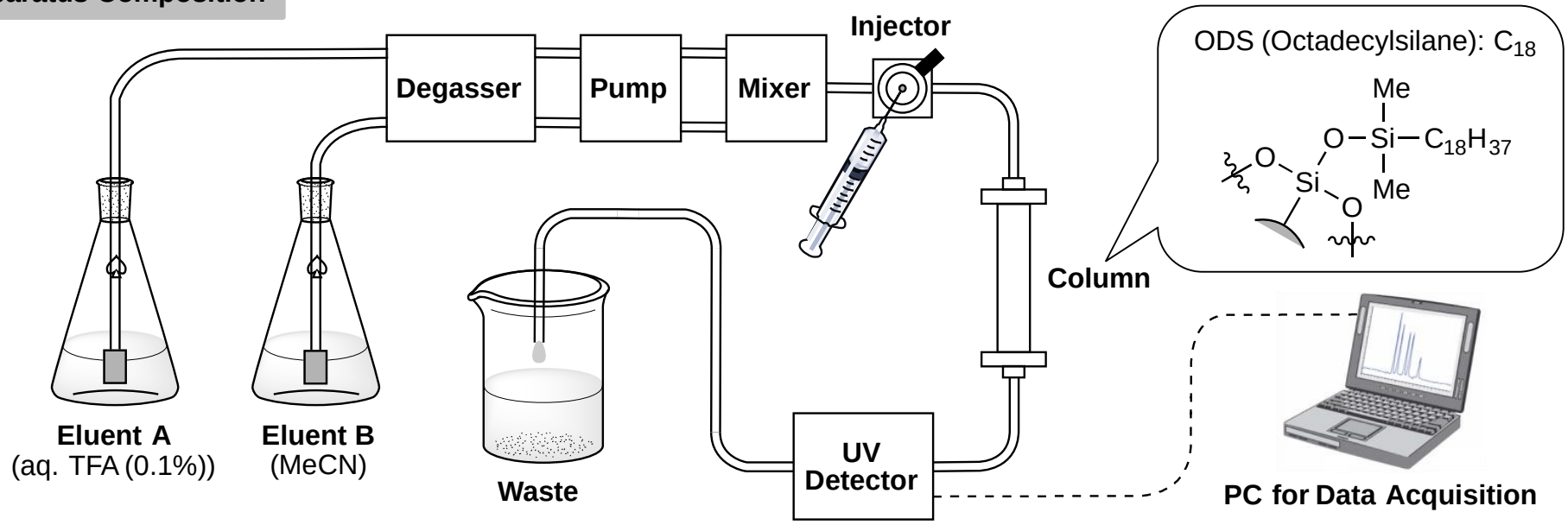
Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

2017/02/04
K. Shinoda

Apparatus Composition

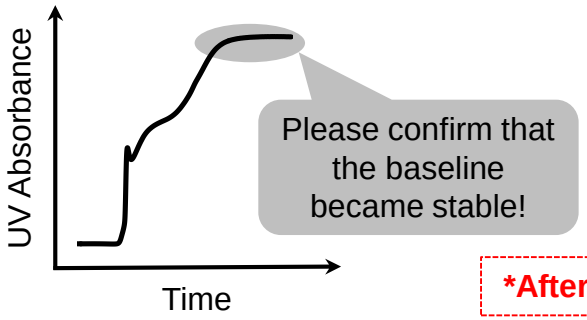


✓ two types of HPLC: "**analytical HPLC** (to analyze your sample)" and "**preparative HPLC** (to purify your sample)"

How to Use

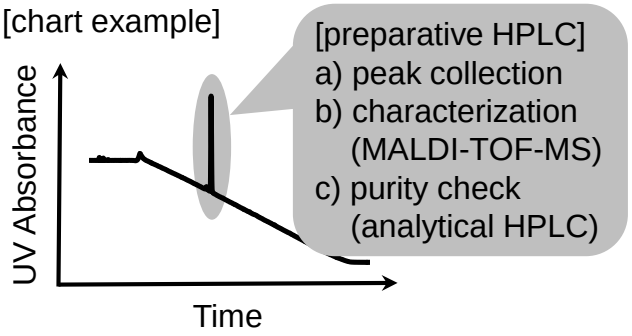
1) **wash** by eluent B → **initialize** by eluent A
✓ 10 − 15 min each

[chart example (initializing)]



