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Improving Extraction Processes Of Crustacean Chitin Using Solid State Analytical Techniques

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Abstract

Solid state analytical techniques are becoming more widely used for the analysis of a range of organic products which demonstrate very poor solubility in both common organic and polar solvents and as such cannot be accurately characterised using solution based techniques. Primarily used as a secondary technique for qualitative analysis of insoluble intermediates and products in organic synthesis, ^{13}C CP-MAS NMR can be utilised in tandem with a targeted extraction and clean up procedure for accurate quantitative analysis of insoluble bio-molecules of interest. Here solid state ^{13}C CP-MAS NMR is utilised as the primary analytical technique in the characterisation of crustacean sourced chitin whereby Cancer pagurus crab shell chitin and Pandalus borealis shrimp shell chitin are shown to have a degree of acetylation greater than 90%. FTIR spectroscopy, Raman spectroscopy and DSC provide secondary structural, molecular and thermal analysis of the raw materials and extracted chitin.

Keywords — *Chitin, crab, shrimp, enzymatic, extraction, solid-state, analysis.*

I. INTRODUCTION

Rapid accurate analysis of crustacean sourced chitin is sought after increasingly in industry as 70% annual global shellfish production ends up in waste streams in landfill, incineration or dumped at sea. In recent years the valorisation of fisheries waste streams for chitin by enzymatic, bacterial or chemical treatment has become especially sought after due to the anti-inflammatory and anti-bacterial properties of the bio-polymer as well as due to the large potential for its use in bio-plastics and in value added products in the food and nutraceuticals sector [1]–[4].

The percentage degree of acetylation (%DA) of chitin (β-(1,4)-N-acetyl-D-glucosamine) dictates properties such as solubility, particle size and thermal stability. Fig 1 shows the structure of chitin and the de-acetylated derivative chitosan (β-(1,4)-amino-D-glucosamine).

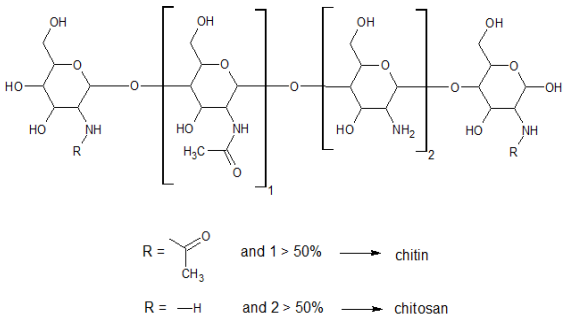

Fig 1: Monomer Structures of Chitin & Chitosan

^{1}H NMR is well established in the literature for accurate determination of low %DA in chitosan [5]. The liquid state technique requires dissolution of chitosan in 2%v/v deuterated acetic acid or 2%v/v DCl for samples that are closer to 50%DA. Determining accurate integration values for peaks can be problematic due to convolution or obstruction by large deuterium oxide solvent peaks. This is overcome by using a temperature programme allowing for analysis at 85˚C, whereby the solvent peaks are shifted and no longer obscure the peaks of interest [6]. Samples require extensive chemical clean up prior to analysis in order to produce clean spectra with well resolved peaks allowing for accurate integrations for use in calculation of the %DA.

Due to dense hydrogen bonding between polymer chains the major challenge in characterising chitin is its poor solubility in any polar or organic solvent [2], [5], [7] - thus ^{1}H NMR is not viable for analysis of chitin and so ^{13}C Cross Polarization - Magic Angle Spinning (CP-MAS) NMR is explored as an alternative, comparably accurate and sensitive solid state technique [8].

FTIR and Raman spectroscopy are well documented as common solid state techniques utilised in identification of chitin [9]–[12]. They are limited to qualitative analysis due to the convolution of peaks and non-linear responses to changes in %DA between samples [10], [11], [13]. These techniques give a good indication of the %DA of a

sample and are particularly useful as rapid tools for indicating how pure and clean a sample is before and after any clean-up is applied.

