

1 **Host-guest molecular interactions in vanillin/amylose**
2 **inclusion complexes**

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15 (FTIR), Circular Dichroism (CD), Differential Scanning Calorimetry (DSC).

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17
18 **Abstract**

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20 The interaction of 4-hydroxy-3-methoxybenzaldehyde (vanillin) and Hylon VII
21 due to the formation of an inclusion complex is studied by Fourier transformed infrared
22 spectroscopy (FTIR), differential scanning calorimetry (DSC) and circular dichroism
23 (CD). The results confirm the close interaction among the different functional groups of
24 vanillin and its host. In addition, a second case study was carried out with an amylose
25 from a different source (100% amylose, APTIII). As a result, remarkable differences

26 were found in the vanillin complexation capability of this amylose, which is only
27 evidenced in solution by circular dichroism spectroscopy studies through a clear Cotton
28 effect. This finding confirm the value of using CD studies, which allow finding that,
29 depending of the amylose source, inclusion complexes can be found only in solution, or
30 both in solution and the coexisting precipitates, when this is evidenced by other
31 techniques such as X-ray diffraction (XRD) or Differential Scanning Calorimetry
32 (DSC). Moreover, solubility assays and complexation of both starches with iodine and
33 subsequent absorption spectroscopy studies gives more information regarding the
34 possible source of the starch encapsulation capability. Thus, Hylon VII evidences higher
35 capacity as vanillin encapsulant than APTIII, showing the formation of inclusion
36 complexes both in solution and solid phase, whereas APTIII complexes are only
37 perceivable in solution.

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44 **Introduction**

45 Cereal grains like wheat, rice, corn, oats, barley and also tubers like potatoes are
46 rich in starch, a glucose polysaccharide used as reservoir of energy. Natural starches are
47 mixes of two polymers: the linear amylose (~10-25%) and the branched amylopectin
48 (~75-90%), both made of α -1,4 linkages with a small proportion of α -1,6 bonds. About
49 5% of the glucose units are linked in amylopectin in the form α -1,6, and 1% in the case
50 of amylose, explaining the branched or the linear conformations respectively.¹ Amylose
51 adopts a helical conformation known as V-amylose, giving the possibility to host
52 molecules such as flavor compounds inside the cavity formed by the helix.^{2,3} These
53 structures, called inclusion complexes, provide protection to the guest molecules against
54 degradation processes.^{4,5} Useful techniques to investigate amylose inclusion complexes
55 in solid phase are differential scanning calorimetry (DSC) and X-ray diffractometry
56 (XRD), while circular dichroism (CD) has proved to be a valuable technique when
57 studying inclusion complexes in solution.^{6,7}

58 Vanillin (4-hydroxy-3-methoxybenzaldehyde), the main component of natural
59 vanilla extracts, is one of the most used compounds in food and pharmaceutical
60 industries as flavoring, antioxidant and masking agent.⁸ In a work done previously,⁹
61 demonstrated the ability of the amylose-rich commercial starch Hylon VII (70%
62 amylose) to form an inclusion complex with vanillin molecule. In that work, the
63 formation of the complex was addressed for the first time both in the soluble fraction as
64 well as in the precipitate obtained, using CD and XRD. Moreover, the results obtained
65 by CD were corroborated by theoretical simulations using quantum mechanical hybrid
66 approaches, which showed the energetic stability of the complex and suggested that the
67 chiro-optical changes observed arises from the geometric distortion undergone by the
68 vanillin molecule when included in the amylose helical cavity.

