

Personal report

One century of liquid chromatography From Tswett's columns to modern high speed and high performance separations

Heinz Engelhardt*

Institute of Instrumental Analysis, University of Saarland, D-66123 Saarbrücken, Germany

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On 21 March 1903, Tswett presented to the Biological Section of the Warsaw Society of Natural Sciences a lecture "On a new category of adsorption phenomena and their application to biochemical analysis" [1]. This is seen as the starting point of liquid chromatography, nowadays the most widely used chromatographic technique. Tswett (or Tsvett) was born in Italy 1872 as the son of a Russian father and an Italian mother. He was educated in Switzerland (Lausanne) and received a Ph.D. in botany at the University of Geneva in 1896. After returning to Russia and some quarrels about his qualification, he became Professor of Botany at the University of Warsaw (at this time under the government of the Russian Czar) [2]. As an "European" scientist he published 13 papers in French, 15 in Russian and 28 in German. The most important paper [3] in respect of chromatography, was "Adsorptionsanalyse und chromatographische Methode. Anwendungen auf die Chemie des Chlorophylls" (Adsorption analysis and chromatographic method. Applications on the chemistry of chlorophyll). In this paper on "chromatographic analysis", he described the "chromatogram" (the developed zones on the column) and its development by using different eluents. In most of the cases, he stopped when the fastest moving colored zone reached the end of the "chromatographic column". In his work, he detected the different yellow zones (carotinoids) and he always found two green zones of the two chlorophylls a and b. His great competitor in chlorophyll research, Willstätter, professor of organic chemistry in Munich, had a chlorophyll obtained by fractionated crystallization, which gave only a single zone employing Tswett's method. Believing only in the classical way

of purification, he argued on decomposition of chlorophyll during the adsorption process [4]. We know nowadays that Tswett was right with the two different forms of chlorophyll, however, Willstätter's verdict was one of the reasons, why chromatography was forgotten for almost 25 years.

The increasing interest of organic chemists in natural products resulted in a rediscovery of chromatography in the twenties of the last century. It is a kind of gratitude of history, that three of the most famous scholars of Willstätter, known primarily as organic chemists and less as chromatographers started the renaissance of chromatography with studies on carotins [5,6], and general application of chromatography in the purification of naturally occurring organic products [7]. Colorless organic solutes were detected and isolated in most cases by unpacking the column and performing color reactions at the adsorbent (brush technique). Elution chromatography was described in detail by another Nobel laureate: Steiger and Reichstein [8], who used the stepwise change of a series of eluents (later compiled in eluotropic series [9]) to elute the components, subsequently evaporated the eluent and made a quantitative analysis by weighing the individual fractions. The now mainly used gradient elution with a continuous change of elution strength was discussed later by Tiselius and coworkers [10], who started his scientific career by applying electrophoresis for the separation of proteins already in 1930 in his Ph.D. thesis.

The increasing of importance of chromatography can be deduced from the fact that very soon monographs on chromatography appeared [11,12], and first theoretical approaches applying equilibrium theory and isotherms to describe the development of the zones were published [13].

Tswett's technique, nowadays described as normal phase (NP) chromatography, could not be applied successfully when water soluble analytes had to be separated. This

* Tel.: +49-681-302-2433; fax: +49-681-302-2963.

E-mail address: iaua@rz.uni-sb.de (H. Engelhardt).

lead to the invention of partition chromatography (LLC; liquid–liquid chromatography) by Martin and Synge in 1941 [14]. In this paper entitled “A new form of chromatogram employing two liquid phases: (1) a theory of chromatography; (2) application to the micro-determination of the higher monoamino-acids in proteins” the height equivalent to a theoretical plate (HETP) concept in analogy to distillation has been introduced. The successful separation of amino acids with on-column detection (the stationary aqueous phase contained an indicator which turned yellow due to the acids within the zones) was shown and also a prediction nobody noticed was made: “. . . the mobile phase need not to be a liquid but may be a vapor. Very refined separations of volatile substances should therefore be possible in a column in which a permanent gas is made to flow over a gel impregnated with a non-volatile solvent, where the substances are separated approximately according to Raoult’s law”. In this paper, the foundation of LLC was laid, gas–liquid chromatography (GLC) anticipated, and finally introduced in 1952 [15].

