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3^2 Factorial design and response surface analysis optimization of *N*-carboxybutylchitosan synthesis

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Abstract

A 3^2 factorial design investigation to optimize *N*-carboxybutylchitosan was carried out varying the levulinic acid/chitosan molar ratio between 1.00 and 3.00 and the sodium borohydride/chitosan molar ratio from 0.50 to 1.00. ^{1}H NMR spectra signals at 1.0 and 2.5 ppm were used to monitor the reaction system. A quadratic model having significant terms for both molar ratios was found for *N*-carboxybutylchitosan substitution degree whereas a linear model best describes the substitution degree of 5-methylpyrrolidinone chitosan. Maximum *N*-carboxybutylchitosan and minimum 5-methylpyrrolidinone chitosan substitution degrees occur for levulinic acid/chitosan molar ratios between 2.00 and 3.00 and a 0.50 for sodium borohydride/chitosan molar ratio.

Keywords: Factorial design; Response surface analysis; Chitosan; *N*-Carboxybutylchitosan; 5-Methylpyrrolidinone chitosan

1. Introduction

Chitin, the second most abundant natural polysaccharide and the major constituent of the exoskeleton of crustaceous species and insects, is a linear homopolymer composed of β(1,4)-linked *N*-acetyl glucosamine (Li, Dunn, Grandmaison, & Goosen, 1997) and is insoluble in water and most organic solvents (Ravi Kumar, 2000). Partial deacetylation of chitin results in the production of chitosan, which is a polysaccharide comprising copolymers of glucosamine and *N*-acetyl glucosamine (Singla & Chawla, 2001).

Chitosan has been investigated for pharmaceutical applications because these natural polymers have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorptivity properties, film-forming ability, antimicrobial activity against fungi, bacteria and viruses (Chirkov, 2002; Lim & Hudson, 2003; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan has been used as an excipient in the pharmaceutical industry (in direct tablet compression, as a tablet disintegrant, for the production of controlled release solid dosage forms or for the improvement of drug dissolution) for production of controlled release implant systems for delivery of hormones over extended periods of time, for nasal and oral delivery of polar drugs and for vaccine delivery (Illum, 1998). These biopolymers are also being studied in food applications owing to their wide range of unique applications including the formation of biodegradable films, immobilization of enzymes, preservation of foods from microbial deterioration, and also as additives (clarification and deacidification of fruits and beverages, emulsifier agents, thickening and stabilizing agents, color stabilization) and dietary supplements (Agullo, Rodriguez, Ramos, & Albertengo, 2003).

N-carboxybutylchitosan is an amphoteric chitosan derivative that is soluble under acidic, neutral and basic conditions whereas chitosan itself is only soluble under acidic conditions (Rinaudo, Desbrières, Le Dung, Thuy Binh, & Dong, 2001). The bacteriostatic activity and wound healing properties of the *N*-carboxybutylchitosan (Biagini, Muzzarelli, Giardino, & Castaldini, 1991), together with other favorable properties, such as its viscous action, enhanced film-forming ability, moisturizing effect and

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emulsion stability, make these novel modified chitosans most suitable as functional cosmetic ingredients (Muzzarelli, Weckx, Filippini, & Lough, 1989).

It is obtained by the reaction of levulinic acid on chitosan (Muzzarelli et al., 1989). But, depending on the chemical conditions the reaction tends to form *N*-carboxybutylchitosan or 5-methylpyrrolidinone chitosan (Muzzarelli, Ilari, & Tomasetti, 1993; Muzzarelli et al., 1989). The solubility range of 5-methylpyrrolidinone chitosan, a cyclic derivative, is restricted to acidic conditions, as is chitosan (Rinaudo et al., 2001).

