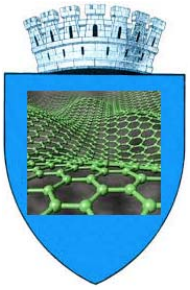

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Article

COMPARISON OF DIFFERENT EXTRACTION METHODS OF ESSENTIAL OILS FROM *LAVANDULA ANGUSTIFOLIA* AND DETECTION OF TARGET COMPOUNDS WITH UPLC-MS/MS

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ABSTRACT

Three extraction methods were used comparatively to extract linalool and linalyl acetate from lavender flowers, namely hydrodistillation, Soxhlet extraction (in ethanol) and accelerated solvent extraction (ASE) (with water or methanol, 80ºC, 1500 psi). The best results were obtained with ASE (methanol). The fresh flowers gave higher yields of linalool and linalyl acetate. The possibility of using UPLC-MS-MS for rapid and precise identification (based on spectra of fragments of target compounds) of essential oils was successfully demonstrated. UPLC-MS analysis of the isolated oils revealed that linalool and linalyl acetate are the major components of all the samples.

Keywords: *Lavandula angustifolia*, hydrodistillation, Soxhlet extraction, accelerated solvent extraction, essential oil, UPLC-MS-MS, linalool, linalyl acetate.

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1. INTRODUCTION

Essential oils are volatile aromatic compounds found in a wide range of terrestrial plants, e.g. flowers, leafs, roots, stems and seeds[1]. They are a combination of highly hydrophobic organic compounds that can be extracted by various techniques[2] among them, the most used being hydrodistillation[3], Soxhlet extraction[4], microwave extraction[5], cold pressing[6] and supercritical fluid extraction[7]. One of the most published technique for the extraction of the essential oil from plants is hydrodistillation[3]. On the other hand, hydrodistillation is a very time-consuming method, during which highly volatile components as well as water-soluble components can get lost[8]. Usual extraction techniques of essential oils and the other chemicals that are present in the plant materials include techniques that use solvents at atmospheric pressure or at high pressure and high temperature [9-11]. The methods that use solvents for extraction can be very selective, as the solvent properties are selected to extract only a few compounds that are more soluble in the chosen solvent. The drawback of these approach is the fact that together with the volatile components of the essential oils will be extracted other compounds, equality soluble in that solvent. Furthermore, the temperature and the pressure can influence not only the extraction yields of the target compound but also can contribute to the degradation of these molecules. This can be avoided by the technique called accelerated solvent extraction (ASE), in which the extraction period can be controlled and the deleterious effects of high temperature and high pressure can be minimized[12].

Some of lavender essential oil constituents present beneficial biological activities[13] such as: the treatment of anxiety [14] and sleep disorders [15], or antimicrobial[16] and antifungal[17] effects or natural preservatives in cosmetic products [18].

The genus *Lavandula* from *Labiatae* family, comprise three species utilized for their production of essential oils: *L. angustifolia* Miller contains essential oil of the highest quality, *L. latifolia* Medicus has the lowest yields, and *L. hybrida* (*L. angustifolia* × *L. latifolia*) has the highest yields, but not the highest quality oil[19].

Essential oils of *L. angustifolia* Miller is a colorless to pale yellow liquid, with a floral fragrance. Over 300 compounds have been found in species of *Lavandula*. The two main compounds in *Lavandula* are linalool and linalyl acetate[20].

The chemical composition of *L. angustifolia* oil have been the subject of several publications[21-23]. Most of the data concerning the investigations of the chemical composition of essential oils of *Lavandula* species were obtain by GC and GC-MS analysis[21-23]. In the present paper the chemical composition of the essential oils of *L. angustifolia* cultivated in Romania was analyzed by using liquid chromatography coupled to mass spectrometry (UPLC-MS).

The aim of this study was to compare different sample preparation methods for their suitability for the subsequent UPLC/MS-MS determination of major compounds of lavender essential oils. Three sample preparation procedures for the extraction of essential oils were compared, namely, hydrodistillation which is the most usual method for the isolation of

essential oils, Soxhlet extraction, and accelerated solvent extraction. The possibility of using UPLC-MS was also tested, as traditionally these compounds are analyzed by gas-chromatography techniques.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Methanol (Sigma, #34966) and ethanol (Sigma, #32221) were purchased from Amex SRL, Bucharest, Romania.

2.2. Plant material

Freshly picked flowers of *Lavandula angustifolia* cultivated in Timiş (Romania) in 2015 and 2016 were purchased from the local market. The fresh material was dried at constant temperature (23ºC), in a dark place[24].

2.3. Preparation of lavender flower samples.

Known amounts of fresh or dried lavender flowers (usually around 1 g) were manually grinded in a mortar. To achieve reproducible extraction yields the samples were passed through a sieve with mesh sizes between 20 and 30 (particle diameters ranging over 0.60-0.85 mm). The dried samples were kept within sealed bag in the cold and dry place until they were used[25].

2.4. Hydrodistillation

The hydrodistillatin extraction was carried for 150 min with 200 mL water and 30 g of dried or fresh lavender flowers, using a Clevenger-type apparatus[3,5]. The oil phase was separated through a separatory funnel.

2.5. Soxhlet extraction

Four experiments of traditional Soxhlet extraction[16] were carried for 300 min with 45 mL of ethanol and (a) 2 g of dried lavender flowers or (b) 2 g of fresh lavender flowers, and with 45 mL of water and (c) 2 g of dried lavender flowers or (d) 2 g of fresh lavender flowers.

The obtained extracts were centrifuged at 5000 rpm for 5 min and then the oil was separated through a separatory funnel.

2.6. Accelerated solvent extraction

Two type of solvents were used for ASE[4] methanol and water. Around 1 g of fresh flowers was loaded into extraction cell of an ASE200 extraction system (Dionex Corporation, USA). In both cases, the volume of solvent used for extraction was 16 mL. The extraction was carried out at 1500 PSI and 80°C for 10 minutes. After extraction, the extracts were mixed with mobile phase (1:1), filtered on 0.2 μm syringe filters and injected in the chromatographic system.

2.7. UPLC-MS analysis

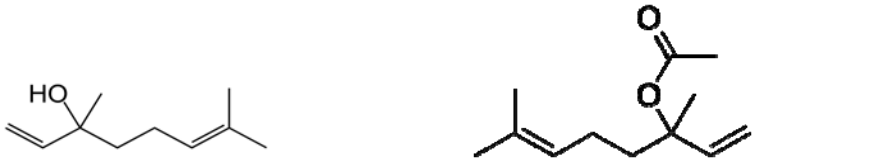
The chromatographic analysis was carried out on a Waters Acquity UPLC-MS system (Binary Solvent Manager, Xevo TQD MS-detector equipped with an electrospray ionization interface)[26] with a UPLC BEH C18, 1.7 μm (2.1×100 mm) column, using a gradient elution procedure. Mobile phase A consisted in 0.02% formic acid in 5% methanol and mobile phase B was 0.02% formic acid in methanol. The gradient profile was: 0 – 0.2 min, 30% A and 70% B; 0.2 – 1 min, linearly increase untill 100% B; 1 – 2.9 min, hold 100% B; 2.9 – 3.0 min, linearly decrease untill 70% B (initial condition). The column temperature was set at 30°C. The analyses were run at a flow rate of 0.3 mL/min, and the sample volume injected was 10 μL.

The electrospary ionization (ESI) parameters for Xevo TQD MS detector were fixed as follows: capillary voltage at 3.0 kV, source temperature at 150°C, desolvation temperature at 400°C, and desolvation gas at 500 L/h. Nitrogen was used as the desolvation gas, and argon was employed as the collision gas.

3. RESULTS AND DISCUSSIONS

In the absence of appropriate standards, the optimization of the extraction procedure has to rely on qualitative results. From the literature regarding the composition of lavender oil results that linalool and linalyl acetate are the major compounds of essential oils extracted from this plant[16]. In fact, in many papers dealing with the yield of extraction of essential oils from lavender, these two compounds are considered as marker molecules[18,27]. The chromatographic analytical method, based on detection with a triple-quadrupole was optimized to separate and to detect the compounds with m/z of 155 ($M+1^+$, for linalool) and

197 ($M+1^+$, for linalyl acetate) and to search the daughter fragments of these parent ions. The chemical structures of the target analytes are presented in Figure 1.



Linalool (MW 154.25 g/mol) Linalyl acetate (MW 196.29 g/mol)

Figure 1. The chemical structures of the two major compound from lavender oil

Based only on the information about the target compounds (molecular mass, log P values) we have undertaken a separation procedure and a detection method to allow the identification of linalool and linalyl acetate in the absence of standards. For setting up a MS-MS detection method, in some preliminary results were search the best conditions of detection and fragmentation to find the ion daughters of positive charged parent ions, i.e. $M+1^+$ = 155 m/z for linalool and $M+1^+$ = 197 m/z for linalyl acetate, respectively. Instead of real standards, a commercial lavender oil was used. The oil was diluted with weak mobile phase (10% MeOH, containing also 0.02% formic acid, to promote positive ionization) and the signal was recorded at various values of parameters of interface and triple-quadrupole: capillary voltage 2 – 4 kV, source temperature at 150°C, desolvation temperature at 200 - 500°C, desolvation gas velocity at 200 - 800 L/h, cone voltage 10 – 70 V, collision energy 0 – 70 V. The results have shown that the best values of the parameters of the interface and triple-quadrupole for detection of the parent ions and one major fragment are: capillary voltage 3.0 kV, source temperature at 150°C, desolvation temperature at 400°C, desolvation gas velocity at 500 L/h, cone voltage 30 V, collision energy 20 V.

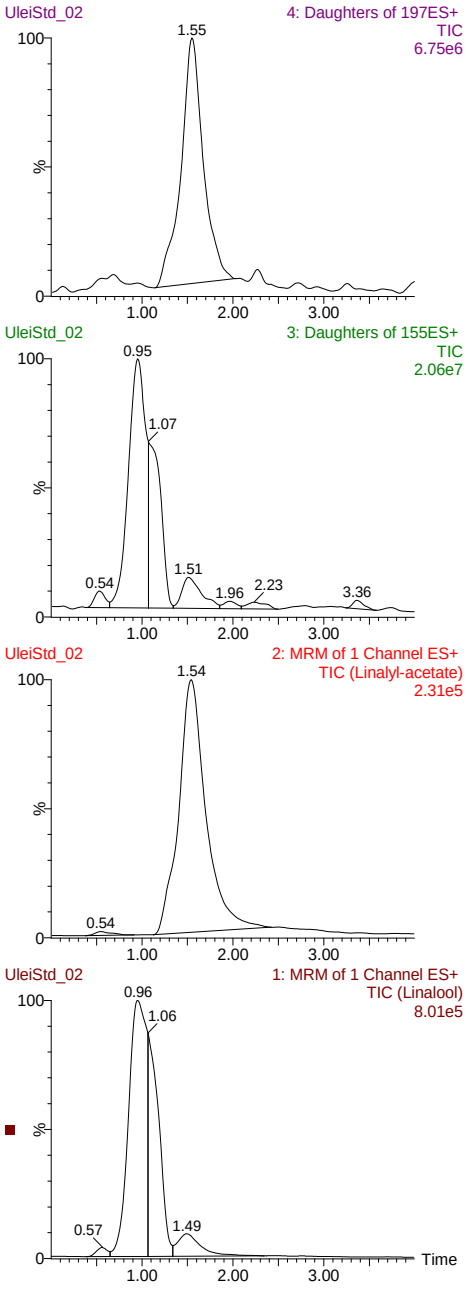


Figure 2.

The chromatographic separation of linalyl acetate and linalool from a sample of commercial lavender oil.

The first upper panel present the chromatogram of linalyl acetate, when the MS-MS method was settled to search the daughter fragments of ion with m/z 197, corresponding to linalyl acetate. Similarly, the second panel present the chromatogram of linalool, were the detector was programed to search the daughter fragments of ion with m/z 155.

The lower panels present the chromatograms obtained when the detector was programed in multiple reactions monitoring mode, searching only for the fragment with m/z 137 of the parent ion 197, for linalyl acetate and with m/z 137 of the parent ion 155, for linalool, respectively.

The next step was the optimization of the chromatographic separation of the target compounds, again, first from the commercial lavender oil product. After searching several elution gradient formulations, the optimal separation was achieved when the gradient profile was: 0 – 0.2 min, 30% A and 70% B; 0.2 – 1 min, linearly increase untill 100% B; 1 – 2.9

min, hold 100% B; 2.9 – 3.0 min, linearly decrease untill 70% B (initial condition). Figure 2 presents the separation of linalool and linalyl acetate from commercial lavender oil.

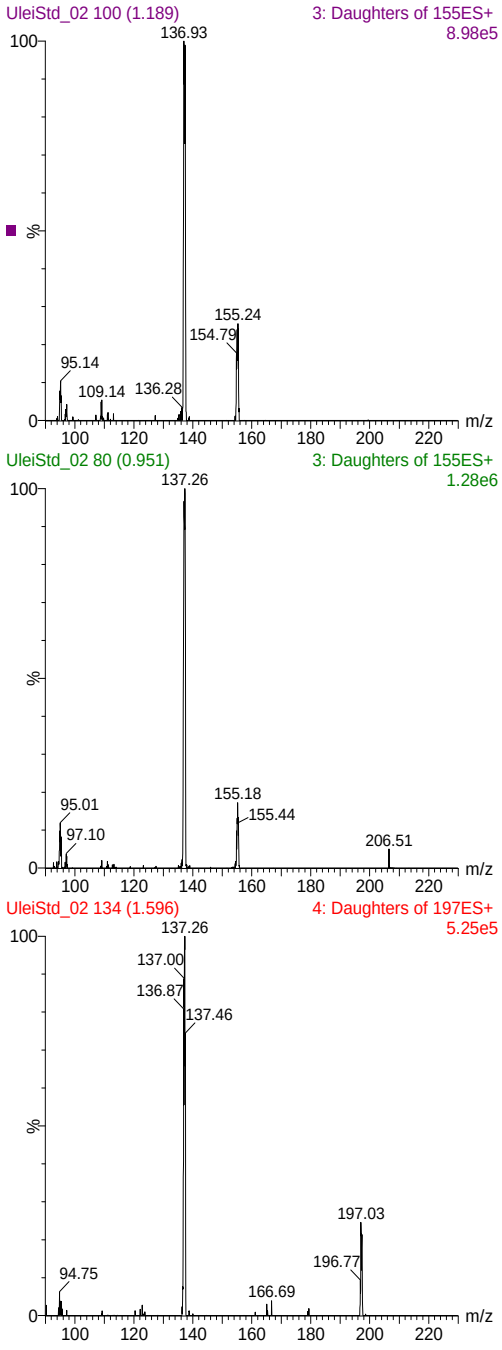


Figure 3.

The spectra of the daughter fragments of compounds separated in Figure 2..

The lower panel presents the ion fragments when the detector was settled to search for daughter of ion with m/z 197.

The first two panels present the ion fragments of parent ion with m/z 155 and these spectra are a confirmation of the fact that the compounds that coelute at 0.95 and 1.07 min have the same mass and produce the same daughter when subjected to the same collision energy.

The evidence that the eluted compounds from Figure 2 are linalool and linalyl acetate is presented in Figure 2 where the spectra of daughter fragments of parent ion with m/z 155 corresponding to linalool and of parent ion with m/z 197 corresponding to linaly acetate, respectively are presented.

In Figure 2 the second and the fourth panels present the chromatograms of linalool, and because the peaks present a shoulder, it is an indication that the peaks are not pure, i.e. there are two compounds that co-elute at close retention times. In Figure 3 the first two panels represent the spectra recorded at 0.98 and 1.18 minutes, respectively, i.e. of the first part and the one of the major peak from Figure 2 the second panel. As both these spectra are very similar, we may conclude that the two-peaks that co-elute in from Figure 2 belong to the same compound. It is well known that linalool has a stereogeneric center at C3 and for this, there are two stereoisomer: the R-isomer is known as licareol and the S-isomer as coriandrol.

To extract the essential oils from lavender in this study were used three methods: hydrodistillation, Soxhlet extraction and ASE. Figure 4 compares the peak areas of the linalool and linalyl acetate from essential oils obtained by Soxhlet extraction and ASE with the commercial product, used as imitation standard.

