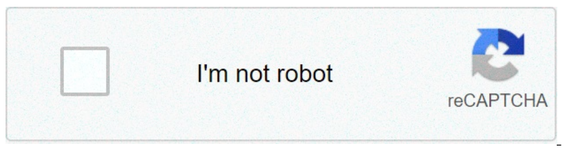

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Protein estimation by kjeldahl method pdf

The total protein content of a bioproduct can be determined by the Kjeldahl method. This method is applicable to any type of medium: food (solids, liquids) ingredients (liquid extracts, pastes, powders) vegetable and animal raw materials (cereals, insects) This method will not take nitrates and nitrites into account. Transcript : French English Nitrogen-containing organic compounds (proteins and nucleic acids in certain matrices) are heated and decomposed using sulfuric acid and a catalyst. This catalyst contains potassium sulfate (K2SO4), which increases the boiling temperature of sulfuric acid, and copper sulfate (CuSO4), which acts as a catalyst for the reaction. Nitrogen will quantitatively give ammonium sulfate: this is the mineralization stage. The ammonia is subsequently displaced from its salt by sodium hydroxide, distilled by steam distillation and collected in a known quantity of excess hydrochloric acid. This step is called distillation. The hydrochloric acid that has not reacted is dosed in return by sodium hydroxide. This is the dosing step.   Two samples will be prepared : a sample containing the bioproduct, liquid or previously ground a sample that does not contain the bioproduct, which will be the control The following calculation will then be applied, considering a conversion factor of 6.25 (16% nitrogen on average in proteins): This experiment requires: suitable equipment (mineralizer, distiller, specific to the Kjeldahl method). suitable individual and collective protection (laboratory coat, gloves, glasses, fume hood). time: 2 half-days, part-time (not necessarily successive). Skip Nav Destination Dairy Products You do not currently have access to this article. The Kjeldahl method was developed by a brewer called Johann Kjeldahl in 1883. The protocol is built on the principle that strong acid helps in the digestion of food so that it releases nitrogen which can be determined by a suitable titration technique. By observing the nitrogen concentration of the food, the amount of protein present is then calculated. This method is particularly ideal for insoluble proteins, protein in foods and protein covalently immobilized on chromatographic supports. Solutions/Reagents: selenium reaction mixture for nitrogen determination according to Wieninger sulfuric acid (98% w/w) Sulfuric Acid (Used to digest the samples –) 60% NaOH, 10% Na2S2O3 (w/v) in ddH2O Sodium Hydroxide Reagent (For the dilution of reagents –) Distilled Water (Used in the dilution of reagents –) 2% boric acid (w/v) in ddH2O Boric Acid (Used in the preparation of solutions –) Tashiro indicator (2 vol. 0.2% methyl red in 90% ethanol + 1 vol. 0.2% methylene blue in 90% ethanol) 010 N HCl (standard solution) Hydrochloric Acid (It activates pepsinogen and converts it to the enzyme pepsin –) Preparation of Reagents and Experiment Protocol: Step 1: Take 1.5 g catalyst A and mix it with accurately weighed 100–250 mg sample. After that, add 3 ml of concentrated sulfuric acid B. Step 2: At the temperature of boiling sulfuric acid (about 180 ◦C), heat the mixture for 2 hours. Note that the acid condenses in the middle of the neck of the Kjeldahl flask. Step 3: After cooling put the flask into the distillation apparatus; after that slowly add 12 ml ddH2O followed by 12 ml of Solution. C. Step 4: To nearly 100◦C heat the mixture to liberate ammonia which is distilled by steam for about 10 minutes through a condenser, the tip of which is submerged in a flask containing 5 ml of Solution D. Step 5: After the distillation is finished (total volume about 25 ml), titrate the ammonia with Soln. F. followed by the addition of three drops of E. Conclusion/Calculations: The results are calculated as follows: 1.0 ml 0.010 N HCl = 10 μMol N = 0.14 mg N By means of the Kjeldahl factors F the amount of protein is: mg protein = mg N · F and the protein content “c” of the sample; c [%] = (mg protein) · 100 weight of sample [mg] Since no additional incubations or reagents are required, quantifying protein by directly measuring absorbance is fast and convenient. No protein standard needs to be prepared, and the procedure does not consume the protein. The relationship of protein concentration to absorbance is linear. When different dilutions of a compound are compared, this should be taken into consideration. By reading the UV absorption, the concentration of an aqueous protein solution can be estimated. With the absorbance maxima at 280 and 200 nm proteins in solution absorb the ultraviolet light. For the absorbance peak at 280 nm, amino acids with aromatic rings are the primary reasons for that. Peptide bonds are usually responsible for the peak at 200nm -215 nm. Protein structures like secondary, tertiary, and quaternary all affect absorbance; therefore factors such as ionic strength, pH, etc. can change the absorbance spectrum. The measurements of the absorbances have to be done against the protein-free solvent (buffer). The absorbance of this blank has to be subtracted from that of the protein solution if a single-beam photometer is used. The following equations are made for 10.0 mm: Warburg and Christian equation: mg protein/ml = 1.55 · A 280− 0.76 · A 260 This estimation of protein concentration is valid up to 20% (w/v) nucleic acid or an A280/A260 ratio < 0.6. Kalckar and Shafran equation: mg protein/ml = 1.45 · A 280 − 0.74 · A 260 Whitaker and Granum equation: mg Protein/ml = (A235− A 280) : 2.51 The concentration of immunoglobulins: mg IgG/ml = A280 : 1.38 Aλ = log10 I0 = ελ · c · d I Where; Aλ: absorbance at wavelength λ; I0: the intensity of incident light; I: the intensity of transmitted light; ελ: (molar) absorption coefficient at wavelength λ; c: concentration; d: length of optical path within the cuvette. Similarly, if solutions of pure proteins with known amino acid sequence or composition are measured, the concentration c (mol/l) is calculated from the absorbances at 280 nm (A280), 320 nm (A320), 350 nm (A350), and the number of tryptophan (nTrp) and tyrosine residues (nTyr) and the number of disulfide bridges (nS−S) according to the following equation: c = ___E280 − 10(2.5 · lgE320−1.5 · E350) 5540 · nTrp + 1480 · nTyr + 134 · nS− Stoscheck, CM. (1990). Quantitation of protein. Methods Enzymol. 