University of Greenwich
Faculty of Engineering & Science
Central Avenue
Chatham Maritime
Kent, ME4 4TB

Samantha Booth BSc PhD PGCert MRSC
Email: s.e.booth@gre.ac.uk
Tel: 02083 319965

Chitosan Fat Binding Analysis and Trace Metal Determination

Dr Samantha Booth

Senior Lecturer in Inorganic Chemistry
Medway Sciences
University of Greenwich at Medway
Chatham Maritime
Kent
ME4 4TB

Author Biography

Dr Samantha E Booth BSc, PhD, MRSC

Senior Lecturer, Inorganic Chemistry

Department of Pharmaceutical, Chemical & Environmental Sciences

Faculty of Engineering & Science

E-mail: S.E.booth@gre.ac.uk

Dr Samantha Booth started her research career studying acid-base materials for dental repair using a combination of cements and bioactive glasses. She has been working at the interface of cell biology and inorganic chemistry ever since. Dr Booth has gone on to develop expertise in the development of biomaterials for bone repair. She maintains an active interest in outreach and currently runs several workshops for schools on different aspects of chemistry.

Research/scholarly interests

Expertise:

Antimicrobial/antibacterial bioactive materials, in vitro bioactivity, materials characterisation, textural analysis, infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), compressive and flexural strength testing, surface hardness analysis and inductively coupled plasma optical emission spectroscopy (ICP-OES).

Patent: Booth SE, Nicholson JW. (2012) Restorative Materials. Patent No. WO2012101432.

Publications:

Gjorgievska, Elizabeta, Van Tendeloo, Gustaaf, Nicholson, John W., Coleman, Nichola J., Slipper, Ian J. and Booth, Samantha (2015) The incorporation of nanoparticles into conventional glass-ionomer dental restorative cements. Microscopy and Microanalysis. ISSN 1431-9276 (Print), 1435-8115 (Online) (In Press) (doi: 10.1017/S1431927615000057)

Gjorgievska, Elizabeta S., Nicholson, John W., Apostolska, Sonja M., Coleman, Nichola J., Booth, Samantha E., Slipper, Ian J. and Mladenov, Mitko I. (2013) Interfacial properties of three different bioactive dentine substitutes. Microscopy and Microanalysis, 19 (6). pp. 1450-1457. ISSN 1431-9276 (Print), 1435-8115 (Online) (doi: 10.1017/S1431927613013573)

Lewis, Selma M., Coleman, Nichola J., Booth, Samantha E. and Nicholson, John W. (2013) Interaction of fluoride complexes derived from glass-ionomer cements with hydroxyapatite. Ceramics-Silikáty, 57 (3). pp. 196-200. ISSN 0862-5468 (Print), 1804-5847 (Online)

Hurt, Andrew P., Vine, George J., Booth, Samantha E. and Coleman, Nichola J. (2012) Preparation and antibacterial properties of Ag+-Exchanged Tobermorite-Chitosan films. International Scholarly and Scientific Research & Innovation. International Science Index , 6 (12):210. pp. 1178-1181.

Coleman, Nichola J., Bishop, Alistair H., Booth, Samantha E. and Nicholson, John W. (2009) Ag+- and Zn2+-exchange kinetics and antimicrobial properties of 11 angstrom tobermorites. Journal of the European Ceramic Society, 29 (6). pp. 1109-1117. ISSN 0955-2219 (doi: 10.1016/j.jeurceramsoc.2008.08.015)

Pawluk, K., Booth, S.E., Coleman, N.J. and Nicholson, J.W. (2008) The interaction of zinc oxide-based dental cements with aqueous solutions of potassium fluoride. Journal of Materials Science: Materials in Medicine, 19 (9). pp. 3035-3039. ISSN 0957-4530 (Print), 1573-4838 (Online) (doi: 10.1007/s10856-008-3443-0)

Fat Binding Report 1

Rapeseed oil and oleic acid were chosen for this fat binding study as they have been used for fat binding studies in the scientific literature for many years.

Examples:
Knorr, D. (1982), Functional Properties of Chitin and Chitosan. J Food Sci, 47: 593–595. doi: 10.1111/j.1365-2621.1982.tb10131.x

Sarbon, N.M., Sandanamsamy, S., Kamaruzaman, S.F.S. and Ahmad, (2015) F. Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties. J Food Sci Technol, 52(7):4266–4275. DOI 10.1007/s13197-014-1522-4

Von Der Haar, D. et al (2014) Rapeseed – tremendous potential for added value generation? Rapeseed proteins – Production methods and possible application ranges. J Oilseeds and Fats, Crops and Lipids, 21(1) DOI: 10.1051/ocl/2013038

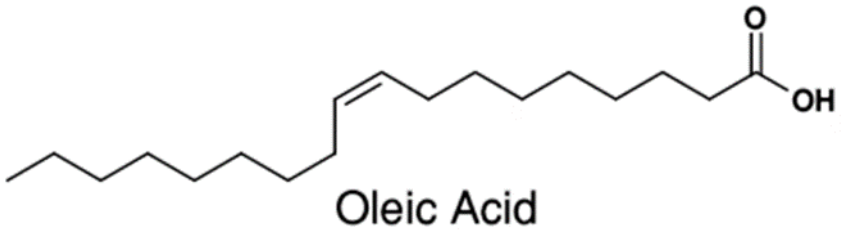
Cistola, D.P. et al (1988) Interactions of oleic acid with liver fatty acid binding protein: a carbon-13 NMR study. Biochemistry, 27 (2), pp 711–717 DOI: 10.1021/bi00402a033

