DEPARTMENT OF BIOCHEMISTRY

BIC 8X01

Advanced Analytical Techniques

EXAM - MEMO

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TIME: 3 HOURS

TOTAL MARKS: 100

This paper consists of two main parts, 1 and 2. Answer all questions. Use relevant equations and diagrams to add to your answers where applicable.

PART 1

QUESTION 1 [10]

If you have to assess and compare the performance of two analytical methods, which performance indicators would you evaluate? List the **5 most important parameters** with a short **description** of each.

Precision (imprecision and variability): a measure of the reproducibility of a particular set of analytical measurements on the same sample of test analyte. If the replicated values agree closely with each other, the measurements are said to be of high precision.

Accuracy (trueness, bias and inaccuracy): this is the difference between the mean of a set of analytical measurements on the same sample of test analyte and the 'true' value for the test sample.

Analytical range (AR): - range of concentrations of test analyte that can be measured reproducibly. Detector response, R vs [C]

Analytical sensitivity (AS): - Change in response of the method to a defined change in the quantity of analyte = slope of linear calibration curve $(\Delta y / \Delta x) = (\Delta R / \Delta [c])$

Analytical specificity (selectivity): this is a measure of the extent to which other substances that may be present in the sample of test analyte may interfere with the analysis and therefore lead to a falsely high or low value.

Detection limit or Limit of detection (LOD) = smallest amount of analyte that can be distinguished from zero (but not accurately quantified) and Limit of quantification (LOQ) = smallest amount of an analyte that can be quantified with a defined degree of confidence.

QUESTION 2 [10]

The basis of all forms of chromatography is the distribution or partition coefficient.

2.1 Define this coefficient and explain how it relates to the retention factor (2)

>>> Distribution/partition coefficient is an expression that describes the way in which a compound (analyte) distributes between two immiscible phases in chromatography. The value of this coefficient is a constant at a given temperature and is given by the expression: K_d = conc. in phase A / conc. in phase B.

>>> The distribution/partition coefficient, K_d, is related to the retention factor, *k*, by the following expression below, involving the mass of analytes in both phases (M_S and M_M), which can be expressed in the volume of the analytes in the two phases >>> V_S/V_M is referred to as the volumetric phase ratio, β

$$k = \frac{t'_R}{t_M} = \frac{M_S}{M_M} = K_d \times \frac{V_S}{V_M}$$

OR $k = K_d \beta$

The retention factor for an analyte will increase with both the distribution coefficient between the two phases and the volume of the stationary phase.

2.2 How can the retention factor be determined by analyzing a chromatogram? (2)

>>> A typical chromatogram with retention time details
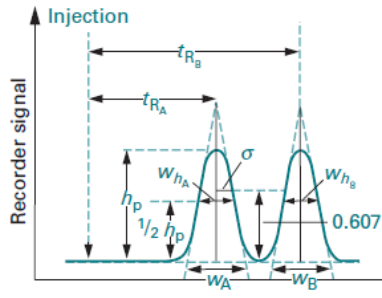
And the retention factor is given by the expression:

$$k = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M}$$

where t_M = the dead time, which can be measured by using an analyte that does not interact with the stationary phase. t'_R = adjusted retention time.

2.3 What is the selectivity factor of a chromatographic system and what influences this factor? (2)

>>> The selectivity factor of a chromatographic system is a measure of its inherent ability to discriminate between two analytes and it's given by the following expression:

$$\alpha = \frac{k_A}{k_B} = \frac{K_{d_A}}{K_{d_B}} = \frac{t'_{R_A}}{t'_{R_B}}$$

>>> The selectivity factor is influenced by the chemical nature of the stationary and mobile phases.

