# Laboratory #6

**Chem 6614 Instrumental Methods of Chemistry**

**SUNY Alfred State College**

**HPLC Component Analysis and Detection Limits (DL)**

**6.1. Background**

Despite its prevalence, simplicity and popularity, gas chromatography (GC) has serious limitations. First, samples must have substantial vapor pressure at temperatures not exceeding ~ 400 oC. Second, they must not decompose at these elevated temperatures. These two strictures eliminate GC as a way to analyze many compounds of medical or biological significance.

For this reason the method of High Pressure Liquid Chromatography (HPLC) has become a mainstay of many chemical laboratories. HPLC is conceptually similar to GC. Both GC and HPLC separate samples by forcing them through specially designed, coated columns which differentially bind. Detection in both GC and HPLC techniques also occurs **after** separation, as the individuated compounds exit.

Unique to HPLC, however, is the strategy of using a reciprocating high pressure (up to 60 MPa ~600 atm) pump which –if it is working- smoothly forces a solution of carrier solvent and sample across the (packed) HPLC column with reasonable speed, e.g. 1-10 mL/min. The pump eliminates the need to use high temperatures and gas phase motion to transport sample, and so virtually all compounds capable of solubilizing in aqueous or organic solvents at room temperature can be separated and detected. The drawback-as you may encounter- is that the high pressure (~ 60-100 X that in a GC) needed to force solvents across the very tightly packed HPLC columns requires use of a high pressure sample interface loop of fixed volume (10 uL- 2 mL). Both the interface and the HPLC plumbing after the interface are often compromised by bubbles, column inhomogeneities and leaks.

The detection system of an HPLC is also markedly different from a GC. The latter often ionizes or burns the target compounds to create a signal since the carrier gas is inert. In HPLC, the carrier is generally a mixture of organic liquids. Hence, the target compounds are generally detected by measuring a difference between the solvent and the solvent + target. This can take the form of an electrochemical current, refractive index, fluorescence, or, as is the case with the Alfred PE HPLC, a UV absorption.

Finally, while HPLC is normally done at room temperature, separation of complex mixtures is often attained using a programmed variation in solvent mixtures which –like a temperature ramp- provides advantages in resolving components.

**6.2. Purpose**

1) An unknown mixture of aromatic compounds (benzene, toluene and ethyl benzene) will be separated and the volume composition of the mixture deduced using an isocratic (constant flow rate) HPLC schedule on the ASCOT lab’s PE HPLC and a known mixture of the same compounds

2) The HPLC detection limit for one of components , ethyl benzene in methanol will be estimated.

**6. 3. Procedure**

**6.3.1. Standard Reference Mixture (provided)**

Amixture of containing benzene, toluene and ethyl benzene all at 4% v/v concentrations (1 mL of each in a total of 25 mL) will be provided

**6.3.2. Ethyl Benzene Calibration Solutions. (provided by N. Preston)**

Standard solutions containing the volume % ethyl benzene in methanol will be provided

As prepared from a stock solution of 5 % v:v ethyl benzene in methanol (2.5 mL ethyl benzene diluted to 50 mL with HPLC grade methanol) using the schedule shown in Table 1.

**Table 1:**

**Standard Preparations for Ethyl Benzene Detection Limit Determination**

|  |  |  |
| --- | --- | --- |
| Standard solution | mL 5% ethyl benzene /50 ml methanol | Volume % |
| 1 | 0.5 | 0.05 |
| 2 | 1.0 | 0.10 |
| 3 | 2.0 | 0.20 |
| 4 | 4.0 | 0.40 |

**6.3.2. Operation of the Alfred HPLC**

The instructor will demonstrate operation of the PE HPLC and the isocratic conditions for obtaining a `standard’ chromatogram (1 mL/min; λ = 280 nm sensitivity= 0.001, response=FAST; 70% methanol/30 % water ). Students will then be asked to `start from scratch’ with the instrument off, and establish a flow schedule and a satisfactorily pumping system. The `guard’ column will be used to produce separation. **The conditions of flow, analysis wavelength, sample loop volume and preparative sparging protocol should be noted in your** `**Procedure’ but you don’t have to describe how you used the instrument.**  You will, however, need to provide details of what samples you ran (see below)

**6.3.3. Standard Reference Mixture (prepared by N. Preston)**

Using the conditions established above each team will inject the standard, 5% reference mix’ of components provided in 6.3.1 and record the observed peak maximum absorbances , **ak [ref]** (where k=1,2...; refers to the kth component) from the screen output in a table as shown below. You may need to `reintegrate’ your peaks for **ak** if the selected baseline chosen by the computer looks stupid. See separate handout entitled:

Instructions for manually re-integrating HPLC peaks on PE Nelson system

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Put **Table 2** in `**Observations.** Peak assignment will be provided . Make sure to record the file name assigned to the standard run.e.g. INST10xx. The printer is down, so the only source of `hard data’ will be stored on the computer hard drive.

**Table 2: Retention times and Integrated Areas (Ak[ref]) of Standard Reference Mixture**

|  |  |  |
| --- | --- | --- |
| **Component** | **retention time, tr** | **ak [ref], absorbance** |
| **Benzene** |  |  |
| **Toluene** |  |  |
| **Ethyl benzene** |  |  |

Computer file name where data is stored: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**6.3.4. Unknown Mixture**

Immediately following the standard reference run and under the same conditions established in 6.3.3. each team should run their unknown mixture and record the observed peak areas, **Ak [unk]** in **Table 3** within **Observations.** As before, you may need to re-integrate manually. Collect the unknown chromatogram and place them in your `**Observations.** Make sure to record the filename assigned to the unknown run.

