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# *"AUTHENTICATION AND DIFFERENTIATION OF CHITOSAN ORIGINS"*

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## 1. Introduction

Chitosan is a valuable biopolymer derived from chitin, a copolymer of D-glucosamine and N-acetyl-D-glucosamine monomeric units linked with β-(1 → 4) glycosidic bonds where N-acetyl-D-glucosamine are predominant in the polymeric chain [1,2]. Chitin is largely represented in animal reign as structural component of exoskeleton of arthropods (crabs, shrimps, …) or endoskeleton of cephalodods (octopus, calmar, …) and is also present in various fungi [3]. Today the main production at industrial scale comes from crustacean by-products or residues, fungi representing a less used alternative, despite their natural abundancy. Chitosan are obtained by the deacetylation (removal of the acetyl group) of chitin under alkaline or enzymatic hydrolysis, whatever its source (animal or fungal). Both chitin and chitosan are largely used in various industries such as pharmaceutical, cosmetic, agricultural, food and non-food industries, as they are easily biodegradable and non-ecotoxic.

The polycationic character of these biopolymers provides a wide range of functional (eg. film-forming) and biological (antioxidant, anti-inflammatory, would-healing, …) properties, making them suitable for various application fields including cosmetic [4]. Many actors of the cosmetic industry are more and more searching for natural and non-animal sources of cosmetic ingredients; leading to the needs to replace animal-derived chitin or chitosan by fungal-derived ones. In this context, the OIV recommends the use of 3 different methods to ensure the fungal origin of chitosan in the chitosan monograph: the settled density, viscosity of 1 % chitosan solution and content of residual glucans [5]. Nevertheless, the official methods have specific limits since the 3 mentionned parameters may be falsified to comply with the specifications (eg. addition of exogenous glucose or glucan).

Considering the limitation of the OIV methods to detect falsification, 3 technical alternatives to differentiate chitosan origins were evaluated: the Isotopic Ratio Mass Spectrometry (IRMS), the Thermogravimetric Analysis (TGA) and the Near Infrared (NIR) spectroscopy. These technical alternatives will be evaluated by analyzing chitosans from certified origins, the aim being to propose a strategy based on one or more technics to ensure an unambiguous chitosan origins differentiation.

## 2. Materials and Methods

### *2.1. Sampling*

The samples of chitosan used for this study were from various crustacean and fungal origins and suppliers. 23 samples from certified origins were used to build the model and evaluate the various techniques: 9 samples from crustacean origins (7 from shrimp shell, 2 from crab shell) provided by Sigma-aldrich (St-Louis, MO, USA) and 14 samples from certified fungal origins (*A. niger* and *A. bisporus;* 8 native chitosans and 6 chitosans depolymerized by mecanochemistry). 23 samples from claimed fungal origins (11 from *A. niger*, 10 from *P. ostreatus*, and 2 from unknown sources) from new suppliers were also analyzed to control the supplier claims. Since the detailed industrial process is confidential, the exact recipes have not been provided. For NIR analysis, 9 samples from certified crustacean origin, 2 samples from certified fungal origins (*A. niger* and *A. bisporus*) and 7 from claimed fungal origin (*A. niger* or unknown) were selected.

For most of the samples, water solubility and native pH were determined and the mean molecular masses (Mw, Mn) and Polydispersity Index (Ip) were calculated based on Size Exclusion Chromatographic analysis.

For TGA, 6 samples from certified crustacean origin, 12 samples from certified fungal origins (*A. niger* and *A. bisporus*) and 21 from claimed fungal origin (*A. niger, P. ostreatus* or unknown) were selected.

For IRMS, 6 samples from certified crustacean origin, 10 samples from certified fungal origins (*A. niger* and *A. bisporus*) and 17 samples from claimed fungal origin (*A. niger, P. ostreatus* or unknown) were selected.

