1 STRUCTURAL EFFECTS OF MICROALGAE ADDITIVES ON THE STARCH
2 GELATINISATION PROCESS

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13 Abstract

14 This work presents a detailed structural characterisation of the starch gelatinisation process
15 and the effect of the addition of three microalgae species, *Nannochloropsis gaditana* sp.,
16 *Scenedesmus almeriensis* and *Spirulina*, by means of an advanced approach consisting of
17 temperature-resolved simultaneous SAXS/WAXS experiments, combined with DSC.
18 Furthermore, regular and high amylose corn starch were utilised to evaluate the impact of
19 the amylose content.

20 The presence of microalgae has been seen to limit water accessibility towards the interior of
21 starch granules, reducing granule swelling and, thus, hindering the arrangement of
22 amylopectin helices into highly ordered crystalline structures. As a result, more
23 heterogeneous lamellar structures, with reduced apparent crystallinity, are attained. Despite
24 the existence of lipidic compounds in the three microalgae species, the tough cell walls in
25 *Nannochloropsis* and *Scenedesmus* impede their release towards the aqueous medium. In
26 contrast, the weak cell walls in *Spirulina* are disrupted by stirring, allowing cell components
27 to be released. The diffused lipids form helical inclusion complexes with the amylose chains
28 and promote the crystallisation of V-type structures. The presence of amylose-lipid
29 complexes counteracts the limited water swelling effect and results in the formation of more
30 crystalline and homogeneous lamellar structures. This result is relevant for the food industry
31 due to the potential of *Spirulina* to affect the processability and nutritional characteristics of
32 starch-based products.

33

34 **Keywords:** starch; microalgae; gelatinisation; SAXS; WAXS; DSC

35

36 **1. Introduction**

37 Starch is one of the most widely known dietary polysaccharides since it is the main
38 component in raw and processed foods. Native starch is found in vegetal resources forming
39 water-insoluble semi-crystalline granules which present a complex hierarchical architecture
40 characterised by at least four structural levels: (i) granules, (ii) semi-crystalline amorphous
41 growth rings, (iii) lamellae (i.e. stacks of alternating amorphous and crystalline structures)
42 and (iv) linear amylose and branched amylopectin chains. The crystalline regions are thought
43 to consist mainly of amylopectin side chains which are organized in double helices (Imberty,
44 Buléon, Tran, & Pérez, 1991), whereas the amorphous regions are mainly composed of linear
45 amylose chains and less ordered amylopectin branch points (Pérez & Bertoft, 2010).
46 Depending on several factors such as the amylose/amylopectin ratio and the amylopectin
47 chain length, these helices can be arranged differently into A-type (mainly found in cereal
48 starches and related to short double helices) or B-type (long double helices found in tubers
49 and high amylose starches) crystalline unit cells (Cheetham & Tao, 1998; Hoover, 2001;
50 Paul J Jenkins, Cameron, & Donald, 1993; Salman, et al., 2009). Water has a great influence
51 in the long range order of starches, constituting up to 4-7% and 25-27% of the crystalline
52 unit cell in the A and B polymorphs, respectively (Imberty, Chanzy, Pérez, Buléon, & Tran,
53 1988; Imberty & Perez, 1988; Sarko & Wu, 1978).

54

55 Many food processing methods involve the use of combined heat and humidity, leading to
56 starch gelatinisation which involves the break-up of the starch structure. Essentially, the
57 gelatinisation process is initiated by the access of water towards the interior of starch
58 granules, which swell and absorb water. Subsequently, structural changes occur at the
59 different architectural levels, leading to crystallinity losses, disruption of the crystalline and
60 lamellar structure of starch and amylose molecules leaking out of the starch granules forming

61 a continuous gel. This complex gelatinisation phenomenon is governed by a number of
62 factors such as starch botanical origin, plant growth conditions, extraction methods, water
63 content, presence of additives, heating rate and thermal history (Imberty, et al., 1991; Liu,
64 Yu, Xie, & Chen, 2006; Tester & Morrison, 1990; Varavinit, Shobsngob, Varanyanond,
65 Chinachoti, & Naivikul, 2003; Waigh, Gidley, Komanshek, & Donald, 2000). A detailed
66 understanding of the structural changes taking place during starch gelatinisation, at the
67 different length-scales, as well as the effect of different parameters, is highly relevant to
68 several scientific and industrial fields. For instance, in many food processing methods such
69 as baking of bread, extrusion of cereal-based products, thickening, and gelling of sauces,
70 starch gelatinisation is a key factor to produce a desirable texture or consistency of the end
71 product (Biliaderis, Maurice, & Vose, 1980). From the human nutrition perspective, the
72 degree of starch gelatinisation in food products is known to affect its digestion rate (Joergen
73 Holm, Lundquist, Björck, Eliasson, & Asp, 1988). Furthermore, the gelatinisation process is
74 the basis for the processing methods applied to produce starch-based biopolymeric materials
75 (Li, et al., 2011; Liu, Xie, Yu, Chen, & Li, 2009).

