Carbohydrate Polymers 92 (2013) 564–570

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

The interaction of chitosan and olive oil: Effects of degree of deacetylation and degree of polymerization

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ARTICLE INFO

Article history:
Received 23 August 2012
Received in revised form
18 September 2012
Accepted 20 September 2012
Available online 28 September 2012

Keywords:
Chitosan
Degree of deacetylation
Degree of polymerization
pls
Chemometrics
Oil-binding capacity

ABSTRACT

The combined effects of degree of deacetylation (DD) and degree of polymerization (DP) on the ability of chitosan to interact with olive oil was studied. The oil-binding test, a method that makes use of olive oil as a representative fat, was adopted as a measure of the interaction of chitosan and olive oil. The oil-binding capacities of twelve chitosan samples with DPs ranging from 470 to 1450 and DDs of 75% to 95% were determined. The oil-binding capacities were then correlated to the DD and DP using partial least squares (PLS) regression. The generated PLS model had a root mean square error of prediction (RMSEP) of 9.1%. Results indicated that oil-binding capacity is a function of DD more than of DP. For chitosan with DD at the interval 50% < DD < 90%, a negatively sloped linear correlation was obtained for DD and oil-binding capacity suggesting that hydrophobic intermolecular forces of attraction dominates the interaction of chitosan with olive oil. For chitosan with DD > 90%, the observed deviation from the linear correlation increased. In this interval, free fatty acid anions facilitate the interaction of chitosan and olive oil. Free fatty acids form a stable ionic interaction with the former and a strong hydrophobic interaction with the latter.

1. Introduction

Chitosan has been widely used for a variety of purposes. Commercially available chitosan is mainly obtained from chitin, an abundant carbohydrate polymer (Aranaz et al., 2009; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Chitin is composed mainly of N-acetyl-D-glucosamine monomer units and the rest are D-glucosamine monomers linked by β-D-(1 → 4) glycoside bonds. Alkali-catalyzed deacetylation of N-acetyl-D-glucosamine in chitin leads to the formation of more glucosamine monomers. The extent of this reaction of chitin is described by the property degree of deacetylation (DD). DD is the ratio (usually expressed in percent) of the amount (in moles) of D-glucosamine units to the total D-glucosamine and N-acetyl-D-glucosamine units. Chitosan is formed when the DD reaches a certain value (Kasaai, 2009). For example, chitosan contains a minimum of 60% D-glucosamine units (Aiba, 1992).

Chitosan is a mixture of homologues at a wide range of degree of polymerization (DP) and molecular weight (MW). Different average molecular weight parameters are often used to describe the average size of the polymer depending on the method used to estimate them. Weight-average molecular weight (M_w) and number-average molecular weight (M_n) are estimated using gel permeation chromatography (GPC) while viscosity molecular weight (M_v) is derived from the measurement of the intrinsic viscosity of the polymer solution in a particular solvent. The use of GPC in polymer analysis especially for polycations like chitosan can be complex. A GPC method that uses neutral stationary phase and a high ionic strength, acidic mobile phase was shown to be robust for the determination of the molecular weight of chitosan (Bernhard, Flato, Dimzon, Hofe, & Knepper, submitted for publication).

Both the DD and DP are basic physico-chemical properties on which the other properties of chitosan depend. For example, the DP and DD influence the viscosity of chitosan solutions. The Mark–Houwink equation (Eq. (1)) shows that the intrinsic viscosity ($[\eta]$) of a polymer solution changes with the molecular weight. A study by Wang, Bo, Li, and Qin (1991) showed that for chitosan samples of the same DD, the logarithm of intrinsic viscosity is a linear function of the logarithm of M_w, consequently is directly proportional to the logarithm of DP. On the other hand, DD influenced the Mark–Houwink constants k and α (Wang et al., 1991) and the non-Newtonian flow properties of chitosan solutions (Wang & Xu, 1994).

$$[\eta] = kM^{\alpha} \quad (1)$$

DD and DP also influence chitosan solubility in aqueous solutions. Chitosan solubility in water increases with the decrease in

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http://dx.doi.org/10.1016/j.carbpol.2012.09.035

the DP and with the increase in DD. The ability of the amine group to gain a positive charge in acidic environment makes the deacetylated chitosan easily dissolve in aqueous acidic medium (Qin et al., 2006; Sannan, Kurita, & Iwakura, 1976; Vårum, Ottøy, & Smidsrød, 1994). It was also reported that chitosan behaviors in aqueous solutions of high ionic strength like refractive index and gyration radius are functions of DD. The interactions of chitosan in aqueous solutions can be divided into three domains based on the DD: (1) at DD > 80%, electrostatic interaction predominates; (2) at 50% < DD < 80%, hydrophobic and hydrophilic interactions are counter balanced; and (3) at DD < 50%, stable aggregates are favored (Schatz, Viton, Delair, Pichot, & Domard, 2003). Additionally, chitosan in dilute aqueous solutions has a semi-flexible rod conformation (Morris, Castile, Smith, Adams, & Harding, 2009). The rigidity in the structure of chitosan and chitin is mainly due to the interchain H bonds (Muzzarelli, 2012).