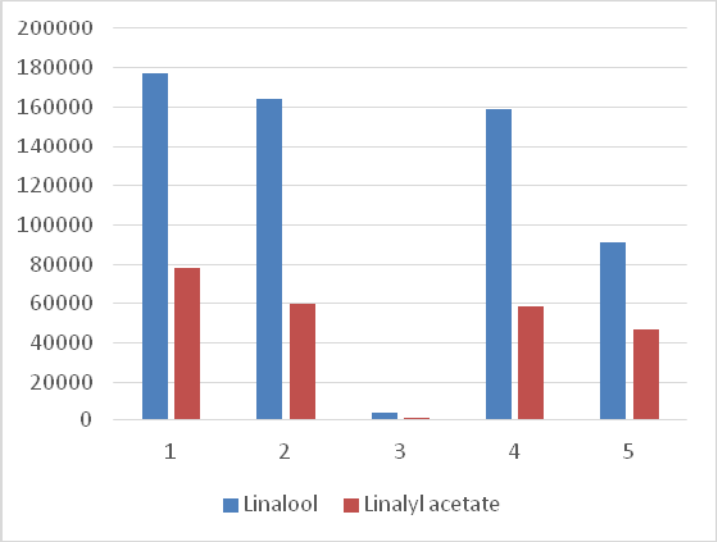


Figure 4. Comparison of yields of extraction, expressed as areas of the separated peaks, of different extraction methods: 1 = commercial lavender oil, used for comparison; 2 = extraction from dried flowers with methanol using ASE method; 3 = extraction from dried flowers with water using ASE method; 4 = Soxhlet extraction from fresh flowers with ethanol; 5 = Soxhlet extraction from dried flowers with ethanol.

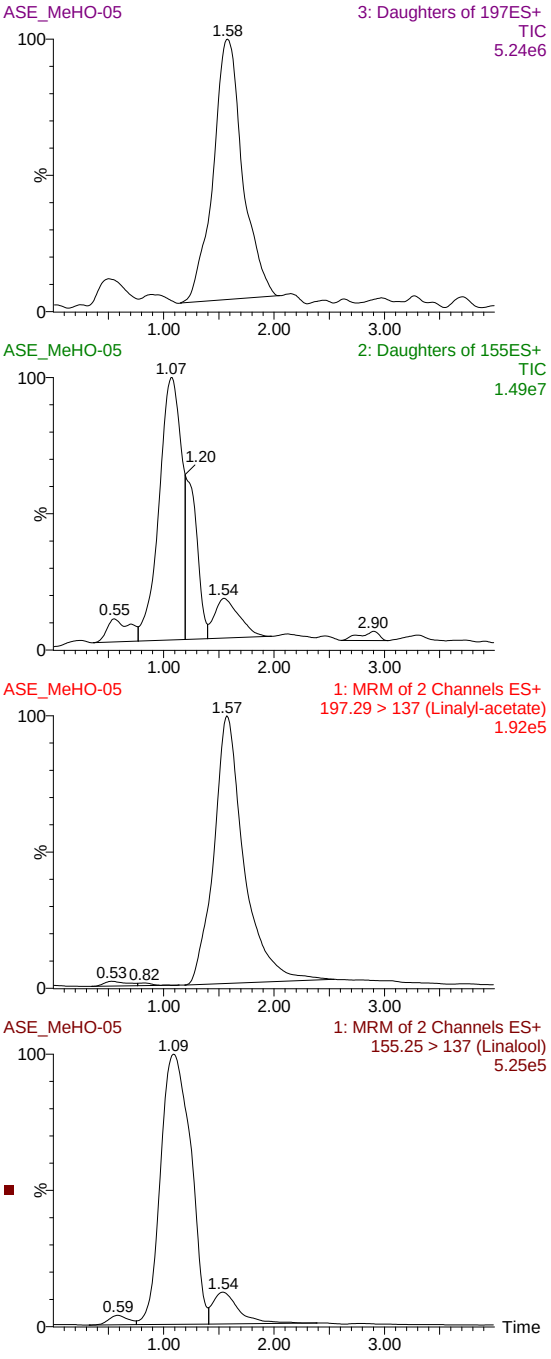


Figure 5.

The chromatograms of the ASE extract realized with methanol. For details of realization of the extraction see the text. For details of MS-MS method see Figure 2.

In Figure 5 there is presented an example of the chromatograms of the essential oil obtained using the ASE technique applied to dried flowers of lavender. Comparing the chromatograms with those of the commercial oil (Figure 2) one may see that the ASE extract contain both target compounds in high amounts.

The results from Figure 4 confirm that the best extraction method for the essential oils (linalool and linalyl acetate) is accelerated solvent extraction, when the extraction solvent is methanol. When water was used as extraction solvent, the extraction yield was very low (column 3 in Figure 4). The fresh flowers provide higher amount of essential oil than the dried flowers, as it can be seen by comparing the columns 4 and 5 from the same figure. The extraction methods should be further optimized as now the extraction yields for the target compounds are lower that the commercial product.

This is the first publication of use of an UPLC-MS-MS method for the separation of linalool and linalyl acetate from essential oils of lavender. The method can identify these compounds by their MS-MS spectra even in the absence of calibration standards. Using a gradient elution profile, the target compounds were separated in less than 3 minutes, with a resolution of 1.72.

4. CONCLUSIONS

Results presented in the present study showed a great variation in the yield, of essential oils obtained by different solvents and between fresh and dried flowers of *Lavandula*. The data obtained indicate that the content of main compounds (linalool and linalyl acetate) was similar with the content of the commercial product.

In the case of lavender, the amount of extracted compounds seems to be higher in fresh flowers, comparing with the dried material.

The optimized UPLC-MS-MS method can identify by MS spectra the linalool and linalyl acetate and can separate them in less than 3 minutes.

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Article

USE OF NINHYDRIN REACTION FOR ESTIMATION OF ACETYLATION DEGREE OF CHITOSAN

Bianca Vanesa Boros[1,2], Gheorghița Menghiu[1], Adina Matica[1], Vasile Ostafe[1,2]♣

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Abstract

The adaptation to ELISA plate format of the method of reaction of ninhydrin with the amino groups of chitosan and optimization of some reaction parameters is presented. The influence of the temperature of reaction, time of reaction, stability in time of the colored final product were studied, using glucosamine as standard compound. For this chemical, linear calibration curves were obtained. The results showed that the miniaturized method is sensitive and reproducible. The miniaturized method was used to estimate the quantity of amino groups of chitin and chitosan subjected to alkaline deacetylation.

Keywords: ninhydrin, glucosamine, chitin, chitosan, acetylation degree.

1. Introduction

After cellulose, chitin is the second most abundant polysaccharide, with annual production estimated to be larger than 25,000[1]. One of the major sources of chitin is the exoskeletons of crustaceans, like shrimps and crabs. Chitin is a linear polymer consisting of poly β(1–4) N-acetyl-d-glucosamine (NAG). Chitosan is the most important derivative of

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chitin (see Figure 1). Chitosan is a linear polymer of β-(1,4)-linked D-glucosamine (GN) and N-acetyl-D-glucosamine (NAG). Chitosan can be obtained by (partial) deacetylation of chitin. Deacetylation reaction can be realized in alkaline conditions (concentrated solutions of NaOH) or using specialized hydrolytic enzymes, like chitin deacetylase[2] when the number of acetylated units decrease in favor of un-acetylated glucosamine (GA). Ideally, when all the amino groups of the polymer are acetylated the degree of acetylation (DA) is 100% (or the degree of deacetylation is 0%) and this polymer is called chitin. Natural chitins have a degree of acetylation between 70 – 98%[3].

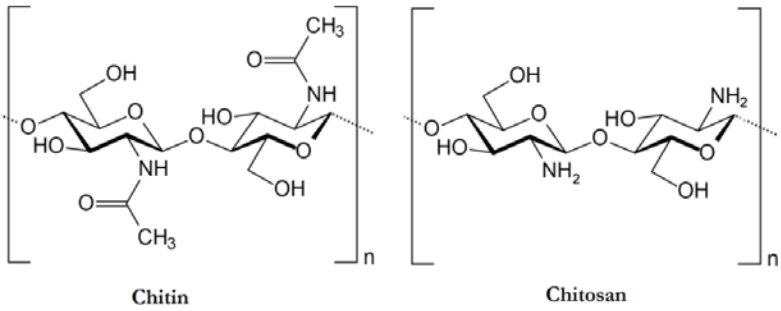

Figure 1. Chemical structures of chitin and chitosan.

The main drawback of using chitin in various types of application is its low solubility in aqueous solution. When the degree of acetylation is about 50%, the polymer becomes more soluble in aqueous (acidic) media due to the protonation of the free amino groups. This makes chitosan a more suitable natural polymer for many applications. The amount of NAG and the molecular weight of the polymer, that naturally can vary between 10 to 10^3 kDa[4], largely determine chitosan properties, and consequently its applications.

Although there were countless research activities regarding the properties of chitin and chitosan, a simple method for direct quantitative analysis of molecular weight of the polymer, of the degree of acetylation and of its structure is still missing. There are many methods used to characterize chitosan but they require specialized instrumentation and most of them have high costs of operation[5]. A simple and inexpensive method for quantitative analysis or characterization of chitosan, should realize the assay with low costs, in short time and with an acceptable precision. At least for the characterization of chitosan, i.e. the estimation of the degree of acetylation, the method based on the reaction of ninhydrin with the amino group of GN seems to be a good candidate[4].

It is well known that the reaction of ninhydrin with a primary amino group will form a colored reaction product, called Ruhemann's purple. This method was extensively use for amino acid analysis and derived peptides[6], but less applied to other compounds bearing amino groups, like chitosan[7]. In this paper, starting from some previous published

studies[4,7] we studied some parameters that may influence the reaction of ninhydrin with amino groups from chitosan and in order to make as simple as possible this assay, we have adapted the reaction to ELISA plate format.

2. Materials and Methods

2.1. Materials and Reagents

The chemicals used in this study were acquired from Sigma Aldrich or Carl Roth via Redox Lab Supplies Com S.R.L: ninhydrin (Carl Roth, #4378.2), potassium phosphate(Carl Roth, #3904.1), disodium phosphate (Carl Roth, # T106.2), glucosamine hydrochloride (Sigma Aldrich, # G1514), chitin (Carl Roth, #8845.1), low molecular weight chitosan (Sigma Aldrich, # 448869), medium molecular weight chitosan (Sigma Aldrich, #448877), yeast nitrogen base (Fluka, #51483), yeast extract (Carl Roth, #2363.3), yeast synthetic drop-out medium supplements without uracil (Sigma Aldrich, #Y1501), casein hydrolysate (Carl Roth, #A157.1), casamino acids (Sigma Aldrich, #22090), acetic acid (Sigma Aldrich, #27225), sodium hydroxide (Sigma Aldrich, # 367176), Tris hydrochloride (Carl Roth, # 9090.3). ELISA plates were purchased from Sarstedt (#82.1581).

For the determination of the optical density of the reaction product, a Tecan Sunrise microplate reader (Tecan Trading AG, Männedorf, Switzerland) with a Magellan Data Analysis software was used. The samples were heated up to 100°C with a HLC Heating-ThermoMixer MHR 11 (DITABIS - Digital Biomedical Imaging Systems AG, Pforzheim, Germany). A Hettich MIKRO 22R Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany) was utilized to clarify the samples and separate the precipitated chitin and chitosan. For the determination of absorption spectra, a UV/Visible Spectrometer T90+ (PG Instruments, Leicestershire, United Kingdom) was used.

2.2. Methods

2.2.1. Solutions

Three solutions of phosphate buffers (KH_2PO_4 / Na_2HPO_4, 0.05M) were prepared in dH_2O, each with a different pH: 6, 7 and 8. Ninhydrin stock solutions with 0.8%, 1%, 1.5% and 2% concentrations were prepared in each phosphate buffer solutions, and kept in the dark, at 4°C.

Stock solutions of 10 mM glucosamine in phosphate buffers were prepared and diluted as required.

Other solutions used in this study were 10 M NaOH and 15% acetic acid, both made in dH_2O.

One type of chitin and one type of chitosan were submitted to deacetylation in alkaline medium by mixing 0.2 g of each polymer with 12 mL of 10 M NaOH. The mixtures were heated up to 110°C under constant stirring. At defined intervals of time, samples of 500 µL were taken for analysis. Each of these collected samples was "washed" three times with 1000 µL of dH_2O and centrifuges between "washings". After the last centrifugation, instead of water, 500 µL of 0.5M Tris-HCl, pH 6.8 were added. The samples were kept at -20°C until use.

2.2.2 Reaction of ninhydrin with glucosamine

The reactions of ninhydrin with compounds carrying amino groups were carried out in ELISA plates. Serial dilutions along the plate column or various volumes of solutions were pipetted in order to put in the desired well the required quantity of sample, analytes of ninhydrin reagent. During heating and cooling stages, the plates were covered in order to minimize the evaporation of the liquids. If not mentioned different, to seed-up the chemical reaction of ninhydrin, the plates were heated up to 100°C for 15 minutes. After a cooling time of 15-20 minutes (usually at room temperature), the optical densities of the solutions from the wells were recorded at 540 nm.

2.2.3 Reaction of ninhydrin with chitin and chitosan

To monitor the reaction of ninhydrin with chitin and chitosan solutions, the experiments were realized in ELISA plates. In each well 50 µL sample, containing the known amount of chitin or chitosan, and 50 µL of ninhydrin reagent were pipetted. After heating the plate at 100°C for 15 minutes, portions of 100 µL of 15% acetic acid were added to ensure the solubilization of chitin and chitosan. Then the plates were read at 540 nm.

2.2.4 Determination of absorption spectra

As the spectra of the product of reaction with ninhydrin were recorded with a spectrophotometer that read cuvettes, the reactions were performed in test tubes. In each tube 500 µL dH_2O, 500 µL of each sample and 1000 µL ninhydrin were pipetted. There were used the following categories of samples: 10 mM glucosamine, low molecular weight chitosan, yeast nitrogen base, yeast extract, uracil drop out mix, casein hydrolyzate and casamino acids. Each test tube was boiled in water bath for 15 minutes. After cooling, each solution was introduced into the cuvette of UV/Vis spectrophotometer for recording of their absorption spectrum from 350 nm to 800 nm.

3. Results and Discussions

One of the aims of this study was to prove that the ninhydrin reaction with compounds bearing amino groups could be miniaturized, i.e. can be realized in ELISA plates. The main drawbacks of ninhydrin reactions is the fact that the reactions mixture has to be heated at 100ºC for several minutes in order to conclude the reaction in a convenient interval of time. The "classical" way to perform this reaction is to make the reaction mixture in glass test tubes and to put these tubes in a vessel with boiling water. This step cannot be performed with ELISA plates. In this study, we have proved that "normal" ELISA plates can be used not only to mix the reagents but also to speed-up the chemical reaction by heating the plates were heated on a thermal block at 100º for more than 20 minutes, without damaging the plates and without disturbing the results.

Taking the advantages of using ELISA plates as reaction vessels, in a first series of experiments, the influence of the pH of the reaction media and the influence of concentration of ninhydrin were realized on a single ELISA plate.

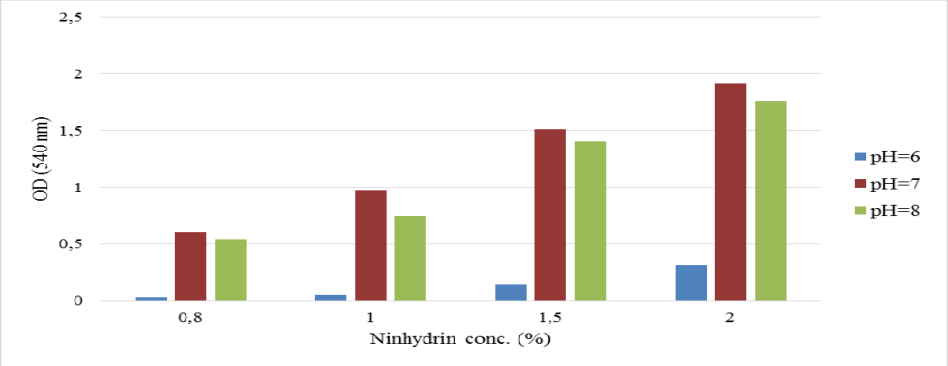

Figure 2. The influence of pH and concentration of ninhydrin on optical density of the colored product

From the results presented in Figure 2 one can see that the highest optical densities of the colored reaction product are obtained when the reaction is performed at pH 7. This is why, in the remaining experiments the reactions were carried out at pH 7.

Figure 2 presents also the influence of concentration of ninhydrin on the optical density of the final product. Although a plateau was not reached with the influence of concentration of ninhydrin used in this series of experiments, the reagent with the concentration of 2% ninhydrin was selected for the most of the rest of the experiments.

The influence of ninhydrin concentration on the optical densities of the reaction product was used to study the possibility to obtain standard curves with different linearity ranges, to better fit the curves to the concentration of the target analyte in the samples. In Figure 3 there

are presented only two such standard curves, realized at 0.8% and 2% ninhydrin concentration, respectively.