69 In this work, a detailed description of the molecular interaction between vanillin
70 and Hylon VII is given on the basis of the results obtained by Fourier transformed
71 infrared spectroscopy (FTIR) and DSC. The results confirm the existence of the
72 inclusion complex and remark the close interaction among the different functional
73 groups of vanillin and its host. In addition, a second case study was carried out with an
74 amylose from a different source (100% amylose, APTIII); as a result, remarkable
75 differences were found in the vanillin complexation capability of this amylose, which is
76 able to form inclusion complex with vanillin in solution as revealed by a clear Cotton
77 effect observed by circular dichroism, but do not show complex signals in either DSC
78 and XRD analysis, indicating that no complex is present in the solid phase. In order to
79 study the possible structural differences that cause this disparity in the encapsulant
80 capability, limiting solubility studies and complexation of both starches with iodine and
81 subsequent absorption spectroscopy studies were assessed, confirming that the
82 complexing ability of an amylose containing starch to form inclusion complexes with
83 vanillin is strongly dependent on the amylose type and source.

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85 **Materials and methods**

86 *Materials*

87 High amylose (70%) maize starch Hylon VII was provided by the National
88 Starch & Chemical Company (Bridgewater, NJ, U.S.A.). Pure 100% amylose (APTIII)
89 and 4-hydroxy-3-methoxybenzaldehyde (vanillin, 99% purity) were purchased from
90 Sigma-Aldrich. All the other reagents were of analytical degree and used as received.

91

92 *Experimental*

93 **Preparation of the Hylon VII and pure amylose dispersions**

94 1.00 g of Hylon VII was dispersed in 150ml of Milli-Q water and heated at
95 130°C for 90 min in a flask with a screw cap. The suspension was then left to cool down
96 to 50°C, at this temperature vanillin was added as indicated in each case. The same
97 procedure was followed when using APTIII.

98

99 **Inclusion Complexes: sample preparation**

100 1.20 g of vanillin (54.5% w/w vanillin/starch) dissolved in 1.50 ml of ethanol
101 was added to 150 ml of the starch dispersion. The mixture was allowed to rest at room
102 temperature for 24 h. The precipitated obtained was filtered, washed, centrifuged, and
103 intensively dried. The supernatant was used for the CD studies.

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105 **Solubility determination**

106 The limiting (saturated) solubility of each starch was calculated by heating an
107 excess of starch in a given volume of Milli-Q water at heated at 130°C for 90 min in a
108 flask with a screw cap, with agitation. After that, the dispersion was cool down at room
109 temperature (20°C) several hours, centrifuging the suspension at 9000 g and discarding
110 the pellet. The supernatant (a completely translucent solution) was separated, 8700 g
111 during 30 minutes at 20°C, and an aliquot of this solution was freeze-dried and
112 weighted, allowing calculating the amount of dissolved starch in each case.

113

114 **FTIR spectra**

115 About 5 milligrams of solid were mortared and mixed with 200 mg of KBr (IR
116 grade) to obtain the pellet. The Fourier transform infrared spectra (FTIR) were recorded
117 on a Thermo Nicolet 8700 spectrometer, in the range of 660-4000 cm⁻¹. Thirty two
118 scans were accumulated for each measurement. As a control, a mechanical mixture was

119 prepared by mixing the amounts of starch and vanillin, matching the relative amounts of
120 each component to the one used in the inclusion complex preparation.

121

122 **DSC measurements: experimental conditions**

123 The dried material obtained (see Inclusion Complexes: sample preparation) was
124 used to carry out the high pressure differential scanning calorimetry (HP-DSC,
125 *Shimadzu DSC-50*) using sealed aluminum capsules. 1 mg of dried sample was placed
126 into a high pressure DSC pan adding 5 μ l of Milli-Q water to ensure an adequate
127 moisture amount. The DSC pan was closed and leave to rest 1 hour until the
128 measurement. The heating rate was 5°C/min from 30 °C until 190°C, and the stage of
129 cooling (heat dissipation) of each sample was also recorded.

130 Regular DSC scans were performed with the same equipments under a rate of 10
131 °C /min using standard aluminum sealed capsules. Pans are crimped, but not
132 hermetically sealed, supporting a maximum pressure of 0.3 MPa (Shimadzu, #201-
133 53090).

