크로마토그래피의 원리와 분석법

HPLC의 분석법-3

Soonchunhyang University

Department of Chemical Engineering

Prof. Jungkyun Im

순천향대학교

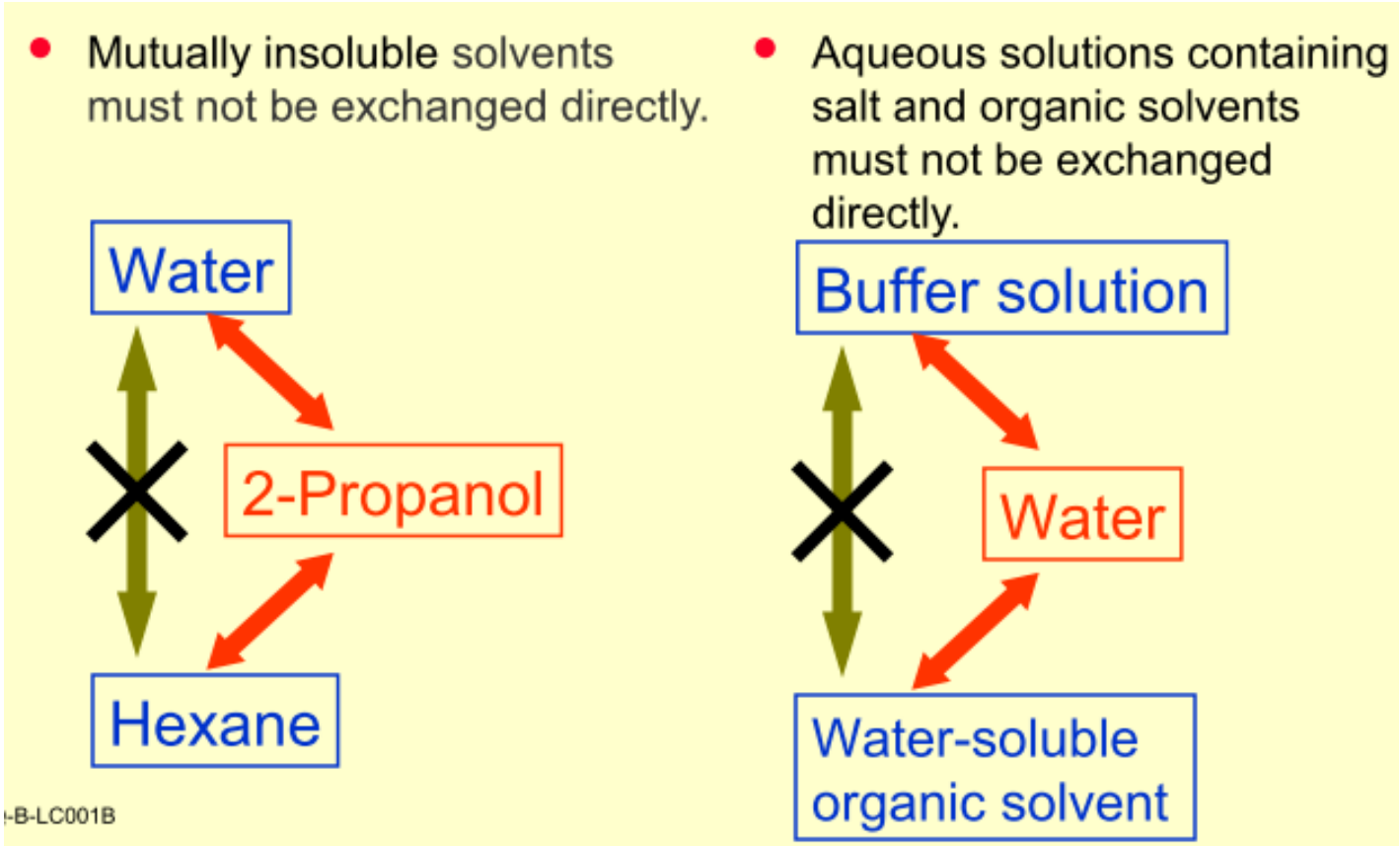
SCH 순천향대

순천향대

나노화학공학과

임 정 균 교수

Changing the mobile phase



Buffer 용액의 경우는 바로 organic solvent로 교체해서는 안됨

Precipitation의 방지가 필요함

How to prepare a buffered mobile phase?

