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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]♣***

[1]West University of Timisoara, Timisoara, Faculty of Chemistry – Biology –Geography, Advanced Research Environmental Laboratories, Oituz 4, Timisoara, 300086, Romania
[2]West University of Timisoara, Timisoara, Faculty of Chemistry – Biology –Geography, Department of Biology – Chemistry, Pestalozzi 16, Timisoara, 300115, Romania

## 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

♣ Correspondent author: vasile.ostafe@e-uvt.ro

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].

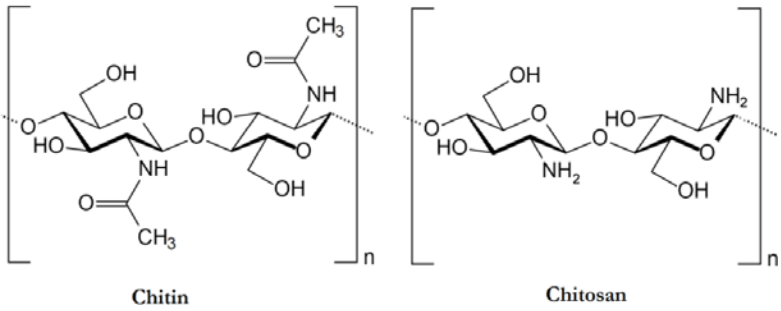


**Figure 1.** Chemical structures of chitin and chitosan.

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.

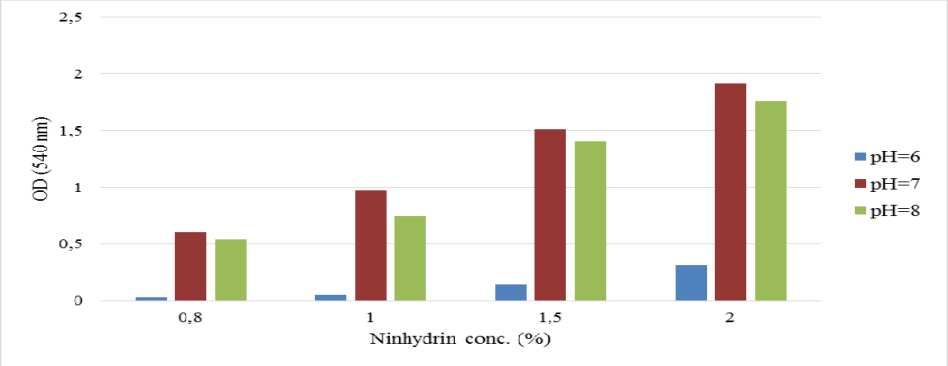


**Figure 2.** The influence of pH and concentration of ninhydrin on optical density of the colored product

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.

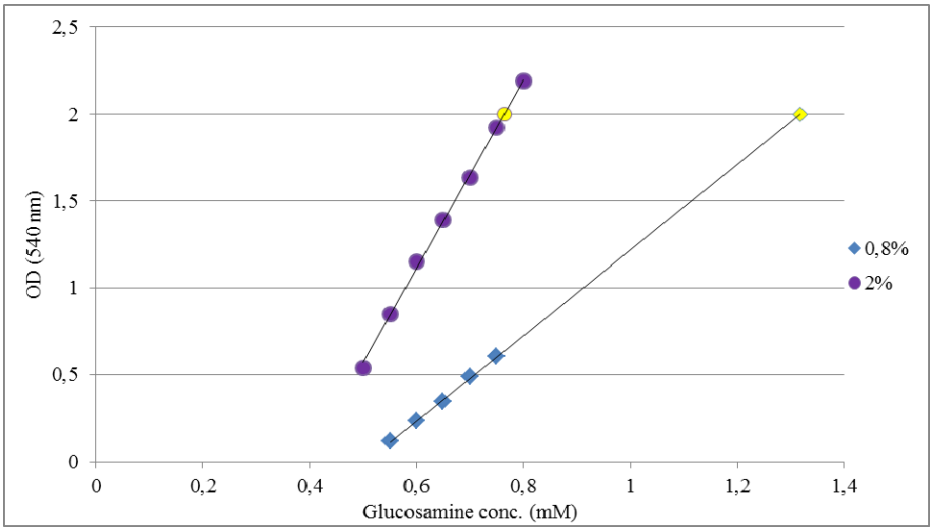


**Figure 3.** Standard calibration curves for glucosamine at two ninhydrin concentrations: 0.8% and 2%, respectively.

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.