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135 **Circular dichroism measurement of APTII complex**

136 An appropriate dilution (0.15 ml into 3.00 ml of Milli-Q water) of the
137 supernatant obtained (see Inclusion Complexes: sample preparation) was used to
138 determine the CD and UV spectra, recorded using a Jasco-815 spectrometer under a 5
139 L/min flow of nitrogen (99.998%). The scans were done at 20 nm/min with a response
140 of 8 seconds, using a band width of 1 nm and averaging 5 accumulated spectra for each
141 measurement.

142

143 **X-Ray diffraction measurement of complexes**

144 X-ray diffraction patterns of the powders obtained (see Inclusion Complexes:
145 sample preparation) were recorded in a Siemens D5000 diffractometer using a Cu K α
146 radiation. The operating conditions were a 0.154 nm radiation wavelength, a voltage of
147 40 kV and a current of 30 mA. Diffractograms were scanned over the 2 θ range of 3.5 -
148 35°, with a scan rate of 0.022°/s.

149

150 **Iodine complexation of Hylon VII and APTII**

151 In order to measure the UV/VIS spectrum of the amylose/iodine complex
152 different aliquots of the supernatant solutions obtained for Hylon VII and APTIII (see
153 Solubility determination) were added to 3.00 ml of a 2×10^{-3} M KI/ 5.2×10^{-5} M I₂
154 solution, obtaining the typical blue coloration. The spectra were recorded from 700 to
155 300 nm with a PG T60 spectrometer.

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157

158 **Results**

159 *FTIR studies of Hylon-vanillin inclusion complexes*

160 FTIR spectra were recorded for samples of the starch Hylon VII, pure vanillin
161 (4-Hydroxy-3-methoxybenzaldehyde), the vanillin-starch inclusion complex and the
162 mechanical mixture of starch and vanillin, matching in this last sample the relative
163 amounts of each component used in the complex preparation (Figure 1). For clarity, the
164 figure was divided in part A, which shows the spectra of the starch Hylon VII (black
165 line) and the mechanical mixture starch+vanillin (green line). Figure 1B shows the
166 spectra of pure vanillin (red line) and the one of the inclusion complex (blue line).

167 In part A, the spectrum of the pure starch shows the typical absorptions of the
168 polysaccharides. The region of frequencies around 1000 cm⁻¹, attributed to different
169 types of transitions: two of the most intense are assigned to the C–C stretching

170 vibrations from 1200 to 1103 cm^{-1} , and to the C–O bending vibrations from 1047 to
171 994 cm^{-1} ; at longer wavenumbers, the noticeable O-H stretching vibration band centered
172 at 3402 cm^{-1} .¹⁰ The mechanical mixture displays high absorption intensity in the whole
173 fingerprint region between 700 and 1700 cm^{-1} , and a broad O-H band between 3100 and
174 3600 cm^{-1} . The overall spectrum and in particular this very broad O-H stretching
175 vibration band represents, as expected, the sum of the individual spectra of the starch
176 and vanillin, as both components were mixed just before running the spectrum.

177 In Figure 1B, the pure vanillin spectrum displays a wide fingerprint region
178 between 700 and 1700 cm^{-1} with several peaks of high absorption intensity, and the
179 phenol O-H stretching vibration band centered at 3170 cm^{-1} . For the Hylon VII-vanillin
180 inclusion complex, remarkable differences can be observed in the spectrum. First, the
181 vanillin's O-H band at 3170 cm^{-1} is not observed, appearing only a unique band
182 centered at 3415 cm^{-1} . In addition, several intense bands of the vanillin spectrum
183 originally found between 700 and 1700 cm^{-1} are in the complex depleted or their
184 absorption intensity significantly diminished.

185 To better describe the numerous changes observed in the fingerprint region of
186 the inclusion complex, the Figure 2 plots exclusively this region showing
187 simultaneously the bands of the four samples studied, and each band is particularly
188 depicted. At first glance, it is noticeable that some bands (pointed out with black dotted
189 arrows) observed in the vanillin spectrum vanish in the spectrum of the inclusion
190 complex (compare the red and blue curves, respectively).

