The Analysis Techniques Of Amino Acid And Protein In Food And Agricultural Products

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Abstract: The protein content in food and agricultural products affects the physicochemical and nutritional properties of these products. This review aims to discuss the analysis techniques of protein and amino acid in food and agricultural products. The qualitative analysis can be conducted using the Hopkins-Cole, Xanthoproteic, Millon, Nitroprusside, and Sakaguchi test. In contrast, the quantitative analysis of proteins can use the KjehIdahl, Biuret, Lowry, UV Spectrophotometry, and Turbidimetry. It also discussed the immunohistochemical techniques to identify cellular or tissue constituents (antigens) by staining techniques, while Formol titration measures the hydrolysis of proteins and N-amino quickly. The amino acids can be analyzed by microbiological methods, colorimetric, high-performance liquid chromatography (HPLC), and gravimetric techniques. These methods/techniques can be chosen according to the type of sample and the purpose of the analysis so that the results can be obtained accurately.

Index Terms: Protein, amino acid, analysis technique, food, agricultural product

1. INTRODUCTION

Protein is one of the main macromolecular components that composed of amino acids through peptide bonds in specific sequences and types. Amino acids contained several main elements, such as C, H, O, and N. In addition, proteins also contain other elements such as sulfur, phosphorus, iron, and copper [1]. Amino acids contained in proteins are linked by a bond called a peptide bond [2]. Protein functions to form body tissue, growth, and form various bioactive compounds [3]. Specifically, proteins contain carbon atoms (50-55%), oxygen (20-23%), nitrogen (12-19%), hydrogen (6-7%), and sulfur (0.2-3%) [4]. The amino acid has two functional groups, a namely amino group and a carboxyl group [5]. Amino groups provide alkaline properties, whereas carboxyl groups provide acidic properties. In liquid or solution form, amino acids have amphoteric properties, which tend to become acidic when in the base solution, and turn into the base when in acidic solution. This is due to amino acids being able to become zwitterions [6]. The characteristics of a protein are determined by the type of amino acids and their sequence in polypeptides. Proteins have types of structures, which include the structure of primary, secondary, tertiary, and quaternary [7]. The primary structure shows the amount and sequence of amino acids in a polypeptide. The structure of secondary shows the structural content of a polypeptide which is influenced by the hydrogen bonding between oxygen (O) of the carbonyl group (C=O) and hydrogen (H) of the amino group (N-H) of the peptide chain framework [8]. The tertiary structure of proteins is formed by additional bonds between R groups in amino acids. Disulfide bonds are the only covalent bonds involved in tertiary structures, formed by oxidation of sulfhydryl groups from two cysteinyl residues. The quarter structure involves the interaction between two or more polypeptide chains that are specifically associated with forming proteins [2]. Based on the source, protein can be divided into two groups, namely vegetable protein and animal protein [9]. Vegetable proteins are the protein derived from vegetable materials, such as nuts, soybean, and other cereals. Animal proteins are the protein derived from animal products such as milk, eggs, meat, and fishery products. Animal protein has a complete and high-quality protein because it contains essential amino acids, especially amino acids that contain sulfur [10]. Both vegetable and animal proteins can also be modified into bioactive peptides that are beneficial both in the field of food and pharmaceutical technology [11], [12]. To determine the content of protein and amino acids in food and agricultural products, a quantitative or qualitative analysis must be conducted. Therefore, an appropriate analysis method is needed to identify the levels of protein and amino acids in food materials. This review discusses various analytical methods to determine the protein and amino acid content, both qualitative and quantitative analysis. The qualitative analysis includes several reactions, including Xanthoproteic reaction, Hopkins-Cole reaction, Millon reaction, Nitroprusside reaction, and Sakaguchi reaction. Quantitative analysis includes several methods, namely the Kjeldahl, Lowry, Biuret, and Spectrophotometry, Turbidimetry, and Formol titration methods. Analysis of amino acid levels can be determined by several methods, namely the Colorimetric, Gravimetric, Chromatographic, and Microbiological methods.

2 QUALITATIVE ANALYSIS OF PROTEINS

Qualitative analysis proteins consisted of the Hopkins Cole test, Millon test, Xanthoproteic test, Nitroprusside test, and Sakaguchi test. The specificity of the qualitative analysis techniques of protein can be seen in Table 1.

