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1 **Amylose and Amylopectin in Starch by Asymmetric Flow Field-Flow**
2 **Fractionation with Multi-Angle Light Scattering and Refractive Index**
3 **Detection (AF4-MALS-RI)**

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11
12 *Keywords:* Starch, Amylose, Amylopectin, AF4-MALS

13
14 *Abbreviations:* AF4, asymmetric flow field flow fractionation; FFF, field-flow fractionation;
15 AMP, amylopectin; AMY, amylose; DMSO, dimethylsulphoxide; MALS, multi-angle light
16 scattering; M_w , weight-average molar mass; M_w/M_n , polydispersity; $\langle r_g^2 \rangle$ mean-square radius,
17 gyration radius; R_z , z-average root-mean-square radius; RI, refractive index; SEC, size-exclusion
18 chromatography.

19

20 **Abstract**

21 The rheological and functional properties of starch are influenced by the size and molar mass
22 distribution of the polymer, the ratio of amylose (AMY) to amylopectin (AMP), and branching
23 characteristics. Asymmetric Flow Field-Flow Fractionation (AF4) was applied to fractionate five
24 different starch hybrids of varying AMY:AMP ratio. When coupled to detection by multi-angle
25 light scattering and refractive index (MALS-RI), it was possible to determine mass percentage
26 and the average weight-average molar mass (M_w) without the need for calibration standards.
27 Sufficient resolution of amylose and amylopectin was achieved by applying a gradient cross-
28 flow on a 17 cm trapezoidal channel with a 350 μm spacer. The observed M_w ranged from about
29 2×10^5 to 4×10^5 for amylose and from 1×10^8 to 4×10^8 for amylopectin. The corresponding z-
30 average root mean-square radii (R_z) for AMP ranged from 145 to over 300 nm. Low recoveries
31 from the AF4 channel were found to be due primarily to the focusing step. The calculated mass
32 percent of AMY and AMP from integrated RI peak areas agreed well with nominal values for the
33 individual starch hybrids. Both qualitative and quantitative data were reproducible. The results
34 show AF4-MALS-RI method to be well suited for routine molecular characterization of starch.

35 **1. Introduction**

36 In addition to being the most common source of digestible carbohydrate in the diet, starch is
37 a naturally abundant, non-toxic, relatively inexpensive raw material. Starch and starch-derived
38 materials find use in a variety of industrial applications including foods and beverages,
39 pharmaceuticals, household products, cosmetics, paper and packaging, etc., where they may be
40 employed as adhesives, absorbents, encapsulates, additives or binders. Starch and modified
41 starch materials also show promise as low-cost replacements for synthetic polymers in some
42 applications. Starch consists of large polydisperse glucose homopolymers of amylopectin (AMP)
43 and amylose (AMY) that are arranged in crystalline and amorphous regions in granules within
44 plant cells. The rheological and functional properties of starch are influenced by the ratio and
45 average molar masses of these macromolecular constituents. Studies of AMP have shown it to be
46 a highly branched structure containing a mixture of (1→4)- α -D-glucose and (1→6)- α -D-glucose
47 linkages with molecular weights that can reach up to 10^8 . AMY is more linear, consisting
48 primarily of long chains of (1→4)- α -D-glucose linked residues and molecular weights in the 10^5
49 to 10^6 range (Aberle et al., 1994; Whistler and BeMiller, 1997). The AMY:AMP ratio differs
50 among starches; levels of AMY and AMP in normal corn (*Zea mays* L.) starch are 25–28 and
51 72–75%, respectively. However, the starches of some mutant plants contain either an increased
52 AMY content (e.g., high amylose or amylostarch with up to 70% AMY) or an increased AMP
53 content (e.g., waxy starch with 99–100% AMP). The different AMY:AMP ratios of these
54 starches lead to variations in granular structure, physicochemical properties and quality of end-
55 use products. For example, the AMY content of rice (*Oryza sativa* L.) starch ranges from 0 to
56 30% (w/w). Cooked rice texture and rice starch functional properties are reported to be primarily
57 impacted by AMY content (Bhattacharya et al., 1982). However, evidence is building that

58 variations in other aspects of starch are also main determinants of cooking and processing
59 quality, such as the molar mass of AMY, the weight- and molar-based distributions of degree of
60 polymerization (DP) of AMY, and the fine structure of AMP (Tsai and Lii, 2000; Umemoto et
61 al., 2002; Vandeputte et al., 2003a; Vandeputte et al. 2003b; Bergman et al., 2004; Chen and
62 Bergman, 2007). It is known that the physical characteristics of starch can vary with botanical
63 source, geographical origin, crop year and environmental conditions. Therefore, it is important
64 to be able to determine the values of certain quality indicators, AMP:AMY ratio and molar mass
65 distribution, for example.

66 Since many of the applications of starch involve dissolution or dispersion in aqueous systems,
67 analytical investigation of starch properties in aqueous solution is especially relevant for
68 understanding structure-function in these systems (Fishman and Hoagland, 1994). However,
69 there are difficulties associated with the separation and characterization of the macromolecular
70 components of starch in aqueous systems. These include, but are not limited to, solubilization,
71 degradation, and the potential for aggregation and retrogradation. Dissolution procedures should
72 dissolve all of the starch material without degradation and checks need to be made to ensure that
73 neither aggregation nor retrogradation occurs. Any viable separation technique has to be able to
74 provide resolution over a broad molar mass range without the loss of significant amounts of
75 material or changes induced by the separation method itself (Gidley et al., 2010).