182:50-68. Whitaker, JR., Granum, PE. (1980). An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal Biochem; 109(1):156-9. Harris, DA. CL, Bashford. (1987). Spectrophotometric assays. In, eds, Spectrophotometry and Spectrofluorimetry: a Practical Approach. IRL Press, Oxford, pp 59-61 K., Smith., R.I., Krohn., G.T., Hermanson et al. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem;150(1):76-85. The protein content of foods can be determined by numerous methods. The Kjeldahl method and the nitrogen combustion (Dumas) method for protein analysis are based on nitrogen determination. Both methods are official for the purposes of nutrition labeling of foods. While the Kjeldahl method has been used widely for over a hundred years, the recent availability of automated instrumentation for the Dumas method in many cases is replacing use of the Kjeldahl method.This article would be throwing light on Protein Estimation By Kjeldahl Method.Principle Of Kjeldahl MethodThe Kjeldahl procedure measures the nitrogen content of a sample. The protein content then, can be calculated assuming a ratio of protein to nitrogen for the specific food being analyzed. The Kjeldahl procedure can be basically divided into three parts:(1) Digestion(2) Distillation(3) Titration In the digestion step, organic nitrogen is converted to an ammonium in the presence of a catalyst at approximately 370°C. In the distillation step, the digested sample is made alkaline with NaOH and the nitrogen is distilled off as NH3 . This NH3 is “trapped” in a boric acid solution. The amount of ammonia nitrogen in this solution is quantified by titration with a standard HCl solution. A reagent blank is carried through the analysis and the volume of HCl titrant required for this blank is subtracted from each determination.ReagentsSulfuric Acid (concentrated, N-Free)Catalyst/Salt Mixture (Kjeldahl digestion tablets) Contains potassium sulfate, cupric sulfate, and titanium dioxide. Note: There are several types of Kjeldahl digestion tablets that contain somewhat different chemicals.Sodium Hydroxide Solution, 50%, w/v, NaOH in deionized distilled (dd) water. Dissolve 2000 g sodium hydroxide (NaOH) pellets in ~3.5 L dd water. Cool. Add dd water to make up to 4.0 LBoric Acid Solution- In a 4-L flask, dissolve 160 g boric acid in ca. 2 L boiled, and still very hot, distilled water. Mix and then add an additional 1.5 L of boiled, hot distilled water. Cool to room temperature under tap water (caution: glassware may break due to sudden cooling) or leave overnight. When using the rapid procedure, the flask must be shaken occasionally to prevent recrystallization of the boric acid. Add 40 ml of bromocresol green solution (100 mg bromocresol green/100 ml ethanol) and 28 ml of methyl red solution (100 mg methyl red/100 ml ethanol). Dilute to 4 L with water and mix carefully. Transfer 25 ml of the boric acid solution to a receiver flask and distill a digested blank (a digested catalyst/salt/acid mixture). The contents of the flask should then be a neutral gray. If not, titrate with 0.1 N NaOH solution until this color is obtained. Calculate the amount of NaOH solution necessary to adjust the boric acid solution in the 4-L flask with the formula:Add the calculated amount of 0.1 N NaOH solution to the boric acid solution. Mix well. Verify the adjustment results by distilling a new blank sample. Place adjusted solution into a bottle equipped with a 50-ml repipettorStandardized HCl solution** Dilute 3.33 ml conc. HCl to 4 L with dd water. Empty old HCl solution from the titrator reservoir and rinse three times with a small portion of the new HCl solution. Fill the titrator with the new HCl solution to be standardized. Using a volumetric pipet, dispense 10 ml aliquots of the THAM solution prepared as described below into three Erlenmeyer flasks (50 ml). Add 3–5 drops indicator (3 parts 0.1% bromocresol green in ethanol to 1 part of 0.2% methyl red in ethanol) to each flask and swirl. Titrate each solution with the HCl solution to a light pink endpoint. Note the acid volume used and calculates the normality as described below.Calculation to standardize HCl solutionTris (hydroxymethyl) aminomethane (THAM) Solution – (0.01 N) ** Place 2 g of THAM in a crucible. Leave in a drying oven (95°C) overnight. Let cool in a desiccator. In a 1-L volumetric flask, dissolve 1.2114 g of oven dried THAM in distilled water. Dilute to volume.Procedure I. DigestionTurn on digestion block and heat to appropriate temperature.Accurately weigh approximately 0.1 g corn flour. Record the weight. Place corn flour in digestion tube. Repeat for two more samples.Add one catalyst tablet and appropriate volume (e.g., 7 ml) of concentrated sulfuric acid to each tube with corn flour. Prepare duplicate blanks: one catalyst tablet+volume of sulfuric acid used in the sample+weigh paper (if weigh paper was added with the corn flour samples).Place rack of digestion tubes on digestion block. Cover digestion block with exhaust system turned on.Let samples digest until digestion is complete. The samples should be clear (but neon green), with no charred material remaining.Take samples off the digestion block and allow to cool with the exhaust system still turned on.Carefully dilute digest with an appropriate volume of dd water. Swirl each tube.II. DistillationFollow appropriate procedure to start up distillation system.Dispense appropriate volume of boric acid solution into the receiving flask. Place receiving flask on distillation system. Make sure that the tube coming from the distillation of the sample is submerged in the boric acid solution.Put sample tube in place, making sure it is seated securely, and proceed with the distillation until completed. In this distillation process, a set volume of NaOH solution will be delivered to the tube and a steam generator will distill the sample for a set period of time.Upon completing distillation of one sample, proceed with a new sample tube and receiving flask.After completing distillation of all samples, follow manufacturer’s instructions to shut down the distillation unit.III.TitrationRecord the normality of the standardized HCl solution as determined by the teaching assistant. 