Carrots and Cancer:

The Bioavailability of Polyacetylene from Carrots and their Association with Biomarkers of Cancer Risk

Sarah Rosalind Warner

Human Nutrition Research Centre

Faculty of Medical Sciences, Newcastle University

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Abstract

Diets high in fruit and vegetables are correlated with better health outcomes and lower risk of chronic diseases such as cardiovascular disease and cancer. Bioactive phytochemicals, including polyphenols, carotenoids and isothiocyanates, in these foods are thought to be at least partly responsible for these protective effects. Specific foods also correlate well with these outcomes, such as carrots. As carrots are high in β-carotene, this compound is commonly thought to be the bioactive substance eliciting the anti-cancer effect, and there are many observational data to suggest higher intakes, and higher plasma levels, confer a reduced risk of cancer. However, supplement studies have little effect and can even increase the risk of cancer in certain populations. The polyacetylene group of compounds, also present in carrots, are gaining interest due to their anti-cancer and anti-inflammatory actions *in vitro* and in rodent studies. However, little is known about their effect in humans.

This work provides novel analysis of the polyacetylene content of carrots, related vegetables, and mixed dishes containing them to create a database of polyacetylene values for commonly eaten foods. The resulting database was used to investigate the intake in a population of adults from the UK. The effect of cooking was also investigated to ensure the retention of compounds during processing. Little is known about the bioavailability of these compounds and so a human trial was conducted to investigate whether polyacetylenes could be seen in blood plasma after consumption of either 100g or 250g of boiled carrots. Finally, a dietary intervention trial was conducted, investigating the effect of consumption of 100g of boiled white carrot (containing polyacetylenes but not β-carotene), served with butter, on biomarkers of cancer risk compared to a fibre-matched control (oatcakes).

Cooked carrots were the most important source of polyacetylenes in the diet of the UK population investigated. Therefore, carrots were chosen to be a viable method of polyacetylene intake for a dietary intervention study. Boiled carrot retained phytochemicals better than fried carrot, and cooking the carrot whole rather than in disks or quarters could offer protection from losses during cooking. Falcarinol and falcarindiol-3-acetate were detected in the blood plasma after consumption of carrot. This is the first study to show the presence of polyacetylenes in blood plasma after consumption of whole boiled carrot. The results of a dietary intervention showed a trend for a reduction in prostaglandin E2 metabolite in the carrot group ($p=0.07$) but not the oatcake control group. There was no effect on any other biomarker measured (IL-6 or lymphocyte DNA damage). Regular consumption of a moderate amount of carrot can reduce a marker of inflammation in healthy adults.

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Abbreviations

13-HODE: 13-hydroxyoctadecadienoic acid

AA: arachidonic acid

ABCG2: ATP (adenosine triphosphate)-binding cassette (ABC) transporters

AC_{50}: concentration to induce 50% apoptosis

ACF: aberrant crypt foci

AOM: azoxymethane

APC^{min}: adenomatous polyposis coli multiple intestinal neoplasia

ARE: antioxidant response element

ATL: aspirin triggered lipoxins

ATP: adenosine triphosohate

CB1: cannabinoid receptor type 1

CCL2/MCP1: chemokine (C-C motif) ligand 2/monocyte chemoattractant protein 1

cGPx: glutathione peroxidase

CHD: cardiovascular heart disease

CI: confidence Interval

COX: cyclooxygenase

CRC: colorectal cancer

CRP: C-reactive protein

Cys: cysteine

DHA: docosahexaenoic acid

DM: dry matter

DNA: deoxyribonucleic acid

DPPH: 2,2,diphenyl-1-picrylhydrazyl

EC_{50}: 50% effective concentration

EDTA: ethylenediaminetetraacetic acid

ED_{50}: effective dose to elicit a 50% reduction in cell growth/cell proliferation

EGCG: epigallocatechin gallate

EPA: eicosapentaenoic acid

FaDOH: falcarindiol

FaD3Ac: falcarindiol-3-acetate

FaOH: falcarinol

GABA: gamma-amino butyric acid

GAE: gallic acid equivalents

GI: gastrointestinal

GSH: glutathione

GST: glutathione *S*-transferase

HETEs: hydroxyeicosatetraenoic acid

HHT: hydroxyheptadecatrienoic acid

HO-1: hemeoxygenase

HPETEs: hydroperoxyeicosatetraenoic acid

HPLC: high performance liquid chromatography

HPV: human papilloma virus

HR: hazard ratio

hsCRP: high specificity C-reactive protein

IC_{50}: concentration to induce 50% inhibition of growth

IgE: immunoglobulin E

IκB: inhibitor of kappa B

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase (gene)

ITC: isothiocyanates

JAK: Janus kinase

Keap1: Kelch-like ECH-associated protein-1

LCMS: liquid chromatography mass spectrometry

ln: natural log

LD_{50}: concentration needed to kill 50% of a sample (lethal dose)

LOX: lipoxygenase

LTA_4, LTD_2, LTB_2: leukotrienes A, D, B

LPS: lipopolysaccharide

MDA: malondialdehyde

mDNA: mitochondrial DNA

mg: milligram

µg: microgram

MMP: metalloprotease

MRM: multiple reaction monitoring

NFκB: nuclear factor κB

MW: molecular weight

NO: nitric oxide

NOS: nitric oxide synthase (enzyme)

NQO1: NAD(P)H quinone oxireductase

Nrf2: nuclear factor E2-related factor

NSAIDs: Non-steroidal anti-inflammatories

OR: odds ratio

PBMC: peripheral blood monocytes

PGDH: 15-hydroxyprostaglandin

PGE_2: prostaglandin E_2

PGE_2M: prostaglandin E_2 Metabolite

PGH_2: prostaglandin H_2

PUFA: polyunsaturated fatty acid

RAW: Ralph, Raschke, and Watson cells

RCT: randomised controlled trials

RNS: reactive nitrogen species

ROS: reactive oxygen species

RR: relative risk

SD: standard deviation

SEM: standard error of the mean

SOD: superoxide dismutase

SSB: single strand breaks

STAT3: signal transducer and activator of transcription

TNF-α: tumour necrosis factor α

TPA: 12-O-tetradecanoylphorbol-13-acetate

TXA_2: thromboxane A_2

TXB_2: thromboxane B_2

WHO: World Health Organisation

Publications arising from this research

Conference and Symposia abstracts:

Nutrition Society Summer meeting 2016, Retention of polyacetylenes and carotenoids in carrot during cooking, Dublin, July 2016 (Warner *et al.*, 2016b)

Nutrition Society Spring meeting 2016, Database of polyacetylene-containing foods for estimation of population intake, Edinburgh, March 2016 (Warner *et al.*, 2016a)

Original Articles:

S. Warner, C. Seal, S. Haldar, G. Stewart and K. Brandt (2017) A project to investigate the effects of carrot and other polyacetylene-rich vegetable consumption on biomarkers of chronic disease risk. Acta Horticulturae, 1153, 149-154.

Chapter 1. Introduction and literature review

1.1 Prevalence of cancer

Cancer is a collection of diseases characterised by uncontrolled DNA replication and cell division of abnormal cells which lead to the development of tumours that can metastasise to invade other tissues, blood or the lymphatic system.

In 2015, nearly 360,000 people were diagnosed with cancer, and there were 163,444 deaths from cancer in 2014 in the UK. There were over 14.1 million people worldwide with the disease in 2012 (Cancer Research). Cancer cases are predicted to increase to 24 million worldwide by 2035 but estimates suggest that a third of the 13 most common cancers could be prevented with improved diet, physical activity and body weight control (World Cancer Research Fund, 2012).

1.2 Pathogenesis of cancer

Cancer occurs in 3 stages: initiation, promotion and progression (Figure 1.1). During initiation, a mutation occurs within the DNA. The growth, development and function of cells are tightly regulated to ensure they progress normally and changes in DNA are usually found and repaired at check points during cell division, or the cell can undergo apoptosis and the mutation will not be passed on. However, these repair/protect mechanisms are not infallible and the mutation can be retained. The second stage is cancer progression, where the mutation gains a promoter and the gene is expressed. Not all mutations get expressed, depending on where they occur in the genetic material. When the ‘oncogene’ is expressed and it causes uncontrolled cellular replication, a tumour forms.

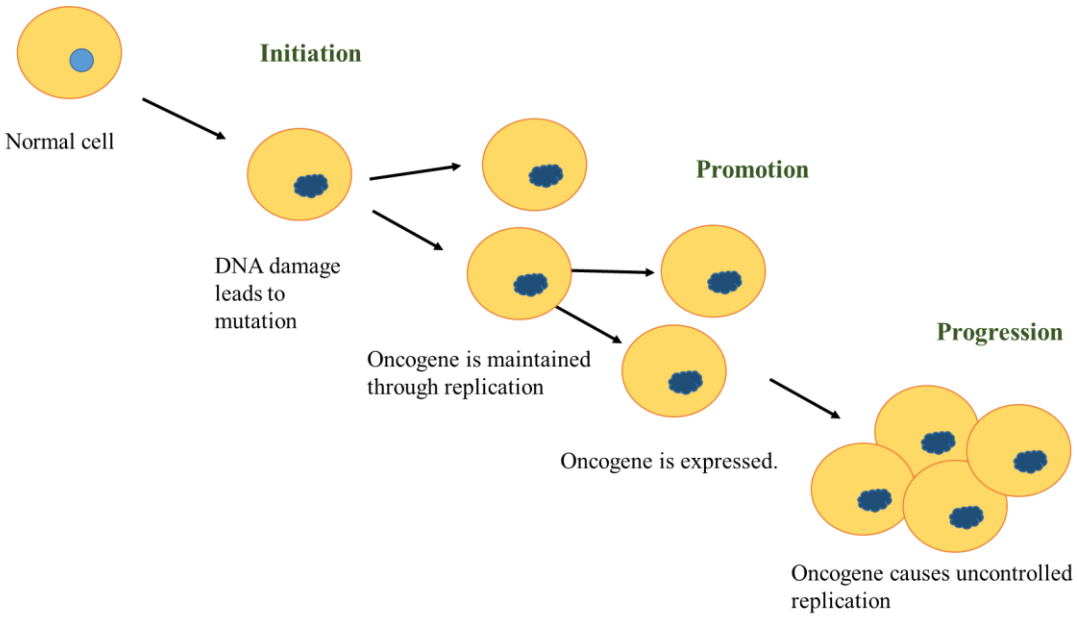


Figure 1.1: The 3 stages of cancer development (modified from (Trejo-Solis *et al.*, 2013)).

Mutations can occur by chance when DNA is inaccurately replicated during cellular division or can be affected by an increase in reactive oxygen species (ROS), exposure to UV light, carcinogens in the environment e.g. from smoking and pollution, and due to diet and/or lifestyle. Reactive oxygen species can come from exogenous sources such as UV, radiation, and certain drugs but are also are generated naturally in the body due to oxidative metabolism and inflammation as discussed in more detail below. Endogenous systems are in place to neutralise the ROS generated during these processes but these systems can be overwhelmed. Exogenous antioxidants such as certain drugs and dietary components can also help to neutralise ROS.

1.3 **The role of inflammation in cancer pathogenesis**

Inflammation is the body's natural response to invading pathogens and tissue damage. Whilst this response is beneficial to protect the body, uncontrolled inflammation can lead to a host of inflammatory conditions such as rheumatoid arthritis, atherosclerosis, asthma and allergies. Inflammation has also been implicated in a number of diseases such as cancer (Coussens and Werb, 2002; Il'yasova *et al.*, 2005) and cardiovascular disease (Libby *et al.*, 2002).

Normal Inflammation:

Normal inflammation involves the release of chemical signals which progress down multi-step pathways and result in the 'healing' of the tissue. The main response is to recruit white

blood cells (leukocytes) including neutrophils, monocytes and eosinophils, from the blood system to the affected tissue. Cytokines and chemokines - chemotactic cytokines - are released, resulting in the movement of cells towards or away from a chemical signal. Neutrophils are recruited to the site of damage first, then monocytes. Monocytes are immobilised at the site then migrate through the damaged tissue behind the endothelium. They then differentiate into macrophages which produce cytokines and growth factors which affect cells in the surrounding area. These cells also form a provisional extracellular membrane which acts as a scaffold on which endothelial cells can attach and repair the damage to the endothelium. This results in the re-building and repairing of the damaged tissue. In normal inflammation, pro-inflammatory cytokines will be switched off and anti-inflammatory signals will be activated when tissue repair is complete thus quenching the reaction. Dysregulation of any of the control mechanisms that switch off the initiating factors or induce the terminating factors of inflammation leads to chronic inflammation. As chemokines attract leukocytes which in turn attract specific downstream effector cells, they determine the natural path of the inflammatory response. Therefore, the cytokines at an inflammatory site are an important determinant of the development and progression of chronic inflammation (Gabay, 2006).

Cancer and Inflammation:

Inflammation and cancer are closely linked, with higher levels of inflammation leading to higher cancer risk. Once diagnosed, higher inflammation can indicate more aggressive cancer and higher mortality in cancer patients (Coussens and Werb, 2002). It is well established that many cancers arise from chronic infection and parasitism, especially in the developing world. Infections with *Helicobacter pylori* can lead to gastric cancers, liver cancer arises from hepatitis B and C infection, cervical cancer from Human Papilloma Virus (HPV) and Epstein Barr viruses can lead to Burkett's Lymphoma. In fact, 18% of cancers worldwide can be attributed to infection (WHO, 2011). Some viruses are able to insert oncogenes into the host genome. In the case of Epstein-Barr viruses, this can lead to sustained B lymphocyte proliferation. They also interact with transcription factors – Hepatitis C and *H.Pylori* both activate STAT3 leading to chronic inflammation (Yu *et al.*, 2009). Cancers can arise at sites of chronic inflammation such as inflammatory conditions of the bowel (Crohn's disease, irritable bowel syndrome, inflammatory bowel disease) which have all been shown to lead to colorectal cancer (Yu *et al.*, 2009). In cases where environmental factors like infection are not involved, leukocytes are still seen in the tumour microenvironment and genetic and epigenetic events in the cells are thought to be the mechanism for this (Yu *et al.*, 2009).

As well as chronic inflammation, disregulation of the inflammatory response has implications in cancer. Macrophages can be recruited to a tumour and the cancer cells exploit the 'tools' that macrophages have at their disposal to help them evade apoptosis, replicate, and move away from the primary tumour site (Sica *et al.*, 2008). Macrophages secrete reactive oxygen species (ROS) and reactive nitrogen species (RNS) as part of their mechanism of fighting infection, increasing DNA damage (point mutations, deletions and rearrangements). They also secrete growth factors to help repair this damage but this rapid dividing of cells encourages uncontrolled growth as some of the mechanisms involved in checking the integrity of the DNA is downregulated in the inflammatory environment. Macrophages use metalloproteases (MMP), matrix-degrading enzymes, which enable cells to move across tissue boundaries and release factors that encourage angiogenesis, the formation of new blood vessels, to increase blood flow and therefore oxygen to the site. These two actions allow tumours to overcome the limitations of growth by hypoxia and allow movement away from the original tumour site. Cancer cells highjack the normal functions of the macrophages to mutate, grow and move through tissues during metastasis (Coussens and Werb, 2002).

To understand how chronic, uncontrolled, inflammation can affect cancer, the mechanism of normal inflammation must first be examined, and then determine how these mechanisms can be altered to result in chronic inflammation and the effect this has on disease states. The two pathways of inflammation - infection and tissue damage - have distinct initiators and progress down unique pathways.

1.3.1 *Tissue injury pathway: LOX/COX enzyme/arachidonic acid cascade*

The Lipoxygenase (LOX) and Cycloxygenase (COX) enzymes are integral to the 'injury' inflammatory response. When a cell is injured, the cell membrane is compromised and releases arachidonic acid as it breaks down. This fatty acid induces a cascade through the LOX and COX enzymes which metabolise polyunsaturated fatty acids such as arachidonic acid to create inflammatory cytokines including prostaglandins (Tuncer and Banerjee, 2015). Figure 1.2 describes the reaction pathway of these enzymes.

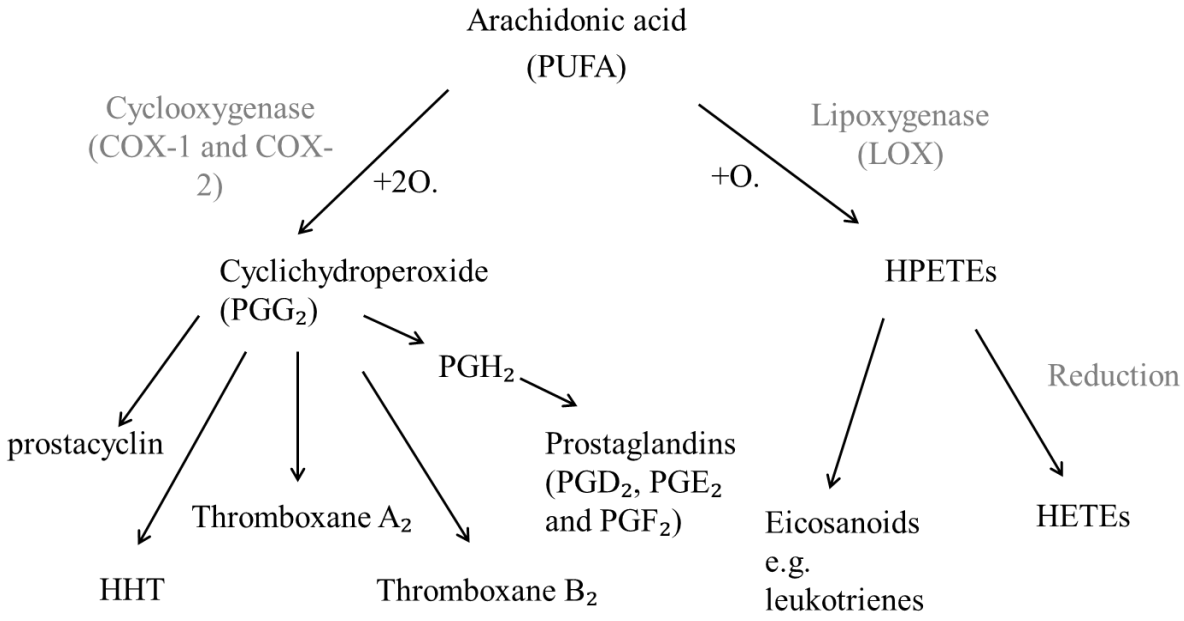


Figure 1.2: Reactions that occur with LOX and COX in the presence of arachidonic acid in the leukocytes (adapted from (Greene *et al.*, 2011)). HHT – hydroxyheptadecatrienoic acid; PGH2 – prostaglandin H_2; PUFA – polyunsaturated fatty acid; HPETE – hydroperoxyeicosatetraenoic acid; HETE - hydroxyeicosatetraenoic acid

LOX are a family of non-heme iron containing enzymes that incorporate one molecule of oxygen into arachadonic acid initiating the production of 5-, 12-, or 15-hydroperoxyeicosatetraenoic acids (HPETEs) which are reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) or converted to eicosanoids such as leukotrienes. There are three types of lipoxygenases named according to the position in arachidonic acid into which the oxygen molecule is inserted: 5, 12- and 15-lipoxygenase (Serhan *et al.*, 2008). The COX enzymes introduce 2 molecules of oxygen into arachadonic acid initiating the creation of prostaglandins, prostacyclin and thromboxane (Greene *et al.*, 2011). The end products of these cascades are implicated in inflammation, pain perception (Liu *et al.*, 1998), allergic reactions, smooth muscle contraction, fever, blood platelet aggregation (Alanko *et al.*, 1994) and cancer cell growth (Flower, 2003). There are 2 types of COX enzyme. COX-1 appears to be basally expressed and produce prostaglandins to maintain physiological functions such as platelet aggregation and gastric mucosa protection whereas COX-2 is induced during inflammatory insult (Flower, 2003; Tuncer and Banerjee, 2015).

1.3.2 *Tissue infection pathway: NFκB/IL-6/STAT3/iNOS cascade*

The 'infection' inflammatory response is mediated through the two closely interrelated pathways involving the NFκB (nuclear factor-κB) and STAT3 (signal transducer and activator protein) transcription factors (Bromberg and Wang, 2009; Yu *et al.*, 2009). The stimulus for the reaction is lipopolysaccharide (LPS), found on the surface of gram negative bacteria, which acts as a trigger for the LPS-induced production of inflammatory markers. This cascade is detailed in Figure 1.3. LPS stimulates the release of NFκB transcription factor which leads to the expression of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (Kang *et al.*, 2011). It also binds to the promoter of the inducible nitric oxide synthase (iNOS) gene to create the enzyme which catalyses the production of nitric oxide (NO), a strongly oxidising free radical capable of causing damage to the epithelial cells by attacking LPS cell membranes and is used to destroy the invading pathogen. The compounds produced in the activation of these genes (IL-6, TNF-α, and NO) can upregulate NFκB activation creating a positive feedback loop (Metzger *et al.*, 2008). They also upregulate the STAT3 pathway, through IL-6, to further stimulate the production of IL-6 and iNOS (Bromberg and Wang, 2009).

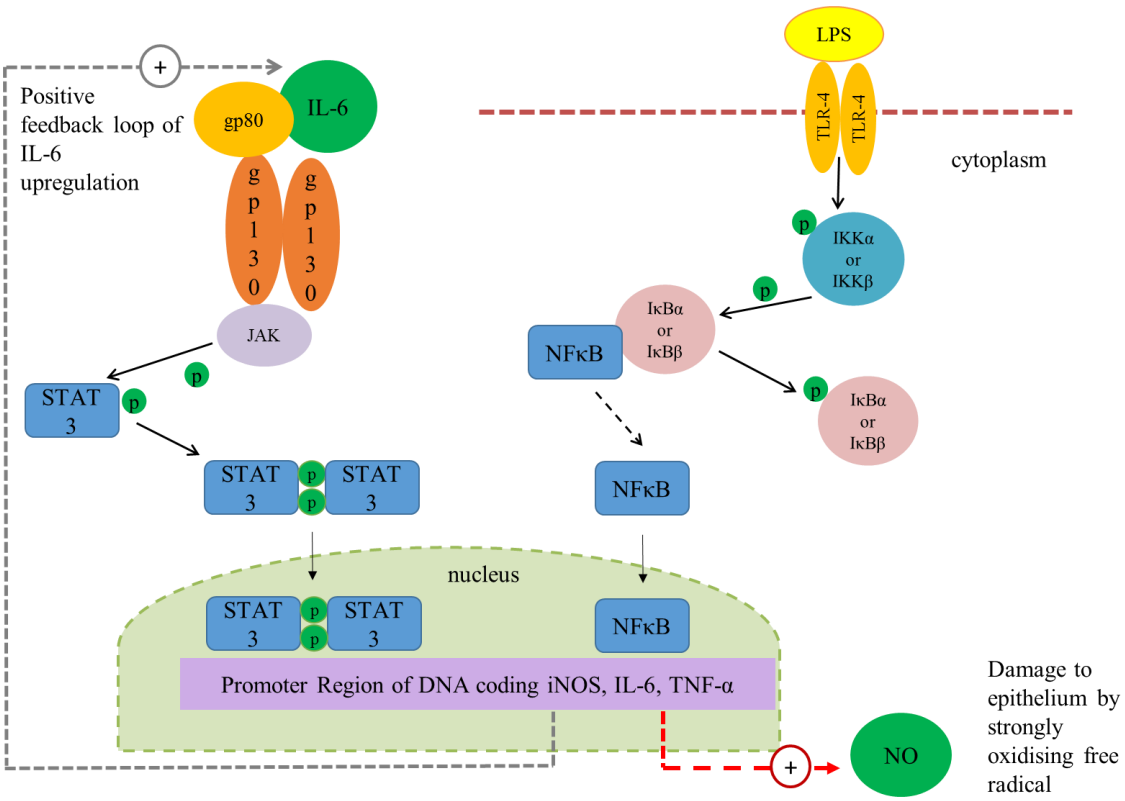


Figure 1.3: LPS induced inflammation: LPS stimulates toll like receptor-4 (TLR-4) in the membrane of a cell. Cytoplasmic adaptor proteins then create a cascade (not shown) that stimulates the phosphorylation of IκB. Once phosphorylated, the IκB releases NFκB transcription factor which is then active and free to move into the cytoplasm to bind to the promoter of several genes including iNOS, IL-6 and TNF-α. The IL-6 that is released from this reaction can then stimulate the STAT3 transcription factor pathway. IL-6 binds to its receptor, gp80, which is on the cell surface or in solution. Gp80 then binds to gp130, a common cell signalling receptor on many cell types, which initiates homodimerisation of gp130. Once dimerised, it activates the associated JAK (janus kinase). JAKs phosphorylate STAT3 which dimerises, forming the active transcription factor. The active STAT3 then translocates into the nucleus to regulate gene transcription of a number of genes, including iNOS and IL-6, further enhancing the inflammatory response. NO production is upregulated as a result (based on information from (Bromberg and Wang, 2009; Yu *et al.*, 2009).

1.3.3 *Uncontrolled inflammation*

When there is sustained expression of any of the inflammatory markers, chronic inflammation can occur. There are also acute cases where inflammation is constantly fed-forward resulting in the system becoming overwhelmed and inflammatory cytokines attack and damage the tissues they are aiming to protect. NO is implicated in septic shock, autoimmune diabetes and

rheumatoid arthritis; TNF-α causes tissue damage, atherosclerosis, systemic inflammatory response syndrome and septic shock. Inhibition of NFκB can be used as a target to therapeutically reduce inflammatory conditions such as rheumatoid arthritis, ischemia and septic shock (Kang *et al.*, 2011).

1.4 **Inflammation and cancer**

The NFκB and STAT3 transcription factors are important in inflammation-related cancer. The role of NFκB to induce those changes in the cell that allow cancer to replicate unchecked (tumour proliferation, survival, angiogenesis and invasion) leads to more aggressive and resistant tumours (Sica *et al.*, 2008). Especially important is the role NFκB has in expression of IL-6 and the subsequent activation of STAT3. Higher concentrations of IL-6 are seen both in human colon cancer patients and mice with AOM-induced colon cancer (Greten *et al.*, 2004), as well as models of prostate, breast and lung cancers (Knüpfer and Preiß, 2007). IL-6 is also important in colon cancer progression as has been demonstrated in STAT3 knockout mice that have dramatic reductions in colitis-associated cancer (CAC) tumour size and incidence. Introduction of exogenous IL-6 into these mice reversed the suppressive effects, increasing tumour burden (Bollrath *et al.*, 2009; Grivennikov *et al.*, 2009). Overactive STAT3 has been observed in a whole host of different cancers (Hodge *et al.*, 2005) and is often found on the edge of tumours, interacting with cells of the immune system (Bromberg and Wang, 2009). A gene that silences STAT3, SHP1, is hyper-phosphorylated and thus inactive in 79% of myeloma patients (Hodge *et al.*, 2005). STAT3, as well as transcribing iNOS and IL-6, also activates the transcription of Bcl-2, an anti-apoptotic protein that allows cells to avoid cell death in very toxic environments e.g. during normal inflammation when high levels of ROS and RNS are experienced. Under normal circumstances this helps mitigate the damage to normal cells during inflammation, however, this can encourage the survival and replication of cells that have undesirable mutations and thus encourage the development of tumours (Hodge *et al.*, 2005).

The COX and LOX pathways also have important roles in tumorigenesis. COX-2 enzyme was first discovered to be upregulated in colon cancers (Eberhart *et al.*, 1994) and has since been a target for therapy in many other types of cancer (Rizzo, 2011). Increased COX-2 expression in cancer cells has been associated with decreased survival rates (Wang and DuBois, 2010), and blocking COX-2 with aspirin, and other non-steroidal anti-inflammatories (NSAIDs), decreases tumour activity and incidence of many types of cancer (Flower, 2003; Tuncer and Banerjee, 2015), with long term users of aspirin having lower colon cancer risk (Bosetti *et al.*,

2012). The downstream products of COX, the prostaglandins PGD_2 and PGE_2, have both been shown to have pro-cancer actions but PGE_2 is the most common prostaglandin found in colon, breast, lung and head and neck cancers (Greene *et al.*, 2011). PGE_2 has tumour-related immunosuppressive effects such as inhibiting macrophage, T-cells and natural killer cells. PGE_2 is a well-known inducer of IL-6 (Hodge *et al.*, 2005) and the differentiation of macrophages into tumour associated macrophages is stimulated by PGE_2 and IL-6 (Sica *et al.*, 2008). Deleting the PGE_2 synthesis genes has been shown to suppress cancer growth by 95-99% (Nakanishi *et al.*, 2008). PGE_2 can also be reduced by over expressing the enzyme that grades PGE_2, 15-prostaglandin dehydrogenase (15-PGDH), or blocking PGE_2 receptors, all of which have been shown to have a tumour suppressing role (Greene *et al.*, 2011).

The LOX enzymes have also been shown to stimulate tumorigenesis. Both 5-, 12- and 15-LOX are able to stimulate tumour growth, angiogenesis and metastasis. 5-LOX is over-expressed in lung, prostate, colon and breast cancers, 12-LOX in cancers of the ovaries and prostate, and 15-LOX in Hodgkin's lymphoma and colorectal cancers (Greene *et al.*, 2011). Downstream products of the LOX enzymes have also been seen to correlate with tumorigenesis. Urinary 12-HETEs, produced by 12-LOX, correlate to progression of prostate cancer (Nithipatikom *et al.*, 2006), and 5-LOX makes leukotrienes (HETE and LTB_4) that can recruit and activate inflammatory cells and increase permeability of vascular epithelium (Peters-Golden and Brock, 2003; Chen *et al.*, 2004).

Reducing LOX enzyme products, including the leukotrienes (LTA_4, LTB_4 and LTD_4), 5-HETE and 12-HETE, have been shown to reduce various aspects of tumorigenesis (Greene *et al.*, 2011). Inhibiting 5-LOX has prevented leukaemia stem cells from being able to differentiate, divide and survive (Chen *et al.*, 2009). Similarly promising results have been seen when targeting 12-LOX. Inhibiting this enzyme has been shown to induce apoptosis through Bcl-2 (Tang *et al.*, 1996; Wong *et al.*, 2001; Pidgeon *et al.*, 2002), arrest the cell cycle at the G1/S-phase and affect the expression of NFκB (Pidgeon *et al.*, 2007) in cancer cells.

This evidence indicates that both the STAT3 and NFκB transcription factors and the LOX and COX enzymes are important in inflammation mediated cancers. This gives us the opportunity to target these mechanisms as a route to cancer treatment. For NFκB caution must be taken as it is responsible for inducing immune responses that are anti-tumour and suppressing it may have effects that are the opposite of what is intended (Yu *et al.*, 2009). Equally, the 15-LOX enzyme has been shown to have both anti- and pro-cancer actions and using this as a target could have detrimental effects (Schneider and Pozzi, 2011). STAT3, therefore, has been the focus of much work as a cancer therapy target.

1.5 **Inflammation as an opportunity to prevent or treat cancer**

Blocking the STAT3 pathway is becoming very important in cancer therapy. There are a number of ways in which it has been suggested that STAT can be inhibited. This includes: blocking the IL-6 receptor with an antagonist, inhibiting Janus Kinase (JAK), disrupting STAT dimerization, inhibiting STAT translocation into the nucleus, and employing STAT transcription factor decoy RNA which STAT binds to instead of the promotor region of the target gene (Hodge 2005; Yu and Jove 2009). Not only do these methods reduce the production of IL-6, for example Bortezomib can control the production of IL-6 through NFκB (Baud and Karin, 2009), but they also have the ability to re-sensitize cells to chemotherapy drugs.

Controlling the activity of the LOX and COX enzymes is also an important therapeutic target in cancer treatment. As LOX contains a non-heme iron atom in the active site of the enzyme, a simple antioxidant can act as an inhibitor of this pathway by reducing the ferric iron and interrupting the catalytic site (Schneider and Bucar, 2005b). However, drugs are being developed to target and inhibit the LOX and COX enzymes specifically, to allow more powerful inhibition of these enzymes. NSAIDs are well known for their COX inhibition and have been used widely for treatment of inflammatory conditions. The COX-2 inhibitors, such as aspirin, are now often used as combination therapy for cancers. They have been associated with lower risk of colorectal, esophagus, lung, stomach and ovarian tumours (Sica *et al.*, 2008). However, caution must be taken in the long-term use of these drugs as they have negative effects both in the gastric mucosa (Smyth *et al.*, 2009) and on cardiovascular health (Mukherjee, 2008). Both of these negative effects are due to the reduction of prostacyclin, derived from the pro-inflammatory prostaglandins (especially PGE_2), which has a protective effect on the endothelium (Grosser *et al.*, 2006). However, there have been some promising COX-2 inhibition from components of foods and herbal medicines, such as glycyrrizic acid (from liquorice), resveratrol (from red wine) and EGCG (epigallocatechingallate from green tea), that can block the enzyme whilst avoiding some of the harmful gastrointestinal and heart effects seen in NSAIDs (Cerella *et al.*, 2010; Schneider and Pozzi, 2011).

A number of 5-LOX inhibiting drugs have been developed recently to treat conditions such as allergies, inflammation, cardiovascular disease and cancer (Pergola and Werz, 2010). Rev5901 prevents *in vitro* and *in vivo* colorectal carcinomas (Melstrom *et al.*, 2008) and Zyflamed, a herbal preparation, has been shown to reduce hyperplasia and dysplasia in oral cancers in mice with significant reductions in LTB_4 (Metzger *et al.*, 1995), di-O-prenylated chalcone derivatives reduce breast cancer cell proliferation *in vitro* (Reddy *et al.*, 2010), and

MK591 has increased apoptosis in human prostate cancer cells (Sarveswaran *et al.*, 2010). 12-LOX has been inhibited with baicalein which reduces proliferation in prostate cells and tumorigenesis *in vivo* and increases apoptosis via caspase in breast cancers (Tong *et al.*, 2002; Agarwal *et al.*, 2009). Baicalein and a general LOX inhibitor, nordihydroguaiaretic acid, have also been shown to reduce pro-angiogenic factors (Nie *et al.*, 2006).

LOX enzymes, much like the COX enzymes, are also inhibited in the presence of some dietary components. Curcumin (from turmeric) has been shown to be a potential LOX and COX inhibitor (Huang *et al.*, 1991). Fatty acids, which have a similar structure to arachidonic acid, can competitively bind to enzymes, producing different metabolites and reducing eicosanoids. Omega-3 fatty acids have therefore displayed anti-cancer effects (Gleissman *et al.*, 2010). Linoleic acid is metabolised by 15-LOX to form 13-HODE (hydroxyoctadecadienoic acid) which has anti-inflammatory and anti-cancer effects in colon cancer cells. Not only does 13-HODE have anti-inflammatory actions of its own, the metabolism of linoleic acid blocks the metabolism of arachidonic acid and further reduces inflammation (Schneider and Pozzi, 2011). It is therefore important that blanket LOX inhibitors are used with caution as these protective effects of 13-HODE would be lost if 15-LOX was inhibited along with 5- and 12-LOX.

1.6 **Cancer and lifestyle**

A World Cancer Research Fund report suggests that development and progression of 37% of cancers are as a result of diet and lifestyle factors (World Cancer Research Fund/American Institute for Cancer Research, 2007). The main lifestyle risk factors for cancer include smoking, high alcohol consumption (over 30g/day), obesity (BMI above 30), consumption of low amounts of fruit and vegetables, and low amounts of physical activity (3.5 hours or less per week) (Khaw *et al.*, 2008; Ford *et al.*, 2009; Orenstein *et al.*, 2016; Elwood *et al.*, 2018).

1.6.1 ***Foods that have associations with cancer risk***

Epidemiological studies can be used to examine specific foods and explore their association with the risk of certain diseases. A large dataset of dietary intake data along with the health status of the population can be used to examine trends and associations between certain foods or nutrients and disease risk. These epidemiological studies form the basis of the idea that certain foods/food groups/nutrients have positive or negative effects on health and risk of certain diseases.

Diet and lifestyle can affect risk of cancer in many ways. Figure 1.4 details the processes involved in the development of cancer and highlights the ways in which risks at each step in the process can be mitigated by dietary factors.

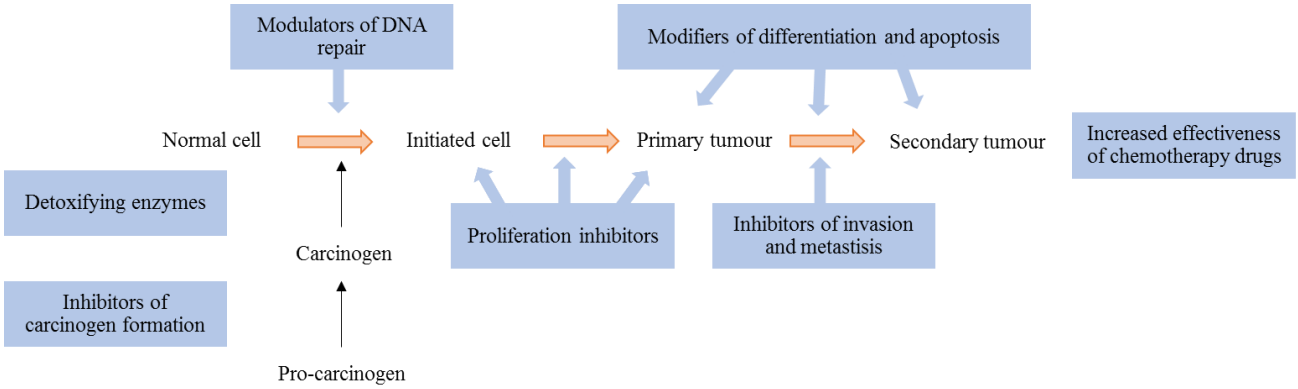


Figure 1.4: How dietary components can affect the initiation or progression of tumours (modified from Johnson (2012)).

Foods high in fat and sugar and lower rates of physical activity are, indirectly, associated with an increased risk of some cancers as they lead to higher body weight in individuals. Higher body weight is thought to increase risk of cancer because body fatness can stimulate hormones such as oestrogen and insulin which create an environment in which carcinogenesis can occur. Excess body fat can also increase inflammation leading to the initiation and progression of cancers (World Cancer Research Fund International/American Institute for Cancer Research, 2017).

Other foods prevent or encourage cancer by the physical actions they have in the body e.g. processed meats are thought to increase the risk of colon cancer by exposing the cells of the digestive tract to carcinogenic N-nitroso compounds (Bouvard *et al.*, 2015). Conversely, increased soluble fibre in the diet can reduce cancer risk by forming a barrier between harmful constituents of the diet and the gut wall (Zeng *et al.*, 2014).

Endogenous detoxifying and antioxidant enzymes: Some foods/drinks are considered to be carcinogenic because they produce toxic products on breakdown e.g. consumption of red and processed meats leads to the production of nitrosamines and alcohol is broken down to toxic acetaldehyde (Larsson *et al.*, 2006; Seitz and Becker, 2007). Alcohol also produces ROS during metabolism. Detoxifying enzymes, such as phase I enzyme cytochrome P450, are able to modify carcinogenic compounds by oxidation, reduction or hydrolysis and the products are then used as substrates for the phase II enzymes, such as glutathione-S-transferase and

NAD:quinone reductase. The phase II enzymes further modify and reduce the activity of these harmful substances so that they are neutralised and excreted (Seitz and Becker, 2007). Certain food components can stimulate the upregulation of the phase I and II enzyme expression. Flavanols, such as epigallocatechingallate (EGCG) found in green tea, and glucosinolates, found in cruciferous vegetables such as broccoli and watercress, have been seen to increase phase II enzyme expression (Johnson, 2007).

Exogenous antioxidants: ROS can also be reduced by exogenous antioxidants such as those found in fruits and vegetables, tea and wine e.g. vitamin C, E, flavanols and carotenoids. They are free radical scavengers that have the ability to donate or receive electrons to form stable by products from ROS (Young and Woodside, 2001).

Modulating DNA repair: Fruits and vegetables, along with their antioxidant effects, also contain certain compounds that have been shown to affect the ability of DNA to repair itself. Consuming certain foods have been shown to increase DNA repair after *ex vivo* oxidative insult using, for example H_2O_2. Fruit and vegetables high in antioxidants, such as carrots and kiwi fruit (Collins *et al.*, 2003; Astley *et al.*, 2004; Bøhn *et al.*, 2010; Brevik *et al.*, 2011), plus food components such as and β-carotene and flavonoids (Torbergsen and Collins, 2000; Astley *et al.*, 2004; Guarrera *et al.*, 2007) have all been shown to increase DNA repair. However, results are not always consistent and no effect was seen when supplementing the diet with fruits and vegetables or broccoli (Moller *et al.*, 2003; Guarnieri *et al.*, 2008; Riso *et al.*, 2010).

Apoptosis: Some food components have been seen to induce apoptosis in cancer cells lines. If the slowing of mitosis in induced cells is not effective in reducing the progression of cancer, some food constituents have been shown to cause cell death and therefore abort the progression of a cell to a tumour. Isothiocyanates (ITCs) from cruciferous vegetables and organosulphur compounds from garlic have been shown to induce apoptosis in cancer cell lines (Smith *et al.*, 2005; Xiao *et al.*, 2005; Tang *et al.*, 2006).

The study of nutrigenomics, the response of the gene expression pathway to nutrients, is incredibly complicated. Bioactive nutrients are hugely diverse, with over 25,000 known bioactives, and these compounds may have different effects in normal and cancer cells. Quantity, timing and length of exposure of nutrients can all have an effect on gene expression and all parts of the translation process can be affected (Johnson, 2007; World Cancer Research Fund/American Institute for Cancer Research, 2007).

1.7 Carrots and cancer risk

Carrots are members of the Apiaceous vegetable family, comprising of carrot, celery, parsnip, celeriac, fennel, and herbs such as parsley (Zidorn *et al.*, 2005). The root of the carrot is the most commonly consumed part of the vegetable. They are a good source of fibre and contain high amounts of β-carotene, a compound which is metabolized into vitamin A in the body. They also contain other important phytochemicals, as well as vitamin C, potassium and iron (Singh *et al.*, 2012).

Different coloured carrots contain differing types and amounts of carotenoids, for example the most common carrots in the UK, orange varieties, predominantly contain β-carotene, red carrots contain lycopene, purple contain anthocyanin, and white carrots do not contain carotenoids at all (Metzger and Barnes, 2009; Singh *et al.*, 2012).

Consumption of carrots in the UK is estimated to be around 101g fresh carrot per person, per week (14.4g/person/day from 2010-2015, estimated from purchasing data) and this intake has been reasonably stable since the 1970s (DEFRA, 2015). The amount of carrots purchased are higher (by weight) than any other vegetable measured in the Defra Family Food survey, demonstrating this vegetable to be an important component of the UK diet.

1.8 Epidemiological studies

1.8.1 *Associations between carrot consumption and cancer risk*

The results of large cohort investigative trials were the first to suggest that carrots can reduce the risk of chronic diseases such as cardiovascular disease (Oude Griep *et al*., 2011) and certain types of cancer (discussed below).

Both prospective and case-control studies have found reductions in cancer risk, incidence and mortality with increased carrot consumption. The sites that have been most widely investigated and have the most compelling evidence are cancers of breast, prostate, lung and stomach (see Table 1.1) but there are also a small number of studies which show effects of higher carrot intake on cancers of the pancreas (Chan *et al.*, 2005), head and neck (Maier *et al.*, 2002; Freedman *et al.*, 2008; Liu *et al.*, 2012), vulvar (Parazzini *et al.*, 1995), bladder (Zeegers *et al.*, 2001; Sakauchi *et al.*, 2004; Wakai *et al.*, 2004), colon and rectum (Franceschi *et al.*, 1998), and skin (Fortes *et al.*, 2008).

For breast cancer, studies conducted in the US (Boggs *et al.*, 2010), China (Zhang *et al.*, 2009), Japan (Hirose *et al.*, 2003), and Italy (Franceschi *et al.*, 1998) have all been shown to

have lower odds ratios (OR) with increasing carrot consumption, either alone or in combination with other vegetables. Two trials conducted in Sweden (Larsson *et al.*, 2010) and China (Guo *et al.*, 1994) did not show any clear effect of carrot consumption in the populations studied. However, carrot intake in the Swedish population was generally very high, 1 serving per day being the highest and 1-2 per week being the lowest, and the converse was true of the Chinese population, with intakes being very low, median intakes being 3.9g per year. This lack of diversity within the population may not allow for differences to be seen between high and low intakes. In lung cancers, 8 studies have been conducted with only one (Takezaki *et al*., 2001) showing no association between cancer and carrot consumption.

For urothelial cancer, carrot intake was protective in a case-control study in a Japanese population (Wakai *et al.*, 2004) but in a Japanese prospective study, the effect of carrots was not seen between cases and controls but the number of cases in this trial was much less than in the case-control study (88 vs 247) (Sakauchi *et al.*, 2004). A Dutch study also found protective effects of carrots in bladder cancer (Zeegers *et al.*, 2001) and in this study the authors had additionally separated the carrots into cooked and raw and found a stronger association with cooked carrots. This agrees with the studies by Chan *et al.*, Longnecker *et al*. and Franceschi *et al*. who observed the same in breast cancer (Longnecker *et al.*, 1997; Franceschi *et al.*, 1998; Chan *et al.*, 2005). Franceschi also observed this in colon cancer but raw and cooked carrot had similar effects on rectal cancer.

Meta-analyses, looking at all available studies together to determine overall effect, have also shown that both gastric cancer (Fallahzadeh *et al.*, 2015) and prostate cancer (Xu *et al.*, 2014) are inversely associated with carrot intake. Increasing carrot intake by 10g per day reduced risk of prostate cancer by 5%.

In the studies that specified the number of servings of carrots that gave a protective effect, the amount of carrots consumed is a manageable amount to consume regularly. Overall, 25-30g carrot (around half a medium carrot) per day appears to reduce cancer risk.

Table 1.1: Epidemiological studies that have investigated carrot consumption related to cancer risk

Type of cancer	Type of study	Population	n Cases	n Controls	OR/RR/ IRR/HR	95% CI	p (trend)	Servings	Food	Reference
Breast (cancer mortality)	prospective	China	n/s		No clear assoc'n		nr	median in population 3.9 servings per year	carrot	(Guo *et al.*, 1994)
Breast	prospective	US	1268	50660	IRR=0.83	0.67-1.04	0.02	>3 servings per wk vs <1 serving per month	carrot	(Boggs *et al.*, 2010)
Breast	prospective	Sweden	1008	38219	OR=0.88	0.70-1.12	0.3	1 or more servings per day vs 1-2 per week	carrot	(Larsson *et al.*, 2010)
Breast	case-control	US	3543	9406	OR=0.85	0.75-0.97	0.004	never vs 2 servings per week	carrot (raw)	(Longnecker *et al.*, 1997)
					OR=0.63	0.50-0.80	0.0003	"	carrot (cooked)	"
Breast	case-control	Italian	2569	5155	RR=0.7	0.6-0.9	nr	3.0 vs 0.5 servings per week	carrots (cooked)	(Franceschi *et al.*, 1998)
"					RR=0.8	0.6-0.9	nr	4.0 vs 0.5 servings per week	carrots (raw)	"
Breast	case-control	Japan (premenopausal)	184	1536	OR=0.81	0.65-1.00	<0.006	almost never vs 5 or more times a week	carrots	(Hirose *et al.*, 2003)
"	"	Japan (postmenopausal)	174	1023	OR=0.72	0.58-0.91	<0.0006	almost never vs 5 or more times a week		"

Type of cancer	Type of study	Population	n Cases	n Controls	OR/RR/ IRR/HR	95% CI	p (trend)	Servings	Food	Reference
Breast	case-control	Chinese	438	438	OR=0.58	0.38-0.88	0.001	Q1 (11.37g) to Q4 (48.48g); median intake 26.78g/day	carrot and tomato	(Zhang *et al.*, 2009)
Nasal pharangeal	case-control	China	600	600	OR=0.53	0.35-0.79	0.001		carrot, pepper and tomatoes	(Liu *et al.*, 2012)
Head and neck	case-control	German (males)	209	110	OR=0.17		<0.001		carrot	(Maier *et al.*, 2002)
Head and neck	prospective	US	787	490802	OR=0.73	0.60-0.89	0.001	2 servings vs 0 servings of carrot per 100Kcal	carrot	(Freedman *et al.*, 2008)
Prostate	case-control	Malaysia	35	70	nr		0.8	Carrot intake higher in controls compared to cases (147.8 vs 157.3g per week respectively)	carrot	(Shahar *et al.*, 2011)
Prostate	meta-analysis (10 papers up to 2006)	n/a	n/a	n/a	OR=0.82	0.70-0.97	nr	highest vs lowest intake in each paper	carrot	(Xu *et al.*, 2014)
Lung	case-control	Spain	103	206	OR=0.37	0.19-0.74	0.007	highest vs lowest tertiles	orange/yellow vegetables (mostly carrot)	(Agudo *et al.*, 1997)
Lung	case-control	Stockholm, Sweden (never smokers)	124	235	RR=0.55	0.27-1.11	0.05	less than weekly vs daily or almost daily	carrot	(Nyberg *et al.*, 1998)

Type of cancer	Type of study	Population	n Cases	n Controls	OR/RR/ IRR/HR	95% CI	p (trend)	Servings	Food	Reference
Lung	case-control	Europe multi-centre (non-smokers)	506	1045	OR=0.80	0.5-1.1	0.18	never/less than monthly vs several times weekly	carrot	(Brennan *et al.*, 2000)
Lung (squamous and small cell carcinoma)	case-control	Japan	1045	4153	OR=1.49	0.94-2.36	0.02	<1 serving a week vs over 5 servings per week	carrot	(Takezaki *et al.*, 2001)
Lung	case-control	UK	982	1486	OR=0.49	0.31-0.78	<0.001	more than weekly vs never	carrot	(Darby *et al.*, 2001)
Lung	case-control	Poland (women)	242	352	OR=0.13	0.06-0.26	0.066	consumption at least 5x per week vs rarely consumed	carrot	(Rachtan, 2002)
Lung	case-control	Italy	342	292	OR=0.67	0.42-1.05	0.066	Never vs weekly or more	carrot	(Fortes *et al.*, 2003)
Lung	case-control	China	218	436	OR=0.84	0.47-0.80	0.006	highest vs lowest tertiles	carrot	(Galeone *et al.*, 2007)
Pancreas	case-control	US	532	1701	OR=0.56	0.41-0.76	0.0001	Q1 vs Q4	carrot	(Chan *et al.*, 2005)
Bladder	prospective	Netherlands	538	2953	RR=0.66	0.47-0.92	nr	per 25g carrot	carrots (cooked)	(Zeegers *et al.*, 2001)
Bladder	"	"	"	"	RR=1.16	0.89-1.50	nr	per 25 g carrot	carrots (raw)	"
Bladder (cancer mortality)	prospective	Japan	88	114517	HR:1.53	0.66–3.55	0.92	<1-2/month vs>3-4 times per week	carrots and squash	(Sakauchi *et al.*, 2004)

Type of cancer	Type of study	Population	n Cases	n Controls	OR/RR/ IRR/HR	95% CI	p (trend)	Servings	Food	Reference
Bladder	case-control	Japan	247	640	OR=0.41	0.16-1.01	0.24	5 or more times per week vs 1-3 times per month or less	carrot	(Wakai *et al.*, 2004)
Lymphoma	case-control	Japan (females)	333	55904	OR=0.63	0.45-0.90	nr	3-4 servings a week vs occasionally or never	carrots	(Matsuo *et al.*, 2001)
	"	Japan (males)			OR=0.86	0.68-1.09	nr	"	"	"
Vulvar	case-control	Milan, Italy	125	541	RR=1.5	1.0-2.4	nr	less than one serving vs more than 2 servings per week	carrots	(Parazzini *et al.*, 1995)
Colon	case-control	Italy	1225	5155	RR=0.7	0.6-0.9	nr	3.0 vs 0.5 servings per week	cooked carrots	(Franceschi *et al.*, 1998)
					RR=0.9	0.7-1.1	nr	4.0 vs 0.5 servings per week	carrots (raw)	"
Rectum	case-control	Italy	728	5155	RR=0.7	0.6-0.9	nr	3.0 vs 0.5 servings per week	carrots (cooked)	(Franceschi *et al.*, 1998)
					RR=0.7	0.5-0.9	nr	4.0 vs 0.5 servings per week	carrots (raw)	
Stomach	prospective	Japan (males)	859	110792	HR=1.14	0.80-1.61	0.6	<1-2 per month vs 1 per day	carrot and pumpkin	(Tokui *et al.*, 2005)
"	"	Japan (females)			HR=1.10	0.76-1.60	0.24	"	"	"
Gastric	meta-analysis	5 papers	n/a	n/a	OR=0.74	0.68-0.81	0.0001	25g/day vs 2-4 per week	carrot	(Fallahzadeh *et al.*, 2015)
Cutaneous melanoma	case-control	Italy	304	305	OR=0.57	0.38–0.84	0.013	less than weekly vs weekly or more	carrot	(Fortes *et al.*, 2008)

Type of cancer	Type of study	Population	n Cases	n Controls	OR/RR/ IRR/HR	95% CI	p (trend)	Servings	Food	Reference
Lymphoma		Japan (women)			OR=0.63	0.45-0.90	nr	3-4 servings a week vs occasionally/never	carrot	(Matsuo *et al.*, 2001)
"	"	Japan (men)			OR=0.86	0.68-1.09	nr	3-4 servings a week vs occasionally/never	carrot	"

n/s: not specified; OR: Odds ratio; HR: Hazard ratio; RR: relative risk; nr: not reported

1.9 **Effect of carrot consumption on human health: Dietary intervention trials**

1.9.1 *Effect of carrot consumption on antioxidant capacity*

A number of trials have investigated the antioxidant effect of consuming carrot juice. A reduction of oxidative stress (measured by 8-iso-PGF2-α) was observed after overweight breast cancer survivors consumed 330mL fresh carrot juice for 3 weeks (Butalla *et al.*, 2012) and increases in antioxidant capacity and decreases MDA (malondialdehyde, a marker of ROS in the blood) were seen after consumption of 470mL fresh carrot juice daily for 3 months by healthy adults (Potter *et al.*, 2011a). The endogenous antioxidant molecules appear to also be affected as GSH and oxidized GSH in whole blood were reduced after 2 weeks' supplementation with 330mL carrot juice (Bub *et al.*, 2000). In contrast, 2 weeks of consumption of 330mL/day carrot juice did not reduce plasma MDA concentration compared with a 2-week carotenoid washout in healthy adults (Briviba *et al.*, 2004b) and Lee and colleagues (2011) found no changes in catalase, GSH or superoxide dismutase (SOD) after smokers were supplemented with either 300mL carrot juice daily, β-carotene supplement or placebo for 8 weeks (Lee *et al.*, 2011).

1.9.2 *Effect of carrot consumption on inflammatory markers*

Despite the reduction in antioxidant status, both the Butalla and Potter trials found no effect on inflammatory markers TXB2, PGE2 and high sensitivity C-reactive protein (hsCRP) (Butalla *et al.*, 2012) or IL-1α ad CRP (Potter *et al.*, 2011a). However, another trial observed non-significant reductions of IL-6 and CRP inflammatory markers after consumption of 200mL carrot juice/day for 8 weeks in diabetics. A second group, consuming carrot juice supplemented with a further 10mg β-carotene saw significant reductions in the inflammatory markers but there was no difference between the two groups. The β-carotene therefore appears to be important for the lowered inflammatory status (Ramezani *et al.*, 2014).

1.9.3 *Effect of carrot consumption on DNA damage*

Carrot juice supplementation has also been seen to reduce DNA damage. The Lee *et al.* (2011) study above found a 13% reduction in lymphocyte DNA damage (measured by %DNA in tail using the comet assay) which was similar to the effect of the β-carotene supplement. Endogenous strand breaks and oxidative pyrimidine base damage were reduced after consumption of 330mL/day carrot juice for 2 weeks. However, this trial also included two weeks of tomato juice and 2 weeks of spinach intake either side of the carrot juice with no wash out in between so these effects could be cumulative or confounded by these other carotenoid rich vegetables (Pool-Zobel *et al.*, 1997).

Investigating the health of the gut during these trials, lipid peroxidation in faeces was not affected by the carrot juice intervention (Briviba *et al.*, 2004a) and faecal water from the trial had no effect on the cytotoxicity or anti-proliferative activity on HT29 colon adenocarcinoma cells in cell culture after consuming carrot juice (Schnabele *et al.*, 2008). However, studies using juices exclude the benefits of the fibre fraction of the carrot which has been shown to reduce the effect of harmful compounds on the intestinal lumen by reducing the toxic by-products of colonic bacteria and faecal ammonia output (Chau *et al.*, 2005) so trials with whole carrots may have a different effect.

These trials have shown mixed effects, either positive or no effect but there have been no trials showing a negative effect on health. These trials had differing participant characteristics and lengths of intervention making the dietary intervention trials difficult to compare and may be the reason for the lack of consistency of results.

1.10 Effect of carrot components on cancer risk

Health promoting phytochemicals in the carrot include carotenoids, phenolic acids (5-*O*-caffeoylquinic acid), ascorbic acid (vitamin C), and the falcarinol-type polyacetylenes (falcarinol, falcarindiol and falcarindiol-3-acetate) (Seljåsen *et al.*, 2013a).

1.10.1 *Phenolic acids*

Phenolics, or polyphenols, have garnered considerable attention for their antioxidant and anti-cancer effects. Phenolic acids are phenolic compounds with a single aromatic ring. While they have no nutritive value, the phenolics are considered to be one of the main sources of antioxidants in the diet. Carrots have a reported total phenolic content of about 27±2µg/g (Oviasogiel *et al.*, 2009), with around 50% of this in the form of chlorogenic acid (5-*O*-caffeoylquinic acid), derived from cinnamic acid (Zhang and Hamauzu, 2004). Chlorogenic acid intake has been associated with a reduction of chronic diseases such as cancer (Arscott and Tanumihardjo, 2010). It has been shown to be a strong inhibitor of MMP-9 (Jin *et al.*, 2005), and has been shown to reduce TPA (12-O-tetradecanoylphorbol-13-acetate)-induced tumour promotion in mouse skin, although the same study showed it had little effect on COX and LOX activity (Conney *et al.*, 1991). However, carrots are considered to be 'low' in phenolic acids, 12mg catechin equivalents/100g fresh vegetable, compared with some dietary sources such as spinach (82mg) and red cabbage (213mg) (Mélo *et al.*, 2009) and chlorogenic acid specifically, with levels of 0.1mg/g carrot (Sun *et al.*, 2009) compared with 1.3mg/mL in

strong coffee (Olthof *et al.*, 2001) so it would be expected that other foods would have stronger associations with cancer risk reduction than carrot if the effects were due to chlorogenic acid alone.

The effect of processing on phenolics shows a detrimental effect of boiling as they are not heat stable and are water soluble so can be lost into cooking fluid. Carrots have been seen to retain only 60% of their raw amount of gallic-acid equivalents (GAE), and losses of 87.6% antioxidant potential after boiling (Guillén *et al.*, 2017). Around 30% of chlorogenic acid is absorbed from the gut (Arscott and Tanumihardjo, 2010), therefore consumption after cooking (as carrots are commonly consumed) would lead to very low intakes.

1.10.2 *Ascorbic acid*

Ascorbic acid, or vitamin C, is a potent antioxidant. The concentration in carrots is approximately 4-6mg/100g (Szeto *et al.*, 2002; Sharma *et al.*, 2012). Carrots are not considered to be a major source of vitamin C compared with many other fruits and vegetables with much higher levels and 95% of the antioxidant ability of the carrot is thought to be due to other compounds (Szeto *et al.*, 2002).

1.10.3 *Carotenoids*

Carotenoids are fat-soluble pigments produced in plants that in the plant they trap energy from sunlight for photosynthesis and protect chlorophyll from photodamage. Carotenoids are therefore present in many fruits and vegetables. There are many types of carotenoid but the major carotenoids in carrots are α- and β-carotene. Both α- and β-carotene are pro-vitamin A carotenoids, meaning that they are metabolized by the body to produce retinol. Carrots contain high amounts of β-carotene (8.3mg/100g) and α-carotene (3.8mg/100g) (USDA, 2018) making them a major source of both carotenoids in the UK diet. The carotenoids have a structure of conjugated double bonds which give them high reduction potential and thus they have antioxidant properties (Sies and Stahl, 1995).

1.10.4 *Associations between carotenoid consumption and cancer risk (α- and β-carotene)*

The cancer-protective effect of carrots (and other vegetables) was originally thought to be related to the carotenoid content, as carrots are rich in α- and β- carotene, and lutein. β-carotene is a potent scavenger of singlet oxygen and has pro-vitamin A activity which are thought to be the reason for its health benefits (Jomova and Valko, 2013) but the biological

actions of β-carotene go beyond these effects, and they have been shown to be able to modulate phase I and II drug detoxifying enzymes, regulate cell growth, modulate gene expression and immune responses which could all contribute to their anti-inflammatory actions (Rao and Rao, 2007). It has been known for a long time that low levels of β-carotene in the blood are associated with increased mortality (Greenberg *et al.*, 1996; De Waart *et al.*, 2001; Li *et al.*, 2011). Having lower plasma β-carotene is also associated with higher lung cancer risk, especially in smokers (Wald *et al.*, 1988) and this, along with the results of epidemiological studies looking at carrots, is what made researchers hypothesise that β-carotene has protective effects against cancer. There is certainly a lot of observational data to corroborate this assumption with many epidemiological studies investigating the associations between cancer and carotenoid consumption. Indeed, some of the epidemiological studies mentioned previously have investigated the effect of carrots grouped with other carotenoid-rich vegetables like tomatoes (Zhang *et al.*, 2009; Liu *et al.*, 2012), or those foods specifically higher in β-carotene such as pumpkin (Tokui *et al.*, 2005), as a result of this assumption. To assess the effect of carotenoids on cancer, carotenoid intake from the diet can be specifically examined using databases specifying the carotenoid content of a variety of foods and estimating intake according to the amounts of those foods eaten. However, it is seen as more accurate to look at carotenoid concentrations in blood plasma, to avoid errors in dietary recall. Despite this being a more objective measure, neither method controls for all confounding factors. In particular, it is impossible to distinguish effects of any dietary component that is found in foods or diets rich in β-carotene. So, in order to assess any causal associations, it is necessary to conduct placebo-controlled supplement trials, where the carotenoid of interest or a placebo is taken in capsule form, then biomarkers of cancer risk are measured over time or cancer occurrence is observed.

Many observational studies have shown inverse associations with cancer risk and carotenoid intake or plasma concentrations. Many recent meta-analyses looking at data for different types of cancer have shown inverse associations with α- and β- carotene dietary intake and plasma concentrations. Two meta-analyses looking at the effect of dietary intake (Yu *et al.*, 2015) or plasma concentrations of carotenoids (Abar *et al.*, 2016) showed inverse effects on lung cancer risk. Assessing 18 publications, β-carotene reduced relative risk (RR) when comparing the highest vs lowest plasma concentrations, (RR=0.71(CI:0.56-0.91)). Plasma α-carotene had the strongest inverse effect on lung cancer risk (RR=0.70 (CI:0.48-1.01)) for the highest vs lowest plasma concentrations with a 34% reduced risk (CI:20-45%) per 5 μg/100 mL plasma concentration. When the cohort was divided by sex, the risk reduction of α-carotene remained but for β-carotene, the effect was only seen in men (Abar *et al.*, 2016). A meta-analysis of 19

studies of dietary intakes of β-carotene showed a reduced risk of lung cancer in those who had the highest vs lowest intake of β-carotene (RR=0.77 (CI: 0.68-0.87)) (Yu *et al.*, 2015) and the same was seen for cervical cancer in a meta-analysis of 22 studies (OR=0.68 (CI:0.55-0.84)), but there was no effect of β-carotene if HPV infection was present (Myung *et al.*, 2011). Oesophagus cancer risk was reduced with both α- and β-carotene dietary intake, (OR=0.81 (CI:0.70-0.94)) and OR=0.58 (CI:0.44-0.77), respectively (n=10)) (Ge *et al.*, 2013). In head and neck cancer studies (n=16), α-carotene intake reduced risk consistently, and the effect was especially strong for oral cavity and pharynx cancer (OR 0.57 (CI:0.41-0.79)) but with the β-carotene, no significant risk reduction was seen overall. β-carotene intake both increased and decreased risk when looking at the different areas of the head and neck separately (Leoncini *et al.*, 2015). Breast cancer risk was reduced with increasing dietary β-carotene intake (RR=0.93 (CI:0.88-0.98)) with 5mg/day increments reducing risk by 5% (CI:1-4%) but only a weak reduction was seen with α-carotene (n=13) with dietary intakes ~1.5mg/d reducing risk by 9% (3-15%) with no further risk reduction following higher intake. Blood β-carotene (n=15) had a significant association with breast cancer (RR=0.82 (CI: 0.65-1.04)) with 26% reduction in risk (CI:3-43%) per 50 μg β-carotene/dL. Contrary to the dietary intake in the same study, plasma concentrations of α-carotene were associated with a reduced risk of breast cancer comparing high vs low concentrations (RR=0.80 (CI:0.68-0.95)) and a 18% reduction in risk (CI:8-27%) per 10 μg α-carotene/dL (Aune *et al.*, 2012). In a prostate cancer meta-analysis (n=34), both dietary intake and plasma concentrations of α-carotene reduced risk of prostate cancer, (RR:0.87 (CI:0.76-0.99)) for dietary α-carotene. A 2% reduction in risk was seen with every 0.2mg/day consumed (CI:1-4%). However, for β-carotene there was no association with prostate cancer risk (Wang *et al.*, 2015).