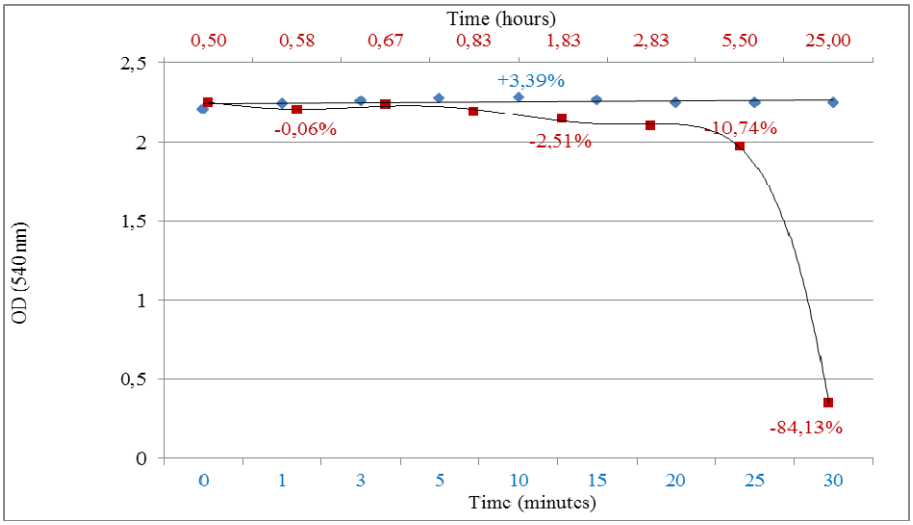


**Figure 4.** The stability of the colour of the reaction product. Lower horizontal axis, corresponding to the blue experimental points is in minutes, while the upper horizontal axis, for the red experimental points is in hours.

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.

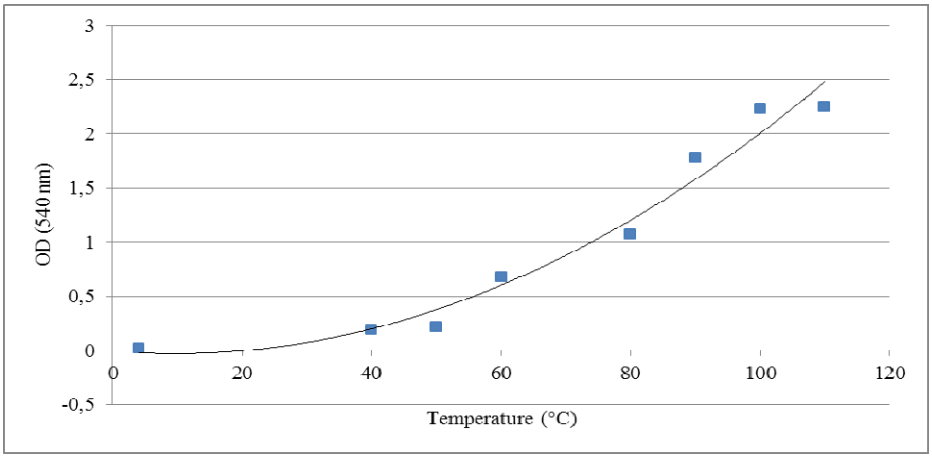


**Figure 5.** The influence of temperature on the optical density of the colored final product

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.