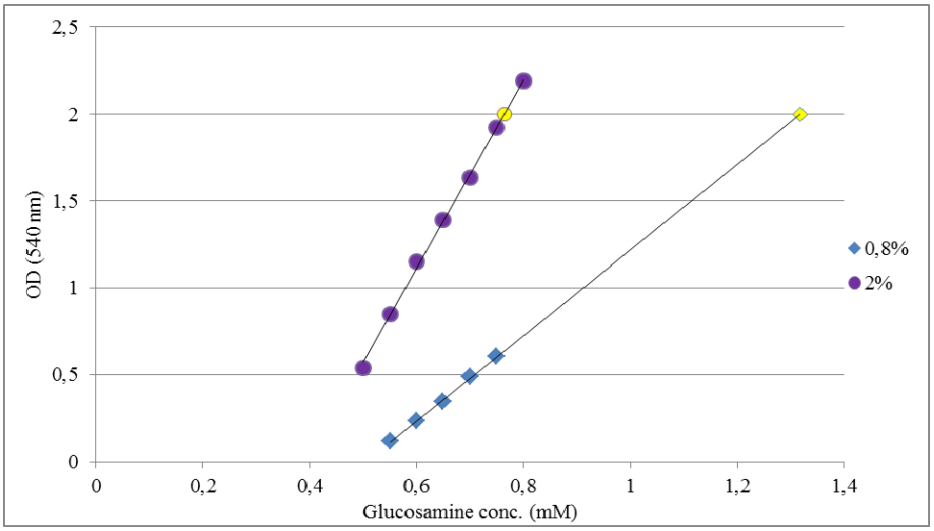

Figure 3. Standard calibration curves for glucosamine at two ninhydrin concentrations: 0.8% and 2%, respectively.

If we look into the values of regression lines (y=a·x+b, with a=slope, b=intercept of linear dependence of y=optical density on x=concentration of glucosamine) of the two standard curves presented in Figure 3 one can see that the concentration of ninhydrin have a major influence on the absorbance of final colored product, as the values of slopes of these two curves are in accordance with the differences in ninhydrin concentration (see Table 1). For example, for the standard curve realized with the reagent having the concentration of ninhydrin 0.8%, the optical density equal with 2 absorbance units is reached when the concentration of glucosamine is around 1.3 mM, while the same value of optical density is reached at approximately 0.7 mM glucosamine, when the ninhydrin concentration is 2% (value marked with yellow in Figure 3).

Using different concentrations of ninhydrin, beside changing the linearity range, it can be adjusted also the limit of detection (LOD) and of quantification (LOQ) in some extent.

Table 1. Values of slope, interception and regression coefficient for ninhydrin concentrations of 0.8% and 2%, respectively. Limits of detection (LOD) and quantification (LOQ) are expressed in mM glucosamine.

Ninhydrin conc.	a	b	R^2	LOD	LOQ
0.8%	2.46	-1.242	0.9984	0.205	0.621
2%	5.412	-2.136	0.9985	0.051	0.156

Another parameter of the reaction that was studied is the stability of the color of the final product. For this, after the plate was heated at 100ºC, the optical densities were recorded at various time intervals. From Figure 4 one can see that the color of the final reaction product have a linear decrease in the first 30 or 60 minutes, with a loss of the absorbance at 540 nm of about 4%, but suffer a huge decrease 20 hours.

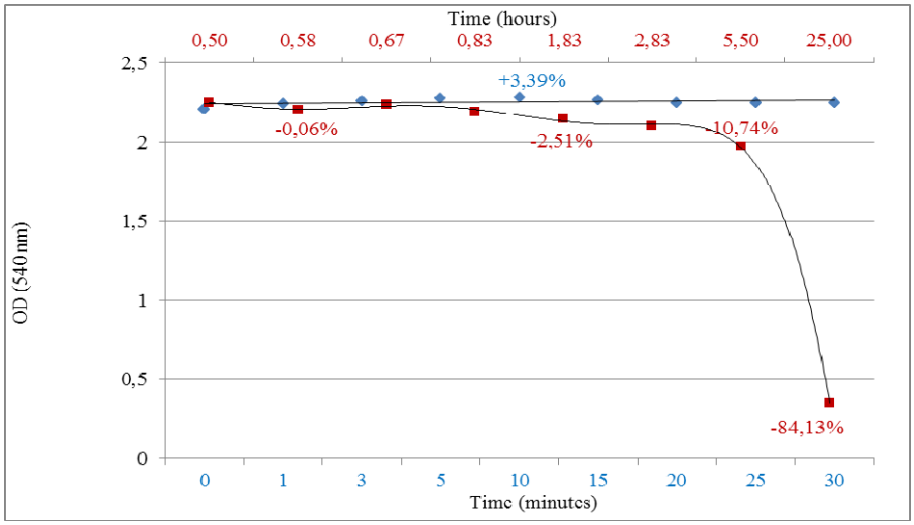

Figure 4. The stability of the colour of the reaction product. Lower horizontal axis, corresponding to the blue experimental points is in minutes, while the upper horizontal axis, for the red experimental points is in hours.

From the results presented in Figure 4 it is obvious that the optical densities of the plates hat to be recorded in the first 30 minutes after the heating step was ended.

Another objective of this study was to make the assay as short as possible. Taking into account that the chemical reaction of ninhydrin with the compounds bearing amino groups depends on the temperature, the influence of this parameters on the color intensity of the final product of the reactions was studied. Although it was selected as a regression curve a polynomial one, from Figure 5 it seems that after 100ºC there will be a plateau in the dependence of the color intensity of the final product on reaction temperature.

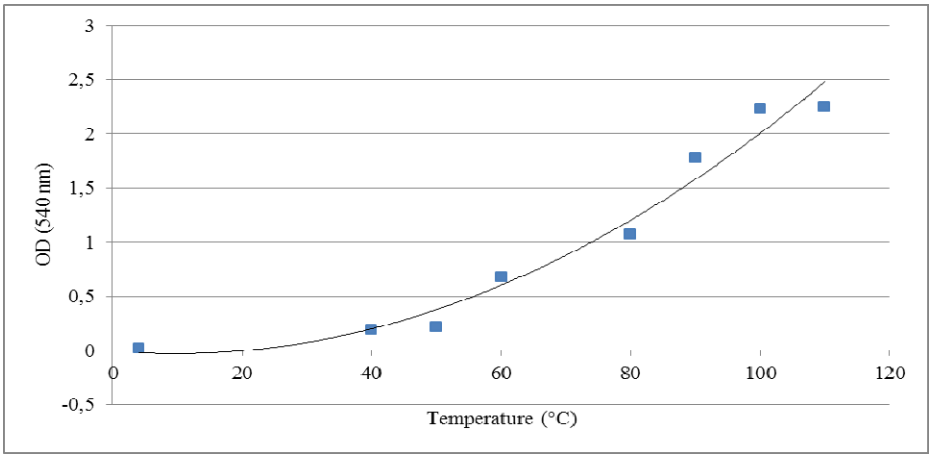

Figure 5. The influence of temperature on the optical density of the colored final product

Corroborating the results from Figure 5 with those from Figure 6, where the influence of the time of reaction on the optical densities of the colored final product is presented, it becomes clear that the reaction time cannot be decreased below 15 minutes without an important decrease of color intensity of the product. An increase of the reaction time above 20 minutes is not justified neither by color intensity nor by duration of the assay.

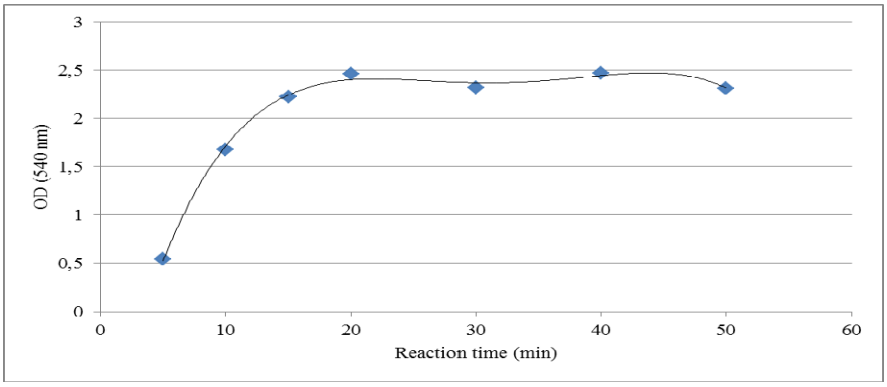

Figure 6. The influence of the reaction time on the optical density of the colored final product.

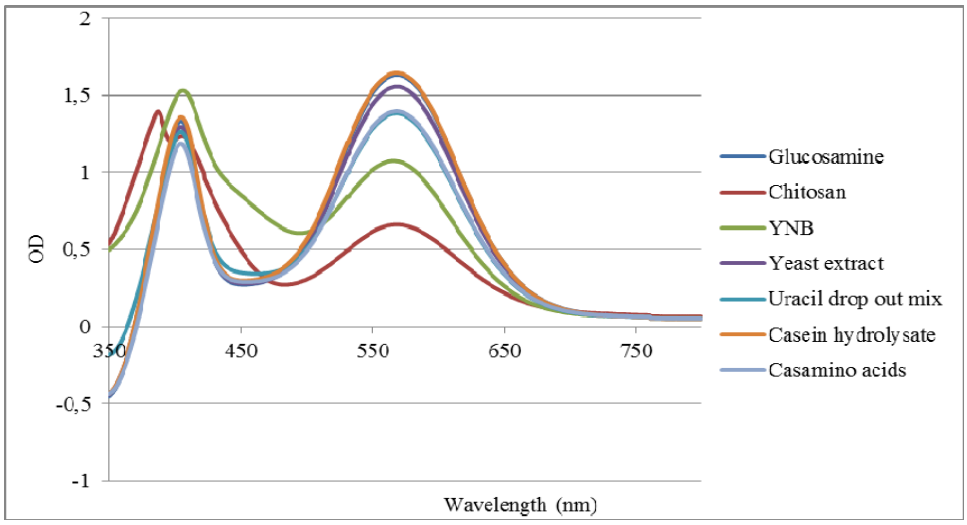

Figure 7. Absorption spectra of glucosamine, chitosan and some common reagents commonly used in molecular biology laboratories.

As the original method of the reaction of ninhydrin was applied to amino acids and peptides, it was checked the possibility that the final colored product to have different spectrum depending the chemical structure of the compound that have the amino group that react with ninhydrin. For this, the spectra of the final colored compounds resulted from the reaction of ninhydrin with several compounds and mixtures of chemicals, frequently used in molecular biology laboratories were recorded (Figure 7).

Unfortunately the chemicals and mixtures of chemicals and materials used to record the spectra of the colored products resulted after the reaction with ninhydrin do not present major difference in their spectra. All the tested reagents produce colored compounds with maximum absorbance around 568 nm and 405 nm, respectively. Only chitosan has a third absorption maximum at 387 nm, but the absorption of chitosan at 405 is high enough to not permit a discrimination of this compound in comparison with other chemicals tested. Considering this, we have to consider that the tested chemicals can interfere with the estimation of amino groups of chitosan and their presence in the reaction mixture has to be avoided, if the analysis of chitosan is the purpose of the assay.

The other aim of this study was to use the miniaturized assay of ninhydrin reaction for estimation of the concentration of amino groups of chitosan and further to estimate the degree of acetylation of chitosan. For this, chitin and chitosan samples were subjected to alkaline deacetylation reaction and samples were analyzed at selected time intervals.

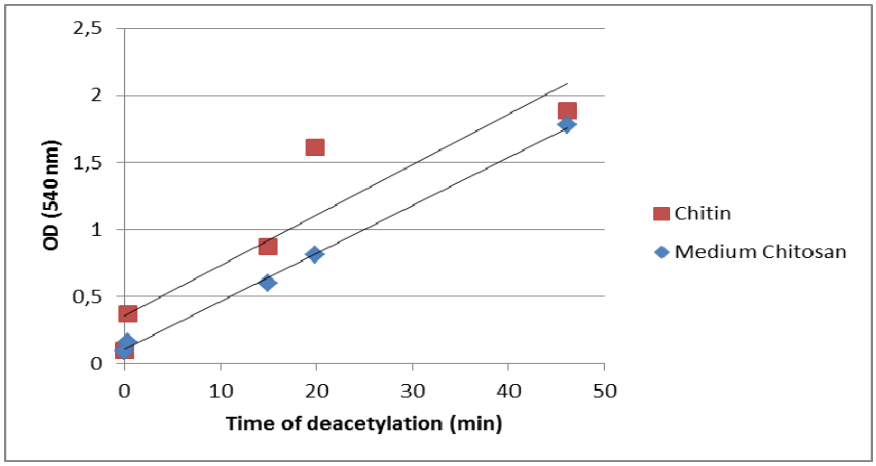

Figure 8. The estimation of degree of deacetylation of chitin and chitosan, using the reaction with ninhydrin.

Although the experimental errors are ranging between 10 – 15%, perhaps to some technical pitfalls that has to be solved in a future study, the results presented in Figure 8 clearly shows that the miniatures assay with ninhydrin can be used for the estimation of the degree of acetylation / deacetylation of chitin and chitosan. The large errors arise from the fact that the deacetylation reaction has to be realized in strong alkaline media, where chitin and chitosan are insoluble. Moreover, the reaction with ninhydrin take place at pH 7, but chitosan has its maximum solubility around pH 5. In some extent, the high values of errors can be reduced by increasing the numbers of repetition of the experiments, that can be easily accomplished when the assay is performed in ELISA plates that have 96 “reaction vessels”.

4. Conclusions

The method based on the reaction of ninhydrin with amino groups of chitosan was adapted to be realized in ELISA plates. The heating step was realized on a thermo-block. Using as analyte glucosamine, the dependence on the ninhydrin concentration of the linear range, slope of the calibration curve, limit of detection and quantification were studied. The dependence of the chemical reaction on temperature (optimal at 100ºC) and time of reaction (optimal 15 minute) were analyzed and the stability of the color of the final product was established (around 30 minutes). The miniaturized method was applied to study the deacetylation of chitin and chitosan in alkaline conditions.

Acknowledgement

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Short Communication

PRELIMINARY OUTCOMES REGARDING THE OPTIMIZATION OF SEPARATION OF PLASMIDS BY AGAROSE GEL ELECTROPHORESIS

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Abstract

The aim of this study was to find some of the necessarily conditions to improve the separation of plasmids (DNA molecules) by agarose gel electrophoresis. The preliminary results here presented shown that the separation of electrophoretic bands depend on the potential applied to the electrodes and on the composition and concentration of the running buffers. The separation was optimized for the following plasmids: pYES2, pCTCON2, Taq_pET22b+ and ChiA_pUC57. These plasmids have been in two forms, digested by restriction enzymes and undigested, in supercoiled form. By using 0.5X TBE running buffer and 180 V potential applied to the electrodes, the running time was decreased from 60 until 20 minutes.

Keywords: agarose gel electrophoresis, DNA plasmid, DNA electrophoresis, TAE, TBE.

1. Introduction

Agarose gel electrophoresis is the method that is most used for the separation of DNA fragments ranging from 100 bp to 25 kbp [1]. Agarose gel electrophoresis is a simple and

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inexpensive technique [2]. In order to separate DNA fragments by agarose gel electrophoresis, the samples containing DNA are loaded into pre-cast wells in the agarose gel and an electric current is applied. As DNA molecule contain phosphate groups, that have negative charges, the DNA fragments will run toward positive electrode. Due to the fact that DNA fragments have a uniform ratio of their molecular masses to total charges, these molecules will be separated according with their size, in a pattern such that the distance traveled is inversely proportional to the log of their masses[3].

When electrophoresis is running, there is a balance between beauty and efficiency; if the gel is run at high voltage, the samples move faster, but the bands are smeared and the gel can melt if the voltage is high enough. If the gel is running at low voltage, it takes long time, but the bands are clearer and nicer [4].

In DNA electrophoresis it is commonly used a constant voltage. During the running of the electrophoresis, the current (mA) increases and it warms the running buffer. To avoid this it is indicated to keep the voltage to a low value (10 v/cm of the gel). To a small size gel (7-10 cm length), the voltage used is ranging from 70 to 100V and the migration of the bands will run for 30-50 minutes. The voltage increasing over this ranging, using TAE or TBE buffers at normal concentration (1X), will increase migration speed of the DNAs, but the buffer and the gel will heat asymmetrically [5]. Increasing the time of electrophoresis do not necessarily produce a beneficial effect on the length or intensity of the comet tail of supercoiled form of DNA subjected to separation. These tails seems to consist of loops of DNA that are attached to the nuclear matrix, as the DNA fragments that are not supercoiled produce clear bands, without tails[6].

To visualize the results after electrophoresis, it is necessary to keep the gel in a buffer that contains a fluorophore or to include the DNA-binding fluorophore in gel before polymerization. Ethidium bromide is an usual DNA binding fluorophore. The disadvantage is that EtBr is its toxicity [4].

In this study, we present some preliminary results of the optimization of electrophoretic separation of plasmids in agarose gels. The optimal composition and concentration of the running buffer were selected and the influence of the potential applied to the electrodes on plasmid separation was investigated.

2. Materials and Methods

2.1. Materials and Reagents

The chemicals were of analytical grade or better and were purchased from Sigma via Redox Lab Supplies Com S.R.L: Tris (Tris(hydroxymethyl)aminomethane, #10708976001),

acetic acid (#320099), boric acid (#B7901), EDTA (Ethylenediaminetetraacetic acid # E9884), agarose (#A9539), ethidium bromide (#E7637).

