DEBRECENI EGYETEM

PRACTICAL EXERCISES FOR THE COURSE OF SEPARATION TECHNIQUES

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A Debreceni Egyetem fejlesztése a felsőfokú oktatás minőségének és hozzáférhetőségének együttes javítása érdekében
EFOP-3.4.3-16-2016-00021

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Exercise 1.

1. Introduction to separation techniques

Question and problem definition: Identify six reasons you might need to determine certain chemical characteristics of a food product as part of a quality management program.

Answer: Formulating and developing new products, evaluating new processes for making food products, identifying the source of the problem for unacceptable products, nutritional labelling, quality of raw ingredients, check composition during processing.

2. Adopting a new analytical method

Question and problem definition: You are considering the use of a new method to measure Compound X in your food product. List six factors you will consider before adopting this new method in your quality assurance laboratory.

Answer: Specificity, precision, accuracy, sample size required, reagents required, equipment required, cost of assay, speed, reliability, safety, procedures.

3. Evaluation of analytical data

Question and problem definition: Method A to quantitate a particular food component was reported to be more specific and accurate than method B, but method A had lower precision. Explain what this means.

Answer: Method A has measured value closer to true value (more accurate), better at detecting only compound of interest (more specific), replicate measurements are not as reproducible (less precise).

4. Determine the precision of the new method and compare it to the old method

Question and problem definition: You are considering adopting a new analytical method in your lab to measure moisture content of cereal products. How would you determine the precision of the new method and compare it to the old method? Include any equations to be used for any needed calculations.

Answer: Make approximately 10 moisture determinations by old and new method, on cereal products of various moisture content. Calculate and compare standard deviation and coefficient of variation.

5. Precision, accuracy and specificity of a method

Questions and problem definitions and answers: A sample known to contain 20 g/L glucose is analyzed by two methods. Ten determinations were made for each method and the following results were obtained:

Method A	Method B
Mean = 19.6	Mean = 20.2
Std. Dev. = 0.055	Std. Dev. = 0.134

Precision and accuracy: Which method is more precise? Why do you say this? Method A has lower standard deviation.

Which method is more accurate? Why do you say this? Method B, because 20.2 is closer to 20 than is 19.6.

You have determined that values obtained using Method B should not be accepted if outside the range of two standard deviations from the mean. What range of values will be acceptable? 20.2 ± [0.134 × 2] = 19.932 to 20.468.

Do the data above tell you anything about the specificity of the method? Describe what "specificity" of the method means, as you explain your answer. No; Specificity is how well the assay detects only the compound of interest.

6. Absolute error, relative error

Question and problem definition: Differentiate the terms "absolute error" versus "relative error." Which is more useful? Why?

Answer: Absolute error: difference between the experimental value and the true value, which is x – T. Relative error: difference between the experimental value and the true value, relative to the true value, which is (x – T)/T. Relative error is more useful because the value is related to the magnitude of the true value.

7. Sensitivity and detection limit

Question and problem definition: Differentiate the terms "sensitivity" and "detection limit."

Answer: Sensitivity: the magnitude of change of a measuring device with changes in compound concentration; how little a change we can make before we can note a difference. Detection limit: the lowest possible increment that we can detect with some degree of confidence.

8. Correlation coefficient, coefficient of determination

Question and problem definition: The correlation coefficient for standard curve A is reported as 0.9970. The coefficient of determination for standard curve B is reported as 0.9950. In which case do the data better fit a straight line?

Answer: Data better fit straight line for standard curve B. Correlation coefficient = r. Coefficient of determination = r^2. Standard curve A, r = 0.9970. r^2 = 0.9940. Standard curve B, r^2 = 0.995.

Exercise 2.

9. Sampling and Sample Preparation

Question and problem definition: As part of your job as supervisor in a quality assurance laboratory, you need to give a new employee instructions regarding choosing a sampling plan. Which general factors would you discuss with the new employee? Distinguish between sampling for attributes versus sampling for variables. Differentiate the three basic sampling plans and the risks associated with selecting a plan.

Answer: General factors to consider: Purpose of the inspection, nature of the product, nature of the test method, nature of the population being investigated.

Sampling for attributes: Sampling is performed to decide on acceptability of a population based on whether sample possesses a certain characteristic (i.e., is or is not present).

Sampling for variables: Sampling is performed to estimate quantitatively the amount of a substance or a characteristic on a continuous scale; obtain actual value and compare to expected value.

Sampling plans: Single sampling plan allow accept/reject decisions to be made by inspection of one sample of specified size. Doubling sampling plan requires selection of two sample sets; Decision can be based on data from first sample set, or on data from both sample sets. At multiple sampling plan the amount of sampling depends on overall lot quality; relate cumulative number of defects to number of samples taken from a lot.

Risks: Consumer's risk: The probability of accepting a poor quality population. Vendor's risk: The probability of rejecting an acceptable product

10. Equipments for collecting a representative sample for analysis

Question and problem definition: Identify a piece of equipment that would be useful in collecting a representative sample for analysis. Describe precautions to be taken to ensure a representative sample is taken and a suitable food product that could be sampled with this device. Identify a piece of equipment that would be useful for preparing a sample for analysis. What precautions should be taken to ensure the sample composition is not changed during sample preparation?

Answer: Collecting sample: Piece of equipment – mechanical (automatic) sampling device. Types of products – powders, granules, pellets, liquids. Precautions – sampling device working properly.

Preparing a sample for analysis: Piece of equipment – grinders. Types of products – dry or moist samples. Precautions – avoid overheating; avoid metal contamination.

11. Sample bias, change in composition, metal and microbial contamination

Question and problem definition: For each of the problems identified below that can be associated with the collection and preparation of samples for analysis, state one solution for how the problem can be overcome.

Answers: Sample bias: Use probability sampling, choose appropriate sampling plan, understand the population distribution.

Change in composition during storage of sample prior to analysis: Store under conditions that prevent degradation (e.g., protect from light, oxygen; use low temperatures; add preservative).

Metal contamination in grinding: Adjust grinder to prevent metal loss, or avoid use of bare metal mills.

Microbial growth during storage of product prior to analysis: Freeze or refrigerate product, dry product, use chemical preservative.

Exercise 3.

12. Protein Analysis

Question and problem definition: What factors should one consider when choosing a method for protein determination?

Answer: Sample preparation, principle, basis of method; what is measured, sensitivity, amount of sample required, speed, cost of equipment, technical expertise.

13. The steps of the Kjeldahl method

Question and problem definition: The Kjeldahl method of protein analysis consists of three major steps. List these steps in the order they are done, and describe in words what occurs in each step. Make it clear why milliliters of HC1 can be used as an indirect measure of the protein content of a sample.

Answers: Digestion – digest protein with H_2SO_4 in presence of catalyst to give N in form of $(NH_4)_2S0_4$. Neutralization/distillation – neutralize with NaOH to convert $(NH_4)_2SO_4$ to NH_3; distil NH_3 into boric acid to get NH_4 and borate anions. Titration – titrate borate anions with dilute HC1 to pH or colorimetric endpoint; volume of HC1 → moles borate anions → moles NH_3 → moles $(NH_4)_2SO_4$ → moles N → protein content.