76 A dissolution method whereby starches are first dissolved in dimethylsulfoxide (DMSO),
77 precipitated with ethanol, followed by dissolution in water with the aid of microwave heating
78 under pressure, has been shown to provide nearly complete solubilization without degradation
79 under optimized conditions (Bello-Perez et al., 1996; Bello-Perez et al., 1998; Fishman and
80 Hoagland, 1994; Fishman et al., 1996; Roger et al., 1999; Roger et al. 2001; Rolland-Sabate et

81 al., 2007; Tetchi et al., 2007). This dissolution technique was applied earlier for the preparation
82 of starch solutions for subsequent analysis by size-exclusion chromatography (SEC). While SEC
83 is well established as a method for the analysis of polymers, problems associated with its use for
84 very large macromolecules such as AMP (You et al., 2002) can include exceeding the exclusion
85 limit of the column packing, irreversible interaction with the column and subsequently low
86 recovery, and shear degradation (Cave et al, 2009; Gidley et al., 2010). For this reason, field-
87 flow fractionation (FFF) methods have been investigated for the separation of intact starch
88 polysaccharides. In the first investigation applying flow FFF to starch (Roger et al., 2001), corn
89 starches of varying amylose content were separated on a symmetrical 30 cm channel equipped
90 with a frit outlet, the purpose of which was to reduce the effect of sample dilution and increase
91 detection signal to noise ratio.

92 In this paper, we report on a practical application of asymmetric flow field-flow fractionation
93 with multi-angle light scattering and refractive index detection (AF4-MALS-RI) for the
94 separation and characterization of AMY and AMP in commercial corn starch using water as
95 eluent. AF4 is a sub-type of the larger family of FFF techniques (Giddings, 1993) and offers the
96 advantage of an open-channel separation without shear degradation and/or column adsorption
97 effects that can arise with SEC. Unlike SEC, resolution can be controlled by varying the cross-
98 flow. Once sufficient resolution is achieved, detection by MALS-RI enables calculation of the
99 mass percent of AMY and AMP and determination of weight-average molar mass (M_w) and root
100 mean-square radii (R_z) without the need for calibration standards. AF4 has been applied to the
101 separation and characterization of starch previously (Roger et al., 2001; van Bruijnsvoort et al.,
102 2001; You et al., 2002; Kim et al., 2007). Resolution could potentially be improved by using an
103 asymmetric channel of enhanced design and flow control. Higher resolution would enable more

104 reliable molecular characterization and differentiation of cultivars where variations may be
105 small. The aim of the present study was to evaluate the capability of AF4-MALS-RI to resolve
106 AMY and AMP for both qualitative and quantitative determination of AMY:AMP mass ratio, as
107 well as other molecular characteristics for different starch hybrids.

108 **2. Materials and methods**

109 *2.1 Asymmetric flow field-flow fractionation (AF4)-multi-angle light scattering (MALS)-* 110 *refractive index (RI)*

111 AF4 separation occurs in an open channel as shown in Figure 1. Two plates are separated by a
112 spacer that determines the channel thickness and breadth. The upper plate is impermeable, while
113 the bottom plate is constructed of a porous frit. An ultrafiltration membrane covers the bottom
114 plate and serves as the accumulation wall to prevent penetration of sample through the bottom of
115 the channel. Asymmetric flow FFF differs from symmetric flow in that channel flow is
116 constantly lost through the single porous wall of the channel, thus there is a decrease in
117 longitudinal flow velocity along the channel, compensated in part by the trapezoidal shape of the
118 channel spacer (Litzén and Wahlund, 1991). The inflow solvent is introduced at the channel inlet
119 at a volumetric flow rate, V_{in} . This inflow rate is divided into the channel or outlet flow rate, V_c ,
120 and the cross-flow rate, V_x , such that $V_{in} = V_x + V_c$. The cross-flow is thus the excess flow
121 created by limiting the channel outflow to the detectors so that $V_c < V_{in}$. The cross-flow velocity
122 is zero at the upper wall and increases nonlinearly toward the accumulation wall (Wahlund and
123 Giddings, 1987). This cross-flow exits the channel through the porous accumulation wall
124 creating a flow field perpendicular to the longitudinal flow and forcing analyte molecules
125 downward. Under laminar flow conditions the channel flow has a parabolic profile where the
126 velocity is greatest at the center of the channel and lowest at the walls (Giddings, 1993). The
127 downward force induced by the cross-flow is balanced by diffusional forces and differences in

128 diffusion coefficients of analyte molecules are the basis for the technique's selectivity. Lower
129 masses with higher diffusion coefficients establish a steady state closer to the center of forward
130 flow and thus elute first, while higher masses traverse more slowly.