76

77 Microalgae constitute one the most promising sustainable feedstocks for the manufacture
78 of plant-derived products. Due to their ease of cultivation, high growing rates and
79 productivity and possibility of adapting the harvesting conditions to modify their
80 composition, microalgae have gained a great deal of interest for their use in a wide range
81 of applications such as the production of biofuels (Mata, Martins, & Caetano, 2010) and
82 food commodities (Draaisma, et al., 2013; Spolaore, Joannis-Cassan, Duran, & Isambert,
83 2006). Their interest from the human nutrition perspective lays in their interesting chemical
84 composition. In particular, the high protein content of several species and their interesting
85 lipidic profile, with relatively high amounts of $\omega 3$ and $\omega 6$ fatty acids (Spolaore, et al., 2006),

86 make them attractive for the production of nutritional supplements or food additives. The
87 incorporation of microalgae into pasta and bakery products to enhance their nutritional
88 profile is currently being investigated and has been reported in several recent works (De
89 Marco, Steffolani, Martínez, & León, 2014; Monica Fradique, et al., 2010; Mónica
90 Fradique, et al., 2013; Kadam & Prabhasankar, 2010). The components present in the
91 microalgae may interfere with the gelatinisation process in starchy foods and, thus, the
92 processing conditions may also be affected. Therefore, investigating the effect of different
93 microalgae on the gelatinisation of starch may be of interest to determine their impact on
94 the processability and digestibility of starch-microalgae blends. In this work, we have
95 investigated the structural changes undergone by two starches, with different amylose
96 contents, as well as the effect of three different microalgae species, during the gelatinisation
97 process. To this end, an advanced approach of temperature-resolved simultaneous small
98 angle and wide angle X-ray scattering (SAXS/WAXS) experiments, combined with DSC
99 characterisation, has been utilised. The use of scattering techniques is particularly
100 advantageous to study the starch gelatinisation process, since it enables the characterisation
101 of different structural levels (crystalline and lamellar structures) in starch as the temperature
102 is raised and at high relative humidity conditions.

103

104 **2. Materials and methods**

105 **2.1 Materials**

106 Corn starch (27-28% amylose) and high amylose starch (70% amylose) powders were
107 supplied by Roquette (Roquette Laisa España, Benifaió, Spain). The three different
108 microalgae species, i.e. *Nannocloropsis gaditana* sp., *Spirulina* and *Scenedesmus*
109 *almeriensis*, in the form of dry powders, were kindly donated by Dr. Ación from the
110 University of Almeria (Spain).

111

112 **2.2 TEM characterisation of microalgae cell walls**

113 The raw microalgae were dispersed in water by vortex stirring at a concentration of 4 g/L.
114 One drop (8 μ l) of the prepared suspensions was allowed to dry on a carbon-coated grid (200
115 mesh). The microalgae cell walls were stained with a 2 % (w/w) solution of uranyl acetate.
116 TEM was performed using a JEOL 1010 equipped with a digital Bioscan (Gatan) image
117 acquisition system at 80 kV.

118

119 **2.3 Preparation of starch and starch/microalgae dispersions**

120 Starch dispersions (samples designated as “corn” and “amylo” for the corn starch and high
121 amylose starch, respectively) were prepared by adding 0.5 g of starch into 1 mL of water
122 and subjecting the samples to vortex stirring for 2 min. For the starch/microalgae samples,
123 0.004 g of the microalgae powder were also added prior to stirring. The dispersions were
124 immediately used for the SAXS/WAXS and the DSC experiments.

125

126 **2.4 Temperature resolved SAXS/WAXS experiments**

127 Combined small and wide angle X-ray scattering (SAXS and WAXS, respectively)
128 experiments were carried out in the Non Crystalline Diffraction beamline, BL-11, at ALBA
129 synchrotron light source (www.albasynchrotron.es). The starch and starch/microalgae
130 dispersions were placed in sealed 2 mm quartz capillaries (Hilgenburg GmbH, Germany).
131 The energy of the incident photons was 12.4 KeV or equivalently a wavelength, λ , of 1 Å.
132 The SAXS diffraction patterns were collected by means of a 9 CCD detector Quantum
133 ADSC 315r with an active area of 315 x 315 mm², an effective pixel size of 102 x 102 μ m²
134 and a dynamic range of 16 bits. The sample-to-detector distance was set to 6488 mm,
135 resulting in a q range with a maximum value of $q = 0.25 \text{ \AA}^{-1}$. Additionally, the WAXS

136 diffraction patterns were collected by means of a 3 CCD detector Rayonix LX255-HS with
137 an active area of $85 \times 255 \text{ mm}^2$, an effective pixel size of $44 \times 44 \mu\text{m}^2$ and a dynamic range
138 of 16 bits. In this case, the sample-to-detector distance was set to 144.9 mm, corresponding
139 to a maximum q value of $7.87 \text{ \AA}^{-1}$. This detector was tilted with a pitch of 26.4 degrees.
140 Based on previous experiments, an exposure time, of 2 seconds was selected for both
141 detectors.

142

143 Samples were heated from 30°C to 110°C at a heating rate of $2^\circ\text{C}/\text{min}$. Data were collected
144 in frames of 30 seconds, followed by a period of 30 seconds in which the samples were
145 protected from the beam by a local shutter. Each data frame thus corresponds to a
146 temperature range of 1°C , with one data frame every 2°C . The data reduction was treated
147 by pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by ALBA beamline
148 staff, to do on-line azimuthal integrations from a previously calibrated file. The calibration
149 files were created from well-known standards, i.e. Silver behenate (AgBh) and Cr_2O_3 for
150 SAXS and WAXS respectively. The intensity profiles were then represented as a function
151 of q (SAXS) and 2θ (WAXS) using the IRENA macro suite (Ilavsky & Jemian, 2009)
152 within the Igor software package (Wavemetrics, Lake Oswego, Oregon).