Although the strength of the affect appears to differ in the different types of cancer, an overall protective effect on cancer risk with α- and β-carotene is apparent, as measured by dietary intake or plasma concentration. To determine whether the associations are truly due to the carotenoids, supplement studies are necessary to exclude the effects of the other components in food that may also mediate the health effects under investigation. Plasma carotenoids are considered a good biomarker of fruit and vegetable consumption in general (Abar *et al.*, 2016) so supplement studies are especially important for these compounds, to try to exclude the effect of the myriad of other components in these foods.

1.10.5 *Beta carotene supplementation studies*

Several clinical trials have been conducted with antioxidant supplements including vitamin A, E and β-carotene with mixed results. In some studies, there was no effect of β-carotene on biomarkers of health (Gallicchio *et al.*, 2008; Druesne-Pecollo *et al.*, 2010). In other populations, especially smokers and those who consumed high levels of alcohol, increased risks of cancer with supplementation have been seen. The Alpha-Tocopherol Beta Carotene Cancer Prevention Study (ATBC) was the first trial in which β-carotene supplementation (20mg) was shown to increase the risk of lung cancer in smokers (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study, 1994). The Carotene and Retinol Efficiency Trial (CARET) had to be stopped early as the researchers showed that the 30mg/day β-carotene supplement was associated with increased lung cancer risk in smokers (Omenn *et al.*, 1996). Other trials with colorectal cancer have since agreed with this (Baron *et al.*, 2003) and a systematic review and meta-analysis of RCT with antioxidant supplements showed both increased and decreased risks with supplementation and concluded that no supplements were of benefit in reducing colorectal cancer risk (Papaioannou *et al.*, 2011). Similar meta-analyses came to the same conclusions for prostate cancers – with no benefit of any antioxidant supplements on occurrence, severity or death from prostate cancers (Stratton and Godwin, 2011), and there tended to be an increase in mortality with β-carotene supplements in cancers of the gastric system RR=1.05 (CI: 0.99-1.11) which increased when β-carotene was taken in combination with vitamins A and E (Bjelakovic *et al.*, 2007). A further meta-analysis (n=13) found there was an increased risk in gastric (RR=1.34 (CI: 1.06–1.70)) and lung (RR=1.13 (CI: 1.04–1.24)) cancers with 20-30mg/day β-carotene and this risk was increased in high risk populations of smokers and asbestos workers (RR=1.20 (CI: 1.07–1.34) and RR=1.54 (CI: 1.08–2.19) for lung and gastric cancer respectively) (Druesne-Pecollo *et al.*, 2010). The carcinogenic environments involved with smoking, heavy drinking and exposure to asbestos in combination with β-carotene supplementation appear to mediate the observed increased cancer risk (Albanes *et al.*, 1996). Carcinogenic substances are usually neutralised by glutathione (GSH), an endogenous detoxifying compound. Glutathione S-transferases (GST) are enzymes that catalyse the binding of the carcinogen to GSH, but in the presence of high concentrations of β-carotene, GST is downregulated, allowing carcinogens to cause DNA damage and increase the risk of cancers (Vrolijk *et al.*, 2015). Therefore, high β-carotene is not recommended for those who are exposed to carcinogenic environments. Additionally, a large meta-analysis looking at all-cause mortality in healthy participants showed that β-carotene supplements significantly increased mortality if taken singly (RR=1.0 (CI: 1.01-1.09)) or in combination with other antioxidants (RR=1.04 (CI: 0.01-1.07)) (Bjelakovic *et al.*,

2014). This demonstrates that it is not just high-risk groups that do not benefit from β-carotene supplementation.

As antioxidant supplement trials have largely shown no benefit to, and even increase risk of cancer (and mortality by all causes), there is a lack of convincing evidence to justify the use of these supplements (Goodman *et al.*, 2011; Papaioannou *et al.*, 2011) so there is a discrepancy seen between the protective effects of fruit and vegetables and the pro-cancer effects of antioxidant supplements. This could be because carotenoids are most effective when in combination with multiple other components and phytochemicals within the carrot, or with fruit and vegetables in general. Their complex interactions would not be seen in purified, high dose, synthetic supplements. However, to the best of the authors' knowledge, no published data are available that specifically support this hypothesis compared with other hypotheses (Liu, 2004; Linnewiel-Hermoni *et al.*, 2015). Alternatively, the anti-cancer effect could be due to other phytochemicals that until now have been understudied for their health benefits. Carrots are the major source of β-carotene in the diet of Europeans and North Americans, with 50% of the intake of the compound coming from this vegetable (O'Neill *et al.*, 2001). Plasma β-carotene would therefore correlate well with carrot intake. However, β-carotene is also present in many other fruits and vegetables e.g. sweet potato, butternut squash and mango (McCance and Widdowson, 2002). Alpha-carotene, also present in large concentrations in carrot, is not as common in other foods. α-carotene intake is therefore more strongly correlated with carrot intake than β-carotene (O'Neill *et al.*, 2001). Also, α-carotene concentration in blood plasma is a much better biomarker of carrot consumption than β-carotene in plasma (Al-Delaimy *et al.*, 2005). Some studies have found a stronger negative correlation of lung cancer with intake of α-carotene than β-carotene (Ziegler *et al.*, 1996; Michaud *et al.*, 2000; Wright *et al.*, 2003; Abar *et al.*, 2016) suggesting that the dietary source of the effect comes specifically from carrots. The anti-cancer effects of β-carotene are stronger than α-carotene: more potent antioxidant, contributes more to vitamin A production and has a stronger anti-cancer effect *in vitro.* This would suggest that another compound inside the carrot contributes to the anti-cancer effect but α-carotene, as a biomarker for carrot consumption, is strongly correlated because it also correlates with these other carrot compounds.

1.11 **Polyacetylenes**

Food-plants of the Apiaeae family, including carrots, contain the bioactive C_{17} - polyacetylenes. They were first discovered when investigating traditional medicines such as

ginseng, Queen Anne's lace, Devil's Club and *Angelica japonica,* all members of the closely related Apiaceae and Araliaceae families of plants (Christensen *et al.*, 2002). Traditional herbal medicines have been used for many centuries for 'more energy and vitality' in the elderly, inflammatory conditions such as arthritis and fever as well as a host of other illnesses such as coughs, bronchitis and pain relief, and cancer (Christensen *et al.*, 2006; Sun *et al.*, 2010; Kang *et al.*, 2012). The active components of these plants have been investigated and there is growing understanding of which compounds are responsible for these effects. Extracts have been investigated for their anti-inflammatory and cytotoxic actions *in vitro* and the polyacetylene family of compounds have shown promise as the phytochemical eliciting these effects, as described in further detail below. It was discovered that the edible members of the Apiaceae family such as carrot, parsnip, celery, celeriac, fennel and parsley also contained the falcarinol-type polyacetylenes (Zidorn *et al.*, 2005) and it is speculated that consumption of these compounds could have beneficial effects on inflammation and cancer, however, this needs to be investigated with *in vivo* studies.

1.11.1 *Chemical structure*

There are over 1400 types of polyacetylene and related compounds that have been identified from plants (Christensen *et al.*, 2002). The polyacetylenes are characterized by two conjugated triple bonds, and two non-conjugated carbon-carbon double bonds, one on either side of the triple bonds and are formed from fatty acids, including oleic/linoleic acid (Minto and Blacklock, 2008). The most commonly occurring polyacetylenes in food plants are those of the falcarinol-type (see Figure 1.5). The types of polyacetylene and their concentration can vary widely between species and carrots have been shown to have concentrations that vary markedly by cultivar (Yates *et al.*, 1983; Metzger and Barnes, 2009), growing conditions (Seljåsen *et al.*, 2013a), harvest year (Yates *et al.*, 1983; Søltoft *et al.*, 2010) and harvest date (Kjellenberg, 2007; Kjellenberg *et al.*, 2010). Three major polyacetylenes identified in carrots are falcarinol, falcarindiol and falcarindiol-3-acetate. Fennel also contains these 3 polyacetylenes, but celery and parsnip contain just falcarinol and falcarindiol (Christensen *et al.*, 2002).

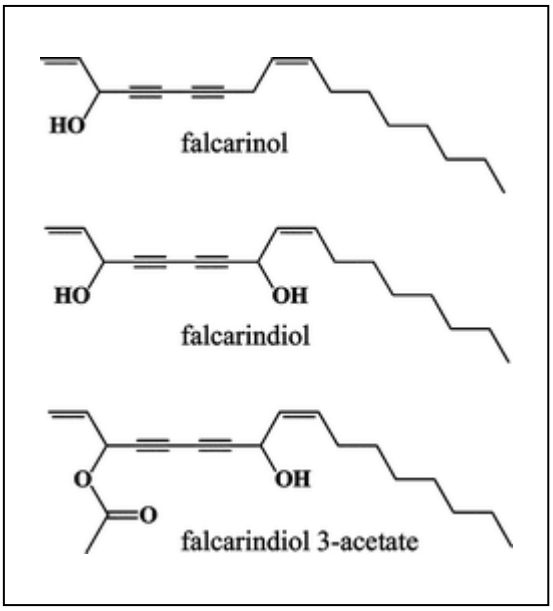


Figure 1.5: Falcarinol-type polyacetylenes found in edible vegetables of the Apiaceae family (Baranska and Schulz, 2005).

1.11.2 *Function of polyacetylenes in plants*

In many plant species, polyacetylenes are phytoalexins - chemicals that are released in response to stress (Imoto and Ohta, 1988). However, in the Apiaceae family of plants they are pre-infectional, that is, they are always present in the vegetable, but they can rise in response to a stress stimulus such as growth in drought conditions (Lund and White, 1986) exposure to infection (Davies and Lewis, 1981) or after tissue damage, as demonstrated by scratching the surface of the skin of a carrot (Czepa and Hofmann, 2003). In vegetables that do not produce polyacetylene at basal levels, they can be induced in response to microbial stress, such as aubergine, which produce falcarindiol (Imoto and Ohta, 1988) and tomatoes which express both falcarinol and falcarindiol (De Wit and Kodde, 1981). Carrots appear to produce polyacetylenes as a protection mechanism for insults during growth, such as attack by pests, fungi or bacteria. When carrots are exposed to carrot psyllid *Trioza apicalis* Förster, a type of jumping plant louse, falcarindiol concentrations increased by over 300% (Seljåsen *et al.*, 2013b). The expression of polyacetylenes is not only seen in the field; the root remains metabolically active after harvest and polyacetylene concentrations have been seen to rise and fall during storage (Hansen *et al.*, 2003; Kidmose *et al.*, 2004a; Kjellenberg *et al.*, 2012). Resistance to storage pathogens is an advantage for the roots, and anti-fungal actions of the polyacetylenes have been studied with the different microorganisms that affect carrots roots. Isolated falcarindiol has been shown to inhibit growth of *Mycocentrospora acerina*, a storage pathogen known as liquorice rot, and the fungus *Cladosporium cladosporioides* (Garrod *et*

al., 1978). Carrots have been shown to inhibit the growth of *M. acerina* on both the periderm and on wounded tissue (Davies and Lewis, 1981) and this inhibition follows the gradient of falcarindiol through the root tissue (Olsson and Svensson, 1996). Falcarinol has also been shown to inhibit the growth of the fungus *Botrytis cinerea* (Harding and Heale, 1980). Additionally, falcarindiol has been shown to have anti-bacterial effects against *Bacillus cereus* and *Micrococcus luteus* (Meot-Duros *et al.*, 2010). The concentration of polyacetylene through the root, at higher concentrations at the surface to decreasing concentrations towards the middle, corresponds to a gradient of resistance to pathogens where it is needed the most (Figure 1.6), keeping the carrot root protected from a variety of insults.

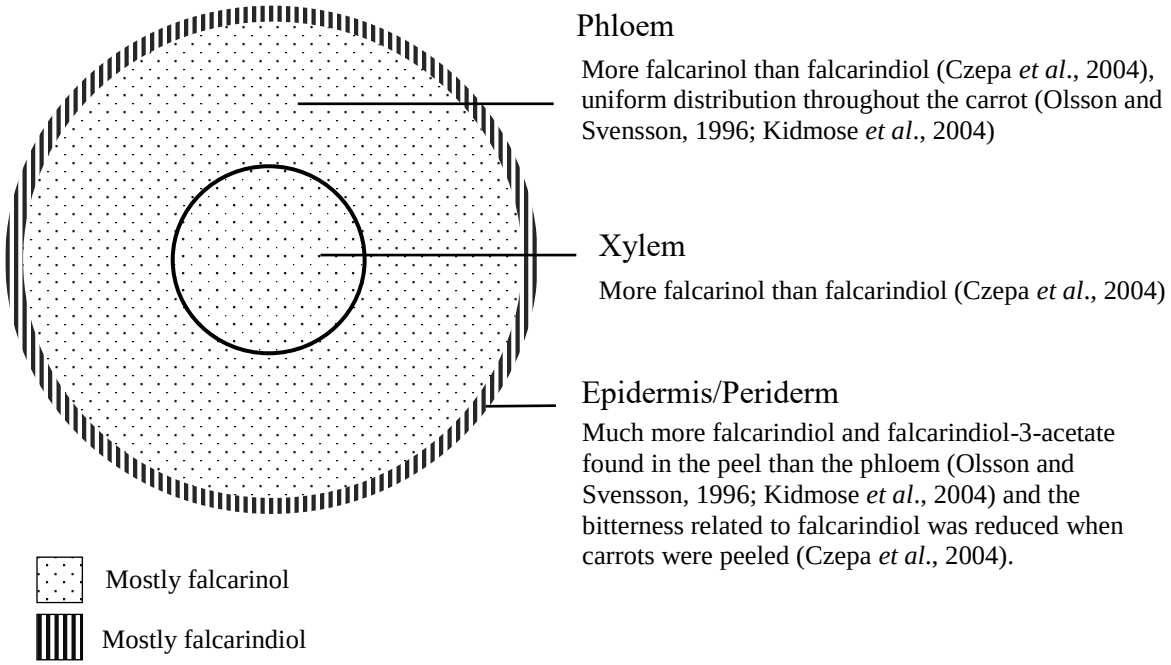


Figure 1.6: Distribution of polyacetylenes in carrot root

1.11.3 ***Polyacetylenes in animals/humans: digestion, metabolism and excretion***

Unlike other phytochemicals, little is known about digestion and metabolism of polyacetylenes in the body. The bioavailability of polyacetylenic substances was observed *in vitro* by using a caco-2 cell monolayer and measuring the amount of the compounds moving from one side of the membrane to the other. Around 30% of the polyacetylenes appeared on the other side of the monolayer, suggesting they are bioavailable, and that there may be some retention of the compounds by the cells (Chicca *et al.*, 2008). MK-1 (human epithelium) cells

have also been seen to absorb panaxytriol (Saita *et al.*, 1993). Although these two studies did not involve the polyacetylenes found in carrots, the compounds are similar in structure.

In vivo, polyacetylenes have been shown to be bioavailable in one rat and two human trials. Panaxytriol, a polyacetylene closely related to falcarinol, was administered orally to rats at 1mg/kg, and peak plasma concentrations of 50ng/mL were seen 25 minutes later (Saita *et al*., 1994). The first small (n=2) human trial fed participants 800mL carrot juice, containing 28mg of falcarinol, with bread and butter. This led to a peak concentration of 6-15ng/mL falcarinol in blood plasma. The researchers also detected falcarindiol in the blood plasma but the concentration was not reported (Hansen-Møller *et al.*, 2002). In a further trial, 14 subjects were fed carrot juice in 300, 600 and 900mL doses (with falcarinol of 4, 8, 12mg respectively) and regular blood samples were taken for falcarinol blood plasma analysis by LC-MS. Falcarinol was detectible after 30 minutes, at peak concentrations after 2 hours, and had returned to baseline by 8 hours. Peak plasma concentrations were between 1 and 2.5ng/mL (Haraldsdóttir *et al.*, 2002). This demonstrates that falcarinol can be detected in blood plasma at 'biologically relevant' concentrations i.e. those concentrations that have a cytotoxic effect on cancer cells and anti-inflammatory action *in vitro*.

The fate of polyacetylene compounds after the blood plasma has not been explored therefore it is not known where the compounds go after they are absorbed into the blood, whether they are taken up into tissues, or stored, whether they are broken down into other active metabolites or how they are excreted.

Both of the human studies were conducted with carrot juice as the source of polyacetylenes. While this may be convenient way to administer an accurate dose, carrots are usually consumed whole, either raw or cooked, in the general population. Bioavailability of a compound in a food is more complex than in a purified form, or a juice which has had the fibre removed. To determine whether polyacetylenes are bioaccessible from whole vegetables and what concentrations are seen in biofluids, bioavailability studies should be carried out with whole vegetables. As polyacetylenes have been seen to affect the risk of colorectal and bladder cancers, analysis of urine and faecal samples for polyacetylenes would also be of interest to determine the concentrations potentially in contact with the cells of these parts of the body.

1.12 Potential effect of polyacetylenes on health

As with many phytochemicals, polyacetylenes are not only beneficial to the plant but could also have benefits in humans, such as anti-bacterial, cardiovascular protective, anti-inflammatory, and anti-cancer effects; they also have some negative effects, such as allergic reactions, and, in high doses, neurotoxic effects.

1.12.1 *Anti-bacterial/anti-viral actions*

As well as being protective against pathogens that attack stored carrots, polyacetylenes (falcarindiol) are also effective against human bacteria and viruses. *Eschericha coli, Staphylococcus aureus* and *Helicobacter pylori* have been inhibited with falcarindiol at 1.67µg/mL (Cho *et al.*, 2013). *Micrococcus luteus* and *Bacillus cereus,* two food-borne pathogens that cause diarrhoea and vomiting, have been inhibited with IC_{50} values (concentrations that cause 50% inhibition) of 20µM of falcarindiol after a 48hr incubation (Meot-Duros *et al.*, 2010). The ability of polyacetylenes to prevent the growth of these bacteria at non-toxic concentrations presents an interesting pharmacological potential for these compounds. Certain bacteria/viruses have an association with cancer as mentioned earlier, due to the pro-inflammatory mechanisms associated with infection. Therefore, the implications of these results extend beyond the observed antibacterial effects.

1.12.2 *Antioxidant activity*

The antioxidant effect of the polyacetylenes appears to be mainly due to their effects on phase II drug metabolising enzymes such as glutathione-*S*-transferase (GST) and NAD(P)H: quinone oxidoreductase (NQO1) and endogenous antioxidant molecules such as GSH and catalase rather than the compounds having radical quenching abilities itself.

Muscle cells treated with hydrogen peroxide (H_2O_2), to induce ROS, were protected against ROS when pre-treated with low dose falcarinol or falcarindiol. There was a slight acceleration in the production of ROS but an increased expression of glutathione reductase (cGPx), an endogenous antioxidant, allowed for more efficient quenching of ROS. There was also a concurrent decrease in heat shock proteins, involved with cell protection and repair, indicating less repair was needed as less damage to the cells was occurring. However, protection was lost at higher concentrations of polyacetylenes (Young *et al.*, 2008). In a further study, a falcarinol-rich oil extract was incubated with whole blood cells and H_2O_2. A concentration-dependant reduction in DNA damage was seen, with lower concentrations of falcarinol

resulting in lower DNA damage. All incubations containing falcarinol had lower damage than the H_2O_2 control (Živković *et al.*, 2016). Treatment of liver cells from rats with falcarindiol increased the expression of the phase 2 enzymes GST and NQO1, and the endogenous antioxidant molecules GSH, catalase (Ohnuma *et al.*, 2009) and heme oxygenase (HO-1) (Uto *et al.*, 2015). This appeared to be through the activation of the transcription factor nuclear factor 2E-related factor 2 (Nrf2) and its binding site antioxidant response element (ARE) which is positioned before the detoxifying genes on the chromosome. Nrf2 is sequestered by Kelch-like ECH-associated protein 1 (Keap2) under non-oxidation conditions but releases it under stimulation with oxidants or electrophiles, allowing it to move into the nucleus. In the presence of falcarindiol, Nrf2 has been seen to accumulate in the nucleus (Ohnuma *et al.*, 2009). The conjugated diacetylene carbons of falcarindiol are electrophilic and form adducts with the cysteine (cys) in Keap1. The alkylated cys residues alter the structure and ultimately the function of Keap1 resulting in the release of Nrf2 which activates ARE (Ohnuma *et al.*, 2010).

In vivo mouse feeding experiments demonstrated that GST and NQO1 drug metabolising enzymes increase in a variety of tissues after ingesting falcarindiol, in a dose dependant manner. Catalase and GSH antioxidant molecules were also increased. Tissue injury was less when the mice received pre-treatment of falcarindiol before being given a hepatotoxic insult and lipid peroxidation was reduced after the insult. Falcarindiol was not able to suppress lipid peroxidation alone and it could not be detected in the 2,2, diphrenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay suggesting it has no antioxidant ability alone (Ohnuma *et al.*, 2011). This data suggests that falcarinol and falcarindiol can stimulate endogenous mechanisms to help detoxify carcinogens and suppress oxidative stress, acting as cytoprotective compounds and helping to maintain DNA integrity. The dose appears to be important, with lower doses having more positive effects on cytoprotection. This could be due to the polyacetylenes stimulating the endogenous expression of antioxidant enzymes by acting as mild oxidants. This would explain why it is protective at low concentrations, stimulating the expression of enzymes, but harmful at high concentrations, overwhelming the detox/antioxidant mechanisms (Young *et al.*, 2008). Antioxidant effects of reducing the amount of NO by inhibiting iNOS is discussed below.

1.12.3 *Cardiovascular heart disease (CHD) and atherosclerosis*

Epidemiological studies have shown that carrot intake is inversely associated with cardiovascular heart disease (CHD). In a prospective study, a 25g/day increase in the intake of

carrots was associated with a 32% lower risk of 10-year incidence of CHD (HR 0.68, 95% CI 0.48, 0.98). The same study found no association with total fruit and vegetable intake or any other fruit and vegetable alone (Oude Griep *et al.*, 2011).

One way the polyacetylenes could be affecting CHD risk is through modulation of platelet activity in the blood. Platelets have a role in detecting damage in the endothelium of blood vessels and stimulating the release of inflammatory cytokines to repair the damage. As the formation of platelets into a blood clot is mediated by the immune system, the anti-platelet aggregatory abilities of polyacetylenes are most likely due to their anti-inflammatory activity in relation to COXs, LOXs and NFκB, explained in further detail in section 1.12.4. Thrombin breaks down cell membranes, releasing arachadonic acid and allowing a reaction with COX enzymes to form thromboxanes (TX) which are platelet aggregatory agents (Teng *et al.*, 1989). TX interact with membrane receptors to increase cytosolic-free calcium which in turn enhances platelet activation (Park *et al.*, 1995).

Falcarinol-rich extracts from ginseng and *Saposhnikovia divaricate* have been shown to inhibit the formation of HHT and TXB_2 in platelets when stimulated with arachidonic acid and, to a lesser extent, thrombin. ATP release from platelets and intracellular calcium was also reduced. Falcarinol did not change the TXB_2 concentration in resting platelets (Baba *et al.*, 1987; Teng *et al.*, 1989). A further experiment with a ginseng extract containing falcarinol showed a decrease in thrombin-induced platelet aggregations in a concentration-dependant manner. The extract also resulted in an increase in cAMP and cGMP which can decrease the concentration of cytosolic-free calcium and thus reduce aggregation. The extract strongly reduced the production of thrombin-induced TXA_2 suggesting that the platelet anti-aggregatory action was due to the reduced expression of TXA_2 (Park *et al.*, 1995). These experiments did not measure the effect on the COX enzyme but as COX catalyses the formation of TXA_2, TXB_2 and HHT it could be the inhibition of COX that is leading to the reduced expression of the platelet aggregatory factors (see section 1.12.4).

1.12.4 *Anti-inflammatory actions of polyacetylenes* in vitro

NSAIDs work by inhibiting the COX-1 and COX-2 enzymes to act as antipyretic and analgesic drugs for conditions such as arthritis and rheumatism (Dang *et al.*, 2005). The polyacetylenes have been shown to affect both the LPS/NFkB and STAT3 pathway and the COX/LOX enzyme pathways.

LOX and COX inhibition:

As previously mentioned, interest in the polyacetylenes first began when looking for the active ingredients of herbal remedies used in the treatment of inflammatory conditions. Therefore, the majority of studies looking into the effects of polyacetylenes on COX and LOX enzymes, have focussed on plant leaves, flowers and roots that have been used for centuries as traditional medicines. For the purposes of this literature review, only the major polyacetylenes found in the edible vegetables of the Apiaceae family - falcarinol, falcarindiol and falcarindiol-3-acetate - will be explored, but the compounds tested are present in, and have been extracted from, a variety of plants. Falcarinol and falcarindiol have been tested on the COX and LOX enzymes with varying results. In enzyme inhibition studies, researchers look for the concentrations that can reduce the activity of the enzyme by half, known as the IC_{50} value.

COX enzymes:

For the COX enzymes, Prior *et al.* (2007) showed a very potent inhibition effect of falcarindiol from ground elder (*Aegopodium podagraria* L.) with an IC_{50} of just 0.3µM. This was more effective than indomethacin, a known COX-1 inhibitor that is used as a positive control in many studies, which had an IC_{50} of 9.0µM (Prior *et al.*, 2007). Similar results were also found with a falcarindiol extract from *Saposhnikoviae* Radix. (Baba *et al.*, 1987) which showed IC_{50} values of 0.386µM and 0.242µM for HHT and TXB_2, respectively, which are the downstream products of COX-1. Others found the IC_{50} values to be higher at 66.0µM (Liu *et al.*, 1998) and 170.3µM (Dang *et al.*, 2005). Alanko *et al*. (1994) also tested falcarinol but no inhibition was seen, up to 100µM (Alanko *et al.*, 1994). This was also the case in cells from rabbit gut mucosa where falcarinol did not inhibit the COX enzymes as seen by the continued production of the prostaglandins E_2, $F_{2\alpha}$ and D_2 after treatment with up to 200µM falcarinol. Contrary to expectations, falcarinol was also shown to inhibit 15-hydroxyprostaglandin (PGDH), an enzyme that breaks down the prostaglandins, thus allowing these pro-inflammatory molecules to remain in the system for longer (Fujimoto *et al.*, 1998).

LOX enzymes:

The LOX enzymes, which are implicated in tumour progression and atherosclerosis, can also be inhibited by both falcarinol and falcarindiol. Falcarinol inhibited purified LOX enzyme with IC_{50} values of 2, 1, 67 and 4µM for 5-, 12- leukocyte type, 12- platelet type and 15-LOX respectively. Falcarindiol also had an inhibitory effect on the LOX enzymes but with less potency than falcarinol. The IC_{50} values for falcarindiol were 7, 48, >100 and 18µM for 5-, 12- leukocyte type, 12-platelet type and 15-LOX respectively (Alanko *et al.*, 1994). This was similar to the results in the Liu *et al.* study where 5-LOX was inhibited by falcarindiol with

IC_{50} of 5.4µM (Liu *et al.*, 1998). Falcarinol appears to have a greater inhibitory effect than falcarindiol on LOX enzymes but it is the converse for COX enzymes.

The mechanism by which the polyacetylenes exert these anti-COX and LOX enzyme effects is thought to be due to competitive inhibition of the COX/LOX enzymes. The falcarinol-type polyacetylenes have 1,9-dien-4,6-diyn structure which is an analogue of the structure of arachidonic acid (Figure 1.7) so it is assumed that these compounds bind in preference to the arachidonic acid and prevent the downstream reactions occurring (Alanko *et al.*, 1994). Some LOX inhibitors are shown to be antioxidants that react with the non-heme iron atom in the active site of the enzyme, reducing the ferric iron and subsequently interfering with the catalytic cycle (Schneider and Bucar, 2005a).

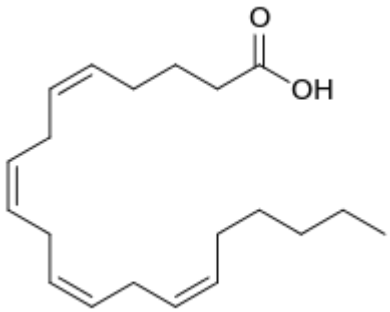


Figure 1.7: Structure of arachidonic acid

LPS/IL-6/STAT3/iNOS pathway:

Two studies have looked at polyacetylene-rich carrot extracts on LPS-induced inflammation. A variety of carrot cultivars were incubated with mice macrophages (RAW cells) to investigate LPS-induced NO synthesis. Seven out of the sixteen varieties tested showed inhibition of NO synthesis, 5 of these 7 had the highest polyacetylene concentrations in their extracts. IC_{25} values for NO production were between 257.7 and 774.6µg/mL of the extract which correlated with the falcarindiol content, but not any other polyacetylene. The remaining varieties also showed inhibition of NO with their extracts but concurrently affected cell viability (Metzger and Barnes, 2009). In another study, exposure to a purple carrot extract, containing falcarinol, falcarindiol and falcarindiol-3-acetate, resulted in a reduction in the expression of iNOS, and the inflammatory markers IL-1β and IL-6 in RAW mouse macrophage cells, and this was dependant on dose. At a concentration of 6.6µg/mL, iNOS and IL-1β were reduced by 77.6±7.9 and 46.9±9.8% respectively. At 13.3µg/mL they were reduced by 84.6±5.5 and 61.4±11.0% respectively and IL-6 was also reduced by around 45%

at this higher concentration. Using porcine endothelial cells, the LPS-induced expression of the inflammatory markers TNF-α and IL-6 was also inhibited. TNF-α was downregulated by 34.2±9.0 and 50.0±10.4% and IL-6 by 59.7±3.1 and 22.9±6.0% with 6.6 and 13.3μg/mL purple carrot extract respectively (Metzger *et al.*, 2008).

Purified individual polyacetylenes can be used to distinguish their actions from each other and other plant bioactives present in plant extracts. When the polyacetylenes were isolated from the purple carrot extract, falcarinol, falcarindiol and falcarindiol-3-acetate all inhibited NO production in macrophages at a concentration over 5μM. Falcarinol was the most active, giving an inhibition of 65.3±2.0% at 10μM. There was no effect on cell viability up to 20μM except in falcarindiol which significantly reduced cell numbers at the highest concentration (Metzger *et al.*, 2008).

Falcarindiol has been shown to inhibit the LPS-induced production of NO in a concentration dependant manner, with IC_{50} values of between 4.3 and 21.2 μM (Matsuda *et al.*, 1998; Shiao *et al.*, 2005; Um *et al.*, 2010; Uto *et al.*, 2015; Choi *et al.*, 2016). A reduction in iNOS mRNA and iNOS protein was also seen. Basal iNOS activity measured did not change, signifying that the activity was not affected but the amount of iNOS produced was lower (Shiao *et al.*, 2005). The inflammatory cytokines IL-6 and TNF-α have also been seen to be reduced by falcarindiol at concentrations of 12.5 and 25μM (Uto *et al.*, 2015). Falcarinol reduced NO production with IC_{50} values of 4.8μM, respectively (Matsuda *et al.*, 1998) and it has been shown to downregulate the production of COX-2 and iNOS mRNA in HT29 colon cancer cells (Um *et al.*, 2010).

To discover how falcarindiol can affect these biomarkers, the individual factors that affect transcription of iNOS were investigated. There was increased degradation of IκB-α and IκB-β, and IKK-α and IKK-β activation was reduced which also led to a reduction in NFκB activation (32% after a 30-minute incubation with 50μM falcarindiol). A reduction in JAK1 and JAK2 was seen and STAT-1 phosphorylation was also reduced, so movement of STAT into the nucleus was inhibited (22.5, 33.6 and 20.7% with 1, 2, 3-hour treatment). Therefore, both pathways that activate the iNOS gene (Figure 1.3) can be downregulated by falcarindiol (Shiao *et al.*, 2005).

These studies show that both falcarinol and falcarindiol have the potential to affect the LPS-mediated inflammatory pathway. The differences in potency between compounds may be due to the different cell types used although there are not enough studies to determine this. Both of these compounds are present in carrots (and other Apiaceae vegetables) so consumption of

these vegetables could give the benefit of both of these compounds. While there are available drugs that have been very effective at reducing inflammation, current NSAIDs can have negative gastric effects which makes their long term use unfavourable. If alternatives can be found that do not have the associated negative effects they would be of benefit as new drugs for inflammatory conditions.

1.12.5 *Anti-inflammatory actions in non-human* in vivo *trials*

The anti-inflammatory actions of whole food (carrots) *in vivo* can be explored quite easily in humans (detailed later in this review) so there appears to be a lack of literature on carrot feeding in rodents with respect to inflammation. In one trial, there was no significant difference in TNF-α in rats fed 40% carrot, either conventional or organic, or rats fed a conventional chow (but levels increased by between 14 and 45%) (Jensen *et al.*, 2012) but rats predisposed to diabetes had lower inflammation after feeding with carrot juice than a β-carotene fed control (Mahesh *et al.*, 2016).

1.12.6 *Anti-cancer actions of polyacetylenes in* in vitro *trials*

Cytotoxicity to cancer cells in vitro:

The effect of falcarinol and falcarindiol on cancer cells has been explored in a large variety of cancer cells including gastric, skin, intestine, colorectal, lymphoma, leukaemia, breast and lung (see Figure 1.2). Both falcarinol and falcarindiol have been shown to have toxicity against these cells but, overall, falcarinol appears to be more potent than falcarindiol in gastric (Matsunaga *et al.*, 1990; Fujioka *et al.*, 1999), skin (Matsunaga *et al.*, 1990; Nakano *et al.*, 1998; Fujioka *et al.*, 1999), intestinal (Young *et al.*, 2007; Purup *et al.*, 2009), leukaemia (Zidorn *et al.*, 2005; Zaini *et al.*, 2012) and colorectal (Zidorn *et al.*, 2005; Sun *et al.*, 2010) cell types.

Interestingly, these compounds appear to not only kill cancer cells but also to maintain healthy cells exposed to the same concentrations. Purup *et al.* (2009) have shown that falcarinol can reduce cell proliferation of Caco-2 cells at 2.5µg/mL but maintains healthy FHs74Int cells at the same concentration. This was the case up to 10µg/mL at which concentration it significantly reduced proliferation of both types of cell. Falcarindiol was less potent in this experiment but reduced Caco-2 proliferation up to 20µg/mL with no effect on healthy cells (Purup *et al.*, 2009). Falcarinol has also been shown to reduce cell proliferation of a variety of cancer cells including cervical (HeLa and Wish), myeloma (K562), lymphoma (Raji) and lung (Calu-1) with IC_{50} values of 40.0, 47.5, 62.5, 34.4, 45.6µg/mL respectively.

The majority of these cells were inhibited at concentrations which were lower than those found to inhibit healthy kidney cells IC_{50} 70.0µg/mL (Kuo *et al.*, 2002) and a much larger differential effect was seen in another study with as little as 0.027, 1.23 and 2.5µg/mL falcarinol reducing cell proliferation by 50% of gastric (MK-1), skin (B16) and fibroblast (L929) cells whereas healthy cells were subjected to much higher amounts 17.1 and 22.5µg/mL falcarinol, for mesothelial and MCR-5 cells, before 50% inhibition was reached (Matsunaga *et al.*, 1990).

These compounds have also been shown to be biphasic in their actions in healthy cells, having beneficial effects at low concentrations but toxic at high concentrations. In healthy bovine epithelial cells, falcarinol has been shown to stimulate cell proliferation at concentrations of 0.01 and 0.05µg/mL falcarinol but over 5 and 10µg/mL cell proliferation was significantly reduced compared with basal levels (Hansen *et al.*, 2003). The same has been seen in porcine myotubes (muscle cells) with falcarindiol which increased viability of these cells at 0.15 and 2.5ng/mL compared with control. No significant increases in viability were seen in the presence of falcarinol but both polyacetylenes decreased cell viability at higher concentrations, from 0.6µg/mL and 1.3µg/mL with falcarinol and falcarindiol respectively and in a dose response manner up to a concentration of 40µM (9.8 and 10.4µg/mL falcarinol and falcarindiol respectively) (Young *et al.*, 2008). As well as healthy cells, this biphasic effect has been seen in one of the cancer cell lines, Caco-2, where DNA strand breakage was reduced when cells were in contact with <2.4µg/mL falcarinol but increased with higher concentrations. Cell number was also increased with 0.12-2.4µg/mL falcarinol but decreased with 12.2 and 24.4µg/mL (Young *et al.*, 2007).

Table 1.2: Polyacetylene actions in *in vitro* cancer cell experiments

Cancer type	Cell Type	Compound	Cytotoxic effect	Reference
Gastric	MK-1	FaDOH	IC_{50} 3.9µg/mL	(Fujioka *et al.*, 1999)
	MK-1	FaOH	ED_{50} 0.027µg/mL	(Matsunaga *et al.*, 1990)
	AGS	FaDOH-rich extract	53.23% cell viability reduction with 100µg/mL of extract	(Cho *et al.*, 2013)
Cervical	HeLa	FaDOH	IC_{50} 38.7µg/mL	(Fujioka *et al.*, 1999)
	HeLa	FaOH	IC_{50} 40.0µg/mL	(Kuo *et al.*, 2002)
	Wish	FaOH	IC_{50} 47.5µg/mL	(Kuo *et al.*, 2002)
Lymphoblast leukemia	CEM-C7H2	FaOH	IC_{50} 0.855µg/mL	(Zidorn *et al.*, 2005)
		FaDOH	IC_{50} 7.2µg/mL	(Zidorn *et al.*, 2005)
	MOLT-3	FaOH	IC_{50} 8.6µg/mL (35µM)	(Zaini *et al.*, 2012)
		FaDOH	IC_{50} not reached	(Zaini *et al.*, 2012)
		FaD3Ac	IC_{50} 21.4µg/mL (71µM)	(Zaini *et al.*, 2012)
	CCRF-CEM	FaOH	IC_{50} 2.9µg/mL (12µM)	(Zaini *et al.*, 2012)
		FaDOH	IC_{50} 7.6 µg/mL (29µM)	(Zaini *et al.*, 2012)
		FaD3Ac	IC_{50} 13.6 µg/mL (45µM)	(Zaini *et al.*, 2012)
Peripheral blood T cell leukemia	Jurkat	FaOH	IC_{50} 3.7µg/mL (15µM)	(Zaini *et al.*, 2012)
		FaDOH	IC_{50} 11.7µg/mL (45µM)	(Zaini *et al.*, 2012)
		FaD3Ac	IC_{50} 23.3µg/mL (77µM)	(Zaini *et al.*, 2012)
Myeloma (leukemia)	RPMI-8226	FaOH	IC_{50} 6.7µg/mL (27.4µM)	(Zidorn *et al.*, 2005)
		FaDOH	IC_{50} 7.7µg/mL (29.6µM)	(Zidorn *et al.*, 2005)
	K562	FaOH	IC_{50} 62.5µg/mL	(Kuo *et al.*, 2002)
Melanoma (skin)	B16	FaOH	ED_{50} 1.23µg/mL	(Matsunaga *et al.*, 1990)
	B16F10	FaOH	ED_{50} 3.7µg/mL	(Nakano *et al.*, 1998)
		FaDOH	ED_{50} 23.2µg/mL	(Nakano *et al.*, 1998)
	B16F10	FaDOH	IC_{50} 23.2µg/mL	(Fujioka *et al.*, 1999)

Cancer type	Cell Type	Compound	Cytotoxic effect	Reference
Lymphoma	Raji	FaOH	IC_{50} 34.4µg/mL	(Kuo *et al.*, 2002)
Lymphoma	U937	FaOH	IC_{50} 7.36µg/mL (30.1µM)	(Zidorn *et al.*, 2005)
		FaDOH	IC_{50} 8.27µg/mL (31.8µM)	(Zidorn *et al.*, 2005)
Fibroblast tumour	L929	FaOH	ED_{50} 2.5µg/mL	(Matsunaga *et al.*, 1990)
Intestine	Caco-2	FaOH	cell no. increased with 0.12-2.4µg/mL (0.5-10µM) but decreased at 12.2 and 24.4µg/mL (50 and 100µM); DNA strand breakage decreased at <10µM but increased with higher concentration	(Young *et al.*, 2007)
	Caco-2	FaOH	>5.0µg/mL inhibited	(Purup *et al.*, 2009)
	Caco-2	FaDOH	>20.0µg/mL inhibited	(Purup *et al.*, 2009)
Colorectal	HRT18	FaOH	IC_{50} 10.34µg/mL (42.3µM)	(Zidorn *et al.*, 2005)
		FaDOH	no effect up to 26.03µg/mL (100µM)	(Zidorn *et al.*, 2005)
	HT2912	FaOH	IC_{50} 15.4µg/mL (63µM)	(Zidorn *et al.*, 2005)
		FaDOH	no effect up to 26.03µg/mL (100µM)	(Zidorn *et al.*, 2005)
	HT29	FaDOH	IC_{50} 9.11µg/mL (35µM)	(Um *et al.*, 2010)
		FaOH	IC_{50} 24.4µg/mL (100µM)	(Um *et al.*, 2010)
	HCT116	FaDOH	75% inhibition with 2.6µg/mL	(Sun *et al.*, 2010)
	SW480	FaDOH	90% inhibition with 2.6µg/mL	(Sun *et al.*, 2010)
	HCT116	FaDOH	dose response inhibition with 1.6 and 2.6µg/mL	(Jin *et al.*, 2012)
	SW480	FaDOH	dose response inhibition with 1.6 and 2.6µg/mL	(Jin *et al.*, 2012)
Breast	MCF-7	FaDOH	20% inhibition with 7.8µg/mL	(Sun *et al.*, 2010)
Lung	Calu-1	FaOH	IC_{50} 45.6µg/mL	(Kuo *et al.*, 2002)
Healthy cells	**Cell Type**	**Compound**	**Cytotoxic effect**	**Reference**
Mesothelial		FaOH	ED_{50} 17.1µg/mL	(Matsunaga *et al.*, 1990)
	MCR-5	FaOH	ED_{50} 22.5µg/mL	(Matsunaga *et al.*, 1990)
Intestine	FHs74Int	FaOH	>5.0µg/mL inhibited	(Purup *et al.*, 2009)
Kidney	Vero	FaOH	IC_{50} 70.0µg/mL	(Kuo *et al.*, 2002)

Healthy cells	Cell Type	Compound	Cytotoxic effect	Reference
Epithelial cells		FaOH	0.01-0.05µg/mL stimulated cell proliferation but 5 and 10µg/mL significantly reduced cell proliferation	(Hansen *et al.*, 2003)
Colon epithelial cells	FHC	FaDOH	No inhibition up to 2.6µg/mL, some inhibition at 5.2µg/mL	(Jin *et al.*, 2012)

FaOH – falcarinol; FaDOH – falcarindiol; FaD3Ac – falcarindiol-3-acetate; IC_{50} is the concentration that causes 50% inhibition of growth; ED - effective dose to elicit a 50% reduction in cell growth/cell proliferation

1.12.7 *Anti-cancer actions of polyacetylene* **in vivo** *in animals*

A small number of trials with rodents have been conducted to investigate the anti-cancer effects of polyacetylenes. A xenograph model of LOX melanoma – in which melanoma cells were injected into the peritoneum of mice – was used to test the effect of injecting falcarinol into the tumour site on the life span of mice. Injections of 3.51mg/kg showed high toxicity and mice died within 2-6.5 days, demonstrating the importance of accurate dosing. However, lower concentrations of 3mg/kg or less, whilst not able to reduce cancer incidence, did extended life span by 13% ($p<0.05$). Dehydrofalcarinol was also used in this experiment and appeared to be more potent than falcarinol, extending lifespan by up to 47% and in some cases even reducing the number of mice with tumours (Bernart *et al.*, 1996). A similar study used HTC116 human colon cancer cells injected under the flank of mice to test the effect of falcarinol infused into the peritoneal space. Doses of 10 and 15mg/kg/day were infused which led to a significant decrease in tumour growth by day 19 with the 15mg/kg/day infusion and a dose response inhibition in growth at day 28 compared with control (Jin *et al.*, 2012).

Rodent studies have also looked at the effect of both carrots and isolated polyacetylenes as supplement to the diet in feeding studies. Rats fed a diet of normal chow mixed with 10% freeze dried carrots containing a total of 35µg/g falcarinol and those fed a diet with a supplementary 35µg/g pure falcarinol in 10% maize starch had a lower incidence of azoxymethane (AOM)-induced large aberrant crypt foci (ACF) and tumours compared with rats fed a standard diet. The number of small ACF was unchanged and there was only a small effect on the medium sized ACF but the number of large ACF and tumours was reduced compared with control ($p=0.027$ for trend) (Kobaek-Larsen *et al.*, 2005). A similar study was conducted in APC^{min} mice which are genetically pre-disposed to cancers of the small and large intestine. The diet of the mice was composed of 20% freeze dried blanched carrot or a control diet of standard chow. The intestines of the mice was examined after 3 months and significant reductions in the number of tumours in the intestine ($p=0.021$) was seen and there was a trend for a reduction in tumour size ($p=0.057$) (Saleh *et al.*, 2013). A trial investigating the combined effect of falcarinol and falcarindiol used 7mg of each compound per g of chow in rat feed. The results showed that AOM-induced ACF and tumours of all sizes were reduced in the intervention compared to the control rats after 18 weeks of AOM treatment (Kobaek-Larsen *et al.*, 2017). These studies show that the polyacetylenes have an effect even when consumed, so they either survive digestion to reach the site of the intestinal/colon cancer cells or the products of their breakdown are also bioactive against cancer cells. Compared with the

in vitro tests, these studies are more indicative of the effects that may also occur with consumption of polyacetylenes in the human diet.

1.13 **Mechanism of polyacetylene anti-cancer effects**

While there is a wealth of evidence to suggest that the polyacetylenes have anti-cancer effects, the mechanism of this effect has not been so well explored. While there is no definitive answer to this, many studies have tried to offer an explanation. The induction of apoptosis has been seen in colorectal (HCT116) and intestinal (Caco-2) cancer cells. HCT116 cells were incubated with falcarindiol and apoptosis followed in a dose response manner with 31% and 33.5% cell death in early phase and 40.2% and 48.4% in the late phase for 2.6 and 7.8µg/mL falcarindiol respectively (Sun *et al.*, 2010). HT29 colon cancer cells had lower levels of Bcl-2 mRNA, which encodes an anti-apoptosis protein, when treated with falcarindiol and falcarinol. There was also lower mRNA for COX-2 and iNOS suggesting inflammatory modulation which could also contribute to reducing cancer potency (Um *et al.*, 2010). In Caco-2 cells, the caspase-3 protein, which is expressed during apoptosis, was up-regulated at higher falcarinol concentrations. DNA damage was also higher in the presence of falcarinol and apoptosis may be related to the higher levels of DNA damage (Young *et al.*, 2007). The same was seen in HCT116 and SW480 colon cancer cells in the presence of falcarinol, with upregulation of caspase-dependant apoptosis which was related to endoplasmic reticulum stress (Jin *et al.*, 2012). Increased caspase related apoptosis was also seen in CCRF-CEM, Jurkat and MOLT-3 leukaemia cell lines with all 3 major carrot polyacetylenes. Interestingly, in this study the falcarindiol-3-acetate was the most potent inducer of apoptosis which is mostly overlooked in other trials, possibly due to its relatively lower levels in the carrot compared with the other two polyacetylenes (Zaini *et al.*, 2012). DNA replication has also been seen to be disrupted in the presence of falcarinol in a variety of cancer cells. The cells appear to be blocked at the G0/G1 stage of the cell cycle and cyclin E mRNA expression is decreased. As cyclin E is required to take the cell from G1 into S phase of the cell cycle, the cells cannot replicate so will undergo apoptosis (Kuo *et al.*, 2002). Two studies have also shown that the polyacetylenes can help enhance the effectiveness of chemotherapy drugs. Polyacetylenes are able to bind to and inhibit the ATP (adenosine triphosphate)-binding cassette (ABC) transporter (ABCG2 transporter), a protein involved in translocating a variety of substances across the cell membrane. When this transporter is over-expressed it is implicated in chemotherapy drug resistance as it will pump the drug out of the cell, allowing cancer cells to evade treatment. Falcarinol, falcarindiol and falcarindiol-3-acetate have all

been shown to bind to and inhibit the actions of this protein, allowing the drug to remain in cells and have an effect (Tan *et al.*, 2014). In a mouse model, falcarinol infused intraperitoneally at 10 or 15mg/kg/day in the presence of 5-fluorouracil, an approved chemotherapy drug, increased the efficacy of the drug against colon cancer (Jin *et al.*, 2012). These studies suggest the polyacetylenes have potential as a novel cancer treatment, as a chemotherapy drug sensitizer, or both.

1.14 **Potential negative effects on health**

1.14.1 ***Allergic Reactions***

The polyacetylenes can induce allergic effects (type IV allergenicity) such as contact dermatitis. Skin prick tests show that falcarinol induces this response. The allergenic activities have been shown to act in 2 different ways but both are due to the strong alkylating ability of falcarinol. Falcarinol is considered to be a hapten, a small molecule that binds to proteins to elicit an immune response. Haptens penetrate the skin and bind to a skin protein carrier, becoming allergenic and elicit a T-cell mediated immune response and allergy contact dermatitis (Christensen *et al.*, 2006; Erkes and Selvan, 2014). The effects of some polyacetylenes can be potent on skin contact, but while contact dermatitis has been a problem for farm workers harvesting celery (Hansen *et al.*, 1986), it is not common in normal handling of polyacetylene-rich vegetables. Wild and ornamental plants are the main culprits of allergenicity due to higher levels of polyacetylene (Christensen and Brandt, 2006). Falcarinol can also alkylate and bind to the CB1 cannabinoid receptor, inducing the pro-allergic chemokines IL-6 and CCL2/MCP-1 (Leonti *et al.*, 2010). Stimulation of the CB1 cannabinoid receptor can induce T-cell mediated hypersensitivity and blocking the receptors could treat inflammatory pain (Clayton *et al.*, 2002). This cannabinoid stimulated allergenicity has not been well explored for the polyacetylenes. Despite these cell-mediated immune responses, allergenicity to the Apiaceae edible vegetables after eating is rare. This is a different type of allergenicity (Type I allergy) that is related to proteins within the carrot, not the polyacetylenes (Ballmer-Weber *et al.*, 2001). In fact, mice fed freshly squeezed carrot juice had reduced allergic responses to type 1 allergens, with lower IgE production, and increased IL-4, IL-12 (anti-inflammatory cytokines) production in the spleen (Akiyama *et al.*, 1999). Blocking of the leukotriene synthesis pathway (potentially through LOX) is considered to reduce the response to allergens. However, these effects could be due to β-carotene, as β-carotene supplements have been shown to reduce IgE, significantly lowering IL-6 and increasing IL-2 (Sato *et al.*, 2004).

1.14.2 *Neurotoxicity*

Polyacetylenes have been shown to be neurotoxic in high doses. The highest potency of toxin is found in wild plant species such as in the water hemlock family, cicutoxin being the polyacetylene identified in these plants (Uwai *et al.*, 2000). Eating normal amounts of edible Apiaceae vegetables will not have a toxic effect but purified, high dose falcarinol has been seen to be toxic in animal trials with a 50% lethal dose (LD_{50}) injection of 100mg/kg (Crosby and Aharonson, 1967) and mice died within days of receiving intraperitoneal injections of doses as low as 3.5mg/kg (Bernart *et al.*, 1996). Falcarindiol is less toxic with LD_{50} injection of >200mg/mL (Uwai *et al.*, 2000). The toxicity is related to the blocking of GABA channels in the brain, inducing convulsions and eventual death (Uwai *et al.*, 2000).

It is highly unlikely that these concentrations could ever be achieved by normal dietary intake of carrots as falcarinol and falcarindiol are present in carrot at around 30 and 20mg/kg respectively, so to achieve toxic concentrations the polyacetylenes would likely have to be as an extract. As the bioavailability of these compounds is also not well understood, the full amount of polyacetylene may not be absorbed into the blood stream so the concentrations injected into the mice are even less likely to be achieved. Trying to elucidate the effects of the polyacetylenes in isolation in humans cannot be done without strict pharmaceutical protocols with carefully controlled dosages. Attempts to investigate the health effects of the polyacetylenes should therefore be done in foods rather than in isolation but this then leads to the confounding of other phytochemicals present in those foods.

1.15 **Outline of PhD Studies**

Higher vegetable intakes are associated with reduced incidence of disease, reduced risk of disease and higher well-being in epidemiological research. The reasons why certain foods have these protective effects on health needs to be investigated in order to identify healthy foods, identify which foods are of the most benefit in particular contexts and to substantiate health claims. Polyacetylenes have been shown to be anti-inflammatory and cytotoxic to cancer cells *in vitro* but have not been well studied for the same effects *in vivo* in humans. This thesis aimed to investigate the potential for carrots to impact on biomarkers of cancer risk. Particular emphasis was placed on the polyacetylene group of compounds, and trying to differentiate their effects from that of the carotenoids.

To observe the effect of polyacetylenes in humans *in vivo* as an isolated substance, they would need to undergo toxicology assessment because of their potential neurotoxicity.

However, using the edible foods that contain them could be used in lieu of a supplement. There have been a small number of trials examining the effect of carrot juice consumption on biomarkers of human health and these trials have shown mixed effects, either positive or no effect but importantly, unlike supplement trials, there have been no trials showing a negative effect on health. The confounding effects of carotenoids in the carrot should be taken into consideration and excluded if possible. With a number of different coloured carrots available, there is the possibility to take advantage of this wide variety to use a cultivar that does not contain carotenoids, thus reducing the confounding effects of these bioactives.

To determine the most appropriate polyacetylene-containing vegetable to use for the dietary intervention study, this thesis aimed to investigate which vegetables contain the largest amounts of polyacetylene and which foods are most commonly eaten i.e. the acceptability of the vegetable. The preparation of vegetable that offers good retention of polyacetylene after cooking was also investigated to ensure the polyacetylenes were present in the dietary intervention.

Bioavailability of polyacetylenes has not been well elucidated and previous studies used carrot juice rather than whole carrots which may not reflect the most commonly consumed sources of polyacetylenes. A further study to investigate the presence of polyacetylene in biofluids after carrot consumption was conducted.

1.15.1 *Aims and objectives*

The aims of this thesis were:

1. Chapter 2: To develop a database of polyacetylene content of foods obtained from experimental and literature values to estimate intake of these compounds based on intakes from the Newcastle 85+ study. Results from this trial were used to determine which food and preparation were used in the dietary intervention.
2. Chapter 3: To determine the effect of cooking on polyacetylene content in foods using 22 different preparations of carrots. Results of this trial were used to determine the preparation of carrot for the dietary intervention.
3. Chapter 4: To determine the bioavailability of polyacetylenes after consumption of a portion of cooked carrots (100g or 250g).
4. Chapter 5: To investigate whether a 6-week dietary intervention using polyacetylene-rich vegetables or matched control food can affect biomarkers of disease risk.

This PhD thesis is presented as separate chapters explaining the rationale, methods and materials, results, discussion and conclusions for each trial. A short discussion at the end brings together the ideas as a whole and discusses the implications within the wider context of phytochemical and cancer research.

Chapter 2. **Database of Polyacetylene-Containing Foods for Estimation of Population Intake**

2.1 **Introduction**

To understand associations between dietary constituents and certain diseases it is important to know how much of these substances are present in the foods consumed. Databases of food constituents have been compiled for phytochemicals such as carotenoids (McCance and Widdowson, 2002) and phenols (Neveu *et al.*, 2010) but there is currently no database available for the polyacetylenes. If a database of polyacetylene content of foods was available, the diets of individuals could then be analysed to see how much polyacetylene-containing food is consumed and how frequently, then an estimate of how much polyacetylene this amounts to. Once calculated, this data can be used to explore associations between consumption of these compounds and risk of certain diseases in epidemiological studies.

To date, the polyacetylene content of certain whole vegetables of the Apiaceae family (carrots, celery, and parsnip) has been studied in the context of raw and industrially processed vegetables. This is often not representative of preparation in the home and does not fully represent the many ways in which carrot and other vegetables are consumed. For example, the different types of cooking or vegetables included in mixed meals. This lack of information on polyacetylene content of foods prepared as they would be in a domestic setting make it difficult to make a comprehensive database of polyacetylene values for many of the sources of polyacetylene that could be used to accurately predict intake.

This means there is also little information about the population intake of polyacetylenes. Only one study has tried to estimate intake in a population (Tiwari *et al.*, 2013) . The study used published data on polyacetylene content in carrots and parsnips and the effect of industrial processing on polyacetylenes in those vegetables, to create a computer model to estimate polyacetylene concentrations of vegetables processed in different ways. The results from the model estimated the amount of polyacetylene in processed cooked and raw carrots but did not include other Apiaceae vegetables, nor did it include any foods with Apiaceae vegetables as components. The results from the model were then used to estimate intake of polyacetylene in an Irish population using the Irish University Nutritional Alliance survey (IUNA, 2011) but again only estimated intake of polyacetylene based on intake of cooked or raw carrots. This may overlook other important sources of polyacetylene in the diet and a more comprehensive look at sources of polyacetylene and the amounts in which these foods are eaten would be of benefit.

The aims of this experiment were therefore:

- To use previously collected dietary intake data from a population of adults living in the UK to estimate the major sources of Apiaceae vegetables in the diet.
- Using the list of Apiaceae vegetables and foods containing them, consumed by this population, conduct a systematic literature search to collate data on the polyacetylene content of these vegetables and foods, taking into consideration different processing techniques.
- To identify the foods/processing techniques that have no polyacetylene values, or very few, in the literature and prepare these foods and analyse them for polyacetylene content.
- To use both the experimental and literature values to create a database of polyacetylene content in foods.
- To assess the amount and frequency of consumption of Apiaceae vegetables in the original dataset and combining this data with the polyacetylene content of the foods to estimate polyacetylene intake in a population.
- To identify the most popular preparation of Apiaceae vegetable (taking into consideration both the highest source of polyacetylene concentration as well as the amount and frequency of consumption) to identify the type and preparation of vegetable to be used in the dietary interventions.
- The database can be used to make comparisons between differently prepared vegetables. This is useful to investigate the effects of processing on polyacetylenes, however this will be explored more fully in the next chapter.

The resulting database could be used to assess intake in individuals where sufficient dietary intake data are available and to make associations between intake of these compounds and disease risk in free-living populations.

2.2 Materials and Methods

2.2.1 *Dietary intake data*

The dietary intake data used was collected as part of the Newcastle 85+ study (Collerton *et al.*, 2007). The study was designed to record many indicators of health and lifestyle in a population of adults who were 85 years old at the time of recruitment. Dietary intake was

assessed with two 24-hr multiple pass dietary recalls. Interviews were conducted with participants in their homes by research nurses trained in conducting dietary recalls. Intake data was complete for 792 participants (participants were excluded if they did not have complete diaries for 2x 24-hour periods). Data from this population was used as it was readily available to the researcher and, although may not be typical of the general population, was assumed to represent a 'traditional' eating pattern in a UK population.

2.2.2 *Ascertaining the major sources of polyacetylene to include in the database*

The edible Apiaceae vegetables include: carrot, celery, parsnip, celeriac, fennel, parsley (Zidorn *et al.,* 2005). Whole Apiaceae vegetables from the dietary intake data from the Newcastle 85+ cohort were identified.

Mixed dishes may contain Apiaceae vegetables, depending on the recipe, for example stews and soups. To investigate whether a mixed dish would typically contain Apiacaea vegetables, the vegetables were searched on the ingredients list of McCance and Widdowson's standard recipes (McCance and Widdowson, 2002), or if not available in this resource, using recipes found on UK based websites (e.g. BBC Good Food). For retail meals, the ingredient lists of at least 3 retail ready-meals were searched. If the foods contained Apiaceae vegetables, they were included in the list of sources of Apiaceae vegetable.

A full list of Apiaceae vegetables, and foods containing them, eaten by this cohort was then compiled. A total of 57 foods (with unique food codes from the Newcastle 85+ trial) were identified (some of these were similar items such as low-fat versions of a dish or vegetables cooked in salted or cooked in unsalted water). The frequency of consumption was estimated by using the number of portions eaten by the entire cohort over the 2 days of intake data. Those foods eaten most frequently (highest number of portions eaten) were prioritised for preparation and analysis. The aim was to prepare those foods that corresponded to at least 80% of the number of portions of Apiaceous vegetables or mixed meals eaten.

2.2.3 *Systematic literature review*

The systematic literature review aimed to gather previously published data on polyacetylene content in foods and identify where more data would be required to assemble the database.

The vegetables selected to be used in the search were those that were commonly eaten by the Newcastle 85+ cohort and therefore excluded Apiaceae vegetables that may be eaten more widely in other populations e.g. fennel. A systematic literature search was performed using Web of Knowledge and PubMed using the following search terms: ((*carrot OR celery OR*

parsnip)) AND (polyacetylene OR falcarinol OR falcarindiol OR falcarindiol-3-acetate OR falcarin).*

Selection criteria:

The searches from the 2 databases returned 153 papers and, after removing those duplicated in both searches, a total of 99 papers were checked for relevant data. Papers were excluded if: polyacetylene content of the vegetable was not measured or did not report actual concentrations (29 papers), it was a review paper (9 papers), data was repeated in more than one paper (6 papers), they did not involve vegetables listed in the McCance and Widdowson database (9 papers), data were for juices rather than whole vegetables (2 papers), wild carrot/plants, or species not commonly eaten in UK (e.g. research varieties, coloured carrots) (8 papers), carrots were grown under stress, or grown or stored under unusual conditions (6 papers) or if the full text was not available (4 papers). Methods were checked to ensure preparation of the vegetable would not have adversely affected the polyacetylene content (e.g. cutting and storing for long periods of time).

2.2.4 *Data extraction*

Mean values reported in the resulting papers were used in the database. If multiple values were reported, each value was checked to ensure the varieties were those commonly consumed and that they were orange varieties. Where more than one variety was reported in a paper, the mean values of each variety was added as an individual line of the database. The final table of results were reported as mg/kg fresh weight. If papers reported the concentrations per dry matter, the values were converted to fresh weight by using the % dry weight from the cooking experiment – carrot 10%, parsnip 19.6% and celery 5%. Fennel dry matter was 7.4% determined from (Koudela and Petříková, 2008).

2.2.5 *Preparation and analysis of foods for polyacetylene content*

Those foods identified as most commonly consumed in the Newcastle 85+ study were prepared and analysed for polyacetylene content. These foods could then be compared to corresponding literature values.

Samples and food preparation:

A full list of foods analysed is shown in Table 2.2. The foods were purchased from multiple sources to ensure representative samples were included. A minimum of 3 different supermarkets were used, where possible, for fresh vegetables. Pre-prepared meals were

purchased from different shops or from different ranges within a shop e.g. budget, mid-range and luxury. All foods were prepared as they would be in a domestic setting and according to the food codes used in the Newcastle 85+ study dietary database. These were also matched to McCance and Widdowson's "Composition of Foods" database (McCance and Widdowson, 2002). Homemade dishes were cooked according to recipes in McCance and Widdowson or, where not available, using recipes found on UK based websites (e.g. BBC Good Food). Frozen and canned vegetables were cooked according to packet instructions. Retail foods, frozen and canned vegetables were cooked according to packet instructions. Foods were finely chopped and well mixed before a representative sample was frozen, stored at -20^{o}C until freeze drying, then ground into a powder.

Laboratory analysis:

The freeze-dried powdered foods were analysed for their falcarinol, falcarindiol and falcarindiol-3-acetate content by high performance liquid chromatography (HPLC). The same HPLC method was also able to determine carotenoid content, but these results are not reported.

HPLC Method:

All solvents used were HPLC grade and purchased from VWR, UK. Carotenoid and polyacetylene concentrations were analysed by HPLC using the method of Rashed (Rashed, 2009) with a few modifications as follows: 1g freeze dried vegetable material was extracted in 10mL ethyl acetate, ultraturraxed and left overnight in the dark at 4^{o}C (both polyacetylenes and carotenoids are light sensitive). The extracts were then centrifuged at 1400rpm for 10 minutes at 4^{o}C, room temperature. A sample of supernatant was removed into an amber HPLC vial and analysed by high performance liquid chromatography (HPLC).