131 The retention time, t_r , of a polymer in field-flow fractionation is approximated by,

$$\frac{t_r}{t_0} = \frac{w^2 V_x}{6DV^0} \quad (1)$$

132 where w is the channel thickness, V_x and V^0 are the volumetric cross-flow and channel volume,
133 respectively, and D is the diffusion coefficient of the polymer. Greater retention, and thus
134 increased resolution, can be achieved by increasing cross-flow. Note that retention time
135 decreases with increasing diffusion coefficient. The relationship between the diffusion
136 coefficient and the hydrodynamic radius, r_h , is given by the Stokes-Einstein expression,
137
138
139

$$D = \frac{kT}{6\pi\eta r_h} \quad (2)$$

140 that when substituted into (1) shows that t_r and r_h are in direct proportion. The AF4 technique is
141 capable of fractionating a broad molar mass range. The lower mass limit is defined by the
142 porosity of the ultrafiltration membrane at the accumulation wall (typically 10,000 Da) and a
143 practical upper limit is an r_h of about 20 μm .
144
145

146 Molar mass and radius can be obtained by MALS combined with RI concentration
147 measurements by applying the Rayleigh-Gans-Debye approximation (Wyatt, 1993),
148

$$\frac{K^* c_i}{R_\theta} = \frac{1}{M_i P(\theta)} + 2A_2 c$$

149 where R_θ is the excess Rayleigh scattering over and above that of the solvent due to a solute of
150 concentration c_i for a slice i of the fractionated sample. K^* is an instrumental constant dependent
151
152
153

154 on the wavelength and the refractive index increment (dn/dc) of the solute in the particular
155 solvent. M_i is the weight-average molar mass for the solute. $P(\theta)$ is the scattering function that
156 describes the angular dependence of the scattered light intensity from which the mean-square
157 radius $\langle r_g^2 \rangle$ is derived. A_2 is the second virial coefficient and captures non-ideality due to
158 solvent-solute interactions; it is expected to be vanishingly small in a dilute fractionated sample.
159 The average weight-average molar mass ($M_w = \sum c_i M_i / \sum c_i$) is derived by measuring R_θ at multiple
160 angles for each slice i of the fractionated sample. The concentration of each slice, c_i , is
161 conveniently measured by RI detection. The z-average root mean-square radius ($R_z =$
162 $\sqrt{\sum \langle r_g^2 \rangle c_i M_i / \sum c_i M_i}$) is derived from the mean-square radii.

163 *2.2 Apparatus and procedures*

164 We carried out AF4 separation on a 17 cm trapezoidal channel mounted on a PEEK
165 (polyetheretherketone) lower block with a stainless steel frit (Wyatt Technology, Santa Barbara
166 CA). The upper block was aluminum with a polycarbonate window. The channel thickness
167 spacer was 350 μm with a breadth of 22 mm at the inlet and 2 mm at the outlet. The
168 accumulation wall was a regenerated cellulose membrane with a cut-off molecular weight of
169 10,000 Da (Wyatt). Flows and injection were accomplished with an Agilent 1100 HPLC
170 equipped with a degasser, isocratic pump and autosampler. AF4 flow control was maintained
171 with a Wyatt Eclipse 3 AF4 Separation System controller. Detection was accomplished with a
172 Wyatt DAWN HELEOS 18-angle MALS and Optilab REX RI detectors, both with a
173 wavelength, $\lambda = 690$ nm. Data were collected and processed with ASTRA Software (v. 5.3.4.13).
174 The MALS detector was calibrated with toluene. The RI detector was calibrated with sodium
175 chloride and the temperature was set at 35°C. A dn/dc value of 0.147 ml/g was determined for
176 the waxy maize starch Hylon VII in our carrier liquid. This value was obtained experimentally

177 by direct injection into the RI detector of five different concentrations of Hylon VII using an
178 external 2 ml loop. The latter allowed us to recover all of the injected mass. The consistency of
179 our experimentally determined dn/dc with the generally accepted value of 0.146 ml/g for starch
180 (Roger et al., 2001; Bello-Perez et al., 1998) verified complete dissolution.

181 The mobile phase was de-ionized water with 0.02% NaN_3 added as preservative. The eluent
182 was passed through a degasser and a 0.22 μm inline filter (Millipore, Billerica, MA) then into the
183 AF4 channel. The sample injection volume was varied between 10 and 50 μL , depending on the
184 amount of AMP in the sample. The sample was injected at 0.2 ml/min for 1 min, followed by a 1
185 min focusing step just prior to elution. At the start of elution, the detector flow and cross-flow
186 were at 1.0 ml/min. The cross-flow rate was decreased linearly from 1.0 to 0.1 ml/min over a 10
187 minute period, after which the detector flow was maintained at 1.0 ml/min with a cross-flow of
188 0.0 ml/min.

189 *2.3 Sample Preparation*

190 Pullulan (Polymer Laboratories, Amherst, MA) solutions, used for initial operational testing
191 of the AF4 system, were prepared by dispersing approximately 0.2 g of the solid material in 100
192 mL water to prepare solutions at about 2 mg/mL. Samples of five different maize hybrids
193 (National Starch Food Innovation, Plant Genetics Group, Indianapolis, IN) covering a range of
194 AMY:AMP mass ratios were shared between laboratories (The Procter & Gamble Co., Mason,
195 Ohio and Institut Polytechnique LaSalle Beauvais, France) for the AF4 experiments. A sample
196 preparation procedure was adapted from the literature (Bello-Perez et al., 1998; Roger et al.,
197 1999), whereby the starch samples were first treated with 95 % dimethylsulphoxide (DMSO) for
198 1 h at 100 °C, precipitated with 5 volumes of ethanol, filtered and washed with acetone, diethyl
199 ether, and finally solubilized in de-ionized water with the aid of microwave heating (900 W, 2 to

200 10 minutes at 130°C) in a pressurized vessel, followed by dilution to 2 mg/ml (Bello Perez et al.,
201 1998). The solutions were filtered through a 5 µm cellulose nitrate filter before injection.

