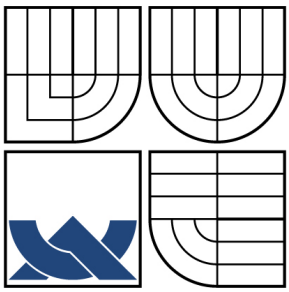

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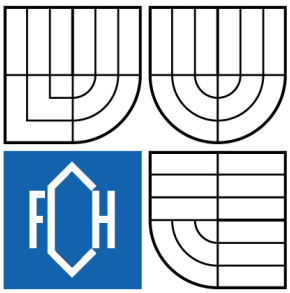

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

STABILITY OF CROSS-LINKED COLLAGEN

STABILITA SÍŤOVANÉHO ALFA-KOLAGENU

BAKALÁŘSKÁ PRÁCE
BACHELOR'S THESIS

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3) Characterization of cross-linked collagen films via spectrophotometric method
4) Determining the amount of crss-linked agent after purification of collagen samples
5) Conclusion

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Bachelor's thesis is necessary to deliver to a secreatry of institute in three copies and in an electronic way to a head of bachelor's thesis. This assignment is enclosure of bachelor's thesis.

Gabriela Součková	prof. RNDr. Josef Jančář, CSc.	
student	Leader	Head of institute

In Brno, 1.9.2006

doc. Ing. Jaromír Havlica, CSc.
Dean

ABSTRACT

Theoretical part of this bachelor thesis is based on general information about collagen, most common fibrous protein in body, collagen cross-linking; leading to its better mechanical and chemical properties; and widely used crosslinking agents, mainly based on 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC). Furthermore it deals with several methods of quantifying EDC in aqueous solutions.

Experimental part is concerned with the determination of EDC remained in water used for washing samples in order to purify them after the cross-linking. The goal is to find out if used washing method is adequate enough for certain application or if it needs some adjusting. Even if EDC is practically non-toxic, it provides side effects on colour of the collagenous material after the sterilization by irradiation. Since it has not been found in the literature what specific amount of EDC can stay in a sample or if it is necessary to be completely washed out. According the state-of-the-art, it is difficult to assessed if the washing method is sufficient or not. Moreover, there are also other side products of cross-linking collagen in the final collageneous products that have to be taken into account. However, the aim of this bachelor thesis is to evaluate washing EDC from the collagenous sample only.

As for cross-linking method, three concentrations of EDC followed by washing samples for different periods of time were used. Following this method, 48 samples were prepared and three times measured using UV-VIS colorimetric assay.

As a result it was found out that for commonly used concentration of cross-linking agent (50 $mmol.l^{-1}$ of EDC) it is sufficient to wash samples for 30 min in solution of disodium phospnonate (Na_2HPO_3) followed by washing with water 4 times for 15 min with changing the water at each step. After these steps, the concentration of EDC in washing water was reduced up to 1 $\mu mol.l^{-1}$. For lower concentrations of cross-linking EDC solution it is sufficient even shorter washing time.

Considering the preliminary results of this bachelor thesis, further investigation dealing with this topic should follow.

KEYWORDS

Collagen, cross-linking, EDC, colorimetric assay, UV-VIS.

ABSTRAKT

Teoretická část této bakalářské práce pojednává o obecných vlastnostech kolagenu, který je nejčastěji rozšířeným vláknitým proteinem v těle, o síťování kolagenu, které vede ke zlepšení mechanických i chemických vlastností, a o široce využívaných síťovacích činidlech, především 1-ethyl-3-(3-dimethylaminopropyl) karbodiimidu (EDC). Dále se zabývá několika metodami stanovení EDC ve vodném roztoku.

Experimentální část je zaměřena na praktické stanovení množství EDC, které zůstalo ve vodě použité k promývání vzorků po síťování. Cílem bylo zjistit, jestli současně používaná metoda promývání je dostatečná pro jisté aplikace nebo je potřeba ji upravit. I přesto, že sloučenina EDC je prakticky netoxická, může způsobovat zbarvení kolagenních materiálů po sterilizaci ozařováním. Vzhledem k tomu, že nebylo zjištěno jaké množství EDC může ve vzorku zůstat nebo jestli je nezbytné vymýt veškeré EDC, je obtížné rozhodnout jestli je promývací metoda dostatečná nebo není. Navíc je potřeba brát do úvahy i ostatní vedlejší produkty síťování kolagenu. Nicméně cílem této práce bylo pouze zhodnotit účinnost vymývání EDC z kolagenních vzorků.

Pro síťování byly použity tři různé koncentrace EDC a následně byly vzorky vymývány po různě dlouhou dobu. Takto bylo získáno 48 vodných vzorků, které byly následně třikrát spektrofotometricky stanoveny.

Výsledně bylo zjištěno, že pro běžně používanou koncentraci síťovacího činidla (50 $mmol.l^{-1}$ EDC) postačuje promývat vzorky po dobu 30 minut v roztoku hydrogenfosforečnanu sodného (Na_2HPO_3) a následně promývat ve vodě čtyřikrát po dobu 15 minut s výměnou vody při každém kroku. Po těchto krocích klesla koncentrace EDC v promývací vodě pod 1 $\mu mol \cdot l^{-1}$, pro nižší koncentrace síťovacího roztoku byla dostatečná i kratší doba promývání.

Výsledky této bakalářské práce jsou pouze předběžné, a proto by mělo následovat další zkoumání této problematiky.

KLÍČOVÁ SLOVA

Kolagen, síťování kolagenu, EDC, kolorimetrické stanovení, UV-VIS.

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Head of Bachelor thesis: prof. RNDr. Josef Jančář, CSc.

DECLARATION

I declare that bachelor thesis has been worked out by myself and that all quotations from the used literary sources are accurate and complete. The content of the bachelor thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

……
Student´s signature

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1. INTRODUCTION

Collagen is unique fibrous protein present in every living cell in human body. It is almost overwhelming how many function can one protein with variety of possible structures have. But mostly collagen serves as mechanical support both in cells and tissues. This is also reason why there is no known allergy to collagen and therefore it is widely used in biomaterials engineering. For medicine purposes it can be used as scaffold for living cells when designing artificial artery, as films for treating minor injuries and also as gels and haemostatics. The research on preparing such materials is extensive ever since Weinberg and Bell succeeded in preparing a blood vessel using collagen[1].

The biggest advantage of collagen is its ability to form firmed fibres which are intermolecularly cross-linked resulting in a network. Collagen is cross-linked naturally but it is also possible to cross-link it by using different cross-linking agents both chemical and physical. Chemical methods often use bi-functional chemicals (aldehydes, epoxides, carbodiimides, etc.). Physical methods use mainly drying or irradiating. However, most of these cross-linking agents are toxic and therefore material cross-linked by them can not be used in medicine. One, practically non-toxic cross-linking agent, is 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC) often combined with *N*-hydroxysuccineimide (NHS).

Based on theoretical part the aim of this bachelor thesis is to investigate washing of EDC from sample after cross-linking. For the experimental part, method developed by Wilchek[2] in 1981 was selected. It is colorimetric assay of carbodiimides, where EDC in solution of pyridine and barbituric acid forms a purple red colour complex, which can be determined by means of UV-VIS spectrophotometer. Experimental part is engaged in evaluation of currently used washing technique. In the conclusion, next steps improving washing of EDC from cross-linked samples are suggested.

2. THEORETICAL PART

2.1. Collagen

2.1.1. Collagen in general

Collagen occurs in all multicellular animals and is the most abundant protein of vertebrates. It is an extracellular protein that is organized into insoluble fibres of great tensile strength and it is major stress-bearing component of connective tissues such as bone, teeth, cartilage, tendon, ligament, and fibrous matrices of skin and blood vessels. Collagen abounds virtually in every tissue[3].

Mammals have at least 20 distinct types of collagen that occurs in different tissues of same individual. The most prominent of these are listed in Table 1 below.