A Shimadzu HPLC system, fitted with a PDA detector, measuring in the UV-Vis range (190-700nm) was used with a gradient elution method, separating compounds by their hydrophobicity. The column used was a Phenomenex HyperClone reverse phase C18 (250x4.6mm, 5µm) column (Phenomenex, Maccelsfield UK) operating under the following conditions: flow rate 1mL/min, column temperature 40^{o}C and an injection volume of 20µL. Polyacetylenes were detected by measuring absorbance at a wavelength of 205nm. Carotenoids were detected in the same extracts at 450nm (data not shown). The mobile phase was methanol (A), deionised water (B) and ethyl acetate (C) with a gradient profile: 0-6 minutes: 50% A, 50% B; 11 minutes: 70% A, 30% B; 30 minutes: 85% A, 15% B; 35 minutes: 100% A; 38 minutes: 90% A, 10% C; 56 minutes: 60% A, 40% C; 62-64 minutes:

100% C; 70 minutes: 100% A; 75 minutes: 50% A, 50% B; 90 minutes: end. The more polar polyacetylenes were eluted first, followed by the more hydrophobic carotenoids at the latter end of the gradient. Example chromatograms are shown in Figure 2.1. The retention times of the polyacetylene peaks changed across sample runs as the HPLC equipment had undergone maintenance. The peaks were checked to ensure they were the polyacetylenes of interest by checking their absorption spectra were the same as those shown in Figure 2.2. These absorption spectra were unique to each of the three polyacetylenes falcarinol, falcarindiol and falcarindiol-3-acetate.

Polyacetylene concentrations were calculated by using the following equations:

1) Calculate % dry weight (g dry matter/100g food):

$$\frac{weight\ after\ freeze\ drying}{weight\ before\ freeze\ drying}\ \text{x}100$$

2) Calculate concentration in freeze dried (FD) food in mg/g of FD powder:

$$\frac{\frac{peak\ area}{std\ peak\ area}\ (mg\ ml^{-1}\ )\ x\ volume\ of\ extraction\ (ml)}{wt\ of\ FD\ powder\ (g)}$$

3) Calculate the concentration of the original food (mg/100g food):

$$mg\ g^{-1} FD\ food\ (2)\ \text{x}\ \%\ dry\ matter\ (g\ 100g^{-1}) in\ carrot\ (1)$$

Results were multiplied by 10 to give mg per kg fresh food.

2.2.6 *Combining the literature and experimental values to form the database*

The experimental and relevant values from the literature were combined to create the full database, summarised in Table 2.3. Each literature value and the mean of the experimental values were assigned one line in the table and the final mean of the data was calculated for each food.

Foods were listed according to the food codes in McCance and Widdowson. The food codes used in the Newcastle 85+ study were more detailed than in McCance and Widdowson, for example, ‘carrots boiled in salted water’ (Newcastle 85+ code 13201) and ‘carrots boiled in unsalted water’ (Newcastle 85+ code 13202) could be combined to ‘carrots, old, boiled’ in the McCance and Widdowson database (MW code: 13-497). As McCance and Widdowson is widely used, the polyacetylene database was standardised to the McCance and Widdowson food codes and definitions. The polyacetylene concentrations of boiled carrots and mixed

vegetables were the same whether boiled in salted or unsalted water and reduced fat coleslaw and beef stew made with lean beef were the same as their full fat counterparts so the same concentrations were assigned in the database. This accounted for 19 food codes in the McCance and Widdowson database. The full database values including the relevant literature search results (with references) can be found in Appendix A.

Values of polyacetylene content determined in the experiment were compared to those from the literature for agreement.

2.2.7 *Estimating polyacetylene in food groups and frequency of intake*

To investigate the importance of certain foods in the diet as sources of polyacetylene, foods were grouped into categories including the individual cooked and raw vegetables, soups and mixed meals. Portion sizes and frequency of consumption were estimated using dietary intake data from the Newcastle 85+ study. For this analysis, foods were grouped into categories: raw and cooked vegetables (cooked carrots include boiled, microwaved, canned and frozen), soups (including dried soup mixes, canned and homemade soups), and mixed meals (including beef stew, cottage/shepherd's pie and coleslaw). Where more than one type of preparation was used in a group (e.g. 'cooked carrots' include boiled, microwaved, canned and frozen carrot preparation), the mean portion sizes, weighted to frequency of consumption of each food, were combined to give an overall mean portion size (g) for the group. The frequency of consumption (number of times eaten by the entire cohort over the 2-day dietary intake period) and mean portion size in the Newcastle 85+ cohort was used together with the polyacetylene content data to estimate intake per person per day.

2.2.8 *Estimation of polyacetylene intake in Newcastle 85+ cohort, using disaggregated data*

As the polyacetylene database aimed to cover at least 80% of the foods containing Apiaceae vegetables eaten by the Newcastle 85+ cohort, an alternative method of polyacetylene intake estimation was also used for comparison. The total amount of carrot, celery and parsnip (in g) consumed by the Newcastle 85+ cohort was estimated by taking this gram weight from the diet diaries for the whole vegetables. This was added to the amount of these vegetables found in mixed meals, estimated by disaggregating mixed meals into their principle components and calculating how much of the dish was carrot, celery or parsnip. The disaggregation of mixed dishes was conducted in accordance with National Diet and Nutrition Survey (NDNS)

methods (Fitt *et al.*, 2010). For homemade dishes, this was estimated using standard recipes in McCance and Widdowson (McCance and Widdowson, 2002) where available, or using an average of at least 5 recipes from British websites e.g. BBC good food. The amount of vegetables in retail foods was estimated from packet ingredients lists of at least 3 different retail products, where available. A conversion factor was then applied to all portions of mixed dishes containing these vegetables to estimate each individual's intake of these vegetables. Once the total weight of each vegetable consumed by each individual was estimated, this gram weight was used with the polyacetylene values of the whole vegetables from the polyacetylene database to estimate intake. The results of the two methods could then be compared to determine whether only analysing 86% of the portions of Apiaceae-containing foods would underestimate the intake of polyacetylenes in this population.

2.2.9 *Investigating the effect of processing of vegetables on polyacetylene content of foods*

To investigate whether foods that had been prepared fresh and foods that were prepared in an industrial setting had different polyacetylene contents, carrots that were boiled and microwaved from whole fresh carrots were compared to carrots that were reheated after being canned and frozen. Comparisons were also made between soups that had been prepared fresh and those canned or powder mixes, and freshly prepared mixed meals compared to ready meals.

2.2.10 *Statistical analysis*

Statistical analysis was performed using SPSS (Version 22, SPSS Inc., Chicago IL). To investigate the agreement between the average polyacetylene intake estimated by the 86% of the foods for which there are values in the polyacetylene database, and the intake estimated by using disaggregated data, a Bland-Altman test was used. The mean of the two values of total polyacetylene intake and the difference in these means were calculated and then plotted on a graph. The mean difference and ±1.96x the standard deviation of the mean difference were drawn on the graph to visualise the variability of the two datasets.

To investigate if there was a difference in the means of the polyacetylene values of foods determined by the literature and those determined in the experiment, the data was first tested for normality using the Shapiro Wilk test. This data was not normally distributed so Mann-Whitney U-tests were used to compare medians of data sets.

To investigate if there was a difference in the means of the polyacetylene values of foods prepared fresh compared to retail prepared whole vegetables, soups and mixed meals, the data from each type of food were tested for normality using the Shapiro Wilk Test. The data was normal so means were compared by one-way ANOVA with a Tukey post-hoc test.

2.3 **Results**

2.3.1 ***Intake of Apiaceae vegetables and foods containing them in the Newcastle 85+ cohort***

The foods eaten by the Newcastle 85+ cohort that contained Apiaceae vegetables are shown in Table 2.1. There were 57 foods, comprising 933 of portions. When sorted for frequency of consumption, boiled carrots are eaten most often by this group. The top 86% of these foods, indicated by those in bold in the table, were analysed for polyacetylene for the database.

Table 2.1: Individual foods eaten by the Newcastle 85+ cohort, sorted for frequency of consumption

Food	Freq	Ave. portion size (g)	Min portion size (g)	Max portion size (g)	% of number of portions eaten
Carrots, old, boiled in unsalted water	360	52	10	240	38.59
Mixed vegetables, frozen, boiled in salted water	57	67	26	145	6.11
Vegetable soup	51	247	75	738	5.47
Vegetable soup, canned	41	246	33	540	4.39
Coleslaw, with mayonnaise, retail	40	52	3	103	4.29
Celery, raw	35	31	8	90	3.75
Parsnip, boiled in unsalted water	24	57	16	180	2.57
Mixed vegetables, frozen, steamed/micro	24	98	30	234	2.57
Beef stew	23	268	107	416	2.47
Instant soup powder, as served	20	206	50	284	2.14
Vegetable soup, dried, as served	20	211	36	313	2.14
Cottage/Shepherd's pie, chilled/frozen, reheated	19	299	163	440	2.04
Carrots, fresh, steamed/micro	19	65	27	180	2.04
Carrots, frozen, boiled in unsalted water	15	63	21	124	1.61
Lentil soup	15	219	145	405	1.61
Carrots, old, boiled in salted water	13	49	14	124	1.39
Carrots, old, raw	8	26	8	51	0.86
Celery, boiled in salted water	6	38	6	90	0.64
Coleslaw, with reduced calorie dressing, retail	5	71	45	90	0.54

Table 2.1 cont'd **Food**	**Freq**	**Ave. portion size (g)**	**Min portion size (g)**	**Max portion size (g)**	**% of number of portions eaten**
Carrots, canned, re-heated, drained	4	135	38	210	0.43
Beef stew, made with lean beef	2	210	140	280	0.21
Mixed vegetables, frozen, unsalted	2	85	84	85	0.21
Chicken casserole	12	268	85	416	1.29
Lamb/Beef hot pot with potatoes, chilled/frozen, retail, reheated	9	302	93	416	0.96
Scotch broth	8	254	89	415	0.86
Minced beef with vegetables, stewed	8	190	54	352	0.86
Beef stew and dumplings, retail, cooked	7	296	48	481	0.75
Cottage/Shepherd's pie	7	330	236	455	0.75
Oxtail soup, canned	6	277	135	405	0.64
Lasagne, chilled/frozen, reheated	6	362	150	420	0.64
Pasty, vegetable	5	142	84	163	0.54
Lancashire hot pot	5	288	222	400	0.54
Casserole, vegetable	4	259	131	416	0.43
Shepherd's pie	4	193	90	400	0.43
Spaghetti bolognese, chilled/frozen, reheated	4	400	300	500	0.43
Mixed vegetables, canned, re-heated, drained	3	134	90	170	0.32
Mixed vegetables, stir-fry type, frozen, fried in blended	3	96	90	108	0.32
Lentil soup, canned	3	259	220	300	0.32
Minestrone soup, canned	3	342	201	425	0.32
Beef curry	3	243	173	350	0.32
Irish stew	3	246	79	330	0.32
Lasagne	3	387	341	420	0.32
Vegetable bake	2	300	300	300	0.21
Vegetable stir fry mix, fried in corn oil	2	90	90	90	0.21
Vegetable stir fry mix, fried in vegetable oil	2	147	90	204	0.21
Minestrone soup	2	231	201	260	0.21
Minestrone soup, dried, as served	2	240	220	260	0.21
Beef casserole, made with canned cook-in sauce	2	338	260	416	0.21
Irish stew, canned	2	297	198	396	0.21
Spaghetti bolognese	2	208	76	340	0.21
Carrots, frozen, steamed/micro	2	44	29	60	0.21
Carrots, young, boiled in unsalted water	1	30	30	30	0.11
Casserole, bean and mixed vegetable	1	260	260	260	0.11
Salad, Waldorf, retail	1	45	45	45	0.11

Oxtail soup, dried, as served	1	150	150	150	0.11
Beef casserole, canned	1	300	300	300	0.11
Chicken in sauce with vegetables, chilled/frozen, reheated	1	287	287	287	0.11

Foods in bold were analysed for the database.

2.3.2 *Systematic literature review results*

25 relevant papers were found during the systematic literature search. 2 papers were excluded, 1 for containing coloured carrots and another for cutting and storing vegetables for long periods. 23 relevant papers were used to compile the database. This amounted to 113 individual mean values of polyacetylene (see Table 8.1, Appendix A for a full list with references). The majority of values in the systematic review were whole vegetables, raw or boiled (and in the case of carrots, steamed and frozen). Gaps in the database included mixed meals and soups (both homemade, canned and dried), canned carrots and mixed vegetables. The frozen carrot polyacetylene values from the literature would not reflect what happened in the home as they are tested in the factory after freezing and not after an additional boil which would happen when they were reheated. The most common whole vegetables, boiled carrots for example, were also prepared for comparison to values found in the literature.

2.3.3 *Experimental values of polyacetylene in foods*

A total of 19 different foods (comprising 86% of foods containing Apiaceae vegetables consumed by the Newcastle 85+ cohort) were analysed in replicates. This was a total of 143 independently prepared samples. Examples of chromatograms are shown in Figure 2.1. The peaks that were considered to be polyacetylene compounds had their absorbance spectra checked to ensure the characteristic peaks were (see Figure 2.2 for characteristic spectra) in accordance with previous literature (El-Houri *et al.*, 2015). The mean values for the polyacetylene content in these foods are displayed in Table 2.2. A table of individual values can be found in Appendix A, Table 8.1. The samples analysed constituted 86% of the portions of foods containing polyacetylene-rich vegetables in the Newcastle 85+ study. The highest total polyacetylene was contained in parsnip but for the individual polyacetylenes, the highest concentrations of falcarinol and falcarindiol-3-acetate were raw carrot, and falcarindiol levels were highest in boiled parsnip. The lowest amounts were found the mixed meals due to being made of multiple ingredients. There was no falcarindiol-3-acetate in celery and parsnip.

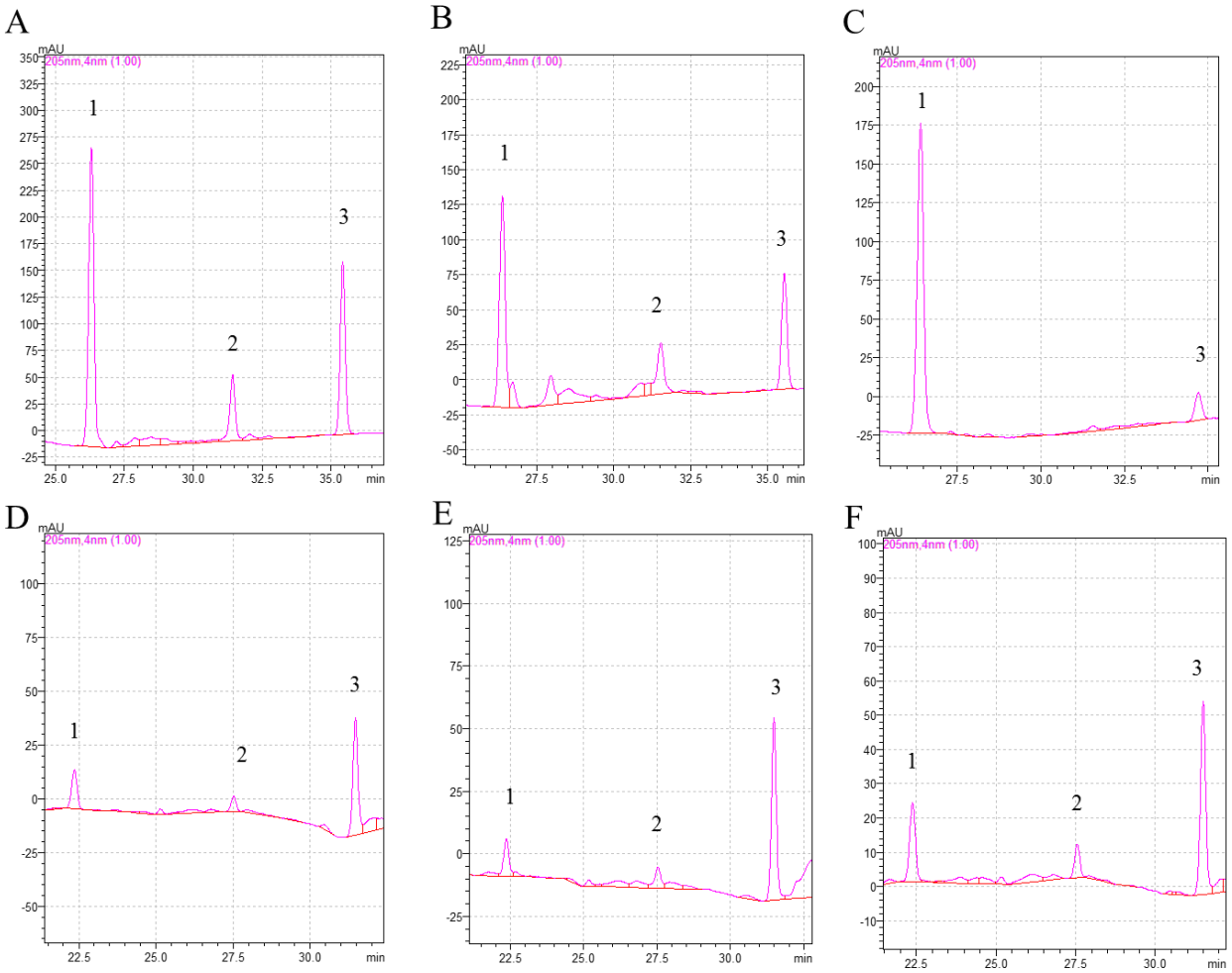
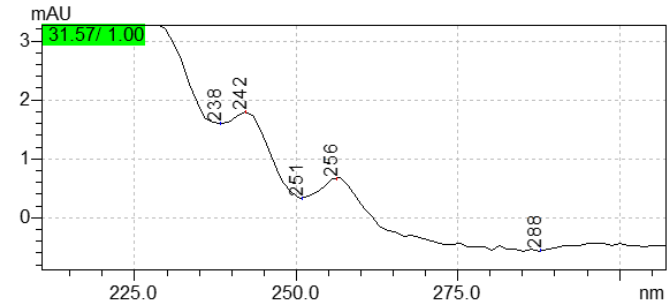
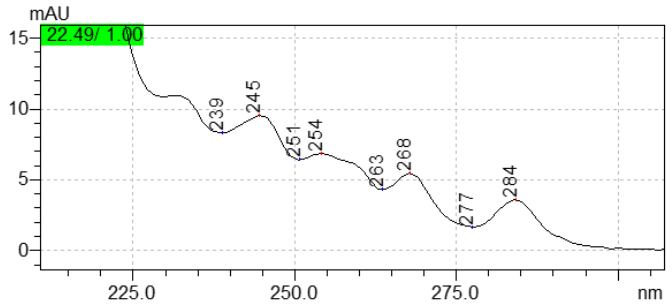


Figure 2.1: Examples of HPLC chromatograms: A) boiled carrots, B) canned carrots, C) boiled parsnip, D) beef stew E) cottage pie F) vegetable soup. 1: falcarindiol, 2: falcarindiol-3-acetate, 3: falcarinol

Falcarinol



Falcarindiol



Falcarindiol-3-acetate

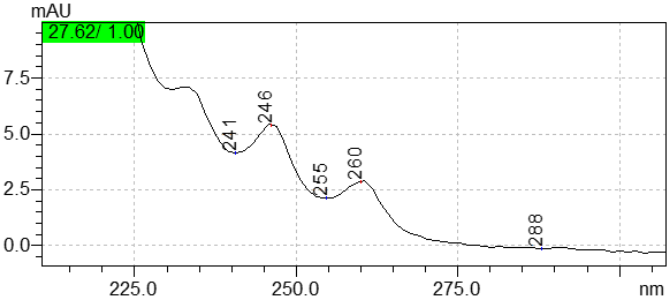


Figure 2.2: Absorption spectra of the polyacetylenes

Table 2.2: Falcarinol, falcarindiol, falcarindiol-3-acetate and total polyacetylene content of foods analysed in experiment.

				Concentration (mg/kg FW)			
85+ food code	**Food description (MW code)**	***n*[1]**		**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
13202	Carrots, boiled in unsalted water (13-497)	16	Mean	32.02	20.48	9.54	62.04
			SD	11.35	6.16	4.08	18.66
			SEM	2.84	1.54	1.02	4.66
13201	Carrots boiled in salted water (13-497)	6	Mean	22.93	23.41	6.62	52.95
			SD	8.85	8.56	1.38	11.68
			SEM	3.61	3.50	0.57	4.77
21559	Carrots, fresh microwaved (13-498)	10	Mean	42.96	47.56	13.74	104.27
			SD	25.49	20.42	6.13	39.51
			SEM	8.06	6.46	1.94	12.49
13207	Carrots, canned, reheated (13-653)	8	Mean	13.17	23.99	6.52	43.67
			SD	5.91	7.64	1.16	12.17
			SEM	2.09	2.70	0.41	4.30
13206	Carrots, frozen, boiled (n/a)	8	Mean	14.60	23.62	7.35	45.57
			SD	5.47	11.89	2.91	18.73
			SEM	1.93	4.20	1.03	6.62
13200	Carrots, raw (13-565)	11	Mean	54.82	40.39	17.61	112.83
			SD	13.57	25.98	3.87	34.03
			SEM	4.09	7.83	1.17	10.26
13221	Celery, raw (13-451)	2	Mean	18.85	107.48	0.00	126.33
			SD	3.22	4.58	0.00	7.80
			SEM	2.27	3.24	0.00	5.51
13222	Celery, boiled (13-544)	15	Mean	7.37	23.82	0.00	31.20
			SD	6.33	17.01	0.00	23.13
			SEM	1.63	4.39	0.00	5.99
13314	Parsnips, boiled (13-454)	9	Mean	44.93	109.97	0.00	154.90
			SD	31.01	46.34	0.00	75.70
			SEM	10.34	15.45	0.00	25.23
13281	Mixed vegetables, boiled in salted water (13-543)	5	Mean	10.81	9.71	3.70	24.22
			SD	6.41	5.64	1.83	9.72
			SEM	2.86	2.52	0.82	4.35
21594	Mixed vegetables, boiled in unsalted water (13-543)	7	Mean	6.90	18.43	9.41	34.74
			SD	3.60	15.16	8.20	26.50
			SEM	1.36	5.73	3.10	10.02
21579	Mixed vegetables, frozen, micro (n/a[2])	3	Mean	6.90	11.55	2.85	21.30
			SD	4.00	4.41	0.95	9.07
			SEM	2.31	2.55	0.55	5.23

Table 2.2 Cont'd				Concentration (mg/kg FW)			
85+ food code	**Food description (MW[1] code)**	***n*[1]**		**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
17283	Vegetable soup, homemade (17-684)	3	Mean	14.42	3.64	2.76	20.82
			SD	0.27	0.40	1.08	1.18
			SEM	0.16	0.23	0.62	0.68
17284	Vegetable soup, canned (17-712)	8	Mean	5.81	2.12	1.38	9.30
			SD	4.05	1.09	0.91	5.83
			SEM	1.43	0.39	0.32	2.06
17264	Lentil Soup, homemade (17-808)	11	Mean	8.64	4.62	1.92	15.19
			SD	6.77	1.77	1.97	9.57
			SEM	2.04	0.54	0.59	2.88
17260/17286	Powdered soup, vegetable (17-660)	5	Mean	3.77	1.69	0.40	5.86
			SD	2.38	1.64	0.14	3.67
			SEM	1.06	0.73	0.06	1.64
19175	Beef stew, homemade (19-473,19-470)	10	Mean	16.16	10.40	3.52	30.08
			SD	13.20	3.54	2.95	17.83
			SEM	4.17	1.12	0.93	5.64
19216	Shepherd's Pie, chilled (19-494)	3	Mean	12.84	0.95	0.33	14.13
			SD	2.67	0.14	0.16	2.80
			SEM	1.54	0.08	0.09	1.62
15077	Coleslaw (15-635,15-636)	3	Mean	17.36	3.39	2.35	23.10
			SD	8.42	1.52	0.81	10.16
			SEM	4.86	0.88	0.47	5.87

[1] number of samples analysed; [2] does not have individual code in database; MW – McCance and Widdowson database codes; FaOH -Falcarinol; FaDOH - Falcarindiol; FaD3Ac - Falcarindiol-3-acetate; PA – polyacetylene.

2.3.1 *Compiled database*

The full database combined the values from the literature with the values from the experiment and is shown in Table 2.3. The database shows that the major dietary sources of the polyacetylenes were the whole vegetables: carrot, celery and parsnip. The highest total polyacetylene concentration was found in the raw celery. The highest concentrations of falcarinol, falcarindiol and falcarindiol-3-acetate were boiled parsnip (54.9mg/kg), raw celery (37.1mg/kg), and boiled carrots (16.5mg/kg) respectively. The lowest amounts were found in shepherd's pie, and other mixed meals were similarly low in polyacetylene due to being made of multiple ingredients.

Table 2.3: Full database of polyacetylene values in foods, combining experimental and literature values.

			Concentration (mg/kg) fresh weight			
Food description (MW food code)		**n[1]**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
Carrots, old, boiled (13-497)	Mean	4	19.5	21.9	8.08	34.5
	SD		8.7	1.47	1.46	23.3
	SEM		4.4	0.73	0.73	11.6
Celery, raw (13-565)	Mean	3	37.1	146.7	0.0	183.8
	SD		31.2	58.2	0.0	89.4
	SEM		18.0	33.6	0.0	51.6
Parsnip, boiled in unsalted water (13-454)	Mean	3	54.9	61.9	0.0	116.8
	SD		8.7	34.0	0.0	27.5
	SEM		5.0	19.6	0.0	15.9
Carrots, fresh, steamed/micro (13-498)	Mean	4	19.7	36.3	11.2	75.5
	SD		13.5	11.3	2.5	28.8
	SEM		6.7	5.6	1.3	14.4
Carrots, frozen (N/A[2])	Mean	9	28.8	21.2	11.8	54.4
	SD		12.4	10.4	3.6	22.7
	SEM		4.1	3.5	1.2	7.6
Carrots, old, raw (13-565)	Mean	93	23.9	35.5	14.8	69.4
	SD		12.9	19.1	19.4	33.9
	SEM		1.5	2.5	3.1	4.9
Carrots, canned, reheated (13-653)	Mean	1	13.2	24.0	6.5	43.7
	SD		5.9	7.6	1.2	12.2
	SEM		2.1	2.4	0.4	4.3
Celery, boiled (13-544)	Mean	1	7.4	23.8	0.0	31.20
	SD		6.3	17.0	0.0	23.13
	SEM		1.6	4.4	0.0	5.97
Mixed vegetables, boiled (13-543)	Mean	1	8.2	14.1	6.2	28.5
	SD		5.1	11.8	6.5	20.3
	SEM		1.3	3.0	1.7	5.2
Lentil Soup, homemade (17-808)	Mean	1	8.6	4.6	1.9	15.2
	SD		6.8	1.8	2.0	9.6
	SEM		2.0	0.5	0.6	2.9
Vegetable soup, homemade (17-684)	Mean	1	14.4	3.6	2.8	20.8
	SD		0.3	0.4	1.1	1.2
	SEM		0.2	0.2	0.6	0.7
Vegetable soup, canned (17-712)	Mean	1	5.8	2.1	1.4	9.3
	SD		4.0	1.1	0.9	5.8
	SEM		1.4	0.4	0.3	2.1
Powdered soup, vegetable (17-660)	Mean	1	3.8	1.7	0.4	5.9
	SD		2.4	1.6	0.1	3.7
	SEM		1.1	0.7	0.1	1.6

Table 2.3 Cont'd			Concentration (mg/kg) fresh weight			
Food description (MW food code)		**n[1]**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
Coleslaw (15-635,15-636)	Mean	1	17.4	3.4	2.4	23.1
	SD		8.4	1.5	0.8	10.2
	SEM		4.9	0.9	0.5	5.9
Beef stew, homemade (19-473,19-470)	Mean	1	16.2	10.4	3.5	30.1
	SD		13.2	3.5	3.0	17.8
	SEM		4.2	1.1	0.9	5.6
Shepherd's Pie, chilled (19-494)	Mean	1	12.8	1.0	0.3	14.1
	SD		2.7	0.1	0.2	2.8
	SEM		1.5	0.1	0.1	1.6
Parsnip raw (13-312)	Mean	3	54.7	91.1	0.0	145.8
	SD		21.3	59.3	0.0	38.5
	SEM		10.7	29.7	0.0	19.2
Fennel, raw (13-241),	Mean	1	30.0	5.0	ND[3]	35.0
	SD		1.4	0.0		

[1] number of samples (means); [2] does not have individual code in database; [3] not determined. MW – McCance and Widdowson database codes; FaOH -Falcarinol; FaDOH - Falcarindiol; FaD3Ac - Falcarindiol-3-acetate; PA – polyacetylene

2.3.2 *Using the database to estimate population intake*

Both portions sizes and frequency of consumption were used to estimate intake in the Newcastle 85+ population. Figure 2.3 shows the estimated amount of polyacetylene per average portion in each of these groups as consumed by the Newcastle 85+ population. These estimates show that the highest amounts per portion were in the boiled parsnips and raw celery. Amounts of polyacetylene per portion of the individual foods can be found in Appendix A, Table 8.3. Portions of mixed meals, soups and mixed vegetables delivered higher total polyacetylene amounts than some whole vegetables once the portion sizes were taken into consideration (Figure 2.3).

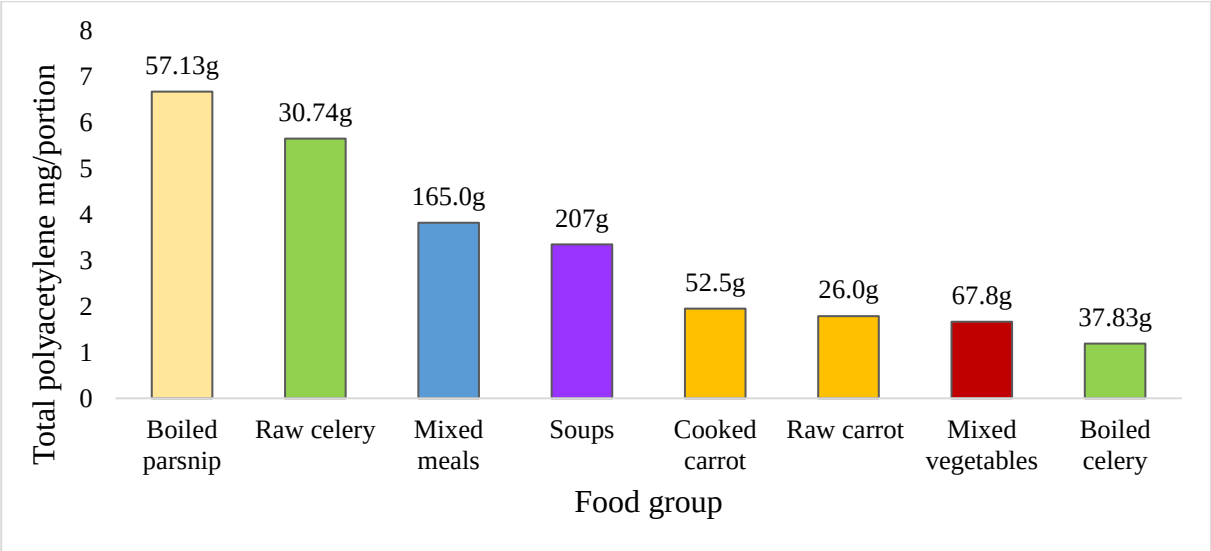


Figure 2.3: Amounts of total polyacetylene per portion. Numbers above bars are portion sizes.

The frequency of intake of the food groups is shown in Figure 2.1. When the frequency of consumption was taken into account, it can be seen that cooked carrots were the biggest contributor of polyacetylene to the diet in this population (0.50mg/person/day) (Figure 2.4). The next largest intakes were from soups which were half the amount, at around 0.31mg/person/day, then mixed meals, raw celery and boiled parsnip which were all between 0.10-0.21mg/day with the other groups delivering <0.062mg/day. Using the database to estimate intake in each participant, the mean intake of total polyacetylene in this population was 1.36mg/day; 42% from falcarinol, 38% from falcarindiol and 20% from falcarindiol-3-acetate.

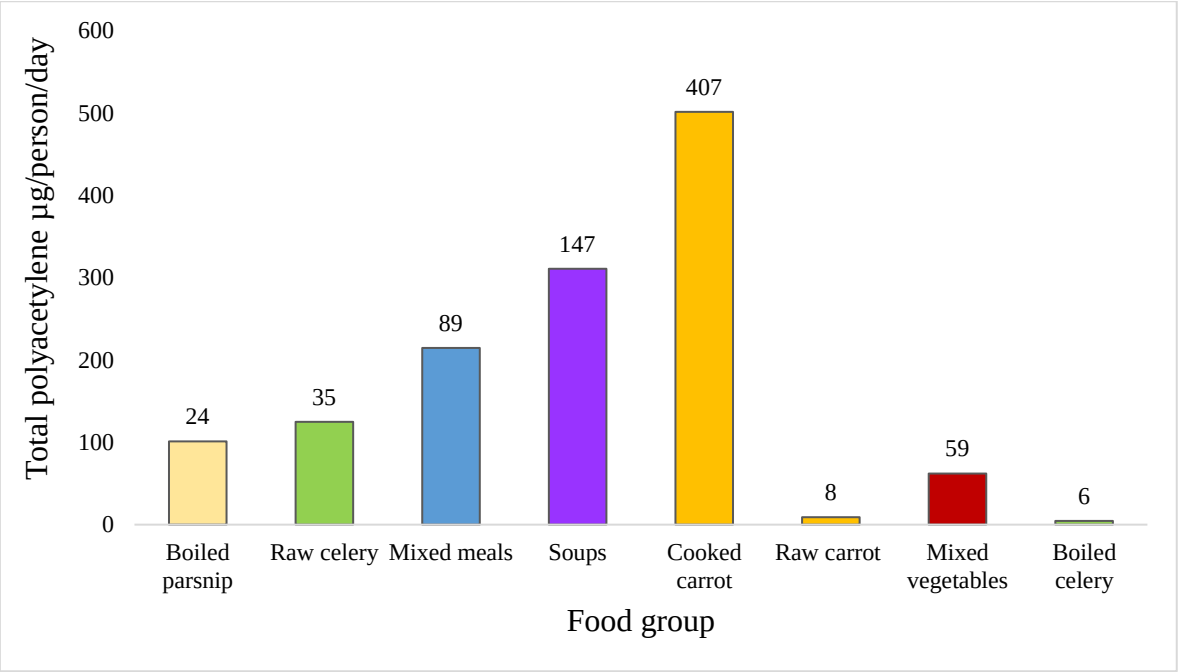


Figure 2.4: Intake of polyacetylene per person per day. Numbers above graphs are total number of portions per person per day for the whole study population (n=792).

2.3.3 *Comparison of methods to determine intake*

Using the disaggregated data, mean intake in the population was 1.50mg/person/day. This amounts to 34% from falcarinol, 47% from falcarindiol and 19% from falcarindiol-3-acetate. Compared to 1.36mg/day from the database foods, the database accounts for around 90% of the amount determined by disaggregating the foods. A Bland-Altman test of similarity showed the majority of values were within 2 standard deviations of the mean difference, indicating that there was good agreement between the values generated by the two different methods. However, the difference becomes larger with increasing mean so the two methods agree less with each other with larger intakes of polyacetylene.

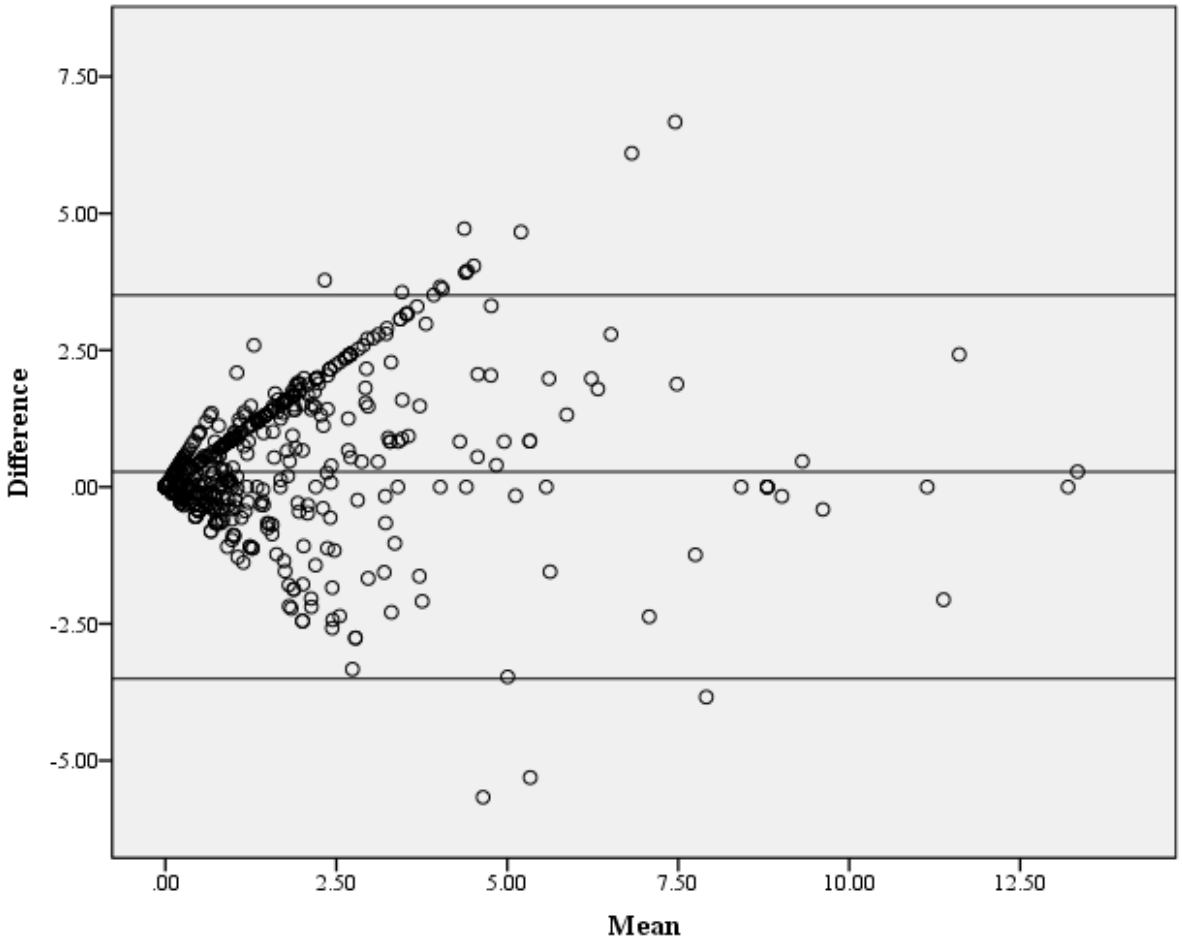


Figure 2.5: Bland-Altman plot of mean difference between total polyacetylene intake over 2 days determined by the polyacetylene database or by disaggregating the data. Lines are mean difference and +/- 1.96x standard deviations of the difference.

2.3.4 *Effect of processing on polyacetylene content of foods*

Comparing carrots that had been processed before cooking (canned, frozen) to those that were cooked from fresh (boiled, microwaved) the freshly prepared carrots had significantly higher falcarindiol ($p=0.0001$), falcarindiol-3-acetate ($p=0.019$) and total polyacetylene ($p=0.003$) than those carrots prepared prior to cooking (boiled and microwaved) but falcarinol was not different.

When looking at whole dishes, processed soups were lower in polyacetylene concentration than soups made in the home ($p=0.031$, 0.0004, 0.070, and 0.008 for falcarinol, falcarindiol and falcarindiol-3-acetate respectively) and retail chilled shepherd's pie had significantly lower falcarindiol than homemade beef stew ($p=0.001$). However, the homemade stew was

19.0% carrot and 6.0% celery, and the retail foods were 4.6-8.0% carrot, so higher values in the home-made foods could simply be due to more fresh vegetable being used in the recipe.

Comparing cooked to raw vegetables also gave an indication of how stable the compounds were during mild processing. Cooked celery had lower concentrations of falcarinol and falcarindiol than raw celery in the database but for carrots, when the data were collated with literature values, only falcarindiol-3-acetate was significantly lower in cooked carrots than in raw ($p=0.03$).

Looking at the experimental values, the raw carrots had significantly higher falcarindiol ($p<0.0001$), falcarindiol-3-acetate ($p<0.001$) and total polyacetylene ($p<0.002$) than cooked carrots (boiled, microwaved). Falcarinol was non-significantly higher in the raw carrot. Also in the experiment, raw celery had higher falcarinol ($p=0.035$) and falcarindiol ($p<0.0001$) and total polyacetylene ($p<0.001$) concentrations than cooked celery which agrees with the combined database.

2.4 **Discussion**

To the knowledge of the author, this is the first study to analyse a comprehensive list of food items to create a database of polyacetylene values for commonly eaten foods, prepared in a domestic setting.

The amount of polyacetylene in vegetables is known to be highly variable, ranging from 12.8-206.0mg/kg in literature values of common varieties of raw carrot (Metzger and Barnes, 2009; Rawson *et al*., 2010a). Basal levels of polyacetylenes appear to be genetically determined as relative concentrations in the different varieties are maintained over growing years and locations (Yates et al., 1983), but they are phytoalexins, i.e. produced in response to a stress stimulus, so in carrots they have been seen to rise during microbial attack, damage to the root (Czepa and Hofmann, 2003), excess or too little water (Lund and White, 1986) and over storage time (Hansen *et al*., 2003; Kidmose *et al*., 2004a; Kjellenberg *et al*., 2010). Polyacetylenes accumulate not only while the carrot is growing (Lund and White, 1986), but also after harvesting (Kjellenberg *et al*., 2010). Variation in concentrations have also been seen in other phytochemicals such as carotenoids, where concentrations have been observed to differ by 2.2-fold in carrots, and ascorbic acid which had 2-3 fold differences in fruits (Kalt, 2005).

2.4.1 *Estimated dietary intake*

The only other study that has estimated intake of polyacetylene used a computer model using published data on polyacetylene content in industrially processed raw and cooked carrots (Tiwari *et al.*, 2013). The results of the model were used to estimate intake of polyacetylene in an Irish population (IUNA, 2011). The model was run with polyacetylene concentrations of raw carrot as the input. These were 10.4, 22.4 and 6.6mg/kg for falcarinol, falcarindiol, and falcarindiol-3-acetate respectively. These values were lower than the values in the present database (23.9, 35.5 and 14.8mg/kg fresh weight) but despite this, the estimated intake in the Irish population was around 21mg total polyacetylenes per day for unprocessed carrot and 12mg/day for processed carrots (based on 249µg/kg body weight/day in males), which is much higher than the average daily intake in the Newcastle 85+ population. Average intake of carrots in the Irish population was 13g/day in both sexes (IUNA, 2011) and if using the concentration values of the present database to calculate total polyacetylenes intake, it would result in a much more conservative 0.90mg/day of polyacetylene. Therefore, the estimates in the Tiwari paper appear to be overstated. The estimated intake per day (20mg) reported in the paper would correspond to the total polyacetylene present in 580g of boiled and 290g of raw carrot from the database values in the present study, much more than the 13g/day reportedly consumed in the population studied (IUNA, 2011). This would suggest that there was an error in this paper regarding the estimated intake/day.

2.4.2 *Comparison of literature and experimental values*

The majority of results from the literature search were for raw carrot. The values obtained for raw carrot from the experiment were higher than the average literature values for falcarinol ($p=0.002$) but falcarindiol, falcarindol-3-acetate and total polyacetylene were not different. The experimental values for carrots were similar to those determined by Metzger and Barnes, and Rawson and colleagues (Metzger and Barnes, 2009; Rawson *et al.*, 2012b). Only one published paper was found containing values for each of fresh boiled carrot, boiled parsnip and raw celery, and the other foods analysed in this experiment had not been tested previously, highlighting the importance of this work. The experimental values of raw celery were similar to one variety of celery reported previously (Zidorn *et al.*, 2005), but the parsnip values in the literature were higher for falcarinol and lower for falcarindiol than in this study (Rawson *et al.*, 2010b). Literature values for boiled carrot only reported falcarinol content (Hansen *et al.*, 2003). Lack of published data make it difficult to make comparisons between the current study and literature values. For frozen carrots, falcarinol and falcarindiol-3-acetate

were higher in the literature (p=0.002, 0.007 respectively) than in the experiment but falcarindiol was not different. In previous studies, carrots, either blanched or unblanched, were frozen and then analysed, which would correspond to store bought frozen carrots. However, a consumer would heat the frozen carrots prior to consuming them which is reflected in the experiment but was not reported in previous studies. The extra cooking step could lead to further losses of compounds which could explain the lower results in this study. This suggests that more processing of a vegetable can lead to higher losses of polyacetylenes, which also agrees with the values from canned carrots.

2.4.3 *Effect of processing on vegetables*

The processing of vegetables appears to have a determinantal effect on the amount of polyacetylene in vegetables. Frozen and canned carrots had lower polyacetylene content than but they had very similar concentrations to each other. The canning and freezing of vegetables can adversely affect both water soluble compounds like ascorbic acid and the phenolics (Rickman *et al.,* 2007a) and fat soluble compounds such as β-carotene (Rickman *et al.*, 2007b). The ascorbic acid losses were seen to be around 90% in canned but a more modest 0-35% in frozen carrots. Phenolic acids in canned vegetables have been seen to change by +5 to -91% and frozen carrots have been seen to lose 0 to 30% compared with fresh (Rickman *et al.*, 2007a). Beta-carotene is also affected by processing, with losses of 53% and 31-69% seen in canned and frozen carrots respectively, compared with fresh carrots (Rickman *et al.*, 2007b). The carotenes are easily oxidised during processing and storage and have been seen to decrease with longer exposures to heat and light (Pinheiro Sant'Ana *et al.*, 1998; Miglio *et al.*, 2008). Whilst blanching could be a source of phytochemical loss, it is thought to be necessary for the preservation of frozen carrots as it sterilizes the vegetable and preserves the texture by softening the cell matrix to protect against splitting. Blanching also reduces the oxidative breakdown of phytochemicals (Hansen *et al.*, 2003; Kidmose *et al.*, 2004a; Rawson *et al.*, 2012b) and denatures the enzymes that metabolise and degrade phytochemicals such as acetylenase, dehydrogenase, desaturase, lipoxygenase and peroxireductase (Minto and Blacklock, 2008). Blanching can lead to losses of 35-50% falcarinol, but no further losses were seen over storage time (60-120 days), whereas the same amount of losses were seen in frozen carrots that had not been blanched (Hansen *et al.*, 2003). However, another study showed losses of falcarinol during frozen storage (60 days) regardless of whether the carrot had been blanched or not (Rawson *et al.*, 2012b).

One limitation of this study is that when comparing values for cooked carrot to raw carrot in the database, the effect of cooking on polyacetylene content may not be seen as the carrots did not all come from the same source, and there were many more samples of raw carrot than cooked carrot in the literature for comparison. However, using the cooked and raw data from the study gives a better idea of the effect of processing as they were from similar starting material. The results of this study show that raw carrots had significantly higher falcarindiol, falcarindiol-3-acetate and total polyacetylene than cooked carrot. Previous literature on the changes of polyacetylene contents during processing have shown inconsistent results, with both increases and decreases observed during hydrothermal processing. In one trial, there were losses of up to 70% falcarinol from carrot cubes ($1cm^3$) after a 12 minute boil (Hansen *et al.*, 2003) but another trial saw no significant changes in polyacetylenes with boiling times of 2-5 minutes. However, longer boiling times of 20-60 minutes were reported to increase the concentration of the three polyacetylenes (actual figures not reported) (Rawson *et al.*, 2010a). Boiled parsnip disks lost 8 and 20% of their falcarinol and falcarindiol respectively during a 2 minute boil (Rawson *et al.*, 2010b) but boiling fennel for 30 minutes increased the concentrations of falcarinol and falcarindiol by 38% and 33% respectively (Rawson *et al.*, 2013b). Steam and water blanching, with shorter cooking times and exposure to water, as well as lower temperatures than boiling, are thought to be less damaging to phytochemicals but this appears not to be the case for polyacetylenes. Water blanched parsnips lost 25% falcarinol and falcarindiol (Rawson *et al.*, 2010b) and water blanched carrots lost 10 and 15% falcarinol and falcarindiol respectively, but saw an increase of falcarindiol-3-acetate by 30% (Rawson *et al.*, 2012a). Steam blanching, with reduced contact with water, induced losses of 25-50% of falcarinol in carrot after 2 minutes (Hansen *et al.*, 2003). Differences in the size/shape of the vegetable pieces as well as the time of cooking may explain some of the differences in results seen in these trials. Whilst the cooking times are shorter and less water is involved in blanching, the losses may not be from leaching but simply the washing away of the polyacetylenes from the cut edge of the carrot. A trial looking at the industrial processing of carrots showed that cutting carrots into shreds, cubes and batons followed by washing, led to significant losses of polyacetylenes (30-44%), without any heat treatment which were mitigated if washing was performed prior to cutting. Mechanical damage to the cells, by peeling and cutting, and the subsequent washing away of the compounds that were released was thought to be leading to these losses (Koidis *et al.*, 2012). The changes during processing are discussed further in the next chapter.

This study highlights the importance of the Apiaceae vegetables, especially cooked carrots, in the diet as a source of polyacetylenes. It has shown that cooked carrots are widely eaten by a population of older people in the UK and that the amount in a portion of cooked carrots can contribute 1.95mg/portion of total polyacetylene. Whilst this was not the highest concentration of polyacetylene per portion of the foods tested, it was the most frequently consumed of the polyacetylene-rich foods. Investigations into the bioavailability of these compounds would allow predictions of whether this is a biologically meaningful amount either in blood plasma or in the gut.

2.4.4 *Limitations of this study*

As this is the first database reporting the polyacetylene content of foods derived experimentally, it has fewer data points than would be expected from larger databases such as 'Phenol Explorer' (phenol-explorer.eu) which uses data from hundreds of studies to create a database of phenol values of foods and drinks. On the other hand, the databases are of similar quality when looking at the number of data points per type of food, since polyacetylenes occur in only a few plant species, while polyphenols are found in almost every plant species and thus in most plant derived foods, requiring orders of magnitudes more data points to obtain an adequate coverage of the diet. The current database will be available as a resource for researchers to use in epidemiological studies, to assess the associations of various outcomes and biomarkers with dietary intakes of polyacetylenes based on existing or future data on dietary intake of the corresponding foods. However, as the present study focussed on the foods that were consumed by the Newcastle 85+ study, there may be a few foods that are commonly eaten in other populations which were not included, so further work is needed to expand the database with additional food types as more data arise.

Dietary intake was measured by 2x24 hour dietary recalls. This could limit the amount of foods captured that may not be eaten most days by an individual. The initial idea for the use of this data was to correlate polyacetylene consumption with cancer frequency in this cohort. However, the data from the 85+ study is not suitable for assessment of associations with disease at an individual level as 2x24 hour dietary recalls would not be sufficient to distinguish between high and low intake of polyacetylene, although at population level the information will give an indication of overall intake. A food frequency questionnaire would have been better at capturing this information. Another limitation is that dietary choices in 85-year olds may be different to the general population. Previous research has shown that people over 65 years old reported low raw vegetable intake due to difficulty in chewing raw vegetables, raw carrot in particular, (Sheiham and Steele, 2001) and stringy and crunchy

foods were avoided in older populations (Hildebrandt *et al.,* 1997). Choice of foods and cooking methods may be different in an older population than in a younger population, as might portion sizes and frequency of consumption. However, intake of carrots in the Newcastle 85+ study was 12.9g/day which was very similar to 13g/day (unspecified cooking/preparation) in an Irish population aged 18-64yrs (IUNA), but comparison to UK population is difficult as NDNS data reports carrot intake in combination with 'yellow, red and dark green leafy vegetables' which amounts to 27g/day in men and 28g/day in women aged 19-64yrs and 27g/day in men and 29g/day in women aged 65 and over (NatCen Social Research *et al.*, 2015) so it is difficult to know how much of these is specifically carrot.

There were not many literature values for comparison for some types of food, so for these the database relied on just one or two studies for their values and therefore the data may be less robust than for other databases. One way this had an effect in this study is when comparing raw and cooked vegetables from different studies. For example, microwaved carrots had very similar polyacetylene concentrations to raw carrots in the experiment, but the values for raw carrots in the literature were much lower than those in the experiment. Because many raw carrots had been studied, most with lower concentrations than those in the experiment, the combined mean was lowered in the database. The lack of literature data on microwaved carrots meant the database concentration remained high, skewing the results so that the concentrations of microwaved carrots looked higher than raw when in fact they were similar when comparing the same source of carrot. Therefore, it is important that databases should be kept up to date as further work on polyacetylenes is published.

2.5 **Further research**

Further research into the effect of cooking on polyacetylene-containing vegetables would be of benefit. The full database suggests there are no differences between raw and cooked carrots, but experiments investigating the change from raw to cooked using the same batch of carrots, reducing the variability between genotypes and growing conditions, have found differences. This variability is not accounted for in the database. Investigating potential changes in polyacetylene content of carrots prepared as they would be in the home would be of benefit to determine sources of losses of phytochemicals to make recommendations to the consumer on how to reduce these losses.

The database can be used in epidemiological studies to investigate associations between polyacetylene intake and health conditions. Further collaborations with the gatekeepers of

large datasets would be an advantage to investigate the potential health benefits of these compounds. This will also allow the intake to be estimated in populations of different ages to give more robust data on population intake.

Related Conference Proceedings: Warner, S., Seal, C., Haldar, S. and Brandt, K. (2016a) 'Database of polyacetylene-containing foods for estimation of population intake', *Proceedings of the Nutrition Society*, 75(OCE3).

Chapter 3. **Retention of polyacetylenes and carotenoids in carrot during cooking under different conditions**

3.1 **Introduction**

The results of the database work in chapter 2 highlighted that there are differences in the phytochemical content of raw and cooked vegetables, with cooked celery and carrots having lower concentrations of polyacetylenes than raw.

The effect of processing and cooking on the composition of the phytochemicals in vegetables is a complicated process; cutting and peeling breaks open cells, allowing enzymatic degradation and oxidation; heat degradation and leaching of phytochemicals into cooking fluid may further decrease concentrations. However, concentrations in the vegetable may still increase due to the leaching of soluble solids or losses of water from the vegetable, in some cases concentrating the remaining phytochemicals (Svanberg *et al.*, 1997; Nyman *et al.*, 2005). Changes in the structure of the vegetable after cooking may also increase the extractability of the compounds by breaking down the cell matrix (McDougall *et al.*, 1996) and releasing phytochemicals from protein complexes (Aman *et al.*, 2005) leading to an apparent increase in concentration. Leaching occurs when compounds move out of the matrix of the vegetable and into the cooking medium. This is a common way in which water soluble compounds, like vitamin C, are lost from vegetables (Rickman *et al.*, 2007a; Miglio *et al.*, 2008) but being more hydrophobic than these compounds, it would be expected that the polyacetylenes would be less susceptible to leaching into water, as is the case with the fat-soluble carotenoids (Rickman *et al.*, 2007b). From the database work it was observed that microwaved carrots, cooked with very little water, had 55% higher polyacetylene concentrations than those that had been boiled, and were similar in concentrations to the raw carrot, therefore leaching into cooking water could be mitigated by using cooking methods that use less water. The results from the database work suggest processing that involves multiple water immersion steps is the most detrimental to polyacetylene concentrations, especially if the water is discarded, such as the blanching, freezing then boiling of frozen carrots, whereas cooking the vegetable within a dish has the potential to retain much of the phytochemical. Interestingly, the dishes in chapter 2 that were cooked with the polyacetylene-rich vegetables in them, e.g. stews and soups, appeared to have polyacetylene levels that reflected the amount in the vegetable with little degradation, despite being cooked for longer amounts of time. This suggests that polyacetylenes are reasonably stable at the temperatures they are exposed to during cooking and any leached polyacetylene would remain within the

dish and be consumed rather than being discarded. As discussed in the previous chapter, inconsistent changes in polyacetylene concentration have been seen during hydrothermal processing of carrots, possibly due to the differences in the processing of the vegetable, for example, how long the carrots were cooked for and the size/shape of the carrots during cooking. In previous publications, the carrots that had been cooked for longer amounts of time (over 20 minutes), may have increased in polyacetylene concentration due to loss of soluble solids, such as sugars and soluble fibre, leaching from the vegetable into water during cooking so the remaining phytochemicals became concentrated in the vegetable (Svanberg *et al.*, 1997; Nyman *et al.*, 2005). This has been seen in a number of studies during cooking, especially for fat soluble chemicals (Puupponen-Pimiä *et al.*, 2003; Imsic *et al.*, 2010; Chiavaro *et al.*, 2012; Bongoni *et al.*, 2014). The carrot cubes in the Hansen (2003) study would have a large surface area from which polyacetylenes could leach into the cooking water leading to large losses. Larger pieces of vegetable in the other trials (Rawson *et al.*, 2010a) had a smaller surface area, in relation to the volume, and showed an increase in concentrations of phytochemical, therefore preparing bigger pieces of carrot could reduce the amount of phytochemicals in the inner tissue from leaching out. Studies using larger chunks of carrot might show higher retention of concentrations of phytochemicals.

Published literature on the effect of cooking on phytochemicals, as well as the database, suggest that time of cooking, surface area of vegetable pieces, and the cooking medium (the presence of water or oil) could affect the retention of polyacetylenes. However, it is not clear how best to prepare carrots to retain polyacetylenes. Previous papers mainly investigated the effects of industrial processing, and do not necessarily reflect what would happen in the home. Additionally, these studies were observational, aiming to investigate the effect on quality under certain conditions, and did not attempt to understand the full processes taking place, including leaching and degradation. Results of these studies varied widely, especially for boiled carrot, the most popular way to consume carrot in the population studied in chapter 2.

The aims of this study were to investigate if time of cooking and the shape of the vegetable during cooking either boiling or frying, can affect the concentrations of phytochemicals in the resulting vegetable. Using the same data, the true retention of phytochemicals after cooking was also investigated to determine how much phytochemical remained in the carrot compared to the amount in the raw carrot. Controlled simulations of domestic cooking were conducted to observe the effect of different preparations of carrot on the concentration of phytochemicals in carrot as well as in the cooking fluid (oil or water).

Hypotheses: The loss of phytochemicals from vegetables will increase with increasing cooking time and with a higher surface area of vegetable. There will be a larger amount of leaching of hydrophilic compounds into oil compared to water.

The results from this study will allow us to ascertain a method of cooking that results in retention of phytochemicals in carrots. The results were intended to inform the method of carrot preparation for the human bioavailability and dietary intervention trials.

3.2 **Methods and Materials**

British carrots were purchased in a local supermarket (Asda, UK). These carrots were of the variety 'Nairobi', grown in sandy soil in Scotland, harvested late April/early May 2015. They were peeled, topped and tailed then cut into three different shapes/sizes: whole (W), quarters (Q) or disks (D). The (W) were left whole, (Q) were cut lengthways into 4 long quarters and (D) were cut into approximately 5mm disks. Each shape/size of carrot was then either boiled or fried. Carrots were weighed before and after cooking.

3.2.1 ***Boiling of carrots and sample preparation***

Boiling was performed inside a boil-in-the-bag with a ratio of 1:3 carrot to water (e.g. 100g carrot with 300g water). The bag was sealed and placed in a boiling pan of water. Once the pan returned to the boil, a timer was started and the bags were removed after 5, 10, 15 or 20 minutes. Once the bag was removed, the water was immediately drained from the boil-in-the-bag, weighed, frozen then freeze dried. The carrot was weighed, chopped into small pieces, frozen then freeze dried. The carrot samples were prepared for extraction and analysed by HPLC for polyacetylenes and carotenoids as previously described in chapter 2.

After freeze drying the water, the dry matter that remained was analysed for phytochemical content. The phytochemicals were extracted from the dry matter by combining 5mL ethyl acetate with 0.5g dry matter. 3mL of water was added to solubilise the sugars, aiding extraction into the solvent. He samples were vortexed for 5 minutes, then centrifuged for 5 minutes (1400rpm, room temperature) and a sample of the ethyl acetate layer was removed into an amber HPLC vial. HPLC analysis of polyacetylenes and carotenoids was performed as described in chapter 2.

Calculating phytochemicals in water:

To calculate the amount of phytochemicals in water, the dry matter from the freeze dried water was calculated per 100g of raw carrot.

1) Calculate dry matter (DM) of water per 100g of raw carrot (g DM in cooking water/100 g raw carrot):

$$\frac{DM\ in\ water\ (g/portion)}{raw\ carrot\ (g/portion)}\ \text{x}\ 100$$

2) Calculate concentration in dry matter (DM) from the water (mg/g DM of FD powder):

$$\frac{\frac{peak\ area}{std\ peak\ area}\ (mg\ mL^{-1})\ \text{x}\ volume\ of\ solvent\ in\ extraction\ (mL)}{wt\ of\ DM\ in\ carrot\ powder\ (g)}$$

3) Calculate the amount of phytochemical in water (mg/100g raw carrot):

concentration in DM from water (mg per g) (2)	*x*	*dry matter of water (g per 100g raw carrot) (1)*

3.2.2 *Frying of carrots and sample preparation*

Frying was performed with 160g carrot, fried in 30g rapeseed oil. Carrots were cooked for either 5, 8 or 10 minutes over a medium heat. After cooking, the oil was weighed and retained for analysis. The carrots were weighed, chopped into small pieces, frozen, then freeze dried. The carrot samples were prepared for extraction and analysed by HPLC for polyacetylenes and carotenoids as previously described in chapter 2.

The oil was frozen and analysed for phytochemicals directly, along with a sample of uncooked rapeseed oil to ensure no polyacetylenes were present in the cooking oil.

Calculating phytochemicals in oil

The amount of phytochemicals in oil was calculated in mg/mL per 100g raw carrot:

$$\frac{\frac{peak\ area}{std\ peak\ area}\ (mg\ mL^{-1})\ x\ volume\ of\ oil\ per\ portion\ after\ cooking\ (mL)}{wt\ of\ raw\ carrot\ used\ per\ portion\ in\ cooking\ (g)}\ \text{x}\ 100$$

3.2.3 *Calculating true retention (change from raw) and estimating degradation of compounds during cooking*

The previous calculation of phytochemical concentration in the carrot, does not account for changes in weight of the carrot during cooking. This could be hiding the true change in amount of phytochemicals in the vegetable as losses in soluble solids and water can artificially 'concentrate' the phytochemicals in the remaining weight of carrot. To estimate the true retention, the following calculation was used.

For boiled carrots:

1) Calculate dry weight (g dry matter (DM)/100g cooked carrot):

$$\frac{weight\ after\ freeze\ drying\ (g)}{weight\ before\ freeze\ drying\ (g)}\ \text{x}100$$

2) Calculate DM of carrot retained after cooking raw carrot (g DM in cooked carrot/100 g raw carrot):

$$\frac{\%\ dry\ weight\ g/100g(1)}{raw\ carrot\ (g)}\ \text{x}\ cooked\ carrot\ (g)$$

3) Calculate concentration in dry matter of carrot (mg/g DM of FD powder):

$$\frac{\frac{peak\ area}{std\ peak\ area}\ (mg\ mL^{-1})\ x\ volume\ of\ extraction\ (mL)}{wt\ of\ FD\ carrot\ powder\ (g)}$$

4) Calculate the concentration of the original carrot (mg/100g raw carrot):

$$concentration\ in\ DM\ carrot\ (mg/g)\ (3)\ \text{x}\ dry\ matter\ in\ carrot\ (g/100g)(2)$$

For fried carrot:

To calculate the true retention in the fried carrot samples, the amount of oil remaining in the carrot was removed from the weight of the dry matter to give % weight with the oil removed. This figure was then used for dry matter in the calculation above (2) for boiled carrot. To estimate the oil content of the carrot, oil was extracted from a sample of the dry matter using hexane. Using the method of Proctor and Bowen (1996), 10mL of hexane was added to 1g freeze dried carrot powder and homogenised. The samples were vortexed for 10 minutes and then centrifuged (5mins, 1400rpm, room temperature). The liquid was removed from the freeze dried carrot powder into weighed clean, glass tubes. The hexane was then evaporated

and the oil content was determined by weighing the remaining liquid (Proctor and Bowen, 1996).

The calculation of true retention in the vegetable can be used to determine which of the preparations tested most effectively retained phytochemicals.

