Strategies for the Removal of Raffinose Family Oligosaccharides from Navy Bean Flour

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Abstract

Navy beans are legumes with highly nutritious qualities. However they are underutilised in the processed food industry due to the undesirable bloating, abdominal discomfort and excessive flatulence associated with ingestion of the raffinose family oligosaccharides (RFOs) present in them. If a suitable technology were available to sufficiently reduce the concentration of RFOs then navy bean flour could find application in many food production processes.

The traditional method for decreasing RFO content in navy beans and other similar legumes has been through soaking in large quantities of water, thereby leaching the RFOs from the bean. However this is a slow process and results in the depletion of all water soluble solids (up to 25% of the dry weight of the bean).

The leaching process can be sped up dramatically through the decrease in particle size achieved by milling the beans into navy bean flour prior to the addition of water. However this process makes dewatering of the flour difficult.

Rates of moisture uptake and RFO leaching were characterised for navy bean flour and de-hulled navy bean cotyledons demonstrating that RFO leaching is slower but of a similar magnitude as moisture uptake and that these rates are dramatically increased with decrease in particle size.

The addition of α-galactosidase to the leaching water enables the rapid removal of RFOs from navy bean flour without the need to separate the flour from the leaching water because the RFOs leached out of the flour are hydrolysed into simple sugars.

Galactose is a product of the hydrolysis of RFOs and its presence at high concentrations can result in the inhibition of α-galactosidase. However at the concentrations likely to be experienced during RFO reduction in bean flour the inhibition effect is minimal. Rates of hydrolysis were studied for raffinose and stachyose hydrolysis by α-galactosidase in the concentration range expected during processing of navy bean flour.

Enzymatic processing using very small amounts of moisture addition was investigated demonstrating partial RFO removal at moisture contents as low as 28% (wet basis). Reduction in RFO content to a level at which the flatus response is negligible can be achieved at moisture contents as low as 38% (wet basis) which significantly minimises the drying required to produce a dry navy bean ingredient or could allow direct application in an extrusion process.

The application of α-galactosidase at low moisture content is a novel process for the depletion of RFOs in navy bean flour. The mechanism for this process is explained by preliminary modelling of moisture and RFO diffusion which demonstrates how partial RFO removal is possible.

From these mechanisms a series of commercially practical methods for RFO depletion of navy bean flour were explored for several targeted product applications. This process also has potential applications for low moisture enzyme processing in a range of food and other biological systems.

The simple processes developed in this work open up the commercial use of navy bean flour for the food industry to develop products that take advantage of its functional and nutritional properties without the negative nutritional problems usually associated with this material.

Acknowledgements

A PhD is very much like a marathon, it requires a large amount of effort, perseverance and in the end a dogged determination to see it through. I could not have made it through without the help of a number of people.

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Nomenclature

a	Characteristic length for diffusion	(m)
A	Substrate for enzyme reaction	-
$[A]$	Concentration of substrate A	(mM)
a_W	Water Activity	-
C_{bf}	Maximum concentration of bean flour	(g/g)
C_{RFO}	RFO concentration	(g/g)
C_{RFOp}	Maximum concentration of RFOs in the product	(g/g)
$C_{RFO\text{-}residue}$	RFO content in the residue	(g/g)
$C_{SS\text{-}flour}$	Soluble solids content of navy bean flour	(g/g)
C_{St}	Concentration of stachyose in bean flour	(g/g)
C_w	Moisture content	(g/g)
C_{wC}	Critical moisture content for diffusion	(g/g)
C_{wf}	Final moisture content of particle	(g/g)
C_{wH2O}	Moisture Content of enzyme solution	(g/g)
$C_{wi\text{-}Cotyledon}$	Initial moisture content of navy bean cotyledons	(g/g)
$C_{wi\text{-}flour}$	Initial moisture content of navy bean flour	(g/g)
$C_{wS\text{-}Cotyledon}$	Saturated moisture content of navy bean cotyledons	(g/g)
$C_{wS\text{-}flour}$	Saturated moisture content of navy bean flour	(g/g)
C_{wt}	Moisture content at time t	(g/g)
D_0	Moisture diffusivity at infinite temperature	(m^2/s)
D_{RFO}	Effective diffusivity of RFOs	(m^2/s)
D_W	Effective moisture diffusion coefficient	(m^2/s)
E	Enzyme	-
E_A	Activation Energy	(kJ/mol)
EA	Enzyme-substrate complex	-
EGa	Enzyme-Galactose substrate	-
EP	Enzyme-Product substrate	-
ERf	Enzyme-Raffinose complex	-
ESt	Enzyme-Stachyose complex	-
E_x	Intersection of N_y versus y with the tie line from leaching stage x	-
Ga	Galactose	-
$[Ga]$	Concentration of galactose	(mM)
K_{Ga}	Inhibition constant for galactose	(mM)
K_M	Michaelis Menten constant for an enzyme reaction	(mM)
K_{MRf}	Michaelis Menten constant for raffinose	(mM)
K_{MSt}	Michaelis Menten constant for stachyose	(mM)
K_P	Inhibition constant for product inhibition	(mM)
m_{flour}	Mass of navy bean flour	(g)
m_{H2Ox}	Mass of water added for leaching stage x	(g)
m_{IS}	Mass of insoluble solids in	(g)
MR	Moisture ratio	-
MR_{Calc}	Calculated moisture ratio	-
$m_{residue}$	Mass of the residue	(g)
MR_{expt}	Measured moisture ratio	-
m_{RFOi}	Initial mass of RFO	(g)
m_{RFOt}	Mass of RFO remaining at time t	(g)
$m_{RFO\infty}$	Mass of RFO remaining when leaching process reaches completion	(g)
$m_{starch\text{-}water}$	Mass of water held in starch granules within the flour	(g)

$m_{supernatant}$	Mass of the supernatant	(g/g)
m_w	Mass of enzyme solution surrounding a particle	(g)
m_{wi}	Initial mass of enzyme solution surrounding a particle	(g)
M_x	Mixture point for leaching stage x	-
n	Number of terms in the summation	-
N_M	Concentration of insoluble solids in the leaching slurry (insoluble free basis)	(g/g)
N_{Mx}	Concentration of insoluble solids in the leaching slurry for stage x (insoluble basis)	(g/g)
N_x	Concentration of insoluble solids in the supernatant (insoluble free basis)	(g/g)
N_y	Concentration of insoluble solids in the residue (insoluble free basis)	(g/g)
P	Product of enzyme reaction	-
$[P]$	Concentration of product P	(mM)
r	Distance from centre of particle	(m)
R	Ideal Gas Constant	(J/mol.K)
R_0	Initial concentration of insoluble solids versus initial concentration of RFO in the leaching water (insoluble free basis)	-
Rf	Raffinose	-
$[Rf]$	Concentration of raffinose	(mM)
ρ	Density of navy bean particles	(kg/m^3)
R_p	Radius of a particle	(m)
R_x	Intersection of N_x versus x with the tie line from leaching stage x	-
St	Stachyose	-
$[St]$	Concentration of stachyose	(mM)
Su	Sucrose	-
t	time	(s)
T	Temperature	(K)
v	Rate of enzyme reaction	$\left(\frac{mol}{mg.min}\right)$
v_{iRf}	Initial rate of stachyose hydrolysis	$\left(\frac{mol}{mg.min}\right)$
v_{iSt}	Initial rate of stachyose hydrolysis	$\left(\frac{mol}{mg.min}\right)$
V_{max}	Maximum rate of enzyme reaction	$\left(\frac{mol}{mg.min}\right)$
V_{max0}	Maximum reaction rate at infinite temperature	$\left(\frac{mol}{mg.min}\right)$
V_{maxRf}	Maximum rate of raffinose hydrolysis	$\left(\frac{mol}{mg.min}\right)$
V_{maxSt}	Maximum rate of stachyose hydrolysis	$\left(\frac{mol}{mg.min}\right)$
V_p	Volume of a particle	(m^3)
V_{Shell}	Volume of hydrated shell	(m^3)
x	Concentration of RFO in the supernatant (insoluble free basis)	(g/g)
X	Proportion of RFOs removed	(g/g)
y	Concentration of RFO in the residue (insoluble free basis)	(g/g)
y_M	Concentration of RFO in the leaching slurry (insoluble free basis)	(g/g)

Chapter 1

Introduction

Navy beans are a type of common bean that is pea-sized and creamy white in colour, mild in flavour and dense and smooth in texture. Navy beans are grown extensively in China, the US and Canada but despite many positive nutritional characteristics they are under utilised in many processed food applications.

Navy beans contain high levels of carbohydrate, protein and dietary fibre and are an excellent source of iron, potassium, selenium, thiamine, vitamin B6, and folic acid (Barampama & Simard 1994, Chau et al 1998, Daveby & Aman 1993, Guillon & Champ 2002, Gujska & Khan 1990, Lee et al 1983).

The protein present in navy beans is gluten free making them appealing to sufferers of celiac disease. Also, when they are eaten in combination with grain cereal proteins such as rice, they provide an inexpensive and non carnivorous source of "whole protein" (Joseph & Swanson 1993). Navy beans have a low glycaemic index (GI) due to their dietary fibre content (Guillon & Champ 2002). Navy beans also contain high levels of saponin which has been shown to exhibit antibacterial and antifungal properties and to lower cholesterol levels and inhibit cancer cell growth (Wang et al2009).

Raffinose family oligosaccharides (RFOs) are complex sugars containing chains of α-galactose which are unable to be digested in the human upper intestine due to the absence of α-galactosidase, the enzyme required to break the links in the α-galactose chains. The RFOs therefore survive through to the lower intestine where they encounter bacteria which use them as the substrate for fermentation. This produces methane, carbon dioxide and hydrogen gas, which can cause bloating, abdominal discomfort and excessive flatulence (Akinyele & Akinlosotu 1991, Barampama & Simard 1994, Guillon & Champ 2002, Sanchez-Mata, et al. 1998, Shimelis & Rakshit 2005). While research indicates that in populations with a steady diet of dry beans the microflora of the gut can adapt to cope with the digestion of RFOs their presence is a significant deterrent to populations that have not developed such coping mechanisms.

In particular the symptoms associated with consumption of dry beans render them untenable as an ingredient in baby food. Traditional cooking methods including soaking of navy beans for long periods of time prior to cooking have been shown to significantly decrease RFO content in the beans. There are also products available that contain enzymes designed to aid in digestion of common beans, however reliance on access to these types of products to facilitate consumption of navy bean products seems impractical.

The predominant application of navy beans currently in the processed food industry is as whole beans in products such as baked beans and ready to eat meals. Production methods for these types of products usually include an initial blanching step to hydrate the beans followed by a final cooking step usually carried out by retorting the final product in the sealed can.

Navy beans could also potentially be used to boost the nutritional content of many other types of processed food if they were added in the form of a flour or paste. A significant amount of navy beans are damaged as a result of mechanical navy bean harvesting, drying and transport methods. These “broken beans” represent an inexpensive source of raw material for the production of navy bean flour. The potential applications for navy bean flour include the replacement or fortification of wheat and maize flours as fillers and thickeners in products such as ready to eat meals, sauces and baby food. Recently a number of new bean based foods have been developed using extrusion cooking technology; for example extruded snacks and breakfast cereal products (Berrios 2010, Berrios et al 2010).

To date the application of milled navy bean flour as an ingredient in processed foods has not found wide spread use. A reason for this is that addition of navy bean flour directly into the formulation of a processed food necessitates the exclusion of the blanching step described above which is essential in depleting the RFO content of the beans via a leaching mechanism. While it is reasonable to assume that the cooking step in the production of any processed food should sufficiently reduce the levels of the other anti nutritional components of the bean flour through thermal degradation it is unlikely to have a significant effect on the RFO content.

It is therefore of interest to develop a process to remove or significantly reduce the concentration of these compounds in the beans or components thereof prior to use of navy

bean flour as an ingredient. The target raw material for navy bean flour is broken beans which are available at a cost of about $0.69 per kilogram and so any process developed should be able to utilise this cheap material and should aim keep processing costs reasonable with respect to the cost of the ingredient.

The objectives of the current work were to;

- To investigate the mechanisms by which the flatulence causing effect of RFOs in navy beans can be neutralised.
- To determine which of these mechanisms is the most effective for RFO reduction in navy bean flour produced from broken beans.
- To define a hierarchy of processing methods for inclusion of navy bean flour into food formulations such that RFO levels are reduced to acceptable levels.

Chapter 2

Literature Review

In order to gain an understanding of the various disciplines required to achieve the objectives outlined in chapter 1 a review of the relevant literature on the following subject matter has been undertaken:

- The nutritional benefits of consumption of legume seeds focussing on common beans and on navy beans in particular.
- The occurrence of anti-nutritional factors in common beans (focussing on navy beans) and the strategies and techniques currently employed to mitigate them.
- Raffinose family oligosaccharides (RFOs) and their role in causing flatulence.
- Methods investigated for the removal and/or reduction of RFOs in legumes and legume flours.

2.1 The Navy Bean (*Phaseolus vulgaris*)

The navy bean is a grain legume with significant commercial importance. Navy beans are small, pea-sized beans that are creamy white in colour, mild in flavour and dense and smooth in texture. The navy bean got its current popular name because it was a staple food of the United States Navy in the early 20th century.

Navy beans (also called pea beans, white beans or haricot beans) belong to a group of pulse legumes called “the common bean” with the scientific name *Phaseolus vulgaris*. Other types of dry beans that also belong to this group include: kidney beans, pinto beans, black turtle beans, field beans, black beans, borlotti beans, pink beans and yellow beans. Among legumes for human food, dry beans are the most consumed as whole beans (Barampama and Simard 1994).

Aside from soybeans, *Phaseolus vulgaris* are the most important legumes worldwide with respect to economic value (Berrios, 2010). *Phaseolus vulgaris* seeds are of particular interest to the food production industry because they represent a low cost high nutrition food source that is currently under utilised in many areas of processed food production.

Archaeological, botanical and historical data suggest that the origin of all domesticated varieties of the common bean can be traced to wild common ancestors in Central and South America (Gepts and Debouck 1991) and it is from the sharing of this common ancestor that the common bean derives its name. Cultivated by the native inhabitants of the area, common beans were dispersed throughout South and Central America by migratory trades. Common beans were introduced into Europe and subsequently to Africa and Asia in the 15th century by Spanish explorers returning from their voyages to the New World. Due to the common beans ability to thrive under a wide range of climatic conditions they have come to represent a relatively inexpensive source of protein and have therefore become popular in many cultures throughout the world (Gepts and Debouck 1991). Today, the largest commercial producers of dried common beans, including the navy bean, are India, China, Indonesia, Brazil and the United States.

Common bean seeds are exalbuminous dicotyledons, meaning that they contain two of the structures called cotyledons and consume the endosperm during seed maturation. Figure 2.1 illustrates the general structure of a bean seed.

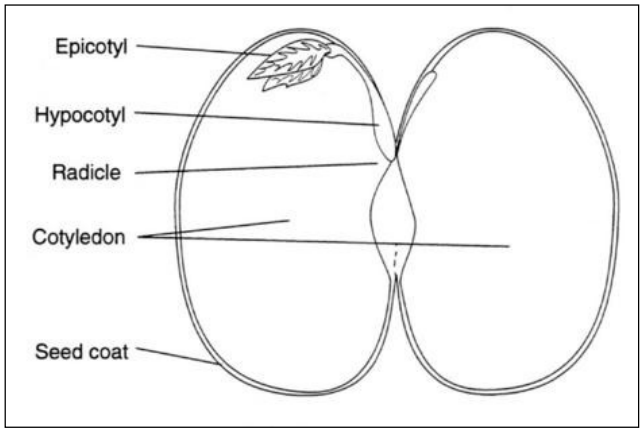


Figure 2.1 General Structure of a Common Bean Seed (sourced from http://bio-ditrl.sunsite.ualberta.ca/detail/?P_MNO=5745)

The various parts of the bean seed labelled in figure 2.1 are:

Epicotyl: The epicotyl forms the first leaves of a new bean plant growing from a seed.

Hypocotyl: The hypocotyl helps form the stem of a new bean plant, and will eventually pull the seed out of the ground.

Radicle: The radicle forms the roots of a new bean plant.

Cotyledon: The cotyledons are large and waxy because they supply the embryo with nutrients and energy until the true leaves (formed from the epicotyl) start photosynthesising and the roots (formed from the radicle) start absorbing soil nutrients. Cotyledons in beans contain a large amount of starch which is the primary form of energy storage in legume seeds. The cotyledons account for the vast majority of the mass of dry bean seeds.

Seed Coat: Also called the 'testa' the seed coat is the seeds outer layer; it is commonly composed of cuticle, malphigian layer and parenchyma cells. The seed coat is only semi-permeable to water and contributes to the slow rate of water uptake of dry beans during soaking.

2.2 Microstructure of Navy Bean Seeds

Figure 2.2(a) is a scanning electron microscopy (SEM) image of a cut section of a raw navy bean cotyledon. The image illustrates the cotyledon cells containing starch granules which are embedded in and surrounded by a thick proteinaceous matrix derived from the cell contents (Berg et al 2012).

Figure 2.2(b) is a SEM image of milled navy bean flour and shows that a significant number of starch granules are freed from disrupted cotyledon cells during the milling process. Despite the inference from the image in figure 2.2(b) the starch content of the navy bean flour was measured to be 36.1% (Berg et al 2012).

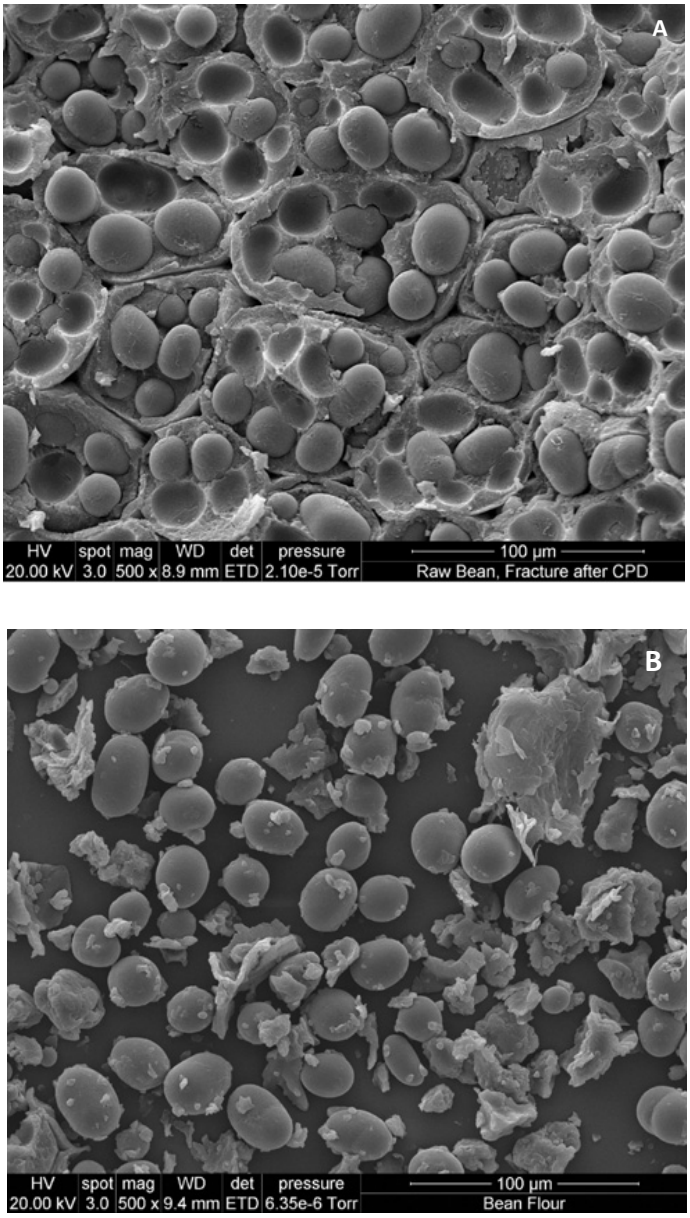


Figure 2.2 **Scanning electron microscope images of A: navy bean cotyledon cells and B: navy bean flour (images sourced from Berg et al 2012).**

2.3 Nutritional Benefits of Legume Seeds

The composition of navy bean seeds varies with cultivar as well as environmental conditions during growth. In general they are composed of differing levels of protein, starch, fibre, sugars, moisture and fat as well as a number of vitamins and minerals. There have been many compositional studies on legume seeds; Table 2.1 summarises the compositions of a number of legume seeds.

Table 2.1 Compositional data for legumes reported in the literature.

Legume	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Starch (%)	Glucose (%)	Sucrose (%)	Verbascose (%)	Raffinose (%)	Stachyose (%)	Reference
Navy bean	8.6-10.6	1.67-4.93	23.1-25.94	3.45-5.25	30.4-60.46	0.04-1.8	2.23-3.47	0.13-0.50	0.35-0.67	2.1-3.53	Boloorforoos-han & Markakis 1979; Fleming 1981; Kereliuk & Kozub 1995; Lee *et.al.* 1983; Naivikul & D'Appolonia 1978; Snauwaert & Markakis 1976; Srisuma *et.al.* 1994..
Haricot bean		0.9	25.1	4.04	34.9	0.07	1.727-3.70		0.235-0.443	1.238-1.841	Bravo *et.al.* 1999; Shimelis & Rakshit 2005.
White bean	9.65	0.25	25.90		57.80		1.448-2.009		0.270-0.513	1.650-2.491	Sanchez-Mata *et.al.* 1998.; Sathe & Salunkhe 1981(c).
Great Northern bean	9.2	1.5-1.7	24.0-24.5	3.7-4.0	40.3-44.0		2.02-3.775	ND	0.56-0.667	2.40-3.75	Iyer *et.al.* 1980; Sathe & Salunkhe 1981(b); Sosulski *et.al.* 1982; Sosulski & Youngs 1979.
Common bean	13.7	1.34	23.1		36.6						Hermida & Rodriguez 2006.
Pinto bean	10.8	3.60	20.2	3.07	56.5	0.09	2.19-3.487	ND-0.15	0.248-0.63	1.960-2.97	Iyer *et.al.* 1980; Naivikul & D'Appolonia 1978; Sanchez-Mata *et.al.* 1998.
Kidney bean							1.92	0.06	0.93	2.44	Iyer *et.al.* 1980.
Red Kidney bean	11.3	1.27	23.94	3.63	32.8-46.95		1.64-2.15	Trace-0.40	0.31-0.38	0.40-4.0	Fleming 1981; Kuo *et.al.* 1988; Labaneiah & Luh 1981.
Lima bean	8.4-10.2	0.9-1.32	20.35-24.65	3.8-4.73	45.5-50.0		1.68-3.60	Trace-0.12	0.69-1.11	1.87-3.03	Kuo *et.al.* 1988; Revilleza *et.al.* 1990; Sosulski *et.al.* 1982; Sosulski & Youngs 1979.
Red Lima bean							0.806	0.246	0.297	2.829	Oboh *et.al.* 2000
White Lima bean							0.770	0.194	0.277	3.157	Oboh *et.al.* 2000
Mung bean	9.0-9.1	0.8-2.58	23.7-26.5	2.1-4.30	40.4-56.8	0.05-0.1	1.28-1.8	1.73-2.77	0.3-0.37	1.5-1.67	Aman 1979; Fleming 1981; Naivikul & D'Appolonia 1978; Sosulski *et.al.* 1982; Sosulski & Youngs 1979.
Green Mung bean	15.2	1.3	23.0	3.4							Watson 1977

Legume	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Starch (%)	Glucose (%)	Sucrose (%)	Verbascose (%)	Raffinose (%)	Stachyose (%)	Reference
Faba bean	8.6-10.3	1.3-2.98	27.9-31.35	2.25-4.40	41.2-52.7	0.34	1.55	1.94	0.24	0.80	Bramsnaes & Olsen 1979; Morad *et.al.* 1980; Naivikul & D'Appolonia 1978; Sosulski *et.al.* 1982; Sosulski & Youngs 1979.
Winged bean	5.4-12.58	15.8-18	27.7-37.5	3.6-4.9	36.2	1.17	0.26	0.04	0.18	0.13	Ekpenyong & Borchers 1980; Okezie & Martin 1980; Rockland *et.al.* 1979; Sajjan & Wankhede 1981; Watson 1977
Broad bean		1.93	30.7-35.4	3.1.3.23	40.0-46.6		1.67-2.7	1.9	0.1	0.48-0.5	Colonna *et.al.* 1980; Daveby and Aman 1993
Garbanzo bean		5.63	21.38	2.95	37.2			0.43	0.67	2.16	Fleming 1981
Hyacinth bean	6.78-7.10	1.70-2.06	21.46-23.24	4.05-4.10			1.62-1.79	0.36-0.44	1.13-1.20	2.07-2.12	Revilleza *et.al.* 1990
Pole bean							2.67	Trace	0.43	2.62	Kuo *et.al.* 1988
Moth bean		0.69	25.3	3.51	39.5	0.80	1.52		5.07		Bravo *et.al.* 1999
Brown bean		1.65	21.8	2.90	44.8		2.35	0.23		2.35	Daveby and Aman 1993
Green bean							1.94-2.90	Trace	0.22-0.25	2.81-3.43	Kuo *et.al.* 1988
Pink bean							1.75		0.20	0.22	Silva & Luh 1979
Gloria pink bean	10.4				42.31		1.473		0.435	0.390	Labaneiah & Luh 1981
Black eye bean	9.9				41.18		2.594-3.00		0.410-0.95	0.420-0.93	Labaneiah & Luh 1981; Silva & Luh 1979.
Pea bean	6.4	1.4	33.4	4.0	36.9						Sosulski *et.al.* 1982
Yam bean	11.2	0.5	19.1	2.4							Watson 1977
African Yam bean							1.090	0.317	0.664	2.863	Oboh *et.al.* 2000
Rice bean	8.97	3.46	16.82	4.31			1.86	0.74	1.14	0.37	Revilleza *et.al.* 1990
Swordbean	8.76	2.51	28.05	4.70			2.57	Trace	0.91	1.44	Revilleza *et.al.* 1990
Jackbean	8.4-8.56	2.46-2.66	27.82-29.41	3.95-4.59			1.443-2.47	0.01-0.248	0.284-0.86	1.44-2.298	Oboh *et.al.* 2000; Revilleza *et.al.* 1990.
Soybean	8.8	0.6-24.9	39.1-55.5	4.52-7.0	0.68-4.7		4.1-5.4	0.53	0.2-2.38	2.6-3.1	Daveby and Aman 1993; Hymowitz *et.al.* 1972; Sosulski *et.al.* 1982; Sosulski & Youngs 1979.

Legume	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Starch (%)	Glucose (%)	Sucrose (%)	Verbascose (%)	Raffinose (%)	Stachyose (%)	Reference
P.angularis	12.3		25.2	3.20							Chau *et.al.* 1998
P.calcaratus	11.7		26.5	3.94							Chau *et.al.* 1998
D.lablab	11.4		24.9	3.76							Chau *et.al.* 1998
Sam-sampling	6.20	2.20	30.12	6.41			4.34	0.26	2.11	2.42	Revilleza *et.al.* 1990
Sabawel	7.40-7.76	2.48-2.67	29.36-29.42	4.00-4.70			2.37-2.60	1.20-1.22	1.12-1.40	0.97-1.02	Revilleza *et.al.* 1990
Phaseolus mungo		1.4	24.3								Patwardhan 1962
Phaseolus aureus		1.2	24.6								Patwardhan 1962
Lentil	7.8-10.5	1.1-2.82	21.0-27.81	2.54-3.2	42.5-59.1	Trace-0.07	0.78-2.50	1.20-3.10	0.39-1.03	1.639-2.60	Bhatty & Slinkard 1979; Goel & Verma. 1980; Iyengar & Kulkarni 1977; Morad *et.al.* 1980; Naivikul & D'Appolonia 1978; Sanchez-Mata *et.al.* 1998; Sosulski *et.al.* 1982; Sosulski & Youngs 1979.
Green lentil		1.25	18.75	3.59	41.0			0.70	0.60	1.70	Fleming 1981
Lupine	6.7	1.2	47.4	3.3							Sosulski *et.al.* 1982
Sweet light lupin		7.95	45.9	3.65	0.5		2.30	1.35		2.55	Daveby and Aman 1993
Horse Gram		0.58	24.9	3.31	36.0		1.21-2.6	3.4	0.7	1.9	Bravo *et.al.* 1999; Iyengar & Kulkarni 1977.
Black Gram		0.45	23.6	3.51	37.9		1.24-1.46	3.44	Trace-1.29	0.89-3.03	Bravo *et.al.* 1999; Goel & Verma. 1980; Reddy & Salunkhe 1980.
Green gram		0.71	24.5	3.45	38.3-39.9		1.16-1.97	3.46-3.50	1.10-2.60	1.26-2.83	Bravo *et.al.* 1999; Goel & Verma. 1980; Iyengar & Kulkarni 1977; Jaya & Venkataraman 1981.
Red gram							1.40	4.00	1.05	3.00	Iyengar & Kulkarni 1977
Bengal gram							2.30	4.50	1.10	2.10	Iyengar & Kulkarni 1977

Legume	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Starch (%)	Glucose (%)	Sucrose (%)	Verbascose (%)	Raffinose (%)	Stachyose (%)	Reference
Cowpea	9.6-15.2		21.2-28.1	3.3-4.7	40.6	0.2-0.55	0.8-2.2	0.9-3.6	0.7-2.4	2.7-3.56	Akinyele 1989; Akinyele & Akinlosotu 1991; Akpapunam & Markakis 1979; Longe 1980-81.
Yellow pea	7.9		27.88	3.04	55.8						Morad *et.al.* 1980
Swedish light pea		2.37	24.4	2.78	45.3		1.88	1.5		1.26	Daveby and Aman 1993
Swedish dark pea		2.12	27.4	2.88	43.0		1.95	1.95		1.33	Daveby and Aman 1993
Danish light pea		2.10	21.8	2.75	45.0		2.47	1.85		1.42	Daveby and Aman 1993
Brown Pigeon pea							1.186	1.067	0.423	0.857	Oboh *et.al.* 2000
Cream Pigeon pea							1.666	1.562	0.620		Oboh *et.al.* 2000
Dry pea						0.071	2.452		0.483	0.640	Sanchez-Mata *et.al.* 1998
Field pea	9.3	1.3	25.0-25.7	2.7-2.9	43.7-54.1						Bramsnaes & Olsen 1979; Sosulski *et.al.* 1982.
Wrinkled field pea		2.20	25.37	3.73	24.0			2.70	1.47	4.50	Fleming 1981
Smooth field pea		1.52	19.04	3.49	37.5			2.19	0.34	2.91	Fleming 1981
Wrinkled pea			26.5	3.1	36.6		4.2	4.0	1.2	5.5	Colonna *et.al.* 1980
Chickpea	9.1	0.7	20.7-23.9	2.1-2.8	34.1-54.9	0.065-0.1	1.092-4.3	Trace-3.80	0.569-1.44	0.743-2.8	Aman 1979; Jaya & Venkataraman 1981; Sanchez-Mata *et.al.* 1998; Sosulski *et.al.* 1982.
P.angularis hull	5.10		12.2	2.48	1.31		0.78		0.60	0.74	Chau *et.al.* 1998
P.calcaratus hull	7.40		10.8	2.63	2.50		0.83		0.60	0.99	Chau *et.al.* 1998
D.lablab hull	4.55		11.7	3.97	4.30		0.35		0.28	0.28	Chau *et.al.* 1998
P.angularis cotyledon	4.08		30.2	3.65	51.4		1.57		1.19	3.53	Chau *et.al.* 1998
P.calcaratus cotyledon	4.17		31.9	4.34	45.9		1.44		1.03	3.34	Chau *et.al.* 1998
D.lablab cotyledon	4.32		30.0	4.31	49.1		1.49		0.84	2.68	Chau *et.al.* 1998

Navy beans have been found to contain (wt%, dry basis) 21-26% protein, 30-60% starch, 0.04-2% glucose, 2-3.5% sucrose and 1.5-5% fat. Navy beans are usually dried after harvest to increase their shelf life and so the moisture content of the bean is dependent on the extent to which it has been dried, however this is usually around 10-15% (wt%).

Navy beans are also a good source of some essential vitamins and minerals including iron, potassium, selenium, thiamine, vitamin B6, and folic acid (Barampama & Simard 1994; Chau et al 1998; Daveby & Aman 1993; Guillon & Champ 2002; Gujska & Khan 1990; Lee et al 1983).

Researchers (Naivikul & D'Appolonia 1978) have found that navy beans have between 33% and 112% higher protein content than wheat flour which indicates that navy bean flour could be used in combination with wheat flour to produce a high protein composite flour.

Joseph and Swanson (1993) investigated the bioavailability of protein from diets consisting of common beans, rice, and a combination of beans and rice in populations of rats. The study demonstrated that blends of common beans and rice have improved protein quality compared to either individually and the authors attribute this to the superior amino acid makeup of complementary limiting amino acids from the cereal and legume mixtures. This type of study clearly demonstrates the potential of navy beans as an ingredient in food formulations if the problems with respect to RFOs can be negated.

2.4 Anti Nutritional Factors in Common Beans

Common beans have been found to contain various “anti nutritional” components. These components of common beans can be divided into two general types: those that can act to decrease the nutritional quality of the bean, and those that can impact detrimentally on the health of the people that consume them.

Anti nutritional components of the first type include trypsin inhibitors, tannins and phytic acid.

Trypsin inhibitors and tannins (when they form complexes with proteins) can decrease protein digestibility and phytic acid lowers the bioavailability of minerals (Wang et al 2009). Trypsin inhibitors and tannins have poor heat stability and can be broken down to negligible levels during most cooking processes.

While the reduction/removal or deactivation of these components may increase the nutritional benefit of the beans, their presence does not reduce the nutritional quality of the common bean by a significant margin.

Anti nutritional components of the second type include phytohemagglutinin and the raffinose family of oligosaccharides (RFOs).

Phytohemagglutinin is a lectin and can cause gastroenteritis, nausea and diarrhoea following consumption of common beans that are raw or have been incorrectly processed (Nasi et al 2009).

Incidences of food poisoning following ingestion of raw kidney beans have been reported and attributed to the action of the phytohemagglutinin in the beans (Nasi et al 2009).

While phytohemagglutinin may cause significant problems for products produced using raw kidney beans, the concentration of this compound in navy beans is significantly lower and therefore less likely to have any detrimental effect on consumers of similar products produced from raw navy beans. Furthermore it has been found that traditional cooking processes provide sufficient heat treatment to reduce these to safe levels for human consumption. Lectins are proteins and display poor heat stability meaning that any product containing raw navy beans that undergoes a significant heating step will also necessarily render any phytohemagglutinin harmless.

The raffinose family of oligosaccharides (RFO's) are complex tri-, quadra- and penta-saccharide sugars which have been identified as a major contributor of the flatulence causing effect of many legumes. These sugars consist of a linear chain of either 1 (raffinose), 2 (stachyose), or 3 (verbascose) galactose molecules attached to a sucrose molecule through (1-6) α-glucosidic linkages (see figure 2.2). As such RFOs are sometimes referred to as α-galactosides.

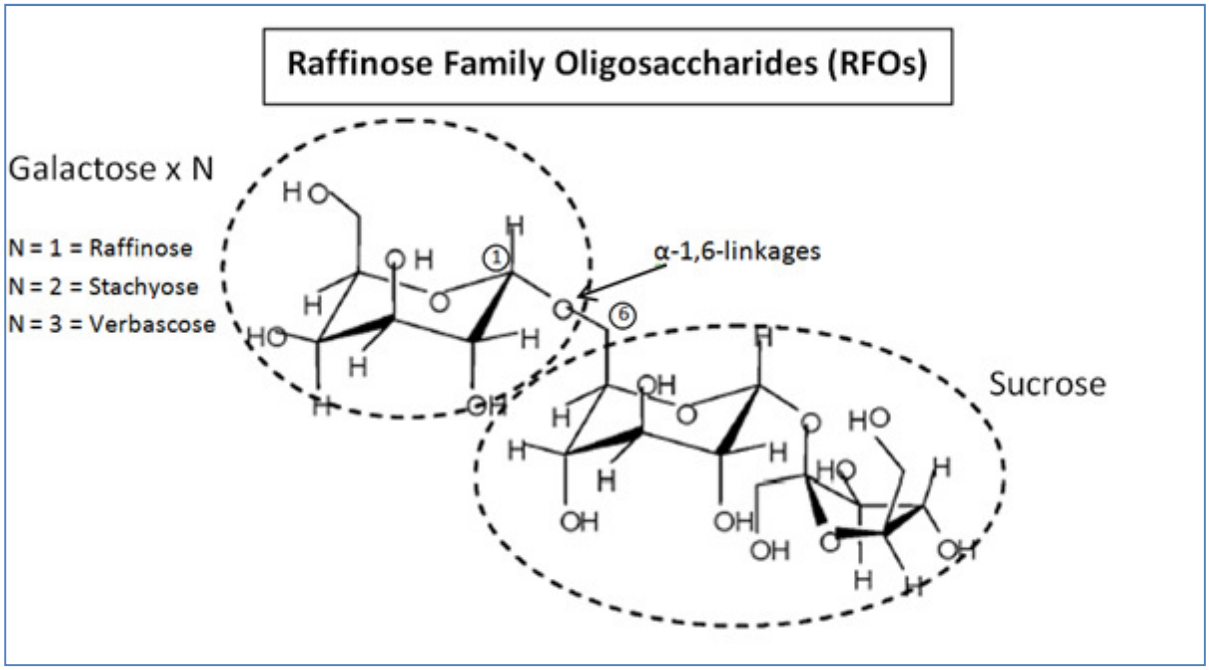


Figure 2.3 Molecular structure of Raffinose Family Oligosaccharides (RFOs) adapted from Martinez-Villaluenga and Vidal-Valverde (2008).

The concentration of RFOs in legumes varies with cultivar and environmental conditions during growth. Table 2.1 gives some concentrations of raffinose family oligosaccharides in legumes reported in the literature. They range from 0.35-0.67% raffinose, 2.1-3.53% stachyose and 0-0.50% verbascose in navy beans; compared to 0.1-2.60% raffinose, 0.13-5.5% stachyose and 0-4.50% verbascose for legumes in general (wt%, dry basis).

The raffinose and stachyose concentrations have been measured in the hull fraction of some legumes and range between 0.28-0.60% and 0.28-0.99% respectively compared to 0.84-1.19% and 2.68-3.53% respectively for the cotyledon fractions of the same legumes (wt%, dry basis). For more detailed oligosaccharide compositions refer to table 2.1.

2.5 Raffinose Family Oligosaccharides (RFOs) and Their Role in Causing Flatulence

RFOs are broadly distributed in higher plants (Dey 1985, French 1954, Kandler & Hopf 1982) and accumulate in leaves during photosynthesis (Senser & Kandler 1967) and in seeds during maturation (Amuti & Pollard 1977).

It is thought that the primary function of RFOs in higher plants is to store or transport carbohydrates (Dey 1985, Kandler & Hopf 1982) and may play a role in protection during seed desiccation and aging (Minorsky 2003). One mechanism by which di and oligo-saccharides may protect cells during desiccation is by glass formation (Ingram & Bartels 1996). During desiccation sugars present in the cytoplasm can form a glass structure which protects the cells against collapse. This suggests that in a dry bean cell the RFOs are present in the proteinaceous intracellular material illustrated in figure 2.2 in which the starch granules are embedded.

Table 2.1 contains sugar composition data from many legumes and demonstrates that RFOs are present in most legume seeds. Navy beans have been shown to contain stachyose and raffinose and possibly trace amounts of verbascose.

Consumers often draw a correlation between consumption of legumes and the likelihood of a subsequent episode of flatulence and/or intestinal discomfort. Symptoms can include excessive burping and flatulence, abdominal pain and bloating (Levitt 1980, Levitt 1985, Reddy et al 1984) and can be exacerbated in those suffering from pre-existing conditions like irritable bowel syndrome (Guillon & Champ 2002).

This public perception has been validated to some extent through a number of studies involving the in vivo testing of intestinal gas production of both humans and animals. An in-vivo bioassay method has been developed to measure the flatus in rats after feeding with various bean diets (Gumbmann & Williams 1971).

This method entailed the collection and quantification of hydrogen produced by bacterial fermentation in the intestines of rats over a 20 hour period following ingestion. The quantity of hydrogen produced was interpreted as an indication of the total flatus for that test diet. The results showed an increase in hydrogen production with increasing consumption of beans.

There have been many studies linking the presence of RFOs to the flatulence causing effect commonly caused by ingestion of many types of legumes (Granito et al 2001, Reddy et al 1984, Wagner et al 1976, Wagner et al 1977).

Humans are unable to digest RFO molecules because of the absence in human intestinal mucosa of appropriate enzyme activity to hydrolyse RFOs which are unable to pass through the intestinal wall whilst still intact (Cristofaro et al 1974, Reddy et al 1984).

Alpha-1,6-galactosidase is an enzyme which breaks the α-1,6 bonds between the galactose and sucrose molecules in RFOs. The absence of this enzyme leads to the oligosaccharides continuing through to the lower intestine undigested. Endogenous microflora in the lower intestinal tract metabolize the undigested oligosaccharides producing the hydrogen, carbon dioxide and methane that is responsible for flatulence (Guillon & Champ 2002, Minorsky 2003, Reddy et al 1984, Wagner et al 1976, Wagner et al 1977).

However, in some cases the removal of RFOs from beans has not completely eliminated their flatus causing effect. Reddy et al (1984) presents data showing a 64% reduction in flatulence activity over that of a basal diet caused by a bean diet following reduction of RFO content to 5-15% of the original. Wagner et al (1976) observed a 52% reduction in hydrogen production in rats over that of a basal diet caused by a bean following extraction of RFOs from the beans. It has been suggested that soluble fibre may also contribute to the flatus causing effect of beans (Granito et al 2001).

Fleming (1981) found a positive correlation between the flatus potential of a number of different legume seeds and the concentration of raffinose and stachyose present in the seed.

Suarez, *et al*. (1999) carried out a double-blind, randomised, crossover study to compare the intestinal gas production (measured as breath-hydrogen) and flatus frequency in healthy human subjects following ingestion of meals containing 80g of two types of soybeans. The first was a conventional soybean containing 3.33% stachyose and 0.51% raffinose; the second was a soybean that was naturally lower in RFO content with 0.46% stachyose and 0.16% raffinose.

Both types of soybean contain the same amount of crude fibre (0.19%) and similar levels of dietary fibre (10.87% and 10.50% respectively). White rice was used as a negative control because preliminary studies showed that ingestion of white rice resulted in minimal breath-hydrogen production.

The study demonstrated that ingestion of the conventional soybean produced markedly more (about 300%) intestinal gas than the low RFO soybean. Ingestion of the conventional soybean resulted in an increased flatus frequency compared to the low RFO soybean (about 190%).

Analysis of variance was carried out on the data and indicated that there was no significant difference between the amount of intestinal gas or the flatus frequency produced following ingestion of the low oligosaccharide soybean and the white rice control. This study therefore suggests that reducing the RFO levels to about 0.6% should be sufficient to reduce the flatus caused by ingesting RFOs in navy beans to the point where it is indistinguishable from the background level.

Low levels of non-digestible oligosaccharides (2g/day) may even have some benefits via their effect on the make-up of colonic bacterial populations (Tomomatsu 1994). Tomomatsu (1994) also reported that consumption of a carbonated drink containing raffinose and stachyose over a two week period did not produce an increase in the incidence of flatulence or diarrhea when compared with a baseline diet.

Non-digestible oligosaccarides have a prebiotic effect and promote the growth of Bifidobacterium and Lactobacillus in the lower intestine that inhibit pathogenic growth (Berrios et al. 2010, Martinez-Villaluenga et al. 2008). They also provide protective effects against colorectal cancer by inhibition of putrefactive (*Clostridium perfringens*) bacteria (Guillon & Champ 2002). It should be noted however that the majority of prebiotic reseach has focussed on fructooligosaccharides with little attention directly on legume RFOs. This suggests that there is potentially some benefit in retaining a low concentration of RFOs in processed navy beans.

2.6 Methods for RFO Reduction and/or removal from Legumes

There have been many studies carried out investigating the effectiveness of different processing methods on the removal of RFOs from beans. The following is a summary of the methods used and their effectiveness in removing RFOs.

2.6.1 Soaking and Boiling

The effect of soaking and boiling of whole legume seeds on the concentration of RFOs has been investigated in a number of studies (Barampama & Simard 1994, Iyer et al 1980, Kon 1979, Matella et al 2005, Revilleza et al 1990, Song & Chang 2006).

Kon (1979) found that increasing the temperature of the soaking water increased the rate of water up-take and the percentage of oligosaccharides removed from small white Californian beans. Beans soaked in water at 20°C took 16 hours to reach saturation during which period only 5% of the stachyose and raffinose content of the beans was extracted into the soaking water. In contrast beans soaked in water at 90°C only took 50 minutes to reach saturation and resulted in the extraction of 57% of the stachyose and raffinose from the beans.

Kon (1979) observed a significant jump in both the rate of water uptake and the amount of soluble solids (including the RFOs) extracted for soaking temperatures above 50°C. Interestingly, the increase in mass of the beans at saturation was around 10% greater for beans soaked at temperatures of 50°C and below than for those soaked at higher temperatures. The difference in saturation mass is attributed to the greater proportion of soluble solids extracted during soaking at temperatures 60°C and above.

Kon (1979) proposes that at temperatures of 60°C and above the inter- and intra-cellular membranes begin to break down and therefore become more permeable both to the imbibition of water into, and the diffusion of solutes out of the bean. Whether the mechanical disruption of these membranes caused by milling the beans into flour serves to similarly increase the extraction rate of RFOs was not investigated.

Iyer et al (1980) found that soaking whole great northern, kidney and pinto beans in water at 22˚C for 18 hours resulted in 25-40% reduction in raffinose and 30-40% reduction in stachyose. Boiling the same three beans for 90 minutes resulted in 80-90% reduction in raffinose and 70-80% reduction in stachyose.

Barampama and Simard (1994) found that soaking of *Phaseolus vulgaris* seeds for 12 hours followed by one hour of boiling resulted in a 60% reduction in raffinose and a 70% reduction in stachyose, while boiling for 90 mins resulted in a 50% reduction in raffinose and a 50% reduction in stachyose. Soaking for 12 hours resulted in a 35% reduction in raffinose and a 30% reduction in stachyose.

Han and Baik (2006) investigated the effect of applying ultrasound or high hydrostatic pressure during soaking of various legumes on the amount of RFOs removed. Soaking soybeans for 3 hours removed 33.3% of the raffinose and 46.6% of the stachyose. Application of ultrasound during the 3 hour soaking period increased the amount of raffinose removed to 55.7% but reduced the amount of stachyose removed to 28.6%. Application of high hydrostatic pressure (621 MPa) while soaking for 1 hour removed between 33.9% of the raffinose and 7.4% of the stachyose.

Song and Chang (2006) found that soaking of pinto beans resulted in a 9.8% reduction in RFOs, while soaking followed by boiling for 90 minutes resulted in a 57.6% reduction in RFOs.

Obulesu and Bhagya (2006) found that preparation of soy protein concentrate via 2 stage aqueous leaching (2 x 2 hour leaching stages) of de-hulled defatted soy bean flakes (0.3mm thickness) reduced raffinose content by 96% and stachyose content by 90% compared to flour milled from the de-hulled defatted soybeans. This indicates that increasing the surface area of the legume prior to leaching (i.e flakes vs whole beans) significantly increases the removal of RFOs during leaching.

These studies suggest that the rate at which RFOs leach out of legume seeds during soaking is significantly affected by the temperature of the soaking water. However, it is likely that the increase in temperature may not be directly influencing the rate of leaching as much as it appears to be. When the soaking water reaches a high enough temperature the membranes and cell walls of the bean begin to break down, which eases the access of the water into the cells and therefore increases the rate of leaching.

Leaching of RFOs via soaking of navy beans is a simple process which has been successful in reducing the RFO content in beans and can be easily applied to processing broken beans. The time frames involved in leaching significant proportions of RFOs from whole beans are slower than desired for a commercial process and so the leaching mechanism should be further investigated.

2.6.2 Hydrolysis using α-galactosidase

Guimaraes et al (2001), Matella et al (2005) and Song and Chang (2006), have investigated the reduction of RFOs in various legume seeds due to incubation with α-galactosidase.
Song and Chang (2006) found that two hours of incubation with α-galactosidase at 55-60°C removed 100% of RFOs in pinto beans. This is a significantly greater reduction than was achieved by soaking followed by boiling for 90 minutes (54.2%).

Guimaraes et al (2001) found that incubation of soymilk with α-galactosidase for 8 hours at 30°C resulted in a 73.3% reduction in raffinose and a 40.6% reduction in stachyose.

Matella et al (2005) found that incubation of black, red and navy beans with α-galactosidase for 1 hour at 23°C was more effective (30% - 50% reduction) in the reduction of RFOs than soaking of beans for 5 hours at 23°C (1% - 35% reduction).

All three of these studies used bean samples which were ground into flour prior to incubation with α-galactosidase in water, where the soaking and boiling data were all measured on whole seeds. As indicated by the work of Obelesu and Bhagya (2006) discussed above a reduction in particle size of the legume drastically increases the RFO removed during leaching. This means that it is entirely feasible that the reduction in RFOs observed during incubation of the legumes with α-galactosidase is in fact due to leaching.

