High Performance Liquid Chromatography (HPLC)

Agilent HP1100

http://www.laboratory-journal.com/applications/analytics/hplc-analysis

January 9th – 10th, 2017

Instrument Center
Faculty of Science and Technology
Prince of Songkla University
Pattani campus
Schedule of
“Using High Performance Liquid Chromatography (HPLC) Agilent HP1100” training
January 9th - 10th, 2017
At Instrument Center, 2nd floor, Building 71 (Pre-Clinical Building)
Faculty of Science and Technology, Prince of Songkla University

January 9th, 2017
<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>08.15 - 08.30 am</td>
<td>Registration</td>
</tr>
<tr>
<td>08.30 - 08.45 am</td>
<td>Inauguration</td>
</tr>
<tr>
<td>08.45 - 10.15 am</td>
<td>Introduction of HPLC Dr. Weeraya Khummoong</td>
</tr>
<tr>
<td>10.15 - 10.30 am</td>
<td>Coffee break</td>
</tr>
<tr>
<td>10.30 am – 12.00 pm</td>
<td>Application of HPLC Dr. Weeraya Khummoong</td>
</tr>
<tr>
<td>12.00 – 01.00 pm</td>
<td>Launch</td>
</tr>
<tr>
<td>01.00 – 02.30 pm</td>
<td>Using and Maintenance of HPLC (Agilent HP1100) Ms. Paphatchaya Kornthattalim</td>
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<tr>
<td>02.30 – 02.45 pm</td>
<td>Coffee break</td>
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<tr>
<td>02.45 – 03.00 pm</td>
<td>Work shop 2 groups</td>
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<tr>
<td>03.00 – 04.30 pm</td>
<td>Work shop # 1.1 Preparation and filtration of mobile phase and sample Work shop # 1.2 Primary using Agilent HP1100 HPLC</td>
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January 10th, 2017
<table>
<thead>
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<th>Time</th>
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<tr>
<td>08.45 – 10.15 am</td>
<td>Work shop # 2.1 Determination of Sodium benzoate by HPLC Work shop # 2.2 Program installation, Frit changing, Guard column changing</td>
</tr>
<tr>
<td>10.15 – 10.30 am</td>
<td>Coffee break</td>
</tr>
<tr>
<td>10.30 am – 12.00 pm</td>
<td>Work shop # 3 Practical HPLC using</td>
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<tr>
<td>12.00 – 01.00 pm</td>
<td>Launch</td>
</tr>
<tr>
<td>01.00 – 03.30 pm</td>
<td>Work shop # 4 Maintenance Agilent HP1100 HPLC</td>
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<tr>
<td>03.30 – 03.45 pm</td>
<td>Coffee break</td>
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<tr>
<td>03.45 – 04.30 pm</td>
<td>Test HPLC principle and using</td>
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Training of
“Using High Performance Liquid Chromatography (HPLC) Agilent HP1100” training
January 9th - 10th, 2017
At Instrument Center, 2nd floor, Pre-Clinical Building
Faculty of Science and Technology, Prince of Songkla University

<table>
<thead>
<tr>
<th>No</th>
<th>Name - Surname</th>
<th>Year/Degree/Major</th>
<th>Code</th>
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<tbody>
<tr>
<td>1</td>
<td>Miss Jennifer Osamede</td>
<td>1st year, Master’s degree, Food Science and Nutrition</td>
<td>1Aα</td>
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<tr>
<td>2</td>
<td>Mr. Alam Surya Wijaya</td>
<td>1st year, Master’s degree, Sustainable Energy Management</td>
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<td>3</td>
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<td>1st year, Master’s degree, Applied Chemistry</td>
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<tr>
<td>4</td>
<td>Miss Sitihaya Malibo</td>
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<tr>
<td>5</td>
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<tr>
<td>7</td>
<td>Mr. Sampan Sengliang</td>
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<td>8</td>
<td>Miss Hasmas Karuemor</td>
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<tr>
<td>9</td>
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<td>10</td>
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<td>11</td>
<td>Miss Rerai Madsaai</td>
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<td>12</td>
<td>Miss Phanita Vonghirundecha</td>
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<tr>
<td>13</td>
<td>Miss Rotnani Thipmat</td>
<td>4th year, Bachelor’s degree, Chemistry (Education)</td>
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<tr>
<td>14</td>
<td>Mr. Anak Pollachat</td>
<td>Lecturer</td>
<td>2BΩ</td>
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Part 1
Introduction and Application of HPLC
Introduction to HPLC

Dr. Weeraya Khummueng

SAT-HPLC training, 9-10th January 2017

What is chromatography?

“Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves in a definite direction.”

Official definitions of chromatography by IUPAC
- Mobile phase = liquid or gas or SCF
  - Liquid Chromatography (LC)
  - Gas Chromatography (GC)

- Stationary phase = column packing material or liquid eg. silica, alumina, silica bond with C8, C18

![Diagram of chromatography techniques]
What is HPLC?

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry* used to separate, identify, and quantify each component in a mixture.


Steps in a measurement process

- Sampling
  - Sample preservation
  - Sample preparation
    - Homogenization
    - Extraction
    - Clean-up
    - Concentration
  - Analysis
Analysis

• Instrumental analysis

Chromatographic analysis

- Non-volatile compounds → HPLC
- Volatile compounds → GC
- Semi-volatile compounds......??

Chromatographic Analysis

Sampling

Sample preservation

Extraction

Clean-up

Concentration

HPLC

High Performance Liquid Chromatography

Sampling

Sample preservation

Extraction

Clean-up

Concentration

GC

Gas Chromatography
Chromatographic techniques

Partition chromatography

Partition chromatography is a method of separation in which the components present in the mixture get distributed more likely into two liquid phases because of differences in partition coefficients.

In Partition chromatography, the molecules are separated in between two phases i.e. both stationary phase and mobile phase are in same phase.

http://chromatography.conferenceseries.com/events-list/partition-chromatography
In the figure, we observe a chromatogram showing the separation of components in a flow system. The injected sample band moves through the stationary phase, and different components are eluted at different times, as indicated by the peaks on the chromatogram. The stationary phase is often a porous material like silica or alumina, which can be seen in the images of these materials in the diagram.

Silica and alumina are commonly used in chromatography due to their high surface area and porosity, which facilitate the adsorption of molecules and enable efficient separation. The images of silica and alumina particles illustrate the typical appearance of these materials.

The chromatogram demonstrates the principle of chromatography, a technique used to separate mixtures into their constituent parts based on differences in their interactions with the stationary and mobile phases.
Partition Chromatography

- **Normal phase**
  - Analyte is non or less-polar organic compounds.
  - Stationary phase is more polar than the mobile phase.

- **Reverse phase**
  - Analyte is polar organic compounds.
  - Stationary phase is less polar than the mobile phase.
**Reversed Phase**
Reversed Phase (RP) is with more than 90% share by far the most often used HPLC mode.


**Normal Phase**
Normal Phase (NP) was the first established HPLC mode and is still used in a lot of cases today.

Elution process

- Isocratic elution: use of a constant mobile phase composition during the entire elution process.
- Gradient elution: composition of the mobile phase is changed (continuously or stepwise) during the elution process.

Technical term in HPLC

1. Chromatogram is a graph showing the detector response as a function of elution time.
2. Peak shape

3. Retention time ($t_R$): time that required for the analyte to reach the detector

$$t'_R = t_R - t_m$$

$t'_R$ = Adjust retention time
where $t_m$ is void time (time of unretained peak)
4. Theoretical plate (N) and Height Equivalent to a Theoretical Plate (HETP):

Plate count (N) becomes greater
Plate height (HETP) becomes smaller.

\[
N = 16 \left( \frac{t_R}{W_b} \right)^2 = 5.54 \left( \frac{t_R}{W_h} \right)^2
\]

5. Peak width

- Width of Gaussian peak at various height as a function of the standard deviation (\(\sigma\)) of the peak.