One of the recent uses of chitosan is as an active ingredient in "fat-binder" tablets or anti-obesity supplements (Woodgate & Conquer, 2003). It is claimed that chitosan has the ability to "bind" with fatty substances in the intestines making them unavailable for metabolism and they are consequently excreted. Many studies substantiate these claims. In vivo studies show that chitosan has hypocholesterolemic effect in humans (Bokura & Kobayashi, 2003; Maezaki et al., 1993) and in other animal subjects such as mice or rats (Bokura & Kobayashi, 2003; Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Liu, Zhang, & Xia, 2008). Chitosan was also associated with the increase in fecal fat (Deuchi, Kanauchi, Imasato, & Kobayashi, 1995; Deuchi, Kanauchi, & Kobayashi, 1994; Gallaher et al., 2000; Han, Kimura, & Okuda, 1999; Kanauchi, Deuchi, Imasato, Shizukuishi, & Kobayashi, 1995) and fecal bile acid (Gallaher et al., 2000) excretion. In vitro studies, on the other hand, reveal that the interaction of chitosan with triacylglycerides contained in olive oil, is highly influenced by the physico-chemical properties of the former. These studies rely mainly on measuring the oil-binding capacity (called fat-binding capacity in some papers) of chitosan. In the test, olive oil is made to form a stable complex with a test substance under a simulated gastrointestinal condition (Zhou, Xia, Zhang, & Yu, 2006). The unbound oil is separated from the stable complex and is measured. The difference between the initial amount of oil added and the measured amount of unbound oil gives the amount of the oil bound to the complex and is taken as a measure of the oil-binding capacity (Cho, No, & Meyers, 1998). It was shown that the oil-binding capacity of chitosan is negatively related to its bulk density and is directly related to viscosity (Cho et al., 1998). It was also shown that for a series of chitosan with increasing MW but constant DD, highest oil binding was achieved in an optimal MW (No, Lee, Park, & Meyers, 2003). Despite the efforts, however, only a number of significant correlations have been demonstrated (Aranaz et al., 2009). This can be due to the fact that the mechanism of interaction of triacylglyceride and chitosan is a complex process.

In this paper we investigate the effects of DD and DP on the interaction of chitosan with olive oil. To achieve this, DD, DP and oil-binding capacity of different chitosan samples should be determined. Partial least squares (PLS), novel chemometric technique, are used to elucidate the relationships.

2. Materials and methods

2.1. Chitosan samples and reagents

The chitosan samples (labeled S1–S10 in this paper) used to derive the chemometric model were from Heppe Medical Chitosan (HMC) GmbH (Halle an der Saale, Germany). The DD of the samples were known and were shown in the certificates of analysis (COA). DD ranged from 73.6% to 94.3% (see Table 1). The MW of the chitosan samples written in the COA was approximate values. GPC was used to derive the M_w.

Table 1
Degree of deacetylation and weight-average degree of polymerization of the chitosan samples. Data for the degree of deacetylation of S1–S10 were directly lifted from the certificate of analysis while for U1 and U2 were obtained experimentally using FT-IR and partial least squares.

Sample code	DD (%)	DP_w
S1	94.3	474
S2	73.6	589
S3	91.8	583
S4	89.7	802
S5	84.7	905
S6	94.2	912
S7	78.1	1076
S8	89.4	987
S9	93.4	1063
S10	92.7	1440
U1	83.8	542
U2	82.2	539

Two chitosan samples of unknown DP and DD from Certmedica International GmbH (Aschaffenburg, Germany) were also analyzed (labeled U1 and U2 in this paper). Fourier transform-infrared (FT-IR) spectroscopy with the Heppe chitosan samples as standards was used to determine the DD of the unknown. GPC was used to derive the M_w.

The extra virgin olive oil (Villa Gusto, Italy) used was purchased from a German supermarket. The oil was submitted to Prof. Dr. Georg Kurz GmbH (Cologne, Germany) for determination of fatty acid distribution and free fatty acids. Analytical reagent grade acetic acid, sodium acetate, sodium chloride, potassium chloride, potassium dihydrogenphosphate and trifluoroacetic acid (TFA) were from Carl Roth (Karlsruhe, Germany). The analytical reagent grade disodium hydrogenphosphate was from Riedel-de Haën (Seelze, Germany). Ultrapure water (Milli-Q water) was prepared by a Milli-Q system with Simpak2 ion exchanger (Millipore, Milford, MA, USA).

