adsorbent candidates than liquids. Hesse succeeded in separating cyclohexane and benzene using gas chromatography (GC) principles with a distillation setup. In his subsequent publications, however, he named his method adsorption distillation, not chromatography. Cremer's research group used equipment based on a silica-filled column to separate a mixture of gases, thus developing the first gas chromatograph at the University of Innsbruck in 1944. However, their work was not published until 30 years later when it first appeared in "Chromatographia" [1–3] (Fig. 8.1).

During the 1940s and 1950s, various leading researchers such as Cremer, Hesse, and Phillips worked on the development of gas adsorption chromatography. In 1952, Martin and James invented gas–liquid chromatography through an experiment that involved separating volatile fatty acids using nitrogen gas (mobile phase) and silicone oil/stearic acid supported on diatomaceous earth (stationary phase). They received the Nobel Prize in Chemistry for the invention of gas–liquid chromatography. To date, it is still the most extensively used analytical technique in modern chemistry. In 1955, American and British companies built the first commercial GC with a thermal conductivity cell as a detector coupled with a column for the separation and detection of analytes. During the 1950s, the oil industry widely used GC to analyze the components present in petroleum followed by fields such as biochemistry and food industry where the technique was greatly explored. Around those years, the British Petroleum Institute organized "the International Symposium on Chromatography" to discuss the further advancements of this technique. By 1958, three discoveries were presented at this event that had significantly improved GC's performance: separation by capillary columns, the development of the flame ionization detector (FID), and the electron capture detector (ECD) which used the argon ionization detector as a precursor. The katharometer, a detector constructed by Ray, was the most used in GC until the invention of the FID, characterized by its low cost and high sensitivity compared with the katharometer. The FID provided low detection limits and response times, while it detected almost all organic compounds. By using ECD, analytes containing aromatic compounds or halogens can be detected which is particularly useful in environmental analysis. The operation of the capillary column was demonstrated by Golay in 1958. This column overcame the problems presented by packed columns,

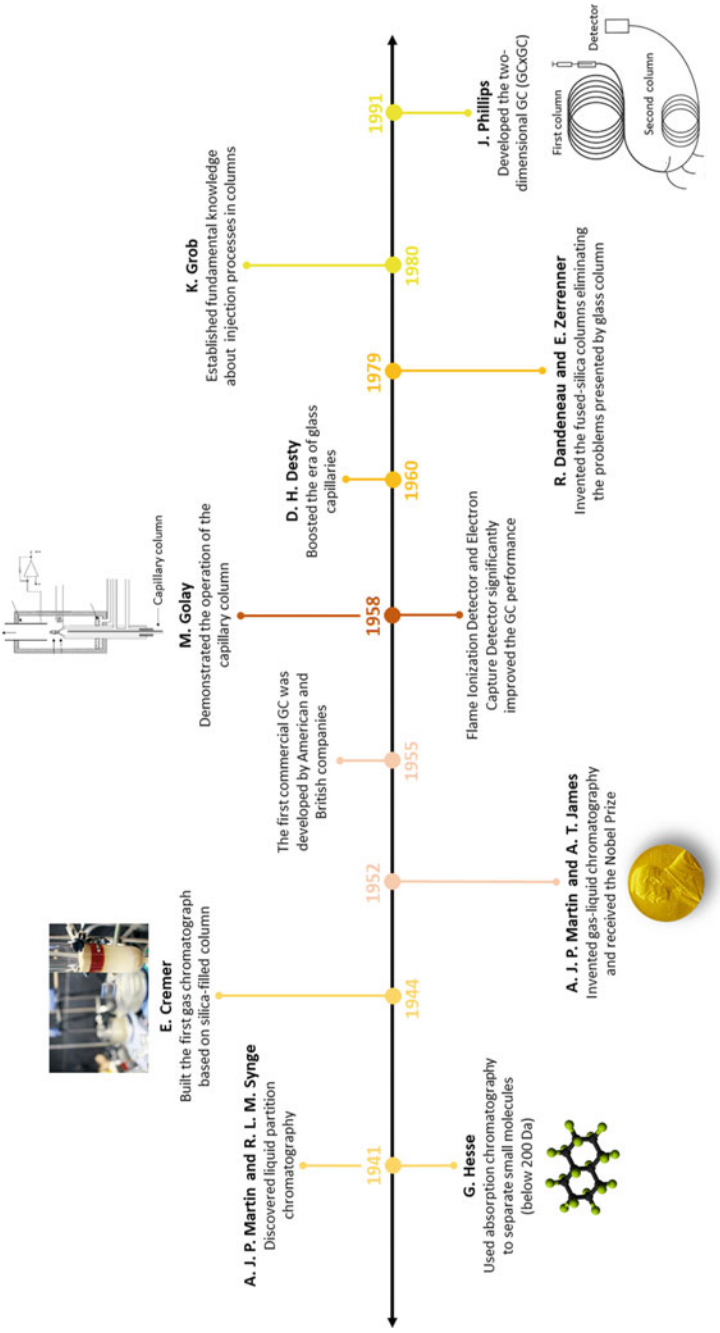

Fig. 8.1 History of GC

for instance, pressure drop. With the improvements of the 1950s, GC was used not only for hydrocarbon analysis but also for the analysis of fatty acids in medicine, environmental analysis, pharmacology, chemical industry, flavor and fragrance analysis, and toxicology, among others [1–3].

In 1960, Desty revolutionized the era of glass capillaries by building equipment capable of manufacturing spiral glass capillaries. However, the coiled glass capillaries were not well accepted at first since they broke easily and had low gas rates, and the deposition of the stationary phase on the inner walls of the column was deficient. It was not until 1979 when Dandeneau and Zerrenner developed the fused silica columns (FS columns) to avoid the troubles presented in glass columns including the interaction between glass and highly polar samples. These columns were treated with a protective layer of polyimide on the outer wall, and the low amounts of metal oxides made them inert, while the low wall thickness served their flexibility. The studies of fatty acid esters and fatty acids were very popular in the decade of 1960. Moreover, the reaction kinetics was studied by Purnell using GC in 1962. Around 1963, Grobs developed the idea of avoiding sample loss during vaporization by directly injecting the analyte into the inlet of the column. Griffiths proposed a method for samples with several volatile components, and the temperature in the column could gradually increase during the analysis. The developed methodology was officially published in 1966 [1–3].

Samples that are highly diluted required a stable solvent and sophisticated injection techniques. In the 1980s, Grobs generated valuable insights on injection processes and column heating during the feeding of liquid samples into the system (e.g., programmed temperature vaporizer (PTV)). During that decade, the in situ cross-linking of polymer systems was used to immobilize the stationary phase in films with a thickness of about 5 μm. To overcome the problems of automation, cost, speed of analysis, accuracy, and reliability of GC, it was necessary to rely on computer technology to handle the task of analyzing and storing the data obtained, as well as electronic control of the temperature and flow rate of the gases. In 1991, the maximum separation of components was carried out by Phillips using a system called GCxGC, where the separation of essential oil, fuels, and air pollutants was effectively performed. The GCxGC was considered the most innovative technological development in GC after the discovery of FS columns. With this technological progress, it was possible to separate complex samples. In 1993, solid-phase microextraction was commercially introduced based upon a concept developed by Belardi and Pawliszyn in 1989. This discovery further facilitated sample analysis without the need for solvent [1–3].

8.1.2 Mechanism of Operation of Gas Chromatography

Chromatography analysis involves a group of separation techniques that allow the identification and quantification of chemical substances from a mixture. The sample,

or the analyte, runs through a matrix where the separation takes place. Chromatography separation occurs when a sample is presented in a flowing mobile phase with a cross section with a stationary phase [4].

The GC is a physicochemical method characterized by a separation of a homogeneous mixture with the help of a gas flow by distribution among two phases (stationary and mobile). The stationary phase will remove molecules that are inclined to stay at a lower velocity; in contrast, the mobile phase will favor molecules that have the tendency to move forward. Therefore, separation occurs from frequent sorption and desorption processes as the samples move along the stationary phase. The key mechanism of the separation is based upon the intermolecular interaction of the solutes migrating through a column located on the way of the stationary phase [5, 6]. The stationary phase usually consists of a porous, granular powder that has sorbents with a high surface area in the form of a homogeneous layer filled within a column. The mobile phase of chromatography is always filled with a gas such as hydrogen (H_2), helium (He), nitrogen (N_2), or argon (Ar) that are commonly used. The composition of the mobile phase does not significantly contribute to the separation procedure as the gases are relatively inert. The mobile phase travels through the layers at a constant average linear velocity prompted by capillary forces [5, 7].

8.1.2.1 Instrumentation

A GC instrument is exemplified in Fig. 8.2, which comprises four main modules: The gas supply which provides the necessary gas is the first module; followed by the injection device; typically, a gas sampling valve is used for gases. The column where the separation takes place is the third module, while the fourth module is the detector which is commonly more than one (mass-dependent or concentration-dependent). A final vital module, which might be considered external to the GC system, is the chromatography data analysis system. All modules are controlled by different temperature-control systems that transfer the information to one or more microprocessing systems [8].

The gas is the medium that carries the compounds from the injector along the column and leads them to the detector (Fig. 8.2). The flow of the gas is controlled by

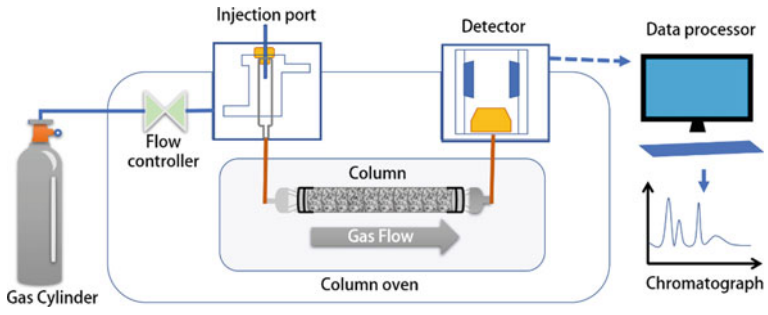

Fig. 8.2 Schematic of a GC system

pressure regulators and gas metering valves. The solutes move at the same average speed as the gas. Since diverse compounds have varied affinities to the stationary phase, compounds separate according to the window of time they take to travel through the stationary phase. The injection port is attached to the GC column, which provides the samples with temperatures sufficient to ensure vaporization of all components. The detector is directly connected to the opposite end of the column. The detector records the chromatogram as a response to the eluted arriving samples [7, 9].

Injection

Injection of the sample is critical in a GC analysis. It can contribute to the band, or peak, broadening. Inadequate injection techniques may decrease column resolution, hence impacting the quantitative results. Different methods can be used to inject the samples into the GC column including split injection, split-less injection, direct injection, and on-column injection. The type of injector design will depend on the column within the system [7, 9]. The sample is introduced into the carrier gas and is mixed with it. Subsequently, the sample is inserted into the injection port and held at high temperature, thus vaporizing the sample. Consequently, the carrier gas flows and sweeps the vaporized sample into the column [8, 9].

Separation Columns and Column Oven

Columns, without a doubt, are the most important part of the chromatography systems where the separation actually takes place. There are two categories of columns, packed and open tubular, used for GC differentiated by the way the stationary phase is held within the column. The choice of the column depends on the target compounds, their molecular weight, the number of components, and the instrument configuration [8–10].

Packed columns contain the stationary phase in powder form. The powder could be incorporated via coating in a liquid stationary phase to fill the space or a powder that is directly in the column. Packed columns are used to inject larger volumes without congesting the column which is beneficial to tracing contaminants in gases of high purity. Typically packed columns are 2–5 m in length with an internal diameter of 2 mm [9, 11].

