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Review Article

Scientific Tools and Techniques for Qualitative and Quantitative Analysis of Bacterial Proteins

Shah Dhrumi, Shukla Pooja, Meghani Sonali, Patel Charmi, Rathod Zalak R* and Saraf Meenu S

Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, India

***Corresponding Author:** Rathod Zalak R**,** Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, India.

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Abstract

Protein content can be measured using a variety of ways. The determination of nitrogen, peptide bonds, aromatic amino acids, dye binding capacity, ultraviolet absorptivity of proteins and light scattering properties among the core concepts of those approaches. Protein analysis and their application are essential for understanding the molecular logic of living cells, as they are one of the most impressive families of macromolecules. Some of the parameters like sensitivity, accuracy, precision, speed and cost of analysis all be taken into account when choosing a suitable method. Proteins are made up of amino acids but they are surrounded by multiple additional configurations creating an overcrowded environment that causes them to operate differently.

Keywords: Analysis; Zwitter Ion; Van-der-waals; Rf Value; HPLC; Reverse Phase Chromatography

Introduction

Amino acids are building blocks of protein. Proteins differ in size, molecular structure and physiochemical properties from one another. Protein analysis and characterization can be accomplished by separation and identification according to these distinctions. Amino acid reacts to all of the standard chemical process occur in compounds containing amino and carboxylic group but only in little amounts when the zwitter ion form is present. Protein analysis is important for understanding protein function and comprehending the effects of their presence, absence, and modification. Protein analysis is used to determine the content of amino acid, peptides and protein amount present in the sample. Protein identification is a main step in determining a protein's characteristics for subsequent research. Amino acid performs essential role in all physiological processes in human body. This is crucial for the advancement of illness knowledge, as is allows for the discovery of biomarkers and the development of therapeuticals.

Qualitative analysis of protein

There are number of qualitative tests to detect the presence of amino acids and these are largely depending on the nature of R-group. Qualitative analysis of protein includes Hopkin's, Millon test, Xanthoproteic test, Nitroprusside test, and Sakaguchi test.

Hopkin's cole test

This test is a confirmatory test the presence of amino acid tryptophan (indoyl group) [11].

Principle

The test is based on the principle that the layering of concentrated sulfuric acid over a mixture of tryptophan contains proteins

with a Hopkin's cole reagents results in the formation of purple or violet color ring at the interface. The formation of the test color is due to the presence of indoyl group. Gelatin does not respond to this test due to absence of amino acid tryptophan [4].

Reagents

Hopkin's cole reagent, Concentrated H_2SO_4, Sample.

Procedure

Take 1 ml of sample solution and add 1 ml of Hopkin's cole reagent in a test tube and mix it well. Later carefully, add concentrated H_2SO_4 along the side of the test tube, keeping the tube in an inclined position (do not shake the test tube, while adding the acid in test tube). As a result, purple – violet ring appears at the junction of the amino acid solution and the Concentrated H_2SO_4. The formation of ring confirms the presence of tryptophan amino acid in the test sample [21].

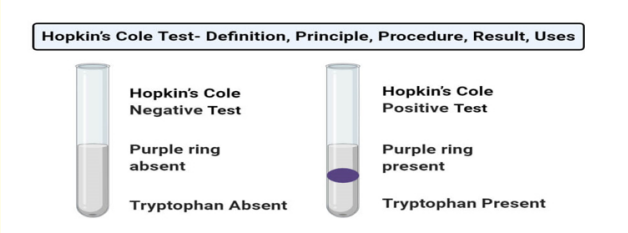

Figure a:

https://microbenotes.com/hopkins-cole-test/

Millon's test

This test is used to detect the presence of phenolic group containing amino acids like tyrosine [11].

Principle

The reaction is because of the hydroxyphenyl group and any phenolic compounds which are unsubstituted in the 3,5 positions such as phenol, tyrosine and thymol will give the reaction. Phenolic group containing amino acids react with acidified mercuric sulphate solution and form yellow precipitate of mercury-amino acid complex. After that, when sodium nitrate solution is added and heat it until red colored complex of mercury phenolate is produced.

Reagent

Test sample, Millon's reagent.

