Automatic Analyzer of Amino Acids AAA500

Chemical part

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1. INTRODUCTION

1.1 Purpose of this documentation

This documentation is designed for the staff dealing with the operation of the automatic analyser of amino acids AAA500. It has been worked out as a supporting manual for the beginning operators and beginners in the field of ionex chromatography. It will make it easier to understand the principle of the apparatus as a whole as well as its individual parts. The understanding of and making familiar with all activities connected with the operation of the apparatus is a basic precondition of its correct utilisation and its trouble-free operation. Introductory chapters will serve as a guide.

2 Basic chemical issues of amino acids

2.1 Separation system

The analyzer operation is based on a method of ion-exchange chromatography with post-column derivatization. During analysis the mobile phases – buffers change so that pH decreases and ion strength increases. Separation is also influenced by temperature in the analytical column. Individual amino acids elute in it based on different values of isoelectric points. In addition to the above-mentioned "acidity", space and hydrophobic effects have also influence on elution of amino acids from the column. Separated amino acids react with ninhydrin contained in the derivatizaion agent and the product that forms is detected.

2.2 Stationary phase – ionex

Amino acid analyzers use high acidic katex for the columns. The katex consists of porous and to a certain extend compressible beads of cross-linked and additionally sulphonated polystyrene. Interaction between negatively charged sulpho groups and positively charged amino groups is the basic element of their separation in the column.

2.3 Buffers – mobile phase

Amino acid analyzers use sodium or lithiun citrate buffers.

2.5 Amino acids

Amino acids are organic compounds containing both the amine and carboxyl groups. Most amino acids has a general structure in which the primary amines and the group of carboxylic acid are connected to one atom of carbon. Another link of this carbon links a hydrogen tom, and the fourth link with the connected group determines the properties of each amino acid. This side chain also contributes to the specific distribution of the charges of individual amino acids. The structures of side chains and common nomenclature of the most important amino acids and ninhydrin-positive substances are provided for in the annex.

2.6 Occurrence of amino acids

Amino acids can be found in nature either in their free form or as polymerised chains referred to as peptides or proteins which can be connected with various substrates as e.g. carbohydrates, lipids, etc. All of these amino acids can be analysed by using chromatographic methods after a suitable treatment of the sample. Peptides are polymeric chains of amino acids. Peptide links are created by means of the condensation of an alpha amino group of one amino acid and alpha carboxylic group of the other amino acid. A link of two amino acids forms dipeptides, a link of three amino acids forms tripeptides and links of more amino acids form polypeptides. Protein is a long polypeptide containing approximately 20 amino acid residues, it usually has a specific biological function (e.g. enzyme). An analysis of composition of amino acids in a peptide or protein is carried out after a hydrolysis of peptide links. By means of a combination of amino acids with other substrates it is possible to use many derivatives. These two groups, i.e. amino acids and substrates are often analysed separately after a hydrolysis of the conjugate in question.

2.7 Reaction of ninhydrin with amino acids

The reaction of ninhydrin (indantrion hydrate) with amino acids and imino acids. Ninhydrin is a strong oxidation agent which reacts with alpha amino groups, releases ammonia, carbon dioxide, aldehyde and a reduced form of ninhydrin - hydrindantin. The ammonia released then reacts with hydrindantin and another molecule of ninhydrin, which produces a purple substance (Ruheman red). Hydrindantin, which is present in the detection agent, prevents from occurring a side reaction which would reduce the appearing Ruheman red. Secondary amino acids create various chromophores.

2.8 Detection

Ruheman red has its absorption maximum at 570 nm. This absorption capacity is a linear function of the quantity of alpha amino groups presented. The reaction is a suitable basis for the determination of all organic compounds containing amino acids. The reaction of ninhydrin with iminoacids has its absorption maximum at 440 nm. The measurement at the wave lengths under 440 nm provides too large complementary absorption kapacity from the non-reacted ninhydrin.

3. CHEMICAL AND TECHNOLOGICAL ISSUES

3.1 Ionexes (Ion exchangers)

Three types of ionexes are used for the analysers of amino acids. All of them are produced by the company Spolek pro chemickou a hutní výrobu Ústí nad Labem. They are marketed under the trademark OSTION. The identification LG means that the konec is designed for analytical purposes. The first two digits identify the level of cross-linking and the second group of two

digits means the size of the grain. This is common for analytic ionexes, for our purposes this identification is only used for the pre-column ionex. The ionexes designed for analytical columns of our analysers of amino acids have a slightly different identification. For the filling of the analytic column designed for the determination of protein hydrolysates Ostion LG FA delivered in the Li-cycle is used. The identification of ionexes is different from ordinary analytic ionexes because they are tested according to different criteria compared to ordinary ionexes.

3.1.1 Pre-column ionex

The pre-column ionex is delivered under the identification Ostion LG KS 0804, in an Na-cycle. For the utilisation in the Li operation it is necessary to recycle it according to chapter 2.1.5. The ionex is stored in the fridge in a humid state.

3.1.2 Poly 8 for the determination of hydrolysates, free amino acids or biogenic amines

Poly 8 is a strongly acid catex (cation exchange resin) with a medium size of grain of about 8 µm. It is delivered exclusively in the Na or Li form in the packing by 10 ml. It is stored in the fridge in a humid state. **Ostion ANB** for detrmination of hydrolysates or biogenic amines.

3.1.3 Ostion LG FA for the determination of free amino acids

It is a ionex specially developed for our apparatus. It is delivered exclusively in the Li form. The medium size of grain of about 8 µm. It is stored in the fridge in a humid state. The the packing is 15 ml.

3.1.4 Work with ionexes

Before the filling of the column or after a longer time of storage (several months) we recommend to recycle the ionex, because during the storage time some residual products from the synthesis are releasing and they can worsen the separation properties of the ionex and the quality of the zero line. During the operation some impurities originating from the samples tend to be linked with the ionex, and in most cases the pressures tend to rise to an unacceptable extent, or the separation capacity of the pairs which are difficult to separate worsens substantially. If the ionex is contaminated in this way, it may not mean the lowering of its duality but it needs cleaning. This cleaning can be performed by repeated transformation to the H-cycle and then back to the Na- or Li-cycle. While working with analytic ionexes you must observe certain rules which are described below.

1. You must never let the ionex dry out. If it happens, it is necessary to recycle the ionex, dilute it strongly - 20 ml of the ionex mix in a measuring cylinder with approximately 250 ml of distilled water. Wait about 20 to 30 minutes for the ionex to stabilise and suck away the upper, turbid part of H2O (decantation). This procedure can be repeated, if needed, twice or three times. By means of this operation you will get rid of crushed material which arose as a consequence of the drying of the ionex. The amount will be smaller, it is true, but the quality will improve and the pressures within the systém will be reduced.
2. Ionex must always be stored in a humid state. It means that after recycling or after another type of handling (column filling, etc.) its quantity should be flushed to a storage vessel with distilled water. After the stabilisation it should be decanted. Let a small amount of water stand over the ionex (1 to 2 mm).
3. Ionex should be stored, if possible, in the fridge for you to prevent it from being contaminated (by bacteria, moulds).

4. While handling ionex you must mix with a glass rod, the mixing by means of shaking is unacceptable. If you used shaking, it would mean that air would be linked to individual grains of ionex, which could result in high pressures and worsened separation of the fresh column.
5. During the cycling procedures you must always avoid the sieving of air through the layer of ionex on fritted glass. If it happens that, despite your carefulness, the konec has bonded some air, it is necessary to repeat cycling at least once.

3.1.5 Ionex cycling - cleaning

If a ionex is contaminated by ballast substances and its properties get worse, it is necessary to recycle it. Now a practical procedure for the cycling of ionexes will be described. Before beginning it is necessary to read the instructions provided for in chapter
2.1.4.
1. Transformation to the H-form. Mix one volume share with 5 to 6 volume shares of 15% HCl (1:1). Mix occasionally and heat to a temperature of 80°C, keep it for the time of 60 minutes. Then wash on the fritted glass S4 (or on such a frit whose pore size does not exceed 4(m) with distilled water up to the neutral reaction. The konec processed in this way will be in the H-form.
2. Transformation to the Na-form. Mix the ionex in the H form with 16% NaOH in the ratio of 1 volume share of the ionex +5 to 6 volume shares of NaOH. Let the mixture stand for about 30 minutes at room temperature. Mix the entire content from time to time with a glass rod. Then filter again through the fritted glass S4, wash with distilled water up to a neutral reaction.
3. Transformation to the Li-form. Mix the ionex in the H form with a five times larger amount of the saturated LiOH solution. Mix from time to time with a glass rod and let stand for approximately 1 hour. Then filter through the fritted glass S4, wash withdistilled water up to a neutral reaction. For the purpose of filling the analytic column or pre-column dilute with the first analytic buffer 0.18 N Li pH 2.9. For storage purposes move the mixture into a PE bottle and store in a humid state and in the fridge.

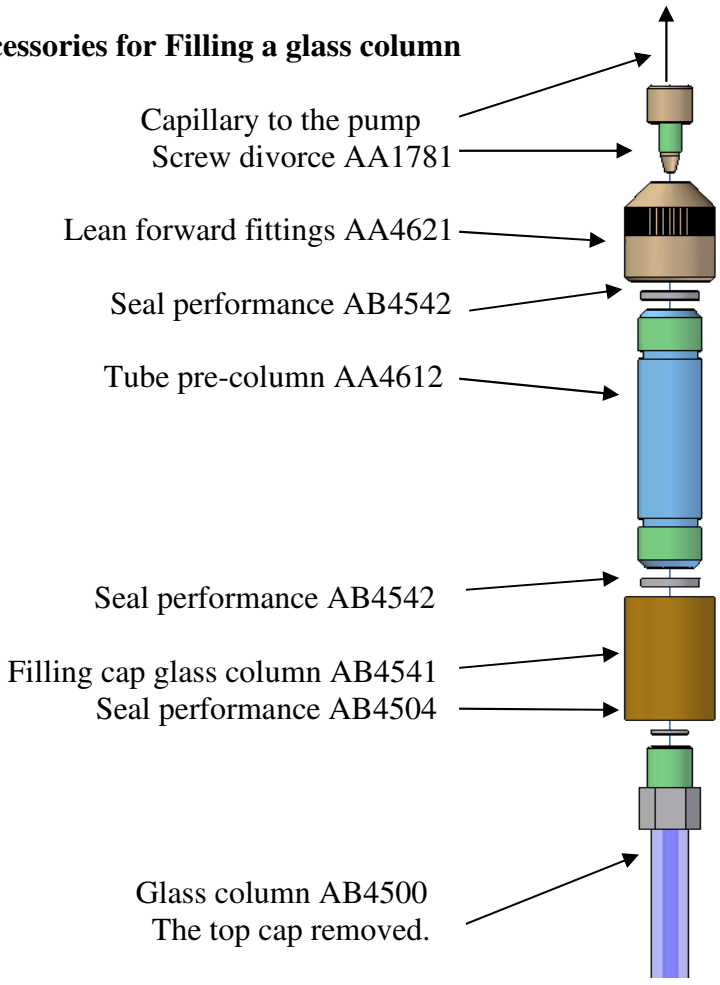
4. Ion exchanger storage. After transferring the ion exchanger into a podium or lithium form on sintered glass wash it thoroughly with the first buffer, and store it in this condition in a PE bottle in the refrigerator. Never store the ion exchanger, after the transferring into a sodium or lithium form, if it has been washed by using a liquor, because this liquor will be released from the pores of the ion exchanger, which means that the ion exchanger will be virtually stored in the liquor, and it will result in its swelling and loss of hardness. Therefore it should be stored in the acid environment of the first buffer. The humidity of vapours can be sufficiently maintained by means of H_2O.