In open tubular columns or capillary columns, the stationary phase is either coated or attached to the inside surfaces of the column. The internal diameter of the column is from 0.1 to 0.53 mm and is commonly made from fused silica and deactivated metals. Open tubular columns offer higher efficiency and greater separation capabilities than packed columns. Depending on the position and function of the stationary phase, capillary columns are classified as: support coated open tubular (SCOT) columns, porous layer open tubular (PLOT), and wall-coated open tubular (WCOT) columns [9, 11].

The column is mounted within an oven that has a precise and stable temperature regulator. The heat rate can be carefully controlled during the process to obtain efficient results. At the primary phase of analysis, the early eluting components are separated by adjusting the temperature low and are systematically increased to further separate the components that are rather more retained. The temperatures are predetermined and set on the data system and within microprocessor-controlled, as the analysis moves forward [8].

Detectors

The detector in the GC system monitors the separation and responds to the components and/or fractions of the components, while they elute from the column. The volume of the mobile phase must not exceed the detector's volume as the analyte peak will be broadened, the concentration will be reduced, and it will became more difficult to have an effective detection. Since the detector chamber allows compounds to expand into a larger volume, this can also act as a mixing chamber. Therefore, an effective detector should benefit from reasonable sensitivity, a broad linear dynamic range, and a small cell volume to avoid the distortion of the GC peak [7, 9, 10].

Multiple detector types for GC systems exist with distinctive operating parameters and performances. Table 8.1 provides a comparison of the characteristics of GC detectors. In most cases, the detector output is analog and is further electronically translated to a digital signal to be processed by a computer [8, 9].

8.1.3 Advantages and Disadvantages of Gas Chromatography

As GC was introduced, it swiftly found popularity as it was a timely invention at the time when analytical control was a major need in the petrochemical industries. GC constitutes the technique for separating volatile materials. It provides efficient complete analysis and high-resolution results within minutes and even seconds [13, 14]. Additionally, it is a sensitive and non-destructive method for sample analysis. This non-destructive nature not only prevents damage to samples but also allows online coupling of additional characterization techniques. The highly accurate quantitative analysis that GC offers, which is among the 1–5% of relative standard deviations, permits its use in a wide range of applications. Moreover, only a small quantity of sample (at the μL scale) is needed for this characterization. As listed in Table 8.2, other favorable aspects of this technique include a reliable column temperature control, simple instrumentation handling, and a relatively low cost of implementation [13, 15].

One of the limitations of GS is that its application is constrained to volatile samples. Furthermore, spectroscopy systems, such as mass spectroscopy, are required for peak identity confirmation. In addition, large samples (above 1000 Daltons) are complicated to analyze with GC, while it is not adequate for thermally unstable

Table 8.1 Characteristics of different detectors

Detector		Principle	Selectivity	Range
Universal	Thermal conductivity detector (TCD) [9]	Measures alteration of thermal conductivity among the carrier gas and the elution carrier gas [9, 12]	All compounds except for the carrier gas [9, 12]	10 ppm [9, 12]
	Flame ionization detector (FID) [9]	Measures ions created when the compound is burned in H_2/O_2 flame at 2000 °C [9]	Organic compounds [9]	0.1 ppm [12]
	Pulsed discharge helium ionization detector (PDHID) [12]	Measures the energy used for the ionization of analytes eluting from the column [12]	All compounds except for helium and neon [12]	0.07 ppm [12]
Selective high sensitivity	Electron capture detector (ECD) [9]	Measures changes in electron current caused by a reaction of an organic compound with electrons [9]	Organic halogen compounds; electronegative compounds [12]	0–01 ppb [12]
	Flame photometric detector (FPD) [12]	Uses a hydrogen-rich flame. Compounds entering the flame decompose, and the detector measures the light emitted by the formed radicals [12]	Sulfur compounds; organic phosphorus compounds; organic tin compounds [12]	10 ppb [12]
	Sulfur chemiluminescence detector (SCD) [12]	Measure the chemiluminescence as a result of the reaction of the combusted eluate with ozone (O_3) [12]	Sulfur-containing compounds [12]	1 ppb [12]

ppm parts per million; *ppb* parts per billion

samples (the upper-temperature limit for GC is ~380 °C). For that reason, samples must have a substantial vapor pressure (60 torrs or greater) within that temperature range. It is noteworthy, however, that the solutes regularly do not surpass the boiling points of 500 °C [13]. Another important drawback is that the conventional instrumentation is bulky. This results in high power consumption and low heating

Table 8.2 Advantages and disadvantages of GC

Advantages	Disadvantages
Separates volatile materials [13, 14]	Constrained to volatile samples [13]
Rapid analysis (within minutes or seconds) [13]	Spectroscopy is required for peak identity confirmation [13]
Efficient and high resolution [13]	Analysis of samples above 1000 Daltons is complicated [13]
High accuracy of quantitative analysis (RSDs of 1–5%) [13]	Not adequate for thermally unstable samples [13]
Non-destructive, sensitive, simple instrumentation handling, low cost [13]	Conventional instrumentation is bulky, not suitable for in situ analysis [14, 15]
Allows online coupling and small sample size [13]	Has low heating rates and requires high power consumption [14, 15]

RSD relative standard deviation; *GC* gas chromatography

rates and precludes in situ analysis. However, alternative GC systems, such as miniature laboratory-on-a-chip GC systems, resolve those challenges presented by the voluminous instrumentation of the conventional method [14, 15].

8.1.4 Applications of Gas Chromatography

As an ever-growing technique, GC has proven to be a useful tool for analyzing complex samples. Multiple reports explore the diverse range of applications of GC including pharmaceutical and cosmetics industries as well as environmental analysis. Commonly, samples that can be analyzed by GC are volatile. In the human body, blood, saliva, breath, and other types of body products that contain organic volatiles can be subjected to GC analysis. The knowledge of the mixture's components and the concentration of the elements within the mixture is of great importance in the field of medicine. This section offers a number of applications for GC with the main focus on biomolecular identification [16–19]. The study of metabolomics, obtaining fingerprints from pathogenic microorganisms, and profiling tissues and fluids to identify the volatile patterns within them, in turn, permits timely and adequate diagnosis and treatment [20]. GC can also be used for biomarker identification in pneumonia, tuberculosis, cystic fibrosis, asthma, and lung cancer. Hill et al. proposed a two-dimensional (2D) gas chromatography (GC×GC) separation technique for the diagnosis of tuberculosis (TB). This method was reported to be rapid, straightforward, and non-invasive for instantaneous diagnosis and concentration identification of species attributed to tuberculosis (Fig. 8.3) [21]. This technique successfully detected a number of biomarkers within breath that allowed differentiating TB from other respiratory infections (Fig. 8.3).

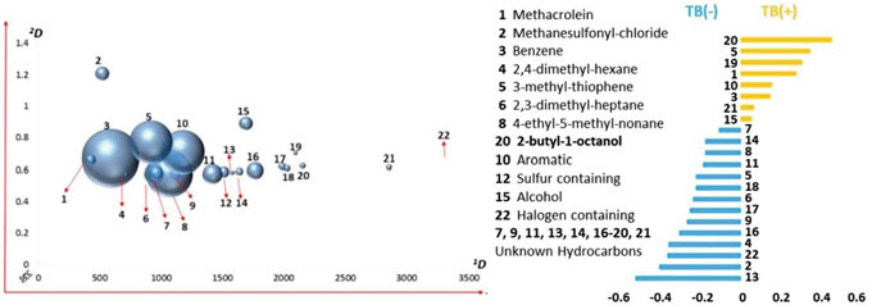

Fig. 8.3 Bubble illustration of discriminatory elements attributing to positive TB (TB+) and negative TB (TB−). Reproduced (or adapted) from [19], ©2018, with permission from Elsevier

A number of reports focus on urine [22], serum, and tissues [23] to explore the impact of diet, exercise, as well as metabolic procedures on an individual's health. In that regard, Cox et al. used GC together with time-of-flight mass spectrometry (GC× GC–TOFMS) to demonstrate a chemical profile of kidney tissues as well as serum samples. This coupled approach facilitated the biorecognition of approximately twice as many metabolites within a serum than what other techniques would be capable of achieving. Furthermore, 35 arterial lesion biomarkers have shown a strong correlation with diet-induced atherosclerosis that not only allowed expanding knowledge on the progression of this condition but also shed light on the sex specificity and heritable characteristics of this problem [23]. Moreover, Groessl et al. utilized a GC×GC–TOFMS to analyze steroid metabolites in urine which plays a key role in multiple physiological activities and hormonal disorders. Although varied in type, steroids commonly have similar chemical structures and are available in small quantities, hence the need for high-resolution and ultrasensitive analytical methods for identification [22]. The study reported GC×GC–TOFMS to be capable of identifying and quantifying 40 steroids within human urine in addition to 10 progesterone metabolites found in newborn urine. Furthermore, the study showed that the method can be also used for untargeted analysis which is highly beneficial for biorecognition of unknown metabolites.

Forensic discipline leverages scientific methods and tools to address legal matters and arguments. Forensic chemistry is the knowledge of analyzing a vast number of forensic samples aimed at extracting and interpreting the data with enough science-based evidence and rigor to be presented in civil and judicial settings. GC is a capable analytical technique that can have multiple uses in forensic research. Sample collection and preparation are of great importance in GC analysis, and incorrect methodology may impact the integrity of GC analysis. When handling traces and/or ultratraces of a given analyte within biological, environmental, fire debris, and explosive residues, it is important to collect samples of sufficient volume and valid nature. For that reason, in forensic chemistry, a number of protocols are developed for sample collection, storage, and analysis that should be strictly followed prior to GC [24]. Figure. 8.4 demonstrates some of the main application areas

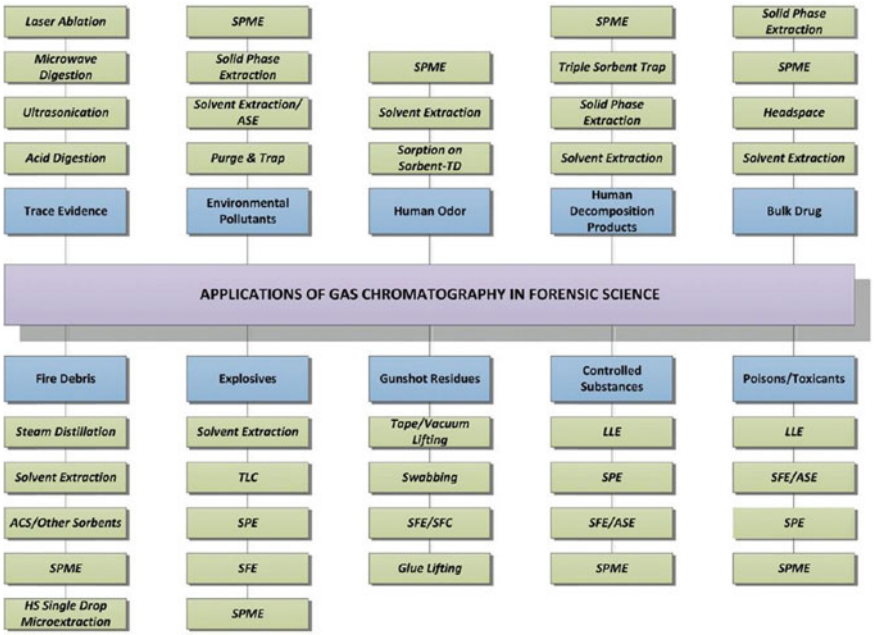

Fig. 8.4 Main applications of GC in the forensic domain. Reproduced (or adapted) from [25], ©2012, with permission from Elsevier

of GC in forensic sciences. Some of the most common uses of GC within this domain can be listed as follows: drug screening and analysis, profiling human odor, tracing evidence, artificial environmental contamination, toxicology, ignitable and explosives examination.