Column LLC was not very widely used, probably because of lack in robustness due to the physical removal of the stationary phase by the mobile phase. However, the planar form on filter paper sheets impregnated with water or another liquid, also introduced by Martin and coworkers [16], became the first chromatographic microanalytical technique. In describing paper chromatography he introduced the retardation factor R_F (ratio to front) and used the two dimensional technique with different phase systems to enhance the separation capacity. Because of the slow separation process (movement of mobile phase by capillary forces) the use of paper chromatography faded with the rise of thin layer chromatography (TLC). The technique of applying a thin layer of an adsorbent on glass plates originated in Russia [17]. “Chromatostrips” were described by Kirchner et al. [18] where the layer was fixed on the glass plate by using starch as binder. The real break through of this technique can be ascribed to the work of Stahl [19], who replaced starch by calcium sulfate (aggressive reagents could now be used for analyte detection), standardized layer thickness and chromatographic development [20]. TLC soon became the standard analytical chromatographic method in organic and pharmaceutical laboratories, and has not yet been completely displaced by other methods because of its simplicity, speed and universality. It permits multiple separations in parallel in the same run, thus being the ideal screening method in biological and chemical analysis with qualitative and semi quantitative determination. As disadvantages may count the lack in automation, reproducibility (e.g. influence of humidity) and accuracy in quantitation, where now HPLC has its prominent field. Nevertheless, TLC is and will survive as a fast and simple micro technique of chromatography, especially for qualitative and semi-quantitative studies.

With the rise of gas chromatography simultaneously with petrochemistry the theoretical description and physicochemical discussion of the chromatographic process received re-

consideration from the kinetic points of view. The influence of diffusion, resistance to mass transfer on equilibrium conditions [21], random walk [22] of molecules within the chromatographic column was described, leading to equations, generally known as “van Deemter equation”, which permit to discuss the influence of intermolecular diffusion, flow rate of mobile phase, layer thickness of stationary phases, etc. on the efficiency of the chromatographic column. Also a pure mathematical approach by Golay, based on electro-techniques, gave a description of the chromatographic process and led to the prediction of capillary gas chromatography [23]. All the theoretical approaches have been summarized by Giddings in his book “dynamics of chromatography” [24]. In the final chapter of this book, while comparing the limits of separation and speed of gas versus liquid chromatography, he stated: “in this region of extremely difficult separations, that liquid chromatography has the advantage by virtue of the fact that gas chromatography cannot make such separations at all”. Around this time several scientists, who were already experts in gas chromatography, started with research on instrumental liquid chromatography, firstly called high pressure liquid chromatography, because the viscosity of liquids is by a factor of 100 lower than that of gases. Anticipating identical conditions (column length, flow rate) a 100 times higher pressure would be required. But soon “pressure” has been exchanged by “performance” because smaller particles and shorter columns could be used, improving the performance of the chromatographic process. With the acronym HPLC, modern instrumental liquid chromatography became the most widely used chromatographic technique.

It is difficult to state who was first. Without any doubt, Horváth et al. reported at the International Symposium on Chromatography in Rome, 1966 on the use of pellicular ion exchangers for the separation of nucleotides. The paper was published in 1967 [25]. At the same time, Huber and his group were applying LLC with small particles. The results have also been published in 1967 [26]. Small particles of ion exchange resins have been applied to analyze constituents of body fluids in the same year by Scott et al. [27].

At this stage two of the pioneers used ion exchange chromatography for the separation of polar analytes. Ion exchange chromatography has been primarily used in inorganic analysis with highly successful separations of rare earth elements, transuranes and their fission products [28]. Derivatized polystyrenes [29] had been used as stationary phases. But also soon analytes of biological interest have been separated. It is worthwhile to mention, that the first automatic liquid chromatograph with gradient elution and post column derivatization was the automatic amino acid analyzer designed by Moore and coworkers [30]. This technique is principally still in use, however, improvements acquired in HPLC in respect of instrumentation and column efficiency have been incorporated.

As commercial instrumentation was not yet available several authors described in detail how to modify available components and to construct HPLC equipment [31,32]. At

the 5th Symposium on “Advances in Chromatography” in Miami 1969 a special session on HPLC was organized, and some companies already presented instrumentation for HPLC. With this meeting the breakthrough of HPLC started. In 1971, a special meeting was organized in Wilmington, leading to the first book on HPLC [33]. In 1973, one of the most successful series of international symposia was inaugurated at Interlaken as a meeting on “Column Liquid Chromatography”. The most recent, the 27th symposium, extended to “High Performance Liquid Phase Separations and Related Techniques” took place this June in Nice, still drawing more than 1000 attendees.

In this period (early 1970s), the rise of HPLC was not foreseeable. Of course, one knew that less than 20% of organic compounds can be separated by gas chromatography. In liquid chromatography, two phases are active in separation. The wide variety of stationary and mobile phases should give a large potential of unique selectivities for separations. However, in the beginning only relatively large particles were available. To improve mass transfer, film-coated (pellicular) or surface etched glass beads or porous layer beads (impenetrable core coated with a thin active layer) were used. Liquid–liquid partitioning systems were used, causing principal problems with column stability (the shear forces were much higher than in LLC columns operated under gravity flow) and selectivity due to the required immiscibility of mobile and stationary liquid. Because of the small phase ratios the range of retention was tiny, k values hardly exceeded values of one [34].