To cast and run the gels, a horizontal electrophoretic system from BioRad (Bucharest, Romania) and a power supply (Consort EV202, BioRad, Bucharest, Romania) were used. The gels were photographed using a documentation gel system (ECX-F15.M, Suebia, Germany). The images were analyzed with GenAnalyzer software (http://www.gelanalyzer.com/).

2.2. Methods

Running Buffers. In this study two type of running buffer were used: TAE (Tris/Acetate/EDTA) and TBE (Tris/Borate/EDTA). Stock solutions of 50 times more concentrated than the working solution (50X) of TAE running buffer was prepared in dH_2O, mixing 48.4 g Tris, 11.44 mL acetic acid (glacial), 20 mL 0.5M EDTA to 200 mL final volume. Stock 10X TBE running buffer was prepared dissolving 108g Tris, 55g boric acid, 7.5g EDTA disodium salt in dH_2O to 1000 mL final volume.

Agarose gel preparation. Agarose gels with 0.8% agarose in running buffer were prepared by purring the hot solution (60^oC) in the casting mold (7 x 6 x 0.5 cm) of the electrophoretic system (Bio-Rad).

Sample preparation. As samples, there were used four plasmids, isolated using MINIPREP protocol[7], with molecular mass ranging from 5000 bp to 8000 bp: pYES2 (5.9 kbp), pCTCON2 (7,2 kbp), Taq_pET22b+(8 kpb), ChiA_pUC57 (5.1 kbp). For comparison reasons, in the electrophoretic experiments, the plasmids were used in native, supercoiled state and after digestion with EcoRI restriction enzyme. For this, at 30µL plasmid solution (2.696 µg/µL), 10 µL sterile H_2O, 3 µL restriction enzyme EcoRI (10u/µL) and 6µL 10X EcoRI buffer were incubated at 37^oC for 16h.

Agarose gel electrophoresis. Gel electrophoresis experiments were performed using 0.8% agarose gels and 7 X 6 cm mini-sub cell GT 8 gel rigs (Bio-Rad). Electrophoresis was run until the dye (bromophenol blue) arrived at less than 0.5 cm from the positive edge of gel. The gels were stained with EtBr (0.1 mg/mL) for 15 minutes and rinsed with dH_2O for 3 times. After staining, the gel is viewed and photographed in UV light.

3. Results and Discussions

A typical agarose gel electrophoresis of nucleic acids (plasmids) is presented in Figure 1. The first lane is the molecular weight scale (in kpb). The samples containing the plasmids digested by EcoRI migrate faster than the same plasmids which are not digested.

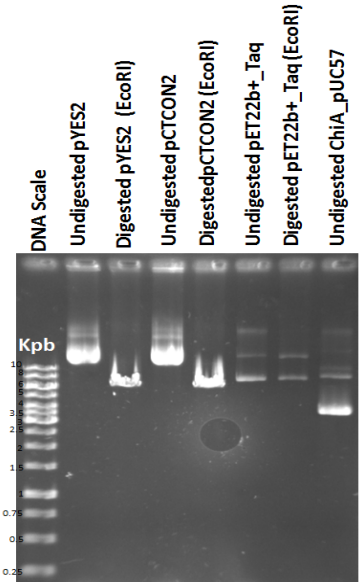

Figure 1. DNA agarose gel electrophoresis of supercoiled and digested plasmids. DNA scale is in the first column and plasmids in the others lanes. The voltage applied to the electrodes was 80V, running buffer TBE 1X and migration time was 60 minutes.

As one on the aims of this study was to find the conditions that lead to a reduction of the total running time of the electrophoresis, the first series of experiments were directed to reveal the influence of the potential (voltage) applied to the gel on the velocity of migration of nucleic acids.

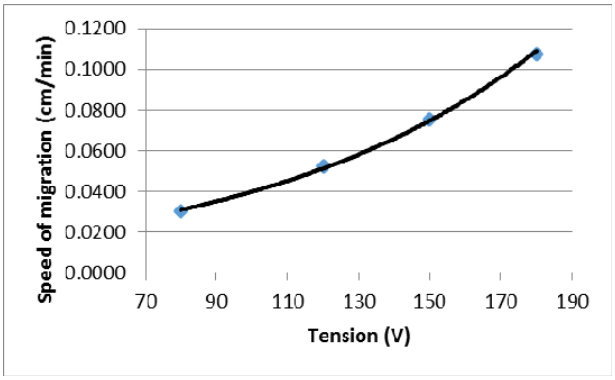

Figure 2 The variation of the speed of migration of undigested plasmid pYES2 according to the potential applied to the electrodes, using 0.5X TBE running buffer

From **Figure 2**, one can see that the higher the voltage, the higher is the migration speed of nucleic acids. The best correlation with these results is an exponential equation ($y=a\cdot e^{b\cdot x}$) where y is the speed of migration of nucleic acids (in mm/min) and x is the voltage applied to the agarose gel. In **Table 1** there are presented the values of coefficients **a** and **b** for the equations obtained for the plasmids used as samples. For the samples pYES2 and pCTCON2, in native state, that are supercoiled, the values of parameters **a** (quite similar with the slope in the case of linear regression line) are smaller than in the case of the same plasmids, but

analyzed after digestion with EcoRI. In these cases, the enzyme cuts the double helix and the nucleic acid chain become uncoiled.

From **Figure 2** one may see that when the voltage is increased from 80 to 160V, the speed of migration rise from 0.3 to 0.8 mm/min. At high voltage, the temperature of the gel can increase above 40ºC and the agarose gel can melt. Without a proper device to thermostat the system, the voltage cannot be increased above 180 V and, consequently, the velocity of the nucleic acids cannot be higher than 1 mm/min.

Table 1. Parameters of the exponential equations that express the dependence of the speed of migration of plasmids (mm/min) on the voltage applied to the agarose gel

Plasmid	a	b	R^2
pYES2 (undigested)	0.113	0.012	0.9986
pYES2 (digested)	0.147	0.011	0.9998
pCTCON2 (undigested)	0.114	0.012	0.9975
pCTCON2 (digested)	0.148	0.011	0.9997
Taq_pET22b+ (undigested)	0.145	0.011	0.9991
Taq_pET22b+ (digested)	0.145	0.011	0.9993

The experiments done with running buffers TBE and TAE have revealed that with TAE, the temperature of the gel is higher than with TBE, at the same buffer concentration. For this reason, the rest of experiments were performed in TBE. Another factor that was taken into consideration was the concentration of the running buffer. As it is depicted in Figure 3, the best results, here expressed as higher migration speed of the nucleic acid used as sample, were obtained when the concentration of the TBE running buffer was 0.5X.

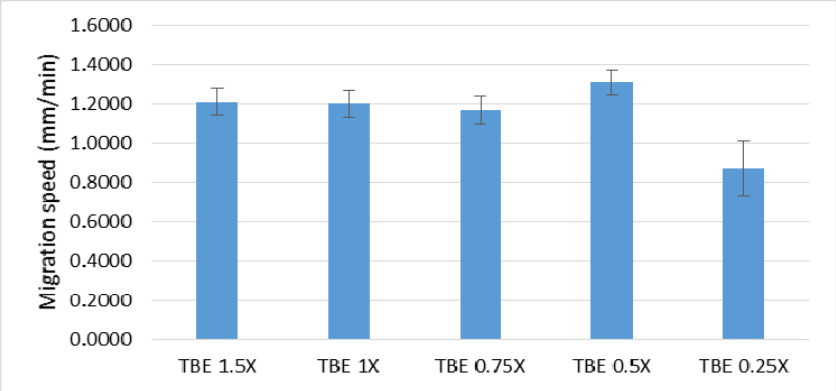

Figure 3 Migration speed of plasmid pCTCON2, digested with EcoRI, in different TBE concentrations

Considering only these two modifications, i.e. the use of 0.5 X TBE running buffer instead of 1X TBE or 1X TAE and a potential applied to the electrodes of 180 V, we have reduced the running time from 60 to 20 minutes, and, in the same time, we have decreased the price of the analysis with 25%. In the experiments yet to come, we will optimize other parameters of the agarose gel electrophoresis, aiming to increase the selectivity and further decrease the price of the analysis.

4. Conclusions

For the separation of some plasmids with chain length ranging between 5000 bp and 8000 bp the best results were obtained when 0.5X TBE running buffer was used. Using this buffer and a potential of 180V applied to the electrodes, the running time of separation was reduced until 20 minute. Beside a 3 times reduction of the time of electrophoretic separation, we have to consider a reduction with 25% of the price of analysis, when half of the concentration of normal buffer was used.

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Short Communication

IMPROVEMENT OF STAINING / DESTAINING STEPS OF PROTEINS SDS - PAGE

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ABSTRACT

The polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate is one of the most used method for separation and identification of proteins in biochemistry laboratories. The time of the total analysis and the sensitivity of this technique are important parameters for optimal activities in this field. The purpose of this paper is to improve the sensitivity, i.e. to obtain a stronger signal of the bands of separated proteins and to reduce as much as possible the total run of the method. For this, the composition of the running buffer and the potential applied to the electrodes was studied. The intensity of the color of the bands of separated proteins, transformed by GelAnalyser software in areas of peaks, was optimized by changing some parameters of the staining and destaining procedures.

Keywords: Polyacrylamide gel electrophoresis, SDS, SDS-PAGE, Coomassie, protein electrophoresis.

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1. INTRODUCTION

One of the most used technique to study the separation and to identify proteins is polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). This method is rapid, simple and have a good sensitivity. The principle of separation of proteins is that in the presence of SDS, that bind to the hydrophobic part of the protein molecules, the proteins become negatively charged due to SO_3^- groups of SDS molecules. In an electric field, the proteins covered with SDS will migrate towards positive electrode, irrespective of their initial charge. The velocity of the migration of proteins covered with SDS will depend mostly on the molecular mass of the polypeptide[1,2].

The proteins separated on the gel (SDS-PAGE) in order to be detected have to be colored. There are several methods of staining the proteins in the SDS-PAGE gels: coloration with Amidoblack[3], Coomassie Brilliant Blue[4,5], Silver stain[6,7], fluorescent dyes[8], negative stains[9] with organic dyes[10] or with copper chloride[11], zinc chloride[12], potassium acetate[13], and many others.

Most of the methods used to color the proteins stain also the gel matrix. A destaining procedure is required to clear the gel and to reveal with high contrast the proteins bands. Most of the destaining methods use hazardous solvent like methanol and / or acetic acid[4].

In this study we focus on the optimization of the staining / destaining procedures, aiming to reduce the number of solutions used for these steps and the total time for coloration / discoloration of proteins bands.

2. MATERIALS AND METHODS

All chemicals used in this study were of analytical grade or purer. Most of them were bought from Sigma (Redox Lab Supplies Com S.R.L,Bucharest, Romania): Tris (#77-86-1), Glycine (#56-40-6), Sodium docecyl sulfate (#151-21-3), Tris-HCl (#1185-53-1), Glycerol (#56-81-5), 2-Mercaptoethanol (#60-24-2), Bromophenol Blue (#115-39-9), Bovine serum albumin (#9048-46-8), Acrylamide (#79-06-1), N,N′-Methylenebis(acrylamide) (#110-26-9), N,N,N′,N′-Tetramethylethylenediamine (#110-18-9), Ammonium persulfate (#7727-54-0), Coomassie Brilliant Blue (#6104-58-1), Acetic acid (#6104-58-1), Ispropanol (#67-63-0)

The electrophoresis was run in a Mini-protean III cell (Bio-Rad) at a constant voltage of 200V per slab gel using a Power PAC 300. Other items used were: casing gel mold, 8x10 cm glass plates.

Buffers preparation. Running buffers = 250 mM Tris, 1.92 M Glycine, 1% SDS, pH 8-8.5, made as 10 times concentrated (10X) stock solution, comparing with the working solution and sample buffer = 250 mM Tris-HCl, 25% Glycerol 1M 2-Mercaptoethanol, 0.05%

Bromophenol Blue, pH 6.8. Before electrophoresis, the running buffer was diluted to desired concentration.

Samples preparation. For comparison reasons, all the analyzed samples have contained bovine serum albumin (BSA) at 3 mg/mL in phosphate-buffered saline (PBS) and before running the solutions were diluted 1:2 with sample buffer.

Polyacrylamide gels preparation. In all the experiments from this study, 10% polyacrylamide with an acrylamide:BIS ratio of 30:0.8 separation gels (100 mm×80 mm×0.75 mm) and 4,5% stacking gels, prepared after Laemmli [14] with small modifications, were used. For 10% separation gels there were mixed 2.78 mL dH_2O, 3.75 mL 1 M Tris HCl (pH 8.8), 100 µL 10% SDS, 3.33 mL acrylamide/bis-acrylamide (30%/8%), 100 µL TEMED and 30 µL 20% APS. To prepare the stacking gels the following solutions were mixed: 3.645 mL dH_2O, 625 µL 1 M Tris HCl (pH6.8), 50 µL 10% SDS, 830µL acrylamide/bis-acrylamide (30%/8%), 5 µL TEMED and 15 µL 20% APS.

Staining / destaining the gels. The gels were immersed in solution A (0.05% CBB, 25% isopropanol, 10% acetic acid), heated for 30 seconds in microwave oven, shacked for 15 minutes at room temperature. Then the gels were immersed in solution B (0.05% CBB, 10% isopropanol), heated 30 seconds in microwave oven and heated again for 30 seconds but in solution C (0.002% CBB, 10% acetic acid). For destaining it was used a solution of 10% acetic acid (solution D) in which the gels were shaken for 15 minutes.

Gels documentation. After migration, gels were stained, photographed and the images were analyzed with GelAnalyzer software (http://www.gelanalyzer.com/). This software was used to transform electrophoretic bands into peaks and to calculate theirs areas. It was also used to measure the distance of migration of various peaks and to calculate Rf or to estimate the molecular weight of proteins.

3. RESULTS AND DISCUSSIONS

In Figure 1 a typical analysis of an image of SDS-PAGE gel is presented. This software transform the bands of colored proteins into peaks and the quantity of dye associated with one band into area of that peak, considered as conventional units (pixels).

This software is useful to create calibration curves and, based on them, to estimate the quantity of the protein from a band. For example, in Figure 2 there is presented the calibration curve that make a correspondence between the quantity of the protein loaded on each lane and the areas of the peaks of each lane (the software name the area of the peak as raw volume and the conventional units are in pixels). The correlation coefficients of such calibration curves were better than 0.99 for proteins with molecular masses ranging from 12 until 116 kDa.

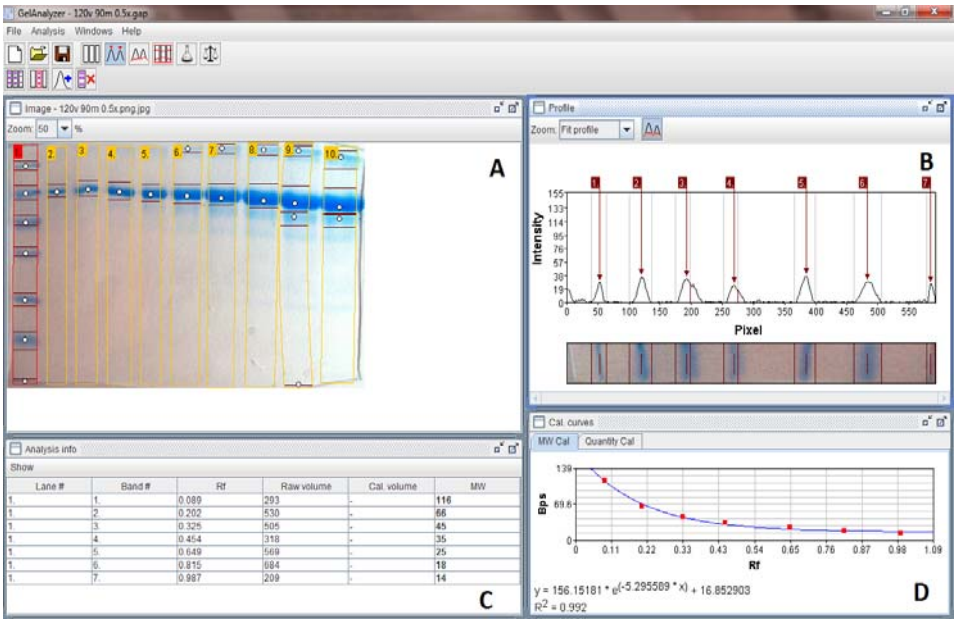

Figure 1. The main panels of GelAnalyser software. Panel A - the image of the entire electrophoresis gel. Panel B – the bands from the selected lane from panel A are transformed in peaks and their area are computed in panel C, as raw volume (conventional units). Based on the molecular weights of the markers, in panel D, the correspondence between the molecular mass and the migration (as Rf) of the protein is presented.