### *2.2. Chitosans characterization: Size Exclusion Chromatography (SEC), pHmetry and water solubility*

Size Exclusion Chromatographic (SEC) analysis were performed using LC20 HPLC system (Shimadzu, Noisiel, France) coupled to an OPTILAB (NEON) 3894-OP1 refractometer and a DAWN-HELEOS II -1438-H2TR light scattering detector (Waters / Wyatt Technology Corporation, Goleta, CA, USA). The system was coupled with the Astra 8.1.0.16 64-bit software (Waters / Wyatt Technology Corporation, Goleta, CA, USA). Prior to analysis, the samples were solubilized in mobile phase at 1 g/L under stirring at room temperature for 48 hours to ensure a complete dissolution and then filtered on 0,45µm syringe filters. Chromatographic separation were done on 3 column branched in serie OHpak SB-803, OHpak SB-804, OHpak SB-805 (300 × 8 mm, 6/10/13 *µ*m, Showa Denko., Tokyo, Japan) under the following conditions: flow-rate: 0,5 mL/min, injection-volume: 100µL, column temperature: 40 C, mobile phase: 95 min isocratic of acetic acid 0.3M / ammonium acetate 0.1M, spontaneous pH 4,2.

In addition, water solubility of chitosan was determined at 1 % by precisely weighing 150 mg in a 20-mL glass vial follow by addition of 15 mL water and 24 hours stirring. The solubility was visually evaluated and the pH of the solutions were measured.

### *2.3. Near Infrared Spectroscopy (NIR) Analysis*

The Near Infrared (NIR) analyses were performed using an Antaris II FT-NIR Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The wave-number range was 12000–3800 $cm^{-1}$ and

the resolution were 4 $cm^{-}1$, with 3 scans performed on each sample. The system was coupled with the Result 3.10.14 software (Thermo Fisher Scientific, Waltham, MA, USA).

Spectra were directly recorded on 1g of samples in transparent borosilicate glass bottle for FT-NIR analyses.

### *2.4. Thermogravimetric Analysis*

Thermogravimetric analysis (TGA) was performed using a TGA 5500 control with the TRIOS V5.1.1 Software (Waters / TA Instruments, Guyancourt, France). A quantity of 10–20 mg of each sample was placed in an open crucible of 80µL and the temperature was raised from 20°C to 700°C at a heating rate of 10°C per minute under air. Measures were replicated 3 times on each sample.

### *2.5. Stable isotope analysis*

The stable isotope ratios of H, C, N and O were measured in pure (95%) bulk lyophilized and ground chitosan were subcontracted to CIRAM. This approach is considerably fast and automated (<10 min for each analysis).

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were measured in one run (around 0.5 mg) using an isotope ratio mass spectrometer (IRMS) Elementar – isoprime precisION coupled to elemental analysers Elementar – vario ISOTOPE select or Elementar – vario PYRO cube (Isoprime Ltd., Cheadle Hulme, UK).

Based on the IUPAC protocol, the different stable isotope ratios were expressed in the delta scale ($\delta$‰) against the international V-PDB (Vienna PeeDee Belemnite) standard according to Equation (1):

$$\delta^{i}E_{S/Ref}\,(‰) = 1000^{*}\,(R_S - R_{Ref}) / R_{Ref}$$

where, *s* is the sample, *ref* is the international measurement standard, *i* the value of the heavier isotope, *E* the element, and $R_s$ and $R_{Ref}$ the isotopic ratio of sample and international standard, respectively.

The sample analysis was carried out in duplicate.

For $\delta^{13}C$ and $\delta^{15}N$, the isotopic values were calculated against working in-house standards (protein), which were themselves calibrated against international reference materials: cafein USGS-61 (U.S. Geological Survey, Reston, VA, USA, $\delta^{13}C$ = -35,05‰ and $\delta^{15}N$ = -2,87‰) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$, Glycine USGS-65 (U.S. Geological Survey, Reston, VA, USA, $\delta^{15}N$ = 20,68‰) for $^{15}N/^{14}N$ and Glucose BCR-657 (Sigma-aldrich, St-Louis, MO, USA, , $\delta^{13}C$ = -10,76‰) for $^{13}C/^{12}C$.