The chemical properties of these two derivatives are very different, so it is important to be able to control the reaction to produce one or the other of these polymers. Several factors are important in forming chitosan derivatives. The content of levulinic acid is one that must be in excess to allow both the dissolution of chitosan and the formation of enough of the intermediate ketimine form. An excess of the reducing agent is also necessary but too much may cause degradation of the polymer (Rinaudo et al., 2001) and could explain the cyclization of the substituent on 5-methylpyrrolidinone chitosan. Muzzarelli et al. (1993) reported that a high chitosan concentration, close to 20 g kg^{-1}, high pH values during the reduction step, high sodium borohydride concentrations and slow sodium borohydride delivery rates lead to the cyclic form. On the other hand, water-soluble chitosan derivatives are obtained when the molar ratio of levulinic acid to amino groups is high and during short time periods for the reduction reaction (Muzzarelli et al., 1989; Rinaudo et al., 2001).

Multivariate statistical experimental designs (Barros Neto, Scarminio, & Bruns, 2002; Box, Hunter, & Hunter, 1978) are very efficient for optimizing systems that depend on several factors. Not only do they result in an economy of experiments they permit the evaluation of possible synergic or antagonistic interactions that may exist between factors. They have been employed to increase synthesis yields of a variety of reactions (Duenas, Munduate, Perea, & Irastorza, 2003; Morris & Mallouk, 2002; Tagliabue, Carluccio, Ghisletti, & Perego, 2003). Here they are used to maximize the substitution degree of *N*-carboxybutylchitosan while attempting to minimize it for 5-methylpyrrolidinone chitosan. A small number of replicate experiments are included in the design to determine if the effects of changing factor levels are significantly larger than the effects of experimental error on the substitution degrees.

2. Material and methods

2.1. Material

Chitosan of medium molecular weight was purchased from Aldrich Chemical Company, Inc. and characterized with a 0.15 acetylation degree and a viscosimetric average molecular weight of 145,000 Da. ^{1}H NMR spectra were obtained on an ADPX 300 MHz spectrometer from Brüker and intrinsic viscosity [η] determination was performed on an AVS-350 viscometer coupled to an AVS-50 automatic dilution module, both from Schott-Geräte. Levulinic acid and sodium borohydride were purchased from Aldrich Chemical Company, Inc. and Acros Organics, respectively. The chitosan derivatives were freeze-dried on Alpha 1–2 from Christ for isolation.

2.2. Synthesis

Chitosan (1.0 mol) was stirred overnight in water in the presence of levulinic acid (1.0–3.0 mol) at room temperature and then stirred for another 2 h at 80 °C. Sodium borohydride (0.5–1.0 mol), previously dissolved in deionized water (10 ml), was added and the reaction stirred, first for 30 min at room temperature and then for 2 h at 80 °C. The product was dialyzed with deionized water in membranes of cellulose acetate with a cutoff of 12,000 Da for 3 days and freeze-dried.

2.3. Characterization

^{1}H NMR spectrum of chitosan and its derivatives were recorded at 70 °C in D_2O, but some HCl was needed to help solubilize the samples of chitosan and 5-methylpyrrolidinone chitosan. The acetylation degree as well as the substitution

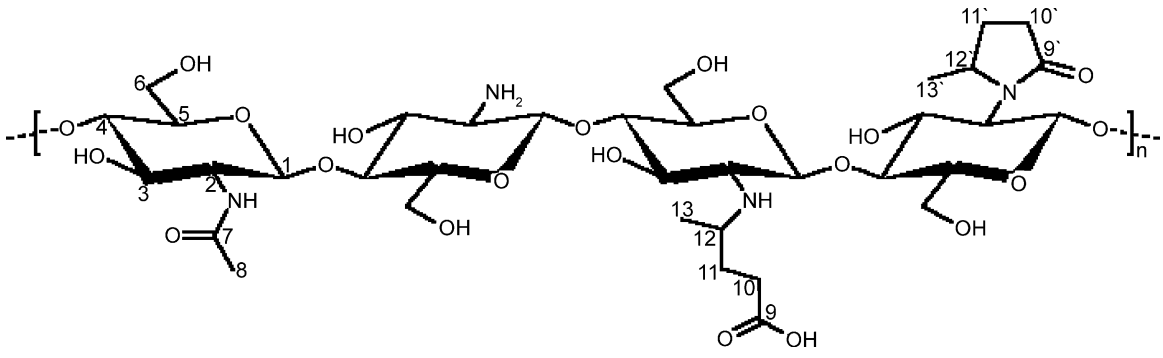

Fig. 1. Chitosan derivative structure which represents, from left to right, acetylated monomer, deacetylated monomer, *N*-carboxybutyl derivative and 5-methylpyrrolidinone derivative.