2. If using an automated pH meter titration system, follow manufacturer’s instructions to calibrate the instrument. Put a magnetic stir bar in the receiver flask and place it on a stir plate. Keep the solution stirring briskly while titrating, but do not let the stir bar hit the electrode. Titrate each sample and blank to an endpoint pH of 4.Record volume of HCl titrant used.If using a colorimetric endpoint, put a magnetic stir bar in the receiver flask, place it on a stir plate, and keep the solution stirring briskly while titrating. Titrate each sample and blank with the standardized HCl solution to the first faint gray color. Record volume of HCl titrant used.CalculationMoles of HCl = moles of NH3 = moles of N in the sampleA reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.% N = N HCl × Corrected acid volume g of sample ×14 g N mol ×100A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).% N/0.16 = % proteinKjeldahl Method Video Written By ~ Harshali Patil Mtech(Food Safety And Quality Management) NIFTEM Connect To Her : harshalipatil40@gmail.com 6. Analysis of Proteins 6.1 Introduction Proteins are polymers of amino acids. Twenty different types of amino acids occur naturally in proteins. Proteins differ from each other according to the type, number and sequence of amino acids that make up the polypeptide backbone. As a result they have different molecular structures, nutritional attributes and physiochemical properties. Proteins are important constituents of foods for a number of different reasons. They are a major source of energy, as well as containing essential amino-acids, such as lysine, tryptophan, methionine, leucine, isoleucine and valine, which are essential to human health, but which the body cannot synthesize. Proteins are also the major structural components of many natural foods, often determining their overall texture, e.g., tenderness of meat or fish products. Isolated proteins are often used in foods as ingredients because of their unique functional properties, i.e., their ability to provide desirable appearance, texture or stability. Typically, proteins are used as gelling agents, emulsifiers, foaming agents and thickeners. Many food proteins are enzymes which are capable of enhancing the rate of certain biochemical reactions. These reactions can have either a favorable or detrimental effect on the overall properties of foods. Food analysts are interested in knowing the total concentration, type, molecular structure and functional properties of the proteins in foods. 6.2. Determination of Overall Protein Concentration 6.2.1. Kjeldahl method The Kjeldahl method was developed in 1883 by a brewer called Johann Kjeldahl. A food is digested with a strong acid so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the food. The same basic approach is still used today, although a number of improvements have been made to speed up the process and to obtain more accurate measurements. It is usually considered to be the standard method of determining protein concentration. Because the Kjeldahl method does not measure the protein content directly a conversion factor (F) is needed to convert the measured nitrogen concentration to a protein concentration. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) is used for many applications, however, this is only an average value, and each protein has a different conversion factor depending on its amino-acid composition. The Kjeldahl method can conveniently be divided into three steps: digestion, neutralization and titration. 6.2.1.1. Principles Digestion The food sample to be analyzed is weighed into a digestion flask and then digested by heating it in the presence of sulfuric acid (an oxidizing agent which digests the food), anhydrous sodium sulfate (to speed up the reaction by raising the boiling point) and a catalyst, such as copper, selenium, titanium, or mercury (to speed up the reaction). Digestion converts any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia, and other organic matter to C02 and H20. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH4+) which binds to the sulfate ion (SO42-) and thus remains in solution: N(food) ® (NH4)2SO4 (1) Neutralization After the digestion has been completed the digestion flask is connected to a recieving flask by a tube. The solution in the digestion flask is then made alkaline by addition of sodium hydroxide, which converts the ammonium sulfate into ammonia gas: (NH4)2SO4 + 2 NaOH ® 2NH3 + 2H2O + Na2SO4 (2) The ammonia gas that is formed is liberated from the solution and moves out of the digestion flask and into the receiving flask - which contains an excess of boric acid. The low pH of the solution in the receiving flask converts the ammonia gas into the ammonium ion, and simultaneously converts the boric acid to the borate ion: NH3 + H3BO3 (boric acid) ® NH4+ + H2BO3- (borate ion) (3) Titration The nitrogen content is then estimated by titration of the ammonium borate formed with standard sulfuric or hydrochloric acid, using a suitable indicator to determine the end-point of the reaction. H2BO3- + H+ ® H3BO3 (4) The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen that was in the original food (Equation 3). The following equation can be used to determine the nitrogen concentration of a sample that weighs m grams using a xM HCl acid solution for the titration: (5) Where vs and vb are the titration volumes of the sample and blank, and 14g is the molecular weight of nitrogen N. A blank sample is usually ran at the same time as the material being analyzed to take into account any residual nitrogen which may be in the reagents used to carry out the analysis. Once the nitrogen content has been determined it is converted to a protein content using the appropriate conversion factor: %Protein = F´ %N. 6.2.1.4. Advantages and Disadvantages Advantages. The Kjeldahl method is widely used internationally and is still the standard method for comparison against all other methods. Its universality, high precision and good reproducibility have made it the major method for the estimation of protein in foods. Disadvantages. It does not give a measure of the true protein, since all nitrogen in foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The use of concentrated sulfuric acid at high temperatures poses a considerable hazard, as does the use of some of the possible catalysts The technique is time consuming to carry-out. 6.2.2. Enhanced Dumas method Recently, an automated instrumental technique has been developed which is capable of rapidly measuring the protein concentration of food samples. This technique is based on a method first described by a scientist called Dumas over a century and a half ago. It is beginning to compete with the Kjeldahl method as the standard method of analysis for proteins for some foodstuffs due to its rapidness. 