**INSTRUMENTAL ANALYSIS LABORATORY**

**NAME OF THE EXPERIMENT:** HPLC

**DATE OF THE EXPERIMENT:** 10.04.2023 – 11.04.2023

**NAME OF THE ASSISTANT:** Mehmet Seçkin Kesici

**NAME OF THE STUDENT:** Elif Nazenin GİRAY

**GROUP MEMBERS:**Batuhan GÜNEŞ,Berkay YAPICI,Alper İREZ,Bekirhan ERDAŞ **INSTRUMENTATION:**

Sample Photomultiplier

Emission Wavelength Selector

SAMPLE

Excitation Wavelength Selector

SOURCE

Beam attenuator

Beam attenuator

Reference beam

**INSTRUMENTAL AND EXPERIMENTAL PART**

**INSTRUMENTATION:**

**Sample**

**Solvent**

**Pump**

**Injector**

**Detector**

**Readout**

**Column**

**Waste**

The brand of the device used in the HPLC experiment is SOR-100 DIONEX P680 HPLC Pump Series. The solvent used in the experiment is a buffer containing 0.5 percent ammonium acetate and methanol with a pH of 5.5. The solvent is called the mobile phase and is sent to the device. The solvent is connected to the device via a cable and is continuously drawn. he solvent reaches the device and then goes to the pump. The device's pump is a Dionex P680 HPLC piston pump containing two pumps. The mobile phase is delivered to the column with high output pressure. The next step is the injection process. The injector used is Rheodyne Model 7125. Four solutions are prepared for injection. These are respectively. It is Methyl Paraben, Propyl Paraben, Ambroxol HCl, and Syrup. While the injection position is the valve position, it is injected using the syringe to allow the solvent to flow into the column.

The column used in the experiment is a reversed phase chromatography called Thermo Scientific AcclaimTM 120 C18 (4.6 x 150 mm) with apolar properties. The signals detected by the detector, which name is Dionex UVD 170U UV-vis PDA are transmitted to the computer by the detector. With the information obtained from the computer and software, the peaks belonging to the solutions are examined and measured. Due to the composition of the syrup, it has been observed that there are three peaks, while other solvents have one peak. Chromatographic conditions are taken into account in the HPLC experiment. The first of these is that the column temperature is at room temperature, the injection volume is 20 μL, and the flow rate of the moving phase is 1.0 mL/min.

**DATA SHEET**

**Table 1**. Retention Times and Widths

|  |  |  |
| --- | --- | --- |
|  | **Retention time (tR)** | **Width (w)** |
| **Methyl Paraben**  **(260 nm)** | 3.78 min | 0.23 |
| **Propyl Paraben**  **(260 nm)** | 9.08 min | 0.50 |
| **Ambroksol HCl**  **(240 nm)** | 4.62 min | 0.30 |

|  |  |  |
| --- | --- | --- |
| **Syrup**  **(260 nm)** | **Retention time (tR)** | **Width (w)** |
| **Peak 1**  **(Methyl Paraben)** | 3.78 min | 0.22 |
| **Peak 2**  **(Ambroksol HCl)** | 4.65 min | 0.29 |
| **Peak 3**  **(Propyl Paraben)** | 9.09 min | 0.51 |

**Table 2**. Areas of Different Concentration Mixtures

|  |  |  |  |
| --- | --- | --- | --- |
| **Concentrations** | **Methyl Paraben**  **Area (mAU x min)** | **Ambroksol HCl**  **Area (mAU x min)** | **Propyl Paraben**  **Area (mAU x min)** |
| **5 ppm** | 8.89 | 2.05 | 3.23 |
| **10 ppm** | 15.52 | 2.54 | 4.21 |
| **15 ppm** | 21.12 | 3.59 | 4.38 |
| **20 ppm** | 28.52 | 5.09 | 5.44 |
| **25 ppm** | 40.45 | 6.70 | 8.54 |

**GRAPHS AND CALCULATIONS**

* N = L/H N: Number of plates

H: Height

L: Length of column (15 cm)

* N = 16( tR / W)2 N: Number of plates

tR: Retention time

w: Peak width

1. **Number of Theoretical Plates and Heights**

**Methyl Paraben**

**N=** 16×**(** )^(2)= 4321.6

**H=** = 3.47×10-3

**Propyl Paraben**

**N=** 16×**(** )^(2)= 5276.6

**H=** = 2.82×10-3

**Ambroksol HCl**

**N=** 16×**(** )^(2)= 3794.6

**H=** = 3.95×10-3

**Syrup**

**Peak 1 for Methyl Paraben**

**N=** 16×**(** )^(2)= 4723.4

**H=** = 3.18×10-3

**Peak 2 for Ambroksol HCl**

**N=** 16×**(** )^(2)= 4113.7

**H=** = 3.65×10-3

**Peak 1 for Propyl Paraben**

**N=** 16×**(** )^(2)= 5082.8

**H=** = 2.95×10-3

**GRAPHS AND CALCULATIONS**

1. **Areas of Peaks of Different Concentrations of Solutions**

**Table 3.** Table of Peak Areas Measured with Different Concentrations of Solutions

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Area**  **(mAU x min)** | **Area**  **(mAU x min** | **Area**  **(mAU x min** |
| **Concentrations** | **Methyl Paraben** | **Ambroksol HCl** | **Propyl Paraben** |
| **5 ppm** | 8.89 | 2.05 | 3.23 |
| **10 ppm** | 15.52 | 2.54 | 4.21 |
| **15 ppm** | 21.12 | 3.59 | 4.38 |
| **20 ppm** | 28.52 | 5.09 | 5.44 |
| **25 ppm** | 40.45 | 6.70 | 8.54 |