### *2.6. Statistical Analysis*

The NIR data were evaluated statistically with an in-house Matlab1 code using the same algorithm as SIMCA-P (Umetrics, Umeå, Sweden). Principal component analysis (PCA) of samples was carried out to investigate the differences between the fungal and animal origin of chitosan based on NIR data. Finally, supervised Orthogonal Partial Least Squares (OPLS) discriminant analysis was carried out on NIR data and both unsupervised (PCA) and supervised (OPLS) statistical models were evaluated to clusterize samples by origin (non-animal vs animal).

## 3. Results

### *1.1. Chitosans characterization (SEC, water solubility and native pH)*

Water solubility determination and native pH measurement of chitosan at 1 % in water reveal that both parameters are closely linked as we observe a full solubilization when the native pH is acids (below pH 6).

Most of the chitosans (from crustacean or fungal origin) are not soluble in water at 1 % (after 24 hours stirring at room temperature) and reveal neutral to basic pH: crustacean chitosan present pH value of 7,2 ± 0,3 while certified fungal chitosans present pH values of 8,9 ± 0,7 and part of claimed fungal chitosans present pH values of 8,0 ± 0,4. Depolymerization of chitosan by mecanochemistry lead to an acidification of chitosans (pH 6,8 ± 0,3 vs pH 8,8 for native chitosan). Part of claimed fungal chitosan are soluble at 1 % in water and reveal acidic pH values (pH 5,1 ± 0,7).

SEC analysis of chitosan reveal that chitosan from certified crustacean origin present is composed of polymers of higher molecular masses (286 < mw < 649 kDa) and polydispersity indexes (3,2 < Ip < 6,3) than those of certified fungal origin (18 < Mw < 113 kDa, 1,55 < Ip < 2,37). Depolymerization by mecanochemistry lead to a decrease of molecular weight and polydispersity index (from 1,63 to 1,26 – 1,55). For chitosans from claimed fungal origins, we observe 2 tendancies which depend on the physico-chemical paramerters (water solubility, native pH): the non acidic chitosans present higher molecular masses (71 < Mw < 304 kDa) and polydispersity indexes (1,7 < Ip < 3,4) than those of certified fungal origin while acidic chitosans present highly variable molecular masses (less than 3 < Mw < 304 kDa) and lower polydispersity indexes (1,6 < Ip < 1,8) than those of certified fungal origin.

### *1.2. Near Infrared Spectroscopy (NIR) Analysis*

Infrared spectroscopy is a simple and low expensive technique widely available, allowing to generate compositional fingerprint of sample without sample preparation. FTIR has been described to study the composition and structure of chitin and chitosan [6] and to differentiate chitosan sources (crustacean vs fungal origins) [7]. In order to gain flexibility and able the analysis directly in the pack, we have evaluated the capacity of NIR to differentiate chitosan sources as NIR portable device are available on the market.

The comparative NIR analysis of 5 chitosans from crustacean (3 samples) vs fungal (2 samples) origins reveal 4 zone of divergence (corresponding to 6 bands) which vary significantly, as shown on the figure below: the 4 bands at 9500 $cm^{-1}$, 8100 $cm^{-1}$, 6500 $cm^{-1}$ and 4900 $cm^{-1}$ are specific to crustacean origin and the 2 bands at 6200 $cm^{-1}$ and 4600 $cm^{-1}$ are more intense in fungal chitosans.

