Report for the Russian Federation:
West Siberian Regional Venture Fund

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- **Professor Dr. Heinz Engelhardt**

What is Chromatography.

The Russian scientist Michael Tsvett was the first to name and describe the analytical technique called "Chromatography". The principle of this technique is that molecules can be separated from each other based on the fact that their distribution between two phases (stationary and mobile) is different. The typical chromatographic system consists of a column packed with a solid and rigid stationary phase penetrated by a mobile phase which can be either a gas (Gas Chromatography - GC) a supercritical fluid (Super critical Fluid Chromatography - SFC) or a liquid (Liquid Chromatography - LC) or to differentiate the modern high performance version from the classical Tsvett process: HPLC = High Performance Liquid Chromatography.

The separation is achieved by the distribution of the solutes between the stationary phase and the mobile phase due to differences in their adsorption and/or solubility on/in the stationary phase. The longer the solute stays in the stationary phase the later it reaches the end of the column, where the solutes are detected in the mobile phase by (usually) a photometric detector. A chromatogram is the recorded traces of the detector signal along the time axis. The signal of the detector is proportional to the concentration of the solute in the mobile phase (quantitative measure), whereas the time at which the solutes reach the detector is specific for their chemical nature (qualitative identification). Because in a single analysis the individual solutes can be separated from sample mixtures and identified qualitatively and their concentration in the mixture can be measured quantitatively, chromatography has become the most widely used analytical technique. Gas chromatography is restricted to volatile solutes.

As EcoNova only is concerned with HPLC, in the following only the aspects of HPLC will be discussed in detail.

Requirements for HPLC instruments

In HPLC one commonly uses as stationary phases high purity silica gels, which are usually surface modified by chemical reaction to enhance separation selectivity. In most of the separations in HPLC so called "reversed phase", CRP columns are used, where the surface of silica is chemically modified with octadecyl – or octyl silanes. As mobile phases (eluents) mixtures of water with methanol or acetonitrile are applied. As a general rule for this system: the lower the solubility of an organic compound in water is, the longer it will stay in the stationary phase, the later it will reach the detector. The higher the organic content of the mobile phase the shorter the analysis time of organic solutes becomes. To be able to separate also mixtures with large differences in solubility one changes continuously the composition of the mobile phase by increasing the concentration of the organic component. This technique is called gradient elution. For this technique two different pumps are required in an instrument.

The samples to be analysed are injected at high pressure into the stream of the mobile phase. Usually a few microliters are sufficient. It should be possible to change easily the volume injected for each sample and for automatic over-night analysis a sample tray with various sample vials should be available (autosampler).

The stationary phases for analytical separations are packed in columns with 2 to 4.6 mm inner diameter. HPLC differs from classical LC by using particles with smaller diameter. The particle diameters used for standard HPLC columns are 5 µm to 10 µm. The smaller the particle diameter the shorter the columns can be to achieve the necessary plate numbers for an efficient separation. This leads additionally to a reduction of analysis time. With decreasing particle diameter the concentration at peak maximum increases, thus leading to improved detection sensitivity. Most of the columns used are packed with 5 µm particles and have a length between 10 cm and 15 cm. Further reduction of the particle diameter to 3 µm and 1.5 µm is presently in introduction. Because of pressure limitations of standard HPLC equipment the column length has to be reduced further to 5 cm and 3.3 cm respectively.

To improve detection sensitivity additionally, and to reduce volume flow rates when coupling HPLC to mass spectroscopy the column diameter is decreased further. Narrow bore columns (inner diameter 2 mm or smaller) pose problems with standard HPLC equipment. Lower flow rates (below 500 µL/min), smaller volumes in the piping within the instrument and lower detector cell volumes (below 5 µl) are

required. Standard HPLC instruments may cause problems when narrow bore columns are used. Standard equipment have to be modified for use with short narrow bore-columns.

Biotechnology opened another broad application field for HPLC. Control of biotechnology reactors, isolation of active ingredients, identification and purity control of bioproducts are mainly achieved by HPLC. It can be estimated that 60 % of the price of a biotechnology product stems from the use of the various chromatographic isolation and identification methods.

Environmental analysis for polyaromatic hydrocarbons, polychlorinated biphenyls, pesticides etc. are also fields for HPLC application, as is there use in food control for illegal additives, pesticides, dyes etc.

