POLYPHENOLS IN APPLES AND THEIR INTERACTIONS WITH VASCULAR ENDOTHELIAL CELLS

Christina Willemina Anne Moyle

Institute of Food Research

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Abstract

Hypothesis: Polyphenols in apples cause specific molecular changes in vascular endothelial cell which can lead to improvements in vascular function.

Results: First, changes in polyphenol concentrations in apples with respect to fruit growth and maturity and seasonal variations were assessed in order to allow the optimisation of harvest dates and predict the effects of weather on polyphenol yields and composition. The data showed that the polyphenol concentration decreases during fruit growth while the content increases due to an increase in fruit weight. It was also shown that the mean temperature is negatively correlated with polyphenol content and concentration while the total amount of rainfall and the total hours of sunshine are positively correlated.

Next, simple strategies to achieve a high-yielding polyphenol-rich apple extract were tested. Heat treatment of whole apples prior to pressing failed to decrease the loss of polyphenols observed during pressing. However, hot water extraction of apple pomace, a by-product of apple juice production, was shown to be a very effective method of extracting polyphenols retained in the pomace (extraction yield 51 % with 17 % of the polyphenols retained in the pomace). In a randomised human intervention trial, it was shown that a pre-commercial apple polyphenol extract caused small but significant improvements in vascular stiffness index, in keeping with the original hypothesis.

Treatment of human umbilical vein endothelial cells with vascular endothelial growth factor (VEGF) and apple procyanidin, but not epicatechin, resulted in an inhibition of VEGF-induced migration. It was therefore hypothesised that apple procyanidins inhibit VEGF signalling. It was shown that certain polyphenols specifically inhibited VEGF-induced VEGFR-2 phosphorylation at physiological concentrations, and it was shown for the first time that polyphenols were able to inhibit VEGF signalling by interacting with the VEGF molecule and not the receptor. The interaction between the polyphenols and VEGF was the result of a non-covalent slow binding of one or more molecules of polyphenol to the VEGF protein resulting in the formation of a tightly bound VEGF-polyphenol complex. Using a human whole genome gene array, it was confirmed that the apple procyanidin fraction completely negated the cellular effects of VEGF. Further, the procyanidin fraction regulated the expression of many other genes (e.g. KLF2) that are involved in angiogenesis and inflammation independently of VEGF.

Conclusions: These findings are significant because they provide a plausible link between consumption of dietary polyphenols and an improvement of vascular function. More importantly this data shows a novel mechanism of how polyphenols inhibit VEGF-induced VEGFR-2 phosphorylation. While this research has only shown an interaction with VEGF, it raises the possibility that polyphenols can affect other signalling pathways by interacting with other signalling peptides such as growth factors and hormones.

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Abbreviations

A2M	Alpha-2-macroglobulin
ACE	Angiotensin 1 converting enzyme
AKT	Serine/theronine kinases
ANGPT1	Angiopoietin-1
ANGPT2	Angiopoietin-2
ANOVA	Analysis of variance
APLN	Apelin
AUC	Area under the curve
Bcl-2	B-cell lymphoma
BDK	Bradykinin
BMI	Body mass index
BMP4	Bone morphogenic protein 4B
BP	Blood pressure
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
CASP	Caspase, apoptosis-related cysteine peptidase
CDF	Chip definition file
CHD	Coronary heart disease
C_{max}	Maximal plasma concentration
COX2	Cycloxygenase 2
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
CYR61	Cysteine-rich angiogenic inducer 61
DAVID	Database for Annotation, Visualization and Integrated Discovery
dp	Degree of polymerization
DMSO	Dimethyl sulfoxide
DSCR-1	Down syndrome critical region 1
DTT	DL-Dithiotheritol
DUSP5	Dual specificity phosphatase 5
DVP	digital volume pulse
EC_{16}-C	Epicatechin$_{16}$ (4→8) catechin
ECG	Epicatechin gallate
EDN1	Endothelin-1
EFSA	European Food Safety Authority
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGM-2	Endothelial cell growth medium-2
EGR	Early growth response
ELISA	Enzyme-linked immunosorbent assay
ErbB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERK 1/2	Extracellular signal regulated kinase 1/2
ETV	Ets variant
FBS	Foetal bovine serum
FMD	Flow mediated dilation

HB-EGF	Heparin binding epidermal growth factor
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
HUVEC	Human umbilical vein endothelial cell
IC_{50}	Half inhibitory concentration
ICAM1	Intercellular adhesion molecule 1
IL8	Interleukin 8
JNK	c-Jun N-terminal kinase
KIT	KIT ligand
KLF	Krüppel like factor
LDL	Low density lipoprotein
LPH	Lactase phloridzin hydrolase
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MEK5	Mitogen-activated protein kinase 5
MWCO	Molecular weight cut off
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor-(erythroid-derived)-related factor 2
NUSE	Normalised unscaled standard error
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffered saline
PCA	Pulse contour analysis
PDGF	Platelet derived growth factor
PERI	Period homolog 1 (Drosophila)
PGG	-1,2,3,4,6-penta-*O*-galloyl-D-glucose
pI	Isoelectric point
PLAU	Urokinase plasminogen activator
PLCγ	Phospholipase C gamma
PRP	Proline rich protein
PVDF	Polyvinylidene fluoride
PWV	Pulse wave velocity
RI	Reflective index
RIPA	Radioimmunoprecipitation assay buffer
RLE	Relative log expression
RMA	Robust multiarray average
RNA	Ribronucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SELE	E-selectin
SGLT1	Sodium-dependent glucose transporter
SI	Stiffness index
TFA	Trifuloroacetic acid
THBD	Thrombomodulin
T_{max}	Time to reach the maximal plasma concentration

TNF-α	Tumour necrosis factor-alpha
VA	Vascular age
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Symbols

CO_2	Carbon dioxide
ºC	Degrees Celsius
cells/cm^2	Cells per centimetre squared
g	Gram
h	Hour
i.d.	Internal diameter
kg	Kilogram
kg/m^2	Kilogram per metre squared
m/s	Metres per second
mg	Milligram
mg/kg	Milligram per kilogram
mg/ml	Milligram per millilitre
mg/L	Milligram per litre
min	Minute
ml	Millilitre
ml/min	Millilitre per minute
mm	Millimetre
mm^2	Millimetre squared
mM	Millimolar
ng	Nanogram
ng/ml	Nanogram per millilitre
nm	Nanometre
nM	Nanomolar
µg	Microgram
µg/g	Microgram per gram
µg/ml	Microgram per millilitre
µm	Micrometre
µM	Micromolar
µl	Microlitre
%	Percent
v/v	Volume per volume
yrs	Years

List of Publications

Papers

Moyle, C.W.A., Wood, R., Kroon, P.A. Intra- and inter-seasonal variations in individual polyphenols during growth and ripening of polyphenol-rich apple fruits. *Manuscript for the Journal of Agricultural and Food Chemistry*. In Preparation.

Moyle, C.W.A., Wood, R., Green, R., Kroon, P.A. The fate of apple polyphenols during apple pressing. *Manuscript for the Journal of Agricultural and Food Chemistry*. In Preparation.

Moyle, C.W.A., Winterbone, M., Needs, P.W., Bacon, J., Hollands, W., Alexeev, Y., Kroon, P.A. Specific polyphenols potently inhibit vascular endothelial growth factor (VEGF) signalling by binding to the receptor-binding region of VEGF. *Manuscript for Arteriosclerosis, Thrombosis, and Vascular Biology*. In Preparation.

Moyle, C.W.A. and Kroon, P.A. Induction of KLF2 mRNA expression by apple procyanidin fraction dp 4 is independent of PI3K/AKT activation. *Manuscript for Biochemical Journal*. In Preparation.

Posters

Moyle, C.W.A. and Kroon, P.A. 2011. Induction of Krüppel-like factor 2 mRNA expression by apple tetrameric procyanidin fraction is independent of phosphatidylinositol 3-kinase/AKT activation. *Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference 2011*, Chicago, Illinois, April 28 – 30, 2011.

Moyle, C.W.A., Hollands, W., Winterbone, M., Bacon, J., Needs, P.W., Wood, R. & Kroon, P.A. 2010. How does an apple a day keep the doctor away? *SET for Britain*, London, England, March 8, 2010.

Moyle, C.W.A., Hollands, W., Winterbone, M., Bacon, J., Needs, P.W., Wood, R. & Kroon, P.A. 2009. Inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) by tea polyphenols and related phenolics. *4th International Conference on Polyphenols and Health*, Harrogate, England, December 7 – 11, 2009.

Other

Moyle, C.W.A & Kroon, P.A. 2011. 2009 and 2010 harvest and test orchard apple analysis. *Technical report for Coressence*.

Moyle, C.W.A., Hollands, W., Winterbone, M., Bacon, J., Needs, P.W., Wood, R. & Kroon, P.A. 2010. How does an apple a day keep the doctor away? *Institute of Food Research Student Showcase*, Oral presentation, Awarded best presentation prize, Norwich, England, May 6, 2010.

Moyle, C.W.A & Kroon, P.A. 2009. Coressence apple samples: red extract material and freeze-dried apple powder. *Technical report for Coressence*.

Moyle, C.W.A & Kroon, P.A. 2009. Coressence apple sample: Week 1 2009 harvest. *Technical report for Coressence*.

Moyle, C.W.A & Kroon, P.A. 2009. Coressence apple samples: Weeks 3 – 5 2009 harvest and the Chaucer food sample. *Technical report for Coressence*.

Moyle, C.W.A & Kroon, P.A. 2008. Ciderkin analysis. *Technical report for Coressence*.

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CHAPTER ONE

GENERAL INTRODUCTION

Chapter 1 : General introduction

1.1 Structure of the thesis

This thesis contains the results of a research project that spanned apple polyphenol content and composition and the effect of seasonal variation, through apple processing techniques for extracting polyphenols, to *in-vitro* and human studies focussed on biological effects of apple polyphenols and mechanisms of action at the cellular and molecular levels. The purpose of this general introduction is to introduce polyphenols; their bioavailability, metabolism and absorption; their health related properties; and to detail the overall aim and specific objectives of the thesis. As the objectives of each of the results chapters focus on different aspects of apple polyphenols and their impact on vascular endothelial cells, each results chapter will also provide an overview of the literature relevant to the research aims of that chapter.

1.2 What are polyphenols?

Polyphenols are one of the most numerous and widely distributed groups of secondary metabolites in the plant kingdom (Bravo, 1998; Ross and Kasum, 2002). Polyphenols are the result of secondary metabolism in plants and are derived from both the shikimate (phenylalanine) pathway and the acetate-malonate (malonyl-CoA) pathway via several enzymatic steps (Bravo, 1998; Awad et al., 2001a; Golding et al., 2001; Ross and Kasum, 2002; Arts and Hollman, 2005). The transformation of phenylalanine to cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) is the first step in the biosynthesis of polyphenols (Figure 1.1) (Awad et al., 2001a).

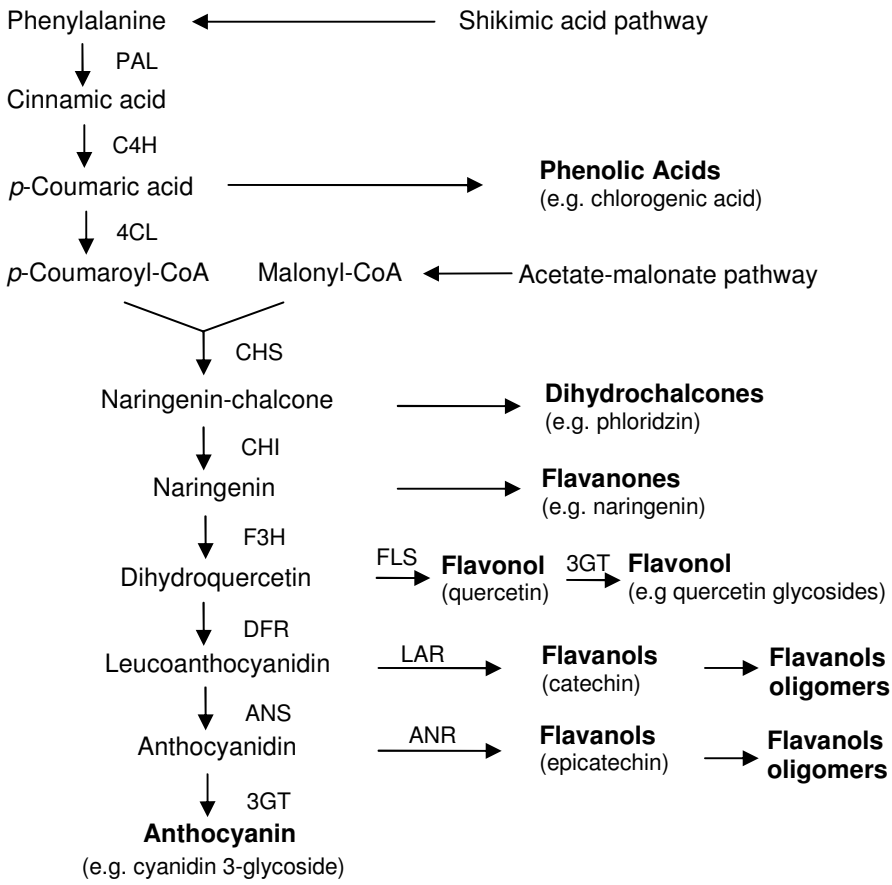


Figure 1.1: A schematic representation of the biosynthetic route of polyphenols in plants. Genes encoding enzymes for each step are shown in italics. The shared genes of phenolic acid synthesis are PAL = phenylalanine ammonia-lyase; C4H = cinnamate hydroxylase; and 4CL = 4-coumarate CoA ligase. The shared genes for anthocyanin synthesis are CHS = chalcone synthase; CHI = chalcone flavanone isomerise; F3H = flavanone-3-hydroxylase; DFR = dihydroflavonol-4-reductase; ANS = anthocyanidin synthase; and 3GT = cyanidin/flavonol 3-*O*-glucosyl transferase. The branch genes: FLS = flavonol synthase and 3GT for flavonol synthesis; LAR = leucoanthocyanidin reductase and ANR = anthocyanidin reductase for synthesis of catechin and epicatechin oligomers.

1.2.1 Classification of polyphenols

There are more than 8000 polyphenol structures which can be divided into different groups based on their chemical structure (the number of phenol rings that they contain and to the structural elements that bind these rings to one another) (Manach et al., 2004; D'Archivio et al., 2007). The main groups of polyphenols are flavonoids, phenolic acids, lignans and stilbenes (Figure 1.2).

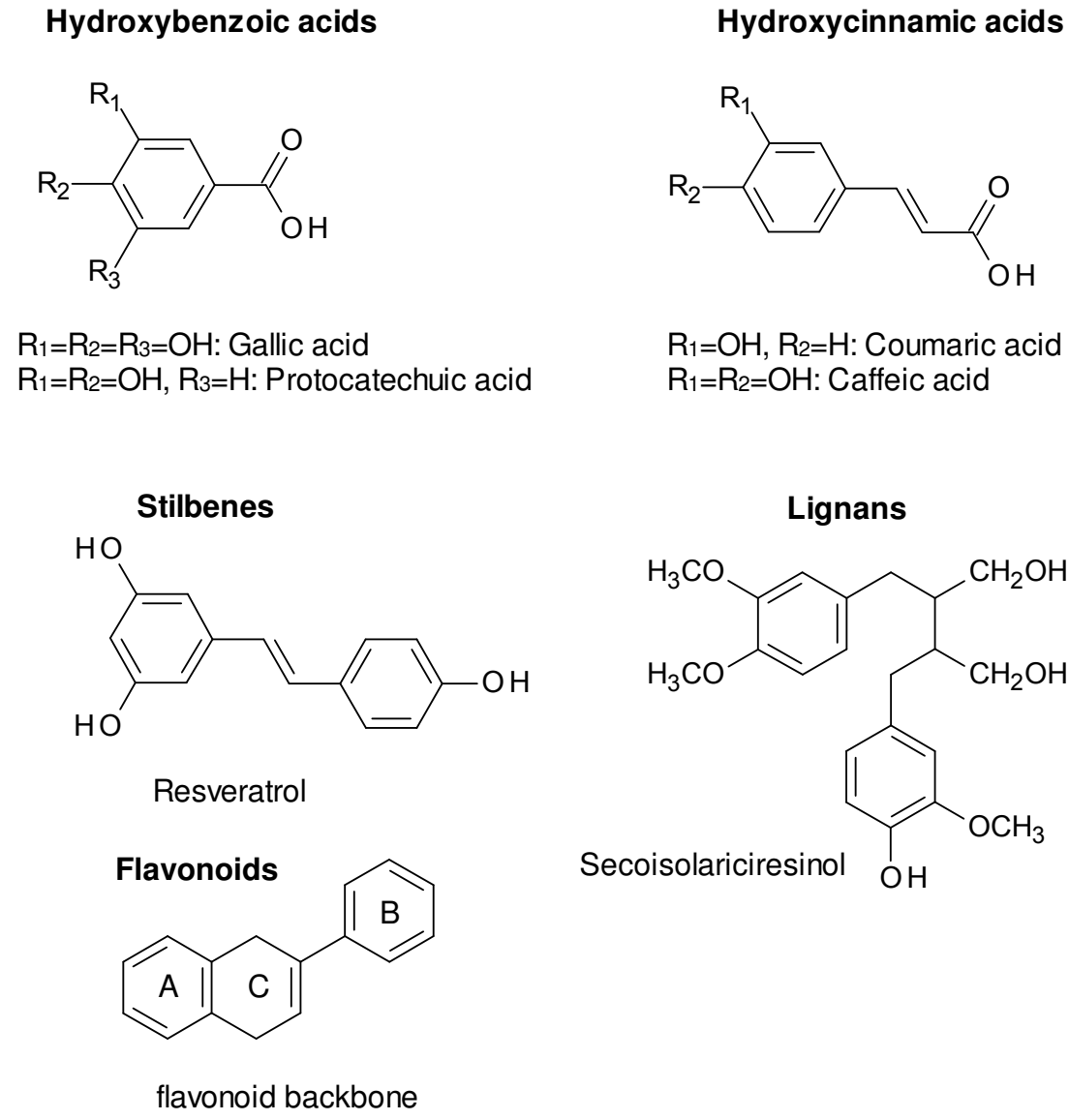


Figure 1.2: Chemical structures of polyphenols.

1.2.1.1 Flavonoids

Flavonoids are the most common and widely distributed group of polyphenols that are present in plants, with more than 4000 different varieties of flavonoids. They are also the most abundant polyphenols in our diet accounting for two-thirds of the total polyphenol intake (Scalbert and Williamson, 2000). Flavonoids share a common basic carbon backbone structure, the diphenylpropane skeleton. The diphenylpropane skeleton (C_6-C_3-C_6) consists of two benzene rings (A and B) linked by a three-carbon chain. This three carbon chain forms a closed pyran ring (C) (Figure 1.2). Different

degrees of hydroxylation and oxidation of the heterocyclic C pyran ring gives rise to six subclasses of flavonoids: flavonols, flavones, flavanols, flavanones, anthocyanins and isoflavones (Figure 1.3).

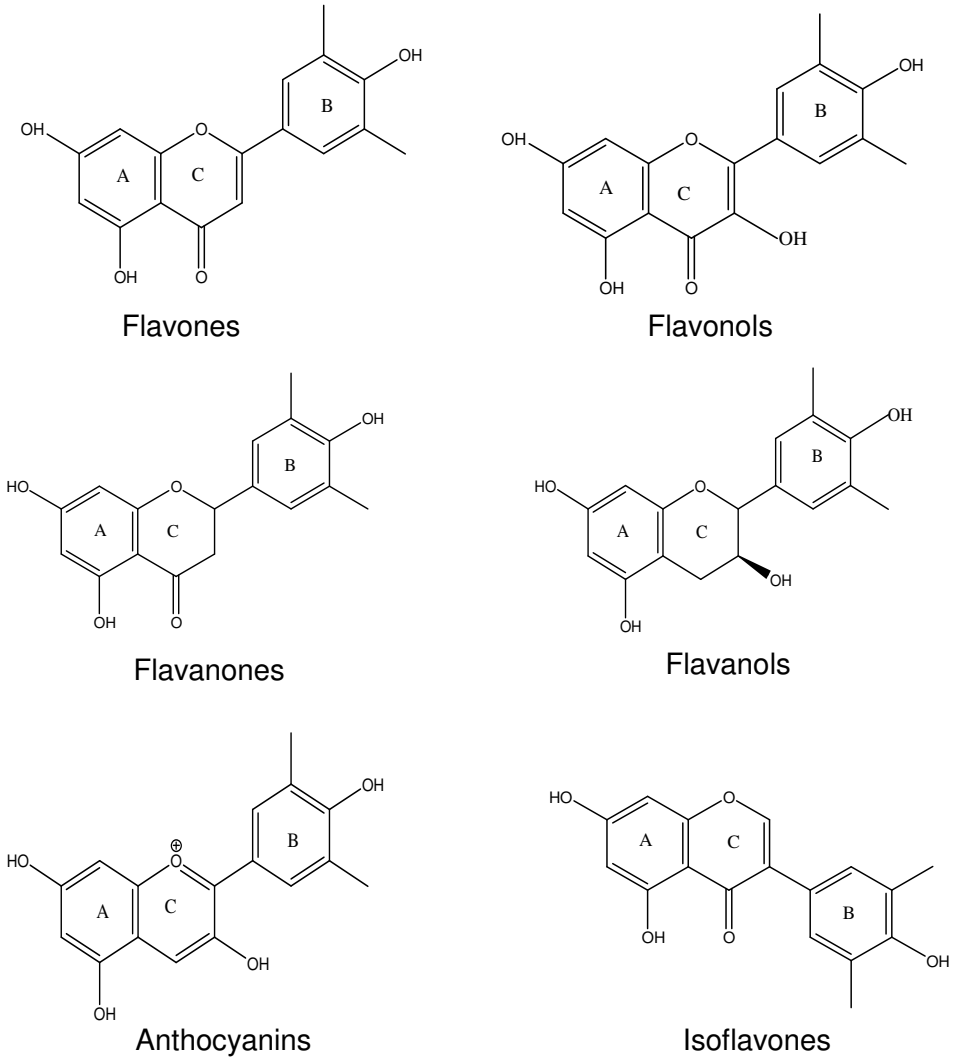


Figure 1.3: Chemical structures of flavonoids

Flavonols are the most commonly occurring flavonoids in foods with quercetin being the main flavonol found in the human diet. Other flavonols include kaempferol, fisetin and myricetin. In plants, flavonols are present in their glycosylated forms and the commonly attached sugars are glucose or rhamnose but other sugars may be involved such as galactose, arabinose and xylose (Manach et al., 2004). Flavonol synthesis is stimulated by light; therefore they accumulate more so in the skin and leaves of fruits

and vegetables (Manach et al., 2004; D'Archivio et al., 2007). The richest sources of flavonols in the human diet are blueberries, onions, leeks, broccoli, curly kale, red wine and tea (Scalbert and Williamson, 2000; D'Archivio et al., 2007).

Flavones are the least common flavonoid and they are not widespread in the human diet. The most common flavones found in plants consist of glycosides of luteolin which are found in sweet red peppers and artichokes and apigenin which are found in celery (Scalbert and Williamson, 2000; Manach et al., 2004; D'Archivio et al., 2007). The flavones apigenin and luteolin are also common in cereal grains and aromatic herbs (parsley, rosemary and thyme) (Peterson and Dwyer, 1998; Pietta, 2000).

Flavanols exist in both the monomer form (catechins) and the polymer form (proanthocyanidins) and unlike other flavonoids they are not glycosylated in foods (Scalbert and Williamson, 2000). Catechins are widely distributed in plants and are found in many fruits such as apples, apricots and cherries as well as in chocolate, tea and red wine (Pietta, 2000). Catechin and epicatechin are the main flavanols found in fruits while gallocatechin, epigallocatechin and epigallocatechin gallate are the main flavanols found in tea (Manach et al., 2004). Proanthocyanidins, which are also known as condensed tannins, are dimers, oligomers and polymers of catechins that are bound together by links between C4 and C8 or C6. Proanthocyanidins are found in fruits (e.g. apples, grapes, and cranberries), cocoa and beverages (e.g. red wine and tea).

Flavanones are found in high concentrations in citrus fruit, but are also present in tomatoes and certain aromatic plants such as mint (Manach et al., 2004). The main flavanones are naringenin (found in grapefruit), hesperidin (found in oranges) and eriodictyol (found in lemons). They are generally glycosylated by either neohesperidose (which gives a bitter taste) or rutinose (which is flavourless) (Peterson and Dwyer, 1998).

Anthocyanins are water soluble pigments that are responsible for the red, blue and purple colours of plants and fruits (D'Archivio et al., 2007). Anthocyanins are the glycosylated forms of anthocyanidins. Anthocyanins are widespread in the human diet; they are most abundant in fruits but are also found in red wine, certain cereals, certain leafy and root vegetables (Manach et al., 2004; D'Archivio et al., 2007). Anthocyanins include cyanidin (most common anthocyanidin in foods), delphinidin, petunidin, pelargonidin, peonidin and malvidin.

Isoflavones are classified as phytoestrogens because of their structural similarities to estrogens and their ability to bind to estrogen receptors (Peterson and Dwyer, 1998; Manach et al., 2004; D'Archivio et al., 2007). Isoflavones are found most often in leguminous plants including soybeans, black beans, green beans and chickpeas with soya and its products being the main source in the human diet (Peterson and Dwyer, 1998; Pietta, 2000; Manach et al., 2004). The main isoflavones are genistein, daidzein and to a lesser extent glycitein.

1.2.1.2 Phenolic acids

Phenolic acids are abundant in foods and account for one-third of the total intake of polyphenols in the diet (Scalbert and Williamson, 2000). Phenolic acids present in plants are hydroxylated derivatives of benzoic acid and cinnamic acid (Manach et al., 2004; D'Archivio et al., 2007).

Hydroxybenzoic acids are found in a few plants that are consumed in the human diet. Edible plants containing hydroxybenzoic acids generally have very low contents of hydroxybenzoic acids with the exception of certain red fruits (e.g. blackberries), black radishes and onions (Manach et al., 2004; D'Archivio et al., 2007). Hydroxybenzoic

acids include gallic acid and protocatechuic acid. Tea is an important source of gallic acid and raspberries are an important source of protocatechuic acid.

Hydroxycinnamic acids consist mainly of *p*-coumaric, caffeic, ferulic and sinapic acids. They are rarely found in their free form and their bound forms are glycosylated derivatives or esters of quinic, shikimic or tartaric acid (Manach et al., 2004). The most frequently encountered hydroxycinnamic acids are caffeic and ferulic acid and caffeic acid is the most abundant phenolic acid in fruits while ferulic acid is the most abundant phenolic acid in cereals. Caffeic and quinic acid combine to form chlorogenic acid which is found in many types of fruits as well as in coffee.

1.2.1.3 Lignans

Lignans are formed by oxidative dimerization of two phenylpropane units. Lignans include secoisolariciresinol, matairesinol, pinoresinol and lariciresinol. The richest dietary source of lignans are linseed and linseed oil, which contain secoisolariciresinol and matairesinol (Manach et al., 2004). Other cereals, grains, fruits and certain vegetables also contain traces of these lignans but in smaller concentrations than that found in linseed. Lignans are also phytoestrogens due to their estrogen agonist and antagonist properties (Scalbert and Williamson, 2000).

1.2.1.4 Stilbenes

Stilbenes are not widespread in food plants and are only found in low quantities in the human diet. The main representative of stilbenes is resveratrol which has received a lot of interest due to its anti-carcinogenic properties (Scalbert and Williamson, 2000; Manach et al., 2004). Resveratrol is found in grapes, red wine and grape juice.

1.2.2 Dietary intake of polyphenols

Several studies have estimated the polyphenol intakes in various countries including Australia (Johannot and Somerset, 2006), Denmark (Justesen et al., 1997), Finland (Hirvonen et al., 2000; Knekt et al., 2002; Ovaskainen et al., 2008), France (Commenges et al., 2000), Greece (Vasilopoulou et al., 2005; Dilis and Trichopoulou, 2010), Japan (Arai et al., 2000), Ireland (Beking and Vieira, 2011), Mexico (Hervert-Hernández et al., 2010), Spain (Garcia-Closas et al., 1999), The Netherlands (Hertog et al., 1993b; Arts et al., 2001a; Boker et al., 2002), the United Kingdom (Beking and Vieira, 2011) and the United States (Rimm et al., 1996; de Kleijn et al., 2001; Wu et al., 2002; Gu et al., 2004; Chun et al., 2007; Wang et al., 2011). A summary of the reported polyphenol intakes in these countries is described in Table 1.1. The reported polyphenol consumption varies substantially between these populations groups. The actual polyphenol intakes are likely to be higher than the reported values because the data provided in these reports do not represent the total polyphenol intake but only estimates of the intake of selected analysed polyphenols. Therefore the dietary intake methodologies, the foods analysed, the method used for quantification of dietary intake of polyphenols and the selected polyphenols analysed all contribute to the differences observed between countries (Johannot and Somerset, 2006; Chun et al., 2007). Dietary habits and food preferences of different cultures also play a part in the variability of the mean polyphenol intakes between countries (Beecher, 2003). The growing conditions (e.g. cultivar, climate conditions, seasonal variations and date of harvesting) of the fruit and vegetable also contribute to the variability in polyphenol consumption between countries. For further information on how the cultivar, climate conditions and seasonal variations affect the polyphenol content in apples, refer to Chapter 2 (Introduction).

Table 1.1: Estimated mean polyphenol consumption (mg/day) in different countries.

Country	Estimated mean polyphenol intake (mg/day)	Polyphenols analysed	Richest sources of polyphenol intake	Reference
Australia	454	Flavanols (93 % of intake), flavonols (4.6 %), flavanones (1.6 %), anthocyanidins (0.6 %) and flavones (0.1 %)	Tea, oranges, citrus fruits, grapes, wine, apples and leaf and stalk vegetables	Johannot and Somerset (2006)
Denmark	26	Flavanones and Flavonols	Tea, onions, apples, oranges and orange juice	Justesen et al. (1997)
Finland	8	Flavonols and Flavones	n/a	Hirvonen et al. (2000)
	24.2	Flavones, flavonols, flavanols and isoflavones	Apples	Knekt et al. (2002)
	863	Phenolic acids (75 % of intake), flavanols (14 %), anthocyanidins (6 %) and other flavonoids (4 %)	Phenoic acids = coffee Anthocyanidins = berries and berry products Flavonoids = apples, berries, tea, chocolate and citrus fruit	Ovaskainen et al. (2008)
France	14.4	Flavones and flavonols	Fruits, vegetables, wine and tea	Commenges et al. (2000)
Greece	118.6	Flavanones (32 % of intake), flavanols (28 %), flavonols (22 %), anthocyanidins (9 %), flavones (8 %) and isoflavones (1 %)	Oranges, red wine and apples	Vasilopoulou et al. (2005)
	161	Flavonoids = 53% of intake (flavones, flavanols, flavanones, flavanols, anthocyanidins, isoflavones), Procyanidins = 47% of intake	Grapes, wine and peaches	Dilis and Trichopoulou (2010)
Ireland	177	Flavanols, flavanols, flavanones, flavones, and anthocyanidins	n/a	Beking and Vieira (2011)

Table 1.1: Estimated mean polyphenol consumption (mg/day) in different countries continued.

Country	Estimated mean polyphenol intake (mg/day)	Polyphenols analysed	Richest sources of polyphenol intake	Reference
Japan	63.9	Flavonoids = 2.0 – 42 mg/day (flavones and flavonols) Isoflavones = 12 – 119 mg/day	Flavonoids = onions, molokheya and apples Isoflavones = tofu, natto and miso	Arai et al. (2000)
Mexico	842	Analysed extractable polyphenols, hydrolysable polyphenols and proanthocyanidins	n/a	Hervert-Hernández et al. (2010)
Spain	9	Flavonols	n/a	Garcia-Closas et al. (1999)
The Netherlands	23	Flavonols and flavones	Tea, onions and apples	Hertog et al. (1993b)
	50	Flavanols	Tea, apples and pears	Arts et al. (2001)
	< 1	Isoflavones	n/a	Boker et al. (2002)
United Kingdom	182	Flavanols, flavanols, flavanones, flavones, and anthocyanidins	n/a	Beking and Vieira (2011)
United States	20.1	Flavonols, flavones	Tea, onions, apples and broccoli	Rimm et al. (1996)
	< 1	Isoflavones	n/a	(de Kleijn et al. (2001)
	12	Isoflavones	n/a	Wu et al. (2002)
	57.7	Procyanidins (polymers = 73.9 %, trimers = 7.8 %, dimers = 11.2 % and monomers = 7.1 % of intake)	Apples, chocolate and grapes	Gu et al. (2004)
	189.7	Flavanols (83.5 % of intake), flavanones (7.6 %), flavonols (6.8 %), anthocyanidins (1.6 %), flavones (0.8 %), and isoflavones (0.6 %)	Tea, citrus fruit juices, wine and citrus fruits	Chun et al. (2007)
	95	Procyanidins (polymers = 30 %, monomers = 22 %, dimers = 16 %, 4-6 mers = 15 %, 7-10 mers = 11 % and trimers = 5 % of intake)	Tea, legumes and wine	Wang et al. (2011)

1.3 Polyphenols found in apples

Polyphenols are ubiquitous in plants and plant derived foods, but in the majority of Western populations the main dietary sources of polyphenols are fruits, tea, red wine and chocolate. This thesis is concerned with apple polyphenols. Apples are widely consumed and are one of the main contributors to polyphenol intakes in several countries including the UK (Hertog et al., 1993a; Rimm et al., 1996; Hertog et al., 1997b; Justesen et al., 1997; Knekt et al., 1997; Price et al., 1999; Arai et al., 2000; Arts et al., 2001a; Knekt et al., 2002; Boyer and Liu, 2004; Gu et al., 2004; Vasilopoulou et al., 2005; Johannot and Somerset, 2006; Ovaskainen et al., 2008; Perez-Jimenez et al., 2010). The consumption of apples has also been associated with a reduced risk of cancer, cardiovascular disease, asthma and diabetes (Boyer and Liu, 2004). Therefore further investigation of polyphenols derived from apples may be beneficial in the understanding of how dietary polyphenols reduce the risk of chronic diseases.

There are five main classes of polyphenols found in apples: (1) flavanols (present in their monomeric [epicatechin and catechin] and in their oligomeric forms [procyanidins]); (2) hydroxycinnamic acids (caffeoylquinic acid and *p*-coumaroylquinic acid); (3) dihydrochalcones (phloretin glycosides = phloridzin and phloretin aglycone); (4) flavonols (quercetin-3-glycosides [galactoside, glucoside, xyloside, arabinoside and rhamnoside]); and (5) anthocyanins (cyanidin3-galactaside) (Awad et al., 2000; Awad et al., 2001b; Tsao et al., 2003; Alonso-Salces et al., 2004; Chinnici et al., 2004; Marks et al., 2007a; Renard et al., 2007). The anthocyanins are present in higher quantities in the apple skin of red varieties (Guyot et al., 2003). The flavanols are the most abundant polyphenol in apples, followed by hydroxycinnamic acids, and present in lower concentrations are the flavonols, dihydrochalcones and anthocyanins (Alonso-Salces et al., 2004; Vrhovsek et al., 2004).

Polyphenols found in apples play an important role in the nutritional, organoleptic and commercial properties of apples and apple products (Podsędek et al., 2000; Alonso-Salces et al., 2004; Renard et al., 2007). Polyphenols are responsible for the taste (bitterness and astringency), flavour and colour of apples and their derived foodstuffs (Chinnici et al., 2004; Marks et al., 2007a). They are also involved in the formation of hazes and sediments in apple juices and ciders and in the browning phenomena contributing to their colour (Alonso-Salces et al., 2004). The dihydrochalcones, flavonols and anthocyanins contribute to the pigmentation of apples (Guyot et al., 1998; Alonso-Salces et al., 2004) and in fruit processing, and the hydroxycinnamic acids and catechins are involved in the browning phenomena contributing to the colour of apple juices and ciders (Guyot et al., 1998; Marks et al., 2007b; Renard et al., 2007).

1.4 Bioavailability, absorption and metabolism of polyphenols

The health benefits of polyphenols are affected by their absorption, metabolism and bioavailability in the human body. For polyphenols to induce their protective effects *in-vivo*, they must be absorbed from the diet and transported into the blood in a bioactive form to their target sites. The following sections will give a brief overview of the bioavailability, absorption and metabolism of polyphenols.

1.4.1 Bioavailability

An important aspect when considering the role of polyphenols in human health is the evaluation of their bioavailability from foods. Bioavailability is defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation and specific sites where it can exert its biological action (Porrini and Riso, 2008). Polyphenol bioavailability is usually assessed from the plasma pharmacokinetics and / or urinary

excretion. Evidence from bioavailability studies has shown that polyphenols are present in systemic circulation after the ingestion of a polyphenol-rich meal. This indicates that at least a portion of the ingested dose can be absorbed.

An excellent review of polyphenol bioavailability is reported by Manach et al. (2005). The authors report mean values of the pharmakonetic data (maximal plasma concentration [C_{max}], time to reach the maximal plasma concentration [T_{max}], area under the plasma concentration-time curve [AUC], elimination half-life and relative urinary excretion) from human bioavailability studies for 18 major polyphenols. To allow for comparisons between polyphenols, the data was normalised such that the pharmakonetic data was representative of a 50 mg dose (aglycone equivalent). Gallic acid represented the most bioavailable polyphenol (C_{max} = 4 µM) followed by the isoflavones (C_{max} = ~2 µM) and anthocyanins were the least bioavailable polyphenol (C_{max} = 0.03 µM) (Manach et al., 2005). The time to reach C_{max} for the majority of the polyphenols is approximately 2 hours, however for rutin and isoflavones T_{max} is reached approximately 6 hours after ingestion (Manach et al., 2005). Urinary excretion is also used to measure the bioavailability of polyphenols. The urinary excretion ranged from 0.3 % to 42 % for the analysed polyphenols (Manach et al., 2005). Therefore a major proportion of the polyphenols ingested are not found in the urine which implies that they have either not been absorbed through the gut barrier; have been absorbed but have been excreted via the bile; or have been metabolized by colonic microflora (Scalbert and Williamson, 2000). As a large proportion of polyphenols are excreted in the bile, the urinary excretion values underestimate the bioavailability of polyphenols (Manach et al., 2005). The elimination half-lives for catechins are also low indicating that the catechins do not accumulate in the plasma with repeated ingestion (Manach et al., 2005).

The bioavailability of flavanol monomers has been widely reported and the majority of the flavanol bioavailability studies with human volunteers involved the consumption of flavanol monomers from cocoa or tea. Manach et al. (2005) reported that the mean C_{max}, from the available bioavailability studies, is 0.4 µM for epicatechin, 0.12 µM for epigallocatechin gallate (EGCG), 1.10 µM for epigallocatechin (EGC), 4 µM for gallic acid, and 0.02 µM for proanthocyanidin dimers. However the values for the flavanol monomers are underestimated in this review because the methylated metabolites were not taken into account in all bioavailability studies (Rein et al., 2000; Manach et al., 2005). Following the ingestion of flavanol monomers from cocoa, the concentration of flavanol monomers have been reported to be between 4.92 and 5.92 µM for epicatechin and 0.16 µM for catechin (Baba et al., 2000; Holt et al., 2002a). Concentrations of epicatechin, EGCG and EGC, after the consumption of four cups of green tea, have been reported to be between 0.28 and 0.65 µM, 0.17 and 0.71 µM, and 0.67 and 1.79 µM, respectively (Lee et al., 1995; Yang et al., 1998b; Lee et al., 2002). The wide variability of the plasma concentrations of flavanol monomers are probably a result of different doses given in the intervention trials as well as the lack of a standardized method available for analysing plasma concentration levels and problems with the detection of certain metabolites.

The bioavailability of flavanol polymers has been less widely investigated. Human plasma concentrations of procyanidin dimer B2, after the consumption of cocoa, has been reported to be 0.041 µM (Holt et al., 2002a) and human plasma concentrations of procyanidin dimer B1, after the consumption of a grape seed extract, has been reported to be 0.011 µM (Sano et al., 2003). It was previously assumed that the procyanidins were degraded into monomers in the acidic environment of the stomach, however it was shown, *in-vivo*, that procyanidins from cocoa were not degraded in the stomach and that the procyanidins were able to reach the small intestine intact (Rios et al., 2002b). It was also shown that after the consumption of grape seed extract, in rats,

the procyanidins were not degraded into monomers and they reached the small intestine (Tsang et al., 2005). It has also recently been shown, in the small intestine of rats, that procyanidins are slightly absorbed but are not conjugated or methylated like their flavanol monomer counterparts (Appeldoorn et al., 2009b). The bioavailability of procyanidin trimers have been determined in rat plasma with a concentration of 4 µM after a high dose (1000 mg/kg of an apple procyanidin extract) (Shoji et al., 2006a), after the consumption of 300 mg/kg grape seed extract (amount not quantified) (Prasain et al., 2009) and after the consumption of 1 g/kg grape seed procyanidin extract (8.55 µM detected in plasma) (Serra et al., 2009). Procyanidin dimers and trimers have been shown to be absorbed through a Caco-2 cell monolayer (used as a model of intestinal epithelium), thus suggesting that these compounds may be absorbed (Déprez et al., 2001). However, human plasma concentrations of procyanidin trimers, tetramers and higher oligomers have yet to be detected. Low bioavailability of procyanidins with increasing (epi)catechin units (Déprez et al., 2001) and limited sensitivity of mass spectrometry analysis (Shikha Saha, IFR, unpublished work) are some of the factors involved with the difficulty in detecting human procyanidin plasma concentrations.

1.4.2 Absorption

Polyphenols exist in foods and beverages in various chemical forms, and the chemical structure plays an important role in the rate and extent of the absorption, metabolism and bioavailability of polyphenols circulating in the plasma (Scalbert and Williamson, 2000). The first step in the absorption of dietary polyphenols is deglycosylation as flavonoids, with the exception of flavanols, are present in the diet as glycosides. Depending on the sugar residue attached, hydrolysis and absorption of flavonoids may occur in the small intestine (Hollman et al., 1999; Arts et al., 2004). In the small intestine, lactase phloridzin hydrolase (LPH) and cystolic β-glucosidase (CBG) are the

two β-glucosidases present which are capable of hydrolysing flavonoid glycosides (Day et al., 2003; Németh et al., 2003). LPH is exposed to the lumen and is situated on the brush boarder of the small intestine epithelial cells and hydrolysis of the flavonoid glycosides occurs extracellularly prior to passive diffusion of the flavonoid aglycone across the membrane (Day et al., 1998; Day et al., 2000; Day et al., 2003; Németh et al., 2003). On the other hand, CBG is located intracellularly. It has been proposed that glucose transporters such as the sodium-dependent glucose transporter (SGLT1) transport the polyphenol-glucose molecules across the membrane before hydrolysis by CBG can occur (Gee et al., 1998; Day et al., 2003; Németh et al., 2003). In the case of flavonoids which are not absorbed in the small intestine (e.g. flavonoids with rhamnose-substitutions [e.g. rutin] and phenolic acid-esters), these pass through the small intestine to the colon and then are hydrolysed by colonic microflora before being absorbed (Hollman et al., 1997). A schematic drawing of the absorption and metabolism of polyphenols is provided in Figures 1.4 and 1.5.

