STRUCTURAL MODIFICATION OF CHITOSAN WITH PHTHALIC ANHYDRIDE AND PHYSICOCHEMICAL AND FILM FORMING PROPERTIES

By

Miss Sunitda Khawthong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Program of Pharmaceutical Sciences

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การดัดแปรโครงสร้างของไคโตซานด้วยทาลิกแอนไฮไดรด์และสมบัติทางเคมีฟิสิกส์และการก่อฟิล์ม

โดย
นางสาวสุนิตดา ขาวทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Structural Modification of Chitosan with Phthalic Anhydride and Physicochemical and Film Forming Properties” submitted by Miss Sunitda Khawthong as a partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

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The aim of this study was to modify N-phthaloyl chitosan (N-PhCS) as an enteric polymer by acylation at amino groups of chitosan (CS) via ring-opening reactions of phthalic anhydride (PA). The suitable conditions for preparation and appropriate factors, i.e. temperature, stirring time, mole ratio of CS:PA, neutralization pH (step 3 of preparation process) and different molecular weights of CS (20 and 200 kDa) were studied. Physicochemical properties of N-PhCS were evaluated, i.e. chemical structure, powder X-ray diffraction, thermal property and solubility of N-PhCS in pH 1-10 media. The results showed that the preparation under high temperature resulted in the cyclization of phthalimido moieties. Thus, the suitable conditions to prepare N-PhCS was at 25˚C under stirring for 4 h, CS:PA at 1:5 mole ratio and neutralization pH at pH 5. The obtained N-PhCS from CS 20 kDa was chitosan-N-phthalamidate sodium (sodium 2-(chitosan-N-carbonyl) benzoate) with the highest degree of substitution and the solubility best fitted to enteric polymer property. The study of stability and cytotoxicity of the polymer suggested that cyclization of N-PhCS occurred during storage at high temperature and it was non-toxic and compatible to Caco-2 cells when used at concentrations of 0.01-1 mg/mL. N-PhCS films exhibited a good enteric property and the solubility in simulated gastric fluid was 12.43% with complete dissolution in simulated intestinal fluid. The moisture barrier property and tensile strength of the films were closed to chitosan acetate film but they were more brittle. Physicochemical properties and solubility of N-PhCS prepared from different molecular weights of CS were similar except for the higher viscosity of the solution of the higher molecular weight salt. In conclusion, chitosan-N-phthalamidate sodium can be applied in enteric and colonic drug delivery system.

Program of Pharmaceutical Sciences Graduate School, Silpakorn University
Student's signature …….…… Academic year 2011
Thesis Advisors' signature ……

52361207 : สาขาวิชาวิทยาการทางเภสัชศาสตร์
คำสำคัญ : ไคโตซาน / ทาลิกแอนไฮไดรด์ / เอ็น-ทาโลอิลไคโตซาน / การดัดแปรโครงสร้าง / สมบัติการก่อฟิล์ม / เอนเทอริก พอลิเมอร์.

สุนิตดา ขาวทอง : การดัดแปรโครงสร้างของไคโตซานด้วยทาลิกแอนไฮไดรด์และสมบัติทางเคมีฟิสิกส์และการก่อฟิล์ม. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : รองศาสตราจารย์ ดร.จุไรรัตน์ นันทานิช. 100 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือการดัดแปรอนุพันธ์ไคโตซานในรูปแบบเอ็น-ทาโลอิลไคโตซานเพื่อพัฒนาสมบัติการเป็นเอนเทอริกพอลิเมอร์ โดยปฏิกิริยาเอซิเลชันที่หมู่อะมิโนของไคโตซานด้วยการเปิดวงแหวนของทาลิกแอนไฮไดรด์ ศึกษาสภาวะและปัจจัยที่เหมาะสม ได้แก่ อุณหภูมิ ระยะเวลาการ เตรียม อัตราส่วนโมลระหว่างไคโตซานและทาลิกแอนไฮไดรด์ พีเอชของขั้นตอนการทำให้เป็นกลาง (ในกระบวนการเตรียมขั้นตอนที่ 3) และน้ำหนักโมเลกุลของไคโตซานที่ 20 และ 200 กิโลดาลตัน โดยประเมินสมบัติทางเคมีฟิสิกส์ของอนุพันธ์ที่เตรียมได้ ได้แก่ โครงสร้างทางเคมี การเลี้ยวเบนรังสีเอกซ์ สมบัติทางความร้อน และค่าการละลาย ที่สภาวะพีเอช 1-10 พบว่าการเตรียมที่อุณหภูมิสูงมีผล ทำให้เกิดไซไคลเซชั่นของหมู่ทาโลอิ ล ดังนั้นสภาวะที่เหมาะสมได้แก่ การเตรียมที่อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 4 ชั่วโมง ที่อัตราส่วนโมลระหว่างไคโตซานต่อ ทาลิกแอนไฮไดรด์เป็น 1:5 และปรับสภาวะพีเอชในขั้นตอนที่ 3 เป็นพีเอช 5 ซึ่งอนุพันธ์ที่เตรียมได้จาก ไคโตซาน 20 กิโลดาลตันแสดงโครงสร้างในรูปแบบไคโตซาน-เอ็น-ทาลิมิเดทโซเดียม (โซเดียม สอง-(ไคโตซาน-เอ็น-คาร์บอนิล) เบนโซเอท) ที่มีค่าความสามารถในการแทนที่ สูงสุดและมีสมบัติการละลายเป็นเอนเทอริกพอลิเมอร์ จากนั้นนำอนุพันธ์ที่ได้มาทดสอบผลของความคงตัว ความเป็นพิษต่อเซลล์คาโคทู (Caco-2) และสมบัติการเป็นสารก่อฟิล์ม พบว่าเกิดไซไคลเซชั่นของหมู่ทาโลอิลเมื่อเก็บที่อุณหภูมิสูง และไม่พบความเป็นพิษ ต่อเซลล์คาโคทูของเอ็น-ทาโลอิลไคโตซานในช่วงความเข้มข้น 0.01-1 มิลลิกรัมต่อมิลลิลิตร เมื่อเตรียมเป็นแผ่นฟิล์มพบว่ามีสมบัติเป็นฟิล์มเอนเทอริกที่ดีเนื่องจากมีค่าการละลายเพียงร้อยละ 12.43 ในของเหลวจำลองน้ำย่อยในกระเพาะอาหารและละลายได้หมดในของเหลวจำลองน้ำย่อยในลำไส้เล็ก ส่วนสมบัติในการป้องกันความชื้นและความทนแรงดึงมีค่าใกล้เคียงกับฟิล์มไคโตซานอะซิเทต แต่มีความเปราะมากกว่า และอนุพันธ์ที่เตรียมได้จากไคโตซานน้ำหนักโมเลกุลต่างกันมีสมบัติทางเคมีฟิสิกส์และค่าการละลายใกล้เคียงกัน เพียงแต่สารละลายของสารที่มีน้ำหนักโมเลกุลสูงกว่าจะมีความหนืดมากกว่า โดยสรุป ไคโตซาน-เอ็น-ทาลิมิเดทโซเดียมเป็นพอลิเมอร์ที่สามารถนำไปประยุกต์ใช้ในระบบนำส่งยาสู่ลำไส้ต่อไป

สาขาวิชาวิทยาการทางเภสัชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ลายมือชื่อนักศึกษา ..... ปีการศึกษา 2554
ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ .....

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I also record my special appreciation for the kind assistance, support and friendship granted me by all my friends, my colleagues and fellow graduate students. Finally, I wish to declare my infinite gratitude to my family for their endless love, encouragement, care and precious spiritual support throughout my life.

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CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the research problem

Thailand is a great source of marine food industry. Therefore, a huge number of wastes and bio-wastes e.g. squid pens and shrimp shells and crab shells, etc., were regarded as a source of pollution. Nevertheless, the bio-wastes composed of biopolymers such as chitin, chitosan, protein and astaxanthin with high economical values. Currently, chitin and chitosan have been used in many other industries, such as agricultural, pharmaceutical, water treatment, food science and cosmetic applications [1]. However, the utilization of chitin has been restricted by its intractability and insolubility because it is highly hydrophobic and insoluble in water and most organic solvents. Chitin is soluble only in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjunction with aqueous solutions of mineral acids, thus chitosan is considerably more versatile than chitin [2].

Chitosan (2-amino-2-deoxy-(1-4)-β-D-glucopyranan) is a natural linear biopolyaminosaccharide. Its structure is very similar to cellulose and chitin, except that the amino group replaces the hydroxyl and acetyl amide group on the C-2 position of cellulose and chitin, respectively [3]. Chitosan is the N-deacetylated derivative of chitin by chemical or enzyme reaction [4]. Chitin is the second abundant polysaccharide next to cellulose in nature, which is the mainly component of exoskeleton of insects, crustaceans such as crabs, shrimps, lobsters, squid pens and cell walls of some fungi such as *Zygomicetes*, *Aspergillus* and *Mucor* [5]. In addition, the characteristics of chitosan especially, degree of N-deacetylation (40-98%) and molecular weight (50-2,000 kDa) are very important to their biological properties [6, 7]. Chitosan has a number of properties, such as biocompatibility, biodegradability and non-toxicity, which suitable for usage in biomedical and pharmaceutical formulations [8-11]. It is practically insoluble in water or aqueous solutions at pH

above 6.5 because of the semi-crystalline structure which limits its applications [12-14]. Although chitosan is soluble in dilute organic acid, such as acetic acid, citric acid, malic acid and some inorganic acid, such as hydrochloric acid, due to the presence of free amino groups along the polymer [15], it may not desirable in many applications of chitosan e.g. food, cosmetics, biomedical. However, the amino functionality is a strong nucleophilic and reactive at higher pH values which could be suitably modified by various chemical reactions [16], resulting in N-substituted derivatives to enhance solubility and impart desired properties. Some N-substituted reactions include N-alkylation, N-acylation, N-Sulfation and N-hydroxyacylation [16-21].

N-acylation of chitosan is the most typical and extensively studied modification reaction. It can be obtained from acyl halide and anhydride [22]. Cyclic acid anhydrides e.g. succinic, maleic, glutaric, itaconic and phthalic anhydrides are also used for acylation of chitosan via ring-opening reaction giving N-carboxyacyl chitosans. This reaction is easy to prepare under mild condition [23, 24]. There are many reports about the development and physicochemical characterization of N-succinyl chitosan by the introduction of succinyl groups into amino groups of glucosamine units of chitosan in various solvent systems, such as acetic acid/ethanol, methanol, acetone, dimethylsulfoxide (DMSO) and dimethylformamide (DMF) [25-30]. N-succinyl chitosan displays good water soluble property at various pHs. It is initially developed as wound dressing materials. It is currently also applied as cosmetic material and drug carrier. Currently, only a few study of the use of other cyclic acid anhydrides such as phthalic anhydride has been reported. N-acylation of chitosan with various cyclic anhydride such as phthalic anhydride were reported [31]. The result showed that the N-phthaloyl chitosan with 34% degree of substitution showed the solubility even in the basic region above pH 7 and acidic region pH below 4. Although, it enhanced the solubility in basic region, the applications of colonic drug delivery are restricted due to the solubility in acidic region. Moreover, the physicochemical properties of N-phthaloyl chitosan such as FTIR, crystalline state and thermal property were not clarified sufficiently and there is no report about the toxicity of this derivative before. In addition, chitosan esters as chitosan succinate

and chitosan phthalate have been used successfully as potential matrices for the colon-specific oral delivery.

Therefore, the aim of this study was to prepare N-phthaloyl chitosan as an enteric polymer by acylation at amino groups of chitosan via ring-opening reactions of phthalic anhydride. The suitable conditions for preparation and appropriate factors, i.e. temperature, stirring time, mole ratio of chitosan and phthalic anhydride, neutralization pH (step 3 of preparation process) and different molecular weights of CS (20 and 200 kDa) were studied. Physicochemical properties of N-phthaloyl chitosan, i.e. chemical structure, powder X-ray diffraction, thermal property and solubility in pH 1-10 media, were evaluated. The degree of substitution, cytotoxicity to Caco-2 cells, stability and film forming properties including mechanical properties, water vapor permeability, moisture content and pH solubility in SGF and SIF of N-phthaloyl chitosan were also investigated.

1.2 Objectives of this research

1. To study suitable conditions for preparation and appropriate factors for enteric polymer property of N-phthaloyl chitosan, i.e. temperature, stirring time, mole ratio between chitosan and phthalic anhydride and molecular weight of chitosan.
2. The physicochemical properties of N-phthaloyl chitosan, i.e. chemical structure, powder X-ray diffraction, thermal property, solubility and degree of substitution were studied.
3. To investigate cytotoxicity of N-phthaloyl chitosan in Caco-2 cells.
4. To evaluate the film forming properties of N-phthaloyl chitosan including tensile strength, water vapor permeability, moisture content and pH solubility.

1.3 Hypotheses of this research

1. N-phthaloyl chitosan can be prepared via ring-opening reactions of phthalic anhydride with amino groups of chitosan under the suitable condition.
2. N-phthaloyl chitosan with high degree of substitution can enhance the solubility of chitosan.

3. N-phthaloyl chitosan has low toxicity and cell compatibility.
4. The films of N-phthaloyl chitosan have good enteric film property.

CHAPTER 2
LITERATURE REVIEWS

2.1 Chitosan

Chitosan is a cationic biopolymer, one of the most important partially deacetylated derivatives obtained from chitin by chemical or enzymic reaction, which is the second abundant polysaccharide next to cellulose in nature. Chitin is found in the exoskeleton of insects, crustaceans such as crabs, shrimps, lobsters, squid pens and the cell walls of some fungi such as *Zygomicetes*, *Aspergillus* and *Mucor* [5]. However, the utilization of chitin has been restricted by its intractability and insolubility, because it is highly hydrophobic and insoluble in water and most organic solvents. Chitin is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjunction with aqueous solutions of mineral acids, thus chitosan is considerably more versatile than chitin [2].

2.1.1 Chemical structure of chitosan

Chitosan (2-amino-2-deoxy-(1-4)-β-D-glucopyranan) is a copolymer consists of D-glucosamine and N-acetyl-D-glucosamine units, randomly or block distributed throughout the biopolymer. It structure very similar to cellulose and chitin, except that the amino group replaces the hydroxyl group and acetylamide group on the C-2 position of the glucose rings [32]. The structural details of cellulose, chitin and chitosan are shown in Figure 1. The characteristics of chitosan that consist of degree of N-deacetylation (40-98%), defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular weights (50-2,000 kDa) are very important to the physicochemical properties of chitosan utilization and they have been a major effect on the biological properties [6, 7, 33].

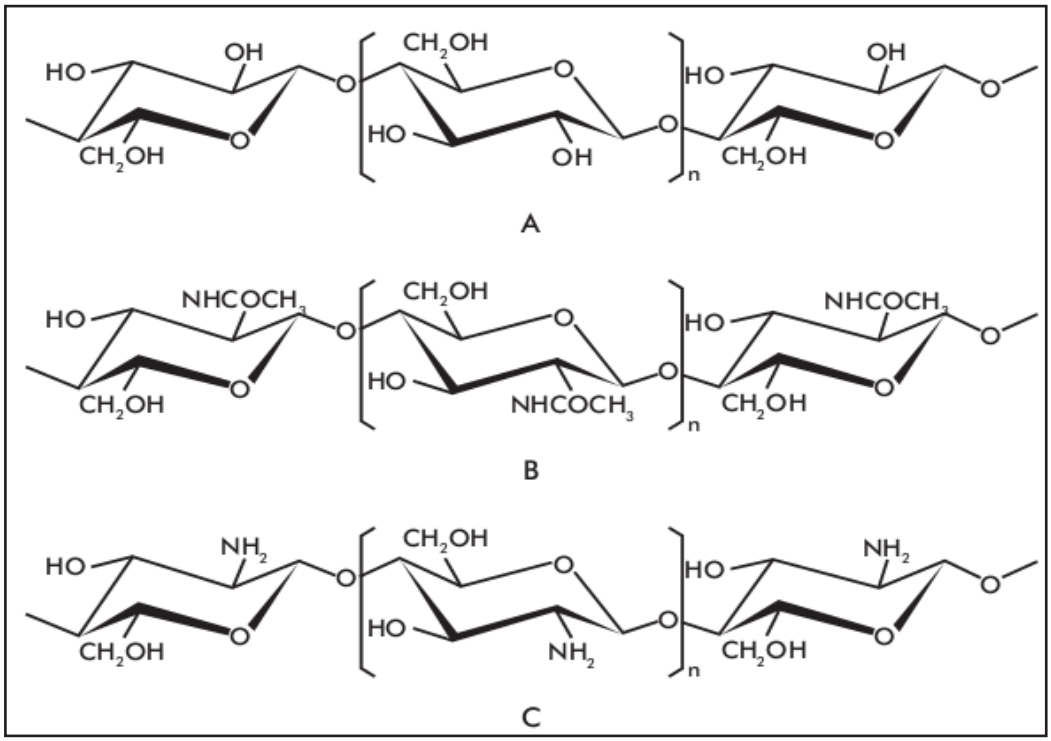


Figure 1 Structural representation (A) cellulose, (B) fully acetylated chitin and (C) fully deacetylated chitosan, evidencing their structural similarity.

Source: Ramírez, M. A. et al., (2010). "Chitin and its derivatives as biopolymers with potential agricultural applications." **Biotecnología Aplicada** 27: 271.

2.1.2 Properties of chitosan

Most of the naturally occurring polysaccharides such as cellulose, pectin, alginic acid, agar, agarose and carragenas are natural and acidic in nature. Meanwhile, chitin and chitosan are examples of highly basic polysaccharides. Their properties include solubility in various media, solution, viscosity, polyelectrolyte behavior, and ability of form films, metal chelations and structural characteristics [34]. The chemical and biological properties of chitosan were reported in Tables 1 and 2 [6]. These properties make chitosan as a good candidate for the development of conventional and novel drug and gene delivery systems [35]. Chitosan acetate was combined with hydroxypropyl methylcellulose, showed the potential of a new combination coating material for colonic drug delivery [10]. It has also been used as a pharmaceutical excipient, as a diluents compression of tablets and a binder in wet granulation [15]. Chitosan performs as an absorption enhancer in the intestine by

increasing the residence time of dosage forms at mucosal sites, inhibiting proteolytic enzymes and increasing the permeability of protein and peptide drugs across mucosal membranes. Chitosan is degraded by the microflora that is available in the colon. As a result, this compound could be promising for colon-specific drug delivery [36]. Chitosan esters, such as chitosan succinate and chitosan phthalate have been used successfully as potential matrices for the colon-specific oral delivery [31].

Film forming property of chitosan

Chitosan has been well known for its good film-forming property as a result of intra- and intermolecular hydrogen bonding. Films from aqueous acidic chitosan solution are clear, tough, flexible and good oxygen barriers [37]. Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution alleviates this problem [38]. A number of researches report the preparation of chitosan derivative films and study of mechanical and physical properties. For example, the alkyl-chitosan derivatives appear to be more plastic than chitosan films but less resistant [39]. N, N, N,-Trimethyl chitosan demonstrated a good film forming ability and a significant reduction in contact angle corresponds to an increase in the degree of hydrophilicity [40]. Carboxymethyl chitosan film was reported that the vapor permeability value was higher than chitosan film [41]. Moreover, chitosan films can be used in widely applications such as materials in food packaging, antimicrobial packaging, coating and wound dressing as well as drug release membranes [42-45]. Although, chitosan has been investigated for colon-specific delivery of drugs because of its biodegradability by colonic bacteria [46], the application of chitosan as enteric coating was less successfully due to its readily dissolving in acidic conditions [47].

Table 1 Chemical properties of chitosan.

Chemical properties of chitosan
Cationic polyamine
High charge density at pH<6.5
Adheres to negatively charged surfaces
Film-forming ability
Forms gels with polyanions
High molecular weight linear polyelectrolyte
Viscosity, high to low
Chelates certain transitional metals
Amiable to chemical modification
Reactive amino/ hydroxyl groups

Table 2 Biological properties of chitosan.

Biological properties of chitosan
Biocompatibility
Natural polymer
Biodegradable to normal body constituents
Safe and non-toxic
Hemostatic, bacteriostatic and fungstatic
Spermicidal
Anticancerogen
Anticholesteremic
Reasonable cost
Versatile

Source: Adapt from Hejazi, R. and M. Amiji, (2003). "Chitosan-based gastrointestinal delivery systems." **Journal of Controlled Release** 89: 153-154.

2.1.3 Solubility of chitosan

Chitosan is soluble only in acidic media and part inorganic acid, such as acetic acid, citric acid, malic acid and hydrochloric acid [15], due to the presence of free amino groups along the polymer chains make chitosan is a cationic polyelectrolyte ($pK_a \approx 6.5$). It is normally insoluble in water, alkali or aqueous solutions above pH 6.5 and organic solvents make its utilization limited in the pharmaceutical field as protein delivery is the easy dissolution of chitosan in the low pH of stomach [33]. Because its semi-crystalline structure, which is attributed to extensive intramolecular and intermolecular hydrogen bonding between the chains and sheets, respectively (Figure 2) [12-14]. Despite this limitation, various applications of chitosan and modified chitosan have been reported. Hence, improving the solubility of chitosan is important if this abundant resource is to be utilized across a wide pH range [49].

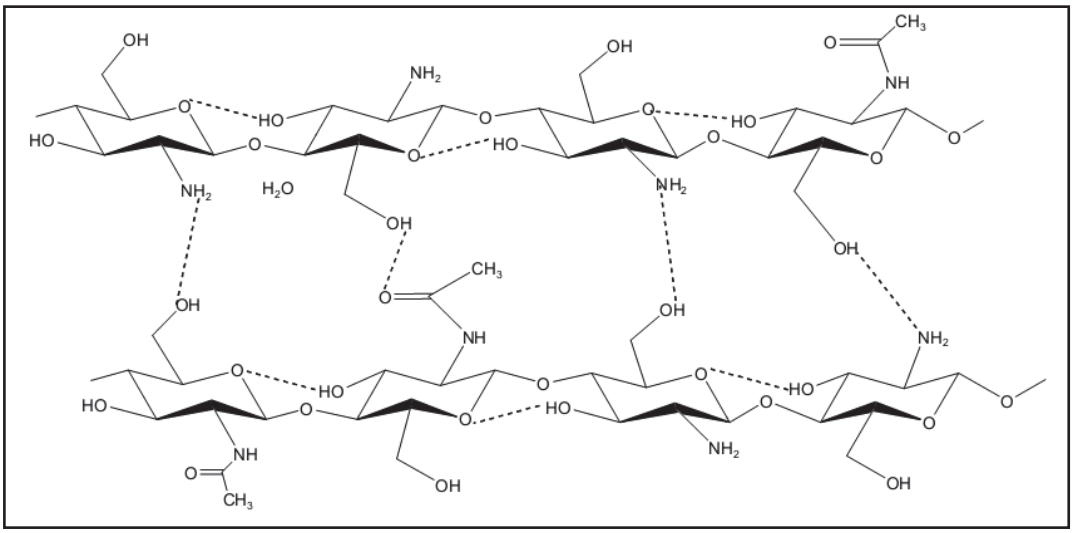


Figure 2 Crystalline structure of chitosan.

Source: Champagne, L. M. (2008). "The synthesis of water soluble / n-acyl chitosan derivatives for characterization as antibacterial agents." PhD thesis, Chemistry, Louisiana State University and Agricultural & Mechanical College: 14.

2.2 Chemical modifications of chitosan

In order to improve or impart new properties to chitosan, chemical modification of chitosan is important and necessary. The chemical modification of chitosan is interesting because it not only unchanged the fundamental skeleton of chitosan but also keep the original physicochemical and biochemical properties and finally could bring new or improved properties [21]. Chitosan chains compose of three attractive reactive sites for chemical modification as two hydroxyl groups (a primary hydroxyl at C-6 and a secondary hydroxyl at C-3) and a highly reactive amino group (at C-2) (Figure 3) [13]. The site of modification is dictated by the desired application of the final chitosan derivative [4]. The scope for preparing a lot of derivatives are shown in Figure 4 [13]. The presence of free amine groups along the chitosan chain, which could be suitably modified by various chemical reactions to impart desired properties and enhance solubility in water as well as in organic solvents [32, 50]. The amino functionality gives starting chemical reactions such as alkylation, acylation, quaternization, hydroxyalkylation, carboxyalkylation, thiolation, sulfation, phosphorylation, graft copolymerization, etc [14, 24, 40, 51-53]. Moreover, the derivatives provided a variety of products with properties such as antibacterial, anti-fungal, anti-viral, anti-acid, anti-ulcer, non-toxic, non-allergenic, total biocompatibility and biodegradability, etc [32, 54, 55].

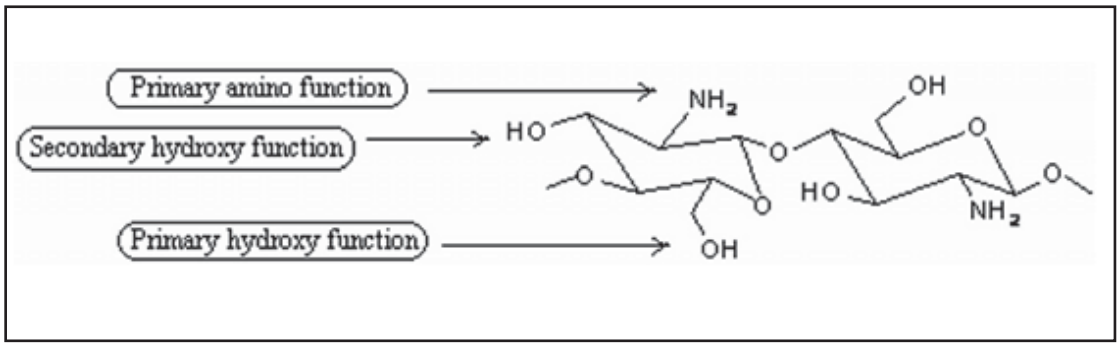


Figure 3 Functional groups of chitosan.

Source: Mourya, V.K., and N. N. Inamdar. (2008) "Chitosan-modifications and applications: Opportunities galore." **Reactive & Functional Polymers** 68: 1017.

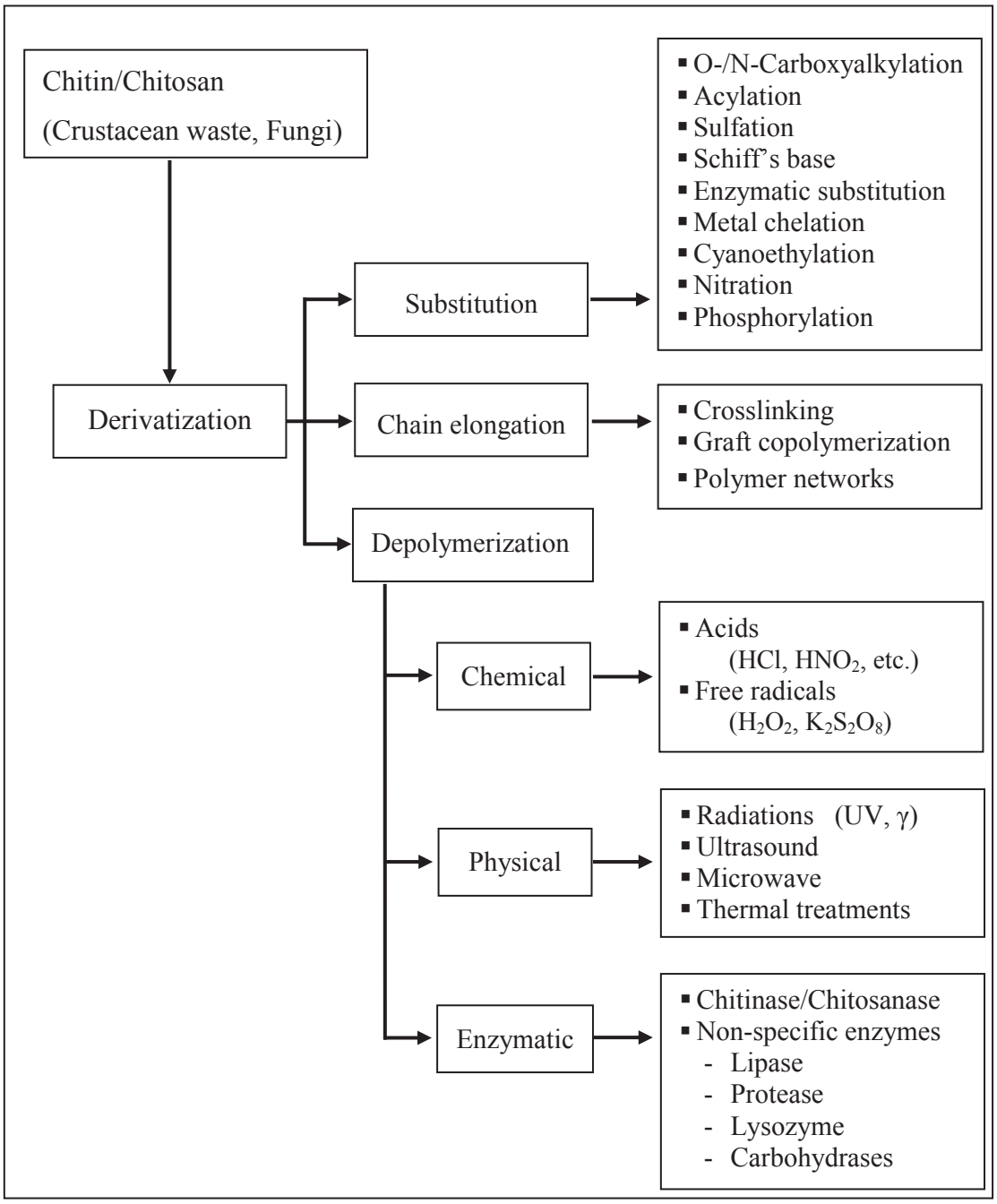


Figure 4 Multifaceted derivatization potential of chitin and chitosan.

Source: Adapt from Prashanth, K.V.H. and R.N. Tharanathan, (2007). "Chitin/ chitosan: modifications and their unlimited application potential-an overview." **Trends in Food Science & Technology**, 18: 119.

2.2.1 N-Acylation chitosan

N-Acyl derivatives of chitosan are the most typical and extensively studied modification reaction. Since, chitosan is a strong nucleophile because of the presence of nonbonding pair of electrons on its primary amino groups [56]. Chitosan reacts readily with most aldehydes to produce imines. It also reacts with acid anhydride and acyl halide to form the corresponding N-Acyl derivatives (Figure 5) [21, 57].

N-acylation of chitosan has been achieved with various kinds of acid anhydride. Since most acid anhydrides exhibit very little solubility in aqueous media, the reactions between chitosan and acid anhydrides are conducted under heterogeneous experimental conditions. In a general way, acylation reactions lead frequently in mediums as aqueous acetic acid/methanol, pyridine/chloroform, trichloroacetic acid/dichloroethane, ethanol/methanol mixture, methanol/formamide or DMA-LiCl [23]. N-acylation with acetic anhydride was reported to give an improved method of preparing water-soluble chitosan. The haft N-acetylated chitosan exhibited good water solubility. Thus, the amount of acetic anhydride was the most important factor affecting the substitution degree of the chitosan [58].

2.2.2 Cyclic acid anhydride

Acid anhydrides are formed from the dehydration reaction of two carboxylic acid groups. Anhydrides are highly reactive toward nucleophiles and are able to acylate a number of the important functional groups of proteins and other macromolecules as well as amino groups of chitosan [57]. Cyclic acid anhydrides are used for acylation reaction of chitosan via ring-opening reaction giving N-acyl chitosans, e.g. succinic, maleic, glutaric, itaconic and phthalic anhydrides, this reaction is easily to prepare under mind condition [23, 24].

N-acylation via ring opening reactions of partially deacetylated chitosan (88% DD) with various cyclic anhydrides in aqueous methanol system was reported [24]. The authors reported that all of the derivatives (DS = 0.21-0.80) displayed solubility in water at various pH. All of the products exhibited solubility in the pH region below 4.0 and above pH 7.0 (Table 3). The solubility in the acidic

region would be caused by the protonation of the N-amino groups ($–NH_2$ to $–NH_3^+$), and the solubility in the basic region would be caused by the change of the carboxy groups to carboxylate ions (–COOH to $–COO^–$). The derivatives did not exhibit complete solubility in the pH range of 4.0 – 7.0, which corresponded to the isoelectric point of the products. In this pH range, an equimolar of $–NH_3^+$ and $–COO^–$ groups existed in the macromolecule [24].

