Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 66 (2006) 363–371
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Chitosan taurocholate capacity to bind lipids and to undergo enzymatic hydrolysis: An in vitro model

Riccardo A.A. Muzzarelli [a,*], Federica Orlandini [a], Deborah Pacetti [c], Emanuele Boselli [c], Natale G. Frega [c], Giorgio Tosi [b], Corrado Muzzarelli [a]

[a] *Institute of Biochemistry, Faculty of Medicine, University of Ancona, Via Ranieri 67, I-60131 Ancona, Italy*
[b] *Department of Materials Sciences, Faculty of Engineering, University of Ancona, Via Brecce Bianche, I-60131 Ancona, Italy*
[c] *Department of Food Science, Faculty of Agriculture, University of Ancona, Via Brecce Bianche, I-60131 Ancona, Italy*

Received 3 March 2006; received in revised form 10 March 2006; accepted 10 March 2006
Available online 4 May 2006

Abstract

Upon mixing a solution of sodium taurocholate with a solution of chitosan acetate, both at pH values close to 4, immediate precipitation of chitosan taurocholate salt is observed. The salt obtained with nearly stoichiometric amounts of both reagents was characterized by infrared spectrometry, and found to exhibit new bands indicative of its ionic nature. Similar compounds were obtained from Na taurodeoxycholate and Na glycocholate. When exposed to a number of hydrolases at pH values close to neutrality and 20 and 37 °C they were found to be poorly susceptible to enzymatic degradation: only *Trichoderma reesei* cellulase, egg white lysozyme and barley malt α-amylase were effective on chitosan taurocholate and glycocholate. The observed capacities of the freeze-dried salts for olive oil were 22 g oil/g of chitosan taurocholate, 60 g oil/g of chitosan glycocholate, and 27 g oil/g of chitosan taurodeoxycholate. The capacities were 22.1 g butter oil/g of chitosan taurocholate and 22.1 g of corn oil/g chitosan taurocholate. These data, substantially much higher than similar data published for plain chitosan and various oils, mean that the lipid uptake mainly takes place by hydrophobic interactions with the insoluble salts formed by chitosan upon contact with bile. The profile of fatty acids and some components of the unsaponifiable (cholesterol, α-tocopherol and γ-tocopherol) were not different in treated and untreated lipids. A model for the action of chitosan in vivo is discussed.

Keywords: Bile; Taurocholate; Glycocholate; Chitosan; Lipids; Cholesterol; Tocopherol

1. Introduction

It is claimed that chitosan reduces liver and serum cholesterol concentrations by one-half or more in cholesterol-fed rats. Chitosan has also been shown to reduce plasma cholesterol in cholesterol-fed broiler chickens at dietary concentrations of 1.5–3.0%. Animal studies, however, might not be predictive of results in humans because of the occurrence of chitinases in the digestive systems of many animals, as a point of difference from humans (Muzzarelli, Mattioli-Belmonte, Pugnaloni, & Biagini, 1999).

For the oral administration to humans, chitosan is generally recognized as safe (Harrison, 2002; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004); it reduced serum cholesterol in adult males who experienced a significant decrease of 6% in total cholesterol in 2 weeks. Further studies have reported serum cholesterol reductions with chitosan treatment: obese women consuming 1.2 g of chitosan per day for 8 weeks demonstrated significant reductions in low-density lipoproteins, although not total serum cholesterol (Wuolijoki, Hirvela, & Ylitalo, 1999). Eighty-four female subjects with mild to moderate hyper-

* Corresponding author. Tel.: +39 071 2204684; fax: +39 071 2204683.
E-mail address: muzzarelli@univpm.it (R.A.A. Muzzarelli).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.03.021

cholesterolemia receiving 1.2 g of chitosan per day experienced a significant decrease in total serum cholesterol (Bokura & Kobayashi, 2003). Twenty-one overweight normo-cholesterolemic subjects were fed a supplement containing equal amounts of glucomannan and chitosan for 28 days: the observed serum cholesterol reduction was mediated by increased fecal steroid excretion and was not linked to fat excretion. Greater fecal excretion of neutral sterols and bile salts was observed. The topic has been reviewed by Muzzarelli (1996, 1998), Pittler and Ernst (2004) and Ni Mhurchu et al. (2005a, 2005b).

Bile salts are formed from cholesterol in the liver and are secreted into the duodenum by the enterohepatic circulation: the bile salt pool is maintained stable, the newly ingested cholesterol compensating the excreted quantities. If, however, bile salts are sequestered by any suitable compound, some cholesterol is oxidised to produce more bile salts (Brady, 1999). Bile is produced at the rate of 700–1200 ml/day, bile salts accounting for 1.24–1.72%, cholesterol for 0.86–1.76 g/L; the average pH is 7.3. For the various intestinal tracts, the pH values are as follows: duodenum 4.7–6.5, upper jejunum 6.2–6.7, lower jejunum 6.2–7.3, ileum 6.1–7.3, colon >7.3. It is worth noting that these values tend to keep the bile salts in solution, while depressing the chitosan solubility (chitosan pK 6.3). Rodriguez and Albertengo (2005) have shown that chitosan flocculates at pH 6.5–7.5 in experimental duodenal environment.

The uptake of bile salts into chitosan–alginate gel beads was observed by Murata, Toniwa, Miyamoto, and Kawashima (1999). It seems that the presence of weak acids (orotic, citric, folic, and ascorbic) does not hinder the uptake; rather, chitosan orotate salt was found to enhance it. Various chitosans and heavily modified Chitopearl® chitosans were studied by Murata, Kojima, and Kawashima (2003) (see also Kumar et al., 2004) and found to have capacities in the range 0.53–1.20 mmol taurocholate per gram of chitosan (various degrees of acetylation) and 0.2–0.9 mmol taurocholate for Chitopearl® products; the capacity of Questran® was ca. 1.0 mmol taurocholate/g. Higher capacities were observed for taurodeoxycholate (1.8 mmol taurodeoxycholate/g chitosan). Of course, these capacities depend on the initial bile salt concentration, grain size, and other parameters. One millimole of taurocholate corresponds to 515 mg, thus the weight ratio taurocholate/chitosan orotate is 0.515:1.000, which appears to be a high ratio, notwithstanding the uncertainties related to the description of the experimental conditions.