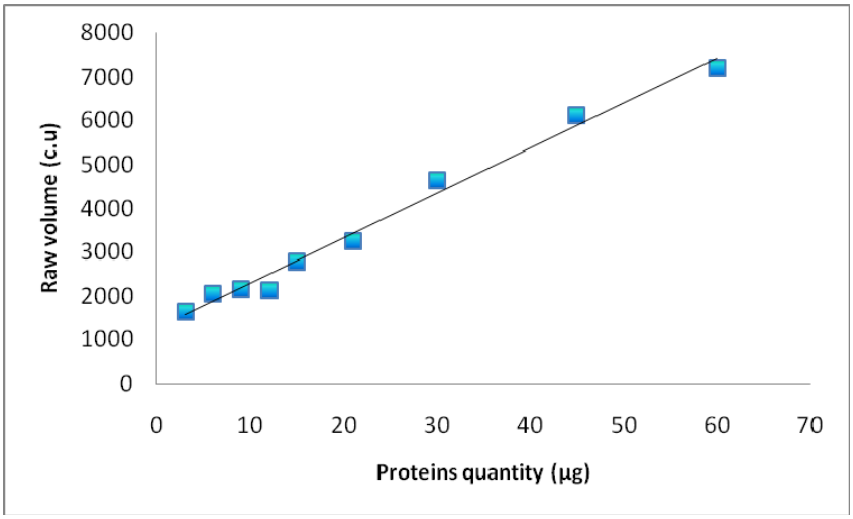

Figure 2. Variation of peak areas (color intensity of bands) on quantity of protein loaded per lane.

In order to reduce as much as possible the total running time of electrophoresis, there were studied the influence of the composition and concentration of the running buffer. As Figure 3 shows, at buffer concentration higher than 1X the migration of protein is higher than when weaker buffers are used. Considering this, the following experiments were realized with running buffer having the concentration 1X (normal dilution).

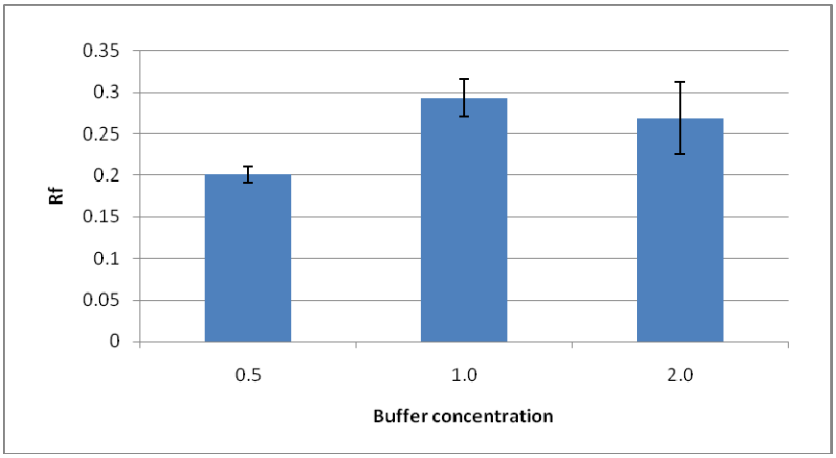

Figure 3. The dependence of Rf of protein on the running buffer concentration

The main objective of this study was to improve or to optimize the steps of the staining procedure of the gels. The most used protocol to stain the proteins separated by SDS-PAGE is staining with Coomassie Brilliant Blue R-250 (CBB) dye[15]. Currently, there are two type of procedures to stain the SDS-PAGE gels for proteins using Coomassie Brilliant Blue, the so call "classic" method, when the gels are stained overnight[5] and the "rapid" technique[4], when the gels are stained only several minutes. There are authors that have concentrated their efforts to reduce the time of the staining step and other to reduce the destaining phase[16].

For staining stage, there are necessary three solutions: solution A (0.05% CBB, 25% isopropanol, 10% acetic acid), solution B (0.05% CBB, 10% isopropanol), solution C (0.002% CBB, 10% acetic acid). For destaining, usually, it is used only a solution of acetic acid (solution D is10% acetic acid).

In the "rapid" method the gel is immersed in solution A, heated in microwave oven for 30 seconds and kept at room temperature 15 minutes, under constant shake. After 15 minutes, the gel is immersed in solution B and for 30 seconds is heated in microwave oven. The same operations are performed with C. The gel colored in this way is destained with solution D, heating it again in microwave oven for 30 seconds and that kept at room temperature for 15 minutes, on shaker, with a paper napkin placed on gel. In total, this "rapid" procedure takes around 40 minutes.

In order to speed up the staining / destaining procedures, we have applied the strategy to omit one-step at the time. From the standard "rapid" protocol, we have omitted one step and recorded the results, in this case the area of the peaks. Based on the results presented in Figure 4, we have concluded that the solution A cannot be omitted from the staining protocol, but the other two (i.e. solution B and C) may be absent, without decreasing very much the areas of the peaks.

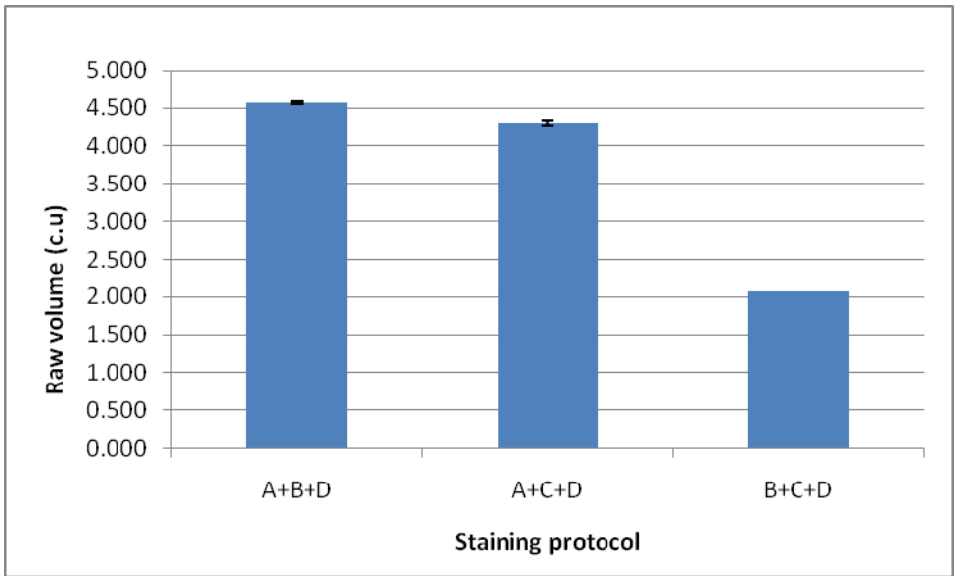

Figure 4. The dependence of the areas of the peaks on the use of the staining solutions (for details see the text)

Renouncing to use the solutions B and C, we have monitored the influence of the time of coloration of the gels with solution A. From Figure 5 one can see that a staining time of 15 minute will be enough to properly stain the gels. Larger time intervals will not increase the intensities of the bands and for the rest of the experiments the staining procedure was to heat the gels for 30 seconds in microwave oven and to color the gel for 15 minutes, under constant shaking.

Due to the fact that SDS inhibits the staining[17], we have introduced before the staining procedure a step in which the gel was simply washed with water. First the gel immersed in water was heated in microwave oven for 30 seconds, and then the gel was shaken for 1 to 10 minutes. As it can be seen from Figure 6, the stage when the gel was shaken in water can last only 3 minutes to obtain the best results.

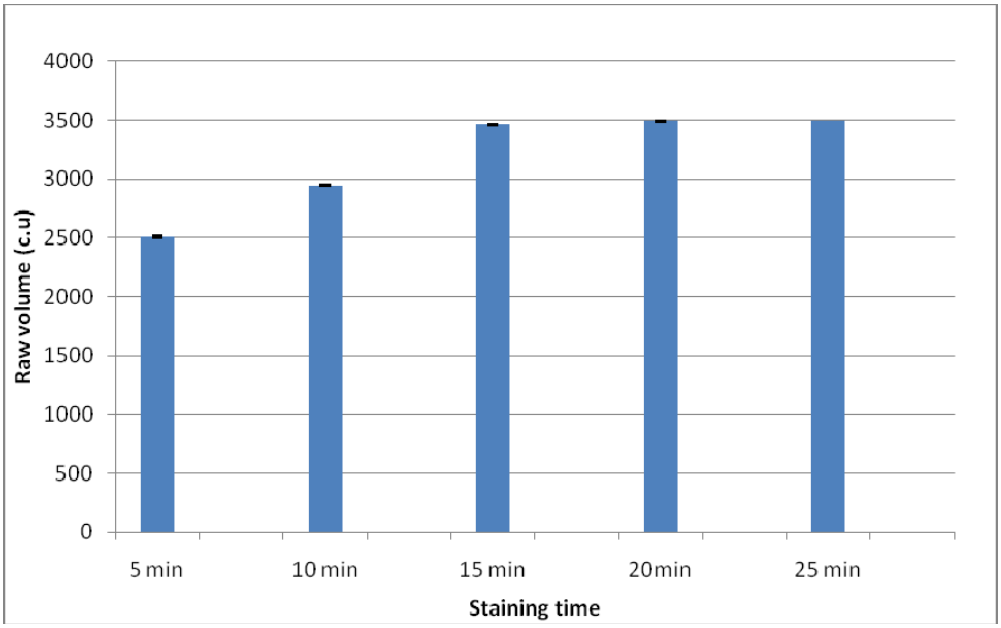

Figure 5. The dependence of the areas of the peaks on the time used for staining procedure, when only solution A was used for coloration of the gel.

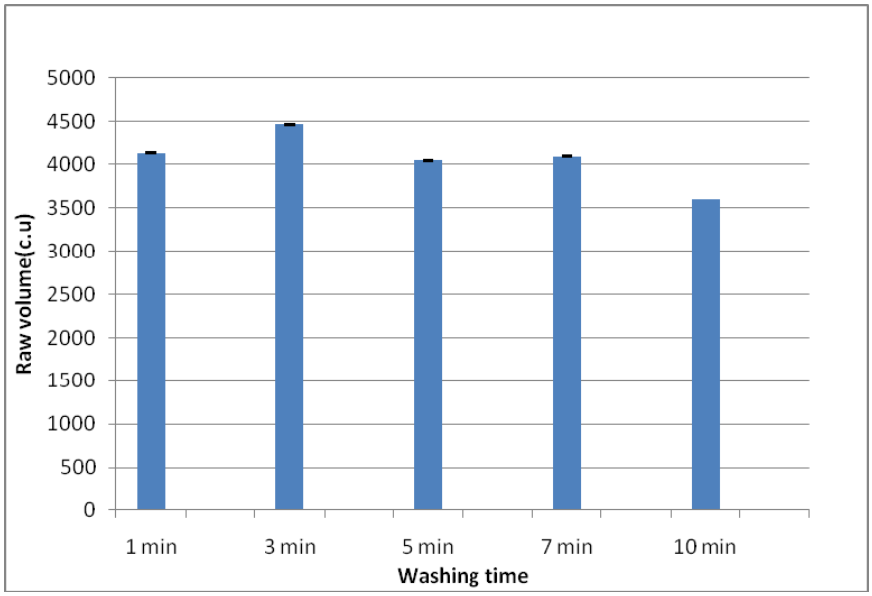

Figure 6 The influence of the time of washing step applied to the gel before staining procedure, to washout the SDS

The attempts to optimize or reduce the time of destaining procedure were addressed to observe the influence of various combination of destaining solutions, made from acetic acid, methanol, water or combination of them. From the results presented in Figure 7 one may see that comparing with the published procedure[18] when the destaining was carried out with mixture methanol / acetic acid (50% / 10%), better results, i.e. higher areas of the peaks, are obtain when the concentrations of these solvents are lower. These results conduct us to the idea to use only water for destaining and, indeed, the best results are obtained in this case. For this, we have used in the remaining experiments, water as solvent for destaining step. The gel was immersed in water, heated in microwave oven for 30 seconds and shaken for 15 minutes at room temperature.

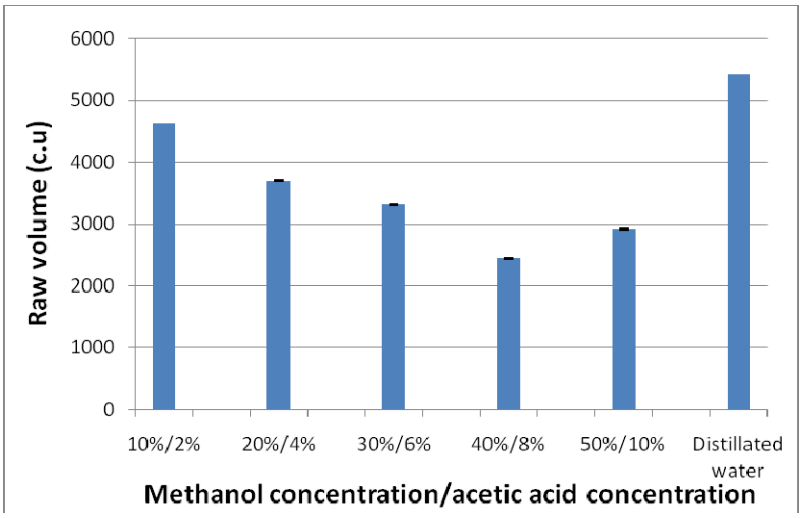

Figure 7. The influence of composition of destaining solution on raw volume (peak area) of protein bands.

This last step was further improved by changing the water repeatedly. In fact, the importance of repeating the washing procedure for destaining is presented in Figure 8, when the first figure represent the total time of the destaining procedure and the second figure represent the time of each step when the gel was shaken at room temperature. For example, the best results are obtained when the gel was maintain in water, after heating in microwave oven for 30 second 5 minutes and this procedure was repeated for 3 times (5x3).

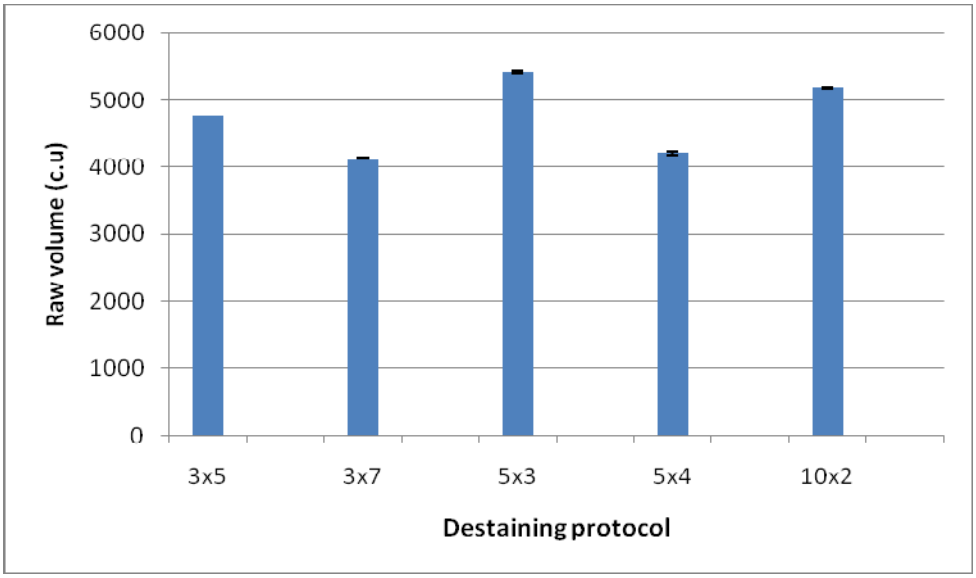

Figure 8 The influence of washing / destaining step on the areas of the peaks (bands): first figure represent the time when the gel was shaken in water, after a 30 seconds heating in microwave oven and the second figure is the number of repeated steps.

The SDS-PAGE is an analytical technique used very often for protein separation. The time of the total run and the sensitivity of this technique are important parameters for the productivity in biochemistry laboratories. In this study, we have optimized the staining / destaining procedures.

4. CONCLUSIONS

The steps of staining procedure of the gel obtained in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were studied and optimized. The staining procedure was reduced from 3 steps to only 1 and the time from 40 to 30 minutes. Beside this some reagents and solutions were also eliminated that conducts to reduction of the price of analysis.

The influence of the concentration of the running buffer and of the voltage applied to the electrodes was also studied. By increasing the voltage to 170 V the running time of the electrophoretic separation was reduced from 90 to 60 minutes.

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Short communication

PRELIMINARY RESULTS ON ESTIMATION OF THE DEGREE OF ACETYLATION OF CHITOSAN BY UV SPECTROPHOTOMETRY

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ABSTRACT

The degree of acetylation of chitosan is an important parameter to be taken into consideration when chitosan is used as starting material for realization of derived products with applications in pharmacy and medicine. Although considered a reliable method, the determination of the degree of acetylation of chitosan by UV spectrophotometry has as main drawback: the lack of accuracy. Starting from an already published method, based on the UV spectrophotometry of N-acetylglucosamine and glucosamine molecules and considering that UV absorbance of chitosan is due to the absorbance of the two monomers, some preliminary results of an improved method for estimation of the degree of acetylation of chitosan are proposed. The improvements are based on the use of phosphoric acid as solvent for chitosan (and of monomer molecules - N-acetylglucosamine and glucosamine) and the use of optical density at 197 nm.

