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Degree of Chitosan Deacetylation: Potential of Experimental Equations Application

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Abstract

In recent years, research has extensively explored the broad industrial potential of chitosan, with the degree of deacetylation being a pivotal chemical attribute that significantly influences its physical and biological properties crucial for its various applications. Numerous methods have been developed to determine the degree of deacetylation: linear potentiometric titration, infrared spectroscopy, nuclear magnetic resonance spectroscopy, pyrolysis-mass spectrometry, UV spectroscopy, and titrimetry. The challenge for researchers lies in selecting an appropriate method due to factors like time consumption, costliness (notably nuclear magnetic resonance spectroscopy), and the potential for sample destruction inherent in certain methods. Among these, infrared spectroscopy has emerged as a preferred method due to its speed and non-destructive nature. This study investigated the use of experimental equations, as documented in the literature, to determine the degree of chitosan deacetylation under laboratory conditions using three chitosan samples differing in viscosity, each having a documented degree of deacetylation above 75 %. Three distinct methods — potentiometric titration, acid-base titration, and Fourier transform infrared spectroscopy — were employed to calculate chitosan's deacetylation degree. While acid-base and potentiometric titration showcased simplicity in terms of equipment, the latter proved more time-consuming. In contrast, infrared spectroscopy demands more intricate instrumentation but requires only minimal samples, ensuring rapid analysis. The results showed that the methods of infrared spectroscopy and acid-base titration, using reported experimental equations, can be used to determine the degree of chitosan deacetylation. However, potentiometric titration did not validate its efficacy for this purpose.

Keywords

Chitosan, degree of deacetylation, experimental equations, FTIR, titrimetry

1 Introduction

Chitin is a linear, highly crystalline polysaccharide composed of acetylglucosamine monomer units linked by β-(1→4) glycosidic bonds. It is considered the second most abundant organic resource on Earth, found in plants, marine invertebrates, insects, the cell walls of some fungi, and microorganisms.[1] The partial deacetylation of chitin yields chitosan. Due to its non-toxic, biodegradable, biocompatible, biofunctional, and antimicrobial properties, chitosan holds potential for a wide range of applications.[2,3] Many of these properties are closely related to its degree of deacetylation. The degree of deacetylation of chitosan is often cited as a fundamental parameter influencing chitosan's performance, making its determination essential when examining samples. Numerous methods can be used to assess the degree of deacetylation, and choosing the appropriate method can be a challenging task for researchers.[4–6] Selecting the best standard technique among those described in the literature is not straightforward, as the variability of sources, isolation, and preparation procedures for chitin and chitosan means that chemical reactions suitable for one sample may not be suitable for another.[4] Additionally, certain limitations of the methods, such as execution time, the requirement for sophisticated equipment, high costs, sample destruction, and irreproducibility of results, need to be considered when conducting routine analyses. A work summarising the most current methods for evaluating the degree of deacetylation would serve as a valuable reference for researchers in the field of chitosan characterisation.[5]

This paper aims to review three adopted methods for determining the degree of chitosan deacetylation: acid-base titration, potentiometric titration, and the FTIR method. The first two methods involve dissolving chitosan in an acetic acid medium, followed by titration with an alkali. After the neutralisation of H^+ ions from a known excess of acetic acid, the number of free amino groups can be determined using the acid-base titration approach. In potentiometric titration, the process continues until neutralization of the NH_3^+ ions.

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This process can be time-consuming and labour-intensive, leading researchers to often rely on established experimental equations for convenience. This approach simplifies and accelerates the research and characterisation of unspecified samples. When it comes to determining the degree of chitosan deacetylation, various experimental equations are commonly employed in the literature. These equations have often been tested in comparison to absolute analytical methods and have been validated.[7]

By comparing these experimental formulas, it becomes evident that not all are equally reliable, as some use the same reference bands but apply different coefficients. In this study, the reproducibility of the proposed experimental equations was tested. Based on the results obtained in this work, the different methods were compared in terms of their advantages, disadvantages, and possible limitations.

2 Experimental

2.1 Materials

Commercial chitosan of low, medium, and high viscosity was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The chitosan samples were used as received without further purification.