191 The bands at 1465 cm^{-1} and 1429 cm^{-1} are assigned to the asymmetric
192 deformation of the methyl group belonging to the $-\text{OCH}_3$ substituent.^{11,12} The two
193 strong bands at 1265 and 732 cm^{-1} correspond to the C-OCH₃ stretching mode.¹³
194 Additionally, the bands at 812 cm^{-1} and 856 cm^{-1} assigned to C-H out-of-plane bending

195 vibrations, also vanished.¹⁴ It is worth to recall that many of these bands belonging to
196 the vanillin molecule fall in regions where no absorption bands are present in the pure
197 starch spectrum, which facilitates the comparison of and allows seeing which of these
198 remain in the starch-vanillin mechanical mixture and which disappear after obtaining
199 the inclusion complex.

200 The vanillin's bands which still remain, although significantly lowered in their
201 intensity and some of them slightly shifted, are those bands centered originally at: 1300
202 cm^{-1} (shifted to 1292 cm^{-1} , assigned to the phenolic OH bending), 1510 cm^{-1} (shifted to
203 1516 cm^{-1}) and 1589 cm^{-1} (shifted to 1593 cm^{-1}) both assigned to C=C and C-C
204 stretching mode vibrations of the benzene ring; and 1664 cm^{-1} (shifted to 1672 cm^{-1} ,
205 corresponding to the aldehyde C=O stretching mode).

206

207 *DSC studies of Hylon-vanillin inclusion complexes*

208 Figure 3 (main frame) shows two HP-DSC thermograms obtained by scanning
209 the temperature from 30°C to 190°C for the Hylon VII-vanillin inclusion complex
210 sample (full black line) and the Hylon VII control sample (dashed red line). The
211 thermogram obtained for Hylon VII-vanillin shows a unique well-defined endothermic
212 peak at 108°C , which is assigned to the dissociation of the inclusion complex. In fact,
213 90°C - 110°C is the range of temperatures reported for the "melting" peaks of most of the
214 starch inclusion complexes with different ligands, which are listed in Table I,
215 incorporating the present case.

216 Instead, the thermogram obtained with Hylon VII control sample (dashed red
217 line) presents a well defined peak at 177°C . Such transitions at elevated temperatures
218 (above 140°C) have been previously assigned to changes in the amylose fraction of the
219 starch due to endothermic transitions of amylose crystals,¹⁵ formed in our case probably

220 during the experimental procedure followed to obtain the complex (starch
221 gelatinization, then subsequent precipitation by slow cooling). During cooling processes
222 no signals were observed, either for the Hylon VII control or the Hylon VII-vanillin
223 samples, indicating that the melting process of the amylose-vanillin complex is not
224 reversible; and either the transition originally found for the control sample, at least
225 under the scanning conditions assayed. A pure vanillin control sample presented no
226 signals by HP-DSC during the whole heating and cooling cycle, indicating that vanillin
227 is dissolved in the water and chemically stable in the range of temperatures tested.
228 These transitions were observed in thermograms obtained in high pressure aluminum
229 capsules with an excess of water added to the solid sample. However, some works have
230 also been reported in which the formation of inclusion complexes are studied analyzing
231 the dried solid samples obtained, focusing on the transitions (melting) occurring to the
232 guest ligands before and after complexation. Therefore we also performed a DSC using
233 regular crimped aluminum capsules and run scans of dried samples of pure vanillin
234 crystals (blue solid line) and the Hylon VII-vanillin inclusion complex (blue dotted
235 line), which are displayed in the inset of Figure 3. In both cases the amount of vanillin
236 introduced in the samples was the same. The first run shows a strong endothermic peak
237 assigned to the melting at 82°C of pure vanillin crystals,¹⁶ and the second run, in
238 contrast, remarks the disappearance of the peak at 82°C, leaving a remaining small and
239 broad peak at lower temperatures.