This also raises the question of whether the α-galactosidase can hydrolyse RFOs while they are still within the bean structure or if it can only access them once they have leached out of the bean. This question has not been addressed in any of the literature reviewed here.

Matella et al (2005) also proposed a method for the commercial removal of RFOs from legume flours. The method includes extraction of soluble sugars from ground beans using ethanol, treatment of the sugars with α-galactosidase to hydrolyse the RFOs, recovery of the ethanol and addition of the reduced-RFO sugars to the bean slurry prior to drying and milling.

Kon (1979) found that soaking beans in water at temperatures ranging from 20-90°C failed to extract any protein material from the beans, even at temperatures greater than 60°C at which the cellular membranes begin to break down. Kon proposes that when the cell membranes are intact they are impermeable to molecules as large as proteins and at temperatures at which the cell membranes are breaking down the proteins are denatured by the heat, become insoluble, and are therefore unable to diffuse out of the bean.

It should therefore be expected that the cell membranes would be similarly impermeable to enzymes present in the soaking water. Therefore, it is likely that in order for the enzyme to hydrolyse the RFOs in navy beans the RFOs must first be extracted into the bulk solution so that they can come into contact with the enzymes. However, this may not be the case if the cellular membranes have been disrupted by some prior process, for example milling.

Enzymatic hydrolysis is an effective technique for the reduction of RFOs in navy bean flour and can be applied to flour milled from broken beans. However the cost of the enzyme used must be weighed against the cost of the raw ingredients. This technique warrants further investigation.

2.6.3 Autolysis using Endogenous α-galactosidase

Becker et al (1974) and Kon et al (1973) optimised the conditions for the endogenous bean α-galactosidase to hydrolyse the RFOs in various bean products. Under their conditions, 37.8 – 95.4% hydrolysis of the RFOs occurred when several types of bean were incubated for 24-48h at 45˚C. However in order for α-galactosidase to be produced endogenously in meaningful concentrations the beans must undergo germination, which renders this process time consuming and also limits its application to whole viable beans thereby excluding the use of broken beans for production of low RFO bean flour. For this reason autolysis and germination have been excluded from further investigation.

2.6.4 Extrusion and Roasting

The high temperature, high pressure environment to which a material is exposed during extrusion cooking can cause desirable chemical changes in the material. The effect of extrusion cooking on RFO content of some beans has therefore been investigated further. Extrusion of a high starch fraction of pinto bean flour using a Wenger twin screw extruder with a moisture content of 21.44% and temperature 157˚C produced a 43% decrease in raffinose and a 22% decrease in stachyose concentration (Borejszo & Khan 1992).

Loss of RFOs during extrusion cooking may be due to the Malliard reaction (Borejszo & Khan 1992). Revilleza et al (1990) found that dry-roasting hyacinth beans for 2 minutes resulted in the complete removal of RFOs. The reduction of RFOs is thought to be caused by a non-enzymatic browning reaction, oxidation of sugars, or pyrolysis. However, this type of treatment will result in denaturation of a large proportion of the bean proteins which may not be desirable for some applications.

Fasina et al (2001) found that heating of various legume seeds to a surface temperature of 140°C via infrared heating followed by soaking for 24h reduced raffinose concentration by 75.7% - 84.4% compared to reductions of 20.8% - 67.1% for soaking alone. The same treatment reduced stachyose concentration by 62.3% - 80.7% compared to reductions of 13.9% - 54.6% for soaking alone. The authors also report small increases in the rate of water absorption for the infrared treated seeds and attribute this and the higher leaching rates of RFOs and other solutes to cracking of the seeds facilitating access of water to the inner regions of the seeds.

Chiang and Johnson (1977) found that extrusion of red wheat flour increased the levels of fructose and melibiose. The authors suggested that the increase in these components was due to the breaking of the glucose – fructose bonds in raffinose, which leaves one molecule of melibiose and one molecule of fructose.

The cause of the degradation of raffinose is thought to be due to the high temperature, pressure and shear in the extruder. Since melibiose molecules still contain the galactose glucose bond that requires α-galactosidase for digestion, this method of RFO reduction is unlikely to reduce the flatulence causing effect of bean flour. This process also results in the gelatinisation of the starch and possible denaturation of the protein components in the flours, which may not be desirable for all applications. For these reasons extrusion and roasting have been excluded from further investigation.

2.7 Conclusion

The literature shows that common beans possess many nutritional benefits which make their inclusion in new formulated and processed food types desirable. Common beans also contain a number of anti-nutritional factors that can decrease the nutritive value of the beans and/or cause adverse effects if consumed. Most of these anti-nutritional factors are protein based and as a result they are generally denatured at temperatures used during cooking processes.

One exception to this are the raffinose family of oligosaccharides (RFOs) which are sugars and are therefore stable at higher temperatures. RFOs have been linked to the flatulence causing effect of legumes which is thought to be a major barrier to consumer acceptance of food products containing beans.

A number of techniques have been investigated for the reduction in RFO content of beans; including leaching, germination, high temperature/shear and hydrolysis using α-galactosidase. In order to take advantage of broken beans as a cheap source of navy bean flour germination has been excluded from further investigation. High temperature and high shear treatment has also been excluded due to the propensity for production of melibiose as a by product of the degradation of RFOs.

The following techniques have therefore been targeted for further investigation: leaching and hydrolysis with α-galactosidase. In addition to these techniques the physical separation of navy bean seed components will also be investigated as a possible process by which the RFO content could be reduced.

Chapter 3

Evaluation of the Mechanisms for RFO Removal from Navy Bean Flour

A number of different mechanisms were identified from the literature to remove or reduce the levels of RFOs in legumes. Those that can be readily adapted to industrial food processes are; physical separation of bean structures, leaching and enzyme hydrolysis. Each of these methods are explored in this chapter.

3.1 Materials

The materials used in the experiments presented in this work were:

- Whole raw navy beans were supplied by Heinz Watties Ltd and transferred to a PC-2 micro-organism containment lab (registered to MAF standard 154.03.02) in which all experiments were conducted.
- Milled navy bean flour was supplied by Heinz Watties Ltd transferred to a PC-2 micro-organism containment lab (registered to MAF standard 154.03.02) in which all experiments were conducted.
- Stachyose Hydrate 98.5% supplied by Acros Organics
- D(+)-Raffinose pentahydrate supplied by Acros Organics
- D(+)-Galactose supplied by Sigma Chemical Company
- D(-)-Fructose supplied by Ajax Finechem
- Sucrose (D(+) Saccharose) supplied by Fisher Scientific UK
- D(+)-Glucose supplied by Sigma Chemical Company
- Ethylenediamine Tetraacetic Acid Disodium Calcium Salt supplied by Sigma Chemical Co.
- α-galactosidase supplied by Novozymes
- Enzidase® AGS supplied by Zymus®

3.2 The Measurement of RFO Content

3.2.1 Measurement of RFO Concentration

Before these mechanisms can be put to the test it is important to develop a method whereby the concentration of RFOs in the navy bean samples can be reliably measured.

By far the most commonly used method for the quantification of RFO concentrations in recently published research is high performance liquid chromatography (HPLC). This method involves the injection of the sample into a stream of a continuous phase which is being pumped at a controlled flow-rate through a packed ionic column. The time it takes for each individual component of the sample to pass through the column is proportional to the affinity of that component to the packed media of the column. The outflow from the column is passed through a refractive index detector which gives an intensity reading that is proportional to the concentration of the solute passing through it. The area under a peak in the plot of intensity versus time can be correlated (through the use of a calibration curve measured for each sugar) to the concentration of the correlating sugar.
The system used to make HPLC measurements in all experiments outlined in this work is as follows:

- Dionex P680 HPLC Pump
- Dionex ASI-100 Automated Sample Injector
- Dionex Thermostated Column Compartment TCC-100
- Waters Guard-Pak sacrificial guard column.
- Waters Sugar-Pak 1 column with internal dimensions 6.5 x 300mm packed with micro-particulate (10µm) calcium cation-exchange gel.
- Shodex RI-101 Refractive Index Detector.

The HPLC setup described above was run isocratically with a mobile phase consisting of a 50mg/L solution of the calcium disodium salt of ethylenediaminetetraacetic acid (CaEDTA) which was being pumped through the HPLC column at a constant flow-rate of 0.2 mL/min and column temperature of 80°. 50 µL of the sample was then injected into the mobile phase and the resulting chromatogram recorded.

3.2.2 Calibration of the HPLC Method Using Sugar Standards

In order to calculate the concentration of the individual sugars in bean samples a calibration curve must be constructed by running sugar solutions of known concentration through the HPLC and plotting the area under the response peak against a range of concentrations for each sugar.

A solution containing a mixture of the following sugars was prepared at a known concentration of approximately 5g/L per sugar:

- Stachyose
- Raffinose
- Sucrose
- Glucose
- Galactose
- Fructose

Note that verbascose was not tested for due to the extreme cost of a purified sample and the large number of studies in the literature indicating that it is either not present or present in trace amounts in navy beans (see table 2.1). Dilutions of this solution were then prepared such that 5 standard solutions each containing a known amount (approximately 1, 2, 3, 4, and 5 g/L) of each sugar were produced.

Figure 3.1 displays the chromatogram measured for the 5g/l standard with each of the peaks labelled with its corresponding sugar as determined by the Sugar-Pak guidelines (available on the Waters website http://www.waters.com/waters/library.htm

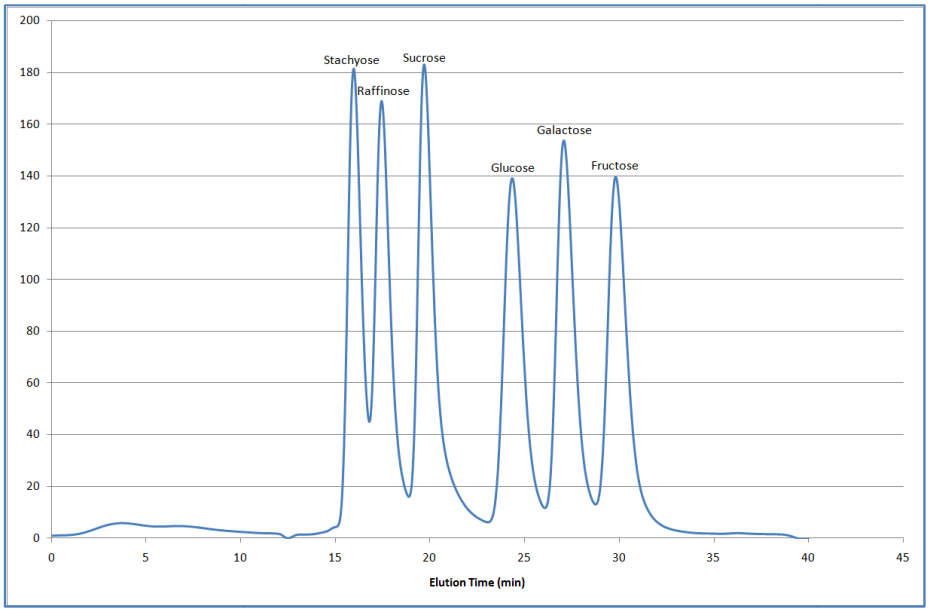


Figure 3.1 Chromatogram of standard sugar solution containing approximately 5g/L of each sugar. Peaks are labelled with their corresponding sugar.

The concentration of each sugar in the sample is proportional to the area under its corresponding peak. The HPLC system software (Chromeleon; Thermo Scientific, Sunnyvale, CA.) can be used to determine the area under each peak, however the degree to which it can accurately separate overlapping peaks is not ideal and can lead to inaccuracies when two peaks of significantly different size are overlapping.

HPLC data measured in this work was therefore analysed using software called PeakFit (Systat Software Inc. San Jose CA.) which provides much better de-convolution of overlapping peaks. Figure 3.2 demonstrates the difference between the two chromatogram analysis techniques.

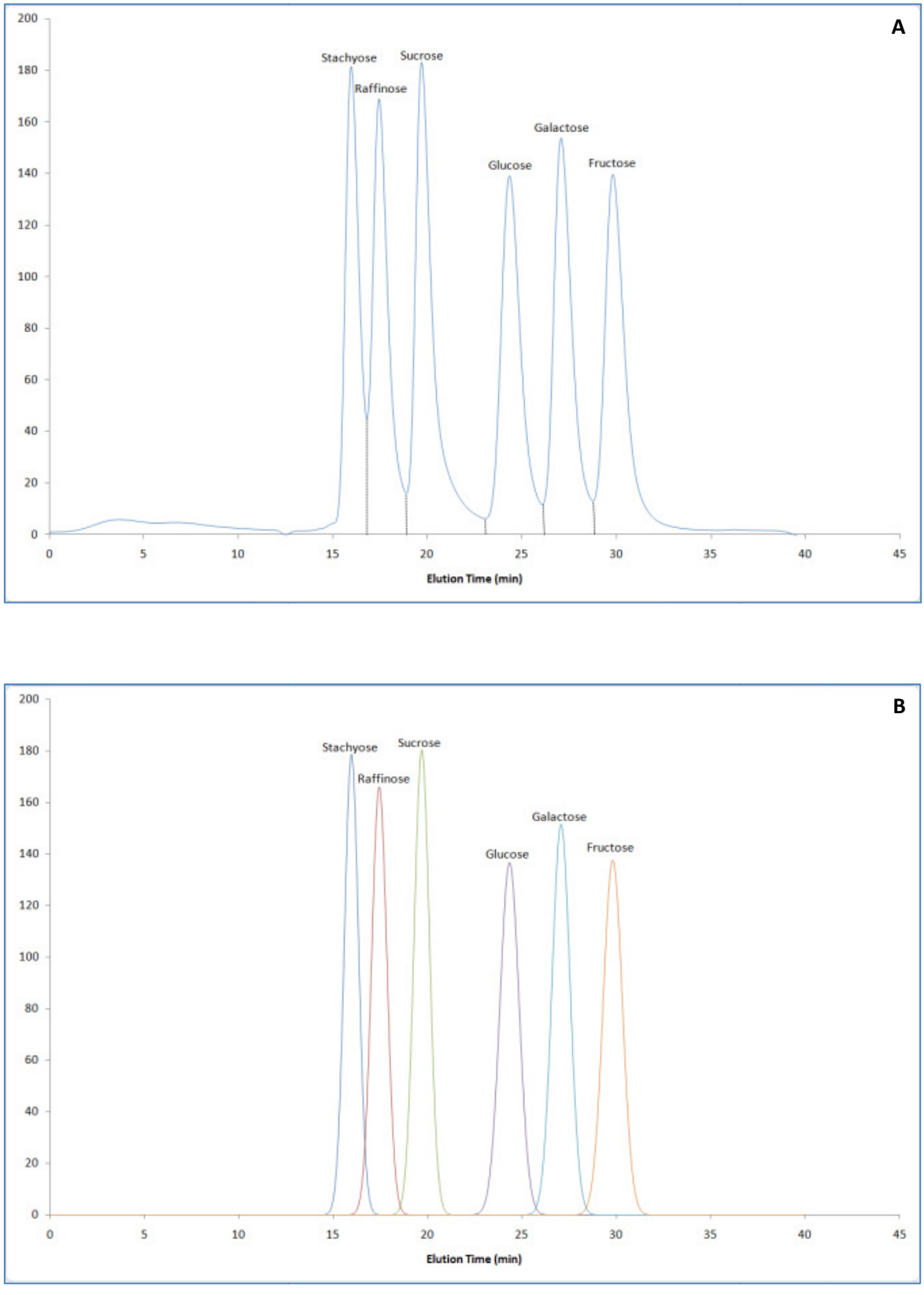


Figure 3.2 **A**: Peak separation using Chromeleon software. **B**: Peak separation using PeakFit software.

Each sugar standard was analysed in triplicate (i.e. 3 chromatograms were measured for each sugar standard) and the area under each peak was calculated for each of the standard sugar solutions and plotted against the concentration to produce calibration curves for each sugar. Figure 3.3 shows the calibration curves for each sugar.

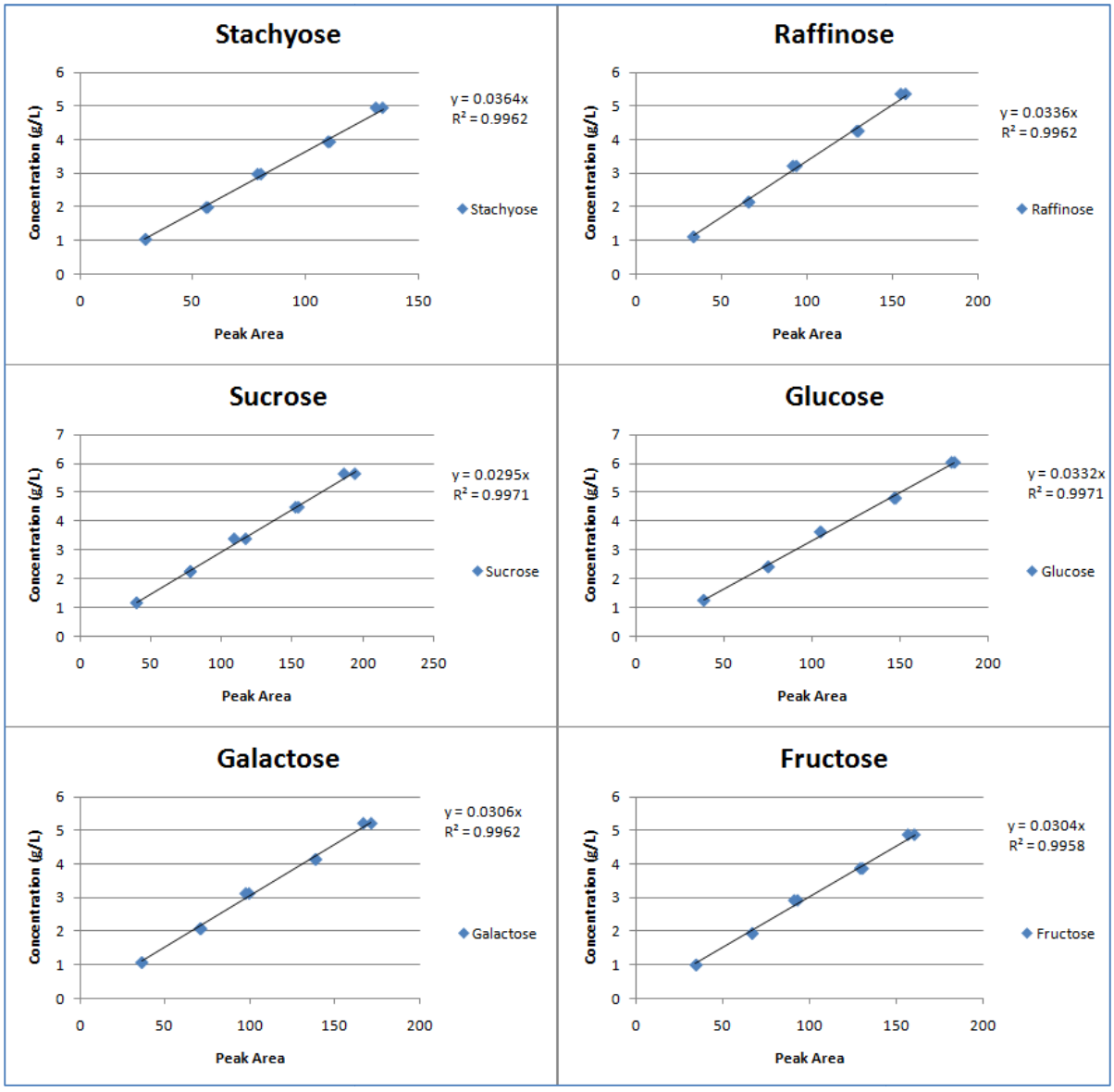


Figure 3.3 Calibration curves for Stachyose, Raffinose, Sucrose, Glucose, Galactose and Fructose

Figure 3.3 illustrates that the relationship between sugar concentration and chromatogram peak area is linear. The R^2 values for the fitted lines displayed are all greater than 0.99 which indicates a very good fit and gives confidence that the HPLC measurement technique can be used to reliably determine sugar concentration.

3.3 RFO Extraction, Solvent Removal and Recovery of Residue

Many researchers have measured the concentration of RFOs in legumes using techniques (Lee et al 1983, Naivikul & D'Appolonia 1978, Sanchez-Mata et al 1998, Shimelis & Rakshit 2005) that are variations on the following general process:

- The RFOs in the sample are extracted using a non-aqueous solvent for example ethanol or methanol.
- The solvent is evaporated off and the residue is re-dissolved into a small amount of distilled water.
- The extract is then assessed using some form of chromatography (paper chromatography, liquid chromatography and gas chromatography have all been used, however HPLC is the preferred method in more recent studies).

Sanchez-Mata et al (1998) have evaluated a number of solvents for the extraction of mono-, di- and oligosaccharides from several types of legume samples. They concluded that an 80% solution of ethanol with water at 55-60°C was the most effective for extraction of these sugars. A method for the quantitative extraction of sugars from navy bean samples and subsequent recovery of sugars from the solvent has been adapted from that outlined by Sanchez-Mata et al (1998):

The bean sample was crushed into a powder/paste using a mortar and pestle. 1.5g of sample was weighed into a centrifuge tube with 40 mL of 80% ethanol. The centrifuge tube was inverted few times until the sample was well suspended and then placed in a water bath at 55˚C and constantly stirred for 45 minutes using a magnetic stirrer. The tube was then centrifuged for 30 min at 3000rpm (eppendorf 5702 with rotor radius 14cm) and the supernatant collected. The extraction step was repeated and the supernatants pooled.

The solvent was then evaporated using a rotary vacuum evaporator at 70˚C. The residue was re-dissolved in 10 mL of distilled water and passed through a 0.45 µm syringe filter into a 1.5mL HPLC vial with a rubber slit septum. The sample was then ready for injection into the HPLC via an auto-sampler.

The sugar extraction method described by Sanchez-Mata et al (1998) consists of 2 successive extraction steps using 80% ethanol as the solvent. The solvent from each extraction step is then combined, the ethanol is evaporated and the residue is dissolved in 10ml of distilled water. It was considered prudent to validate this extraction method by comparing the measured concentration of stachyose and raffinose in navy bean samples with those reported in the literature. The appropriate number of extraction steps to extract the sugars also requires validation and this was investigated experimentally.

3.3.1 Validation of Sugar Extraction Method

In order to validate the method of sugar extraction outlined above the number of extraction steps using 80% ethanol at 55°C was investigated. In each extraction step the maximum amount of sugar extracted from the bean sample will be equal to the amount necessary to bring the concentration in the bean into equilibrium with the concentration in the ethanol solution. One should therefore expect to see slightly larger amounts of sugars extracted as additional extraction steps are added; however given the large volume of extraction solution compared to the mass of bean sample it is expected that this increase should be negligible after two extractions. In order to confirm this, measurements of RFO concentration using 2 and 3 extraction steps were carried out on multiple samples of bean flour from the same batch and the measured concentrations were compared.

Three replicate 1g samples of navy bean flour were extracted with either 2 or 3 extraction steps. Each replicate was analysed in triplicate using the HPLC method described above. The results from each triplicate were averaged to give an average concentration for each sample. The average values for the replicate samples were then averaged to give an overall average concentration for each sugar for each extraction method.

Figure 3.4 shows the average measured concentrations of stachyose, raffinose, sucrose and galactose for each extraction method including standard error bars to indicate uncertainty in each value.

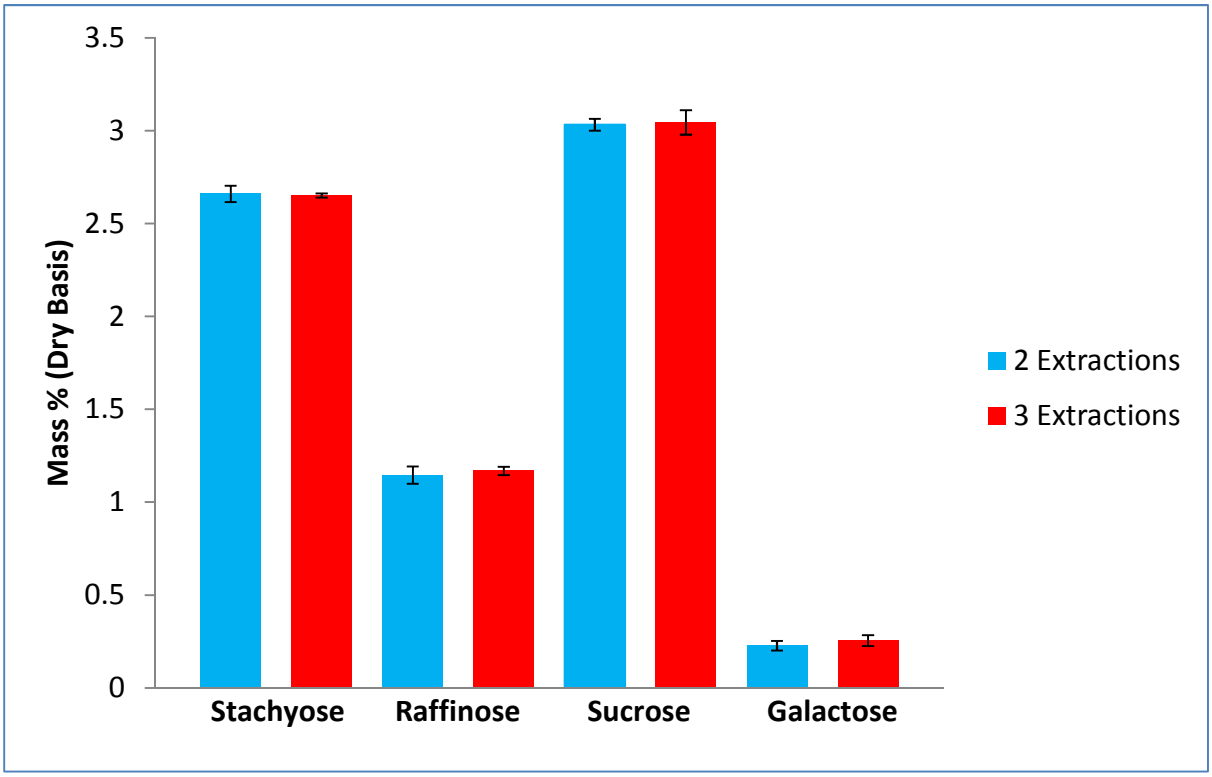


Figure 3.4 Measured sugar concentrations in navy bean flour using 2 and 3 80% ethanol extraction steps.

Figure 3.4 demonstrates that the difference in measured concentration between 2 extraction steps and 3 extraction steps was negligible. The average values for concentration of stachyose and raffinose were reasonable when compared to the literature (see table 3.1) and the method can therefore be considered valid for the measurement of these sugars. The extraction method with 2 extraction steps was therefore employed for the subsequent determination of sugar concentrations in all bean samples.

Table 3.1 Comparison of RFO concentrations measured for navy beans and the ranges reported in the literature for legumes. See table 2.1 for literature references.

RFO	Measured Value	Range in Literature
Stachyose	2.66% (DB)	0.1 - 2.6% (DB)
Raffinose	1.15% (DB)	0.13 - 5.5% (DB)

3.4 Characterisation of Navy Bean Flour

In order to carry out analysis of the data presented in this chapter the following properties of navy bean flour were determined.

- Initial moisture content of the flour
- Saturated moisture content of the flour
- Soluble solids content of the flour
- Particle Size Distribution of the flour

3.4.1 Initial Moisture Content

1g samples of bean flour were weighed onto pre-weighed watch glasses. The watch glasses were placed in a vacuum oven at 105°C for 20 hours. The watch glasses were then placed in a desiccator for 5 minutes to cool down. The watch glasses were then weighed. The watch glasses were then returned to the vacuum oven for 1h before being cooled in a desiccator and re-weighed. The mass of the watch glass + sample measured after each period in the vacuum oven were compared to ensure that no further evaporation was taking place. The final mass of the flour was compared to the initial mass to give the initial moisture content of the navy bean flour. Samples were repeated in triplicate. The mean of the results from each replicate was calculated and used in subsequent calculations as the value for initial moisture content of the navy bean flour ($C_{wi\text{-}flour}$).

Table 3.2: Initial moisture content of navy bean flour on both wet and dry basis.

Replicate	Wet basis	Dry basis
1	14.9%	17.5%
2	14.8%	17.4%
3	14.8%	17.4%
$C_{wi\text{-}flour}$	14.8%	17.4%

3.4.2 Saturated Moisture Content

1g samples of navy bean flour were placed in a 40 ml centrifuge tube with 30ml of distilled water at 23°C and stirred for 30 minutes using a magnetic stirrer. The tubes were then centrifuged at 4000rpm for 5 minutes and the supernatant decanted off. The mass of the residue was measured before being transferred to a pre-weighed watch glass. The samples were then dried in a vacuum oven at 105°C as above until no change in mass is observed between drying periods. The dry mass of the sample was measured and compared to the mass of the wet residue to determine saturated moisture content. Samples were repeated in triplicate. The mean of the results from each replicate was calculated and used in subsequent calculations as the value for saturated moisture content of the navy bean flour ($C_{wS\text{-}flour}$).

Table 3.3 Saturated moisture content of navy bean flour on both wet and dry basis.

Replicate	Wet Basis	Dry Basis
1	76.0%	315.6%
2	76.0%	316.7%
3	76.2%	319.3%
$C_{wS\text{-}flour}$	76.1%	317.2%

3.4.3 Soluble Solids Content

1g samples of navy bean flour were placed in a 40 ml centrifuge tube with 30ml of distilled water at 23°C and stirred for 30 minutes using a magnetic stirrer. The tubes were then centrifuged at 4000 rpm for 5 minutes and the supernatant decanted off. 30 ml of fresh distilled water at 23°C was added to the residue, the tube was shaken to disperse the residue, and the sample was stirred for 30 minutes using a magnetic stirrer and centrifuged at 4000rpm for 5 minutes. This process was repeated for a third time before the mass of the residue was measured and transferred to a pre-weighed watch glass. The samples were then dried in a vacuum oven at 105°C as above until no change in mass is observed between drying periods.

The dry mass of the sample was measured and compared to the initial dry mass of the sample (calculated assuming an initial moisture content of 14.8%) to determine the soluble solids content of the flour. Samples were repeated in triplicate. The mean of the results from each replicate was calculated and used in subsequent calculations as the value for soluble solids content of the navy bean flour ($C_{SS\text{-}flour}$).

Results:

Table 3.4 Soluble solids content measured in navy bean flour on a wet and dry basis.

Replicate	Wet Basis	Dry basis
1	20.5%	25.8%
2	21.3%	27.0%
3	19.4%	24.0%
$C_{SS\text{-}flour}$	20.4%	25.6%

3.4.4 Particle Size Distribution

The particle size distribution (PSD) of the navy bean flour was measured using a Malvern Mastersizer S (long-bed model). The flour samples were presented using a manual dry powder feeder and the unit was run using Malvern Mastersizer S software version 2.19.

The PSD measurement was performed using a 1000F lens; the active beam length was 2.4mm; the analysis mode was polydisperse and the presentation was 3RHA. The sample size for the measurement was 15000 and the lower and upper obscuration limits were set at 1 and 6 respectively.

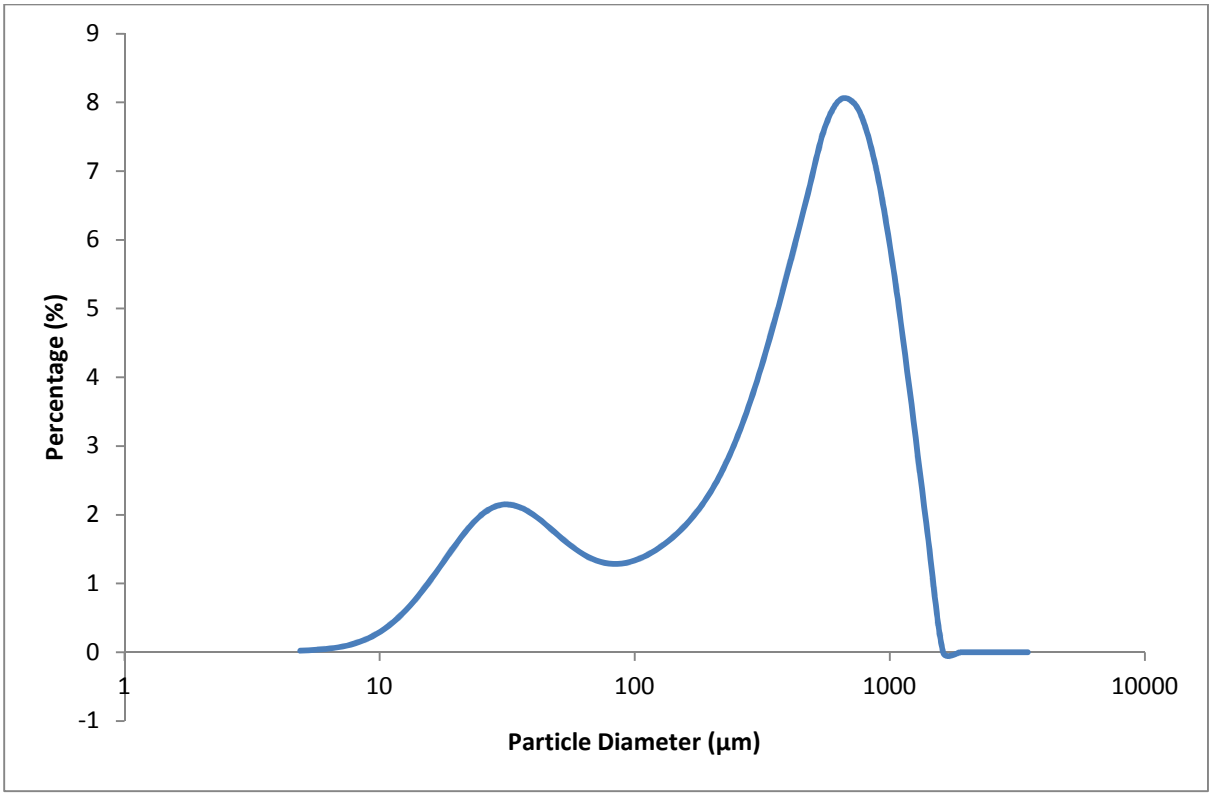


Figure 3.5 Particle size distribution of navy bean flour.

The median particle size is 400 µm and figure 3.5 clearly shows that the navy bean flour displays a bimodal PSD. This correlates well with the SEM images of navy bean flour in figure reported by Berg et al (2012) which indicate than a significant proportion of the starch granules may be released via disruption of the cotyledon cells during milling. Berg et al (2012) report PSD for navy bean starch granules between 10 and 80µm in diameter. This correlates well with the first peak in figure 3.5, implying that this peak is due in large part to the presence of individual starch granules which have been liberated from their cells during milling. Berg et al (2012) report a size range of 50 - 100µm for individual navy bean cotyledon cells and therefore the majority of the volume of the flour as represented by the second peak in figure 3.5 must consist of clusters of intact cells bound together.

3.5 Techniques for the Reduction/Removal of RFOs from Navy Bean Flour

The literature reviewed in chapter 2 identified several techniques for the reduction or removal of RFOs from navy beans. The purpose of the remainder of this chapter is to test the most promising of these techniques for the processing of milled navy beans. The techniques to be investigated are:

- Physical separation of navy bean components
- Leaching of RFOs into solution
- Hydrolysis of RFOs using α-galactosidase

3.5.1 Physical Separation of Navy Bean Components

The literature reviewed in chapter 2 contained little information pertaining to the distribution of RFOs within the various structures of the navy bean seed. The major functional components of milled beans are the cotyledons, in that they comprise by far the largest mass fraction of the bean and contain the majority of the starch and protein. Therefore if the majority of the RFOs reside in some other structural component of the bean then a process could be designed to remove that component with its attendant RFOs prior to milling.

To determine if this strategy is feasible an experiment was carried out to determine whether there is a significantly higher concentration of RFOs in either the hull or the plumule compared to the cotyledons of navy beans.

To this aim a number of navy beans were manually de-hulled and the plumules removed using a scalpel and tweezers such that 1g samples of each component were produced. The RFO content was measured in the samples of each component using the methods outlined in sections 3.2 and 3.3 above.

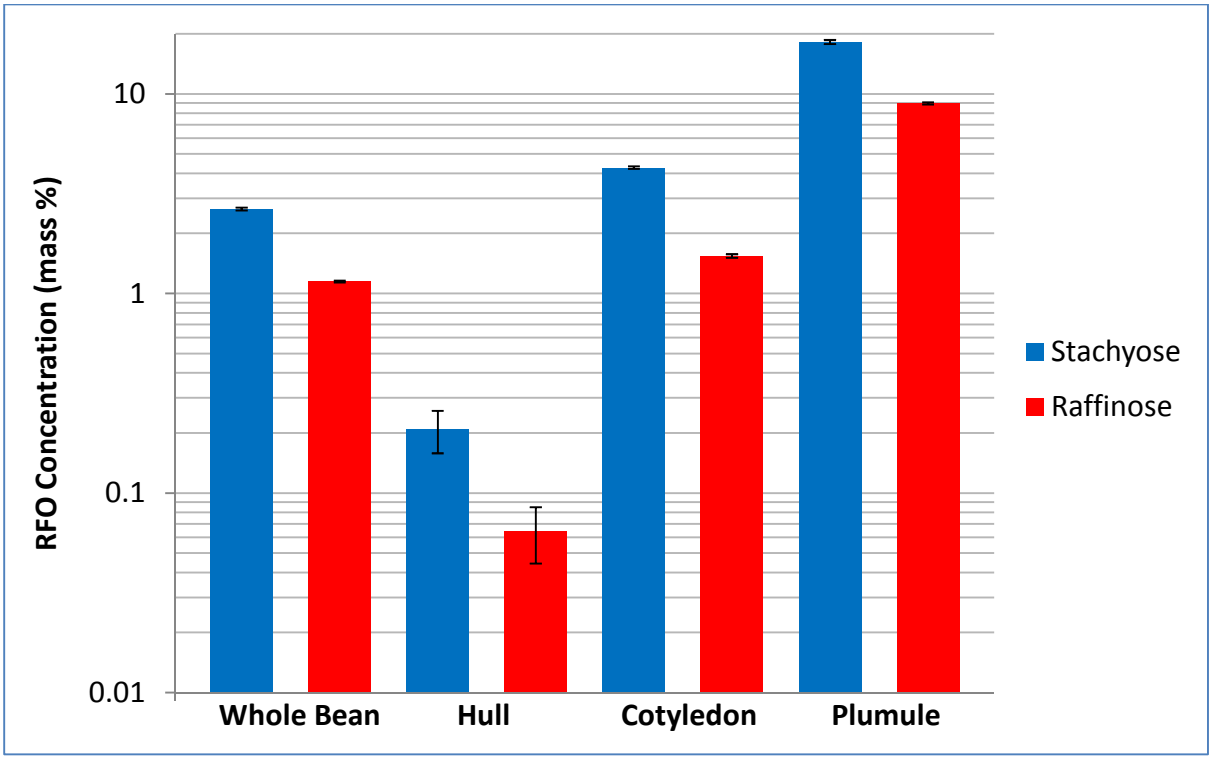


Figure 3.6 RFO content of structural components of navy beans. Note y axis is on a log scale.

Figure 3.5 shows that compared to the whole bean flour sample the hull has significantly lower concentrations of both stachyose and raffinose, the cotyledons have moderately higher concentrations of both stachyose and raffinose and the plumule has significantly higher concentrations of both stachyose and raffinose. Although the plumule contains 300-400% higher concentration of RFOs than the cotyledons, they account for less than 2% of the mass of the bean and therefore contain less than 10% of the total mass of RFOs in the bean. This coupled with the difficulty of separation means that the removal of plumules is an impractical and ineffective mechanism for RFO reduction in navy beans.

3.5.2 RFO Leaching

The literature reviewed in chapter two contained a large number of studies investigating the effectiveness of leaching as a mechanism for the removal of RFOs from legume seeds. Kon (1979) found that extraction of RFOs into soaking water at temperatures below 50°C is both slow (4-16 hours) and ineffective (5-15% removal). Even at 90°C the extraction rate was slower than desired for an industrial process (50 minutes) and only resulted in 57% removal of RFOs. This is unfortunate as an elegant process would be to leach RFOs from whole beans, thereby making it relatively easy to separate the beans from the water. The slow and incomplete RFO removal in this way makes it commercially unfeasible.

Obulesu and Bhagya (2006) found that two stages (2h) of leaching of de-hulled defatted soybean flakes resulted in 91.5% RFO removal demonstrating that an increase in surface area can significantly improve the effectiveness of leaching for RFO removal. While this technique results in RFO removal at or exceeding the target the four hour leaching time is again significantly slower than desired for an industrial process. This technique would also require several processing operations prior to RFO removal and would require drying of the leached flakes prior to milling into flour.

Milling the beans into flour prior to the leaching step will dramatically increase the contact surface area between the bean and water as well as potentially disrupting some of the cellular membranes in the bean through mechanical stress. The rate of extraction is therefore expected to be faster in the milled bean flour.

To investigate this an experiment was carried out to determine whether the extraction rate of RFOs from navy bean flour is significantly faster than the extraction rates for whole beans reported in the literature, and to determine the maximum proportion of RFOs that can be removed from the bean flour using this mechanism.

2g samples of navy bean flour were added to 18ml of distilled water at room temperature (23°C) in a 40mL centrifuge tube. The samples were continuously stirred using a magnetic stirrer for 2, 5, or 10 minutes as well as 15 hours (to determine the maximum amount of RFO that can be removed by leaching under these conditions). Triplicate samples were tested for each leaching time. Following the leaching time the samples were centrifuged and the supernatant poured off. The sugars remaining in the residue were extracted using the method described in section 3.3. The concentration of RFOs in each extracted sample as well as in the corresponding leaching water was measured using the HPLC method described in section 3.2.

The values displayed in figure 3.6 were calculated on the initial dry matter basis assuming that the total amount of dry matter in the sample remains constant throughout the leaching process. This is obviously not the case as some proportion of the soluble solids will have dissolved during the leaching period (including the RFOs that were measured), and a greater proportion will have dissolved the longer the period of leaching.

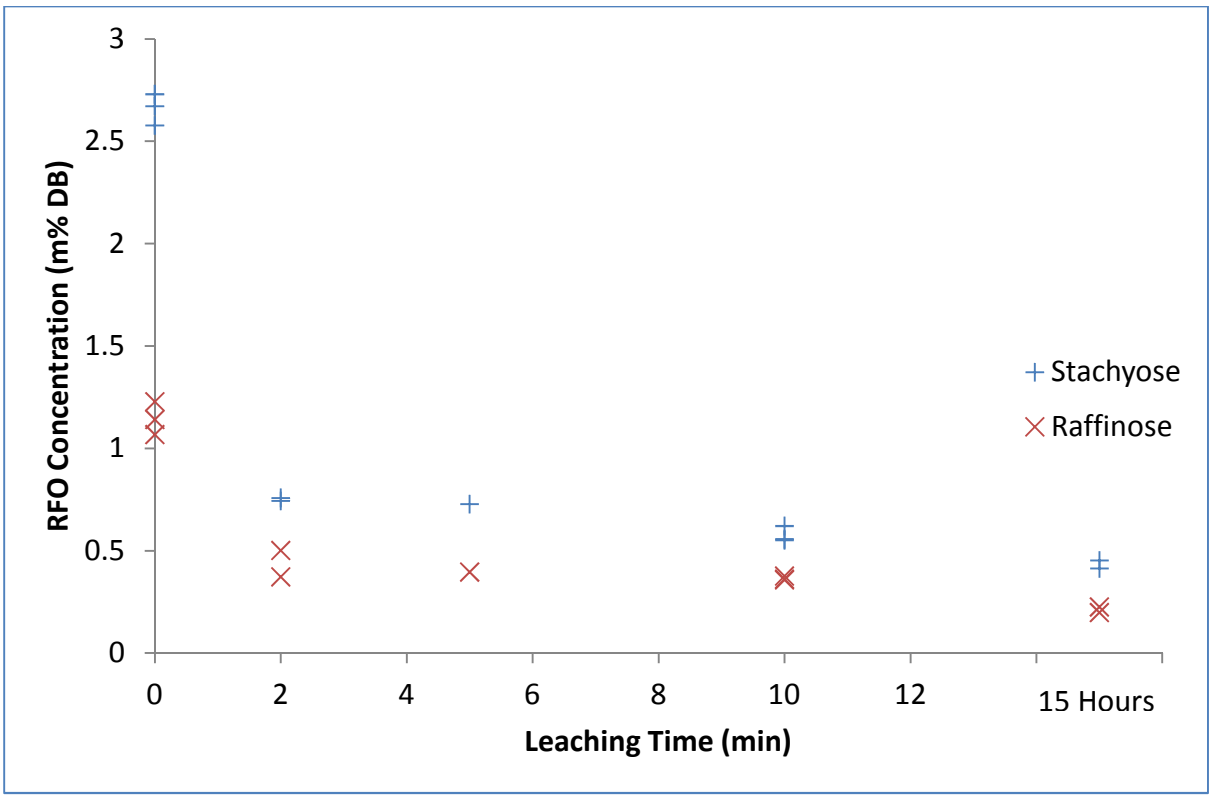


Figure 3.7 Leaching Rates of stachyose and raffinose at 23°C in slurry containing 10% navy bean flour

Figure 3.7 indicates that the leaching of RFOs from navy bean flour progresses at a much faster rate than those reported in the literature for whole navy beans (Kon 1979). This result was as expected and is due to the vastly increased surface area of the flour compared to whole beans, which provides much greater opportunity for the leaching water to penetrate the bean particles and leach the RFOs out.

Interestingly the proportion of total RFOs removed from the bean flour samples is also significantly higher than those removed from whole navy beans (Kon 1979). Kon attributed the low amount of total RFOs leached at low temperatures to inter and intra-cellular membranes acting as diffusion barriers for both moisture and solutes. As discussed in section 3.4.4 the PSD of navy bean flour suggests that the majority of the flour is composed of pieces of bean cotyledon containing multiple undisrupted cells.

While the disruption of cotyledon cells may have some impact on the leaching rates of RFOs from the bean flour the proportion of cell membranes that are disrupted as a result of milling does not seem to be significant enough to account for the dramatic increase in the total amount of RFOs leached from the flour.

This suggests that the diffusion of RFOs in navy beans is highly dependent on particle size, due to both the increase in contact area between the bean particle and the leaching water and the decrease in the distance over which the RFOs must diffuse to exit the particle. The diffusion of RFOs out of a bean particle is also dependent on the moisture content of the particle because of its effect on the mobility of the RFO molecule. In diffusion through a porous solid the diffusion coefficient depends not only on the concentration gradient between one point and another, but also on the mobility of the solute and the tortuosity or obstructive resistance to diffusion of the porous path through which the solute must diffuse.

At very low moisture contents the RFO molecules are not mobile and so cannot diffuse to an area of lower concentration, therefore the faster the leaching water can diffuse into the bean the faster the RFOs can become mobile enough to begin diffusing out.

At low moisture contents the solubility of the RFOs may also play a role in leaching rates. When the water available to dissolve a given mass of RFOs is less than a critical amount then not all of the RFOs will be able to dissolve. Below this critical moisture content the concentration of dissolved RFOs will be the saturation concentration. At first glance it would seem as though this should maximise diffusion rate from this region of the bean because the saturation concentration is the maximum concentration possible in the leaching water and the concentration gradient driving diffusion will therefore be at its maximum.

During moisture penetration into a bean particle the particle can be described in terms of three regions. The inner region of the bean particle is at the initial moisture content and no RFO diffusion is possible. A low moisture region will exist immediately surrounding the inner region over which the moisture content will range from the initial moisture content (at the boundary with the inner region) to the critical moisture content for solubility. The leaching water present in this region will all be at the saturated concentration of RFO and therefore no RFO concentration gradient for diffusion will exist within this region.

The moisture content of the outer region is above the critical moisture content for solubility and a gradient will develop across this region as the RFO concentration varies with moisture content and as RFOs are diffused across the surface of the particle into the bulk water.

A concentration gradient will exist across the surface of the low moisture region and so diffusion can take place from the boundary of this region into the outer region of the particle. The concentration of RFOs in this surface layer will be maintained at saturation via dissolution of the un-dissolved RFOs present. The layer within the low moisture region from which this diffusion takes place will move inwards as the un-dissolved RFOs in the outer layers of this region are exhausted and the concentration drops below saturation.

Values reported for the solubilities of stachyose and raffinose are given in table 3.5.

Table 3.5 Reported values for solubility of stachyose and raffinose. Solubility values were taken from the website Sigma-Aldrich who supplies these compounds at HPLC grade purity.

RFO	Solubility (mg/mL H_2O)	Reference
Stachyose	50	www.sigmaaldrich.com
Raffinose	100	www.sigmaaldrich.com

These solubilities correspond to critical moisture contents of 53.2% and 11.5% (db) below which the concentration of RFOs in solution will be saturated for stachyose and raffinose respectively.

Also, as the moisture content of the bean particle increases, the particle begins to swell, opening up more accessible channels within the particle and thereby decreasing the tortuosity of the diffusion path. Because the rate at which the leaching water penetrates to the centre of the particle is dramatically increased by the increase in surface area and reduction in diffusion distance in the bean flour, both the rate at which the particles swell and the rate at which the innermost RFO molecules become mobile increases proportionately.

