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Analysis of starch amylolysis using plots for first-order kinetics

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ABSTRACT

Investigators often study product release from starches during prolonged incubations with α-amylase *in vitro*. The reaction time courses usually fit to a linear form of a first order rate equation, i.e., $\ln[(C_\infty - C_t)/C_\infty] = -kt$. This equation calls for an accurate estimate of C_∞, i.e., the concentration of product at the end of the reaction. Estimates of C_∞ from digestibility curves can be unreliable. The Guggenheim method does not require prior knowledge of C_∞ but seems not to have been applied to starch hydrolysis data. An alternative method is also available in which the logarithm of the slope (LOS) of a digestibility curve at various time points is plotted against time. This allows estimations of both k and C_∞ and can also reveal whether changes occur in digestion rate from rapid to slow as digestion proceeds. We describe the Guggenheim and LOS methods and provide examples of their application to starch digestibility data.

1. Introduction

Starch, which consists of two α-glucan polymers amylose and amylopectin, is the main source of 'digestible' carbohydrate in the human diet. Therefore a marked increase in blood glucose concentration following a starch-rich meal can result from the amylolysis of starch in the gastrointestinal tract to mainly maltose, maltotriose and α-limit dextrins. These products are then hydrolysed by brush border carbohydrases to glucose, which is absorbed by intestinal enterocytes and finally transferred to the peripheral blood circulation via the hepatic portal vein. It is well known however, that ingestion of different foods containing identical amounts of starch can produce very different postprandial blood glucose and insulin responses (Crapo, Reaven, & Olefsky, 1977; Truswell, 1992). These different metabolic responses have been largely attributed to marked differences in the rate at which starches are digested (Butterworth, Warren, & Ellis, 2011; Holm, Lundquist, Bjorck, Eliasson, & Asp, 1988).

Limiting the fluctuations in postprandial glycaemia and insulinaemia has been shown to be important in the prevention of life-style related conditions such as diabetes mellitus and cardiovascular disease (Ells, Seal, Kettlitz, Bal, & Mathers, 2005; Jenkins et al., 2002; Judd & Ellis, 2006; Mann, 2007; Seal et al., 2003). The potential health benefits that can accrue from a dietary choice of starch-containing foods that are digested relatively slowly have prompted a considerable amount of research seeking explanations for observed differences in digestion rates. With such information to hand, and with knowledge of the starch origin and physicochemical properties of foods, it may become possible to make better predictions of the likely rate of digestion of a particular starch-containing food.

The reasons for the differences in the rates of digestion of starch in a food matrix are not fully understood, but many factors are of importance (Svius, Uhlen, & Harstad, 2005). These include: (1) the physical and chemical structure and properties of the starch at the molecular and granular levels (e.g. granule surface area and structure, the amount of crystalline α-glucan polymer; (2) the structural architecture and properties of the food matrix (e.g. plant cell wall barrier restriction of enzyme access, mechanical disruption of food) and (3) the presence of other dietary components such as proteins, lipids, phenolic compounds and water-soluble non-starch polysaccharides. Many of these factors will be affected to a greater or lesser extent by food processing conditions such as milling and hydrothermal treatments. The latter process gelatinises starch and makes it more susceptible to amylolysis (Buleon, Colonna, Planchot, & Ball, 1998; Dhital, Shrestha, & Gidley, 2010; Holm et al., 1988; Roder et al., 2009; Svius et al., 2005; Tahir, Ellis, & Butterworth, 2010; Yook & Robyt, 2002).

Studies of starch digestion *in vitro* using pancreatic α-amylase preparations acting upon various starchy foods and extracted starches have been performed by many workers. By taking account of certain of the various factors listed above, attempts have been made to model *in vivo* digestion and permit predictions of the likely rise in blood glucose following meals containing a particular kind

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of starch. These predictions include empirical estimates of the glycaemic index of foods (Goni, Garcia-Alonso, & Saura-Calixto, 1997).

The enzyme-based approach for prediction of digestibility is easier and cheaper to perform than studies using experimental animals and/or human subjects that involve blood sampling and appropriate ethical authorisation. The enzyme experiments have included studies of Michaelis–Menten kinetic parameters (Dona, Pages, Gilbert, & Kuchel, 2011; Roder et al., 2009; Slaughter, Ellis, & Butterworth, 2001; Tahir, Ellis, Bogracheva, Meares-Taylor, & Butterworth, 2011; Tahir et al., 2010) and also analysis of digestibility curves determined over relatively lengthy periods by fitting to first-order kinetics (Al-Rabadi, Gilbert, & Gidley, 2009; Dhital et al., 2010; Goni et al., 1997; Mahasukhonthachat, Sopade, & Gidley, 2010).

The relative merits of the different kinetic approaches have been discussed (Butterworth et al., 2011; Dona, Pages, Gilbert, & Kuchel, 2010; Tahir et al., 2011). A recent publication discusses likely differences between starch digestion *in vivo* and *in vitro* because of the greater complexity of the intestinal digestion process (Hasjim, Lavau, Gidley, & Gilbert, 2010). This is a fair enough warning of possible dangers inherent in over-interpretation of *in vitro* digestibility studies, but such studies are so useful that they are likely to continue to be popular. Investigators need to be aware however, that the *in vitro* conditions prevalent in the digestibility studies do not mimic the *in vivo* process completely.

We discuss available methods for fitting of amylase-starch digestibility curves to obtain parameters that are useful in investigations of the rate and extent of starch digestion. The methods are illustrated both by re-plots of data published in the literature and by plots of results obtained in our laboratory.

