Electronic Supplementary Material (ESI) for Chemical Communications. This journal is © The Royal Society of Chemistry 2015

**Electronic Supplementary Information (ESI)** 

## Rapid Method for Quantifying the Molecular Order of Starches

# Analysed by <sup>13</sup>C CP/MAS NMR Spectroscopy

Bernadine M. Flanagan, Michael J. Gidley and Frederick J. Warren\*

Centre for Nutrition and Food Sciences, ARC Centre of Excellence in Plant Cell Walls, Queensland

Alliance Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia

\*Tel ++61(0)733467373 <u>f.warren@uq.edu.au</u>

Keywords: starch, molecular order, multivariate analysis, NMR, crystallinity, solid state.

#### 1. Materials and Methods

#### **1.1 Materials**

Commercially available starches: waxy maize, raw maize, gelose 80 and Mazaca were purchased from National Starch Pty Ltd. (Lane Cove NSW 2066, Australia) and used without further treatment. All other starches were gifts from collaborators and were from a range of botanical sources including native barley and barley mutants<sup>1, 2</sup> and wild rice and rice mutants.<sup>3</sup> Other starches were subjected to different drying conditions,<sup>4</sup> or extruded (Zhang et al, submitted). Starches were also subjected to in vitro enzymic digestion for different lengths of time<sup>5</sup> or extruded and then digested.<sup>6</sup> Wheat starch (Cerestar, cv. GL04) and pea starches (WT, r and lam<sup>7</sup>) were gifts from Prof. T. Bogracheva and Prof. C. Hedley (formerly of the John Innes Centre, Norwich, UK), waxy rice starch (cv. Remyrise) was a gift from Dr. P. Rayment (Unilever, UK); these starches are described in detail elsewhere.<sup>8</sup> Native starch powders from tapioca (Penford, AU), maize (Penford, NZ), waxy maize (Tate and Lyle, Decatur, IL), high amylose maize (HylonVII, Penford, AU), potato, and wheat (both commercial material, supermarket, Sydney, AU) were gifts from Dr. E.P. Gilbert (ANSTO, Australia).<sup>9</sup> Amorphous starches were prepared following the method of Gidley and Bociek,<sup>10</sup> with minor adjustments. Starches were boiled in water (1% w/v) for 30 minutes and precipitated with ethanol or rapidly frozen in liquid nitrogen and lyophilised.

## 1.2<sup>13</sup> C CP/MAS NMR spectroscopy

The solid-state 13C CP/MAS NMR experiments were performed at a 13C frequency of 75.46 MHz on a Bruker MSL-300 spectrometer. Approximately 200-300 mg of starch was packed in a 4-mm diameter, cylindrical, PSZ (partially-stabilized zirconium oxide) rotor with a perfluorinated polymer (KelF) end cap. The rotor was spun at 5-6 kHz at the magic angle (54.7°). The 90° pulse width was 5 µs and a contact time of 1 ms was used for all starches

with a recycle delay of 3 s. The spectral width was 38 kHz, acquisition time 50 ms, time domain points 2000, transform size 4000 and line broadening 50 Hz. At least 1200 scans were accumulated for each spectrum. Spectra were referenced to external adamantane.

The NMR spectra were analysed by separating the spectrum of the native starch into its respective amorphous and ordered sub-spectra as detailed by Tan et al. <sup>13</sup>

## 1.3 Wide Angle X-Ray Diffractometry

X-ray diffraction (XRD) measurements were performed with an X'Pert Pro X-ray diffractometer (PANalytical, Almelo, the Netherlands) operating at 40 kV and 40 mA with Cu K $\alpha$  radiation ( $\lambda$ ) at 0.15405 nm. The scanning region was set from 3 to 40° of the diffraction angle 2 $\theta$  with a step interval of 0.02° and a scan rate of 0.5°/min. The crystalline peak area and amorphous area were separated by PeakFit software (Version 4.12, Systat Software Inc., San Jose, CA, USA) following the method of Lopez-Rubio, et al. <sup>11</sup> Relative crystallinity was calculated as the ratio of the crystalline peak area to the total diffraction area.

