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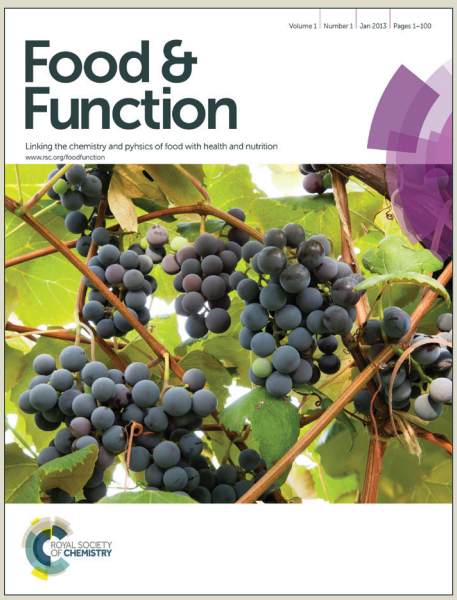

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A novel method for classifying starch digestion by modelling the amylolysis of plant foods using first-order enzyme kinetic principles

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Abstract

Studying starch amylolysis kinetics *in vitro* is valuable for predicting the post-prandial glycaemic response to starch intake. Prediction of starch amylolysis behaviour is challenging however, because of the many physico-chemical factors which influence amylolysis. The Logarithm of Slope (LOS) method for analysis of digestibility curves using first-order enzyme kinetics can identify and quantify nutritionally important starch fractions. The early stages of *in vitro* amylolysis of hydrothermally processed chickpea and durum wheat with variable degrees of structural integrity were studied. The end-point product concentration (C_∞) and the pseudo first-order digestibility rate constant k, obtained from LOS analysis, were then used to compute predictive digestibility curves for evaluation of the model performance. LOS analysis enabled rapid identification of nutritionally important starch-fractions. It was clear that purified starches and flours were digested by a single-phase process, but starch amylolysis in macroparticles occurred by a two-phase system that reflected differences in substrate accessibility. The model gave an excellent fit to data obtained from a range of heterogeneous materials. It provides a rigorous means of studying the mechanisms of starch amylolysis in samples of varying complexity, and we strongly recommend its use for the rapid and accurate predictions of amylolysis. Such predictions have implications for prevention and management of type 2 diabetes mellitus and obesity.

1 Introduction

The rate and extent of starch amylolysis is an important determinant of the magnitude and duration of the glycaemic response. *In vitro* studies provide popular and cost-effective means of predicting postprandial outcomes [1, 2]. However, many current methods require the rate and extent of starch amylolysis to be established by running *in vitro* digestions to completion- a process which often requires assaying over several hours and which can introduce unacceptable errors due to product inhibition and enzyme inactivation [3]. Moreover, despite the accumulating evidence that food structure is a major factor which influences the post-prandial response [4] many *in vitro* methodologies are not necessarily ideal for analysis of the structurally complex food matrices that are likely to be present post-mastication. Masticated particle sizes can range from several microns to cm and frequently contain structurally intact plant tissue that may encapsulate starch [5-7]. The mechanisms of starch amylolysis and the factors that influence the metabolic effects are still not fully understood [4]. A new and improved approach to studying and interpreting starch digestibility *in vitro* should assist in applying enzyme mechanistic understanding to physiological predictions.

The Englyst classification system, which uses acronyms RDS, SDS and RS for rapidly and slowly digestible and resistant starch respectively, has been widely adopted [8]. This system, however, is based on the extent of *in vitro* starch digestion at selected time points reflecting the blood glucose response peak following a starch-rich meal. The classification of RDS and SDS has proved very popular with nutritionists, thus explaining the frequency in which Englyst is cited in the literature. Since it is already known that starch amylolysis follows a first-order kinetic reaction [9], or more correctly a pseudo-first order reaction, the division of the starch into RDS and SDS fractions on the basis of a slowing in the amylolysis rate as the reaction proceeds is unsound. The rate naturally becomes slower because the concentration of substrate falls continuously as the reaction proceeds [3]. We advocate the use of Log of Slope (LOS) analysis of

digestibility curves because it is based on the well-documented 1st order reaction kinetics of amylolysis.

We argue that LOS analysis provides a more rigorous means of analyzing digestibility curves [3, 10]. LOS plots are very sensitive to changes in the digestibility rate constant, and therefore enhance differences in starch digestibility which are not immediately obvious from conventional digestibility curves. LOS plots have the potential of clearly revealing and quantifying differences in rate processes during starch amylolysis and also enabling the product concentration at the end of amylolysis to be predicted without the need to carry out prolonged digestions. This end product is the total amount of starch digested in the food and is referred to as *C* infinity (C_∞).

To date, LOS analysis has been applied successfully to previously published digestibility curves of purified starches and homogenised food materials [3]. However it has not yet been applied to the more complex food structures that can be present in the small intestine post-mastication [6, 11]. In many foods, plant cell walls which encapsulate starch may hinder α-amylase access, with implications for the release of starch hydrolysis products (e.g. maltose and maltotriose) during luminal digestion and thereby postprandial glycaemia. [5, 6, 12]. The low glycaemic index of chickpeas and other pulses, for instance, could be attributed to their resilient cell walls, which appear to protect intracellular starch from digestion *in vivo* [6, 12-14]. Comparison of plant materials with contrasting cell wall structure (i.e. Type 1 cell walls in legumes versus Type 2 cell walls in cereals) provides a valuable means of elucidating the mechanisms by which cell walls influence amylolysis. In theory, it should be possible to use LOS analysis to compute predictive digestibility curves of amylolysis, and if more than one digestible fraction is present, to assess the contribution of different digestible starch fractions (i.e. cell wall encapsulated or available starch) to total starch breakdown.