Keywords: UV spectrophotometry, chitin, chitosan, degree of acetylation.

1. INTRODUCTION

Chitin is considered to be the second most abundant natural synthetized compound, after cellulose[1]. The most important source of natural chitin are invertebrates[2], of which crustaceans are by far the most available source[3]. In addition to its main importance as

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structural and building material in many types of organisms, chitin importance is rising due to unnumbered applications in almost all the fields of human activities. The success of chitin is, in fact, due to chitosan, the more soluble derivative of chitin. Considering the natural abundance that makes any application sustainable, chitin, together with chitosan, has been proposed for a broad range of industrial applications, including separation techniques[4], biotechnological processes[5], wastewater treatment[6], food, agriculture[7], cosmetics[8], pharmaceutical[9], medical, tissue engineering[10], biomaterials[11], and the list can continue almost endlessly. Through chemical modification, chitosan can be considered as a precursor to produce other materials, increasing its applications further.

Chitin and chitosan are poly[β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose], simply named N-Acetyl-D-glucosamine (NAG) and poly[β-(1→4)-2-amino-2-deoxy-D-glucopyranose], named also glucosamine (GN) respectively. Chitosans are the fully or partially N-deacetylated derivatives of the chitin polymers that are usually produced by treatment with alkali[12]. The molar fraction of the NAG units is defined as the degree of N-acetylation (DA)[13]. Chitin is the polymer with the highest DA, virtually equal to 1 (or 100%), and chitosan is the polymer with the lowest DA (ideally 0, or 0%). Solubility is greatly dependent on DA, as chitin is virtually insoluble and chitosan is quite soluble, at least in in dilute acidic solutions. When DA is around or below 50%, the polymer becomes soluble in weak acidic media as the amino group ($-NH_2$) will ionize as $-NH_3^+$. Beside solubility, many other chemical and biochemical properties of these polymers depend on DA[14]: mechanical properties, especially of the films of these polymers, cytotoxicity, cellular uptake, crystallinity, supramolecular aggregation, and so on.

Although at first glance considered simple, the methods used to determine DA of chitosan possess inherent drawbacks and limitations. Many methods have been published for determining the DA, including spectroscopy[15], Fourier-transform infrared[16], nuclear magnetic resonance spectroscopic methods[17], dye absorption[18], potentiometric and conductometric titrations[19], ninhydrin assay[19], elemental analysis[20], circular dichroism[21], chromatographic separations (GPC)[22], and many others. Some of the above-mentioned methods require high-priced instrumentation and high-skilled personnel and some of them have a high level of errors. Consequently, there is a need for an uncomplicated, trustworthy and accurate method for determining the DA of chitosan. Spectrometric methods generally enter in this category and many spectrometric techniques were proposed for calculation of the DA of chitosan. The group of Liu [23] have determined DA of chitin and chitosan by UV spectrophotometry using dual standards, proving that their method is a simple and accurate alternative. In this study, some preliminary results regarding the estimation of the degree of acetylation of chitosan based on direct UV spectrophotometry, similar with the method of Liu[23] with some minor modifications, are presented.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

The following chemicals were used in this study: glucosamine (Sigma, #G1514), N-Acetyl-D-glucosamine (Sigma, #A8625), chitin from shrimp shells (Sigma, #C7170), chitosan from shrimp shells, ≥75% (deacetylated)(Sigma, #C3646), partly deacetylated chitin (Roth #5375), orthophosphoric acid (Sigma, #438081). All other chemicals were of analytical reagent grade.

2.2. UV Spectra

UV spectra of standard and sample solutions were recorded on a double beam scanning spectrophotometer, model T90, from PG Instruments (Leicester, UK). The standards and samples were dissolved in 0.1 M orthophosphoric acid.

2.3. Methods

Stock solutions of glucosamine (GN) and N-Acetyl-D-glucosamine (NAG) with the concentration of 0.5 mM were realized in phosphoric acid 0.1 M. For standard curves, these solutions were diluted in the range 0.5 – 0.05 mM.

Stock solutions with concentrations between 0.1 – 0.15% of chitin and chitosan (2 batches from Sigma and 1 for Roth) were dissolved in phosphoric acid 0.1 M. Dilutions to have around 1 – 1.5 mg/100 mL chitin or chitosan in phosphoric acid 0.1 mM were performed and considered as unknown samples.

A chitin solution was subjected to deacetylation procedure, mixing it with 40 % NaOH, (final concentration of chitin 155 mg per 100 mL solution). The reaction mixture was boiled under reflux, and samples (0.5 mL) were taken at 0, 1, 2, 4, and 8 hours. The samples were dissolved in 4.5 mL of 0.1 M phosphoric acid and UV spectra was recorded. The final concentration of chitin, respectively chitosan, in the analyzed samples was considered to be 15.5 mg/100 mL solution.

All experiments were performed in duplicate, standard deviations and errors being calculated using MS Excel software.

3. RESULTS AND DISCUSSIONS

Usually, chitosan is obtained by deacetylation of chitin by treatment with hydrolytic enzymes, like chitinaze[13] or chitosanase[24], or with concentrated alkali at high temperature[12]. If the starting material, i.e. chitin, is highly purified, the chitosan obtained by these methods is quite pure, having very low levels of contaminations. Chitosan molecule is composed of two monomeric units N-acetylglucosamine (GNA) and glucosamine (GN). Both these units are far UV chromophoric groups. The results have shown that these two

groups, and especially amino and N-acetylamine functions, do not interact with the polymeric chain in a manner that will affect its adsorption in the UV region[23].

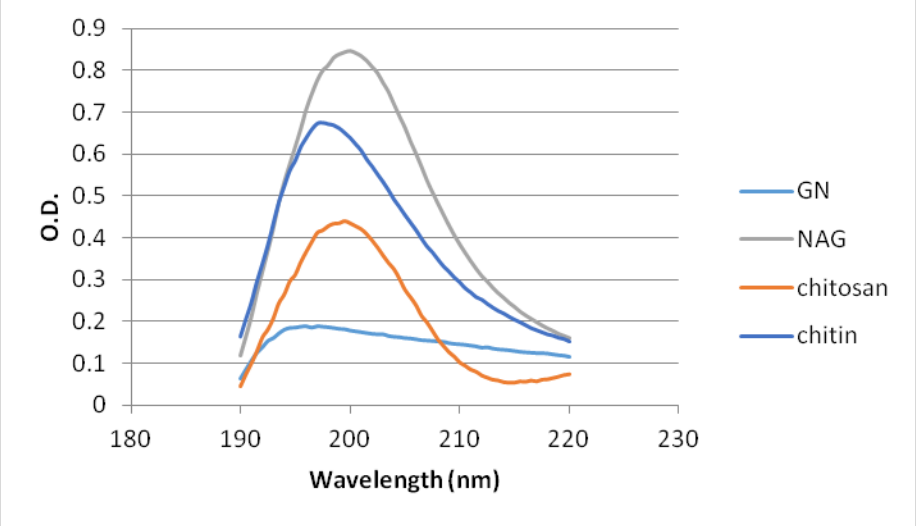

Figure 1. UV spectra of N-acetylglucosamine and glucosamine, chitosan and chitin

In this study, GNA and GN were used as standards to represent these units from chitosan. In Figure 1 there are presented the UV spectra of solutions of N-acetylglucosamine, glucosamine, chitin and chitosan. When comparing the spectra of GN and NAG at the same molar concentrations, one can observe that the molar absorptions (ϵ coeficients) have different values, i.e. $\epsilon_{NAG} > \epsilon_{GN}$. In fact, at low concentration, the contribution of NG can be neglected.

What is important to see from Figure 1 is that both chitosan and NAG have the same λ_{max}, their spectra being similar. These results are similar with those of Liu[23].

Based on the results presented above one may suppose that the monomer units, GNA and GN, contribute in a simple additive way to the total absorbance of the entire polymer. That means the optical density of a chitosan sample ($DO_{chitosan}$) will additively depend on the concentration (C_x) and absorptivities (ϵ_x) of GNA and GN, according with the following equation, made in accordance with Liu[23]:

$$DO_{chitosan} = C_{GNA} \cdot \epsilon_{GNA} + C_{GN} \cdot \epsilon_{GN} \quad \text{Eq. 1}$$

One may define de degree of acetylation (DA) of chitosan as the mole fraction of acetylated units in the polymer chain:

$$DA = \frac{C_{GNA}}{C_{GNA} + C_{GN}} \quad \text{Eq. 2}$$

Considering that the total concentrations of the two chromophoric groups is

$$C_T = C_{GNA} + C_{GN} \quad \text{Eq. 3}$$

one may transform these equations to obtain:

$$\frac{DO_{chitosan}}{C_T} = (\epsilon_{GNA} - \epsilon_{GN})DA + \epsilon_{GN} \quad \text{Eq. 4}$$

This can be considered as the equation of a straight line $y=ax+b$, where y is the absorbance of chitosan at a designated wavelength divided by the total concentration of polymer, DA is x, coefficient a is the term ($\epsilon_{GNA} - \epsilon_{GN}$) and the term ϵ_{GN} is the coefficient b. In fact, one may transform these equations to obtain a simplified one:

$$\frac{DO_{chitosan}}{C_T} = a \cdot DA + b \quad \text{Eq. 5}$$

Based on these, solutions with known concentrations of NAG and GN were prepared in phosphoric acid and their UV absorbance at λ_{max} =197 nm were recorded. In fact, the calculations have shown relatively high errors when the values at 197 nm were used, but lower errors when for calculations were used the averaged values recorded in the range 195 – 200 nm. These averaged values were labeled as the optical densities values at 197 nm.

The results presented in Table 1 and in Figure 2 show that the degree of acetylation is correlated with the concentrations of NAG and GN from the analyzed sample. In this case, DA is the concentration of NAG divided by the sum of concentrations of NAG and GN. One may assume that this will be true in the case of chitin and chitosan, as these polymers are from monomers of NAG and GN.

Table 1. Optical densities at 197 nm of standard mixtures of NAG with GN

No.	C_{GNA} (mM)	C_{GNA} (mM)	DA	$DO_{197\ nm}$	$DO_{197\ nm}/C_T$
1	0.05	0	1	0.795	1.590
2	0.045	0.005	0.9	0.755	1.510
3	0.04	0.01	0.8	0.701	1.402
4	0.03	0.02	0.6	0.547	1.094
5	0.02	0.03	0.4	0.358	0.716
6	0.01	0.04	0.2	0.147	0.294
7	0.005	0.045	0.1	0.185	0.370
8	0	0.05	0	0.078	0.156

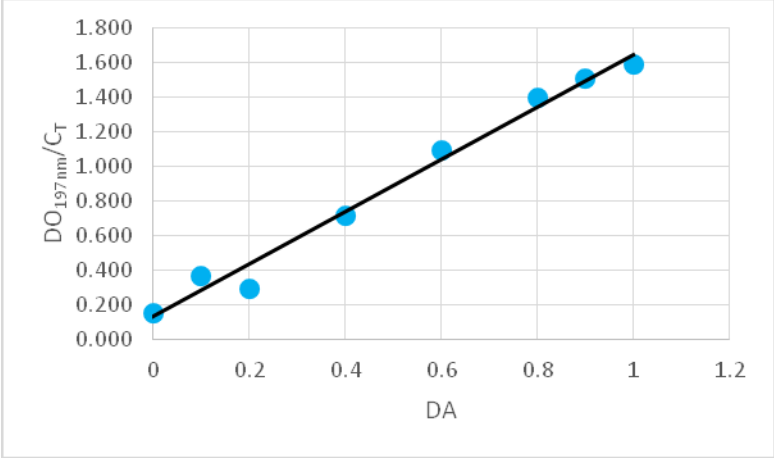

Figure 2. Dependence of the factor $DO_{207\ nm}/C_T$ on DA

The plot from Figure 2 proves that there is a linear correlation (R^2 = 0.992) between the optical density of NAG plus NG, divided to their total concentration and DA (slope = 1.512, intercept = 0.135).

To apply the standard curve (in fact the coefficients of the regression line) to chitin and chitosan samples, some calculations based on the equations presented above have to be performed. For this, one has to consider that the molecular weights of the monomer molecules are 203.2 for NAG monomer (instead of 221.2, which is the molecular weight of acetyl-D-glucosamine as single molecule, but in polymer, by condensation with other monomer molecules will lose a water molecule), and 161.1 for GN. For convenience, we have considered the concentration of chitin or chitosan in samples expressed as mg product per 100 mL solution (V factor in Eq. 6). Other factors from Eq. 6 are the slope *a* and the intercept *b* from curve presented in Figure 2. In these conditions, the equation used, very similar with that of Liu[23], to calculate the DA of chitin or chitosan samples is:

$$DA = \frac{161.1 \cdot DO_{chitosan} \cdot V - b \cdot m}{a \cdot m - 41.2 \cdot DO_{chitosan} \cdot V} \qquad \text{Eq. 6}$$

The Eq. 6 was used to calculate the degree of acetylation of chitin or chitosan samples. In Table 2 the DA was calculated for 3 chitosan samples (two different batches from Sigma and one from Roth) and a chitin sample. The samples were analyzed in duplicate and the error (as % from the average DA) was calculated. Considering that we are working with polymers with relatively low solubility, errors under 20% are considered as acceptable.

Table 2. DA of some samples of chitosan (2 batches from Sigma, 1 from Roth) and of chitin

No.	Sample	m (mg)	V (L)	$DO_{chitosan}$	DA	% Error
1	Chitosan (source 1)	12.5	0.1	0.715	0.61	7.85
2	Chitosan (source 2)	14.1	0.1	0.865	0.68	14.54
3	Chitosan (source 3)	17.7	0.1	0.842	0.48	8.74
4	Chitin	15.5	0.1	1.247	0.98	11.74

In Table 3 the DA of chitin subjected to deacetylation reaction in the presence of NaOH concentrated solutions are presented. The experiment was performed in duplicate and the errors (except for low DA value) are in the acceptable range. Taking into account that the values of DA calculated with Eq. 6 are in accordance with the time of deacetylation reaction, i.e. the DA decrease with the reaction time, one may consider that the results presented in Table 3 are accurate, even in the absence of results obtained with an alternative method.

Table 3. DA of some samples of chitosan subjected to deacetylation with 40% NaOH. The mass of chitin was 15.1 mg dissolved in 100 mL 0.1 M phosphoric acid

No.	Deacetylation time (h)	$DO_{chitosan}$	DA	STD	% Error
1	0	1.198	0.93	0.07	5.85
2	1	1.102	0.83	0.03	2.95
3	2	0.792	0.52	0.07	8.98
4	4	0.675	0.42	0.03	4.30
5	8	0.488	0.26	0.13	26.25

Following the method proposed by Liu[23], with some minor modifications, i.e. use of phosphoric acid as solvent for chitin and chitosan, use of other wavelengths for measurement of optical densities of samples (197 nm instead 207 nm, in fact the averaged values recorded in the range 195 – 200 nm), it was proved that the UV spectra of mixtures of NAG and GN are quite similar to the UV spectra of chitosan and the DA chitosan can be estimated with sufficient accuracy based on spectrophotometry measurements.

4. CONCLUSIONS

Some preliminary results of the attempt to improve the published method of determination of degree of acetylation of chitosan by UV spectrophotometry using dual standards were presented. The method is based on the fact that the UV absorption of chitosan depends on absorbencies of N-acetylglucosamine and glucosamine monomers that form the polymeric chain of chitosan. In order to improve the errors of this method, the UV absorbance values recorded on the range 195-200 nm, instead the absorbance value at a single wavelength was used. Another modification of the published method was the use of phosphoric acid as solvent for chitosan, N-acetylglucosamine and glucosamine, instead hydrochloric acid, modification that also seems to contribute to the stability of the optical densities of the samples and to decrease in a certain degree the magnitude of errors.

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Article

ESTIMATION OF THE MOLECULAR WEIGHT OF CHITOSAN BY PAGE

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Abstract

The estimation of the molecular weight of chitosan, a natural polymer consisting of molecules with masses ranging from 10 to 10^3 kDa, using polyacrylamide gel electrophoresis was investigated. The polyacrylamide gel was adapted for the migration of positive charged polymers. As application of electrophoresis of chitosan, time course of the hydrolysis of chitosan with hydrochloric acid and with some enzymes (chitinase, cellulase and α-amylase) used alone or in mixture, was studied. The preliminary results have shown that cellulase has a relatively small chitinolytic activity and that in the presence of cellulase and α-amylase, chitinase has an increased activity towards the glycosidic bounds of monomers from chitosan.

Keywords: polyacrylamide gel electrophoresis, chitosan hydrolysis, chitinase, α-amylase, cellulase.