2. Experimental

2.1. Fitting to 1st order kinetics

When starch or starch-containing foods are digested *in vitro* with relatively high concentrations of pancreatic α-amylase and for long time periods, i.e., extending over several hours, the rate of reaction decreases as the time is extended and plots of the concentration of product formed (or quantity of starch digested) against time are logarithmic. The plots approach an end point where no further reaction is measurable no matter how much longer incubation times are prolonged. This response is predictable based on the assumption that the concentration of available starch substrate decreases with time as starch is converted to products. The end point has been described erroneously as a state of equilibrium (Goni et al., 1997). There is no back reaction under the conditions of the digestions and so the end point represents a situation in which no more starch substrate is available for digestion (Butterworth et al., 2011). There is a possibility that product inhibition, particularly by maltose, can contribute to the slowing down of reaction rates, so many workers include amyloglucosidases in their reaction mixtures to remove maltose and maltotriose by conversion to non-inhibitory glucose. A recent publication reports that inhibition by maltose seems to be negligible, however, under the conditions prevailing in most digestibility studies performed on pure starches and various foods (Dona et al., 2011).

Englyst, Kingman, and Cummings (1992) introduced a classification system to describe the various phases of starch digestion with reference to specific time frames. The fraction digested within 20 min is denoted as rapidly digestible starch (RDS), the fraction digested between 20 and 120 min represents slowly digested starch (SDS), and the material remaining undigested after 120 min is resistant starch (RS). This system implies that starch granules as purified samples or as part of a food matrix, contain fractions that differ in susceptibility to digestion by α-amylase. Rather unfortunately, this classification system has proved popular and been widely quoted by authors. A recent review (Zhang & Hamaker, 2009) seems to add to the confusion somewhat, although these authors discuss usefully, the factors that are important in determining whether a particular starch is digested relatively rapidly or slowly.

The literature now contains examples of digestibility data being fitted to 1st order kinetic equations (Dhital et al., 2010; Goni et al., 1997) and the results reveal that for hydrothermally processed (cooked) starches and starch-containing foods, regardless of degree of 'Englyst' digestibility, the kinetics can be described by a single rate constant. Such a result reveals that all digestible fractions are inherently of the same reactivity. The decrease in the observed rate of release of product over the time course of the reaction is a natural feature of an exponential reaction. There is no doubt that some starch substrates are digested more rapidly than others (see the figures below) and that certain fractions may be resistant to amylase action. It is questionable however, that individual starches possess fractions of rapidly digested and more slowly digested material unless the starch is in a native granular state.

Bertoft and Manelius (1992) showed that digestion of granular starch catalysed by α-amylase from *B. subtilis* occurred in two stages with an initial rapid rate followed by a slower rate. Planchot, Colonna, and Buleon (1997) reported similar findings for the digestion of starch and starch lintners. They conducted very long incubations however, and seemed to identify the slow phase with the stage at which the reactions were approaching the end point, i.e., the point of substrate exhaustion. Planchot et al. (1997), did not present their data as 1st order plots, but Evans and Thompson (2008) showed that amylose and amylopectin dispersions displayed discontinuities in semi-logarithmic kinetic plots. This finding seemed to give clear evidence of fractions that are digested at different rates, although the direct relevance to the studies of native and gelatinised starches that we report here is not immediately obvious. Also, in both of the Evans and Thompson (2008) and the Planchot et al. (1997) studies, the incubations were prolonged and the slower phase probably arose from substrate depletion, so that the substrate conversion rates had become almost zero.

α-Glucan chains at the periphery of a granule are more accessible to amylase and are therefore likely to be digested more rapidly than material located within the granule particularly as the peripheral starch has a greater preponderance of amorphous material (Jane & Shen, 1993). Even potentially available α-glucan chains located within the granule will have to rely on the slow diffusion of α-amylase into the granule before amylolysis can take place (Mahasukhonthachat et al., 2010) and will therefore be hydrolysed more slowly than peripheral material.

It can be argued that an analysis method that can test for the existence of RDS and SDS, as exemplified by a change in rate constant as the reaction proceeds, would be a useful investigative tool.

Digestibility curves can be fitted to a familiar first-order equation (Goni et al., 1997):

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

C_t is the concentration of product or reactant at time t, C_∞ is the corresponding concentration at the end point and k is a pseudo-first order rate constant. For ease of plotting, the equation is usually cast in the form:

$$\ln\left[\frac{(C_\infty - C_t)}{C_\infty}\right] = -kt \quad (2)$$

Thus, a plot of $\ln[(C_\infty - C_t)/C_\infty]$ against t is linear with a slope of $-k$. The rate constant is a function of the fixed amylase concentration used in the digestion and is therefore pseudo-first order. A problem in using this simple equation comes from the need of an accurate

estimate of C_∞. Unless the enzyme-catalysed digestion is allowed to run for a long time, digestibility curves cannot be guaranteed to have reached a true end point. Because logarithms are plotted, C values do not have to be concentrations. They can be any measured parameter that is directly related to concentration, but if C_∞ values are to be calculated from suitable plots (see below) actual concentration values will be needed.

Guggenheim introduced a method for determining the rate constant of 1st order reactions where C_∞ is unknown and the method has been widely used by chemists for decades (Frost & Pearson, 1961; Niebergall & Sugita, 1968). C is measured at times that are separated by a constant time increment Δt (i.e., the intervals between, t_1, t_2, t_3 etc. are all Δt).