Differential Scanning Calorimetry (DSC) is similarly documented as a solid state technique which allows for analysis of the thermal profile of a chitin sample [14]–[16]. DSC does not allow for quantitative analysis of %DA nor does it indicate purity well. The main property indicated by DSC analysis is the polymorphic state and thermal stability of a sample. Chitin naturally occurs in two polymorphs; the most common α-chitin, whereby the polymer lies in tightly compacted alternating sheets of antiparallel chains, or β-chitin, whereby the polymer lies in less compacted parallel chains [6], [9], [12], [17].

FTIR, Raman and DSC are used in this study as secondary solid state techniques for the qualitative analysis of chitin samples to supplement the quantitative analysis via solid state NMR.

The optimisation of the chemical extraction techniques required for isolation of high purity chitin from crustacean sources is significant regarding industrial waste streams and scalability, especially when comparing the products with chitin from the same source isolated by enzymatic treatment. Analysis of raw samples determines the necessity of the demineralisation, deproteinisation and depigmentation steps to allow for accurate analysis by each analytical technique. Chemical extraction is optimised under the principles of green chemistry allowing for savings in time and materials required and reduction in environmental impact when scaled-up. Optimisation is performed in combination by review of literature and in-house replicate studies.

II. MATERIALS AND METHODS

A. *Optimised Chemical Extraction Procedure*

a). Portioning of Shell Samples:

10g of each sample to be extracted was thawed in a fridge at 4˚C overnight. Once thawed the samples were placed in an oven at 60˚C overnight to remove excess water.

b). Demineralisation:

0.5M HCl was added to each sample in the ratio of 5cm^3 per 1g of dry sample. Each mixture was placed in a 250cm^3 conical flask. The mixtures were stirred at 240rpm at room temperature for 2hrs. After 2hrs each sample was centrifuged at 2800RCF at room temperature for 10mins. The supernatant, which contains the mineral fraction, was decanted off as waste and the pellets were retained. The steps above were repeated twice more, to give a total of three demineralization washes. The sample pellets were then filtered and washed with deionized water using a vacuum filtration apparatus. Samples were filtered to dryness as much as possible. Samples were then transferred to sample vials and stored in a fridge at 4˚C.

c), Deproteinisation:

Samples were transferred into 250cm^3 conical flasks using 0.5M NaOH in the ratio 5cm^3 per 1g of dry sample. The mixtures were stirred at 240rpm at >85˚C for 1hr. After 1hr each sample was cooled to room temperature using an ice bath and then was centrifuged at 2800RCF for 10mins at room temperature. The supernatant, which contains the protein fraction, was decanted off as waste and the pellets were retained. The steps above were repeated twice more, to give a total of three deproteinisation washes. The sample pellets were then filtered and washed with deionized water using a vacuum filtration apparatus. Samples were washed until pH of 7 was reached as indicated by an electronic pH meter. Samples were filtered to dryness as much as possible. Samples were then transferred to sample vials and stored in a fridge at 4˚C.

d). Depigmentation:

Mild oxidising reagent, 30w/w% H_2O_2:0.5M HCl in a ratio of 9:1, was added to each sample in the ration of 10cm^3 per 1g in 250cm^3 conical flasks. The mixtures were stirred at 240rpm at room temperature until visible absence of pigment was observed. This required the reaction to run overnight for 15hrs until each sample was completely absent of pigment. Samples were then filtered and washed with 3 x 100cm^3 of deionised water. Samples were filtered to dryness and transferred to sample vials and stored in a fridge at 4˚C.

e). *Freeze Drying:*

All extracted samples were freeze dried for 48 hours prior to analysis to ensure no excess water remained.