- Tangents drawn to the inflection points

- Inflection points

- Widths: \(w_1 = 2\sigma\), \(w_b = 2.355\sigma\), \(3\sigma\), \(4\sigma\), \(5\sigma\)
6. **Resolution (Rs):** ability of column to separate two analytes.

\[
R_s = \frac{2[t_2 - t_1]}{W_1 + W_2}
\]

Baseline resolution is achieved when \( R = 1.5 \)

7. **Retention factor or Capacity factor \((k')\)** is often used to describe the migration rate of an analyte on a column.

\[
k' = \frac{m_s}{m_m} \quad \text{or} \quad k' = \frac{t_r - t_m}{t_m}
\]

Ideally, \( k' \) for an analyte is between 1 to 5.

8. **Selectivity factor \((\alpha)\):** describes the separation of two species (A and B) on the column

\[
\alpha = \frac{k'_B}{k'_A}
\]
Effect of $k'$, $N$ and $\alpha$ to separation power

Adjust $k'$ and $\alpha$ is more convenience than adjust $N$ value

### $k'$, $N$ and $\alpha$

<table>
<thead>
<tr>
<th>Efficiency, $N$</th>
<th>Effect to peak width</th>
<th>$N\uparrow \iff R_s\uparrow$ (decrease peak width)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity, $\alpha$</td>
<td>Effect the position between two peaks</td>
<td>$\alpha\uparrow \iff R_s\uparrow$ (increase peak distance)</td>
</tr>
<tr>
<td>Capacity, $k'$</td>
<td>Effect resident time of analyte in column</td>
<td>$k'\uparrow \iff R_s\uparrow$ (increase peak distance)</td>
</tr>
</tbody>
</table>
To obtain high resolution, the three terms must be maximised.

1. Increasing $N$, by lengthening the column (leads to an increase in retention time and band broadening) or reducing the size of the stationary phase particles.

2. Controlling $k'$, which improve separations by changing the composition of the mobile phase.

3. Adjusting $\alpha$ to unity can also be manipulated to improve separations.

Basically, adjusting $N$ is not convenience. Thus, $k'$ is optimised first, and then $\alpha$ is increased by one of the following procedures:

- Changing mobile phase composition
- Changing column temperature
- Changing composition of stationary phase

Flow rate??
Changing $k'$
1. Changing mobile phase composition
2. Decrease/increase the amount of stationary phase
3. Changing flow rate
4. Changing temperature

**Peak area:** The area measured under a chromatographic peak; usually measured by an integrator or data system; the peak area is related to the amount of substance eluted in a peak.

**Peak height:** The height of a chromatographic peak as measured from the baseline to the peak apex; the peak height is related to the amount of substance eluted in a peak.
The other terms:

- **Degassing**: removing dissolve gas from mobile phase
- **Elution chromatography**:
  - Gradient elution
  - Isocratic
- **Flow rate**: the volumetric rate of flow of a mobile phase through LC column.
- **Guard column**: a small column placed between injector and analytical column.
- **Octadecylsilane (ODS)**: is a bonded phase between silica and C18
- **Silanol**: The Si-OH group found on the surface of silica gel.
- **Siloxane**: The Si-O-Si bond.
- **Solvent strength**: ability of a solvent to elute compounds from column.
High Performance Liquid Chromatography (HPLC)
Ultra Performance Liquid Chromatography (UPLC)

Block Diagram of HPLC

Mobile phase reservoir → Pump → Injection system → Column → Detector

Guard column

Data system

HPLC

UPLC
1. Mobile phase reservoir: mobile phase container, normally placed on the top of HPLC and has Milipore filter to remove the impurity before pump into the system.

Milipore filter  
Tubing  
Polyetheretherketone (PEEK)  
Teflon

Sonicator Degassing and filtrate
2. Pump: to introduce eluent into the system and control the flow rate of mobile phase

HPLC is a high pressure system
25 – 100 bar (1 bar = 105 Pa = 14.5 psi)

Type
1. Piston reciprocating pump
2. Diaphragm or Membrane reciprocating pump
   - Syringe pump
   - Pneumatic pump

3. Sample injection system is a system that introduce standard or sample into the system.

   Loop
   Syringe
   Injection port
   Automatic sample injector

http://www.restek.com/info_sixport.asp
4. Column: an essential part of HPLC system where the separation of the analyte is occurred. HPLC columns are packed with fine particle in stainless steel tube.