2.2 Acid-base titration

Chitosan (0.3–0.5 g) was dissolved in 30 ml of 0.1 M HCl while stirring on a magnetic stirrer, followed by the addition of two drops of methyl orange indicator. The solution was titrated with 0.1 M NaOH. The final point of titration was determined by a colour change from pink to orange-yellow. The method was extended to include the use of a pH meter to determine more precisely the final point of titration.[5] To calculate water content, 0.5 g of chitosan was heated at 105 °C until a constant weight was reached. The percent of free amino groups in chitosan was calculated using the following equations:[6,8,9]

$$NH_2\% = [(c_1V_1 - c_2V_2) \cdot 0.0016] / [m\,(1 - w)] \cdot 100 \quad (1)$$

$$\text{Free } NH_2\% = (NH_2\% / 9.94\%) \cdot 100 \quad (2)$$

The theoretical value of amino group (NH_2) content for chitosan is calculated as (16/161) · 100 % = 9.94 %. In this calculation, c_1 represents the concentration of HCl (M), c_2 is the concentration of NaOH (M), V_1 is the volume of HCl added (ml), and V_2 is the volume of NaOH used in the titration (ml). The sample weight is denoted as *G* (g), and *w* refers to the water content of the sample (%). Additionally, 0.0016 g corresponds to the NH_2 content in 1 ml of 1 M HCl.

2.3 Potentiometric titration

Chitosan (0.3–0.5 g) was dissolved in 20 ml of 0.3 M HCl. After adding 400 ml of distilled water, the solution was titrated with a 0.1 M NaOH solution. A titration curve (pH *vs*. NaOH volume) was generated, and the inflection points for each indicated transition were identified. The volume of NaOH at each inflection point was applied to the following equation:[6]

$$NH_2\% = 16.1 \cdot (y - x)/m \quad (3)$$

where *m* is the weight of chitosan used, *x* is the first inflection point on the graph of measured pH *vs*. titration volume, and *y* is the second inflection point.

2.4 Fourier transform infrared spectroscopy

A thin film of chitosan was cast from a solution obtained by dissolving chitosan (0.4 g) in 50 ml of 1 % (*w*/*w*) acetic acid. After casting, the chitosan films were left to dry, and three parallel samples were used. Fourier transform infrared spectroscopy (FTIR) analysis of the samples was performed in the wavelength range specified in the literature, with a resolution of 4 cm^{-1}, using a Nicolet iS10 IR spectrometer equipped with an attenuated total reflection (ATR) accessory. The results were processed using Omnic software, and the recorded absorbance values were then incorporated into various experimental equations for calculating the degree of acetylation, as proposed in the literature:[5,10,11]

$$DA = (A1655/A3450) \cdot 100/1.33 \quad (4)$$

$$DA = (A1655/A3450) \cdot 115 \quad (5)$$

$$A1320/A3450 = 0.03146 + 0.00226 \cdot DA \quad (R^2 = 0.95) \quad (6)$$

$$A1320/A1420 = 0.3822 + 0.03133 \cdot DA \quad (R^2 = 0.99) \quad (7)$$

$$A1560/A2875 = 0.0125 \cdot DA + 0.2 \quad (R^2 = 0.99) \quad (8)$$

The degree of deacetylation was determined by subtracting the obtained degree of acetylation from 100.[5]

3 Results and discussion

3.1 Acid-base titration

The degrees of deacetylation of the tested samples, determined by acid-base titration, are shown in Table 1. Based on the results presented in Table 1, it is evident that the obtained values for all three tested chitosan samples fall within the expected range of degree of

deacetylation. This suggests that the method could be suitable for assessing deacetylation degrees in laboratory settings without the need for complex instrumental analysis. Moreover, the method demonstrated rapidity, indicating its potential for routine deacetylation assessments.

Table 1 – Degree of deacetylation (DD) values obtained using the acid-base titration method for chitosan samples of varying viscosities

Tablica 1 – Stupanj deacetilacije (DD) određen kiselo-baznom titracijom uzoraka kitozana različitih viskoznosti

Sample Uzorak	DD/%
CLV (low-viscosity chitosan)	77.8
CMV (medium-viscosity chitosan)	76.6
CHV (high viscosity-chitosan)	81.1

Consequently, this method could also be well-suited for industrial applications, facilitating routine analyses during chitosan production. By adopting this efficient approach, industries can enhance quality control while reducing the costs associated with more complex analytical techniques.