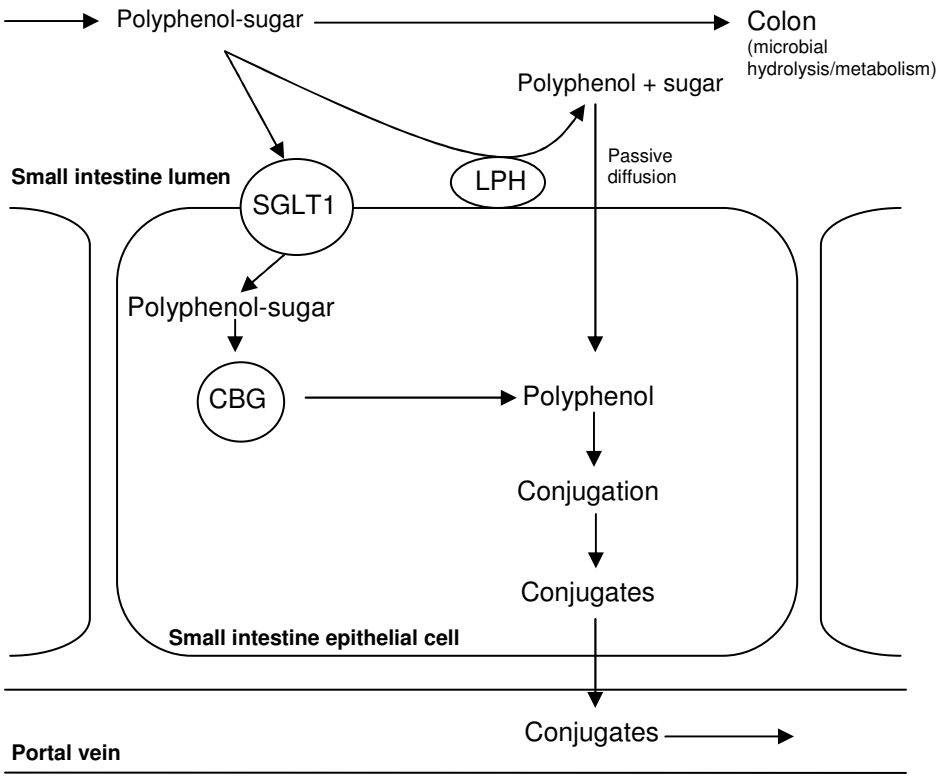


Figure 1.4: Absorption and metabolism of polyphenols in the small intestine.

Polyphenols are either hydrolysed in the small intestine by LPH before passively diffusing through the membrane or are transported into the cells by SGLT1 (or other sugar transporters) and hydrolysed intracellularly by CBG. Conjugation (methylation, sulfation, glucuronidation) of the flavonoid aglycones occurs in the small intestine epithelial cells before passing into the blood supply.

1.4.3 Metabolism

Following absorption, polyphenols (which are now aglycones) are subjected to conjugation in the small intestine and/or in the liver. All polyphenols are subjected to three main types of conjugation: methylation, sulfation and glucuronidation. The primary site of polyphenol metabolism is the small intestine (Spencer et al., 1999; Crespy et al., 2001; Donovan et al., 2001). Flavonoids are metabolized by uridine-5'-diphosphate glucuronosyl-transferases, sulfotransferases and catechol-*O*-methyltransferases in the small intestine (Rice-Evans, 2001; Kroon et al., 2004). The conjugates are then transported via the portal vein to the liver and are subjected to further conjugation (Olthof et al., 2003). Following conjugation in the liver, flavonoid

metabolites are transported around the body in the blood bound to plasma proteins (Manach et al., 1995). Conjugated polyphenols can also be secreted from the liver into the bile resulting in the transport back into the small intestine for further deconjugation or passage into the colon (Das and Griffiths, 1969; Donovan et al., 2001). Once reaching the colon, polyphenols are metabolised by the colonic microflora where the resulting metabolites are reabsorbed or are excreted in the faeces (Sfakianos et al., 1997). Flavonoid metabolites can also be secreted by the kidneys via the urine.

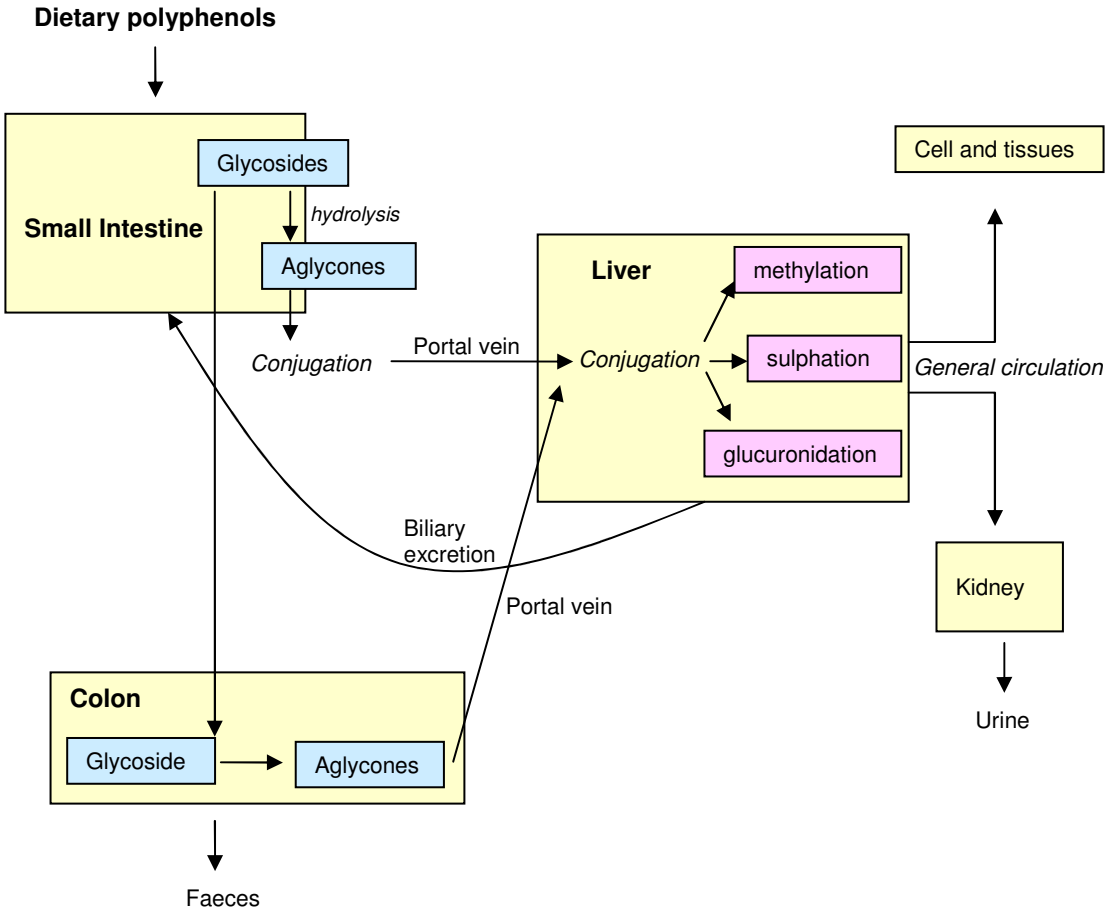


Figure 1.5: Absorption and metabolism of polyphenols.

Polyphenols are either hydrolysed in the small intestine or the colon depending on their chemical structure and the resulting aglycones are absorbed. Conjugation (methylation, sulfation and glucuronidation) occurs in the small intestine before passing into the liver where further conjugation occurs. From the liver the polyphenols are either secreted into the bile and transported back into the liver for further deconjugation or the polyphenol metabolites are transported around the body in the blood bound to plasma proteins and/or are secreted by the kidneys via the urine.

1.5 Polyphenols and cardiovascular disease

A high consumption of fruits and vegetables has been linked with a reduced risk of chronic diseases including cardiovascular disease (CVD). Polyphenols present in fruits and vegetables have been implicated as playing a significant role in the protective effects of these foods (Bravo, 1998). Therefore understanding how polyphenols reduce the risk of CVD is important as CVD is one of the primary chronic diseases afflicting industrialised countries (Epstein and Ross, 1999; Steinberg et al., 2003; Boyer and Liu, 2004).

1.5.1 Epidemiological associations

Higher intakes of polyphenols have been associated with a reduced risk of CVD in several epidemiological studies (Hertog et al., 1993a; Knekt et al., 1996; Hertog et al., 1997b; Yochum et al., 1999; Geleijnse et al., 2002; Knekt et al., 2002; Mink et al., 2007; Janszky et al., 2009; Buijsse et al., 2010) while other studies have shown no association between the consumption of polyphenols and a reduced risk of CVD (Rimm et al., 1996; Hertog et al., 1997a; Sesso et al., 2003).

In the Zutphen Elderly Study, flavonoid intake was inversely associated with coronary heart disease (CHD) and total mortality (Hertog et al., 1993a; Hertog et al., 1997b) and apples, which contributed 10% of the flavonoid intake, were also inversely related to coronary heart disease (CHD) mortality but with a weaker association (Hertog et al., 1993a). In the Finnish Mobile Clinic Health Examination Survey, the consumption of flavonoids and apples were inversely associated with total mortality and coronary mortality (Knekt et al., 1996) and ischemic heart disease and cerebrovascular disease (Knekt et al., 2002). The intake of flavanols and flavones was also inversely associated with CHD mortality in the Iowa Women's Health Study (Yochum et al., 1999) and

recently further investigation of the Iowa Women's Health Study showed that the intake of flavanones and anthoycanidins and apples were also inversely associated with CHD and CVD mortality after multivariate adjustment (Mink et al., 2007). An inverse relationship between the consumption of tea and flavonoids and myocardial infarction was observed in the Rotterdam Study cohort (Geleijnse et al., 2002). In the Stockholm Heart Epidemiological Program (Janszky et al., 2009) and in a middle-aged German cohort (Buijsse et al., 2010) the consumption of chocolate was related to a lower risk of CVD. In the Stockholm Heart Epidemiological Program, chocolate consumption had a strong inverse relationship with cardiac mortality (Janszky et al., 2009) and in the German cohort chocolate consumption had a stronger inverse relationship with stroke than myocardial infarction (Buijsse et al., 2010).

In contrast, three cohort studies found no association between flavonoid consumption and CVD risk (Hertog et al., 1997a). In the Health Professionals Follow-up Study (Rimm et al., 1996) and the Women's Health Study (Sesso et al., 2003), the intake of flavonoids was not associated with a reduced risk of CVD. The intake of flavonols, from tea to which milk was added, was also not associated with the incidence of ischemic heart disease in the Caerphilly Study (Hertog et al., 1997a). No association found between flavonoid consumption and CVD risk in these cohorts may be attributed, in part, on younger ages, greater ratio of women to men, lower risk factors, population group studied (Graf et al., 2005) and/or other factors such as the tendency to consume tea with milk in the Caerphilly Study and not accounting for appropriate confounding factors (Hertog et al., 1997a; Graf et al., 2005). Milk has been suggested to impair the bioavailability of flavonoids (Graf et al., 2005). Therefore, the majority of the epidemiological studies indicate that higher intakes of flavonoids are associated with a reduced risk of CVD.

1.5.2 Intervention studies

Cardiovascular disease is associated with the development of atherosclerotic plaques and thrombosis and atherosclerosis is the main cause of myocardial infarction and stroke. Endothelial dysfunction, inflammatory response, modified lipids and lipoproteins, and activated platelets are all involved in the development of CVD (Epstein and Ross, 1999; Steinberg et al., 2003). A number of human intervention trials have shown a positive relationship between the consumption of flavonoids and reductions in biomarkers of cardiovascular risk (Taubert et al., 2007; Hooper et al., 2008). A systematic review of the effectiveness of different flavonoid subclasses and flavonoid-rich food sources on CVD risk factors (endothelial function, blood pressure and lipoproteins) was conducted by Hooper et al. (2008). Endothelial function was one of the risk factors assessed because the vascular endothelium plays an important role in the regulation of vascular homeostasis and endothelial dysfunction is an indication of the initial development of atherosclerosis (Hashimoto et al., 1999; McEniery et al., 2006). Assessing endothelial function by flow-mediated dilation (FMD) is the gold standard, for further information regarding endothelial function refer to Chapter 3. The oxidation of low density lipoproteins (LDL) has also been implicated in the development of atherosclerosis (Hollman and Katan, 1997; Yochum et al., 1999) and the formation of foam cells and atherosclerotic plaques is due to the oxidation of LDL cholesterol which is taken up more quickly by macrophages (Yochum et al., 1999).

The majority of the dietary intervention trials, described in the systematic review by Hooper et al. (2008), assessed the effects of soy- or soy isoflavone-products or the effects of flavanols from red wine, grapes, chocolate, cocoa, black tea or green tea. The consumption of chocolate or cocoa increased FMD and reduced systolic and diastolic blood pressure after acute and chronic consumption but had no effect on low density lipoprotein (LDL) or high density lipoprotein (HDL) cholesterol (Hooper et al.,

2008). Reduced systolic and diastolic blood pressure was also shown in a meta-analysis of five randomized controlled studies (Taubert et al., 2007) and ten randomized controlled intervention trials after the consumption of flavanol-rich cocoa (Desch et al., 2010). While the meta-analysis of 13 intervention trials showed that the consumption of dark chocolate reduced systolic blood pressure in hypertensive subgroups and diastolic blood pressure in pre-hypertensive subgroups (Ried et al., 2010). Reduction of LDL cholesterol after cocoa consumption was only significant in subjects with CVD risk factors as shown in a meta-analysis of eight controlled intervention trials (Jia et al., 2010).

Acute consumption of black tea caused an increase in systolic and diastolic blood pressure, increased FMD response, but had no effect on LDL or HDL cholesterol while the consumption of green tea reduced LDL cholesterol but had no effect on systolic or diastolic blood pressure and no published data on FMD effects were available (Hooper et al., 2008). The consumption of tea (black or green) showed no significant effect on blood pressure in a meta-analysis of five randomized controlled studies (Taubert et al., 2007) but tea consumption was found to substantially enhance FMD in a meta-analysis of nine human intervention studies (Ras et al., 2011). Soy protein isolate, but not other soy groups, significantly reduced diastolic blood pressure and LDL cholesterol and the consumption of grapes or red wine had no significant effects on FMD, systolic or diastolic blood pressure, and LDL or HDL cholesterol (Hooper et al., 2008).

The results from the meta-analyses suggest that some flavonoid subclasses or flavonoid-rich foods have clinically relevant effects on CVD risk factors. However, there is still limited intervention trial data for some flavonoid subclasses and flavonoid-rich foods.

1.5.3 *In-vitro* studies and cellular mechanisms

In order to understand the mechanisms related to the clinically relevant effects of dietary polyphenols the *in-vitro* investigation of polyphenols have been conducted. Numerous reports of the potential cellular mechanisms of polyphenols have been reported, but the most convincing *in-vitro* results of the cellular mechanisms of polyphenols come from their ability to (1) increase nitric oxide (NO) by inducing nitric oxide synthase (NOS) and inhibiting NADPH oxidase activity; (2) induce cellular antioxidant mechanisms and scavenge free radicals; and (3) inhibit vascular endothelial growth factor (VEGF) induced angiogenesis.

1.5.3.1 Polyphenols increase nitric oxide synthase

The ability of polyphenols to improve endothelial function, *in-vivo*, has been attributed to their ability to increase NO-dependent vasorelxation (Heiss et al., 2003; Grassi et al., 2005b). NO is produced from L-arginine in a reaction catalyzed by the enzyme NOS (Leikert et al., 2002; Appeldoorn et al., 2009a). This enzyme is present in different isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). Endothelial-dependent NO is produced by eNOS. Therefore the *in-vitro* investigation of NO expression focuses on the capability of polyphenols to increase eNOS. EGCG (Lorenz et al., 2004; Appeldoorn et al., 2009a; Lorenz et al., 2009b), epicatechin (Ramirez-Sanchez et al., 2010), epicatechin gallate (Appeldoorn et al., 2009a), theaflavins (theaflavin-3-monogallate, theaflavin-3'-monogallate and theaflavin-3,3'-digallate) (Lorenz et al., 2009b), black tea polyphenols (Anter et al., 2004), resveratrol (Wallerath et al., 2002; Nicholson et al., 2008; Appeldoorn et al., 2009a), red wine polyphenols (Wallerath et al., 2003; Wallerath et al., 2005), red wine polyphenol extract (Leikert et al., 2002), grape skin extract (Madeira et al., 2009) and procyanidins (Karim et al., 2000) have been shown to increase eNOS expression in endothelial cells.

Procyanidins were also found to exert a non-significant increase in eNOS in endothelial cells (Appeldoorn et al., 2009a).

The increase of NO bioavailability by polyphenols has been reported to be due to their ability to inhibit NADPH oxidase activity (Steffen et al., 2007). Epicatechin metabolites (3'- and 4'-*O*-methyl epicatechin), epicatechin glucuronide, procyanidin dimer B2, ECG, EGC, EGCG and resveratrol were shown to inhibit NADPH oxidase (Steffen et al., 2007; Steffen et al., 2008). However, the majority of the *in-vitro* investigation of the NO preserving-effect of polyphenols has been shown at concentrations ranging from 10 µM to 100 µM (Karim et al., 2000; Wallerath et al., 2002; Appeldoorn et al., 2009a; Lorenz et al., 2009b) and concentrations of 4 µM to 16 µM for their NADPH oxidase activity inhibition (Steffen et al., 2008). The physiological concentrations of dietary polyphenols do not exceed 10 µM and thus the effects of *in-vitro* concentrations of > 10 µM are generally not valid (Williamson and Manach, 2005). However, the NO-preserving effect of epicatechin has been observed at a concentration of 1 µM (Ramirez-Sanchez et al., 2010) and the ability of certain polyphenols to inhibit NADPH oxidase activity has been observed at concentrations less than 10 µM (Steffen et al., 2008). Therefore this mechanism is applicable *in-vivo* and the intervention studies assessing endothelial function support this mechanism. The ability of polyphenols to increase NO bioavailability and inhibit endothelial NADPH oxidase activity is therefore an important mechanism in the cardio-protective effects as NO has also been shown to improve insulin sensitivity, decrease platelet aggregation and adhesion, decrease oxidation of LDL cholesterol and increase HDL cholesterol (Holt et al., 2002b; Wallerath et al., 2003; Grassi et al., 2005b; Baba et al., 2007).

1.5.3.2 Antioxidant effects of polyphenols

The ability of polyphenols to protect against CVD may be due to their ability to act as antioxidants. Polyphenols, especially flavonoids, have been reported to act as antioxidants through their ability to (1) scavenge free radicals and (2) suppress reactive oxygen species formation through the inhibition of enzymes responsible for superoxide anion production and chelating trace elements involved in free radical production (Bravo, 1998; Pietta, 2000). Polyphenols interfere with the oxidation of lipids and other molecules by donating a hydrogen atom to the free radicals and the efficiency of polyphenols to act as antioxidants is therefore dependent on their structure and number of hydroxyl groups (Bravo, 1998). The following foods rich in flavanols are an example of some of the foods which have been reported to be powerful antioxidants: black and green tea (Henning et al., 2003), cocoa (Vinson et al., 1999), red wine (Fuhrman et al., 2001) and apples (Eberhardt et al., 2000). The antioxidant effects of flavanols are due to their ability to stimulate nuclear factor-(erythroid-derived)-related factor 2 (Nrf2) protein expression (Yeh and Yen, 2006; Jung and Kwak, 2010). Nrf2 is a transcription factor that has been identified as an antioxidant response element-binding protein (Yeh and Yen, 2006; Na and Surh, 2008). EGCG (Na et al., 2008; Na and Surh, 2008), epicatechin (Granado-Serrano et al., 2010), gallic acid (Yeh and Yen, 2006), and resveratrol (Kode et al., 2008) have been reported to increased Nrf2 expression *in-vitro*.

The antioxidant capability of these foods have been investigated *in-vitro*, however the ability of the flavanols to inhibit LDL oxidation only resulted through the use of high doses that do not take into account what is seen *in-vivo*. For example, green or black tea inhibited LDL oxidation in plasma after the incubation of very high amounts of green or black tea *in-vitro* and the consumption of six cups per day for four weeks of green or black tea did not affect serum lipid concentrations or resistance to LDL cholesterol but only slightly increased the total cholesterol antioxidant activity of plasma *in-vivo* (van

het Hof et al., 1997). The consumption of a green tea supplement also only resulted in a small but significant increase in plasma antioxidant activity compared with when tea polyphenols were consumed as black tea or green tea (Henning et al., 2004).Therefore based on this evidence, the antioxidant effects of polyphenols is not a plausible mechanism.

1.5.3.3 Inhibition of vascular endothelial growth factor-induced angiogenesis by polyphenols

Physiological and supraphysiological concentrations of polyphenols have been found to have an effect on and influence VEGF-induced phosphorylation of VEGF receptor-2 in endothelial cells (Kondo et al., 2002b; Lamy et al., 2002; Tang et al., 2003; Neuhaus et al., 2004; Labrecque et al., 2005; Lamy et al., 2006; Mojzis et al., 2008; Wen et al., 2008; Lu et al., 2010). Further information regarding VEGF and its receptors and the effect of polyphenols on inhibition of VEGF-induced VEGF receptor-2 (VEGFR-2) phosphorylation is described in Chapter 4 and 5.

1.6 Aims of the thesis

The overall aims of this thesis were to (1) assess the potential of apples as a rich source of extractable bioactive polyphenols and (2) investigate the bioactivity of apple polyphenols with a focus on understanding mechanisms of action in the vascular endothelium.

Polyphenols found in different apple cultivars have been well documented in the literature; however it has become evident that there is a lack of information (i.e. the quantification of the full apple polyphenol profile) on how apple polyphenols are affected by growth and maturity and seasonal variations. Therefore, the quantification of the full profile of polyphenols found in apples was conducted over an eight week growing season over a three year period (Chapter 2). The knowledge of how individual polyphenols are affected by growth and seasonal variations is useful in identifying the harvest time that is optimal for polyphenol yield or polyphenol concentration for fruit consumption, fruit processing or if the fruit were to be used as a source of polyphenols for extraction.

As polyphenols are known to be associated with a reduced risk of cardiovascular disease and have been shown to: improve endothelial function by increasing the bioavailability of NO by inhibiting NADPH oxidase; exhibit antioxidant effects; and inhibit VEGF-induced angiogenesis the supplementation of apple polyphenols in foods may provide health protecting effects. In order to supplement foods with polyphenols, the polyphenols first needs to be efficiently extracted from the apples. The current extraction methods available do not provide an overall high extraction yield or affect the characteristics of the juice. Therefore the investigation of an efficient extraction method of polyphenols from apple pomace, a by-product of apple juice production, was conducted and the effect of the consumption of an apple polyphenol-rich extract

supplemented drink on the improvement of vascular function in human subjects was also studied (Chapter 3).

From the data presented by García-Conesa et al. (2009), it was shown that the majority of biological processes influenced by an apple procyanidin fraction were most likely related to VEGF signalling. Certain polyphenols have been previously shown to inhibit VEGF-induced VEGFR-2 signalling, however the mechanisms of this action has not been investigated. As a result the examination of the ability of procyanidins, isolated from apples, and other flavanols to inhibit VEGF-induced VEGFR-2 signalling and the underlying mechanism(s) behind the inhibitory action were investigated (Chapter 4). It was discovered that certain flavanols were able to inhibit VEGF-induced VEGFR-2 phosphorylation by interacting with the VEGF molecule. This finding led to the subsequent characterization of the binding interactions between certain flavanols and VEGF (Chapter 5).

The results also showed that a procyanidin fraction isolated from apples was able to completely inhibit VEGF-induced VEGFR-2 phosphorylation at physiological concentrations (Chapter 4). Hence, the global analysis of the genes influenced by VEGF and the procyanidin fraction were investigated (Chapter 6). It was revealed that the apple procyanidin fraction not only blocks VEGF-regulated genes but also regulates genes that are independent of VEGF. One of the genes that was induced by the procyanidin fraction was Krüppel-like factor 2 (KLF2). As KLF2 is an important transcription factor implicated in the suppression of pro-inflammatory and pro-atherosclerotic genes as well as inducing antithrombotic genes and regulating angiogenesis, further investigation of how the apple procyanidin fraction induces KLF2 expression was conducted (Chapter 7).

Finally the main findings are summarized in Chapter 8 and suggestions for the direction of future research are given in this chapter as well.

CHAPTER TWO

APPLE POLYPHENOL ANALYSIS OVER THE GROWING SEASON

Chapter 2 : Apple polyphenol analysis over the growing season

2.1 Abstract

The understanding of how the content and composition of apple polyphenols changes during the growing season and between seasons is beneficial as it allows the identification of when the total or individual content is the greatest and how variable the polyphenol content is between seasons. This knowledge is useful in identifying the harvest time that is optimal for polyphenol yield or polyphenol concentration for fruit consumption, fruit processing or if the fruit were to be used as a source of polyphenols for extraction. The effect of seasonal variation on the polyphenol concentration and content and percentages of individual polyphenols in the apple fruit were therefore investigated in the years 2007, 2009 and 2010 from an apple orchard located in Herefordshire. Apples were collected from the same three trees in each year and were sampled from the north, south, east, west, top, bottom, inner and outer positions of the tree. This allowed for the direct comparison between the years as well as assessing the variability in the polyphenol concentration of the apples located on the same tree. The results from this chapter have shown that the polyphenol concentration, content and percentage of quercetin glycosides and total flavanol monomers in the apple fruit of two varieties grown in an orchard in Herefordshire are affected by (1) growth and maturity (the polyphenol concentration decreases during growth while the content increases due to an increase in fruit weight); (2) seasonal variations (mean temperature is negatively correlated with polyphenol content and concentration while total amount of rainfall and total hours of sunshine are positively correlated with content and concentration); (3) location in the orchard; and (4) may be affected by the location on the tree as a large variation is observed from apples collected from the same tree. This is also the first

time that a complete quantitative HPLC analysis of the polyphenols found in apples was conducted as well as the first time an analysis of the direct effect of seasonal variation on individual apple trees has been investigated.

2.2 Introduction

The understanding of how the content and composition of apple polyphenols changes during the growing season and between seasons is useful because it allows the identification of when the total or individual polyphenol content is the greatest and the determination of how variable this is between seasons. This knowledge could be used to identify the harvest time that is optimal for polyphenol yield or polyphenol concentration for fruit consumption, fruit processing or if the fruit were to be used as a source of polyphenols for extraction.

The concentrations of polyphenols in apples and apple derived products show a large variation depending on the cultivar (Pérez-Ilzarbe et al., 1991; Guyot et al., 1998; Podsędek et al., 2000; Guyot et al., 2003; Vrhovsek et al., 2004; Cheynier, 2005; Marks et al., 2007a; Marks et al., 2007b; Renard et al., 2007), distribution within the fruit (Guyot et al., 1998; Chinnici et al., 2004; Cheynier, 2005; Lata, 2007; Marks et al., 2007a; Renard et al., 2007), growth and maturation (Lister et al., 1994; Mayr et al., 1995; Awad et al., 2001a; Kondo et al., 2002a; Guyot et al., 2003; Takos et al., 2006; Renard et al., 2007), seasonal variations (Awad et al., 2001b; van der Sluis et al., 2001; Guyot et al., 2003), climate conditions (Awad et al., 2000; Awad et al., 2001a; Awad et al., 2001b; Takos et al., 2006) and food preparation, processing and storage (Ju et al., 1996; Awad and de Jager, 2000; Golding et al., 2001; Lattanzio et al., 2001; Aherne and O'Brien, 2002). These literature reports relating to various agronomic, climatic and genetic factors to the polyphenol content and composition of apples are described in the following sections.

2.2.1 Cultivar

Several reports have shown that there is substantial inter-cultivar variation in the concentration of polyphenols in apples (Pérez-Ilzarbe et al., 1991; Escarpa and

Gonzalez, 1998; Guyot et al., 1998; Price et al., 1999; Podsędek et al., 2000; Guyot et al., 2003; Vrhovsek et al., 2004; Cheynier, 2005; Marks et al., 2007a; Marks et al., 2007b; Renard et al., 2007). Cider apples and dessert apples contain the same polyphenols but cider apples contain higher concentrations of polyphenols than dessert apples. There is also a large variation in polyphenol concentration within the dessert and cider apple varieties. According to the data presented in Table 2.1, the reported polyphenol concentrations in dessert apples range from 662 mg/kg to 3240 mg/kg fresh weight while the polyphenol concentration in cider apples range from 1899 mg/kg to 10183 mg/kg fresh weight.

2.2.2 Distribution within the fruit

The composition and concentration of polyphenols in apples also vary greatly between the apple peel and the apple flesh (Escarpa and Gonzalez, 1998; Guyot et al., 1998; Price et al., 1999; Lata et al., 2005; Marks et al., 2007a). The apple flesh and peel contain the same polyphenols, with the exception of anthocyanins which are predominately located in the apple peel. However the apple flesh has higher concentrations of flavanols and dihydrochalcones while the apple peel has higher concentrations of flavonols, hydroxycinnamic acids and anthocyanins (Chinnici et al., 2004; Cheynier, 2005; Lata et al., 2005; Renard et al., 2007). Overall, higher concentrations of polyphenols are found in the apple peel (546 mg/kg to 6306 mg/kg) compared to the apple flesh (230 mg/kg to 5065 mg/kg) (Table 2.1). However, when examining the content of polyphenols in the entire apple (Kermerrien cider apples) the greatest polyphenol content was located in the apple flesh (65 %) than the skin (24 %), core (10 %) or seeds (1 %) (Guyot et al., 1998). This is because there is a greater proportion of flesh in an apple than skin, core or seeds and therefore the largest amount of polyphenols would be located in the flesh.

Table 2.1: Polyphenol content in apple varieties (mg/kg fresh weight)

Variety	Total phenolics	Flesh	Peel
Dessert Apples			
Alwa[1]	2800 ± 300	n/a	n/a
Braeburn[3]	754	n/a	n/a
Boskoop[1]	3570 ± 340	n/a	n/a
Close[1]	2290 ± 250	n/a	n/a
Elstar[1]	2520 ± 290	n/a	n/a
Fuji[3]	662 ± 131	n/a	n/a
Idared[1]	2730 ± 340	n/a	n/a
Jonagold[1]	3100 ± 190	n/a	n/a
Golden Delicious[4]	776	230 ± 16	546 ± 43
Golden Delicious[3]	863 ± 75		
Granny Smith[3]	1210	n/a	n/a
Lobo[1]	2900 ± 250	n/a	n/a
Morgenduft[3]	1058	n/a	n/a
Papierówka[1]	3240 ± 300	n/a	n/a
Red Delicious[3]	1311 ± 138	n/a	n/a
Renetta[3]	2119 ± 138	n/a	n/a
Royal Gala[3]	839 ± 120	n/a	n/a
Szampion[1]	2970 ± 70	n/a	n/a
Warta[1]	3240 ± 310	n/a	n/a
Cider Apples			
Ashton Bitter[4]	6903	2798 ± 29	4105 ± 254
Avrolles[2]	--	3182 ± 297	n/a
Bedan[2]	--	3441 ± 223	n/a
Brown Snout[4]	4484	1693 ± 37	2791 ± 50
Browns Apple[4]	3680	893 ± 28	2787 ± 120
Broxwood Foxwhelp[4]	2379	640 ± 42	1739 ± 80
Bulmers Norman[4]	7315	2294 ± 19	5021 ± 113
Chisel Jersey[4]	6967	2474 ± 15	4493 ± 65
Dabinett[4]	5163	1653 ± 57	3510 ± 88
Dous Möen[2]	--	3161 ± 433	n/a
Ellis Bitter[4]	4116	1243 ± 39	2873 ± 114
Harry Masters Jersey[4]	6007	2862 ± 101	3145 ± 14
Kermerrien[2]	--	5065 ± 550	n/a
Major[4]	3197	1418 ± 50	1779 ± 25
Medaille d'Or[4]	10448	4920 ± 20	5528 ± 46
Michelin[4]	4145	1754 ± 38	2391 ± 162
Petit Jaune[2]	--	1899 ± 186	n/a
Reine des Hâtives[4]	2746	931 ± 19	1815 ± 34
Somerset Redstreak[4]	3273	1372 ± 35	1901 ± 120
Sweet Coppin[4]	1251	485 ± 4	766 ± 12
Taylors Sweet[4]	3958	1122 ± 23	2836 ± 103
Tremletts Bitter[4]	6685	2746 ± 24	3939 ± 102
Vilberie[4]	4933	2093 ± 59	2840 ± 143
Yarlington Mill[4]	10183	3877 ± 103	6306 ± 117

Data obtained from (Podsędek et al., 2000; Guyot et al., 2003; Vrhovsek et al., 2004; Marks et al., 2007a)

n/a = not available

[1] Polyphenol concentration determined by the Folin-Ciocialteu method and (+)-catechin was used as the standard (Podsędek et al., 2000)

[2] Polyphenol concentration determined by HPLC analysis and the procyanidins were determined by thiolysis followed by HPLC analysis. Quantified (+)-catechin, (-)-epicatechin, procyanidin B2, total procyanidins, caffeoylquinic acid, *p*-coumaroylquinic acid, phloretin xyloglucoside, phloridzin (Guyot et al., 2003).

[3] Polyphenol concentration determined by the Folin-Ciocialteu method (Vrhovsek et al., 2004).

[4] Polyphenol concentration determined by HPLC analysis. Quantified (+)-catechin, (-)-epicatechin, procyanidin B2, cyanidin-3-*O*-galactoside, 5-*O*-coumaroylquinic acid, 4-*O*-*p*-coumaroylquinic acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-xyloside, phloretin-2'-*O*-(2''-*O*-xylosylglucoside), quercetin-3-*O*-arabinopyranoside, quercetin-3-*O*-arabinofuranoside, and phloretin 2'-*O*-glucoside (phloridzin) (Marks et al., 2007a).

2.2.3 Growth and maturity

The composition and concentrations of polyphenols in apples is influenced by growth and the level of fruit maturity. In general, reports have shown that the highest concentrations of polyphenols occur early in fruit development and decline throughout fruit growth and maturation. The polyphenol concentration of Granny Smith and Splendour apples (Lister et al., 1994), Golden Delicious apples (Mayr et al., 1995), Jonagold and Elstar apples (Awad et al., 2001a), five French cider apple varieties (Avrolles, Dous Möen, Petit Jaune, Kermerrien and Bedlan) (Guyot et al., 2003), Cripps Red apples (Takos et al., 2006), Dous Moen, Kermerrien, Elstar and Gala apples (Renard et al., 2007) and Fuji, Oorin and Redfield apples (Kondo et al., 2002a) were highest in the early stages of fruit development and significantly decreased during fruit development. However, in Splendour apples an increase in quercetin glycosides and anthocyanin concentrations was observed during ripening (Lister et al., 1994). The observed decline in the concentration of polyphenols during fruit growth and maturation may be attributed to a dilution effect due to fruit growth (Takos et al., 2006).

2.2.4 Seasonal variations

Seasonal variations (differences in weather conditions between harvest years) may influence the polyphenol concentration in apple cultivars. Certain apple cultivars have been shown to have stable polyphenol concentrations between harvest years (Lister et al., 1994; Guyot et al., 2003) while other cultivars are more susceptible to seasonal variations (van der Sluis et al., 2001; Lata et al., 2005; Lata and Tomala, 2007; Stracke et al., 2009). In Granny Smith and Splendour apples (Lister et al., 1994) and five French apple varieties (Avrolles, Dous Möen, Petit Jaune, Kermerrien and Bedlan) (Guyot et al., 2003) there were no significant differences in the polyphenol concentrations between harvest years. van der Sluis et al. (2001) investigated the seasonal variation on flavonoid concentration in four apple varieties (Jonagold, Golden

Delicious, Cox's Orange and Elstar) for the harvest years 1996 to 1998. In Jonagold apples no significant differences were observed in the concentrations of chlorogenic acid, total catechins, total quercetin glycosides, phloridzin and cyanidin 3-galatacoside between harvest years while Golden Delicious had some variation in total quercetin glycosides and phloridzin concentrations and Elstar apples varied in phloridzin concentration (van der Sluis et al., 2001). Cox's Orange apples were most sensitive to seasonal variation and a significant difference in polyphenol concentrations was observed. A significant variation between the harvest years 2003 and 2004 was shown in 56 apple varieties (Lata et al., 2005). Lata and Tomala (2007) showed a significant variation between the harvest years 2004 and 2005, from the same tree, in 19 apple varieties and Stracke et al. (2009) also found a significant variation in the polyphenol concentration of Golden Delicious apples between the harvest years 2004 and 2006. Therefore differences in polyphenol concentrations between harvest years may depend on the cultivar investigated as well as the climate conditions between harvest years.

2.2.5 Environmental factors

Environmental factors such as climate and soil conditions also have an influence on the polyphenol concentration of apples. Climate refers to the weather conditions of the geographical region. The amount of light (sunlight) is considered to be one of the most important factors in the control of flavonoid synthesis (Awad et al., 2001b; Takos et al., 2006). In Jonagold and Elstar apples, greater concentrations of cyanidin 3-galactoside and quercetin glycosides were found in apples positioned on the outer tree while those in the inner/shaded positions, with less amount of light received, had significantly lower concentrations of these compounds (Awad et al., 2000; Awad et al., 2001b). The concentration of catechins, phloridzin and chlorogenic acid were unaffected by light conditions. Therefore, the differences in light conditions (quantity and quality) in the

outer and inner positions of the tree explain the higher concentrations of anthocyanins and quercetin glycosides found in the outer fruit (Awad et al., 2001a).

Temperature has also been shown to effect anthocyanin formation in apples. Low evening temperatures and high sunlight levels during autumn has been reported to increase anthocyanin content formation (Saure, 1990). Although it has been suggested that increases in the amount of rainfall can result in lower polyphenol concentrations (Stracke et al., 2009), there is a lack of literature concerned with how rainfall affects polyphenol content in apples.

Soil nutrients are another environmental variable influencing polyphenol content in apples. In general, higher amounts of nitrogen in soil has a negative effect on anthocyanin formation in apples and the effect of other nutrients (e.g. potassium and phosphorous) on anthocyanin formation is inconsistent (Awad and de Jager, 2002).

Therefore the difference in environmental factors between geographical regions may also influence the total polyphenol content of apples of the same variety. The polyphenol concentrations of certain cultivars (Pacific Beauty, Pacific Queen, Pacific Rose and Granny Smith) showed significant variation between the three regions investigated in New Zealand, while other cultivars (Braeburn, Royal Gala, Jazz and Red Delicious) showed no significant variation between regions (McGhie et al., 2005). Variables within the geographical region that may influence the polyphenol content of apples include the climate and soil nutrients.

2.2.6 Storage, food preparation and processing

Storage conditions can increase, decrease or have no effect on the polyphenols concentrations in foods depending on the method of storage (Awad and de Jager,

2000; Aherne and O'Brien, 2002). However, the polyphenol content in apples during cold storage is relatively stable. In Golden Delicious, Cox's Orange, Elstar and Jonagold apples, cold storage did not have a significant effect on the total quercetin glycosides, phloridzin and cyanidin galactoside concentrations (van der Sluis et al., 2001). In Delicious and Ralls apples no significant changes occurred in the concentrations of chlorogenic acid, flavonoids and anthocyanins during 4 – 5 months of cold storage (Ju et al., 1996). However, after transfer from cold storage to 20˚C, a rapid decrease in chlorogenic acid and flavonoids concentrations was observed. Lattinzo et al. (2001) reported that the polyphenol concentration in Golden Delicious apples increased in cold storage with a maximum increase after 60 days, afterward fruit stored for a prolonged period showed a decrease in polyphenol concentration while Golding et al. (2001) showed that the polyphenol concentration of Granny Smith, Lady Williams and Crofton apples remained relatively stable in cold storage.

Food preparation and processing can also lead to a significant decrease in polyphenol concentration (Aherne and O'Brien, 2002; Cocci et al., 2006). Polyphenols are resistant to heat, oxygen and moderate degrees of acidity (Aherne and O'Brien, 2002), however kitchen preparation will result in polyphenol losses. Cutting or slicing apples causes enzymatic browning to occur through the mixture of polyphenol compounds with polyphenol oxidase (PPO) and the diffusion of oxygen into the tissue, which causes the total polyphenol content in the flesh to decrease (Cocci et al., 2006). The processing of apples results in a significant loss of polyphenols in the resulting apple products. For example, losses in polyphenols in apple juice can occur due to (1) pressing and (2) oxidation (Awad et al., 2000; Treutter, 2001; van der Sluis et al., 2002; Guyot et al., 2003). During pressing, the polyphenols may bind to the cell-walls and be retained in the pomace (Guyot et al., 2003). Enzymatic oxidation of chlorogenic acid and procyanidins contribute to the colour of the juice or cider, however enzymatic oxidation decreases the final polyphenol concentration (Awad et al., 2000; Treutter, 2001).