Wydro, P., Krajewska, B. and Hąc-Wydro, K. (2007) Chitosan as a Lipid Binder: A Langmuir Monolayer Study of Chitosan−Lipid Interactions. Biomacromolecules, 8 (8), pp 2611–2617 DOI: 10.1021/bm700453x

Nauss, J.L., Thompson J.L., and Nagyvary, J. (1983) The binding of micellar lipids to chitosan. Lipids. 18 (10) pp 714-719.

Oleic acid

Oleic acid is an unsaturated fatty acid that occurs naturally in various animal and vegetable fats. Oleic acid has the chemical formula $C_{18}H_{34}O_2$.

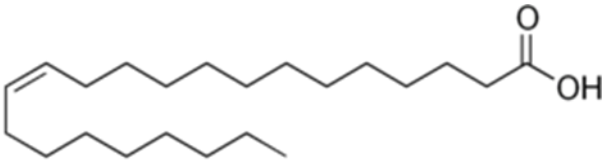


Oleic Acid

Fatty acids are the main components of food fats, oils and fat deposits in animals. The highest sources of oleic acid are avocados, olive oil, olives and canola oil. It is also found in beef tallow, lard and sunflower oil. Oleic acid is often used in food preparation to make products safe to eat for longer periods. These foods include bakery goods such as breads, cakes and pies. http://www.livestrong.com/article/438717-what-is-oleic-acid/

Rapeseed oil

Rapeseed oil, sometimes called vegetable or canola oil, is from the third most important crop grown in the UK after wheat and barley, and along with linseed are the only oils grown and bottled in the UK.
Rapeseed oil comes from the black seeds of the rapeseed plant, Brassica napus, from the same Brassica family as the vegetables broccoli, cabbage and cauliflower. http://www.rapeseedoilbenefits.com/guide-to-rapeseed-oil/what-is-rapeseed-oil.aspx
Natural rapeseed oil contains approximately 50% erucic acid. Erucic acid is an unsaturated fatty acid with the chemical formula $C_{22}H_{42}O_2$.



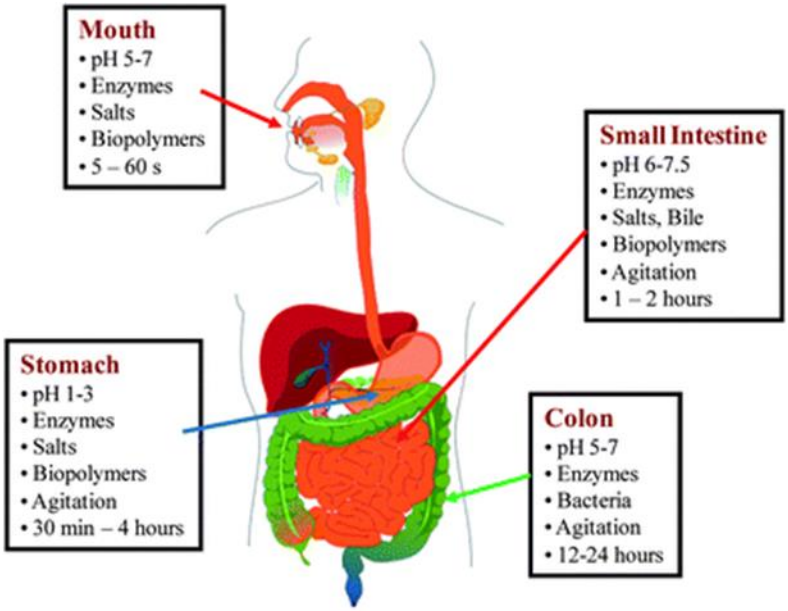
Erucic acid

Rapeseed oil has in the past been in the shadow of its better known Mediterranean counterparts, olive and sunflower oil, however, worldwide production of rapeseed (including canola) has increased sixfold between 1975 and 2007. http://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf The increased production of rapeseed has opened up the edible oil market for rapeseed oil making it one of the most popular edible oils found on the supermarket shelves. http://www.telegraph.co.uk/foodanddrink/11710297/Goodbye-olive-oil-why-weve-all-fallen-in-love-with-rapeseed.html

Methods for the fat binding study were taken from:
R. Czechowska-Biskup, B. Rokita, P. Ulanski and J.M. Rosaik. Radiation-induced and sonochemical degradation of chitosan as a way to increase its fat-binding capacity. Nuclear Instruments and Methods in Physics Research B, Vol. 236, 2005, p. 383-390.

J. Meler, J. Pluta, P. Ulanski, M. Krotkiewski, in H. Struszczyk (Ed.), Progress on Chemistry and Application of Chitin and Its Derivatives, Vol. IX, Polish Chitin Society, Lodz, 2003, p. 129.

For the determination of the fat-binding capacity of chitosan, a modification of the model developed by Meler was applied, allowing for in vitro simulation of the human digestive tract conditions (see diagram below for pH changes).



http://pubs.rsc.org/en/content/articlehtml/2010/fo/c0fo00111b

This process was performed for each batch of chitosan.