Thongngam and McClements (2005a) focused on the binding of Na taurocholate to chitosan and provided thermodynamic data by isothermal titration calorimetry. At 30 °C, Na taurocholate binds strongly to chitosan to form an insoluble complex containing ca. 4 mmol Na taurocholate/g chitosan at saturation, that means taurocholate/chitosan molar ratio ca. 2:3 and weight ratio ca. 2:1. Ionic strength had scarce influence; the enthalpy changes went from endothermic (at 10 °C) to exothermic (at 40 °C) indicating the importance of changes of hydrophobic interactions leading to the formation of micelle-like clusters within the chitosan structure. The binding capacities of sodium glycocholate to chitosan, diethylaminoethyl chitosan, quaternized diethylaminoethyl chitosan, and cholestyramine were 1.42, 3.12, 4.06, and 2.78 mmol/g, respectively. The capacity of dialkylaminoalkyl chitosans increased with the number of carbons in the alkyl groups, indicating that hydrophobic interaction plays a major role in the sequestration of bile acids (Lee, Kim, & Kim, 1999); similarly, the capacity of 6-oxychitosan for cholic acid decreases with increasing degree of oxidation, i.e., loss of cationicity (Yoo et al., 2005).

This kind of evidence, however, does not include data on the nature of the reaction between a bile salt and chitosan, nor the instrumental characterization of the product. The tendency of chitosan to precipitate when the hydrolysis of sodium salts rises the pH value of the medium (pH >6) has prevented so far the unambiguous description of the formation of a salt of chitosan with a bile anion.

This article is intended to demonstrate the spontaneous formation of insoluble chitosan salts from bile acids such as chitosan taurocholate, whose hydrophobic nature should permit the collection of cholesterol and lipids via hydrophobic interaction, and should depress the enzymatic hydrolysis of the salts as well as their complexes.

2. Materials and methods

2.1. Chemicals

Chito-clear FG90, a food grade chitosan manufactured from crustaceans by Primex, Drammen, Norway, was used (degree of acetylation 0.13, average MW 150 kDa, viscosity of 1% solution in 1% acetic acid 100 mPa s, ashes 0.3%). Na taurocholate, Na taurodeoxycholate and Na glycocholate as well as common chemicals were supplied by Aldrich, Milano, Italy. Butter, olive oil and refined corn oil enriched with α-tocopherol (90 mg/100 g) were purchased in a local retail shop.

2.2. Preparation of chitosan salts

The chitosan powder (55 mg) was dissolved in acetic acid (10 ml, 0.5%), the resulting pH being 4.0. The Na bile salts (160 mg) were dissolved separately in water (10 ml) with resulting pH values between 5 and 6. Each of the latter solutions was poured in the respective chitosan solution thus producing a white precipitate; under these conditions (20 ml) the bile salt concentration was 8 g/L, very close to 16 mM, that would be the best concentration according to Thongngam and McClements (2005a). Each suspension was introduced into a dialysis tube and kept against water for 24 h; the final pH values were: chitosan taurocholate 5.8; chitosan glycocholate 5.9; chitosan taurodeoxycholate 6.5. Upon freeze-drying at −92 °C, the salts were obtained in dry form, in

the following amounts: chitosan taurocholate 0.159 mg; chitosan glycocholate 0.149 mg; chitosan taurodeoxycholate 0.150 mg.

2.3. Emulsion stability

Oil-in-water emulsions were prepared by stirring a mixture of olive oil and aqueous taurocholate solution (4:6), the latter containing 0.5% of Na taurocholate as an emulsifier, with the aid of a Silverson emulsifier. Chitosan lactate solution (1%) was added to observe the de-stabilization of the emulsions.

2.4. Enzymatic hydrolysis of chitosan salts

The following hydrolases were used: cellulase from *Trichoderma reesei* (Sigma, Milano, Italy), pectinase from *Aspergillus niger* (Novozyme, Copenhagen, Denmark), α-amylases from barley malt and from porcine pancreas, lipases from wheat germ and porcine pancreas, and hen egg white lysozyme (Fluka, Milano, Italy). Enzyme solutions were prepared by dissolving the desired enzyme in water; when necessary phosphate buffer was added. The weighed chitosan salts were introduced separately in each enzyme solution (40 ml) and kept on a rolling bar machine for 48 h with periodical observations. The final pH values were read with a pH-meter.

2.5. Treatment of oils and butter with chitosan taurocholate

An aliquot of 10.0 g butter was liquefied at 40 °C and thoroughly mixed with 240 mg chitosan taurocholate in a test tube. The tube was closed and shaken at 40 °C in a thermal bath for 24 h. The olive and corn oils (10.0 g) were thoroughly mixed with 240 mg chitosan taurocholate in a test tube and kept at 40 °C for 24 h. The oil-bearing materials were weighed and kept for 6 months at room temperature with no visible alteration or oil loss.

The determinations of total cholesterol and fatty acids were performed in the samples of untreated corn oil and butter as well as in the aliquot of lipids which were not retained by chitosan taurocholate after a 24-h contact. Squalane (10 μl of 10% w/v benzene solution) was added to samples of butter and corn oil (1.0 g) prior to saponification (Stazione Sperimentale Grassi, 1976). For the determination of cholesterol, an aliquot of the unsaponifiable matter (50 mg) obtained after extraction with diethyl ether was dried and silylated according to Sweeley, Bentley, Makita, and Welles (1963) prior to gas chromatography–mass spectrometry.

2.6. Instruments

A Perkin-Elmer Spectrum GX FT-IR spectrometer equipped with a Perkin-Elmer Multiscope system infrared microscope (MCT-SL detector) was used to record Attenuated Total Reflection, ATR, spectra. The microscope was equipped with a movable 75 × 50 mm X–Y stage. In some cases, it was necessary to adopt the following procedure: small amounts of the sample, cooled in liquid nitrogen, were ground with KBr and the spectra were obtained by using a Spectra Tech Diffuse Reflectance (DRIFT) accessory. In both cases, the spectral resolution was 4 cm^{-1}. The absorption spectra were the results of 16 scans. Treatments of the data were achieved with a Perkin-Elmer Spectrum and with a Grams/32 Galactic Corp., software package.