2.4 Chromatographic columns are often compared with regards to the number of 'theoretical plates'. Explain what this is and why two well-separated analytes will have different values for the number of theoretical plates. (2)

>>>> Theoretical plats represent a hypothetical division of chromatographic columns, and each plate represents an equilibrated partitioning of the analyte between the stationary and mobile phases. The <u>more theoretical plates available within a column</u>, the more the equilibrations between the stationary and mobile phases are possible and the <u>better the quality of the separation</u>

$$R_S = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k_2}{1 + k_{av}} \right)$$

2.5 Why are HPLC/UHPLC chromatographic columns housed in a column oven? (explain your answer by referring to chromatographic concepts) (2)

>>> HPLC/UHPLC chromatographic columns are housed in a column oven for <u>temperature control/regulation</u>.
>>> Change in column temperature will change / affect the distribution coefficient, K_d, affecting (positively or negatively) the resolution (R_S).

QUESTION 3 [10]

3.1. In protein research, chromatography is often used in conjunction with electrophoresis to characterize the physico-chemical properties of an isolated/purified protein. With the focus on chromatography, mention **three** different techniques that can be used and indicate the **properties** of the protein that can be determined. (3)

- Gel filtration / Molecular (size) exclusion chromatography – size
- Ion exchange chromatography – charge / pI estimate
- Adsorption Chromatography, particularly Hydrophobic interaction chromatography (HIC) – hydrophobicity

3.2. Reverse phase liquid chromatography is one of the most widely used separation/ analytical techniques. Describe the composition of such as system and mention the order of elution of polar / mid-polar / non-polar analytes (4)

- RP-LC – stationary phase is a chromatographic column and mobile phase is a liquid; and isocratic or gradient elution is applied, and detectors can be UV, Fluorescence, MS
- The property of the stationary phase = non-polar (e.g. C18); the property of mobile phase = relatively polar (but less polar than water)
- The order of elution of analytes (using gradient elution) = (i) the polar analytes elute first because such analytes (polar) are less retained on the non-polar column, (ii) the mid-polar analytes elute second since these analytes are relatively retained on the column and lastly (iii) non-polar analytes are more retained on the non-polar column and elute last

3.3. You work in a laboratory that performs analyses of environmental samples and must write a motivation for the use of gas chromatography (GC) as an analytical technique for the analysis of chlorinated pesticides. As part of your motivation, **explain** how chromatographic principles are applied in the case of GC and **which type** of analytes are suitable for analysis by GC (3)

>>> <u>Chromatographic principles in GC</u>: As in all other forms of chromatography, the basis of gas chromatography (GC) is the partition coefficient. GC exploits differences in the partition coefficients between a stationary liquid phase and a mobile gas phase of the volatilized analytes as they are carried through the column by the mobile gas phase. The partition coefficients are inversely proportional to the volatility of the analytes so that the most volatile elute first. The temperature of the column is raised to 50 – 300 °C to facilitate analyte volatilization. The

stationary phase = a high-boiling-point liquid material such as silicone grease or wax that is either coated onto the internal wall of the column or supported on an inert granular solid and packed into the column. The mobile phase = carrier gas, an inert gas, e.g. nitrogen (for packed columns) or helium or argon (for capillary columns)
>>> Type of analytes = volatile (low polarity / non-polar) and thermally stable analytes

QUESTION 4 [10]

Mass spectrometry, often regarded as the science of ions, has wide range of applications in life sciences. Please answer the following questions.

a) Explain (briefly) how the MALDI technique functions (3)

Matrix assisted laser desorption ionization (MALDI) >>> (**1**) sample test is mixed with matrix (X) and dried on plate; (**2**) laser flash ionizes matrix molecules; (**3**) sample molecules (M) are ionized by proton transfer ($XH^+ + M \rightarrow MH^+ + X$).

b) Explain how the electrospray ionization (ESI) source functions (3)

Electrospray ionization (ESI) >>>> (**1**) formation of charged droplets; (**2**) drastic reduction of droplet size; and production of desolvated ions from highly charged micro-droplets; (**3**) transport of free gas phase ions into the mass analyser.

c) What **types of ions** that can be formed during the ESI process of an analyte **M** (4)

Type of ions that could be formed during ESI: **(i)** protonation = $[M+H]^+$; **(ii)** deprotonation = $[M-H]^-$; **(iii)** adducts = $[M+A] +/-$; **(iv)** in-source fragments.

