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Interactions of chitin, chitosan, *N*-lauryl chitosan and *N*-dimethylaminopropyl chitosan with olive oil

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Abstract

Chitin, chitosan and the newly synthesized and fully characterized *N*-lauryl chitosan and *N*-dimethylaminopropyl chitosan, endowed with higher hydrophobicity and cationicity, respectively, were tested for their capacity to alter the composition of olive oil upon percolation of the latter through a bed of their respective powders. The oil samples were extracted, saponified and submitted to gas-chromatography. Results indicated that the percentages of 12 fatty acids were not modified, but the diacylglycerol and steroid concentrations were greatly altered. The percolated oil was depleted of C34 and C36 diacylglycerols (lowered to 42% of the control) when the oil was contacted with chitosan and *N*-lauryl chitosan, whilst the oil fraction percolated through chitin became 30% enriched. *N*-Dimethylaminopropyl chitosan was also effective in retaining diacylglycerols. The direct analysis of the unsaponifiable fraction revealed that campesterol, stigmasterol and avenasterol were enriched in the oil fraction retained by chitin and *N*-lauryl chitosan, while β-sitosterol increased slightly in the fraction retained by chitosan and *N*-lauryl chitosan. Triterpene alcohols were higher in the oil fraction retained by chitin. This work indicates that chitin might be more suitable than chitosan for sequestering steroids, and that, in general, the chitin derivatives discriminate among the various lipids.

Keywords: Chitin; Chitosan; Olive oil

1. Introduction

Chitosan is being marketed in various countries as a dietary supplement for human consumption. The claimed efficacy of chitosan in reducing hypercholesterolemia, hypertension and body weight stimulated industrial production of chitosan tablets in spite of inadequate scientific knowledge about the reactivity of chitosan towards lipids. It is claimed that the cationic nature of chitosan makes it particularly suitable for sequestering bile acids and fatty acids. Nevertheless, concern was expressed about the fact that certain promotional campaigns presented chitosan as a fat-binder active toward all kinds of lipids with no supporting scientific evidence, thus rising the protest of consumer associations.

Information about the interaction of chitosan with lipids is scanty: the removal of lipids (mainly lecithin) from cheese whey was studied by Hwang and Damodaran (1995), and chitosan lecithin interactions were studied by Magdassi, Bach and Mumcuoglu (1997). The subject was addressed more directly by Sathirakul (1999) who compared the lipid binding capacity of chitosan in term of oil holding capacity, cholesterol binding capacity and bile acid capacity.

Crosslinked *N*,*O*-carboxymethyl chitosan was used by Yu and He (1997) to adsorb triglycerides from serum. The capacity of the polymer was 3.35 mg triglyceride/g, sufficient for abatement of triglycerides concentration to one half. While the reasons for selecting this modified chitosan are unclear, it is evident that there is interest in examining the lipid collection ability of the chitin itself, of highly cationic chitosans, and hydrophobic chitosans, to verify whether cationicity is a key aspect. On the other hand, the formation of hydrophobic aggregates has been indicated as a cause of lipid sequestering by chitosans or by chitosan–bile acid associations.

Emulsans formed by *Acinetobacter calcoaceticus* are natural examples of fatty acid substituted chitosans (Gorkovenko, Zhang, Gross & Kaplan, 1999). Chitosan laurate salt was described by Wong, Gastinou, Gregorski, Tillin and Pavlath (1992) and lauric acid was claimed to assist dry grinding of chitosan powder (Fukumori et al., 1998); *N*-lauryl carboxymethyl chitosan was studied by Miwa et al. (1998) in connection with delivery of taxol to cancerous tissues. Physico-chemical studies were conducted by Kjoniksen,