8.2 High-Performance Liquid Chromatography for Material Characterization

8.2.1 History of High-Performance Liquid Chromatography

Liquid chromatography (LC) is a class of chromatography aimed at analyzing individual components of a mixture that are separated in solution. In specific, high-performance LC (HPLC) is a useful separation technique widely used for complex polymer characterization. Figure 8.5 presents the evolution of science until the invention of HPLC and provides an overview of the significant contributions that have led to the development of this technique [5, 26, 27].

In the early 1900s, Tswett invented chromatography to separate plant pigments. Years later, in 1941, Martin and Synge shared their advancements in an article on

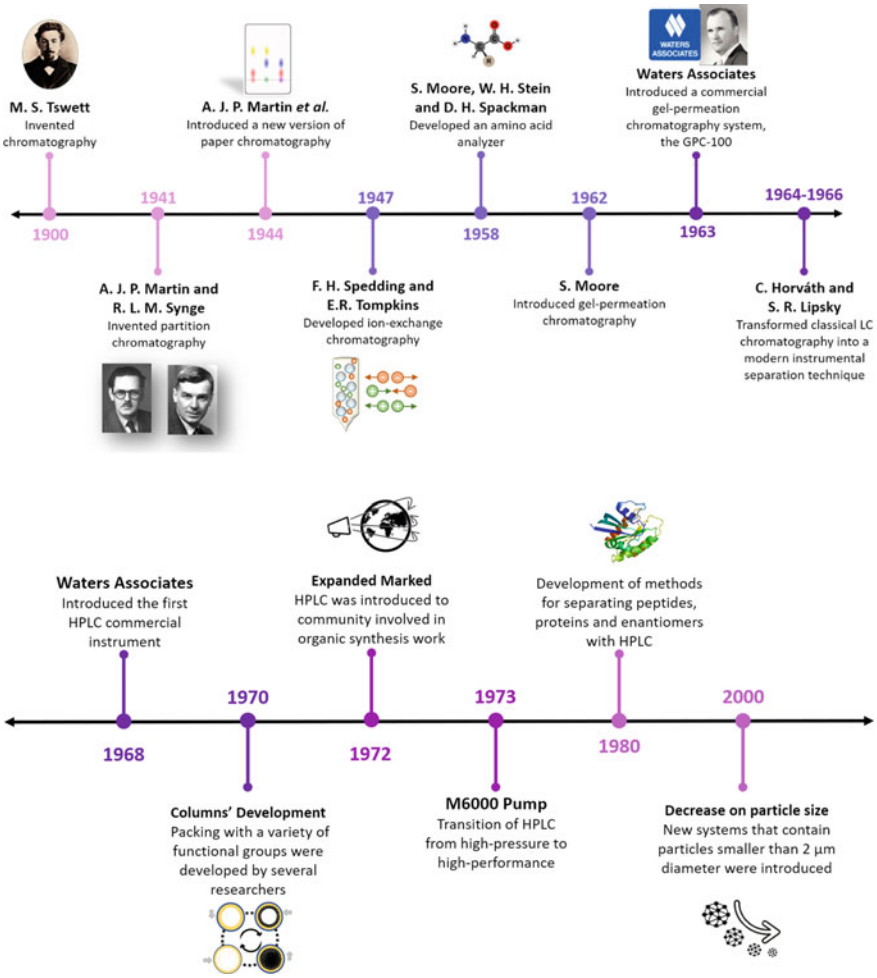

Fig. 8.5 Timeline of relevant events for the development of HPLC

liquid partition chromatography where they described that a mixture of small particles and high pressure lead to an efficient separation. Another historically important event in the development of HPLC was the progress in ion-exchange chromatography in 1947 along with the amino acid analyzer in 1958. The advances of gel permeation chromatography at the beginning of the 1960s were an achievement for early liquid chromatographs. However, the development of gas chromatography played a major role in the foundations of LC [27–29].

HPLC was proposed and primarily developed in the 1960s. Prior to this development, scientists applied the knowledge of GC to improve liquid column chromatography. Giddings in 1963 was the first to establish the theoretical basis for the changes required for LC to be performed successfully. In 1964, Horváth and Lipsky began

developing a system for modern LC which later laid the foundation for the commercial HPLC. Moreover, Horváth developed a pellicular packing in 1976, which became the mainstay column in several laboratories for over a decade. In a similar fashion, Huber focused his work on the study of column performance under different conditions based upon his primary findings in 1965. Thus, advances in HPLC were marked by theoretical development and by the development and improvement of equipment and columns over several decades [27, 28].

In 1972, Woodward Hamberger contacted Waters Associates to request help in the separation of isomers of intermediates in the synthesis of vitamin B12. Waters used a model ALC-100 liquid chromatography to accomplish this task. Their success opened the market of HPLC to the organic synthesis community. The HPLC technique, by the time, was also used for separating peptides and proteins as well as enantiomers [28, 30].

After the first prototype was introduced by Waters Associates in 1963 for gel permeation chromatography, in 1964, Waters contributed to the development of a liquid chromatography system for Shell Development Co. Horvath's approach was principally used to change the column to a solid column packing using the adoption of an LC system for fabricating high-pressure performance. The result was the ALC-100 analytical liquid chromatography, which was introduced in 1968. The system was equipped with a pump made by the Milton Roy Company. Years later, the pump was changed to Waters' system, and in 1973, a pump providing pulseless flow at 600psi (M6000 pump) was introduced [27, 28, 31].

Advances in pumping systems for HPLC devices were further driven by the field of microfluidics and micrototal analysis systems (μ-TAS) with the development of electroosmotic pumps capable of producing pulse-free flow with no mobile parts involved in the design. Moreover, micropillar array columns (μPACs) have advanced the fabrication of alternative columns via lithography techniques presenting high permeability and low dispersion as their main beneficial features. Additionally, 3D printing systems can greatly contribute to the increased efficiency of chromatographic columns [32, 33].

8.2.2 Mechanism of Operation of High-Performance Liquid Chromatography

8.2.2.1 Liquid Chromatographic Methods

As previously mentioned, LC is one of the main chromatography methods in which the movable phase is a liquid; thus, a solution mixture is separated into its discrete constituents [5, 26]. Common procedures in HPLC comprise a liquid phase that is mobile and travels through a stationary phase. Different HPLC techniques can be categorized based upon their retention, separation, or operation mechanism. The

major modes of classification are adsorption, partition, ion-exchange, size-exclusion, and affinity chromatography presented in Table 8.3 [4, 5, 26].

Table 8.3 Separation methods used in HPLC

Separation method	Principle	Stationary phase	Mobile phase	Retention mechanism
Adsorption [26, 34]	Based on the molecular exchange between the liquid mobile phase and the neutral, solid stationary phase [4, 26, 34]	Polar with hydroxyl groups. Porous surface covered with an active bonded phase [4, 35]	Nonpolar solvent [4]	Polarity; retention of molecules on the solid stationary phase [4]
Partition [26, 34]	The solute molecules are distributed among two non-miscible liquid phases [4, 26, 34]	A nonpolar liquid is immobilized on a solid support. Usually with alkyl, phenyl, cyano-, or amino groups [4, 35]	Polar solvent [4]	Polarity; hydrogen bond formation [4]
Size exclusion [26, 34]	Separation according to the hydrodynamic volume of substances occurs by using a molecular filter [26, 34]	Porous structures [4, 34]	Organic solvent [34]	Gel filtration and gel permeation [4, 34]
Affinity [26]	Lock and key mechanism using the specific binding of protein [4, 26]	Biological complement immobilized on the solid support [36]	A target biological compound specific to the complementary segment [36]	Reversible attachment of a protein to a ligand covalently bounded to a solid surface [36]
Ion exchange [26, 34]	The exchange process among an ion species in the solution and another ion species within the stationary phase takes place through ionic interactions [34]	Ionic or counterionic groups [34]	Ionic or anionic species [34]	Cation-exchange or anion-exchange chromatography [34]

8.2.2.2 Instruments

The HPLC has been the premier method for the characterization of polymers: a technique that pumps a solution mixture mechanically through a column and guides it through the stationary phase. Common instruments involved in this analytical technique are injectors, pumps, columns, and detectors. Figure 8.6 shows a schematic representation of a basic HPLC system [5, 26].

Mobile Phase

In HPLC systems, the right selection of a mobile phase is crucial to its effective operation. Moreover, the inherent characteristics of the analytes, the stationary phase, and the detection method to determine the type of solvents that are adequate for the system. The selected solvent is aimed to offer separation of the component mixtures within the minimum required time, and the delivery of analytes to the detection point without affecting the measurement. An ideal mobile phase is a solvent that elutes analytes within shorter retention time periods [4].

Stationary Phase

The thermodynamic aspects of the separation process can be controlled by the chemical class of the stationary phase. Thus, by adjusting the physical and chemical characteristics of the stationary phase, the performance of the HPLC can be manipulated. The packing material of the column would directly impact the chemical and physical interaction with the analyte of interest, while such interaction can be further influenced by changing the solvent strength. When the interaction between the stationary phase and analyte increases, the retention of solutes also increases [5, 26].

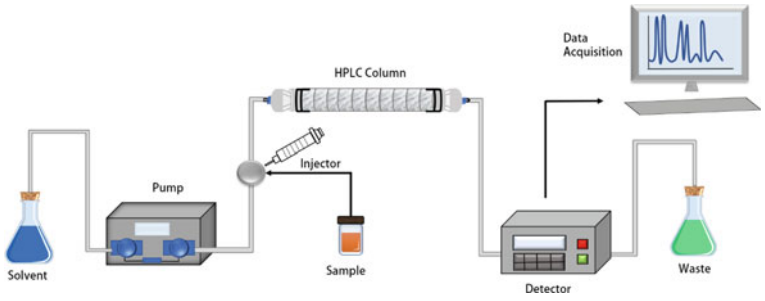

Fig. 8.6 Schematic representation of an HPLC system

8.2.2.3 Mechanism of Retention

In HPLC, solutes are injected in the mobile phase which travels through the column with the help of a pump to inject the solutes at the head of the chromatographic column. The pressure exerted by the pump moves the mobile phase along the column, and the eluded molecules that leave the column are then detected by a certain type of detector, according to the physicochemical properties of the solution. Finally, the received signal will be processed to produce the chromatogram [34].

The displayed peaks in the chromatograph correspond to diverse components that exist within the mixture. The height and peak specifications of the chromatogram are direct functions of multiple elements including the concentration of the compound within the mixture, the quantity of the injected samples, in addition to the detection sensitivity of the system. Therefore, the system's efficiency can be adjusted by frequent optimization of these factors to obtain better separation outcomes and minimize band broadening. In HPLC, the variables of selectivity and capacity of the column are principally designed and developed by the manufacturers, while the efficiency and resolution can be optimized by the chromatography technicians. Table 8.4 provides general insights on different formulations used to control the efficiency and resolution along with other essential parameters involved in HPLC [26, 34].

8.2.3 Advantages and Disadvantages of High-Performance Liquid Chromatography

While a wide variety of fields use HPLC as a powerful technique for the separation and quantification of mixture components, this chromatography technique, like others, has both advantageous and disadvantageous features. HPLC is known to be a versatile technique as it can be applied in a wide extent of applications [38, 39]. Moreover, it is suitable to analyze various samples, whereby between 60 and 80% of the existing compounds can be successfully separated, detected, and identified [38]. Another advantageous characteristic of this technique is the precision of the analysis (of <0.5% relative standard deviations) and rich quantitative outcomes [38, 39]. HPLC features a simple operation process using a unique automated system. The data system embedded within the instrument permits analysis and report of the outcomes without user's attendance and autosamplers [38]. In addition, HPLC provides high-resolution results and constitutes a sensitive characterization technique capable of performing detection within nanogram, picogram, and femtogram levels while facilitating rapid analysis and a significant sample recovery rate [38, 40].

