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Determination of amino acid composition of foods by photometric methods, Part 1 Determination of tyrosine, tryptophan and phenylalanine

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1. SUMMARY

Today to determine the amino acid composition of foods, the most commonly used technics are the ion exchange column chromatography (IEC) with post-column derivatization with ninhydrin and the high-performance liquid chromatography (HPLC) with pre-column derivatization. Both technics are excellent in their own right, either for solving special tasks or for determining all amino acids in a protein. However, the methods require special purpose instruments (IEC) or special equipment (HPLC), which are expensive and require highly qualified analysts to operate, which cannot be afforded by smaller laboratories and in-process inspection units. The methods we propose, after proper sample preparation, can be implemented with a UV-VIS spectrophotometer capable to measure the aromatic amino acids and the coloured amino acid derivatives in the range of 200-800 nm.

In this paper, we give a report on the photometric methods developed for the determination of total amino acids with ninhydrin, determination of aromatic amino acids, measurement of the combined amount of tyrosine and tryptophan, separate determination of tyrosine, tryptophan and phenylalanine. We plan to quantify the most suitable of the mentioned methods and make them applicable to food testing laboratories. Since the indole group of tryptophan is virtually completely degraded during the 6 M hydrochloric acid hydrolysis used in the hydrolysis of proteins, this publication protein hydrolysis methods that are suitable for determining the tryptophan content of foods is described.

2. Introduction

Amino acids are organic compounds whose molecules contain both carboxyl and amino groups. In nature they occur in relatively small amounts as free compounds, but they have outstanding importance to living organisms in proteins, as the major source of amino acids in heterotrophic organisms comes from the breakdown of dietary proteins. In living organisms, during digestion, the proteins are broken down into 20 types of amino acids, some of which are essential for the body (phenylalanine, isoleucine,

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leucine, lysine, methionine, threonine, tryptophan, valine). The essential amino acid that occurs in the smallest amount in the diet compared to the needs of the body is the limiting amino acid **[4, 5, 8, 20, 49]**.

The amino acid composition of foods in most laboratories is determined by ion exchange column chromatography (IEC) or an automatic amino acid analyser based on it **[7, 9, 16, 20, 28]** or high performance liquid chromatography (HPLC) **[9, 20]**. However, obtaining and operating the necessary equipment is very expensive, which a smaller laboratory cannot afford. Therefore, the aim of our studies is to develop photometric methods that can be used to measure amino acids easily without the use of highly sophisticated analytical instruments. Using the reported methods below, it may be possible to determine some essential amino acids in laboratories without expensive analytical instruments.

3. Hydrolysis of proteins

Before determining the amino acid composition of a protein, the protein must be hydrolyzed to free amino acids. An internationally accepted method of protein hydrolysis was developed by Moore and Stein **[35]**, which weighs 20-200 mg of food and feed sample, depending on the protein content of the sample, and hydrolyses with 6 M hydrochloric acid at 110±1 °C for 24 hours. Under these conditions, tryptophan (Trp) is partially degrading in 40-60% but completely degraded in the presence of carbohydrates. That is the reason why in lot of cases the acidic hydrolysis methods have been modified. To avoid the degradation of the Trp the researschers added different soultions to the 6M HCl: Freelender and Haber **[12]** added 1,4-butanedithiol, Matsubara and Sasaki **[33]** added 2-4% thioglycolic acid, Gruen and Nichols **[15]** 3-(3-indolyl)-propionic acid, and James **[26]** was able to partially inhibit Trp degradation by alternating thioglycolic acid, mercaptopropionic acid, mercaptosuccinic acid and oxalic acid. Liu and Chang **[30]** with 3 M para-toluenesulfonic acid containing 0.2% 3- (2-aminoethyl) indole (tryptamine), Liu **[31]** with 4 M methanesulfonic acid, Penke et al. **[40]** hydrolyzed the protein with 3 M mercaptoethanesulfonic acid prior to Trp determination. Csapó et al. **[6]** hydrolysed the protein with 3 M mercaptoethanesulfonic acid at high temperatures (160, 170, 180 °C) and for a short time (30-60 minutes), with the highest Trp yield compared to all acidic methods, and the result of hydrolysis was same for the methionine content too. Perfect success could not be achieved with acidic methods, therefore according to Liu **[31]** the Trp content can only be quantified by alkaline hydrolysis.