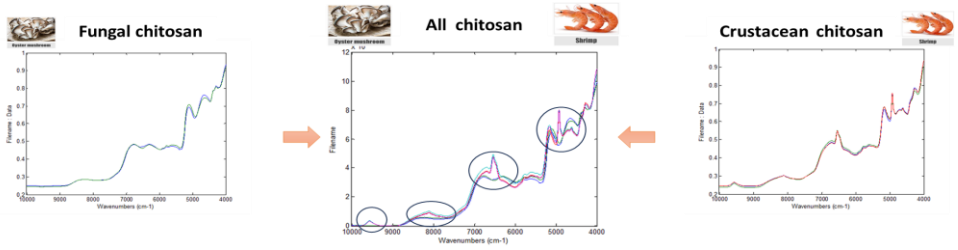



*Figure 1: NIR spectra of fungal (left) and crustacean (right) chitosans and representation of all spectra to highlight zone of divergence (surrounded).*

Considering the clear difference between spectra, a second analysis was done on a larger cohorte of samples to evaluate the statistical treatment of spectra to differentiate chitosans sources. The following process was applied: 1/ NIR spectra recording (triplicate), 2/ spectra pretreatment using MSC method for global effect attenuation followed by Savitzky-Golay derivatization methods for local effect correction, 3/ Statistical treatment using unsupervised (PCA) and supervised (OPLS) statistical models.

As shown on the figure below, we observe that pre-treatment of spectra is a crucial step enabling the attenuation of differences linked to spectra acquisition. The Multiplicative Scatter Correction (MSC) provide an alignment of spectra revealing differences between them and successive derivatization steps (Savitzky-Golay derivatization methods) able to attenuate non-significant differences and exert significant ones.

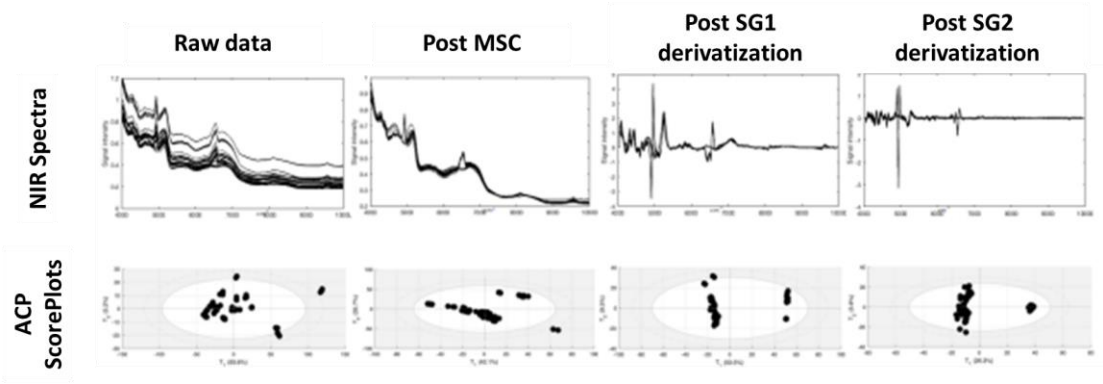



*Figure 2: NIR spectra and ACP ScorePlots from Raw data to pre-treated data.*

As shown on the figure below, the ACP ScorePlots reveals 2 clusters: one (right) grouping all acidic chitosans (claimed fungal) and the other one grouping all neutral/basic chitosans, whatever their origins (crustacean, fungal or claimed fungal). This clusterization reveals that the main differences previously observed on NIR spectra seems to be directly linked to physico-chemical characteristics of chitosans (native pH/water solubility). Considering it, the data were analyzed using supervised (OPLS) statistical model in order to attenuate spectral differences linked to physico-chemical properties of chitosans. The OPLS ScorePlots (Figure 3b) reveals 3 clusters: one cluster (at the bottom left) grouping all acidic chitosans (as previously observed on ACP ScorePlots), a second cluster (at the top left) grouping all fungal and claimed fungal chitosans (neutral/basic pH) and a third one (at the right) grouping all crustacean chitosans (neutral/basic pH).