153

154 **2.5 SAXS/WAXS data fitting**

155 SAXS data were fitted using the Igor NIST analysis macro suite (Kline, 2006) and applying
156 a mathematical function consisting of a power-law term plus one Gaussian-Lorentzian
157 peak, similar to that previously reported for several starch samples (A. Lopez-Rubio, et al.,
158 2007; Salman, et al., 2009):

$$I(q) = A \cdot q^{-m} + \left[R \cdot \left[I_{max} \cdot \left(1 + \left(\frac{2 \cdot (q - q_{max})}{\Delta q} \right)^2 \right)^{-1} \right] + \left[(1 - R) \cdot \left[I_{max} \cdot \exp \left[-\frac{1}{2} \cdot \left(\frac{q - q_{max}}{\Delta q} \right)^2 \right] \right] \right] \right] + bkg \quad (1)$$

The first term in equation (1) corresponds to the power-law function (where A is a prefactor and m is the power-law exponent) to account for the underlying diffuse scattering, the second and third terms correspond to the Lorentzian and Gaussian functions used to describe the starch lamellar peak (where q_{max} is the peak position, I_{max} is the intensity of the peak, Δq is the full width at half maximum and R is the Lorentzian to Gaussian ratio of the peak shape) and the fourth term accounts for the incoherent background.

167

WAXS peak fitting was performed in Igor, following the procedure described in a previous work (Amparo Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \sum \left(\frac{y - y_i}{\sigma_i} \right)^2 \quad (2)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i . The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2 . The crystallinity index X_C was determined from the obtained fitting results by applying the following equation:

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (3)$$

where A_{Total} is the sum of the areas under all the diffraction peaks and $\sum A_{Crystal}$ is the sum of the areas corresponding to the crystalline peaks.

181

182 **2.6 Differential Scanning Calorimetry (DSC)**

183 DSC measurements of starch and starch/microalgae dispersions were performed on a Perkin-
184 Elmer DSC 8000 thermal analysis system using N₂ as the purging gas. Approximately 10
185 mg of samples were weighted and added into hermetically sealed aluminium sample pans.
186 The sample treatment consisted of heating step from 0 °C to 180 °C at a heating rate of 2
187 °C/min. Before evaluation, similar runs of an empty pan were subtracted from the
188 thermograms. The DSC equipment was calibrated using indium as a standard. Measurements
189 were done, at least, in triplicate.

190

191 **3. Results and Discussion**

192 **3.1 Thermodynamic characterisation of the starch gelatinisation process**

193 The gelatinisation process of starch and starch-microalgae suspensions in excess water was
194 studied by means of DSC characterisation by heating the samples at a rate of 2°C/min.
195 Typical DSC profiles for the corn starch and high amylose based samples are shown in
196 Figures 1A and 1B, respectively, and the extracted gelatinisation parameters are summarised
197 in Table 1. All the samples show a broad melting peak that corresponds to the gelatinisation
198 endotherm. In the case of corn starch the endotherm extends over a temperature range of ca.
199 60-70°C with the maximum at 64 °C, whereas the process takes place within the range of ca.
200 70-90°C, with the maximum at 78°C, for the high amylose starch. Similar gelatinisation
201 temperatures have been previously reported for corn starch (Liu, et al., 2006; Yoshimura,
202 Takaya, & Nishinari, 1996) and high amylose starch (Liu, et al., 2006) samples. The higher
203 gelatinisation temperature for the high amylose starch is partly a result of the amylose
204 fraction being less prone to swelling than the amylopectin (Hermansson & Svegmarm, 1996;
205 Yuryev, Kalistratova, van Soest, & Niemann, 1998). Moreover, despite the higher packing

206 density of the A-type polymorph, B-type starches have been seen to be more enzymatically
207 resistant than A-type starches due to the greater crystallinity of the former ones in the outer
208 granule regions (Sevenou, Hill, Farhat, & Mitchell, 2002). This could be an additional factor
209 limiting water accessibility and therefore, hindering the gelatinisation process in high
210 amylose starch.

211

212 It should be noted that the gelatinisation endotherm clearly presents a distinct shape of two
213 overlapped peaks in the case of corn starch (see inset in Figure 1A). A similar behaviour has
214 been previously reported for corn starch samples (46% w/w suspensions in water) with
215 comparable amylose content (Biliaderis, Maurice & Vose, 1980). This is related to the
216 existence of two different transitions that have been described to take place in the presence
217 of moderate/high water contents: (i) the disruption of the inter-molecular hydrogen bonds
218 leading to the dissociation of the amylopectin double helices from the sides of the
219 crystallites, known as the smectic to nematic/isotropic phase transition and (ii) the unwinding
220 of the amylopectin helices at higher temperatures, known as the helix-coil transition (Waigh,
221 Gidley, Komanshek & Donald, 2000). In the case of the high amylose starch, the
222 gelatinisation transition is characterised by a broad peak, similar to that previously detected
223 for corn starch with 70% amylose content. This broad peak has been described to account
224 for several processes: the disruption of amylopectin double helices, the dissociation of the
225 amylose-lipid complexes and the melting of single-helical V-type crystallites (Matveev, et
226 al., 2001).