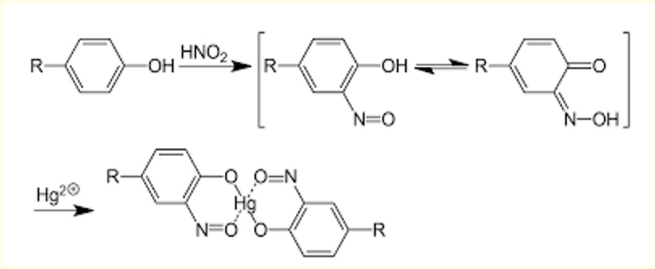

Figure b:

https://www.egyankosh.ac.in/bitstream/123456789/68528/1/Experiment-6.pdf

Procedure

Take 2 ml of test sample and add 2 ml Millon's reagent in it. Then boil it for 2-5 minutes in water bath to develop red color precipitate. Observation of red colored precipitates indicates presence of protein in the test solution. However, excess of reagent produces yellow color which does not indicate positive reaction.

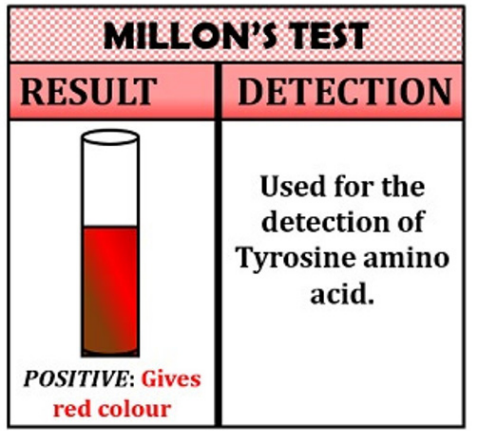

Figure c

https://biologyreader.com/qualitative-analysis-of-amino-acids.html

Xanthoproteic test

This test is identification test for the presence of aromatic amino acids.

Principle

Aromatic amino acid (tyrosine and tryptophan) reacts with concentrated nitric acid and produce yellow colored nitro-derivatives. Addition of NaOH to this solution, ionization of the phenolic group is occurred which produced orange color. Phenylalanine possesses aromatic nucleus, but does not give positive reaction because in normal condition, nitration of phenylalanine is difficult [29].

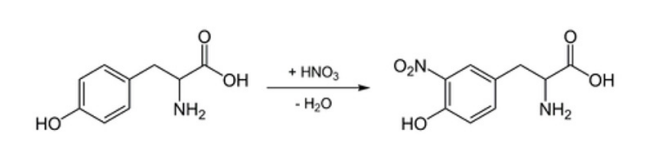

Figure d

https://www.wikiwand.com/en/Xanthoproteic_reaction

Reagent

Test sample, concentrated HNO_3, 40% NaOH.

Procedure

After adding 1 ml test sample in dry test tube, add equal amount of conc.HNO_3 and mix well. White precipitates are formed which are converted into yellow color upon heating for few minutes. Allow it to cool for some time and then add 1-2 ml of 40%NaOH. Orange color is observed if test is positive.

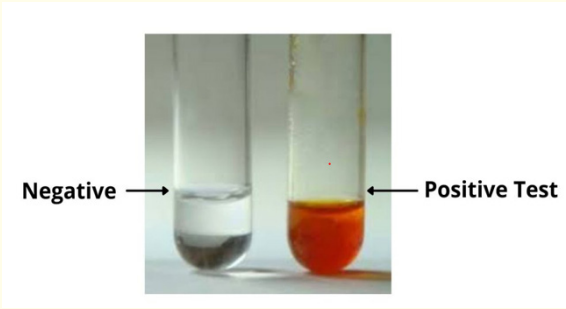

Figure e

https://microbiologynote.com/xanthoproteic-test/

Nitroprusside test

This test is used to detect Sulphur containing amino acids. For instance, cysteine, cysteine and methionine.

Principle

A free sulphahydral group containing amino acids reacts with sodium nitroprusside in the presence of excess ammonium hydroxide and produce red colored compound. Thiol group plays important role here [29].

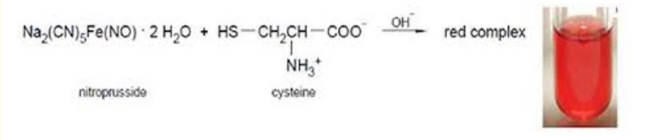

Figure f

http://nuwanthikakumarasinghe.blogspot.com/2011/05/tests-for-proteins-2.html?m=1

Reagents

Test sample, freshly prepared 2% sodium nitroprusside, ammonium hydroxide.