B. *Solid State Analysis*

a). ^{13}C CP-MAS NMR:

CP-MAS NMR was performed using a Bruker 400MHz Ultrashield NMR with solid state CP-MAS probe. Optimised parameters are 128 scans, spin rate of 10kHz, 60kHz carbon polarisation with contact time of 1ms at 25˚C [9], [30]–[32]. Output data is plot of Signal Intensity (Rel. units) vs. Chemical Shift (ppm). Run time is 20min per sample.

b). Raman Spectroscopy:

Raman analysis was performed over the frequency range of 3600 – 200cm^{-1} using a Horiba HR800 UV Spectrometer, with a laser line of 785nm and laser line of 532nm. The output data is a plot of Intensity vs. Wavenumber (cm^{-1}). Optimised parameters for analysis with laser line 785nm are: Acquisition time of 200s, Accumulation of 3 and x10

Objective [5], [10], [11], [24]. Run time is 30min per sample.

c). FTIR Spectroscopy:

FTIR Analysis was performed over the frequency range of 4000 – 550cm^{-1} using a Perkin Elmer Spectrum 100 FTIR Spectrometer with ATR attachment. The output data is a plot of %Transmission vs. Wavenumber (cm-1). Parameters used were 4 scans per sample. Run time is approximately 60s per sample [6], [20], [28].

d). Differential Scanning Calorimetry:

DSC was carried out using an Instrument Specialists Inc. DSC 650 in an atmosphere of air. The sample cell and the empty reference cell were heated from 40° to 350°C at a rate of 10°C/min. There was no hold time. The output data is a plot of Heat Flow (mW) vs. Temperature (˚C). Run time is 50mins per sample [14], [16].

III. RESULTS AND DISCUSSION

A. Extraction

The extraction of chitin from crustacean shells is well documented in the literature [6], [16], [18–22]. Extraction consists of three major steps; demineralisation, deproteinisation and depigmentation.

Demineralisation is the removal of CaCO3, calcium carbonate, from the organic matrix of the shell. Demineralisation is achieved by washing the shell samples with dilute HCl at room temperature. When washed with acid calcium ions, carbon dioxide gas and carbonic acid gas are liberated. The calcium, phosphate and carbonate ions remain in solution whereas the insoluble chitin does not. Thus using centrifugation and filtration the mineral content is removed from the chitin. The molarity of the acid and the length of time washing is performed for, are dependent on the taxonomy, source, environment and pre-treatment of the sample. Thus for each sample type the extraction procedure must be optimised. For the initial extraction technique the template detailed in Tolaimate, et al. [22] is used. Reference [22] reports the most comprehensive volume of information regarding treatment of multiple different types of crustacean samples. Thus, 0.5M HCl is used, washings last 2hrs with a total of 3 washes performed. This is the recommended approach for red crab shell samples. The end point of the demineralization reaction is deemed by the cessation of carbonic acid gas and carbon dioxide gas being released from the solution. The vast majority of the shell mineral is CaCO3 as calcite, amorphous $CaCO_3$ and $Ca_3(PO_4)_2$ as hydroxyapatite [23].

Deproteinisation is achieved by washing the samples in 0.5M NaOH solution at >85˚C for 1hr. This wash is performed 3 times, whereby the end point is indicated by a lack of colour in the reaction medium and the filtrate. The molarity of the base and the length of time washing is performed for, are again dependent on the taxonomy, source, environment and pre-treatment of the sample. Thus for each sample the extraction procedure must be optimised. For the initial extraction technique the template detailed in Tolaimate, et al. [22] is once again used.

Depigmentation is the final step of the extraction of chitin from crab shells. It is not commonly discussed in the literature, thus, it is suspected that depigmentation may only be performed in certain cases for aesthetic reasons. The depigmentation is achieved by treating the samples with a mild oxidising reagent consisting of H_2O_2 : HCl in a 9 : 1 ratio. This oxidation cleaves and substitutes bonds along the conjugated system of the pigment molecule. The predominant pigment molecule in crab shells is astaxanthin, a carotenoid, the structure of which is shown in Fig 2. The deproteinisation step can also result in the loss of pigment as the layers of asthaxanthin, known as crustacyanin (2 layers = β, 8 layers = α), are degraded by the basic wash at >85˚C [8].

