

Parallel HPLC Approach for Solving the Purification Bottleneck of Synthesized Oligonucleotides



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1 Introduction

MWG Biotech AG is Europe's leading producer of oligonucleotides. At MWG currently up to 10.000 oligonucleotides per day are being synthesized and purified. Using automated techniques for high throughput purification is very essential. For standard and amino-modified oligonucleotides, the proprietary HPSR® technology is used. For other modified primers, it is necessary to use other purification strategies. HPLC purification is the method of choice to obtain high quality products. Whenever a large number of samples are to be processed, sequential purification procedures using liquid chromatography represent a major bottleneck.

Using Sepmatix - the parallel HPLC system from Sepiatec - eight samples can be processed simultaneously to increase the throughput. The samples are processed in an array of eight columns using a single pump (Fig. 1). The flow of this pump is distributed by the Sepmatix FlowControl, which is a module with flow measurement and control for eight channels. A specially developed autosampler for simultaneous injection of eight samples and a parallel photodiode array detector unit completes the eightfold HPLC-system. The preparative version also has a fraction collector with eight independent channels. A dedicated software controls the chromatographic procedure and allows independent collection of the substances controlled by online peak detection. A data management system provides the information about the collected fractions.



Fig. 1: Pictures of the fraction collector and the HPLC columns

The operators' only two commands to this box are "flow control on" and "off". A typical setup is with four or eight separation columns within the range of 0.25 to 1.5 ml/min, 1.5 to 6 ml/min and 6 to 20 ml/min per branch. Especially in the setup with eight columns the flow control unit is significantly less expensive and space requiring than individual HPLC pumping systems. The positive effect of levelling out retention times when switching on the flow control unit can be seen in Fig. 2.

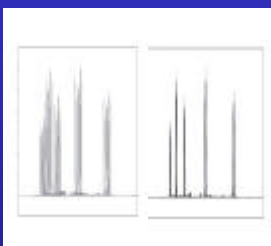


Fig. 2: Parallel HPLC runs without (left) and with (right) flow control

In high-throughput purification (HTP) the 96 well microtiterplate is the most common format in sample handling. Mainly deep well or the shallow well plates with a sample capacity of approx. 1 ml or 50 µl per well are used. Thus the autosampler must be able to deal with these microtiterplates and to inject samples from the plates either in a parallel mode or independently in the so called sample picking mode.

The handling of microtiterplates is solved by a robotic arm which stacks the plates onto each other in three magazines, whereas two are normally filled with new microtiterplates, one is empty to store the already used plates and one is used to present a microtiterplate to another robotic arm with eight independently operating injection needles. This arm can be moved in x and the table with the fixed plate in y direction. By this mechanism it is possible to fill the sample loops of the injection valves in parallel with one row of eight samples or to pick the samples independently by a specific needle from a selected well.

2 Discussion

When looking at HPLC separation speed itself there is some physical borderline which is difficult to overcome with respect to the desired high resolution of the separated chromatographic peaks. In a simplified way for a gradient separation this can be illustrated by Eq. (1) which is applicable for analytical separations but can approximately be used for preparative HPLC as well:

$$k' \sim t_R \cdot F / V_0 \quad (1)$$

The capacity factor k' and therefore the resolution of the chromatographic peaks is proportional to the gradient time t_R and the flow rate F at a given interparticle volume, i.e. the void volume V_0 of the column. For a given resolution the gradient time t_R though can only be shortened when increasing the flow rate F and / or decreasing the void volume V_0 of the column at the same time. Both these measures will increase the backpressure of the column dramatically and therefore shorter columns with lower void volume, i.e. smaller particles in stationary phase have been used for speeding up gradient separations¹².

Another aspect of speeding up HPLC which is not interfering with an existing gradient separation method itself is the parallel operation of several HPLC columns. The initial development started a couple of years ago^{3,4} and was combined with fast gradient separation and mass spectrometry-based detection. In this first system it was difficult to enhance the degree of parallelization and especially the detection system was a bottleneck¹.

Today several so called "parallel" HPLC systems have been developed for the market and also for the individual laboratory approach^{4,5}. The degree of parallelization of this equipment varies a great deal and there is no clear definition existing for the meaning of "parallel HPLC".

From an operators point of view it is quite obvious that parallelization should lead to easier handling, enhancing the sample throughput and reducing the workload. According to this definition a simple hardware concept would be the simultaneous operation of several HPLC systems by using only one computer with a user friendly software interface for instrument control, data acquisition and sample tracking.

The injection needles are operated by a syringe pump. A washing station cleans the needles after each injection. The injection valves with sample loops are directly plugged between the flow control unit and the separation columns. During the injection process these valves are switched simultaneously into the eluent flow and the samples in the filled sample loops are flushed onto the columns. Similar to the flow control unit the detection system is an important matter in parallel HPLC. Photodiode array and mass spectrometric detection provide very characteristic data for almost any compound and therefore are the two preferred detection systems in analytical and preparative HPLC. However the comparably high cost of a photodiode array detector (PDA) or mass spectrometric detector with an HPLC interface is a major drawback when trying to use these systems in parallel HPLC. It is not economically feasible to install several of these detectors in parallel. Thus a solution for parallelizing these detection systems is inevitable. In both the detection systems the analyzer part is the most expensive and should not be duplicated. Due to this a multiplex solution is the most feasible way to go in this development.