6.2.2.1. General Principles A sample of known mass is combusted in a high temperature (about 900 oC) chamber in the presence of oxygen. This leads to the release of CO2, H2O and N2. The CO2 and H2O are removed by passing the gasses over special columns that absorb them. The nitrogen content is then measured by passing the remaining gasses through a column that has a thermal conductivity detector at the end. The column helps separate the nitrogen from any residual CO2 and H2O that may have remained in the gas stream. The instrument is calibrated by analyzing a material that is pure and has a known nitrogen concentration, such as EDTA (= 9.59%N). Thus the signal from the thermal conductivity detector can be converted into a nitrogen content. As with the Kjeldahl method it is necessary to convert the concentration of nitrogen in a sample to the protein content, using suitable conversion factors which depend on the precise amino acid sequence of the protein. 6.2.2.2. Advantages and Disadvantages Advantages: It is much faster than the Kjeldahl method (under 4 minutes per measurement, compared to 1-2 hours for Kjeldahl). It doesn't need toxic chemicals or catalysts. Many samples can be measured automatically. It is easy to use. Disadvantages: High initial cost. It does not give a measure of the true protein, since all nitrogen in foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The small sample size makes it difficult to obtain a representative sample. 6.2.3. Methods using UV-visible spectroscopy A number of methods have been devised to measure protein concentration, which are based on UV-visible spectroscopy. These methods use either the natural ability of proteins to absorb (or scatter) light in the UV-visible region of the electromagnetic spectrum, or they chemically or physically modify proteins to make them absorb (or scatter) light in this region. The basic principle behind each of these tests is similar. First of all a calibration curve of absorbance (or turbidity) versus protein concentration is prepared using a series of protein solutions of known concentration. The absorbance (or turbidity) of the solution being analyzed is then measured at the same wavelength, and its protein concentration determined from the calibration curve. The main difference between the tests are the chemical groups which are responsible for the absorption or scattering of radiation, e.g., peptide bonds, aromatic side-groups, basic groups and aggregated proteins. A number of the most commonly used UV-visible methods for determining the protein content of foods are highlighted below: 6.2.3.1. Principles Direct measurement at 280nm Tryptophan and tyrosine absorb ultraviolet light strongly at 280 nm. The tryptophan and tyrosine content of many proteins remains fairly constant, and so the absorbance of protein solutions at 280nm can be used to determine their concentration. The advantages of this method are that the procedure is simple to carry out, it is nondestructive, and no special reagents are required. The major disadvantage is that nucleic acids also absorb strongly at 280 nm and could therefore interfere with the measurement of the protein if they are present in sufficient concentrations. Even so, methods have been developed to overcome this problem, e.g., by measuring the absorbance at two different wavelengths. Biuret Method A violet-purplish color is produced when cupric ions (Cu2+) interact with peptide bonds under alkaline conditions. The biuret reagent, which contains all the chemicals required to carry out the analysis, can be purchased commercially. It is mixed with a protein solution and then allowed to stand for 15-30 minutes before the absorbance is read at 540 nm. The major advantage of this technique is that there is no interference from materials that adsorb at lower wavelengths, and the technique is less sensitive to protein type because it utilizes absorption involving peptide bonds that are common to all proteins, rather than specific side groups. However, it has a relatively low sensitivity compared to other UV-visible methods. Lowry Method The Lowry method combines the biuret reagent with another reagent (the Folin-Ciocalteau phenol reagent) which reacts with tyrosine and tryptophan residues in proteins. This gives a bluish color which can be read somewhere between 500 - 750 nm depending on the sensitivity required. There is a small peak around 500 nm that can be used to determine high protein concentrations and a large peak around 750 nm that can be used to determine low protein concentrations. This method is more sensitive to low concentrations of proteins than the biuret method. Dye binding methods A known excess of a negatively charged (anionic) dye is added to a protein solution whose pH is adjusted so that the proteins are positively charged (i.e. < the isoelectric point). The proteins form an insoluble complex with the dye because of the electrostatic attraction between the molecules, but the unbound dye remains soluble. The anionic dye binds to cationic groups of the basic amino acid residues (histidine, arganine and lysine) and to free amino terminal groups. The amount of unbound dye remaining in solution after the insoluble protein-dye complex has been removed (e.g., by centrifugation) is determined by measuring its absorbance. The amount of protein present in the original solution is proportional to the amount of dye that bound to it: dyebound = dyeinitial - dyefree. Turbimetric method Protein molecules which are normally soluble in solution can be made to precipitate by the addition of certain chemicals, e.g., trichloroacetic acid. Protein precipitation causes the solution to become turbid. Thus the concentration of protein can be determined by measuring the degree of turbidity. 