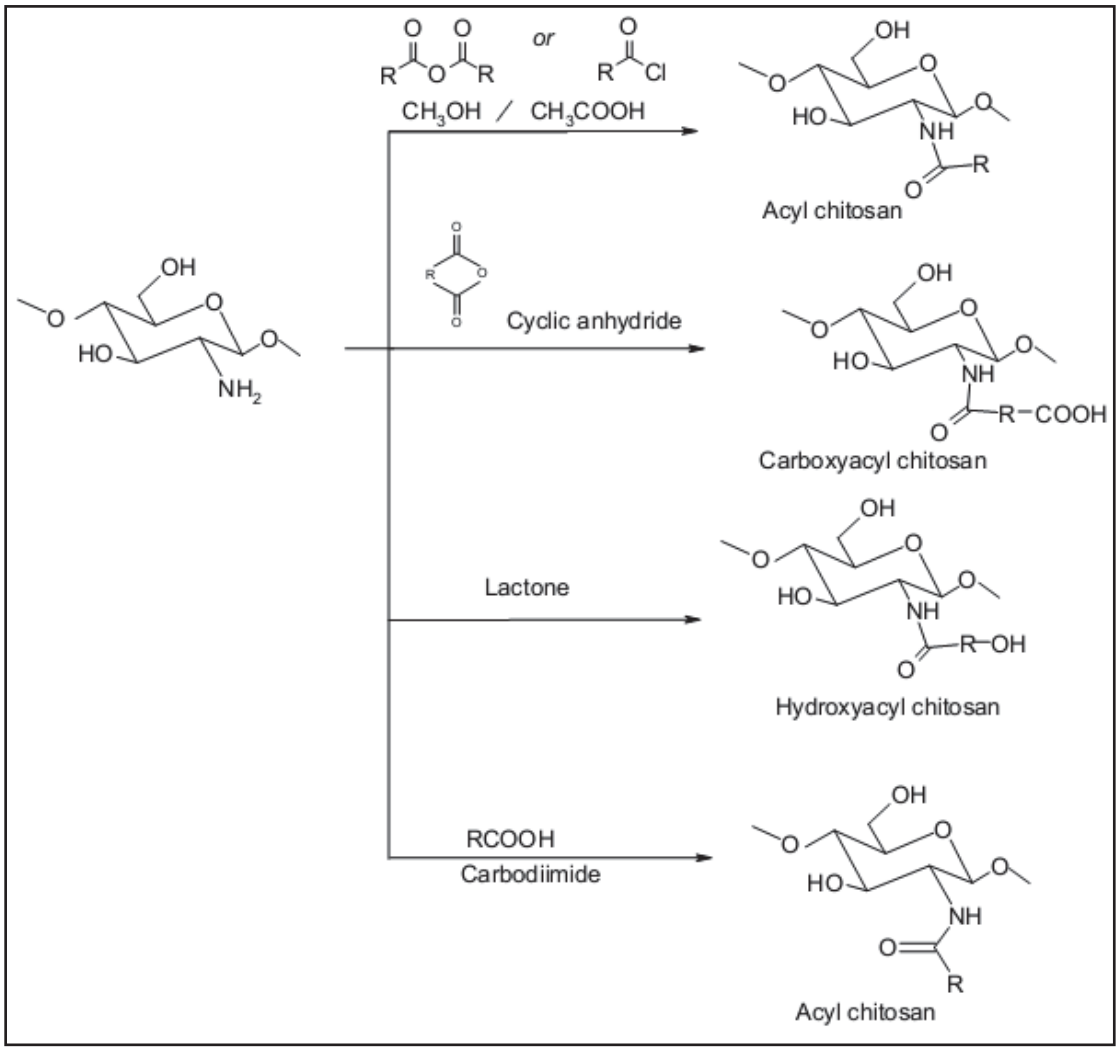


Figure 5 Acylation of chitosan.

Source: Mourya, V.K. and N. N. Inamdar. (2008) “Chitosan-modifications and applications: Opportunities galore.” **Reactive & Functional Polymers** 68: 1026.

Table 3 Solubility of N-acylated chitosan (88%DD) in water of various pH[a].

Sample[b]	DS	Solubility[c] pH: 1 3 5 7 9 11 13
Suc	0.32	
Gltl	0.43	
Phth	0.34	
THP	0.32	
Norb	0.33	
Cycl	0.36	
Trim	0.20	
CS-88		

a. Solid sample (100 mg; 88%DD) was dispersed in H_2O (20 mL). The pH of the solution was adjusted with 0.5% (w/v) aqueous HCl and NaOH.

b. Suc, succinic; Gltl, glutalic; Phth, phthalic; THP, cis-1,2,3,6-tetrahydrophthalic; Norb, 5-norbornyl-endo-2,3-dicarboxylic; Cycl, cis-1,2-cyclohexyl dicarboxylic; Trim, trimellitic anhydride; CS-88, chitosan (88%DD).

c. white bar, soluble; black bar, insoluble.

Source: Adapt from Sashiwa, H. and Y. Shigemasa, (1999). "Chemical modification of chitin and chitosan 2: preparation and water soluble property of N-acylated or N-alkylated partially deacetylated chitins." **Carbohydrate Polymers** 39: 129.

Succinic anhydride

N-succinyl chitosan has been reports by many researchers. It has been obtained via ring-opening reaction by introduction of succinyl groups into N-terminal of the glucosamine units of chitosan (Figure 6) [28]. Succinylation degree of N-succinyl chitosan could be easily modified by changing reaction conditions using succinic anhydride in various solvent systems, such as acetic acid/ethanol, methanol, acetone, dimethylsulfoxide (DMSO) and dimethylformamide (DMF). The reaction temperature ranged from room temperature to 65˚C for 3-24 h. Moreover, chitosan

was used in their reactions have the ranging of deacetylation degree from 85 to 90 %DD and molecular weight ranging from10 to 300 kDa [26-30, 59]. The structure changes of derivatives were confirmed by ^{1}H-NMR, ^{13}C-NMR and FTIR spectroscopy and their physical properties were analyzed by powder X-ray diffraction (PXRD) and differential scanning calorimetry (DSC) to investigate the crystallinity of the derivatives. The comparison between the FTIR spectra and the PXRD patterns of chitosan and N-succinyl chitosan were reported (Figure 7-8). In the FTIR spectrum of N-succinyl chitosan, the absorption bands of stretching vibration of the –OH and –NH_3 (3300–3500 cm^{-1}) became narrower and shifted to lower wave number after introducing succinyl groups. Another major change could be observed at the new absorption band of 1413 cm^{-1}, corresponding to symmetric stretching of the –COO group. The peak at 1650 cm^{-1} (Amide I) increased and no absorption bands appeared at 1720–1750 cm^{-1}, indicating that the succinyl derivation reaction took place at the N-position and –NH–CO– groups have been formed. Meanwhile, the peak at 1597 cm^{-1} (–NH_2 bending) decreased greatly, and the new signal at 1566 cm^{-1} (assigned to the secondary amines) further suggested that the amino groups of chitosan were substituted. The X-ray diffraction pattern of chitosan showed distinct crystalline peaks at around 11˚ and 20˚ compared to N-succinyl chitosan. A lot of strong intermolecular and intramolecular hydrogen bonds (H- bonds) make chitosan to form crystalline regions easily and be insoluble in water. However, the peak at 11˚ in N-succinyl chitosan X-ray diffraction spectrum disappeared and the peak at 20˚ weakened obviously. This result suggests that with the substitution of N-succinyl resulting in the hydrogen bonding capacity decreased. The solubility of N-succinyl chitosan in various pH was reported, it has degree of substitution of 33% showed the solubility of both acidic and basic region (Figure 9). As the derivative has both amino and carboxy group, the solubility in acid region (pH 1-3) would be caused by the protonation of amino group. The solubility in alkaline region (pH 7-13) would also be caused by the change of carboxy group to carboxylate ion. In addition, the solubility between pH 4.5-6.8 would be owing to the isoelectric point which exists equimolar of -NH_3^+ and -COO^- groups in the molecule [30]. Whereas N-succinyl chitosan with high degree of substitution (degree of substitution > 65%) exhibits the

opposite behavior of chitosan [21]. The application of N-succinyl chitosan has been widely researched in cosmetics and pharmaceutical field such as drug carrier, film or membrane formation, enzyme immobilization [25, 29, 59]. However, it was initially developed as wound dressing materials, it is currently also applied as cosmetic materials (Moistfine liquids) [59].

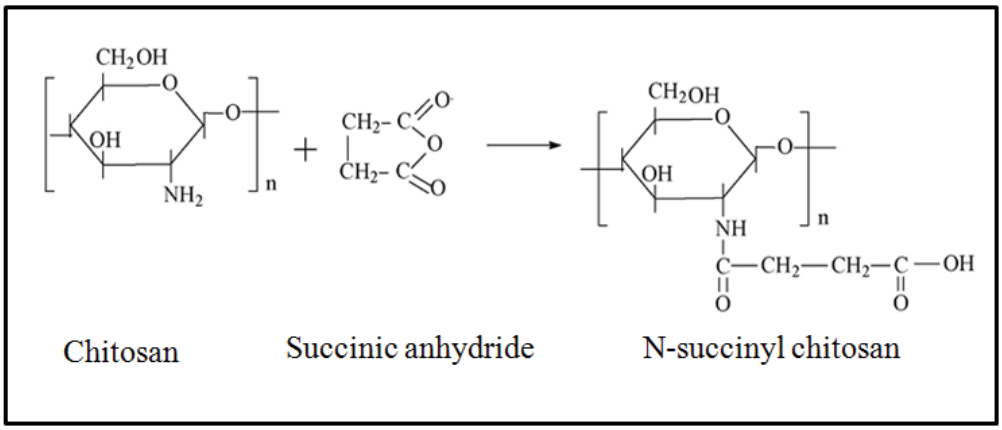


Figure 6 Modification of N-succinyl chitosan.

Source: Sui, W. et al. (2008). "Preparation and properties of an amphiphilic derivative of succinyl-chitosan." **Colloids and Surfaces A: Physicochem. Eng. Aspects** 316: 172.

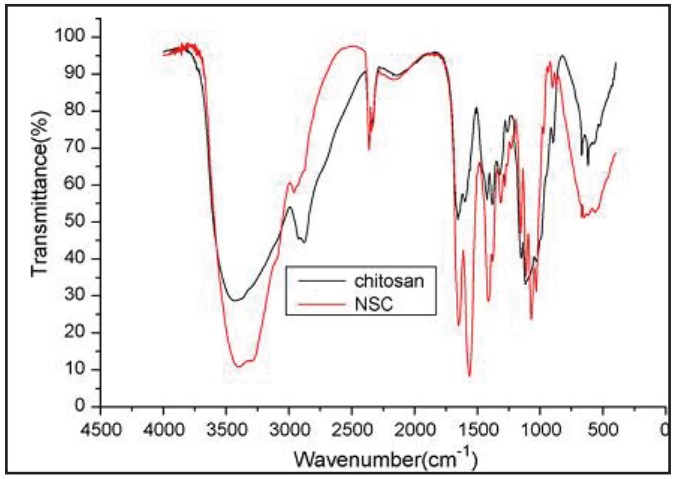


Figure 7 FTIR spectra of chitosan and N-succinyl chitosan (NSC).

Source: Zhou, J. Q. and J. W. Wang (2009). "Immobilization of alliinase with a water soluble–insoluble reversible N-succinyl-chitosan for allicin production" **Enzyme and Microbial Technology** 45: 301.

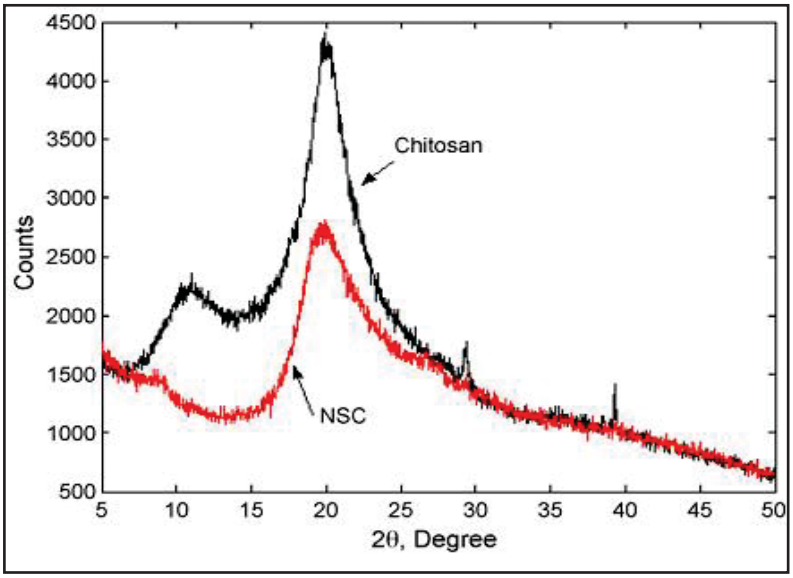


Figure 8 The X-ray diffraction patterns of chitosan and N-succinyl chitosan (NSC). Source: Zhou, J. Q. and J. W. Wang (2009). "Immobilization of alliinase with a water soluble–insoluble reversible N-succinyl-chitosan for allicin production" **Enzyme and Microbial Technology** 45: 301.

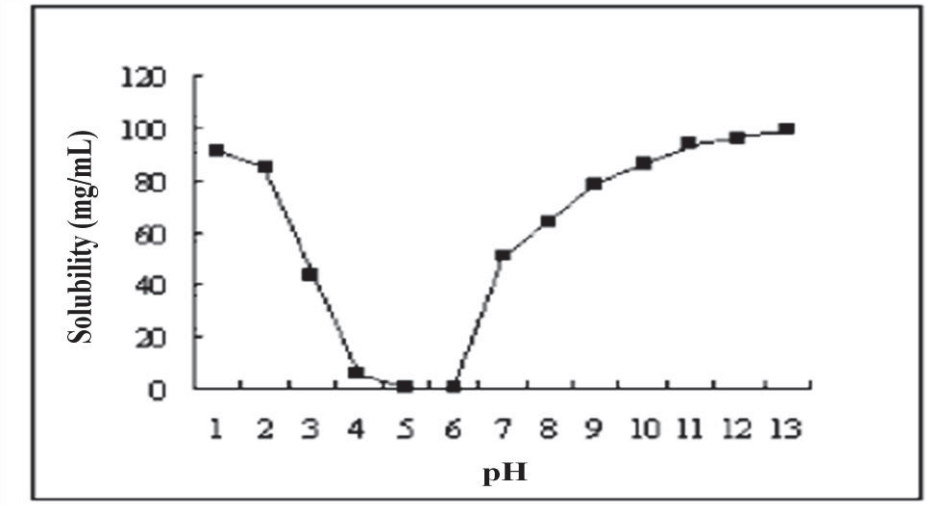


Figure 9 Solubility of N-succinyl chitosan (DS=0.33) in water of various pH. Source: Yan, C., et al. (2006). "Preparation of N-succinyl chitosan and their physical-chemical properties as a novel excipient." **Yakugaku Zasshi: Journal of the Pharmaceutical Society of Japan** 126 (9): 791.

Phthalic anhydride

Phthalic anhydride is a member of cyclic acid anhydrides that utilized in this study by introduction of phthaloyl groups into amino groups of the glucosamine units of chitosan. Phthalic anhydride itself is used as a monomer for synthetic resins such as glyptal, the alkyd resins and polyester resins [60]. Phthalates, in combination with various polymers, may be used as plasticizers and film coating agents in orally ingested solid pharmaceutical dosage forms and in numerous types of modified-release drug delivery systems such as enteric-coated and delayed-release tablets, pelletized delayed-release capsules, enteric-coated capsules, and controlled-release transdermal films [61]. Several different phthalates are currently used as excipients in approved pharmaceutical formulations:

1. Cellulose acetate phthalate (CAP)

CAP is produced by reacting the partial acetate ester of cellulose with phthalic anhydride in the presence of a tertiary organic base such as pyridine, or a strong acid such as sulfuric acid. CAP is used as an enteric film coating material, or as a matrix binder for tablets and capsules. Such coatings resist prolonged contact with the strongly acidic gastric fluid, but dissolve in the mildly acidic or neutral intestinal environment. CAP is commonly applied to solid-dosage forms either by coating from organic or aqueous solvent systems, or by direct compression. Concentrations generally used are 0.5–9.0% of the core weight. The addition of plasticizers improves the water resistance of this coating material, and formulations using such plasticizers are more effective than when CAP is used alone [62].

2. Dibutyl Phthalate (DBP)

DBP is produced from n-butanol and phthalic anhydride in an ester formation reaction. DBP is used in pharmaceutical formulations as a plasticizer in film-coatings. It has been evaluated as a pore-forming agent in novel delivery systems. It is also used extensively as a solvent, particularly in cosmetic formulations such as antiperspirants, hair shampoos, and hair sprays [63].

3. Diethyl Phthalate (DEP)

DEP is produced by the reaction of phthalic anhydride with ethanol in the presence of sulfuric acid. It is used as a plasticizer for film coatings on tablets,

beads, and granules at concentrations of 10–30% by weight of polymer. DEP is also used as an alcohol denaturant and as a solvent for cellulose acetate in the manufacture of varnishes and dopes [64].

4. Dimethyl Phthalate (DMP)

DMP is produced industrially from phthalic anhydride and methanol. DMP is used in pharmaceutical applications as a solvent and plasticizer for film-coatings such as hydroxypropyl methylcellulose, cellulose acetate and cellulose acetate–butyrate mixtures. In addition to a number of industrial applications, DMP is also widely used as an insect repellent with topical preparations typically applied as a 40% cream or lotion.

5. Hydroxypropyl methylcellulose phthalate (HPMCP)

HPMCP is prepared by the esterification of hypromellose with phthalic anhydride. The degree of alkyloxy and carboxybenzoyl substitution determines the properties of the polymer and in particular the pH at which it dissolves in aqueous media. It is widely used in oral pharmaceutical formulations as an enteric coating material for tablets or granules. HPMCP is insoluble in gastric fluid but will swell and dissolve rapidly in the upper intestine. Generally, concentrations of 5–10% of HPMCP are employed with the material being dissolved in either a dichloromethane : ethanol (50 : 50) or an ethanol : water (80 : 20) solvent mixture. HPMCP can normally be applied to tablets and granules without the addition of a plasticizer or other film formers, using established coating techniques [65].

6. Polyvinyl Acetate Phthalate (PVAP)

Polyvinyl acetate phthalate is a reaction product of phthalic anhydride, sodium acetate, and a partially hydrolyzed polyvinyl alcohol. The polyvinyl alcohol is a low molecular weight grade, and 87–89 mole percent is hydrolyzed. Therefore, the polyvinyl acetate phthalate polymer is a partial esterification of partially hydrolyzed polyvinyl acetate. PVAP is a viscosity-modifying agent that is used in pharmaceutical formulations to produce enteric coatings for products and for the core sealing of tablets prior to a sugar-coating process. PVAP does not exhibit tackiness during coating and produces strong robust

films. Plasticizers are often included in PVAP coating formulations to enable a continuous, homogeneous, noncracking film to be produced [66].

Moreover, phthalic anhydride is used as protecting amino groups by heating the amino group with phthalic anhydride [67]. The study chitosan ester as chitosan phthalate was prepared by reacting chitosan with phthalic anhydride. The esteric form with a different solubility profile was insoluble in acidic condition and provided sustained release in basic condition, suggesting its suitability for colon-specific drug delivery systems [50]. In addition, shellac ester as shellac phthalate demonstrated to improve the thermal stability as compared to native shellac [68].

2.3 Degree of substitutions

Degree of substitution (DS): represents the number of the substitution groups which are in the molecular unit of the glucosamine units [69]. The DS is an important parameter when assessing the conversion of chitosan into one of its derivates, that influences the physicochemical properties such as solubility, chemical reactivity and biodegradability [9, 40, 53]. For example, the bioadhesive property of chitosan was enhanced by N-acylation with fatty acid chlorides. Chitosan modified with oleoyl chloride showed better mucoadhesion properties than chitosan modified with lower of %DS [70]. The release of drug is controlled by diffusion, or by swelling followed by diffusion, depending on both the acyl chain length and the degree of acylation. The resulted showed that palmitoyl chitosan has a substitution degree 40 – 50% the best mechanical characteristics and drug release properties [16]. The water-soluble chitosan-N-arginine with various DS from 8.7 to 28.4%, there were able to inhibit almost all the bacteria (*Staphylococcus aureus* and *Escherichia coli*) at a concentration higher than 150 ppm [55]. The preparation of amphiphilic derivatives of chitosan, propyl-succinyl-chitosan (HBP-SCCHS) by chemical modification. The DS of derivative was estimated by elemental analysis. The results showed that the increase of DS of propyl groups resulting in the surface tension decreases at the same concentration of the derivatives for there are more hydrophobic groups get to the surface of the solution (Figure 10) [28].

N-succinyl chitosan were prepared by reaction of chitosan with succinic anhydride at 1:1 w/w (SC1:1) and 1:3 w/w (SC1:3), respectively. DS of SC1:1 and SC1:3 were determined by FTIR and ninhydrin assays, presented the DS is 10 and 20%, respectively (Figure 11). The solubility of chitosan and its derivative were measured at three different pH. The result showed that chitosan was perfectly soluble in acid media but precipitated at neutral and alkaline solutions. The higher DS derivative (SC1:3) appeared insoluble in pH 4.0 due to the predominance of carboxylic groups compared to amino groups but completely soluble at high pH while the lower DS derivative (SC1:1) become partially soluble in the entire pH range due to the increasing substitution of the amino groups by carboxylic groups, which become negatively charged above pH 6.0 (Table 4). The result indicated that the ratio of reagent was factor affecting of DS [29].

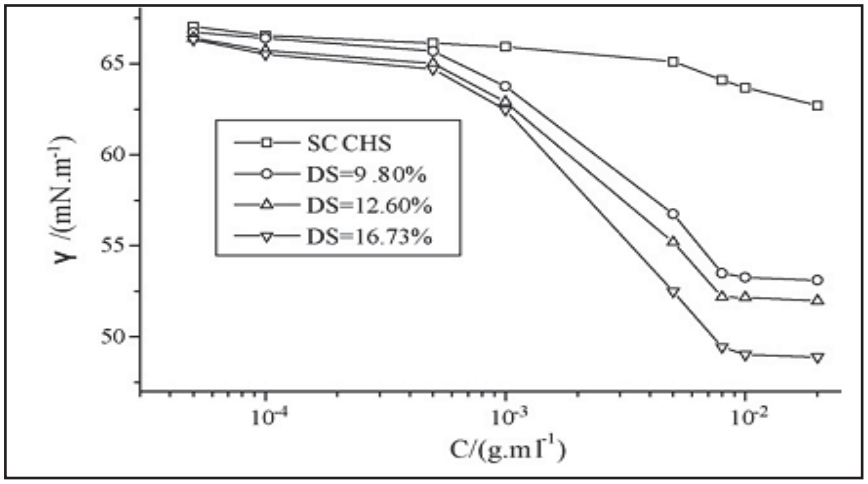


Figure 10 Surface tension–concentration plots of propyl-succinyl-chitosan (HBP-SCCHS) at different DS and N-succinyl chitosan (SC CHS).

Source: Sui, W. et al. (2008). "Preparation and properties of an amphiphilic derivative of succinyl-chitosan." **Colloids and Surfaces A: Physicochemical and Engineering Aspects** 316: 174.

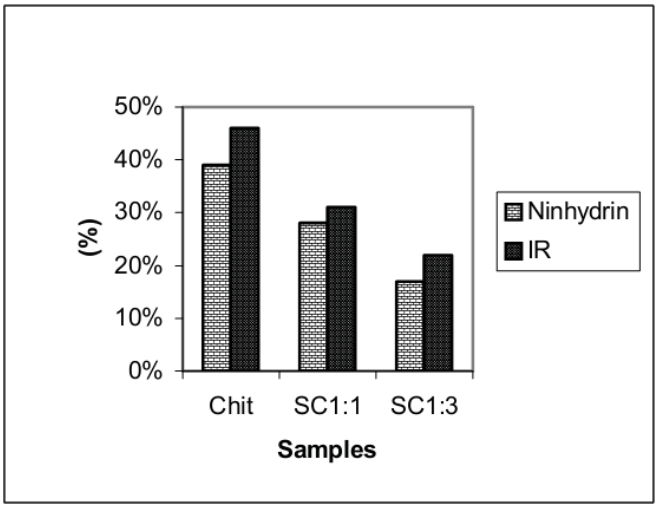


Figure 11 Degree of deacetylation of chitosan (Chit) and degree of substitution of N-succinyl chitosan prepared at 1:1 w/w (SC1:1) and 1:3 w/w (SC1:3) of chitosan and succinic anhydride determined by ninhydrin titration and infrared spectroscopy.

Source: Mello, K., et al. (2006). "Synthesis and physicochemical characterization of chemically modified chitosan by succinic anhydride." **Brazilian archives of biology and technology** 49(4): 667.

Table 4 Solubility tests of chitosan and N-succinyl chitosan prepared at 1:1 w/w (SC1:1) and 1:3 w/w (SC1:3) of chitosan and succinic anhydride in different solutions and pH.

Samples	**Solubility**		
	pH = 4.0	**pH = 7.0**	**pH = 10.0**
Chitosan	++++	-	-
SC1:1	++	++	+++
SC1:3	-	++	++++

(+, soluble; -, insoluble)

Source: Adapt from Mello, K., et al. (2006). "Synthesis and physicochemical characterization of chemically modified chitosan by succinic anhydride." **Brazilian archives of biology and technology** 49(4): 666.

The methods should be established to determine the exact DS, which can be used in manufacture, research and application of chitosan [71]. The chemical method, such as titration (ninhydrin assay) [72] or instrument methods, such as FTIR [16, 58, 73], NMR [24, 27, 40] and CHN elemental [55] analysis were used to determine the DS. However, all the methods have some limitation. In the case of chemical method, the operation is complicated and time-consuming. The instrument methods are normally expensive, but it is easy to be available, fast and can use solid sample directly [71]. The preparation of N-acylation of chitosan with various fatty acid chlorides for increasing hydrophobic character of derivatives [16]. The DS values were compared with ninhydrin and FTIR methods. The results showed no significant differences were noticed between the values obtained by these two assays (Table 5).

Table 5 Estimation of degree of substitution by ninhydrin and FTIR assays.

Samples	**Degree of substitution (%)**	
	Ninhydrin assay	**FTIR**
Nonmodified chitosan	15.4 ± 3.6	18.9 ± 2.1
Caproyl chitosan	43.6 ± 3.2	46.2 ± 4.2
Octanoyl chitosan	41.8 ± 3.3	43.9 ± 3.8
Myristoyl chitosan	45.6 ± 3.8	47.1 ± 2.7
Palmitoyl chitosan	44.4 ± 4.1	47.1 ± 3.6

Source: Adapt from Tien, C. L., et al. (2003). “N -acylated chitosan: hydrophobic matrices for controlled drug release.” **Journal of Controlled Release** 93: 4.

2.4 Toxicity of chitosan and its derivatives

Chitosan is widely regarded as a nontoxic, biologically compatible polymer [74]. Chitosan has low oral toxicity with an LD_{50} in mice exceeds 16 g/kg [75]. Moreover, chitosan was compared to common sugars and concluded that chitosan is less toxic than these substances [76]. Toxicity of chitosan might depend on different factors such as degree of deacetylation, molecular weight, purity and route of administration [6]. Although, chitosan has been proved as a non-toxic polymer, but the modifications made to chitosan could make it more or less toxic and any residual reactants should be carefully removed before using it for biomaterials as drug delivery systems etc. The summary of toxicity of chitosan and its derivatives is shown in Table 6 [77].

For example, chitosan in the form of nano/microparticles was not affect the cell viability of the Caco-2 cells when using in the concentration 0.01-0.1 mg/mL [78]. Trimethyl chitosan has relative low cytotoxicity when it is an oligomer (3-6 kDa) with IC_{50}> 10 mg/mL for DS below 55%. Nevertheless, it was shown that trimethyl chitosan of increasing degree of trimethylation increased cytotoxicity as did higher molecular weight derivatives (100 kDa) [79]. In addition, *In vitro* cell toxicity testing of N-succinyl chitosan was not shown the effect on the activity of 3T3 fibroblasts when the concentration in the range of 0–0.25 mg/mL of N-succinyl chitosan nanospheres. These results demonstrate that N-succinyl chitosan is non-toxic, and cell-compatible. It can be safely used as the drug matrix (Figure 12) [26].

Table 6 Toxicity of chitosan and chitosan derivatives.

Chitosan details (DD, MW)	**Modification**	**Assessment**	**IC_{50}**
95% DD, 18.7 kDa	Steric acid conjugation micelle	*In vitro*, A549 cells	369 ± 27 μg/mL
95% DD, 18.7 kDa	Steric acid conjugation and entrapment in micelle	*In vitro*, A549 cells	234 ± 9 μg/mL
97% DD, 65 kDa	N-octyl-O-sulphate	*In vitro*, primary rat hepatocytes	> 200 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, aspartic acid salt	*In vitro*, Caco-2 cells, pH 6.2	0.67 ± 0.24, 0.61 ± 0.10, 0.65 ± 0.20, 0.72 ± 0.16 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, glutamic acid salt	*In vitro*, Caco-2 cells, pH 6.2	0.56 ± 0.10, 0.48 ± 0.07, 0.35 ± 0.06, 0.46 ± 0.06 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, Lactic acid salt	*In vitro*, Caco-2 cells, pH 6.2	0.38 ± 0.13, 0.31 ± 0.06, 0.34 ± 0.04, 0.37 ± 0.08 mg/mL
87% DD, 20, 45, 200, 460 kDa	None, hydrochloride salt	*In vitro*, Caco-2 cells, pH 6.2	0.23 ± 0.13, 0.22 ± 0.06, 0.27 ± 0.08,0.23 ± 0.08 mg/mL
78% DD, < 50 kDa	None, lactic acid salt	*In vitro* B16F10 cells	2.50 mg/mL
82% DD, 150-170 kDa	None, lactic acid salt	*In vitro* B16F10 cells	2.00 ± 0.18 mg/mL
>80% DD, 60-90 kDa	None, glutamic acid salt	*In vitro* B16F10 cells	2.47 ± 0.14 mg/mL
77% DD, 180-230 kDa	None, lactic acid salt	*In vitro* B16F10 cells	1.73 ± 1.39 mg/mL
85% DD, 60-90 kDa	None, hydrochloric acid salt	*In vitro* B16F10 cells	2.24 ± 0.16 mg/mL
81% DD, 100-130 kDa	None, hydrochloric acid salt	*In vitro* B16F10 cells	0.21 ± 0.04 mg/mL
100% DD, 152 kDa	Glycol chitosan	*In vitro* B16F10 cells	2.47 ± 0.15 mg/mL
100% DD, 3-6 kDa	94% Trimethyl chitosan, chloride salt	*In vitro*, MCF7, 6 h	1.402 ± 0.210 mg/mL
100% DD, 100 kDa	36% Trimethyl chitosan, chloride salt	*In vitro*, COS7, 6 h	> 10 mg/mL
97% DD, 65 kDa	N-octyl-O-sulphate	*In vivo* , IV, mice	102.59 mg/kg
97% DD, 65 kDa	N-octyl-O-sulphate	*In vivo* , IP, mice	130.53 mg/kg

Source: Kean, T. and M. Thanou. (2010). "Biodegradation, biodistribution and toxicity of chitosan." **Advanced Drug Delivery Reviews** 62: 8.

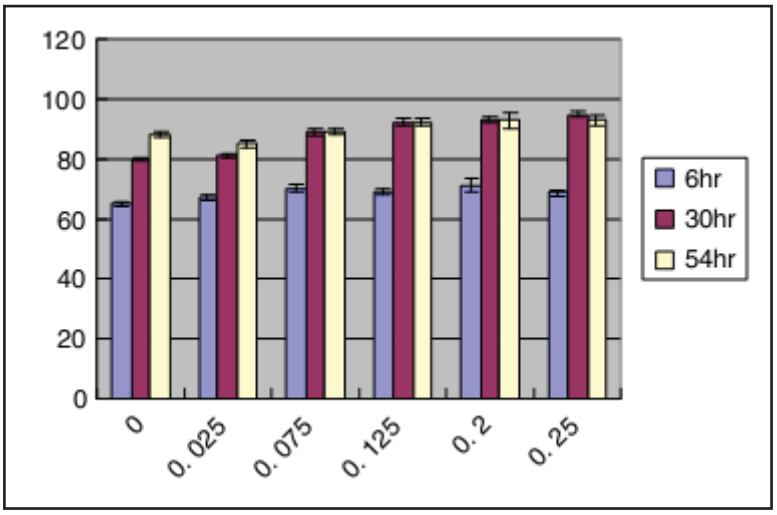


Figure 12 The dependence of the concentration of N-succinyl chitosan colloidal dispersion on the relative cell activity for a different period of cell culture.

Source: Aiping, Z., et al. (2006). "Synthesis and characterization of N-succinyl-chitosan and its self-assembly of nanospheres." **Carbohydrate Polymers** 66: 278.

2.5 Enteric coating

An enteric coating is a barrier applied to oral medication that controls the location in the digestive system where it is absorbed. Enteric refers to the small intestine, therefore enteric coatings prevent release of medication before it reaches the small intestine. Most enteric coatings work by presenting a surface that is stable at the highly acidic pH found in the stomach, but breaks down rapidly at a less acidic (relatively more basic) pH [80].

Purpose of enteric coating

Enteric coating is the most established of delayed-release products, designed to pass through the stomach unaltered, later to release their medication within the intestinal tract. There are three reasons for putting such a coating on a tablet or capsule ingredient:

1. To protect the stomach from the drug.
2. To protect the drug from the stomach, e.g. antibiotics, proteins, and peptides.
3. To release the drug after the stomach, e.g. in the intestines.

Enteric coating materials are also used to prevent release of the drug substance in the stomach if the drug is either an irritant to the gastric mucosa or unstable in gastric juice. List of enteric coating polymers commonly used in tablet formulations is shown in Table 7. The choice of enteric coating material depends on its solubility [81].

Table 7 Enteric coating polymers commonly used in tablet formulations.