227

228 Both types of starch present a similar gelatinisation enthalpy, although the peak height index
229 (PHI), defined as the ratio between the enthalpy and the difference between the peak
230 maximum and onset temperatures (Krueger, Knutson, Inglett, & Walker, 1987), which

231 provides a measure of the uniformity in the gelatinisation process, is greater for corn starch.
232 This suggests that although a comparable amount of energy is needed to melt the
233 amylopectin crystallites in both samples, a more heterogenous process takes place in the case
234 of the high amylose starch, supporting the co-existence of different types of crystallite
235 structures.

236

237 As deduced from Figure 1A and Table 1, the incorporation of microalgae into the corn starch
238 suspensions does not lead to significant changes on the gelatinisation parameters estimated
239 from the DSC thermograms. In contrast, the presence of microalgae seems to induce an
240 increase in the maximum of the gelatinisation endotherm for the high amylose starch
241 samples, although given the large associated standard deviation values the differences cannot
242 be considered as significative. This effect is more evident for the *Spirulina* and *Scenedesmus*
243 species and in the latter, it is accompanied by an increase in the gelatinisation enthalpy and
244 the peak height index (i.e. a larger amount of energy is required to complete the phase
245 transition and the gelatinisation process is more homogeneous). These results suggest that
246 certain compounds in the microalgae are able to interact with the amylose fraction in the
247 high amylose starch promoting the formation of more thermodynamically stable
248 suspensions.

249

250

251

252 **Figure 1.** DSC thermograms from (A) corn starch samples and (B) high amylose starch
 253 samples. Curves have been offset for clarity.

254

255 **Table 1.** Onset (T_O), peak maximum (T_P) and conclusion (T_C) temperatures, enthalpy (ΔH)
 256 and peak height index (PHI) values for the gelatinisation endotherm of pure starch and
 257 starch-microalgae suspensions. Enthalpy values are expressed in J/g of dry starch. Standard
 258 deviation values on the last digit are shown in parantheses.

	T_O (°C)	T_P (°C)	T_C (°C)	ΔH (J/g)	PHI
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Corn	62 (1)	64 (2)	67 (1)	10 (6)	5.0
Corn- <i>Spirulina</i>	62 (1)	64 (1)	69 (1)	9 (1)	4.5
Corn- <i>Nannochloropsis</i>	62.2 (8)	65.3 (5)	68.3 (1)	10.8 (8)	3.5
Corn- <i>Scenedesmus</i>	61.2 (2)	64.0 (1)	66 (2)	10.7 (6)	3.8
High Amylose	73 (1)	77.7 (8)	85.4 (5)	13 (6)	2.8
High Amylose- <i>Spirulina</i>	69 (6)	81 (3)	84 (6)	19 (5)	1.6
High Amylose- <i>Nannochloropsis</i>	73 (5)	79.7 (5)	82 (4)	8 (4)	1.2
High Amylose- <i>Scenedesmus</i>	77.4 (5)	80.5 (7)	88 (4)	22 (2)	7.1

259

260 3.2 Evolution of the starch crystalline and lamellar structure during the gelatinisation 261 process

262 To investigate the structural changes undergone by starch during the gelatinisation process,
263 the pure starch and starch-microalgae suspensions were characterised by means of
264 temperature-resolved simultaneous SAXS/WAXS experiments. While SAXS is useful to
265 characterise the lamellar structure of starch, WAXS provides information on the crystalline
266 structure.

267

268 3.2.1. Crystalline structure

269 The crystalline structure of the starch and starch-microalgae samples was investigated by
270 analyzing the WAXS patterns collected when heating the samples from 30°C up to 110°C.
271 Figures 2 and 3 show the temperature-resolved WAXS curves (temperature steps of 10°C)
272 for the corn and high amylose starch samples, respectively. As shown in Figure 2, all the
273 corn starch samples display typical A-type diffraction patterns, characterized by the main
274 diffraction peaks located at $2\theta = 15.0^\circ$, 17.3° , 18.0° and 23.0° . Interestingly, a weak peak
275 located at ca. 20° , characteristic from the V-type crystalline structure (Amparo Lopez-
276 Rubio, Flanagan, Gilbert, et al., 2008), appears in the spectra of the corn starch loaded with