Barium hydroxide hydrolysis of proteins was first used by Homer **[21]** to determine Trp content. Jorpes **[27]** hydrolyzed the protein with 5 M NaOH and recovered Trp in 78-98%. Holyler **[24]** hydrolyzed the protein with sodium hydroxide and found that both free and peptide-bound Trp are partially degraded during basic hydrolysis. According to Shizuko-Isole **[45]** and Dreeze **[11]**, hydrolysis under a nitrogen atmosphere can prevent the decomposition of free Trp.

According to Warner **[48]**, sodium hydroxide hydrolysis is slower than barium hydroxide, and sodium is much more difficult to remove from the system than barium. Using barium hydroxide as the hydrolyzing agent, Dreeze **[11]** found that the hydrolysis is faster and Trp does not degrade even in the presence of starch. Miller **[34]** removed barium in the form of barium sulphate from the solution, with minimal Trp adsorption of the precipitate. The protein was hydrolyzed with 4 M sodium hydroxide and then the alkali was neutralized with hydrochloric acid, after which the hydrolyzate was chromatographed directly according to Dévényi et al. **[10]**.

Noltmann et al. **[37]**, barium hydroxide hydrolysis seems to be the most expedient in determining tryptophan content, since here the hydrolysis takes place relatively quickly and the barium can be removed much more easily than sodium. Removal of barium in sulphate form appears to be more expedient because the precipitation of barium with sodium sulphate from a neutral solution does not cause tryptophan adsorption.

4. Determination of the amino acid composition of a proteins using photometric methods

4.1. Determination of total amount of amino acids by colour reaction in ninhydrine (ninhydrine positive compounds)

The ninhydrin (2,2-dihydroxyindane-1,3-dione) converts amino acids to aldehyde, carbon dioxide, and ammonia in the pH range of 4 to 8, is excellent for determining the total amino acid content of a hydrolyzed sample. Ammonia reacts with the excess of ninhydrin to form an intense blue and purple compound, hydrindantine, with α-amino acids, with a maximum light absorption of 570 nm, where the amount of amino acids can be determined. Imino acids, proline, and hydroxyproline form a yellow compound with ninhydrin under similar conditions, with a light absorption maximum at 440 nm **[43, 50]**. The reaction measures all other ninhydrin-positive compounds in the sample in addition to amino acids, but since their amount in food is negligible, the reaction is suitable for measuring all amino acids with approximate accuracy.

4.2. Determination of aromatic amino acids tyrosine, phenylalanine, tryptophane) by spectrophotometry in ultraviolet range

Of the protein-forming amino acids, only three aromatic amino acids (tyrosine, phenylalanine, tryptophan) have absorption maximum in the ultraviolet range at 280 nm. The amount of the three amino acids, after appropriate sample preparation, can be measured without derivatization. Its advantage is that

it is quick and easy, as it is not necessary to subject the proteins to a chemical reaction for the measurement. Thus, the combined amount of the three amino acids can be determined by this method if the protein concentration of the solutions is between 20 and 3000 µg/ml **[25, 44, 46]**. However, it is very important to select the right solvent, as many solvents have significant UV absorption in the light absorption band of aromatic amino acids.

4.3. Determination of the total amount of tyrosine and tryptophan

During the xantoprotein reaction, concentrated nitric acid reacts with the aromatic nucleus that forms the side chain of the amino acid, resulting in a yellow product that can be quantified photometrically. The aromatic ring of phenylalanine does not react with nitric acid, so only the combined amount of tyrosine and tryptophan can be determined by this reaction. Hassan **[17]** used 16 M nitric acid, 5 M sodium hydroxide, and ethyl alcohol to determine the amount of tyrosine and tryptophan. The absorbance of the solutions was studied at 360 and 430 nm.