240

241 *Characterization of a vanillin inclusion complex obtained with another amylose* 242 *source*

243 To evaluate the complexing ability of amylose obtained from a different source
244 we assayed the amylose APTIII (from Sigma-Aldrich, see materials). The APTIII-

245 vanillin inclusion complex was prepared with exactly the same methodology and
246 host/guest ratios used in the case of the starch Hylon VII.

247 The first study was to record the circular dichroism spectra of the solution after
248 centrifugation and separation of the precipitate. The experimental procedure is clearly
249 detailed in methods (see Inclusion Complexes: sample preparation) and in a previous
250 work.⁹ The obtained absorption and associated circular dichroism spectra of the APTIII-
251 vanillin solution are shown in Figure 4; being similar to those obtained for the Hylon
252 VII-vanillin inclusion complex. The CD spectrum shows the same two negative bands
253 at 205 and 230 nm, centered at the same wavelengths observed in UV spectrum. Notice
254 that the vanillin molecule has no chiral centers, thus, a pure aqueous vanillin solution
255 does not show any signal in CD, and the water soluble fraction of pure amylose have no
256 absorption above 180 nm.

257 As in the case study with Hylon VII, X-Ray diffraction studies were performed
258 with the solid isolated and dried after obtaining the APTIII-vanillin complex. However,
259 no differences between the complex and the control samples were found in the x-ray
260 pattern. Moreover, HP-DSC measurements showed no peaks in heating-cooling cycles,
261 confirming that no APTIII-vanillin complex was present in the solid phase. CD, XRD
262 and HP-DSC experiments were repeated with different batches, arriving to the same
263 results, that is, CD yielded evidence of complex formation in solution, but lack of
264 signals in XRD and DSC, indicating that no complex is found in the solid phase.

265

266 *Comparison of the ability of Hylon VII and APTIII to form inclusion complexes*
267 *studied by titration of an iodine solution.*

268 With the aim of characterizing the capability of inclusion complex formation of
269 both amylose sources, both Hylon VII and APTIII were complexed with iodine

270 solutions according to the method developed by Gilbert and Spragg.¹⁷ First, saturated
271 solutions at room temperature (20°C) of both starches were obtained, and the limiting
272 solubility of each starch in water was determined (see Solubility determination). The
273 results showed that the limiting solubility in pure water was 172 mg/ml for Hylon VII
274 and 226 mg/ml for APTIII amylose.

275 Two well measured volumes of iodine solutions from the same batch (see Iodine
276 complexation of Hylon VII and APTII) were titrated separately by adding successive
277 aliquots of the Hylon VII and APTIII saturated solutions. As a result, a linear increasing
278 formation of the iodine-amylose complex is clearly observed with both starches. In
279 Figure 6 the absorption at the maximum absorption wavelength (λ_{\max}^{abs}) of the iodine
280 complexes with Hylon VII and APTIII is plotted as a function of polysaccharide
281 concentration. Two straight curves with excellent linear correlation coefficients (higher
282 than 0.99) were obtained. It is remarkable that the λ_{\max}^{abs} was invariant, being always 602-
283 603 nm irrespective of the starch identity and concentration.

284 Noticeable, a pronounced difference among both starches is observed in the
285 slope of the linear regressions, indicating that for a similar concentration of starch, the
286 absorbance of the Hylon complex is about 10 times higher than the absorbance of the
287 APTIII iodine complex. This difference is observed for polysaccharides concentrations
288 well below the limiting solubility of both starches.

289 A valuable parameter for the characterization of a starch ability to form
290 inclusion complexes with iodine is the so called "blue value",¹⁷ which is a calculation
291 based on the absorption in the far red side (680 nm) of the iodine complex spectrum,
292 which is directly proportional to the concentration of said complex,

293
$$BV = \frac{0.4 \times Abs_{680nm}}{C_{starch} (mg/ml)} \quad \text{Eq. 1}$$

294 To make a reliable calculation of BV is relevant performing calibration curves as a
295 function of the concentration of the tested starches, as shown in Figure 5. The results
296 described in Table II shows a very good reproducibility in the BV value obtained, which
297 for Hylon VII is 12 times larger than for APTIII.