14. The conversion factor from Kjeldahl nitrogen

Question and problem definition: Why is the conversion factor from Kjeldahl nitrogen to protein different for various foods, and how is the factor of 6.25 obtained?

Answer: Average MW of N relative to MW of an amino acid is 16%. 100/16 = 6.25.

15. Nesslerization

Question and problem definition: How can Nesslerization or the procedure that uses phenol and hypochlorite be used as part of the Kjeldahl procedure, and why might they best be put to use?

Answer: These procedures can be used after an initial Kjeldahl digestion, to replace typical neutralization and titration. These procedures are faster and more sensitive.

16. Different techniques for the determination of the protein content

Question and problem definition and answers: Differentiate and explain the chemical basis of the following techniques that can be used to quantitate proteins in quality control/research:

Kjeldahl method −	presence of N, determined by method involving digestion, neutralization, distillation, and titration
Dumas method (N combustion) −	presence of N, released upon combustion of sample; N measured by GC using TCD
Biuret method −	presence of peptide bond, which complexed with cupric ions in alkaline conditions, to give violet-purple colour
Lowry method −	presence of peptide bond (biuret reaction), combined with reduction of Folin-Ciocalteau phenol reagent by tyrosine and tryptophan
Bradford method −	specific dye changes colour when it binds to protein; colour is measured by absorbance
Absorbance at 280 nm −	presence of tryptophan and tyrosine; absorbance is related to concentration, by Beer's law
Absorbance at 220 nm −	presence of peptide bond

Exercise 4.

17. Basic principles of chromatography; adsorption, partition, normal phase, reversed phase, cation and anion exchanger, external and internal standards, thin layer and column-liquid chromatography

Question and problem definition and answers: For each set of two (or three) terms used in chromatography, give a brief explanation as indicated to distinguish between the terms.

Adsorption versus Partition Chromatography

	Adsorption	Partition
Nature of stationary phase	solid	Liquid
Nature of mobile phase	liquid or gas	liquid or gas
How solute interacts with the phases	van der Waals forces, electrostatic interactions, hydrogen bonding, hydrophobic interactions	Solute partitions between liquid and stationary phases, according to partition coefficient

Normal-Phase versus Reversed-Phase Chromatography

	Normal phase	Reversed-phase
Nature of stationary phase	Polar	Nonpolar
Nature of mobile phase	Nonpolar	Polar
What elutes last	Most polar compounds	Most nonpolar compounds

Cation versus Anion Exchangers

	Cation exchanger	Anion exchanger

Charge on column	Negative	Positive
Nature of compounds bound	Cations	Anions

Internal Standards versus External Standards

	Nature of Stds.	How stds. are handled in relation to samples	What is plotted on std. curve
Internal Standard	Not present in sample	Added in constant amount to sample and standard compounds	Plot ratio of peak height, area (or mass) of internal std. versus other standard compounds (y-axis) vs. concentration of standard compounds (x-axis)
External Standard	Same as present in sample	Prepare standards of different concentrations and inject separate from sample	Plot peak height, area (or mass) of each standard versus concentration of standards

Thin-Layer Chromatography versus Column Liquid Chromatography

	Thin-layer	Column liquid
Nature and location of stationary phase	Solid stationary phase in thin layer on plate or sheet	Liquid (or solid) inside column
Nature and location of mobile phase	Liquid at bottom (and/or top) of chamber	Liquid passes through column
How sample is applied	Apply as dot on stationary phase at bottom of plate	Apply to top of column
Identification of solutes	Visualize bands by chemical	Detectors

separated	reaction or fluorescence

HETP versus N versus L (from the equation HETP = L/N)

HETP – height equivalent of a theoretical plate, N – number of theoretical plates, L – length of column.

18. Bonded support, coated support

Question and problem definition: What is the advantage of bonded supports over coated supports for partition chromatography?

Answer: With bonded supports, the stationary phase may be covalently attached to the support material, so the stationary phase is not stripped off with continued use of the column.

19. Anion exchange column chromatography of the proteins

Question and problem definition and answers: You applied a mixture of proteins, in a buffer at pH 8.0, to an anion-exchange column. Based on some assays you performed, you know that the protein of interest adsorbed to the column.

Does the anion-exchange stationary phase have a positive or negative charge? Positive charge.

What is the overall charge of the protein of interest that adsorbed to the stationary phase? Negative.

Is the isoelectric point of the protein of interest (adsorbed to the column) higher or lower than pH 8.0? Lower.

What are the two most common methods you could use to elute the protein of interest from the anion-exchange column? Explain how each method works. Change the mobile phase pH, to change charge on protein so it no longer binds to the column. Increase the ionic strength (e.g., add NaCl) to mobile phase, to weaken the electrostatic interactions.

20. Size exclusion chromatography (SEC) for determination of the molecular mass of proteins

Question and problem definition: Explain how you would use SEC to estimate the molecular weight of a protein molecule. Include an explanation of what information must be collected and how it is used.

Answers: Need to calibrate column by running: Blue Dextran, to determine V_o, coloured low molecular weight compound, to determine V_t, proteins of known M.W. (read A_{280}), then use their V_e to calculate their K_{av} [$(K_{av} = (V_e - V_o)/(V_t - V_o)]$.

Make a plot of K_{av} vs. log M.W., for proteins of known M.W.

Run protein of unknown M.W., obtain V_e, calculate K_{av}, then use plot to get estimate of M.W.

Exercise 5

21. Stationary phases for protein separation

Question and problem definition: Would you use a polystyrene- or a polysaccharide-based stationary phase for work with proteins? Explain your answer.

Answer: Use polysaccharide-based (vs. polystyrene-based) stationary phase for work with proteins, since they are softer (vs. more rigid), hydrophilic (vs. hydrophobic), and have larger pore size and lower charge densities.

22. The principle of affinity chromatography; spacer arm

Question and problem definition: Explain the principle of affinity chromatography, why a spacer arm is used, and how the solute can be eluted.

Answer: Principle: A type of adsorption chromatography, in which separation is based on the specific, reversible interaction between a solute molecule and a ligand immobilized on the stationary phase.

Why spacer arm is used? To hold the ligand away from the support surface, enabling it to reach into the binding site of the analyte.

How the solute is eluted? Nonspecific methods – disrupting ligand analyte binding by changing the mobile-phase pH, ionic strength, dielectric constant, or temperature. Biospecific methods – free ligand is added to mobile phase, so it competes for binding sites on the analyte.

23. Isocratic and gradient elution

Question and problem definition: What is gradient elution from a column, and why is it often advantageous over isocratic elution?

Answer: Gradient elution refers to changing the mobile phase (e.g., increasing the ionic strength or pH) during elution to enhance resolution and decrease analysis time.

24. Quantitate sample components

Question and problem definition: How can chromatographic data are used to quantitate sample components?

Answer: Use peak height, peak area, or peak mass, to plot against concentration.

25. Internal standard, external standard

Question and problem definition: Why would you choose to use an internal standard rather than an external standard? Describe how you would select an internal standard for use.

Answer: Use of internal standards can minimize errors due to sample preparation, apparatus, and operator technique. Injection volumes need not be accurately measured, and detector response need not remain constant. Internal standard must not interfere chromatographically with components of interest in the sample. It must not be naturally present in the sample, but it should have a chemical structure similar to compounds of interest.