The polyphenol concentration of apples can significantly change based on the different variables discussed above, however, in this chapter, the variability concerning seasonal variation will be further investigated. Although there have been a number of reports (Lister et al., 1994; van der Sluis et al., 2001; Guyot et al., 2003; Lata et al., 2005; Lata and Tomala, 2007; Stracke et al., 2009) concerned with variations in polyphenol content and composition of apples in relation to seasonal variability, these studies lack a complete analysis of the full apple polyphenol profile. Lister et al. (1994) quantified flavanols (catechin, epicatechin), quercetin glycosides (quercetin-3-rhamnoglucoside, quercetin-3-glucoside, quercetin-3-xyloside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside and quercetin-3-rhamonside) chlorogenic acid, phloridzin, cyanidin-3-*O*-galactoside, and proanthocyanidins by using a water solvent delivery system. van der Sluis et al. (2001) quantified quercetin glycosides (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xyloside, quercetin-3-arabinoside and quercetin-3-rhamnoside), epicatechin, catechin, chlorogenic acid, phloridzin and cyanidin galactoside by HPLC. Guyot et al. (2003) quantified epicatechin, catechin, procyanidin B2, caffeoylquinic acid, *p*-coumarolyquinic acid, phloretin xyloglucoside and phloridzin by HPLC, and total procyanidins by thiolysis of the samples followed by HPLC analysis. Total phenolics (gallic acid standard), flavonols (quercetin standard) and anthocyanins (cyanidin-3,5-diglucoside standard) were measured spectrophotometerically by Lata et al. (2005) and Lata and Tomala (2007). Finally, Stracke et al. (2009) quantified chlorogenic acid, hydroxycinnamic acids (4-caffeoylquinic acid, 3-coumaroylquinic acid, 4-coumaroylquinic acid and 5-coumaroylquinic acid) and dihydrochalcones (phloretin 2'-xyloglucoside and phloretin-2'-glucoside), flavanols (epicatechin, catechin and procyanidin B2) and quercetin glycosides (quercetin-galactoside, quercetin-glucoside, quercetin-xyloside, quercetin-arabinoside and quercetin-rhamonoside) by HPLC analysis.

There are also flaws in the sampling design in some of the reports: in one report (van der Sluis et al., 2001) the sampling design included sampling the fruit from the outer layer of the tree only and avoided sampling fruit located on the top, bottom or inner positions of the tree; another report (Guyot et al., 2003) only selected the predominant fruit for further analysis; and finally another report (Lata and Tomala, 2007) only selected fruit of similar size or diameter located on the outer layer of the tree. This chapter, therefore, presents results of a full apple polyphenol profile between three seasons and the sampling preformed was representative of the whole tree.

2.3 Objectives

The overall aim of the research presented in this chapter was to investigate changes in the content and composition of apple polyphenols during the growing season for several growing seasons. The key aspects of the study design were: 1) apples were harvested from the same individual trees and analysed as such to allow direct comparisons to be made between seasons for individual trees; 2) sampling of individual trees was representative of the whole tree; 3) on selected occasions, individual apples were analysed to allow within-tree variation to be assessed; and 4) all of the different apple polyphenols were quantified. The resulting data was used to address the following questions concerning apple polyphenol content, concentration and composition: 1) the changes/differences in total polyphenol concentration, content and composition between seasons; 2) the changes/differences in total polyphenol concentration, content and profile within a single sampled tree between seasons; 3) the relationship between growth and maturity of the apple fruit and the concentration and content of polyphenols within a season; and 4) the extent of within-tree variation in polyphenol concentration.

2.4 Materials and methods

2.4.1 Materials

Methanol, acetonitrile and dichloromethane were obtained from Fisher-Scientific and trifuloroacetic acid, (-)-epicatechin, (+)-catechin and chlorogenic acid were purchased from Sigma-Aldrich (Poole, UK). The NIST Standard Reference Material 2384 baking chocolate (NIST chocolate standard) was sourced from the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland). All solvents were HPLC grade. The polyphenol standards, quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, phloridzin and galangin were purchased from Extrasynthase (Genay, France).

2.4.2 Apple samples

The analysed apple samples were provided and collected by Richard Wood of Coressence Ltd (Herefordshire, UK). The apples were collected every week for eight weeks during the apple growing season. The apples were collected approximately eight weeks post bud burst. The 2007 apples were collected from the week beginning July 22, 2007, the 2009 apples were collected from the week commencing July 19, 2009 and the 2010 apples were collected from the week starting July 19, 2010. Three trees, on the southern end of the apple orchard, were used to collect the samples. The treatment (i.e. use of fertilizers, pesticides, etc) of the orchard was consistent for each year. The orchard represents a mixed orchard, with the presence of the main cultivar (tree code: 07003102) and a pollinator cultivar (tree code: 0700392). Both the main cultivar and the pollinator cultivar were a cross of three bittersweet apple varieties. The main apple cultivar is planted in a ratio of 4:1 to the pollinater apple cultivar. Trees A and B are the main variety and tree C is the pollinator variety. The apples were

collected from three consecutive rows and columns and sampled from the north, south, west, east, top, bottom and inner positions of the tree. Once collected, the apples were immediately frozen and were subsequently transported to IFR using refrigerated transport (-20 ºC).

In 2007, the apples were delivered frozen and seven apples were collected from each tree in Week 1, followed by six apples for the remaining weeks. In 2009, seven apples were collected from each week. The 2009 Week 1 apples were delivered fresh and the apples from Weeks 2 – 8 (2009) were delivered frozen. The 2009 Week 5 apples were not collected and analysis on 2009 Week 7 apples for trees A and B and 2009 Week 8 apples for trees A, B and C were lost due to a failure in the freeze-drying equipment causing the fruit to defrost and rot. In 2010, seven apples were collected in each week.

2.4.3 Meteorological information

The apple orchard where the apples were harvested is located in Dewsall, Herefordshire (West Midlands). Meteorological information on the mean temperature, total rainfall and total hours of sunshine in the Midlands (Figure 2.1) was obtained from the Met Office (www.metoffice.co.uk). The Midlands area includes the Cotswold Hills to the south, the Northamptonshire uplands to the east, the Peak District to the north and is bounded by the Welsh border to the west (www.metoffice.co.uk). Therefore the presentation of the average climatic data from the Midlands is a limitation in this study because the climatic data covers a large geographical region rather than focusing on the specific geographical region where the orchard is located.

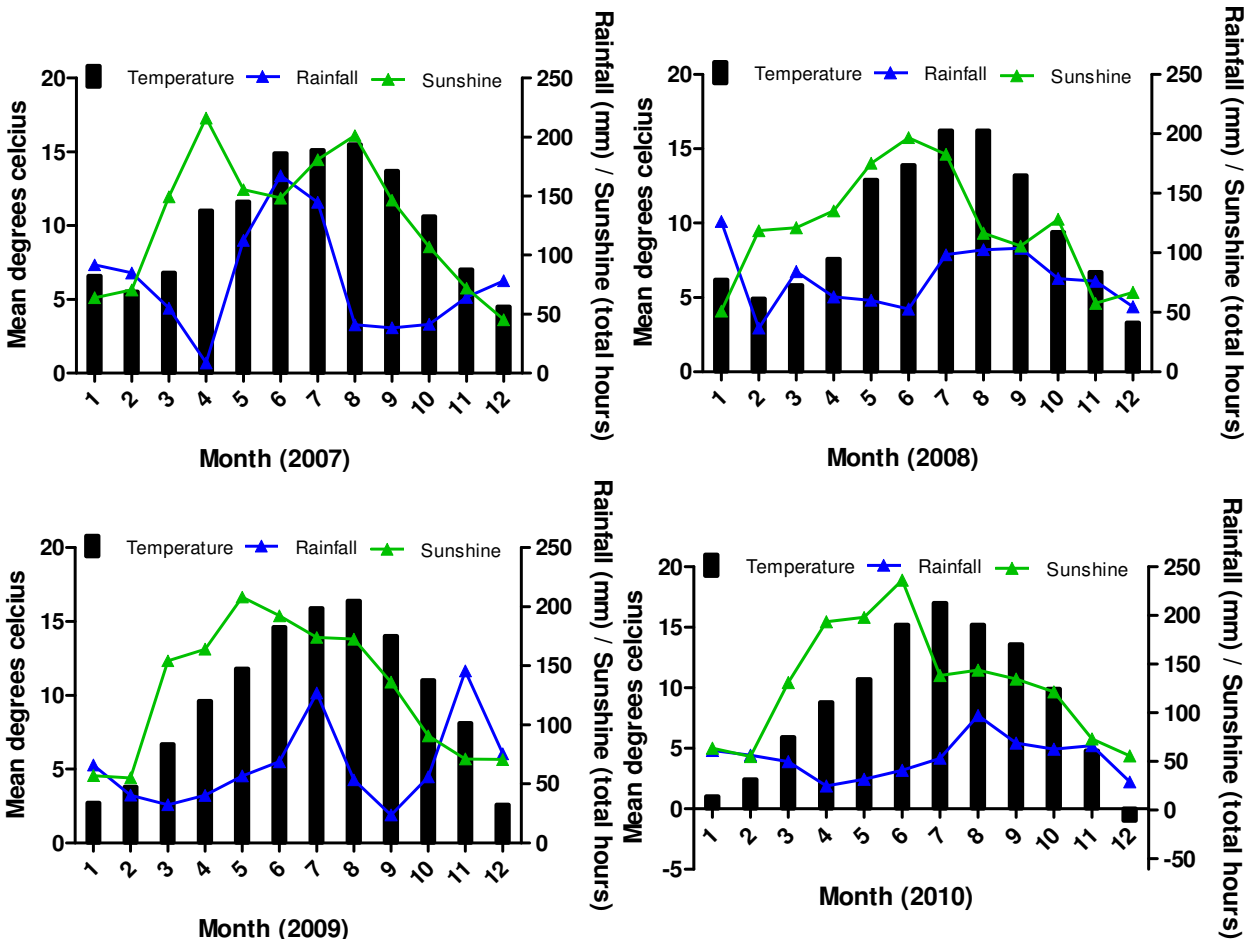


Figure 2.1: Mean temperature (ºC), total rainfall (mm) and sunshine (total hours) in the Midlands, England during 2007 to 2010.

2.4.4 Sample preparation

2.4.4.1 Preparation of apples after delivery

Once delivered, the apples from each collection week and tree code were weighed individually. The apples were then cut into eight segments and four alternating segments were bagged for individual analysis and the other four alternating segments were bagged into a group bag for composite analysis. The bagged apples were re-weighed and subsequently freeze-dried for seven days. The weight of the freeze-dried apples was recorded and the apples were ground into a powder using a domestic food processor and the powder was transferred to zip-lock plastic bags and stored at room temperature in the dark.

2.4.4.2 Apple polyphenol extraction

Samples (40 mg, in triplicates) of freeze-dried whole apple powder were extracted using 950 µl of 70 % (v/v) methanol and 50 µl of internal standard (0.1 mg/ml galangin in methanol) at 70 ºC for 20 min. The samples were centrifuged for 10 min at 13000 x g. The supernatants were filtered (0.22 µM) and the filtrate from each sample was transferred to a HPLC vial. Samples were stored at -20 ºC for up to 48 hours subsequent to HPLC analysis.

2.4.5 High-performance liquid chromatography analysis

Each sample was analysed using two different high-performance liquid chromatography (HPLC; Agilent HP1100) methods: (1) reverse-phase HPLC analysis and (2) normal-phase HPLC analysis. The coefficient of variation of the HPLC methods was below 10 % (intra-assay) for approximately 80 % of the samples analysed.

2.4.5.1 Reverse-phase HPLC analysis with UV Diode Array Detection

The reverse-phase analysis method is used for the separation and quantification of phenolics and flavonoids and was used to quantify the following phenolics: epicatechin, quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, phloridzin, and 3-*O*-caffeoylquinic acid (chlorogenic acid). Phenolics and flavonoids were analysed using a Luna C18 column (250 x 4.5 mm^2, 5 µM particle size) (Phenomenex, Macclesfield, UK) and a mobile phase consisting of a 65 min gradient (0 – 15 , 0 % B; 15 – 22 min, 17 % B; 22 – 30 min, 25 % B; 30 – 35 min, 35 % B; 35 – 40 min, 50 % B; 40 – 55 min, 100 % B; 55 – 65 min, 0 % B) where solvent A is 0.1 % aqueous TFA and solvent B is 0.1 % TFA in acetonitrile. Phenolics

and flavonoids were detected by diode array detection (DAD) and the detection of phenolics present was established at 270 nm. Identification of phenolics and flavonoids were established by running standards for each flavonoid of interest. Example chromatograms of the standards and apple samples are shown in Figure 2.2. The limits of detection of the polyphenol compounds quantified are described in Table 2.2.

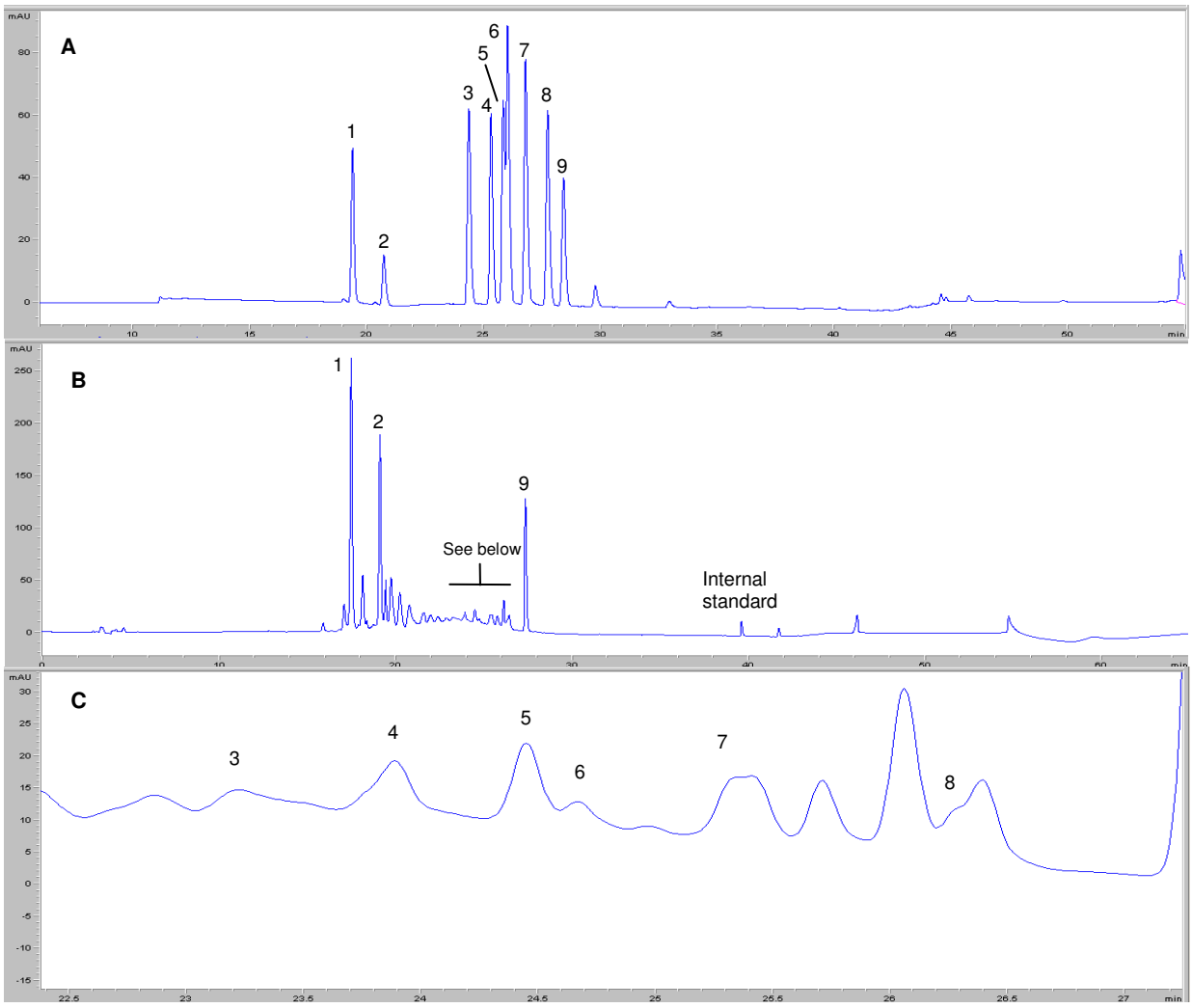


Figure 2.2: Example reverse-phase HPLC chromatograms of low MW phenolics at 270nm. Mixed standard (**A**) and apple example (**B**: all phenolics and **C**: zoomed section). Peak ID: 1 = chlorogenic acid, 2 = epicatechin, 3 = quercetin-3-*O*-arabinose, 4 = quercetin-3-*O*-rutinoside (rutin), 5 = quercetin-3-*O*-galactoside, 6 = quercetin-3-*O*-glucoside, 7 = quercetin-3-*O*-xyloside, 8 = quercetin-3-*O*-rhamnoside and 9 = phloridzin.

2.4.5.2 Normal-phase HPLC analysis with Fluorescence Detection

The normal-phase method is used for the quantification of polymeric flavan-3-ols (polymeric catechins, also termed procyanidins or condensed tannins). Procyanidin oligomers up to dp 10 (degree of polymerisation of 10 units) can be separated while those of higher dp are eluted as a 'hump' following the dp10 peaks. Samples of the NIST chocolate were run alongside apple samples to confirm peak retention times and were used as a quality control standard. Epicatechin and catechin standards were run to quantify the polymeric fractions. In the normal-phase method, flavanols were separated according to degree of polymerisation using a Luna silica column (250 x 4.6 mm^2, 5 µM particle size) (Phenomenex, Macclesfield, UK) and a mobile phase consisting of a 65 min gradient (0 – 30 min, 14.0 – 28.4 % B; 30 – 45 min, 28.4 – 39.6 % B; 45 – 50 min, 39.6 – 86.0 % B; 50 – 55 min, 86.0 % B; 55 – 65 min, 14.0 % B; with a constant 4 % of C maintained throughout the gradient) where solvent A is dichloromethane, B is methanol and C is acetic acid/water (1:1, v/v). Flavanols were detected by fluorescence emission at 316 nm, following excitation at 276 nm. The LC-fluorescence detection method is that described by (Gu et al., 2002) with the additional relative fluorescence response data by (Prior and Gu, 2005). Example chromatograms of the standards, NIST chocolate standard and apple samples are shown in Figure 2.3. The limits of detection of the polyphenol compounds quantified are described in Table 2.2.

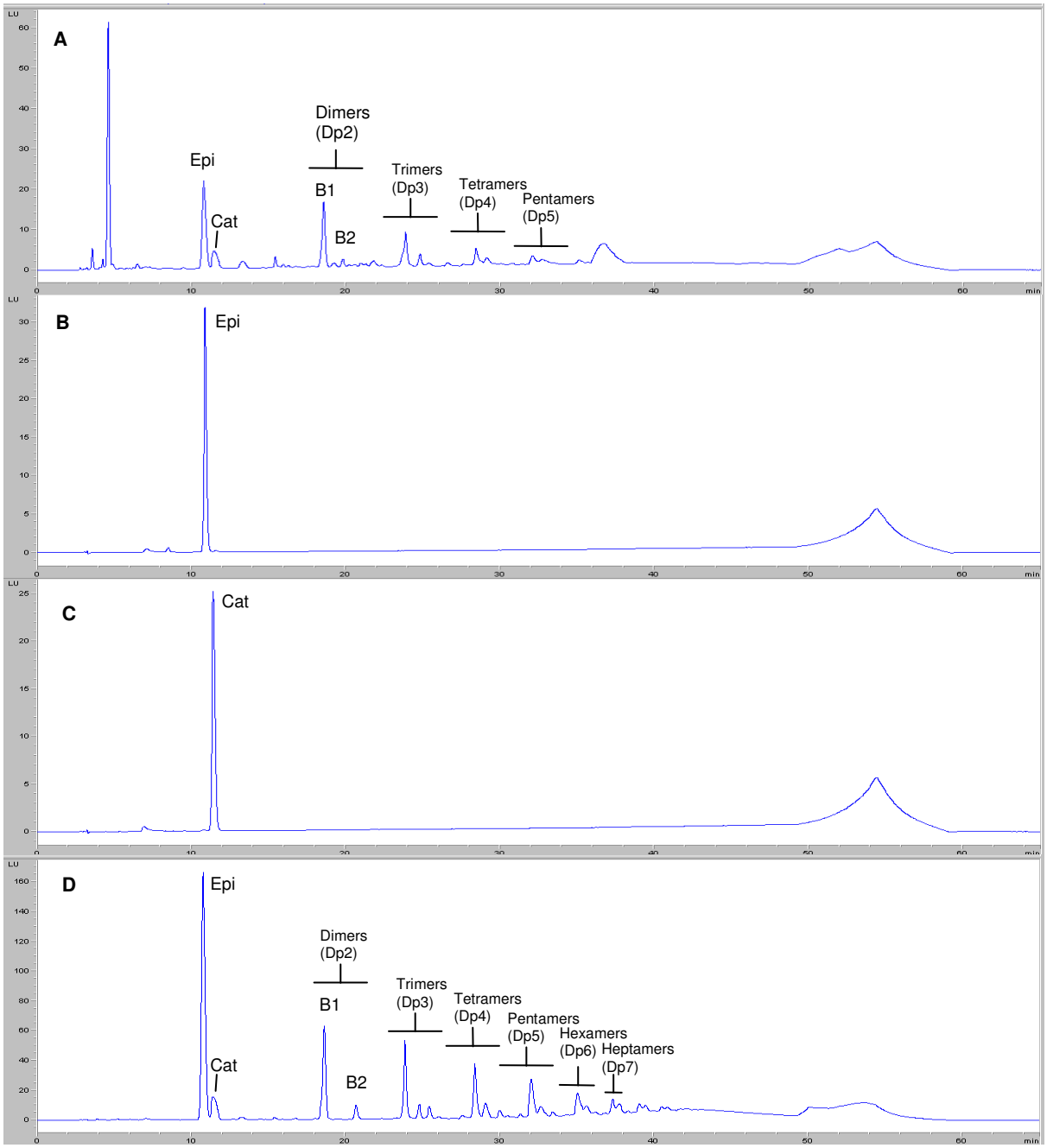


Figure 2.3: Example normal-phase HPLC chromatograms of flavanols.

NIST chocolate standard **(A)**, epicatechin standard **(B)**, catechin standard **(C)**, and apple example **(D)**. Peak ID: epi = epicatechin and cat = catechin.

Table 2.2 Limit of detection of the polyphenol compounds quantified using normal- and reverse-phase HPLC methods

Polyphenol compounds	Limit of detection (ng/ml)
Reverse phase	
Epicatechin	19.6
Chlorogenic acid	5.2
Quercetin-3-*O*-arabinose	2.3
Quercetin-3-*O*-rutinoside (rutin)	1.3
Quercetin-3-*O*-galactoside	1.9
Quercetin-3-*O*-glucoside	2.1
Quercetin-3-*O*-xyloside	1.6
Quercetin-3-*O*-rhamnoside	3.5
Phloridzin	3.8
Normal phase	
Epicatechin	23
Catechin	23
Dimers (dp 2)	36
Trimers (dp 3)	33
Tetramers (dp 4)	38
Pentamers (dp 5)	41
Hexamers (dp 6)	52
Heptamers (dp 7)	38

2.4.6 Statistical analysis

Year and tree variation were analysed by analysis of variance (ANOVA). A *p*-value of < 0.05 was considered significant. The Tukey all pair-wise comparison was preformed if the *p*-value for the overall test was < 0.05. The significance of weather (temperature, rainfall and sunshine) was analysed by ANOVA. A *p*-value of < 0.05 was considered significant. The correlation between the area under the curve and weather variables (mean temperature, total rainfall, and total hours of sunshine) were analysed using Pearson's correlation in SPSS (correlate: bivariate).

2.5 Results

2.5.1 The effect of growth and maturity and seasonal variation on total polyphenol concentration and content

Apple fruits, from one orchard, were collected during three years to investigate the effect of growth and maturity and seasonal variation on polyphenol concentration and content. For the purposes of this chapter, total polyphenols include the following quantified polyphenols: flavanol monomers (epicatechin and catechin), flavanol polymers (dp 2 – 7), quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xyloside), chlorogenic acid and phloridzin. The effects of seasonal variation and growth and maturity on total polyphenol concentration and content were determined between the three harvest years 2007, 2009 and 2010.

In the case of total polyphenol concentration, each harvest year follows the same trend. A greater polyphenol concentration was observed in the beginning of the growing season (week 1) followed by a steady decrease until the end of the growing season (week 8) (Figure 2.4). In each year a statistically significant decrease in polyphenol concentration was observed at the end of the growing season compared to the first week of the growing season (2007, $p < 0.0001$; 2009, $p = 0.0022$; 2010, $p < 0.0001$). A significant difference ($p < 0.0001$) was observed in polyphenol concentration over the different years analysed. However when comparing specific years, a significant difference was only observed between the years 2007 and 2009 ($p < 0.0001$) and the years 2009 and 2010 ($p < 0.0001$). The year 2007 was not significantly different from the year 2010 ($p = 0.752$).

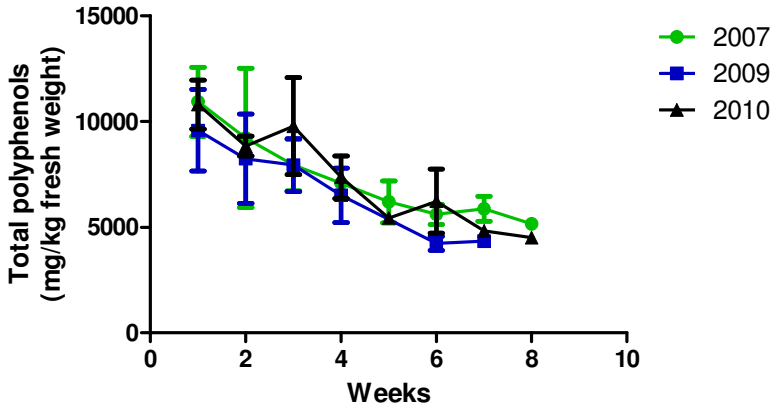


Figure 2.4: Total polyphenol concentration in the tested apple orchard in 2007, 2009 and 2010.

The total polyphenol concentration was calculated on a fresh weight basis and the polyphenol content. Total polyphenols include: flavanol monomers (epicatechin and catechin), flavanol polymers (dp 2 – 7), quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), chlorogenic acid and phloridzin. Each data point represents the mean ± the standard deviation of the three trees analysed (triplicate analysis).

The differences between the years may be attributed to the differences in weather conditions (mean temperature, total rainfall and total sunshine) between the three growing seasons. The weather relating to the months in the growing seasons (July to September) were used for statistical analysis. The differences in mean temperature (º C) and total rainfall (mm) were correlated with total polyphenol concentration, while there was no correlation in the total hours of sunshine ($p = 0.392$). Mean temperature was negatively correlated with polyphenol concentration ($p < 0.0001$, $r^2 = 0.213$, Pearson's correlation = -0.461) and total rainfall was positively correlated with polyphenol concentration ($p = 0.013$, $r^2 = 0.518$, Pearson's correlation = 0.720) (Figure 2.5). Therefore, the explanation why there was no significant difference between the years 2007 and 2010 may be due to the fact that they have a similar mean temperature and total amount of rainfall values. However the climatic data has to be interpreted with some care as the climatic data covered a wide geographical region rather than focussing on the location of the orchard.

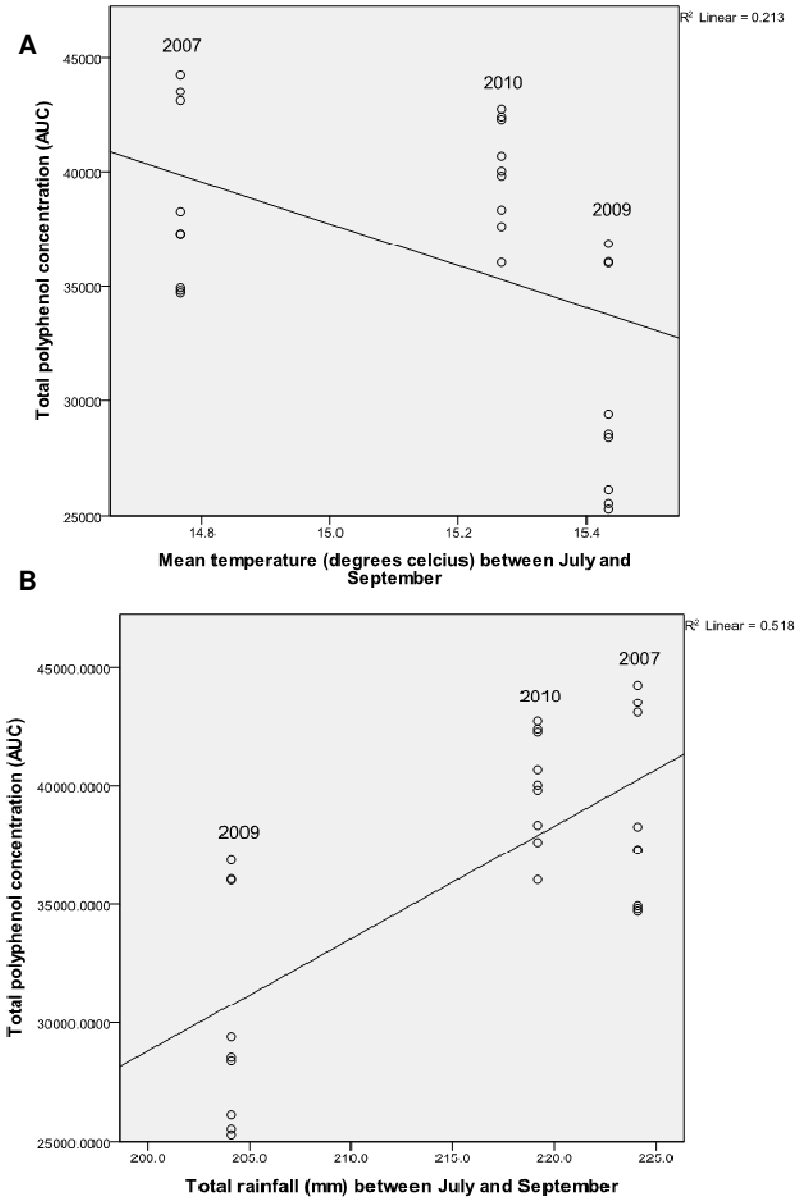


Figure 2.5: Correlation between total polyphenol concentration and mean temperature and total rainfall during the months July and September.

(A) Correlation between total polyphenol concentration (area under the curve = AUC) and mean temperature (ºC), and **(B)** correlation between total polyphenol concentration (AUC) and total rainfall (mm). The correlation between the AUC and weather variables (mean temperature and total rainfall) were analysed using Pearson's correlation in SPSS (correlate: bivariate).

For the total polyphenol content, the three years do not follow a similar trend. In 2007, the polyphenol content increases from week 4, while the years 2009 and 2010 followed a similar trend with an apparent stable amount of total polyphenol content during the

growing season (Figure 2.6). In 2007, a statistically significant increase ($p < 0.0001$) in polyphenol content was observed in week 8 compared to week 1, while in 2010 the amount of total polyphenol content was not significantly different ($p = 0.578$) between week 1 and week 8. In 2009 a borderline significant difference ($p = 0.048$) was observed between the last week analysed (week 7) in the growing season and the first week in the growing season. Therefore the polyphenol content did not change during the growing season in 2010.

There is a significant difference ($p < 0.0001$) in the polyphenol content over the three analysed growing seasons. When evaluating differences between the specific years the polyphenol content in the year 2007 was significantly different to the polyphenol content in the year 2009 ($p < 0.0001$) and 2010 ($p < 0.0001$). The polyphenol content was highest in 2007. There was no difference between the years 2009 and 2010 ($p = 0.138$).

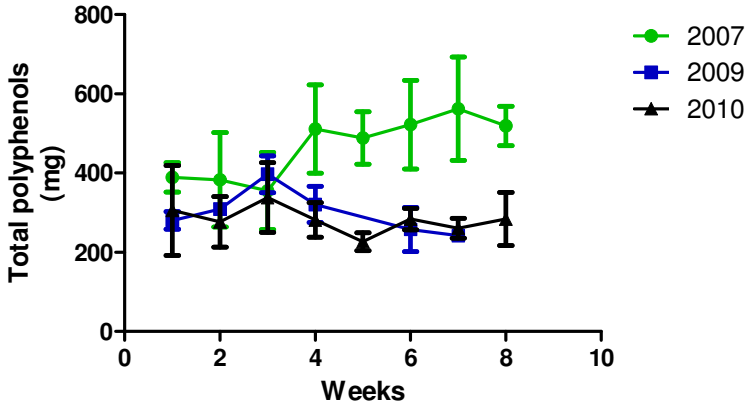


Figure 2.6: Total polyphenol content in the tested apple orchard in 2007, 2009 and 2010.

The polyphenol content was calculated from the fresh weight data and the mean apple weight from each week. Total polyphenols include: flavanol monomers (epicatechin and catechin), flavanol polymers (dp 2 - 7), quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), chlorogenic acid and phloridzin. Each data point represents the mean ± the standard deviation of the three trees analysed (triplicate analysis).

The differences in total polyphenol content in 2007 may be attributed to the fruit size and the difference in weather conditions (temperature, rainfall and sunshine). In the

case of fruit size, 2007 had larger average fruit sizes than 2009 and 2010 (Table 2.3). A significant difference ($p < 0.05$) between average apple weights in 2007 and 2009 was observed in weeks 2, 4, 6 and 7 and a significant difference ($p < 0.05$) in average apple weights was observed between 2007 and 2010 in weeks 3, 4, 5 and 7. Weather is also another variable that can account for the difference in polyphenol content in 2007. Mean temperature (ºC), total rainfall (mm) and total sunshine (hours) during the growing season period (July to September) were correlated with an increase in polyphenol content. Temperature was negatively correlated (weak association) with polyphenol content ($p < 0.0001$, $r^2 = 0.650$, Pearson's correlation = -0.806) while total rainfall ($p = 0.013$, $r^2 = 0.223$, Pearson's correlation = 0.472) and total sunshine ($p < 0.0001$, $r^2 = 0.666$, Pearson's correlation = 0.816) were positively correlated (Figure 2.7). The climatic data has to be interpreted with some caution as the climatic data covered a wide geographical region rather than focussing on the location of the orchard.

Table 2.3: Apple weights (g) of Trees A, B and C collected over the eight week growing period in 2007, 2009 and 2010.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
2007								
A[1]	39	48	57	59	79	101	106	92
B[1]	34	38	50	91	76	74	75	105
C[1]	35	40	39	67	83	104	106	106
Average	*35.8*	*42.0*	*48.6*	*72.3*	*79.1*	*92.9*	*95.6*	*100.6*
2009								
A[2]	39	55	70	66	n/a	73	n/a	n/a
B[2]	42	50	65	62	n/a	78	n/a	n/a
C[2]	26	32	44	45	n/a	56	63	n/a
Average	*35.7*	*45.5*	*59.9*	*57.8*	*n/a*	*68.9*	*63*	*n/a*
2010								
A[3]	36	37	45	52	49	50	60	75
B[3]	26	32	33	37	38	47	47	48
C[3]	22	25	27	29	40	39	56	66
Average	*27.9*	*31.2*	*35.1*	*39.2*	*42.0*	*45.4*	*54.3*	*62.9*

[1] Mean apple weight (g) of the 7 apples collected in week 1 and the 6 apples collected in week 8 in 2007.

[2] Mean apple weight (g) of the 7 apples collected in weeks 1 – 4 and week 6 and 7 in 2009.

[3] Mean apple weight (g) of the 7 apples collected in weeks 1 – 8 in 2010.

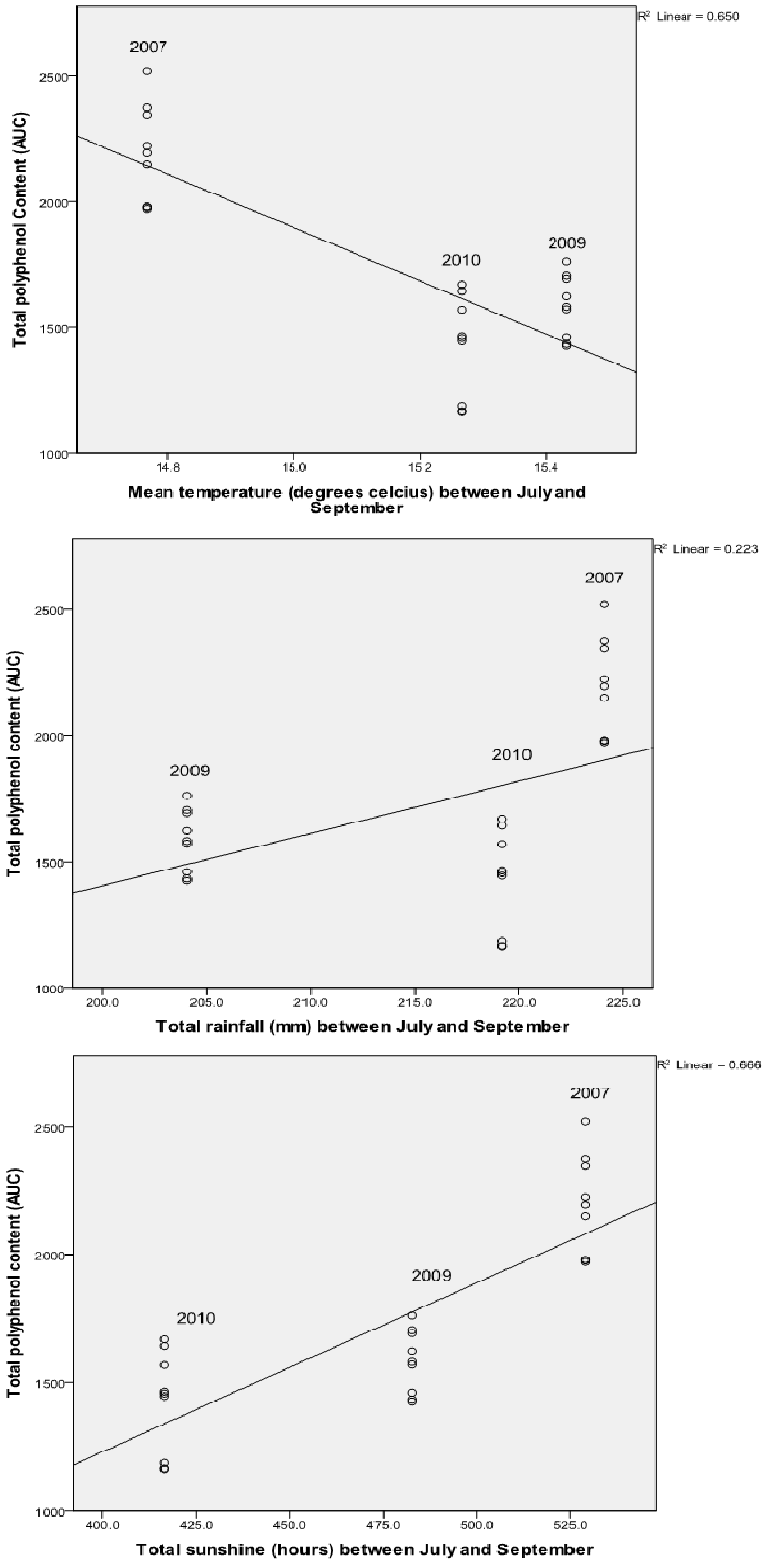


Figure 2.7: Correlation between total polyphenol content and mean temperature, total rainfall and total sunshine during the months July and September.

(A) Correlation between total polyphenol content (area under the curve = AUC) and mean temperature (ºC), **(B)** correlation between total polyphenol content (AUC) and total rainfall (mm), and **(C)** correlation between total polyphenol content (AUC) and total sunshine (hours). The correlation between the AUC and weather variables (mean temperature, total rainfall, and total hours of sunshine) were analysed using Pearson's correlation in SPSS (correlate: bivariate).

2.5.2 The effect of growth and maturity and seasonal variation on individual polyphenols

The apples in 2007, 2009 and 2010 were analysed for seventeen different polyphenolic compounds that were grouped together as total polyphenol concentration and content in Section 2.5.1. It is therefore also interesting to determine how the individual polyphenols are affected during growth and maturity and between seasons. The polyphenols were grouped into categories to which they are closely related to: total quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), total flavanol monomers (epicatechin and catechin), total polymers (dp 2 – 7), chlorogenic acid and phloridzin. In this section the individual polyphenols are expressed as a percentage of the total amount of polyphenols present in the apples.

The percentages of individual polyphenol (total quercetin glycosides, total flavanol monomers, total flavanol polymers, chlorogenic acid and phloridzin) for each of the three years follow the same pattern (Figure 2.8). In 2007, the percentage of total quercetin glycosides ($p = 0.019$), total flavanol polymers ($p = 0.017$) in the apples were significantly greater in week 8 compared to week 1, while the percentage of chlorogenic acid ($p = 0.010$) was significantly greater in week 1 compared to week 8. The percentage of total flavanol monomers ($p = 0.153$) and phloridzin ($p = 0.349$) in the apple fruit did not change significantly during the growing season.

In 2009, the percentage of total flavanol polymers ($p = 0.009$), chlorogenic acid ($p = 0.002$), and quercetin glycosides ($p = 0.040$) in the apples was significantly greater at the end of the growing season (week 7) compared to the beginning of the growing season, while the percentage of total chlorogenic acid ($p = 0.042$), total flavanol

monomers ($p = 0.003$) and phloridzin ($p = 0.007$) was significantly greater at the beginning of the growing season compared to the end of the growing season.

In 2010, the percentage of total quercetin glycosides ($p = 0.013$) and total flavanol polymers ($p < 0.001$) in the apple fruits was significantly greater in week 8 compared to week 1, while the percentage of total flavanol monomers ($p = 0.0002$) and chlorogenic acid ($p = 0.028$) was significantly greater in week 1 compared to week 8. The percentage of phloridzin ($p = 0.108$) in the apple fruit did not change significantly over the growing season.

The overall effect of seasonal variation was significant for the percentage of total quercetin glycosides ($p = 0.003$) and total flavanol monomers ($p < 0.0001$) and not significant for the percentage of total flavanol polymers ($p = 0.190$), chlorogenic acid ($p = 0.905$) and phloridzin ($p = 0.540$). When analysing the specific seasons, the percentage of total quercetin glycosides in the year 2010 was significantly different to the year 2007 ($p = 0.017$) and 2009 ($p = 0.003$). The year 2010 had a lower amount of percentage of total quercetin glycosides in the fruit compared to 2007 and 2009. There was no significant difference in the percentage of total quercetin glycosides between the years 2007 and 2009 ($p = 0.767$). For the percentage of total flavanol monomers, the year 2007 was significantly different between the years 2009 ($p < 0.0001$) and 2010 ($p < 0.0001$) with a greater percentage of total flavanol monomers in 2007. There was no significant difference between the years 2009 and 2010 ($p = 0.794$).