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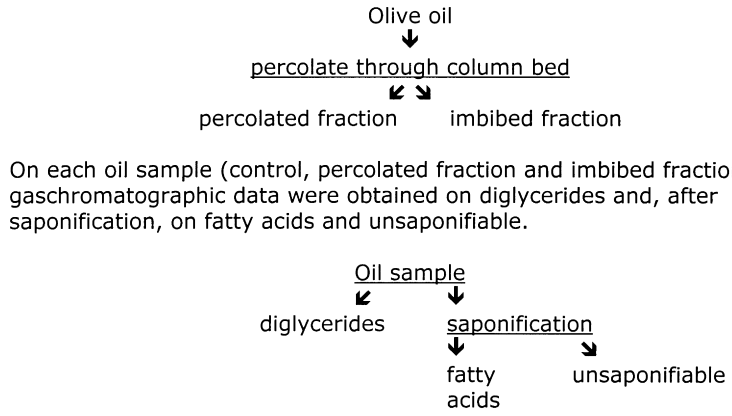

Scheme 1.

Iversen, Nystrom, Nakken and Palmgren (1998) and Nystrom, Kjoniksen and Iversen (1999) who described the association phenomena in aqueous systems of *N*-lauryl chitosan obtained from lauryl aldehyde via Schiff reaction. Desbrières, Martinez and Rinaudo (1996) and Desbrières, Rinaudo and Chtcheglova (1997) examined the role of charge and temperature on the formation of hydrophobic aggregates of *N*-lauryl chitosan, a subject addressed by Muzzarelli, Tanfani, Emanuelli and Mariotti (1983) and Hirano et al. (1999).

Acyl derivatives of chitosan of related interest were the *O*-stearoyl derivatives (Hirano & Osaka, 1983), and the palmitoyl glycol chitosan, which assembles into unilamellar polymeric vesicles in the presence of cholesterol, useful as drug carrier in view of its documented biocompatibility and hemocompatibility (Uchegbu, 1998; Uchegbu et al., 1998). Lauroyl derivatives were prepared from the anhydride by Seo, Ohtake, Kanbara, Yonetake and Iijima (1991) and the hydrophobic films were found suitable for the selective sorption of amino acids. Lower fatty acyl *N*-derivatives were found to resist degradation by lysozyme (Hirano, Kaneko & Kitagawa, 1991).

Chitosans with higher content of amino groups have been sought for the purpose of increasing their cationicity, their antimicrobial action and their chelating power. *N*-Dimethyl chitosan and *N*-trimethyl chitosan iodide were prepared by using formaldehyde and Na borohydride (Muzzarelli & Tanfani, 1985). *O*-Diethylaminoethyl chitosan was prepared by Kurita (1989) (see also Kim & Chun, 1999; Kim & Lee, 1993), who used *N*,*N*-dimethylaminoethyl chloride to modify chitosan at the 6 position. The Eschweiler–Clarke reaction was also used, but it requires ethanol as a medium and high temperature (Nikolaev, Prokopov, Shul'gina & Chudnova, 1985); the quaternization was carried out in *N*-methyl-2-pyrrolidone (Domard, Rinaudo & Terrassin, 1986). The Chitopearl® chitosans include a strongly basic quaternary cross-linked chitosan (Yoshida, Nishihara & Kataota, 1994). The capacity for cholesterol of *N*-alkyl chitosans was studied by Chandler and Curatolo (1992).

Scope of the present study was the verification of the effect of chitosan, chitin, *N*-lauryl chitosan and the new *N*-dimethylaminopropyl chitosan on olive oil, i.e. their respective capacity to retain preferentially some component when contacted with the oil.

2. Experimental

2.1. Contacting olive oil with chitosans

Olive oil was percolated (15 g, 1.5 g/min) through a bed of powder (2 g, 3 cm high, at 25°C). Both the effluent and the imbibition fraction were retained for analysis. The analytical procedure is presented in Scheme 1 (analytical procedure adopted for olive oil contacted with chitin derivatives). Squalane, $C_{30}H_{62}$, internal standard (10 μl of 10% benzene solution) and nonadecanoic acid C19:0, internal standard (200 μl of 1% benzene solution) were added to the olive oil (1 g). Then the saponification procedure was applied (Stazione Sperimentali Grassi, 1976). The fatty acids were methylated with diazomethane in ether, and analysed with a Carlo Erba Mega 5160 gas chromatograph equipped with a Mega 2 integrator. The analytical conditions are in Table 1.