3.2 Potentiometric titration

The results obtained from potentiometric titration for evaluating the degree of deacetylation in low- and high-viscosity chitosan samples are illustrated in Figs. 1 and 2. The inflection points necessary for calculating the degree of deacetylation were determined from the first derivative of the pH *vs*. V function, with the calculated values summarised in Table 2.

Table 2 – Degree of deacetylation (DD) values obtained using the potentiometric titration method for chitosan samples of varying viscosities

Tablica 2 – Stupanj deacetilacije (DD) određen potenciometrijskom titracijom uzoraka kitozana različitih viskoznosti

Sample Uzorak	DD/%
CLV (low-viscosity chitosan)	40.25
CMV (medium-viscosity chitosan)	–
CHV (high-viscosity chitosan)	32.85

The first inflection point corresponds to the neutralisation of free H^+ ions from the acid, while the second inflection point corresponds to the neutralisation of protonated amino groups of chitosan. The difference in the volume of alkali consumed between these two inflection points correlates with the number of free amino groups in chitosan, *i.e.*, the degree of deacetylation. Since the used chitosan samples had a known degree of deacetylation (≥ 75 %), the results in Table 2 indicate that potentiometric titration did not yield values within the expected range. One possible explanation is that, in the range above chitosan's pK_a (approximately 6.5[3]), chitosan precipitates may not be effectively dissolved, causing the second inflection point to inaccurately represent the actual state. This could lead to an underestimated number of free amino groups, and consequently, a lower degree of deacetylation. Additionally, Table 2 reveals that it was not feasible to determine the degree of deacetylation for the medium-viscosity chitosan sample.

3.3 Fourier-transform infrared spectroscopy

The principle behind determining the degree of acetylation using FTIR methods is based on the ratio between the probe band, which is sensitive to the N-acetyl or amine group, and a reference band, selected from bands that do not change with variations in the degree of acetylation.[12,13] There is no universal reference range that can be used as a reference for the entire DA range because the chitosan spectrum changes as a function of DA; therefore, the appropriate reference range depends on the DA itself. The absorption range can be selected as an internal reference.[5]

Peak broadening and overlapping of two or more peaks are phenomena often observed in spectroscopy. Using sharp, well-separated peaks for determining the degree of acetylation (DA) results in more precise data compared to using broad and overlapping peaks.[5]

Consequently, selecting an appropriate baseline in a suitable manner is crucial, yet this often presents a challenge. Figs. 3 and 4 show two methods of reading absorbance values used in this study.

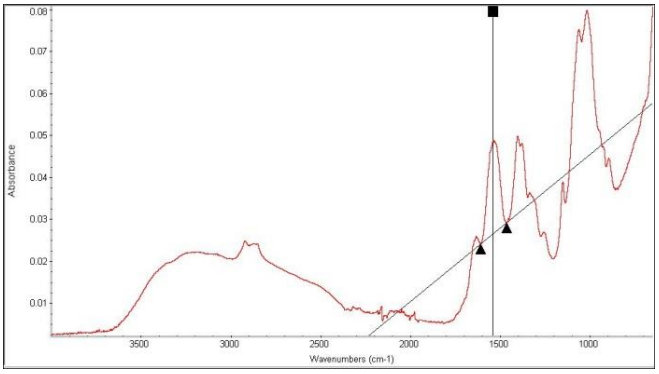

Fig. 4 – Example of reading absorbance values when the baseline is observed in the narrower part of the peak

Slika 4 – Primjer očitanja apsorbancije prilikom odabira užeg maksimuma na snimljenom spektru

The third method of absorbance reading used in this study involved setting the baseline to encompass the entire peak height.