Tabel 1. *The specificity of the qualitative analysis techniques of protein.*

No	Analysis techniques	Analysis specificity
1	Hopkins Cole test	Identify the tryptophan based on Indole ring group.
2	Millon test	Identify the phenolic amino acids such as tyrosine and its derivatives.
3	Xanthoproteic test	Identify the proteins and amino acids that have aromatic rings or benzene groups such as phenylalanine, tyrosine, tryptophan, etc.

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4	Nitroprusside test	Detect the presence of cysteine amino acids.
5	Sakaguchi test	Identify the amino acid arginine based on guanidine group in its side chain.

2.1 Hopkins Cole test

Hopkins Cole Test is one of the qualitative test methods to determine differences in types of proteins and amino acids. This test is used to identify the presence of the amino acid tryptophan, which is the only amino acid that has an indole ring group [13]. Tryptophan belongs to the group of essential amino acids. Tryptophan is a precursor of niacin vitamin and an introduction to the serotonin nerve. Tryptophan functions to maximize the use of vitamin B complex, improve nerve health, stabilize emotions, increase feelings of calm, prevent insomnia, and increase the release of growth hormone. One of these amino acids can be found in egg whites. Hopkins Cole reagents which contain glyoxylic acid ($C_2H_2O_3$) react with sulfuric acid. Tryptophan in the protein solution will be condensed with the aldehyde group on glyoxylic acid with the help of strong oxidizing sulfuric acid. Positive results are indicated by the formation of purple rings between 2 separate layers [13], [14]. According to Elzagheid [15], the principle of testing tryptophan by the Hopkins Cole test method is by adding 1 mL of Hopkins Cole reagent into a test tube that already contains a 2% protein solution, then the concentrated sulfuric acid solution is added as much as 1-2 mL. The solution is homogeneous with vortex, and then color changes are formed.

2.2 Millon test

Millon test is one of the qualitative test methods to determine differences in types of proteins and amino acids, namely the type of phenolic amino or amino acids that have phenol groups such as tyrosine and its derivatives [13]. Tyrosine is a non-essential amino acid that has a phenyl group and is a weak acid. One of these amino acids can be found in milk casein. Millon test is not a specific test, because this test identifies all types of phenol compounds, so to ensure that testing is needed by other means [16]. In this test, Millon reagents are used, which are solutions containing mercury (Hg) dissolved in nitric acid [17]. These mercury compounds bind with hydroxyphenyl groups to produce a white precipitate in a protein solution [18]. Tyrosine in protein solution will form a solution or reddish-brown sediment when heated. The principle of testing tyrosine with the Millon test method is to homogenate 1-3 drops of Millon reagents into a test tube that already contains a 2% protein solution. After that, the solution is heated using a water bath and observed the changes in color and the formed deposits [15].

2.3 Xanthoproteic test

Xanthoproteic test aims to determine differences in types of proteins and amino acids that have aromatic rings or benzene groups such as phenylalanine, tyrosine, tryptophan, etc. [13]. In this test used a solution of concentrated nitric acid and a base in the form of ammonia or sodium hydroxide (NaOH). Phenylalanine, tyrosine, and tryptophan will form white deposits that can turn yellow when reacting with nitric acid in the presence of heat [17]. This yellow color is called Xantho protein. Addition of a basic solution such as HNO_3 or NaOH will create a very alkaline so that the nitro compound that has been previously formed can be ionized and the solution changes color to dark yellow or orange. The visible color appears based on the nitration reaction on the aromatic ring of amino acids [18]. The principle of testing phenylalanine, tyrosine, and tryptophan by the Xanthoproteic test method is by homogenizing 1 mL of HNO_3 solution that already contains a 2% protein solution. After that, the sample is heated using a water bath for 15-20 minutes or on fire for 2 minutes, then a change in the color of the solution is observed. The test tube is incubated at room temperature until the temperature decreases then NH_3 solution or 20-40% NaOH is added to it, and a change in the color of the formed solution is observed again [15].

