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Determination of Phytochemicals in Vegetable Juices and Their Effects on Postprandial Glycaemia

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A thesis submitted in part-fulfilment of the requirements for the degree of

Doctor of Philosophy at Oxford Brookes University

October 2012

Abstract

High fruit and vegetable intake correlates well with positive health outcomes and reduced rates of chronic diseases such as cardiovascular diseases, type 2 diabetes, cancers, neurological decline and metabolic diseases. Bioactive phytochemicals such as polyphenols, carotenoids, vitamins, minerals and others present in fruit and vegetables may be at least partly responsible for this effect. The precise mechanisms of action for several groups of compounds, and their potential impacts upon each sphere of health have not yet been fully elucidated. This work provides novel analysis of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices which are available on the UK market, utilising 6 biochemical assays, before and after *in vitro* digestion. Beetroot juice had the highest total antioxidant capacity and total polyphenol content regardless of the method of analysis, and both measures either increased or remained stable following *in vitro* digestion. A commercially available beetroot juice shot was selected as a viable method to increase bioactive phytochemical intake in the general population and its carbohydrate and phytochemical profiles were obtained by HPLC and GCMS analysis. The impact of consuming beetroot juice (70 mL) as part of a mixed meal or consuming beetroot juice alone (225 mL) on postprandial glucose and insulin responses was then assessed to investigate a potential role for these phytochemicals in the control of diseases featuring insulin resistance as a primary symptom, such as type 2 diabetes and metabolic syndrome.

Consumption of 70 mL of beetroot juice as part of a mixed meal containing a total of 50g available carbohydrate resulted in a significant ($P<0.05$) reduction in postprandial insulin concentration at 15 minutes compared to a matched control meal. Consumption of 50g available carbohydrate as 225 mL beetroot juice resulted in a

significant (P<0.05) lowering of blood glucose in the 0-30 minute segment of the glucose response and a significant (P<0.05) lowering of the insulin response in the corresponding 0-60 minute segment, compared to a matched control beverage. Insulin sensitivity was estimated using a mathematical model and non-significantly increased with the dose of beetroot juice. Phytochemicals in beetroot juice, namely betanin and its degradation products, alone or in combination with polyphenolic compounds, may improve the postprandial glycaemic state with relevance to diseases characterised by insulin resistance in a similar pattern to other investigated foods such as berries and cinnamon. Further research should aim to further quantify the effects of beetroot juice phytochemicals on postprandial insulinaemia using larger cohorts and diseased populations. Phytochemicals such as neobetanin should also be given in isolation to clarify the compounds responsible for the observed effects. The potential role of phytochemicals in potentiating endogenous nitrate conversion is also worthy of further investigation.

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Acknowledgements

The realisation that the completion of your PhD is going to take more than just your stubbornness and hard work arrives very quickly after you begin. I think it may have even arrived slightly before I began! You may think that I am referring to the elements of luck and co-incidence which never quite develop at the times you wish they would, but you would be wrong. The rest of this work is an expression of me, displaying all the things that I have learnt and discovered throughout this journey so let this part be about the people who got me here.

My first thanks are for my supervisors, Dr Lisa Ryan, Professor David Fell and Professor Jeya Henry. Lisa, you have been a tremendous and vital source of support, guidance and inspiration. Your door has always been open for me, and although it has cost me a lot of coffee over the last years, I will be forever in your debt. David and Jeya, you have both provided much needed encouragement and support together with your unrivalled technical expertise. To have worked with you both during this first part of my career is a genuine honour and privilege.

Many thanks to my collaborator at Newcastle University; Dr Kirsten Brandt, who is a wonderful source of knowledge and inspiration and kindly agreed to provide the HPLC-GCMS analysis for this work, and to Mr Lawrence Mallinson of James White Drinks Ltd, who provided the beetroot juice samples.

I would also like to thank the members of the Graduate School; Professor David Evans, Dr Susan Brookes and Dr Jane Appleton who examined my work as I progressed through my studies and provided useful help and guidance which informed the decisions I made throughout.

A number of academic colleagues have offered advice, information and friendship during my work and I would like to thank my Functional Food Centre colleagues: Dr Michelle Venables, Dr Helen Lightowler, Dr Simon Wheeler, Dr Richard Craven, Dr Viren Ranawana, Dr Sangeetha Thondre, Dr Miriam Clegg, Mrs Megan Ronan, Ms Ann Fraser, Ms Sarah Hillier and

Ms Shelly Coe. To the latter two, very best wishes for the successful completion of your PhD projects.

It has been vital to know Dr David Carter, Dr Charlie Simpson, Dr Rhod Kinch, Dr Roger Ramsbottom, Dr Lesley Smith, Dr Ryan Pink, Dr Teddy Seyoum and Dr Hooshang Izadi, for the bending of their ears and the clarity of their thinking. It was also vital for me to meet my undergraduate supervisor Dr Simon Jobson, who gave me the self confidence to continue in academia. I have had the pleasure of having some students work with me during my time at Oxford Brookes and I should like to extend a special thanks to Ms Sarah Warner and Ms Aisling Moran, who both remain great friends of mine.

The following people are very important to me and their understanding, perseverance and love is a continual source of strength. To my close friends Dr Daniel Caley, Nurdin Topham, Matt Donoghue, and Zoe Birch, I appreciate your enthusiasm and encouragement during this period. It has been so important to have you to talk to when things have not been going my way and to celebrate with you on the rare occasions that they have!

Thanks to my family at home; Mum, Ian, Abi and Anna who try their best to put up with me trying to turn them all into nutritionists and boring them repeatedly with stories about aromatic carbon rings and hydroxyl groups, and to my Dad and Grandparents who get the same treatment when we meet. Thanks also to Anthony, Siân and Delyth in Wales who have allowed me to stay with them and work, providing some much needed peace and quiet during the final stages.

Finally the biggest debt of gratitude which I owe is to my partner, Dr Rhianedd Jewell. You know when you find the right person because they have unlimited patience with your idiosyncrasies and your moods, they pick you up without you ever telling them you were down, and they keep you going when you feel like standing still.

There is no obligation for any of you to read on, but if you do, I hope you feel that it has all been worth it.

Abbreviations

µM	=	Micromolar
AAPH	=	2, 2'-azobis (2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	=	2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
AICR	=	American Institute of Cancer Research
ANOVA	=	Analysis of variance
AP-1	=	Activator protein 1
ARE	=	Antioxidant response element
ASK-1	=	Apoptosis signal regulating kinase 1
AUC	=	Area under the curve
iAUC	=	Incremental area under the curve
sAUC	=	Segmental area under the curve
BEET	=	Beetroot meal in study 3
	=	Beetroot beverage in study 4
BH_4	=	Tetrahydrobiopterin
BP	=	Blood pressure
β-PE	=	β-phycoerythrin
BR	=	Bread meal in study 3
CAD	=	Coronary artery disease
CE^{IV}	=	Cerium IV
CERAC	=	Cerium reducing antioxidant capacity
CHD	=	Coronary heart disease
CHO	=	Carbohydrate
CHAOS	=	Cambridge Heart Antioxidant Study
CI	=	Confidence interval
COMA	=	Committee on Medical Aspects of Food and Nutrition Policy
CT	=	Catalase

CU^{II}	=	Copper II
CUPRAC	=	Copper reducing antioxidant capacity
CVD	=	Cardiovascular disease
dH_2O	**=**	Distilled water
$DPPH^{\bullet}$	**=**	2, 2-diphenyl-1-picrylhydrazyl
DR	**=**	Dietary recall
EC	=	European Commission
ECG	**=**	Epicatechin gallate
EGCG	**=**	Epigallocatechin gallate
eNOS	=	Endogenous nitric oxide synthase
EPIC	=	European Prospective Investigation into Cancer and Nutrition
ERK 1/2	**=**	Extracellular signal-related kinases
ESI-MS	**=**	Electron spray-ionisation mass spectrometry
FAE	**=**	Ferulic acid equivalents
FC	=	Folin Ciocalteu reagent method/Folin Ciocalteu
$FE^{II}/^{III}$	**=**	Iron $^{II/III}$
FFQ	=	Food frequency questionnaire
FL	=	Flourescein
FMD	=	Flow mediated dilatation
FRAP	=	Ferric reducing antioxidant power
FSA	=	Food Standards Agency
GAE	=	Gallic acid equivalents
GC-MS	=	Gas chromatography mass spectrometry
GLUT 1-5	=	Glucose transporter 1-5
GIP	**=**	Gastric inhibitory polypeptide
GLP	=	Glucagon-like peptide 1
GLUC	=	Glucose beverage in study 4
GSK	=	Glycogen synthase kinase

GTP	=	Glutathione peroxidase
H_2O_2	=	Hydrogen peroxide
HAT	=	Hydrogen atom transfer
HPLC	=	High performance liquid chromatography
HPLC-DAD	=	HPLC with diode array detection
RPHPLC	=	Reverse phase HPLC
ICAM-	=	Intracellular adhesion molecule 1
IL-6	=	Interleukin 6
LCMS	=	Liquid chromatography mass spectrometry
LL	=	Low density lipoprotein
oxLDL	=	Oxidised LDL
$LOO^{\bullet}$	**=**	Peroxyl radical
MAPK	=	Mitogen activated protein kinase
MCI	=	Mild cognitive impairment
MCON	=	Matched control meal in study 3
	=	Matched control beverage in study 4
MCP-1	=	Monocyte chemotactic protein 1
MEK1	**=**	MAPK phosphorylator
MJ	=	Mega joule
Nc	=	Neocuproine
NDNS	=	National Diet and Nutrition Survey
NF-κβ	=	Nuclear factor kappa-light-chain-enhancer of beta cells
NICI	**=**	Negative-ion chemical ionization
nM	=	Nanomolar
NO	=	Nitric oxide
Nrf2	=	Nuclear factor (erythroid derived 2)-like 2
$O^{\bullet}$	=	Singlet oxygen
$O_2^{\bullet-}$	**=**	Superoxide radical

OH	=	Hydroxyl group
$OH^{\bullet}$	**=**	Hydroxyl radical
$ONOO^{\bullet}$	**=**	Peroxynitrate
OR	=	Odds ratio
ORAC	=	Oxygen radical absorbance capacity
pAkt	=	Protein kinase B
PFB-Br	=	2,3,4,5,6-Pentafluorobenzyl bromide
PI3-K	=	Phosphatidylinositol 3-kinase
PKC	=	Protein kinase C
PTK	=	Protein tyrosine kinase
RCT	=	Randomised controlled trial
RQ	=	Respiratory quotient
RT	=	Retention time
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
RONS	=	Reactive oxygen and nitrogen species
RR	=	Risk ratio
RW	=	Red wine
RWS	=	Red wine serum
SEM	=	Standard error of the mean
SET	=	Single electron transfer
SGLT-1	=	Sodium glucose co-transporter 1
SOD	=	Superoxide dismutase
TAC	=	Total antioxidant capacity
TAG	=	Triacyl glyceride
TE	=	Trolox equivalents
TEAC	=	Trolox equivalent antioxidant capacity
TNF-α	=	Tumor necrosis factor alpha

TOSC	=	Total oxygen scavenging capacity
TP	=	Total polyphenol
TPTZ	=	Tripyridyltriazine
TRAP	=	Total radical-trapping antioxidant parameter
USA	=	United States of America
USDA	=	United States Department of Agriculture
VCAM-1	=	Vascular cell adhesion molecule 1
WCRF	=	World Cancer Research Fund

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Publications Arising From the Thesis

Conference and symposia abstracts

Wootton-Beard, P.C. & Ryan, L. (2010). Total antioxidant capacity of 10 commercially available tomato juices before and after in vitro digestion. *Proceedings of the Nutrition Society*, 69 (OCE6), E436.

Wootton-Beard, P.C., & Ryan, L. (2010). Stability of the total antioxidant capacity and total polyphenol content of commercially available tomato juices and beetroot juices. *Oxford Nutrition Group Conference Abstracts Booklet.*

Wootton-Beard, P.C., & Ryan, L. (2010). Stability of the total antioxidant capacity and total polyphenol content of 10 commercially available vegetable juices subjected to an *in vitro* digestion procedure. *Proceedings of the 1st International Conference on Functional Foods*.

Wootton-Beard, P.C., & Ryan, L, (2011). A beetroot juice shot is a significant and convenient source of bioaccessible antioxidants. *Proceedings of the Nutrition Society*, 70 (OCE4), E135.

Wootton-Beard, P.C. (2011). Identification of the potential of fruit and vegetable juices to increase plasma antioxidant capacity in humans. *Postgraduate Symposium Abstract Booklet*. Oxford Brookes University.

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Wootton - Beard, P.C., Fell, D., Henry, C.J.K., & Ryan, L. (2012). A beetroot juice meal elicits a lower postprandial insulin response compared to a matched control in 17 healthy adults. *Postgraduate Symposium Abstract Booklet*. Oxford Brookes University.

Wootton-Beard, P.C., & Ryan, L. (2012). Potential influence of phytochemicals from beetroot juice on postprandial glycaemia. *Proceedings of the Nutrition Society*, accepted.

Original Articles

Wootton-Beard, P.C., Moran, A. & Ryan, L. (2011). Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion, measured by FRAP, DPPH, ABTS and Folin Ciocalteu methods. *Food Research International*, 44 (1), 217-224.

Wootton-Beard, P.C., & Ryan, L. (2011). A beetroot juice shot is a significant and convenient source of bioaccessible antioxidants. *Journal of Functional Foods*, 3, 329-334.

Wootton-Beard, P.C., & Ryan, L. (2012). The combined use of multiple methodologies for the measurement of total antioxidant capacity in UK commercially available vegetable juices. *Plant Foods for Human Nutrition*, 67, 142-147.

Wootton - Beard, P.C., Fell, D., Warner, S., & Ryan, L. (2012). Effects of a beetroot juice meal on postprandial glucose and insulin - A pilot study. *Food and Function* (submitted).

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Wootton-Beard, P.C., & Ryan, L. (2011). Improving public health? The role of antioxidant rich beverages. *Food Research International*, 44(10), 3135-3148.

1. Introduction

This thesis aims to investigate the potential for novel vegetable beverages to impact upon postprandial glycaemia. A huge challenge has been presented to human nutrition researchers by their counterparts in the field of epidemiology. Plant foods are consistently associated with reduced incidence of disease, reduced risk of disease, and general well-being in epidemiological research. The challenge is therefore clear; there is a need for research to discover why these effects are evident using rigorous scientific methodology in order to clearly articulate health claims, identify healthy foods, and discover in which context of health they may be most effective. Whilst undertaking this investigation it is important to remember the relevance of it, to the lives of the world's citizens, by asking ourselves questions like 'do people consume enough of this food?', 'can people afford to consume this food?', 'can this food be presented to consumers in a way that is attractive but not misleading?' and 'is it likely that reasonable consumption of the food will result in the desired effect?'.

One growing risk to health is disturbance to the postprandial glycaemic response, implicated in insulin resistance, type 2 diabetes mellitus, and metabolic syndrome, as well as, by association, cardiovascular disease (CVD), neurological decline, and cancer. A number of studies have identified some compounds, traditionally thought of as antioxidants, which may alter the postprandial glycaemic response, leading to reduced glucose uptake, more efficient insulin kinetics or a reduction in the associated inflammatory response. A small number of foods and compounds have been

identified and further research has been crucial to elucidate other foods and compounds which may be therapeutic in cases of disturbed postprandial glycaemia, particularly those which are consumed widely at a population level.

This thesis is the first work to investigate the effects of compounds contained in beetroot juice on postprandial glycaemia. Within the thesis, the efficacy of beetroot juice is identified by comparison with a wide variety of other juices. Its efficacy following digestion is assessed, its phytochemical and carbohydrate composition are obtained and two human intervention trials articulate its relevance to conditions characterised by insulin resistance. A number of peer-reviewed publications have been generated during the course of this research. There has also been a collaboration pertaining to the identification and quantification of the phytochemical profile of the beetroot juice.

Beetroot juice represents an interesting test food because of its phytochemical profile, widespread use and ease of consumption. The use of beetroot as a functional ingredient would appear to be highly viable. It can be produced very economically as crops are viable in a wide variety of conditions and are easily selected for yield, flavour and other characteristics. Beetroot is widely consumed throughout the world although perhaps not in large quantities currently. Beetroot can be presented to the consumer in a wide variety of forms such as whole cooked, raw, juice, powder and capsules. Furthermore, thermal processing appears to produce bioactive secondary products. This thesis therefore aims to present a series of experiments which suggest that a modest intake of beetroot juice (225 mL) may be sufficient to observe a reduction in the postprandial insulin

excursion after consumption of a standard carbohydrate bolus. Further research questions are proposed by this work and this further investigation may lead to the potential for new functional products and/or altered dietary advice for persons afflicted by conditions characterised by insulin resistance.

The hypothesis tested in this thesis is "phytochemicals contained within vegetable juice are capable of surviving the digestion process, and can reduce the postprandial glucose excursion". The thesis aims to:

1. Identify the best vegetable juice sources of bioactive phytochemicals using a wide range of biochemical analyses
2. Isolate and describe the phytochemicals contained in the selected juice using HPLC and GCMS methods
3. Measure the effect of the selected juice on postprandial glucose and insulin responses to a standard consumption protocol.

The results of these experiments will determine the potential efficacy of vegetable juice products as healthful options for the prevention of diet related diseases characterised by insulin resistance,

1.1. Bioactive phytochemicals and health

The Food Standards Agency (FSA) has established its message that a minimum of five portions of fruit and vegetables a day contributes towards a healthy diet (FSA, 2010). However, in the most recent report from the National Diet and Nutrition Survey (NDNS), it was revealed that two thirds of the UK population are still not consuming the recommendation for fruit and vegetables (Bates, 2010). Some of the responsibility for addressing this disparity obviously lies with the consumer, but there is also an inherent challenge to the food industry to develop new, exciting and convenient food products to help inspire people to make a positive change to their diet. Beverages have been used habitually to deliver high concentrations of functional ingredients (i.e. sports and performance beverages, ready to drink teas, vitamin enhanced water, soy beverages and energy beverages). This is due, in part, to their ease of delivery but also to the innate human requirement for fluid.

Beverages represent an appropriate medium for the dissolution of functional components, but also a convenient and widely accepted method of consumption. The influx of functional beverages and novel ingredients has prompted improved legislation (Regulation No. 258/97) from the European Commission (EC) which now requires an extensive safety document to be submitted before novel ingredients can be incorporated into food. The EC has been willing to grant approval and support the industry, provided that their development is based on evidential science and has been validated in human populations (Wilkinson and Hall, 2008). Within the emerging functional food paradigm, functional beverages may contribute toward increasing the consumption of fruit and vegetables to re-equate the

balance between recommendations and actual intake. Juice intake does not always equate to intake of whole fruits or vegetables, for example; a glass of carrot juice is not equal to eating a portion of carrots since nutrients are lost during processing.

Epidemiological evidence still forms the basis of the supposition that antioxidants and other phytochemicals contained in fruit and vegetables can help to prevent or affect the development of disease. Mechanistically, strong *in vitro* evidence exists for these roles, particularly in CVD and neurological conditions. Additionally we are beginning to understand that antioxidants may have a role in type 2 diabetes regulation (Hanhineva et al., 2010). Translating these findings into tangible benefits during human intervention trials however, has been difficult to achieve. As such, little *in vivo* evidence exists to substantiate the potential for direct antioxidant action in the body, and the over-arching notion of exogenous phytochemicals as protectors against certain diseases is being critically and correctly questioned in the literature (Halliwell, 2007, 2008, 2011). Certainly it would appear that there is little or no benefit to be gained from the intake of high-dose antioxidant supplements (Hennekens et al., 1996; Czernichow et al., 2009) on *in vivo* antioxidant functions. This raises an important question; does a diet rich in antioxidants and other phytochemicals from fruit and vegetables complement health without any deleterious consequences, and could it support the response to human diseases through non-antioxidant functions? Despite a great deal of scepticism, the mechanistic roles of phytochemical compounds are numerous *in vitro* and continue to be implicated in an ever-lengthening list of biological functions.

The contemporary definition of health is broad, complex and nuanced, perhaps more so than the definition of any other modern concept. This research is therefore set in the arena of investigations pertaining to the effects of antioxidant compounds and other phytochemicals on diseases such as CVD, neurological decline, type 2 diabetes and cancer. CVD and type 2 diabetes are linked, as they often occur as co-morbidities in metabolic dysfunction. Details of the evidence within these areas are given in this introduction, and short summaries of the research in the fields of neurological decline and cancer are also included to provide a wider context for the current research. The metabolic syndrome, which combines aspects of insulin resistance, abdominal obesity and cardiovascular complications, has facets which manifest in each arena of serious disease prevention in western and developing nations. The focus of this research was therefore narrowed to investigate the effects of novel, phytochemical-rich produce on aspects related to blood glucose and blood insulin control, which may have particular implications within the prevention and/or treatment of type 2 diabetes and the metabolic syndrome, but which remain pertinent to the wider context of the public health paradigm.

1.1.1. Antioxidants and cellular redox balance

Research aimed at fully elucidating the mechanisms by which antioxidants contribute to good health is a relatively young discipline which has intensified over the last three decades. Investigations aiming to understand the potential for produce to be beneficial towards health often begin with a determination of the antioxidant content, since most compounds suggested to be beneficial for health have antioxidant properties. The term 'antioxidant' encompasses a vast array of substances which are divided into endogenous and exogenous antioxidants (figure 1.1). Fruit and vegetable juices are predominately rich sources of polyphenols, carotenoids, vitamins and minerals. Huang and colleagues (2005) defined dietary antioxidants as

"Substances which can (sacrificially) scavenge reactive oxygen/nitrogen (ROS/RNS) to stop radical chain reactions, or can inhibit the reactive oxidants from being formed in the first place".

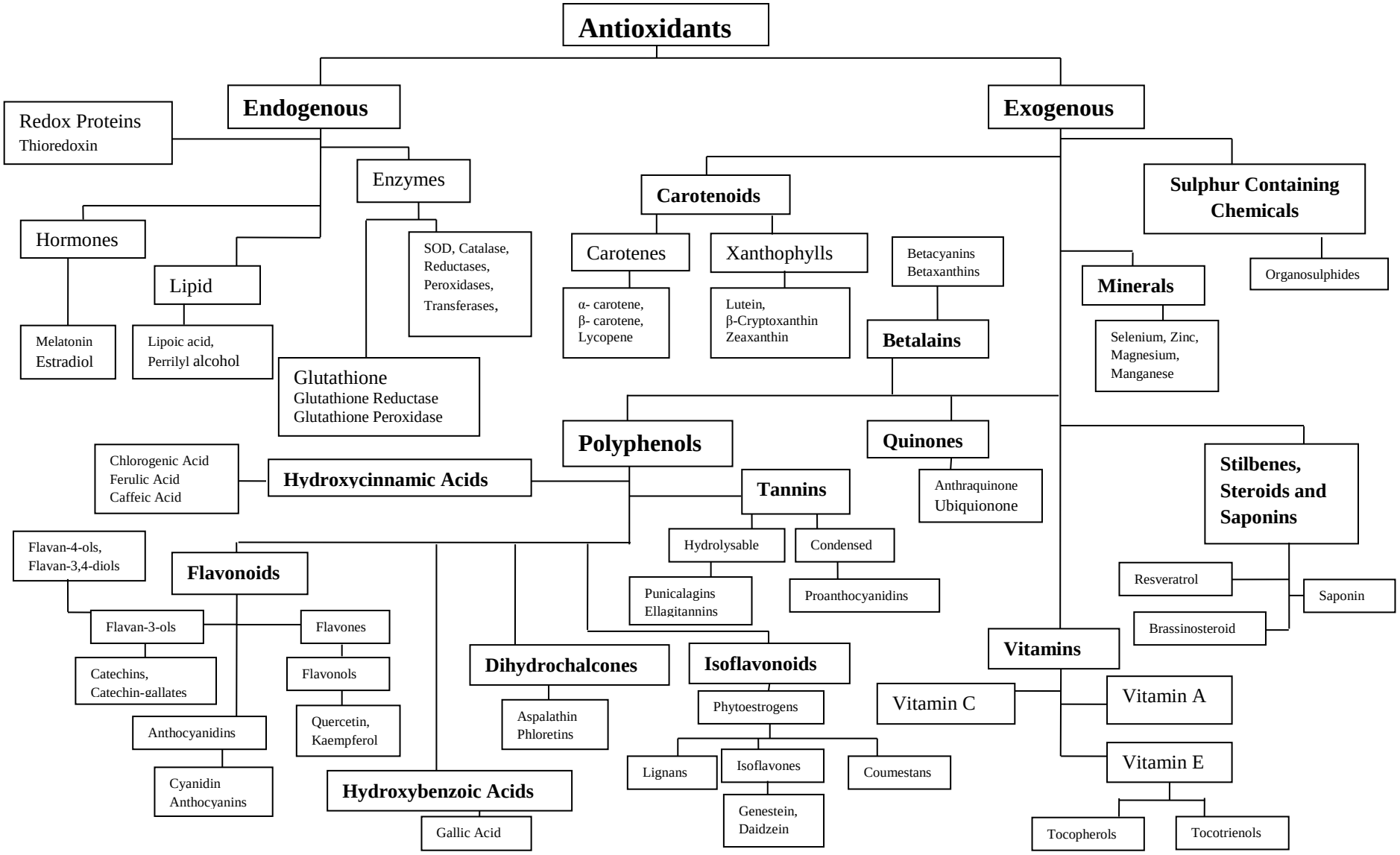


Figure 1.1 Classes of endogenous and dietary antioxidant compounds.
N.b. Stilbenes may be considered as polyphenolic compounds

Some antioxidants perform this function by being oxidised themselves, thus performing a rate limiting role in initiation, propagation and termination of radical chain reactions where the resulting 'antioxidant radical' is less reactive, such as vitamin E. Antioxidants differ in their efficacy against differing substrates; some are potent free radical scavengers whilst others have stronger metal chelation effects, for example; carotenoids are particularly effective at inhibiting the oxidation caused by singlet oxygen (Niki and Noguchi, 2000). Direct antioxidant action of dietary compounds is now considered unlikely in most conditions due to low bioavailability. It is however widely accepted that many compounds traditionally thought of as antioxidants have important non-antioxidant functions including maintenance of redox balance (Forman et al., 2002), stimulation of the antioxidant response element (ARE) and various signalling roles (Hensley et al., 2000), as well as some impact on the oxidation of lipids, proteins and DNA (Valko et al., 2007). It has been suggested that some molecules which are traditionally thought of as antioxidants (such as polyphenols) may also have pro-oxidant effects in certain tissues (Azam et al., 2004). This position raises the question as to whether dietary antioxidants act most beneficially as antioxidant or pro-oxidant molecules in maintaining an optimum level of ROS for health.

Within the biological system the primary ROS in question is the superoxide radical ($O_2^{\bullet-}$) which is created by a premature electron 'leak' to oxygen in the electron transport phase of aerobic metabolism. The unpaired electron in the valence shell of the superoxide radical makes it reactive and it subsequently reacts with other molecules to form secondary radicals such as the hydroxyl radical ($OH^{\bullet}$), peroxynitrate ($ONOO^{\bullet}$), hydrogen peroxide H_2O_2 and the peroxyl radical ($LOO^{\bullet}$). It can also be split to form singlet oxygen ($O^{\bullet}$). Under normal conditions the removal of ROS is effectively regulated by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GTP) and catalase (CT) (figure 1.1), rendering most additional antioxidant

consumption less important (Valko et al., 2007). However, in periods of excess ROS production, or down-regulation of antioxidant enzymes induced by CVD (Van Berkel et al., 2003) for example, it is proposed that increased intake of antioxidants may be of benefit to maintain an optimum balance of ROS.

A rich area of research has developed to investigate effects of bioactive plant food components which are not related to traditional direct antioxidant action. It is argued that antioxidant activity should be considered simply as a chemical property and not automatically related to an equivalent function *in vivo* (Azzi, 2007; Virgili and Marino, 2008). Greater emphasis is placed upon cellular redox status with relation to cellular signalling cascades, representing a broadening of the oxidative stress hypotheses (Cadenas and Packer, 2007). RONS are capable of affecting a number of cellular pathways by activating/inactivating both regulatory kinases and phosphatases, since most protein kinases are activated by phosphorylation and 'switched off' by phosphatases, both of which are redox sensitive (Fraga and Oteiza, 2011). They also affect pro-inflammatory signal cascades and influence redox sensitive gene promoters (Benzie and Wachtel-Galor, 2010).

Dietary phytochemicals in high nanomolar (nM)/low micromolar (μM) concentration such as vitamin C and flavonoids are believed to mediate the activation of p38 mitogen-activated protein kinases (MAPKs) such as apoptosis signal regulating kinase (ASK1), as well as protein tyrosine kinase (PTK), extracellular signal-related kinase (ERK1/2) and its MAPK phosphorylator MEK1, protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3-K) which are primarily inhibited via the oxidation of cysteine groups (Fraga and Oteiza, 2011). Dietary phytochemicals are also thought to inhibit phosphatases leading to further kinase activation. In a study by Briviba et al. (2002) red wine polyphenols were shown to inhibit the growth of colon carcinoma cells *in vitro* through the activation of MAPK and the inhibition of ERK1/2 at 6 mmol/L

concentrations. Other studies have shown inhibition of the ERK1/2 pathway following administration of (-)-epicatechin (Lee et al., 2010), inhibition of MEK1 following administration of flavonoids (anthocyanidin and delphinidin) (Kang et al., 2008a) and modulation of MEK, p38 MAPK, and ERK1/2 as well as several transcription factors by the procyanidin B2 dimer (Kang et al., 2008b).

Furthermore, it has been proposed that compounds such as polyphenols can regulate redox-sensitive transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB), activator protein 1 (AP-1) and nuclear factor (erythroid derived 2)-like 2 (Nrf2) which are essential to the biological pro-inflammatory response (Manson et al., 2006). Cell adhesion factors such as vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1) and monocyte chemotactic protein 1 (MCP-1) may also be modulated by phytochemicals, potentially regulating aspects of vascular health (Virgili and Marino, 2008). A novel model of some of these polyphenol actions was devised by Canali et al. (2007) which differentiated between the actions of 'intact polyphenols' and their metabolites. The authors administered red wine 'as drunk' (RW) and red wine serum (RWS) (obtained by drawing blood 40 minutes after red wine consumption) and displayed a large increase in mRNAs which encode for VCAM, ICAM and MCP-1 with RW whilst RWS dosage resulted in decreases for all except MCP-1 in primary endothelial cells, suggesting very different actions for intact polyphenols and their metabolites. In the same study, following an inflammatory stimulus using tumour necrosis factor alpha (TNF-α) there was nuclear translocation of both NF- κB and AP-1 at 12h suggesting the secondary metabolites in RWS delay the activation of these transcription factors and alter the pro-inflammatory response. An excellent review by Na and Surh (2008) described the role of epigallo-catechin-3-gallate (EGCG - present in tea) in the activation of Nrf2 which has been shown to be activated by a number of flavonoids (Fraga and Oteiza, 2011).

1.1.2. Cardiovascular disease

The link between CVD and phytochemicals was established because of the rate-limiting role of antioxidant phytochemicals in lipid peroxidation (Griendling and Fitzgerald, 2003). Lipid peroxidation in turn has been shown to have a pivotal role in atherogenesis (Diaz et al., 1997). Atherogenesis is believed to occur at sites of endothelial damage whereby plaques are formed by the attachment of activated monocytes and T lymphocytes to the endothelial cell layer at the site of damage (Thomson et al., 2007). The transformation of these monocytes into active macrophages produces ROS, leading to the oxidation of low density lipoprotein (LDL) and stimulation of foam cell production (macrophages which have engulfed the oxidised LDL) (Rimm and Stampfer, 2000).

The oxidation of LDL (OxLDL) follows a typical lipid peroxidation pattern, whereby LDL reacts with the ROS creating water and an OxLDL radical (reactive aldehydes); this reaction is then propagated to the surrounding LDL molecules and endothelial lipids causing increased cellular damage (Steinberg, 1993). The pro-inflammatory immune response to this damage causes the increased adhesion of macrophages and platelets to the walls of the vessel and the stimulation of cytokines such as interleukin-6 (IL-6) and TNF-α (Mullan and Sattar, 2009). In a cyclic fashion the process is repeated and an atherosclerotic plaque can be formed. It is known that the formation of plaques can lead to occlusion of blood vessels and ischemia (Steinberg, 1993; Mashima et al., 2001).

Evidence also suggests that phytochemicals and dietary nitrate may play an early protective role against the development of CVD. Atherosclerotic plaques begin at a site of endothelial damage and it has been widely proposed that phytochemicals with antioxidant properties have a more pivotal role in preventing endothelial dysfunction. The mechanism for this action is based on the production of nitric oxide (NO) by the

vascular endothelium for the maintenance of vascular homeostasis (Thomson et al., 2007). The functionality of the vascular endothelium is crucial for maintaining NO production and preventing the previously described pre-state of atherosclerosis (Ganz and Vita, 2003). A number of studies have conclusively shown that endothelial function is closely associated with atherogenesis and is a predictor for future atherosclerotic events (Schachinger et al., 2000; Schachinger and Zeiher, 2000; Verma et al., 2003). Furthermore, endothelial function is routinely used as a clinical tool in the diagnosis and prognosis of CVD (Bonetti et al., 2003). ROS, particularly $O_2^{-\bullet}$, have a negative impact on NO bioavailability by scavenging it to produce peroxynitrite, which is in turn implicated in lipid peroxidation and endothelial damage. When this process occurs, NO production is catalysed by endothelial nitric oxide synthase (eNOS) which is reliant on L-arginine and tetrahydrobiopterin (BH_4) for its activity (Wever et al., 1998). In a situation whereby L-arginine or BH4 are deficient, NO production is decreased and endothelial damage can occur. Studies have shown that vitamin C improves endothelial function in clinical settings, in patients with CVD (Levine et al., 1996), due to the stimulation of BH_4 and eNOS activity (Huang et al., 2000). Additionally, Webb and colleagues (2008) have shown that dietary nitrate supplementation in the form of beetroot juice can have similar effects on endothelial function.

Mechanistically there is clear potential for oxidative stress to induce higher risk of CVD. However, few studies have established a clear link between markers of oxidative stress and increased CVD risk, or shown any effect of phytochemical supplementation on CVD risk. Some studies have even reported adverse effects of vitamin A, vitamin E and β-carotene supplementation (Bjelakovic et al., 2008). This may be due, in part, to the lack of appropriate biomarkers of oxidative stress, the lack of inter-group standardisation of antioxidant trials in human subjects across large scale

prospective studies, or the use of a limited number of isolated phytochemicals (Mullan and Sattar, 2009).

A Cochrane review of 77 trials concluded that as yet there is no clear evidence for the use of antioxidant vitamins in the treatment of CVD (Bjelakovic et al., 2008). Most of these studies suffered some limitations, which were magnified by the between-study methodological differences, specifically, the diseased/disease free state of the participants and the supplemental dose of antioxidant vitamins administered. The Chief Medical Officer's Committee on Medical Aspects of Food and Nutrition Policy in the United Kingdom (COMA) report in 1995 suggested that evidence was *" persuasive but not yet conclusive"* for the protective effect of fruits and vegetables on CVD (de la Hunty, 1995). The Scientific Advisory Committee on Nutrition (SACN) have since replaced COMA, and are yet to release a report detailing their current position with regards to phytochemicals and cardiovascular disease. Hennekens et al. (1996) showed now effect of long-term supplementation with β-carotene on cardiovascular disease. Over 22,000 males were recruited to the study and β-carotene was given over a 12 year period. There were 338 deaths from cardiovascular disease in the supplementation group and 313 in the placebo group ($P>0.05$). Incidents of myocardial infarction were also not significantly different (468 vs. 489). In a further large scale study, Brown et al. (2001) gave a combination of antioxidant vitamins including 800 IU vitamin E, 1000mg vitamin C, 25mg β-carotene and 100µg selenium per day to 160 patients with coronary disease over a 3 year period. The authors observed no effect of antioxidant vitamins on cardiovascular events (risk ratio = 0.98 (95% CI 0.96-1.06)) although the authors acknowledged a trend towards a slowing of stenosis ($P=0.16$). The authors also gave simvastatin and niacin as another treatment in this study and then combined simvastatin and niacin with the antioxidants to investigate interaction effects between the two treatment regimes. The authors noticed a significant interaction between the therapies

(*P*=0.02) suggesting that antioxidants may interact with simvastatin and niacin in a way which ameliorates their effects.