3.2.4 *Calculating total retention of phytochemicals (amount in carrot plus cooking fluid)*

The total amount of phytochemical retained in both the carrot and the cooking fluid can be combined to estimate the total retention of phytochemicals. This allows us to investigate whether compounds have degraded. The amount of phytochemical in the water and oil, calculated above, was already determined per g raw carrot, as the concentration in the fluid depends on how much carrot was present during cooking. Therefore the same values can be used. Combining the amount retained in the carrot and the amount in the cooking fluid (mg/g raw carrot), will allow an estimation of total phytochemical retention.

3.2.5 *Statistics*

To investigate the effect of time of cooking on the phytochemicals within the carrot or in the cooking medium, the mean polyacetylene and carotenoids in each shape of carrot were compared between time points in both boiled and fried carrots.

To investigate the effect of shape on phytochemicals within the carrot or in the cooking medium, the mean phytochemicals in the different shapes of carrot/cooking medium were compared at each time point in both boiled and fried carrots.

The same analysis was performed using the means of phytochemicals calculated to give true retentions of the phytochemicals.

The data were first checked for normality using the Shapiro-Wilk test. The majority of the data were normally distributed and due to the small sample size (n=3), those data that were not normally distributed were able to be analysed by parametric one-way analysis of variance (ANOVA) to compare means. Differences between means were considered significant at a 95% confidence level ($p \leq 0.05$). Values expressed are means of 3 replications (n=3). All statistics were performed using SPSS statistical software (version 22, SPSS Inc., Chicago, IL, USA).

3.3 **Results**

Example chromatograms from this experiment are shown in Figure 3.1.

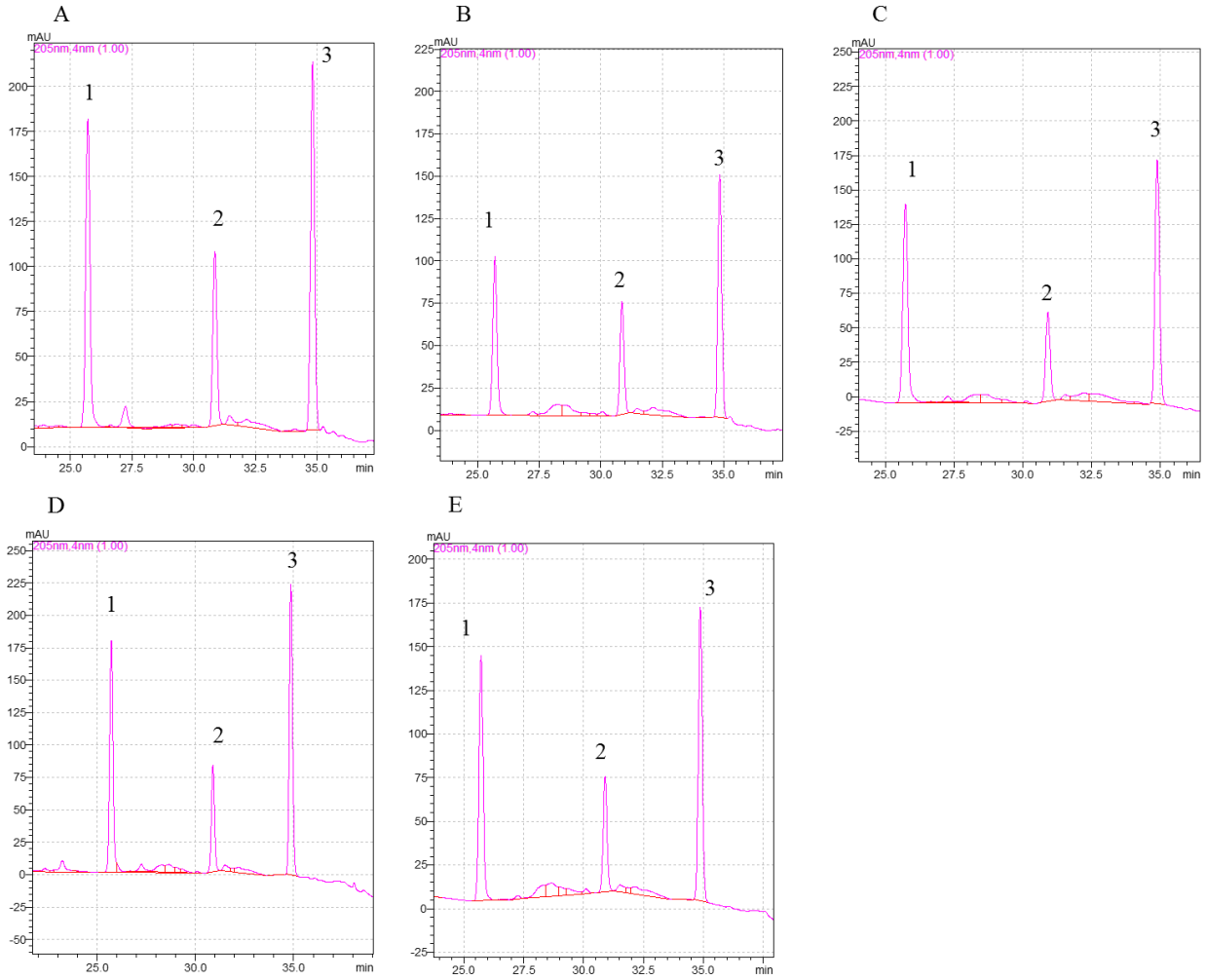


Figure 3.1: Chromatograms from cooking experiment. A: Raw carrot, B-E: carrot boiled for 5, 10, 15 and 20 minutes respectively. Peaks are: 1: falcarindiol, 2: falcarindiol-3-acetate, 3: falcarinol.

3.3.1 *Absolute concentrations of phytochemicals*

The size/shape of carrots appeared to be an important factor in the change of polyacetylene concentration during boiling (Figure 3.2). The concentrations in carrots cooked whole (W) did not change significantly from raw for any polyacetylene after boiling. For the disks (D) and quarters (Q) however, the polyacetylene concentrations decreased over time of cooking. The largest change in concentration occurred in the first 5 minutes of cooking, between raw and 5 minute samples in Q and D (only significant in Q, and in D only for falcarinol – see Appendix B for p-values), but after 5 minutes there were no differences between carrots cooked for different lengths of time. Looking at differences in shape at each time point, carrots cut into D, saw reductions in falcarinol, falcarindiol-3-acetate and total polyacetylenes compared with W from 10 minutes of boiling. Q were only significantly different from W in falcarinol and total polyacetylene at 20 minutes. See Table 9.1 for significant differences between the means of polyacetylene concentration in boiled carrot between time points in each shape.

In water, the largest change was also in the first 5 minutes, which mirrored what was happening in the carrot (Figure 3.3). There was no significant effect of shape on the amount in the water at any time point but there tended to be lower phytochemicals in the water from W in falcarinol and falcarindiol-3-acetate (W<Q<D). The amount of falcarinol and falcarindiol-3-acetate in water decreased between 5 and 10 minutes in Q and continued to decrease over time (non-significantly). A similar trend was seen in W but this was also not significant. The amount of polyacetylenes seen in water did not explain all the losses from carrot as there was a 2.3mg/100g carrot reduction in falcarinol from the D carrot in the first 5 minutes but only 0.4mg/100g carrot was seen in the water. See Table 9.2 for significant differences between the means of polyacetylene concentration in water from boiling between time points in each shape.

For carotenoids, there were no significant differences in concentrations between any of the carrot shapes at each time point in any of the carotenoids (Figure 3.4). There were also no differences in concentration over time. The carotenoids were not detectible in water from cooking, despite the observed colour of the water becoming more orange as cooking time increased (Figure 3.5).

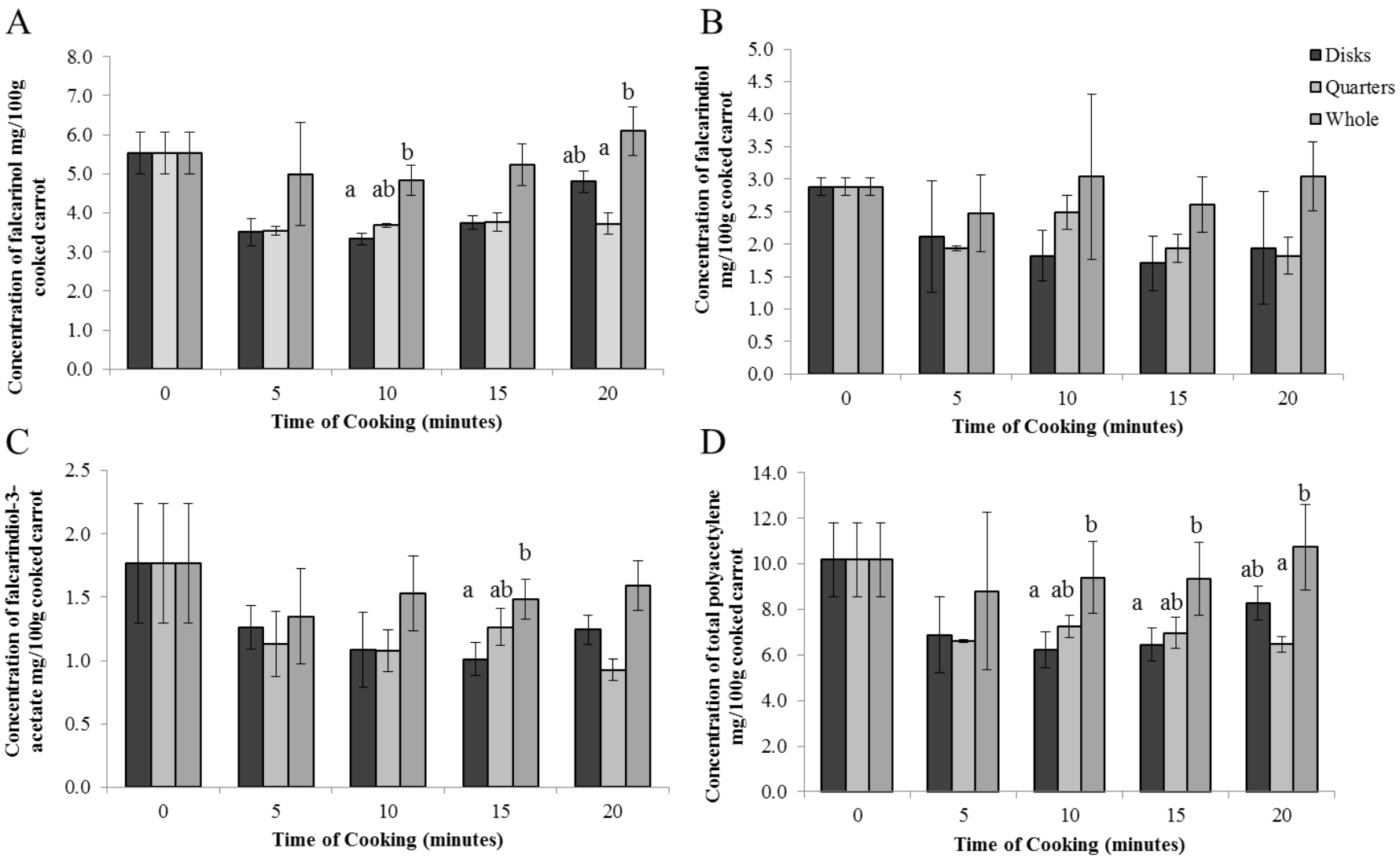


Figure 3.2: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in carrot after boiling (mg/100g cooked carrot). Error bars are confidence intervals (CI). Letters indicate differences between shapes.

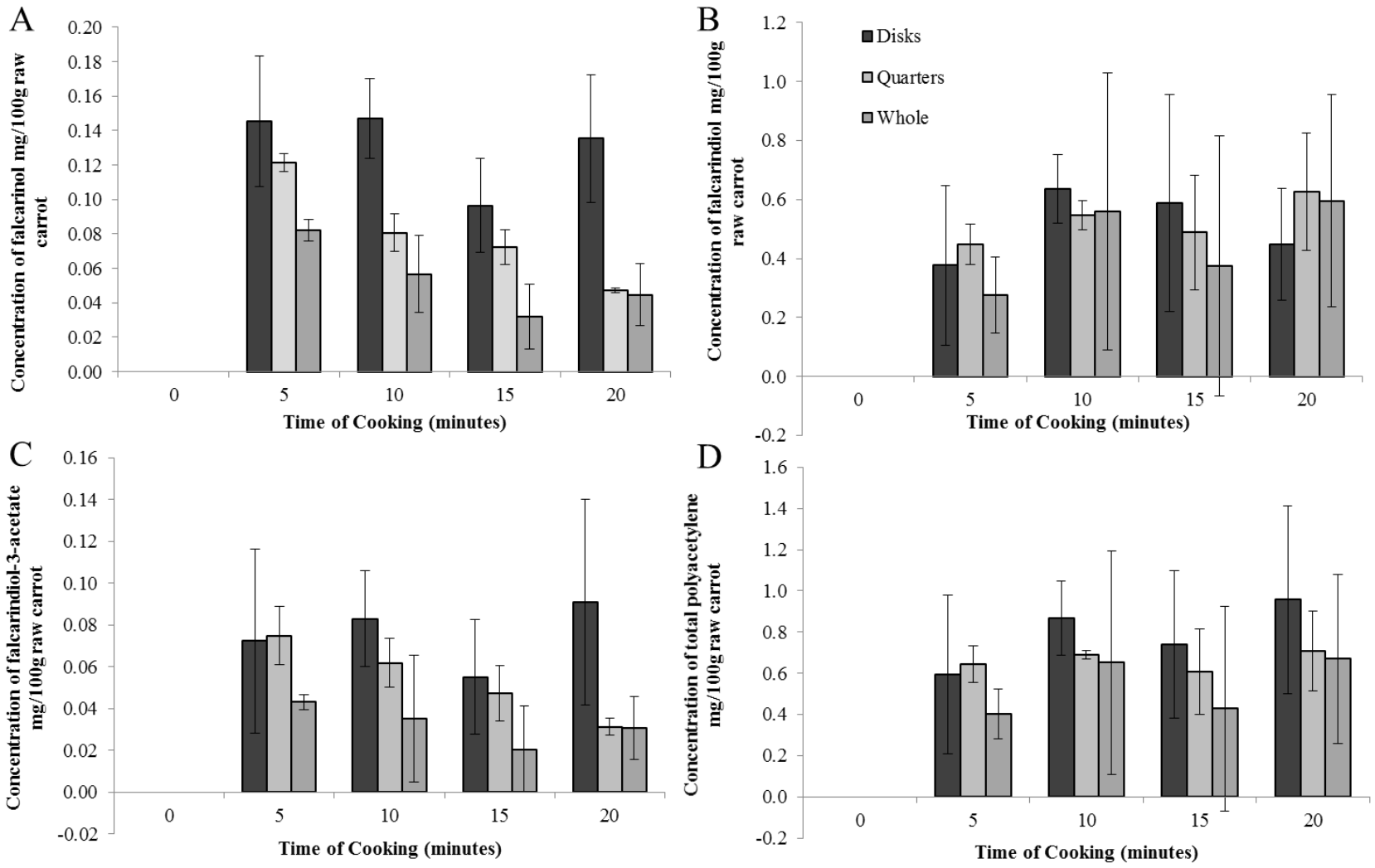


Figure 3.3: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in water after boiling carrot (mg/100g raw carrot). Error bars are CI. Letters indicate differences between shapes.

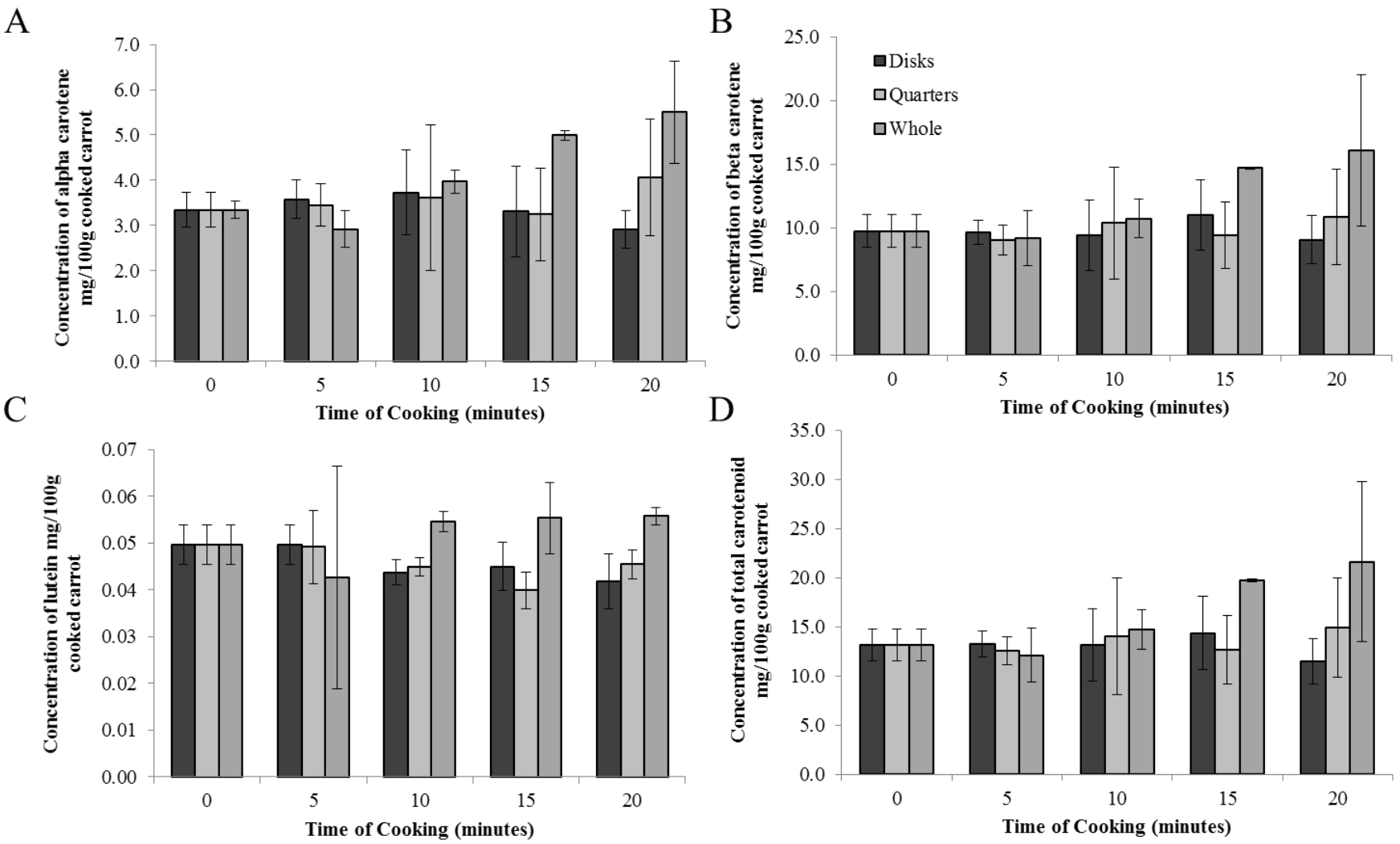


Figure 3.4: Mean concentrations of A) α-carotene, B) β-carotene, C) lutein and D) total carotenoid in carrot after boiling (mg/100g cooked carrot). Error bars are CI.

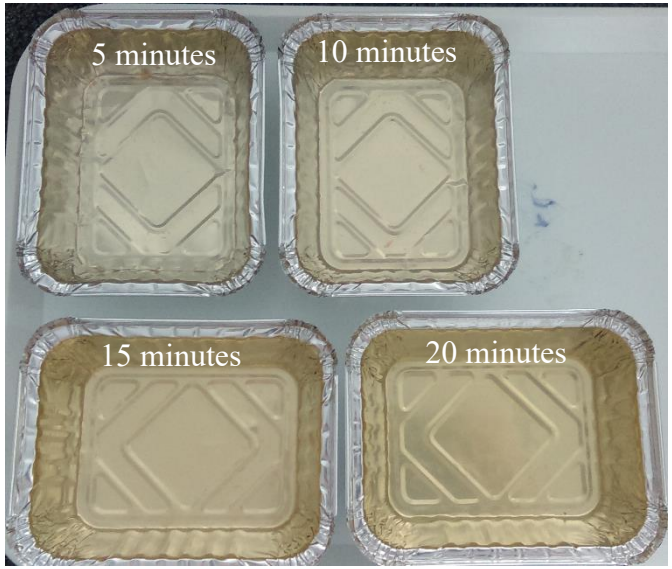


Figure 3.5: Water from boiled carrots at 5, 10, 15 and 20 minutes of cooking.

In fried carrot, there were no differences in the amount of polyacetylenes in the different shapes but they were affected by time of cooking as there was a trend for amount of polyacetylene to increase in the carrot over time (Figure 3.6). This was only significant for falcarinol and total polyacetylene in Q when comparing raw to 10 minutes (see Appendix B for p-values). Falcarindiol decreased (non-significantly) in the first 5 minutes then significantly increased from 5 to 10 minutes in D and Q, and from 5 to 8 minutes in W but there was no change from raw in this phytochemical. The mean polyacetylene values for the W fried for 8 minutes were higher than the 5 and 10 minute samples and higher than raw, suggesting that these values should be treated with caution.

Similar to the water, the majority of leached phytochemical entered the oil in the first 5 minutes of cooking (Figure 3.7). There were no significant differences in the concentration of polyacetylenes in the frying between shapes of carrot nor over time but there was a non-significant trend for less polyacetylenes in the oil from the carrots cooked W compared with the D and Q samples.

Of the carotenoids, only lutein was significantly affected during frying (Figure 3.8). W carrots were not affected but in D and Q samples lutein concentration increased over time. At 10 minutes Q had significantly higher lutein concentrations than W but otherwise there were no differences between shapes at any time point. As with water from boiling, intact carotenoids were not found in the oil after frying carrots but the oil became more orange with longer cooking times.

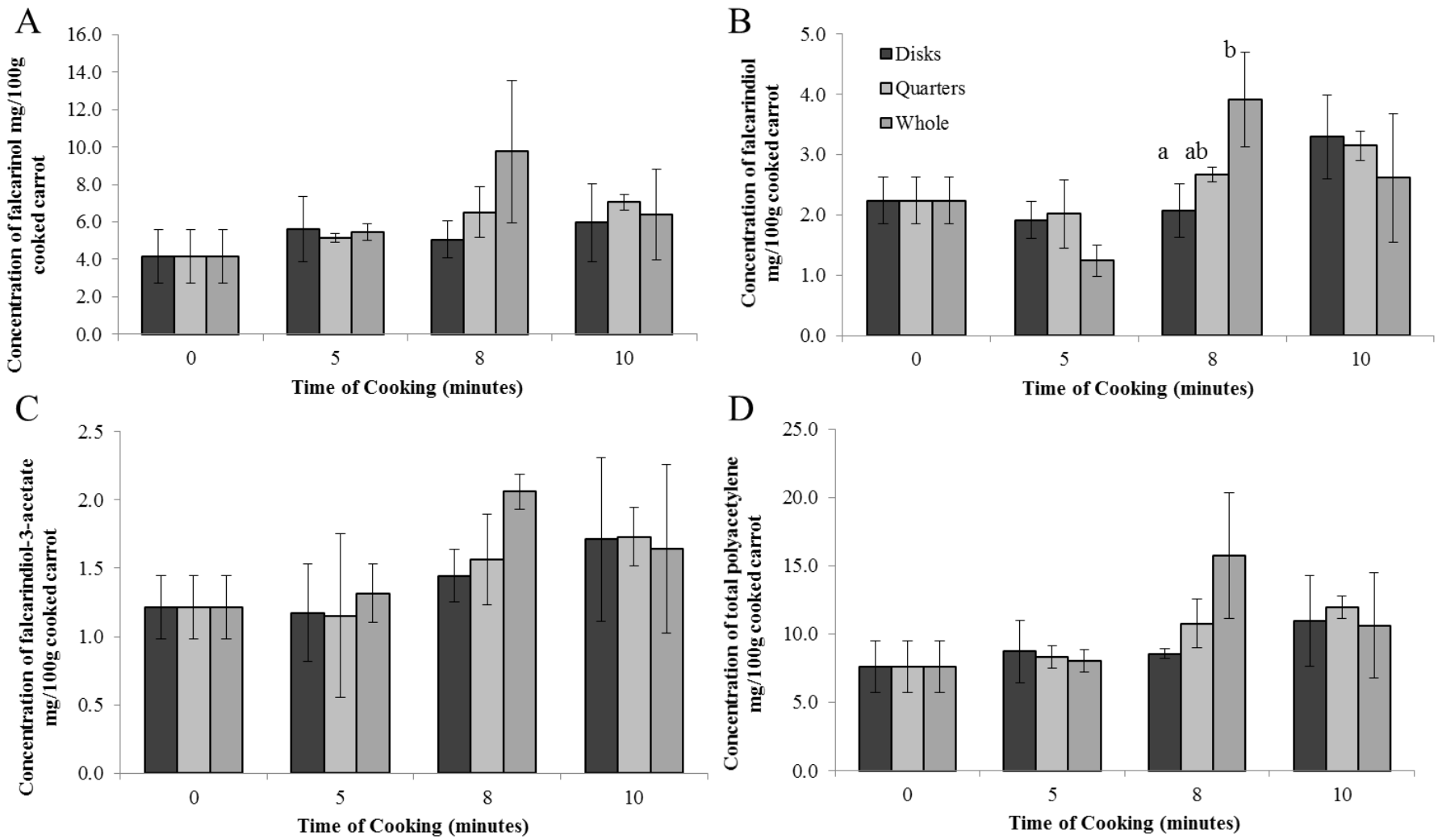


Figure 3.6: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in carrot after frying (mg/100g cooked carrot). Error bars are CI. Letters indicate differences between shapes.

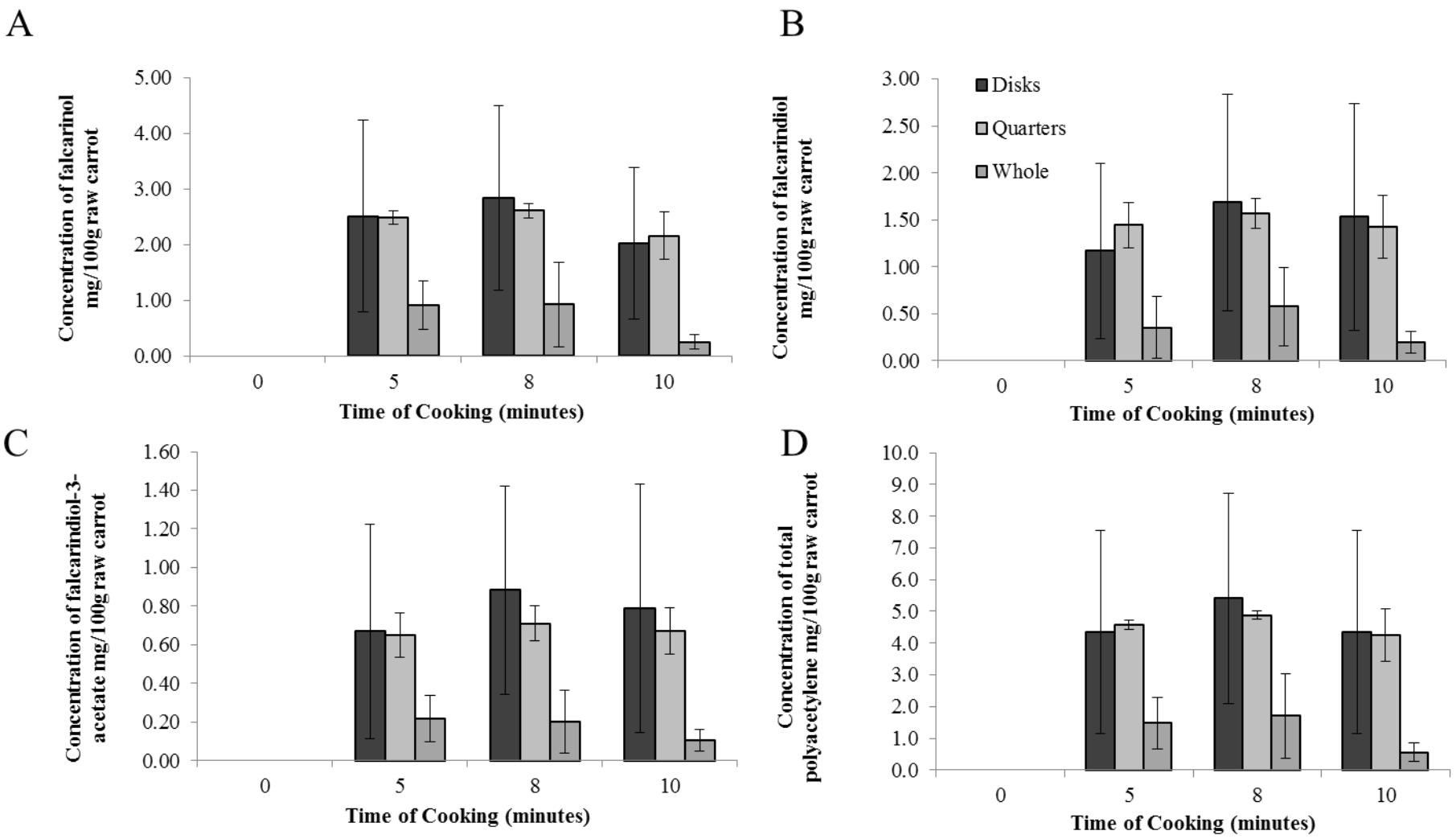


Figure 3.7: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in oil from frying carrot (mg/100g raw carrot). Error bars are CI.

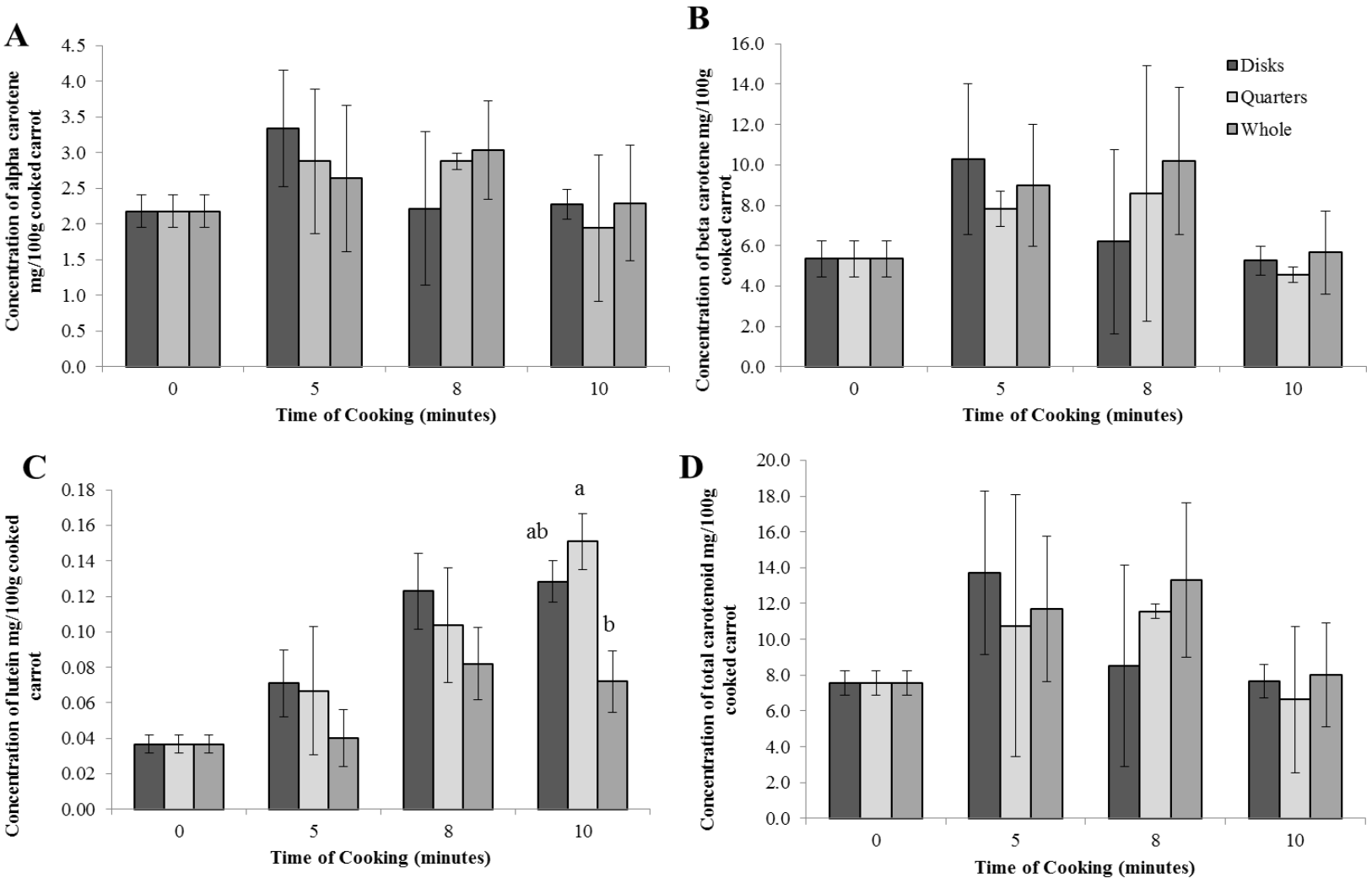


Figure 3.8: Mean concentrations of A) α-carotene, B) β-carotene, C) lutein and D) total carotenoid in carrot after frying (mg/100g cooked carrot). Error bars are CI. Letters indicate differences between shapes.

3.3.2 *Calculating the true retention of phytochemicals (amount per raw weight of carrot)*

The true retention of phytochemicals takes into consideration the changes in weight of the carrot during cooking. Figure 3.9-Figure 3.12 show the amount of phytochemicals in boiled and fried carrot when adjusting for the change in dry matter of carrot during cooking and thus the concentration measured as mg/100g raw carrot (true retention).

The amount of dry matter in the cooking water after boiling was around 32% of the dry matter in raw carrot in D and Q, and 16% in W. Dry weight of carrot decreased from 8.5% to 5.9, 6.3 and 7.7% in D, Q and W respectively after 20 minutes of boiling. The amount of polyacetylenes and carotenoids per 100g raw carrot was around 10% lower than per 100g cooked carrot as a result of this. The concentration according to the raw weight of carrot showed further significant differences between the different shapes, and the differences between raw and cooked carrots were larger/more significant. The same was seen in lutein which had differences in concentrations between raw and cooked carrot as well as differences between shapes. Alpha- and β-carotene remained unchanged during boiling.

In the fried carrot, the % dry weight increased from 8.8% in raw to 25.6, 27.4 and 13.7% in D, Q and W respectively after 10 minutes of frying. Some of this change in dry matter was the uptake of oil into the carrot. The hexane extraction demonstrated around 10-11% of the weight of dry matter in D and Q and 3% in W carrot was the weight of oil. Removing this, and adjusting for the amount per raw carrot, a more accurate estimation of loss of phytochemicals from the fried carrot can be calculated. The dry weight percentage almost doubled from 9 to 19% in Q and D, but only increased to 13% in W, excluding the weight of the oil, so a major change during frying was loss of water.

When the concentration of polyacetylene in the fried carrot was calculated per 100g of raw carrot (Figure 3.11), the concentration was lower than raw in all 3 polyacetylenes, in contrast to the concentration per 100g cooked weight which was higher than raw. The true retention in carrot was as low as 36% of the values per 100g cooked carrot after 10 minutes, suggesting that the losses of polyacetylenes during frying were disguised by the loss of water. The concentration of polyacetylenes changed in the first 5 minutes of cooking but longer cooking time did not significantly reduce any of the three polyacetylenes further (see Appendix B for p-values). Alpha- and β-carotene concentrations appeared to be more significantly affected by frying as the concentration continued to decrease over cooking time (but this was only significant in α-carotene). Lutein concentrations, however, remained constant throughout cooking (Figure 3.12).

These results highlight that the actual concentration of phytochemicals in cooked carrot (mg/100g cooked) did not fully describe the losses from the vegetable during cooking or the differences between shapes. Changes in dry matter and water content of the vegetable disguised the losses which became apparent when true retention was calculated.

Both boiling and frying led to losses of phytochemicals. True retention of polyacetylenes was around 60% in both the boiled and fried carrot for D and Q. Retention in W carrot was higher (86% and 85% in boiled and fried respectively). For the carotenoids, boiling was better for retention (90% in D and Q and 108% in W) in the D and Q carrot than frying (66% in D and Q, and 104% in W) but there was little difference between boiling and frying in the W carrots. Boiled carrots were therefore better than fried carrots for overall phytochemical intake but if carrots were cooked W this mitigated the losses.

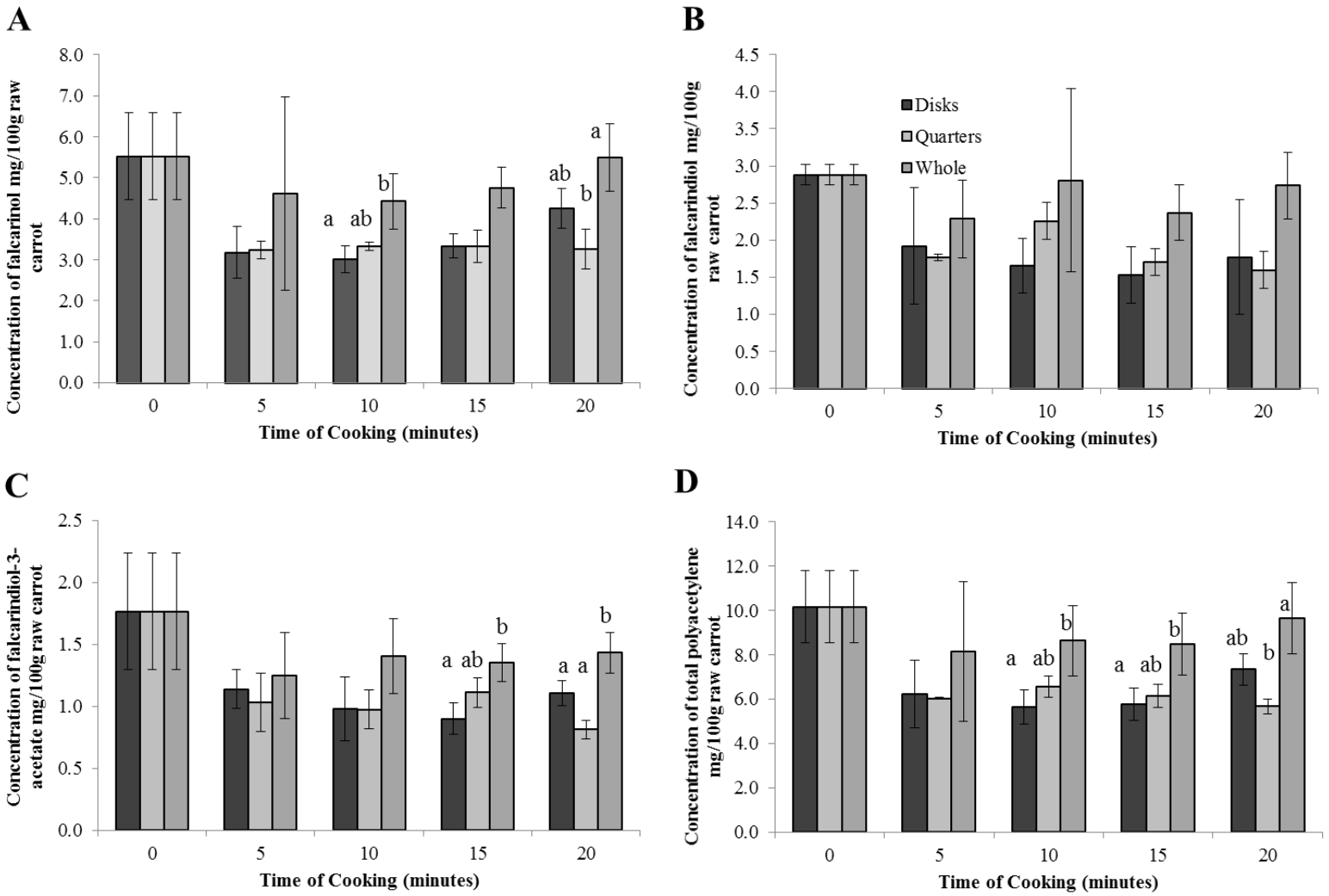


Figure 3.9: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in carrot after boiling (mg/100g raw carrot). Error bars are confidence intervals (CI). Letters indicate differences between shapes.

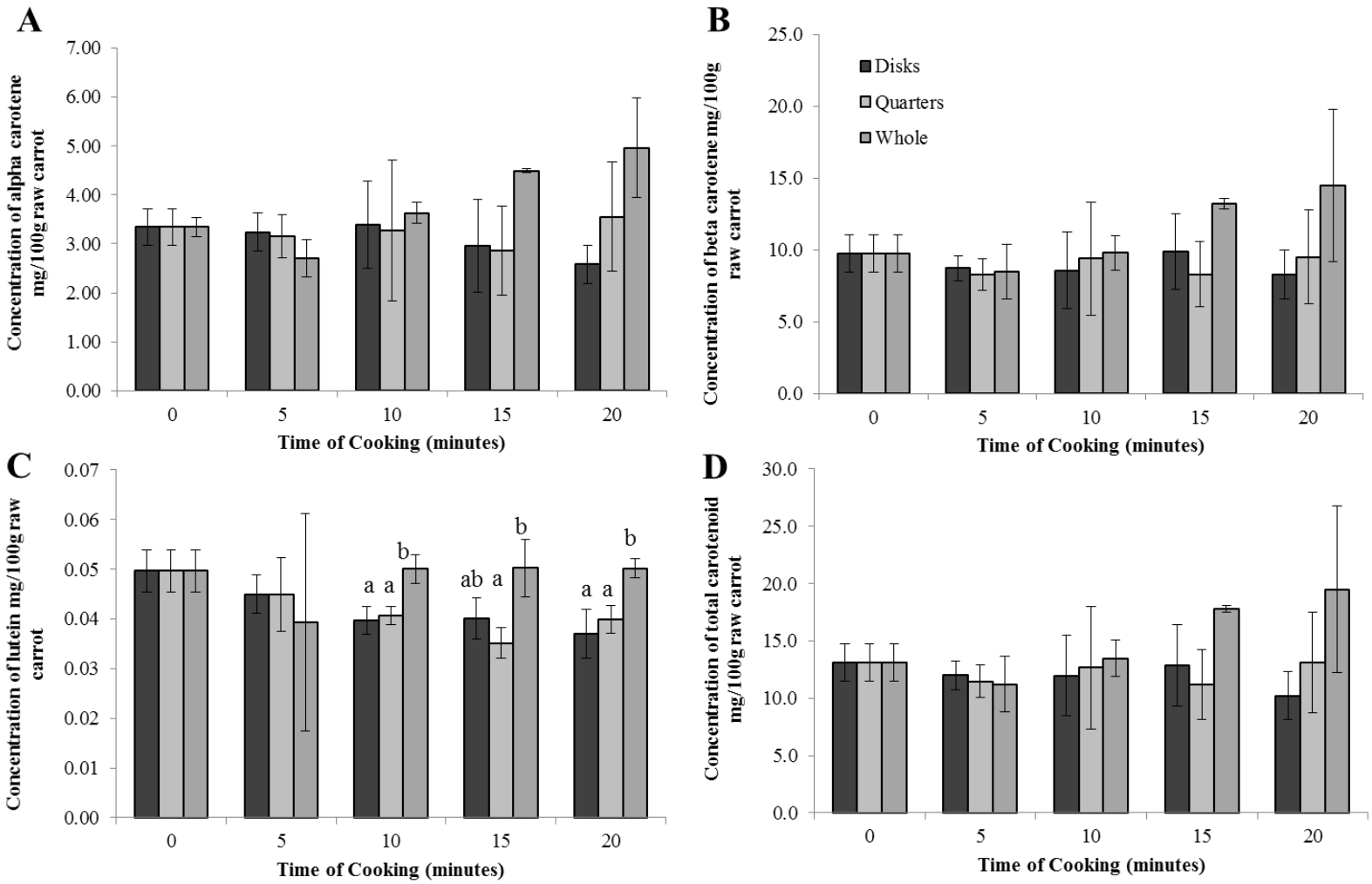


Figure 3.10: Mean concentrations of A) α-carotene, B) β-carotene, C) lutein and D) total carotenoid in carrot after boiling (mg/100g raw carrot). Error bars are CI. Letters indicate differences between shapes.

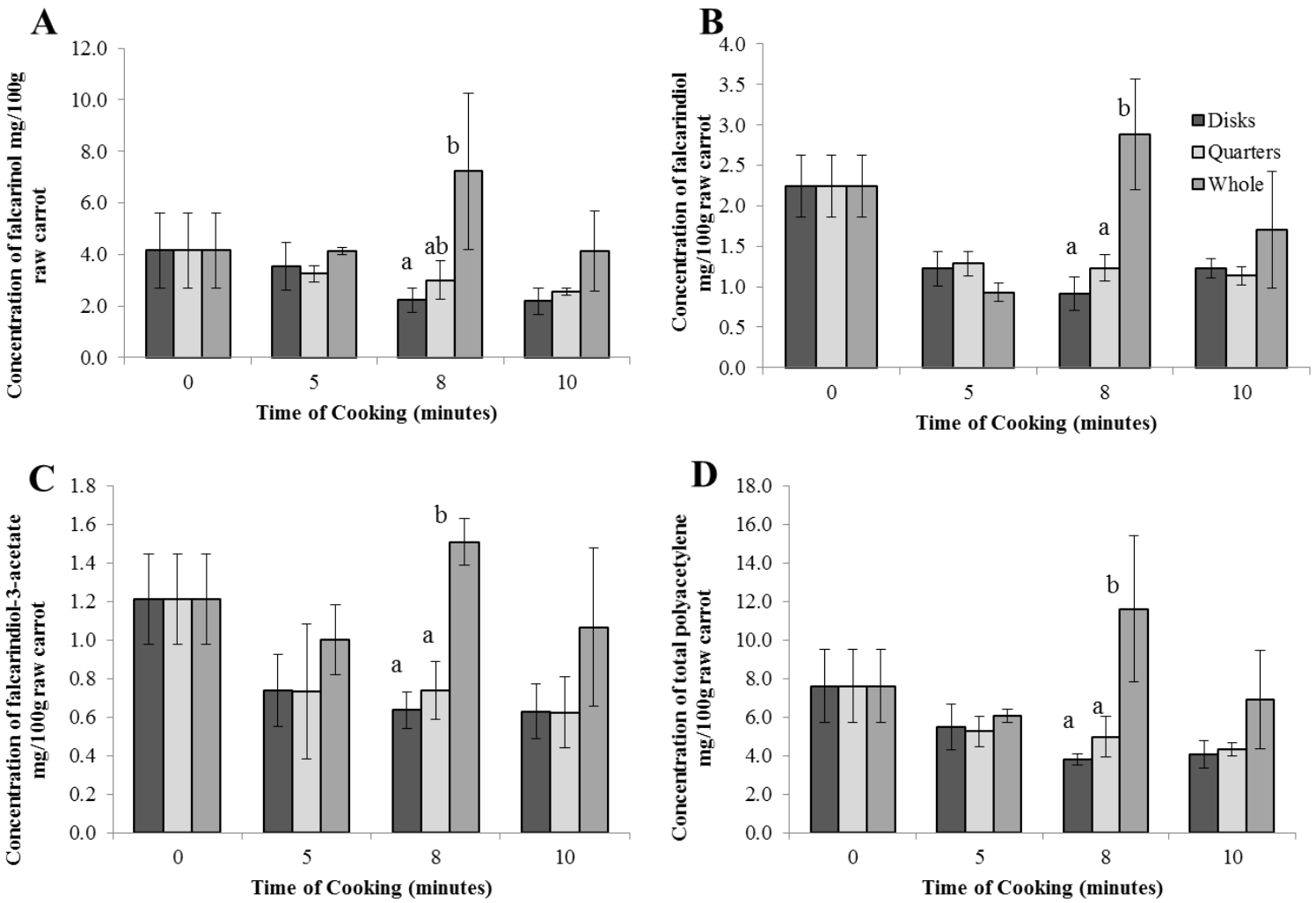


Figure 3.11: Mean concentrations of A) falcarinol, B) falcarindiol, C) falcarindiol-3-acetate and D) total polyacetylene in carrot after frying (mg/100g raw carrot). Error bars are confidence intervals (CI). Letters indicate differences between shapes.

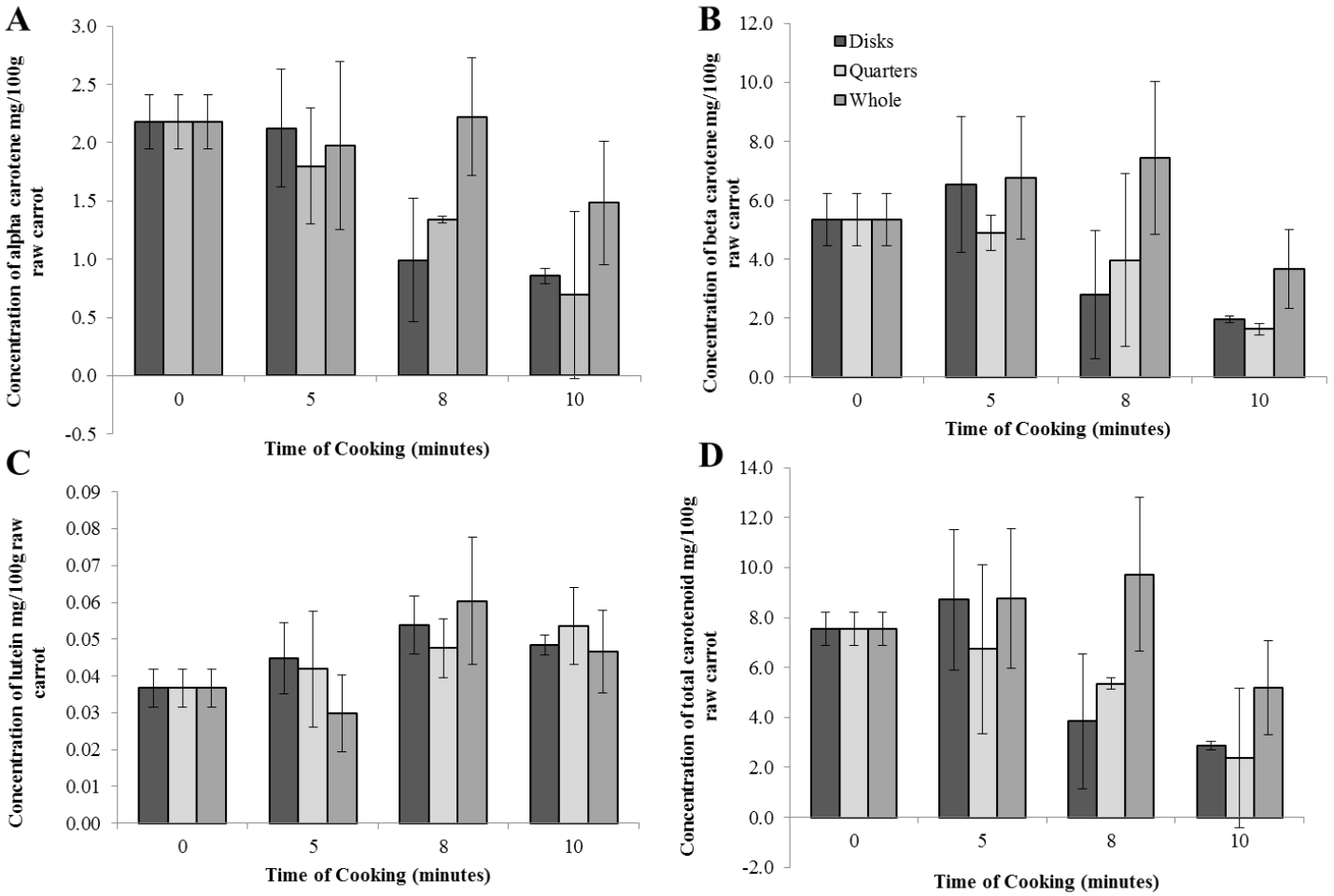


Figure 3.12: Mean concentrations of A) α-carotene, B) β-carotene, C) lutein and D) total carotenoid in carrot after frying (mg/100g raw carrot). Error bars are CI. Letters indicate differences between shapes.

3.3.3 *Total retention of polyacetylenes (amount in carrot plus cooking fluid)*

When the amount in the cooking fluid was combined with the amount in the carrot, total retention of total polyacetylene was higher in the fried carrot (110-130% in D, 113-130% in Q, and 97-175% in W) than the boiled (81-103% in D, 81-89% Q and 96-122% in W) (Figure 3.13 and Figure 3.14). True retention was different for the 3 polyacetylenes as the amount of falcarinol and falcarindiol-3-acetate in boiled carrot and water was 51-82% of raw in D and Q and 77-101% in W but a larger amount of falcarindiol was retained in all shapes (between 102-148% of raw). In the fried carrot, the amount of total polyacetylenes retained in the carrot plus oil was 98-130% of raw (but 175% of raw for W 8 min sample). There were only small differences between polyacetylenes, and only slightly more was retained in the W carrots (with the exception of the W 8-minute sample).

There was a small apparent increase in amount of total polyacetylene after frying (around 130% of raw in the majority of samples but 175% in W 8-minute sample), falcarindiol after boiling (102-148%), and total polyacetylene in whole carrots after boiling (up to 122%), even when accounting for changes in the weight of the carrot but these increases were not significant.

The total retention allows us to make estimates of phytochemical degradation in each of the conditions as phytochemicals that are not accounted for in either the carrot or cooking fluid are assumed to be degraded. For boiling, up to 39% of falcarinol and 49% of falcarindiol-3-acetate in D and Q was degraded but a more modest 0-17% of falcarinol and 14-23% falcarindiol-3-acetate was lost from the W carrots. During frying up to 23% falcarinol, 17% falcarindiol and 44% falcarindiol-3-acetate was lost from D and Q. W appeared to lose more to degradation with up to 21, 66, and 44% of falcarinol, falcarindiol and falcarindiol-3-acetate being unaccounted for after frying. However, this may be due to less release of phytochemicals from the carrot matrix in the W carrot compared to D and Q during cooking, as discussed further below.

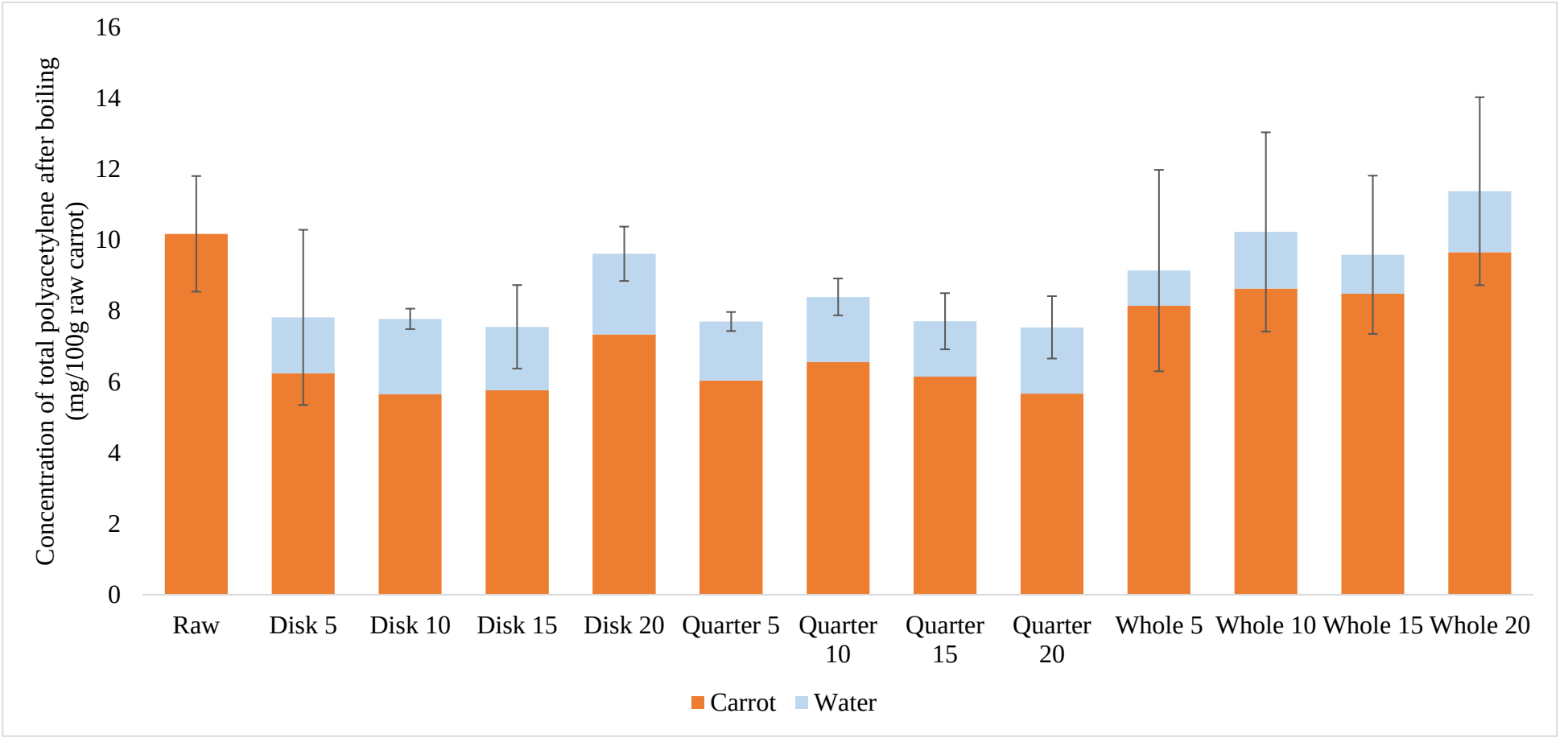


Figure 3.13: Mean amount of total polyacetylenes in carrot and water (mg/100g raw carrot) after boiling. Error bars are CI.

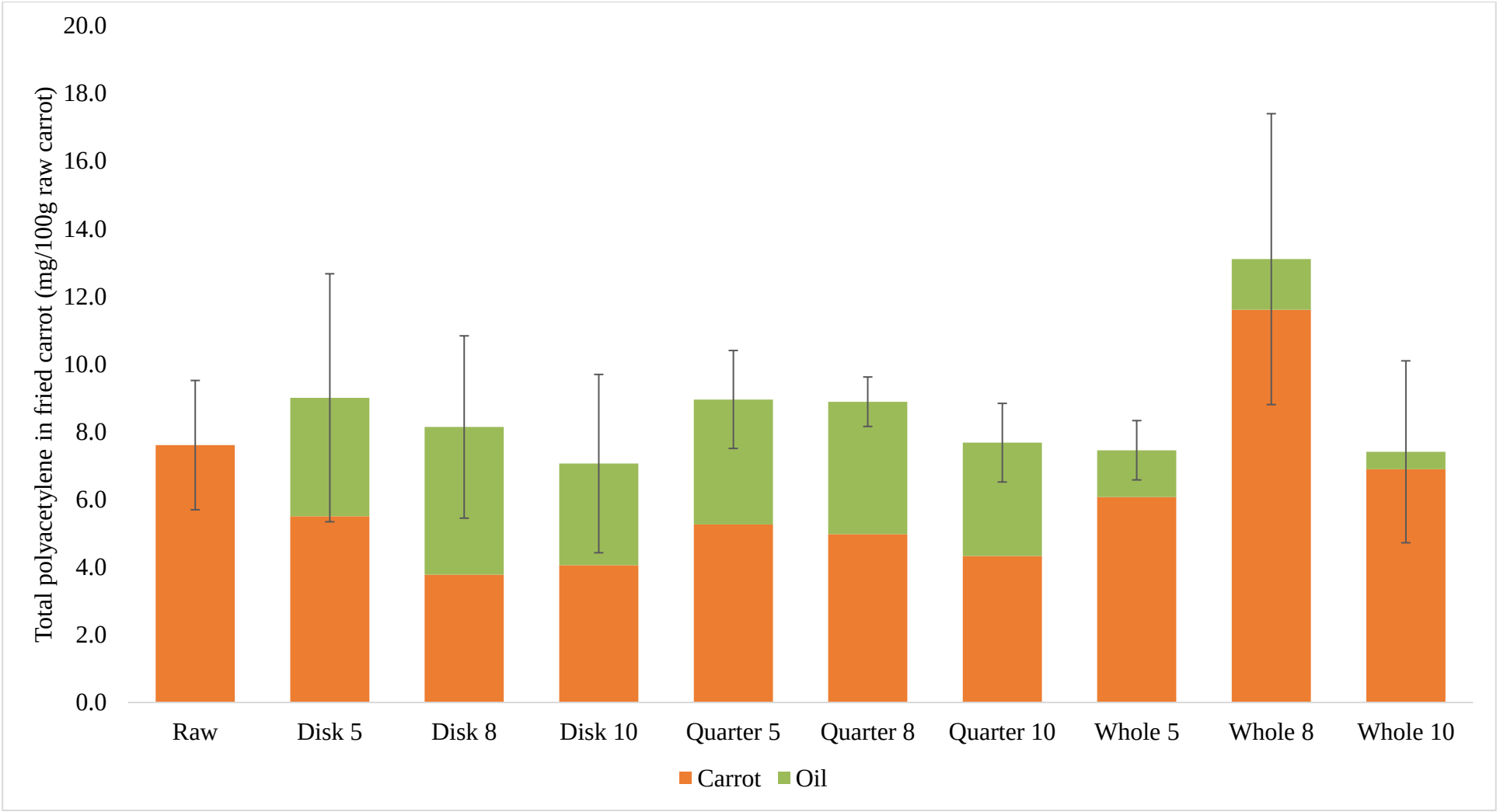


Figure 3.14: Mean amount of total polyacetylenes in carrot and oil (mg/100g raw carrot) after frying. Error bars are CI.

3.4 **Discussion**

To the knowledge of the author, this is the first trial to show the effect of surface area on polyacetylenes in carrot during domestic cooking, to detect polyacetylenes in cooking water and oil and to investigate polyacetylene changes in fried carrot.

As with the database studies, the carrot in this trial had higher concentrations of polyacetylenes than the majority of literature values but they are comparable to the values found in 2 papers (Metzger and Barnes, 2009; Rawson *et al.*, 2012b). The carotenoid concentrations in the literature are found in ranges 2.2-5.8, 4.6-27.6 and 0.1-0.6mg/100g fresh for α- and β-carotene and lutein respectively (Dietz *et al.*, 1988; Granado *et al.*, 1992; Pinheiro Sant'Ana *et al.*, 1998; Sulaeman *et al.*, 2001; Borowska *et al.*, 2003; Nunn *et al.*, 2006; Imsic *et al.*, 2010). This is comparable to those found in this trial for α- and β- carotene (mean concentrations of 3.6 and 10.3mg/100g respectively) but lutein appeared to be a factor of 10 lower than many literature values with averages of 0.05mg/100g fresh weight compared with 0.3-0.6mg/100g (Granado *et al.*, 1992; Nunn *et al.*, 2006). However, some varieties in the literature were as low as 0.06-0.09mg/100g (Nicolle *et al.*, 2004) and differences in genetic variability and growing conditions may have led to lower concentrations in the carrots from the experiment.

3.4.1 ***The effect of boiling on phytochemicals***

Concentrations of polyacetylenes and lutein were lower in cooked D and Q carrots compared with raw. The loss from carrot and appearance in water seemed to be dependent on surface area of the carrot as D and Q carrots were significantly affected but W carrots did not change from raw. It would therefore appear that the losses from the carrot are at least in part explained by transfer into the water either by diffusion as the cells die and release their turgor, or the mechanical release of phytochemicals during cutting and peeling, and then washing into the water from the surface. In both cases, the larger the surface area of the carrot, the more phytochemicals were lost into the cooking fluid. After 5 minutes of boiling, the polyacetylene in water either remained the same, or decreased in concentration in the case of falcarinol and falcarindiol-3-acetate, and no further losses were seen from the carrot. With diffusion, it would be expected that the phytochemical loss from vegetables would continue over cooking time as cells continue to soften and release their contents. This and other studies (Svanberg *et al.*, 1997; Nyman *et al.*, 2005) have demonstrated sugar and fibre loss into water during boiling continues throughout the cooking time (see Appendix B). It could therefore be the case that some of the appearance in water is due to phytochemicals simply being washed

off the surface as seen in the Koidis *et al.* (2012) paper. Losses of 30-44% of total polyacetylenes occurred during washing, similar to the 32-35% reduction in the first 5 minutes from D and Q in this trial. Only 13% total polyacetylene was lost from W after 5 minutes suggesting a smaller surface area per unit volume can help to reduce the losses of phytochemicals. However, the amount that is lost from carrot is not entirely explained by the amount found in water with just 17% of the losses of falcarinol seen in the water at 5 minutes. Oxidation, due to exposure to heat or light (Boon *et al.*, 2010) and enzymatic degradation can occur on cut edges of the carrot and would therefore also lead to higher degradation in carrots with a higher surface area (Bolin and Huxsoll, 1989). These enzymes are denatured when heated so degradation of phytochemicals would only occur until the carrot reached the temperature of denaturation. This could explain some of the losses seen between raw and the 5 minute samples that did not continue with cooking time. Falcarinol and falcarindiol-3-acetate were seen in the water during the first 5 minutes but fell in concentration after this, suggesting they were degraded in an aqueous environment. Falcarindiol appeared at higher concentrations in the water and remained at the same concentration over time. It is more polar than falcarinol and falcarindiol-3-acetate, suggesting the stability of the compounds in liquid may depend on the polarity of the compound.