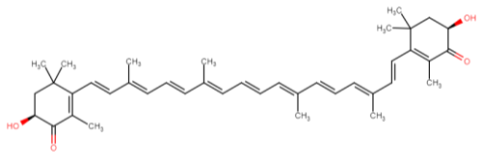

Fig 2: Astaxanthin Pigment

Extraction was performed in replicate. The preliminary extraction included demineralisation, deproteinisation and depigmentation. The optimised extraction process flow, seen in Fig 3, does not include the depigmentation step as the presence of the pigment is shown not to impede accurate analysis by FTIR or Raman spectroscopy. The presence of astaxanthin does not cause the peaks of interest for characterisation of chitin to be obscured on spectra from either qualitative technique. Pigment is seen to be in such low concentration that its presence similarly does not affect analysis by DSC or ^{13}C CP-MAS NMR.

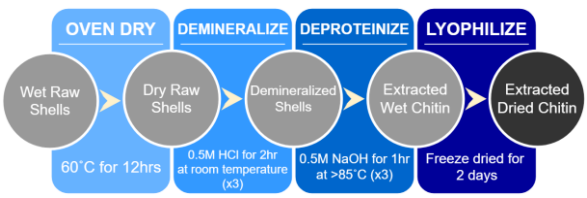

Fig 3: Optimised Extraction Process Flow

All samples were pre-dried before extraction to maximise yields and reduce the amount of solvent used per gram of raw sample. Chitin is known to constitute 15-25% of dry crustacean shell weight [5]-[9], [11]. The increase in yields of the crab shell chitin due to pre drying is shown in Table 1. It is reasonable to see low yield for the sample containing

tissue after drying as excess protein and fats remain in high quantities. However, the samples of solely shell material come up to the level of expected chitin content.

TABLE I

%YIELD OF PRELIMINARY CRAB SHELL EXTRACTION PRODUCTS AND %YIELD OF OPTIMISED CRAB SHELL EXTRACTION PRODUCTS.

Sample	Preliminary %Yield	Optimised %Yield
Fine Ground Shell	6.13%	19.74%
Very Fine Ground Shell and Tissue	2.96%	7.11%
Coarse Ground Shell	8.08%	17.73%

B. ^{13}C CP--MAS NMR

%DA is determined via ^{13}C CP-MAS NMR by relative comparison of the integral of the C-1 peak with integral of the C-H3 peak using Equation 1.[30,31] The carbons as labelled by CP-MAS NMR are shown in Fig 4 [12], [31], [32]. The C-1 peak is used for comparison as the ratio of carbons represented is 1:1 with the C-H3 peak. The C-4, C-5, and C-3 peaks are convoluted, with a 3:1 ratio of carbons represented compared to the C-H3 peak. The C-6 and C-2 peaks are also convoluted with a ratio of 2:1. For relative comparison of the integrals of these peaks with the integral of the C-H3 peak, normalisation is required whereby the integrals are divided by 3 and 2, respectively, to give ratios of carbons represented of 1:1. This introduces error into the calculation of %DA as the normalised integral values are approximations of individually resolved peaks. Both the C-1 and C-H3 peaks show good resolution, with baseline resolution achieved for all extracted samples. This resolution allows for more accurate integral values and therefore more accurate %DA determination.

$$\frac{\text{C–H3 Integral}}{\text{C–1 Integral}} \times 100 \qquad \text{(Equation 1)}$$

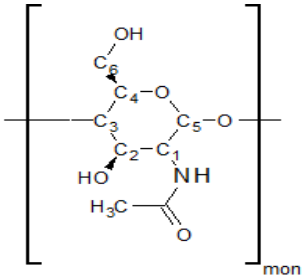

Fig 4: CP-MAS Labelled Carbons in the Chitin Monomer Structure

Listed in Table II are the three standards analysed via ^{13}C CP-MAS NMR, all of which have been previously standardised by multiple ^{1}H NMR techniques and have been used as QC standards for %DA determination of chitosan via ^{1}H NMR. Due to the poor solubility of chitin, the SA_CH standard has not been standardised via ^{1}H NMR and thus has an unknown %DA. It is therefore not considered a standard for the purposes of the ^{13}C CP-MAS analysis, but rather regarded as a sample with unknown %DA. %DA of the FungalCS, ShrimpCH5 and SA_CS standards, see Fig 5, were experimentally determined with a %recovery within the acceptable limits for accuracy of 90-110% of the known %DA [33]. The ^{13}C CP-MAS technique is therefore deemed to accurately determine the %DA of the standards.

