an important one (Peter T. Kissinger, *c.* 1975): it can be used for analytes with readily oxidizable or reducible functional groups. An alternative to refractive index detection is the light-scattering detector for nonvolatile analytes. This instrument is able to detect all types of molecules irrespective of the presence or lack of functional groups; it does not respond to changes in eluent composition or temperature in the way that the refractive index detector does. LC–mass spectrometry (LC–MS) is no longer in its infancy and could become one of the most important detectors, even for routine analyses. LC–nuclear magnetic resonance (LC–NMR) has been developed into an excellent tool for structure elucidation. Depending on analyte concentration, NMR spectra can be obtained online or offline after storage of the relevant chromatographic peaks in loops.

Despite new techniques such as capillary electrophoresis, HPLC continues to be probably the most important analytical method, with more 100 000 instruments in daily use worldwide.

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Infrared Detectors in Liquid Chromatography

See **II / CHROMATOGRAPHY: LIQUID / Detectors: Infrared**

Instrumentation

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The Instrumental Set-Up

Modern high performance liquid chromatography (HPLC) uses high pressure to force the mobile phase and an analyte through a closed column packed with micron-size particles, which constitute the stationary phase. HPLC instrumentation is made up typically of nine basic components: mobile phase/solvent reservoir; solvent delivery system; sample introduction device; column; post-column apparatus; detector; data collection and output system; post-detector eluent processing; and connective tubing and fittings. All components except for the post-column apparatus are essential to performing HPLC. **Figure 1** shows a schematic diagram of a generic high performance liquid chromatography system.

Mobile-Phase Reservoir

The mobile-phase reservoir can be any clean, inert container. It usually contains from 0.5 to 2 L of solvent, and it should have a cap that allows for a tubing inlet line, which feeds mobile phase to the solvent-delivery system. The cap also serves to keep out dust, reduce solvent evaporation, allow for pressurization of the bottle, offer ports for additional inlet lines, and sparging (i.e. dispersing He or Ar into the

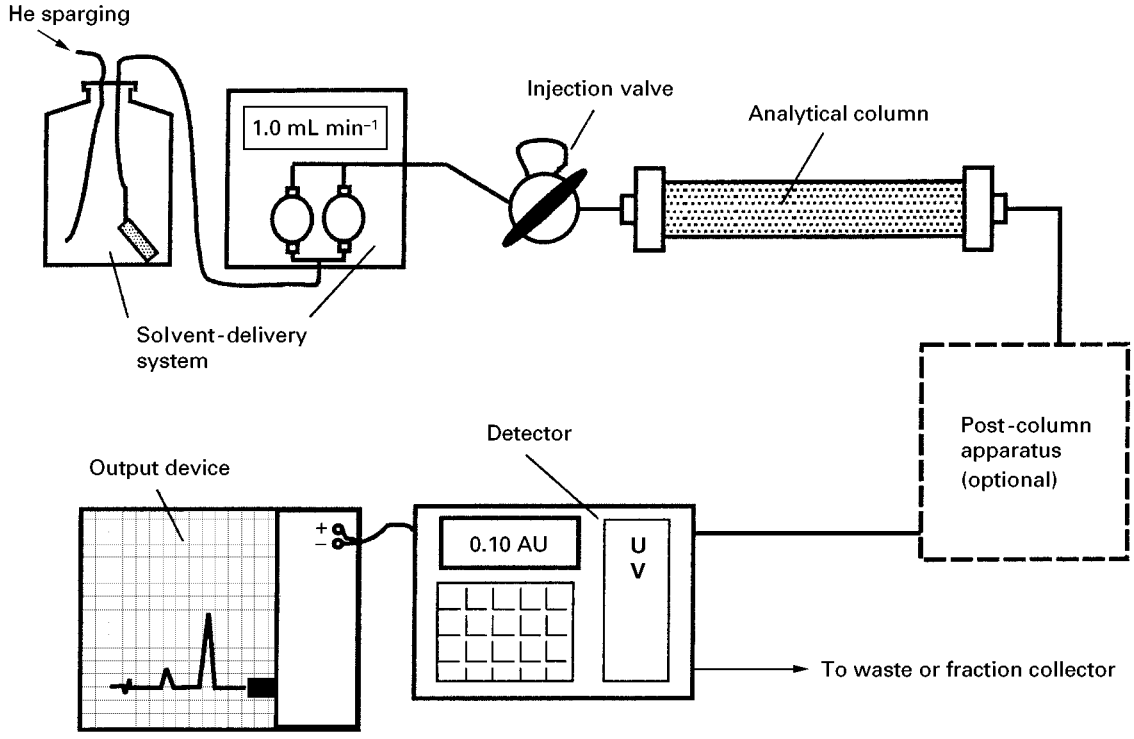

Figure 1 Generic HPLC system.