Other trials that have looked at the concentration of polyacetylenes in boiled vegetables had mixed results. As mentioned previously, increases and decreases have been seen during boiling. Decreases have been seen most often in vegetables that are hydrothermally treated for short amounts of time (Hansen *et al.*, 2003). In vegetables treated for longer periods, 20-60 minutes, the concentrations increased (Rawson *et al.*, 2013b). However, increases were not seen in the current trial up to 20 minutes, possibly because 20 minutes was not enough time for the loss of solids to concentrate the remaining phytochemicals and outweigh the losses into water, or to release phytochemicals from structures that may be binding them.

The carotenoids did not change in actual concentration after boiling at any time point in any shape in the trial. However, the true retention showed that lutein, but not α- and β- carotene, was lost over time. Lutein is more polar than α- and β- carotene so may be more easily lost into the aqueous environment, but as the carotenoids were not detected in the water, it is not possible to definitively determine this. Carotenoids have been widely investigated for their retention in carrot during boiling. As they are fat soluble compounds, it is expected that they would mostly remain in the vegetable during boiling rather than leaching out, but oxidation and heat degradation could reduce concentrations. The concentration of α- and β-carotene has been seen to increase between 4.9% to 73% during boiling if the change in soluble solids was

not taken into account (Puupponen-Pimiä *et al.*, 2003; Imsic *et al.*, 2010; Chiavaro *et al.*, 2012; Bongoni *et al.*, 2014) but decreases of 9-20% have also been observed (Sulaeman *et al.*, 2001; Nunn *et al.*, 2006). When losses of soluble solids were taken into consideration, the concentrations all fell to below those in raw carrots, with 22-36% of α-carotene and 11-40% of β-carotene lost from the carrot during boiling (Dietz *et al.*, 1988; Pinheiro Sant'Ana *et al.*, 1998; Borowska *et al.*, 2003). In all three trials where concentrations fell, the boiling time was 21-32 minutes and the carrots were cut into cubes/small pieces, suggesting carotenoids are lost more when cut into shapes with high surface area and long cooking times.

Determination of leaching of phytochemicals into cooking water has been attempted in previous trials with little success. Polyacetylenes from fennel were not detected in water after boiling (Rawson *et al.*, 2013a) but the method the authors used was not fully described and if the polyacetylenes were left in the water for long periods, their potential instability in an aqueous environment could mean they were degraded before analysis. Freeze drying in the current experiment appears to have preserved them. To our knowledge, carotenoids have not been measured in water from boiling but cooking carrots with minimal or no water retains carotenoids more than if they were immersed in water (Guillén *et al.*, 2017) suggesting that leaching does occur.

3.4.2 *The effect of frying on phytochemicals*

The measured concentration of phytochemicals slightly increased in carrot after frying but the % dry weight increased over 3-fold in D and Q. The true retentions show that the phytochemicals were degraded during frying but loss of water from the carrot disguised this loss. Shape was important during frying as losses of polyacetylenes and α- and β-carotene occurred from D and Q but not W carrot over time. Lutein did not change significantly compared with raw suggesting that lutein could be more heat stable than α-and β-carotene during frying, or leached less into oil as it is more non-polar. However as none of the carotenoids were detected in oil this cannot be known for certain.

The majority of polyacetylenes in oil appear in the first 5 minutes and the concentrations did not change up to 10 minutes suggesting polyacetylenes are reasonably heat stable in oil even at the high heats experienced during frying (Figure 3.14). To our knowledge, this is the first trial to investigate the effect of frying on polyacetylenes in carrots. Carotenoids have been seen to decrease during frying, with stir fried carrots retaining around 72% of β-carotene after 10 minutes (Veda *et al.*, 2010) and carrots deep fried retained 34-36%, 27.9-76% and 57% of

α- and β-carotene and lutein respectively (Dueik *et al.*, 2010). Although this is only a small number of trials to compare, losses were higher from vegetables that were deep fried compared with those that were stir fried, suggesting contact with oil is important in phytochemical retention. However, attempts to measure the amount of carotenoids in oil after cooking has proved difficult. Around 5% of the amount of β-carotene in the raw vegetable has been detected in oil (Renquist *et al.*, 1978) which could be due to low stability in oil during frying. The amount in oil during vegetable cooking has been reported to be highest after 2 minutes, after which the amount falls with increasing cooking time (Kidmose *et al.*, 2006). Temperature appears to play a part in stability as β-carotene in fortified soy bean oil was stable up to 100^{o}C but degraded above this temperature (Dutra-de-Oliveira *et al.*, 1998) and temperatures lower than 35^{o}C have shown a positive correlation between degradation and temperature with isolated carotenoids (Chen *et al.*, 1996). The lack of carotenoid in the oil in the current experiment suggests that any carotenoid leached could have completely broken down in the high temperature of frying (around 180^{o}C in this experiment). Carotenoid extracts from carrot have been seen to degrade by 44% in 5 minutes at 80^{o}C (Kadian *et al.*, 2013). This would explain why the colour of the oil became increasingly orange but carotenoids were not detected in the analysis. However, when carotenoids are within the matrix of a vegetable they appear to be fairly stable. They are found in a crystalline form, bound to pectin cellulose and proteins within chromoplasts which protect them from degradation during heating as they are unable to be dissolved into lipids. Even when the crystal structures are within a liquid, e.g. mango juice, the carotenoids appear to be protected (Marx *et al.*, 2003; Vasquez-Caicedo *et al.*, 2007). For lutein, if bound to an ester, maltodextrin or sucrose, the compound is more stable that the pure compound (Kuang *et al.*, 2015). As well as being protected from oxidation and light, this may explain why they are reasonably retained in the vegetable matrix but are not present in the cooking fluids.

3.4.3 *Total retention of polyacetylenes (amount in carrot plus cooking fluid)*

The total amount of polyacetylene retained in the fried carrots plus oil was higher than the amount in boiled carrot plus water. The amount retained in boiled and fried carrot flesh were similar so the differences appear to be due to the amount in the cooking fluid. This could potentially be due to stability of these compounds in the cooking fluid, with polyacetylenes being more stable in oil than in water and therefore overall retention being better in fried carrots and oil. The amount of falcarindiol in water was higher than the amounts of falcarinol and falcarindiol-3-acetate and falcarindiol is more polar than the other two polyacetylenes,

suggesting the polarity of these compounds may be affecting their stability, as mentioned previously.

The increases in total amount of polyacetylene in W carrots suggests that cooking may have improved the extractability of the polyacetylenes. The W boiled carrots had less surface area for leaching so improvements in extractability would stay within the carrot. Apparent increases in phytochemicals compared with raw have been seen in previous publications when measuring absolute concentrations in the vegetable, which does not account for loss of soluble solids. In this trial, taking account of the changes in dry matter does not seem to fully resolve this increase. The apparent large increase of polyacetylene in the W, 8 minute, fried sample could be due to the improved extractability but this is not carried over into the 10 minute sample. It is possible that by 10 minutes the compounds start to degrade, therefore there could be a fine balance between improvements in extractability and degradation over cooking times, but there are not enough individual time points during the cooking in this experiment to accurately predict these mechanisms occurring during cooking. As the increases in amount of polyacetylene were not significant in this study, it is possible that there was no real improvement in extractability. However, some previous research has concluded that the increases in the concentration of phytochemicals such as carotenoids are due to greater extractability of compounds after cooking (van het Hof *et al.*, 2000). Heating breaks down proteins and fibre which could be binding phytochemicals (Aman *et al.*, 2005) and structural changes have been seen in the cells of vegetables after cooking compared with raw. Water swells the cells during boiling and starch expands during heating which breaks open the cell walls (Tumuhimbise *et al.*, 2009; Iborra-Bernad *et al.*, 2015), and pectins in the cell wall break down on heating, resulting in thinning of the cell walls (Tumuhimbise *et al.*, 2009). Cooked carrot will therefore more easily release compounds into the extraction solvent. Improvements in bioaccessibility and bioavailability of compounds from cooked vegetables compared with raw have been seen for the same reason. Two trials that used *in vitro* models of digestion saw better bioaccessibility of carotenoids from fried carrot compared with raw (Tumuhimbise *et al.*, 2009; Veda *et al.*, 2010) and these improvements in extractability could improve digestibility, and thus bioavailability, when consumed. In carotenoid bioavailability trials, higher blood plasma concentrations of β-carotene have been achieved after regular consumption of boiled carrots compared with raw (Rock *et al.*, 1998; Livny *et al.*, 2003), and lycopene is more bioavailable from processed tomato products than fresh (Dewanto *et al.*, 2002). However, the bioavailability of polyacetylenes from different food matrices has not been tested *in vitro* or *in vivo*.

3.4.4 *Changing habits of processing*

Keeping the cooking water to incorporate into a dish is a common recommendation for improving phytochemical intake. The amount in the water accounts for 22-35% of total polyacetylene from D and Q, and 11-16% in W (depending on time of cooking) in cooked carrot. Changing the shape of carrots from D to W could increase intake of total polyacetylenes by 14-33% (dependent on time of cooking) after boiling. This demonstrates both keeping the cooking water or changing the shape of the carrot from disks to whole could similarly lead to reduced loss of phytochemical. Accounting for the amount in both carrot and water, total polyacetylene lost to degradation is much lower in W (0-18% and 14-24% of falcarinol and falcarindiol-3-acetate respectively, dependent on cooking time) than in D and Q (18-39% falcarinol and 25-49% falcarindiol-3-acetate), showing that simple changes to cooking technique could mitigate losses during cooking. For the carotenoids, regardless of the amount lost to leaching, incorporating the fluid would be of no benefit if the compounds are degraded once they have left the vegetable. Consumer behaviour in processing of vegetables suggests that the length of cooking time is predominantly determined by the textural properties of the carrot. The most common boiling method is 6-10 minutes in a pan with a high amount of water (Bongoni *et al.*, 2014). Whole carrots would take longer to soften than disks and the cooking time may need to be adjusted upwards accordingly to satisfy the textural requirements of a consumer. However, longer cooking times did not have an effect on the polyacetylenes or carotenoids up to 20 minutes in this trial so the longer cooking time did not adversely affect the nutritive value of the carrots.

Whilst it is interesting to explore the effect of frying, carrots fried for 8-10 minutes became very brown (see Figure 3.15) and probably would not be consumed this way in the home. The palatability and acceptability of the resulting cooked carrots was not formally assessed, however, the fried carrots did not look appetizing. The result was dark brown pieces that were quite oily. The whole carrots were still slightly hard in the middle, especially carrots that were large in diameter. However, as the time of cooking seemed to have less of an effect on the concentration of phytochemicals than shape, the whole carrots could be cooked for longer than disks to achieve the same texture with little resulting loss of phytochemicals.

Frying was chosen as one of the cooking methods to investigate as vegetables cooking in oil had been seen to improve bioavailability of fat soluble compounds (Fernández-García *et al.*,

2012). It was an intention for the cooked vegetables to be digested in an *in vitro* system to explore the bioaccessibility of polyacetylenes, however, due to issues with equipment failure, this work was unable to be realised. A comparison could have been made with juicing as another preparation of carrot and the compounds within, but as this experiment was going to inform the dietary intervention, juicing was discarded as an option. Juicing had been explored both for bioavailability (Haraldsdóttir *et al.*, 2002) and effect on DNA damage (Butalla *et al.*, 2012) previously and the intention of this project was to look at whole carrots. Juicing also does not represent how the majority of people consume carrot (not represented in NDNS survey (NatCen Social Research *et al.*, 2015)).

To ensure enough oil was available for analysis, more oil was used than typical for stir frying but shallow frying is similar to the way vegetables would be cooked prior to them being incorporated into a dish, for example, the sweating of vegetables for mixing into a casserole. It is interesting to see that the compounds would not be adversely affected in this stage of cooking with the added benefit of leached compounds remaining in the dish. Stability of polyacetylenes in cooking oil suggests that the compounds will remain intact if there is a source of fat present for the polyacetylenes to be dissolved into. This may in part explain why polyacetylenes in mixed dishes (from chapter 2) were well retained despite long cooking times in soups and stews. As well as issues of palatability, fried carrots would add fat into the diet that could be avoided if they were boiled. As there are associations between excess body weight and cancer (World Cancer Research Fund/American Institute for Cancer Research, 2007), regular consumption of shallow fried carrots is not recommended.

Figure 3.15: Carrot disks fried for 8 minutes

3.4.1 *Limitations:*

With the large variability in both the cooked and raw carrots, more samples should have been analysed. However, due to the large number of samples that were created during this experiment, preparation, cooking, freeze drying, extraction and HPLC analysis of the carrot samples, plus the cooking fluid, it was not feasible to repeat the experiment in the time frame of the PhD. Additionally, as the variability in the samples was not realised until HPLC analysis was completed, the repeated samples would have been performed with carrots that were harvested later in the harvest period, or in a different harvest year, which have both been shown to change the polyacetylene concentrations and could therefore introduce more variability into the data set. A power calculation could have been completed prior to this work, using the results of the combined database. Using the figures for total polyacetylene and comparing the means from boiled carrot and raw carrot, this sample size would have been 20 for 80% power. Future work to reduce variability could include either using more uniform samples, for example, selecting carrots that had all come from the same field, or increasing the number of the samples in the experiment.

3.5 **Conclusions**

Changes of phytochemicals during cooking is a complicated process, with fluctuations both up and down dependant on a myriad of factors that occur simultaneously, often countering each other's effects. Around 60% of polyacetylenes are retained in carrots after both boiling and frying. Polyacetylenes are lost during cooking in the first 5 minutes, but after this there is little change. Therefore, raw is different to cooked but time of cooking is not important for retention. More carotenoids are retained in boiled carrots than fried carrots, therefore to get the full benefit of the phytochemicals in carrots, boiling is recommended. The surface area is important for losses of phytochemicals, and preparing carrots whole or in big pieces could mitigate these. Retaining the cooking fluid for incorporation into a dish is recommended to increase intake of polyacetylenes, but not carotenoids, which appear to be degraded.

Calculating the amount of phytochemicals in carrot both according to cooked weight and raw weight demonstrated that the concentration in 100g of cooked carrot was higher than in 100g of raw carrot in this trial. However, if a consumer were to prepare one carrot, regardless of its weight, the raw carrot may have more phytochemicals. Improvements in extractability and therefore bioavailability of polyacetylenes may be higher in the cooked carrot but this has yet to be tested *in vivo*.

3.6 **Further Work**

Whilst this work allows us to describe the changes during processing, it is not known how much is absorbed from whole carrots when consumed. Polyacetylenes have been seen to be bioavailable from carrot juice, but bioavailability from whole carrots is unknown. To determine whether the concentrations of polyacetylene that are seen to be cytotoxic to tumour cells *in vitro* are achieved in the body after eating whole carrot, the bioavailability of these compounds from carrot will need to be assessed.

Related Conference Proceedings: Warner, S., Seal, C., Haldar, S. and Brandt, K. (2016b) 'Retention of polyacetylenes and carotenoids in carrot during cooking', *Proceedings of the Nutrition Society*, 75(OCE2).

Chapter 4. **Investigating the occurrence of polyacetylenes in blood plasma after consumption of 100g or 250g of whole boiled carrots.**

4.1 **Introduction**

Following on from the previous experiments in which the amount of polyacetylenes in foods was estimated, it is important to consider whether these compounds survive the digestion process intact. Bioavailability is often defined as the amount of a compound (or its metabolites) that reaches the circulation, and depends on stability, digestibility and assimilation (Fernández-García *et al.*, 2012; Carbonell-Capella *et al.*, 2014). Sometimes the term also encompasses bioactivity within the definition: the uptake by tissues, potential metabolism to an active compound, and a physiological response (Carbonell-Capella *et al.*, 2014) but for the purposes of this study, bioavailability refers only to the occurrence of compounds within blood plasma. If the polyacetylene compounds are detectible and quantifiable in the biofluids after consumption, then the concentrations at target sites within the body can be estimated. Polyacetylenes have been seen to affect colon and breast cancer cells (and others) *in vitro* (Zidorn *et al.*, 2005; Sun *et al.*, 2010; Um *et al.*, 2010; Zaini *et al.*, 2012) and carrot consumption has been associated with lower risk of colon, bladder and breast cancers (Franceschi *et al.*, 1998; Zeegers *et al.*, 2001; Sakauchi *et al.*, 2004; Wakai *et al.*, 2004; Boggs *et al.*, 2010) so measuring plasma, urine and stool samples would be of interest to determine if the target cells could reasonably be exposed to polyacetylenes, and the concentrations that are observed adjacent to these tissues.

Liquid chromatography mass spectroscopy (LCMS) has been used in past to identify the polyacetylenes (Haraldsdóttir *et al.*, 2002; Rai *et al.*, 2011) from blood plasma. This method is used because the LCMS allows both quantitation and identification of compounds. It is also much more sensitive than HPLC and can accurately detect compounds at the picogram level as opposed to the microgram to nanogram level in HPLC. The peak areas from the chromatography can quantify the amount of a compound and the MS can identify the peaks. In samples where the compounds of interest may undergo metabolism or breakdown, the MS can help to identify fragments of the parent compound or metabolites generated during digestion. LCMS is used in cluster analysis, whereby blood is analysed after consumption of certain foods and the MS is used to detect increasing levels of metabolites that could be attributed to compounds within the original foods. As described in Chapter 1, the metabolism of polyacetylenes has not been well investigated and it is not known how they are absorbed, metabolised or excreted. LC-MS analysis could be used to identify metabolites as compounds

are changed during transit through the digestive tract based on structural similarity to the parent compound (Bohn *et al.*, 2015).

Bioavailability trials have been conducted in the past with carrot juice (Hansen-Møller *et al.*, 2002; Haraldsdóttir *et al.*, 2002). However, boiled carrots were the most commonly consumed polyacetylene-rich vegetable preparation in a UK population (chapter 2) so it is important to investigate the bioavailability of polyacetylene from this preparation. Whilst polyacetylene bioavailability has not been compared between raw and cooked carrots, β-carotene and lycopene plasma concentrations were observed to be higher after consuming cooked vegetables compared to raw after a chronic feeding period (Stahl and Sies, 1992; Rock *et al.*, 1998; Livny *et al.*, 2003). This would suggest other fat-soluble compounds might also be more bioavailable from cooked than raw vegetables, despite the changes that occur during cooking (chapter 3). Boiled carrots were the chosen preparation for the dietary intervention trial based on results from chapter 1 and 2, it was therefore important to investigate whether polyacetylenes are bioavailable from this preparation of carrots.

Hypothesis: There will be an increase in polyacetylene concentrations in blood plasma compared to baseline levels and the larger 'dose' of carrots will give a significantly higher concentration of polyacetylenes in the blood than the lower dose.

Aims:

1. Extract polyacetylenes from blood plasma and identify them by LCMS.
2. Determine if there a detectible increase in polyacetylene concentrations in the blood plasma after consumption of a 'normal' amount of carrots compared to baseline.
3. Determine if there a detectible increase in polyacetylene concentrations in the blood plasma after consumption of a 'large' amount of carrots (which would indicate they are adequately absorbed during digestion).
4. Determine if there is a 'dose effect', i.e. if there is a significantly higher amount of polyacetylene in the blood plasma after consuming the larger dose of carrots.
5. Determine if 'exposure' to polyacetylenes is higher after consuming a larger dose of carrot i.e. the levels in the blood remain higher for longer.

To investigate this, participants were fed two different doses of carrot, and blood samples were collected over 24 hours. Concentration of polyacetylenes was determined by LCMS to detect changes in polyacetylene concentration in blood over time, and to detect differences between doses of carrot.

Bioactive effects of the polyacetylenes have been seen in *in vitro* experiments but it is not known if these effects are possible in the body as it is not known whether the polyacetylenes are seen in the tissues after carrot consumption. Investigating whether the polyacetylenes are seen in blood plasma is the first step in determining whether they could be having an effect in the human body by demonstrating the absorption of these compounds after consumption.

4.2 **Methods and Materials**

Ethical approval for this trial was sought and obtained from the Newcastle University, Faculty of Science, Agriculture and Engineering committee (approval number: 15-WAR-023, Appendix C) and was conducted with the informed consent of the volunteers. The trial was registered on www.clinicaltrials.gov (NCT02696473).

4.2.1 ***Subjects and Study Design***

Sample size determination:

Power calculations were conducted to determine the sample size needed for this study. Data from a previous human trial using carrot juice (Haraldsdóttir *et al.*, 2002) was used for the calculation.

1. To detect a difference between baseline and low dose carrot, the data for consumption of 300mL of juice was used (containing a similar amount of falcarinol). The following figures were used for the calculation: Mean A: 0.37ng/mL (baseline concentration); Mean B: 1.01ng/mL (peak concentration); Standard Deviation: 0.3; Power: 0.8; Type 1 error rate: 5%. The sample size calculated was n=4.
2. To detect a difference between baseline and high dose carrot, the data for consumption of 600mL juice was used (containing a similar amount of falcarinol). The following figures were used for the calculation: Mean A: 0.29ng/mL (baseline concentration); Mean B: 1.49ng/mL (peak concentration); Standard Deviation: 0.5; Power: 0.8; Type 1 error rate: 5%. The sample size calculated was n=3.
3. To detect a difference between the two doses, the data for 300mL and 600mL peak concentrations were used: Mean A: 1.01ng/mL (peak concentration after 300mL juice); Mean B: 1.49ng/mL (peak concentration after 600mL juice); Standard Deviation: 0.3; Power: 0.8; Type 1 error rate: 5%. The sample size calculated was n=7

Sample size was calculated using an online calculator (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality)

The largest sample size to achieve predicted power was 7 so the trial aimed to recruit 9 participants, allowing for drop outs.

All participants were provided with a participant information sheet and provided written informed consent (Appendix C).

Participants were asked to attend the laboratory on three separate occasions. Before the first testing day, participants attended a screening session and were asked to complete a health and lifestyle questionnaire (Appendix C) to ensure they met the eligibility criteria: aged 18-30 years, BMI 18-30 kg/m^2, no metabolic or gastrointestinal conditions, no food intolerances/allergies; no antibiotic use in the last 3 months which could affect the bacteria in the gut, allergies to carrot or bread, not taking any medication that affects metabolism or digestion, non-smokers. After obtaining informed consent and upon confirming suitability, participants were anonymised prior to enrolment into the study.

4.2.2 *Study protocol*

The second and third visits were the testing days and were separated by at least a week to avoid carry over effect (based on falcarinol levels returning to baseline 12 hours after consumption of large quantities of carrot juice). Three days prior to the testing day, participants were asked to avoid all polyacetylene containing foods (list provided – Appendix C). At the start of each visit, participants arrived fasted (no food or drink except water for 10-12 hours), provided their 24-hour urine samples and a faecal sample from that morning or the evening before. A food diary from 3 days prior to the study was checked to ensure no polyacetylene containing foods had been consumed. They then had a cannula inserted into a vein in their arm and a fasting blood sample was taken into a lithium heparin coated vacutainer (Becton Dickinson, Oxford, UK). In a randomised cross-over design, one of two doses of carrots was served to the participants to eat; doses were either 100g or 250g of boiled carrot. The carrots (Tesco, UK) were served with white bread (Hovis, UK) and 10g butter (Lurpak spreadable, Lurpak, UK) as a source of fat, to ensure absorption of fat soluble compounds, and the whole breakfast was eaten within 15 minutes. Further blood samples were taken at 1, 2, 4, 5, 6, 8 and 24 hours after the start of consumption of the test meal. Urine samples were collected up to 72 hours after carrot consumption and faecal samples were collected from 4 hours, up to 72 hours after consumption, however the urine and faecal samples were not analysed due to time constraints. Plasma was separated from whole blood by centrifugation (1000xg for 10 minutes, 4^{o}C) and stored at -80^{o}C until analysis (<3 months). Figure 4.1 summarises the test protocol.

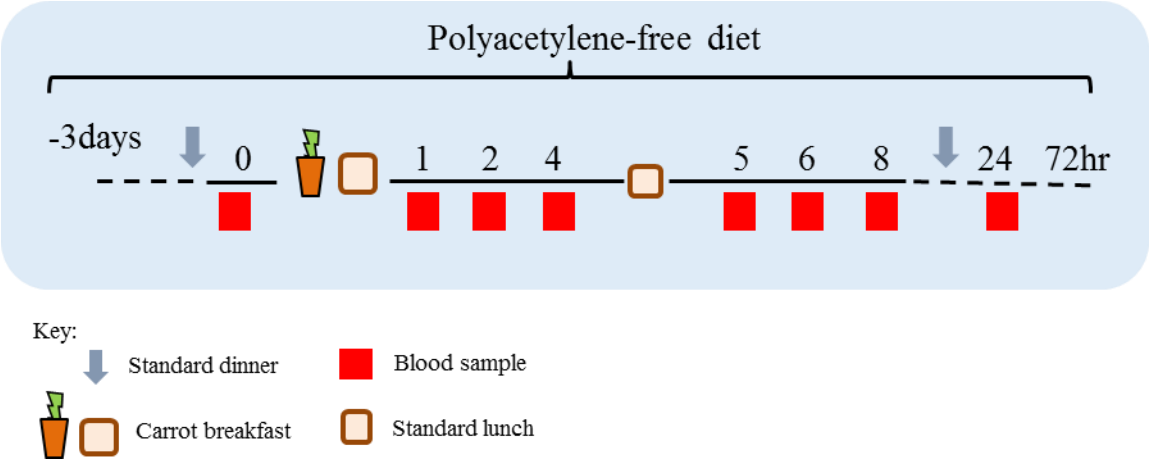


Figure 4.1: Summary timeline of bioavailability study test protocol

4.2.3 *Food preparation*

All carrots were purchased from the same supermarket and prepared fresh on the morning of the test sessions. The carrots were topped and tailed then boiled whole for 10 minutes. They were then drained and weighed after cooking. Participants were provided with a standard dinner (pasta in tomato sauce ready meal and broccoli) to consume the night before the testing visit. A standard lunch (tomato soup, cheese and tomato sandwich (Hovis, UK; Lurpak, UK), cereal bar (Cadbury's, UK), apple), snack (banana), and dinner (cheese pizza with cauliflower) on the day of testing and came back fasted in the morning. All foods were Tesco own brand, except where specified (Tesco, UK) (see section 10.7 for full food list). After the 24-hour fasted blood sample they were free to eat any foods as long as they did not contain polyacetylenes. The standard meals selected did not have any Apiaceae vegetables as ingredients, and were analysed by HPLC to ensure they contained no polyacetylenes. They were also selected to be vegetarian to ensure all participants could eat the same test food. A sample of carrots, cooked at the same time as the test carrots, was immediately frozen for analysis of polyacetylene by HPLC to estimate intake (as chapter 2).

Chemicals:

Ethyl acetate, ethanol, formic acid, and acetonitrile were all of HPLC grade and purchased from Fisher Scientific (Loughborough, UK).

4.2.4 *Polyacetylene standard preparation*

Polyacetylene compounds were extracted from carrot using flash chromatography by Othman Qadir, Newcastle University as part of a separate project. The method used was based on PhD research by Huda Saleh, (unpublished). Freeze dried and ground carrot (1kg) was combined with ethyl acetate (2.5L) and left to extract overnight in the dark at room temperature. The solvent was removed and the extraction of the material was repeated a further 5 times with 500mL ethyl acetate. The solvents were combined and evaporated to dryness. The remaining solid (19g) was combined with dichloromethane to just dissolve the solid (170mL). This extract was analysed by HPLC (same method as chapter 2) to ensure polyacetylenes were present. Separation 1: The polyacetylenes were then separated by automated flash chromatography using Agilent 971-FP UV-VIS system. A hand packed, normal phase, silica gel polypropylene cartridge (SF 65-400g) was loaded with the liquid extract by syringe. The mobile phase consisted of petroleum ether (A) and acetone (B) and was run with the following gradient: time 0: 100% A; 2mins: decrease to 60% A over 1 minute, hold for 5 minutes; 6 minutes: decrease to 20% A over 2 minutes, hold for 2 minutes; 10 minutes: stop. Flow rate was 100mL/min. The chromatographic separation was monitored at 205nm (Søltoft *et al.*, 2010) and 256nm (Kidmose *et al.*, 2004b) and fractions were collected which corresponded to signals that occurred at these wavelengths. The fractions were then tested for falcarinol, falcarindiol and falcarindiol-3-acetate by HPLC. The 50mL fractions from flash chromatography were dried under nitrogen and resuspended in 50mL acetonitrile. HPLC analysis was used to determine which fractions contained the falcarinol, falcarindiol and falcarindiol-3-acetate. The fractions containing the 3 polyacetylenes were combined and evaporated using rotary evaporation. The solid was then resuspended 1:20 in ethyl acetate. Flash chromatography separations were performed with Agilent 971-FP UV-VIS system and the column SuperFlash C18 (22-25g; 28.2 x 164mm, Merck). Injection volume 5mL. A gradient method was used with mobile phase methanol:water. The gradient conditions were as follows: time 0: 50% methanol:water increasing to 100% methanol over 8 minutes; 100% methanol for 7 minutes; stop (15 minutes). Fractions of the separated material were collected when signal was present at 205nm and 256nm. Fractions were then analysed by the same method as in chapter 2 to determine which polyacetylenes were contained in each fraction. The samples derived from this procedure were solutions of falcarindiol (0.57g/mL), falcarinol (0.15g/mL) and falcarindiol-3-acetate (0.47g/mL).

4.2.5 *Detection of polyacetylene standards by LCMS*

The mass spectrometer, API4000 triple quadrupole (Applied Biosystems, Carlsbad, CA), was tuned via infusion to develop a Multiple Reaction Monitoring (MRM) method. The HPLC

method was a gradient method of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) with a flow rate of 0.5mL/min using a Zorbax eclipse plus C18 column (Agilent) 4.6µm x 100mm x 3.5µm (see Table 4.1 for gradient conditions). The injection volume was 10µL. Approximately 1µg/mL of the solutions were used in this method for detection of fragments in the MRM method. Previous publications that had used LCMS to find polyacetylenes were consulted to help target the mass of interest (Hansen-Møller *et al.*, 2002; Rai *et al.*, 2011). Each solution was run on the LCMS method in turn and potential peaks were identified. Probable peaks were identified from these solutions at masses of: falcarinol: 141; falcarindiol: 131; falcarindiol-3-acetate: 117.

4.2.6 *Preparation of blood plasma samples for polyacetylene analysis by LCMS*

First extraction method:

Using the extraction method described by Hansen-Møller *(*Hansen-Møller *et al.*, 2002), 0.5mL plasma was added to 1mL acetonitrile and the proteins were precipitated. The samples were then cenrifuged at 10,000rpm for 10 minutes at 4^{o}C and the acetonitrile removed into amber glass vials for analysis by LCMS. There were no polyacetylenes detected in the resultant extract. The plasma samples were then analysed for β-carotene (using the method described below) and, again, no compounds were detected. This was surprising as carrots should provide a large dose of β-carotene and even baseline levels in the plasma should have been detectible.

Second extraction method:

An alternative method of extraction was then used, based on Oxley *et al*. (2014), previously used to extract β-carotene from blood plasma. Briefly, 10mL ethanol/ethyl acetate (1:1) solvent was applied to 1mL plasma. Samples were placed on an orbital shaker for 10 minutes then centrifuged at 10,000rpm for 30 mins at 4^{o}C. The supernatant was transferred to a clean glass tube and evaporated to dryness under a stream of nitrogen, resuspended in 1mL of acetonitrile/water with 1% formic acid (1:1), vortexed briefly and transferred to amber glass vials for LCMS analysis.

4.2.7 *LCMS method*

Based on Hansen-Møller *et al.* (2002), and with the kind assistance of Phil Berry, Northern Institute for Cancer Research, Newcastle University, the polyacetylenes were separated by chromatography using a Perkin Elmer Series 200 LC (Perkin Elmer, UK) equipped with a

SecurityGuard C18 cartridge pre-column (4x3mm) and Zorbax Eclipse C18 column from (Phenomenex, UK) (100mm x 4.6mm i.d., 3.5µm) (Agilent, UK) maintained at 40°C. This was a reverse phase binary gradient system, performed with 0.1% formic acid in water (A) and acetonitrile (B) mobile phases. Gradient conditions are described in Table 4.1. Flow rate was 0.5ml/min and injection volume was 10µL.

Table 4.1: HPLC gradient conditions for LCMS analysis of polyacetylenes in blood plasma

Time	**0.1% formic acid in water (%)**	**Acetonitrile (%)**
0	90	10
3	5	95
5	5	95
6	90	10
10	90	10

The LC/MS system, API4000 triple quadrupole (Applied Biosystems, Carlsbad, CA), was used for analysis with atmospheric pressure chemical ionization (APCI) performed in positive ion mode using nitrogen gas with the following MS interface settings: collision gas, 6; curtain gas, 20; ion source gas 1, 40; ion source gas 2, 20. Heated nebulizer temperature of 300°C, ion source voltage of 5,500.
The instrument was set to detect compounds at masses of 141, 131 and 117 to detect the probable peaks of falcarinol; falcarindiol; and falcarindiol-3-acetate, respectively. The peak of interest was identified by the retention time (4.6 minutes and 5.9 minutes for falcarindiol-3-acetate and falcarinol respectively). Peak areas were determined from the area under the peak at the retention times in each mass channel (Figure 10.1).

4.2.8 *Polyacetylene plasma concentrations data transformation*

The responses of each individual participant were very different, as demonstrated in Table 4.3. The data was not normally distributed, with some very high peak areas skewing the data, and the resulting means were misleading. The data was therefore natural log (ln) transformed to normalise the data distribution and make the interval between data points consistent. This allowed the use of parametric tests on the resulting data.
The ln peak areas were plotted to explore the amounts in blood plasma at given time points. This data was used to detect differences between baseline and peak values. Figure 4.4 demonstrates the change in ln peak areas from baseline in all participants, showing the range of responses. Two of the volunteers had little or no change in their polyacetylene ln peak area after intake of carrots, or even fell below baseline (participant 2 and 3 in the figure). They

were considered ‘non-responders’ and were excluded from the analysis. Only data from the remaining volunteers (n=4) was used for analysis.

The change from baseline was calculated for each individual by subtracting the baseline polyacetylene ln peak area from the ln peak area at the other time points for each individual. The values for each individual were then averaged. The change from baseline data were used to detect differences between peak areas between the 100 and 250g doses.

4.2.9 *Calculating incremental area under the curve (iAUC)*

Area under the curve was calculated using the trapezoid method, multiplying the time increment (hours) by the change in ln peak area and dividing by 2. Where the ln peak area goes from positive to negative over a time increment, resulting in a negative value, the area was changed to a positive and added to the total area. The increments were calculated for each individual and the mean at each time increment was reported. The cumulative AUC was calculated by adding the means at each increment together over increasing time to estimate total exposure to polyacetylene. The 24-hour sample was not included in the AUC as there were not enough incremental measures in the 16-hour period to accurately report the changes. This method should be able to detect differences in the amounts in blood after the different doses that may not occur acutely at each time point i.e. if the concentration is not as high at one time point in the higher dose compared to the lower dose but higher concentrations are sustained over several hours, this is a longer exposure which will be reflected in the cumulative iAUC.

4.2.10 *Statistics*

The data were analysed using SPSS (version 23, Chicago, IL.) Only the data from the 4 ‘responder’ volunteers were analysed. A Shapiro-Wilk test was used to determine whether the peak areas were normally distributed. As they were not, they were natural log (ln) transformed.

To test if there was a detectible increase in polyacetylene concentration from baseline, ln peak area at baseline was compared to the ln peak area at each time point up to 24hrs after consuming 100g or 250g of carrot, using paired t-tests. To determine if the ln peak area at any time point differed from each other, a repeated measures ANOVA with Tukey post hoc tests were performed.

To determine if there was a difference in the ln peak areas between doses, paired t-tests were performed using the change in ln peak area from baseline to compare means between the 100g and 250g doses.

The cumulative incremental areas under the curve (iAUC) were compared at each time point using paired t-tests between 100g and 250g dose to detect differences in total exposure between the doses.

4.3 **Results**

4.3.1 ***Mass Spec identification of peaks***

The falcarinol peak is a fragment of molecular weight (MW) 141, falcarindiol peak MW 131 and falcarindiol-3-acetate peak MW 117 (examples of LCMS peaks shown in Figure 10.2). The probable structures are shown in Figure 4.2 with the following changes during fragmentation: OH is lost from falcarinol ($C_{11}H_{11}O$) [M-85-OH]+, H_2O is lost from falcarindiol ($C_9H_8O_2$) [M-112-H2O]+, and H_2O is lost and Na+ is gained in falcarindiol-3-acetate ($C_6H_7O_2$) [M-191-H2O+Na]+. Na^+ had been seen to attach to falcarindiol during mass spectrometry (Rai *et al.*, 2011).

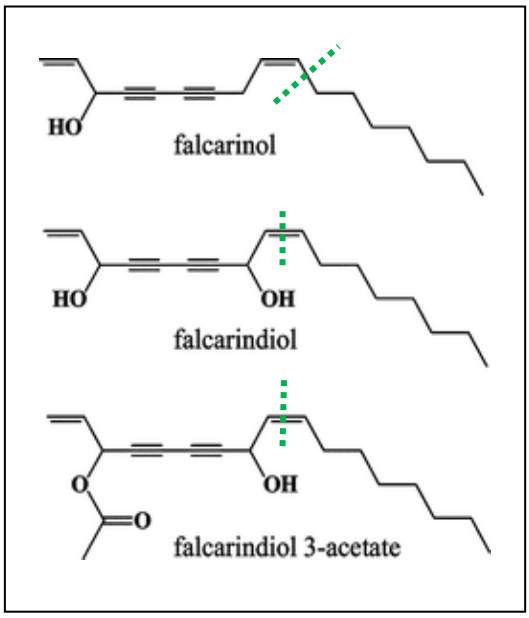


Figure 4.2: Assumed fragmentation of polyacetylenes during LCMS analysis. The dotted line represents where the fragment is assumed to have broken from the parent molecule. The fragment in each case is the structure on the left-hand side of the dotted line.

A separate project to this PhD aimed to produce pure compounds for use in the LCMS method development. The compounds isolated were used to identify probably polyacetylene peaks with the LCMS but they were not of adequate purity to use as standards. It was therefore not possible to accurately quantify the amount of each compound so results are presented as peak areas from the HPLC. The data for the suspected falcarindiol fragment did not respond as expected, with the 100g dose being higher than the 250g dose both in peak area and in AUC. The fragment chosen (MW: 131) may not be the correct compound to represent falcarindiol in the plasma. Without a standard of at least 99% purity, peaks can be wrongly assigned. Previous research into the bioavailability of these compounds have reported that falcarindiol had been detected but MW and actual figures were not presented (Hansen-Møller *et al.*, 2002).

4.3.2 *Study participants*

A total of six healthy adults (5 male, 1 female) were recruited for the study. The subjects were aged between 23 and 33 years old and full demographics can be found in Table 4.2.

Table 4.2: Participant characteristics in bioavailability study.

	Age (yrs)	**Sex (M:F)**	**BMI (kg/m^2)**	**Body fat (%)**
Mean ±SD	26.2±3.9	5:1	22.8±3.5	14.1±4.1

4.3.3 *Polyacetylene consumption*

The estimated average amount of each polyacetylene consumed by participants during the trial is shown in Table 4.3. The amount of falcarinol consumed from 100g dose carrot was less than in previous trials. In the lowest 'dose' of 300mL of carrot juice in the Haraldsdóttir *et al*. trial (2002), 4mg of falcarinol was consumed (concentration of juice was 13µg/mL). The 100g sample of carrots provided 1.72mg falcarinol, which was less than half the amount in carrot juice but it was still detectible in the plasma. The 250g carrot portion contained around the same as the 300mL juice (4.06mg).

Table 4.3: Mean±SD of phytochemical consumed in bioavailability study

	Amount of phytochemical consumed (mg) (mean±SD)		
	Falcarinol	**Falcarindiol**	**Falcarindiol-3-acetate**
100g	1.72 ±0.19	2.17 ±0.62	0.62 ±0.06
250g	4.06 ±0.74	4.79 ±0.47	1.50 ±0.15

4.3.4 *Results of all participants*

The responses of each individual participant, before data transformation are displayed in Figure 4.3. The responses in each individual were very different from each other so the peak areas were natural log transformed as described in the methods.

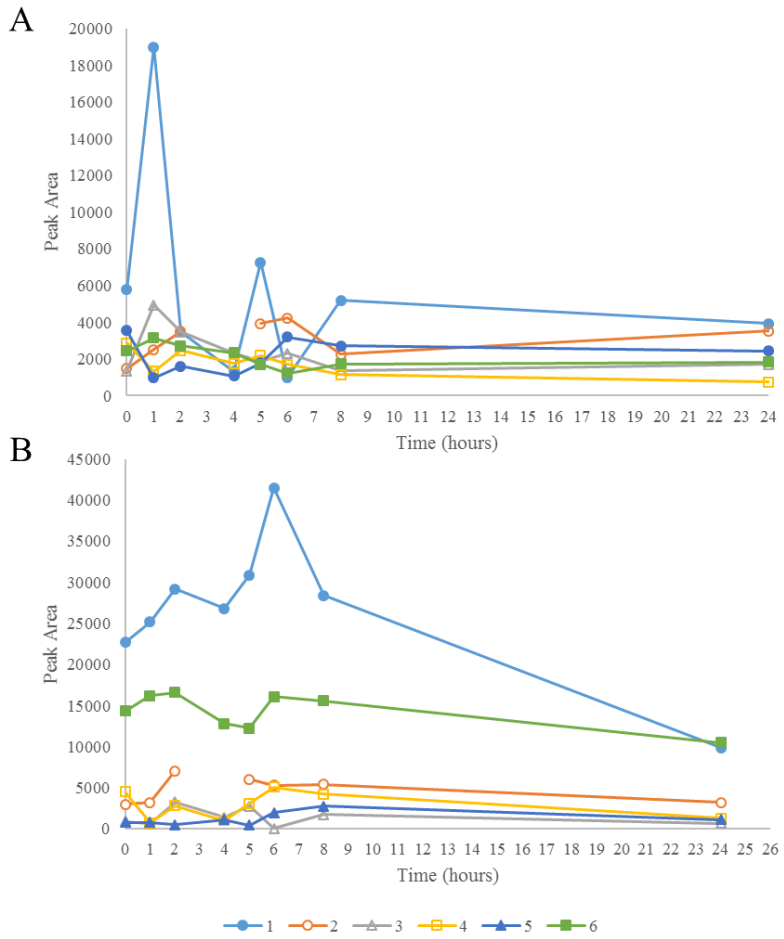


Figure 4.3: Peak areas of falcarinol (A) and falcarindiol-3-acetate (B) in all participants after consuming 100g carrot.

4.3.5 *Individual responses of all participants*

The individual responses (change in ln peak area from baseline) of each participant are shown in Figure 4.4. The results for participants 4 and 5 showed little or no response after eating carrot, with peak areas falling below baseline levels. These two participants were excluded from further analysis. The demographics of the remaining participants are shown in Table 4.4.

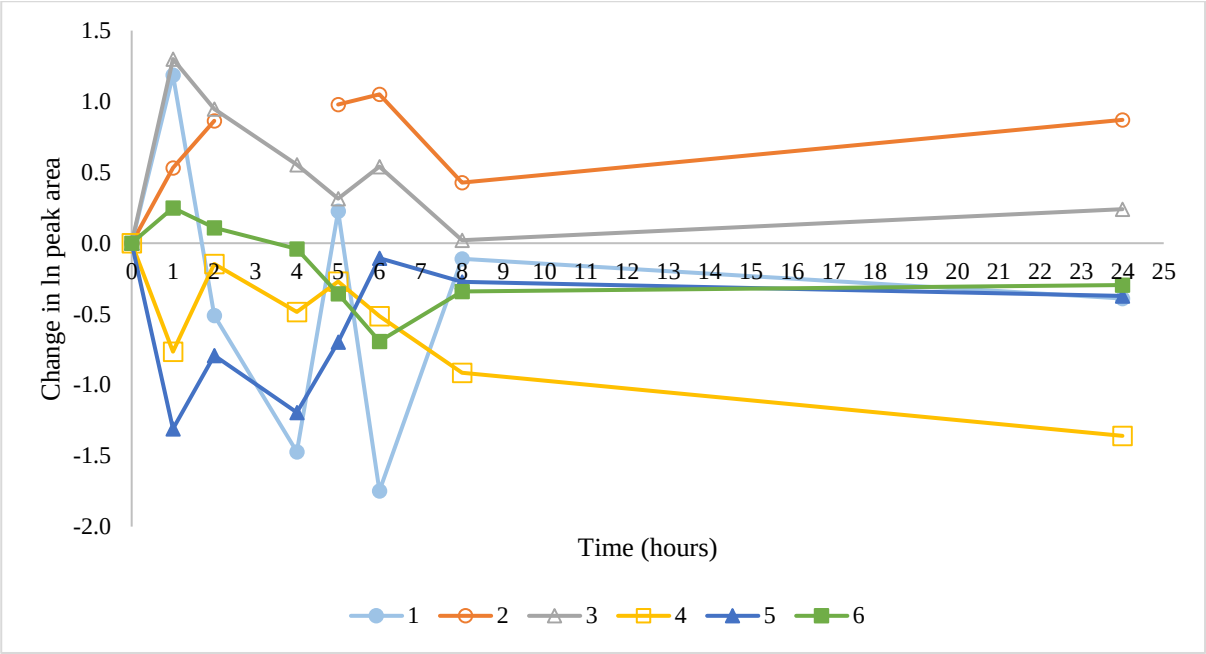


Figure 4.4: Individual response in falcarinol blood plasma peak area after consuming 100g carrot. Participants 4 and 5 were considered 'non-responders' and excluded from further analysis.

Table 4.4: Participant characteristics of the responders only (n=4).

	Age (yrs)	Sex M:F	BMI (Kg/m^2)	Body fat (%)
Mean ±SD	25.0±2.7	3:1	21.9±2.8	12.9±2.2

4.3.1 *Changes in plasma polyacetylene concentration from baseline*

The primary aim of this trial was to investigate whether consumption of carrots could cause a significant increase in polyacetylene plasma concentration from baseline concentrations. Falcarinol and falcarindiol-3-acetate increased to peak concentrations between 1-2hrs after breakfast which then decreased before a further peak was seen after lunch at around 5-6hrs.

To investigate whether a significant change in polyacetylene concentration from baseline could be detected, ln peak areas were used, as shown in Figure 4.5. There were significant increases in ln peak area of falcarinol after consuming 250g (p=0.003) dose of carrot compared to baseline after 1, 6 and 24 hrs (p=0.045, 0.028, 0.035 respectively) but no difference after consuming 100g. For falcarindiol-3-acetate, plasma concentrations were significantly higher than baseline after 1, 2, 5, 8 and 24 hrs after consuming 100g (p=0.003, 0.007, 0,031, 0.004 and 0.025 respectively) and higher than baseline after consuming 250g

after 2, 5, and 6 hours (p=0.016, 0.002 and 0.045 respectively). The after-lunch peaks were seen in both doses in both compounds at 5 hours for falcarindiol-3-acetate in both doses and for falcarinol only at 6 hours in the 250g dose.

Table 4.5: Mean (±SEM) ln peak areas of falcarinol and falcarindiol-3-acetate in plasma over time (n=4).

	Mean FaOH ln peak area				Mean FaD3Ac ln peak area			
Time (hrs)	100g	SEM	250g	SEM	100g	SEM	250g	SEM
0	7.7	±0.3	7.4	±0.2	6.6	±0.6	7.8	±0.3
1	8.6	±0.4	7.9	±0.2*	8.6	±0.7*	7.8	±0.5
2	8.1	±0.0	7.8	±0.2	9.2	±0.4*	8.7	±0.2*
4	7.6	±0.1	7.6	±0.2	6.7	±0.7	7.9	±0.3
5	8.0	±0.3	7.6	±0.2	9.1	±0.4*	8.4	±0.2*
6	7.5	±0.3	7.9	±0.3*	8.3	±1.2	7.4	±0.9*
8	7.7	±0.2	7.2	±0.4	9.0	±0.5*	8.0	±0.5
24	7.8	±0.2	7.6	±0.1*	8.3	±0.5*	7.5	±0.3

FaOH - falcarinol; FaD3Ac - falcarindiol-3-acetate, * denotes significant difference from baseline (p≤0.05).

4.3.2 *Dose response*

The secondary aim of this trial was to detect a dose response. The 250g dose produced higher change in peak areas than the 100g dose at each time point up to 6 hours as shown in Table 4.5, however, they did not differ significantly from each other, and there was only a trend for higher falcarinol at 6 hours (p=0.084) after the 250g dose compared to the 100g dose.

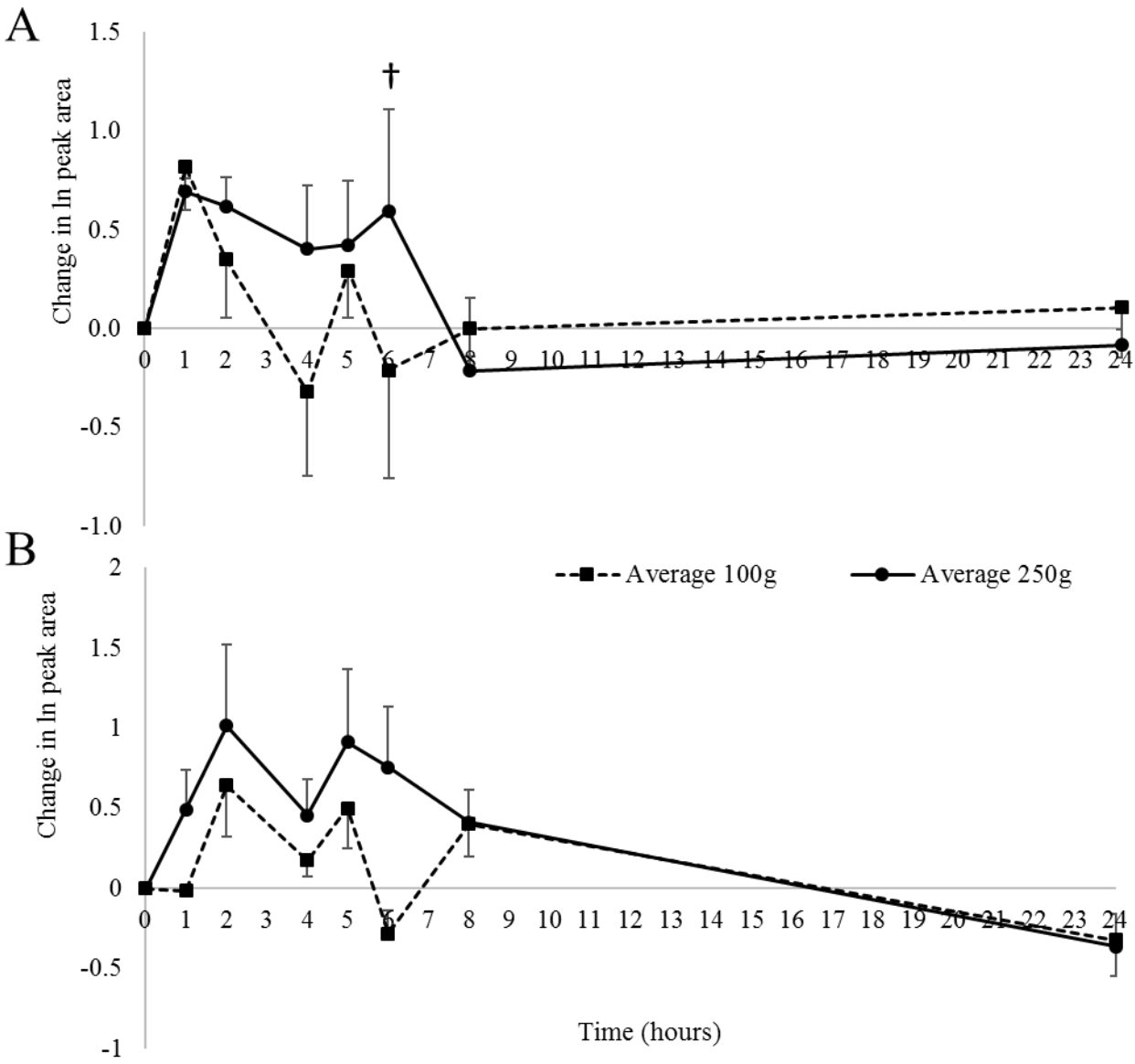


Figure 4.5: Change from baseline in A) falcarinol and B) falcarindiol-3-acetate ln peak area after consuming 100g or 250g carrot for 'responders' only (n=4). Error bars are SEM † signifies p=0.08 trend in difference between 100g and 250g doses.

4.3.3 *Exposure to phytochemicals*

The cumulative incremental area under the curve (iAUC) for the response up to 8 hours is shown in Figure 4.6. The iAUC for the 250g dose was not significantly higher than the iAUC for the 100g dose for falcarinol. For falcarindiol-3-acetate there was a trend for higher iAUC in the 250g dose at 1 hr (p=0.056) and 5 hours (p=0.096). The total iAUC was 1.3 and 2.1 times higher in the 250g dose compared with the 100g dose in falcarinol and falcarindiol-3-acetate, respectively, after 8 hours.

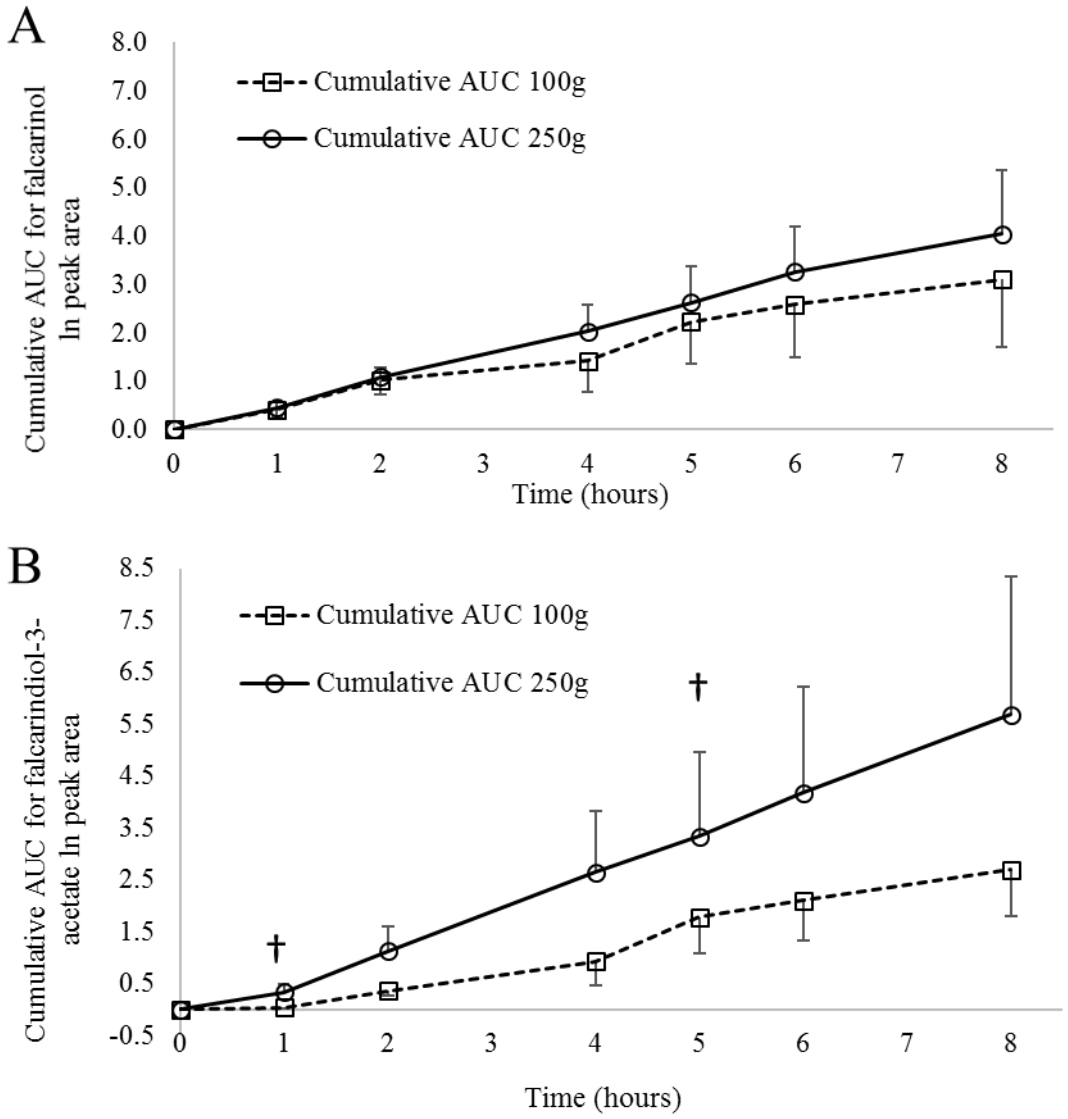


Figure 4.6: Cumulative ln AUC for falcarinol (A) and falcarindiol-3-acetate (B) ln peak area. † signifies trend $p<0.08$. Error bars are SEM.

4.4 **Discussion**

These results show that polyacetylenes (falcarinol and falcarindiol-3-acetate) are detectible in the blood after consumption of boiled carrot. To the knowledge of the author, this is the first trial that has detected falcarinol and falcarindiol-3-acetate in blood plasma after consumption of whole carrot. Without a standard of adequate purity, it has not been possible to quantify the concentration of these compounds but it is promising for the potential biological effects of the polyacetylenes that these compounds are detectible *in vivo*.

Bioavailability studies are notoriously difficult to conduct, with individual differences in digestion efficiency, gut microflora and absorptive ability all affecting the appearance of food components in the blood. The responses between participants were very different in this trial,

but large variations in responses have also been seen in previous trials with β-carotene. In a trial investigating a single dose of carrots (270g) or a β-carotene supplement, there was a 3-4 fold difference in absorption efficiency (Brown *et al.*, 1989) and a trial investigating β-carotene supplements saw up to 8-fold difference between participants (Törrönen *et al.*, 1996), some of whom had no plasma response to the supplementation. These "non-responders" are often seen in trials of carotenoid bioavailability where no apparent increase in plasma concentration occurs. A trial looking at β-carotene absorption from carrots in a soup mixture saw that as little as 50% of the participants were β-carotene responders (Sánchez-Campillo *et al.*, 2012) and a trial using cooked and fresh tomatoes, β carotene and lycopene plasma concentrations did not significantly rise after ingestion of both test meals (Bugianesi *et al.*, 2004). However, the appearance of β-carotene in the blood may not simply rely on absorption efficiency but also stability in the GI tract, the rate of metabolism and rate of tissue uptake. In an *in vitro* digestion simulation, 45% of β-carotene was degraded during gastric digestion phase, thought to be due to instability of the compounds in an acidic environment (Courraud *et al.*, 2013). Beta-carotene is taken up and stored in the liver, or metabolised to retinoic acid, the active form of the molecule, therefore it can be rapidly removed from the blood. The differences in the efficiency of uptake and metabolism in individuals (Lietz *et al.*, 2012; Oxley *et al.*, 2015) can affect the plasma concentration to the extent that there is no apparent rise in plasma concentrations when the compounds are rapidly metabolised (Bugianesi *et al.*, 2004). It is therefore reasonable to expect large inter-individual differences in plasma concentrations. It is not known how the conditions inside the GI tract affect the stability of polyacetylenes, nor how they are metabolised either in the gut or once they reach the circulation, or whether they are stored in tissues. Further work, initially with *in vitro* models, is needed in this area.

As the trial aimed to investigate whether consumption of normal portion sizes of carrot could affect the plasma concentration of polyacetylenes, 100g was chosen as the low 'dose', as it was close to the recommendation of one portion (80g) in the 5-a-day recommendations (NHS, 2015). Also, keeping the dietary intervention trial in mind, it was important to choose a portion size that would not be a burden to participants in the longer dietary intervention trial. It was decided that 250g was the highest manageable amount of carrot to consume in one sitting (within 15 minutes) without experiencing GI distress (as tested by the researcher). The results show that there may not be an advantage of consuming larger doses than 100g on falcarinol blood plasma concentrations as there was no difference in the AUC of falcarinol between 100g and 250g doses, a measure of exposure to the compounds over time. However, there was a trend for higher AUC for falcarindiol-3-acetate at some time points. The lack of a

difference between ‘doses’ is consistent with studies of carotenoids which shows large differences in dose are needed to see a difference in plasma concentrations, even from baseline values (Yeum and Russell, 2002). In a paper exploring bioavailability of β-carotene and lycopene from soup, the soup with a higher concentration of carotenoids did not increase AUC for β-carotene (Sánchez-Campillo *et al.*, 2012).

It is not known how the polyacetylenes are absorbed, but as they are quite hydrophobic, it was assumed for the purposes of this trial that they are micellized, like the carotenoids. However, polyacetylenes are not as hydrophobic as the carotenoids so it could be possible for a proportion of them to diffuse directly into the blood plasma. Even in *in vitro* models of digestion, the amount of β-carotene micellized, and thus available for absorption, is a very small percentage of the amount available. The amount of β-carotene from carrot juice (Courraud *et al.*, 2013), mixed salad (Huo *et al.*, 2007), carrot puree (Reboul *et al.*, 2006), raw pulped carrot (Hedren *et al.*, 2002), and raw carrot (Jeffery *et al.*, 2012) were 1.8, 2.8, 8.9, 21, and 21.6% micellized during *in vitro* digestion respectively, highlighting that even in controlled models of digestion bioaccessibility of compounds can be very low. The body may also only be able to process and absorb a certain amount of a compound at any one time before becoming saturated. Carotenoids are thought to be passively absorbed (Yeum and Russell, 2002) once solubilized in fat (Yonekura and Nagao, 2007) so the amount of absorption may depend on the amount or type of fat present in digestion. In this trial, 10g of fat was served with both portions of carrot so this would equate to 10% of the 100g dose and 4% of the 250g dose. Studies of β-carotene have shown that a relatively small amount of fat (3-5g) is adequate to absorb maximum concentrations of the compound (Fernández-García *et al.*, 2012). Intake of larger amounts of fat than 5g did not increase plasma concentrations. In an *in vitro* digestion trial, no improvement in micellization of β-carotene was seen between 5 and 10% oil added to a cooked carrot digest (Hornero-Méndez and Mínguez-Mosquera, 2007). Another trial saw β-carotene micellization as half that of lutein with lower amounts of fat (2.1-3.5%) but at 10% fat the micellization was equivalent between the two (Yonekura and Nagao, 2007). In a human trial where participants were fed a fat spread supplemented with α- and β-carotene (8mg) and lutein ester (8mg), there was no improvement in plasma β-carotene status after 7 days when meals contained 3g compared to 36g fat. However, plasma lutein did increase with the additional fat, possibly due to needing fat in the diet to stimulate the release of esterases, enzymes needed to hydrolyse the ester and release lutein, which are only secreted in the presence of dietary fat (Roodenburg *et al.*, 2000). Differences in carotenoid uptake have also been seen in the type of fat used. An *in vitro* trial using different types of oil found that β-carotene micellization and uptake into Caco-2 cells was enhanced with increasing unsaturated

fatty acid content of the fat source. Lutein and zeaxanthin were not significantly affected however (Failla *et al.*, 2014), possibly due to polarity. And a further study found lutein and zeaxanthin bioaccessibility was 20% higher in the presence of butter than olive and fish oils *in vitro*. This corresponded with higher plasma lutein concentrations in rats fed spinach for 3 days with butter compared to the two oils (Gleize *et al.*, 2013). In the *in vitro* trial, lutein appeared to move easily between the food matrix and micelles for absorption, possibly due to being less polar (Yonekura and Nagao, 2007). The polyacetylenes and lutein are less lipophilic than β-carotene so it could be that the polyacetylenes respond in the same way as lutein, easily micellizing, and using a fat source with more saturated fatty acid, like butter, should not affect, or may even enhance, the transportation of polyacetylenes compared with a more unsaturated fatty acid. In everyday situations, consumers would most likely consume carrots as part of a meal. The reference intake for fat in the UK is 20% of calories in the diet (NHS, 2014), so most meals would have a source of fat from the other food items that could aid absorption and it should not be necessary to add extra fat to carrots get the full benefit of these compounds.