The availability of small porous silica particles (particle diameter $\sim 10\ \mu\text{m}$) which had to be packed in columns by slurry techniques [35], and the introduction of chemically modified stationary phases [35,36], especially the reversed phase (RP) columns (this kind has also been introduced by Martin and Howard and Martin [37] for classical LLC) marks the real break through of HPLC. The RP (primarily octadecyl RP18, and octyl RP8) systems opened the direct way of injecting analytes from aqueous media. The columns were very stable and robust exhibiting high selectivity, but caused some problems when strongly basic analytes had to be separated. Optimization of mobile phases in RP chromatography is very easy, because in most cases only the ratio of water to organic modifier (acetonitrile or methanol) has to be changed. It is easy to modify the properties of the stationary phase or the analyte by changing the pH value, the ionic strength of the eluent, or via addition of complexing agents like amines, ion pairing agents, etc. Of course, up to now optimized protocols for the preparation of RP columns significantly improved their properties, especially with respect to the reduction of tailing of basic analytes. Standardization of silica surfaces by defined hydroxylation, application of very pure silica, improvement in bonding and end-capping procedures give us now very versatile RP systems with high selectivity, great separation power and robustness, excellent suitability, high stability and efficiency. By introducing functional groups into the bonded alkyl groups, the properties

can easily be modified generating stationary phases with polar, ion exchange, and chiral selectivities.

The introduction of spherical particles enabling the preparation of columns with improved packing stability, a further reduction of the diameter of the stationary phase particles from initially 10–5 μm in 1975, to 3 μm in 1978, and finally to 1.5 μm in 1990 improved column efficiency, and allowed to generate the required plates in much shorter columns thus enhancing speed of analysis and improving detection sensitivity. In the beginning the standard column length had been 25 cm, where 8000 to 10,000 plates/m could be achieved. With 3 μm particles the same plates are now generated in an approximately 6 cm long column, and analysis time is reduced by a factor of approximately 4.

The requirements of pharmaceutical chemistry have been the major driving force for the advancement of HPLC. The quality of pharmaceutical products is determined by the quality of their HPLC analysis. Selectivity and efficiency of the columns are not the only prerequisites for the separation and determination of the active components and their related substances, but also the quality and reliability of instrumentation. The accuracy of quantitation is determined by the flow accuracy of the pumps. Relative standard deviation of the flow below 0.5% are essential, when the maximal permitted R.S.D. values of the content of a minor component in the 1% concentration range has to be below 0.5%. These requirements of the European Pharmacopoeia can only be achieved with HPLC [38].

HPLC was the biggest revolution in analytical chemistry during the past 40 years. Among the instruments present in the analytical laboratories, HPLC has taken the third rank after balances and pH meters. It will certainly fulfill the challenges of the future brought up now by the requirements of proteomics, metabolomics, and all the future “-omics” sciences. HPLC is a technique allowing high throughput separations, at a high speed of analysis with high detection sensitivities to detect low concentrations of analytes present in biological samples. The main challenges of proteomics, separation of multi-component mixtures present in extremely differing concentrations (a dynamic range of concentrations of 10^8 is discussed) and molecular masses (10^5 and more), high complexity (caused by post translational modifications) require automation and multidimensional approaches. There is no doubt that the main analytical technique for these problems will be HPLC with coupling to mass spectrometry for sensitive and selective detection and identification.

As Tswett wrote once [2]: “An essential condition for a fruitful research is to have at one’s disposal a satisfactory technique. “Tout progrès scientifique est un progrès de méthode” as somebody once remarked.¹ Unfortunately, the methodology is frequently the weakest aspect of scientific

¹ “All scientific progress is progress of a method” has been written by the French philosopher René Descartes (1596–1650).

investigation". The future will show how the pending problems will be solved by HPLC and the expected improvements.

A personal view on the history of liquid chromatography has been given, starting with my personal experience with paper chromatography dating back to 1958. If I have forgotten the contribution of a colleague it was not on purpose, but limitations of memory and space may be responsible. For those further interested in the history of chromatography, the following monographs should be referred to [39,40].