#### 1.4 DSC

All DSC data were obtained using a TA Instruments Q2000 instrument, using Tzero hermetically sealed aluminium pans. The sample chamber was purged with nitrogen gas at a rate of 40 mL/min. The instrument was calibrated for temperature using indium and tin standards. Samples were prepared in triplicate by accurately weighing approximately 5mg of starch with 40mg of deionised water into a pan, mixing, sealing and leaving overnight to equilibrate. Samples were heated from 10 to 95°C at a rate of 5°C/min. Subsequent thermograms were analysed using TA instruments Universal Analysis software to obtain gelatinisation enthalpies as described elsewhere.<sup>12</sup>

# 1.5 Data analysis

Preparation of figures, statistical analyses and linear regression fitting was carried out using Sigma Plot 12.5. Principle component analysis (PCA) and PLS model building was carried out using Unscrambler X 10.3.

**Table S1.** Reference data for the complete set of samples. NMR values are calculated using the deconvolution method of Tan *et al.*<sup>13</sup> Crystalline polymorph and XRD crystallinity was calculated according to the method of Lopez-Rubio *et al.*<sup>14</sup>

Sample	NMR	NMR	Crystalline	XRD (%	DSC	Botanical	Sample pre-
No.	A/B (%)	V (%)	polymorph	crystallinity)	(J/g)	origin	treatement
1	17	5	B+V	N.D.	N.D.	maize	extruded
2	16	6	B+V	N.D.	N.D.	maize	extruded
3	28	1	А	N.D.	N.D.	rice	native
4*	37	2	А	N.D.	N.D.	rice	native
5*	15	1	В	10	6.5	rice	native
6	23	2	В	22	7.4	rice	native
7	27	1	В	N.D.	N.D.	rice	native
8	23	1	В	N.D.	N.D.	rice	native
9	26	5	В	17	8.8	rice	native
10*	32	5	В	19	9.4	rice	native
11	31	3	А	27	11.3	wheat	native
12	24	6	В	28		maize	native
13*	43	4	А	45	15.7	maize	native
14	38	7	Α	42	12.9	maize	native
15	40	3	А	39	15.9	tapioca	native

16*	40	4	В	44	17.6	potato	native
17	29	5	А	27	8.5	rice	native
18	30	5	А	23	10.0	rice	native
19*	28	5	А	23	6.4	rice	native
20	33	1	В	N.D.	N.D.	maize	α-amylase digested
21	54	4	В	N.D.	N.D.	maize	α-amylase digested
22*	56	1	В	N.D.	N.D.	maize	α-amylase digested
23	57	1	В	N.D.	N.D.	maize	α-amylase digested
24	45	10	В	N.D.	N.D.	wheat	α-amylase digested
25*	47	3	В	N.D.	N.D.	wheat	α-amylase digested
26	41	4	А	44	14.2	maize	native
27	39	4	А	36	14.0	maize	native
28*	35	3	А	38	N.D.	maize	native
29	11	2	В	13	3.0	maize	extruded
30	8	1	В	13	3.5	maize	extruded
31*	6	1	В	14	2.9	maize	extruded
32	8	0	В	8	4.3	maize	extruded
33	6	0	В	9	4.6	maize	extruded
34*	10	0	В	10	4.4	maize	extruded
35	9	0	В	12	4.4	maize	extruded
36*	6	1	В	N.D.	N.D.	maize	resistant starch

37	6	0	В	N.D.	N.D.	maize	resistant
							starch
38	10	0	В	N.D.	N.D.	maize	resistant
							starch
39	14	2	В	N.D.	N.D.	maize	resistant
							starch
40	62	9	B+V	N.D.	N.D.	maize	extruded and
							$\alpha$ -amylase
							digested
41	17	6	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
42	29	9	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
43*	20	5	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
44*	24	7	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
45	16	5	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
46*	57	9	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested
47	26	5	B+V	N.D.	N.D.	maize	extruded and
							α-amylase
							digested