Despite its advantages, HPLC requires an expensive implementation and large amounts of solvent. The high sensitivity of columns to pH changes in mobile phases represents another limitation of the method [41]. Since some analytes do not absorb UV or are challenging to ionize, the detection is not universal for HPLC [38]. Another disadvantage is the large number of variables that need to be adjusted prior

Table 8.4 Equations used in HPLC to control its variables

Name	Equation	Specifics	Terms
Capacity factor (retention factor) [26, 37]	$k_R = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0}$	Determines retention time of the solute by measuring the strength of the interaction between the sample and the packing substance [26]	k_R = capacity factor t_R = retention time t_0 = holdup time V_R = retention volume V_0 = void volume [26, 37]
Selectivity [26, 37]	$\propto = \frac{t_2 - t_0}{t_1 - t_0} = \frac{k_2}{k_1}$	Describes the effectiveness of a system to separate two compounds by measuring the variance in retention periods of two given peaks [26, 37]	$\propto$ = selectivity of the column t_2 = retention time of component 2 t_1 = retention time of component 1 k_2 = capacity factor of component 2 k_1 = capacity factor of component 1 [26, 37]
Resolution [26, 37]	$R = \frac{1}{4} \frac{\propto - 1}{\propto} \left(N^{\frac{1}{2}} \right) \frac{k}{1+k}$	Characterizes peak separation; R>1 is considered a good peak separation [26, 37]	N = efficiency of the column $\propto$ = selectivity k_R = capacity factor [26, 37]
Efficiency [26, 37]	$N = \left(\frac{t_R}{\sigma} \right)^2 = \frac{t_R^2}{\sigma^2}$	Explains peak broadening in correlation with retention. The extent of peak broadening is determined by σ [26, 37]	N = efficiency t_R = retention time σ = width of the Gaussian curve [26, 37]

to each run which, in turn, requires training [38, 39]. Additionally, the analysis of complex mixtures is rather more demanding. However, it remains relevant to highlight that existing columns and instrumental developments have significantly reduced the constraints of HPLC [38]. Table 8.5 summarizes the advantages and disadvantages of this powerful analytical method.

8.2.4 Applications of High-Performance Liquid Chromatography

It was initially believed that HPLC would act as a complementary technique to GC. Nowadays, however, this powerful analytical method has nearly substituted GC in various disciplines. The properties of the samples are the main determinants for

Table 8.5 Advantages and disadvantages of HPLC

Advantages	Disadvantages
Versatility, high sensitivity, and rapid analysis [38–40]	Expensiveness [41]
Suitable for diverse samples (60% to 80% of all existing compounds can be analyzed) [38]	Requires large amounts of solvent [41]
Precise (<0.5% RSD) and rich quantitative outcomes [38, 39]	Columns sensitive to extreme pH in the mobile phases [41]
Simple operation [38]	Detection is limited to ionizable components [38]
High resolution [38, 40]	A large number of variables must be adjusted before each run [38, 39]
Significant sample recovery [38]	Requires trained technicians [38]
Automated operation [38]	Analysis of complex mixtures is rather demanding [38]

HPLC high-performance liquid chromatography; *RSD* relative standard deviation

the incorporation of the liquid mobile phase which, in turn, allows a great deal of flexibility in sample analysis. The data that could be collected via HPLC involves resolution, compound identification and quantification, and chemical separation, and, in specific cases, sample purification. This analytical technique has various applications in diverse fields such as pharmaceutics, environmental monitoring, forensic research, and clinical diagnosis, to name a few [42].

In recent years, HPLC has attracted considerable attention in the isolation and purification of peptides and proteins. Proteins such as insulin can be detected, and the concentration can be accurately measured via biological assays. Although specific and effective, biological assays fall short in detecting contaminants or decomposition components in a mixture. HPLC can be a great candidate for such assessments. For instance, the straightforward HPLC technique equipped with diode arrays was found to be highly effective in insulin detection (50–500 μg/mL). The study suggests the method to be accurate, sensitive, and stable over time which marks it as an excellent candidate for pharmaceutical preparations. Figure 8.7 represents the triplicated chromatograms for insulin and the blank sample [43].

The field of forensic studies is perhaps the most benefited area of research from HPLC analyses. From detection to the identification of biological traces and drug and poison recognition, a wide range of forensic reports were published in the literature highlighting identification of acute or lethal toxins, workplace drug check, change of driver's performance under drug's influence, and doping test for sports.

Heroin is a diacetyl derivative of morphine that is considered to be an unlawful drug obtained from the opium poppy. HPLC is a useful method in the detection of heroin's metabolites in addition to identifying other synthetic drugs including methadone and tramadol, and semisynthetic drugs including buprenorphine opioids. In 2007, HPLC coupled with diode array detection (DAD) was proposed by Fernandez et al. to detect heroin, methadone, cocaine, and other metabolites within

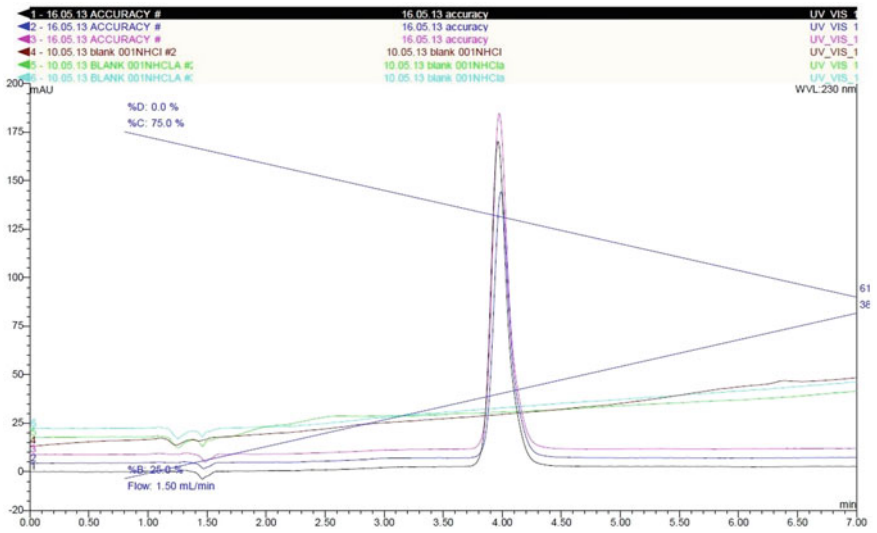

Fig. 8.7 Sample chromatograms for insulin detection as opposed to blank samples. Reproduced (or adapted) from [43], ©2018, with permission from the Journal of Advances in Biotechnology

the plasma. Unlike GC–MS, HPLC–DAD involved no earlier analyte derivatization and could be used for the analysis of non-volatile components as well. In addition to detecting the above-mentioned analytes, this technique has also proven to be effective for monitoring the commitment of drug addicts to the methadone detoxification programs. Figure 8.8 demonstrated the chromatogram ($\lambda = 285$ nm) of drug-free plasma spiked with 10 mg/mL of each analyte [44].

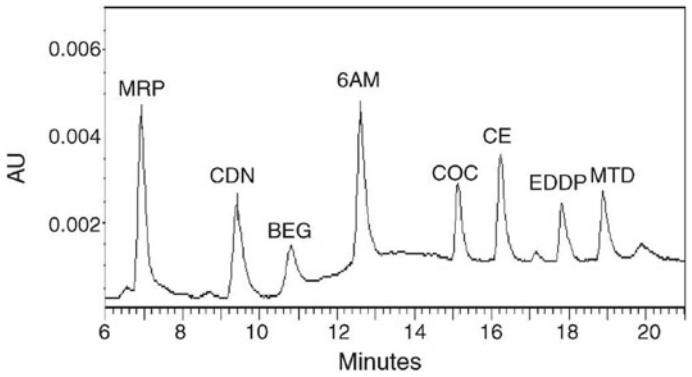

Fig. 8.8 Chromatogram from spiked plasma (conc.: 10 mg/mL; l = 285 nm). Reproduced (or adapted) from [44], ©2006, with permission from Elsevier

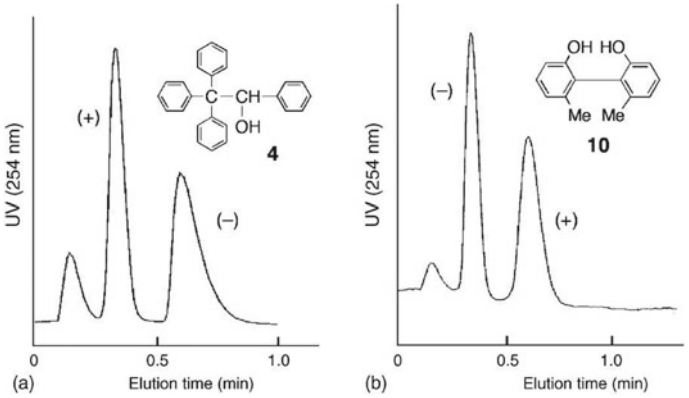

Fig. 8.9 The enantioseparation of **a** 1,2,2,2-tetraphenylethanol and **b** 2,2′-dihydroxy-6,6′-dimethylbiphenyl via a customized capillary column. Reproduced (or adapted) from [46], ©2006, with permission from Elsevier

Historically, the majority of bioanalytical chiral drug analyses were established based upon the use of HPLC with UV or fluorescence detection as well as mass spectrometry (MS) analysis. In specific, for examining complex matrices, the sensitive and selective nature of MS is highly favorable and acts as a great complementary element to HPLC analysis in offering a complete set of information. This coupled methodology saves operation time and expenses as it avoids column switching and thorough sample cleanup processes. In that perspective, a timely examination of enantiomers for both chiral purity testing and enantioselective checking is essential [45]. In 2006, Chankvetadze et al. used a customized capillary column that consisted of monolithic silica modified with amylose tris(3,5-dimethylphenylcarbamate) and conducted enantioseparations in approximately 1 min. This successful coupled analytical method examined two chiral compounds (1,2,2,2-tetraphenylethanol and 2,2′-dihydroxy-6,6′-dimethylbiphenyl) in a highly effective manner (Fig. 8.9) [46].

8.3 Ion Chromatography for Material Characterization

8.3.1 History of Ion Chromatography

Ion chromatography (IC) was primarily presented in 1975, a technique that was laid upon decades of foundational advancement. Rare soils were first separated by displacement ion-exchange chromatography around the mid-1940s followed by the work of Kraus and Nelson who later demonstrated the metal ion separation by anion-exchange chromatography with a focus on chloride, fluoride, nitrate, or sulfate complexes [47, 48].

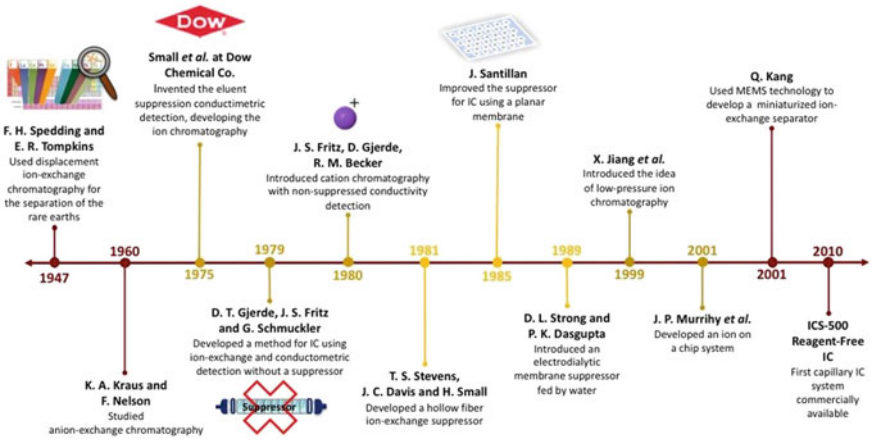

Fig. 8.10 Timeline of the development of IC

As part of Dow Chemical Co., Small, Stevens, and Bauman created a milestone in ion chromatography development by conceiving a system that leveraged suppressed conductivity detection for separation (Fig. 8.10). This system needed a large suppressor column that required frequent regeneration and broadening of the peak [47, 49].