3.1.6 Packing the glass column

1. Work outside the PC-operated mode. Use a wash bottle, beaker (50 ml), stirring rod, syringe with a capillary (see the accessory kit), packing end fitting, and the resin. Clean the inside of the column (use wire with a gauze swab on one end). Proceed as follows:
Make sure all connection lines are well purged with buffer before attaching column to the system.
2. Insert the outlet-end capillary (frit, white rubber O-ring, stainless steel ring, screw) into the glass tube and screw it up.
3. Mark the expected bed length on the outside of the glass tube (make two marks, measured from the outlet-end frit: 26 and 24 cm for hydrolyzates, 22 and 20 cm for free amino acids.

4. Hang the tube on the telescopic column holder (in the top of the column oven compartment). Screwed a metal shaft (the same piece as that used for the pre-column).
5. Using the syringe, place 1 cm water on the bottom.
6. Dilute the resin with buffer Nr. 1 in a ratio 1 : 2. Stirr gently with a rod, never shake!
7. Draw the diluted resin, place the capillary on the column bottom, and start discharging the content of the syringe. Pull out the capillary as the suspension level rises. Fill up to the brim.
8. Put on the packing end fitting (the same as used with the guard column, see Accessory Kit), turn on the buffer pump and set it to 0.3 ml/min.
9. Turn off the pump when the sediment level reaches 2 cm below the suspension level. Wait until the backpressure drops, unscrew the end fitting, remove the buffer (the transparent layer), and add more resin. Repeat this until the level of resin sediment reaches the high mark. Do not remove the buffer layer this time. If the sediment level reaches above the high mark, simply stirr the sediment with the tip of the capillary and remove it.
10. Put on the inlet-end fitting; for the free amino acid method, use also several steel spacer tubes. Be careful not to break the peek capillary over the spacers.
11. Place the column in the oven compartment by stretching the parts of the oven with one hand while inserting the column with the other. Turn on the buffer pump. Start the and continue it for 10 min then switch to buffer Nr. 1. After 20 min, turn off the pump and wait until the beckpressure drops. Screw down the inlet-end frit to leave a void of 0.5 mm between the frit and the resin.
12. Observe the void for the first few runs; if it widens markedly, turn off the pumps and screw down the fitting. An excessive void can result in peak tailing with the result of compromised integration and quantification.

Accessories for Filling a glass column



3.1.7 Filling of metal or peek column

Filling of columns: performed by the manufacturer

3.1.8 Filling of pre-column

The pre-column shall be filled with the OSTION 0804 ion exchanger in the same way as the analytical column. For the purpose of the filling of the column within the framework of the Li cycle (free amino acid specification) the ion exchanger should theoretically be cycled into the Li cycle. Nevertheless, our experience says that it is not necessary because the ion exchanger itself is subject to cycling during the phase of filling. It is, however necessary to change (at least twice, after 10 minutes) the passage through the LiOH column, and on the upper part you may see a small hollow. This will result in a worse separation of arginine, which does not get to the base line after its evolution. In such a case it is sufficient to open the upper closure and fill the column with a fresh ion exchanger, the best method is to pour the buffer down from the ion exchanger stored and to complete (to putty) with the ion exchanger by using a dense sediment and a spařila (lanceolate weighing spoon). If you register, after a certain period of time, that the arg detection quality on the base line became worse, while the column is properly filled, it usually means that the upper layer of the ion exchanger is spoiled. It can be verified after the removal of the upper closure. There is a ion exchanger of a dark colour on the surface. This ion exchanger shall be removed up to the clear ion exchanger, and then fill with a fresh one. If you are closing the pre-column, it is necessary that its border be always cleaned properly, otherwise the sealing of the column could be insufficient, which is a serious defect.

3.1.9 Emptying of columns

The column is removed from the thermostat and hung on the holder. The bottom cap is unscrewed and the capillary going out of the cap is closed so that it is screwed in the coupling with blinded screwing stopper. Then the buffer pump is started (with buffer No. 1) which forces out frit and ionex which is let drain to a beaker. Then the peek capillary (without the frit!) is mounted onto the free part of the column and the entry to the column from the pump and the exit of the column are interposed. The sealing screw on the column exit is unscrewed. The capillary going out of this part is sealed like in the previous case. The pump is started and using pressure the second frit is forced out. The cap on the entry is unscrewed and upon being washed the column is prepared for re-fill.

3.2 Buffers

The chromatographic separation uses mobile phases that are divided into elution buffers and regeneration solution. One more solution is used for dissolving and diluting of samples, called **diluting buffer**. Overview of solutions is mentioned below.

Solution		Function
Diluting buffer		Buffer in which samples are dissolved and diluted is more acidic than elution buffers (pH 2.2).
Mobile phase	Elution buffers	The buffers, sequence of which causes gradual separation of individual amino acids
	Regeneration solution	Alkaline solution of sodium hydroxide or hydroxide which washes out all residuals of the sample out of the column and prepares the column for stabilization.

Mobile phases and their functions in ion chromatography of amino acids

For determination in amino acids in hydrolyzates sodium-citrate solutions are used while for determination of free amino acids lithium-citrate solutions are used. Analyses in the sodium system are faster and are used for determination of 17 amino acids in protein hydrolyzates but they do not enable separation of e.g. amides (asparagine and glutamine). The lithium system offers longer times of analyses but it enables separation of a wider range of ninhydrin-positive substances, and that is why it is used for analyses of physiological liquids, e.g. urine.

Elution buffers consist of main components determining the chromatographic environment and complementary components that prevent air oxidization and microbial contamination (→ Appendix A).

The required acidity and ion strength of buffers is made up by combination of the following main components:

- **Citric acid**
- **Sodium or lithium citrate**
- **Sodium of lithium chloride**.

In addition to the main components the buffers also contain

thiodiglycol as antioxidant for stabilizing of methionine and
sodium azide as preservative (caprylic acid or phenol can be used as well).
Boric acid is used in buffer No. 4 in which the citrate system is not effective any more

Weighed quantities for all buffers can be obtained using a "PreBuffer" programme which is a part of each delivery and it is contained in the Clarity window. For each elution buffer an "Instruction" window can be opened. It indicates which amino acids elute in the respective buffer and which are more dependent on a change of temperature and pH of elution buffer. If the user decides to prepare a buffer of a pH value other than set in default, the programme re-calculates automatically all weighed amounts of chemicals of which the buffer consists of.

3.2.1 Practical remarks on the Li operation

1. Li buffers are much more aggressive than Na buffers, that is why it is suitable to dinze approximately once a month with distilled water at the maximum through flow of the pump. This water should not be released into the hydraulic system.

2. For the corrections of time changes of the switching of buffers use the cursor with the help of which you can read time values of the distances of individual peaks from the graph.
3. It is necessary to remember that the time shifts of the switching of lower buffers must be performed also for buffers with higher numbers. E.g. buffer number 3 will be shifted by 5 minutes, it means that also the 3rd and 4th buffers will be shifted in the same direction and also regeneration will be shifted by 5 minutes.
4. In the case of a serious contamination of buffers by ammonia or in the case of work with strongly contaminated samples it can happen that the zero line will be difficult to stabilise. It will be demonstrated by a slow drop to the baseline. The start of the analysis will be carried out and the zero line will not be stabilised yet. In this case it will not be sufficient to perform a simple regeneration only. It will be necessary to perform the regeneration twice. Thus you will manage to reach perfect repeatability. We recommend the application of the following procedure:
a. regeneration of 0.3 M LiOH for the time of 10 minutes at the temperature of 60°C.
b. stabilisation by means of buffer no. 1 for the time of 20 minutes, temperature 60°C.
c. regeneration of 0.3 M LiOH for the time of 15 minutes at the temperature of 60°C.
d. stabilisation by means of buffer no. 1 for the time of 50 minutes at the temperature of 38°C.
6. In order to improve the repeatability parameters we recommend a continuous operation.
7. All treatments and tuning of buffers are only performed at the beginning during the introduction of the program or in the case of a change in analytic conditions. Here they are described in a more detailed way in order not to avoid later possible delays in the work of operators. In practice no buffer treatments are carried out any more, they are only subject to balancing, or their concentrates are produced as it was described in the chapter dealing with the preparation of the Na buffers.

3.2.2 Safety regulations for the work with Li buffers

These regulations are identical with those applying to the work with the Na buffers, however, the Li buffers are more aggressive towards all metals, and thus it is not suitable to leave them for longer times in contact with surfaces of varnishes and metals.

3.2.3 Switch-over from sodium buffers to lithium ones and vice versa

Regeneration of the pre-column and column and rinsing of the system from one system of buffers to the other one is realized via connecting of the respective regeneration solution for approx. 30 min. E.g. if you switch over from hydrolyzates (sodium buffers) to free amino acids (lithium buffers), change buffers and column and wash the system with regeneration solution No. 6 for the specified time.

3.2.4 Procedure for the preparation of elution solutions (hereinafter

referred to as buffers only)

Dissolve gradually individual components of buffer in hot distilled water (40 to 60°C) while mixing the mixture permanently. After dissolving fill it to the required volume. The buffers prepared this way will not ”mature”, they can be used immediately. Several practical remarks for the preparation of buffers. For the introduction of a new system, e.g. during the replacement of a ionex first prepare 1000 ml of buffer, if this buffer is not suitable (i.e. if it is too fast or too small), do not lose time with its preparation, but prepare a new buffer with changed parameters according to a new computation. The change for the first buffer should not generally be higher than by 0.1 pH. If you know the parameters of a suitable buffer, it is not necessary to compute it for the second time, it will be sufficient to write its pH and weighing quantities which will later be repeated in practice. We recommend to prepare concentrates of suitable buffers in the amount of approximately 5000 ml, i.e. a five-times higher volume of concentrate. Then the complete preparation of the buffer is based on the measuring of 200 ml of concentrate and filling to 1000 ml with distilled water. Example of concentrate preparation: The concentrate will be produced in such a way that approximately in 2500 ml of distilled water you will gradually dissolve a 25-times higher amount of individual buffer components. After the dissolving fill with distilled water to 5000 ml.

3.3 Preparation of ninhydrin agent

You will use for preparation: a 250ml beaker, a rod for stirring and a metal rod for blocking of a hose. The procedure is as follows:

1. Prepare reduction of the hose from the pressure bottle of 0.5mm metal capillary of the ninhydrin bottle stopper.
2. Pour the content of the vial with ninhydrin (NHD, 40 g) in the bottle.
3. Pour with approx. 1.3 l of methylcellsolve, put a cap loosely on the NHD bottle (make sure that both inlets are not clogged. Red-labelled outlet is a supply of nitrogen from a pressure vessel, bubble through with mild flow till NHD is dissolved completely; dissolving can be accelerated by stirring.
4. When NHD is dissolved, add 0.5 l of Na-acetate buffer (4M, pH 5.5) and bubble through with mild flow of nitrogen for approx. 5 min.
5. Hydrindantine (2 g) is desolved in 200 ml methylcellsolve by stirring glass rod durring 1-2 minutes.
6. This solution is quickly added into bottle of ninhydrin solution.
7. Put the stopper tightly in the neck, press it down with one hand while tighten the screw with the other; when tightened the stopper in the neck must not be loose. The bottle with the agent, still connected to nitrogen, is put in the analyzer.
8. Connect a nitrogen storage bin (bag) through a rubber bin to the green outlet of the stopper and let it fill. The bag has a pressure fuse, you needn’t be afraid that it will burst.