2) **inject your sample*** and **start the run**

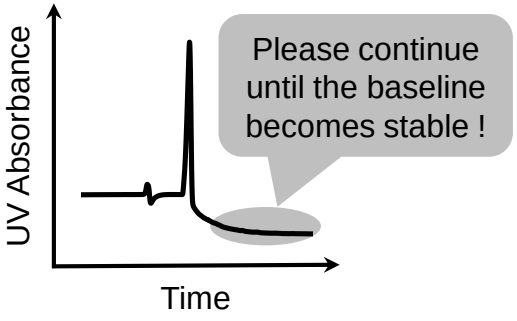
[chart example]



***After the injection, please wash the injector by MeOH.**

3) **wash** by eluent B again
✓ 10 − 15 min

[chart example]



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Difference between Analytical HPLC and Preparative HPLC

✓ **flow rate**
- ○ analytical (150 × 4.6 mm): ca. 1 mL/min*
- ○ semi-prep. (250 × 10 mm): ca. 3.0 mL/min*
- ○ prep. (250 × 20 mm): ca. 10.0 mL/min*

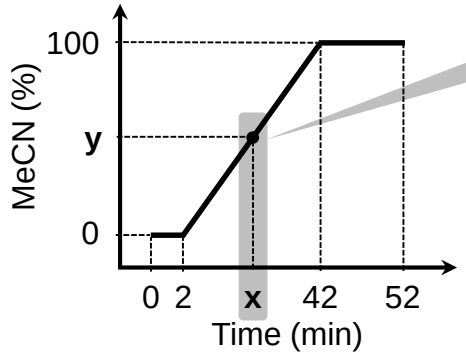
✓ **maximum loading amount**
- ○ analytical: ca. 0.5 mg
- ○ semi-prep.: ca. 10 mg
- ○ prep.: ca. 50 mg

✓ **maximum loading volume**
- ○ analytical: ca. 100 µL
- ○ semi-prep.: ca. 1 mL
- ○ prep.: ca. 5 mL

*In all cases, the pressure should be less than 10 MPa.

✓ **method**

[example of analytical method]

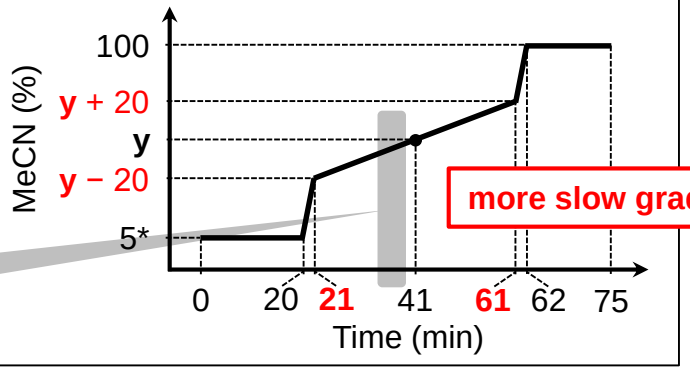


When the target compound is eluted at **x** min, …

y [%] = (**x** − 2 [min]) × 2.5 [%/min]

the target compound will be eluted at ca. 40 min.

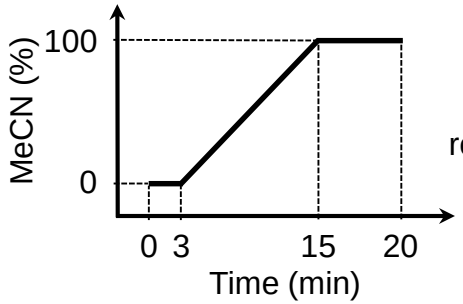
[example of preparative method]



*In preparative scale, 0% of MeCN is not recommended because the compound may be precipitated.

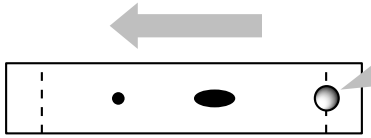
Tips for Analytical HPLC

✓ another example of analytical method with shorter analyzing time



In order to let more people to use analytical HPLC, it is recommended to use the method with short analyzing time.

✓ It is recommended to analyze your sample by reverse-phase TLC (ex. ODS) before injection.



Such compound should be removed before injection as it should damage the ODS column.

ODS short column will be effective to remove it.

