Sultan Qaboos University

College of Medicine & Health Science

Instrumentation and Biomedical Techniques

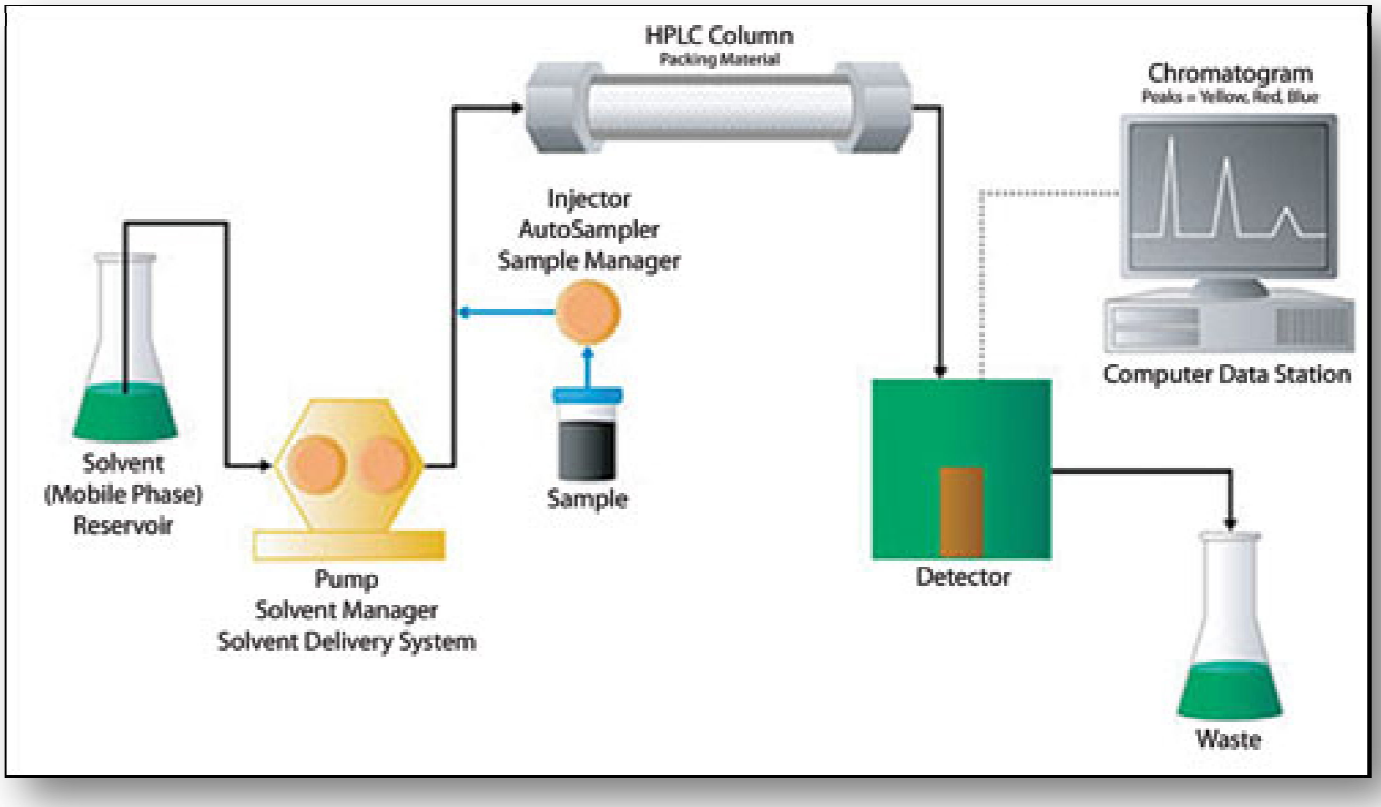
BIOC 6002

Laboratory Report

Practical No. (6): 30/10/2011

Gel Filtration Chromatography

(By using High Performance Liquid Chromatography-HPLC)



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❖ Introduction:

Column chromatography originally used large “soft” stationary phases that required low pressure flow of the mobile phase to avoid compression; separations were usually time-consuming and of low resolution. Subsequently, the production of small, incompressible, homogeneous particulate support materials and high pressure pumps with reliable, steady flow rates have enabled high performance systems to be developed. These systems operate at pressures up to **10 MPa**, forcing the mobile phase through the column at a high flow rate to give rapid separation with reduced band broadening, due to smaller particle size.