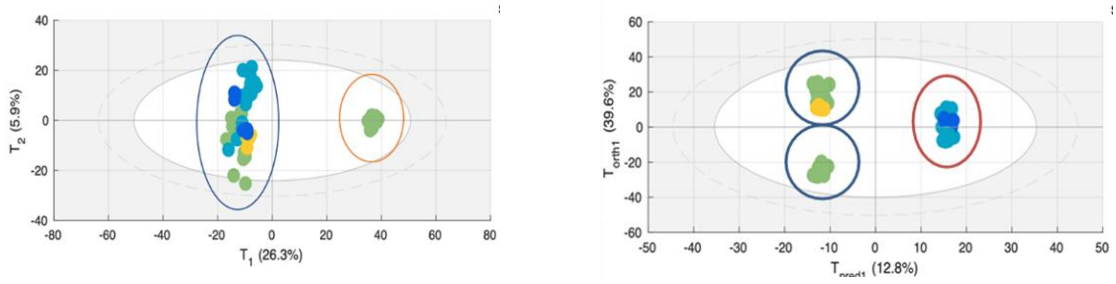



*Figure 3: ScorePlots of statistical analysis of pre-treated NIR spectra of chitosans. (a) ScorePlots of the PCA analysis and (b) ScorePlots of the OPLS analysis. Sources: Crab (dark blue), shrimp (blue), claimed fungal (green) and fungal (yellow).*

### *1.3. Thermogravimetric Analysis*

Thermogravimetric Analysis is a simple and relatively low expensive technique allowing to follow the changes in physico and chemical properties of materials as a function of increasing temperature or as a function of time. This technique is generally used on chitosan to determine water content (due to strong affinity of polysaccharides to water) but recent paper propose TGA approach to differentiate chitosan sources (crustacean vs fungal origins) using DTGmax values (around 300 °C: crustacean chitosan, around 280 °C: fungal chitosan) [7].

As shown on the figure below, according to the literature [7], we observe DTGmax values of 295 ± 5 °C for certified crustacean chitosans and lower DTGmax values of 277 ± 2,0 °C for certified fungal chitosans. The analysis of chitosans depolymerized by mecanochemistry (coming from certified fungal chitosan, DTGmax = 275 °C) reveal lower DTGmax values of 262 ± 7 °C, indicating that processes can significantly impact the DTGmax values. Analysis of chitosans from claimed fungal origin confirm that DTGmax value is not only linked to chitosans origins but also depend on their physico-chemical parameters as two set of DTGmax values were obtained: neutral/basic chitosans having DTGmax values of 289 ± 6 °C (close to DTGmax values of crustacean chitosans) and acidic chitosans which provide DTGmax values of 202 ± 5 °C. Consequently we can suspect that neutral/basic chitosans from claimed fungal origin are in reality chitosan from crustacean origin.

