Fluorescence Detectors in Liquid Chromatography

See **II / CHROMATOGRAPHY: LIQUID / Detectors: Fluorescence Detection**

Historical Development

V. R. Meyer, EMPA, St. Gallen, Switzerland

Tswett's papers on chromatography were published at the beginning of the 20th century but there was little interest in the technique for several decades. It was not until the 1930s that a growing number of researchers used liquid chromatography (LC) in open columns for the successful separation of complex mixtures of natural compounds, and it was only in the 1970s that LC found widespread use in its instrumentalized form. Over this time it developed:

- from a simple set-up to sophisticated instrumentation with computer assistance
- from the separation of coloured compounds which did not need an instrument for detection to an impressive variety of detectors which allow quantitation of analytes in the picomol range and lower
- from adsorption chromatography as the single option to a large number of varied separation principles (adsorption, reversed-phase, polar bonded phase, ion exchange, enantioselective, size exclusion and affinity chromatography)
- from trial-and-error to a deep understanding of theory, separation mechanisms and method development
- from open-column separations to high performance, closed-column chromatography
- from nonacceptance by the greatest scientists of the time to a method of the upmost importance.

It is difficult to name the most important participants in this development; exceptions are Martin and Synge, who published a paper in 1941 that can be looked upon as the beginning of modern chromatography. A large number of other scientists were responsible for various improvements in LC: **Table 1** lists the most important historical papers in the development of the technique.

The Beginning

Open-column LC was invented by Michael (Mikhail) Semenovich Tswett (1872–1919) at the beginning of the 20th century and he was responsible for naming the method. Paper, thin-layer, closed-column liquid and gas chromatography were later invented by other scientists who varied the method set-up described by Tswett.

The father of chromatography was not a chemist but a botanist. Although born in Asti, Italy, and educated in Geneva, Switzerland, he was a Russain national. In 1896 he obtained a PhD from the University of Geneva. Later, when he was an assistant professor at the University of Warsaw, Poland, he needed a method to isolate plant pigments in pure form. At that time the standard technique for this purpose was the partition of plant extracts between immiscible organic solvents. However, Tswett was also interested in the problem of how the pigments are fixed, i.e. adsorbed, within the plant cell. He carried out numerous experiments with adsorbents and tested

Table 1 Important historical papers on liquid chromatography

Tswett MS (1906) Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. *Berichte der Deutschen Botanischen Gesellschaft* 24: 384

Tswett MS (1910) *Khromofilly v Rastitel'nom i Zhivotnom Mire*. Warsaw: Karlassiakov

Palmer LS and Eckles CH (1914) Carotin – the principal natural yellow pigment of milk fat: its relations to plant carotin and the carotin of the body fat, corpus luteum and blood serum. *Journal of Biological Chemistry* 17: 191

Kuhn R, Winterstein A and Lederer E (1931) Zur Kenntnis der Xanthophylle. *Hoppe Seyer's Zeitschrift für Physiologische Chemie* 197: 141

Martin AJP and Synge RLM (1941) A new form of chromatogram employing two liquid phases. *Biochemical Journal* 35: 1358

Glueckauf E (1955) Theory of chromatography. IX. Theoretical plate concept in column separations. *Transactions of the Faraday Society* 51: 34

Van Deemter JJ, Zuiderweg FJ and Klinkenberg A (1956) Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography. *Chemical Engineering Science* 5: 271

Moore S, Sparkman DH and Stein WH (1958) Chromatography of amino acids on sulfonated polystyrene resins. An improved system. *Analytical Chemistry* 30: 1185

Porath J and Flodin P (1959) Gel filtration: a method for desalting and group separation. *Nature* (*London*) 183: 1657