26. Differences between SFC and LC

Question and problem definition: Explain how SFC differs from LC, including the advantages of SFC.

Answers: Supercritical fluid chromatography (SFC) uses a supercritical fluid (vs. liquid) mobile phase, conditions that can be varied include pressure, the stationary phase is similar to those used for HPLC, and detectors can be of many types (including those designed for GC).

SFC gives decreased analysis time and improved resolution, and makes possible the analysis of nonvolatile, thermally labile compounds (that cannot be analyzed by GC).

Exercise 6.

27. High performance liquid chromatography

Question and problem definition: Why might you choose to use HPLC rather than traditional low-pressure column chromatography?

Answer: Speed, improved resolution, greater sensitivity, reusable columns, ideal for ionic species and large molecules, easy sample recovery.

28. Guard columns

Question and problem definition: What is a guard column and why is it used?

Answer: A guard column is a short, expendable column used to protect the analytical column from strongly adsorbed sample components. It is installed between the injector and analytical column. It contains the same packing material as in the analytical column, but the particle size may be larger.

29. Requirements of HPLC column packing materials

Question and problem definition: Give three general requirements for HPLC column packing materials. Describe and distinguish among porous silica, bonded phases, pellicular, and polymeric column packings, including the advantages and disadvantages of each type.

Answer: General requirements: Availability in a well-defined particle size, with a narrow particle size distribution. Sufficient mechanical strength to withstand pressure. Good chemical stability. Serves role as support and/or stationary phase.

Porous silica: Silicon dioxide (SiO_2) has surface hydroxyl groups that can be modified. Can select particle size and pore diameter. Dissolves slowly in aqueous solutions.

Bonded phases: Hydrocarbon groups are covalently bonded to the surface of silica particles via surface silanols. Dissolves slowly in aqueous solutions.

Pellicular: A thin layer or coating (with desired functional groups) is deposited onto the surface of an inert, usually nonporous microparticulate core (organic or inorganic). Rigid core gives good physical strength. Thin stationary phase layer gives rapid mass transfer and good column efficiency. The thin layer surface coating limits the number of interactive sites, so binding capacity is low (has been used also on porous supports; useful for large molecules). Polymeric

column packing: Synthetic organic resins of two types: microporous and macroporous. Microporous (microreticular) – crosslinked copolymers in which the apparent porosity is determined by degree of crosslinking. Macroporous (macroreticular) – highly crosslinked, and consist of a network of microspheric gel beads joined together to form a larger bead. Advantages – good chemical stability, possibility to vary interactive properties through direct chemical modification.

30. HPLC detectors

Question and problem definition: What is the primary function of an HPLC detector (regardless of type)? What factors would you consider in choosing an HPLC detector? Describe three different types of detectors and explain the principles of operation for each.

Answers: Primary function of detector: Translates concentration changes in the HPLC column effluent into electrical signals.

Factors to consider in selecting detector: Solute type and concentration, sensitivity, linear range, selectivity, compatibility with the solvent, effect of temperature or flow rate changes, possibility of use with gradient elution, initial and operating costs.

UV-Vis absorption detector (fixed wavelength, or continuously variable wavelength and photodiode-array instrument): Measures absorption of radiation in UV and visible wavelength range, according to Beer's law. Is a selective detector.

Fluorescence detector: Measures the emission of electromagnetic radiation by molecules that fluoresce (i.e., absorb at one wavelength and emit at another). More sensitive and selective than UV detector.

Refractive index detector: Measures change in refractive index of the mobile phase due to presence of solutes. Universal detector, since it responds to all solutes. Sensitive to changes in temperature and flow rates. Cannot be used with gradient elution.

Amperometric detectors: Measure the change in current as analyte is oxidized or reduced by the application of voltage across electrodes of the flow cell. Sensitivity and selectivity improved by adding pulse techniques (i.e., pulsed amperometric detection).

Conductivity monitor:_Responds to presence of ions eluting from the column (e.g., ion chromatography).

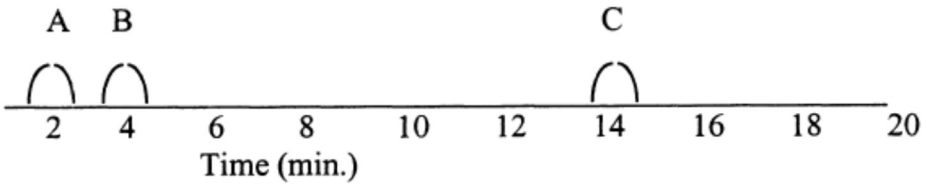
31. Stationary phase with a polar, nonionic functional group

Question and problem definition: You are performing HPLC using a stationary phase that contains a polar nonionic functional group. What type of chromatography is this, and what could you do to increase the retention time of an analyte?

Answer: This is normal phase chromatography. You could increase the retention time of the analyte by decreasing the polarity of the mobile phase, which will make the solute want to interact more with the column and not the mobile phase.

32. HPLC analysis using a column packed with silica gel

Question and problem definition and answers: A sample containing compounds A, B, and C is analyzed via HPLC using a column packed with a silica-based Cig bonded phase. A 1:5 solution of ethanol and H_20 was used as the mobile phase. A UV detector was used and the following chromatogram was obtained.



Assuming that the separation of compounds is based on their polarity.

Is this normal- or reversed-phase chromatography? Explain your answer. Reversed-phase chromatography, because stationary phase is nonpolar and mobile phase is polar.

Which compound is the most polar? Compound A.

How would you change the mobile phase so Compound C would elute sooner, without changing the relative positions of Compounds A and B? Explain why this would work. After ~ 6 min., change to less polar (i.e., more nonpolar) solvent (i.e., less H_2O). This would make Compounds A and B elute as normal, but then would make the mobile phase to be more like the stationary phase, so Compound C, which is nonpolar, would elute sooner.

33. HPLC and external standards

Question and problem definition and answers: You have used HPLC to achieve good chromatographic separation of Compounds A and B in your sample. You now plan to use external standards to help quantitate Compound A and B. You have purchased standards identical to what

you believe are Compounds A and B. To describe how using external standards work for this purpose, answer the following questions.

What specifically will you do with the standards? Individually inject each standard (A and B) at several concentration, at times others than when sample is injected.

What do you actually measure? Measure peak height or area

What do you plot? For both standards A and B, plot peak height versus concentrations of standard.

How do you use the plot? Calculate the equation of the line for the standard curves for Standards A and B. Determine peak height of Compounds A and B in sample, then use those values in equations to calculate concentrations of Compound A and B in sample.

Why are external standards commonly used for HPLC (unlike in GC, for which internal standards are more commonly used)? HPLC uses larger sample volumes, and autosampler or sample loading loop allow for uniform volumes injected.

34. Ion chromatography in food analysis

Question and problem definition: Ion chromatography has recently become a widely promoted chromatographic technique in food analysis. Describe ion chromatography and give at least two examples of its use.

Answer: Ion chromatography is high-performance ion-exchange chromatography performed using a relatively low-capacity stationary phase (either anion or cation exchanger) and a conductivity detector (usually).