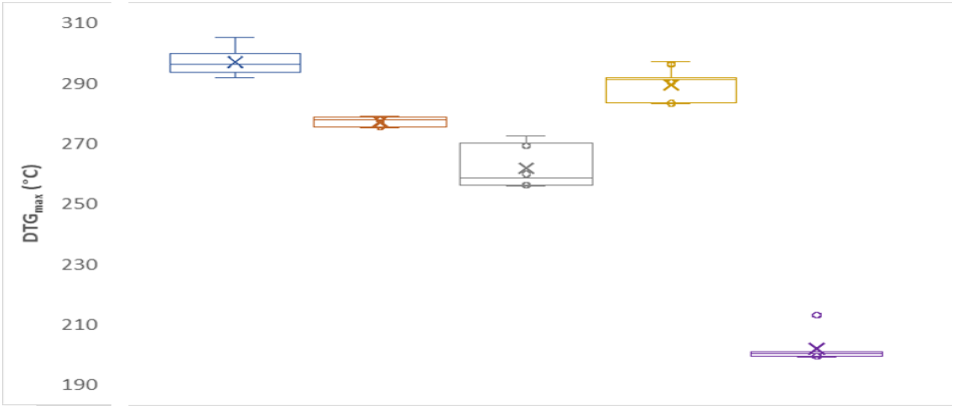
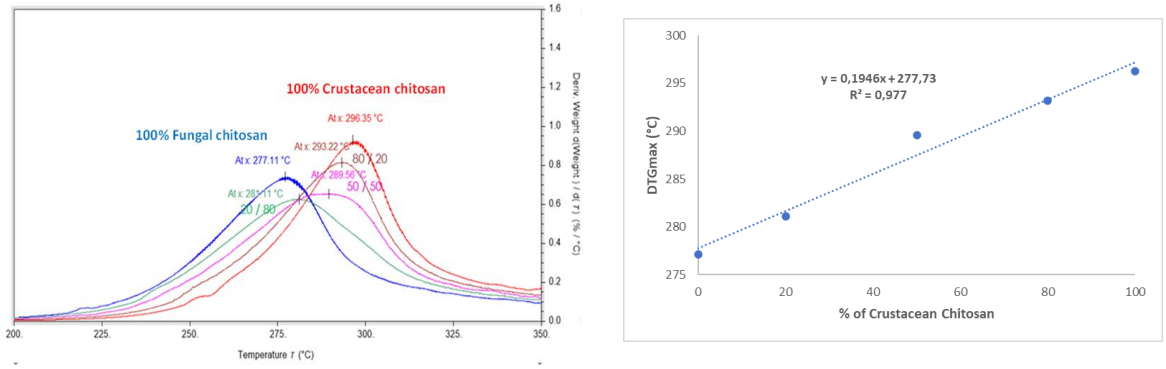



*Figure 4: BoxPlot of DTGmax values obtained for the various population of chitosans. Blue: certified crustacean chitosans, orange: certified fungal chitosans, grey: certified fungal chitosans depolymerized by mecanochemistry, yellow: claimed fungal chitosans (neutral/basic) and violet: claimed fungal chitosans (acidic).*

To evaluate the capacity of TGA to identify fungal chitosan adulteration (using chitosan from animal sources), mixtures of crustacean and fungal chitosan were prepared and analyzed to compare the thermograms and DTGmax values. Results are given on the figure below. For all mix, we only detect one signal in the zone of pyrolytic decomposition of chitosan (enlarged compare to pure chitosan) and DTGmax vary linearly in function of proportion of the mix (as shown on the graph below).




**(a)** **(b)**

*Figure 5: (a) Thermograms (zoom on the signal of pyrolytic decomposition of chitosan) of certified crustacean, fungal chitosan and mix of both. Blue: 100 % fungal chitosan, red: 100 % crustacean chitosan, green: mix of 20 % fungal/80 % crustacean, pink: mix of 50 % fungal/50 % crustacean and orange: mix of 80 % fungal/20 % crustacean*

### *1.4. Stable isotope analysis*

Reproducibility of litterature data [7,8] was evaluated by determining the stable isotope ratio $\delta^2$H, $\delta^{13}$C, $\delta^{15}$N and $\delta^{18}$O on six chitosan from certified crustacean origin (4 from shrimps, 2 from crabs) and validated as all the values we obtained are in the range of described ones (Cf figure below). Replication of IRMS analysis of crustacean chitosans reveals that measurements of $\delta^{13}$C, $\delta^{15}$N are reproducibles (standards deviation of respectively 0.04 ‰ and 0.17 ‰) while measurement of $\delta^2$H, $\delta^{18}$O present significant variations (standards deviation of respectively 7.18 ‰ and 1.41 ‰; probably due to the hygroscopic properties of chitosans which will adsorb atmospheric water, impacting the $\delta^2$H, $\delta^{18}$O values).