TABLE II

CP-MAS DETERMINED %DA OF STANDARDS.

Standard	Known %DA	Experimental %DA	%Recovery
FungalCS	16.00	15.81	98.81
ShrimpCH	81.00	80.39	99.25
SA_CS	18.40	19.07	103.56

The spectra of all extracted samples, shown in Fig 5, had excellent signal to noise ratios, far greater than the 10:1 minimum requirement for accurate quantitative analysis. As the determination of %DA of the standards is shown to be accurate, the determined values of %DA of each of the extracted samples, shown in Fig 6, are therefore considered accurate.

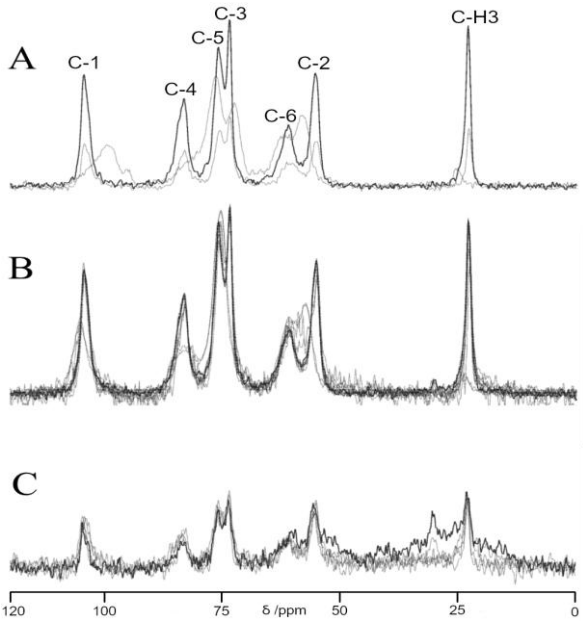

Fig 5: Overlayed CP-MAS Spectra of Standards (A), Extracted Samples (B), and Raw Samples (C)

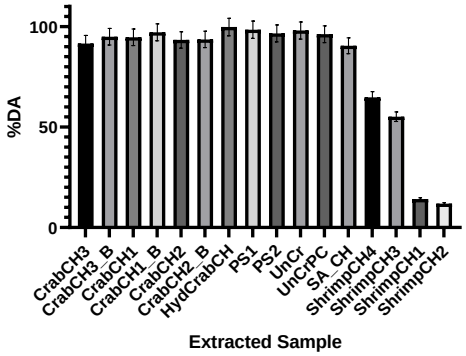

Fig 6: CP-MAS Determined %DA of Extracted Samples

To determine the necessity of the extraction procedure for accurate %DA determination, raw samples were also analysed via ^{13}C CP-MAS. The spectra of these raw samples, shown in Fig 5, have very poor signal to noise ratios, with peaks often below the 3:1 limit of detection. Similarly, a hump is seen in the spectra which is indicative of the presence of protein in the sample [6]. This also decreases accuracy as peaks are not baseline resolved. The experimentally determined values of %DA for the raw samples, shown in Fig 7, are greater than 100%DA which demonstrates the poor accuracy.

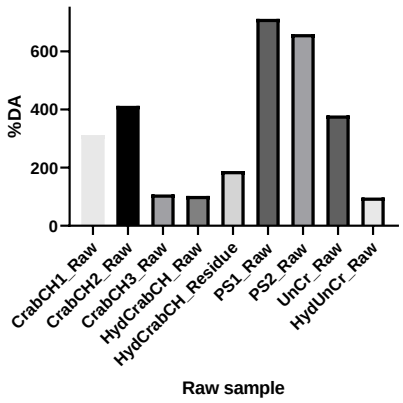

Fig 7: CP-MAS Determined %DA of Raw Samples

There is one exception to this, the HydUnCr_Raw sample has received sufficient clean up by enzymatic hydrolysis to allow for accurate integration and comparison of peaks using half height parameters, as shown in Fig 8. The %DA determined for the HydUnCr_Raw sample is 96.95%DA whereas the %DA determined for the chemically extracted HydUnCr sample is 96.17%DA. Although extraction is deemed necessary for accurate analysis of the majority of raw samples, analysis of the HydUnCr_Raw indicates well the power of the ^{13}C CP-MAS NMR technique in accurately characterising samples that retain protein fractions and mineral fractions. Similarly the technique demonstrates the efficacy of enzymatic treatment in comparison to chemical extraction/clean up [34]–[37].