202 **3. Results and discussion**

203 *3.1 System suitability*

204 In order to verify that the AF4 system was operating properly, pullulans covering molar mass
205 range from 47 to 788 kDa were used in this study. Pullulan is a linear polysaccharide of α -(1→4)
206 and α -(1→6) linked maltotriose units synthesized by the fungus *Aureobasidium pullulans*. It is
207 available in monodisperse fractions over a range of molar mass. The 112 kD pullulan was used
208 to normalize the diodes of the MALS detector. Figure 2 and Table 1 summarize AF4 results for
209 five pullulans of different molar mass eluted with water as the mobile phase. Elution behavior
210 was satisfactory with the exception of the highest molar mass (788 kD) which shows delayed
211 retention, possibly due to interaction with the membrane material. Generally, the calculated M_w
212 were reasonably close to nominal values. The slightly high bias in the lower M_w samples is
213 possibly due to loss of low molar mass (<10 kD) material through the membrane.

214 *3.2 Solubilization and fractogram analysis*

215 The treatment with 95% DMSO followed by dispersion with the aid of microwave heating in
216 a pressurized vessel allowed us to obtain the maximum solubilization of starch in water. This
217 was verified by direct injection of the starch solutions into the RI detector, measuring Δn and
218 calculating concentration from the known dn/dc (= 0.146 mL/g) for starch. The dissolution
219 recovery was near 100% for all of the starch hybrids used in this investigation, covering a range
220 in AMY from 1 to 75%. This is in accordance with results obtained in literature (Rolland-Sabate
221 et al., 2007; Tetchi et al., 2007). Several methods have been tested previously using autoclave,
222 boiling water or oven at high temperature after pre-gelatinization (Vorwerk and Radosta, 1995;

223 van Bruijnsvoort et al., 2001; Yoo and Jane, 2002). None of these achieved a total dissolution of
224 starch. The technique used here appears to be the most efficient, rapid and reproducible method.

225 A filtered solution containing solubilized starch was injected into the AF4 system and
226 analyzed with MALS-RI detectors. The resulting fractograms shown in Figure 3 are typical of
227 AF4-MALS-RI fractograms. The LS and RI peaks are not comparable and do not superimpose
228 due to the polydisperse nature of the polysaccharide. The RI peak is seen to be comprised of two
229 polysaccharide fractions while the LS peak is dominated by one fraction. The first fraction seen
230 in the RI signal eluted rapidly just after the end of focusing/relaxation steps and corresponds to
231 AMY. The second fraction, corresponding to AMP, begins to elute just after 15 min, the time
232 about which the cross flow was reduced to 0.0 ml/min. The small spike in both detector signals
233 observed at about 6 min is an artifact caused by the pressure drop at the start of elution. This
234 peak, corresponding to the void time, was excluded from the analysis. The fractograms clearly
235 show the separation of the AMY and AMP components in the five starch hybrids. It has been
236 noted that higher cross-flows delay the elution of high molecular weight starch polymers (You et
237 al., 2002). Thus, we found that we could optimize separation of the starch macromolecules by
238 applying a linearly decreasing cross-flow gradient, thereby delaying the elution of the higher
239 molar mass AMP until the cross-flow approached zero. The normal AF4 elution, as well as
240 nearly Gaussian peak shapes and reasonably good conformation plots for AMP ($\log\langle r_g \rangle$ vs.
241 $\log M_i$, Figure 4) led us to conclude that there was adequate resolution across the AMP peak.
242 There was no evidence of steric mode of elution or abnormally high MALS signal that would
243 suggest higher-order aggregates in solution. (Giddings, 1993). Our separation is comparable to
244 that obtained previously (Roger et al., 2001), although with noticeably better resolution. The use

245 of a single solvent pump, and motor driven, software-controlled valves for precise flow control
246 undoubtedly provided enhanced system performance.

247 *3.3 AF4-RI data analysis and recovery*

248 Integration of RI peak areas and applying the dn/dc enabled determination of the recovery of
249 injected mass and calculation of AMY:AMP ratios. In this investigation, the elution recovery
250 was low (~60%) compared to results from literature. Rolland-Sabate et al. (2007) reported 81.7
251 to 100% recovery from different starch sources while Roger et al. (2001) obtained 84 to 100%
252 recovery from maize starch. We attempted to explain the poor recovery observed at both
253 laboratories (LaSalle Beauvais France, Procter & Gamble Company USA) by using different
254 elution protocols while injecting constant amounts of the same sample. First, the AF4 separation
255 cell was replaced by an external 2 ml loop and sample was injected and eluted with a flow rate of
256 1.0 ml/min. In this case, the recovery was 100%. This experiment verified that analysis system in
257 general and, more specifically, the injector were not implicated in the loss of material. Secondly,
258 sample was injected into the AF4 channel and eluted at 1.0 ml/min without a focusing step and
259 with cross-flow maintained at 0.0 ml/min for the duration of the experiment. Again, 100%
260 recovery was obtained. In a third trial, the sample was analyzed without a focusing step and with
261 cross flow decreased gradually from 1.0 to 0.0 ml/min over a 10 min period. The mass recovery
262 obtained in the absence of a focusing step was 86%. Finally, sample was eluted using our
263 standard method as described, yielding a mass recovery of about 60%. Therefore, it was
264 concluded that the poor recovery was due to the loss of injected material during focusing and
265 elution with cross-flow, with the majority of mass being lost during focusing. This phenomenon
266 could be explained by increased interactions between sample and membrane during focusing, the
267 purpose of which is to concentrate the sample into a narrow zone prior to elution. It is known