2.2. Determination of weight-average degree of polymerization (DP_w)

A robust GPC method was used to estimate the MW of the Heppe chitosan and unknown samples (Bernhard et al., submitted for publication). The polymers were previously dissolved (0.5%, w/v) in 0.3 M acetate buffer (0.2 M CH_3COOH and 0.1 M $Na^+CH_3COO^-$). Chromatographic separation was carried out in Novema 3000 Å and Novema 300 Å dual column (both columns have: internal diameter, 8 mm; length, 30 cm; particle size, 10 μm; material, OH-functionalized methacrylatecopolymer; Polymer Standards Service, Mainz, Germany) using 0.2% trifluoroacetic acid (TFA) (v/v) in 0.3 M aqueous sodium chloride (NaCl) solution as eluent. Detection of the polymers was done in a refractive index detector. The M_w's, were then estimated using the absolute retention time of polyvinyl pyridine (PVP) molecular weight standards (PSS, Mainz, Germany) dissolved in 0.5% TFA (v/v) in 0.3 M aqueous NaCl. The determinations were done in duplicates.

The DP_w was estimated from the M_w and DD using Eq. (2).

$$DP_w = \frac{M_w - 18}{204 - (43 \times DD)} \quad (2)$$

2.3. Determination of DD

FT-IR spectroscopy was used to determine the DD of U1 and U2. Chitosan S1–S10 were used as DD standards. The chitosan samples (S1–S10, U1 and U2) were initially dried at 105 °C for at least

one hour. Five to ten milligram portion of chitosan was mixed and homogenized with 100 mg KBr. The mixture was then made into a pellet using a hydraulic press. The IR spectrum at the wavenumber region 4000–600 cm^{-1} was then obtained using Perkin Elmer Spectrum Bx FTIR System. The IR spectrum of a blank KBr pellet was used as background. The resulting IR spectrum is the average of 10 scans at a resolution of 4000.

The IR data files of the absorbance spectra were extracted as ASCII files using the Spectrum v5.0.1 software. The absorbances within the region 1800–1500 cm^{-1} were used to generate a calibration and to determine the DD of the unknown using partial least squares (PLS). Prior to PLS, the absorbance data were corrected for the baseline and were normalized against the highest absorbance using Microsoft Excel™. The analysis using PLS is discussed in Section 2.5.

2.4. Oil-binding test

The oil-binding capacity of chitosan was determined using a commonly used technique (Zhou et al., 2006) with some modifications. A 0.125–0.250 g chitosan was dissolved in 25 mL 0.1 M HCl. Then, an aliquot was mixed with 11.67 g olive oil. The mixture was stabilized in a water bath for 2 h at 37 °C after pH adjustment to 2.0 using VWR pH 100 pH meter (VWR International, USA) with Schott Instrument Blueline electrode (SI Analytics, Germany). A 10 mM phosphate buffer saline solution was then added and the pH was adjusted to 7.0. The resulting mixture was stabilized in the water bath for 30 min at 37 °C. The mixture was then centrifuged at 2000 rpm speed and 25 °C for 20 min. The amount of unbound oil is weighed and the oil-binding capacity is calculated in percentage relative to the initial amount of olive oil added. The determination was done two to eight times per sample for Heppe chitosan and in duplicate for the unknown chitosan.

2.5. Statistical analysis

The free software, 'R' (The R Foundation for Statistical Computing) was used in the statistical evaluation of the results. The 'pls' package was used to perform partial least squares (PLS) analysis on the derived data (Bjorn-Helge & Wehrens, 2007). PLS was used in two ways in this study.

First, PLS was used to determine the DD of U1 and U2. The 'plsr' function was performed on the calibration dataset (absorbances of S1–S10 at the region 1800–1500 cm^{-1}) using the default kernel algorithm and with the number of components set to a maximum of eight. Internal validation using the leave-one-out (LOO) technique was done. Based on the calculated root mean square error of prediction (RMSEP), PLS prediction was done with the optimal number of components.

Secondly, PLS was also used to investigate the interaction of chitosan and olive oil as affected by DD and DP. The oil-binding capacities of the chitosan samples were tabulated against the DD and DP. Ten datasets (S1–S10) were initially selected to be the training set while the two remaining datasets (U1 and U2) were selected to be the test set. All datasets were scaled prior to analysis. The 'plsr' function was performed on the training set using the default kernel algorithm and with the number of components set to a maximum of two. Internal validation using the LOO technique was done and results were evaluated by calculating the RMSEP.

The 'scatterplot3d' package, also from 'R' was used to generate the 3D plot to visualize the combined effects of DP and DD on the oil-binding of chitosan. All data pre-treatments were done using Microsoft Excel™ (v. 2007).