As stated above there are no real limitations for the application of HPLC in all fields where the identification and quantitation of inorganic and especially organic compounds is required. There is no sign that HPLC will be displaced by another technique. It is to be expected that with the newly arising improved coupling techniques for mass spectroscopy to HPLC, the application of HPLC to molecules without a chromophor will be extended. To this class of compounds belong e.g. detergents, who cannot be analysed so far at concentration levels required in environmental analysis.

Worldmarket of chromatography

The amount of worldwide sales of HPLC systems is difficult to define exactly, because sales in complete equipment, sales of components, sales of accessories (fraction collectors etc.) and consumables, like columns, solvents, reagents etc. can be included in the published and estimated sums. It is generally agreed that the total sales connected with HPLC are in the range of US $ 2 billions. The percentage growth rate of the market had been in the 70's and early 80's around 15 % - 20 % annually. At the present times growth rates around 8 % - 10 % are still to be reached.

Approximately one third of the market volume is handled in the USA, a second third in Japan, Germany, France, United Kingdom, Italy and Spain (ordered for declining market share). The market for Eastern Europe amounts only to 1 % of the US market share. (Only figures of western companies have been compiled). Only a few companies have a market share in HPLC sales around or over 20 %. These are Waters, Hewlett-Packard and Beckman. Other manufacturers are Biorad, Perkin Elmer, ThermoQuest and Varian with a market share around 5 %. In Germany only Bischoff Analysentechnik (Leonberg), and Dr. H. Knauer Wissenschaftlicher Gerätebau (Berlin), manufacture HPLC instrument components like pumps and detectors.

From these data it can be concluded that the Eastern European market for HPLC is underdeveloped, concerning imported instruments. An instrument developed and distributed in this area should achieve excellent sale rates. It is of great importance for the economy of the Russian Federation to follow here the western lead with the application of HPLC.

Chromatographic columns are one of the most important consumables in HPLC. The usual column lifetime is between 1 and 12 month, depending on the type of samples to be analysed. There are figures available for the US market concerning column consumption. With approximately 25.000 laboratories applying HPLC the purchase of 23 columns per laboratory annually is estimated. This alone amounts to a market in the range of US $ 200 millions. It can be assumed that in average 5 columns are used per instrument annually. Column sales is a market not neglected by instrument manufacturers as the recent acquisitions of companies preparing HPLC columns and stationary phases by US instrument companies show (Phase Separations by Waters, Rockland Technologies (Zorbax) by Hewlett-Packard, Hypersil by ThermoQuest).

History of EcoNova and MiLiChrom

MiLiChrom is a well known trademark in Russia produced since 1979 by Nauchpribor at Oryol according to the specifications of EcoNova. The specifications given to the production plant lead to a continuous improvement of the instrument. Before the macroeconomical change in early 90-ties approximately 800 instruments have been distributed annually because of the availability of governmental money. In total about 6500 instruments were produced by a typical Russian plant with insufficient quality control. (I saw such an instrument in the former GDR not repairable after 2 weeks of use.) The market share of these instruments in Russia is estimated to be around 60 %.

In the last years the production of the MiLiChrom instruments has been transferred to Novosibirsk In cooperation with Dr. H. Knauer Wissenschaftlicher Gerätebau, Berlin, western technology and components became available. This opening of the gate to the west allowed an significant step forward in the improvement of the latest version of the instrument: "MiLiChrom A-02". The purchase of components in Germany, including the painting powder, is still continued.

All mechanical parts are produced by "Berdsk Electromechanical Plant". This is a well equipped fabrication unit, now a joint-stock company, for precision mechanics with a high standard of precision. Fabrication can be done in fully air-conditioned rooms. They are world leader in gyroscope production. The production capacity with this company is presently in the range of 10-15 instruments per month. However, it can easily be extended to larger numbers. There are more than 100 instrumental parts for MiLiChrom A-02 on the shelf in an unfinished status. The problem to finish these products lies in the lack of money in order to purchase the additionally required optical and electronical parts from different companies in the Russian Federation and abroad. Therefore, in average only two instruments per month are compiled. This corresponds to the present sales figures for instruments from EcoNova.