- Analytical column
- Guard column

Stationary phase
- Silica gel: Si-OH group on the surface (Normal phase)
- Silica gel: which Si-OH was bonded with R groups such as C8, C18, CN, phenyl, NH₂, etc. (Reverse phase)

MP: non-polar eg. hexane

MP: polar eg. Water, MeOH

Silica bonded with R group: Reverse phase

R can be C2-C18, Phenyl, CN, NO₂ or Diol
The models for octadecyl bonded silica

eg. C-18 activated with n-hexane

A. Without conditioning

B. Partial conditioned

C. Fully conditioned

Note: Equilibrate column with MP before hand at least 15-20 min

Column for several companies

RESTEK
C18 Columns
Allure® Aqueous C18 5um Columns (USP L1)
Allure® C18 5um Columns
Pinnacle® DB Aqueous C18 1.9µm, 3µm & 5µm Columns (USP L1)
Pinnacle® DB C18 1.9µm, 3µm & 5µm Columns (USP L1)
Pinnacle® II C18 3µm & 5µm Columns (USP L1)
Ultra Aqueous C18 3µm & 5µm Columns (USP L1)
Ultra C18 3µm & 5µm Columns (USP L3)
Viva C18 3µm & 5µm Columns (USP L1)

Agilent
Poroshell 120
ZORBAX Eclipse Plus
ZORBAX Eclipse XDB
ZORBAX StableBond 80A
ZORBAX Rx
ZORBAX Extend C18
ZORBAX Bonus
HPLC Detector

- Sensitivity
- Reproducibility
- Linearity
- Sample non-destructive
- Small internal volume to decrease band broadening

1. Ultraviolet-visible absorbance detectors
   1.1 Fixed-wavelength detector
       Single wavelength detector
       Multi-wavelength detector
   1.2 Variable wavelength detector use deuterium lamp and tungsten lamp as a light source

A UV-visible absorption cell for HPLC.
1.3 Diode Array Detector (DAD) or PDA (Photo Diode Array)

http://www.chromatography-online.org/HPLC-Detectors/UV/Diode-Array/rs50.html
Access on 05/12/16

2. **Fluorescence detector** is probably the most sensitive LC detector that is available, consists of a single wavelength excitation source and a sensor that monitors fluorescent light of all wavelengths.

http://www.chromatography-online.org/HPLC-Detectors/Fluorescence/Single-Wavelength-Excitation/rs57.html access on 05/12/16
3. **Refractive-Index Detector (RI)** is response to nearly all solutes, highly temperature dependent and not compatible with gradient elution method. RI detector is normally used for sugar analysis.

How to start HPLC analysis

- Literature reviews
- Sample
- Analyte
- Standard
- Sample preparation
- Column
- Mobile phase
- Flow rate
- Injection volume
- Detector
- Calibration curve
- Quantitation
- Validation
Troubleshooting

• Lower pressure
• Higher pressure
• Leaking
• Air bubble

การวิเคราะห์โดยวิธีเทียบมาตรฐาน

1. External standard เป็นวิธีเทียบมาตรฐาน โดยเตรียมสารมาตรฐานชนิดเดียวกับสารที่ต้องการวิเคราะห์และทราบความเข้มข้นที่ถูกต้องแน่นอนเหมาะสมเมื่อตัวอย่างที่ประกอบด้วย matrix และวิธีเงื่อนที่ใช้ไม่รบกวนการวิเคราะห์

สร้างกราฟมาตรฐาน สมการเชิงเส้น หาความสัมพันธ์เชิงเส้นระหว่างตัวแปร 2 ตัว รูปแบบสมการมักเป็นสมการเส้นตรง คือ

\[ y = mx + b \]