2.4 Nitroprusside test

Nitroprusside test is a test used to detect the presence of cysteine amino acids. This test uses sodium nitroprusside reagents and ammonia solution. The testing principle is that the free -SH group called thiol or mercapto, which is owned by the amino acid cysteine will react with nitroprusside in the case of excess ammonia to form red compounds. The S-S-group in cysteine will give positive results in nitroprusside testing if it is reduced first. Nitroprusside testing is conducted by entering 0.5 mL of sodium nitroprusside 1% into the test tube then add 2 mL of the sample to be tested, then add 0.5 mL of NH_4OH solution. The sodium nitroprusside solution used must be in a new condition and made just before the test [19]. Nitroprusside reactions can be applied in various tests, one of which is to detect the content of cysteine in urine clinically [20]. The test was carried out by adding of sodium nitroprusside 20% (w/v), then the color intensity was measured using a spectrophotometer at a wavelength of 521 nm in 1 minute, and the cysteine concentration was calculated using a standard curve [21].

2.5 Sakaguchi Test

The Sakaguchi test is a chemical test that includes a colorimetric reaction for the identification and quantification of the amino acid arginine. Arginine is an amino acid that has a guanidine group in its side chain, which is the C atom that binds N_2 with a single bond and binds N with a double bond. Sakaguchi reaction is carried out using naphthol and sodium hypobromite or sodium hypochlorite reagents. The guanidine group in arginine, which is oxidized by sodium hypochlorite, will react with alpha-naphthol and produce red compounds under alkaline conditions; the absorption spectrum produced by the Sakaguchi reaction at a wavelength of 520 nm [22]. In general, Sakaguchi testing is carried out by inserting about 2 mL of the sample into a test tube then adding two drops of 1% α-naphthol in alcohol, 4% sodium hydroxide and 8-10 drops of bromine water. Testing gives positive results if red complexes are formed [23]. Adding hypobromite to the arginine solution that has been given α-naphthol in an alkaline state will produce a red complex immediately, followed by rapid fading of the color [24].

3 QUANTITATIVE ANALYSIS OF PROTEIN

Quantitative analysis proteins consisted of the Kjeldahl, Biuret, Lowry, UV-Spectrophotometry, Turbidimetry, Immunohistochemistry, Formol titration. The specificity of the quantitative analysis techniques of protein can be seen in Table 2.

Tabel 2. *The specificity of the quantitative analysis techniques of protein.*

No	Analysis techniques	Analysis specificity
1	Kjeldahl	Determine the total protein based on total nitrogen.
2	Biuret	Determine the soluble protein content based on peptide bonds.
3	Lowy	Determine the soluble protein content in small amounts based on peptide bonds. More sensitive than Biuret
4	UV-Spectrophotometry	Determine the soluble protein based on the interaction of the sample with UV light.
5	Turbidimetry	Determine the protein based on the measurement of light scattering in a cloudy protein solution.
6	Immunohistochemistry	Determine the cellular antigens (protein) through antigen-antibody interactions
7	Formol titration	Determine the protein hydrolysis to show N-amino levels

3.1 Kjeldahl method

Determination of total protein in various fields such as biology, pharmacy, environment, and food is generally carried out by the Kjeldahl method. This method is still recognized by AOAC International as the official method for protein analysis. The principle of the Kjeldahl method is to measure the total protein based on the nitrogen content, which represents the protein in the materials [25]. In general, the Kjeldahl method is carried out through 3 stages, namely destruction, distillation, and titration. The destruction stage involves the destruction of organic compounds in the sample using sulfuric acid or potassium sulfate with a catalyst to convert nitrogen in protein into ammonium sulfate quantitatively. Then the solution is distilled using excess sodium hydroxide to free ammonia and then absorb it in boric acid [26]. Ammonia distillate in boric acid is then titrated using hydrochloric acid to measure nitrogen in ammonia that reacts with acids [27]. Nitrogen levels obtained from the titration results represent the amount of crude protein present in the sample [28]. In general, the conversion factor used in determining total protein using the Kjeldahl method is 6.25, which is based on the assumption that the general nitrogen content is 16% in food proteins, and all nitrogen in food is bound to protein. This assumption is a fairly crude and inaccurate assumption because of the relatively variable nitrogen content between the varying protein and amino acids content of food products. The presence of various other compounds containing non-protein nitrogen such as free amino acids, nucleic acids, urea, ammonia, nitrate, chlorophyll, and alkaloids in food products is one proof that the conversion factor 6.25 is less accurate in determining total protein. Specific conversion factors for various types of food have been made to overcome this problem so that now the calculation of the conversion of nitrogen into protein is more precise [29].