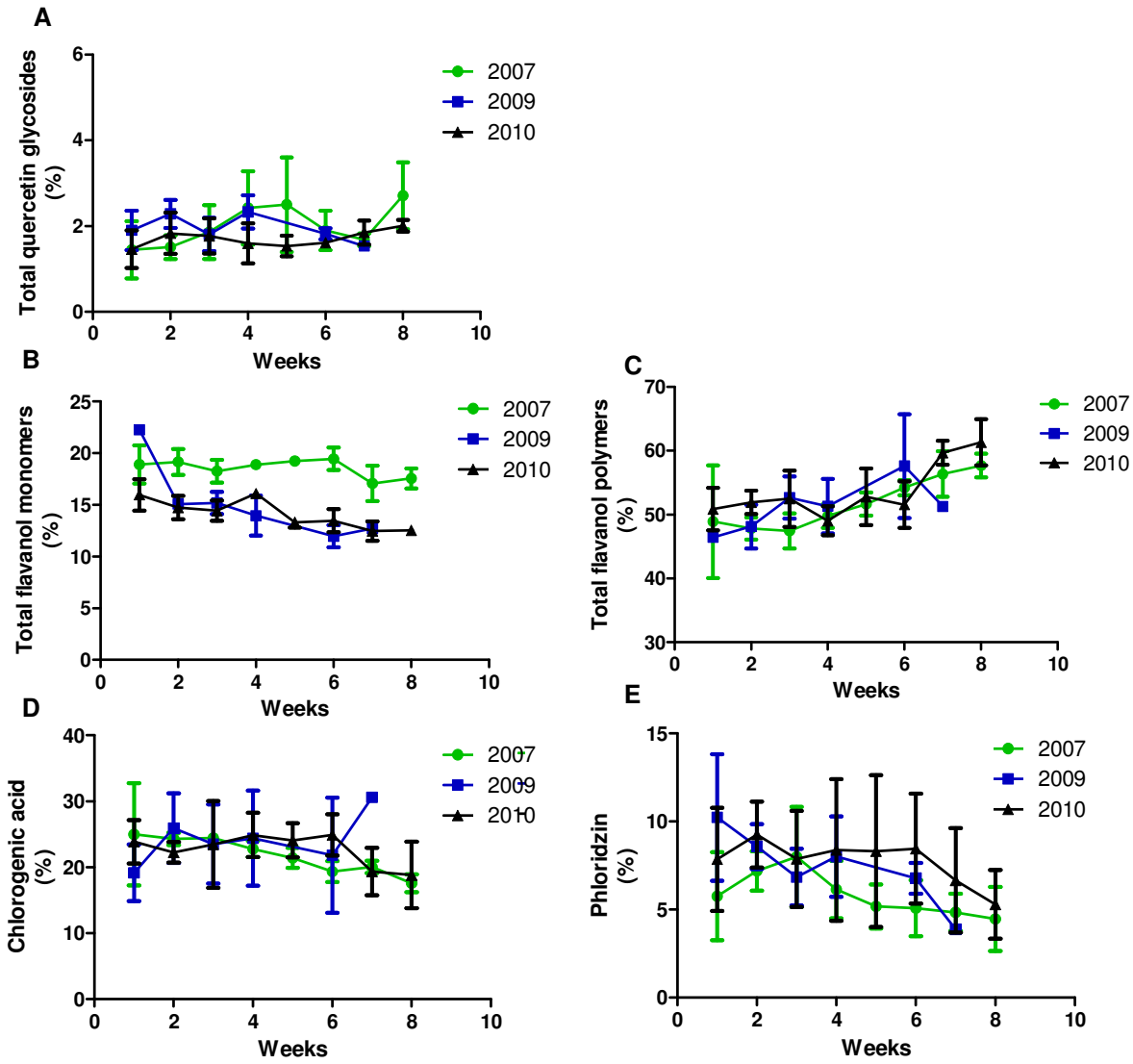


Figure 2.8: The percentage of total quercetin glycosides, total flavanol monomers, total flavanol polymers, chlorogenic acid and phloridzin in relation to the total polyphenol content in the tested apple orchard in 2007, 2009 and 2010.

The individual polyphenols are expressed as a percentage of the total amount of polyphenols present in the apples. **(A)** total quercetin glycosides percentage (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), **(B)** total flavanol monomers percentage (epicatechin and catechin), **(C)** total flavanol polymers percentage (dp 2 - 7), **(D)** chlorogenic acid percentage, and **(E)** phloridzin percentage. Each data point represents the mean ± the standard deviation of the three trees analysed (triplicate analysis).

The differences in the percentages of total quercetin glycosides and total flavanol monomers might be related to the variations in weather conditions (mean temperature, total rainfall, total sunshine) over the growing seasons (July to September). In the case of the percentage of total quercetin glycosides, sunshine was the only weather factor that was correlated with the percentage of total quercetin glycosides; the relationship

was positively correlated ($p < 0.0001$, $r^2 = 0.266$, Pearson's correlation = 0.516) (Figure 2.9). This is in keeping with previous reports indicating that the amount of quercetin glycosides present in the fruit is dependent on the amount of light that the fruit receives (Awad et al., 2001b). With the percentage of total flavanol monomers in the fruit, the effect of mean temperature, total rainfall and total sunshine were correlated with the percentage of total monomers. Mean temperature ($p < 0.0001$, $r^2 = 0.839$, Pearson's correlation = -0.916) is negatively correlated with the total monomers ratio while total rainfall ($p < 0.0001$, $r^2 = 0.396$, Pearson's correlation = 0.629) and total hours of sunshine ($p < 0.0001$, $r^2 = 0.634$, Pearson's correlation = 0.796) are positively correlated with the total flavanol monomers ratio (Figure 2.10). However the climatic data has to be interpreted with some caution as the climatic data covered a wide geographical region rather than focussing on the location of the orchard.

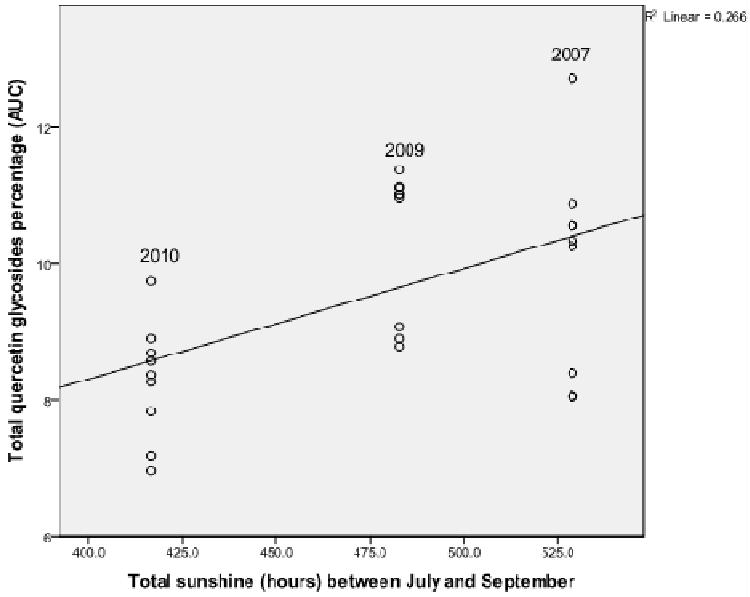


Figure 2.9: Correlation between total quercetin glycosides percentage and total hours of sunshine between the months July and September.

Correlation between total quercetin glycosides percentage (area under the curve = AUC) and total sunshine (hours). The correlation between the AUC and weather variables (total hours of sunshine) were analysed using Pearson's correlation in SPSS (correlate: bivariate).

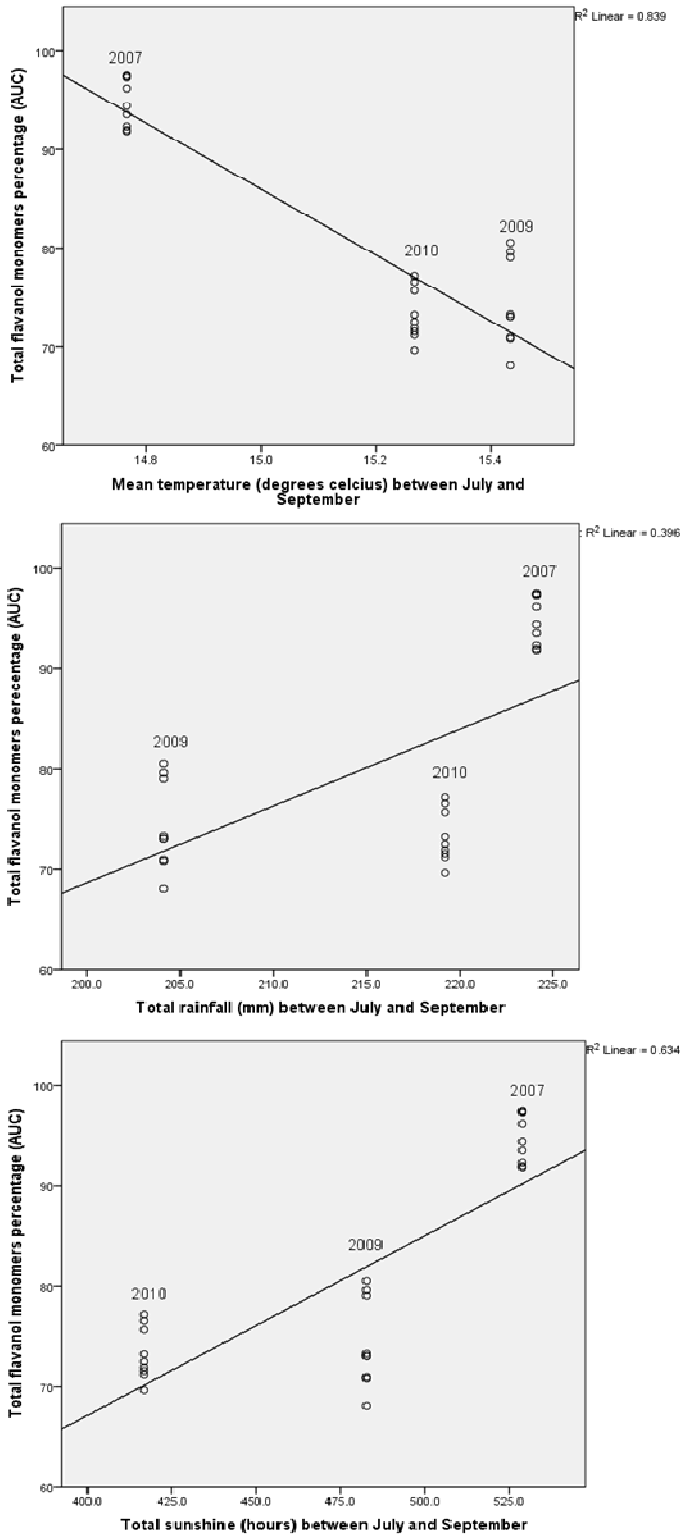


Figure 2.10: Correlation between total flavanol monomers percentage and mean temperature, total rainfall and total sunshine between the months July and September.

(A) Correlation between total flavanol monomers percentage (area under the curve = AUC) and mean temperature (ºC), **(B)** correlation between total flavanol monomers percentage (AUC) and total rainfall (mm), and **(C)** correlation between total flavanol monomers percentage (AUC) and total sunshine (hours). The correlation between the area under the curve and weather variables (mean temperature, total rainfall, and total hours of sunshine) were analysed using Pearson's correlation in SPSS (correlate: bivariate).

2.5.3 Changes in total polyphenol concentration and content and individual polyphenols within a single season between the three sampled trees

2.5.3.1 Changes in total polyphenol concentration and content in the apples within a single season and between the three sampled trees

The apples that were collected from the orchard in Herefordshire were collected from the same three trees (tree A, B and C) in the southern end of the orchard in 2007, 2009 and 2010. Therefore the effect of growth and maturity and on polyphenol concentration and content on the three analysed trees within a season can also be investigated.

The polyphenol concentration, in the three investigated trees (A, B and C), follow the same trend with a significantly greater ($p < 0.01$) concentration in the beginning of the growing season followed by a slow, general decline in the concentration to the end of the growing season (Figure 2.11A, B and C). In the years 2007, 2009 and 2010, there was a statistical difference ($p < 0.05$) between the polyphenol concentration in the three analysed trees (A, B and C).

The polyphenol content, in the three investigated trees (A, B and C) did not follow the same trend (Figure 2.11D, E and F). In 2007, the general trend in polyphenol content is an increase in total polyphenol content (Figure 2.11D). Tree A has a borderline significant ($p = 0.049$) increase in polyphenol content between week 1 and week 8, while the polyphenol content in tree B is significantly greater ($p = 0.007$) at the end of the growing season compared to the beginning of the growing season. There is no significant difference ($p = 0.096$) in the polyphenol content of tree C between the beginning of the growing season and the end. Trees A, B and C are also statistically different ($p < 0.01$) from one another in 2007.

In 2009, the general trend is a decrease in polyphenol content from the beginning of the growing season to the end of the growing season (Figure 2.11C). However there is no significant difference ($p > 0.05$) in the polyphenol content from the beginning of the growing season to the end of the growing season. Trees A, B and C are statistically different ($p < 0.01$) from one another in 2009.

In 2010, the general trend appears to be a relatively stable amount of polyphenol content during the growing season (Figure 2.11D). However, the polyphenol content was significantly greater ($p < 0.01$) at the beginning of the growing season compared to the end of the growing season for trees A and B, while there was no significant difference ($p = 0.011$) between the beginning and the end of the growing season in tree C. Trees A, B and C are statistically different ($p < 0.001$) from one another in 2010.

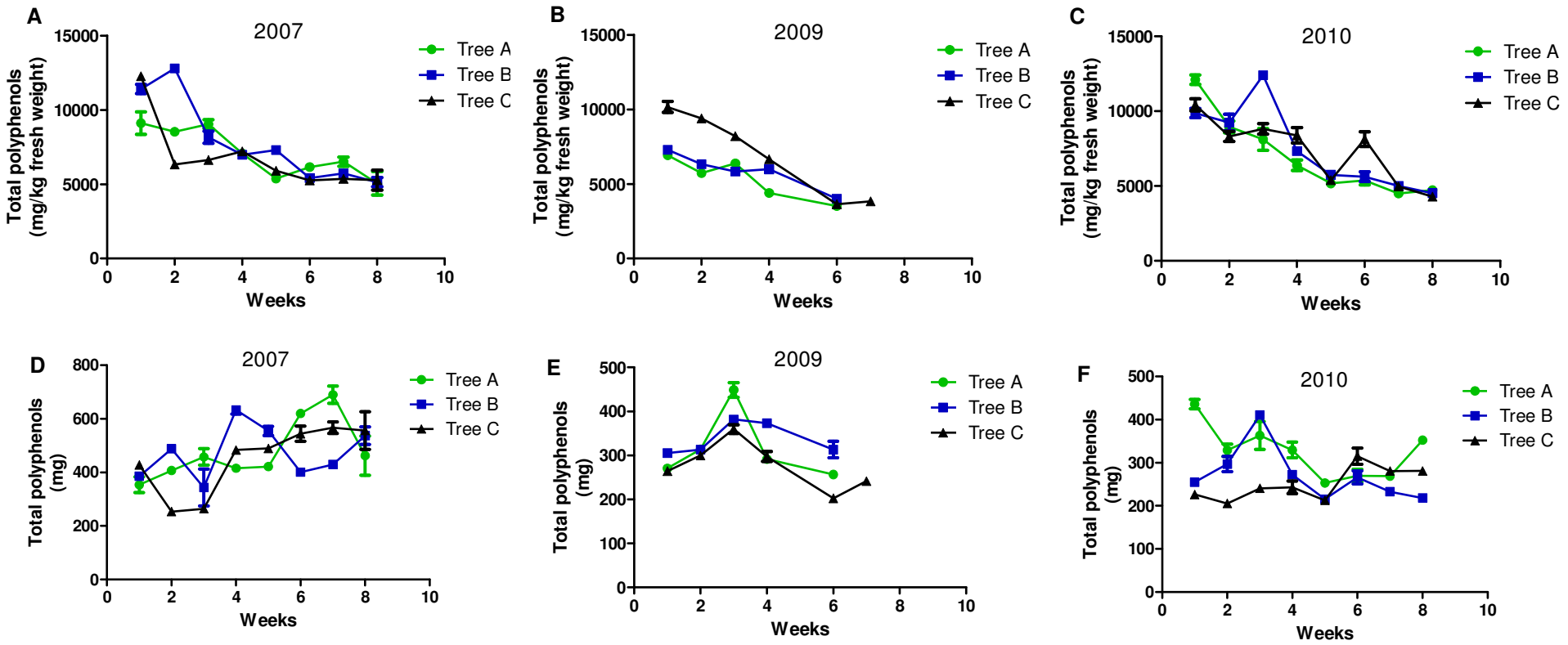


Figure 2.11: Total polyphenol concentration and content of Tree A, Tree B and Tree C in 2007, 2009 and 2010.

The total polyphenol concentration was calculated on a fresh weight basis and the polyphenol content **(A, B and C)** and the total polyphenol content was calculated from the fresh weight data and the mean apple weight from each week **(D, E and F).** Total polyphenols include: flavanol monomers (epicatechin and catechin), flavanol polymers (dp 2 – 7), quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), chlorogenic acid and phloridzin. Each data point represents the mean ± the standard deviation of the triplicate analysis of the time point.

2.5.3.2 Changes in the percentage of individual polyphenols in the apples within a single season between the three sampled trees

When looking at the percentages of quercetin glycosides over the three years in the individual trees, the percentage of quercetin glycosides appears to be either stable during the growing season or increase slightly at the end of the growing season (Figure 2.12). In 2007, the percentage of quercetin glycosides in tree B was significantly different ($p < 0.05$) from trees A and C, while there was no difference ($p = 0.608$) in the percentage of quercetin glycosides between trees A and C. In 2009, the percentage of quercetin glycosides in tree C was significantly different ($p < 0.0001$) from trees A and B, while there was no difference ($p = 0.728$) in the percentage of quercetin glycosides between trees A and B. In 2010, the percentage of quercetin glycosides in tree A was significantly different ($p = 0.008$) between tree C, while there was no difference ($p > 0.05$) in the percentage of quercetin glycosides between trees A and B and between trees C and B.

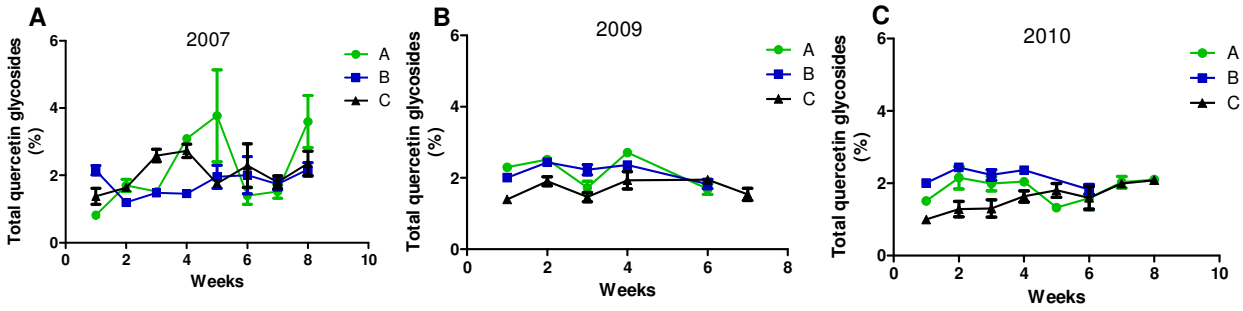


Figure 2.12: The percentage of total quercetin glycosides in relation to the total polyphenol content of the apples from trees A, B and C in 2007, 2009 and 2010.

Total quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide) are expressed as a percentage of the total amount of polyphenols present in the apples from tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

When looking at the percentages of total flavanol monomers over the three years in the individual trees, there appears to be either a stable amount of flavanol monomers during the growing season or they appear to be decreasing slightly at the end of the growing season (Figure 2.13). In 2007 and 2009, the percentages of flavanol

monomers in all trees were significantly different ($p < 0.05$), while in 2010 the percentage of flavanol monomers tree C was significantly different ($p < 0.001$) from trees A and B, while there was no difference ($p = 0.090$) in the percentage of flavanol monomers between trees A and B.

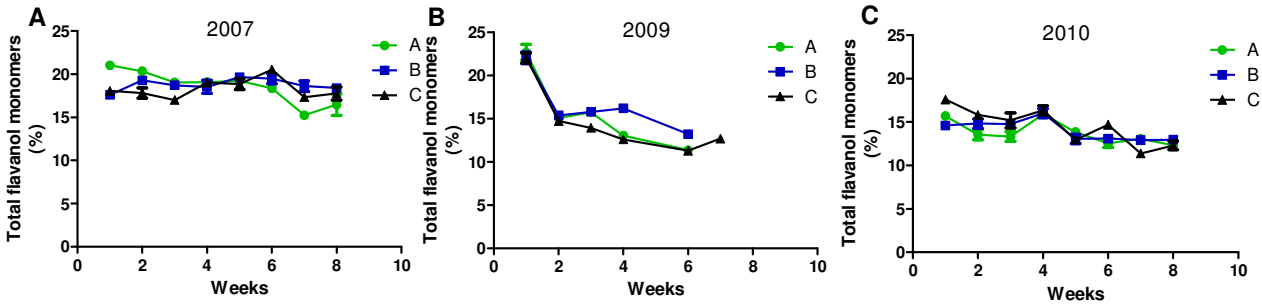


Figure 2.13: The percentage of total flavanol monomers in relation to the total polyphenol content of the apples from trees A, B and C in 2007, 2009 and 2010.

Total flavanol monomers (epicatechin and catechin) are expressed as a percentage of the total amount of polyphenols present in the apples from tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of total flavanol polymers increases from the beginning of the growing season to the end of the growing season (Figure 2.14). In 2007, there was no significant difference ($p > 0.05$) in the percentage of flavanol polymers in the three trees. In 2009, the percentage of total flavanol polymers was significantly different ($p < 0.0001$) in tree C compared to trees A and B, while there was no significant difference ($p = 0.848$) between tree A and B. In 2010, there was a significant difference ($p < 0.05$) in the percentage of total flavanol polymers in all trees.

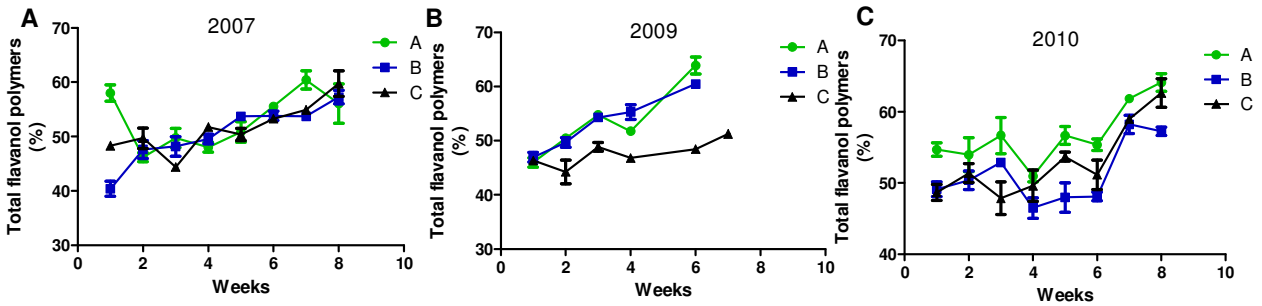


Figure 2.14: The percentage of total flavanol polymers in relation to the total polyphenol content of the apples from trees A, B and C in 2007, 2009 and 2010.

Total flavanol polymers (dp 2 -7) percentage are expressed as a percentage of the total amount of polyphenols present in the apples from tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The general trend of the percentage of chlorogenic acid in the apples over the growing season is to decline from the beginning to the end of the growing season (Figure 2.15). In 2007, the percentage of chlorogenic acid in tree A was significantly different ($p = 0.016$) between tree B, while there was no difference ($p > 0.05$) in tree C between trees A and B. In 2009, the percentage of chlorogenic acid was significantly different ($p < 0.0001$) in tree C between trees A and B, while there was no difference ($p = 0.127$) in the percentage of chlorogenic acid between tree A and B. In 2010, there was a significant difference ($p < 0.05$) in the percentage of chlorogenic acid in all trees.

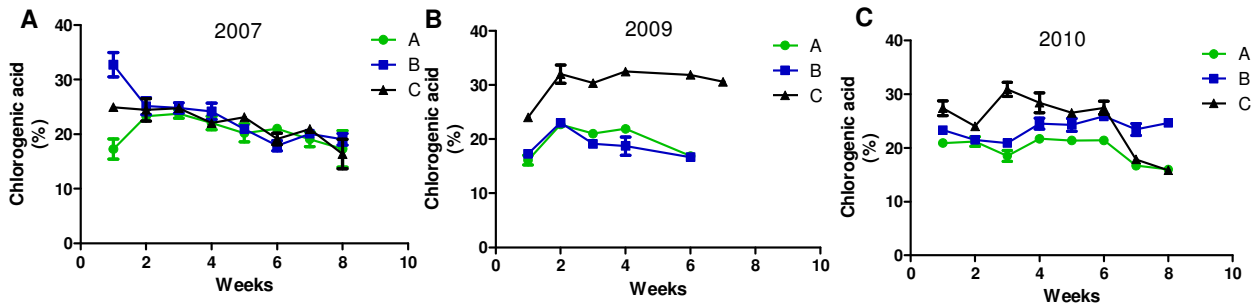


Figure 2.15: The percentage of chlorogenic acid in relation to the total polyphenol content in apples from trees A, B and C in 2007, 2009 and 2010.

Chlorogenic acid is expressed as a percentage of the total amount of polyphenols present in the apples from tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of phloridzin in the apples is generally stable throughout the growing season (Figure 2.16). In 2007, there was no significant difference ($p > 0.05$) in the percentage of phloridzin in the three trees. In 2009, the percentage of phloridzin was significantly different ($p < 0.0001$) in tree C between trees A and B, while there was no significant difference ($p = 0.480$) between tree A and B. In 2010, there was a significant difference ($p < 0.0001$) in the percentage of phloridzin in all trees.

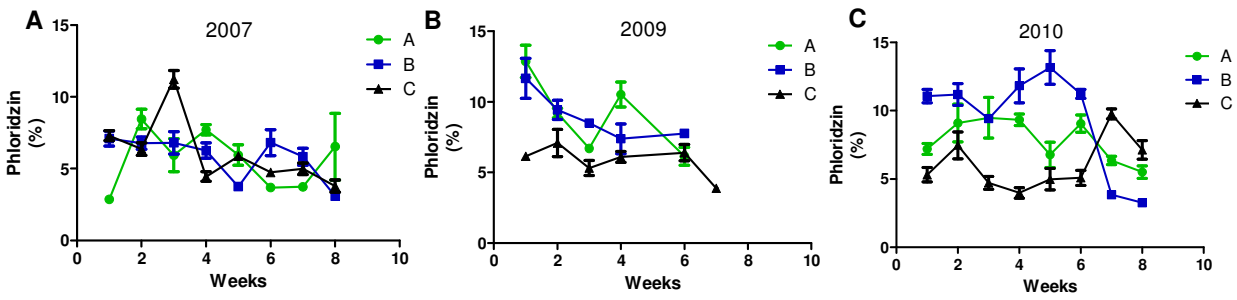


Figure 2.16: The percentage of phloridzin in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

Phloridzin is expressed as a percentage of the total amount of polyphenols present in the apples from tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

2.5.4 Changes in total polyphenol concentration and content and percentages of individual polyphenols within a single tree between the three sampled seasons

2.5.4.1 Changes in total polyphenol concentration and content within a single tree between the three sampled seasons

The apples were collected from the same three trees (tree A, B and C) in the southern end of the orchard in 2007, 2009 and 2010. Therefore the effect of growth and maturity on polyphenol concentration and content on the individual analysed trees over the three seasons can also be investigated.

The polyphenol concentration, in the all three trees (A, B and C) over the three season (2007, 2009 and 2010) follow the same trend with a significantly greater ($p < 0.01$) concentration in the beginning of the growing season followed by a slow, general decline in the concentration to the end of the growing season (Figure 2.17 A, B and C). In trees A and B, the polyphenol concentration was significantly different ($p < 0.0001$) in 2009 between the years 2007 and 2010, and there was no difference ($p > 0.05$) between the year 2007 and 2010 (Figure 2.17A and B). In tree C, the polyphenol concentration was significantly different ($p < 0.01$) in all years (Figure 2.17C).

The polyphenol content, in the three investigated years (2007, 2009 and 2010) did not follow the same trend (Figure 2.17D, E and F). In tree A, the polyphenol content in the years 2009 and 2010 were generally stable throughout the growing season, while in 2007 there was an increase in polyphenol content from week 5 up to week 7 (Figure 2.17D). The polyphenol content in the year 2009 at the end of the growing season was not significantly different ($p > 0.05$) from the beginning of the growing season, while in 2010 the beginning of the growing season had a significantly greater ($p = 0.003$) amount of total polyphenol content that the end of the growing season. In 2007, there was a borderline significant increase ($p = 0.049$) in polyphenol content from the beginning of the growing season to the end. In tree A, the polyphenol content in 2007 was significantly different ($p < 0.0001$) between the years 2009 and 2010, and there was no difference ($p = 0.561$) between the years 2009 and 2010.

In tree B, the polyphenol content in the years 2009 and 2010 are general stable throughout the growing season, while in 2007 there was an increase in polyphenol content (Figure 2.17E). In the year 2007, there was a significant increase ($p = 0.007$) in total polyphenol content from the beginning of the growing season to the end of the growing season while in the 2009 the beginning of the growing season had a significantly greater ($p = 0.003$) polyphenol content than the end of the growing season. There was no difference ($p = 0.55$) between the first week and last week in the growing season in the polyphenol content in the year 2009. In tree B, the polyphenol content in 2009 was significantly different ($p < 0.0001$) between the years 2007 and 2010, and there was no difference ($p = 0.057$) between the years 2007 and 2010.

In tree C, the polyphenol content in the years 2009 and 2010 was generally stable throughout the growing season, while in 2007 there was an increase in polyphenol content from the beginning to the end of the growing season (Figure 2.17F). In the year

2010, there was a significant increase ($p = 0.010$) in total polyphenol content from the beginning of the growing season to the end of the growing season while in the years 2007 and 2009 there was no difference ($p > 0.05$) between the first week and last week in the growing season. In tree C, the polyphenol content was significantly different ($p < 0.01$) between all the analysed years.

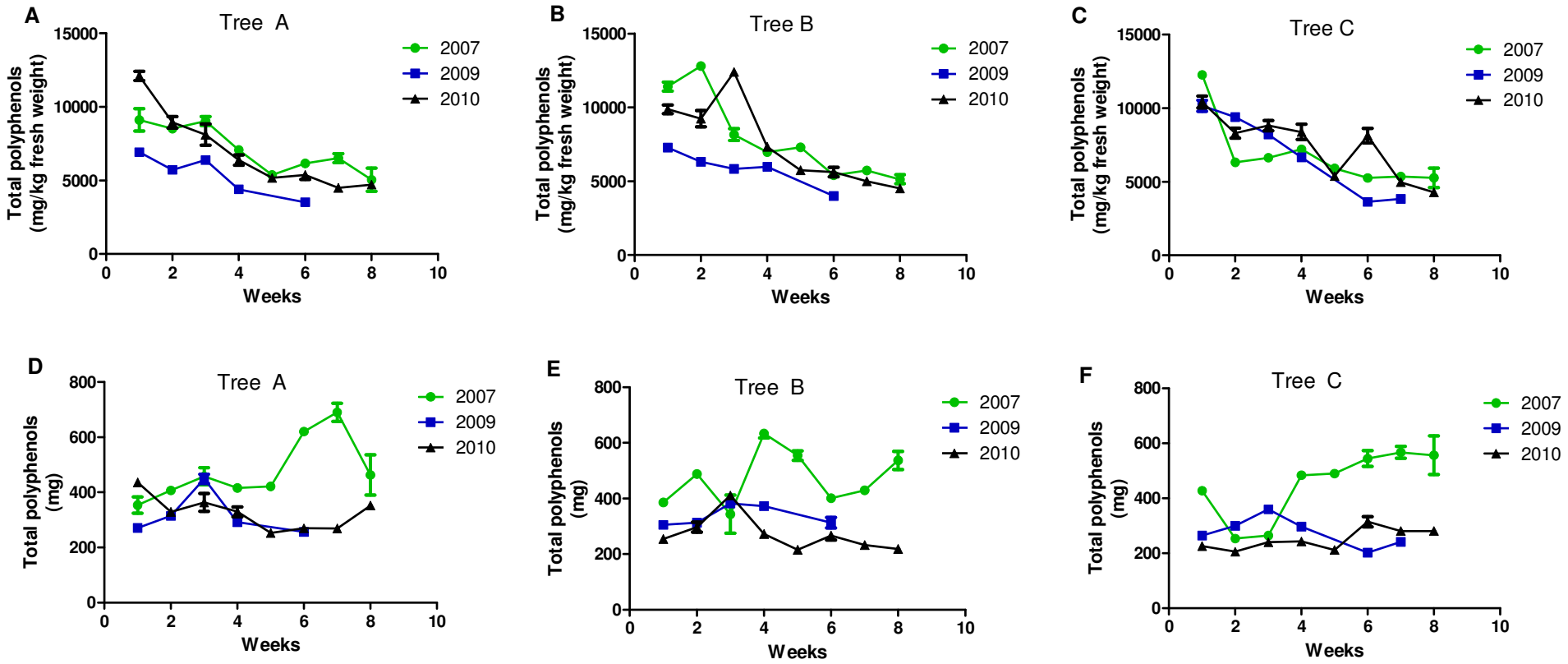


Figure 2.17: Total polyphenol concentration and content of Tree A, Tree B and Tree C in 2007, 2009 and 2010.

The total polyphenol concentration was calculated on a fresh weight basis and the polyphenol content **(A, B and C)** and the total polyphenol content was calculated from the fresh weight data and the mean apple weight from each week **(D, E and F).** Total polyphenols include: flavanol monomers (epicatechin and catechin), flavanol polymers (dp 2 – 7), quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide), chlorogenic acid and phloridzin. Each data point represents the mean ± the standard deviation of the triplicate analysis of the time point.

2.5.4.2 Changes in the percentage of individual polyphenols in the apples within a single tree between the three sampled seasons

When looking at the percentages of quercetin glycosides over the three years in the individual trees, the percentage of quercetin glycosides appears to be either stable during the growing season or increases slightly at the end of the growing season (Figure 2.18). In tree A, the percentage of quercetin glycosides between the years 2007 and 2010 were borderline significantly different (p = 0.049) and there was no difference (p > 0.05) in the year 2009 between the years 2007 and 2010. In tree B, the percentage of quercetin glycosides in 2009 was significantly different (p < 0.0001) between the years 2007 and 2010, while there was no difference (p = 0.219) in the percentage of quercetin glycosides between the years 2007 and 2010. In tree C, the percentage of quercetin glycosides was significantly different (p < 0.01) in all years.

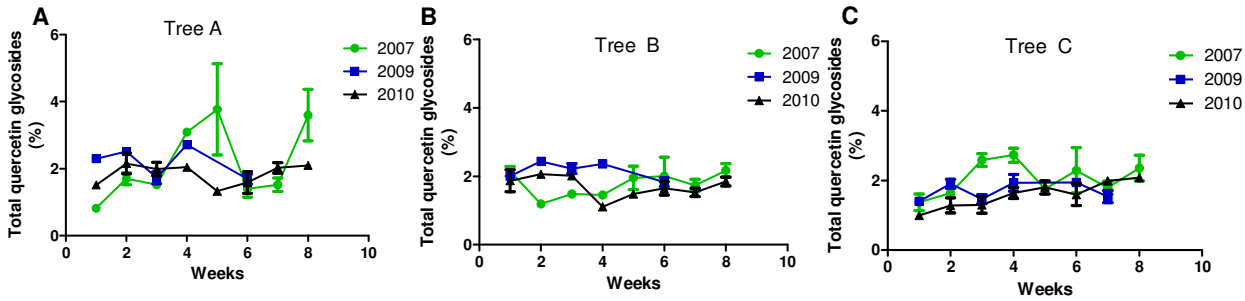


Figure 2.18: The percentage of total quercetin glycosides in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

The total quercetin glycosides (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xylsoide) are expressed as a percentage of the total amount of polyphenols present in the apples from Tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of total flavanol monomers in the apples either increases slightly from the beginning to the end of the growing season or remains relatively stable (Figure 2.19). In trees A, B and C, there is a significant difference (p < 0.01) in the percentage of total flavanols between all the years investigated.

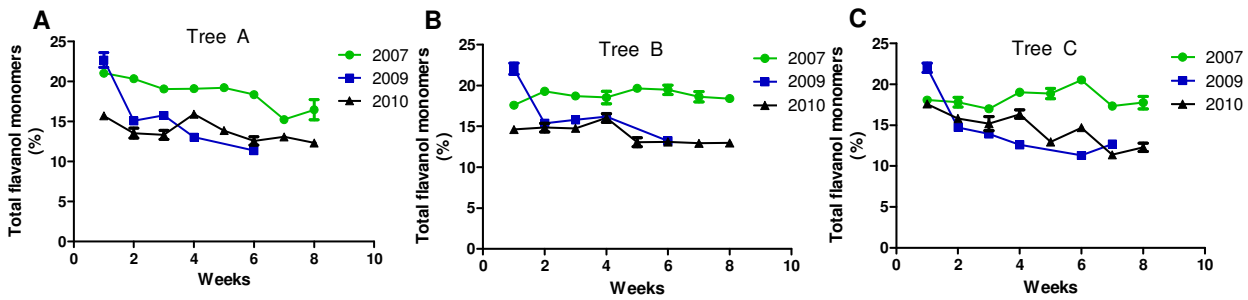


Figure 2.19: The percentage of total flavanol monomers in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

The total flavanol monomers (epicatechin and catechin) are expressed as a percentage of the total amount of polyphenols present in the apples from Tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of total flavanol polymers increases from the beginning of the growing season to the end of the growing season (Figure 2.20). In tree A, the percentage of total flavanol polymers in 2007 was significantly different ($p < 0.0001$) between the years 2009 and 2010 while there was no significant difference ($p = 0.301$) between the years 2009 and 2010. In tree B and C, the percentage of total flavanol polymers was significantly different ($p < 0.01$) in the year 2009 between the years 2007 and 2010, while there was no difference ($p > 0.05$) between the years 2007 and 2010.

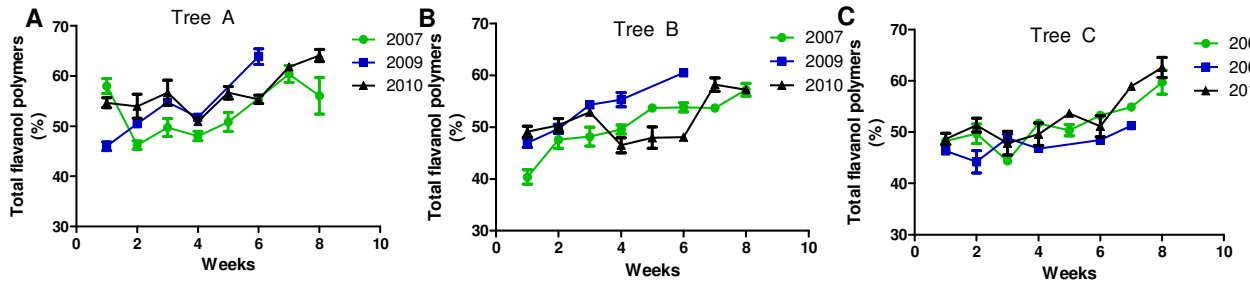


Figure 2.20: The percentage of total flavanol polymers in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

The total flavanol polymers (dp 2 – 7) are expressed as a percentage of the total amount of polyphenols present in the apples from Tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of chlorogenic acid in the apples either declines from the beginning to the end of the growing season or remains stable throughout the growing season (Figure 2.21). In tree A, the percentage of chlorogenic acid in the year 2007 was significantly different ($p = 0.039$) between the year 2009, while there was no difference

(p > 0.05) in the year 2010 between the years 2007 and 2009. In tree B, the percentage of chlorogenic acid was significantly different (p < 0.0001) in the year 2009 between the years 2007 and 2010, while there was no difference (p = 0.265) in the percentage of chlorogenic acid between the years 2007 and 2010. In tree C, there was a significant difference (p < 0.01) in the percentage of chlorogenic acid in all years.

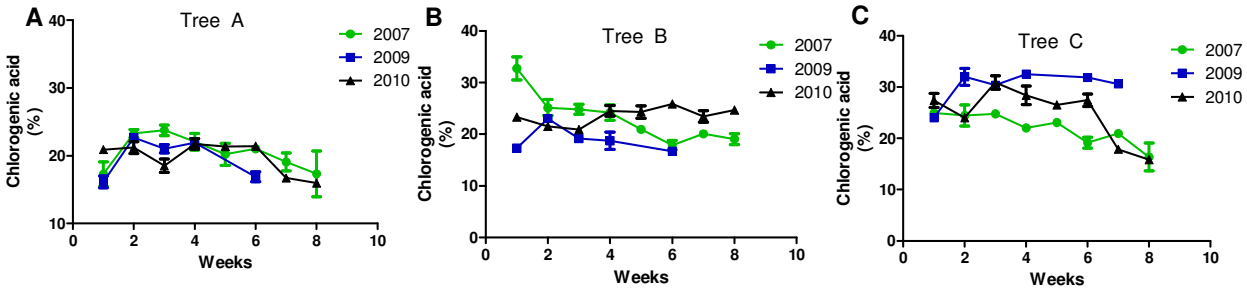


Figure 2.21: The percentage of chlorogenic acid in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

Chlorogenic acid is expressed as a percentage of the total amount of polyphenols present in the apples from Tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

The percentage of phloridzin in the apples is generally stable throughout the growing season (Figure 2.22). In tree A, there was a significant difference (p < 0.01) in the percentage of phloridzin in 2007 between the years 2009 and 2010 while there was no difference (p = 0.639) between the years 2009 and 2010. In tree B, the percentage of phloridzin was significantly different (p < 0.01) in all three years. In tree C, there was a significant difference (p < 0.01) in the percentage of phloridzin in 2010 between the years 2007 and 2009 while there was no difference (p = 0.059) between the years 2007 and 2009.