It was therefore proposed that the low levels of RFO removal achieved by Kon (1979) at low temperatures was due less to the condition of the cell membranes and more to slow rates of moisture penetration into the beans and subsequently very low diffusion rates of RFOs out of the beans.

Therefore had Kon (1979) increased the duration of immersion of the beans at low temperatures they would eventually have come to the same equilibrium concentration of RFOs as the beans soaked at higher temperatures.

The higher temperatures increased the rate of moisture uptake of the beans and therefore decreased the amount of time between immersion and the onset of solute diffusion from the centre of the bean. The increase in temperature also has a significant increase in the diffusion rate due to the increase in kinetic energy of the RFO molecules, meaning that once they become mobile they can diffuse out of the particle at an increased rate.

Figure 3.8 shows the extent of the leaching on a proportion of absolute mass of RFO removed from the bean flour. This assumes that the total amount of RFO that can be leached from the bean flour has done so within the 15 hour leaching period. Figure 3.7 plots *X* versus leaching time where *X* represents the proportion (g/g) of the total mass of RFOs that have been leached at the end of a given leaching period and is given by:

$$X = \frac{(m_{RFOi} - m_{RFOt})}{(m_{RFOi} - m_{RFO\infty})} \tag{3.1}$$

Where m_{RFOi} = initial mass of RFO in the bean flour sample, m_{RFOt} = mass of RFO remaining in the bean flour sample at time = *t*, and $m_{RFO\infty}$ = mass of RFO remaining in the bean flour sample when the leaching process reaches completion. i.e. *X* = 0 when $m_{RFOt} = m_{RFOi}$, and *X* = 1 when $m_{RFOt} = m_{RFO\infty}$.

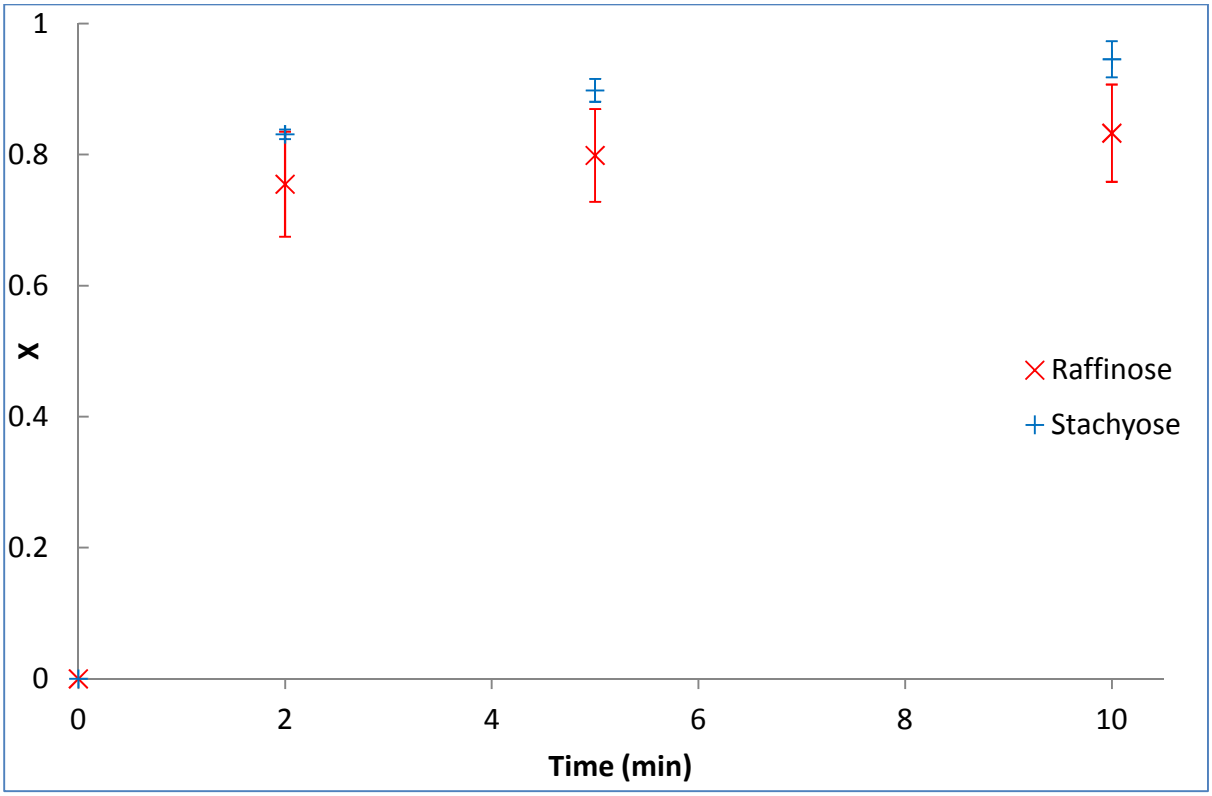


Figure 3.8 Extent of leaching of stachyose and raffinose at 23°C in a slurry containing 10% by mass navy bean flour.

The values for *X* displayed in figure 3.8 were calculated using the mean concentration of each RFO measured in the raw bean flour (table 3.1) to calculate m_i for each sample. Likewise the mean % RFO remaining after 15 hours leaching was used to calculate m_∞ for each RFO. The values plotted in figure 3.8 are the means for each leaching period and the error bars are the standard deviation of those means. It should be noted that the larger standard deviations in the raffinose data are likely due to the fact that the raffinose peaks in the chromatograms are often partially overlapped with the stachyose peak.

The error inherent in the process of resolving these peaks using the peak fitting software has a larger proportional impact on the calculated area of the raffinose peak because it has a smaller area than the stachyose peak.

In figures 3.7 and 3.8 the rate of leaching of RFOs from the navy bean flour is shown to be very high initially but then rapidly tails off. The driving force for the diffusion of RFOs out of the bean particles is the concentration gradient between the particle and the leaching water.

Initially the difference in concentration is large and the leaching rate is therefore high but as the concentration of RFOs in the bean particles decreases and the concentration in the leaching water increases the difference between the two rapidly approaches equilibrium and the leaching rate decreases proportionately.

Because the leaching rate is dependent on the concentration gradient between the bean particles and leaching water it stands to reason that the larger the volume of leaching water to which a given mass of bean flour is exposed, the lower the concentration of RFOs remaining in the bean flour at equilibrium.

To illustrate this theory, an experiment was carried out investigating the total amount of RFOs leached from navy bean flour using varying ratios of bean flour to leaching water.

2g samples of navy bean flour were weighed into 40 mL centrifuge tubes. The appropriate amount of distilled water was added to each sample to produce mixtures at the following concentrations of dry matter:

Table 3.6 Concentration of bean flour in leaching water to be investigated

Concentration of Bean Flour (db) in Leaching Surry
5%
10%
20%

Each sample was prepared in triplicate. The samples were continuously mixed for 15 hours at 23°C using magnetic stirrers. The samples were then centrifuged at 4000rpm for 5mins. The supernatant was discarded, and the mass of the residue was measured. The RFOs remaining in the residue were then extracted using the method described in section 3.3 and analysed using the HPLC method described section 3.2. The mass of raffinose and stachyose remaining in the residue was then compared to the initial mass of these components in the bean flour samples.

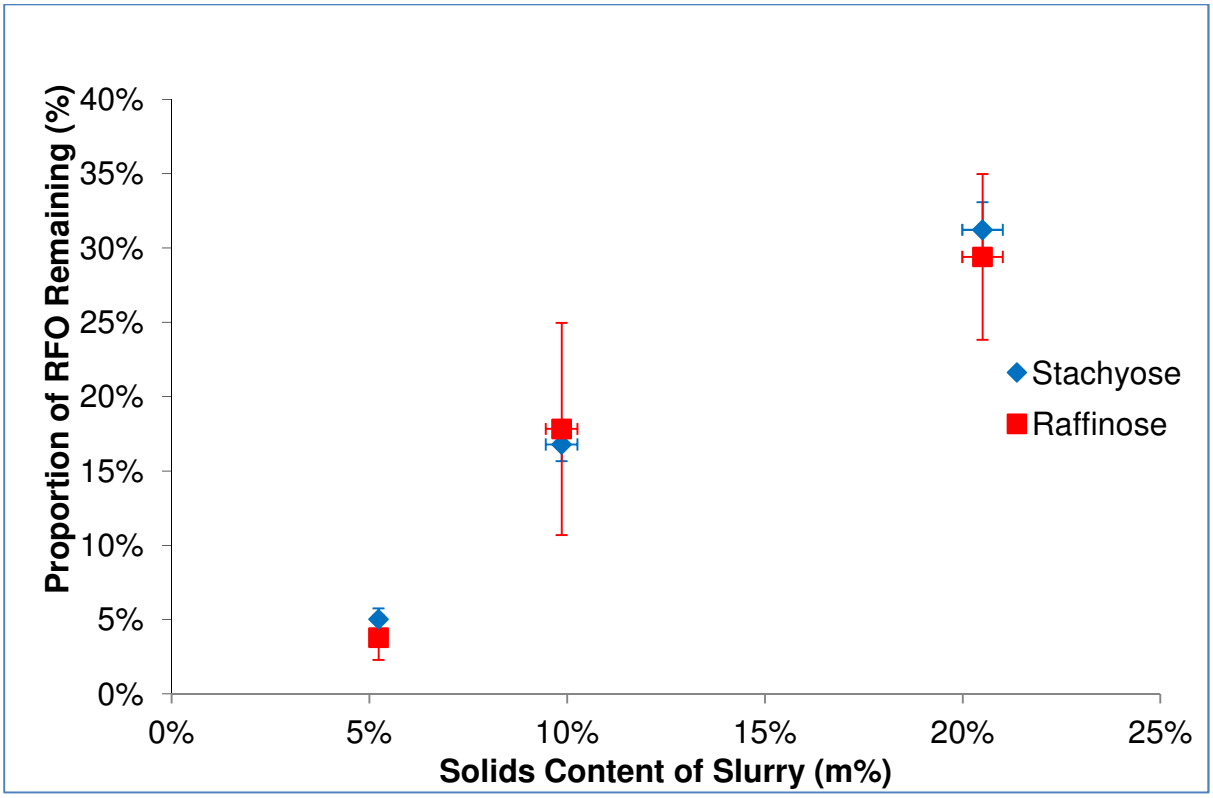


Figure 3.9 Proportion of initial RFO remaining in bean flour following leaching overnight (13h) at 23°C as a function of the solids content of the leaching slurry. Data plotted are the average of three replicates; error bars represent 1 standard deviation.

Figure 3.9 demonstrates that the greater the amount of leaching water to which a given amount of navy bean flour is exposed, the greater the proportion of the total mass of RFOs present in the flour is leached out. The fact that the proportions of stachyose and raffinose removed at each level of bean solids are approximately the same lends credence to the idea that the total amount of RFO leached out in a given system is determined by the concentration of RFOs in the leaching water when the system is at equilibrium.

The results shown in figure 3.9 demonstrate the dependence of the proportion of RFOs able to be leached from a given mass of navy bean flour on the amount of leaching water present. The consequence of this is that in order to achieve close to 100% removal a relatively large amount of leaching water must be used. An alternative to this is to employ multiple successive leaching stages using smaller amounts of water. If each stage is allowed to approach its equilibrium concentration then the initial concentration for the following leaching stage will significantly lower than that for the previous stage.

Depending on the ratios of bean flour to leaching water employed, it should be possible to approach total RFO removal within a handful of leaching stages.

In order to aid in predicting the effectiveness of multiple leaching stages the data presented in figure 3.9 can be presented in the form of a leaching equilibrium diagram. In order to do this the following data analysis was undertaken.

The mass of the insoluble solids in each bean flour sample can be calculated using the following equation:

$$m_{IS} = m_{flour}\left(1 - C_{SS-flour}\right) \quad (3.2)$$

Where m_{IS} = mass of insoluble solids in the bean flour sample (g)

m_{flour} = mass of the flour sample (g)

$C_{SS\text{-}flour}$ = soluble solids content of the flour (g/g) (see table 3.4)

The initial mass of a given RFO in the flour sample can be calculated using the following equation:

$$m_{RFO} = m_{flour} \times C_{RFO} \quad (3.3)$$

Where m_{RFO} = mass of a given RFO (either stachyose or raffinose) in the bean flour sample (g)

C_{RFO} = RFO content (either stachyose or raffinose) of the bean flour (g/g) (see table 3.1)

Assuming that no undissolved solids were present in the supernatant then the concentration of insoluble solids in the residue can be calculated on an insoluble free basis using the following equation:

$$N_y = \frac{m_{IS}}{m_{residue} - m_{IS}} \quad (3.4)$$

Where N_y = concentration of insoluble solids in the residue on an insoluble free basis (g/g)

$m_{residue}$ = mass of the residue following centrifugation of the leaching slurry (g)

The concentration of insoluble solids in the supernatant on an insoluble free basis (N_x) is assumed to be 0.

The concentration of a given RFO in the residue on an insoluble free basis can be calculated using the following equation:

$$y = \frac{C_{RFO-residue} \times m_{residue}}{m_{residue} - m_{IS}} \quad (3.5)$$

Where y = concentration of a given RFO (either stachyose or raffinose) in the residue on an insoluble free basis (g/g).

$M_{RFO\text{-}residue}$ = RFO content (either stachyose or raffinose) in the residue following centrifugation of the leaching slurry (g/g)

The concentration of a given RFO in the supernatant on an insoluble free basis can be calculated using the following equation:

$$x = \frac{m_{RFO} - (C_{RFO-residue} \times m_{residue})}{m_{supernatant}} \quad (3.6)$$

Where x = concentration of a given RFO (either stachyose or raffinose) in the supernatant on an insoluble free basis (g/g)

$m_{supernatant}$ = mass of the supernatant following centrifugation of the leaching slurry (g)

The concentration of insoluble solids in the leaching slurry on an insoluble free basis can be calculated using the following equation:

$$N_M = \frac{m_{IS}}{m_{residue} + m_{supernatant} - m_{IS}} \quad (3.7)$$

Where N_M = concentration of insoluble solids in the leaching slurry on an insoluble free basis (g/g)

The concentration of a given RFO in the leaching slurry on an insoluble free basis can be calculated using the following equation:

$$y_M = \frac{m_{RFO}}{m_{residue} + m_{supernatant} - m_{IS}} \quad (3.8)$$

Where y_M = concentration of a given RFO (either stachyose or raffinose) in the leaching slurry on an insoluble free basis (g/g)

Plotting y vs N_y, x vs N_x and y_M vs N_M for each of the leaching slurry concentrations yields the equilibrium diagrams given in figure 3.10(a) and 3.10(b).

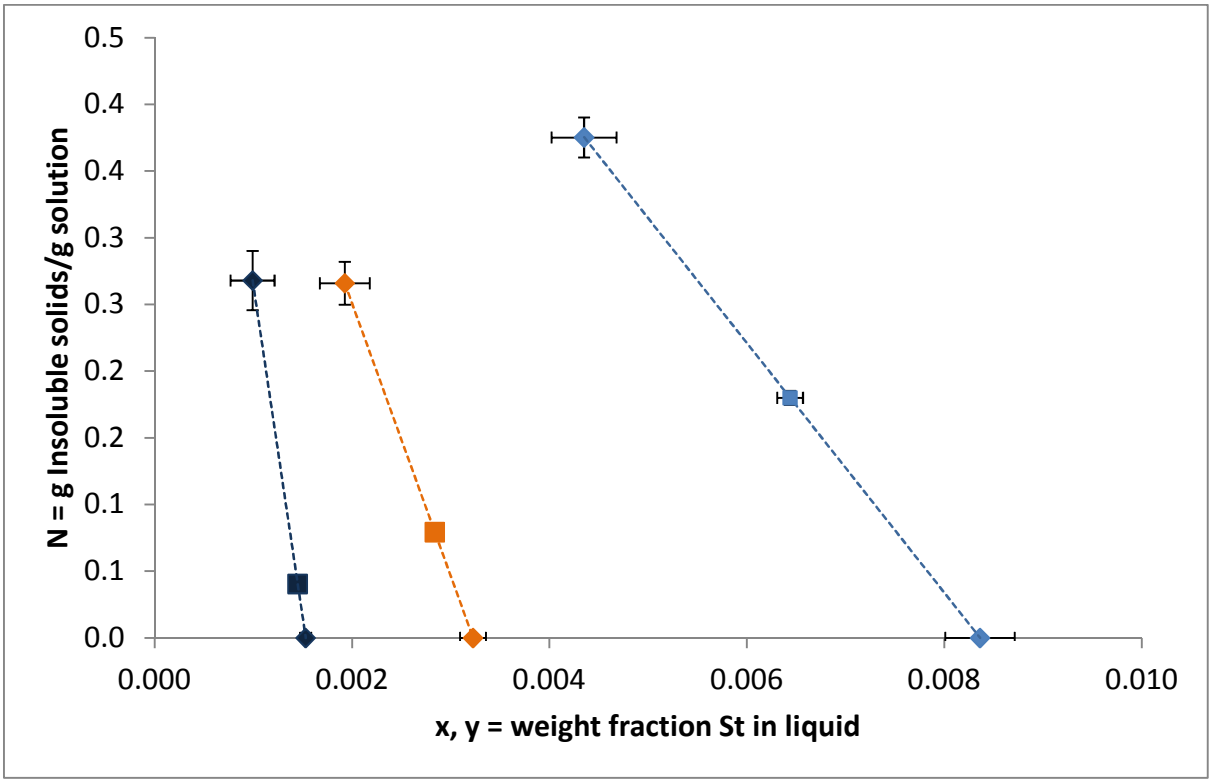


Figure 3.10(a) Equilibrium diagram for stachyose in leaching samples. Data is presented on an insoluble free basis. Plotted data are averages of 3 replicates and error bars represent 1 standard deviation.

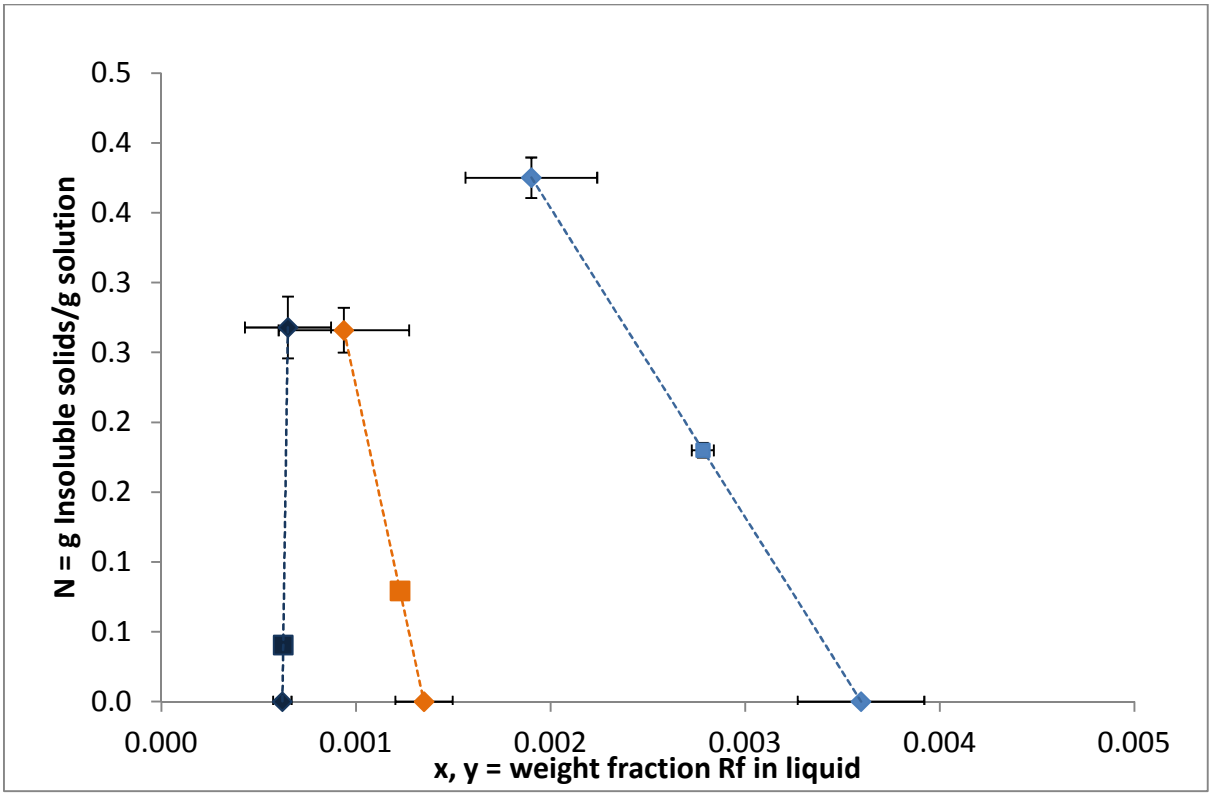


Figure 3.10(b) Equilibrium diagram for raffinose in leaching samples. Data presented on an insoluble free basis. Plotted data are averages of three replicates and error bars represent one standard deviation.

The tie lines plotted in figures 3.10(a) and 3.10(b) all have negative slopes indicating that the concentration of RFOs in the supernatant is higher than the concentration in the water associated with the residue. This result is counter intuitive and requires explanation.

As discussed in section 3.4 the navy bean flour consists of a combination of free starch granules, broken remnants of the intra cellular proteinaceous matrix and clusters of intact cells. The starch granules present in the flour are hydrophilic and according to Sathe and Salunkhe (1981a) can absorb up to 293% of their dry mass in water. Because the leaching slurries have been left for sufficient time for the flour particles to become saturated the starch granules in the flour (both free and bound within the intact cells) will have absorbed approximately 3 times their dry mass of water. It is therefore likely that a significant proportion of the water content of the residue is bound within the starch granules and therefore not available for leaching of RFOs.

Berg et al (2012) measured the starch content of navy beans supplied by Heinz Watties Ltd to be 36.1% (db). Since the beans used in this work were also sourced from Heinz Watties Ltd it is sensible to assume a similar starch content for these beans.

Re-analysis of the above data by including the water absorbed by the starch granules ($m_{starch\text{-}water}$ = 2.93 x 0.361 x m_{flour} (db)) as contributing to the insoluble content of the residue results in equilibrium diagrams given in Figures 3.11(a) and 3.11(b).

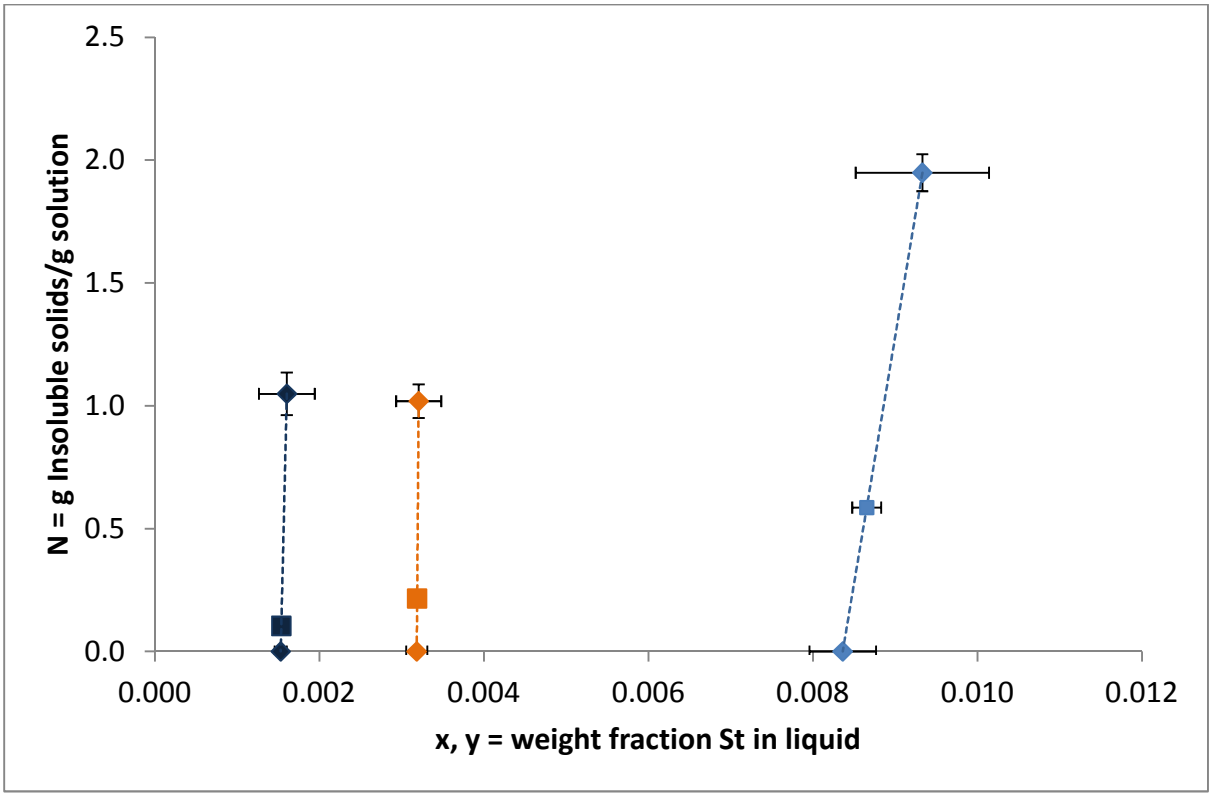


Figure 3.11(a) Equilibrium diagram for stachyose in leaching samples – corrected for unavailable water bound in the starch granules. Data is presented on an insoluble free basis. Points plotted are the average of three measurements and the error bars represent 1 standard deviation.

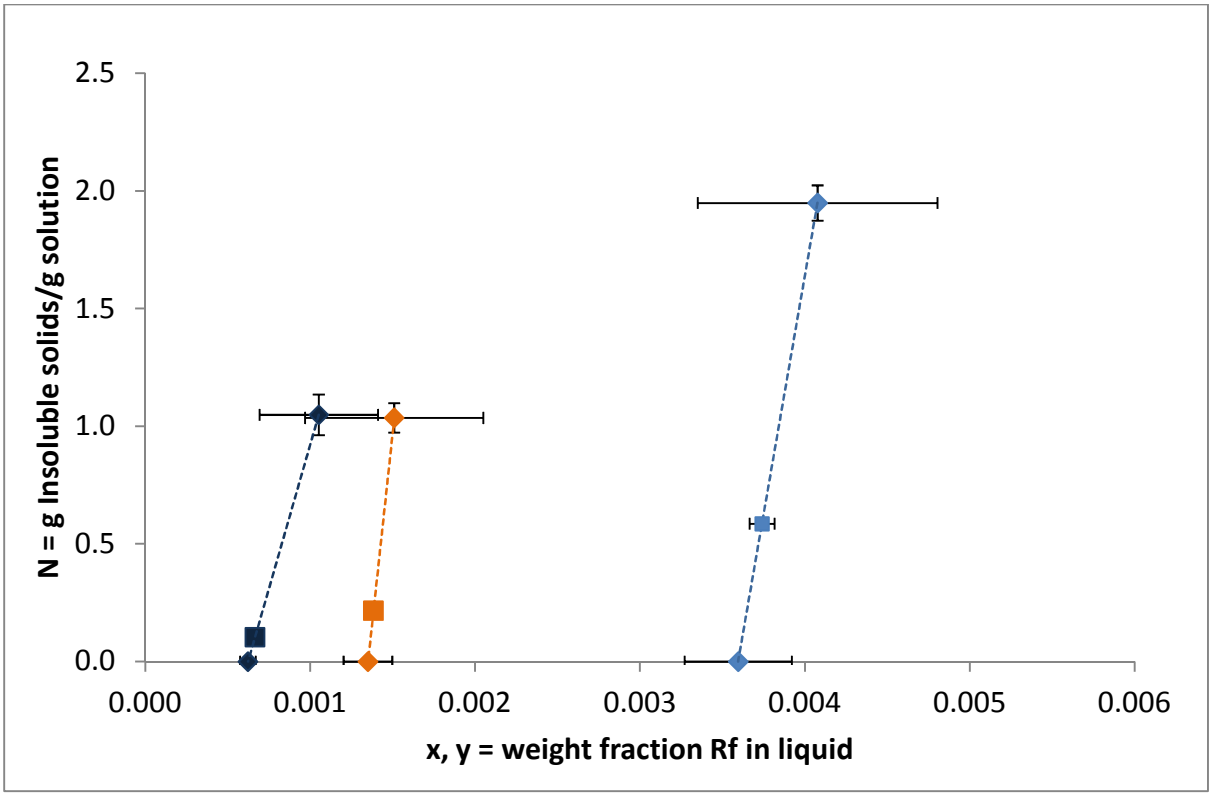


Figure 3.11(b) Equilibrium diagram for raffinose in leaching samples – corrected for unavailable water bound in the starch granules. Data is presented on an insoluble free basis. Points plotted are the average of three measurements and the error bars represent 1 standard deviation.

It can be seen in figures 3.11(a) and 3.11(b) that when the unavailable water bound in the starch granules is included in the insoluble fraction the slope of the tie lines in the equilibrium diagrams become positive. This indicates either that some of the water associated with the starch granules contribute to RFO leaching or that there is some preferential adsorption of the RFOs to the insoluble fraction of the bean flour.

Equilibrium diagrams of the sort displayed in figures 3.11(a) and 3.11(b) can be used to predict the efficiency of a stage-wise leaching process. However it is unusual that the N_y value for the 20% bean slurry is greater than those for the more dilute slurries. A possible cause for this may have been some carryover of insoluble solids into the supernatant for the 20% slurry. In order to be able to use the data presented in figures 3.11(a) and 3.11(b) to predict the efficiency of a stage-wise leaching process the average N_y value for the two dilute slurry concentrations was calculated and applied to the 20% slurry data. This is displayed in figures 3.12(a) and 3.12(b).

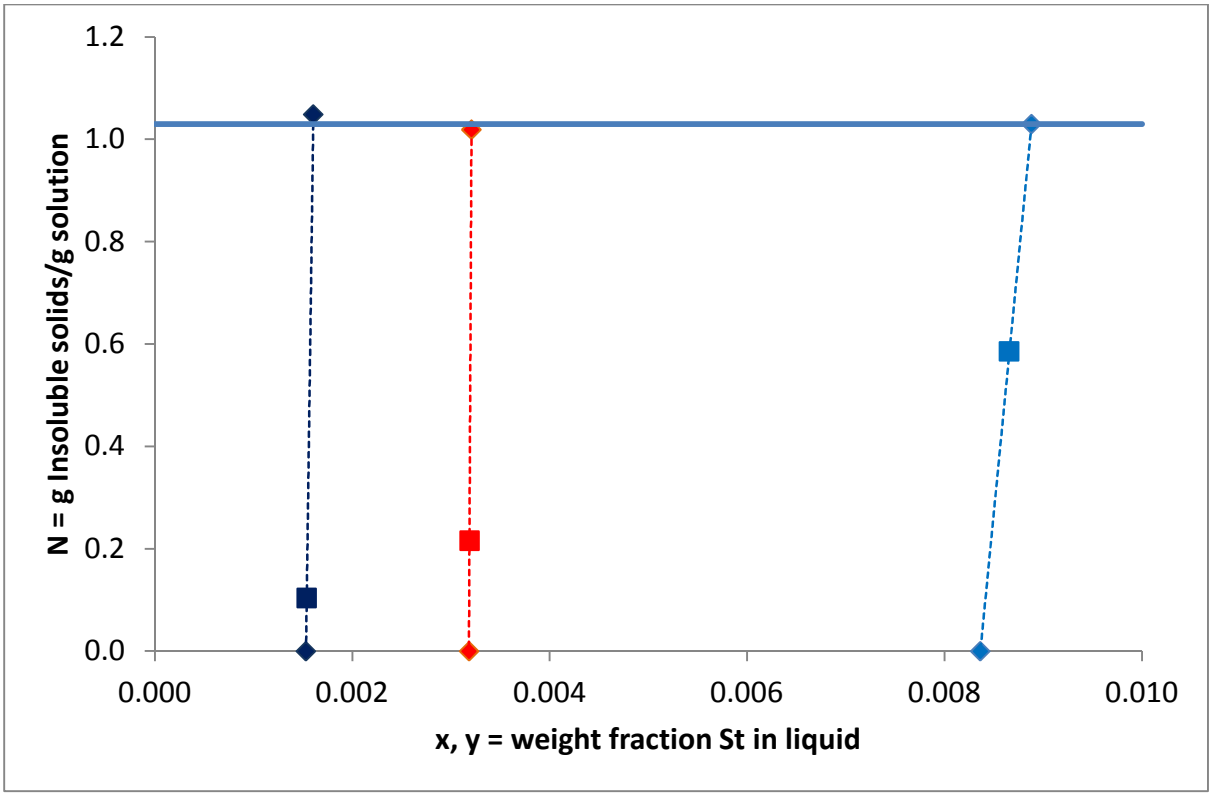


Figure 3.12(a) Equilibrium diagram for stachyose in leaching samples – corrected for unavailable water bound in the starch granules and insoluble particle carryover into the supernatant. Data is presented on an insoluble free basis. Points plotted are the average of three measurements.

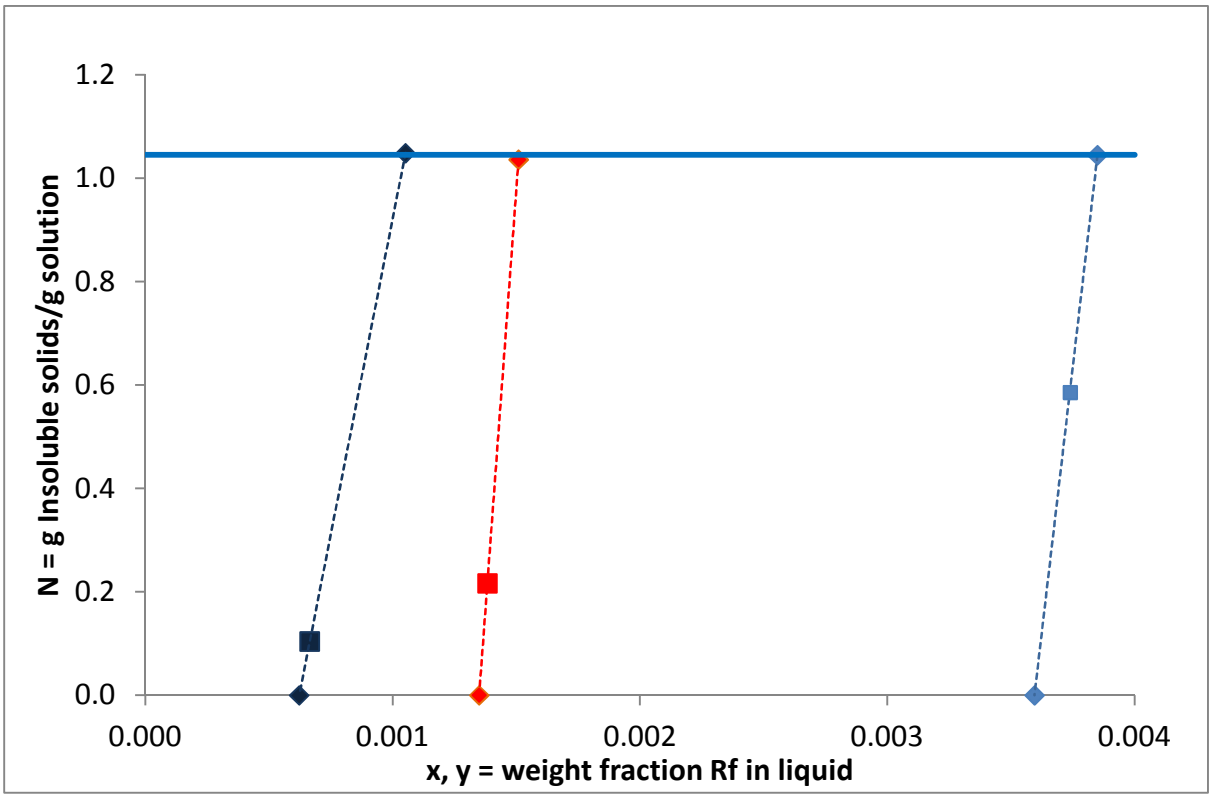


Figure 3.12(b) Equilibrium diagram for raffinose in leaching samples – corrected for unavailable water bound in the starch granules and insoluble particle carryover into the supernatant. Data is presented on an insoluble free basis. Points plotted are the average of three measurements.

Using figures 3.12(a) and 3.12(b) the amount of RFOs expected to be removed in a stage-wise leaching system can be determined.

For a leaching slurry in which the dry bean solids comprise approximately 15% of the total mass, the mixture point (M_1) can be found by plotting y_M vs N_m (equation 3.7 and 3.8). A tie line can then be drawn through M_1.

Point E_1 can be found at the intersection of the tie line with the N_y vs y line and represents the concentration of RFO in the residue versus the concentration of insoluble solids in the residue (on an insoluble free basis).

Plotting the initial concentration of RFO in the water versus the initial concentration of the insoluble solids in the water gives the point R_0 at the origin. A straight line can then be drawn between points E_1 and R_0.

If the 15% solids bean slurry is allowed to reach equilibrium prior to being centrifuged and the supernatant is removed and replaced with fresh water, the value of N_M can be determined for the resulting slurry using the following equation:

$$N_{Mx} = \frac{m_{IS}}{m_{reisdue} + m_{H20x} + m_{IS}} \tag{3.9}$$

Where N_{Mx} = Concentration of insoluble solids in a given stage leaching slurry on an insoluble free basis (g/g)

m_{H2Ox} = mass of water added to the residue from the previous stage to make up the given stage leaching slurry (g)

The point on the straight line plotted between E_1 and R_0 at which the N value equals N_{M2} corresponds to the mixture point for the second leaching stage (M_2). A tie line can be drawn through M_2 and the point where the tie line intersects the N_y vs y line corresponds to point E_2.

N_{M3} can be calculated using in equation 3.9 and the point on a straight line plotted between E_2 and R_0 at which the N value equals N_{M3} corresponds to the leaching point for the third leaching stage (M_3). This process can be continued for as many leaching stages as required.

Figures 3.13(a) and 3.13(b) illustrate the process for four leaching stages.

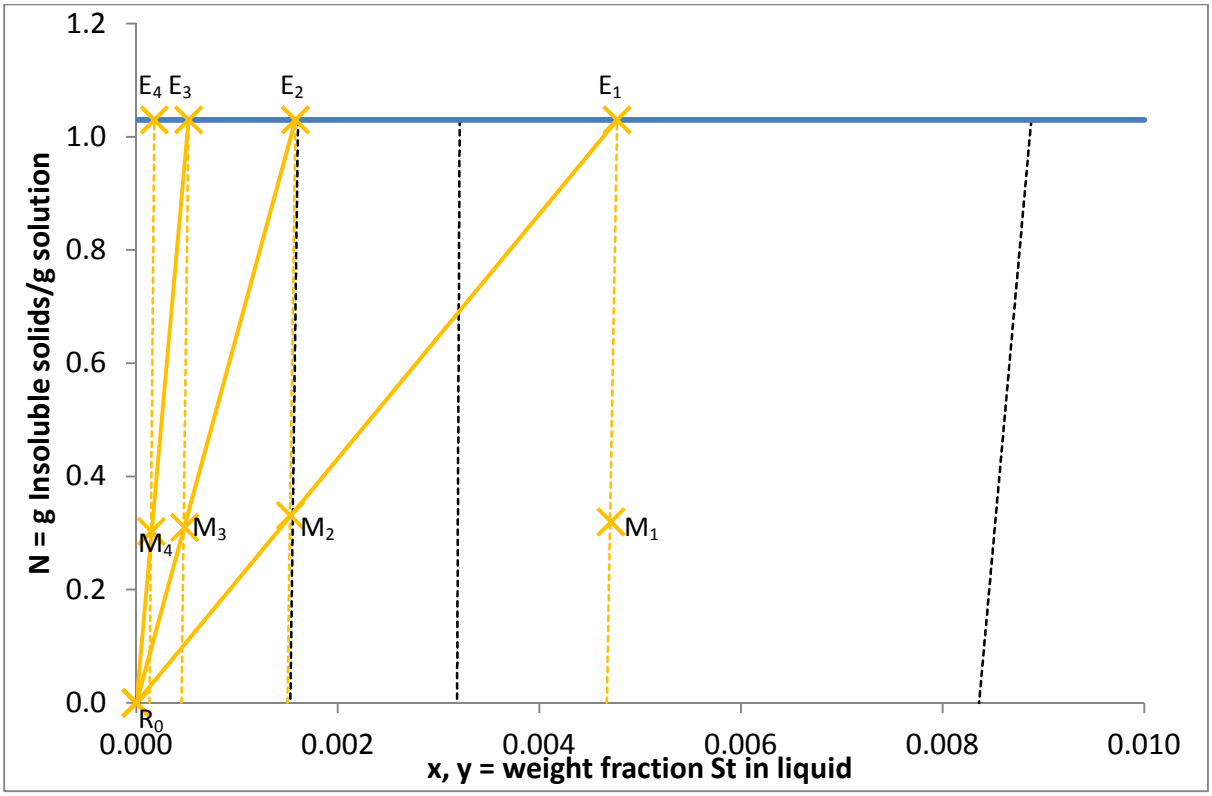


Figure 3.13(a) Equilibrium diagram illustrating the calculated stachyose removal during a 4 stage leaching process. Orange lines represent the 4 stage leaching operation; black lines represent the measured tie lines from figure 3.11(a).

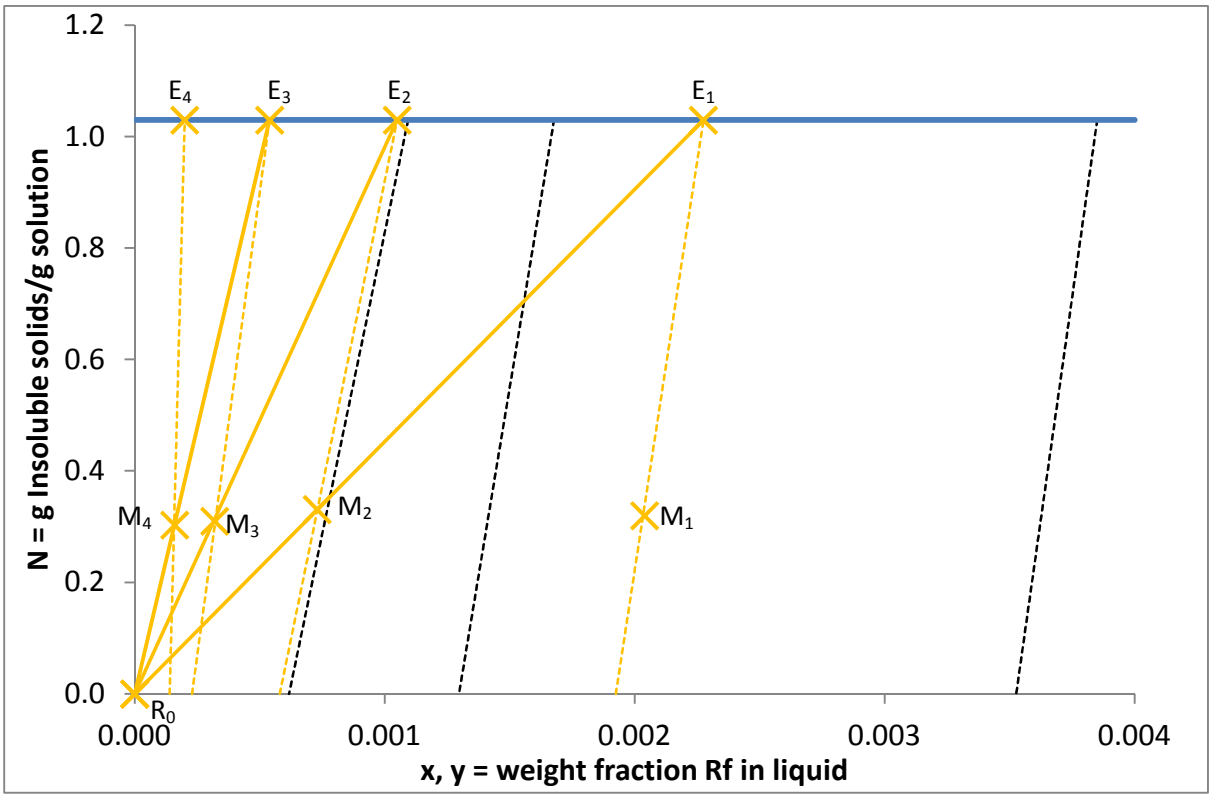


Figure 3.13(b) Equilibrium diagram illustrating the calculated raffinose removal during a 4 stage leaching process. Orange lines represent the 4 stage leaching operation; black lines represent the measured tie lines from figure 3.11(b).

The value of y at the E point corresponding to each leaching stage can be used to calculate the concentration of RFO remaining in the residue following that leaching stage using the following equation:

$$C_{RFO} = y\left(1 - \frac{m_{IS}}{m_{residue}}\right) \tag{3.10}$$

Application of the above calculation for the E_4 points in figures 3.12(a) and 3.12(b) results in predictions of 99.73% removal of stachyose and 99.3% removal of raffinose.An experiment was carried out to validate the above predictions.

A 2g sample of navy bean flour was placed in a pre-weighed 40ml centrifuge tube with approximately 10 ml of distilled water at 23°C. The mixture was agitated for 30 minutes using a magnetic stirrer. The sample was then centrifuged at 3000 rpm for 30 minutes (eppendorf 5702 with rotor radius 14cm). The supernatant was collected and the tube + residue were weighed. The supernatant was passed through a 0.45 micron syringe filter and analysed for RFOs using the HPLC method described in section 3.2.

Fresh distilled water was added to the tube to bring the total mass of the system back to approximately 12g. These steps were repeated such that the sample was leached through 4 stages in total. Following the final leaching stage the RFOs remaining in the residue were extracted using the method described in section 3.3 and measured using the HPLC method described in section 3.2.

These results are shown in figure 3.14 below, which shows near complete removal of RFOs was achieved after 3-4 leaching stages.

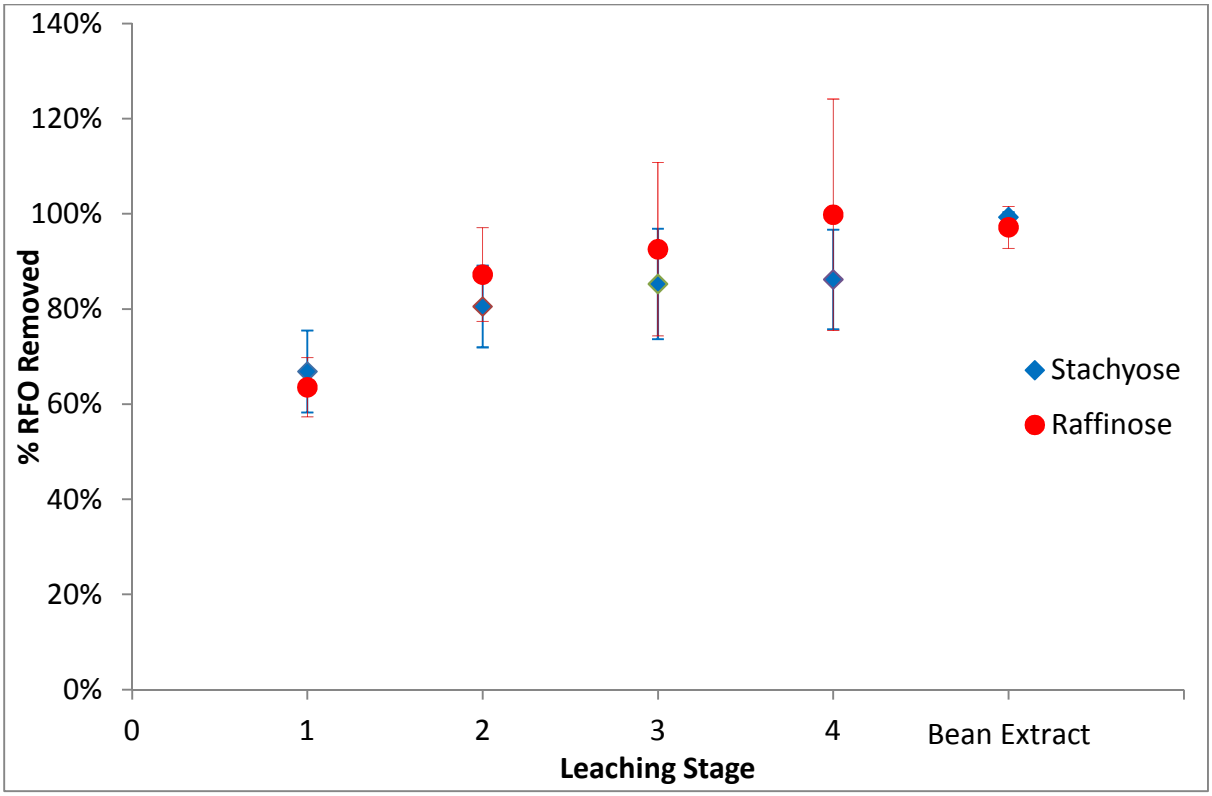


Figure 3.14 RFO removal by successive leaching stages using fresh water at each stage. At each stage the flour was agitated in the leaching water for 30 minutes at 23°C. Values displayed are the mean of 3 measurements and the error bars represent one standard deviation.