6.2.3.2. Advantages and Disadvantages Advantages: UV-visible techniques are fairly rapid and simple to carry out, and are sensitive to low concentrations of proteins. Disadvantages: For most UV-visible techniques it is necessary to use dilute and transparent solutions, which contain no contaminating substances which absorb or scatter light at the same wavelength as the protein being analyzed. The need for transparent solutions means that most foods must undergo significant amounts of sample preparation before they can be analyzed, e.g., homogenization, solvent extraction, centrifugation, filtration, which can be time consuming and laborious. In addition, it is sometimes difficult to quantitatively extract proteins from certain types of foods, especially after they have been processed so that the proteins become aggregated or covalently bound with other substances. In addition the absorbance depends on the type of protein analyzed (different proteins have different amino acid sequences). 6.2.4. Other Instrumental Techniques There are a wide variety of different instrumental methods available for determining the total protein content of food materials. These can be divided into three different categories according to their physicochemical principles: (i) measurement of bulk physical properties, (ii) measurement of adsorption of radiation, and (iii) measurement of scattering of radiation. Each instrumental methods has its own advantages and disadvantages, and range of foods to which it can be applied. 6.2.4.1. Principles Measurement of Bulk Physical Properties Density: The density of a protein is greater than that of most other food components, and so there is an increase in density of a food as its protein content increases. Thus the protein content of foods can be determined by measuring their density. Refractive index: The refractive index of an aqueous solution increases as the protein concentration increases and therefore RI measurements can be used to determine the protein content. Measurement of Adsorption of Radiation UV-visible: The concentration of proteins can be determined by measuring the absorbance of ultraviolet-visible radiation (see above). Infrared: Infrared techniques can be used to determine the concentration of proteins in food samples. Proteins absorb IR naturally due to characteristic vibrations (stretching and bending) of certain chemical groups along the polypeptide backbone. Measurements of the absorbance of radiation at certain wavelengths can thus be used to quantify the concentration of protein in the sample. IR is particularly useful for rapid on-line analysis of protein content. It also requires little sample preparation and is nondestructive. Its major disadvantages are its high initial cost and the need for extensive calibration. Nuclear Magnetic Resonance: NMR spectroscopy can be used to determine the total protein concentration of foods. The protein content is determined by measuring the area under a peak in an NMR chemical shift spectra that corresponds to the protein fraction. Measurement of Scattering of Radiation Light scattering: The concentration of protein aggregates in aqueous solution can be determined using light scattering techniques because the turbidity of a solution is directly proportional to the concentration of aggregates present. Ultrasonic scattering: The concentration of protein aggregates can also be determined using ultrasonic scattering techniques because the ultrasonic velocity and absorption of ultrasound are related to the concentration of protein aggregates present. 6.2.4.2. Advantages and Disadvantages A number of these instrumental methods have major advantages over the other techniques mentioned above because they are nondestructive, require little or no sample preparation, and measurements are rapid and precise. A major disadvantage of the techniques which rely on measurements of the bulk physical properties of foods are that a calibration curve must be prepared between the physical property of interest and the total protein content, and this may depend on the type of protein present and the food matrix it is contained within. In addition, the techniques based on measurements of bulk physicochemical properties can only be used to analyze foods with relatively simple compositions. In a food that contains many different components whose concentration may vary, it is difficult to disentangle the contribution that the protein makes to the overall measurement from that of the other components. 6.2.5. Comparison of methods As food scientists we may often be in a position where we have to choose a particular technique for measuring the protein concentration of a food. How do we decide which technique is the most appropriate for our particular application ? The first thing to determine is what is the information going to be used for. If the analysis is to be carried out for official purposes, e.g., legal or labeling requirements, then it is important to use an officially recognized method. The Kjeldahl method, and increasingly the Dumas method, have been officially approved for a wide range of food applications. In contrast, only a small number of applications of UV-visible spectroscopy have been officially recognized. For quality control purposes, it is often more useful to have rapid and simple measurements of protein content and therefore IR techniques are most suitable. For fundamental studies in the laboratory, where pure proteins are often analyzed, UV-visible spectroscopic techniques are often preferred because they give rapid and reliable measurements, and are sensitive to low concentrations of protein. Other factors which may have to be considered are the amount of sample preparation required, their sensitivity and their speed. The Kjeldahl, Dumas and IR methods require very little sample preparation. After a representative sample of the food has been selected it can usually be tested directly. On the other hand, the