EC = European Chitosan
CC1 = Forza Chitosan batch 1 (G00735)
CC2 = Forza Chitosan batch 2 (G00736)

Rapeseed Oil - Commercially available, Borderfields, UK.
Steric Acid - Fisher, Analytical Reagent (saturated fatty acid)
Oleic Acid - Fisher, Analytical Reagent (unsaturated fatty acid)

Stage 1

12 ml of chitosan solution (7.5g/L, set with 0.1M HCL to pH 2 to mimic the natural stomach environment) and 3g of plant oil or fatty acid were placed into a 100ml conical flask and shaken at 300 rpm for 2 h at 37 °C in an orbital shaker.

After the time had elapsed, 0.1M NaOH was added to adjust the pH to 6.4, which corresponds to the pH of the duodenum fluid. Shaking was continued for 0.5 h.

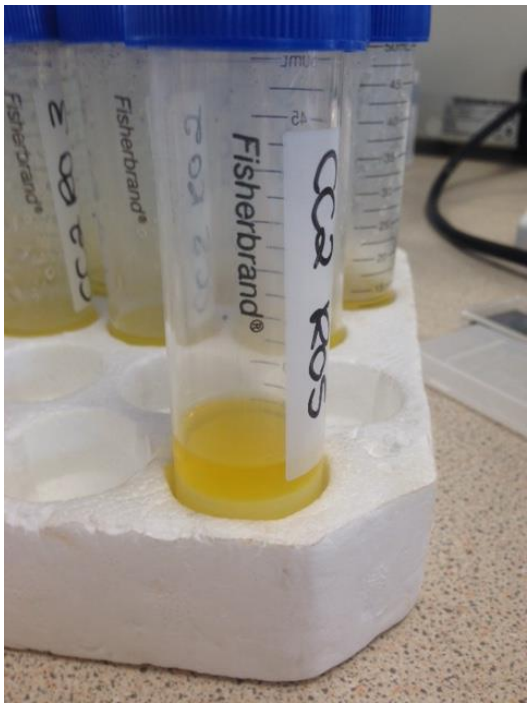
The pH was further adjusted to 7.0-7.6 to mimic the intestine and colon and shaken for a final 2.5 h.

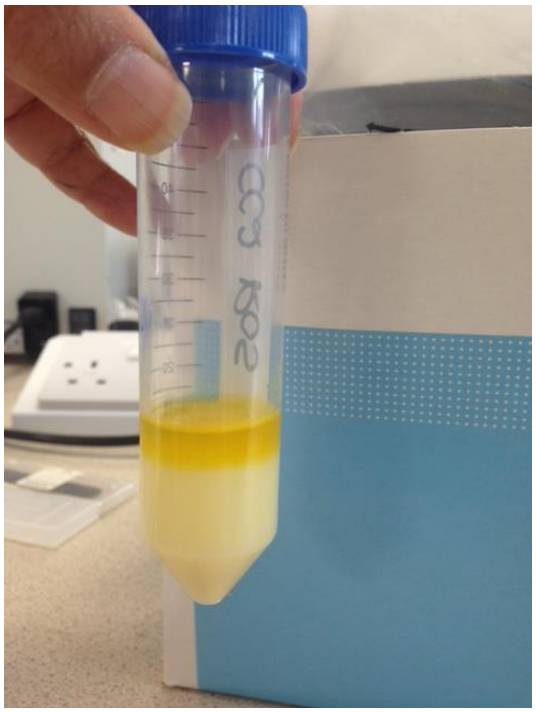
The solution was allowed to cool to room temperature and was centrifuged at 200 x g for 20 min. The layer of free, unbound oil/fatty acid was carefully, quantitatively removed.

This mass was used to calculate the fat binding capacity of the chitosan.

For each type of chitosan, five independent experiments were performed. And the presented results are the average values.

Rapeseed Oil Results – 3g





A supernatent layer of unbound rapeseed oil was present above the bound chitosan-oil layer.

	Unbound Mean (g)	Bound Mean (g)	Standard deviation	Binding capacity (%)	Ratio
EC	**1.9763**	**1.0237**	**0.0269**	**34.1227**	**1:11**
CC1	**1.7350**	**1.2650**	**0.1277**	**42.1653**	**1:14**
CC2	**1.7065**	**1.2935**	**0.1986**	**43.1160**	**1:14**

To work out the ratio:
There is 7.5g chitosan in the stock solution (1000ml)
There is 0.0075g in 1 ml of the stock solution.
The sample size was 12 ml, this equates to (0.0075x12) = 0.09g.
0.09g of EC binds 1.0237g oil - equal to 1g EC binding 11.37g oil
0.09g of CC1 binds 1.2650g oil – equal to 1g CC1 binding 14.05g oil
0.09g CC2 binds 1.2935g oil – equal to 1g CC2 binding 14.37g oil.

A t-test was used to determine if the CC batches were significantly different to the EC batch.

The CC1 batch is significantly different to EC ($P<0.01$)
The CC2 batch is significantly different to EC ($P<0.05$)

Czechowska-Biskup et al used rapeseed oil for their fat binding study. They estimated that 1g of chitosan could bind between 8-20g of rapeseed oil. The values in this study fall between the predicted range.