The proportion of the original RFO removed from the bean flour after four leaching stages was measured to be 99.34% for stachyose and 97.22% for raffinose. These values agree with the values predicted above within the experimental uncertainty.

Figures 3.15(a) and 3.15(b) below show the data from the four stage leaching measurements plotted on equilibrium diagrams for comparison with figures 3.13(a) and 3.13(b).

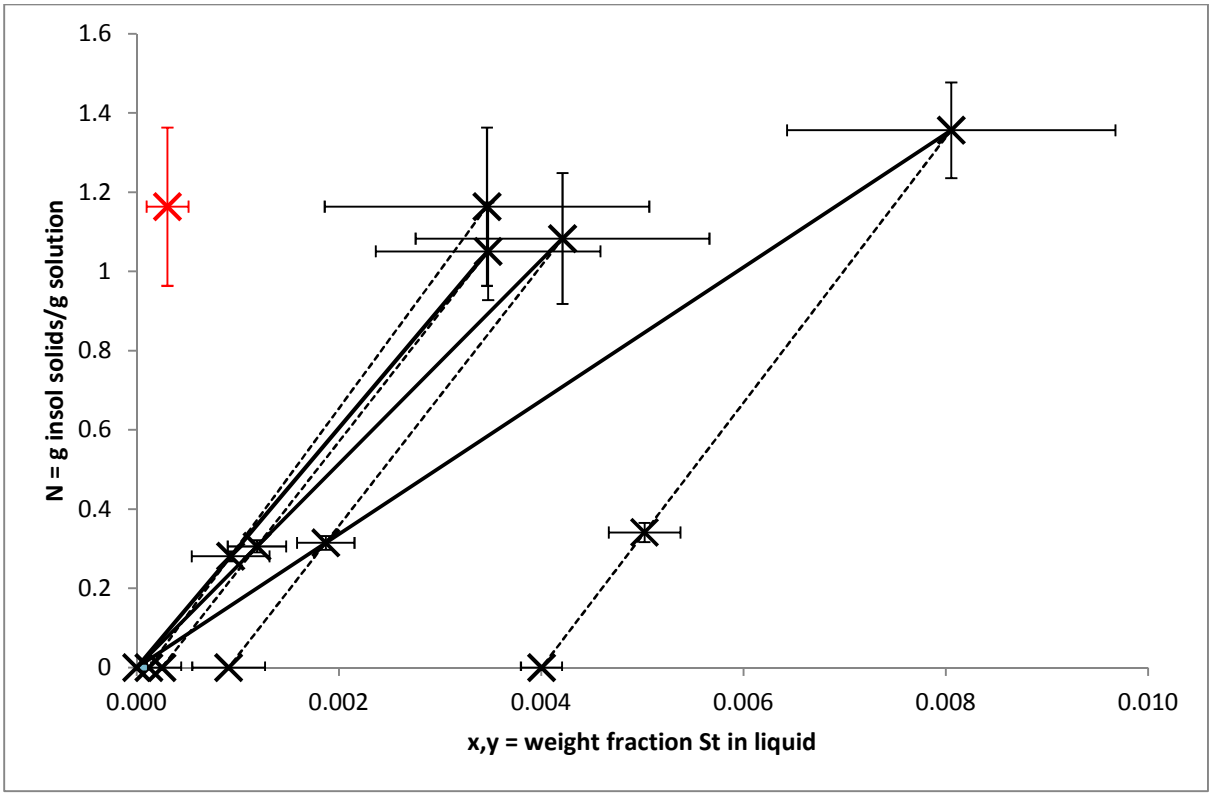


Figure 3.15(a) Equilibrium diagram illustrating the measured stachyose removal during a 4 stage leaching process. Black lines represent the data based on stachyose concentration measured in the supernatant; red cross shows the position of the E_4 value based on the stachyose concentration measured in the residue. Plotted data are averages of three replicates and error bars represent one standard deviation.

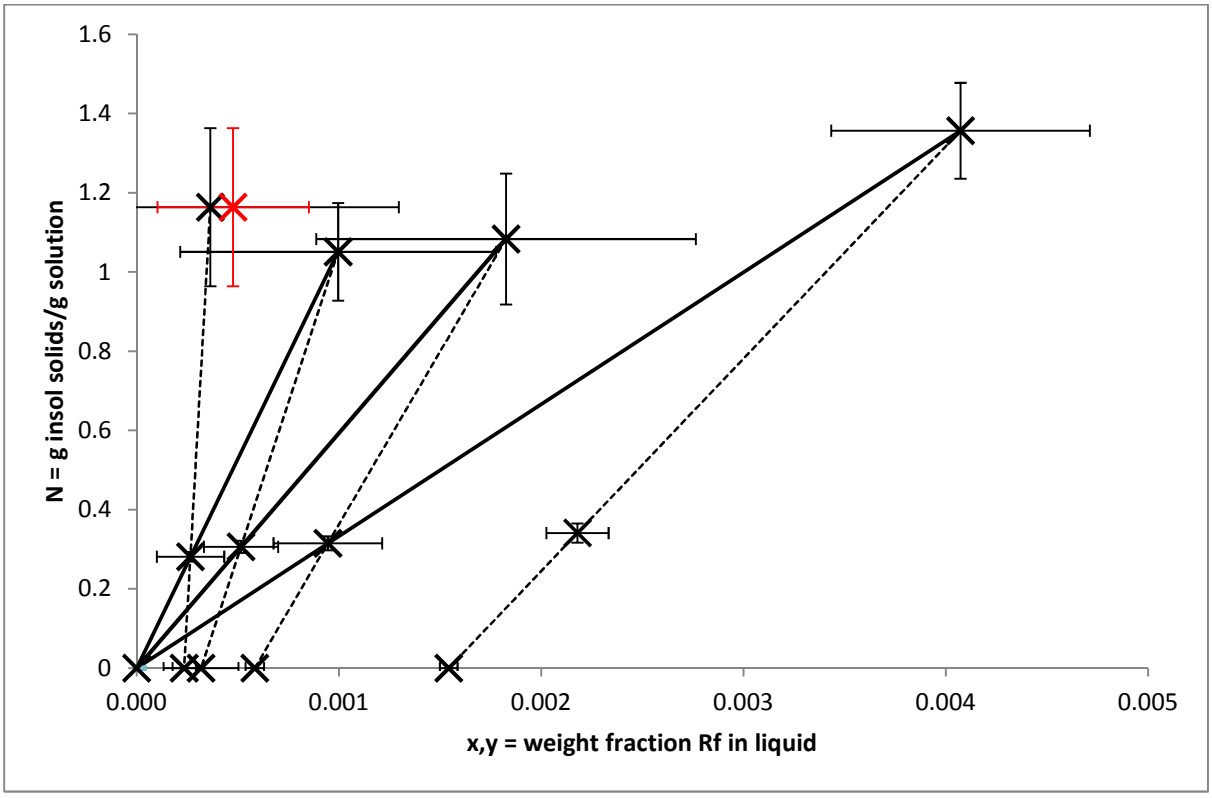


Figure 3.15(b) Equilibrium diagram illustrating the measured raffinose removal during a 4 stage leaching process. Black lines represent the data based on raffinose concentration measured in the supernatant; red cross shows the position of the E_4 value based on the raffinose concentration measured in the residue. Plotted data are averages of three replicates and error bars represent one standard deviation.

Comparison of figures 3.15(a) and 3.15(b) with figures 3.13(a) and 3.13(b) shows that the measured stage-wise leaching data display significantly larger slopes on the tie lines than the predicted data. There is also poor agreement between the E_4 values measured using the supernatant and those measured using the final residue.

In general the chromatograms generated for measurement of the RFO concentrations in the supernatant display more variable baselines and are therefore less accurate that measurements of RFOs extracted from the residue. This observation is borne out to a certain extent in the smaller standard deviations for the E_4 values calculated from the residue concentration compared to those for the E_4 values calculated from the supernatant concentration. Furthermore the concentration of RFO measured in the final residue compares well with the value predicted using the data presented in figures 3.13(a) and 3.13(b).

It is therefore expected that the true E values are smaller than those calculated from the measured concentration of RFOs in the supernatant at each stage and hence that the tie lines are in reality closer to those predicted in figures 3.13(a) and 3.13(b).

Nevertheless the data presented in figures 3.14, 3.15(a) and 3.15(b) demonstrates that multiple leaching stages can be successfully employed to achieve very close to complete removal of RFOs from navy bean flour.

3.5.3 Enzyme Hydrolysis

The data presented figure 3.9 has demonstrated that the ratio of bean flour to water in a leaching slurry plays a large role in the amount of RFOs that can be leached from the flour.

It has also been observed in figures 3.10 through to 3.15(b) that the replacement of leaching water with fresh water allows the leaching process to continue past the point of the original equilibrium concentration.

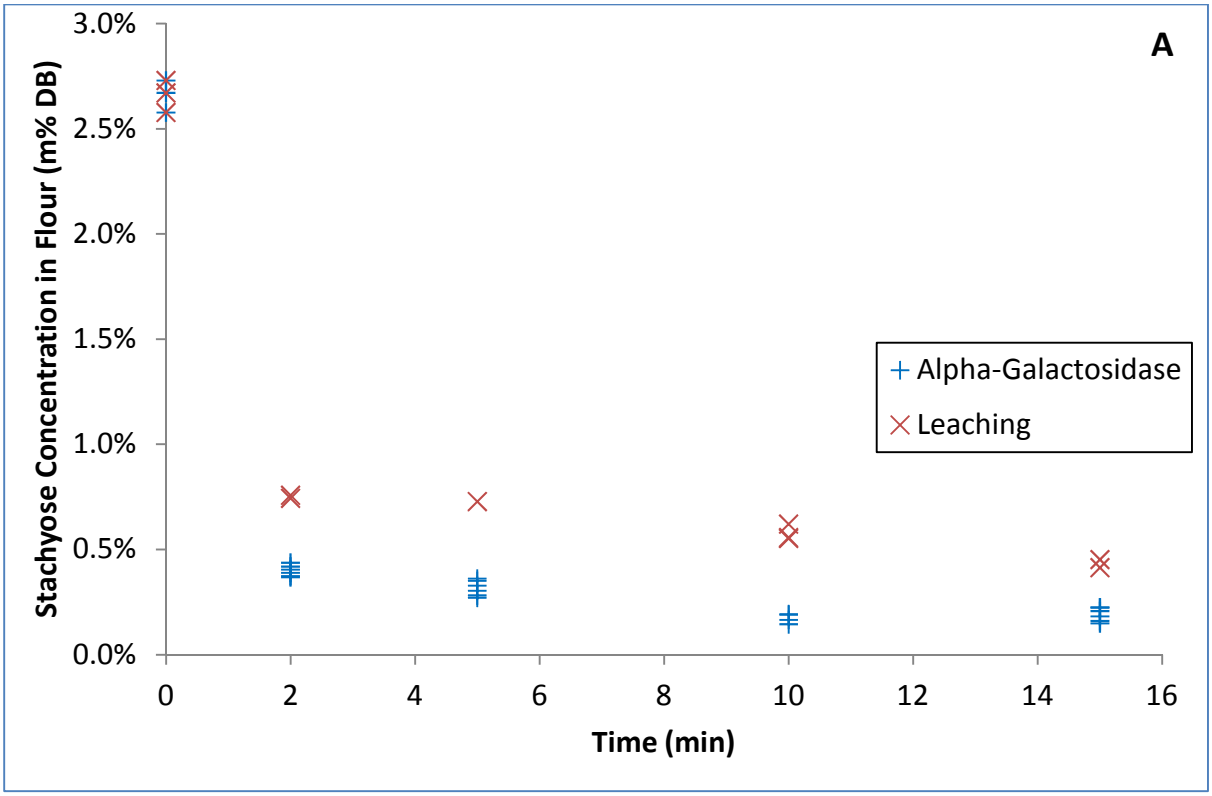
The driving force for diffusion of a solute in solution is the concentration gradient between a high concentration region and a low concentration region. It follows from this that if the RFO concentration in the leaching water could be kept close to zero then the leaching would proceed at close to the maximum rate until all of the RFOs have leached out. One way to achieve this would be the use of continuous running water.

This is the extreme limit of the multiple leaching stage technique. However, this could be difficult to achieve in practise as the navy bean powder would need to be continuously filtered from the running water.

Another way to maintain RFO concentrations close to 0 in the leaching water is to add an enzyme such that it hydrolyses the RFOs at the same rate or faster than the rate at which they can leach out of the flour. The literature reviewed in chapter 2 identified α-galactosidase as the appropriate enzyme to hydrolyse stachyose and raffinose.

An experiment was conducted to determine whether the addition of α-galactosidase to the leaching water increases the rate of RFO removal from navy bean flour and increases the overall amount of RFOs removed from the flour.

The method used to produce figure 3.7 was repeated with the exception that the leaching water contained α-galactosidase (supplied by Novozymes®) at a concentration of 5% by mass. The results are plotted with the data from figure 3.7 with no enzyme.



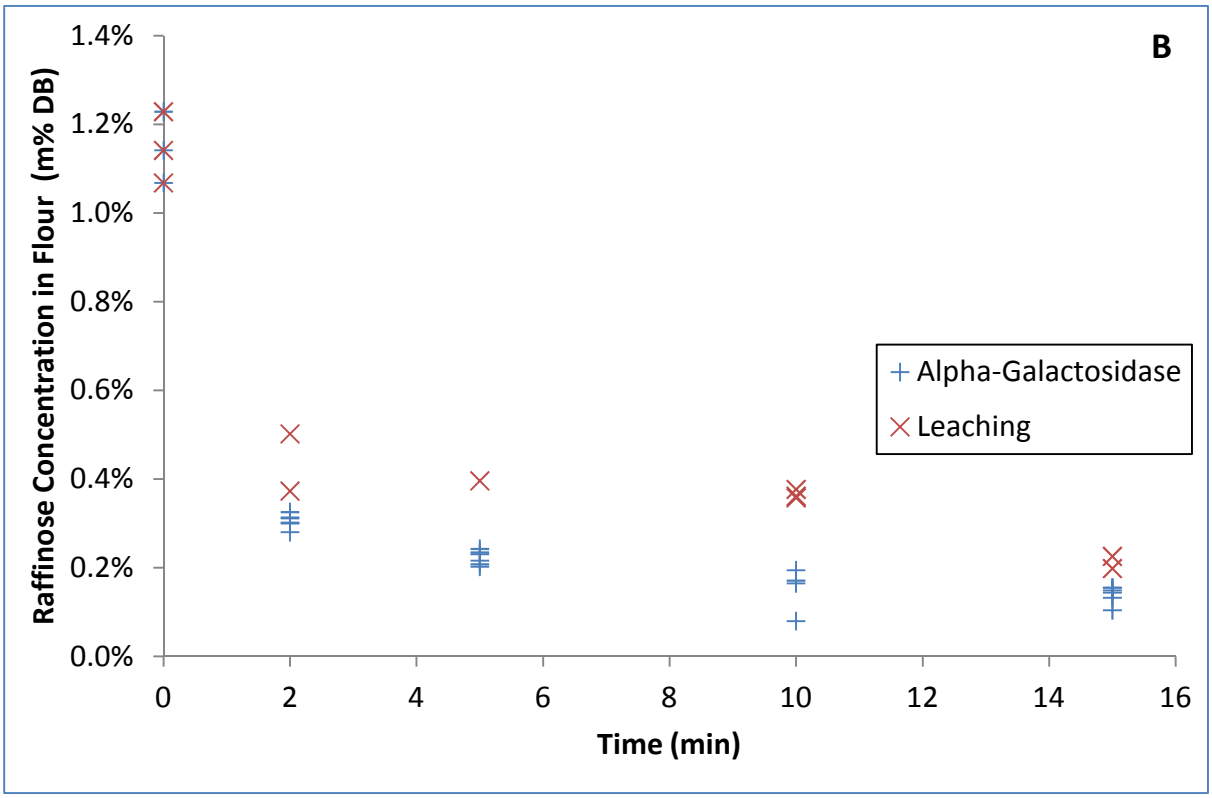


Figure 3.16 Rate of hydrolysis of **A:** stachyose and **B:** raffinose in a slurry containing 10% navy bean flour and 5% alpha-galactosidase by mass at 23°C. Leaching data for the same conditions without enzyme is included for comparison.

Figure 3.16 shows a clear increase in both the rate of RFO removal and the total amount of RFOs removed for the flour slurries containing α-galactosidase over those without the enzyme. The total amount of RFO remaining in the enzyme treated samples at each time point is roughly half that of the corresponding point for the leaching samples.

The rate of removal of RFOs follows a similar trend to those observed in figure 3.7 in that the initial rate of removal is very high with approximately 80% of the RFO removed within the first two minutes. Again the rate then tapers off as the concentration of RFOs remaining in the flour, and therefore the concentration gradient between the flour and the bulk solution decreases. This indicates that the ability of the α-galactosidase to hydrolyse the RFOs is in some way dependent on the rate of RFO leaching out of the bean flour.

3.6 Conclusion

The concentration of RFOs in the plumule of the navy bean was found to be approximately four times that in the cotyledons. However, the plumule accounts for less than 2% of the total mass of the bean, and therefore the removal of these components is not an efficient means by which to significantly reduce the concentration of RFOs in navy beans.

Leaching of raffinose family oligosaccarides from navy bean flour is much faster than reported for whole beans; this is probably due to the decreased particle size and increased surface area of the flour. While disruption of cells may contribute to the increase removal rates a significant proportion of the navy bean flour consists of groups of intact cells. Leaching therefore can be considered a rapid and effective mechanism for the removal of RFOs from navy bean flour. However there are several major draw backs to this process, those being the need to recover the flour from the leaching water, the high moisture content of the resulting product and the high proportion of total solids lost through leaching of soluble solids including soluble vitamins and minerals. These problems all increase the both the cost of processing to produce a dry RFO-free navy bean flour and the capital cost to set up the plant. This includes the capital and running costs of a separator or filter unit to separate the solids from the leaching slurry and a fluidised bed or other type of drying unit. The cost in lost solids if all of the soluble content of the bean were lost this would effectively increase the raw ingredient cost of the RFO free flour from $0.69 to $0.93 per kilogram, this constitutes a 35% increase over the cost of the broken beans.

The enzymatic hydrolysis of RFOs by α-galactosidase is also an effective mechanism for the decrease of RFO content in navy bean flour. An advantage in the use of α-galactosidase to hydrolyse RFOs is that there is no loss in soluble solids; the RFOs are simply converted into digestible sugars. This means that the vitamins and minerals that confer nutritional quality to the navy beans remain in the final product and the raw material cost for the flour remains at $0.69 per kilogram.

The overall rate of hydrolysis of RFOs in navy bean flour is thought to be highly dependent on the rate of diffusion of the solutes out of the bean particle. The testing and application of this hypothesis and its various implications will be the focus for the remainder of this work.

Chapter 4

Measurement of Leaching Rates

The previous chapter outlined several possible mechanisms by which the concentration of RFOs can be decreased in navy bean flour. The conclusions arrived at in chapter three are that when the size of the bean flour particles are very small in comparison with the size of a whole bean then the rate at which RFOs are leached from particles when they are submerged in water is rapid compared to the leaching time for whole beans.

The experiments reported in chapter three also show that the total decrease in concentration of RFOs in the bean flour following leaching is proportional to the ratio of the mass of leaching water and the mass of bean flour. This is because the leaching mechanism continues to extract RFOs from the bean flour only until an equilibrium concentration is reached between the flour and the leaching water. As discussed in section 3.5 the concentration of RFOs in the bean flour may not be equal to that in the leaching water at the equilibrium point, due to preferential adsorption of the RFOs to the flour.

Furthermore the leaching rate of the RFOs at any given time is proportional to the difference in concentration of RFOs between the bean flour and the leaching water at that moment in time.

It follows therefore that a higher rate of leaching is maintained for a longer period if the mass of leaching water is much larger than the mass of bean flour (i.e. it takes a larger absolute mass of RFOs to significantly increase the concentration in the leaching water). Therefore the overall time it takes for the system to approach equilibrium will be shorter and the final concentration of RFOs in the bean will be lower than that for a system in which the relative amount of water is small.

At first glance it would therefore seem that the ideal solution to the problem of high RFO content in navy bean flour is to perform a leaching operation on the flour using a high ratio of water to flour. However, as discussed in chapter three there are some major drawbacks to this approach. Unless the particular application to which the navy bean flour is to be put requires a relatively dilute slurry there will be the need to remove a large fraction of the water phase of the resulting slurry, adding both cost and complexity to the process.

If the water phase is removed via an evaporation step then the resulting concentrate will retain all of the RFOS that have been leached out of the bean flour. However if the water phase is separated from the solids, for example via filtration, then the leached RFOs will be removed along with the water. However all of the other soluble solids that have been extracted will be removed along with the RFOs (approximately 25% of the dry mass of the bean flour including many water soluble vitamins and other nutrients – see section 3.4.3).

RFO removal from a bean particle through diffusion would be maximised if the concentration gradient between the leaching water and the bean flour remains at a maximum. This could be achieved by using a very large volume of leaching water, however this would result in the loss of all of the soluble solids. To that end it is proposed that a combination of leaching and enzymatic hydrolysis be employed. The presence of α-galactosidase in a small volume of leaching water would hydrolyse the RFOs as they were leached out of the bean flour, effectively keeping the concentration of RFOs in the leaching water at zero.

This would allow the maximum leaching rate whilst minimising the ratio of leaching water to bean flour. The bean flour/water mixture could then be dried so that all of the soluble solids are retained with the RFOs having been converted into di- and monosaccharides.

In such a system the rate at which the RFOs can diffuse to the surface of the bean particle will be critical in determining the rate of RFO hydrolysis. The overall rate of RFO leaching from the bean particle at any given time during the leaching process will be dependent on the moisture content of the bean, the particle size, the concentration gradient within the bean particle/water system and the temperature.

In order to develop an understanding of the leaching component of the proposed system the rate of water uptake and the rate of RFO removal from navy bean particles needs to be measured.

The data presented in chapter three does a good job of illustrating how quickly the RFOs can be leached from navy beans when they are milled into a flour, however this extremely high leaching rate does not allow for sufficient samples at intermediate concentrations to define the leaching rate with reasonable precision.

In order to slow the leaching to a rate at which a reasonable number of samples can be collected over the course of the leaching the size of the bean particles must be significantly increased. The obvious solution to this problem is to use whole beans, however the seed coat or ”hull” of the whole bean is a moisture barrier which could lead to the measurement of leaching rate data that doesn’t accurately represent the flour particles.

4.1 Preparation of De-Hulled Navy Beans

There are a number of techniques that have been developed for the de-hulling of cereal grains and legume seeds, however these techniques usually employ the use of large scale specialist equipment for use in industrial applications, for example the de-hulling of soya beans in the production of soya bean oil. Other techniques involve hydrating the bean to soften the hull to ease its removal.

Lack of access to specialised equipment and the small volume of de-hulled beans required for this work preclude the former approach and the need to keep the bean dry rules out the latter. Therefore after much trial and error a small scale technique was developed to produce dry, de-hulled single cotyledons. Each cotyledon accounts for close to 50% of the total mass of the bean and was therefore large enough to provide leaching over a more easily measurable timescale.

The de-hulling involves the use of a food processor (Breville Super Wizz Duo 800W) with a "chipping disk" attachment which substitutes the blade in the food processor for a solid plate with raised serrations. The beans are introduced to the food processor chamber while the grater is spinning and the high speed impacts between the serrations and the beans cause the beans to break in half forming the two cotyledons. Approximately 50g (1 handful of beans were processed per run and the blade was stopped after approximately 20 – 30 seconds. This process also strips the hulls from a significant proportion of the cotyledons. The de-hulled cotyledons are then manually separated from the broken cotyledons and those that still retain their hulls. Figures 4.1 A – C show the beans before and after processing and following separation of the intact de-hulled cotyledons.

Figure 4.1 Navy bean before de-hulling (A), after de-hulling process (B) and final de-hulled cotyledon (C)

4.2 Characterisation of De-Hulled Navy Bean Cotyledons

There are several characteristics of de-hulled navy bean cotyledons that are required for subsequent analysis of the leaching rate. These include:

- Initial moisture content
- Saturated moisture content
- Density

Some of these parameters were measured for the milled bean flour in chapter three; however they were re-measured for the de-hulled cotyledon. The flour also includes milled hull and thus has a slightly different composition, so some variation might be expected.

4.2.1 Initial Moisture Content

2g samples of de-hulled cotyledons were weighed onto pre-weighed watch glasses. The watch glasses were placed in a vacuum oven at 105°C for 20 hours. The watch glasses were then placed in a desiccator for 5 minutes to cool down, before weighing. The watch glasses were then returned to the vacuum oven for 1h before being cooled in a desiccator and re-weighed. The mass of the watch glass + sample measured after each period in the vacuum over were compared to ensure that no further evaporation was taking place. The final mass of the cotyledons was compared to the initial mass to give the initial moisture content of the navy bean cotyledons. Samples were repeated in triplicate. The mean of the results from each replicate was calculated and used in subsequent calculations as the value for initial moisture content of the navy bean cotyledons ($C_{wi\text{-}Cotyledon}$).

Table 4.1 below, shows the initial moisture content of the de-hulled cotyledons. They were dryer than observed in the flour samples shown in table 3.2 (11.4% versus 14.8% WB).

Table 4.1 Initial moisture content of de-hulled navy bean cotyledons on both wet and dry basis.

Replicate	Wet basis	Dry Basis
1	11.3%	12.7%
2	11.5%	13.0%
3	11.4%	12.9%
$C_{wi\text{-}Cotyledon}$	11.4%	12.9%

4.2.2 Saturated Moisture Content

1.5g samples of de-hulled navy bean cotyledons were placed in 30 ml vials with 20ml of distilled water and left to hydrate for 24 hours. The contents of the vial were then emptied through a sieve and the recovered cotyledons were patted dry using absorbent paper. The mass of the hydrated cotyledons was then measured before being transferred to a pre-weighed watch glass. The samples were then dried in a vacuum oven at 105°C as above until no change in mass was observed between drying periods. The dry mass of the sample was measured and compared to the mass of the hydrated cotyledons to determine saturated moisture content. Samples were repeated in triplicate at ambient (23°C), 35°C and 55°C.

The results of these measurements are summarised in table 4.2

Table 4.2 Moisture content of de-hulled navy bean cotyledons after 24h soaking at 23, 35 and 55°C. Values presented are the mean of 3 measurements ± SE. Values are reported on both wet and dry basis.

	Temp	Wet Basis	Dry Basis
$C_{wS\text{-}Cotyledon}$	23°C	55.7% ± 0.2%	125.7% ± 0.4%
$C_{wS\text{-}Cotyledon}$	35°C	55.2% ± 0.3%	122.3% ± 0.6%
$C_{wS\text{-}Cotyledon}$	55°C	56.5% ± 1.9%	130.6% ± 4.5%

The moisture content measured for the de-hulled cotyledons after 24 hours soaking are significantly lower than the saturated moisture content measured for the navy bean flour in table 3.3. The saturated moisture content measured for bean flour is likely to be higher than the actual saturated moisture content due to inter-particulate water within the residue cake following centrifugation. It is also possible that the moisture content for the de-hulled cotyledons after 24 hours has not reached the saturated moisture content, and that water absorption was still occurring in those samples. However the fact that there is no significant difference between the values measured across the three temperatures suggests that saturation has been achieved.

Interestingly the amount of soluble solids lost by the samples at each temperature increases significantly with temperature. 11.25% (DB) is lost from the 23°C samples, 13.86% (DB) is lost from the 35°C samples and 17.82% (DB) is lost from the 55°C samples. This is the same trend reported by Kon (1979) and as discussed in chapter two this indicates that the diffusion rates of the soluble solids are slower than the rate of water diffusion into the particle, and that they are highly dependent on temperature.

4.2.3 Density

The density of 1g (approximately 18 cotyledons) samples of de-hulled navy bean cotyledons was determined using displacement of canola oil in a 10mL volumetric flask. The cotyledons were placed in the flask and the mass of oil required to fill the flask to the mark was measured.

The volume of the cotyledons was calculated by comparison of the data to the density of the oil measured using an empty volumetric flask. Density measurements were carried out in triplicate. The mean of the results from each replicate was calculated and used in subsequent calculations as the value for density of the navy bean particles (ρ).

Table 4.3 summarises these results.

Table 4.3 Density of de-hulled navy bean cotyledons on both wet and dry basis.

Replicate	WB	DB
1	1395 g/L	1336 g/L
2	1358 g/L	1203 g/L
3	1392 g/L	1233 g/L
ρ	1382 g/L	1225 g/L

4.3 Water Absorption Rates in De-Hulled Navy Bean Cotyledons

As discussed in chapter 3 the moisture content of the bean has a large effect on the rate at which the RFOs leach out because of its effect on molecular mobility of the RFOs in low moisture systems and hence on the rate at which they can diffuse through the bean matrix. This in turn means that the rate of moisture diffusion into the bean particle during leaching is very important. As such, an experiment was carried out to determine the rate at which water is absorbed into de-hulled navy bean cotyledons.

1.5g samples of de-hulled cotyledons were weighed into 30mL vials. 20mL of distilled water was added to each vial and the samples were left to leach for different amounts of time (2.5, 5, 10, 15, 20, 30, 45, 60 and 90min). At the conclusion of the leaching period the contents of the vials were passed through a sieve and the cotyledons patted dry with absorbent paper. The mass of the leached cotyledons were then measured. Samples were prepared in duplicate and repeated for leaching temperatures of ambient (23°C), 35°C and 55°C (55°C samples were only measured up to 20min leaching time). The moisture content of the cotyledons after leaching was calculated using the initial moisture content of 11.39% WB as presented in table 4.1.

Figure 4.2 below shows the rate of moisture uptake into the beans.

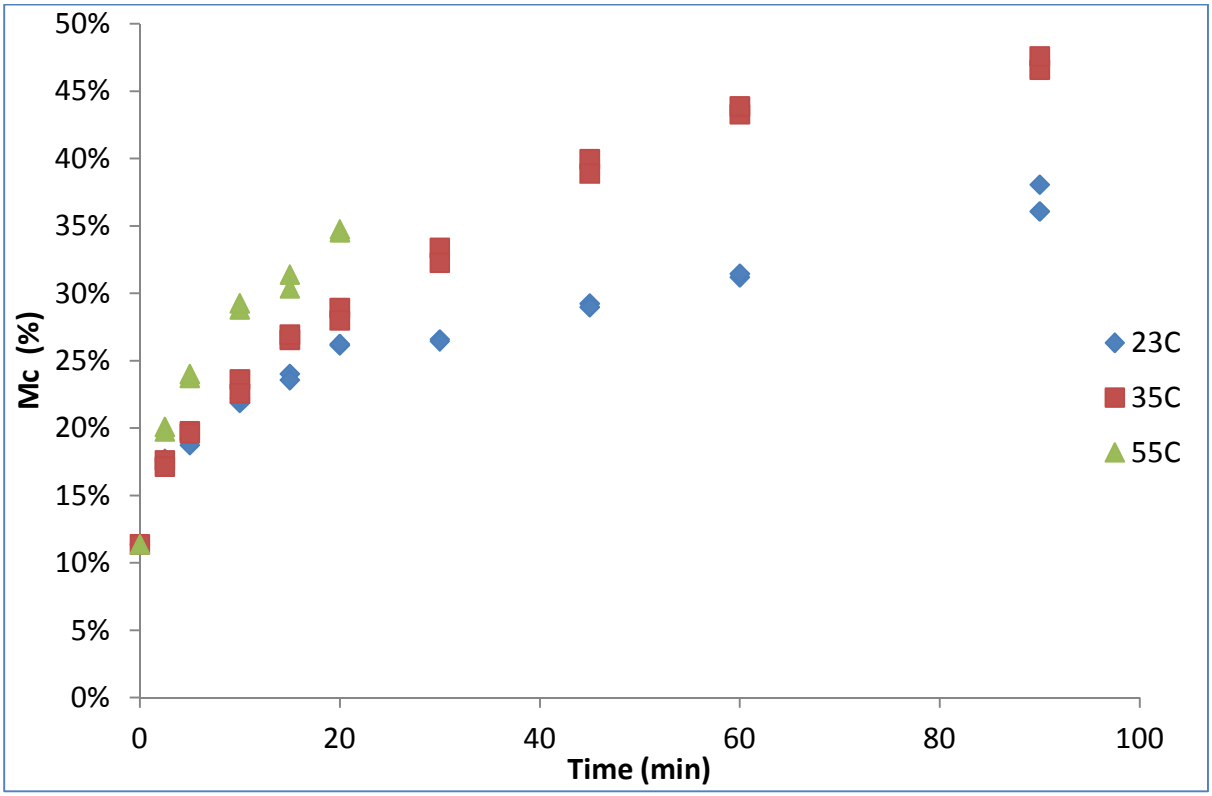


Figure 4.2 Moisture content vs leaching time for de-hulled navy bean cotyledons at 23, 30 and 55°C. Moisture contents are reported on a total mass basis and were calculated assuming initial moisture content of 11.39%

Figure 4.2 illustrates the temperature dependence of moisture diffusion into the navy bean particle. This data has ignored the effect of mass of solute leaching out and assumed that the change in mass of the particles following leaching is due entirely to water absorption. As demonstrated in section 4.2 this will affect the data measured at 55°C to a greater extent than the data measured at lower temperatures.

This means that as the leaching time increases the moisture content reported will be slightly lower than the actual moisture content and this will become more significant the longer the leaching time and the higher the leaching temperature. However, given that the soluble solids content of navy beans (as measured for navy bean flour) is approximately 25.6% DB (table 3.4) and the amount of solids lost over 24 hours in section 4.2 the effect of solute loss over the leaching times reported in Figure 4.2 should be minimal.

The rate of diffusion of water into the bean particle is dependent on the moisture gradient within the particle, the temperature, and the diffusion coefficient or diffusivity.

Seyhan-Gurtas *et al* (2001) described a method of fitting a constant effective water diffusion coefficient to leaching data based on the analytical solution of one-dimensional Fick's law of diffusion in a sphere given by Crank (1975):

$$MR = \frac{C_{wt} - C_{wi}}{C_{wS} - C_{wi}} = 1 - \sum_{n=1}^{\infty} \frac{6}{\pi^2 n^2} exp\left[\frac{D_W n^2 \pi^2 t}{a^2}\right] \quad (4.1)$$

Where:

- MR is the moisture ratio
- C_{wt} is the average moisture at time t (g/g)
- C_{wi} is the initial moisture (g/g)
- C_{wS} is the saturated moisture (g/g)
- n is the number of terms in summation
- D_W is the effective diffusion coefficient (m^2/s)
- a is the characteristic length for diffusion (m)
- t is the time over which the leaching has taken place (s)

A value for D_W that best fits the experimental data can then determined by formulating the problem as a root finding equation:

$$f\left(D_{eff}\right) = MR_{expt} - MR_{calc} \quad (4.2)$$

Where :

- MR_{exp} = measured moisture ratio
- MR_{calc} = calculated moisture ratio

The optimum value for D_W is then found using least squares linear regression to maximise the R^2 value for the fit of the predicted data with the measured data.

Applying this technique to the data from figure 4.2 yields the following:

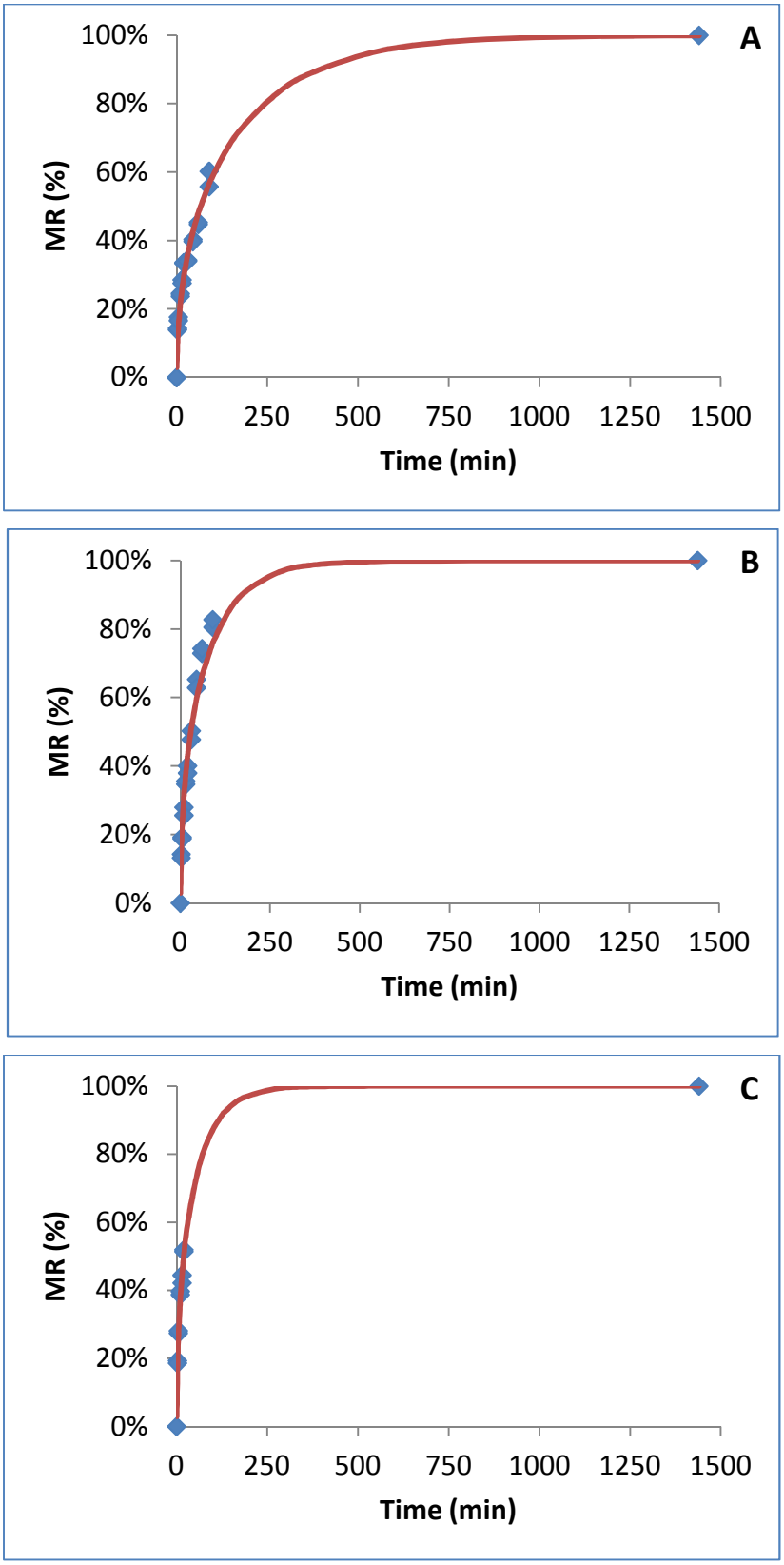


Figure 4.3 Moisture ratio versus time for de-hulled navy bean cotyledons leached at A: 23°C; B: 35°C and C: 55°C (assuming saturated moisture contents of 55.7%, 55.15% and 56.45% respectively). The points represent measured data and the solid line represents calculated data based on values of D_W determined using least squares regression. The R^2 values for the fit of the calculated line with the measured data are 0.985, 0.973 and 0.996 for A, B and C respectively.

The calculated data plotted in figure 4.3 was calculated using values for D_W determined using least square regression to maximise the R^2 value for the fit of the calculated data with the measured data. The value of n was set to 20 after numerical error checking revealed no difference in the values MR_{expt} to four significant figures between $n = 20$ and $n = 100$.

The value for a used in the calculations was 2.6 mm which is the radius of a sphere with the equivalent of the average volume of a cotyledon. The volume of the cotyledons was determined using the mass of the sample, the number of cotyledons per sample and the density of raw cotyledons determined in section 4.2.

The optimum values found for D_W at each temperature are given below:

Table 4.4 D_W values determined for diffusion at 23°C, 35°C and 55°C

Temperature (°C)	D_W (m^2/s)	R^2
23	4.88×10^{-11}	0.985
35	1.11×10^{-10}	0.973
55	1.65×10^{-10}	0.996

The diffusion coefficients determined here fit the measured data well and are comparable to those reported by Seyhan-Gurtas et al (2001) for Seker beans (1.18×10^{-11} at 25°C and 2.35×10^{-10} at 40°C).

The temperature dependence of D_W is expected to follow the diffusion form of the Arrhenius equation:

$$D_W = D_0 e^{\frac{-E_A}{RT}} \tag{4.3}$$

The Arrhenius equation can be re-arranged to give:

$$\ln(D_W) = \ln(D_0) - \frac{E_A}{R} \times \frac{1}{T} \tag{4.4}$$

E_A and D_0 were obtained from the linear regression of $\ln(D_{eff})$ versus $\frac{1}{T}$.

Table 4.5 E_A *and* D_0 values determined for moisture diffusivity in navy bean particles

$E_A =$	24.6	kJ/mol
$D_0 =$	1.37 x 10^{-6}	m^2/s

The R^2 value for the regression analysis was 0.92 indicating a relatively good fit and confirming that the Arrhenius equation does provide an adequate description of the temperature dependence of the diffusion coefficients for water in de-hulled navy bean cotyledons. The value of E_A determined in this work is comparable to those reported by Seyhan-Gurtas et al (2001) for several Turkish beans (33.6-50.8 kJ/mol) and the values reported by Singh and Kulshrestha (1987) for soybean (44.3 kJ/mol) and pigeon pea (51.4 kJ/mol).

This model, using a constant effective diffusion coefficient, performs remarkably well for predicting the overall water absorption rate of large bean particles and can be used to demonstrate the effect that particle size has on the absorption rate. While the disruption of cells during milling may have some impact on the water absorption rate the significance of this is not expected to be large due to the proportion of the bean flour that is composed of large clumps of intact cells (see section 3.4).

Figure 4.4 demonstrates the large effect that particle size has on the rate of water absorption into navy bean particles correlates well with the fast rate of RFO leaching from navy bean flour presented in chapter 3. The majority of the particles in the bean flour are between 200 and 500µm in radius and the first order time constant (time taken for 63.2% removal) is less than two minutes. This correlates well with the first order time constants predicted for water absorption in particles of 200µm and 500µm, which were approximately 40s and 180s respectively.

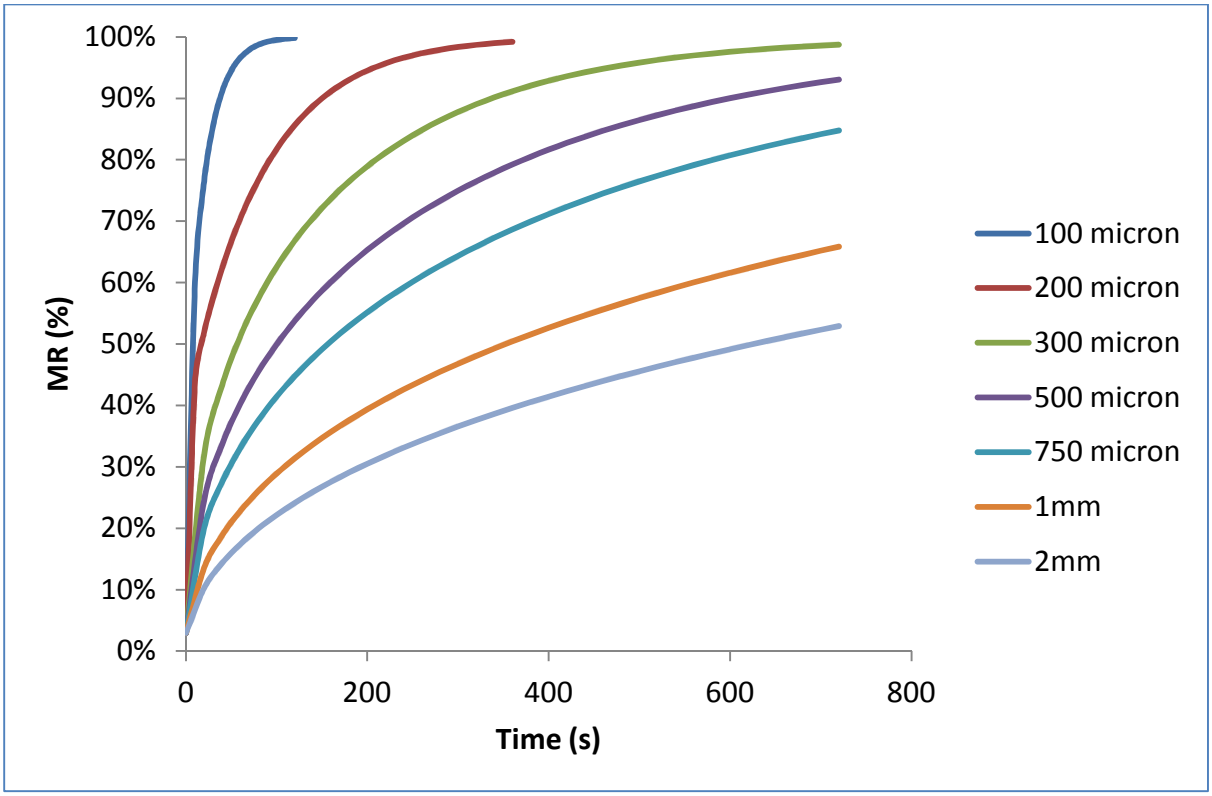


Figure 4.4 Predicted rates of moisture absorption for navy bean particles of different radii. Data was predicted using the effective diffusion coefficient at 23°C and the model described in section 4.3.

4.4 RFO Leaching Rates in De-Hulled Navy Bean Cotyledons

The samples from the moisture absorption experiment were also analysed for stachyose and raffinose concentrations to measure the leaching rates of RFOs from de-hulled navy bean cotyledons at 23°C, 35°C and 55°C.

Following the measurement of the change in mass that occurred during leaching the samples from experiment 4.2 were analysed for stachyose and raffinose concentration. The leached cotyledons were then crushed into a course powder/paste using a mortar and pestle and the sugars were extracted using the method described in section 3.2. The sugar extracted were then analysed for raffinose and stachyose concentration using the HPLC method described in section 3.1.

Figure 4.5 shows the RFO leaching results from the large cotyledon particles.

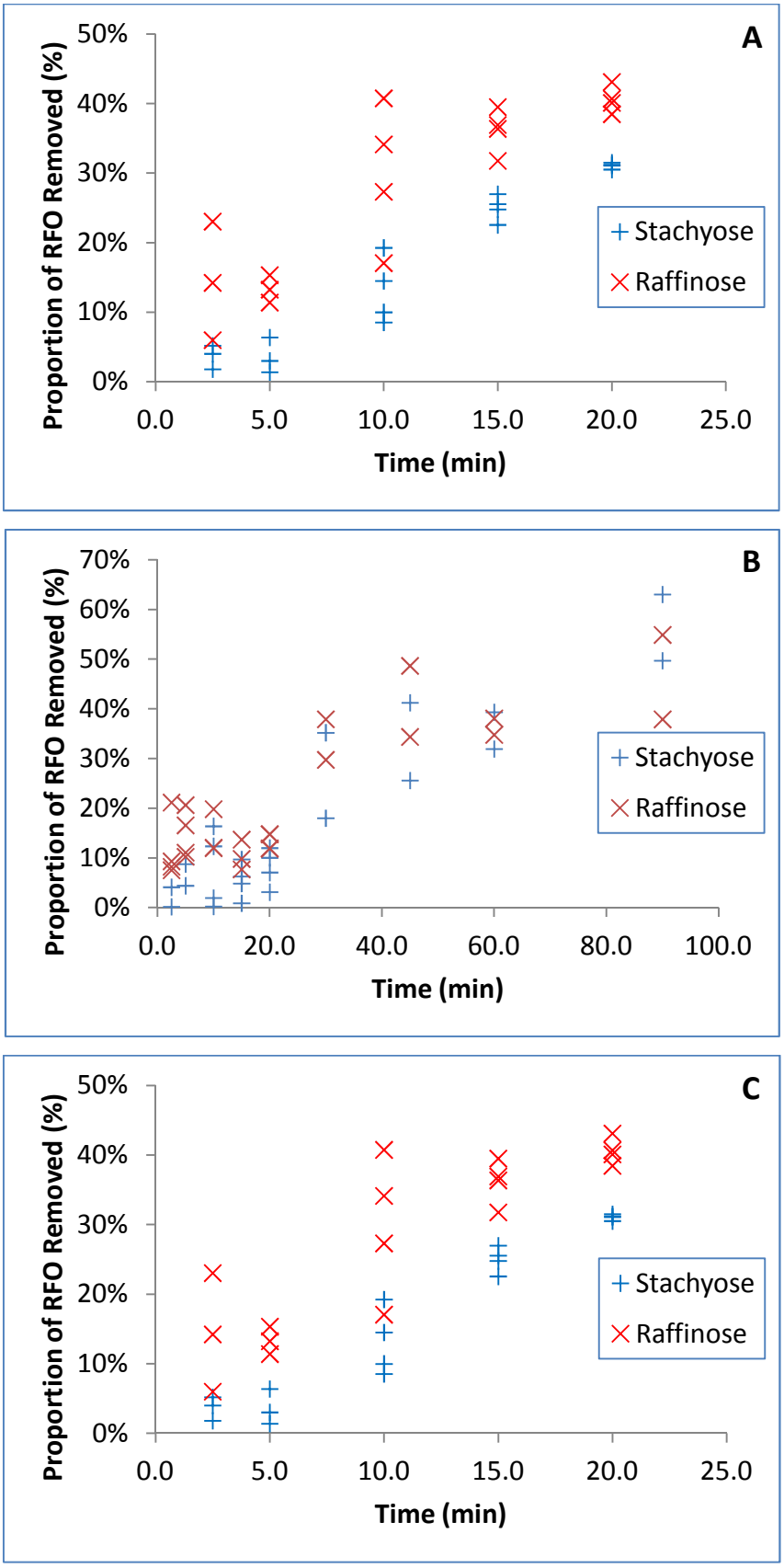


Figure 4.5 Stachyose and raffinose removal via leaching at A: 23°C; B: 35°C and C: 55°C. Note that the 55°C data is plotted on shorter timescale than the other temperatures.