As the ^{13}C CP-MAS analysis is carbon specific, the C-H3 peak intensity and integral increases proportionally with increase in acetylation. Thus the technique is most viable for use with samples of high %DA chitin samples. The technique is shown here to be accurate to as low as 10% DA. However below this the signal to noise ratio of the C-H3 peak is below the minimum requirement of 10:1 for quantification and thus does not accurately determine %DA for very low %DA samples. It is also noted that the lower the %DA of a sample, the greater the convolution of C-4, C-5, and C-3 peaks as well as the C-6 and C-2 peaks. The poor accuracy of the technique for samples below 10%DA is overcome by complimenting the ^{13}C CP-MAS NMR technique with the established ^{1}H NMR technique which is accurate for low %DA chitosan samples [6], [38], [39]. This is due to low %DA samples being soluble in mildly acidic conditions and thus being viable for analysis in solution via ^{1}H NMR. Using the techniques in tandem allows for accurate determination of the %DA of any extracted crustacean chitin or chitosan sample across the entire range of 0-100%DA.

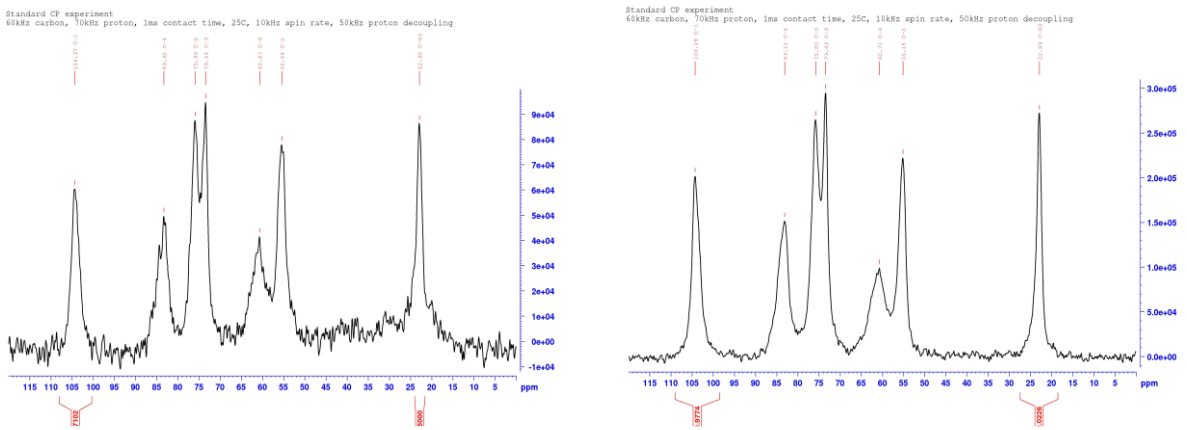

Fig 8: Chitin Product from Enzymatic Hydrolysis (Left) & Chitin Product from Enzymatic Hydrolysis After Clean Up by Chemical Extraction (Right)

C. FTIR Spectroscopy

The use of FTIR spectroscopy as rapid qualitative analysis for indication of %DA of extracts and sample purity is demonstrated well in Fig 9. The spectra of the standards are clean with good signal to noise ratios. The main peaks of interest for use in indicating %DA are the C=O [Amide] stretch peak at approx. 1655cm^{-1} and the N-H [1°, 2° Amine/Amide] bend at approx. 1620cm^{-1}. After the preliminary extraction, the spectra of samples CrabCH1, CrabCH2, CrabCH3 and their depigmented derivatives were elucidated. Upon comparison with the four standards; SA_CH, SA_CS, FungalCS and ShrimpCH5 it was determined that the extracts were pure chitin [6], [20], [28]. The depigmented samples do not produce cleaner spectra or enhanced peaks compared with the pigmented samples. There are no extra peaks on the spectra of pigmented samples, implying that the pigment is in small concentration in the samples after extraction. These small peaks are likely convoluted with the noise signals between the large peaks for the chitin. Thus the depigmentation step is deemed un-necessary for accurate analysis via FTIR.