References

- [1] Proc. Warsaw Soc. Nat. Sci., Biol. Sect. XIV (6) (1903). This paper is hardly available in libraries. An English translation has been published by G. Hesse, H. Weil in 1954 (Woelm Mitteilungen AL8, M. Woelm, Eschwege).
- [2] The most recent and extensive biography on the life and work of M.S. Tswett, in: E.M. Senchenkova (Ed.), Michael Tswett—The Creator of Chromatography, Russian Academy of Science, Moscow, 2003. Easier accessible: K.J. Sakodyskii, J. Chromatogr. 73 (1972) 303.
- [3] M. Tswett, Ber. Deutsch. Bot. Ges. 24 (1906) 384.
- [4] There are many versions on the quarrel between Tswett and Willstätter (see [2]). This version has always been told by my advisor in chromatography, G. Hesse. Artefact formation was certainly a problem in the early days of chromatography stemming from the long analysis times and the not well characterized adsorbents sometimes acidic or basic in nature. See G. Hesse, Z. anal. Chem. 211 (1965) 5.
- [5] R. Kuhn, E. Lederer, Ber. Deutsch. Chem. Ges. 64 (1931) 1349.
- [6] P. Karrer, W. Schlienz, Helv. Chim. Acta 17 (1934) 7.
- [7] A. Winterstein, G. Stein, Z. physiol. Chem. 220 (1933) 247, 263.
- [8] M. Steiger, T. Reichstein, Helv. Chim. Acta 20 (1937) 817.
- [9] W. Trappe, Biochem. Z. 305 (1940) 150.
- [10] R.S. Alm, R.J.P. Williams, A. Tiselius, Acta Chem. Scand. 6 (1952) 826.
- [11] G. Hesse, Angew. Chem. 49 (1936) 315.
- [12] L. Zechmeister, L.V. Cholnoky, Die chromatographische Adsorptionssmethode, Springer, Wien, 1938.
- [13] J.N. Wilson, J. Am. Chem. Soc. 62 (1940) 1583.
- [14] A.J.P. Martin, R.L.M. Synge, Biochem. J. 35 (1941) 1358.
- [15] A.J.P. Martin, A.T. James, Biochem. J. 50 (1952) 679.
- [16] R. Consden, A.H. Gordon, A.J.P. Martin, Biochem. J. 38 (1944) 224.
- [17] N.A. Izmailov, M.S. Shraiber, Farmatsia (Russ.) 3 (1938) 1.
- [18] J.G. Kirchner, J.M. Miller, G.J. Keller, Anal. Chem. 23 (1951) 420.
- [19] E. Stahl, Pharmazie 11 (1956) 633.
- [20] E. Stahl, Chem.-Ztg. 82 (1958) 323.
- [21] J.J. van Deemter, F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sci. 5 (1956) 271.
- [22] J.C. Giddings, H. Eyring, J. Phys. Chem. 59 (1955) 416.
- [23] M.J.E. Golay, in: D.H. Desty (Ed.), Gas Chromatography, Institute of Petroleum, London, 1958.
- [24] J.C. Giddings, Dynamics of Chromatography, Marcel Dekker, New York, 1965.
- [25] Cs. Horváth, B.A. Preiss, S.R. Lipsky, Anal. Chem. 39 (1967) 1422.
- [26] J.F.K. Huber, J.A.R.J. Hulsman, Anal. Chim. Acta 38 (1967) 305.
- [27] C.D. Scott, J.E. Attrill, N.G. Anderson, Proc. Soc. Exp. Biol. Med. 125 (1967) 181.
- [28] Multiple authors (Manhattan project) J. Am. Chem. Soc. 69 (1947) 2769–2881.
- [29] O. Samuelson, Z. Anal. Chem. 116 (1939) 328.
- [30] D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30 (1958) 1190.
- [31] I. Halász, A. Kroneisen, H.O. Gerlach, P. Walkling, Z. Anal. Chem. 234 (1968) 81.
- [32] J.J. Kirkland, Anal. Chem. 40 (1968) 391.
- [33] J.J. Kirkland (Ed.), Modern Practice of Liquid Chromatography, Wiley, New York, 1971.
- [34] B.L. Karger, K. Conroe, H. Engelhardt, J. Chromatogr. Sci. 8 (1970) 242.
- [35] R.E. Majors, Anal. Chem. 44 (1972) 1722.
- [36] J.J. Kirkland, J.J. De Stefano, J. Chromatogr. Sci. 8 (1970) 309.
- [37] G.A. Howard, A.J.P. Martin, Biochem. J. 46 (1950) 532.
- [38] M. Renger, J. Chromatogr. B 745 (2000) 167.
- [39] L.S. Ettre, A. Zlatkis (Eds.), 75 Years of Chromatography—A Historical Dialogue, J. Chromatogr. Libr., vol. 17, Elsevier, Amsterdam, 1979.
- [40] C.W. Gehrke et al. (Eds.), Chromatography: A Century of Discovery 1900–2000, J. Chromatogr. Libr., vol. 64, Elsevier, Amsterdam, 2001.