ANALYSIS OF AMINO ACIDS-CONTAINING DRUGS

Manual developed by Piotr Cyganowski, MSc

Attention!

During the exercises 2 and 3 students will be handling aggressive chemical reagents, such as hot, concentrated sulfuric acid, and sodium hydroxide. For that reason every participant must possess personal protection equipment:

1. Laboratory gloves (acid-resistant; nitrile or other with certificate).
2. Eye-protection.
3. Apron with long sleeves.

Students who will not possess mentioned above equipment **will not be able to attend the practical.**

Issues

1. Chemical formulas of basic amino acids
2. Functional groups in organic chemistry.
3. Distillation using water steam.
4. Lambert-Beer Law.
5. Calculating concentrations and preparing dilutions

Introduction

The human body is a very complex system in which many actions, associated with life functions take place. They allow processing a food into energy and provide for the body essential micronutrients. Among them, biologically important products of protein metabolism (sulfur amino acids) have taken special place. They play an important role in the processes associated with diseases of the cardiovascular system, arthritis and AIDS. Amino acids such as methionine, are essential to the functioning of the human body – they are responsible for creating proteins that are the building blocks of muscle mass, as well as for the synthesis of compounds enabling the transport of fatty acids into body cells and convert them into energy.

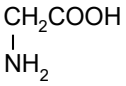
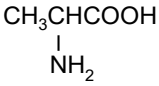
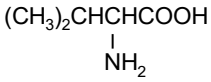
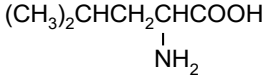
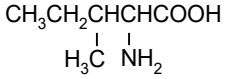
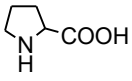
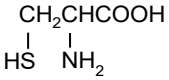
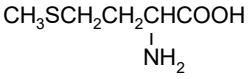
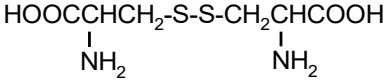
There are two types of amino acids: exogenous, taken up with food and endogenic, that organism can synthesize on its own. These compounds are essential in treatment

therapies of muscle, ligaments, and tendons damages as well as maintaining in good condition hair, nails and skin.

The cause of amino acids deficit may possess multiple sources, including low-protein diet, stress, infections or advanced age.

Pharmacology knows over 300 different amino acids. From definition each contain amino and carboxyl functionalities. Moreover species that additionally contain phosphone, sulfonic as well as thiol groups are also recognized. Table 1 gives examples of the most popular amino acids' chemical formulas.

Table 1. Chemical formulas of selected amino acids

	Name	Formula	3-digit code
Aliphatic amino acids	glycine		*Gly*
	alanine		*Ala*
	valine		*Val*
	leucine		*Leu*
	isoleucine		*Ile*
	proline		*Pro*
Sulfur amino acids	cysteine		*Cys*
	methionine		*Met*
	cystatin		*(Cys)₂*

The functional groups (see Table 1) may be a base of potential analyses, that could be helpful in determining witch type of amino acid is placed in a drug. On the other hand, knowing a chemical formula of a specific compound quantitative analysis is also possible. For that reason the purpose of the present exercises is to proceed two parallel analyses that will allow to determine presence of amino acids and sulfur amino acids in different pharmaceutical formulations.

Exercise 2

Determination of the presence of amino groups [1]

Nitrogen content determination has a long history. The element Nitrogen is a primary growth ingredient found in numerous chemical compounds such as fertilizers, foods, oils, water/wastewater, amino acids and much more.

Nitrogen occurs in many forms including ammonia, organics, nitrate and nitrites; therefore, selection of a suitable analysis method depends on a specific moiety that will be handled.

In the present, the most popular method for determination of nitrogen content in organic and inorganic samples is Kjeldahl's procedure, that was first announced in 1883 and since then was significantly improved and automated.

Due to its versatility, the method covers a wide range of samples. It is especially useful in analysis of food and pharmaceuticals because of its ability to detect organic nitrogen as well as, both, ammonia (NH_3) and ammonium (NH_4^+) moieties.

The Kjeldahl's procedure can be resolved in the following steps:

1. **Sample digestion**

The operation allows to convert organic nitrogen to ammonia. This requires boiling the sample with concentrated sulfuric acid in the presence potassium sulfate and copper catalyst (most usually its sulfate). The reaction is relatively slow; therefore its rate is usually being increased by applying the digestion temperature of 395 ˚C.