Polymer	**Solubility Profile**	**Comments**
Shellac	Above pH 7	Original enteric coating material, originally used in sugar-coated tablets; high pH required for dissolution may delay drug release; natural product which exhibits batch-to-batch variability
Cellulose acetate phthalate (CAP)	Above pH 6	High pH required for dissolution a disadvantage; forms brittle films, so must be combined with other polymers
Polyvinylacetate phthalate (PVAP)	Above pH 5	–
Hydroxypropyl methylcellulose phthalate (HPMCP)	Above pH 4.5	Optimal dissolution profile for enteric coating
Polymers of methacrylic acid and its esters	Various grades available with dissolution occurring above pH 6	–

Source: Gad, S.C. (2008). **Pharmaceutical manufacturing handbook: production and processes**. North Carolina: John wiley & sons, Inc.: 894.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

1. Acetone (Lot No. 10100223, LAB-SCAN, USA)
2. Calcium chloride dried granular (Code No. 328757, Carlo Erbra, Germany)
3. Chitosan 20 kDa, 87%DD (Lot No. COA050507, Seafresh Co. Ltd., Thailand)
4. Chitosan 200 kDa, 87%DD (Lot No. COA240720, Seafresh Co. Ltd., Thailand)
5. Dimethyl sulfoxide (Lot No. 2216B073, Amresco, USA)
6. Dulbecco's modification of Eagle's medium (DMEM, Lot No. 861491, Gibco™,USA)
7. Fetal Bovine Serum (Lot No. 41F6394K, Gibco, EU)
8. Glacial acetic acid (Lot No. 6M387197A, CARLO ERBA, Italy)
9. Hydrochloric acid 36.5-38% (Lot No. E15W66, J.T. Baker, USA)
10. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Lot No. D00121274, Calbiochem, Germany)
11. Potassium bromide (KBr, Lot No. B01351607 905, Merck, Germany)
12. Potassium chloride (Lot No. AF501338, Ajax Finechem, Australia)
13. Sodium acetate (Lot No. AF604190, Ajax Finechem, Australia)
14. Sodium hydroxide (Lot No. B0035298, Merck, Germany)
15. Tris (hydroxymethylaminomethane) (Lot No. Y70885, Research organic, INC, USA)
16. Phthalic anhydride (Lot No. S5335992 917, Merck, Germany)

3.2 Equipments

1. Aluminum pans (Perkins, P/N SSC0000E030 open sample pan, Japan)
2. CO_2 Incubator (Heraeus, Germany)
3. Desiccators (Biologix Reseach Company, USA)
4. Differential scanning calorimeter (DSC 6200; SII Seiko instruments Inc., Japan)
5. Disintegration testing apparatus (Sotax DT3, Switzerland)
6. Fourier transform infrared spectrophotometer (Nicolet 4700, USA)
7. Freeze dryer (Free Zone2.5, Labconco, USA)
8. Hot air oven (Heraeus, Germany)
9. Laminar air flow cabinet (Hera Safe, Heraeus, Germany)
10. Magnetic stirrer and Magnetic bar (Mettler-toledo GmbH, Germany)
11. Microplate reader (Packard BioScience AOPUS01, USA))
12. Moisture balance (Sartorius YTX01L, Germany)
13. Nuclear magnetic resonance spectroscopy (ADVANCE 300, Bruker, Germany)
14. pH meter (Mettler Toledo seveneasy, Switzerland)
15. Powder X-ray diffractometer (Miniflex II, Rigaku, Japan)
16. Schott DURAN (250, 500, 1000 mL)
17. Spectrophotometer (Lamda2, Perkin-Elmer, USA)
18. Texture analysis (TA.XT. Plus, UK)
19. Thickness meter (Minitest 600B, Typ 80-121-0306, Germany)
20. UV-VIS spectrophotometer (Lambda 2, Perkin Elmer, USA)
21. Viscometer (Brook field digital viscometer, DV-III ULTRA, USA)
22. Vortex mixer (Gibthai VX-100, Thailand)
23. Water bath (SANYO, Walk-Ins, Japan)
24. 96 well cell culture plates (Costar®, USA)

3.3 Methods

3.3.1 Preliminary study

CS (20 kDa, 87%DD), 10% w/v in 20 mL of 2.5% v/v aqueous acetic acid solution was prepared (Figure 13). The proposed reaction between chitosan and phthalic anhydride is shown in Figure 14. The very low amount of medium was used to prevent the hydrolysis of phthalic anhydride (PA). PA, 5% w/v in acetone was dropped in CS acidic solutions at 1:1 mole ratio of CS:PA under stirring for 4 and 24 h. The temperature was controlled at 25 and 40˚C. The excess acid in viscous dispersion was neutralized with 0.5 N NaOH. Then, the dispersion was dropped in acetone followed by filtration. The precipitates were washed with mixture of acetone:water, 8:1 v/v and then with excess acetone. Finally, the precipitates were dried in oven at 40˚C for 2 h and the obtained powder was collected. The chemical structure using FTIR spectroscopy and the solubility by measurement of % transmittance of solutions of the obtained powder was characterized.

3.3.1.1 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of CS and N-PhCS samples were recorded with FTIR spectrophotometer (Nicolet 4700, USA) using the KBr disc method. Each sample was pulverized and blended with KBr powder and then compressed with pressure of 5 tons for 60 seconds. The KBr discs were placed in the sample holder and scanned from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.

3.3.1.2 Solubility study

The solubility of N-PhCS in various pH media was determined by measurement of %transmission of solution using UV spectrophotometry. The sample, 50 mg, was dissolved in 5 mL of pH 1-10 buffer solutions and %transmission of the solution was measured using a UV-VIS spectrophotometer (Lambda 2, Perkin Elmer, USA) at wavelength of 600 nm.

The results from preliminary study showed that the suitable condition to prepare N-PhCS was at 25˚C under stirring for 4 h. Therefore, we used these conditions for our further experiments.

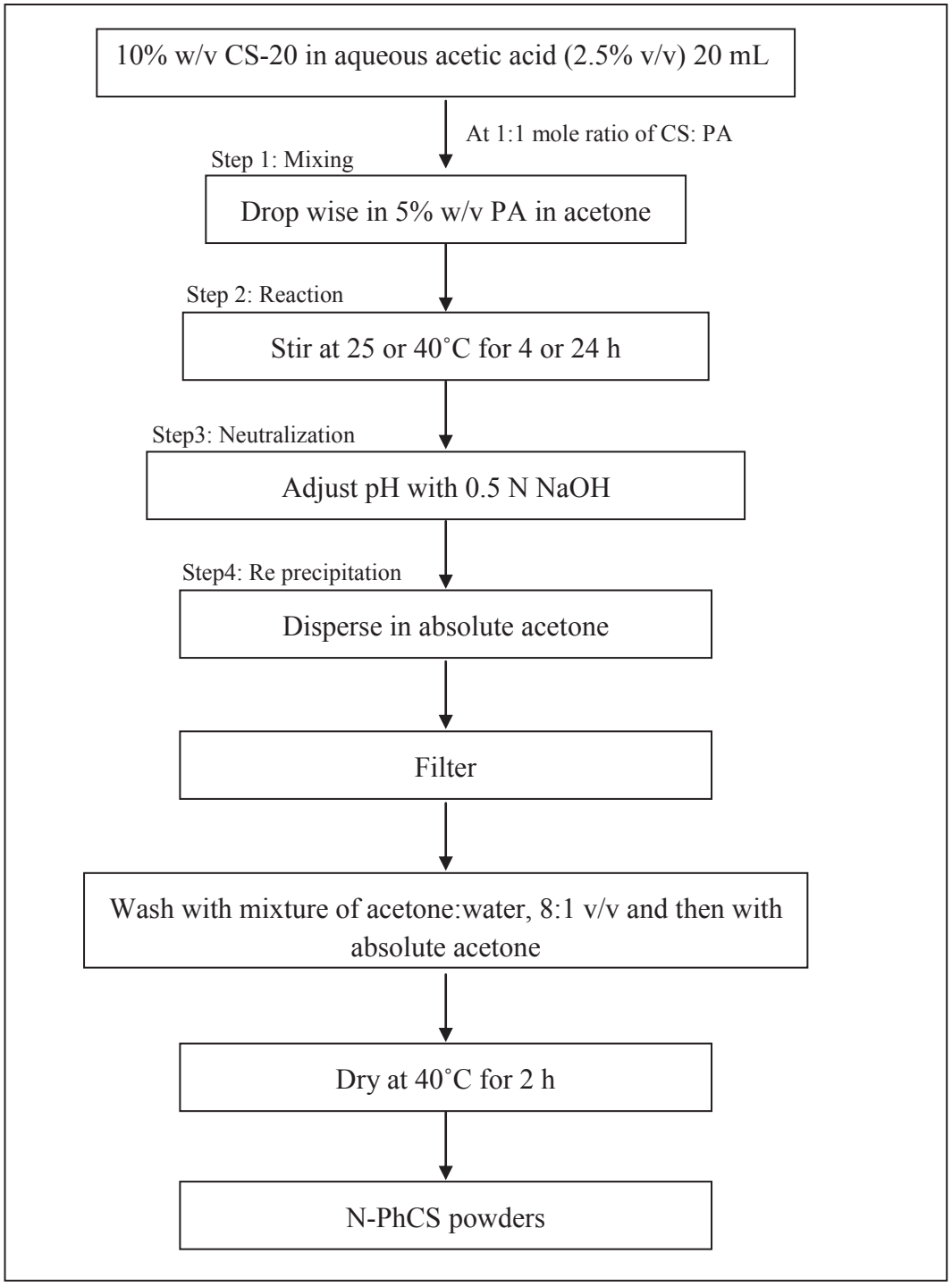


Figure 13 Preparation process of N-PhCS prepared under preliminary study.

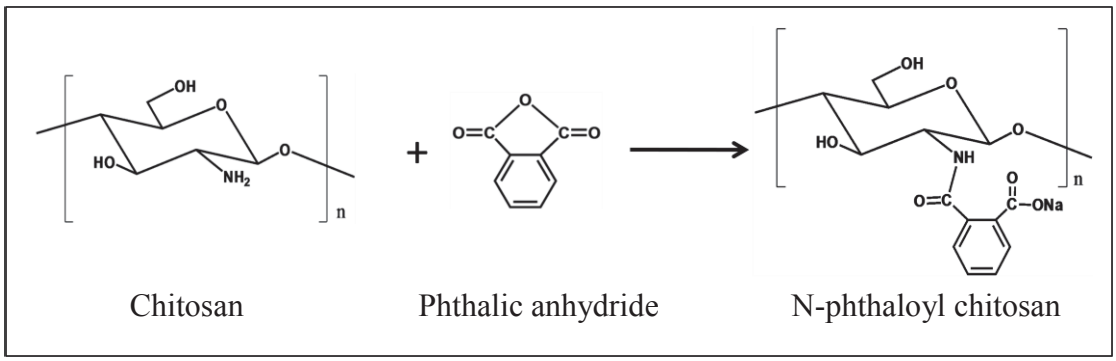


Figure 14 Proposed reaction between chitosan and phthalic anhydride.

3.3.2 Preparation of N-PhCS prepared under various conditions

N-PhCS was prepared from CS 20 kDa, 87%DD (CS-20) and 200 kDa, 87 %DD (CS-200) under varying mole ratio at 1:1, 1:3 and 1:5, respectively (Figure 15). The effect of neutralization pH was also varied at pH 4, 5 and 6, because it was found in the preliminary study that the characteristic of the precipitates depended on the amount of the added NaOH solution.

The physicochemical properties and degree of substitution of the obtained samples were characterized as well as cytotoxicity and film forming properties as follows:

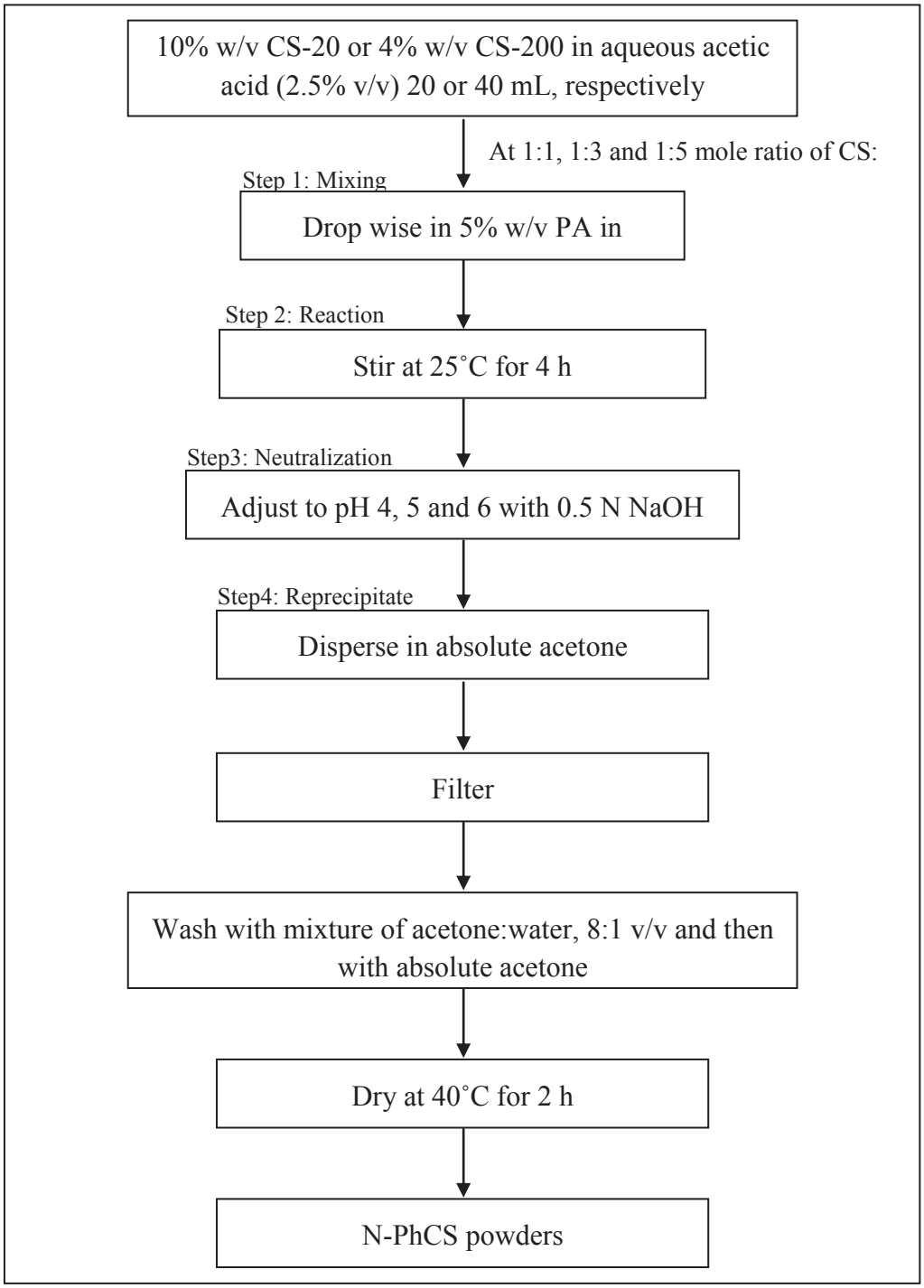


Figure 15 Preparation process of N-PhCS prepared under various conditions.

3.3.3 Characterization of N-PhCS prepared under various conditions

3.3.3.1 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of N-PhCS were studied following the method as described in section 3.3.1.1.

3.3.3.2 Powder X-ray diffractometry (PXRD)

N-PhCS and CS powder was loaded into PXRD plate and scanned by powder X-ray diffractometer (Rigaku, Miniflex II, Japan). The PXRD data were recorded on a Rigaku Miniflex System using Ni-filtered, Cu-K (alpha) radiation, 30 kV, 15 mA with a scanning ratio: 20˚= 4˚/min.

3.3.3.3 Differential scanning colorimetry (DSC)

The DSC thermograms of N-PhCS and CS samples were determined by differential scanning calorimetry (DSC 6200; SII Seiko instruments Inc., Japan) using indium as a standard. About 3-4 mg of powder sample were accurately weighed and placed in a closed aluminum solid pan. The aluminum pan was then transferred into the furnace. The thermal behavior of the samples was determined at heating rate of 10°C per min from 60-350°C using an empty closed aluminum solid pan as a reference. The measurement was done under nitrogen gas at a flow rate of 10 mL per min.

3.3.3.4 Thermogravimetric analysis (TGA)

The TGA thermograms of N-PhCS and CS samples were measured using a thermogravimetric analyzer (TG/DTA 6200; SII Seiko instruments Inc., Japan). About 3-4 mg of powder samples were accurately weighed into an aluminium pan. The measurements were conducted over 60-350˚C at a heating rate of 10 °C/min under nitrogen purge.

3.3.3.5 Solubility study

Solubility study of N-PhCS was studied following the method as described in section 3.3.1.2.

3.3.3.6 Determination of degree of substitution (DS)

3.3.3.6.1 FTIR method

The degree of substitution of all samples was screened by calculation of the ratio of absorbance at 1645 cm^{-1} (amide I band) and the hydroxyl band at 3450 cm^{-1} of FTIR spectra (as described in section 3.3.3.1) of all N-PhCS following the Equation (1) proposed by Moore and Roberts [82]. The FTIR measurement of three discs (n=3) was used to obtain a statistical evaluation.

$$DS(\%) = \left(\frac{A_{1645}}{A_{3450}}\right)_{N-PhCS} - \left(\frac{A_{1645}}{A_{3450}}\right)_{CS} \times 100 \qquad \text{……..(1)}$$

Where

- A_{1645} is the absorbance of the amide-I band as a measure of the N-acyl group content
- A_{3450} the absorbance of hydroxyl band as an internal standard to correct for film thickness or for differences in chitosan

3.3.3.6.2 ^{1}H-NMR method

According to the high accuracy of the DS investigation [28], ^{1}H NMR was used to confirm the DS of N-PhCS prepared under the suitable pH neutralization with mole ratio of CS:PA at 1:1, 1:3 and 1:5 mole ratio. N-PhCS was placed into dialysis bag (MWCO: 6000) for dialysis against excess distilled water to remove the impurities. The solution in dialysis bag was dried using freeze dryer (FreeZone 2.5, Labconco, USA). The ^{1}H NMR spectra of the obtained samples was recorded using nuclear magnetic resonance spectroscopy. The DS was determined from the ratio of area between the protons of substituted group and methyl proton of monosaccharide residue (H_3, H_4, H_5 and H_6). The chemical shifts were reported in δ (ppm). The degree of substitution was calculated using the following Equation (2):

$$DS\ (\%) = \left[\left(\frac{I_{substitution\ \ group}}{number\ \ of\ proton}\right) / \left(\frac{I_{\mathrm{H3+H4+H5+H6}}}{\text{number of proton}}\right)\right] \times 100 \qquad \text{……..……(2)}$$

where I was the integral of the hydrogen atom.

3.3.4 Stability study

N-PhCS samples from section 3.3.3.6 with highest %DS were kept in the hot air oven at 60, 80 and 120 ± 2°C for 6, 12 and 24 hours, respectively. Then remove the sample from the oven, cool down to room temperature and determine by FTIR spectra.

3.3.5 Cytotoxicity test

Cytotoxicity of N-PhCS samples from section 3.3.3.6 with highest %DS was studied by the MTT cytotoxicity assay. Caco-2 cells were harvested and seeded in 96-well plates at a seeding density of 1 x 10^4 cells per well and pre-incubated for 24 h. Then, the cells were treated with N-PhCS sample at various concentrations ranging from 0.01-10 mg/mL in medium (pH 7.4) and incubated for 24 h. After treatment, the solutions were removed. Finally, the cells were incubated with MTT containing medium (0.05 mg/mL) for 3 h. The medium was removed and the formazan crystal that formed in the living cells was dissolved in 100 µL DMSO/well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Packard BioScience AOPUS01, USA). Viability was determined by comparing absorbance in wells containing treated cells with that of untreated cells. Eight replicates were measured for each concentration. The relative cell viability was calculated according to the following Equation (3).

$$\text{Relative cell viability} = \frac{[OD_{550,sample} - OD_{550,blank}]}{[OD_{550,control} - OD_{550,blank}]} \times 100 \qquad \text{.....(3)}$$

where $OD_{550,\ sample}$, $OD_{550,\ control}$ and $OD_{550,\ blank}$ are optical density at 550 mM of N-PhCS sample, control and blank solution, respectively.

3.3.6 Preparation and characterization of N-PhCS and chitosan acetate films

The films of N-PhCS from section 3.3.3.6 with highest %DS were prepared by casting method. N-PhCS was dissolved in distilled water at concentration of 4% w/v. The solution, 100 mL, was then poured on a silicone coated

glass plate (15 x 15 cm^2) and dried at 50˚C for 4-5 h. The films were peeled off and kept in a desiccators containing dried silica gel to control the moisture for all films prior to test. Chitosan acetate (CSA) films were also prepared in the same manner by dissolving CS-20 at concentration of 2.5% w/v in 0.5 N acetic acid solution.

3.3.6.1 Films thickness

The film thickness was determined at ten points by using a thickness gauge Mini-Test 600 (Elektro Physik Dr. Steingroever GmbH & Co. KG, Germany).

3.3.6.2 Mechanical properties of films

Mechanical properties of films were measured by texture analyzer; model TA.XT plus (Stable Micro Systems Ltd., United Kingdom). A maximum load of 50 N was used. The prepared films were cut in a dumbbell shape with length of 25 mm and width of 6.25 mm. The thickness of the films was measured using a micrometer. The test speed was 1 mm/sec. The tensile strength, the elongation at break and gradient stress-strain of 10 samples were reported [83].

3.3.6.3 Water vapor permeability of films

Water vapor permeability (WVP) was conducted using modifications of the standard procedure of the ASTM standard method E96-95 (ASTM, 1995) described by Zavareze et al. [84]. Each film sample was sealed over the circular opening of a permeation cell containing with 30 g dried-granular calcium chloride. These cells were then placed on desiccators with a saturated sodium chloride solution (75% RH) at 25˚C. After the samples reached steady-state conditions, the cell weight was recorded every 24 h for 10 days. The WVP of at least six cells for all films was calculated using the following Equation (4):

$$\text{WVP coefficient} = \frac{(W \times t)}{(A \times \Delta P)} \qquad \text{.....(4)}$$

where WVP coefficient is water vapor permeability coefficient (g h^{-1} m^{-1} Pa^{-1}),
W is the amount of water permeated through the film (g/h),
t is the thickness of film (m),
A is exposed area of film (m^2),
ΔP is the vapor pressure difference (ΔP = 5386.21 Pa)

3.3.6.4 Moisture content

The film of N-PhCS was accurately weighed (W_0) and dried by loss on drying measurement (W_1). The weight of film before and after drying was calculated using the following Equation (5). All samples were performed in triplicate.

$$\text{Moisture content (\%)} = 100 \times \frac{(W_0 - W_1)}{W_0} \quad \text{.....(5)}$$

where W_0 and W_1 were the constant weight before and after drying, respectively.

3.3.6.5 Percentage dissolved of the films

The film was cut in a square of 1.5 cm × 1.5 cm, weighed and placed in each tube of the basket of USP disintegration apparatus. The simulated gastric fluid (SGF, pH 1.2) was used as immersion fluid for the first 2 h. After immersion in SGF, the film was transferred to simulated intestinal fluid (SIF, pH 6.8 Tris buffer) for the next 3 h. The temperature was controlled at 37 ± 2 °C. The rest film was dried at 70˚C for 3 h, and reweighed. The percentage of dissolved film was calculated from the percent weight loss of the film. In case of the film was completely dissolved within 3 h in SIF solutions, the dissolving time was recorded.

3.3.7 Statistic analyses

Data of research were expressed as mean ± standard deviation (SD). The statistical analysis was carried out using analysis of variance at the 0.05 significance level.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preliminary study

4.1.1 FTIR spectroscopy

FITR spectra of CS and N-PhCS prepared under 1:1 mole ratio of CS:PA at different temperatures and stirring times are presented in Figure 16. The spectrum of CS showed characteristic peaks at 1650 and 1600 cm^{-1} assigned to the C=O stretching (amide I) and the bending of amine ($–NH_2$) functional group, respectively (Figure 16a). The absorption band at 1155 cm^{-1} was an asymmetric stretching of the C-O-C bridge. The peaks at 1081 and 1033 cm^{-1} assigned to the skeletal vibration of C-O stretching [14]. In all the spectra of N-PhCS (Figure 16 b-e), the stretching vibration of –OH overlapped with -NH at 3500-3400 cm^{-1} became narrower and shifted to lower wavenumber, indicating the substitution at amino groups of CS [25]. The new peak at 1647 cm^{-1} assigned to the carbonyl stretching (amide I) of N-phthaloyl amide was also observed, while the peak of ($-NH_2$) bending at 1600 cm^{-1} disappeared. The strong peaks at 1560 cm^{-1} and 1383 cm^{-1} regions attributed to an asymmetric and a symmetric carboxylate anion stretching ($-COO^-$), respectively, indicated that N-PhCS was chitosan-N-phthalamidate sodium (sodium 2-(chitosan-N-carbonyl) benzoate) (Figure 17a). The spectra of N-PhCS prepared under higher temperature (40˚C) for 4 and 24 h (Figure 16 d-e), showed the characteristic peaks at 1712 cm^{-1} and 1772 cm^{-1} regions attributed to carbonyl group of phthalimido moieties due to cyclization (Figure 17b) [85] while it was not observed in those prepared at 25˚C for 4 and 24 h (Figure 16 b-c). In addition, there was no effect of the stirring time on the FTIR spectra of N-PhCS.

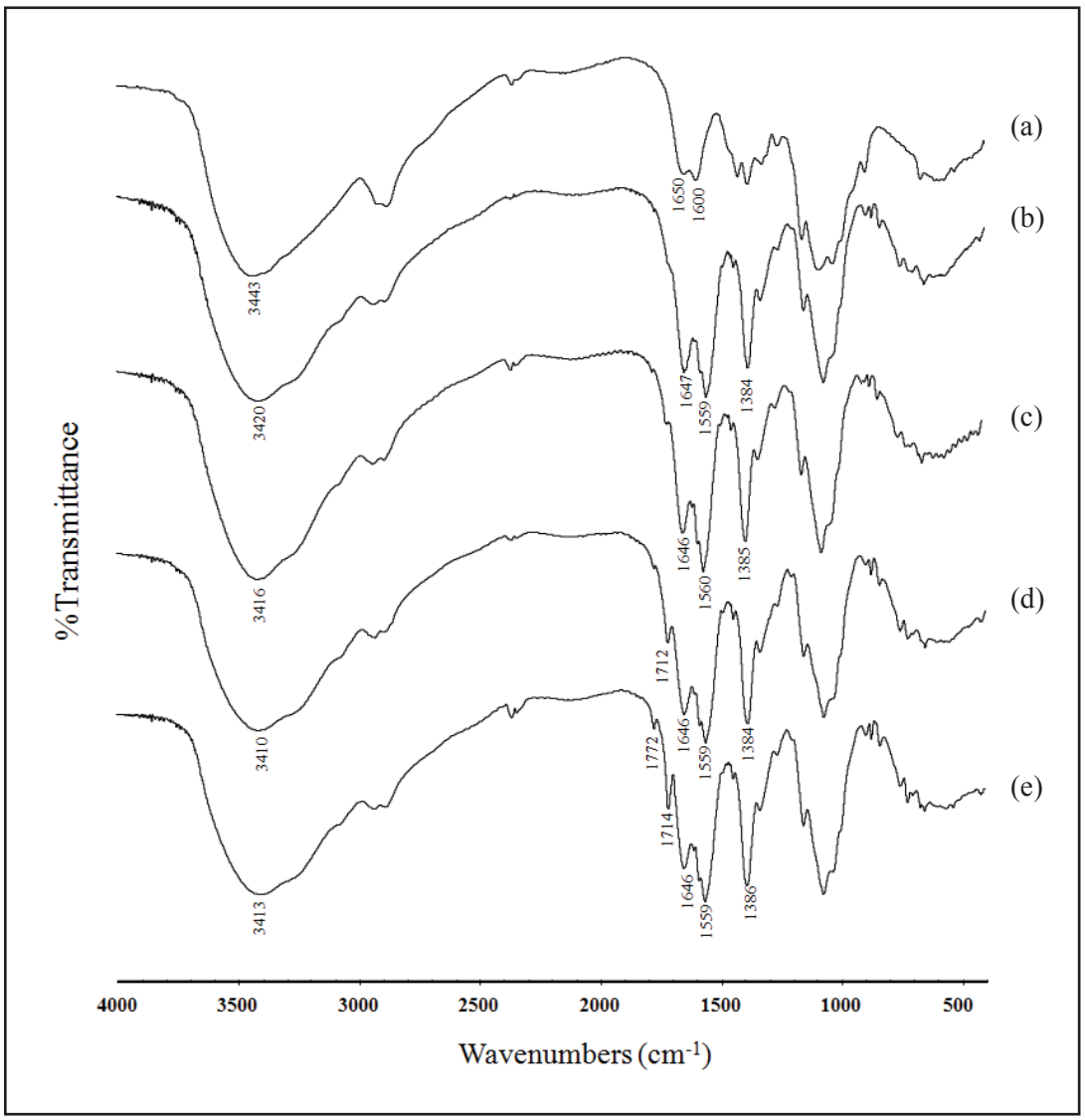


Figure 16 FTIR spectra of N-PhCS prepared under different temperatures and stirring times: (a) CS, N-PhCS at 25˚C for (b) 4 h and (c) 24 h, at 40˚C for (d) 4 h and (e) 24 h.

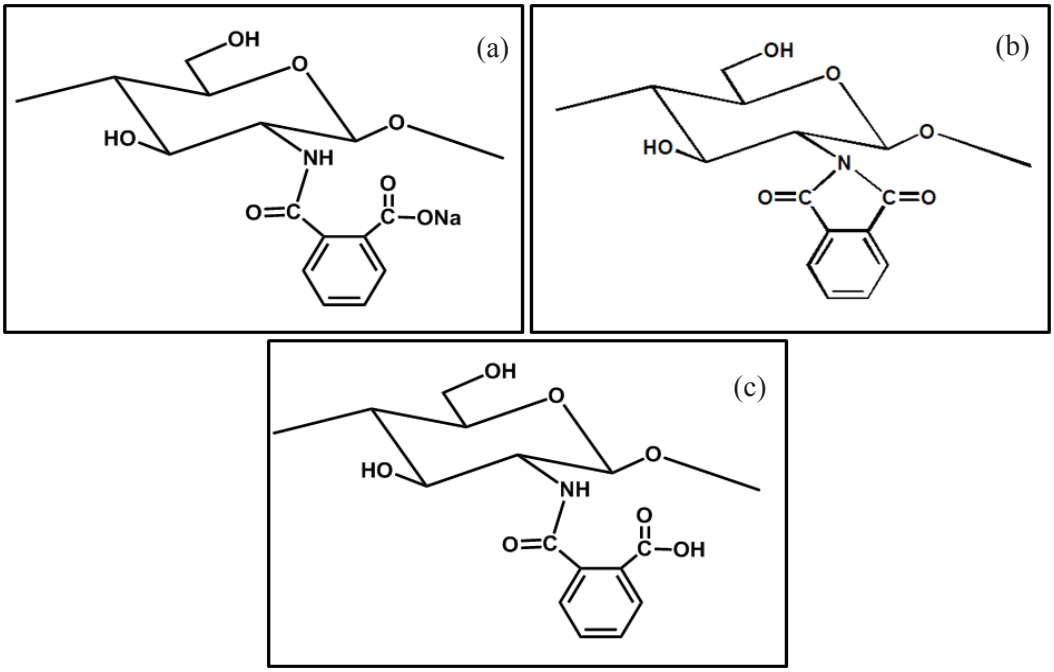


Figure 17 Proposed molecular structure of (a) chitosan-N-phthalamidate sodium, (b) N-phthalimido-chitosan and (c) chitosan-N-phthalamidic acid.

4.1.2 Solubility study

The %transmittance of all N-PhCS in pH 1-10 media are shown in Figure 18. At pH 1, the swelling of N-PhCS due to the protonation of unsubstituted amino group was observed and the %transmittance was around 20%. At pH 2-6, N-PhCS was precipitated according to the decrease of free amino groups. At pH above 7, the solubility of N-PhCS was increased as the % transmittance increased due to salt formation at carboxyl groups of N-PhCS as described in section 4.1.1. The % transmittance of N-PhCS prepared at 25˚C was around 90%. At 40˚C for 24 h, the solubility of N-PhCS was decreased due to the cyclization of phthalimido moieties and the %transmittance was less than 20%. Therefore, we chose the suitable condition to prepare N-PhCS at 25˚C under 4 h stirring for further experiment. In addition, it was also found that the characteristic of N-PhCS depended on the amount of NaOH added in the neutralization pH (step 3 of preparation process) and the effect of pH condition was also studied.