Table 1
A 3^2 factorial design with center point replicated four times

Run	Execution order	X_1	X_2	Moles per mol of chitosan	
				Levulinic acid	Sodium borohydride
1	1	−1	−1	1.00	0.50
2	3	+1	−1	3.00	0.50
3[a]	8	+1	−1	3.00	0.50
4	5	−1	+1	1.00	1.00
5	7	+1	+1	3.00	1.00
6	9	−1	0	1.00	0.75
7	13	+1	0	3.00	0.75
8	12	0	−1	2.00	0.50
9	10	0	+1	2.00	1.00
10[b]	2	0	0	2.00	0.75
11[b]	4	0	0	2.00	0.75
12[b]	6	0	0	2.00	0.75
13[b]	11	0	0	2.00	0.75

[a] Assay 3 is a replicate of the assay 2.
[b] Assays 10, 11, 12 and 13 are replicates of the central point.

degree was determined using the peak integrals obtained. The chitosan acetylation degree was determined from the signal integrals corresponding to $–CH_3$ of the acetamide group (H-8) at 1.8 ppm and that of the H-1 proton (Fig. 1). The substitution degree of *N*-carboxybutylchitosan was determined from the signal integral corresponding to $–CH_2$ group closest to carboxyl group of the butyl substituent (H-10) at 2.5 ppm whereas the substitution degree of 5-methylpyrrolidinone chitosan was obtained from the one corresponding to $–CH_3$ group of the cyclic substituent (H-13′) at 1.1 ppm.

2.4. Factorial design

Of all the factors that can effect the substitution degrees of *N*-carboxybutylchitosan and 5-methylpyrrolidinone chitosan preliminary experiments showed that the molar ratios of levulinic acid and sodium borohydride relative to chitosan appear to be the most important.

A 3^2 full factorial design with replicated center point was carried out in order to study how the substitution degrees depend on these molar ratios. This design permits determining both linear and quadratic models for substitution degree as well as determining their accuracies by comparing lacks of fit of model predictions to experimental points with experimental error estimated from replicates at the central point. Response surfaces can be graphed for statistically significant models with insignificant lacks of fit at desired confidence levels. These can be used to find optimum values of levulinic acid and sodium borohydride quantities for substitution degree and to plan new experiments expected to increase substitution degree even more.

3. Results and discussion

The dissolution of chitosan is certainly due to protonation by levulinic acid; however, in the 3.7–4.5 pH range, ketimine formation is expected to take place (Muzzarelli et al., 1993, 1989).

When chitosan is in the presence of an excess of levulinic acid, it dissolves and the respective ketimine is formed, which is then reduced to form the *N*-carboxylated derivative or 5-methylpyrrolidinone chitosan (Rinaudo et al., 2001), depending on reaction conditions.

Table 1 contains details of the experiments carried out for the 3^2 factorial design. Levulinic acid and sodium borohydride levels are given for each experiment, in real and scaled values, as well as the chronological order in which the experiments were run. The corresponding signal integrals and the substitution degrees for *N*-carboxybutylchitosan and 5-methylpyrrolidinone chitosan are given in Table 2.