mobile phase to remove dissolved air). All mobile phases/solvents should be freshly filtered and preferably degassed. Online degassers, which are primarily used to remove small gas bubbles and reduce the amount of dissolved air, are now popular additions to many HPLC systems, and they eliminate the need to degas mobile phase offline. An additional filter is often placed at the end of the mobile-phase inlet line to remove any precipitants that may form in the mobile phase during its use. Sparging control and the ability to blanket the solvents with inert gases is highly recommended to eliminate carbonate formation in alkaline solvents and to maintain extremely low levels of dissolved oxygen, when performing electrochemical or fluorescence detection.

Solvent-Delivery System

The high-pressure pump can operate at pressures from 500 to 5000 psi. The purpose of the pump is to deliver a precise, accurate, reproducible, constant, and pulse-free flow of mobile phase to the column. Three major classes of HPLC pumps are currently in use: constant-pressure pumps; syringe-based or displacement pumps; and constant flow pumps. Neither constant pressure or syringe-based pumps are easily adapted to gradient solvent delivery; hence, constant-flow pumps are by far the most common.

The majority of commercial high-pressure pumps available today are designed around a simple reciprocating piston pump. The rotational energy of a motor is transferred into the reciprocal movement of the piston by an eccentric cam or gear. The piston is driven in and out of a solvent chamber in the pump head, which typically has a volume of 10 to 100 μL. A pair of check valves control the direction of flow through the pump head. A piston seal keeps the mobile phase from leaking out of the pump head. On the intake stroke, the piston is withdrawn from the pump head, creating a low-pressure zone. The low pressure causes the inlet check valve (i.e. from the mobile-phase reservoir) to open and the outlet check valve (i.e. delivery to the column) to close, allowing the mobile phase to fill the pump head. On the delivery stroke, the piston moves into the solvent chamber, which increases the pressure. The high pressure closes the inlet check valve and opens the outlet check valve, allowing the mobile phase to flow to the column. In a single-head reciprocating pump, the solvent chamber is delivering mobile phase to the column only half the time. The other half of the time it is being used to fill the solvent chamber. With a twin-head reciprocating pump, two pump heads operate simultaneously but at 180° out of phase with each other. As a result, mobile phase flows to the column 100% of the time. The twin-head design gives essentially pulseless flow as compared to the single-head design.

Many separations can be done isocratically, which means that solvent composition being delivered to the

column is not changing in composition over the course of the separation. For more complex separations, gradient elution is required. Most commonly, gradient elution is performed by altering the proportion of the eluents over the course of the separation. In doing so, the early eluting compounds remain well resolved, while the more highly retained compounds elute quicker. Gradient elution is simply the programming, or changing of the solvent strength over the course of a separation. A gradient can be linear, convex, concave, stepped, or a complex sequence of each to achieve the desired separation. Computer-controlled pumping is required to generate a gradient flow. Three major approaches are used to produce a gradient flow: (i) controlled amounts of each eluent (up to four may be used) are metered into a mixing chamber before reaching the high-pressure pump; (ii) a proportioning valve, which is controlled by a microprocessor, regulates the amount of up to four eluents and the eluent mixture is sent to the high-pressure pump; and (iii) the outputs of multiple high-pressure pumps, which are controlled individually by a programming device, are mixed together in a high-pressure mixing chamber after the pump. Approaches (i) and (ii) are known as low-pressure mixing, and approach (iii) is high-pressure mixing. Low-pressure mixing is less expensive than high-pressure mixing since only one high-pressure pump is used versus the two or more required for high-pressure mixing, and the maintenance of one pump is much easier than the maintenance of two or more pumps. The main problem with low-pressure mixing is that it is more susceptible to bubble formation because the solvents are being mixed at atmospheric pressure. Hence, the common use of online degassers.