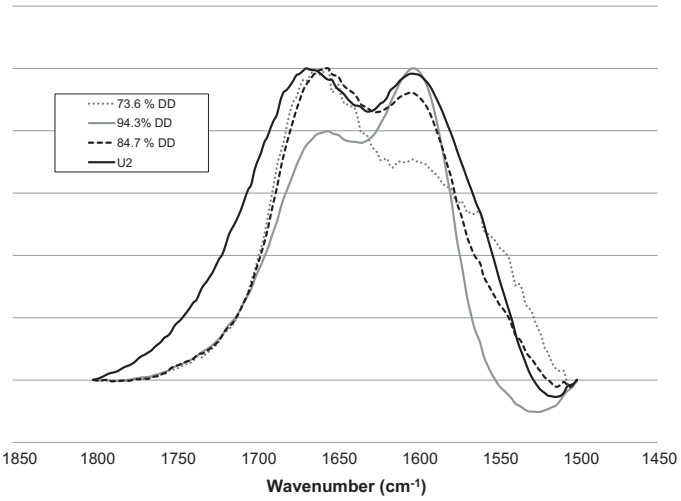

Fig. 1. FT-IR absorption spectra (normalized to relative to the highest absorbance) at the wavenumber region 1800–1500 cm^{-1} of chitosan samples with known degree of deacetylation (73.6%, 94.3% and 84.7%) and of a chitosan sample with unknown degree of deacetylation (U2). The spectra were corrected for the baseline drawn from 1800 to 1500 cm^{-1} and were normalized relative to the highest corrected absorbance.

3. Results and discussion

3.1. DD and DP_w of chitosan

Chitosan samples S1–S10 were pure chitosan samples with respective COA specifying the DD of the polymers. The DD values are summarized in Table 1. Chitosans S1–S10 were also used as calibration standards to determine the DD of U1 and U2 with FT-IR in combination with PLS chemometric technique. Traditionally, in determining the DD using FT-IR, the absorbances of chitosan standards with known DD at two wavenumbers are selected. One is the characteristic wavenumber which is related to the amide vibrations and can be correlated to the number of the N-acetyl-D-glucosamine monomers. The other is the reference wavenumber which is related to either the hydroxyl groups or the bridge oxygen–carbon bond in the main pyranose ring and can be correlated to the total number of N-acetyl-D-glucosamine and D-glucosamine monomers. The absorbance ratio of the characteristic wavenumber to the reference wavenumber is plotted against DD generating a negatively sloped linear calibration curve that can be used to determine the DD of an unknown chitosan (Brugnerotto et al., 2001; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996).

An alternative method is to consider the whole amide absorption band and correlate the changes in its shape with the change in DD. Fig. 1 shows how the infrared absorption band shape in the wavenumber region 1800–1500 cm^{-1} varies with DD. The absorption at approximately 1660 cm^{-1} is due to the amide I bond vibrations (C=O stretching). Only the N-acetyl-D-glucosamine monomers will exhibit the absorption, thus, as DD is increased, the absorption at this wavenumber is decreased relative to the neighboring band. This creates a change in the shape of the normalized bands. By visual inspection, one can easily deduce that the U2 has a DD between 84.7% and 94.3%.

The PLS method provides a way of estimating more accurately the DD of the unknown samples. PLS was specifically chosen from a number of other multivariate regression techniques like the principal component regression (PCR) because of its robustness to factors with high variance but not at all correlated to the dependent property (Bjorn-Helge & Wehrens, 2007; Vandeginste et al., 1998). In this chemometric technique, the absorptions of the individual wavenumber within the infrared spectral region 1800–1500 cm^{-1}

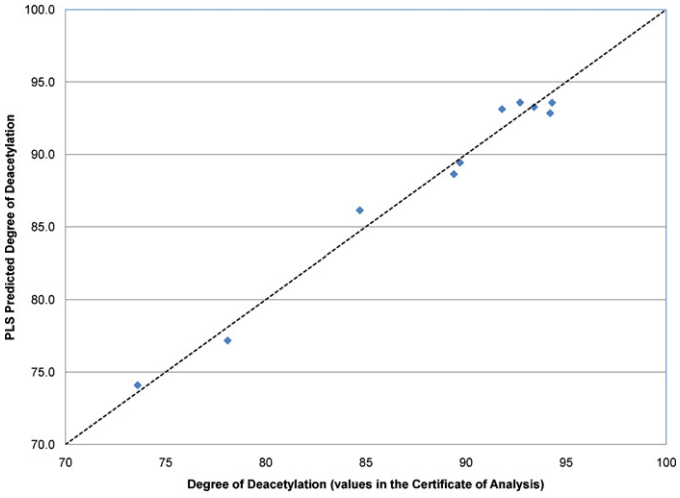

Fig. 2. External validation of the partial least squares (PLS) model to predict the degree of deacetylation (DD) of chitosan. The dotted line represents: certified DD = PLS – predicted DD.

are taken as unique variables. Through the algorithm specified in the PLS package for R (the default Kernel algorithm was used (Bjorn-Helge & Wehrens, 2007)), the number of original variables are reduced to only a few components (latent variables). Features reduction is done by linear combination of the original variables (Massart et al., 1998). Characteristic of any algorithm used in this family of techniques, the first component is always chosen so that it explains the greatest variation in the spread of data points, component 2 explains the 2nd greatest variation and so on. In the end all the components should be able to explain 100% of the variations in the data. One or more of these components can be used to generate the 'scores' that have a linear relationship with the property being predicted, thus can be used as *x*-variables in a regression model.

