Ensuring precise isolation of macromolecules by hyphenating UHPLC separation power with exact fractionation for analytical and semi-preparative LC purification

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Abstract

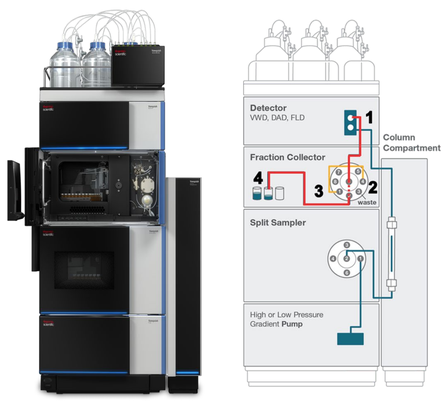
The ultimate goal of fractionation is to isolate compounds of interest or impurities, all while conserving fraction integrity. Ensuring that the resolution achieved by the column is preserved throughout the process is essential for successful fractionation. This work details a complete purification workflow with re-analysis of the purified fractions to illustrate the fraction collection resolving power, new fraction collector features, and the various parameter settings. Performance was evaluated experimentally using a mixture of oligonucleotides (ON) with incremental lengths (12mer to 40mer). Initially discussed is the delay volume (DV), which is defined by the volume between the detector used to trigger the fraction collector device and the tip of the dispensing needle and is a critical factor affecting collection efficiency. Next, fractions containing the differing oligomer lengths were collected using either time-based or peak-based fractionation. The advantages and disadvantages of both approaches are discussed in detail. The recovery as the ratio between injected amount versus the resulting amount in the fraction of the optimized system was investigated. The final experiment demonstrates the new flush function feature and its impact on the final fraction purity. The results are correlated with the versatile options for fractionation parameters in modern instrumentation. Due to the low dispersion characteristics of the instrument, pure fractions as well as neighboring impurities could be readily isolated for further analysis.

Introduction

The technique of fraction collection (FC) is continuously evolving and becoming more refined as it must keep pace with the ever-growing separation power of the HPLC and UHPLC instrumentation. FC is the process of capturing the eluting fluid from a liquid chromatographic separation into vessels, with high recovery and low carry-over. The presence and concentration of desired analytes in the fractionation vessels is confirmed using re-injection analysis applying different detection methods, ranging from UV detectors to charged aerosol detectors and mass spectrometers. These detectors, in combination with the chromatographic data system (CDS), can be used to determine the fraction window, based either on detector signal or retention time.

Crucial for the accurate fractionation of the analytes of interest is understanding the importance of the DV and how it can be optimized. Several factors contribute to the DV (see 1-4 Figure 1). The DV can be calculated manually from the volumes of the installed fluidic parts or, more conveniently, determined precisely using the automated delay volume determination (DVD) (see below).

Figure 1. Delay volume in the LC instrument. The delay capillary (1), internal volume of the fraction collection valve (2), needle capillary (3), and fraction collection needle (4) combine to create the delay volume resulting from the fluidic connection.



A key component of the FC system is the FC valve as its internal volume and switching time both impact the DV. It also plays two major roles: First, it is responsible for directing the flow to waste or to the collection vessel. Second, it includes flush, wash and rinse functions to actively improve resolution and minimize carry over, as discussed in greater detail below.

This poster focuses on the Thermo Scientific™ Vanquish™ Fraction Collector (VFC) system[1] operating at analytical scale fraction collection (0.05–10 mL/min) and discusses factors to consider for optimal translation of chromatographic resolution to fraction purity. Topics include:

- The use of time-based or peak-based fractionation (illustrated using ON chemistry).
- The measurement of recovery.
- The effects of flush/wash on performance (illustrated using RP5 chemistry).
- The convenience of automated DVD.

Materials and methods

To demonstrate the required settings for a typical fractionation, an ON mixture was first purified by reversed-phase chromatography and then fractionated using different fractionation parameters such as a time-based trigger and a peak-based trigger. Next, a 5-substance test mixture (RP5) was fractionated with and without flush. All fractions were then reanalyzed using the same chromatographic conditions to evaluate fractionation performance.