QUESTION 5 [10]

Mass spectrometry is a very powerful analytical technique with wide applications in the biological sciences.

5.1. Explain in detail the events that happen during electron impact ionization of analytes that lead to the generation of a mass spectrum of an analyte. (7)

1. A stream of high energy electrons is generated from a heated filament
2. Gaseous analytes in ionization chamber are bombarded with electrons, leading to
3. A loss or gain of electrons (*i.e.* radical $M^{.+}$ cations or $M^{.-}$ anions)
4. = molecular ions / precursor ions (unstable due to excess energy),
5. Excess energy leads to disintegration of precursor ion to unstable fragment ions (and radicals) that can
6. further break down to product ions (and neutral radicals),
7. which are separated in the mass analyzer and recorded as a mass spectrum = fragment fingerprint

5.2. To obtain structural information on biomolecules and sequence information (in the case of proteins and peptides), tandem MS experiments are performed. **Explain** how quadrupole technology has been applied to make tandem MS possible (3)

By using 3 quadrupoles in series, an ion of specific *m/z* can be trapped (selected) in Q1, subject to further fragmentation through CID (Argon gas) in Q2 before further separation of generated fragments (resulting from the selected / trapped ion) in Q3

Total marks for Part 1: [50]

PART 2

SECTION A (UV/Vis spectroscopy)

[10]

QUESTION 1 **[6]**

1.1. Ultraviolet-visible (UV/Vis) light spectroscopy is one of the analytical techniques used in analytical chemistry and biochemistry for routine analyses and research work. This technique, like any other spectroscopic techniques, is fundamentally based on the interaction of light (electromagnetic radiation) with matter, a quantum phenomenon. Describe an **electromagnetic radiation**, and make a reference to UV/Vis spectroscopy (6 lines max). (3)

Fig 12.1. Electromagnetic radiation can be described as a wave propagating transversally in space and time. It is composed of both the electric (*E*) and magnetic (*M*) field vectors, which are directed perpendicular to each other. For **UV/Vis** spectroscopy, the electric field vector is of most importance. For UV/Vis spectroscopy, this radiation on the electromagnetic spectrum, is in the region of (about) 10^2 to 10^3 nm wavelength (Fig 12.2).

1.2. In UV/Vis spectroscopy, structural components of molecules that are responsible for interaction with electromagnetic radiation are known as chromophores. What are the types of chromophores found / explored in proteins? (3)

(i) peptide bonds (amide bond); **(ii)** certain amino acid side chains (mainly tryptophan and tyrosine); **(iii)** certain prosthetic groups and coenzymes (e.g. porphyrine group such as in haem).

QUESTION 2 [4]

2.1. The A solution with a concentration of 0.10M is measured to have an absorbance of 0.42. Another solution of the same biochemical analyte is measured under the same conditions and has absorbance of 0.32. What is its concentration? (2)

C1/C2 = A1/A2 >>> C2 = C1 / (A1/A2) >>> C2 = 0.10M / (0.42/0.32) = **0.076 M OR** using the equation of A = Ɛ.C.l, with the assumption that l and Ɛ are the same, **calculate Ɛ** from C1 and A1, then use it to calculate C2, given A2

2.2. A solution of protein B shows a transmittance of 20%, when taken in a cell of 2.5 cm thickness. What is the molar concentration of this solution, if the molar absorption coefficient is 12000 $dm^3\,mol^{-1}\,cm^{-1}$. (2)

A = log 1/T >>> T = 20% >>> A = log (1/20%) >>> A = log 5 = 0.698; And A = Ɛ.C.l >>> C = A / (Ɛ.l) >>> C = 0.698 / (12000 L $mole^{-1}\,cm^{-1}$. 2.5 cm) >>> **C = 2.33 × 10^{-5} M**

SECTION B (Spectroscopic techniques II)