Tab 1 *The most abundant types of collagen*

Type	Chain composition	Distribution
I	$[\alpha1(I)]_2\alpha2(I)$	Skin, bone, tendon, blood vessels, cornea
II	$[\alpha1(II)]_3$	Cartilage, intervertebral disk
III	$[\alpha1(III)]_3$	Blood vessels, fetal skin

2.1.2. Collagen structure

Collagen has distinctive amino acid composition. Nearly one third of its residues are Glycin (Gly), another 15-30% of them are prolin (Pro) and 4-hydroxyprolyl (Hyp). Hyp confers stability on collagen, probably through intramolecular hydrogen bonds that involve bridging water molecules. The amino acid sequence of bovine collagen α1(I), which is similar to that of other collagens, consists of monotonously repeating triplets of sequence Gly-X-Y over a continuous 1011-residue stretched of its 1042 residue polypeptide chain (Fig. 1). Here X is often Pro and Y is often Hyp[3].

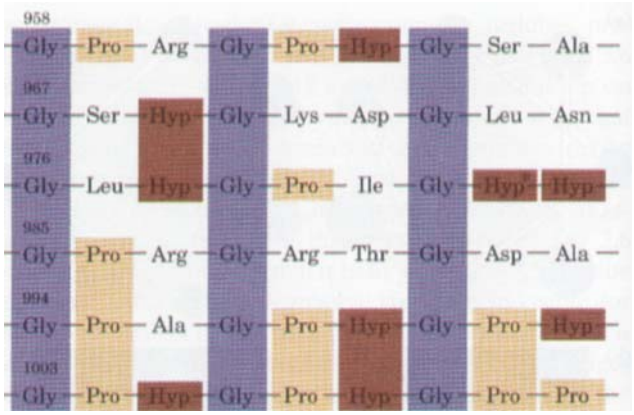

Fig 1 *The amino acid sequence at the C-terminal and of triple helical region of bovine α1(I) collagen chain*

The high Gly, Pro, and Hyp content of collagen suggests that its polypeptide backbone conformation resembles those of the polyglycine II and polyproline II helices. Fig. 2 shows how the left-handed polypeptide helices are twisted together to form a right-handed superhelical structure.

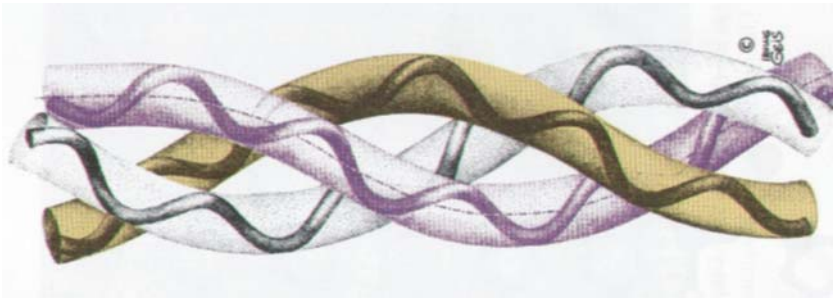

Fig 2 *The triple helix of collagen*

Collagen is well-packed and rigid. Triple helical structure is responsible for its characteristic tensile strength. As with the twisted fibres of a rope, the extended and twisted polypeptide chains of collagen convert a longitudinal tensile force to more easily supported lateral compression forces on the almost incompressible triple helix[3].

2.1.3. Collagen organized into fibrils

Types I, II, III, V and XI collagens form fibrils (Fig 3) that are mostly, if not entirely, composed of several different types of collagens. These fibrils have a periodicity of 680 Å and diameter of 100 to 2000 Å depending on the types of collagen they contain and the origin of tissue. The driving force for assembly of collagen molecules into fibrils is apparently provided by the added hydrophobic interactions within fibrils in a manner analogous to the packing of secondary structural elements to form a globular protein[3].

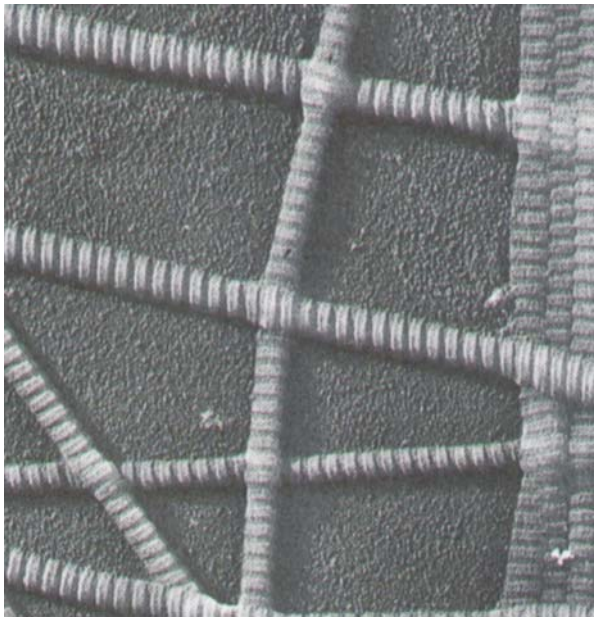

Fig 3 *Electron micrograph of collagen fibrils from skin*

2.1.4. Naturally occurred cross-linking

Insolubility of collagen in solvents that disrupt hydrogen bonding and ionic interactions is explained by the observation that it is both intramoleculary and intermoleculary covalently cross-linked. The cross-links are probably formed from Lys and His side chains in reactions such as in Fig 4.

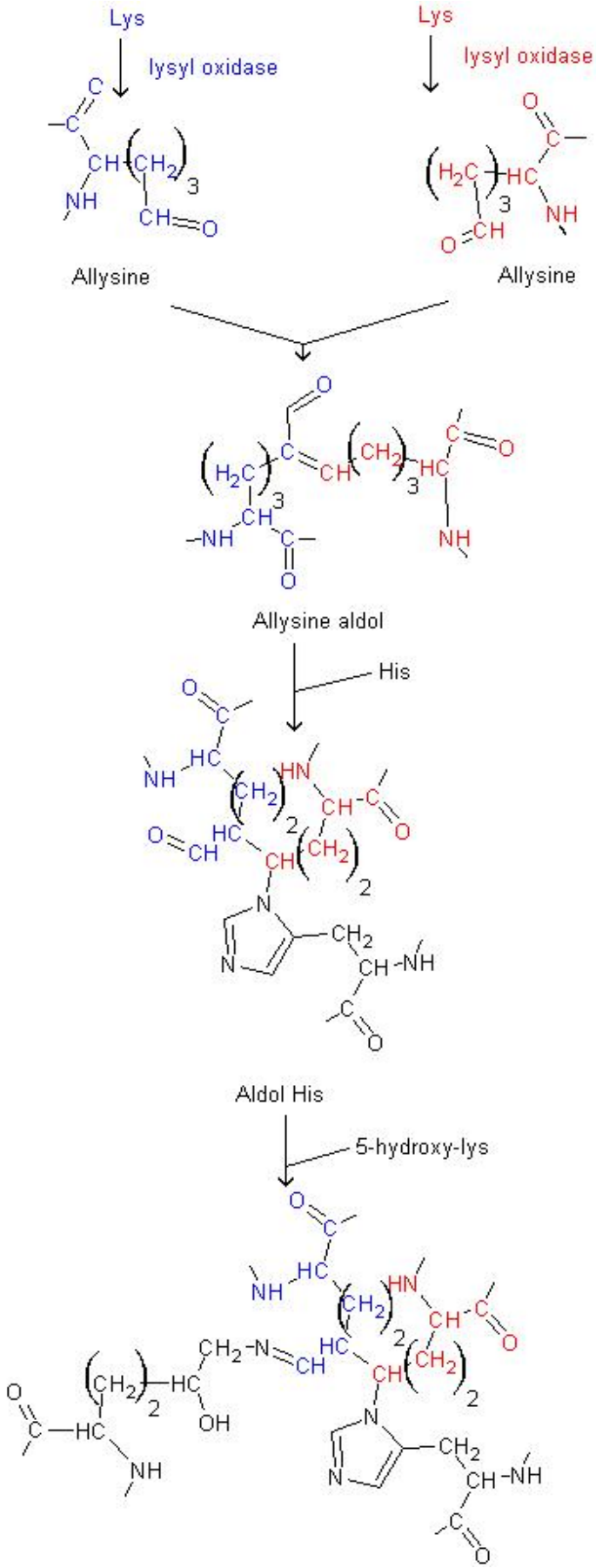

Fig 4 *A biosynthetic pathways for cross-linking Lys, Hyl and His side chains in collagen*

The degree of cross-linking of the collagen from a particular tissue increases with its age thus individual molecules of collagen and tropocollagen can only be extracted from very young animals.