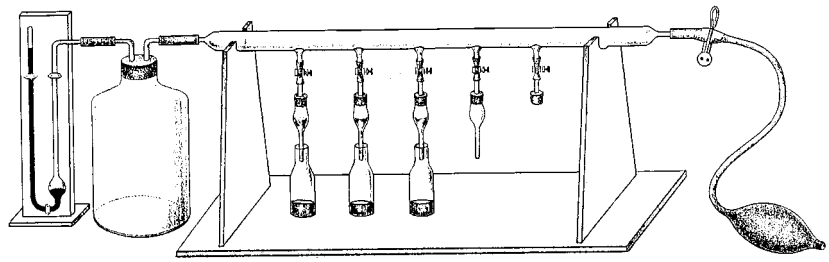

Figure 1 Tswett's chromatographic apparatus. (Reproduced from Tswett, 1906; see Table 1.) *Ber. Deutsch. Bot. Ges.* 24: 384.

more than 100 different powdered materials. Finally he found that inulin, sucrose and calcium carbonate were best suited for the separation of the pigments of green leaves – chlorophylls (two compounds) and xanthophylls.

Unfortunately the new method was only described in lectures, in a paper in a German botanical journal (1906) and in a book in the Russian language (1910). It is obvious that most chemists did not read either botanical journals or a Russian book published in Warsaw. Moreover, when reading these texts today, many details of the experimental set-up are missing. These facts, together with the outbreak of World War I in 1914 and Tswett's untimely death at the age of 47 in 1919, hindered the acceptance of the new method.

Tswett mainly used small glass columns of 2–3 mm diameter packed with adsorbent to a height of *c.* 30 mm. The plant extract was applied on top of the column and transported into the packing by a solvent – the mobile phase – which was added on top and sucked through the adsorbent by a slight vacuum or forced through with a slight pressure. The best eluents were found to be benzene and carbon disulfide; solvent mixtures and even gradients could also be used. Several columns could be used in parallel (**Figure 1**).

Tswett did not publish more than three 'chromatograms'; one is shown in **Figure 2**. Experimental details of all these drawings are missing. The pigments were not eluted but the whole packing was pushed out of the glass tubing and the different zones were then cut apart with a knife. The pigments were extracted from the adsorbent by an appropriate solvent and identified by UV spectroscopy (which was a tedious and time-consuming method). **Figure 3** makes clear how similar the structures of chlorophylls a and b are and, from our current perspective, we get the impression that Tswett was a very skilled experimenter. It was difficult to reproduce his separations using only his descriptions.

The First Followers

A small handful of researchers used chromatography with great success despite the obstacles outlined above: Gottfried Kränzlin, Charles Dhéré and co-workers, and Leroy Sheldon Palmer. They were all able to isolate pure compounds from natural mixtures.

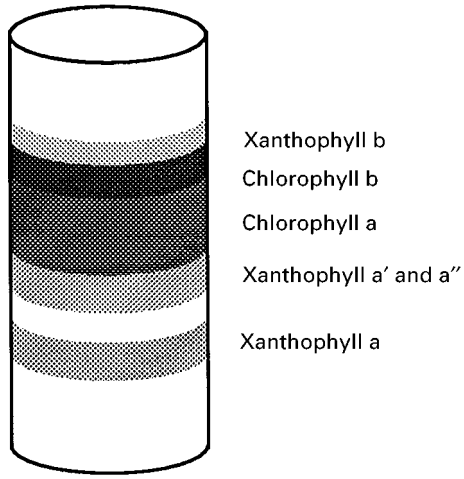

Figure 2 Separation of plant pigments as obtained by Tswett. (Redrawn after Tswett, 1906; see Table 1.)

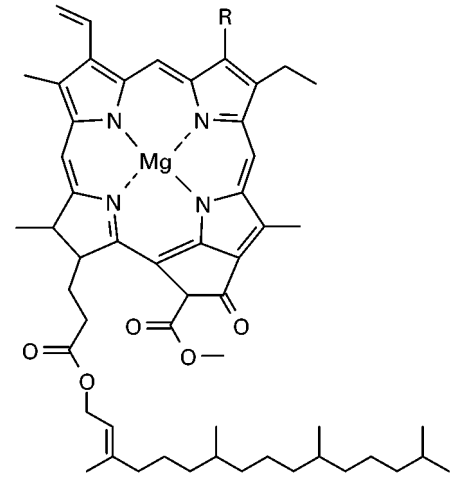

Figure 3 The structures of the chlorophylls separated by Tswett. Chlorophyll a: R = CH_3; chlorophyll b: R = CHO.