Examples of applications: Inorganic ions in milk, organic acids in coffee extract and wine, chlorine in infant formula, trace metals, phosphates, and sulphites in foods.

35. Ion exchange and size exclusion chromatography

Question and problem definition and answers: Describe one application each for ion-exchange and size-exclusion HPLC.

Ion-exchange HPLC: charged molecules by ion chromatography, carbohydrates, amino acids. Size-exclusion HPLC: Average molecular weight and degree of polydispersity of polysaccharides (amylose, amylopectin, etc.), assessing plant cultivars by proteins present, determining polymerized triacylglycerols in oils and fats.

Exercise 7.

36. Gas Chromatography

Question and problem definition and answers: For each of the following methods to isolate solutes from food prior to GC analysis, describe the procedure, the applications, and the cautions in use of the method:

Headspace methods: Procedure – directly inject the headspace vapours above a food product; may instead concentrate headspace by cryogenic or adsorbent trap. Applications – measurement of hexanal as indicator of oxidation; other compounds as indicators of nonenzymatic browning, determination of volatiles in packaging materials.

Cautions: adsorbent traps have differential adsorption affinity and limited capacity, but adsorbent traps are often needed to get H_2O-free flavour isolate.

Distillation methods: Procedure: product moisture or outside steam is used to heat and codistill the volatiles from the food, then a solvent extraction of the distillate is needed to get concentrate. Applications : distill volatiles from a food.

Cautions: volatile isolate may be contaminated by artefacts from solvents used in extraction, antifoam agents, steam supply, heat induced chemical changes, and leakage of lab air into system.

Solvent extraction: Procedure: use organic solvent (usually) to extract solutes, then concentrate as necessary. Applications: fat-free foods (e.g., wines, some breads, fruits and berries some vegetables, alcoholic beverages), unless additional procedure is used to separate the extracted fat from the isolated volatiles.

Cautions: choose solvent carefully, based on solubility of solutes being extracted – watch for interference from fats.

37. Solid phase extraction

Question and problem definition: What is solid-phase extraction and why is it advantageous over traditional liquid-liquid extractions?

Answer: In solid-phase extraction, a liquid sample is passed through a column filled with chromatographic packing. Solutes with affinity for the packing are retained and others elute. Then rinse packing with an eluent that will remove solute of interest. Advantages: less solvent required,

faster, less glassware needed, better precision and accuracy, minimal solvent evaporation for GC analysis, readily automated.

38. Derivatization before GC analysis

Question and problem definition: Why must sugars and fatty acids be derivatized before GC analysis, while pesticides and aroma compounds need not be derivatized?

Answer: Compounds that must be derivatized are those that are thermally unstable, too low in volatility, or yield poor chromatographic separation due to polarity.

39. Temperature of the injection port at GC analysis

Question and problem definition: Why is the injection port of a GC at a higher temperature than the oven temperature?

Answer: Injection port is at higher temperature to rapidly vaporize the sample, so the sample can pass through the column for separation.

40. Physical characteristics of packed and capillary columns

Question and problem definition: Differentiate packed columns from capillary columns (microbore and megabore) with regard to physical characteristics, and column efficiency.

Answers:

Parameter	Packed columns	Capillary columns
Column material	stainless steel or glass	hollow fused or silica glass
Position of stationary phase	on solid support	on walls
Length	0.5–5 m	5–100 m
Diameter	1.6–12.7 mm (o.d.)	0.1 mm(i.d.) microbore 0.2-0.32 mm (i.d.) normal 0.53 mm (i.d.) megabore
Column efficiency N	5,000	100,000–500,000

41. Rises of the baseline

Question and problem definition: You are doing GC with a packed column and notice that the baseline rises from the beginning to the end of each run. Explain a likely cause for this increase.

Answer: Column bleeding – liquid coating on column is coming off the solid support at high temperatures.

Exercise 8.

42. Differentiation between the GC detectors

Question and problem definition: The most common detectors for GC are TCD, FID, ECD, FPD, and PID. Differentiate each of these with regard to the operating principles. Also, indicate below which detector(s) fits the description given.

Answer: Least sensitive: TCD, most sensitive: ECD, least specific: TCD, greatest linear range: FID, PID, nondestructive to sample: TCD, commonly used for pesticides: ECD, commonly used for volatile sulphur compounds: FPD.

TCD – As carrier gas passes over a hot filament (tungsten), it cools the filament at a certain rate depending on carrier gas velocity and composition. The temperature of the filament determines its resistance to electrical current. When a compound elutes with the carrier gas, it has less of a cooling effect, so temperature increases and resistance increases.

FID – As compounds elute from the column, they are burned in a hydrogen flame. A potential is applied across the flame. The flame will carry a current across the potential that is proportional to the organic ions present in the flame from the burning of an organic compound. The current flowing across the flame is amplified and recorded.

ECD – The ECD contains a radioactive foil coating that emits electrons as it undergoes decay. The electrons are collected on an anode, and the standing current is monitored by instrument electronics. As an analyte elutes from the column, it passes between the radioactive foil and the anode. Compounds that capture electrons reduce the standing current and give a measurable response.

FPD – Works by burning all analytes eluting from the analytical column, and then measuring specific wavelengths of light that are emitted from the flame using a filter and photometer. The wavelengths of light that are suitable in terms of intensity and uniqueness are characteristic of sulphur and phosphorus.

PID – Uses UV radiation to ionize analytes eluting from the analytical column. The ions are accelerated by a polarizing electrode to a collecting electrode. The small current formed is magnified by the electrometer of the GC to provide a measurable signal.

43. Different separation methods for GC

Question and problem definition: What types of chromatography does GC rely upon for separation of compounds?

Answer: Size-exclusion chromatography, adsorption chromatography, partition chromatography. (Also relies on solute boiling point).

44. Connection between efficiency and capacity

Question and problem definition: In GC, explain why a balance has to be maintained between efficiency and capacity. Also, give an example situation in which you would sacrifice capacity for efficiency.

Answer: In any separation, to have high efficiency, capacity is sacrificed. Likewise, to have high capacity, you sacrifice efficiency. However, you can improve both capacity and efficiency by using a small column with a thick film. If you had a sample with numerous compounds that needed to be separated, and it was not necessary to collect the solutes, you could sacrifice capacity for efficiency.

45. Using internal standard in GC

Question and problem definition and answers: You plan to use GC to achieve good chromatographic separation of Compounds A, B, and C in your food sample. You plan to use an internal standard to quantitate each compound. By answering the following questions, describe how using an internal standard works for this purpose.

How do you choose the internal standard for your application? Not a compound present in the samples to be tested. Has the same characteristics as compounds to be quantitated. Elutes at time different from compounds of interest.

What do you do with the internal standard, relative to the standard solutions for Compounds A, B, and C, and relative to the food sample? Be specific in your answer. Add a constant amount of internal standard to food sample and to solutions of mixed standard compounds. Inject constant volume of sample of each type.

What do you measure? Measure peak height, area or mass.

If you were to prepare a standard curve, what would you plot? [Peak height of internal standard versus Peak height of Standards A, B, and C] versus Concentration of Standards A, B, and C.