9. Close supply of nitrogen, choke the hose between the bad and the bottle (with forceps, clamp etc.) and replace the hose of nitrogen supply with a hose to the analyzer.

Fig. 23 *Last phase of preparation of the reagent: filling of the gas storage with inert gas. Gas comes from a pressurized bottle to a dark bottle through the red-labelled outlet, goes through the prepared reagent and leaves through the green-labelled outlet to the gas storage. This operation can be carried out with the dark bottle and gas storage already placed in the analyzer.*

Note 1:

The ninhydrin reagent must be cooled and stored without access of oxidizing reagents. Residuals of oxygen in used inert gas will cause gradual oxidization of the reagent and reduced response of the detector.

Note 2:

You should keep in mind that the ninhydrin solution fodes away and is not used detector response decreases. If a break from frech ninhydrin preparetion lasts more than one week, the reagent must be regenerated or a new one prepared.

Note 3:

Oxidized reagent can be regenerated with addition of solution 0.5g hydrindantin in 50 ml methylcellsolve. At first, bubble through the content of the bottle thoroughly with inert gas (15 min). Meanwhile, let the gas out from the gas storage.

***Warning!** This operation must be followed by analysis of standard.*

Note 4:

The frequency of ninhydrin check ups using a standard.

a) Reactivity of fresh nihydrin solution solution is verified using standard for each amino acid and ninhydrin positive compound. Makes the first two analysis of standard. For calibration second analysis is used.

b) When starting work with the instrument. The instrument was in stage Stanby or On. For calibration second analysis is used again.

c) When is changed some bottle of buffer.

d) The life of nihydrin solutin is limited. Therefore check up must bedone during life of nihydrin solution. Maximaly after 4 days of preparation fresh nihydrin solution and then daily. For better results it is better to do check up daily after preparation.

e) For calculation of sample the standard which closest to the time of the sample is used.

Note 5:

It is possible to use 0.8g of $SnCl_2$ or 7.5 ml solution of 15% $TiCl_3$ in HCl instead of 2g hydrindantin

4. PREPARATION OF SAMPLES

4.1 Introduction

The correctly selected method of preparation of a sample is the basic precondition for correct and repeatable results in the automatic analysis of amino acids. An erroneous method of

sample preparation can not only lead to errors in the qualitative analysis and inexact determination during the quantitative analysis, but it can also endanger the operation of the analyser. For a successful preparation of a sample it is desirable first to make familiar with the available documentation sources dealing with its range of amino acids and other substances which can play an important role during the processing of samples and interpretation of chromatogram. The prior knowledge of the approximate content of amino acids is convenient for the selection of optimum weighing. For dispensing into the automatic analyser use only pure solutions which are free of solid and colloidal admixtures. The preparation of samples can be divided into two parts: releasing of amino acids linked in proteins, peptides, etc. by means of hydrolysis and preparation of symplex containing free amino acids (biological liquids, tissue extracts) from which proteins and other disturbing substances are removed. In complex biological materials it is often required to determine both linked and free amino acids. Given the unusual variety of natural materials and given the wide range of utilisation of the automatic analysis of amino acids in a number of branches it is only possible to provide for some orientation data concerning the preparation of samples in this part, and we strongly recommend to compare these facts with the available literature in the branch in question. When selecting methods for the preparation of samples we have preferred, in this part, simple procedures whichhave been proven in practice and which are not demanding for the apparatus equipment. However, if it is only possible, prepare especially those samples which contain free amino acids at low temperatures (use cooled separators, try to replace vacuum evaporation by freeze drying (lyophilisation), etc.)

4.2 Proteins and peptides

4.2.1 Characteristics of material

The resistance of peptide links against hydrolysis differs depending on the type of amino acid and structure of the protein. Amino acids released from the peptide link can be subjected to various decomposition changes caused by the effects of hydrolysing agent or other components of the reaction mixture. Therefore it is not possible to obtain any absolute data about the contents of amino acids during the sole conditions of hydrolysis.

4.2.2 Hydrolysis by hydrochloric acid (1)

For this most common method of hydrolysis by 6N HCl which can be obtained ether by diluting a concentrated acid in the ratio 1:1 with distilled water, or by distillation of azeotropic mixture of HCl with water (1:1) in a glass set with the addition of a small amount of $SnCl_2$. The hydrolysis will be carried out in the test tube of Sial or Pyrex, gradually rinsed with a chromium sulphur mixture (40 g of CrO_3 into 600 ml of H_2O, after dissolving + 400 ml of H_2SO_4), distilled water and 1 N HCl. The HCl residue will be removed in a drying facility at a temperature of 100°C. Keep the test tubes in polyethylene bags to avoid the deposition of NH_4Cl. Use differential weighing when putting a sample to the bottom of the test tube to avoid material depositions on the walls. Then pipette 6N HCl in a two-hundred times higher surplus into the tube. To the liquid samples add 12N HCl in the volume of the sample. During the determination of weighing amounts base your operations on the orientation fact that 1 mg of protein contains 0.3 - 1 μmol of individual amino acids. Narrow the top of the test tube on the oxygen flame to an internal diameter of about 2 mm in such a way that there is no weakening of its walls. Then freeze the solution down in a freezing bath (acetone and dry ice (solid carbon dioxide)) or in an undercooled alcohol. Then evacuate the sample through a valve, close and fuse-in the test tube at its neck. Insert the test tube into the hydrolysing block or to an air thermostat and leave it for the time of 20 hours at the temperature of 110°C + 1°C. Another share of the sample, prepared in the same way, will be hydrolysed for 70 hours. After

the hydrolysis cool the test tubes down, slightly cut and let them crack by applying the end of a hot rod. Evaporate the acid in a vacuum rotary evaporator. Do not open the samples which cannot be analysed immediately after the hydrolysis and store them in the fridge or in the freezer. If the sample contains cysteine, it is necessary to dissolve the evaporation residue in distilled water and after the treatment by means of a phosphate buffer to the pH value 6.5 leave it for 4 hours at the laboratory temperature. After this oxidation of cysteine to cystine, acidify the sample by 1N HCl and fill it to the necessary volume by a buffer of the pH value 2.2. If you do not perform the cysteine oxidation, dissolve the evaporation residue directly in this buffer.

4.2.3 Sources of errors

The presence of heavy metals in the hydrolysing agent, where they can be brought for example with HCl, will leads to the increasing of the losses of threonine, serine, sulphur amino acids and tyrosine. The losses of sulphur amino acids and chlorination of tyroxine is also supported by an imperfect evacuation of the sample and especially by the presence of oxidation agents (e.g. methionine - sulphoxide, dimethylsulphoxide, NO3 ions, etc.). At the presence of nitroarginine also phenylalanine is chlorinated. The losses of threonine and serine by means of esterification are supported by the presence of a sulphate ion, and by the older method of HCl removal by evaporation in an exicator over lye, which will be indicated by the presence of small peaks in various parts of chromatogram. Glutamic acid will be partially cycling on these conditions. The incomplete yields of certain amino acids are given by the HCl hydrolysis Metod used and they are not practically removable. Cystine is recemising during the hydrolysis, and thus provides a flat, asymmetric peak on the chromatogram, which is difficult to integrate. More exact results of the determination of sulphur amino acids, also with regard to possible oxidation during the HCl hydrolysis, can be obtained by means of sample oxidation before hydrolysis For the determination of exact contents of isoleucine and valine, which are difficult to release from peptide links a 70-hour-long hydrolysis is used. In the case of threonine and serine it is possible, in the opinion of some authors, to register losses amounting to 3-15% per 20-24 hours of hydrolysis, serine being less stable than threonine according to most sources. The tyrosine losses presented in the literature for the same times of hydrolysis move within the range of 1-14%. Tryptophan is during the acid HCl hydrolysis destroyed almost completely, the peaks of its oxidation products may, however, appear on the chromatogram before the position of tryptophan. Other amino acids are, according to most literature sources, considered as stable dutiny 20-24 hours of hydrolysis, or their losses are negligible with regard to the exactness of the determination, respectively.

4.2.4 Preparation of a sample for the determination of sulphur amino acids (4).

You can prevent the unspecified redox reactions of sulphur amino acids during the HCl hydrolysis from their arising by transforming cystine and methionine by means of performic acid to stable, oxidised derivatives - cysteic acid and methioninesulphon. Oxidation mixture preparation: mix 1 part of 30% H_2O_2 with 9 parts of 88% of formic acid, leave standing for the time of 30 minutes at laboratory temperature and cool off to 0°C. Then add the mixture to the sample in the ratio of 1 ml per 0.04 to 0.08 mg of cystine (i.e. about 2 mg of proteins) and leave standing for 4 hours at a temperature of 0°C. The samples which will not be dissolved during this time should be left at the same temperature over night. Then evaporate the oxidation mixture on a rotary vakuum evaporator at 40°C up to a syrup consistence - complete evaporation of the sample will lead to losses of cystine. After the adding of a 200-times higher surplus of HCl the Sample will be subjected to a standard acid hydrolysis 4.2.2. The oxidised hydrolysate will not generally be used for the specification of other amino acids, even though they are, excerpt for tyrosine and tryptophan, stable against oxidation. The yield

of the cysteic acid can be slightly increased by means of the reduction of the oxidation mixture residue efore vacuum evaporation with the help of HBr - see (5) for details.

4.2.5 Accelerated evaporation of samples after hydrolysis, both oxidation and common

After the hydrolysis, filter the sample through a black tape into the measuring flask of a volume of 200 or 250 ml. Wash filter thoroughly with hot water and after cooling complete it. For evaporation use an aliquot amount (20 or 25 ml) which you flush to a 10 ml measuring flask and complete with a diluting buffer of pH=2.2. This way you can significantly accelerate the evaporation of the sample because the evaporation of avolume of about 150 ml of sample is very long. The evaporation of 20 ml lasts several minutes.

4.2.6 Preparation of samples for the determination of amino sugars

Amino sugars which are linked in glycoproteins can be well determined by the analyse of amino acids because they are substances reacting with ninhydrin. During the application of ordinary methods of hydrolysis some larger parts are, however destroyed and they form a part of humins which are to be removed from the hydrolysates by means of centrifugation or filtration. For the specification of amino sugars the hydrolysis by means of 4N HCl is used for the time of 1 to 4 hours, or 2N HCl for the time of 12 hours at a temperature of 100°C. Proceed in accordance with the method specified in the part 4.2.2, the exclusion of oxygen and heavy metals is fully necessary.