The entire unsaponifiable fraction, after treatment with CH_2N_2 to methylate the free fatty acids, was treated with a silanizing mixture to transform the hydroxyl groups into trimethylsilyl derivatives (Sweeley, Bentley, Makita & Welles, 1963). The chromatographic analyses were carried out as indicated above.

The composition of the diacylglycerols, identified by comparison with literature data, was determined similarly, after adding to each sample (50 mg) squalane, diazomethane and silanizing mixture (Frega, Bocci & Lerker, 1992; Frega, Garzi, Mancuso & Rinaldelli, 1995).

2.2. Preparation of N-dimethylaminopropyl chitosan

3-Dimethylaminoacrolein, $(CH_3)_2N–CH=CH–CHO$, offers a pre-formed dimethylamino function, and high reactivity

Table 1
Gas chromatographic working conditions (FID = flame ionization detector; TAP = Chrompack)

	Fatty acids	Diacylglycerols	Unsaponifiable[a]
Stationary phase	SP 2330	TAP	TAP
Internal diameter (mm)	0.25	0.32	0.32
Length (m)	60	25	25
Film thickness (μm)	0.25	0.1	0.1
Injection technique	Split system	Split system	Split system
Carrier gas (kPa)	1.3	2	1
Split ratio	1/60	1/80	1/80
Carrier gas	He	He	He
Oven temperature (°C)	150 → 230	200 → 355	200 → 300
Temperature rate	Isoth. 1′; 3°C/min	3°C/min	Isoth. 1′; 3°C/min
Detector temperature (°C)	240	360	340
Injector temperature (°C)	240	360	340
Detector	F.I.D.	F.I.D.	F.I.D.

[a] Analytical determination made on the whole unsaponifiable.

being an unsaturated aldehyde. It would easily lend itself to Schiff reaction: preparations have been carried out under homogeneous and heterogeneous conditions, the latter being devised to obtain a powder of the same grain size as the chitin and chitosan.

2.2.1. Homogeneous conditions

Chitosan (30 g) was dissolved with the aid of acetic acid, and dimethylaminoacrolein was added under stirring, to reach a molar ratio 1:1 (chitosan amino group:aldehyde). Stirring was protracted for 2 h. A 5% Na borohydride solution (ca. 150 ml) was added with the aid of a peristaltic pump to reach pH 5.2. The product was dialyzed for 70 h against three changes of demineralized water. The freeze dried material was used for analytical purposes.

2.2.2. Heterogeneous conditions

Chitosan (10 g, 150–200 μm) was suspended in 1:1 water:ethanol mixture (100 ml). Dimethylaminoacrolein (5 g) was then added and stirring continued for 2 h. A Na borohydride solution (5 g in 100 ml) was added with the aid of a peristaltic pump. The product was filtered and washed with water and ethanol: yield 11.2 g. This powder was used for contacting the oil.

2.3. Characteristic properties of DMA-chitosan prepared under homogeneous conditions

2.3.1. Alkalimetry

A solution aliquot (0.5 g polymer in 65.8 ml) was mixed with 0.3 N HCl (20 ml) and titrated with 0.1 N NaOH. A second titration was carried out on the freeze-dried material (0.5 g) in spite of its relatively scarce solubility. In the first case the inflection points were at 41.0 and 68.1 ml (27.1 difference) and p*K* was 5.8 (solution aliquot). In the second case the inflection points were at 43.5 and 70.0 ml (26.5 difference) and p*K* was 5.3 (dissolved freeze-dried material). The titration of the same amount of chitosan gave inflection points at 39.8 and 59.2 (19.4 difference) and p*K* was 6.1.