The collagen fibrils in various tissues are organized in ways reflecting the function of the tissue (Tab 2). Tendon, skin and cartilage must support stress in predominantly one, two, and three dimensions and their component collagen fibrils are arranged accordingly[3].

Tab 2 *The arrangement of collagen fibrils in various tissues*

Tissue	**Arrangement**
Tendon	Parallel bundles
Skin	Sheets of fibrils layered at many angles
Cartilage	No distinct arrangement
Cornea	Planar sheets stacked crossways so as to minimize light scattering

2.2. Cross-linking collagen

The construction biomaterials using natural products have been performed by many researches world wide. Based on a fact that biological tissues such as skin or tendon are mainly composed of collagen, many researchers have attempted to prepare a collagen material to construct an extracellular matrix which could be used in several applications[4].

Among those applications belong scaffolds for corneal tissue engineering[5] where collagen scaffold for the cells acts as mechanical support and creates a microenviroment to which the cells can respond. The reason why collagen can be used in vascular engineering is that artery can be regarded as a fibre-reinforced structure composed of a protein fibre network and cells, which determine the particular mechanical properties of that vessel[6].

The critical aspect in using collagen gel is that its mechanical strength is too small and easily deforms its triple-helix structure into random coil structure when heated. The low mechanical strength and easy deformability make collagen shrink easily due to external stimuli. These aspects make it difficult to use collagen as an extracellular matrix[4].

By cross-linking collagen triple-helices, it is possible to maintain its mechanical strength and suppress any deformation caused by external stimuli. However, it is also important to consider biological respond and toxicity.

There are several options how to cross-link collagen both chemical and physical. Chemical methods often uses bi-functional chemicals (aldehydes, epoxides, carbodiimides, etc.). Physical methods use mainly drying or irradiating.

Water-soluble carbodiimides, especially 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), are often used as zero-length cross-linkers in organic chemistry or peptide synthesis or for immobilization of different biological molecules on solid supports. In a typical approach, a carbodiimide molecule reacts with carboxylic group of a molecule, thus giving a highly reactive *O*-acylurea, which then reacts with nucleophiles such as primary amines to form an amide (peptide) bond (Fig 5). The reaction is simple and can be performed in aqueous solution at room temperature, which is especially important for manipulations with biological molecules. However, carbodiimides are very unstable. They can easily react with many different components. The lifetime of carbodiimides can be increased considerably in the presence of *N*-hydroxysuccinimides (NHS) [7].

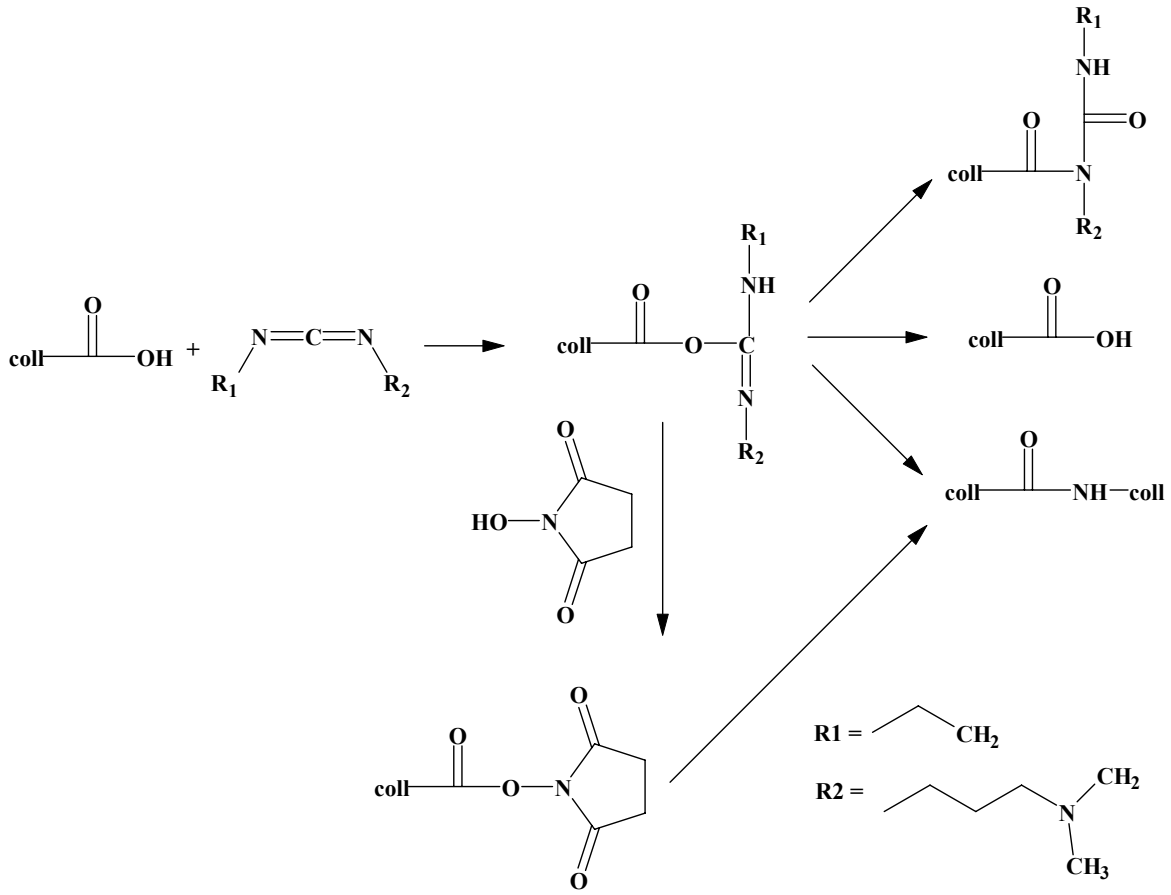

Fig 5 *Scheme explains how NHS influences the cross-linking reaction between collagen and EDC*

2.3. Carbodiimides assay

Even though EDC/NHS cross-linking agent is practically non-toxic it is necessary to know amount of EDC washed from sample after cross-linking. Since EDC serves only as a catalyst it can be presumed that all EDC can be washed from sample after cross-linking. Several methods how to measure amounts of EDC in aqueous solutions were investigated and possible ways of determination were narrowed down to following five methods.

2.3.1 Method by Jacobson and Fairman[8]

Method is based on the formation of chromophore involving carbodiimide, pyridine:HCl, and diamine (1,2-diaminoethane for water soluble). The maximum absorbance in the visible spectrum for the chromophore was 400 nm for carbodiimides in aqueous assay. The optimum pH for assay was 7.0 and the absorbance of chromophore was linearly dependent with concentration of carbodiimide. Concentrations as low as 50 μM were quantifited. The assay was specific for the carbodiimide and not for intermediates or products formed when carbodiimide was used in coupling reactions to form amide bonds.

2.3.2 Method by Wilchek[2]

A highly sensitive colorimetric method has been developed for determination of carbodiimides in solution. The method is based on the „König“reaction of carbodiimides with pyridine and barbituric or dimethylbarbituric acid to yield a purple red product with λ_{max} at 595 nm and molar extinction coefficient ε equal to150 000. Due to sensitivity of the method,

trace amounts of carbodiimide (1μM solutions) can be quantified. The method is fast, reliable, and free of side reactions. Precautions have to be taken when carbodiimides are used in pyridine solutions are also discussed.

2.3.3. Method by Williams, Hill and Ibrahim[9]

Carbodiimides maybe assayed by reacting with aqueous anilinium chloride and reading the absorbance at 230 nm after quenching in 1 M HCl. The basis of the assay is the formation of *N*-phenylguanidine which has a very large ultraviolet absorption; the limit of detection is 0.2 μM. A second method of assay involves reaction of the carbodiimide with acetic acid to yield anhydride which is assayed by hydroxamic acid test based on colour development at 540 nm with ferric chloride. The limit of detection in later test is 20 μM and the methods are free from interference from proteins or nucleic acid.