In the analysis, the 'plsr' function generated a PLS model with 3 components. To find out which of the 3 components or their combinations have a linear relationship with DD, model validation was simultaneously performed using the LOO validation technique. In LOO, one sample is taken out and the model is generated using the remaining samples. The one taken out is then used as a test sample. This process is repeated until a substantial amount of validation points are gathered. The predicted values of the validation points are then compared to the experimental or labeled values. The RMSEP can be calculated based on the difference between the predicted and experimental values. In this study, results revealed that if only the first component was taken into account in the prediction, the RMSEP was 2.35%. This value was lowered to 1.81% if the first and second components were taken. The value was increased again if the rest of the components were added. This implies that two is the optimum number of components in the regression to predict DD. Fig. 2 shows the external validation using the PLS model with only two components. The low deviation from the dotted line (representing values in the COA = PLS – predicted values) is consistent with the computed RMSEP of 1.78%. The model was thus used to predict the DD of U1 and U2. The results gave 83.8% and 82.2% DD for U1 and U2 respectively (also shown in Table 1). The relative standard deviation was below 3% for the two to four trial runs.

The COA showed only approximate values for the MW. It was thus necessary to determine experimentally the M_w by GPC. Most of the chitosan samples are only partially soluble in the mobile phase used. It was thus necessary to use another solvent system to dissolve the chitosan with. The use of 0.3 M acetate buffer as a solvent system for chitosan was shown to give an M_w that is not statistically different from the M_w if the mobile phase 0.2% TFA

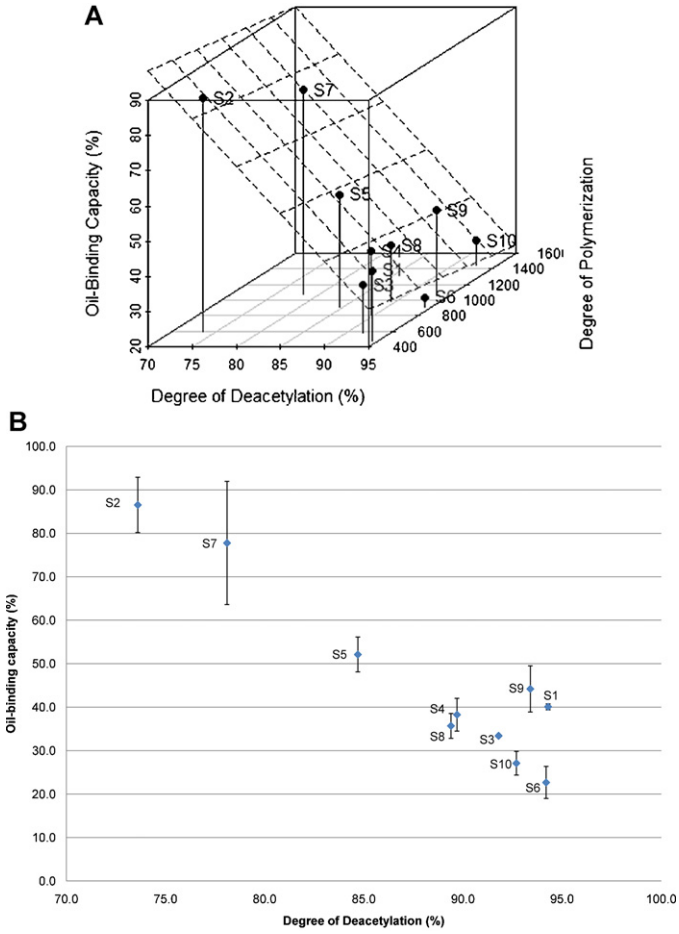

Fig. 3. Relationship between weight-average degree of polymerization (DP_w), degree of deacetylation (DD) and oil-binding capacity. Shown are: (A) 3D-graph showing the combined effects of DP_w and DD on the oil-binding capacity; and (B) the 2D graph representing the projection on the DD versus oil-binding capacity plane. The error bars represent ±1 standard deviation.

in 0.3 M NaCl in Milli-Q water is used (Bernhard et al., submitted for publication). From the M_w and DD data, the DP_w of S1 to S10 was calculated using Eq. (2). The calculated DP_w of the samples are summarized in Table 1.

3.2. Oil-binding capacity of chitosan

The oil-binding capacity measures the ability of chitosan to interact with oil. To do this, chitosan is made to form a stable emulsion complex with olive oil. The excess olive oil (unbound oil) is separated from the stable complex and is measured gravimetrically.