The final assembling of the instrument takes place at EcoNova, after quality control of the components. Each instrument is tested for two weeks to assure the quality. Tests are performed at two temperatures of +7°C and +60°C to assure proper functioning under extreme climatic conditions. Finally the instruments are checked by governmental authorities for quality control, which issue a governmental certificate.
These high standards for quality control lead to an effective performance of the instruments produced. In 1997, a total number of 34 instruments have been produced and sold, only 2 of them were not functioning to total satisfaction.
It should be mentioned that in the production GLP rules are followed. The accreditation of the company by ISO 9000 rules is intended.

Present Sales of Instruments

As mentioned above, in 1997 a total number of 34 instruments has been sold to various governmental and industrial institutions in the Russian Federation and abroad. These instruments are used for typical HPLC applications in pharmaceutical quality control, food control and animal feed control. They are used in criminological, ecological, veterinary, sanitary an epidemiological laboratories, for biotechnological production control, vitamin production and in power stations to measure the content of polychlorinated biphenyls, dioxins and furanes in transformator oils. According to the management there are additional pending orders within Russian Federation on the various markets for HPLC.

The management is well aware of the present and future applications of chromatography. It knows that in HPLC it is not only necessary to just sell an instrument, but also to give practical support to the customers. This is essential in all fields of chromatographic applications not only for advises how to run the instrument. Support in method development is required, ranging from selection of the proper column and eluent combination to the adjustment of instrumental parameters. The well-trained staff knows that in HPLC it is better to sell an application to customers, where the instrument MiLiChrom A-02 has to be used, than to sell just the instrument per se to somebody. The boom of HPLC in the 70's did not start with the selling of instruments but with selling of practical applications to people who were not aware that their analytical problems may be solved by HPLC.

Preparing applications, teaching potential customers in seminars and courses could be one of the important advantages of EcoNova compared to their Western competitors, because the latter have not yet installed a service and training system for their customers in the Russian Federation. The native market should be, at least in the next future, the main field of sales for EcoNova.

MiLiChrom A-02 Instrument

The MiLiChrom A-02 instrument is unique compared to all other available LC instruments. It is the only one designed primarily for the use of microbore columns. To get maximum performance out of this columns the dead volume in the system has been reduced, the pump is able to deliver the required low flow rates, and the detector cell has the necessary low volume for optimal sensitivity. The instrument is very compact and weighs only 17 kg, making on the road analysis easy to achieve.

The instrument is equipped with a double syringe pump with a volume of 2500 µL for each cylinder. The volume displaced per step of the motor is 0.05 µL. The volume flow rate can be adjusted in discrete 1 µL steps, the minimum applicable flow rate being 5 µL/min. For the usually used columns with 2 mm inner diameter flow rates between 100 µL/min and 400 µL/min are optimal. With the two heads of the pump high pressure gradients can be generated. The dead volume between mixer and column is only 100 µL, thus an extremely low dwell volume (delay of gradient) is achieved.

The standard instrument is equipped with an autosampler containing maximum 48 vials with 200 µL vial volume. The injection volume can be varied between 1 µL and 99 µL. The introduction of the sample is achieved by disconnecting the flow from the column, transferring the tube into the vial and reversal of the flow direction. To wash the needle and for waste two additional positions are available.

As detector a double-beam multi-wave length spectrophotometer with an oscillating mirror is installed. This allows to measure simultaneously at 8 different wavelengths. In stop-flow mode the full UV-spectra of the samples can be obtained. The cell volume is very low with 1.26 µL at an optical path length of 1.56 mm.

A column oven is also installed with operating temperatures between 35 and 90°C.

For running the instrument a user-friendly software running under Windows is supplied with the instrument, requiring a standard computer or laptop.

The advantages of this instrument for microbore column technology are in the pulseless eluent flow (no check valves and dampeners are in the instrument) from the syringe type pumps, the compact design, where the volume of the connection pipes and the number of connecting fittings has been minimized. This leads to less influence of extra-column band broadening and reduces leakage problems. The advantage of microbore column technology are obvious: less solvent consumption and hence, less waste production (reduction by a factor of 10 compared to conventional columns) leads to a significant saving in expenditures for solvents. The compact instrument is ready to use immediately after connection to the power supply and the computer. No additional set-up is required. The warm-up time of the detector is extremely short due to the double-beam optics. For servicing the instrument can be send back to the service station by mail, because of the compactness and low weight (17 kg !).