2.3.4. Method by Wrobel, Schinkinger and Mirsky[7]

Carbodiimides posses considerable absorbance in the ultraviolet region; the extinction coefficient of EDC in water is $\varepsilon_{214} = 6.3.10^3$ L. $mol^{-1}.cm^{-1}$. It is very simple method for testing possible side reactions of carbodiimides. This technique was used to study effects of pH, different buffers, and other components typically present in biological samples on EDC stability. The results can be used to optimize carbodiimide-mediated reactions of peptide bond formation in organic chemistry or peptide synthesis or during immobilization or cross-linking of biological molecules

2.3.5 Method by Gilles, Hudson and Borders[10]

A dimethylbarbituric acid reagent has been used to follow the kinetics of loss of two water soluble carbodiimides, EDC and the structurally related 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide (EAC), in aqueous solution as a function of pH and added chemical reagents. In 50 mM 2-(*N*-morpholino)ethanesulfonic acid at 25°C, EDC has $\tau_{1/2}$ values of 37, 20 and 3,9 h at pH 7.0, 6.0, 5.0, respectively, while the corresponding values for EAC are 12, 2.9 and 0.32 h. Iodine, bromide, or chloride, at 0.1 M glycine methylester or 0.1 M ethylenediamine causes a significant increase in the rate of loss of EAC and EDC, while the presence of 0.1M phosphatate, 0.1M hydroxylamine, or 0.01M ATP decreases the half-lives to <0.4 h at all pH values.

2.3.6 Comparing methods

The methods mentioned above were compared according to several criteria (Tab 3). Among the most important belong sensitivity of method, amount of chemicals needed for the experiment, instrumental demands for actual measuring and also amount of time needed for making the experiment. Method A is unfortunately not sensitive enough so it was rejected along with method C. Method E is similar to method B which was eventually selected for carrying out the experimental part.

Tab 3 *Comparison of mentioned methods*

Method	Used chemicals	pH	Sensitivity
A	Pyridine	7	50 μM
	HCl		
	1,2-diaminoethene		
B	pyridine	6.9	1 μM
	dimethylbarbituric acid		
C	aniline hydrochloride	7	0,2 μM
	acetic acid		20 μM
	$FeCl_3$		
D	HCl	5.8	5 μM
	NaOH		
E	barbituric acid	6.9	1 μM
	dimethylbarbituric acid		
	pyridine		

3. Experimental part

3.1. Chemicals

- Collagen type I, purchased from VÚP, a.s., used as received
- *N*-(3-dimethylaminopropyl)-N´-ethyl-carbodiimide hydrochloride – EDC
  $C_8N_{18}N_3HCL$98% purchased from Sigma Aldrich Germany, stored in freezer under -17°C
- *N*-hydroxysuccineimide – NHS
  $C_4H_5NO_3$ $C_4H_5NO_3$ 98% purchased from Sigma Aldrich Germany
- Disodium Hydrogenphosphate dodecahydrate
  Na_2HPO_4 . $12H_2O$ purchased from LachNer, s.r.o., Czech Republic, used as 0,1M solution
- 1,3-dimethylbarbituric acid
  $C_6H_8N_20_3$ 98% purchased from Acros Organics , Belgium
- Pyridine
  C_5H_5N 99,5%, purchased from LachNer, s.r.o, Czech Republic.

3.2. Instruments

Absorption spectrometer Thermo Spectronic UNICAM UV 500 (Cambridge, United Kingdom) was used for measuring the absorbance of samples. This two-beam scanning spectrophotometer of classical construction uses a photomultiplier as a detector. Measuring was performed in one-centimeter glass cuvettes delivered with instrument. All data were processed by softwares VISION (© 1999) and Microsoft Excel..

3.3. Used Method

3.3.1. Colorimetric method

This method for carbodiimides determination was discovered by Wilchek et.al[2] by accident during a study on cyanogen bromide activation of Sepharose. It was observed that carbodiimides gives a purple red colour with pyridine and barbituric or dimethylbarbituric acid. The use of this reaction as a colorimetric test for determination of the carbodiimides is based on scheme below (Fig 6). The vital step in the entire sequence is the attack of carbodiimide on the pyridine ring and its subsequent cleavage yields glutaconic aldehyde which reacts with barbituric acid.

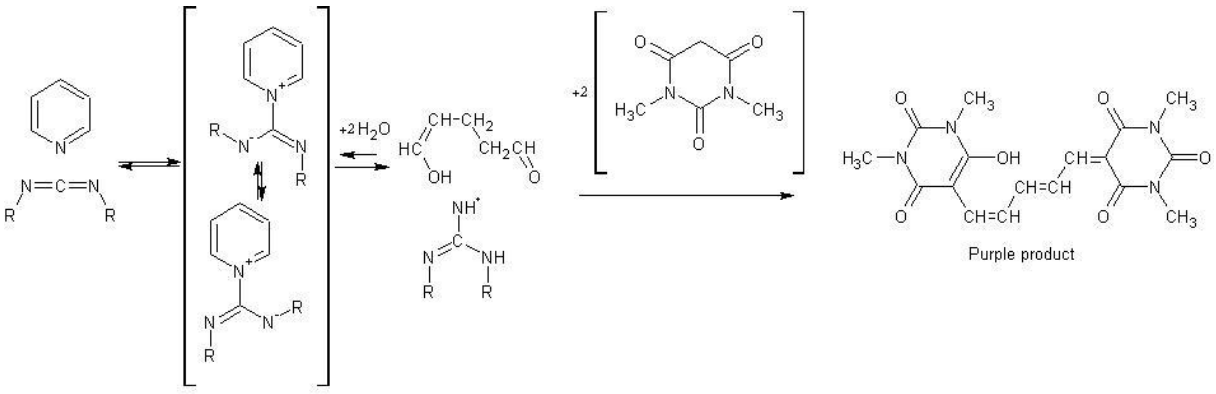

Fig 6 *Principle of forming colorimetric product*

3.3.2 Preparation of solutions

At first, three series of solutions for accomplishing a calibration curve were prepared. These solutions were made by dissolving EDC in distilled water. Due to very low detection concentrations three extra concentrated solutions were prepared for further dilution to 25 ml graduated flasks. Concentrations were used as followed in Tab 4.

Tab 4 *Dilution of solutions*

	c (mol/l)	**Preparation**	
Solution nr.1	0.004	0,7668g of EDC dissolved in 1l of distilled water (1)	
Solution nr.2	0.00004	10 ml of solution nr.1 dissolved in 1l of distilled water	
Solution nr.3	0.0004	100 ml of solution nr.1 dissolved in 1l of distilled water	
Samples	**c (mol/l)**	**n/25ml (mol)**	**Dilution**
1	0.000001	0.00000004	0.625 ml of solution nr.2
2	0.00001	0.0000004	6.25 ml of solution nr.2
3	0.000025	0.000001	15.625 ml of solution nr.2
4	0.00005	0.000002	3.125 ml of solution nr.3
5	0.0001	0.000004	6.25 ml of solution nr.3
6	0.00015	0.000006	9.375 ml of solution nr.3
7	0.0002	0.000008	12.5 ml of solution nr.3
8	0.0003	0.000012	1.875 ml of solution nr.1
9	0.0004	0.000016	2.5 ml of solution nr.1
10	0.0005	0.00002	3.125 ml of solution nr.1

$MW_{EDC} = 191.7$ $c_{sol} = 0.004 mol \,.\, l^{-1}$ $V_{sol} = 1\ l$

$$m = c \cdot M \cdot V$$
$$m = 0.004 \cdot 191.7 \cdot 1 \quad (1)$$
$$m = 0.7668 g$$

The barbituric acid reagent was prepared by dissolving 500 mg of barbituric acid in mixture of 8 ml of pyridine and 2 ml of water. Since only 98% barbituric acid was available, 510 mg per 10 ml of mixture were used. For dissolving barbituric acid magnetic stirrer was used. As recommended in literature[2], fresh solution was prepared before every series of measuring. The barbituric acid reagent was used as reagent blank.

2.3.3 Preparation of samples and washing samples

48 samples of bovine collagen cut to pieces of approximately same size (14 mm x 10 mm x 3 mm) were used. Those samples were cross-linked by three different concentrations of EDC/NHS (2, 3).

