Improving the Quality and the Throughput of Oligonucleotides by Parallel HPLC Purification

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1 Introduction

As currently up to 10.000 oligonucleotides per day are being synthesized and purified at MWG Biotech AG, using automated techniques for high throughput purification of compounds is very essential. For standard and aminomodified oligonucleotides, the proprietary HPSF® technology is used. For other modified primers, it is necessary to use other purification strategies. Reversed phase HPLC purification is the method of choice to obtain high quality products. High purities and yields of "trityl on" purified oligonucleotides are achieved, confirmed by analytical HPLC and MALDI-TOF MS. Even oligonucleotides with the same sequence but different secondary structures could be fractionated.

Whenever a large number of samples are to be processed, sequential purification procedures using liquid chromatography represents 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. The flow of this pump is spread 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 eight-fold HPLC-system. The preparative version 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.

Compared to the previous HPLC devices used at MWG Biotech AG the Sepmatix system provides a much better solution than before. One of these devices replaces eight conventional preparative HPLC devices. This yields to less required laboratory space, less expenses for laboratory space and less costs for laboratory personnel. The purchasing costs are also significantly lower than for the corresponding number of conventional HPLCs. Due to this new technology, separations could also be improved, leading to higher product quality and throughput.

2 Experimental

A comparison of three HPLC systems from different suppliers that were available in the purification laboratory of MWG Biotech AG was performed. A mixture of methyl, ethyl, propyl and butyl parabene was used to determine the resolution of the various HPLC systems. The dead volume was determined by injection of uracil. In order to compare the separation performance several oligonucleotides that were labeled with fluorescence markers were used. For the comparison the following HPLC systems were available in our labs:

1) Sepiatec Sepmatix 8x FlowControl with 8x channel DAD and 8x fraction collector and a Knauer Well Chrom Preparative Pump K-1800 with a preparative dynamic mixing chamber from Knauer (high pressure gradient system)

2) Biocad Sprint Integrated System from Applied Biosystems with a variable wave length UV detector and a 231 XL sampling injector/fraction collector from Gilson (low pressure gradient system)

3) Kontron System 525 form Bio-Tek Kontron with a HPLC 535 variable wave length UV detector and a 233 XL sampling injector/fraction collector from Gilson (low pressure gradient system)

The injection volume was 500 µl each. As stationary phase we used Grom-Sil 120 ODS4 60x8 mm with a particle size of 5 µm. As mobile phase A 0.05 M aqueous triethyl ammonium acetate with 10% methanol and as mobile phase B methanol was used.

For the comparison the following gradient was applied (flow rate 5 ml/min):

Time [min]	Flow [ml/min]	%B
0,0	5,0	10
17,0	5,0	50
17,5	5,0	90
18,0	5,0	90
18,5	5,0	10
20,0	5,0	10

The collected fractions were investigated using analytical HPLC and MALDI-TOF analysis. For the analytical HPLC a Beckman-Coulter System Gold device with an 168 DAD, a 126 pump and a LC-508 autosampler was used. The injection volume was 5 µl using a standard gradient with mobile phase A containing 0.05 M aqueous TEAA with 1% acetonitrile and mobile phase B using acetonitrile. Flow rate: 1.2 ml/min. As stationary phase Sunfire from Waters (3x50 mm, 3.5 µm) was used.

MALDI-TOF analysis was performed with a Biflex III device from Bruker: The mass spectra were generated using the linear mode with a matrix containing diammoniumhydrogene citrate / 3-hydroxy picolinic acid.

3 Results and Discussions

In order to compare the properties of the three HPLC devices, in the first step the dead volumes were determined by injecting uracil:

3.15 ml for the Sepmatix device from Sepiatec
4.38 ml for the Biocad Sprint System from Applied Biosystems
3.90 ml for the Kontron System from Bio-Tek

The dead volume of the Sepmatix system is significantly lower than at the other two HPLC devices.

In the second step a mixture of parabens was injected and afterwards the resolutions between methyl parabene (A) and ethyl parabene (B) were calculated via the following formula:

$$R_s = 2(t_R(A) - t_R(B))/(w_B(A)+w_B(B))$$

where t_R = retention time and w_B = peak width at baseline

The resolutions were determined to be 1.8 for the Kontron device, 4.6 for the Biocad device and 11.3 for the Sepiatec device. The Sepmatix HPLC showed the best resolution of the three devices.

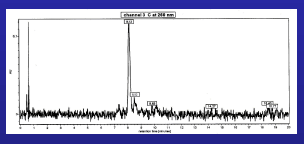
In the next step several modified and unmodified oligonucleotides with a length between 20 and 40 nucleobases were used to determine the practical separation capacity of the three HPLC devices. A 0.2 micromolar synthesis was performed for each oligonucleotide using phosphoramidite chemistry[1,2]. After the synthesis the oligonucleotides were cleaved from the controlled porous glass beads (CPG) with aqueous ammonia and dried in vacuum centrifuges. Before purification the residues were each dissolved in 1500 microliters of water. These solutions were each divided into three parts with the same volume. An overview of the purity of the main fractions determined by analytical HPLC is shown in Fig. 1.

The superiority of the Sepmatix device could be demonstrated by comparing the analytical results of the product fractions: The products separated with the HPLC from Sepiatec had an average purity of 92.0% whereas the products purified by the other instruments had a purity of 86.9% and 87.0%, respectively.