Sample-Introduction Device

In many instances, the limiting factor in the precision of an HPLC system lies in the reproducibility of the sample-introduction system. The sample-introduction device, also known as a 'sample injector', is used to introduce the sample into the HPLC system without depressurizing it. The most widely used method of sample injection is based upon a sample loop that can be placed in and out of the mobile phase flow path by merely switching a valve (**Figure 2**). When the valve is in the load position, the sample loop is filled at atmospheric pressure. Sample sizes often range from 5 to 500 μL. For best results, an excess of sample (i.e. two to five times the injection volume) is flushed through the loop to ensure that no trace of the previous sample remains. By turning the valve from the load to the inject position, the sample loop is connected to the high-pressure mobile-phase stream and the sample is then carried to the column.

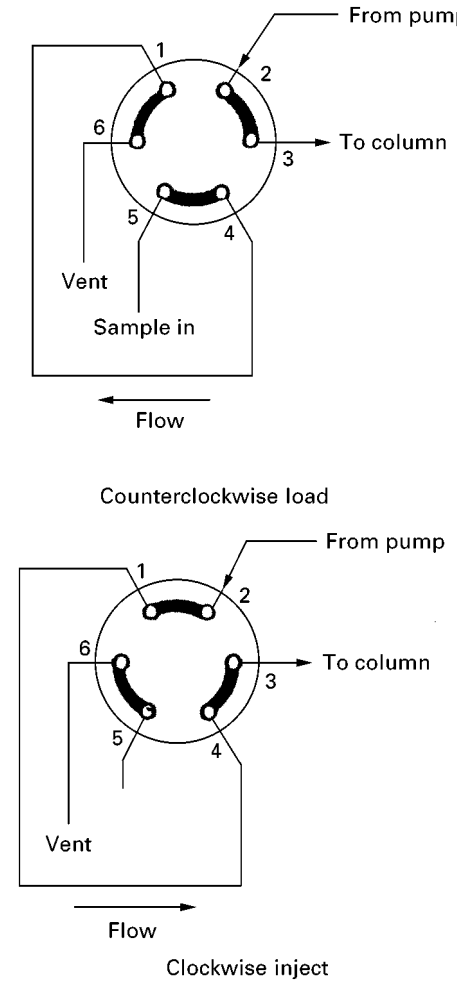

Figure 2 Generic high-pressure injection valve shown in the load and inject positions.

The valve-based sample-introduction system is easily automated using simple robotic technology. The use of auto-injectors not only improves injection reproducibility, but they allow for the continuous processing of numerous (i.e. tens to thousands) of samples at a time. Auto-injectors have also been used for the implementation of pre-column derivatization protocols, especially for amino acid analyses. Pre-column derivatization is used to improve the chromatographic and/or detection properties of analytes.

Column

The column is the part of the HPLC in which the separation occurs. HPLC columns are mainly constructed from smooth-bore stainless-steel tubing due to the high-pressure aspects of HPLC. Nowadays columns are sometimes constructed from heavy-walled glass, titanium, or plastic (e.g. PEEK®) to offer the analyst better performance for particular analytes. Common dimensions for analytical-scale

columns are in the range of 10 to 30 cm long and 4 to 10 mm inner diameter. The common particle sizes of packings are 3, 5, and 10 μm. Columns of the these dimensions often have efficiencies of 40 000 to 60 000 plates per metre. The current trend has been the use of higher performance, high-speed columns, which have smaller dimensions than those described. Such columns have efficiencies of 100 000 plates per metre and have the advantage of speed and minimal solvent consumption. Hundreds of packed columns in differing size and packing material are available from numerous manufacturers.

It is important to read the manufacturers' literature relating to the maintenance, handling, and limitations of the column (e.g. silica-based columns are only compatible with pH values from 2 to 7). In addition to chemical limitations of the packing material, columns are easily degraded by the irreversible adsorption of impurities from samples and solvents. Hence, a small guard column is often used to protect the integrity of the analytical column, which is much more expensive. Also, for analytes, which may contain particulates, an inline filter can be placed between the injector and guard column. It should be noted that with the addition of each component after the injector, the efficiency of the separation is degraded. Hence, judicious use of inline devices is necessary.

For many applications, close control of column temperature is not necessary and HPLC separations are performed under ambient conditions. However, temperature control can enhance chromatographic reproducibility and afford opportunities to improve separation efficiency. Modern instruments can be equipped with column heaters/ovens that control column temperature to a few tenths of a degree from near ambient to 150°C.