3.2 Biuret method

Biuret method is a method used to determine the protein content based on peptide bonds in the material being tested. Peptide bonds obtained indicate that the protein contained because amino acids bind to other amino acids through peptide bonds. The reagents used in this method are NaOH and $CuSO_4$ [30]. The principle of the biuret method is that the protein solution is converted into alkalis with NaOH, then a $CuSO_4$ solution is added so that the protein reacts with Cu^{2+} to form a blue-purple complex under alkaline conditions. The more or the longer the peptide bonds contained in the protein, the stronger the purple color produced [31]. Biuret reagents can be prepared by dissolving 150 mg of copper (II) sulfate ($CuSO_4.5H_2O$) and potassium tartrate ($KNaC_4H_4O_6.4H_2O$) in 50 mL of distilled water. The next step, add 30 mL of 10% sodium hydroxide while shaken, then add distilled water to the limit of the measuring flask line [32]. According to Janairo et al. [33], testing by the Biuret method can be conducted by taking a few samples of dissolved protein, such as albumin. Albumin needs to be precipitated before being used by adding crystalline ammonium sulfate until it approaches the saturation of ammonium sulfate in solution. The precipitating protein is separated by centrifuge at 11,000 rpm for 10 minutes. The precipitate, which is a protein, is then dissolved with acetic acid (pH 5) of 10 mL. The solution is then taken and biuret reagent added and incubated for 10 minutes. The solution then reads its absorbance at a wavelength of 550 nm to the blank containing the biuret reagent and acetate pH 5. Dilution factors must be considered, and the absorbance of the sample should be within the absorbance range of the standard curve.

3.3 Lowry method

Lowry method is a development of the Biuret method. The principle of the Lowry method is the reaction that occurs between Cu^{2+} with peptide bonds and the reduction reaction of phosphotungstic acid and phosphomolybdic acid by tryptophan and tyrosine contained in a protein [34]. Protein in the alkaline condition then added phosphomolybdic and phosphotungstic acid to produce a blue color whose thickness depends on the concentration of protein contained in the materials. The resulting bluish color can be measured at wavelengths of 500-750 nm [29]. There are two kinds of reagents used in the Lowry method, namely solution A and solution B. Solution A is a mixture of phosphotungstic acid and phosphomolybdic acid in a ratio of (1:1). Solution B can be made by mixing 2% sodium carbonate in 100 mL of 0.1 N sodium hydroxide, then adding with 1 mL of 1% copper (II) sulfate and 1 mL of potassium sodium tartrate 2% [35]. Determination of protein content by the Lowry method requires a standard curve that describes the relationship between protein concentration and optical density (OD) [35]. A standard curve can be made by preparing a bovine serum albumin (BSA) solution with a concentration of 300 µg/ mL. Next, add 8 mL of Lowry B reagent into each of the tubes with different concentrations and leave it for 10 minutes, then add 1 mL of Lowry A reagent, shake and let stand for 20 minutes. The solution obtained was then measured for its absorbance at a wavelength of 600 nm against the blank [29]. The test step of the Lowry method is almost the same as the protein testing step of the Biuret method, the difference at the end is determined by the addition of 8 mL of Lowry A reagent and so on as in the standard curve.

3.4 UV-Spectrophotometry method

Determination of protein content by the UV Spectrophotometry method is based on the interaction of the sample with UV light [36]. UV light has a wavelength of around 100-400 nm. UV rays cannot be seen in the human eyes; therefore, compounds that can absorb UV rays are compounds that have a clear and transparent color. The principle of the UV spectrophotometer

method is that the tested sample must be clear and completely dissolved; there are no colloidal particles or suspensions [37]. Tryptophan, tyrosine, and phenylalanine are amino acids making up proteins that have aromatic groups. Tryptophan has a maximum absorption at 280 nm [38]. Tyrosine has a maximum absorption at 278 nm [39]. Phenylalanine has less strong light absorption and absorption at shorter wavelengths. Estimation of protein concentration in a test solution can be seen absorbance at 280 nm. For more accurate results, it is necessary to correct possible contamination of nucleic acid content at 260 nm. The absorption ratio of 280/260 nm can be used to determine the correction factor [40].