The aim of this study is to provide a re-appraisal of starch classification systems that is based on a mechanistic understanding of starch amylolysis kinetics, and apply this to edible starch-rich plant tissues for prediction of nutritional responses. In the present work, we utilise LOS plot analysis of experimentally obtained starch digestibility curves to identify and quantify potential nutritionally important fractions, providing extensive information on the rate processes that contribute to the amylolysis of starch in a food matrix. Our use of this analysis to study amylolysis of hydrothermally-processed particles of durum wheat and chickpeas (containing variable proportions of cell wall encapsulated starch and of different particle sizes), serves to introduce LOS analysis as a novel experimental tool with broad applications.

2 Materials and Methods

2.1 Food Materials

Chickpeas (*C. arietinum L.;* Russian cv.*),* were donated from Poortman Ltd., London, UK. Durum wheat grains (*Triticum durum L.*; Svevo cv.), were provided by Millbo S.p.A., Trecate, Italy. Chickpeas and Durum wheat were selected for study because of their known differences in cell wall properties and glycaemic potential [13, 14].

2.2 Starch purification

Food materials were steeped in ~0.2% (w/v) sodium bisulphite overnight at 25 °C, and homogenized using an Ultra-Turrax® (IKA T25 digital). Starch was isolated from these materials as described elsewhere [15, 16], except that purification was carried out in 80% ethanol, rather than in NaOH or water.

2.3 Milling

Durum wheat grains were de-branned (Satake TM-05C, equipped with a medium abrasive roller No. 40, roller speed: 1450 rpm) and chickpeas were manually de-hulled (following a 2 h soak in distilled water at room temperature) to obtain relatively pure endosperm tissue. These materials were then roller-milled (Satake Test Roller Mill ST-100, equipped with 10.5 fl/in break rolls, 250 mm diameter) using a sharp-sharp disposition and sieved into nine distinct particle size fractions with diameters ranging from flour (<0.21 mm) to coarse particles (up to 3.15 mm). This size range attempts to represent particle sizes that occur following *in vivo* mastication of food.

Durum wheat endosperm cells have approximate dimensions of 0.25 x 0.05 x 0.05 mm and chickpea parenchyma cells are 0.14 x 0.04 x 0.04 mm. By geometric principles it follows that the larger particles contain a greater proportion of intact cells, in which the starch is encapsulated and therefore less accessible to amylase, whereas smaller size fractions contain a greater proportion of ruptured cells, so that a higher proportion of starch is exposed on particle surfaces. These predictions, based on geometry, were confirmed by microscopy (*micrographs not shown*). Particle sizes are defined on the basis of median sieve aperture range.

2.4 Characterisation of plant food materials

The starch content of all materials was determined using a modified version of the DMSO format of the Megazyme Total Starch Procedure (AOAC 996.11) in which the duration of the DMSO heat solubilisation step was extended to 16 min, and 6 mL of 1:60 diluted thermostable amylase was used instead of 3 mL of 1:30 diluted amylase. These modifications were introduced to ensure complete solubilisation and conversion of starch to maltodextrin and thus provide a more reliable estimate of total starch content. The total starch content (means ± SD) of milled chickpea (de-hulled) and durum wheat (de-branned) was 45 ± 1.07 and 71 ± 3.1,

114 respectively, expressed on a g/100 g dry weight basis. No significant differences were observed
115 between the starch content of the different milled fractions.

116 *2.5 In vitro* amylolysis

117 Porcine-pancreatic α-amylase of a high purity (Grade 1-A) was obtained from Sigma-Aldrich Co.
118 Ltd, Poole, Dorset, United Kingdom (A6255, EC 3.2.1.1). The enzyme was supplied as a
119 suspension in 2.9 mol/L NaCl containing 3 mmol/L $CaCl_2$. The purity of the enzyme was
120 confirmed by denaturing gel electrophoresis, in which the enzyme formed a band at 56 kDa, and
121 no other contaminants were observed. The total protein content (determined by bicinchoninic
122 assay) and activity (determined by assaying hydrolysis of purified wheat starch) of the enzyme
123 was found to be within the range specified by the manufacturer (1333 U/mg protein). One unit of
124 activity, as defined by the manufacturers, releases 1 mg of maltose from starch in 3 min at
125 20 °C. This is approximately equivalent to 1 IU/mg protein at 20 °C [17].

126 Milled materials were suspended in 30 mL of PBS (Oxoid tablets, pH 7.4 at 37 °C) and
127 hydrothermally processed at 100 °C for 85 min with gentle stirring. In preliminary experiments
128 no birefringence remained in the milled fractions after processing, thus establishing that these
129 processing conditions ensured sufficient gelatinisation of starch in all size fractions, and were
130 strictly adhered to *(micrographs not shown)*. The amount of milled material used was adjusted,
131 based on total starch content (see previous section), to contain 117 mg starch in each tube.

132 Once processed, the suspensions were equilibrated in a water bath at 37 °C for 20 min,
133 and then incubated with 8 nmol/L α-amylase (in PBS, pH 7.4) on a rotary mixer at 37 °C. Other
134 studies, not reported here, have demonstrated that with our assay system, there is no loss of
135 activity through precipitation of calcium phosphate. Although pre-treatment with protease or
136 other digestive secretions does not preclude the application of LOS analysis, pre-treatment was
137 omitted in our procedure. It is recognised that the inclusion of pepsin can, in some instances

(e.g., pasta), increase starch hydrolysis rates by removal of a dense protein matrix that may hinder amylase access [2]. Nevertheless the overall effect on amylolysis appears to be very small [2]. During the incubation period, aliquots were collected into tubes on ice containing 0.3 mol/L Na_2CO_3, pH 9, to stop the reaction. Only the first 60 min of amylolysis was assayed, as this provided sufficient information for application of the LOS method. Aliquots were centrifuged at 16,200 x *g* (Haraeus Pico, Thermo Scientific) for 6 min to exclude any starch remnants. No amyloglucosidase was present in our assay and so reducing equivalents would be mainly maltose with some maltotriose and very small amounts of glucose [18]. The starch hydrolysis products in the supernatant were quantified using a scaled-down version of the previously described Prussian blue assay [18], performed in 1.5 mL Eppendorf ® safe-lock™ tubes. To test for endogenous reducing sugars or enzyme activity, the addition of amylase was omitted for control assays. The amount of reducing sugar freshly produced in these controls was found to be negligible; subsequently, endogenous sugar was accounted for by subtraction of blank values taken for each assay prior to enzyme addition.