277 *Spirulina* (pointed out by an arrow in Figure 2B). Similarly, V-type crystallites have been
278 reported to originate from the formation of complexes between the polar lipids found in
279 native starches and the amylose fraction (Zobel, 1988). In addition, weak but sharp peaks,
280 located at ca. 15.6°, 20.3°, 26.5°, 29.2° and 29.6°, are visible in the spectra of the sample
281 loaded with *Spirulina* and two peaks at 22.9° and 29.6° appear in the sample containing
282 *Scenedesmus* (indicated by discontinuous lines in Figures 2B and 2D). These peaks could
283 arise from crystalline components, such as fatty acids, which are known to be found in the
284 microalgae (Ahlgren, Gustafsson, & Boberg, 1992; James, Al-Hinty, & Salman, 1989; Ma,
285 et al., 2017; Matos, et al., 2016). In fact, as shown in Figure S1, the X-ray diffraction
286 patterns of the microalgae present several peaks, with the most intense ones located at 2 θ
287 values of ca. 16.6°, 20.3°, 29.0°, 30.8° and 31.6° for *Spirulina*, 27.3 ° and 31.5° for
288 *Nannochloropsis* and 18.5°, 23.2° and 29.5° for *Scenedesmus*. The appearance of these
289 peaks in the WAXS patterns from the aqueous suspensions (even at 30°C) is indicative of
290 the presence of crystallised lipids in the *Spirulina* and *Scenedesmus* microalgae. However,
291 only those lipids contained in the *Spirulina* microalgae seem to be able to establish strong
292 interactions with amylose, leading to the formation of V-type crystallites. The formation of
293 amylose-lipid complexes has been reported to depend on the chain size (with smaller lipid
294 molecules being more prone to form complexes (Cui & Oates, 1999)) and the concentration
295 of the lipids (Tang & Copeland, 2007). Wheat starch has been shown to form complexes
296 with stearic acid up to a certain lipid concentration, above which the X-ray diffraction peaks
297 characteristic of the V-type crystallites are accompanied by the appearance of additional
298 peaks arising from the presence of lipid aggregates (Tang & Copeland, 2007). Thus, our
299 results seem to indicate that the concentration of lipids in the *Spirulina* microalgae is above
300 this critical point at which some of the lipids tend to self-associate.

301

302 The estimated crystallinity index values for the samples at the initial temperature of 30°C,
303 listed in Table 2, evidence a structural effect of the microalgae in decreasing the apparent
304 crystallinity of the starch granules, being this effect more obvious for the *Nannochloropsis*
305 species. Hydration has been shown to be a relevant factor affecting starch crystalline
306 structure, with a positive linear relationship between moisture content and crystallinity
307 index (Cheetham, et al., 1998; Qiao, et al., 2017). This is due to the fact that water favors
308 molecular motion and chain organization, thus promoting the formation of more ordered
309 structures (Qiao, et al., 2017). Therefore, the decreased apparent crystallinity observed for
310 the starch-microalgae suspensions is most likely a consequence of a limited moisture
311 accessibility towards the interior of starch granules, i.e. a certain amount of the added water
312 is taken up by the microalgae. The WAXS apparent crystallinity drop is in contrast with the
313 DSC results, which show that the gelatinisation enthalpy of corn starch is unaffected by the
314 presence of microalgae. The discrepancy between DSC and WAXS arises from (i) the lower
315 sensitivity of DSC to determine crystalline transitions as compared to WAXS and (ii) the
316 susceptibility of these techniques to identify crystalline or molecular order. With regards to
317 (i), it has been shown that X-ray scattering is indeed able to detect residual crystallinity at
318 temperatures higher than those at which the DSC gelatinisation endotherm is completed
319 (Wang, Zhang, Wang, & Copeland, 2016). Furthermore, in relation to (ii), whereas the
320 thermal transition determined by DSC accounts for the dissociation and unwinding of
321 helical structures (amylopectin double helices, amylose-lipid complexes and V-type single
322 helices), WAXS is only susceptible to those helical structures which are packed forming
323 crystallites. Thus, the presence of microalgae does not perturb the molecular order (i.e.
324 amount of helical structures) but restrains water access towards the interior of the granules,
325 hindering the organisation of these helices into crystallites and hence, reducing the apparent
326 crystallinity estimated by WAXS. The temperature range at which the crystallinity starts

327 decreasing for all the samples, i.e. 60-70°C (cf. Figure 4A), is well correlated to the
 328 gelatinisation temperature range determined from the DSC characterization (cf. Table 1).

329

330

331 **Figure 2.** Temperature-resolved WAXS patterns of corn starch samples. (A) pure corn
 332 starch, (B) corn starch-*Spirulina*, (C) corn starch-*Nannochloropsis* and (D) corn starch-
 333 *Scenedesmus*.

334

335 As shown in Figure 3, the WAXS patterns from the high amylose corn starch samples differ
 336 significantly from the corn starch patterns, with peak positions characteristic of the B-type

