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Determination of amino acid composition of foods by photometric methods, Part 2 - Determination of methionine, cystine, lysine and arginine

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1. SUMMARY

In the second part of our review paper on the photometric determination of the amino acid content of foods, methods of determination of two essential amino acids (methionine, lysine) and two semi-essential amino acids (cystine, arginine) reported in the literature are described. Selective determination of the two sulfur-containing amino acids is made possible by the fact that both the methylmercapto group of methionine and the sulfhydryl group of cysteine formed by the reduction of cystine can be subjected to specific derivatization reactions which are characteristic of only these amino acids. The same can be said for the ε-amino group of lysine and the guanidine group of arginine, which allow the specific determination of the amino acids in question with the help of special colour reactions.

The reaction most suitable for the determination of methionine is one in which the discolouration of platinum and palladium complexes is catalyzed by methionine, and from which the amount of methionine can be deduced. Under appropriate conditions, organic sulfides and cystine do not interfere with the reaction. The first step in the determination of cystine and cysteine is the reduction of cystine to cysteine, followed by a reaction between cysteine and a derivatizing agent, most commonly 5,5'-dithiobis(2-nitrobenzoic acid). Quantification can be performed by the photometric measurement of the coloured compound formed. In the case of lysine, the free ε-amino group of lysine is reacted with the derivatizing agent, most commonly 1-fluoro-2,4-dinitrobenzene, and then colour intensity is measured. In the case of arginine, most often the reaction between the guanidine group, α-naphthol and sodium hypobromide serves as the basis for the determination.

2. Introduction

Of the protein-building amino acids, methionine and lysine are essential for humans, while cysteine and arginine are semi-essential amino acids, because cysteine can be produced by the body from an essential amino acid, methionine, while arginine can be produced by the body as well, but under certain physiological conditions it can become essential to the body. Lysine and methionine are two limiting amino

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acids of our food raw materials, so when determining the nutritional value of foods, when calculating the biological value of a protein, it is essential to know the amount of these two amino acids.

Cystine and cysteine occur only in low concentrations in food proteins, so their amounts are difficult to measure in the presence of all the other amino acids. In addition, both amino acids are sensitive to oxidation, so they are usually determined by instrumental analytical techniques after oxidation as cysteic acid. The sulfhydryl group of cysteine is more reactive than the disulfide bridge in cystine, so reduction is an essential step in the determination, during which cystine is converted to cysteine, so that cystine can be determined as cysteine. Compared to human needs, arginine is usually present in food proteins in optimal amounts, but it may become essential in younger age groups or in individuals with certain diseases, therefore it is important to be able to ascertain its amount **[2, 9, 12, 18, 33]**.

In most laboratories, the amino acid composition of foods is determined by ion exchange column chromatography (IEC), by an automated amino acid analyzer based on the same principle **[11, 13, 17, 21]**, or by high performance liquid chromatography (HPLC) **[13, 18]**. However, obtaining and operating the necessary instruments is very expensive, which smaller laboratories cannot afford. In some cases, it is not even necessary to determine all amino acids, so it may be unnecessary to separate and quantify all amino acids by costly large instrumental techniques, it may be sufficient to measure the concentrations of one or two essential or possibly limiting amino acids. The objective of our research was therefore to develop photometric methods that can easily determine the methionine, cystine, lysine and arginine contents of proteins without the application of large instruments. By using such methods, the measurement of essential or semi-essential amino acids may be possible in laboratories that do not possess large instruments. In our paper, a compilation of methods reported in the literature for the photometric determination of methionine, cystine, lysine and arginine is presented.

2.1. Hydrolysis of proteins

Before the determination of the amino acid composition of proteins, they must be hydrolyzed to free amino acids. The internationally accepted method of protein hydrolysis, during which in the case of foods 20 to 200 mg of the substance is weighed in depending on the protein content of the sample, and the hydrolysis is carried out with 6 M hydrochloric acid at 110±1 °C for 24 hours, was developed by Moore and Stein **[26]**. Prior to the hydrolysis, complete removal of oxygen must be ensured, which can be achieved by evacuating the space above the frozen liquid, purging with nitrogen gas, or a combination of the two. The optimum temperature for hydrolysis is 110±1 °C, fluctuations of which should be avoided, since during hydrolysis performed at lower temperatures (105 °C) the breakdown of peptide bonds is no longer quantitative, while at higher temperatures the degradation of more sensitive amino acids can be expected. Following hydrolysis, the hydrochloric acid is removed by rotary vacuum distillation or lyophilization.