Buffered mobile phases can basically be prepared in two ways:

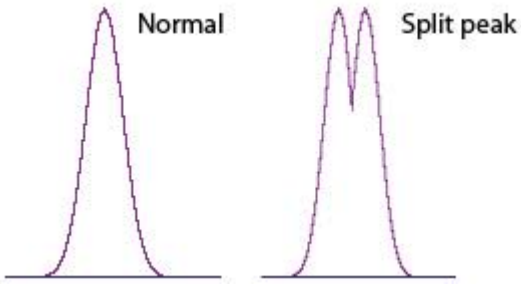
1. preparing the buffer, including pH adjustment prior to mixing with organic modifier.
2. mixing buffer and organic modifier prior to pH adjustment.

While the pH is a well defined parameter in aqueous systems, it is not as straight forward to define or measure proton concentration in partially organic solvent systems.

Attention should be given to the following points:

- buffers are effective within +/- 1 pH unit from their pKa $\Rightarrow$ choose correct buffer system
- consider the risk of precipitation if high buffer concentrations are mixed with organic solvents
- always filter buffers through 0.4 mm filter prior to use
- be aware of the risk for bacterial or fungal growth in pure aqueous buffers
- the pH generally shifts upon the addition of an organic modifier (pH increases for inorganic buffers (e.g. phosphates) and decreases for organic buffers (e.g. acetates)

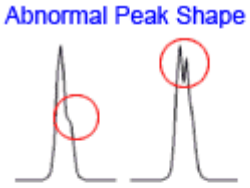
Why does the chromatogram show split peaks?



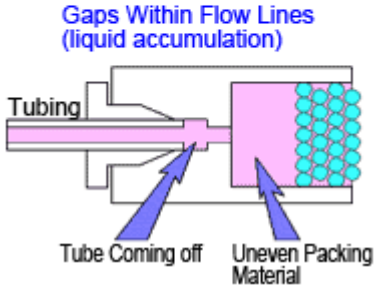
Split Peaks can be caused by:

- Column contamination
- Partially plugged frit
- Column void
- Injection solvent effects
- Co-eluting compounds

Column contamination or partially clogged frits are generally caused when unfiltered samples are injected or when the sample solvent is different to the mobile phase. If the latter is the case, constituents of the sample may precipitate at the column inlet upon mixing with the mobile phase or by contact with the metallic surface of the frit.



A column void is formed either by hydrodynamic stress (high flow rate, high viscosity of the mobile phase) of a poorly packed column, by mechanical impact (the column was dropped on the floor) or by chemical dissolution of the packing material, e.g. pH > 12.
When using injection solvents that have higher elution strength than the mobile phase split or broader peaks can appear. The effect is most pronounced for early eluting peaks.



Moreover, one compound can elute in two states, e.g. if not sufficient ion-pairing reagent is present in the mobile phase or the buffer capacity is deficient.

Peak Problem

피크의 모양에 관련된 문제는 평가하고 바로 잡는 것이 모두 어렵다.

피크 모양에 관련된 문제점은 결과 데이터에 영향을 줄 수 있기 때문에, 분석 결과를 문제 해결하기 이 전의 결과와 실제로 일치하는지 결정해야 한다.

(1) Peak 갈라짐과 Shoulder

Peak 갈라짐이나 Shoulder 발생은 프릿이 막히거나 가드컬럼 결함 또는 컬럼의 빈 공간 때문이다.
작은 입자들이 축적되기 쉬운 장소인 Column inlet 부분 프릿을 교체한다. 이동상의 불순물을 거르기 위해 Injector 바로 뒤에 0.2-0.5um의 in-line 필터 사용을 추천한다.
가드컬럼을 교체하고, 문제가 지속되면 컬럼을 교체해야 한다. 크로마토그램 상에 하나 또는 두 개의 피크만이 갈라진다면 시료가 녹지 않았거나 방해 물질이 있기 때문이다. 알 수 없는 피크를 분석하기 위해 이동상의 성분을 교체할 수 있다. 또한 분석 전에 시료준비과정을 재검토하면 문제가 제거될 수 있다.

(2) Peak Broad와 Fronting
피크가 넓어지거나 (Broad) 앞 쪽으로 기울게 되는 것(Fronting)은 **시료의 농도가 진해서이다**. 시료를 10-20배로 희석하고 재주입하면 적절하게 변할 것이다. 크로마토그램 전반에 걸쳐 뾰족해진 피크는 고농도 시료 때문이다. 차후 비슷한 문제 방지를 위해서 실험 방법과 시료준비 과정을 조정한다.