268 that concentration effects can lead to aggregation and interaction with the membrane at the
269 accumulation wall (Schimpf and Wahlund, 1997). Better control over focusing conditions to
270 allow for control of zone width, reduced concentration effects, and minimal sample interactions
271 would be a significant improvement (Johann et al., 2011). It is noteworthy that we obtained the
272 same results with both regenerated cellulose and polysulphone membranes. Blank injections
273 before and after sample injections were superimposable, regardless of membrane used,
274 demonstrating that the membranes did not release material retained from previous injections.

275 Despite a low mass recovery, we injected the waxy maize starch (Hylon VII) several times
276 ($n=5$) in order to further examine the system performance and its capability to produce reliable
277 qualitative and quantitative data. The mass percentage of AMY was calculated by ratio of the
278 integrated RI peak area for AMY relative to the RI signal for the entire fractogram. The within-
279 day coefficient of variation (CV) was below 2%, while intermediate precision for different days
280 and between laboratories was better than 4% CV. Thus, although recoveries were poor, the AF4
281 technique was very reproducible. Furthermore, the AMY mass percentage was in good
282 agreement with the nominal value of 75%, verifying that the loss of starch material during
283 analysis did not affect the qualitative nor quantitative nature of the data. Concentration effects,
284 and the resulting loss of mass during focusing, were apparently non-selective as the low recovery
285 did not bias one fraction over the other. No observable bias for AMP suggests that selective
286 retrogradation of AMY did not occur during the course of analysis. Both AMY and AMP
287 apparently lost the same fraction of material during the focusing and elution steps. In order to
288 verify that the AF4-MALS-RI system could be reliably used for the determination of AMY:AMP
289 ratios, all five hybrids of varying composition were tested with results shown in Table 2. The
290 nominal AMY:AMP ratios were provided by the National Starch Plant Genetics Group

291 (Indianapolis, IN). They were determined by quantifying AMY according to an iodine-binding
292 colorimetric method (Zhu et al., 2008) whereby the absorbance (610 nm) was measured and
293 compared against a calibration curve constructed from solutions prepared from purified AMY
294 fractions from regular corn, Hylon V, or Hylon VII types, as appropriate. The nominal
295 AMY:AMP ratios and those obtained here by AF4 were in good agreement.

296 *3.4 Analysis of AF4-MALS data*

297 A Debye Plot (Zimm fit) of $R_{\theta}/K*c$ vs. $\sin^2(\theta/2)$ for the AMY component enabled an estimate
298 of M_w , however a low signal to noise ratio precluded derivation of the root-mean-square radii, R_z ,
299 with suitable precision. For the AMP, we employed a 2nd-order Berry fit to account for curvature
300 The Berry plot has been reported by other researchers to be the most appropriate fit for AMP
301 molecules (Bello-Perez et al., 1998; Rolland-Sabate et al., 2007). For the high AMP starches it
302 was necessary to exclude low angle data points for which R_{θ} was off scale. This likely reduces
303 the precision of the calculated results and may introduce error. There is also the possibility of a
304 non-zero A_2 in the case of these large macromolecules. For these reasons, results should be taken
305 as approximate. Notably however, the weight-average molar mass (M_w) ranged from 2×10^5 to
306 4×10^5 g/mol for amylose and from 1×10^8 to 4×10^8 g/mol for amylopectin. The R_z values for
307 amylopectin ranged from 145 to 316 nm. The values obtained agree reasonably with those cited
308 in the literature for AMY and AMP (Banks and Greenwood, 1975; Aberle et al., 1994; Fishman
309 and Hoagland, 1994; Vorwerg and Radosta, 1995; Fishman et al., 1996; Hanselmann et al., 1996;
310 Bello-Perez et al., 1996; Bello-Perez et al., 1998; Millard et al., 1999; Roger et al., 1999;
311 Murphy, 2000; Roger et al., 2001; Yoo and Jane, 2002; Elfstrand et al., 2004; Han and Lim,
312 2004a, 2004b; Han et al., 2005; Bowen et al., 2006; Yang et al., 2006; Tetchi et al., 2007;
313 Rolland-Sabate et al., 2007). Any discrepancies between our results and those published

314 previously could be due to the possible sources of error cited above and/or differences in the
315 analytical system employed. For example, others have used an external column calibration for
316 determining M_w or have employed different separation systems and detectors. Differences could
317 also be attributed to the starch solubilization process and the source of starch material.