Four years later, in 1979, Gjerde et al. published a technique for anion chromatography without the need for suppressed conductivity detection. The system used an eluent that had significantly lower conductance; therefore, the separated sample ions were easily identified by solely using a conductivity detector. The following year, a comparable technique offered cation chromatography [47, 49].

In 1981, Stevens et al. introduced a membrane suppressor which was used within the chromatography system and the concept of chemically regenerated eluent suppressor was born from their advancement. Later, membrane devices were linked to electrochemically regenerated devices that used only water and an electrical current to keep the system functioning. In this way, previous shortcomings of the suppressor were addressed and the evolution of the suppressor took place [49–51].

In search of miniaturizing the IC systems, Jiang et al. proposed a low-pressure ion chromatography (LPIC). Furthermore, separation using a microchip-based system was examined in 2001 when the first microchip for ion separation was announced. In the same year, Kang et al. used microelectromechanical systems (MEMS) to develop a miniaturized ion-exchange separator, and in 2010, the first capillary IC system was commercially produced and introduced to the market [51, 52].

The trends of IC developments continued with new designs of ion exchangers to increase their capacity, the reduction of particle size to increase the column's efficiency, and the coupling of sensitive and selective spectroscopic and spectrometric techniques. Moreover, the incorporation of nanoparticles to the columns for dynamic coating was widely explored thereafter [52].

8.3.2 Mechanism of Operation of Ion Chromatography

The term IC is given to a collection of techniques used for ion mixture separation. IC utilizes liquid chromatography for the separation process; therefore, the mobile phase is a liquid of small charged solutes. IC principally operates based on electrostatic interactions that occur between oppositely charged sites/groups within the stationary phase. Separations by IC involve the use of ion-exchange resins. The separation mechanism utilizes the reversible ion interchange between a functionalized insoluble resin (the ion-exchange component or stationary phase) and an ionizable material in the solution. In the separation process, the ion exchanger is brought to equilibrium with eluent ions by pumping the eluent through the column [53–56].

Figure 8.11 presents different techniques used in IC. The most common methodologies for IC separations are conducted by ion-exchange chromatography (IEX). The mechanisms used by the IEX technique can be categorized into two groups. The first is non-suppressed ion chromatography, in which an ion-exchange column is directly joined to the detector. The second group is the suppressed ion chromatography which consists of an ion-exchange chromatography, an eluent suppressor, and

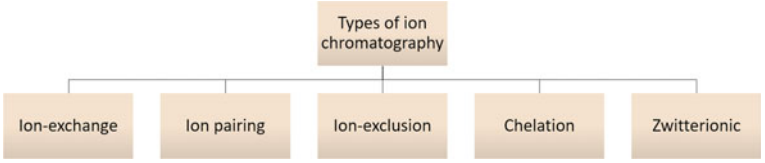

Fig. 8.11 Chromatographic methods using IC

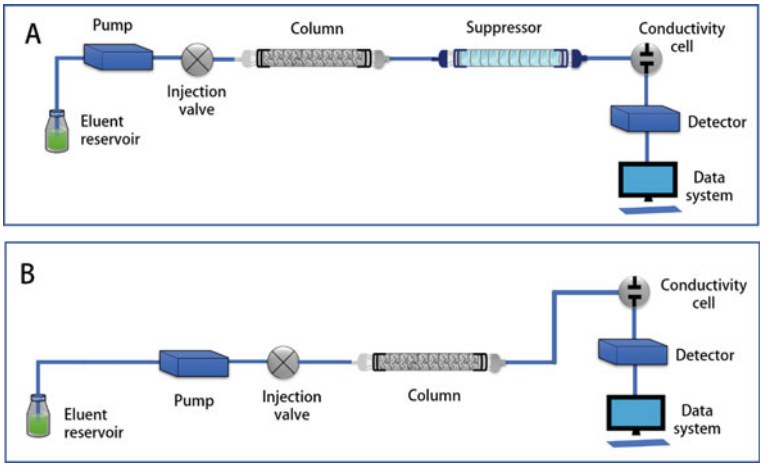

Fig. 8.12 Schematic representation of an IC: **a** suppressed and **b** non-suppressed

a conductometric detector. Figure 8.12 shows a schematic representation of the major components of IEX [55–57].

The mechanism of separation used in IEX benefits from changes in ion affinity for the fluid ion-exchange phase that allows ion separation based on ionic interaction with the stationary phase and eluent. The stationary phase, also known as ion exchanger, has ionic sites mounted on a polymer backbone equipped with a mobile counterion charged oppositely. When the solution that surrounds the ion-exchange is free of charge, the counterions stay where they are within the ion-exchange material. If the counterions and solution have the same ion type, it leads the ions into a dynamic equilibrium, hence swiftly traveling from the aqueous phase to the ion-exchange phase [56, 57].

However, if ions are of different types, the ion of the highest affinity would accumulate within the ion-exchange material while those with the lowest affinity would expectedly concentrate in the surrounding aqueous phase. Noteworthy, as the name suggests, the counterion is swapped as a result of the ion-exchange course. Therefore, ions that move faster through the column will reach the detector first, for which the detector provides separate bands. Ion-exchange resin can act as a cation-exchange resin if negatively charged or as an anionic exchange when positively charged [56, 57].

Ion-exclusion chromatography (IEC) forms a pseudo-semipermeable membrane around the ion-exchange resin, known as Donnan membrane equilibrium. The separation mechanism, as the name implies, is based on "ion-exclusion" leveraging electrostatic repulsion. Neutral species are separated when they are retained by the resins' pores, whereas ionic solutes of the same charge as the stationary phase undergo repulsion from the resin surface, preventing the permeation into the stationary phase. The retention control is manipulated by the pH of the mobile phase. If the pH decreases, the retention increases [53, 57].

Ion pair chromatography (IPC) is a reversed-phase liquid chromatographic technique that utilizes ion pair reagents to produce in situ ion-exchange sites via adsorption of ion pairs on the reversed-phase surface which is commonly composed of a hydrophobic chromatographic media. This technique is typically used when the analyte is difficult to elute from ion-exchange materials [57, 58].

Chelation ion chromatography (CIC) is distinguished by how the stationary phase interreacts. The stationary phase is where chelating functional groups are used, and, apart from ion separation by ion-exchange means, it promotes coordination bonds based on chelate formation with the solute ions [53].

Zwitterionic ion chromatography (ZIC) uses ion exchangers, whereby positive and negative charges are located in close proximity. Therefore, this technique has dual functionality for the concurrent separation of anionic and cationic solutes [53].

8.3.2.1 Instrumentation

Columns

Ion Exchangers

The most significant features of the ion exchangers are their porous matrix and how the functional groups (charged exchange groups) are generated within them. These features control the mass transfer kinetics within the chromatographic column. Ion exchangers can be categorized into the following groups: (i) ion exchangers with grafted functional groups; (ii) ion exchangers with grafted ionogenic polymer layers; (iii) polymer-coated ion exchangers; (iv) and agglomerated ion exchangers or dynamically modified ion exchangers. The structure and preparation method in each type explains the category and its functions [53].

Materials used in IEX can be divided into four groups: (i) strong base anion-exchange components with quaternary ion sites; (ii) strong acid cation-exchange materials with sulfonate ion-exchange sites; (iii) weak acid cation-exchange components with carboxylate or phosphonate ion-exchange sites; and (iv) weak base anion-exchange materials with primary, secondary, or tertiary amine ion-exchange sites. Nowadays, the majority of exchange materials incorporate carboxylic acid ion-exchange sites [57].

Suppression Columns

The function of the suppressor system is to chemically decrease the background conductivity of the electrolytes within the eluent while increasing the conductivity of the sample ions. The suppressor is mounted immediately after the column [57]. The basic suppressor functions are based on the dialysis reactions that occur in ion-exchange membranes. Modern suppressors have a flat sheet as a membrane. The eluent contacts one side of the membrane; meanwhile, the regenerant solution streams in the opposite direction at the other side of the membrane. The sandwich configuration equipped with gaskets helps defining the anticipated flow paths. The eluent flows in the central layer with ion-exchange membrane sheets above and below its current. The regenerant, however, streams in a countercurrent track over the configuration's outer layers. Mesh screens are composed of polymeric ion-exchange materials that are introduced to the eluent cavity and those of the flowing regenerant solution [55]. Post-column reactions that result in a decrease in the background conductance of the eluent are also known as suppression reactions [55].

Eluent

The eluent solution in IC is principally water; however, this can diverge from dilute electrolytes to complex multicomponent buffer solutions based on the operating IC technique. Eluents can be classified using the list below [53, 57].

- Anion-exchange eluents for suppressed IC.
- Anion-exchange eluents in non-suppressed IC.
- Cation-exchange eluents in suppressed IC.
- Cation-exchange eluents in non-suppressed IC.
- Eluents in ultraviolet–visible detection IC of transition metals.
- Ion pair eluents in IC.
- Ion-exclusion eluents in IC.

Eluent Generator

Eluent generators produce the IC eluent as an ion source by means of a deionized water flow generated by the IC pump. It is located before the sample injector. The concentration of produced ions is a function of the ions' current to a charged membrane and the flow rate of the receiver solution. Since the two parameters can be precisely controlled, the eluent can be produced with accuracy as well [53, 57].

Detection

Detection methods are diverse for IC, and some of the most common techniques are listed in Table 8.6. The majority of IC applications operate based on conductivity detectors since ions are conductive in a solution which can, in turn, cover a wide range of components. Ions can be detected with direct or alternating current modes. However, direct current results in oxidation or reduction of analyte ions during conductivity assessment. Thus, alternating current is used in conductometric detections since it can overcome the anomalous measurements of direct current by quickly changing the direction of the electric field, canceling errors correlated to oxidation and reduction [53, 56, 57].

8.3.3 Advantages and Disadvantages of Ion Chromatography

The principle of the IC analytical method is that the substances are separated and preferentially distributed among a stationary and a mobile phase. With the advancements of the technology over the years, the IC has become much more sensitive, while the technique still holds a number of benefits and shortcomings (summarized in Table 8.7). One of the beneficial features of IC is that it allows the determination and separation of a vast variety of ionic components within samples including cations, anions, large/small ions, and organic/inorganic ions [59].

Furthermore, the high degree of selectivity which is confirmed by the appropriate choice of separation and detection systems simplifies the identification of unknown sample components. In addition, high sensitivity detection has been achieved by the implementation of efficient stationary phases and microprocessor technology. This facilitated the detection of ionic constituents at a significantly low concentration

Table 8.6 Detection methods used for IC

Detection	Subtype detection	Principle	Remarks
Conductometric [53]	Non-suppressed conductivity [53]	The conductivity of the elute and the transient bands of elute ions are assessed [53]	Most commonly employed technique [53, 57]
	Suppressed conductivity [53]	The suppressor reduces the background conductivity [53]	
Electrochemical [53]	Charge detector [53]	Measures the charge to which the signal is relative [53]	Changes with the residence time of analyte ions within the change between the anion and cation exchanges [53]
	Amperometry detection [53]	Through applying a potential solute undergo oxidation and reduction when passing over the working electrode [53]	Used for electroactive solutes [53]
Spectroscopic [53]	Photometric detection [53]	The measurements occur by having absorbing eluent ions in the system [53]	Operates only with solutes that have a substantial absorbance profile [53]
	Post-column reaction detection [53]	A chemical reaction between the analyte and a chromophore takes place, and the species are converted after passing the column [53, 56]	The system reacts with most metals and quickly forms kinetics [56]

Table 8.7 Advantages and disadvantages of IC

Advantages	Disadvantages
Determination of various ionic components with simultaneous detection [59–61]	Frequent incompatibility of samples with IC system [59]
High selectivity and sensitivity [59–61]	Requires cautious sample preparation [59, 60]
Low cost, rapid analysis (≈5–15 min), and large dynamic working range [59–61]	High acidity or alkalinity may require further workload and augmented analysis time [59]
Safety and environmental friendliness [61]	Loss of analytes might be observed in small sample concentration [59]

IC Ion chromatography

range (microgram/Liter) without the need for pre-concentration. The ability to simultaneously separate and detect multiple sample components is also an advantageous feature offered by IC [59–61].