However, a meta-analysis of 9 cohort studies (221,080 participants, 5,007 events) was conducted which showed a 4% reduction in coronary heart disease (CHD) risk (risk ratio (RR) 0.96 (95% confidence interval (CI): 0.93-0.99) *P*=0.0027) for each portion of vegetables consumed (Dauchet et al., 2006). This rose to a 7% reduction in risk for portions of fruit alone (RR= 0.93 (0.89-0.96) *P*<0.001). The authors also observed marked reductions in cardiovascular mortality (RR= 0.74 (0.65-0.84) *P*<0.001) and myocardial infarction (RR= 0.95 (0.92-0.99) *P*=0.0058); the reduction in risk may be due to increased vitamin C intake. Boekholdt et al. (2006) showed that higher plasma vitamin C is associated with lower risk of coronary artery disease (CAD). In a case-control design (979 cases, 1794 controls) a RR of 0.67 (0.52-0.87, *P*<0.001) was observed for subjects in the highest quartile of plasma vitamin C compared to the lowest quartile. Boekholdt and colleagues (2006) also showed that the effect of vitamin C was independent from classical CVD risk factors (high blood pressure, cholesterol etc) and not related to increased inflammation. Stephens and colleagues (1996) showed a significant reduction in cardiovascular events in the Cambridge Heart Antioxidant Study (CHAOS) following α-tocopherol supplementation. Furthermore, a significant reduction in intima-media arterial thickness following acute supplementation with vitamin C and α-tocopherol in hypercholesterolaemic patients was observed by Salonen et al. (2003), and an inverse relationship between atherosclerosis and fruit and berry consumption was observed by Ellingsen et al. (2008). For a comprehensive review please see Bjelakovic et al. (2008).

1.1.3. Type 2 Diabetes

The global incidence of type 2 diabetes is predicted to reach 360 million cases by 2030 (Wild et al., 2004). Effective dietary modifications to ameliorate such a figure are therefore, of the upmost importance. It has been proposed that phytochemicals may play a role in either increasing insulin sensitivity or modulating the rise in blood glucose following carbohydrate (CHO) consumption through their interaction with digestive enzymes. Early animal studies indicated that supplementation with green and black tea (rich in polyphenolic compounds) decreased postprandial blood glucose concentrations of Sprague-Dawley rats (Zeyuan et al., 1998). Further *in vivo* studies revealed a reduction in glycated haemoglobin (a marker of habitual blood glucose concentration) (Fukino et al., 2008) and an increase in insulin activity (Richarda and Dolansky, 2002) following consumption of tea extracts. A randomised controlled trial (RCT) examining the impact of acute (3g dry weight) instant black tea consumption on postprandial glucose concentrations following an oral glucose load (75g) in 16 healthy fasted subjects was published by Bryans and colleagues (2007). The results of this trial indicated that a significant reduction ($P<0.01$) in postprandial blood glucose concentration at 120 minutes post ingestion was attributable to a significant ($P<0.01$) rise in insulin production caused by the stimulating effects of the black tea on pancreatic β cells and the competitive binding of polyphenolic compounds, particularly catechins, with the sodium-glucose co-transporter 1 (SGLT-1) at 90 minutes post ingestion (Bryans et al., 2007). Fruit and vegetable intake has been shown to lower markers of oxidative stress and inflammation in a cohort of type 2 diabetes sufferers (Asgard et al., 2007). Furthermore, a recent meta-analysis of epidemiological studies concluded that there was sufficient evidence to recommend an increased consumption of green leafy vegetables in order to prevent the development of type 2 diabetes mellitus. The authors reported a hazard ratio of 0.86 (95% CI = 0.77-0.97, $P=0.01$) for consumption of 1.35

portions per day (highest) compared with 0.2 portions per day (lowest) (Carter et al., 2010). Carter and colleagues (2010) suggest that this effect may be due to the actions, alone or in synergism, of vitamin C, β-carotene, polyphenols, magnesium and α-linolenic acid which can be found in green leafy vegetables.

Studies investigating phytochemical supplementation in the treatment of type 2 diabetes have been somewhat equivocal. A recent meta analysis of 13 clinical trials which provided either vitamin C or vitamin E to type 2 diabetic patients by Akbar et al. (2011) reported that the pooled study results showed no difference in blood glucose following intervention (standardised mean difference = 0.013; 95% CI = -0.14 to 0.17). There was also no pooled difference in insulin (standardised mean difference = -0.58; 95% CI = -1.64 to 0.47). The studies did however, reveal a significant reduction in glycated haemoglobin (standardised mean difference = -0.57; 95% CI = -0.93 to -0.21; $P = 0.002$)

1.1.4. Inflammation and the Metabolic Syndrome

Inflammatory pathways are heavily implicated in a wide range of disease aetiologies, including all those mentioned in this work. Inflammation is a very necessary phenomenon because it allows the immune system to respond to invading pathogens. Conversely, prolonged inflammation is pathological and if it is not adequately controlled, it can lead to considerable morbidity or premature mortality. Flavonoids, in particular, have been implicated in the regulation of inflammation and immunomodulatory processes (Gonzalez et al., 2011). Flavonoids are all based upon a 3 carbon ring structure, the addition of hydroxyl groups, double bonds and sugar moieties differentiate between individual compounds. Structure is a fundamental element when considering the influence of polyphenols on inflammatory pathways.

Insulin resistance, obesity and cardiovascular complications are the associated co-morbities which make up syndrome X or 'the metabolic syndrome'. Inflammation is characteristic of this condition and is implicated in each of its facets. Inflammation associated with the condition is thought to be largely due to impaired hormone production and increased secretion of pro-inflammatory cytokines such as TNF-α and the interleukin group or a reduction in anti-inflammatory factors (Rivera et al., 2008). TNF-α in particular appears to have a central role in the inflammatory processes which lead to cardiovascular complications (Zhang and Zhang, 2012). Although the prevention of lipid peroxidation via antioxidant action is perfectly plausible, inflammation also plays a critical role in the progression of CVD since foam cells are only produced following the release of pro-inflammatory cytokines. Indeed, polyphenols such as resveratrol have been shown to reduce the expression of these cytokines (Yoshida et al., 2007). TNF-α causes the up-regulation of various adhesion molecules such as VCAM-1 and ICAM-1 which are crucial in the adhesion of monocytes to the cell wall of the vasculature and a key step in development of atherosclerotic plaques. Ahn and colleagues (2008) showed that EGCG significantly reduced the production of adhesion molecules via the inhibition of TNF-α, providing evidence of non-antioxidant functions for molecules traditionally thought of only as antioxidants.

The most extensively studied flavonoid in relation to inflammation is quercetin. Rivera and colleagues (2008) investigated the effects of high-dose quercetin on factors related to the metabolic syndrome in an obese rat model, showing that quercetin reduced the levels of each cytokine studied, and largely corrected insulin resistance. Furthermore, quercetin has been shown to reduce the production of vascular adhesion molecules (Kobuchi et al., 1999). Resveratrol, a polyphenol extracted from grapes, has also been extensively studied in relation to its effects on inflammation and cardiovascular health (Bertelli and Das, 2009). The interest in resveratrol was ignited by

the association of the Mediterranean diet, which commonly includes the habitual consumption of red wine, with reduced incidence of CVD (Simopoulos, 2005). Resveratrol has been shown to inhibit adhesion molecules, TNF-α expression and further downstream pro-inflammatory signals such as the activation of NF-κB and the expression of pro-inflammatory genes (Csiszar et al., 2006; Zhang et al., 2009). Curcumin has also attracted a large amount of attention due to its inhibition of TNF-α, suppression of the same vascular adhesion molecules and inhibition of downstream pro-inflammatory promotion (Zhang and Zhang, 2012). However, the study of curcumin revealed the potential for direct inhibition of the binding of AP-1 to its DNA binding site and the inhibition of further downstream factors in the inflammatory pathway such as MAPK (Kim et al., 2007).

Human trials assessing the role of antioxidant compounds in metabolic syndrome risk have been conducted using individual compounds and multiple compounds. In a study of 5220 subjects who consumed either a multi-compound supplement (containing vitamin C, vitamin E, β-carotene, zinc and selenium) or a placebo revealed that supplementation did not decrease the risk of metabolic syndrome (Czernichow et al., 2009). The authors did however report that baseline serum antioxidant concentrations of β-carotene (0.34 (95% confidence interval (CI) 0.21 - 0.53), $P = 0.0002$) and vitamin C (0.53 (95% CI 0.35-0.80) $P = 0.01$) were important for reducing the risk of metabolic syndrome, suggesting that habitual consumption of plant foods may be critical. Suzuki et al. (2011) investigated the association between serum carotenoid levels and the odds ratio for metabolic syndrome in a Japanese cohort (931 individuals) with metabolic syndrome. Serum β-cryptoxanthin had an odds ratio of 0.45 (95% CI 0.21 - 0.95), whilst serum zeaxanthin (0.37 (95% CI 0.16-0.84)), and serum β-carotene (0.41 (95% CI 0.21-0.95)) were also associated with reduced risk of metabolic syndrome when highest and lowest tertiles were compared.

1.1.5. Cognition

Neurological decline encompasses recognised neurological diseases such as Parkinson's disease and Alzheimer's disease as well as others such as mild cognitive impairment (MCI). In similarity to the evidence for other diseases, direct causal effects of phytochemicals on neurological decline have been difficult to obtain and largely inconsistent. In terms of overall cognitive function, three large epidemiological studies (the Nurses' health study, moVIES and the women's antioxidant and cardiovascular study) examining elderly subjects found no association between antioxidant consumption and cognitive function (Mendelsohn et al., 1998; Kang et al., 2009; Devore et al., 2010). In contrast, during the four-year follow up 'EVA' study, which examined 1166 patients aged 60-70 years, Berr and colleagues (2000) concluded that oxidative stress and endogenous antioxidant deficiency could be classified as risk factors for neurological disease. It is generally accepted that ROS may contribute towards the pathological progression of neurological decline in the elderly and that phytochemicals could reverse these effects (Mattson, 2006).

Additionally, although data from the Nurses' health study did not reveal any overall effect of phytochemicals on cognitive function, a study focussing on a separate cohort of 14, 968 women from the same study found that the women who consumed a high level of phytochemical supplements containing vitamin C and E did have higher cognitive function test scores (Grodstein et al., 2003). Grodstein and colleagues (2003) concluded that women who were regular users of vitamin E and vitamin C supplements (4,961 vitamin E+C, 1,364 Vit C only, 2,969 vitamin E only) had a significantly higher mean cognitive function test score than those who had never used supplements (P=0.03). The authors did not report the specific doses used but suggested they were well above dietary requirements. Additionally, the study revealed that there was a trend for increases in the improvement depending on the duration of supplement usage.

1.1.6. Cancer

Cancer or malignant neoplasm is characterised by uncontrolled DNA replication and cellular division of abnormal cells leading to the development and/or metastasis of cancerous tumours which invade and destroy adjacent tissues, blood or the lymphatic system. Early research by Doll & Peto (1981) estimated that 30% of all cancers could be caused by dietary factors alone and the joint World Cancer Research Fund (WCRF) and American Institute of Cancer Research (AICR) report highlighted the importance of diet (particularly western diets) and lifestyle factors in the development and progression of 37% of cancers (WCRF/AICR, 2007). Cancerous cellular proliferation can be initiated by ROS-mediated cell damage, immune dysfunction, and the regulation of gene expression. Dietary phytochemicals can play a role in the pathological progression of this process, particularly in the case of carcinoma; however, there is little evidence to suggest that supplementation with isolated phytochemicals can significantly decrease the initiation or progression of cancer. Regulation of oxidative stress by phytochemicals may suppress the genes involved in cancer cell replication thus inhibiting proliferation. Phenolic compounds and isothiocyanates are also capable of generating H_2O_2 via their pro-oxidant activities described in detail elsewhere (Middleton et al., 2000; Abou Samra et al., 2011).

In the Hiroshima/Nagasaki prospective life span study (38,540 men and women), daily fruit consumption was associated with a 12% reduction in total cancer mortality (Sauvaget et al., 2003) compared with consumption once a week or less (RR= 0.88 (95% CI= 0.80-0.96)). Data from Sauvaget and colleagues (2003) specifically linked consumption of green and yellow vegetables (high in β-cryptoxanthin) with a 0.75 relative risk (95% CI = 0.60-0.95) of liver cancer, and fruit intake with a 0.80 relative risk (95% CI = 0.65-0.98) of stomach and lung cancer. Additionally, lycopene has been shown to decrease the relative risk of prostate and digestive tract cancers in large

prospective studies (LaVecchia, 1997; Gann et al., 1999), and it has been observed that high plasma concentrations of lycopene decrease biomarkers of oxidation in prostate cancer patients in case-control studies (Giovannucci et al., 1995; Rao et al., 1999). A meta-analysis of 16 case-control studies revealed a 49% reduction in the incidence of oral cancers for each portion of fruit consumed per day (odds ratio (OR) = 0.51 (95% CI= 0.40-0.65)) based on pooled data from 65, 802 subjects and a 50% reduction for each portion of vegetables (OR = 0.50 (95% CI = 0.38-0.65)) from data for 57,993 subjects (Pavia et al., 2006). Larger prospective studies such as the European Prospective Investigation into Cancer and Nutrition (EPIC) have revealed mixed overall results in the relative risk of developing cancer with increased fruit and vegetable consumption, reporting null findings for prostate (Key et al., 2004), pancreatic (Vrieling et al., 2009), renal (Weikert et al., 2006), breast (van Gils et al., 2005), lymphoma (Rohrmann et al., 2007) and bladder cancer (Buchner et al., 2009), a small inverse effect on total cancer incidence (Boffetta et al., 2010), and positive results for total mortality (Agudo et al., 2007), lung cancer (Linseisen et al., 2007), colorectal cancer (van Duijnhoven et al., 2009) and cancer of the upper aero-digestive tract (Boeing et al., 2006) in various different cohorts throughout Europe.

1.2. Chemistry of total antioxidant measurement

It is known that antioxidant phytochemicals can quench the proliferation of free radicals known as ROS. ROS are implicated in the oxidative damage of lipids, proteins and nucleic acids (including DNA) which contribute to the progression of the previously mentioned diseases. A large body of studies have attempted to quantify the total antioxidant capacity (TAC) of a great many fruits and vegetables, which gives an indication of their phytochemical content. The majority of these studies have provided

conflicting information on the actual antioxidant capacity of the food stuffs they have measured, principally due to the lack of any one, standard, acceptable method for the quantification of TAC (Frankel and Meyer, 2000; Sanchez-Moreno, 2002; Macdonald-Wicks et al., 2006). Biochemical assays have been developed and continuously modified in order that TAC can be more accurately measured. The most widely used and accepted of these assays are the Oxygen radical absorbance capacity (ORAC) (Cao et al., 1993), Trolox-equivalent antioxidant capacity (TEAC) (Rice-Evans and Miller, 1994), Ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996b), 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging capacity ($DPPH^{\bullet}$) (Brand-Williams et al., 1995), Copper II reducing capacity (CUPRAC) and Cerium IV reducing capacity (CERAC) assays.

Biochemical assays are separated roughly into two categories; those which rely on hydrogen atom transfer (HAT) reactions and those which rely on single electron transfer (SET) reactions (Sanchez-Moreno, 2002; Huang et al., 2005). Most HAT based assays employ a competitive reaction system whereby both an indicator and antioxidant compete to scavenge artificially generated peroxyl radicals from the decomposition of azo compounds (Frankel and Meyer, 2000; Huang et al., 2005). HAT assays include the ORAC assay. SET assays measure the reduction capacity of an antioxidant in an oxidant which changes colour when it is reduced (Huang et al., 2005; Sun and Tanumihardjo, 2007). The degree of colour change is quantified spectrophotometrically and is correlated with the antioxidant capacity of the sample (Huang et al., 2005).

1.3. Methods for the measurement of antioxidant content in foods

1.3.1. Oxygen Radical Absorbance Capacity (ORAC)

ORAC is largely used in the USA for the determination of antioxidant capacity. It requires a specified dark room and a fluorescence plate reader, which were not available at Oxford Brookes University. For these reasons details of the ORAC assay are given here in brief and the method was not utilised in this work. A thorough review of the ORAC methodology was completed by Prior and Cao (2000). For further details please refer to this work. The ORAC assay was developed by Cao et al. (1993). The antioxidant capacity of a sample is determined by the total inhibition and time to inhibition of a fluorescent probe (Cao et al., 1993; Cao et al., 1995; Huang et al., 2002). The degree to which a sample preserves the fluorescence of the probe in the presence of peroxyl radicals determines its TAC (Huang et al., 2002; Huang et al., 2005). The value for TAC is obtained by comparing the area under curve (AUC) (time × inhibition percentage) of the sample compared with the AUC of a blank sample containing no antioxidant. The antioxidant activity of the sample is measured by comparison with Trolox, a stable analogue of vitamin E which gives known standard values. The antioxidant capacity is then expressed as Trolox equivalents (TE).

1.3.2. Electron Transfer Assays

1.3.2.1. Trolox Equivalent Antioxidant Capacity

The TEAC assay measures the ability of the antioxidant sample to quench the 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical and is as such often referred to as the $ABTS^{\bullet+}$ assay. It was initially developed by Rice-Evans and Miller (1994) after the popular TRAP assay (Wayner et al., 1985) was discovered to be severely flawed. Rice-Evans and Miller (1994) showed that the oxygen electrode endpoint of the TRAP assay was not sufficiently stable over the two hour period required to measure each sample. Originally, the antioxidant capacity of a sample was based on the inhibition of the $ABTS^{\bullet+}$ radical cation, which was formed by the interaction of $ABTS^{\bullet+}$ with the ferrylmyoglobin radical species (created by the activation of metmyoglobin by hydrogen peroxide) (Rice-Evans and Miller, 1994; Rice-Evans, 2000).

The $ABTS^{\bullet+}$ method was developed in such a way as to allow for several different analytical strategies to be employed. The TAC of samples using $ABTS^{\bullet+}$ are quantified using the second method: inhibition at a fixed time point. The reaction solution is completed with the omission of hydrogen peroxide or metmyoglobin, either of these is then used as the initiator. The absorbance is then measured at a defined time point together with a buffer blank. During assessment of plasma the absorbance is measured at maximal wavelengths of 660, 734 or 820 nm, specifically to prevent spectral interference of heme proteins such as metmyoglobin itself or bilirubin, which are readily absorbed at lower wavelengths (Rice-Evans and Miller, 1994). In the improved format, the assay utilises a decolourisation process whereby the radical is generated in stable form before the addition of the antioxidant. The blue/green $ABTS^{\bullet+}$ chromophore is created by combining $ABTS^{\bullet+}$ with potassium persulfate ($K_2S_2O_8$)

which has maximal absorption wavelengths of 645, 734 and 815 nm but can also be analysed at 415 nm which is more commonly used (Re et al., 1999).

The $ABTS^{\bullet+}$ decolourisation ($ABTS/H_2O_2/HRP$) assay can be performed in aqueous mixtures as well as organic media in order to quantify both hydrophilic and lipophilic antioxidant activity, making the $ABTS^{\bullet+}$ assay more relevant for use in complex fruit and vegetable samples (Arnao et al., 1999, 2001). In order to quantify antioxidant capacity using this method, percentage inhibition of the $ABTS^{\bullet+}$ radical cation (a function of concentration and time) is correlated with the standard reactivity of Trolox under the same conditions, and can thus be expressed as TE and compared with the outcome measures of other biochemical assays. Although the majority of antioxidants fully react within one minute (Ozgen et al., 2006), flavonones like narigenin, and flavones like luteolin may take more than four minutes to fully react (Re et al., 1999). In such cases antioxidant capacity can also be quantified using an area under the curve (AUC) technique using the gradient of the plot of percent inhibition against the concentration of antioxidant (Van den Berg et al., 1999; Arnao et al., 2001). A further update of the described methods produced by Ozgen et al. (2006) is utilised in this work.

1.3.2.2. Ferric Reducing Antioxidant Power

The FRAP assay was initially developed to measure the 'antioxidant power' of plasma (Benzie and Strain, 1996b). The assay is based on the ability of an antioxidant containing sample to reduce a ferric-tripyridyltriazine (Fe^{III} –TPTZ) to its ferrous (Fe^{II}) form under conditions of 37°C and pH 3.6 (Benzie and Strain, 1999; Langley-Evans, 2000; Pellegrini et al., 2003). The antioxidant or reductant in this case, reduces the Fe^{III} TPTZ complex, which must be present in stoichiometric excess to its FE^{II} form. The FE^{III} to FE^{II} ion reduction causes a colour change in the sample with an increase in

spectrophotometric absorbance at 593nm. The change in absorbance gives a measure of the combined ferric-reducing power of the antioxidants present in the sample. FRAP values are calculated by comparing the absorbance change in the sample at 593nm compared with that of a known standard such as ferrous-sulphate, ascorbic acid or Trolox. Results are often expressed in TE (Benzie and Strain, 1999) or whole values. Ou and colleagues (2002) suggest that a low pH such as that used for the FRAP assay can inhibit one electron transfer from the antioxidant to the ferric ion. As such the FRAP assay measures antioxidant reducing capacity based on the ferric ion and not necessarily TAC per se.

Several of the authors who repeatedly criticise the FRAP assay are themselves concerned with the development of other assay procedures leading to some bias in opinion. Several authors (Pulido et al., 2000; Ou et al., 2002) suggest that the slow rate of reaction of some antioxidants like glutathione limits the applicability of FRAP. However, Guo et al. (2003) point out that only very limited amounts of plant glutathione are absorbed by humans. Indeed it is unlikely that values obtained from different methods would ever be able to be correlated with each other because they focus on different principles. Between studies of the antioxidant capacity of various fruit pulps using FRAP there is a marked difference in results. Guo and colleagues (2003) found no positive relationship between the results which they obtained and those obtained in a similar study using the same method (Halvorsen et al., 2002). As the authors suggest, this may not be a product of the assay used but of the region of harvest, cultivar, or storage conditions.

1.3.2.3. DPPH•

The DPPH• free radical method was originally developed by Blois (1958) to elucidate the reaction kinetics of chain breaking antioxidants. The original method was discovered to be over-simplified because of the complexity and reversibility of the observed reactions. It was shown that the stoichiometry of the reaction need not be a whole number integer and that in some cases the antioxidant- DPPH• reaction was reversible (Brand-Williams et al., 1995; Bondet et al., 1997). It was improved by Brand-Williams and colleagues (1995) using a variety of phenolic compounds. Studies by Kurechi et al. (1980) and Shimada et al. (1992) suggested the use of a method whereby a stable free radical substance be directly used to measure the antioxidant activity of a sample. DPPH• can be categorised as a 'stable' free radical because its spare electron is delocalised across the whole molecule, which means that it does not dimerise like many other free radicals (Molyneux, 2004).

DPPH• is prepared in a methanol solution. Samples are added to the solution and the reaction is allowed to occur. The degree to which DPPH• is reduced is a measure of the antioxidant activity of the sample. In its radical form, DPPH• is readily absorbed at a wavelength of 515nm. When it is reduced this absorption no longer occurs. It is therefore very simple to determine an outcome measure of the assay by measuring the reduction in absorbance at 515nm. The DPPH• free radical method has been used extensively to calculate antioxidant capacity in fruit and vegetable samples. Most recently Tabart et al. (2009) used a variety of methods including DPPH• to evaluate the antioxidant activity of fruit and vegetable juices, ice green tea and wine, as well as an extensive range of standard compounds including flavonols, anthocyanins, flavanones, flavan-3-ols, phenolic acids, ascorbic acid, Trolox and reduced glutathione. The results of the investigation by Tabart and colleagues (2009) showed that DPPH• was the least sensitive assay when assessing the activity of the standard compounds with the

exception of some flavan-3-ols. The major criticism of the DPPH free radical method is the difference in the reaction kinetics between various different antioxidants and $DPPH^{\bullet}$. Rather than individually assessing each kinetic case, it may be just as appropriate to follow each reaction to completion, provided this is accurately defined (Molyneux, 2004).

1.3.2.4. Copper II reducing antioxidant capacity

The CUPRAC method was developed in response to criticism of the reaction speed of other reducing methods. The method was designed to measure a broad range of different antioxidants which might be contained in vegetable extracts (Apak et al., 2004) and purports to accurately quantify polyphenols, vitamin C and vitamin E. The method is based on the reduction of an oxidising combination of copper II chloride (Cu^{II}) and neocuproine (nc). This method offers some advantages over other reducing assays such as FRAP because Cu^{II}-nc has faster reactions kinetics than FE^{III}. Cu^{II} is combined with neocuproine in an ethanolic mixture and diluted with an ammonium acetate buffer at pH 7, which develops a dark green colour. Decolourisation occurs during incubation with antioxidant containing samples and can be quantified spectrophotometrically at a wavelength of 450 nm, and compared to an appropriate standard such as Trolox or ascorbic acid. Apak et al. (2008) characterised the reaction of the Cu^{II} reagent with polyphenols using the following equation:

$$2n\ \mathrm{Cu}\ (\mathrm{nc})_2{}^{2+} + \mathrm{Ar(OH)_n} = 2n\ \mathrm{Cu}\ (\mathrm{nc})_2{}^{+} + \mathrm{Ar}\ (=\mathrm{O})_\mathrm{n} + 2n\ \mathrm{H}^{+}$$

Where Ar is the compound in question and OH_n represents the number of hydroxyl groups present in the structure (H= hydrogen).

The research paper presenting the CUPRAC method by Apak et al. (2004) tested the method using a variety of isolated substances and food products. The authors predicted that single hydroxy-substituted compounds would display a lower TAC than those with more complex, conjugated structures as reported elsewhere (Robards et al., 1999). In practice this was successfully achieved. They also showed that aglycones have a higher TAC than their corresponding glycosides (for example, naringenin was higher than naringin) which was also predicted, based on the information provided by Robards and colleagues (1999). Using these principles, the most effective compounds were epicatechin gallate (ECG), EGCG, quercetin, fisetin and others with highly conjugated structures. The authors also reported that synthetic mixtures of compounds produced a TAC which was within 5% of the expected value based on their individual TAC, suggesting that there were no interactions between compounds during the reaction.

There is one particular drawback to using CUPRAC. The Cu^{II}-nc reagent is unable to react with compounds which have isolated hydrocarbon double bonds such as ferulic acid. Furthermore, Apak et al. (2004) acknowledge that other antioxidants like β-carotene which has alternating hydrocarbon double bonds without hydroxyl (OH) groups, would not be measured unless dichloromethane was used as the solvent in a separate procedure. A comprehensive review of the CUPRAC method, applied to a number of phenolic compounds and compared to other TAC assays is available (Apak et al., 2007).

1.3.2.5. Cerium IV reducing antioxidant capacity

The majority of reducing methods can be criticised for over-estimating TAC due to the interference of other reducing substances in sample mixtures, such as simple reducing sugars and citric acid. Such substances are not defined as antioxidants. The contribution of such substances to TAC is reported to be in the order of 25% (Ozyurt et al., 2010). The CERAC assay was developed by Ozyurt and colleagues (2007) because the reduction of Ce^{IV} to Ce^{III} has selectivity over reducing sugars and citric acid, limiting the overestimation of TAC which can be attributed to these substances. The results of the CERAC assay were shown to be comparable to $ABTS^{\bullet+}$, CUPRAC and Folin Ciocalteu. Quercetin, catechin, gallic acid and caffeic acid were consistently ranked highly by the assays. Narigenin, narinigin and ascorbic acid were consistently ranked lowest (Ozyurt et al., 2010). The authors report that the redox potential of the Ce^{IV} reagent allows the oxidisation of only true antioxidants and not pharmaceutical substances. Furthermore, they report that the cerium reduction causes the hydrolysis of certain flavonoid glycosides to their corresponding aglycones, such that the TAC of corresponding pairs is similar and the measurement of the glycoside is not underestimated as in CUPRAC.

In summary, the TAC assays selected represent a diverse range of reaction kinetics and are included to provide the greatest resolution regarding the TAC of the produce to be tested. The description of the ORAC method is included for comparison as it is widely used in the USA, but is not included within the analyses presented in this work. The FRAP assay provides a reference point for results to be compared with existing data from other studies and a measure of metal chelation, whilst the CUPRAC and CERAC assays are included to provide values which offer selectivity over common confounding factors such as citric acid and reducing sugar content. The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays have been included to represent radical scavenging capacity.

1.4. Methods for the specific measurement of polyphenol content

1.4.1. High Performance Liquid Chromatography

Measurement of individual polyphenols centres around two key questions. Firstly, what is the total amount of extractable polyphenols? And secondly, when intervention trials produce a link between foods containing polyphenols and health outcomes, what are the compounds which are responsible for the effect? The isolation and quantification of individual polyphenols is most commonly achieved by high performance liquid chromatography (HPLC). An HPLC system consists of a column containing both a 'stationary phase' and a 'mobile phase'. The technique commonly applied to polyphenol measurement is reverse-phase liquid chromatography (RP-HPLC) which is so called when the stationary phase is a non-polar substance, commonly C^{18} silica particles which create a hydrophobic environment. HPLC techniques have been commonly applied to the quantification of polyphenolic compounds in fruit and vegetable juices. Lugasi and Hovari (2000) quantified flavonoid aglycones such as quercetin, myricetin and kaempferol in vegetables including peppers, broccoli, celery, turnip, onions and lettuce using RP-HPLC. Similar techniques have also been used for the measurement of phenolic acids (Ping et al., 1993), carotenoids (Huck et al., 2000), and total polyphenols (Sakakibara et al., 2003).

Sakakibara and colleagues (2003) compared samples of 63 vegetables, fruits and teas with a library of retention times and spectral peaks obtained from 100 standard compounds. Food extracts were first compared in their natural form and then after hydrolysis to identify each compound present as an aglycone if it had been present as a glycoside. The authors constructed a typical system for this type of analysis, utilising a 250 × 4.6 mm column with a further guard column, gradient elutions with 50 mM sodium phosphate, 10% methanol and 70% methanol at a flow rate of 1.0 L/min (which comprised the mobile phase). The sample injection volume was 10 µL. Some

compounds, such as EGC had a very high detection limit suggesting that the system would need to be fine tuned if measuring EGC had been the sole aim of the study. Elution times for the various polyphenols were between 5 and 95 minutes, and a vast number of individual compounds were identified.

1.4.2. Mass Spectrometry (MS)

If unknown retention times or peak spectra are identified using RP-HPLC, or in cases where the structure of polyphenolic compounds is required, HPLC can be coupled to electron spray ionisation mass spectrometry (ESI-MS) or, alternatively, gas chromatography mass spectrometry (GC-MS) can be used (Self et al., 2005; Wu and Prior, 2005; Ignat et al., 2011). Mass spectrometry converts substances into gaseous phase ions by heating and then bombarding the gas with electrons. Ions created from different compounds have a specific mass to charge ratio (m/z) which is detected and converted to a graph which displays peaks corresponding to specific compounds. When conducted alongside RP-HPLC or GC, more specific compound identification is possible, and differentiation can be made between similar compounds such as aglycones and their glycosides or oxidised and non-oxidised compounds.

1.4.3. Folin Ciocalteu Reagent Method

HPLC remains the ‘benchmark’ standard for the measurement of individual polyphenols and for measuring the total extractable polyphenols in a sample through addition. In some situations, particularly when analysing digested samples, including complex foods or human fluids, it may be advantageous to use a method with less selectivity over the compounds which are included in the measurement such as the Folin Ciocalteu (FC) reagent method (Singleton et al., 1999). The FC method has been

developed and modified to replace the Folin-Denis phenol reagent described in their original work (Folin and Denis, 1912). The method was adapted to measure total phenolic compounds in alcoholic beverages (Scott, 1922), and improved several times before being accepted as the Folin Denis procedure.

The most commonly used version of the FC appears in Singleton et al. (1999) although adaptations continue to be made. The procedure now involves a commercially produced Folin reagent which is combined with the sample and sodium carbonate as the alkali, before incubation at room temperature for 90 minutes. There have been a number of criticisms of the FC method as a measure of total polyphenols (TP), as it can lead to an over-estimation of polyphenol content due to the interference of water soluble non-phenolic substances such as vitamin C, which can also reduce the Folin Ciocalteu complex. Improvements have been suggested in order to compensate for these weaknesses in FC and produce a more accurate method by reducing the interference of ascorbic acid in the assay (Vinson et al., 2001; George et al., 2005; Prior et al., 2005). Despite these amendments, the nature of the reagent still results in interference from reducing substances. The FC method is therefore a reducing substance measure with a higher specificity for polyphenols, rather than a measure of polyphenols per se.

The FC method is routinely used for the determination of TP, whilst acknowledging its limitations, in a vast number of fruit and vegetable beverages. It is used as a method for the compilation of the European phenol explorer database (Neveu et al., 2010) which has been applied to the determination of phenolics in a wide variety of foods (Perez-Jimenez et al., 2010a; Perez-Jimenez et al., 2010b). It has also been used extensively for the determination of phenolic compounds in commonly consumed foods in the USA (Seeram et al., 2008). Although expressed in catechin equivalents rather than the more common gallic acid equivalents, Vinson and colleagues (1998) assessed the phenolic content of 23 vegetables including beetroot, legumes, greens,

alliums and tubers. Beetroot was ranked highest for polyphenol content based on dry weight measures whilst red kidney beans replaced it when the samples were ranked based on wet weight measures. Świderski et al. (2009) assessed the polyphenol content of organic and non-organic tomato and carrot juices using the FC method. The authors reported that both were good sources and that organic juices had a somewhat lower TP content than their non-organic counterparts.

1.5. Consumption of phytochemical-rich fruit and vegetables in the UK

Following the general consensus that consumption of fruits and vegetables which are rich in phytochemical compounds may improve health outcomes related to a number of diseases, it is important to consider the intake of such produce in the UK. The most recent NDNS report highlights the disparity between the recommendation for consumption of 5 portions of fruit and vegetables per day and the actual estimated intake in the population (Bates, 2010). From these statistics, it was deduced that two thirds of the population still fail to achieve the recommended intake of fruits and vegetables. The achievement of a consistent increase in fruit and vegetable consumption across the UK population would inherently lead to an increase in phytochemical intake which may decrease the incidence of the aforementioned diseases.

One method of increasing total intake is to increase the consumption of fruit and vegetable beverages. Beverages can be modified to alter some of the sensory barriers to fruit and vegetable consumption (e.g. bitter taste, grainy texture), and provide a convenient method of ingestion. The NDNS survey noted that fruit juice consumption had increased markedly since the last survey (Bates, 2010). However this increase was observed only in children; adult fruit juice consumption actually decreased. No data were provided for the consumption of vegetable juices. For comparison, in the USA it is

estimated that fruit juice accounts for 50% of the total intake of fruit by children (Dennison, 1996). Fruit juice consumption in particular has raised concerns because it contains naturally high levels of sugar, which has implications in diabetes management, obesity and dental health (Dennison, 1996; Baker et al., 2001; Bazzano et al., 2008). Juices can only be seen as supplemental to whole produce consumption due to the removal of fibrous material during processing. Nevertheless, fruit and vegetable juices continue to grow in popularity, potentially driven by an increased public interest in 'preventative healthcare'.

1.5.1. Fruit and Vegetable beverages

Fruit juices and vegetable juices have been shown to be a rich source of bioaccessible phytochemicals (Lichtenthaler and Marx, 2005; Sun et al., 2005; Seeram et al., 2008; Swatsitang and Wonginyoo, 2008; Fraternale et al., 2009; Piljac-Zegarac et al., 2009; Ryan and Prescott, 2010) (table 1). In terms of public health, it is generally accepted that moderately increased consumption of any type of fruit or vegetable juice is beneficial. Furthermore, consumption of a wide range of different products provides the most comprehensive access to the vast number of compounds which they contain.

The number of commercially available vegetable juices and vegetable/fruit blends appears to be increasing in the UK. Commonly available vegetable juices in the UK include tomato, carrot, beetroot, mixed vegetable and a number of fruit and vegetable blends. Several studies have reported the phenolic content and antioxidant capacity of vegetables and vegetable juices measured by a variety of biochemical methods (Vinson et al., 1998; Lugasi and Hovari, 2003; Lichtenthaler and Marx, 2005; Swatsitang and Wonginyoo, 2008; Perez-Jimenez et al., 2010b). A comparison of the TAC, ascorbic acid content and phenolic content of some commercially available

vegetable juices measured can be viewed in table 1. Lichtenthaler and Marx (2005) measured the total oxidant scavenging capacity (TOSC) of beetroot, carrot and tomato juices alongside a variety of fruit juices and sauerkraut. Carrot and tomato proved relatively inefficient radical scavengers and were similar to orange and apple juice. Beetroot juice however, was a particularly efficient radical scavenger in the TOSC assay (Lichtenthaler and Marx, 2005) ahead of both blueberry and cherry juices.