Why are internal standards commonly used for GC? Sample volumes injected for GC are very small, so it is difficult to always inject the exact same volume.

46. Compare the HPLC and GC chromatographic techniques for separation and determination of different food components

Question and problem definition and answers: A fellow lab worker is familiar with HPLC for food analysis but not with GC. As you consider each component of a typical chromatographic system (and specifically the components and conditions for GC and HPLC systems), explain GC to the fellow worker by comparing and contrasting it to HPLC. Following that, state in general terms the differences among the types of samples appropriate for analysis by GC versus HPLC, and give several examples of food constituents appropriate for analysis by each.

Parameter	GC	HPLC
Mobil phase	inert gas	liquid
Stationary phase	liquid or solid	liquid or solid
Column length	0.5 – 100 m	10 – 25 cm
Phase of analytes		
– injected	gas or liquid	liquid
– detected	gas	liquid
Temperature of column	100 – 300 °C	ambient → 85 °C
Detectors	TCD, FID, ECD, PID, ELCD, NPD, FPD	UV-VIS, R.I., fluorescence, amperometric, conductivity
Gradient	temperature	mobile phase
Nature of sample	volatile	not volatile
Identification of peaks	same	same
Quantification of peaks	same	same

Description of column	same	same efficiency

GC and HPLC applications for separation of different food samples.

GC applications: fatty acids, triglycerides, cholesterol, pesticides, herbicides, polychlorinated biphenyls, drugs, flavour compounds, volatiles in packaging materials, separation of stereoisomers, headspace analysis.

HPLC applications: carbohydrates, proteins, fat-soluble and water-soluble vitamins, caffeine, aspartame, pigments, organic acids

Exercise 9.

47. Mass Spectrometry

Question and problem definition: What are the basic components of an MS?

Answer: Sample Introduction → Ion Source → Mass Analyzer → Detector → Data System

48. Unique data an MS provide

Question and problem definition: What are the unique aspects of data that an MS provides? How is this useful in the analysis of foods?

Answer: Provides for detection and identification of an unknown compound. Useful when you need to identify a specific component of food.

49. EI and CI ionization

Question and problem definition: What is EI ionization? What is CI ionization?

Answers: Electron impact (EI) ionization a fragmentation method. Once in the ion source, the compound is exposed to a beam of electrons emitted from a filament composed of rhenium or tungsten metal. When a direct current is applied to the filament, it heats and emits electrons that move across the ion chamber toward a positive electrode on the other side. As the electrons pass through the source region, they come in close proximity to the sample molecule and extract an electron, forming an ionized molecule. Once ionized, the molecules are unstable and, through a series of reactions, breaks into smaller molecular fragments.

Chemical ionization (CI): A fragmentation method. A gas is ionized (e.g., methane), which then directly ionizes the molecule. "Soft ionization". Only a few fragments are produced. Most important use is to determine the molecular ion.

50. Base peak, molecular ion peak at MS

Question and problem definition: What is the base peak on a mass spectrum? What is the molecular ion peak?

Answer: Base peak (base ion): The fragment that has the highest abundance or intensity.

Parent ion (molecular ion) peak: Peak that has the highest mass number and represents the positively charged intact molecule with an m/z equal to the molecular mass.

51. Major ions in the in the EI mass spectrum

Question and problem definition: What are the major ions (fragments) expected in the EI mass spectrum of ethanol (CH_3-CH_2-OH)?

Answer: CH_3-CH_2-OH^+, CH_2-OH^+, CHO^+, CH_3^+

52. Major differences between the different mass analysers

Question and problem definition: What are the major differences between the magnetic sector, quadrupole, and ion trap mass analyzer? What are the advantages of using each analyzer?

Answer: Magnetic sector – Uses a magnet to separate ions, it is slower and takes up more lab space, but can have higher resolution (i.e., can measure ion fragment masses down to 0.001 or lower mass units in special MS instruments).

Quadrupole – Uses four rods with varying electrical potentials that selectively filter ions very rapidly to scan a range of masses. It is fast and the detector can be made very small (relative to a magnetic sector) which explains its popularity in bench top MS instruments. However, resolution is not very good (about 0.5 mass units).

Ion trap – Has been called a 3D quadrupole and is somewhat similar except ALL ions are trapped and then released over time to produce the MS spectra. It is also small in size and fast. Resolution is not as good as the magnetic sector and is about the same as quadrupoles. With a good resolution ion trap can perform tandem MS experiments (i.e., multiple MS/MS).

Exercise 10.

53. Analysis of pesticide, mycotoxin, and drug residues in foods

Question and problem definition: What is meant by the "tolerance level" for a pesticide on a fresh agricultural product?

Answer: Tolerance level - legal limit of pesticide residue at harvest, expressed in units of concentration (e.g., mg/kg), must be established prior to registration.

54. Analytical methods provide only estimates

Question and problem definition: Why is it that all analytical methods provide only estimates of the level(s) of an analyte within the sample? In your opinion, how exact are these estimates?

Answer: Numbers generated are subject to uncertainties, even if sample is homogenous and representative. Estimates are ± 10% if present at 1–10 mg/kg, and ± 30–60% if present at 1–10 µg/kg.

55. Multiresidue, single-residue, and screening methods

Question and problem definition: Multiresidue, single-residue, and screening methods are the three most commonly used approaches to pesticide residue analysis. Differentiate these three types of methods with regard to their purpose/application, and give a comparative advantage of each type of method.

Answer:

	Purpose/Application	Advantage
Multiresidue	To detect and measure multiple residues in a range of foods; Provide reliable estimates	Multi-pesticide application
Single-residue	To measure a single residue (its principle metabolites and transformation products); To support application for registration	Very accurate

Screening	Semiquantitative and qualitative; Assay large number of samples for limited number of pesticides in a short time	Fast

56. Five major steps in pesticide analysis

Question and problem definition: Briefly outline the five major steps involved in a quantitative single-residue method for residues of a pesticide in a fresh plant food.

Answer: Sample preparation – separate by edible and nonedible, chop, grind, macerate sample. **Extraction** – solubilize pesticide residues in suitable solvent, by blending chopped sample with solvent in a homogenizer; filter sample. **Cleanup** (isolation) – remove coextracted compounds that would interfere with a separation and detection steps. **Separation** – separate components in extract by differential partitioning between mobile and stationary phases. **Detection and quantitation** – use detector to measure some physical parameter of the separated compounds; use signal to quantitate.

57. Pesticide, mycotoxin and drug residue analysis

Question and problem definition: What strategies can be followed in an attempt to corroborate the presence of a pesticide, a mycotoxin, or a drug residue in a sample? What is the value, if any, of these approaches?

Answer: Ideal to use two independent methods based on entirely different analytical principles, if available. Use GC-MS to record the mass spectrum of the analyte and estimate quantity. Use a selective detector different from the one used in the original analysis, or use a column with a different stationary phase. Chemically alter compound then rechromatograph to ensure signal has moved. Value of these approaches is to increase the level of confidence concerning the identity and amount of the analyte present.

58. Immunoassay based analytical methods

Question and problem definition: For immunoassay-based analytical methods, what is meant by the term cross-reactivity?

Answer: Crossreactivity – the affinity the antibody shows for related chemical structures.