เมื่อ \( m \) เป็นสัมประสิทธิ์การถดถอย (regression coefficient) หรือความชัน 
\( b \) คือ จุดตัดแกน \( y \) หรือค่าของ \( y \) เมื่อ \( x \) เป็น 0
External standard

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<th>ug/L of Pb</th>
<th>Instrument response</th>
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<td>0.00</td>
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</tr>
<tr>
<td>1.00</td>
<td>125</td>
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<tr>
<td>2.00</td>
<td>246</td>
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<tr>
<td>5.00</td>
<td>619</td>
</tr>
<tr>
<td>10.00</td>
<td>1250</td>
</tr>
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</table>

แทนค่า $y$ หรือ instrument response ลงในสมการเพื่อหาค่า ความเข้มข้น ของ analytes ในสารตัวอย่าง (ถ้า $y = 1019$ และ $x = 8.17 \mu g/L$)
2. Internal standard method คือการเติมสารเคมีชนิดกับสารที่ต้องการวิเคราะห์ลงไปในชุดตัวอย่าง sample blank และ ชุด calibration standards ในปริมาณคงที่ internal standard ที่เติมลงไปจึงใช้เป็นตัวเปรียบเทียบในการวิเคราะห์

Internal standard method เป็นวิธีที่ให้ความถูกต้องแม่นยำสูงกว่าวิธี external standard

ขณะบัดของ Internal standard???
### Internal standard

<table>
<thead>
<tr>
<th>No.</th>
<th>Vol. of std (mL)</th>
<th>Vol. of IS (mL)</th>
<th>Peak area of Std</th>
<th>Peak area of IS</th>
<th>Ratio of Std/IS</th>
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</thead>
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<tr>
<td>1</td>
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<td>70655</td>
<td>123563</td>
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<td>96218</td>
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<tr>
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<td>25.00</td>
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<td>126783</td>
<td>0.9449</td>
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<tr>
<td>4</td>
<td>30.00</td>
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<td>151310</td>
<td>127889</td>
<td>1.1831</td>
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<td>5</td>
<td>35.00</td>
<td>25.00</td>
<td>166673</td>
<td>125436</td>
<td>1.3287</td>
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### Internal standard

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<td>5</td>
<td>35</td>
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<tr>
<td>Sample</td>
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<td>0.9662</td>
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</table>
Internal standard curve

\[ y = 0.0386x - 0.0055 \]

\[ R^2 = 0.9962 \]
Part 2
Using and Maintenance of HPLC
(Agilent HP1100)
Using
Agilent HP 1100 HPLC
Paphatchaya Kornthatthalim
Scientist
Instrument Center
Faculty of Science and Technology

Mobile Phase
and
Sample Filtration
Mobile Phase Filtration

Standard filter set
Put filter set and sieve respectively

- Rinse with mobile phase before switch on vacuum pump
- Pour filtered mobile phase into mobile phase bottle
- 20 min degasses
- Remove the filter set and clean by surfactant, tap water, DI water and overturn to dry
- Please plan the preparation of glass and equipment before

**Sample Filtration**

Ready-use filter
Manual consisted filter

Assemble the filter respectively

1 2 3 4 5 6
Tighten the filter set

Connect to syringe
Pour sample into syringe and press pistol to filter sample into vial. Now sample ready to analyse

Using
Agilent HP 1100 HPLC
**Turn on**

**Turning on HPLC**

1. Turn on UPS by pressing SWITCH ON
2. Power on 5 modules of HPLC
   - Degasser module
   - Pump module
   - Auto Sampler module
   - Column compartment module
   - Detector module

3. Turn on computer and monitor
Turning on HPLC

4. Wait until Auto sampler module finish for set up (the light on autosampler will be disappear)

5. Open “Chem Station” software by double click at Instrument 1 Online icon

**Method & Run Control will appear on screen**

6. Turn on the HPLC system by click ON at right bottom of window
6. Turn on the HPLC system by click ON at right bottom of window

7. Purging: open purge valve by twist it anticlockwise direction for 1 round (remove column before) to substitute the old solution with mobile phase
Right click at pump arrow and select “Set up pump” to set flow rate from 0 to 3 mL/min increment by 0.5 mL/min