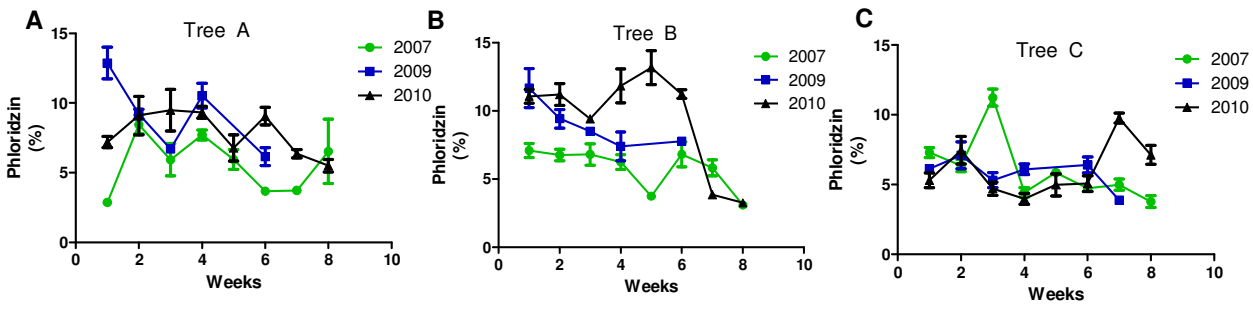


Figure 2.22: The percentage of phloridzin in relation to the total polyphenol content in the apples from trees A, B and C in 2007, 2009 and 2010.

Phloridzin is expressed as a percentage of the total amount of polyphenols present in the apples from Tree A, B and C in 2007 **(A)**, 2009 **(B)** and 2010 **(C)**.

2.5.5 Total and individual polyphenol concentrations and contents within in a tree

For each of the weeks in the growing seasons seven apples were collected from each tree, with the exception of 2007 where seven apples were collected in week 1 and six apples were collected in weeks 2 to 8. As polyphenol synthesis is dependent on the amount of sunlight received due to the location of fruit on the tree (Awad et al., 2001b), the analysis of the seven (or six) individual apples collected from week 1 and week 8 were analysed to assess the variation in polyphenol concentration from apples collected from different positions on the same tree.

In the week 1 individual analysis (data not shown), the individual apple analysis average was higher in all years for all of the following measurements: total polyphenol, total quercetin glycosides, total flavanol monomers, total flavanol polymers, chlorogenic acid and phloridzin concentrations compared to the composite apple analysis due to a systematic error. The systematic error was due to inadequate homogenization of the freeze-dried apple because of the small sample size. This error was not seen with the week 8 individual and composite apple analyses.

The week 8 individual apple sample averages and the week 8 composite apple sample averages are described in Table 2.4 for the year 2007 and Table 2.5 for the year 2010. There is no data for week 8 in 2009 due to a freeze-drier failure. In 2007, a greater than 10% variation was observed in the triplicate analyses of the composite samples in tree A and C while in tree B and all the trees in 2010, a variation of less than 10% was observed between the triplicate analyses of the composite samples. The large variation in the composite samples in tree A and C in 2007 was difficult to determine because a high variation did not occur in the tree B samples which were extracted and analysed at the same time.

In 2007 (11 – 40 %) and 2010 (14 – 47 %) a large variation was seen in the data for the individual apples. The large variation observed in the analysis of the individual apples is due to the location of the fruit on the tree. Apples were picked from the north, south, west, east, top, bottom and inner positions of the tree. The apples picked from different locations on the tree were not kept separate; therefore a link between polyphenol concentration and tree position can not be derived. However, it can be stated that the polyphenol concentration varies depending on the position of the apple fruit on the tree.

Table 2.4: Within tree variation of the total amount of polyphenols, quercetin-glycosides, flavanol monomers, flavanol polymers, chlorogenic acid and phloridzin in week 8 in the 2007 growing season.

	2007					
	A		B		C	
	Week 8 (Individual)	Week 8 (composite)	Week 8 (Individual)	Week 8 (composite)	Week 8 (Individual)	Week 8 (composite)
Total polyphenols						
Mean ± standard deviation[1]	5337 ± 603	5046 ± 312	6161 ± 909	5137 ± 312	6519 ± 993	5269 ± 666
Range (min-max)[1]	4432 – 5958	4797 – 5411	4538 – 6993	4797 – 5411	5101 – 8171	4766 – 6024
Variance	11%	15%	15%	6%	15%	13%
Total quercetin-glycosides						
Mean ± standard deviation[1]	152 ± 36	185 ± 65	135 ± 54	111 ± 4	116 ± 28	124 ± 19
Range (min-max)[1]	118 – 206	120 – 250	71 – 206	106 – 113	71 – 143	102 – 138
Variance	24%	35%	40%	4%	24%	16%
Total flavanol monomers						
Mean ± standard deviation[1]	875 ± 119	838 ± 197	1102 ± 171	946 ± 74	3543 ± 793	939 ± 160
Range (min-max)[1]	700 – 1035	678 – 1058	914 – 1498	873 – 1020	2216 - 4427	829 – 1123
Variance	14%	24%	16%	7%	22%	17%
Total flavanol polymers						
Mean ± standard deviation[1]	2882 ± 426	2849 ± 642	3428 ± 455	2944 ± 240	3483± 586	3157 ± 528
Range (min-max)[1]	2398 – 3435	2384 – 3581	3158 – 4459	2679 – 3148	3401 – 5456	2792 – 2917
Variance	15%	23%	13%	8%	15%	17 %
Chlorogenic acid						
Mean ± standard deviation[1]	1084 ± 151	857 ± 61	1212 ± 222	978 ± 10	1096 ± 218	851 ± 68
Range (min-max)[1]	797 – 1221	799 – 921	789 – 1439	967 – 988	969 – 1527	803 – 928
Variance	14%	7%	18%	1%	20%	8%
Phloridzin						
Mean ± standard deviation[1]	343 ± 93	318 ± 71	229 ± 72	158 ± 6	290 ± 75	197 ± 5
Range (min-max)[1]	258 – 466	254 – 394	106 – 315	152 – 164	145 – 352	191 – 202
Variance	27%	22%	31%	4%	26%	3%

[1]Mean and standard deviation are expressed in mg per kg fresh weight.
Week 8 (individual) is the mean, range and variance of seven apples. Week 8 (composite) is the mean, range and variance of triplicate analysis of the composite sample.

Table 2.5: Within tree variation of the total amount of polyphenols, quercetin-glycosides, flavanol monomers, flavanol polymers, chlorogenic acid and phloridzin in week 8 in the 2010 growing season.

	2010					
	A		B		C	
	Week 8 (Individual)	Week 8 (composite)	Week 8 (Individual)	Week 8 (composite)	Week 8 (Individual)	Week 8 (composite)
Total polyphenols						
Mean ± standard deviation[1]	4882 ± 1027	4722 ±54	4779 ± 1208	4513 ± 108	4703 ± 1107	4281 ± 90
Range (min-max)[1]	3063 – 5740	4660 – 4754	3479 – 6821	4402 – 4617	3668 – 6764	4192 – 4373
Variance	21%	1%	25%	2%	24%	2%
Total quercetin-glycosides						
Mean ± standard deviation[1]	96 ± 35	99 ± 5	92 ± 36	83 ± 5	91 ± 42	89 ± 6
Range (min-max)[1]	55 – 154	94 – 102	33 – 130	79 – 88	39 – 138	85 – 95
Variance	35%	5%	39%	6%	47%	6%
Total flavanol monomers						
Mean ± standard deviation[1]	587 ± 167	582 ± 18	614 ± 89	585 ± 9	574 ±109	527 ± 32
Range (min-max)[1]	338 – 737	561 – 596	423 – 851	575 – 591	460 – 769	491 – 554
Variance	28%	3%	14%	1%	19%	6%
Total flavanol polymers						
Mean ± standard deviation[1]	3166 ± 734	3027 ± 35	2738 ± 802	2584 ± 84	2901 ± 729	2680 ±30
Range (min-max)[1]	1860 – 3891	2987 – 3053	1813 – 4044	2510 – 2675	2285 – 4285	2659 – 2714
Variance	23%	1%	29%	3%	25%	1%
Chlorogenic acid						
Mean ± standard deviation[1]	789 ± 163	754 ± 31	1159 ± 272	1113 ± 24	758 ± 198	679 ± 46
Range (min-max)[1]	522 – 989	721 – 781	904 – 1674	1085 – 1132	509 – 1062	629 – 721
Variance	21%	4%	23%	2%	26%	7%
Phloridzin						
Mean ± standard deviation[1]	264 ± 38	260 ± 23	176 ± 37	146 ± 12	386 ± 118	306 ± 118
Range (min-max)[1]	211 – 317	243 – 286	127 – 220	137 – 160	225 – 530	270 – 342
Variance	14%	9%	21%	8%	31%	11%

[1]Mean and standard deviation are expressed in mg per kg fresh weight.
Week 8 (individual) is the mean, range and variance of seven apples. Week 8 (composite) is the mean, range and variance of triplicate analysis of the composite sample.

2.6 Discussion

The data presented in this chapter show that the polyphenol concentration, content and percentage of individual polyphenols in apples of two varieties grown in an orchard in Herefordshire are affected by (1) growth and maturity; (2) seasonal variation; (3) location in the orchard; and (4) may be affected by the location on the tree as a large variation is observed from apples collected from the same tree. In general, the concentration of total polyphenols (the mass amount of polyphenols per unit mass of apple tissues) decreases, while the absolute amount of polyphenols increases over the growing season. This is also the first time that a complete quantitative analysis of the polyphenols found in apples was conducted as well as the first time an analysis of the direct effect of seasonal variations on individual apple trees has been investigated.

The concentration of polyphenols (mg per kg fresh weight) was greatest at the beginning of the growing season and declined during fruit growth. Whereas the polyphenol content (the mass amount of polyphenols per unit mass of apple tissues) in the apples showed different trends based on the year of investigation. The polyphenol content in 2007 increased during the growing season from week 5 up to week 8. While in 2009 and 2010, the amount of polyphenols in the apples fluctuated slightly during the growing season but remained generally stable. The individual polyphenol compounds also did not behave uniformly. The percentage of quercetin glycosides and total flavanol polymers in the apples increased from the beginning of the growing season to the end of the growing season. The percentage of total flavanol monomers or phloridzin was either relatively stable during the growing season or had a greater percentage in the apple fruit at the beginning of the growing season compared to the end based on the year of investigation. The percentage of chlorogenic acid in the apple fruit was greatest at the beginning of the growing season and declined during fruit growth.

The results presented agree with a number of studies reporting that the concentration of polyphenols decreases during fruit growth and maturation (Lister et al., 1994; Mayr et al., 1995; Awad et al., 2001a; Kondo et al., 2002a; Guyot et al., 2003; Takos et al., 2006; Renard et al., 2007) while the content increases during fruit growth and maturation (Lister et al., 1994; Awad et al., 2001a; Kondo et al., 2002a; Takos et al., 2006; Renard et al., 2007). It can be concluded that the decrease in the concentration of polyphenols is due to a dilution effect of these compounds caused by the progressive growth of the fruit (Lister et al., 1994; Awad et al., 2001a; Takos et al., 2006; Renard et al., 2007). Therefore the reason why the polyphenol content in the apples in 2009 and 2010 did not increase during the growing season was because the size of the fruit did not increase as substantially during the growing season as it did in 2007.

The results presented for individual polyphenols also agree with the profile of individual polyphenols as described by Awad et al. (2001a). In their analysis of Jonagold and Elstar fruit, it was found that the amount of quercetin glycosides and chlorogenic acid increased during fruit growth; the amount of flavanol monomers slightly decreased before remaining relatively constant; and the amount of phloridzin increased during fruit growth before remaining relatively stable (Awad et al., 2001a). The only deviation between the results presented in this chapter and those with Awad et al. (2001a) is the profile of chlorogenic acid; the percentage of chlorogenic acid decreased during fruit growth in the results presented in this chapter whereas in those presented by Awad et al. (2001a) the amount of chlorogenic acid increased during fruit growth. In addition, data presented here show that percentage of procyanidins increased during the growing season.

The differences in the accumulation of polyphenols during fruit growth may be attributed to the polyphenol biosynthetic pathway. The biosynthesis of polyphenols is derived from two pathways, the shikimate and the acetate-malonate pathways, as described in Chapter 1, Figure 1.1. In the biosynthetic pathway, quercetin glycosides, flavanol monomers and polymers and anthocyanins are derived from phenolic acids and dihydrochalcones via several enzymatic steps. The decrease in the percentage of chlorogenic acid and phloridzin may therefore be responsible for the greater percentage of quercetin glycosides. However as the percentage of quercetin glycosides increases during fruit growth, their biosynthetic process may be regulated slightly differently to the other polyphenols as the accumulation of catechins, phloridzin and chlorogenic acid was not affected by the synthesis of quercetin glycosides (Awad et al., 2001a; Awad et al., 2001b). The increase in flavanol polymers corresponded with a decrease in flavanol monomers as the flavanol polymers are derived from the monomers in the polyphenol biosynthetic process. The decrease of chlorogenic acid and flavanol monomers in favour of quercetin glycosides and flavanol polymers was also observed by Mayr et al. (1995). Lister et al. (1994) concluded that the parallel changes in the concentration of quercetin glycosides, procyanidins and cyanidin glycosides during fruit growth correspond to the levels of flavonoid enzymes which alter in response to environmental and/or developmental changes.

Environmental factors such as differences in weather (temperature, rainfall and amount of sunshine) can have an influence on the amount of polyphenols in the apple fruit. The results presented in this chapter show that the polyphenol concentration and content of the two apple varieties from an orchard in Herefordshire were significantly different between growing seasons. When investigating the effect of weather on polyphenol concentration, the mean temperature and total amount of rainfall, during the three months of the growing season, were correlated with the polyphenol concentration. Mean temperature was negatively correlated while total amount of rainfall was

positively correlated with the concentration of polyphenols in apples. In the case of polyphenol content, mean temperature, total amount of rainfall and total hours of sunshine were all correlated with the content. Mean temperature was negatively correlated while total amount of rainfall and total hours of sunshine were positively correlated with the amount of polyphenols in the apple fruit. The quercetin glycosides and total flavanol monomers were the only two individual polyphenol groups that were significantly different between growing seasons. The total hours of sunshine was positively correlated with the percentage of quercetin glycosides in the apple fruit. Mean temperature was negatively correlated while total amount of rainfall and total hours of sunshine were positively correlated with the percentage of total flavanol monomers in the apple fruit. Therefore these results show for the first time that the concentration and content of polyphenols in apples and the percentage of quercetin glycosides and total flavanol monomers are different between seasons and this difference is correlated with the differences in weather conditions over the years. However the climatic data should be interpreted with some care because the climatic data was represented the mean values of the geographical region of the Midlands rather than focussing on the precise location of the orchard.

These results agree with those studies stating that certain cultivars are more susceptible to seasonal variations (van der Sluis et al., 2001; Lata et al., 2005; Lata and Tomala, 2007; Stracke et al., 2009). While there have been previous studies reporting the effects of the concentration and content of total and individual polyphenols, this is the first time that a complete quantitative HPLC analysis of the polyphenols found in apples was conducted. The apples were collected from all locations on the tree to ensure a representative sampling.

Weather has also been reported to influence the accumulation of polyphenols in apples. It is widely known that the amount of light received is an important factor in the

synthesis of polyphenols (Awad et al., 2001b; van der Sluis et al., 2001; Stracke et al., 2009). It has been shown that apple fruit located on the outer positions of the tree canopy had a greater concentration of cyanidin-3-galactoside and quercetin glycosides than fruit which were located on the inner positions of the tree canopy due to the amount of light received by the fruit (Awad et al., 2001b). The concentration of catechins, phloridzin and chlorogenic acid were unaffected by the amount of light (Awad et al., 2001b). The results presented in this chapter also show that the percentage of quercetin glycosides in the apple fruit was correlated with the amount of sunshine received. However in contradiction with Awad et al. (2001b), the data in this chapter show that the content of polyphenols and the percentage of total flavanol monomers are also positively correlated with the amount of sunlight received. The data in this chapter also show that the there is a large variation of polyphenols sampled from different positions on the tree (north, east, west, south, top, bottom, outer and inner positions of the tree) which may also be due to the amount of sunlight received.

The effect of temperature and rainfall on the content and concentration of polyphenols in apples has not been widely reported. It has been postulated that the differences in polyphenol concentration between two harvest seasons were due to the differences in rainfall between the two years, with a greater concentration of polyphenols in the year with the least amount of rainfall (Stracke et al., 2009). Low evening temperatures coupled with high sunlight levels during the autumnal season have been reported to increase the anthocyanin content formation in apples (Saure, 1990), however no investigation of other polyphenols has been conducted. The results presented in this chapter show that mean temperature, during the growing season months, was negatively correlated with polyphenol content and concentration and that total amount of rainfall was positively correlated with the concentration and content of polyphenols in apples.

During the sampling process, apples were collected from the same three trees over the years of investigation. This sampling procedure allowed for the direct comparison of how variable the concentration and content of polyphenols are within an orchard and how variable the concentration and content of polyphenols are in a tree over three years. The three trees that were selected comprised two of the main cultivar in the orchard and one of the pollinator variety; both were high polyphenol varieties. Despite the fact that two of the trees were the same variety, there was a significant difference in the concentration and content of polyphenols and percentages of individual polyphenols in all three years. A significant difference was also observed in the polyphenol content and concentration and percentages of individual polyphenols in the apple fruit in one tree over the three years investigated. Therefore, this suggests that further variability arises in the accumulation of polyphenols in different trees located in the same section of the same orchard as well as variability, in one tree, introduced by seasonal differences. This is the first time that the direct effect of seasonal variations on individual apple trees has been investigated.

2.7 Conclusions

The data presented in this chapter have shown that the polyphenol concentration, content and percentage of individual polyphenols in the apple fruit of two varieties grown in an orchard in Herefordshire are affected by (1) growth and maturity; (2) seasonal variations; (3) location in the orchard; and (4) may be affected by the location on the tree as a large variation is observed from apples collected from the same tree. The polyphenol concentration of apples decrease during growth and maturity, while the polyphenol content of apples generally increases during growth and maturity due to an increase in fruit size. Weather, during the growing season, also influences the concentration and content of polyphenols in apples. Mean temperatures are negatively correlated with polyphenol content and concentration, while total amount of rainfall and

total hours of sunshine are positively correlated with content and concentration. The location of the apple on the tree and ultimately the amount of sunlight the fruit received, causes a large variation in the concentration of polyphenols in the apple fruit, and significant differences in the polyphenol content and concentration were also observed between the trees located next to one another. Therefore the accumulation of polyphenols in apples is not a simple process and is dependent on a number of factors.

CHAPTER THREE

NOVEL ROUTES FOR PRODUCING POLYPHENOL-RICH APPLE EXTRACTS AND THE EFFECTS OF AN APPLE POLYPHENOL EXTRACT ON VASCULAR FUNCTION IN HUMANS

Chapter 3 : Novel routes for producing polyphenol-rich apple extracts and the effects of an apple polyphenol extract on vascular function in humans

3.1 Abstract

Apples are eaten fresh as well as being processed into different apple products. However, the processing of apples into various products has been shown to substantially affect the polyphenol content of apple products such as apple juice. In apple juice production the losses occur in the pressing of the apples and the majority of the polyphenols are retained in the apple pomace. Therefore an effective method of extracting polyphenols from the apple pomace, a by-product of apple juice production, for the supplementation into food products is desirable as epidemiological and *in-vivo* studies have shown protective effects of dietary polyphenols. Therefore the objectives of this chapter were to investigate how to reduce the losses observed with apple juice production, determine if a hot water extraction of the apple pomace efficiently extracts polyphenols as well as investigating the effects of an apple polyphenol-rich extract supplemented apple juice improves vascular function in an intervention trial. The results from this chapter have shown that (1) a microwave-heat treatment of whole apple slices was not effective in increasing the yield of polyphenols in the first press juice or the recovery in the first press pomace; (2) a hot water extraction of the apple pomace was a very effective method of extracting the polyphenols retained from the first press and yielded a polyphenol-rich aqueous extract; (3) the hot water extraction of the apple pomace conducted at room temperature compared to 4 ºC was more effective in extracting the polyphenols retained in the apple pomace and similar losses of polyphenols were observed at both temperatures; and (4) consumption of an apple polyphenol-rich extract supplemented apple juice resulted in a small but significant

improvement in arterial stiffness and vascular age but not in reflective index (endothelial function) compared to the low polyphenol apple juice. Therefore future studies should investigate: (1) how to decrease the losses observed in the production of apple juice; (2) the possibility of using the polyphenol-rich extract as a food supplement; and (3) the consumption of apple polyphenol-rich foods and apple polyphenol supplement foods in different food matrices (i.e. solid food versus a drink) which will provide further evidence regarding apple polyphenols and their vasodilatory effects on endothelial function.

3.2 Introduction

High intakes of fruits and vegetables have been linked with a reduced risk of chronic diseases such as cardiovascular disease and cancer and it has been suggested that this protective effect is due, at least partly, to polyphenols especially flavonoids. Epidemiological evidence of the effects of flavonoid intake on cardiovascular risk is mixed with some supporting studies (Hertog et al., 1993a; Knekt et al., 1996; Hollman and Katan, 1999; Arts et al., 2001b; Mink et al., 2007) and some studies not demonstrating the positive effects (Rimm et al., 1996; Yochum et al., 1999; Sesso et al., 2003). However, there have been a number of human intervention trials that have shown that consumption of flavonoids reduces risk of CVD (Hooper et al., 2008; Desch et al., 2010; Jia et al., 2010). Based on a meta-analysis of randomised controlled intervention studies, it was shown that some flavonoids or foods rich in flavonoids such as chocolate or cocoa and black tea reduced important risk factors of cardiovascular disease such as a reduction in flow-mediated dilation (FMD), reduction in systolic and diastolic blood pressure, and a reduction in the amount of low-density lipoprotein (LDL) cholesterol (Hooper et al., 2008). A meta-analysis of ten controlled intervention trials, has also shown that the consumption of flavanol-rich cocoa products caused a reduction in blood pressure (Desch et al., 2010) while a meta-analysis of eight controlled intervention trials showed an LDL cholesterol lowering effect in subjects with cardiovascular disease risk factors who consumed a low dose of cocoa (Jia et al., 2010).

Based on the published evidence, polyphenols (especially flavonoids) have been shown to reduce cardiovascular risk biomarkers. A reduction of FMD and blood pressure by flavonoids has been suggested to be attributed to the vasodilating effects of flavonoids by causing an increase in nitric oxide (NO) (Hooper et al., 2008; Desch et al., 2010). Flow-mediated dilation assesses the arterial diameter, by high-resolution

ultrasound, during a period of induced ischemia and is a direct method of assessing endothelial function (Hashimoto et al., 2003; Kobayashi et al., 2004; Wilson et al., 2004). Endothelial function is a key determinant of vascular health, as dysfunction of the endothelium is an indication of the initial development of atherosclerosis (Hashimoto et al., 1999; McEniery et al., 2006). Flavonoids have been shown to improve endothelial function through an increase in nitric oxide synthase (NOS) expression and NO-dependent vasorelaxation (Heiss et al., 2003; Grassi et al., 2005b). NO has also been shown to cause improvements in insulin sensitivity (Grassi et al., 2005b), improvements in platelet function (Holt et al., 2002b), inhibition of LDL oxidiation (Baba et al., 2007) and an increase in HDL cholesterol concentration (Baba et al., 2007). Flavanols have also been reported to increase NOS expression and activity *in-vitro* (Persson et al., 2006).

Therefore the investigation of the effects of an apple-derived epicatechin-rich extract on vascular function was conducted in a randomised controlled human intervention pilot study. Apples were chosen as the fruit of interest as apples are one of the most popular fruits in the UK (Weichselbaum et al., 2010) and they are one of the five foods in the UK that supplies the majority of the flavonoids intake in the diet (Beking and Vieira, 2011). Apple consumption has also been associated with a reduced risk of cardiovascular disease risk (Sesso et al., 2003), coronary mortality (Knekt et al., 1996) and coronary heart disease and cardiovascular disease mortality (Mink et al., 2007).

The polyphenols found in apples include flavanols, hydroxycinnamic acids, dihydrochalcones, flavonols and anthocyanins as described in Chapter 1, Section 1.4. The polyphenol profile and content of apples are affected by several factors including cultivar, distribution within the fruit, growth and maturation, seasonal variations, geographical region, and food preparation, processing and storage as described in Chapter 2 (Introduction). As apples are consumed fresh as well as processed apple

products (e.g. apple juice, apple sauce and apple cider), it is important to understand how the processing of apples affects the final polyphenol content of the product. The processing of apples into apple juice has been shown to substantially affect the polyphenol content. Apple juice contains significantly lower concentrations of polyphenols compared to the fruit and a large proportion of the polyphenols remain in the post-press apple pomace (Pérez-Ilzarbe et al., 1991; Guyot et al., 2003; Boyer and Liu, 2004). During pressing, the main polyphenols to be transferred into the juice are the water-soluble polyphenols such as dihydrochalcones or hydroxycinnamic acids whereas the extraction of other polyphenols is more difficult (Bellion et al., 2010). For example, in the pressing of five French cider apple varieties, it was shown that 42 % of the polyphenols were extracted from the apples to the juice, indicating that the rest remained in the pomace (Guyot et al., 2003). However, analysis of the pomace was not conducted. In a separate study, the juice produced from three varieties of cider apples was shown to contain a small proportion of the flavanols (3.2 – 5.0 µg/g) while the majority of the flavanols (87 –113 µg/g) remained in the pomace (Price et al., 1999). In apple juice production, enzymatic treatment of the apple pulp is widely used to improve the ease of pressing, increase the juice yield and to clarify the juice (Van Buren et al., 1976; Schols et al., 1991; van der Sluis et al., 2002). However, juices obtained by enzymatic treatments have been shown to have a lower content of polyphenols and have characteristic differences in the colour and aroma of the juice compared to the conventionally pressed apple juice (Van Buren et al., 1976; Spanos et al., 1990; Schols et al., 1991; van der Sluis et al., 2002).

Different methods have been applied in apple juice production to successfully increase the yield of polyphenols in the juice from the apple pomace. The different methods include: alcohol extraction (van der Sluis et al., 2004), diffusion extraction (Spanos et al., 1990), liquefaction (Schols et al., 1991; Will et al., 2000; Mihalev et al., 2004) and microwaving (Gerard and Roberts, 2004). However these methods have certain

disadvantages which include loss of juice characteristics (e.g. aroma and taste), a greater susceptibility to browning, and having to comply with food laws (van der Sluis et al., 2004). Diagrams of the different apple juice production methods are shown in Figure 3.1. It has been suggested that a hot water extraction of the apple pomace may prove to be a favourable method of extraction than the other methods currently applied (van der Sluis et al., 2004). Due to the high levels of polyphenols present in the apple pomace after pressing, researchers have concluded that the extraction of polyphenols from the apple pomace could be commercially exploited (Lu and Foo, 1997; Price et al., 1999; van der Sluis et al., 2001; Schieber et al., 2003). Therefore the investigation of hot water extraction was undertaken on the apple pomace to determine whether an efficient extraction of epicatechin and other polyphenols could be achieved.

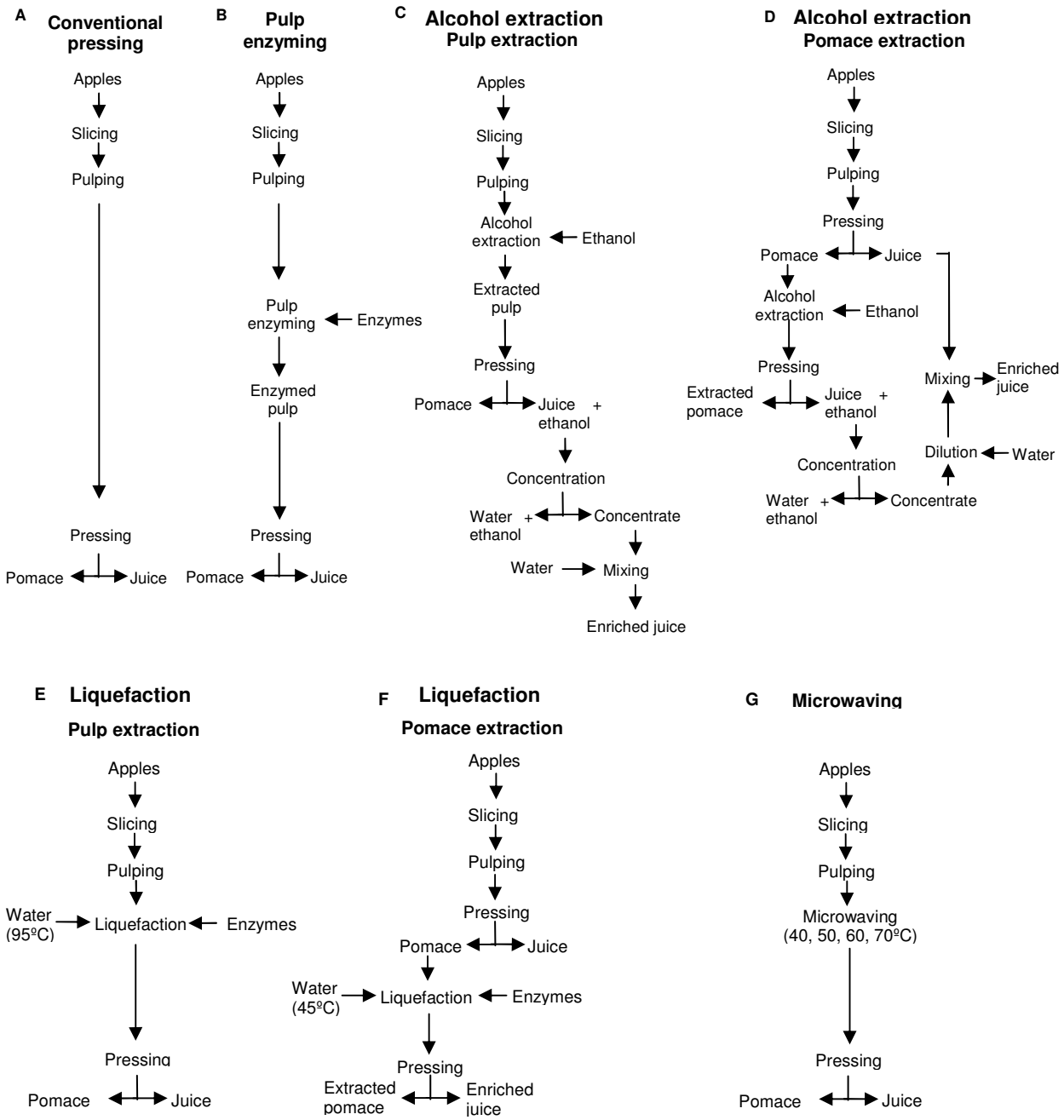


Figure 3.1: Apple juice extraction methods.

(**A**) conventional pressing, (**B**) pulp enzyming, (**C**) alcohol pulp extraction, (**D**) alcohol pomace extraction, (**E**) liquefaction of the pulp, (**F**) liquefaction of the pomace, and (**G**) microwaving

3.3 Objectives

The specific aims of this chapter were to: (1) determine if heat treatment before pressing can reduce the loss of polyphenols observed during pressing; (2) determine if hot water extraction of the apple pomace is an efficient method of extracting polyphenols; (3) determine if steeping the pomace being extracted at a lower temperature can reduce the loss observed during extraction; and (4) assess the effects of an apple polyphenol-rich extract supplemented apple juice versus a low polyphenol apple juice on vascular function by non-invasively assessing arterial function through the use of photoplethysmography.

3.4 Materials and methods

3.4.1 Materials

Methanol, acetonitrile and dichloromethane were obtained from Fisher-Scientific and trifuloroacetic acid (TFA), hydrochloric acid (HCl) (-)-epicatechin, (+)-catechin and chlorogenic acid were purchased from Sigma-Aldrich (Poole, UK). All solvents were HPLC grade. The phenolic standards, quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, phloridzin and galangin were purchased from Extrasynthase (Genay, France). The Harry Masters and Yarlington Mill cider apples were provided by Richard Wood of Coressence Ltd from the 2007 harvest year. TESCO-value apples were purchased from TESCO and Idared, Cox and Jonagold apples were from the Apple & Pear People (Hoveton, Norfolk).

3.4.2 Apple juice production

The conventional method of apple juice production involves the slicing and pulping of apples followed by the pressing of the apple pulp into juice (Figure 3.1A). The experiments conducted in this chapter modify this process to try and increase the amount of polyphenols in the juice.

3.4.2.1 Apple juice production modification one

A 4.5 L wooden press was used for the production of apple juice (Winepress4u; Cheshire, UK). The apples were microwaved for 1 min to increase the core temperature to 100 ºC. The apples were cut into segments and pulped using a food processor (Magimix Cuisine System Automatic 5100) in order to increase juice yield. The apple pulp was then pressed and the juice collected. Samples of the apple pomace and juice were collected for analysis. The method is described in Figure 3.2A.

3.4.2.2 Apple juice production modification two

The apples were cut into segments and pulped using a food processor in order to increase juice yield. The apple pulp was then pressed and the juice collected. The apple pomace (the residue of the pulped and pressed apples) was then steeped in boiling water for 24 h at room temperature. The amount of boiling water added to the pomace was approximately two litres, enough water to cover all of the pomace. After steeping, the second press was performed. Samples of the apple pomace and juice were collected for analysis at the first and second pressing. The method is described in Figure 3.2B.

3.4.2.3 Apple juice production modification three

The apples were then cut into segments and pulped using a food processor in order to increase juice yield. The apple pulp was then pressed and the juice collected. The

apple pomace was steeped in boiling water for 24 h at 4 ºC. The amount of boiling water added to the pomace was approximately two litres, enough water to cover all of the pomace. After steeping, the second press was performed. Samples of the juice and pomace were collected for analysis at the first and second pressing. The method is described in Figure 3.2C.

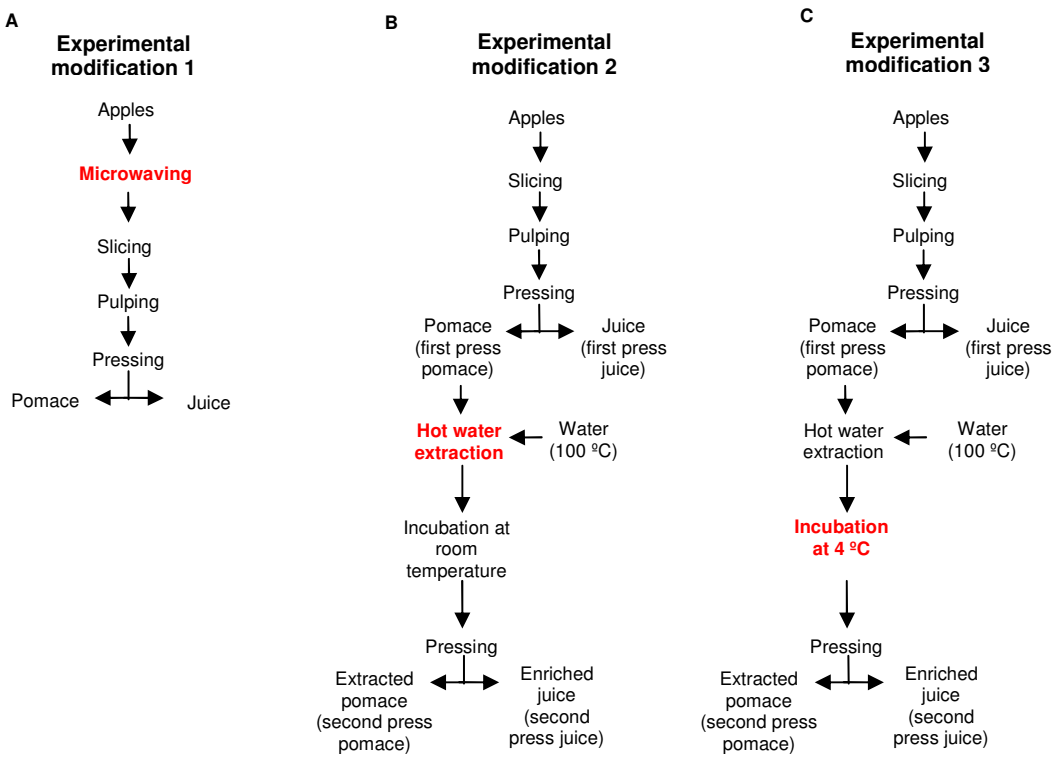


Figure 3.2: Apple juice production experimental procedures.
(**A**) experimental modification 1 – microwave step prior to slicing, (**B**) experimental modification 2 – hot water extraction of the apple pomace followed by a 24 incubation at room temperature and re-pressing of the apple pomace; and (**C**) experimental modification 3 – hot water extraction of the apple pomace followed by a 24 h incubation at 4 ºC prior to the re-pressing of the apple pomace.

3.4.2.4 Sample preparation for analysis

3.4.2.4.1 Juice

The juice was treated with HCl (final concentration 0.1 %) to prevent further enzymatic oxidation.

3.4.2.4.2 Apples and pomace

A sample of five apples, from each analysis, were weighed individually and cut into eight segments. The apples were bagged into a group bag for composite analysis and were re-weighed. The pomace samples were collected from the first press and the second press production steps and were weighed and bagged. This process was performed quickly so as to not cause an increase in enzymatic browning. The apples and pomace samples were then frozen (-20 ºC). Subsequently, the frozen apples and pomace samples were freeze-dried for seven days. The freeze-dried apples and pomace samples were homogenised into a powder using a domestic food processor and the powder was transferred to zip-lock plastic bags and stored at room temperature.

3.4.2.5 Sample extraction for analysis

3.4.2.5.1 Juice

Juice samples were extracted using two methods. Two methods were applied as further work, by a member in the group, in the extraction of polyphenols from juice was found to be more precise if a greater volume was used in the extraction.

Method 1: samples (300 µl, in triplicates) of the juice produced from Harry Masters and Yarlington Mill cider apples, TESCO value-range apples and gala apples were added to 700 µl of 70 % v/v methanol and 50 µl of the internal standard (0.1 mg/ml galangin in

100 % methanol). The samples were incubated at 70 ºC for 20 min and then centrifuged for 10 min at 13 000 x g. The supernatants were filtered (0.22 µm) and the filtrate was transferred to an HPLC vial. Samples were stored at -20 ºC for up to 24 h prior to HPLC analysis.

Method 2: samples of Idared, Cox and Jonagold apples (3 ml, in triplicate) were added to 7 ml of 100 % methanol and 50 µl of the internal standard (0.1 mg/ml galangin in 100 % methanol). The mixture was placed in an ultrasonic water bath to disperse the sample, incubated in an air oven at 70 º C for 20 min and then sonicated in the water bath for a final time. The sample was then made up to volume (10 ml) and a 1.5 ml sample was taken and centrifuged at 13 000 x g for 20 min. The supernatant was filtered (0.22 µm) and the filtrate was transferred to an HPLC vial. Samples were stored at -20 ºC for up to 24 h prior to HPLC analysis.

3.4.2.5.2 Apples and pomace

Apple and pomace samples were extracted using two methods. Two methods were applied as further work, by a member in the group, in the extraction of polyphenols from freeze-dried material was found to more precise if a greater mass was used in the extraction.

Method 1: samples (40 mg, in triplicates) of freeze-dried whole apple powder or pomace powder from Harry Masters and Yarlington Mill cider apples, TESCO value-range apples and gala apples were extracted using 950 µl of 70 % v/v methanol and 50 µl of the internal standard (0.1 mg/ml galangin in 100 % methanol) at 70 ºC for 20 min. The samples were centrifuged for 10 min at 13 000 x g. The supernatants were filtered (0.22 µm) and the filtrate was transferred to an HPLC vial. Samples were stored at -20 ºC for up to 24 h prior to HPLC analysis.

Method 2: samples (500 mg, in triplicate) of freeze-dried whole apple powder or pomace powder from Idared, Cox and Jonagold apples were extracted using 40 ml of 70 % v/v methanol in a 50 cm^2 volumetric flask. The mixture was placed in a ultrasonic water bath to disperse the sample, incubated in an air oven at 70 ºC for 20 min and then sonicated in the water bath for a final time. The sample was then made up to volume (50 ml) and a 1.5 ml sample was taken and centrifuged at 13 000 x g for 20 min. The supernatant was filtered (0.22 µm) and the filtrate was transferred to an HPLC vial. Samples were stored at -20 ºC for up to 24 h prior to HPLC analysis.

3.4.2.6 High-performance liquid chromatography analysis

Each sample was analysed using two different high-performance liquid chromatography (HPLC) methods (reverse-phase HPLC analysis and normal-phase HPLC analysis) as described in Chapter 2, Section 2.4.5.

3.4.2.7 Statistical analysis

Where indicated, comparisons between the apples, first press pomace, first press juice, second press pomace and second press juice were carried out using a two tailed t-test. A *p*-value < 0.05 was considered significant.

3.4.3 Human Study

3.4.3.1 Ethics

The human study was approved by the Institute of Food Research Human Research Governance Committee and the Norfolk Research Ethics Committee (reference: 07/H0310/156).

3.4.3.2 Subjects

Ten healthy, non-smoking males (aged between 19 and 64 years) were recruited by advertisements placed on the Norwich Research Park and from the Volunteer Database held at the Human Nutrition Unit (HNU) at IFR. Exclusion criteria were based on the following criteria: depressed or elevated blood pressure (<90/50 or 99/55 if symptomatic or >160/100); BMI <20 or >35; history of stroke; gastrointestinal problems; diagnosis of a long-term illness (e.g. diabetes, cancer); diagnosis of a metabolic disease; allergy to apples or birch pollen; on medications; unwillingness to discontinue dietary supplements one month prior and for the duration of the intervention; immunisation required one month prior and during the intervention; antibiotic use during the intervention; those who have given blood 16 weeks prior to the intervention and intend to donate less than 16 weeks after the last study sample; and parallel participation in another research project involving dietary intervention and sampling of blood. Potential subjects were screened for suitability and measurements of blood pressure, pulse, height, body weight, urine analysis, full blood count, glucose analysis and urea and electrolyte level were taken after an overnight fast.

3.4.3.3 Design

A randomised, double-blind, crossover study was used to compare the consumption of a standard apple juice (control) and apple juice supplemented with an epicatechin-rich apple concentrate (Evesse^{TM} Fructose). Each of the phases lasted four days. Volunteers were asked to refrain from consuming foods and drinks containing high levels of polyphenols starting on study day one. The intervention began on study day three and a 24 h sample was collected on study day four. There was a minimum wash out period of 14 days between the two phases of the intervention.

3.4.3.4 Test Drink

The flavonoid-rich apple concentrate, Evesse™ Fructose, was provided by Coressence Ltd. The Evesse™ Fructose was received in two batches, the first batch was produced on 20/02/2008 and the second batch was produced on 09/04/2008. Standard value-range apple juice was purchased from TESCO. The apple juice was purchased in bulk with the same batch number and a fresh apple juice was opened on each study day to ensure that there were no differences in the apple juice between study days.