Table 2
Integrals of ^{1}H NMR spectra signals used to determine the substitution degree of *N*-carboxybutylchitosan (CBC) and 5-methylpyrrolidinone chitosan (MPC)

Assay	Integral[a]			Degree of substitution	
	H-1[b]	H-10	H-13′	CBC	MPC
1	1.0000	0.5494	0.2721	27.47	9.070
2	1.0000	0.5310	0.0536	26.55	1.787
3	1.0000	0.4372	0.0847	21.86	2.823
4	1.0000	0.0339	0.1781	1.695	5.937
5	1.0000	0.4261	0.1629	21.31	5.430
6	1.0000	0.1180	0.2695	5.900	8.983
7	1.0000	0.4502	0.1658	22.51	5.527
8	1.0000	0.5332	0.1433	26.66	4.777
9	1.0000	0.2817	0.3651	14.09	12.17
10	1.0000	0.4424	0.1227	22.12	4.090
11	1.0000	0.6685	0.2006	33.43	6.687
12	1.0000	0.4571	0.1585	22.86	5.283
13	1.0000	0.5458	0.2234	27.29	7.447

[a] Each of the integrals is expressed as the average of three measurements of the same spectra.
[b] The H-1 proton signal was given a unit value since it was used as a reference to calibrate the integration procedure.

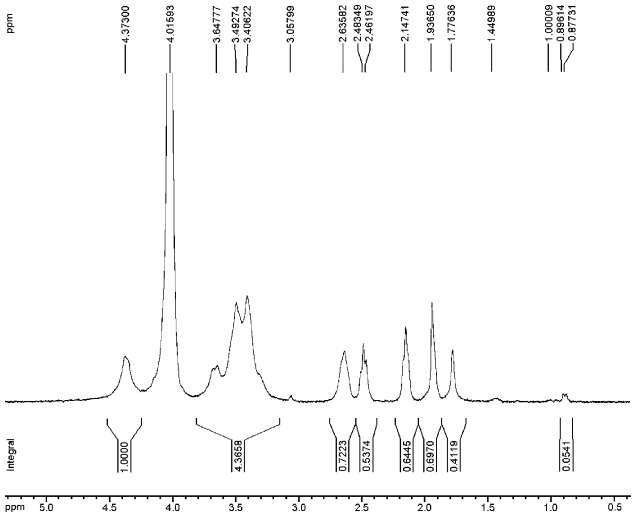

Fig. 2. ^{1}H NMR spectrum for *N*-carboxybutylchitosan (assay 2).

The ^{1}H NMR spectrum given in Fig. 2 was obtained for assay 2, which represents the best substitution degree obtained for *N*-carboxybutylchitosan (*N*-carboxybutylchitosan/5-methylpyrrolidinone chitosan ratio is 14.9). The signals at 2.4 and 2.1 ppm correspond to H-10 and H-11, respectively. The methyl group (H-13) is represented by the signal at 1.9 ppm. The small signal at 0.8 ppm, which represents the methyl group of the cyclic derivative (H-13′), demonstrates the presence of traces of 5-methylpyrrolidinone chitosan. The best substitution degree for the cyclic derivative (*N*-carboxybutylchitosan/5-methylpyrrolidinone chitosan ratio is 0.29) was obtained for assay 4 (Fig. 3). The signal at 1.1 ppm, which describes the methyl group of 5-methylpyrrolidinone chitosan (H-13′), is significantly higher than the corresponding signal in assay 2. The other signals are attributed to chitosan (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996): 4.5 ppm (H-1), 3.5 ppm (H-2 of *N*-acetyl-glucosamine, H-3, H-4, H-5 and H-6), 2.8 ppm (H-2 of glucosamine) and 1.8 ppm (H-8).

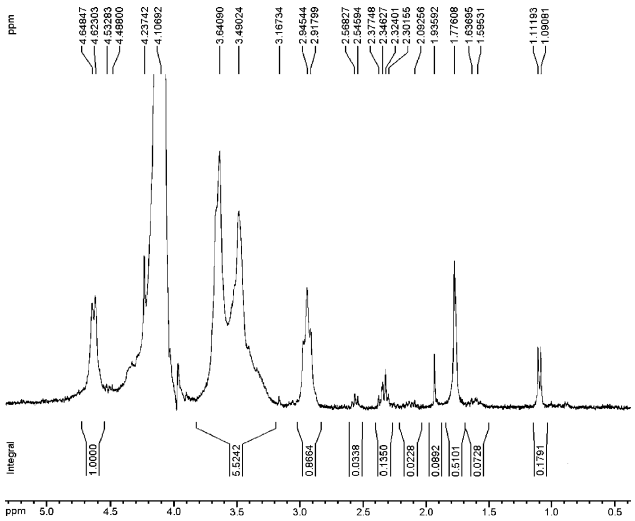

Fig. 3. ^{1}H NMR spectrum for 5-methylpyrrolidinone chitosan (assay 4).