1. **GRAPHS**

**Graph 1**. Different Concentration of Methyl Paraben Solutionsvs Peak Area Graph

**Graph 2.** Different Concentration of Ambroksol HCl Solutionsvs Peak Area Graph

**Graph 3.** . Different Concentration of Propyl Paraben Solutionsvs Peak Area Graph

**Graph 4.** Different Concentration of Unknown Solutionvs Peak Area Graph

From the Graph 4, y = 7,8571x and R² = 0,9878.

For Methy Paraben Area equal to 34,07 then using the formula 34,07=7,8571x then x equal to 4.718

For Ambroksol HCl Area equal to 4,25 then x equal to 0.5409

For Propyl Paraben Area equal to 4,02 then x equal to 0,5116

**POST-LAB QUESTIONS**

1. Different wavelengths are used in HPLC analysis to detect and measure analytes in mixtures. Differences in wavelengths depend on the absorption properties of the analytes and the solvent used. Each analyte has its unique absorption properties. For the optimization of the analytes' wavelengths, the analyte's maximum wavelength must be considered. Methyl paraben and propyl paraben are known as esters of p-hydroxybenzoic acid. Differences in the lengths of the carbon chains in their molecular structures can affect their polarity, solubility, and interaction with the stationary phase in the PLC column.
2. On the other hand, Ambroxol HCl is a brominated phthalimide with a side chain containing an amino and a hydroxy group, which complicates its molecular structure. It can be challenging to separate and detect in HPLC analysis. Syrup is a complex mixture of various sugars, water, and other additives, and a single compound is insufficient to define molecular structure. Having a complex structure can affect its separation and detection in HPLC analysis.
3. The order of polarity:

**Hydrocarbons < ethers < esters < ketones < aldehyde < amides < amines < alcohols**

The column used in the experiment is Thermo Scientific AcclaimTM 120 C18 (4.6 x 150 mm), and "C18" is a long column consisting of only carbons and nonpolar ones. If the polarity increases, the retention times decrease.

**The order of retention time;**

tR of propyl paraben=9.08 min

tR of ambroksol HCl =4.62 min

tR of methyl paraben=3.78 min

**Relative polarities:**

methyl paraben > ambroksol HCl > propyl paraben

1. In HPLC, the retention times of the compounds are equal to the time it takes for the compounds to elute from the column after they are injected into the mobile phase.

The retention times of ethyl paraben, propyl paraben, and ambroxol HCl will decrease with the mobile phase's polarity increase. The hydrophobicity of these compounds is considered the reason for this. The hydrophobic property of a combination plays a role in determining its interaction with the stationary and mobile phases in chromatography. If the phase is more polar, the hydrophobic compounds will interact less with the stationary phase, increasing their decomposition rate.

1. **Improved Fundamental Stability**: Online degasser is used to remove dissolved gases from the mobile phase, which causes the formation of fundamental fluctuations and has the power to affect the accuracy and precision of the peak area measurements. The elemental stability can be improved by eliminating the dissolved gases, and noise in the chromatogram can be suppressed.

**Improved Column Life**: bubbles may form due to gases in the mobile phase, which can cause backpressure in the column and reduce column efficiency. Column life and separation performance can be maintained by an online degasser removing dissolved gases.

**Increased Sensitivity**: band broadening causes lower chromatographic resolution, and dissolved gases cause Sensitivity. An online degasser can increase Sensitivity by removing gases.

**Kind Of Precautions:**

Using containers without air intake can minimize the effect of atmospheric gases, and solvents that do not contain dissolved gases can be used. If the degassing system is manual, sonication or sputtering can be done with an inert gas to degas the solvents .adequately.

Exposure to air can be minimized by using degassing caps in the preparation phase of the mobile phase.

1. Propylparaben has the least polarity and the most extended retention times, which causes increasing the broadening.
2. tm peak is also known as the valid peak. The reasons for this peak can be insufficient stabilization, column voids or leaks, preliminary solvent degassing, sample matrix interference, and detector saturation, such as controlling column leaks, proper dilution of the sample, or preventing sample matrix interference. The width of the tm peak can be reduced by taking precautions.
3. Pulse-free output is obtained by using a pulse damper or an accumulator, preventing pressure fluctuations or pulses that may occur in the moving phase flow. High-quality pipes and proper system maintenance contribute to pulse-free output. At the same time, suitable operating conditions optimize flow rate and temperature to provide pulse-free output and contribute.
4. Since the column is very thin in HPLC, if injected into the instrument without removing large particles, particles or impurities from the sample, the column may become clogged, preventing accurate and reproducible results. The filtering process contributes to reducing background noise and increasing the efficiency and Sensitivity of the analysis.
5. Isocratic elution is defined as the elution between a single solvent or a mixture of solvents of fixed composition. If two (and sometimes more) solvent systems that differ significantly in polarity are used and their composition changes during separation, it is called Gradient elution.

**REFERENCES**

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