Then it can be shown that:

$$C_{i+1} - C_i = C_\infty e^{-kt}(1 - e^{-k\Delta}) \quad (3)$$

Hence:

$$\ln(C_{i+1} - C_i) = \ln[C_\infty(1 - e^{-k\Delta})] - kt \quad (4)$$

The first term on the right hand side of the equation is constant because both C_∞ and the time interval Δ are constant, and so a plot of $\ln(C_{i+1} - C_i)$ against t is linear with a slope of $-k$. In practice a plot is made using data points of $\ln(C_2 - C_1)$, $\ln(C_3 - C_2)$, …, $\ln(C_{n+1} - C_n)$, etc. against $t_1, t_2, \ldots, t_{n-1}$ etc., respectively.

Poulsen, Ruiter, Visser, Jorgen, and Iversen (2003) extended the Guggenheim approach to situations where the interval between measurements does not have to be constant. These authors also point out that their method will demonstrate whether the data are truly of logarithmic form and should also reveal whether the rate constant remains unchanged throughout the whole of the reaction.

Differentiation of Eq. (1) gives:

$$\frac{dC}{dt} = C_\infty k e^{-kt} \quad (5)$$

This first derivative represents the slope of a digestibility curve at time t.

Expressing Eq. (5) in logarithmic form gives:

$$\ln\left(\frac{dC}{dt}\right) = \ln(C_\infty k) - kt \quad (6)$$

Thus, a plot of $\ln(dC/dt)$ against t is linear with a slope of $-k$. The intercept on the y axis equals $\ln(C_\infty k)$ and so C_∞ can be calculated from the value of k obtained from the slope of the plot. Poulsen et al. (2003) refer to this plot as a 'log of slope' or logarithm of the slope (LOS) plot, and as has already been mentioned, the slope is sensitive to changes in k occurring during a reaction. Such changes will be revealed by discontinuities in the linear plot. As mentioned above, this potential has relevance to starch digestibility curves because of suggestions that various starch sources contain fractions that are digested at different rates (Bertoft & Manelius, 1992; Englyst et al., 1992).

To use the LOS plot requires determination of the slope of a digestibility curve at several time points. Provided that the time points on the curve are not too far apart, the slope may be estimated from the fraction $(C_2 - C_1)/(t_2 - t_1)$, $(C_3 - C_2)/(t_3 - t_2)$ etc. and the natural logarithms plotted against the relevant, mean time, i.e., $(t_2 + t_1)/2$, $(t_2 + t_3)/2$ etc. A spread sheet can be set up to perform these relatively simple calculations. Our experience of using the LOS plot suggests that the time intervals should be sufficiently spaced to allow reliable measurement of the changes in product concentration occurring between time points chosen for slope determination. This helps to overcome the disadvantage mentioned by Poulsen et al. (2003) that noise can have a large influence on the slope between two data points and hence introduce large errors. At the last stages of digestion, the slope of the digestibility curve becomes essentially zero and the experimental points in this region are not useable for a LOS plot.

Reliable estimates of k and C_∞ values are required for calculating hydrolysis indices (HI) which equal the area under digestibility curves (AUC) between time t_0 and a selected time t_x. The AUC is obtained from integration of Eq. (1) between bounds of t_0 and t_x:

$$\text{AUC} = C_\infty(t_x - t_0) + \left(\frac{C_\infty}{k}\right)[(e^{-kt_x} - e^{-kt_0})] \quad (7)$$

If $t_0 = 0$, this equation simplifies to:

$$\text{AUC} = C_\infty t_x + \left(\frac{C_\infty}{k}\right)(e^{-kt_x} - 1) \quad (8)$$

HI values provide another means of comparing the relative rates of digestion of different botanical starches and of various starch-containing foods (Goni et al., 1997; Mahasukhonthachat et al., 2010). LOS plots allow estimates of both k and C_∞ required for the calculation (see above).

2.2. Sources, characterisation and digestion of wheat and potato starches

PBS tablets (phosphate buffered saline, Dulbecco A, catalogue number BR0014G) were purchased from Oxoid Ltd., Basingstoke, Hampshire UK. The constituted saline consists of 137 mM NaCl, 2.7 mM KCl, and 10 mM total phosphate, pH 7.3 at 25 °C. Porcine pancreatic α-amylase (type 1A, DFP treated) was obtained from Sigma–Aldrich Co. Ltd. The manufacturers specify an activity for the preparation of 1333 units per mg protein. A unit defined by Sigma–Aldrich corresponds to 0.97 IU at 25 °C (Tahir et al., 2010). The protein concentration of the α-amylase preparation was determined using the bicinchonic acid method (Sigma–Aldrich Chemical Company, catalogue number B6943) and the high purity of the enzyme was verified by SDS-PAGE.

Wheat starch was provided by Prof. C. Hedley (formerly of the John Innes Centre, Norwich, UK) and wild type pea starch was extracted from seeds (Tahir et al., 2011). The chemical and physical characteristics of the starch samples used in this study have been reported in detail elsewhere (Tahir et al., 2011, 2010). The amylose fraction of the wheat and pea starch samples was 28.3% and 30% respectively. For wheat, the percentage moisture, lipid and protein contents were respectively, 11.7, 0.4 and 0.31. The equivalent percentage values for pea starch were 14, 0.25 and 0.25. Thus the total level of impurity represented by lipid plus protein was <1% (w/w). Starch damage values for the same samples were found to be <0.5% of the total starch content, when determined by Congo red dye exclusion and microscopy (Slaughter et al., 2001). The enzyme assay based Megazyme® K-SDAM starch damage kit (Megazyme International Ltd., County Wicklow, Ireland) was also tested as a method for measuring starch damage, but it proved unreliable for estimating the very small degree of damage present in our starch samples. The kit was developed to monitor starch damage levels in milled cereal flour samples (Gibson, Al Qalla, & McCleary, 1992). A further complication was presented by the different botanical sources, i.e., wheat and pea, of our samples.