The gas chromatograph for the determination of total cholesterol was a HRGC Mega 2 series from Fisons (Milan, Italy) equipped with a flame ionization detector (FID). A DB-5MS fused silica column (30 m× 0.25 mm i.d., 0.25 μm film thickness) from J & W Scientific (Folsom, CA, USA) was used. The oven temperature raised from 250 to 320 °C at a rate of 3 °C/min. The injector temperature was 300 °C and the split ratio was 30:1. The carrier gas flow (helium) was 2 ml/min.

The fatty acids were methylated with diazomethane in ether and analysed by gas chromatography using the method reported by Pacetti, Hulan, Schreiner, Boselli, and Frega (2005).

The determination of α- and γ-tocopherol was conducted by injecting a diluted solution of treated and untreated corn oil (10 mg/ml in the HPLC mobile phase) in HPLC with fluorescence detection (λ_{exc} 290 nm, λ_{em} 330 nm). A Prodigy 5 μm Si column (250 mm × 4.6 mm i.d.) from Phenomenex was used. The analysis was run under isocratic conditions using *n*-hexane/2-propanol/acetic acid (99.5:0.5:0.1, v/v) at a flow of 1.3 ml/min; the injection loop was 20 μl.

3. Results and discussion

3.1. Precipitation of chitosan salts

The pK_a values for taurocholic acid and glycocholic acid are 1.40 and 4.35, respectively, and therefore these compounds are primarily occurring in the ionized form at concentrations below their critical micelle concentration (14.0 and 10.0 mM, respectively). This also means that the aqueous solutions of the corresponding salts at concentrations of interest for the present purposes are slightly acidic, for example, pH 5 for Na taurocholate, and 6 for Na glycocholate.

Because a chitosan acetate solution is inherently acidic, typically pH 4.5, the mixing of a Na taurocholate solution with a chitosan lactate solution certainly leads to an acidic mixture, i.e., a solution where the precipitation of chitosan cannot occur. Therefore, the insoluble chitosan taurocholate salt formation is perceived by naked-eye observation. On the opposite, previous workers did not follow this straightforward approach, but preferred to introduce solid particles of chitosan, substituted chitosans, cross-linked chitosans and resins into bile salt solutions.

According to the present research, the homogeneous reaction of chitosan acetate with Na taurocholate leads

actually to the precipitation of what is found instrumentally to be a chitosan taurocholate salt, at slightly acidic pH values, typically 5.8–5.9 where chitosan coagulation cannot occur.

3.2. Infrared spectrometry

3.2.1. Chitosan taurocholate salt

The spectra of plain chitosan, Na taurocholate and chitosan taurocholate salt are shown in Fig. 1. The spectrum of the chitosan taurocholate appears to result from contributions of both reagents, with a higher incidence of taurocholate bands mainly in the fingerprint region. Peak fitting in the Amide I and Amide II region better highlights differences between the spectral pattern of chitosan taurocholate on one side and the plain chitosan and Na taurocholate on the other side.

Concerning the Amide I, the chitosan band at 1662 cm^{-1} falls at 1667 in chitosan taurocholate while the one at 1594 cm^{-1} is absent. In chitosan taurocholate, the Na taurocholate bands at 1691 and 1637 are shifted to 1718 and 1627 cm^{-1} while the one at 1599 is not evident. In the chitosan taurocholate spectrum the vibrational modes of chitosan at 1594 and of taurocholate at 1560 appear as a single band at 1557, and the 1542 chitosan band is red shifted at 1526 cm^{-1} (Fig. 2). In the region 1500–1000 cm^{-1}, the spectral profile of chitosan taurocholate is the sum of the component modes with a broadening of the taurocholate band around 1200 cm^{-1}. In the region 1000–400 cm^{-1}, the spectral pattern of the complex is greatly influenced by taurocholate absorptions.

The chitosan selected for the present investigation was essentially amorphous, even though the wide-angle diffuse signal centered around 0.45 nm pointed to a certain degree of short-range ordering; said chitosan was appropriate for detecting any peak arising from reactions with the bile acids. On the other hand, Na taurocholate showed two relatively sharp diffraction peaks in the small-angle region and a dominant signal in the wide-angle region. The spectrum for the chitosan taurocholate salt exhibited a relatively sharp diffraction peak in the small-angle region, corresponding to a layered structural organization of the molecules in one dimension, featuring a repeat distance of about 2.4 nm, accompanied by two broad diffuse halos centered about 0.61 and 0.43 nm, corresponding to two different short-order correlation lengths in the plane orthogonal to the layers. The modest crystallinity of Na taurocholate was therefore depressed by salt formation with chitosan. Similar observations were made with chitosan glycocholate (Fig. 3).

Chitosan taurocholate salt appears to be a well-defined and stable water-insoluble substance that readily forms by electrostatic and hydrophobic interactions. Our instrumental data are in agreement with the thermodynamic data by Thongngam and McClements (2005a), and in line with data on chitosan dodecyl sulfate complex salt formed at pH 3 that does not release dodecyl sulfate even at pH 7 (Thongngam & McClements,