4.2.7 Preparation of hydrolysates for the determination of tryptophan

The preparation of a sample for the determination of tryptophan linked in protein is difficult with regard to low stability of this amino acid in the usual hydrolysing media, especially at the presence of non-protein components. In the case of pure proteins not containing sugars it is possible to prevent, at least in part, tryptophan (hereinafter referred to as trp only) from its decomposing during the acid hydrolysis by adding mercaptide compounds to HCl or by using aryl- and alkyl-sulphon acids to hydrolysis. For the samples containing sugars the basic hydrolysis is used, during this hydrolysis the tryptophan result is being reduced through oxidation by air oxygen, releasing of silicates from the walls of glass hydrolysis vessels (especially during the use of NaOH) and by means of tryptophan absorption on possible precipitates and insoluble shares of reaction mixture. According to most sources the highest yields of trp are provided by the hydrolysis performed by means of barium hydroxide, the removal of Ba(OH)2 before konec chromatography is, however, quite difficult and it can be a source of further losses. Below you will find two examples of seriously verified methods of hydrolysis for the purpose of tryptophan determination.

4.2.8 Acid hydrolysis (6)

Add 4% of thioglycollic acid p.a. to HCl and proceed in the same way as in the case of a standard acid hydrolysis (part 4.2.2). The tryptophan yield is 90%, the sample, however, must not contain sugars. Cysteine arising during this method of hydrolysis interferes with the determination of proline. Thioglycollic acid provides two peaks on the chromatogram, one of them being in the area between the peaks of cysteic acid and aspartic acid, the other one being in the place of carboxymethylcysteine.

4.2.9 Basic hydrolysis (7)

Tryptophan is released by the effects of 4.2N NaOH on the sample placed in the evacuated propylene measuring cell at the addition of partially hydrolysed starch. Preparation of partially hydrolysed starch: add 50 g of potato starch to a mixture of 99 ml of acetone and 1 ml of concentrated HCl. The mixture will be warmed up for 2 hours up to a temperature of 50°C. After the addition of 25 ml of 1M sodium acetate the mixture will be moved on a fritted

glass and washed gradually by 2 l of distilled water and 2 l of acetone. The product will be dried in the exicator. It is also possible to use a commercially available starch preparation designed for gel electrophoresis. For hydrolyses first prepare a protein solution in 0.005N HCl or NaOH, containing 0.1 - 0.5 mol trp per 1 ml, pipette 0.1 ml of this solution into a polypropylene centrifuga measuring cell (11 x 50 mm), add 25 mg of partially hydrolysed starch and 0.5 ml of 5n NaOH, freshly prepared of 50% NaOH. Place the measuring cell into a thin-walled test tube (16 x 150 mm) and add 5(l of 1% solution of octanol in toluene for avoiding foaming. The test tube will be pulled out approximately at one half over the oxygen flame to a diameter of about 2 mm. In the case of a thin-walled test tube it can be carried out without the plastic insert being damaged. Then cool off the bottom part of the test tube in a mixture of acetone and dry ice for the time of 90 s in such a way that the content will not get frozen. Then evacuate several times by using oil vacuum pump, while interrupting several times the connection with the source of vacuum, and in the case that the Sample is foaming too much, immerse the test tube again to a cooling bath. After the reaching of the evacuation the test tube should be fused-in after 50(m Hg. The sealed test tube will be hydrolysed at a temperature of 110 + 1oC. After a perfect cooling down open the test tube by cutting it slightly and letting it crack by applying the end of a hot glass rod. Add 0.5 ml of sodium citrate buffer (its pH being 4.25, it should not contain BRIJ - 35) to the sample and mix the mixture thoroughly. Then the solution will be quantitatively transferred by means of this buffer to a 5 ml measuring vessel, containing 420 (l of 6N HCl and placed in the dry ice. Then fill the content of the flask up to the mark. Any possible turbidity will be removed by centrifuging for the time of 30 minutes at 40,000 g.

4.2.10 Sources of errors

The yields of tryptophan reach, during the application of this method, 100 + 2% on the condition of a sufficient time of hydrolysis. For a majority of proteins 16 hours are sufficient, however the link Val - Trp or Ile - Trp requires as much as 98 hours or 48 hours at a temperature of 135oC. For the specification of other amino acids the basic hydrolysis is not used (with the exception of methioninesulphoxide), because there are significant changes for a number of them. Arginine is partially transformed to ornithine, cystine is transformed to lanthionine, by means of the reaction of the decomposing products of cystine and serine with lysine there arises lysinealanine, etc. For the separation of tryptophan from lysinealanine we recommend to use the Hugli and Moore method (7).

4.3 Blood plasma

4.3.1 Characteristics of material

Blood plasma or serum containing free amino acids, proteins removed by deproteination and linked amino acids in ninhydrin-negative forms which are not removable by deproteination. The ultra-filtration, gel filtration, ultra-centrifugation after acidifying, etc. were suggested as means of deproteination, but the most frequent are chemical deproteination methods, especially the methods using sulphosalicylic and picric acids.

4.3.2 Taking and preparation of plasma for deproteination

The blood taken will be transferred to the a centrifugal test tube containing 5 mg of heparin per 25 ml of blood, centrifuge for about 10 minutes at 21,000 g and take supernatant for the analysis in such a way that you will prevent its being contaminated by deposits. All of these operations including deproteination should be performed immediately after the taking.

4.3.3 Deproteination by sulphosalicylic acid

Add solid sulphosalicylic acid to plasma in the ratio of 30 mg of acid per 1 ml of plasma, shake and centrifuge for 10 minutes at 21,000 g. For the purpose of the elimination of errors arising as a result of changes in the liquid state volume it is possible to add, during deproteination an internal standard of norleucine (10 μmol) ml in 0.02N HCl in the ratio of 0.2 ml per 4 ml of plasma. This procedure provides for a solution whose concentration of amino acids (0.05 - 0.6 μmol / ml) is sufficient for direct dispensing into the analyser. The utilisation of sulphosalicylic acid can, however, slightly worsen the separation of acid and neutral amino acids in the area from the beginning up to the peak of serine in some cases.

Examples of sample preparation:

1) 250 l serum + 150 ml buffer 5 (pH 4.8) + 100 ml of 20% SSA
total volume of 500 ml - centrifuge 5 minutes (3000 rpm) and download deproteinized
pH of about 2.0; 2 times dilution; just one analysis!

2) 300 l serum + 180 ml buffer 5 (pH 4.8) + 120 ml of 20% SSA
total volume 600 ml - centrifuge 5 minutes (3000 rpm) and deproteinized download (about 480 ml)
pH of about 2.0; 2 times dilution; enough for two analysis

3) if the sample is small:
125 l + 125 ml serum dilution buffer + 150 ml 5 buffer (pH 4.8) + 100 ml of 20% SSA
total volume of 500 ml - 5 minutes centrifuged (3000 rpm) and deproteinized download (about 400 ml) and pH of about 2.0; 4 times dilution; just one analysis!

Also it can add an internal standard e.g norleucine
SSA = 5-Sulfosalicylic acid

4.3.4 Deproteination by picric acid (10).

Add 20 ml of 1% solution of picric acid to 4 ml of plasma. After shaking carefully centrifuge, and apply 20 ml of supernatant to the little column of the diameter of 2 cm, filled with 6 ml of a medium basic ionex (Ionex Bio Rad AG 2 - X 10 used in the original work can be substituted by Dowex 2, Amberlit IRA - 410 or Ostion AD. A suitable graining is 200-400 or 100-200 mesh.). The ionex serves for the removal of picric acid. Ionex column preparation: mix ionex several times in an amount of distilled water approximately ten times larger, and by means of repeated decantation remove dust particles. Then wash the ionex on the fritted glass thoroughly by means of 1N HCl in a volume ten times larger, and by means of distilled water up to a neutral reaction of the eluent and fill it into the column in water suspension (2 parts of water per 1 part of ionex). The specified amount of ionex will form a column, approximately 2 cm high. Its surfaře should be protected by applying a circle of filtration paper. Wash the column by using 15 ml of 1N HCl, and after a new washing with water up to the neutral reaction the column will be prepared. Leave 2-5 mm of liquid over the surface of the ionex - the surface must not get dry!! During the passage through the column of a sample it is necessary to collect all eluent. After the full absorption of the sample (you must, however, prevent air from its penetrating into the ionex column) wash the walls with 3 ml of 0.02N HCl. After the absorption of the acid repeat this procedure still twice with the same dose of HCl, and in the end with 1 ml of 0.02N HCl. If lyophilisation is not available, proceed in

accordance with Stein and Moore (12) in such a way that the sample will be evaporated in vakuum and after the dissolving in water of the sample and approximate neutralisation to a pH value of 7-8 oxidise cysteine to cystine by leaving it to stand at a laboratory temperature for 4 hours. Then acidify the sample (pH 2.2).

4.3.5 Preparation of a plasma sample for the determination of tryptophan

If you want to determine all free tryptophan in plasma including the share of the tryptophan which is reversibly linked to proteins, use deproteination by means of trichloroacetic acid (10).

4.3.6 Determination of all non-protein amino acids in plasma

For the specification of all amino acids including the conjugated acids use 1 ml of the aliquot solution obtained after the deproteination by picric acid and cleaning on an anion exchange resin. Proceed according to the part 3.2.2, the evaporation residue after the hydrolysis will be dissolved in 2 ml of the buffer (pH 2.2).

4.3.7 Sources of errors

In the case of a repeated venipuncture the plasma taken features a reducing kontent of taurine and glutamic acid. The delayed deproteination brings about some losses of cystine, homocystine and mixture disulphides of sulphur amino acids. During the contamination of the taken plasma by means of thrombocytes and leucocytes the contents of taurine, aspartic acid and glutamic acid are rising. Hemolysis is proven by the finding of glutathione and by a rising content of ornithine, while the contents of aspartic acid and cystine are declining. The storage of plasma at temperatures above -68^{o}C leads to an increase in the contents of aspartic acid and glutamic acid, while the content of corresponding amides is lower. Also the content of tryptophan is lower the content of amides is reduced also when the sample is being processed at a temperature exceeding 40°C and when an acidified sample is standing at a low value of pH (glutamine is substantially less stable than asparagine). By using ethylenediamine (tetra)acetic acid as a dispersion stabiliser it is possible that some ninhydrin-positive impurities will be introduced into the sample. As far as the selection of an optimum deproteination agent is concerned with the view of the yields of free amino acids and their repeatability, the conclusions in the literature are not unambiguous.

4.4 Tissue extracts

4.4.1 Characteristics of material

Besides free amino acids it is also possible to find some typical peptides in the tissues of animal origin. The occurrence of some of them (carnosine, anserine, homocarnosine, etc.) is linked only to certain tissues, while the peptide glutathione can be found in both oxidised and reduced forms in all tissue formulas. Glutathione is present in the liver in a high concentration. A reduced glutathione is eluted in the area of the aspartic acid peak, while the oxidised glutathione is washed out as a very wide peak after glutamine. The preparation of a tissue sample includes extraction connected with deproteination and removal of glutathione.