Table 1.1 - Comparison of total antioxidant, ascorbic acid and total phenolic content of commonly consumed whole fruit and vegetables and fresh juices

Fruit	**TAC using FRAP method**		**Total ascorbic acid content**		**Phenolic content (Folin)**	
	Whole Fruit (µmol.kg)	*Fresh Juice (µmol.L)*	*Whole Fruit (mg.g.fw)*	*Fresh Juice (mg.ml.fw)*	*Whole Fruit (mg.g.fw)*	*Fresh Juice (mg.ml.fw)*
Orange	9420	4700 - 5828	0.53	0.5	2.8	0.5
Apple	4200 - 6300	2536 - 9946	0.05	0.009	2.1	0.34
Grapefruit	8080	7268 - 7668	0.33	0.38	1.6	0.53
Pineapple	3480	5689 - 8576	0.48	0.1	1.5	0.36
Cranberry	32900	6733 - 7582	0.13	0.09	3.15	1.7
Pomegranate	19400	8557 - 10232	0.06	<0.0001		2.3
Red Grape	4160	5653 - 6697	0.11	0.001	1.8	0.68
Blueberry	18500	4200	0.1		6.6	2.3
Mango	5060		0.28		1.4	
Strawberry	15940	4300	0.59		2.9	
Blackcurrant	5490		1.81		8.2	1.2
Cherry	3500	3400	0.07		1.7	2.1
Acai	n/a	3800				2.1
Mixed Vegetable	n/a	5000	n/a	0.28		0.32
Tomato	1600	1843 - 3104	0.13	0.18	0.45	
Beetroot	16800	8355 - 9500	0.04		1.6	0.53
Carrot	600	1369 - 1533	0.06	0.08	0.57	0.26

Values obtained from Vinson et al. (1998) ; Gardener et al. (2000); Vinson et al. (2001); Szeto et al. (2002); Lugasi & Hovari (2003); Carlsen et al. (2010) ; Perez-Jiménez et al. (2010a); Ryan & Prescott (2010); Wootton-Beard et al. (2011). FRAP = ferric reducing antioxidant power, TAC = total antioxidant capacity.

Swatsitang and Wonginyoo (2008) created a variety of juices from whole produce and assessed their efficiency in scavenging the 2, 2, diphenyl-1-picryhydrazl (DPPH) radical. Results indicated that cabbage juice was a particularly efficient radical scavenger together with sweet pepper and broccoli. Some carotenoids (α-carotene and lutein) have been shown to be more bioavailable from vegetable juice than from either cooked or raw vegetables, with no difference observed for other carotenoids (McEligot et al., 1999). Shenoy et al. (2010) conducted a randomised controlled trial to assess the suitability of vegetable juice to bridge the gap between actual vegetable intake and recommended vegetable intake, following an earlier study which established vegetable juice as a viable method to increase vegetable intake (Shenoy et al., 2009). The authors concluded that just 1-2 cups of vegetable juice a day could significantly increase vegetable intake, which was in turn associated with a reduction in blood pressure in hypertensive subjects.

1.5.2. Effects of juicing and processing

High pressure, thermal and physical processing techniques used to produce most commercial fruit and vegetable juice products may have profound effects on the levels of a variety of phytochemicals, mostly due to the localisation of phytochemicals within the plant and the separation of parts such as peel and seeds. Processing has been shown to result in both increases and decreases in antioxidant phytochemical activity. Heating, separation and storage techniques can result in oxidation, thermal degradation and leaching to the surrounding water. In particular, studies have reported an advantage of high pressure processing over thermal treatments in both fruit and vegetable juices. Brunton et al. (2009) analysed the effects of high pressure (400, 500, 600 MPa/15min/10-30°C) treatment in comparison to a more conventional thermal treatment (70°C) on TAC as well as ascorbic acid, anthocyanin and polyphenol content

of strawberry and blackberry purées. High pressure treatment resulted in negligible losses in ascorbic acid, anthocyanins and polyphenols, whereas thermal treatment (2 mins) resulted in significant losses for all three ($P < 0.05$). The same research group also analysed the effects of the same treatments on tomato and carrot juices. High pressure treatment (600 MPa/15min/10-30°C) resulted in the retention of 90% more ascorbic acid than thermal processing at 70°C ($P < 0.05$), although no effect was seen on phenolic content for either treatment (Brunton et al., 2009). Keenan and colleagues (2010) meanwhile, reported significant reductions ($P < 0.001$) in both the antioxidant activity and phenolic content of fruit smoothies after 5 minutes of high hydrostatic pressure treatment (450 MPa). The authors suggested that a higher pressure treatment of >450 MPa might result in greater retention of phytochemicals.

A comprehensive study of processing techniques by Gil-Izquierdo et al. (2002) examined the effects of five industrial techniques (squeezing, mild pasteurisation, standard pasteurisation, concentration and freezing) on the phenolic content. L-ascorbic acid content and TAC in orange juice separated into soluble and cloud fractions. Commercial squeezing resulted in 22% greater phenolic compound extraction than hand squeezing, whilst freezing led to marked reductions in phenolic compounds. The concentration process caused some precipitation of phenolic compounds into the cloud fraction, whilst pasteurisation led to ~30% losses for these compounds. L-ascorbic acid content was increased by 25% in the commercial squeezed product compared with domestic squeezing, and was slightly enhanced by pasteurisation. There was no change in L-ascorbic acid concentration in either the concentration or freezing conditions. None of the methods had a significant effect on TAC of orange juice.

Conversely, the phenolic concentration and antioxidant activity of apple juices have been shown to decrease by 30% and 75-88% respectively following pressing, but increased following treatment with pectolytic enzymes (Zhang et al., 2008). For further

information, a detailed review by Kalt (2005) can be consulted which discusses in detail the effects of processing techniques on vitamin C, carotenoids and phenolics. In general phenolics are highly affected by pre-harvest environmental factors, more so than carotenoids or vitamin C. Vitamin C is most prone to losses during fresh storage, whilst carotenoids and phenolic content may actually increase under appropriate conditions. Carotenoids are the most stable compounds during processing, whilst vitamin C and phenolics are often highly degraded by modern processing techniques.

1.5.3. Bioaccessibility and bioavailability

One important factor when considering the potential impact of dietary phytochemicals on disease states is the circulating levels of the phytochemicals in the body following digestion. In this respect the term 'bioavailability' is commonly used. The term 'bioaccessibility' is also commonly used to represent the outcome measure of *in vitro* studies. It is important to differentiate between the terms bioaccessibility and bioavailability within the paradigm of phytochemical research. Bioaccessibility refers specifically to the amount of a given nutrient(s) which is/are potentially presented to the intestinal brush border for absorption. Bioavailability generally refers to the amount of a given nutrient which is available for normal physiological functions and storage within the body. Bioaccessibility is commonly used to rank produce in a laboratory setting with the theoretical implication that a better ranking suggests greater potential bioavailability (Karppinen et al., 2000), although this may not always be the case. Bioavailability of phytochemicals is generally reported to be low, however, the bioavailability of a number of polyphenolic compounds in beverages may be underestimated due to a failure or inability to measure the secondary catabolites created during the digestion process (Manach et al., 2004).

Certain phytochemicals such as lycopene become more bioavailable following processing (Paetau et al., 1998a; Karakaya and Yilmaz, 2007). Serrano and colleagues (2007) showed that basic antioxidant capacity is underestimated in whole produce and generally increases following interactions which occur in the digestion process. TAC is therefore higher when the compounds are presented to the intestinal lumen. Using a thorough *in vitro* digestion system, Serrano et al. (2007b) analysed a wide variety of phytochemical containing produce found in the Spanish diet including cereals, vegetables, nuts, fruits, and legumes. The samples were subjected to an enzymatic digestion which mimicked the gastric and duodenal phases of digestion, after which TAC was assessed using the FRAP and $ABTS^{\bullet+}$ assays. The samples were then subjected to colonic fermentation using rat faeces under anaerobic conditions, after which, TAC was once assessed again. The results obtained showed that TAC was markedly increased by enzymatic digestion and colonic fermentation; furthermore, if absorption were low in the intestinal lumen, then the phytochemicals released may also reach the colon.

The absorption of most phenolic compounds appears to occur mostly in the small intestine, where the enzyme, lactase phloridizin hydrolase, removes the glucose moiety from glycosides to release the aglycone. Lactase phloridizin hydrolase is present in the brush border of the intestine; hydrolysis here means that the lipophilic aglycone passes into the epithelial cells by passive diffusion (Day et al., 2000). Gee and colleagues (2000) also showed that quercetin glycosides can be transported into the epithelial cells, possibly via the sodium glucose co-transporter (SGLT-1) intact and are then hydrolysed by cytosolic β-glucosidase. The appearance of the aglycones in epithelial cells was therefore believed to occur by one of these two mechanisms, although some questions have been posed as to whether or not SGLT-1 can transport the glycosides, as some studies have shown that they inhibit glucose uptake by binding

to SGLT-1 or to GLUT transporters (Kottra and Daniel, 2007). This is discussed in detail in section 1.6 and it is likely that there is some specificity as to which glycosides can be transported.

Once absorbed, sulphotransferases, catechol-*O*-methyltransferases and UDP-glucoronosyltransferases add sulphate, methylate or glucuronide groups to form the secondary metabolites (Del Rio et al., 2010). If metabolism does not occur in the small intestine, some intact glycosides may proceed into the colon. The colon is populated by a diverse range of microflora, which are specific to the individual in terms of species diversity and in number. The interaction of the glucosides with the gut microflora cleaves the glucose moieties, liberating the aglycones, which go on to form phenolic acids and other catabolites via the process of ring fission. It has been suggested that the process of colonic fermentation, particularly of phenolic compounds, may produce a great many secondary catabolites, the bioactivity of which is yet to be fully elucidated (Manach et al., 2004). The only method by which bioavailability can be truly assessed is by physically measuring the metabolites of digestion in human subjects.

Gastric and duodenal digestion are replicated with relative ease *in vitro*, and colonic fermentation is simulated using either defined bacterial strains, or via the use of ileostomy fluid/faeces. A comprehensive review of *in vitro* digestion models which have been used to measure the bioaccessibility of compounds from food products has been recently presented by Hur et al. (2011). Saura-Calixto et al. (2007) assessed the intake and bioaccessibility of polyphenolic compounds in a whole diet noting that non-extractable polyphenols represent a far greater proportion of the total intake than extractable polyphenols. It was also estimated that 48% of the total polyphenol intake was bioaccessible in the small intestine whilst 42% were bioaccessible in the colon. This proportion of polyphenol release in the gastric phase of digestion is supported by Bouayed et al. (2011) who showed that approximately 65% of apple flavonoids and

other polyphenols were released during the gastric phase of digestion, with a further 10% becoming accessible in the colon. Toor et al. (2009) also report similar figures with 71-77% bioaccesibility of total phenolics and flavonoids from tomatoes during *in vitro* digestion. Bouayed et al. (2011) also assessed the amount of dialyzable polyphenols using cellulose membrane and found that these comprised 55% of the original concentration which was reflected in their antioxidant capacity. A second study by these authors showed that the digestive process had caused noteworthy structural changes and considerable changes to the concentration of polyphenols in apples (Bouayed et al., 2012).

In comprehensive reviews of this topic, Crozier et al. (2010) and Williamson and Clifford (2010) discuss the bioavailability of flavonoids and other phenolics, and whether or not the secondary catabolites of polyphenols from colonic fermentation might have considerable biological activity. Crozier et al. (2010) conclude, after considering the bioavailability of flavan-3-ols, flavonols, isoflavones, anthocyanins, ellagitannins and phenolic acids, that colonic metabolites are becoming an increasingly important factor when calculating the potential bioavailability of phytochemical compounds from food products. Williamson and Clifford (2010) explain that colonic fermentation has been shown to produce low molecular weight phenolic acids which do not exist in the intact plant. These phenolic acids, depending on their structural arrangement, may be more readily absorbed than the corresponding intact aglycones or glucosides, potentially making them more available to other cells. In their discussion of the factors affecting polyphenol metabolism, Williamson and Clifford (2010) also noted that the human gut contains 10^{13} - 10^{14} individual microbes and that the composition of these microbes varies considerably depending on the composition of the diet, the age of the participants and the geographical locality. Serra et al. (2012) studied the metabolic pathways of flavonoids and phenolic acids. Individual compounds such as quercetin,

naringenin, kaempferol and myricetin showed wide variation in the number of colonic metabolites which they produce, with quercetin producing the most diverse range of metabolites.

1.6. Carbohydrate (CHO) metabolism in humans

CHO is, at its most simple, a source of substrate for metabolism. CHO is arranged as saccarides, chains of glucose molecules which vary in length and arrangement to create everything from simple disaccharides such as fructose and sucrose to complex polysaccharides such as β-glucans and starch. The breakdown of these chains and the subsequent absorption and metabolism of individual molecules of glucose is fundamental to understanding and manipulating the production and storage of energy. CHO uptake is largely regulated by the peptide hormone, insulin. Inefficient or malfunctioning processes which are implicated in the digestion or absorption of CHO result in an increased risk of morbidity and mortality, particular from complications such as type 2 diabetes, obesity and the metabolic syndrome. Alteration in postprandial CHO metabolism caused by other dietary factors, such as phytochemicals, may therefore have profound implications for the prevention and/or treatment of these diseases. Complex CHOs like starch are composed of the glucose polymers amylose and amylopectin. These polymers are made from glucose ($C_6 H_{12} O_6$) chains linked and branched at the 1^{st} and 4^{th} carbon atoms or at the 1^{st} and 6^{th} carbon atoms known as α-1,4-linked and α-1,6-linked chains. A shortened version of the full process first involves salivary enzymes such as α-amylase which hydrolyse some of the α-1,4 linkages and release shorter glucose chains such as oligomers. Further enzyme catalysed digestion takes place when these chains reach the intestine, where the primary enzyme is α-glucosidase. At the end of this process, simple glucose molecules are available for

absorption, whilst any compounds which cannot be broken down (insoluble fibre) pass onwards to add bulk to the faeces.

In the intestine, absorption of glucose occurs via an active transport mechanism. The SGLT-1 utilises a concentration gradient to move both sodium and glucose molecules from the lumen into enterocytes in the intestinal endothelium. Glucose is allowed to bind and be transported by conformational changes in SGLT-1 initiated by the prior binding of two Na^+ ions. The basolateral membrane which is the border between the intestinal enterocytes and the circulation then has its own, high capacity transporter (GLUT 2) to complete the absorption process. GLUT 2 also regulates transport into, and out of, the liver. The process of glucose uptake into adipocytes, skeletal muscle cells, liver storage or neuronal cells is mediated by insulin. A plethora of signalling pathways communicate with glucose transporters and sensors known usefully as GLUT 1 (erythrocytes), GLUT 3 (neuronal), and GLUT 4. The most widely studied pathway in humans is the GLUT 4 pathway which regulates the uptake of glucose into skeletal muscle. At the surface of the cell, specific receptors allow insulin to bind. This binding triggers the phosphorylation of the intracellular domain of the receptor. A signalling cascade then ensues which involves the further phosphorylation of insulin receptor substrates and downstream kinases including PI3-K. Inside the cell, and located at the end of this cascade is an intracellular pool of GLUT 4 vesicles. In response to the signalling cascade begun by the binding of insulin to the membrane receptor, GLUT 4 translocates to the plasma membrane, and another conformational change allows glucose to enter the cell. Various insulin signalling pathways have been characterised and a comprehensive review was compiled by Saltiel and Khan (2001).

1.6.1. Phytochemical compounds in the regulation of glycaemia

Disturbances in the postprandial response to glucose and insulin sensitivity are now known to be critical for good health. In the case of type 2 diabetes mellitus, a number of compounds which may limit oxidative stress have also been suggested to either reduce postprandial glycaemia or to affect the postprandial insulin response, although the evidence is somewhat inconsistent (de Bock et al., 2012). Polyphenols including flavonoids, phenolic acids, proanthocyanidins and tannins have been suggested to be able to modify glucose metabolism. Polyphenols may alter glucose metabolism by inhibiting CHO digestion, reducing CHO absorption in the intestines, stimulation of insulin release from the pancreatic β cells, modulation of hepatic glucose output, activation of insulin receptors, or modulation of glucose uptake in insulin sensitive cells (Hanhineva et al., 2010). Isoflavonoids (soy), condensed tannins such as EGCG from tea, phenolic acids (coffee), resveratrol (grapes), apple flavonoids, terpenoids (herbs), as well as cranberry, strawberry, and blueberry anthocyanin metabolites are commonly studied compounds.

Digestive enzymes such as α-amylase and α-glucosidase are the first potential targets for polyphenol action (Matsui et al., 2002; Matsui et al., 2007), and the second is intestinal absorption of glucose by SGLT-1 and by sodium-independent facilitated transport via the GLUT 2 glucose transporter. Both SGLT-1 and GLUT 2 transporters have been inhibited by polyphenols in experimental conditions. Inhibition is proposed to occur via one of four mechanisms. Either by competitive binding, a non-competitive mechanism whereby polyphenols are transported by SGLT-1 instead of glucose and are then available to inhibit GLUT 2 (Ader et al., 2001), by causing a reduction in transport through steric hindrance (Johnston et al., 2005) or by preventing the SGLT-1 transporter, the GLUT 2 transporter or both from effectively transporting high amounts of glucose using an independent binding site. Flavonoids such as quercetin have been

shown to reduce the transport of glucose and fructose by the GLUT 2 transporter without being transported (Kwon et al., 2007). Kwon and colleagues (2007) injected xenopus oocytes expressing GLUT 2, GLUT 5 and SGLT-1 transporters with a selection of isolated flavonoids including quercetin, isoquercetrin, fisetin and myricetin alongside 250μM ^{14}C glucose and fructose. Transport of glucose and fructose was reduced by ~50% by 10μM quercetin and complete inhibition was achieved at a dose of 50μM. Furthermore the authors demonstrated that glucosides formed as a result of metabolism by the gut microflora were also able to inhibit GLUT 2 transport whilst the fully glycosylated form (rutin) was not. SGLT-1 and GLUT 5 transport was not inhibited, suggesting that flavonols are specific to GLUT 2.

However, inhibition of SGLT-1 by glycosides such as phloridzin is well documented historically (Wright et al., 1998). Johnston and colleagues (2005) showed that neohesperidin dihydrochalcone also caused a significant inhibition of glucose transport under sodium-dependent conditions in Caco-2 cells. Naringin, rutin and arbutin all resulted in non-significant inhibition. Johnston et al. (2005) continue, reporting inhibitory effects for catechin gallates from green tea in both sodium dependent and sodium-independent conditions suggesting that both SGLT-1 and GLUT 2 are potential targets either by conformational inhibition or by disruption of the surrounding lipid bilayer due to the incorporation of these compounds. The more likely target is perhaps GLUT 2 because it has a relatively high transport capacity and it can transport both glucose and fructose.

The preparation of the cells for glucose absorption relies on hormonal signalling, utilising insulin released by the pancreatic β cells, in response to CHO in a meal and the subsequent glucose content of the blood stream. The stages of the insulin signalling cascade are further potential targets for bioactive compounds which elicit a change in the postprandial glucose condition. In response to high blood glucose, the peptide

hormone insulin, released from the pancreatic β cells, binds to the insulin specific receptor on the surface of fat, muscle or brain cells. The insulin receptor itself is the first potential target in this series of events because its activity regulates the frequency and efficiency of the rest of the process. The binding of insulin causes the autophosphorylation of the intracellular domain of the receptor beginning a signal transduction cascade involving the phosphorylation by kinases (including (depending on the cell type) PI3-K, protein kinase B (pAkt), MAPK, glycogen synthase kinase (GSK) and others) of a number of different proteins resulting in the sequential amplification of the insulin signal relating to the extracellular glucose concentration. Beyond this, the insulin-mediated signalling cascade continues to regulate transcription factors and inflammatory mediators as well as a number of other functional aspects of cellular processes which account for some of the more long-term effects that polyphenols (such as those found in black tea) may have on metabolic events in the ageing human (Cameron et al., 2008).

Cinnamon has been reported to improve the postprandial glucose profile in human subjects (Khan et al., 2003). Polyphenols such as those found in cinnamon, and others found in tea, blueberry, grape seed and bitter melon are reported to be 'insulin-like' in nature, acting as insulin in the previously described signalling cascade. Evidence suggests that these compounds may increase the autophosphorylation rate of insulin receptors, and reduce the phosphorylation of tyrosine (Imparl-Radosevich et al., 1998) as well as regulating downstream kinase activity that leads to amplification of the glucose uptake signals (Jarvill-Taylor et al., 2001). Cao et al. (2007) showed that cinnamon extract potentiated the amount of proteins involved in insulin signalling and glucose uptake by increasing tristetraprolin mRNA levels in mouse adipocytes. A number of human studies have been designed to investigate the potential for compounds

in teas to affect either glycaemic or insulinaemic response following the consumption of a standard CHO bolus, and these have been recently reviewed by de Bock et al. (2012).

Johnston and colleagues (2002) pointed a tentative finger at phloridzin and other polyphenols to explain why they observed significant effects on the postprandial response to a 25g glucose load with cloudy apple juice. Apple juice resulted in lower concentrations of glucose, and impacted upon insulin, gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) in a manner suggesting delayed intestinal absorption in 9 healthy subjects. Törrönen et al. (2010) showed that a sucrose sweetened purée containing several polyphenol-rich berries significantly lowered postprandial glucose at 15 and 30 minutes ($P<0.05$) and delayed the postprandial glucose peak compared with a control sucrose load. The authors indicate that this was due to reduced digestion or absorption of the sucrose in the berry condition. They did not quantify the actual polyphenol content of the berries but postulated that the most likely mechanism was inhibition of α-glucosidase by anthocyanins, previously reported by others (McDougall et al., 2005b; Wilson et al., 2008). Further studies by Törrönen et al. (2012a, b, and c) have demonstrated similar effects with different combinations of berries added to a sucrose load.

1.6.2. Beetroot juice and glycaemia

Beetroot juice has received attention in the scientific literature recently, particularly due to its nitrate content (Webb et al., 2008). Nitrate from beetroot juice enjoys bioconversion to nitric oxide in the body which exerts a positive influence over oxygen use in prolonged aerobic exercise and in the treatment of hypertension. However, nitrate is only one component of this traditional root vegetable, and other inquiries have unmasked its macronutrient, micronutrient, and phytochemical composition. In terms of

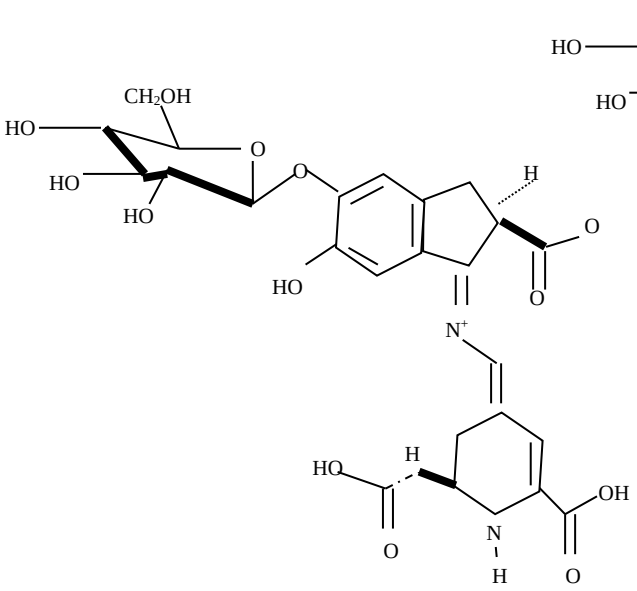
available CHO, previous reports indicate that beetroot is composed of approximately 80% sucrose, 12% galactose, 5% glucose and 4% fructose with small amounts (<0.1g/100g) of maltose and lactose, although galactose was not present in another report (unpublished results). Beetroot also contains a number of bioactive compounds including phenolic acids and betalains. Kujala and colleagues (2002) reported the phenolic and betalain content of four beetroot cultivars. Beetroot was shown to contain several function groups of compounds including phenolic acids, flavonoids, betalains and vitamins. Beetroot juice is therefore, an interesting food model to investigate any influence of its bioactive components on the glycaemic response either by direct inhibition of glucose uptake or by indirect action affecting insulin sensitivity.

In several of the studies mentioned above, beetroot was found to contain a particularly high amount of phytochemicals. Beetroot juice contains betalains; pigments which are classified as betacyanins and lend beetroot its deep red colouration; the structures of these are somewhat similar to anthocyanins and are shown in figure 1.2. The predominating betalain is betanin which is a glycoside composed of glucose and betanidin. Both betanin and betanidin have been independently shown to have strong antioxidant properties (Tesoriere et al., 2004b; Tesoriere et al., 2009). Beetroot juice may be a favourable product amongst the general public because it has a relatively sweet taste due to its high sugar content whilst maintaining a low glycaemic load (Brand-Miller et al., 2008). Products displaying these characteristics may have the potential to affect the postprandial glycaemic response and/or to alter the insulin response. Beetroot juice is also a rich source of dietary nitrate, which has a variety of functional interactions within the human body.

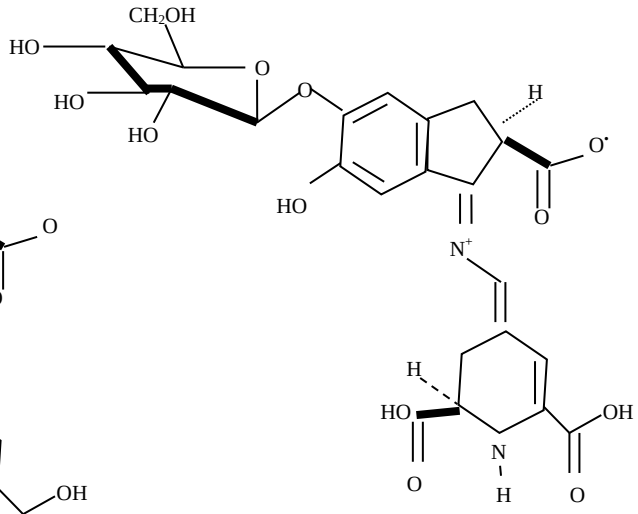
1.6.3. Effects of beetroot juice related to nitrate content and interactions with glycaemia

Beetroot and its juice are dietary sources of nitrate. Dietary nitrate is converted to nitrite on the tongue before being further reduced to NO in the stomach. NO is a crucial molecule in vascular biology; it is produced endogenously through the action of eNOS. NO produced in this way is utilised in vasodilatation, which in turn regulates blood pressure (BP) and reduces the incidence of atherosclerosis and stroke which are both caused by blockages associated with plaques. NO is also heavily implicated in the inhibition of inflammation and platelet aggregation associated with atherosclerosis (Webb et al., 2008). Recent investigations have sought to understand how NO produced via the reduction of dietary nitrate may provide NO for cardiovascular functions in a mechanism independent of eNOS. Moreover, endogenous NO production is mediated by insulin, implicating insulin resistance in potential NO deficiency. Insulin resistance and cardiovascular complications combine with obesity to form a triad of co-morbidities which comprise metabolic syndrome. Webb and colleagues (2008) investigated the vasoprotective effects of dietary nitrate by feeding volunteers 500 mL of red beetroot juice before assessing aspects of vascular function such as flow mediated dilatation (FMD), BP, and platelet aggregation. Reductions in both systolic and diastolic BP were also observed, with systolic pressure remaining lower over 24 hours after consumption. Beetroot juice also attenuated the drop in FMD associated with ischemic insult and prevented endothelial dysfunction. A significant reduction in BP has also been recently reported by Hobbs et al. (2012) using beetroot juice and a beetroot enriched bread (containing 100g beetroot). Consumption of the beetroot juice or beetroot enriched bread resulted in a significant reduction in both systolic and diastolic BP ($P< 0.05$) over a 24 hour period in normotensive subjects.

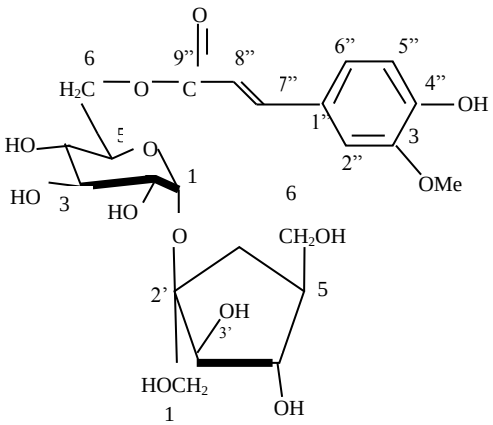
The vascular benefits of NO are predicated on the activation of signalling pathways which regulate the relaxation of smooth muscle cells in the vasculature. A cascade, beginning with the activation of soluble guanylyl cyclase and mediated by insulin, culminates in the eventual regulation of intracellular calcium, lowering of contractility and vasodilation via the activation of the PI3-K and MAPK signalling pathways (Doronzo et al., 2011). Several studies have reported that the production of NO is reduced in patients with diseases characterised by insulin resistance (Anfossi et al., 2009; Potenza et al., 2009). Doronzo et al. (2011) showed that insulin resistance results in increased oxidative stress which interferes with NO-mediated relaxation of smooth muscle cells in the vasculature, linking the effects of dietary nitrate to both CVD and diseases characterised by insulin resistance, such as type 2 diabetes and metabolic syndrome. When oxidative stress affects a process, there is always the potential for reducing compounds to re-dress the balance. Volk and colleagues (2009) showed that the production of NO from nitrate is potentiated by the addition of reducing compounds. Under simulated gastric conditions NO protected against lipid peroxidation. Volk et al. (2009) suggest that in order to maximise the protection of NO against lipid peroxidation, nitrate rich foods should be co-ingested together with foods rich in polyphenols. Beetroot juice is a rich source of both nitrate and polyphenols.



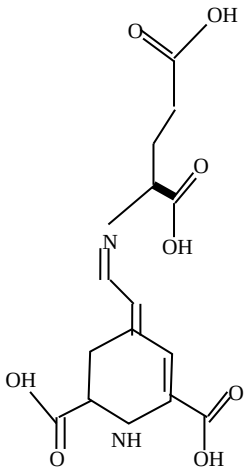
(a) Betanin



(b) Isobetanin



(c) β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside)



(d) Vulgaxanthin I

Figure 1.2 - Structures of Betanin, Isobetanin, β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) and Vulgaxanthin I

2. Study 1 - Determination of total antioxidant capacity and total polyphenol content in commercial vegetable juices

2.1. Justification

A large number of studies have focussed on the determination of phytochemical compounds in fruit juices and teas over the past decade. Some have also measured a small selection of vegetable juices. To date, no study has solely measured a broad range of commercially available vegetable juices in the UK. Furthermore, no study has used a wide range of methodologies to assess antioxidant capacity in vegetable juices. The focus of health policy in the UK has always been, and continues to be, that a diet rich in fruit and vegetables is healthful. An increasing number of vegetable juices and vegetable/fruit juice blends are being presented to the consumer in commercial outlets, and the quantification of their potential impact on health is required. The prevalence of phytochemical compounds in produce is thought to indicate their value in the diet, and as such the measurement of the antioxidant capacity of new vegetable juice products can be considered useful knowledge to be gained. No single method has been accepted in the literature for the determination of antioxidant capacity. It is therefore equally vital that a variety of methods are utilised and compared to obtain clarity.

The hypothesis to be tested is "vegetable juices represent a valuable source of phytochemicals, regardless of the reaction kinetics within any specific biochemical assay". The aim of this study was to analyse a variety of vegetable juices using a selection of common biochemical methods to discover which juice performs best across the assays and therefore contains the highest concentration of phytochemicals. All analysis was carried out by the author at Oxford Brookes University.

2.2. Materials and Methods

All chemicals were of analytical grade and were purchased from Sigma Aldrich (Poole, UK). Table 2.1 displays details of 23 vegetable juices which were purchased from local commercial outlets and included Big Tom spiced tomato juice (James White Drinks Ltd, Ipswich, UK), Sainsbury's tomato juice from concentrate, Sainsbury's Basic tomato juice from concentrate (Sainsbury's Supermarkets Ltd, London, UK), Sunraysia pure squeezed tomato juice (Sunraysia, London, UK), Princes tomato juice from concentrate (Princes Ltd, Liverpool, UK), Tesco tomato juice from concentrate, Tesco Value tomato juice from concentrate (Tesco, UK), Waitrose fresh pressed tomato juice, Waitrose long life tomato juice from concentrate (Waitrose, Bracknell, UK), ASDA tomato juice from concentrate (ASDA stores Ltd, Leeds, UK), Del Monte tomato juice from concentrate (Del Monte Europe Ltd, Staines, UK), Sunpride tomato juice (Gerber Juice Co. Ltd, Somerset, UK), The Co-operative tomato juice from concentrate with added salt (The Co-operative, Manchester, UK), Eden organic carrot juice (Granovita, UK), Sunraysia organic carrot juice (Sunraysia, London, UK), James White organic beetroot juice (James White Drinks Ltd, Ipswich, UK), Eden organic beetroot juice (Granovita, UK), James White organic vegetable juice (James White Drinks Ltd, Ipswich, UK), Eden organic vegetable cocktail (Granovita, UK), V8 100% vegetable juice original, V8 100% fruit and carrot juice tropical (Campbell Foods, Rijksweg, Belgium), Cawston Press apple and rhubarb juice, Cawston Press apple and beetroot juice (Cawston Press, Wokingham, UK). Both value and premium brands were selected which included fresh, concentrated and long life varieties. The prices of the juices ranged from 84p to £3.49/litre. Experiments were conducted on a single batch of juices whereby one carton of each juice was used; juices were refrigerated for no more than 1 week once open. In all experiments amber bottles were used throughout to prevent the photodecomposition of antioxidants and efforts were made to exclude oxygen contact

by working in a timely manner and covering the samples at all times. Samples were analysed on a minimum of three separate occasions and in triplicate for each experiment.

Table 2.1 - Time to expiry of the tested vegetable juices

Vegetable Juice	Time to expiry (unopened)
Big Tom Spiced Tomato Juice	20 Months
Sainsbury's Tomato Juice from Concentrate	11 Months
Sainsbury's Basic Tomato Juice from Concentrate	11 Months
Sunraysia Pure Squeezed Tomato Juice	20 Months
Princes Tomato Juice from Concentrate	6 Months
Tesco Tomato Juice from Concentrate	6 Months
Tesco Value Tomato Juice from Concentrate	5 Months
Waitrose Fresh Pressed Tomato Juice	1 Month
Waitrose Longlife Pressed Tomato Juice	20 Months
ASDA Tomato Juice from Concentrate	5 Months
Del Monte Tomato Juice from Concentrate	8 Months
Sunpride Tomato Juice	10 Months
The Co-operative Tomato Juice from Concentrate	5 Months
Eden Organic Carrot Juice	19 Months
Sunraysia Organic Carrot Juice	10 Months
Eden Organic Beetroot Juice	21 Months
'Beet It' - James White Organic Beetroot Juice	20 Months
James White Organic Vegetable Juice	19 Months
V8 100% Vegetable Juice (Original)	10 Months
Eden Organic Vegetable Cocktail	5 Months
V8 100% Fruit and Carrot Juice (Tropical)	13 Months
Cawston Press Apple and Rhubarb Juice	14 Months
Cawston Press Apple and Beetroot Juice	13 Months

2.2.1. Ferric-Ion Reducing Antioxidant Power

The TAC of the samples was determined using a modification of the FRAP assay (Benzie and Strain, 1996a). The FRAP assay was performed by adding 25 µL of sample and 1 mL of freshly prepared FRAP reagent to clean test tubes containing 1 mL distilled water (dH_2O). In order to produce the working FRAP reagent, 0.156g tripyridyltriazine (TPTZ) was dissolved in 50 mL of 40 mM HCL to a concentration of 10 mM TPTZ. This was then combined with 20 mM ferric chloride solution (0.270g ferric chloride per 50 mL dH_2O) and 300 mM sodium acetate buffer, containing 16 mL glacial acetic acid (pH 3.6) at a ratio of 1:1:10 v/v/v, in order to form the ferric-TPTZ (Fe^{III}-TPTZ)

complex. The resulting dark blue solution was then incubated together with the sample at 37°C for 4 min. The reduction of the Fe^{III} complex to Fe^{II} is proportional to the absorbance at 593 nm using a UV-vis spectrophotometer. The absorbance of each sample was then determined relative to a reagent blank and compared with a 0-1 mM Trolox standard curve. Results were then expressed in TE.