The after-lunch peak observed in this trial is also seen in studies of β-carotene bioavailability and has been attributed to additional fat from the lunchtime meal solubilising the β-carotene remaining in the gut, allowing it to be transported into the blood stream (Sánchez-Campillo *et al.*, 2012). This makes fat intake important, not only at the time of eating fat soluble compounds, but also at further meal times after the original intake.

Another factor that affects bioavailability of carotenoids is the presence of fibre. The 250g dose of carrots contained more phytochemicals but also more fibre than the 100g dose. It is thought that the presence of fibre would affect the accessibility compared with juice, however due to not being able to quantify the amount of polyacetylenes in the plasma, it is not possible to directly compare the plasma concentrations of this trial to those seen in the Haraldsdóttir *et al.* (2002) trial, which would give an indication of the effect of additional fibre from whole carrots compared with juice. Phytochemicals have been seen to be less accessible from fibre-rich vegetables as they can be bound within the fibre of the cell-matrix (van het Hof *et al.*, 2000). Adding extra fibre into a food has also been shown to reduce bioavailability when the same source of available carotenoids is present. Carrot fibre added into a muffin containing tomato paste increased plasma lycopene less (35% increase) than a muffin containing tomato paste alone (45% increase) (Horvitz *et al.*, 2004) and purified carotenoid supplements eaten with mixed meals containing different amounts of dietary fibre reduced absorption by 40-74 % compared with no fibre. The reduction of absorption was especially pronounced in the presence of soluble fibres (Riedl *et al.*, 1999). Soluble fibres have been shown to bind to bile

acids which are released in the presence of fat during digestion, creating a fat emulsion that enables fat to be absorbed in the intestine. Soluble fibres have a gel-like consistency which bind to bile acids and they can no longer be re-absorbed in the intestines so will pass through and be excreted (Rock and Swendseid, 1992), along with any fat soluble dietary components. High amounts of fibre have also been shown to delay gastric emptying (Benini *et al.*, 1995) which could explain why the after-breakfast falcarinol peak was slightly lower after the 250g dose of carrots than 100g, and concentrations remained high for longer between breakfast and lunch after 250g of carrot.

Despite the negative effects of fibre on bioavailability of some compounds, the bioavailability of the polyphenol quercetin is almost 2.8 times higher when soluble fibre (a source of easily accessible energy for gut bacteria) is present compared with insoluble fibre. Gut bacteria are needed to cleave a sugar group from polyphenols before they can be absorbed through the intestine, so as the microflora become more active in the presence of fermentable fibre, the bioavailability of polyphenols increases (Tamura *et al.*, 2007). Bacterial digestion in the large intestine could be breaking down fibre, releasing polyacetylenes from the cellular matrix and making them available for absorption with the fat from the lunchtime meal, contributing to the after-lunch peak. However, it is not known how gut microflora affect the digestion of polyacetylenes and this would need further work.

While there does not appear to be an effect of consuming large doses of carrot in one sitting to increase plasma concentrations of falcarinol, if the polyacetylenes are not fully absorbed and some remained in the digestive tract, this could have an impact on the health of the intestine. If polyacetylenes survive digestion and pass through the gut, they could potentially expose the intestinal cells to concentrations of polyacetylene that have been seen to kill colon cancer cells *in vitro* (Zidorn *et al.*, 2005; Um *et al.*, 2010). However, there was not enough time in the project to develop a method for the extraction and LCMS analysis of faecal samples from this experiment so the fate of these compounds in the GI tract is not known.

The peak area for falcarindiol-3-acetate was much higher (range: 4468-11675) than that for falcarinol (range: 1775-5317) which was surprising given that the concentration of falcarindiol-3-acetate in the carrot is 2.5x lower than falcarinol. This could result under several circumstances: if falcarinol is not as bioavailable as, or less stable than falcarindiol-3-acetate, if falcarindiol-3-acetate is synthesized during digestion, or if falcarinol is quickly metabolised and converted to another compound during digestion. Falcarinol is a precursor to both falcarindiol and falcarindiol-3-acetate and can be easily converted (Hansen and Boll, 1986; Kidmose *et al.*, 2004a; Rawson *et al.*, 2012a) so it is possible that falcarinol is being

converted to falcarindiol-3-acetate during digestion, which could be why the peak concentrations are so much higher. However, in the carrot, it is usually in the presence of an enzyme that this conversion takes place (Kidmose *et al.*, 2004a) and as the carrots were boiled before being consumed, that particular enzyme is unlikely to still be active unless additional enzymes of bacterial origin in the gut can be used for this conversion. To determine whether the falcarindiol-3-acetate is being made from other compounds, other methods such as isotope labelling of the suspected parent compound could be employed. Further analysis of faecal samples from this experiment could also identify metabolite concentrations and enzyme levels.

The potential for falcarindiol-3-acetate to have health promoting effects has been not been well investigated. One study looked at the cytotoxicity of falcarindiol-3-acetate in CCRF-CEM, MOLT-3 (lymphoblastic leukaemia) and Jurkat (peripheral blood T cell leukaemia) cells which showed that the compound had cytotoxic properties but at higher IC_{50} than falcarinol and falcarindiol for the same cells. However, the AC_{50} was lower for falcarindiol-3-acetate than for the other 2 compounds in leukemia cell lines CCRF-CEM and MOLT-3 (Zaini *et al.*, 2012). Further studies are needed to elucidate the effects of this compound to determine if there is a benefit of consuming more falcarindiol-3-acetate. This is especially important if falcarindiol-3-acetate is the major compound in the blood plasma, and created from falcarinol during digestion. Knowing the concentrations of both compounds would enable the comparison to plasma falcarinol concentrations in the literature but without a pure enough standard this was not possible, further work with an adequate standard would be of benefit.

Falcarinol has been seen to be bioavailable in previous trials from carrot juice. As a preparation, carrot juice is quick and easy to administer, enabling large doses of compounds to be consumed without having the burden of chewing large portions of fibrous vegetable, feeling excessively full, or having other plant components interfering with the digestion of compounds. For example, 250g carrot in this trial contained the same amount of falcarinol as 300mL juice but the juice would be much easier to consume. However, juice has had the insoluble dietary fibre removed so is not considered to have the full health benefits of whole carrots. The effect of juicing on the polyacetylenes is also not known. The juice in the Haraldsdóttir *et al*. (2002) trial, which was freshly prepared, had similar amounts of falcarinol per mL as the carrots had per g (1.3mg/100mL and 1.7mg/100g), but the polyacetylene content of commercially produced juice is unknown. From the previous chapters, it can be seen that the effects of processing are damaging to polyacetylenes and therefore the commercial juicing process may lead to decreases in polyacetylenes. In chapter 3 it was

observed that falcarinol and falcarindiol-3-acetate in cooking water decreased over cooking time, so are thought to be unstable in the aqueous environment, especially at high temperatures. Carrots are typically blanched and/or pasteurised before juicing and further heated during the canning process, conditions which may lead to a decrease in polyacetylene content. This has been seen with β-carotene, which decreased by around 30% during high heat exposure in the canning of carrot juice (Kim and Gerber, 1988; Chen *et al.*, 1995). However, in a trial comparing 6 weeks supplementation of carrot in the diet, plasma β-carotene was actually 20% higher from juice than from raw carrots after 6 weeks of supplementation, but this difference was not significant (Törrönen *et al.*, 1996). As juicing removes insoluble fibre, the benefits of fibre on gut health will also be lost. Consuming carrot juice in preference to whole food is therefore not recommended in everyday situations and, despite the difference in matrix, results from this trial demonstrate that polyacetylenes can be detected in the blood plasma from cooked carrots.

The detection of polyacetylenes in the blood plasma demonstrates that they are available to have a potential biological effect. However, after appearing in the plasma, their fate in the body is not known. The small number of bioavailability and pharmacokinetic investigations conducted previously suggest that the polyacetylenes are rapidly removed from blood plasma. Falcarinol was shown to return to baseline concentrations over 8 hours in humans (Haraldsdóttir *et al.*, 2002) and panaxytriol, a related polyacetylene, had a mean serum α-half-life (initial clearance from blood into tissue) of 5 minutes in the plasma and β-half-life (elimination due to metabolism and excretion) half-life of 51.1 minutes (Saita *et al.*, 1994). However, in this study, even at baseline the falcarinol and falcarindiol-3-acetate were detectible. Polyacetylenes could be taken up into tissues and stored (as they are fat soluble), rapidly metabolised to another compound, or cleared from the body. An *in vitro* trial using MK-1 tumour cells showed that the closely related panaxytriol was rapidly taken up into cells from the cell culture medium reducing from 1µg/mL to 0.53µg/mL after 1 minute. It did not decrease further, possibly because the same trial showed MK-1 cytotoxicity at 0.07µg/mL (Saita *et al.*, 1993). It is not known if they reach the tissues of interest, affecting those cell types that have been seen to react to polyacetylenes in *in vitro* studies. Unfortunately, due to lack of time, the faecal and urine samples collected in this study were not analysed for polyacetylene content so it is not known if the polyacetylenes remain in the gut or enter the bladder and expose the cells to biologically relevant amounts. Further work with labelled polyacetylene molecules could help to investigate the fate of the compounds after blood plasma.

4.5 **Limitations**

A cohort of young adults 18-33 years was chosen for this trial as digestive action and ability to absorb nutrients declines with age (Russell, 2001). Whilst this may not reflect the bioavailability in the general population, the study was designed to give the best chance of being able to detect these compounds. Even so, the individual responses were very different between participants.

There was an unequal distribution of males and females in the trial, with 5 males and 1 female. Not enough is known about the digestive processes of the polyacetylenes to know if there would be an effect of sex on digestion.

4.6 **Conclusions**

The results from this human trial have shown that polyacetylenes (falcarinol and falcarindiol-3-acetate) are detectible in the blood after consumption of a manageable amount (100g) of carrot. Without a standard of adequate purity, it was not possible to quantify the concentration of these compounds but it is promising for the potential biological effects of the polyacetylenes that these compounds are detectible *in vivo*. Further research into distribution, metabolism and elimination will provide detail on metabolic actions of the polyacetylenes and allow further research into polyacetylenes (and carrots as a food source) for their potential beneficial actions in the human body.

Chapter 5. **Randomised dietary intervention trial to determine the effects of polyacetylene-rich vegetables on markers of DNA damage and chronic inflammation**

5.1 **Introduction**

Carcinogenesis takes a long time to develop, it is a multi-step process comprising initiation, promotion and progression. Results from epidemiological and pathological studies suggest that dietary components can reduce the risk of cancer (World Cancer Research Fund/American Institute for Cancer Research, 2007). These chemo-preventative components could target the initiation, promotion or progression stage of carcinogenesis, or combinations of these. They can do this by limiting the exposure of cells to carcinogenic substances, increasing their detoxification and removal, or by affecting the signalling pathways that lead to oxidative stress (Klaunig and Kamendulis, 2004), inflammation (Karin, 2006), and cellular proliferation. Fruits and vegetables are rich sources of compounds such as polyphenols, isothiocyanates (ITCs) and carotenoids, which potentially modulate the signalling pathways and therefore the endpoints of the pathway (Wang *et al.*, 2012). These 'biomarkers' are specific cells, hormones or enzymes from biological fluids, and can be used to predict the risk for certain diseases.

Common biomarkers for predicting cancer risk include the lymphocytes, isolated from blood, analysed for DNA damage using the 'comet assay' (single cell alkaline gel electrophoresis), and inflammatory markers (Mayne, 2003. The comet assay is a widely used measure of exposure to genotoxic events/environments and the resulting DNA damage can be quantitated when compared to a control (EFSA NDA Panel, 2017). Lymphocytes can be used as a surrogate tissue to predict the effects occurring in target tissues that are inaccessible or would require more invasive biopsy procedures to obtain samples. These cells are easily accessible from the blood and occur throughout the body so offer a less invasive method by which to measure the systemic exposure to insults (Collins, 2004). They have been used to measure the effect of consumption of foods or food components on cancer risk in previous studies including watercress (Gill *et al.*, 2007), green tea polyphenols (Morley *et al.*, 2005; Bakuradze *et al.*, 2015; Choi *et al.*, 2015), coffee (Bakuradze *et al.*, 2015), and carotenoids (Collins *et al.*, 1998). The use of lymphocyte DNA damage in epidemiology for predicting cancer risk has been considered (Berwick and Vineis, 2000), and elevated lymphocyte DNA damage has been observed in individuals exposed to occupational and environmental carcinogens such as air pollution, smoking and UV light (Møller, 2006). However, there are

not enough studies available for comparison to definitively use the simple comet assay in biomonitoring for cancer risk. Case-controlled studies have shown that the cases have higher lymphocyte DNA damage than controls but these studies do not separate the cause effect and the tumour environment may suppress DNA repair ability. Prospective studies may be more informative on the use of lymphocytes on biomonitoring but it will take time before enough data is available from biobank samples for prediction of risk using this technique (Ladeira and Smajdova, 2017).

Several studies have shown that carrot consumption can affect the risk of cancer. Epidemiological studies have observed that carrot intake has an inverse association with certain cancers (Galeone *et al.*, 2007; Boggs *et al.*, 2010; Liu *et al.*, 2012), and rodents predisposed to colon cancer that are fed a high carrot diet have a reduced incidence of tumours (Kobaek-Larsen *et al.*, 2005; Saleh *et al.*, 2013; Garti, 2016; Kobaek-Larsen *et al.*, 2017). While these anti-cancer and anti-inflammatory actions were originally thought to be due to the high β-carotene content of carrots, supplement studies showed that increasing β-carotene has little effect on risk of cancer and even increases the risk of cancer under certain circumstances (Omenn *et al.*, 1996; Bjelakovic *et al.*, 2014). Investigations into the polyacetylenes, another bioactive group of compounds in the carrot, have shown promising anti-inflammatory and cancer cytotoxic actions *in vitro* and in rodents *in vivo* (as discussed in chapter 1). Despite these widely documented *in vitro* effects, few studies have investigated the effect of polyacetylenes on humans *in vivo* so it is unknown whether the biological actions seen in isolated cells and animal studies will also be seen in humans. As boiled carrots are the source of the majority of polyacetylenes in a UK population (from Chapter 2), it would be sensible to investigate whether this food source can affect the biomarkers of disease.

To exclude the confounding effect of β-carotene, it is possible to take advantage of the many different coloured carrots and use a white variety that does not contain β-carotene. The polyacetylenes are present in all varieties of carrot previously analysed, regardless of colour (Metzger and Barnes, 2009). The other studies in this thesis used orange carrot as the basis for the experiment as this is the most common colour of carrot in the UK. The results of the studies were intended to be as generalizable as possible to UK eating habits, especially the database study. Whilst the polyacetylenes are present in all varieties of carrot, the amount is different between different varieties and therefore different colours of carrot (Metzger and Barnes, 2009), so the cooking and bioavailability experiments in chapter 3 and 4 were performed with the widely available orange varieties. Using white carrots for this study

allows us to exclude the confounding effects of the β carotene while investigating the effect of carrot consumption on biomarkers.

The aim of this research was to determine if consuming a moderate portion of white carrots every day for 6 weeks could affect biomarkers of inflammation and DNA damage compared to consuming fibre-matched oatcakes in the same period. It also aimed to determine whether carrot consumption can affect the biomarkers compared to baseline, and a washout period where no Apiaceae vegetables were eaten. The researcher hypothesises that there will be reductions in these biomarkers after consuming carrot compared to consuming oatcakes, and compared to baseline and washout periods where carrots are consumed less frequently or not at all. To investigate this, biomarkers of inflammation were chosen that have been seen to be affected in *in vitro* studies when investigating polyacetylenes and inflammation (discussed in detail in Chapter 1). Basal lymphocyte DNA damage, a biomarker of overall DNA damage, was analysed by comet assay. A 6-week dietary intervention trial was then conducted, supplementing a normal diet with either carrots or oatcakes, followed by a 6-week washout where neither Apiaceous vegetables nor oats were consumed. Blood plasma (and urine and faeces) was collected to observe changes in biomarkers selected.

The study was designed to investigate whether carrot consumption, without β-carotene, can have specific health benefits which will give an indication whether other constituents of the carrot are likely to be mediating effects on health.

5.2 **Methods and Materials**

Ethical approval for this trial was sought and obtained from the Newcastle University, Faculty of Science, Agriculture and Engineering ethics committee (approval number: 15-WAR-092, see Appendix D) and with the informed consent of the volunteers (see Appendix D for consent form). The trial was registered on www.clinicaltrials.gov (NCT02696811).

5.2.1 ***Sample size***

The sample size was calculated based on a study which had a similar study design (parallel trial) with a different vegetable (cruciferous sprouts) but looking at H_2O_2-induced oxidised DNA damage rather than DNA single strand breaks (SSB) as this study intended (Gill *et al.*, 2004). The figures used were for the change in H_2O_2 -induced oxidative DNA damage in lymphocytes before and after the intervention. Change in the mean of 13.82 and standard deviation of 18.03 were entered into an online power calculator for parallel trials

(http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html). Using 0.05 as the significance level and a power of 0.8, the result gave a participant number of 56 (28 per group). This is how many participants the study aimed to recruit.

This was a single-blind, randomised parallel trial involving the consumption of either a portion of white carrots (intervention) with 10g of butter or oat cake biscuits (control), which were matched for fibre to carrots served with butter (see Table 5.1). Blood, faecal and urine samples were taken at the beginning and end of the 6-week intervention or control period and again after a 6-week wash-out period to see the effect on inflammatory and DNA damage markers in these biofluids.

Participants were recruited via posters in the university, public places such as libraries, cafes and metro stations. Letters or emails were sent to the members of the HNRC (Human Nutrition Research Centre), Newcastle University, mailing list. Targeted emails were also sent to the gatekeepers of the mailing lists of organisations with a typical demographic over 40 years old such as the Women's Institute and Voice North. Interested volunteers were sent a participant information sheet with the details of the trial (Appendix D) and if they were still interested in taking part, attended a screening session where it was determined whether they were eligible based on the inclusion and exclusion criteria. Inclusion Criteria: healthy adults, aged over 45 years, BMI between 18.5 and 30 kg/m^2, males and females. Exclusion Criteria: Metabolic, malignant or gastrointestinal conditions, allergies to carrot or oats, taking any medication that affects metabolism or digestion (including antibiotics in the last 3 months), smokers, taking aspirin or aspirin-like drugs, common cold or other inflammatory illness at the time of bio-sample collection. Anthropometric measurements (height, weight, BMI, body fat) were taken and they filled out a Health and Lifestyle Questionnaire (as section 10.5) to ensure they met the eligibility criteria. After obtaining informed consent, the participant was anonymised and randomised to either the control (oatcakes) or intervention (carrots) at the time of blood sample collection.

5.2.2 *Dietary intervention trial*

In the week before the dietary intervention, volunteers were asked to complete a 3-day food diary, recording everything they ate and drank for 3 days (2 week days and 1 weekend day). For the 24 hours prior to the start of the study, participants collected their urine into timed containers, recording the volume of urine each time, and a stool sample. At the start of the dietary intervention, participants gave fasted venous blood samples (10mL into a serum tube,

6mL into a lithium heparin tube and 20mL into EDTA tubes) and a finger prick sample onto a protein saver card (Whatman, UK). They were then given either white carrots and butter, or oat biscuits to take home. If they had been randomised to receive the carrots, they were instructed to consume a 100g portion (cooked weight) of vegetable every day. Specific cooking instructions were given to the participants: carrots were topped, tailed and peeled but kept whole and not cut in any further way. They were put into boiling water and, once the water had returned to the boil, cooked for exactly 10 minutes. Cooked carrot was weighed to ensure approx. 100g were consumed. More or less could be eaten in one day as long as the weight was compensated for the next day so an average of 100g per day was eaten over the course of the intervention. Participants randomised to the oats group were instructed to eat 3 oatcakes a day (commercially available oat biscuits, Nairn's UK). Compliance was recorded on a 'test food recording sheet' where participants were asked to record the time they ate the portion of vegetables or oat cakes each day. A comparison of test foods is shown in Table 5.1. The oatcakes were chosen as a control food as they were well matched for fibre. The carbohydrate in the oat biscuits is double that in the carrots and had 25% more energy overall, however 37 Kcal over 24 hours is negligible and the oatcakes were chosen as other fibre-rich vegetables may have other biomarker affecting phytochemicals that would make them inappropriate as a control.

Table 5.1**:** Comparison of nutritional components of carrots with butter and oatcakes.

	100g carrot and 10g butter[1]	3 x Oatcakes[2]
Energy (Kcal)	35.0+72.0=107	144
Fat (g)	0.18+8.11=8.29	5.7
Carbohydrate (g)	8.22+0.01=8.23	19.8
Total fibre (g)	2.8+0.0=2.8	2.7

[1]From McCance and Widdowson database of foods (McCance and Widdowson, 2002) and [2]Nairn's, UK.

Participants were not allowed to consume any polyacetylene containing foods or drinks, or oats, except those provided by the researchers, during the intervention period. The list of excluded foods comprised: carrots, parsnips, celery, celeriac, fennel bulb, parsley leaf, coriander leaf, ginseng and oats (see full list in Appendix D). They were also asked not to consume any vitamin or mineral supplements. 3-day food diaries were collected at week 3 and 6 of the intervention to test compliance and determine any major changes in normal dietary habits during the intervention. Compliance of consumption of the test foods was also monitored by asking the participants to record whether the supplementary foods had been

eaten, time of day they were eaten and if there were any left overs. The participants collected 24-hour urine samples and a stool sample the day prior to giving venous and finger prick blood samples in week 7 of the intervention. From week 7 the participants were in the 6-week 'wash-out' period where they no longer had any supplementary foods in the diet but continued to avoid polyacetylene containing foods and oats. They completed further food diaries in week 9 and 12 and again collected urine and stool samples the day before returning to have the final fasted blood sample taken in week 13. The washout period was used to create a 'true' baseline, i.e. what we would observe if no polyacetylene containing foods and no oats were present in the diet. For the oatcake group the effect of consuming no polyacetylenes was being measured at the end of the 6-week intervention period as they were required to avoid polyacetylene containing foods in this arm of the trial. As the participants were unaware which group was the 'intervention' and which was the 'control', all of the participants were asked to follow the same procedure and complete the 6-week washout. See Figure 5.1 for a summary of the trial.

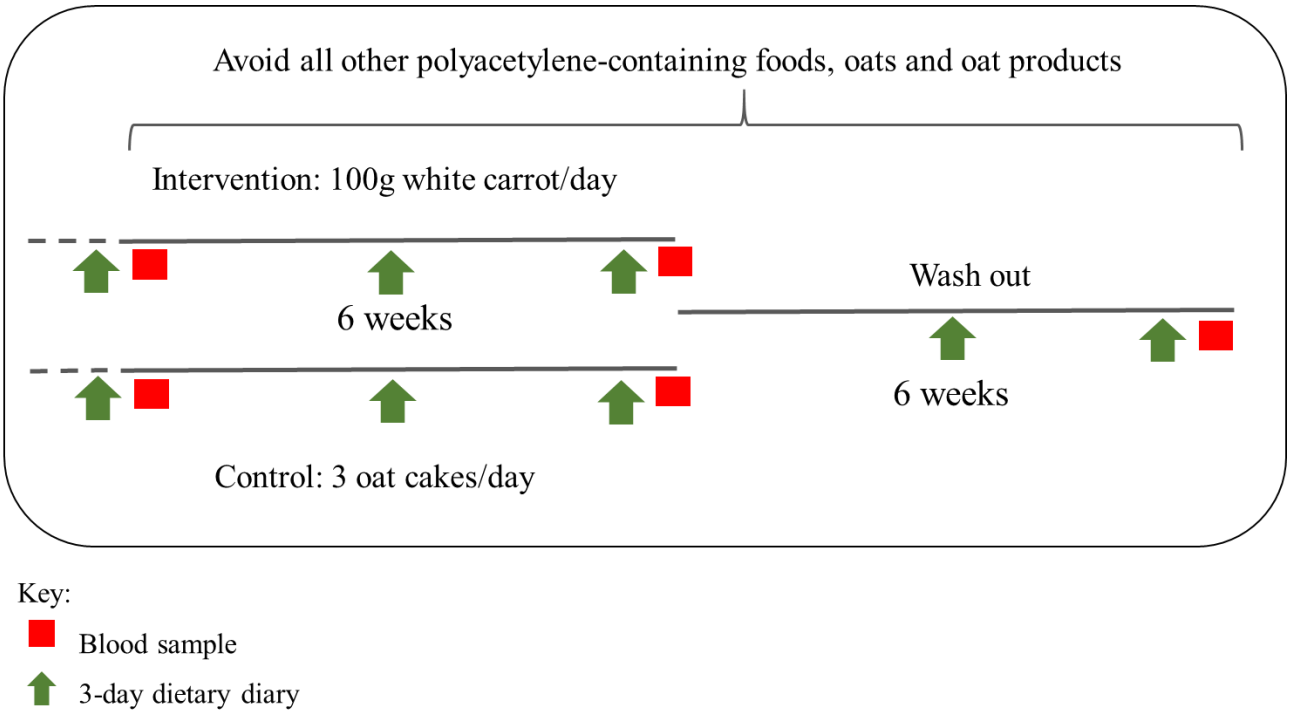


Figure 5.1**:** Timetable of the dietary intervention study

The majority of materials were purchased from Sigma Aldrich, UK; leucocep tubes were from Greiner, UK; Oxiselect™ comet assay kits from Cell Biolabs, Inc, UK; ELISA kits were purchased from Cambridge Biosciences, UK.

5.2.3 *Supply of carrots*

Due to problems with supply, white carrots were obtained from different sources throughout the trial. In the first 3 months, the carrots came from Scotland, UK, the following month the carrots were supplied from York, UK, and the remainder of the trial the carrots were sourced from Europe (origin unknown). The batches of carrots were tested for polyacetylene content throughout the trial. Samples of carrot from each batch were cooked as per the instructions for participants (peeled, topped and tailed and boiled whole in water for 10 minutes) and then immediately frozen. They were then freeze dried and analysed by HPLC (method as in Chapter 2).

5.2.4 *Analysis of dietary intake data*

Diet diaries were analysed using WinDiets (2015, Robert Gordon University, Aberdeen, UK). The 3-day diet diaries were grouped into 'baseline' (1 diary, 3 days), 'intervention' (2 diaries, 6 days) and 'washout' (2 diaries, 6 days). All foods and their recorded weights were coded into WinDiets. Where weights were not recorded in the diary, estimates were made using Food Portions Guide for standard reference portion sizes of common foods (Food Standards Agency, 1994). The data was then analysed for macronutrients, and micronutrients that are considered to affect the risk of cancer such as fibre, polyunsaturated fatty acids (PUFA) and vitamins A, E and C. The data from the diaries were analysed for differences in baseline diets between the carrot and oatcake groups. Data were also analysed for changes in diet over the course of the intervention, comparing baseline, intervention and washout periods in each of the groups.

5.2.5 *Sample processing*

Urine and faecal samples provided by the participant were immediately put on ice. The urine samples were centrifuged for 10 minutes at 300xg then pooled and frozen at -20^{o}C in aliquots. The faecal sample was frozen whole at -20^{o}C. Finger prick blood samples were collected onto a protein saver card as per the manufacturer's instructions (Whatman, UK) and stored in plastic bags at -20^{o}C until analysis. However, due to time constraints, the urine, faecal and finger prick blood samples were not analysed.

Blood samples collected into EDTA tubes were placed on a blood sample roller, covered in foil and left at room temperature for at least 20 minutes. They were then diluted 2 parts blood to 1 part RPMI media kept in a water bath at 37.5^{o}C, and poured onto the frit layer of a leucocep tube (Greiner Bio One, UK). Lymphocytes were removed according to manufacturer's instructions. In brief, the samples were centrifuged at 800xg for 15 minutes at room temperature, buffy layer was removed and the lymphocyte cells from the buffy layer were carefully washed in warm RPMI media, gently aspirated and centrifuged again at 400xg for 5 minutes at room temperature. This was repeated, then the supernatant was removed and the final pellet was diluted in 7mL freeze down medium (90% fetal calf serum, 10% glycerol) and aspirated to ensure an even spread of the cells. Approximately 1mL was aliquoted into cryotubes and placed into -80^{o}C freezer for 8-48 hours before being placed under liquid nitrogen for long term storage (Gill *et al.*, 2007). Blood samples collected into lithium heparin tubes were placed on ice until they were centrifuged (10 minutes at 1000xg at 4^{o}C), then the plasma was removed and stored in aliquots at -80^{o}C until analysis for inflammatory markers. Serum samples were left to stand for 30 minutes at room temperature until clotted and then centrifuged for 10 minutes at 200xg. Serum was removed into aliquots and stored at -80^{o}C until analysis.

5.2.6 *Basal lymphocyte DNA damage using the comet assay*

All samples were anonymised and referred to only by number, as per the information sheet, but also further randomised before analysis to ensure complete blinding of the researcher during analysis. Single strand breaks (SSB) in the DNA were measured using the single cell gel electrophoresis (comet) assay (Collins, 2004; Gill *et al.*, 2007). All activities were performed in dimmed light. Lymphocyte cells were defrosted at room temperature then placed in 37.5^{o}C water bath to fully defrost. The cells were twice washed in warm RPMI media and centrifuged at 350xg for 10 minutes at room temperature to remove the freeze down medium. The cells were then resuspended in 3mL ice cold PBS, centrifuged again (same conditions), supernatant discarded and finally resuspended in ice cold PBS to a cell suspension of $1x10^{5}$ cells/mL. Comet assay slides and reagents were made up using Oxiselect™ comet assay kit according to the manufacturer's instructions. Briefly, cells were combined with comet agarose (from kit) at 1:10 ratio (v/v) (0.5mL agarose with 50µL cells), aspirated then 75µL was immediately pipetted onto the Oxiselect™ comet slide. Slides were chilled in the dark for 15 minutes before transferring to a slide holder containing pre-chilled lysis buffer (14.6g NaCl, 20mL EDTA solution, 10mL 10x lysis solution (kit), 10mL DMSO, DI H_2O to 90mL) for 60 minutes at 4^{o}C in the dark. The lysis buffer was then removed and replaced with pre-chilled

alkaline solution then left for 30 minutes at 4°C in the dark. Pre-chilled alkaline electrophoresis buffer (12.0g NaOH, 2mL EDTA solution (from kit), adjusted to 1000mL with DI H_2O), was poured into a pre-chilled electrophoresis tank to just cover the slides. Voltage was applied to the chamber for 30 minutes at 1volt/cm (28 volts) and a current of 300mA. Slides were returned to the slide holder, and washed with pre-chilled DI H_2O for 2 minutes, aspirated then washed a further 2 times. The slides were then immersed in 70% ethanol for 5 minutes and allowed to air dry completely. 100µL of vista green dye (1X vista green DNA staining solution (from kit), diluted 1:10,000 in TE buffer (10mM Tris, pH 7.5, 1mM EDTA)) was added to each gel and incubated for 15 minutes. Slides were viewed by epifluorescence microscopy using FITC filter and Comet Assay IV software (Perceptive Instruments, Bury St Edmunds, UK). The % tail intensity, reflecting the number of single strand breaks in the DNA, was used as a measure of basal lymphocyte DNA damage (Figure 5.2). 100 cells per gel (each sample in duplicate) were counted and the mean of each set of data was used in the statistical analysis.

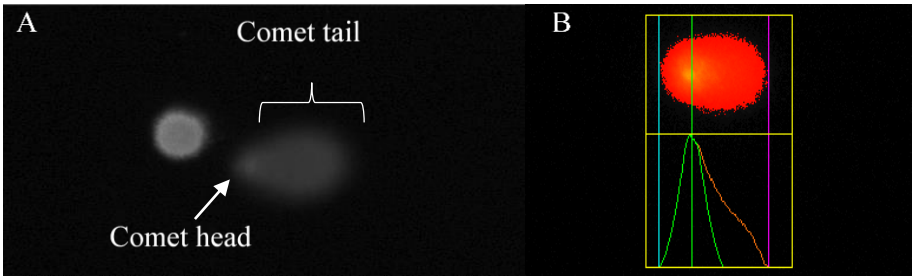


Figure 5.2: Examples of comets. A) cells suspended in agarose gel: a minimally damaged cell (left) and a damaged cell (right). The undamaged DNA is seen as the comet head and damaged SSB of DNA are seen as a tail. B) software identifies the head and tail regions of the cells and calculates the % DNA in the tail as a measure of damage.

5.2.7 *ELISA inflammatory marker analysis*

Inflammatory markers were analysed by ELISA. Interleukin-6 (IL-6) (Cambridge Biosciences, UK) and prostaglandin E metabolite (PGEM) (Cayman Chemical, Ann Arbor, MI, USA) assays were performed according to manufacturer's instructions. All inter- and intra-assay CVs for all inflammatory markers were <20%. Intra assay %CV for PGEM, IL-6 were 10.0, and 2.5 respectively. Inter assay %CV was 17.6 and 13.2 respectively.

5.2.8 *Data and Statistics*

All statistics were performed using SPSS (Version 23, Chicago, IL).
Independent samples T-tests were used to measure the difference in means between groups for baseline measures (biomarkers, macro- and micro nutrient intake). To determine if there was an effect of carrot or oatcake intake on the biomarkers between periods within the carrot group or the oatcake group, paired T-tests were used to compare means between baseline and intervention periods, and intervention and wash out periods. Biomarker values from baseline and washout periods were also combined to be used as a 'non-intervention' sample to determine if there was a difference between biomarkers in the 'non-intervention' periods compared to 'intervention' periods.
To investigate if there was a difference in biomarkers after the carrot intervention compared with after the oatcake intervention, the change in biomarkers between baseline and intervention (wk6-wk0) and intervention and wash out (wk6-wk12) were calculated for each individual and the means of these differences were compared between carrot and oatcake groups using independent samples T-test. This method takes into consideration the potential differences in baseline between the two groups.

5.3 **Results**

5.3.1 *Volunteers*

Figure 5.3 shows the study volunteer progression.

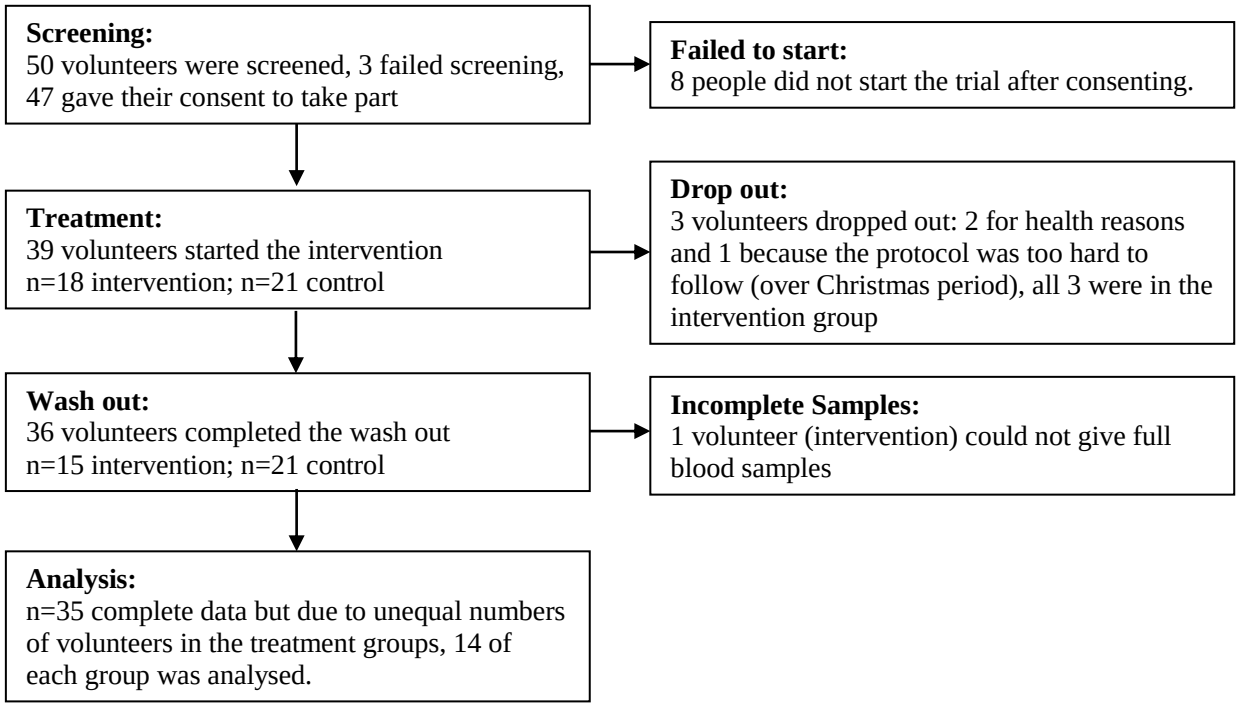


Figure 5.3**:** Progression of volunteers throughout the dietary intervention trial

In the time available, 36 volunteers completed the trial but, due to unequal numbers of volunteers in each treatment group and blood samples for 1 participant being incomplete, 14 people in each group were analysed for a total of 28 volunteers. The first 14 people that completed the intervention in each arm were selected for analysis. The rest of the samples were stored until the study could be continued and the rest of the volunteers were recruited. This would allow an equal number of intervention and control group samples to be analysed in the same analysis period. As the trial was going to be continued past the period of time available for this thesis, the remaining samples could be analysed with those obtained with further recruitment up to 56 volunteers.

Volunteer demographics are displayed in Table 5.2. There were no differences in any of the volunteer characteristics at baseline. There was a slightly lower % body fat in the oat group but this was not statistically significant.

Table 5.2**:** Baseline characteristics of participants in the carrot and oatcake groups

	Carrot group		Oatcake group	
n=28	Mean	±SD	Mean	±SD
Gender (M:F)	2:12		5:9	
Age (years)	58.9	±8.53	60.1	±4.79
BMI (kg/m$^{2)}$	25.23	±2.99	24.64	±2.92
% Body fat	29.2	±8.77	26.9	±8.56

5.3.2 *Compliance and acceptance*

The test food recording sheets suggested compliance to the intervention was excellent with the majority of people consuming the correct amount of intervention foods with a mean of 97% and 99% of doses consumed in the carrot and oatcake groups respectively. Often, if a portion of carrots was missed on one day, the amount was consumed the next day in addition to the daily dose. Food diaries indicated that the compliance at avoiding polyacetylene containing foods was excellent. Anecdotally, the carrots and oatcakes were well tolerated by the groups but 'study fatigue' was experienced by some of the participants by the end of the 6 weeks, indicating that they were either tired of eating the intervention foods or were missing some of the foods on the Foods to Avoid list, especially after the full 12-week trial.

5.3.3 *Dietary intake*

The dietary intake in each period is shown in Table 5.3. Baseline diets were similar (as estimated using diet diaries) in the intervention and control groups, the only difference being vitamin C which was consumed in higher amounts in the intervention group than the control group (131 vs 80mg p=0.01) at baseline. There was no significant change in consumption of vitamin C between baseline and intervention in either group (but the carrot group consumed significantly less vitamin C in the washout period compared to baseline). For both the carrot and oatcake groups, carotenoid intake was significantly lower in both the intervention and washout compared with the baseline diet but intake in the intervention and washout were similar to each other. The carrot group had significantly lower fibre intakes during the washout compared to the intervention period but there were no other differences between baseline or washout and the intervention periods in either group.

Table 5.3**:** Mean daily dietary intake values in each period, separated into carrot and oatcake groups.

	Carrot group			Oatcakes group		
Mean daily intake (range)	Baseline	Intervention	Washout	Baseline	Intervention	Washout
Energy (Kcal)	1882 (847-3087)	1773 (561-3765)	1844 (415-3841)	1839 (566-5002)	1748 (689-3294)	1705 (502-3031)
Fat intake (g)	73 (27-191)	74 (30-145)	76 (19-181)	64 (19-145)	62 (2-145)	60 (8-132)
Protein intake (g)	76 (32-146)	74 (21-276)	79 (37-189)	77 (23-160)	72 (6-125)	70 (17-109)
CHO intake (g)	224 (105-361)	200 (42-434)	203 (17-415)	238 (78-1034)	225 (65-520)	216 (46-493)
Dietary fibre intake (g)	18^{ab} (5-41)	19^{a} (5-39)	16^{b} (3-38)	21 (7-98)	18 (0-48)	18 (4-43)
Vit C intake (mg)	131^{a} (3-394)	108^{ab} (0-529)	95^{b} (2-425)	80 (4-490)	76 (2-298)	90 (4.3-257)
Vit E intake (mg)	9 (1-26)	8 (1-34)	9 (1-24)	8 (1-39)	8 (0-39)	8 (2-27)
PUFA (g)	13 (4-35)	10 (3-34)	12 (3-425)	11 (2-47)	10 (0-47)	10 (2-29)
Carotene (µg)	3649^{a} (129-18866)	1574^{b} (91-7094)	1186^{b} (57-5811)	3263^{a} (3-28688)	1129^{b} (9-9391)	1227^{b} (4-9391)

Letters signify intra-group differences in nutrient within the carrot group or the oat group across the different periods of the trial (but do not compare inter-group differences).

Analysis of white carrots showed that the average concentrations of falcarinol, falcarindiol and falcarindiol-3-acetate in the test carrots were 0.81, 3.84 and 1.92 mg/100g respectively. Each batch of carrots was tested for amount of polyacetylenes which showed different results throughout the trial. The polyacetylene values from each batch is shown in Table 5.4.

Table 5.4: The polyacetylene concentrations in each of the batches of white carrots for the trial.

Batch concentrations (mg/100g)				
Batch	FaOH	FaDOH	FaD3Ac	Total PA
1	0.84	1.14	1.11	3.10
2	0.83	1.75	1.84	4.42
3	0.82	2.35	2.57	5.75
4	1.59	2.41	3.25	7.26
5	1.02	3.90	2.80	7.72
6	1.06	4.18	2.70	7.95
7	0.58	6.53	1.54	8.65
8	0.69	7.71	2.02	10.41
9	0.38	4.05	0.62	5.05
10	0.32	2.27	0.66	3.25
11	0.35	3.16	0.64	4.15

Batches 1-5 were from Scotland, batch 6 was from York and batches 7-11 were from France.

Batches 1-5 appeared to increase in polyacetylene over the growing season which is not uncommon in polyacetylenes (Kjellenberg *et al.*, 2010). Batches 7-11 also fluctuated over the period, generally decreasing over the time period. The compounds differed by 5-7x across the trial which could lead to inconsistent results in the volunteers if these compounds are having an effect on the biomarkers chosen. The amount of falcarindiol and falcarindiol-3-acetate in the white carrots was comparable to the database figures, but falcarinol was lower in the white carrots compared to the database. White carrots (white satin, lunar white, snow white, crème de lite and an experimental white carrot B10720 varieties) have been measured previously with concentrations ranging from 0.94-5.4 mg/100g falcarinol (Yates *et al.*, 1983; Metzger and Barnes, 2009) so it is not unusual to see lower values and large ranges of falcarinol concentration in carrot. Due to large differences in polyacetylene concentrations over the harvesting period, and thus throughout the period of the trial, the amount of polyacetylene consumed was estimated for each participant individually depending on which batches of carrot they consumed. The mean daily intake of polyacetylenes in both the baseline (as estimated from the diet diaries) and intervention periods is shown in Table 5.5. There were no

significant differences between the intake of polyacetylene containing vegetables (g) or the intake of individual polyacetylenes (mg) between the carrot group and the oatcake group at baseline (as measured by diet diaries). Participants in the carrot group ate significantly more falcarindiol ($p=0.01$) and falcarindiol-3-acetate ($p<0.001$) during the intervention period compared to baseline but falcarinol intake was not different ($p=0.28$) as shown in Table 5.5.

Table 5.5**:** Mean polyacetylene intake (mg/day) during pre-intervention and intervention periods (no polyacetylene was consumed by either group during the wash out period).

	Baseline				Intervention			
	Carrot group		Oatcake group		Carrot group		Oatcake group	
	Intake (mean±SD)							
Polyacetylene-rich veg (g/day)	29.1	±39.0	23.5	±30.7	100	±0.0	0.0	±0.0
Falcarinol (mg/day)	1.1	±1.6	0.6	±0.8	0.8	±0.3	0.0	±0.0
Falcarindiol (mg/day)	1.5	±2.4	0.5	±0.7	3.6	±1.7	0.0	±0.0
Falcarindiol-3-acetate (mg/day)	0.2	±0.4	0.2	±0.2	2.0	±0.8	0.0	±0.0
Total polyacetylene (mg/day)	2.8	±4.2	1.4	±1.8	6.4	±1.8	0.0	±0.0

5.3.4 ***Change in inflammatory markers***

Inflammation:

The standard curves for the ELISAs can be found in Appendix D.

Comparing baseline measures between carrot group and oatcake group.

Table 5.6 shows the results of the biomarkers measured at each time point in the carrot and oatcake groups. There were no significant differences in any of the baseline measures between the carrot and oat groups. The median values of IL-6 in all groups was zero suggesting IL-6 levels in the participants was generally low. The carrot group IL-6 levels were not above the level of quantitation (7.8pg/mL) using this ELISA assay (and thus were considered to be zero) so the oatcake group had non-significantly higher levels of IL-6 than the carrot group at baseline and throughout the trial.

Comparison of inflammation in each trial period (baseline, intervention and washout) within the carrot group and within the oatcake group.

There were no significant differences between the means of IL-6 between any of the trial periods in the carrot group or in the oatcake group. There were also no significant changes in

PGEM concentration in the oatcake group between any of the periods. However, there was a 28% reduction in PGEM concentration between the baseline and intervention periods in the carrot group (p=0.08). During the washout period, the PGEM concentration returned to similar levels as at baseline, which was higher than during the intervention period (p=0.072). If the baseline and washout periods were counted as two independent 'non-intervention' periods, then the PGEM concentration is significantly different to the intervention period in the carrot group (p=0.043) but not the oatcake group.

Table 5.6**:** Biomarkers at baseline, intervention and control periods in the carrot and oatcake groups.

	Carrots						Oatcakes					
	Baseline		Intervention		Washout		Baseline		Intervention		Washout	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Tail intensity (%)	43.7	±14.8	40.7	±15.5	41.1	±13.8	49.8	±10.0	44.4	±13.5	42.8	±15.1
IL-6 (pg/mL)	0.0	±0.00	0.0	±0.0	0.0	±0.0	7.74	±15.8	3.02	±10.9	2.62	±9.46
PGEM (pg/mL)	44.2	±28.4a	30.3	±11.2b	42.5	±26.1a	38.2	±22.1	36.2	±19.1	33.4	±16.2

Letters that differ in the same row signifies a trend toward significance (p=0.072) in means between intervention periods within the carrot group.

Comparing inflammatory markers between time periods in carrot and oatcake groups:

Table 5.7 shows the mean change in biomarkers between baseline and intervention, and intervention and washout periods (see also Figure 11.3). Comparing the mean change in PGEM concentration between the carrot and oatcake groups, there is a large reduction in PGEM in the carrot group but less of a reduction in the oatcake group, compared to baseline (-13.9pg/mL in the carrot group and -2.1pg/mL in the oatcake group), but comparing the means of the change shows that this was not a statistically significant difference. Comparing the means of the change in PGEM concentration from intervention period to washout period, the mean change over this time period for the carrot group (+12.1pg/mL) was significantly larger ($p=0.04$) compared to the change in the same time period in the oatcake group (-2.8pg/mL). The increase in PGEM concentration in the carrot group after the washout period returned PGEM concentration to baseline levels. There was very little change in the oatcake group across the 12 weeks of the trial so baseline levels were maintained. There was also no difference in the means of the change in IL-6 concentration between the carrot and oatcake groups either between the intervention and baseline or between intervention and washout periods.

Table 5.7**:** Change in biomarkers (wk6-wk0 and wk6-wk12) in the carrot and oatcake groups.

	Intervention-Baseline				Intervention-Washout			
	Carrots		Oatcakes		Carrots		Oatcakes	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Change in tail intensity (%)	-3.0	±9.1	-5.5	±10.6	0.4	±7.6	-1.5	±9.9
Change in IL-6 (pg/mL)	-0.2	±0.6	0.4	±2.1	0.0	±0.6	0.2	±0.5
Change in PGEM (pg/mL)	-13.9	±27.9	-2.1	±24.0	12.1	±22.4[a]	-2.8	±19.4[b]

Letters that differ on the same row signify a difference in mean ($p<0.05$) between the carrot and oatcake groups.

5.3.5 *Change in Lymphocyte DNA damage (comet)*

There was no difference in DNA damage to lymphocytes as measure by % tail damage in the comet assay between the carrot group and oatcake group at baseline (Table 5.6). There was also no change in mean % tail damage between trial periods in the carrot group or the oatcake group. There were no significant differences between the means of the change in % tail intensity between carrot and oatcake groups either after the intervention or after the washout periods (Table 5.7, Figure 11.4).

5.4 **Discussion**

5.4.1 *Inflammatory markers*

Polyacetylenes have been shown to have anti-inflammatory actions *in vitro* (as discussed in chapter 1) and this trial aimed to investigate whether anti-inflammatory actions could also be seen *in vivo* after consumption of a source of polyacetylene-rich vegetables. This trial observed a 28% reduction in PGEM after 6 weeks of consuming white carrots compared with a baseline diet (trend p=0.08) and a 6-week washout (trend p=0.07) when no polyacetylene-containing foods were eaten. Although this did does not reach statistical significance, there is a clear effect. PGEM concentrations returned to baseline levels after the 6-week washout, without the inclusion of carrots or related vegetables in the diet. There was very little change in PGEM concentration between the 3 time periods in the oatcake group. Comparing the differences in mean change in PGEM concentration between intervention and washout periods between the carrot and oatcake groups, there was a statistically significant difference between the two groups (p=0.04). The oatcake group had been on the 'carrot-free' diet for 6 weeks longer than the carrot group by the end of the trial (12 weeks) which could explain this difference. However, as there was no difference in PGEM concentration between time periods in the oatcake group, the difference between the change in concentration between the two groups suggests some component/s of the carrot is mediating the change in PGEM concentration in the carrot group compared with the fibre matched control oatcakes. IL-6 did not significantly change over the trial in either group.

Only a few trials have investigated the effect of carrot on inflammatory markers in humans and all of these used carrot juice rather than the whole vegetable. Supplementing the diet of breast cancer survivors (n=69) with 330mL juice every day for 3 weeks had no significant effect on the inflammatory markers TXB_2, PGE_2 and hsCRP compared with 3 weeks consuming habitual diet but with only fruit and vegetables low in carotenoids (Butalla *et al.*,

2012). Consumption of 470mL carrot juice daily for 3 months did not affect IL-1α or CRP compared with normal habitual diet (n=17) (Potter *et al.*, 2011a). IL-6 and CRP also did not change in diabetics (n=44) after consumption of 200mL carrot juice/day for 8 weeks compared with habitual diet (Ramezani *et al.*, 2014). The lack of change in inflammatory markers in these trials suggests that intake of carrot juice cannot significantly affect inflammation. This may be due to the processing effect of juicing, reducing active phytochemicals. Freshly prepared carrot juice has been shown to have a falcarinol content of 13-35µg/mL (Hansen-Møller *et al.*, 2002; Haraldsdóttir *et al.*, 2002), but as polyacetylenes are easily oxidised, they could quickly be destroyed if the juice is not consumed straight away. The (non-significant) change in PGEM in this trial but not the Butalla *et al.* (2012) trial may be due to whole carrots being used.

Inflammatory marker PGEM:

The only inflammatory marker that was affected during the intervention was PGE_2, as determined by PGEM in serum. Prostaglandin E_2 is created during the 'injury' pathway of inflammation. As described in chapter 1, arachidonic acid (AA) is released when a cell is damaged. This is metabolised by the COX and LOX enzymes to initiate an inflammatory response to repair the damaged tissue. The COX enzymes start a cascade that ultimately leads to the release of prostaglandins including PGE_2. A reduction in PGEM suggests that the COX enzyme or its substrate is being affected. *In vitro* trials have shown that falcarindiol can reduce the activity of COX-1 enzymes, with inhibition observed with an IC_{50} of 0.3-170.3µM, (Prior *et al.*, 2007). The downstream products of COX-1, HHT and TXB_2, were also inhibited in human platelets with IC_{50} values of 0.386µM and 0.242µM, respectively (Baba *et al.*, 1987). Falcarinol has also been investigated for modulation of COX-1 or -2 activities but no inhibition was seen in the presence of concentrations up to 100µM (Alanko *et al.*, 1994). This was also the case in cells from rabbit gut mucosa where the continued production of the prostaglandins E_2, $F_{2\alpha}$ and D_2 was seen after treatment with concentrations of up to 200µM falcarinol (Fujimoto *et al.*, 1998). A reduction in COX-2 mRNA expression was seen when concentrations of 100µM falcarinol were incubated with HT-29 colon cancer cells but inhibition was less than that seen when exposed to falcarindiol at much lower concentrations of 1µM (Um *et al.*, 2010). The *in vitro* results suggest falcarindiol has the strongest effect on COX mediated inflammation. The falcarindiol (and falcarindiol-3-acetate) intake in this trial significantly increased between baseline and intervention periods in the carrot group, but not the oatcake group, and PGEM decreased in the carrot but not oatcake group between these periods. PGEM then returned to similar levels as baseline during the washout period when no

polyacetylenes were consumed. This suggests falcarindiol (or falcarindiol-3-acetate) may be mediating this effect. As falcarinol appears to have little or no effect on COX enzymes, it is not important that the white carrots had lower amounts of this compound than orange carrots. In fact, falcarinol has been seen to inhibit the enzyme that metabolises prostaglandins (Fujimoto *et al.*, 1998) so having less falcarinol may have even been beneficial to the anti-inflammatory effect. Falcarindiol-3-acetate has not been investigated for its anti-inflammatory effects *in vitro* but as significantly more was consumed during the carrot intervention, it is possible it could also be contributing to the reduction in PGE_2. Further studies would be needed to investigate this.

Mechanisms of COX activation/modification:

1. *Changing the COX active site:*

The mechanism by which COX is affected by the polyacetylenes has not fully been elucidated. However, based on the effects of known COX modifiers, there are several potential mechanisms for their action. The COX enzyme could be completely inhibited like, for example, in the presence of aspirin, a commonly used NSAID that irreversibly alkylates the COX-1 enzyme active site (Morton and Hall, 1999; Patrignani and Patrono, 2016). Alkylation is a common way for proteins to be modified, changing the function of or inhibiting an enzyme. The COX enzyme could also be modified so that it is still active but the function, and therefore the metabolites produced, are different. As well as acting on COX-1, aspirin also modifies the COX-2 active site so that it can no longer produce prostaglandins. Instead, 18-HEPE is produced which can be further metabolised into lipoxins, protectins or resolvins, which are anti-inflammatory signalling molecules that stop the inflammatory reaction (Serhan *et al.*, 2002; Serhan *et al.*, 2008; Seki *et al.*, 2009). Polyacetylenes form a strong carbocation (an unstable carbon atom with three bonds and a positive charge) by losing water through liberation of the hydroxyl group on the C3 (Purup *et al.*, 2009), therefore are highly hydrophobic. They can bind strongly to amino and mercapto groups in proteins, acting as an alkylating agent (Christensen and Brandt, 2006) so may have a similar effect to aspirin.

2. *Polyacetylenes as analogues to arachidonic acid:*

It is also speculated that, due to the polyacetylenes being AA analogues, they could competitively bind to the COX enzyme, preventing AA binding and progression down the

inflammatory pathway to produce prostaglandins (Alanko *et al.*, 1994). The polyacetylenes are synthesised from linoleic acid as are AA, and the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Minto and Blacklock, 2008). The structures of the 4 molecules are similar (Figure 5.4) in that they are long chain hydrocarbons with one or more double bond.

PUFAs such as DHA and EPA also compete with AA for the COX active site (Gleissman *et al.*, 2010). Due to this effect of PUFA on inflammation, PUFA intake in the participants of this trial was analysed across the time periods by diet diary. PUFA intake did not significantly change during the intervention between any trial period in either group so the dietary intake of PUFA is unlikely to be mediating the effect on PGE_2 observed. Due to similarities in the structure of polyacetylenes and PUFAs the polyacetylenes could bind to the COX enzyme in the same way as EPA and DHA, potentially acting as a competitive inhibitor. EPA and DHA can be both pro- and anti-inflammatory. When they are metabolised through COX-1 and 2 and 5-LOX, pro-inflammatory metabolites are released. However, when metabolised through aspirin-modified COX-1 and 15-LOX, anti-inflammatory metabolites are produced, including the lipoxins, resolvins and protectins, signalling the end of inflammation and downregulating the inflammatory response (Serhan *et al.*, 2002; Serhan *et al.*, 2008; Poorani *et al.*, 2016). Blocking these enzymes completely can therefore result in complications due to interference in the natural resolution of inflammation which can delay the healing process (Poorani *et al.*, 2016). It is not known whether polyacetylenes completely inhibit COX enzymes and therefore whether they would prevent the production of resolvins. It would be of interest to further investigate the interaction of COX with polyacetylenes, specifically with respect to the effect on generation of molecules that can resolve inflammation.

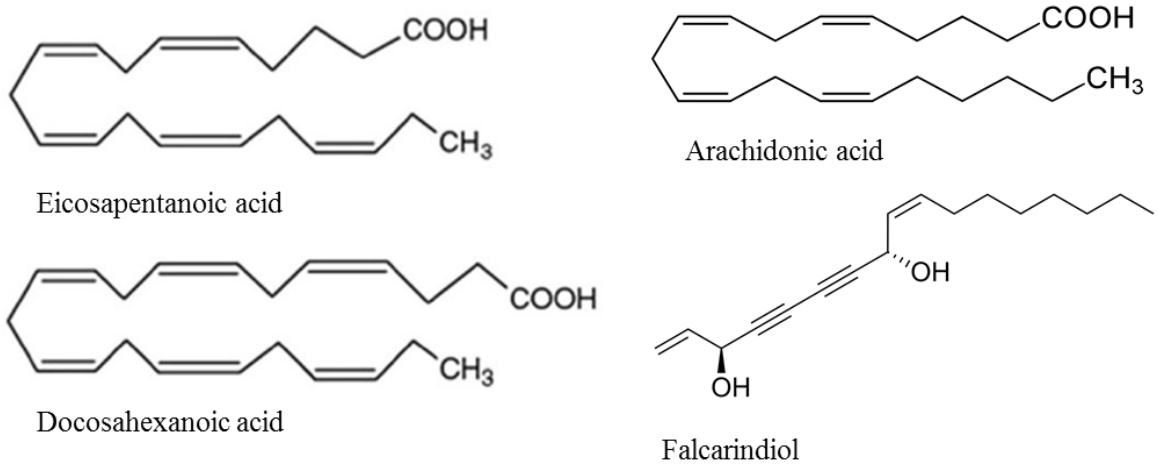


Figure 5.4**:** Structures of falcarinol, AA, EPA and DHA

Inflammatory marker IL-6:

The iNOS and LPS/STAT3/IL-6 inflammatory response pathway has been inhibited by polyacetylenes *in vitro* (Uto *et al.*, 2015). However, no effect on IL-6 was observed with additional dietary polyacetylenes in this trial. One pathway for IL-6 metabolism, through NFκB transcription factor, is stimulated by LPS which is present in the cell walls of gram negative bacteria. This 'infection' pathway may not have been active in the healthy participants. IL-6 also occurs at higher concentrations during chronic inflammatory conditions such as rheumatoid arthritis (Shrivastava *et al.*, 2015) but often interventions to reduce inflammatory markers fail to detect a difference in these markers because they act locally and may not be picked up in the systemic circulation (Mozaffarian and Wu, 2011). Interventions with Omega-3 PUFA, for example, have observed a reduction of eicosanoids, such as the prostaglandins, but other inflammatory markers such as IL-1 and TNF-α have more mixed results (Mozaffarian and Wu, 2011). The concentrations of IL-6 in this trial were quite low, with previously recorded values being between 1.7-2.5pg/mL in healthy adults (Acharjee *et al.*, 2015; Migliori *et al.*, 2015). An older cohort (over 45 years old) was selected for the current trial, as inflammation increases naturally with age (Chung *et al.*, 2009), and the aim was to have a higher baseline to be better able to detect a difference. However, mean concentrations of IL-6 were low in this healthy cohort, with median values of 0.0pg/mL, and most not reaching the LOD of 7.8pg/mL and therefore it was not surprising that no significant lowering was seen over the intervention. Previous studies observed that PUFA interventions in people who have higher inflammation due to medical conditions can significantly lower inflammatory markers after supplementation with Omega 3, whereas supplementation in healthy participants appeared not to (Nakamura *et al.*, 2005; Tayyebi-Khosroshahi *et al.*, 2012). It could be that patient populations may benefit most from anti-inflammatory interventions and further work is needed to investigate this.

5.4.2 *Anti-cancer effects of polyacetylenes*

Oxidative DNA damage:

Oxidative DNA damage in humans is considered to be an important mutagenic and carcinogenic factor (Valko *et al.*, 2004). Cancer preventative mechanisms of dietary components related to oxidative DNA damage include the prevention of oxidant-induced DNA strand breaks by direct antioxidant effects (quenching free radicals), by increasing the

expression of endogenous defence mechanisms (Valko *et al.*, 2004), or by reducing the sources of ROS, for example, in the inflammatory response to invading pathogens.

This trial investigated the potential for white carrot supplementation in the diet to reduce lymphocyte DNA damage. Lymphocytes act as a minimally invasive surrogate that can be used to assess genotoxicity in tissues that may be affected by oxidative stress in lieu of more invasive techniques such as biopsies (Gill *et al*., 2004). In this trial, there was no effect of white carrot or oatcake consumption on basal damage to lymphocyte DNA as measured by the comet assay, despite a reduction in a biomarker of inflammation.

ROS are released as a result of infection, with macrophages releasing NO to destroy the invading pathogen. IL-6 can stimulate the iNOS gene, leading to production of NO for this purpose, but this free radical can also damage surrounding healthy cells, leading to oxidative DNA damage (Metzger *et al.*, 2008). In this trial, IL-6 did not change, suggesting there was no change in NO generation by this mechanism. As the concentrations of these cytokines were low, the oxidative damage due to NO could also be low and therefore no change in DNA damage was detected.

Previous research in humans (n=23) saw reduced endogenous DNA strand breaks as well as lower oxidative pyrimidine base damage after consumption of a supplementary 300mL carrot juice per day for 2 weeks compared with a diet depleted in carotenoids (Pool-Zobel *et al.*, 1997). The trial was also testing the effect of other high carotenoid foods (tomato juice and spinach) with no wash out between the different food periods so results were potentially confounded by these other vegetables. As DNA damage was reduced when eating a variety of carotenoid-rich vegetables compared with the low carotenoid period, the protective effect appeared to be dependent on the carotenoid content of these vegetables. In another trial, consumption of 200g minced carrot or with matched carotenoids supplements (containing 3.7mg α-carotene and 8.2mg β-carotene) per day for 3 weeks (n=31) increased the ability of lymphocytes to repair strand breaks after H_2O_2 insult. The results suggest the carotene content was modulating the repair ability as both carrot juice and the supplement had the same effect. However, single strand breaks, reflective of the net DNA damage occurring over the intervention were unchanged after 3 weeks of the intervention in either group (Astley *et al.*, 2004).

One disadvantage of using lymphocytes to measure basal DNA damage is that they have the capacity to repair strand breaks and so the damage seen in the assay will represent the net damage that is occurring: the amount of damage occurring plus the endogenous repair

activity. Therefore, it is not possible to determine if reductions in net DNA damage are occurring because the cells are in a less damaging environment or whether the endogenous enzymes have improved their repair ability. An indirect way to investigate the potential for endogenous antioxidant mechanisms to be stimulated in the presence of oxidative stress is to perform the comet assay after exposing isolated cells to H_2O_2, a potent oxidant (Gill et al., 2004). Unfortunately this was not done in the current trial due to lack of time. Another method to assess DNA damage is to use mitochondrial DNA (mDNA) which is less able than nuclear DNA to repair itself (Kazak *et al.*, 2012) and will therefore better reflect the burden of oxidative damage on the system as a whole (Hunter *et al.*, 2010). Blood samples were collected onto protein saver cards for the purposes of measuring mDNA in this trial but unfortunately there was not enough time to analyse these.