Native wheat or pea starch at a concentration of 5 mg/ml in phosphate buffered saline (PBS) was incubated at 37 °C with 4.5 nM porcine pancreatic α-amylase (i.e., 0.252 μg/ml of protein or approximately 0.33 IU/ml) and with constant mixing. Samples were withdrawn at timed intervals up to 120 min and transferred to ice-cold 0.3 M Na_2CO_3 stop solution. Non-reacted starch was sedimented by centrifugation. The reducing sugar present in the supernatant was determined by a Prussian blue method (Slaughter et al., 2001) and expressed as maltose equivalents by reference to a standard curve prepared with this sugar. An almost identical procedure was used for the digestion of processed wheat and pea

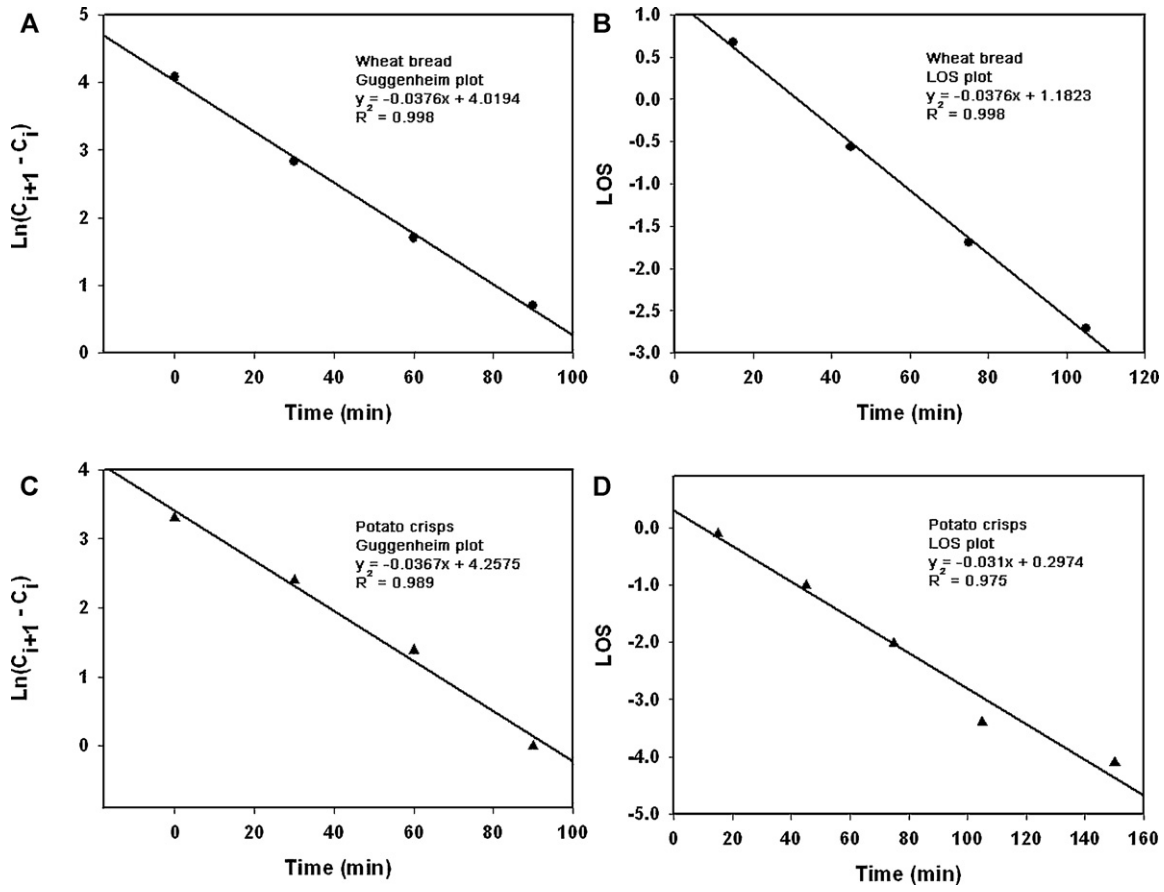

Fig. 1. Starch digestibility data taken from Goni et al. (1997) re-plotted as Guggenheim and log of slope (LOS) plots. (A) White bread, Guggenheim plot; (B) white bread, LOS plot; (C) potato crisps, Guggenheim plot; (D) potato crisps, LOS plot.

starches, but the samples were pre-treated at 90 °C for 30 min and then cooled to 37 °C before the addition of amylase at a final concentration of 2.25 nM (0.126 μg/ml) to start the reaction. The 90 °C processed samples are referred to as 'gelatinised' in subsequent sections of this paper. The enzyme concentrations were calculated assuming a molecular weight of 56,000 for porcine pancreatic α-amylase (Tahir et al., 2011, 2010).

3. Results

3.1. Application of 1st order fitting to digestibility data

Figs. 1 and 2 show Guggenheim and LOS plots of data extracted by callipers and ruler from figures published by Goni et al. (1997). It can be seen that the digestibility curves for white bread, potatoes, potato crisps, peas and beans are well fitted by Guggenheim and LOS plots. The values of the pseudo-first order rate constants generated by these plots agree with each other and also agree well with the values published by the original authors (Table 1). The plots reveal no evidence for a change in rate constant as the digestion of these food samples proceeds and so the starch substrates do not seem to consist of distinct fractions that differ in digestion rate, i.e., there is no evidence for the presence of separate rapidly digested and slowly digested starch components. Values for C_∞ generated by the LOS plots (Table 1) agree very well with the data from the 180 min point in Goni et al. (1997). Reaction half times calculated from $(\ln 2)/k$ values are shown in Table 2.