3. Determination of the amino acid composition of proteins by photometric methods

3.1. Photometric determination of methionine

The nitroprusside sodium method, which is the simplest to apply, has been described for the photometric determination of methionine by McCarthy and Sullivan **[25]**, however, its sensitivity is poor and the presence of histidine or tryptophan interfere with the colour reaction. The interfering effect can be eliminated by the addition of excess glycine and the method can be made automatic. During the procedure, 1 ml of 14.3 M sodium hydroxide solution, 1 ml of 1% glycine solution and 0.3 ml of 10% nitroprusside sodium solution are added to 5 ml of the test solution, while the mixture was mixed each time. It was placed in a water bath at 35-40 °C for 5 to 10 minutes, then it was cooled for two minutes with a mixture of water and ice at 0 °C, 5 ml of a mixture of hydrochloric acid and phosphoric acid (8 parts of concentrated hydrochloric acid and 1 part of 85% phosphoric acid by volume) was added with constant stirring. After vigorous stirring, the mixture was cooled in water and kept at room temperature for 5 to 10 minutes. A methionine calibration series was prepared in a similar manner. It was achieved by the use of concentrated sodium hydroxide solution that glycine and histidine did not give a colour reaction, while the combined use of hydrochloric acid and phosphoric acid resulted in a solution with a clearer colour than derivatization without the addition of phosphoric acid. The absorbance of the samples was measured in the wavelength range of 520 to 580 nm.

The reaction is specific for methionine, as the other amino acids do not form coloured compounds under these conditions. Also, oxidized derivatives of methionine, homocysteine, cysteine and cystine do not react, nor does tryptophan, if the acid is added cold to the reaction mixture.

Pieniazek et al. **[29]** reacted methionine with sodium nitrite and trisodium pentacyanoaminoferrate in an acetic acid medium. Cystine and cysteine do not interfere with the reaction, while the interference of histidine can be eliminated with a pH=1.5 glycine buffer. The sensitivity of this method is not very high either, however, an automated determination with a flow-through system was developed based on the colour reaction. The absorbance of the solutions was measured at a wavelength of 520 nm.

Tonkovic and Hadzija **[32]** used lactic acid, copper sulfate and p-hydroxydiphenyl for the quantitative determination of methionine. The colour reaction which serves as the basis for the analysis was first described by Barker and Summerson **[1]**. The essence of the method is that methionine is an inhibitor of the reaction between lactic acid and p-hydroxydiphenyl (PHD), therefore, in the presence of methionine, the reaction results in a colour reaction with diminished intensity proportional to the concentration of methionine. The decrease in colour intensity is linear in the 5 to 45 μg methionine range, so it can be used to measure the concentration of methionine. Protein-forming amino acids do not interfere with the reaction, so the method can be applied to either pure methionine or protein hydrolyzates. The coloured product obtained during the reaction of lactic acid and PHD, apart from higher lactic acid concentrations, follows the Lambert-Beer law, the decrease in colour intensity is proportional to the methionine concentration, therefore the method is well suited to the measurement of methionine concentration.

During the analysis, 20 μg of lactic acid was added to a sample containing 5 to 45 μg methionine, then 1 ml of water, 0.05 ml of copper sulfate solution and 6 ml of concentrated sulfuric acid was added in a test tube, and the hot mixture was cooled under running water. It was kept in hot water for five minutes, cooled to 20 °C and 0.1 ml of PHD solution was added. Using a capillary, it was stirred at 30 °C for 30 minutes in a stream of air. If the PHD reagent was not completely dissolved, the solution was again placed in hot water for 90 seconds, and then the absorbance of the cooled sample was measured at 560 nm against the reagent blank.