2.6 Theory of Predictive Model

Starch amylolysis data [9, 19] can be fitted to a first-order equation (Equation 1)**:**

$$C_t = C_\infty (1 - e^{-kt}) \quad (1)$$

where C_t is the concentration of product at a given time (t), C_∞ is the concentration of product at the end of the reaction, and k is the digestibility rate constant. For ease of interpretation, C_t may be expressed as the amount of starch digested as a percentage of the total starch content of sample, calculated assuming that all polysaccharide is converted to maltose.

A Logarithm of Slope (LOS) plot is obtained by expressing the first derivative of the first-order equation in logarithmic form (Equation 2). This gives a linear plot in which the values of

digestibility constants, k and C_{∞}, are calculated from the slope ($-k$) and y-intercept ($ln[C_{\infty}k]$), respectively (for full details refer to reference [3]).

$$\ln\left(\frac{dC}{dt}\right) = -kt + \ln(C_{\infty}k) \quad (2)$$

where ln (dC/dt) represents the logarithm of the slope, and the equation describes a linear relationship between LOS and time of amylolysis, t.

In foods containing starch fractions that are digested at different rates, LOS plots reveal two or more distinct linear phases, in which the slope of each distinct phase provides a rate constant, denoted k_1, k_2... etc., enabling the end-point of starch amylolysis (denoted $C_{1\infty}$, $C_{2\infty}$... etc.) to be computed for each phase

We propose the following modification to Equation 1, which includes time identifiers to indicate the period over which each consecutive reaction occurs, i.e. the rapid phase is considered to have become negligible at the intersection of the different phases in the LOS plot, where the slower phase commences (Equation 3).

$$C(t) = \begin{cases} C_{1\infty}\left(1 - e^{-k_1 t}\right), & if\ t \le t_{int} \\ C_{int} + C_{2\infty}\left(1 - e^{-k_2 \times (t - t_{int})}\right), & if\ t \ge t_{int} \end{cases} \quad (3)$$

where identifiers define the time-limits of each first order reaction, t_{int} is the time of intersection of the two plots, C_{int} is the concentration of product at t_{int} and is therefore added to the second term to describe total product formation. The value of C_{int} may be determined computationally by solving equation 1 letting $t = t_{int}$ using substituted values for $C_{1\infty}$ and k_1. When the LOS plot consists of a single linear phase, t_{int} does not exist, and only the first part of this equation applies.

2.7 Statistics

All data are presented as mean ± SEM unless otherwise specified. Replicate values obtained experimentally from digestibility assays were fitted to Equation 1 or 3, depending on whether one or two distinct rates were observed in the LOS plot, respectively. For comparative purposes, the 'best-fits' to experimental data were obtained by iterative Maximum Likelihood Estimation (MLE) of the parameters ($C_{1\infty}$, k_1, $C_{2\infty}$, k_2, C_{int} and t_{int}), whereas the 'model-fits' were computed from the same equation, but with all the variables defined on the basis of LOS plot estimates. Residual analysis was used to assess the performance of the model in accurately predicting the concentration of product over time for experimental data obtained from 10 particle sizes of durum wheat and chickpea tissue (including the purified starch equivalent). Repeated Measures Analysis of Variance was used to compare experimentally obtained digestibility curves, with time as a 'within-sample' factor, and particle size and botanical source as 'between-sample' factors. Tukey's *post-hoc* analysis was carried out to identify homogenous subsets among particle sizes. Statistically significant differences were accepted at $P < 0.05$. The analysis was performed using IBM SPSS Statistics 20.0 (©IBM Corp. 2011). All other analyses were performed using SIGMAPLOT 12.0 (©Systat software 2011) statistical and graphical software.

3 Results

3.1 *In vitro* digestibility

Experimentally obtained digestibility curves are shown for all particle size fractions of chickpea and durum wheat in Fig. 1**.** Purified starches (extracted from durum wheat or chickpea) were the most digestible, with no statistically significant differences observed between the starch or flour

digestibility curves of durum wheat and chickpea. As the particle size, and therefore the proportion of encapsulated starch, increased, a significant reduction in starch digestibility was observed, giving rise to obvious differences in the amount of starch amylolysis achieved after 60 min.

3.2 Logarithm of Slope analysis

LOS analysis was applied to the experimentally obtained digestibility curves to establish whether amylolysis followed a single-phase or two-phase pseudo-first order process, and to estimate values for the variables in Equations 1 or 3 (see above). LOS analysis of flour and purified starch digestibility data revealed linear plots (r^2 > 0.90) characterised by a single rate constant (Fig. 2**)**. Thus, in hydrothermally processed starch and flour fractions, amylolysis occurs by a single-phase process, and may be described by Equation 1, in which the values of k and C_∞ can be determined from the slope and y-intercept of the LOS plot, respectively.

When LOS analysis was applied to digestibility data of the more coarsely milled fractions, two linear phases were apparent in a number of the LOS plots (Fig. 3). Amongst the durum wheat fractions, two-phases were observed only for the largest milled fractions (>1.29 mm), whereas all milled fractions of chickpea >0.50 mm displayed this behaviour. As starch amylolysis in these more complex materials followed a two-phase process, two sets of C_∞ and k values were required to describe each amylolysis phase. Estimated values describing the rapid-phase ($C_{1\infty}$ and k_1) were obtained from the y-intercept and slope, as described previously. It became clear however, that for the slower second phase, obtaining $C_{2\infty}$ from the y-intercept was not satisfactory, as this resulted in a substantial overestimation of product formation, characterised by largely negative residuals (mean residual = 4.2%, with a SD of 5%). Instead, a much improved fit to the experimental data was achieved when the two digestive phases were

described by two consecutive, rather than simultaneous, reactions, as represented in Equation 3. Therefore, $C_{2\infty}$ was estimated from the intersection between the two-phases in the LOS plot (i.e., $t = t_{int}$), rather than from the y-intercept ($t = 0$).