Post-Column Apparatus

If post-column modification of the mobile phase or analyte is required, then the system will have a post-column apparatus in the flow path. Modification of the mobile phase (e.g. addition of buffers, changing the pH, and solvent strength) may be needed to enhance the compatibility of the mobile phase with the detector, while post-column derivatization of the analyte may be needed to improve the detection properties of the analytes after their separation. In either case, a post-column addition system consists simply of a reagent-delivery pump, a mixing-tee, and a mixing coil. Typically, a pressurized reservoir is commonly used to deliver a pulseless flow of the reagent. The vessel is usually fitted with a check value to prevent reagent back-up. The major drawback of using a pressure-based delivery system is that it cannot handle a great deal of system back-pressure. Hence, close attention must be paid to the minimization of post-column back-pressure sources. Any single-piston pump, even with extensive pulse dampening, is usually inadequate for high-sensitivity work.

Delivery of the post-column reagent to the chromatographic eluent flow is accomplished via a mixing tee. The mixing tee should be a low dead-volume fitting. Probably the most crucial component in the post-column system is the mixing coil, which connects the mixing tee to the detector. It is essential that the mixing coil produces a homogeneous solution in the most efficient manner, in other words, with minimal band-broadening. The best mixing is obtained with a woven/knitted reaction coil. The three-dimensional weave achieves efficient mixing and effectively reduces band-broadening effects by preventing laminar flow patterns. In addition, their open-tubular nature produces less back-pressure than packed-bed reactors, and woven reactors are easy to make using commercially available Teflon® tubing.

Detector

By passing the column effluent through the detector, some chemical or physical property of the analyte is transduced to an electrical signal, and the solutes are monitored as they are eluted from the column. The electrical signal, which can be amplified and manipulated by suitable electronics, is proportional to the level of some property of the mobile phase or solutes.

HPLC detectors are classified as either bulk-property detectors, which respond to a bulk property of the eluent such as refractive index or conductivity, or solute-property detectors, which respond to some property of the analyte such as UV absorbance. In either case, the response of the detector is modulated by the presence and amount of the analyte. Solute property detectors tend to be more sensitive than bulk property detectors (in the order of 1000 times or more).

Ideal characteristics of an HPLC detector are high sensitivity, good stability, linearity, short response time, reliability, non-destructiveness, ease of use, and low dead volume. Many types of analytical techniques have been applied to HPLC with varying degrees of success. It has been estimated that HPLC detection is divided as follows: 70% by UV absorption, 15% by fluorescence, 5% by refractive index, 5% by electrochemical methods, and 5% by other measurements. **Table 1** lists some of the common HPLC detectors and their relevant properties. Only a brief review of the most common detectors is presented here.

Table 1 Performance characteristics of common HPLC detectors

Parameter	Detector				
	UV/vis absorption	Fluorescence	Electrochemical	Conductivity	Refractive index
Classification	Solute property	Solute property	Solute property	Bulk property	Bulk property
Response	Selective	Selective	Selective	Selective	Universal
Sensitivity	Nanogram	Picogram	Picogram	Nanogram	Microgram
Gradient compatibility	Yes	Yes	Limited	Yes (suppressed) No (nonsupressed)	No
Flow sensitivity	No	No	Yes	Yes	Yes
Temperature sensitivity	No	No	Yes	Yes	Yes

UV/vis absorbance detectors In UV/vis detectors, the mobile phase is passed through a small flow cell, where the radiation beam of a UV/vis photometer or spectrophotometer is located. As a UV-absorbing solute passes through the flow cell, a signal is generated that is proportional to the solute concentration. Only UV-absorbing compounds, such as alkenes, aromatics, and compounds that have multiple bonds between C and O, N, or S are detected, The mobile-phase components should be selected carefully so that they absorb little or no radiation. Absorption of radiation is a function of concentration, c, as described by the Beer–Lambert law: $A = \varepsilon bc$, where A absorbance, ε = molar extinction coefficient, and b = flow cell path length.