Table 3
Analysis of variance table for substitution degrees of *N*-carboxybutylchitosan

Variation source	SS	ν	MS	F calculated	F critical
Regression	764.0223	5	152.8045	5.6066	3.97
Residuals	190.7819	7	27.2546		
Lack of fit	98.7234	3	32.9078	1.4299	6.59
Pure error	92.0585	4	23.0146		
Total	954.8042	12			

% of explained variance: 80.02; maximum % of explainable variance: 90.36.

Linear and quadratic models for each substitution degree of *N*-carboxybutylchitosan and 5-methylpyrrolidinone chitosan as a function of levulinic acid and sodium borohydride molar ratios were determined. For *N*-carboxybutylchitosan a quadratic model

$$\mathrm{SD} = \underset{(\pm 2.18)}{24.66} + \underset{(\pm 1.87)}{5.55}\, x_1 - \underset{(\pm 1.87)}{6.93}\, x_2 - \underset{(\pm 2.90)}{6.92}\, x_1^2 - \underset{(\pm 2.90)}{0.75}\, x_2^2 + \underset{(\pm 2.23)}{5.63}$$

was found to be superior to the linear one. The analysis of variance table for this regression is given in Table 3. The $\mathrm{MS_{lack\ of\ fit}}/\mathrm{MS_{pure\ error}}$ ratio of 1.43 is smaller than the 95% critical $F_{3,4,95\%}$ value of 6.59 showing that this model does not have significant lack of fit at this confidence level. Furthermore, the $\mathrm{MS_{regression}}/\mathrm{MS_{residual}}$ ratio of 5.61 is larger than the tabled $F_{5,7,95\%}$ value of 3.97 indicating that the regression is statistically significant at the 95% confidence level. Both the gradients for x_1 and x_2 are significant at this confidence level whereas the x_1^2 curvature is significant at the 90% level. The other

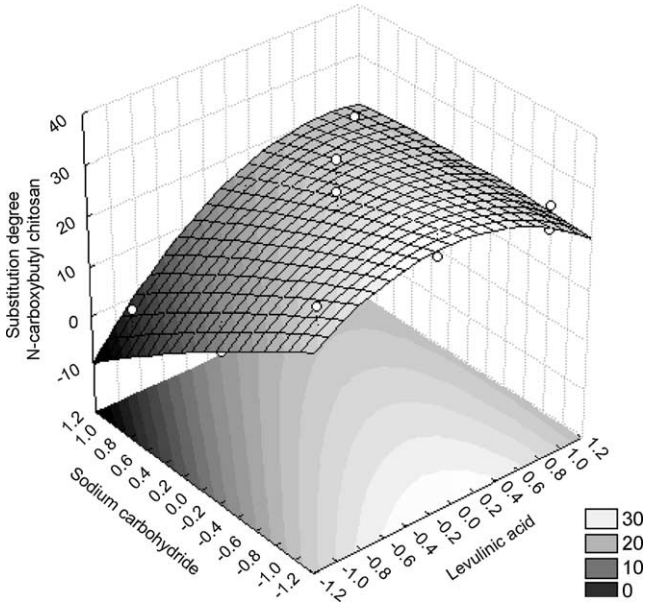

Fig. 4. Quadratic model response surface for *N*-carboxybutylchitosan substitution degree as function of levulinic acid/chitosan and sodium borohydride/chitosan molar ratios.