Remarkably, the titration end points for the modified chitosan did not fall at 60 ml as expected (they were close to 70 in both cases) whilst with plain chitosan the titration end point was close to 60 ml as always in chitosan titrations. Here, the span between inflection points was larger. These results show that a significant alteration of the chemical nature of chitosan occurred as a consequence of the reaction with 3-dimethylamino acrolein, leading to really unusual alkalimetric curves.

2.3.2. Viscometry

The flow-curve for dimethylaminopropyl chitosan at pH 4.8 shows a somewhat higher viscosity compared to chitosan. Values remained constant during 8 days of measurements and were identical for two preparations, the first one made with molar ratio 1:1 and the second one with molar ratio 2:1 chitosan/dimethylamino acrolein.

2.3.3. Infrared spectrometry

Compared to the chitosan spectrum, the infrared spectrum of dimethylaminopropyl chitosan showed alterations in the 1600–1650 cm^{-1} region, and, to a minor extent, in the 1100–1150 cm^{-1} region corresponding to main bands in the 3-dimethylamino acrolein spectrum.

2.3.4. UV spectrophotometry

The UV spectrum recorded in the absorbance mode showed a distinct band at 282.0 nm which was not found in other water-soluble modified chitosans. The absorbance of the 282.0 nm band was the double of that for the chitosan backbone 194 nm band. Absorption readings at 282.0 were plotted vs dimethylaminopropyl chitosan concentration and the straight reference curve thus obtained was found useful for the determination of *N*-dimethylaminopropyl chitosan.

Table 2
Quantity of oil percolated and retained by 2 g of chitin derivatives

Polysaccharide	g	Oil (g)	Percolated (g)	Retained (g)
Chitosan	2	15	11	4
Chitin	2	15	4.2	10.8
Lauryl chitosan	2	15	11	4
DMA chitosan	2	15	9.5	5.5

2.4. Preparation of N-lauryl chitosan

Chitosan (10 g) was suspended in a water–methanol 1:1 mixture (134 ml), lauryl aldehyde (15 g) was added and stirring protracted for 30 min. Reduction was carried out as indicated above, and the preparation was left overnight. After filtration and washing with water–methanol and methanol, the powder was dried at 50°C. Yield: 25 g.

3. Results and discussion

The reaction of chitosan with 3-dimethylamino acrolein permits to introduce the pre-formed dimethylamino group in the chitosan using the amino group of chitosan for anchoring the substituent rather than for methylation. The *N*-dimethylaminopropyl chitosan, here reported for the first time, is a more basic compound than plain chitosan.

When olive oil was percolated through a bed of chitin or modified chitin of comparable mesh size, the amounts of oil retained in the dry powder varied depending on the chemical functions carried by the polysaccharide. In practice, when 15 g of oil were percolated at 25°C, 10.8 g were retained in the chitin, 5.5 g in the *N*-dimethylaminopropyl chitosan and 4.0 g in chitosan or in *N*-lauryl chitosan, as shown in Table 2 for 2 g of each powder.

The fatty acid composition of the oil was not appreciably altered, as a consequence of the contact with the polysaccharides: Table 3 shows that the concentrations of 12 fatty acids remained substantially the same in the percolated and in the retained fractions for all systems tested, in spite of the different amine nitrogen content, moisture content and hydrophobicity of the powders, and regardless of the degree of unsaturation of the fatty acids (compare for instance C18:0, C18:1 and C18:2 acids in Table 3).

On the other hand, the diacylglycerol content of the percolated oil was deeply altered. The percolated oil was depleted of diacylglycerols (lowered to 42% of original concentration) when contacted with chitosan and *N*-lauryl chitosan, while the oil fraction percolated through chitin was 30% enriched with diacylglycerols; *N*-dimethylaminopropyl chitosan was the most effective in retaining them. It might be possible that the alcohol function present in the diacylglycerols is important for establishing an interaction with the polysaccharides.