59. Microbiological assays for determination of mycotoxin contamination?

Question and problem definition: Why haven't microbiological assays been developed to detect the presence of toxicogenic fungi in fresh or stored produce and used as an indicator of possible mycotoxin contamination of that product?

Answer: There is not a good correlation between the level of fungal infection and the level of mycotoxin(s) in the contaminated produce.

60. Sampling procedures for different analyses

Question and problem definition: Why are sampling procedures for pesticide residues appreciably different from sampling procedures for mycotoxins even when dealing with the same sample matrix?

Answer: Typically, the method of sampling is directed/optimized not only to the target food crop but also for the target analyte(s) as well. The great majority of pesticides are not systemic (they are not translocated throughout the plant) and occur as surface residues. They are not distributed evenly across the surfaces of the fresh produce. Mycotoxins (toxic fungal metabolites) present a more difficult sampling problem in that they can occur at very high concentration(s) but are located sporadically on the contaminated surface.

61. Screening procedures for mycotoxin, pesticide and drug analysis

Question and problem definition: What are the advantages and disadvantages of analytical screening procedures for pesticide, for mycotoxin, and for drug residues?

Answer: Advantages: A large number of samples can be analyzed in a short time. Methods are generally robust and not limited to highly controlled lab environments. Rapid. Low cost. Simple methods.

Disadvantages: Are not specific to a particular analyte. (Typically, give a positive response to any one of a group of compounds). Provide only an estimate of the concentration range (i.e. positive response if at or above a critical concentration). In some procedures, other compounds can interfere with detection and quantitation. In some procedures, several factors affect the results.

62. Mycotoxin analysis by mini-column, commercial kits and HPLC

Question and problem definition: Selected food products can be screened for certain mycotoxin residues using a simple mini-column and for antibiotic residues by using a commercial kit such as the Charm II test. A different approach for aflatoxin or for certain sulfonamide antibiotic residues would be to perform an HPLC separation coupled with a selective, yet sensitive, detection process. Briefly explain how each of these three methods might be applied to solve these analytical problems.

Answers: Minicolumn: Application – Intended to provide a preliminary indication of the presence of certain compounds that fluoresce naturally. Principle – After sample cleanup, an extract is applied to a small chromatography column packed with layers of different adsorbents. The aflatoxins, zearalenone and ochratoxins, which are naturally fluorescent, adsorb to the column while many other extracted compounds do not. A second mobile phase combination is used to move the mycotoxin residues from the upper part of the column into a Florisil-lover layer, where these analytes appear as a blue fluorescent band (when viewed at 365 nm). Limitations: Only semiquantitative. Doe not distinguish between the types of analytes identified above.

Charm II Test: Application – Intended as simple and rapid screening assay for antibiotic residues that may be present at or above a critical concentration. Principles – A radioreceptor assay, based on competition between labelled drug and the drug residues in milk, both of which will react with limited number of specific binding sites on bacteria. With more antibiotic residues present in a milk sample, less radiolabelled tracer is bound to the microorganism. Limitations: Assay is nonspecific, responding to numerous antibiotic groups. HPLC: Application – Intended for quantitative analysis of specific aflatoxins and drug residues. Principle: Like with analysis for pesticide residues, the HPLC procedures for mycotoxins and drug residues often involve separate steps of extraction, filtration, cleanup, concentration, chromatographic separation, detection/ quantitation, and confirmation. The chromatographic separation may be reversed-phase HPLC, which uses a nonpolar stationary phase and a polar mobile phase, so analytes separate based on polarity. Limitations: Procedure is time consuming, requires the use of expensive equipment, results are dependent on operator expertise, and requires that conditions be rigorously controlled.

Exercise 11.

63. Vitamin Analysis

Question and problem definition: What factors should be considered in selecting the assay for a particular vitamin?

Answer: Accuracy, precision, economic factors – time, instrumentation, personnel, number of samples to be analyzed, applicability of certain methods for a particular matrix – (i.e., type of sample), need for bioavailability information.

64. Extract the vitamins from foods

Question and problem definition: To be quantitated by most methods, vitamins must be extracted from foods. What treatments are commonly used to extract the vitamins? For one fat-soluble vitamin and one water-soluble vitamin, give an appropriate extraction procedure.

Answer: Commonly used: heat, acid, alkali, solvents, enzymes. Fat-soluble vitamin (e.g., A, D or E): extract with organic solvent; saponify fats with ethanolic KOH; reextract with organic solvent. Water-soluble vitamin (e.g., niacin): autoclave in acid (noncereal products) or alkali (cereal products).

65. Microorganisms for quantitate vitamins

Question and problem definition: Explain why it is possible to use microorganisms to quantitate a particular vitamin in a food product, and describe such a procedure.

Answer: Growth of microorganisms is proportional to their requirement for a specific vitamin. Growth of microorganism in extract of vitamin-containing sample (unknown concentration) is compared to growth in known amount of vitamin. Growth is measured by turbidity, acid production, gravimetry, or by respiration.

66. Niacin and folate determination

Question and problem definition: Niacin and folate both can be quantitated by microbiological methods. What extra procedures and precautions are necessary in the folate assay compared to the niacin assay, and why?

Answer: In folate analysis, samples should be protected from oxidation and photochemical degradation, and strict adherence to microbiological assay techniques is necessary.

67. Fluorometric and titrimetric methods for vitamin C content determination

Question and problem definition: There are two commonly used methods to measure the vitamin C content of foods. Identify these two methods; then compare and contrast them with regard to the principles involved.

Answer: 2,6-Dichloroindophenol titrimetric method. L-ascorbic acid is oxidized to L-dehydroascorbic acid by the indicator dye 2,6-dichloroindephenol. At the endpoint, excess unreduced dye in rose-pink in acid solution.

Fluorometric method. Ascorbic acid, is oxidized to dehydroascorbic acid then reacted with o-phenylenediamine, to form a quinoxaline compound that can be quantitated by fluorescence spectroscopy.

68. Vitamin C forms determination

Question and problem definition: During processing and storage of foods, L-ascorbic acid can be oxidized to L-dehydroascorbic acid. Using the 2,6-dichloroindophenol titrimetric method for vitamin C, how could you quantitate total vitamin C and each form individually?

Answer: Use titration procedure, to give L-ascorbic acid content, then use a reducing agent to convert any L-dehydroascorbic acid to L-ascorbic acid and repeat titration. Difference between two assays gives L-dehydroascorbic acid content.

69. Using HPLC for vitamin analysis

Question and problem definition: What are the advantages and disadvantages of using HPLC for vitamin analysis?

Answer: Advantages: HPLC methods are relatively simple, accurate and precise. It is applicable to most vitamins and in some cases can be used to analyze several vitamins and/or isomers of vitamins.

Disadvantages: HPLC is a separation method and not an identification method, so peak identity and purity must be established for any method developed or adapted for vitamin analysis of a particular food. Also, HPLC requires a high initial capital outlay.

Exercise 12.

70. Protein separation and characterization procedures

Question and problem definition and answers: For each of the techniques listed below, identify the basis by which it can be used to separate proteins within a protein solution (e.g., precipitation, adsorption, size, charge) and give a brief explanation of how/why it works in that way.