298

299 **Discussion**

300 The molecular structure of the amylose, a linear polysaccharide having 99% of
301 α -(1 \rightarrow 4) links among glucose monomers with almost no branching, gives the
302 possibility of forming different types of helices, such as the double helices observed in
303 the diffraction patterns A and B in the starch granules, and also a single helical
304 conformation, characteristic in the case of inclusion complex formation, with a
305 diffraction pattern called type V.¹⁸

306 The amylose molecule builds an helical internal cavity which, when hosting the
307 vanillin molecules forms an inclusion complex and shrinks wrapping around the guest,
308 as depicted in the sequence depicted in Figure 6 parts a and b, thus decreasing the
309 interatomic distances favoring the interaction among vanillin and amylose functional
310 groups.⁹ This close interaction becomes evident in the changes observed in the FTIR
311 spectrum of the complex, when comparing with the spectra obtained with pure vanillin,
312 amylose, or a simple mechanical mixture of both.

313 In general, in Figures 1 and 2, we can observe that the host-guest interaction
314 significantly reduce the absorption intensity of the vanillin, the guest in this case. First,
315 the vanillin's O-H band originally at 3170 cm^{-1} is not observed, appearing only a unique
316 band centered at 3415 cm^{-1} . This suggests a close interaction of this functional group
317 with the amylose in the complex, leading to a noticeable shift to higher frequencies (a
318 faint shoulder can be seen at 3260 cm^{-1}), being overlapped by the O-H stretching band

319 of the starch, which was in fact also shifted from 3350 to 3415 cm^{-1} .²¹ This is supported
320 by a previous theoretical approach, which also allowed understanding the circular
321 dichroism spectrum of the amylose-vanillin inclusion complex. That study mentions the
322 hydroxyl phenolic group among the chemical substituents of vanillin which experience
323 the shortest interatomic distances ($<3\text{\AA}$) with the atoms of the amylose (see Table II and
324 Figure 5 in Rodríguez et al.⁹) Similar changes were observed in the obtaining of the
325 inclusion complex of vanillin with cyclodextrin, as reported by Rajendiran &
326 Balasubramanian,¹⁹ who also attributed this change to a more internal and compromised
327 position of the OH group within the helical cavity.

328 Among the other differences observed in the FTIR spectra, the bands at 1465
329 cm^{-1} and 1429 cm^{-1} (asymmetric deformation of the $-\text{OCH}_3$ methyl group) and the two
330 bands at 1265 and 732 cm^{-1} ($\text{C}-\text{OCH}_3$ stretching mode) disappear in the inclusion
331 complex spectrum. This is also consistent with the changes induced by the host when
332 including the guest, suggested by theoretical modeling: the Figure 6 c shows a torsion
333 out of plane of the $-\text{OCH}_3$ methyl group which, by simulation, gives place to a
334 theoretical CD spectrum similar to the experimentally found, supporting the proposed
335 structure. The bands observed in the CD spectra are then assigned to the chirality
336 induced by the geometrical distortion of the vanillin molecule after entrapment into the
337 amylose helix, leading to a new chiral conformation and producing the signals in the
338 CD spectra (Rodríguez et al., 2011,⁹ and this work). This represents a clear Cotton
339 effect, which, in the case of this complex is induced by the molecular distortion of the
340 vanillin when included in the amylose cavity.

341 The formation of the complex is also undoubtedly ascertained by using the
342 thermoanalytical DSC analysis, one of the most widespread techniques to study the
343 formation of amylose inclusion complexes. When applied to polymers, DSC detects