4.4.2 Preparation of a sample with the utilisation of picric acid (13)

Tissues designed for analysis should be removed from a bled animal and processed as fast as possible. If they are to be transported or stored, they must be frozen by means of dry ice and stored at a temperature of -45°C. Weigh the tissue deprived of fat and connecting ligament, cut it into doses put them into a mixer and pour on it a ten times larger weight amount of 1 % of water solution of picric acid. In the case of brain tissuesuse a five times larger surplus. For

the purpose of homogenisation in the mixer centrifuge quickly the separated proteins, and apply the liquid share to the column, filled with a medium basic anion exchange resin (see part 3.4.4.). For less than 80 ml of extract use a column which has an internal diameter 2 cm, filled 6 ml with ionex. The walls of the columns will be washed out by 3x5 ml of 0.02N HCl, all liquid coming from the column during the application of the sample and rinsing of the column will be collected. For a larger amount of extract use a double amount of ion exchanger, and for brain extracts use a column of the diameter 7.5 cm, filled with ionex up to the height of 2 cm. For a changed amount of ionex accordingly treat also the amount of HCl designed for the rinsing of the column. Lyophilise the elute from the column or vacuum evaporate it to a volume of about 1 ml. If there appears a precipitate, add approximately 4 ml of waters and several mg of Celite and filter the suspension on a filter thoroughly washed by 1N HCl and water. Evaporate the filtrate again. Wash the concentrate out into a 5 ml measuring flask in such a way that the volume will not exceed 3 ml. In this phase it is possible to store the sample in a frozen state over night. The following day modify the pH value of the solution to 7-8 by means of 1N NaOH (inspection by means of an indication paper) and perform a volume treatment by means of distilled water to 5 ml. Take an aliquot part from the sample, which will serve for the specification of a total content of free and linked amino acids after the hydrolysis (see part 3.2.2) and mix another 2-ml aliquot part with 0.5N sodium sulphite which will be added in the ratio of 0.2 ml per each 2.5 g of tissue in the aliquot part of the extract. Both the parts of the sample non subjected to hydrolysis (with an addition of Na2SO3 and without this addition) leave to stand for 4 hours at laboratory temperature. In the part which does not contain sulphite there will be a cysteine oxidation to cystine, while in the aliquot part with the sulphite addition both of the glutathionine forms will be transformed to glutathione - S - sulphonate, eluted during the automatic analysis before aspartic acid. Also cysteine to cystine will be transformed in this part of the sample to an appropriate - S - sulphonate, resulting at the beginning of the analysis with a dead volume of the column. For the determination of cystine therefore use a share without any addition of Na2SO3. The final part of the preparation of the sample is the treatment of pH with 1N HCl to 2.2 in such a way that the final concentration of the sample will correspond (approximately) to 0.5 g of the original tissue per 1 ml.

4.4.3 Sources of errors

The above described procedure provides lowered yields of citrulline, homocitrulline and tryptophan, and according to Saifer (15) it also leads to reduced yields of basic and sulphur amino acids. It is also possible to use other agents for deproteination from which the best results for brain extracts were represented by perchloric acid, which can simply be removed by adding KOH and cooling the solution off (15). The tissue extracts are sometimes also cleaned on a cation exchange resin (see part 3.6.2), if you want to obtain
pure amino acids.

Cerebrospinal fluid (liquor)

Examples of sample preparation:

1) 500 ml of cerebrospinal fluid + 25 ml of 20% SSA, centrifuge 5 minutes (3000 rpm) and download deproteinized pH 1.9; dilution of 1.05 times; enough for two analysis

2) when there is little liquor:
250 ml CSF + 12.5 ml of 20% of SSA, centrifuge 5 minutes (3000 rpm), and download deproteinized pH 1.9; dilution of 1.05 times; just for one analysis!

It can also add an internal standard e.g. Norleucine
SSA = 5-Sulfosalicylic acid

4.5 Urine

4.5.1 Characteristics of material

Urine is a very complicated material from the viewpoint of automatic analysis of amino acids. Prevailing ninhydrin-positive components are urea and ammonia, more than 50% of amino acids in urine are present in various conjugated forms. Proteins are not present in standard urine.

4.5.2 Taking and processing of urine samples

The sample presented to analysis generally represents a 24-hour-old collection which should be preserved by the addition of several ml of toluene and stored in the fridge. A longer storage of the sample (several days) requires the depositing in a polyethylene bottle and storage in the freezer. Before analysis warm the sample to the laboratory temperature and find out its total volume and specific weight. If you do not remove ammonia from urine, the only treatment for the analysis is the acidifying of the sample to a pH value 2 by means of 6N HCl. If a wide peak of ammonia prevents from the determination of neighbouring substances, add 2-4N NaOH by drops to 10 ml of urine in a flask, up to the reaching of a pH value 11.5-12. Check the reaction by means of indication paper. Insert the flask with urine into the exicator and evacuate by using a water vacuum pump for 6 hours. Then remove urine from the exicator, treat the pH value to 2.2 with the help of 6N HCl and adjust the volume by means of the buffer the pH value of which is 2.2 (8). For the specification of all amino acids in urine including the conjugated amino acids hydrolyse 1 ml of aliquot part of urine with the same volume of 12N HCl according to the part 3.2.2. Dissolve the residuum after the vacuum evaporation in 3 ml of buffer (pH value being 2.2), transfer to a 5 ml measuring flask and fill with the buffer up to the mark.

Examples of sample preparation:

1) The urine creatinine of 1.0 mmol / l thinned
1 ml of urine, acidify about 2 ml zřeď.HCl, check with pH paper (should be around 2.0)

2) Urine with creatinine 1.0 to 2.0 mmol / l must be diluted 2 times
0.5 ml of urine + 0.5 ml of dilution buffer pH 2.2

3) Urine with creatinine above 2.0 mmol / L was diluted 4 times
250 ml + 750 ml of urine dilution buffer pH 2.2

It can also add an internal standard e.g. Norleucine

4.6 Vegetable extracts

4.6.1 Characteristics of material

Vegetable extracts are also a very complicated material from the viewpoint of the analysis of amino acids because they are an exclusive source of most of the several hundreds of free

amino acids which are known nowadays (16, 17). A majority of these amino acids are, however, limited in terms of their appearance to several related plants, and most economically important plants do not contain these rare amino acids. Besides amino acids the vegetable extracts may, however, contain also other ninhydrin-positive substances such as amines, acid - glutamyl peptides, vegetable colouring agents and nitrogen substance reacting with ninhydrin (e.g. reducing sugars). The preparation of vegetable samples for the purpose of the specification of free amino acids requires disintegration of vegetable cells, extraction of amino acids and possibly also the removal of interfering components of extracts.

4.6.2 Extraction of vegetable samples and extract treatments

The most common extraction agent is ethanol, which does not have any aggressive influences on amino acids from the chemical point of view, it can easily be evaporated, and during the boiling it deproteins the extract at the same time. Fill 50 g of finely cut sample or ground dry sample (for vegetable materials with a low content of water and firm cell walls it is necessary to pulverise the sample in a pulverising tray with an abrasive material, e.g. crushed glass) with 200 ml of boiling alcohol by means of the concentration of which it is necessary to tread hot water according to the content of water in the sample in such a way that the final concentration after the mixing with the sample will be 80 % of volume. Boil the mixture for 5 minutes under a reflux condenser and then let it cool down to laboratory temperature. Then transfer the mixture by usány alcohol (80 % vol.) and homogenise it for the time of 20 minutes. Then let the solid state deposit and add the liquid part to the supernatant after centrifugation. Repeat the extraction in the mixer still three times with new 100 ml doses of 80 % alcohol. Let the combined liquid shares stand over night in the fridge, filter the following day. Rinse the filter by using 80 % alcohol and proceed with further processing depending on the nature of the sample. Materials with a high content of free amino acids or with a low kontent of colouring agents (e.g. above ground parts) and the samples in which it is necessary to specify the content of glutamine and glutamic acid exactly must be processed as follows:

Evaporate the filter extract at a temperature of 50°C on a rotary vacuum evaporator to a concentration of about 2.5 - 25 g of sample per 1 ml of solution (depending on the expected amount of the content of amino acids). It is possible to store the concentrate in the fridge. Before analysis take 1 - 2 ml from the concentrate tempered to laboratory temperature, transfer this amount to a measuring flask and add 0.5 ml of the inner standard (norleucine in 10 % isopropanol, 10 μmol/ml) and 1 ml of 1.5N lithium-citrate buffer. This buffer will be prepared according to the instructions for the preparation of 0.3N lithium-citrate buffer with the pH value 2.2, it is, however, necessary to use five times higher amounts for weighing. Fill the solution up to the mark with distilled water and leave it standing in the fridge for one hour. Then, if necessary, centrifuge the solution and use it immediately for analysis the sample is not stable after the adding of the buffer - there is a hydrolysis of glutamine as a result of a low pH value. Also peaks of other substances mentioned in the part 4.6.1 can appear in the sample prepared in the above described way during the chromatography, besides amino acids. The peaks of sugars and vegetable colouring agents are localised on the chromatogram in the area of the cysteic acid peak. The samples containing an increased amount of pigments cannot be successfully concentrated in vacuum without any failure of solid state. Besides, these substances can coagulate in a capillary reactor and they can cause a bad record, possibly they can clog the reactor. Almost pure mixtures of amino acids it is possible to obtain by cleaning extract on a cation exchanger in the H+ form. It is possible to use any strongly acid sulphonated polystyrene cation exchange resin (Dowex 50W, Amberlite IT-120, Kationit KV-2, Bio Rad AG-50W, etc.). Pour 100 ml of extract through the column containing 16 ml of ionex in the form H+ (to this form the ionex will be transferred by 2N HCl and then rinsed

with water to a neutral reaction). After the absorption of the sample wash the column with 300 ml of distilled water with which you will wash out a part of vegetable colouring agents, sugars and both inorganic and organic cations. Then wash out the amino acids (18) by means of a gradual elution with 100 ml of 2N NH4OH and 100 ml 4N NH4OH at a flow rate about 5 ml/min. Ammonia will be removed by evaporation on a vacuum rotary evaporator at a temperature of 40°C. Dissolve the dry evaporation residue in 2.5 ml of 10 % isopropanol (it is convenient, if the solution contains the inner standard - norleucine in the concentration of 1 μmol/ml). Store the dissolved evaporation residue in the fridge. One hour before the analysis take
2 ml from the solution tempered to laboratory temperature, add 1 ml of 1.5N lithiumcitrate buffer and fill with distilled water up to the mark. Leave the sample in the fridge for one hour. Before dosing into the analyser centrifuge any possible precipitate. 4.6.3 Preparation of samples for the determination of amino acids in various fractions of the nitrate regime of a plant During the utilisation of extraction agents other than boiling alcohol also vegetable proteins (18, 19) pass together with free amino acids to the solution, to a greater or leader extent. For the purpose of a correct interpretation of results it is therefore necessary to take into consideration the separation of the low- and high-molecular parts. From the extract of the protein there will be some acetone condensing with a seven times larger surplus during the standing at a temperature of -20°C or through the mixing with a 10 % solution of trichloroacetic acid. In the ratio 1:1. The condensing products will be dried after a good washing, and then they will be weighed for the hydrolysis, during which the losses are increased by the presence of sulphate, heavy metals and residue of organic agents.