3.5 Turbidimetry method

Turbidimetry is the analysis method based on the measurement of light scattering in solution. The method of measurement is by reducing the intensity of the light after passing through the suspension solution. Turbidimeters apply a continuous scattering of light or 180°, in contrast to a nephelometer that uses light scattering with an angle of 90° [41]. Basically, turbidimetry measures the ratio between the intensity of the light that is passed on with the intensity of the initial beam. The continued measurement of light intensity as a function of concentration is a basic principle of turbidimeter equipment. Protein analysis using the turbidimetry method can use benzethonium chloride (BZ) from cationic detergents. The results showed that turbidity is dependent on pH and is reversible. However, when BZ concentrations in sediment are low, turbidity decreases with increasing pH at a higher pH range. Turbidity formation by aromatic organic acids such as sulfosalicylic acid is reversible and can only be observed in the lower pH range [42]. The formation of turbidity in the turbidimetry method uses BZ because the positively charged cationic detergent will bind to the negatively charged protein in the pH range of 5-6 with the dissociated and complex carboxyl groups produced together by their respective intermolecular forces. However, when the BZ concentration is low, the principle of reaction that has been reported is not enough to explain the phenomenon that turbidity decreases with increasing pH in a higher pH range. According to Boumaza et al. [43], the turbidimetry method is suitable to be implemented to evaluate protein from milk and cereal wastewater. This has proven to be effective in terms of analysis time and precision, so as to avoid the disadvantages of using other classical methods to determine proteins that usually take a long time and are expensive.

3.6 Immunohistochemistry method

Immunohistochemistry (IHC) is the method that can be used for identifying cellular antigens through antigen-antibody interactions, where binding of antibodies are identified by direct labeling of antibodies or by using a secondary labeling method [44]. Immunohistochemical staining methods related to the use of antibodies labeled enzymes (immunoperoxidase) and labeled fluorophore (immunofluorescence) to identify proteins in cells. In general, the mechanism of the immunohistochemical method begins with deparaffinization. Then proceed with the collection of antigens. The unspecified binding site is blocked and primary antibody bound. Then, the biotinylated secondary antibody is bound as well. In this method, detection uses the peroxidization-anti peroxidase method, the biotin-avidin conjugate, the peroxidation complex or the two-step polymer labeling method, which is more widely used. Then, additional chromogen substrate, usually DAB, and the final stage is counterstaining and dehydration. There are two basic methods of using immunohistochemistry to identify antigens in tissues, namely the direct method and the indirect method. The principle of the direct method is the use of labeled primary antibodies so that they will bind directly to the antigen directly. Meanwhile, the principle of the indirect method is the use of primary antibodies that are not labeled, but this method also uses secondary antibodies that already have a label and will react with Immunoglobulin G (IgG) from the primary antibody. The advantages of immunohistochemical methods are the location and distribution of visible proteins, which can be detected in the biopsy of small and large tissues as well as fixed tissues, and validation of other high yield studies (DNA microarrays). While the shortcomings of this method are limited ability to measure protein content, problems with antibody types, limited ability to detect protein modification, limited or less evidence-based criteria, no method of normalizing, the results are limited, and limited capacity for creating clinical biomarker profiles (only with tissue microarrays) [45].