Steric Acid Results – 3g
Steric acid remained as a solid in the shaker. The chitosan could not thoroughly mix with the steric acid (apart from surface contact) on shaking so using steric acid was deemed unfeasible and eliminated from the study.

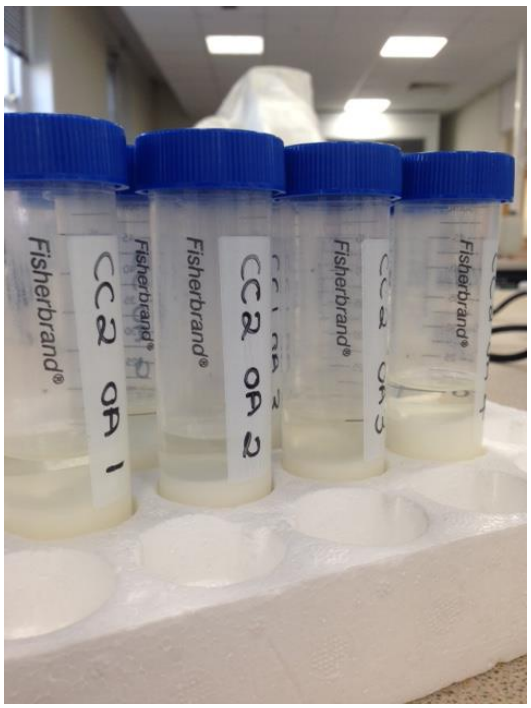
Oleic Acid Results – 3g
There was no supernatent layer of unbound oil when using 3g of oleic acid. This indicated all of the acid had been bound to all three types of chitosan. In addition, the samples had physically solidified, indicating that there was no unbound acid. To determine a binding capacity it was necessary to scale up the quantity of acid used.

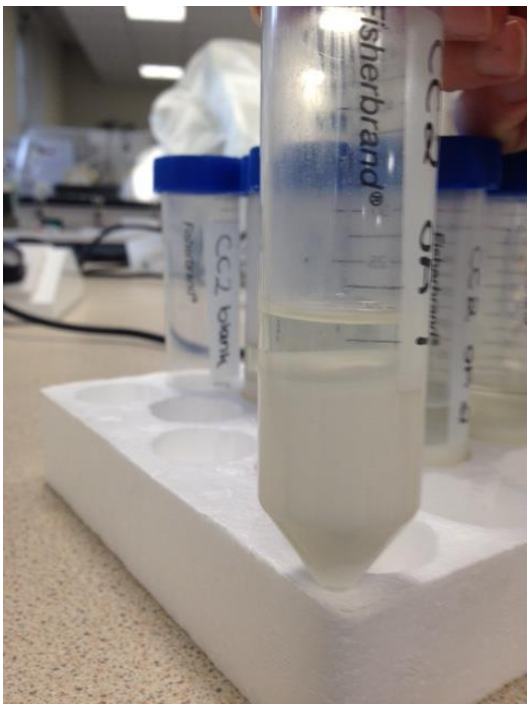
<u>Stage 2</u>
The procedure in stage 1 was followed with the exception of the mass of oleic acid being changed to 6g.

An additional parallel study was set up using 9g of oleic acid.

Oleic Acid Results – 6g
A supernatent layer of unbound oleic acid was present but was so small it was difficult to quantitatively remove and accuracy was reduced. These results were discarded.

Oleic Acid Results – 9g





A clear supernatent layer of unbound oleic acid was present above the bound chitosan-acid layer.

	Unbound Mean (g)	Bound Mean (g)	Standard deviation	Binding capacity (%)	Ratio
EC	**4.8807**	**4.1193**	**0.0671**	**45.7698**	**1:45**
CC1	**3.8051**	**5.1949**	**0.1233**	**57.7218**	**1:57**
CC2	**3.5727**	**5.4273**	**0.1392**	**60.3031**	**1:60**

To work out the ratio:
There is 7.5g chitosan in the stock solution (1000ml)
There is 0.0075g in 1 ml of the stock solution.
The sample size was 12 ml, this equates to (0.0075x12) = 0.09g.
0.09g of EC binds 4.1193g acid - equal to 1g EC binding 45.77g acid
0.09g of CC1 binds 5.1949g acid – equal to 1g CC1 binding 57.72g acid
0.09g CC2 binds 5.4273g acid – equal to 1g CC2 binding 60.30g acid.

A t-test was used to determine if the CC batches were significantly different to the EC batch.

The CC1 batch is significantly different to EC ($P<0.001$)
The CC2 batch is significantly different to EC ($P<0.001$)

Summary:
The binding capacity of the Forza Chitosan (CC) batches is higher than the EC batches. The increase in capacity varies between the rapeseed oil and oleic acid tested.

Recommendations for future work:
A replica study should be conducted using a different fatty acid and oil to ensure the fat binding capacities are in a similar range.

Fat Binding Report 2 – Prickly Pear (Litramine)

Methods were taken from:
R. Czechowska-Biskup, B. Rokita, P. Ulanski and J.M. Rosaik. Radiation-induced and sonochemical degradation of chitosan as a way to increase its fat-binding capacity. Nuclear Instruments and Methods in Physics Research B, Vol. 236, 2005, p. 383-390.