Values estimated from LOS plots for the variables in Equation 3 are summarised for all size fractions in Table 1. Discontinuous LOS plots, observed for larger macroparticles (Fig. 3), consist of two linear phases, each identified by a rate constant. The rate of the first reaction (k_1) was always greater than the second. For chickpeas, the value of k_1 increased with particle size, whereas for durum wheat, the value of k_1 did not appear to be influenced by size. The rate constant of the second phase, k_2, was similar for all milled fractions ($k_2 = 0.06 \pm 0.006$ min^{-1} for chickpea and 0.05 ± 0.006 min^{-1} for durum wheat), and is comparable to the single rate constant obtained where amylolysis occurs as a single-phase process, i.e., starch, flour and smaller size-fractions (Fig. 2).

The point at which the slower phase becomes the predominant reaction is represented by the intersection between the two linear phases of the LOS plot, and seemed to occur after 7 to 15 minutes of amylolysis under the digestibility conditions used in the experiment (Fig. 3). The estimated values for $C_{1\infty}$ and $C_{2\infty}$ provide an indication of the contribution of each amylolysis phase to the total starch breakdown. For chickpea materials, the rapid reaction, where it exists, was the greater contributor to total starch amylolysis, whereas for durum wheat particles, the two reactions contributed fairly equally. The total extent of amylolysis ('*Total* C_∞', which is the sum of $C_{1\infty}$ and $C_{2\infty}$) was reduced by increasing particle size and therefore the proportion of cell wall encapsulated starch. The largest reductions were observed for chickpea materials, where *Total* C_∞ decreased by nearly 50 % (i.e. from flour to larger macroparticles), whereas in durum wheat a 14 % reduction was observed.

3.3 Modelling of digestibility data

Predicted values for variables of starch amylolysis (e.g. k and C_∞) were obtained from the LOS plots and entered into either Equation 1 or 3, depending on whether one or two phases were observed. This enabled computation of curves showing product formation over assay incubation time (C_t). Overall, the computed digestibility curves provided a very good fit (see representative example, Fig. 4A) to all experimental data, with $R^2 > 0.9$ and Standard Error of Estimates (SEE) <6% for all particle size fractions and starches..

Model performance was evaluated by residual analysis, which indicates how well the model-computed curves describe the experimental data The mean residual values and SDs observed were low (1.65 ± 3.5 for durum wheat and 0.95 ± 2.4 for chickpea, expressed as % starch digested), indicating a very good fit to the experimental data (Fig. 4B). Somewhat larger residuals were observed for durum wheat, which suggests that the model is more likely to overestimate the digestibility somewhat of fractions from this starch source. However, no systematic error was observed with increasing amylolysis time or particle size, and taking account of the likely experimental error associated with obtaining digestibility curves from such complex materials, a deviation of such small magnitude may be considered negligible. Overall, the strong correlations between best-fit and model-fit residual values (R^2 <0.99 in chickpea and R^2 = 0.92 in durum wheat) confirms that this model is an excellent predictor of starch amylolysis in the materials examined.

4. Discussion

We have introduced a novel method of analysing first-order kinetic data, to describe the amylolysis of edible plant tissue containing starch. Unlike the current classification system, LOS

analysis is based on sound enzyme-kinetic principles, and employs only two variables, C_∞ and *k,* to accurately predict the release of hydrolysed products from amylolysis [8]. In particular, this method provides a sensitive, more rigorous and less arbitrary means of identifying fractions of starch-containing food particles that are digested at different intrinsic rates and to different extents. Uniquely, it also allows the contribution of these distinct fractions to total product formation to be modelled over time. This feature not only provides mechanistic insight about digestion of heterogeneous substrates, but should prove of great relevance to predicting glycaemia and insulinaemia [1, 20].

The strong correlation between the predictive digestibility curves and experimental data confirmed that the estimates of C_∞ and *k* obtained from LOS plots are valid predictors, and that the equations developed provided a representative description of the processes leading to the release of starch hydrolysis products. Previously, LOS analysis of digestibility curves obtained for hydrothermally-processed starches, and homogenised, food products (i.e. loss of structural integrity) revealed that these materials followed a *single-phase* amylolysis process [3, 9]. However, using the Englyst classification system, these food materials would be subdivided into RDS and SDS, even though the slower rate in the later stages of amylolysis is a natural consequence of the fall in the concentration of available substrate and is not indicative (as already explained) of *intrinsic* differences in rate. This example demonstrates the questionable value of the existing starch classification system, which results from a misinterpretation of starch digestibility data.

In the current study, discontinuities were clearly evident in the LOS plots of materials which contained cell wall encapsulated starch (particle sizes ≥ 1.02 mm in durum wheat and particles ≥ 0.55 mm in chickpea), indicating that amylolysis in these materials occurred in two-phases. Notably, other researchers have recognised that a single-first order reaction does not always provide a suitable description of starch amylolysis, and have suggested that bi-phasic equations provided better fits to digestibility data [19]. Of course, when curve fitting is performed

using an iterative process, increasing the number of variables in an equation inevitably increases the likelihood of obtaining a good fit to an experimental curve, and so results should be interpreted cautiously. Here, application of a bi-phasic model to raw data was justified, because the LOS plots clearly indicated a bi-phasic digestion process. Moreover, the values of parameters obtained using the LOS model are based on the well-established properties of first-order reactions, and therefore provide confidence from a scientific viewpoint. Indeed, the various k and C_∞ values obtained from the LOS plots were used directly to define parameters of the two-phase consecutive model, which was then found to provide an excellent description of experimental data.