various UV-visible methods require extensive sample preparation prior to analysis. The protein must be extracted from the food into a dilute transparent solution, which usually involves time consuming homogenization, solvent extraction, filtration and centrifugation procedures. In addition, it may be difficult to completely isolate some proteins from foods because they are strongly bound to other components. The various techniques also have different sensitivities, i.e., the lowest concentration of protein which they can detect. The UV-visible methods are the most sensitive, being able to detect protein concentrations as low as 0.001 wt%. The sensitivity of the Dumas, Kjeldahl and IR methods is somewhere around 0.1 wt%. The time required per analysis, and the number of samples which can be run simultaneously, are also important factors to consider when deciding which analytical technique to use. IR techniques are capable of rapid analysis (< 1 minute) of protein concentration once they have been calibrated. The modern instrumental Dumas method is fully automated and can measure the protein concentration of a sample in less than 5 minutes, compared to the Kjeldahl method which takes between 30 minutes and 2 hours to carry out. The various UV-visible methods range between a couple of minutes to an hour (depending on the type of dye that is used and how long it takes to react), although it does have the advantage that many samples can be run simultaneously. Nevertheless, it is usually necessary to carry out extensive sample preparation prior to analysis in order to get a transparent solution. Other factors which may be important when selecting an appropriate technique are: the equipment available, ease of operation, the desired accuracy, and whether or not the technique is nondestructive. 6.3. Protein Separation and Characterization In the previous lecture, techniques used to determine the total concentration of protein in a food were discussed. Food analysts are also often interested in the type of proteins present in a food because each protein has unique nutritional and physicochemical properties. Protein type is usually determined by separating and isolating the individual proteins from a complex mixture of proteins, so that they can be subsequently identified and characterized. Proteins are separated on the basis of differences in their physicochemical properties, such as size, charge, adsorption characteristics, solubility and heat-stability. The choice of an appropriate separation technique depends on a number of factors, including the reasons for carrying out the analysis, the amount of sample available, the desired purity, the equipment available, the type of proteins present and the cost. Large-scale methods are available for crude isolations of large quantities of proteins, whereas small-scale methods are available for proteins that are expensive or only available in small quantities. One of the factors that must be considered during the separation procedure is the possibility that the native three dimensional structure of the protein molecules may be altered. A prior knowledge of the effects of environmental conditions on protein structure and interactions is extremely useful when selecting the most appropriate separation technique. Firstly, because it helps determine the most suitable conditions to use to isolate a particular protein from a mixture of proteins (e.g., pH, ionic strength, solvent, temperature etc.), and secondly, because it may be important to choose conditions which will not adversely affect the molecular structure of the proteins. 6.3.1. Methods Based on Different Solubility Characteristics Proteins can be separated by exploiting differences in their solubility in aqueous solutions. The solubility of a protein molecule is determined by its amino acid sequence because this determines its size, shape, hydrophobicity and electrical charge. Proteins can be selectively precipitated or solubilized by altering the pH, ionic strength, dielectric constant or temperature of a solution. These separation techniques are the most simple to use when large quantities of sample are involved, because they are relatively quick, inexpensive and are not particularly influenced by other food components. They are often used as the first step in any separation procedure because the majority of the contaminating materials can be easily removed. Salting out Proteins are precipitated from aqueous solutions when the salt concentration exceeds a critical level, which is known as salting-out, because all the water is "bound" to the salts, and is therefore not available to hydrate the proteins. Ammonium sulfate [(NH4)2SO4] is commonly used because it has a high water-solubility, although other neutral salts may also be used, e.g., NaCl or KCl. Generally a two-step procedure is used to maximize the separation efficiency. In the first step, the salt is added at a concentration just below that necessary to precipitate out the protein of interest. The solution is then centrifuged to remove any proteins that are less soluble than the protein of interest. The salt concentration is then increased to a point just above that required to cause precipitation of the protein. This precipitates out the protein of interest (which can be separated by centrifugation), but leaves more soluble proteins in solution. The main problem with this method is that large concentrations of salt contaminate the solution, which must be removed before the protein can be resolubilzed, e.g., by dialysis or ultrafiltration. Isoelectric Precipitation The isoelectric point (pI) of a protein is the pH where the net charge on the protein is zero. Proteins tend to aggregate and precipitate at their pI because there is no electrostatic repulsion keeping them apart. Proteins have different isoelectric points because of their different amino acid sequences (i.e., relative numbers of anionic and cationic groups), and thus they can be separated by adjusting the pH of a solution. When the pH is adjusted to the pI of a particular protein it precipitates leaving the other proteins in solution. Solvent Fractionation The solubility of a protein depends on the dielectric constant of the solution that