J. Meler, J. Pluta, P. Ulanski, M. Krotkiewski, in H. Struszczyk (Ed.), Progress on Chemistry and Application of Chitin and Its Derivatives, Vol. IX, Polish Chitin Society, Lodz, 2003, p. 129.

For the determination of the fat-binding capacity of chitosan, a modification of the model developed by Meler was applied, allowing for in vitro simulation of the human digestive tract conditions.

This process was performed for the prickly pear tablets.

PP = Prickly Pear
The prickly pear was tested against rapeseed oil and oleic acid.
Rapeseed Oil - Commercially available, Borderfields, UK.
Oleic Acid - Fisher, Analytical Reagent (unsaturated fatty acid)

Experimental

12 ml of PP solution (7.5g/L, set with 0.1M HCL to pH 2 to mimic the natural stomach environment [see report 1 for stomach diagram]) and 3g of plant oil or fatty acid were placed into a 100ml conical flask and shaken at 300 rpm for 2 h at 37 °C in an orbital shaker.

After the time had elapsed, 0.1M NaOH was added to adjust the pH to 6.4, which corresponds to the pH of the duodenum fluid. Shaking was continued for 0.5 h.

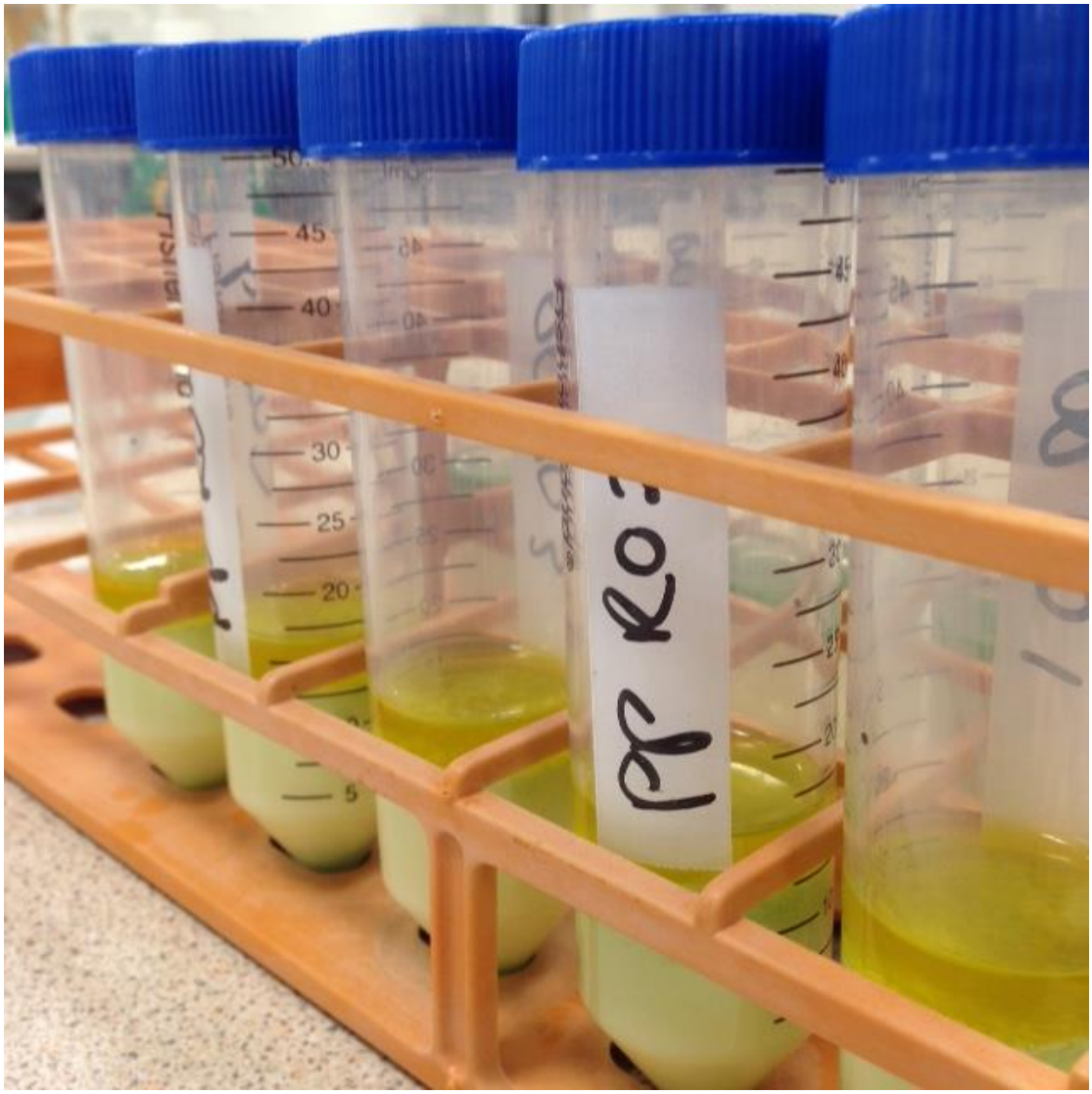
The pH was further adjusted to 7.0-7.6 to mimic the intestine and colon and shaken for a final 2.5 h.