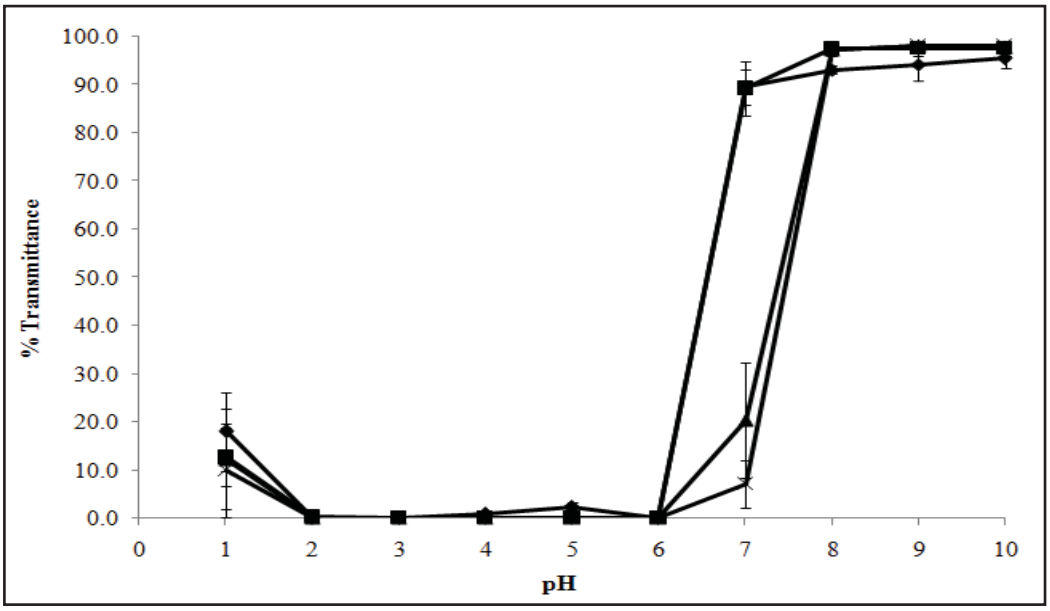


Figure 18 The %transmittance of N-PhCS prepared at different temperatures and stirring times in pH 1-10 media: N-PhCS at 25˚C for (♦) 4 h and (■) 24 h, at (▲) 40˚C for 4 h and (x) 24 h.

4.2 Characterization of N-PhCS prepared under various conditions

4.2.1 FTIR spectroscopy

The FITR spectra of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are presented in Figures 19 and 20, respectively. All the spectra of N-PhCS-20 demonstrated the characteristic peaks at 1647, 1560 and 1385 cm^{-1} region assigned to the phthalate moieties of chitosan-N-phthalamidate sodium. At pH 4, the peak at 1716 cm^{-1} attributed to carbonyl stretching of carboxylic functional group [86] indicating the structure of chitosan-N-phthalamidic acid (Figure 17c) was observed only in the spectra of N-PhCS at 1:3 and 1:5 of CS:PA (Figure 19 b-c). It was indicated that at higher mole ratio of CS:PA, the substitution with phthaloyl group was increased resulting in less salt formation of chitosan-N-phthalamidic acid at the same pH of the neutralization step. At pH 6 under 1:5 mole ratio of CS:PA, the intense and narrow band at 3615 cm^{-1} assigned to the stretching vibrations of O–H bonds which might be inferred to the excess NaOH

residues (Figure 19i). In addition, the spectra of N-PhCS-200 showed the results similar to those of N-PhCS-20.

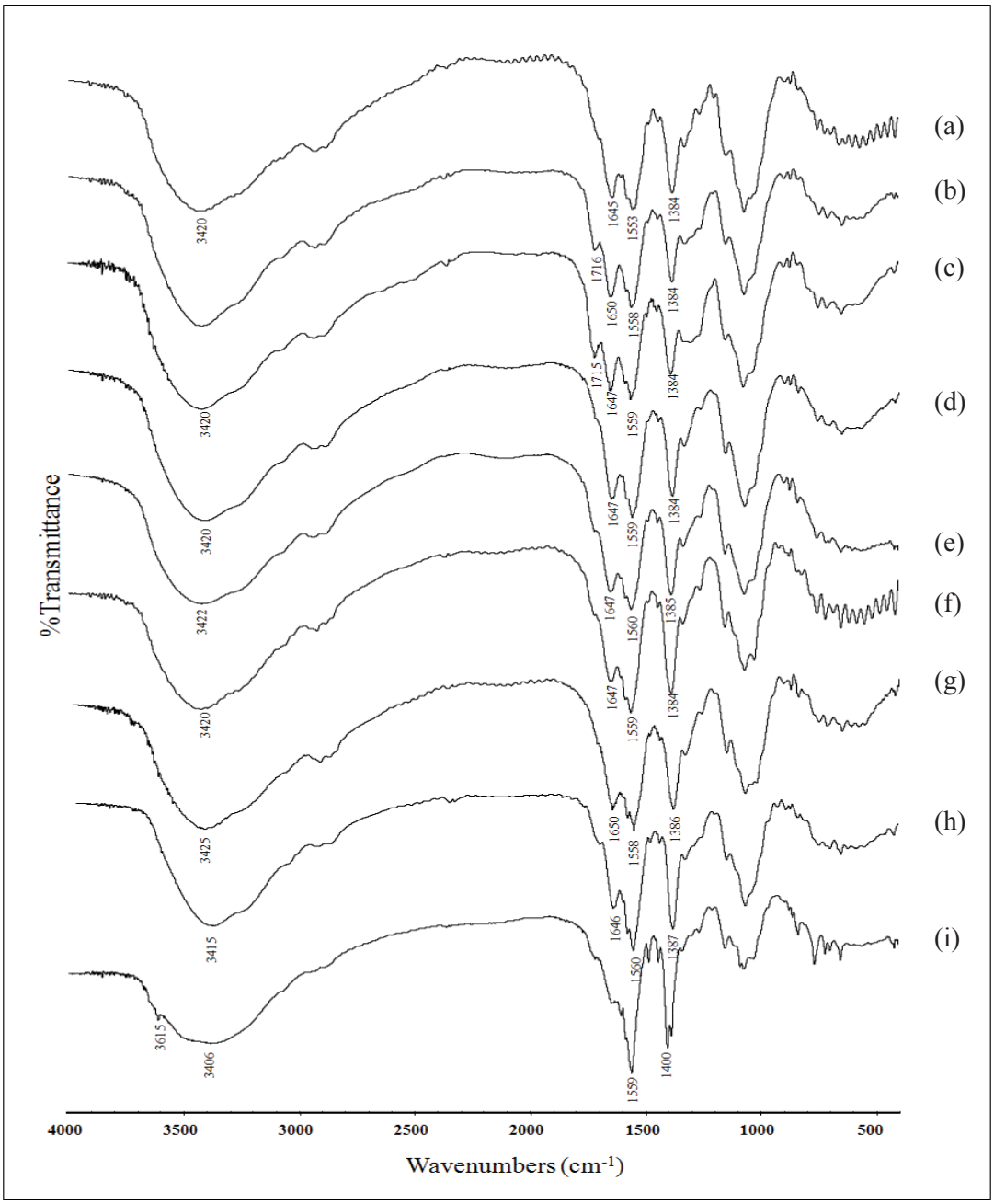


Figure 19 FTIR spectra of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: at pH 4 under CS:PA; (a) 1:1, (b) 1:3 and (c) 1:5; at pH 5 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 6 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5.

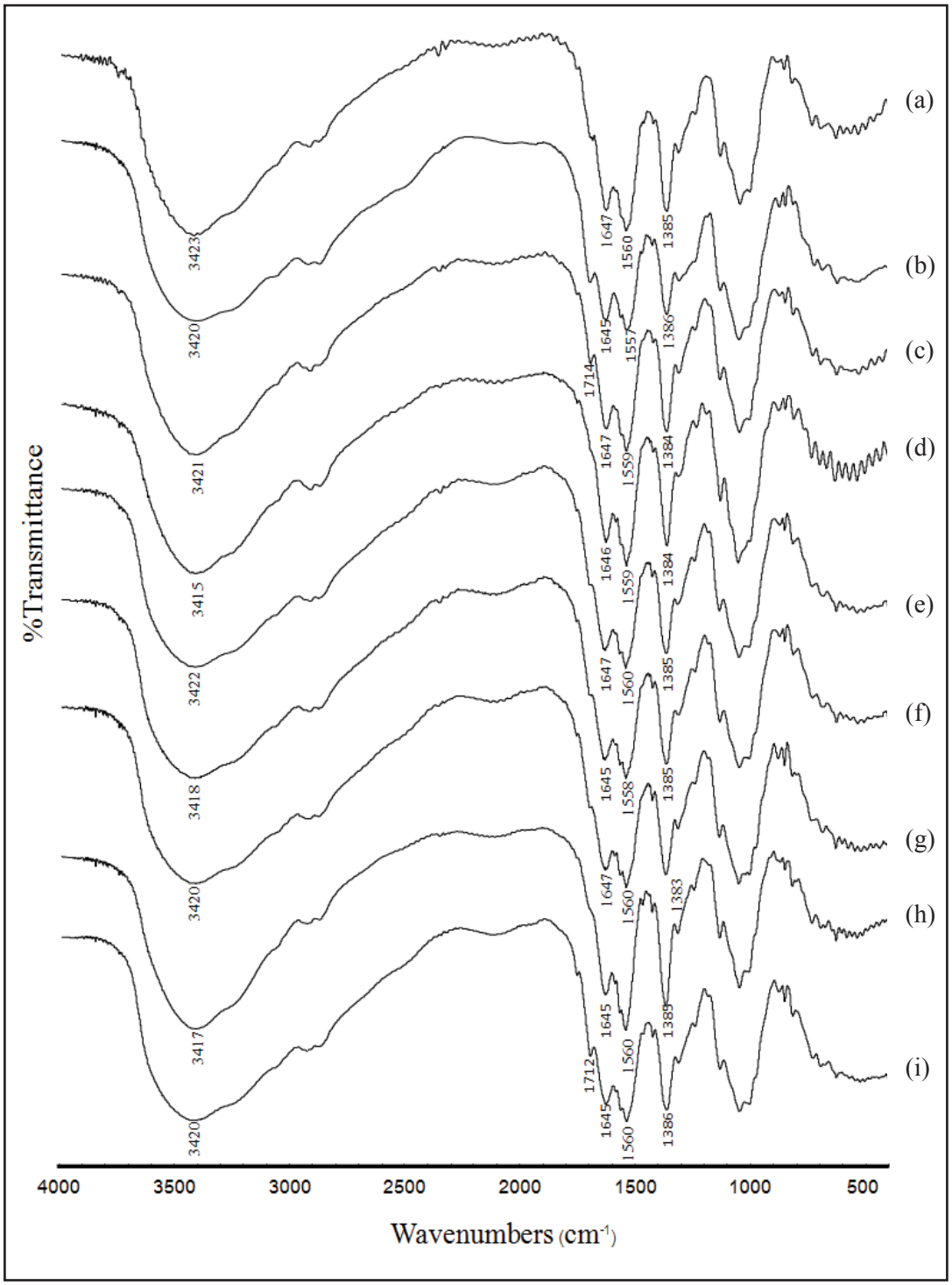


Figure 20 FTIR spectra of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: at pH 4 under CS:PA; (a) 1:1, (b) 1:3 and (c) 1:5; at pH 5 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 6 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5.

4.2.2 Powder X-ray diffraction characterization

Powder X-ray diffraction (PXRD) patterns of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are presented in Figures 21 and 22, respectively. The PXRD patterns of CS-20, CS-200, PA and physical mixtures of CS-20:PA, CS-200:PA are also demonstrated. The crystalline peaks at around 11° and 20° (2θ) were observed in the PXRD pattern of CS while physical mixtures expressed the sharp peaks of PA at 13°, 17°, 20°, 22°, 23°, 27° and 28° (2θ) and the broad peaks related to the peaks of CS. A lot of strong intermolecular and intramolecular hydrogen bonds (H-bonds) make CS form crystalline regions and results in being insoluble in water [25]. After modification, halo diffraction patterns all of N-PhCS were observed. It was suggested that its ability of forming hydrogen bond might be decreased after the amino groups of CS were substituted with phthaloyl groups, resulting in the formation of amorphous solids of N-PhCS.

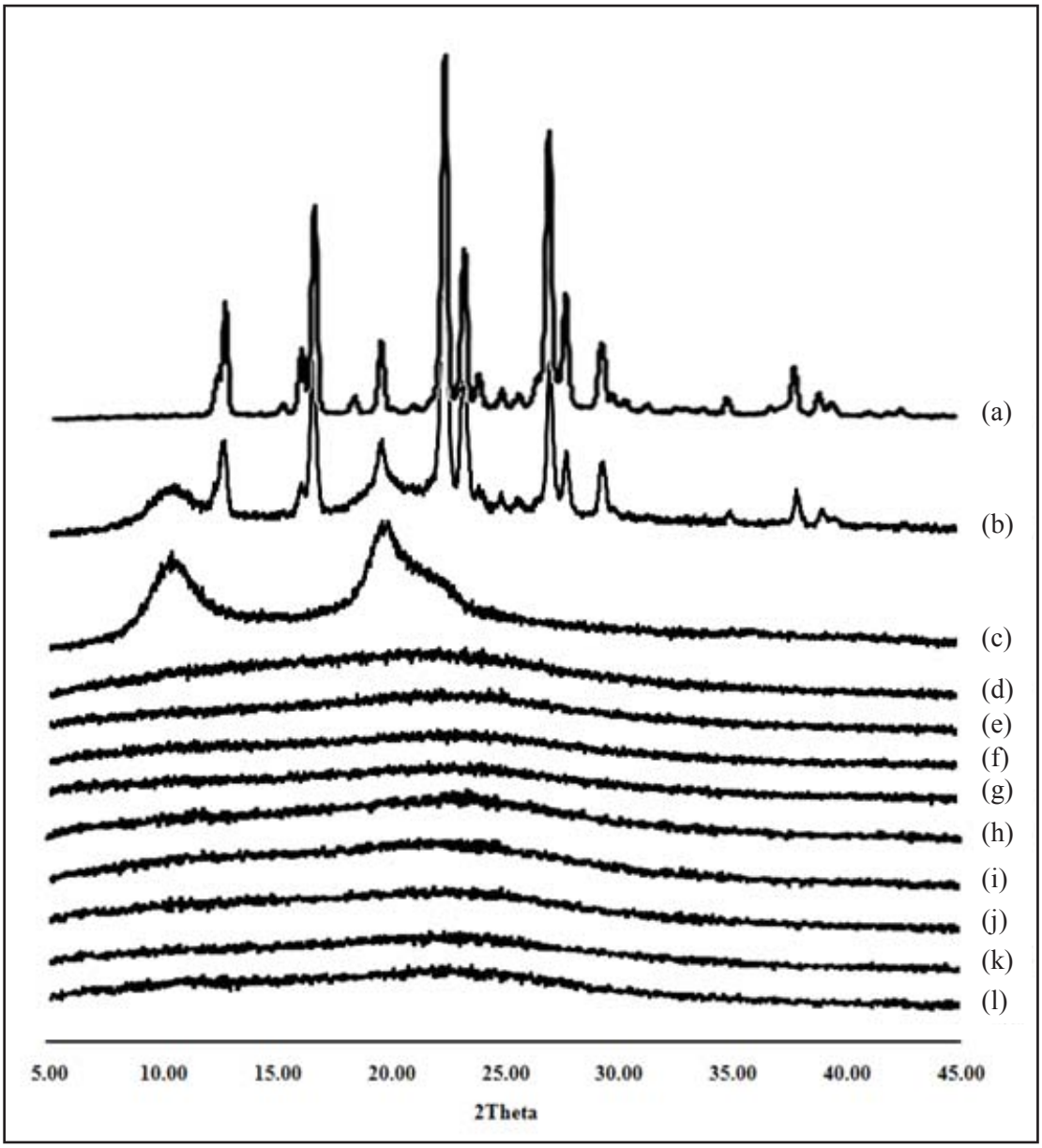


Figure 21 PXRD patterns of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) PA, (b) physical mixture of CS:PA, 1:1, (c) CS-20, at pH 4 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 5 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5; at pH 6 under CS:PA; (j) 1:1, (k) 1:3 and (l) 1:5.

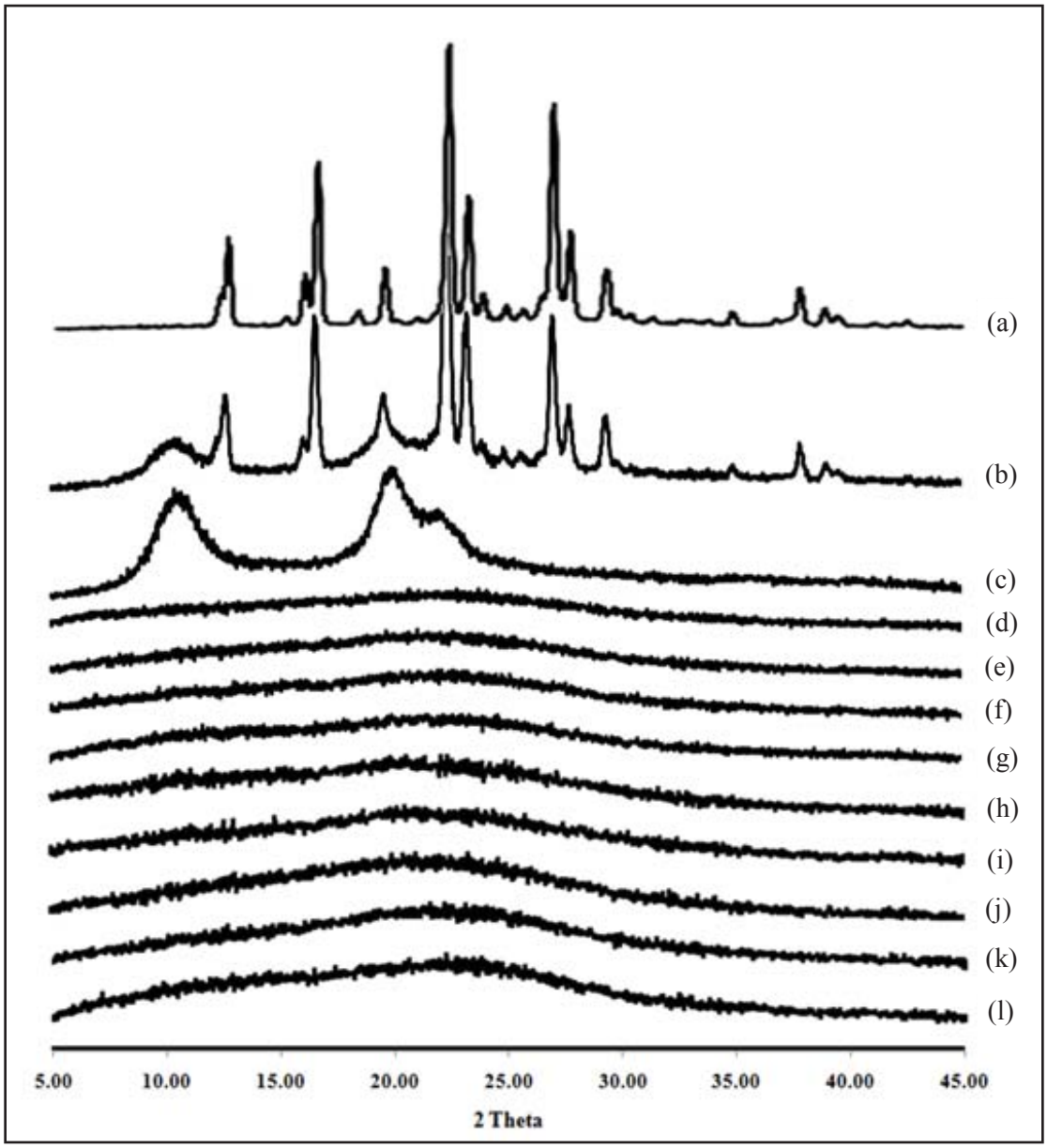


Figure 22 PXRD patterns of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) PA, (b) physical mixture of CS:PA, 1:1, (c) CS-200, at pH 4 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 5 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5; at pH 6 under CS:PA; (j) 1:1, (k) 1:3 and (l) 1:5.

4.2.3 Differential scanning calorimetry (DSC)

DSC thermograms of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are illustrated in Figures 23 and 24, respectively. The thermograms of CS 20 and 200 kDa showed the broad endothermic peak around 90˚C due to the water vapor that the CS contains and the exothermic decomposition peaks at onset at 290-320˚C [87]. The DSC thermograms all of N-PhCS expressed endothermic dehydration peaks around 60-80˚C and the decomposing peak at onset around 200-250˚C were observed, correspond to its thermal decomposition. The results indicated that the decomposition temperature of N-PhCS was lower than CS base due to the structure of CS chains has been changed after the introduction of phthaloyl groups and the reduced ability of crystallization was in agreement with PXRD results.

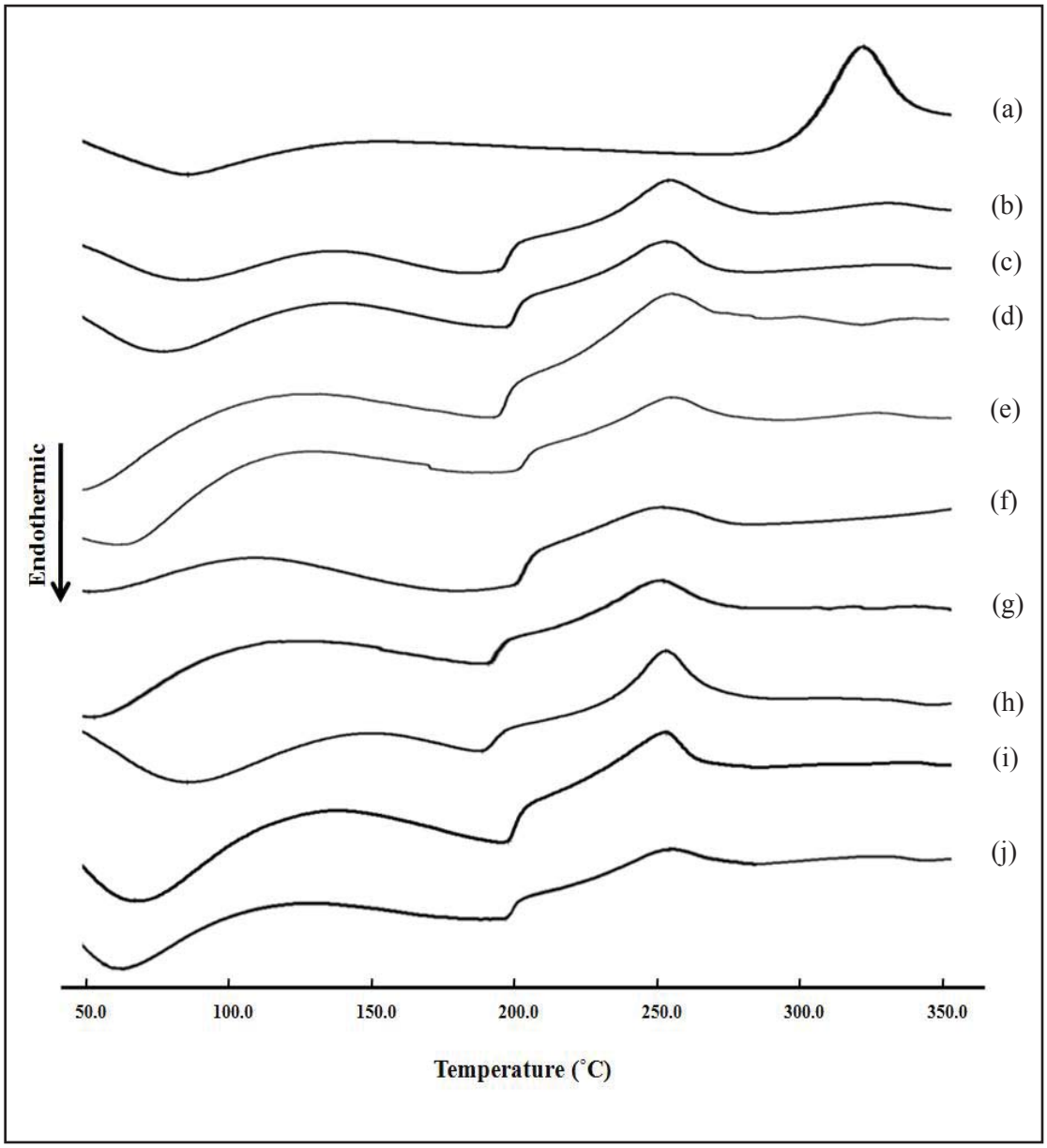


Figure 23 DSC thermograms of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-20, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1; (i) 1:3 and (j) 1:5.

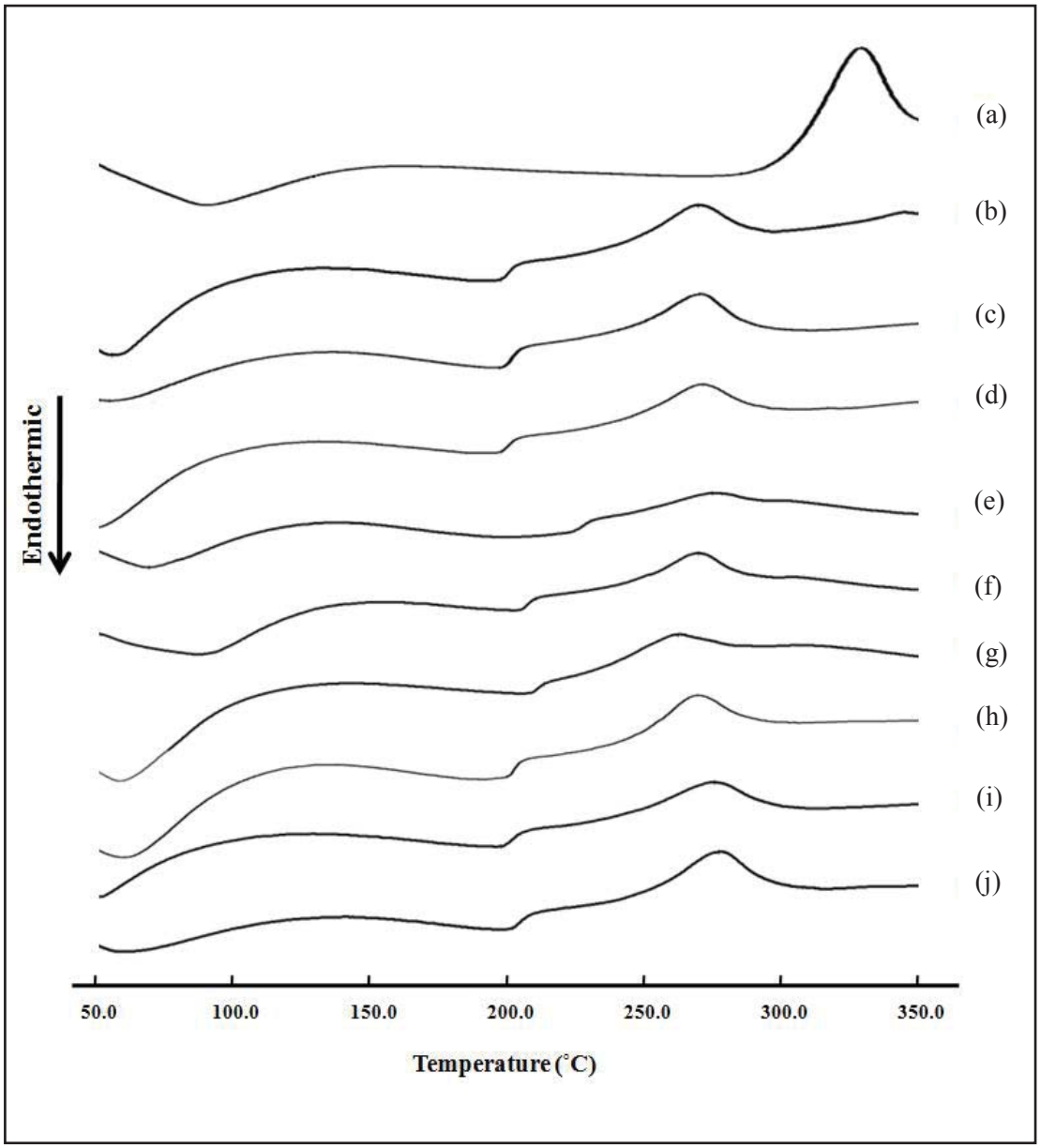


Figure 24 DSC thermograms of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-200, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1, (i) 1:3 and (j) 1:5.

4.2.4 Thermogravimetric analysis (TGA)

TGA thermograms of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are illustrated in Figures 25 and 26, respectively. The thermogram of CS showed two steps of weight loss in which, first step at about 90˚C corresponded to the dehydration of water. The second step at around 280-320˚C, was attributed to the decomposition of the polymer [85]. The thermograms of all of N-PhCS showed three steps of weight loss related to the dehydration of bound water around 60-100˚C, the melting behavior of impurities such as, sodium phthathalate, phthalic acid sodium acetate and acetic acid around 100-200˚C and the decomposition of N-PhCS at around 200-250˚C (as shown in Figures 36-53 of Appendix). It was indicated that N-PhCS was less stable than CS, which was due to the weakening of the hydrogen bonding as a result of introduction of substitution groups [25]. These behaviors were in good agreement with the DSC results.

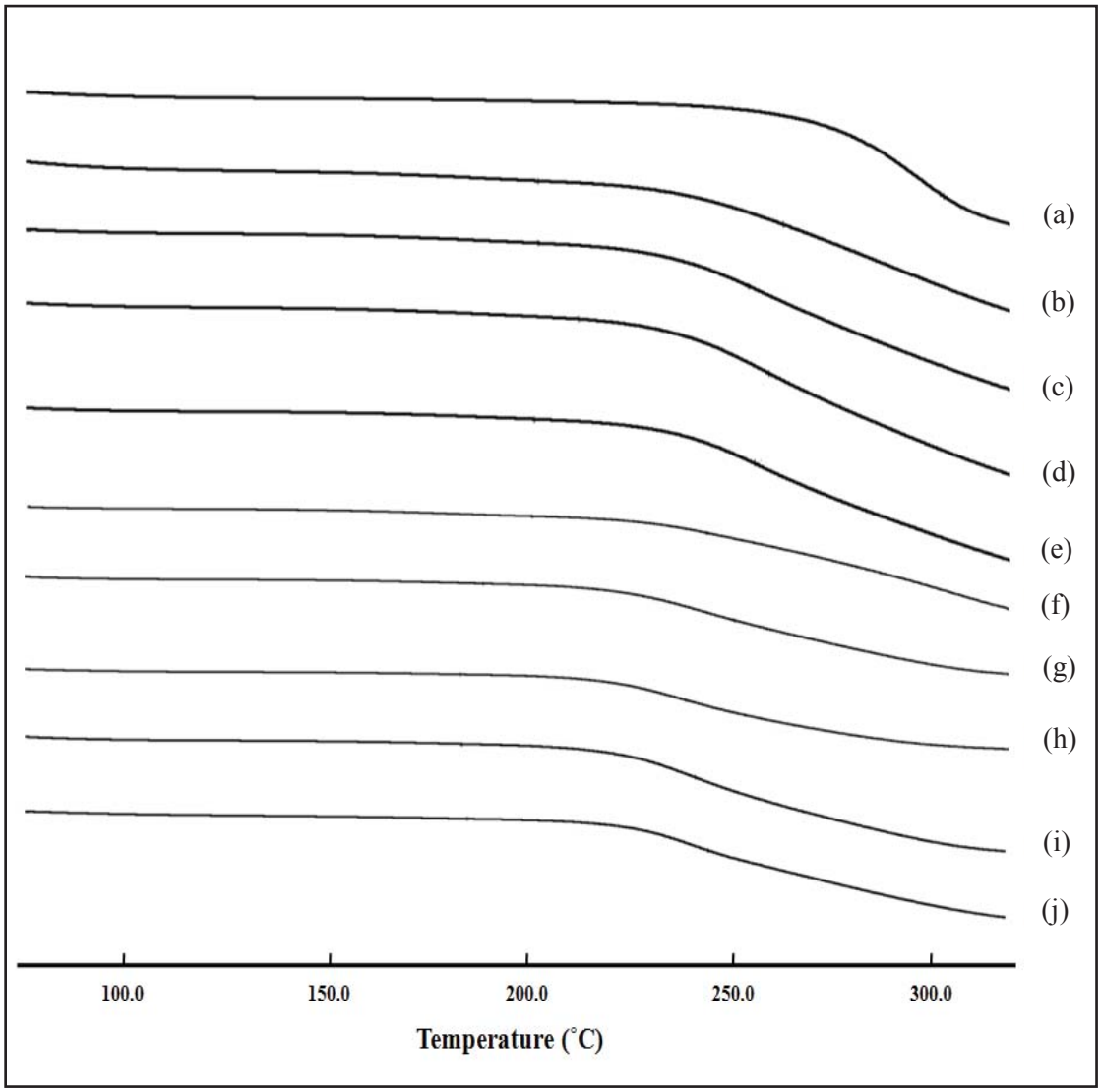


Figure 25 TGA thermograms of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-20, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1; (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1, (i) 1:3 and (j) 1:5.