Perhaps, one of the main strengths of IC is the rapid analysis of the samples which takes 5–15 min for a complete test [59–61]. This, in turn, allows a greater quantity of samples to be analyzed in each session, hence positively impacting the manufacturing costs of products. The recent introduction of highly efficient separator columns has further contributed to this rapid analysis [60]. In addition, the high stability of separator columns is an outstanding feature of this analytical method. The resin materials (e.g., styrene-based polymers) that are filled within the column enable high pH stability. This, in turn, permits strong acids and bases to be analyzed as eluents expanding the application areas of IC [60, 61]. Other remarkable characteristics of IC include its great dynamic working range, the low consumption of sample solution and reagents, the availability of low-cost systems, and its safe and environmentally friendly nature that commonly uses water-soluble compounds (e.g., Na_2CO_3/$NaHCO_3$, HCl, HNO_3) [59–61].

The incompatibility of samples with the IC system is one of the most frequently reported limitations of IC. Additionally, in specific cases, sample preparation may require careful and detailed processing. For instance, significantly low analyte concentrations and high acidity or alkalinity occasionally limit the overall analysis of ionic components, while highly accentuated differences in ion concentration limit simultaneous ion quantification [59, 60]. This involves further workload and augmented analysis time, and may contribute to error in sample analysis including conversion, loss of analytes, and contamination [59].

8.3.4 Applications of Ion Chromatography

As a well-established characterization method, IC is a great candidate for analyzing various sample types in their amphoteric form. Benefiting from the stationary and mobile phases, IC presents a vast range of applications in different research and industry lines. The non-suppressed IC is recognized as a generally less sensitive system than suppressed IC which marks it as a more suitable choice for analyzing samples that contain medium-to-high solute concentrations (1–100 mg/L). For lower solute concentrations, advanced suppressed IC can be a better choice as it accurately quantifies inorganic anions in aqueous media (10 μg/L −10 mg/L). Both IC systems (suppressed and non-suppressed), however, have been reportedly utilized characterization of organic and inorganic cations in addition to alkali and alkaline metal ions as well as organic amines [62].

Among various metabolites, organic acids (OAs) play a crucial role as hosts of diverse regulatory pathways. Having a polar nature makes OAs challenging to characterize by many of the existing characterization techniques. In one of the reports of the literature, C18 reversed-phase chromatography was used for quantifying ionic or polar metabolites [63], where derivatization was used as a method for enhancement

of the retention of such specimens. In the study of metabolomics, IC has also been shown to effectively retain multiple polar metabolites [64, 65]. Petucci et al. in 2016 proposed that when IC is coupled with MS, it can act as a complementary separation method to HPLC for retention and quantification of polar OAs. The key advantage of IC in this study is the separation of highly polar OAs that could not be otherwise retained by reversed-phase chromatography [66]. Generally, ion chromatography-mass spectrometry (IC/MS) demonstrates a wide range of applications for quantifying various polar compounds. In another study, the IC/MS was applied to measure the OA alterations within quadriceps muscles for the purpose of comparing sedentary mice with fatigued mice. The fatigued mice were subject to treadmill exercise following the low-intensity long duration (LILD) or high-intensity short duration (HISD) scheme. As illustrated in Fig. 8.13, this combined method clearly indicates baseline for narrow Gaussian OA peaks with almost no background noise, and the following were separated from the analyzed samples: monocarboxylic acids such as lactic acid, dicarboxylic acids such as succinic acid, and tricarboxylic acids such as citric acid. The result of this study showed that 11 out of 28 OAs presented lower concentrations within the mouse quadriceps muscle homogenate.

The pharmaceutical industry is closely connected to various characterization techniques for analyses of the newly developed drugs. Advances in therapeutic and biosimilar proteins demand solid chromatography methods to thoroughly investigate multiple involving factors prior to release. These include identity, heterogeneity,

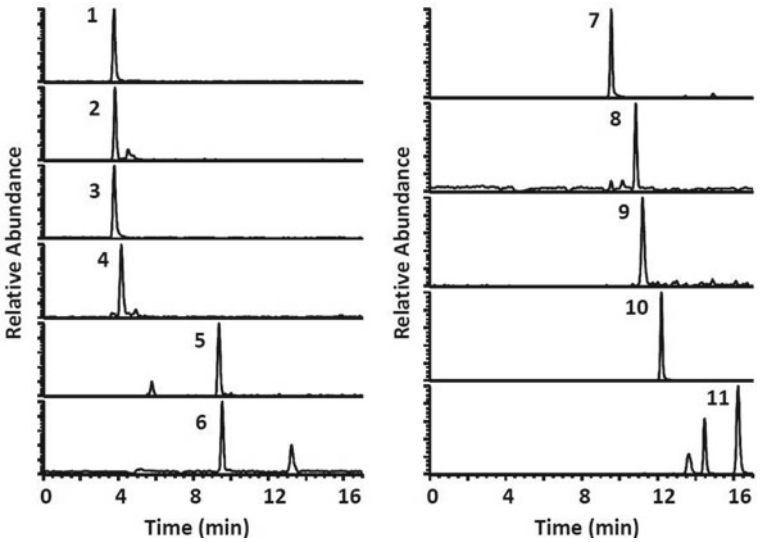

Fig. 8.13 Ion chromatography coupled with mass spectroscopy for analysis of metabolites in mouse quadriceps muscle. The consecutive elution peaks correspond to (1) lactate, (2) pantothenate, (3) 3-hydroxyisobutyrate, (4) 2-hydroxybutyrate, (5) hippurate, 96) succinate, 97) malate, 98) fumarate, (9) pyruvate, (10) citrate, (11) 2-ketoglutarate. Reproduced (or adapted) from [66], ©2016, with permission from ACS publication

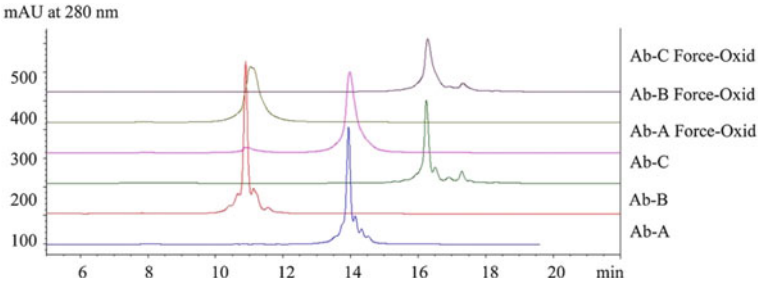

Fig. 8.14 Cation-exchange HPLC performed on XOMA 3AB antibodies showing multiple isoforms of mAbs including Ab-A, Ab-B, and Ab-C. Reproduced (or adapted) from [70], ©2010, with permission from Elsevier

impurity, and activity of the compounds that have to be carefully assessed. A diverse range of analytical approaches, including IEX and reversed-phase liquid chromatography (RPLC), can serve this purpose. For instance, IEX can be a great candidate for separating protein charge variants and isoforms. This technique can support processes such as (i) intact protein analysis, (ii) partially digested large fragments of protein analysis, and (iii) tryptic digest analysis and peptide mapping [67].

A monoclonal antibody (mAb) is an identical copy of an antibody that can bind to a complementary antigen in a very specific manner. In 1997, Moorhouse et al. were among the first to suggest IEX can be used for mAb detection [68]. Reports of the literature confirm IEX to be capable of analyzing intricate degradation processes that involve immunoglobulin-1 (IgG1) antibodies [69]. In another study presented by Teshima et al., IEX was highlighted as a powerful method for characterization of oxidized mAbs [70]. Cation-exchange (CEX) and anion-exchange (AEX) chromatography techniques have successfully separated the oxidized variations of the mAbs. Figure 8.14 exhibits several acidic and basic isoforms of mAbs resulting from CEX analysis.

8.4 High Temperature-Gel Permeation Chromatography for Material Characterization

8.4.1 History of High Temperature-Gel Permeation Chromatography

In the early 1930s, the porosity property of zeolites was named by McBain as a "molecular sieve" which laid the foundation of the high temperature-gel permeation chromatography (HT-GPC). Around 1944, Synge and Tiselius were the first to observe the separation of small molecules according to their molecular size using zeolites with relatively small pores. In 1959, Porath and Flodin used cross-linked

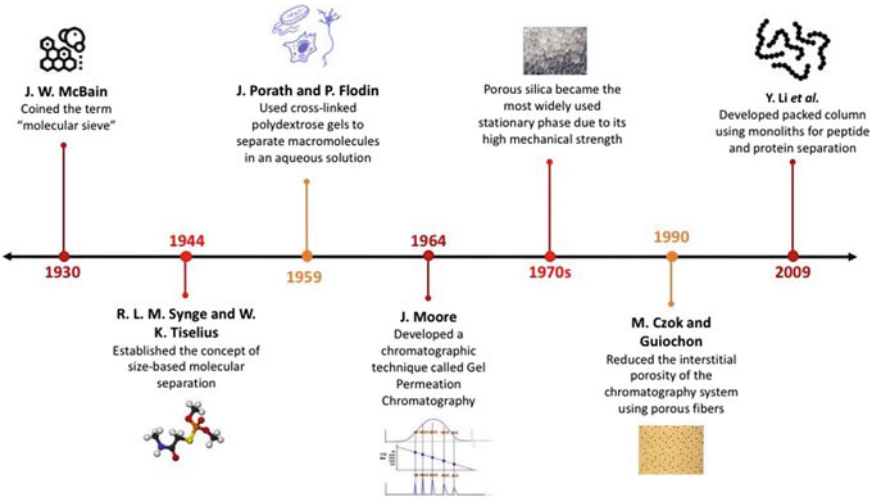

Fig. 8.15 History of HT-GPC

polydextrose gels to separate macromolecules in an aqueous solution and demonstrated that it was possible to separate molecules based on their sizes (Fig. 8.15). When these gels became commercially available, scientists began to employ them to separate biomolecules using GPC at low pressures. Moore first developed the GPC technique in 1964, and he worked for the Dow Chemical Company and used synthetic polymers on cross-linked polystyrene (PS) as a separation medium in a mobile organic phase. Over time, this technique was called by different names such as size-exclusion chromatography, steric-exclusion chromatography, liquid-exclusion chromatography, gel filtration chromatography, and restricted-diffusion chromatography. This chromatographic technique soon became popular for the separation of species such as polymers, aggregates, micelles, and single molecules according to their size dissolved in solution. In addition, it could obtain the molar mass of polymers and analyze aggregation phenomena. The technique was also used for the analysis of phenomena where a change in the hydrodynamic volume was recorded including polymerization, degradation, hydrolysis, and refolding of proteins (Fig. 8.15) [71, 72].

In 1955, Lindqvist and Storgards were successful in separating peptides from amino acids using packed columns containing starch. This study was the first to demonstrate that biomolecules can be separated using size-exclusion processes. Around 1956, Lathe and Ruthven used the "molecular sieve" effect to separate a range of compounds comprising peptides and proteins through a packed column containing corn starch. Two years later, in 1958, Clark carried out a series of studies in which he demonstrated that alcohols that form sugar can be removed by a potent cation-exchange resin. Nonetheless, starch limited the rate at which the molecules separated

due to its low mechanical strength. Proposed materials that later overcame this limitation by minimal protein interaction were dextrans cross-linked with epichlorohydrin (commercially called Sephadex). Subsequently, several polymeric resins were developed to substitute starch, e.g., polyvinylpyrrolidone and polyvinylethylcarbitol gels. The Bio-Rad Company later offered polyacrylamide-based commercial gels (called biogel). Flodin commented that to improve data acquisition speed and better resolution the particle size should be reduced; however, reducing the particle size in polymer resins is limited by manufacturing processes as such particles are compressed under pressure and flow [72, 73].