Sample handling

Vial and closures: Polypropylene, 0.3 mL vials
(Thermo Scientific™, P/N 055428)

Chemicals

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ Acetonitrile, LC-MS grade (P/N A955-4)
- Applied Biosystems™ Triethylamine acetate (TEAA) 2.0 M (P/N 400613)
- Fisher Scientific™ Optima™ Acetic acid, LC-MS grade (P/N A11350)
- Integrated DNA Technologies, Inc. Oligonucleotide standard. 8-combo DNA containing: 12mer (GACT)3; 16mer (GACT)4; 20mer (GACT)5; 24mer (GACT)6; 28mer (GACT)7; 32mer (GACT)8; 36mer (GACT)9; and 40mer (GACT)10
- RP5 Test mixture: Uracil (40µg/mL), p-nitroaniline (200µg/mL), methyl benzoate (1000µg/mL), phenetole (3500µg/mL), o-xylene (5500 µg/mL)

Sample preparation

- Stock solutions were prepared by dissolving the ONs to 200 µM with deionized water (DI) and stored at 5 °C. Equivalent amounts of each ON were mixed to prepare 25 µM for each ON. The sample was further diluted five-fold with DI water to make 5 µM mixed standard working solution.
- Stock solution of RP5 mixture was prepared by diluting the standards in 1:1 water:acetonitrile.

Instrumentation

Thermo Scientific™ Vanquish ™ Flex Analytical Purification System consisting of:

- Thermo Scientific™ Vanquish™ Quaternary Pump F (VF-P20-A)
- Thermo Scientific™ Vanquish™ Split Sampler FT (VF-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (VH-C10-A-02)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F (VF-D40-A) with Semi-Micro Flow cell, 2.5 µL (7 mm), SST (P/N 6077.0360)
- Thermo Scientific™ Integral Fraction Collector FT (VF-F20-A-01) with delay capillary depending on fractionation trigger: – for time-based fractionation (0.1 mm x 350 mm, P/N 63042.23040) – for peak-based fractionation (0.25 mm x 1500 mm, P/N 6706.1110) Software
- Thermo Scientific™ Chromeleon™ CDS software, Feature Release 7.3.1

Method parameters

Table 1. LC method for separation ON mixture and re-analysis of fractions

Parameter	Value		
Column	Thermo Scientific™ DNAPac RP, 2.1 × 50 mm, 4µm (P/N 088924)		
Eluent	A – 100mM TEAA, pH 7.0 B – 100mM TEAA, pH 7.0 / acetonitrile (75:25 v/v)		
Flow rate	0.6 mL/min		
Gradient	Time (min)	%A	%B
	-3.0	85	15
	0	85	15
	4	61	39
	4.1	10	90
	6.0	10	90
Autosampler temp.	5°C		
Column temp.	60°C forced air 60°C active preheater		
Injection volume	10 µL		
Injection wash solvent	10% methanol in water		
Detection	260 nm, 20 Hz, 0.2 s response time, 4 nm bandwidth, Slit width Wide		

Table 2A. Fraction collector instrument methods settings for time-based fractionation

Parameter	Value
Wash solvent	10% Methanol in Water
Flush solvent	Eluent A – 100mM TEAA, pH 7.0
Fraction collector compartment Temperature	5°C
Collect by Time - collection period	3.0 s
Collection Time Frame	3.0 – 5.2 min
Flush	Inactive
Max. tube volume	0.125 mL
Minimum time for tube change	2 s
Delay volume	7.7 µL - determined by automated Delay Volume Determination with delay capillary 0.10 x 350 mm (PN 6042.2340)
Collection Path Mode	Horizontal
Collection Valve mode	Continue
Needle Positioning Mode	AboveVial
Use Safe Needle Height	Active
Wash Mode	Both
Wash Speed	100.0 µL/s
Wash Time	3.0 s
Rinse Mode	Both
Puncture Offset	0.0 mm

Table 2B. Fraction collector instrument methods settings for peak-based fractionation. Other settings the same as in Table 2A

Parameter	Value	
Flush	Active	
Delay Volume	35.5 µL determined by automated Delay Volume Determination with delay capillary 0.25 x 1500 mm (PN 6706.1110)	
Peak Detection Options	Show advanced settings	
	Peak Start Threshold	0.00 mAU
	Peak Start Slope	6.000 mAU/s
	Peak Start True Time	0.20 s
	Peak End Threshold	2.00 mAU
	Peak End Slop	-5.000 mAU/s
	Peak End True Time	0.2 s
	Derivative Step	0.02 s

Table 3A. Fraction collector instrument methods settings for flush/no flush of RP5 and re-analysis of fractions