4.4. Determination of tyrosine content by photometry

The Millon test is specific for tyrosine as it is the only amino acid that contains a phenyl side chain. During the reaction, the phenyl group of tyrosine first reacts with nitric acid and then forms a brick red colour reaction with a complex with mercury ions. The colour intensity of the resulting product can be measured spectrophotometrically at a wavelength of 500 nm, and the amount of tyrosine can be calculated from the absorbance. However, the assay is specific for tyrosine only if the sample does not contain a compound with other phenolic groups **[32, 41].**

Grau **[14]** used a modified Millon reaction to determine the tyrosine content of the protein. The amount of tyrosine in the sample was determined using mercury sulphate reagent and sodium nitrite, and the absorbance of the resulting red solution was examined at a wavelength of 475 nm. No colour reaction was observed when the method was attempted to determine the amount of phenylalanine, histidine, or tryptophan.

4.5. Determination of tryptophan content by photometry

Free or peptide-bound tryptophan forms a coloured product with para-toluene-sulfonic acid, para-dimethyl-amino-benzaldehyde and N-bromo-succinimide, which are the reagents that form the basis of the photometric determination of tryptophan, in which the colour obtained in the quantitative determination compared to a standard colour of a well-known concentration, which usually consists of free tryptophan. Spies and Chambers **[47]** determined the Trp content of the samples in 19 M sulfuric acid medium with para-dimethyl-amino-benzaldehyde (DAB). The absorbance of the solutions was studied in the 580-620 nm wavelength range. The Trp content of the meat was determined was also determined with para-dimethyl-amino-benzaldehyde (DAB) in 9.5 M sulfuric acid, in which the colourless condensation product was oxidized by sodium nitrite to a blue compound, the absorbance of which was studied at 590 nm by Rékásiné et al. **[42]**. Hydrolysis of the test protein simplifies the situation, as Trp goes into solution and its colour can be determined in a similar way.

Among the photometric methods, the determination of N-bromosuccinimide is not widespread in practice, and the same can be said for the colour reaction with glacial acetic acid (III) chloride reagent. In the latter case, the Trp content of cereal grains was determined by photometry at 545 nm of the resulting red solution **[38]**. The para-dimethyl-amino-benzaldehyde method, on the other hand, has been used by several to determine the Trp content of both unhydrolyzed protein and hydrolysate. An interesting method for the photometric determination of Trp is reported by Basha and Roberts **[3]**, who oxidized Trp with sodium nitrite and then reacted the oxidized product with N-1(naphthyl)-ethylene-diamino-dihydrochloride and then obtained the purple-pink material was photometrized at 550 nm.

The indole group of tryptophan shows light absorption in the ultraviolet range, which can also be used to determine its amount. For soluble proteins, UV absorbance is measured at 280-288 nm. Ultraviolet absorbance is also suitable for measuring the absorbance of a protein in solution by any method, as well as the absorbance of a hydrolyzate.

The amount of tryptophan can be determined using the Hopkins-Cole test **[13]**, which is based on the fact that the indole ring of tryptophan reacts with a mixture of Hopkins-Cole reagent (glyoxylic acid) and sulfuric acid to give a violet or purple product. The absorbance of the resulting coloured product was studied at 545 nm. Koshland et al. **[2, 22]** used 2-hydroxy-5-nitrobenzyl bromide as a special reagent for the photometric determination of tryptophan content. The absorbance of the resulting coloured products was studied between 300 and 410 nm wavelength range. Horton and Tucker **[23]** showed that dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonium salt can be used for spectrophotometric determination of tryptophan content in proteins at pH 3. This water-soluble sulfonium salt allows a more advantageous determination than water-insoluble 2-hydroxy-5-nitrobenzyl bromide.

Mulder and Bakema **[36]** estimated tryptophan content by the Brummer method. The method is based on the formation of a red-violet colour with vanillin in a strongly acidic solution. The method is only applicable to protein hydrolysates because other sub-

stances in the sample interfere with colour development. In addition to vanillin, sulfuric acid and sodium sulphide were used to determine tryptophan content. The absorbance of the solutions was measured at 605 nm.