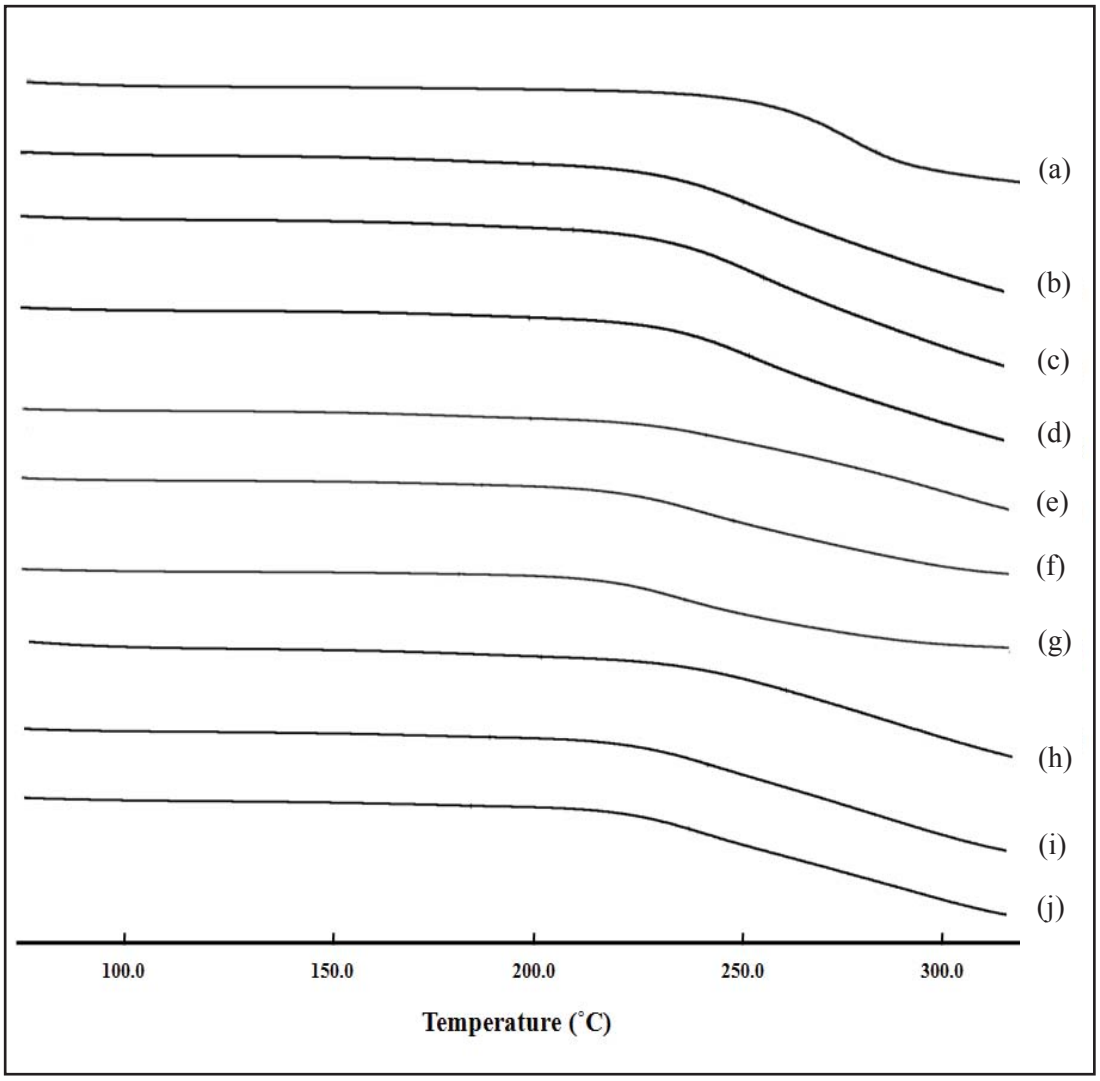


Figure 26 TGA thermograms of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-200, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1,CS:PA; (i) 1:3 and (j) 1:5.

4.2.5 Solubility study

The %transmittance of N-PhCS prepared by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5mole ratio, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 in pH 1-10 media are presented in Figures 27 and 28, respectively. As the mole ratio of CS:PA increased, the precipitation of N-PhCS was observed at pH range 1-5 with zero %transmittances. N-PhCS gradually swelled at pH 5-6 and the % transmittance was around 10-30%. The solubility of N-PhCS was increased at pH above 7 with nearly 100% transmittances. At 1:1 mole ratio, the partial swelling of N-PhCS at pH 1 was observed, indicating the protonation of unsubstituted amino group as already described in section 4.1.2. At pH 5-6, the partial swelling was not observed which might be due to the less substitution of N-phthaloyl groups of N-PhCS [29]. In case of pH 4 in neutralization pH, the %transmittance of N-PhCS at pH range 6-7 was lower than those of pH 5 and 6 which might be due to the less salt formation of chitosan-N-phthalamidic acid as already described in section 4.2.1. The % transmittance of N-PhCS prepared from both different molecular weights demonstrated the similar results but the N-PhCS with high molecular weight exhibited the higher viscosity [88].

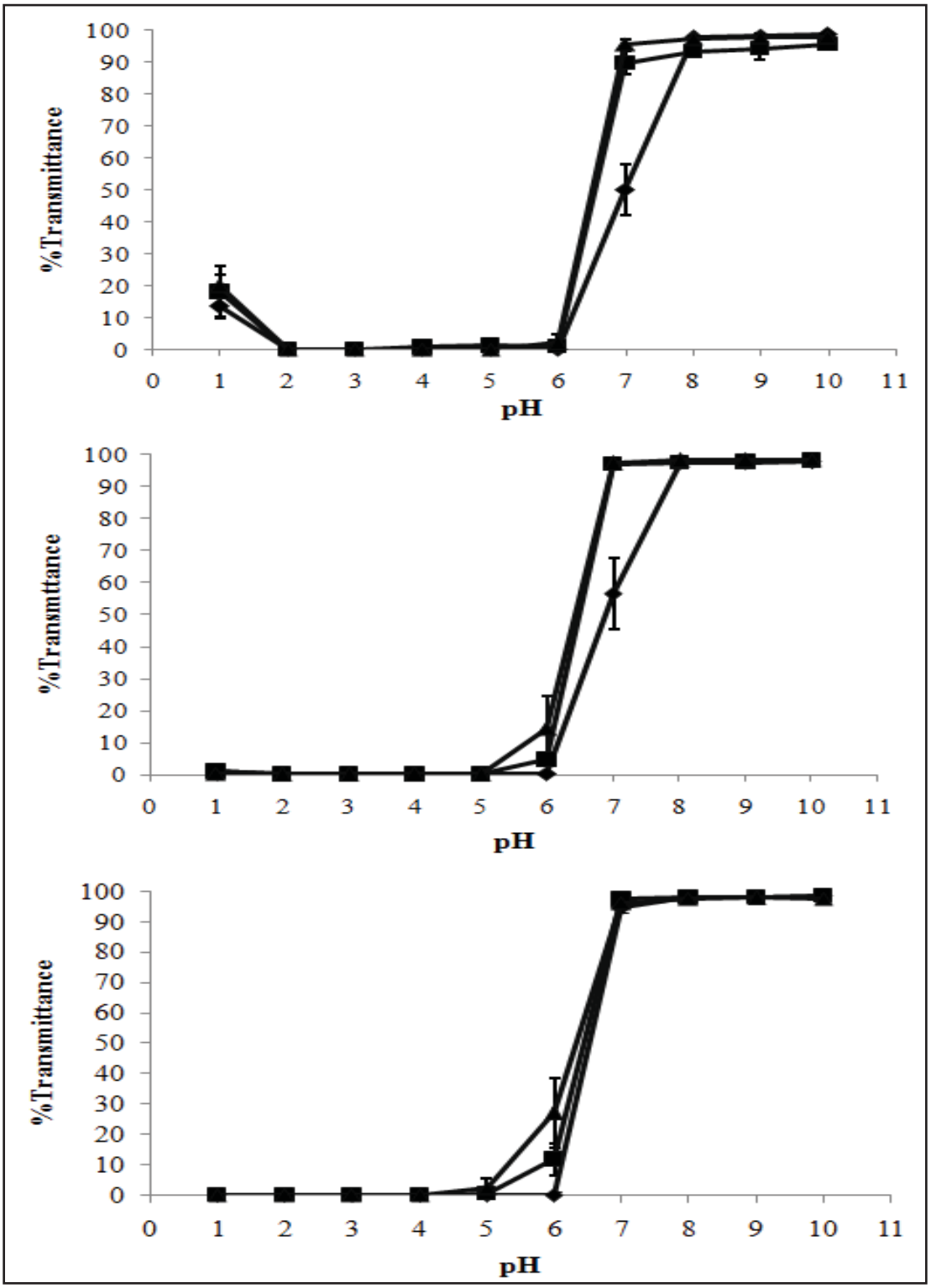


Figure 27 The %transmittance of N-PhCS-20 kDa prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) 1:1, (b) 1:3 and (c) 1:5 mole ratio of CS:PA and (♦) pH 4, (■) pH 5 and (▲) pH 6.

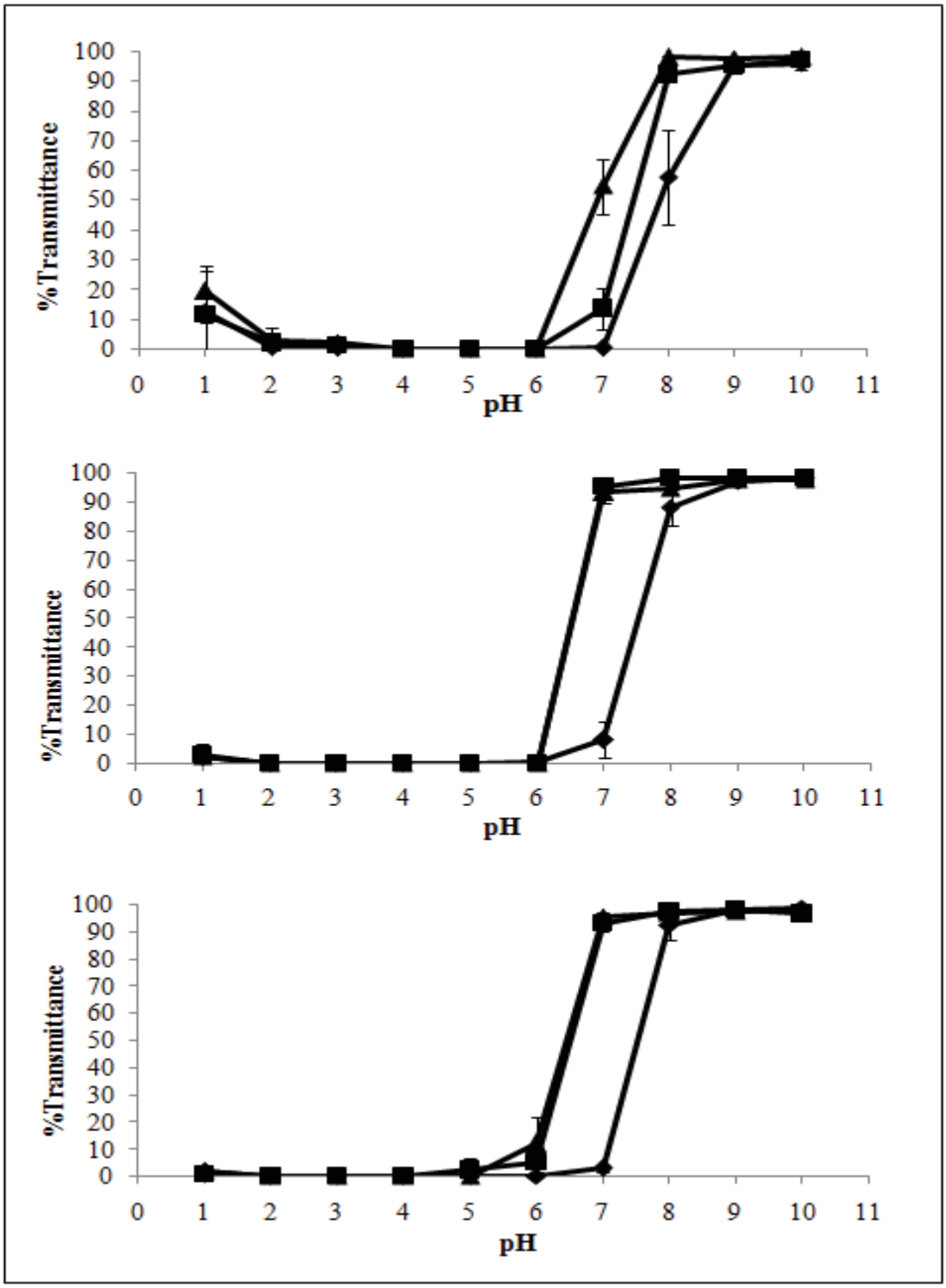


Figure 28 The %transmittance of N-PhCS-200 kDa prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) 1:1, (b) 1:3 and (c) 1:5 mole ratio of CS:PA and (♦) pH 4, (■) pH 5 and (▲) pH 6.

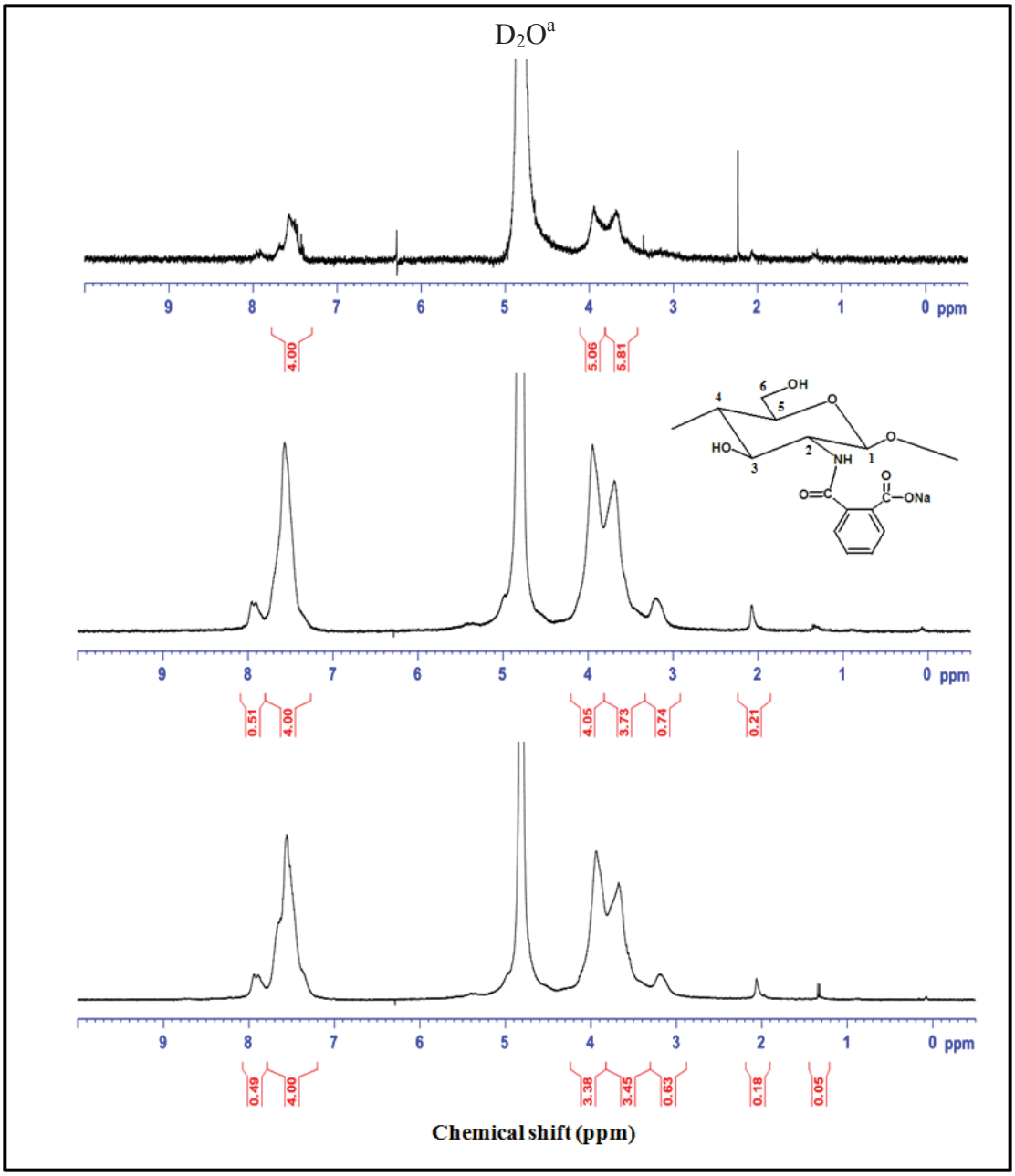
4.2.6 Measurement of degree of substitution (DS)

The DS of all N-PhCS determined by FTIR assay is shown in Table 8. The %DS was around 10-20% and not correlated with mole ratio of CS:PA. It was suggested that no significant difference was noticed between the values obtained by FTIR assay. This was because the %DS of chitosan derivatives was calculated from the ratio of absorbance at 1650 cm^{-1} (described to amide I as probe band) and the hydroxyl band at 3450 cm^{-1} (reference band) [16, 45, 71]. In the spectra of N-PhCS, the hydroxyl band became narrower and shifted to lower wavenumber as described in section 4.1.1. Thus, FTIR assay was not suitable for estimation the DS of N-PhCS.

From the results of FTIR and solubility study, it was suggested that N-PhCS prepared from CS-20 under the condition of pH 5 in neutralization and 1:5 mole ratio of CS:PA gave the solubility best fitted to enteric polymer property. Therefore, the exact %DS of N-PhCS with 1:1, 1:3 and 1:5 mole ratio of CS:PA under pH 5 in neutralization pH was confirmed by ^{1}H-NMR assay (Figure 29). The assignments of ^{1}H in the NMR spectra of N-PhCS were as follows, the signals at 3.1 ppm (H_2) and 3.5-3.9 ppm (H_3, H_4, H_5, H_6) were corresponding to the ring methenyl protons of CS [26]. The signals at 7.4-7.9 ppm were assigned to the aromatic proton of phthaloyl group [85]. The %DS of N-PhCS with 1:1, 1:3 and 1:5 mole ratio of CS:PA, calculated from $[(I_{\text{aromatic proton}}/ 4) /(I_{(H3+ H4+ H5+H6)} / 5)]$ x100, were 46.0%, 64.3% and 73.2%, respectively. It was suggested that the substitution of N-phthaloyl group increased, as the mole ratio of CS:PA increased. Therefore, N-PhCS prepared at 1:5 mole ratio of CS:PA was chosen for stability and cytotoxicity as well as film forming properties study.

Table 8 The degree of substitution of N-PhCS prepared under various conditions using FTIR and ^{1}H-NMR methods.

Sample	%DS	
	FTIR assay	^{1}H-NMR assay
N-PhCS-20 1:1;CS:PA, pH4	19.58 ± 0.39	
N-PhCS-20 1:1;CS:PA, pH5	18.70 ± 0.15	46.0
N-PhCS-20 1:1;CS:PA, pH6	13.61 ± 0.57	
N-PhCS-20 1:3;CS:PA, pH4	20.07 ± 0.39	
N-PhCS-20 1:3;CS:PA, pH5	17.98 ± 0.33	64.3
N-PhCS-20 1:3;CS:PA, pH6	14.99 ± 0.39	
N-PhCS-20 1:5;CS:PA, pH4	16.78 ± 0.85	
N-PhCS-20 1:5;CS:PA, pH5	16.94 ± 1.01	73.2
N-PhCS-20 1:5;CS:PA, pH6	-	
N-PhCS-200 1:1;CS:PA, pH4	12.34 ± 0.81	
N-PhCS-200 1:1;CS:PA, pH5	14.53 ± 1.26	
N-PhCS-200 1:1;CS:PA, pH6	12.45 ± 1.07	
N-PhCS-200 1:3;CS:PA, pH4	15.79 ± 0.55	
N-PhCS-200 1:3;CS:PA, pH5	12.04 ± 1.54	
N-PhCS-200 1:3;CS:PA, pH6	13.71 ± 1.86	
N-PhCS-200 1:5;CS:PA, pH4	12.32 ± 1.37	
N-PhCS-200 1:5;CS:PA, pH5	13.95 ± 0.71	
N-PhCS-200 1:5;CS:PA, pH6	8.73 ± 1.22	



[a]The chemical shifts at 4.8 ppm referred to D_2O [89].

Figure 29 ^{1}H NMR spectra of N-PhCS prepared at 25˚C for 4 h under various mole ratios: (a)1:1, (b) 1:3 and (c)1:5.

4.3 Stability study

Figure 30 demonstrates the FTIR spectra of N-PhCS stored at 60, 80 and 120 ± 2˚C for 6, 12 and 24 h. After storage at 120˚C, the peaks at 1712 cm^{-1} and 1772 cm^{-1} attributed to carbonyl stretching of phthalimido moieties were observed and tended to increase when the exposure time increased. This indicated the cyclization of chitosan-N-phthalamidic acid after exposure to high temperature. The results suggested the applications of N-PhCS should be aware of dealing with high temperatures.

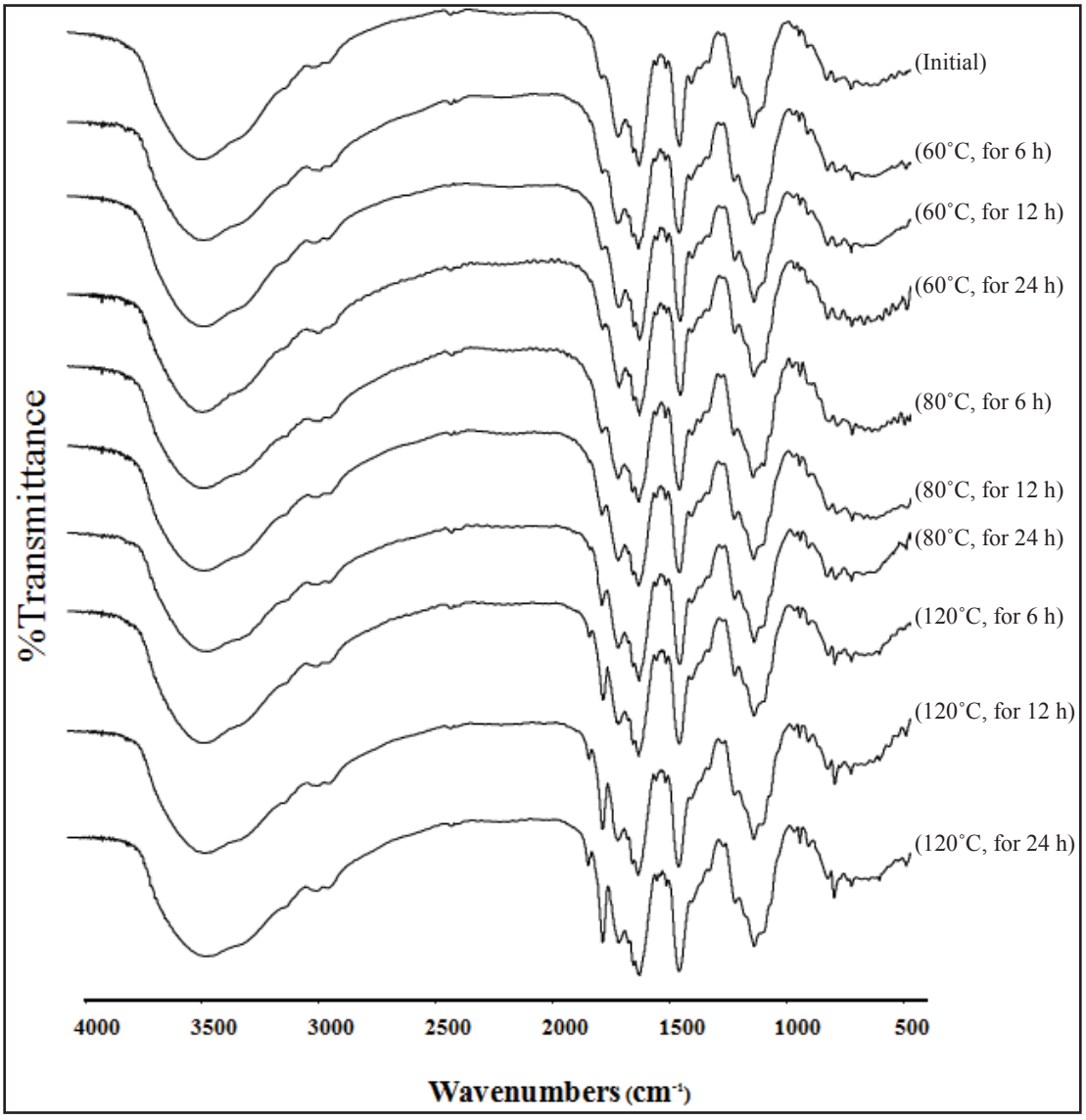
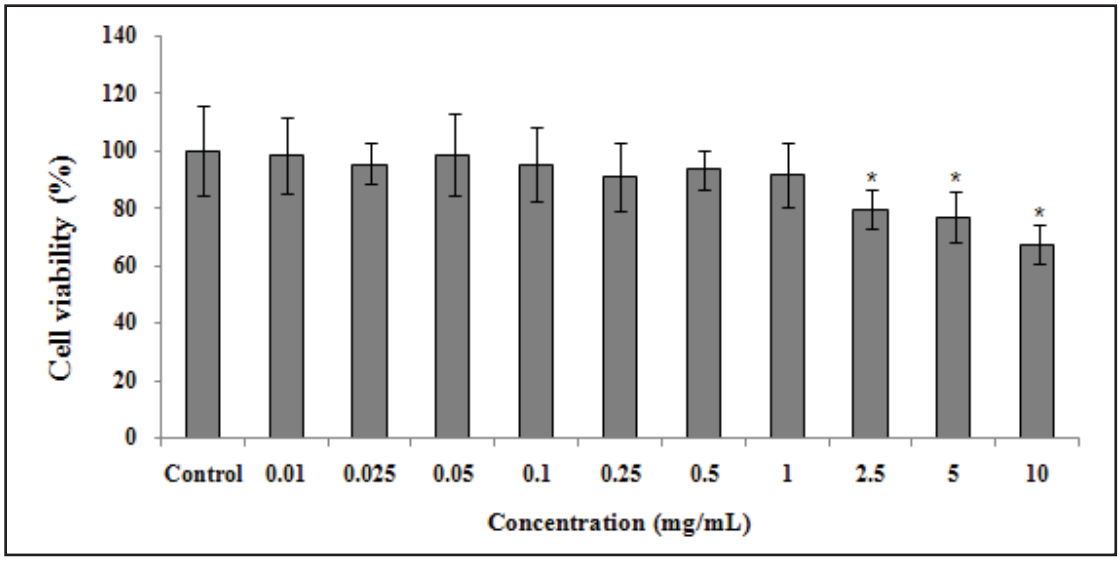


Figure 30 FTIR spectra of N-PhCS under storage at various conditions (stability study).

4.4 Cytotoxicity test

The MTT assay was performed to assess the cytotoxic activity of the N-PhCS in Caco-2 cell lines used. As presented in Figure 31, N-PhCS in concentrations ranges 0.01-1 mg/mL exhibited cell viability around 90% and it was not significantly different from the control (p>0.05). As the concentrations of N-PhCS increased higher than 2.5 mg/mL, the cell viability decreased lower than 80%. The cytotoxicity result agrees with a previous research by Huanbutta [78] that CS in the form of nano/microparticles was non-toxic, and cell-compatible when using in the concentration 0.01-0.1 mg/mL.



*p<0.05 (Turkey)

Figure 31 Cytotoxicity of N-PhCS in Caco-2 cells under the MTT assay.

4.5 Characterization of N-PhCS films

The films prepared from N-PhCS were light yellow clear transparent films with no odor and the thickness was controlled in a range of 110 ± 20 μm (Figure 32).

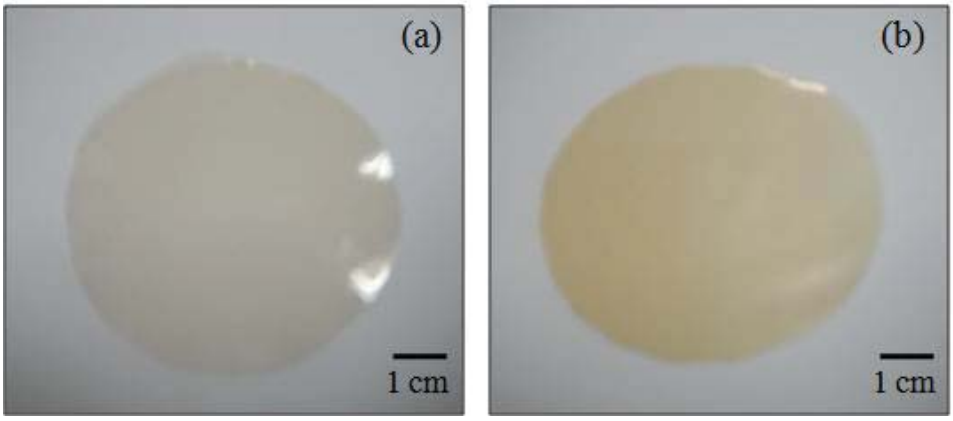


Figure 32 Images of (a) N-PhCS film and (b) CSA film (digital camera).

4.5.1 Mechanical properties

The tensile strength, including the elongation at break and gradient stress-strain of N-PhCS and CSA films, are summarized in Table 9. The tensile strength of N-PhCS films were not significantly different from CSA films (p>0.05) whereas percent of elongation at break of N-PhCS was significantly lower than that of CSA films (p< 0.05) due to the rigid aromatic ring (phthaloyl moiety) reduced the polymer chain mobility [90]. In addition, gradient stress-strain of N-PhCS was significantly higher than that of CSA films (p< 0.05). The results demonstrated that N-PhCS films were more brittle than CSA films, characterized by having small elongation and high values of gradient stress-strain.

Table 9 Mechanical properties of N-PhCS and CSA films.

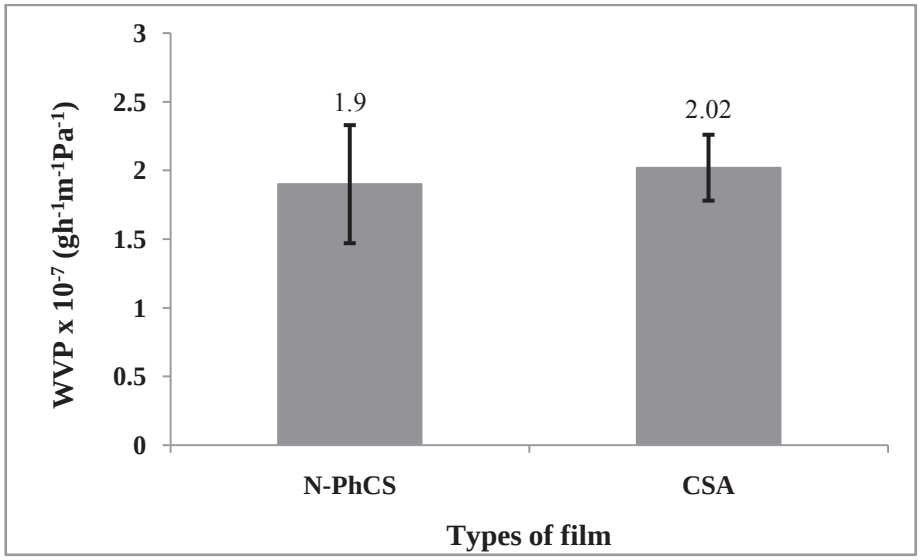
Sample	Thickness[a] (mm)	Tensile strength[a] (MPa)	elongation at break[a] (%)	Gradient St-Strain[a] (MPa/%)
N-PhCS	0.117±0.008	49.891±11.4	2.881±0.79*	19.534±1.96*
CSA	0.113±0.004	46.672±5.5	8.802±2.45	17.665±1.52

[a]All values were mean ± SD of ten samples.

*p<0.05 (t-test)

4.5.2 Water vapor permeability

The water vapor permeability (WVP) of N-PhCS and CSA films are illustrate in Figure 33. N-PhCS films demonstrated not significantly different moisture permeability (1.90 x 10^{-7} g × h^{-1} × m^{-1} × Pa^{-1}) as compared to that of CSA film (2.02 x 10^{-7} g × h^{-1} × m^{-1} × Pa^{-1}) (*p*>0.05). It was indicated that moisture protection of N-PhCS films was not deteriorated after introducing the phthaloyl group.



[a]All values were mean ± SD of six samples.

[*]*p*>0.05 (t-test)

Figure 33 Water vapor permeability of N-PhCS and CSA films.

4.5.3 Moisture content and pH solubility

The moisture content and percent dissolved of N-PhCS and CSA films in SGF (pH 1.2) and SIF (pH 6.8) are presented in Table 10. The result showed that the moisture content of N-PhCS was significantly higher than that of CSA film ($p< 0.05$). The percent dissolved of N-PhCS films in SGF was 12.43 ± 1.28 while in SIF, the films were completely dissolved. The results were in consistent with the solubility study of N-PhCS powder as described in section 4.2.5. On the other hand, CSA films were completely dissolved in SGF and 23.21 ± 3.11% in SIF. It was suggested that N-PhCS film may be suitable for using as a film-forming polymer for gastro-resistant or enteric coating.

Table 10 Moisture content and percent dissolved of N-PhCS and CSA films in SGF and SIF (pH 6.8).

Films	% Moisture content	Percent dissolved[a]	
		in SGF pH 1.2	in SIF pH 6.8
N-PhCS	11.51 ± 0.93*	12.43 ± 1.28	100
CSA	9.54 ± 0.55	100	23.20 ± 3.11

[a]All values were mean ± SD of six samples.

*p<0.05 (t-test)

CHAPTER 5
CONCLUSIONS

In this study, N-phthaloyl chitosan (N-PhCS) as an enteric polymer was successfully prepared by acylation at amino groups of chitosan via ring-opening reactions of phthalic anhydride (PA). The effects of various conditions by varying temperature at 25 and 40˚C, stirring time for 4 and 24 h, mole ratio of chitosan:phthalic anhydride at 1:1, 1:3 and 1:5 mole ratio, neutralization pH (step 3 of preparation process) at pH 4, 5 and 6 and different molecular weights of chitosan 20 and 200 kDa could be summarized as follows:

1. The suitable condition for preparing N-PhCS was at 25˚C under stirring time for 4 h. The preparation under high temperature resulted in the cyclization of phthalimido moieties.