337 crystalline allomorph. A transition from A-type to B-type crystallinity has been previously
338 reported for corn starches when increasing the amylose content across a range of 0-84%
339 (Cheetham, et al., 1998). This is a consequence of longer amylopectin helical chains found
340 in high amylose starches, which are preferentially crystallised into the B-type allomorph
341 (Gernat, Radosta, Anger, & Damaschun, 1993). As observed for the corn starch, the peak
342 indicative of V-type crystallinity is detected in the sample containing *Spirulina* and sharp
343 peaks are visible in the scattering patterns of high amylose starch with *Spirulina* (peaks
344 located at ca. 15.0°, 20.4°, 26.6° and 29.2°) and *Scenedesmus* (peaks located at ca. 23.0 °
345 and 29.7°). The formation of V-type crystallites as a result of amylose-lipid complexes may
346 have important implications not only for the industrial processing of starch-based products,
347 but also from the nutritional perspective. For instance, it has been reported that when
348 amylose is complexed with lipids it is hydrolyzed and absorbed in the gastrointestinal tract
349 to the same extent as free amylose but at a slower rate (J. Holm, et al., 1983). Additionally,
350 the V-type amylose-lipid complexes have been shown to produce a decrease in the amylose
351 solubility, hence increasing the gelatinisation temperature (Eliasson, Carlson, & Larsson,
352 1981) and postpone retrogradation, thus enabling longer storage times (Krog, 1971). It
353 should be mentioned that the fact that a strong impact on the gelatinisation temperature is
354 not clearly observed in this work is most likely related to the low microalgae loadings added
355 into the starch aqueous suspensions (only 0.8 wt.-% with regards to the starch weight). The
356 calculated crystallinity values at 30°C are 21.5%, 26.8%, 13.9% and 17.9% for the high
357 amylose, high amylose-*Spirulina*, high amylose-*Nannochloropsis* and high amylose-
358 *Scenedesmus* samples, respectively. It is also worth noting that, as seen in Figure 4B, the
359 crystallinity of all the samples decreases significantly within the temperature range of ca.
360 70-90°C, which is in agreement with the temperature range determined from the DSC
361 gelatinisation endotherms. However, it should be noted that, in agreement with previous

362 studies (Wang, et al., 2016), the samples still present residual structural order at
363 temperatures higher than the DSC gelatinisation endotherm end temperature. It is also
364 worth noting that even though at the final temperature of 110°C the starch crystalline
365 structure has been almost completely destroyed, the peaks characteristic of the crystallised
366 lipids present in the *Spirulina* and *Scenedesmus* microalgae are still detected, indicating the
367 high thermal stability of these compounds.

368

369 The fact that the corn and high amylose starch present similar crystallinity values contrasts
370 with previous works stating that the crystallinity index is negatively correlated with the
371 amylose content (Gernat, et al., 1993). This may be true for low to intermediate amylose
372 contents, where the A-type allomorph is preserved; however, it could be hypothesised that
373 the longer helical structures present in starches with greater amylose contents and which
374 are crystallised into the B-type conformation, do not significantly affect the overall
375 crystallinity index. In fact, it has been shown that increasing the amylose content in B-type
376 starch does not have a strong impact on the crystallinity as it does in A-type starch
377 (Cheetham, et al., 1998). The complexation of amylose with the lipids from the added
378 microalgae seems to have a positive impact on the starch crystallinity. Thus, while the
379 general trend when incorporating microalgae into the suspensions is a reduction in the
380 apparent crystallinity (especially in the case of the *Nannochloropsis*) due to a reduced
381 swelling of the starch granules, this effect is reverted in the case of the high amylose starch
382 loaded with *Spirulina*, as in this case a greater amount of lipid-amylose complexes are
383 formed.

384

385

386 **Figure 3.** Temperature-resolved WAXS patterns of high amylose starch samples. (A) pure
 387 high amylose starch, (B) high amylose starch-*Spirulina*, (C) high amylose starch-
 388 *Nannochloropsis* and (D) high amylose starch-*Scenedesmus*.

389

390 **Table 2.** Lamellar peak parameters and crystallinity (X_c) for the native samples ($T=30^\circ\text{C}$).
 391 The lamellar peak parameters were obtained by fitting the experimental SAXS data to the
 392 sum function of a power-law plus a Gaussian-Lorentzian peak and crystallinity was obtained
 393 from the peak fitting of the WAXS patterns. Standard deviation values on the last digit are
 394 shown in parentheses.

	Lamellar peak position ($\text{\AA}^{-1}$)	Intensity of the lamellar peak (a.u.)	Width of the lamellar peak ($\text{\AA}^{-1}$)	Lamellar repeat distance ($\text{\AA}$)	X _C (%)
Corn	0.06358 (1)	110.4 (1)	0.0258 (1)	9.88	21.3 (3)
Corn- <i>Spirulina</i>	0.06335 (1)	72.6 (1)	0.0233 (1)	9.92	16.7 (4)
Corn- <i>Nannochloropsis</i>	0.06418 (2)	53.9 (1)	0.0269 (1)	9.79	13.9 (2)
Corn- <i>Scenedesmus</i>	0.06390 (1)	62.8 (1)	0.0257 (1)	9.83	18.0 (3)
High Amylose	0.06508 (1)	21.2 (1)	0.0239 (2)	9.65	21.5 (2)
High Amylose- <i>Spirulina</i>	0.06575 (2)	16.2 (1)	0.0197 (1)	9.56	26.8 (3)
High Amylose- <i>Nannochloropsis</i>	0.05239 (2)	24.6 (1)	0.0322 (3)	11.99	13.9 (2)
High Amylose- <i>Scenedesmus</i>	0.06456 (2)	14.4 (1)	0.0250 (2)	9.73	17.9 (2)

395

396

Figure 4. Evolution of the crystallinity index for the corn starch (A) and high amylose starch (B) samples.