The solution was allowed to cool to room temperature and was centrifuged at 200 x g for 20 min. The layer of free, unbound oil/fatty acid was carefully, quantitatively removed.

This mass was used to calculate the fat binding capacity of the chitosan.

Five independent experiments were performed for each oil or acid. The presented results are the average values.

Rapeseed Oil Results – 3g



A supernatent layer of unbound rapeseed oil was present above the bound PP-oil layer.

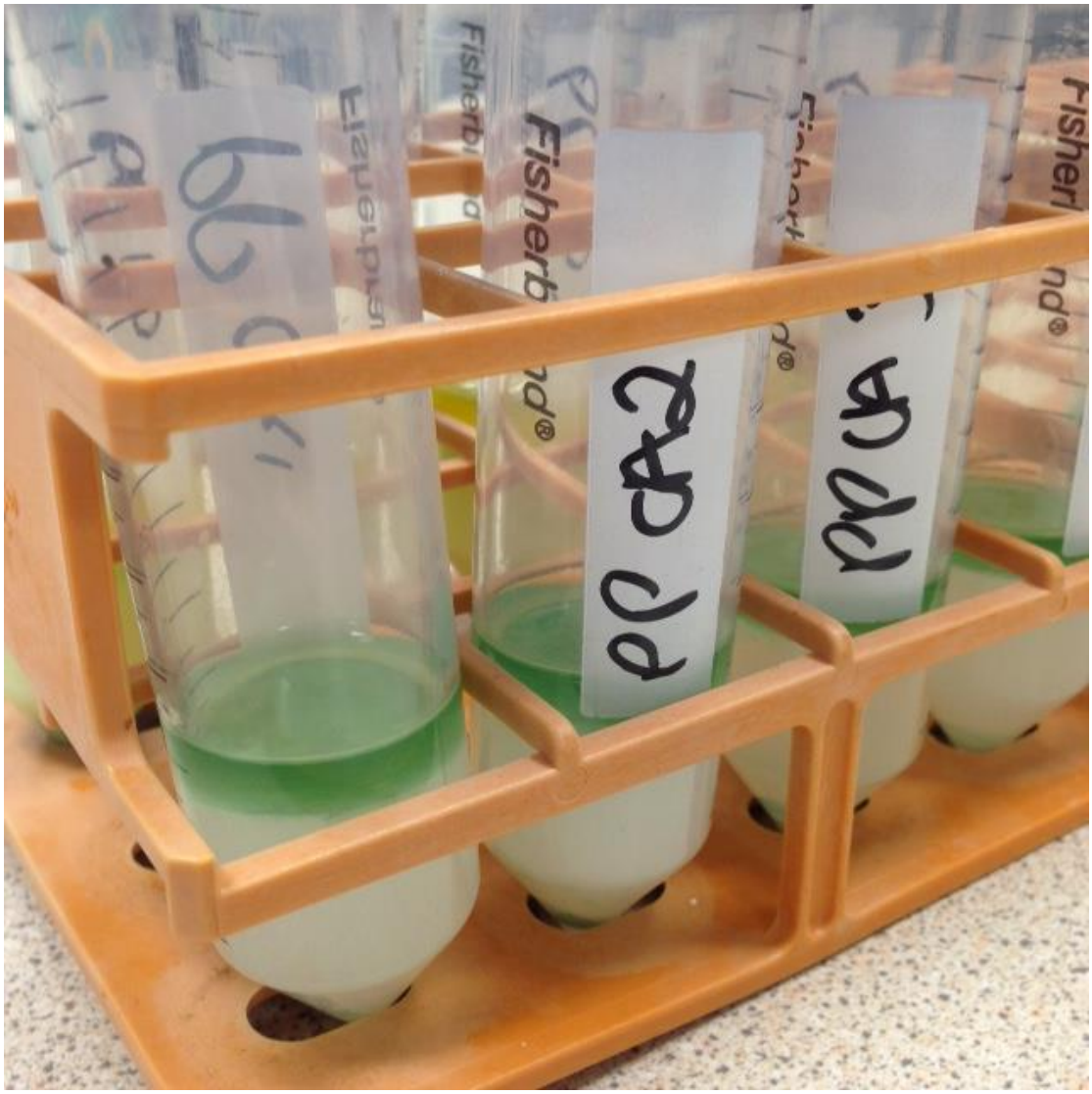
	Unbound Mean (g)	Bound Mean (g)	Standard deviation	Binding capacity (%)	Ratio
PP	**1.9714**	**1.0286**	**0.0601**	**34.2866**	**1:11**

To work out the ratio:
There is 7.5g PP in the stock solution (1000ml)
There is 0.0075g in 1 ml of the stock solution.
The sample size was 12 ml, this equates to (0.0075x12) = 0.09g.
0.09g of PP binds 1.0286g oil - equal to 1g PP binding 11.43g oil

A t-test was used to determine if the Forza Chitosan data (fat binding report 1) were significantly different to the PP batch.

The binding capacity of Forza Chitosan is significantly different to the PP ($P>0.05$ but less than 0.01)

Oleic Acid Results – 3g



A supernatent layer of unbound oleic acid was present above the bound PP-acid layer.