1. Introduction

Chitosan is a polymer that is obtained by the deacetylation of chitin which is the second most abundant polysaccharide from the world after cellulose. Chitosan is a polymer formed

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from monomeric units of glucosamine (GA) and N-acetyl glucosamine (NAG) linked by glycosidic bonds in different ratio [1]. The number of monomeric units in a polymeric chain, i.e. the molecular weight, greatly depend on the natural source of chitin[2]. By chemical or enzymatic hydrolysis, shorter chains of oligosaccharides can be obtained. For chemical hydrolysis of chitosan, strong acids such as hydrochloric acid [3-6], phosphoric acid [7,8], chemical compounds like hydrogen peroxide [9-11] are used, sometime in ionic liquids [12-14]. Enzymatic hydrolysis of chitosan for the production of oligosaccharides involves enzymes like chitinase and chitosanase that are enzymes with a high specificity towards the chemical structure of chitosan. There are mentioned examples when other types of enzymes, like pectinases, cellulases, α-amylases, β-amylases[15], pepsin, proteases, lipases[16], lysozyme and even some tissue extracts from different organisms[3,16] can hydrolyze chitosan.

Although many papers have aimed to identify the degree of acetylation and molecular weight of chitosan by different methods this goal remains a challenge for research on chitin and chitosan.

In this work the determination of depolymerisation degree of chitosan by an electrophoresis procedure was studied.

The aim of this study was the determination of depolymerisation degree of chitosan by electrophoresis procedure. The paper of Audy and Asselin [3] was used as starting point, where some preliminary results regarding the migration of chitosan oligomers on polyacrylamide gel electrophoresis were presented. Mainly, PAGE is used for separation of proteins, but in this study it was investigated the migration in the electric field of charged chains of chitosan. As application, the time course of the hydrolysis reaction of chitosan in the presence of hydrochloric acid or of some enzymes was studied.

2. Materials and Methods

2.1. Reagents

Most of the reagents were purchased from Carl Roth or Sigma Aldrich: urea (Sigma Aldrich, #15604, acetic acid (Sigma Aldrich ,#27225) acrylamide (Fluka, #01700), N, N' – methylenebis acrylamide (Sigma Aldrich, #146072), N, N, N', N' – tetramethylethylenediamine (Sigma Aldrich, #T22500), coomassie brilliant blue R250 (Carl Roth, #3862.1), 2-propanol (Sigma Aldrich, #33539), brilliant green (Carl Roth, #0324.1), ammonium persulphate (Sigma Aldrich, #215589), sodium acetate (Carl Roth, #6773.1), sodium hydroxide (Sigma Aldrich, #30620) chitosan low molecular mass (Sigma Aldrich, #448869).

2.2. Enzymes

The enzymes used for hydrolysis of chitosan were: chitinase from *Streptomyces griseus* (Sigma Aldrich, #6137), cellulase from *Aspergillus niger* (Sigma Aldrich, #22178), α-amylase from bacteria (Merck, #1329).

2.3. Buffers and solutions

The following solutions were realized: *Chitosan solution:* 0.5% chitosan in 2% acetic acid, pH 4.6 adjusted with NaOH; *Running buffer*: 5% acetic acid; *Sample buffer*: 1,8 mL 10 M urea, 100 μL dH_2O, 100 μL 100% acetic acid, 2 mg green brilliant; *Staining solution*: 0.05% coomassie brilliant blue (CBB), 25% isopropanol, 10% acetic acid; *Destaining solution*: 10% acetic acid; *Enzymes solutions*: *chitinase solution*: 1mg/mL chitinase 0.2 U/mg in potassium phosphate buffer, 0.5M, pH 6, *cellulase solution*: 1mg/mL celluase 1.14 U/mg in sodium acetate buffer 0.1M, pH 4.5, *α-amylase solution*: 1mg/mL α-amylase 130 U/mg in H_2O.

2.4. Instruments

A Mini-protean III cell (Bio-Rad) electrophoresis system was used to run the electrophoresis at a constant voltage (80V per slab gel), using a Power PAC 300 source. The gels were realised using a casing gel mold and 8x10 cm glass plates.

For performing the hydrolysis reaction at various temperatures, the samples were incubated in a thermal cycler (Applied Biosystems).

2.5. Methods

2.5.1. Chitosan hydrolysis with hydrochloric acid

Portions of 0.5 g chitosan were hydrolysed in 10 mL of 37% hydrochloric acid at 70^0C for 123 hours. Samples were taken at various time intervals: 0, 2, 4, 8, 24, 29, 52, 76, 100 and 123 h. The reaction was stopped by keeping the samples at -20^0C until used in experiments PAGE.

2.5.2. Chitosan hydrolysis with enzymes

To 1 mL of 0.5% chitosan in 2% acetic acid, 200 μL of solution of each of the studied enzyme (chitinase, cellulase and α-amylase) was added and incubated at 37ºC, under constant

mixing (250 rpm). Combination of two or of all three enzymes were also used for hydrolysis of chitosan. From each reaction mixture, at define time intervals (0, 1, 2, 4, 6, 8, 24, 48 and 72 hours) samples were collected. To inactivate the enzymes, these samples were warmed at 100ºC in PCR thermoblock, for 10 minutes. The samples were kept at - 20ºC until analyzed by PAGE.

2.5.3. Acid acetic-urea PAGE

To separate the chitosan chains by electrophoresis in polyacrylamide gels, the starting point was the work of Audy and Asselin [3]. The acrylamide gels with 12% concentration were prepared as follows: 2.12 mL H_2O, 3.75 mL 7 M urea, 0.2 mL 5,5% acetic acid, 4 mL 30% acrylamide, 20 µL TEMED, 40 µL 20% APS were mixed and poured in the casing gel mold. Before loading on the gel, the samples were mixed 2:1 with sample buffer and boiled for 3 minutes. A volume of 5 µL of each sample was loaded in sample wells and the electrophoresis was run at 80 V, for about 55 minutes, until the dye reached near the edge of the gel.

2.5.4. Staining and destaining of the gels

For staining, the gels were immersed in the staining solution, boiled for few seconds (in microwave oven) and cooled at room temperature for 10 minutes, under slow shaking. Destaining was realized in 10% acetic acid, boiling the gels in microwave oven for 1-2 seconds and cooled under gentle shaking for 10 minutes. These step was repeated for few times to obtain clear gels with well colored bands.

3. Results and Discussions

The main aim of this study was to find a simple, rapid and reliable method to assess the degree of polymerization or the molecular weight of chitosan polymers. The selected method has to be fitted to study the hydrolysis of chitosan in chemical or enzymatic reactions. Among the studied method, the electrophoresis in polyacrylamide gels drew our attention as PAGE is chiefly used for separation of proteins, rarely for separation of nucleic acids and seldom for separation of other type of polymers. In fact, only one article was published on strictly the subject of separation of chitosan using PAGE[3], paper that was used as starting point in this study.

In Figure 1 there is presented the electrophoresis of a chitosan sample subjected to hydrolysis with hydrochloric acid. The first observation is that in contrast with the protein PAGE, the electrophoretic bands of chitosan are very wide. This is due to the fact that chitosan is a natural product (derived from chitin) and like the majority of the polysaccharides

do not have a unique molecular weight. The same sample of chitosan contains polymeric chains with the molecular mass ranging from 10 to 10^3 kDa, depending on the natural source of chitin[17].

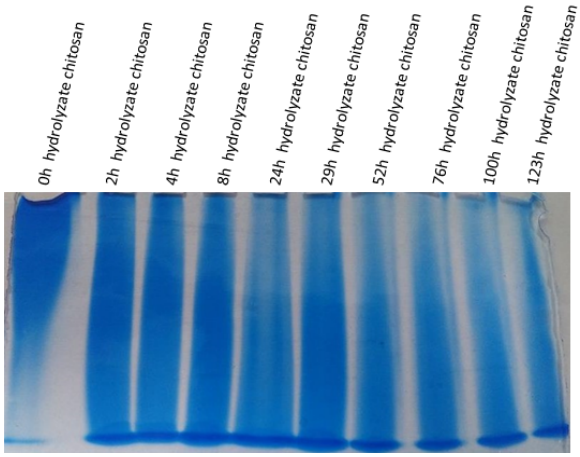

Figure 1. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with HCl at different reaction time intervals.

In the first lane of electrophoresis presented in Figure 1 the band although is wide, proving that this chitosan sample contains chains having the molecular weight in a large range, do not contains polymeric chains with low molecular weight as the first lane is quite uncolored at its bottom. That means at the beginning of the hydrolysis experiment, the studied sample of chitosan contained only polymeric chains with relatively high molecular weights. Looking at the other lanes from the gel, one can see that as the hydrolysis time increases, the concentration of polymeric chains with low molecular weight increases too, as the bands become more colored in their lower part. This is a prove that PAGE can be a simple, rapid and convenient technique for the estimation of the hydrolysis reaction of chitosan.

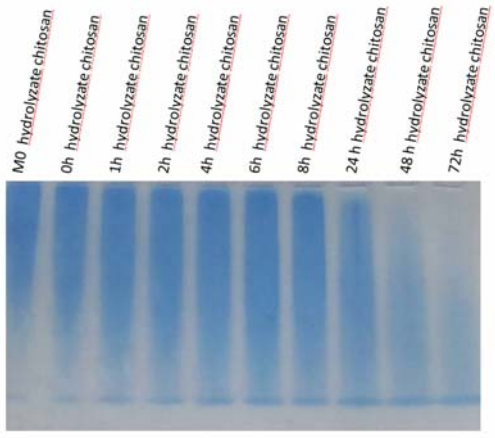

Figure 2. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with chitinase at different reaction time intervals.

A similar electrophoresis pattern is obtained when the same type of chitosan is hydrolyzed with a commercial chitinase (Figure 2). On the first lane, corresponding to zero reaction time, the band is colored in the upper part and uncolored in the lower part, proving that the loaded sample contained polymeric chains with relatively high molecular weight.

As the reaction time increases, the representation of the polymeric chains tend to reverse, i.e. the lanes correspond to samples that contain less polymeric chains with high molecular weight and more small molecules, like oligochitosaccharides. From the lane corresponding to 72 hours of hydrolysis with chitinase, it becomes obvious that the sample has contained only small molecules and no long polymeric chains.

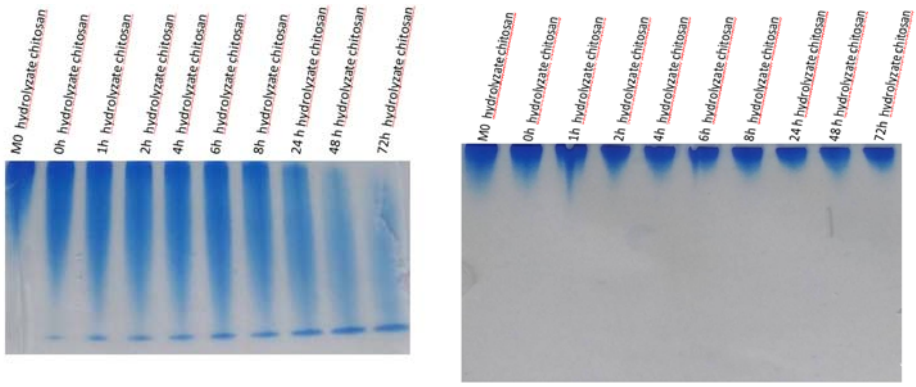

Figure 3. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase and α-amylase (left electrophoresis) and with α-amylase alone (right electrophoresis), at different reaction time intervals.

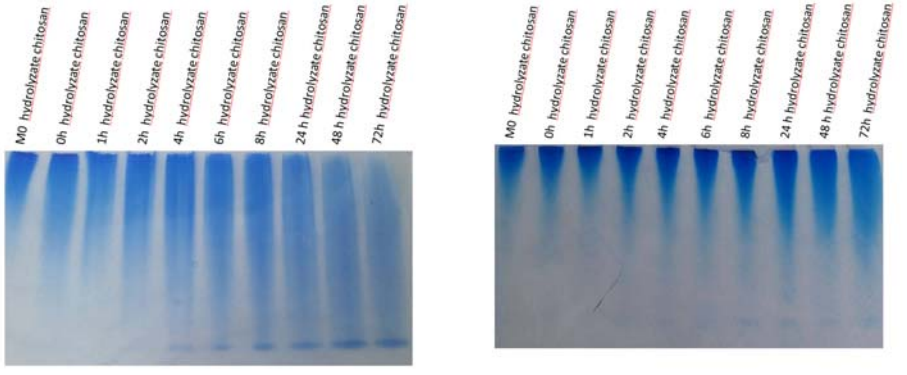

Figure 4. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase and cellulase (left electrophoresis) and with cellulase alone (right electrophoresis), at different reaction time intervals.

In another series of experiments it was studied the hydrolysis of chitosan with chitinase, α-amiylase and cellulase and combination of them. In Figure 3 the two electrophoresis present the reaction course of hydrolysis of chitosan with a mixture of chitinase and α-amylase and, for comparison the reaction course of hydrolysis of the same type of chitosan with α-amylase alone. From these two electrophoresis is clear that α- amylase do not hydrolyze chitosan, as the patterns of the lanes from the right electrophoresis, corresponding to the hydrolysis with α-amilase, do not change.

A different situation is when a similar experiment was carried out with cellulase (data presented in Figure 4). Although the mixture of chitinase and cellulase has a higher hydrolytic activity, as the right electrophoresis from Figure 4 shows, cellulase has a chitinolytic activity. In this stage of the research we cannot assign this chitinolytic activity to the cellulase, as the presence of a chitinase as an impurity of a chitinase cannot be excluded. Nevertheless, based on the results presented in Figure 4, the most plausible hypothesis is that cellulase can hydrolyse (with low activity) the glycosidic links between monomer units of chitosan.

To find out if the above assumption is correct, another experiment of hydrolysis of chitosan was realized with a mixture of all three enzymes: chitinase, cellulase and α-amylase (see Figure 5). As it was expected, the chitosan is hydrolyzed when it is mixed with these enzymes, with a higher velocity than when it was hydrolyzed only with chitinase or with binary mixture of chitinase and cellulase. Although this conclusion is based on a semi-quantitative estimation of the color intensities of the bands (specialized software Gel Anayser), it can be considered to be correct, if we take into account the observation that cocktails of hydrolytic enzymes have higher activities than one enzyme alone[18].

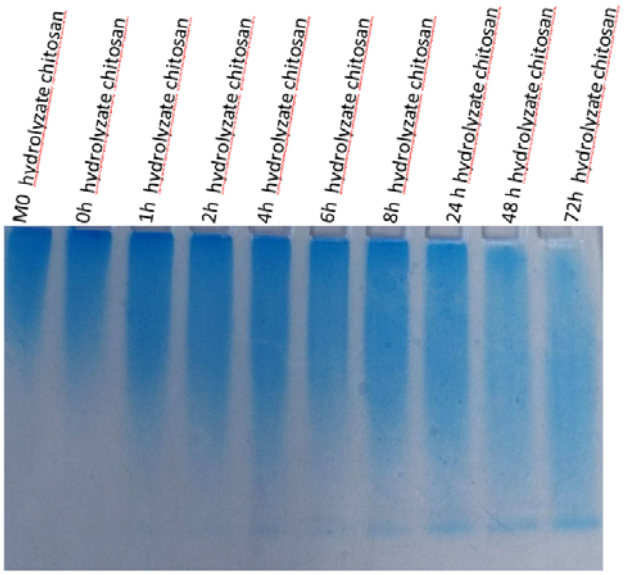

Figure 5. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase, cellulose and α-amylase, at different reaction time intervals.

The main drawbacks of the use of PAGE for estimation of the molecular weight of chitosan chains, as for any other polysaccharide bearing charged groups, is the fact that these natural polymers are synthetized by the organisms with chains with different lengths, sometime the range of the values of molecular weight range covering three or more degree of magnitude. Nevertheless, PAGE can be used to estimate the variation of the molecular mass of these polymers, as it is happen when the hydrolysis (depolymerization) reaction is studied.

4. Conclusions

A polyacrylamide gel electrophoresis was adapted to estimate the molecular weights of chitosan molecules and to study the reaction of hydrolysis of this polymer. The time course of hydrolysis of chitosan with hydrochloric acid, chitinase, α-amylase, cellulase, as well as with mixtures of these enzymes was examined.

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Short Communication

USE OF 3,5-DINITROSALICYLIC ACID REACTION TO STUDY THE CHITOSAN HYDROLYSIS

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Abstract

Chitin, one of the most abundant polymer in the biosphere, and its main derivative, chitosan have many applications in which the hydrolysis of the polymer chain has to be performed. To control the hydrolysis reaction a method to assess the course of reaction is required. Among other methods, DNS (3,5-dinitrosalicylic acid) assay is most used. The main drawback of the classical DNS method is that the heating step, i.e. boiling in water, required glass test tubes. The miniaturization of the method, i.e. performing the reaction in ELISA plates have the technical problem of heating the microtitter plates. This work present an improved DNS method, in which the DNS reaction is performed in ELISA plates, heated on thermoblock. The new improved method was applied to the hydrolysis of chitosan with chitinase.