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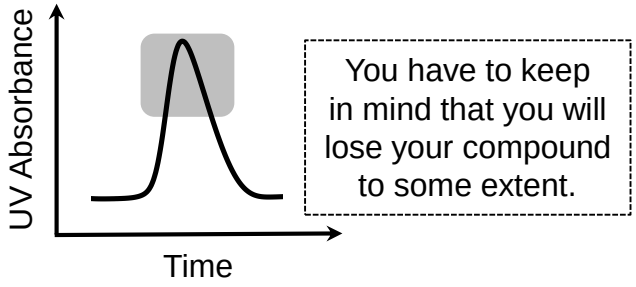
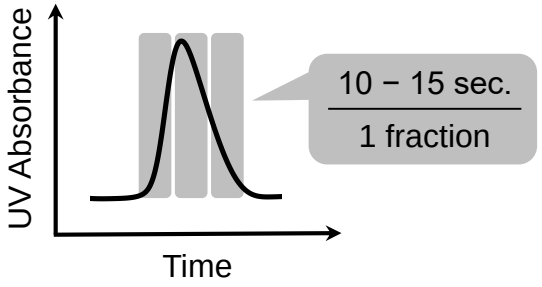
Several Strategies of Purification

✓ If you want to obtain the single peak, it is recommended to collect the upper half of the peak.



✓ If you don't want to lose your compound, it is recommended to collect the whole peak.



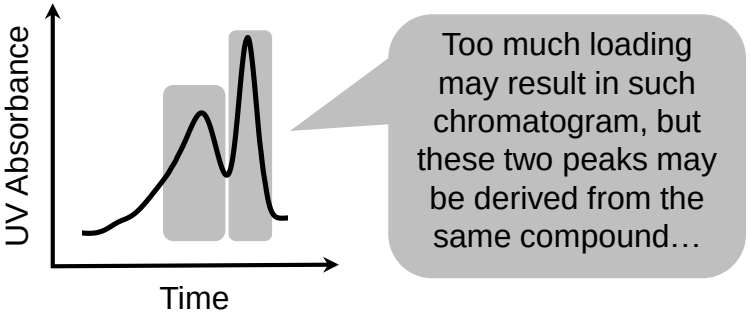
✓ If you couldn't purify your compound in one time purification, it will be effective to conduct the second purification.

[key point in the second purification]

- ○ changing the type of the column (e.g. YMC-Triart → ODS-Pack AM)
- ○ using slower gradient method

Tips for Preparative HPLC

✓ **Please don't load too much.**



✓ **Before the purification, please filter your sample solution to remove particulates, which may damage the column.**

✓ Generally speaking, the amount of your compound will be decreased to less than 50% after the purification.

✓ As in the case of analytical HPLC, it is better to use the method with shorter time.

✓ **It is recommended to dissolve your compound in solvents which are used as eluents** (e.g. aq. TFA (0.1%) or MeCN).

○ Polar solvents with strong dissolving power (e.g. DMSO, DMF) may inhibit the capture of your compound to the column.

✓ We have two ways to collect peaks (manual and automatic (fraction collector)). Please choose whatever you'd like.

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Tips for Eluent

✓ other candidates for eluent A: **aq. HCO_2H (0.1%)**

✓ other candidates for eluent B: **MeOH** (stronger eluent than MeCN)

✓ It is recommended to degas both eluents just after their preparation (air bubble may be generated in flow paths).

Tips for Injector

✓ **The injection volume of your sample should be less than the maximum of the loop volume; 80% will be the limit.**

✓ When you want to inject much more volume than the limit above, you may follow the following procedure.
1) inject a part of your sample; 2) wait 10 – 20 min (keep flowing with the initialization condition); 3) inject again;
4) repeat the sequence above (1 – 3) until the whole of your sample is injected

Tips for Column

✓ other candidates for C_{18} column: C_4, C_8, etc.

✓ You may use the column oven to heat the column (40 ºC, 60 ºC, etc.).

✓ **When a column is clogged, please follow the following procedure.**
1) Please connect the column in the opposite direction and wash with strong eluent such as *i*-PrOH.
2) Wash should be continued for O/N or 1 day with a 10% flow rate of the usual flow rate.

Tips for UV detector

✓ general UV wavelength for peptides: 230 nm

✓ general UV wavelength for proteins: 280 nm

✓ other candidates for UV wavelength:
- 214 nm (aromatic residues-poor peptides)
- 254 nm (aromatic residues-rich peptides)
- 301 nm (indole ring (Trp residue))

✓ We also have fluorescence detector and refractive index detector.

✓ When air bubble is contaminated, the chart will become as follows.

If you see such chart, please purge the system.

During the purge, please take the column off so as not to damage the column.