4.6.3 Sources of errors

Extraction: No extraction agent provides fully quantitative yields. For the increasing of the yields it is more effective to increase the number of repeating than to increase the volume of extraction. The utilisation of acid extraction agents containing alcohol can lead to the esterification of some amino acids (at higher temperatures), while a low pH value causes the hydrolysis of glutamine. A long-term heating of the sample during a neutral reaction (especially if it concerns the presence of phosphate) causes the cycling of glutamine to pyrolidoncarboxylic acid, which cannot be detected by ninhydrin. At a presence of proteins there can appear some links of sulphur amino acids to a protein or proteolysis. Separation on cation exchanger resin: It causes 16 - 50 % of loss of glutamic acid by cycling. There can also be some losses of other amino acids caused by hydrolysis, incomplete detection on the column (aspartic acid), incomplete elution, or as a result of a basic reaction. You can verify the losses by a model attempt with a standard mixture of amino acids and standardise them by means of maintaining carefully the constant times of individual operation. Instead of ammonia, whose presence in the evaporation residue can cancel neighbouring peaks, it is also possible to use trimethylamine (20). It is also possible to wash out from the cation exchanger resin the ninhydrin-positive substances which are contained in it and which are washed out during the automatic analysis approximately in the place of ammonia.

4.7 Foodstu_s and fodders

4.7.1 Characteristics of material

It covers a very wide group of samples of vegetable, animal and possibly also microbiological origin. For the specification of the nutrition value it is usually necessary to determine the whole content of both free and linked amino acids after the hydrolysis of the sample. After the assessment of the influence of the production and storage technologies it is often purposeful to follow also the content of free amino acids. During the technological processing also

ninhydrin-positive substances which did not appear in the original natural material can be formed in the samples.

4.7.2 Preparation of foodstu_ samples for the determination of free amino acids The preparation of samples is determined by their origins. In the case of the samples

of animal origin proceed in the same way as during the preparation of samples designed for the determination of free amino acids in tissue extracts (part 3.4), in the case of the samples of vegetable origin it is necessary to proceed in the same way as during the preparation of vegetable extracts (4.6). If the samples are in a liquid form, or in a form which is fully soluble in water, deprotein the solution by using trichloroacetic acid (4.6.3),
condensed proteins wash thoroughly with water, dry out and use for the preparation of the hydrolysate(part 3.2 an further information from 4.7.3). If a larger amount of foreign substances was introduced into the sample during the technological processing, the sample can be cleaned on a cation exchanger resin (4.6.2).

4.7.3 Hydrolyses of foodstu_s and fodders

The methodology of the preparation of foodstuff and fodder hydrolysates differs from work with pure proteins, because with regard to a heterogeneous nature of material it is usually necessary to work with large weighing amounts and the evacuation of the Sample is often omitted and replaced by a nitrogen atmosphere. Samples with a high kontent of fats (oil plants) are weighed before hydrolysis in a dry state (it is a precondition), as finely ground samples with a known content of nitrogen. Procedure for hydrolysis with 6N HCl: Weigh 0.5-2.5 g of a finely ground dry Sample corresponding to 0.04 g of N (0.25 g of nitrate substances) into a 500 ml three-neck linking flask, pour 250 ml of 6N HCl over it, introduce a stream of nitrogen by means of one of the side arms and by using a capillary tube, and hydrolyse for 24 hours under the reflex condenser. Filter cooled hydrolysate through the fritted glass S2. Wash the hydrolysing flask and fritted glass with 0.1N HCl. Transfer quantitatively the connected filtrates and washing waters into a measuring flask of a volume of 500 ml and fill with distilled water up to the mark. Take 50 ml of the aliquot part from the solution, which corresponds to 0.004 g of N, which will be evaporated at 50°C in the vacuum rotary evaporator into a syrup consistence. Transfer the evaporation residue by using the 0.2N sodium-citrate buffer of a pH value of 2.2 to a 50 ml measuring flask and fill. Alternative method of hydrolysis: More comfortable than the hydrolysis under a reflex condenser is the utilisation of special ampoules. Weigh the same amount of the sample, add 150 ml of 6N HCl and bubble the content of the ampoule with the help of a capillary tube and by means of analytically pure N2 for the time of 10 minutes (foaming is suppressed by 0.2 ml of caprylic acid) and seal (fuse-in). The sealed ampoules should stand in a vertical position in a thermostat tempered to 100 + 1°C for the time of 22 hours. After cooling down perfectly slightly cut the narrowed part of the ampoule immediately behind the sealing, break the top away and transfer the content onto the fritted glass S2. Flush the ampoule and fritted glass with 0.1N HCl, and then proceed in the same way as in the case of previous method. The ampoule can be used several times, if its stem is at least 2 cm long. The hydrolysis can be significantly accelerated by means of a higher temperature and pressure (by using an autoclave). According to the literature (2) four hours at a temperature of 145oC are equivalent to 26 hours at 110°C.

4.7.4 Sources of errors

From the literature it is possible to state that in the case of samples with a high content of non-protein substances the hydrolysis method (reflux, hydrolysis in a dealer flask under vacuum or under nitrogen) does not have any significant influence on results. The amino acid losses can,

however, be significantly influenced by the composition of the sample. If serial samples of similar composition are to be analysed (e.g. cereals), it is suitable, for a representative sample, on the basis of several times of hydrolysis (see part 3.2.3) to find out the losses of non-stable amino acids, which can be determined after the HCl hydrolysis (threonine, serine, tyrosine). For other serial analyses then the only time of hydrolysis is used (usually 20 to 24 hours) and the losses are corrected with the help of the correction factors computed (23). During the interpretation of the hydrolysate chromatogram some problems can be caused by the presence of free amino acids in the sample before the hydrolysis. Some of these substances are stable during the hydrolysis (aminoadipic acid, aminobutyric acid, ornithine and a number of less frequent amino acids of vegetable origin), non-stable substances can decompose to ninhydrin-positive decomposition products. For samples with a significant representation of free amino acids it is therefore possible to recommend to perform first the analysis of a hydrogenated sample by means of a free amino acid method and only then, after the identifying of the range of the ninhydrinpositive substances, to use it for serial analyses of the hydrolysate methodology. The influences of alkaline substances on the sample before the hydrolysis can be demonstrated by a partial transformation of arginine to ornithine and by the creation of lanthionine from cystine. The presents of sugars does not increase losses of amino acids during the hydrolysis according to most authors. Some decomposition products of sugars are eluted during the automatic analysis in the area behind the cysteic acid, another share of saccharides is transformed to humines which can be partially removed by filtration on a fritted glass. The utilisation of decolouring agents for the removal of dark colour humine substance cannot be recommended because of the possibility of absorption of some amino acids.

4.7.5 Preparation of foodstu_ samples for the determination of sulphur amino acids

For the determination of sulphur amino acids in fodders and foodstuffs it is necessary to use exclusively oxidation methodologies (see part 3.2.4), for especially in the case of the samples of vegetable origin during the HCl hydrolysis it is not possible to avoid damages. Notes to methodology: For the weighing of the sample corresponding to 0.04 g of N use 100 ml of oxidation mixture. The foaming of the sample during evaporation will be suppressed by adding 0.2 ml of caprylic acid. After the evaporation of performic acid hydrolyse the samples directly in the flask, in which the sample was oxidised, under a reflux condenser. No inlet of nitrogen is necessary. The hydrolysis in closed ampoules is not recommended because there could be an explosion in the case of an imperfekt evaporation of the oxidation mixture.
The error at the determination of cysteic acid can be caused by the presence of a ferric ion, which will be eluted in the same place of chromatogram. However, it can be removed on a cation exchanger resin in the H+ form (4.6.2). With using cysteic acid it is also possible to wash the oxidation products of other sulphur amino acids appearing e.g. in a free form in some plants.

4.7.6 Preparation of foodstu_ and fodder samples for the determination of tryptophan

With regard to the presence of sugars it is necessary to use, while determining linked tryptophan in fodders and foodstuffs, exclusively the basic hydrolysis (part 3.2.9). The part of the sample which has not been dissolved during the hydrolysis will be removed by centrifuging. There can be absorbed a part of tryptophan on the precipitate - it is recommended to verify the information about 4 % of losses (7) by adding a well-known amount of tryptophan, possibly by an analysis of a non-centrifuged sample

4.8 References

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4.9 BIOGENIC AMINES

Biogenic amines arise mostly by means of decarboxylation of amino acids (see several examples in Chapter 4.2). They can be divided into two groups – monoamines and polyamines. Some of them are beneficial for health, others are harmful and serve as evidence of the beginning of foodstuff deterioration. The best known ones include tyramine (arising by decarboxylation of tyrosine), dopamine (3,4-dihydroxyphenylalanine, derived from histidine), serotonin (derived from 5-hydroxytryptophan), putrescine (from ornithine) cadaverine (from lysine) and a number of others. With regard to the fact that the firm INGOS would like to offer to its customers further use of amino acid analyser, without any demanding modifications, it has drawn the methodology of the identification of the most important biogenic amines. For this determination we offer all necessary chemicals, including standards designed for apparatus calibration.

4.9.1 Basic biogenic amines

Histamine is a biogenic amine, stimulates secretion of gastric juices, widens blood capillaries and reduces blood pressure. It increases the permeability of cellular membranes and provokes contraction of unstriped musculature of the digestive tract, uterus and bronchi. Histamine is considered to be a tissue hormone. Serotonine is an animal hormone, biologically active substance, so-called tissue hormone which arises by means of hydrolysing and subsequent decarboxylation of tryptophan. It is formed in many animal as well as vegetable tissues, and operates as neurotransmitter in the places of its origin. It has, for example, vasoconstrictive effects (it gets released from thrombocytes in the case of injury, see vasoconstriction), in intestina mucosa it supports intestinal peristalsis, in the central nervous system its secretion is connected with the states of attenuation (sleeping). Serotomine is a precursor of the hormone melatonine.
Tyrosamine is a decarboxylation product of tyrosine.
Dopamine is a transmitter of synapses in the central nervous system. A shortage of dopamine leads to the arising of parkinsonism (see catecholamine).
Putrescine is a toxic organic amine, which arises by means of microbial decomposition of proteins.
Cadaverine is an alkaline organic substance which arises during the enzyme decarboxylation lysine.

4.9.2 AAA500 treatment for determination of biogenicamines

For determination of biogenic amines it is not necessary to use a pre-column because amines are virtually eluted after amino acids. This means that first it is necessary to expel amino acids from the column and only then to divide amines. The pre-column serves only for the delaying of the elution of ammonia contained in elution solutions, and that is why it is not necessary.
1. Disconnect the pre-column from the apparatus and close it with a closure so that it will not get dry.
2. Connect the analytic column directly to the air release valve instead of the precolumn.
3. Empty the analytic column or use an empty one.

4. Fill the column (in an ordinary manner) with ion exchanger Poly 8 up to a height of 10 cm and tighten the upper closure on the column. The height of the ion exchanger column should move within a range from 5.5 to 10 cm. A higher height of the column will increase the division of some amines. In our experiences the best height of the column for ordinary operation is 10 cm.
5. Prepare buffers (elution solutions for the identification of biogenic amines), see preparation of el. solutions in next sections.
6. Preparation of the detection sensor of ninhydrin is identical with the preparation for amino acids.
7. Flow rates, reactor temperature and other parameters remain the same.