344 transformations that can be primarily ascribed to fusion, inter-conversion between
345 different crystalline states, and sub-T_g transitions of glassy or crystalline polymers. In
346 the case of HP-DSC applied to starches and their complexes with diverse guest
347 molecules, this technique reveals endothermic transitions assigned to the dissociation of
348 the complex when the solid is subjected to heating in the presence of water, a process
349 commonly called "melting" of the inclusion complex.¹⁸ The peak at 108 °C found by
350 HP-DSC and the disappearance of the melting peak of the pure ligand (Figure 3) are
351 unequivocal evidences of the amylose-vanillin inclusion complex existence. Similar
352 findings were reported in the cases of β-cyclodextrin/vanillin and β-
353 cyclodextrin/flavonoid inclusion complexes.^{20,21} On the other hand, the transitions
354 observed on pure starches at elevated temperatures (above 140 °C) have been assigned
355 to changes in the amylose fraction of the starch, probably due to an endothermic
356 transition of amylose crystals, in our case formed during the experimental procedure to
357 obtain the complex (starch gelatinization, then vanillin addition and subsequent
358 precipitation by slow cooling). We found such a transition at 177 °C (see Figure 3, red
359 dashed line), a similar to the one observed by Heussen et al.¹⁵

360 The examples of inclusion complexes and their melting points shown in Table I
361 remark that melting temperatures fluctuate depending on factors such as the source of
362 amylose used, its native or processed nature, and the guest molecule incorporated in the
363 helical structure. Factors depending particularly on the amylose source are evidenced
364 by the inclusion complexes obtained with geraniol and fenchone, whose reported
365 melting temperatures are 91°C/107°C and 92°C/114°C, for the case of complexation
366 using native potato starch and using potato amylose, respectively (see Table I; Nuessli
367 et al.²²; Nuessli et al.⁶). Another example is the melting temperatures recorded when
368 using native and pre-processed starch, like in the complexes obtained with Hylon VII by

369 Lay Ma et al.²³. The authors observed differences in the temperatures of dissociation of
370 the complexes formed among Hylon VII and three different fatty acid esters (ascorbyl
371 palmitate, retinyl palmitate and phytosterol esters), when the starch is used in its native
372 form or after lipid extraction, informing temperatures of 100°C/ 98°C, 102°C/ 80°C and
373 126°C/ 98°C, respectively.

374 Thus, the key influence of the starch source, its composition and pre-treatment
375 before complexation with a flavor ligand help to explain why in this work we can show
376 that Hylon VII forms an inclusion complex both in the solid, as in the solution obtained
377 by centrifugation and filtration; whereas pure APTIII amylose evidences the formation
378 of inclusion complex with vanillin only in solution. To better understand starch
379 properties which may account for these differences, we tested the complexing ability of
380 the starches by obtaining iodine inclusion complexes, a well known spectroscopic tool.

381 Remarkable differences were found between both Hylon VII and APTIII
382 starches. The lowest complexation capacity displayed by APTIII, besides the fact that
383 the solubility of APTIII is greater than the one observed for Hylon VII, suggest that
384 APTIII has higher proportion of short chains, which do not form iodide complex,
385 together with a lower proportion of soluble chains with a length similar to those found
386 in the Hylon VII solutions. This last statement is suggested by the fact that the
387 maximum absorption wavelength (λ_{\max}^{abs}) of the complexes with iodine is the same for
388 both Hylon VII and APTIII. According to Wulff et al.²⁴ (and references therein), the
389 λ_{\max}^{abs} shifts to longer values as the average chain length of the amylose increases. In
390 addition, the finding of a “blue value” for Hylon VII twelve times higher than the one
391 obtained for APTIII further supports all the previous statements.

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396 **Conclusions**

397 Summarizing, Hylon VII evidences higher capacity as vanillin encapsulant than
398 APTIII, showing the formation of inclusion complexes in the solid phase whereas
399 APTIII complexes are only appreciable in solution by circular dichroism. These
400 conclusions are reached on the basis of a joint analysis of the spectroscopic evidences
401 provided by Circular Dichroism, FTIR, XRD analysis and iodine complexation, as well
402 as thermoanalytical DSC analysis and the previous support shown by theoretical
403 modeling. These studies confirm the ability of the amylose to form complexes with
404 vanillin and the close interaction of the corresponding host/guest functional groups.