50 mmol . l^{-1} EDC

$MW_{EDC} = 191.7$ $c_{sol} = 0.05\ mol \,.\, l^{-1}$ $V_{sol} = 250\ ml$

$$m = c \cdot M \cdot V$$
$$m = 0.05 \cdot 191.7 \cdot 0.25 \quad (2)$$
$$m = 2.39625 g$$

25 mmol . l^{-1} NHS

MW_{NHS} = 115.09 c_{sol} = 0.025 mol . l^{-1} V_{sol} = 250 ml

$$m = c \cdot M \cdot V$$
$$m = 0.025 \cdot 115.09 \cdot 0.25 \quad (3)$$
$$m = 0.7193g$$

For each concentration 16 pieces of collagen were used and subsequently cross-linked by EDC/NHS system for 2 hours. After it the same amount of Petri dishes filled with 20 ml of 0.1M solution of Na_2HPO_4 were prepared (Fig 7). Since the cross-linking has been finished, samples were washed in solution of sodium phosphate dibasic followed by water. All samples were washed for a different periods of time and in different amount of water and Na_2HPO_4 as shows Tab 5.

The procedure of washing samples is very simple. After cross-linking, all samples were squeezed to get rid of cross-linking agent and than put into solution of Na_2HPO_4 in Petri dishes. Washing is based on diffusion and therefore no further manipulation with sample is required. After passing given time of washing in Na_2HPO_4, samples were squeezed again and than transported into distilled water. Water from last step was stored in a fridge for two days and than amount of EDC was determined using UNICAM UV 500 spectrophotometer.

Fig 7 *series of Petri dishes prepared for washing samples*

Tab 5 *Scheme of washing samples*

number	c (mmol/l)	Washing in Na2HPO4	Washing in water 1	Washing in water 2	Washing in water 3	Washing in water 4
1	50	120 min	30 min			
2	50	120 min	30 min	30 min		
3	50	120 min	30 min	30 min	30 min	
4	50	120 min	30 min	30 min	30 min	30 min
5	50	120 min	15 min			
6	50	120 min	15 min	15 min		

7	50	120 min	15 min	15 min	15 min	
8	50	120 min	15 min	15 min	15 min	15 min
9	50	30 min	30 min			
10	50	30 min	30 min	30 min		
11	50	30 min	30 min	30 min	30 min	
12	50	30 min	30 min	30 min	30 min	30 min
13	50	30 min	15 min			
14	50	30 min	15 min	15 min		
15	50	30 min	15 min	15 min	15 min	
16	50	30 min	15 min	15 min	15 min	15 min

The same scheme was used for other two concentrations of 25 and 15 mmol.l^{-1}. Only washing-water from the last washing step was used for measuring, which is highlighted in Table 5.

3.3.4. Determination of carbodiimide

0.9 ml of dimethyl barbituric acid reagent was added to the 0.1 ml of EDC solution in concentration ranging between 1 μM and 0.5 mM (Fig 8). The reaction was then allowed to continue at room temperature for 25 minutes until sufficient colour was developed. After 25 minutes absorption spectra were measured between 400 nm and 700 nm against reagent blank.

Fig 8 *Solution of barbituric acid dissolved in pyridine and sample of EDC with barbituric acid – pyridine agent in cuvette*

4. RESULTS AND DISCUSSION

4.1. Absorption spectrum

The highest absorbance was obtained at the wavelength of 598 nm (Fig 9, Tab 1(attachment)), which is of 3 nm difference from the wavelength published by Wilchek[2]. According to Wilchek[2], the highest absorbance should be obtained at the wavelength of 595 nm. This negligible deviation might be caused by using different spectrophotometer.

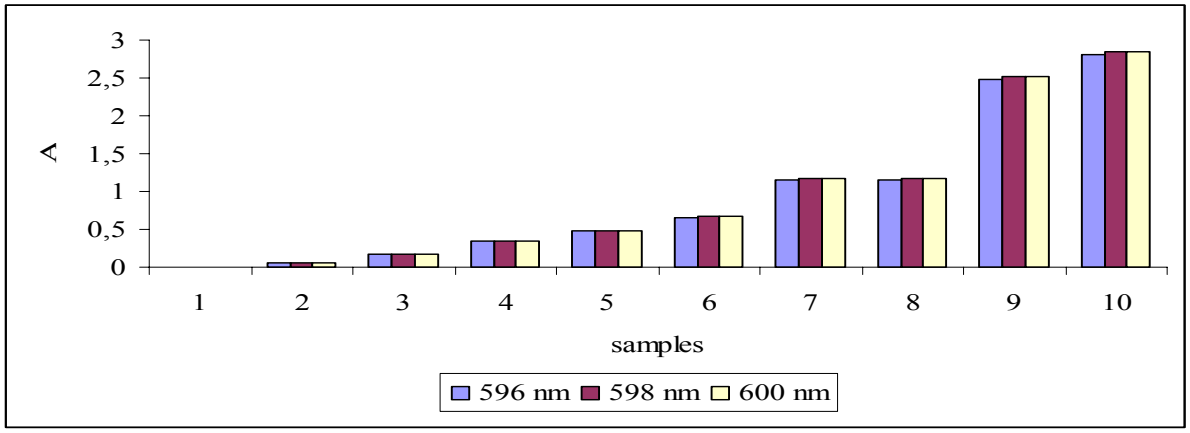

Fig 9 *Comparison of absorptions at different wave length for calibration series nr. 1*

Three absorption spectra obtained from three calibration series are shown in Fig 10, 11 and 12. In graphs of each series at least one sample was left out because it did not correspond with linear trend, however those values were implicate into accounts. Example of obtained data from UNICAM UV 500 is in Tab 2 in attachment.