Procedure

Initially, take 2 ml test sample in test tube and then add 0.5 ml freshly prepared sodium nitroprusside by shaking test tube thoroughly. At the end 0.5 ml ammonium hydroxide is add. Red color indicates positive test.

Sakaguchi test

This test is given by guanidine compounds for example arginine [30].

Principle

Specifically arginine gives red color by reacting with α-naphthol in presence of oxidizing agent like bromine water or sodium hypochlorite. Other than arginine, some guanidine containing non-amino acids also give this reaction [13].

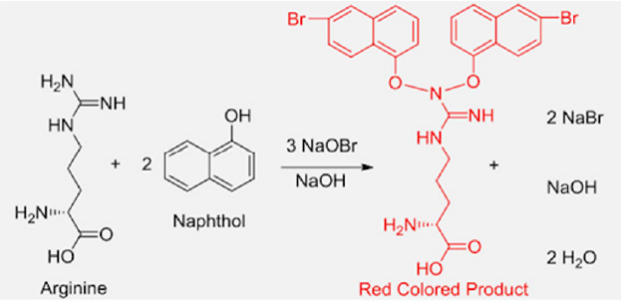

Figure g

http://biocheminfo.com/2020/04/15/sakaguchi-test-principle-reaction-reagents-procedure-and-result-interpretation/

Reagents

Sample, 40%NaOH, α-naphthol solution (in 1% alcohol), bromine water or sodium hypochlorite.

Procedure

Prepare mixture of 2 ml test sample and 1 ml of 40%NaOH. After that add 2-3 drops of α-naphthol. Mix it well and add 6-8 drops of bromine water or 2-3 drop of sodium hypochlorite. Observe red color at the end.

Thin layer Chromatography: - Variety of chemical substances can be separated on thin layer of absorbent material adhered to support like glass plates or glass slides [27].

Principle

A thin layer of absorbent acting as stationary phase allows rapid flow of solvent system. A polar absorbent, such as finely ground alumina (Al_2O_3) or silica (SiO_2) particles, is coated on a glass slide or plastic sheet to form a thin layer of the particular stationary phase in TLC. Adsorption occurs because silica contains certain free – OH groups that form hydrogen bonds or other Van-der-Waals interactions with the analyte components. To make the coating easier, a little amount of a binder, such as plaster of Paris, is sometimes combined with the absorbent. If the sample's components are coloured, they can be seen right away. If not, they can be seen by putting ultraviolet light on the plate or spraying the plate with a reagent (such as ninhydrin) that reacts with one or more of the sample's components. Compounds are estimated from their characteristics Rf values.

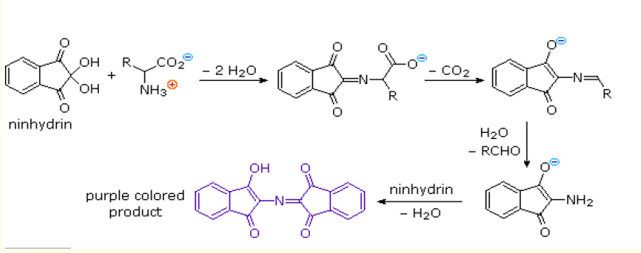

Figure h

https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Organic_Chemistry_(McMurry)/26%3A_Biomolecules_Amino_Acids_Peptides_and_Proteins/26.06%3A_Amino_Acid_Analysis_of_Peptides

Materials

TLC plate, solvent system according to sample, standard amino acid solutions like arginine, phenylalanine, leucine, isoleucine, developing agents like ninhydrin, TLC chamber, test sample, hot air oven, micropipette.

Procedure

Take a TLC sheet and load the amino acid standard and unknown sample along the line of spotting. Place the plate in a chromatography chamber previously saturated with the solvent system. Allow the solvent system to move in ascending direction until the boundary remains 2cm from the upper edge of the plate and mark the solvent front line. Remove the plate and dry it after that. Spray the detection reagent like ninhydrin solution gently and place the TLC plates at 80ºC for 5 minutes. Observe the spot developed and mark it and then calculate the Rf value by comparing with standard amino acid solutions [27].