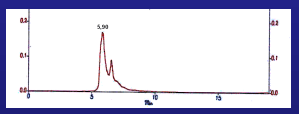
Oligonucleotide		HPLC purity		
Sample Name	Sequence	Sepiatec (Area%)	Biocad (Area%)	Kontron (Area%)
84251	5'-FAMTCA TTC CTT CTA CCC TTT CA-3'	94.3	91.2	92.0
84252	5'-FAMTGC AAT TTC TGA ATT CTT CGC TTA GAT TCA -3'	failed	failed	failed
84253	5'-FAMCAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A -3'	88.2	86.1	85.9
84254	5'-FAMMT-TCA TTC CTT CTA CCC TTT CA-3'	69.4	74.0	79.2
84255	5'-(CO)MMT-TCG AAT TTC TGA ATT CTT CGC TTA GAT TCA-3	80.3	63.0	78.1
84256	5'-(CO)MMT-CAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A-3	87.1	82.3	86.6
84257	5'-Bio-TCA TTC CTT CTA CCC TTT CA-3'	98.7	91.0	no fraction
84258	5'-Bio-TGC AAT TTC TGA ATT CTT CGC TTA GAT TCA-3'	97.0	96.0	96.5
84259	5'-Bio-CAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A-3'	93.4	no fraction	no fraction
84260	5'-(CO)-TCA TTC CTT CTA CCC TTT CA-3'	100.0	96.0	95.8
84261	5'-(CO)-TGC AAT TTC TGA ATT CTT CGC TTA GAT TCA-3'	98.1	99.7	98.0
84262	5'-(CO)-CAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A-3'	99.0	99.7	99.4
84263	5'-(CO)-TCA TTC CTT CTA CCC TTT CA-3'	94.5	87.6	89.9
84264	5'-(CO)-TGC AAT TTC TGA ATT CTT CGC TTA GAT TCA-3'	99.9	99.0	99.9
84265	5'-(CO)-CAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A-3'	99.7	99.1	98.8
84266	5'-TCA TTC CTT CTA CCC TTT CA-3'	82.1	76.0	71.9
84267	5'-TGC AAT TTC TGA ATT CTT CGC TTA GAT TCA-3'	84.4	67.9	50.7
84268	5'-CAT TCG ATC CTT TAG TTC CAT TCT GTT AGT CAA TTC ATC A-3	97.1	81.3	81.7
	Average HPLC purity (Area%)	**92.0**	**86.9**	**87.0**

Fig.1: Purity of the product fractions determined by HPLC

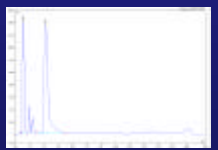
Examples of the separation performance of the three devices are shown in Fig. 2. The different retention times of the same product (8.1 min, 5.9 min and 3.2 min) can be explained by the different dead volumes and the eightfold splitting mechanism of the Sepmatix device.



Sepiatec product fraction: 8.01 – 8.27 min



Biocad product fraction: 5.52 – 6.29 min



Kontron product fraction: 2.25 – 5.25 min

Fig.2: Chromatograms of sample 8425-3

With the new HPLC device from Sepiatec it is even possible to separate oligonucleotides with different secondary structures by using a standard gradient of 20 minutes. Before using this technology, secondary structures could never be separated and fractionated at MWG Biotech AG.
Secondary structures are normally disturbing purification as they behave chromatographically very similar and therefore are leading to broad peaks. To overcome this problem one could use ion exchange chromatography[3,4] or denaturing HPLC[5] but in both cases the corresponding buffer ions disturb mass spectrometric analysis and also some biological applications. Using ion pair reversed phase chromatography[6] with triethylammonium acetate as buffer system, MALDI-TOF analysis without disturbing alkali ions is possible and because of the volatility of the TEAA buffer only traces remain in the product after the drying process.
In order to obtain oligonucleotides with high purity, it is necessary to separate the oligonucleotides from failure sequences, protecting group derivatives and salts. To obtain a better selectivity the separations are performed with the dimethoxytrityl protecting group „on".

Fig.3: HPLC Chromatogram showing secondary structures

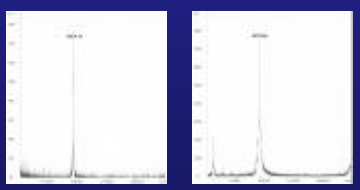


Fig.4: MALDI-TOF Analysis of fraction 1 and 2

A chromatogram of 5'-ATT CAG ATT CAG TTC ATT CAG TTC TGA CTC AGT TCA GCT TCA GTG TCT TAC-3' is shown in Figure 3. There are several peaks with a retention time between 10 and 13 minutes. The MALDI-TOF MS revealed that these peaks all have the same mass. The MALDI-TOF MS of fraction 1 and 2 are shown in Fig. 4.
As the masses of these peaks are identical they are supposed to be secondary structures of the sequence mentioned above. This was supported by analytical HPLC with a column oven temperature of 40°C: Due to the destruction of the secondary structures at this temperature only one peak could be identified for each sample, having the same retention times and UV spectra. For high throughput separations of oligonucleotides where only one fraction is desired, either a column oven can be used or the gradient could be optimized.

4 Summary

Due to the low dead volume of the system, the separations could be improved, using even shorter gradients than before. Oligonucleotides with the same sequence but different secondary structures could be separated and fractionated. Using this new technology the costs could be decreased and more laboratory space is available now. The throughput could be enhanced dramatically as the HPLC methods could be shortened for many practical applications. Also by using microtiter plates instead of vials the throughput could be increased. 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. A basic version of the autosampler can handle up to 10 deep well microtiterplates. This is equivalent to injection of 960 samples, respectively. Due to parallel operation of the instrument these samples can be processed in 120 HPLC runs in an unattended mode which is typically the sample load for one day. Sample collection is triggered by the multiplex PDA detector and the fraction collector can collect up to 9.216 fractions which corresponds to 8 x 12 = 96 deep well microtiterplates.

Literature

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