surrounds it because this alters the magnitude of the electrostatic interactions between charged groups. As the dielectric constant of a solution decreases the magnitude of the electrostatic interactions between charged species increases. This tends to decrease the solubility of proteins in solution because they are less ionized, and therefore the electrostatic repulsion between them is not sufficient to prevent them from aggregating. The dielectric constant of aqueous solutions can be lowered by adding water-soluble organic solvents, such as ethanol or acetone. The amount of organic solvent required to cause precipitation depends on the protein and therefore proteins can be separated on this basis. The optimum quantity of organic solvent required to precipitate a protein varies from about 5 to 60%. Solvent fractionation is usually performed at 0oC or below to prevent protein denaturation caused by temperature increases that occur when organic solvents are mixed with water. Denaturation of Contaminating Proteins Many proteins are denatured and precipitate from solution when heated above a certain temperature or by adjusting a solution to highly acid or basic pHs. Proteins that are stable at high temperature or at extremes of pH are most easily separated by this technique because contaminating proteins can be precipitated while the protein of interest remains in solution. 6.3.2. Separation due to Different Adsorption Characteristics Adsorption chromatography involves the separation of compounds by selective adsorption-desorption at a solid matrix that is contained within a column through which the mixture passes. Separation is based on the different affinities of different proteins for the solid matrix. Affinity and ion-exchange chromatography are the two major types of adsorption chromatography commonly used for the separation of proteins. Separation can be carried out using either an open column or high-pressure liquid chromatography. Ion Exchange Chromatography Ion exchange chromatography relies on the reversible adsorption-desorption of ions in solution to a charged solid matrix or polymer network. This technique is the most commonly used chromatographic technique for protein separation. A positively charged matrix is called an anion-exchanger because it binds negatively charged ions (anions). A negatively charged matrix is called a cation-exchanger because it binds positively charged ions (cations). The buffer conditions (pH and ionic strength) are adjusted to favor maximum binding of the protein of interest to the ion-exchange column. Contaminating proteins bind less strongly and therefore pass more rapidly through the column. The protein of interest is then eluted using another buffer solution which favors its desorption from the column (e.g., different pH or ionic strength). Affinity Chromatography Affinity chromatography uses a stationary phase that consists of a ligand covalently bound to a solid support. The ligand is a molecule that has a highly specific and unique reversible affinity for a particular protein. The sample to be analyzed is passed through the column and the protein of interest binds to the ligand, whereas the contaminating proteins pass directly through. The protein of interest is then eluted using a buffer solution which favors its desorption from the column. This technique is the most efficient means of separating an individual protein from a mixture of proteins, but it is the most expensive, because of the need to have columns with specific ligands bound to them. Both ion-exchange and affinity chromatography are commonly used to separate proteins and amino-acids in the laboratory. They are used less commonly for commercial separations because they are not suitable for rapidly separating large volumes and are relatively expensive. 6.3.3. Separation Due to Size Differences Proteins can also be separated according to their size. Typically, the molecular weights of proteins vary from about 10,000 to 1,000,000 daltons. In practice, separation depends on the Stokes radius of a protein, rather than directly on its molecular weight. The Stokes radius is the average radius that a protein has in solution, and depends on its three dimensional molecular structure. For proteins with the same molecular weight the Stokes radius increases in the following order: compact globular protein < flexible random-coil < rod-like protein. Dialysis Dialysis is used to separate molecules in solution by use of semipermeable membranes that permit the passage of molecules smaller than a certain size through, but prevent the passing of larger molecules. A protein solution is placed in dialysis tubing which is sealed and placed into a large volume of water or buffer which is slowly stirred. Low molecular weight solutes flow through the bag, but the large molecular weight protein molecules remain in the bag. Dialysis is a relatively slow method, taking up to 12 hours to be completed. It is therefore most frequently used in the laboratory. Dialysis is often used to remove salt from protein solutions after they have been separated by salting-out, and to change buffers. Ultrafiltration A solution of protein is placed in a cell containing a semipermeable membrane, and pressure is applied. Smaller molecules pass through the membrane, whereas the larger molecules remain in the solution. The separation principle of this technique is therefore similar to dialysis, but because pressure is applied separation is much quicker. Semipermeable membranes with cutoff points between about 500 to 300,000 are available. That portion of the solution which is retained by the cell (large molecules) is called the retentate, whilst that part which passes through the membrane (small molecules) forms part of the ultrafiltrate. Ultrafiltration can be used to concentrate a protein solution, remove salts, exchange buffers or fractionate proteins on the basis of their size. Ultrafiltration units are used in the laboratory and on a commercial scale. Size Exclusion Chromatography This technique, sometimes known as gel filtration, also separates proteins according to their size. A protein solution is poured into a column which is packed with porous beads made of a cross-linked polymeric material (such as dextran or agarose). Molecules larger than the pores in the beads are excluded, and move quickly through the column, whereas the movement of molecules which enter the pores is retarded. Thus molecules are eluted off the column in order of decreasing size. Beads of different average pore size are available for separating proteins of different molecular weights. Manufacturers of these beads provide information about the molecular weight range that they are most suitable for separating. Molecular weights of unknown proteins can be determined by comparing their elution volumes Vo, with those determined using proteins of known molecular weight: a plot of elution volume versus log(molecular weight) should give a straight line. One problem with this method is that the molecular weight is not directly related to the Stokes radius for different shaped proteins. 