48*	39	8	B+V	N.D.	N.D.	maize	extruded and $\alpha$ -amylase
49	28	6	B+V	N.D.	N.D.	maize	digested extruded and α-amylase
50*	12	3	B+V	N.D.	N.D.	maize	digested extruded and $\alpha$ -amylase
51	40	8	B+V	N.D.	N.D.	maize	extruded and α-amylase digested
52*	14	5	B+V	N.D.	N.D.	maize	extruded
53	23	7	B+V	N.D.	N.D.	maize	extruded
54	25	5	В	25	6.2	rice	native
55*	28	4	А	25	8.3	rice	native
56	22	4	А	22	4.6	rice	native
57	30	0	В	21	4.9	rice	native
58*	32	8	А	25	7.8	rice	native
59	40	0	В	35	12.7	pea	native
60	41	4	А	40	14.5	maize	native
61*	41	4	А	38	14.7	maize	native
62	43	4	А	35	13.2	maize	native
63	36	3	А	38	N.D.	maize	native
64*	33	3	А	21	12.3	maize	native
65	34	4	А	32	5.5	rice	native
66	18	2	А	18	6.5	rice	native
67*	5	1	А	0	0.0	maize	extruded
68	34	5	В	41	19.0	potato	native

69	44	2	В	N.D.	N.D.	potato	native
70	44	5	В	51	19.2	potato	native
71*	30	0	В	30	13.7	potato	native
72	35	9	В	N.D.	N.D.	maize	α-amylase digested
73*	32	8	В	N.D.	N.D.	maize	α-amylase digested
74	24	11	В	N.D.	N.D.	maize	α-amylase digested
75*	23	12	В	N.D.	N.D.	maize	α-amylase digested
76	28	6	В	N.D.	N.D.	maize	α-amylase digested
77	20	6	В	N.D.	N.D.	maize	α-amylase digested
78*	23	7	В	N.D.	N.D.	maize	α-amylase digested
79	26	4	В	N.D.	N.D.	maize	α-amylase digested
80	22	3	В	N.D.	N.D.	maize	α-amylase digested
81*	21	6	В	N.D.	N.D.	maize	native
82	33	7	А	N.D.	N.D.	maize	α-amylase digested
83*	30	11	А	N.D.	N.D.	maize	α-amylase digested
84	34	8	А	N.D.	N.D.	maize	α-amylase digested

85	22	8	В	N.D.	N.D.	maize	α-amylase digested
86	22	9	В	N.D.	N.D.	maize	α-amylase digested
87	5	1	В	N.D.	N.D.	maize	α-amylase digested and reprocessed
88	4	0	В	N.D.	N.D.	maize	α-amylase digested and reprocessed
89*	10	0	В	N.D.	N.D.	maize	α-amylase digested and reprocessed
90	6	0	В	N.D.	N.D.	maize	α-amylase digested and reprocessed
91	11	1	A+V	N.D.	N.D.	maize	extruded
92	0	0			0.0	maize	cooked
93*	13	0	В	18	4.4	pea	native
94*	32	2	А	N.D.	N.D.	rice	native
95	34	2	А	N.D.	N.D.	rice	native
96	37	2	С	N.D.	N.D.	rice	native
97*	34	3	С	N.D.	N.D.	rice	native
98	21	1	А	17	4.2	rice	native

99	18	0	А	17	5.9	rice	native
100	33	2	А	N.D.	N.D.	rice	native
101	37	2	А	N.D.	N.D.	rice	native
102*	5	4	А	16	5.0	barley	native
103	10	1	А	15	3.9	barley	native
104	28	5	А	23	N.D.	rice	native
105*	23	5	А	24	7.2	rice	native
106	44	6	А	44	10.2	rice	native
107	24	2	А	25	6.9	rice	native
108*	30	0	А	46	10.6	rice	native
109	35	4	А	38	13.6	wheat	native
110	31	4	А	30	12.3	barley	native
111*	32	0	В	22	13.4	pea	native
112	4	0	В	0	0.0	maize	extruded
113	0	0	N.A.	N.D.	N.D.	wheat	resistant
							starch
114*	0	0	N.A.	N.D.	N.D.	wheat	resistant
							starch

\* denotes samples used in the validation dataset. All other samples were used in the calibration dataset.