Dialysis: Size: Semipermeable membrane permits passage of small molecules but not larger molecules; pores in membrane have certain size, specified certain molecular weight cutoff.

Adjustment of pH to pI: Precipitation (i.e., differential solubility characteristics). Protein has no net charge at pI, so it aggregates and precipitates from solution.

Addition of ammonium sulphate: Precipitation. Proteins are precipitated from solution as ionic strength increases, using a neutral salt, such as $(NH_4)_2SO_4$.

Ultrafiltration: Size. Pressure is applied to solution sitting on semipermeable membrane with certain molecular weight cutoff. Small molecules pass through and large ones are retained.

Heating to high temperature: Precipitation. Proteins are denatured to different extents by high heat, to precipitate from solution.

Addition of ethanol: Precipitation. Water miscible organic solvents decrease the dielectric constant of an aqueous solution and decrease the solubility of most proteins, so proteins precipitate from solution.

Affinity chromatography: Adsorption. Protein is adsorbed to chromatographic matrix that contains a ligand covalently bound to a solid support; The ligand used has reversible, specific, and unique binding affinity for the protein of interest; Protein that binds to ligand can be unbound by changing the pH, temperature, or concentration of salt or ligand in the eluting buffer.

Size-exclusion chromatography: Size. Porous beads with specific average pore size is used in a chromatography column; Large molecules are excluded from the pores and move quickly through the column; Small molecules enter the pores and are retarded, to move slowly through the column.

71. Separation of four different proteins from others

Question and problem definition: You have a protein system with the following characteristics:

Protein	Solubility in	Solubility in	pI	Denaturation

	$(NH_4)_2SO_4$	ethanol		temperature
1	10 – 20%	5 – 10%	4.6	80 °C
2	70 – 80%	10 – 20%	6.4	40 °C
3	60 – 75%	10 – 20%	4.6	40 °C
4	50 – 70%	5 – 10%	6.4	70 °C

Describe how you would separate protein 4 from the others.

Answer: Precipitate protein 1 by adding 45% $(NH_4)_2SO_4$, perform dialysis to remove salt, heat to 50 °C to precipitate proteins 2 and 3, then separate by filtration. You now have protein 4 isolated.

72. Compare the principles of SDS-PAGE and IEF

Question and problem definition: Compare and contrast the principles and procedures of SDS-PAGE versus isoelectric focusing to separate proteins. Include in your explanation how and why it is possible to separate proteins by each method and what you can learn about the protein by running it on each type of system.

Answer: **SDS-PAGE.** Separate based on size. Proteins bind SDS to become negatively charged, so they move through the gel matrix toward the anode (pole with positive charge) at a rate based on size alone, since all molecules are highly negatively charged. Can determine protein composition of sample, purity of a protein extract, subunit composition, and molecular weight (estimate).

IEF: Separate based on charge. Proteins are separated by charge in an electric field on a gel matrix in which a pH gradient has been generated using ampholytes. Proteins are focused long enough (so size is not a factor) so they migrate to the location on the gradient at which pH equals the pi of the protein. Can determine isoelectric point of proteins, and determine purity.

73. Differences between capillary electrophoresis and SDS-PAGE

Question and problem definition: Explain how capillary electrophoresis differs from SDS-PAGE.

Answer: In capillary electrophoresis, capillary tubing is used in place of the acrylamide gels cast in tubes or slabs as in SDS-PAGE; Separation of proteins in capillary electrophoresis is based on size or charge, but also can be influenced by electroosmotic flow within the capillary.

Exercise 13.

74. Characteristics of the proteins of interest

Question and problem definition and answers: Briefly describe what each of the following tells you about the characteristics of the proteins of interest described in the statement (note: protein is not the same one in each statement):

When subjected to dialysis using tubing with a molecular weight cutoff of 3,000 Daltons, a protein of interest is found in the retentate (i.e., not in the filtrate). Protein is larger than 3,000 Daltons.

When subjected to ultrafiltration using a membrane with a molecular weight cutoff of 10,000 Daltons, a protein of interest is found in the filtrate (i.e., not in the retentate). Protein is smaller than 10,000.

When the protein was subjected to ion exchange chromatography using an anion exchange column and a buffer of pH 8.0, a protein of interest bound to the column. Protein has pI < pH 8.0.

When a protein of interest was subjected to IEF, the protein migrated to a position of approximately pH 7.2 in the pH gradient of the gel. pI point of protein is ~ pH 7.2.

When a protein of interest was subjected to SDS-PAGE in both the presence and absence of mercaptoethanol, the protein appeared as three bands at molecular weight 42,000, 45,000, and 48,000 Daltons. Protein has three subunits, not held together by disulfide bonds.

When a solution with various proteins was heated to 60 °C, the protein of interest was found in the precipitate obtained upon centrifugation of the solution. Protein is heat stable to 60 °C.

75. Determination of the amino acid composition of a soy protein

Question and problem definition: You are submitting a soy protein sample to a testing laboratory with an amino acid analyzer (ion-exchange chromatography) so that you can obtain the amino acid composition. Explain how the sample will be treated initially and the amino acids will be quantified as they elute from the ion exchange column. Describe the procedures. (Note: You want to quantify all the amino acids.)

Answers: How samples will be treated initially: Hydrolyze protein to amino acids with 6 M HC1 for 24 hours; Cleave at peptide bonds. Exceptions: Losses of threonine and serine by hydrolysis can be estimated by hydrolysis for several time periods. Valine and isoleucine can be best estimated from a 72 hr. hydrolysate. Cysteine and cystine are converted to cysteic acid by

hydrolysis in performic acid, followed by 6 M HC1 hydrolysis before chromatography. Tryptophan is base hydrolyzed.

How amino acids will be quantified: Quantified by reaction with ninhydrin to produce a coloured product that is measured spectrophotometrically; or derivatize hydrolyzed amino acids before separation then quantitate by UV spectroscopy.

76. Cation exchange column chromatography for separation of amino acids

Question and problem definition and answers: In amino acid analysis, a protein sample hydrolyzed to individual amino acids is applied to a cation-exchange column. The amino acids are eluted by gradually increasing the pH of the mobile phase.

Describe the principles of ion-exchange chromatography. Charged molecules and ions are reversibly adsorbed to a charged solid support matrix; Opposite charges attract each other; Proteins bound to the exchangers are eluted by gradually changing the ionic strength or the pH of the eluting solution.

Differentiate anion- versus cation-exchangers. Anion-exchangers have positively charged matrix, that binds negatively charged ions or molecules. Cation-exchangers have negatively charged matrix, that binds positively charged ions or molecules.

Explain why changing the pH allows different amino acid to elute from the column at different times. By increasing the pH of the mobile phase, the positive charge on each amino acid is eliminated (in order of pI); When amino acid charge changes to neutral then negative with increasing pH, it is desorbed from the column matrix.

77. The amino acid profiles of protein supplements sold to body builders

Question and problem definition: You work for a manufacturer of protein supplements sold to body builders. You need to screen several proteins that may be used in a new protein supplement. You have three samples (A, B, and C) to evaluate. The amino acid profiles of these three samples and the reference profile (i.e., amino acid requirements of preschool age children) are shown below.