2. The adjusted pH in the neutralization pH at pH 5 provided the structure of chitosan-N-phthalamidate sodium (sodium 2-(chitosan-N-carbonyl) benzoate) while the less sodium salts formation and the excess NaOH residue was observed at pH 4 and 6, respectively.

3. The degree of N-phthaloyl substitution increased as the mole ratio of chitosan: phthalic anhydride increased resulting in the increase of solubility of enteric polymer property of chitosan-N-phthalamidate sodium.

4. N-PhCS prepared from different molecular weights exhibited similar physicochemical properties except for the higher viscosity of the solution of the higher molecular weight salt.

5. The % degree of substitution of N-PhCS with 1:1, 1:3 and 1:5 mole ratio of chitosan: phthalic anhydride (46.0%, 64.3% and 73.2%, respectively), was calculated by ^{1}H-NMR assay whereas the FTIR assay was restricted according to the change of the hydroxyl band at 3450 cm^{-1} which was used as the reference band in

the calculation of % degree of substitution, during the substitution of N-phthaloyl groups.

6. The stability study suggested that N-PhCS was unstable when exposed to high temperature. Therefore, the applications of N-PhCS should be aware of dealing with high temperatures.

7. N-PhCS was non-toxic and compatible to Caco-2 cells when used at concentration of 0.01-1 mg/mL.

8. N-PhCS films exhibited a good enteric property and the solubility in simulated gastric fluid was 12.43% with complete dissolution in simulated intestinal fluid. The moisture barrier property and tensile strength of the films were closed to chitosan acetate film but they were more brittle as characterized by less elongation and higher gradient stress-strain value.

In conclusion, the suitable conditions to prepare N-PhCS was at 25˚C under stirring for 4 h, CS:PA at 1:5 mole ratio and neutralization pH at pH 5. The obtained N-PhCS from CS 20 kDa was chitosan-N-phthalamidate sodium with the highest degree of substitution and the solubility best fitted to enteric polymer property. It was non-toxic with good film forming properties. Thus, chitosan-N-phthalamidate sodium can be applied in pharmaceutical dosage forms, especially in enteric and colonic drug delivery system.

REFERENCES

[1]. Khanafari, A., R. Marandi, and S. Sanatei. (2008). "Recovery of chitin and chitosan from shrimp wasteby chemical and microbial methods " **Iranian Journal of Environmental Health, Science and Engineering** 5(1): 19-24.

[2]. Dutta, P.K., M. N. V. Ravikumar, and J. Dutta. (2002). "Chitin and chitosan for versatile applications." **Journal of Macromolecular Science, Part C: Polymer Reviews,** 42(3): 307-354.

[3]. Roberts, G. (1992). "Solubility and solution behaviour of chitin and chitosan." In **Chitin Chemistry**. London: Macmillan Press: 72-73.

[4]. Aranaz, I., et al. (2009). "Functional characterization of chitin and chitosan." **Current Chemical Biology** 3: 203-230.

[5]. Sinha, V.R., et al. (2004). "Chitosan microspheres as a potential carrier for drugs." **International Journal of Pharmaceutics** 274: 1-33.

[6]. Hejazi, R. and M. Amiji. (2003). "Chitosan-based gastrointestinal delivery systems." **Journal of Controlled Release** 89(2): 151-165.

[7]. Pedro, A.S., et al. (2009). "Chitosan: An option for development of essential oil delivery systems for oral cavity care?" **Carbohydrate Polymers** 76(4): 501-508.

[8]. Rekha, M.R. and C.P. Sharma. (2009). "Synthesis and evaluation of lauryl succinyl chitosan particles towards oral insulin delivery and absorption." **Journal of Controlled Release** 135(2): 144-151.

[9]. Opanasopit, P., et al. (2009). "Methylated N-(4-N,N-dimethylaminobenzyl) chitosan as effective gene carriers: Effect of degree of substitution." **Carbohydrate Polymers** 75(1): 143-149.

[10]. Nunthanid, J., et al. (2008). "Development of time-, pH-, and enzyme-controlled colonic drug delivery using spray-dried chitosan acetate and hydroxypropyl methylcellulose." **European Journal of Pharmaceutics and Biopharmaceutics** 68(2): 253-259.

[11]. Puttipipatkhachorn, S., et al. (2001). "Drug physical state and drug-polymer interaction on drug release from chitosan matrix films." **Journal of Controlled Release** 75: 143-153.

[12]. Cravotto, G., et al. (2005). "Chemical modification of chitosan under high-intensity ultrasound." **Ultrasonics Sonochemistry** 12: 95-98.

[13]. Prashanth, K.V.H. and R.N. Tharanathan. (2007). "Chitin/chitosan: modifications and their unlimited application potential—an overview." **Trends in Food Science & Technology** 18: 117-131.

[14]. Ma, G., et al. (2008). "Preparation and characterization of water-soluble N-alkylated chitosan." **Carbohydrate Polymers** 74(1): 121-126.

[15]. Nunthanid, J., et al. (2004). "Characterization of chitosan acetate as a binder for sustained release tablets." **Journal of Controlled Release** 99(1): 15-26.

[16]. Tien, C.L., et al. (2003). "N-acylated chitosan: hydrophobic matrices for controlled drug release." **Journal of Controlled Release** 93(1): 1-13.

[17]. Shelma, R. and C. Sharma. (2010). "Acyl modified chitosan derivatives for oral delivery of insulin and curcumin." **Journal of Materials Science: Materials in Medicine** 21(7): 2133-2140.

[18]. Yang, T., C. Chou, and C. Li. (2002). "Preparation, water solubility and rheological property of the N-alkylated mono or disaccharide chitosan derivatives." **Food Research International** 35(8): 707-713.

[19]. Tian, Q., et al. (2011). "Self-Assembly and liver targeting of sulfated chitosan nanoparticles functionalized with glycyrrhetinic acid." **Nanomedicine: Nanotechnology, Biology and Medicine** (Article in press).

[20]. Champagne, L.M. (2008). "The synthesis of water soluble /n-acyl chitosan derivatives for characterization as antibacterial agents." Ph.D. Chemistry, Louisiana State University and Agricultural & Mechanical College.

[21]. Mourya, V.K. and N.N. Inamdar. (2008). "Chitosan-modifications and applications: Opportunities galore." **Reactive and Functional Polymers** 68(6): 1013-1051.

[22]. Francesko, A. and T. Tzanov. (2011). "Chitin, chitosan and derivatives for wound healing and tissue engineering." **Advances Biochemical Engineering and Biotechnology** 125: 1-27.

[23]. Shigemasa, Y., et al. (1999). "Chemical modification of chitin and chitosan 1: preparation of partially deacetylated chitin derivatives via a ring-opening reaction with cyclic acid anhydrides in lithium chloride/N,N-dimethylacetamide." **Carbohydrate Polymers** 39(3): 237-243.

[24]. Sashiwa, H. and S. Aiba. (2004). "Chemically modified chitin and chitosan as biomaterials." **Progress in Polymer Science** 29(9): 887-908.

[25]. Zhou, J.Q. and J.W. Wang. (2009). " Immobilization of alliinase with a water soluble-insoluble reversible N-succinyl-chitosan for allicin production." **Enzyme and Microbial Technology** 45(4): 299-304.

[26]. Aiping, Z., et al. (2006). "Synthesis and characterization of N-succinyl-chitosan and its self-assembly of nanospheres." **Carbohydrate Polymers** 66(2): 274-279.

[27]. Hou, Z., et al. (2010). "Synthesis and evaluation of N-succinyl-chitosan nanoparticles toward local hydroxycamptothecin delivery." **Carbohydrate Polymers** 81(4): 765-768.

[28]. Sui, W., et al. (2008). "Preparation and properties of an amphiphilic derivative of succinyl-chitosan." **Colloids and Surfaces A: Physicochemical and Engineering Aspects** 316: 171-175.

[29]. Mello, K., et al. (2006). "Synthesis and physicochemical characterization of chemically modified chitosan by succinic anhydride." **Brazilian Archives of Biology and Technology** 49(4): 665-668.

[30]. Yan, C., et al. (2006). "Preparation of N-succinyl-chitosan and its physical-chemical properties as a novel excipient." **Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan** 126(9): 789-793.

[31]. Aiedeh, K. and M.O. Taha. (1999). "Synthesis of chitosan succinate and chitosan phthalate and their evaluation as suggested matrices in orally administered, colon-specific drug delivery systems." **Archiv der Pharmazie - Pharmaceutical and Medicinal Chemistry** 332(3): 103-107.

[32]. Pillai, C.K.S., W. Paul, and C.P. Sharma. (2009). "Chitin and chitosan polymers: Chemistry, solubility and fiber formation." **Progress in Polymer Science** 34(7): 641-678.

[33]. George, M. and T.E. Abraham. (2006). "Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan - a review." **Journal of Controlled Release** 114(1): 1-14.

[34]. Dutta, P.K., J. Dutta, and V.S. Tripathi. (2004). "Chitin and chitosan: Chemistry, properties and applications." **Journal of Scientific and Industrial Research** 63: 20-31.

[35]. Paños, I., N. Acosta, and A. Heras. (2008). " New drug delivery systems based on chitosan." **Current Drug Discovery Technologies** 5: 333-341.

[36]. Wang, M.-J., et al. (2009). "A Novel, Potential Microflora-Activated Carrier for a Colon-Specific Drug Delivery System and Its Characteristics." **Industrial & Engineering Chemistry Research** 48(11): 5276-5284.

[37]. Caner, C., P.J. Vergano, and J.L. Wiles. (1998). "Chitosan Film Mechanical and Permeation Properties as Affected by Acid, Plasticizer, and Storage." **Journal of Food Science** 63(6): 1049-1053.

[38]. Kalia, S. and L. Averous. (2011). **Biopolymers: Biomedical and Environmental Applications**. John Wiley & Sons, Inc.

[39]. Britto, D. and O.B.G. Assis. (2007). "Synthesis and mechanical properties of quaternary salts of chitosan-based films for food application." **International Journal of Biological Macromolecules** 41(2): 198-203.

[40]. Britto, D. and O.B.G. Assis. (2007). "A novel method for obtaining a quaternary salt of chitosan." **Carbohydrate Polymers** 69(2): 305-310.

[41]. Pang, H., et al. (2008). "Preparation and function of composite asymmetric chitosan/CM-chitosan membrane." **Journal of Materials Science: Materials in Medicine** 19(3): 1413-1417.

[42]. Srinivasa, P.C., M.N. Ramesh, and R.N. Tharanathan. (2007). "Effect of plasticizers and fatty acids on mechanical and permeability characteristics of chitosan films." **Food Hydrocolloids** 21(7): 1113-1122.

[43]. Bigin, A. and M.-R. Van Calsteren. (1999). "Antimicrobial films produced from chitosan." **International Journal of Biological Macromolecules** 26(1): 63-67.

[44]. Bourtoom, T. (2008). "Edible films and coatings: characteristics and properties." **International Food Research Journal** 15(3): 1-12.

[45]. Khan, T.A. and K.K. Peh. (2003). "A preliminary investigation of chitosan film as dressing for punch biopsy wounds in rats." **Journal of Pharmaceutical Sciences** 6(1): 20-26.

[46]. Kaur, K. and K. Kim. (2009). "Studies of chitosan/organic acid/Eudragit RS/RL-coated system for colonic delivery." **International Journal of Pharmaceutics** 366: 140-148.

[47]. Ilium, L. (1998). "Chitosan and Its Use as a Pharmaceutical Excipient." **Pharmaceutical Research** 15(9): 1326-1331.

[48]. Hejazi, R. and M. Amiji. (2003). "Chitosan-based gastrointestinal delivery systems." **Journal of Controlled Release** 89(2): 151-165.

[49]. Chung, Y., C. Tsai, and C. Li. (2006). "Preparation and characterization of water-soluble chitosan produced by Maillard reaction." **Fisheries Science** 72(5): 1096-1103.

[50]. Bansal, V., et al. (2011). "Applications of Chitosan and Chitosan Derivatives in Drug Delivery." **Advances in Biological Research** 5(1): 28-37.

[51]. An, N.T., et al. (2009). "Water-soluble N-carboxymethylchitosan derivatives: Preparation, characteristics and its application." **Carbohydrate Polymers** 75(3): 489-497.

[52]. Choi, C.Y., et al. (2007). "Effect of N-acylation on structure and properties of chitosan fibers." **Carbohydrate Polymers** 68(1): 122-127.

[53]. Kittur, F.S., et al. (2002). "Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry." **Carbohydrate Polymers** 49(2): 185-193.

[54]. Xie, Y., X. Liu, and Q. Chen. (2007). "Synthesis and characterization of water-soluble chitosan derivate and its antibacterial activity." **Carbohydrate Polymers** 69(1): 142-147.

[55]. Xiao, B., et al. (2011). "Preparation and characterization of antimicrobial chitosan-N-arginine with different degrees of substitution." **Carbohydrate Polymers** 83: 144–150.

[56]. Shahidi, F. and R. Abuzaytoun. (2005). "Chitin, Chitosan, and Co-Products: Chemistry, Production, Applications, and Health Effects." In **Advances in Food and Nutrition Research**, Volume 49 London: Academic Press: 93-135.

[57]. Hermanson, G.T. (1996). **Bioconjugate techniques**. San Diego: Elsevier Inc.

[58]. Qin, C., et al. (2006). "Water-solubility of chitosan and its antimicrobial activity." **Carbohydrate Polymers** 63(3): 367-374.

[59]. Kato, Y., H. Onishi, and Y. Machida. (2004). "N-succinyl-chitosan as a drug carrier: water-insoluble and water-soluble conjugates." **Biomaterials** 25(5): 907-915.

[60]. Agency, U.S.E.P. (2000). "Phthalic anhydride." Accessed October 21, 2010. Available from Technology Transfer Network Air Toxics Web Site http://www.epa.gov/ttn/atw/hlthef/phthalic.html.

[61]. Kelley, K.E., et al. (2012). "Identification of phthalates in medications and dietary supplement formulations in the United States and Canada." **Environ Health Perspect** 120(3): 379-384.

[62]. Raymond, C.R., et al. (2003). "Cellulose Acetate Phthalate." In **Handbook of Pharmaceutical Excipients**. London: Pharmaceutical Press: 143-146.

[63]. Raymond, C.R., et al. (2003). "Dibutyl Phthalate." In **Handbook of Pharmaceutical Excipients**. London: Pharmaceutical Press: 225-227.

[64]. Raymond, C.R., et al. (2003). "Diethyl Phthalate." In **Handbook of Pharmaceutical Excipients**. London: Pharmaceutical Press: 230-231.

[65]. Raymond, C.R., et al. (2003). "Hypromellose Phthalate." In **Handbook of Pharmaceutical Excipients**. London: Pharmaceutical Press: 333-336.

[66]. Raymond, C.R., et al. (2003). "Polyvinyl Acetate Phthalate." In **Handbook of Pharmaceutical Excipients**. London: Pharmaceutical Press: 562-563.

[67]. Wuts, P.G.M. and Theodora W. Greene. (2007). "Protection for the Amino Group." In **Greene's Protective groups in organic synthesis**. John Wiley & Sons, Inc.

[68]. Panchapornpon, D., et al. (2011). "Fabrication of thermally stabilized shellac through solid state reaction with phthalic anhydride." **Materials Letters** 65(8): 1241-1244.

[69]. Amtex. (2005). "Analytic method for determining degree of substitution in the product (A.S.T.M. Method)." Accessed January 15, 2010. Available from http://www.amtex.com.mx/docs/DS.pdf.

[70]. Sonia, T.A. and C.P. Sharma. (2011). "Chitosan and its derivatives for drug delivery perspective." **Advances in Polymer Science** 243: 23–54.

[71]. Dong, Y., et al. (2001). "Determination of degree of substitution for N-acylated chitosan using IR spectra." **Science in China Series B: Chemistry** 44(2): 216-224.

[72]. Leane, M.M., et al. (2004). "Use of the ninhydrin assay to measure the release of chitosan from oral solid dosage forms." **International Journal of Pharmaceutics** 271: 241-249.

[73]. Velde, K.V.d. and P. Kiekens. (2004). "Structure analysis and degree of substitution of chitin, chitosan and dibutyrylchitin by FT-IR spectroscopy and solid state 13C NMR." **Carbohydrate Polymers** 58(4): 409-416.

[74]. Thanou, M., J.C. Verhoef, and H.E. Junginger. (2001). "Oral drug absorption enhancement by chitosan and its derivatives." **Advanced Drug Delivery Reviews** 52(2): 117-126.

[75]. Yu, S., et al. (2004). "Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies." **International Journal of Pharmaceutics** 281: 11-23.

[76]. Prajapati, B.G. (2009). "Chitosan A Marine Medical Polymer And Its Lipid Lowering Capacity." **The Internet Journal of Health** 8: (available at http://www.ispub.com/journal/the_internet_journal_of_health/volume_9_number_2_13/article/chitosan-a-marine-medical-polymer-and-its-lipid-lowering-capacity.html).

[77]. Kean, T. and M. Thanou. (2010). "Biodegradation, biodistribution and toxicity of chitosan." **Advanced Drug Delivery Reviews** 62(1): 3-11.

[78]. Huanbutta, K. (2010). "Development of chitosan nano/microparticles for colonic drug delivery." Ph.D. Pharmaceutical Technology, Silpakorn University.

[79]. Kean, T., S. Roth, and M. Thanou. (2005). "Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency." **Journal of Controlled Release** 103(3): 643-653.

[80]. Chakraborty, S., S. Sarkar, and S.K. Debnath. (2009). "Formulation Development and Evaluation of Pantoprazole Enteric Coated Tablets." **International Journal of ChemTech Research** 1(3): 663-666.

[81]. Gad, S.C. (2008). **Pharmaceutical manufacturing handbook: production and processes**. North Carolina: John wiley & sons, Inc.

[82]. Moore, G.K. and G.A.F. Roberts. (1980). "Determination of the degree of deacetylation of chitosan." **International Journal of Biological Macromolecules** 2(2): 115-116.

[83]. Luangtana-anan, M., et al. (2007). "Effect of salts and plasticizers on stability of shellac film." **Journal of Agricultural and Food Chemistry** 55: 687−692

[84]. Zavareze, E.d.R., et al. (2012). "Development of oxidised and heat-moisture treated potato starch film." **Food Chemistry** 132(1): 344-350.

[85]. Zhang, C., et al. (2003). "Synthesis and characterization of water-soluble O-succinyl-chitosan." **European Polymer Journal** 39(8): 1629-1634.

[86]. Hirano, S. and T. Moriyasu. (2004). "Some novel N-(carboxyacyl)chitosan filaments." **Carbohydrate Polymers** 55(3): 245-248.

[87]. Nunthanid, J., et al. (2001). "Physical properties and molecular behavior of chitosan films." **Drug Development and Industrial Pharmacy** 27(2): 143–157.

[88]. Huanbutta, K., et al. (2011). "Swelling kinetics of spray-dried chitosan acetate assessed by magnetic resonance imaging and their relation to drug release kinetics of chitosan matrix tablets." **European Journal of Pharmaceutics and Biopharmaceutics** 77(2): 320-326.

[89]. Casy, A.F. and G.H. Dewar. (1994). "Captopril and its probable contaminants: NMR and MS features of analytical value." **Journal of Pharmaceutical and Biomedical Analysis** 12(7): 855-861.

[90]. Mustafa, R., et al. (2009). "Synthesis and characterization of rigid aromatic-based epoxy resin." **Malaysian Polymer Journal** 4(2): 68-75.

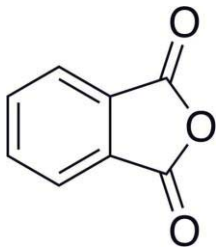
[91]. Kim, J.H. and O. World Health. (2009). **Cyclic acid anhydrides: human health aspects**. Stuttgart: World Health Organization.

APPENDIX

Phthalic anhydride [91]

Formula: $C_8H_4O_3$

Molecular structure:



Molecular Weight: 148.12 g/mol

Synonyms: Phthalic acid anhydride
1,3-Isobenzofurandione
Isobenzofuran-1,3-dione
1,2-Benzenedicarboxylic acid anhydride
1,2-Benzenedicarboxylic anhydride
1,3-Dihydro-1,3-dioxoisobenzofurane
1,3-Dioxophthalane
1,3-Phthalandione
Phthalandione

Physical state: White flakes or needles

Melting point: 131.6 ºC

Boiling point: 284.5 ºC

Density: 1.527 g/cm^3

Vapor density: 6.6 (vs air)

Flash point: 152 ºC

Solubility: 0.62 g/100 mL water at 20 ºC
Very slightly soluble in cold water
Soluble in alcohol
Soluble in ether

Toxicity: Oral rat LD_{50}: 4020 mg/kg
Inhalation rat LC_{50}: > 210 mg/m^3/1 h
Skin rabbit LD_{50}: > 10 gm/k

Example: The Evaluation of degree of substitution (DS)

FTIR method

The DS of N-PhCS prepared at 25˚C under stirring for 4 h, CS:PA at 1:5 mole ratio and neutralization pH at pH 5 by calculation from the ratio of absorbance at 1645 cm^{-1} (amide I band) and the hydroxyl band at 3450 cm^{-1} of FTIR spectra of N-PhCS (Figure 34a) and chitosan (Figure 34b). The degree of substitution was calculated using the following Equation (1):

$$\%\text{DS of N} - \text{PhCS} = \left(\frac{A_{1645}}{A_{3450}}\right)_{N-PhCS} - \left(\frac{A_{1645}}{A_{3450}}\right)_{CS} \times 100 \qquad \text{.....(1)}$$

$$\%\text{DS of N} - \text{PhCS} = \left(\frac{0.090}{0.311}\right)_{N-PhCS} - \left(\frac{0.025}{0.200}\right)_{CS} \times 100$$

$$\%\text{DS of N} - \text{PhCS} = \quad 16.43$$

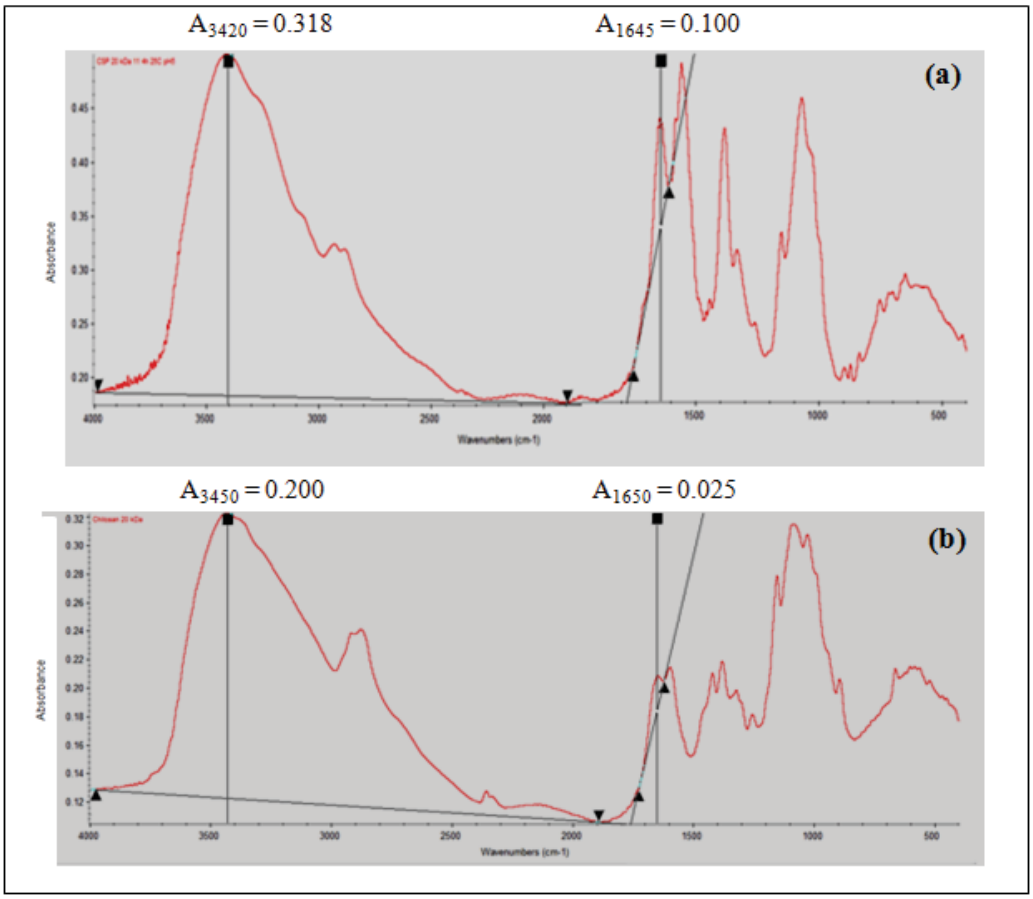


Figure 34 The FTIR spectra of (a) N-PhCS and (b) chitosan showing the baselines for calculating the amide I band absorbance for the ratio A_{1645} /A_{3420}.

^{1}H-NMR method

The degree of substitution of N-PhCS prepared at 25˚C under stirring for 4 h, CS:PA at 1:5 mole ratio and neutralization pH at pH 5 by calculation from the ratio of area between the aromatic protons of phthaloyl group at 7.4-7.9 ppm and methyl proton of monosaccharide residue (H_3, H_4, H_5 and H_6) at 3.5-3.9 ppm (Figure 35). The degree of substitution was calculated using the following Equation (2):

$$\%\text{DS of N} - \text{PhCS} = \left[\left(\frac{I_{aromatic\ \ proton}}{number\ \ of\ proton}\right) / \left(\frac{I_{\text{H3+H4+H5+H6}}}{\text{number of proton}}\right)\right] \times 100 \quad \text{.....(2)}$$

$$\%\text{DS of N} - \text{PhCS} = \left[\left(\frac{4.0}{4}\right) / \left(\frac{3.38 + 3.45}{5}\right)\right] \times 100$$

$$\%\text{DS of N} - \text{PhCS} = \ 73.2\%$$

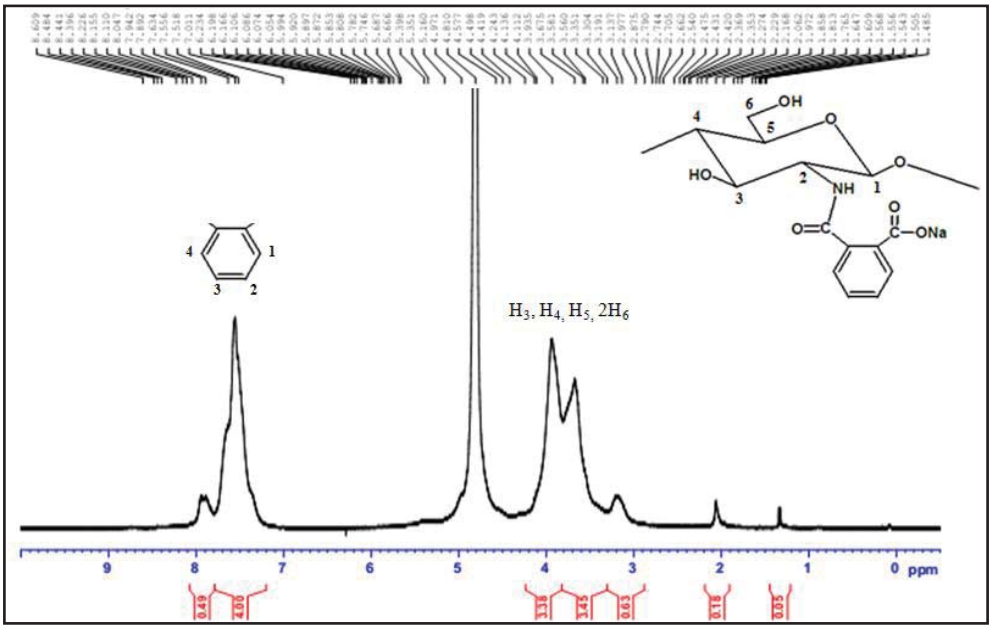


Figure 35 ^{1}H NMR spectra of N-PhCS prepared at 25˚C under stirring for 4 h, CS:PA at 1:5 mole ratio and neutralization pH at pH 5.

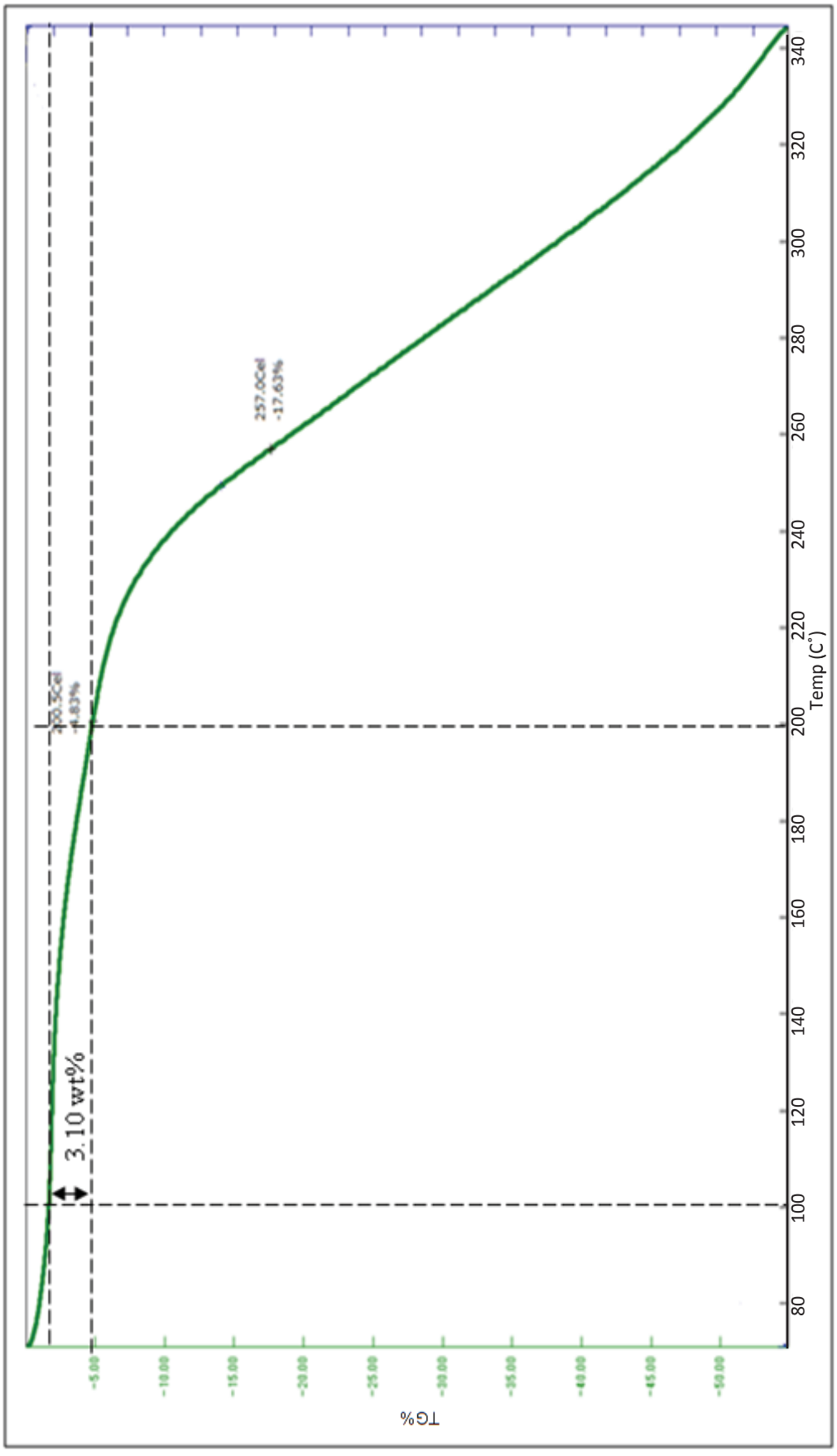


Figure 36 TGA thermogram of N-PhCS-20 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 4.