In summary, the photometric determination of Trp is best performed by the colour reaction of para-dimethyl-amino-benzaldehyde, but if necessary the photometry of the colour between acetic acid and glyoxal or n-1 (naphthyl) ethylene-diamino-hydrochloride and Trp also recommended.

4.6. Determination of phenylalanin using photometry

The colorimetric determination of the phenylalanine content of a protein hydrolyzate is based on two basic principles described by Kapeller-Adler **[29]**: eliminating the interference of tyrosine and histidine and quantitative nitration and reduction of phenylalanine in the violet ammonium salt of diacyl-o-dinitrobenzoic acid. Photometry was performed in the wavelength range of 520 and 580 nm.

Albanese **[1]** further developed the Kapeller-Adler method, eliminating histidine interference with permutit (synthetic zeolite), and using ammonium sulphate and sodium hydroxide instead of concentrated ammonium hydroxide. This is because ammonium hydroxide was considered dangerous to the health of the experimenter person, and daily changes in the ammonia concentration of the reagent affected the colour intensity.

According to the research of Hess and Sullivan **[19]**, the colourimetric determination of phenylalanine requires its nitration, reduction of dinitrophenylalanine with zinc and hydrochloric acid, and reaction with 2-naphthoquinone-4-sodium sulfonate in a slightly acidic solution, resulting in a red colour compound. The absorbance of the solutions was tested at 560 nm. Histidine and tyrosine do not interfere with the reaction. Pretreatment with potassium permanganate solution prevented tryptophan from being disrupted without affecting the colour formation from phenylalanine.

To determine the amount of phenylalanine, Grau **[14]** dried the flask containing the sample in an oven at 110-120 °C until the sample was completely dryness, or a thick syrup was obtained. A solution of potassium nitrate in concentrated sulfuric acid was then added to the sample. The nitration was performed at 110-120 °C for 30 minutes. After cooling the flask, hydroxylamine hydrochloride, ammonium sulphate and sodium hydroxide were used to determine the amount of phenylalanine. The absorbance of the solutions was studied at 550 nm.

Henry et al. **[18]** modified the Kapeller-Adler method, hydrolysis of proteins was performed with picric acid. Concentrated sulfuric acid solution of potassium nitrate, hydroxylamine hydrochloride and concentrated ammonium hydroxide solution were used for the determination after picric acid hydrolysis. Photometry was performed in the wavelength range of 550 and 560 nm.

Pan and Perlman **[39]** had made two modifications to the original Kapeller-Adler method. During the process, when concentrated ammonium hydroxide was added to the concentrated sulphuric acid, a large amount of heat was generated, which caused the solution bubbled out from the test tubes, causing significant losses. In their experiments, they solved the problem by adding a smaller amount of nitrating reagent. The second modification, to facilitate the performance of the test, consisted of a reduction in the time required to evaporate the samples to dryness. The tubes containing the sample solutions were immersed in a water bath at 50–70 °C and a gentle stream of air was passed through the surface of the liquid. As a result, numerous samples can be dried simultaneously using a few glass blowers.

5. Conclusions

Prior to the determination of amount of each amino acid, the protein is hydrolyzed with 6 M hydrochloric acid, followed by photometric determination of the amino acids. The Trp content of the protein is degraded under these conditions due to the lability of the indole group, so during the determination of Trp either special acidic protein hydrolysis methods (3 M mercapto-ethane-sulfonic acid, 3 M para-toluene-sulfonic acid, 4 M methane sulfonic acid) are used or special protective reagents (1,4-butanedithiol, thioglycolic acid, 3-(3-indolylpropionic) acid, 3-(2-minoethyl) indole should be used. The other opportunity of determinating the Trp is the alcalic hydrolysis which is performed with 4 M sodium-hydroxide or barium-hydroxide. In case of high protein content, the sodium ion content of the hydrolysate does not interfere with the determination, and the advantage of the barium hydroxide determination is that barium is easier to remove from the hydrolysate than sodium.