HPLC columns are usually made of stainless, and all components valves, etc., are manufactured from materials which can withstand the high pressures involved. The two main systems are:

1. **Isocratic separation:** a single solvent is used through the analysis

2. **Gradient elution separation:** the composition of mobile phase is altered using a microprocessor-controlled gradient programmer, which mixes appropriate amounts of two different substances to produce the required gradient

Most HPLC systems are linked to a continuous monitoring detector of high sensitivity, e.g. proteins may be detected spectrophotometrically by monitoring the absorbance of the eluent at 280 nm as it passes through a flow cell (cuvette). Other detectors can be used to measure changes in fluorescence, current or potential. Most detection systems are non-destructive, which means that you can collect eluent with an automatic fraction collector for further study.

The speed and sensitivity of HPLC make this the method of choice for the separation of many small molecules of biological interest, normally using reverse phase partition chromatography. Separation of macromolecules (especially proteins and nucleic acid) usually requires “biocompatible” systems in which stainless steel components are replaced by titanium, glass or fluoroplastics, using lower pressures to avoid denaturation, e.g. the Pharmacia FPLC system. Such separations are carried out using ion-exchange, gel permeation and/or hydrophobic interaction chromatography.

❖ Aim:

In this practical session, we are going to run our previously extracted, eluted and prepared egg with sample fractions. We are using gel filtration chromatography by the means of HPLC. We also prepared our calibration standards from commercially available lysozymal enzyme stock of 1.0 mg/mL. Overview about the HPLC principle and operation together with programming the software (Millennium) which operates the HPLC analyzer.

❖ **Materials:**

1. HPLC:
    a. Column: Bio-Silect®SEC 250-5, 300 mmX7.8mm
    b. HPLC pump, vials, and sample holders
    c. Mobile phase: buffer 0.10 M Sodium Phosphate, 0.15 M NaCl
    d. UV-detector attached to measure protein at 280 nm (photo-diode array)
    e. PC unit attached to HPLC
    f. System specification: 20 uL injection volume, 1.0 mL/mintues flow rate

2. Standard lysozyme solution of 1.0 mg/mL

3. Sample fractions. (F0, F1, and F2) and ion exchange elution's (4,5,6,7,and 8) and fractions from GFC (71,26,27,79,64,49, and 66)

4. Tubes

❖ **Methodology:**

1. We have first prepared our calibration standards from 1.0 mg/mL (10 mg/10 mL) of commercially available lysozyme as follow:

Serial No	C_1 in mg/mL	V_1 in uL	C_2 in mg/mL	V_2 in mL
1	1.0	*37.5*	0.025	1.5
2	1.0	*202*	0.135	1.5
3	1.0	*367*	0.245	1.5
4	1.0	*532*	0.355	1.5
5	1.0	*697*	0.465	1.5
6	1.0	*862*	0.575	1.5
7	1.0	*1192*	0.795	1.5

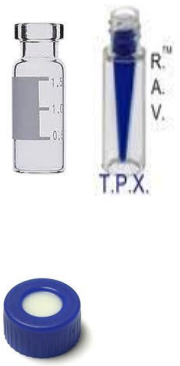


Table: (1) Dilution to prepare calibration standards from 1.0 mg/mL

2. After that we have collected specified saved samples from refrigerator at + 4 °C and let them outside to warm up at room temperature