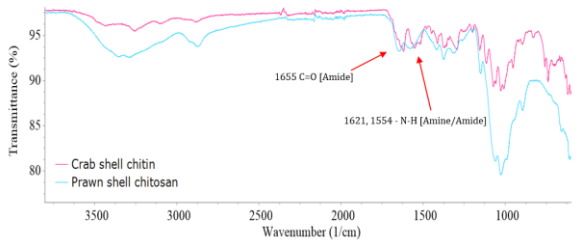

Fig 9: Overlayed FTIR Spectra of Crab Shell Chitin and Shrimp Shell Chitosan Standard.

All preliminary and optimised extraction samples display convolution of peaks, including the main peaks of interest. The convolution of peaks is enhanced by the sensitivity of FTIR to water. Broad, medium intensity –OH peaks are seen above 3200cm^{-1} and they convolute with the other peaks of interest; the C=O overtone at approx. 3430cm^{-1} and the N-H [1°, 2° Amine/Amide] stretch at approx. 3108cm^{-1}. These peaks are hard to isolate due to the hygroscopic nature of chitin and chitosan. Even when freeze dried, the peaks remain convoluted due to both trapped water in the polymer matrix but also due to the large abundance of -OH groups along the polymer chains [10]-[13]. Thus FTIR is not viable for determination of %DA by relative comparison of peaks. Raw samples display massive convolution of peaks compared to the extracted samples. Thus extraction, as described in the Experimental section, is deemed necessary for analysis.

D. Raman Spectroscopy

The use of Raman spectroscopy as rapid qualitative analysis for indication of %DA of extracts and sample purity is demonstrated well in Fig 10. The technique is limited to qualitative analysis due to the convolution of peaks and non-linear responses to changes in %DA between samples. %DA determination via Raman spectroscopy is complex due to convolution of the peaks representative of the ring carbons in the chitin structure [10], [11], [24]. Thus, there is no individual peak with which to relatively compare the C-H3 stretch peaks.

Spectra required application of FLAT post processing correction to remove a sloping baseline in the low wavenumber range of the spectrum. This slope is due to the glass beneath the sample fluorescing when exposed to the laser light. The FLAT correction is an automated fluorescence removal algorithm specifically designed for Raman spectra analysis [25]. Thus, the fluorescence slope is easily corrected for and spectra produced for the extracted samples are clean and well resolved through the entire wavenumber range.

The spectra of the raw samples display massive convolution of all peaks as well as a large hump in the baseline, which is indicative of a complex organic matrix [26]. This convolution and lack of baseline resolution deems the extraction procedure necessary for accurate analysis.

Comparison of the data from FTIR and Raman for samples from the preliminary extraction and the optimised extraction show that the extraction procedure is successful in isolating chitin from the crustacean shells. FTIR and Raman data implies that the extracts are pure chitin when compared with literature spectra [6], [9], [16], [20], [27], [28].

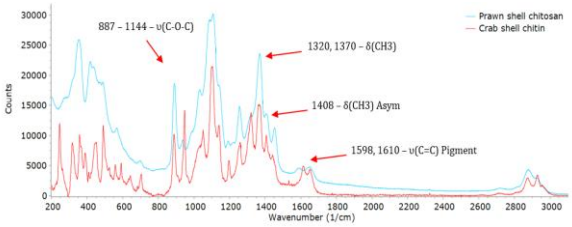

Fig 10: Overlayed Raman Spectra of Crab Shell Chitin and Shrimp Shell Chitosan Standard.