Amino acid	Reference Profile	Sample A	Sample B	Sample C
Isoleucine	28	50	55	35

Leucine	66	65	46	32
Lysine	58	80	92	80
Methionine/cysteine	25	70	48	50
Phenylalanine/Tyrosine	63	70	90	85
Threonine	34	51	40	39
Tryptophan	11	16	22	25
Valine	35	60	64	42
Histidine	19	26	35	24

Calculate the PDCAAS for each supplement. (Assume that the true digestiblities of Sample A, B, and C are 87%, 93%, and 64%, respectively.). PDCAAS = amino acid score for 1st limiting amino acid x true digestibility. The 1st limiting amino acid for all three samples is leucine. (Determine by determining ratio of each amino acid compared to reference profile.)

Sample A = (65/66) × 0.87 = 0.85

Sample B = (46/66) × 0.93 = 0.65

Sample C = (32/66) × 0.64 = 0.31

Which sample would you use if Sample A costs $1.25/lb., Sample B costs $3.25/lb., and Sample C costs $1.15/lb.? The cost to protein quality ratio for each sample is:

Sample A = ($1.25 /0.85) = $1.47

Sample B = ($3.25/0.65) = $5.00

Sample C = ($1.15/0,31) = $3.71

Sample A provides the highest amount of usable protein per dollar, suggesting it would be best to use.

78. Protein quality assay methods

Question and problem definition: For each of the protein quality assay methods listed below, state what are the method measures, and briefly describe an appropriate application of the method (e.g., for what situations/food samples is each required and/or ideally used).

Answers:

What is measured? Application

PDCAAS	Amino acid content of 1st limiting amino acid compared to requirements of preschool age children, and true digestibility based on rat feeding experiment	Nutrition labelling for all but infant foods (to express g protein as % Daily Value)
PER	Weight gain of rats per g protein consumed	Nutrition labelling for infant foods (to express g protein as % Daily Value)
pH stat	Amount of standardized sodium hydroxide needed to maintain constant pH during enzymatic digestion of protein under standard conditions	Rapid test of protein digestibility
DNFB method for available lysine	Amount of lysine that has not already reacted with other food constituents, to become unavailable as an essential amino acid	Determine effect of heat treatment on availability of lysine

79. Differences between protein quality assay procedures

Question and problem definition and answers: Briefly describe the differences between the following assay procedures:

Amino acid score versus essential amino acid index: Amino acid score utilizes only first limiting amino acid, while essential amino acid index utilizes the 8 essential amino acids plus histidine.

PDCAAS versus amino acid score: Protein digestibility-corrected amino acid score vs. amino acid score PDCAAS includes both amino acid score and % true digestibility, based on N balance study with rats. Amino acid score alone may compare amino acid content of test protein with that of reference protein or reference pattern, but in PDCAAS method the amino acid score must be based on reference pattern for preschool age children.

pH-shift versus pH-stat method for in vitro digestibility: pH-shift method measures drop in pH when enzymes digest protein under controlled conditions, while pH-drop method measures the

volume of a standard alkali required to maintain a constant pH when enzymes digest protein under controlled conditions.

80. Determine the protein quality of a snack food under various processing

Question and problem definition and answers: You are helping to develop a new process for making a high-protein snack food from cereal grains and soy. You want to determine the protein quality of the snack food under various processing (toasting and drying) conditions. Considering the number of samples to be tested, you cannot afford an expensive in vivo assay, and you cannot wait more than a few days to get the results.

What method would you use to compare the protein quality of the snack food made under different processing conditions? Include an explanation of the principles involved. Use pH-stat method, measuring amount of standardized NaOH required to maintain a constant pH during enzymatic hydrolysis of test protein; More NaOH required means more extensive digestion. This method would not include any assay for amino acid composition, because composition of product is presumably constant. (Chose pH-stat method vs. pH-shift method because in pH-stat method the pH is maintained at a pH optimum for the enzymes used in the assay.)

You suspect that certain time-temperature combinations lead to overprocessed products. Your testing shows that these samples have a lower nutritional quality. What amino acid(s) in the snack food would you suspect to be the most adversely affected by thermal abuse? Lysine.

What test(s) could you use to confirm that amino acid(s) have become nutritionally unavailable by the overprocessing? How are these tests conducted? Use assay with l-fluoro-2,4-dinitrobenzene (DNFB). The DNFB reacts with free e -amino groups in lysine. Any lysine that does not react with DFFB has been chemically reacted with other food constituents. The DNFB-reactive lysine, upon protein hydrolysis of the sample with acid, can be measured spectrophotometrically.

Exercise 14.

81. Carbohydrate Analysis

Question and problem definition: Describe the principle behind anion-exchange chromatography of carbohydrates.

Answer: At a high pH, some of the hydroxyl groups of carbohydrates become ionized, providing negative charges ($R{-}O^-$) that bind to an anion-exchange column (positive charge on the surface of the stationary phase).

82. Determination of the sugars by GC

Question and problem definition: Describe the general procedure for preparation of sugars for GC. What is required for this method to be successful?

Answer: Neutral sugars are reduced to alditols (using sodium borohydride). The alditols are acetylated with acetic anhydride to yield alditol peracetates, which can be subjected to GC analysis.

To be successful, the two preparation steps: Reduction of aldehyde groups to primary alcohol groups, and conversion of the reduced sugars into a volatile peracetate ester must be 100% complete.

83. HPLC vs. GC for carbohydrate analysis

Question and problem definition: Why has HPLC largely replaced GC for analysis of carbohydrates?

Answer: HPLC analysis requires no prior derivatization of carbohydrates, as does GC. Derivatization is needed for GC analysis because carbohydrates are nonvolatile. Each of the two steps involved in derivatization must be 100% complete.

84. RI and PAD detectors in carbohydrate analysis

Question and problem definition and answers: Compare and contrast RI and PAD detectors.

RI: Refractive index (RI) detectors measure changes in refractive index of a solution (due to carbohydrate present) eluting from an HPLC column. The RI detector is linear over a wide range of carbohydrate concentrations and can be applied universally to all carbohydrates. However, it is

sensitive to changes in flow, pressure, and temperature, and is insensitive to low concentrations. A gradient elution cannot be used with RI detection.

PAD: Pulsed amperometric detectors (PAD) rely on oxidation of carbohydrate hydroxyl groups. The PAD is used universally with anion-exchange chromatography, and can be used with gradient elutions. The PAD is more sensitive than RI detectors.

85. Separation of cellulose, water soluble gums and starch

Question and problem definition and answers: Describe the principles behind separation and analysis of cellulose, water-soluble gums, and starch.

Cellulose. Analysis relies on the prior removal of free sugars, then solubilization of starch and water-soluble gums, so cellulose in the insoluble fiber fraction can be hydrolyzed to D-glucose that is quantitated by HPLC or GC.

Water-soluble gums. Analysis relies on the extraction of the gums, and fractionation to remove free sugars and other water-soluble polysaccharides, such as starch. The isolated gum is acid hydrolyzed and its constituent sugars are quantitated by HPLC or GC.

Starch. Analysis relies on removal of free sugars, then enzymatic digestion of the starch to D-glucose, and enzymatic quantitation of the glucose.