The potential antioxidant activities of the polyacetylenes, discussed in Chapter 1, are in part due to being able to upregulate the expression of endogenous detoxifying molecules rather than being radical quenching themselves, affecting the expression of GST, GSH, NQO1, HO-1 and catalase (Young *et al.*, 2008; Ohnuma *et al.*, 2009; Uto *et al.*, 2015). In fact, falcarinol and falcarindiol have been seen to be pro-oxidative, increasing ROS in cultured muscle cells but leading to an increase of the endogenous antioxidant enzyme glutathione peroxidase (cGPx) and an overall net effect of a reduction in oxidative damage (Young *et al.*, 2008). Despite the results of these *in vitro* trials (Young *et al.*, 2008), no changes were seen in catalase and SOD expression in red blood cells after a diet was supplemented with 300mL carrot juice daily for 8 weeks (Lee *et al.*, 2011). A marker of lipid peroxidation (i.e. oxidative stress) was not changed after 3 weeks of carrot juice consumption (237mL per day) compared with a low carotenoid diet washout period (Butalla *et al.*, 2012) but a trial using a larger amount of juice (450mL) saw reductions in MDA, and increased antioxidant status (Potter *et al.*, 2011a). The biomarkers chosen for the assessment of the effect of oxidative damage (% tail DNA) in the current trial were inadequate to look for changes in endogenous detoxifying mechanisms. Investigating the expression of cGPx, catalase or SOD after feeding whole white carrot may be of interest and could be investigated in further studies.

Other anti-cancer effects of polyacetylenes:

Another potential anti-cancer effect of polyacetylenes is the apparent cytotoxic activity specific to cancer cells. These cytotoxic effects were demonstrated in *in vitro* trials where proliferation of cancer cells was inhibited but healthy cells remained unaffected at the same concentration of polyacetylene (Purup *et al.*, 2009). The polyacetylenes would therefore have no demonstrable effect on the healthy participants in this trial. There is some suggestion that

polyacetylenes act on cells that are in the initiation and progression phases of cancer development. Thus, anti-cancer mechanisms of polyacetylenes are not only potentially preventative but also actively target initiated cancer cells. They have been shown to induce apoptosis (Sun *et al.*, 2010), increasing the expression of an apoptosis protein, caspase (Young *et al.*, 2007; Jin *et al.*, 2012; Zaini *et al.*, 2012), and reducing the expression of an anti-apoptosis protein, Bcl-2 (Um *et al.*, 2010) in cancer cells. In rodent trials of falcarinol feeding, AOM-induced small ACF number did not change but large ACF and tumour number decreased after feeding with either carrot or falcarinol supplemented diet compared with control (Kobaek-Larsen *et al.*, 2005). A further study with both falcarinol and falcarindiol supplemented feed (7mg/g feed for both compounds) reduced AOM-induced ACF and tumours of all sizes in rats (Kobaek-Larsen *et al.*, 2017). The combined effect of falcarinol and falcarindiol was stronger than that of falcarinol alone in the researcher's previous trial (Kobaek-Larsen *et al.*, 2005). Mice with a genetic predisposition to intestinal cancers also had lower tumour number with a diet containing 20% carrot powder (Saleh *et al.*, 2013). In both cases, the strength of the inhibition was correlated to tumour size. In the Kobaek-Larsen (2017) trial there was an 88% reduction in large tumours and 34% reduction in small ACF compared to control demonstrating the negative effect on cancer progression was stronger than the effect on cancer initiation.

Inflammation also has effects in cancer progression separate to the DNA damage that could occur due to release of ROS. Cancer cells can take advantage of other cancer enhancing factors that occur in the inflammatory environment, such as inhibition of macrophages, T-cells and natural killer cells (Hodge *et al.*, 2005), the differentiation of macrophages to tumour associated macrophages (Sica *et al.*, 2008), the production of signals that downregulate apoptosis and encourage angiogenesis (Coussens and Werb, 2002), and increased expression of MMPs (Hodge *et al.*, 2005). These actions would be beneficial to the cells in which cancer is already initiated rather than preventing initiation as measured by oxidative stress and resulting DNA damage in healthy cells. COX-2 enzyme activity has been shown to increase risk of proliferation, angiogenesis and metastasis and decrease apoptosis in CRC cells (Tuncer and Banerjee, 2015) suggesting COX-2 inhibition, and the reduction of associated prostaglandins, would prevent the progression of cancer.

As healthy volunteers were used in this trial, the effects of the carrot intervention on cancer cells was not investigated. The polyacetylenes appear to be cytotoxic against cancer cells and have effects after initiation of cancer. Lymphocyte DNA damage of healthy cells would therefore not change if the polyacetylenes were specifically targeting cancer cells thus they

are not the most relevant biomarker to measure cancer risk with these compounds, or white carrot, in healthy participants. Further work in cancer patients who are undergoing treatment would be of interest to investigate whether consumption of carrots can improve outcomes of treatment. This is especially pertinent as falcarinol has been shown to increase the efficacy of chemotherapy drugs (Jin *et al.*, 2012; Tan *et al.*, 2014).

Falcarinol is thought to have the most potent cytotoxic activities. As falcarinol levels were very low in the white carrots used in this trial, this highlights that the polyacetylenes could have been measured in the white carrots prior to the intervention and future studies using white carrots should measure polyacetylene content and perhaps find a variety with similar concentrations to orange carrots. Published data on white carrot polyacetylene demonstrated the white satin variety of carrot has 1.82mg/100g falcarinol which is slightly lower than some of the orange carrot varieties (Metzger and Barnes, 2009). To compensate for the lower concentration a portion size of 100g was chosen, 25% more than the 80g portion recommended for the 5-a-day (NHS, 2015). However, the white carrots in this trial had 40% lower falcarinol than the database boiled carrot so this did not increase the total intake to the equivalent of orange carrot as desired. The daily intake of the other 2 polyacetylenes were increased during the white carrot intervention. A manageable portion size of carrot was chosen for this trial as this would allow generalisation of effects to a normal daily intake.

Although falcarindiol was not as potent as falcarinol at killing cancer cells in some *in vitro* studies, they are still actively cytotoxic in many cancer cell lines (Table 1.2 in chapter 1). Falcarindiol-3-acetate has not been as well investigated as the other two compounds but it has been seen to be cytotoxic to several leukemia cancer cells and even had an IC_{50} lower than falcarindiol in MOLT-3 cells. Falcarindiol-3-acetate was also the most potent apoptosis inducer of the 3 polyacetylenes tested in leukemia cells (Zaini *et al.*, 2012). Falcarindiol-3-acetate also inhibited LPS induced NO at concentrations not cytotoxic to healthy cells (Metzger *et al.*, 2008) suggesting it also has anti-inflammatory and antioxidant activities.

5.4.3 *Synergistic effects of phytochemicals*

Whilst a normal portion size was used to investigate the effects that could be occuring with usual intake, the results may not truly reflect orange carrot consumption as the phytochemicals in white carrots are not the same. The polyacetylenes in this trial occurred in different proportions, with very low falcarinol, and they did not contain β-carotene which may influence the biomarkers, though it is not clear in what way. Synergism of phytochemicals has

been previously explored with combinations of phytochemicals such as carotenoids, polyphenols and DHA and EPA omega 3 fatty acids which show a higher inhibition of cancer cells with different carotenoids in combination, carotenoids with polyphenols, and/or other compounds in low concentrations (Liu, 2004; Lupton *et al.*, 2014; Linnewiel-Hermoni *et al.*, 2015). Synergistic activities have also been seen with the polyacetylenes from carrot. Falcarindiol in low doses inhibits cell proliferation. When low doses of falcarinol and falcarindiol are present together (1:1), the inhibition is greater. Increasing the ratio of falcarindiol concentration increases inhibition of proliferation (up to 1:10). The same is not true with increasing concentrations of falcarinol so having lower concentrations of falcarinol in comparison to falcarindiol in the carrot in this trial may even be of benefit (Purup *et al.*, 2009). The anti-tumour effects seen in rats consuming falcarinol and falcarindiol together were also stronger than with falcarinol alone (though concentrations were also higher in the former) (Kobaek-Larsen *et al.*, 2005; Kobaek-Larsen *et al.*, 2017). Another trial showed there was no change to LPS-induced NO production when falcarinol, falcarindiol and falcarindiol-3-acetate were incubated together in macrophages compared to each compound alone. However, they were added in equimolar concentrations which is not reflective of the ratios found in carrots (Metzger *et al.*, 2008).

Synergy of polyacetylenes with carotenoids has not been well explored so it is not known whether presence of carotenoids in the carrot would have resulted in a different effect in the intervention trial. Carrots appear to be a major source of carotenoids in the diet of this cohort as carotenoids were significantly lower in the intervention and washout periods (around 2.5x lower) in both groups compared with baseline, and not replaced by another source during the intervention and washout phases. There was no detectible change in biomarkers in response to the decrease in carotenoid intake. The reduction in PGEM seen in the carrot intervention returned to baseline by the end of the wash out period, despite the carotenoid intake remaining low. PGEM did not change significantly in any period in the oatcake group despite similar carotenoid intake in both groups and significantly lower carotenoid intake in both intervention and washout. DNA damage did not change in any group in any time period, despite significant decreases in carotenoids between baseline and the other periods in both groups.

5.5 **Limitations**

The study did not reach the planned power (n=56) and if there had been more people completing the trial, the effect on PGEM reduction may have been more significant. The power calculation was based on results from a study using H_2O_2-induced oxidative stress on

the SSBs as an outcome. However, as endogenous SSBs only were measured in this study, without further oxidative stress, values from a paper measuring these outcomes should have been used to calculate sample size for the desired power. The outcome of endogenous SSB may be less powerful than using further induction with oxidative stress and therefore the samples size should have been adjusted upwards as appropriate. Cross over trials are seen as more powerful than parallel trials (Wellek and Blettner, 2012), requiring fewer participants, as each person acts as their own control which as considered in this trial, but the cross over trial would have lasted 18 weeks if each participant had completed the carrot and oatcake intervention with a washout period between them. Each period in this trial (intervention and washout) was 6 weeks long to allow for enough time for lymphocytes to completely regenerate in an environment either containing carrot phytochemicals or not (Asquith *et al.*, 2007; de Boer *et al.*, 2012). Comet assay results have been shown to be effected by the seasons with increasing sunlight hours leading to increasing DNA damage due to UV (Møller and Loft, 2002). A 12-week parallel trial was therefore chosen to reduce trial fatigue, participant burden and seasonal effects. As the parallel trial contained baseline, intervention and washout periods, the participants acted as their own control between periods but having each person take part in both the oat and carrot intervention could have made the trial more powerful.

Compliance to the intervention could have been attempted by measuring polyacetylene concentrations in the blood. However, this was not designed into the trial because polyacetylene in blood plasma samples returns to baseline levels after 8 hours of consumption of a high polyacetylene food (Haraldsdóttir *et al.*, 2002) and after a 12 hour fast it was thought that the levels in the blood would not accurately reflect intake. This is the case with many of the fat-soluble vitamins, such as vitamin A, the blood levels do not accurately reflect the nutritional status of the individual. Storage in the organs, such as the liver, can maintain vitamin A levels and ensure nutritional status is high but this may not be reflected in the blood plasma (Olson, 1984). As discussed in the previous chapter, future work into the fate of polyacetylenes in the body could help determine if, and where, polyacetylenes are stored in the body and if blood plasma is the most relevant way of assessing intake.

5.6 **Conclusions**

Regular consumption of a normal amount (100g) of white carrot can reduce a biomarker of inflammation (PGEM) compared with baseline ($p=0.08$) and washout periods ($p=0.07$), and the change in PGEM between intervention and washout was significantly different compared

with an oatcake control (p=0.04) This effect was probably due to the significantly increased falcarindiol and falcarindiol-3-acetate content of the diet in the white carrot group. Whilst there was an observed effect on inflammation, there was no resulting effect on lymphocyte DNA damage in healthy participants. To further investigate the potential effects of polyacetylenes in humans, trials in patient groups, such as those with cancer and/or high levels of inflammation, is recommended.

Chapter 6. **Final Discussion**

6.1 **Summary**

This thesis has demonstrated that boiled carrots are widely eaten by a UK population of older adults, providing a higher contribution of polyacetylenes to the diet than all the other polyacetylene-rich vegetables. This makes the carrot the most relevant vegetable to investigate for the effects of polyacetylene-rich vegetables on biomarkers of health. The results of chapter 3 have shown that making small changes to the preparation of carrots during cooking can mitigate some of the phytochemical losses seen in traditional preparation. The dietary interventions have shown that a manageable amount of cooked carrot (100g) can increase plasma concentrations of falcarinol and falcarindiol-3-acetate and that inter-individual differences are seen in response of blood plasma concentrations, with some volunteers not having an increase in blood plasma concentration after consumption up to 250g. When eaten regularly (every day) 100g of carrot can also reduce a marker of inflammation in healthy adults.

6.2 **Discussion**

To investigate a health effect of a certain food or compounds in foods, it is important to consider the full lifecycle of the food to ensure that compounds of interest a) are present in a food; b) survive common processing that the food undergoes before being consumed; c) survive digestion, and enter the circulation (or come into contact with the tissues of interest e.g colon cells); and d) have the desired health effect in humans after consumption. Figure 6.1 summarises some of the considerations within this lifecycle.

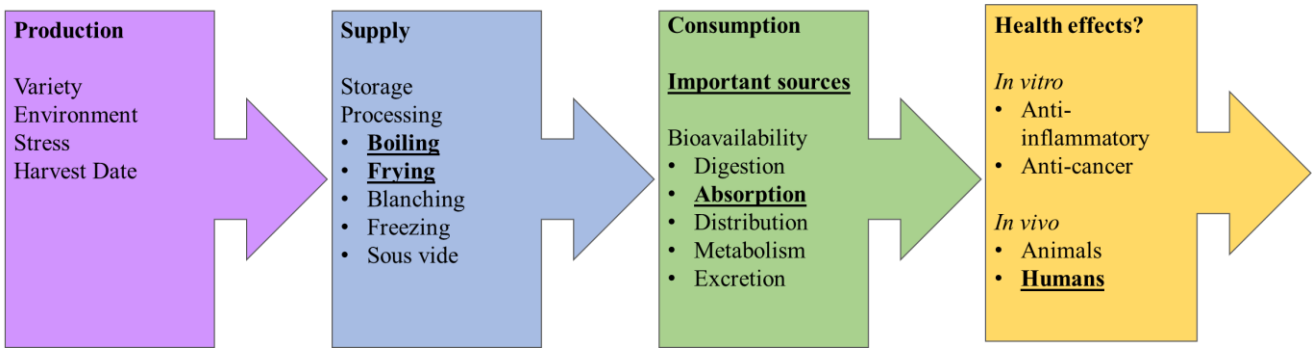


Figure 6.1: The food chain of polyacetylene-containing foods and the areas in which they have been studied or need to be studied.

From the beginning of the life cycle, polyacetylene concentrations in vegetables can be affected by the way the vegetables are produced. Concentrations in carrots appear to be genetically determined and therefore levels are dependent on the variety (Yates *et al.*, 1983; Metzger and Barnes, 2009). Concentrations can increase in response to stress during growing (Seljåsen *et al.*, 2013a) and can change over the growing season (Kjellenberg, 2007; Kjellenberg *et al.*, 2010). After harvesting, polyacetylene concentrations can increase or decrease due to storage conditions and length of storage (Hansen *et al.*, 2003; Kidmose *et al.*, 2004a; Kjellenberg *et al.*, 2012). Once the vegetable has been grown, transported and purchased by the consumer, the vegetable can be subject to further processing which could affect the polyacetylene concentrations. Boiling, blanching, and freezing have all been shown to have large effects on retention of the compounds (Hansen *et al.*, 2003; Rawson *et al.*, 2010a; Rawson *et al.*, 2010b; Koidis *et al.*, 2012; Rawson *et al.*, 2012b). The preparation/source of foods will therefore affect the amount of polyacetylene consumed.

When investigating whether certain compounds have the potential to have a health effect, it is important to know how they are processed in the body. If the compounds are thought to affect the tissues of the colon, are the compounds retained in the gut? If it is speculated that they affect tissues throughout the body, are they absorbed and detectible in blood plasma or other tissues?

In vitro experiments have described the potential effects of the compounds in certain cells of the body (Chapter 1, Table 1.2) and *in vivo* studies in rodents have begun to investigate which of the *in vitro* effects may actually occur (Kobaek-Larsen *et al.*, 2005; Garti, 2016; Kobaek-Larsen *et al.*, 2017), specifically in the gut. This allows us to speculate that health benefits may also occur in humans.

There are many questions about the polyacetylenes throughout the food chain that remain unanswered. The aim of this thesis was to investigate the issues underlined in Figure 6.1 as follows:

- determine the amount of polyacetylenes in foods as they are consumed in the home to create a database (chapter 2);
- investigate the most important sources of polyacetylenes in the diet in a UK population (chapter 2);

- determine the effects of selected processing methods (the effects of boiling, frying and shapes of carrot) on polyacetylenes (chapter 3);
- determine whether polyacetylenes are detectible in blood plasma after consumption of boiled carrot (chapter 4);
- investigate the effect of regular carrot consumption on biomarkers of disease risk in humans (chapter 5).

6.3 Summary of the main findings of the research

6.3.1 *Amount of polyacetylene in foods and determining important sources of polyacetylenes*

The database created in chapter 2 has attempted to create a comprehensive list of polyacetylene containing foods, combining literature and new experimental data. The database includes data on polyacetylene values of commonly eaten foods that had not previously been investigated so that there are now estimates of polyacetylene content in mixed meals such as soups, stews and ready meals, as well as expanding on already investigated vegetables such as carrot, celery and parsnip, and including new preparations of those vegetables. The work highlights the importance of the Apiaceae vegetables, especially boiled carrot, as a source of polyacetylenes in a UK diet. This preparation of polyacetylene containing food was chosen going forward in the dietary intervention studies as it was the most common source of polyacetylene. This indicated it would be most acceptable to the consumer, and it was therefore used for the dietary intervention as it was assumed that compliance would be higher if the test food was well liked. The research would also be more generalisable to eating habits of the UK population. Whilst carrots are the most common source of polyacetylenes, there are high amounts of polyacetylene in parsnip and celery, and even some mixed meals deliver large amounts of polyacetylene per portion, so these foods should not be overlooked as good sources of polyacetylene.

The database, combining published data with further experimental data, is now available as a tool for epidemiological studies and was used in chapter 2 to estimate the intake of polyacetylenes in a UK population. The dietary data suggested that mean polyacetylene intakes amount to around 1.36mg/day. Further work is needed to translate this figure into a meaningful recommendation.

6.3.2 *The effect of processing on polyacetylene concentrations*

Both the database and the cooking experiment showed that vegetables that were more highly processed, such as canning and freezing, had lower concentrations of polyacetylenes. The processing experiments, simulating cooking carrots from the raw fresh vegetable, demonstrated that polyacetylenes are reasonably well retained during mild cooking and processing. Boiling and frying had similar effects on polyacetylene retention but boiling was better for carotenoid retention. These results suggest that polyacetylenes are reasonably heat stable at temperatures experienced during cooking. Longer cooking times may even 'increase' the concentration of polyacetylenes due to compositional changes in the carrot such as loss of water and of soluble solids.

Simple changes in processing can mitigate the losses seen during boiling and frying and it is recommended to cook carrots whole to reduce the surface area through which losses may occur. Polyacetylenes, but not carotenoids, are detectible in water and oil from cooking and therefore using the cooking water or oil in a meal may increase intake of polyacetylenes. The polarity of compounds could affect their stability in either water or oil but this needs further clarification. Some health promoting compounds do not survive the full lifecycle of a food and suffer significant losses during cooking, such as water-soluble vitamin C (Rickman *et al.*, 2007a; Miglio *et al.*, 2008), heat unstable polyphenols (Rickman *et al.*, 2007a) and the isothiocyanates which rely on an enzyme for bioconversion that would be denatured during cooking (Rouzaud *et al.*, 2004). It is therefore promising for the potential health benefits of polyacetylenes that they are reasonably retained during common processing, especially in the most common source of polyacetylenes, boiled carrots. In some epidemiological studies, raw carrots appeared to be more protective than cooked carrots (Chan *et al.*, 2005) but in others, the converse seemed to be true (Longnecker *et al.*, 1997; Zeegers *et al.*, 2001) which could be a balance between the heat destruction of bioactives and the increased digestibility/absorption of bioactives when the cell matrix is broken down during cooking. More work needs to be conducted to investigate how the processing methods can affect digestibility and bioavailability as some phytochemicals, such as β-carotene appear to be more available after mild processing.

The work in chapter 3 demonstrates that calculating phytochemicals in the carrot after cooking according to their raw weight gives a more accurate estimation of the changes in phytochemical in the carrot. It is therefore recommended that future studies looking at the

effect of processing on phytochemicals should take into consideration the change in dry weight of the vegetable.

6.3.3 *Absorption of polyacetylene*

The results from chapter 4 suggest that polyacetylenes (falcarinol and falcarindiol-3-acetate) are detectible in the blood after consumption of a manageable amount (100g) of whole carrots. Although falcarinol has been detected in blood plasma after consumption of carrot juice, this is the first study that has detected falcarinol and falcarindiol-3-acetate in blood plasma after consumption of whole carrot. There is a peak in falcarinol and falcarindiol-3-acetate concentration in blood plasma after breakfast, as expected, as well as an after-lunch effect with compounds increasing in peak area after eating a polyacetylene-free meal, which suggests that the compounds are fat soluble and the fat in the lunch time meal is important for absorption and can increase bioavailability. Not all participants had detectible changes in blood plasma polyacetylenes, and changes in concentration were very different between individuals after carrot consumption. The polyacetylene is removed from the blood, returning to baseline over the course of 24 hours. There appears to be little benefit of a larger dose of carrots compared to a normal amount on exposure to falcarinol or falcarindiol-3-acetate and therefore the dietary advice of consuming moderate amounts of fruit or vegetables but with a large variety appears to be justified (Conklin *et al.*, 2014; Publich Health England, 2016).

6.3.4 *Potential health effects*

The second human study, the dietary intervention, showed that 100g of carrot, when eaten regularly (every day), can lead to a moderate reduction in a marker of inflammation, PGEM ($p=0.07$) in healthy adults compared with a washout period where no carrots were eaten. However, no effect on lymphocyte DNA damage was detected in this study. The anti-cancer actions of the polyacetylenes in healthy humans could be related to their ability to reduce eicosanoid-related inflammation. As prostaglandin E_2 has been implicated in the development of cancers (Eberhart *et al.*, 1994; Rizzo, 2011; Tuncer and Banerjee, 2015), and decreased survival rates (Wang and DuBois, 2010). Blocking production of PGE_2 can decrease incidence of some types of cancer (Flower, 2003; Bosetti *et al.*, 2012; Tuncer and Banerjee, 2015) and suppress cancer growth (Nakanishi *et al.*, 2008). The lowering of PGE_2 production is therefore seen to be beneficial in reducing cancer risk and therefore regular consumption of a source of polyacetylene-rich vegetables may be able to reduce the risk of cancer in healthy humans.

The biomarker of DNA damage chosen to be examined in the dietary intervention may not have been adequate to investigate the anti-cancer effects of polyacetylenes in a healthy population. Suggestions of other potential biomarkers are discussed below.

The results from chapter 5 can be combined with previous studies to build a picture of the potential mechanisms of the effects of polyacetylenes on cancer risk. Figure 1.4 from chapter 1 summarises the potential modes of action that dietary components can have in cancer initiation, progression and potential resolution (Johnson, 2007) and the polyacetylenes/carrots have demonstrated the potential to act in these areas as summarised below.

Detoxifying enzymes: Falcarinol and falcarindiol upregulate detox molecules SOD, GSH and catalase, *in vitro* (Young *et al.*, 2008; Ohnuma *et al.*, 2009; Uto *et al.*, 2015; Živković *et al.*, 2016) but carrot juice could not achieve this *in vivo* (Lee *et al.*, 2011; Butalla *et al.*, 2012).

Inhibitor of carcinogens (ROS)/proliferation inhibitor: Reduces infection induced inflammation (IL-6) and therefore the production of ROS released by macrophages in the destruction of invading pathogens *in vitro* (Shiao *et al.*, 2005; Uto *et al.*, 2015). Decreasing oxidative stress *in vivo* (Potter *et al.*, 2011b). Decreasing ROS means cancer cells are unable to take advantage of the high ROS environment to hijack growth factors, encourage angiogenesis and enable cells to cross tissue boundaries (Coussens and Werb, 2002).

Proliferation inhibitor: Falcarinol and falcarindiol reduce PGE_2 through COX *in vitro* (Baba *et al.*, 1987; Prior *et al.*, 2007; Um *et al.*, 2010) and PGEM was reduced after white carrot consumption *in vivo* (trend p=0.07) in this trial, therefore potentially preventing proliferation and ability to metastasize.

Modifier of differentiation and apoptosis: Falcarindiol, falcarindiol and falcarindiol-3-acetate are cytotoxic to cancer cells *in vitro,* by stimulating apoptosis (Kuo *et al.*, 2002; Young *et al.*, 2007; Sun *et al.*, 2010; Um *et al.*, 2010; Jin *et al.*, 2012; Zaini *et al.*, 2012).

Modulators of DNA stability and repair: From the dietary intervention we observe no effect on basal DNA damage in lymphocytes (Chapter 5) which agrees with the research by Pool-Zobel (1997) and Astley (2004) where DNA damage is not seen to change after consuming carrots or carrot juice (Pool-Zobel *et al.*, 1997; Astley *et al.*, 2004) but DNA repair might be improved (Astley *et al.*, 2004). However, carotenes are confounding in these trials.

Increased effectiveness of chemotherapy drugs: Falcarinol, falcarindiol and falcarindiol-3-acetate have all improved effectiveness of the chemotherapy drug 5-flurouracil and inhibits a transporter that removes chemotherapy drugs from cells (Jin *et al.*, 2012; Tan *et al.*, 2014).

Cytotoxicity to cancer cells: The ability of polyacetylenes to specifically induce apoptosis in cancer cells whilst leaving healthy cells intact shows that the polyacetylenes may have anti-cancer effects that are independent of their ability to affect the DNA repair ability. *In vivo* rodent trials describe lower incidences of intestinal tumours after carrot, falcarinol, or falcarinol and falcarindiol feeding in genetically predisposed or AOM-induced rodents (Kobaek-Larsen *et al.*, 2005; Saleh *et al.*, 2013; Garti, 2016; Kobaek-Larsen *et al.*, 2017). The precise mechanism of action of cytotoxicity cannot be inferred by the methods used in these trials, however, the results highlight that the lack of effect in healthy populations does not mean that there might not be an effect in a cancer model.

6.3.5 *Main strengths and limitations of the research*

The database created in chapter 2 is a comprehensive accumulation of the current appropriate literature values, which has been further strengthened by experimental work in the thesis to include mixed meals and other preparations of vegetable. This results in a useful tool to use for predicting intake. Predictions of intake were made using the polyacetylene database from a population of people 85 years old and may not be representative of the UK population as a whole. Therefore, preparation of carrot may be different to reflect changing tastes or preferences.

The carrot cooking experiment attempted to measure phytochemicals in cooking fluid to help to determine the different avenues of losses during cooking. This demonstrated that polyacetylenes but not carotenoids are retained within the cooking fluid. It also estimated the content of phytochemicals by both the cooked weight and raw weight, this reflects the amount in the carrot and can help to estimate losses and therefore how ‘damaging’ each method of processing was to phytochemicals, allowing recommendations about how to prepare vegetables to preserve phytochemicals. The methods used could not determine how losses occurred if the amount in the vegetable and amount in the fluid did not equate to the amount in the raw. Other changes during cooking such as oxidation or improving extraction were unable to be determined using the current methods. Exploration of the effect of

vegetable shape on the retention of compounds has led to a simple recommendation to change processing to improve the health benefits of the carrot.

The bioavailability study differs from other bioavailability investigations because whole vegetables were used, rather than juice, and is therefore much more reflective of the intake of carrots, and therefore polyacetylenes, in the UK. As two of the volunteers did not have a blood plasma response, it would have been beneficial to recruit more people to the trial so as to observe the absorption profiles of a larger number of responders. A limitation to this trial was that without a standard of adequate purity, it was not possible to quantify the concentration of these compounds but it is promising for the potential biological effects of the polyacetylenes that these compounds are detectible *in vivo*. It would also have been beneficial to analyse the faceal and urine samples collected during the study to attempt to perform a mass balance of the polyacetylenes.

This work attempted to investigate the effect of polyacetylenes on the risk of cancer. The use of white carrot, while not commonly consumed in the UK, allowed the research to draw some conclusions about the effect of carrots on disease risk that excluded the effect of β-carotene. Supplement studies could be used in lieu of carrots to exclude the effects of other components of the carrot but supplement studies should be treated with caution as the polyacetylenes can be toxic, albeit at concentrations much higher than those found naturally. Supplementation of other dietary components, such as β-carotene and vitamin E, have had adverse effects in the past (Omenn *et al.*, 1996) and may not adequately reflect the effect that would occur if the compound was eaten as part of a whole food.

As with many dietary intervention studies, a specific subset of a population will be used to investigate effects of a particular dietary factor, to reduce the variation within the population being studied. Whilst this is justifiable within the limits of a particular study, it makes the findings hard to generalise to the wider population. There are numerous factors that could affect food choice, digestion, absorption and metabolism and these should be considered before trying to apply the findings of the research on food choice/intake, bioavailability and the effect of consumption on biomarkers to other populations.

6.4 Implications and recommendations for research and nutritional practice

The ability of whole carrot to have a small effect where juice studies did not show much effect on inflammation suggests that the choice of foods/drink matrix that are used in dietary interventions are important and it cannot be assumed that all sources of a phytochemical will have the same effect. This is especially pertinent with compounds such as the polyacetylenes and carotenoids which are sensitive to oxidation and UV degradation and may have a different stability within a vegetable and in a juice or extract.

The results from chapter 3 demonstrate that the calculation of loss/retention of compounds during processing can differ markedly depending on whether the loss of dry weight in vegetables was taken into consideration. It is recommended that future studies looking at the effect of processing on concentration of phytochemicals takes this change into account as the concentration of the vegetable per weight may not wholly reflect the losses of compounds, and can even appear to increase concentration of compounds.

If future work indicates that polyacetylenes can have a health effect, either for inflammation or cancer risk, these compounds should be considered to be confounding variables in studies investigating these diseases.

6.5 Future studies

While this research aimed to address some of the gaps in the knowledge of polyacetylene research, it is obvious that there is a long way to go before there is a comprehensive understanding of how polyacetylene can affect health. Further work is suggested in the following areas:

6.5.1 *Expanding the database*

The database can be added to, expanding the breadth of vegetables that are included, for example fennel and celeriac, that may be more appropriate to other populations, as more data is generated on polyacetylene content of foods and drinks. It could even be expanded to include ‘herbal’ preparations such as ginseng. Differently processed vegetables can also be added to the database to strengthen the information on the effect of certain processes on vegetables, e.g. microwaving and steaming, and including other preparations such as stir-frying.

As the experiment on cooking carrots (chapter 2) has shown, there can be large variability in polyacetylene concentrations among carrots in shop bought material (and potentially also in other material) which would require larger sets of samples to be analysed. This should be taken into consideration, not only when analysing foods to expand the database, but also in any further investigations into the effect of cooking on polyacetylene content. Obtaining vegetable samples from a more uniform source, such as harvesting material from the same part of the field and on the same day, could be considered before undertaking these sorts of experiments to ensure variability in starting material is kept low and any variability seen is as a result of the cooking conditions.

As there is more information gathered on whether polyacetylene can affect health and, if so, how much polyacetylene is needed to have an effect, the population intake can be used to make recommendations about whether populations in the UK are consuming enough to gain a health benefit. Comparison could also be made to other populations, for example Mediterranean diets that are more likely to include vegetables such as fennel which were not found in the UK population studied, or the diets of populations more likely to regularly consume herbal remedies or teas such as ginseng.

6.5.2 *Use of the database in prospective cohorts*

The database of polyacetylene-containing foods is an extremely useful tool to investigate potential associations between consumption of these foods and health conditions. Using databases of food intake and disease outcomes that have already been collected would be a convenient way to investigate associations of polyacetylene intake and disease state. The database can be used in large prospective cohort studies that have recorded dietary intake and health outcomes. The intake of polyacetylene for individuals could be estimated and this could be used to correlate intake of polyacetylene with disease outcomes.

Future research studies investigating the effect of lifestyle on disease risk should consider polyacetylene, or polyacetylene-rich vegetables, as a confounding factor. The polyacetylene database will be a useful tool in estimating polyacetylene intake in research cohorts.

6.5.1 *Investigating the fate of compounds during digestion*

A lot more work could be done to determine the fate of compounds in the body during digestion, metabolism and excretion. This research showed that they were able to be detected in the blood plasma, though plasma concentrations were unable to be determined. However, little is known about how the polyacetylenes are affected by the conditions they would be exposed to during digestion, or how bioavailability is affected by the food matrix, or whether the vegetable is eaten raw or cooked. *In vitro* digestion experiments could investigate how they react to environments like the stomach acid, whether other food components, such as different types of fats or fibre, affect their incorporation into micelles, and how they are affected by colonic fermentation. The colonic bacteria could produce enzymes that help to break down the polyacetylenes, and this would be of particular interest in investigating the unexpectedly high levels of falcarindiol-3-acetate compared to falcarinol in the blood plasma.

Falcarindiol has been shown to affect inflammation more than falcarinol (and falcarinol intake did not significantly increase during the carrot intervention) but as falcarindiol was unable to be detected in blood plasma in the bioavailability trial, this raises questions about which of the polyacetylenes, if any, was eliciting the reduction in PGEM. Further work on the anti-inflammatory effects of falcarindiol-3-acetate are of interest as this compound was detected in the highest amounts in the blood. The lack of adequate standards could mean that falcarindiol was not properly identified in the blood plasma, or it could be that this compound may not reach the blood plasma during digestion. As falcarindiol has been shown to be cytotoxic to colon cancer cells *in vitro*, the lack of absorption could be beneficial to gut health if the falcarindiol remains in the gut, however, there is not enough information on the stability of polyacetylenes through the gut to know if they are unchanged during the passage through the digestive tract. A mass balance approach using faecal samples could attempt to investigate the fate of these compounds in the GI tract.
The polarity of compounds could affect their stability in either water or oil, as suggested by the cooking experiment, and this could affect their digestion, which may differ between the different polyacetylenes, but this needs further clarification.

Investigations to determine why polyacetylenes were not detected in the blood of some of the participants in the bioavailability trial and why there may be differences in response would also be of interest.

6.5.2 *Investigating the fate of compounds after digestion: metabolism and excretion*

The presence of falcarinol and falcarindiol-3-acetate in blood plasma after consumption of whole carrot indicates that polyacetylenes survive digestion and are absorbed, however we do not know what happens to the compounds after entering the blood. Further investigations into the pharmacokinetics of these compounds would help us to better understand whether the polyacetylenes can reach tissues of interest, such as those in which the risk of cancer is lower with increasing carrot consumption Table 1.1. It is not known if they are taken up into tissues, whether they are stored or quickly removed from the body, or how they are metabolised and excreted. To investigate whether they are taken up into tissues, *in vitro* studies could first be conducted with cells from the tissue of interest to see if the compounds can be absorbed into the cells. Animal feeding studies could then be used to try to detect the polyacetylenes in tissues after supplementing their diet with carrot. This would give an indication of whether polyacetylenes are stored in the body tissue as well as whether they can be detected in the tissues where cancer risk has been seen to be reduced after consumption of carrot, for example, colon, breast, lung and bladder. While animal studies offer a model of human metabolism, investigations in humans would be of interest but would require invasive biopsy samples which would be much harder to conduct. Isotope labelling of compounds could instead be used for this purpose which would help to investigate the distribution, metabolism and elimination of these compounds in humans, and their potential sites of action.

How the polyacetylenes are metabolised will also allow the investigation of whether the blood plasma polyacetylene levels reflect overall intake of polyacetylenes. For the carotenoids, and other components such as calcium, the blood plasma levels do not adequately reflect the nutritional status as plasma levels are tightly regulated. It is not known how fasting polyacetylene concentrations correlate to intake.

6.5.3 *Different ways of analysing the effect of anti-cancer mechanisms in healthy volunteers*

Using % tail damage in basal lymphocytes as a marker of DNA damage/repair may not have been adequate to investigate the anti-cancer effects of polyacetylenes in a healthy population. Other ways that the effects of phytochemicals on cancer risk could be explored in healthy

populations include analysing changes in endogenous antioxidant expression, determining whether there are changes in repair ability of DNA in lymphocytes after an oxidative stress in ex vivo experiments. Mitochondrial DNA damage can also be investigated as the DNA from mitochondria is less able to repair itself that DNA from cells.

Investigations into the effect of polyacetylenes in feeding studies with rodents that are genetically predisposed to colon cancer have shown promising anti-cancer effects. The carrot feeding affected the size of tumours, rather than the number of initiated tumours which indicates the effect of polyacetylenes is likely to be in initiated cancer cells rather than healthy cells. As the dietary intervention in this thesis used healthy volunteers, an effect on DNA damage may not have been seen and therefore future work in a clinical setting would be of interest. Future work could explore the clinical impact of consuming polyacetylene-rich foods in patient groups as discussed further below.

6.5.4 *Health effects of falcarindiol-3-acetate*

Future research could further investigate the effect of falcarindiol-3-acetate for anti-inflammatory and anti-cancer affects *in vitro.* The low levels of this compound in vegetables in comparison to the other two polyacetylenes could mean it has been overlooked in the past but the little amount of research that has looked into its effect *in vitro* shows promising anti-inflammatory and cancer properties (Metzger *et al.*, 2008; Zaini *et al.*, 2012). If it is metabolised in the body from the other two compounds it could be the main bioactive polyacetylene from carrot.

6.5.5 *The effect of polyacetylenes in clinically relevant participants*

Anti-inflammatory effects:

Research using healthy participants is useful to investigate the effects of an intervention in potential prevention of disease states, and it is easier to conduct a trial in participants with no underlying disease state for ethical reasons and ease of recruitment. However, the biomarkers chosen to be measured are often within the healthy range and therefore any differences seen are very small. In patient groups, such as those with chronic inflammation, there may be a more significant benefit of consuming carrots as baseline levels of inflammation are higher than in the healthy population. Dietary interventions in patients with chronic inflammatory

conditions such arthritis could be used to investigate the lowering of inflammatory markers in people with higher overall inflammation.

Anti-cancer effects:

It would be of interest to investigate the effect of consumption of polyacetylene-rich foods on potential changes in disease status in patients undergoing standard cancer treatment such as radiation or chemotherapy. A comparison could be made between those undergoing standard treatments whilst consuming a portion of carrots a day compared to standard treatment alone. This could be used to assess effectiveness of therapy with and without polyacetylene-rich vegetable supplementation. Previous research has suggested that polyacetylenes can improve the effectiveness of chemotherapy drugs so research in patient groups could assess whether this also happens *in vivo* during regular chemotherapy treatment.

Further animal experiments could be conducted using different models of cancer. Colonic cancers could be more likely to be affected by anti-cancer compounds from food as they are directly exposed to the compounds in the food bolus, regardless of whether the compounds are absorbed into the blood. However, carrot consumption has been seen to affect cancers of other tissues, not directly exposed. A rodent model of breast or other tissue cancers, if available, could be used to investigate this. This may also tell us something about the ability of the compounds to move from the blood plasma into tissues which is currently unknown.

6.5.6 *Amount of polyacetylene needed to consume to have a health effect*

Following directly on from the work in this thesis, adequate purification of standards would enable the quantitation of polyacetylene in the blood plasma samples from this trial to estimate the concentrations in the blood and thus the concentrations that tissues are exposed to. Additionally, analysis of the faecal and urine samples that have already been collected during this work could be examined to estimate the amount of polyacetylene to which GI and urinary tract tissues are exposed. Investigations could then be conducted to determine whether these concentrations could have a health effect. This would allow suggestions for the recommended amount of polyacetylene in the diet and how much of a polyacetylene containing food is required to provide this amount.

In epidemiological studies that specified the number and portion size of servings of carrots that gave a protective effect, the amount of carrots consumed is a manageable amount to

regularly consume, around 25g/day (Zeegers *et al.*, 2001; Zhang *et al.*, 2009; Fallahzadeh *et al.*, 2015).

6.5.7 *Interactions of polyacetylenes with other phytochemicals*

There is currently little known about how the polyacetylenes interact either with each other, or with other compounds in carrots or in the diet in general. Work to explore the interactions between the polyacetylenes should be conducted to determine if their activity is enhanced when they are present in combination with each other and at what ratios. Also, whether their activity is enhanced in combination with other phytochemicals, particularly those phytochemicals found in carrots, such as carotenoids.

Carrots offer a cheap, readily available source of polyacetylenes that have demonstrated a modest lowering of a biomarker of disease risk with a normal intake. This thesis, in combination with previous studies, builds a compelling case for the anti-cancer effects of the polyacetylenes.

Chapter 7. **References**

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Chapter 8. **Appendix A**

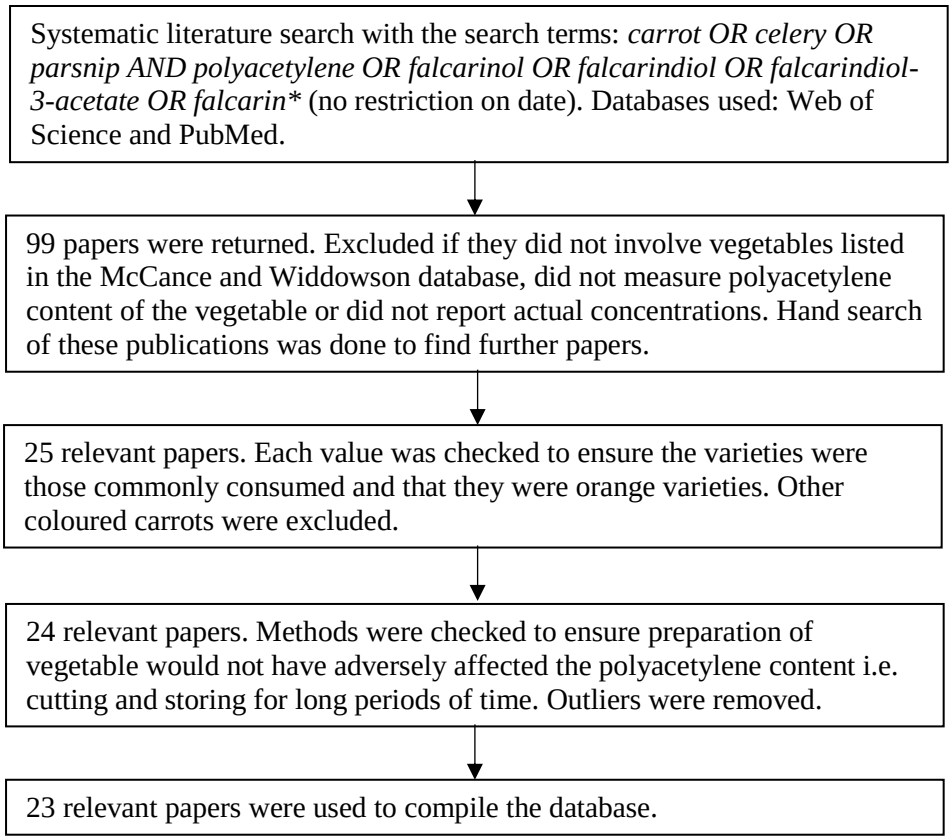

Figure 8.1: Flow chart to describe the Systematic Review Protocol and selection of data points for the polyacetylene database.

Table 8.1: Individual sample values of polyacetylene concentrations from experiment

		Concentration (mg/kg) in sample			
85+ Food code	**Food description (MW[1] food code)**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
13202	Carrots, boiled in unsalted water (13-497)	17.91	32.56	5.42	55.89
		25.20	20.01	8.11	53.31
		16.73	16.78	3.59	37.09
		14.22	14.21	3.14	31.57
		30.06	25.97	5.84	61.87
		26.00	14.47	9.06	49.53
		44.06	24.09	14.98	83.13
		25.03	15.88	8.49	49.40
		37.80	18.77	11.28	67.84
		60.52	35.22	20.26	116.00
		30.09	17.64	8.98	56.71
		32.83	13.90	11.18	57.91
		36.41	18.86	10.60	65.86
		37.05	21.04	10.09	68.19
		35.19	22.90	11.13	69.23
		43.28	15.35	10.46	69.09
	Mean	32.02	20.48	9.54	62.04
	SD	11.35	6.16	4.08	18.66
	SEM	2.84	1.54	1.02	4.77
13201	Carrots boiled in salted water (13-497)	17.96	32.47	7.31	57.74
		23.68	15.29	5.93	44.90
		12.53	18.11	4.35	34.98
		27.54	14.59	6.55	48.67
		16.40	37.04	6.66	60.10
		39.45	22.95	8.94	71.34
	Mean	22.93	23.41	6.62	52.95
	SD	8.85	8.56	1.38	11.68
	SEM	3.61	3.50	0.57	4.77
21559	Carrots, fresh microwaved (13-498)	60.45	32.98	11.86	105.30
		39.35	26.24	10.30	75.89
		20.32	27.89	7.18	55.39
		20.76	33.91	9.50	64.17
		36.37	67.36	13.11	116.84
		25.08	32.58	7.11	64.77
		86.42	32.73	12.91	132.05
		88.47	75.17	27.78	191.42
		38.30	72.36	20.43	131.10
		14.14	74.34	17.26	105.74
	Mean	42.96	47.56	13.74	104.27
	SD	25.49	20.42	6.13	39.51
	SEM	8.06	6.46	1.94	12.49

Table 8.1 cont'd		Concentration (mg/kg) in sample			
85+ Food code	**Food description (MW[1] food code)**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
		13.40	15.56	5.89	34.85
		4.21	29.31	8.40	41.92
13207	Carrots, canned,	16.46	21.19	6.93	44.59
	reheated	15.66	18.86	5.46	39.98
	(13-653)	11.74	17.89	5.43	35.06
		23.41	39.66	7.82	70.88
		15.58	29.66	7.23	52.48
	Mean	13.17	23.99	6.52	43.67
	SD	5.91	7.64	1.16	12.17
	SEM	2.09	2.70	0.41	4.30
		11.66	17.72	4.72	34.10
		9.13	26.69	6.37	42.19
		25.81	53.84	14.79	94.44
13206	Carrots, frozen,	12.46	18.09	7.25	37.80
	boiled	11.11	16.16	7.17	34.43
	(n/a)	21.64	15.76	6.05	43.45
		12.33	19.05	6.40	37.78
		12.70	21.61	6.03	40.34
	Mean	14.60	23.62	7.35	45.57
	SD	5.47	11.89	2.91	18.73
	SEM	1.93	4.20	1.03	6.62
		37.23	23.01	12.08	72.31
		77.86	32.40	17.55	127.82
		47.14	31.68	17.62	96.44
		42.55	27.36	11.80	81.71
		58.09	30.25	20.22	108.57
13200	Carrots, raw	65.15	28.80	21.01	114.96
	(13-565)	41.84	32.72	12.55	87.11
		54.77	118.13	18.52	191.41
		79.22	57.74	24.68	161.64
		45.10	32.15	17.76	95.01
		54.10	30.08	19.96	104.14
	Mean	54.82	40.39	17.61	112.83
	SD	13.57	25.98	3.87	34.03
	SEM	4.09	7.83	1.17	10.26
13221	Celery, raw	22.07	112.06	0.00	134.13
	(13-451)	15.63	102.90	0.00	118.53
	Mean	18.85	107.48	0.00	126.33
	SD	3.22	4.58	0.00	7.80
	SEM	2.27	3.24	0.00	5.51
13222	Celery, boiled	5.32	22.45	0.00	27.77
	(13-544)	12.33	34.56	0.00	46.89
		15.56	35.15	0.00	50.71

		8.22	16.72	0.00	24.94
		5.29	18.26	0.00	23.55
		8.26	29.89	0.00	38.15
		26.78	81.11	0.00	107.88
13222	Celery, boiled	2.29	11.70	0.00	13.98
	Cont'd	3.24	12.40	0.00	15.64
	(13-544)	3.53	17.93	0.00	21.46
		3.42	17.99	0.00	21.42
		2.05	16.53	0.00	18.58
		4.66	10.71	0.00	15.37
		4.54	15.52	0.00	20.06
		5.07	16.44	0.00	21.51
	Mean	7.37	23.82	0.00	31.20
	SD	6.33	17.01	0.00	23.13
	SEM	1.63	4.39	0.00	5.97

Table 8.1 cont'd

		Concentration (mg/kg) in sample			
85+ Food code	**Food description (MW[1] food code)**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
		48.44	116.41	0.00	164.84
		113.43	195.89	0.00	309.32
		3.50	77.97	0.00	81.47
13314	Parsnips, boiled	46.36	81.11	0.00	127.47
	(13-454)	23.72	76.12	0.00	99.84
		17.71	56.25	0.00	73.96
		54.60	124.39	0.00	179.00
		26.78	81.11	0.00	107.88
	Mean	44.93	109.97	0.00	154.90
	SD	31.01	46.34	0.00	75.70
	SEM	10.34	15.45	0.00	25.23
		1.88	3.65	1.59	7.11
		8.13	14.05	2.68	24.86
13281/	Mixed vegetables,	10.97	18.27	5.24	34.49
21594	boiled	11.38	8.14	2.55	22.07
	(13-543)	21.67	4.43	6.46	32.55
		2.05	6.37	1.14	9.57
		5.49	7.81	1.25	14.56
		6.12	8.84	2.38	17.34
		14.79	53.84	25.81	94.44
		7.25	18.09	12.46	37.80
		7.17	16.16	11.11	34.43
		5.43	17.89	11.74	35.06
	Mean	8.53	14.79	7.03	30.36
	SD	5.33	12.88	6.97	21.82
	SEM	1.54	3.72	2.01	6.30
	Mixed vegetables,	1.61	5.39	2.15	9.14
21579	frozen, micro	7.82	13.82	2.21	23.85

	(n/a)	11.27	15.44	4.19	30.90
	Mean	6.90	11.55	2.85	21.30
	SD	4.00	4.41	0.95	9.07
	SEM	2.31	2.55	0.55	5.23

Table 8.1 cont'd

		Concentration (mg/kg) in sample			
85+ Food code	**Food description (MW[1] food code)**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
17283	Vegetable soup, homemade (17-684)	14.20	3.08	2.24	19.52
		14.26	3.85	4.27	22.38
		14.80	3.99	1.78	20.57
	Mean	14.42	3.64	2.76	20.82
	SD	0.27	0.40	1.08	1.18
	SEM	0.16	0.23	0.62	0.68
17284	Vegetable soup, canned (17-712)	14.35	4.72	3.20	22.26
		10.03	2.26	2.10	14.38
		2.89	0.86	0.12	3.88
		5.94	1.95	0.60	8.50
		1.48	1.41	0.91	3.80
		5.17	1.63	1.62	8.42
		3.05	1.61	0.89	5.54
		3.55	2.50	1.59	7.64
	Mean	5.81	2.12	1.38	9.30
	SD	4.05	1.09	0.91	5.83
	SEM	1.43	0.39	0.32	2.06
17264	Lentil Soup, homemade (17-808)	22.67	8.53	5.18	36.38
		20.14	4.80	5.80	30.74
		14.79	2.86	2.71	20.36
		4.82	5.03	0.44	10.29
		3.06	3.20	0.36	6.62
		4.76	3.12	0.41	8.29
		3.99	3.12	0.34	7.45
		4.20	3.19	1.19	8.59
		3.74	5.10	0.22	9.06
		5.43	4.58	3.53	13.54
		7.46	7.32	0.98	15.75
	Mean	8.64	4.62	1.92	15.19
	SD	6.77	1.77	1.97	9.57
	SEM	2.04	0.54	0.59	2.88
17260/ 17286	Powdered soup, vegetable (17-660)	0.18	0.47	0.18	0.83
		3.35	0.22	0.39	3.97
		3.63	4.05	0.45	8.14
		4.05	0.42	0.37	4.84
		7.65	3.29	0.60	11.54
	Mean	3.77	1.69	0.40	5.86
	SD	2.38	1.64	0.14	3.67
	SEM	1.06	0.73	0.06	1.64

Table 8.1 cont'd		Concentration (mg/kg) in sample			
85+ Food code	**Food description (MW[1] food code)**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**
		23.37	4.89	2.17	30.42
		37.01	13.60	10.30	60.91
		43.56	14.70	6.97	65.24
		6.78	10.12	1.30	18.20
19175	Beef stew, homemade (19-473,19-470)	4.75	6.38	0.80	11.93
		10.72	12.42	2.90	26.03
		4.26	5.12	1.00	10.38
		12.59	12.71	5.57	30.87
		11.25	13.90	1.85	27.00
		7.31	10.19	2.32	19.82
	Mean	16.16	10.40	3.52	30.08
	SD	13.20	3.54	2.95	17.83
	SEM	4.17	1.12	0.93	5.64
		10.59	0.76	0.49	11.84
19216	Shepherd's Pie, chilled (19-494)	16.60	1.09	0.39	18.07
		11.34	1.02	0.12	12.47
	Mean	12.84	0.95	0.33	14.13
	SD	2.67	0.14	0.16	2.80
	SEM	1.54	0.08	0.09	1.62
		16.84	5.09	2.70	24.64
15077	Coleslaw (15-635,15-636)	7.31	1.41	1.23	9.96
		27.93	3.65	3.12	34.70
	Mean	17.36	3.39	2.35	23.10
	SD	8.42	1.52	0.81	10.16
	SEM	4.86	0.88	0.47	5.87

[1] McCance and Widdowson, FaOH - falcarinol, FaDOH - falcarindiol, FaD3Ac - falcarindiol-3-acetate, PA-polyacetylene

Table 8.2: Combined database of literature and experimental mean concentrations of polyacetylenes in food.

		Concentration (mg/kg) fresh weight[1]				Reference
Food Code	**Food Description**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**	
13202	Carrots, old, boiled (13-497)	9.50			9.50	(Hansen *et al.*, 2003)
		13.50			13.50	
		32.02	20.48	9.54	62.04	Experiment
		22.93	23.41	6.62	52.95	Experiment
	Mean	**19.49**	**21.94**	**8.08**	**34.50**	
	SD	**8.73**	**1.47**	**1.46**	**23.26**	
	SEM	**4.36**	**0.73**	**0.73**	**11.63**	
13221	Celery, raw (13-565)	11.50	103.50	0.00	115.00	(Zidorn *et al.*, 2005)
		81.00	229.00	0.00	310.00	
		18.85	107.48	0.00	126.33	Experiment
	Mean	**37.12**	**146.66**	**0.00**	**183.78**	
	SD	**31.18**	**58.25**	**0.00**	**89.37**	
	SEM	**18.00**	**33.63**	**0.00**	**51.60**	
13314	Parsnip, boiled in unsalted water (13-454)	66.10	38.20	0.00	104.30	(Rawson *et al.*, 2010b)
		53.70	37.50	0.00	91.20	
		44.93	109.97	0.00	154.90	Experiment
	Mean	**54.91**	**61.89**	**0.00**	**116.80**	
	SD	**8.68**	**34.00**	**0.00**	**27.47**	
	SEM	**5.01**	**19.63**	**0.00**	**15.86**	
21559	Carrots, fresh, steamed/micro (13-498)	13.00	25.00	8.70	46.70	(Kidmose *et al.*, 2004a)
		9.50				(Hansen *et al.*, 2003)
		13.50				
		42.96	47.56	13.74	104.27	Experiment
	Mean	**19.74**	**36.28**	**11.22**	**75.48**	
	SD	**13.50**	**11.28**	**2.52**	**28.78**	
	SEM	**6.75**	**5.64**	**1.26**	**14.39**	
13206	Carrots, frozen (n/a)	9.50			9.50	(Hansen *et al.*, 2003)
		13.50			13.50	
		29.60	7.20	18.60	55.40	(Kreutzmann *et al.*, 2008)
		35.20	10.10	12.90	58.20	
		40.68	20.66	13.34	74.68	(Rawson *et al.*, 2012b)
		40.23	20.47	12.85	73.55	
		30.51	41.66	7.56	79.73	
		44.98	24.52	10.05	79.55	
		14.60	23.62	7.35	45.57	Experiment
	Mean	**28.76**	**21.18**	**11.81**	**54.41**	
	SD	**12.41**	**10.39**	**3.62**	**22.72**	
	SEM	**4.14**	**3.46**	**1.21**	**7.57**	

Table 8.2 cont'd		Concentration (mg/kg) fresh weight				Reference
Food Code	**Food Description**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**	
13200	Carrots, old, raw	29.00	24.00		53.00	(Zidorn *et al.*, 2005)
	(13-565)	44.98	24.52		69.50	(Rawson *et al.*, 2012b)
		27.70	15.83	5.55	49.08	(Koidis *et al.*, 2012)
		38.00				(Wulf *et al.*, 1978)
		36.00				
		32.00				
		47.00				
		28.10	69.00		97.10	(Yates *et al.*, 1983)
		28.20	59.30		87.50	
		22.20	59.90		82.10	
		24.00	53.30		77.30	(Yates and England, 1982)
		19.10	43.20		62.30	
			41.00		41.00	(Czepa and Hofmann, 2003)
			45.00		45.00	
		45.30	60.80	7.90	114.00	(Kjellenberg *et al.*, 2010)
		24.00	45.90	5.40	75.30	
		23.30	49.80	7.90	81.00	
		11.50	36.40	4.70	52.60	
		24.40	44.70	6.60	75.70	
		16.50	30.80	4.40	51.70	
		18.70	53.10	11.90	83.70	
		13.80	40.50	8.10	62.40	
		23.60	14.20	30.80	68.60	(Metzger and Barnes, 2009)
		60.20	36.80	109.00	206.00	
		56.60	22.80	48.40	127.80	
		55.20	25.10	60.50	140.80	
		18.80	23.80	15.40	58.00	(Killeen *et al.*, 2013)
		43.40	34.50	8.70	86.60	
		19.10	24.10	10.80	54.00	
		33.60	27.10	8.50	69.20	
		26.60	20.00	7.10	53.70	
		33.40	22.20	6.80	62.40	
		31.60	38.70	15.20	85.50	
		36.80	24.00	12.30	73.10	
		27.40	40.10	9.80	77.30	
		30.50	40.00	9.00	79.50	
		11.20	49.70	10.70	71.60	
		19.50	30.00	12.30	61.80	

		24.20	55.70	25.40	105.30	

Table 8.2 cont'd		Concentration (mg/kg) fresh weight				Reference
Food Code	**Food Description**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**	
13200	Carrots, old, raw cont'd (13-565)	27.50	31.10	12.60	71.20	
		2.50	18.80	7.90	29.20	
		38.10	15.70	5.10	58.90	
		16.20	0.00	0.80	17.00	
		24.80				(Hansen *et al.*, 2003)
		22.30				
		8.70	39.00	13.80	61.50	(Kidmose *et al.*, 2004a)
		4.40	19.30	8.90	32.60	
		7.00				(Pferschy-Wenzig *et al.*, 2009)
		7.60				
		8.50				
		10.80				
		10.90				
		10.90				
		11.20				
		12.40				
		13.00				
		13.70				
		13.80				
		15.30				
		15.80				
		16.70				
		17.60				
		18.00				
		20.20				
		20.30				
		21.60				
		23.10				
		25.20				
		25.60				
		26.00				
		28.90				
		32.20				
		37.30				
		40.60				
13200	Carrots, old, raw cont'd (13-565)		16.20			(Czepa and Hofmann, 2004)
			16.00			
			24.90			
			31.70			
			32.40			
			38.50			
			45.00			

			43.50			
			46.40			
Table 8.2 cont'd		**Concentration (mg/kg) fresh weight**				**References**
Food Code	**Food Description**	**FaOH**	**FaDOH**	**FaD3Ac**	**Total PA**	
			45.70			
			84.30			
			76.00			(Garrod *et al.*, 1978)
			93.00			(Garrod and Lewis, 1979)
		3.59	1.54	7.70	12.83	(Rawson *et al.*, 2010a)
		11.00	26.00	3.60	40.60	(Søltoft *et al.*, 2010)
		11.26	6.45	1.19	18.90	(Rawson *et al.*, 2011)
		25.36	4.82	18.81	48.99	(Rawson *et al.*, 2012b)
		5.52	7.37	2.83	15.72	(Aguiló-Aguayo *et al.*, 2014)
		54.82	40.39	17.61	112.83	Experiment
	Mean	**23.92**	**35.50**	**14.84**	**69.40**	
	SD	**12.86**	**19.06**	**19.38**	**33.92**	
	SEM	**1.46**	**2.48**	**3.14**	**4.95**	
13207	Carrots, canned, reheated (13-653)	13.17	23.99	6.52	43.67	Experiment
	Mean	**13.17**	**23.99**	**6.52**	**43.67**	
	SD	**5.91**	**7.64**	**1.16**	**12.17**	
	SEM	**2.09**	**2.44**	**0.41**	**4.30**	
13222	Celery, boiled (13-544)	7.37	23.82	0.00	31.20	Experiment
	Mean	**7.37**	**23.82**	**0.00**	**31.20**	
	SD	**6.33**	**17.01**	**0.00**	**23.13**	
	SEM	**1.63**	**4.39**	**0.00**	**5.97**	
13281/ 21594/ 21579	Mixed vegetables, boiled (13-543)	8.20	14.15	6.20	28.54	Experiment
	Mean	**8.20**	**14.15**	**6.20**	**28.54**	
	SD	**5.13**	**11.76**	**6.47**	**20.26**	
	SEM	**1.32**	**3.04**	**1.67**	**5.23**	
17264	Lentil Soup, homemade (17-808)	8.64	4.62	1.92	15.19	Experiment
	Mean	**8.64**	**4.62**	**1.92**	**15.19**	

	SD	**6.77**	**1.77**	**1.97**	**9.57**	
	SEM	**2.04**	**0.54**	**0.59**	**2.88**	

Table 8.2 cont'd		**Concentration (mg/kg) fresh weight**				**Reference**
Food Code	**Food Description**	**FaOH**	**FaDOH**	**FaDOH**	**Total PA**	
17283	Vegetable soup, homemade (17-684)	14.42	3.64	2.76	20.82	Experiment
	Mean	**14.42**	**3.64**	**2.76**	**20.82**	
	SD	**0.27**	**0.40**	**1.08**	**1.18**	
	SEM	**0.16**	**0.23**	**0.62**	**0.68**	
17284	Vegetable soup, canned (17-712)	5.81	2.12	1.38	9.30	Experiment
	Mean	**5.81**	**2.12**	**1.38**	**9.30**	
	SD	**4.05**	**1.09**	**0.91**	**5.83**	
	SEM	**1.43**	**0.39**	**0.32**	**2.06**	
17260/ 17286	Powdered soup, vegetable (17-660)	3.77	1.69	0.40	5.86	Experiment
	Mean	**3.77**	**1.69**	**0.40**	**5.86**	
	SD	**2.38**	**1.64**	**0.14**	**3.67**	
	SEM	**1.06**	**0.73**	**0.06**	**1.64**	
15077	Coleslaw (15-635,15-636)	17.36	3.39	2.35	23.10	Experiment
	Mean	**17.36**	**3.39**	**2.35**	**23.10**	
	SD	**8.42**	**1.52**	**0.81**	**10.16**	
	SEM	**4.86**	**0.88**	**0.47**	**5.87**	
19175	Beef stew, homemade (19-473,19-470)	16.16	10.40	3.52	30.08	Experiment
	Mean	**16.16**	**10.40**	**3.52**	**30.08**	
	SD	**13.20**	**3.54**	**2.95**	**17.83**	
	SEM	**4.17**	**1.12**	**0.93**	**5.64**	
19216	Shepherd's Pie, chilled (19-494)	12.84	0.95	0.33	14.13	Experiment
	Mean	**12.84**	**0.95**	**0.33**	**14.13**	
	SD	**2.67**	**0.14**	**0.16**	**2.80**	
	SEM	**1.54**	**0.08**	**0.09**	**1.62**	
Not eaten by Newcastle 85+ cohort	Parsnip, raw (13-312)	64.82	49.41	0.00	114.23	(Koidis *et al.*, 2012)
		74.20	49.00	0.00	123.20	(Rawson *et al.*, 2010b)
		25.00	175.00	0.00	200.00	(Acworth *et al.*, 2011)

Mean	**54.67**	**91.14**	**0.00**	**145.81**	
SD	**21.33**	**59.30**	**0.00**	**38.49**	
SEM	**10.66**	**29.65**	**0.00**	**19.25**	
Fennel, raw (13-241)	30.00	5.00	ND[1]	35.00	(Zidorn *et al.*, 2005)

FaOH - falcarinol, FaDOH - falcarindiol, FaD3Ac - falcarindiol-3-acetate, PA – polyacetylene, [1] Dry weights have been converted to fresh weights using the % dry weight from the cooking experiment – carrot 10%, parsnip 19.6% and celery 5%. Fennel dry matter was 7.4% determined from Koudela and Petříková (Koudela and Petříková, 2008), [2] not determined.