The metals of the platinum group of the periodic table (platinum, palladium) form coloured complex iodides, which are discoloured by organic sulfides or mercapto compounds, because the sulfur-containing compounds act as ligands and form complexes with platinum and palladium. Methionine and cysteine react similarly with the palladium phenazinotriazo complex with a decrease in colour intensity. The decolourization reaction is suitable for the determination of methionine content **[19]**. The effect of cysteine on the platinum complex is significantly smaller that that of methionine. Cysteine can be captured in the form of thiazolidine-4-carboxylic acid by the addition of excess formaldehyde. During the determination, the coloured reagent solution becomes colourless in proportion to the methionine content. The decrease in colour intensity can be measured at 490 nm in a cuvette of 5 to 15 mm.

3.2. Photometric determination of cystine-cysteine

In most cases, in the photometric determination of cystine and cysteine, cystine must be converted to cysteine by reduction. Sulfite, sodium borohydride or mercaptoethanol are used for the reaction. For the reduction of cystine, dithioerythritol (erythro-2,3-dihydroxy-1,4-butanedithiol) and dithiothreitol (threo-2,3-dihydroxy-1,4-butanedithiol) were introduced by Cleland **[10]**. The advantage of reduction with dithioerythritol (DTE) and dithiothreitol (DTT) is that due to the formation of a cyclic disulfide, the oxidized form of the Cleland reagent predominates, shifting the equilibrium of the reaction to the cysteine side. Due to the low redox potential of DTE and DTT, only a small excess of reagent is required for the complete reduction of cystine to occur.

Quantitative determination of cysteine was performed by Gaitonde in an acetic acid-hydrochloric acid medium by photometry at 570 nm of the coloured reaction product of ninhydrin and cysteine formed at 100 °C over 6 to 10 minutes **[15]**. In the presence of other naturally occurring amino acids, the concentration of cysteine can also be determined at 411 nm by measuring the decolourization of the noradenochrome compound prepared by the oxidation of noradrenaline bitartrate with potassium ferricyanide **[30]**. A colour reaction was also performed using cysteine, brucine and a 50% sulfuric acid solution of potassium persulfate, after which the amount of cysteine was measured at 660 nm by photometry **[27]**. Measurement of the absorbance of the coloured product formed in the reaction between sodium naphthoquinone-4-sulfonate and cysteine, showing a light absorption maximum at 520 nm, can also be used for the determination of cysteine **[22]**. When reacting with cysteine, sodium nitrite, sulfanylamide and N-(1-naphthyl)ethylenediamine are also suitable for a colour reaction, resulting in the formation of nitroisocysteine, the absorbance of which can be measured at 650 nm **[24]**.

Cysteine also forms coloured compounds with thiofluorescein, 5,5'-dithiobis (2-nitrobenzoic acid), 2-vinylquinoline and 4,4'-bisdimethylaminodiphenyl-carbinol. Reactions of cysteine with nitrilotriacetic acid ferric chloride and 1,10-phenanthroline have also been reported in the literature, and these are also suitable for the determination of cysteine **[4]**.

Holz **[19]** considered the method of Ellman, during which cystine was reduced to cysteine by DTE or DTT according to Cleland's method and the excess reducing agent was reacted with sodium arsenite, to be the most suitable for the automated determination of cysteine. The absorbance of the solutions was measured by Ellman **[14]** at a wavelength of 412 nm. The amount of cysteine was determined by the absorbance of the yellow trinitrobenzoic acid after derivatization with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB). Reduction with DTE or DTT, as well as determination using Ellman's reagent are based on a thiol-disulfide exchange, therefore, the method for the determination of cysteine or cystine is specific, other amino acids do not interfere with it.

Studies have shown that the reducing abilities of DTE and DTT can be considered the same, as cysteine and cystine colour intensities were the same for both compounds. For the reduction of cystine, a reaction time of 10 to 12 minutes is recommended, although some consider a time of 30 minutes to be appropriate. Cysteine determination results of cysteine-containing samples without the addition of a reducing agent were found to be significantly lower, because cysteine is sensitive to oxygen and is partially oxidized to cystine during storage. Without the addition of a reducing agent, the oxidized cysteine does not participate in the reaction, so the determination leads to a cysteine value lower than the actual one. The photometric method is suitable for the determination of the cystine content of large series of food and feed samples.