Parameter	Value
Column	Thermo Scientific™ Hypersil GOLD™, 2.1 × 50 mm, 1.9µm (P/N 25002-052130)
Eluent	A – Water B – Acetonitrile
Flow rate	0.5 mL/min
Gradient	50% B Isocratic
Autosampler temp.	5°C
Column temp.	30°C forced air 30°C active preheater
Injection volume	10 µL
Injection wash solvent	10% methanol in water
Detection	254 nm, 20 Hz, 0.2 s response time, 4 nm bandwidth, Slit width Wide

Table 3B. Fraction collector instrument methods settings for flush/no flush fractionation

Parameter	Value	
Flush	1. De-active 2. Active	
Delay Volume	35.5 µL determined by automated Delay Volume Determination with delay capillary 0.25 x 1500 mm (PN 6706.1110)	
Peak Detection Options	Show advanced settings	
	Peak Start Threshold	200 mAU
	Peak Start Slope	off
	Peak Start True Time	0.00 s
	Peak End Threshold	200 mAU
	Peak End Slop	off
	Peak End True Time	0.0 s
	Derivative Step	0.02 s

Results and discussion

Time-based fractionation

The chromatogram of the ON separation and the visualization of time-based fractionation obtained using the settings described in Tables 1 and 2A, is shown in Figure 2A. Following their initial chromatographic separation, the individual fractions were then re-analyzed (Figure 2B).

Figure 2A. Time-based fractionation of ON mixture and re-analysis of collected fractions

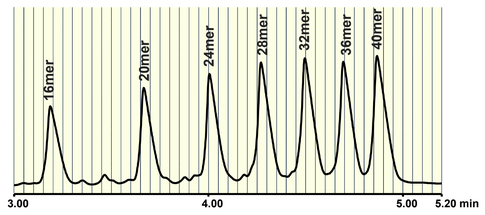
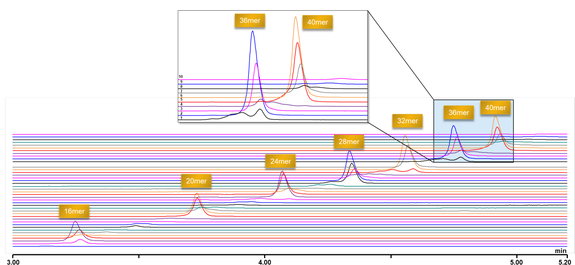


Figure 2B. Re-analysis of collected time-based fractions



When comparing the individual chromatograms of the reanalyzed fractions with the source chromatogram, one can draw a few conclusions regarding the efficacy of the fraction collector.

- Impurities can be isolated for further analysis due to the low dispersion and efficient fractionation resolution in combination with the optimized fluidics of the delay capillary.
- Fraction #2 and #3 for the 36mer and fraction #5 and #6 for the 40mer are the best choice, if high yield fractions at good purity are needed.
- Pure fractions of target analytes can be pooled and used for further workflow applications.

Peak-based fractionation

The same ON mixture was fractionated in a peak-based fashion as described in Tables 1 and 2B. The resulting fractionation of the source chromatogram and subsequent re-analysis is shown in Figure 3. The re-analysis shows efficient purification of the individual peaks based on ON length. The high resolution of the ON mixture and efficient fractionation resulted in collection and detection of a single ON peak only. This is achieved by the properties of the VFC such as low dispersion from the precisely determined low delay volume, as well as by the flush, rinse, and wash functions, which eliminated carry-over from previous fractions and has a high impact on purity and recovery. Selecting the best mode of fraction collection is detailed in Table 4.

Figure 3. Peak-based fractionation of ON mixture (upper chromatogram) and re-analysis (lower chromatograms) of collected fractions

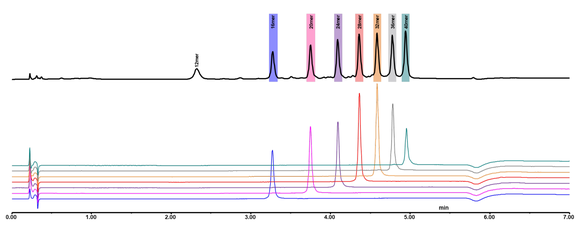


Table 4. Recommendations for when to use peak-based fractionation and when to use time-base fractionation

Attributes	Time-based	Peak-based
Known stable retention times	●	
Known stable peak shape	●	
Unknown retention times		●
Peak shoulders		●
High resolution		●
Low resolution	●	
Very complex samples	●	

Recovery

The recovery is the ratio between injected amount versus the resulting amount in the fraction. For example, when an absolutely pure sample is injected with a known concentration and is fractionated into one single vial, it is expected that the resulting fraction in the vial contains all of the amount injected from the sample. While travelling through the fluidic pathway of the HPLC, inherently the injected sample volume is diluted through the dispersion effect. Therefore, one needs to account for this dilution effect in terms of the resulting peak volume in the chromatogram calculated by the peak width and flow rate. The simplest method to calculate the recovery is to compare the peak area of the fractionation chromatogram to the area of the fraction's reanalysis.