3.2.2. Lamellar structure

The crystalline regions constituted by the tightly packed helical structures are alternated with amorphous regions, forming the next structural level known as lamellae. Due to their different physical density, the crystalline and amorphous domains present distinct X-ray scattering length density and, thus, the presence of these lamellar structures gives rise to the appearance of scattering features in the SAXS patterns of starch samples. To investigate the structural changes taking place in the lamellar arrangement during the gelatinisation process, as well as the effect of the microalgae addition, the SAXS patterns were collected simultaneously to the WAXS patterns when heating up the samples. As shown in Figure 5, at the initial temperature of 30°C all the samples present a well-defined peak located at ca. 0.06 Å⁻¹, which corresponds to the lamellar peak characteristic of hydrated native starch (Blazek & Gilbert, 2011; Chanvrier, et al., 2007; Salman, et al., 2009). The position of this peak is related to the thickness of crystalline and amorphous lamellae. Additionally, the peak

414 area depends on the scattering length density difference between the crystalline and
415 amorphous domains. To extract detailed structural information from the obtained SAXS
416 patterns, the experimental data within the q range of 0.045-0.2 $\text{\AA}^{-1}$ were fitted using a
417 mathematical function based on the sum of a power-law plus a Lorentzian-Gaussian peak
418 (cf. section 2.5) and the main parameters extracted from the fits are gathered in Table 2. The
419 first observation is that while the lamellar distance is not strongly affected by the starch
420 amylose content, remaining within the range of 9-10 $\text{\AA}$ typically reported for native starches
421 (P. J. Jenkins & Donald, 1995), the intensity of the lamellar peak is greatly reduced for the
422 high amylose starch. This is probably originated by a lower scattering length density contrast
423 in this sample. In fact, it has been reported that while the lamellar distance remains constant,
424 the size of the crystalline regions increases and the electron density difference between the
425 crystalline and amorphous regions decreases with the amylose content (P. J. Jenkins, et al.,
426 1995). This is a consequence of the larger amylopectin chain length observed for high
427 amylose starches, which results in the formation of the less densely packed B-type
428 crystallites. A-type structures are closely packed with water molecules between each double
429 helical structure, whereas B-type are more open and water molecules are located in the
430 central cavity formed by six double helices (Imberty, Buléon, Tran, & Pérez, 1991). Thus,
431 in low amylose starches (with short amylopectin chains which are crystallised into the A-
432 type structure) the crystalline lamellae are smaller but more densely packed, whereas in high
433 amylose starches (with B-type crystallites formed by large amylopectin chains) crystalline
434 lamellae become larger but more loosely packed.

435

436 As clearly observed in Figure 5 and confirmed by the fitting parameters, the incorporation
437 of microalgae into the corn starch suspensions leads to a marked decrease in the relative
438 intensity of the lamellar peak. Conversely, the peak position and width are not significantly

439 affected. This indicates that the addition of microalgae reduces the scattering length density
440 contrast but does not affect the lamellae structural parameters. Together with the DSC and
441 WAXS characterisation, this suggests that the presence of microalgae does not affect the
442 molecular order but impairs the organisation of these helical structures into densely packed
443 and ordered crystalline domains by limiting to some extent the swelling of starch granules.
444 The reduced apparent crystallinity would then contribute to the observed decrease in the
445 scattering length density contrast. In the case of the high amylose starch, the presence of
446 microalgae does not have a strong effect on the lamellar peak intensity, but the peak position
447 and width are affected differently depending on the microalgae species. A significantly
448 broader lamellar peak, with a greater associated lamellar repeat distance of ca. 12Å is
449 observed upon incorporation of *Nannochloropsis*, being indicative of more heterogeneous
450 lamellae in the presence of this microalgae species. This effect can also be explained by a
451 decreased water accessibility towards the interior of starch granules, since it has been
452 reported that in the dry state only a small fraction of starch helices can be aligned and packed
453 to construct the semi-crystalline lamellar structure (Qiao, et al., 2017). Together with the
454 reduced crystallinity estimated from the WAXS patterns, this result indicates that the
455 presence of *Nannochloropsis* has a strong impact on the swelling mechanism of starch
456 granules. In contrast, the addition of *Spirulina* has the opposite effect, i.e. a greater degree
457 of crystalline order is attained (as indicated by the higher crystallinity index) and more
458 homogeneous lamellae are formed (as suggested by the reduced lamellar peak width). The
459 distinct effect of these microalgae on the starch structure and the gelatinisation process may
460 be explained based on their different composition and, mostly, on their distinct cell wall
461 structure. The total lipidic contents have been reported as ca. 6% for *Spirulina* (Matos, et al.,
462 2016), 8-16% for *Nannochloropsis* (Matos, et al., 2016) and up to 30% for *Scenedesmus*
463 microalgae (Jiang, et al., 2017; Ma, et al., 2017). However, the *Nannochloropsis* microalgae

464 have been shown to possess a tough cell wall, formed by a cellulosic inner layer and an outer
465 layer rich in algaenans, which requires intense sonication treatments to be disrupted (Fabra,
466 et al., 2017; Scholz, et al., 2014). The existence of such resistant cell walls would then
467 impede the release of lipids from the cells towards the aqueous medium. The cell wall
468 structure of the *Scenedesmus* species also contains an inner cellulosic layer, which is then
469 covered by a thin middle layer and an outer pectic layer (Bisalputra & Weier, 1963). On the
470 other hand, the cell walls in *Spirulina* seem to be richer in proteins and they present porous
471 features which may facilitate the release of cell components (Van Eykelenburg, 1977). To
472 corroborate their different cell wall integrity, raw microalgae were characterised by TEM
473 and the crystalline components in their cell walls were stained with uranyl acetate. As
474 observed in Figure S2, while the *Nannochloropsis* microalgae presented intact cell walls,
475 intensely stained and with clearly defined edges, some of the cell walls in *Spirulina*
476 microalgae seemed to be disrupted as a result of the sample preparation process. Therefore,
477 despite the lower lipidic content of *Spirulina*, these components could more easily be
478 released through the weak cell walls, being then able to interact with the amylose fraction in
479 starch and forming complexes which induce a higher degree of crystallinity, as well as
480 promoting the formation of more homogeneous lamellae.