Kränzlin, at the Friedrich-Wilhelms University of Berlin, Germany, used Tswett's method in his PhD thesis work in the years 1906 and 1907 – just after Tswett's paper had been published. In a semiquantitative manner he determined the content of chlorophylls and carotenes in various plants from the width of the chromatographic bands. Dhéré was a professor of physiology at the University of Fribourg, Switzerland. In two PhD theses carried out under his guidance, chromatography was used to solve analytical problems: the spectroscopic investigation of the chlorophylls (Wladyslaw de Rogowski, 1914) and the isolation and characterization of invertebrate pigments (Guglielmo Vegezzi, 1916). Palmer, an agricultural chemist, used chromatography for his PhD thesis work at the University of Missouri, USA, in 1909–13. He investigated the pigment content in cattle food, i.e. grass, hay, and carrots, as well as in milk and butter. He proved that the carotene in cow's milk comes from the food plants and is not synthesized by the animal.

The work of these pioneers had only a minor influence on the later development of chromatography, perhaps because they were not chemists (or pure chemists) but were engaged in related fields. The great authorities of natural compound chemistry of the 1910s and later, most of all, the Nobel prize winner Richard Willstätter, were sceptical about the usefulness of chromatography.

The Renaissance

In fact, Willstätter had first-hand knowledge of chromatography because he owned a German translation of Tswett's book (the translator is unknown). Later the translated text came into the possession of Richard Kuhn, a professor of chemistry at the Kaiser Wilhelm Institute for Medical Research at Heidelberg, Germany; Kuhn was a former student of Willstätter. In 1930, Edgar Lederer joined Kuhn as a postdoctoral fellow. His task was to separate xanthophyll (today called lutein) and zeaxanthin (**Figure 4**), and he was able to solve this problem after reading Tswett's book. Lederer used what we would call today preparative columns – glass tubes with a diameter of 7 cm. The results were published in three papers which appeared in leading German scientific journals in 1931. These articles mark the breakthrough and acceptance of chromatography. The method was successfully used in the laboratories of Nobel prize-winners Paul Karrer and Leopold Ruzicka (who received the award in 1937 and 1939, respectively; Kuhn was awarded it in 1938). All these scientists were active in the isolation and structure elucidation of natural compounds, and chromatography was an invaluable help to them.

The main problem in these years was how to find suitable stationary phases. Systematic tests and good luck were necessary to establish adsorbents such as alumina, magnesia and silica. Some researchers began to elute the chromatographic bands from the column instead of cutting the packing; however, it is not clear who used this technique first. It was proved that chromatography can be used on a large scale; Winterstein was able to obtain gram quantities of chlorophylls by using 12.5 cm i.d. columns.

The first application of chromatography to compounds other than organic molecules was done by Georg-Maria Schwab in Munich, Germany. In 1937 he separated inorganic cations on alumina. The first enantioselective separation was performed by Geoffrey M. Henderson and H. Gordon Rule, also in 1937. They were able to partially resolve (*R*,*S*)-phenylene-bis(iminocamphor) on lactose with petroleum–benzene 8 : 1 as the mobile phase.

From Empirism to Science

In 1940, Archer J. P. Martin and Richard L. M. Synge, two researchers at the Wool Industries Research Association in Leeds, England, were trying to separate the amino acids of wool proteins. They