Figure 4.5 shows the leaching rates of stachyose and raffinose at 23°C, 35°C and 55°C. The data points show significant scattering but the general trends can be seen. The scatter of the data points is an artefact of the noise of the chromatogram baseline for these samples. This makes it difficult to accurately quantify small differences in the area under the sample peaks.

A comparison of the data from figure 4.5 with the data from figure 4.2 shows that the diffusion rates of RFOs in navy beans are slower than those of moisture within the bean. For the 23°C data the moisture ratio reaches approximately 60% over the 90 min leaching period, and yet only 20% of the RFOs have leached out over the same period. The values for 35°C and 55°C for moisture diffusion are 80% and 50% while the corresponding values for RFO diffusion are 60% and 40% respectively. The values for the 55°C data were only measured over 20 minutes.

Another explanation for this observed phenomenon is that the rate of RFO diffusion is heavily dependent on the moisture content of the bean particle. It would therefore be expected that over the period during which the bean particle is absorbing water and therefore increasing its moisture content, the RFO leaching rate would be slow. Once the moisture content in the bean particle gets to a particular level the RFO diffusion could become closer to that of moisture, however the proportion of RFOs removed will at any time after that point still be less than the moisture ratio of the particle.

4.5 Conclusion

The rate of moisture diffusion into de-hulled navy bean cotyledons was measured at three temperatures and an analytical solution of the one dimensional Fick's law was used to fit a constant effective diffusion coefficient to the data at each temperature. The moisture diffusion rates calculated using this model fit well with the experimental data over the initial period and the effective diffusion coefficients that were determined seem to be sensible and within the range of values reported for similar materials in the literature.

The effective moisture diffusion coefficients at the three temperatures (4.9 x 10^{-11}, 1.11 x 10^{-10}, and 1.65 x 10^{-10} m^2/s for 23, 35 and 55°C respectively) were used to calculate D_0 and E_A (24.6 kJ/mol and 1.37 x 10^{-6} m^2/s respectively) for the diffusion form of the Arrhenius equation using linear regression. The values arrived at fit the data with an R^2 of 0.92 which indicates a reasonable fit and the value of E_A is somewhat lower but of the same order of magnitude as values reported in the literature for other legume seeds. These findings support the conclusion that the Arrhenius equation gives a good description of the temperature dependence of effective moisture diffusion coefficients in navy beans.

The RFO leaching rates were also measured for the de-hulled navy bean cotyledons, and despite relatively noisy data, the plots of RFO removal versus time can be used to support the hypothesis that RFO leaching is slower than moisture diffusion and/or depends greatly on the moisture content of the bean particle.

Chapter 5

The Kinetics of α-Galactosidase Hydrolysis of Stachyose and Raffinose

The objective of this chapter is to define the kinetic parameters required to predict the hydrolysis rate of stachyose and raffinose by α-galactosidase under varying conditions of temperature, enzyme concentration and substrate concentration. Reported values for the Michaelis Menten kinetic parameters of K_m and V_{max} for a number of different forms of α-galactosidase produced by different organisms vary widely (Adya & Elbein 1977, Civas et al 1984, Ferreira et al 2011, Gherardini et al 1985, Katrolia et al 2012). It is therefore important that these parameters be determined for the particular form of α-galactosidase being tested in this work (Enzidase® AGS supplied by Zymus®).

Enzidase® AGS is a high activity α-galactosidase supplied in the form of a powder and available in industrial quantities of 10kg pails. Enzidase® AGS is produced by the controlled fermentation of *Aspergillus niger* and complies with FCC and FAO/WHO JECFA recommended specifications for food grade enzymes. Enzidase AGS has an advantage over enzymes supplied in liquid form in that it is more compact and can be added to a dry ingredient and become active on the introduction of water. If stored in cool dry conditions the expected loss of enzyme activity is less than 10% per year, and the product life can be extended by refrigeration at < 5°C.

Ferreira et al (2011) reported that some forms of alpha-galactosidase exhibit galactose inhibition. This phenomenon is where galactose produced as a product of the hydrolysis of stachyose and raffinose binds to the active site of the enzyme, preventing access of the substrates and thereby inhibiting the rate at which the substrates are hydrolysed. In these situations the further the hydrolysis of the substrate progresses the higher the concentration of galactose in the system and the greater the inhibition effect.

Accumulation of galactose will be more significant as the volume of leaching water to flour volume decreases. By studying the limiting galactose concentration it will be possible to place limits on the extent to which this ratio can be reduced.

Product inhibition can be accounted for by adding additional terms to the Michaelis Menten rate equation; however it is first necessary to determine whether galactose inhibition is in fact taking place with the Enzidase® AGS enzyme. It has also been noticed (see experiment 6.2) that sucrose is hydrolysed into its component sugars glucose and fructose during the hydrolysis of stachyose and raffinose with Enzidase® AGS. It is possible that this hydrolysis is being performed by a different active site on the enzyme, in which case there should be no noticeable effect on hydrolysis rate of stachyose and raffinose. It was therefore prudent to determine whether sucrose inhibition is also taking place.

5.1 Inhibition by Sucrose and Galactose

A series of experiments were carried out to determine whether Enzidase® AGS α-galactosidase is susceptible to inhibition by either sucrose or raffinose.

5.1.1 Sucrose Inhibition

1.5 ml of distilled water at 50°C containing 0.03% Enzidase® AGS by mass was added to 30mg samples of raffinose pentahydrate in 2mL centrifuge tubes. The samples were shaken to ensure homogenous mixing and then held at 50°C for varying amounts of time (hydrolysis periods tested were 2.5, 7.5 and 10 min). At the conclusion of the hydrolysis period the samples were placed in boiling water for 5 minutes to denature the enzyme and halt the hydrolysis. The samples were then passed through a 0.45µm syringe filter and analysed for raffinose concentration using the HPLC method described in section 3.1. Samples were repeated in duplicate. The effect of pH on the enzyme activity has been ignored in all kinetics experiments in this chapter because the change in sugar concentration has no effect on the pH of the solution and pH control would only add increased complexity to any industrial process derived from this work.

The 7.5 and 10 minute samples were repeated again, however in this iteration 30mg of sucrose was added as well as the 30mg of raffinose pentahydrate. This resulted in raffinose and sucrose concentrations of 20g/L in each sample.

Figure 5.1 clearly demonstrates that there is no observable change in hydrolysis rate over 10 minutes when a relatively large concentration of sucrose is present. This indicates that the sucrose hydrolysis is taking place at a different active site on the enzyme and has no inhibitory effect on the hydrolysis rate of raffinose.

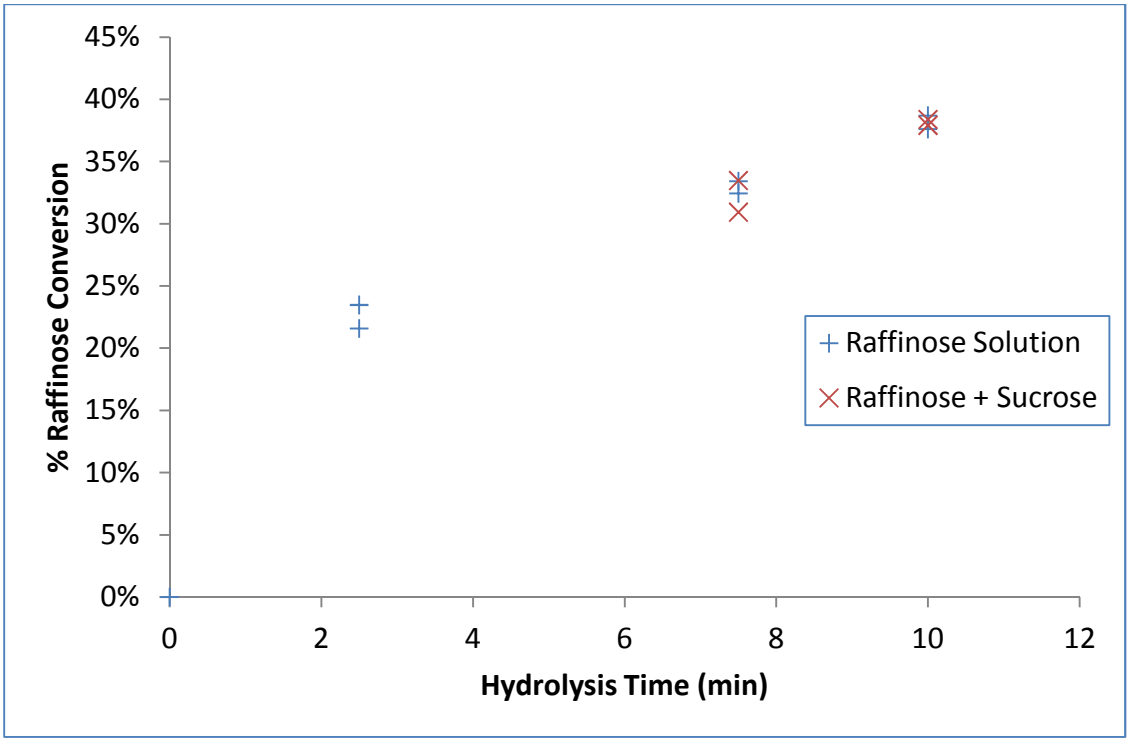


Figure 5.1 Hydrolysis rates of 30mg of raffinose in 1.5mL of 0.03% Enzidase® solution at 23°C with and without the presence of 30mg of sucrose.

5.1.2 Galactose Inhibition

1.5 ml of distilled water at 23°C containing 0.03% Enzidase® AGS by mass was added to 5 mg samples of raffinose in 2mL centrifuge tubes. The samples were shaken to ensure homogenous mixing and then held at 23°C for varying amounts of time (hydrolysis periods tested were 7.5, 10 and 12.5 min).

At the conclusion of the hydrolysis period the samples were placed in boiling water for 5 minutes to denature the enzyme and halt the hydrolysis. The samples were then passed through a 0.45 µm syringe filter and analysed for raffinose concentration using the HPLC method described in section 3.1. Samples were repeated in duplicate.

The samples were also repeated with the addition of 6 mg of galactose to the raffinose samples prior to addition of the enzyme solution. This resulted in concentrations of raffinose and galactose of 3.3 g/L and 4 g/L respectively.

Figure 5.2 shows that the rate of raffinose hydrolysis is significantly decreased when the galactose is added. This indicates that Enzidase® AGS is susceptible to inhibition by galactose and that the enzyme rate kinetics will therefore need to include a term to account for it.

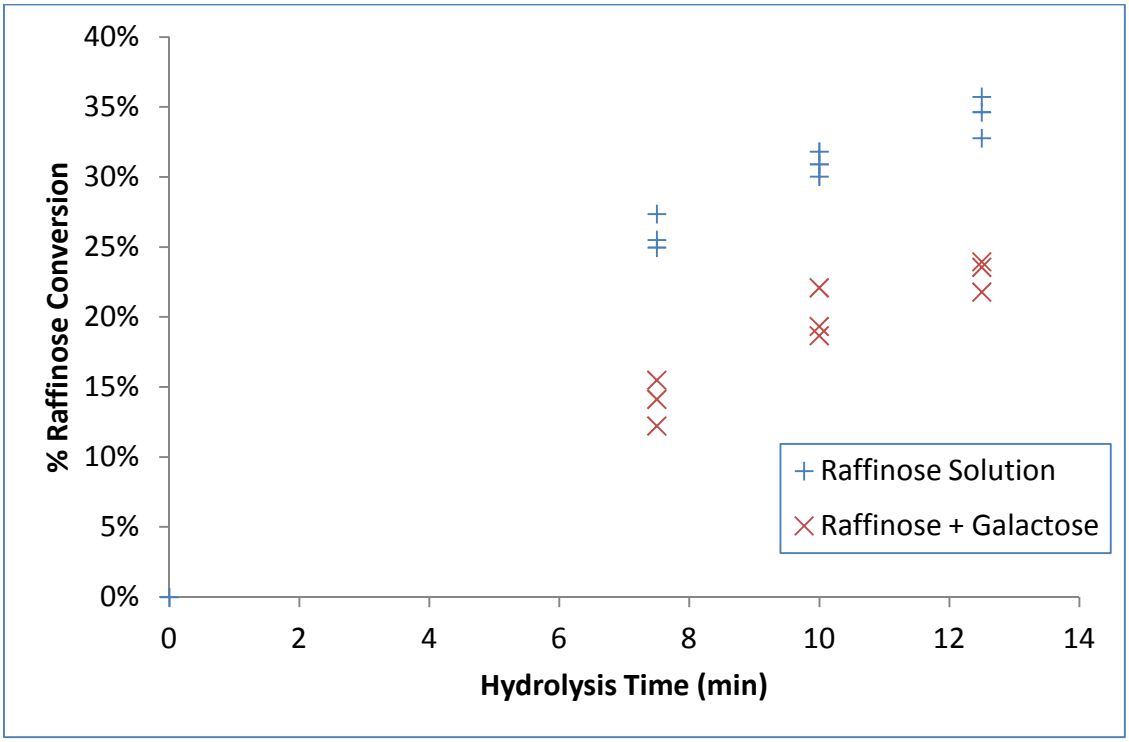


Figure 5.2 Hydrolysis rate of 5.3mg of raffinose in 1.5mL of 0.03% Enzidase® solution at 60°C with and without the presence of 5.8mg of galactose.

5.2 Michaelis Menten Enzyme Kinetics

In a system in which a substrate A is converted into a product P through the catalytic action of an enzyme E the process can be described by the following reaction:

$$A + E \rightleftharpoons EA \rightarrow E + P \tag{5.1}$$

This model of enzyme action assumes that the substrate forms an enzyme-substrate complex at an active site with which only a narrow range of molecules can attach. The narrower the range of molecules with which an enzyme can form a substrate the higher the specificity of the enzyme.

The formation of the enzyme substrate complex is reversible, and so the substrate can be released back into solution without being hydrolysed. If the enzyme substrate complex holds together for sufficient time the enzyme can catalyse the hydrolysis of A into P and the product is released into solution freeing the enzyme to form a complex with another molecule of A. The formation of P from the enzyme substrate complex is usually irreversible, and therefore it cannot be recombined to form A.

For the system described above where the concentration of the substrate is significantly greater than the concentration of the enzyme the overall rate of reaction is given by the rate of formation of product P and is governed by the following relationship:

$$v = \frac{V_{max}\,[A]}{K_M + [A]} \tag{5.2}$$

Where

- v is the reaction rate in $\frac{mol}{mg.min}$ and refers to either the rate of consumption of A or the rate of formation of P divided by the concentration of the enzyme.
- V_{max} is the maximum rate of reaction where all enzyme molecules are present in the enzyme-substrate complex state and has the same units as v.
- $[A]$ is the concentration of substrate A in mM

- K_M is the Michaelis Menten constant and is equal to $\frac{k_2+k_3}{k_1}$ where k_1= rate constant for the formation of the enzyme-substrate complex; k_2 = rate constant for the reverse reaction of *EA* to *A* + *E*; k_3 = rate constant for the formation of product *P* from the enzyme-substrate complex. K_M has the same units as A as is equal to the concentration of A at which the initial velocity of reaction is half V_{max}

The active site on the enzyme may also have an affinity for one or more of the products of the hydrolysis, and this is the case with Enzidase® AGS and galactose. This affinity results in the galactose forming a complex with the enzyme at the same active site used to catalyse the hydrolysis of stachyose and raffinose.

Because galactose is a monosaccharide, the enzyme is unable to catalyse its hydrolysis into any other molecules and so the galactose is released unaltered back into solution. In the period during which galactose and enzyme have formed a complex, the enzyme is not available to complex with the substrates (stachyose and raffinose).

In this way the presence of the galactose inhibits the access of stachyose and raffinose to the Enzidase® AGS and thereby slows the rate of hydrolysis. The higher the concentration of the product compared to the concentration of enzyme and substrate, the larger the proportion of enzyme molecules forming product complexes and the larger the inhibitory effect will be. This situation can be represented by the following reaction:

$$\begin{array}{l} A + E \rightleftharpoons EA \rightarrow E + P \\ \qquad\qquad\quad \downarrow\uparrow \\ \qquad\qquad\quad EP \end{array} \tag{5.3}$$

The rate equation for product inhibition of this sort is as follows:

$$v = \frac{V_{max}\,[A]}{K_M\left(1+\frac{[P]}{K_P}\right)+\,[A]} \tag{5.4}$$

Where

- $[P]$ is the concentration of the product P in mM
- K_P is the inhibition constant with units of mM and is equal to $\frac{k_5}{k_4}$ where k_4= rate constant for the formation of the product-enzyme complex; k_5= rate constant for the reverse reaction of EP to $E + P$

For the hydrolysis of stachyose and raffinose by Enzidase® AGS the rate equation is even more complex. The stachyose and raffinose are both hydrolysed at the same active site on the enzyme and so as well as product inhibition this system also exhibits competitive substrate inhibition. The situation is further complicated because raffinose is also a product of the hydrolysis of stachyose. The following reaction equation describes the stachyose/raffinose/Enzidase® AGS system:

$$\begin{array}{ccccccc} St + E & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ESt & \xrightarrow{k_3} & Ga + Rf + E & \underset{k_7}{\overset{k_6}{\rightleftharpoons}} & ERF \xrightarrow{k_8} Ga + Su \\ & & + & & & & + \\ & & E & & & & E \\ & & {\scriptstyle k_5}\uparrow\downarrow{\scriptstyle k_4} & & & & {\scriptstyle k_5}\uparrow\downarrow{\scriptstyle k_4} \\ & & EGa & & & & EGa \end{array} \quad (5.5)$$

The sucrose produced in this reaction is also hydrolysed by the Enzidase® AGS, however as demonstrated in figure 5.1 this does not have a measureable effect on the rate of the RFO hydrolysis and can therefore be ignored in the rate equation. The rate of the above reaction can be defined in terms of either the rate of hydrolysis of stachyose, rate of hydrolysis of raffinose, or rate of formation of galactose. The rate equations are given below:

$$\frac{d[St]}{dt} = \frac{-V_{maxSt}E[St]}{K_{MSt}\left(1+\frac{[Rf]}{K_{MRf}}+\frac{[Ga]}{K_{Ga}}\right)+[St]} \quad (5.6)$$

$$\frac{d[Rf]}{dt} = \frac{V_{maxSt}E[St]}{K_{MSt}\left(1+\frac{[Rf]}{K_{MRf}}+\frac{[Ga]}{K_{Ga}}\right)+[St]} - \frac{V_{maxRf}E[Rf]}{K_{MRf}\left(1+\frac{[St]}{K_{MSt}}+\frac{[Ga]}{K_{Ga}}\right)+[Rf]} \quad (5.7)$$

$$\frac{d[Ga]}{dt} = \frac{V_{maxSt}E[St]}{K_{MSt}\left(1+\frac{[Rf]}{K_{MRf}}+\frac{[Ga]}{K_{Ga}}\right)+[St]} + \frac{V_{maxRf}E[Rf]}{K_{MRf}\left(1+\frac{[St]}{K_{MSt}}+\frac{[Ga]}{K_{Ga}}\right)+[Rf]} \quad (5.8)$$

Where

- $\frac{d[St]}{dt}$ is the change in stachyose concentration with time ($\frac{mol}{mg.min}$)
- $\frac{d[Rf]}{dt}$ is the change in raffinose concentration with time ($\frac{mol}{mg.min}$)
- $\frac{d[Ga]}{dt}$ is the change in galactose concentration with time ($\frac{mol}{mg.min}$)
- $[St]$ is the concentration of stachyose (mM)
- $[Rf]$ is the concentration of raffinose (mM)
- $[Ga]$ is the concentration of galactose (mM)
- V_{maxSt} is the maximum rate of stachyose hydrolysis and is equal to $[E]_{Total}k_3$ ($\frac{mol}{mg.min}$)

- V_{maxRf} is the maximum rate of raffinose hydrolysis and is equal to $[E]_{Total}k_8$ $(\frac{mol}{mg.min})$
- K_{MSt} is the Michaelis Menten constant for stachyose and is equal to $\frac{k_2+k_3}{k_1}$ (mM)
- K_{MRf} is the Michaelis Menten constant for raffinose and is equal to $\frac{k_7+k_8}{k_6}$ (mM)
- K_{Ga} is the galactose inhibition constant and is equal to $\frac{k_5}{k_4}$ (mM)

In order to be able to use the above expressions to predict the hydrolysis rates of stachyose and raffinose the values for K_{MSt}, K_{MRf}, K_{Ga}, V_{maxST}, and V_{maxRf} must be determined.

For a typical enzyme catalysed reaction where the concentration of substrate is significantly higher than that of the enzyme, the change in substrate concentration with time will initially be linear. The reaction rate during this period is referred to as the initial rate v_i. As the concentration of the substrate decreases the reaction rate begins to tail off, this tailing off continues until the reaction ceases when the substrate concentration reaches zero.

When a comparatively small amount of enzyme is added to a concentrated stachyose solution the initial rate (v_{iSt}) can be measured before the concentration of raffinose and galactose can become significant enough to have a measureable effect. In this situation the rate equation simplifies back to the following:

$$v_{iSt} = \frac{-V_{maxSt}[St]}{K_{MSt}+[St]} \tag{5.9}$$

Non linear regression can be used to determine optimum values for K_{MSt} and V_{maxSt} to the above equation to provide the best fit to a set of data of v_{iSt} measured at different values of initial $[St]$.

Similarly when a comparatively small amount of enzyme is added to a concentrated raffinose solution in the absence of stachyose, the initial rate of raffinose hydrolysis can be measured before the concentration of galactose is significant enough to have a measureable effect. The rate equation for this situation is:

$$v_{iRf} = \frac{-V_{maxRf}[Rf]}{K_{MRf}+[Rf]} \tag{5.10}$$

Again non linear regression can be used to determine the values for K_{MRf} and V_{maxRf} that give the best fit to measured data for v_{iSt} at different values of initial $[St]$.

This leaves only K_{Ga} remaining to be determined for the kinetic expression for the stachyose/raffinose/Enzidase® AGS system. If the test used to determine the values for K_{MRf} and V_{maxRf} were repeated with a known concentration of galactose added to the system the rate of raffinose hydrolysis would be given by:

$$v_{iRf} = \frac{-V_{maxRf}[Rf]}{K_{MRf}\left(1+\frac{[Ga]}{K_{Ga}}\right)+[Rf]} \tag{5.11}$$

Using the previously determined values for K_{MRf} and V_{maxRf} the value for K_{Ga} that best fits the measured v_{iRf} for different values of $[Rf]$ can be determined by non-linear regression.

5.3 Determination of Hydrolysis time for Measurement of Initial Rate

The first step in designing an experimental plan to measure the kinetic parameters for Enzidase® AGS is to determine an appropriate length of time over which to measure initial hydrolysis rate. The appropriate length of time will be one that causes the minimum decrease in substrate concentration that can be accurately and repeatably measured.

To determine the optimal hydrolysis time for the measurement of v_i, 1.5ml sample solutions were prepared containing 4.5 mM stachyose and 200 mg/L Enzidase®AGS in 2ml centrifuge tubes. The samples were left to hydrolyse at room temperature (23˚C) for different lengths of time (2.5, 5, 7.5, 10 or 15 minutes). Samples for each hydrolysis time were prepared in duplicate. Following the hydrolysis time, each sample was placed in a water bath at 100˚C for 5 minutes to deactivate the enzyme.

The samples were then filtered using 0.45 µm syringe filters and the final stachyose concentration was measured using the HPLC method described in section 3.1. The proportion of stachyose hydrolysed was plotted against the hydrolysis time as shown in figure 5.3

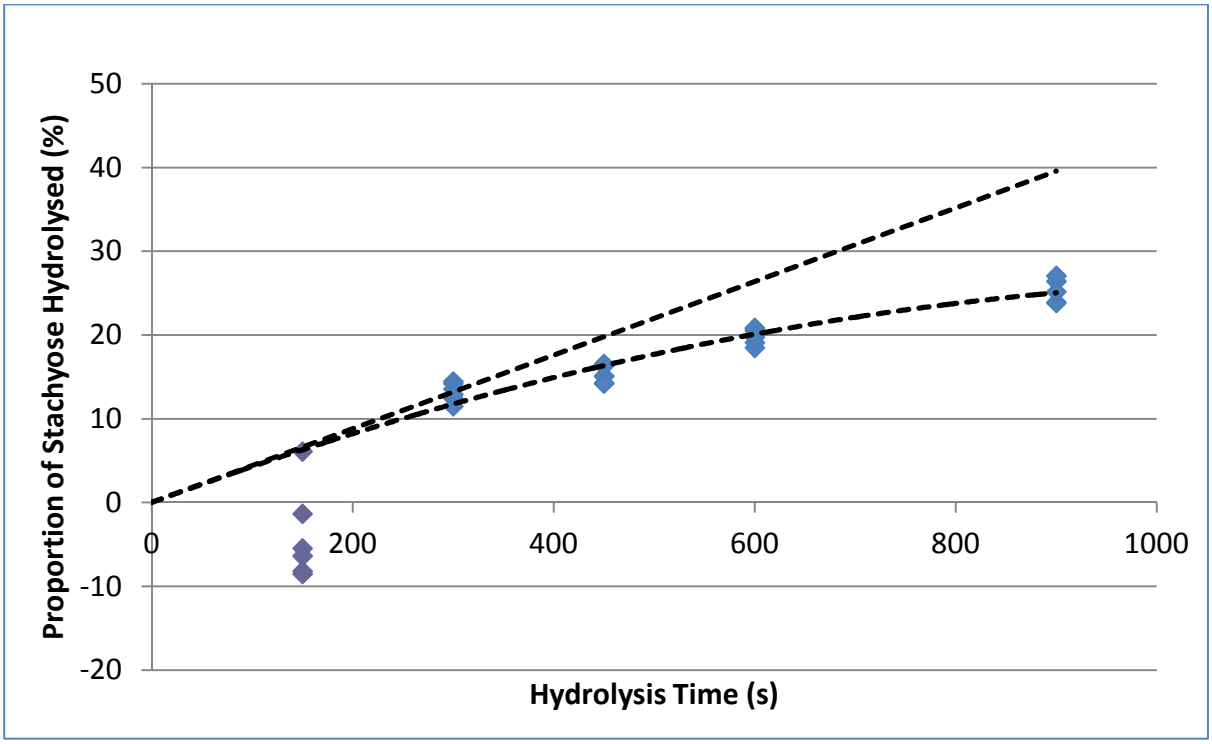


Figure 5.3 Hydrolysis versus time for standard stachyose solution with 0.2 g/L Enzidase® AGS

Figure 5.3 above shows the increase in the percentage of stachyose that has been hydrolysed versus the amount of time hydrolysis has been taking place. The data points for the 2.5 minute readings are widely scattered.

The major causes of the scattering are the inherent variability in the chromatograms and the error introduced when separating the baseline of the chromatogram from the peak. Due to the scattering of the data points at 2.5 minutes, the lowest hydrolysis time measured with good repeatability was 5 minutes.

The other concern aside from repeatability is that the rate of hydrolysis will decrease as the concentration of the substrate decreases and the concentration of inhibiting products (in this case galactose) increases.

Figure 5.3 shows that as hydrolysis time increases the slope of the slope of the measured data decreases indicating that the hydrolysis rate is being affected by the mechanisms discussed above. The data points at 5 minutes lie close to the point at which the slope of the data begins to deviate from a straight line and therefore that hydrolysis is still occurring at, or close to, the initial rate.

The data measured at 5 minutes hydrolysis time satisfy both the need for repeatability of measurements and the requirement that hydrolysis rate is still close to the initial rate. Therefore the following experiments requiring the determination of initial hydrolysis rate in subsequent experiments were run using a 5 minute hydrolysis time.

5.4 Determination of Kinetic Parameters for α-Galactosidase

To characterise the kinetics of the enzymatic hydrolysis of RFOs an experiment was conducted to measure the initial hydrolysis rate as a function of the initial substrate concentration for Enzidase® AGS with stachyose and raffinose as the substrates. The constants required for the Michaelis Menten kinetic equations outlined above could then be calculated from the measured data using non linear regression.

Table 5.1 shows the concentrations of raffinose, stachyose and galactose that were prepared for hydrolysis in 200 mg/L Enzidase® AGS at 23°C.

Table 5.1 Concentrations of substrates prepared to measure enzyme kinetic parameters.

Sample	Stachyose (mM)	Raffinose (mM)	Galactose (mM)
1	7.5	0	0
2	15	0	0
3	22.5	0	0
4	30	0	0
5	0	8.5	0
6	0	17	0
7	0	25.5	0
8	0	34	0
9	0	4.2	55 Gal
10	0	8.4	55 Gal
11	0	12.6	55 Gal
12	0	16.8	55 Gal

The samples were allowed to hydrolyse at 23°C for 5 minutes before being immersed in boiling water for 5 minutes to denature the enzyme and halt the hydrolysis. The samples were then passed through a 0.45 µm syringe filter and analysed for RFOs using the HPLC method described in section 3.2.

Figure 5.4 shows the measured data for v_i versus initial substrate concentration for each set of substrates.

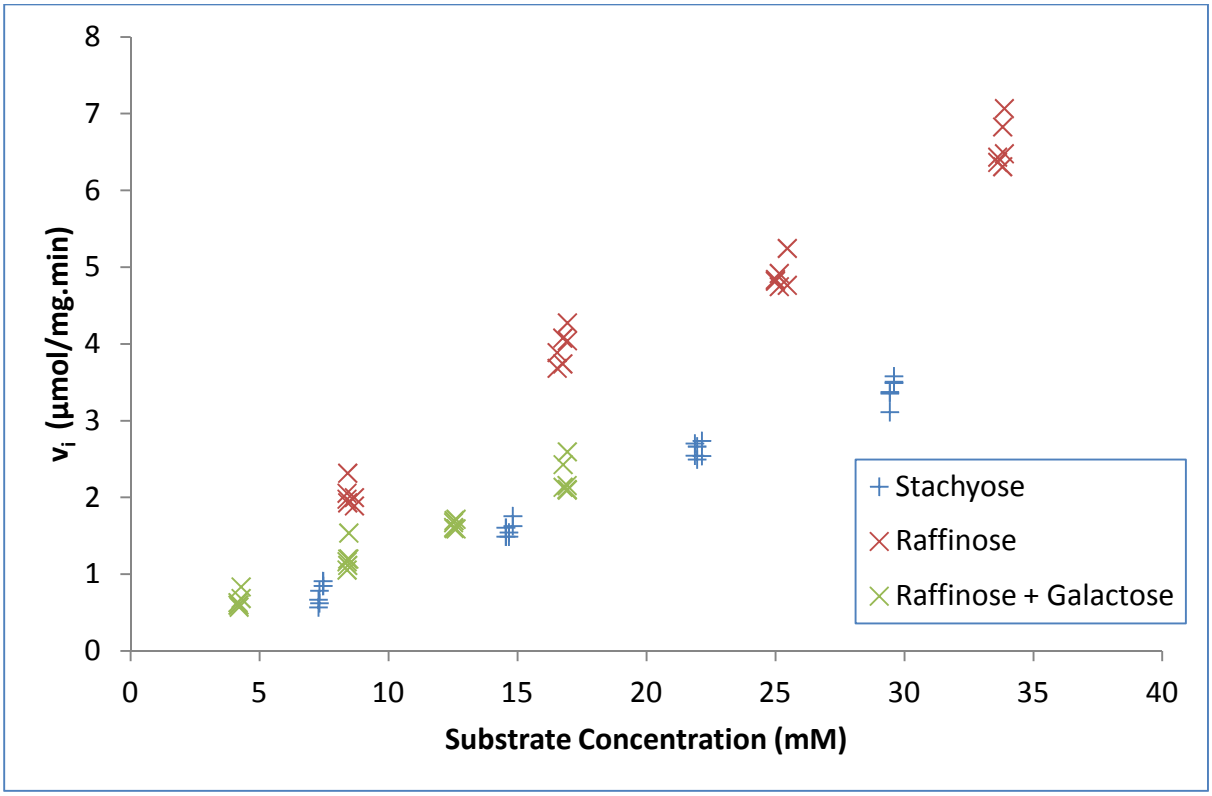


Figure 5.4 v_i versus initial substrate concentration for solutions of stachyose, raffinose and raffinose + galactose.

The relevant kinetic expressions were then used to calculate the predicted v_i using the initial concentrations from the measured data and best estimates for K_m and V_{max}. A non-linear regression technique using the solver function in Microsoft® Excel was employed to determine the values of K_m and V_{max} which resulted in the maximum R^2 value for the overall fit of the calculated values for v_i with the experimentally measured values. The fits of the kinetic model to the experimental data is shown in figures 5.5 – 5.7 below.

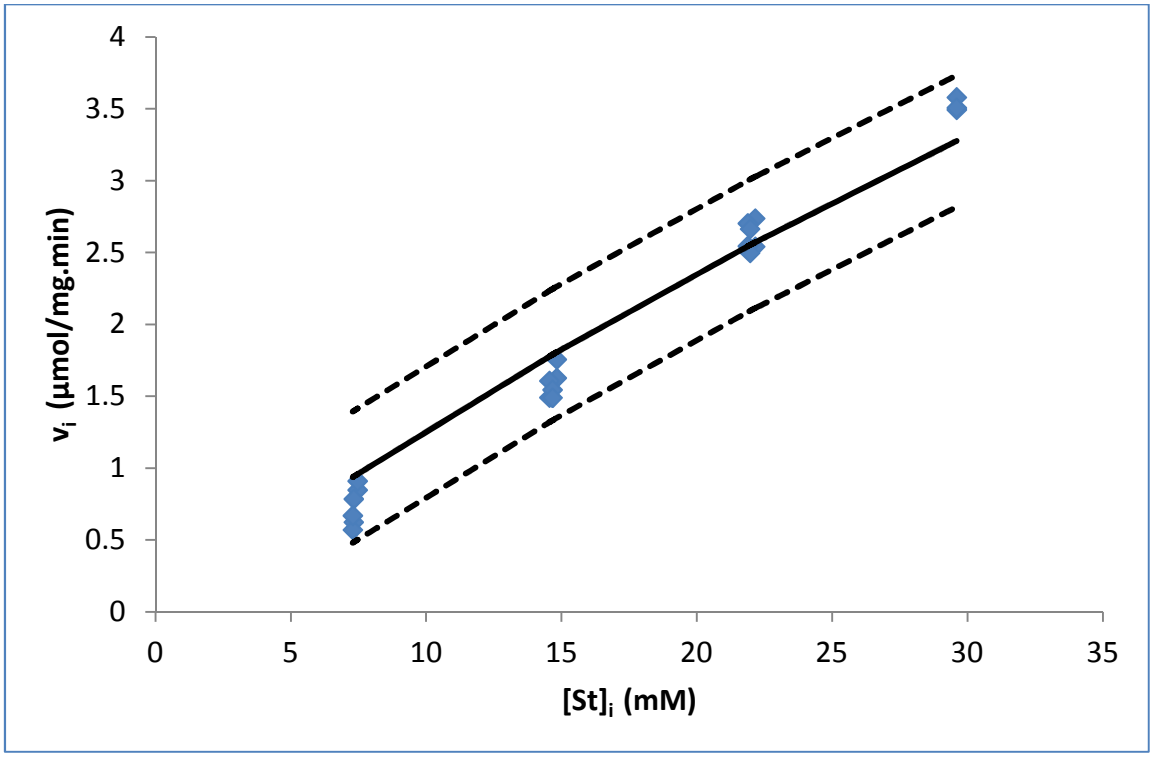


Figure 5.5 Initial rate of reaction versus stachyose concentration at 23°C. Points represent measured data; the solid line shows the line of best fit for the kinetic expression using values of K_{MSt} = 130.5 mM and V_{maxSt} = 17.7µmol/mg.min as determined by non linear regression. The dotted lines represent the 95% confidence interval for the predicted line; the R^2 value for the fit of the predicted line is 0.95.

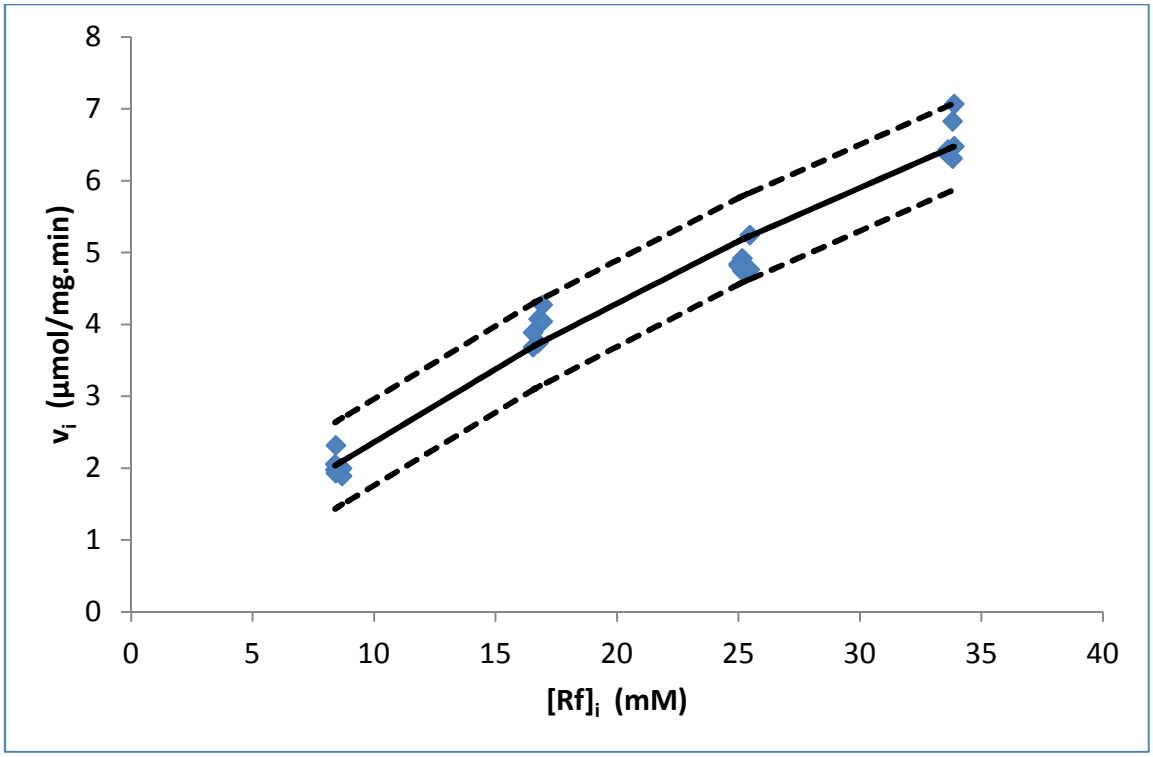


Figure 5.6 Initial rate of reaction versus raffinose concentration at 23°C. Points represent measured data; the solid line shows the line of best fit for the kinetic expression using values of K_{MRf} = 86.8 mM and V_{maxRf} = 23.1 µmol/mg.min as determined by non linear regression. The dotted lines represent the 95% confidence interval for the predicted line; the R^2 value for the fit of the predicted line is 0.97.

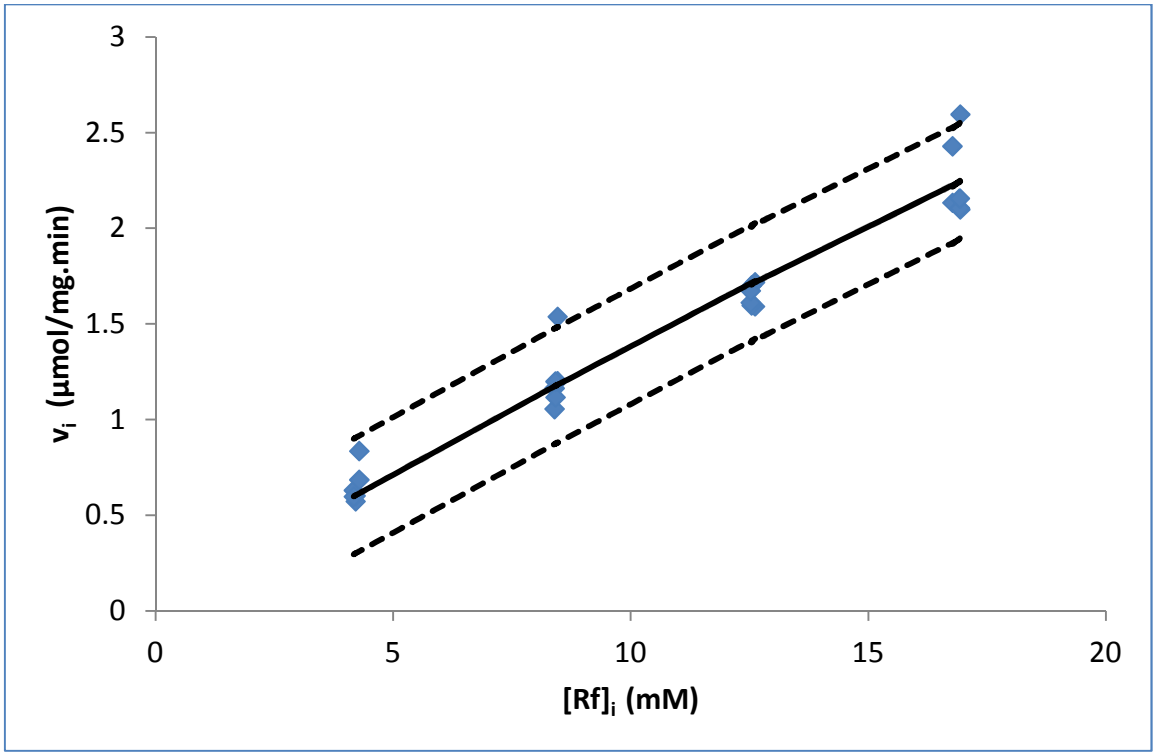


Figure 5.7 Initial rate of reaction versus raffinose concentration at 23°C with initial galactose concentration 55mM. Points represent measured data; the solid line shows the line of best fit for the kinetic expression using values of K_{MRf} = 86.8 mM, V_{maxRf} = 23.1 µmol/mg.min and $K_{Ga} = 69.1$ as determined by non linear regression. The dotted lines represent the 95% confidence interval for the predicted line; the R^2 value for the fit of the predicted line is 0.95.

Figures 5.5 – 5.7 show the measured data for initial reaction rate versus substrate concentration plotted against the values calculated using the values determined for K_{MSt}, K_{MRf}, K_{Ga}, V_{maxST}, and V_{maxRf} using non linear regression. The values for the kinetic constants were determined using non linear regression to fit the kinetic equation to the measured data. The values determined were:

Table 5.2 Kinetic parameters measured for α-galactosidase hydrolysis of stachyose and raffinose at 23°C

K_{MSt}	130.5	mM
V_{maxST}	17.7	µmol/mg.min
K_{MRf}	86.8	mM
V_{maxRf}	23.1	µmol/mg.min
K_{Ga}	69.1	mM

The values of K_M and V_{max} for α-galactosidase hydrolysis of stachyose and raffinose that have been reported in the literature (Adya & Elbein 1977, Civas et al 1984, Ferreira et al 2011, Gherardini et al 1985, Katrolia et al 2012) vary widely depending on the source of the enzyme. Values for the kinetic parameters reported in the literature range between:

Table 5.3 Values reported in the literature for kinetic parameters of α-galactosidase hydrolysis of stachyose and raffinose.

K_{MSt}	0.3 - 72	mM
V_{maxST}	1.1 - 381	µmol/mg.min
K_{MRf}	0.5 -98.1	mM
V_{maxRf}	3.0 - 252	µmol/mg.min
K_{Ga}	11	mM

The values determined above are for the most part within the range reported in the literature and in all cases are of the same order of magnitude. The kinetic values determined fit well with the measured data with R^2 values of 0.95 or greater.

5.5 Validation of Kinetic Parameters

It is prudent to validate this kinetic expression against data that played no part in the determination of the parameters of the kinetic expressions. To do this 1.5mL samples containing 11mM stachyose hydrate were exposed to different concentrations (100, 200, 300, 400, 500 mg/L) of Enzidase® AGS and allowed to hydrolyse at 23°C for five minutes. After 5 minutes of hydrolysis the samples were immersed in boiling water for five minutes to denature the enzyme and halt the hydrolysis. The samples were then passed through a 0.45 µm syringe filter and analysed for RFOs using the HPLC method described in section 3.2.

The rate of stachyose hydrolysis is given by

$$\frac{d[St]}{dt} = \frac{-V_{maxSt}E[St]}{K_{MSt}\left(1+\frac{[Rf]}{K_{MRf}}+\frac{[Ga]}{K_{Ga}}\right)+[St]} \tag{5.12}$$

A spreadsheet was created to solve this ODE through iterative calculations using Euler's method based on the kinetic expressions outlined above and using the parameters determined in section 5.4. The calculations were based on 1 second intervals.

A numerical error check was performed on the 100 mg/L and 500 mg/L calculations using a time step of 0.1 seconds. The difference between the 1 second and 0.1 second values for the stachyose conversion after 5 minutes was negligible; therefore only the 1 second interval data is presented here.

The comparison of predicted and measured data is shown in figure 5.8 below.

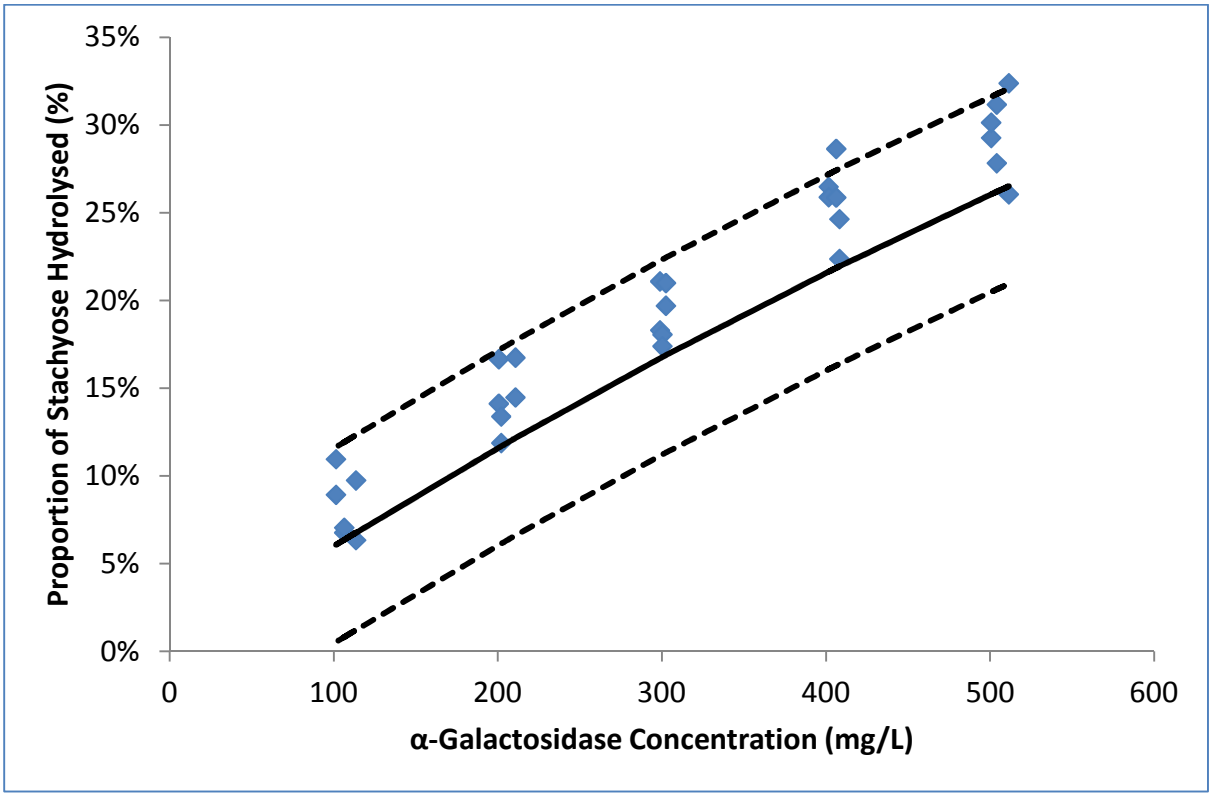


Figure 5.8 Stachyose hydrolysis over 5 minutes as a function of enzyme concentration. The points represent measured data; the solid line represents values calculated using the kinetic parameters determined in experiment 5.3; the dotted lines represent the 95% confidence interval for the calculated line. The R^2 value for the fit of the calculated line is 0.91

Figure 5.8 demonstrates that the kinetic parameters presented in table 5.2 perform reasonably well in the prediction of stachyose hydrolysis rates. The predicted curve follows the same trend as the measured data but is consistently on the lower side of the spread at each enzyme concentration. That being said, the R^2 value for the fit between the calculated line and the measured data is 0.91 indicating that it is a reasonable fit. Since the overall purpose of this exercise is to predict the enzyme concentration/time profile required to reach a minimum level of stachyose hydrolysis, basing process variables on calculated values that are slightly low only adds to the margin of safety.