3. We have filled the vials with standards and our sample fraction

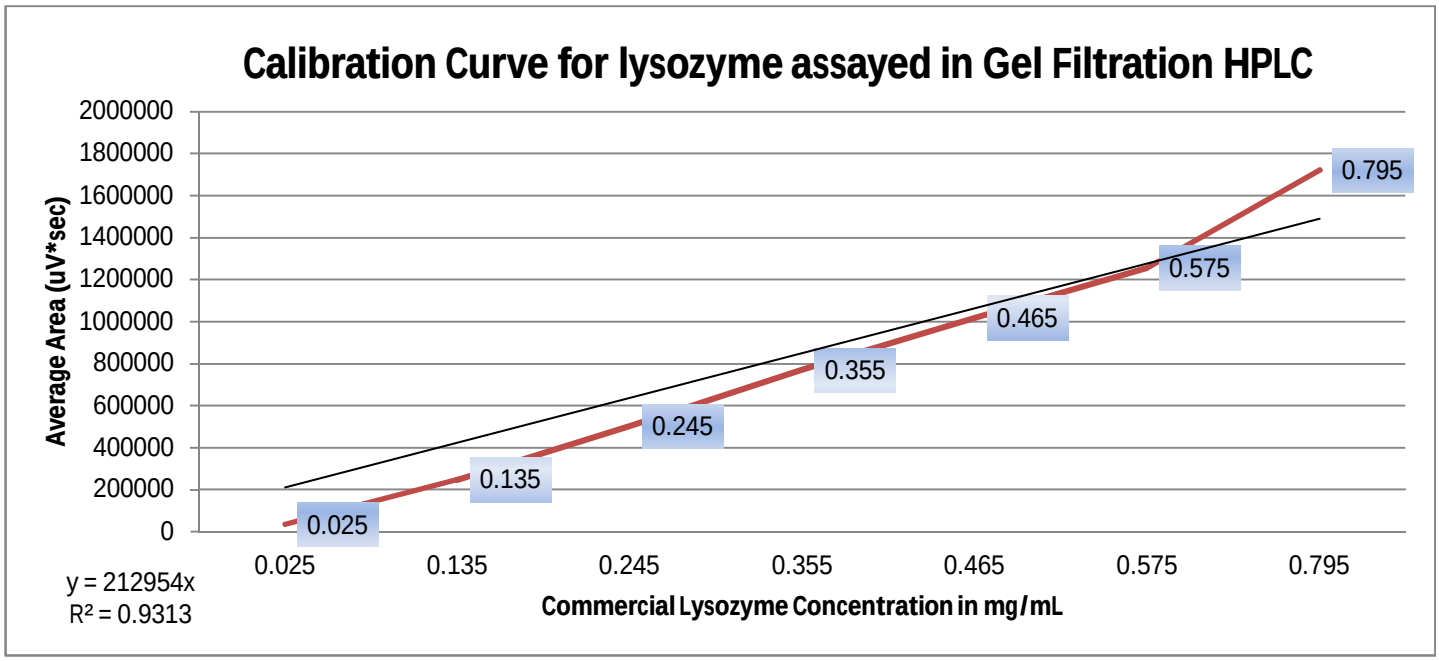
4. Then finally vials loaded into sectors carousal inside HPLC pump with programming

❖ **Results, Calculations & Interpretation:**

Below are the results for commercially prepared lysozyme calibration standard. As observed from the table the lysozyme retention time **(RT) = 10.3 mintues**

#	Concentration	Sample Name	Rt (min)	Area (uV*sec)	Average Area	Height (uV)
1	0.025	Standard-1	10.341	36061	**36244.5**	1610
1	0.025	Standard-1	10.333	36428		1611
2	0.135	Standard-2	10.337	234890	**249633**	9427
2	0.135	Standard-2	10.296	264376		9080
3	0.245	Standard-3	10.317	495645	**503026.5**	18203
3	0.245	Standard-3	10.319	510408		18895
4	0.355	Standard-4	10.317	767112	**770968**	27215
4	0.355	Standard-4	10.313	774824		28917
5	0.465	Standard-5	10.315	1017073	**1019299.5**	36604
5	0.465	Standard-5	10.311	1021526		37276
6	0.575	Standard-6	10.306	1237096	**1256675**	46198
6	0.575	Standard-6	10.304	1276254		47170
7	0.795	Standard-7	10.305	1722156	**1721228.5**	63250
7	0.795	Standard-7	10.305	1720301		64203
-	**Average=**	**-**	**10.31564**	**-**	**-**	**-**
-	**SD=**	**-**	**0.013258**	**-**	**-**	**-**
-	**CV%=**	**-**	**0.128527**	**-**	**-**	**-**