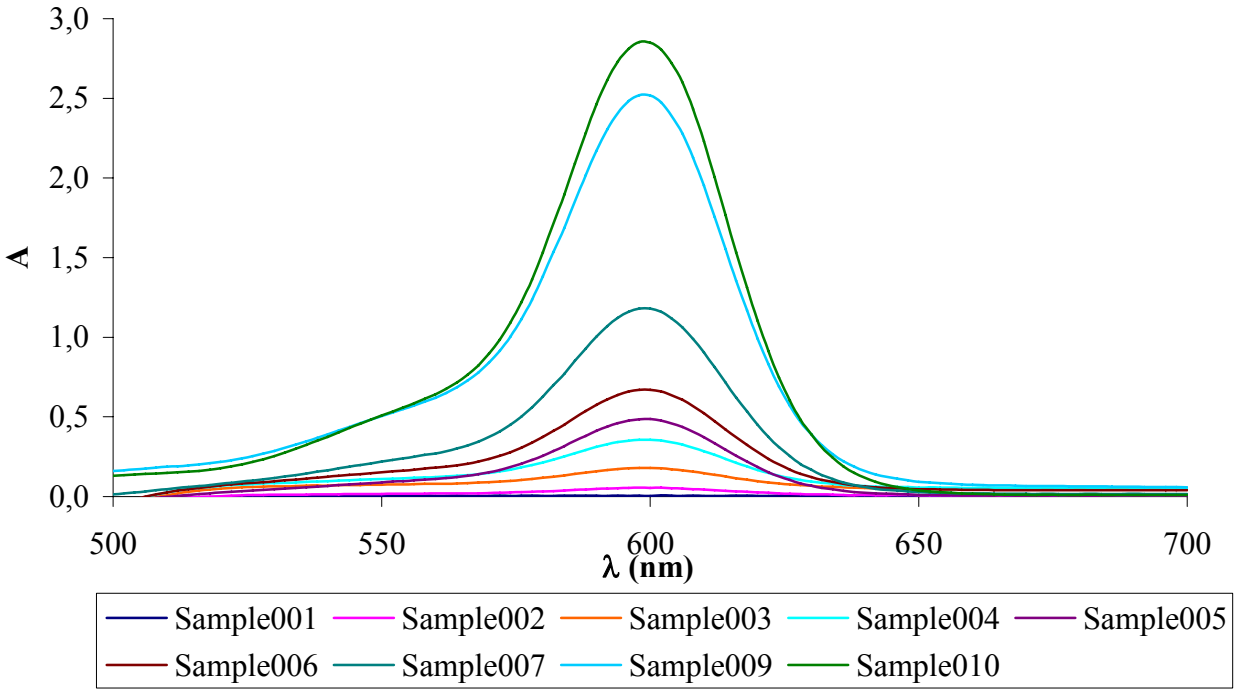

Fig 10 *Absorption spectrum for calibration series nr. 1*

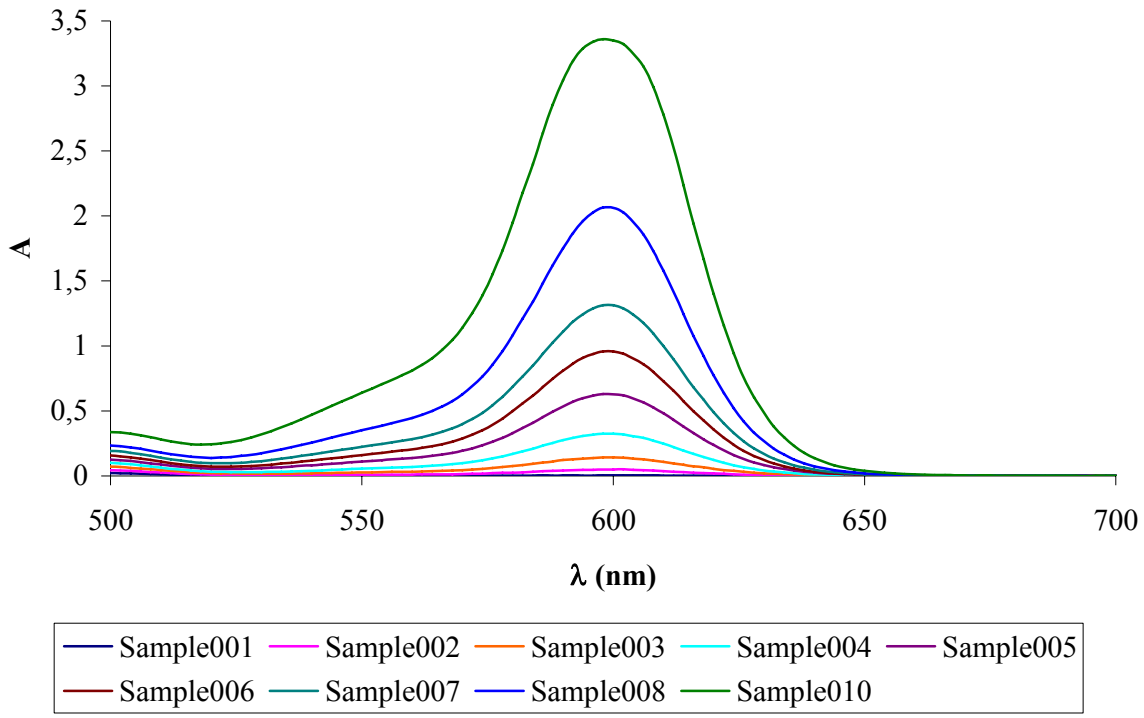

Fig 11 *Absorption spectrum for calibration series nr. 2.*

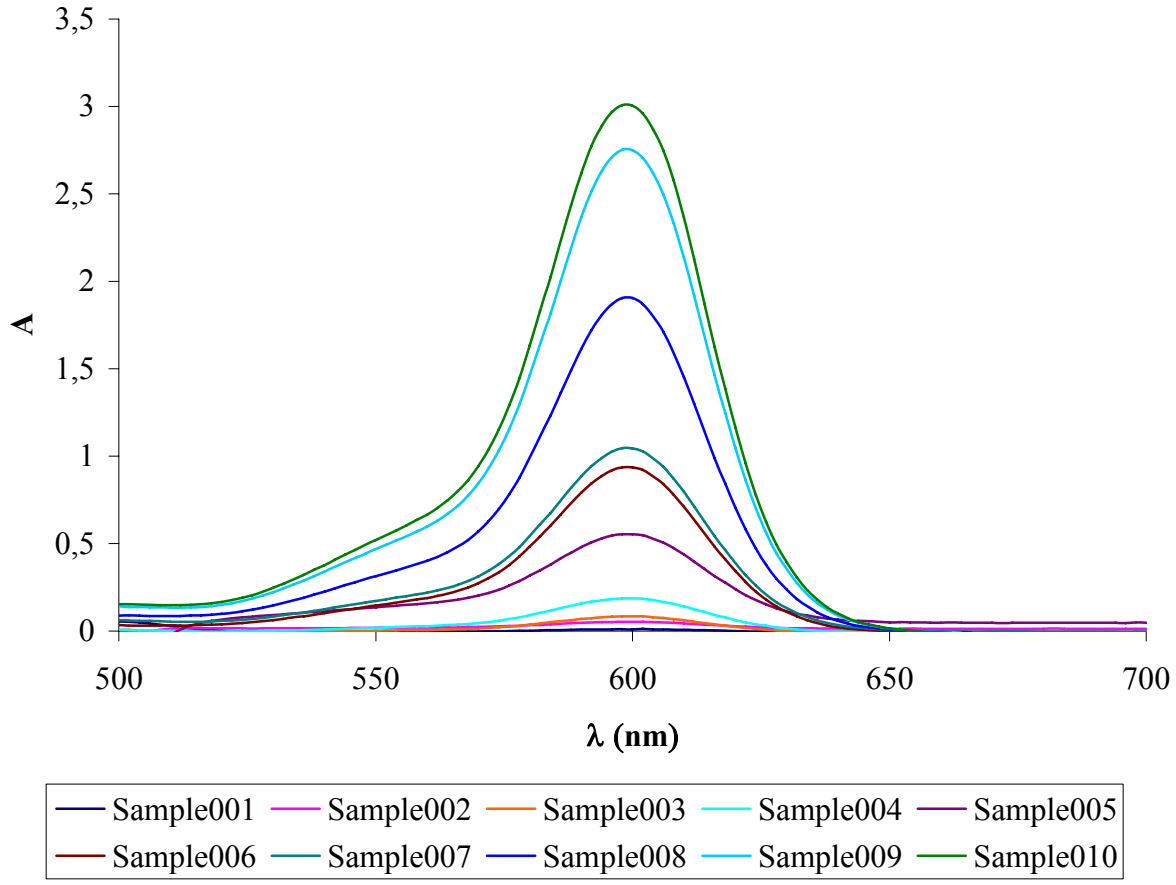

Fig 12 *Absorption spectrum for calibration series nr. 3*

4.2. Calibration curve

Absorbance of samples from each series for wave lengths 596 nm, 598 nm and 600 nm were measured and used for assembling a calibration curve (Fig 13). Table with values used for making calibration curve are in attachment (Tab 3). Calibration series were taken after 25 minutes of colour development (see Fig. 14).

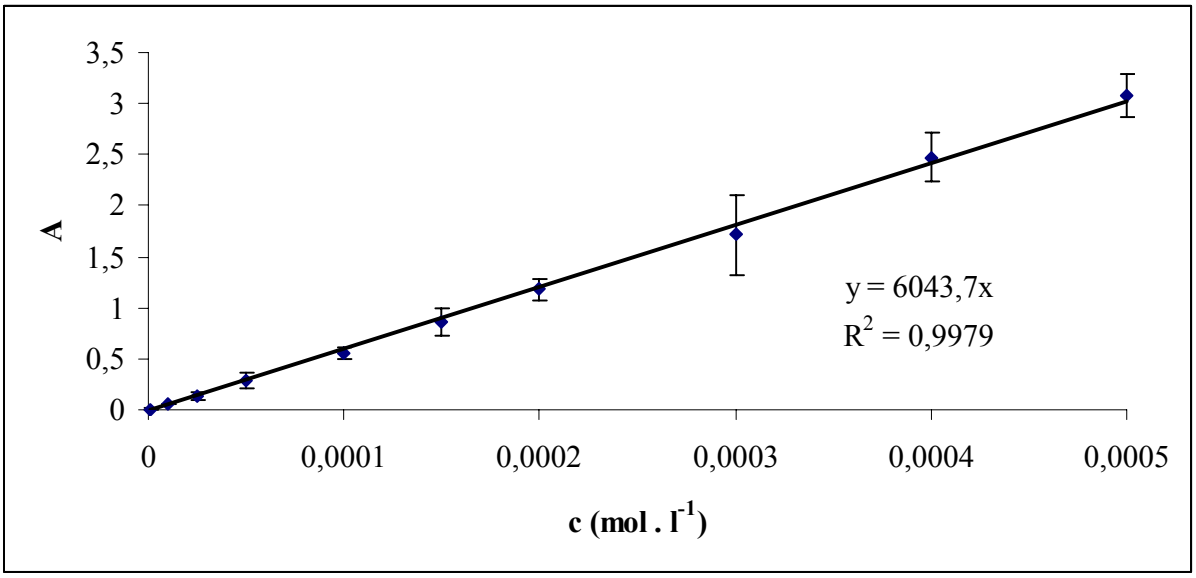

Fig 13 *Calibration curve at 598 nm from 16.4.2008*

Fig 14 *Calibration series after 25 minutes of colour development*

Absorbance of calibration series was measured again in 7 days to see if calibration curve course and values is affected. The new calibration curve made from these data does not show any significant diference confirming one week stability of prepared samples (Fig 15). Table with values used for making calibration curve are in attachment (Tab 4).