Peak Problem

(3) Peak 겹침에 의한 영향

먼저 용출되는 피크가 더 뒤의 피크에 영향을 준다면 컬럼 이외의 영향(extra-column effect)이거나 주입 시 사용된 용매 관련 문제이다.

이 문제가 지속된다면 주입 시 사용되는 용매의 농도와 시료의 크기를 확인한다. 고농도 용매는 시료가 이동상과 재평형을 이루어 컬럼의 처음 부분을 지나 빠르게 이동하도록 하기 때문에 피크가 넓어지거나 나뉘어진다.

이런 문제를 피하기 위해서는 **실험을 시작할 때 사용하는 이동상과 함께 시료를 주입하는 것이 최선**이다.

밴드가 넓게 퍼지는 것은 컬럼의 분해나 오염물질 때문이다. 컬럼의 효율이 낮다면 컬럼을 교체하기 전에 강한 용매로 세척한다. 컬럼이 잘 작동하고 있다면, 문제점은 부분적으로 삽입된 인-라인 필터, 주입장치 또는 디텍터의 문제, 또는 잘못 설치된 튜빙과 관련되어 있을 것이다.

이러한 문제점들은 너무 높은 압력, 샘, 또는 일정치 못한 용매의 흐름과 같은 다른 징후들도 나타나게 할 것이다.

(4) Peak tailing

Peak Tailing은 보통 분석물에 있응 염기성 기능 그룹 (예를 들어 아미노그룹)과 컬럼 충진 물질에 있는 결합되지 못 하고 노출되어 있는 시라놀과의 상호작용에 의한 지연 때문이다.

이러한 형태의 상호 작용에 의한 tailing 피크는 20-30mM의 낮은 농도의 트리에틸아민 (TEA)과 같은 이동상 개선제를 사용하는 것으로 최소화 할 수 있다. 단, 이러한 종류의 이동상 modifier의 사용은 컬럼을 되돌릴 수 없게 변화시켜, 이와 비슷한 modifier를 사용하지 않는 분석에는 컬럼을 사용하지 못하게 할 것이다.

Ion-paring 시약, 샘플의 변형, 그리고 강한 완충제도 역시 peak tailing을 감소시킨다.

Practical Implications

The characteristics of the column and of samples in general, lead to a recommendation to start method development with a mobile phase in the pH 2-3 range. At this pH, the ionization of most organic acids will be suppressed, as will the ionization of any silanol groups on the column. Bases will be ionized under these conditions, but the pKa of most basic compounds is >7, so operating at a sufficiently high pH to suppress ionization will be detrimental to most columns. So, all other things being equal, it is best to start out at a low pH. If you need to operate the column at a high pH, be sure to select a column known to be stable in the pH region you choose.

If low-pH ion suppression does not provide acceptable results, the mobile phase pH can be adjusted to help obtain the desirable separation. It usually is most fruitful to adjust the mobile phase organic content (%B-solvent) to obtain acceptable retention for neutral and non-ionized compounds, then to adjust the pH to fine-tune retention of ionic analytes.

Switch to water/organic mobile phase (e.g. replace 50/50 buffer/MeOH with 50/50 water/MeOH) and flush 10-20 mL through the system to remove buffer prior to a strong-solvent flush to remove strongly retained material from the column. With reversed-phase columns, flushing with 100% water is not an effective way to remove buffer – many stationary phases will undergo dewetting (sometimes called phase collapse) with 100% water, defeating the washing process.

Evaluation of the Reliability of Analysis

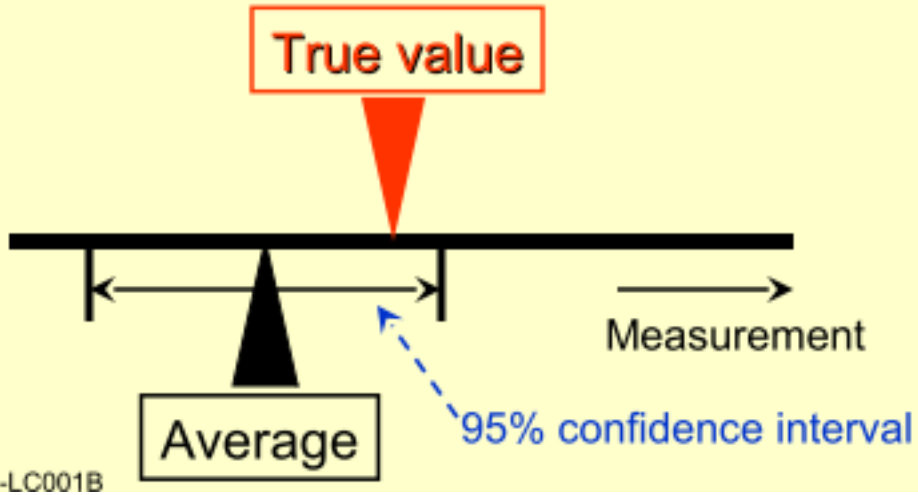
Validation of Analytical Methods

What Is "Validation of Analytical Methods"?