Table: (2) Calibration standard run in HPLC for commercial lysozyme



Graph: (1) Calibration Standard for commercial lysozyme in Gel-Filtration HPLC

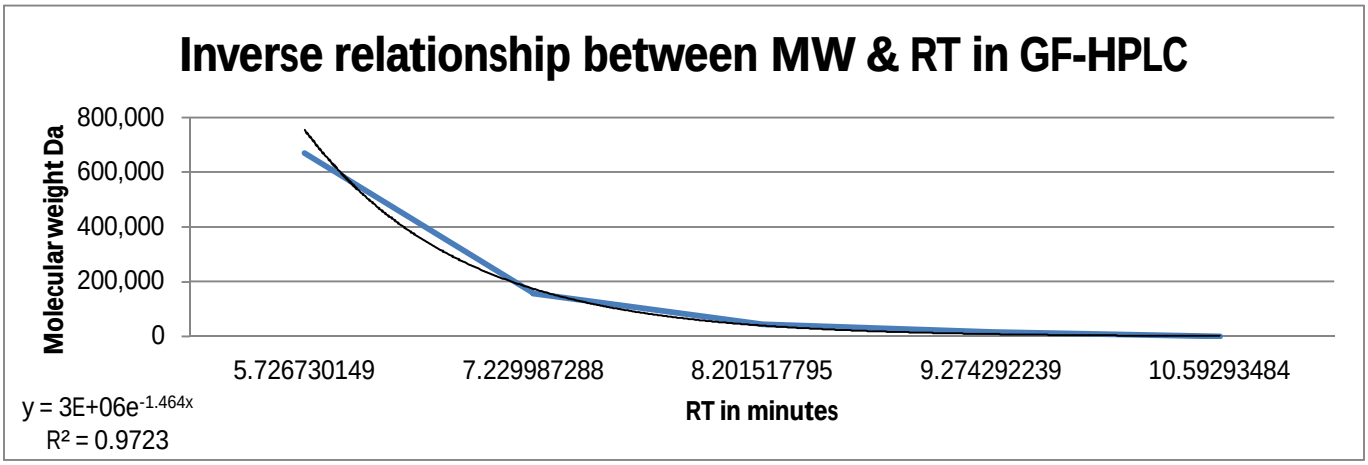
By analyzing our sample fractions we got the following results:

Sample Name	Rt (min)	Area (uVXsec)	Height (uV)	Comments
F_0(X50)	**10.399**	61180	2179	**Lysozyme detected**
F_1(X50)	No peak	No Area	No Height	No result
F_2(X50)	No peak	No Area	No Height	No result
71	No peak	No Area	No Height	No result
26	*8.302*	123225	3833	RT? Ovalbumin
27	*8.307*	114476	3689	RT? Ovalbumin
79	No peak	No Area	No Height	No result
64	No peak	No Area	No Height	No result
49	No peak	No Area	No Height	No result
66	No peak	No Area	No Height	No result
E8	**10.341**	46023	3072	**Lysozyme detected**
E7	**10.318**	569615	20785	**Lysozyme detected**
E4	**10.395**	146642	4689	**Lysozyme detected**
E5	**10.363**	2158156	68680	**Lysozyme detected**
E6	**10.348**	7341	652	**Lysozyme detected**

Table: (3) Sample fractions analysis by Gel-Filtration Chromatography

❖ **Observations & Conclusion:**

From table no 3 we can easily detect our samples which contain lysozyme by comparing the retention times (10.3 minutes) peaks eluted from the size exclusion column chromatography. Fractions (F0, E4, E5, E6, E7, and E8) contain lysozyme enzymes. However; no lysozyme enzymes been found in F1 and F2 fractions which is obvious. These results match with our purification table constructed in the previous lab sessions.Unusual RT values been also found in our chromatogram results in the fractions 26 and 27.