The analysis of the unsaponifiable fraction revealed that the concentrations of the steroids present in the olive oil were altered as a consequence of the contact with the polysaccharides. Remarkable cases were those of campesterol, stigmasterol and avenasterol, which were enriched in the fraction retained on chitin and on *N*-lauryl chitosan, while the β-sitosterol concentration increased slightly in the fraction retained on chitosan and *N*-lauryl chitosan. α-Tocopherol did not appear to be retained by chitin and chitosan. As for the triterpene alcohols 24-methylenecycloartanol and citrostadienol, their concentrations were high in the fraction retained by chitin. The steroid profile of the unsaponifiable fraction (first line, Table 4) was remarkably altered as a consequence of the passage through the polysaccharide powders.

It would be hard to find molecular motifs that would

Table 3
Composition (%) of fatty acids in the percolated and retained fractions, determined as methyl esters. Percent calculated on the total fatty acids, based on the relevant HRGC areas. $C_{n:m}$ (n = number of carbon atoms, m = number of double bonds). $C_{14:0}$ = mirystic acid; $C_{16:0}$ = palmitic acid; $C_{16:1}$ = palmitoleic acid; $C_{17:0}$ = margaric acid; $C_{17:1}$ = eptadecenoic acid; $C_{18:0}$ = stearic acid; $C_{18:1}$ = oleic (ω-9) acid; $C_{18:2}$ = linoleic (ω-6) acid; $C_{18:3}$ = linolenic (ω-3) acid; $C_{20:0}$ = arachidonic acid; $C_{20:1}$ = gadoleic acid; $C_{22:0}$ = beenic acid

Sample	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$
Oil	< 0.1	12.9	1.2	< 0.1	0.1	2.6	72.7	9.2	0.6	0.4	0.2	0.1
Chitosan												
Percolated	< 0.1	12.9	1.2	< 0.1	0.1	2.6	72.6	9.2	0.6	0.4	0.3	0.1
Retained	< 0.1	12.9	1.2	< 0.1	0.1	2.6	72.5	9.2	0.6	0.4	0.3	0.1
Chitin												
Percolated	< 0.1	12.9	1.2	< 0.1	0.1	2.6	72.8	9.3	0.6	0.4	0.3	0.1
Retained	< 0.1	12.9	1.2	< 0.1	0.1	2.6	72.7	9.2	0.6	0.4	0.3	0.1
Laurylchit												
Percolated	< 0.1	11.9	1.2	< 0.1	0.1	2.1	74.0	9.4	0.6	0.3	0.3	0.1
Retained	< 0.1	13.1	1.2	< 0.1	0.1	2.5	72.4	9.2	0.6	0.4	0.3	0.1
DMA Chitos												
Percolated	< 0.1	12.5	1.1	< 0.1	0.1	2.6	72.9	9.3	0.6	0.4	0.3	0.1
Retained	< 0.1	12.8	1.2	< 0.1	0.1	2.6	72.6	9.2	0.6	0.4	0.3	0.1

Table 4
Composition (%) of the unsaponifiable fractions. Percent calculated on the whole of HRGC areas in terms of TMS. DMAPC = *N*-dimethylaminopropyl chitosan

Sample	Squalene	α-Tocopherol	Campesterol	Stigmasterol	β-Sitosterol	Δ^5-Avenasterol	24-Methylene cycloartanol	Citrostadienol	o.p.
Olive oil	57.8	0.2	< 0.1	0.9	24.0	4.1	9.0	4.0	< 0.1
Chitosan									
Percolated	57.5	0.2	1.0	0.9	24.5	2.0	9.0	3.5	1.4
Retained	64.3	< 0.1	< 0.1	< 0.1	29.6	1.8	1.5	0.8	2.0
Chitin									
Percolated	67.7	0.4	1.0	0.9	25.0	< 0.1	4.1	0.5	0.4
Retained	58.1	< 0.1	1.0	0.9	25.9	1.7	8.6	3.2	0.6
Laurylch									
Percolated	60.0	0.2	< 0.1	0.4	25.7	< 0.1	2.7	0.3	10.7
Retained	56.4	0.2	0.9	1.0	33.9	1.9	0.9	3.9	< 0.1
DMAPC									
Percolated	60.0	0.1	1.0	0.9	25.4	1.7	8.0	2.9	< 0.1
Retained	73.6	0.1	0.8	0.4	19.9	< 0.1	4.1	1.1	< 0.1