**Supplementary Figure S1.** PLS models constructed using the C2,3,5, C4 and C6 regions of the 13C CP/MAS NMR spectra f or starch. Reference vs. predicted ordered helical structure for calibration samples (closed circles) and validation samples (open circles). a. C2,3,5; b. C4; c. C6. Model correlation loadings for PC1 (black line) and PC2 (broken line). d. C2,3,5; e. C4; f. C6.



S1a.



















**Supplementary Figure S2.** Relationship between NMR ordered structure determined from the reference deconvolution method, or using the prediction macro, and two alternative measures of ordered starch (XRD and DSC). a. Predicted NMR vs. XRD. b. Reference NMR vs. XRD. c. Predicted NMR vs. DSC. d. Reference NMR vs. DSC. e. XRD vs. DSC.



S2a.









S2e.



# References

- 1. A. Regina, J. Blazek, E. Gilbert, B. M. Flanagan, M. J. Gidley, C. Cavanagh, J.-P. Ral, O. Larroque, A. R. Bird and Z. Li, *Carbohydrate polymers*, 2012, **89**, 979-991.
- 2. J. E. Higgins, B. Kosar-Hashemi, Z. Li, C. A. Howitt, O. Larroque, B. Flanagan, M. K. Morell and S. Rahman, *Journal of the Science of Food and Agriculture*, 2013, **93**, 2137-2145.
- 3. V. M. Butardo, M. A. Fitzgerald, A. R. Bird, M. J. Gidley, B. M. Flanagan, O. Larroque, A. P. Resurreccion, H. K. Laidlaw, S. A. Jobling and M. K. Morell, *Journal of Experimental Botany*, 2011, err188.
- 4. B. Zhang, K. Wang, J. Hasjim, E. Li, B. M. Flanagan, M. J. Gidley and S. Dhital, *Journal of agricultural and food chemistry*, 2014, **62**, 1482-1491.
- 5. A. K. Shrestha, J. Blazek, B. M. Flanagan, S. Dhital, O. Larroque, M. K. Morell, E. P. Gilbert and M. J. Gidley, *Carbohydrate polymers*, 2012, **90**, 23-33.
- 6. A. K. Shrestha, J. Blazek, B. M. Flanagan, S. Dhital, O. Larroque, M. K. Morell, E. P. Gilbert and M. J. Gidley, *Carbohydrate polymers*, 2015, **118**, 224-234.
- 7. T. L. Wang, T. Y. Bogracheva and C. L. Hedley, *Journal of Experimental Botany*, 1998, **49**, 481-502.
- 8. F. J. Warren, P. G. Royall, S. Gaisford, P. J. Butterworth and P. R. Ellis, *Carbohydrate Polymers*, 2011, **86**, 1038-1047.
- 9. J. Doutch and E. P. Gilbert, *Carbohydrate polymers*, 2013, **91**, 444-451.
- 10. M. J. Gidley and S. M. Bociek, *Journal of the American Chemical Society*, 1985, **107**, 7040-7044.
- 11. A. Lopez-Rubio, B. M. Flanagan, A. K. Shrestha, M. J. Gidley and E. P. Gilbert, *Biomacromolecules*, 2008, **9**, 1951-1958.
- 12. T. Bogracheva, Y. Wang, T. Wang and C. Hedley, *Biopolymers*, 2002, **64**, 268-281.
- 13. I. Tan, B. M. Flanagan, P. J. Halley, A. K. Whittaker and M. J. Gidley, *Biomacromolecules*, 2007, **8**, 885-891.
- 14. A. Lopez-Rubio, B. M. Flanagan, E. P. Gilbert and M. J. Gidley, *Biopolymers*, 2008, **89**, 761-768.