5.6 Temperature Effects on Kinetic Parameters

The kinetics of enzyme catalysed reactions are also affected by temperature in that the V_{max} values are typically dependent on the temperature of the system. According to collision theory for chemical reactions the rate increases as the temperature increases due to the increase in molecular velocity, which corresponds to an increased frequency of collisions between molecules and thus an increase in the rate at which they react together.

For enzyme catalysed reactions this corresponds to an increased frequency of the active site on an enzyme molecule coming into contact with a substrate molecule in a given time period and therefore an increase in the reaction rate. The temperature dependence of V_{max} can typically be described using the following form of the Arrhenius equation:

$$V_{max} = V_{max0} exp\left(\frac{-E_A}{RT}\right) \quad (5.13)$$

Where:

- V_{max0} is the maximum reaction velocity at infinite temperature in µmol/mg.min
- E_A is the activation energy of the reaction in J/mol
- R is the ideal gas constant = 8.31 J/mol.K
- T is the temperature in units of Kelvin

However, because enzymes are proteins they are often sensitive to moderate to high temperatures, under which conditions they begin to denature and lose their catalytic activity. Therefore the Arrhenius equation should only hold up to the point where the enzyme begins to denature, at which point any further increase in temperature will decrease the activity of the enzyme.

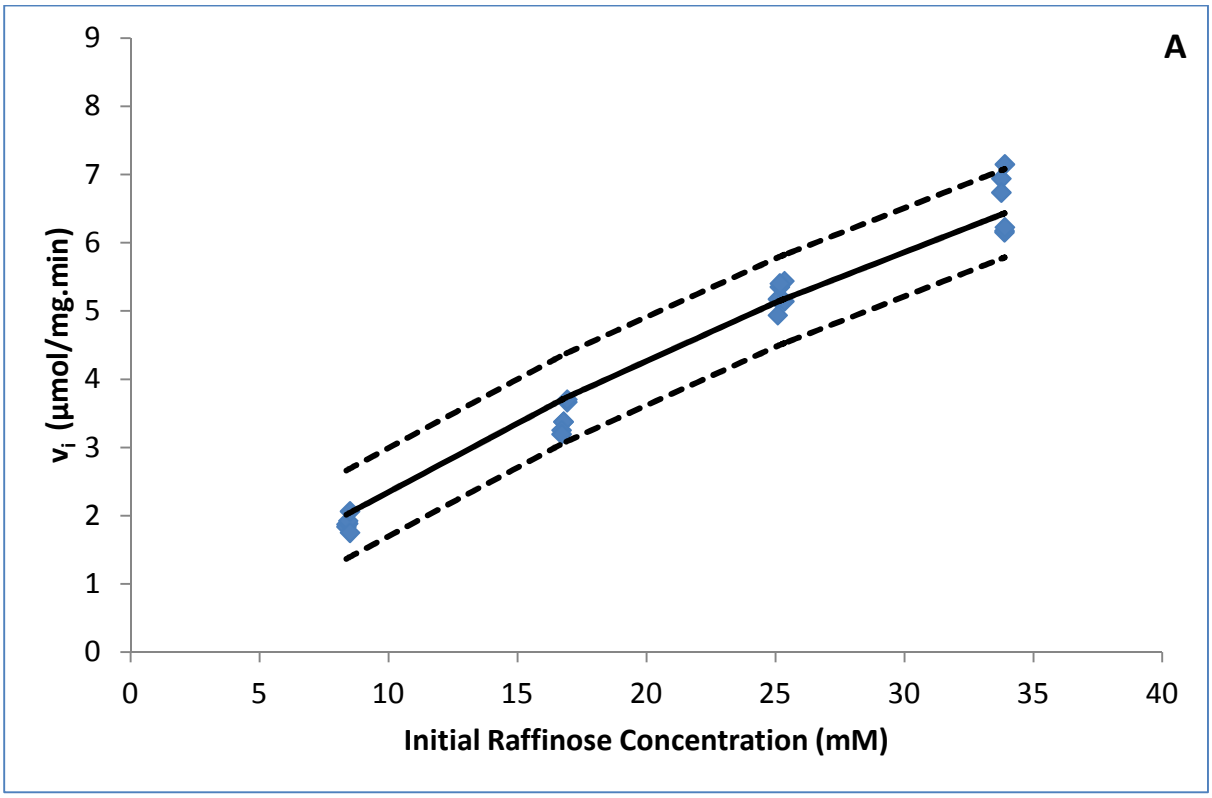
In order to enable the calculation of optimum conditions for the hydrolysis of RFOs in navy bean flour processing the effect of temperature on V_{maxSt} and V_{maxRf} must be determined. The optimum temperature range reported by Zymus® for Enzidase® AGS activity is 45°C to 60°C with an effective temperature range of up to 60°C.

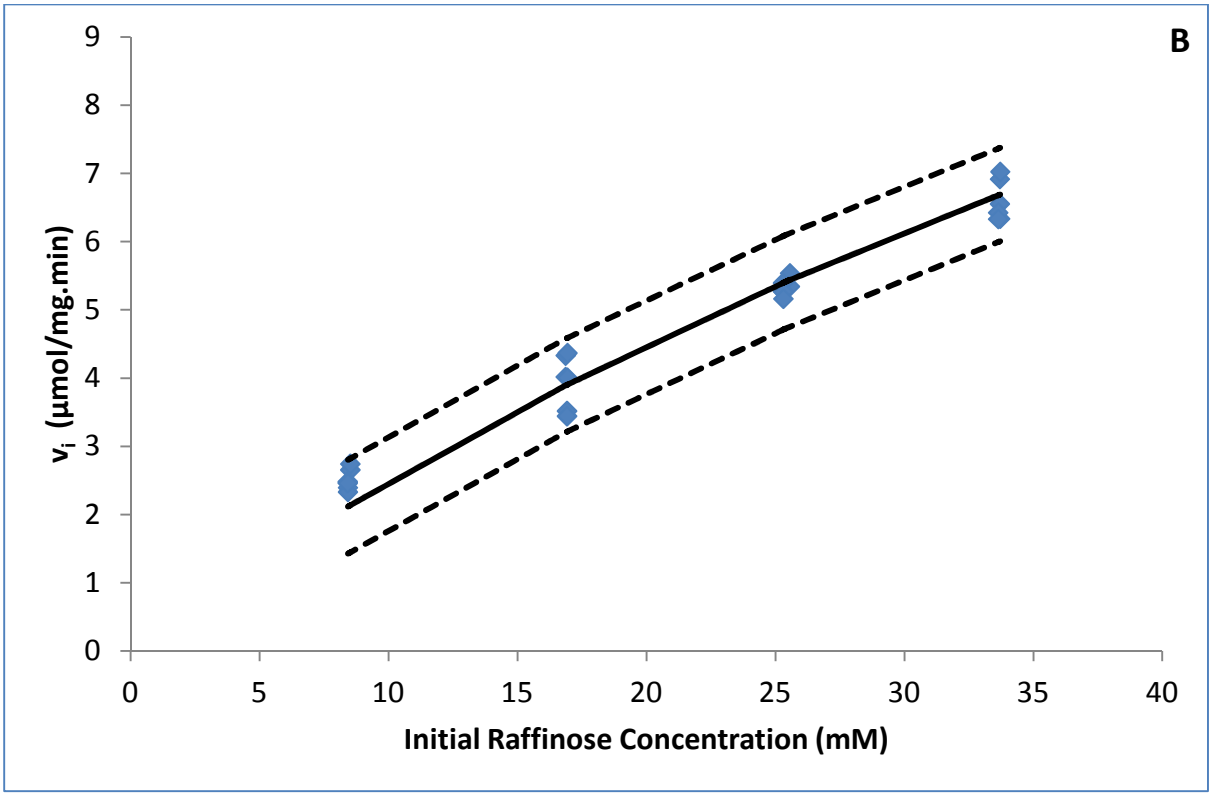
Experiments were undertaken to measure the effect of temperature on the kinetic parameters V_{maxSt} and V_{maxRf}. The methods undertaken in section 5.4 were repeated at

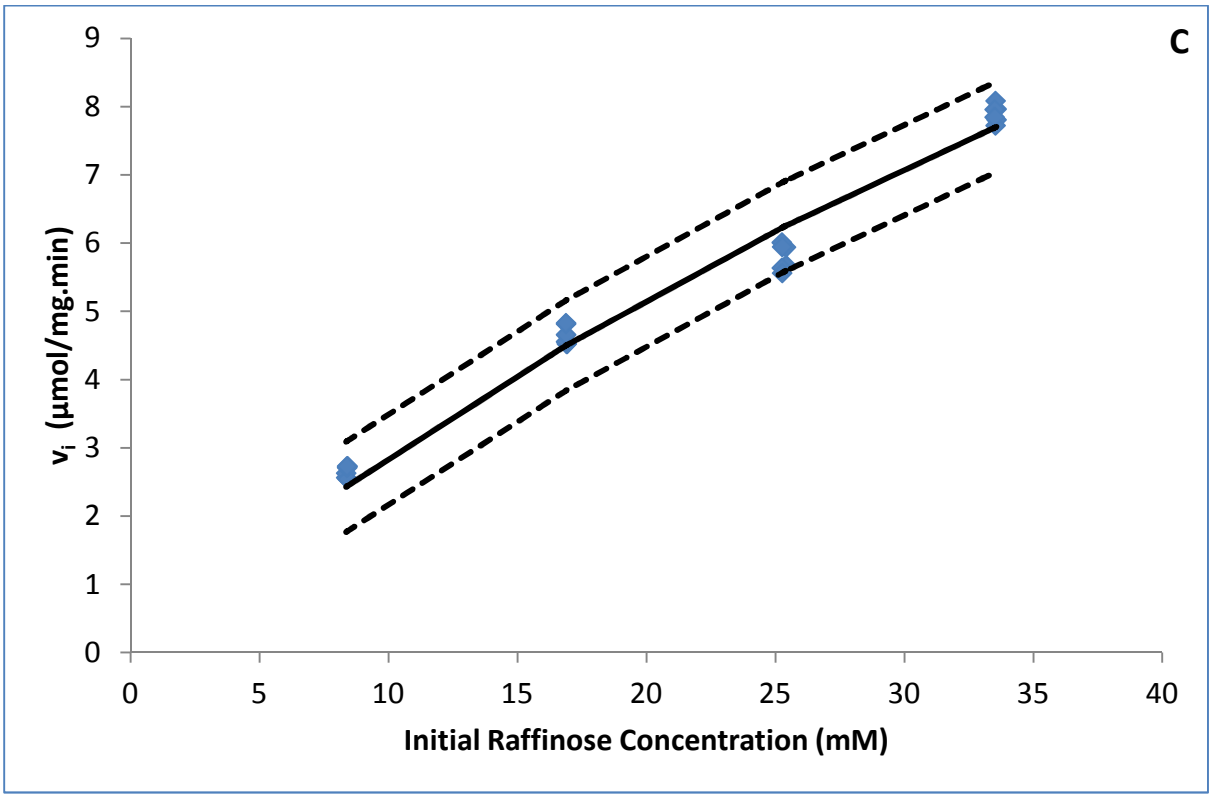
30, 40, 50, and 60°C using a water bath to pre-heat the samples and maintain the target temperature during the hydrolysis period.

The data is presented in the same fashion as figures 5.5 – 5.7 for each temperature and substrate. The relevant kinetic expressions were then used to calculate the predicted v_i using the initial concentrations from the measured data and the values determined in section 5.4 for K_m and V_{max}.

Non-linear regression was again employed to determine the value of V_{max} (K_M is not affected by temperature and so was held constant) which resulted in the maximum R^2 value for the overall fit of the calculated values for v_i with the experimentally measured values. Figure 5.9 below shows the collected data and Michaelis Menten fits.




B
9
8
7
6
5
4
3
2
1
0
v_i (µmol/mg.min)
0
5
10
15
20
25
30
35
40
Initial Raffinose Concentration (mM)


C
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6
5
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2
1
0
v_i (µmol/mg.min)
0
5
10
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30
35
40
Initial Raffinose Concentration (mM)

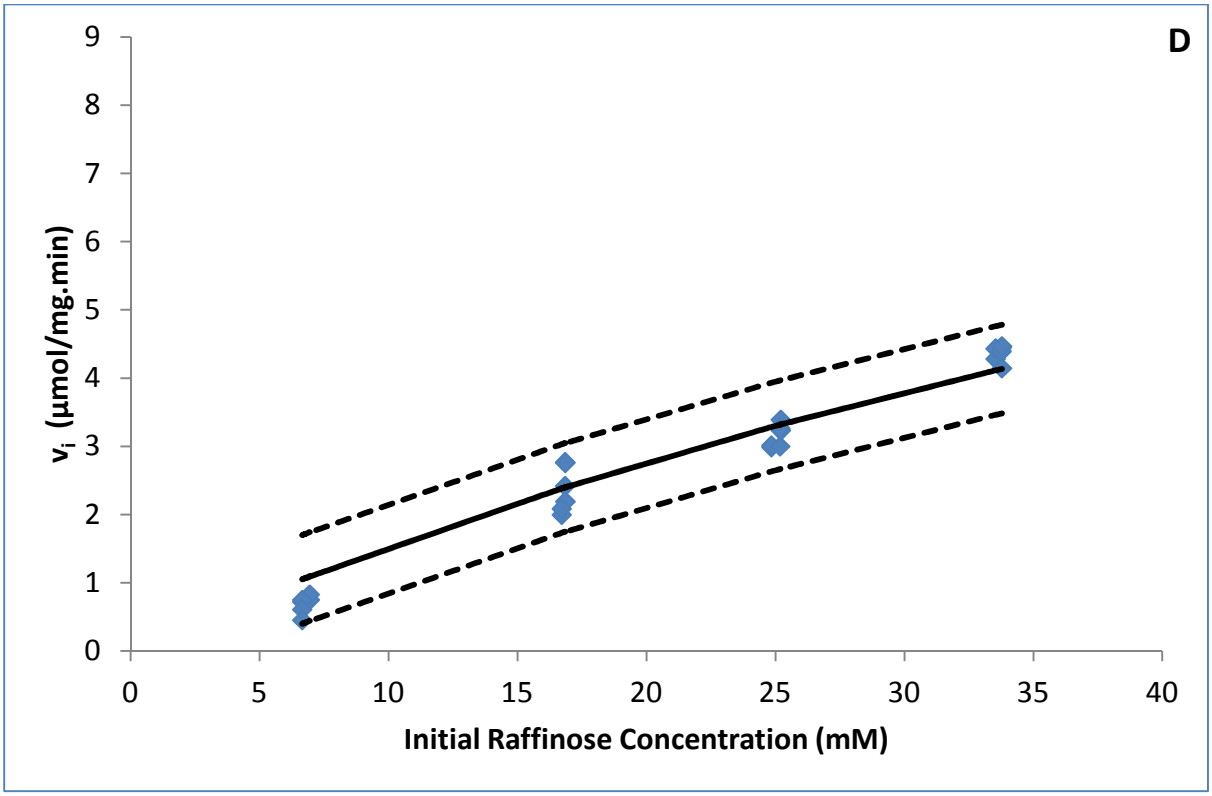
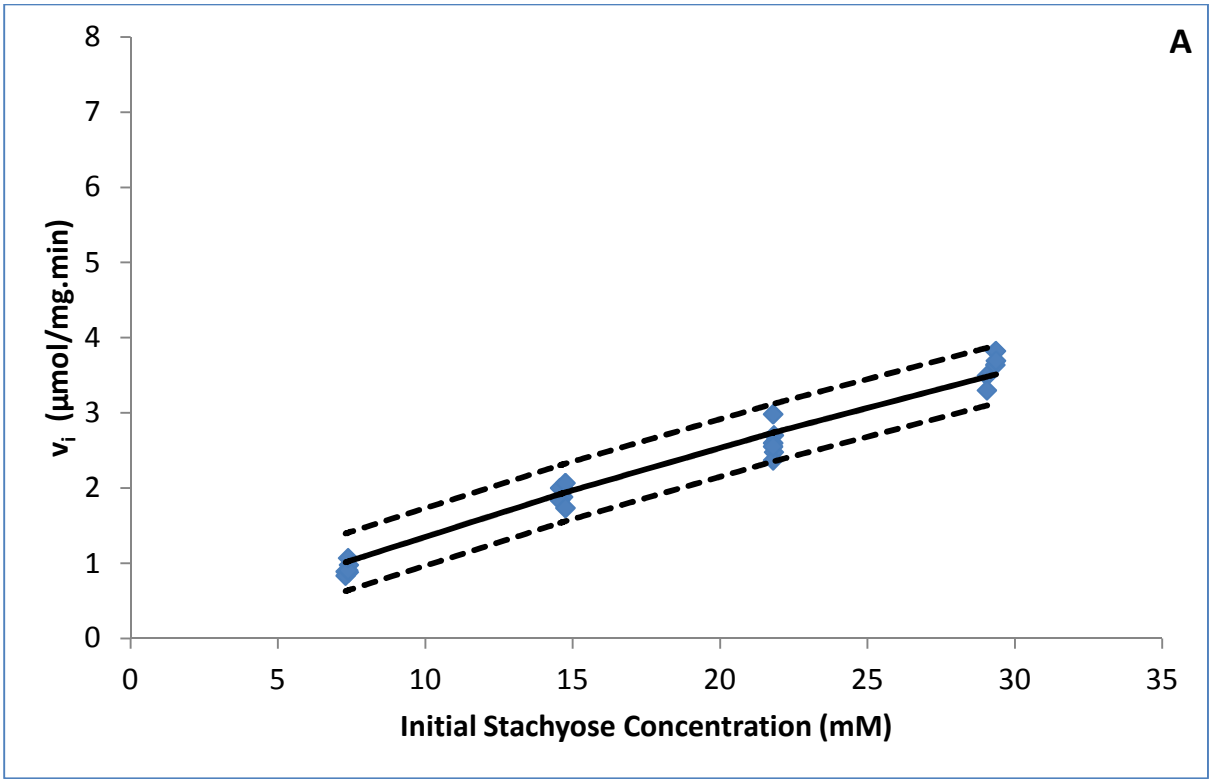


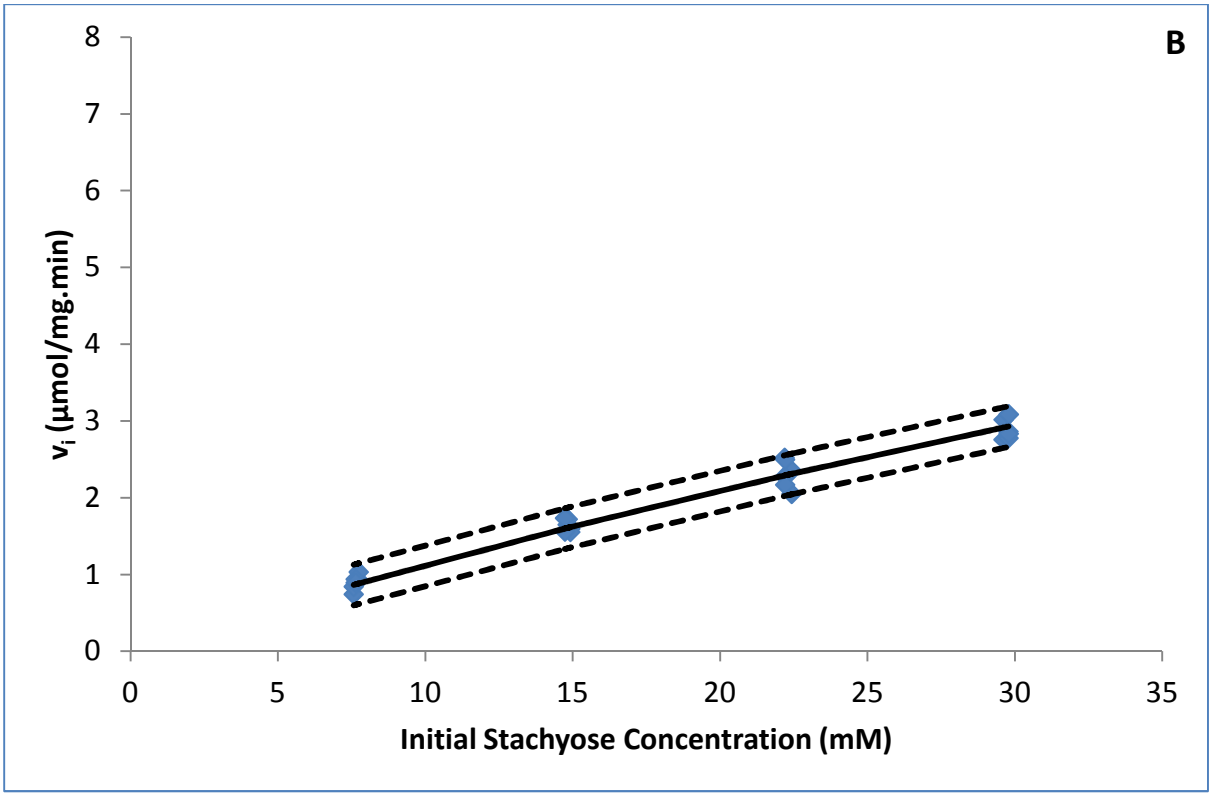
Figure 5.9 Initial rate of reaction versus raffinose concentration at A = 30°C; B = 40°C; C = 50°C; D = 60°C. Points represent measured data; the solid line shows the line of best fit for the kinetic expression using values of K_{MRf} = 86.8 mM as determined in section 5.4. Values for V_{maxRf} were determined for each temperature by non linear regression. The dotted lines represent the 95% confidence interval for the predicted line; the R^2 values for the fit of the predicted lines are A = 0.97; B = 0.96; C = 0.97 and D =0.95

Figure 5.9 shows the initial rate of reaction versus raffinose concentration for hydrolysis of raffinose by Enzidase® AGS at several temperatures ranging from 30°C to 60°C. The predicted v_i was calculated using K_{mRf} = 86.8 as determined in section 5.4. Non linear regression was employed to determine the optimum value for V_{maxRf} at each temperature. The predicted values for v_i fit well with the measured data, the R^2 values ranging from 0.95 to 0.97 across the temperature range. The values for V_{maxRf} determined in these experiments are given below:

Table 5.4 Measured values of V_{maxRf} at several temperatures.

T (°C)	V_{maxRf} (µmol/mg.min)
30	22.9
40	23.9
50	27.6
60	14.8


A
8
7
6
5
4
3
2
1
0
v_i (µmol/mg.min)
0
5
10
15
20
25
30
35
Initial Stachyose Concentration (mM)


B
8
7
6
5
4
3
2
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v_i (µmol/mg.min)
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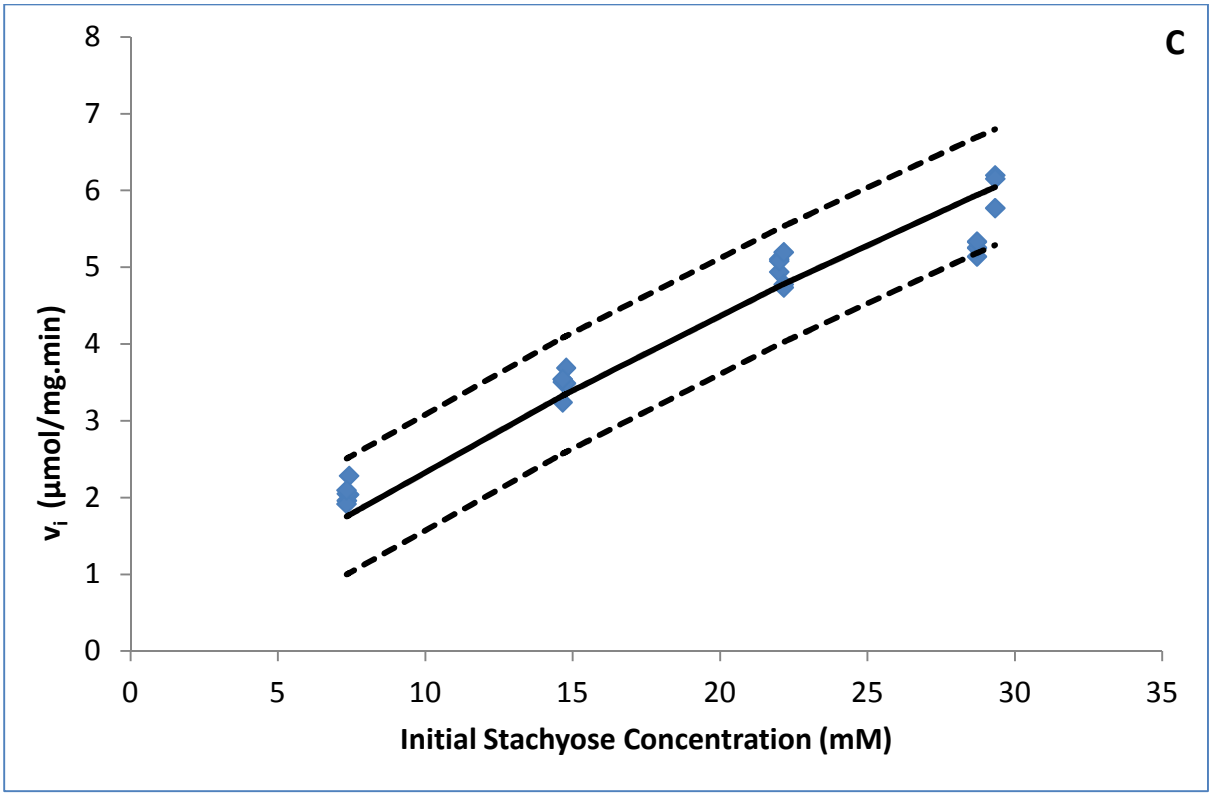
20
25
30
35
Initial Stachyose Concentration (mM)



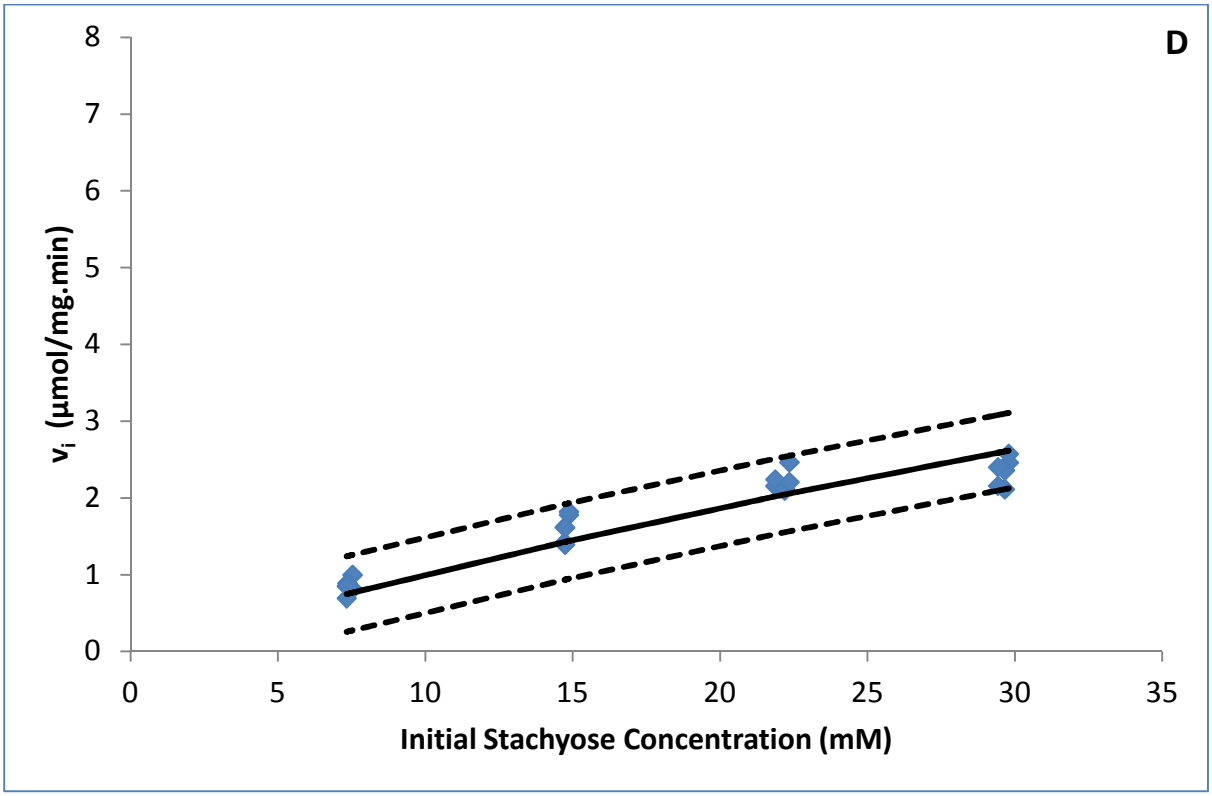


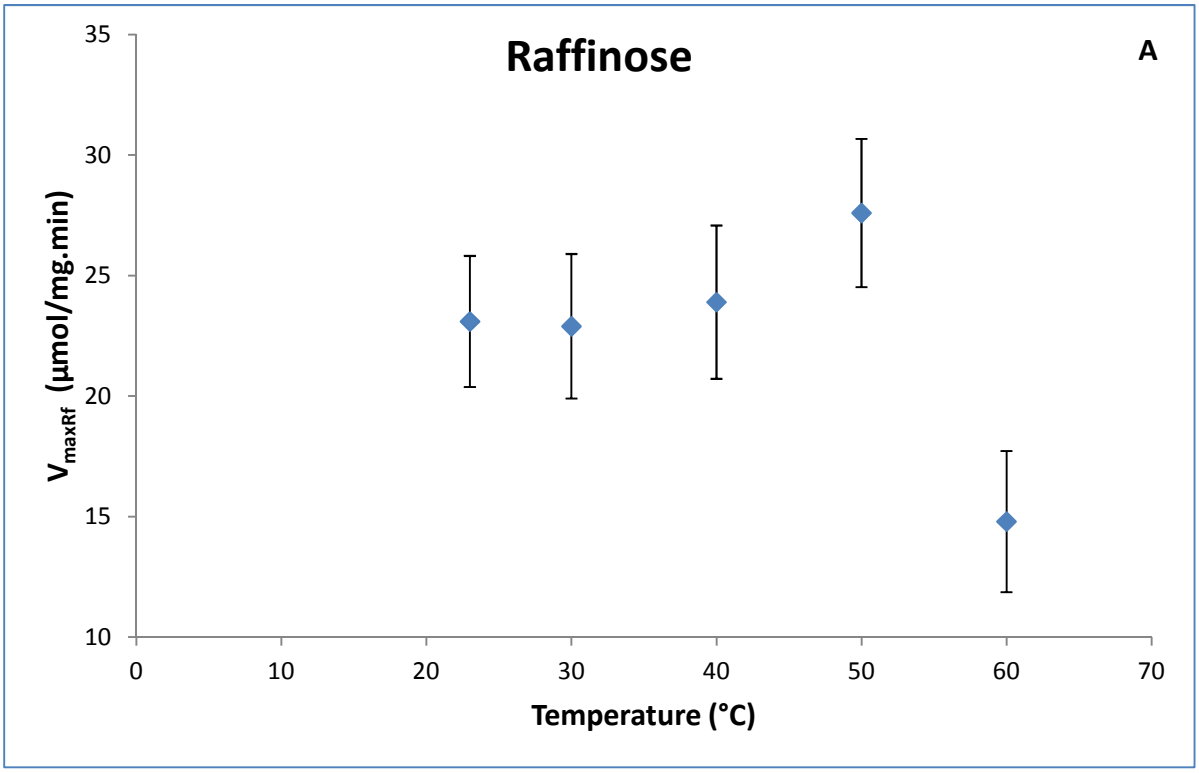
Figure 5.10 Initial rate of reaction versus stachyose concentration at A = 30°C; B = 40°C; C = 50°C; D = 60°C. Points represent measured data; the solid line shows the line of best fit for the kinetic expression using values of K_{MSt} = 130.5 mM as determined in section 5.4. Values for V_{maxSt} were determined for each temperature by non linear regression. The dotted lines represent the 95% confidence interval for the predicted line; the R^2 values for the fit of the predicted lines are A = 0.98; B = 0.97; C = 0.94 and D =0.86

Figure 5.10 shows the initial rate of reaction versus stachyose concentration for hydrolysis of raffinose by Enzidase® AGS at several temperatures ranging from 30°C to 60°C. The predicted v_i was calculated using K_{mSt} = 130.5 as determined in section 5.4. Non linear regression was employed to determine the optimum value for V_{maxSt} at each temperature.

The predicted values for v_i fit well with the measured data for 30 - 50°C, the R^2 values ranging from 0.94 to 0.98 across this temperature range. The R^2 value for the 60°C data is a bit lower at 0.86, the calculated values being a poorer fit for the measured data at this temperature. The values for V_{maxSt} determined in these experiments are given below:

Table 5.5 Measured values of V_{maxSt} at several temperatures.

T (°C)	V_{maxSt} (µmol/mg.min)
30	19.1
40	15.8
50	32.9
60	14.1



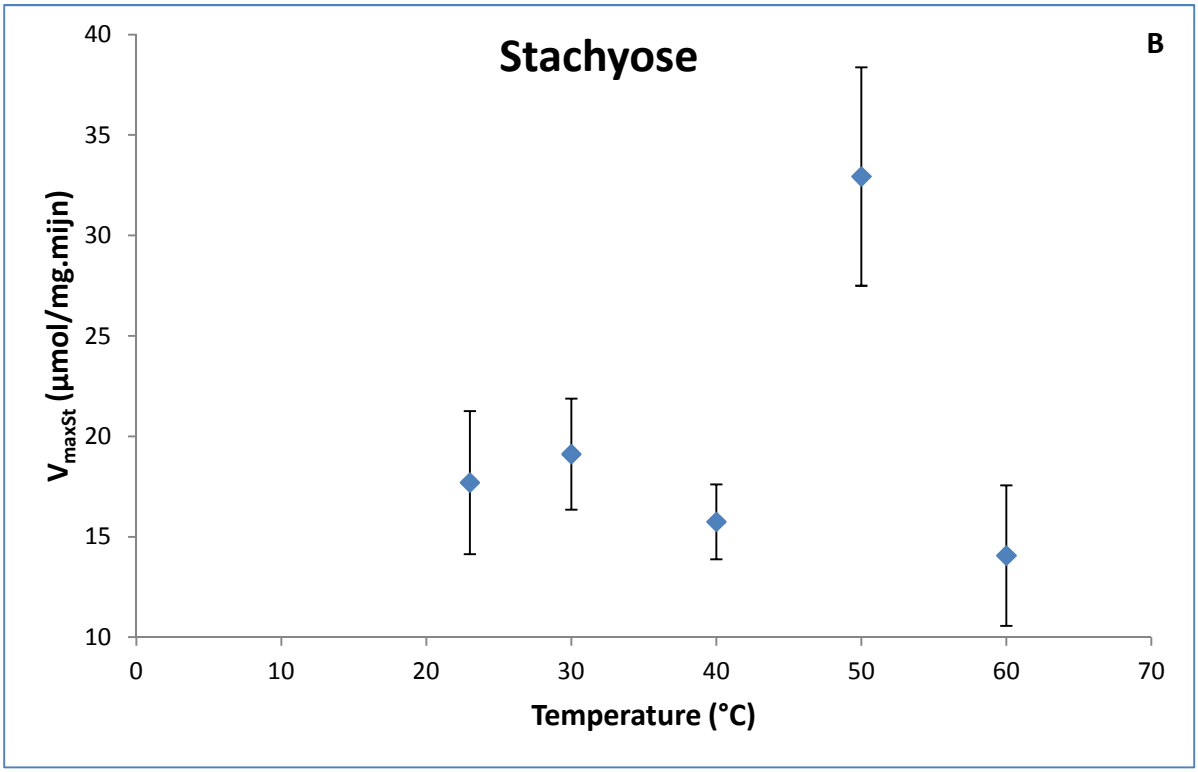


Figure 5.11 V_{max} versus temperature for hydrolysis of raffinose (A) and stachyose (B) by Enzidase® AGS. Error bars represent the V_{max} values corresponding to the 95% confidence interval for the data predicted in figures 5.9 & 5.10.

Figure 5.11 shows the values for V_{maxRf} and V_{maxSt} as a function of temperature. This data clearly shows that the enzyme has begun to denature at 60°C as the V_{max} values for both raffinose and stachyose drop significantly from those at 50°C. This data doesn't follow the trend expected by the Arrhenius equation; in fact the V_{max} values at temperatures below 50°C are relatively constant. The 40°C value for stachyose is lower than those at 23 and 30°C but agree with them within the range of the error bars. The data at 50°C was the only point measured within the optimum temperature range stated by the supplier of Enzidase (with the exception of the 60°C (top of stated optimum range) data in which enzyme denaturation has obviously set in).

Enzyme catalysed reactions can be more complicated than standard chemical reactions because the enzyme is a complex biological molecule and thus its own conformation can be affected by temperature. It is possible that some temperatures might enlarge the number of conformations of enzyme intermediates in equilibrium with those at standard temperature and that one or more of the new conformations may react at a different rate, or not react at all (Taylor 2002).

The failure of the measured data to conform to the Arrhenius equation could therefore be due to some types of the aforementioned conformational changes to the enzyme as the temperature changes. It is therefore recommended that for calculation of expected hydrolysis rates the values for V_{maxRf} and V_{maxSt} determined at 23°C be used for all temperatures aside those that are close to 50°C and that temperatures approaching 60°C be avoided due to denaturation of the enzyme.

5.7 Calculation of Critical Limits for Galactose Concentration

The kinetic equations developed in sections 5.2 and 5.4 can be used to predict the effect of galactose inhibition as a function of galactose concentration. Figure 5.12 shows this relationship for two concentrations of stachyose that are of the order of the maximum concentration if all of the stachyose in a saturated bean particle were dissolved in the water phase. The y axis of figure 5.12 is the relative rate of hydrolysis calculated as the rate of hydrolysis divided by what the rate would be with no galactose present.

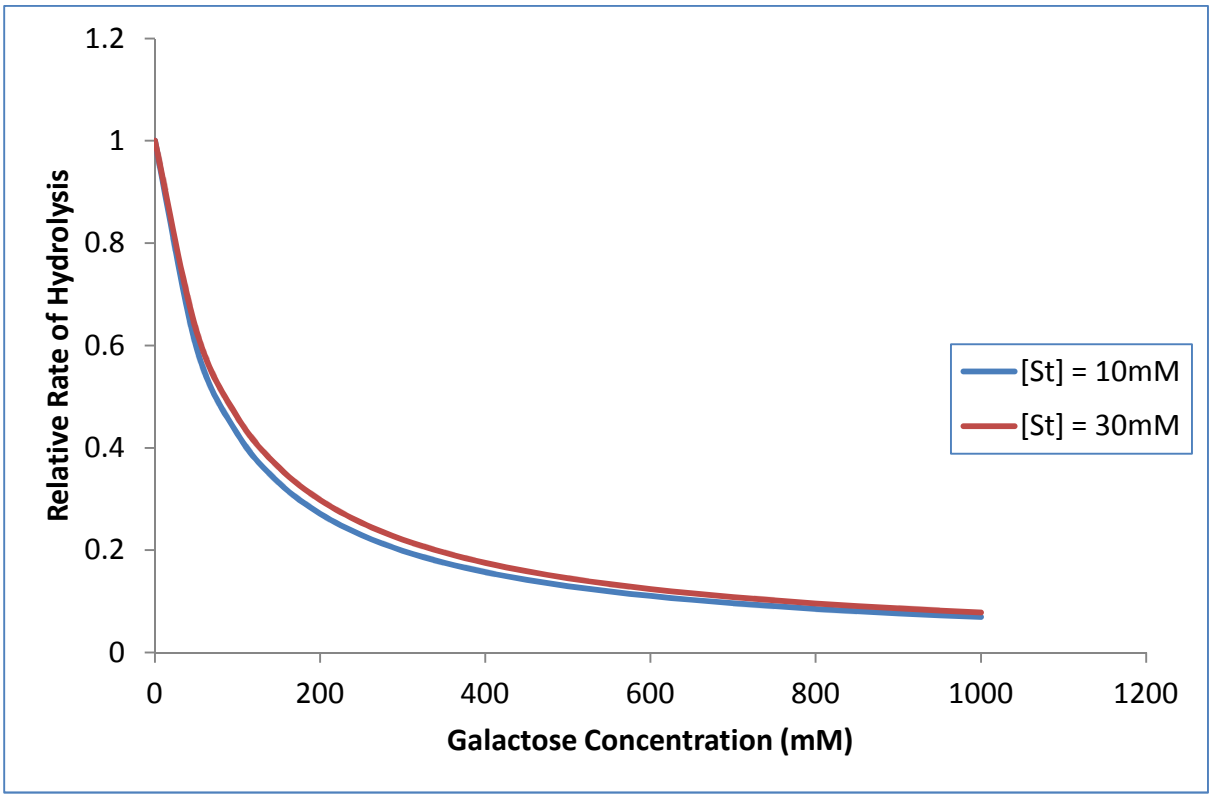


Figure 5.12 Relative rate of stachyose hydrolysis versus galactose concentration for two stachyose concentrations.

The maximum concentration of galactose likely in a bean flour/enzyme system is when the flour particles are saturated and all of the RFOs have been hydrolysed. In this worst case scenario the galactose concentration will only reach approximately 85mM. It can be clearly seen in figure 5.12 that at this order of galactose concentration the inhibitory effect is only sufficient to approximately halve the hydrolysis rate. A decrease of 50% in hydrolysis rate does not preclude the use of α-galactosidase hydrolysis at these concentrations, but will only serve to increase potential processing times.

5.8 Conclusion

The enzyme kinetics for the hydrolysis of stachyose and raffinose by Enzidase® AGS α-galactosidase were investigated and values for the kinetic parameters K_{MSt}= 130 mM , K_{MRf} = 86.8 mM, K_{Ga} =69.1 mM , V_{maxSt} = 17.7 $\frac{\mu mol}{mg.min}$ and V_{maxRf} = 23.1 $\frac{\mu mol}{mg.min}$ were determined and validated at ambient temperature (23°C).

The effect of temperature on V_{maxRf} and V_{maxSt} did not follow the Arrhenius equation, V_{maxRf} and V_{maxSt} remained relatively constant (V_{maxRf} = 22.9-23.9 $\frac{\mu mol}{mg.min}$ and V_{maxSt} = 15.8 – 19.1 $\frac{\mu mol}{mg.min}$) at temperatures below 50°C, increased significantly at 50°C (V_{maxRf} = 27.6 $\frac{\mu mol}{mg.min}$ and V_{maxSt} = 32.9 $\frac{\mu mol}{mg.min}$) and dropped off at 60°C (V_{maxRf} = 14.8 $\frac{\mu mol}{mg.min}$ and V_{maxSt} = 14.1 $\frac{\mu mol}{mg.min}$) due to denaturation. It was recommended that for prediction of reaction rates at temperatures between ambient and 50°C the V_{maxRf} and V_{maxSt} values determined at ambient be used. For reactions at close to 50°C the V_{maxRf} and V_{maxSt} values determined at 50°C should be used, and temperatures approaching 60°C should be avoided.

Chapter 6

Low Moisture Enzyme Processing

6.1 Introduction

It was found in chapter 3 that leaching is an effective mechanism for the removal of RFOs from navy bean flour as long as a concentration gradient is maintained to act as the driving force for diffusion, (i.e. as long as the concentration of RFOs in the leaching water is lower than that of the navy bean flour). These conditions are easily achieved by applying a large ratio of leaching water to navy bean flour.

There are however some significant drawbacks to this approach. Unless the particular application to which the navy bean flour is to be put requires a relatively dilute slurry there will be the need to remove a large fraction of the water phase, adding both cost and complexity to the process. If the water phase is removed via an evaporation mechanism then the resulting concentrate will retain all of the RFOs that have been leached out of the bean flour. If the water phase is removed via some other mechanism, for example filtration, then the leached RFOs will be removed along with the water, as will all of the other soluble solids that have been extracted, amounting to approximately 25% loss of the dry mass of the bean flour including all water soluble nutrients (see chapter 3.4.3).

The ideal system for RFO removal would be such that the concentration gradient between the leaching water and the bean flour remains at a maximum, but that required a minimal mass of leaching water and didn't require the loss of all of the soluble solids. To that end it is proposed that a combination of leaching and enzymatic hydrolysis be employed. The presence of α-galactosidase in the leaching water would hydrolyse the RFOs as they were leached out of the bean flour, effectively keeping the concentration of RFOs in the leaching water at zero. This would allow the maximum leaching rate whilst minimising the ratio of leaching water to bean flour.

The bean flour/water mixture could then be dried so that all of the soluble solids are retained with the RFOs having been converted into di- and monosaccharides. This approach was demonstrated in figure 3.7 where the flour to water ratio was 1 part flour to 9 parts water.

In applications where a slurry of bean flour is required (for example to add to a formulation as a thickener), addition of an enzyme can allow RFO removal as long as sufficient time is allowed for leaching and hydrolysis before the solution is heated to greater than 60°C where enzyme denaturation becomes significant. For details on leaching rates refer to chapter 4 and hydrolysis rates refer to chapter 5.

There are however many other applications in which the flour would be used in a dry form. An example of this is the extrusion of bean navy bean flour for which the flour moisture contents of 30% or less are required (Van De Ven 2009).

It would be expected that there must be some limiting moisture addition required to allow mobility of the RFOs through the bean matrix to the surface of the flour particles where the enzyme is applied, or to allow migration of the enzyme into the particle where the RFOs are located. Experiments were therefore carried out to determine the minimum moisture addition required for the hydrolysis of RFOs by α-galactosidase.

6.2 Low Moisture Enzyme Hydrolysis

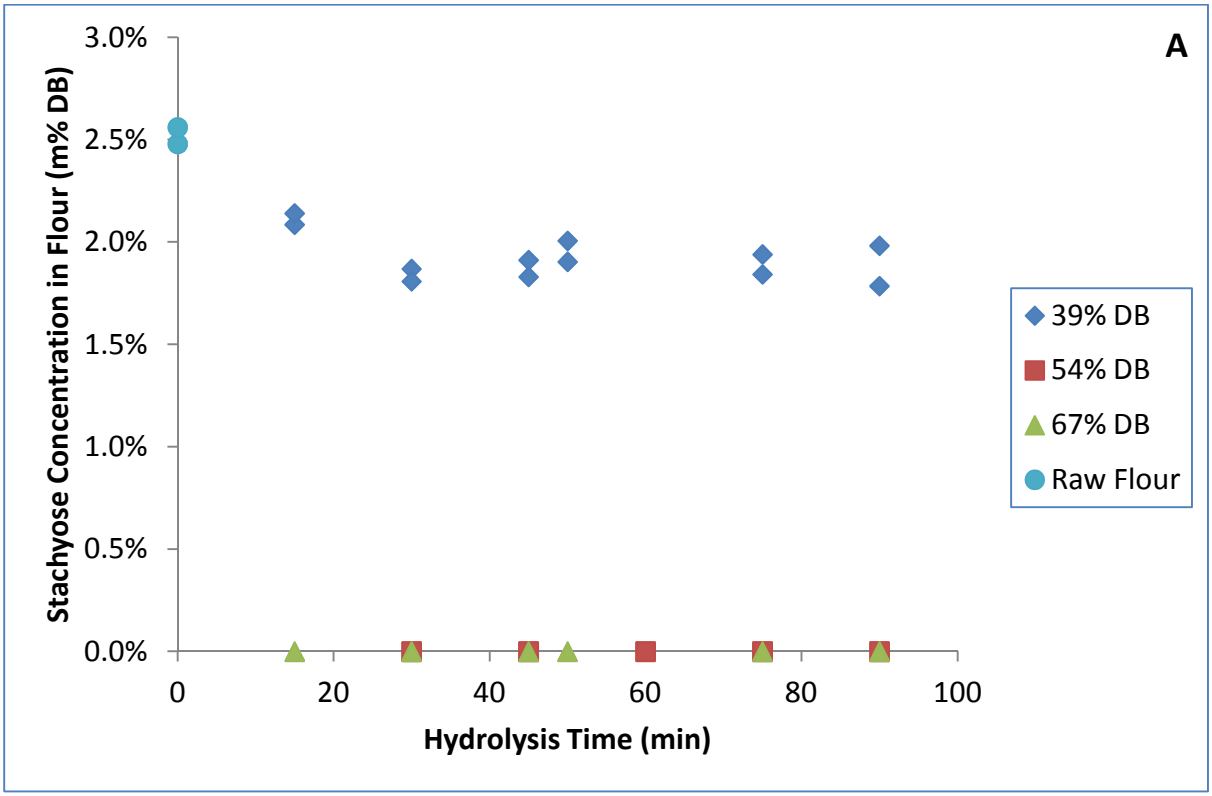
An experiment was conducted to determine the effectiveness of α-galactosidase in the hydrolysis of raffinose and stachyose from navy bean flour at low moisture contents.