2.2.2. DPPH$^{\bullet}$ Radical Scavenging Capacity

The DPPH$^{\bullet}$ method was conducted using a modification of the method of Brand-Williams et al. (1995). DPPH$^{\bullet}$ has an intense violet colour with a maximum absorbance at 517 nm, but turns colourless as unpaired electrons are scavenged by antioxidants. Reaction mixtures containing 0.1 mL of sample and 3.9 mL of 50 µM DPPH$^{\bullet}$ (0.0039g prepared in 200 mL methanol) were incubated in a water bath at 37°C for 30 minutes. After incubation, an aliquot of the sample from each test tube was removed and placed into a cuvette to prevent occlusion of the light pathway by sediment in the juice, and the absorbance was measured at 517 nm. The percentage inhibition was calculated against a control and compared with a Trolox standard curve (0 – 1 mM). Results were then expressed in TE.

2.2.3. ABTS$^{\bullet+}$ Radical Cation Scavenging Capacity

The ABTS$^{\bullet+}$ scavenging capacity was assessed using a modified methodology previously reported by Ozgen et al. (2006). When combined with 2.45 mM potassium persulfate (0.0166g/25 mL dH_2O), 7 mM ABTS (0.192g/50 mL 20 mM sodium acetate buffer, pH 4.5) reacts to create a stable, dark blue-green radical solution following 12-16 hours of incubation in the dark (4°C). The solution was then diluted to an absorbance of 0.7 ± 0.01 at 734 nm to form the test reagent. Reaction mixtures containing 20 µL of sample and 3 mL of reagent were incubated in a water bath at 30°C for 30 minutes. As unpaired electrons are sequestered by antioxidants in the sample the test solution turns

colourless and the absorbance at 734 nm is reduced. An aliquot from each test tube was removed and placed into a cuvette to prevent occlusion of the light pathway by sediment in the juice before the absorbance of each sample was measured. The percentage inhibition was compared with a Trolox standard curve 1 - 5 mM. Results were then expressed in TE.

2.2.4. Copper (II) Reducing Capacity (CUPRAC)

A modification of the CUPRAC assay of Apak et al. (2004) was also used to determine the TAC of the samples. The CUPRAC method comprises mixing of the antioxidant solution (directly or after acid hydrolysis) with a Cu^{II} solution (0.170g/100 mL dH_2O), a neocuproine alcoholic solution (0.156g/100 mL ethanol), and an ammonium acetate aqueous buffer (7.708g/ 100 mL dH_2O) at pH 7 and subsequent measurement of the developed absorbance at 450 nm after 30 - 60 minutes. 1 mL of 0.1 M Cu^{II} prepared in dH_2O, 1 mL ammonium acetate (pH 7) and 7.5 mM neocuproine, prepared in ethanol were added to clean test tubes containing 1 mL of dH_2O and 10 µL of sample to create a final volume of 4.1 mL. Samples were incubated in the dark for 1 hr at room temperature and absorbance at 450 nm was quantified spectrophotometrically and compared with a Trolox standard curve (0 – 1 mM). Results were then expressed in TE.

2.2.5. Cerium (IV) Reducing Capacity (CERAC)

TAC of the tested samples was also determined using a modification of the Cerium (IV) assay of Ozyurt et al. (2010). A 2 mM solution of Ce^{IV} (0.081g/ 100 mL) was prepared in dH_2O and transferred to a volumetric flask where 17 mL of concentrated sulphuric acid was added to the mixture to prevent ceric ion hydrolysis; a total volume of 100 mL was then completed using dH_2O. A second solution of 1 M sodium sulphate (71g/500 mL dH_2O) was prepared before they were combined in a ratio of 1:7 with 25 µL of sample. Samples were incubated at 20 ± 2°C for 30 minutes and absorbance at

320 nm was determined spectrophotometrically and compared with a Trolox standard curve 1 - 20 mM. Results were then expressed in TE.

2.2.6. Folin Ciocalteu Method for Total Polyphenols

Commercially prepared Folin Ciocalteu reagent was diluted 1:10 in dH_2O immediately prior to analysis. The vegetable juice aliquot (0.2 mL) was added to 1.5 mL of freshly prepared Folin Ciocalteu reagent and allowed to equilibrate for 5 minutes. 1.5 mL of sodium carbonate (60g/L) solution was added to the equilibrated Folin mixture. The samples were then incubated in the dark at room temperature for 90 minutes. The absorbance of the mixture was read at 725 nm using Folin Ciocalteu reagent and sodium carbonate alone as a blank and compared with a ferulic acid standard curve 0 - 0.1 M. The results were expressed as μg ferulic acid equivalents (FAE) per mL of sample.

2.2.7. Statistical Analyses

All data are presented as means (± SEM) of at least three independent experiments (n = 3); each experiment had a minimum of three replicates of each sample. For comparisons between samples, data were analysed by Pearson's product moment correlation after homogeneity of variance and normality were confirmed, using the results from each method against each other method in turn (SPSS, version 17). A probability of 1% or less was accepted as statistically significant. Data were not transformed.

2.3. Results

2.3.1. Total Antioxidant Capacity and Total Polyphenol Content

All of the juices were a significant source of antioxidants and in particular, polyphenols. Table 2.2 shows that Beetroot juice had the highest TAC and TP of the vegetable juices in all of the assays FRAP (1.01 - 1.17 mM TE), $DPPH^{\bullet}$ (1.16 mM TE), $ABTS^{\bullet+}$ (3.89 – 3.90 mM TE), CUPRAC (1.81 – 2.13 mM TE), CERAC (13.81 – 15.14 mM TE) and FC (2126.4 – 3024.8 µg FAE/ mL) followed by mixed fruit and vegetable, mixed vegetable, tomato and carrot juices.

2.3.2. Methodological Comparison

2.3.2.1. FRAP

All of the juices were a significant source of antioxidants. FRAP results for the juices were compared with a Trolox standard curve (0 – 1 mM) which displayed a positive dose response relationship ($r^2 = 0.95$). Trolox showed a high TAC in the FRAP assay (7588 µmol/L at 1 mM concentration). Beetroot juice showed the highest TAC of the vegetable juices measured by FRAP (1.01 - 1.17 mM TE) followed by mixed fruit and vegetable juices (0.13 – 0.74 mM TE), mixed vegetable (0.19 – 0.21 mM TE), tomato (0.10 – 0.28 mM TE) and carrot (0.03 – 0.06 mM TE) (table 2.2). The results for FRAP correlated significantly with those for $ABTS^{+\bullet}$ ($r = 0.87$, $P<0.01$), CUPRAC ($r = 0.95$, $P<0.01$), CERAC ($r = 0.79$, $P<0.01$) and FC ($r = 0.96$, $P<0.01$) but not with $DPPH^{\bullet}$ ($r = 0.51$, $P = 0.12$).

2.3.2.2. $DPPH^{\bullet}$

All of the juices were efficient scavengers of the pre-formed $DPPH^{\bullet}$ radical. Additionally Trolox proved to be a particularly efficient scavenger of the $DPPH^{\bullet}$

radical, reaching the reaction endpoint whereby the entire spectrophotometrically detectable radical had been scavenged at a concentration of 1 mM. The Trolox standard curve displayed a positive dose response relationship (r = 0.99). Beetroot juice displayed the highest TAC, fully inhibiting the $DPPH^{\bullet}$ radical on each occasion (1.16 mM TE). Beetroot juice was followed by tomato (0.78 – 1.01 mM TE), mixed fruit and vegetable (0.70 - 1.01 mM TE), mixed vegetable (0.94 – 0.95 mM TE) and carrot juice (0.62 – 0.93 mM TE) (table 2.2). There was no significant correlation between the results obtained for $DPPH^{\bullet}$ and FRAP (r = 0.51, *P* = 0.12); there were also no significant correlations observed between $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ (r = 0.41, *P* = 0.05), CUPRAC (r = 0.45, *P* = 0.03) or FC (r = 0.48, *P* = 0.02). There was a significant correlation between the results for $DPPH^{\bullet}$ and those obtained for the CERAC method (r = 0.66, *P* = 0.01).

2.3.2.3. $ABTS^{\bullet+}$

Samples were analysed for TAC using the $ABTS^{\bullet+}$ /potassium persulfate radical scavenging method. Results were compared with a Trolox standard curve 0.1 – 4 mM, which displayed a positive dose response relationship (r = 0.99). Trolox was a relatively efficient scavenger of the $ABTS^{\bullet+}$ radical but required a 4 mM concentration to take the reaction to its spectrophotometric endpoint. Beetroot juice displayed the highest $ABTS^{\bullet+}$ radical scavenging TAC (3.89 – 3.90 mM TE), followed by the mixed fruit and vegetable (1.66 – 3.84 mM TE), tomato (0.89 – 2.96 mM), mixed vegetable (1.07 - 2.35 mM TE), and carrot juice (0.47 – 0.98 mM TE) (table 2.2). The results obtained by the $ABTS^{\bullet+}$ method were significantly correlated with those obtained by FRAP (r = 0.87, *P*<0.01). There were also significant correlations between $ABTS^{\bullet+}$ and CUPRAC (r = 0.85, *P*<0.01), CERAC (r = 0.74, *P*<0.01) and FC (r = 0.89, *P*<0.01).

2.3.2.4. CUPRAC

The juices required dilution at a 1:5 ratio with dH_2O for analysis in the CUPRAC assay since pure beetroot juice caused a reduction which measured beyond the maximum absorbance reading of the UV spectrophotometer (4.0+). As a result the reducing capacity of the juices was theoretically extrapolated to whole juice samples by multiplying the results by 5 to give the final TE. Results were compared with a Trolox standard curve 0 – 1 mM, which displayed a positive dose response relationship (r = 0.99). Trolox effectively reduced the copper (II) complex to copper (I) yielding an absorbance of 3.66 at 450 nm for a concentration of 1 mM. Beetroot juice displayed the highest TAC as measured by CUPRAC (1.81 – 2.13 mM TE), followed by the mixed fruit and vegetable (0.38 – 1.30 mM TE), tomato (0.25 – 0.65 mM TE), mixed vegetable (0.44 – 0.59 mM TE) and carrot juices (0.40 – 0.45 mM TE) (table 2.2). Significant correlations were observed between the data for CUPRAC and both FRAP and $ABTS^{\bullet+}$, whereas there was not a significant correlation between CUPRAC and $DPPH^{\bullet}$ (3.2). Additionally significant correlations were observed between CUPRAC and CERAC (r = 0.82, $P<0.01$) and between CUPRAC and FC (r = 0.96, $P<0.01$).

2.3.2.5. CERAC

All of the juices proved effective at reducing the cerium (IV) complex. The results were compared with a Trolox standard curve (0 – 20 mM), which displayed a positive dose response relationship (r = 0.98). Trolox proved to be particularly inefficient at reducing the cerium (IV) complex, requiring a Trolox concentration of 20 mM to reach the spectrophotometric endpoint of the reaction. Beetroot juice displayed the highest TAC (13.81 – 15.14 mM TE) followed by the mixed fruit and vegetable (6.21 – 10.62 mM TE), tomato (4.97 – 10.50 mM TE), mixed vegetable (7.76 – 8.26 mM TE) and carrot

juices (5.61 – 7.62 mM TE) (table 2.2). The CERAC results were significantly correlated with those obtained by FRAP, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and CUPRAC. Additionally a significant correlation was observed between the results obtained using the CERAC method and those obtained using FC ($r = 0.80$, $P<0.01$).

2.3.2.6. Folin Ciocalteu Method for Total Polyphenols

All of the juices were a significant source of polyphenols. Results were compared to a ferulic acid standard curve (0 – 1 mM) and expressed as micrograms of ferulic acid equivalents per millilitre of sample (µg FAE/ mL). There was a positive dose response relationship ($r = 0.99$). Beetroot juice had the highest amount of polyphenols (2126.4 – 3024.8 µg FAE/ mL) followed by the mixed fruit and vegetable (813.5 – 1985.8 µg FAE/ mL), tomato (590.9 – 1140 µg FAE/ mL), mixed vegetable (627.1 – 840.1 µg FAE/ mL) and carrot juices (484.8 – 604.6 µg FAE/ mL) (table 2.2). There were significant correlations observed between the results for FC and those obtained by FRAP, $ABTS^{\bullet+}$, CUPRAC and CERAC. There was not a significant correlation observed between results obtained by FC and those obtained by the $DPPH^{\bullet}$ method.

Table 2.2 - Comparison of FRAP, DPPH$^{\bullet}$, ABTS$^{\bullet+}$, CUPRAC, CERAC methods for total antioxidant capacity (mM TE) and total polyphenols using FC method (µg FAE/ mL) [a]

Vegetable Juices	**mM TE**					**µg FAE/ mL**
	FRAP	**DPPH**	**ABTS**	**CUPRAC**	**CERAC**	**TP**
Big Tom Spiced Tomato Juice	0.16 ± 0.01	0.92 ± 0.01	1.36 ± 0.02	0.41 ± 0.02	8.02 ± 0.04	820.85 ± 40.8
Sainsbury's Tomato Juice Concentrate	0.12 ± 0.00	0.90 ± 0.01	1.11 ± 0.04	0.44 ± 0.04	7.06 ± 0.04	923.60 ± 66.8
Sainsbury's Basic Tomato Juice	0.19 ± 0.00	1.01 ± 0.00	1.35 ± 0.05	0.46 ± 0.05	10.5 ± 0.21	991.98 ± 139.1
Sunraysia Pure Squeezed Tomato Juice	0.10 ± 0.00	0.78 ± 0.01	0.89 ± 0.04	0.35 ± 0.04	7.49 ± 0.08	624.89 ± 48.1
Princes Tomato Juice from Concentrate	0.23 ± 0.02	0.94 ± 0.01	1.30 ± 0.01	0.42 ± 0.01	8.54 ± 0.07	851.98 ± 33.9
Tesco Tomato Juice from Concentrate	0.25 ± 0.03	0.95 ± 0.01	1.61 ± 0.01	0.45 ± 0.01	7.36 ± 0.16	910.07 ± 64.7
Tesco Value Tomato Juice	0.28 ± 0.01	0.99 ± 0.01	1.07 ± 0.01	0.41 ± 0.01	4.97 ± 0.12	590.92 ± 30.8
Waitrose Fresh Pressed Tomato Juice	0.18 ± 0.01	0.96 ± 0.00	1.26 ± 0.03	0.25 ± 0.03	7.61 ± 0.03	704.33 ± 25.1
Waitrose Longlife Pressed Tomato Juice	0.22 ± 0.01	0.93 ± 0.00	1.61 ± 0.01	0.35 ± 0.01	8.74 ± 0.08	871.89 ± 16.9
ASDA Tomato Juice from Concentrate	0.19 ± 0.01	0.96 ± 0.01	2.96 ± 0.02	0.65 ± 0.02	9.76 ± 0.19	1140.00 ± 4.1
Del Monte Tomato Juice	0.15 ± 0.01	0.92 ± 0.00	1.26 ± 0.04	0.49 ± 0.04	8.16 ± 0.26	955.89 ± 37.8
Sunpride Tomato Juice	0.19 ± 0.00	0.88 ± 0.00	1.32 ± 0.02	0.40 ± 0.02	9.27 ± 0.15	668.09 ± 48.6
The Co-operative Tomato Juice	0.28 ± 0.00	1.01 ± 0.00	1.25 ± 0.01	0.51 ± 0.01	9.48 ± 0.06	933.83 ± 33.9
Eden Organic Carrot Juice	0.06 ± 0.00	0.93 ± 0.00	0.98 ± 0.01	0.45 ± 0.01	7.62 ± 0.04	604.63 ± 17.1
Sunraysia Organic Carrot Juice	0.03 ± 0.00	0.62 ± 0.04	0.47 ± 0.01	0.40 ± 0.01	5.61 ± 0.02	484.85 ± 39.9
Eden Organic Beetroot Juice	1.17 ± 0.00	1.16 ± 0.00	3.90 ± 0.01	2.13 ± 0.01	13.81 ± 0.02	3024.79 ± 82.1
James White Organic Beetroot Juice	1.01 ± 0.01	1.16 ± 0.00	3.89 ± 0.00	1.81 ± 0.00	15.14 ± 0.13	2126.28 ± 113.9
James White Organic Vegetable Juice	0.21 ± 0.00	0.95 ± 0.00	2.35 ± 0.04	0.59 ± 0.04	8.26 ± 0.19	840.08 ± 16.3
V8 100% Vegetable Juice (Original)	0.20 ± 0.01	0.95 ± 0.00	1.07 ± 0.00	0.44 ± 0.00	7.76 ± 0.12	826.11 ± 37.2
Eden Organic Vegetable Cocktail	0.19 ± 0.01	0.94 ± 0.00	1.11 ± 0.01	0.46 ± 0.01	7.15 ± 0.21	627.06 ± 17.0
V8 100% Fruit and Carrot Juice	0.13 ± 0.01	0.92 ± 0.04	1.66 ± 0.04	0.38 ± 0.04	6.21 ± 0.01	813.54 ± 21.0
Cawston Press Apple and Rhubarb Juice	0.68 ± 0.01	1.02 ± 0.00	3.84 ± 0.01	0.87 ± 0.01	10.62 ± 0.05	1782.43 ± 95.2
Cawston Press Apple and Beetroot Juice	0.74 ± 0.01	0.70 ± 0.01	3.81 ± 0.01	1.30 ± 0.01	8.55 ± 0.04	1985.85 ± 23.1

[a] Results are displayed as mean ± SEM for three separate experiments, each performed in triplicate. ABTS$^{\bullet+}$ = 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid scavenging capacity, CERAC = Cerium IV reducing antioxidant capacity, CUPRAC = Copper II reducing antioxidant capacity, DPPH$^{\bullet}$ = 2, 2-diphenyl-1-picryhydrazyl scavenging capacity, FAE = Ferulic Acid Equivalents, FRAP = Ferric Reducing Antioxidant Power, TE = Trolox Equivalents, TP = Total polyphenols.

2.4. Discussion

Twenty three commercially available vegetable juices were analysed for TAC using 5 spectrophotometric quantification methods (FRAP, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, CUPRAC, and CERAC) as well as for TP using the FC method. The beetroot juices ranked either 1 or 2 respectively in every assay. Beetroot is rich in a variety of different phytochemicals including polyphenolic compounds, carotenoids and ascorbate. In particular it contains a significant concentration of betalains, a class of betalamic acid derivatives. Betanin and its aglycone betanidin have shown strong antioxidant activity *in vitro* (Butera et al., 2002) and *ex vivo* (Tesoriere et al., 2004a), and are relatively stable throughout the digestion process (Pavlov et al., 2005). Georgiev et al. (2010) measured the antioxidant activity and phenolic content of betalain extracts from red beetroot. The particularly high antioxidant capacity observed in this study was attributed to potential synergistic effects of betalains and polyphenolic compounds such as caffeic acid and epicatechin. Furthermore, Tesoriere et al. (2004b) showed that cactus fruit pulp containing 16 mg betanin and 28 mg indicaxanthin significantly inhibited oxidation of LDL and inhibited the $ABTS^{\bullet+}$ radical.

The 3 mixed fruit and vegetable juices ranked just below the beetroot juices in almost all of the assays followed by either tomato juices or mixed vegetable juices which interchangeably ranked from 5 – 22. The James White vegetable juice cocktail contained 38% tomato, 25% carrot, 5% cucumber, 4% beetroot as well as celeriac, apple, and lemon juices in smaller quantities. It was flavoured with lovage, dill, parsely and basil. The V8 vegetable juice contained 87% tomato juice and smaller quantities of beetroot, celery, carrot, lettuce, parsely, watercress and spinach. Juices which contain a combination of antioxidant classes (beetroot, mixed fruit and vegetable and mixed vegetable) were more efficient across all of the assays. Carrot juices routinely displayed the lowest TAC.

Lichtenthaler and Marx (2005) reported the antioxidant potential of beetroot, carrot and tomato juices assessed using the TOSC which is based on the yield of ethylene from reactions involving peroxyl and hydroxyl radicals and peroxynitrite. Beetroot juice was reported to be 8-10 times more effective than carrot or tomato, which is in accordance with the results presented here. Lugasi and Hovari (2003) measured mixed vegetable juices, beetroot juice, and carrot juice using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FC. Lugasi and Hovari (2003), report that the reducing power of beetroot was 3 times higher than mixed vegetable juice and 6 times higher than carrot juice, which is supported in the current study. TP content is reported to be relatively low for beetroot (529 mg/L) whereas the mixed vegetable juice (tomato, beetroot, onion, sauerkraut, celery, cucumber, sweet pepper, cumin, dill, parsely and wheat bran) had higher polyphenol content (696 mg/L), potentially due to the inclusion of cumin and sweet pepper. Hydrogen donating capacity ($DPPH^{\bullet}$) was considered very low for beetroot whereas carrot and mixed vegetable were considered to be some 30 times higher. This finding is not supported in the current study. It is possible that the pigmentation of the beetroot juice in its pure form affected the spectrophotometric measurement of the sample in the $DPPH^{\bullet}$ assay since $DPPH^{\bullet}$ also has a deep purple colouration. In the current study, the beetroot juice was diluted to prevent this issue.

Results varied considerably between assays. Using the example of James White beetroot juice, results varied from 1.01 mM TE (FRAP) to 15.14 mM TE (CERAC); the differences were proportionally similar between the other juices (table 2.2). The most significant contributor to this variation is the use of Trolox as the standard compound, an analogue of α-tocopherol. It is an effective scavenger of the $DPPH^{\bullet}$ radical since a 1 mM concentration is sufficient to reach the reaction endpoint, but it is far less effective at reducing the Cerium (IV) complex in the CERAC assay since a concentration of 20 mM was required to reach the reaction endpoint. It may be necessary to utilise more

than one standard compound during future assessments of TAC, such that the most appropriate standard is used rather than the most common. In addition a number of the juices used in this study were organic. Świderski et al. (2009) have suggested that the antioxidant capacity of organic vegetable juices is lower than that of conventional juices. This cannot be supported conclusively in this study although organic juices did tend to display slightly lower values in general.

In the current study there was broad agreement in terms of the direction and proportion of TAC in 5 of the methods. Significant positive, linear correlations were observed between FRAP, $ABTS^{\bullet+}$, CUPRAC, CERAC and the FC method ($0.74 \leq r \leq 0.96$, $P < 0.01$) (table 2.3). Previous research has found that there are positive correlations between FRAP and TP (Benzie and Szeto, 1999), FRAP and $ABTS^{\bullet+}$ (Ozgen et al., 2006), as well as $ABTS^{\bullet+}$ and FC (Sreeramulu and Raghunath, 2010). The current study reveals positive correlations between the more recently developed methods such as CUPRAC and CERAC and more traditional methods such as FRAP and $ABTS^{\bullet+}$, suggesting that these methods can be selected for the evaluation of food products.

Modest relationships were found between the majority of these methods and $DPPH^{\bullet}$ ($0.41 \leq r \leq 0.51$, $0.05 > P > 0.01$) with the exception of CERAC which showed a significant correlation with $DPPH^{\bullet}$ ($r = 0.66$, $P < 0.01$). There are some fundamental differences in the reaction kinetics which may account for the observed differences between the $DPPH^{\bullet}$ method and the other methods of analysis. Each of the other 5 assays essentially follows the same ET mechanism; $DPPH^{\bullet}$ on the other hand, utilises the nitrogen radical rather than the more transient peroxyl radical, which predominates in biological systems, a methodological limitation discussed by Foti and colleagues (2004).

Table 2.3 - Pearson's product moment correlation coefficients (r = 0 - 1) for each assay compared with each of the other assays.

Method	FRAP	DPPH	ABTS	CUPRAC	CERAC	TP
FRAP	X	0.51*	0.87**	0.96**	0.79**	0.96**
DPPH	0.51*	X	0.41*	0.45*	0.66**	0.48*
ABTS	0.87**	0.41*	X	0.85**	0.74**	0.89**
CUPRAC	0.96**	0.45*	0.85**	X	0.82**	0.95**
CERAC	0.79**	0.66**	0.74**	0.82**	X	0.80**
TP	0.96**	0.48*	0.89**	0.95**	0.80**	X

*P<0.05, **P<0.01.

The CUPRAC assay purports to provide more accurate quantification of TAC than the FRAP assay due to greater selectivity and faster reaction kinetics (Apak et al., 2004). At least, in terms of the correlation between the data, this does not appear to impact upon the assay selection. It is not possible to say which of the data sets (either CUPRAC or FRAP) is potentially the more accurate; however, assuming that the claims of Apak et al. (2004) are accurate there is no evidence to disprove them in this study. The same research group have also developed the CERAC assay (Ozyurt et al., 2010), designed to have selectivity over simple reducing sugars and citric acid (preservative) which are not classified as true antioxidants and may account for approximately 25% of the TAC value of products (Tezcan et al., 2009). The strong correlations observed between the CERAC assay and the other ET assays suggests that either there were small amounts of citric acid used or that it does not contribute significantly to the TAC of these juices. Citric acid could therefore be considered a worthy addition to these juices since it has been shown to prevent the formation of hydrogen peroxide when the juices are opened (Aoshima and Ayabe, 2007).

2.5. Conclusion

This study has revealed that all of the assessed juices, particularly beetroot juice, are valuable sources of phytochemicals as measured by all of the assays. A wide variation in the reactivity of Trolox with each of the reactants in the studied assays caused a wide variation in the reported TE values of the juices. Correlation analysis revealed that there is chronological agreement between all SET methods excluding only $DPPH^{\bullet}$. This suggests that combined rank order can be effectively used for TAC in products which contain a variety of different antioxidant compounds and when measuring diet quality. These biochemical methods remain a useful method for screening produce and identifying those with a high TAC, which is often the first step in the assessment of potential phytochemical effects in humans.

Related publication: Wootton-Beard, P.C., & Ryan, L. (2012). The combined use of multiple methodologies for the measurement of total antioxidant capacity in UK commercially available vegetable juices. *Plant Foods for Human Nutrition*, 67, 142-147.

3. Study 2 - Determination of Total Antioxidant Capacity and Total Polyphenols following *in vitro* digestion in commercial vegetable juices

3.1. Justification

Following the determination of antioxidant capacity using multiple methodologies in the previous study, it is also vital to consider the potential for phytochemicals contained in vegetable juice to reach the intestinal lumen intact. Simulated digestion models offer the opportunity to measure the bioaccessibility of phytochemicals from various food matrices. Although bioaccessibility does not infer bioavailability, as previously discussed, it provides a useful proxy measure of the potential for phytochemicals to be absorbed and provides further resolution to the information on their content. Bioavailability is a near impossible concept to measure accurately due to the vast differences in individual digestion processes. The abundance and diversity of interactive substances such as the gut microflora in the lower intestine and the lack of any improved resolution that simulated bioavailability assays can add to antioxidant capacity measurement.

The hypothesis to be tested was "the bioaccessibility of vegetable juice phytochemicals increases following *in vitro* digestion". The aim of this study was to ascertain which of the tested products provided the most realistic opportunity to increase phytochemical consumption in the general public by examining the likely impact of digestion on the reducing capacity of the juices by employing multiple methodologies.

3.2. Materials and Methods

All chemicals were of analytical grade and were purchased from Sigma Aldrich (Poole, UK). Vegetable juices were purchased from local commercial outlets (Tesco, Oxford, UK; Sainsbury's, Oxford, UK; ASDA, Oxford, UK; Londis, Oxford, UK; Waitrose, Oxford, UK; Holland and Barrett, Oxford, UK; The Co-operative, Oxford, UK). All analysis was carried out by the author at Oxford Brookes University. The previous study showed that there was no measureable advantage to be gained from utilising the more recently developed CUPRAC and CERAC assays over the more established methodologies. This study therefore utilised FRAP, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods since these are the most commonly utilised methods in the literature and allow for the best comparison between this and other similar studies whilst still representing differing reaction kinetics.

3.2.1. Sample Preparation

Twenty three vegetable juices were selected on the basis of commercial availability. Both value and premium brands were selected which included fresh, concentrated and long life varieties. The prices of the juices ranged from 84p to £3.49/litre. The juices selected were as detailed in section 2.2 and were prepared in the same way.

3.2.2. *In vitro* digestion procedure

The *in vitro* digestion model was adapted from Ryan et al. (2008). Vegetable juice samples were transferred to clean amber bottles and mixed with Hanks' balanced salt solution (with $NaCO_3$, without phenol red, sterile filtered, Sigma Aldrich, Poole, UK) to create a final volume of 20 mL. The samples were acidified to pH 2.0 with 1 mL of a porcine pepsin preparation (0.04 g pepsin in 1 mL 0.1M HCl) and incubated at 37°C in

a shaking water bath at 95 rpm for 1 h. After gastric digestion, a 500 µl aliquot of each sample was stored at ¯20°C. The pH was then increased to 5.3 with 0.9 M sodium bicarbonate followed by the addition of 200 µL of bile salts glycodeoxycholate (0.04 g in 1 mL saline), taurodeoxycholate (0.025 g in 1 mL saline), taurocholate (0.04 g in 1 mL saline) and 100 µL of pancreatin (0.04 g in 500 µL saline). The pH of each sample was increased to 7.4 with 1M NaOH. Samples were incubated in a shaking water bath (95 rpm) at 37°C for 2 h to complete the intestinal phase of the *in vitro* digestion process. After the intestinal phase, a 500 µL aliquot of each sample was stored at ¯20°C. Samples were analysed within 48 hours.

3.2.3. FRAP

The TAC of the samples, before and after the gastric and duodenal phases of digestion, was determined using a modification of the FRAP assay of Benzie & Strain (1996) as previously described in section 2.4.1. Absorbance at 593 nm was determined relative to a reagent blank also incubated at 37°C. The TAC of samples was determined against a standard of known FRAP value, ferrous sulphate (1000 µM). Results are displayed as whole values relative to the standard.

3.2.4. DPPH$^{\bullet}$

Antioxidant activities of the samples before and after the gastric and duodenal phases of digestion were also analysed by investigating their ability to scavenge the DPPH$^{\bullet}$ free radical using a modification of the method by Brand-Williams et al. (1995) as previously described in section 2.4.2. After incubation, an aliquot of the sample from each test tube was removed and placed into a cuvette to prevent occlusion of the light pathway by sediment in the juice, and the absorbance was measured at 517 nm. The percentage inhibition was calculated against a control and compared with an ascorbic acid standard curve (0–1000 µM). The effect of digestion on the ability to scavenge the

DPPH$^{\bullet}$ radical was expressed as fold change compared with the sample prior to digestion. For accurate comparisons to be drawn a 1:5 dilution of the juices prior to digestion was made and tested in order that the digested samples be compared to the original samples at the same dilution ratio.

3.2.5. ABTS$^{\bullet+}$ radical cation scavenging activity

Antioxidant activities of the samples before and after the gastric and duodenal phases of digestion were also analysed by investigating their ability to scavenge the ABTS$^{\bullet+}$ free radical using a modified methodology previously reported by Ozgen et al. (2006) as previously described in section 2.4.3. An aliquot from each test tube was removed and placed into a cuvette to prevent occlusion of the light pathway by sediment in the juice before the absorbance of each sample was measured. The percentage inhibition was calculated against a control and compared with a Trolox standard curve 10-100 mM and expressed as fold change compared with the sample prior to digestion. Again, digested samples were compared with 1:5 dilutions of the original juices

3.2.6. Folin-Ciocalteu method for total polyphenols

Samples were analysed for TP content before and after the gastric and duodenal phases of digestion using the methodology of Singleton et al. (1999) as previously described in section 2.4.6. After incubation at room temperature for 90 minutes, the absorbance of the mixture was read at 725 nm using the respective solvent as blank. The results were expressed as μg FAE per mL of sample.

3.2.7. Statistical Analyses

All data are presented as means (± SEM) of at least 3 independent experiments (n=3), each experiment had a minimum of 3 replicates of each sample. For comparisons between samples, data were analysed by ANOVA and Tukey's multiple comparison test after homogeneity of variance and normality were confirmed (SPSS, version 17). A probability of 5% or less was accepted as statistically significant. Data were not transformed.

3.3. Results

3.3.1. FRAP analysis

The initial FRAP values of the tested vegetable juices varied considerably (1369 – 9500 μmol/L) (table 3.1). Within categories, beetroot juices displayed the highest FRAP values (8355 – 9500 μmol/L) followed by mixed fruit and vegetable juices (2060 – 6047 μmol/L), tomato juices (1843 – 3139 μmol/L), mixed vegetable juices (2468 – 2601 μmol/L) and carrot juices (1369 - 1533μmol/L). Following both the gastric and duodenal phases of digestion, the FRAP values of all 23 juices were significantly (P<0.05) increased. High importance is placed upon the post digestion values for these juices because the *in vitro* digestion model gives an indication as to the availability of vegetable juice phytochemicals in a biological system, since the model is designed to simulate *in vivo* digestion. Beetroot juices in particular showed a near 2 fold increase in FRAP following the gastric phase of digestion, although this was not maintained after the duodenal phase for Eden organic beetroot juice. This pattern was evident for the majority of the juices, whereby the greatest increase was observed after the gastric phase with a subsequent small decrease after the duodenal phase, although still remaining higher than their respective levels prior to digestion. The only exception was

Sainsbury's tomato juice from concentrate which increased a little further between gastric and duodenal phases.

Importantly the FRAP values of all the juices remained relatively stable following both phases of digestion and none were decreased compared with the juices prior to digestion. Thirteen tomato juices were analysed comprising both value and premium brands. All were long life (non-refrigerated) products with the exception of Waitrose fresh pressed tomato juice. In comparison this fresh juice displayed a significantly (P<0.05) higher FRAP value than Waitrose long life. In most cases the value brands had a higher reducing capacity than the premium brands in the FRAP assay.

Table 3.1 – FRAP values for tested juices before, and after the gastric and duodenal phases of *in vitro* digestion

Vegetable Juice	***FRAP Prior*** *(μmol/L)*	***FRAP Gastric*** *(μmol/L)*	***FRAP Duodenal*** *(μmol/L)*
Big Tom Spiced Tomato Juice	2251 ± 95	3799 ± 308*	3687 ± 257*
Sainsbury's Tomato Juice from Concentrate	1971 ± 21	3668 ± 298*	3687 ± 327*
Sainsbury's Basic Tomato Juice	2497 ± 19	3890 ± 83*	3743 ± 149*
Sunraysia Pure Squeezed Tomato Juice	1843 ± 15	2841 ± 71*	2638 ± 132*
Princes Tomato Juice from Concentrate	2750 ± 111	3724 ± 120*	3190 ± 83*
Tesco Tomato Juice from Concentrate	2683 ± 74	3287 ± 69*	3077 ± 5*
Tesco Value Tomato Juice from Concentrate	2905 ± 223	3882 ± 105*	3308 ± 302*
Waitrose Fresh Pressed Tomato Juice	3139 ± 96	5008 ± 159*	3772 ± 239*
Waitrose Longlife Pressed Tomato Juice	2415 ± 88	3172 ± 87*	2959 ± 174*
ASDA Tomato Juice from Concentrate	2497 ± 84	3547 ± 138*	3289 ± 92*
Del Monte Tomato Juice from Concentrate	2202 ± 40	3417 ± 87*	3111 ± 45*
Sunpride Tomato Juice	2463 ± 28	4427 ± 541*	3880 ± 112*
The Co-operative Tomato Juice	3104 ± 32	3599 ± 33*	3059 ± 119*
Eden Organic Carrot Juice	1533 ± 11	2926 ± 89*	2732 ± 28*
Sunraysia Organic Carrot Juice	1369 ± 22	3734 ± 64*	3916 ± 36*
Eden Organic Beetroot Juice	9500 ± 27	18639 ± 184*	8890 ± 92*
'Beet It' - James White Organic Beetroot Juice	8355 ± 85	15014 ± 146*	12397 ± 326*
James White Organic Vegetable Juice	2601 ± 22	3852 ± 180*	3592 ± 308*
V8 100% Vegetable Juice (Original)	2573 ± 49	4206 ± 349*	3704 ± 79*
Eden Organic Vegetable Cocktail	2468 ± 93	4157 ± 118*	3689 ± 16*
V8 100% Fruit and Carrot Juice (Tropical)	2060 ± 49	5160 ± 272*	4950 ± 203*
Cawston Press Apple and Rhubarb Juice	6017 ± 54	8758 ± 131*	8399 ± 434*
Cawston Press Apple and Beetroot Juice	6407 ± 73	8936 ± 129*	8958 ± 275*

*$P<0.05$, denotes significant increase from the Ferric Reducing Antioxidant Power (FRAP) value prior to *in vitro* digestion [ANOVA, Tukey's test]. Values represent mean ± standard error of the means (SEM) of three independent experiments (n=3).