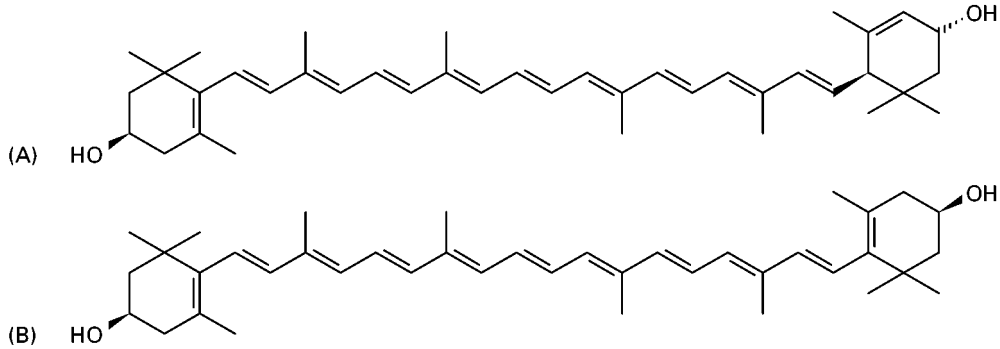

Figure 4 The structures of the carotenes separated by Lederer. (A) Lutein (xanthophyll); (B) zeaxanthin.

attempted to solve the problem by countercurrent extraction of the acetyl derivatives with water and chloroform. This process was time-consuming (one run took a week), tedious and required large volumes of solvents. With ingenuity they came upon a completely different procedure; they immobilized the water phase on silica, packed this material into columns and used the chloroform (with 1% *n*-butanol) as the mobile phase. Although this was chromatography, the technique was different to what had been done previously. It was not based on adsorption but on the partition between two liquid phases (water being the stationary phase). Martin and Synge had invented liquid–liquid partition chromatography.

In addition to the practical applications of liquid–liquid partition chromatography, Martin and Synge's paper also included:

- a theory of the chromatographic separation process which used the theoretical plate as a hypothetical compartment within the column where the solutes are equilibrated between the mobile and stationary phase (this concept was taken from the theory of distillation)
- the statement that a chromatographic process is governed by the diffusion coefficient of the analyte, which leads to specific problems for the separation of large molecules such as proteins
- the insight that there is an optimum flow rate for any chromatographic separation
- the prediction that the finer the particles of the stationary phase, the more efficient the column (this led to high performance liquid chromatography (HPLC) some 25–30 years later)
- the proposal that the mobile phase could also be a gas (this led to gas chromatography (GC), which was first successfully pursued by James and Martin some 10 years later).

In 1952 Martin and Synge were given the Nobel prize for their truly remarkable prescience.

The plate theory of chromatography was defined in the 1950s by Eugen Glueckauf (who worked for the UK Atomic Energy Authority) and others. It became clear how the separation performance can be influenced, i.e. which prerequisites are necessary for efficient chromatography. The prediction of Martin and Synge was quantified by Calvin Giddings: it would be necessary to use stationary-phase packings with particle diameters of 10 μm or less in order to get faster and better LC separations. In 1955, Jan J. van Deemter presented a rate theory approach to describe the chromatographic process and discussed the influence of mobile-phase velocity on the height of a theoretical plate to give relationships known today as van Deemter curves. They show that there is an optimum mobile-phase flow rate for separations performed by gas, liquid or supercritical fluid chromatography.

A fruitful concept for the judgement and comparison of LC separations was introduced in 1977 by John H. Knox with reduced (dimensionless) expressions for theoretical plate height, mobile-phase velocity, pressure drop and overall efficiency. An excellent column should have a reduced plate height of not higher than 3, irrespective of its size, and the reduced flow velocity must not be smaller than 3, irrespective of the chemical natures of the phase system and the analytes.

Towards HPLC

The pre-HPLC era can be defined as the decades when particles of 20 μm and larger were used as stationary phases. High pressure was not necessary and the separations were usually performed with gravity flow or with moderate vacuum at the column outlet, i.e. without a pump. Hindered first by World War II and later, to a certain extent, by the impressive rise of GC, progress was slow but steady.

In the 1930s, the first ion exchange resins were synthesized and became commercially available in the next decade. They could be used not only for batch processes (e.g. desalting) but also as stationary phases, thus opening a new separation principle to LC. During World War II the ability to separate and identify the rare earth ions was of utmost importance in the research activities which led to the construction of nuclear bombs.