A two-phase *consecutive* reaction implies that unless extremely high concentrations of α-amylase are present, the 'available' α-glucan chains of starch must complex with virtually all of the amylase. Therefore, negligible enzyme remains free to react with the 'less available' α-glucan chains and promote a simultaneous reaction. Only when amylase becomes free following hydrolysis of the available starch, can sufficient enzyme interact with the less accessible starch and so enable a second reaction to proceed at a detectable rate. This result is also compatible with how amylase interacts with native, granular, starch as reported in our recent study [3]. We believe this is an interesting and significant observation that provides a new understanding of the mechanism by which amylase acts on hydrothermally-processed plant tissues.

With this new insight, the question arises as to what type of mechanisms the different rate constants represent. This is a challenging question, as the mechanisms may differ depending on the nature of the substrate examined. For the macro-particles of durum wheat and chickpea included in this study, amylase is likely to encounter first the most accessible, and therefore potentially available, starch in ruptured cells on the particle surfaces. To access starch in the underlying cell layers of the plant tissue (i.e. cell wall encapsulated starch), the enzyme

would have to diffuse across the cell wall barrier of intact cells and other structural elements e.g., a protein network. Alternatively or in addition, if a degree of cell degradation occurs during the enzyme assays, digestion of previously encapsulated starch would become possible. Thus, $C_{1\infty}$ is likely to represent amylolysis of immediately accessible starch occurring predominantly on the fractured surfaces, whereas $C_{2\infty}$ probably represents amylolysis of encapsulated starch in the underlying cell layers. In durum wheat materials, each fraction contributed more or less equally to total starch breakdown, whereas for chickpeas the second reaction contributed considerably less. This result is consistent with our expectation that chickpea cell walls protect encapsulated starch from digestion, and therefore explains their low glycaemic index. The durum wheat cell walls, on the other hand, appear to be less effective enzyme barriers, permitting the digestion of intracellular starch

Interestingly, the single rate constant of purified starches and flour is similar to the rate constant of the second, slower phase of amylolysis found in the plant tissues. This was unexpected, as the rate constant is an inherent property of the enzyme, independent of substrate concentration, and the greatest reaction efficiency would be expected for the most accessible substrate, i.e. the purified starches.

We considered a number of possible explanations for this size-dependant increase in k_1: First and foremost, an increase in the rate constant suggests that an activator may be present. Chloride and calcium are both known activators of α-amylase, and plant tissue does contain these compounds. The enzyme preparation itself contained 3 mmol/L $CaCl_2$, and 2.9 mol/L NaCl, and the assay conditions in PBS (approx 140 mmol/L Cl^- ion concentration would be expected to more than satisfy the requirements for these ions [21, 22]. Plant materials are also known to contain endogenous enzymes, which could contribute to the overall release of reducing sugar. Enzyme-free control runs (data not shown) established that no increase in reducing sugars concentration was produced during the timed assays, and in any case, it is

almost certain that any endogenous enzymes would be denatured by 85 min of hydrothermal processing.

Phenolic compounds, which are found in wheat and chickpeas, are reported to inhibit the action of α-amylase [23, 24]. Paradoxically however, a recent paper reported that a polyphenolic compound (i.e. lignin) is an even more effective activator of α-amylase than chloride [25]. Furthermore, lignins with a larger molecular weight were found to be considerably more efficient at stimulating α-amylase [25]. Also, the amylose polymer of starch is a good substrate for α-amylase and is known to leach from starch granules during hydrothermal processing [26]. Under these conditions of high amylase activity, the effects of any potential activator would be very noticeable. Accordingly, the rapid phase may represent hydrolysis of solubilised amylose chains in the presence of the suspected activator. It is worthwhile noting that the $C_{1\infty}$, which, of course, is unlikely to be affected by the presence of an activator, could usefully represent the leached amylose portion of starch. The possibility of activation clearly warrants further investigation.

The nutrient-release behaviour during amylolysis of carbohydrate foods is of metabolic and clinical importance. Consumption of starches which are digested slowly, and therefore elicit a smaller glycaemic and insulinaemic response, is associated with a reduced risk of developing diabetes and cardiovascular disease [27], and also has implications for the management of obesity [28]. Additionally, starch which escapes amylolysis in the upper-intestinal tract ('resistant starch') provides a substrate for fermentation to short chain fatty acids (e.g. butyrate) by microbial organisms in the colon, and therefore exerts pre-biotic effects [29]. Thus, some carbohydrate foods may have potential therapeutic effects; however, predicting nutritional properties presents a formidable challenge due to the many physico-chemical factors that influence starch amylolysis [4]. LOS analysis of digestibility data is particularly well-suited to mechanistic studies, as it provides a rapid and accurate means of predicting the rate of amylolysis and the total amount of digestible starch. This method may therefore facilitate

identification and development of so-called slow release functional ingredients and foods for use in the dietary management of metabolic disorders such as type 2 diabetes.

5 Conclusions

The model described in this paper enables accurate predictions of the release of starch hydrolysis products from a complex food matrix. LOS plots are recommended for precise identification of nutritionally relevant starch fractions, and have important advantages over the popular but flawed Englyst classification system. One particular benefit arising from the use of the LOS approach is that the contribution of each phase to total starch breakdown can be estimated from C_∞ values - an attribute which is of significant value when working with complex food materials. LOS plots are also advantageous for universal, quantitative comparisons, because each amylolysis phase is represented by a rate constant. This method can be applied to both new data and previously published digestibility curves for estimation of C_∞ and can be performed conveniently in an Excel spreadsheet [3]. Overall, LOS analysis provides a superior and more rigorous method for classification of starch amylolysis. We believe that LOS plot analysis of starch amylolysis data will prove to be a useful and cost-effective tool for studying the many factors that influence starch digestibility.

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Author Disclosure

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Tables

Table 1. Values of variables estimated from LOS analysis for all size fractions of hydrothermally processed chickpea and durum wheat [a].