3.3.2. DPPH$^{\bullet}$ radical scavenging activity

DPPH$^{\bullet}$ radical scavenging activity was quantified in terms of percentage inhibition of a pre-formed free radical by antioxidants in each sample. There was a significant variation in the percentage inhibition of the DPPH$^{\bullet}$ radical by the juices (57.8 – 100% inhibition) (Table 3.2). Again beetroot juice exhibited the highest antioxidant capacity with both juices fully inhibiting the radical. Tomato juices (67.3 - 88.5%), carrot juices (57.8 - 82.2%), mixed fruit and vegetable juices (64.4 – 89.1%) and mixed vegetable

juices (82.2 – 83.5%) exhibited similar DPPH$^{\bullet}$ scavenging activity. Eden organic carrot juice (82.2%) showed greater scavenging DPPH$^{\bullet}$ scavenging capacity than Sunraysia organic carrot juice (57.8%). Concurrently, the Sunraysia tomato juice also had a considerably lower DPPH$^{\bullet}$ scavenging capacity (67.3%) than any of the other tomato juices despite costing the most. Interestingly there was a trend for the radical scavenging capacity to be slightly increased following the gastric phase and slightly decreased following the duodenal phase, although there were some exceptions to this trend. Overall, DPPH$^{\bullet}$ radical scavenging capacity remained consistently high throughout *in vitro* digestion.

Table 3.2 – DPPH$^{\bullet}$ inhibition of the tested juices before, and after the gastric and duodenal phases of *in vitro* digestion

Vegetable Juice	***DPPH*** *(% Inhibition)*	***DPPH Gastric*** *(Fold Change)*	***DPPH Duodenal*** *(Fold Change)*
Big Tom Spiced Tomato Juice	83.5 ± 0.2	0.61*	0.96
Sainsbury's Tomato Juice from Concentrate	79.5 ± 1.1	1.01	0.71*
Sainsbury's Basic Tomato Juice	88.5 ± 0.2	1.24*	0.86*
Sunraysia Pure Squeezed Tomato Juice	67.3 ± 0.9	1.02	0.70*
Princes Tomato Juice from Concentrate	81.0 ± 0.6	1.13*	0.82*
Tesco Tomato Juice from Concentrate	81.2 ± 0.5	1.29*	0.85*
Tesco Value Tomato Juice from Concentrate	81.8 ± 0.2	1.02	0.67*
Waitrose Fresh Pressed Tomato Juice	85.7 ± 0.2	1.21*	0.76*
Waitrose Longlife Pressed Tomato Juice	82.5 ± 0.2	1.27*	0.92*
ASDA Tomato Juice from Concentrate	83.9 ± 0.4	1.25*	0.94
Del Monte Tomato Juice from Concentrate	80.4 ± 0.4	1.15*	0.88*
Sunpride Tomato Juice	76.6 ± 0.1	1.14*	1.03
The Co-operative Tomato Juice	87.6 ± 0.1	1.09*	0.80*
Eden Organic Carrot Juice	82.2 ± 0.1	0.84*	0.84*
Sunraysia Organic Carrot Juice	57.8 ± 1.9	0.80*	0.81*
Eden Organic Beetroot Juice	100 ± 0.0	1.02	1.12*
'Beet It' - James White Organic Beetroot Juice	100 ± 0.0	1.23*	0.69*
James White Organic Vegetable Juice	83.5 ± 0.2	0.80*	0.78*
V8 100% Vegetable Juice (Original)	83.1 ± 0.3	1.08*	0.98
Eden Organic Vegetable Cocktail	82.2 ± 0.2	1.03	0.79*
V8 100% Fruit and Carrot Juice (Tropical)	81.8 ± 2.3	0.74*	0.97
Cawston Press Apple and Rhubarb Juice	89.1 ± 0.2	0.63*	0.84*
Cawston Press Apple and Beetroot Juice	64.4 ± 0.6	0.64	0.79

*$P<0.05$, denotes significant increase from the DPPH percentage inhibition value prior to *in vitro* digestion [ANOVA, Tukey's test]. Values represent mean ± standard error of the means (SEM) of three independent experiments (n=3). Fold change refers to the fold increase or decrease in TAC of digested samples (1:5 dilution in saline) compared with the % inhibition of a 1 in 5 saline dilution of the original juice prior to digestion.

3.3.3. $ABTS^{\bullet+}$ radical cation scavenging activity

ABTS activity was quantified in terms of percentage inhibition of the $ABTS^{\bullet+}$ radical cation by antioxidants in each sample. There was a significant variation in the percentage inhibition of the juices (10.9 – 92.3% inhibition) (Table 3.3). Beetroot juice was once again the most efficient scavenger of the radical (92.1 – 92.3% inhibition). Interestingly both of the Cawston Press fruit and vegetable juices (apple and rhubarb/apple and beetroot) were also particularly effective scavengers of the $ABTS^{\bullet+}$ radical (90.1 – 90.7% inhibition). The other mixed fruit and vegetable juice (V8 100% fruit and carrot juice tropical) had a more modest inhibitory effect at 39.1%. Tomato juices ranged from 22.4% for Sunraysia (the most expensive brand) to 39.1 and 38.4% for Tesco own brand and value juices. There was one notable exception amongst tomato juices in that ASDA tomato juice from concentrate inhibited the radical by 70.5%, by far the most potent of the tomato juices. Within the mixed vegetable juices, James White organic vegetable juice (55.9%) displayed a greater $ABTS^{\bullet+}$ scavenging capacity than the other mixed vegetable juices (25.5 – 26.1%). Carrot juice proved the most inefficient scavenger of the $ABTS^{\bullet+}$ radical at 10.9 – 23.9% inhibition. Following the gastric phase of the *in vitro* digestion model, 18 of the juices increased their inhibitory effects significantly ($P<0.05$).

The only juice to show a significant decrease following the gastric phase of digestion was Tesco tomato juice from concentrate; concurrently Sainsbury's Basic tomato juice from concentrate displayed the greatest increase at 2.06 fold. Contrary to the results from the $DPPH^{\bullet}$ assay 12 of the juices maintained this significant increase after the duodenal phase. Despite this finding, beetroot juice although remaining high, showed a significant decrease ($P<0.05$) in $ABTS^{\bullet+}$ inhibition following the duodenal phase (0.79 and 0.56 fold change respectively). Similarly both Cawston Press juices (apple and rhubarb/apple and beetroot) decreased significantly ($P<0.05$) after the duodenal phase. Despite these observed decreases, both beetroot and the mixed fruit and

vegetable juices still had a higher $ABTS^{\bullet+}$ scavenging capacity than all the other juices, even if they had increased significantly.

Table 3.3 - $ABTS^{\bullet+}$ inhibition of the tested juices before, and after the gastric and duodenal phases of *in vitro* digestion

Vegetable Juice	***ABTS*** *(% Inhibition)*	***ABTS Gastric*** *(Fold Change)*	***ABTS Duodenal*** *(Fold Change)*
Big Tom Spiced Tomato Juice	32.7 ± 0.3	1.37*	1.49*
Sainsbury's Tomato Juice from Concentrate	27.3 ± 1.1	1.79*	1.38*
Sainsbury's Basic Tomato Juice	32.9 ± 0.9	2.06*	1.51*
Sunraysia Pure Squeezed Tomato Juice	22.4 ± 0.6	1.96*	1.32*
Princes Tomato Juice from Concentrate	31.8 ± 0.4	1.78*	0.98
Tesco Tomato Juice from Concentrate	39.1 ± 0.4	0.77*	0.94
Tesco Value Tomato Juice from Concentrate	38.4 ± 0.3	1.06	1.00
Waitrose Fresh Pressed Tomato Juice	26.5 ± 0.8	1.33*	1.65*
Waitrose Longlife Pressed Tomato Juice	30.9 ± 0.3	1.33*	1.49*
ASDA Tomato Juice from Concentrate	70.5 ± 0.5	1.42*	0.96
Del Monte Tomato Juice from Concentrate	30.0 ± 1.0	1.25*	1.21*
Sunpride Tomato Juice	31.4 ± 0.2	1.32*	1.00
The Co-operative Tomato Juice	29.9 ± 0.2	1.54*	1.55*
Eden Organic Carrot Juice	23.9 ± 0.3	1.38*	1.58*
Sunraysia Organic Carrot Juice	10.9 ± 0.2	0.91*	0.79*
Eden Organic Beetroot Juice	92.1 ± 0.2	1.14*	0.56*
'Beet It' - James White Organic Beetroot Juice	92.3 ± 0.1	1.02	0.92
James White Organic Vegetable Juice	55.9 ± 0.9	1.18*	1.18*
V8 100% Vegetable Juice (Original)	25.5 ± 0.3	1.09*	1.09*
Eden Organic Vegetable Cocktail	26.1 ± 0.2	1.48*	1.41*
V8 100% Fruit and Carrot Juice (Tropical)	39.1 ± 0.1	1.29*	1.01
Cawston Press Apple and Rhubarb Juice	90.7 ± 0.3	1.00	0.71*
Cawston Press Apple and Beetroot Juice	90.1 ± 0.3	0.93	0.70*

*$P<0.05$, denotes significant increase from the ABTS percentage inhibition value prior to *in vitro* digestion [ANOVA, Tukey's test]. Values represent mean ± standard error of the means (SEM) of three independent experiments (n=3). Fold change refers to the fold increase or decrease in TAC of digested samples (1:5 dilution in saline) compared with the % inhibition of a 1 in 5 saline dilution of the original juice prior to digestion.

3.3.4. Total polyphenols analysis

The TP content of each juice was quantified using Folin Ciocalteu reagent. All 23 juices were a significant source of polyphenols; however the total amount varied significantly between juices (514 – 3025 µg FAE/mL) (table 3.4). Prior to digestion results showed that Eden organic beetroot juice contained the highest amount of polyphenols (3025 µg FAE/mL). Sunraysia organic carrot juice contained the lowest amount of polyphenols (514 µg FAE/mL). After the gastric phase of digestion there was a significant ($P<0.05$) increase in TP content for all 23 juices. The greatest increase was seen in Sunpride tomato juice (a value brand) with an increase in total polyphenol content from 668 – 1117 µg FAE/mL. Eden organic beetroot juice showed a modest increase in TP following the gastric phase, increasing from 3025 – 3124 µg FAE/mL and remained the juice with the highest polyphenol content. After the duodenal phase of digestion, 19 of the juices showed a further increase in TP content. The Waitrose fresh tomato juice increased most significantly at this stage (257 µg FAE/mL). Four of the 23 juices decreased in TP content after the duodenal phase of digestion. The most notable of which was once again observed for Eden organic beetroot juice, which despite remaining high, decreased from 3124 µg FAE/mL (after the gastric phase) to 1619 µg FAE/mL (after the duodenal phase).

Table 3.4 – Total Polyphenol measurement of the tested juices before, and after the gastric and duodenal phases of *in vitro* digestion

Vegetable Juice	***Polyphenols*** *(μg FAE/mL)*	***Polyphenols Gas*** *(μg FAE /mL)*	***Polyphenols Duo*** *(μg FAE/mL)*
Big Tom Spiced Tomato Juice	821 ± 44	1025 ± 7*	1137 ± 14*
Sainsbury's Tomato Juice from Concentrate	924 ± 43	1225 ± 22*	1251 ± 9*
Sainsbury's Basic Tomato Juice	992 ± 74	1188 ± 9*	1249 ± 7*
Sunraysia Pure Squeezed Tomato Juice	575 ± 17	892 ± 16*	1045 ± 14*
Princes Tomato Juice from Concentrate	852 ± 36	1031 ± 12*	1077 ± 8*
Tesco Tomato Juice from Concentrate	872 ± 33	1142 ± 20*	1301 ± 27*
Tesco Value Tomato Juice from Concentrate	931 ± 45	1126 ± 32*	1260 ± 33*
Waitrose Fresh Pressed Tomato Juice	573 ± 21	904 ± 41*	1161 ± 23*
Waitrose Longlife pressed Tomato Juice	704 ± 13	1068 ± 33*	1286 ± 23*
ASDA Tomato Juice from Concentrate	1140 ± 33	1549 ± 19*	1422 ± 12*
Del Monte Tomato Juice from Concentrate	976 ± 27	1300 ± 11*	1347 ± 19*
Sunpride Tomato Juice	690 ± 27	1117 ± 6*	1211 ± 20*
Co-Operative Tomato Juice	934 ± 22	1113 ± 16*	1239 ± 8*
Eden Organic Carrot Juice	605 ± 12	956 ± 15*	1094 ± 8*
Sunraysia Organic Carrot Juice	449 ± 11	859 ± 23*	1065 ± 11*
Eden Organic Beetroot Juice	3025 ± 53	3124 ± 45*	1619 ± 22*
'Beet It'-James White Organic Beetroot Juice	2126 ± 66	2490 ± 23*	1985 ± 32*
James White Organic Vegetable Juice	840 ± 21	1123 ± 9*	1220 ± 19*
V8 100% Vegetable Juice (Original)	826 ± 26	1166 ± 11*	1241 ± 10*
Eden Organic Vegetable Cocktail	627 ± 15	924 ± 16*	1080 ± 16*
V8 100% Fruit and Carrot Juice (Tropical)	790 ± 21	1253 ± 15*	1407 ± 14*
Cawston Press Apple and Rhubarb Juice	1782 ± 52	2064 ± 43*	1923 ± 20*
Cawston Press Apple and Beetroot Juice	1986 ± 59	2173 ± 65*	2019 ± 54*

*$P<0.05$, denotes significant increase from the total polyphenol content of the juice prior to *in vitro* digestion [ANOVA, Tukey's test]. Values represent mean ± standard error of the means (SEM) of three independent experiments (n=3). Gas = measurement after the gastric phase, Duo = measurement after the duodenal phase. FAE = Ferulic acid equivalents.

3.4. Discussion

Previous publications detailing the antioxidant capacity of vegetable juices are sparse. Some studies have presented information on immune modulation, *in vivo* lipid peroxidation and carcinogenesis following consumption of tomato and carrot juices (Paetau et al., 1998b; Bub et al., 2000; Watzl et al., 2003) but no studies to date have provided information regarding beetroot juice, mixed fruit and vegetable juices or mixed vegetable juices. These studies have provided information about whole juices but no studies to date have reported the stability of vegetable juice phytochemicals following digestion. By measuring the TAC of the juices after the *in vitro* digestion it is possible to quickly and cost-effectively provide more biologically relevant data detailing the phytochemical properties of these juices.

Crucially the results of this study showed that antioxidant capacity is relatively stable throughout the digestive process in the vast majority of vegetable juices. This is in contrast to previous research examining fruit juices which have consistently shown large decreases in TAC post digestion (McDougall et al., 2005a; McDougall et al., 2005b; Bermudez-Soto et al., 2007), suggesting that vegetable juices may be a greater source of bioaccessible phytochemicals than the more popular fruit juices. In the study by Bermudez-Soto et al. (2007) the researchers reported a reduction in various polyphenols following a similar two phase digestion of a variety of fruit juices. The fruit juices displayed largely increased polyphenol content after the gastric phase of digestion (similar to the current study) but fell to below pre-digestion levels after the duodenal phase, a trend which was not observed in the current study of vegetable juices. Ryan and Prescott (2010) suggested that this may have been due to a structural transformation in the polyphenols which render them undetectable by the individual HPLC polyphenol analysis which Bermudez-Soto and colleagues (2007) had used. The authors did not measure total polyphenols. The current study shows that this does not occur in

vegetable juices since the polyphenol content was much higher after both stages of digestion compared with the levels prior to digestion. It also highlights the efficacy of multiple methods of analysis to prevent under-reporting of the antioxidant content of juices.

The results of the three assays which were conducted are comparable in magnitude for each category of juice. There were some exceptions to this trend whereby particular juices performed markedly better in one or more of the assays. Overall beetroot juice displayed the strongest radical scavenging capacity in both the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, and also displayed the highest reducing capacity as measured by FRAP. Beetroot juices also ranked amongst the highest juices in terms of TP content. The TAC of beetroot juice was greater than or comparable with that of pomegranate or cranberry juice (Ryan and Prescott, 2010). Beetroot is afforded its vibrant red and purple colourations by the betacyanin; betanin. Betacyanins are one of the two sub-categories of betalains which may reduce LDL cholesterol oxidation and therefore impact upon cardiovascular diseases (Escribano et al., 1998; Tesoriere et al., 2004a). Beetroot is also a source of many other health promoting constituents such as folic acid, iron, magnesium, selenium, potassium, calcium, zinc, phosphorus, sodium, biotin, niacin and β-carotene as well as vitamins A, B_6 and C. The high antioxidant capacity of beetroot juice, together with its other nutritional value may make beetroot juice a very positive addition to the diet.

Interesting comparisons emerged between the two beetroot juices following FRAP analysis. The first beetroot juice, Eden organic, is produced by combining pure beetroot juice with lactic acid fermented beetroot juice and ascorbic acid as a preservative. The second, James White organic beetroot juice (‘Beet it’), is made from 90% pressed beetroots and 10% pressed apple juice. Initially Eden organic beetroot juice displayed a higher FRAP value (9500 μmol/L) than the James White juice (8355

μmol/L). Principally due to the higher beetroot content, since beetroot has a higher FRAP value compared with apple juice (Ryan and Prescott, 2010), and perhaps in part to the addition of ascorbic acid, a powerful reducing agent in itself. This trend is continued after the gastric phase where the Eden organic juice increased almost 2 fold to 18639 μmol/L and the James White juices, similar in magnitude, increased to 15014 μmol/L. Following the duodenal phase of digestion where the pH is first adjusted to 5.3 and then to 7.4 following the addition of bile salts and pancreatin, both juices show a decrease in FRAP. The James White beverage remains well above pre-digestion at 12397 μmol/L. The Eden beverage however, displayed a reducing capacity that fell below even its pre-digestion value (8890 μmol/L).

Since this result is shown consistently across three separate experiments, it is reasonable to assume that the phytochemicals in this juice are destroyed, inhibited, transformed or otherwise far less available during this phase. A similar trend was observed for the $ABTS^{\bullet+}$ assay, and for the James White juice, in the $DPPH^{\bullet}$ assay. There are several key differences between the juices, which could account for these results. Firstly, Eden organic beetroot juice is at least partially lactic acid fermented. Lactic acid fermentation involves the addition of probiotic lactic acid bacteria, which consume reducing sugars in the raw produce causing a rapid but modest acidification of the juice. This process extends the shelf life of the juice and provides additional nutritional value in terms of probiotic bacteria (Moraru et al., 2007). It has also been suggested to increase its nutritional content in terms of phytochemicals (Hugenholtz and Smit, 2002). Potentially, the lacto-fermentation of this particular juice induces a structural transformation, perhaps by the action of the acidification of the product. The pH of a substance is known to affect racemisation of molecules, possibly creating two chiral enantiomers with different reactivity in the respective reagents. In theory this could alter their biological reactivity and may render the antioxidants more reactive

early in the digestive process, particularly at acidic pH in the gastric phase and less reactive at pH 7.4 in the duodenal phase as racemisation can increase with pH in other compounds (Jamali et al., 2008). As a result there are either fewer reactive phytochemicals available at the duodenal phase or their reducing capacity is impaired. There is also the suggestion that dependant on any structural changes which occur, resulting metabolites may react differently across different assays. The Eden organic beetroot juice did not show a reduction in reducing capacity following digestion when assessed using the $DPPH^{\bullet}$ radical scavenging assay.

Differences were observed between the two radical scavenging assays ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$). Both assays measure the ability of all of the antioxidants in a sample to scavenge a pre-formed radical. The $DPPH^{\bullet}$ radical is stable at formation; DPPH in powder form is simply dissolved in methanol and the resulting purple solution is added to the sample. In contrast the $ABTS^{\bullet+}$ radical cation is created by reacting ABTS (7 mM in 20 mM acetate pH 4.5) with the oxidant, potassium persulfate (2.45 mM). The reaction proceeds during a 12-16 hour incubation at a stoichiometric ratio of 1:0.5 to form the radical cation. Previous work has shown that this method allows formation of a radical which remains stable for several days due to the optimised pH (Cano et al., 1998). In fact in the current study the radical remained stable for a week or more. Further fundamental differences can be observed between the species of radical which is created in each of these methods and the methodologies by which they are executed.

Firstly, the $ABTS^{\bullet+}$ assay is an electron transfer, end-point assay whereby different antioxidant compounds donate one or two electrons to reduce the radical cation. Regardless of the donating potential of individual antioxidants they all have time to react fully giving an accurate measurement of TAC at the end-point of the assay (Huang et al., 2005). The $DPPH^{\bullet}$ assay is based on the normal HAT reaction that occurs between antioxidants and the peroxyl radical. Instead of peroxyl radicals, more stable

and less transient nitrogen radicals are created, with which some antioxidants react more slowly than they would with the peroxyl radical in a biological system (Huang et al., 2005). Additionally, the reaction then proceeds in a manner which bears more similarity to a SET reaction (Foti et al., 2004). In the current study this does not seem to affect the TAC measurement of the juices prior to digestion, since most of the juices display a high inhibition of the radical. However following the gastric phase of *in vitro* digestion, when structural transformations may occur in the phytochemicals, the TAC is relatively unchanged in the $DPPH^{\bullet}$ assay but increases significantly ($P<0.05$) for a number of the juices in the $ABTS^{\bullet+}$ assay. Theoretically, alterations in the structure of phytochemicals following digestion may affect their reactivity with the less biologically relevant nitrogen radical formed in the $DPPH^{\bullet}$ assay, leading to underestimation of TAC at this time point. Similar mechanisms may also contribute to explaining the differences observed between the measurements of TAC in the two assays after the duodenal phase. A number of juices remain significantly higher ($P<0.05$) than their pre-digestion value in the $ABTS^{\bullet+}$ assay, whilst all of the juices show a similar or reduced TAC after the duodenal phase in the $DPPH^{\bullet}$ assay. There was a relatively weak correlation observed between the results for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ ($r = 0.45$); differences in the rank order between the 13 tomato juices may have skewed this statistic, weakening what may otherwise have been a stronger correlation.

Despite these observed differences, both assays rank the juices in a similar order. Both give beetroot juice as the most effective free radical scavenger, both rank the mixed fruit and vegetable juices second, and both rank carrot juices as the most inefficient scavenger. This information is actually more relevant to the pursuit of public health than knowing the exact chemical reactivity of each component of a sample in each different assay. By obtaining consistent information about the rank order of different products, in terms of their TAC, from multiple methodologies, accurate

information about the most potent juices can be disseminated to a wider audience. Comparisons were also drawn between the results obtained by the FRAP assay and those obtained for TP by FC. Both of these assays utilise similar SET reaction kinetics and are both essentially measures of reducing capacity. Where FRAP measures the reducing capacity of all the antioxidants in the mixture, FC records only those classed as polyphenolic compounds (Huang et al., 2005). As a result previous researchers have often found very good linear correlations between FC and one or more SET based assays such as FRAP or $ABTS^{\bullet+}$ (Stratil et al., 2006; Jimenez-Alvarez et al., 2008; Molan et al., 2009; Sreeramulu and Raghunath, 2010). The same relationship is observed in the current study, where a very strong positive linear correlation ($r = 0.96$) is observed between FRAP and FC. Additionally, there were strong, positive, linear correlations between other assays utilising similar SET reaction kinetics. FRAP also correlated well with $ABTS^{\bullet+}$ ($r = 0.87$), whilst $ABTS^{\bullet+}$ showed a strong positive association to FC ($r = 0.89$). The methodological anomaly in these comparisons was $DPPH^{\bullet}$, which also correlated poorly with FRAP ($r = 0.53$) and FC ($r = 0.50$).

3.5. Conclusion

This research has shown that all vegetable juices may be a significant source of phytochemicals including antioxidants and polyphenols, regardless of price, storage or processing conditions. However, there is a wide variation in TAC between different types of juice. Beetroot juice displayed by far the highest antioxidant capacity across all of the assays conducted, and has an antioxidant capacity similar to, or greater than that of pomegranate or cranberry juice. Additionally, this research has provided the first measurement concerning the stability of commercial vegetable juice antioxidants following *in vitro* digestion. All of the juices were either stable or enhanced in terms of TAC following *in vitro* digestion, although there was a large variation in the responses

of individual juices to this procedure. This research highlights the need for further investigation in several areas. Firstly, it is important to consider the contribution of structurally transformed molecules and other antioxidant metabolites to TAC. It is likely that a number of methods underestimate TAC due to a failure to measure these. Further to this, it is important that research aims to provide biologically relevant information on phytochemicals by providing data concerning the bioaccessibility and bioavailability of phytochemicals in a human system. Finally this study highlights the importance of using multiple methods of analysis in the absence of any single accepted assay for the measurement of TAC, and that measurement of TAC provides useful guidance as to the potential beneficial effects of a wide range of produce containing bioactive phytochemicals.

Related publication: Wootton-Beard, P.C., Moran, A. & Ryan, L. (2011). Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion, measured by FRAP, DPPH, ABTS and Folin Ciocalteu methods. *Food Research International*, 44 (1), 217-224.

3.6. The Beetroot Juice Shot

3.6.1. Justification

Convenience is considered an important marketing tool in the food industry (Drewnowski and Darmon, 2005), and conscientious food manufacturers are looking for ways to make healthy food, particularly fruit and vegetables, more convenient to consume in order that public health may be improved. There has been a significant increase in the number of fruit based beverages which are available in UK commercial outlets over the past decade and they have become an important method of fruit intake, particularly for children. By examining the shelves in local supermarkets it is clear to see that vegetables juices are beginning to expand in the same way. Fruit and vegetable blends in particular appear to have become more popular, given the emergence of brands such as V8 (Campbell Foods, Belgium), Cawston Press (Cawston Press, Wokingham, UK) and Sunraysia (Sunraysia UK Ltd, London, UK). Vegetable juices are considered to be a viable method of bridging the gap between actual and observed vegetable intake (Shenoy et al., 2010).

However, beetroot juice consumption may not be as popular as other fruit and vegetable juices such as tomato, carrot, apple or mango, perhaps due to perceived issues of taste, texture and urinary colouration. In reality beetroot juice has a relatively pleasant taste in comparison with other vegetable juices due to its relatively high sucrose content (Thakur and Das Gupta, 2006). A small increase in the habitual consumption of phytochemical-rich beverages such as beetroot juice may have a significant positive effect on public health. Recently, a number of more convenient vegetable juice products have been created to try to increase consumption, including popular brands of mixed vegetable juice which are available in cans and small bottles (V8, Campbell Foods, Belgium). Additionally a beetroot juice ‘shot’ (70 mL) has been

developed (James White Drinks, Ipswich, UK). This particular product offers an opportunity for beetroot juice to be consumed with ease and convenience by the general public and may contribute positively toward increasing consumption of phytochemical-rich produce.

3.6.2. Materials and Methods

All chemicals were of analytical grade and were purchased from Sigma Aldrich (Poole, UK). Beetroot juice shots were provided by James White Drinks Ltd, Ipswich, UK. The beetroot shot was analysed in its original form. In all experiments, the beverages were prepared using a standard protocol. Amber bottles were used throughout to prevent the photodecomposition of antioxidants and efforts were made to exclude oxygen contact with the samples. All experiments were carried out on a minimum of three separate occasions. Samples were analysed in triplicate for each experiment. All analysis was carried out by the author at Oxford Brookes University.

3.6.2.1. Determination of total antioxidant capacity and total polyphenols

The TAC of the samples was determined using a modification of the FRAP assay of Benzie and Strain (1996a) as previously described in section 2.4.1. The TAC of samples was determined against a standard of known FRAP value, ferrous sulphate (1000 µM). TP content was determined using the FC method (Singleton et al., 1999) as previously described in section 2.4.6. After incubation at room temperature for 90 minutes, the absorbance of the mixture was read at 725 nm using the respective solvent as blank. The results were expressed as mg of gallic acid equivalents (mg GAE).

3.6.2.2. *In vitro* digestion procedure

Analysis was repeated following *in vitro* digestion. The *in vitro* digestion model was adapted from Ryan et al., (2008) as previously described in section 3.2.2.

3.6.2.3. Statistical Analyses

All data are presented as means (± SEM) of at least three independent experiments (n=3), each experiment had a minimum of three replicates of each sample. For comparisons between samples, data were analysed by ANOVA and Tukey's multiple comparison test after homogeneity of variance and normality were confirmed (SPSS, version 17). Statistical significance was set at $P < 0.01$. Data were not transformed.

3.6.3. Results and Discussion

The beetroot juice shot delivers a significant amount of phytochemicals in a small, convenient volume as measured by the FRAP assay (697.9 ± 1.6 µmol/70 mL) (Table 3.5). The TAC of the shot increased significantly ($P < 0.01$) following the *in vitro* digestion procedure (1740.3 ± 21.1 µmol/70 mL) despite a decrease in TAC between the gastric (2361.2 ± 20.9 µmol/70 mL) and duodenal phases. The differences observed between the gastric and duodenal phase of digestion were in agreement with previous studies (Bermudez-Soto et al., 2007; Wootton-Beard et al., 2011). In both of these studies the greatest increase in FRAP was seen following the gastric phase with a marked decrease after the duodenal phase, which may result from biotransformation of phytochemicals caused by interaction with the enzymes present in the system (Ryan and

Prescott, 2010). The increase in FRAP observed for the beetroot juice shot, following the individual phases of digestion, was proportionally much higher than that of other vegetable juices, most notably the other beetroot juice product produced at the same location, suggesting differences in the processing conditions between the two products. Both beetroot juice products started at relatively similar values prior to digestion and despite significant increases in both, the shot produced a particularly large increase in TAC following both the gastric and duodenal phases, around 2 fold higher than the standard product (Table 3.5). The results clearly demonstrate that phytochemicals contained in this particular product become more accessible following digestion than those in other vegetable juice products.

Table 3.5 - Total antioxidant capacity and total polyphenol content of the beetroot juice shot and other beverages

Product	FRAP (µmol)		FRAP post digestion (µmol)		TP (mg GAE)		TP post digestion (mg GAE)	
	Per L	Per serving	Per L	Per serving	Per L	Per serving	Per L	Per serving
James W Beetroot Shot	9971 ± 22	697.9 ± 1.6	24862 ± 300*	1740.3 ± 21.1*	977.2 ± 5.2	68.4 ± 0.3	3189.1 ± 77.3*	223.2 ± 5.4*
James W Beetroot Juice[1]	8354 ± 84	584.8 ± 5.9	12152 ± 336	850.6 ± 23.5	1450.3 ± 42.1	101.5 ± 2.9	1527.1 ± 18.0	106.9 ± 1.3
V8 Veg Juice[1]	2573 ± 48	180.1 ± 3.4	3488 ± 223	244.2 ± 15.6	617.8 ± 15.6	43.2 ± 1.1	1053.9 ± 6.4	74.7 ± 0.5
V8 Fruit and Veg Juice[1]	2060 ± 49	144.8 ± 3.4	4986 ± 202	349.0 ± 10.3	537.8 ± 17.9	37.6 ± 1.3	1097.4 ± 7.5	76.8 ± 0.5
Del Monte Tomato Juice[1]	2202 ± 39	154.1 ± 2.8	3071 ± 50	215.0 ± 3.6	695.3 ± 19.3	48.7 ± 1.4	1120.1 ± 12.2	78.4 ± 0.9
Eden Carrot Juice[1]	1516 ± 11	107.3 ± 0.78	2731 ± 27	191.9 ± 1.5	473.7 ± 7.7	33.2 ± 0.5	1022.2 ± 4.8	71.6 ± 0.3

Data adapted from Wootton – Beard et al. (2011), values are given for comparative purposes and are not included in the statistical analysis for this experiment. Results are expressed as mean ± SEM of three experiments each performed in triplicate. 'Post digestion' refers to the levels following the duodenal phase of the *in vitro* digestion procedure (section 2.3). *Significantly increased compared to the juice prior to digestion, $P < 0.01$. FRAP = Ferric reducing antioxidant power. TP = Total polyphenols using Folin Ciocalteu method. GAE = Gallic acid equivalents. One serving represents a volume of 70 mL, equal to the total volume of the beetroot juice shot.

The *in vitro* digestion model gives an indication as to the bioaccessibility of vegetable juice phytochemicals in a biological system, since the model is designed to simulate *in vivo* digestion. Bioaccessibility can be used to suggest the rank order of products, and infers that if more phytochemicals are presented to the intestinal brush border, it is likely that more will be absorbed.

Additionally, beetroot juice compares favourably with other well accepted high-antioxidant products such as pomegranate and cranberry juices, particularly in terms of

bioaccessibility. Research from our laboratory has shown that pomegranate and cranberry juice (both fresh and long life), display FRAP values of 16789 – 20063 µmol/L and 8419 – 8570 µmol/L respectively, after the same *in vitro* digestion procedure (Ryan and Prescott, 2010). This is significantly lower than the value observed for the beetroot juice shot following digestion (24862 ± 300 µmol/L). The high TAC of the beetroot juice shot can be largely attributed to its high polyphenol content. The shot was a concentrated source of dietary polyphenols as measured by the FC method (68.4 ± 0.3 mg GAE/70 mL) (Table 3.5). In similarity with the trends observed in the FRAP assay, the TP content increased 5 fold following the gastric phase of the *in vitro* digestion to 341.6 ± 4.8 mg GAE/70 mL. Following the duodenal phase the TP content was still 3.3 fold higher than the level prior to digestion (223.2 ± 5.4 mg GAE/70 mL). The term polyphenol refers to a particularly wide range of compounds found almost ubiquitously amongst plant foods. In order to be classified as a polyphenol, a compound must simply contain one or more aromatic rings and at least two hydroxyl groups according to a review by Sies (2010). Despite such a broad classification, Sies (2010) comments that any compound matching these criteria can be considered biologically interesting. With criteria such as these it becomes ever more important to quantify the TP content of food products, since all polyphenolic compounds contained in a product may contribute to health (Table 3.6).

Table 3.6 - Reported phytochemical composition of red beetroot, adapted from Kujala et al. (2002).

Classification	Compound
Betalains	
Betaxanthins	Vulgaxanthin I
	Vulgaxanthin II
Betacyanins	Betanin
	Isobetanin
Phenolics	
	5,5',6,6'-tetrahydroxy-3,3'-biindolyl
Ferulic acid conjugates	feruloylglucose
	β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside)
Phenolic Amides	*N-trans*-Feruloyltyramine
	N-trans-Feruloylhomovanillylamine
Flavonoids	Betagarin
	Betavulgarin
	Cochliophilin A

Opinions had once focussed on polyphenols primarily as phytochemicals (such as polyphenols) with a direct action against RONS, and to a degree this may well be the case. However, due to the relatively low concentrations of free circulating phytochemicals found in the body amongst other factors such as biotransformation and a lack of clinical data from intervention trials, researchers have begun to focus on other mechanisms to define the health benefits inferred by increasing fruit and vegetable consumption. In recent years, phytochemicals, including polyphenols, have been shown to inhibit alpha-glucosidase/maltase, as well as potentially stimulating insulin secretion which may reduce the absorption of glucose into the blood stream, having profound implications for diabetes management (McDougall and Stewart, 2005). Additionally, they have been shown to display important indirect functions, acting as a 'switch' in redox-dependant signalling pathways and affecting the pro-inflammatory effects of RONS through such actions as the modulation of NF-κB activation (Rahman et al., 2006).

3.6.4. Conclusion

Given the potential for the multiple health benefits of phytochemical consumption, beverages containing high levels can be considered a positive addition to the diet. Additionally, both sensory characteristics and convenience would appear to be important factors in obtaining an increased level of consumption amongst the general public. Beetroot juice provides a significant source of dietary phytochemicals and in particular, a shot can provide a significant quantity of these bioactive components together with a convenient method for consumption.

Related publication: Wootton-Beard, P.C., & Ryan, L. (2011). A beetroot juice shot is a significant and convenient source of bioaccessible antioxidants. *Journal of Functional Foods,* 3, 329-334.

4. Study 3 - Postprandial glycaemic and insulinaemic responses to a mixed meal containing beetroot juice in healthy humans

4.1. Justification

The combination of nitrate and phytochemicals in beetroot juice gives rise to a possible synergistic effect on postprandial glycaemia, either via the inhibition of glucose uptake or the modulation of insulin utilisation.

The hypothesis tested was “the addition of beetroot juice to a CHO rich meal will reduce the postprandial glycaemic excursion”. The aim of this study was to investigate the potential for beetroot juice to affect postprandial glycaemia when consumed as part of a mixed meal.

4.2. Determination of carbohydrate concentration of beetroot juice

Previous studies relating to the CHO composition of beetroot have reported various concentrations of sucrose, glucose, fructose and galactose. Additionally, the fibre content varies according to the amount of peeling, type of cooking and cooking time of the tested products. In order to assure the accuracy of the matched control beverage (MCON) for the current study and for future studies, the CHO composition of the beetroot juice shot (BEET) was independently measured by an external laboratory (Eurofins Food Testing UK Ltd, Wolverhampton, UK). The outcome of this analysis is shown in table 4.1. It was confirmed that MCON had been accurately composed in terms of CHO. The figures provided by this analysis were subsequently used to compose MCON in the current study.