Table 4
Analysis of variance table for substitution degrees of 5-methylpyrrolidinone

Variation source	SS	ν	MS	F calculated	F critical
Regression	43.1940	2	21.5970	4.4028	4.10
Residuals	49.0532	10	4.9053		
Lack of fit	41.8377	6	6.9730	3.8655	6.16
Pure error	7.2155	4	1.8039		
Total	92.2472	12			

% of explained variance: 46.82; maximum % of explainable variance: 92.18.

terms in the model are not highly significant. The response surface for this model is given in Fig. 4 and shows that maximum substitution degrees for *N*-carboxybutylchitosan are expected for low sodium borohydride and intermediate levulinic acid levels.

A linear model

$$SD_{MPD} = \underset{(\pm 0.38)}{6.44} - \underset{(\pm 0.52)}{1.86}\, x_1 + \underset{(\pm 0.52)}{1.44}\, x_2$$

most adequately represents the 5-methylpyrrolidinone chitosan substitution degree. The analysis of variance for this model is given in Table 4. An $MS_{lack\ of\ fit}/MS_{pure\ error}$ ratio of 3.87 compared with the $F_{6,4,95\%}=6.16$ critical value indicates that this model does not have significant lack of fit at the 95% confidence level. Furthermore, the $MS_{regression}/MS_{residual}$ ratio of 4.40 is slightly larger than the $F_{2,10,95\%}=4.10$ critical value and is statistically significant at the 95% confidence level. The response surface for this model is shown in Fig. 5. Even though high lacks of fit are observed for two experimental points with low levulinic acid and high sodium borohydride levels in this figure it does seem clear that low 5-methylpyrrolidinone chitosan degrees of substitution are expected for high levulinic acid and low sodium borohydride levels.

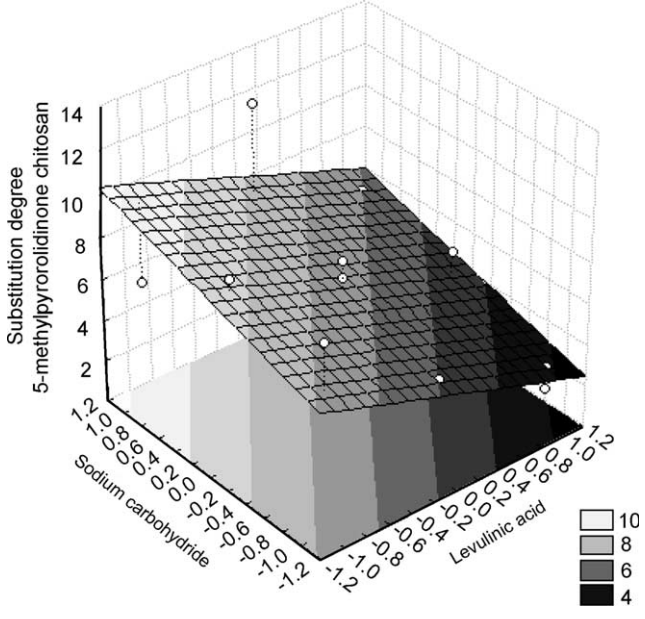

Fig. 5. Linear model response surface for 5-methylpyrrolidinone substitution degree as function of levulinic acid/chitosan and sodium borohydride/chitosan molar ratios.

4. Conclusions

The 3^2 factorial design and response surface analysis are found to be efficient tools for the optimization of *N*-carboxybutylchitosan synthesis. A quadratic response surface for *N*-carboxybutylchitosan substitution degree predicts that maximum substitution occurs for a levulinic acid/chitosan molar ratio of 2.00 and a sodium borohydride/chitosan ratio of 0.50. A linear response surface for 5-methylpyrrolidinone chitosan predicts minimum substitution degrees for levulinic acid/chitosan and sodium borohydride/chitosan molar ratios of 3.00 and 0.50, respectively. Of the nine different experiments performed the two resulting in highest yields of *N*-carboxybutylchitosan and lowest yields of 5-methylpyrrolidinone occur for 2.00 and 3.00 levulinic acid/chitosan molar ratios and a 0.50 sodium borohydride/chitosan molar ratio.

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