CHICKPEA

	RAPID PHASE		SINGLE OR SLOWER PHASE		INTERSECTION		TOTAL
Size (mm)	**$C_{1\infty}$ (%)**	**k_1 (min^{-1})**	**$C_{2\infty}$ (%)**	**k_2 (min^{-1})**	**t_{int} (min)**	**C_{int} (%)**	**C_∞ (%)**
Starch	N/A	N/A	73.5*	0.05*	N/A	N/A	73.5
< 0.21	N/A	N/A	64.7*	0.07*	N/A	N/A	64.7
0.38	N/A	N/A	51.7*	0.09*	N/A	N/A	51.7
0.55	33.2	0.16	15.9	0.06	11.1	27.3	49.2
0.73	27.4	0.16	12.2	0.05	12.4	23.7	39.6
1.02	19.5	0.26	9.0	0.06	8.5	17.4	28.5
1.29	18.2	0.17	4.3	0.06	15.3	16.8	22.5
1.55	12.4	0.32	3.3	0.07	9.2	11.7	15.7
1.85	13.6	0.32	6.0	0.08	6.8	12.1	19.5
2.58	11.3	0.44	10.3	0.02	7.1	10.8	21.6

DURUM WHEAT

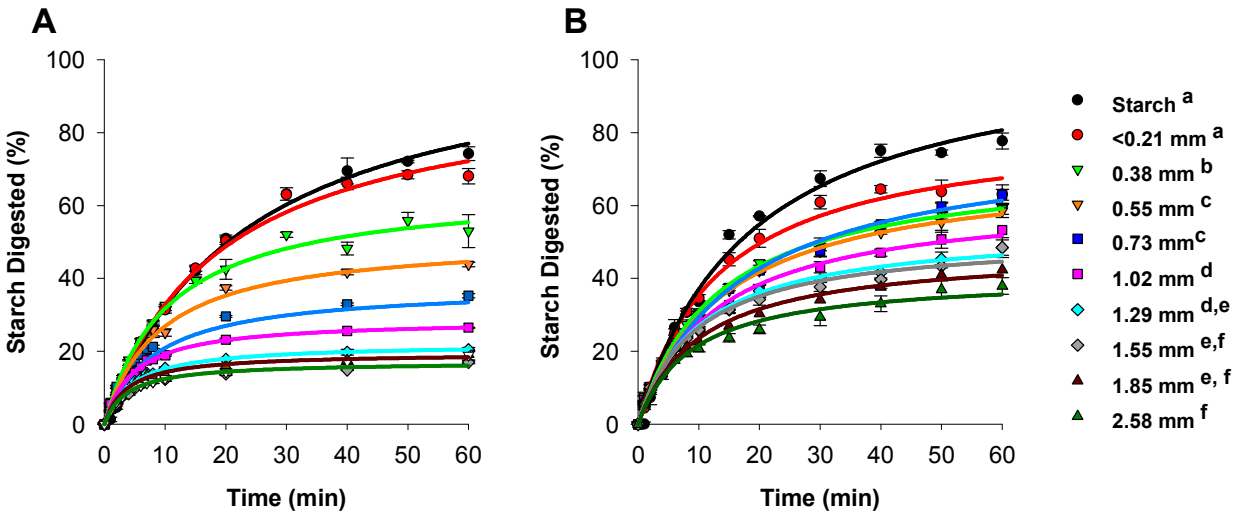
	RAPID PHASE		SINGLE OR SLOWER PHASE		INTERSECTION		TOTAL
Size (mm)	**$C_{1\infty}$ (%)**	**k_1 (min^{-1})**	**$C_{2\infty}$ (%)**	**k_2 (min^{-1})**	**t_{int} (min)**	**C_{int} (%)**	**C_∞ (%)**
Starch	N/A	N/A	78.9*	0.06*	N/A	N/A	78.9
< 0.21	N/A	N/A	63.7*	0.09*	N/A	N/A	63.7
0.38	N/A	N/A	58.9*	0.05*	N/A	N/A	58.9
0.55	N/A	N/A	60.7*	0.05*	N/A	N/A	60.7
0.73	N/A	N/A	59.5*	0.05*	N/A	N/A	59.5
1.02	N/A	N/A	52.0*	0.05*	N/A	N/A	52.0
1.29	29.4	0.22	24.9	0.05	7.8	23.9	54.3
1.55	30.0	0.17	21.5	0.04	11.2	25.5	51.5
1.85	23.8	0.16	25.7	0.03	9.5	18.8	49.5
2.58	21.6	0.22	33.0	0.02	9.8	19.2	54.6

[a] Values are estimated from LOS plots with one or two-phases.

* Indicates that amylolysis occurred by a single-phase process, and that no rapid phase was observed. Values for the rapid phase and the intersection are therefore not applicable (N/A).

C_∞ is the extent of starch amylolysis for each digestive phase. *Total* C_∞ is the sum of $C_{1\infty}$ and $C_{2\infty}$ and represents the total extent of starch amylolysis. k is the rate constant of each phase. C_{int} is the extent of starch amylolysis at the time of intersection, t_{int}.

Figures



464

465 **Fig. 1** Digestibility curves obtained for milled particle sizes and purified starches of
466 hydrothermally processed (A) chickpea and (B) durum wheat. Particle size is defined on the
467 basis of material retention in sieves of known aperture. Values are means ± SEM. Legend
468 applies to both panels, and different superscript letters indicate significant differences ($P < 0.05$)
469 between curves for both chickpea and durum wheat.