2. Sample distillation

The digested drug should be distilled in presence of a concentrated NaOH. At a pH of 9.5 ammonia gas is being created and, then, entrapped in absorbing solution where it converts back to ammonium.

3. Titration

Once the sample is distilled, nitrogen content may be determined using classical titration procedure.

Execution of the analysis

The analysis will be performed using automated distillation unit manufactured by Büchi Co. Due to the nature of the whole procedure, the students will be performing only 2nd and 3rd step (see above) on their own. The 1st step will be presented by a tutor.

1) Indicator preparation

a) Prepare a 1 dm^3 of 2 % solution of H_3BO_3.

b) Add 8 cm^3 of methyl red in ethanol and 10 cm^3 of bromocresol green in ethanol, respectively. As the result you will receive gray-green solution.

c) Using a solution of 0.1M NaOH carefully adjust the color of the H_3BO_3 solution to gray.

2) Samples preparation

You will receive a set of distillation tubes that will contain digested in sulfuric acid samples.

a) Add 50 cm^3 of distilled water to each of them. Leave the samples to cool down.

b) Prepare a set of Erlenmeyer's flasks. To each of them add 60 cm^3 of the indicator prepared in 1)

3) Distillation

Figure 1 displays the automated distillation unit.

a) Insert the Distillation Tube **(1)** prepared in 2) to a slot. Make sure that it is fitted correctly.

b) Place the Erlenmeyer's flask prepared in 2) under sample collector **(5)**

c) Open a cooling water tap.

d) Turn the power switch **(2)** on. Wait a moment, while a steam generator heats up.

e) Using pump **(3)** add NaOH, untill the solution in Distillation Tube **(1)** turns to gray. Make sure that the volume of the sample is not exceeding 150 cm^3.

f) Turn the steam generator **(4)** on. Distill the sample for exactly 5 min. Add more NaOH **(3)** if necessary.

g) Distill distilled water for 5 min. in order to clean the system (repeat the procedure before every distillation of a sample).