4.9.3 Method for determination of biogenic amines

Time Temperature Buffer Command Note

Time (min)	Column T(°C)	Buffer Nr.	AA Command	Duration (min)
0	76	1	Inject	3
3	76	NoChange	Zero	30
33	76	2	None	14
47	76	NoChange	None	25
72	76	NoChange	StartEquil	4
76	76	6	None	5
81	76	1	H2O	2
83	76	1	None	3
86	76	NoChange	Load	2
88	76	NoChange	NHD	9
97	76	NoChange	None	0

4.9.4 Composition of buffers for determination of bio-

genic amines

The dilution buffer is a common Na buffer (pH being 2.2), which is used for the dilution of standards and samples for the analysis of hydrolysis products. Its composition is identical and can be found in the technological screen AAA 500 pod F1.
buffer 1 2

	Č. 1	*Č. 2*
Kyselina citronová monohydrát (g)	1,05	14,00
Citronan sodný dihydrát (g)	21,00	-
Chlorid sodný (g)	5,00	-
Chlorid draselný (g)	-	171,50
Bromid draselný (g)	41,65	-
Hydroxid draselný, 50 % roztok (ml)	-	10,0
2-propanol (ml)	250,0	

Standards for this determination will be prepared either in molar concentration common for amino acids, which means 2.5 micromol for 1 ml, or in weight amounts in an exact

concentration of 50 mg/ml. The firm INGOS is preparing a standard in molar concentration, at customer's request we can produce a different concentration as well. With regard to high acquisition costs of solid (loose) amines we do not recommend the customers to prepare these standards by themselves. The standards are to be kept in a refrigerator at a temperature of +5°C. They are very stable, approximately as the standards of amino acids. The standards of our firm are stabilised.

4.9.5 Notes to the identification of biogenic amines

Preparation of buffers is not demanding (in terms of both pH and ion concentration). It is sufficient to weight on ordinary pre-weighting devices and to complete in the cylinder to the prescribed volume. With regard to a large buffer 1 consumption we recommend to replace the 1,000 ml bottle in the apparatus with a 2,000 ml bottle and to přepade directly 2,000 ml of buffer 1. Buffer 2 shows, from time to time, a deviation of the zero line from the base, because with regard to its ion concentration it is usually slightly coloured in yellow, which does not mean any defect, but somehow increases the zero line only. We do not recommend to identify tryptamine by using this method, with begard to its low response. It is possible to identify it reliably only in the case of a higher concentration in the samples. If you need to determine tryptamine, remember that its peak rises immediately after cadaverine, and our firm will prepare for you a solution in a higher concentration as a standard for this purpose. Also agmatine has a low response for common concentration, and that it is subject to the same rules as tryptamine. It rises from the proximity of spermine. If you do not need to identify spermine and spermidine, it is possible to shorten the entire determination approximately by 40 minutes. In this case we recommend you to contact our technicians who will solve these issues with you. Our firm will also provide you with assistance during the implementation of this program, by way of telephone, fax and e-mail, free of charge, or by means of a paid service directly at your workplace.

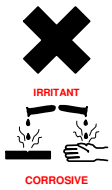
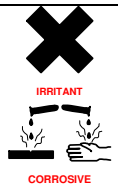
APPENDIX A

Chemicals for AAA500

Name	MW	Function	Purity, appearance	Safety	Other
Ninhydrin	178.14	Main component of detection agent	p.a.; light yellow crystals		
Hydrindantin dihydrate	358.31	Ninhydrin stabilizer	p.a.; white crystals		Poorly soluble in water
Citric acid monohydrate	210.14	Components of elution solution	p.a.	-	
Sodium citrate dihydrate	294.10			-	
Sodium chloride	58.44			-	
Sodium hydroxide	40.00				Absorbs air CO_2 and

Name	MW	Function	Purity, appearance	Safety	Other
Potassium hydroxide	56.10			 CORROSIVE	humidity
Boric acid	61.83			-	
Lithium citrate tetrahydrate	281.99			Li^+ - components toxic when therapeutic dose is exceeded	
Lithium chloride anhydrous	42.39				
Lithium hydroxide mohohydrate	41.96			 IRRITANT  CORROSIVE	Absorbs air CO_2
Potassium chloride	74.55			-	
Potassium bromide	119.01			-	
Sodium azide	65.02	Preservative of buffers		 POISON	
Sodium acetate trihydrate	136.08	Component of det. agent buffer			
Methylcellosolve (2-methoxy-ethanol)	76.10	Solvent of ninhydrin and hydrindantin	p.a.; stinking colourless liquid d=0.966[1]	 FLAMMABLE LIQUID  POISON	
Ethanol	46.07		p.a., d=0.789	 FLAMMABLE LIQUID	
2-propanol	60.10	Component of buffers for biogenic amines	p.a., d=0.785	 FLAMMABLE LIQUID	
Thiodiglycol	122.19	Methionine stabilizer	p.a., d=1.221	 IRRITANT	
4 mol/l acetate buffer pH5.5		Buffer of ninhydrin agent	Acrid colourless liquid	 IRRITANT	
Titanium chloride	154.23	Ninhydrin stabilizer	In hydrochloric acid	 IRRITANT	

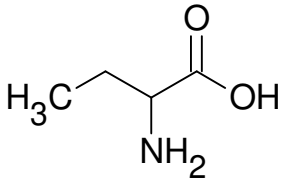
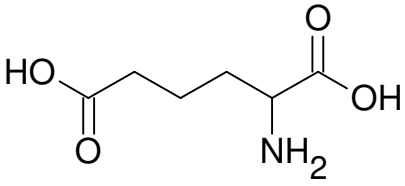
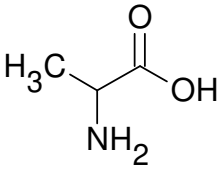
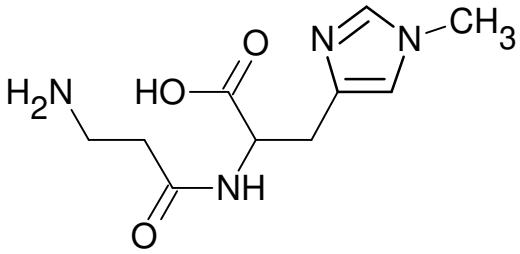
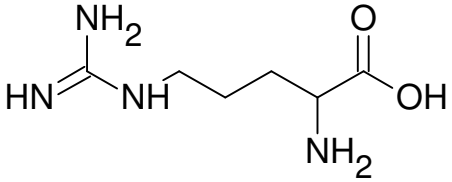
[1] d – specific gravity (g/cm^3) at 25°C.

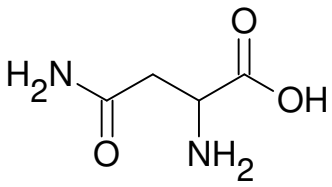
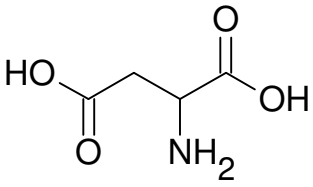
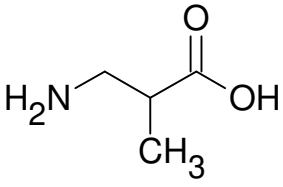
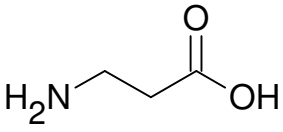
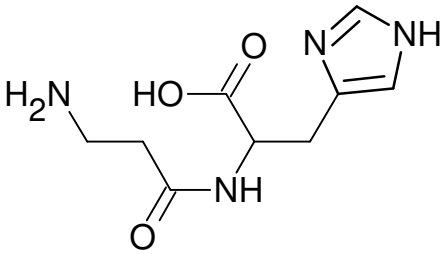
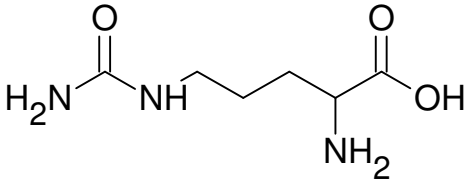
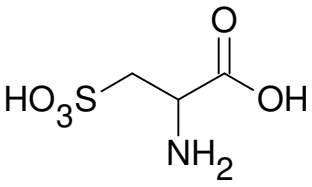
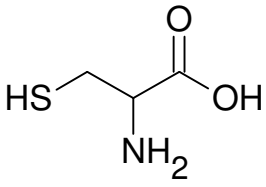
Name	MW	Function	Purity, appearance	Safety	Other
					
Ninhydrin agent		For detection of amino acids	Acrid, purple liquid		Stains skin
Hydrochloric acid, conc.	35.46	For adjustment of buffers, extraction and hydrolysis	p.a., d=1.096		Interferes with determination
Formic acid, conc.	46.03	For oxidative hydrolysis	p.a., d=1.22		
Hydrogen peroxide, 30%	34.01		p.a., d=1.11		
NaOH solution, 50%		For adjustment of buffers			Store in plastic bottle; it absorbs air CO_2
KOH solution, 50 %					
Na-/Li- diluting buffers pH 2.20		For diluting of solutions			Stire in cooled dark bottle
Na-/Li- elution buffers					
Mixed standard of amino acids (hydrolyzates, sulphurous, saline)			2.5 µmol/ml solution in diluting buffer		Store cooled
Mixed standard of amino acids (Asn+Gln)					Store frozen[2]
Water (demi or distilled)	18.02		Conductivity < 10 µS/cm		

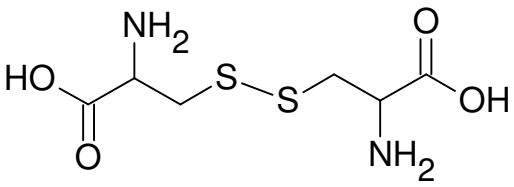
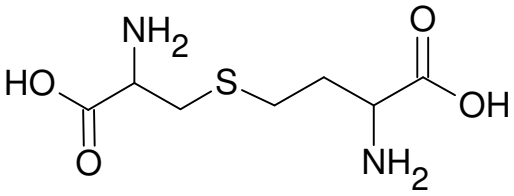
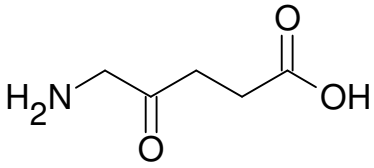
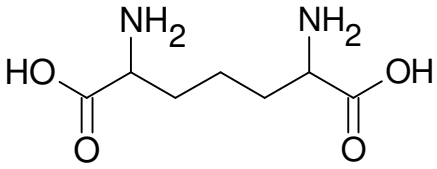
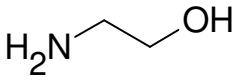
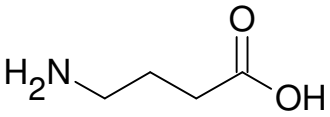
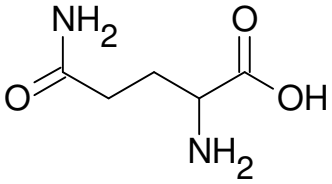
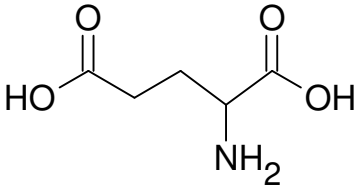
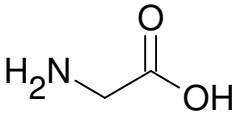
[2] Prepare 250 µmol/l working solution of mixed standard (physiological + Asn+Gln), divide by 1 ml into micro-tubes and keep frozen under – 20 °C (→ 2.5.2).