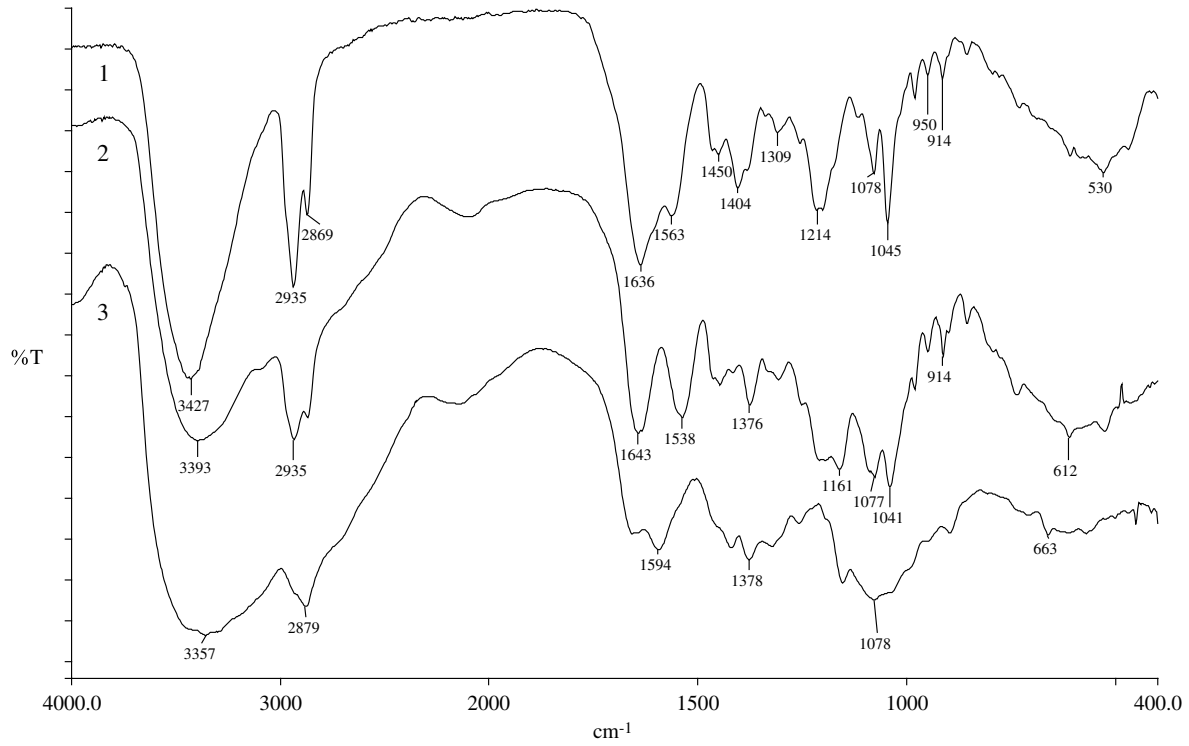

Fig. 1. Infrared spectra of: 1, Na taurocholate; 2, chitosan taurocholate; 3, chitosan.

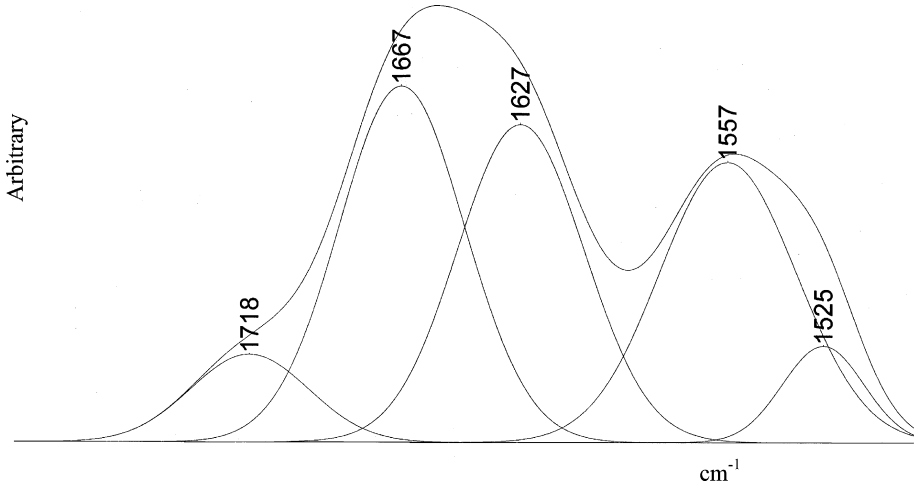

Fig. 2. Deconvolution of a portion of the infrared spectrum of chitosan taurocholate.

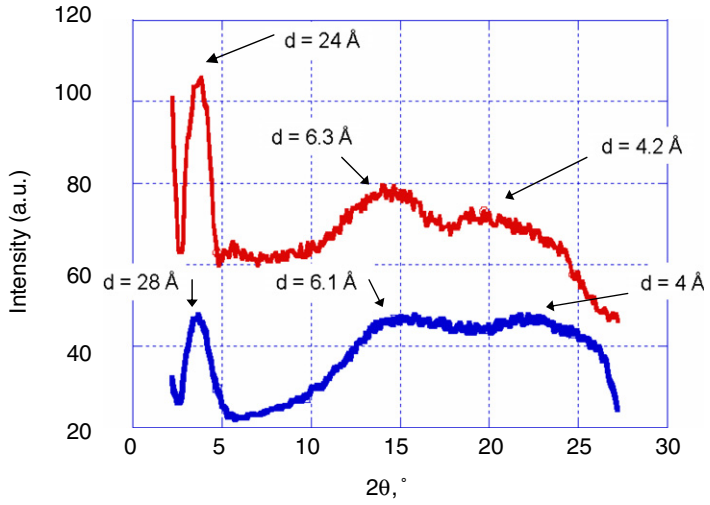

Fig. 3. X-ray diffraction spectra. (Upper curve) Chitosan taurocholate. (Lower curve) Chitosan glycocholate.

2005b). In these cited studies, both taurocholate and dodecyl sulfate complexes contain nearly equal weights of chitosan and surfactant.

3.2.2. Chitosan glycocholate salt

The spectra of chitosan, Na glycocholate and chitosan glycocholate are shown in Fig. 4. The spectral pattern of chitosan glycocholate exhibits the absorptions of the components. Some differences can be found in the region 1800–1500 cm^{-1} after Fourier self-deconvolution and curve fitting (Fig. 5). In chitosan glycocholate, a weak band is found at 1711 cm^{-1} while the chitosan band at 1662 and the glycocholate band at 1644 are condensed in a band at 1657 cm^{-1}; the modes of the chitosan glycocholate at 1593, 1542 and 1520 cm^{-1} fall at the same wavenumbers as those of both components. Below 1500 cm^{-1}, the spectral pattern of glycocholate is predominant.