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517 Figure Captions:

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519 Figure 1: FTIR spectra of A) Hylon VII (black solid line) and the mechanical mixture of
520 Hylon VII with vanillin (green solid line). B) Inclusion complex between Hylon VII and
521 vanillin (blue solid line) and pure vanillin (red solid line).

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523 Figure 2: FTIR spectra of the Figure 1 showing a selected region between 700-1800 cm^{-1} ,
524 for Hylon VII (black solid line), mechanical mixture of Hylon VII with vanillin
525 (green solid line), inclusion complex between Hylon VII and vanillin (blue solid line)
526 and pure vanillin (red solid line). The vanillin bands which disappear in the complex
527 (and their wavenumbers) are indicated with dashed arrows, whereas the bands which
528 have a significant lowered intensity are indicated by solid arrows. The assignments of
529 the bands are described in the text.

530

531 Figure 3: High pressure DSC thermograms of the inclusion complex between Hylon VII
532 and vanillin (black solid line) and Hylon VII as control sample (red dashed line) in
533 presence of water. Insert : DSC thermograms of the sample containing the solid
534 inclusion complex (blue dashed line) and pure vanillin crystals (blue solid line).

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536 Figure 4: CD and UV spectra of the inclusion complex between ATPIII and vanillin in
537 solution. For scanning conditions details, see Circular dichroism measurement of APTII
538 complex.

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540 Figure 5: Scatter plot of the absorbances measured at the maximum absorption
541 wavelength $\lambda_{\text{max}}^{\text{abs}}$ of iodine complexes formed with increasing concentrations of Hylon
542 VII (green triangles) and APT III (red squares).

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544 Figure 6: a) top view of a 10 units amylose helix alone ; b) top view of the inclusion
545 complex of the helix and vanillin, and c) representation of the molecular distortion of
546 vanillin upon inclusion, which gives place to CD spectrum.

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556 Table I: phase transition temperatures (melting) of inclusion complexes between
 557 amylose and various guest compounds.

Guest molecule	Amylose source	Transition temperatures (°C)	Reference	
Hexanal	native potato starch	107	Jouquand et al. ²⁵ , 2006	
γ-hepta-lactone		90	Heinemann et al. ²⁶ , 2001	
γ-nona-lactone		91		
γ-deca-lactone		95		
δ-deca-lactone		105		
δ-dodeca-lactone		115		
Decanal		90		Nuessli et al. ⁶ , 1997
(-) fenchone		107		
(-) carvone		91		
Geraniol		91		
(+) campher		76		
Thymol		105		
1-naphthol		109		
Fenchone		potato amylose	114	Nuessli et al. ²² , 2003
Geraniol			92	
Menthone	114			
ácido láurico	Hylon VII	109	Zhang et al. ²⁷ , 2012	
vanillin		108	This work	
ascorbyl palmitate		100 y 98*	Lay Ma et al. ²³ , 2011	
retinyl palmitate		102 y 80*		
phytosterol ester		126 y 98*		

558 *Two temperatures reported for complexes obtained with native and lipid-free starch,
 559 respectively.

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567 Table II: Blue values obtained for Hylon VII-iodine and APTII-iodine complexes at
 568 each starch concentration, according to Eq. 1.

Hylon VII iodine complex $\lambda_{\max}^{abs} = 603 \text{ nm}$			APTIII iodine complex $\lambda_{\max}^{abs} = 602 \text{ nm}$		
C (starch mg /ml)	Absorption at 680 nm	Blue value	C (starch mg/ml)	Absorption at 680 nm	Blue value
1.14	0.201	0.070	3.70	0.058	0.0063
1.70	0.292	0.069	7.29	0.111	0.0061
2.26	0.381	0.069	14.13	0.213	0.0060
2.82	0.470	0.067	26.59	0.384	0.0058
3.37	0.566	0.067	32.29	0.470	0.0058
		$BV_{\text{Hylon VII}} \approx 0.07$			$BV_{\text{APTIII}} \approx 0.006$

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