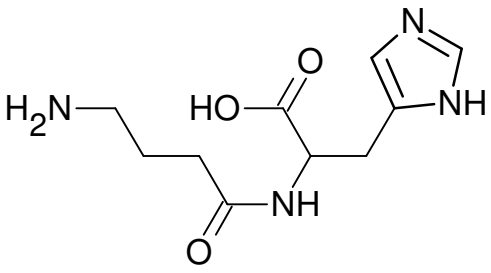
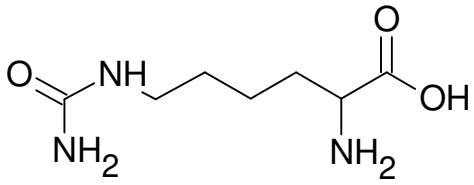
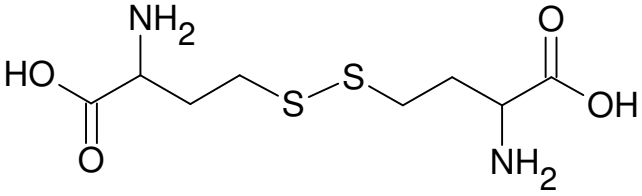
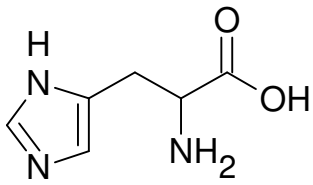
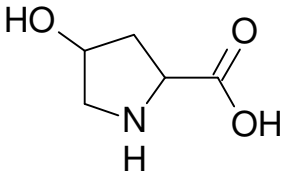
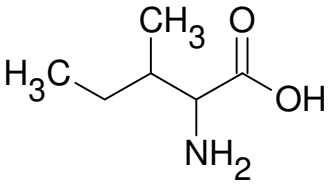
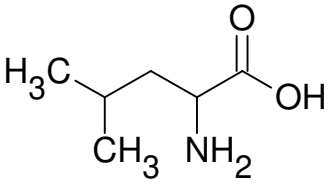
Name	MW	Function	Purity, appearance	Safety	Other
Nitrogen	28.01		>4.0		
Argon	39.95		>4.0		Heavier than air

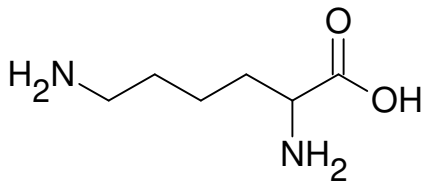
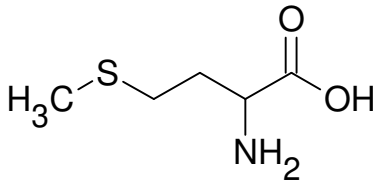
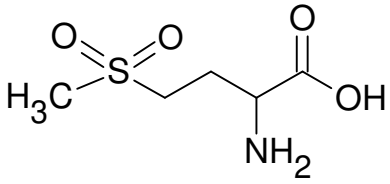
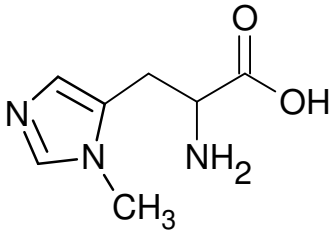
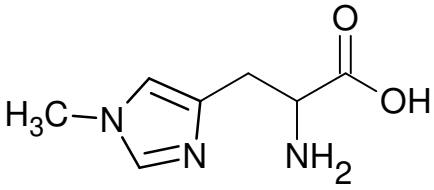
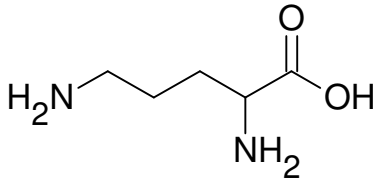
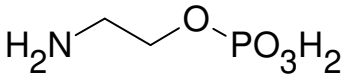
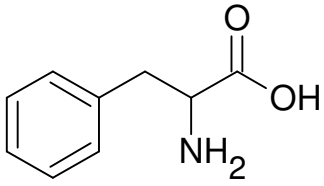
B: Ninhydrin-pozitive amino acides

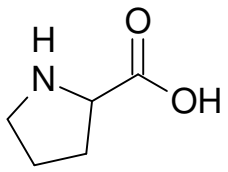
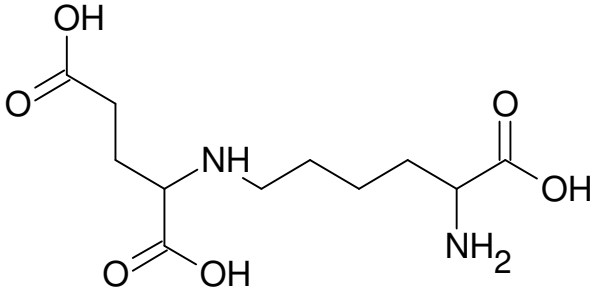
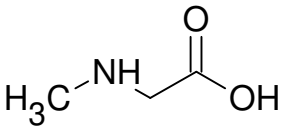
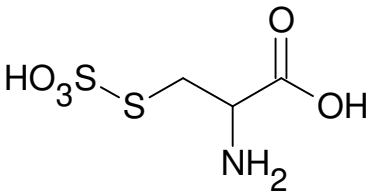
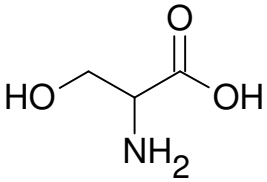
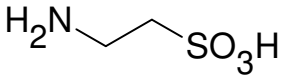
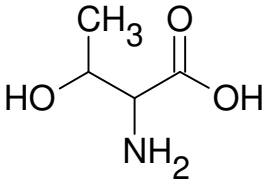
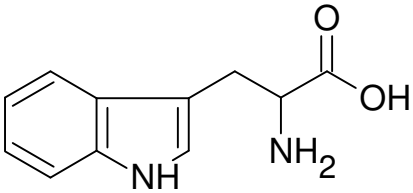
Abbrev.	*Amini acid name*	*MW*	*Structural formula*
AAB, Abu	α- amino-n-butyric acid	103,12	
AAD, Aad	α-Amino adipic acid	161,16	
Ala	Alanine	89,09	
Ans	Anserine (N-β-alanyl-3-methylhistidin)	240,26	
Arg	Arginine	174,2	

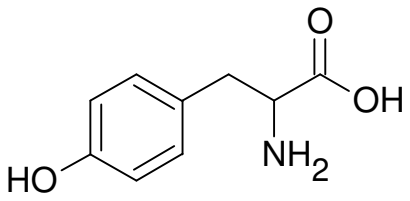
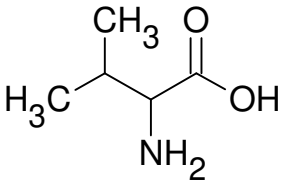
Asn	Asparagine	132,12	
Asp	Aspartic acid	133,1	
Baib, BAIBA	β-Amino butiric acid	103,12	
Bala	β-Alanine	89,09	
Car, Carn	Carnosin (N- β-alanyl-histidin)	226,23	
Cit	Citruline	175,19	
Cya, CysH, HCys	Cysteoic acid	169,16	
Cys, 1/2Cys	Cysteine	121,16	

Cys	Cystine	240,3	
Cyst, Cysta	Cystathionine (S-2-amino-2-karboxyethyl-homocystein)	208,24	
Dala, dAL	δ-Aminolevulová kyselina	131,11	
Dapa, Dpm	2,6-Diaminopimelic acid	190,2	
EA	Ethanolamine	61,08	
Gaba, gAbu	γ-Aminomáselná kyselina	103,12	
Gln	Glutamine	146,15	
Glu	Glutamic acid	147,13	
Gly	Glycine	75,07	

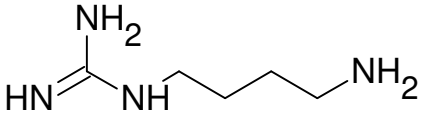
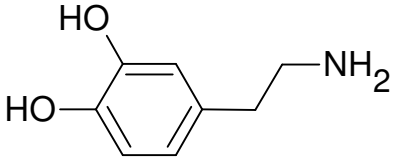
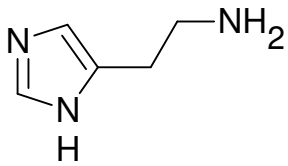
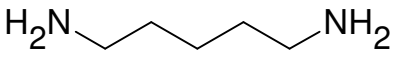
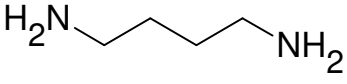
Hcar, HCarn	Homocarnosine (N-γ-aminobutyryl-histidin)	240,22	
HCit, Hcit	Homocitruline	189,20	
Hcy, Hcys	Homocystine	268,38	
His	Histidine	155,16	
Hyp, 4Hyp, OHPro	4-Hydroxyproline	131,13	
Ile	Isoleucine	131,18	
Leu	Leucine	131,18	

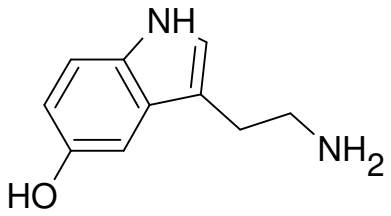
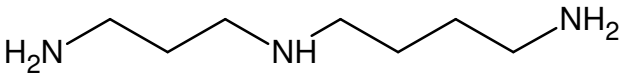
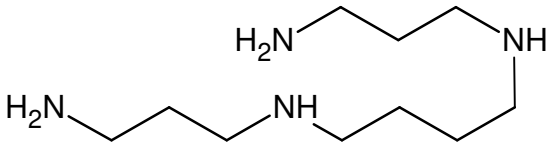
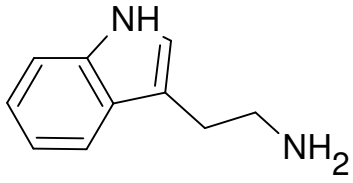
Lys	Lysine	146,19	
Met	Methionine	149,21	
MetS	Methionine sulfon	181,21	
1MHis, 1mHis	1-Methylhistidine	169,18	
3MHis, 3mHis	3-Methylhistidine	169,18	
Orn	Ornithine	132,16	
Pea	O-Fosfoethanolamine	141,06	
Phe	Fenylalanine	165,19	

Pro	Proline	115,13	
Sach, Sacch	Sacharopine (ε-N-L-glutar-2-yl)-L-lysine)	276,29	
Sar	Sarkosine	89,09	
SCys, Scys	S-sulfocysteine	201,22	
Ser	Serine	105,09	
Tau	Taurine	125,14	
Thr	Threonine	119,12	
Trp	Tryptofane	204,23	

Tyr	Tyrosine	181,19	
Val	Valine	117,15	

B2 Ninhyrin-pozitive biogenic amines

Název	***MW***	***Strukturní vzorec***
Agmatine (argamine)	130,19	
Dopamine	153,18	
Histamine	111,15	
Kadaverine	102,20	
Putrescine	88,15	

Název	*MW*	*Strukturní vzorec*
Serotonine (5-hydroxytryptamine)	176,21	
Spermidin	145,20	
Spermine	202,34	
Tryptamine	160,21	
Tyramin (tyrosamin)	137,18