[20]

QUESTION 1 [10]

1.1. In a table format, compare Raman and infrared (IR) spectroscopy techniques, specifically on the following aspects: (i) type / mode; (ii) what is measured; (iii) the criterion for a band/peak to appear in the spectrum; (iv) molecular structural information recorded (8)

(i)   Type/mode: both Raman and IR are <u>vibrational</u> spectroscopy
(ii)   <u>Raman scattered light</u> (Raman) and <u>absorbed light</u>/transmittance (IR)
(iii)   The criterion for a band to appear in the infrared spectrum is that the transition to the excited state is accompanied by a change in <u>dipole moment</u>, i.e. a change in charge displacement. Conversely, the criterion for a peak to appear in the Raman spectrum is a change in <u>polarizability</u> of the molecule during the transition.
(iv)   <u>Functional groups</u>; and fingerprinting (in IR)

1.2. Write briefly about bending vibrations in IR spectroscopy (5 lines max) (2)

An IR spectrometer detects how the absorption of a sample varies with wavenumber (cm^{-1}). The wavenumber is proportional to the energy or frequency of the vibration of the bonds in the molecule. Bending vibrations are <u>form of vibrations that change the angle between two bonds</u>. There are <u>four types</u> of bending vibrations: <u>scissoring, rocking, wagging and twisting</u>.

QUESTION 2 [5]

Signals recorded on a 1D ^{1}H NMR spectrum are chemical shifts representing frequencies from all NMR-visible nuclei (protons, H's) in a sample.

2.1. Explain the use of deuterium oxide and TSP in the sample preparation step, in a proton-NMR experiment (2)

Deuterium oxide: to stabilize the magnetic field strength (locking of the signal), avoiding the proton signal from non-deuterated solvent to overshadow signals from the sample/analytes. TSP: an internal standard, and used as chemical shift reference for the calibration of the NMR data/spectra (at δ 0.0 ppm), and for quantification.

2.2. Write down the expression showing the dependence of the S/N ratio on the number of scan. (1)

The signal/noise-ratio is proportional to the number of scans performed. S/N = square root of NS, where S/N = signal to noise ration; and NS = number of scans

2.3. The fundamental rule governing (first order) multiplet intensities for spin 1/2 nuclei with all couplings identical is Pascal's triangle (n = number of equivalent couplings). Explain (4 lines max) a triplet in a 1D proton-NMR spectrum. (2)

A triplet (in a 1D H-NMR spectrum) will arise from peak splitting into three peaks due to two (un-equivalent) hydrogens on the adjacent atoms, with an area (intensities) in the ratio of 1:2:1 (n+1 rule, Pascal's triangle)

QUESTION 3 [5]

Signals recorded on a 1D ^{1}H NMR spectrum are chemical shifts representing frequencies from all NMR-visible nuclei in a sample. Thus, in 1D ^{1}H NMR metabolite profiling of plant extract samples, the generated spectrum is the result of the superposition of the NMR spectra of all NMR-visible single compounds present in the extracts.

3.1. To extract information from such analyses, data processing is required prior to statistical analyses. Explain (very briefly, 5 lines max) spectral 'bucketing or binning'. (3)

Spectral 'bucketing or binning': procedure that consists in segmenting a spectrum into small areas (buckets or bins) and taking the area under the spectrum for each segment. The size of the bins should be large enough to keep a given peak in its bin despite spectral shifts, but not too large as to include peaks belonging to multiple compounds within a single bin.

3.2. Due to the complexity of such samples, multidimensional NMR methods are often employed. Give two examples (full name) of such methods. (2)

(**a**) correlated spectroscopy (COSY); (**b**) nuclear overhauser effect spectroscopy (NOESY).