Table 2
Reaction half times ($t_{0.5}$) calculated from data in Table 1.

Food or starch sample	$t_{0.5}$ (min) Calculated from data of Goni et al.	$t_{0.5}$ (min) Calculated from LOS plots
Boiled potatoes	17.3	19.8
White bread	17.3	19
Potato crisps	23.1	22.4
Peas	23.1	24.8
Beans	23.1	25.7
Gelatinised wheat	Not applicable	17.3
Gelatinised pea	Not applicable	24.7

Table 1
Pseudo-1st order rate constants for starch amylolysis. Data from Goni et al. (1997) re-plotted using Guggenheim and LOS methods.

Food or starch sample	k (min^{-1}) Goni et al.	k (min^{-1}) by Guggenheim	k (min^{-1}) by LOS method	C_∞ (%) from LOS plot	C_∞ (%) Goni et al.	Relative HI Goni et al.
Boiled potatoes	0.04	0.038	0.038	108	83.3	112
White bread	0.04	0.038	0.038	86.8	77.8	100
Potato crisps	0.03	0.038	0.031	43.4	40.7	51
Peas	0.03	0.032	0.028	62.5	58.6	76
Beans	0.03	0.025	0.025	25.8	27.2	34

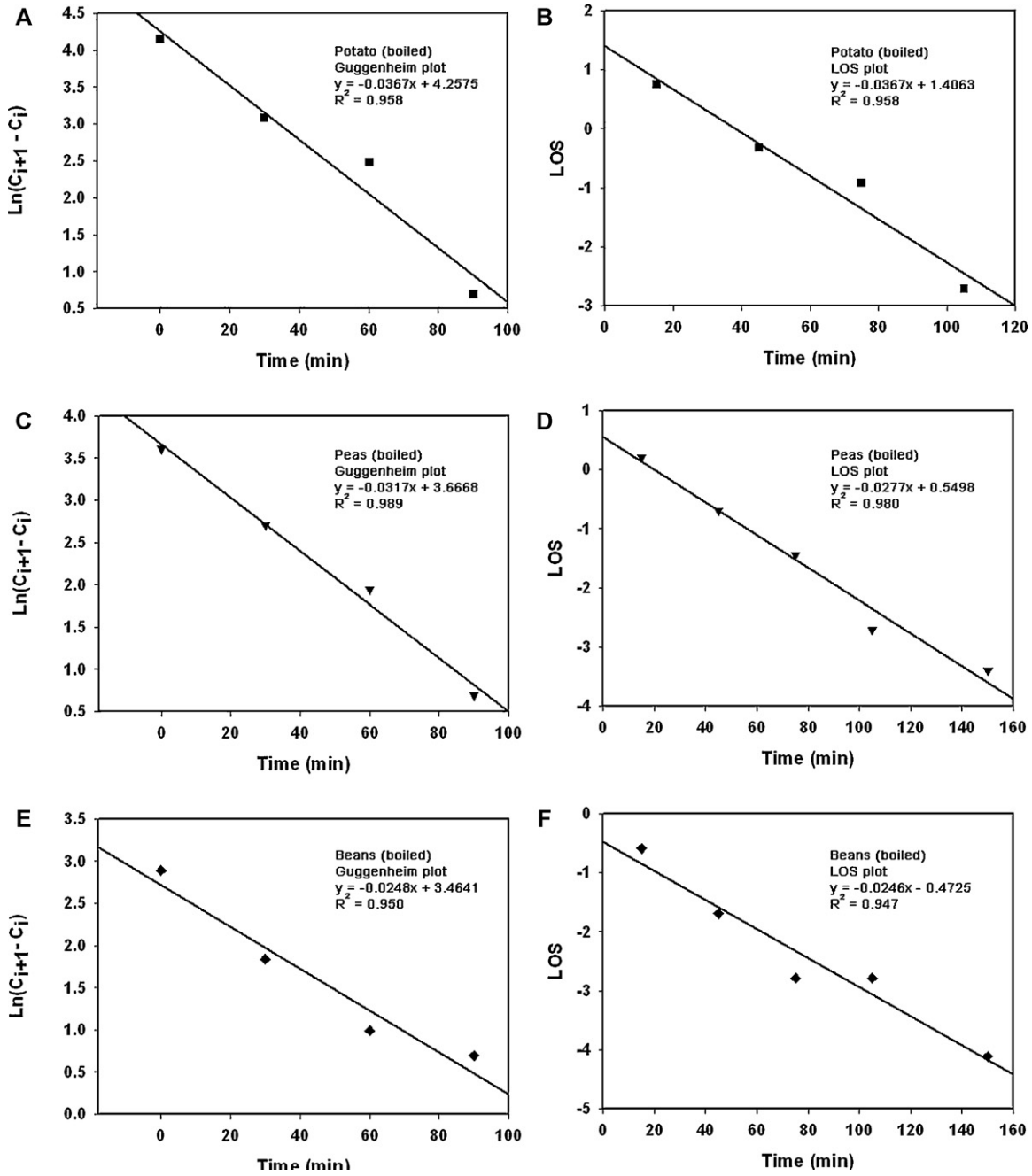

Fig. 2. Starch digestibility data taken from Goni et al. (1997) re-plotted as Guggenheim and LOS plots. (A) Boiled potato, Guggenheim plot; (B) boiled potato, LOS plot; (C) boiled peas, Guggenheim plot; (D) boiled peas, LOS plot; (E) boiled beans, Guggenheim plot; (F) boiled beans, LOS plot.