Quantitative analysis of protein

There are number of quantitative tests to detect the presence of amino acids and these are largely depending on the nature of R-group and based on their absorption efficiency. Quantitative analysis methods are mostly carried out through spectrophotometrically. HPLC can also be used for appropriate detection. Quantitative

analysis of protein includes Folin-Lowry's method, Biuret method, Braford method, Ninhydrin method.

Folin-lowry's method

A biochemical technique for detecting the total amount of protein in any sample is the Folin – Lowry protein assay. A color change in the sample solution in proportion to protein content indicates total protein concentration, which may subsequently be quantified using colorimetric method [5].

Principle

Protein content present in the solution react with alkaline copper tartrate and Folin's reagent to display deep blue color by combination of the two reactions. In the first reaction, blue color is observed due to reaction of alkaline copper ions which is present in alkaline copper reagent with peptide bonds of proteins. Another reaction responsible for that is Folin's reagent which possesses phosphomolybdate and phosphotungstate are reduced by tyrosine and tryptophan present in the protein containing solution. The color intensity of solution is directly proportional to protein concentration and it is measured at 750 nm calorimetrically [32].

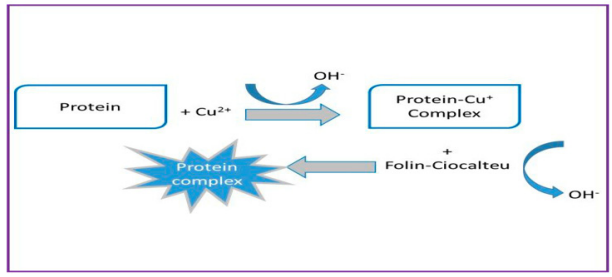

Figure i

https://bioquochem.com/lowry-protein-quantification-assay/

Reagents

Alkaline copper reagent, Folin's Ciocalteau reagent, Standard protein sample (Bovine serum albumin), Test sample.

Procedure

First take different aliquots of standard protein solution ranging from 0.1 to 1.0 ml. Then take suitable aliquots of undiluted and diluted unknown sample and make up final volume to 1.0 ml with distilled water. After that, add 5.0 ml of alkaline copper reagent in all the test tubes. Mix thoroughly and incubate at room temperature for 15 minutes. At the end, prepare final mixture by adding 0.5 ml Folin's Ciocalteau reagent in all the test tubes and mix well. Incubate it at room temperature for 30 minutes. Measure the intensity of blue color colorimetrically at 750 nm. Detect concentration of protein in unknown sample by drawing the standard graph.

Biuret test

The Biuret test is a chemical test that determines the presence of peptide link [26].

Principle

The compounds having two or more peptide bonds, copper salts in alkaline solution create a purple color complex. In other words, the cupric ions Cu^{2+} react with peptide bonds in an alkaline solution in the Biuret test. These ions generate a purple or violet colored complex when they react with the nitrogen in the peptide link. Cupric hydroxide is produced in an alkaline medium from copper sulphate from the biuret reagent, which support in chelating the peptide link with cupric ions, resulting in a violet or purple color complex. This chelate complex is purple color due to its capacity to absorb light with a wavelength of 540 nm. As a result, the presence of proteins in the analyte is indicated by the development of a purple-colored complex. The amount of peptide bonds that are reacting, and hence the number of protein molecules present in the reaction system, determines the absorbance produced. In the biuret test, short-chain peptides frequently produce a blue or pink color. Reaction showing the formation of violet color complex [9].

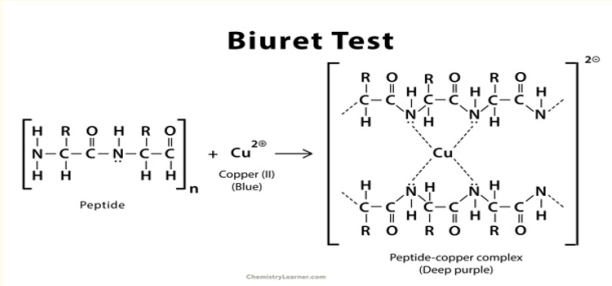

Figure j

https://www.chemistrylearner.com/biuret-test.html

Reagents

0.5% NaOH, 10% $CuSO_4$.

Procedure

Take three test tubes that are clean and dry. Fill the test tubes with 1-2 ml of the test solution, egg albumin and deionized water. Fill all three test tubes with 1-2 ml of Biuret reagent. Shake well and allow the mixtures to stand for 5 minutes. Observe for any color change.