	Unbound Mean (g)	Bound Mean (g)	Standard deviation	Binding capacity (%)	Ratio
PP	**1.5734**	**1.4266**	**0.1232**	**47.55**	**1:15**

To work out the ratio:
There is 7.5g PP in the stock solution (1000ml)
There is 0.0075g in 1 ml of the stock solution.
The sample size was 12 ml, this equates to (0.0075x12) = 0.09g.
0.09g PP binds 1.4266g acid – equal to 1g PP binding 15.85g acid.

A t-test was used to determine if the Forza Chitosan data (from fat binding report 1) were significantly different to the PP batch.

The binding capacity of Forza Chitosan is significantly different to the PP ($P>0.001$)

Summary:
The binding capacity of the Forza chitosan is significantly higher than the PP alternative. However, the level of significance varies with the specific oil/acid used.

Metal Determination Report

Two digestion methods were used to mimic the chitosan at different pH (acidic and alkali)

Alkali Digestion

This process was performed for each batch of chitosan.

EC = European Chitosan
CC1 = Forza Chitosan batch 1 (G00735)
CC2 = Forza Chitosan batch 2 (G00736)

12ml of chitosan solution (7.5g/L, set with 0.1M HCl to pH 2) was pipetted into a 100ml conical flask and shaken at 37 °C for 2 hours at 300rpm to mimic the natural stomach environment.
The pH was adjusted with 0.1M sodium hydroxide to pH 6.4 and shaking was continued for 0.5 hours to mimic the duodenum fluid.
The pH was further adjusted to pH 7.0-7.6 to mimic the intestine and colon and shaken for a final 2.5 hours.
The solution was cooled to room temperature and centrifuged at (2000 x g, 20 min). 1ml of the supernatent layer was removed and placed in a clean 15 ml centrifuge tube in preparation for ICP-OES analysis. 4 ml of de-ionised water was added to the 1 ml of supernatent and the solution inverted several times to ensure thorough mixing. Five replicates were made for each batch of chitosan.

Image shows a chitosan pellet after being centrifuged, with a clear liquor on top (Batch CC1, replicate 3).

Acid Digestion

This process was performed for each batch of chitosan.

EC = European Chitosan
CC1 = Forza Chitosan batch 1
CC2 = Forza Chitosan batch 2

12ml of chitosan solution (7.5g/L pH 2) was centrifuged at 2000 x g for 20 minutes.

1 ml of the solution was placed in a clean 15 ml centrifuge tube in preparation for ICP-OES analysis. 4 ml of de-ionised water was added and the solution inverted several times to ensure thorough mixing.

Five replicates were made for each batch of chitosan.

ICP-OES

Cd, Cu, Ni, Pb, Hg, Al, Cr and Zn

The diluted samples were run through the Optima 4300 Inductively Coupled Plasma Optical Emission Spectrometer at 1.5ml/min. The system was flushed with 0.1M HNO_3 between each sample.

Standards for cadmium, copper, nickel, lead, mercury, aluminium, chromium and zinc were made at 0.1, 1, 5, 12.5 and 25 ppm.

The metals (Cd, Cu, Ni, Pb, Hg, Al, Cr and Zn) were all below the detection limit of the instrument in all of the samples tested.

Ca and Mg

Standards for magnesium and calcium were made at 1, 10, 50 and 100 ppm.

Means and standard deviations of the results are given below:

Ca

	5 ml			
	Alkali		Acid	
	Mean	S.D.	Mean	S.D.
EC	-	-	**0.254**	0.003
CC1	**1.087**	0.187	**3.050**	0.024
CC2	**0.522**	0.256	**3.099**	0.029

After calculating the for the dilution factor:

	5 ml			
	Alkali		Acid	
	Mean	S.D.	Mean	S.D.
EC	-	-	**1.27**	0.003
CC1	**5.44**	0.187	**15.25**	0.024
CC2	**2.61**	0.256	**15.49**	0.029

Mg

	5 ml			
	Alkali		Acid	
	Mean	S.D.	Mean	S.D.
EC	-	-	-	-
CC1	-	-	**0.785**	0.007
CC2	-	-	**0.807**	0.011

After calculating the for the dilution factor:

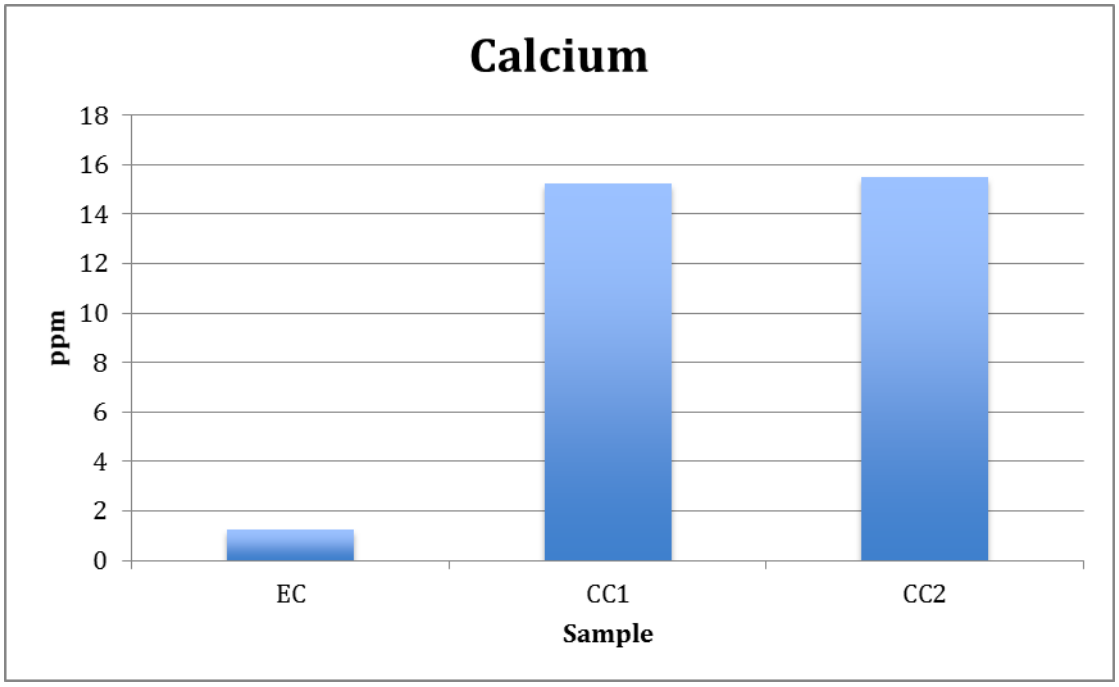
	5 ml			
	Alkali		Acid	
	Mean	S.D.	Mean	S.D.
EC	-	-	-	-
CC1	-	-	**3.93**	0.007
CC2	-	-	**4.04**	0.011

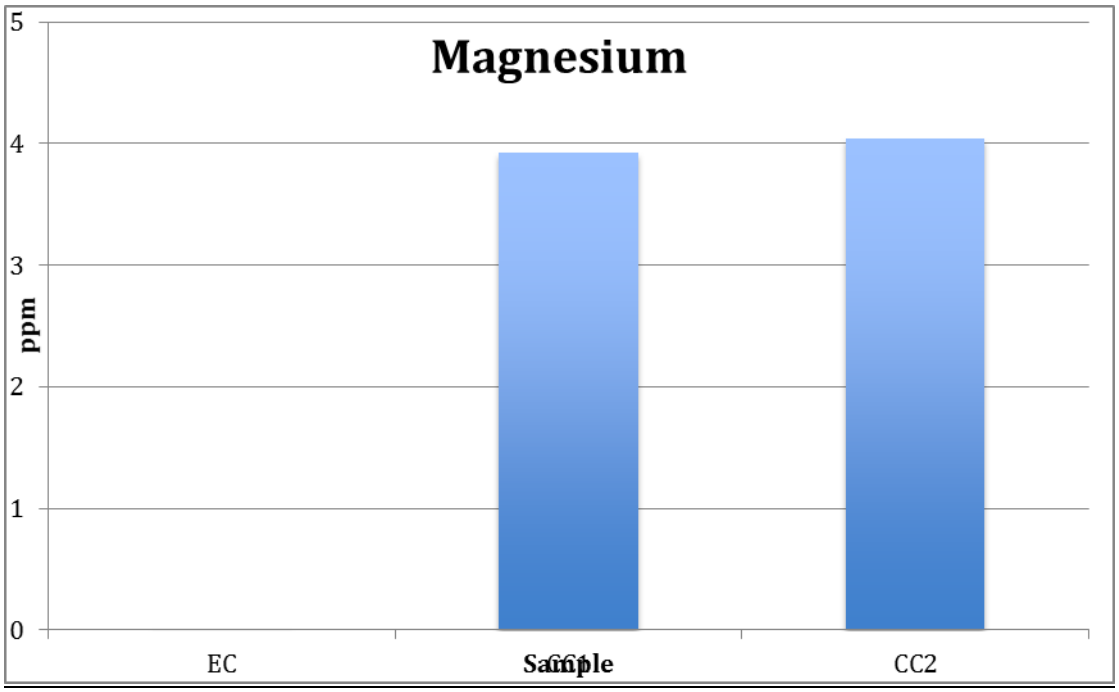
Values are given in ppm. Where there is no value, the result is below the detection limit of the instrument.

Summary:
The results indicate that at an alkaline pH, the Ca and Mg ions are mostly bound to the chitosan, as you would expect, as the chitosan is solid at this pH.

The acidified samples show the results where the chitosan has dissolved at the lower pH. The results indicate that Ca and Mg are released at this low pH, in larger quantities, for the Forza Chitosan batches.

Graphical representation of the acid digested chitosan samples:





Quotes for Forza:

On the Forza Chitosan compared to the European Chitosan:

'The Forza Chitosan binds similar quantities of rapeseed oil to the European alternative'

'The Forza Chitosan binds significantly more (15% more) oleic acid than the European alternative'

'The Forza Chitosan does not contain heavy metals'

On the Forza Chitosan compared to the Prickly Pear alternative:

'The Forza Chitosan binds significantly more (10% more) rapeseed oil than the Prickly Pear alternative.'

'The Forza Chitosan binds almost 4 times (actual = 3.8 times) as much fatty acid compared to the Prickly Pear alternative (380% more)'