A simple and specific analysis for the determination of cysteine was developed by Ohmori et al. **[28]**. Cysteine was reacted with p-dimethylaminocinnamic aldehyde in methanol at 60 °C for 2 hours in the presence of sulfuric acid. Absorbance was measured at 587 nm and the coloured derivative was stable at 60 °C for at least 5 hours. The procedure was specific for cysteine, since the colour reaction did not take place with other amino acids.

3.3. Photometric determination of lysine

To determine the quantity of lysine, 1-fluoro-2,4,-dinitrobenzene was used by Carpenter et al. **[5]**, as well as Lea et al. **[23]**, following acid protein hydrolysis. This procedure was further developed by Carpenter and Ellinger **[6, 7]**, which was proved to be useful for many samples of animal origin, but the determination was significantly influenced by the interfering effect of α-dinitrophenylarginine. Interference was eliminated using methoxycarbonyl chloride **[3]**, but this treatment led to the unexpected formation of a coloured histidine derivative **[8]**. The method was further developed by Carpenter **[9]**, and further experiments were performed with 1-fluoro-2,4-dinitrobenzene.

The essence of the method is that the free ε-amino group of lysine reacts with 1-fluoro-2,4-dinitrobenzene (FDNB) in the first step, resulting in a yellow coloured dinitrophenyl (DNP) derivative. Following this, the protein is hydrolyzed to amino acids with 6 M hydrochloric acid, excess reagent and substances interfering with the photometric determination are removed by extraction with ether, and finally the absorbance of the aqueous residue is measured, from which the amount of free ε-amino groups and the thus the concentration of lysine can be deduced. During the procedure, an 8% sodium hydrogen carbonate solution and then an alcoholic solution of FDNB were added to a sample containing 30 to 50 mg of nitrogen, it was stirred for two hours, and after the reaction took place, the protein was immediately hydrolyzed with 8 M hydrochloric acid for 16 hours at reflux. After appropriate dilution, the hydrolyzate was extracted several times with ether, methoxycarbonyl chloride solution was added to the aqueous phase, it was again extracted with ether and the absorbance of the aqueous phase was measured at 435 nm against a blank. The method was not perfect because both histidine and arginine interfered with the determination. The interfering effect of histidine was smaller because its dinitrophenyl (DNP) derivative did not show a maximum absorbance at 435 nm. This method had been used routinely to determine the recoverable lysine content for a long time, until automated amino acid analyzers have gained widespread use.

3.4. Photometric determination of arginine

Of the amino acids, only arginine has a guanidine group that is subject to colour reactions which make the determination of arginine specific. According to the method of Sakaguchi **[31]**, under alkaline conditions, arginine reacts with α-naphthol and sodium hypobromite or sodium hypochlorite to give a spectrophotometrically measurable reddish-brown compound as a result of the oxidation. The protein hydrolyzate can contain a number of substances that prevent the development of colour, the most common of which is ammonia. The arginine content of the samples was determined by Izumi **[20]** at a wavelength of 520 nm, while it was performed by Gilboe and William **[16]** in the 490-510 nm wavelength range using the same reaction.

4. General review

The photometric determination of amino acids, with the exception of tryptophan, may begin after the hydrolysis of proteins with 6 M hydrochloric acid at 110±1 °C for 24 hours. Selective determination of the two sulfur-containing amino acids is made possible by the fact that both the methylmercapto group of methionine and the sulfhydryl group of cysteine formed by the reduction of cystine can be subjected to special derivatization reactions characteristic of only these amino acids. The same can be said for the ε-amino group of lysine and the guanidine group of arginine, the special colour reactions of which allow their specific determination.

The nitroprusside sodium colour reaction can be used for the photometric determination of methionine **[25]**, however, the presence of histidine and tryptophan interfere with it. Once the interference has been eliminated, the reaction is specific for methionine, since the other amino acids do not form coloured compounds under these conditions, and the oxidized derivatives of methionine, homocysteine, cysteine and cystine do not undergo colour reactions either.