Results

- The solution turns from blue to deep purple – proteins are present (Positive biuret test).
- No color changes., the solution remains blue – Proteins are absent (Negative biuret test).

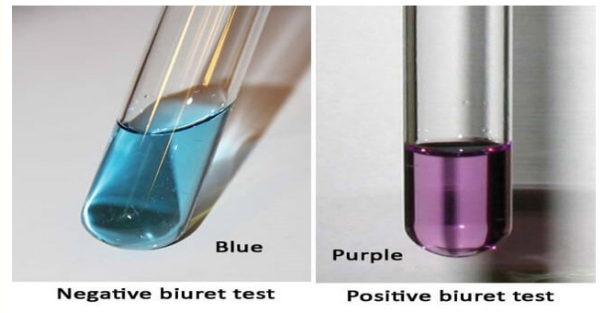

Figure k

https://thebiologynotes.com/testing-for-biological-molecules/

Braford protein assay

Braford assay is analytical procedure used to measure the concentration of protein in a solution. The reaction depends on the amino acid composition of the measured protein [14].

Principle

Coomassie brilliant blue is acidic stain, this test is depending on the fact that when coomassie brilliant blue G-250 is bound to the protein, the absorbance maximum moves from 465 nm to 595 nm in an acidic solution [3]. The anionic form of the dye is stabilized by both hydrophobic and ionic interaction, resulting in noticeable color change from brown to blue. The blue color intensity level can then be measured using a spectrophotometer which determine the concentration of the protein in test sample [34].

Reagents

Test sample, standard protein solution (Bovine serum albumin), Bradford reagent, 1 M NaOH.

Procedure

Before using spectrophotometer, it should be warmed up. You can use any complete protein as a standard, however bovine serum albumin (BSA) is commonly employed as a standard since it is inexpensive and easy to obtain. Prepare at least five dilutions of the BSA standard. The dilutions might be 5, 10, 25, 50, 75, and 100 micrograms of BSA per millilitre, for example. To the BSA dilutions, add reagent (which contains an acid and the Coomassie dye). Incubate for 5 to 10minutes and then measure absorbance at 595 nm by using spectrophotometer. The sample and standard processes are extremely similar. Dilute the sample if necessary and thenadd Bradford reagent. Incubate it for 5 to 10 minutes just like standards and then measure absorbance at 595 nm [14].

Ninhydrin test

This test is used for the detection of all α-L-amino acids.

Principle

Ninhydrin test assay is depend on the reaction of molecules of ninhydrin (2,2-dihydroxyindane-1,3-dione) which react with a free alpha-amino acid to create a deep purple or blue color. This blue or purple color known as Ruhemann's purple. Amino group belonging to free amino acid undergoes a chemical reaction with ninhydrin, which behaves as an oxidizing agent causes deamination and decarboxylation of amino acid. Deamination liberating ammonia, CO_2, aldehyde and reduced form of ninhydrin (hydrindanin). This is followed by the condensation reaction. NH_3 formed from amino group reacts with second molecule of ninhydrin. At the end, a diketohydrin complex is formed. This complex is responsible for the deep blue color [20].

Reagent

Ninhydrin reagent, Test sample.

Procedure

Take 1 ml of the test sample in test tube and take other tube in which add 1 ml of distilled water which act as control. Pour few

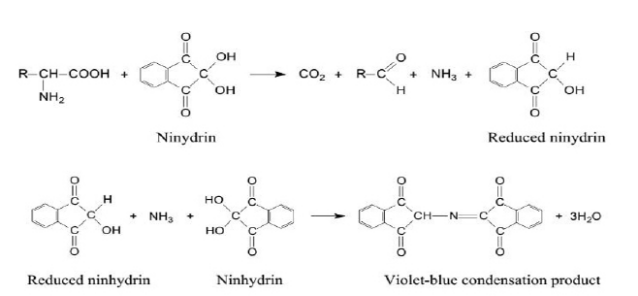

Figure l

https://biocheminsider.com/ninhydrin-test/

drops of 2% ninhydrin reagent in both of the tubes. Keep both of the test tubes in the water bath for 5 minutes and then allow it to cool at room temperature. Look for the development of violet or blue color complex [20].