3.3. Enzymatic hydrolysis

The chitosan salts were suspended in aqueous solutions of hydrolases. The results, listed in Table 1, show that fungal cellulase and lysozyme are most prone to exert hydrolytic action on the chitosan taurocholate and glycocholate salts, while the barley α-amylase has a relatively slower action, leading to complete digestion of the freeze-dried sample within 24 h. The other enzymes tested have no observable hydrolytic activity in the 48-h observation period, this fact being remarkable for pectinase and lipases, in particular, that have been found active on plain chitosan in previous works. In a further set of trials, the enzymes with no activity at 20 °C were found to be inactive at 37 °C as well.

Therefore, while it seems that the digestion of these substrates is mainly due to the hydrolysis of the chitosan component, this is not obvious because it is not observable for a majority of these hydrolases. Of course, the bile acid

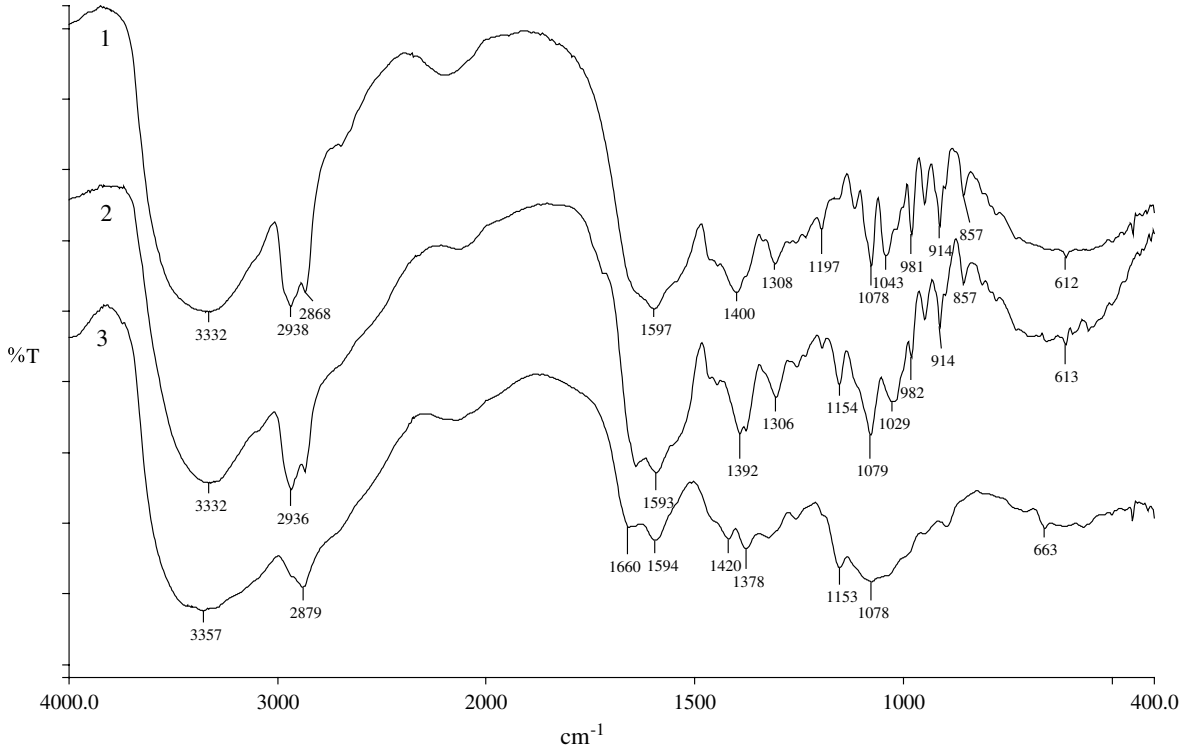

Fig. 4. Infrared spectra of: 1, Na glycocholate; 2, chitosan glycocholate; 3, chitosan.

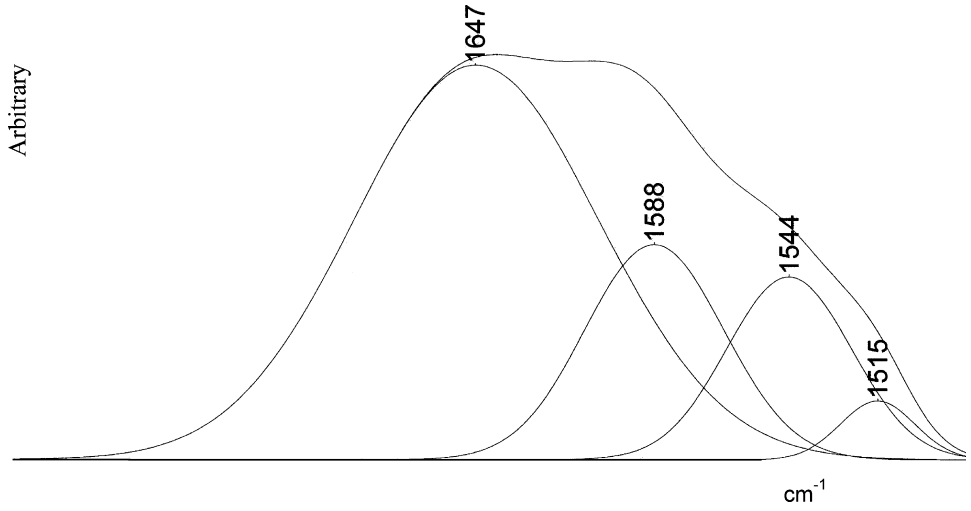

Fig. 5. Deconvolution of a portion of the infrared spectrum of chitosan glycocholate.

component plays major roles, as evidenced by the fact that the chitosan taurodeoxycholate is not susceptible to hydrolytic action by any of the enzymes tested.