The total amino acid content of the hydrolysate can be determined by the ninhydrin colour reaction at 570 nm, and the amount of proline and hydroxyproline can be determined by the absorbance at 440 nm. Aromatic amino acids (tyrosine, phenylalanine, tryptophan) can be measured together at 280 nm in the ultraviolet range. At this wavelength, the determination of the three amino acids is generally not disturbed by other food ingredients.

By the xantoprotein reaction, since the aromatic ring of phenylalanine does not react with nitric acid, the combined amount of tyrosine and tryptophan can be determined at 360 and 430 nm, respectively. Because tyrosine is the only amino acid that contains a phenyl side chain, the Millon reaction, in which tyrosine

gives a brick-red colour reaction with nitric acid and mercury ions, is specific for tyrosine. The absorbance of the solution was tested at 475 nm, where the presence of phenylalanine, histidine or tryptophan was not disturbed.

The tryptophan content of the protein forms a coloured compound with para-toluene-sulphonic acid, para-dimethyl-amino-benzaldehyde, N-bromo-succinimide, sodium nitrite and N-1(naphthyl) ethylenediamine dihydrochloride and the glacial acetic acid ferric chloride reagent, which form the basis of the photometric determination of tryptophan. Among the methods, the most common method is the para-dimethyl-amino-benzaldehyde method, which has been used by several people to determine the tryptophan content of various proteins. Derivatization with a mixture of glyoxylic acid and sulfuric acid (Hopkins-Cole test), 2-hydroxy-5-nitrobenzyl bromide and dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonic acid was also used, however, these methods have not become widespread in practice.

During the determination of the phenylalanine content of the protein we have to eliminate the interfering effect of tyrosine and histidine, which may be followed by nitration of phenylalanine and derivatisation with diacyl-o-nitrobenzoic acid, photometrized at 570 nm. 2-naphthoquinone-4-sodium sulfonate is also suitable for post-nitration derivatization, which is not expected to interfere with histidine and tyrosine, as well as derivatization with hydroxylamine hydrochloride, ammonium sulphate and sodium hydroxide.

Overall, therefore, all three amino acids containing aromatic side chains are known in the literature for a colour reaction that can be developed into a quantitative assay.

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7. References

[1] Albanese, A.A. (1944): Colorimetric estimation of phenylalanine in some biological material. *Journal of Biological Chemistry.* **155** pp. 291-298.

[2] Barman, T.E., Koshland, D.E. (1967): A colorimetric procedure for the quantitative determination of tryptophan residues in proteins. *The Journal of Biological Chemistry.* **242** (24) 5771-5776.

[3] Basha, S.M.M., Roberts, R.M. (1977): A simple colorimetric method for the determination of tryptophan. *Analytical Biochemistry.* **77** (2) pp. 378-386.

[4] Bokori J., Gundel J., Herold I., Kakuk T., Kovács G., Mézes M., Schmidt J., Szigeti G., Vincze L. (2003): A takarmányozás alapjai. Mezőgazda Kiadó, Budapest. pp. 3-11.

[5] Carpenter, K.J. (1960): The estimation of the available lysine in animal-protein foods. *Biochemical Journal*. **77** (3) pp. 604-610.

[6] Csapó J., Lóki K., Csapóné Kiss Zs., Albert Cs. (2005): Az aminosavak elválasztása és meghatározása ioncserés oszlopkromatográfiával, oszlop utáni származékképzés alkalmazásával. *Acta Agraria Kaposváriensis*. **9** (2) pp. 33-51.

[7] Csapó, J., Csapó-Kiss, Zs., Einarsson, S., Folestad, S., Tivesten, A. (1995): Methods for determination of D-amino acid content of foods and feeds. *Acta Alimentaria.* 24. 125-126.

[8] Csapó, J., Csapóné Kiss, Zs., Babinszky, L., Győri, Z., Simonné Sarkadi, L., Schmidt, J. (2006): Élelmiszer- és takarmányfehérjék minősítése. Mezőgazda Kiadó, Budapest pp. 1-451.

[9] Csapó, J., Csapóné Kiss, Zs. (2007): Biokémia állattenyésztőknek. Mezőgazda Kiadó, Budapest, pp. 35-38, pp. 233-235.