The first reversed-phase separations were performed in 1950 by Martin and Howard with a stationary phase of silylated kieselguhr and a

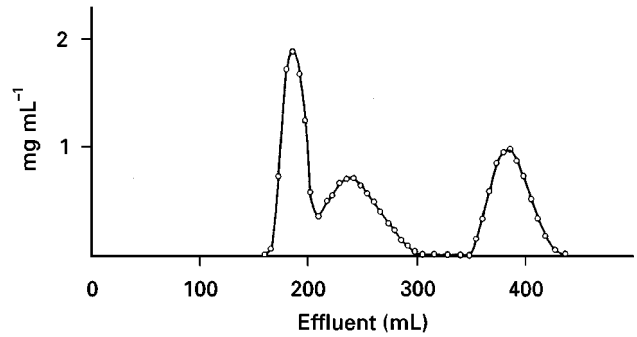

Figure 5 Size exclusion chromatogram of dextran (mol wt 20 000), dextran (mol wt 1000) and glucose as obtained by Porath and Flodin. Column: 36.5 × 4.0 cm i.d.; stationary phase: dextran gel 100–200 mesh; mobile phase: water, 110 mL h^{-1}. (Reproduced from Porath and Flodin, 1959; see Table 1.)

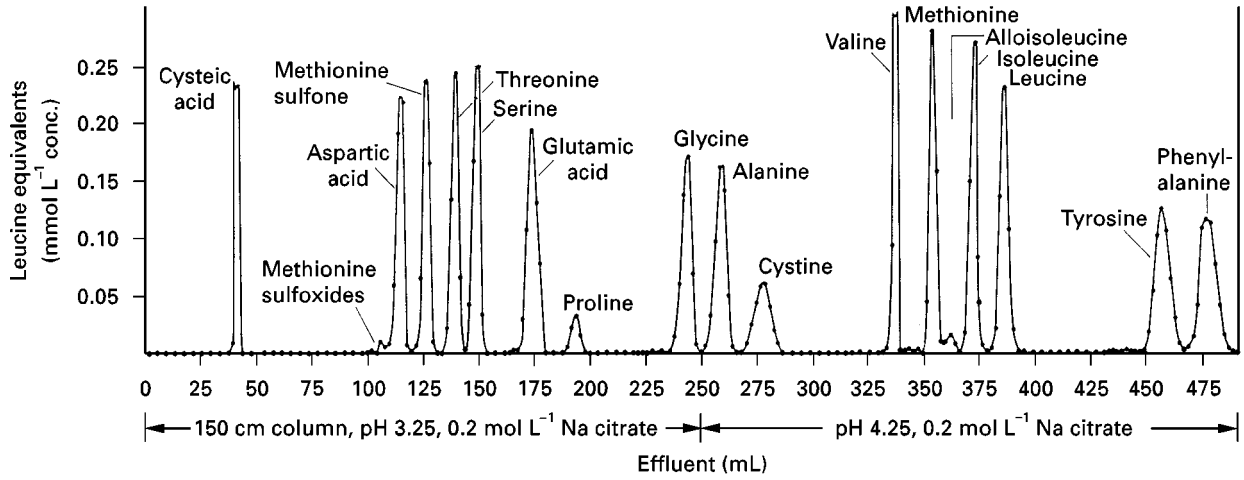

Figure 6 Separation of amino acids as obtained by Moore, Sparkman and Stein. Column: 150 × 0.9 cm i.d.; stationary phase: Amberlite Type III cation exchanger 400–600 mesh; mobile phase: citrate buffer pH 3.25 and pH 4.25, 12 mL h^{-1}. (Reproduced from Moore *et al.*, 1958; see Table 1.)

water–methanol–*n*-octane mobile phase. This system allowed the separation of fatty acids.