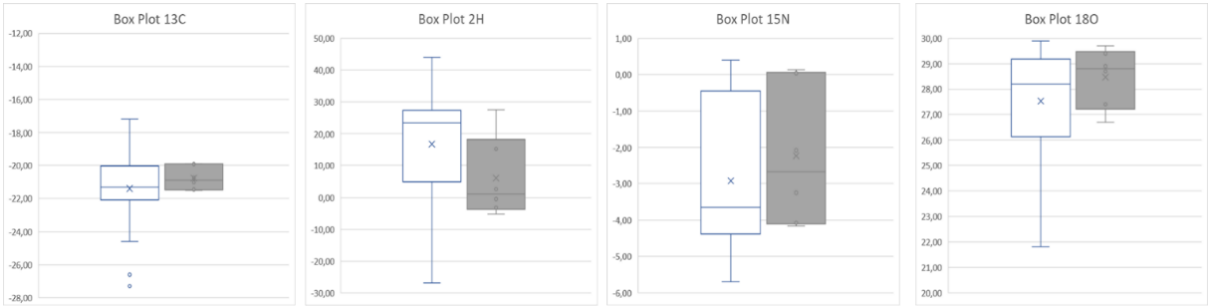



*Figure 6: BoxPots of isotopic deviations of crustacean chitosans. Blue BoxPlots: literature data [7,8], grey BoxPlots: experimental data.*

Based on $\delta^{13}$C and $\delta^{15}$N of chitosans, it was possible to separate not only chitosans from crustacean and fungal sources but also from various fungal sources (*A. niger* vs *A. bisporus*), as shown on the figure below.

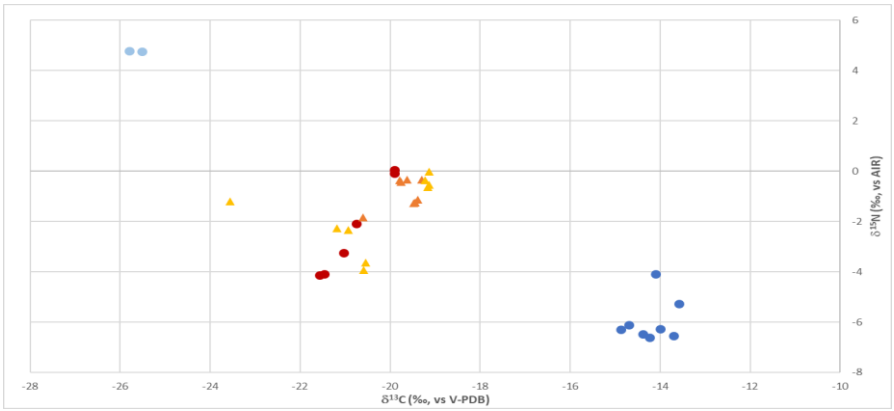



*Figure 7: $\delta^{13}C$ vs $\delta^{15}N$ distribution of chitosans from certified fungal origins (strain A. bisporus and A. niger in light blue and blue, respectively), certified crustacean origins (in red) and of claimed fungal origins (acidic and neutral/basic in orange and yellow, respectively).*

The $\delta^{13}C$ of chitosan from crustacean (n = 6) ranges from -21.56 ‰ to -19.91 ‰ while the $\delta^{15}N$ ranges -3.25 ‰ to 0.04 ‰, which is in accordance with literature data [7,8]; we do not observe significant differences between chitosans from shrimps or crabs exoskeleton. Certified fungal chitosans are divided in 2 clusters function of their origin: The $\delta^{13}C$ and $\delta^{15}N$ of chitosan from *A. niger* ranges from -14.87 ‰ to -13.58 ‰ and -6.55 ‰ to -4.08 ‰ respectively, while mean $\delta^{13}C$ and $\delta^{15}N$ values of chitosan from *A. bisporus* are around -25.65 ‰ and 4.76 ‰ respectively. Analysis of chitosans from claimed fungal origin revealed $\delta^{13}C$ and $\delta^{15}N$ values in the range of chitosan from crustacean origine with $\delta^{13}C$ values ranges from -23.56 ‰ to -19.13 ‰ while the $\delta^{15}N$ ranges -3.93 ‰ to -0.01 ‰; we do not observe differences between acidic and neutral/basic chitosans. Consequently we can suspect that chitosan from claimed fungal origin are in reality chitosan from crustacean origin.