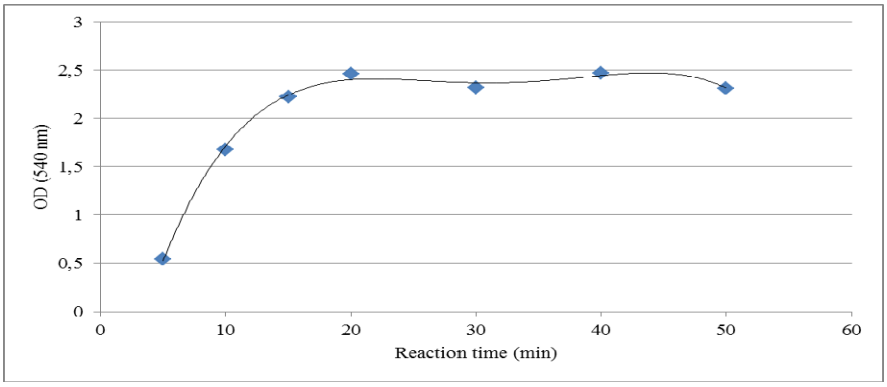


**Figure 6.** The influence of the reaction time on the optical density of the colored final product.

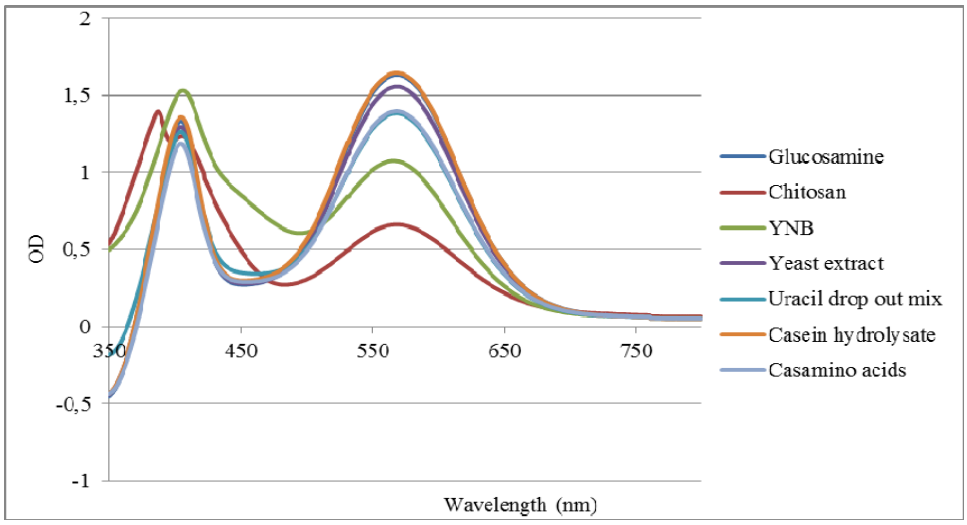


**Figure 7.** Absorption spectra of glucosamine, chitosan and some common reagents commonly used in molecular biology laboratories.

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.

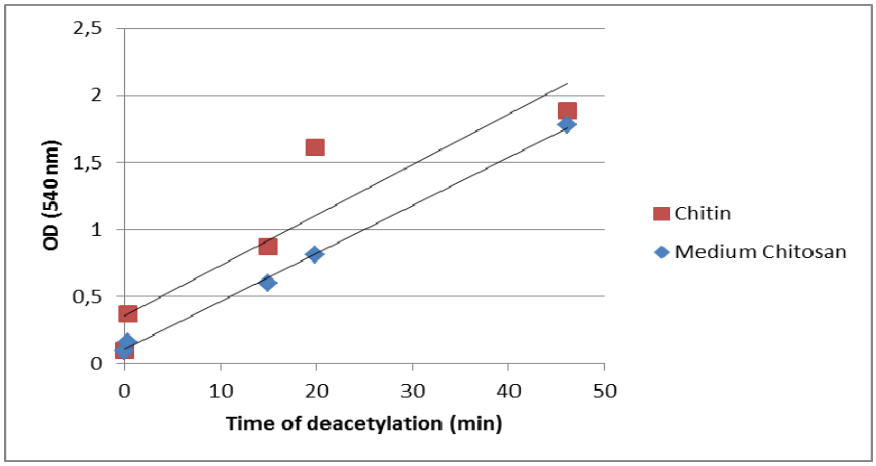


**Figure 8.** The estimation of degree of deacetylation of chitin and chitosan, using the reaction with ninhydrin.

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.

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