318 *3.5 Amylopectin conformation*

319 For further characterization of the five starches, structural conformation of AMP was obtained
320 by plotting the logarithm of root-mean-square radii, $\log\langle r_g \rangle$, vs. the logarithm of the
321 corresponding molar mass, $\log M_i$, for each slice i of the fractogram for each of the samples.
322 Figure 4 shows the resulting conformation plot for the Hylon VII sample, from which a slope of
323 0.40 was obtained. The slopes of the log-log plots for the remaining samples ranged from 0.39
324 (Hi-Maize, Melojel) to 0.41 (Hylon V, Amioca), indicative of a highly branched structure. These
325 values are in good agreement with results reported in literature (Hanselmann et al., 1996; Roger
326 et al., 1999; Rolland-Sabate et al., 2007), with the average value (0.40) corresponding exactly to
327 that determined by Rolland-Sabate et al. (2007) for amylopectins from multiple starch sources.

328 **4. Conclusions**

329 Fractionation of starch by AF4 with MALS-RI detection enabled the qualitative
330 characterization of five different starch hybrids, as well as quantitation of the relative amounts of
331 AMY and AMP, and conformational information for the latter. Despite low recoveries, the
332 results were reproducible. The starch components were resolved by aqueous AF4 on a short
333 (17cm) channel with a gradient cross-flow. Integration of RI peak areas enabled calculation of
334 AMY:AMP ratios, in excellent agreement with the nominal values. Low signal to noise of the
335 MALS data precluded estimation of R_z for AMY. Nonetheless, estimated values for M_w for AMY
336 and AMP, as well as R_z values for AMP fall within the ranges previously reported.

337 Conformational plots ($\log\langle r_g \rangle$ vs. $\log M_i$) for the AMP component yielded slopes 0.39–0.41,
338 consistent with the branched nature of this macromolecule.

339 We have shown the AF4 system to be a practical analytical platform for purpose of starch
340 characterization and quality control. In the future, this technique may find use in correlating
341 molecular structure and function for the many end use applications of these important materials.

342

343

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347

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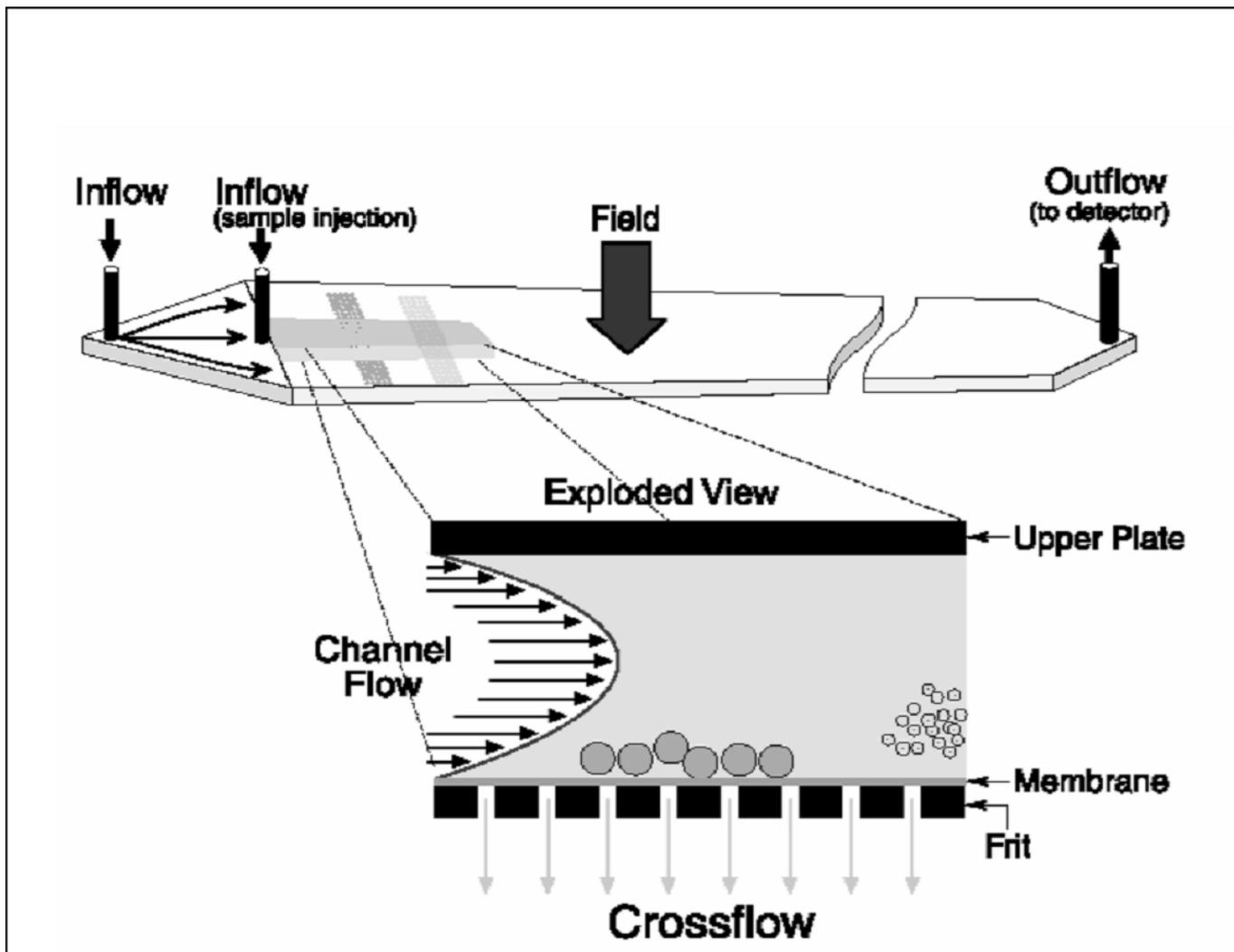
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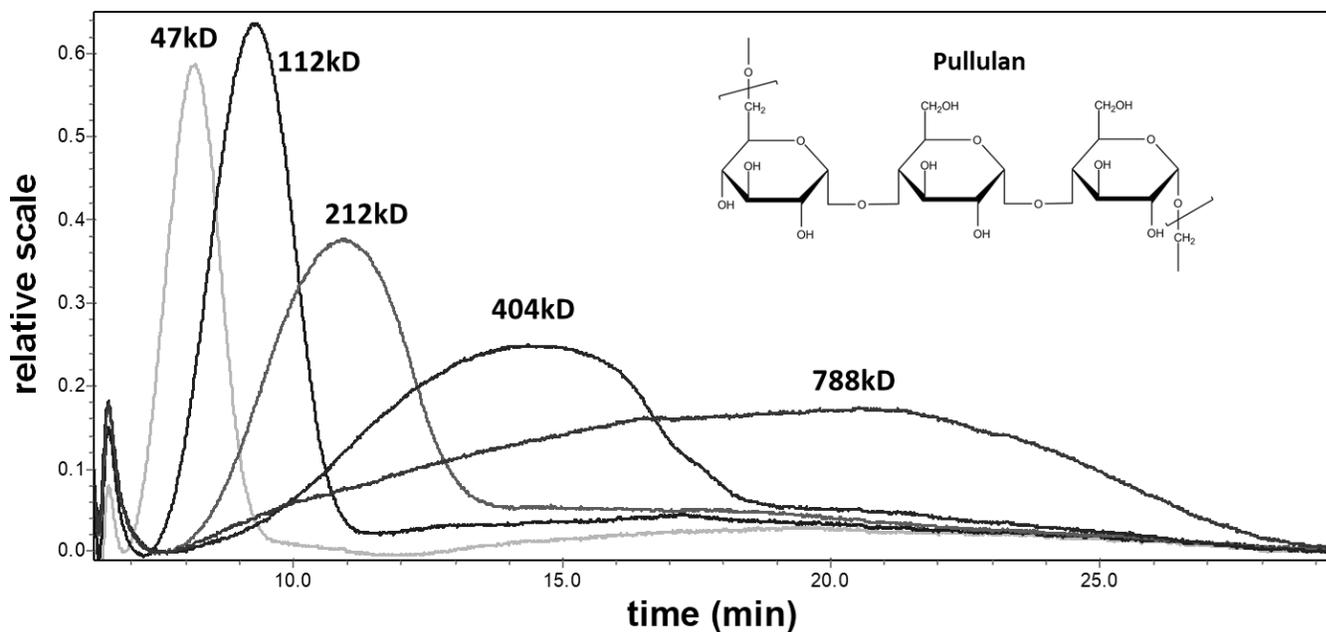
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468 **Figure 1.** Separation mechanism in AF4.