The epicatechin and polyphenol content was analysed using reverse-phase HPLC. Triplicate analysis was performed on three samples of each Evesse™ Fructose Batch 1 and 2, apple juice and test drink. Analysis was preformed as described above for the juice samples (extraction Method 1 as described in Section 3.4.2.2.1 and the HPLC analysis as described in Chapter 2, Section 2.4.3).

The volunteers who received Evesse™ Fructose Batch 1 were given 190 g of apple juice supplemented with either 60 g of water (control) or 60 g of Evesse™ Fructose to give ~70 mg of epicatechin. While for those volunteers who received Evesse™ Fructose Batch 2, 190g of apple juice was supplemented with 70g of water or 70 g of Evesse™ Fructose (to give ~70 mg of epicatechin).

3.4.3.5 Pulse Contour Measurements

Triplicate measurements were taken after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after consumption. Subjects rested for at least 10 min prior to the blood pressure being taken. A photoplethysmography probe (Pulse Trace PCA 2, Micro Medical, Kent) was then placed on the index finger, of the non-cannulated arm to measure the digital volume

pulse (DVP). Subjects were asked to stay in a relaxed state with no strenuous activity for the duration of the measurements as exercise can have an affect on the pulse contour analysis (PCA) measurements. In the case of an unstable reading, PCA measurements were attempted for another two attempts before abandoning the time point.

3.4.3.6 Data Analysis

Subject characteristics are shown as mean ± standard deviation. Differences between the groups were analysed using analysis of variance (ANOVA) and differences due to time were analysed using a Dunnett test. A power transformation of the response variables (SI, RI and VA) was performed in order to conform to the assumptions of ANOVA.

3.5 Results

3.5.1 Heat treatment of apples prior to pressing does not reduce losses of polyphenols and does not increase the juice polyphenol yield

The processing of apples results in a significant loss of epicatechin and total polyphenols. In order to try and reduce the loss of polyphenols in the processing of apples into juice, a modification of the conventional apple juice production method was performed. The modification of the apple juice production method involved the use of a heat treatment (microwave) on the apples prior to pressing (experimental modification 1, Figure 3.2 A). It was hypothesised that a heat treatment would inactivate the polyphenol oxidase and inhibit enzymatic browning and thus reduce the loss of epicatechin and total polyphenols from the apples to the first press pomace and

juice. The application of heat was hypothesised to reduce the rate of enzymatic browning and polyphenol loss because the enzymes, which are proteins, are easily denatured by heat (El-Shimi, 1993). Therefore, whole apples were microwaved for 1 min in order to increase the core temperature from approximately 23 ºC to 100 ºC prior to pulping and pressing.

The polyphenols analysed included: epicatechin, catechin, procyanidin dimer B2, procyanidins (dp 3 – 7), chlorogenic acid, phloridzin, rutin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside. Epicatechin, catechin, dimers and the procyanidins were analysed using normal phase HPLC and the quercetin glycosides, chlorogenic acid and phloridzin were analysed using reverse-phase HPLC.

Pressing the fresh apples resulted in an extraction of 7 % of both epicatechin and total polyphenols into the juice while 59 % epicatechin and 60 % total polyphenols were retained in the pomace (Table 3.1 and Figure 3.3). The pressing of the apples resulted in a 34 % loss of epicatechin (p = 0.007) and a 32 % loss of total polyphenols (p = 0.004). The epicatechin and total polyphenol contents were derived from the fresh weight data and the mass of the apples, pomace or juice. In order to reduce the loss of epicatechin and total polyphenols associated with apple pressing, a microwave heat treatment was applied prior to pressing.

The pre-pressed microwave heat treatment did not have any effect on reducing the pressing-induced losses of the epicatechin content or total polyphenol content. The effect of microwaving the apples resulted in a 35 % loss of epicatechin and a loss of 18 % of total polyphenols in the microwaved apples compared to the fresh apples (Figure 3.3). Therefore the application of the heat treatment increased the loss of polyphenols compared to fresh apples rather than decreasing the polyphenol loss it

also increased the rate of enzymatic browning. Enzymatic browning occurred at a faster rate in the apples that had been mirowaved and a heat ring was apparent in the apples that were microwaved (Figure 3.4).

After pressing the microwaved apples, the majority of the polyphenols was located in the pomace (55 % epicatechin and 48 % total polyphenols) compared to the juice (19 % epicatechin and 14 % total polyphenols). Therefore a further loss (26 % loss of epicatechin and 38 % loss of total polyphenols) of polyphenols occurred during the pressing of the apples. The majority of the polyphenols were also retained in the apple pomace (Table 3.1)

Table 3.1: Polyphenol profile of Jonagold, Idared and Cox apples in the apples, microwaved apples, first press pomace and juice and second press pomace and juice.

	Apples (mg)	**Apples microwaved (mg)**	**First press (mg)**		**First press (microwaved) (mg)**	
			Pomace	**Juice**	**Pomace**	**Juice**
Monomers[1]	244	162	154	17	88	29
Polymers[2]	1712	1493	894	56	619	107
Quercetin glycosides	190	137	128	5	94	14
Chlorogenic acid	437	350	248	67	187	99
Phloridzin	175	123	141	11	101	33
Total	*2759*	*2265*	*1565*	*156*	*1088*	*282*

[1]Monomers include epicatechin and catechin

[2]Polymers include procyanidins dp 2 -7

[3]Quercetin glycosides include the following compounds: rutin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside

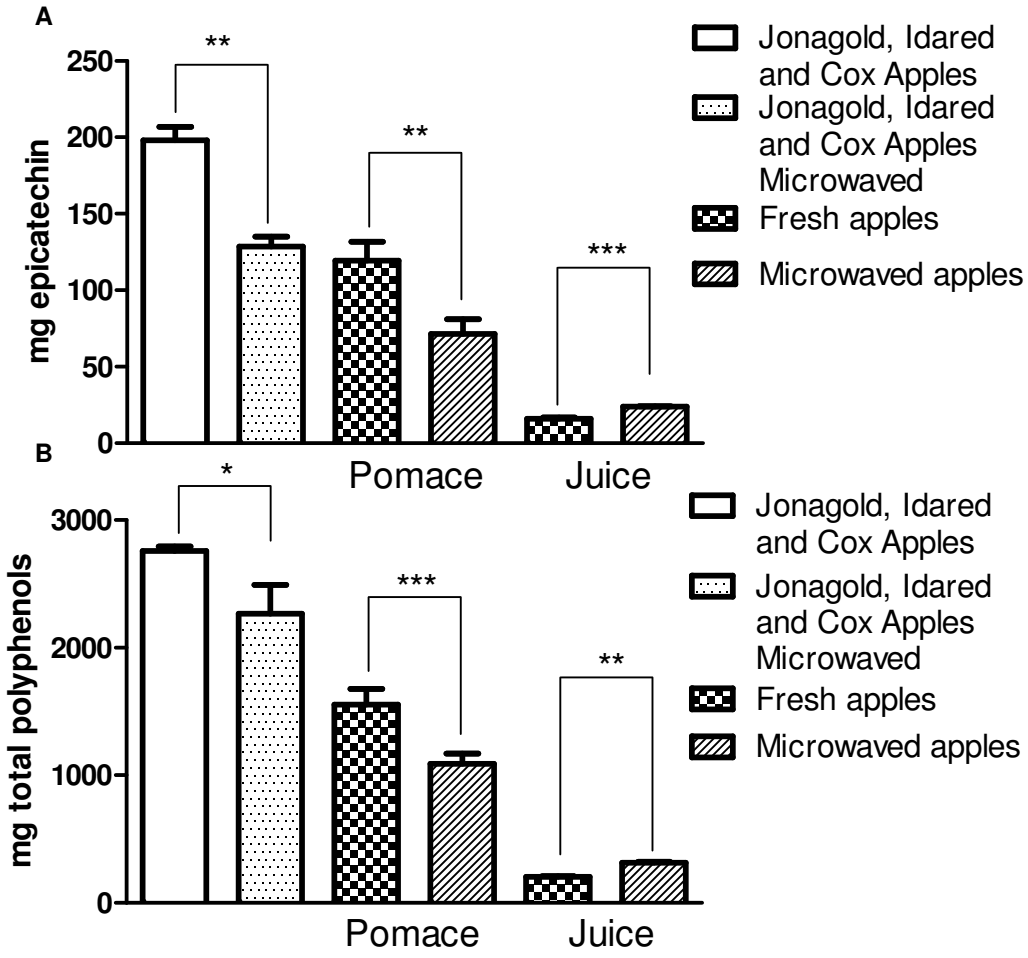


Figure 3.3: Epicatechin content (mg) and total polyphenols content (mg) of the pomace and juice of the first and second press in microwaved apples in comparison to the epicatechin content (mg) of the fresh and microwaved apples.

Jonagold, Idared and Cox apples were first microwaved whole for 1 min, cut into quarters, blended in a food processor to increase juice yield, and then pressed using a wooden press. The pomace was then steeped in boiling water for 24 h at room temperature prior to a second pressing. Samples of the fresh and microwaved apples, 1st press pomace and juice and 2nd press pomace and juice were analysed for epicatechin content (**A**) and total polyphenol content (**B**) using reverse- and normal-phase HPLC. The epicatechin content and total polyphenol content is the total amount of epicatechin/polyphenol in 2500 g of fresh or microwaved apples; the total amount of epicatechin/polyphenol in 1850 g and 650 g in the fresh apple pomace and juice, respectively; and the total amount of epicatechin/polyphenol in 1600 g and 850 g in the microwaved apple pomace and juice, respectively. Total polyphenols = epicatechin, catechin, dimer, procyanidins (dp 3 – 7), chlorogenic acid, phloridzin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, and quercetin-3-*O*-arabinoside. Data represent mean ± standard deviation. Results are representative of triplicate analyses; * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$.

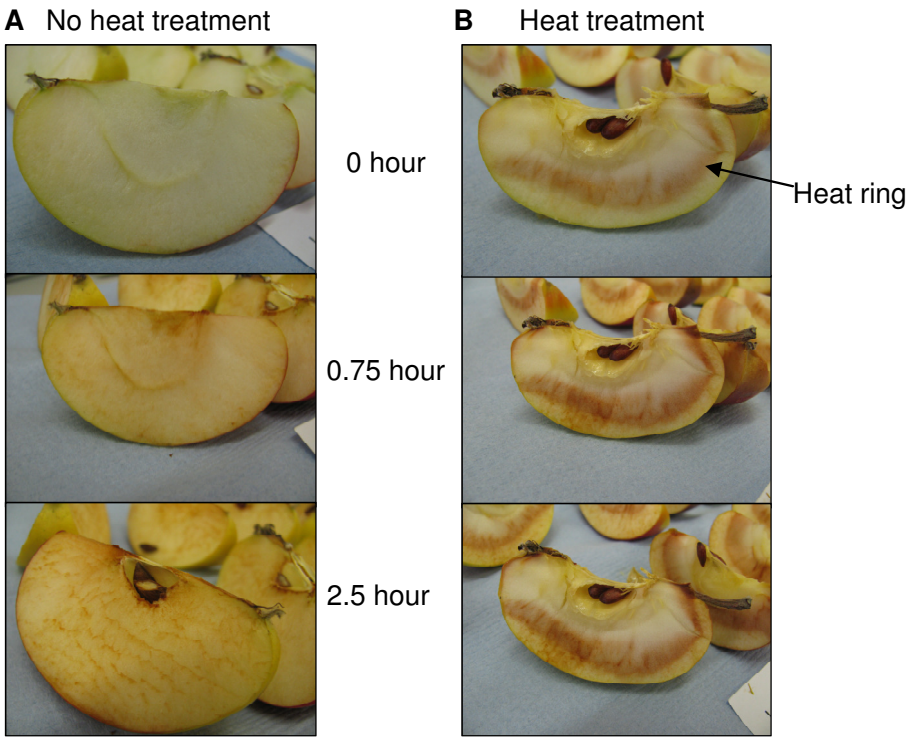


Figure 3.4: Comparison of apples with and without heat treatment.
Apples were cut into eight segments from fresh apples (**A**) or apples that had been microwaved whole for 1 min prior to be cut into segments (**B**). Comparisons were photographed after cutting the apples, after 45 min and after 2.5 h.

3.5.2 Hot water treatment of the apple pomace is an effective means of extracting polyphenols

An adaptation of the conventional method of apple juice production was conducted in order to extract polyphenols from the pomace. The adaptation of the conventional method included the steeping of the apple pomace in boiling water for 24 h, at room temperature, before re-pressing (experimental modification 2, Figure 3.2B). The experiment was conducted on different types of apple varieties: (A) TESCO value apples; (B) a mixture of Harry Masters and Yarlington Mill cider apples; and (C) a mixture of Jonagold, Idared and Cox dessert apples (Figure 3.5). Different varieties of apples were chosen to determine if the type of apple variety had an affect on the hot water extraction yield. The TESCO value apples represented eating apples, the Harry

Masters and Yarlington Mill apples represented apples used for the production of cider and Jonagold, Idared and Cox apples represented varieties used for the production of apple juice.

The dessert apple varieties (TESCO value and Jonagold, Idared and Cox apples) had a similar concentration of epicatechin in the fresh apples (approximately 82 mg/kg) while the cider apples (Harry Masters and Yarlington Mill apples) had a much greater concentration of epicatechin (498 mg/kg). The content of epicatechin was derived from the fresh weight data (mg/kg) and the total mass of the apples, pomace or juice. After the first pressing the extraction yield of epicatechin into the juice (4 %) was similar between the three varieties and therefore was not dependent on the variety or epicatechin concentration. On average 52 % of the epicatechin was retained in the pomace and a statistically significant loss of epicatechin (44 %; $p < 0.0001$) was observed during the processing of the apples.

In all three experimental apple varieties, the hot water extraction efficiently extracted the epicatechin from the pomace into the second press juice. The different apple varieties had a similar extraction yield of epicatechin from the pomace to the second press juice that was not dependent on variety or epicatechin concentration. On average, 64 % of the epicatechin was extracted from the apple pomace into the second press juice with 17 % remaining in the pomace and a significant loss of 19 % ($p < 0.0001$) due to processing losses or oxidation.

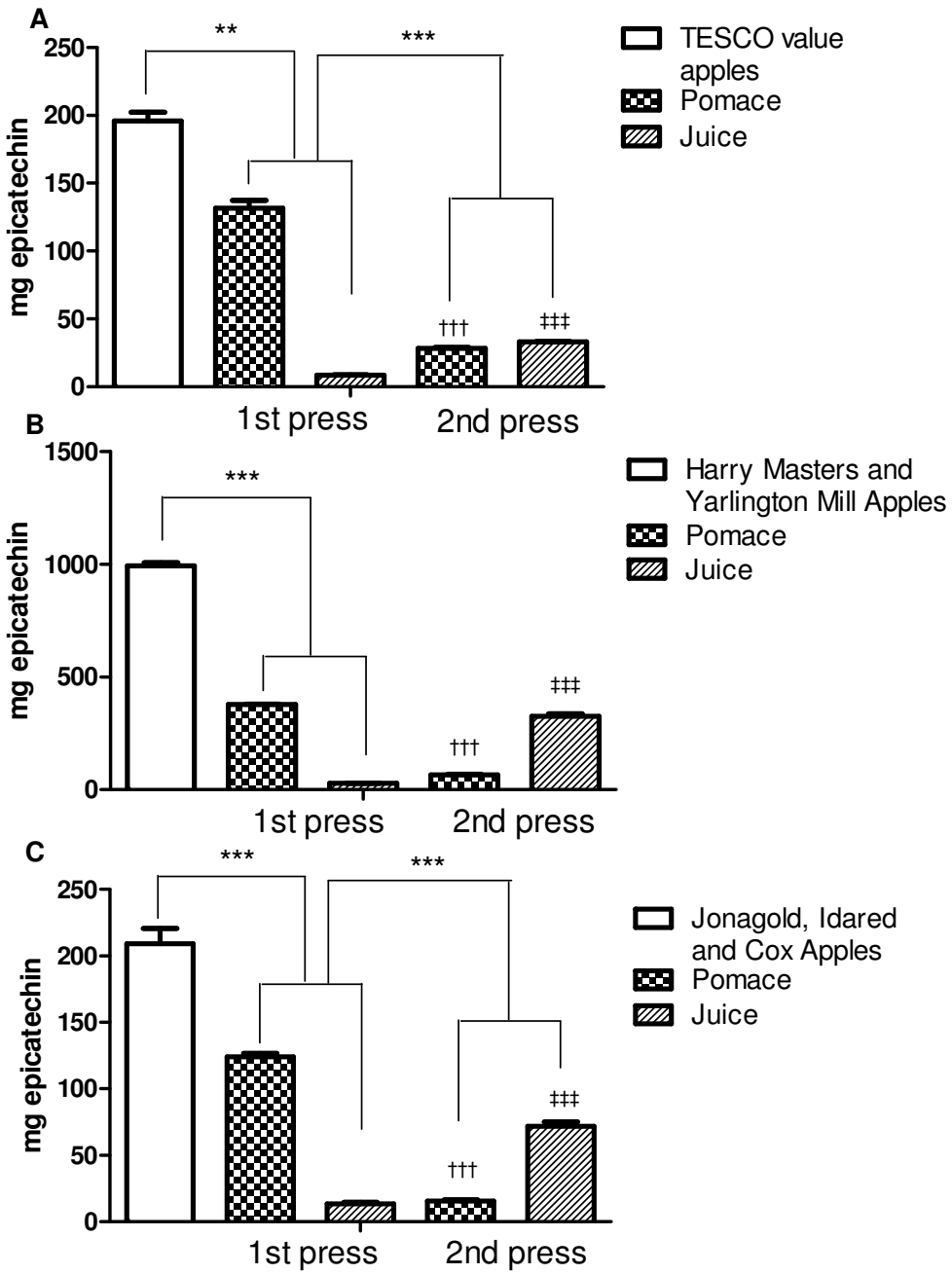


Figure 3.5: Epicatechin content (mg) of the pomace and juice of the first and second press in comparison to the epicatechin content (mg) of the apples.

TESCO value apples (A), Harry Masters and Yarlington Mill apples (B) and Jonagold, Idared and Cox apples (C) were first cut into quarters, blended in a food processor to increase juice yield, and then pressed using a wooden press. The pomace was then steeped in boiling water for 24 h at room temperature prior to a second pressing. Samples of the apples, 1st press pomace and juice and 2nd press pomace and juice were analysed for epicatechin content using reverse-phase HPLC. The epicatechin content in the TESCO value apples is derived from the total amount of apples pressed (2500 g), the weight of the first press products (1450 g pomace; 638 juice), and the weight of the second press products (904 g pomace; 1349 g juice). The epicatechin content in the Harry Masters and Yarlington Mill apples are derived from the total amount of apples (2000 g), the weight of the first press products (1600 g pomace; 265 g pomace) and the weight of the second press products (1260 g pomace; 2340 g juice). The epicatechin content in the Jonagold, Idared and Cox apples are derived from the total amount of apples (2500 g), the weight of the first press products (1850 g pomace; 665 g juice), and the second press products (1000 g pomace; 3111 g juice). Data represent mean ± standard deviation. Results are representative of triplicate analyses and (C) is the average of three experiments. ** $p < 0.01$, *** $p < 0.001$ compared to the fresh apples or the first press, ††† $p < 0.001$ compared to the first press pomace and ‡‡‡ $p < 0.001$ compared to the first press juice.

For the total polyphenol content, the same pattern was observed as seen with the epicatechin content. The TESCO value apples were not analysed using the reverse-phase method, therefore there is no data available on the total polyphenol content.

The dessert apples (2500 mg) had substantially less total polyphenols compared to the cider apples (9500 mg). The extraction yield of total polyphenols in the juice (7 %) and in the pomace (61 %) was greater in the dessert apples compared to the cider apples (3 % in the juice and 35 % in the pomace). This is due to the greater losses of polyphenols observed in the cider apples (63 %) compared to the dessert apples (32 %).

In the two experimental varieties, a hot water extraction efficiently extracted the polyphenols from the pomace into the second press juice in all experimental apple varieties (Figure 3.6). The majority of the polyphenols were retained in the apple pomace after the first press; however, after the hot water extraction of the apple pomace, the majority of the polyphenols were located in the second press juice compared to the second press apple pomace (Table 3.2). On average, 51 % of total polyphenols were extracted from the apple pomace into the second press juice with 19 % remaining in the pomace and a loss of 30 % due to processing losses and oxidation.

Therefore while a hot water extraction efficiently extracts epicatechin and total polyphenols from the apple pomace, a significant loss of epicatechin and total polyphenols are observed between the first press pomace and the second press (pomace plus juice).

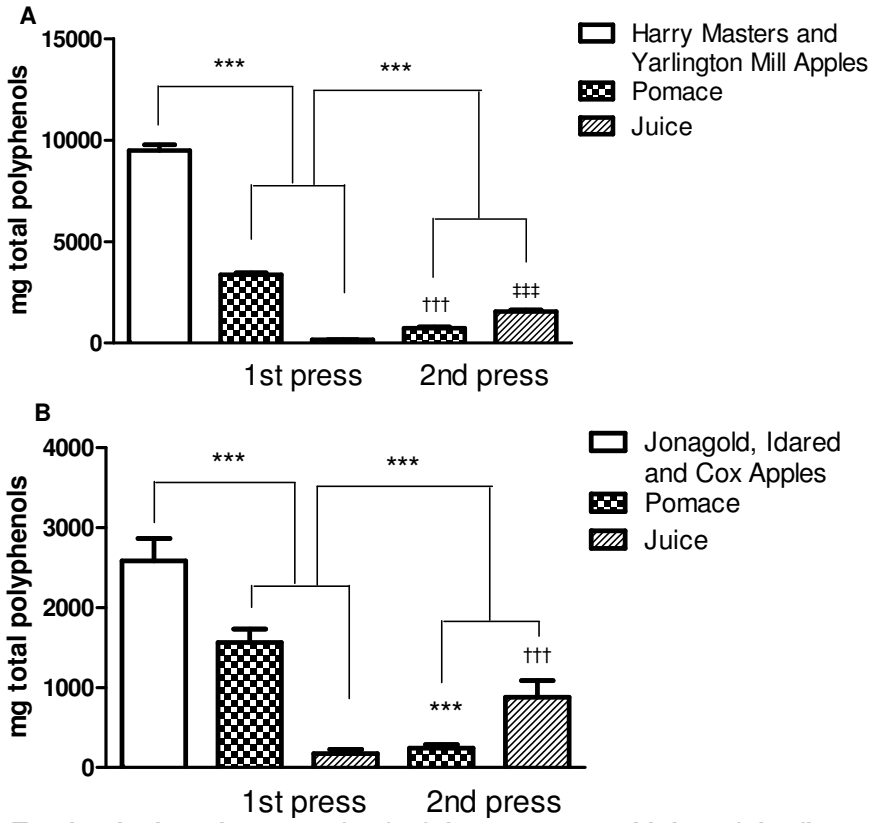


Figure 3.6: Total polyphenol content (mg) of the pomace and juice of the first and second press in comparison to the total polyphenol content (mg) of the apples.

Harry Masters and Yarlington Mill apples (**A**) and Jonagold, Idared and Cox apples (**B**) were first cut into quarters, blended in a food processor to increase juice yield, and then pressed using a wooden press. The pomace was then steeped in boiling water for 24 h at room temperature prior to a second pressing. Samples of the apples, 1st press pomace and juice and 2nd press pomace and juice were analysed for total polyphenol content using reverse- and normal-phase HPLC. Total polyphenols = epicatechin, catechin, dimer, procyanidins (dp 3 – 7), chlorogenic acid, phloridzin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, and quercetin-3-*O*-arabinoside. The total polyphenol content in the Harry Masters and Yarlington Mill apples are derived from the total amount of apples (2000 g), the weight of the first press products (1600 g pomace; 265 g pomace), and the weight of the second press products (1260 g pomace; 2340 g juice). The total polyphenol content in the Jonagold, Idared and Cox apples are derived from the total amount of apples (2500 g), the weight of the first press products (1850 g pomace; 665 g juice), and the second press products (1000 g pomace; 3111 g juice). Data represent mean ± standard deviation. Results are representative of triplicate analyses and (**B**) is the average of three experiments. ** $p < 0.01$, *** $p < 0.001$ compared to the fresh apples or the first press, ††† $p < 0.001$ compared to the first press pomace and ‡‡‡ $p < 0.001$ compared to the first press juice.

Table 3.2: Polyphenol profile of Harry Master and Yarlington Mill apples and Jonagold, Idared and Cox apples in the apples, first press pomace and juice and second press pomace and juice.

	Apples	First Press (mg)		Second Press (mg)	
		Pomace	Juice	Pomace	Juice
Harry Masters and Yarlington Mill Apples					
Monomers[1]	1026	398	29	70	335
Polymers[2]	5829	1947	48	383	877
Quercetin glycosides[3]	116	68	3	43	15
Chlorogenic acid	1591	648	48	155	299
Phloridzin	966	282	11	95	101
Total	*9528*	*3363*	*136*	*746*	*1627*
Jonagold, Idared and Cox apples					
Monomers[1]	260	154	17	20	91
Polymers[2]	1494	894	56	121	469
Quercetin glycosides[3]	190	128	5	41	63
Chlorogenic acid	453	248	67	40	187
Phloridzin	187	141	11	25	70
Total	*2584*	*1565*	*156*	*247*	*880*

[1]Monomers include epicatechin and catechin

[2]Polymers include procyanidins dp 2 -7

[3]Quercetin glycosides include the following compounds: rutin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside

3.5.3 Steeping apple pomace at 4 ºC rather than room temperature does not improve the polyphenol yield

In Section 3.5.2, a significant loss of epicatechin and total polyphenols was observed between the first press pomace due to the second press (pomace plus juice). Therefore a final modification of the conventional apple juice production was preformed in order to try and reduce the losses observed during the hot water extraction of the apple pomace. The experimental modification (experimental modification 3, Figure 3.2C) included at hot water extraction of the apple pomace at 4 ºC overnight to test whether reducing the standing temperature would have an affect on the polyphenol losses.

The extraction yields of epicatechin and total polyphenols in the first press juice was 10 % and 9 %, respectively, and the majority of the epicatechin (47 %) and total polyphenols (52 %) remained in the pomace. A significant loss of epicatechin (43 %,

$p < 0.0001$) and total polyphenols (38 %, $p < 0.0001$) was observed between the first press products (pomace and juice) and the apples (Figure 3.7). The majority of the polyphenols were retained in the apple pomace compared to the first press juice (Table 3.3).

The hot water extraction of the apple pomace and incubation at 4 ºC efficiently extracted epicatechin and total polyphenols, with a significant increase ($p < 0.0001$) in both epicatechin and total polyphenol contents in the second press juice compared to the first press juice. However, the majority of the polyphenols were still retained in the second press pomace (Table 3.3). In the case of monomers and chlorogenic acid, a greater amount was extracted into the juice, while the majority of the polymers, quercetin glycosides were retained in the pomace. The same amount of phloridzin was located in the second press pomace (55 mg) and in the second press juice (56 mg).

The incubation at 4 ºC was not as efficient as the extraction at room temperature. The extraction yield of epicatechin (46 %) and total polyphenols (34 %) in the juice at 4 ºC was significantly lower ($p < 0.001$) than the extraction yield of epicatechin (64 %) and total polyphenols (51 %) in the juice at room temperature. The losses observed from the first press pomace to the second press (pomace plus juice) were similar between the two methods; a 14 % loss of epicatechin and a 27 % loss of total polyphenols occurred at 4 ºC while a 19 % loss of epicatechin and a 30 % loss of total polyphenols occurred at room temperature. Therefore, the incubation at 4 ºC did not reduce the loss in polyphenols from the apple pomace during the incubation period and it was significantly less efficient in extracting epicatechin or total polyphenols from the apple pomace into the second press juice compared to the incubation period at room temperature.

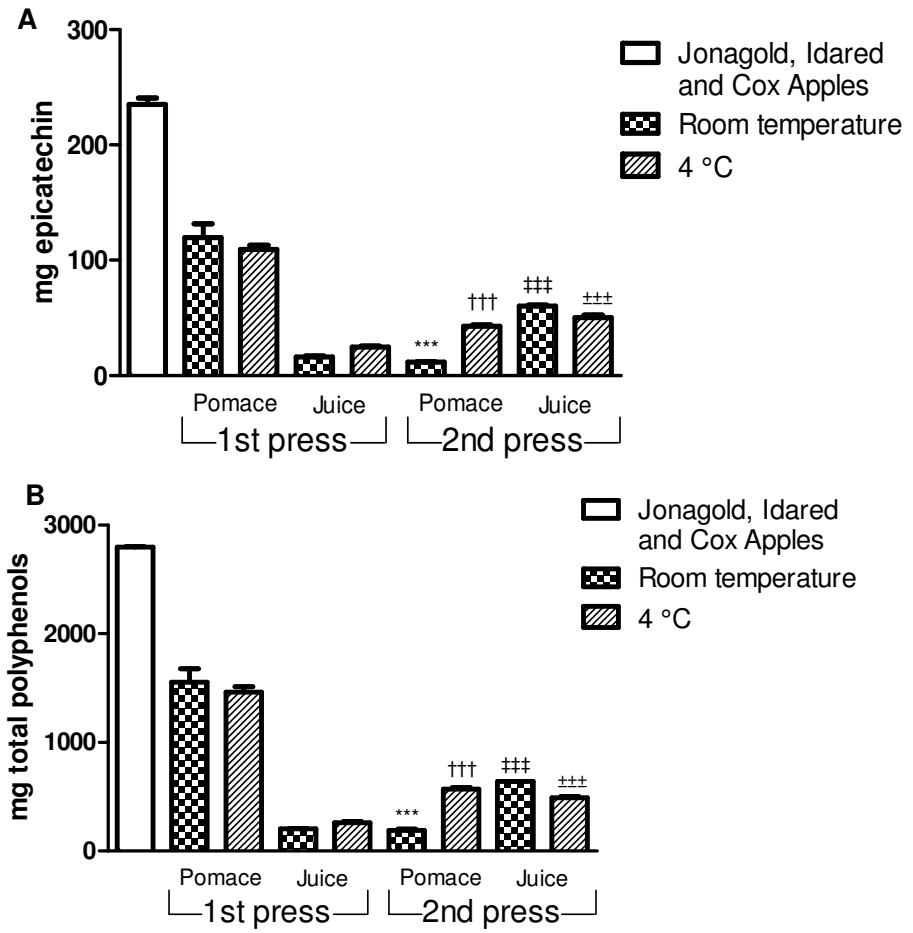


Figure 3.7: Epicatechin content (mg) and total polyphenol content (mg) of the pomace and juice of the second press in comparison to the pomace and juice of the second press after a hot water extraction at 4 ºC.

Jonagold, Idared and Cox apples were first cut into quarters, blended in a food processor to increase juice yield, and then pressed using a wooden press. The pomace was then steeped in boiling water for 24 h at 4 ºC prior to a second pressing. Samples of the apples, 1st press pomace and juice and 2nd press pomace and juice were analysed for epicatechin content (**A**) and total polyphenol content (**B**) using reverse- and normal-phase HPLC. Total polyphenols = epicatechin, catechin, dimer, procyanidins (dp 3 – 7), chlorogenic acid, phloridzin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, and quercetin-3-*O*-arabinoside. The total polyphenol content in the Jonagold, Idared and Cox apples at room temperature are derived from the total amount of apples (2500 g), the weight of the first press products (1850 g pomace; 665 g juice), and the second press products (1000 g pomace; 3111 g juice). The total polyphenol content in the Jonagold, Idared and Cox apples at 4 ºC are derived from the total amount of apples (2500 g), the first press products (1700 g pomace; 1180 g juice), and the second press products (2300 g pomace; 1300 g juice). Data represent mean ± standard deviation. Results are representative of triplicate analysis. *** p <0.001 compared to the room temperature first press pomace, ††† p < 0.001 compared to the 4 ºC first press pomace, ‡‡‡ p < 0.001 compared to the room temperature first press juice, and ±±± p <0.001 compared to the 4 ºC first press juice.

Table 3.3: Polyphenol profile of Jonagold, Idared and Cox apples in the apples, first press pomace and juice and second press pomace and juice that had been incubated at 4 ºC.

	Apples	First press (mg)		Second press (mg)	
		Pomace	Juice	Pomace	Juice
Jonagold, Idared and Cox apples					
Monomers[1]	290	132	68	53	64
Procyanidins[2]	1681	805	52	329	224
Quercetin[3] glycosides	150	133	14	72	49
Chlorogenic acid	479	229	112	64	98
Phloridzin	196	163	23	55	56
Total	*2796*	*1464*	*233*	*572*	*491*

[1]Monomers include epicatechin and catechin

[2]Polymers include procyanidins dp 2 -7

[3]Quercetin glycosides include the following compounds: rutin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside

3.5.4 Total polyphenol and epicatechin concentration and content in the Evesse™ Fructose, apple juice and test drink

The epicatechin concentration and content, and total polyphenol content were analysed for the Evesse™ Fructose Batch 1 and Batch 2, apple juice and test drink using normal- and reverse-phase HPLC analysis. The total polyphenol content included the following analysed compounds: epicatechin, catechin, dimers and procyanidins (dp 2-7), chlorogenic acid, phloridzin, rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-rhamnoside. Quercetin-3-*O*-arabinoside was not detectable in Evesse™ Fructose Batch 1 or 2 or in the apple juice. Catechin, procyanidins, quercetin-3-*O*-xyloside and quercetin-3-*O*-rhamnoside were also not detectable in the apple juice samples.

The epicatechin concentration in the commercial apple juice was low (epicatechin: 25.9 mg/L), and the Evesse™ Fructose Batches 1 and 2 had much higher concentrations of epicatechin; 1212.8 mg/L and 1028.8 mg/L, respectively (Table 3.4). The epicatechin content of Evesse™ Fructose Batch 1 in the test drink volume (250 ml) was 72.7 mg while the epicatechin content of Evesse™ Fructose Batch 2 in the test

drink volume (250 ml) was lower (61.7 mg). Therefore, the volunteers receiving Batch 2 were given an extra 10 g of the apple concentrate (or water, for the control) in order to provide the same amount of epicatechin contents between the two batches. At the end of the study the epicatechin content in the test drink was analysed. The analysis was preformed on triplicate samples of three test drinks for both of the batches. The HPLC analyses showed no significant differences in the epicatechin content of the 60 ml of Evesse™ Fructose Batch 1 (72.7 mg) compared to the test drink (72.6 mg). However, surprisingly the Batch 2 epicatechin content in the test drink (84.0 mg) was apparently 14 mg greater than in the 70 ml of the Evesse™ Fructose (71.9 mg).

The apparent 14 mg increase in the epicatechin content in test drink compared to the Evesse™ Fructose Batch 2 is most likely due to problems associated with the HPLC extraction method used. Following the completion of this study, further investigation on the HPLC extraction method had been conducted. It was found that the mass/volume of material used in current extraction method was sufficient for analysing low-polyphenol apple granules however when analysing high-polyphenol apple fructose the extraction method underestimated the concentration of epicatechin (Dave Hart, Wendy Hollands & Paul Kroon, IFR, unpublished work). It was shown that using a greater mass/volume of high-polyphenol apple fructose or granules resulted in a greater precision and accuracy of the concentration of epicatechin. The analysis of the Evesse™ material could not be re-analysed using the new method as there was no more product remaining. Therefore, the likely error in the measurement of the epicatechin concentration and content arose from the analysis of Evesse™ Fructose than the test drink as the Evesse Fructose was a high-polyphenol extract compared to the diluted test drink.

The subjects were not split into two different groups based on the batch number of Evesse™ fructose that they received because the sample size for each group was too small to perform statistical analysis on the PCA data.

Table 3.4: Total polyphenol and epicatechin concentration and content in reverse-phase HPLC data of Evesse™ Batch 1 and 2, apple juice and test drinks.

Product	Total polyphenol concentration (mg/L)	Epicatechin concentration (mg/L)	Epicatechin content (mg) in 60 g	Epicatechin content (mg) in the test drink	Total polyphenol content (mg) in the test drink
Evesse™ Batch 1	8242.4	1212.8	72.7	n/a	n/a
Evesse™ Batch 2	5810.1	1028.8	61.7	n/a	n/a
Apple juice	102.7	25.9	n/a	n/a	n/a
Test drink with Batch 1	1813.4	290.3	n/a	72.6	453.4
Test drink with Batch 2	2038.5	323.1	n/a	84.0	530.1

n/a = not applicable

Total polyphenol concentration and content only includes the following analysed polyphenols: epicatechin, catechin, dimers and procyanidins (dp 2-7), chlorogenic acid, phloridzin, rutin, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-arabinoside, quercetin-3-xyloside and quercetin-3-rhamnoside.

3.5.5 Baseline characteristics of volunteers

Of the ten subjects recruited into the study, nine completed. One male subject was excluded after the first phase as he was prescribed antibiotics during the study. Paired data were therefore available for analysis on nine subjects. The mean age and BMI of the subjects was 48.3 years and 24.9 kg/m^2, respectively (Table 3.5). The mean of the stiffness index (SI) (7.75 m/s ± 2.24), reflective index (RI) (69.70 ± 11.77) and vascular age (VA) (36.48 yrs ± 18.29) at baseline in the control phase was not statistically different (SI: p = 0.88; RI: p = 0.47; VA: p = 0.97) with the baseline measurements of the SI (7.58 m/s ± 2.21), RI (65.36 ± 13.6) and VA (36.78 yrs ± 17.30) in the treatment phase. There was also no significant difference, at baseline, in both phases between age and BMI.

Table 3.5: Baseline characteristics of the subjects

Characteristics	Mean ± Standard Deviation
Age, *y*	48.3 ± 7.2
Height, *m*	1.77 ± 6.2
Weight, *kg*	78.7 ± 9.4
BMI, kg/m^2	24.9 ± 9.4
Systolic BP[1], *mm/Hg*	124.5 ± 9.7
Diastolic BP[1], *mm/Hg*	77.9 ± 7.9

Values are means ± SD, n = 9

[1]Values are means ± SD of baseline measurements on both phases, n = 18

3.5.6 Assessment of vascular function by pulse contour analysis

Vascular function was assessed by PCA. PCA is one of the methods available to measure vascular function. PCA measures the DVP by measuring the transmission of infrared light absorbed through the finger using a photoplethysmography probe (Millasseau et al., 2002). The DVP consists of two waveforms: the systolic wave and the diastolic wave (Figure 3.8) (Oliver and Webb, 2003; Woodman and Watts, 2003). The systolic wave is a direct measurement of pressure transmission from the left ventricle in the heart to the finger. The diastolic wave is formed during the period when the pressure from the pulse is reflected back up the aorta and down to the finger (Takazawa et al., 1998; Allen, 2007).

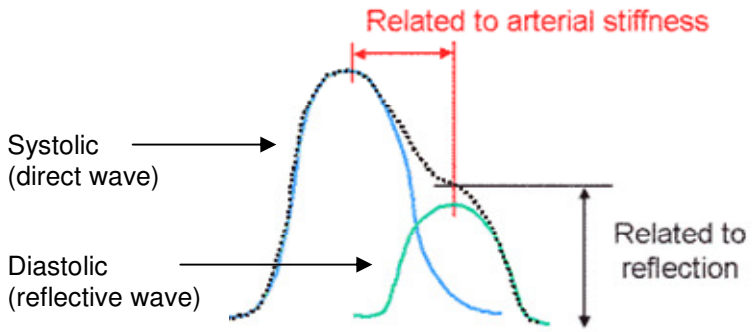


Figure 3.8: Digital volume pulse waveform

Two pressure waves combine to form the finger pulse. The first pressure wave is the systolic wave and it is a direct result of the pressure transmission from the aortic root in the heart to the finger. The second pressure wave is the diastolic wave and it is formed when part of the pulse is reflected back up the aorta and down to the finger. The time between the systolic and diastolic peaks is related to arterial stiffness and the height of the diastolic peak is related to the reflection index.

The DVP waveform has been used to determine the SI and RI (Mackenzie et al., 2002). The transit time (in milliseconds) between the systolic and diastolic wave depends on large artery stiffness. The transit time divided by the subject's height is used to obtain a stiffness index (SI), which correlates strongly with aortic pulse wave velocity (PWV) (Woodman and Watts, 2003). The height of the diastolic wave relates to the amount of wave reflection, which is related to endothelial function. The RI is defined as the relative height of the diastolic peak expressed as a percentage of the systolic peak.

Across both phases of the study, triplicate measurements were recorded 85 % of the time, and repeated unstable readings and time constraints were responsible for two or fewer measurements being recorded at some time points. Unstable measurements which produced no recordings for a specific time point occurred less than 1 % of the time. An example measurement is shown in Figure 3.9.

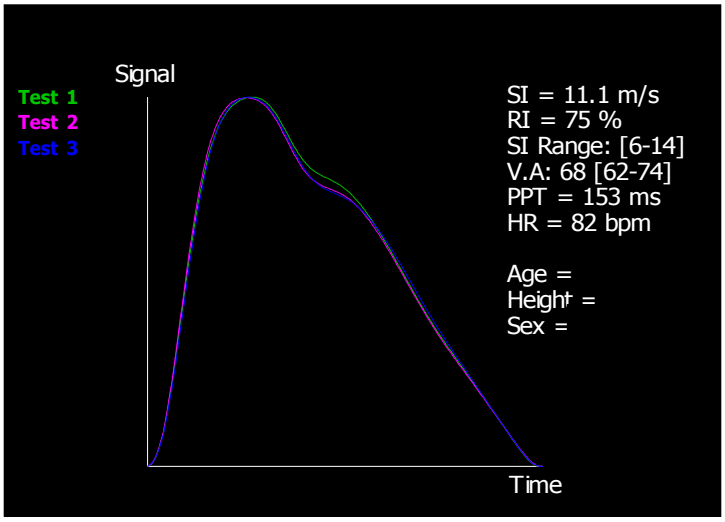


Figure 3.9: Example digital volume pulse waveform.

Triplicate measurements of the digital volume pulse waveform were obtained from subjects after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after the consumption of the control or treatment drink. SI = stiffness index, RI = reflective index, V.A. = vascular age, HR = heart rate. Age, height and sex are the subject's characteristics.

The baseline measurement of SI during the treatment phase was not significantly different ($p = 0.88$) to the baseline measurement in the control phase. During the treatment phase, the SI increased after 15 min followed by a steady decrease until 100 min when the SI slightly increased before finally levelling off back to baseline. The control phase SI shows a similar pattern to the treatment phase; however the increase up to 100 min is greater than for the treatment phase but does not reach significance at any individual time point (Figure 3.10).