The CODEX standard for olive oils and olive pomace oils defines virgin olive oils as the "oils obtained from the fruit of the olive tree solely by mechanical or by physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decanting, centrifuging and filtration" ("CODEX standards for olive oils and olive pomace oils CODEX STAN 33-1981", 1981). Results from Prof. Dr. Georg Kurz GmbH, a third party laboratory showed that the fatty acid content of the olive oil used in the study had the following distributions: palmitic acid, 9.9%; stearic acid, 3.3%; oleic acid, 79.1%; linoleic acid, 4.7%; and the rest of the fatty acids are less than 1%. The oil also contains 0.39% (expressed as %oleic acid) free fatty acid. These results are within the criteria set by CODEX Alimentarius (1981). The sterol content of the olive oil was not determined although CODEX specifiess that virgin olive

Table 2
Oil-binding capacity of the chitosan samples.

Sample code	Oil-binding capacity (%)	
	Value	RSD
S1	40.1	2
S2	86.5	7
S3	33.4	1
S4	38.3	10
S5	52.1	8
S6	22.7	16
S7	77.8	18
S8	35.7	8
S9	44.2	12
S10	27.1	10
U1	62.8	2
U2	69.0	1

oil can contain a minimum of 1000 μg/g sterols composed mostly of β-sitosterol, δ-5-avenasterol, δ-5-23-stigmastadienol, clerosterol, sitostanol and δ-5-24-stigmastadienol (CODEX Alimentarius, 1981). In a separate study using phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy, it was found out that olive oils can contain <0.20% 1-monoglycerides and <4.00% total 1,2- and 1,3-diglycerides (Spyros & Dais, 2000). Given these, it is safe to say that the olive oil used in this study is greater than 95% triacylglyceride with mostly C18 unsaturated and C16 saturated fatty acids.

The reported oil-binding capacity is expressed in percent. Based on its initial definition, oil-binding capacity does not consider the variable initial amount of chitosan used. To allow comparison, the results were normalized to 4.15 mg initial amount of chitosan and to 11.67 g olive oil used. The results of the oil-binding test are given in Table 2.

The oil-binding capacity values represent the means and relative standard deviation (RSD) of 2–8 trial runs. It can be observed that some of the RSD of the data is up to almost 20%. The relatively low precision of the oil-binding test results from the low ruggedness of the method used. The neutral pH to which the chitosan–olive oil complex is to be stabilized was difficult to control.

To study the relationship of DP_w and DD versus oil-binding capacity, the values for chitosan S1–S10 were put in a graph shown in Fig. 3. Fig. 3A represents in a 3D graph the general relationship between the variables DP_w and DD versus oil-binding capacity. A regression plane was drawn to visualize the general trend of the data. It can be observed that DD has a greater effect on the oil-binding capacity than DP_w as indicated by the steepness and direction of the slope of the regression plane. This observation is highlighted in the 2D graph representing the projections of the points on the DD versus oil-binding capacity plane in Fig. 3B. The error bars shown in the figure represent ±1 standard deviation. There is a clear negatively sloped linear relationship between DD and oil-binding capacity despite the samples having a wide range of DP.

3.3. Evaluation of the results using PLS

PLS was used to correlate DD and DP_w to oil-binding capacity. Initially, PLS was generated using the S1–S10 as the training set. The kernel algorithm used with the 'plsr' function fitted the training set with only two variables (DD and DP_w), and the data was pre-treated by scaling prior to analysis (Bjorn-Helge & Wehrens, 2007). Cross-validation of the generated model using the LOO had 9.6% RMSEP if only one component was used and 9.1% if two components were used. The sources of the large prediction errors were the data coming from samples with low oil-binding capacities. The model with two components can only explain 89.2% of the variations in oil-binding capacity.

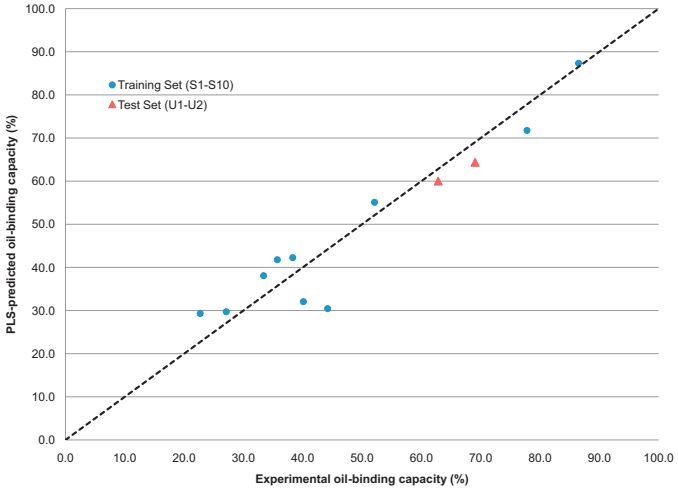

Fig. 4. External validation of the partial least square (PLS) model to predict the oil-binding capacity of chitosan. The dotted line represents: experimental values = PLS – predicted values.