Three types of absorbance detectors are available: fixed wavelength; variable wavelength; and photodiode array. A fixed-wavelength detector uses a light source that emits maximum light intensity at one or several discrete wavelengths (e.g. 254, 280, and 365 nm for a mercury lamp) that are isolated by appropriate filters. A fixed-wavelength detector is the most sensitive and least expensive of the three. A variable-wavelength detector uses a relatively wide bandpass UV/vis spectrophotometer, and has the advantage of wavelength-selection flexibility. It is also more expensive than the fixed-wavelength detector. In order to generate a real-time spectrum for each peak as it is eluted, a photodiode array is used. Comparison of spectra generated chromatographically with a known spectrum is useful for solute identification. In addition, software has been designed to evaluate peak purity with diode array-generated data. The major disadvantage with scanning-wavelength technology is a loss in sensitivity.

Fluorescence detectors Fluorescent detectors exploit the ability of a compound to fluoresce upon irradiation. The emitted light, which is detected at right angles to the irradiation beam to minimize background noise, is then detected similarly to that of the UV/vis detector. The major advantage of a fluorescent detector is selectivity and sensitivity for fluorescent solutes. Unfortunately, relatively few solutes fluoresce naturally, and analytes of interest must often be derivatized with a fluorescent entity. Because both the excitation and emission wavelengths can be varied, the detector can be made highly selective.

Electrochemical detectors Electrochemical detectors measure the current associated with the oxidation or reduction of solutes as they are eluted from the column. The suitability of electrochemical detection depends on the redox characteristics of the solute molecules in the environment of the mobile phase. Electrochemical detectors have the advantages of high sensitivity, high selectivity, and wide linear range. Most naturally occurring electroactive compounds have an aromatic substituent (e.g. catechols, quinines, and aryl amines). Polar aliphatic compounds (e.g. carbohydrates, amines, and thio compounds) can also be detected directly using pulsed electrochemical detection (PED). PED exploits electrocatalytic detection at noble metal electrodes by combining amperometric detection with pulsed potential cleaning to maintain uniform electrode activity.

Conductivity detectors Conductivity detectors are used primarily to detect ions in conjunction with ion chromatography (IC). This type of detector monitors the ability of the eluent to conduct electricity. In non-suppressed ion chromatography, the conductivity of the eluent is minimized via the careful selection of reagents and control of their concentration. Under these conditions, charged analyte ions are more conductive than the eluent and a signal is generated as they are pumped through the detector. In suppressed ion chromatography, the response of the background is neutralized using a post-column suppressor (e.g. eluent hydroxide ions in anion-exchange chromatography and eluent hydrogen ions in cation-exchange

chromatography are neutralized to water). Under these conditions, only the charged analyte ions are detected. Suppressed IC inherently, and in practice, has superior detection limits as compared to non-suppressed IC, despite having a larger dead volume.

Refractive index (RI) detectors Refractive index detectors monitor the difference in refractive index between the column eluent containing analyte and a reference stream containing mobile phase only. These detectors are the closest to universal detectors in HPLC because any solute can be detected as long as its refractive index is different from that of the mobile phase. Unfortunately, RI detection is limited by a number of significant drawbacks. The sensitivity of RI detection (Table 1) is poor owing to the small differences in the absolute refractive indices of many substances commonly analysed by HPLC. RI detection is sensitive to both temperature and pressure changes, and, as a consequence, strict temperature control of the detector and pulseless flow are mandatory. Since RI is a bulk property detector, it is sensitive to changes in the mobile-phase composition, as well as analyte concentration, and it is not amenable to gradient elution. Gradient elution can be performed with RI if the detector is configured with a working and a reference column and two flowing liquid streams – this is easier said than done.

Mass spectrometer Owing to innovations in LC–mass spectrometry (MS) interfacing, MS has become a very important HPLC detector because of its ability to generate structural and molecular weight information about the eluted solutes. The combination of HPLC and mass spectrometry allows for both separation and identification in the same step, an advantage few of the other detectors provide. Two major problems must be addressed when interfacing MS with HPLC. First, the flow rate in HPLC with conventional 4.6 mm i.d. columns is about 1 mL min^{-1}, which is much larger than the flow that can be taken by the conventional mass spectrometer vacuum systems. Second, MS has difficulty in vaporizing non-volatile and thermally labile molecules without degrading them.

Other detectors In addition to the highly popular detectors mentioned above, numerous other detectors have been used in HPLC including Fourier transform infrared (FTIR), radioactivity, element selective, and photoionization. Two detectors which have been gaining in popularity for specific applications are chiroptical for optically active compounds and evaporative light scattering for triglyceride analysis.