Maximum Flow rate at 3 mL/min increment by 0.5 mL/min
- 10 min purging time or wait until no bubble out, then switch from channel A to B → C → D respectively
- Decrease flow rate from 3 to 0.5 mL/min decrement by 0.5 mL/min
- Twist purge valve clockwise direction

8. Column set up
   - Take off the column compartment cover
- Now, pump is flow mobile phase at 0.5 mL/min through column capillary tube, flow until run out of bubble
- Connect to Guard column and wait until no bubble out
- Connect to Analytical column and wait until no bubble out
- Connect the column to complete system

- Put column in thermostat box
- Nip the column clip at column
- Cover of Column compartment cover again
9. Check mobile phase leaking by touch on the in-out of column connection and other connection with hand or tissue paper
- If method has already been set up, click "Method" menu → Load method and choose method name

**New Method**

1. Choose "Method" menu → New method
2. Click Method → Edit entire method for method setting
3. Various method sections will show to edit the value as follows
   - **Edit method: check (✓) all of method sections**

- **Method Information:** type comments (if any)
- Set up pump: type Flow, Stop time and % of Solvents
  - Increment by 0.2 mL/min until flow rate will be used
  - Loop time set up
  - Set up of time table for gradient system

- Setup Injector: choose injection type and injection volume
Use Injector Program: For Post injector using

Column Thermostat Method: choose Temp. control or Not controlled
DAD Signals: set up the wavelength and Bw

- Set up wavelength, Bw
- Click Vis if wavelength higher than 400 nm

FLD Signals: set up the Signal of Excitation and Emission

- Set up Excitation  และ Emission
Signal Details: choose Signals as required and click “Add to Method”

Edit Integration Events: Do not set up
Specify Report: Do not set up

Instrument Curves: check (X) at data that want to be show on screen
Run time checklist: check ✓ at Data Acquisition and Standard Data Analysis

4. Click Method menu → Save Method As, type method name and click Save

Ready to analyze now ^_^
Online Plot Setting

Enlarge of Online Plot blog
Click Change to choose signal plot

Choose Pressure and used Signal to follow pressure of system and chromatogram
Sample Analysis

Sample analysis

HP 1100 Agilent HPLC can analyze sample for 2 styles

- Single Analysis: 1 sample/time but duplication it can
- Sequence Analysis: multi sample or multi method per time for continuous analysis (Automatic)
Sample analysis - Single

1. Click “RunControl” menu → Sample Info

2. Type Operator Name, select Prefix/Counter in Data file, type Subdirectory, Specify Prefix, Vial and Sample Name
3. Put vial in sample tray, click **Start**

![Image of a laboratory instrument interface]

Sample analysis - Sequence

1. Sequence Parameters is create by click “Sequence” menu

![Image of a laboratory instrument interface]
2. Type Operator Name, Prefix/Counter, Subdirectory, click OK

3. Select Sequence Table or click at tray figure
4. Type Vial, Sample Name, choose Method Name, Inj/Vial and Inj Volume, click OK

5. Put vials in the right position
6. Choose Save Sequence and click Start
Standard Curve Plotting

**Standard curve plotting**

1. Click “View” menu and choose Data analysis
2. Click File >> Load signal, select first file of standard
3. Click “Calibration” >> New Calibration Table
4. In “Calibration Instrument 1” select Automatic Setup, type “1” in level (No. of standard)
5. Type concentration in Default Amount, click OK

6. RT of chromatogram will show in Calibration Table, type Compound name and concentration, click OK
7. Load signal again, choose 2\textsuperscript{nd} standard file and do follow as first standard but in “Calibration” choose “Add Level” instead.