SECTION C (Centrifugation & Electrophoresis)

[20]

QUESTION 1 [5]

1.1. A centrifuge rotor is spinning at 25000 rpm. The 'top' of the cell is 5.5 cm from the rotor's central axis, and the 'bottom' of the cell is 9.5 cm from the central axis. What are the *g*-forces on a particle found at the top and at the bottom of the tube? (2)

>>> First, a rotor speed of 25000 rpm is equivalent to an angular velocity $\omega=2\pi s/60 = 2618$ rad sec^{-1}.

>>> Then applying the equation $G = \omega^2 r$, calculate the applied centrifugal forces at different r. At r = 5.5 cm, **G = 38400 ×*g*** and at r = 9.5 cm, **G = 66400 ×*g***

1.2. A protein-RNA complex (partial volume, v = 0.71 cm^3 g^{-1}) gives a sedimentation coefficient of 12.7 S in 10% sucrose, 50 mM Tris buffer, pH 7.4 at 4°C. What will be the velocity of sedimentation of the complex under these conditions when the complex is found 5.5 cm from the central axis of a rotor spinning at 4.0 ×10^4 rpm? (3)

>>> First, a rotor speed of 40000 rpm is equivalent to an angular velocity $\omega=2\pi s/60 = 4186$ rad sec^{-1}.

>>> Sedimentation coefficient *s* is given by the equation (3.7), $s= v/ \omega^2 r$, which implies that ***v = s ω^2r***. **>>>** Knowing that the sedimentation coefficients of biological macromolecules are relatively small, and usually expressed as Svedberg units, S. and One S = 10^{-13} s. This means that the 12.7 S = 12.7×10^{-13} s.

>>> Applying then the velocity of sedimentation equation, ***v = s ω^2r***, then ***v = 12.7 ×10^{-13} × (4186)² × 5.5 = 1.224 ×10^{-4} cm sec^{-1}***

QUESTION 2 [5]

Primary skeletal muscle tissues (from mouse) is sent to your lab for analysis. The client wants you to isolate the proteins and organelles of the tissues for further analysis.

2.1. What technique will you use to ensure successful separation? Mention the major steps in the correct order. (4)

- By using a centrifugation technique (multistep method) called subcellular fractionation
- Steps include: homogenization, differential centrifugation and density gradient centrifugation

2.2. To ensure that the membrane fractions are not contaminated with myosin you include an extra step. Mention this step? (1)

By washing the membrane fractions with mild salts

QUESTION 3 [10]

You are given two liver extracts of which the one was obtained from a healthy patient and the other from a patient diagnosed with hepatocellular carcinoma (a form of liver cancer). A proteomic approach is required to compare the two states (healthy vs cancer) with each other.

3.1. What electrophoretic technique will you use to identify differentially expressed proteins in the cancerous extract? (1)

2D PAGE

3.2. Explain the principle of the technique mentioned in (3.1). (7)

2D PAGE is a combination of IEF (first dimension) and SDS-PAGE (second dimension)
IEF separate proteins in a mixture according to charge (Pi)
SDS-PAGE separates a mixture of proteins according to their size
IEF is carried out in acrylamide gel that has been cast on a plastic strip
The gel contains ampholytes (forming a pH gradient) and 8M urea (a non-ionic detergent).
The ampholytes and 8M urea denature and maintain the solubility of the proteins being analysed
Denatured proteins separate in the gel according to their isoelectric points.
IEF strip is then incubated in a sample buffer containing SDS (SDS binds to denatured proteins)
This strip is placed between the glass plates of a 10% SDS-PAGE gel followed by electrophoresis
The SDS bound proteins run into the gel and separate according to size

3.3. You identify two proteins (using the method in (3.1)) that are absent in the healthy tissue extract but expressed in the cancer tissue extract. How will you isolate the proteins and with what other technique will you be able to identify them? (2)

The two separate spots will be cut out of the resolved 2D-PAGE gels
After being electroeluted the proteins will be tryptic digested, creating a peptide mixture
Peptides will then be analysed using in MS/MS experiments (analyses) to obtain a specific m/z ration for each peptide, and using bioinformatics tools derive the identity of the proteins under study

Total marks for Part 2 (sections A, B, and C): [50]