Data Collection and Output System

A data collection and output device (e.g. computer, integrator, or recorder) is connected to the electronic output of the detector. The data-collection device takes the electronic signal produced by the detector and outputs a plot of response versus time. This resulting chromatogram can then be evaluated for both qualitative and quantitative information. Recorders are rarely used today on their own. Both integrators and computers can integrate the peaks of a chromatogram and have the added advantage of being able to store chromatograms for post-collection processing. The data from computer-based collection systems can also be exported to other software programs and around the world by e-mail or over the Internet. In addition, the computer is typically able to communicate with and control the entire HPLC system. Hence, the majority of modern HPLC systems are fitted with computer control, data collection, and output systems.

Post-Detection Eluent Processing

After the eluent passes through the detector, it is often directed to a waste container. The waste container should be properly labelled for eventual disposable by environmentally acceptable means. If isocratic chromatography is being performed, a mobile-phase recycling device can be added after the detector. While the instrument is running and no injections are being made or during periods of the separation when no peaks are being eluted, the solvent-recycling device will direct clean mobile phase back to the mobile-phase reservoir.

If collection of the eluted peaks is needed, a fraction-collection device is added to the system after the detector. The collection of the peaks by the fraction collector can be done on the basis of a fixed interval of time or mobile-phase volume or via the output signal from the detector. Fraction collectors can also be under the control of an HPLC's computer for ease of use.

Connective Tubing and Fittings

The connective tubing in the HPLC system should be made of material inert to the solvents and constituents of the mobile phase. Often tubing is made of stainless steel or polymer-based materials (e.g. PEEK®, Teflon®, etc.). The connections between the tubings and HPLC components are made with fittings and unions designed to minimize excess volume, or dead volume. Zero dead volume (ZDV) and low dead-volume (LDV) fittings are necessary to reduce band-broadening effects. Also, great care should be used when assembling columns and fittings so that they match properly. Tubing should be cut flat and fit flush, and all fittings should be zero dead volume. Any

extra-column volume compromises separation efficiency. Polymer-based, or PEEK®, tubing and fittings throughout the chromatographic flow path are becoming very popular. PEEK tubing is available in a wide range of sizes, is colour-coded for ease of size selection, is easy to work with, is tolerant to a wide range of buffer and solvent conditions, and is impermeable to oxygen.

The effects of band-broadening are not important prior to injection; therefore, the inner diameter of the tubing from the pump to the injector/autosampler should be as large (i.e. typically 0.030 inch i.d.) as possible (i.e. to reduce system back-pressure) while maintaining high-pressure strength. From the injector to the column to the detector, all tubing should be as short as possible and of the narrowest diameter available to minimize extra-column effects. Post-injector tubing diameters are often 0.01–0.005 inch i.d.

Related HPLC Techniques

The HPLC system described above was based on the dimensions of a typical analytical system, otherwise known as 'normal-bore chromatography'. The same basic components and designs are used in both micro- (i.e. column dimensions of 1–2 mm i.d.) and preparative systems, which have much larger column dimensions.

Microchromatographic techniques include microbore, nanobore, and capillary chromatography, which have column inner diameters of 2 to 1 mm, 1 to 0.3 mm, and less than 300 μm, respectively. Most importantly, microchromatographic systems require smaller extra-column volumes than normal-bore systems. Hence, great pains should be taken to use the smallest inner-diameter tubing available, zero dead-volume fittings, smaller injection volumes, and smaller detection cell volumes. Microchromatographic systems are employed when sample volume is limited.

If compound purification or isolation is intended, preparative chromatography is used. Since a large quantity of material is to be injected in order to isolate a significant quantity of analyte, column capacity and, hence, column diameters are larger. Column diameters in preparative HPLC can be on the order of metres. The high flow rates needed for these large-scale systems require the use of special pumps and larger tubing throughout.

See also: **II/Chromatography: Liquid:** Derivatization; Detectors: Ultraviolet and Visible Detection; Mechanisms: Ion Chromatography.

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Ion Chromatography: Mechanisms

See **II / CHROMATOGRAPHY: LIQUID / Mechanisms: Ion Chromatography**

Ion Pair Liquid Chromatography

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Ion pair chromatography is a widely used reversed phase liquid chromatographic technique for the separation of ionic analytes. Retention of ions in ion pair chromatography is determined by many experimental parameters and it therefore provides an effective tool for obtaining chromatographic separation between ionic analytes. A major part of this article discusses the influence of the most important