Porous silica became the widely used stationary phase during the 1970s due to its mechanical properties and inertness under different conditions. This change of the stationary phase allowed GPC to decrease the particle size and therefore enhance its performance. The diol was used as a functional group in the stationary phase to reduce hydrophobic interactions and prevent proteins from interacting with the stationary phase (silanol activity). However, the efficiency of this chromatographic technique remarkably increased with the development of the bridged ethyl hybrid (BEH) particles, since this advancement involved reduced particle size (1.7 μm), high mechanical strength, as well as drastically reduced ionic interactions. The columns were packed with porous particles that facilitated the sample separation. In 1990, Czok and Guiochon used porous fibers reducing the interstitial porosity by 15% to 18%, even though this advancement did not necessarily translate to the efficiency of the system, as fibers have possibly prevented the radial dispersion within the packed column. In 2009, Li et al. developed monoliths and used them for the separation of biopolymers which permitted the separation of proteins and peptides of above 670,000 Da [73].

8.4.2 Mechanism of Operation of High Temperature-Gel Permeation Chromatography

Gel permeation chromatography (GPC) is commonly used under the term of size-exclusion chromatography (SEC), since the procedure involves separating the components based upon the size of the fragments. GPC is also a type of high-performance LC that can be used for separating polymer chains according to their sizes in addition to its application in determining the molecular weight (MW) and the molecular weight distribution (MWD) of the polymers. The term of high temperature-gel permeation chromatography (HT-GPC) is used when referring to processes that involve high temperature (up to 250 °C) for characterization [72, 74]. Since the system operates at high temperatures, the mobile phase's viscosity is reduced, the analytes diffuse in a more effective manner, and therefore, the system exhibits a higher resolution compared with a simple SEC system. As previously mentioned, HT-GPC is utilized when there is a need for high temperatures. Compared with a

GPC system, the HT-GPC analysis can be carried out for samples that are resistant to degradation and do not initiate reactions during the sample preparation step [74, 75].

8.4.2.1 Chromatography System

A GPC or SEC system is typically composed of a solvent transfer pump, a sample injector, a set of columns, one or more detectors, and software to control the different segments of the tool and analyze and display the results. A regular SEC system can be modified and converted to an HT-GPC system by adding a column oven and a restrictor tubing to control the backpressure [74, 76].

Columns

The columns are, indeed, the heart of chromatography equipment since it is in their interior where separation actually takes place. On GPC systems, columns are packed with a porous cross-linked gel polymer packing where the passing molecules interact with the polymer pores dependent on their size. Several types and sizes of pores for columns are available. For HT-GPC systems, the columns can stand high temperatures, and in order to acquire acceptable results, sets of several columns (three or four) are commonly installed [5, 10].

8.4.2.2 Separation Process

To start a SEC characterization analysis, first, the polymer sample has to be dissolved in a solvent, and then the molecules curl themselves to form a coil with a size dependable on the molecular weight. Higher molecular weight polymers coil up to form larger spheres. The coiled-up molecules are captured by the mobile phase and are introduced to the GPC column (stationary phase). The column is packed with semisolid polymer particles that cross-link to form a gel that retains the molecules according to their size. Once the mobile phase carries the polymer molecules through the column, the larger polymer coils would pass straight while the smaller ones would be temporarily retained by the pores (Fig. 8.16) [10, 76, 77].

Lastly, the components are separated based on their sizes into discrete chromatographic bands and a detector responds to their signals by producing corresponding peaks for different bands. The peak size is proportionate to the concentration of the molecules present within the mixture [77].

8.4.2.3 Calibration

It is necessary to calibrate the HT-GPC system to have adequate precision and linearity which are determined by the upper and lower limits of the calibration curves.

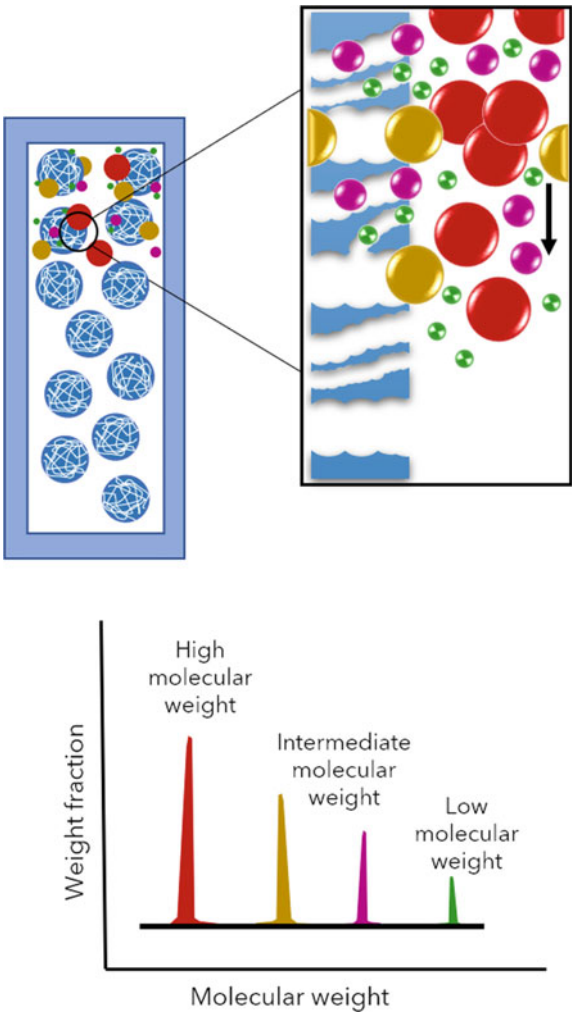

Fig. 8.16 Mechanism of separation in a GPC column

The purpose of calibration is to define the correlation between MW and retention volume in a selective permeation range of the column. For this purpose, standard polymer solutions of known MW and narrow MWD are used to obtain a calibration curve. The solution is injected into the GPC system, and the calibration curve is subsequently plotted on the y-axis using the logarithmic value of MW and on the x-axis the elution volume [5, 10, 77]. In HT-GPC systems, parameters such as retention volume, MW calibration errors, and solvent should be considered as a function of the separation temperature. However, the analysis time is reduced in HT-GPC which, in turn, results in increased separation efficiency [10].

8.4.3 Advantages and Disadvantages of High Temperature-Gel Permeation Chromatography

The characterization of molecular and macromolecular species is mainly done using the HT-GPC technique since the molecular weight and mass sample distribution can be reliably determined through this technique. HT-GPC is a fast and high-resolution technique for analyzing aggregation phenomena as well as the identification of polymeric compounds. By using higher temperatures, the diffusion increases and the viscosity decreases, thus increasing the speed of data acquisition. Samples commonly analyzed with this chromatographic technique are micelles, single molecules, aggregates, and polymers as they are separated according to their size in solution. HT-GPC uses the elution times to produce data in one of the following forms: average molecular weight (M_z), weight average molecular weight (M_w), viscosity average molecular weight (M_v), higher average molecular weight (M_{z+1}), and number average molecular weight (M_n) [74, 78–81].

When the characteristics of the sample do not rely only on the molecular weight, for example, the particle size, using the HT-GPC technique has certain limitations. High molecular weights, insoluble polymers, or samples of complex composition also pose challenges when analyzing the specimen using HT-GPC. The resolution of the obtained peaks directly depend on the molecular weight of the sample. Additionally, pre-treatment is necessary to eliminate unwanted compounds that complicate the interpretation of the results. The operating conditions and experimental setup may vary in different laboratories (e.g., the mobile phase and the column). Common advantages and drawbacks encountered when analyzing samples by HT-GPC are listed in Table 8.8 [71, 74, 81, 82].

8.4.4 Applications of High Temperature-Gel Permeation Chromatography

Extracellular vesicles (EVs) are lipid bilayer-delimited elements that contain several biological components such as proteins and RNA projecting the cell content from which they are secreted. EVs impact physiological and pathological processes by facilitating intercellular communication [83]. For that reason, they attract considerable attention in therapeutic areas such as cell-free methods for drug delivery and regenerative medicine. Multiple methods for isolating EVs are known including differential ultracentrifugation (UC) that separates EVs from other extracellular components based on high-speed sedimentation. Another technique for EVs isolation was recently proposed by Nordin et al. which relies on ultrafiltration and SEC principles [84]. When UC-EVs and SEC-EVs were compared, the EVs were found to be rather more intact using the latter technique since no centrifugation at high speeds was used. Mol et al. in 2017 studied whether the isolation method would negatively

Table 8.8 Advantages and disadvantages regarding HT-GPC

Advantages	Disadvantages
Theoretical plate height tends to be low [78]	Calibration is not easy [82]
The exclusion range and resolution of the technique are high [74, 78, 81, 82]	Determining the volume is complicated [82]
Fast analysis [74, 78, 80–82]	Signals by detectors with different sensitivities and widths are difficult to correlate [81, 82]
The technique has high process control and can be scaled to microsystems [78]	For large molecules, the packaging does not offer reasonable mechanical properties [82]
Determines the mass distribution and molecular weight of the sample [79] [81]	If the molecular weight is high, the resolution decreases [81, 82]
The sample does not interact with the stationary phase [80]	
Interactions between macromolecules can be analyzed [80]	Samples must be soluble [82]
Requires small sample size [74]	At high pressures, the detectors do not have good stability [74]

impact EVs' functionality. In this study, the EVs derived from cardiomyocyte progenitor cells (CPCs) were isolated using UC and SEC. A schematic illustration of the UC and SEC isolation protocols is shown in Fig. 8.17. CPC-based EVs are known for their proangiogenic properties and hence are widely studied for cardiac-related therapies. The results of this study favored the application of SEC over UC hinting that the cells show a more healthy profile and make better candidates for therapeutics testing [85].

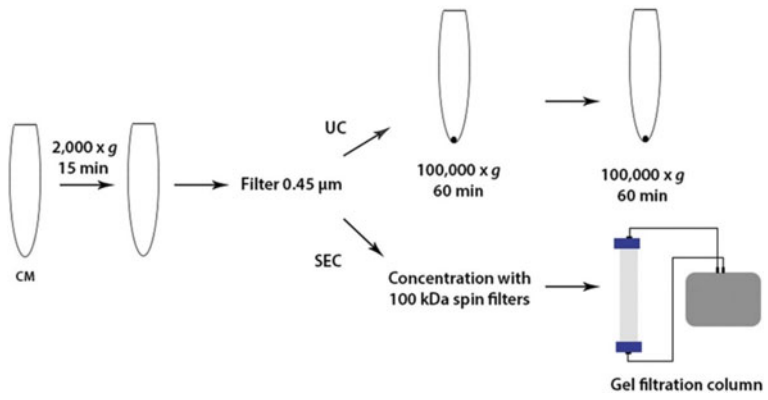

Fig. 8.17 Schematic summary of EVs isolation protocols using UC and SEC. Reproduced (or adapted) from [85], ©2017, with permission from Elsevier

Biotechnological approaches play a significant role in the development of various pharmaceutical products including monoclonal antibodies, vaccines, enzymes, and cytokines with high specificity and potentials that can be crucial in the treatment and control of lethal diseases such as inflammation, infections, cardiovascular diseases, cancer, and immune disorders. To ensure the efficacy and safety of biopharmaceuticals, they have to be thoroughly examined at different development stages ranging from primary steps of the formation, such as amino acid sequencing and post-translational modification (PTM), to secondary and tertiary formation of proteins for the right assembly of multiunit proteins [86]. The evaluation process of protein-based microheterogeneity products can be complex and challenging for the large size and multifaceted structure of the involving proteins. In the case of mAbs, for instance, high MW aggregates of heavy chains may act counterproductive to the mission of the protein and be considered as an impurity or unwanted components that may give rise to immunogenicity [87]. It has been revealed that the characterization of higher MW aggregates is not reproducible using conventional SEC columns. To overcome this limitation, hydrophobic coated-silica SEC columns were suggested as a substitute to achieve reproducible and high-resolution results. Figure 8.18 demonstrates an acceptable consistency between replicated analyses of a biopharmaceutical enzyme, Erwinia chrysanthemi l-asparaginase (ErA) octamer, and monomer. In this study, a ProSEC 300S column with hydrophobic coated-silica was used [88].