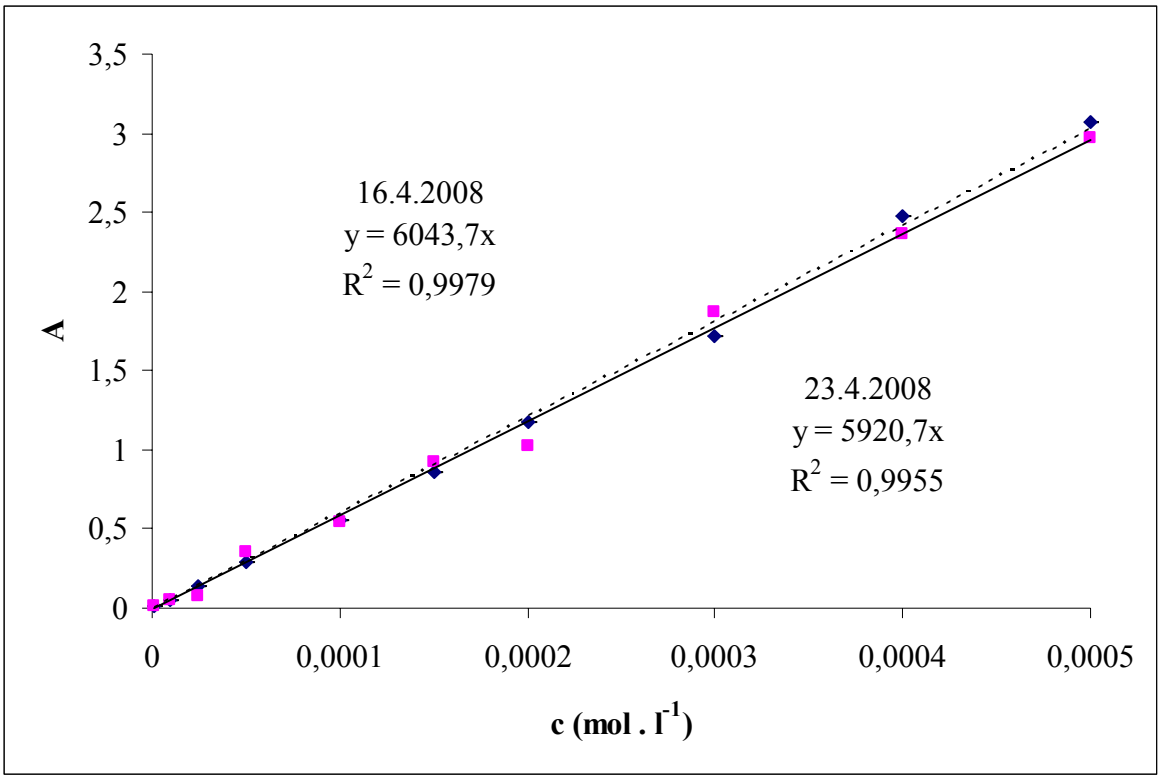

Fig 15 *Calibration curve at 598 nm*

4.3. Sample evaluation

For determination of EDC amount in samples calibration equation for calibration nr.2 at 598 nm (Fig 15) was used (4,5).

$$y = 5982{,}2x \Rightarrow c = \frac{A}{5982{,}2} \tag{4}$$

Example

50 mM cross-linking agent, washed in Na_2HPO_4 for 30 minutes and subsequently in water for 15 minutes:

$$A = 0{,}061$$

$$c = \frac{A}{5982{,}2}$$

$$c = \frac{0{,}061}{5982{,}2} \tag{5}$$

$$c = 1{,}02 \cdot 10^{-4}\ \mathrm{mol \cdot l^{-1}}$$

EDC was measured three times in each sample of washing water from last step of washing. So far as nearly no EDC was detected within the first measurement, the second and third measuring did not continue. As well as if in the sample of certain concentration with shortest washing time no detectable amount of EDC was presented, it was not continued in measuring samples with longer time of washing. Tables with measured and counted values are in attachment (Tab 5, 6). Here follows the graphic evaluation only.

Chart below (Fig 16, Tab 7 in attachment) shows, that cross-linking agent was easier washed out from sample cross-linked by less concentrated agent.

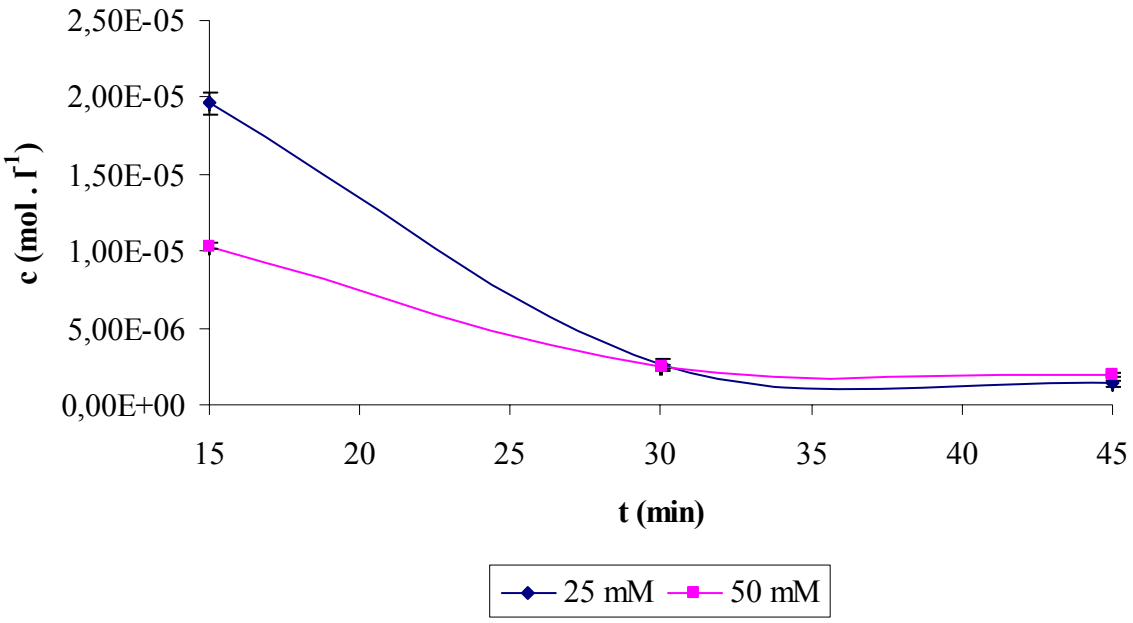

Fig 16 *Comparison of washing EDC from samples after 30 min in Na_2HPO_4 and 15 min as a step*