Table 4.1 - Nutrient composition of the beetroot juice and matched control beverages

Nutrient	Beetroot Juice (per 70 mL)	Matched Control (per 70 mL)
Energy (kcal)	77.00	77.00
Protein (g)	2.50	2.50
Fat (g)	0.35	0.35
Carbohydrate (g)	17.00	17.00
Of which sucrose (g)	15.29	15.29
Of which glucose (g)	0.67	0.67
Of which fructose (g)	0.50	0.50
Fibre	0.50	0.50
Sodium	0.03	0.03

Values based on unpublished data from Eurofins Food Testing UK Ltd

4.3. Modelling of insulin sensitivity

An index of insulin sensitivity (S_I) was calculated using the oral glucose minimal model of Caumo et al. (2000). The actual equation used was reported by Burattini and colleagues (2009) in their investigation of insulin action and secretion which used a model based on the oral glucose tolerance test. It is an amalgamation of the classic minimal model of glucose kinetics coupled with an equation describing the rate of appearance of glucose into the circulation (Caumo et al., 2000). The following equation describes the model:

$$S_I = \frac{f \cdot \frac{D}{W} \cdot \frac{AUC[\,\Delta G(t)/G(t)\,]}{AUC\,[\Delta G(t)]} - GE \cdot AUC\,[\Delta G(t)/G(t)]}{AUC\,[\Delta I(t)]}$$

Where AUC denotes the area under the curve of the quantities in brackets during the total time course (t) 0 - (t) 150, $\Delta G(t)$ and $\Delta I(t)$ are glycaemia and insulinaemia above baseline respectively. GE is glucose effectiveness and f is the fraction of glucose appearing in the systemic circulation, values of 3.7×10^{-2} $min^{-1} \cdot dL \cdot kg^{-1}$ and 0.87 respectively are used in accordance with those reported by Burattini et al. (2009). $\frac{D}{W}$ is

the dose of glucose per kg of body weight and is calculated individually for each subject.

4.4. Human intervention trial

4.4.1. Materials and Methods

4.4.1.1. Subjects and study design

Ethical approval was sought and obtained from Oxford Brookes University Ethics Committee (approval number: 110553; appendix 4). Seventeen healthy adults (7 Male, 10 Female) were recruited by poster for this study; all subjects were provided with a participant information sheet (appendix 7) and provided written informed consent (appendix 6). A power calculation was completed based on an effect size of 0.25 and a mean difference of 0.7 mmol/L in blood glucose which revealed that the selected sample size delivered adequate power to detect differences in glucose (0.97) and insulin (0.92). Subject characteristics are provided in table 4.2. Subjects were aged 18-45 years and were only excluded if they had a history of disturbed postprandial blood glucose or insulin, any chronic disease affecting blood glucose such as CVD, cancer, diabetes or metabolic conditions, were over 45 years of age, obese (BMI>30) or had a fasting blood glucose measurement of >5.6 mmol/L.

Table 4.2: Participant characteristics; data are displayed as mean ± standard deviation (SD).

	Age *(yrs)*	**Height** *(cm)*	**Weight** *(kg)*	**BMI** *(kg/m^2)*	**Fat** *(%)*
Mean ±SD	26.5 ± 4.2	172.1 ± 9.8	70.5 ± 14.6	23.5 ± 2.6	23.5 ± 8.3

Participants were asked to visit the laboratory on three separate occasions. Before the first visit each participant was asked to complete a confidential health questionnaire pertaining to their medical history (appendix 1), a questionnaire detailing habitual physical activity (appendix 2), and a modified food frequency questionnaire (FFQ) designed to provide an estimate of their habitual food and polyphenol intake (appendix 3). The FFQ was based on a previously validated questionnaire and adapted to include foods considered to be rich sources of phytochemicals using the phenol explorer database and the USDA food tables. Each visit was separated by no less than 48 hours. At the start of each visit anthropometric measurements were taken and adherence to a 12 hour fast, abstinence from caffeine and alcohol and adequate hydration were assured verbally. Participants provided two baseline finger prick samples separated by 5 minutes before consuming the test meal. In a randomised, single-blind, crossover design, three test meals were provided to each subject. All testing and analyses were carried out by the author at Oxford Brookes University.

4.4.1.2. Study protocol

The first meal (BEET) consisted of a 70 mL beetroot juice shot (James White Drinks Ltd, Ipswich, UK) containing 71 Kcal, 17g CHO, 2.5 protein, 0.5g fibre and 0.1g sodium and 74g white bread (Hovis medium sliced white bread, Hovis, UK); a total of 50g of available CHO. The second meal (MCON) consisted of a matched control drink (70 mL) containing 79Kcal, 15.294g sucrose, 0.499g fructose, 0.665g glucose (providing 16.624g CHO), 3g pea protein isolate (providing 2.5g protein, 0.33g CHO, 0.09g fat and 0.03g sodium), 0.575g Inulin (providing 0.506g fibre and 0.046g CHO), 0.178g sodium chloride and 74g white bread providing a total of 50g of available CHO. The third meal (BR) contained 112g white bread providing 50g available CHO (70 mL water was available to participants in the third test). On each test day postprandial finger

prick blood samples were taken at 5, 15, 30, 45, 60, 90, 120 and 150 minutes to measure both blood glucose (5 µL) and plasma insulin (300 µL). Plasma was separated from whole blood by centrifugation (4,000 rpm for 10 minutes) and stored at -80°C for no more than 1 month.

FFQs were analysed using the phenol explorer database (Neveu et al., 2010) to estimate habitual polyphenol intake. Habitual physical activity was assessed using the physical activity index proposed by Baecke at al. (1982). Briefly, participants were asked to answer a short questionnaire asking them to list their activities during a typical week. These included questions related to their profession such as time spent sitting, questions relating to their leisure time such as 'time spent watching television' as well as 'time spent cycling/walking to work', and questions relating to sport and exercise such as 'what is your main sport' and 'how often do you take part in it'. Based on a scale, proportionate to the levels of physical exertion required to perform the activity, each question is given a value between 1 and 5, with the exception of sport and exercise which is calculated as a product of the average energy used for the activity (MJ), intensity of performance and the time spent performing. Plasma samples were analysed for insulin using a radio-immuno assay on an automated immunoanalyser (Cobas e 411, Roche Diagnostics, Indianapolis, USA).

4.4.1.3. Statistical Analyses

A number of different parameters were of interest. A mixed model ANOVA with two factors ('meal' and 'time') was conducted but the assumed covariance matrix was discovered to be significantly different from the observed matrix. Therefore, comparisons of the whole data were made by repeated measures ANOVA for glucose and insulin at each time point after homogeneity of variance and normality had been

confirmed. Main effects were compared for the within subjects factor 'meal' and corrected with a bonferroni adjustment. Both incremental area under the curve (iAUC) and segmental area under the curve (sAUC) were also calculated and compared using repeated measures ANOVA. Insulin sensitivity data obtained from the model provided small values owing to the relatively low dosage of CHO administered. Individual variation in glucose and insulin responses were clearly apparent in the model because the outcome values were small and therefore a log transformation was applied to the data to reduce the influence of outliers and reduce the impact of the data skew.

4.5. Results

4.5.1. Glycaemic Response

Incremental AUC analysis revealed no significant differences in the glycaemic response between BEET and MCON. Both BEET and MCON resulted in a significantly lower glycaemic response than BR ($P < 0.05$). Segmental AUC analysis revealed that BEET and MCON only displayed a significantly lower glycaemic response than BR at 0-90 minutes and 0-150 minutes ($P < 0.05$) until this time there was no difference in glycaemic response for the three conditions (Figure 4.1). No significant differences in glycaemic response were detected between BEET and MCON in the sAUC analysis. Repeated measures analysis by time point revealed that blood glucose was lower for BEET than for MCON at 15 minutes although this did not reach significance ($P = 0.06$). MCON was significantly higher ($P < 0.05$) than BR at 15 minutes and significantly lower ($P < 0.05$) than BR at 45, 60 and 90 minutes. BEET was significantly lower ($P < 0.05$) than BR at 60, 90 and 120 minutes (table 4.3).

Table 4.3 - Blood glucose and Insulin response data

Glycaemic Response

Time (mins)	MCON		BEET		BR	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***
-5	4.72	0.37	4.62	0.48	4.68	0.57
0	4.78	0.42	4.78	0.50	4.69	0.54
Mean	**4.75**	**0.36**	**4.70**	**0.44**	**4.68**	**0.55**
5	5.12	0.52	5.13	0.68	4.94	0.35
15	6.50 [a]	0.62	6.08	0.79	5.84 [b]	0.65
30	6.12	0.91	6.16	0.79	6.32	0.87
45	5.31 [a]	0.85	5.48	0.85	5.88 [b]	1.01
60	4.78 [a]	0.75	4.82 [a]	0.63	5.68 [b]	0.78
90	4.99 [a]	0.74	4.83 [a]	0.55	5.39 [b]	0.65
120	4.77	0.42	4.62 [a]	0.53	5.09	0.75
150	4.56	0.50	4.38	0.55	4.74	0.66

Insulin Response

Time (mins)	MCON		BEET		BR	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***
-5	9.12	3.96	8.24	3.09	8.59	3.47
0	9.48	3.50	8.11	2.29	9.17	4.09
Mean	**9.30**	**3.48**	**8.17**	**2.38**	**8.88**	**3.48**
5	25.82 [a]	15.18	18.43	12.68	16.38 [b]	15.20
15	48.03 [a]	20.40	34.40 [b]	13.16	29.62 [b]	21.86
30	43.00	20.41	38.44	14.55	32.84	13.08
45	28.08	13.07	26.84	15.06	29.62	16.95
60	19.57	13.21	19.81	12.57	22.34	12.58
90	14.73	11.54	12.75	7.26	18.09	10.09
120	10.63	7.49	10.28	4.92	13.19	7.57
150	7.81 [a]	5.01	6.48 [a]	3.16	9.75 [b]	5.71

BEET = beetroot meal, MCON = matched control meal, BR = white bread meal. [a] significantly different from BR (P<0.05). [b] significantly different from MCON (P<0.05). Values are given in mmol/L for glycaemic response and µU/mL for insulin response. Data are displayed as mean ± standard deviation (SD, n=17).

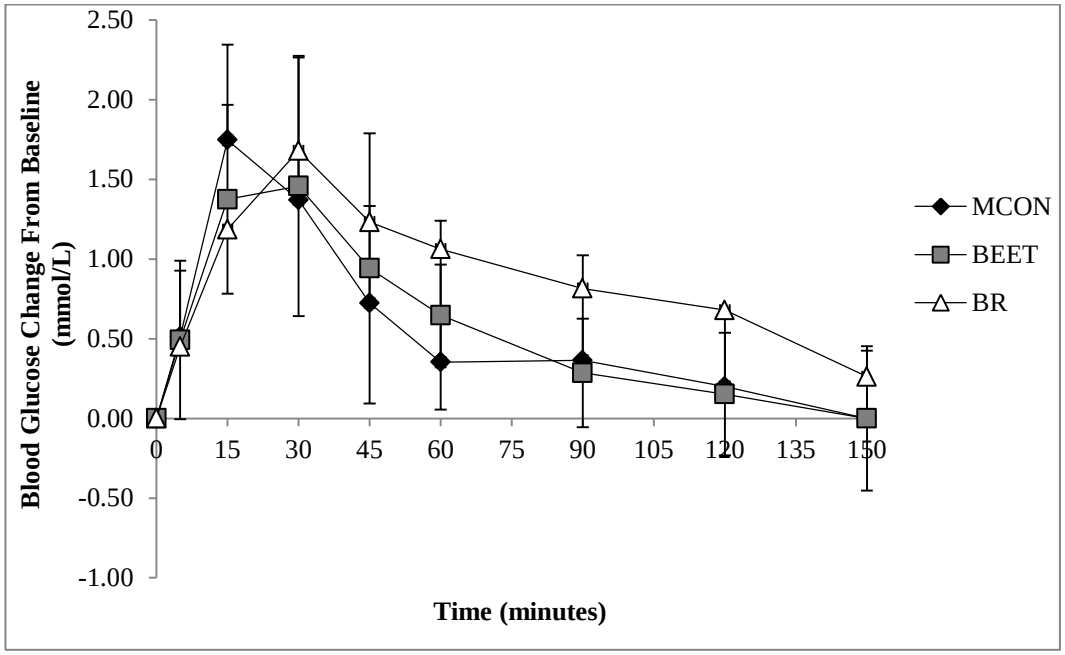


Figure 4.1 - Incremental blood glucose response. Data are displayed as mean ± SD (n = 17), for clarity, negative error bars are shown for 'beetroot' and positive error bars are displayed for 'MCON'. Error bars are not displayed for the bread condition but were comparable in magnitude. BEET = beetroot meal, MCON = matched control meal, BR = white bread meal.

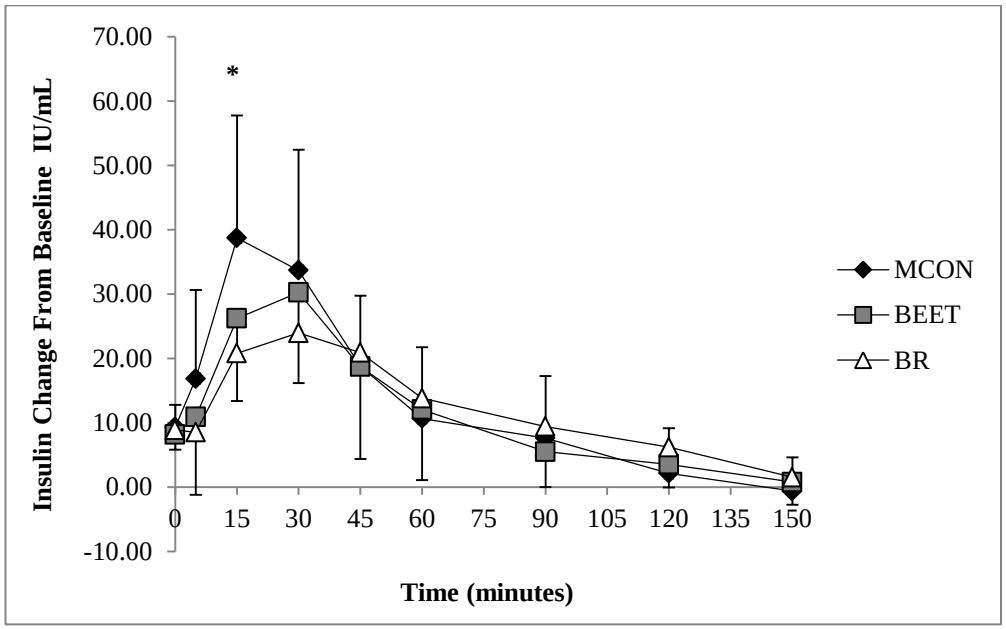


Figure 4.2 - Incremental blood insulin response. Data are displayed as mean ± SD (n = 17), for clarity, negative error bars are shown for 'beetroot' and positive error bars are displayed for 'MCON'. Error bars are not displayed for the bread condition but were comparable in magnitude. BEET = beetroot meal, MCON = matched control meal, BR = white bread meal. * BEET significantly different from MCON (P<0.05).

4.5.2. Insulin Response

There was no significant difference in iAUC or sAUC between BEET and BR or between MCON and BR (Figure 4.2). Further investigation by time point revealed that the insulin response was significantly lower for BEET compared with MCON at 15 minutes ($P < 0.05$) which corresponded to the lower glucose value also observed at this time point. Additionally, insulin response for MCON was significantly higher ($P < 0.05$) than BR at 5 and 15 minutes. BEET and MCON were significantly lower ($P < 0.05$) than BR at 150 minutes (table 4.3).

Table 4.4: Incremental area under the curve analysis for glucose and insulin

Glycaemic Response

Time (mins)	MCON		BEET		BR	
	Mean	***SE***	***Mean***	***SE***	***Mean***	***SE***
5	1.10	0.30	1.22	0.30	0.91	0.27
15	10.62	1.27	8.91	1.19	8.22	1.47
30	23.38	2.41	19.85	2.07	21.10	2.46
45	14.67	2.73	15.92	2.79	21.72	2.91
60	6.60 [a]	2.03	7.43 [a]	2.50	17.01 [b]	2.93
90	9.97 [a]	3.44	6.63 [a]	2.94	27.01 [b]	5.02
120	8.26 [a]	3.12	4.61 [a]	1.86	19.61 [b]	4.06
150	3.48 [a]	1.49	2.13 [a]	1.00	11.67 [b]	2.91
Total	**78.07 [a]**	14.77	**66.71 [a]**	11.54	**127.25 [b]**	17.81

Insulin Response

Time (mins)	MCON		BEET		BR	
	Mean	***SE***	***Mean***	***SE***	***Mean***	***SE***
5	41.73 [a]	8.48	26.44	7.50	20.00 [b]	8.12
15	276.50 [a]	37.08	182.69 [b]	29.44	142.60 [b]	37.01
30	543.28 [a]	62.06	423.74	42.10	335.22 [b]	46.51
45	393.68	50.27	367.00	45.21	335.79	41.07
60	218.24	37.61	228.66	43.67	257.62	43.09
90	251.29 [a]	74.39	252.54	58.74	345.20 [b]	65.41
120	146.74	54.75	115.50	30.81	215.86	55.20
150	65.03	30.05	47.45	15.79	115.63	37.17
Total	**1936.48**	281.82	**1644.00**	214.31	**1767.82**	247.53

BEET = beetroot meal, MCON = matched control meal, BR = white bread meal. [a] significantly different from BR (P<0.05). [b] significantly different from MCON (P<0.05). Data are displayed as mean ± standard error of the mean (SE, n=17).

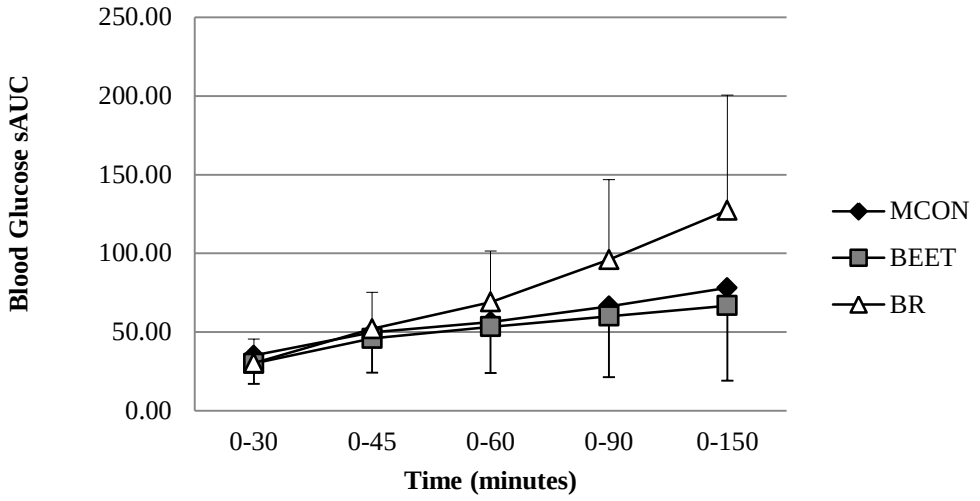


Figure 4.3 - Segmental areas under the blood glucose response curve. Data are displayed as mean ± SD (n = 17), for clarity, negative error bars are shown for 'beetroot' and positive error bars are displayed for 'bread'. Error bars are not displayed for the control condition but were comparable in magnitude. BEET = beetroot meal, MCON = matched control meal, BR = white bread meal.

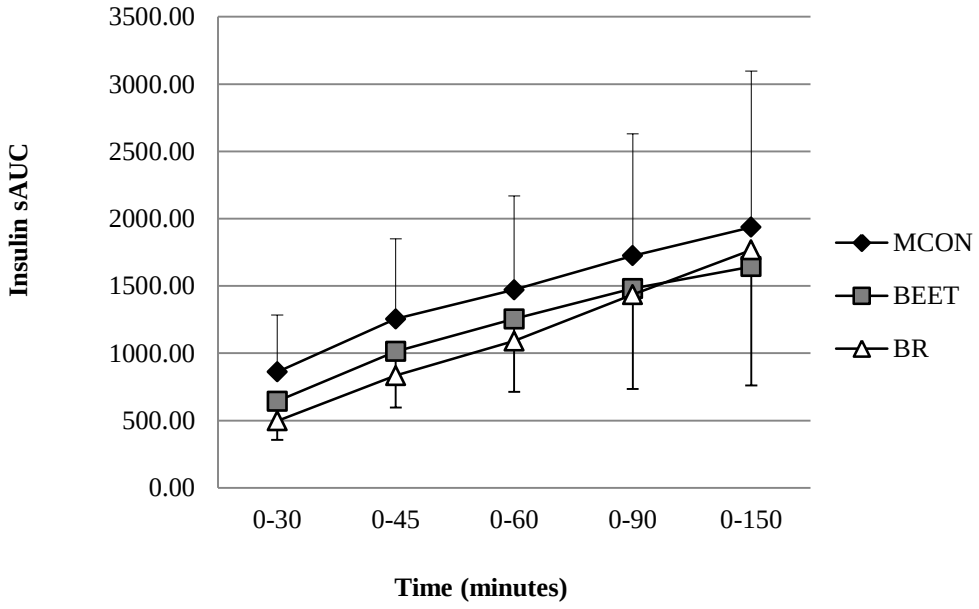


Figure 4.4 - Segmental areas under the blood insulin response curve. Data are displayed as mean ± SD (n = 17), for clarity, negative error bars are shown for 'beetroot' and positive error bars are displayed for 'control'. Error bars are not displayed for the bread condition but were comparable in magnitude. BEET = beetroot meal, MCON = matched control meal, BR = white bread meal.

4.5.3. Physical Activity and Polyphenol Intake

Physical activity was quantified according to Baecke et al. (1982). Physical activity is divided into a 'work' index, a 'sport' index and a 'leisure' index. The mean physical activity index for this cohort was 3.1 ± 0.6. Individually, the work index was 2.4 ± 0.4, the sport index was 3.8 ± 1.5 and the leisure index was 3.1 ± 0.7. These figures indicate an active cohort. Average polyphenol intake is estimated at around 1000 mg per day (Manach et al., 2004). Polyphenol intake for this cohort was 1633.0 ± 533.4 mg per day with a range of 875.6 - 2753.3 mg per day. This suggests a cohort with a relatively high habitual intake of polyphenols.

4.5.4. Insulin Sensitivity

Insulin sensitivity was calculated according to the minimal model proposed by Caumo et al. (2000). Data were log transformed to reduce the impact of inter-individual variability. No significant differences were observed between the conditions however as the standard error was still relatively high, owing to the variability in individual responses, which may have abolished the opportunity to observe differences. Data are displayed as mean and 95% confidence intervals (CI). Mean insulin sensitivity was 1.04 (95% CI = 0.49 - 2.40) × 10^{-5} min^{-1} ·dL· kg^{-1}/ (pmol) for the control condition, 1.14 (95% CI = 0.63 - 2.04) × 10^{-5} min^{-1} ·dL· kg^{-1}/ (pmol) for the beetroot condition and 2.22 (95% CI = 1.46 - 3.38) × 10^{-5} min^{-1} ·dL· kg^{-1}/ (pmol) for the bread condition.

4.6. Discussion

The results of this study did not show a reduction in the postprandial AUC for glucose or insulin. This suggests that either phytochemicals present in the beetroot juice do not influence glycaemic response or that the dose administered in this study (70 mL) was not sufficient for any potential effects to be observed. Some evidence is presented that the BEET meal may produce a lower glucose and significantly lower insulin response 15 minutes after consumption compared with a control, which coincides with the early phase of insulin secretion. This observation suggests that there may be a potential influence of beetroot phytochemicals on postprandial insulin response which was not fully appreciated during this study. Red beetroot juice is a particularly interesting test beverage because of its composition, potentially wide consumption and relatively low production cost. There are several possible mechanisms and substances within beetroot juice which may be individually or collectively responsible for the observed potential changes.

Beetroot contains five classes of antioxidant compounds, contributing to high TAC and TP content as shown in studies 1 and 2 of this thesis. High performance liquid chromatography coupled with electron spray ionisation mass spectrometry (HPLC-ESI-MS) identified examples of betalains (including betaxanthins and betacyanins), phenolics, ferulic acid conjugates, phenolic amides and flavonoids in four beetroot cultivars (Kujala et al., 2002). Previous research has identified flavonoids and hydroxycinnamic acids as compounds which potentially influence CHO metabolism (Welsch et al., 1989; Johnston et al., 2002; Ishikawa et al., 2007). Additionally, betalains have been described as anti-inflammatory inhibitors of cyclooxygenase enzymes (Reddy et al., 2005) and protective against LDL-oxidation (Tesoriere et al., 2004b). Polyphenol intake in this cohort was relatively high (1633.0 ± 533.4 mg/day) in comparison with average intakes, which are reported to be around 1000 mg per day

(Manach et al., 2004), suggesting that polyphenolic control of glycaemia may be commonplace in these individuals.

Digestive enzymes such as α-amylase and α-glucosidase are the first potential targets for polyphenol action (Matsui et al., 2002; Matsui et al., 2007). Furthermore, glucose is absorbed across the membrane of intestinal enterocytes, the enzymes and transporters involved in this process are also potential targets. Beetroot juice is composed of relatively simple mono and disaccharides, limiting the potential influence of α-amylase and α-glucosidase inhibition by polyphenols since these enzymes are primarily involved in starch digestion. The remaining potential targets are other digestive enzymes, aspects within the glucose transport system or aspects within the insulin signalling pathway. Intestinal absorption of glucose relies upon active transport by SGLT-1 and by sodium-independent facilitated transport via the GLUT 2 transporter. There is no direct evidence to suggest that beetroot juice polyphenols are inhibitors of glucose transporters. Based on previous reports it would be reasonable to suggest that these compounds would not be present in sufficient quantities, given the likely concentration and the dosage administered in this study. However, studies have suggested that glucosides formed as a result of metabolism by the gut microflora may be able to inhibit GLUT 2 transport, and this possibility cannot be fully excluded since the microbial metabolites of beetroot phytochemicals have not been well characterised.

A number of other human studies have been designed to investigate the potential for bioactive compounds in a variety of foods to affect either glycaemic or insulinaemic response following the consumption of a standard CHO bolus. Details of these are given in section 1.6.1. Johnston and colleagues (2002) reported that apple juice resulted in lower concentrations of glucose, and impacted upon insulin, GIP and GLP-1 in a manner suggesting delayed intestinal absorption in 9 healthy subjects. Törrönen et al. (2010) showed that a sucrose sweetened purée containing several polyphenol-rich berries significantly lowered postprandial glucose at 15 and 30 minutes ($P < 0.05$) and

delayed the postprandial glucose peak compared with a control sucrose load. The authors indicate that this was due to reduced digestion or absorption of the sucrose in the berry condition. This mirrors the observations made in the current study suggesting that potential alterations in postprandial glycaemia caused by beetroot juice ingestion may occur via a similar process. The preparation of the cells for glucose absorption relies on insulin released by the pancreatic β cells, in response to the glucose content of the blood stream following a meal. Although not significant, and affected by subject variability, a slightly higher mean insulin sensitivity score was observed in the current study for BEET (1.14 (95% CI = 0.63 - 2.04) × 10^{-4} min^{-1} ·dL· kg^{-1}/ (pmol)), compared with MCON (1.04 (95% CI = 0.49 - 2.40) × 10^{-4} min^{-1} ·dL· kg^{-1}/ (pmol)) suggesting that less insulin was released in response to the beetroot meal, or that insulin receptors may not have been responding in the same manner. The stages of the insulin signalling cascade are further potential targets for bioactive compounds which elicit a change in the postprandial glucose condition.

As discussed in section 1.6.3, the insulin signalling cascade also mediates the endogenous production of NO in the vasculature through the PI3-K/MAPK pathway. Interestingly, several studies have reported that the production of NO is reduced in patients with diseases characterised by insulin resistance (Anfossi et al., 2009; Potenza et al., 2009). In theory, it may be possible that NO produced from nitrate in the beetroot juice alters the global utilisation of insulin via interactions in the PI3-K/MAPK pathway, since this method of NO production is independent of the traditional endogenous pathway. Foods rich in phytochemical compounds such as polyphenols have been shown to potentiate NO production from dietary nitrate by scavenging singlet oxygen which would otherwise react with NO to form peroxynitrite and the NO radical (Volk et al., 2009).

4.7. Conclusion

Observations made during the postprandial situation in these healthy subjects suggest that phytochemicals present in beetroot juice did not have a glycaemic lowering effect. Statistical inferences at 15 minutes after consumption did show a significant difference in the plasma insulin response between beetroot juice and a matched control, with a suggestion that glucose may be concurrently lowered. The low dose of beetroot used in this study may not have been sufficient to fully appreciate any potential effects of phytochemicals such as polyphenols of nitrate on postprandial insulin or glucose. The co-ingestion of antioxidant phytochemicals and dietary nitrate in beetroot may lead to interactions which may beneficially impact conditions associated with insulin resistance. However, further research using larger quantities of beetroot juice and without solid-liquid interactions are required to substantiate these hypotheses.

5. Study 4 - Postprandial glycaemic and insulinaemic responses to beetroot juice beverage containing 50g available CHO

5.1. Carbohydrate (CHO) Composition

Carbohydrate composition of BEET and MCON were according to the analysis described in section 4.2. The composition of the beverages is shown in table 5.1.

Table 5.1. Nutrient Composition of the beetroot (BEET) and matched control (MCON) beverages

Nutrient	Beetroot Juice (per 225 mL)	Matched Control (per 225 mL)
Energy (kcal)	222.53	225.37
Protein (g)	5.69	5.69
Fat (g)	0.00	0.08
Carbohydrate (g)	50.00	50.00
Of which sucrose (g)	39.35	39.35
Of which glucose (g)	4.77	4.77
Of which fructose (g)	5.13	5.13
Fibre	1.13	1.13
Sodium	0.09	0.09

Values based on unpublished data from Eurofins Food Testing UK Ltd

5.2. Determination of nitrate, nitrite, betalain and phenolic content of beetroot juice by HPLC-GCMS.

This work was carried out by Dr Kirsten Brandt (Newcastle University) using samples provided. The aim of this research was always to provide the most accurate and insightful conclusions as possible, the equipment, knowledge and skill of Dr Brandt was employed, as this type of analysis could not be accommodated at Oxford Brookes University within the required timescale. The information obtained in this collaboration adds clarity, and allows a greater degree of confidence to be expressed in the results and conclusions of the existing body of work.

5.2.1. Materials and Methods

Nitrate and nitrite concentrations were measured in duplicate in samples from 6 separate bottles by GC-MS as isotope dilution after derivatisation with 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br) according to Tsikas (2000) with minor modifications. Briefly, known concentrations of ^{15}N nitrate and ^{15}N nitrite as internal standards were added to the beetroot juice. 200 µL spiked sample, 800 µL acetone and 20 µL PFB-Br were mixed and incubated for 20 minutes at 50°C in a sealed tube, after which the acetone was evaporated under a nitrogen stream. Two mL toluene and 1 mL water was added and shaken for 1 minute. After phase separation, the upper (toluene) phase was analysed on a GC-MS (Shimadzu Corporation, Japan) with an Optima 17 column (15 m, 0.25 mm i.d., 0.25 µm film thickness), using negative-ion chemical ionization (NICI), splitless mode, helium (70 kPa) as column carrier, methane (200 Pa) as reagent. Initial column temperature was 70 °C, held for 1 minute then increased 30 °C/minute to 280°C, with electron energy 230 eV and electron current 300 µA, ion source 180°C, interface 280 °C and injector 200 °C. ^{15}N and ^{14}N nitrate were measured

at m/z 63 and 62, respectively, with retention time (RT) 3.2 minutes, ^{15}N and ^{14}N nitrite at m/z 47 and 46, respectively, with RT 3.4 minutes.

Betalains and phenolic compounds were measured using the method of Nemzer et al. (2011) on a Shimadzu Prominence HPLC with a Luna C-18 analytical column, 25cm, 3 mm i.d., 5 µm particle size (Phenomenex, Torrance, CA, USA) and an SPD-M20A diode array detector. Solvents were A, acetonitrile and B, 2% formic acid in water. The gradient was 3% A at 0 minutes, 16% A at 17 minutes, 50% A at 30 minutes with 0.5 ml per minute flow rate, 35°C column temperature and 10µl injection volume. Peaks were recorded at 538 nm (betanins), 480 nm (betaxanthins and neobetanin), 505 nm (decarboxylated betanins) and 320 nm (phenolic compounds). Quantification of betanin, isobetanin, 17-decarboxyisobetanin and neobetanin was done by comparison with standards isolated by preparative HPLC from the beetroot juice using the same equipment and solvents, on a Develosil ODS-HG-5 HPLC column (RP-18, 250 × 20 mm i.d.), 5 µm particle size. The gradient was 3% A at 0 minutes, 16% A at 17 minutes, 50% A at 30 minutes with 3 ml per minute flow rate, 35°C column temperature and 400µl injection volume. Quantification of flavonoids and phenolic acids was done by comparison with authentic standards of rutin and chlorogenic acid, respectively, measured at 320 nm. Four peaks had UV-spectra that matched typical flavonoid spectra and 18 peaks were similar to phenolic acids.

5.2.2. Results

Average nitrate concentration was 4.40 g/L, ranging from 3.03 to 5.24 g/L in the 6 individual bottles, with an average relative standard deviation within samples of 2.5%. Average nitrite concentration was 2.34 mg/L, ranging from 2.23 to 2.55 mg/L in individual bottles, with an average relative standard deviation within samples of 2.0%. There was no correlation between nitrate and nitrite concentrations when comparing individual bottles, $R^2 = 0.147$ and $P = 0.781$ (table 5.2).

Table 5.2 - Betalain composition of the beetroot juice beverage

Compound	**Concentration** g/L	**Standard Error of the Mean** g/L	**Relative Standard Deviation** %
Sum of betaxanthins (yellow native pigments)	0.0146	0.0011	17.73
Sum of betanins (purple native pigments)	0.215	0.023	25.78
Sum of betanin degradation products	5.975	0.068	2.81
Sum of flavonoids (rutin equivalents)	0.32314	0.00069	0.52
Sum of phenolic acids (chlorogenic acid equivalents)	0.2482	0.0046	4.50
Betanin	0.08368	0.00796	23.29
Isobetanin	0.1311	0.0148	27.63
17-decarboxy-isobetanin	0.02256	0.00040	4.29
Neobetanin	5.617	0.065	2.86

The value for neobetanin is adjusted for a low recovery (47.5%) measured by quantification of a purified sample with analytical HPLC and compared with the spectrophotometric value. The recovery was even less during semi-preparative HPLC. This compound is possibly degraded during passage through the HPLC column, since all other compounds showed good correlations between absorption on the spectrophotometer and the HPLC peak area.

5.2.3. Discussion

The contents of nitrate and nitrite are within the ranges previously found for processed beetroot products (Tamme et al., 2010). The nitrite levels are relatively low when compared with results obtained by Tamme et al. (2010), indicating that little or no bacterial denitrification has occurred during processing and storage. The content of partially degraded betanins tentatively identified as the orange pigment neobetanin was exceptionally high compared with literature values on other processed beetroot products, which showed values from near 0 to a maximum of 9.5% of the total pigment content (Nemzer et al., 2011). This is, however, in line with the also exceptionally high betanin-isobetanin ratio, 1:1.61, (literature range 1:0.21 – 1:1.27, Nemzer et al., 2011), low content of betaxanthins and high content of 17-decarboxy-isobetanin, indicate extensive degradation of betalain pigments in this product (Herbach et al., 2004b). The much higher coefficient of variation for the native pigments than for the non-betalain types of phenolic compounds indicate that some of the pigment degradation took place after the juice was distributed into the containers, while the non-betalain compounds did not degrade.

5.3. Human intervention trial 2 - justification

This study builds upon study 3 and also employs the principles of the glycaemic index (GI) which ranks carbohydrate rich foods based on their ability to elicit a postprandial rise in blood glucose. The rise in blood glucose is regulated by insulin released from the pancreatic β cells in response to CHO consumption. This rise is attenuated in three situations; the absorption of glucose is reduced, the release of insulin is increased or the sensitivity of cells towards insulin is enhanced. In recent years a number of research

studies have focused on the interaction of plant based bioactive components with each stage of postprandial blood glucose response. The outcome measures of these studies suggest that some plant compounds such as anthocyanins and flavanols may, by virtue of their structure, alter the postprandial glucose response. This is achieved by inhibiting the binding of glucose to its intestinal transporters, by stimulating production of insulin in the pancreatic β cells or by rendering cells more sensitive to insulin release, such that a lesser insulin response is necessary for the same degree of glucose control. Flavanols, specifically catechins found in green tea, have been shown to modify the postprandial blood glucose condition. Fukino et al. (2007) showed that consumption of a green tea extract containing 456mg catechins reduced fasting glycated haemoglobin (measured using haemoglobin A1c) in a 2 month testing period.