8.5 Troubleshooting of Chromatography Analysis Techniques

To identify different gas compounds within a sample, the GC is a useful chromatographic technique that provides the user with excellent accuracy and precision. In general, a mixture of gases is injected into a column and the data obtained is plotted in correlation with the time or volume of the carrier gas. This technique is highly beneficial in pharmaceutical and environmental analyses for the identification of environmental pollutants, residual solvents, and drug products. However, the GC could present several shortcomings during its operation, mainly with respect to operating conditions and equipment maintenance. The troubleshooting of GC is presented in Table 8.9 [89–91].

Within analytical laboratories, and clinical and industrial setups, the HPLC is considered to be a vital chromatography technique for quantitative and qualitative analyses of target samples as it provides high resolution and short time for analysis. This technique identifies molecules present in complex chemical and biological systems and separates molecules with similar physical and chemical properties. The species present in the sample are separated according to the affinity they have to the molecules present within the column. Nevertheless, there are several drawbacks when handling HPLC that mostly depend on equipment components (pumps, seals, detectors, data system, etc.) and/or sample preparation for the mobile phase. One of the recommendations when dealing with HPLC is to clean the column thoroughly

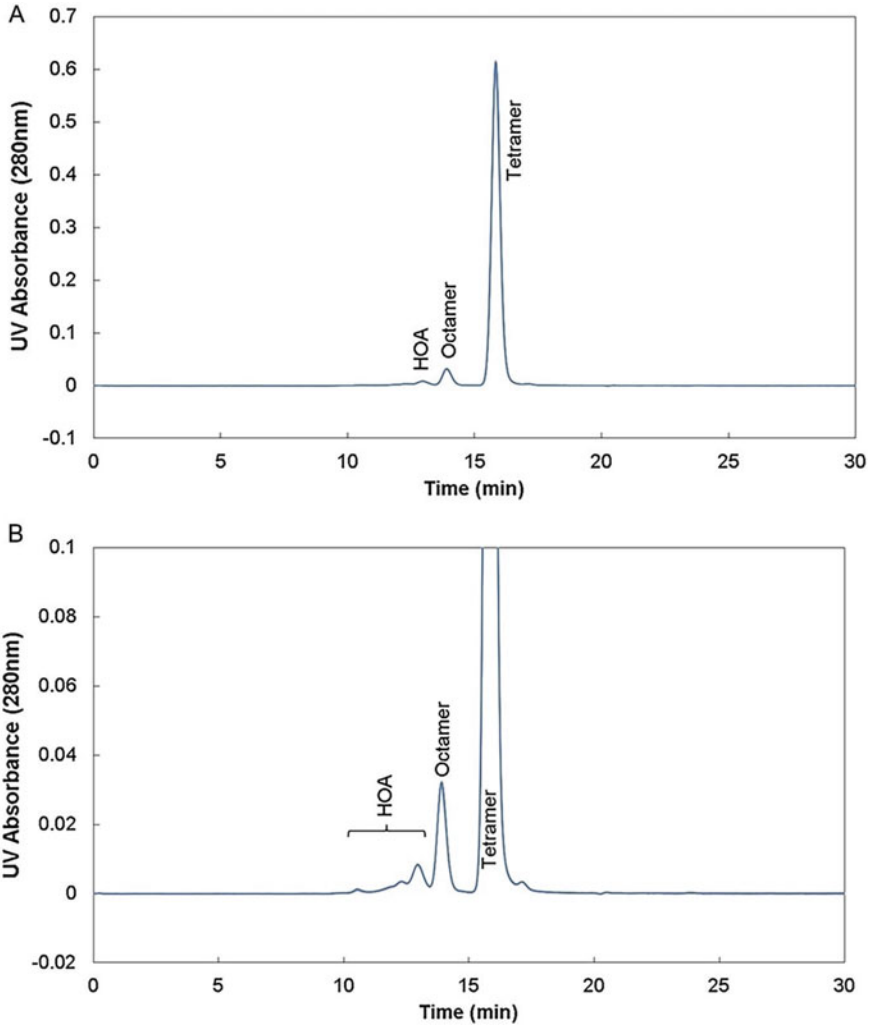

Fig. 8.18 SEC chromatograms of ErA obtained from a coated-silica column in (**a**) full-scale and (**b**) zoomed-in. Reproduced (or adapted) from [88], ©2017, with permission from Elsevier

and frequently. Table 8.9 addresses some of the main challenges users face when working with HPLC, as well as their possible causes and solutions [92].

A commonly employed analytical method for the identification of ions present in various samples is IC. One key element that defines the quality of the results obtained by IC is the pretreatment of the samples. Some components present in sample matrices must be removed prior to IC analysis as they prevent effective separation resulting in poor quantification. Depending on the structure, some organic or inorganic ions may be retained in the column decreasing its capacity, hence affecting the retention time.

Table 8.9 A summarized troubleshooting guide for GC, HPLC, IC, and HT-GPC

Technique	Problem	Cause	Solution
GC	The shape of the earlier-eluting peaks is broad and tailing [89]	The chosen ratio [89]	Split the ratio depending on the column diameter. The lowest recommended ratios are 1:20–1:25 for 0.20 mm; 1:15–1:20 for 0.25 mm; 1:10–1:12 for 0.32 mm; and 1:3–1:5 for 0.53 mm [89]
	A hump peak appears previous to the analyte peak [89]	Degradation of some compound in the column [89]	Decrease both the temperature and the retention time [89]
	Extra peaks appear at the chromatogram [89]	Some species might be condensed in the column [89]	Heat the oven at the highest temperature for 15–30 min and subsequently at 40–50 °C for 60 min [89]
	The peak capacity is deficient [90]	NS	Reduce the column diameter, select a bigger column length, or a mixture of both [90]
	The peaks do not appear [91]	The column is cracked [91]	If the damage is at the edges of the column, it could be carefully cut and reinstalled. On the contrary, if the crack is in the center, it must be replaced [91]
		The temperature in the column is very low [91]	Check the temperature controls [91]
		The carrier gas is not running [91]	Verify if the column has suffered any damage or if there is a sufficient carrier gas flow rate [91]
	Injectors are leaking [91]	The connections of the gas chromatograph are in a bad condition or are not properly installed [91]	Verify all the gas lines in the equipment [91]
	The retention time is too long [91]	Capillaries, tubes, or column are blocked [91]	Verify the flow at inlet and outlet [91]
			Clean the gas lines [91]
		The gas flow rate is very slow [91]	Increase the gas flow rate [91]
	The chromatogram is noised [91]	The column is dirty [91]	Clean the column with a special solvent [91]

(continued)

Table 8.9 (continued)

Technique	Problem	Cause	Solution
		The combustion gases or flow rates are not appropriate [91]	Verify and reset the values of the gas conditions [91]
	The baseline is drifted [91]	Septum bleed is occurring [91]	Use lower injector temperature to analyze the specimen [91]
HPLC	The absent peaks [92]	The detector lamp is switched off [92]	Make sure that the detector lamp is switched on [92]
		There is no mobile phase flow [92]	Verify there is enough liquid in the sampler vials, and air bubbles are not present in the sample [92]
	The HPLC column does not present liquid flow [92]	The pump is not working, or the liquid line is blocked [92]	Turn on the pump and check that the mobile phase has no air [92]
	Detectors are leaking [92]	The cell sealer is damaged, or the cell window is broken [92]	Check and replace components that are in poor condition [92]
	The pressure is low or close to 0 [92]	There are leaks in the system [92]	Verify all the connections in the system, and if necessarily open and then reconnect everything. Also, inject the solvent at twice the previous flow rate [92]
	Inappropriate detector response [92]	Response time is very low, or the noise is very high [92]	Check the response time is correct. Generally, the response time is 1/4 of the peak width at half-altitude of the narrowest peak [92]
		The data points are few [92]	Choose at least 20–30 points regarding reproducible peak integration [92]
	Poor resolution [92]	The mobile phase is polluted. Also, incorrect columns or temperatures are used [92]	Verify and replace the mobile phase. Change the column or the temperature of operation [92]
IC	Retention time is progressively decreasing [93]	Organic or inorganic ions are absorbed to the column [93]	The ions can be removed from the sample using resins (e.g., polyvinylpyrrolidone) [93]
	Low peak efficiency [93]	There is a higher concentration of matrix ions than the ion-exchange column [93]	The conditions under which the chromatography is performed must be changed [93]

(continued)

Table 8.9 (continued)

Technique	Problem	Cause	Solution
			Change the column [93]
	The peak area or peak response shows a low reproducibility [93]	Pulsed or direct current may cause fouling of the detector electrode [93]	Prior to analysis, remove compounds such as fats, proteins, and surfactants from the sample [93]
	Poor peak resolution [94]	Similar retention times [94]	Change the chromatographic conditions [94]
			Pretreatment of the matrix sample may help [94]
	Peak heights reduced [95]	Sample injection problem [95]	Check that the valves are correctly functioning [95]
	Baseline shows deviations [95]	Detector's problem [95]	Pre-treat the sample to avoid impurities [95]
	Low linearity [95]	Electrolytic suppression [95]	Membranes should be regenerated or cleaned [95]
	Air bubbles [95]	Pump's problem [95]	Each time freshwater or eluent is used, the pumps must be purged [95]
HT-GPC	Pressure rapidly increases in the system [96]	The column is damaged, or blockage occurs in the system [96]	Replace or clean the column [96]
	Noise in the chromatogram [96]	Something is trapped in the refractometer cell [96]	Clean the refractometer cell [96]
	The pressure increases [97]	System or column failures [97]	Clean or replace the filters [97]
			Samples should be filtered before use [97]
			Ensure that none of the sample components precipitate during injection [97]
	Peaks do not appear [97]	The injection system does not work properly [97]	Make sure that the injection system is not leaking [97]
	Unexpected peak shapes [97]	The molar mass of the sample or system conditions (tubing length, type of the column) [97]	Use a combination of columns [97]
	Peak splitting [98]	The injection valves are partially clogged [98]	Replace the rotor sealer [98]
	Ghost peaks [98]	The total permeation limit can produce some components that may elute [98]	Change the solvent [98]

(continued)

Table 8.9 (continued)

Technique	Problem	Cause	Solution
	Molecular weight variations [98]	Column's aging [98]	Verify the pump flow rate [98]

NS Not specific; *GC* gas chromatography; *HPLC* high-performance liquid chromatography; *HT-GPC* high temperature-gel permeation chromatography; *IC* ion chromatography

When the sample has an ion concentration that exceeds that of the ion-exchange column, either due to a low sample pH or weak acidity of the column, it causes a low peak acquisition. In Table 8.9, the troubleshooting of the IC technique is presented [93–95].

Calculating the molecular weight of some polymer compounds is complicated when they have low solubility in commonly used organic solvents. HT-GPC is an interesting tool for the characterization (e.g., molar mass) of macromolecules such as proteins and polymers as it is a non-destructive technique. Detectors, injection systems, separation columns, and pumping systems are the main components found in HT-GPC equipment. Despite the advancement of technology in recent years, there are some complications that can occur during the operation of the equipment that affects the quality of the results. Failures in the injection system are among the most frequently observed; as a consequence, the peaks may present strange behaviors [96–98]. The most common problems and their possible causes are summarized in Table 8.9, and a possible solution is presented for each error.

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