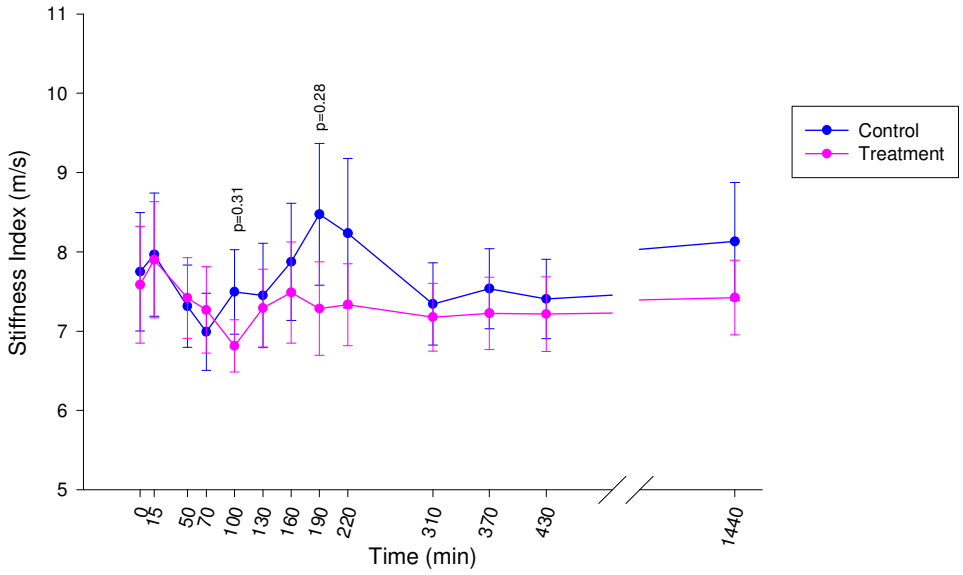


Figure 3.10: Comparison of the stiffness index in the control and treatment phases.

Triplicate measurements of the digital volume pulse were taken using a photoplethysmography probe were taken after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after consumption of the standard apple juice (control) or the Evesse^{TM} fructose-supplemented apple juice (treatment). Values represent mean ± standard deviation of the nine subjects triplicate measurements. Differences between the groups were analysed using ANOVA and differences due to time was analysed using a Dunnett test. A power transformation of the response variable (SI) was preformed in order to conform to the assumptions of ANOVA.

Age is known to be a confounding variable; therefore age was accounted for as a covariate during statistical analysis. The mean SI of the control phase (7.68 m/s ± 1.94) was significantly different (p = 0.031) to the mean SI of the treatment phase

(7.36 m/s ± 1.54). Therefore a statistically significant lower SI was observed after consumption of the test drink.

The RI is an indicator of endothelial function and an increase in RI is due to an increase in peripheral pulse wave reflection (e.g. vasoconstriction) while a reduction in RI indicates vasodialtion (Kneifel et al., 2006; Alty et al., 2007). After consumption of the test drink, a reduction in RI measurements is expected as a NO preserving effect has been observed following treatment with flavonoids (Karim et al., 2000; Leikert et al., 2002; Wallerath et al., 2002; Anter et al., 2004; Lorenz et al., 2004; Nicholson et al., 2008; Appeldoorn et al., 2009a; Lorenz et al., 2009b; Madeira et al., 2009; Ramirez-Sanchez et al., 2010). While a difference in baseline is observed between the RI measurement in the treatment and control phase, the difference is not statistically significant difference (p = 0.47) and the RI measurement at 15 min in the treatment phase increases to the baseline measurement of the control phase (Figure 3.11).

Following the consumption of the Evesse Fructose^{TM}-supplemented drink, the RI increased moderately at 15 min, and then decreased substantially up to 100 min. After this, there was an increase to 130 min, followed by an uneven slow decline. The consumption of lunch (between the measurements at 210 and 310 min) may be the cause of the resulting decrease in RI. There was no statistically significant difference between the RI (all the data) of the control and treatment phases (p = 0.222), with no significant differences at any time point.

However, the RI over time was significantly different (p = 0.022), and the greatest difference occurred at 310 min when compared to the baseline measurement in both the control and treatment phase.

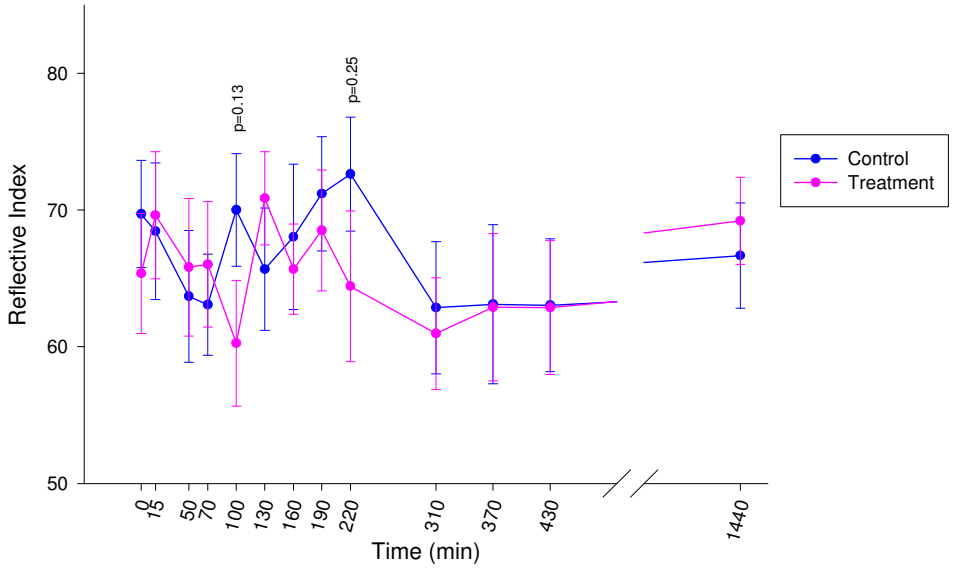


Figure 3.11: Comparison of the reflective index in the control and treatment phases.

Triplicate measurements of the digital volume pulse were taken using a photoplethysmography probe were taken after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after consumption of the standard apple juice (control) or the Evesse™ fructose-supplemented apple juice (treatment). Values represent mean ± standard deviation of the nine subjects triplicate measurements. Differences between the groups were analysed using ANOVA and differences due to time was analysed using a Dunnett test. A power transformation of the response variable (RI) was preformed in order to conform to the assumptions of ANOVA.

Vascular age, which is a calculated value derived from the SI (PulseTrance PCA 2 Operating Manual, Micro Medical), showed a similar pattern to that observed with the SI (Figure 3.10). There was no significant difference (p = 0.97) in the baseline measurements between the control and treatment phase. As age is a confounding variable, age was accounted for as a covariate during statistical analysis of the data. The mean VA of the control phase (36.52 yrs ± 17.21) was significantly different (p = 0.005) to the mean of the treatment phase (33.74 yrs ± 13.93) (Figure 3.12). Therefore a lower VA was observed after consumption of the test drink. The effect of time on VA after the consumption of the treatment phase was also significantly different (p = 0.009) to the control phase and the greatest difference occurred at 70 min when compared to the baseline.

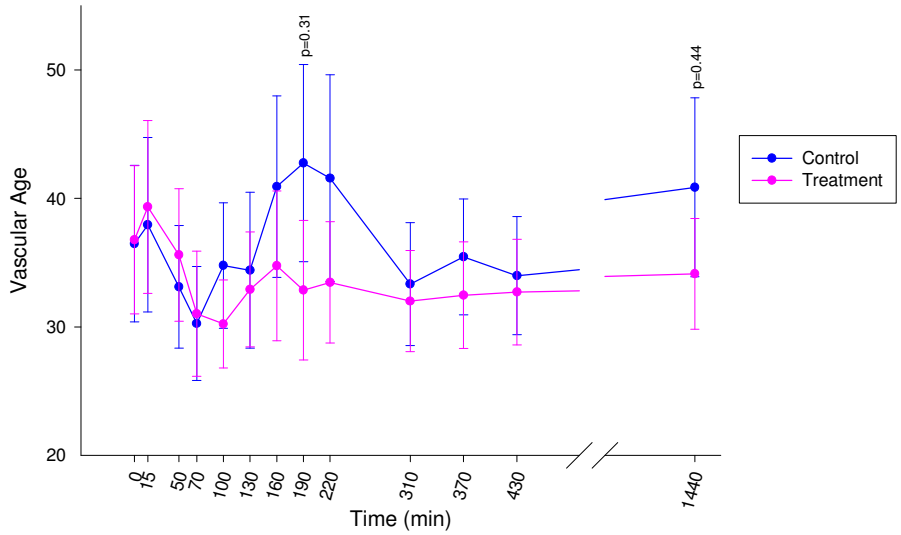


Figure 3.12: Comparison of the vascular age in the control and treatment phases.

Triplicate measurements of the digital volume pulse were taken using a photoplethysmography probe were taken after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after consumption of the standard apple juice (control) or the Evesse^{TM} fructose-supplemented apple juice (treatment). Values represent mean ± standard deviation of the nine subjects triplicate measurements. Differences between the groups were analysed using ANOVA and differences due to time was analysed using a Dunnett test. A power transformation of the response variable (VA) was preformed in order to conform to the assumptions of ANOVA.

The percentage differences of the treatment phase minus the control phase followed a similar pattern for the three measurements (SI, RI and VA) with the exception of a couple of time points (130 min and 1440 min) where the RI had a positive difference and the SI and VA had a negative difference in comparison to the control (Figure 3.13). In general, the treatment caused a decrease in the SI, RI and VA between 100 min and 220 min compared to the control drink.

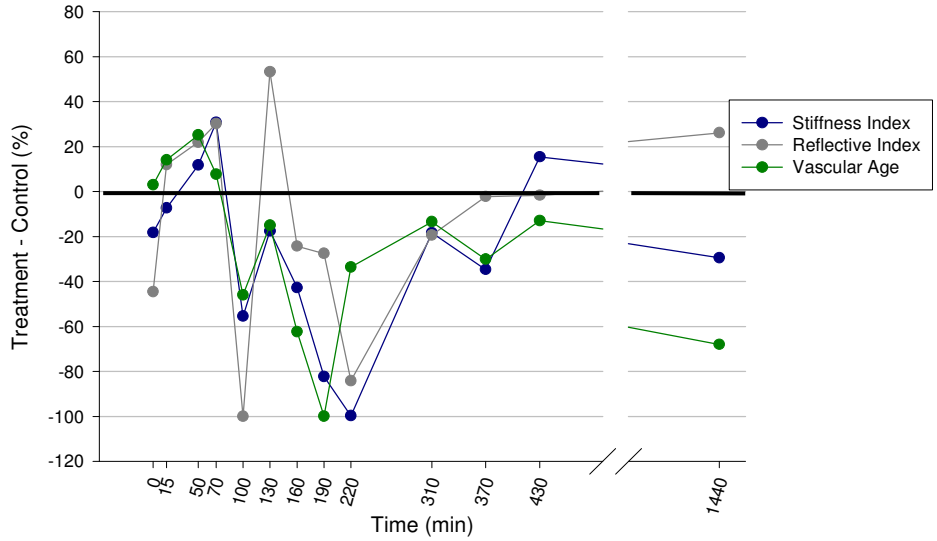


Figure 3.13: Differences between the control and treatment phases.

Triplicate measurements of the digital volume pulse were taken using a photoplethysmography probe were taken after an 8 h fast (baseline) and then at 15, 50, 70, 100, 130, 160, 190, 220, 310, 370, 430 and 1440 min after consumption of the standard apple juice (control) or the Evesse^{TM} fructose-supplemented apple juice (treatment). Values represent the treatment phase values minus the control phase values, so that the change indicates the effect of the test drink, and are normalised to the highest negative value for each parameter.

3.6 Discussion

The data presented in this chapter show that: (1) applying a heat treatment (microwave) to the apples prior to pulping did not reduce the loss of polyphenols observed with apple pressing; (2) a hot water extraction of the pomace prior to re-pressing efficiently extracted the majority of epicatechin and total polyphenols into the second press juice from the apple pomace; (3) allowing the hot water extracted pomace to stand at 4 ºC compared to room temperature was less effective in extracting the majority of the polyphenols from the apple pomace and did not reduce the polyphenol loss in the second press procedure; and (4) a commercially produced polyphenol-rich extract improved vascular function by improving arterial stiffness as assessed by PCA measurements. Therefore the efficient extraction of polyphenols from apple pomace, a by-product of apple juice production, provides a method of

extracting polyphenols for food supplementation which has the potential to improve vascular function.

A number of published reports have been concerned with extracting a greater quantity of polyphenols from the apple pulp or apple pomace in the production of apple juice (Spanos et al., 1990; Schols et al., 1991; Will et al., 2000; Gerard and Roberts, 2004; van der Sluis et al., 2004). Alcohol extraction of the apple pulp and pomace was shown to enhance the polyphenol concentration of the apple juice. The levels of quercetin glycosides and chlorogenic in the enriched apple juice following alcohol extraction were between 1.4 (chlorogenic acid) and 9 (quercetin glycosides) times higher than in conventionally produced apple juice (van der Sluis et al., 2004). However this extraction method had several limitations including limitations on the amount of solvent that could be used in the extraction process in order to comply with food laws, as well as the loss of key juice characteristics (e.g. aroma) (van der Sluis et al., 2004). Diffusion extraction, the extraction of juice by counter-current flow of hot water through the fruit slices, was shown to increase the hydroxycinnamic acid concentration of the juice three fold, the phloretin glycosides by five fold, the flavanols up to 32 fold and the quercetin glycosides concentration up to 52 fold compared to conventional pressing (Spanos et al., 1990). The apple juice produced using this production method results in a more bitter and astringent juice due to the higher polyphenol content (Gerard and Roberts, 2004; van der Sluis et al., 2004). Liquefaction, the enzyme treatment of apple pulp by the use of enzymes (pectinases and cellulases), is another treatment used to increase the polyphenol content in apple juice. Schols et al. (1991) showed that liquefaction (with pectinases) of the apple pulp increased the concentration of catechin, epicatechin, phloridzin and chlorogenic acid up to six-fold in comparison to conventionally pressed apple juice, while Will et al. (1999) demonstrated that apple pomace subjected to liquefaction (with pectinases and cellulases) resulted in an increased polyphenol content (30 – 50 %) in the extracted juice. However the use of

cellulases in fruit juice production is prohibited by European Law (Ribeiro et al., 2010). The use of heat treatment prior to pressing has also been tested as a means of increasing the polyphenol concentration. Apples which were microwaved after pulping generated juices with increased polyphenol concentrations compared to conventional pressing (Gerard and Roberts, 2004). However, while the increase in total polyphenol concentration was significant, the proportion of total apple polyphenols extracted to the juice was low (Gerard and Roberts, 2004).

Therefore, while alcohol extraction, diffusion extraction, liquefaction and a microwave heat-treatment of the apple pulp are effective methods in significantly increasing the apple juice polyphenol concentration compared to a conventional pressing method, these extraction methods also affected the characteristics of the juice or did not provide an overall high extraction yield. These methods also did not report the loss of polyphenols during the production of the juice from the apples to the press products (apple pomace and juice). Therefore, one of the aims of the studies described in this chapter was to investigate whether (1) applying a microwave heat treatment of the apple prior to pulping would reduced the loss of polyphenol observed with apple juice production; (2) to investigate whether a hot water extraction of the apple pomace would efficiently extract the epicatechin and other polyphenols from the polyphenol-rich pomace resulting from the first press; and (3) establish whether incubating the hot water extracted pomace at 4 ºC, rather than at room temperature, would improve the recovery of polyphenols in the second press juice.

The rationale for applying a microwave-heat treatment to the whole apples prior to the pulping and pressing of the apples was to denature the polyphenol oxidases and peroxidases and thus inhibit enzymatic browning, which would be expected to reduce the loss of polyphenols observed in the production of apple juice. The microwave-heat treatment increased the core temperature of the apples from 23 ºC to 98 ºC; however it

was apparently ineffective in denaturing the polyphenol oxidase enzymes. Instead the rate of browning, on the flesh, was substantially increased compared to the non-heat treated apple. The heat treatment of the whole apples resulted in a loss of 35 % epicatechin and 18 % total polyphenols. After pulping and pressing of the heat-treated whole apples, a further loss of 26 % epicatechin and 38 % total polyphenols between the press products (apple pomace and juice) and the heat treated apples. Therefore the application of a heat treatment on the whole apples caused a loss of 61 % epicatechin and 53 % total polyphenols during the juice production process which was substantially greater than using non-heat treated apples (34 % loss of epicatechin and 28 % loss of total polyphenols). Hence, microwave-heat treatment of the whole apples is not an effective method in reducing the loss of polyphenols during the production of apple juice.

The second experimental modification involved the hot water extraction of the apple pomace to extract the polyphenols from the apple pomace as the majority of the polyphenols are retained in the pomace. In the three experimental varieties investigated, a hot water extraction of the apple pomace resulted in a significant increase in the epicatechin and total polyphenol content of the second press juice. The extraction yield of polyphenols was not dependent on the apple variety. A four to eleven fold increase in epicatechin content and a five to nine fold increase in total polyphenol content were observed in the second press juice compared to the first press juice. However a significant loss of 29 – 59 % of epicatechin and 33 – 63 % of total polyphenols was observed between the apples and the first press products and a significant loss of 37 – 56 % of epicatechin and 35 % of total polyphenols was observed between the first press pomace and the second press products. Therefore while a hot water extraction is an efficient method in extracting polyphenols from the pomace, a loss of polyphenols also occurs in the production of the second press products.

A small proportion of the loss is due to procedure losses (e.g. apple pulp remaining in the food processor and apple pomace remaining on the press) in the various processing steps while the majority of the loss is most likely due to enzymatic browning. Therefore in order to try and inhibit enzymatic browning a further modification of the conventional method was conducted. The third experimental modification was to incubate the hot water extracted pomace overnight at 4 ºC rather than at room temperature. In this instance the incubation at 4 ºC was less efficient than incubation at room temperature. The loss of polyphenols between the extraction at room temperature and the extraction at 4 ºC was also similar. Therefore, the incubation at 4 ºC did not reduce the losses of polyphenols from the apple pomace during the incubation period and it was significantly less efficient in extracting epicatechin or total polyphenols from the apple pomace into the second press juice, compared to the incubation period at room temperature.

Investigation in how to reduce polyphenol losses during apple pressing needs to be conducted further. Polyphenol losses during apple processing may be reduced if apple processing is conducted in a controlled atmosphere (reduced oxygen environment) or if the hot water extraction incubation period was reduced to a shorter length of time (e.g. 1 h).

An effective procedure in reducing the loss of epicatechin and other polyphenols from the apples during the production of apple juice and the effective extraction of epicatechin and other polyphenols from the apple pomace is important as the extracted polyphenols can be used for food supplementation. The supplementation of foods or beverages with apple flavanols may have an impact on human health as the intake of apple flavanols and flavonoids have been shown to be inversely associated with CVD mortality (Hertog et al., 1993a; Knekt et al., 1996; Arts et al., 2001a; Mink et al., 2007).

Flavonoids have been shown to have antioxidant activity, inhibit low-density lipoprotein oxidation, inhibit inflammatory responses; inhibit platelet aggregation, and improve endothelial function (Yochum et al., 1999; Boyer and Liu, 2004).

Endothelial dysfunction is defined as the impairment of endothelium-dependent vascular relaxation and is considered to be a risk factor for the development of atherosclerosis. The vascular endothelium is a single layer of cells lining the blood vessels in the body. It is a source of vasodilators (e.g. NO) and vasoconstrictors (e.g. endothelin-1 and angiopoietin-2) that affect vascular tone, growth, platelet function, coagulation and monoycte function (McEniery et al., 2003; Nadar et al., 2004). Normal vascular tone is sustained when the balance between the vasodilators and vasoconstrictors are maintained (Pyke and Tschakovsky, 2005).

Epicatechin and other flavonoids have been shown to improve endothelial function of arterial vessels and to lower blood pressure by increasing the rate of NOS (Schroeter et al., 2006). The effect on the consumption of flavanol-rich foods and beverages on the endothelial function has been previously investigated by FMD (Stein et al., 1999; Agewall et al., 2000; Chou et al., 2001; Duffy et al., 2001; Hodgson et al., 2002; Fisher et al., 2003; Heiss et al., 2003; Engler et al., 2004b; Heiss et al., 2005; Schroeter et al., 2006; Auclair et al., 2010). FMD represents the gold standard for the non-invasive measurement of endothelial function in humans. FMD measures dilation of the vessel in response to altered flow, is expressed as the percentage increase in artery diameter after release of occlusion, is mediated by NO release in response to shear stress, and is used to measure endothelial function (Hashimoto et al., 2003; Kobayashi et al., 2004; Wilson et al., 2004).

The short- (2 h after consumption) and long-term (40 day) consumption of black tea improved brachial artery FMD in subjects with coronary heart disease (Duffy et al.,

2001). In another tea intervention, the consumption of five cups of black tea per day for five weeks improved FMD in hypercholesterolemic subjects (Hodgson et al., 2002). The consumption of purple grape juice for 15 days (Stein et al., 1999) or 56 days (Chou et al., 2001) resulted in an improvement of FMD in subjects with coronary heart disease. The consumption of de-alcoholised red wine also resulted in an improvement in FMD in healthy subjects (Agewall et al., 2000). Consumption of a high flavonoid chocolate or cocoa improved FMD compared to a low flavonoid chocolate (Fisher et al., 2003; Heiss et al., 2003; Engler et al., 2004b; Heiss et al., 2005; Schroeter et al., 2006). Recently the investigation of endothelial function after the consumption of a polyphenol-rich re-hydrated lyophilised apple sample (Auclair et al., 2010). No significant difference was observed in FMD between the low-polyphenol and high-polyphenol phases in the intervention in hypercholesterolemic subjects (Auclair et al., 2010).

Arterial stiffness is another measurement that assesses vascular function. A stiff artery may act as a marker for the development of future atherosclerotic disease or may be a direct factor in the process of atherosclerosis (Woodside et al., 2004). The main method of determining arterial stiffness is by PWV. PWV is calculated from measurements of pulse transit time and the distance travelled by the pulse between two recording sites (e.g. from the carotid artery to the femoral artery) (Asmar et al., 1995). The higher the velocity recorded, the higher the stiffness of the arterial wall (Safar et al., 2002; Woodside et al., 2004). Flavanoid-rich foods have also been found to decrease arterial stiffness (Vlachopoulos et al., 2006b; Vlachopoulos et al., 2007). Consumption of chocolate (Vlachopoulos et al., 2006b) and cocoa (Vlachopoulos et al., 2007) was associated with a significant decrease in arterial stiffness as assessed by carotid-femoral PWV. The chronic consumption of cranberry juice was also associated with a reduction in carotid-femoral PWV compared to the placebo beverage

(Dohadwala et al., 2010). While the consumption of black tea increased arterial stiffness, the consumption of green tea had no effect (Vlachopoulos et al., 2006a).

The human intervention study presented in this chapter assessed vascular function by using PCA. PCA is a non-invasive method that indirectly measures endothelial function and arterial stiffness. The measurements of SI and RI are indirect measurements of arterial stiffness and endothelial function, respectively. A similarity of the relationship between PCA, age and blood pressure and between PWV, age and blood pressure has been described therefore indicating that these two measurements are influenced by similar factors (Millasseau et al., 2002).

The results from the human intervention study described in this chapter show that supplementing a standard apple juice with Evesse™ Fructose (an apple polyphenol-rich extract, epicatechin = 72 mg in Batch 1 and 84 mg in Batch 2) resulted in a significant improvement in the stiffness index as assessed by PCA. A significant difference between the control and treatment phases was observed for the SI ($p = 0.031$) (Figure 3.10). This is in agreement with the evidence in the literature which has shown that the consumption of flavanols causes an improvement in vascular function. A statistically significant difference in VA between the control and treatment phases ($p = 0.005$) was also observed (Figure 3.12). This observation was expected as VA is a derived value calculated from the SI. However, there was no difference between the control and treatment phases in regards to the RI. The RI is an indicator of endothelial function and an increase in RI is due to an increase in the peripheral pulse wave reflection (e.g. vasoconstriction) while a reduction in RI indicates vasodilation (Kneifel et al., 2006; Alty et al., 2007). As PCA is an indirect measure of endothelial function, an effect of the consumption of Evesse™ Fructose may be more observable when using FMD as the measurement. Auclair et al. (2010) recently investigated the FMD response on the consumption of 214 mg polyphenols per day in the low-

polyphenol re-hydrated lyophilised apples phase and 1210 mg polyphenols per day in the high-polyphenol re-hydrate lyophilised apples phase for four weeks in hypercholesterolemic subjects. The FMD measured at the end of each intervention did not differ between the high-polyphenol and low-polyphenol apples (Auclair et al., 2010). There was also no difference in baseline measurements. However, they did not measure the short-term effect of apple polyphenol consumption. Therefore further investigation on the acute consumption of polyphenol-rich extract should be assessed by FMD. Further human intervention trials should investigate the consumption of apple polyphenols between young and old subjects; compromised and healthy subjects; and the effect of chronic and acute consumption of apple polyphenols.

3.7 Conclusions

The results from this chapter have shown that (1) a microwave-heat treatment of whole apples was not effective in increasing the yield of polyphenols in the first press juice or the recovery in the first press pomace; (2) a hot water extraction of the apple pomace was a very effective method of extracting the polyphenols retained from the first press and yielded a polyphenol-rich aqueous extract; (3) the incubation of hot water extracted apple pomace at room temperature compared to 4 ºC was more effective in extracting the polyphenols retained in the apple pomace, although similar losses of polyphenols were observed at both temperatures; and (4) consumption of an apple polyphenol-rich extract supplemented apple juice by human subjects resulted in a small but statistically significant improvement in arterial stiffness and vascular age but not in reflective index (endothelial function) compared to the low polyphenol apple juice.

Therefore future studies should examine alternative strategies aimed at decreasing the losses of polyphenols during the production of apple juice (the pressing of apples and the extraction of the apple pomace) as well as measuring the acute effect of the

consumption of a polyphenol-rich apple extract on endothelial function as assessed by flow-mediated dilation.

CHAPTER FOUR

APPLE FLAVANOLS AND VEGF SIGNALLING IN HUMAN ENDOTHELIAL CELLS

Chapter 4 : Apple flavanols and VEGF signalling in human endothelial cells

4.1 Abstract

Previous work showed that when human umbilical vein endothelial cells (HUVECs) were treated with an apple procyanidin fraction degree of polymerisation (dp) 3.9 there were more than 1000 genes that had their expression levels significantly altered in both resting and cytokine-stimulated cells (García-Conesa et al., 2009). The apple procyanidin fraction dp 3.9 significantly altered the expression levels of several genes that are associated with key angiogenesis related functions in HUVECs. Vascular endothelial growth factor (VEGF) is the most important pro-angiogenic growth factor. Therefore it was hypothesised that apple procyanidin fraction dp 3.9 regulated key angiogenesis related genes by inhibiting the VEGF pathway. The overall aim was to investigate the ability of apple procyanidins and other polyphenols to inhibit VEGF signalling. It was shown that VEGF-induced VEGF receptor-2 (VEGFR-2) phosphorylation was inhibited almost instantly once the polyphenol and VEGF were mixed together and complete inhibition was observed after 5 min of incubation. It was also demonstrated that the inhibitory effect of the polyphenols was due to the polyphenol interacting with the VEGF molecule. An investigation of different polyphenol structures showed that the inhibition of phosphorylation was dependent on key structural features (epicatechin polymers with a degree of polymerisation greater than three; 3-galloylation; and a trihydroxy B-ring). The polyphenol was also able to inhibit VEGFR-2 phosphorylation at physiological concentrations. These findings are significant because they provide a plausible link between consumption of polyphenols and a reduced risk of cardiovascular disease (CVD). While this research has only shown an interaction with VEGF, it raises the possibility that polyphenols can affect

other signalling pathways by interacting with other signalling peptides including growth factors and hormones.

4.2 Introduction

Oligomeric procyanidins have been shown to have cardioprotective attributes (Kenny et al., 2004b; Actis-Goretta et al., 2006; Corder et al., 2006; Ottaviani et al., 2006). In a microarray study investigating the effects of (-)-epicatechin, procyanidin dimer B2 and an apple procyanidin fraction with a mean degree of polymerization of 3.9 (dp 3.9) in HUVECs, it was found that there were no significant gene expression changes in resting or cytokine stimulated cells in response to epicatechin and procyanidin dimer B2 treatments (García-Conesa et al., 2009). However, more than 1000 genes had their expression levels significantly altered in both resting and cytokine-stimulated (tumour necrosis factor-alpha [TNF-α]) cells in response to the apple procyanidin fraction dp 3.9 treatment. The main molecular functions that were influenced by apple procyanidin fraction dp 3.9 were cell death, cell growth and proliferation, cell migration, cell signalling and cell development. Cell growth and proliferation and cell migration are the main molecular functions involved in angiogenesis. The authors concluded that apple procyanidin fraction dp 3.9 significantly changed the expression levels of several genes that are associated with key angiogenesis-related functions in HUVECs (García-Conesa et al., 2009).

Angiogenesis, the formation of new blood vessels from pre-existing ones, has been proven to play an essential role in the pathogenesis of diverse chronic diseases. In cardiovascular disease, it has been suggested that angiogenesis plays an important part in the development and destabilization of atherosclerotic plaques. The role of angiogenesis in cardiovascular disease functions in two different manners. It can either (1) be a causative factor of atherosclerotic plaque formation or (2) it can be a therapeutic way to treat ischemic heart disease (Khurana et al., 2005; Stangl et al., 2007; Ribatti et al., 2008). In the first instance pro-angiogenic growth factors can either promote atherosclerosis and cause plaque growth and destabilization with the plaques

more likely to rupture and cause myocardial infarction or stroke (Tenaglia et al., 1998; Moulton et al., 1999; Celletti et al., 2001; Moreno et al., 2004; Khurana et al., 2005; Holm et al., 2008). In the second situation pro-angiogenic growth factors can be used as a therapeutic factor in ischemia by restoring blood flow through the stimulation of new blood vessel formation (Ware and Simons, 1997; Khurana et al., 2005).

Whether the role of angiogenesis is involved as the causative factor in the destabilization of atherosclerotic plaques or the therapeutic factor in ischemia, it is an important biological process under normal and disease conditions. During embryonic development, any impairment in angiogenic events results in early mortality due to growth retardation (Dulak, 2005). After birth, angiogenesis contributes to organ growth but in adulthood there is a lack of physiological angiogenesis, with the exception of menstrual cycles and pregnancy in females and wound healing and repair (Mojzis et al., 2008). In established blood vessels endothelial cells remain in a dormant, non-proliferative state but they retain their ability to rapidly divide in response to physical stimuli (wounding and inflammation) or other pathological conditions that stimulate angiogenesis (Ware and Simons, 1997). Angiogenesis occurs when there is an imbalance between the pro-angiogenic (e.g. vascular endothelial growth factor [VEGF], basic fibroblast growth factor and TNF-α) and anti-angiogenic (e.g. angiostatin and endostatin) factors. The most important pro-angiogenic factor is VEGF (Giles, 2001; Dulak, 2005; Cebe-Suarez et al., 2006).

4.2.1 Vascular endothelial growth factor

VEGF is a dimeric cysteine-linked secreted glycoprotein of approximately 40 kDa and is characterised by the intrachain and interchain disulfide bonds between eight conserved cysteine residues (Giles, 2001; Matsumoto and Claesson-Welsh, 2001; Cebe-Suarez et al., 2006; Ng et al., 2006; Olsson et al., 2006). The structure of VEGF

is organised so that the two monomers are positioned in an anti-parallel fashion, with the receptor-binding sites located at each pole of the dimer (Muller et al., 1997). The VEGF family consists of five members: VEGF-A, -B, -C and –D and placental growth factor. VEGF-A is the predominant form and is commonly referred to as VEGF.

The gene encoding VEGF, in humans, is located on the short arm of chromosome 6 (6p21.1) (Ng et al., 2006). VEGF gene expression is up-regulated by hypoxia, growth factors (such as epidermal growth factor, TNF-α, transforming growth factor-beta, keratinocyte growth factor, insulin-like growth factor-1, fibroblast growth factor 4 and platelet derived growth factor [PDGF]) and inflammatory cytokines (interleukin-1 and interleukin-6) (Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999; Cebe-Suarez et al., 2006). The VEGF gene comprises of eight exons, and different isoforms of VEGF with different biological activities are produced by alternative exon splicing (Ferrara and Davis-Smyth, 1997; Giles, 2001; Pan et al., 2002; Ng et al., 2006). The predominant isoforms of VEGF are $VEGF_{121}$, $_{145}$, $_{165}$, $_{189}$ and $_{206}$. All VEGF isoforms contain exons 1 to 5, with various combinations of the remaining exons 6 to 8. Exons 3 and 4 contain the VEGF receptor (VEGFR)-1 and VEGFR-2 binding domains, respectively, therefore all isoforms should be able to bind to these receptors and are predicted to be biologically active (Ng et al., 2006). $VEGF_{165}$ is the predominant form of VEGF and is characterised by its heparin binding ability. VEGF regulates cell functions including mitogenesis, permeability, vascular tone and the production of vasoactive molecules by binding to specific endothelial cell surface receptors (VEGF receptors).

4.2.2 Vascular endothelial growth factor receptors

VEGF stimulates cellular responses by binding to type III receptor tyrosine kinases (VEGFRs) on the cell surface and causes the receptors to dimerize and become activated through transphosphorylation. There are three main VEGFRs: VEGFR-1

(Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). The VEGFRs consist of a 750 amino acid extracellular matrix, a single transmembrane region, an intracellular tyrosine kinase domain that is interrupted by a kinase insert domain, and a C-terminal tail (Figure 4.1) (Cebe-Suarez et al., 2006; Olsson et al., 2006). The extracellular immunoglobulin (Ig)-like domains two and three are required for binding of VEGF to VEGFR-1 and -2 and the fourth Ig-like loop contains a receptor dimerization domain (Matsumoto and Claesson-Welsh, 2001; Cebe-Suarez et al., 2006). Similar to VEGF, the expression of VEGFR-1 and -2 are also up-regulated in response to hypoxia (Neufeld et al., 1999; Matsumoto and Claesson-Welsh, 2001).

The VEGF receptors are expressed primarily on vascular endothelial cells, but are also expressed on vascular smooth muscle cells, hematopoietic cells, macrophages, and on some malignant cells (Cebe-Suarez et al., 2006; Ng et al., 2006). VEGFR-1 induces organizational effects on the vasculature and is crucial in embryonic angiogenesis but does not appear to be critical in pathogenic angiogenesis (Pan et al., 2002). VEGFR-3 is important for lymphatic cell development and function while VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF (Giles, 2001; Ferrara and Kerbel, 2005).

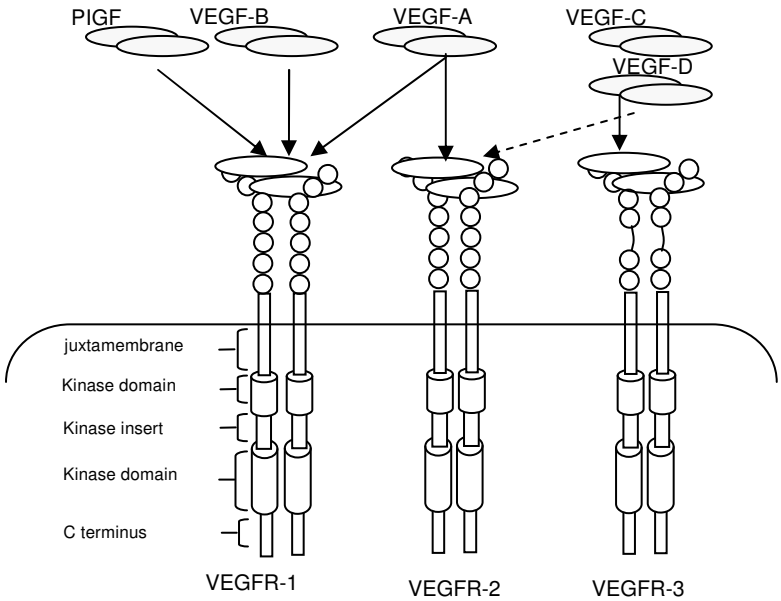


Figure 4.1: VEGF receptor binding properties.

VEGFRs have an approximate 750 amino acid residue extracellular domain, which in VEGFR-1 and -2 is organised into seven immunoglobulin (Ig)-like loops. In VEGFR-3, the fifth Ig domain is replaced by a disulfide bridge. The extracellular domain is followed by a juxtamembrane domain, a tyrosine kinase domain which is interrupted by a 70 amino acid kinase insert and a C-terminal tail (Cebe-Suarez et al., 2006). The different forms of VEGF bind to specific receptors: VEGF-A binds to VEGFR-1 and -2, VEGF-C and –D binds to VEGFR-2 and -3 and PIGF and VEGF-B binds to VEGFR-1.

VEGFR-2 is the major signalling receptor for VEGF. VEGFR-2 is a 200 kDa glycoprotein which is expressed in hematopoietic, neural and retinal cells. VEGFR-2 expression is up-regulated in response to hypoxia and down-regulated and de-phosphorylated through internalization and degradation of the receptor by protein-kinase C (PKC)-dependent phosphorylation of the receptor C-terminal tail (Cebe-Suarez et al., 2006; Rahimi, 2006). There are 19 tyrosine residues present in the intracellular domain of VEGFR-2 and the most prominent phosphorylation sites are tyrosine resides (Tyr) 951, 1054, 1059, 1175 and 1214 (Figure 4.2). Tyr1175 is the most important phosphorylation site as it is implicated in several of the roles of VEGFR-2, including the activation of cell proliferation, cell migration, cell survival, vascular permeability, actin remodelling and gene expression. Other important phosphorylation sites are Tyr951 and Tyr1214. Phosphorylated Tyr951 regulates

vascular permeability and cell migration and phosphorylation of Tyr1214 has been implicated in cell migration, actin remodelling and vascular permeability.

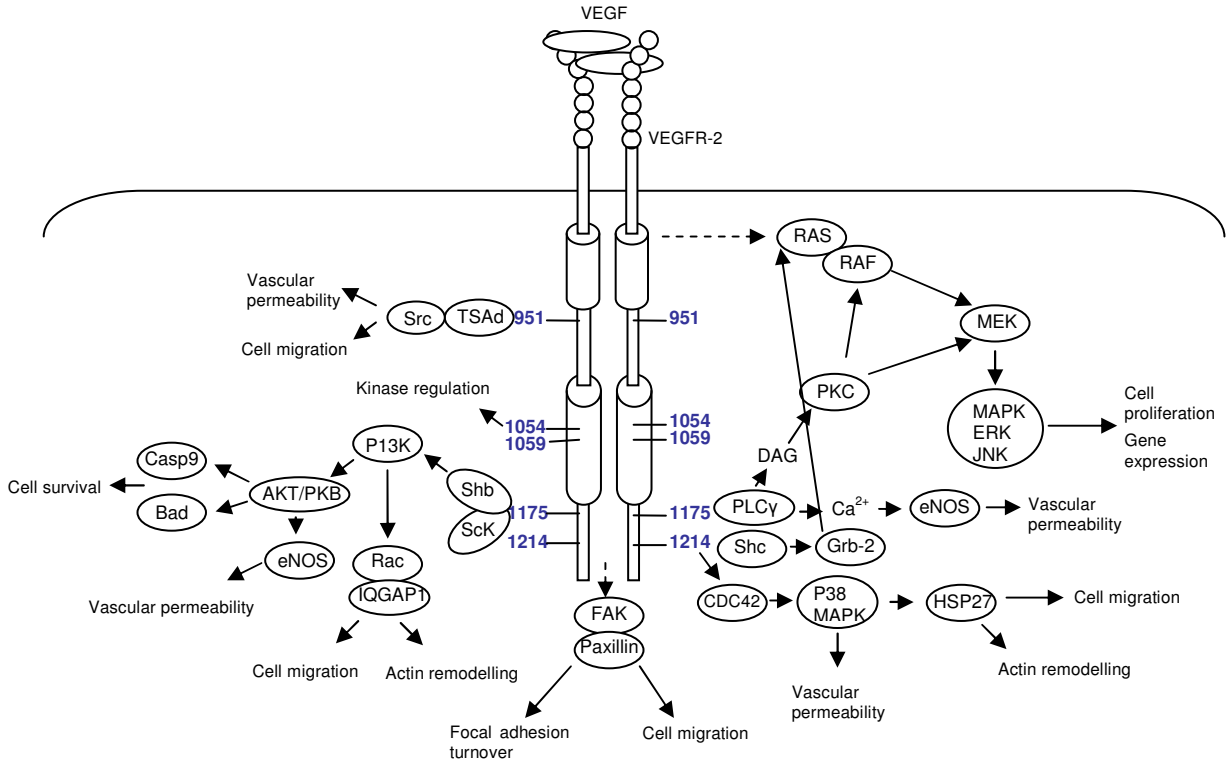


Figure 4.2: VEGFR-2 phosphorylation sites and signalling pathways.

Binding of signalling molecules (Shb, Sck, phospholipase C-γ [PLC-γ], Shc and TSad) to certain tyrosine phosphorylation sites initiates signalling cascades which lead to specific biological responses.

4.2.3 Vascular endothelial growth factor receptor-2 inhibition

Inhibition of angiogenesis has been determined to be an effective strategy in the treatment of chronic diseases, in particular cancer. Therapeutics that function to inhibit the VEGF pathway have been used to target certain types of cancers. Anti-VEGF antibodies, aptamers (peptides that act like anti-VEGF antibodies) and small molecule VEGFR tyrosine kinase inhibitors have been produced and are available for the treatment of colon, lung, breast, kidney and liver cancer and neovascular age-related macular degeneration (Giles, 2001; Ferrara and Kerbel, 2005). These different treatments all work by inhibiting the VEGF signalling pathway.

Various food components also have been found to have an effect on and influence phosphorylation of VEGFR-2. Green tea catechins (with epigallocatechin gallate being the most potent) inhibited VEGFR-2 phosphorylation in a dose- and time-dependent manner as well as reducing VEGF-induced cell migration and proliferation (Kondo et al., 2002b; Lamy et al., 2002; Tang et al., 2003; Neuhaus et al., 2004; Mojzis et al., 2008). Red wine polyphenolic compounds were also able to inhibit proliferation and migration of endothelial and vascular smooth muscle cells as well as inhibiting VEGFR-2 phosphorylation (Mojzis et al., 2008). Ellagic acid completely inhibited VEGF-induced VEGFR-2 phosphorylation in a time dependent manner (Labrecque et al., 2005). The anthocyanin delphinidin inhibited VEGF-induced phosphorylation of VEGFR-2 in a dose- and time-dependent manner (Lamy et al., 2006). A grape seed extract constituting at least 85 % w/w procyanidins (Wen et al., 2008) and purified procyanidin trimer and tetramer from a cinnamon extract (Lu et al., 2010) were also found to inhibit VEGFR-2 signalling in a dose-dependent manner, however, the mechanisms of this effect was not investigated. The aim of the present study was thus to investigate the inhibitory action of certain polyphenols on VEGFR-2 phosphorylation.