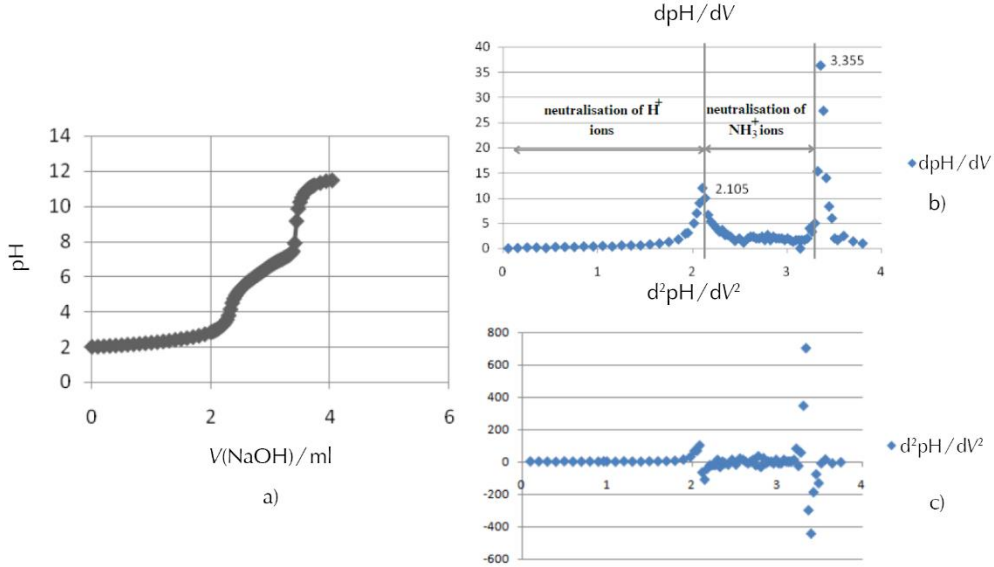

Fig. 1 – a) pH titration curve relative to the volume of NaOH consumed for titrating the low-viscosity chitosan sample, b) first derivative pH *vs*. *V*, and c) second derivative

Slika 1 – a) titracijska krivulja pH u odnosu na volumen utrošenog NaOH za titraciju uzorka kitozana niske viskoznosti, b) prva derivacija pH u odnosu na volumen (*V*), i c) druga derivacija.

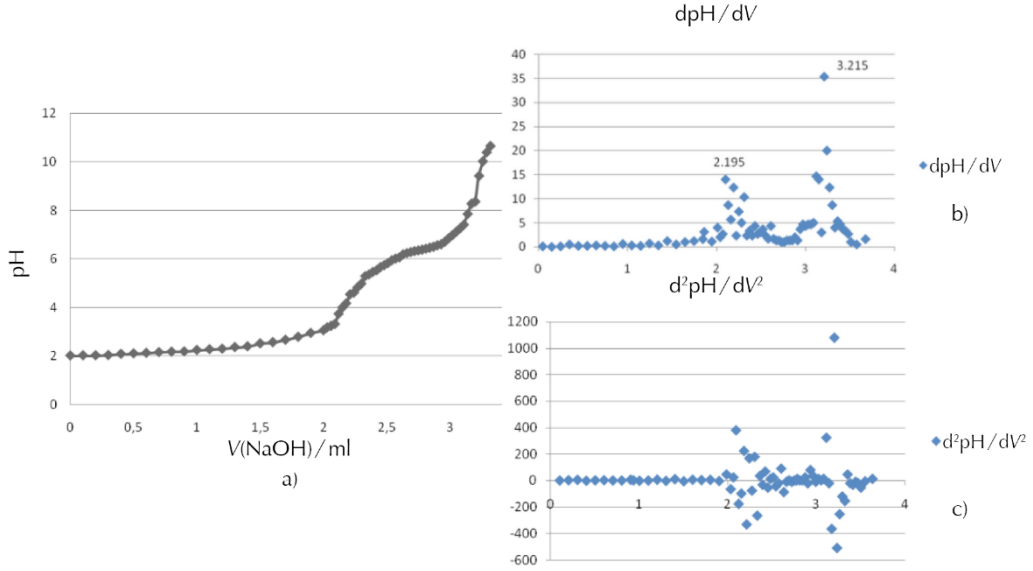

Fig. 2 – a) pH titration curve relative to the volume of NaOH consumed for titrating the high-viscosity chitosan sample, b) first derivative pH *vs*. *V*, and c) second derivative

Slika 2 – a) titracijska krivulja pH u odnosu na volumen utrošenog NaOH za titraciju uzorka kitozana velike viskoznosti, b) prva derivacija pH u odnosu na volumen (*V*), i c) druga derivacija.

Different baseline corrections for the IR spectra of the chitosan samples produced varied absorbance values and corresponding values of degree of deacetylation. These absorbance readings were then integrated into the experimental equations described in the literature[5,10,11] to determine the degree of deacetylation, with the results presented in Tables 3, 4, and 5 for low-, medium-, and high-viscosity chitosan samples, respectively.