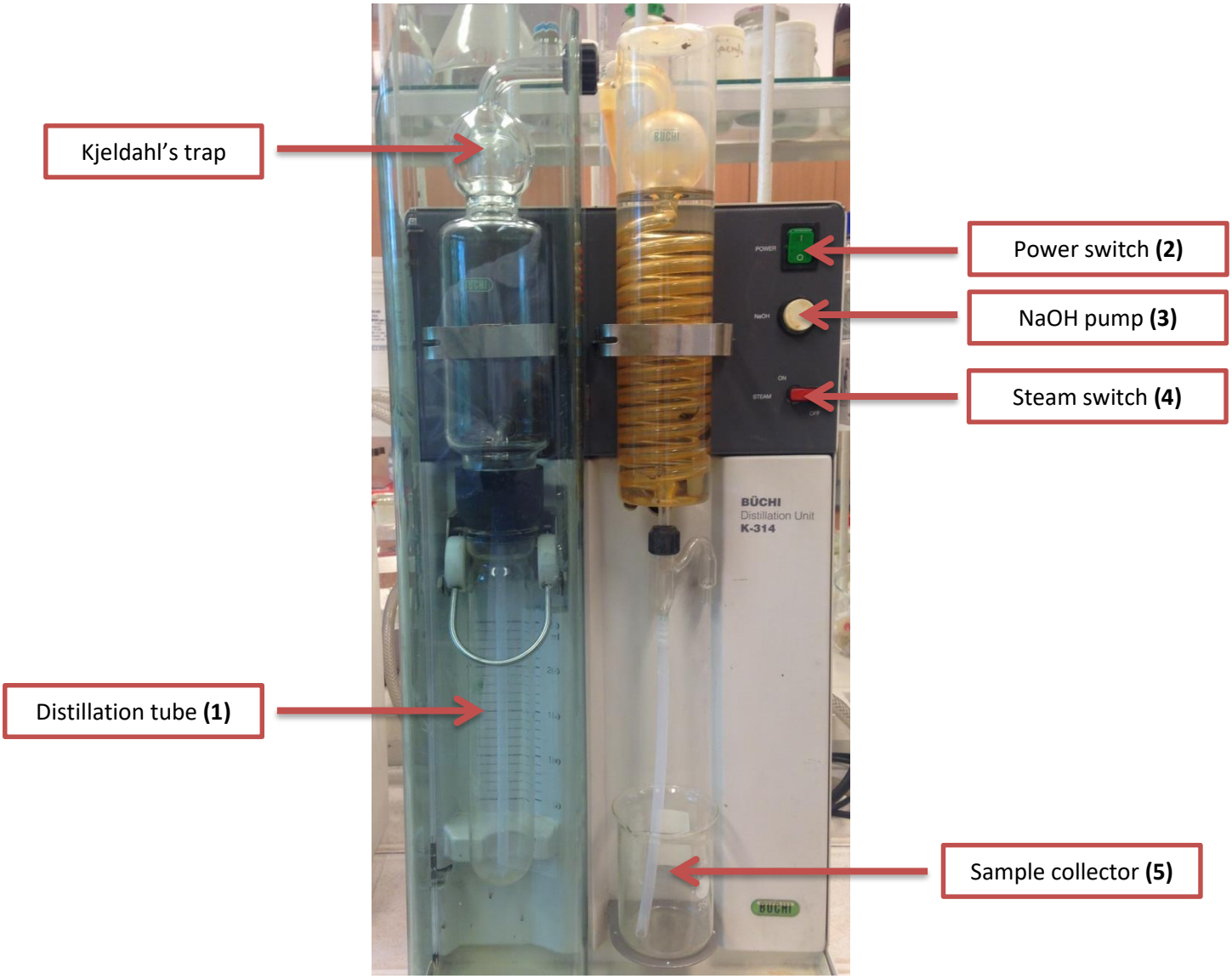


Figure 1. Automated Büchi distillation unit

4) Titration

a) Titrate the collected solution **(5)** using 0.1M HCl until sample turns gray. Make a suitable dilutions if necessary

b) Calculate total nitrogen content using the equation:

$$N\ [mmol\ N/g] = \frac{V_{HCl} \cdot C_{HCl}}{m_s}$$

Where:

$V_{HCl} - volume\ of\ HCl\ used\ for\ titration\ [ml]$.

$C_{HCl} - concentration\ of\ HCl\ [mol \cdot dm^{-3}]$.

$m_s - mass\ of\ the\ sample\ [g]$.

Dissolve the selected samples in concentrated H_2SO_4 at room temperature. Then, repeat the steps **2)-4)**. Compare the results.

Exercise 3

Free sulfhydryl groups in amino acids [2]

In 1959 Ellman introduced 5,5'-dithio-bis-(2-nitrobenzoic acid), also known as DTNB, as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. The analysis relies on the ability of DTNB to reduce in the presence of thiols (Figure 2).

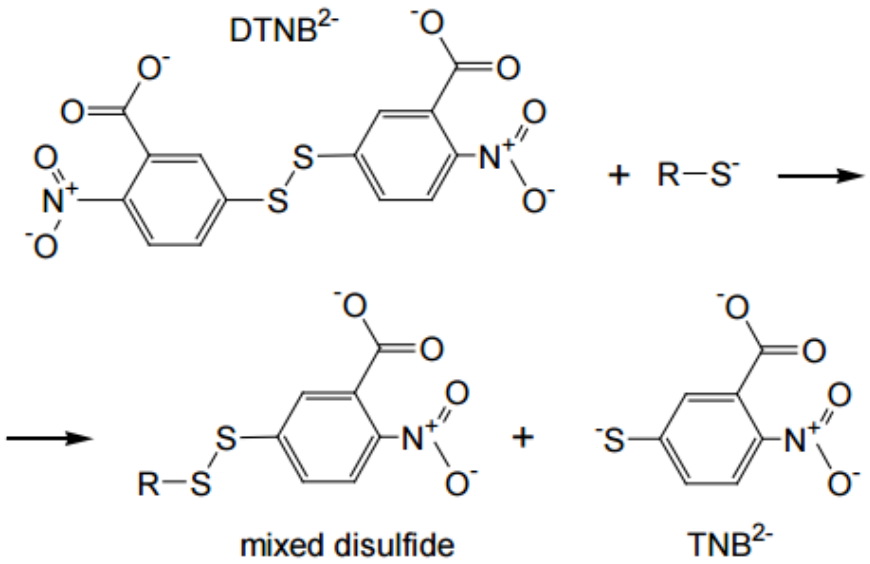


Figure 2. Reduction of the Ellman's reagent [2]

A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls (Figure 2). Moreover, application of the Ellman's reagent allows for selective determining content of –SH groups at neutral pH.

DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (see Figure 2). The target of DTNB in this reaction is the conjugate base R—S^- of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several factors:

1. the reaction pH,
2. the pKa of the sulfhydryl,
3. steric and electrostatic effects.

TNB is the "colored" species produced in this reaction and has a high molar extinction coefficient in the visible range. The molar extinction coefficient of TNB in 0.1 M phosphate and 1 mM EDTA was reported to be 14,150 $M^{-1}cm^{-1}$ at 412 nm and pH 8.0. The extinction of TNB is not affected by changes in pH between 7.6 and 8.6. However, the extinction of TNB is different in other solvents.

Sulfhydryl groups may be estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine. Alternatively, sulfhydryl groups may be quantitated by reference to the extinction coefficient of TNB.

Execution of the analysis

1) Regents preparation

a) Prepare a 50 cm^3 of a reaction buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA

b) Prepare the Ellman's Reagent solution: dissolve 4 mg of Ellman's Reagent in 1 mL of the reaction buffer.

2) Samples preparation

a) Prepare a set of test tubes containing 50 µL of Ellman's Reagent solution and 2.5 mL of the reaction buffer.

b) Dissolve the amino acids containing drugs in 100 cm^3 volumetric flask.

c) Make a hundredfold dilution of the solutions prepared in b).

d) Add a 250 µL of each unknown sample prepared in c) to the test tubes prepared in a). As a blank add 250 µL of reaction buffer to a separate test tube.

e) Mix and incubate the samples for approximately 30 min.

3) Absorbance measurement

a) Set a spectrophotometer to 412 nm. Zero the instrument on the blank.
b) Measure the absorbance of each sample.
c) Calculate the concentration of free sulfhydryls from the molar extinction coefficient of TNB (14,150 $M^{-1}cm^{-1}$, see the example below).

Example of calculations:
A 250 µL sample of the unknown mixed with 2.5 mL of Reaction Buffer and 50 µL of Ellman's Reagent solution gave an absorbance of 0.879 (after subtracting the blank) using a 1cm spectrophotometric cuvette. Calculate the sulfhydryl concentration in mM of the unknown. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB in this buffer system at 412nm is 14,150. Molar absorptivity, E, is defined as follows:

$$E = \frac{A}{bc}$$

Where:
A - absorbance,
b - path length in centimeters,
c - concentration in moles/liter (=M)

Solving for concentration gives the following formula:

$$c = \frac{A}{bE} = \frac{0.879}{1 \cdot 14{,}150} = 6{,}21 \cdot 10^{-5}$$

In the present example, A = 0.879; b = 1 cm; E = 14,150$M^{-1}cm^{-1}$; therefore c=$6{,}21 \cdot 10^{-5}$
This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is:
2.50 mL of Reaction Buffer
+ 0.25 mL of Unknown Sample

+ 0.05 mL of Ellman's Reagent Solution

2.80 mL of solution

If the concentration of the assay solution is 6.21 x 10^{-5} M, then 2.80mL of that solution contains 1.74·10^{-5} M

*That value contributes to the assay solution where sulfhydryl groups were contributed by the original 0.25mL sample. Therefore, the concentration of free sulfhydryl in the original unknown sample should be calculated using dilutions made in **2).***

Creating a report

The report should contain:

1. Short description of the proceeded work
2. Examples of calculations
3. All of the determined concentrations
4. Statement, which drugs contained **amino acids**, **sulfur amino acids** or **neither**

[1] J. Leonard, B. Lygo, G. Procter, Advanced Practical Organic Chemistry, Second Edition, CRC Press, Cheltenham, 2001
[2] Product's instruction No. 22582: Ellman's Reagent, ThermoScientific, Rockford, IL, 2015