470

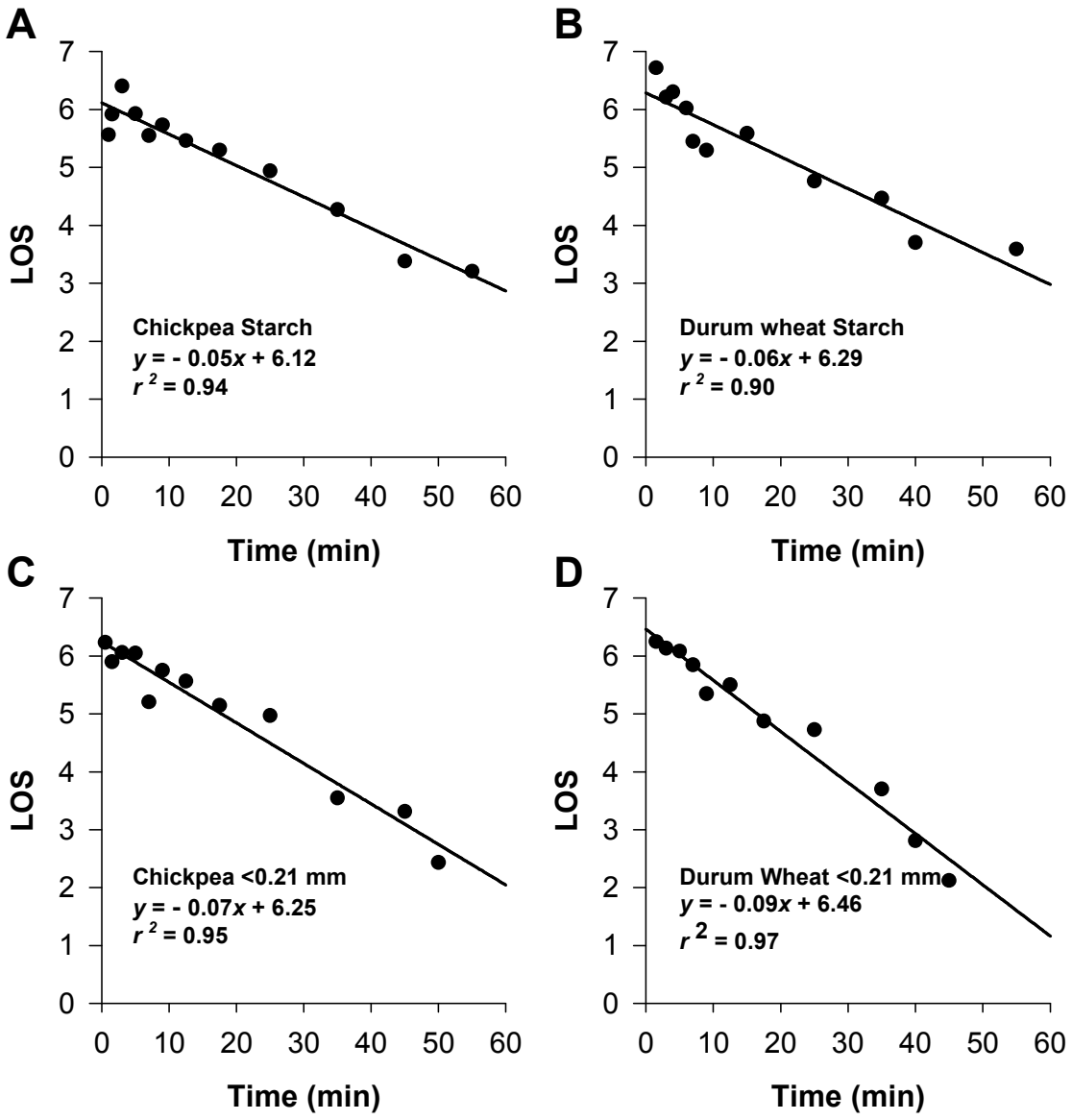


Fig. 2 LOS plots obtained for hydrothermally processed plant materials reveal a single phase of amylolysis, defined by equation 2, from which values of k and $C_{1\infty}$ can be estimated. (A) Chickpea starch; (B) durum wheat starch; (C) chickpea < 0.21 mm; (D) durum wheat < 0.21 mm.