## 4. Discussion

The NIR analysis coupled to statistical analysis of pre-treated data (MSC followed by Savitzky-Golay derivatization methods) could to be a promising approach to differentiate chitosan sources. Nevertheless, as physico-chemical characteristics of chitosans present today the highest impact on clusterisation, this approach is not recommended for chitosan's origins differentiation. This approach would have to be implemented with additionnal chitosan from certified origins to ensure its robustness and make it reliable for origins differentiation.

Thermogravimetric Analysis (TGA) is a simple and relatively low expensive technique allowing to follow the changes in physico and chemical properties of materials as a function of increasing temperature or as a function of time. In this study we have confirmed that TGA could be used to differentiate chitosan from animal (crustacean) and fungal sources as described in recent paper [7], as significantly different DTGmax values were obtained on chitosan from crustacean and fungal sources (295,2 ± 4,6 °C and 277,2 ± 2,0 °C, respectively). We also observed that process to obtain chitosan can significantly impact the DTGmax values (depolymerization of chitosan by mecanochemistry lead to a decrease of DTGmax from 5 to 20 °C) as well as the physico-chemical properties of chitosans (DTGmax values vary up to 85 °C between claimed fungal acidic and neutral/basic chitosans). Evaluation of TGA to detect adulteration (mix of chitosan of animal and fungal sources) reveals that DTGmax can indicate the occurrence of adulteration only if pure fungal and/or animal reference are available.

The determination of $\delta^2$H, $\delta^{13}$C, $\delta^{15}$N and $\delta^{18}$O isotopic deviations of chitosans from certified crustacean sources (crabs, shrimps) reveals values in accordance with literature data. Replication of IRMS analysis indicates good reproducibility for $\delta^{13}$C and $\delta^{15}$N measurements but non reproducible values for $\delta^2$H, $\delta^{18}$O (probably due to hygroscopic properties of chitosans). Considering it, we decide to only used $\delta^{13}$C and $\delta^{15}$N to able raw material analysis without specific sample pre-treatment. $\delta^{13}$C and $\delta^{15}$N able a clear differentiation between chitosan from fungal (*A. niger*, *A. bisporus*) and crustacean origins (shrimps or crabs) and also able a clear differentiation between fungal chitosan from various strains, as described [7,8].

## 5. Conclusion

From the 3 technical approaches evaluated in this study, Isotopic Ratio Mass Spectrometry (IRMS) appears as the most reliable approach to differentiate between fungal and crustacean chitosans using isotopic deviations $\delta^{13}$C and $\delta^{15}$N. This technique provides reproducible measurements of $\delta^{13}$C and $\delta^{15}$N (standards deviation of respectively 0.04 ‰ and 0.17 ‰) and also able to differentiate between chitosans from different fungal sources (*A. niger* vs *A. bisporus*). Thermogravimetric analysis (TGA) allows the differentiation between chitosans of fungal and crustacean origins based on the determination of DTGmax values; nevertheless DTGmax values can be significantly impacted by physico-chemical properties of chitosans (native pH / water solubility) or by the process of fabrication. In this study, we do not succeed to demonstrate the capacity of NIR spectroscopy coupled to chemometric analysis (PCA and OPLS) to differentiate between chitosan origins as the main differences between spectra are linked to phyciso-chemical properties of chitosan. The proposed analytical strategy consist to first analyze chitosans by TGA, which is a simple and relatively low expensive technique, for a partial differentiation between crustacean and fungal chitosans and then by IRMS, which requires more technological expertise and specialized equipment, to confirm the results or go deeper into the differentiation (eg. between fungal chitosans themselves).

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