The available software is at a high level of development. It allows to run the instrument automatically, it is controlling pump flow rate, gradient, auto sampler and detection wavelength. Various methods can be stored by the software, including the calibration curves for quantitation. In general, it is a good working horse for microbore HPLC.

A standard prejudice against syringe type pumps is their limited volume of mobile phase available. This, of course, limits the analysis time to the point when the pumps have to be refilled. However, with a total eluent volume of 5000 µL and usually applied flowrates between 100 µL/min and 400 µL/min, where optimal resolution is achieved (maximum number of plates) the runtime of the instrument, before a refill is required, is between 50 min and 12.5 min. Also with standard HPLC instruments with reciprocating pumps for continuous flow, seldom longer analysis times than 20–30 minutes are used. Consequently, the MiLiChrom system has no real disadvantage compared to standard HPLC systems. The trends in HPLC go to shorter analysis times and the question might be risen: why should an HPLC analysis last always 20 to 30 minutes, when reasonable shorter analysis times are achievable.

Performance of MiLiChrom A-02

The performance of the MiLiChrom A-02 instrument has been described by G. I. Baram in J. Chromatography A, 728 (1996) 387 – 399.

In 1995, we compared the performance of a prototype MiLiChrom A-02 instrument (provided by Dr. H.Knauer Wissenschaftlicher Gerätebau, Berlin) with standard HPLC instruments from different suppliers in our laboratories. In an overall system suitability test the precision of injection, flow constancy, and quantitation was determined via repetitive injections of a standard solution. The best standard HPLC system gave a system repeatability with a standard deviation in peak heights of $\pm$ 0.6 %, and in peak area of $\pm$ 1.2 % RSD. The other HPLC systems gave $\pm$ 1.2 % RSD for peak heights, and $\pm$ 1.5 % RSD for peak areas. This compares very well to the figures obtained with the MiLiChrom

A-02 system with ± 0.75 % for peak heights, and ± 1.25 % RSD for peak areas. Consequently the overall precision of MiLIChrom A-02 is comparable to standard HPLC equipment manufactured in the USA and in Western Europe.

The correlation of flow rate with generated pressure of the MiLIChrom A-02 system is excellent with an accuracy of the linear regression of R = 0.9996 at 110 bar $min \cdot mL^{-1}$, and R = 0.99999 at 43 $bar \cdot min \cdot mL^{-1}$. The maximum pressure is achieved in less than 5 sec if no air bubbles are in the system. The volume to be compressed is so small, that in the absence of air bubbles the time delay until the full flow rate is approached can be neglected. The high pressure gradient showed also a good performance (accuracy of the linear regression R = 0.99988). Only the start of the gradient was slightly delayed due to the compressibility of the eluent, when the second pump starts to deliver. But this is not a significant disadvantage.

The accuracy of sample injection was ± 1.2 %. This value is slightly worse compared to good functioning standard Rheodyne injection valves, where a RSD of ± 0.5 % could be achieved. However, when the rotor seal of this system gets old the precision drops and only RSD > 2 % are obtained. The linearity of the MiLiChrom A-02 auto injection system between 2 µL and 30 µL is excellent (R = 0.9999) and the amounts injected are reproducible as stated above.

The detector performance was measured by a log-bottle experiment (exponential dilution flask). The upper limit of the linear range was 1 AU (absorption units), that of the dynamic range was 2.76 AU. The noise corresponded to $5 \cdot 10^{-5}$ AU at a time constant of 0.34 sec. These values correspond to those obtained with standard UV detectors. Because of the short optical path length (1.56 mm compared to the usual 10 mm) the detection limit is higher than with conventional detectors. The wave length accuracy is within the range of ± 2 nm as with other detectors. (Now specifications for MiLiChrom A-02 require accuracy of the wavelength setting ± 0.5 nm and reprodusibility of wavelength setting better that 0.01 nm).The spectral resolution corresponds to diode array detectors with less than 100 diodes for the whole UV range (slit width 5 nm).

Conclusion:

The performance of the MiLiChrom A-02 instrument is comparable to that of HPLC instruments with Western technology and it can be used for standard HPLC applications without any restrictions and disadvantages. The advantages of the instrument are in the special design for the use of microbore columns, where no comparable instrumentation can be found on the market.