At Uppsala University, Sweden, Arne Tiselius introduced new chromatographic and electrophoretic techniques into biochemical research and invented flow-through refractive index detectors. (Previously, the effluent from a column had been collected as individual fractions which were analysed offline.) The first application of gradient elution chromatography was also made in the laboratories of Tiselius: mixtures of oligosaccharides were separated by a continuous increase of the ethanol content of the aqueous mobile phase. Also in Uppsala, size exclusion chromatography (as gel filtration with an aqueous mobile phase) was invented by Per Flodin and Jerker O. Porath (**Figure 5**). The stationary phase with well-defined pores was a cross-linked dextran.

Gel permeation chromatography, i.e. size exclusion in organic solvents, was introduced in 1964 with cross-linked polystyrenes by John C. Moore. For the convenient use of this analytical method an instrument was also built. In retrospect we can say that this was the first liquid chromatograph, in that it included all the necessary parts, from the pump to a refractive index detector. Several years before, in 1958, a specialized instrument, the automated amino acid analyser, had been introduced by Stanford Moore and William H. Stein. Although the separation of a complex mixture (with up to 50 amino acids and related compounds!) took several days (**Figure 6**), this analytical tool was of utmost value for biochemical research. It was a real breakthrough and a revolution. The separation was based on a polystyrene-type cation exchanger run with citrate or acetate buffers at elevated temperature.

Affinity chromatography, a highly selective bioanalytical technique, goes back to Jerker O. Porath and to Meir Wilcheck in 1967–68. This powerful method can be used for the isolation of proteins (e.g. insulin) or the quantitative analysis of bioactive compounds (e.g. aflatoxins).

High Performance Liquid Chromatography

The term HPLC is appropriate for separations of any size (from microanalytical to preparative) if the particles of the stationary phase are not larger than about 10 μm and if the reduced plate height (after Knox) is not larger than 5. This means that the pressure which is necessary in order to force the mobile phase through the column is a parameter of minor importance (the P in HPLC meant pressure when the abbreviation was first coined). It was a long way from pressure to performance with a wealth of technical improvements. Two obstacles needed to be overcome: it was not easy to pack columns with 5 μm particles, and the construction of pumps that can deliver a constant, pulse-free flow as low as e.g. 1 mL min^{-1} at pressures up to 300 bar was demanding. Today we can choose from hundreds of packed column types and dozens of pumps.

Stimulated by the successful amino acid analyser and by the need of a separation method for molecules not suitable for GC, research activities towards an improved, instrumentalized LC began in the late 1960s. Although the available instruments were less sophisticated and less convenient than those available today and despite the fact that most users had to pack their own columns, it soon became clear that HPLC is