6.3.4. Separation by Electrophoresis Electrophoresis relies on differences in the migration of charged molecules in a solution when an electrical field is applied across it. It can be used to separate proteins on the basis of their size, shape or charge. Non-denaturing Electrophoresis In non-denaturing electrophoresis, a buffered solution of native proteins is poured onto a porous gel (usually polyacrylamide, starch or agarose) and a voltage is applied across the gel. The proteins move through the gel in a direction that depends on the sign of their charge, and at a rate that depends on the magnitude of the charge, and the friction to their movement: Proteins may be positively or negatively charged in solution depending on their isoelectic points (pI) and the pH of the solution. A protein is negatively charged if the pH is above the pI, and positively charged if the pH is below the pI. The magnitude of the charge and applied voltage will determine how far proteins migrate in a certain time. The higher the voltage or the greater the charge on the protein the further it will move. The friction of a molecule is a measure of its resistance to movement through the gel and is largely determined by the relationship between the effective size of the molecule, and the size of the pores in the gel. The smaller the size of the molecule, or the larger the size of the pores in the gel, the lower the resistance and therefore the faster a molecule moves through the gel. Gels with different porosity's can be purchased from chemical suppliers, or made up in the laboratory. Smaller pores sizes are obtained by using a higher concentration of cross-linking reagent to form the gel. Gels may be contained between two parallel plates, or in cylindrical tubes. In non-denaturing electrophoresis the native proteins are separated based on a combination of their charge, size and shape. Denaturing Electrophoresis In denaturing electrophoresis proteins are separated primarily on their molecular weight. Proteins are denatured prior to analysis by mixing them with mercaptoethanol, which breaks down disulfide bonds, and sodium dodecyl sulfate (SDS), which is an anionic surfactant that hydrophobically binds to protein molecules and causes them to unfold because of the repulsion between negatively charged surfactant head-groups. Each protein molecule binds approximately the same amount of SDS per unit length. Hence, the charge per unit length and the molecular conformation is approximately similar for all proteins. As proteins travel through a gel network they are primarily separated on the basis of their molecular weight because their movement depends on the size of the protein molecule relative to the size of the pores in the gel: smaller proteins moving more rapidly through the matrix than larger molecules. This type of electrophoresis is commonly called sodium dodecyl sulfate -polyacrylamide gel electrophoresis, or SDS-PAGE. To determine how far proteins have moved a tracking dye is added to the protein solution, e.g., bromophenol blue. This dye is a small charged molecule that migrates ahead of the proteins. After the electrophoresis is completed the proteins are made visible by treating the gel with a protein dye such as Coomassie Brilliant Blue or silver stain. The relative mobility of each protein band is calculated: Electrophoresis is often used to determine the protein composition of food products. The protein is extracted from the food into solution, which is then separated using electrophoresis. SDS-PAGE is used to determine the molecular weight of a protein by measuring Rm, and then comparing it with a calibration curve produced using proteins of known molecular weight: a plot of log (molecular weight) against relative mobility is usually linear. Denaturing electrophoresis is more useful for determining molecular weights than non-denaturing electrophoresis, because the friction to movement does not depend on the shape or original charge of the protein molecules. Isoelectric Focusing Electrophoresis This technique is a modification of electrophoresis, in which proteins are separated by charge on a gel matrix which has a pH gradient across it. Proteins migrate to the location where the pH equals their isoelectric point and then stop moving because they are no longer charged. This methods has one of the highest resolutions of all techniques used to separate proteins. Gels are available that cover a narrow pH range (2-3 units) or a broad pH range (3-10 units) and one should therefore select a gel which is most suitable for the proteins being separated. Two Dimensional Electrophoresis Isoelectric focusing and SDS-PAGE can be used together to improve resolution of complex protein mixtures. Proteins are separated in one direction on the basis of charge using isoelectric focusing, and then in a perpendicular direction on the basis of size using SDS-PAGE. 6.3.5. Amino Acid Analysis Amino acid analysis is used to determine the amino acid composition of proteins. A protein sample is first hydrolyzed (e.g. using a strong acid) to release the amino acids, which are then separated using chromatography, e.g., ion exchange, affinity or absorption chromatography. Back to McClements Home Page Back to FD SCI 581 Page To Previous Lecture (Lipids) To Next Lecture (Carbohydrates)

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