The ability of the regression model to predict the oil-binding of chitosan given only the information on its DD and DP was tested using U1 and U2. Fig. 4 shows the correlation between the experimental oil-binding capacity and the predicted oil-binding capacity for both the training set and the test set using the PLS model. The data points are scattered around the line experimental oil-binding capacity = predicted oil-binding capacity. The model's prediction of the training set is consistent with the result of the internal validation. The calculated %residuals for the test sets were −4.5% and −6.8% for U1 and U2 respectively. The accuracy of the predicted value can only be as good as the precision of the experimental results used to derive the model.

3.4. Interaction of chitosan and olive oil

The oil-binding capacity can be taken as a measure of the stability of emulsion of chitosan and olive oil in an aqueous environment at neutral pH. The olive oil used in the study is a mixture of at least 95% triacylglycerides (based on estimation) and the rest are diacylglycerides, monoacylglycerides and free fatty acids. The behavior of chitosan of a specific DD and DP under the conditions stated dictates its interaction with the glycerides and fatty acids. This can be explained by a number of mechanisms proposed in the literature (Aranaz et al., 2009).

It was shown that chitosan is capable of direct hydrophobic interaction with nonpolar molecules like cholesterol in the air–water interface and can directly eliminate them from aqueous solution. Simulation studies indicated that there are specific sites for interactions between chitosan and stearic acid or cholesterol. Results from a number of experiments show that cholesterol in aqueous (Liu et al., 2008) and in nonpolar solvents such as chloroform (Parra-Barraza et al., 2005) can interact with chitosan. The increase in chain length (greater DP) increases the capacity of the chitosan for van der Waals interaction and thus the ability to bind with fat. However, for large polymers, conformation and folding in a complex aqueous system can decrease the interactions. It was shown in an in vitro study that at constant DD, high molecular weight chitosan adsorbed less cholesterol. In the same study, however, the effect of DD was unclear (Liu et al., 2008). Zhou et al. (2006) also found the same trend for molecular size and the adsorption of olive oil by chitosan, however they have not found significant correlations between physico-chemical properties like DD and oil-binding capacity. The hydrophobic interaction of chitosan with oil is not necessarily a one-to-one interaction. Chitosan was shown to

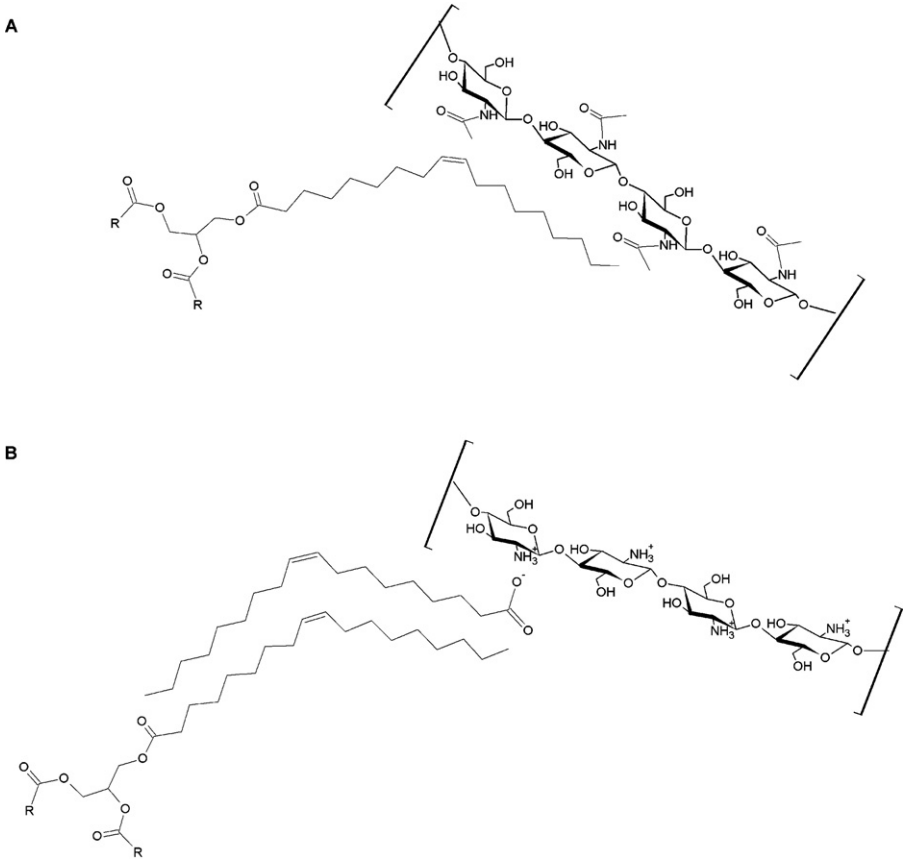

Fig. 5. Interaction of chitosan with olive oil. Shown is a representative triacylglyceride of olive oil with an oleic acid and two other fatty acids represented by R. (A) Chitosan interacts directly with olive oil through the N-acetyl-D-glucosamine monomers; and (B) chitosan interacts indirectly with olive oil with the aid of free fatty acid (oleic acid) that is electrostatically bound to the amine group of the D-glucosamine monomer.

act as an emulsifier providing mechanical and electrostatic stability to the oil droplets resulting to a stable water–oil–water multiple emulsions (Payet & Terentjev, 2008; Pereda, Amica, & Marcovich, 2012; Schulz, Rodríguez, Del Blanco, Pistonesi, & Agulló, 1998).