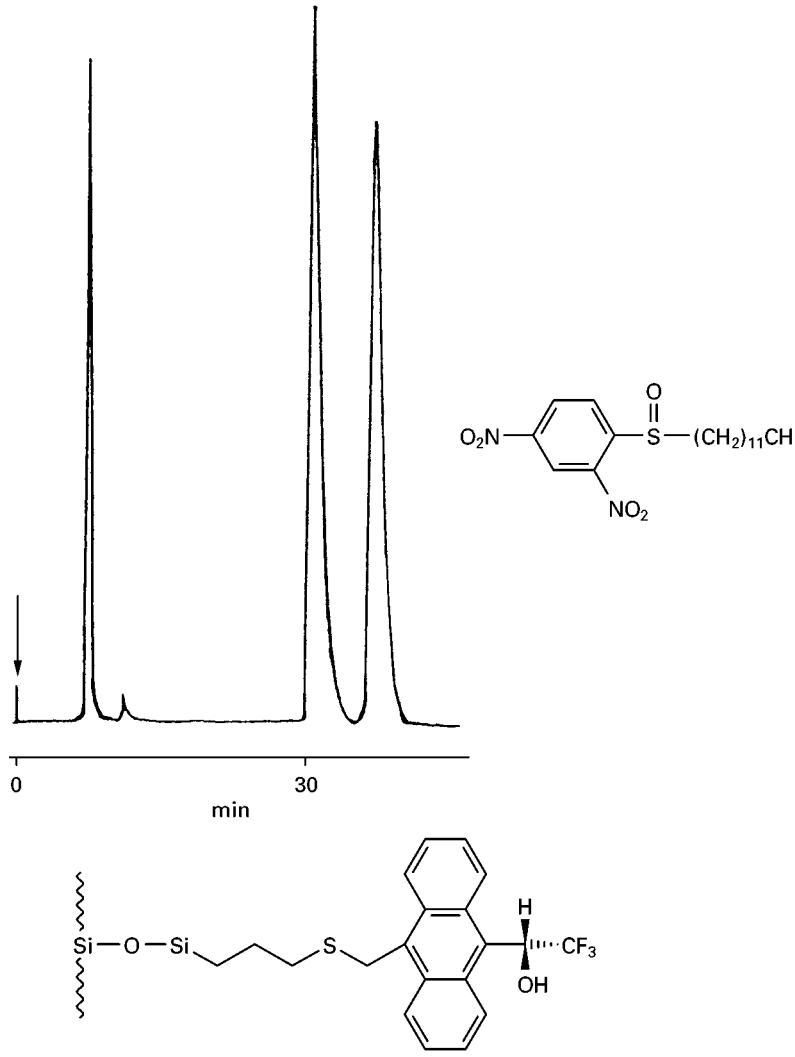

Figure 7 Separation of (*R*,*S*)-*n*-dodecyl-2,4-dinitrophenyl sulfoxide on a trifluoroanthryl ethanol chiral stationary phase as obtained by Pirkle and House. Column: 25.4 × 1 cm i.d.; mobile phase: hexane–2-propanol 4 : 1. (Reproduced with permission from Pirkle WH and House DW (1979) *Journal of Organic Chemistry* 44: 1957.)

a useful method for almost any type of sample. Much of the success of the new method was due to a number of excellent books which soon appeared; the most popular one was *Introduction to Modern Liquid Chromatography* by Lloyd R. Snyder and Jack J. Kirkland (first edition 1974), from which the first generation of users learnt the technique. In 1973, the first International Symposium on Column Liquid Chromatography took place in Interlaken, Switzerland.

Besides the techniques still in use today (adsorption, reversed-phase, ion exchange, size exclusion and affinity chromatography), in the early years of HPLC liquid–liquid partition chromatography was also used, following Martin and Synge's first paper. The stationary phase consisted of a liquid film held in place by a support, i.e. a special type of adsorbent. Although such a separation system can be highly selective, it was inconvenient and temperature-sensitive. Classical liquid–liquid chromatography was abandoned with the rise of chemically bonded stationary phases of almost any polarity and selectivity.

The most important bonded phases are the C_8 and C_{18} reversed phases which are used with an aqueous eluent. The separation principle, first described by Martin and Howard (see above) gained in importance mainly due to the activities of Jack J. Kirkland and Istvan Halász. Today approximately half of all HPLC separations are performed in the reversed-phase mode. New developments in the field of stationary phases are nonporous particles of about 1 μm size; perfusive particles with large throughpores which allow the mobile phase to flow (not diffuse) through the body of the particle; and monolithic, rod-type chromatographic beds which are not built up from

particles but consist of one single, porous piece of stationary phase. All these concepts allow separation speed to increase markedly.

An important class of stationary phases which is now in widespread use are the chiral phases. Enantioselective chromatography can also be done with chiral additives in the mobile phase or with precolumn derivatization to diastereomers but the approach using chiral stationary phases (CSPs) was the most fruitful. Although not the first CSP, the trifluoroanthrylethanol phase by William H. Pirkle opened the door to this new analytical field in 1979 (**Figure 7**). A large number of research groups found numerous other CSPs, including the cellulose and amylose derivatives of Yoshio Okamoto.

The introduction of materials for the separation of proteins and other biomolecules by Fred E. Regnier in 1976 and by William S. Hancock and Milton T. W. Hearn in 1978 marked an important breakthrough. The rise of industrial biotechnology was in fact only possible as HPLC was capable of identifying proteins or peptides exprimed by mutants, i.e. of guaranteeing the true identity of the isolated product (**Figure 8**).