8. Type concentration in Default Amount, click OK and do follow as first step until complete all of standard concentration.
9. Click “Calibration” and choose Calibration Settings
10. Type the using unit (ppm or mg/mL) then, click OK
11. Click “Report” menu >> Specify Report
12. In Quantitative Results >> “Calculate” change to ESTD, click OK
13. Report will show the concentration of sample that has same RT with standard
14. If want to report that no compare with standard, choose Report >> Specify Report and change Calculate from ESTD to be %

Data Analysis
Data Analysis

1. Click “View” menu → Data analysis or click drop down list to choose Data analysis

2. Click “File” menu → Load signal or click

3. Choose file from Folder and Subdirectory
4. Integration adjust by choose
“Integration” menu → Integration events

- Or click

- Chromatogram show as this slide
Approximately Integration

Adjust data by click Report —— Specify Report
Click “Signal Options” to adjust range of chromatogram

Adjust Ranges and choose “All the same Scale” to use it for all of data
5. View data by click

HPLC
Turning Off
HPLC Turning off

1. Flow solvent such as 50% Acetonitrile, Methanol (flow 1 mL/min, 30 min or more) to wash column

2. Bring out of column and cap the both of column edges

3. Keep HPLC inner system by Isopropanol (flow 1 mL/min, 20 min or more)

4. Save method and turn off system by click “off” bottom at right of window
5. Power off all of modules
   - Detector
   - Column compartment
   - Auto Sampler
   - Pump
   - Degasser

6. Shut down computer and monitor

7. Switch of UPS
Conclusion of analytical by HPLC

1. Prepare mobile phase >> filter >> degases >> connect system >> purge system >> connect column
2. Equilibrium column by Flow mobile phase
3. Analyze one of standard to get RT and chromatogram
4. Analyze all of standards to plot Calibration curve
5. Inject sample
6. Integrate chromatogram
7. Clean HPLC system >> Keep column >> Turn off HPLC
Appropriate HPLC Using

- Follow operation manual and understand of usage, technique and maintenance before
- Use HPLC grade solvents and DI water only
- Clean the tubing system before and after used
- Be careful about run out of mobile phase
- Change DI water bottle every 2 days or use brown glass bottle
Avoid to use corrosive solvent such as buffer or halogen acid pH <2.5 and basic solution pH >10

Be careful when mix high reaction solvent

Study UV cut off of solvent before used

Install antivirus program in computer

Do not install another programs in computer and be careful to connect to internet

Usually back up data

HPLC Maintenance
Pump

- PTFE Frit Changing
- Gold Seal Changing
Column and Tubing maintenance

- Use appropriate ferrules, nut and fitting with column and connection

- Column clogging may be cause of high pressure

- Change guard column cartridge when it is decline (be careful and keep right side)

- Causes broad peak
  1. Over loading
  2. Declination of column
Column Care

- Filter all solvents and samples
- Use guard column
- Flush to remove buffer after used
- Cap when it is not use
- Store column in appropriate solvent
- Use column in safety pH
- Do not pressure or solvents shock
- Do not hit and keep in high temperature

Solving of clogging

- Find clogging point from end to top of flow system (detector to pump)
Solving of leaking

- Capillary tube - back pressure flow or change new tube
- Column, Guard column - clean by solvent flow or change new cartridge
- Pump - change frit or clean
- Auto sampler & Column compartment - back pressure flow
- Detector - clean flow cell or flood by solvent

Thank you for your attention
DETERMINATION OF SODIUM BENZOATE

1. Mobile phase preparation

- Acetate buffer preparation
  - Weight ammonium acetate 0.30 g
  - Dissolve with DI water 900 ml
  - Add acetic acid 0.5 ml, mix well
  - Adjust to 1 L in volumetric flask
  - Filter by Nylon filter 0.45 um
  - Degas both buffer and Acetonitrile for 10 min

2. Preparation of sodium benzoate stock standard solution (1,000 mg/L)

- Weight sodium benzoate 100 mg
- Add DI water 50 mL, swirl to dissolve
- Fill into 100 mL volumetric flask and adjust to 100 mL with DI water