The results of this study indicate that using experimental Eq. (7) provides consistent and reproducible values for the degree of deacetylation across all three absorbance reading methods. This is based on the intensity of the band at 1320 cm^{-1} (amid III), which is directly related to the number of acetylated amino groups, and the band at 1420 cm^{-1} that depends solely on the number of CH_2 groups present.[7]

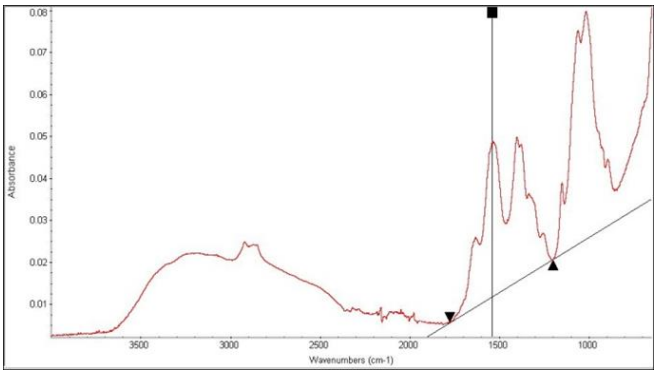

Fig. 5 – Example of reading absorbance values when the baseline is observed in the broader part of the peak

Slika 5 – Primjer očitanja apsorbancije prilikom odabira šireg maksimuma na snimljenom spektru

A significant advantage of this method is that the selected bands are insensitive to the moisture content in the sample.

Table 3 – Degree of deacetylation (DD) values of low-viscosity chitosan samples obtained using different baseline corrections

Tablica 3 – Stupanj deacetilacije (DD) uzorka kitozana niske viskoznosti koristeći različite metode korekcije bazne linije

	DD values of low-viscosity chitosan depending on the applied equation DD vrijednosti za kitozan niske viskoznosti ovisno o primijenjenoj jednadžbi				
Baseline correction Korekcija bazne linije	4)	5)	6)	7)	8)
1	80.87	70.74	−32.69	82.89	−356.00
2	19.37	−23.33	−714.98	83.84	−55.98
3	64.44	45.61	−280.72	86.82	−441.33

However, other experimental equations yielded significantly varying results, both within the same sample and in comparison with other samples. These discrepancies, coupled with a lack of reproducibility, render these alternative equations inadequate for determining the degree of deacetylation. Equations (4)–(6), which rely on the reference band at 3450 cm^{-1} (OH), proved to be unsuitable under the applied testing conditions. This is likely due to the hygroscopic nature of chitosan and changes in the moisture content of the sample during the determination.[7] For these equations to be successfully applied, testing would need to be conducted on completely dry samples, in a controlled atmosphere.

Table 4 – Degree of deacetylation (DD) values of medium-viscosity chitosan samples obtained using different baseline corrections

Tablica 4 – Stupanj deacetilacije (DD) uzorka kitozana umjerene viskoznosti određen koristeći različite metode korekcije bazne linije

	DD values of medium-viscosity chitosan depending on the applied equation DD vrijednosti za kitozan umjerene viskoznosti ovisno o primijenjenoj jednadžbi				
Baseline correction Korekcija bazne linije	4)	5)	6)	7)	8)
1	45.63	16.85	−124.34	95.53	−889.71
2	2.53	−49.07	−689.09	90.01	−56.02
3	27.89	−10.29	−945.13	78.55	−335.85

Table 5 – Degree of deacetylation values of high-viscosity chitosan samples obtained using different baseline corrections

Tablica 5 – Stupanj deacetilacije (DD) uzorka kitozana visoke viskoznosti određen koristeći različite metode korekcije bazne linije

	DD values of medium-viscosity chitosan depending on the applied equation DD vrijednosti o primijenjenoj jednadžbi				
Baseline correction Korekcija bazne linije	4)	5)	6)	7)	8)
1	74.59	61.15	−83.40	81.22	56.00
2	17.26	−26.55	−583.13	88.52	−54.74
3	41.94	11.20	−188.53	86.85	−252.00

Such testing conditions complicate, slow down, and hinder the instrumental determination of the degree of deacetylation. In terms of method performance, FTIR, coupled with an appropriate equation, has demonstrated its efficiency as a rapid technique for assessing the degree of deacetylation.