3.7 Formol titration method

Formol titration is a titration that is usually conducted to determine the protein content in milk quickly. In addition, formol titration can also be used to measure protein hydrolysis to show N-amino levels in a processing or storage process. The principle of the formol titration method is to neutralize the solution with a NaOH added with formaldehyde in which the amino group is bound and does not affect the acid-base reaction of NaOH to form Dimethylol. The indicator commonly used is phenolphthalein (PP), and the color change reaction turns pink at the end of the titration [46]. In general, the determination of protein content by formol titration is conducted by weighing a sample that has been mashed about 10 g, then dissolved in distilled water and shaken out with a stirrer for 15 minutes. The filtrate is filtered and diluted with distilled water, then taken about 10 mL to be added with distilled water, potassium oxalate, and PP indicator. The reaction mixture is then titrated with 0.1 N NaOH until it turns pink. The titrated sample added 2 mL of 40% Formaldehyde, and the PP indicator was added. After that, it was titrated again using 0.1 N NaOH, then calculate the protein content [47].

4 AMINO ACID ANALYSIS

4.1 Colorimetric Method

The colorimetric is a chemical analysis that is included in photometric analysis. The principle of the analysis is to compare the intensity of the color between the sample solution made using a Nessler tube or Dubosque colorimeter with the color of the solution whose concentration is known or is called a standard solution, using polychromatic light as a light source and the eye as a detector. The colorimetric method is widely used in the amino acid analysis because of the low costs incurred, minimal equipment used, the discoloration can be observed easily even at very low concentrations [48]. Color duplication in the analysis using the colorimetric method is conducted by using two solutions in an upright position in the direction of the light or visualization tool that has the same substance in the column with the same cross-section aerometer capabilities. There are several reagents used in

amino acid analysis using the colorimetric method, among which the most common is ninhydrin and several other reagents such as 2,4-dinitrofluorobenzene and their derivatives and sodium nitroprusside [49]. The colorimetric method with the ninhydrin reagent is widely used for the analysis of compounds containing amino groups in the pharmaceutical products and food industry [50]. Amino acid analysis using the colorimetric method can be conducted by adding 1 mL of the ninhydrin reagent to 5 mL of the sample and reacted at 80-100 °C for 4-7 minutes, then cooled to room temperature. The sample is then absorbed using a spectrophotometer and compared with a standard solution.

4.2 High-performance liquid chromatography (HPLC) method

HPLC is one method that is often used to separate heat-resistant compounds such as amino acids [51]. HPLC is a special type of column chromatography. This method uses a high-pressure liquid as the mobile phase [52]. The principle of amino acid analysis using this method is freeing amino acids from protein through hydrolysis by HCl 6 N. Hydrolyzate is then dissolved with a sodium citrate buffer which then these amino acids will be separated by HPLC [53]. Analysis of amino acids by the HPLC method can be conducted using OPA reagents [54]. Provision of OPA reagents can be conducted by preparing 50 mg of OPA, 4 mL of methanol, 0.025 mL of mercaptoethanol, 0.050 mL of 30% Brij-30, and borate buffer 0.5 M, pH 10.4. The second step is to prepare mobile phase A, which consists of 2 g Na-acetate hydrate, 90 mL methanol, 0.5 g Na-EDTA, and 10 mL THF. The reagent mixture was added with water up to 1 liter in a measuring flask; then the pH was adjusted to 6.5 by NaOH. Furthermore, mobile phase B consisting of 95% methanol is made. Both phases were filtered with a 0.45 µl membrane filter. The next step is to do sample preparation. First, insert a sample containing 3 mg of protein into the screw tube; then add 1 mL of HCl 6 N. Hydrolysis by heating the tube in an oven at 110 °C for 24 hours, then cooled. The sample was filtered with sintered glass, rinsed several times with 0.01 N HCl then dried with a vacuum evaporator. Next, re-dissolve the dried sample with 5 ml of 0.01 N HCl. The sample is ready to be injected into HPLC [55]. Injection of the sample at HPLC is conducted by preparing a sample, then adding with potassium borate in a ratio of 1: 1. A total of 1 µl of the sample is put into an empty vial, then 25 µl of OPA reagent is added, leave it for 1 minute so that the derivatization is fully flawed. Furthermore, inject a sample of 5 µl into the HPLC, wait until the amino acid separation is complete, the time required is approximately 30 minutes [56].