Furthermore, Bryans and colleagues (2007) showed that 1g tea extract reduced later phase glucose response with an accompanying rise in plasma insulin. Somewhat more recently, Grussu et al. (2011) and Törrönen et al. (2010) showed that berry polyphenols (namely anthocyanins and proanthocyanidins) inhibited the activity of α-amylase, the principle enzyme concerned with starch digestion, particularly in an extract enhanced with proanthocyanidins. Study 3 showed that when 70 mL of beetroot juice was given to healthy subjects in combination with white bread (a total available CHO intake of 50g), a significantly lower insulin response was elicited at 15 minutes post consumption in comparison with a matched control meal (containing the same amount of CHO) with a corresponding reduction (not-significant) in postprandial glucose at the same time point. Although mechanistically unclear, these results suggested that there was potential for bioactive components of beetroot such as polyphenols or betalains to modify the postprandial glucose condition. Similar results were observed in another study whereby subjects consumed either 1g or 3g of cinnamon together with rice pudding (Hlebowicz et al., 2009). Three grams of

cinnamon elicited a significant reduction in postprandial insulin concentration with no discernible change in blood glucose. The effect was attributed to the signalling effects of chromium and polyphenols (procyanidin and catechin derivatives) in cinnamon. The present study aims to build upon initial research to further explore the potential role of bioactive compounds from beetroot in modifying postprandial blood glucose and insulin.

By comparing beverages containing beetroot with a matched CHO source control together with a positive glucose control, the pharmacokinetics of the beetroot action can be further elucidated. Insulin is released in response to a rise in blood glucose and catalyses the uptake of glucose into the cells for storage or use. The degree of insulin release depends upon two major factors; firstly the sensitivity of the cells to insulin and secondly, the amount of glucose in the blood. Our research showed that there was an attenuated rise in plasma insulin following consumption of the CHO bolus. A significant reduction in insulin, measured using time point analysis was observed at 15 minutes. Hypothetically, this effect may be mechanistically similar to the effects of cinnamon. Cao et al. (2008) and Hlebowicz et al. (2009) proposed that the attenuation in plasma insulin was due to an improvement in insulin receptor function elicited by the effects of cinnamon polyphenols on signalling cascades. The enhancement in insulin receptor function essentially makes the cell more sensitive to insulin and thus improves the efficiency of glucose uptake. An improvement in insulin receptor function has significant implications for both type 2 diabetic patients but also those who suffer from subclinical insulin resistance as a facet of the metabolic syndrome. It is estimated that 1 million people suffer from type 2 diabetes in the UK and many more exhibit some degree of insulin resistance (Diabetes UK, 2011). The aim of this study was to further investigate the effects of beetroot juice phytochemicals on postprandial glycaemia. The potential for a lower insulin response to be elicited by beetroot juice is assessed using a

higher dosage of beetroot juice than in study 3. Furthermore, solid-liquid interactions are ruled out by utilising only beverages.

The hypothesis to be tested in this study was "an increase in the volume of beetroot juice administered, and the removal of the solid food component, will increase the effects of beetroot juice phytochemicals on postprandial glucose and insulin". The study aims to discover whether 225 mL of beetroot juice will deliver sufficient phytochemicals to reduce either the postprandial glucose concentration or reduce the postprandial insulin concentration without affecting blood glucose.

5.4. Materials and Methods

5.4.1. Subjects and Study Design

Ethical approval was sought and obtained from Oxford Brookes University Ethics Committee (approval number: 110596; appendix 5). Sixteen healthy adults were recruited by poster for this study. All subjects were provided with a participant information sheet (appendix 8) and provided informed consent (appendix 6). Subject characteristics are provided in table 5.3. As in the previous study a power calculation was completed based on the mean and standard deviation of glucose and insulin in study 3. An effect size of 0.25 was again selected and the calculation revealed that the selected sample size delivered adequate power to detect statistical differences in both glucose and insulin (0.90; 0.89). Subjects were aged 18-45 years and were only excluded if they had a history of disturbed postprandial blood glucose or insulin, any chronic disease affecting blood glucose such as CVD, cancer, diabetes or metabolic conditions, were over 45 years of age, obese (BMI>30) or had a fasting blood glucose measurement of >5.6 mmol/L.

Table 5.3. Subject Characteristics, data are displayed as mean ± SD.

	Age *(yrs)*	**Height** *(cm)*	**Weight** *(kg)*	**BMI** *(kg/m^2)*	**Fat** *(%)*
Mean ± SD	26.5 ± 4.7	172.4 ± 8.6	69.2 ± 11.7	23.3 ± 2.8	23.0 ± 7.0

Data are provided as mean ± standard deviation (n=16). BMI = body mass index (kg/m^2).

Participants were asked to visit the laboratory on three separate occasions. Before the first visit each participant was asked to complete a confidential health questionnaire pertaining to their medical history (appendix 1), a questionnaire detailing habitual physical activity (appendix 2), and a modified FFQ designed to provide an estimate of their habitual food and polyphenol intake (appendix 3). Each visit was separated by no less than 48 hours. At the start of each visit anthropometric measurements were taken and adherence to a 12 hour fast, abstinence from caffeine and alcohol and adequate hydration were assured verbally. Participants provided two baseline finger prick samples separated by 5 minutes before consuming the test beverage. In a randomised, single-blind, crossover design, three test beverages were provided to each subject.

5.4.2. Study Protocol

The first beverage (BEET) consisted of a 225 mL beetroot juice (James White Drinks Ltd, Ipswich, UK) containing 222.5 Kcal, 50g CHO, 5.69 protein, 1.13g fibre and 0.08g sodium. According to the phytochemical analysis the beetroot juice beverage delivered 3.29 mg total betaxanthins, 48.38 mg total betanins, 1344.38 mg total betanin degradation products, 72.71 mg flavonoids, and 55.85 mg phenolic acids. The second beverage (MCON) consisted of a matched control drink (225 mL) containing 225 Kcal,

39.35g sucrose, 5.13g fructose, 4.77g glucose (providing 49.35g available CHO), 6.94g pea protein isolate (providing 5.69g protein, 0.76g CHO, 0.21g fat and 0.07g sodium), 1.28g Inulin (providing 1.125g fibre and 1.23g CHO), 0.178g sodium chloride. The third beverage (CON) contained 50g glucose in 225 mL of water. On each test day postprandial finger prick blood samples were taken at 5, 15, 30, 45, 60, 90, 120 and 150 minutes to measure both blood glucose (5 µL) and plasma insulin (300 µL). Plasma was separated from whole blood by centrifugation (4,000 rpm for 10 minutes) and stored at -40°C for no more than 1 month.

FFQs were analysed using the phenol explorer database (Neveu et al., 2010) to estimate habitual polyphenol intake and were analysed as previously described in section 4.4.3.2. Habitual physical activity was assessed using the physical activity index proposed by Baecke at al. (1982) as described in section 4.4.3.2. Plasma samples were analysed for insulin using a radio-immuno assay on an automated immunoanalyser (Cobas e 411, Roche Diagnostics, Indianapolis, USA). All testing was carried out by the author at Oxford Brookes University.

5.4.3. Insulin Sensitivity

Measures of insulin sensitivity were calculated using the same method as in section 4.3.

5.4.4. Statistical Analyses

Data were analysed by repeated measures ANOVA. Comparisons were made for total iAUC (0-150 minutes), and by individual time point after homogeneity of variance and normality were confirmed. In order to identify particular sections of the responses which differed, comparisons were also made by segment (sAUC). The segments analysed were 0-30 minutes, 0-45 minutes, 0-60 minutes, and 0-90 minutes. Statistical

outcomes were corrected using a bonferroni adjustment. Insulin sensitivity data from the model by Burattini et al. (2009) was log transformed to reduce the influence of inter-individual variability.

5.5. Results

5.5.1. Glycaemic Response

No significant differences were observed between MCON and BEET during the total glycaemic response (iAUC). Both the MCON and BEET conditions produced a significantly lower glycaemic response than the glucose (GLUC) condition ($P<0.05$) as expected (Figure 5.1, Table 5.4). Analysis of the AUC by segment (sAUC) revealed a significantly lower glycaemic response for BEET compared with MCON in the 0-30 minute segment ($P<0.05$) although this did not remain evident in the 0-45 minute segment analysis ($P = 0.13$) (Figure 5.3). BEET was also significantly lower than GLUC in the 0-30 minute and 0-45 minute segments whilst no significant differences were observed between MCON and GLUC. In the 0-60 minute segment BEET remained significantly lower than GLUC ($P<0.05$) whilst no significant differences were observed between MCON and GLUC or between MCON and BEET. Both MCON and BEET were significantly lower than GLUC ($P<0.05$) in the 0-90 minute segment. Whole data were also analysed by time point (Table 5.4). There were no significant differences between the conditions at T0, BEET was significantly lower ($P<0.05$) than MCON at T5, and no significant differences were observed at T15. BEET was significantly lower ($P<0.05$) than GLUC at T30, BEET and MCON were both significantly lower than GLUC at T45, T60 and T90. BEET remained significantly higher ($P<0.05$) than GLUC at both T120 and T150. No other significant differences were observed at these time points.

Table 5.4 - Whole data for glycaemic and insulin responses

Glycaemic Response

Time (mins)	MCON		BEET		GLUC	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***
-5	4.49	0.40	4.66	0.37	4.55	0.32
0	4.47	0.39	4.63	0.34	4.65	0.28
Mean	**4.48**	**0.38**	**4.64**	**0.35**	**4.60**	**0.29**
5	5.47	0.79	5.15[b]	0.61	5.01	0.63
15	6.57	1.13	6.26	0.79	6.66	0.82
30	6.16	1.43	5.95[a]	0.94	6.94	1.28
45	4.71 [a]	1.11	5.11[a]	0.84	6.15 [b]	1.12
60	4.16 [a]	0.80	4.56 [a]	0.68	5.40 [b]	0.71
90	4.06 [a]	0.45	4.18 [a]	0.56	4.71 [b]	0.59
120	4.19	0.44	4.30 [a]	0.49	3.99	0.51
150	4.16	0.46	4.24[a]	0.48	3.91	0.59

Insulin Response

Time (mins)	MCON		BEET		GLUC	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***
-5	8.77	3.66	8.19	3.75	7.34	5.26
0	7.07	1.93	7.64	3.42	8.32	5.52
Mean	**7.92**	**2.52**	**7.91**	**3.40**	**7.83**	**5.29**
5	32.86	23.18	21.90	12.64	22.31	19.59
15	53.86	29.10	39.28[b]	23.73	37.64	22.85
30	46.87	28.29	35.70	27.25	39.96	34.14
45	23.69	13.32	21.81	14.50	30.13	22.93
60	13.22	6.71	16.89	9.35	19.21	15.24
90	6.08	3.57	9.07	6.97	8.76	8.57
120	4.36	2.18	5.33[a]	3.56	4.11	3.66
150	4.89	2.06	3.75	3.28	3.24	2.10

BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. [a] significantly different from GLUC (P<0.05). [b] significantly different from MCON (P<0.05). Values are given in mmol/L for glycaemic response and µIU/mL for insulin response. Data are displayed as mean ± standard deviation (SD, n=16).

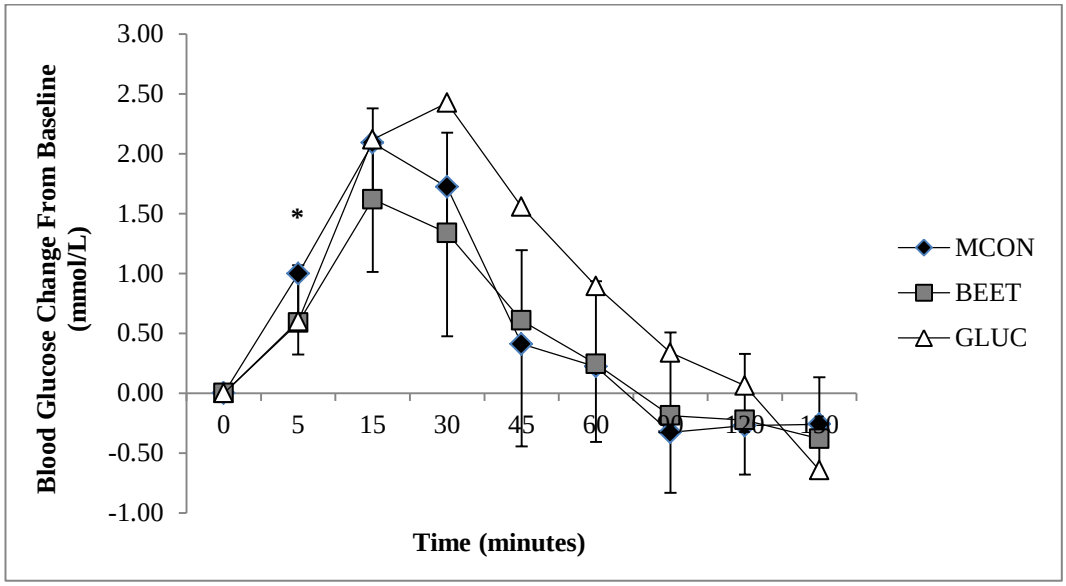


Figure 5.1 - Incremental blood glucose response. Data are displayed as mean ± SD (n = 16), for clarity, negative error bars are shown for 'MCON' and positive error bars are displayed for 'BEET'. Error bars are not displayed for the GLUC condition but were comparable in magnitude. BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. * BEET significantly different from MCON (P<0.05).

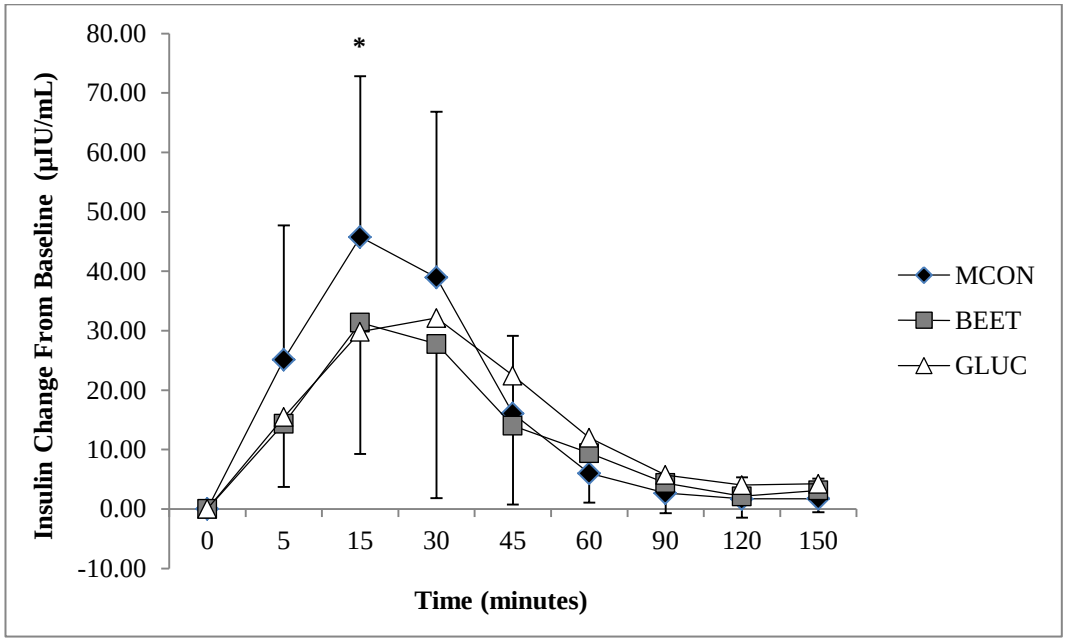


Figure 5.2 - Incremental blood insulin response. Data are displayed as mean ± SD (n = 16). For clarity, negative error bars are shown for 'BEET' and positive error bars are displayed for 'MCON'. Error bars are not displayed for the GLUC condition but were comparable in magnitude. BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. * BEET significantly different from MCON (P<0.05).

5.5.2. Insulin Response

Incremental area under the curve (iAUC) analysis revealed no significant difference between the three conditions for insulin response (table 5.5). The data were separated into the 0-30, 0-45, 0-60 and 0-90 minute segments and further analysed (figure 5.4). The BEET condition elicited a significantly lower insulin response in the 0-30, 0-45 and 0-60 minute segments ($P<0.05$ for each) compared with MCON. Neither MCON nor BEET differed significantly from GLUC in these segments. There were no significant differences observed between the three conditions in the 0-90 minute segment. The whole data were also analysed by time point. There were no significant differences between the three conditions at T0. A non-significant, lower insulin response was observed for BEET compared with MCON at T5 ($P = 0.08$), which reached significance at T15 ($P<0.05$) and remained evident, although not significant at T30 ($P = 0.09$). There were no differences between MCON and GLUC at these time points. There were no significant differences between the three conditions at T45, T60, T90 and T150. The BEET condition was shown to elicit a significantly higher insulin response than MCON at T120 ($P<0.05$), although the actual values are relatively small.

Table 5.5 - Incremental area under the curve for glucose and insulin

Glycaemic Response

	MCON		BEET		GLUC	
Time (mins)	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**
5	2.73	1.91	1.37	1.29	1.27	1.46
15	15.41	7.01	10.72	5.71	14.07	7.38
30	28.29	15.94	21.95	9.96	33.88	12.75
45	15.50	14.58	13.54	10.24	28.74	17.83
60	3.40	8.28	5.61	7.16	17.94	13.72
90	1.60	3.87	3.89	6.67	16.76	16.33
120	0.67	1.90	1.55	3.68	3.55	5.65
150	1.36	2.88	1.29	2.51	0.22	0.49
Total	**68.96**	44.44	**59.92**	33.49	**116.43**	59.63

Insulin Response

	MCON		BEET		GLUC	
Time (mins)	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**
5	62.57	56.74	35.42	27.26	37.42	33.91
15	353.51	206.97	226.97	143.75	221.86	132.57
30	635.30	333.89	443.63	345.71	464.59	348.92
45	397.23	291.62	312.62	268.22	408.31	368.91
60	165.57	115.99	172.51	148.12	253.45	226.94
90	82.15	107.69	169.92	177.42	215.77	289.43
120	8.39	21.32	43.49	93.86	54.57	118.80
150	4.67	18.68	4.37	14.29	5.02	13.42
Total	**1693.91**	**841.70**	**1408.93**	**959.27**	**1660.98**	**1109.22**

BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. Data are displayed as mean ± SD (n=16).

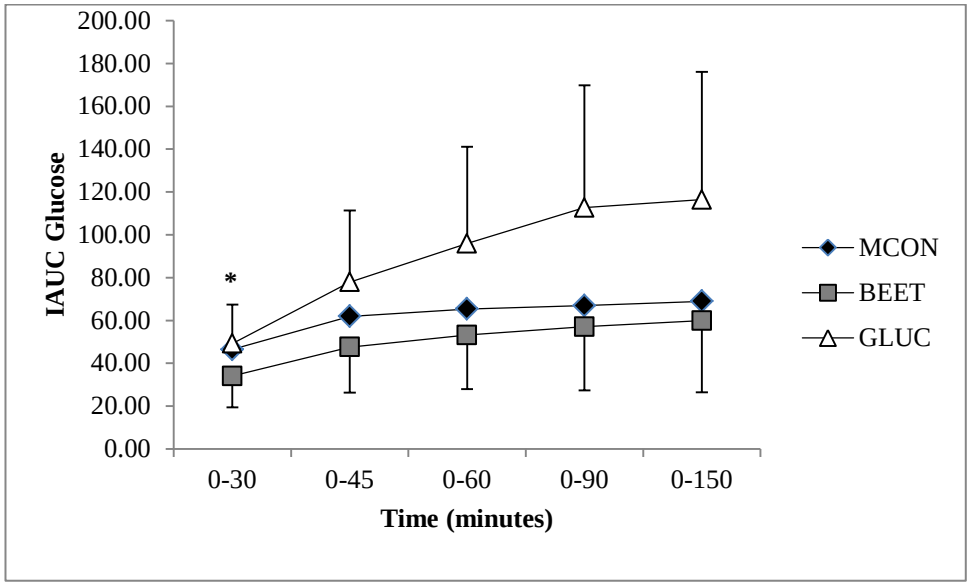


Figure 9 - Segmental areas under the blood glucose response curve. Data are displayed as mean ± SD (n = 16). For clarity, negative error bars are shown for 'BEET' and positive error bars are displayed for 'GLUC'. Error bars are not displayed for the control condition but were comparable in magnitude. BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. * BEET significantly different from MCON (P<0.05).

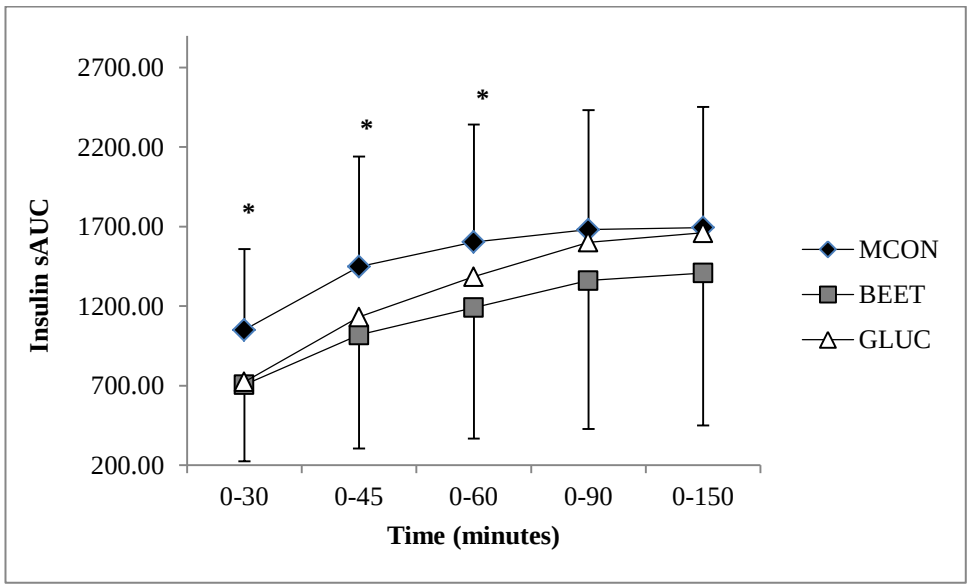


Figure 10 - Segmental areas under the blood glucose response curve. Data are displayed as mean ± SD (n = 16). For clarity, negative error bars are shown for 'BEET' and positive error bars are displayed for 'MCON'. Error bars are not displayed for 'GLUC' but were comparable in magnitude. BEET = beetroot beverage, MCON = matched control beverage, GLUC = glucose beverage. * BEET significantly different from MCON (P<0.05).

5.6. Physical Activity and Polyphenol Consumption

Physical activity was quantified according to Baecke et al. (1982). Physical activity is divided into a 'work' index, a 'sport' index and a 'leisure' index. The mean physical activity index for this cohort was 3.11 ± 0.75. Individually, the work index was 2.44 ± 0.25, the sport index was 3.71 ± 2.06 and the leisure index was 3.19 ± 0.49. These figures indicate an active cohort. Polyphenol intake for this cohort was 1925.7 ± 592.0 mg per day with a range of 853.4 - 2807.3 mg per day. This suggests a cohort with a relatively high habitual intake of polyphenols.

5.7. Insulin Sensitivity

Insulin sensitivity was calculated according to the minimal model proposed by Caumo et al. (2000). Data were log transformed to reduce the impact of inter-individual variability. No significant differences were observed between the conditions however, once again, the standard error was still relatively high. Data are displayed as mean and 95% confidence intervals (95% CI). Mean insulin sensitivity was 1.05 (95% CI = 0.55 - 2.01) $\times 10^{-5}$ min^{-1} ·dL· kg^{-1}/ (pmol) for the control condition, 1.68 (95% CI = 0.86 - 3.29) $\times 10^{-5}$ min^{-1} ·dL· kg^{-1}/ (pmol) for the beetroot condition and 2.10 (95% CI = 1.08 - 4.06) $\times 10^{-5}$ min^{-1} ·dL· kg^{-1}/ (pmol) for the glucose condition.

5.8. Discussion and Conclusion

Study 3 employed a mixed meal composed of white bread and a 70 mL beverage as the test conditions together with a white bread positive control meal. One of the test beverages was beetroot juice and the other was matched, for CHO content, to the beetroot juice. In the current study, the bread was removed to prevent any influence of solid-liquid interactions on the overall responses. The available CHO load was kept constant at 50g and this was achieved by increasing the volume of the beetroot juice and the matched control beverages to 225 mL. The increase in the volume of beetroot juice inevitably increased the amounts of polyphenolic compounds, betalains and nitrate consumed by the participants. The study aimed to provide clarity regarding the differences observed in study 3.

The glycaemic and insulinaemic responses of the participants in the current study followed a similar pattern to study 3. There was a similar glycaemic response across the three conditions with the exception of a significantly lower glycaemic response at 5 minutes ($P<0.05$) which persisted, although not significantly, across the proceeding minutes, sufficient to cause a significantly lower glycaemic response ($P<0.05$) to be detected in the 0-30 minute sAUC for BEET compared with MCON. The moderately attenuated glycaemic response is accompanied by a similarly attenuated early-phase insulin response. A significantly lower insulin response was detected at 15 minutes for BEET compared with MCON with further lower responses measured at 5 and 30 minutes which did not reach significance ($P = 0.08$; $P = 0.09$ respectively). These differences were sufficient to cause significant differences to also be observed in the sAUC segments between 0-30, 0-45 and 0-60 minutes for BEET compared to MCON. These results suggest, although not conclusively, that bioactive compounds within beetroot juice may have the potential to help control postprandial glycaemia.

Beetroot juice contains a number of bioactive components including betaxanthins, betanins, flavonoids, phenolic acids and betanin degradation products produced by thermal processing. The concentration of betaxanthin is relatively low (3.29 mg/225 mL), as is the concentration of intact betanins (48.38 mg/225 mL). The betanins were divided into 18.83 mg betanin and 29.50 mg isobetanin. Both flavonoids and phenolic acids were present in moderately low concentrations (72.71 mg/225 mL; 55.85 mg/225 mL respectively). The most abundant compounds in the beetroot juice were betanin degradation products formed, presumably, by thermal processing techniques. These were divided into two predominant compounds, 17-decarboxy-isobetanin (5.08 mg/225 mL) and the orange pigment; neobetanin (1263.83 mg/225 mL). A recent enquiry demonstrated similarly attenuated glycaemic and insulin responses with combinations of polyphenols.

Törrönen et al. (2012b) fed 14 healthy participants a blackcurrant juice beverage containing 50g sucrose fortified with crowberry powder, which doubled the polyphenol content compared with the placebo beverage. The crowberry powder was a particularly rich source of anthocyanins, a group within the flavonoid family and the test beverage provided 293 mg /100 mL polyphenols. The postprandial glycaemic response in the study by Törrönen et al. (2012b) followed a similar pattern to the current study with no significant differences overall, but a larger (although not significant) glucose and insulin response observed in the early phase (up to 30 minutes) for the control condition compared with the test condition, despite a higher amount of available CHO in the test condition. There was also a slower decline in both glucose and insulin for the test condition compared with the control which reached significance at 90 minutes ($P<0.05$), this effect is visually evident in figure 7, but does not approach significance. Törrönen and colleagues (2012c) suggest that their results are a result of the higher polyphenol

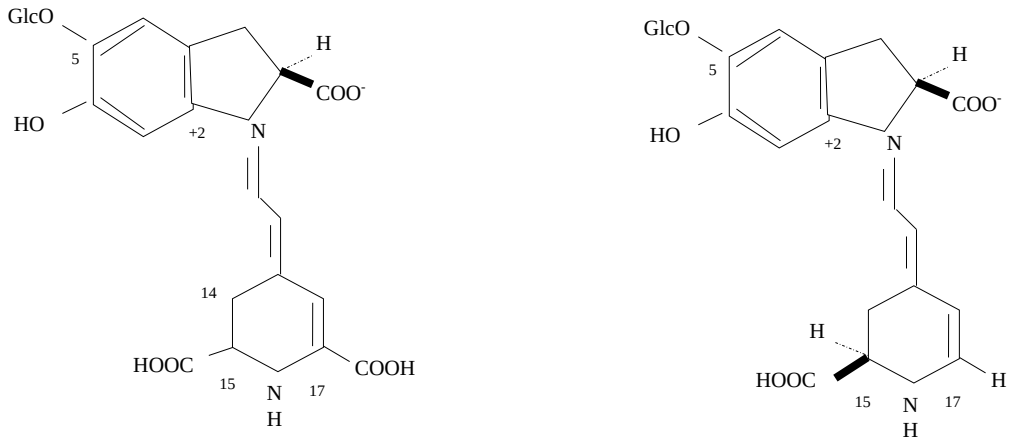
content of the test juice, and assign reduced digestion of sucrose and/or a slower release of glucose in the gut as the likely mechanism(s).

The addition of berries, rich in polyphenols, to a sucrose load has previously been shown to attenuate the postprandial glucose and insulin responses in adults (Törrönen et al., 2010; Törrönen et al., 2012a; Törrönen et al., 2012c). Each of these studies suffered similarly in that inter-individual variation was large, reducing the ability to detect real differences. In the current study, the mean values for the three conditions were actually quite different, but a large standard deviation reduced the significance of the outcome. Törrönen and colleagues (2012c) measured both glucose and insulin responses to a mixture of berries (150g) and 35g sucrose in both capillary and venous samples. No difference was observed between capillary and venous sampling, although capillary sampling has previously been suggested to be more accurate (Brouns et al., 2005). The authors reported the same attenuation of glucose and insulin at 15 minutes with a delayed return to baseline until 90 minutes.

The release and uptake of insulin is central to the absorption and transport of glucose from an ingested meal. Meals containing a large amount of CHO elicit a proportionate rise in plasma glucose which stimulates a rapid rise in blood insulin, termed insulinaemia. Repeated bouts of hyperglycaemia and hyperinsulinaemia may result in insulin resistance or transient hypoglycaemia owing to the rapid rise and fall of blood glucose. Insulin resistance is central to the development of type 2 diabetes and is one pillar of the metabolic syndrome, diseases which place a high economic burden on global societies (Popkin, 2011). Polyphenols and related compounds have been described to reduce both postprandial hyperglycaemia and prevent reactive hyperinsulinaemia by reducing the digestion, absorption and transport of glucose as discussed in sections 4.6 and 5.2 respectively. This study suggests that betanins,

particularly the degradation product neobetanin may have comparable effects, either alone or in combination with flavonoids and phenolic acids contained in beetroot juice.

Previous studies have reported polyphenol intakes of 300-800 mg as sufficient to attenuate postprandial glycaemia when consumed alongside CHO (Törrönen et al., 2012b; Törrönen et al., 2012c). The beetroot juice beverage in the current study provided a total of 129 mg of polyphenols together with 1393 mg of betanins and betanin degradation products, in particular 1263 mg neobetanin. Neobetanin was identified in the 1980's as the yellow- orange pigment 5-*O*-β-D-glucopyranosylneobetanidin isolated from red beetroot (Alard et al., 1985; Wyler, 1986; Strack et al., 1987). Initially it was suggested to be an artefact of the isolation process as Wyler (1986) was not able to detect its presence in fresh beetroot juice. It was not until work by Herbach and colleagues (2004a) assessing the impact of thermal treatments on the colour and pigment patterns in red beetroot that neobetanin was rediscovered as a naturally occurring degradation product produced by prolonged thermal processing at high temperature. Herbach and colleagues (2004a) heated continually stirred red beetroot juice to 85°C in a water bath and took regular aliquots over the proceeding 8 hour period to monitor the effect of the heat treatment on the red betanin pigments. Each aliquot was analysed by HPLC with diode array detection (HPLC-DAD) and LC-MS. Betanin and isobetanin concentrations fell to 10-20% of the original whilst the concentration of neobetanin rose by 50% as a result of dehydrogenation. The ratio of isobetanin to betanin also rose, showing that isomerisation of betanin also occurs with heat treatment. Further isomers of neobetanin were also identified depending on the loss of a carboxyl moiety at different structural locations (Herbach et al., 2004a). The structures of neobetanin and 17-decarboxy-isobetanin which were found in the beetroot juice sample are shown in figure 5.5.



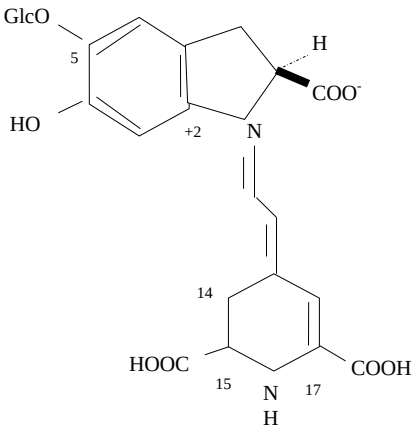
Neobetanin 17-Decarboxy-isobetanin

Figure 5.5 - Structures of neobetanin (yellow pigment) and 17-decarboxy-isobetanin (orange-red pigment) adapted from Herbach et al. (2004).

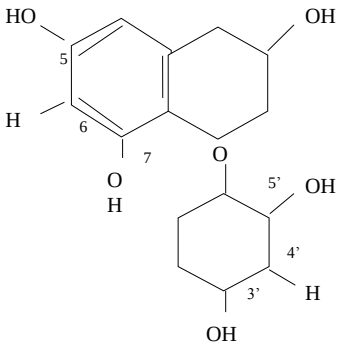
As has been discussed previously, the potential beneficial effects of phytochemicals from plant foods on the control of postprandial glycaemia may be due to a reduced digestion of glucose, reduced absorption of glucose, stimulation of insulin release or alterations in insulin signalling/sensitivity. The reduced glucose response in the 0-30 minute period following consumption of the beetroot beverage and the persistently reduced insulin response over the first 60 minutes also observed following ingestion of the beetroot beverage in this study suggest that either glucose uptake is blunted, resulting in reduced insulin secretion, or that less insulin is required to restore glucose homeostasis. There is not sufficient evidence presented in this study to eliminate any potential mechanism, nor are the differences observed in the responses sufficient to confirm any of the potential mechanisms. The insulin sensitivity model which was applied to the data did not reveal any significant differences between the three conditions, which may be largely due to the apparent inter-individual variability of human glycaemic response data. The beetroot condition did result in a higher insulin sensitivity score (1.68 (95% CI = 0.86 - 3.29) × 10^{-5} min^{-1} ·dL· kg^{-1}/ (pmol)) than the matched control condition (1.05 (95% CI = 0.55 - 2.01) × 10^{-5} min^{-1} ·dL· kg^{-1}/ (pmol))

which did not approach significance (*P*=0.24). However, the difference between the beetroot condition and the control condition in the current study was greater than the difference observed between the mean insulin sensitivity for the same two conditions in the previous study; 0.10 in the previous study compared to 0.63 in the current study. This may suggest that if beetroot juice phytochemicals play a role in improving insulin sensitivity, then this effect is increased in a dose dependent manner.

The high concentration of betalains found in the beetroot juice, particularly betanin degradation products such as neobetanin, suggests that they are the most likely compounds to be responsible for any of the alterations observed. However, the potential influence of polyphenolic compounds cannot be discounted, despite the relatively low (129 mg) concentration present in the juice. Betalains bear certain structural similarities to the anthocyanin group of polyphenolic compounds, a group which has also been shown to alter postprandial glycaemia (Törrönen et al., 2012b). The structures of neobetanin and cyanidin are shown in figure 5.6 for comparison.



Neobetanin



Cyanidin

Figure 5.6 - The structures of neobetanin and cyandin shown for comparison

A review of 97 bioavailability studies showed that ingested polyphenolic compounds reach the low µM range in the plasma (Manach et al., 2005). These concentrations were observed for flavanols and catechins, whilst anthocyanins reached only high nM concentrations. There are no studies to date assessing the bioavailability or metabolism of betalain degradation products in humans. Tesoriere et al. (2004a) examined the absorption, excretion and distribution of betalains from cactus pear fruit in human LDL. Tesoriere and colleagues (2004b) showed that betanin contained in cactus pear fruit reached a maximum plasma concentration of 0.2 µmol/L after approximately 3 hours following an intake of 16 mg which indicates that betalains reach the plasma in relatively high concentrations compared with anthocyanins. Assuming a linear relationship between the intake of betalains and appearance in the plasma, and that betalain degradation products are absorbed at similar rates, the maximum concentration which could possibly be achieved in the current study is 17.45 µmol/L; this does not take into account any potential polyphenolic compounds which may be available.