While FTIR is commonly used to compare the degree of deacetylation values obtained from other methods, it is crucial to note that its application for this purpose necessitates the creation of a calibration curve. This step ensures the verification and validation of the experimental data acquired, making it suitable for scientific research.

4 Conclusion

Following the evaluation of the methods for assessing the degree of deacetylation, a comparative analysis was conducted. Based on the results obtained in this study, both the acid-base titration and FTIR methods were found to be suitable for determining the degree of deacetylation. Conversely, the potentiometric titration method did not prove to be reliable for determining the degree of deacetylation.

The findings of this study indicate that using experimental Eq. (7) in the FTIR method yields consistent and reproducible values for the degree of deacetylation across all three absorbance reading methods employed. When selecting a method, it is important to consider that acid-base titration is straightforward, rapid, and cost-effective, making it suitable for less equipped laboratories. In contrast, while FTIR is relatively fast, it requires more advanced equipment. Moreover, the process of determining absorbance ratios from a single spectrum and analysing the data statistically takes longer compared to acid-base titration. However, FTIR is particularly useful for analysing chitin due to its limited solubility in most solvents.

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List of abbreviations and symbols
Popis kratica i simbola

c_1 – concentration of HCl, M
– koncentracija HCl, M

c_2 – concentration of NaOH, M
– koncentracija NaOH, M

CHV – high viscosity chitosan
– kitozan visoke viskoznosti

CLV – low viscosity chitosan
– kitozan niske viskoznosti

CMV – medium viscosity chitosan
– kitozan umjerene viskoznosti

DA – degree of acetylation
– stupanj acetilacije

DD – degree of deacetylation
– stupanj deacetilacije

G – sample weight, g
– masa uzorka, g

m – weight of chitosan, g
– masa kitozana, g

V_1 – volume of HCl added, ml
– volumen dodane HCl, ml

V_2 – volume of NaOH used in the titration, ml
– volumen utroška NaOH u titraciji, ml

w – water content of the sample, %
– udio vode u uzorku, %

x – first inflection point
– prva točka infleksije

y – second inflection point
– druga točka infleksije

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SAŽETAK

Stupanj acetilacije kitozana: Potencijal primjene empirijskih jednadžbi

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U posljednjih nekoliko godina intenzivno je istraživan industrijski potencijal kitozana, a stupanj deacetilacije predstavlja ključnu kemijsku značajku koja određuje njegova fizikalna i biološka primjenska svojstva. Razvijene su brojne tehnike za određivanje stupnja deacetilacije, poput: potenciometrijske titracije, infracrvene spektroskopije, nuklearne magnetske rezonancijske spektroskopije, pirolize spregnute s masenom spektrometrijom, UV spektroskopije i titrimetrije. Istraživači se prilikom odabira prikladne tehnike suočavaju s izazovima zbog niza čimbenika kao što su trajanje analize, visoki troškovi (posebice kod nuklearne magnetske rezonancijske spektroskopije) te destruktivna narav određenih analiza. Među nabrojanim, infracrvena spektroskopija istaknula se kao preferirana metoda zbog svoje brzine i neinvazivnosti. U ovom istraživanju ispitane su literaturne empirijske jednadžbe za određivanje stupnja deacetilacije kitozana u laboratorijskim uvjetima koristeći tri uzorka kitozana različite viskoznosti, pri čemu je poznato da je u svakom od uzoraka stupanj deacetilacije veći od 75 %. Tri različite metode — potenciometrijska titracija, kiselo-bazna titracija i spektroskopija u infracrvenom području s Fourierovom transformacijom — primijenjene su za izračunavanje stupnja deacetilacije kitozana. Dok su kiselo-bazna i potenciometrijska titracija pokazale jednostavnost u pogledu potrebne opreme za provedbu analize, potonja se pokazala vremenski zahtjevnijom. S druge strane, infracrvena spektroskopija zahtijeva složeniju opremu, ali je potrebna minimalna količina uzorka te omogućuje brzu analizu. Rezultati ukazuju da su infracrvena spektroskopija i kiselo-bazna titracija, primjenjujući odabrane empirijske jednadžbe, mogu uspješno primjenjivati za određivanje stupnja deacetilacije kitozana. Međutim, potenciometrijska titracija nije se pokazala prikladnom u tu svrhu.

Ključne riječi

Kitozan, stupanj deacetilacije, empirijske jednadžbe, FTIR, titrimetrija

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