explain these behaviours: for example the difference between campesterol and stigmasterol is a methylene on the side chain, and the difference between stigmasterol and β-sitosterol is a double bond. Therefore, it would seem that each steroid had its own collection behaviour.

The oil studied was not emulsified, nor was the chitosan dissolved in an aqueous system. The powders contained however a considerable amount of water (5–15%, w/w). Thus, the present results could be of value for interpreting the mechanism of interaction between dietary chitosan and lipids in the early moments after ingestion, when chitosan is not dissolved and lipids are not emulsified.

First of all, chitin had a high olive oil holding capacity. The chitin used did not undergo any treatment intended to depress its degree of crystallinity, therefore it might be expected that fully amorphous chitins could have even higher holding capacity. It is also evident that fatty acids had no particular capacity to form chitosan salts under these conditions, and did not appreciably react with *N*-lauryl chitosan by hydrophobic interaction. This scarce affinity did not prevent them from being retained by these polysaccharides, but their profile remained unaltered.

Diacylglycerols present in the olive oil became more or less enriched in the retained oil, depending on the nature of the polysaccharide: notably, chitin seemed to have scarce affinity to them (Table 5).

Table 5
Concentration (mg/100 g) of diacylglycerols in the percolated oil fractions (D-34 = 34 carbon atom diacylglycerols, D-36 = 36 carbon atom diacylglycerols)

Sample	D-34	D-36
Oil	602.8	2097.1
Percolated on chitin	363.8	1262.4
Percolated on chitosan	796.7	2820.2
Percolated on lauryl chitosan	339.8	1257.0
Percolated on DMAP-chitosan	237.1	1019.6

The fact that steroids interacted effectively with the polysaccharides under study, seems to be of the outmost importance, because it would imply that cholesterol might behave analogously.

Lee, Kim and Kim (1999) have pointed out that chitosan entering the small intestine has 1–3 h to combine with bile acids, but the reaction of chitosan or quaternary chitosan with glycocholate is very fast. It is also assumed that the previously dissolved chitosan would precipitate due to higher pH values of the intestine. As a matter of fact, chitosan, initially as a suspension, has already contacted dietary oils and fats and collected some, and possibly does not completely go into solution. It seems therefore plausible that steroids are already with chitin/chitosan at the time bile acids form chitosan salts in the intestine, rather than the contrary, i.e. steroids are collected by the chitosan bile salts.

4. Conclusions

The present preliminary study provides for the first time analytical data on the interactions between chitin and dietary oil, and indicates that certain olive oil components may be preferentially retained in the oil fraction that imbibes the chitin or the chitin derivative. This is verified in particular for plant steroids. Chitin is more effective in holding olive oil, and enriching the retained oil fraction with steroids.

The present work casts doubts on the need for high cationicity in order to sequester cholesterol, and points out that further research should consider chitin too, because chitin appears to have high capacity for steroids: Lee et al. (1999) as well as other authors indicate that quaternized DEAE-chitosan and other cationic chitosans have enhanced capacity for cholesterol but omit to include data for chitin. Moreover, Herrera and Mata-Segreda (1997) claim that

chitin has higher capacity for bile acids than chitosan: while their values for chitosan are lower than those of Lee et al. (1999), their findings deserve a verification (Jollès & Muzzarelli, 1999; Muzzarelli, 2000).

Work in progress in our laboratories intends to define the selective capacity of chitin and several chitin derivatives for bile acids and steroids.

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