- Scientifically demonstrating that the analytical methods concur with the intended purpose (i.e., that errors are within a permissible range)
- Evaluating required items from the validation characteristics
- Validation characteristics
  - Accuracy / trueness
  - Precision
  - Specificity
  - Detection limit
  - Quantitation limit
  - Linearity
  - Range
  - (Robustness)

Accuracy / Trueness

- Definition
  - Degree of bias in measurements obtained with analytical procedures
  - Difference between true value and grand mean of measurements



- Evaluation Method
  - Comparison with theoretical values (or authenticated values)
  - Comparison with results obtained using other analytical procedures for which the accuracy (trueness) is known
  - Recovery test

Precision

- Definition
  - Degree of coincidence of series of measurements obtained by repeatedly analyzing multiple samples taken from a homogenous test substance
  - Variance, standard deviation, or relative standard deviation of measurements
- Repeatability / Intra-Assay Precision
  - Precision of measurements taken over a short time period under the same conditions
- Intermediate Precision
- Reproducibility

Specificity

- Definition
  - The ability to accurately analyze the target substance in the presence of other expected substances
  - The discrimination capability of the analytical methods
  - Multiple analytical procedures may be combined in order to attain the required level of discrimination
- Evaluation Method
  - Confirmation that the target substance can be discriminated (separated) from co-existing components, related substances, decomposition products, etc.
  - If reference standards for impurities cannot be obtained, the measurement results for samples thought to contain the impurities are compared.

Detection Limit

- Definition
  - The minimum quantity of a target substance that can be detected.
  - Quantitation is not absolutely necessary.
- Evaluation Method
  - Calculated from the standard deviation of measurements and the slope of the calibration curve.
    - $DL = 3.3\ \sigma/slope$
      (σ: Standard deviation of measurements)
      (Slope: Slope of calibration curve)
  - Calculated from the signal-to-noise ratio.
    - Concentration for which S/N = 3 or 2

Quantitation Limit

- Definition
  - The minimum quantity of a target substance that can be quantified
  - Quantitation with an appropriate level of accuracy and precision must be possible. (In general, the relative standard deviation must not exceed 10%.)
- Evaluation Method
  - Calculated from the standard deviation of measurements and the slope of the calibration curve.
    - $QL = 10\,\sigma/slope$
      (σ: Standard deviation of measurements)
      (Slope: Slope of calibration curve)
  - Calculated from the signal-to-noise ratio.
    - Concentration for which S/N = 10

Linearity

- Definition
  - The ability of the analytical method to produce measurements for the quantity of a target substance that satisfy a linear relationship.
  - Values produced by converting quantities or measurements of the target substance using a precisely defined formula may be used.
- Evaluation Method
  - Samples containing different quantities of the target substance (usually 5 concentrations) are analyzed repeatedly, and regression equations and correlation coefficients are obtained.
  - Residuals obtained from the regression equations of the measurements are plotted, and it is confirmed that there is no specific slope.

Range

- Definition
  - The region between the lower and upper limits of the quantity of a target substance that gives appropriate levels of accuracy and precision
- Evaluation Method
  - The accuracy, precision, and linearity are investigated for samples containing quantities of a target substance that correspond to the lower limit, upper limit, and approximate center of the range.

Robustness

- Definition
  - The ability of an analytical procedure to remain unaffected by small changes in analytical conditions.
- Evaluation Method
  - Some or all of the variable factors (i.e., the analytical conditions) are changed and the effects are evaluated.