[10] Dévényi, T., Bati, J., Fábián, F. (1971): Detection and determination of tryptophan by ion exchange chromatography. *Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae*. **6** (2) pp. 133-138.

[11] Dreeze, A. (1960): Determination of tryptophan in natural milieu. 2. Stability of tryptophan during alkaline hydrolysis done in the presence of carbohydrates. *Bulletin De La Societe Chimique De France.* **42** pp. 407-417.

[12] Freedlender, E.F., Haber, E. (1972): Obtaining large variable-region peptides from rabbit light chains. *Biochemistry.* **11** pp. 2362-2370.

[13] Friedman, M., Finley, J.W. (1971): Methods of tryptophan analysis. *Journal of Agricultural and Food Chemistry.* **19** (4) pp. 626–631.

[14] Grau, C.R. (1947): The phenylalanine and tyrosine contents of chicks and eggs. *Journal of Biological Chemistry.* **168** (2) pp. 485-491.

[15] Gruen, L.C., Nicholls, P.W. (1972): Improved recovery of tryptophan following acid hydrolysis of proteins. *Analytical Biochemistry*. **47** pp. 348-355.

[16] Hamilton, P.B. (1963): Ion exchange chromatography of amino acids. A single column, high resolving, fully automatic procedure. *Analytical Chemistry.* **35** (13) pp. 2055–2064.

[17] Hassan, S.S.M. (1975): New spectrophotometric method for simultaneous determination of tryptophan and tyrosine. *Analytical Chemistry*. **47** (8) pp. 1429–1432.

[18] Henry, R.J., Sobel, C., Chiamori, N. (1957): Method for determination of serum phenylalanine with use of the Kapeller-Adler Reaction. *The American Journal of Diseases of Children.* **94** (6) pp. 604–608.

[19] Hess, W.C., Sullivan, M.X. (1944): The determination of phenylalanine in proteins. *Archives of Biochemistry and Biophysics*. **5** pp. 165-173.

[20] Holme, D.J., Peck H. (1998): Analytical Biochemistry Third Edition. Pearson Education Limited, Edinburgh Gate Harlow, pp. 342-405.

[21] Homer, A. (1915): Method for the estimation of the tryptophane content of proteins, involving the use of baryta as a hydrolyzing agent. *The Journal of Biological Chemistry*. **22** pp. 369-389.

[22] Horton, H.R., Koshland, D.E. (1965): A highly reactive colored reagent with selectivity for the tryptophan residue in proteins. 2-Hydroxy-5-nitrobenzyl bromide. *Journal of the American Chemical Society*. **87** (5) pp. 1126–1132.

[23] Horton, H.R., Tucker, D.W.P. (1970): The reaction of dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonium salts with tryptophan ethyl ester. *The Journal of Biological Chemistry.* **245** (13) pp. 3397-3401.

[24] Höller, H. (1958): Zur Frage des Einflusses der alkalischen Hydrolyse von Proteinen auf die nachfolgende chemische und mikrobiologische Tryptophanbestimmung. *Archiv* Für *Tierernahrung*. **8 (**1-6) pp. 182–193.

[25] Ichikawa, T., Terada, H. (1981): Determination of phenylalanine, tryptophan and tyrosine in a mixture of amino acids by second derivative spectrophotometry. *Chemical & Pharmaceutical Bulletin.* **29** (2) pp. 438–444.

[26] James, L. (1972): Amino acid analysis: The humin problem. *Journal of Chromatography.* **68** pp. 123-130.

[27] Jorpes, E. (1932): The protein component of the erythrocyte membrane or stroma. *Biochemical Journal.* **26** (5) pp. 1488–1503.

[28] Jungbauer, A., Hahn, R. (2009): Ion-exchange chromatography. *Methods in enzymology*. **463** pp. 349-371.

[29] Kapeller-Adler, R. (1932): Über eine neue Reaktion zur qualitativen und quantitativen Bestimmungdes Phenylalanins. *Biochemical Zeitschrift.* **252** pp. 185–200.