481

482

483 **Figure 5.** SAXS patterns of starch and starch-microalgae suspensions in water at 30 °C.

484

485 The structural changes undergone by starch during the gelatinisation process were studied
 486 by heating the prepared suspensions up to 110°C and recording the SAXS patterns. The
 487 results for the pure corn starch and microalgae-loaded samples are shown in Figure 6. As
 488 observed from this figure, the relative intensity of the lamellar peak decreases gradually
 489 when the temperature increases, reaching a point at which the well-resolved peak is replaced
 490 by a very broad shoulder, which in some of the samples is eventually lost at the highest
 491 temperatures. A similar broad shoulder has been observed for retrograded starch and it has
 492 been correlated with the growth of crystals in an amorphous matrix (Amparo Lopez-Rubio,
 493 Flanagan, Shrestha, Gidley, & Gilbert, 2008). The distortion of the lamellar structure is
 494 evidenced by a strong increase in the width of the lamellar peak and in the associated lamellar
 495 repeating distance. As deduced from Figure S3, which shows the evolution of the calculated
 496 lamellar repeat distance as a function of the temperature, this transition takes place within
 497 the temperature range of 80-100°C.

Figure 6. Temperature-resolved SAXS patterns of corn starch samples and the corresponding fitting curves. (A) pure corn starch, (B) corn starch-*Spirulina*, (C) corn starch-*Nannochloropsis* and (D) corn starch-*Scenedesmus*.

As shown in Figure 7, increasing the temperature has a similar effect on the lamellar peak in the high amylose starch samples. In that case, the transition from the lamellar peak to the broad shoulder also takes place within a temperature range of 80-100°C for the pure and

microalgae-loaded samples (cf. Figure S1). As opposed to the corn starch samples, the shoulder is still visible for all the high amylose samples at the final temperature of 110°C, indicating that starch still presents a residual long-range structure. This, together with the residual crystallinity detected in the WAXS patterns when raising the temperature above the gelatinisation endotherm end temperatures calculated from DSC, supports the hypothesis presented by Wang et al. that the gelatinization endotherm of starch does not represent complete starch gelatinization (Wang, et al., 2016).

Figure 7. Temperature-resolved SAXS patterns of high amylose starch samples and the corresponding fitting curves. (A) pure high amylose starch, (B) high amylose starch-*Spirulina*, (C) high amylose starch-*Nannochloropsis* and (D) high amylose starch-*Scenedesmus*.

4. Conclusions

This study presents a detailed characterisation of the structural effects undergone by two types of starch with different amylose contents during the gelatinisation process as affected by the addition of different microalgae, carried out by means of temperature-resolved simultaneous SAXS/WAXS experiments, combined with DSC analyses.

The three different microalgae species incorporated into the starch aqueous suspensions, namely *Spirulina*, *Nannochloropsis* and *Scenedesmus*, have shown to induce structural changes during the gelatinisation process. Interestingly, the observed structural effects are strongly dependent on the microalgae cell wall structure and integrity. In general, the presence of microalgae limits water accessibility towards the interior of starch granules, hence impeding the organisation of amylopectin double helices into highly ordered crystalline regions, leading to the formation of more heterogeneous lamellar structures with decreased apparent crystallinity. This effect is more evident in the case of the *Nannochloropsis* species, whose resistant cell wall remains intact and does not allow the release of any component towards the aqueous medium. In contrast, the weak cell walls in the *Spirulina* microalgae are easily disrupted, enabling the release of the lipids contained within the cells. These free lipids form helical inclusion complexes with the starch amylose chains, promoting the formation of a certain fraction of V-type crystallites, increasing the overall crystalline index and resulting in the formation of more homogeneous lamellar structures. The formation of these lipid-amylose complexes is relevant from the processing and nutritional point of view. Starch granules become more thermodynamically stable, i.e. the gelatinisation temperature increases, and the complexed amylose is known to be more slowly digested in the gastrointestinal tract than free amylose.

These results support the potential of scattering techniques in providing a detailed structural information for starch samples, being able to simulate industrial-relevant processes such as the gelatinisation phenomenon by means of temperature-resolved experiments. The incorporation of microalgae with permeable and/or weak cell walls into starch may be interesting to generate more crystalline and thermodynamically stable products through the formation of lipid-amylose complexes.

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Supplementary Material

Figure S1. X-ray diffraction patterns of the raw microalgae (freeze-dried).

Figure S2. TEM images of raw microalgae: (A) *Spirulina*, (B) *Nannochloropsis* and (C) *Scenedesmus*.

Figure S3. Evolution of the lamellar distance extracted from the SAXS fitting parameters for the corn starch (A) and high amylose starch (B) samples.