기 기 명 : 분취용 액체 크로마토그래피

(Prep Liquid Chromatography)

분석용도 : 혼합 물질 분리 후 정제

▶ Prep-LC 컬럼 선택 가이드

	Column Diameter (mm)	Typical Flow Rate (1)	Sample Capacity (2)	Maximum Practical Sample Load (3)
Capillary 	0.075 0.15 0.30 0.50	0.25µL/min 1µL/min 5µL/min 10µL/min	0.05µg 0.2µg 1µg 2µg	
Microbore 	1.0	25–50µL/min	0.05–10µg	
Narrowbore 	2.1	100–300µL/min	0.2–50µg	
Analytical 	4.6	0.5–1.5mL/min	1–200µg	10mg
Semi-preparative 	10	2.5–7.5mL/min	1,000µg	50mg
Preparative 	22	10–30mL/min	5mg	200mg
Process 	50 100	50–100mL/min 150–300mL/min	25mg 125mg	1,000mg 5,000mg

HPLC

Prep-LC

Prominence series

LC-20AD/T

0.001 to 10.000 mL/min

LC-20AR

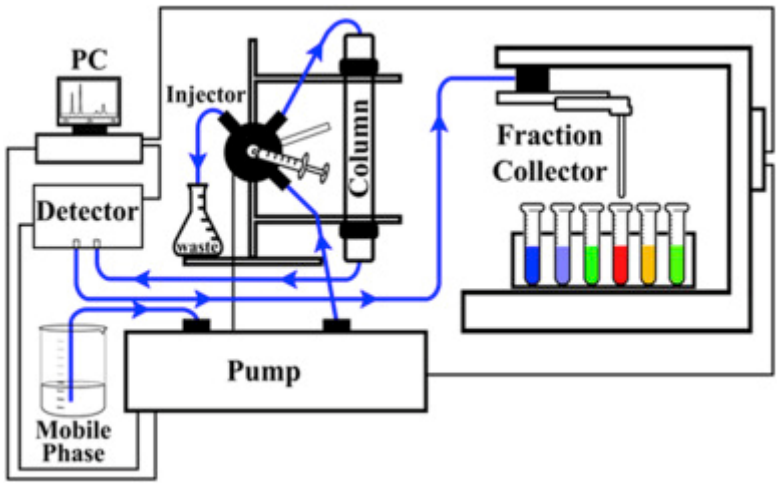
0.01 to 20.00 mL/min

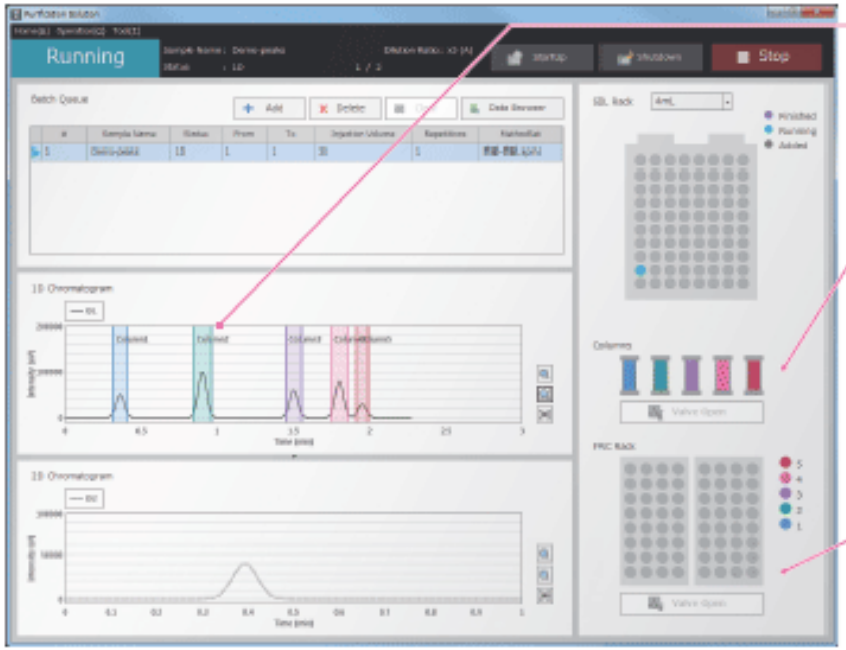
LC-20AP

0.01 to 150.00 mL/min

- 최대 압력 : 42MP
- 유량 : 0.01~150.0000ml/min, Single Flow
- Flow rate accuracy : ±1%

Fractional Collector





Chromatogram

Individual fraction peaks are color-coded.

Trap Column

This shows a diagram of the columns where fraction peaks were adsorbed.
The colors correspond to the color-coded fraction peaks.
Adsorption, purification, and elution processes are each indicated with specialized animated graphics.

Fraction Collector

This displays which vial in the fraction collector was used to collect the eluate recovered from the trap column.

The End.