**Table 3: Retention times and Integrated Areas (Ak[unk] of Unknown Mixture (3 assumed)**

|  |  |  |
| --- | --- | --- |
| **Component** | **retention time, tr** | **Integrated Area Ak**[unk] |
| **Benzene** |  |  |
| **Toluene** |  |  |
| **Ethyl benzene** |  |  |

Computer file name where data is stored: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**6.3.5. Diluted Standard Chromatograms**

Under the same conditions established in 6.3.3. the class will collectively run the four samples prepared in Table 1 (section 6.3.2.) Note that a triplicate of standard 1 is required. Record the observed calibration peak areas, **Acal** for the ethyl benzene component for each of the standard solutions in **Table 4.** You do not need to collect the chromatograms since the printer is malfunctioning. However, In each case, record the file name of the 4 standard chromatograms of ethyl benzene.

**Table 4: Retention times and Integrated Areas (Ak** ) for **Standard Ethyl Benzene Mixtures**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Standard mixture** | Volume % | **retention time, tr** | **Integrated Area Acal** | **File name** |
| **1** | 0.05 |  |  |  |
| **1** | 0.05 |  |  |  |
| **1** | 0.05 |  |  |  |
| **2** | 0.10 |  |  |  |
| **3** | 0.20 |  |  |  |
| **4** | 0.40 |  |  |  |

**6.4. Calculations**

6.4.1. **Component Sensitivities, γk**

The absolute volume of each component of the PE Reference Mixture, Vk[ref] injected is calculable as below:

**1a Vk[ref] = total sample loop volume (μL) \* vol % kth component**

**100**

Since all three components are made up to 5%

**1b Vk[ref] = total sample loop volume (μL) \* 5 = 0.05\* sample loop volume**

**100**

Thus, since the sample loop size is 20 μL, each Vk = 0.05\*20= 1 μL.

The analytical sensitivity, **γk** for that component can then be established according to the formula:

**2** γk **= Ak [ref] /Vk[ref] kth component sensitivity**

If loop volume is 20 uL, then γk **= Ak [ref] /1=** γk **= Ak [ref]**

Compute and collect your ascertained γk in **Table 5** as below:

**Table 5: Computed Analytical Sensitivities of Reference Standard Components**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Component** | **% Vk** | **Vk**  (see equation 1) | **Ak [ref]** | γk **= Ak [ref] /Vk[ref]**  (see equation 2) |
| **Benzene** | **5** |  |  |  |
| **Toluene** | **5** |  |  |  |
| **Ethyl benzene** | **5** |  |  |  |

**6.4.2 Unknown Component Volumes, Vk[unk]**

The foregoing allows computation of the unknown component volumes according to:

**3** **Vk(unk) = Ak[unk]/γk uL volume of kth unknown component**

Compute and collect the deduced Vk[unk] in **Table 6** as below:

**Table 6: Computed Volumes [Vk(unk)] of Unknown Components (3 assumed here)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **Integrated Area k**[unk] | γk **(from table 5)** | **Vk(unk) = Ak(unk)/γk uL** |
| **Benzene** |  |  |  |
| **toluene** |  |  |  |
| **Ethyl benzene** |  |  |  |

**6.4.3. Detection Limit Calculations for Ethyl Benzene**

Use equation 1 in 6.4.1 to calculate the actual detected volume of the standard ethyl benzene in each standard mix and tabulate the results in Table 7. Note that if the loop volume is 20 uL, then standard mixture #1 will have a Vk= (0.05 %/100 )\* 20 = 0.010 μL . Note that the minimum Volume% standard is repeated 3 times to establish an uncertainty, s, in the extracted ak

**Table 7: Standard Ethyl Benzene Mixtures**

Ak =m Vk  +b

**ak**

|  |  |  |  |
| --- | --- | --- | --- |
| **Standard mixture** | Volume % | **Vk ul** | **ak[ref]** |
| **1** | 0.05 |  |  |
| **1** | 0.05 |  |  |
| **1** | 0.05 |  |  |
| **2** | 0.10 |  |  |
| **3** | 0.20 |  |  |
| **4** | 0.40 |  |  |

Vk

.Plot ak vs Vk  in EXCEL with Vk as the x axis and ak as the y axis.

Carry out a linear regression analysis assuming:

Ak = mVk +b

and include a copy of the plot and fit in **Calculations.**

The detection limit, DL in ng is computed as follows:

Using **Table 7**, compute the standard deviation of A1 = s1 for standard mixture #1, the uncertainty in the minimum area of the calibration. (This assumes no variation in s with magnitude of A . The lowest concentration in the calibration is used to at least be nearer to DL)

The detection limit, DL in ng is computed as follows:

**4** **DL** = 3\*s1 \* 0.001 mL\* 0.886 g \*109 **ng**

m uL mL g

DL in uL uL🡪mL ethyl benzene g🡪 ng

density g/mL

**6.5 Results**

* **Unknown mixture volumes**

|  |  |
| --- | --- |
| **Unknown Component**  **Report this:** | **Vk(unk) uL** |
| **Benzene** |  |
| **toluene** |  |
| **Ethyl benzene** |  |

* **DL of ethyl benzene in ng**