The simulated *in vitro* digestion protocol which was employed in study 2 suggests that compounds in beetroot may become more potent in terms of their reducing capability as they pass through the digestive tract, particularly after the gastric phase of digestion. Any influence of polyphenols on glycaemic response clearly occurs in the early phase of glucose uptake and is unlikely, therefore, to be influenced by the duodenal phase of digestion, although any long term improvements which may exist as a result of repeated habitual intake could certainly benefit from duodenal phase metabolites. Betalains have a short half life in the plasma and red blood cells (~5 hours) (Tesoriere et al., 2005) which may limit their potential function to processes lasting only a short period such as glucose digestion and absorption. For this potential effect to be prolonged, the desired compounds would need to be ingested in sufficient quantity, at each eating episode containing CHO.

The potential for bioactive phytochemicals to modify substrate metabolism in the postprandial state is a developing area of interest. A recent report by Murase et al. (2012) investigated the effects of coffee polyphenols (caffeic acid derivatives) on whole body metabolism, including substrate utilisation in a mouse model. Murase and colleagues (2012) were able to demonstrate that co-ingestion of coffee polyphenols with a starch-lipid emulsion significantly lowered blood glucose at 10 minutes without significantly altering the glucose AUC accompanied by a significant lowering of the insulin response, similar to the results displayed in the current study. The authors were also able to demonstrate a suppression of the emulsion induced postprandial respiratory quotient (RQ) value, plasma triglycerides (TAG) and GIP concentrations. Tiwari et al. (2011) investigated the effects of Indian vegetable juices on postprandial hyperglycaemia in rats. The authors noted that whilst modest intake of polyphenols lowered the postprandial glycaemic response, there was a significant correlation ($P<0.01$) between TP content and 2 hour glycaemic load. This suggests that a very high intake may actually have the opposite effect and reverse the anti-hyperglycaemic effects of the juices. The fibres and polyphenols in sea buckthorn have been shown to elicit a delay in lipaemia, producing a delay in TAG increase following a meal without a corresponding total TAG concentration (Linderborg et al., 2012). Hyperglycaemia, hyperinsulinaemia and hyperlipidemia are crucial in the development of CVD, type 2 diabetes, and metabolic syndrome, and whether or not they are able to be modified by dietary components is of considerable ongoing interest.

6. Concluding remarks and implications for future work

This work presents evidence that beetroot juice contains a high amount of reducing compounds capable of scavenging free radicals, chelating metal ions and reducing a variety of chemical compounds compared with other varieties of vegetable juices as shown in study 1. This study was the first to measure the antioxidant capacity and total polyphenol content of commercially available vegetable juices in the UK and the first to analyse these juices using 6 methods including the recently developed CUPRAC and CERAC assays. The reducing power of beetroot juice is comparable with that of established high-antioxidant fruits such as pomegranate and cranberry and contains a high amount of polyphenolic compounds. These results suggested that beetroot juice may contain compounds which could influence the postprandial glycaemic condition in a similar way to berries which contain similar compounds and had previously been shown to influence postprandial glucose and/or insulin. This first study had some limitations. Firstly that the ORAC method was not utilised limiting the comparisons which can be drawn between this study and those conducted in the USA. Secondly the biochemical methods chosen are not necessarily representative of biological processes. Including methods such as lipid peroxidation inhibition may help to make the results more relevant to human biology.

Study 2 showed that these compounds are highly stable throughout an *in vitro* digestion procedure designed to mimic the gastric and duodenal phases of digestion, although the colonic fate of these compounds is yet to be determined. Beetroot juice phytochemicals significantly ($P<0.05$) increased following the gastric phase of digestion and remained above the pre-digestive level following the duodenal phase. The Folin Ciocalteu method to determine polyphenols was also applied to the juice, showing that the beetroot juice contained a high level of compounds capable of reducing the Folin Ciocalteu complex, namely phenolics and ascorbic acid. These results suggested

that beetroot juice phytochemicals may survive digestion and be available to influence biological processes in the postprandial state. The *in vitro* digestion procedure only gives an indication as to the potential bioaccessibility of compounds after digestion and is limited in its applicability to actual bioavailability. A further series of studies utilising cellular models to more closely understand the likely bioavailability of these compounds would give a clearer indication as to whether or not they are available at sufficient concentration to affect biological processes. Furthermore, it would be useful to include a colonic phase investigation to understand the array of secondary metabolites which may be produced after the duodenal phase that may contribute towards any long term impact of beetroot juice phytochemicals on health.

The first human intervention trial (study 3) suggested that beetroot juice significantly lowered the postprandial insulin response at 15 minutes ($P<0.05$) without modification of the glucose response. This was the first investigation of beetroot juice phytochemicals in the modulation of postprandial glycaemia and seemed to suggest that even at modest doses (70 mL) there may be some effect of beetroot juice on postprandial insulin concentration in a similar pattern to that observed when berries are fed alongside a CHO load. The study was limited considerably by the low dose of beetroot juice administered and by the potential for interactions between the solid and liquid components of the meal which may have abolished or accentuated differences which either were, or may have been observed between the conditions.

A second human intervention trial (study 4) showed that beetroot juice elicited a significant suppression of postprandial glycaemia in the 0-30 minute sAUC ($P<0.05$) and postprandial insulinaemia in the 0-30, 0-45 and 0-60 sAUC ($P<0.05$) when compared with a control beverage matched for CHO content, which was comparable with that observed using berries in other investigations. The study replicated the results of study 3 without the interference of solid-liquid interactions and with a higher dosage

of beetroot juice. The higher dosage appeared to make the differences in postprandial insulin more clearly apparent and extended the time when differences could be detected to 60 minutes after consumption. HPLC and GCMS analysis of the beetroot juice revealed that the predominant compounds in this product are the yellow and orange pigments derived from betanin. Neobetanin in particular was identified in large quantities and the analysis also revealed moderate amounts of both intact betanin and polyphenolic compounds. It is suggested; therefore, that neobetanin alone, or in combination with polyphenolic compounds, either reduces the amount of glucose absorbed into the blood stream thus reducing the required insulin response, or improves the sensitivity of the body's cells to insulin. Insulin sensitivity models applied to the data suggested that insulin sensitivity in the tested cohorts increased as the dose of beetroot juice increased, although this did not reach significance.

Additional investigations are warranted to elucidate how beetroot juice phytochemicals are metabolised in the colonic environment and to discover how these compounds interact with the gut microflora. Further human investigations are needed to examine the effects of compounds from beetroot juice in isolation, or in combination with phenolic compounds, on other measures of the postprandial glycaemic, insulinaemic and lipaemic responses such as TAG, cholesterol, GLP, and GIP. Further bioavailability studies utilising both cellular and mathematical models are also required to understand the incorporation of beetroot juice phytochemicals into the blood, and whether this occurs at physiologically relevant concentrations. In these studies it would be important to consider the effects of beetroot juice before and after thermal processing techniques whereby neobetanin would not be produced at such high levels. The impact of dietary nitrate in combination with phenolic compounds is also of considerable interest from a public health perspective, since polyphenols have been shown to potentiate the production of nitrate. It will be important that future studies incorporate

measures of cardiovascular health to align the potential effects of nitrate such as the lowering of blood pressure and improvement in endothelial function with those of phenolic compounds. Conditions such as the metabolic syndrome are key targets for the use of beetroot juice therapeutically since it often manifests with co-morbidities such as cardiovascular complications together with type 2 diabetes and obesity allowing for several parameters to be improved within a single intervention. In order to fully elucidate the efficacy of beetroot products as therapeutic agents in the prevention and treatment of these conditions there will also need to be large scale clinical investigations with sub-clinical and clinical populations.

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Appendices

1. - Copy of Health Screening Questionnaire used in Studies 3 & 4

Confidential Health Screening Questionnaire

OXFORD BROOKES UNIVERSITY

Participant's Name:_____Study Name:_____

Date of Birth:_____

It is important that volunteers participating in research studies are currently in good health and have had no important medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If YES to any question, please describe briefly in the spaces provided

(eg to confirm problem was/is short-lived, insignificant or well controlled.)

1 At present, do you have any health problem for which you are: *(Please tick as appropriate)*

(a) on medication, prescribed or otherwise	**Yes**	☐	**No**	☐
(b) attending your general practitioner	**Yes**	☐	**No**	☐
(c) on a hospital waiting list	**Yes**	☐	**No**	☐

If yes please specify

_____

_____

_____

2 In the past two years, have you had any illness which required you to:

(a) consult your GP	**Yes**		**No**	
(b) attend a hospital outpatient department	**Yes**		**No**	
(c) be admitted to hospital	**Yes**		**No**	

If yes please specify

_____

_____

_____

3 Have you ever had any of the following:

(a) Convulsions/epilepsy	**Yes**		**No**	
(b) Asthma	**Yes**		**No**	
(c) Eczema	**Yes**		**No**	
(d) Diabetes	**Yes**		**No**	
(e) A blood disorder	**Yes**		**No**	
(f) Neurological problems (e.g. Alzheimer's disease)	**Yes**		**No**	
(g) Digestive problems	**Yes**		**No**	
(h) Heart problems	**Yes**		**No**	
(i) Problems with bones or joints	**Yes**		**No**	
(k) Ear / hearing problems	**Yes**		**No**	
(l) Thyroid problems	**Yes**		**No**	
(n) Kidney or liver problems	**Yes**		**No**	
(o) Fainting problems	**Yes**		**No**	
(p) Breathing problems/diseases (e.g. COPD)	**Yes**		**No**	
(q) Cancer	**Yes**		**No**	
(r) Cardiovascular disease (e.g. atherosclerosis)	**Yes**		**No**	

If yes please specify…

_____

_____

_____

4 Do you have allergies to any food types:

Yes ☐ **No** ☐

If yes please specify…..

_____

Signed:_____ Date:_____

2. - Copy of Habitual Physical Activity Questionnaire used in Studies 3 & 4

Subject number:

Habitual Physical Activity Questionnaire

Please circle the response most applicable to each of the statements or answer the questions. **All of the results will be strictly confidential and will be available only to the researcher.** This is a previously validated questionnaire.

Thank You

1. What is your main occupation?

2.	At work I sit	never	seldom	sometimes	often	always
3.	At work I stand	never	seldom	sometimes	often	always
4.	At work I walk	never	seldom	sometimes	often	always
5.	At work I lift heavy loads	never	seldom	sometimes	often	always

6.	After work I'm tired	very often	often	sometimes	seldom	never
7.	At work I sweat	very often	often	sometimes	seldom	never
8.	In comparison with others of my own age, I think my work is physically	much heavier	heavier	as heavy	lighter	much lighter
9.	Do you play sport?	Yes	No			
	If Yes:					
	Which sport do you play most frequently					
	……...……					
	How many hours a week?	<1	1-2	2-3	3-4	>4
	How many months a year?	<1	1-3	4-6	7-9	>9
	If you play a second sport:					

	Which sport is it? ……..					
	How many hours a week?	<1	1-2	2-3	3-4	>4
	How many months a year?	<1	1-3	4-6	7-9	>9
10.	In comparison with other of my own age I think my physical activity during leisure time is	much more	more	the same	less	much less
11.	During leisure time I sweat	very often	often	sometimes	seldom	Never
12.	During leisure time I play sport	never	seldom	sometimes	often	very often
13.	During leisure time I watch television	never	seldom	sometimes	often	very often
14.	During leisure time I walk	never	seldom	sometimes	often	very often

15.	During leisure time I cycle	Never	seldom	sometimes	often	very often
16.	How many minutes do you walk and/or cycle per day to and from work, school and shopping?	<5	5-15	15-30	30-45	>45

3. - Copy of Food Frequency Questionnaire (FFQ) used in Studies 3 & 4

Food Frequency Questionnaire

Subject number:

Instructions:

FOOD AND AMOUNTS	AVERAGE USE LAST MONTH

PLEASE READ THE INSTRUCTION CAREFULLY BEFORE FILLING THE QUESTIONNAIRE.

- **This questionnaire asks for some background information about your diet**
- **We would like you to answer each question as best as you can.**
- **If you are unsure about how to answer a question do the best you can and please do not leave a question blank.**
- **For each food item there is an amount shown, either an average portion size or usual household unit such as 1 slice of bread, 1 teaspoon of sugar. Please put a tick (√) in the box to indicate how often, on average you have eaten the specified amount of each food over the last month.**

Example

For white bread the average amount is one slice, so if you ate 5 slices in a week, you need to put a tick in the column headed 5 per week.

BREAD AND ROLLS	Once a week	2 per week	3 per week	4 per week	5 per week	6 per week	7 per week	Every 2 to 3 weeks	Rarely or never
White bread					√				

BREAD AND ROLLS	**Once a week**	**2 per week**	**3 per week**	**4 per week**	**5 per week**	**6 per week**	**7 per week**	**Every 2 to 3 weeks**	**Rarely or never**
White bread (1 slice)									
Brown bread (1 slice)									
Wholemeal bread (1 slice)									
Chapattis and Naan									
Rye bread (1 slice)									
CEREALS									
Weetabix (1 biscuit)									
Allbran (1 portion)									
Muesli (1 portion)									
Porridge (1 portion)									
Shredded wheat (1)									
Blueberry wheats									
VEGETABLES									
Potatoes (5 medium size)									
Chips (10 average)									
Celery (1 stick)									
Kale (1 handful)									
Carrots (1 handful)									
Red Beetroot (1 average)									
Red Cabbage (1 handful)									

Artichokes (1 whole)									
Aubergines (1 whole)									
Broccoli (4 stems)									
White Onions (1 whole)									
Red Onions (1 whole)									
Soya/soya beans (20g)									
Rhubarb (1 stick)									
Tomatoes (2 whole)									
Capsicum peppers (1/2)									
Salad leaves (1/4 bag)									
Radicchio (1 whole)									
Chillies (1 whole)									
Cauliflower (4 stems)									
Mushrooms (4 whole)									
Cabbage/sprouts (handful)									
Cucumber (1/4)									
Sweetcorn (2 tbsp)									
Spinach (handful)									
Food	**Once a week**	**2 per week**	**3 per week**	**4 per week**	**5 per week**	**6 per week**	**7 per week**	**Every 2 to 3 weeks**	**Rarely or never**
FRUITS									
Melon (1 slice)									
Dark plums (2 units)									
Blackberries (10 units)									

Pomegranates (1/2)									
Cranberries (10 units)									
Acai berries (5 units)									
Blueberries (10 units)									
Cherries (5 units)									
Raspberries (10 units)									
Redcurrents (10 units)									
Ligonberries (10 units)									
Chokeberries (10 units)									
Blackcurrents (10 units)									
Pears (1 whole)									
Apples (1 whole)									
White grapes (10 units)									
Red grapes (10 units)									
Strawberries (5 units)									
Orange/Lemon (1 whole)									
Kiwi									
NUTS AND PULSES									
Peanut butter (1 tbsp)									
Brazil nuts (3 whole)									
Peanuts (10 whole)									
Almonds (6 whole)									
Hazelnuts (6 whole)									
Walnuts (6 halves)									
Coconut (1/2 or 1 tbsp)									

Lentils (boiled - 1 tbsp)									
Chickpeas (1 tbsp – cook)									
Hummus (1 tbsp)									
Red kidney beans (1 tbsp)									
Baked beans (2 tbsp)									
PUDDINGS & SWEET SNACKS (PORTION)									
Milk chocolate (4 units)									
Dark chocolate (4 units)									
Plain Chocolate (4 units)									
Oat Biscuits (2)									
Chocolate Biscuits (2)									
Cereal bars (1)									
Jams/jellies (1 tbsp)									
DRINKS (200ML = 1)									
Pure Orange juice									
Pure Apple juice									
Pure Pomegranate juice									
Pure Cranberry juice									
Pure Blueberry juice									
Pure Mango juice									
Pure Tomato juice									
Pure Beetroot juice									
Pure Prune juice									

Pure Carrot juice									
Other vegetable juice									
Mixed dark fruit juice									
Mixed light fruit juice									
Green tea									
Black tea (English)									
Rooibos,Oolong, other tea									
Fruit juice (not pure)									
Red wine									
Coffee									
HERBS AND SPICES									
Cinnamon (1/2 tsp)									
Turmeric (1/2 tsp)									
Cumin (1/2 tsp)									
Basil (1 tbsp)									
Parsley (1 tbsp)									

Is there any other fruit, vegetable, cereal, bread or drink item (such as smoothies, home baking) or supplement (i.e. vitamin C tablet) that you ate more than once a week? If yes, please list them below

FOOD	**Once a week**	**2 per week**	**3 per week**	**4 per week**	**5 per week**	**6 per week**	**7 per week**	**Every 2 to 3 weeks**	**Rarely or never**
SUPPLEMENTS									

OTHER FOODS									

Thank you for completing this questionnaire. Please take a moment to review each page and answer each question you may have skipped.

4. Ethical approval for the commencement of study 3

Professor Jeya Henry and
Dr. Lisa Ryan, Director of Studies
School of Life Sciences
Gipsy Lane Site
Oxford Brookes University

University Research Ethics Committee
Headington Campus, Gipsy Lane, Headington, Oxford OX3 0BP U
t. +44 (0)1865 483484
ethics@brookes.ac.uk

1st June 2011

Dear Professor Jeya Henry and Dr Lisa Ryan

UREC Registration No: 110553: "The effects of polyphenol-rich beetroot juice consumption on glycaemic and insulinaemic response to high-GI meal"

Thank you for your letter of the 19th May 2011 outlining your response to the points raised in my previous letter about the PhD study of your **research student Peter Wootton-Beard,** and attaching the revised documents.

My apologies for this later reply but the documentation seems to have gone astray in the internal post and only reached me yesterday, 31st May 2011.**I am pleased to confirm, that on this basis, I have given full approval for the study to commence.**

The UREC approval period for this study is two years from the date of this letter, so the1st June 2013. If you need the approval to be extended please do contact me nearer the time of expiry.

In order to monitor studies approved by the University Research Ethics Committee, we will ask you to provide a (very brief) report on the conduct and conclusions of the study in a year's time. If the study is completed in less than a year, could you please contact me and I will send you the appropriate guidelines for the report.

Yours sincerely

Dr Elizabeth T Hurren

Chair of the University Research Ethics Committee

cc Peter Wootton-Beard, PhD student

Louise Wood, UREC administrator

Jill Organ, Graduate School

Dick Craven, UREC Officer, Life Sciences

5. Ethical approval for the commencement of study 4

Dr Lisa Ryan, Director of Studies

Functional Food Centre

Department of Sport and Health Sciences

Faculty of Health and Life Sciences

Oxford Brookes University

Gipsy Lane Campus

19th December 2011

Dear Dr Lisa Ryan

UREC Registration No: 110596: "The effects of beetroot juice consumption on insulin response in healthy adults"

Thank you for your email of the 19th December 2012 outlining your response to the points raised in my previous letter about the PhD study of your **research student Peter Wootton-Beard,** and attaching the revised documents. **I am pleased to inform you that, on this basis, I have given Chair's Approval for the study to begin.**

The UREC approval period for this study is two years from the date of this letter, so the 19th December 2013. If you need the approval to be extended please do contact me nearer the time of expiry.

In order to monitor studies approved by the University Research Ethics Committee, we will ask you to provide a (very brief) report on the conduct and conclusions of the study in a year's time. If the study is completed in less than a year, could you please contact me and I will send you the appropriate guidelines for the report.

Yours sincerely

Dr Elizabeth T Hurren

Chair of the University Research Ethics Committee

cc Peter Wootton-Beard, Research Student

Dick Craven, Research Ethics Officer

Jill Organ, Graduate Office

Louise Wood, UREC Administrator

6. Informed consent form for study 3 and study 4

CONSENT FORM

Full title of Project: The effects of polyphenol- rich beetroot juice consumption on blood glucose and blood insulin response

Mr Peter Wootton – Beard,

PhD Researcher, Functional Food Centre, Oxford Brookes University, Gipsy Lane, Oxford, OX3 0BP

Email: peter.wootton-beard@brookes.ac.uk; **Tel**: 01865 48(3283)

Dr Lisa Ryan

Email: lisaryan@brookes.ac.uk; **Tel**: 01865 48(3199)

Please initial box

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason. ☐

3. I agree to take part in the above study. ☐

		Please tick box	
		Yes	**No**
4.	I agree to have 11 finger prick blood samples taken on on test day 1, test day 2 and on test day 3	☐	☐
5.	I understand that all personal data will be processed for the purposes of research and in conformity with the Data Protection Act	☐	☐
6.	I understand that although every effort will be made, the relatively small sample size in this study means that my complete de-identification may not be possible to maintain	☐	☐

Name of Participant	Date	Signature
Name of Researcher	Date	Signature

7. Participant Information Sheet for Study 3

Principal investigator: Mr Peter Wootton-Beard
PhD Researcher, Functional Food Centre, Oxford Brookes University
Gipsy Lane, Oxford, OX3 0BP

Email: peter.wootton-beard@brookes.ac.uk
Contact: 01865 48(3283)

Participant Information Sheet

Study title

The effects of polyphenol- rich beetroot juice consumption on blood glucose and blood insulin responses to a high GI meal

Invitation paragraph

You are being invited to take part in a research project. This sheet contains information to help you decide whether you wish to participate. Please read it carefully and discuss it with family and friends if you wish. If there is anything that you do not understand, if you would like more information or you have any questions please do not hesitate to contact us. Please take time to decide whether or not you wish to take part.

What is the purpose of the study?

It has been suggested that consuming foods which are rich in polyphenols (a type of antioxidant) may help to improve public health by reducing the incidence of disease such as cardiovascular disease, cancer and neurological decline. Studies in our laboratory have already shown that beetroot juice contains a high amount of polyphenols and that these polyphenols are still available for the body to use even after digestion has taken place. This means that the polyphenols are able to get into the cells and start working to reduce the effects of these diseases. One of the most interesting interactions which polyphenols might have with the body is the reduction of blood glucose (sugar) after eating by to either lowering the blood glucose response or increasing the insulin response to a meal. Both of these effects may reduce how much glucose is in the blood after we eat. We would like to try and find out if this is the case by measuring the blood glucose and blood insulin responses to two meals which have the same nutritional value but one contains polyphenols (in the form of 70 mL of beetroot juice) and one does not. If polyphenols are able to reduce the blood glucose response this may lead to further clinical trials and have implications for people with diabetes who have to manage their blood glucose levels very carefully.

Why have I been invited to participate?

This study is seeking healthy volunteers aged 18-65. You have been invited to participate because you are a healthy adult aged 18-65 with no known chronic disease. We have aimed to recruit around 20 participants from the local area who fall into the same category as you. You will be asked to complete a simple health questionnaire at

the start of the study, since it is important for us to examine only healthy subjects we may exclude you from taking part if you suffer from any serious illnesses including diabetes, past or present cancer, cardiovascular disease (such as atherosclerosis), neurological disorders (such as Alzheimer's disease), are obese (Body Mass Index (BMI) greater than 30) or have a fasting blood glucose level of >6.1 mmol/L. During the study we will also ask you to complete a physical activity questionnaire. Physical activity levels are known to affect the blood glucose and insulin responses so we may exclude you from the study if your physical activity level is very high.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you can contact the researchers in the first instance, you will then be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Following the receipt of you informed consent form we will invite you to the first of two laboratory sessions.

If you are a student who is taught by Dr Lisa Ryan, it is important that you know that choosing to either take part or not to take part in the study will have no impact on your marks, assessments or future studies

What will happen to me if I take part?

You will be asked to attend at least 4 separate sessions at Oxford Brookes University nutrition department. During the first session we would like to welcome you to the study and talk you through the testing procedures, we will provide you with a health questionnaire in order to assess your suitability for the study. We would also like to measure your height, weight and body composition during this session using a stadiometer (height) and tanita scales for weight and body composition. You will also be asked to fill in a physical activity questionnaire and a food frequency questionnaire to measure you activity and diet respectively. You would need to conduct an overnight (12h) fast before this, and each subsequent session. The tanita works by measuring the resistance of various different components of the body to electrical impulse. Fat, muscle and water all have differing levels of resistance which allows us to measure the proportions of these things in each individual person. It is just like standing on a set of normal scales and is not at all uncomfortable. Using you height and weight we can calculate your body mass index (BMI), if this is greater than 30 we may exclude you from taking part in the study. Your first test day will be scheduled for at least 3 days after this first session.

The second session will be your first test day. You will need to conduct a 12h overnight fast before this session also. On this day we would like to take measurements of blood glucose and blood insulin following consumption of one of the two test meals. The first thing to do is taken two baseline measurements of both glucose and insulin, one at the start and another 5 minutes later to establish your fasted levels. The blood samples will be collected via finger prick using a small lancet, the lancet prick feels like a small sharp scratch. We would ask you to complete 11 finger prick samples in total. During each finger-prick sample you would donate around 300 microlitres of blood which is equivalent to a few large drops. There are no major risks with finger prick samples but your fingers may feel a little sore after all 11 samples have been taken. The 11 samples would be taken as follows; two at baseline (5 minutes apart), then you would eat your meal, then 1 finger prick at 5 minutes, one at 15 minutes and then every 15 minutes for

the first hour and 1 every 30 minutes for the two subsequent hours. This means you would be in the lab for a total of about 3 hours and 30 minutes on each test day. The meals we would ask you to consume are very simple, one contains 33g of white bread together with a 70 mL glass of sugary water (made from natural sugar, natural pea protein and natural soluble fibre), one would be the same 33g of white bread together with a 70 mL bottle of beetroot juice and the third would be simply 50g of white bread. In between the measurements you will need to stay at the centre, you will be able to bring a laptop, book or other media to pass the time.

At least 48 hours later you would be able to return for your second test day, this would be exactly the same as the first except you would have a different meal to that which you had on the first test day. For example, if you had the beetroot juice meal on the first test day you might have the sucrose water meal or the white bread meal on the second test day. You would also have conducted a 12h fast before this test day. The third test day would be identical to this except you would consume whichever meal is remaining that you haven't had.

What are the possible benefits of taking part?

There are no direct benefits to you other than receiving £5 in Blackwell's book vouchers. By taking part in this study you will be helping to discover whether or not polyphenols can help in the management of blood glucose levels which may benefit people suffering from diabetes and promote good long term health.

Will what I say in this study be kept confidential?

All information collected about individuals will be kept strictly confidential (subject to legal limitations). Confidentiality, privacy and anonymity will be ensured in the collection, storage and publication of research material. Participants will be de-identified (i.e. they will be referred to by number rather than name) in any published material. Data generated by the study will be retained in accordance with the University's policy on Academic Integrity and kept for a minimum of 10 years. All data will be stored in a locked filing cabinet (accessible only by the primary researchers) if in paper form and in a password protected file (accessible only by the primary researchers) if in electronic form.

What should I do if I want to take part?

If you would like to take part in this study then you need to contact one of the primary researchers (either Mr Peter Wootton – Beard or Dr Lisa Ryan), our contact details can be found at the bottom of the sheet

What will happen to the results of the research study?

The results of this study will contribute towards the PhD thesis of Mr Peter Wootton-Beard, it is anticipated that the results will be published in peer-reviewed journals and may also be presented at relevant conferences. If you would like a copy of the published work then you will be able to contact the lead researcher (Mr Wootton-Beard) and he will provide you with a copy. In addition, you will be able to receive a summary of the

study results as soon as it has been concluded, please feel free to contact the study organisers by email or post at any time to request this and we will advise you when it will be available.

Who is organising and funding the research?

This research project is being organised and run by Mr Peter Wootton – Beard, a PhD student in the school of life sciences at Oxford Brookes University under the supervision of Dr Lisa Ryan. The Beetroot juice shots are being provided by the manufacturer: James White Drinks Ltd, UK.

Mr Peter Wootton – Beard BA. MSc
PhD Researcher
S407 Functional Food Centre,
Oxford Brookes University
Gipsy Lane,
Oxford
OX3 0BP

Tel: 01865 483283
Email: peter.wootton-beard@brookes.ac.uk

Dr Lisa Ryan *RNutr*
Senior Lecturer in Nutrition,
Functional Food Centre,
Oxford Brookes University
Gipsy Lane,
Oxford,
OX3 0BP

Tel: 01865 483199
Email: lisaryan@brookes.ac.uk

Who has reviewed the study?

This research project has been approved by the University Research Ethics Committee, Oxford Brookes University.

Contact for Further Information

For further information please contact Dr Lisa Ryan using the details above. If you have any concerns about the way in which the study has been conducted, you should contact the Chair of the University Research Ethics Committee on ethics@brookes.ac.uk.

Thank you for taking the time to read through this information sheet

Warm Regards

Peter Wootton – Beard

22nd October 2010

8. Participant Information Sheet for Study 4

Principal investigator: Mr Peter Wootton-Beard
PhD Researcher, Functional Food Centre, Oxford Brookes University
Gipsy Lane, Oxford, OX3 0BP

Email: peter.wootton-beard@brookes.ac.uk
Contact: 01865 48(3283)

Participant Information Sheet

Study title

The effects of polyphenol- rich beetroot juice consumption on blood glucose and blood insulin response.

Invitation

You are being invited to take part in a research project. This sheet contains information to help you decide whether you wish to participate. Please read it carefully and discuss it with family and friends if you wish. If there is anything that you do not understand, if you would like more information or you have any questions please do not hesitate to contact us. Please take time to decide whether or not you wish to take part.

What is the purpose of the study?

It has been suggested that consuming foods which are rich in polyphenols (a type of antioxidant) may help to improve public health by reducing the incidence of disease such as cardiovascular disease, cancer and neurological decline. Studies in our laboratory have already shown that beetroot juice contains a high amount of polyphenols and that these polyphenols are still available for the body to use even after digestion has taken place. This means that the polyphenols are able to get into the cells and start working to reduce the effects of these diseases. One of the most interesting interactions which polyphenols might have with the body is the reduction of blood glucose (sugar) after eating by either lowering the blood glucose response or influencing the insulin response to a meal. Both of these effects may reduce how much glucose is in the blood after we eat or how sensitive our bodies are to insulin. We would like to try and find out if this is the case by measuring the blood glucose and blood insulin responses to two beverages which have the same nutritional value but one contains polyphenols (in the form of beetroot juice) and one does not. We will compare both of these drinks to pure glucose. If polyphenols are able to reduce the blood glucose response this may lead to further clinical trials and have implications for people with diabetes who have to manage their blood glucose levels very carefully.

Why have I been invited to participate?

This study is seeking healthy volunteers aged 18-45. You have been invited to participate because you are a healthy adult aged 18-45 with no known chronic disease and are not pregnant. We have aimed to recruit around 30 participants from the local

area who fall into the same category as you. You will be asked to complete a simple health questionnaire at the start of the study, since it is important for us to examine only healthy subjects we may exclude you from taking part if you suffer from any serious illnesses including diabetes, past or present cancer, cardiovascular disease (such as atherosclerosis), neurological disorders (such as Alzheimer's disease), are obese (Body Mass Index (BMI) greater than 30), are currently pregnant/planning to become pregnant during the study or have a fasting blood glucose level of >6.1 mmol/L. During the study we will also ask you to complete a physical activity questionnaire. Physical activity levels are known to affect the blood glucose and insulin responses so we may also exclude you from the study if your physical activity level is very high.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you should contact the researchers in the first instance, you will then be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Following the receipt of your informed consent form we will invite you to the first of four laboratory sessions.

If you are a student who is taught by Dr Lisa Ryan, it is important that you know that choosing to either take part or not to take part in the study will have no impact on your marks, assessments or future studies

What will happen to me if I take part?

You will be asked to attend at least 4 separate sessions at Oxford Brookes University nutrition department. During the first session we would like to welcome you to the study and talk you through the testing procedures, we will provide you with a health questionnaire in order to assess your suitability for the study. We would also like to measure your height, weight and body composition during this session using a stadiometer (height) and tanita scales for weight and body composition. You will also be asked to fill in a physical activity questionnaire and a food frequency questionnaire (this is not a food diary) to measure you activity and diet respectively. You would need to conduct an overnight (12h) fast before this, and each subsequent session. The tanita works by measuring the resistance of various different components of the body to electrical impulse. Fat, muscle and water all have differing levels of resistance which allows us to measure the proportions of these things in each individual person. It is just like standing on a set of normal scales and is not at all uncomfortable. Using you height and weight we can calculate your body mass index (BMI), if this is greater than 30 we may exclude you from taking part in the study. Your first test day will be scheduled for at least 3 days after this first session.

The second session will be your first test day. You will need to conduct a 12h overnight fast before this session. On this day we would like to take measurements of blood glucose and blood insulin following consumption of one of the test beverages. The first thing to do is to take two baseline measurements of both glucose and insulin, one at the start and another 5 minutes later to establish your fasted levels. The blood samples will be collected via finger prick using a small lancet, the lancet prick feels like a small sharp scratch. We would ask you to complete 11 finger prick samples in total. During each finger-prick sample you would donate around 350 microlitres of blood which is equivalent to a few large drops. There are no major risks with finger prick samples but

your fingers may feel a little sore after all 11 samples have been taken. The 11 samples would be taken as follows; two at baseline (5 minutes apart), then you would eat your meal, then 1 finger prick at 5 minutes, one at 15 minutes and then every 15 minutes for the first hour and 1 every 30 minutes for the two subsequent hours. This means you would be in the lab for a total of about 3 hours and 30 minutes on each test day. The beverages we would ask you to consume are very simple; each is 206 mL in volume and contains 50g of carbohydrate. The first is beetroot juice, the second is a drink which contains the same type of carbohydrates as beetroot juice (made from natural sugars, natural pea protein and natural soluble fibre) and the third contains only glucose. In between the measurements you will need to stay at the centre, you will be able to bring a laptop, book or other media to pass the time.

At least 48 hours later you would be able to return for your second test day, this would be exactly the same as the first except you would have a different drink to that which you had on the first test day. For example, if you had the beetroot juice meal on the first test day you might have the matched drink or the glucose drink on the second test day. You would also have conducted a 12h fast before this test day. The third test day would be identical to this except you would consume whichever drink is remaining that you haven't had.

What are the possible benefits of taking part?

There are no direct benefits to you other than receiving £10 in Blackwell's book vouchers. By taking part in this study you will be helping to discover whether or not polyphenols can help in the management of blood glucose levels which may benefit people suffering from diabetes and promote good long term health.

Will what I say in this study be kept confidential?

All information collected about individuals will be kept strictly confidential (subject to legal limitations). Confidentiality, privacy and anonymity will be ensured in the collection, storage and publication of research material. Participants will be de-identified (i.e. they will be referred to by number rather than name) in any published material. Data generated by the study will be retained in accordance with the University's policy on Academic Integrity and kept for a minimum of 10 years. All data will be stored in a locked filing cabinet (accessible only by the primary researchers) if in paper form, and in a password protected file (accessible only by the primary researchers) if in electronic form. You are made aware that it is possible for data to be subject to subpoena, freedom of information requests and mandated reporting although occurrences of this type are rare. Furthermore, this research study uses a relatively small sample size; it is therefore not always possible to guarantee the complete de-identification of participants, for example other participants may be in the same room as you during testing and may be able to identify you. Every effort will be made to avoid this but participants are advised of the existence of this limitation.

What should I do if I want to take part?

If you would like to take part in this study then you need to contact one of the primary researchers (either Mr Peter Wootton – Beard or Dr Lisa Ryan), our contact details can be found at the bottom of the sheet

What will happen to the results of the research study?

The results of this study will contribute towards the PhD thesis of Mr Peter Wootton-Beard, it is anticipated that the results will be published in peer-reviewed journals and may also be presented at relevant conferences. If you would like a copy of the published work then you will be able to contact the lead researcher (Mr Wootton-Beard) and he will provide you with a copy. In addition, you will be able to receive a summary of the study results as soon as it has been concluded, please feel free to contact the study organisers by email or post at any time to request this and we will advise you when it will be available.

Who is organising and funding the research?

This research project is being organised and run by Mr Peter Wootton – Beard, a PhD student in the school of life sciences at Oxford Brookes University under the supervision of Dr Lisa Ryan. The Beetroot juice is being provided by the manufacturer: James White Drinks Ltd, UK (http://www.jameswhite.co.uk/) however the research is not sponsored in any way by this company.

Mr Peter Wootton – Beard BA. M.Sc.
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Who has reviewed the study?

This research project has been approved by the University Research Ethics Committee, Oxford Brookes University.

Contact for Further Information

For further information please contact Dr Lisa Ryan using the details above. If you have any concerns about the way in which the study has been conducted, you should contact the Chair of the University Research Ethics Committee on ethics@brookes.ac.uk.

Warm Regards

Peter Wootton – Beard

19th December 2011