3.4. Lipid uptake and emulsion destabilization

The observed capacities of the freeze-dried salts for olive oil were 22 g oil/g of chitosan taurocholate, 60 g oil/g of chitosan glycocholate, and 27 g oil/g of chitosan taurodeoxycholate. The capacities were 22.1 g butter oil/g of chitosan taurocholate and 22.1 g of corn oil/g chitosan taurocholate. These data mean that the lipid uptake is mainly generated by hydrophobic interactions, and that the ionic reaction between the fatty acid carboxyl group and the chitosan amino group is just marginal, considering also the scarce water content of the system. For a water system such as the emulsions studied by Ham-Pichavant, Sebe, Pardon, and Coma (2005), the interaction of plain chitosan with oleic acid is modest, and confined to oleic acid/chitosan weight ratios as low as 1:1. Similarly, sunflower oil was adsorbed on four different plain chitosans at ratios 1:2.8–1:10 depending on the quantity of oil added to 1 g

Table 1
Time necessary for total digestion of freeze-dried chitosan salts suspended in aqueous solutions of animal, fungal and plant enzymes at 20 °C

Enzyme	CTC		CGC		CTDC	
	pH	Time	pH	Time	pH	Time
Cellulase, *T. reesei*	6.8	60 min	6.6	90 min	6.5	No
α-Amylase, malt	7.1	24 h	7.0	24 h	4.5	No
α-Amylase, pancreas	6.8	No	6.8	No	7.0	No
Pectinase, *A. niger*	7.1	No	6.9	No	6.4	No
Lysozyme, egg white	7.3	30 min	7.0	45 min	7.0	No
Lipase, wheat germ	6.7	No	6.6	No	6.7	No
Lipase, pancreas	7.3	No	7.0	No	6.9	No

CTC, chitosan taurocholate; CGC, chitosan glycocholate; CTDC, chitosan taurodeoxycholate. No, no appreciable digestion after 48 h.

of chitosan at pH 6.8 (Rodriguez & Albertengo, 2005): again these values are much lower than those observed in the present work.

On the other hand, the oil-in-water emulsions made in the presence of Na taurocholate were promptly destabilized by the addition of chitosan lactate in amounts stoichiometrically close to the amount of taurocholate present in the emulsion due to the precipitation of the insoluble salt.

3.5. Cholesterol, tocopherols and fatty acids uptake by chitosan taurocholate

The uptake selectivity of chitosan taurocholate for cholesterol was evaluated in a lipid source of animal origin (butter), whereas the adsorption of α- and γ-tocopherol was evaluated in a source of plant lipids (refined corn oil enriched with tocopherols). In the case of butter, the lipid fraction is partially structured in the fat globules containing phospholipids, whereas in corn oil, lipids are composed by 99% triacylglycerols.

The cholesterol content was not significantly altered, considering the samples of untreated butter oil (2.09 mg/g) and butter oil resulting as a supernatant (2.05 mg/g) in the presence of the chitosan taurocholate–oil mixture (treated oil).

In the untreated corn oil the content of α-tocopherol (155 μg/g) and γ-tocopherol (365 μg/g) was not different with respect to the treated oil (151 and 351 μg/g, respectively).

The fatty acid profile of butter oil and corn oil obtained by means of gas chromatography can be used to point out preferential adsorptions of total lipids after a 24-h contact with chitosan taurocholate. In Fig. 6, the fatty acid profiles of untreated and treated butter oil do not appear to be significantly different from each other; the same happened with corn oil, as reported in Fig. 7. This indicates absence of selective uptake of triacylglycerols phospholipids and free fatty acids, as a point of difference from other modified chitosans (Muzzarelli, Frega, Miliani, Muzzarelli, & Cartolari, 2000).

4. Conclusions

Among the three novel chitosan salts that instantly form from chitosan and bile acids, taurocholate appears to possess infrared spectral characteristics that qualify it as a real ionic, water-insoluble, poorly crystalline, hydrophobic chitosan salt. The insolubilization of taurocholate, a powerful emulsifier, destabilizes emulsions.

In this chitosan taurocholate and homologues, chitosan accounts for no more than one-half by weight, therefore the hydrophobic nature prevails when contacting lipids. Butter and oils are collected to high extents with no discrimination of their components, including tocopherols. For these chitosan salts the lipid uptake is much higher than for plain chitosan. These findings are

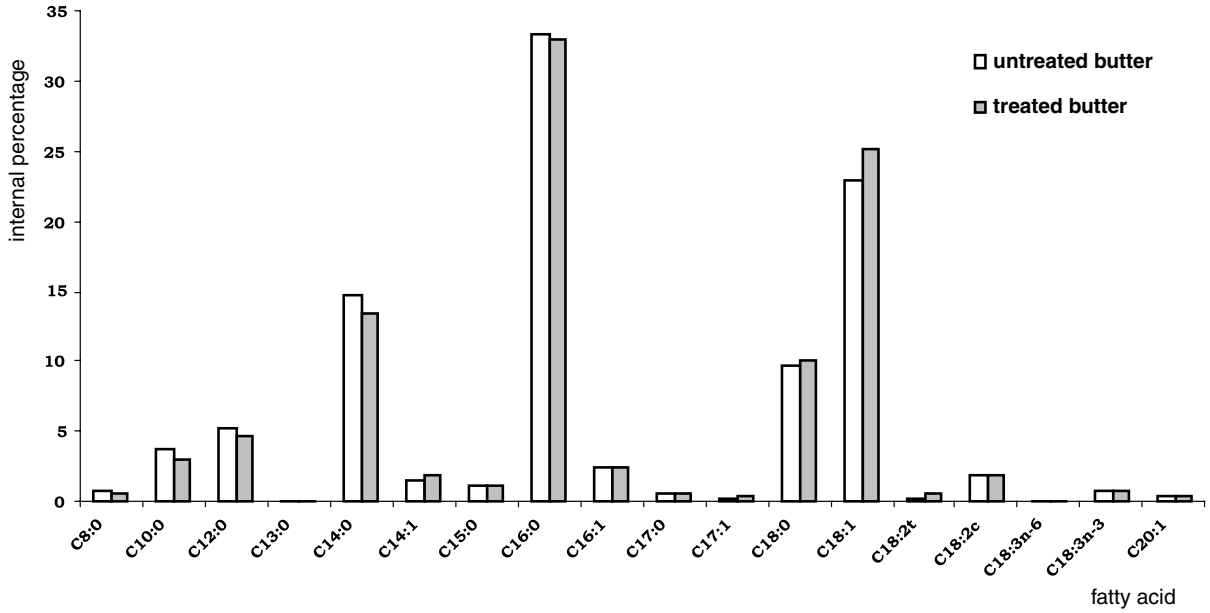

Fig. 6. Fatty acid composition (weight % of total fatty acid) of untreated butter (white bars) and chitosan taurocholate-treated butter. C*n*:*m*, number of carbon:number of double bonds.