A different mechanism suggests that chitosan, through the amino group of the glucosamine units, forms an insoluble salt by ionic interaction with bile acid anions (Muzzarelli et al., 2006). These anions are characterized by an ionic end on one side a hydrophobic region on the other. The resulting insoluble salt is hydrophobic and can trap fats like cholesterol and triacylglycerides. A similar mechanism suggests the formation of micelle-like structure with bile salts that can trap fats in the inside. This is made possible with the amino and hydroxyl groups of chitosan acting as an interface between the bile acid anion and the aqueous environment respectively (Thongngam & McClements, 2004). Like the bile acids, the free fatty acids in the oil can also interact with chitosan. A study on chitosan–lipid interactions in Langmuir monolayers suggests that fatty acids interact with chitosan in two steps: first is for the fatty acid to anchor in chitosan via electrostatic forces, then second hydrophobic interactions occur between the lipid tail of the fatty acid and chitosan forming the complex. The study noted that the bulkiness and saturation of the fatty acid can influence the interactions (Wydro, Krajewska, & Hąc-Wydro, 2007). This resulting complex of free fatty acid and chitosan can further interact with oils.

At first look, there seems to be a contradiction between the two mechanisms discussed above in explaining the interaction of chitosan and olive oil. However, after considering that chitosan behaves differently in aqueous environment depending on its DD (Schatz et al., 2003), one can conclude that the two mechanisms in reality, complement each other. Chitosan in the domain $50\% < DD < 80\%$ behave in such a way that the hydrophobic and hydrophilic interaction in aqueous environment are counterbalanced. This domain is the interface between the highly electrostatic domain and the hydrophobic domain (Schatz et al., 2003). In this context, the first mechanism is favored. Chitosan without any need for additional surfactant can act as an emulsifier and interact directly with the triacylglycerides in olive oil. Based on the PLS model in the previous section, DD has a negatively sloped linear correlation with oil-binding capacity suggesting that the chitosan–triacylglyceride interaction becomes stronger as the chitosan becomes acetylated and less polar. This model is consistent with the results of the study by Muzzarelli et al., where they percolated the olive oil in a column bed with chitin, chitosan or a chitosan derivative. The group found out that chitosan allowed more oil to be percolated through, while chitin on the contrary retained more of the olive oil. The authors also noted that the concentration of diacylglycerides (34 carbon and 36 carbon diacylglycerides) in the olive oil percolated in chitosan decreased compared with the original oil. On the other hand, the concentration of diacylglyceride was enriched in the percolated olive oil in chitin (Muzzarelli, Frega, Miliani, Muzzarelli, & Cartolari, 2000). The trend of oil-binding capacity decreasing with DD can be extended up to DD of 90% based on the PLS model. The PLS model points to hydrophobic interaction as the dominant intermolecular force of attraction that "binds" chitosan via the N-acetyl-D-glucosamine monomers and olive oil triacylglycerides (Fig. 5A).

At the region of very high DD ($DD > 90\%$) the negatively sloped linear correlation tends to be diminished. The amount of N-acetyl-D-glucosamine monomers limits the direct hydrophobic interaction of chitosan to olive oil triacylglycerides. It is inferred that on this region, the 2nd mechanism is favored. The free oleic

acid interacts electrostatically with the amine group of the D-glucosamine monomers while at the same time also interacts via hydrophobic forces with olive oil (Fig. 5B). Under this mechanism, it is expected that as DD is increased, oil-binding capacity should increase. However, in the results of this study (see Fig. 3B), some points were off the line. In the oil-binding test, no additional oleic acid was added and the olive oil contained only 0.39% free oleic acid. This paucity of free fatty acids could be a limiting factor in the interaction of excess chitosan and the olive oil triacylglycerides.

4. Conclusion

The behavior of chitosan in aqueous solutions of high ionic strength governs its interaction with olive oil. In this study, the oil-binding capacity was taken as a measure of the strength of the interaction of chitosan and olive oil. PLS was used to study how the interaction is affected by the basic properties of chitosan as a polymer namely its DD and DP. It was found that DP and DD influence the polymer's capacity to interact with olive oil; with DD having the greater effect. For chitosan with DD in the interval 50% < DD < 90%, oil-binding capacity increased with the increasing hydrophobicity of chitosan with the decrease in its DD. For chitosan with DD > 90%, electrostatic forces dominates the interaction. Free fatty acid anions assist in the chitosan and olive oil by forming a stable ionic interaction with the former and a strong hydrophobic interaction with the latter.

Acknowledgement

This research work is funded in part by the European Commission under the project Environmental Chemoinformatics – Initial Training Network (ITN No. 238701).

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