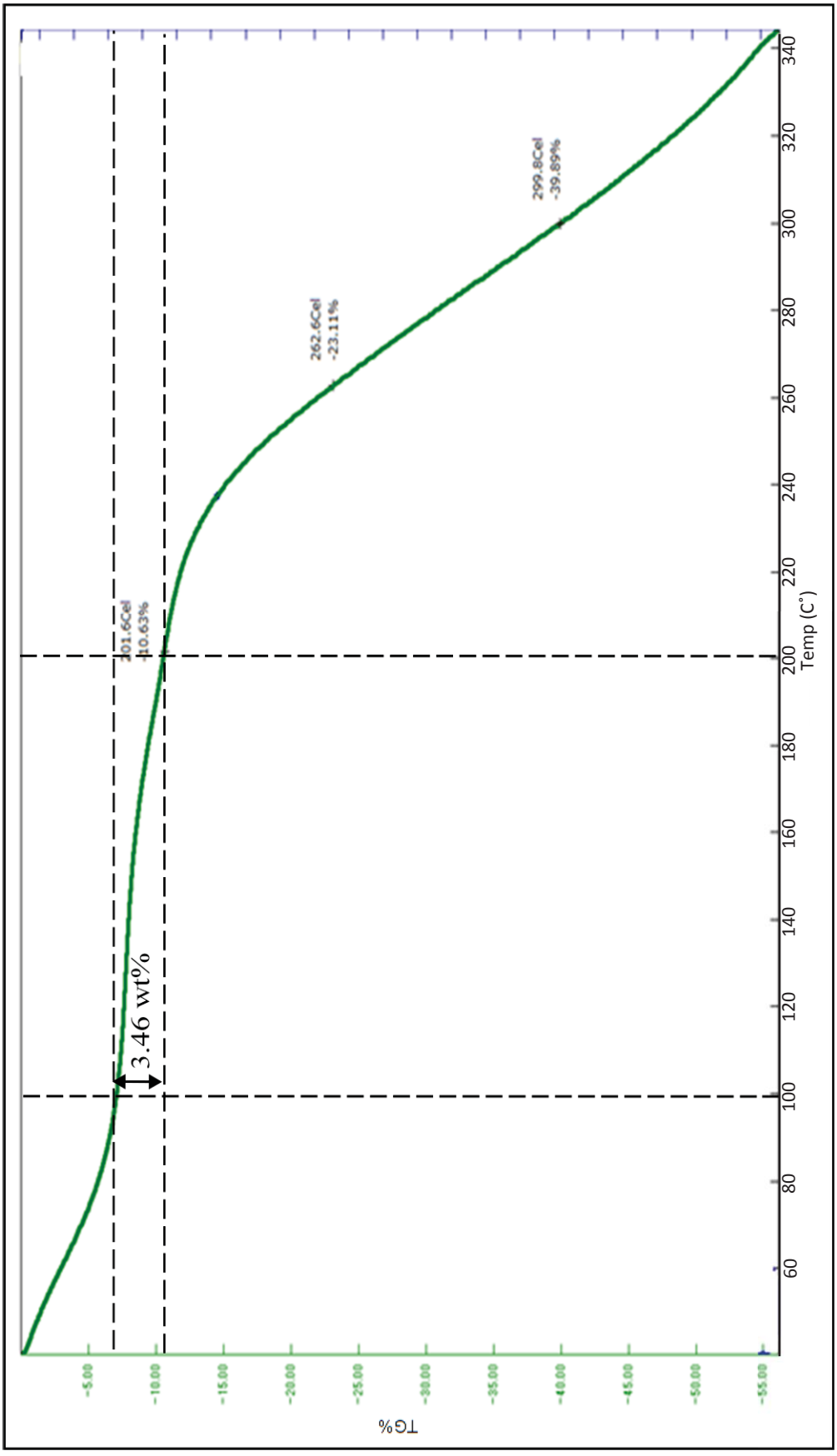


Figure 37 TGA thermogram of N-PhCS-20 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 5.

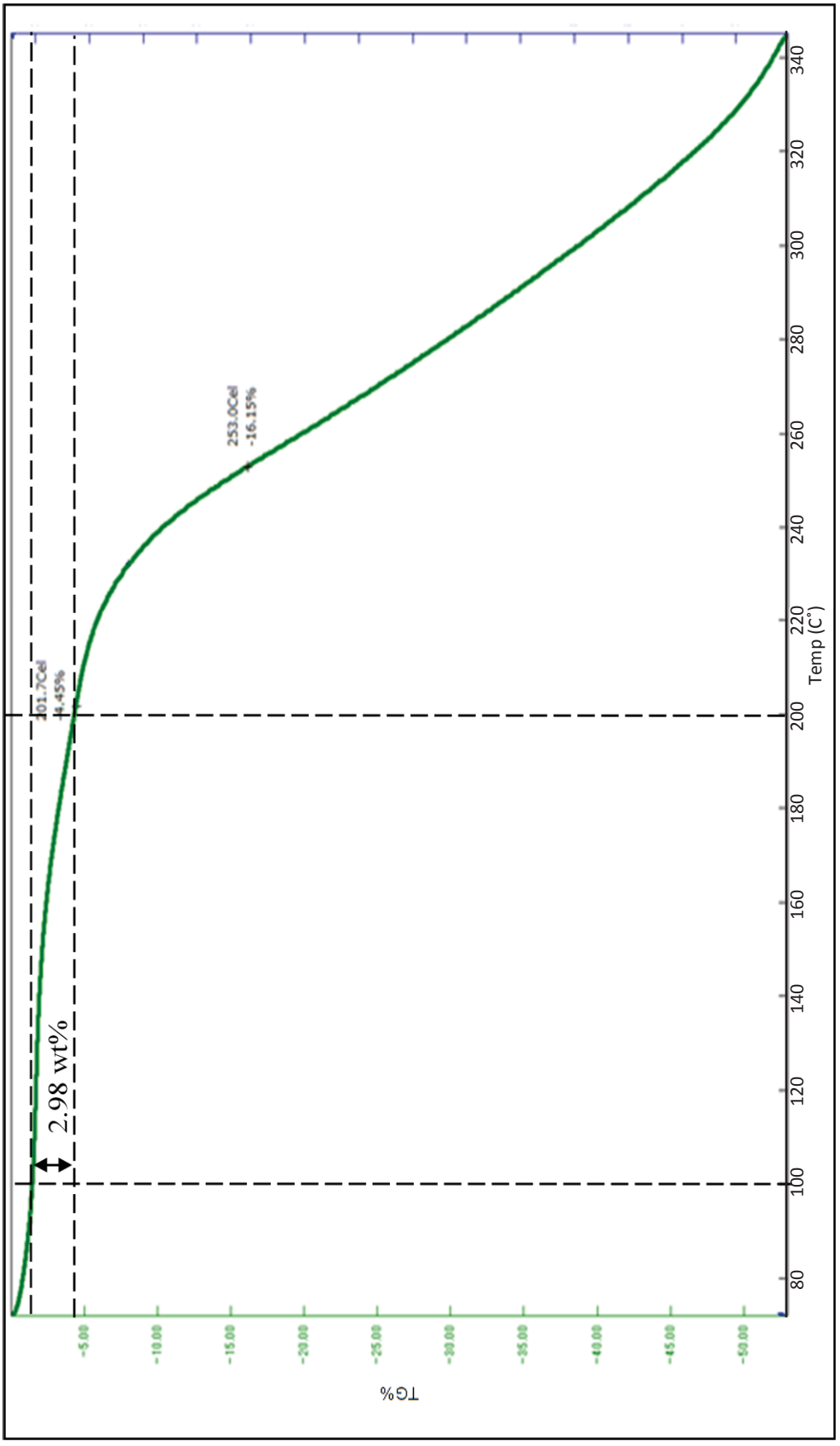


Figure 38 TGA thermogram of N-PhCS-20 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 6.

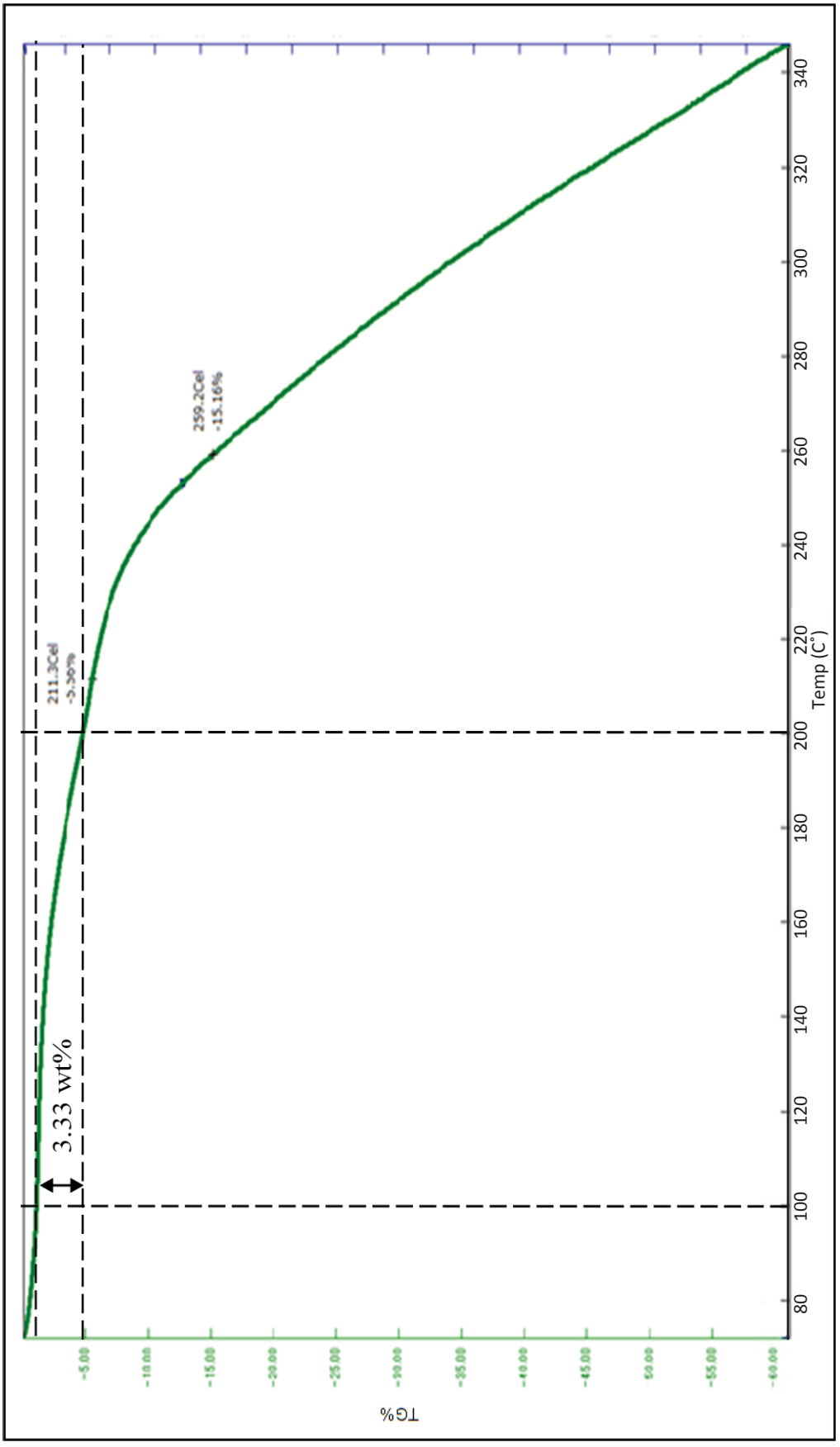


Figure 39 TGA thermogram of N-PhCS-20 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 4.

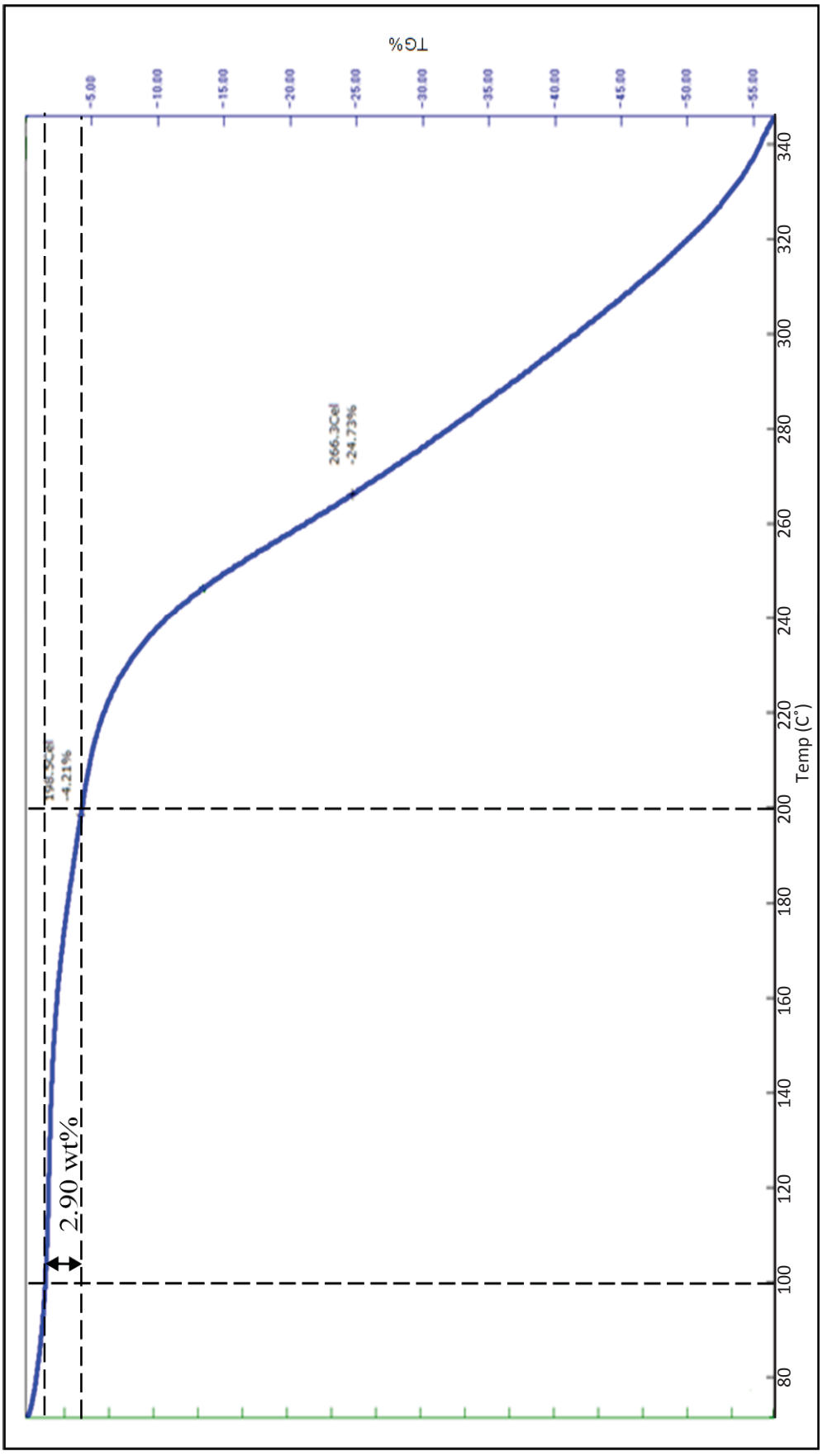


Figure 40 TGA thermogram of N-PhCS-20 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 5.

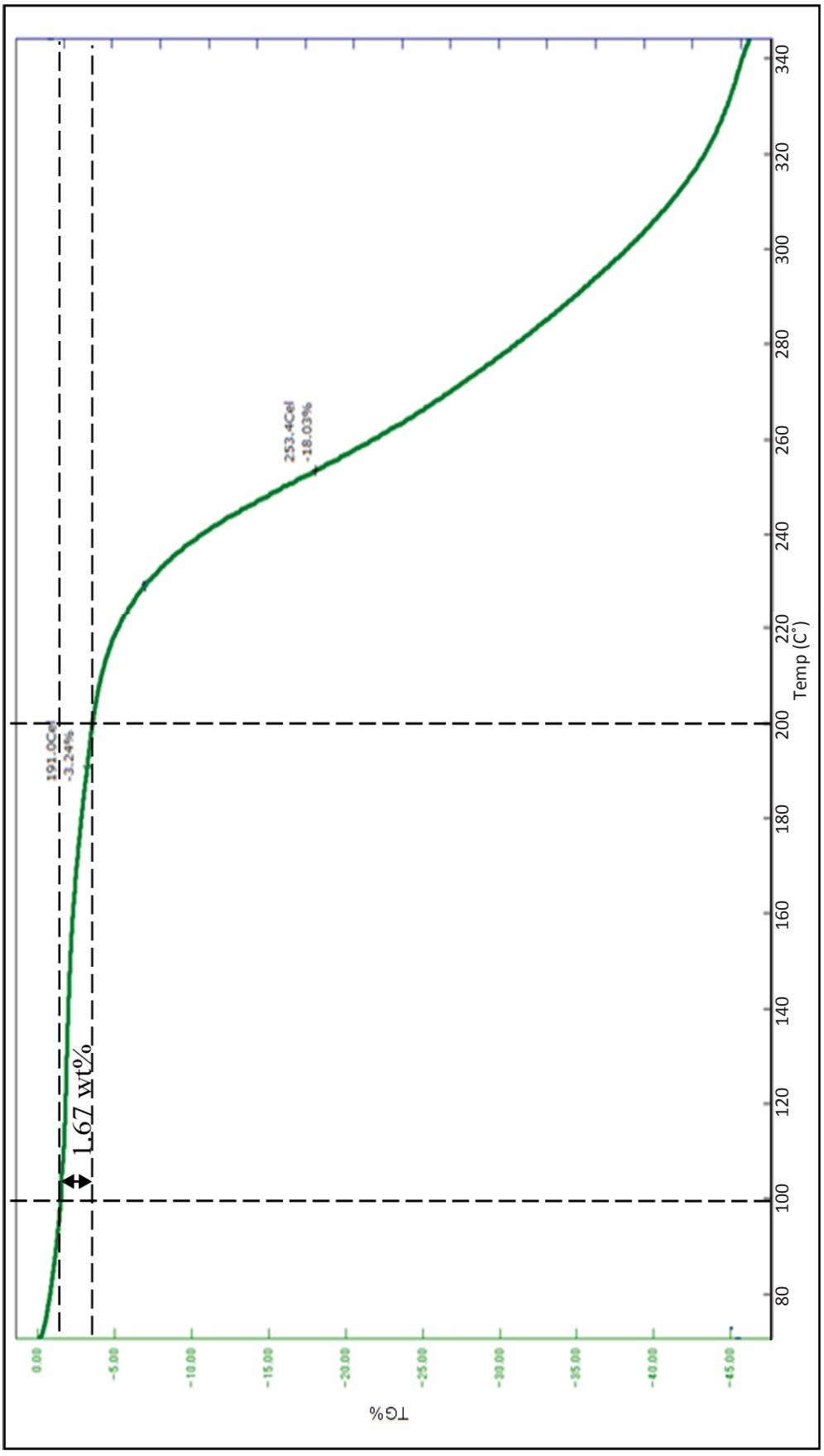


Figure 41 TGA thermogram of N-PhCS-20 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 6.

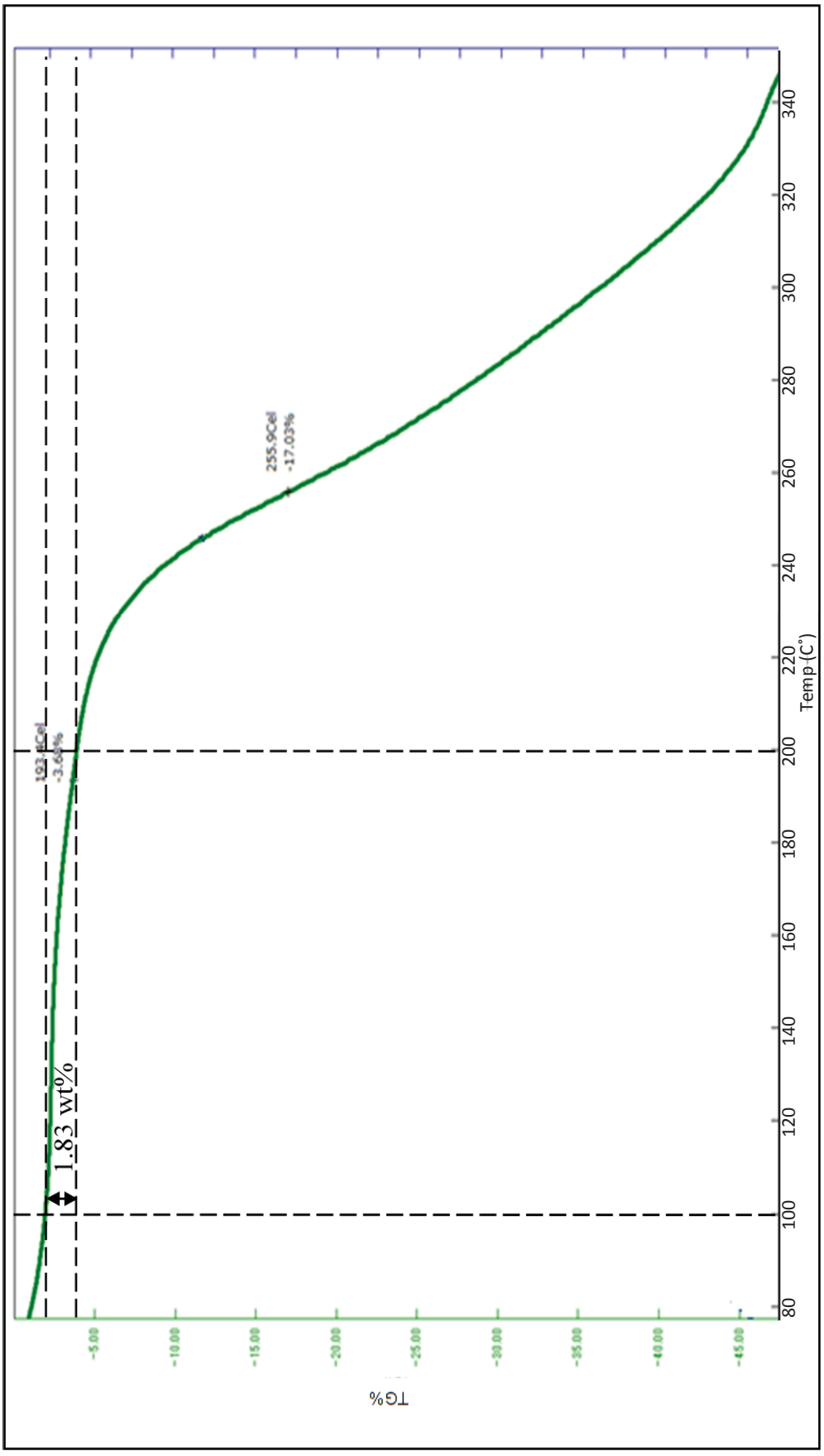


Figure 42 TGA thermogram of N-PhCS-20 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 4.

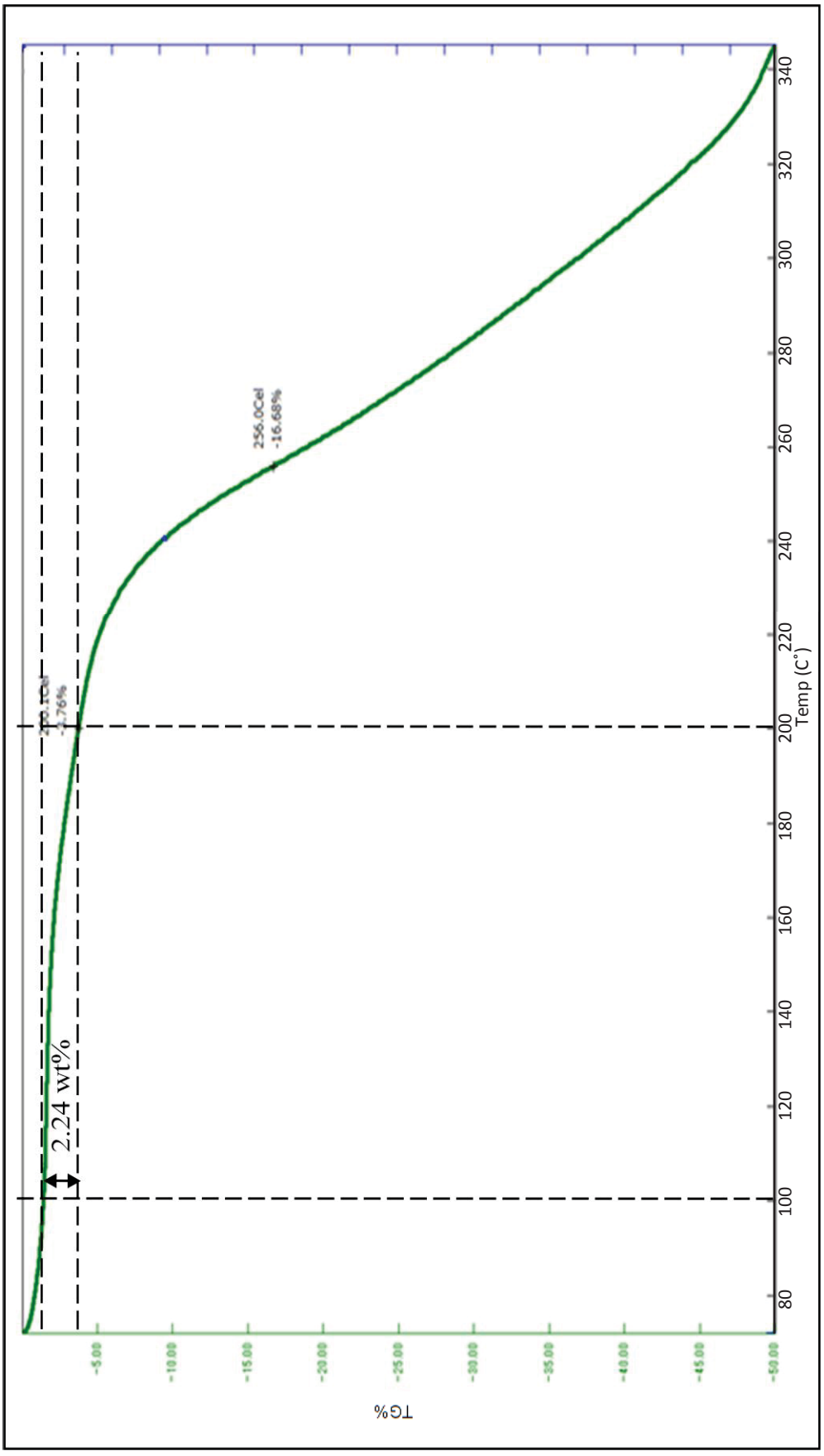


Figure 43 TGA thermogram of N-PhCS-20 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 5.

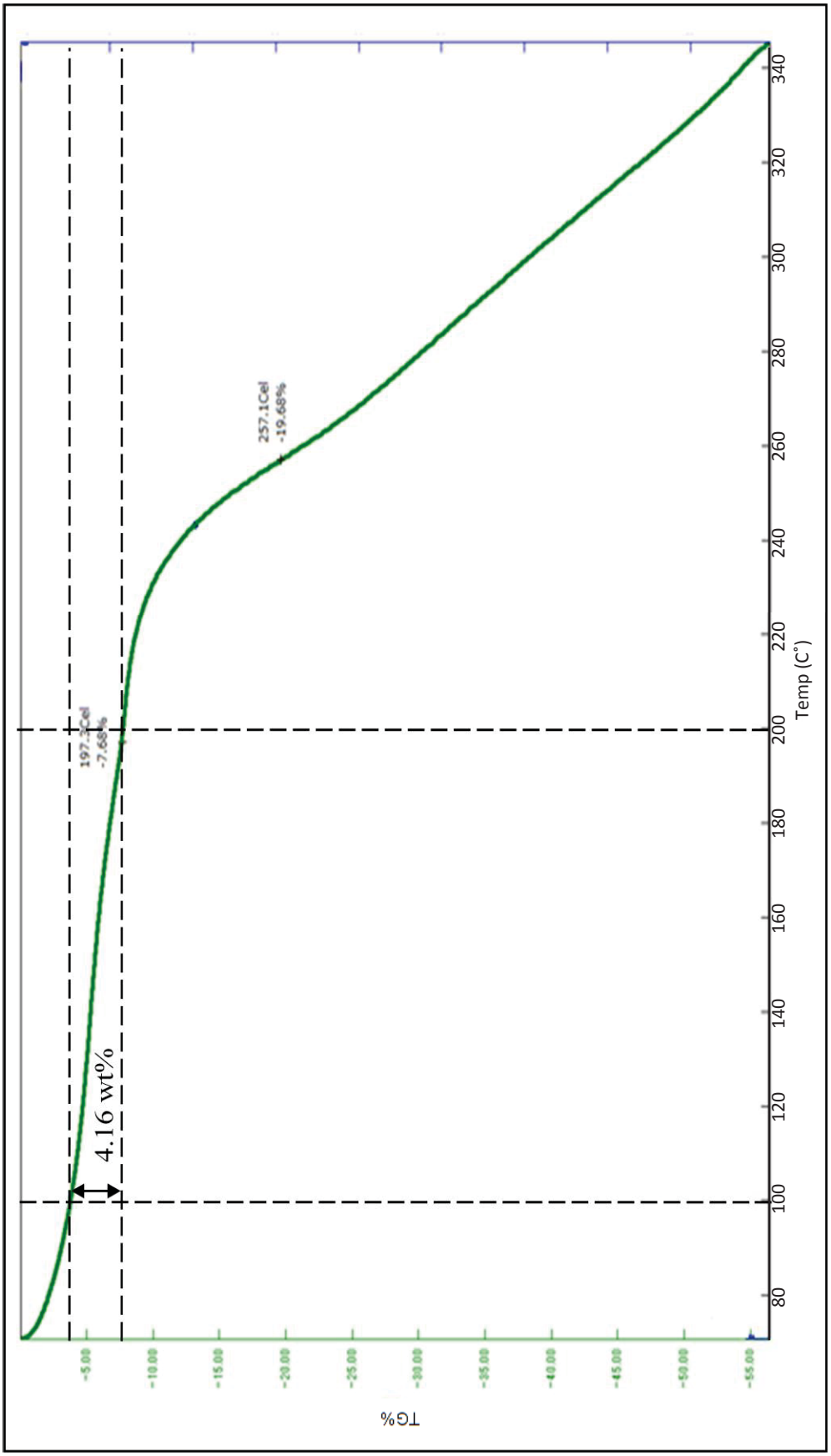


Figure 44 TGA thermogram of N-PhCS-20 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 6.

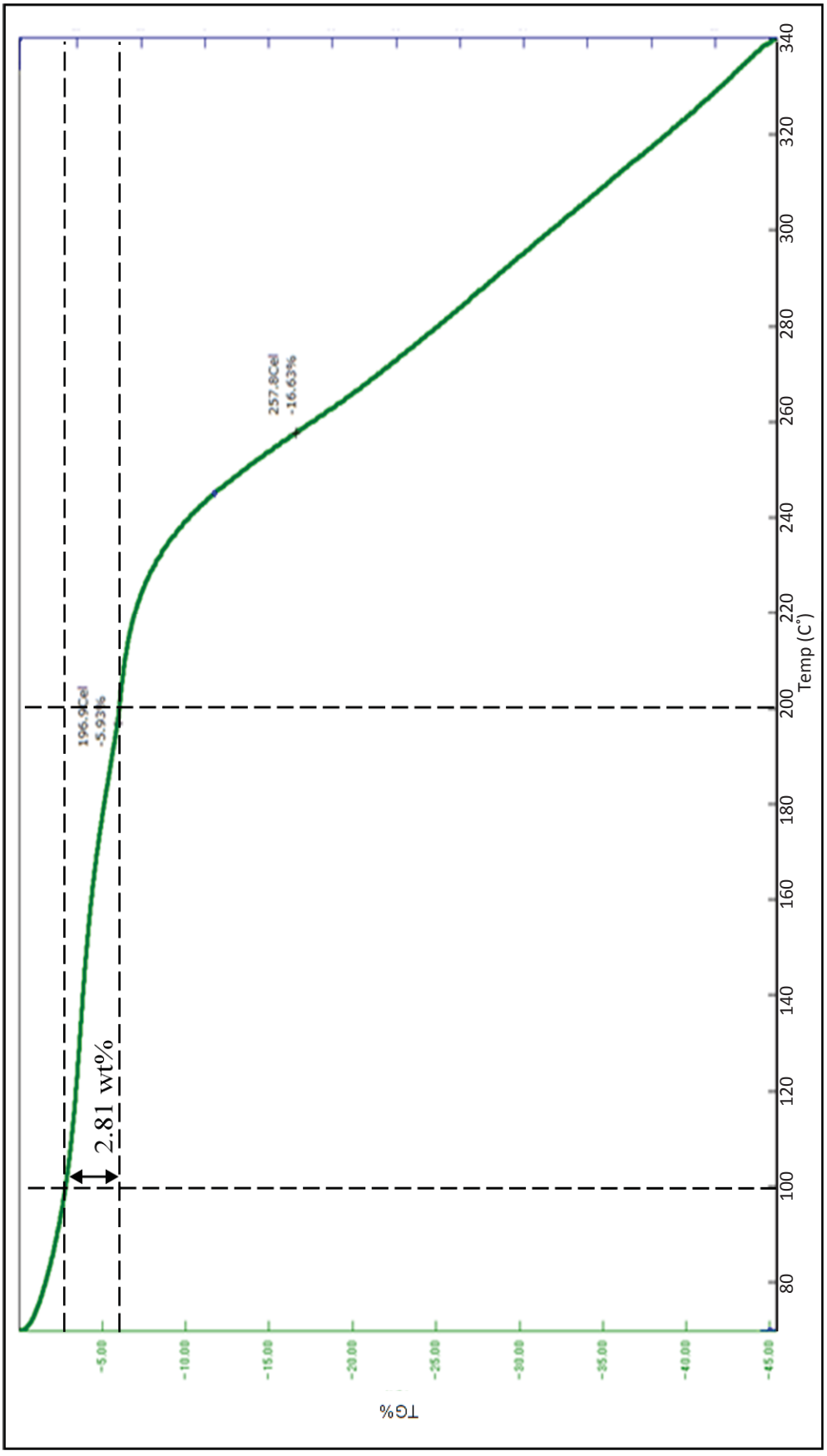


Figure 45 TGA thermogram of N-PhCS-200 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 4.