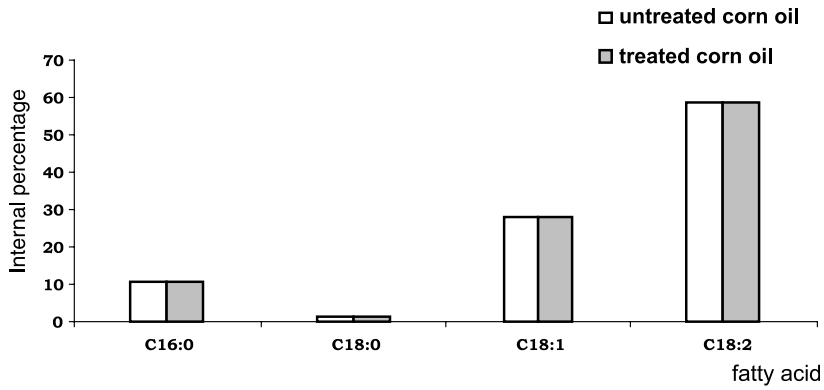

Fig. 7. Fatty acid composition (weight % of total fatty acid) of untreated corn oil (white bars) and chitosan taurocholate-treated corn oil. C*n*:*m*, number of carbons:number of double bonds.

also in agreement with previous data for hydrophobically modified chitosans: for example, *O*,*O*′-dipalmitoyl chitosan, a highly hydrophobic amphiphilic polymeric substance, adsorbs cholesterol from polar and non-polar solvents (Tong, Wang, Xu, Chua, & He, 2005). Similarly, glycol chitosan bearing 5-β-cholanic acid, and deoxycholic acid-modified chitosan form self-aggregates containing compact hydrophobic domains (Kwon, Park, Chung, Kwon, & Jeong, 2003; Lee, Kim, Kwon, & Jeong, 2000). Bile acid-modified glycol chitosan form self-assembled nanoparticles suitable for cancer therapy (Kim et al., 2005) and for gene delivery (Chae, Son, Lee, Jang, & Nah, 2005). The salts studied in the present work are scarcely hydrolysed by a variety of hydrolases, and it is presumed that their complexes with lipids would be even more resistant to enzymatic attack.

If the above findings are extrapolated from the in vitro model to the physiological environment, it seems reasonable to speculate that:

(i) Chitosan glycocholate and chitosan taurocholate insoluble salts subtract bile salts from the circulation thus forcing the organism to replete the bile pool at the expenses of cholesterol.

(ii) The activity of lipases on triglycerides is depressed as a consequence of the poor emulsification of lipids due to the lowered availability of taurocholate, the emulsifier. It is known that the pancreatic lipases require a certain dimension of the oil droplets in the emulsion in order to hydrolyse triglycerides: now, when the bile salts become scarce, inadequate emulsions are formed and then limited hydrolysis of triglycerides takes place. Ample information on digestive lipases supports these views (Cajal, Svendsen, DeBolos, Patkar, & Alsina, 2000; Lombardo, 2001; Miled et al., 2000; Mukherjee, 2003; Wickman, Wilde, & Fillery-Travis, 2002). Lipases work thanks to the presence of bile salts that in one case activate the bile salt-dependent lipases, and in the other case provide the emulsion necessary to the pancreatic lipases for enzymatic activity. Moreover, as soon as the bile salt availability decreases due to chitosan ingestion, the bile salt-dependent lipases are poorly activated, and assimilation of lipids by the organism decreases sharply.

(iii) While pectinase is one of the most representative bacterial enzymes in the intestine, the resistance of the chitosan taurocholate, glycocholate and taurodeoxycholate salts to hydrolysis by this enzyme, as well as by other hydrolases, would certainly be an indication of the capacity of these hydrophobic salts to be excreted, presumably with accompanying adsorbed lipids.

Considering the results of this in vitro investigation and the supporting literature, it appears that chitosan would be most effective in the reduction of cholesterol and body weight provided that chitosan formulations lend themselves to prompt reaction with bile salts.

Acknowledgments

This work was carried out under the auspices of the European Commission, Brussels, Contract 508421 Chitosanperos. Thanks are due to Prof. Oriano Francescangeli for X-ray diffraction measurements, and to Mrs. Carla Conti for recording the infrared spectra.

References

Bokura, H., & Kobayashi, S. (2003). Chitosan decreases total cholesterol in women: a randomized, double-blind, placebo-controlled trial. *European Journal of Clinical Nutrition, 57*, 721–725.

Brady, T. (1999). *Nutritional biochemistry*. New York: Academic Press.

Cajal, Y., Svendsen, A., DeBolos, J., Patkar, S. A., & Alsina, M. A. (2000). Effect of the lipid interface on the catalytic activity and spectroscopic properties of a fungal lipase. *Biochimie, 82*, 1053–1061.

Chae, S. Y., Son, S., Lee, M., Jang, M. K., & Nah, J. W. (2005). Deoxycholic acid-conjugated chitosan oligosaccharide nanoparticles for efficient gene carrier. *Journal of Controlled Release, 109*, 330–344.

Ham-Pichavant, F., Sebe, G., Pardon, P., & Coma, V. (2005). Fat resistance of chitosan-based paper packaging for food applications. *Carbohydrate Polymers, 61*, 259–265.

Harrison, T. A. (2002). The FDA regulation of labelling claims for nutraceuticals in the U.S. *Agro-Food Industry Hi-Tech, 13*, 8–11.