4.3 Microbiological methods

Microbiological method is one of the quantitative test methods for amino acid content in a material [57]. The implementation of amino acid testing using microbiological methods is not more widely used compared to other methods. This is because to determine accurate results, and this testing requires a long time and adequate expertise. But in addition to these shortcomings, this method has very good sensitivity to amino acids, and the process does not cost a lot. The use of microbiology and biotechnology continues to grow rapidly both for analysis and bioprocess purposes [58], [59]. Utilization of microorganism growth is used in microbiological test methods. Some of the most commonly used bacteria are Lactobacillus arabinosus for leucine, isoleucine, valine, and tryptophan; Leuconostoc mesenteroides for histidine, phenylalanine, and methionine; and Streptococcus faecalis for arginine and threonine. Apart from microorganisms, other important components involved in the microbiological test method are the medium in which the microorganisms grow and the protein samples that are to be tested. Before testing, protein samples must be ensured that they have been hydrolyzed to their amino acids [57]. Hydrolysis of this protein can be conducted by two methods, namely precipitation with acetic acid or by ultrafiltration method [60]. In general, the steps in testing amino acids by using this microbiology test method are by entering hydrolyzed protein samples into a test tube. Then, added to the test medium composed of yeast extract, glucose, and agar [57]. The test tube was then sterilized using an autoclave at 121 °C for 10 minutes. The medium in the tube is inoculated with microorganisms corresponding to the amino acids tested and incubated at 37 °C for 17 hours. The optical density produced is then measured against water in the CF4 optical spectrophotometer. Amino acid levels will be obtained after comparison with blanks [60].

4.4 Gravimetric method

Gravimetry is an analytical method that can be used for amino acid analysis. Gravimetric method is a measurement based on changes in mass or weight. This method is the oldest quantitative analysis technique [61]. Gravimetric method is divided into several types, one of which is the most popular is thermo-gravimetry. Thermo-gravimetric analysis (TGA) is commonly used to measure the water that binds to unnatural amino acids and to humectant, which fills as is the case with urea. The number of bound water molecules per hydrotrope molecule of unnatural amino acids can use quantum-mechanical molecular modeling (QMMM) which is used to predict the binding sides and the levels of water molecules associated with each of the unnatural amino acids and urea [62]. Thermo-gravimetric analysis can be conducted using the instrument of TGA Q50 TA, UK. Samples need to be pulverized lightly to separate rough aggregate using mortar, then dried in a vacuum desiccator. The dry sample is then placed into a controlled relative humidity chamber for one week at room temperature. The RH of the desiccator 0% can be achieved by a vacuum desiccator on silica, 33% RH can be achieved by a desiccator containing saturated zinc nitrate solution, 40% RH can be achieved by a desiccator containing saturated potassium carbonate solution, and RH 100 % can be achieved by a desiccator containing water. The saturated zinc nitrate gives an initial RH of 42% at 20 °C [63], but this is balanced at 33% of recorded RH due to unnatural hygroscopic amino acids. The sample is then heated in the instrument at a temperature of 25 - 250 °C for 1 minute [62].

5 CONCLUSION

Protein analysis by qualitative methods focuses on identifying the amino acids contained therein. Tryptophan can be identified by the Hopkins-Cole and Xanthoproteic test methods. Tyrosine and its derivatives can be identified by the Millon and Xanthoproteic test methods. Cysteine can be identified by the Nitroprusside test method. Arginine can be identified by the Sakaguchi test method. Quantitative protein analysis is conducted by measure the total protein content through several methods with different techniques. The Kjeldahl method measures protein content based on its

nitrogen content by the steps of destruction, distillation, and titration. The Biuret method measures the content of peptide bonds by taking into account the dilution and absorbance factors of the sample. The Lowry method is similar to the biuret method, but this method is more specific for determining low protein concentration levels. The UV Spectrophotometry Method measures protein content based on the interaction of the test sample with UV light. The Turbidimetry method measures protein content based on the measurement of light scattering species in solution. Immunohistochemical methods identify cellular or tissue constituents (antigens) by coloring techniques. In comparison, Formol titration measures the hydrolysis of protein and N-amino quickly. Amino acid analysis can be conducted by several methods such as the Microbiology method that utilizes the growth of certain microorganisms for each amino acid, the Colorimetry method that compares the color intensity of the amino acid sample solution, the HPLC method that uses a liquid with high pressure as a mobile phase, and the Gravimetry method that measures changes amino acid mass or weight.

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