Table 3 summarises data obtained in our laboratory for digestion at pH 7.4 and 37 °C of wheat and pea starches, both native and gelatinised samples. The digestibility curves and LOS plots are shown in Fig. 3. A porcine pancreatic amylase concentration of 4.5 nM and 2.25 nM was used for the native and gelatinised samples, respectively. Provided that the same enzyme concentration is used in a comparative study of digestibility curves obtained for different starches, the value for k will not differ greatly. This is

Table 3
Data extracted from LOS plots of wheat and pea starch amylolysis. The concentrations of α-amylase used in incubations with native and gelatinised starches were 4.5 and 2.25 nM, respectively. Estimation of k values from Michaelis–Menten parameters (see text) were based on maltopentaose units of 828 molecular weight.

Starch sample	k (min^{-1}) from LOS plot	k (min^{-1}) from Michaelis–Menten	C_∞ (%) from LOS plot
Native wheat	Rapid phase 0.049		6.3
	Slow phase 0.008		11.7
Gelatinised wheat	0.04	0.032	63.4
Native pea	Rapid phase 0.041		5.9
	Slow phase 0.007		13.3
Gelatinised pea	0.028	0.035	64

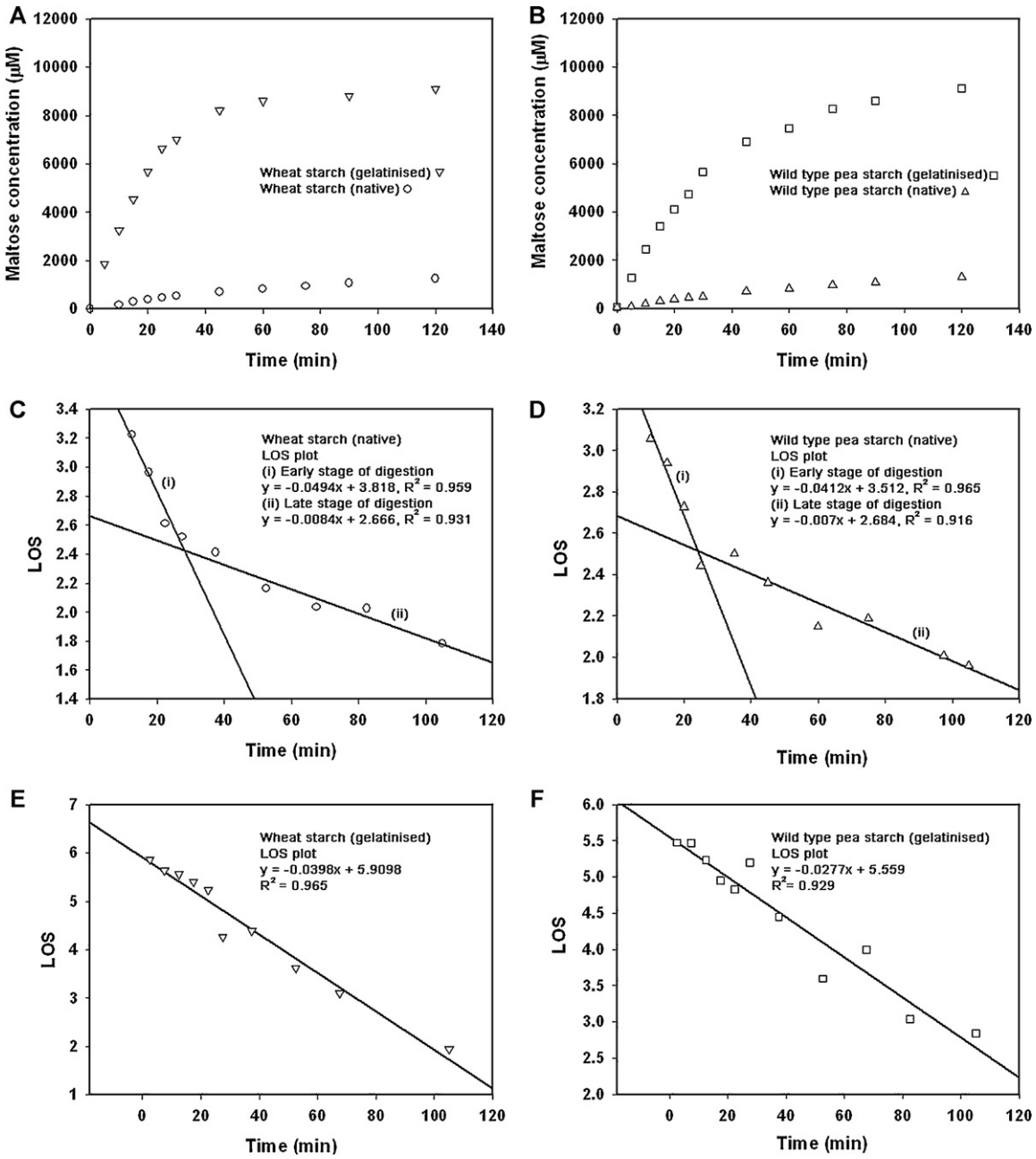

Fig. 3. Digestibility plots for native and gelatinised wheat and pea starches. (A) Digestibility curves of native and gelatinised wheat starch; (B) digestibility curves for native and gelatinised pea starch; (C) LOS plot of digestion of native wheat starch; (D) LOS plot of digestion of native pea starch; (E) LOS plot of digestion of gelatinised wheat starch; (F) LOS plot of digestion of gelatinised pea starch. α-Amylase was present at a concentration of 4.5 nM in digestions of native starches and at 2.25 nM for digestion of gelatinised starches.

expected because the rate constant will be governed by the catalytic properties of the amylase itself, i.e., the catalytic rate constant. The catalytic rate constant (k_{cat}) equals V_{max}/[amylase] (Roder et al., 2009; Slaughter et al., 2001).