[30] Liu, T.Y. (1972): The precision and sensitivity of amino acid analyses. Chemistry and biology of peptides. *Ann Arbor Science Publishers*. **629.**

[31] Liu, T.Y., Chang, Y.H (1971): Hydrolysis of proteins with p-toluenesulfonic acid. determination of triptophan. *The Journal of Biological Chemistry*. **246** pp. 2842-2848.

[32] Mathews, J., Partington, M.W. (1964): The plasma tyrosine levels of premature babies. *Archives of Disease in Childhood.* **39** (206) pp. 371–378.

[33] Matsubara, H., Sasaki, M.: 1969. High recovery of tryptophan from acid hydrolysates of proteins. *Biochemical and Biophysical Research Communications.* **35** pp. 175-181.

[34] Miller, E.L. (1967): Determination of the tryptophan content of feeding stuffs with particular reference to cereals. *Journal of the Science of Food and Agriculture*. **18** pp. 381-387.

[35] Moore, S., Stein, W.H. (1956): Column chromatography of peptides and proteins. *Advances in Protein Chemistry.* **11** pp. 191-236.

[36] Mulder E.G., Bakema K. (1956): Effect of the nitrogen, phosphorus, potassium and magnesium nutrition of potato plants on the content of free amino-acids and on the amino-acid composition of the protein of the tubers. *Plant and Soil*. **7** (2) pp. 135-166.

[37] Noltmann, E.A., Mahowald, T.A., Kuby, S.A. (1962): Studies on adenosine triphosphate transphosphorylases. II. Amino acid composition of adenosine triphosphate-creatine transphosphorylase. *The Journal of Biological Chemistry.* **237** (4) pp. 1146-1154.

[38] Opienska-Blauth, J., Charezinski, M., Berbec, H. (1963): A new, rapid method of determining tryptophan. *Analytical Biochemistry.* **6** (1) pp. 69-76.

[39] Pan S.C., Perlman D. (1954): Determination of Phenylacetic Acid and Phenylacetamides in Samples from Penicillin Fermentations. *Analytical Chemistry*. **26** (9) pp. 1432-1438.

[40] Penke, B., Ferenczy, R., Kovács, K. (1974): A new acid hydrolysis method for determining tryptophan in peptides and proteins. *Analytical Biochemistry*. **60** (1) 45-50.

[41] Rasch, E., Swift, H. (1960): Microphotometric analysis of the cytochemical Millon reaction. *Journal of Histochemistry & Cytochemistry*. **8** (1) pp. 4–17.

[42] Rékásiné Szombati, É., Caballero, O.P., Mihályiné-Kengyel, V., Körmendy, L. (1977): Triptofántartalom meghatározása hús- és húskészítményekben. Élelmiszervizsgálati Közlemények. **24** (1-2) pp. 17-26.

[43] Rosen, H. (1957): A modified ninhydrin colorimetric analysis for amino acids. *Archives of Biochemistry and Biophysics.* **67** (1) pp. 10–15.

[44] Scopes, R.K. (1974): Measurement of protein by spectrophotometry at 205 nm. *Analytical Biochemistry.* **59** (1) pp. 277–282.

[45] Shizuko-Isole, A. (1959): Report National Institute of Nutrition. pp. 45-51.

[46] Simonian, M.H., Smith, J.A. (2006): Spectrophotometric and colorimetric determination of protein concentration. *Current Protocols in Molecular Biology.* **10** p. 10.

[47] Spies, J.R., Chambers, D.C. (1950): Determination of tryptophan with p-dimethylaminobenzaldehyde using photochemical development of color. *Analytical Biochemistry*. **22** (9) pp. 1209–1210.

[48] Warner, R.C. (1941): The alkaline hydrolysis of egg albumin. *The Journal of Biological Chemistry.* **142** pp. 741-756.

[49] Wunderlich, L., Szarka, A. (2014): A biokémia alapjai. Typotex Kiadó, Budapest. pp. 41-45.

[50] Yemm, E.W., Cocking, E.C., Ricketts, R.E. (1955): The determination of amino-acids with ninhydrin. *The Analyst*. **80** pp. 209-214.