Snyder's work on the interaction between stationary and mobile phase led to a better understanding of adsorption chromatography and to a scientific classification of solvents with regard to their dipole, proton donor and proton acceptor properties. Such classification is also important for reversed-phase separations and gives a sound background to the choice of methanol, acetonitrile or tetrahydrofuran in addition to water or buffer as mobile-phase components. Several computer simulation programs have been developed which allow the prediction of separations based on the chemical structures of the analytes and to optimize the separations. It has been recognized how important temperature can be for most HPLC separations; therefore this parameter should be studied and optimized, too. High temperatures, even above the boiling point of the mobile phase, lead to fast separations; however, not all stationary phases are suited to such conditions, and a pressure restrictor at the detector outlet is necessary in order to prevent bubble formation in the cell.

With regard to instrumentation, continuous improvement in all parts of the HPLC system has taken place and is still going on. This concerns degassing systems, pumps, tubing, fittings, injection valves, autosamplers, columns (e.g. the cartridge principle), detectors and data handling. Authorities now demand that analyses are performed in accordance with Good Laboratory Practice or similar guidelines, therefore all aspects of the analysis, including data acquisition by computers and their manipulation by personnel, must be regulated. Concerning detectors, the refractive index principle was invented decades ago and UV and fluorescence detection were rather obvious. The diode array detector (Hewlett-Packard, 1979) was not obvious but it revolutionized the possibilities for analyte identification and peak purity judgement by UV/Vis spectroscopy. Of the less frequently used detectors, the electrochemical detector is

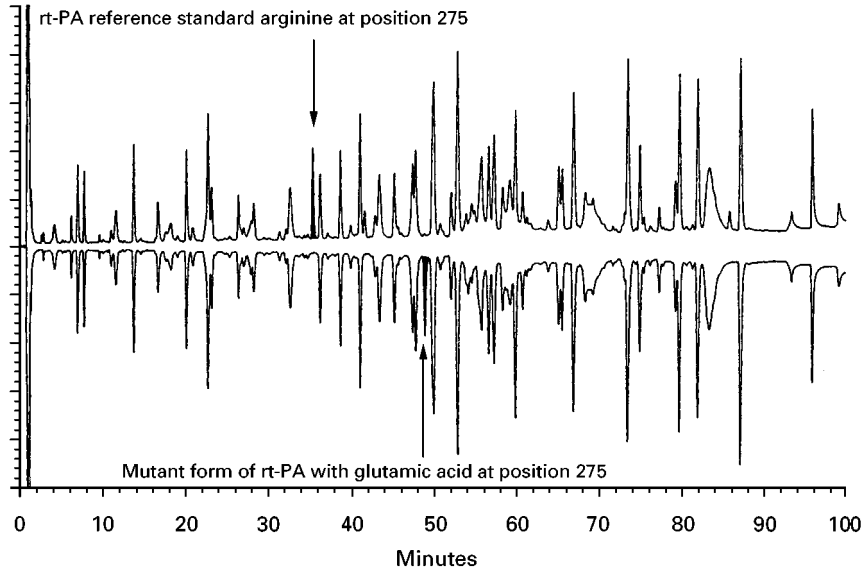

Figure 8 Separation of the tryptic hydrolysate of tissue-type plasminogen activator as obtained by Garnick, Solli and Papa. Top: normal protein with arginine at position 275; bottom: erroneous peptide with glutamic acid at position 275. Stationary phase: Nova Pak C18, 5 μm; mobile phase: phosphate buffer pH 2.8/acetonitrile, step gradient, 1 mL min^{-1}; detector: UV 210 nm. (Reproduced with permission from Garnick RL, Solli NJ and Papa PA (1988) The role of quality control in biotechnology: an analytical perspective. *Analytical Chemistry* 60: 2546.)

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.

Further Reading

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Infrared Detectors in Liquid Chromatography

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

Instrumentation

W. R. LaCourse, University of Maryland, Baltimore, MD, USA

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