Keywords: DNS, reducing sugars, glucosamine, N-acetil-glucozamine, microtiter plate assay

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1. Introduction

It is well known that the modern society has growing needs for materials, energy, chemicals to make advanced final products to improve the quality of life. On the other side, there is a growing concern regarding the global warming, consumption of the fossil resources and disposal of wastes. In this context, more attention was addressed to the renewable raw materials as a source for creation of the advanced final products. Today, many waste products are not used properly used to obtain valuable chemicals. In this category enter also chitin. This material is a polymer synthetized in huge quantities, especially by marine organisms. It is estimated that chitin is the second most abundant polymer synthetized on biosphere, with an annual production of 10^{10} – 10^{12} tons, after cellulose[1]. The annual worldwide commercial production of crustaceans exceed 10 million tons[2]. The huge amount of waste products resulted from processing of seafood become a major environmental issue, as about 45% of the mass of marine organisms used as seafood (shrimps) is waste. About one third of this garbage is chitin[3]. As chitin and its deacetylated derivative chitosan are a renewable resources, these materials become the source of numerous applications in agriculture, foods, waste water treatment, textiles, cosmetics, pharmaceuticals, medicine, biotechnology and many other fields[4,5].

For many applications, after the extraction of the polysaccharide from the waste materials, the polymeric chain has to be hydrolyzed to smaller fragments and in some cases until monomers. Classical hydrolysis is performed with acids, but in order to make the hydrolysis process more environmentally friendly the hydrolysis reaction is realized with hydrolytic enzymes - carbohydrases (glycoside hydrolases, O-glycosidases) – that belong to the class of hydrolases EC 3.2.1.-[6]. Both, hydrolysis with acids or with enzymes, has to be monitored. Most of the assays for determination of hydrolysis of carbohydrate polymers are based on analysis of reducing sugars. Among these methods, the Somogyi-Nelson method[7,8], based on copper and arsenomolybdate reagents and the DNS method[9], are the most used. Less frequently used are the method with potassium ferricyanide[10], sodium 2,2'-bicinchoninate[11] or p-hydroxybenzoic acid hydrazide[12]. Most of the above mentioned methods, as are based on chemical reaction of reducing sugars with some chemicals, require a heating step. This is why in the majority of the published articles the experiments are performed in glass tubes that can be warmed in boiling water. In some previous papers [13,14] we have described the attempt to perform the DNS reaction in ELISA microtitter plates, by performing the heating step in a microwave oven. In this study we have adapted the DNS reaction to microtitter plates, realizing the heating step in a thermoblock. The method was applied to study the hydrolysis of chitin and chitosan.

2. Materials and Methods

2.1. Chemicals and Instruments

The chemicals used in this study were acquired from Sigma Aldrich or Carl Roth: glucosamine hydrochloride (Sigma Aldrich, # G1514), N-acetil-glucosamine (CarlRoth, # 8993.2), glucose anhydrous (Scharlau, #GL01250500), maltose (Carl Roth, #8951.1), galactose (Carl Roth,#4987.2), lactose (Carl Roth, #8921.1), sodium hydroxide (Sigma Aldrich, # 367176), 3,5-dinitrosalicylic acid (DNS; Sigma Aldrich, # D0550) phenol (Carl Roth,# 0040.1), potassium sulphite (Carl Roth, # 7995.1), potassium tartrate (Sigma Aldrich, # 243531), potassium sodium tartrate tetrahydrate (Carl Roth, #8087.1), potassium hydroxide (Carl Roth , # P747.2), ELISA plates were acquired from Sarstedt (#82.1581),

Optical density of the reaction product was determined by using of a Tecan Sunrise microplate reader (Tecan Trading AG, Männedorf, Switzerland) with a Magellan Data Analysis software, A HLC Heating-Thermo Mixer MHR 11 (DITABIS - Digital Biomedical Imaging Systems AG, Pforzheim, Germany) was used for heating of the samples to 100°C .

2.2. Stock solutions

To perform the DNS reaction, the following variants were prepared: ***Recipe 1:*** 1% DNS, 30% potassium sodium tartrate tetrahydrate, 4N sodium hydroxide; ***Recipe 2***: 1% DNS, 1% sodium hydroxide, 0.2% phenol, 0.05% potassium sulphite; *DNS solution **3** **Recipe 3***: 1% DNS, 30% potassium tartrate, 0.5% sodium hydroxide, 0.5% potassium hydroxide, 0.2% phenol.

The following stock solutions were used: 10 mM glucose solution; 10 mM galactose solution; 10 mM lactose solution; 10 mM maltose solution; 50 mM glucosamine hydrochloride solution, 50 mM N-acetil glucosamine solution. All stock solution were prepared in distillated water.

2.3. Methods

2.3.1. DNS reaction in microtitter plates

The reaction of DNS reagent with the solutions containing reducing sugars were performed in microtitter plates. The total volume of DNS reagent (one of the three recipes) was (usually) 100 µL and the maximum volume of the containing the analyte was also 100 µL. The heating step was realized on a microplate heat block. The plates were covered with a plastic cover, to reduce as much as possible the evaporation process.

2.3.2. Calibration curve for reducing sugars

To realize the calibration curves, various volumes of stock solutions (glucose, maltose, galactose, glucosamine, N-acetyl-glucosamine) ranging from 1 to 100 µL were mixed with 100 µL DNS reagents (one of the three Recipes). ELISA plates were incubated for 30 minutes, at 100°C in thermomixer. To avoid evaporation of solutions the plates were covered with a foil. After cooling the plates at room temperature, optical density was measured at 540 nm in the plate reader. All experiments were performed at least in duplicate.

3. Results and Discussions

The mains goals of this study were to miniaturize the DNS method for reducing sugars, i.e. to perform this method in microtitter plates and to optimize this method for the evaluation of hydrolysis of chitin and chitosan.

Although the DNS methods was miniaturized to ELISA plates[13,14], the heating step was performed in the microwave oven, that made the assay to be unpleasant for technical point of view. In this study the heating phase was realized in an ELISA thermic block.

Three types of recipes were tested, in order to make the DNS reagent as simple as possible, but also as sensitive and accurate as possible. In Figure 1 there is presented a comparison of the optical densities of the same solution of glucosamine (GN) when different recipes of DNS reagents were used.

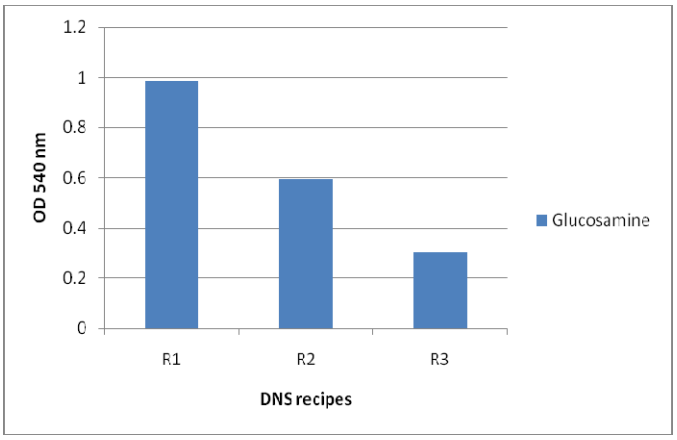

Figure 1 The influence of DNS solution compounds on reaction products absorbance. The concentration of glucosamine and N - acetil - glucosamine was 6.25 mM

Based on the results presented in Figure 1, in the rest of the experiments the DNS reagent presented in recipe 1 was used.

Several mono- and di-saccharides (glucose, galactose, lactose, maltose, glucosamine, N-acetil glucosamine) were tested and standard calibration course were realized (Figure 2 presents some of the standard curves).

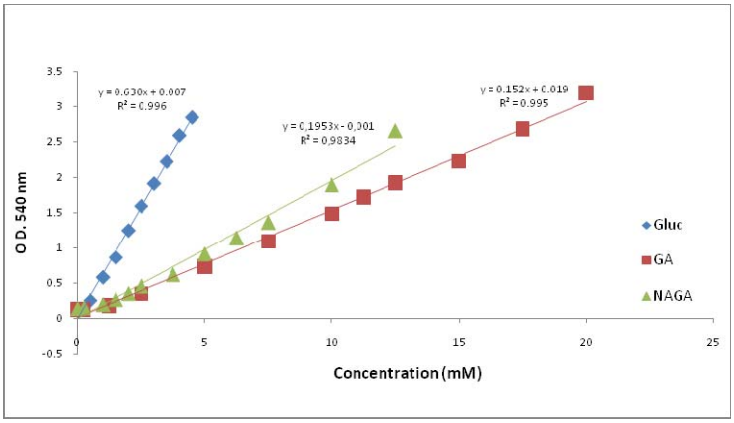

Figure 2 Calibration curves for glucose, glucosamine (GA) and N-acetil-glucosamine (NAGA)

The conclusion that can be drown from Figure 2 and from **Table 1** is that the DNS reagent has different sensibility when the reducing sugars are in their native state, i.e. the –OH groups are not functionalized or blocked, comparing with the case when these –OH groups are substituted with amino or N-acetyl-amino groups.

Table 1 Values of slope, interception and regression coefficient for reducing sugars. Limits of detection (LOD) and quantification (LOQ) are expressed in mM reducing sugars.

Sugar	**a**	**b**	**R^2**	**LOD**	**LOQ**
Glucose	0.6309	0.0073	0.9967	0.317498	0.962115
Galactose	0.6384	0.0048	0.997	0.303391	0.919367
Lactose	0.6589	0.0203	0.9929	0.468246	0.418928
Maltose	0.638	0.006	0.9961	0.346015	1.048531
Glucosamine	0.1953	0.001	0.9834	1.558106	4.721534
N-acetil-glucosamine	0.152	0.0197	0.9958	1.799821	5.494004

The slopes of the standard curves on neat mono- or di-saccharides are higher than when the monosaccharides units contain an amino or N-acetyl-amino group. This information has to be taken into account when the DNS assay hast to be applied to assess the hydrolysis of various types of polysaccharides. If during the hydrolysis of the polysaccharide will result fragments with the reducing ends belonging to neat carbohydrate, like glucose, galactose, manose, then the standard curve can de realized with glucose. If the fragments resulted from

hydrolysis of the polymeric chain will produce reducing sugars that have amino or N-acetly-amino groups, then the standard curves has to be realized with the specified monomers.

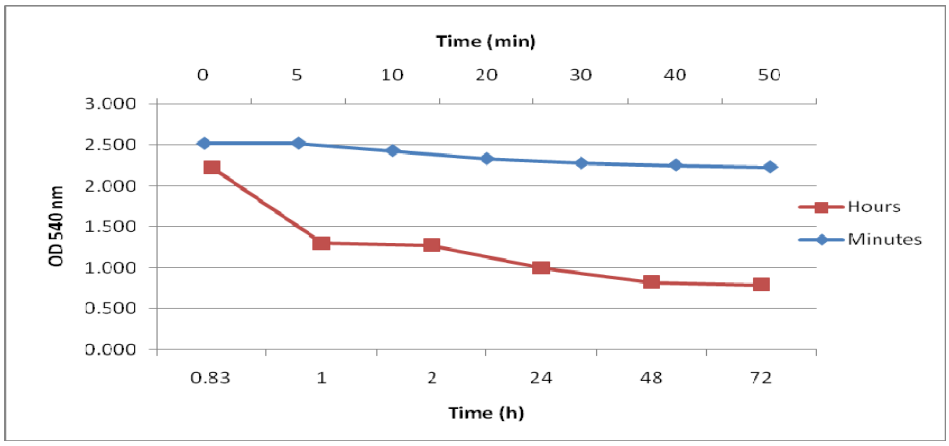

Figure 3 The stability of the colour of the reaction product. Lower horizontal axis, corresponding to the red experimental points, is in hours, while the upper horizontal axis, for the blue experimental points, is in minutes.

As one of the objective of this work was to set-up a method useful for the study hydrolysis of chitosan, the rest of experiments were realized with glucosamine (GN). In Figure 3 there is presented the stability of the colored reaction product. The color intensity of the final reaction product do no decrease in one hour with more than 3%, but after 72 hours, the color fade with more than 60%. It is recommended to read the plates as soon as 30 minute after the heating step of the reaction was ended.

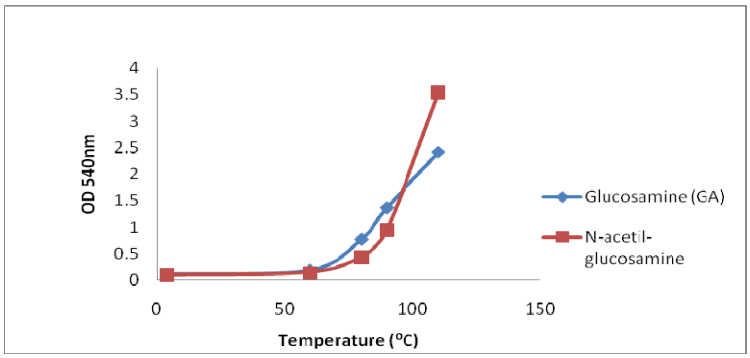

Figure 4 Influence of temperature on reaction product absorbance

From the variation of the color intensity of the reaction product as a function of the temperature of the reaction, we may assume that it is important to perform the heating of the plates at 100°C, as the results presented in Figure 4 show. As these are preliminary results of

a wider study of optimization of DNS method, there are no results at temperature higher than 100°C.

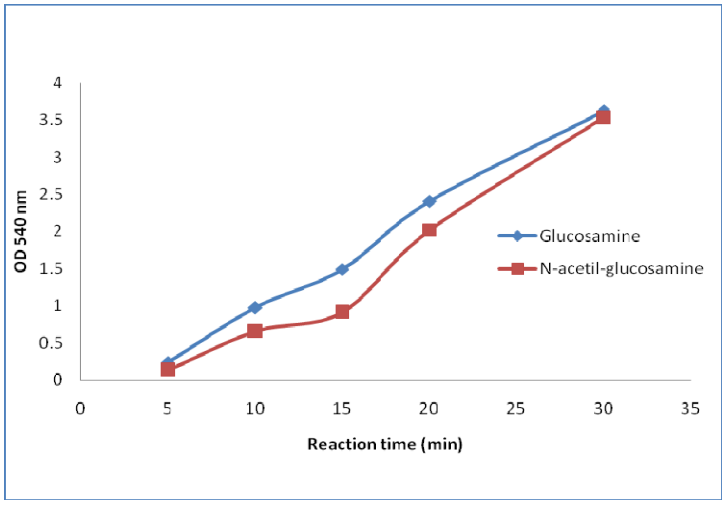

Figure 5. The influence of the time of reaction (DNS reagent with glucosamine and B-acetyl-glucosamine, respectively) upon the color intensity of the final product.

Although based on the preliminary results presented in Figure 5, it become obvious that after 30 minutes of the reaction between DNS reagent and the reducing sugar solution, a plateau was not reached, due to practical reasons, a 30 minutes reaction time (at 100°C) was considered for the rest of experiments.

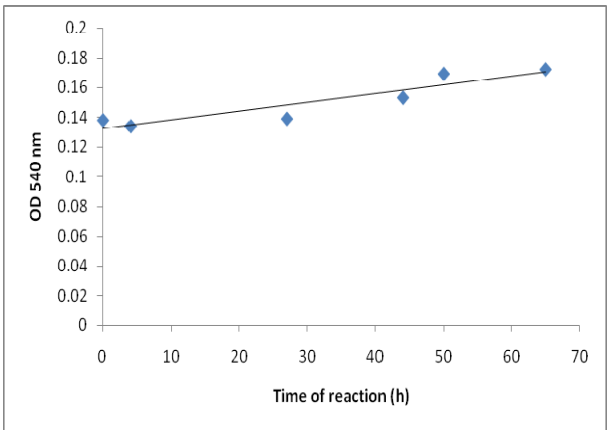

Figure 6 DNS assay for reaction between chitinaze and chitosan

As an application of the DNS method improved to be realized on ELISA microplates, in Figure 6 there is presented the hydrolysis of a chitosan sample with chitinase.

Although the DNS method here presented, in comparison with the previous methods also performed in ELISA plates[13,14] requires a much longer time to be realized, have the advantage to be simpler. In the previous methods, the heating step was performed in vessel with water, in a microwave oven and for technical point of view this was an unpleasant operation. The present method, although longer, is technically simpler. We also expect that the method with the thermic block instead of microwave oven to be more accurate and even sensitive, after an optimization process based on design of experiments approach.

4. Conclusions

The classical DNS method was miniaturized to be performed in ELISA plates. The heating step was performed on ELISA thermic block. The main inconvenient of this new procedure is the rather long time of the reaction (30 min) that has to be realized, in comparison with a previous method when the heating step was realized on microwave oven. The new proposed method was applied to the hydrolysis of chitosan with chitinase.

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