The results for the data taken from Goni et al. (1997) shown in Table 1 indicate that all the digestibility rate constants are indeed very similar. It has been proposed that a low k value reflects the slow diffusion of amylase into a starch granule as digestion proceeds (Dhital et al., 2010). Thus, differences in the rate at which particular starches are digested depend mainly on C_∞, i.e., the total amount of available/digestible starch. We have argued that this conclusion can be reached from Michaelis–Menten kinetic studies of initial reaction rates because changes in measured K_m values as a starch sample is processed, reflect the change in the quantity of digestible starch available (Butterworth et al., 2011; Roder et al., 2009). A lower K_m is a signal of greater availability.

LOS plots of our data obtained for native wheat starch and pea starches revealed a discontinuity (Fig. 3) and therefore provided evidence of a fraction that is digested more rapidly than the remainder of the polysaccharide. The plots are based on the results of 2–3 separate experiments each carried out in duplicate. The material that is digested more rapidly is assumed to consist of α-glucan chains located at regions which are exposed to the bulk solution and so are readily available for attack by amylase (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). The discontinuity in the LOS plots occurred at 20–30 min of incubation which was very much earlier than in the results shown by Evans and Thompson (2008) where we believe the results are complicated by effects arising from

substrate exhaustion. LOS plots for the digestibility of gelatinised wheat and pea starches reveal that the whole digestion process can be well described for each starch by a single digestibility constant (k value). The digestibility constant for pea was a little lower than that for wheat (0.028 and 0.04 min^{-1} respectively). Both were obtained using an α-amylase concentration of 2.5 nM.

It has been mentioned above that the various food samples studied by Goni et al. (1997) are associated with a single digestibility constant and so terms such as RDS and SDS are not particularly useful for describing the digestibility behaviour. With the exceptions of bread and potato crisps, the samples studied by Goni et al. (1997) were boiled before use and so the starch would be expected to be in a readily digestible form. Starch consumed by humans however, can be present in a wide range of physical forms and crystalline material can be present even in processed foods; birefringent starch granules in a food source were observed in a study published more than 30 years ago (Varriano-Marston, Ke, Huang, & Ponte, 1980). Although it is possible therefore, that some RDS and SDS could co-exist in certain foods, the SDS fraction is likely to be minimal in most cooked foods and is unlikely to have a significant impact on the overall digestibility behaviour. If the difference in k is significant, some remaining structural differences in the starches following the hydrothermal treatments could be the main cause of the dissimilarity.

Our results for native wheat and pea starches were obtained using an amylase concentration that was twice that present in the experiments conducted with the gelatinised samples and this has to be borne in mind when comparing the observed digestibility constants. Allowing for the use of the doubled enzyme concentration for the native starch studies, the k value for the 'rapid' wheat fraction (0.049 min^{-1}) becomes 0.025 min^{-1} and therefore lower than the k value of 0.04 min^{-1} obtained for gelatinised. The disparity in the pea starch results for the rapid phase and the gelatinised is less marked when account is taken of the doubled enzyme concentration used for the digestion of native starch. Although this 'rapid' fraction in native starch represents material that is more available to the enzyme than the rest of the starch, its digestibility may still be slower than gelatinised material. Nevertheless, calculation of C_∞ values show that the percentage of freely digestible, available, starch was increased by at least 10-fold following gelatinisation of wheat and pea starches. From changes in the K_m values obtained from Michaelis–Menten fitting of initial rate data for amylolysis of starches, a 14-fold increase in starch availability was estimated for wheat (Tahir, 2009; Tahir et al., 2010). Thus, Michaelis–Menten kinetics applied to initial rates of amylolysis can provide useful estimates of the relative change in available starch brought about by gelatinisation.

Fitting the published data of Mahasukhonthachat et al. (2010) was not successful. Guggenheim and LOS plots were curved rather than linear (data not shown). Close inspection of the digestibility curves presented in the original publication reveals that they diverged very little from linearity with time, i.e., they were not logarithmic. The curved LOS plots proved useful in identifying the non-logarithmic nature of the plots and justified the claim of Poulsen et al. (2003) who introduced the plots to biologists.

4. Conclusions

Guggenheim and LOS plots can be used with advantage for analysis of starch digestibility curves. They call for accurate estimates of product formation (or substrate disappearance) with time, but obviate the requirement of reliable estimates of the product concentration when reactions have reached an end point. Determination of end-point concentrations will nearly always pose difficulties. Text books recommend that for best accuracy in exploiting the Guggenheim method, the constant time increment (Δt) should be at least twice the half-time of reaction (Frost & Pearson, 1961). With a pseudo-first order rate constant (k) of 0.03–0.04 min^{-1} (see Table 2), the half-time is approximately 17–23 min and so the recommended Δt needs to be >30 min. This is a relatively long time and so ideally the concentration of amylase in the digestion mixture should be increased in order to increase k, and thus decrease $t_{0.5}$, accordingly. On the other hand, if the chosen amylase concentration is too great, the digestibility curves may be approaching the end point within the time of the first measurements. The literature contains examples of figures showing data that have almost reached an end point by the time that the first samples have been taken for measurements of digested starch (e.g., Frei, Siddhuraju, & Becker, 2003). Such plots are ill-conditioned and will not provide reliable estimates of the kinetic parameters.