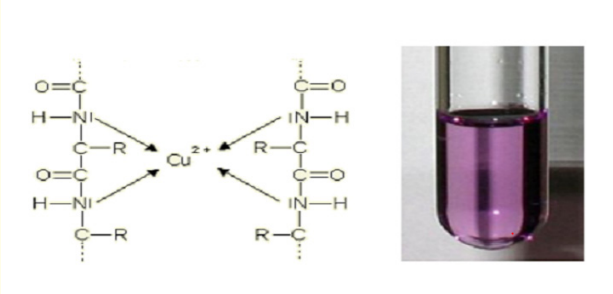

Figure m

https://biotrychemology.wordpress.com/2013/04/10/the-ninhydrin-test-of-proteins/

Note

When analyte contains amino acids like proline, a yellow-colored complex is formed. And when Asparagine is used, as a result brown color is occurring.

High performance liquid chromatography

HPLC stands for high-performance liquid chromatography, which is a type of column chromatography used for biomolecules to purify, identify, analyze, and quantify them. 4 types of HPLC are performed for the protein analysis. They are Reverse phased HPLC(RPC), Ion exchanged chromatography, Affinity chromatography and Size exclusion chromatography. However, reverse phased HPLC is widely used for the analysis of proteins [8].

Principle

RPC is the process of separating protein molecules according on their hydrophobicity. The separation is based on the hydrophobic binding of protein from the mobile phase to the immobilized hydrophobic ligands associated with the stationary phase. In the presence of aqueous buffers, the protein mixture is applied to the stationary phase, and the proteins are eluted by adding organic solvent to the mobile phase. Although all peptides and proteins have a mixture of hydrophilic and hydrophobic amino acids, those with a high net hydrophobicity will be able to interact with the stationary phase in hydrophobic interactions. Polar proteins will elute first, while non-polar proteins will bond to the column as mixtures of proteins are put to the column. By raising the concentration of organic solvent, the bound hydrophobic protein can be eluted.

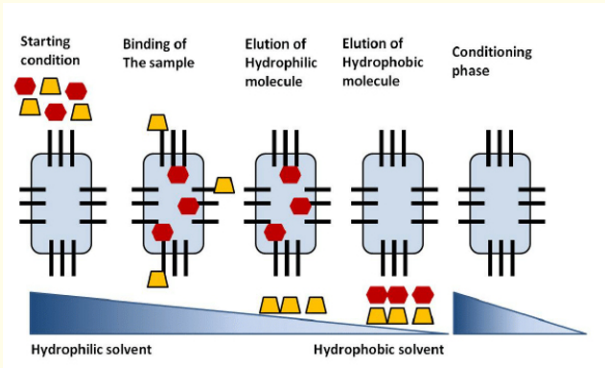

Figure n

https://www.researchgate.net/figure/Steps-of-a-of-reversed-phase-chromatography-separation_fig8_221929569

Method

The RPC experimental setup for peptide and protein analysis typically consists of an n-alkyl silica-based sorbent from which the solutes are eluted with gradients of increasing organic solvent concentrations. The RPC procedure can be broken down into four

phases. The hydrophobic column is primed by administering the specific sample buffer during the equilibration process. The sample protein is then introduced into the system under circumstances that encourage binding, usually with a low concentration of organic modifier. Proteins with a high percentage of exposed hydrophobic amino acid residues in the mixture will be adsorbed to the hydrophobic stationary phase, whereas other proteins will be washed away. During the elution process, the bound hydrophobic protein is eluted by adjusting the buffer conditions. The most typical method is to utilize a gradient that gradually increases hydrophobicity as the concentration of organic solvent increases. The molecules with the highest degree of hydrophobicity will be the ones that are held the most and eluted last. Finally, at the end of elution, a wash step eliminates the majority of the tightly bound molecules.

Conclusion

The goal of qualitative protein analysis is to identify the amino acids that are present. The Hopkins-Cole and Xanthoproteic test methods can both detect tryptophan. The Millon and Xanthoproteic test procedures can be used to identify tyrosine and its derivatives. The Nitroprusside test method can be used to detect cystine. The Sakaguchi test method can be used to identify arginine. Quantitative protein analysis involves determining the total protein content using a variety of methods and procedures. The Biuret method calculates the amount of peptide bonds in a sample by taking into consideration the sample's dilution and absorbance variables. The Lowry method is comparable to the biuret method; however, it is better at determining low protein concentration levels.

Acknowledgement

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Conflict of Interest

Authors have no conflict of interest.

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