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473 **Figure 2.** AF4-RI fractograms of five linear pullulan standards at constant cross flow, $V_x =$
 474 0.5ml/min. Effect of molecular weight (diffusion coefficient).

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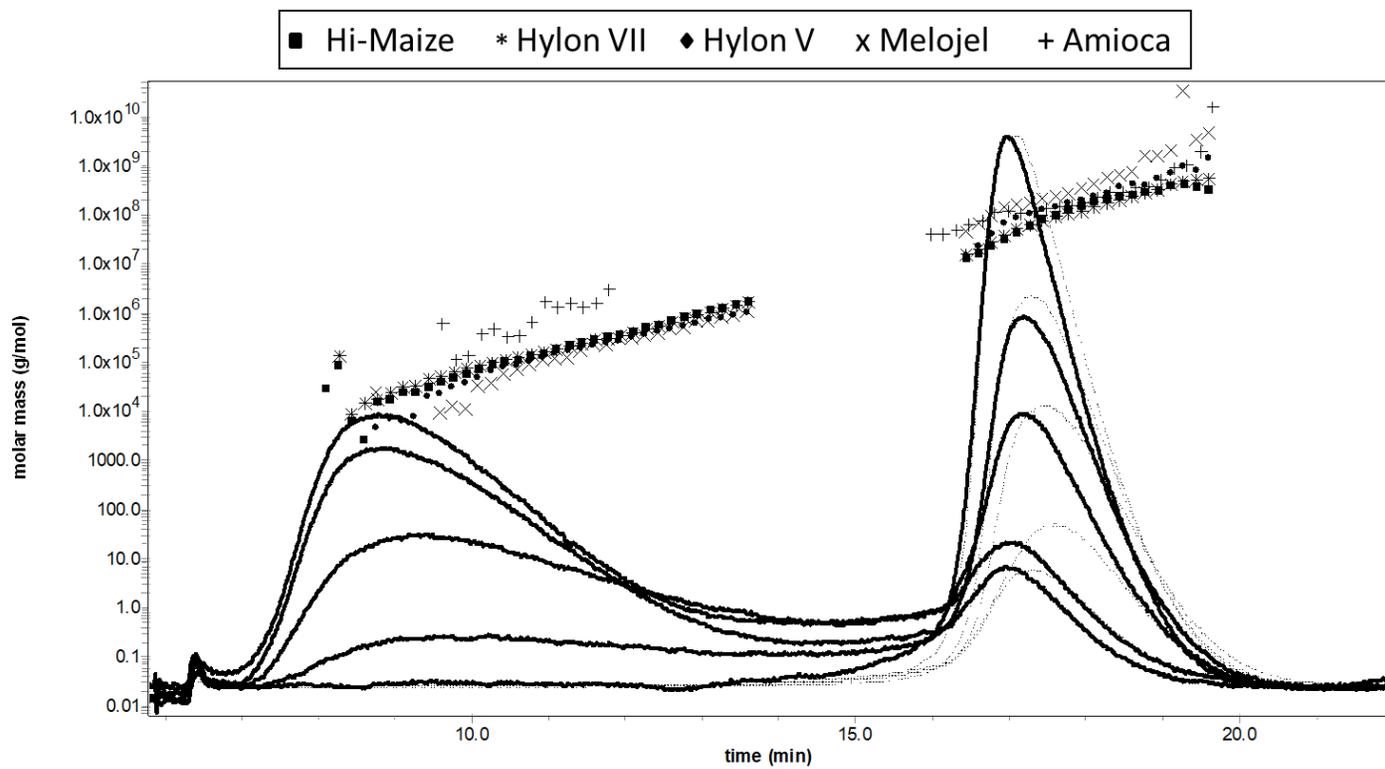
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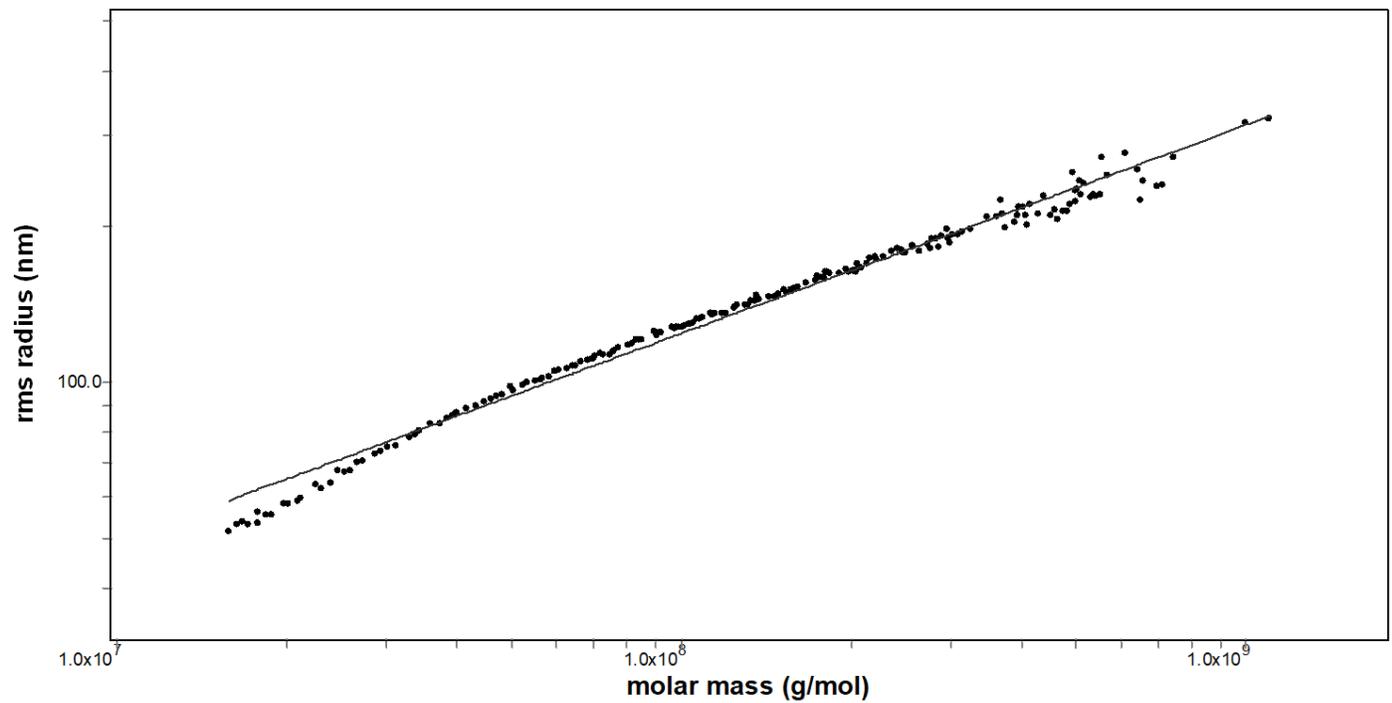
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484 **Figure 3.** AF4-MALS-RI results for five starches of varying AMY:AMP ratio: AF4-
 485 MALS(90°)-RI fractograms with molar mass distribution overlay.



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487 **Figure 4.** Conformational plot ($\log \langle r_g \rangle$ vs. $\log M_i$) for the AMP fraction of Hylon VII starch:

488 slope = 0.40, consistent with branched structure.