Table 8.3: Polyacetylene content per portion of food, full data.

Food code	Food description	*f* [1]	Ave. portion size (g)	FaOH	FaDOH	FaD3Ac	Total PA
13200	Carrots, old, raw	8	26	0.62	0.91	0.38	1.8
21559	Carrots, fresh, steamed/micro	19	65	1.28	2.35	0.73	4.9
13206	Carrots, frozen, boiled in unsalted water	15	63	1.80	1.33	0.74	3.4
13202	Carrots, old, boiled in and unsalted water	360	52	1.01	0.70	0.85	1.8
13206	Carrots, old, boiled in salted water	13	49	0.95	0.66	0.81	1.7
13207	Carrots, canned, reheated drained	4	135	1.77	3.23	0.88	5.87
13314	Parsnips boiled	24	57	3.14	3.54	0.00	6.7
13222	Celery boiled	6	38	0.28	0.90	0.00	1.19
13221	Celery raw	35	31	1.14	4.51	0.00	5.6
13281	Mixed vegetables, boiled salted water	57	67	0.73	0.65	0.25	1.63
21594	Mixed vegetables, boiled unsalted water	2	85	0.58	1.56	0.80	2.94
21579	Mixed vegetables, microwaved	24	98	0.68	1.13	0.28	2.09
17264	Lentil soup	15	219	1.89	1.01	0.42	3.33
17283	Vegetable soup	51	247	3.57	0.90	0.68	5.15
17284	Vegetable soup, canned	41	246	3.55	0.89	0.68	5.12
17260	Instant soup powder	20	206	0.78	0.35	0.08	1.21
17286	Vegetable soup, dried as served	20	211	0.80	0.36	0.08	1.24
15077	Coleslaw	40	52	0.90	0.17	0.12	1.19
	Coleslaw, reduced calorie	5	71	1.24	0.24	0.17	1.64

Food code	Food description	*f*	Ave. portion size (g)	FaOH	FaDOH	FaD3Ac	Total PA
19175	Beef stew	23	268	4.33	2.79	0.94	8.07
	Beef stew, lean beef	2	210	3.39	2.18	0.74	6.32
19216	Cottage/shepherd's pie, chilled/frozen	19	299	3.84	0.29	0.10	4.22

[1] frequency of consumption i.e. number of portions eaten, FaOH - falcarinol, FaDOH - falcarindiol, FaD3Ac - falcarindiol-3-acetate, PA - polyacetylene.

Table 8.4: Frequency of consumption of the food groups by Newcastle 85+ cohort.

Food group	Freq. of consumption[1]	Average (weighted) portion size (g)	Average g per person per day
Cooked carrot	407.0	52.5	13.5
Soups	147.0	207.0	19.2
Mixed meals	89.0	165.0	9.3
Mixed vegetables	59.0	67.8	2.5
Celery raw	35.0	30.7	0.7
Parsnips boiled	24.0	57.1	0.9
Raw carrot	8.0	25.8	0.1
Celery boiled	6.0	37.8	0.1

1- Number of portions eaten over 2 days.

Chapter 9. **Appendix B**

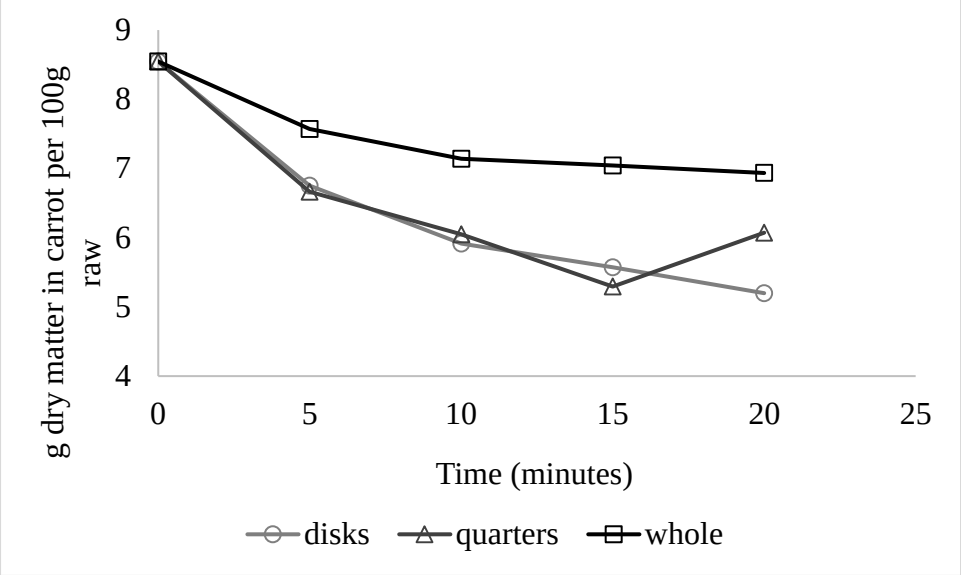

Figure 9.1: Mean amount of dry matter in carrot (g/100g raw) demonstrates the dry matter decreased over cooking time

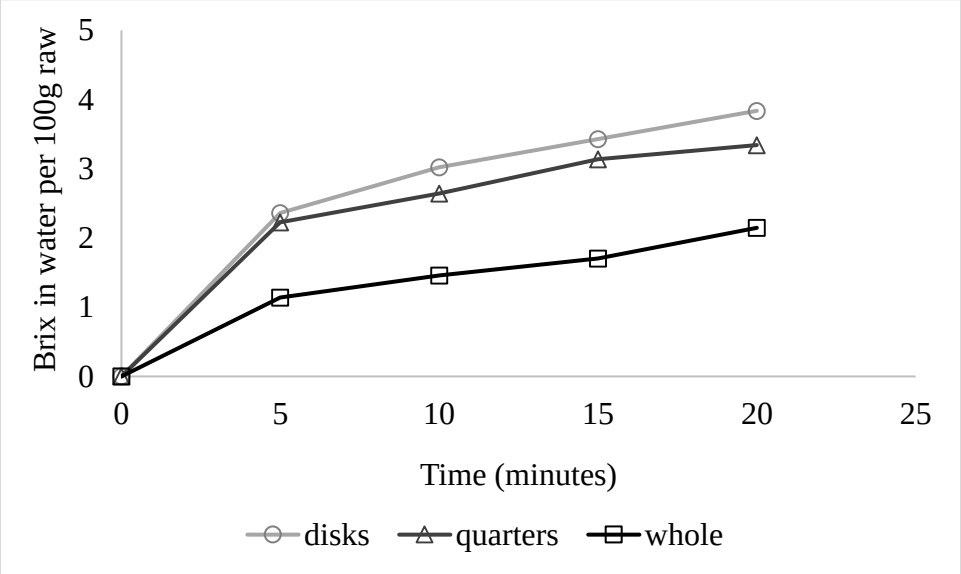

Figure 9.2: Mean Brix (^{o}Bx) in water after cooking (^{o}Bx/100g raw carrot) demonstrates the leaching of sugar into water over cooking time

Table 9.1: Mean concentrations of polyacetylenes in carrot after boiling

		Falcarinol concentration (mg/g cooked carrot)			Falcarindiol concentration (mg/g cooked carrot)			Falcarindiol-3-acetate concentration (mg/g cooked carrot)			Total polyacetylene concentration (mg/g cooked carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	5.53	0.94	a	2.88	0.12	a	1.77	0.42	a	10.17	1.44	a
Disk	5	3.51	0.61	b*	2.12	0.76	a	1.26	0.15	a	6.89	1.48	ab
	10	3.33	0.27	b*	1.82	0.35	a	1.09	0.26	a	6.23	0.70	b*
	15	3.74	0.30	ab	1.71	0.37	a	1.01	0.12	a	6.46	0.63	b*
	20	4.80	0.47	ab	1.94	0.77	a	1.25	0.10	a	8.27	0.66	ab
Quarter	5	3.54	0.21	b*	1.94	0.03	bc**	1.13	0.23	ab	6.61	0.04	b**
	10	3.68	0.08	b*	2.49	0.23	ab**	1.08	0.15	ab	7.25	0.43	b*
	15	3.77	0.41	b*	1.94	0.19	bc***	1.26	0.13	ab	6.97	0.59	b**
	20	3.72	0.47	b*	1.82	0.25	c**	0.93	0.07	b*	6.47	0.30	b**
Whole	5	4.98	2.29	a	2.47	0.52	a	1.35	0.33	a	8.80	3.07	a
	10	4.83	0.69	a	3.04	1.12	a	1.53	0.26	a	9.40	1.38	a
	15	5.24	0.52	a	2.61	0.38	a	1.49	0.14	a	9.33	1.42	a
	20	6.09	0.89	a	3.04	0.47	a	1.59	0.17	a	10.73	1.64	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.2: Mean concentrations of polyacetylene in water after boiling

		Falcarinol concentration (mg/g raw carrot)			Falcarindiol concentration (mg/g raw carrot)			Falcarindiol-3-acetate concentration (mg/g raw carrot)			Total polyacetylene concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (water)**	**SD**	**Sig. diff.[1]**	**Mean (water)**	**SD**	**Sig. diff.[1]**	**Mean (water)**	**SD**	**Sig. diff.[1]**	**Mean (water)**	**SD**	**Sig. diff.[1]**
Disk	5	0.38	0.19	a	1.01	0.65	a	0.19	0.11	a	1.58	0.94	a
	10	0.35	0.08	a	1.57	0.35	a	0.20	0.06	a	2.12	0.48	a
	15	0.23	0.08	a	1.42	0.50	a	0.13	0.03	a	1.79	0.45	a
	20	0.32	0.11	a	1.16	0.44	a	0.21	0.07	a	2.27	0.67	a
Quarter	5	0.31	0.02	a	1.16	0.16	a	0.19	0.03	a	1.67	0.20	a
	10	0.21	0.05	ab*	1.45	0.14	a	0.16	0.02	a	1.83	0.09	a
	15	0.20	0.07	ab*	1.25	0.28	a	0.12	0.03	ab	1.57	0.30	a
	20	0.12	0.01	b**	1.66	0.50	a	0.08	0.01	b**	1.87	0.50	a
Whole	5	0.20	0.03	a	0.69	0.29	a	0.11	0.01	a	1.00	0.28	a
	10	0.14	0.10	a	1.37	1.03	a	0.09	0.07	a	1.60	1.19	a
	15	0.08	0.08	a	0.97	1.02	a	0.05	0.05	a	1.10	1.15	a
	20	0.11	0.09	a	1.53	0.81	a	0.08	0.03	a	1.72	0.92	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.3: Mean concentrations of carotenoids in carrot after boiling

		Alpha carotene concentration (mg/g cooked carrot)			Beta carotene concentration (mg/g cooked carrot)			Lutein concentration (mg/g cooked carrot)			Total carotenoid concentration (mg/g cooked carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	3.35	0.33	a	9.74	1.15	a	0.05	0.00	a	13.14	1.44	a
Disk	5	3.58	0.38	a	9.63	0.82	a	0.05	0.00	a	13.26	1.20	a
	10	3.73	0.83	a	9.41	2.47	a	0.04	0.00	a	13.17	3.27	a
	15	3.31	0.89	a	11.03	2.45	a	0.04	0.00	a	14.38	3.30	a
	20	2.91	0.37	a	9.05	1.68	a	0.04	0.01	a	11.50	2.05	a
Quarter	5	3.45	0.41	a	9.05	1.02	a	0.05	0.01	a	12.55	1.27	a
	10	3.62	1.42	a	10.36	3.86	a	0.04	0.00	a	14.03	5.24	a
	15	3.25	0.90	a	9.41	2.30	a	0.04	0.00	a	12.70	3.09	a
	20	4.06	1.14	a	10.84	3.33	a	0.05	0.00	a	14.95	4.46	a
Whole	5	2.92	0.72	a	9.16	1.91	a	0.04	0.02	a	12.12	2.42	a
	10	3.97	0.46	a	10.71	1.34	a	0.05	0.00	a	14.73	1.75	a
	15	4.49	0.14	a	14.69	0.05	a	0.06	0.01	a	19.74	0.09	a
	20	5.50	1.96	a	16.08	5.27	a	0.06	0.00	a	21.64	7.20	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.4: Mean concentrations of polyacetylene in carrot after frying

		Falcarinol concentration (mg/g cooked carrot)			Falcarindiol concentration (mg/g cooked carrot)			Falcarindiol-3-acetate concentration (mg/g cooked carrot)			Total polyacetylene concentration (mg/g cooked carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	4.15	1.28	a	2.24	0.34	ab	1.21	0.21	a	7.61	1.69	a
Disk	5	5.63	1.54	a	1.92	0.28	b	1.17	0.32	a	8.72	2.01	a
	8	5.05	0.87	a	2.08	0.39	ab	1.45	0.17	a	8.57	0.32	a
	10	5.96	1.83	a	3.30	0.62	a*	1.71	0.53	a	10.97	2.92	a
Quarter	5	5.15	0.20	a	2.02	0.50	b	1.15	0.53	a	8.32	0.72	ab
	8	6.52	1.20	a	2.67	0.11	ab	1.56	0.30	a	10.75	1.58	ab
	10	7.05	0.38	a	3.15	0.21	a*	1.73	0.19	a	11.93	0.72	b*
Whole	5	5.46	0.38	a	1.24	0.23	b	1.32	0.19	a	8.02	0.72	a
	8	9.76	3.35	a	3.91	0.70	a**	2.06	0.11	a	15.73	4.08	a
	10	6.37	2.14	a	2.61	0.94	ab	1.64	0.55	a	10.63	3.40	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.5: Mean concentrations of polyacetylene in oil after frying of carrots

		Falcarinol concentration (mg/g raw carrot)			Falcarindiol concentration (mg/g raw carrot)			Falcarindiol-3-acetate concentration (mg/g raw carrot)			Total polyacetylene concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (oil)**	**SD**	**Sig. diff.[1]**	**Mean (oil)**	**SD**	**Sig. diff.[1]**	**Mean (oil)**	**SD**	**Sig. diff.[1]**	**Mean (oil)**	**SD**	**Sig. diff.[1]**
Disk	5	2.51	1.53	a	1.17	0.82	a	0.67	0.49	a	4.35	2.83	a
	8	2.84	1.46	a	1.69	1.02	a	0.88	0.48	a	5.41	2.94	a
	10	2.03	1.20	a	1.53	1.06	a	0.79	0.57	a	4.34	2.83	a
Quarter	5	2.48	0.10	a	1.44	0.21	a	0.65	0.10	a	4.58	0.12	a
	8	2.61	0.11	a	1.57	0.14	a	0.71	0.08	a	4.89	0.10	a
	10	2.16	0.38	a	1.43	0.29	a	0.67	0.11	a	4.25	0.72	a
Whole	5	0.91	0.38	a	0.35	0.29	a	0.22	0.11	a	1.48	0.72	a
	8	0.93	0.67	a	0.58	0.37	a	0.20	0.14	a	1.70	1.18	a
	10	0.25	0.12	a	0.19	0.10	a	0.11	0.05	a	0.55	0.26	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.6: Mean concentrations of carotenoid in carrot after frying

		Alpha carotene concentration (mg/g cooked carrot)			Beta carotene concentration (mg/g cooked carrot)			Lutein concentration (mg/g cooked carrot)			Total carotenoid concentration (mg/g cooked carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	2.18	0.20	a	5.35	0.79	a	0.04	0.00	a	7.56	0.58	a
Disk	5	3.34	0.72	a	10.29	3.30	a	0.07	0.02	a	13.70	4.03	a
	8	2.22	0.95	a	6.20	4.04	a	0.12	0.02	b**	8.54	4.97	a
	10	2.27	0.18	a	5.25	0.63	a	0.13	0.01	b**	7.65	0.82	a
Quarter	5	2.88	0.89	a	7.81	0.78	a	0.07	0.03	a	10.76	6.45	a
	8	2.88	0.10	a	8.58	5.60	a	0.10	0.03	ab	11.56	0.36	a
	10	1.94	0.91	a	4.54	0.33	a	0.15	0.01	b**	6.63	3.59	a
Whole	5	2.64	0.91	a	9.00	2.67	a	0.04	0.01	ab	11.68	3.59	a
	8	3.03	0.61	a	10.19	3.23	a	0.08	0.02	b*	13.30	3.81	a
	10	2.29	0.71	a	5.66	1.82	a	0.07	0.02	ab	8.03	2.55	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.7: Mean concentrations showing the retention of polyacetylenes in carrot after boiling compared to raw carrot

		Falcarinol concentration (mg/g raw carrot)			Falcarindiol concentration (mg/g raw carrot)			Falcarindiol-3-acetate concentration (mg/g raw carrot)			Total polyacetylene concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	5.53	0.94	a	2.88	0.12	a	1.77	0.42	a	10.17	1.44	a
Disk	5	3.18	0.56	b**	1.92	0.69	a	1.14	0.14	ab	6.24	1.36	b*
	10	3.02	0.29	b**	1.65	0.33	a	0.98	0.23	b*	5.66	0.68	b**
	15	3.33	0.26	b*	1.53	0.33	a	0.90	0.11	b*	5.77	0.63	b**
	20	4.26	0.42	ab	1.77	0.68	a	1.11	0.09	ab	7.34	0.62	ab
Quarter	5	3.24	0.19	b**	1.77	0.04	bc*** b **raw, *15	1.03	0.21	ab	6.04	0.04	b**
	10	3.33	0.09	b**	2.26	0.22	and *20	0.97	0.14	b*	6.56	0.43	b*
	15	3.33	0.34	b*	1.71	0.16	c***	1.11	0.11	ab	6.14	0.47	b*
	20	3.26	0.43	b**	1.60	0.22	c***	0.81	0.07	b*	5.67	0.29	b**
Whole	5	4.61	2.08	a	2.29	0.46	a	1.25	0.31	a	8.14	2.79	a
	10	4.43	0.60	a	2.80	1.09	a	1.41	0.27	a	8.63	1.41	a
	15	4.76	0.44	a	2.37	0.33	a	1.35	0.14	a	8.48	1.23	a
	20	5.49	0.73	a	2.74	0.40	a	1.43	0.15	a	9.66	1.43	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.8: Mean concentrations showing the retention of carotenoids in carrot after boiling compared to raw carrot

		Alpha carotene concentration (mg/g raw carrot)			Beta carotene concentration (mg/g raw carrot)			Lutein concentration (mg/g raw carrot)			Total carotenoid concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	3.35	0.33	a	9.74	1.15	a	0.05	0.00	a	13.14	1.44	a
Disk	5	3.24	0.35	a	8.72	0.75	a	0.04	0.00	ab	12.01	1.10	a
	10	3.39	0.79	a	8.56	2.36	a	0.04	0.00	ab	11.98	3.13	a
	15	2.96	0.83	a	9.86	2.32	a	0.04	0.00	ab	12.86	3.12	a
	20	2.58	0.34	a	8.27	1.53	a	0.04	0.00	b*	10.21	1.87	a
Quarter	5	3.16	0.39	a	8.27	0.57	a	0.04	0.01	ab	11.47	0.71	a
	10	3.27	1.28	a	9.38	2.02	a	0.04	0.00	ab	12.69	2.73	a
	15	2.86	0.80	a	8.30	1.16	a	0.04	0.00	b**	11.20	1.56	a
	20	3.55	0.99	a	9.49	1.67	a	0.04	0.00	ab	13.09	2.23	a
Whole	5	2.70	0.66	a	8.47	1.69	a	0.04	0.02	a	11.21	2.15	a
	10	3.63	0.37	a	9.79	1.07	a	0.05	0.00	a	13.47	1.39	a
	15	4.49	3.05	a	13.23	0.27	a	0.05	0.01	a	17.77	0.22	a
	20	4.96	1.28	a	14.48	4.69	a	0.05	0.00	a	19.49	6.42	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * p≤0.05), **p≤0.01, **p≤0.001

Table 9.9: Mean concentrations showing the retention of polyacetylenes in carrot after frying compared to raw carrot

		Falcarinol concentration (mg/g raw carrot)			Falcarindiol concentration (mg/g raw carrot)			Falcarindiol-3-acetate concentration (mg/g raw carrot)			Total polyacetylene concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	4.15	1.28	a	2.24	0.34	a	1.21	0.21	a	7.61	1.69	a
Disk	5	3.54	0.82	a	1.22	0.19	b**	0.74	0.16	ab	5.50	1.05	ab
	8	2.22	0.42	a	0.91	0.18	b***	0.64	0.08	b*	3.78	0.26	b*
	10	2.20	0.45	a	1.23	0.10	b**	0.63	0.13	b*	4.06	0.64	b*
Quarter	5	3.24	0.26	a	1.28	0.13	a	0.73	0.31	a	5.26	0.70	a
	8	3.01	0.66	a	1.23	0.15	a	0.74	0.13	a	4.98	0.92	a
	10	2.57	0.12	a	1.13	0.10	a	0.62	0.16	a	4.33	0.30	a
Whole	5	4.14	0.12	a	0.93	0.10	a	1.00	0.16	a	6.07	0.30	a
	8	7.22	2.69	a	2.88	0.60	a	1.51	0.11	a	11.61	3.36	a
	10	4.12	1.38	a	1.70	0.64	a	1.07	0.36	a	6.89	2.25	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * $p \leq 0.05$), **$p \leq 0.01$, **$p \leq 0.001$

Table 9.10: Mean concentrations showing the retention of carotenoids in carrot after frying compared to raw carrot

		Alpha carotene concentration (mg/g raw carrot)			Beta carotene concentration (mg/g raw carrot)			Lutein concentration (mg/g raw carrot)			Total carotenoid concentration (mg/g raw carrot)		
Shape	**Cooking time (mins)**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**	**Mean (carrot)**	**SD**	**Sig. diff.[1]**
Raw	0	2.18	0.20	a	5.35	0.79	a	0.04	0.00	a	7.56	0.58	a
Disk	5	2.12	0.45	a	6.55	2.03	a	0.04	0.01	a	8.72	2.48	ab
	8	0.99	0.47	b*	2.80	1.93	a	0.05	0.01	a	3.85	2.40	ab
	10	0.86	0.06	b*	1.97	0.10	a	0.05	0.00	a	2.88	0.15	b*
Quarter	5	1.80	0.44	ab	4.89	0.53	a	0.04	0.01	a	6.73	3.00	ab
	8	1.34	0.03	bc*	3.98	2.60	a	0.05	0.01	a	5.36	0.19	ab
	10	0.69	0.63	c**	1.63	0.17	a	0.05	0.01	a	2.38	2.48	b*
Whole	5	1.97	0.63	a	6.75	1.83	a	0.03	0.01	a	8.76	2.48	a
	8	2.22	0.45	a	7.45	2.29	a	0.06	0.02	a	9.73	2.71	a
	10	1.48	0.47	a	3.67	1.19	a	0.05	0.01	a	5.20	1.67	a

[1]Significant difference, means that share a letter are not statistically different from each other. Significant differences are shown between raw and 5-20 minute time point samples within shapes only, not between shapes. * p≤0.05), **p≤0.01, **p≤0.001

Chapter 10. **Appendix C**

10.1 **Ethical approval for bioavailability study**

15-WAR-23

Applicant Name:	Sarah Warner
Applicant email:	s.r.warner@newcastle.ac.uk
Academic Unit	School of Agriculture, Food and Rural Development
Supervisor email (if available)	kirsten.brandt@newcastle.ac.uk
Category	Student Project (PGR)
Project Title:	Absorption of Naturally Present Compounds in Carrots and their Appearance in Blood and other Biofluids after Consumption (The 'Carrot Study')
Start / End Date	02-03-2015 - 30-09-2014
MyProjects Reference (if available)	BH122642
Reviewer 1	
Name:	Jem Stach (BIO)
Date sent:	11/02/2015
Date comments received:	N/R
Reviewer 2	
Name:	John Vines (COMP)
Date sent:	09/03/2015
Date comments received:	24/03/2015
Date comments provided to researcher:	02/03/2015
Date researcher confirmed amendments made:	09/04/2015
Faculty final approval date:	23/04/2015
Notes	Satisfactory amendments received. Informal approval provided by email 13/04/2015.

1

10.2 **Ethics amendment to include participant aged 33 years**

15-WAR-23

Applicant Name:	Sarah Warner
Applicant email:	s.r.warner@newcastle.ac.uk
Academic Unit	School of Agriculture, Food and Rural Development
Supervisor email (if available)	kirsten.brandt@newcastle.ac.uk
Category	Student Project (PGR)
Project Title:	Absorption of Naturally Present Compounds in Carrots and their Appearance in Blood and other Biofluids after Consumption (The 'Carrot Study')
Start / End Date	02-03-2015 - 30-09-2014
MyProjects Reference (if available)	BH122642
Reviewer 1	
Name:	Jem Stach (BIO)
Date sent:	11/02/2015
Date comments received:	N/R
Reviewer 2	
Name:	John Vines (COMP)
Date sent:	09/03/2015
Date comments received:	24/03/2015
Date comments provided to researcher:	02/03/2015
Date researcher confirmed amendments made:	09/04/2015
Faculty final approval date:	23/04/2015
Notes	Satisfactory amendments received. Informal approval provided by email 13/04/2015. Chair of the ethics committee has approved the inclusion of a 33-year-old participant in the research. Approved 20/02/2018.

Approved / ~~Not Approved~~ by the SAgE Faculty Ethics Committee

Signed by Dr Patrick Degenaar (Chair)

Date: 9/04/2018

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10.3 **Participant information sheet for bioavailability study**

Information sheet

Absorption of Naturally Present Compounds in Carrots and their Appearance in Blood and other Biofluids after Consumption (The 'Carrot Study')

You are being invited to take part in a research study being conducted as part of a PhD research project. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?
Studies have shown that certain compounds inside vegetables can reduce the risk of cancer. Some of these compounds have been studied in isolated cells that have shown a reduction in cancer cells as well as inflammatory markers which have been associated with an increased risk of cancer. These beneficial plant compounds have not been well studied in the human body and it is unclear whether they are able to be digested from the vegetable and reach the blood stream or whether they remain in the digestive system. The aim of this research project is to determine whether eating an amount of carrot can lead to the appearance of the bioactive compounds in the blood stream, in stool samples and in urine.

Why have I been invited to take part?
The participants chosen to take part in the study are healthy adults, aged 18-30 years with a BMI ≥18.5 and ≤ 30kg/m^2. At the beginning of the study, you will be asked to complete a (anonymous) health and lifestyle questionnaire and your height, weight and body composition will be measured. If you smoke, have any metabolic/genetic diseases or take any medications known to interfere with digestion, have an allergy to carrots or wheat, you will be excluded from the study.

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 will 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, without giving a reason, and to withdraw any unprocessed data previously supplied. If you are involved in a dependent relationship (i.e. teacher/student) with any of the researchers working on the project, your involvement in the study will not affect your grades, nor will any decision you make to withdraw from the study.

What will happen to me if I do take part?
This research will involve two separate testing sessions. On average, the time spent taking part in the study will be just over 9 hours per testing session.
You will be asked to come to a screening session where the procedures can be explained to you in more details and you will be provided with materials you will need in the 3 days prior to each testing session.
Screening:

- Measurements such as height, weight and body composition will be taken.
- You will be given a list of foods which you will be asked to **avoid in the 72 hours (3 days) prior to the testing session** and the 48 hours (2 days) following testing. You must also **avoid taking any supplements** during this time.

The day/evening before each testing session:

- The day before each testing session you will be given a pot to collect all urine passed after waking.

- The evening of the day before the test session you will consume a **meal (frozen ready meal for microwave/oven) provided to you by the researchers**.
- You will also be asked to fast overnight (no food or drink for 12 hours, water is allowed).

Each testing session will be conducted in the morning at NUFood, Agriculture Building, Newcastle University.
At the research facility:

- you will be asked to provide a stool and urine sample from that morning (or a stool sample from the night before).
- You will have a cannula (small rubber tube with a tap for sample collection, commonly used in hospital procedures) inserted into a vein in the arm or hand and a fasting blood sample will be taken. The cannula is a small, flexible rubber tube that may cause minimal discomfort when it is fitted but should be painless once in place.
- You will then be asked to consume a meal of boiled carrots with bread and butter.
- Further blood samples will then be collected from the cannula at 1, 2, 4, 5, 6 and 8 hours after the start of consumption of breakfast. Blood samples for determination of the plant compounds will require approx. 16mL blood each (2 medium sized tubes). All measurements will be taken by trained personnel in designated area.
- You will be required to eat lunch (provided) after 4 hours
- You will collect all urine you pass during your stay at the Clinical Research Facility and any stool samples (after 4 hours of eating breakfast)
- You will be given snacks and another frozen ready meal to take home for dinner (oven preparation) that evening
- after dinner you should fast for 12 hours in preparation for returning to the facility the next day.
- You will continue to collect urine and stool samples that evening and the next morning.

The following morning:

- when you return to the testing facility you will provide a further fasting blood sample as well as the urine and stool samples collected overnight and that morning.

The following 48 hours:

- All urine and stool samples will be collected throughout the next 48 hours (or, for stool sample, until the bowel movement nearest to 48 hours after the start of the study) and returned to the researcher

The two test sessions will be undertaken with at least 1 week between sessions.

How many times do I have to take part? There is a minimum of 2 test sessions – each session will last around 9 hours and preparations are required both before and after each testing session. There must be at least a week separating the test sessions.

What is the test food?
You will be required to eat an amount of boiled carrots during the test session. In one session you will be asked to consume a 'normal' amount of carrots and the other session will be a 'large' amount of carrots. Each time the carrots will be served with bread and butter. All foods are normal everyday foods which are fit for human consumption. The standard meals (2x dinner and lunch) should not be shared with friends or family members but are normal, commercially available foods so are safe for consumption if they are accidentally eaten by anyone other than the volunteer.

Are there any risks involved?
Blood sampling will be kept to a minimum and performed by trained personnel in a clean, designated area. Cannulation involves the insertion of a flexible plastic tube into a vein in the arm. There may be a slight discomfort when the cannula is inserted but after insertion, the area is usually pain free and you can move your arm as normal. Blood will be removed from a tap in the cannula and the cannula flushed with sterile saline to prevent blood clotting inside the tube. Only enough saline will be used to flush the tube and this should not enter the blood stream which will ensure infection risk is kept to a minimum. The blood analysis requires approx. 16mL of blood (i.e. 2 medium sized tubes) for each measuring point.

What are the possible benefits of taking part?
You will receive your own body composition measurements, one evening meal and food for a whole day will be provided for free and, at the completion of the study (once all samples have been collected up to 48 hours after the testing session), you will be reimbursed for your time with £30 per session (£60 in total).

What will happen to my personal data?
Confidentiality of information provided can only be protected within the limitations of the law. All samples and records will be coded and will only be available to the researchers involved in the study; your name will never appear in any published work.

What will happen to the results of the study?
All data from the study will be owned by Newcastle University and will be stored securely at the University. The results of the study will be written up as part of a research paper for publication in peer-reviewed journals. Data might also be presented at meetings and international conferences.

Who can I contact if I have any questions?
If you have any questions regarding this study, you can contact either:

Sarah Warner
PhD research Student
07828906990
Email: s.r.warner@ncl.ac.uk
Human Nutrition Research Centre
Agriculture Building,
Newcastle University,
NE1 7RU

Dr Kirsten Brandt
0191 208 5852
kirsten.brandt@ncl.ac.uk
Human Nutrition Research Centre
Agriculture Building,
Newcastle University
NE1 7RU

This research project has received clearance from the SAgE Newcastle University Ethics Committee (15WAR23).

10.4 **Consent form for bioavailability study**

Consent form

Absorption of Naturally Present Compounds in Carrots and their Appearance in Blood and other Biofluids after Consumption (The 'Carrot Study')

Contacts:
Sarah Warner, Postgraduate Research Student, Human Nutrition Research Centre, Newcastle University
Dr. Kirsten Brandt, Senior Lecturer, Food Quality and Health Research Group, Human Nutrition Research Centre, Newcastle University.NE1 7RU

Tel: Sarah: 07828906990 / Kirsten: 0191 208 5852
Email: s.r.warner@newcastle.ac.uk / kirsten.brandt@newcastle.ac.uk

Please ***INITIAL*** the appropriate box	Yes	No
1. I confirm that I have read and understand the information sheet for the above research project (Version_04 dated 16th May 2016).	☐	☐
2. I confirm that I have had the opportunity to ask questions and have received satisfactory answers to all my questions.	☐	☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, or to withdraw any unprocessed data previously supplied.	☐	☐
4. I understand that data will be treated with full confidentiality and that if published, data will not be identifyable to an individual.	☐	☐
5. I understand that venous blood will be taken from me by a trained professional in the process of this research.	☐	☐
6. I agree to take part in the above research	☐	☐

Continued over the page...

7. The potential benefits of keeping blood, urine and faecal samples has been explained to me and: *(please read carefully and choose ONE)*:		
a. I consent to the indefinite storage of my samples and for use in any University-approved future study; OR	☐	☐
b. I do not wish my blood, urine or faecal samples to be used for any purpose other than this study	☐	☐

Name of Participant (block capitals) ..... Date .....

Signature .....

Name of Researcher (block capitals) ..... Date .....

Signature .....

10.5 **Health and Lifestyle Questionnaire for bioavailability study**

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Health and Lifestyle Questionnaire

Anthropometric Measurements	
Gender:	
Date of Birth (age):	
Height (cm):	
Weight (kg):	
BMI:	
Body fat (%):	
Waist Circumference:	

Participant No. ☐

Newcastle University

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Please tick the appropriate box and give details where appropriate	Yes	No
1. Do you have any medical conditions that affect your digestion or metabolism?	☐	☐
2. Do you have any food allergies or intolerances? If yes, which food(s)? _____	☐	☐
3. Are you taking any medication? If yes, which one(s)? _____	☐	☐
4. Are you taking any vitamins or supplements? If yes, which one(s)? _____	☐	☐
5. Do you smoke? If yes, how many per day? _____	☐	☐
6. Do you exercise or participate in any sports? How often a week?_____ Duration:_____ Intensity:_____	☐	☐
7. Are you following a special diet? If yes, which one? _____	☐	☐

Participant No. ☐

10.6 List of polyacetylene containing foods to avoid during bioavailability study

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Foods to Avoid during 'The Carrot Study'

Please do not eat the following foods in the 72 hours (3 days) prior to the testing session and during the following 48 hours (2 days) after the testing session. After the final fasting blood, urine and faecal sample have been taken/provided, you are able to eat these items freely again.

Supplements:

Please avoid taking all vitamins, supplements and herbal remedies during the testing periods.

Carrots:
including any products that contain carrot e.g. carrot cake, carrot soup, carrot juice, coleslaw. Carrot may also be a 'hidden' ingredient in products such as Shepherd's pie, soups, stews, casseroles and vegetable soup etc. Powdered carrot is also a common constituent of powdered soup mixes. Please check the ingredients list carefully before consuming this type of product.

Parsnip:
including any products that contain parsnip e.g. soups, stews and casseroles.

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Celery:
Celery is a common constituent of ready meals and is often contained in powdered soups and other ready meals. Please check the ingredients list carefully before consuming this type of product.

Celeriac
Celeriac is sometimes contained in powdered soups and other ready meals. Please check the ingredients list carefully before consuming this type of product.

Fennel bulb

Fennel is an aniseed flavoured vegetable that can be eaten fresh in salad or is often a constituent in fish soups and stews. Please check the ingredients list carefully before consuming this type of product.

Herbs:

Please check the ingredients list of foods carefully to check for the following herbs.

Parsley Leaf and any products containing parsley leaf.

Coriander Leaf (also known as cilantro) and any products containing coriander leaf

Ginseng
Ginseng is a herbal remedy and often contained in herbal teas/infusions and supplements.
Please ensure you check the ingredients list of teas/infusions and avoid taking supplements.

10.7 **Foods for controlled meals during bioavailability study**

Evening meal (night before testing):

- Tomato and mozzarella pasta bake, Tesco own brand, UK
- 80g frozen broccoli, Tesco own brand, Tesco UK

Breakfast:

- 100 or 250g boiled carrot (weighed after cooking), Tesco UK
- 2 slices medium sliced soft white bread, Hovis, UK
- 10g Lurpak spreadable butter, Lurpak, UK

Lunch:

- 300g tomato and basil soup, Tesco own brand, Tesco UK
- Cheese and tomato sandwich: 2 slices Hovis soft white bread, spread with 10g Lurpak spreadable butter, mature cheddar cheese, Tesco own brand, Tesco UK, fresh tomato, Tesco UK.
- Cadbury's brunch bar with raisins, Cadbury's UK
- Apple, Tesco, UK

Snack:

- Banana, Tesco, UK

Evening meal:

- 3 cheese pizza, Tesco own brand, Tesco UK
- 80g frozen cauliflower, Tesco own brand, Tesco UK

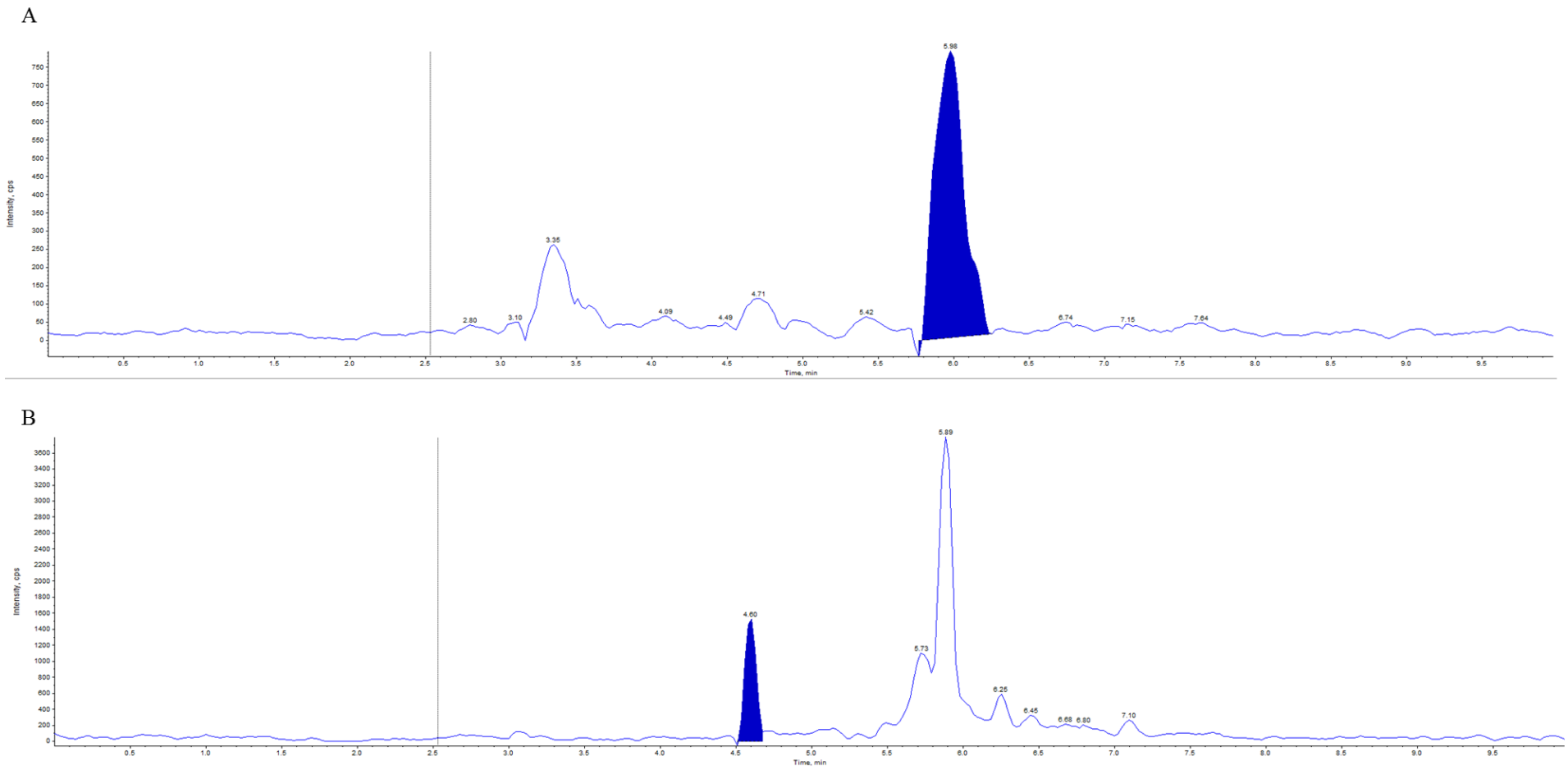

Figure 10.1: Example of peak areas from the LCMS peaks for A) Channel 141: Falcarinol and B) Channel 117: Falcarindiol-3-acetate

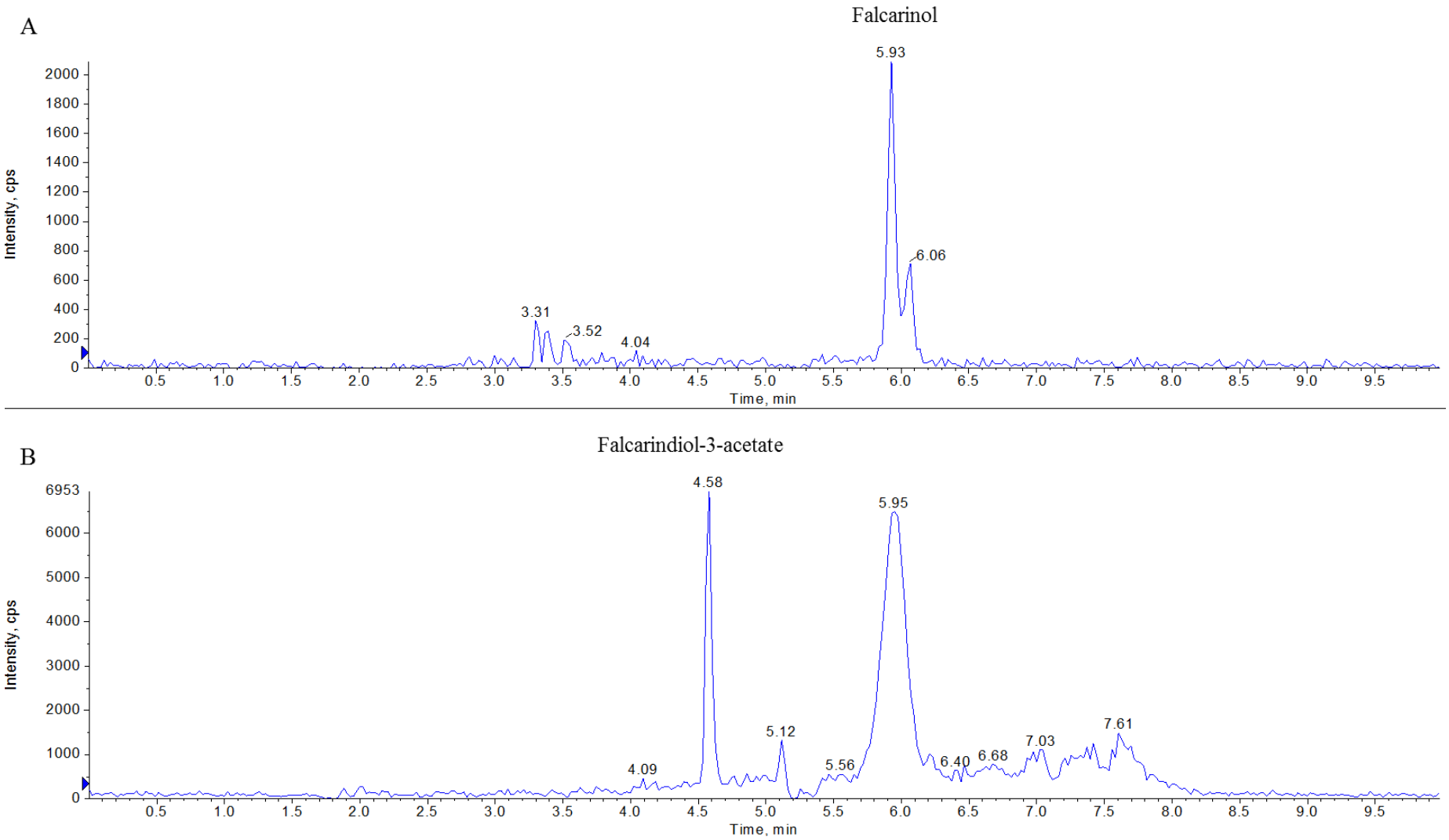

Figure 10.2: Example of peak areas from the LCMS peaks for A) Channel 141: Falcarinol and B) Channel 117: Falcarindiol-3-acetate

Chapter 11. **Appendix D**

11.1 **Ethical approval for dietary intervention study**

15-WAR-92

SAgE Faculty Ethics Committee
Ethics Application Pro-forma

Applicant Name:	Sarah Warner
Applicant email:	s.r.warner@newcastle.ac.uk
Academic Unit	School of Agriculture, Food and Rural Development
Supervisor email (if available)	kirsten.brandt@newcastle.ac.uk
Category	Student Project (PGR)
Project Title:	Randomised 6-week, parallel group, placebo controlled intervention trial to investigate the effects of white carrots on inflammatory markers and lymphocyte DNA damage (Short title: Effect of Foods on Cell Damage)
Start / End Date	07-09-2015 - 31-12-2016
MyProjects Reference (if available)	BH122642 (cc Carol Huntley) Horticultural Development Council Studentship
Reviewer 1 Name: Date sent: Date comments received:	 Per Berrggren (MAST) 01/09/2015 02/09/2015
Reviewer 2 Name: Date sent: Date comments received:	
Date comments provided to researcher:	N/A – no amends required.
Date researcher confirmed amendments made:	N/A
Faculty final approval date:	30/09/2015
Notes	Use of Human Participants (non–clinical). Use of Human Tissue – Standing Operating Procedures demonstrate compliance with the Human Tissue Act. Participants will be recruited through the University and offered a small incentive as compensation for their time. Informal approval granted by email 03/09/15.

1

11.2 **Participant information sheet for dietary intervention study**

Information sheet

Effect of Foods on Cell Damage

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?
Studies have shown that consumption of certain vegetables, such as carrots, is associated with a reduced risk of cancer. Vegetables contain many different substances that may cause this benefit, such as vitamins, fibre and phytochemicals. These beneficial plant compounds have not been well studied in the human body and for most of them it is unclear whether they can directly affect the biomarkers of health. The aim of this research project is to determine whether eating a daily portion of white carrot or a specific type of oat biscuit containing some of the same type of substances that are in the carrot can lead to the reduction of DNA damage and inflammatory biomarkers in the blood and other biofluids. Samples of blood and stools will be used to collect cells and measure how much DNA damage occurred naturally in these cells, while markers of inflammation will be measured in samples of blood and urine.

Why have I been invited to take part?
The participants chosen to take part in the study are healthy adults, aged over 45 years with a BMI between 18.5 and 30kg/m^2. However if you smoke, have any metabolic/genetic diseases or take any medications known to interfere with digestion, if you take anti-inflammatories, aspirin or aspirin-like medication, have taken antibiotics within 3 months of the start of the study or have an allergy to carrots or oats, then you will not be able to take part in this study.

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 will be given this Information Sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time, without giving a reason, and to withdraw any unprocessed data previously supplied.

What will happen to me if I do take part?
The following table outlines the timetable of activities that you will be asked to undertake during the study. A detailed explanation follows below.

Screening	Run in	Intervention Period						Wash out period						End
	Week: 0	1	2	3	4	5	6	7	8	9	10	11	12	13
At NU Food: Questions about health and lifestyle, assessment of eligibility	3 day food diary, 24 hour urine and stool samples	**At NU Food:** Fasting blood, finger prick,		3-day food diary			3 day food diary, 24 hour urine and stool samples	**At NU Food:** Fasting blood, finger prick		3 day food diary			3 day food diary, 24 hour urine and stool samples	**At NU Food:** Fasting blood, finger prick
		Avoid those foods on the list provided												

All activities will take part at the NU Food facility, Agriculture Building, Newcastle University, Newcastle upon Tyne.

Screening:
You will be asked to come to a screening session at the NU Food where the procedures can be explained to you in more detail and your eligibility for the trial will be assessed. Measurements such as height, weight and body composition will also be taken and you will be asked about your habitual diet. You will be given a list of foods which you will be asked to avoid during the study (except if provided by the researchers) and the other materials you will need during the study, such as containers for collection of urine and stool samples. You will be asked to complete an (anonymous) health and lifestyle questionnaire and complete a Food Frequency Questionnaire about how much you normally consume of common foods.

The week before the intervention period (week 0):
You will be asked to complete a food diary where you will record all of the food and drink you consume in a 3 day period (2 week days and 1 weekend day). On the day prior to the start day, you will collect your urine for 24 hours and a stool sample. You will collect these samples at home and bring them with you for the study visits, using the containers we provided to you.

The intervention period (weeks 1-6):
For the first study visit, you will be asked to come to NU-Food in the morning, to provide a fasting blood sample. This means you must not consume any food or drink (except water) in the 12 hours prior to the blood sample being collected. When you arrive, 6 tubes of blood will be collected from a vein in your arm (a total of 60mL), and a single finger prick blood sample will be collected onto a piece of card. You will then be provided with breakfast.

After the blood samples are collected, the intervention period will begin. The intervention period is 6 weeks long and during this time you will be asked to supplement your diet with one daily portion of either white carrots or a specific type of oat biscuits, which will be provided to you. The food will be either 100g white carrots with 10g butter (which you will need to boil at home), or 3 of the oat biscuits. During this time you will be asked to avoid any other foods and drinks containing carrots or oats or vegetables closely related to carrots, **except** those foods provided by the researcher. The foods to avoid include carrot, parsnip, celery, celeriac, oats, ginseng, parsley and certain herbal infusions, supplements and herbs. A full list of foods will be provided to you. In weeks 3 and 6 you will complete another 3-day food diary. At the end of the 6 weeks, you will need to collect your urine for 24 hours and a stool sample in the day prior to coming to NU-Food.

Wash out period (weeks 7-12):
At the start of week 7 (at the end of the 6 weeks), you will return to NU food for the second study visit to repeat the blood sampling procedure that you completed at the start of the study (venous blood and a finger prick sample). There will then be a "wash-out" period of 6 weeks following the intervention period. In this period you will eat as normal **except** you continue avoiding the foods on the Foods to Avoid list. In weeks 9 and 12 you will complete another 3-day food diary. At the end of the 6 weeks wash out (week 12), you will again need to collect your urine for 24 hours and a stool sample in the day prior to coming to NU Food.

Final Visit (week 13):
At the third and final study visit, you will repeat the blood sampling procedure once again.

What is the test food?
During the intervention period you will be asked to consume either a 100g portion of boiled white carrots or a special oat biscuit every day. You will be randomly assigned to eat one of the two test foods.

Are there any risks involved?
Blood sampling will be performed by trained personnel in a clean, designated area. There may be a slight discomfort when the needle is inserted and there may be a small amount of bruising after the procedure.

What are the possible benefits of taking part?
You will receive your own body composition measurements and, at the completion of the study, you will be reimbursed for your time with £40 in gift vouchers.

What will happen to my personal data?

All samples and records will be coded (made anonymous) and will only be available to the researchers involved in the study or representatives of the authorities; your name will never appear in any published work.

What will happen to the results of the study?
All data from the study will be owned by Newcastle University and will be stored securely at the University. The results of the study will be written up as part of a research paper for publication in peer-reviewed journals. Data might also be presented at meetings and international conferences.

Who can I contact if I have any questions?
If you have any questions regarding this study, you can contact either:

Sarah Warner	Dr Kirsten Brandt
07828906990	0191 208 5852
Email: s.r.warner@ncl.ac.uk	kirsten.brandt@ncl.ac.uk
Human Nutrition Research Centre	Human Nutrition Research Centre
Agriculture Building,	Agriculture Building,
Newcastle University,	Newcastle University
NE1 7RU	NE1 7RU

This research project has received clearance from the University Ethics Committee at Newcastle University. If you have any ethical concerns about the conduct of this project, you can contact the AFRD faculty ethics coordinator (jillgolightly@newcastle.ac.uk).

11.3 **Consent form for dietary intervention study**

Newcastle University

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Consent form

Effect of Foods on Cell Damage

Contacts:
Sarah Warner, Postgraduate Research Student, Human Nutrition Research Centre, Newcastle University
Dr. Kirsten Brandt, Senior Lecturer, Food Quality and Health Research Group, Human Nutrition Research Centre, Newcastle University

Tel: Sarah: 07828906990 / Kirsten: 0191 208 5852
Email: s.r.warner@newcastle.ac.uk / kirsten.brandt@newcastle.ac.uk

Please ***INITIAL*** the appropriate box	Yes	No
1. I confirm that I have read and understand the information sheet for the above research project (Version_01 dated 15th April 2015).	☐	☐
2. I confirm that I have had the opportunity to ask questions and have received satisfactory answers to all my questions.	☐	☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, or to withdraw any unprocessed data previously supplied.	☐	☐
4. I understand that data will be treated with full confidentiality and that if published, data will not be identifyable to an individual.	☐	☐
5. I give my permission for my GP to be contacted to inform them that I am taking part in this study	☐	☐
6. I understand that venous blood samples will be taken from me by a trained person in the process of this research.	☐	☐
7. I agree to take part in the above research	☐	☐

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8. The potential benefits of keeping blood, urine and faecal samples has been explained to me and: *(please read carefully and choose **ONE**)*:		
a. I consent to the indefinite storage of my samples and for use in any University-approved future study; **OR**	☐	☐
b. I do not wish my blood, urine or faecal samples to be used for any purpose other than this study	☐	☐

Name of Participant (block capitals) ..... Date .....

Signature .....

Name of Researcher (block capitals) ..... Date .....

Signature .....

11.4 List of foods to avoid whilst taking part in the dietary intervention trial

Foods to Avoid during 'Effect of Foods on Cell Damage' study

Please do not eat the following foods, apart from those provided to you by the researcher, during the 6 week testing period or during the 6 week 'wash-out' phase. After the final blood, urine and faecal samples have been collected, you are able to eat these items freely again.

Supplements:

Please avoid taking all vitamins, supplements and herbal remedies during the testing periods.

Carrots:
including any products that contain carrot e.g. carrot cake, carrot soup, carrot juice, coleslaw. Carrot may also be a 'hidden' ingredient in products such as Shepherd's pie, soups, stews, casseroles and vegetable soup etc. Powdered carrot is also a common constituent of powdered soup mixes. Please check the ingredients list carefully before consuming this type of product.

Parsnip:
including any products that contain parsnip e.g. soups, stews and casseroles.

1

Celery:
Celery is a common constituent of ready meals and is often contained in powdered soups and other ready meals. Please check the ingredients list carefully before consuming this type of product.

Celeriac
Celeriac is sometimes contained in powdered soups and other ready meals. Please check the ingredients list carefully before consuming this type of product.

Fennel bulb

Fennel is an aniseed flavoured vegetable that can be eaten fresh in salad or is often a constituent in fish soups and stews. Please check the ingredients list carefully before consuming this type of product.

2

Herbs:

Please check the ingredients list of foods carefully to check for the following herbs.

Parsley Leaf and any products containing parsley leaf.

Coriander Leaf (also known as cilantro) and any products containing coriander leaf

Ginseng
Ginseng is a herbal remedy and often contained in herbal teas/infusions and supplements. Please ensure you check the ingredients list of teas/infusions and avoid taking supplements.

Oats and oat products

Common oat products include porridge, cereal bars, oat biscuits/cookies, some breads and muffins. Please check the ingredients labels carefully before consuming any of these products.

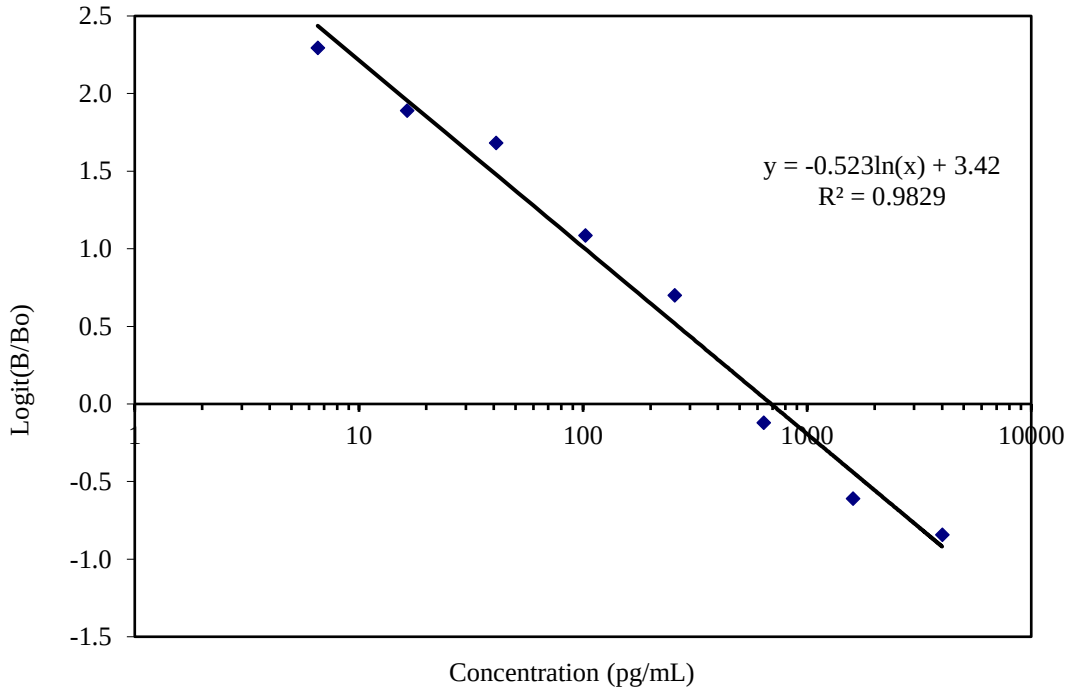

Figure 11.1: PGEM ELISA standard curve. Data analysed using software from Cayman Chemical website: https://www.caymanchem.com/analysisTools/elisa

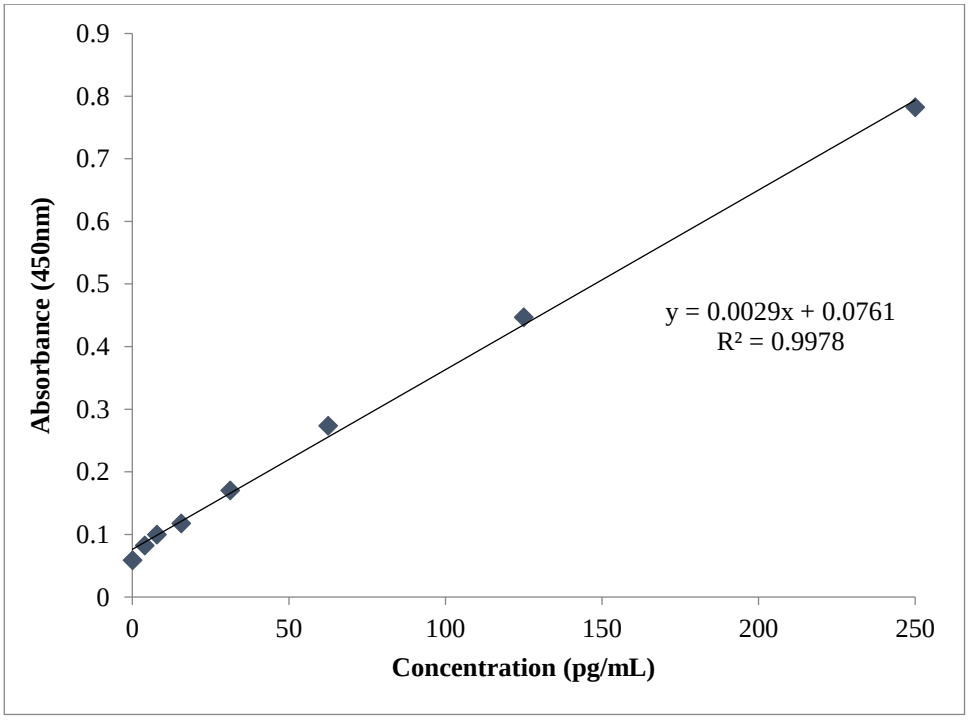

Figure 11.2: IL-6 ELISA standard curve.

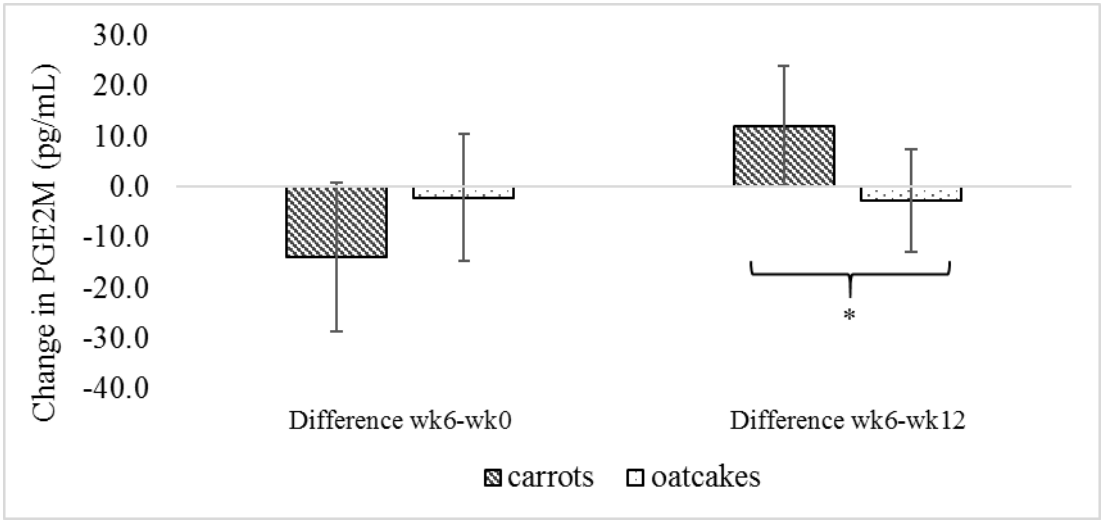

Figure 11.3**:** Change in PGEM between treatment periods. * signifies a statistically significant difference between means of the change in PGEM between carrot group (n=14) and oatcake group (n=14). Error bars are CI.

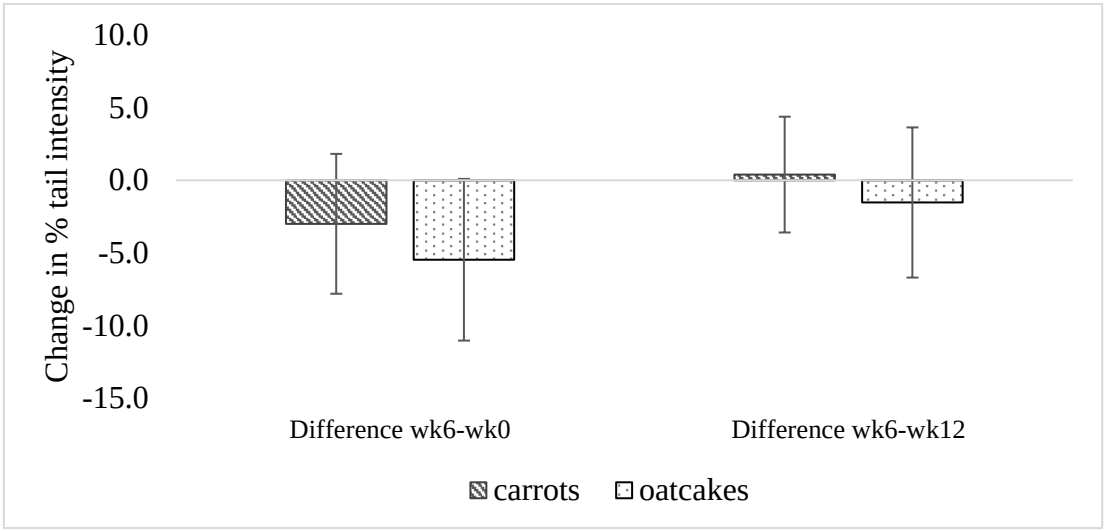

Figure 11.4: Change in % tail intensity between baseline and intervention and between intervention and washout periods in the carrot group (n=14) and oat group (n=14). Error bars are CI.