Methionine was reacted with sodium nitrite and trisodium pentacyanoaminoferrate in an acetic acid medium by Pieniazek et al. **[29]**, while Tonkovic and

Hadzija **[32]** used lactic acid, copper sulfate and p-hydroxydiphenyl to determine methionine content. The essence of the method is that methionine is an inhibitor of the reaction between lactic acid and p-hydroxydiphenyl (PHD), therefore, in the presence of methionine, the reaction results in a colour reaction with diminished intensity proportional to the concentration of methionine.

Platinum and palladium form coloured complex iodides that are discoloured by organic sulfides or mercapto compounds through the formation of complexes of sulfur-containing compounds as ligands with platinum or palladium. Methionine and cysteine react similarly with the palladium phenazinotriazo complex with a decrease in colour intensity, a decolourization reaction suitable for the determination of methionine content **[19]**. The effect of cysteine on the platinum complex is significantly less than that of methionine, and the interfering effect can be eliminated by the addition of excess formaldehyde.

The first step in the photometric determination of cystine and cysteine is the conversion of cystine to cysteine by reduction with sulfite, sodium borohydride or mercaptoethanol. Dithioerythritol (erythro-2,3-dihydroxy-1,4-butanedithiol) and dithiothreitol (threo-2,3-dihydroxy-1,4-butanedithiol) were introduced by Cleland **[10]** for the reduction of cystine, the advantage of which is that due to the formation of a cyclic disulfide, the oxidized form of the Cleland reagent predominates, shifting the equilibrium of the reaction to the cysteine side.

During the photometric determination of cysteine, the reaction between ninhydrin and cysteine in an acetic acid-hydrochloric acid medium was used. The coloured compound formed at 100 °C over 6 to 10 minutes was determined photometrically at 570 nm **[15]**. The decolourization of the noradenochrome compound prepared by the oxidation of noradrenaline bitartrate with potassium ferricyanide was also used for the quantification of cysteine **[30]**. Another colour reaction was performed using cysteine, brucine and a 50% sulfuric acid solution of potassium persulfate **[27].** Sodium naphthoquinone-4-sulfonate **[22]**, sodium nitrite, sulfanylamide and N-(1-naphthyl)ethylenediamine **[24]** have also been used for this purpose. Cysteine also forms coloured compounds with thiofluorescein, 5,5'-dithiobis(2-nitrobenzoic acid), 2-vinylquinoline and 4,4'-bisdimethylaminodiphenylcarbinol, as well as nitrilotriacetic acid ferric chloride and 1,10-phenanthroline **[4].**

By modifying the method of Ellman **[14]**, cystine was reduced to cysteine by Holz **[19]** using DTE or DTT according to the method of Cleland, and then cysteine was measured with the help of the absorbance of the yellow coloured trinitrobenzoic acid, following derivatization with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB). The determination is based on the thiol-disulfide exchange, therefore the method is specific for the determination of cysteine and cystine, other amino acids do not interfere with it.

Cysteine was reacted with p-dimethylaminocinnamic aldehyde by Ohmori et al. **[28]**, and the absorbance was measured at 587 nm. The reaction was specific for cysteine because no coloured derivative was formed with other amino acids.

Following acid protein hydrolysis, 1-fluoro-2,4-dinitrobenzene was used by Carpenter et al. **[5]**, as well as Lea et al. **[23]** to determine the amount of lysine. The essence of the method is that in the first step the free ε-amino group of lysine reacts with 1-fluoro-2,4-dinitrobenzene (FDNB), resulting in a yellow coloured dinitrophenyl(DNP) derivative. Following this, the protein is hydrolyzed to amino acids with 6 M hydrochloric acid, excess reagent and substances interfering with the photometric determination are removed by extraction with ether, and finally the absorbance of the aqueous residue is measured at 435 nm, from which the amount of free ε-amino groups, and from that the concentration of lysine can be deduced.

According to the method of Sakaguchi **[31]**, arginine reacts with α-naphthol and sodium hypobromite or sodium hypochlorite under alkaline conditions and the oxidation results in a reddish-brown compound which can be measured spectrophotometrically at a wavelength of 520 nm **[20]** or in the wavelength range between 490 and 510 nm **[16]**.

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