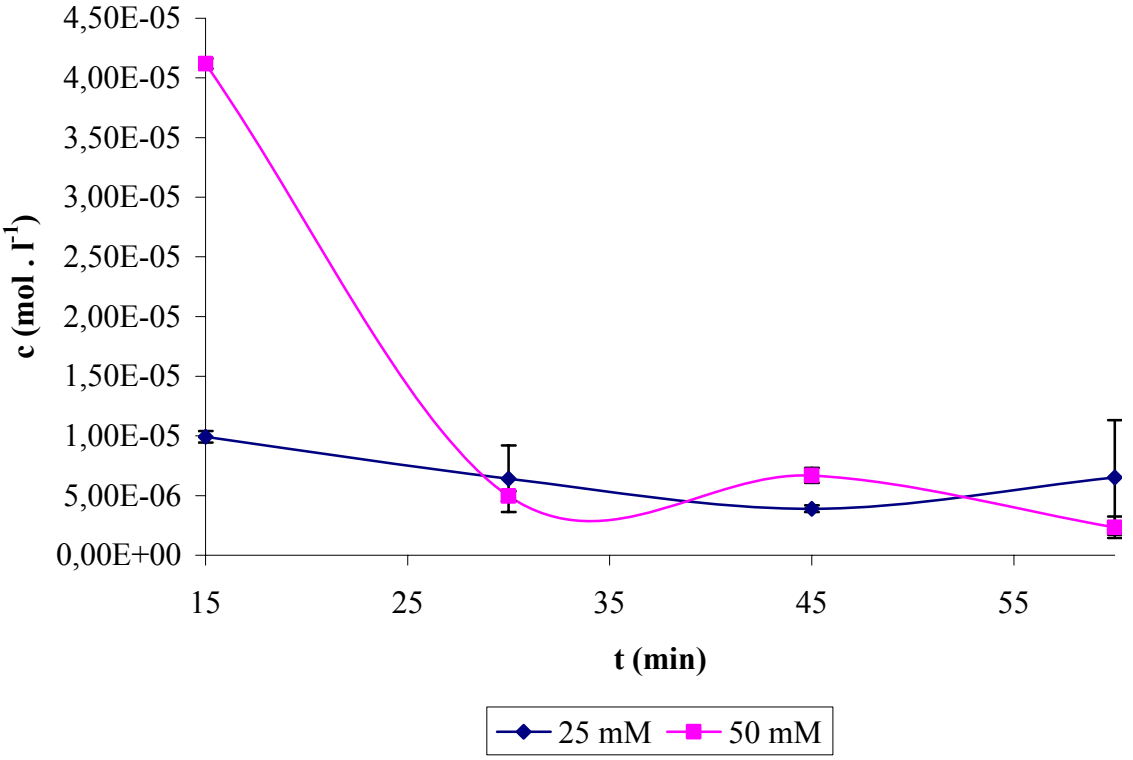

Fig 17 *Comparison of washing EDC from samples after 120 min in Na_2HPO_4 and 15 min as a step*

Chart above (Fig 17, Tab 8 in attachment) shows that most of cross-linking agent is washed out from the sample during 2 hours of washing with Na_2HPO_4. By comparison of chart on Fig

16 and Fig 17 it is possible to see that amount of EDC washed from sample cross-linked in agent of 25 mM concentration is lower after two hours ($1 . 10^{-5}$ mol . l^{-1}) in Na_2HPO_4 than after mere 30 minutes ($2 . 10^{-5}$ mol . l^{-1}). There are several reasons why that are so and they are discussed in conclusion part.

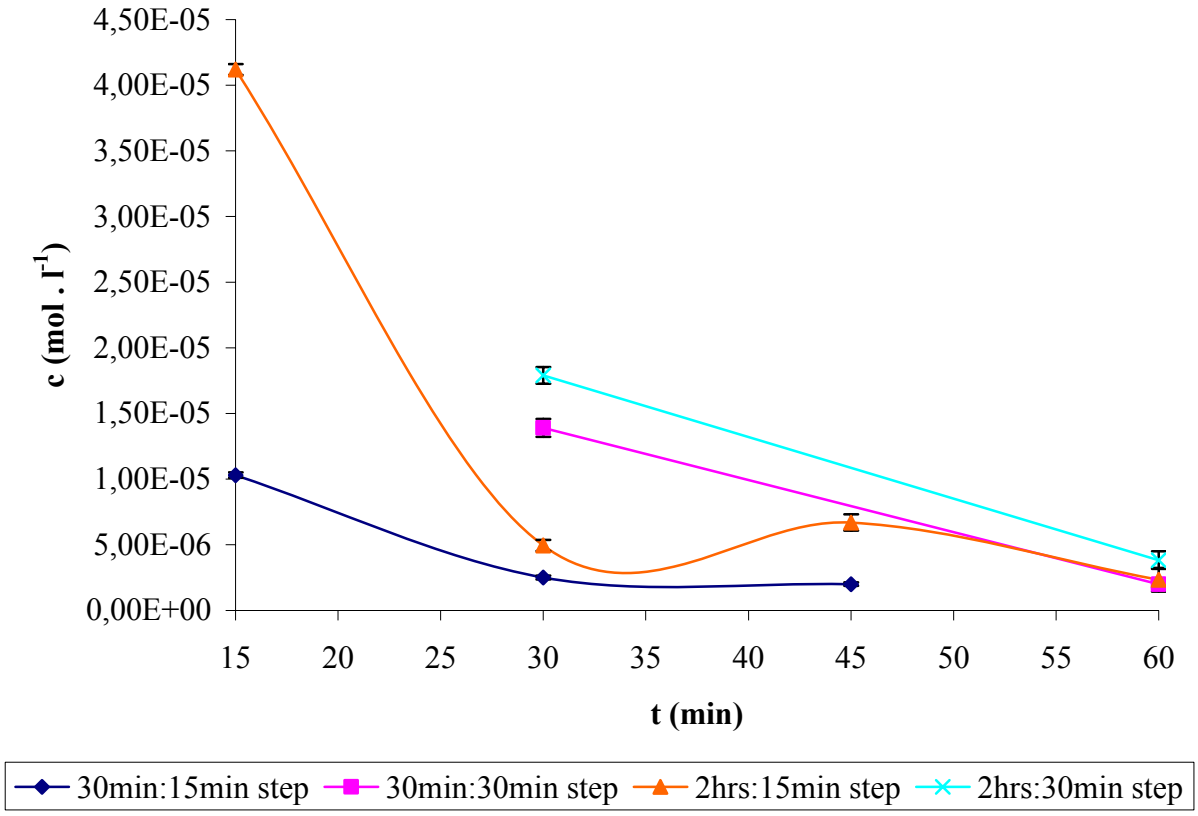

Fig 18 *Washing of EDC from samples cross-linked in agent of concentration 50 mM*

By comparing results for 15 and for 30 min step of EDC washing from sample cross-linked by 50 mM agent (Fig 18, Tab 9 in attachment), it is possible to see that they are similar. In both times, washing water contains more EDC in 2 hours of washing by Na_2HPO_4 than in 30 minutes of washing by Na_2HPO_4 (Fig 18). Table contenting values from those graphs are included in attachment (Tab 7, 8, 9).

5. CONCLUSION

The experimental part was focused on determination of EDC in aqueous solution using Wilcheks´ method[2]. As a first step calibration series were prepared and absorption spectra were measured. From calibration equation a dependence of absorbance on concentration of EDC, which was further used in evaluating samples, was deduced.

By using three different concentrations of cross-linking agents 48 samples were obtained. The same technique of washing but different washing times were applied on these samples. Samples were firstly washed by 0.1 M solution of Na_2HPO_4 and consequently by distilled water. Amount of EDC was measured only in the water from last step of washing.

By evaluating data from the whole experiment, it was found out that currently used washing technique is sufficient enough for becoming non-detectable amounts of EDC in the samples after commonly used washing times (50 mM of EDC cross-linked agent, washing for 2 hours in 0.1 M Na_2HPO_4 followed by 4 times 30 min in distilled water). When the shortest washing time of experiment was used (30 min in 0.1 M Na_2HPO_4 and 15 min in distilled water) concentration of EDC in washing water dropped already up to 10,2 $\mu mol.l^{-1}$. However, these values are not exactly accurate because samples of collagen were not weighted before the actual experiment. That also can be a reason why higher amount of EDC was washed out from the sample cross-linked in less concentrated agent than from sample cross-linked in agent with higher concentration. All results that were obtained are examined closely in experimental part.

It is certainly important to know how much of EDC the washing water contains, but it should be also examined how much of EDC stays in collagen sample. Therefore, the wisest course of action now would be re-examinaniton of the washing technique with more samples and if the results are analytically accurate each sample should be cross-linked individually on Petri dish. Not like in this experiment where 16 samples were cross-linked by same solution of cross-linking agent in the beaker. Also, the repeating of calibration assessment and determining the stability of the dimethylbarbituric reagent (used also as blank) and the stability of samples in longer period of time is needed. If the results are even more accurate, it would be necessary to determine exact amount of EDC in cross-linking solution after cross-linking, amount of EDC washed by Na_2HPO_4 solution and amount of EDC washed out in each step of washing. Unfortunately, while using such low cross-linking concentrations it might happen that amount of EDC in some steps will not be detectable by used method as well as determination of EDC in collagen sample.

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