3. Preparation of sodium benzoate working standard solution and sample

- Prepare 5, 10, 20, 50 and 100 mg/L (10 mL) of sodium benzoate

4. Preparation of sample

- Filter standard and sample by syringe filter 0.45 μm into vial

5. HPLC condition

- Mobile phase: Acetate buffer : Acetonitrile (50:50)
- Column : Eclipse XDB C18, 150x4.6 mm
- Injection volume : 10 μL
- Flow rate : 1.0 ml/min
- Detector : DAD at 225 nm

- Flow mobile phase until base line is smooth
- Inject standard sodium benzoate 5, 10, 20, 50 and 100 mg/L, respectively
- Inject sample
Workshop

Operator name : ___________________________ Code____________________

**Workshop 1** (Alternate group-1, 2)

*Workshop 1.1* Mobile phase, standard and sample preparation  
(First day 02.45-03.30 pm)

- Prepare mobile phase: 100% Ammonium acetate and 100% Acetonitrile 500 mL each 1 bottle
- Prepare stock standard solution of sodium benzoate 1000 mg/l, 100 ml
- Prepare working standard solution of sodium benzoate 5, 10, 20, 50 and 100 mg/l (1 vial/concentration)
- Prepare sample solution

*Workshop 1.2* Demonstrate turn on-off HPLC, various modules, purging valve, column changing, method edition, detector sections

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**Workshop 2** (Alternative group -1, 2)

*Workshop 2.1* Determination of sodium benzoate by HPLC (45 min)

Operator name : ___________________________

Column : Type________________________ Size__________________

Flow rate : ______ mL/min

Mobile phase : ____________________

Detector : Detector ____________________ Wavelength_______ nm

Injection volume : ______ uL

Method name : _______________________

Standard file name : ____________________

Sample file name : ____________________

Linear equation : _______________________

Result of sample determination

: ____________________________________________
  ____________________________________________
  ____________________________________________

*Workshop 2.2* Explanation of program installation, frit changing and guard column changing (45 min)
**Workshop 3** Practical of HPLC using (Circulate 3 group - A, B, C)  
(30 min/workshop)  
**Workshop 3.1** Turn on HPLC / purging valve test  
**Workshop 3.2** Program installation test  
**Workshop 3.3** Set up Condition test  

A #  
- Column: Pinnacle II C18, 250x4.6 mm  
- Mobile phase: 50% Acetonitrile  
- Injection volume: 5 µL  
- Flow rate: 0.5 mL/min  
- Column thermostat: 50°C  
- Detector: FLD at Excitation 250 nm Emission 470 nm  

B #  
- Column: Pinnacle II C18, 250x4.6 mm  
- Mobile phase: DI: Acetonitrile= 0 % Acetonitrile at 0-5 min, 10 % Acetonitrile at 5-7 min and 30 % Acetonitrile at 7-10 min  
- Injection volume: 10 µL  
- Flow rate: 0.7 mL/min  
- Column thermostat: -  
- Detector: DAD at 550 nm BW 16 nm  

**Workshop 4** Maintenance of Agilent HP1100 HPLC (Draw lots of 5 groups- \( \alpha, \beta, \gamma, \Omega, \lambda \))  
(15 min/workshop)  
**Workshop 4.1** Find leak and repair  
**Workshop 4.2** Change guard column  
**Workshop 4.3** Change frit  
**Workshop 4.4** Takeback pressure between autosampler and column compartment  
**Workshop 4.5** Change FLD instead DAD  

*****************************************
Installation of HP ChemStation Program

1. Insert CD program >> Auto run will active, if it's not work >> Open drive and double click at "SETUP" icon

2. Program will automatically run about 5 min - When it is finished, question will be appear, click "Yes"

3. "HP ChemStation" page will show up, click Add/Change to choose programs

4. "Configure Instrument 1" page will show up, choose G2170AA and click Add to get it down into Current Product.

5. Type New License Number and click Add to pairing program and license
6. When added 2 instrument programs already, click **Install** >> then, Run HP Configuration by click “Yes”

7. When “Configuration Edition” page is show, choose Configure >> Instruments..

8. In “Device Configuration” page, choose 1100 System Access (fill “26” in HPIB Address) click “Add” and OK

9. Click “Exit” in file menu >> Happy ending