A multiplex design for a PDA can be achieved by using optical fiber guides. The light of a deuterium UV light source can be split into individual beams by separating the fiber bundle into several individual channels which are connected to an optical multiplex switch. A rotating disc with a small hole is stepping through the channels to open one channel for a short moment while the others are closed. The optical fiber guides on the outlet of the multiplex switch are connected to the detection cells to provide UV light to the optical inlet of the cell while a channel is active. The transmitted light is collected by another fiber guide at the outlet of the detection cell and is transferred onto the entrance slit of a photodiode array. The correlation of spectra and detection cells is guaranteed by an encoding system which is installed on the stepper motor axis of the optical switch. Rotation frequency of the rotating disc is approx. 1 Hz. This means that per channel every second a complete PDA spectrum is recorded. Compared to modern single channel devices the recording frequency of an multiplex eight channel PDA is approximately ten times lower and thus not sufficient for trace analysis, but its sensitivity completely covers all the applications in high-throughput analysis and purification.

From an economical point of view a parallel HPLC system should be more clearly marked by a distinct reduction in the number of building blocks, but a much higher efficiency compared to known stand-alone devices. This goal can only be reached by a parallel or multiplex use of the individual components such as gradient pump, autosampler, detection system and fraction collector. Such equipment can easily be controlled by using only one personal computer. This concept will lead to a reduced footprint and a lower cost of ownership compared to other customary systems.

When trying to use only one pump for the parallel operation of several HPLC columns it is almost impossible to get the same flow conditions in the individual branches, even if the columns are taken from the same manufacturing lot. This is due to the variation in backpressure of the individual columns which is even more predominant in dynamic use and which can dramatically change during the lifetime of a column.

For this reason the basic building block of a parallel HPLC device is a flow control unit which can be plugged to any high pressure isocratic or gradient pumping system. It provides controlled eluent streams to a multi-channel injection system which is connected to individual separation columns. After separation the eluent streams are passing through the detection cells of a multiplex photodiode array detector (PDA). Finally this detection system triggers the fraction collector to collect the separated samples in microtiterplates.

The active flow control unit⁶ is based on a flow measurement device, flow adjustment valves and a feedback control unit. The flow measurement is performed by metering the pressure drop Δp in a defined restrictor capillary using high precision pressure sensors.

According to Hagen-Poiseuille's law for laminar flow in capillary tubes the measured pressure drop is proportional to the flow in each line. Flow adjustment is achieved by specific valves consisting of an orifice and an electromagnetically driven plate to cover the exit slit of the orifice with an adjustable force.

This active flow control unit is plugged to an isocratic or gradient pumping system. It divides the total pump flow F_{total} into exactly equal split flows F_i through F_j by using a sophisticated feedback control circuit. In a simplified way it can be seen as a black box which is plugged between the pump and the injection system.

3 Summary

The presented parallel HPLC system with its specific characteristics in microtiterplate handling, active flow control, simultaneous sample injection and multiplex PDA detection is designed for industrial use whenever a large number of samples has to be analyzed or purified under identical chromatographic conditions. The target was to develop an HPLC system of the second generation exploiting available technology in automation, detection and control to combine this with the high level of existing experience in HPLC separations accumulated during the last three decades. The schematic outline of the overall system is given in Fig. 3.

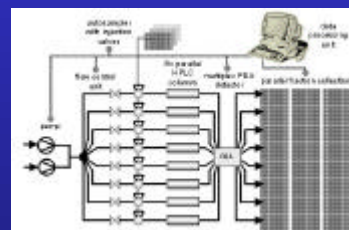


Fig. 3: Schematic overview of the parallel HPLC device

As a result - compared to eight conventional HPLC devices - this system leads to space and cost savings of approximately 80% and 30%, respectively. Due to user-friendly software and small footprint the operation of the instrument, the sample logistics and maintenance are significantly simplified.

Literature

1. Waller, H. N., Young, M. G., Michelczyk, S. J., Reinsauer, G. H., Cooley, R. S., Rahn, P. C., Loyd, D. J., Fiore, D. and Fischlman, S. J., *Molecular Diversity* 3 61-70 (1997).
2. Goetzinger, W. K. and Kyranos, J.N., *American Laboratory* 4 27-37 (1998).
3. Zeng, L., Burton, L., Yang, K., Shushan, B. and Kassel, D. B., *J. Chromatogr. A* 794, 313 (1998).
4. Zeng, L. and Kassel, D. B., *Anal. Chem.* 70, 4380-4388 (1998).
5. Biele, V., de, Hopkins, N., Organ, A., Bateman, R., Giles, K. and Jarvis, S., *Rapid Commun. Mass Spectrom.* 13, 1165-1168 (1999).
6. European Patent Nr. 1133718, US Patent Nr. 6,532,978, L. Mueller-Kuhrt, R. God, H. Gumm, J. Binkele (to SEPIATEC GmbH).