E. DSC

DSC indicates the polymorphic stability of the extracts. The plots of the SA_CH, SA_CS and ShrimpCH5 standards show that the thermal profile is very similar for both chitin and chitosan. However, the degradation event occurs at a higher temperature for chitin. The degradation event is observed on the curve as the point of inflection beyond which the heat flow increases steeply. The higher the %DA of a sample, the higher the temperature at which the degradation occurs. The thermal stability of chitin or chitosan is proportional to the %DA [14].

As seen in Fig 11 the FungalCS standard produces a profile that is indicative of the formation of a polymorph of chitin/chitosan. This sharp drop in heat

flow is due to the transition of the chitosan from its most common polymorph α-chitosan, whereby the polymer lies in tightly compacted alternating sheets of antiparallel chains, to β-chitosan, whereby the polymer lies in less compacted parallel chains [5], [6]. The lack of this transition in the thermal profiles of the crustacean shell chitin and chitosan samples implies that they are more thermally stable than fungal sourced chitin or chitosan. This characterisation of the polymorphic state and thermal stability of the raw waste stream materials and chitin products is significant in the design of upscaled extraction processes, whereby increased temperatures and pressures can occur [40], [41].

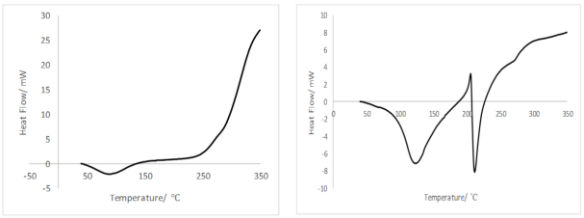

Fig 11: DSC Profile of CrabCH1 Chitin (Left) and FungalCS Chitosan Standard (Right).

Similar to FTIR and Raman analysis, DSC is highly sensitive to structural variability between samples. The sensitivity to structural variance deems the technique inherently inaccurate. Similarly, the temperature range over which the degradation events occur is approximately 50°C, a small range over which there is much variance between samples. Although the thermal profiles of the extracted samples are all very similarly shaped, the slopes of the curves, the exact points of degradation and heat flow are not consistent between the preliminary and optimised extracts. The slope of the curve and the exact point of degradation depend on the variable physical properties of the polymer structure [28], [29]. The raw samples are shown to have similar DSC profile shapes to the extracted samples but again degradation events and heat flow vary significantly between similar %DA samples. Extraction is not necessary for analysis of thermal stability of chitin samples.

IV. CONCLUSION

Accurate characterisation of crustacean sourced chitin by solid state ^{13}C CP-MAS has been shown to be achievable with an optimised extraction procedure. This extraction procedure is not only optimised for improving the accuracy of solid state analytical techniques - it also ensures minimal use of materials, minimal production of waste and maximum yield of chitin.

Treatment of raw samples with this extraction procedure followed by analysis via ^{13}C CP-MAS NMR used in tandem with established ^{1}H NMR techniques allows for accurate %DA determination of any crustacean chitin sample from 0-100%. FTIR, Raman and DSC analysis provide complimentary qualitative analysis of the same samples, before and after extraction, allowing for monitoring of the quality and purity of chitin recovered during extraction.

The chitin extracted from the *Cancer pagurus* (crab) and *Pandulus borealis* (shrimp) waste streams was determined to be of >90%DA, of high purity when compared with literature spectra, with polymorphic stability relative to fungal sourced chitins.

Specifically, for use in characterisation of bio-molecules of interest in waste-stream mixtures, ^{13}C CP-MAS has the advantage of being highly tuneable for specific carbon centres in a sample. This allows for much greater selectivity when analysing raw or unclean samples. ^{1}H NMR exhibits no comparable selectivity with samples requiring extensive clean up to produce any signals useful for quantitative analysis. Similarly by allowing for analysis in the solid state, the sensitivity of the technique is increased compared to solution based ^{1}H NMR as bulk material can be analysed directly and does not required dissolution and dilution.

This work demonstrates that solid state ^{13}C CP-MAS in tandem with optimised extraction can be used for rapid and accurate characterisation of multiple types of crustacean sourced chitin.

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