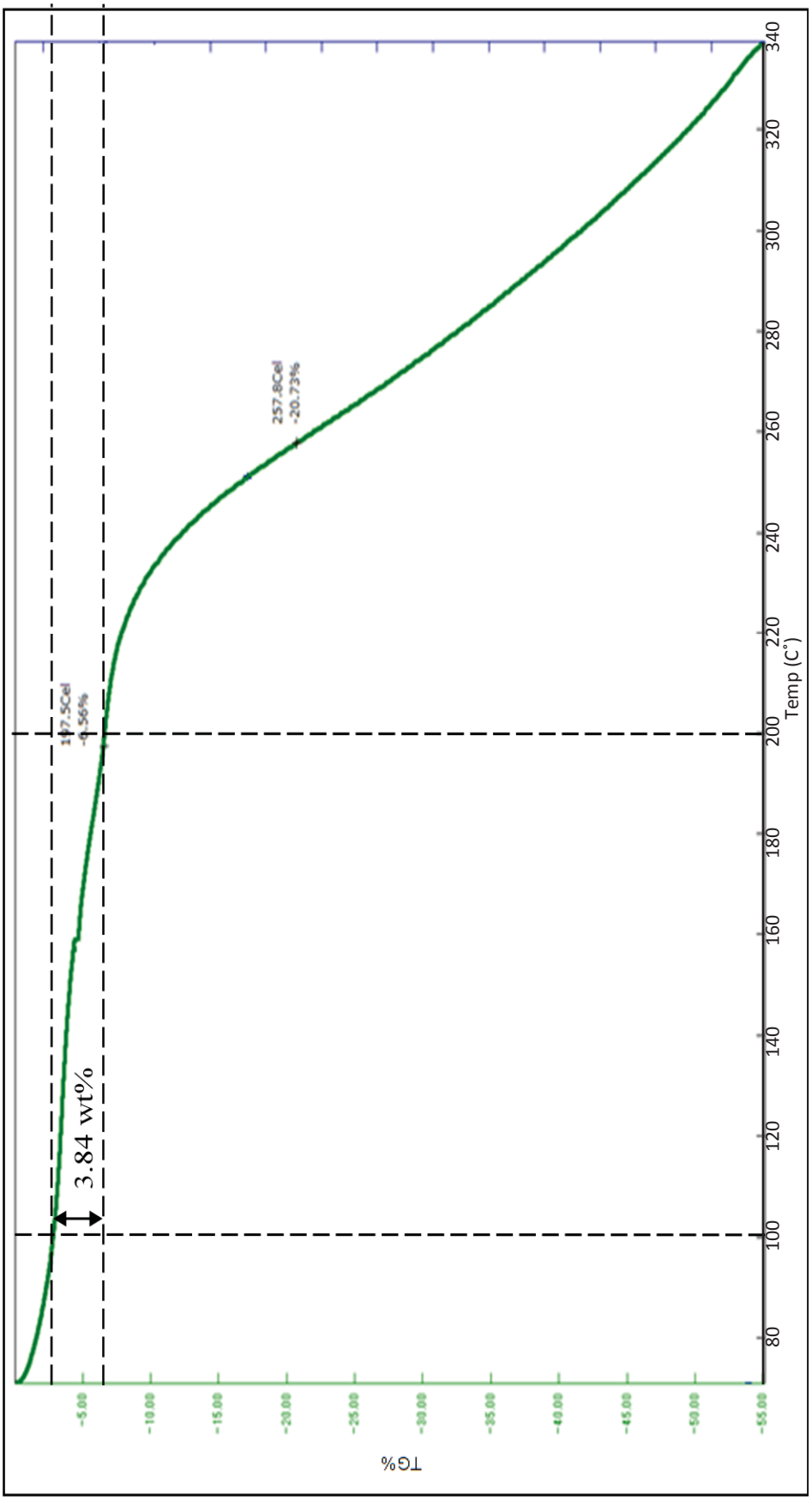


Figure 46 TGA thermogram of N-PhCS-200 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 5.

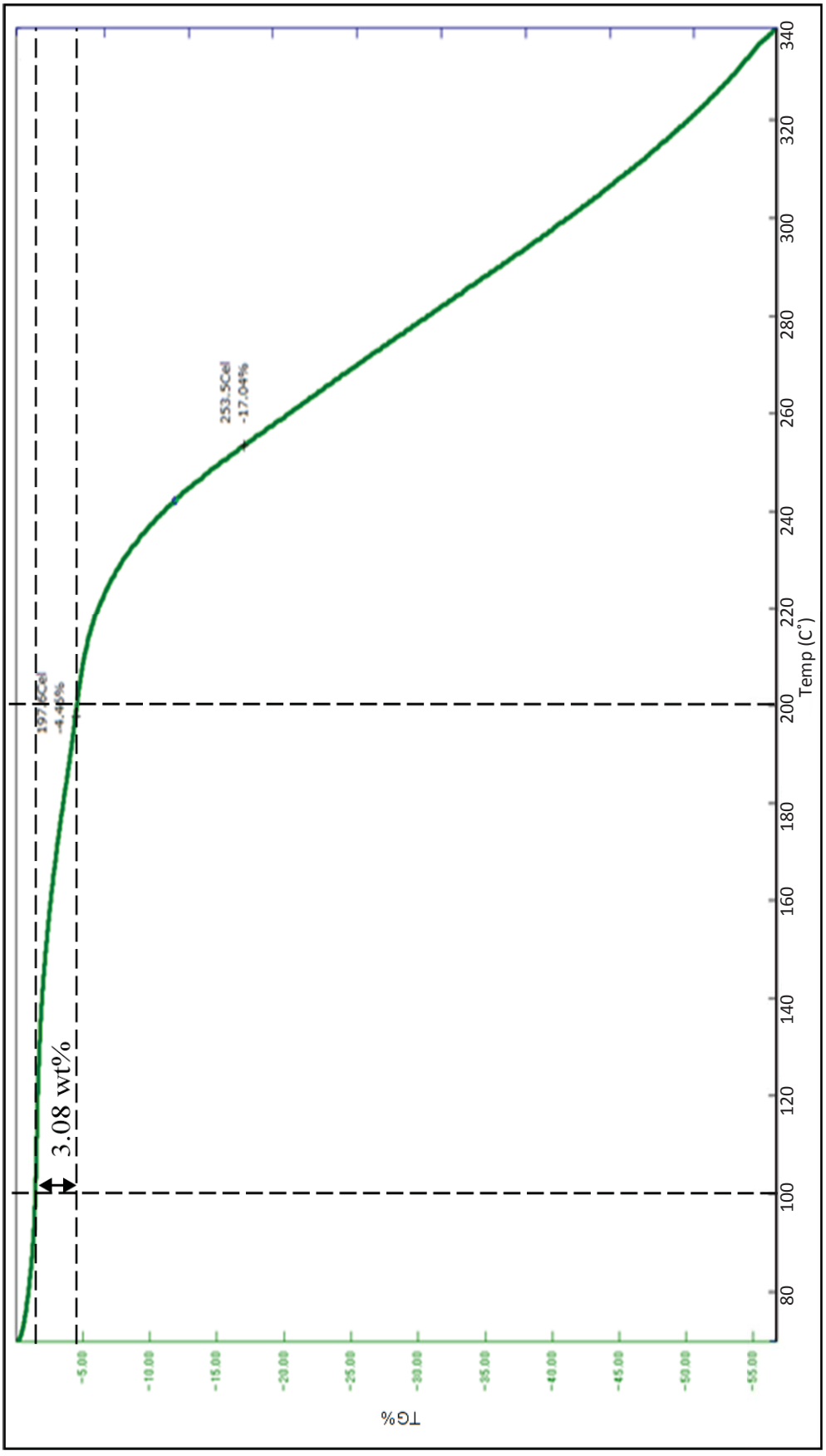


Figure 47 TGA thermogram of N-PhCS-200 prepared at 1:1 mole ratio of CS:PA and neutralization pH at pH 6.

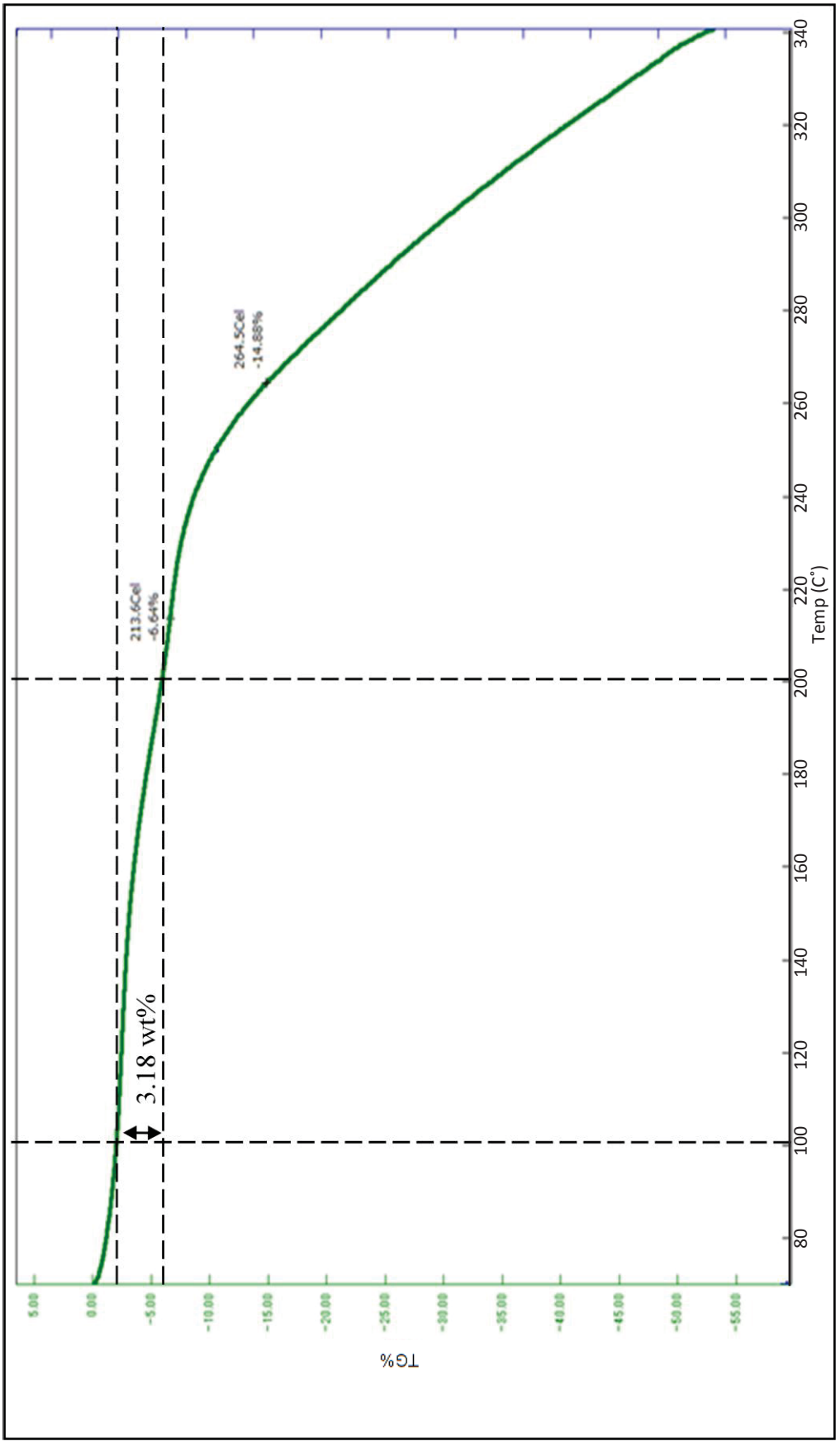


Figure 48 TGA thermogram of N-PhCS-200 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 4.

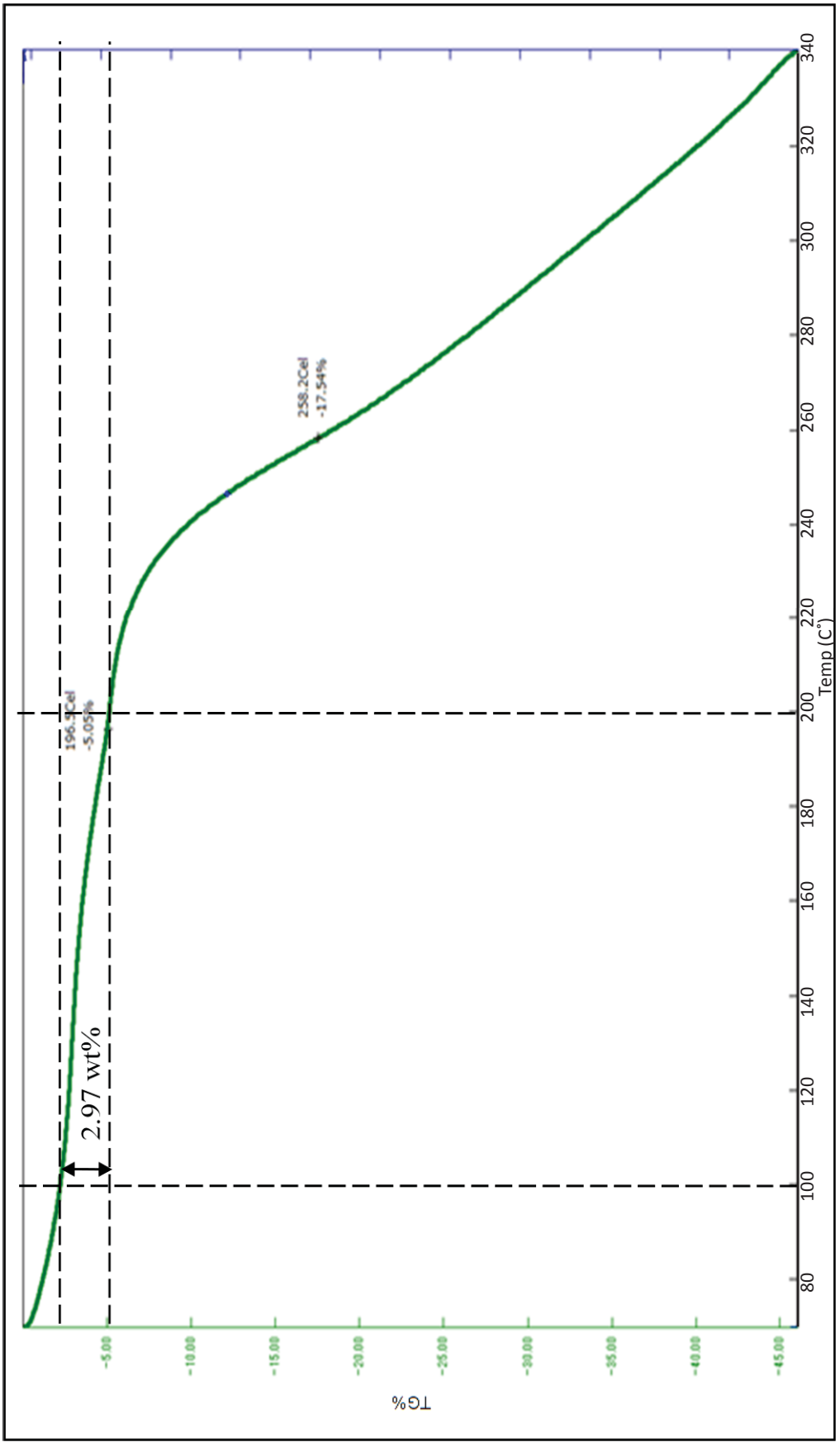


Figure 49 TGA thermogram of N-PhCS-200 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 5.

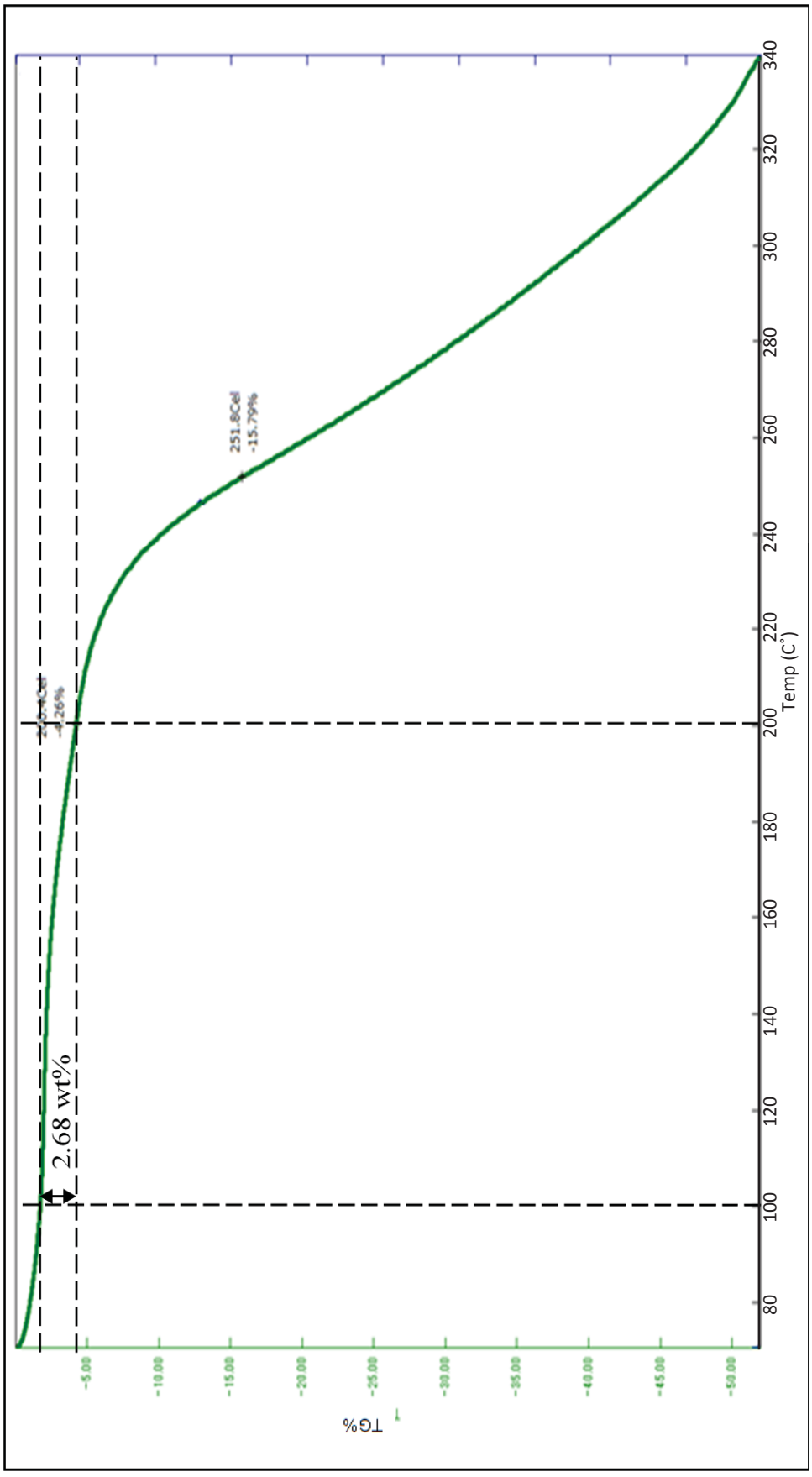


Figure 50 TGA thermogram of N-PhCS-200 prepared at 1:3 mole ratio of CS:PA and neutralization pH at pH 6.

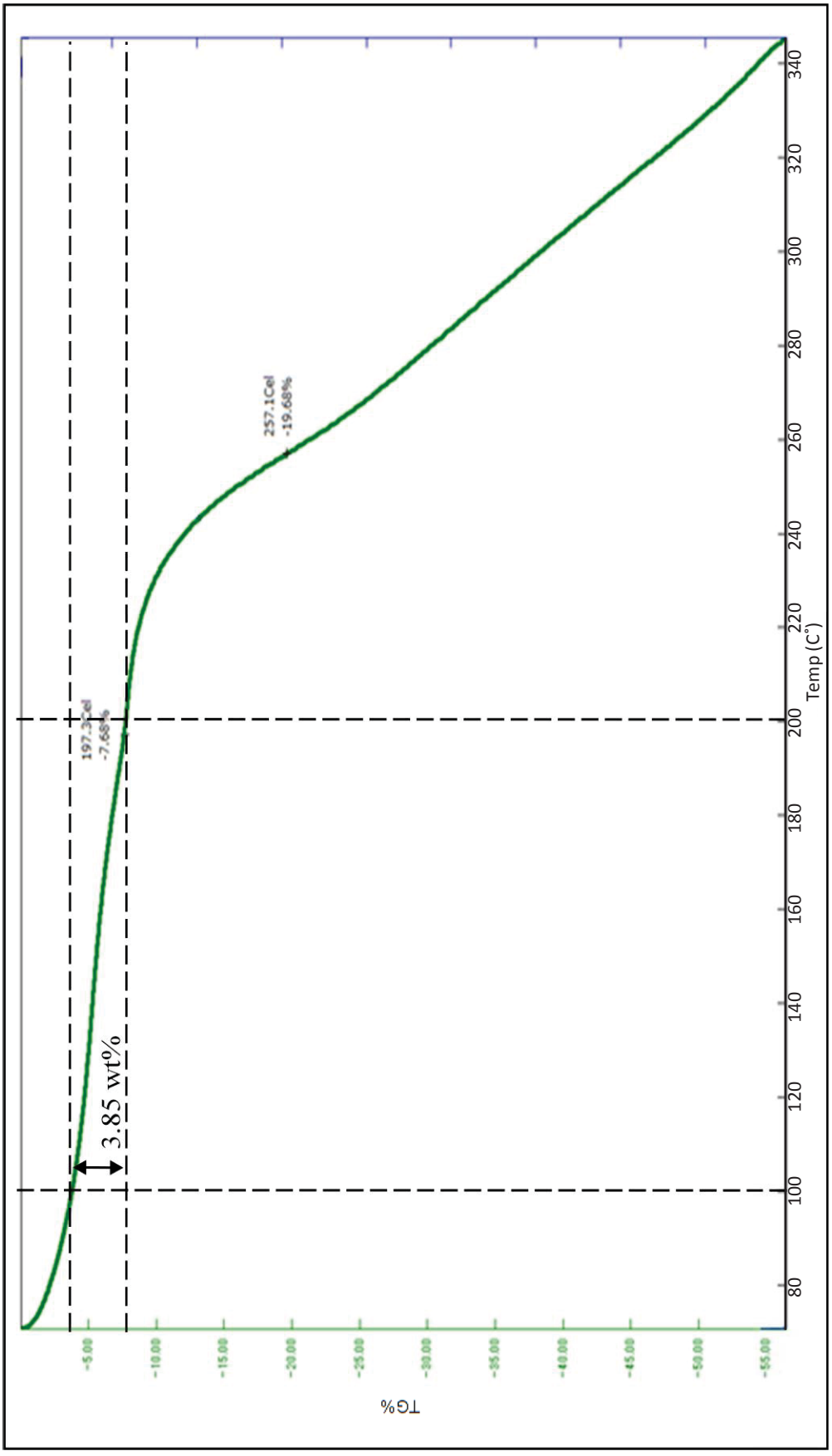


Figure 51 TGA thermogram of N-PhCS-200 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 4.

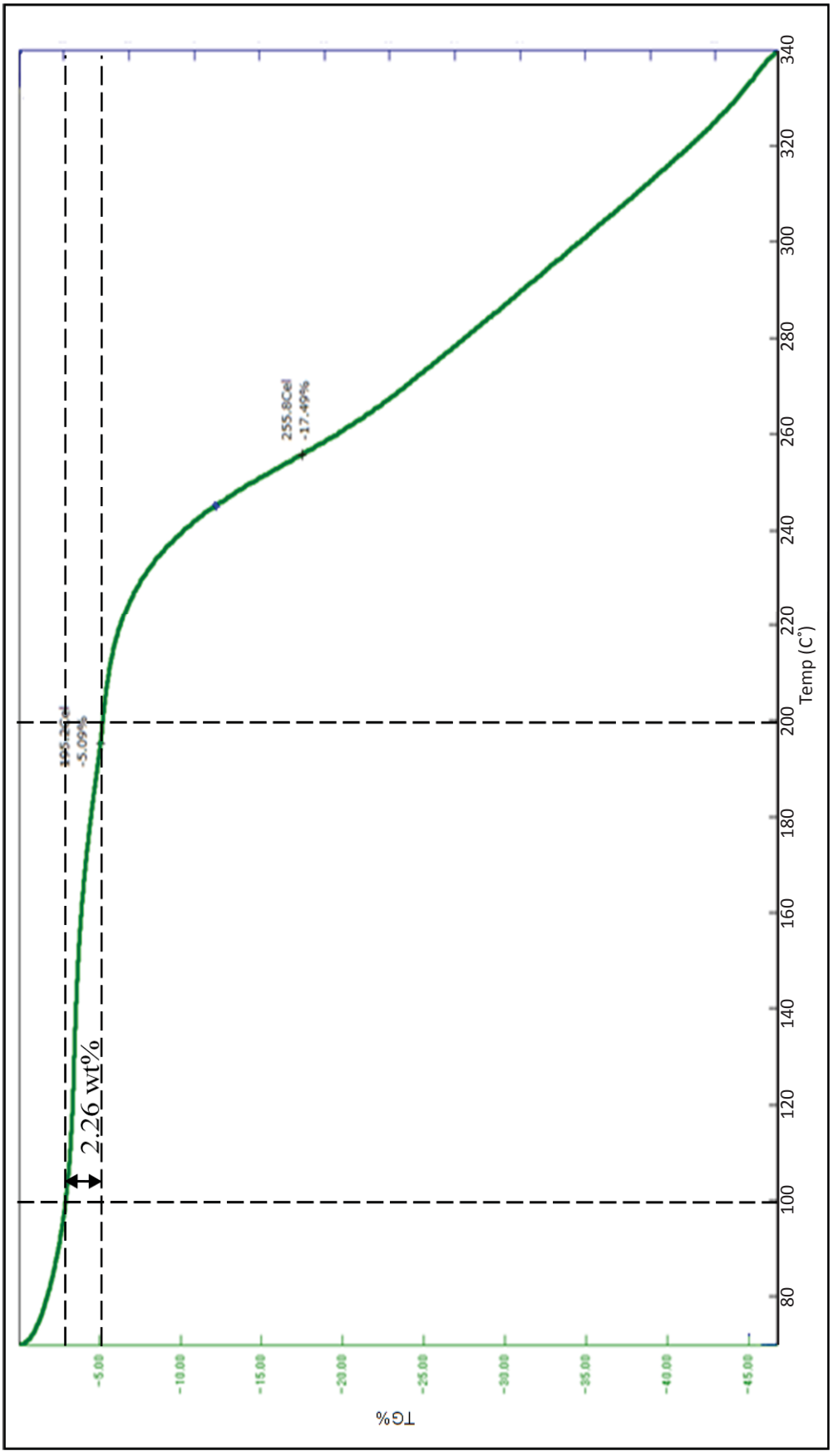


Figure 52 TGA thermogram of N-PhCS-200 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 5.

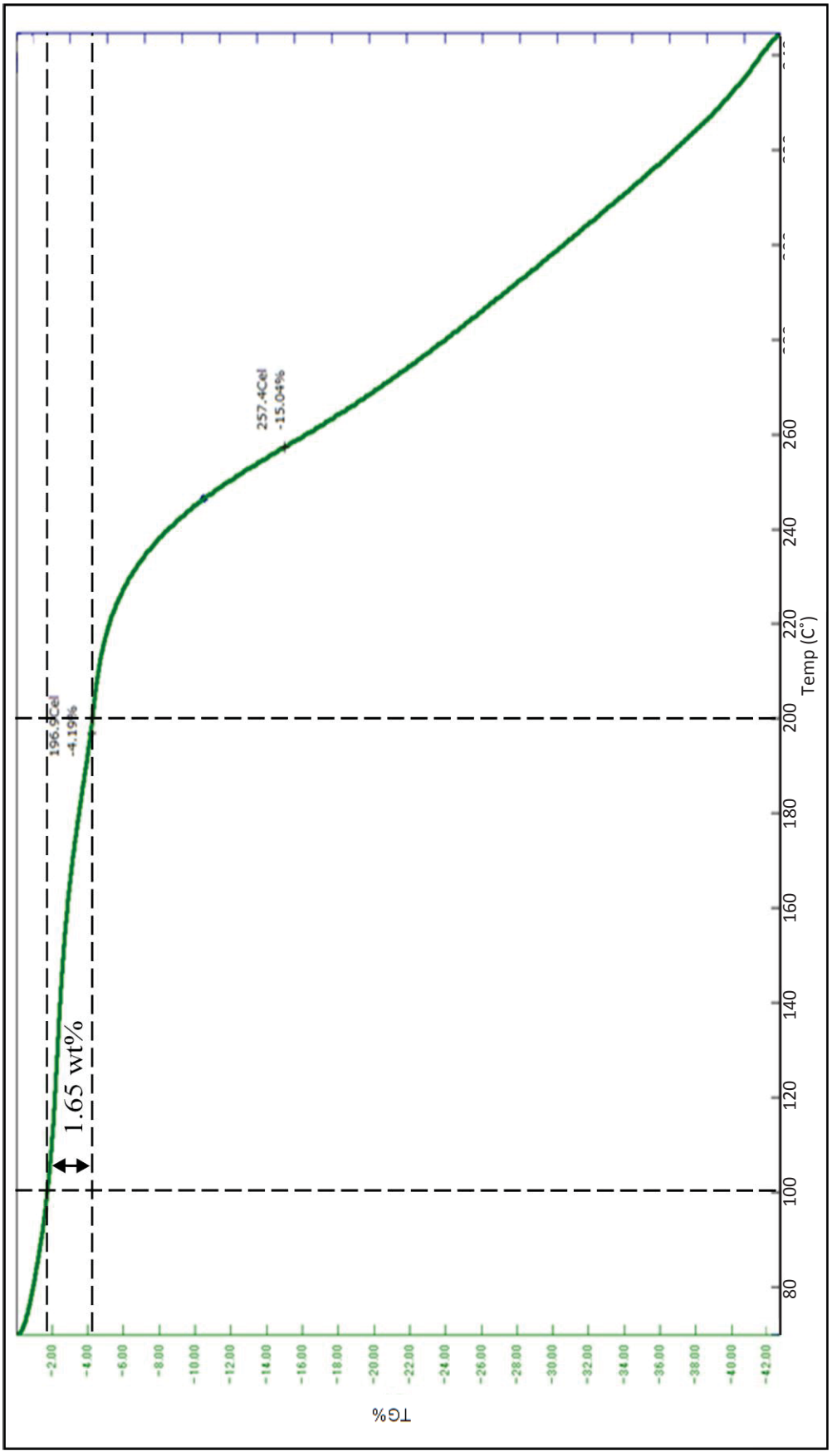


Figure 53 TGA thermogram of N-PhCS-200 prepared at 1:5 mole ratio of CS:PA and neutralization pH at pH 6.

Table 11 List of abbreviations.

Symbol	Definition
%	percent
>	more than
<	less than
˚C	degree celsius
µg	microgram
µL	microliter
^{1}H NMR	proton nuclear magnetic resonance
%v/v	percent volume by volume
%w/v	percent weight by volume
%w/w	percent weight by weight
A	absorbance
cm^{-1}	wavenumbers
cm^{2}	square centimeter
CS	chitosan
CS-20	chitosan 20 kDa
CS-200	chitosan 200 kDa
N-PhCS	N-phthaloyl chitosan
DD	degree of deacetylation
D_2O	deuterium oxide
DS	degree of substitution
DSC	differential scanning calorimetry
e.g.	exemplī grātiā (Latin); for example
et al.	and others
etc.	et cetera (Latin); and other things/ and so forth
FTIR	fourier transform infrared spectroscopy
g	gram
HCl	Hydrochloric acid
h	hours

Symbol	Definition
I	integral (area under curve)
i.e.	id est (Latin); that is
kDa	kilodalton
kg	kilogram(s)
Lot. No.	lot number
mg	milligram
mL	milliliter
MW	molecular weight
N	normality
NaOH	Sodium hydroxide
nm	nanometer
PA	phthalic anhydride
p	p-value
pK_a	the negative logarithm of the dissociation constants
ppm	parts per million
PXRD	powder X-ray diffraction
SD	standard deviation
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
TGA	thermogravimetric analysis
Tris	Tris (hydroxymethyl) aminomethane
W	weight
UV-VIS	ultraviolet -visible

BIOGRAPHY

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Conference proceedings

1. Sunitda Khawthong, Pornsak Sriamornsak, Sontaya Limmatvapirat, Manee Luangtana-anan, Sathit Niratisai, Jurairat Nunthanid and Panjapol Laopoonpat. (2011). "Characterization of chemically modified chitosan with phthalic anhydride prepared by spray drying and solvent precipitation techniques." **Proceeding of the Pure and Applied Chemistry International Conference (PACCON)**: 694-696. [Bangkok, Thailand January 5-7, 2011]
2. Sunitda Khawthong, Pornsak Sriamornsak, Sontaya Limmatvapirat, Manee Luangtana-anan, Sathit Niratisai and Jurairat Nunthanid. "Physicochemical characterization of a novel chitosan derivative, chitosan sodium biphthalate." **Proceeding of the 6th Thailand Materials Science and Technology Conference (MSAT)**: 422-423. [Bangkok, Thailand, August 26-27, 2010]