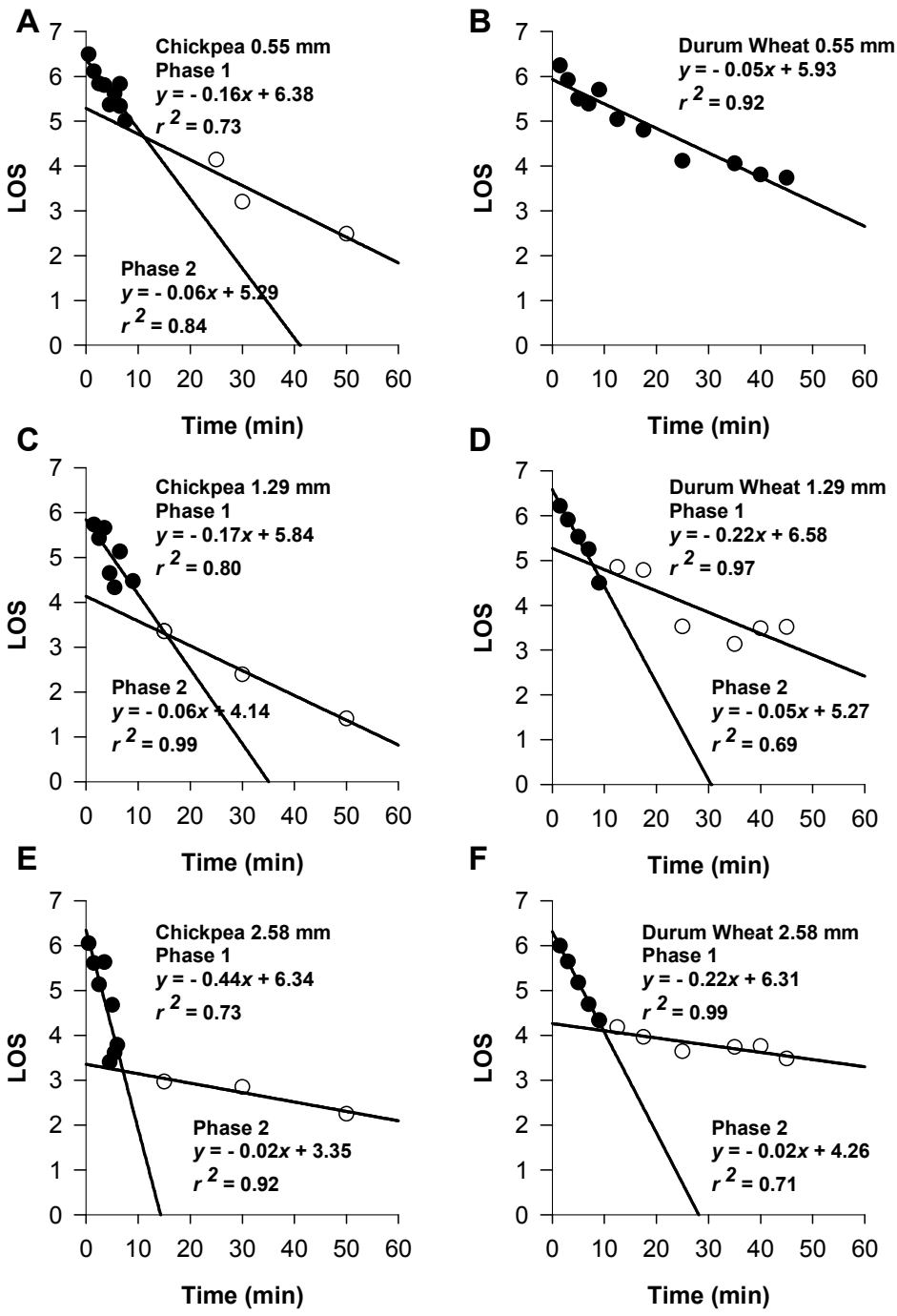


Fig. 3 LOS plots obtained for selected particle sizes of hydrothermally processed plant materials showing one or more phases of amylolysis. Each linear phase is defined by equation 2, from which values of k and $C_{1\infty}$ can be estimated. (A) Chickpea 0.55 mm; (B) durum wheat 0.55 mm; (C) chickpea 1.29 mm; (D) durum wheat 1.29 mm; (E) chickpea 2.58 mm; (F) durum wheat 2.58 mm.

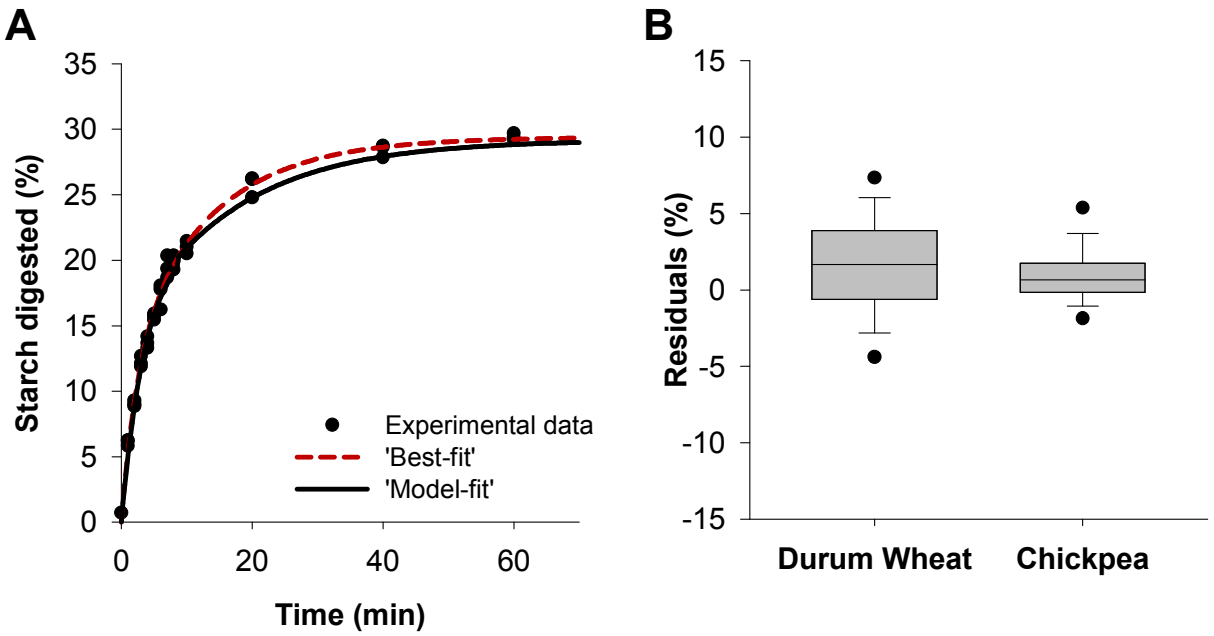
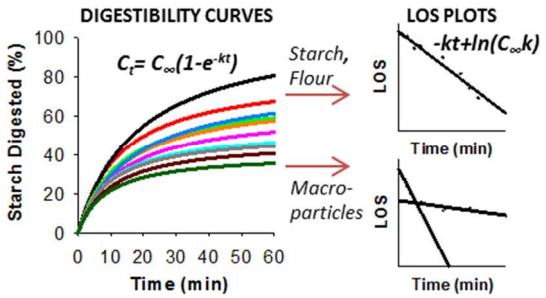


Fig. 4 Example of model computed digestibility curve shown alongside a best-fit to experimental data and box-plot of pooled residuals. (A) Data points are experimentally obtained replicate values from a digestibility assay of chickpea, size 1.02 mm. The model-fit was obtained by substitution of LOS estimated values into equation 3 (R^2 = 0.991, SEE = 0.85). The best-fit was obtained by Maximum Likelihood Estimation (MLE) regression of experimental data (R^2 = 0.995, SEE = 0.64). (B) Box-plot showing pooled residuals for all size fractions of durum wheat and chickpea. Quartiles: 10^{th} to 90^{th}; values outside this range are represented by a single dot.

Graphical Abstract



Highlights

LOS plots of first-order digestibility data enable the rapid identification of nutritionally-important starch fractions, and allow the final extent (C_∞) of starch amylolysis to be accurately predicted.