Kim, K., Kim, J. H., Kim, S., Chung, H., Choi, K., & Kwon, I. C. (2005). Self-assembled nanoparticles of bile acid-modified glycol chitosans and their applications for cancer therapy. *Macromolecular Research, 13*, 167–175.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*, 6017–6084.

Kwon, S., Park, J. H., Chung, H., Kwon, I. C., & Jeong, S. Y. (2003). Physico-chemical characterization of self-assembled nanoparticles based on glycol chitosan bearing 5-beta-cholanic acid. *Langmuir, 19*, 10188–10193.

Lee, J. K., Kim, S. U., & Kim, J. H. (1999). Modification of chitosan to improve its hypocholesterolemic capacity. *Bioscience, Biotechnology and Biochemistry, 63*, 833–839.

Lee, K. Y., Kim, J. H., Kwon, I. C., & Jeong, S. Y. (2000). Self-aggregates of deoxycholic acid-modified chitosan as a novel carrier of adriamycin. *Colloid Polymer Science, 278*, 1216–1219.

Lombardo, D. (2001). Bile salt-dependent lipase: its patho-physiological implications. *Biochimica et Biophysica Acta, 1533*, 1–28.

Miled, N., Canaan, S., Dupuis, L., Roussel, A., Riviere, M., Carriere, F., et al. (2000). Digestive lipases: from three-dimensional structure to physiology. *Biochimie, 82*, 973–986.

Mukherjee, M. (2003). Human digestive and metabolic lipases, a brief review. *Journal of Molecular Catalysis B: Enzymatic, 22*, 369–376.

Murata, Y., Kojima, N., & Kawashima, S. (2003). Functions of a chitosan-orotic acid salt in the gastrointestinal tract. *Biology and Pharmacology Bulletin, 26*, 687–690.

Murata, Y., Toniwa, S., Miyamoto, E., & Kawashima, S. (1999). Preparation of alginate gel beads containing chitosan nicotinic acid salt and the functions. *European Journal of Pharmaceutics and Biopharmaceutics, 48*, 49–52.

Muzzarelli, R. A. A. (1996). Chitosan-based dietary foods. *Carbohydrate Polymers, 29*, 309–316.

Muzzarelli, R. A. A. (1998). Management of hypercholesterolemia and overweight by oral administration of chitosans. In D. Chapman & P. I. Haris (Eds.), *New biomedical materials – Applied and basic studies* (pp. 135–142). Amsterdam: IOS Press.

Muzzarelli, R. A. A., Frega, N., Miliani, M., Muzzarelli, C., & Cartolari, M. (2000). Interactions of chitin, chitosan, *N*-lauryl chitosan and *N*-dimethylaminopropyl chitosan with olive oil. *Carbohydrate Polymers, 43*, 263–268.

Muzzarelli, R. A. A., Mattioli-Belmonte, M., Pugnaloni, A., & Biagini, G. (1999). In P. Jollés & R. A. A. Muzzarelli (Eds.), *Chitin and chitinases* (pp. 251–264). Basel: Birkhauser.

Ni Mhurchu, C., Dunshea-Mooij, C., Bennett, D., & Rodgers, A. (2005a). Effect of chitosan on weight loss in overweight and obese individuals: a systematic review of randomized controlled trials. *Obesity Reviews, 6*, 35–42.

Ni Mhurchu, C., Dunshea-Mooij, C., Bennett, D., & Rodgers, A. (2005b). Chitosan for overweight or obesity. *Cochrane Database of Systematic Reviews, 3*, 1136–1183.

Pacetti, D., Hulan, H. W., Schreiner, M., Boselli, E., & Frega, N. G. (2005). Positional analysis of egg triacylglycerols and phospholipids from hens fed diets enriched with refined seal blubber oil. *Journal of Science Food Agriculture, 85*, 1703–1714.

Pittler, M. H., & Ernst, E. (2004). Dietary supplements for body-weight reduction: a systematic review. *American Journal of Clinical Nutrition, 79*, 529–536.

Rodriguez, M. S., & Albertengo, L. E. (2005). Interaction between chitosan and oil under stomach and duodenal digestive chemical conditions. *Bioscience, Biotechnology and Biochemistry, 69*, 2057–2062.

Stazione Sperimentale per le Industrie degli Olii e dei Grassi (1976). Norme Grassi e Derivati, metodo NGD C-12, Milano.

Sweeley, C. C., Bentley, R., Makita, M., & Welles, W. E. (1963). Gas–liquid chromatography of trimethylsilyl derivatives of sugar and related substances. *Journal of the American Chemical Society, 85*, 2497–2507.

Thongngam, T., & McClements, D. J. (2005a). Isothermal titration calorimetry study of the interaction between chitosan and bile salt (sodium taurocholate). *Food Hydrocolloids, 19*, 813–819.

Thongngam, T., & McClements, D. J. (2005b). Influence of pH, ionic strength and temperature on self-association and interactions of sodium dodecyl sulfate in the absence and presence of chitosan. *Langmuir, 21*, 79–86.

Tong, Y., Wang, S., Xu, J., Chua, B., & He, C. (2005). Synthesis of *O*,*O*′-dipalmitoyl chitosan and its amphiphilic properties and cholesterol adsorption. *Carbohydrate Polymers, 60*, 229–233.

Wickman, M., Wilde, P., & Fillery-Travis, A. (2002). A physico-chemical investigation of two phosphatidylcholine / bile salt interfaces: implications for lipase activation. *Biochimica et Biophysica Acta, 1580*, 110–122.

Wuolijoki, E., Hirvela, T., & Ylitalo, P. (1999). Decrease of serum LDL cholesterol with microcrystalline chitosan. *Methods and Findings in Experimental and Clinical Pharmacology, 21*, 357–361.

Yoo, S. H., Lee, J. S., Park, S. Y., Kim, Y. S., Chang, P. S., & Lee, H. G. (2005). Effects of selective oxidation of chitosan on physical and biological properties. *International Journal of Biological Macromolecules, 35*, 27–31.