150g samples of navy bean flour were placed in a rotating blade type food processor. Enzyme solutions were prepared using 18.3g of α-galactosidase (supplied by Novozymes®) with the following amounts of distilled water:

Table 6.1 Ratios of reactants to be combined for a low moisture enzyme experiment.

Final Moisture Content (WB)	Final Moisture Content (DB)	Mass of Flour (g)	Mass of α-galactosidase (g) (Novozymes®)	Mass of Water (g)
28%	39%	150	18.3	11.7
35%	54%	150	18.3	30.2
40%	67%	150	18.3	46.2

The enzyme solution was added to the food processor and the mixture was blended for 30 seconds until well mixed. Samples were taken from the mixture every 15 minutes and placed in 40mL centrifuge tubes. The hydrolysis reaction was halted by addition of 10 ml of 80% ethanol at 70°C followed by immersion of the tube in boiling water for 5 minutes. Following this a further 30 ml of 80% ethanol was added to the centrifuge tube and the extraction of the sugars was carried out as described in method B1. The sugars were analysed using the HPLC method described in experiment 3.1. Each set of conditions were repeated in duplicate. Figure 6.1 A and B show the reduction in RFO content of the bean flour as a function of time for stachyose and raffinose respectively.



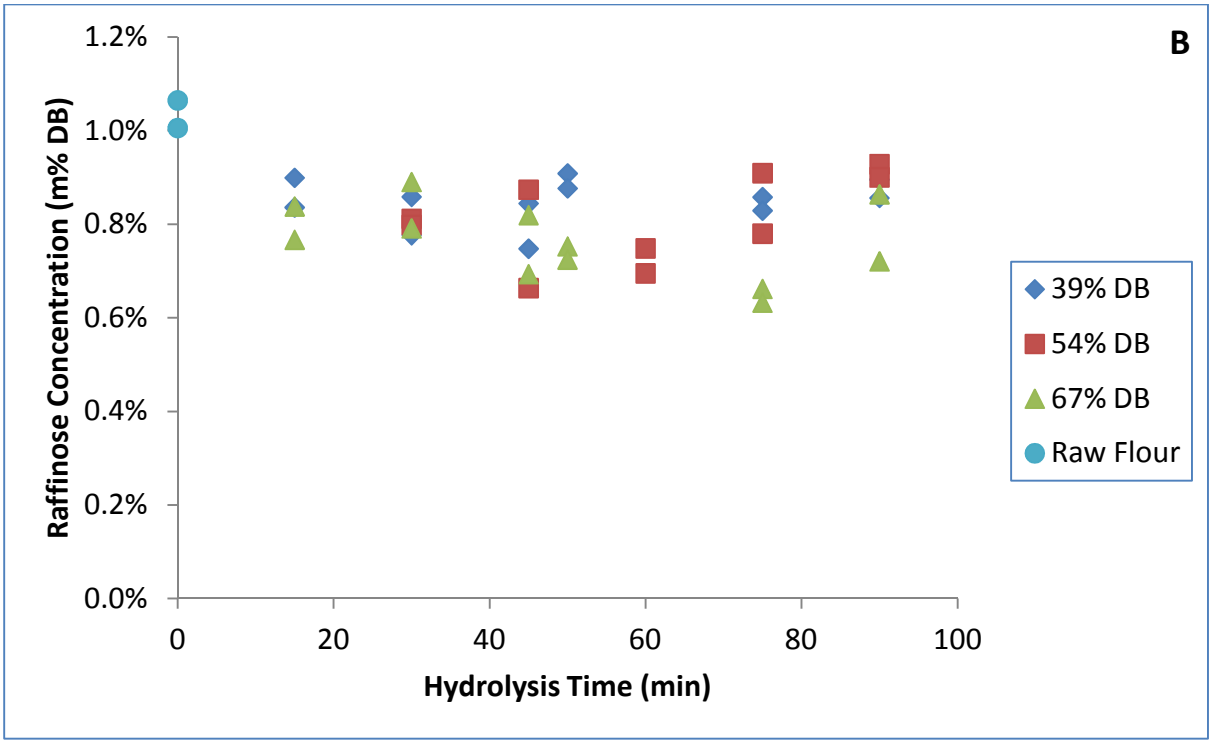


Figure 6.1 Concentrations of **A:** stachyose and **B:** raffinose in navy bean flour during hydrolysis with alpha-galactosidase at several moisture contents. A ratio of 1 part enzyme to 8 parts bean flour (DB) was maintained for all moisture contents.

Stachyose is completely hydrolysed within 15 minutes at all final equilibrated moisture contents of 35% WB and higher (constant ratio of 1 part enzyme to 8 parts bean flour i.e. concentration of enzyme solution increases with decreasing moisture content). Only a small proportion of the raffinose is hydrolysed and hydrolysis seems to have ceased within the first 30 minutes for all samples.

It should be noted that the while the raffinose concentration was only decreased by between 10 and 30% the amount of raffinose hydrolysed is significantly greater than that. Every molecule of stachyose that is hydrolysed produces one molecule of galactose and one molecule of raffinose. Given that all of the stachyose was hydrolysed in the 35% and 40% samples and noting that the initial concentration of stachyose is about 2.5 times that of raffinose this equates to an additional raffinose content of about 1.9% (DB), or a total of nearly 3 times the initial concentration. This means that the proportion of total raffinose hydrolysed ranges from 75% to 95%.

These results show that significant reductions in RFO levels in bean flour can be achieved with the addition of relatively small amounts of water. This presents an opportunity to reduce oligosaccharides without significant reduction in yield (through washing out other solutes) or without excessive dehydration costs.

In order for the hydrolysis of RFOs in navy bean flour to be commercially viable the α-galactosidase must be available in commercial quantities. The α-galactosidase used in the previous experiment and in chapter 3.5.3 was supplied by Novozymes®, is produced as a reagent for assays of α-galactosides and is only available in relatively small quantities. The enzyme kinetics presented in chapter 5 were measured using Enzidase® AGS which is a commercially available, food – safe α-galactosidase. It is also in a powdered form which offers the possibility to dry blend it with the bean flour allowing hydrolysis to take place upon addition of water during a later processing step.

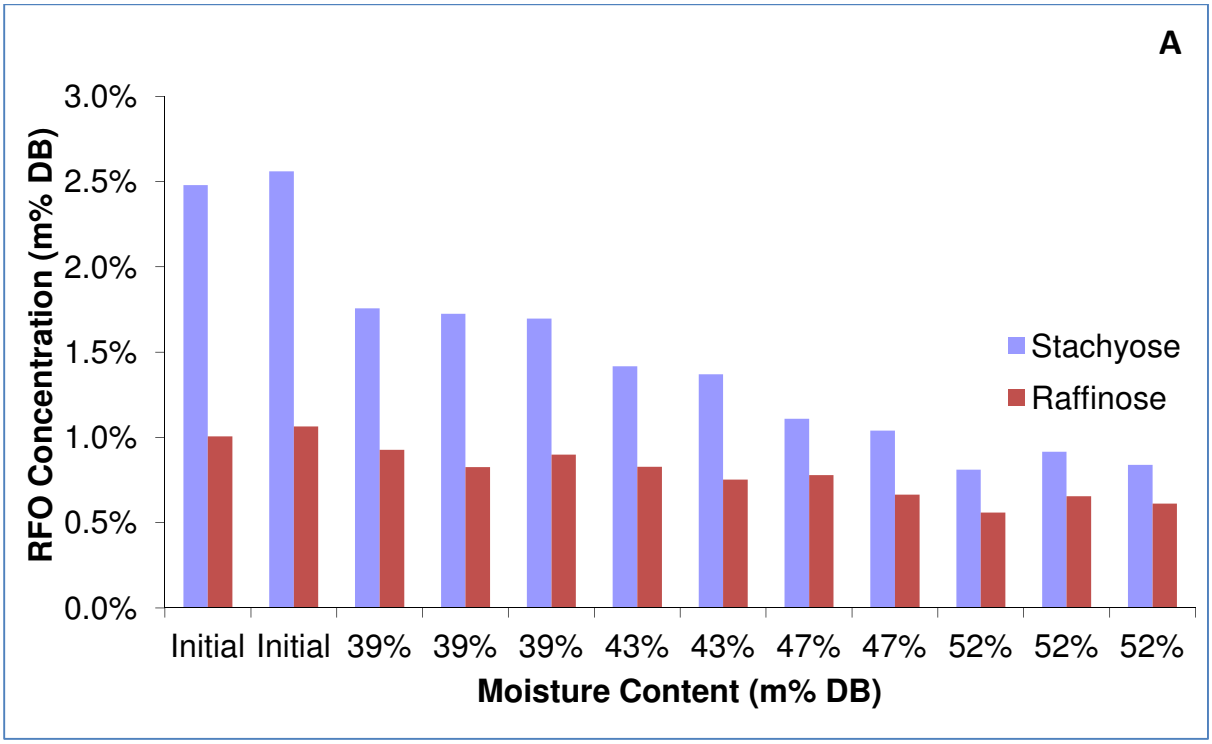
6.3 Low Moisture Hydrolysis of RFOs with Enzidase® AGS

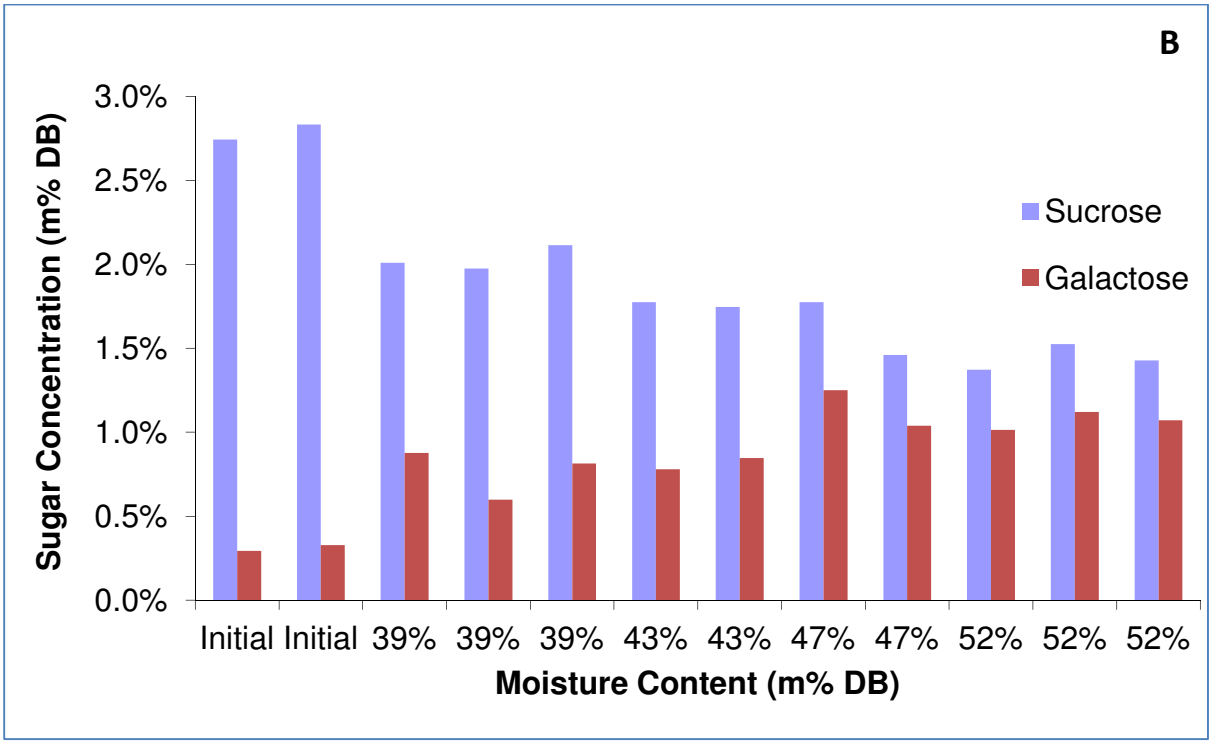
An experiment was carried out to characterise the behaviour of Enzidase® AGS across a range of low moisture contents. Given that the stachyose was completely hydrolysed at moisture contents of 35% (wb) and above (figure 6.1A), moisture contents between 28% and 35% (wb) were investigated. The method employed in experiment 6.2 was repeated with the exception that the powdered enzyme was pre-mixed with the dry flour prior to the addition of the water. The ratio of enzyme powder to flour was held constant at 1 part enzyme to 100 parts flour. The amounts of water added were such that the final moisture contents achieved were as follows:

Table 6.2 Ratios of reactants to be combined for a low moisture enzyme experiment.

Final Moisture Content (WB)	Final Moisture Content (DB)	Mass of Flour (g)	Mass of Enzidase® AGS (g)	Mass of Water (g)
28%	39%	150	1.5	27.5
30%	43%	150	1.5	32.6
32%	47%	150	1.5	38.6
34%	52%	150	1.5	44.3

The hydrolysis was carried out for 90 minutes at 53°C which is the middle of the optimal temperature range for Enzidase® AGS activity recommended by Zymus®. The concentrations of RFOs are given in figure 6.2(A). The hydrolysis products are also presented in figure 6.2 (B-C).





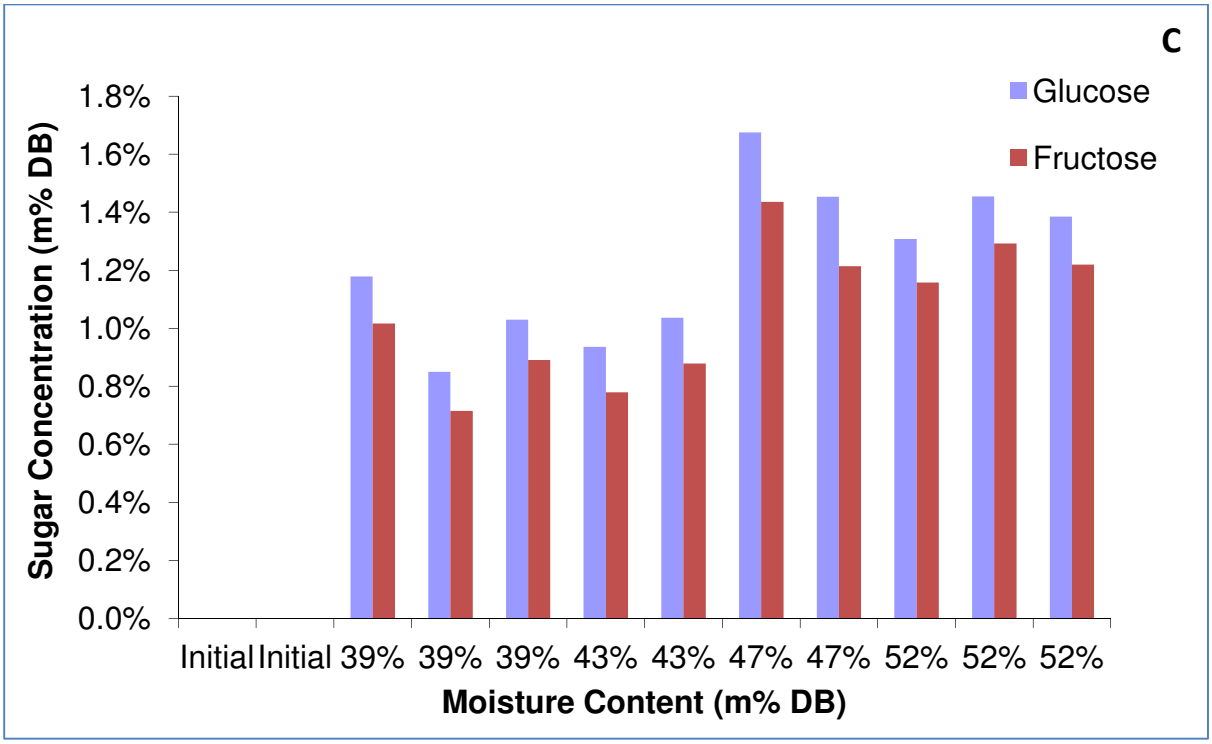


Figure 6.2 Concentration of RFOs and hydrolysis products in navy bean flour following 90 minutes of hydrolysis with alpha-galactosidase at 53°C with constant ratio of 1 part enzyme to 100 parts flour at moisture contents between 28% and 34%. **A:** stachyose and raffinose; **B:** sucrose and galactose; **C:** glucose and fructose

Figure 6.2(A) clearly demonstrates the dependence of Enzidase® AGS activity on the moisture content of the system with a steady increase in the proportion of both stachyose and raffinose hydrolysed with increasing moisture content.

Figure 6.2(B) shows a steady decrease in the concentration of sucrose and a steady increase in the concentration of galactose as moisture content increases. Sucrose and galactose are the hydrolysis products of stachyose and raffinose and so the expected trend is that the concentrations of both of these sugars should increase with moisture content as more stachyose and raffinose is hydrolysed. The fact that the overall concentration of sucrose is decreasing despite the fact that it is being produced by the RFO hydrolysis, indicates that Enzidase® AGS also hydrolyses sucrose.

Figure 6.2(C) shows the concentration of glucose and fructose both increasing with increasing moisture content. Glucose and fructose are the hydrolysis products of sucrose and the presence of these sugars in the hydrolysed flour samples confirms that the sucrose is being hydrolysed.

The data presented in figure 6.2(A) can be re-plotted with a longer x axis and extrapolated via linear regression to predict the moisture content at which a target RFO concentration can be achieved by the enzyme.

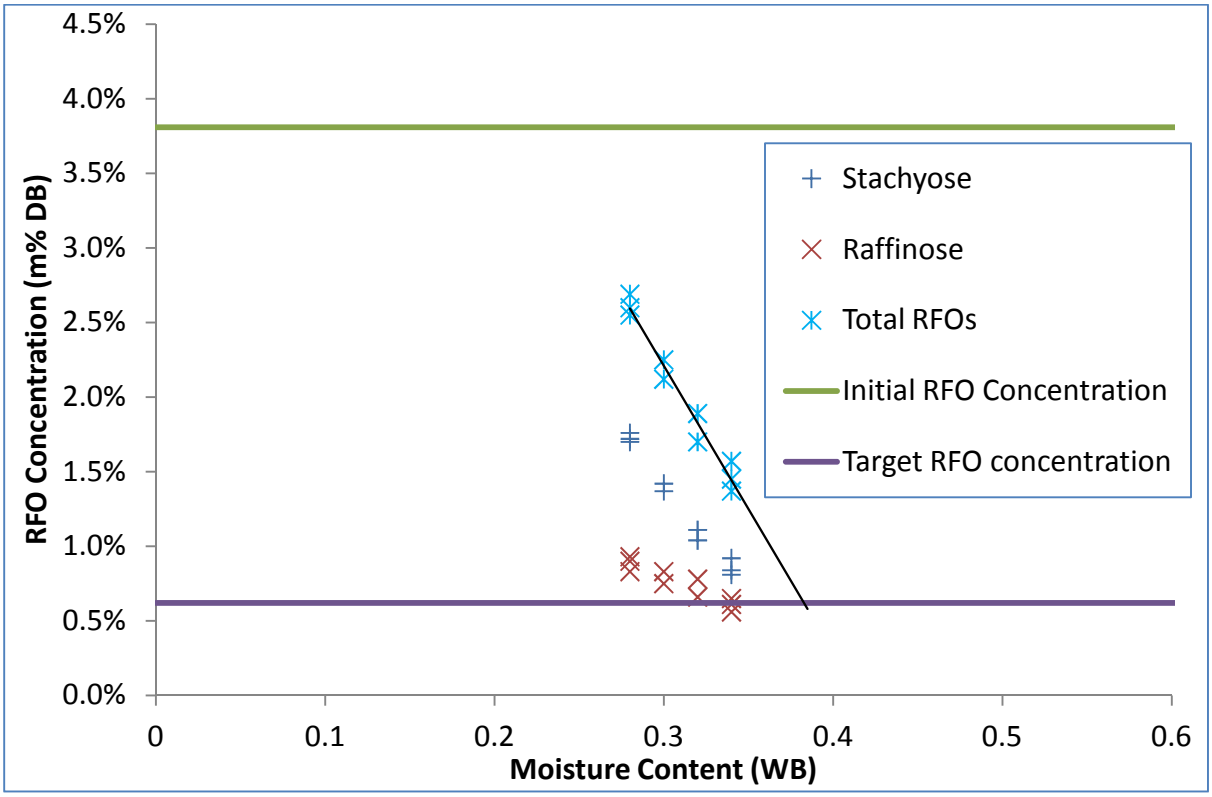


Figure 6.3 Data from figure 6.2-A plotted on a larger x-axis and extrapolated to predict the minimum moisture content at which α-galactosidase can achieve the necessary level of hydrolysis for cessation of measureable flatus response following ingestion.

Figure 6.3 shows the data from figure 6.2(A) for residual raffinose and stachyose concentration in navy bean flour following hydrolysis by Enzidase® AGS for 90 minutes. The total RFO concentration (stachyose + raffinose) is also plotted so that the moisture content at which a given total RFO concentration can be achieved can be predicted by extrapolating the line of best fit from the data ($R^2 = 0.97$).

The target residual RFO concentration in figure 6.3 was set at 0.62% because this was the concentration of RFOs in the low-oligosaccharide soy flour found by (Suarez, et al. 1999) to produce flatus levels barely distinguishable from that produced by a baseline diet of white rice.

The critical moisture content at which the total concentration of RFOs remaining post leaching predicted using this data is 38.3% WB (62.1% DB).

The reason that the RFO hydrolysis doesn't progress to completion could be due to two causes. The first possible cause of incomplete hydrolysis is that the α-galactosidase is inhibited by the galactose produced during the hydrolysis of the RFOs. As the hydrolysis progresses the concentration of galactose steadily increases to the point at which it completely inhibits the hydrolysis of the remaining raffinose. This hypothesis is supported by the data presented in chapter 5 that indicates that Enzidase® AGS is inhibited by galactose; however as demonstrated in chapter 5 the concentration of galactose would need to be extremely high for the inhibition to completely halt the hydrolysis. The maximum concentration of galactose likely in a bean/flour system is when the flour particles are saturated and all of the RFOs have been hydrolysed. In this case the galactose concentration could reach about 85mM and the hydrolysis rate may decrease by up to 50% (figure 5.12).

The other possible cause of incomplete RFO hydrolysis is that the equilibrium moisture content of the flour is too low to allow mobility of the raffinose molecules to access the active sites on the enzyme. While water is still being absorbed into the flour particles there exists a layer of free water around the bean particles in which the enzyme is free to hydrolyse the RFOs as they leach out. Once all of the water has absorbed into the bean flour this is no longer the case and the hydrolysis may cease.

The presence of water is required for the enzymatic hydrolysis of RFOs for two separate reasons. Firstly water is required by the stoichiometry of the reaction. Every molecule of raffinose hydrolysed requires one molecule of water and every molecule of stachyose hydrolysed requires two water molecules. Furthermore the hydrolysis of sucrose also requires one molecule of water per molecule of sucrose. However this amounts to less than 3 mg of water for every gram of flour processed, which is far less than the water content already within in the raw flour.

The second reason that water is required for the hydrolysis to take place is the mobility of both the enzyme and the substrates. If there is insufficient water absorbed into the bean flour then RFOs are immobile and cannot come into contact with the active site of the enzyme. Furthermore, given the large relative size of the enzyme molecule compared to that of the RFOs it is likely that the enzyme is unable to penetrate into the bean flour particles.

This hypothesis is supported by the work of Kon (1979) in which he demonstrates that proteins are unable to leach out of whole beans during leaching, which supports the idea that they are also very slow to diffuse into the beans.

If the enzyme molecules are able to penetrate the bean particles, the rate at which they do so is likely to be significantly lower than the rate at which the solutes can leach out of the bean particles. It is therefore likely that the majority of the RFO hydrolysis takes place at or near to the surface of the bean particles and the overall rate of hydrolysis will be highly dependent on the rate at which the solutes can diffuse to the surface.

The role of moisture in providing molecular mobility for the RFOs and the enzyme in low moisture systems was further explored in a conceptual model.

6.4 A Conceptual Model of RFO Removal in Low Moisture Enzyme Processing

If a single navy bean particle is considered after the application of an enzyme solution it will be surrounded by a given mass of water containing some concentration of α-galactosidase. This system is represented pictorially in figure 6.4.

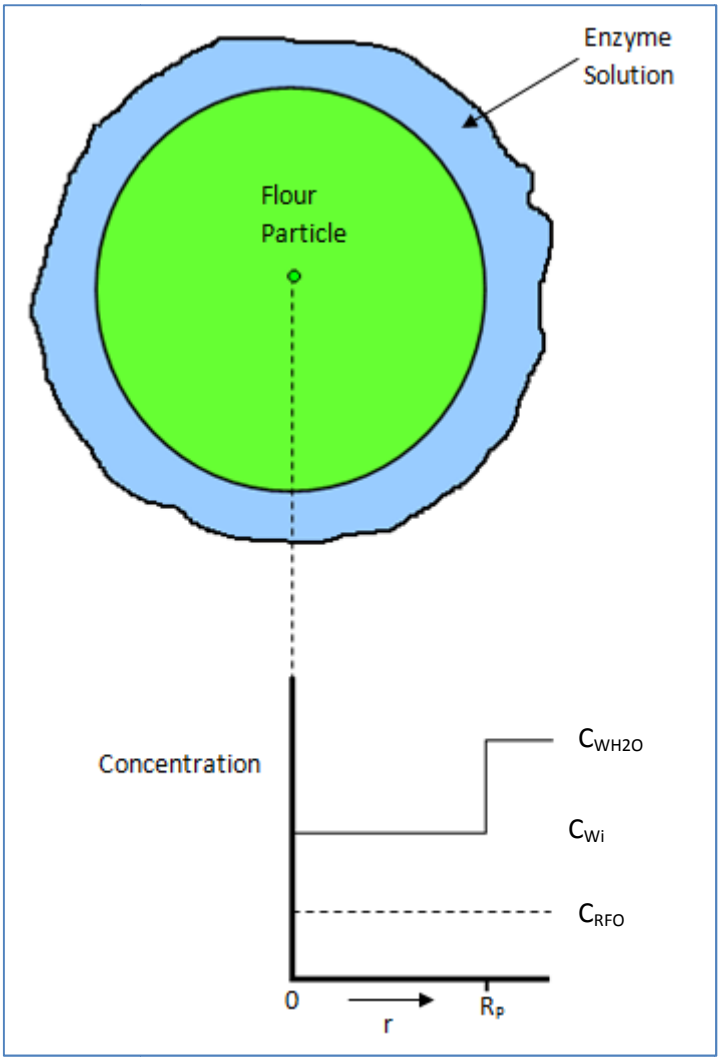


Figure 6.4 Pictorial representation of **step 1** in the conceptual model of a low moisture bean flour/enzyme system.

Step 1: The particle is coated with a layer of enzyme solution. Because the particle is relatively dry, moisture will be absorbed into the interior by diffusion, allowing RFO diffusion out to the surface.

Where C_{wi} = initial moisture content (g/g)

C_{wH2O} = moisture content of enzyme solution (~1 g/g wb)

r = distance from centre of particle (m)

R_p = radius of the particle (m)

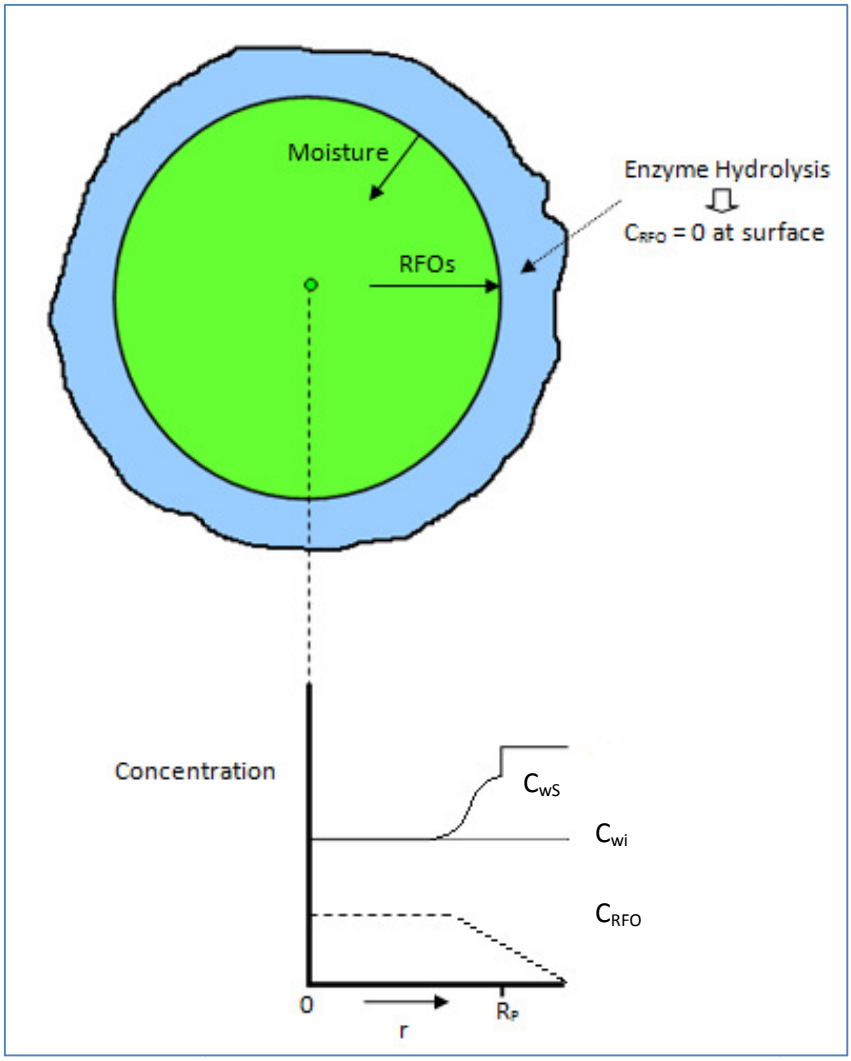


Figure 6.5 Pictorial representation of **step 2** in the conceptual model of a low moisture bean flour/enzyme system.

Step 2: A moisture content gradient develops allowing RFO diffusion to the surface where the enzyme hydrolyses it, maintaining RFO concentration of zero at the surface.

Where C_{wS} = saturated moisture content (g/g)

C_{RFO} = concentration of RFO (g/g)

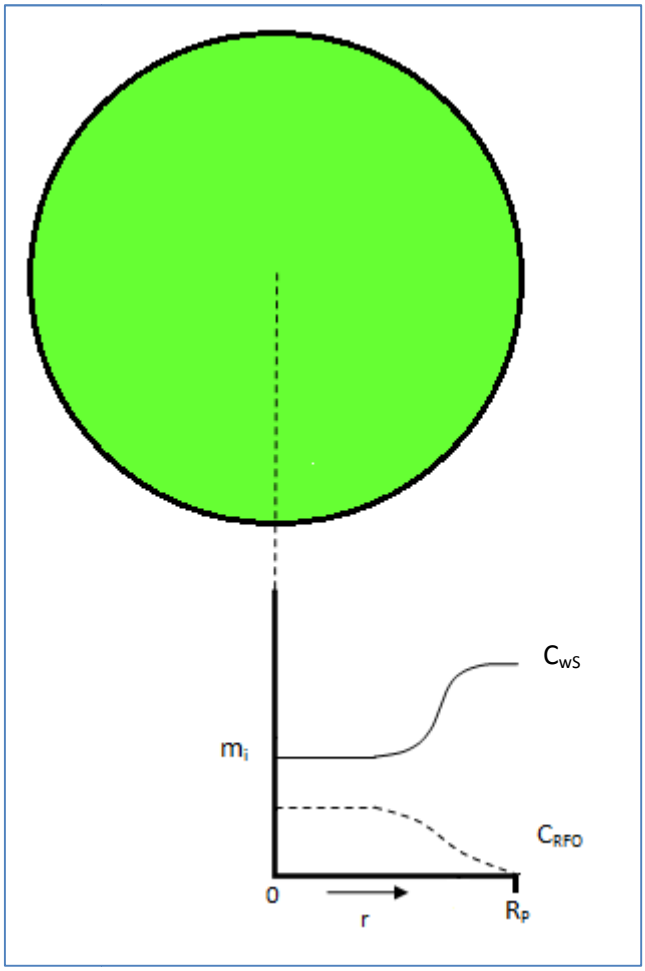


Figure 6.6 Pictorial representation of **step 3** in the conceptual model of a low moisture bean flour/enzyme system.

Step 3: A point is reached where the water at the surface is exhausted. It is likely that enzyme diffusion into the particle is low, however significant enzyme activity continues until the water activity at the surface becomes very low.

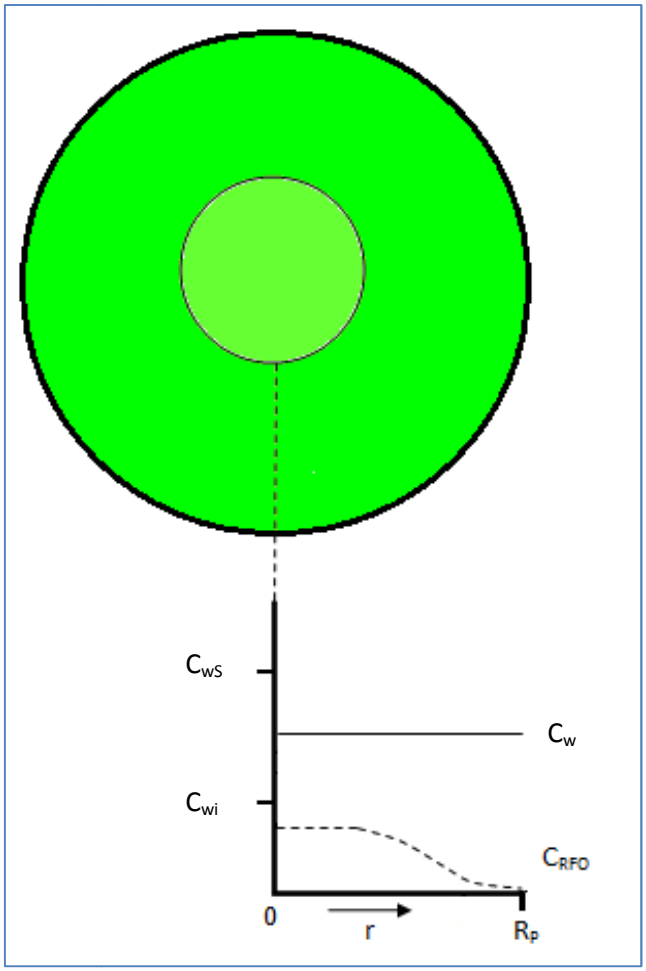


Figure 6.7 Pictorial representation of step 4 in the conceptual model of a low moisture bean flour/enzyme system.

Step 4: Moisture gradients within the particle equilibrate at a moisture content which precludes further diffusion of RFOs and there is therefore no further RFO removal. As a result RFO levels are only significantly reduced at the surface. The depth of RFO leaching into the surface increases with increasing water added.

From the perspective of this conceptual model there are two potential reasons for why the RFO removal may not be complete in low moisture systems.

1. The water activity at the surface reaches a point where the enzyme activity ceases.
2. The diffusivity of RFOs are strongly affected by the moisture content.

Each of these ideas was explored further.

Figure 6.8 is extracted from Roos (2001) and indicates that enzyme activity dramatically decreases at intermediate water activity.

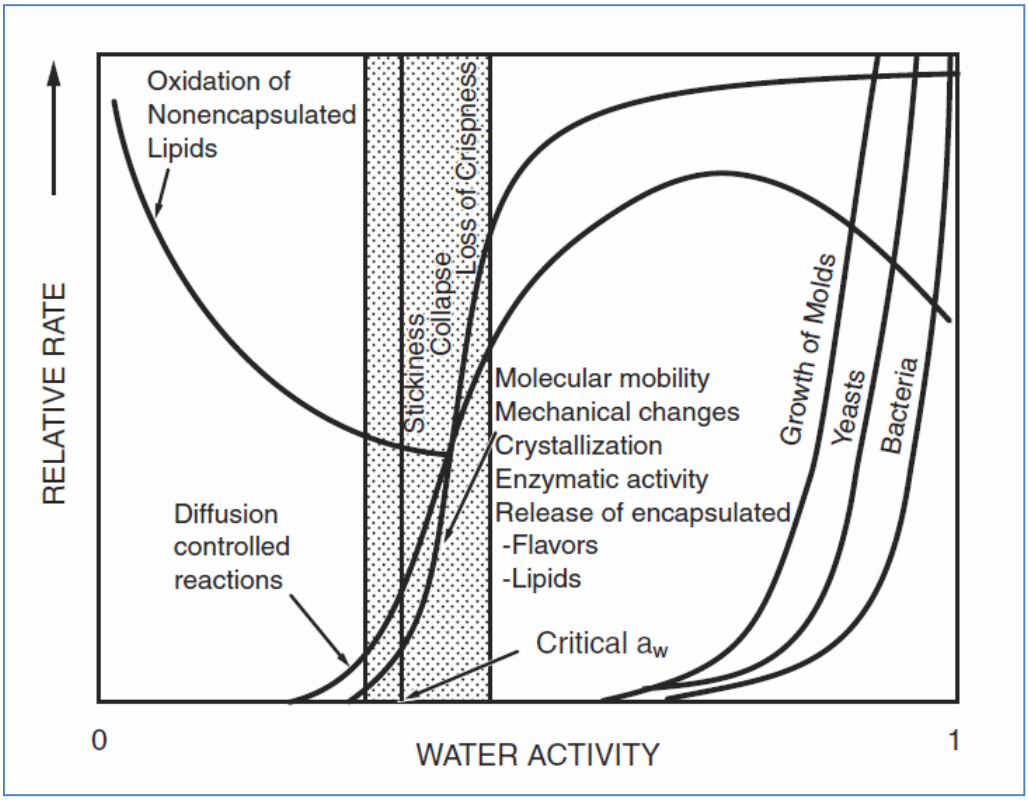


Figure 6.8 A food stability "map" showing the effect of water activity on various time dependent changes and rates of diffusion-controlled reactions as well as the growth of microorganisms in food materials. Figure extracted from Roos (2001).

It has been suggested in the literature (Rahman & Labuza 2007) that reduction in enzyme activity due to water activity limitations can result from:

- Reduced rates of reactant diffusion to the enzyme, or
- Conformational changes in the quaternary structure of the enzyme

Water activity thresholds have be found to vary between different enzymes with thresholds ranging from 0.9 for phytases in grains down to 0.2 for glycoside hydrolases in wheat germ. Drapron (1985) report critical water activities for galactosidase enzymes to be 0.4 to 0.6 (reported in Rahman and Labuza (2007)).

The following moisture sorption isotherm for common beans was extracted from Moreira et al (2009).

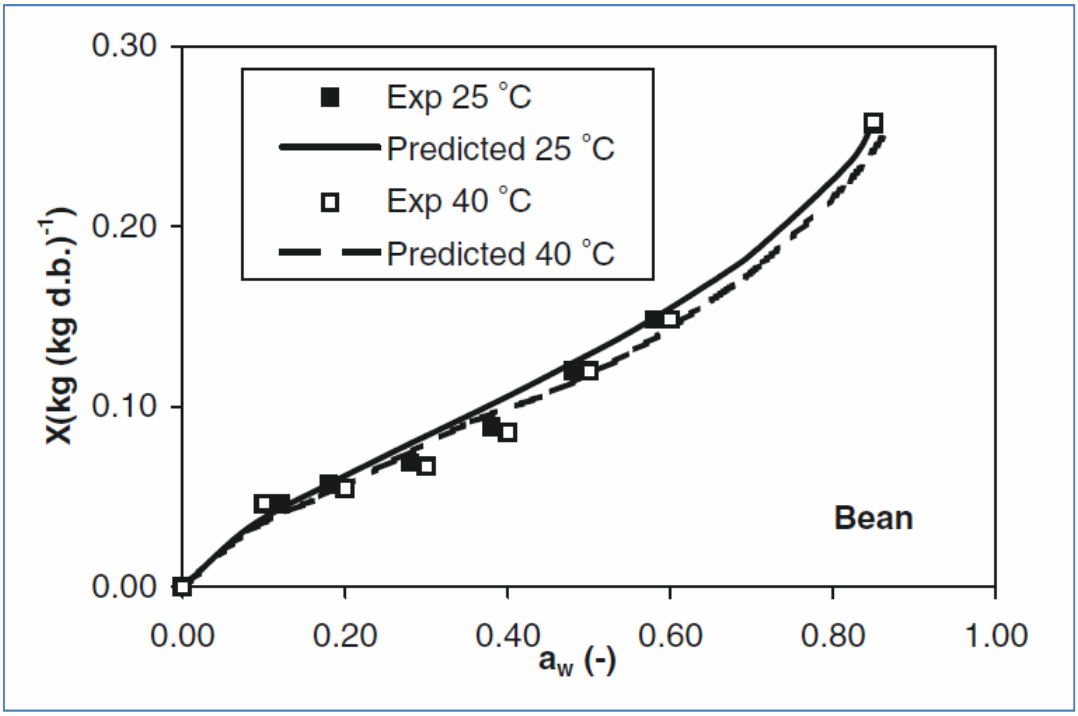


Figure 6.9 Moisture sorption isotherm of common beans. Figure extracted from Moreira et al (2009)

Upon examination of the moisture sorption isotherm presented in figure 6.9 it can be seen that the range of critical water activities reported by Drapron (1985) correspond to moisture contents of approximately 10 -15% DB. This moisture content range for critical water activity is well below the range at which incomplete RFO hydrolysis is observed in this work (38.9-51.5% DB section 6.2 and 6.3). In fact moisture contents of 10-15% DB are on the same order as the initial moisture content of the beans (12.9% DB for the de-hulled cotyledons table 4.1 and 17.4% DB for navy bean flour table 3.2). This suggests that limiting water activity is not the primary cause for the failure of the enzyme to achieve complete hydrolysis at the moisture contents observed in figure 6.2.

The data presented above suggests that if the enzyme was able to diffuse into the bean particles any significant distance, then the water activity once the moisture content within the bean equilibrates should be high enough to allow sufficient enzyme activity to complete the hydrolysis. The fact that the hydrolysis does not reach completion indicates that the enzyme does not penetrate to the centre of the particle.

This leaves the idea that moisture limited diffusion is the cause of incomplete hydrolysis under low moisture conditions. The limiting of diffusion rate could apply to either the diffusion of the enzyme into the particle or to the RFO diffusion out of the particle.

However the relatively large size of the enzyme molecule compared to those of the water and the RFOs means that it is more likely that when there is enough moisture transferred into the particle, the RFOs will be able to diffuse to the surface significantly faster than the enzyme can diffuse in.

It is therefore proposed that the RFOs must first diffuse to the surface of the bean particle before they can be hydrolysed by the enzyme. This would explain the incomplete hydrolysis of the RFOs only if the diffusivity of the RFOs through the bean matrix is dependent on the moisture content.

The only reported data for moisture dependent diffusion in navy beans was reported by Radajewski et al (1992), who obtained data from drying kinetics. From the analysis they found that diffusivity changed by four orders of magnitude over a range of temperatures and moisture contents.

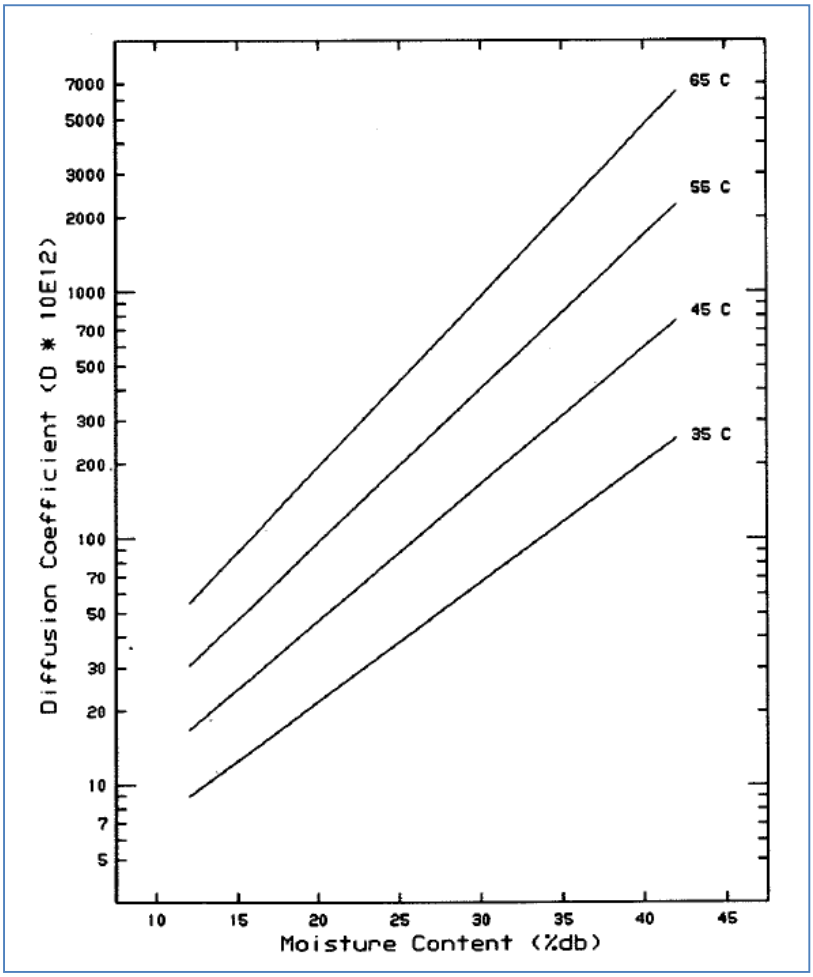


Figure 6.10 Diffusion coefficient versus moisture content for navy beans at four different temperatures. Note that the diffusivity in this figure is given in m^2hr^{-1}. Figure extracted from Radajewski et al (1992)

The following relationship was determined using the 35°C data from figure 6.10.

$$D_W = e^{(11.12C_w - 34.97)} \tag{6.1}$$

Where:

- D_W is the moisture diffusion coefficient in m^2/s
- C_w is the moisture content (% DB)

The saturated moisture content of navy beans as measured in chapter 4.2 was 1.26 g/g DB. If this value for M_C is applied to the above equation the resulting diffusion coefficient is $8x10^{-10}$ m^2/s which is high. Moisture diffusivity in soya beans measured in the literature (Yildiz & Kokini 2006) showed a similar strong moisture content dependency but once above

a certain moisture content the diffusivity remained relatively constant at approximately 1×10^{-11} m^2/s.

The constant diffusivity measured for navy bean cotyledons in at ambient temperature in chapter 4 was 4.9 x 10^{-11}. In the absence of more relevant data, the data from Radajewski et al (1992) was used up until a diffusivity of 4.9×10^{-11} m^2/s. At moisture contents above this value the diffusivity was assumed to remain constant. This corresponds to a moisture content of:

$$C_w = \frac{ln(4.9\times10^{-11})+34.97}{11.12} = 1.010 \qquad (6.2)$$

Therefore the diffusivity of moisture in navy bean flour can be approximated by:

$$D_W = e^{(11.12C_w-34.97)} \qquad \text{For } C_w \leq 101.0\%\ DB$$

$$D_W = 4.9 \times 10^{-11} \qquad \text{For } C_w > 101.0\%\ DB$$

This is illustrated graphically in figure 6.11

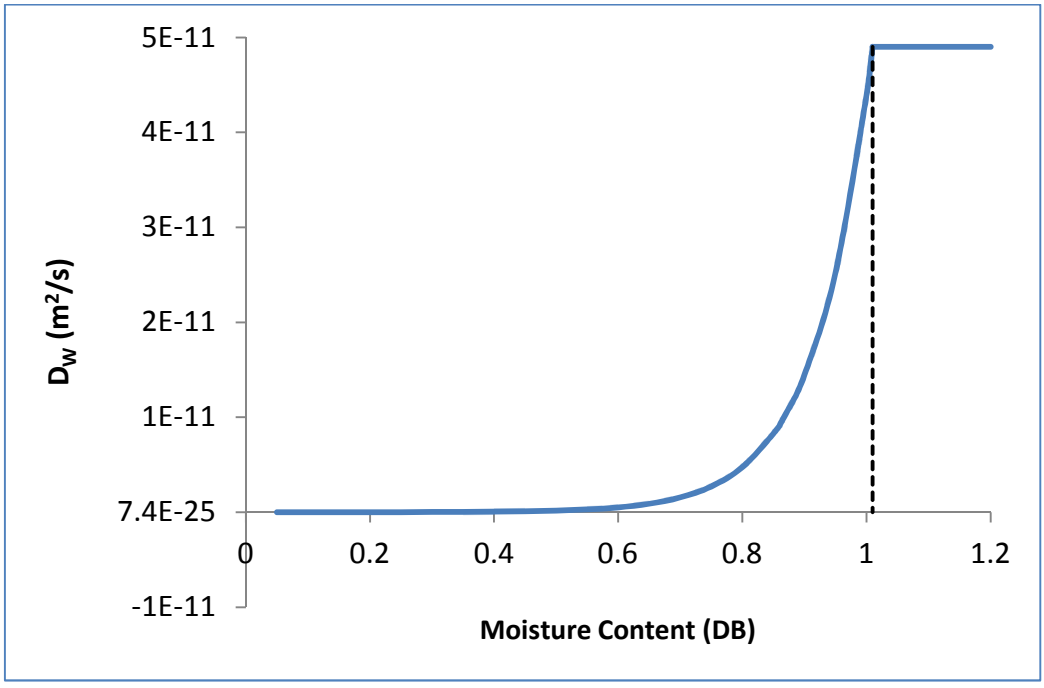


Figure 6.11 Illustration of the assumed relationship between diffusivity and moisture content.