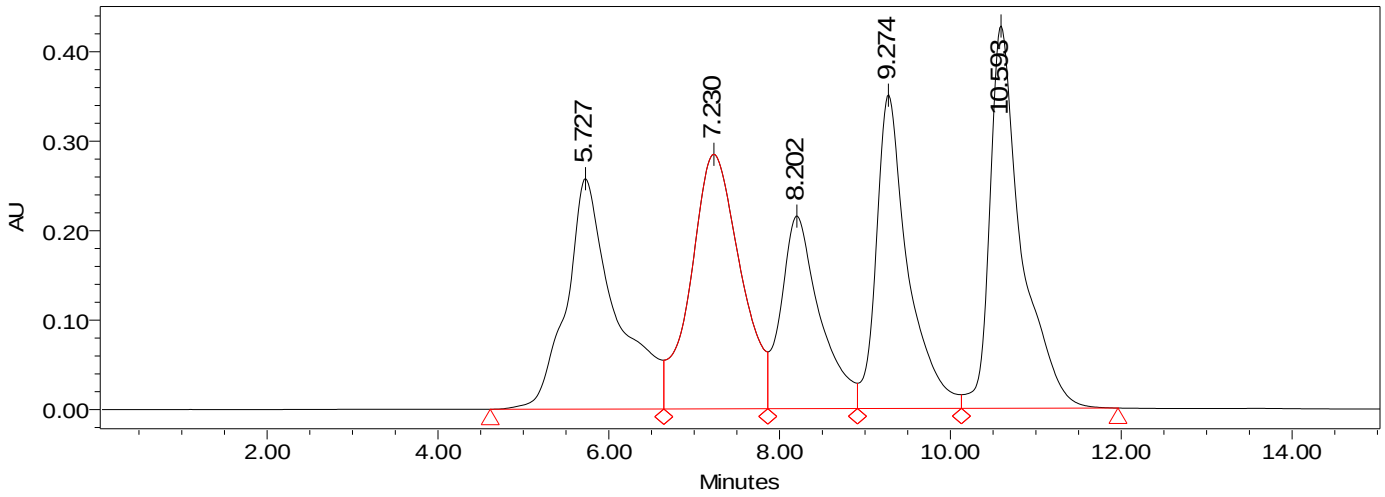


Graph: (2) Shows inverse exponential relationship between MW & RT values

Name	MW	Retention Time	Area	Height
Thyroglobulin (bovine)	670,000	5.726730149	10278274	257633.5
Gamma globulin (bovine)	158,000	7.229987288	11518579	284517.2
Ovalbumin (chicken)	44,000	8.201517795	6814902	215505.9
Myoglobin (horse)	17,000	9.274292239	9030026	350640.3
Lysozyme (Interested)	**14600**	**10.3**	-	-
Vitamin B-12	1,350	10.59293484	10912337	427561.2

Table: (4) Calibration Standard by Gel-Filtration Chromatography*

It can be seen from table above that heavier molecular has lowest RT values which is the characteristic of GFC. Here we should not to be mistaken by the area because its only represents the concentration of molecule and not its size. See the graph below:



Graph: (3) Calibration Standard Chromatogram in Gel-Filtration HPLC

Elution of molecules depends on their size and not concentration. So lysozyme will be eluted nearly 10.3 mintues.

- *If we compared our lysozyme's MW with the calibration standard's components we can see that RT and MW of lysozyme matches the size excluding chromatography of this STD.*

2. **Using the gel filtration standards, what is the expected retention time for lysozyme?**

   RT = 10.3 mintues

3. **Is the expected retention time for lysozyme fits with the obtained from standards? If not, Why?**

Yes it fits with our standards RT values.

4. **Prepare standard curve for lysozyme and calculate lysozyme concentration in each loaded sample? Comment on the results.**

   See graph no 1

Concentrations	area	factor
0.025 mg/mL	36244.5	0.0000006898
0.135 mg/mL	249633	0.0000005408
0.245 mg/mL	503026.5	0.0000004871
0.355 mg/mL	770968	0.0000004605
0.465 mg/mL	1019300	0.0000004562
0.575 mg/mL	1256675	0.0000004576
0.795 mg/mL	1721229	0.0000004619
Average Factor=		**0.0000005077**

Table: (5) Calibration factor for standard curve

Thus the concentration of fraction calculated as followed:

Sample Name	Rt (min)	Area (uV*sec)	Height (uV)	Concentrations
F0(X50)	**10.399**	61180	2179	0.0311 mg/mL
E8	**10.341**	46023	3072	0.0234 mg/mL
E7	**10.318**	569615	20785	0.2892 mg/mL
E4	**10.395**	146642	4689	0.0744 mg/mL
E5	**10.363**	2158156	68680	1.0956 mg/mL
E6	**10.348**	7341	652	0.0037 mg/mL

Table: (6) Showing multiplication of unknown samples with calibration factor

These findings also correlate with our purification table results. Highest concentration found in E5, E7, and E4 fractions.

5. **How to improve peak resolution?**

Of course lowering the flow rate, longer column height, narrow columns and controlling the pore sizes for smaller peptide may enhance the resolution of separated peaks which are very close in MW to each other. Similar or close MW may cause curve overlap and lower the resolution. Increasing the column diameter increases the capacity of the column. Proper column packing is important to maximize resolution: An over packed column can collapse the pores in the beads, resulting in a loss of resolution. An under packed column can reduce the relative surface area of

the stationary phase accessible to smaller species, resulting in those species spending less time trapped in pores. Unlike affinity chromatography techniques, a solvent head at the top of the column can drastically diminish resolution as the sample diffuses prior to loading, broadening the downstream elution.

❖ References:

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