In fitting the data presented in Figs. 1 and 2, Δt was 30 min and thus at the limit of the recommended range. The good agreement between the rate constants obtained by the Guggenheim plots and the published values suggest that the recommended time for Δt can be shorter than two half lives and still produce reasonably reliable estimates of rate constants. The LOS plot has the added advantages that it is not subject to the same constraints on Δt and also, the end point (C_∞) can be readily obtained from the intercept on the vertical axis of the plots. The chosen time points for slope measurement need however, to be taken from curved regions of the digestibility plot i.e., where the reaction is exponential rather than linear. The fewer restrictions on time intervals and the property of generating values for C_∞ render LOS plots particularly valuable. The agreement between the values presented in the original paper and by our replots is remarkably good given that in our examples taken for fitting by Guggenheim and LOS plots, the concentrations of starch hydrolysed shown in the Goni et al. (1997) paper were estimated by ruler from an enlarged version of the published figure.

In our previous studies, we have mainly concentrated on measuring initial rates of reducing sugar released from starch during incubation with relatively low concentrations of amylase. The data have then been fitted to the Michaelis–Menten equation for calculation of kinetic parameters (Roder et al., 2009; Slaughter et al., 2001; Tahir et al., 2011, 2010). Alterations in the kinetic constants as starch is subjected to various hydrothermal treatments can provide sensitive probes of changes in structure that accompany the treatments. Also, comparisons of kinetic parameters obtained for starches from different botanical sources are useful for predicting the relative speeds at which starch is hydrolysed in the early stages of digestion. This information is of value for health and dietary reasons, since large early peaks in blood glucose and insulin concentrations following starch ingestion seem to result in unfavourable outcomes in the long term (Jenkins et al., 2002; Judd & Ellis, 2006; Mann, 2007; Seal et al., 2003). Initial reaction rates allow K_m values to be calculated and thus provide an indication of the amount of available/readily digestible starch. Similarly, C_∞ values calculated from LOS plots point to the same information. The concentration of available starch in a food governs the rate of digestion and this rate is likely to be reflected in the early blood glucose response. Our results obtained with gelatinised starches reveal that approximately 33% and 25% of the total available starch in the wheat and pea samples respectively, are digested within the initial 10 min of reaction (Fig. 3A and B). The C_∞ values for the more rapid digestion phase of the native starches were very low and therefore predict that their digestion would have little impact on blood glucose concentrations in the early postprandial stage following ingestion. We believe that Michaelis–Menten kinetics and logarithmic digestibility curves can complement each other. Whether digestibility studies have any particular advantages over initial reaction rate studies is a matter for debate, especially when

no real evidence exists for the presence of different fractions of starch, i.e., RDS and SDS, within a particular starch source.

The active site of amylase accommodates five glucoside residues (Butterworth et al., 2011; Robyt, 2008). If it is assumed that all the starch can be represented by units of maltopentaose (molecular weight 828), a starch concentration of 5 mg/ml is equivalent to 6 mM maltopentaose. It then becomes possible to make some comparison of results from digestibility data and Michaelis–Menten kinetics if it is assumed that maltose is the sole product (for calculating k_{cat} values) and that the total starch concentration and K_m can be expressed in terms of maltopentaose molarity. Using the Michaelis–Menten equation:

$$v = \frac{k_{cat}E_0S}{(K_m + S)} \quad (9)$$

Values for k_{cat} and K_m for a gelatinised wheat starch sample are respectively, $0.96 \times 10^5\ min^{-1}$ and 0.6 mg/ml. The equivalent values for gelatinised pea starch are $1.18 \times 10^5\ min^{-1}$ and 1.0 mg/ml (Tahir, 2009). Since the rate of digestion can also be written in the form of $v = kS$, then:

$$k = \frac{k_{cat}E_0}{K_m + S} \quad (10)$$

Under the conditions of the digestibility experiment, $(K_m + S)$ for gelatinised wheat equates to 5.6 mg/ml starch, i.e., 6.725 mM maltopentaose and E_0 was 2.25 nM. Using these values for the various parameters generates a k value of $0.032\ min^{-1}$. This agrees remarkably well with the value obtained from digestibility curves given the broad assumptions that all of the starch and K_m are represented in terms of maltopentaose concentration and that the product detected in an assay of reducing sugar is assumed to be maltose. Performing a similar calculation for gelatinised pea starch produces a value of $0.035\ min^{-1}$. These estimates are essentially identical and close to the experimentally determined values for the wheat and pea starches shown in Table 3. The close similarity of the experimental and calculated values is interesting, but it is necessary to remember that the calculations depend on speculative assumptions about the 'real' concentration of substrate.

Digestibility studies are likely to continue to be popular because of the belief that they more accurately replicate *in vitro*, starch digestion that *in vivo* can take several hours to complete. Hydrolysis index (HI) values for comparison amongst different starches can also be useful and 1st order fitting to digestibility curves is a suitable way of obtaining HI. When comparing various k and/or $t_{0.5}$ values obtained from digestion of different starch substrates, it is important to allow for differences in the activity/concentration of α-amylase used in the digests. Comparing the results obtained from different research groups can be difficult because of uncertainties in the amount of enzyme added to digests (see Butterworth et al., 2011). Nevertheless, we hope that this present contribution to analysis of digestibility will be of assistance to many workers in the starch area.

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