Briefly, one compares the source chromatogram peak area with the resulting re-analysis peak area in combination with the dilution effect. When using the flush function, there is an additional 22 µL dilution that needs to be addressed. Since one normally does not re-inject all of the resulting fraction, the volume of the fractionated sample re-injected versus the source injection volume needs to be considered. All these factors result in the equation shown below using the 16mer of the oligonucleotide purification as an example

$$V0 = F^{*}\Delta t = 0.6^{*}3.3135 - 3.1468 = 100.0\ \mu L$$

$$\%\ \text{Recovery} = \frac{\frac{V_0 + Vf}{V_2} \times A_2}{A_1} = \frac{\frac{100 + 22}{10} \times 1.4743}{18.5923} \times 100\ (\%) = \mathbf{96.74\%}$$

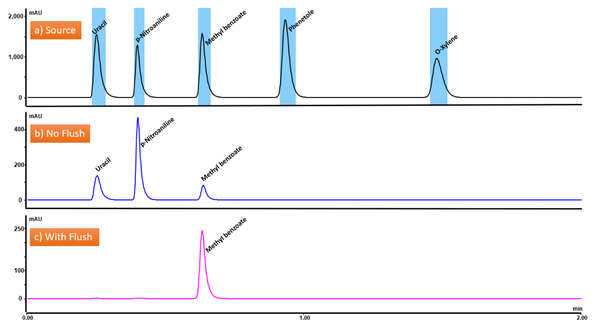
Table 4. Explanation of parameters used in % recovery calculation

Variable	Description	Value
F	Flow rate (mL/min.)	0.6
Δ_t	Collection end time (min.) – Collection start time (min.)	3.3135 - 3.1468
V_0	Collection Volume (µL)	100.0 (see below)
V_1	Injection volume for the source fractionation (µL)	20.0
V_2	Injection volume for the re-analysis of the fraction (µL)	10.0
Vf	Flush volume (µL)	22
A_1	Peak area for the source fractionation (mAU*min.)	18.5923
A_2	Peak area for the re-analysis of the fraction (mAU*min.)	1.4743

Flush, wash, and rinse functions

The new VFC has an additional features adding to the fractionation resolving power, reduction of carry-over, and the increase in recovery. The flush function works to expel the volume of the needle capillary and needle using a specified flush solvent into the collection vial to elute any residual substance still present in the needle capillary and needle. The rinse and wash functions can be executed before and/or after the run or not at all. These functions wash the needle externally and rinse the needle internally with optionally separate selectable solvent sources. An experiment below in Figure 4 was run using the RP5 mixture, the instrument method parameters shown in Table 3A, and the fraction collection settings in Table 3B.

Figure 4. a) The fraction collection of the source chromatogram, b) the re-analysis of the peak 3 without flush, b) the re-analysis of the peak 3 with flush



Measurement of the delay volume

The DV can be calculated manually from the volumes of the installed fluidic parts or, more conveniently, determined precisely using the automated DVD. Here, the time taken for an air bubble to be detected first by an optical detector and then by an integrated air bubble sensor in the fraction collector module, is used to accurately calculate the total delay volume. This simple and effective approach does not need any additional reagent like a dye and works easily with mobile phases available in every laboratory. This is much better and more precise than just estimating the DV based on theoretical calculations.

Conclusions

- High performance fraction collectors enable fractionation with such high resolution that the resulting elution profile exactly matches the corresponding peaks of the sample chromatogram.
- For enhanced performance the DV must be minimized by using optimized fluidics, and a fast switching, low-volume fraction valve.
- Users can choose between peak-based or time-based fractionation depending upon their analytical needs.
- Flush and wash options improve fraction purity and significantly decrease carry over.
- The automated DVD function is both convenient and accurate.

References

1. TN 72940 Principles of fraction collection using the Vanquish HPLC and UHPLC systems.

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