The proposed relationship between diffusivity and moisture content effectively results in a moisture diffusion front into the particle. Note that this relationship predicts almost no diffusion at moisture contents below 53.2% (db) and therefore solubility of RFOs should not affect diffusion rates (see section 3.5.2 for discussion).

There is little literature available on the diffusivity of solutes from legume materials at low moisture content. However at high moisture content the diffusion of disaccharides has been reported to be at a similar order of magnitude as for water in other materials such as papaya (Mendoza & Schmalko 2002). It is therefore likely that the diffusion rates of RFOs are similar to those of water in the bean matrix. It was shown in leaching experiments in section 4.4 that for large bean particles the rate of RFO removal was slower but at a similar magnitude to the rate of bean hydration.

Because moisture and therefore RFO diffusion effectively moves as a front, a simple model linking the amount of water to the extent of RFO removal was developed. It can be assumed that an outer shell of the particle will reach moisture contents at or greater than a critical level (M_{Crit}) required for significant diffusion. M_{Crit} could be taken from figure 6.11 as 101% DB below which diffusivity decreases dramatically.

In actual fact, at the point where the free water at the surface is exhausted, the leading edge of the front may be at M_{Crit} while the outer surface is likely to be saturated. In this shell it is assumed that the RFO can diffuse to the surface and be hydrolysed by the enzyme.

A water balance for this system is given by:

$$\rho V_p C_{wf} = \rho\left(V_p - V_{shell}\right)C_{wi} + \rho V_{shell} C_{wC} \qquad (6.3)$$

Where:

ρ is the density of the bean particle (kg/m^3)

V_p is the volume of the particle (m^3)

C_{wf} is the final moisture content after equilibration (g/g)

V_{shell} is the volume of the hydrated shell (m^3)

C_{wi} is the initial moisture content of the bean (g/g)

C_{wC} is the critical moisture content below which no significant diffusion occurs (101% DB for this exercise) (g/g)

The above equation links the moisture content after equilibrium to the inner particle being at the initial moisture content and the outer shell at M_{Crit}

Rearranging the above equation gives:

$$\frac{V_{shell}}{V_p} = \left(\frac{C_{wf} - C_{wi}}{C_{wi} + C_{wC}}\right) \tag{6.4}$$

If it is assumed that this outer shell has all of the RFOs removed, then $\frac{V_{shell}}{V_p}$ represents the fraction of RFOs removed (*X*).

$$X = \left(\frac{C_{wf} - C_{wi}}{C_{wi} + C_{wC}}\right) \tag{6.5}$$

The results of this calculation are displayed in figure 6.12 for critical moisture contents of 101.0% DB and the bean saturation moisture content (125.7% DB).

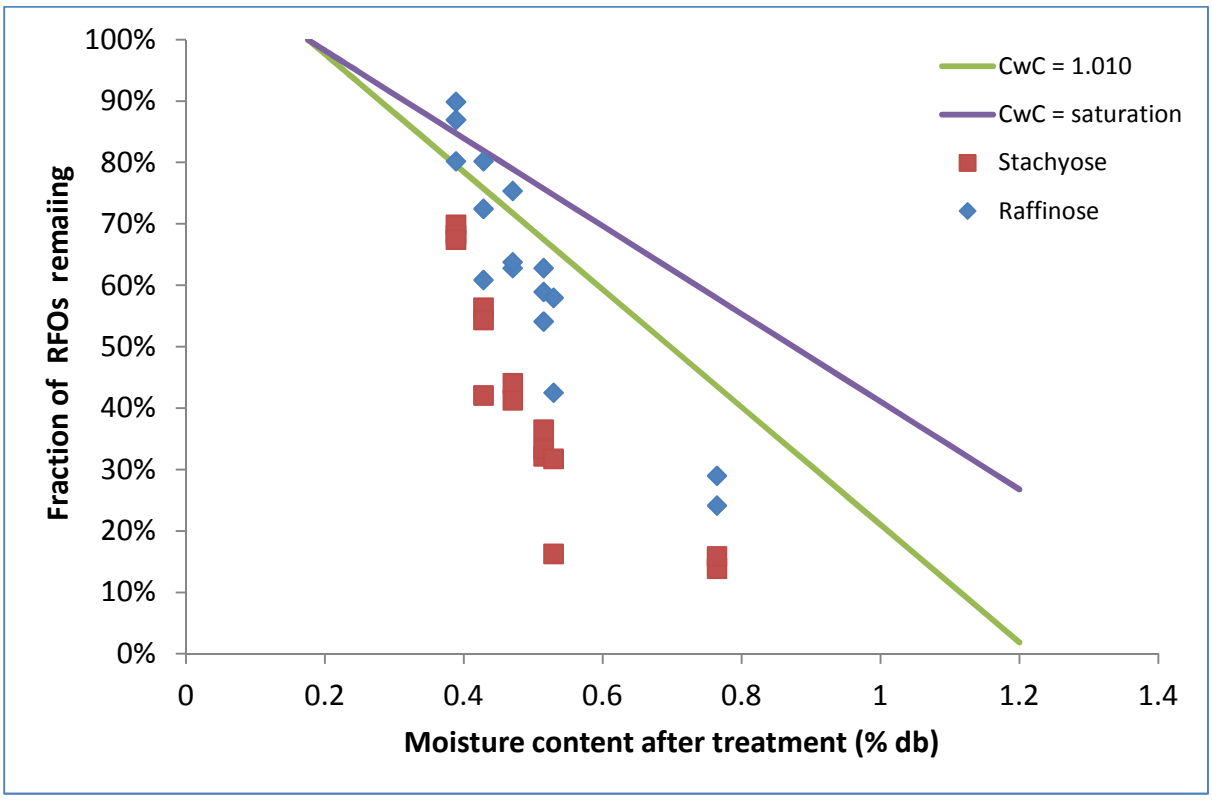


Figure 6.12 Equilibrium moisture content of flour/enzyme system versus fraction of RFOs remaining post treatment. Points represent measured data; lines represent predictions using the simple model developed in section 6.4

The experimental data measured in sections 6.2 and 6.3, was combined with data measured by Clark (2010) (a fourth year engineering student using the same methodology under my supervision) and is included in figure 6.12 for comparison. It can be seen that this simplified approach shows poor agreement with the measured data.

One of the major reasons for this poor fit are the assumptions that RFO diffusivity is similar to moisture diffusivity and that the outer shell is sufficiently hydrated to allow all RFOs within it to diffuse out prior to moisture equilibration. Better estimates should be possible through dynamic modelling of moisture and RFO concentrations within the bean particles during processing.

6.5 Preliminary Dynamic Modelling of RFO Removal in Low Moisture Systems

The hydrated shell model developed above assumed a very sharp front for moisture diffusion into the particle. In reality the diffusivity changes exponentially as a function of moisture content (see figure 6.10) and therefore there will be a gradient (albeit with a sharp front) moving into the particle. When the free moisture at the surface is depleted the internal moisture gradients will equilibrate through the particle and during this time further leaching of RFOs can occur resulting in greater extents of RFO removal than predicted above.

There is limited data available for the diffusivities of RFOs in the bean matrix as a function of moisture content. It is also difficult to model swelling that is likely to occur at high moisture contents in the outer regions of the particle. Despite these short comings it is possible to develop a mathematical model to demonstrate how diffusion limitations could result in the incomplete hydrolysis of RFOs observed in this work.

6.5.1 Conceptual Model Development

A single particle of radius R_p is modelled assuming no swelling. A known quantity of enzyme solution (m_w) is applied to the surface of this particle. The immediate surface will absorb moisture until it reaches its saturation level C_{wS} (125.7% DB, see section 4.2). Moisture will then diffuse, increasing the moisture content C_w in the particle interior allowing RFOs to diffuse out, reducing the local RFO concentration C_{RFO}.

The RFO concentration at the surface is assumed to be zero due to enzyme action by α-galactosidase. Due to the high molecular weight of the enzyme protein it was assumed that no significant diffusion of enzyme into the particle occurs. Although galactose inhibition on the enzyme activity was observed in chapter 5, to keep the model simple this was assumed to be negligible.

The rate of moisture diffusion D_w was assumed to follow the data presented in figure 6.11 where the rate significantly reduces at moisture contents below 101% DB. RFO diffusion rates D_{RFO} were assumed to be strongly correlated with moisture diffusion rates. The data from chapter 4 suggested the mass transfer of RFOs was slower than for moisture. For preliminary modelling purposes and in the absence of reliable data it was assumed to be 1 order of magnitude slower than moisture diffusivity at the same moisture content.

The enzyme solution was assumed to be applied uniformly across the surface of the particle and the particle was assumed to be spherical. These simplifications allow mass transfer to be modelled as diffusion in one dimension in an axi-symmetrical sphere.

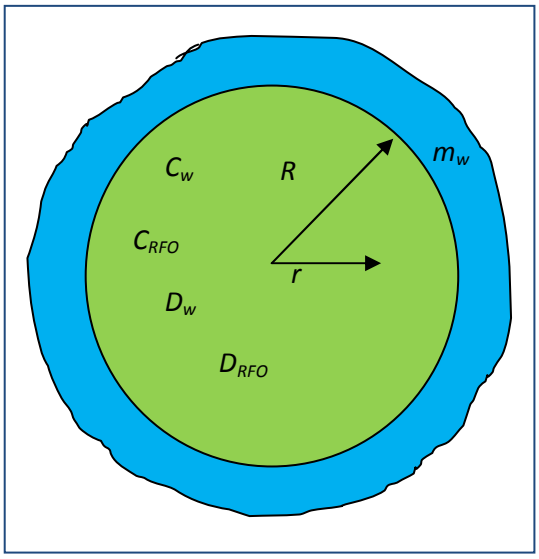


Figure 6.13 Diagram of the system to be modelled.

6.5.2 Model Formulation

Ficks law of diffusion in one dimension in a sphere with variable local diffusivity was used to describe the rate of change of moisture content at radius *r*.

$$\frac{dC_w}{d_t} = \frac{d}{dr}\left(D_w \frac{dC_w}{dr}\right) + \frac{2D_w}{r}\left(\frac{dC_w}{dr}\right) \tag{6.6}$$

For $0 < r < R_p$ and $t > 0$

Similarly the same equation can be used to describe RFO transfer.

$$\frac{dC_{RFO}}{d_t} = \frac{d}{dr}\left(D_{RFO} \frac{dC_{RFO}}{dr}\right) + \frac{2D_{RFO}}{r}\left(\frac{dC_{RFO}}{dr}\right) \tag{6.7}$$

For $0 < r < R_p$ and $t > 0$

Because of the one dimensional spherical geometry, symmetry boundary conditions were used at the particle centre.

$$D_w \frac{dC_w}{dr} = 0 \quad \text{and} \quad D_{RFO} \frac{dC_{RFO}}{dr} = 0 \tag{6.8}$$

At $r = 0$ and $t > 0$

Because the enzyme is assumed to hydrolyse any RFOs arriving at the surface the RFO concentration at the surface was set to zero.

$$C_{RFO} = 0 \tag{6.9}$$

At $r = R$ and $t > 0$

When free moisture is present at the surface the surface moisture content of the bean is set at the saturated moisture content

$$C_w = C_{wS} \tag{6.10}$$

If $m_w > 0$ at $r = R_p$ and $t > 0$

At the point where free moisture at the surface is exhausted there is no more diffusion over the outer surface.

$$D_w \frac{dC_w}{dr} = 0 \tag{6.11}$$

If $m_w \leq 0$ at $r = R_p$ and $t > 0$

A moisture balance results in an ODE describing the depletion of the water at the surface.

$$\frac{dm_w}{d_t} = -D_w \left(\frac{dC_w}{dr}\right)_{r=R_p} \tag{6.12}$$

For $t > 0$

Initial conditions were uniform throughout the particle

$$C_w = 0.17 \ {}^{\mathrm{g}}/_{\mathrm{g}} \,\text{dry solids} \tag{6.13}$$

$$C_{RFO} = 0.038 \ {}^{\mathrm{g}}/_{\mathrm{g}} \,\text{dry solids} \tag{6.14}$$

At $0 \leq r \leq R$ and $t = 0$

$$m_w = m_{wi} \tag{6.15}$$

At $t = 0$

The diffusivity of moisture was discussed above resulting in;

$$D_w = e^{(11.12C_w - 34.97)} \quad \text{For} \quad C_w \leq 101\%\ DB \tag{6.16}$$

$$D_w = 4.9 \times 10^{-11} \quad \text{For} \quad C_w > 101\%\ DB \tag{6.17}$$

The diffusivity of RFOs was set at;

$$D_{RFO} = 0.1 D_w \tag{6.18}$$

6.5.3 Model Solution

Comsol Multiphysics was used to numerically solve the model. Figure 6.14 Shows the geometry used. To achieve the one dimensional spherical geometry in this package it was necessary to use a two dimensional axi-symmetrical cylindrical geometry which when rotated around the $r = 0$ axis results in a sphere.

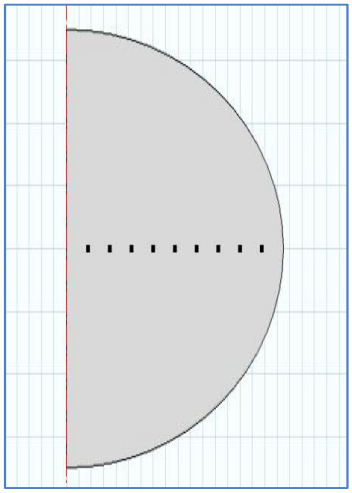


Figure 6.14 Illustration of the two dimensional axi-symmetrical cylindrical geometry used by Comsol Multiphysics to achieve the one dimensional spherical geometry required for the model.

The modelling package uses finite element methods to numerically solve the moisture and RFO concentrations as a function of radius and time. Figure 6.15 Shows the mesh used.

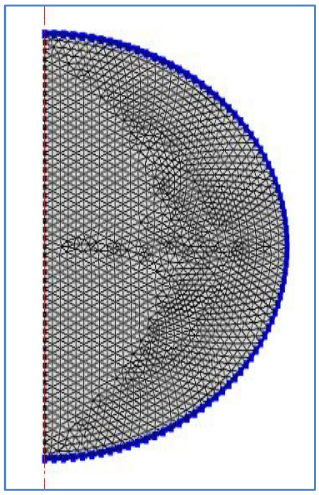


Figure 6.15 Illustration of the finite element mesh used for the numerical solution of the model by Comsol Multiphysics.

6.5.4 Model Predictions

Figures 6.16 – 6.17 show an example simulation where enough water was added at the surface to reach 24% of saturation after equilibration. The figures show moisture and RFO concentration as a fraction of saturation and initial values respectively at the point where moisture at the surface was just exhausted. It shows that only the outer regions have been affected.

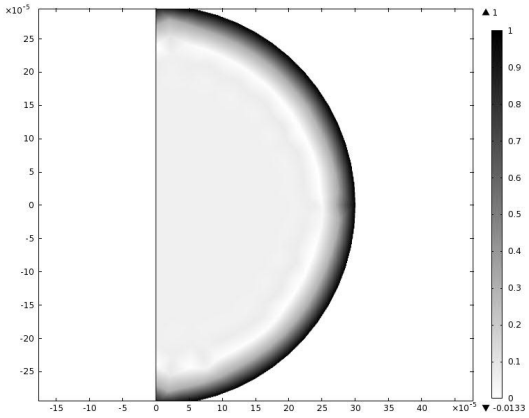


Figure 6.16 Model simulation of the moisture profile of a bean particle at the point when all free water has been absorbed.

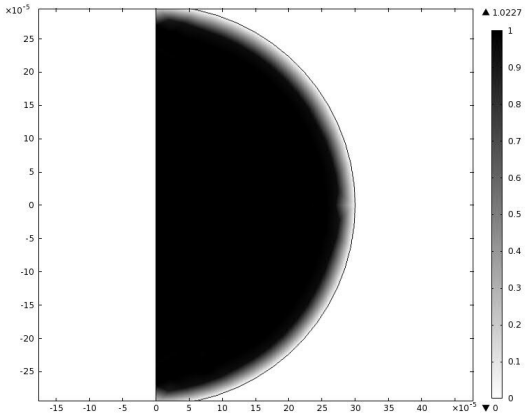


Figure 6.17 Model simulation of the RFO concentration profile of a bean particle at the point when all free water has been absorbed.

After changing the boundary condition so that moisture diffusion no longer occurs over the surface, the moisture equilibration was modelled resulting in figures 6.18-6.19.

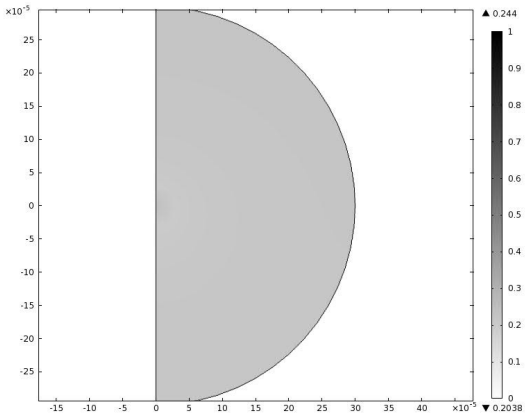


Figure 6.18 Model simulation showing moisture profile within a bean particle after moisture equilibration has been achieved

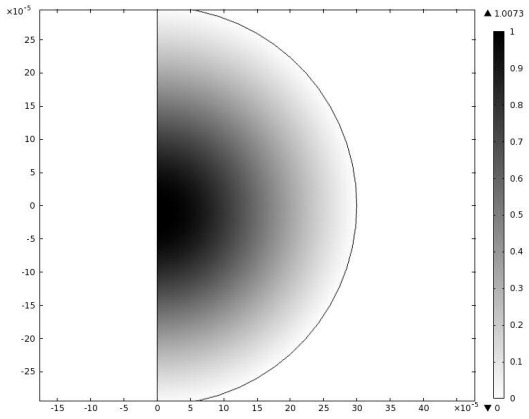


Figure 6.19 Model simulation showing RFO concentration profile within a bean particle after moisture equilibration has been achieved

As these simulations show at low moisture additions a steady state RFO concentration gradient can be achieved where after equilibration RFO diffusion rates are negligible and so RFO removal is incomplete. Three similar model predictions were carried out where different amounts of moisture were added resulting in the predictions of RFO removal shown in figure 6.20 in comparison with experimental data.

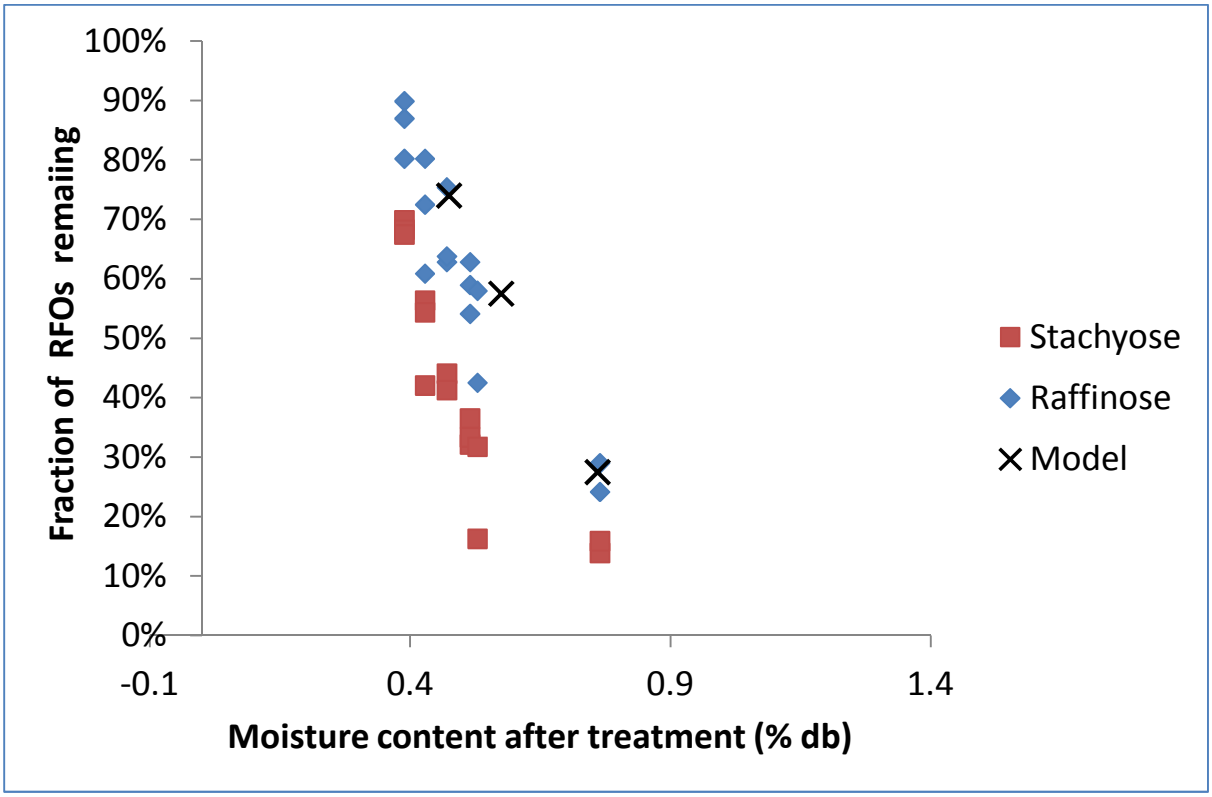


Figure 6.20 Levels of RFO removal predicted by the model at three different equilibrium moisture contents. Measured data is included for comparison.

For the purposes of further validation the dynamic model was used to predict the water absorption rate data measured in figure 4.2. Due to the over-prediction of the absorption rate using a maximum diffusivity of 4.9×10^{-11} model predictions were also carried out for a range of other maximum diffusivities.

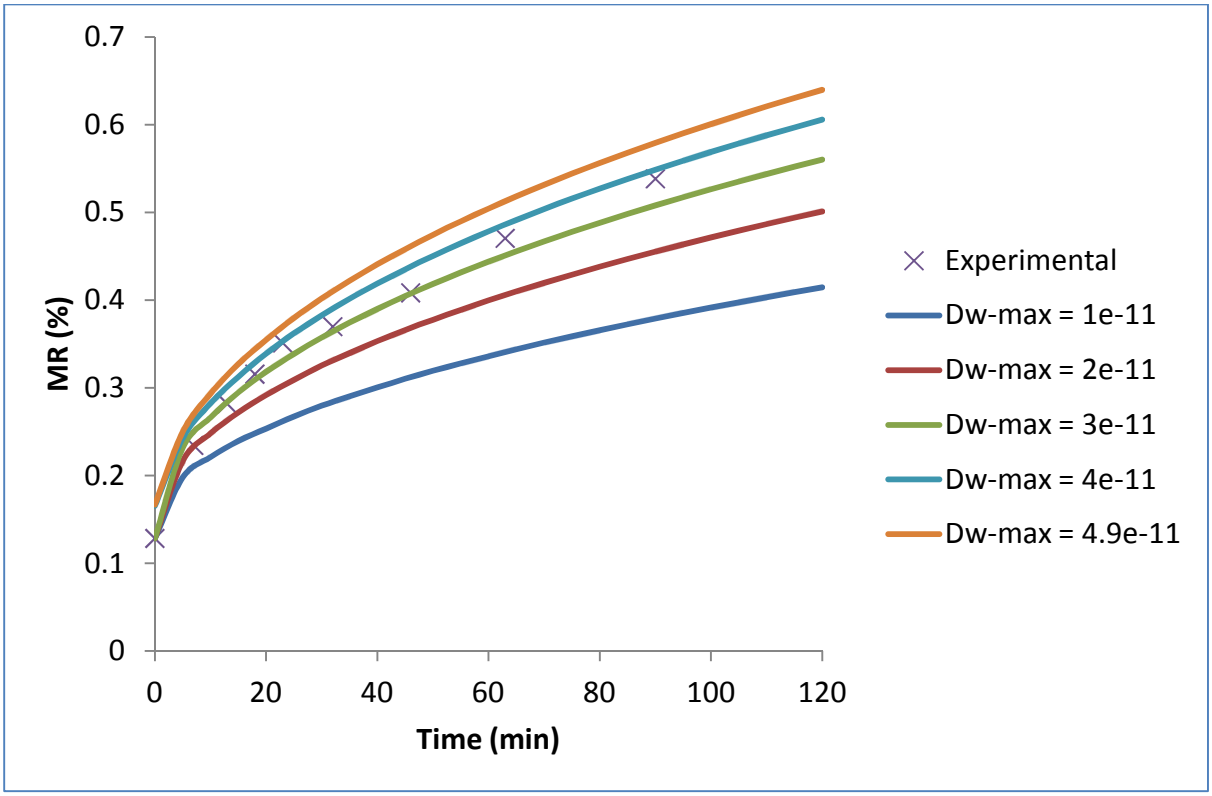


Figure 6.21 Lines represent moisture absorption predicted using dynamic model with a range of maximum diffusivities. Data points represent measured water absorption in navy bean cotyledons at 23°C

Figure 6.21 clearly illustrates that a maximum water diffusivity of 4.9 x 10^{-11} is too high and over-predicts the rate of water absorption. The value for maximum water diffusivity for which the model best fits the measured data over the lower moisture contents at which the model is being applied is 3 x 10^{-11}. The model was therefore re-run using this value for maximum water diffusivity. Due to the lack of data on RFO diffusivities the model was also re-run using a number of different ratios of RFO diffusivity to water diffusivity. The results from these simulations are plotted in figure 6.22.

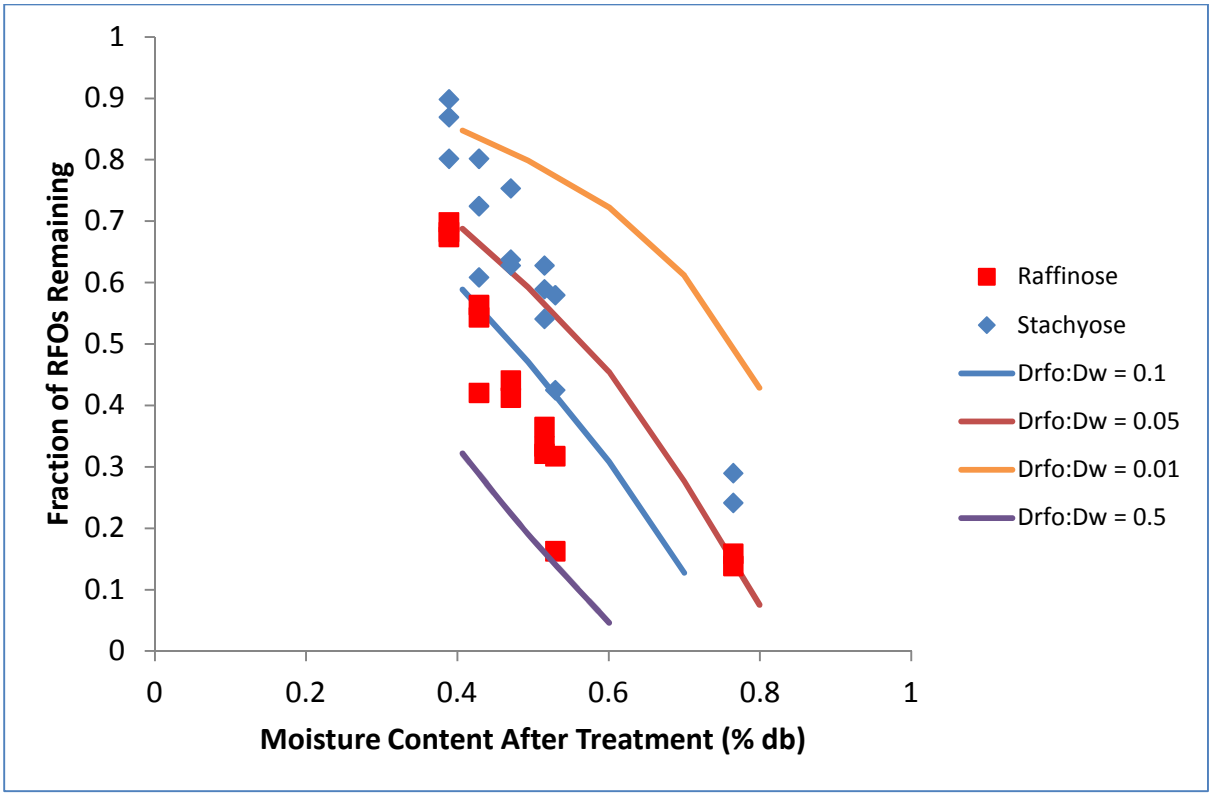


Figure 6.22 Levels of RFO removal predicted by the model using a maximum water diffusivity of 3 x 10^{-11} at four different ratios of RFO diffusivity to water diffusivity. Measured data is included for comparison. The lines represent predicted data and the points represent measured data.

Figure 6.22 demonstrates that ratios of RFO diffusivity to water diffusivity of 0.1 and 0.05 fit the data best and this vindicates the choice of a ratio of 0.1 RFO diffusivity to water diffusivity used for the initial simulation. Despite the simplifications made in the model development and the assumed relative rates of diffusion of RFOs and moisture figure 6.22 shows reasonable agreement with the experimental data.

Better agreement could be achieved through optimisation of the RFO diffusivity relationship. Because the modelling was carried out only to demonstrate that diffusion limitations could explain the incomplete RFO removal further optimisation was not carried out.

It is suggested that future work could be carried out to characterise RFO diffusion and the model should be adapted to include particle swelling if more accurate predictions are required.

6.6 Conclusion

Experiments were carried out at low moisture contents using α-galactosidase to hydrolyse the RFOs in navy bean flour. It was found that at moisture contents of 34% WB and lower only partial hydrolysis of the RFOs was achieved.

The data from these experiments was extrapolated to predict the minimum moisture content at which RFOs can be reduced to levels at which the flatulence response they generate following ingestion is negligible. This minimum moisture content was found to be 38.3% WB.

Several causes for the incomplete hydrolysis of RFOs at low moisture contents were proposed. Closer investigation ruled out galactose inhibition and restriction of enzyme activity by low water activity as the cause.

Limitation of RFO diffusion within the bean particle at low moisture contents was proposed as a cause for the observed incomplete hydrolysis. A simple saturated shell model was formulated to test the plausibility of this hypothesis. The predictions made by this crude model roughly approximated the measured data and so the concept was shown to be plausible.

In order to better demonstrate this concept, a preliminary dynamic model of the moisture and RFO concentrations within the bean during processing with α-galactosidase was developed. A number of simplifications were made during the development of the model and a number of the key model parameters were estimated based on data from somewhat similar systems. Despite these simplifications the model performed well as a tool for demonstrating the concept that diffusion limitation could be responsible for the incomplete RFO removal observed in the low moisture experiments. The dynamic model performed significantly better than the saturated shell model when compared to the measured data.

Future work is suggested to refine the model and characterise the estimated parameters from this model to improve the agreement between predicted and measured data.

Chapter 7

General Discussion and Conclusions

7.1 Introduction

The navy bean is a nutritious legume that is naturally high in protein, carbohydrates and dietary fibre and is also an excellent source of iron, potassium, selenium, thiamine, vitamin B6 and folic acid. Navy beans have a low glycemic index and also contain saponin which has been shown to confer many health benefits including the inhibition of cancer cell growth.

The widespread human consumption of navy beans has traditionally been inhibited due to the flatulence causing effect of the raffinose family oligosaccharides that they contain. The presence of RFOs is also part of the reason that they are not more widely used throughout the processed food industry. Navy bean flour has some significant nutritional advantages over wheat flour and has the potential for use in many processed food applications if the problems associated with the RFOs can be resolved.

Literature suggests that if the RFO content of navy beans could be reduced by about 80% then the flatulence response due to RFOs following ingestion of the beans would become negligible. This work has identified several possible mechanisms that can be employed to reduce the level of RFOs in navy bean flour and has investigated the most promising of these in depth.

From the work presented in this thesis, several strategies have been developed to allow the inclusion of navy bean flour in a range of different processed food applications without the issues associated with the RFO content of the beans. The target applications for which strategies have been developed are:

- Use of navy bean flour at low levels as an ingredient.
- Use of navy bean flour at high concentrations in wet products.
- Use of navy bean flour in low moisture applications.

7.2 Use of Navy Bean Flour at Low Concentrations

The use of navy bean flour at low levels as an ingredient in a formulated product has the effect of distributing the bean flour across a much larger mass of product and essentially diluting the RFO content. Examples of this type of application might be as one of several thickening agents in a soup, or perhaps as a component in a type of mixed grain flour. It is relatively simple to calculate the maximum proportion of the total product mass that navy bean flour can account for without surpassing the RFO threshold of 0.62% DM.

$$C_{bf} = \frac{C_{RFOp}\,(1+C_w)}{(C_{St}+C_{Rf})} \tag{7.1}$$

Where:

- C_{bf} is the maximum concentration of bean flour in the product (g/g)
- C_{RFOp} is the threshold concentration of RFOs in the product (g/g)
- C_{St} is the concentration of stachyose in the bean flour (g/g) DB
- C_{Rf} is the concentration of raffinose in the bean flour (g/g) DB
- C_w is the moisture content of the bean flour (g/g) DB

When the values for moisture, raffinose and stachyose content measured for the bean flour in chapter 3 are input into the above equation and C_{RFOp} is set to 0.62%, the resulting maximum concentration of untreated navy bean flour in a formulated product should be 19.1% by mass.

7.3 Use of Navy Bean Flour at High Concentrations in Wet Products

When the desired product has high moisture content and requires more than 19.1% navy bean content, the addition of α-galactosidase to the product formulation can completely hydrolyse the RFOs from the product. However this is dependent on there being sufficient time allowed after enzyme addition for leaching of the RFOs out of the flour particles and for hydrolysis of the RFOs by the enzyme before the product is subjected to extremes in pH (below 3.5 or above 7) or temperatures above 60°C under which conditions the enzyme begins to denature.

Figure 7.1 below shows the time required for 95% removal of stachyose and raffinose from solution as a function of enzyme concentration. This graph was constructed for processing at 23°C for a starting concentration equivalent to assuming all the RFO's in bean flour become soluble in enough water to saturate the bean. This corresponds to the practical maximum concentration RFO's will be at, therefore figure 7.1 represents the maximum time required. The time for 95% removal was calculated by numerically solving (as in section 5.5) the ordinary differential equations for stachyose, raffinose and galactose changes due to enzyme hydrolysis developed in sections 5.2 and 5.4.

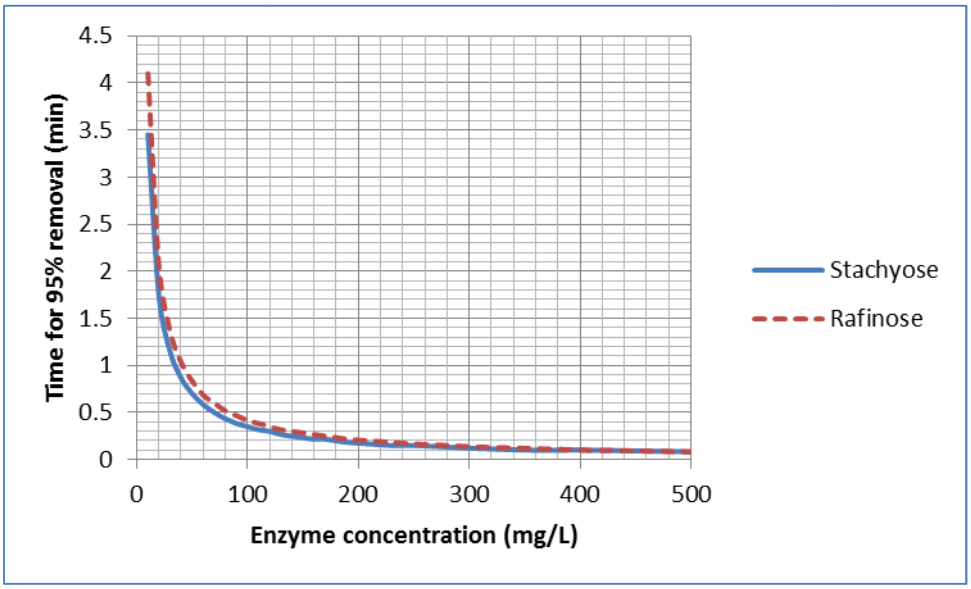


Figure 7.1 Maximum time required for enzyme hydrolysis of RFO's from solution as a function of enzyme concentration.

The work in chapter 4 demonstrated that the amount of time required for the leaching of the RFOs out of the navy bean flour is slower but of similar magnitude as the rate of water absorption into the particle. Both the water uptake rate and the RFO diffusion rates out of the bean particles are highly dependent on the particle size of the bean flour.

The constant moisture diffusion model employed in chapter 4 predicted water uptakes rates accurately for large particles and can be used to give an indication of the leaching time required for a given sized particle. Figure 7.2 shows the amount of time required for a bean particle to reach 50% and 95% of saturation at two temperatures and was calculated using the parameters determined in chapter 4.

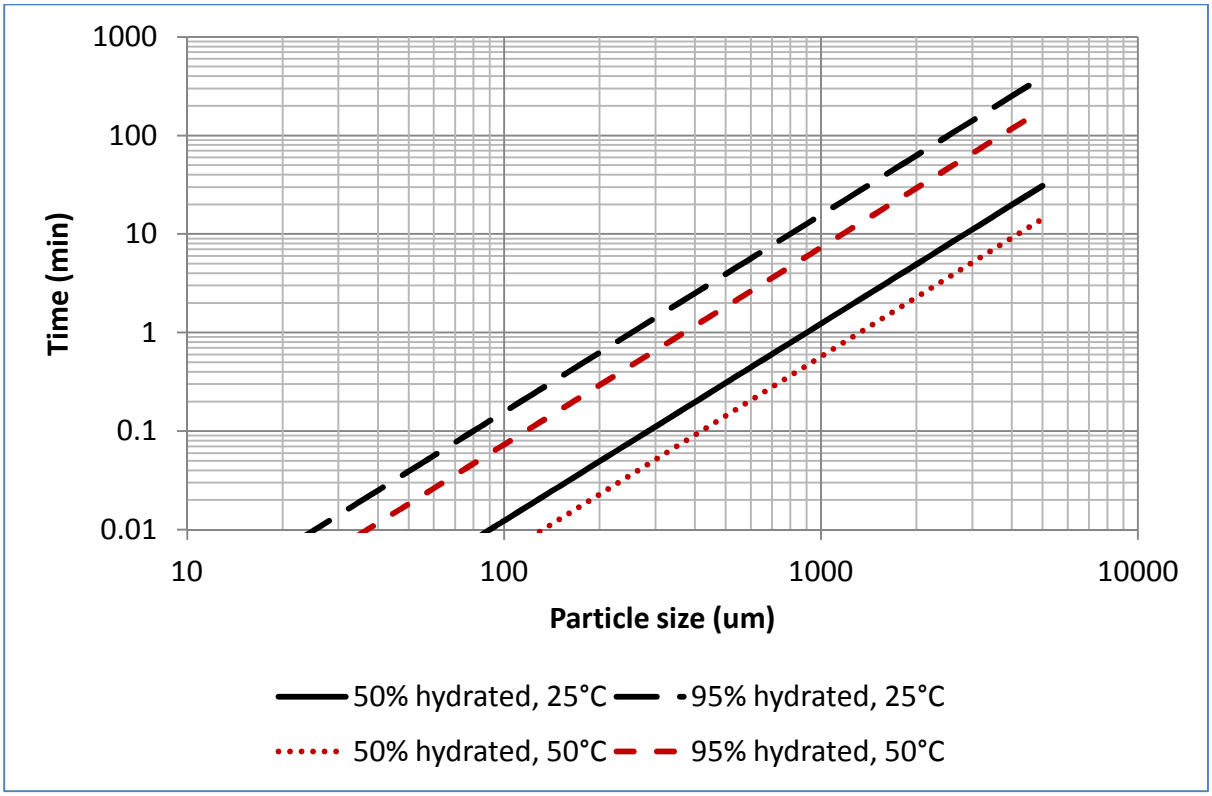


Figure 7.2 Time required to reach 50% and 95% hydration as a function of particle size at 25°C and 50°C. Data was calculated using the model and data presented in chapter 4.

The median particle size of the navy bean flour particles is 400µm (section 3.4) and so the time taken to reach 95% hydration at 25°C should be between two and three minutes.

By comparing figures 7.1 and 7.2 it can be seen that relatively short processing times are required and are likely to be dominated by the enzyme kinetics if low enzyme concentrations are used. As such, integration of a leaching and enzyme processing step into existing processes should be relatively simple. By adding an enzyme, leaching can continue to completion because the concentration gradient and hence diffusion out of the particle can be maintained by RFO removal by hydrolysis.

This process overcomes the serious disadvantages that arise in a traditional leaching/washing process as removal of water by filtration or centrifugation will result in soluble solids loss or water removal by drying will require high energy input.

Using the data plotted in figure 7.1 it can be seen that hydrolysis time of less than one minute can achieve 95% RFO removal at enzyme concentrations of approximately 50 mg/L. Commercial α-galactosidase is available at a cost of approximately $40 per kilogram.

At a concentration of 50 mg/L in the leaching water of volume five times that required to saturate the bean flour (15.85 L/kg flour) the amount of enzyme required is 0.79 g/kg flour. This equates to an enzyme cost of $0.03 per kilo of navy bean flour. This compares well with the $0.69 per kilogram cost of the broken beans and means that enzymatic treatment in this scenario will not add significant cost to the navy bean flour.

7.4 Use of Navy Bean Flour in Low Moisture Applications

There are two major low moisture applications in which navy bean flour has a potential use. These applications are as follows:

- As a pre-treated flour for use as a dry ingredient.
- In the production of extruded snacks and cereals.

In each case it is desirable to achieve significant RFO removal without adding excess water. Extrusion of dried products requires flour moisture contents of less than approximately 30% moisture WB (see section 6.1). Figure 6.3 (reproduced below), shows that total RFO levels can be reduced to appropriate levels at moisture contents around this value.

Research work investigating the maximum moisture content levels for extrusion should be carried out to see if direct low moisture enzyme processing before extrusion is possible. It would certainly be feasible to carry out this process if bean flour was used in conjunction with another low RFO ingredient such as wheat flour or corn grits.

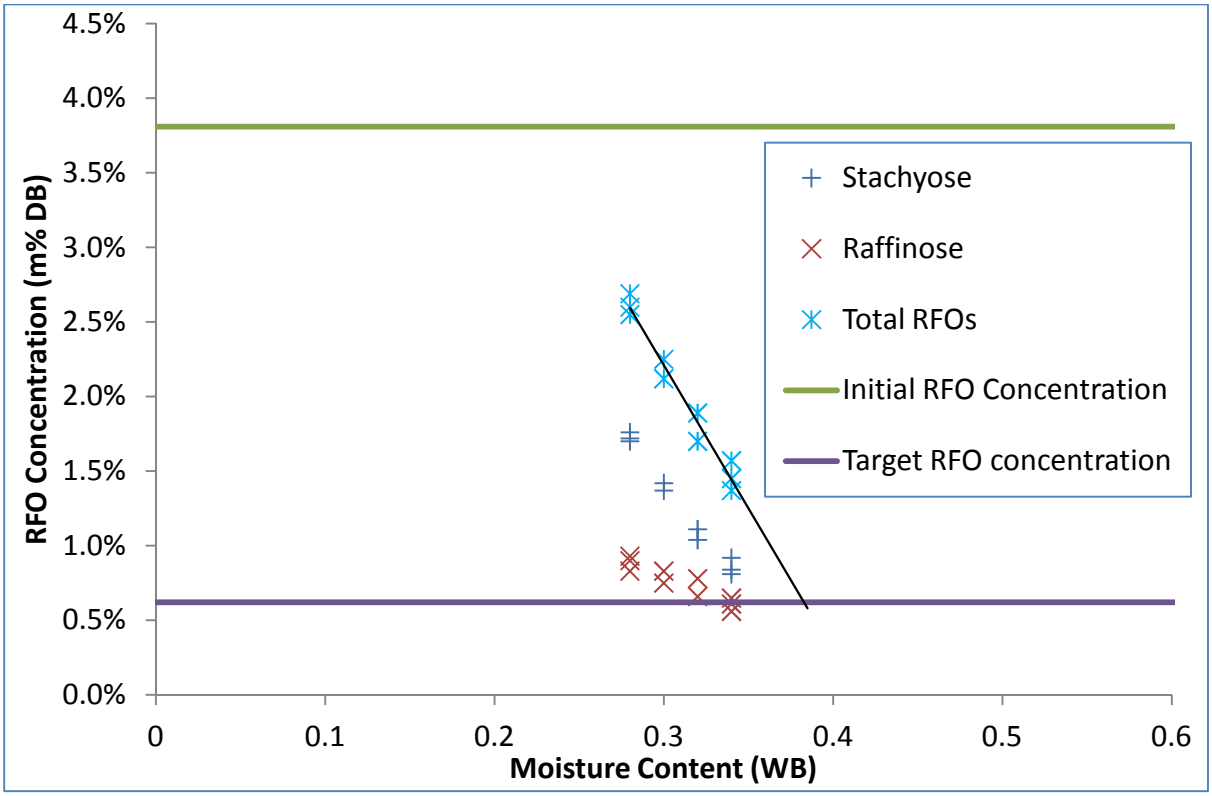


Figure 6.3 Data from figure 6.2-A plotted on a larger x-axis and extrapolated to predict the minimum moisture content at which α-galactosidase can achieve the necessary level of hydrolysis for cessation of measureable flatus response following ingestion.

Commercial scale processing using this method would involve:

- Blending bean flour with dry enzyme powder. Figure 7.1 can be used to select enzyme concentration and processing time. The data presented in figure 6.3 was measured using a ratio of 1 part enzyme to 100 parts bean flour. This equates to an enzyme cost of approximately $0.40 per kilogram of flour.
- The flour/enzyme mixture would then be mixed and enough moisture sprayed in to achieve the desired RFO removal. Figure 6.3 can be used to determine the amount of water required.
- Once mixed, the flour can be rested (or mixing continued if lumping or caking occurs) for leaching and hydrolysis to occur. Because diffusion rates for RFO's are likely to dramatically reduce at lower moisture contents, greater RFO removal will be achieved if the mixture is left for longer periods.
- The treated flour could then be used directly in extrusion if moisture content is low enough, blended with dry flour if required or subsequently dried for later use.

This process is relatively easy to adopt in existing food processing plants and offers significant advantages over traditional leaching and drying methods as described above. The cost of enzyme required for this type of process is over an order of magnitude larger than for the high moisture process described in section 7.3. However this approach would avoid the loss of soluble components that would occur in a high moisture process. The loss of soluble solids effectively raises the cost of the broken beans to $0.93 per kilogram of flour as well as requiring separation and drying steps. Therefore the process outlined in section 7.4 is still a reasonably cost effective approach to low moisture applications and is able to retain beneficial nutrient that would otherwise be lost.

7.5 Conclusions and Recommendations

This work has investigated the various options for processing Navy bean flour to remove raffinose family oligosaccharides. The underlying mechanisms for RFO losses during processing were identified from literature. Initial investigations into leaching showed that to remove enough RFOs from the flour, large water to bean flour ratios are required. Bean hydration and leaching rates were fast in bean flour, however if the wash water is subsequently separated by filtration or centrifugation, significant losses of other solutes (25% dry solids) result.

The use of an enzyme to maintain the concentration gradient and hydrolyse the RFO's into harmless mono-saccharides provides a more practical processing option. A study of the kinetics of α-galactosidase showed that inhibition by galactose produced during hydrolysis occurred at higher concentrations but hydrolysis continued to allow complete RFO removal. From this work, practical guidelines on the use of enzymes to reduce RFOs from bean flour were constructed.

Enzyme processing at low moisture contents was also demonstrated. This processing method has not been reported elsewhere and offers significant advantages for production of reduced RFO low moisture powders for use in extrusion or for cost effective drying and later use. An analysis was carried out to demonstrate why partial hydrolysis could be achieved.

The models produced to understand these mechanisms were of a preliminary nature due to a lack of quality data on RFO diffusion rates at low moisture content. Nevertheless they provide a good platform for further study of this unique process. Other applications for low moisture content enzyme processing using similar methodologies should also be investigated.

The simple processes developed in this work open up the commercial use of navy bean flour for the food industry to develop products that take advantage of its functional and nutritional properties without the negative nutritional problems usually associated with this material. It is important to note that there may be other components of navy beans that also contribute to flatulence (e.g. soluble fibre) and therefore the flatulence response to treated navy bean flour should be investigated.

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