4.3 Objectives

The overall aim of this study was to investigate the ability of apple procyanidins and other polyphenols to inhibit VEGF-induced VEGFR-2 signalling and the underlying mechanism(s) behind the inhibitory actions. The specific objectives were to determine: (1) if apple flavanols and other phenolics have an inhibitory effect on the VEGF pathway; (2) if the apple procyanidin fraction causes the inhibitory action through interacting with the VEGF molecule or its receptors; and (3) to confirm inhibition of downstream signalling pathways in response to the polyphenol treatment.

4.4 Materials and methods

4.4.1 Materials

Human recombinant $VEGF_{165}$ was obtained from R&D Systems Europe Ltd (Abingdon, UK). Purified (-)-epicatechin and (+)-catechin and procyanidin dimer B2 were purchased from Sigma-Aldrich and epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), methyl gallate and gallic acid were purchased from Extrasynthese (Genay, France). Electrophoresis reagents were purchased from Invitrogen. The VEGFR-2, phospho-VEGFR-2 (Tyr 1175), PLCγ1, phospho-PLCγ1 (Tyr 783), AKT and phospho-AKT (Ser 473) monoclonal antibodies, the anti-rabbit IgG, HRP-linked antibody and the PathScan phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit were from Cell Signaling. The apple procyanidin fraction dp 3.9 was purified from a cider apple (*Malus domestica*, variety Antoniette) as previously described (Gossé et al., 2005).

The apple procyanidin fractions dp 3 and dp 4 were purified from the 2007 harvest apples (Chapter 3) provided by Coressence Ltd (Herefordshire, UK) as previously described (Yanagida et al., 1999; Shoji et al., 2005; Shoji et al., 2006b), with some modifications. Briefly 50 g of freeze-dried apple powder was extracted in 1250 ml of 0.1 % potassium disulphite, shaken and left to stand for 10 min. The sample was centrifuged and passed through a Sepabeads SP-850 (Sigma) column (355 mm x 25 mm). The polyphenol fraction was eluted with 200 ml of 50 % (v/v) ethanol and concentrated, to approximately 60 ml, by rotary evaporation at 45 °C. The polyphenol fraction was adjusted to pH 6.5 with 1N sodium hydroxide and was passed through a Diaion HP-20ss (Sigma) column (355 mm x 25 mm). The column was rinsed with distilled water and the procyanidin fraction was eluted with 700 ml of 25 % (v/v) ethanol and concentrated by rotary evaporation. The fraction was reconstituted in 1 ml

of methanol and the remaining quercetin-glycosides, chlorogenic acid and phloridzin compounds in the fraction were removed by passing the fraction through a 50 g Biotage Snap FLASH Purification Cartridge HP-Sil (Biotage) on a Medium Pressure Liquid Chromatography (MPLC) (Gilson). The flow rate was set to 45 ml/min and the method used mobile phases consisting of 82 % dichloromethane, 14 % methanol and 4 % of 50 % (v/v) acetic acid (solvent A) and 96 % methanol and 4 % of 50 % (v/v) acetic acid (solvent B) with a 120 min gradient (0 – 10 min, 0 % B; 10 – 15 min, 18 % B; 15 – 18 min, 30 % B; 18 – 20 min 88 % B; 20 – 120 min, 0 % B). Procyanidins were fractionated according to the degree of polymerization by performing normal-phase chromatography using an Inertsil PREP-Sil (30 mm i.d. x 250 mm) column (GL Sciences Inc) on the preparatory-HPLC (Gilson). The flow rate was 24 ml/min and the method used a mobile phase containing hexane/methanol/ethyl acetate in a 8:3:1 ratio (solvent A) and hexane/methanol/ethyl acetate in a 2:3:1 ratio (solvent B) with a 185 min gradient (0 – 35 min, 0 % B; 35 – 185 min, 100 % B) and was monitored at 280 nm with a diode array detector (DAD). A more detailed description of the method is located in the Supplementary Information (Supplementary Information 1: Purification of apple procyanidin fractions dp 3 and dp 4).

4.4.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) (Cambrex Bio Science, Wokingham, UK) were maintained in Endothelial Cell Growth Medium-2 (EGM-2) bullet kit (basal medium plus SingleQuot kit [foetal bovine serum (FBS); hydrocortisone; ascorbic acid; heparin; gentamicin; VEGF; human fibroblast growth factor; human insulin-like growth factor; human epidermal growth factor]) (Lonza-Cambrex). The cells were cultured at 37 ºC under a humidified atmosphere containing 5 % CO_2. For experimental purposes, cells were used at passage 4 (population doubling 6) and were seeded onto six-well plates at a density of 3500 cells/cm^2. Cells were grown to confluence before serum

starvation and removal of growth factors (serum and growth factor free medium referred to as basal medium).

4.4.3 Flavanol treatments

Flavanol treatments were prepared in Universal tubes prior to adding them to the confluent monolayers. Appropriate concentrations of flavanols (apple procyanidin fractions dp 3.9, dp 3 and dp 4, (-)-epicatechin, catechin, procyanidin dimer B2, EGCG, EGC, ECG, methyl gallate or ellagic acid, structures of these polyphenols are described in Figure 4.18) were added to a Universal tube containing basal medium and VEGF (10 ng/ml or 25 ng/ml) and incubated at room temperature for 5 min. Control treatments were prepared in the same manner. HUVEC confluent monolayers were washed twice with warm phosphate buffered saline (PBS), to remove any residual growth factors, and prepared treatments were added to the respective wells and incubated for 5 min at 37 ºC under a humidified atmosphere containing 5 % CO_2 (Figure 4.3 A). All treatments were prepared in this manner unless otherwise stated.

Two additional experimental procedures were tested in order to determine where the polyphenol is exerting its inhibitory effect. In the first experimental design modification (modification 1), confluent HUVEC monolayers were pre-treated with the polyphenol for 4 h, then the medium was aspirated, the cells were washed with basal medium, and finally they were stimulated with VEGF (Figure 4.3 B). In the second experimental modification (modification 2), HUVEC monolayers were pre-stimulated with VEGF for 5 min and then the VEGF-supplemented medium was treated with the polyphenol (Figure 4.3 C).

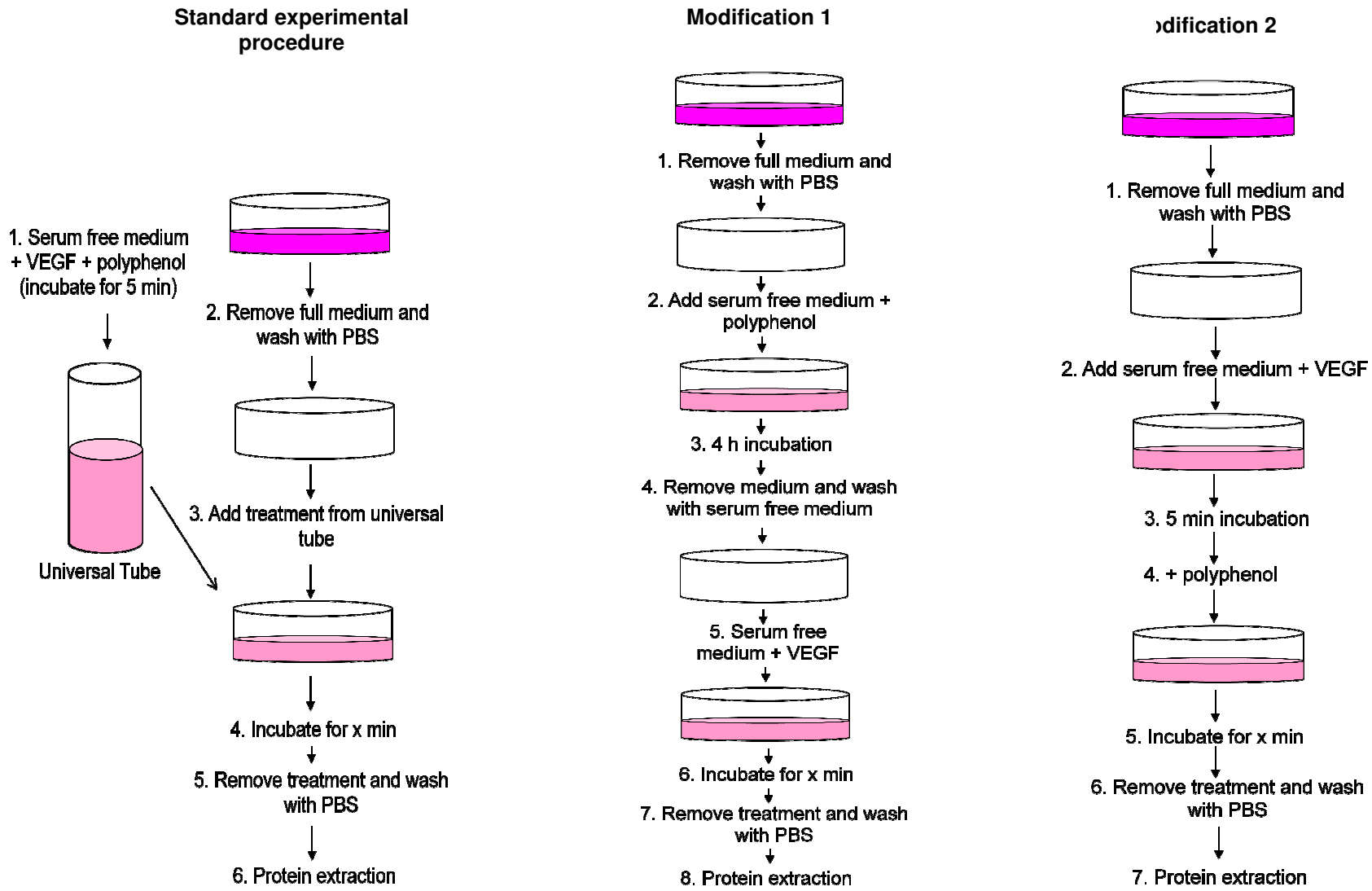


Figure 4.3: Cell culture experimental procedures

4.4.4 Migration wound assay

Confluent HUVEC monolayers were washed with PBS and scraped once horizontally with a sterile 200 µl pipette tip. The wells were washed with PBS for a second time in order to remove the dislodged cells and debris. Fresh medium with or without the treatments were added to the wells to initiate the experiment (treatments as prepared in Section 4.4.3). Randomly selected views along the scraped line were photographed on each well, using a phase contrast inverted microscope and a CCD camera attached to the microscope. Photographs were taken after 0, 4, 24 and 48 h of incubation. The change in the area of the experimental condition was compared, visually, with that of the corresponding control.

4.4.5 Flavanol treatment and VEGF stimulation in the presence of other growth factors

HUVEC monolayers were treated with apple procyanidin fraction dp 3.9 (1 µM) or dp 4 (1 µM) in three different types of medium. Full medium (EGM-2 bullet kit; FBS; hydrocortisone; ascorbic acid; heparin; gentamicin; VEGF; human fibroblast growth factor; human insulin-like growth factor; and human epidermal growth factor); basal medium (EGM-2 plus gentamicin provided in the kit), and basal medium plus bovine serum albumin (BSA) (1 mg/ml of BSA). The stock medium and the basal medium plus BSA were also supplemented with 10 ng/ml VEGF.

4.4.6 Protein extraction

After polyphenol treatment and VEGF stimulation, HUVECs were harvested by using radioimmunoprecipitation assay (RIPA) buffer (50 µl) and scraping the wells with a cell scraper. The cells were washed twice with cold PBS and the protein was extracted using ice cold RIPA buffer (50 µl of 50 mM Tris-Hcl, pH7.4; 150 mM NaCl; 1 % Triton

X-100; 0.5 % sodium deoxycholate; 0.2 % SDS; 200 mM sodium orthovanadate; plus one Roche complete protease inhibitor cocktail tablet, one Roche PhosStop phosphatase inhibitor cocktail tablet and Benzonase [Novagen]). The plates were left on ice for two min to allow the cells to detach. The cells were collected into an Eppendorf and vortexed three times over a 15 min period. Centrifugation was carried out at 13000 x g for 10 min and the supernatant was collected and the pellet discarded. Protein levels, from the supernatant of each lysate, were quantified using a Bicinchoninic Acid Kit (Sigma) according to the manufacturer's instructions.

4.4.7 VEGFR-2 phosphorylation measurement

Phosphorylated VEGFR-2 expression was detected using Western blots or quantified using enzyme-linked immunosorbent assay (ELISA). Phosphorylated VEGFR-2 expression levels were measured by ELISA following the manufacturer's instructions. After the incubation, washing and labelling steps, absorption was measured at 450 nm using a Dynex 96 well microplate reader.

Phosphorylation of VEGFR-2 was also assessed by Western blot. Proteins (30 µg) were reduced using DL-Dithiothreitol (DTT) (Sigma), denatured at 70 ºC for 10 min and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated using NuPAGE Novex 10 % Bis-Tris gels (Invitrogen) in combination with NuPAGE MOPS SDS Running Buffer (Invitrogen) and NuPAGE antioxidant (Invitrogen). The gel was then transferred on to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories). Equal protein loading was confirmed by ponceau red (Sigma-Aldrich) and by either total VEGFR-2, AKT or PLCγ1. Membranes were blocked with 5 % skimmed milk in TBST (50 mM Tris, pH 7.4; 200 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. The membrane was washed with TBST and followed by an overnight incubation with the primary antibody (5 µl of phospho-

VEGFR-2 (Tyr1175) antibody, VEGFR-2, phospho-AKT (Ser 473), AKT, phospho-PLCγ1 (Tyr 783) or PLCγ1 in 5 ml of 5 % BSA in TBST) at 4 ºC. The membrane was washed with TBST and incubated with the secondary antibody (10 µl anti-rabbit IgG, HRP-linked antibody in 10 ml of 5 % skimmed milk in TBST) for 1 h at room temperature. The membranes were developed using Pierce SuperSignal West Pico chemiluminescent substrate (Pierce) and the fluorescence intensity was measured using a BioRad Fluor-S MultiImager. For re-probing of the membranes, the previous antibodies were stripped for 45 min at room temperature using Restore™ Western blot stripping buffer (Pierce).

4.4.8 Statistical analysis

Where indicated, comparisons between mean values from control and treated samples were carried out using a two tailed t-test. A p value < 0.05 was considered significant.

4.5 Results

4.5.1 Apple procyanidin fraction dp 3.9 inhibits endothelial cell migration

Exposure of HUVECs to 8.9 µM apple procyanidin fraction dp 3.9 was shown to inhibit cell migration in comparison to control cells after 24 and 48 h (García-Conesa et al., 2009). However, the data presented in this chapter expand on the García-Conesa et al. (2009) paper by investigating whether the apple procyanidin fraction dp 3.9, epicatechin and procyanidin dimer B2 are able to inhibit cell migration in the presence of 10 ng/ml VEGF.

The most important pro-angiogenic growth factor in vascular endothelial cells is VEGF. Therefore the effect of flavanols on vascular endothelial cell migration was assessed

using a wound healing assay. The effect of flavanols in endothelial cell migration was investigated in two types of medium: full medium and VEGF-supplemented basal medium. The data presented for the full medium migration wound healing assay showed that after a 48 h period, the wound in the control-treated HUVECs was no longer visible and had completely healed (Figure 4.4 – 4.6). The 8.9 µM apple procyanidin fraction dp 3.9 treatment was able to inhibit endothelial cell migration (Figure 4.4) while the 10 µM epicatechin (Figure 4.5) and 10 µM procyanidin dimer B2 (Figure 4.6) treatments were ineffective in inhibiting endothelial cell migration. Based on visual observations, the morphology of the cells was also changed in response to the apple procyanidin fraction dp 3.9 treatment. In the full medium cells, the apple procyanidin fraction dp 3.9 treated cells were more elongated and rod-shaped compared to the rounded-shaped control cells (Figure 4.4).

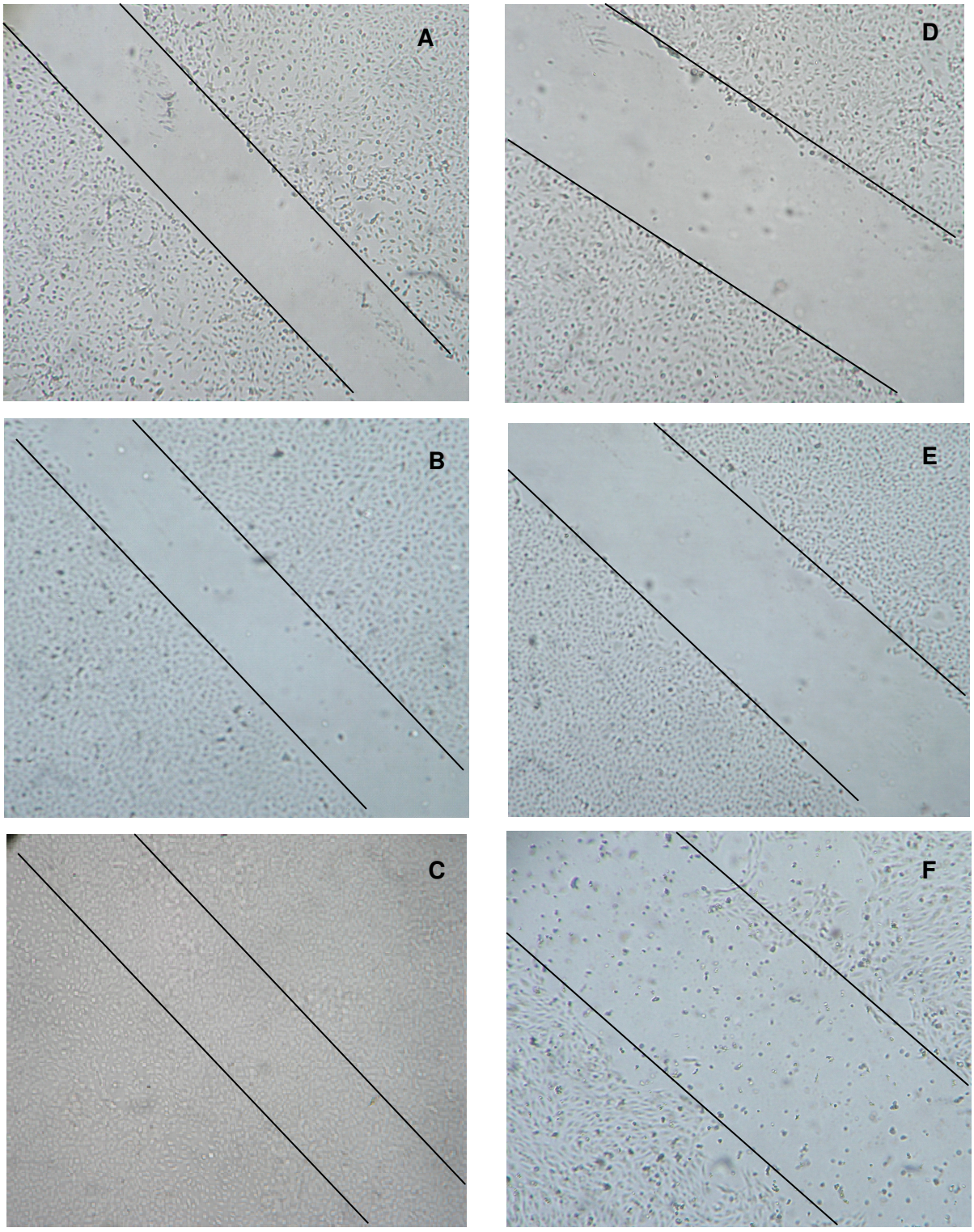


Figure 4.4: Effect of apple procyanidin fraction dp 3.9 on HUVEC migration in full medium examined by migration wound healing assay.

The confluent HUVEC monolayers were wounded at time 0 and cultured with 8.9 µM dp 3.9 in EGM-2 medium (full medium). Photographs of the full medium control **(A, B, C)** and apple procyanidin fraction dp 3.9 **(D, E, F)** treated cells were taken after 0, 4, and 48 h, (top, middle and bottom, respectively). This data are representative of triplicate analysis of three independent experiments.

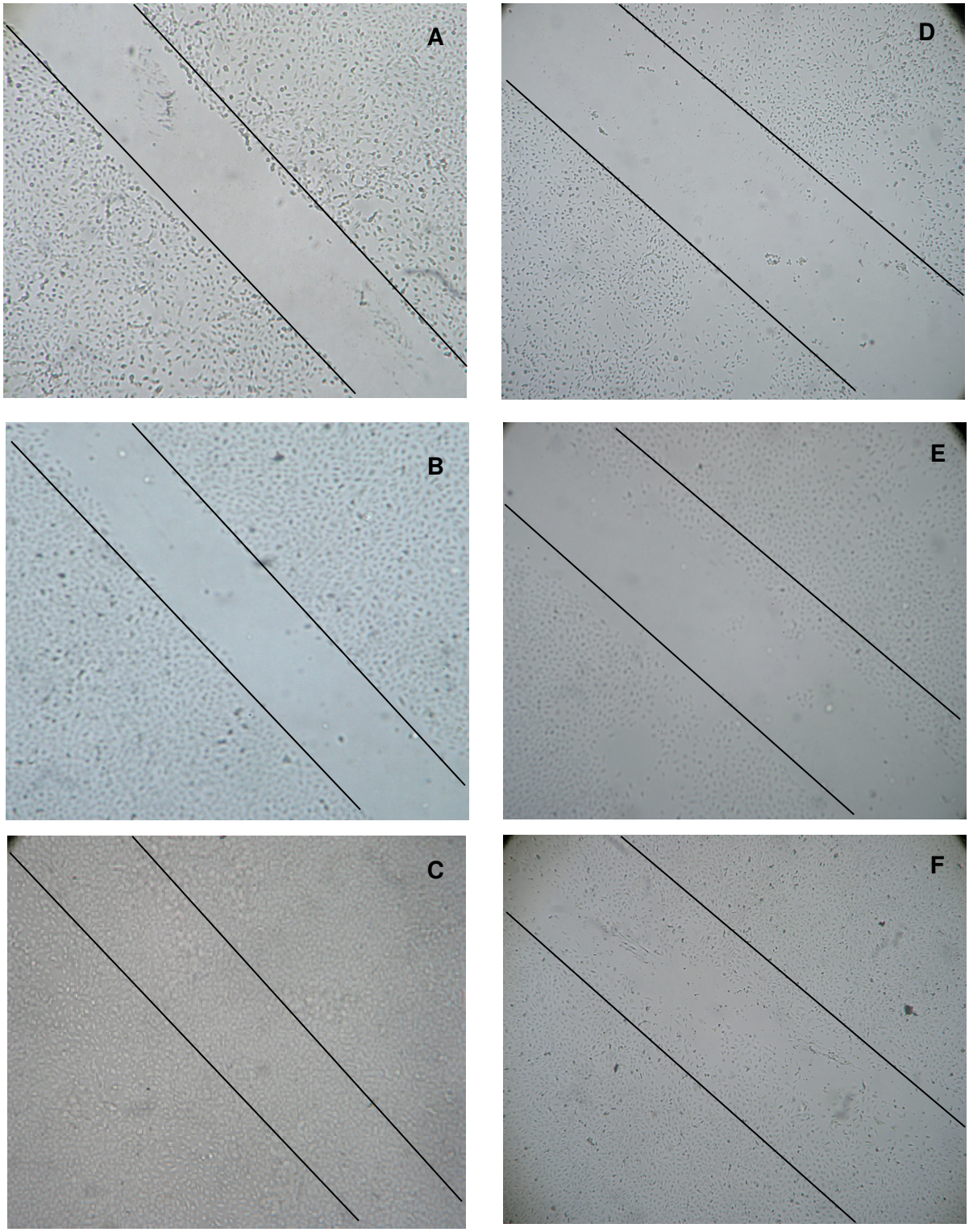


Figure 4.5: Effect of epicatechin on HUVEC migration examined in full medium by migration wound healing assay.

The confluent HUVEC monolayers were wounded at time 0 and cultured with 10 µM epicatechin in EGM-2 medium (full medium). Photographs of the full medium control **(A, B, C)** and epicatechin **(D, E, F)** treated cells were taken after 0, 4, and 48 h (top, middle and bottom, respectively. This data are representative of triplicate analysis of two independent experiments.

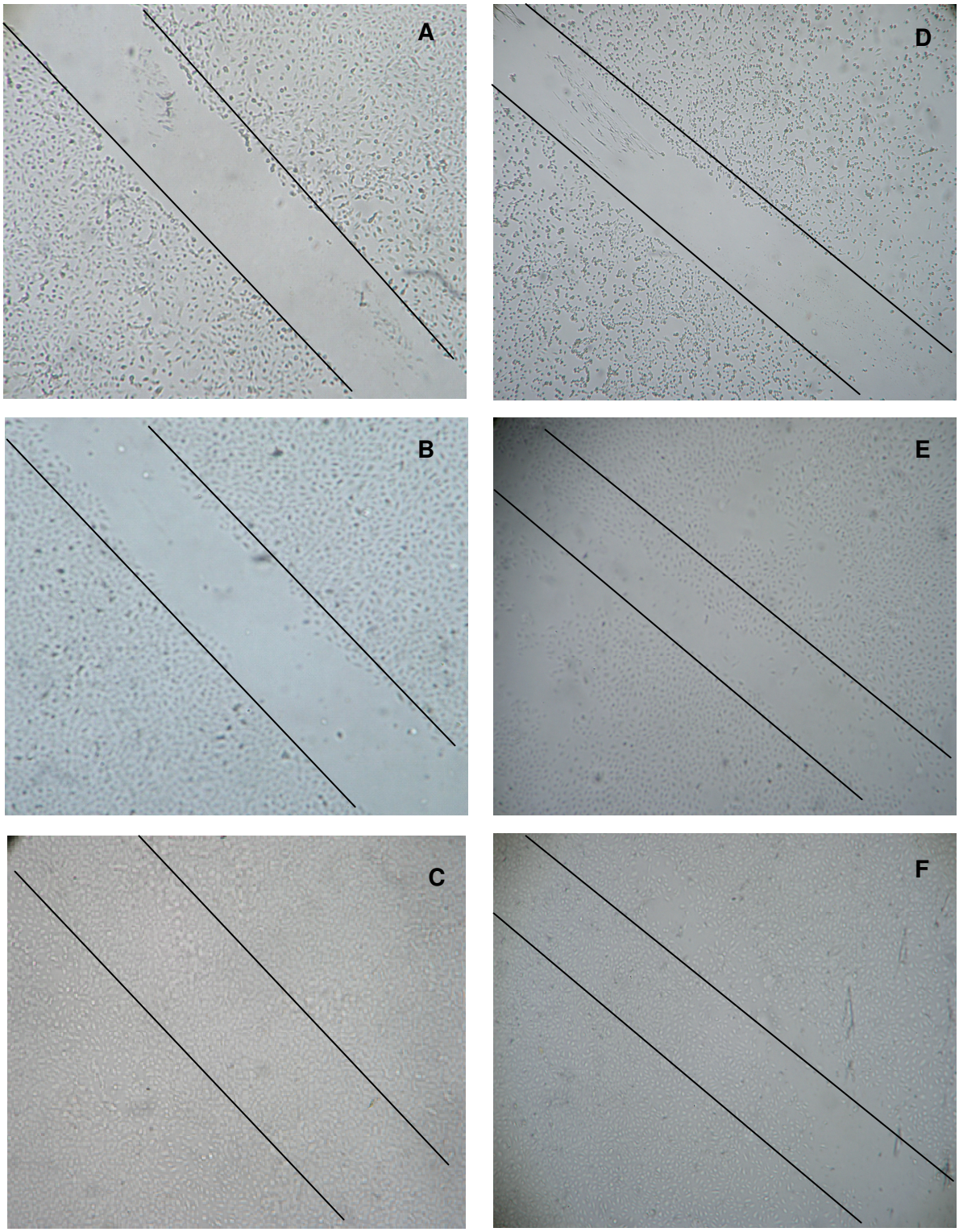


Figure 4.6: Effect of procyanidin dimer B2 on HUVEC migration in full medium examined by migration wound healing assay.

The confluent HUVEC monolayers were wounded at time 0 and cultured with 10 µM procyanidin dimer B2 in EGM-2 medium (full medium). Photographs of the full medium control **(A, B, C)**, and procyanidin dimer B2 **(D, E, F)** treated cells were taken after 0, 4, and 48 h (top, middle and bottom, respectively). This data are representative of triplicate analysis of two independent experiments.

The effect of endothelial cell migration was also investigated in VEGF-supplemented basal medium. The wound in the control-treated HUVECs was no longer visible in the VEGF-supplemented basal medium (Figure 4.7 – 4.9), while the 8.9 µM apple procyanidin fraction dp 3.9 treated cells was able to completely inhibit endothelial cell migration (Figure 4.7). Treatment of 10 µM epicatechin (Figure 4.8) and 10 µM procyanidin dimer B2 (Figure 4.9) were less effective in inhibiting endothelial cell migration. Based on visual observations, the morphology of the cells was also changed in response to the apple procyanidin fraction dp 3.9 treatment. In VEGF-supplemented basal medium, the apple procyanidin fraction dp 3.9 treated cells were rounded with a lot of detached floating cells in the medium, compared to the elongated and rod-shaped cells in the control treated cells (Figure 4.7).

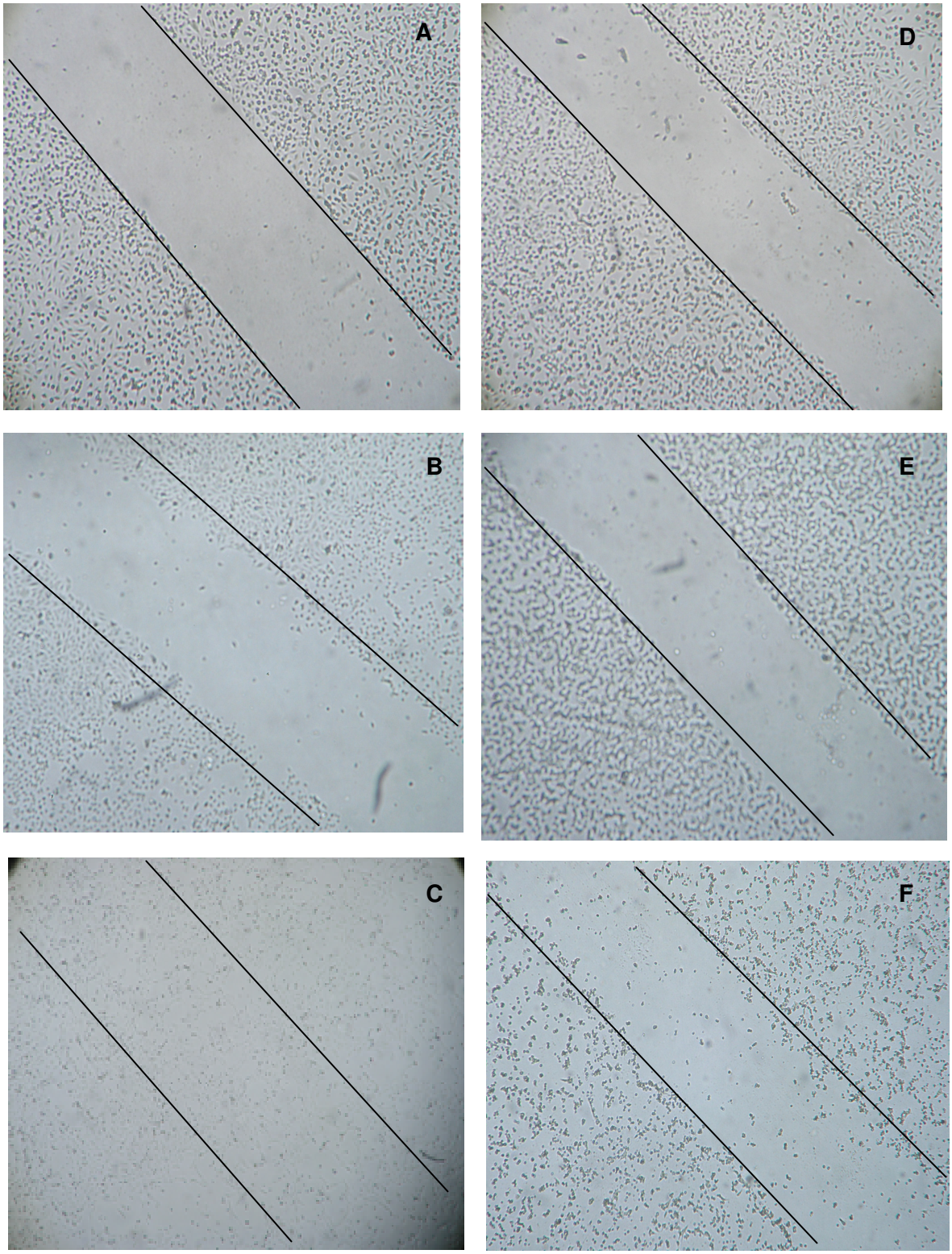


Figure 4.7: Effect of apple procyanidin fraction dp 3.9 on HUVEC migration in VEGF-supplemented medium examined by migration wound assay.

The confluent HUVEC monolayers were wounded at time 0 and cultured with 8.9 µM apple procyanidin fraction dp 3.9 in basal medium plus 10 ng/ml VEGF. Photographs of the VEGF-supplemented basal medium control **(A, B, C)** and apple procyanidin fraction dp 3.9 **(D, E, F)** treated cells were taken after 0, 4, and 48 h (top, middle and bottom, respectively). This data are representative of triplicate analysis of three independent experiments.

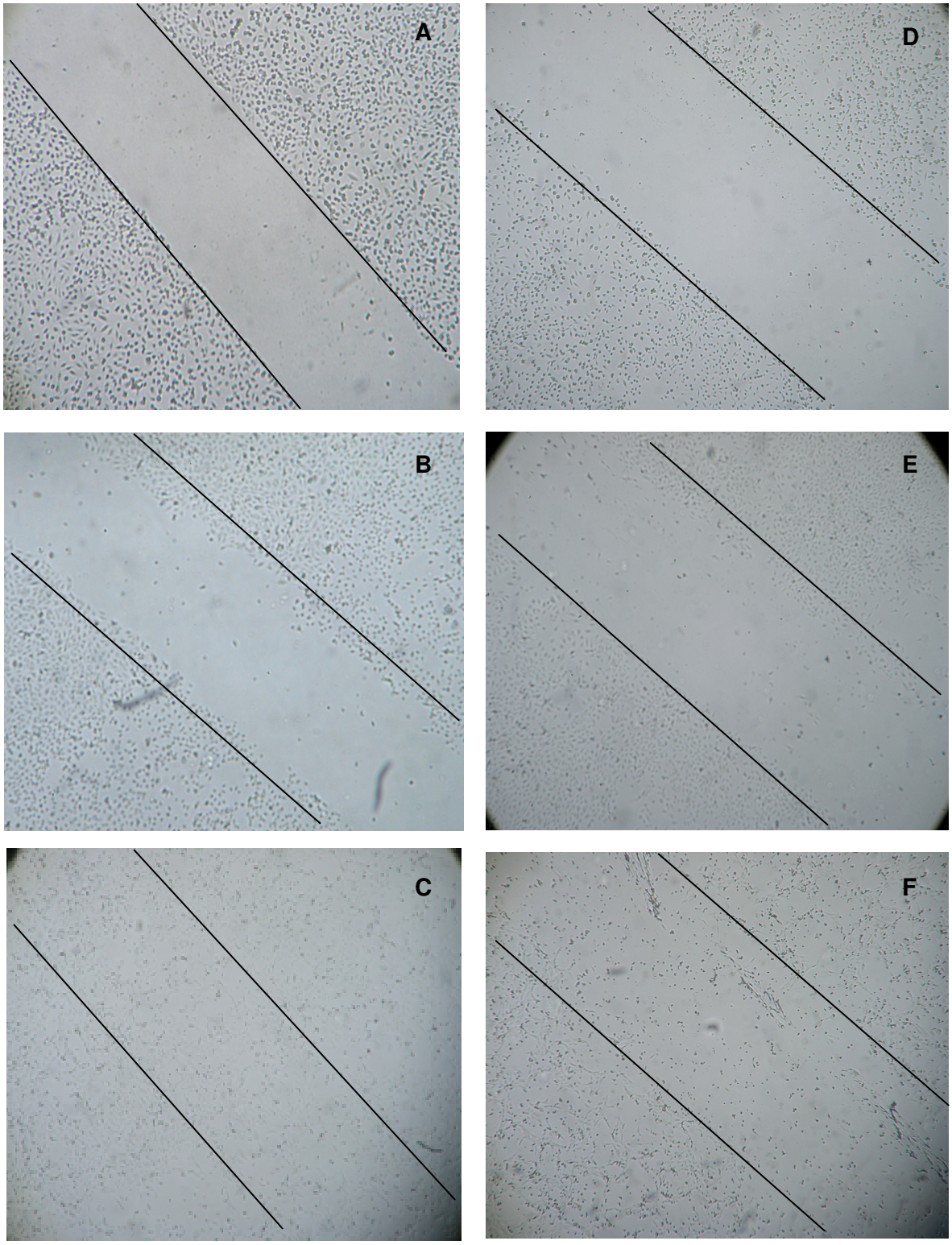


Figure 4.8: Effect of epicatechin on HUVEC migration in VEGF-supplemented medium examined by migration wound healing assay.

The confluent HUVEC monolayers were wounded at time 0 and cultured with 10 µM epicatechin in basal medium plus 10 ng/ml VEGF. Photographs of the basal medium plus VEGF control **(A, B, C)** and epicatechin **(D, E, F)** treated cells were taken after 0, 4, and 48 h (top, middle and bottom respectively). This data are representative of triplicate analysis of two independent experiments.

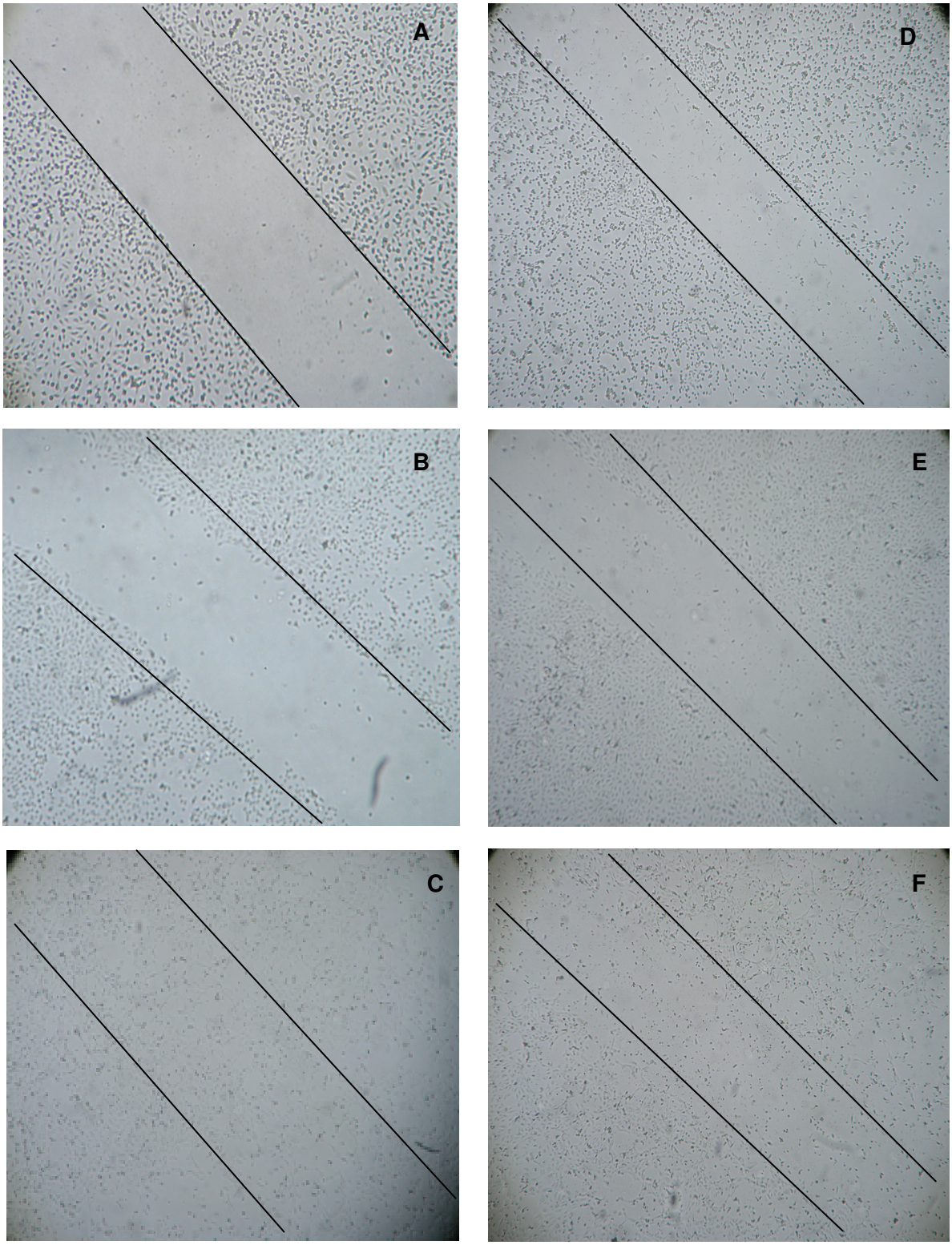


Figure 4.9: Effect of procyanidin dimer B2 on HUVEC migration in VEGF-supplemented medium examined by migration wound healing assay.

The confluent HUVEC monolayers were wounded at time 0 with and cultured with 10 µM procyanidin dimer B2 in basal medium plus 10 ng/ml VEGF. Photographs of the basal medium plus VEGF control **(A, B, C)** and procyanidin dimer B2 **(D, E, F)** treated cells were taken after 0, 4, and 48 h (top, middle and bottom, respectively). This data are representative of triplicate analysis of two independent experiments.

These data confirm the results reported by García-Conesa et al. (2009), namely that apple procyanidin fraction dp 3.9, but not epicatechin or procyanidin dimer B2, inhibits endothelial cell migration, which is an angiogenesis related function. In addition, the data presented show, for the first time, that the apple procyanidin fraction dp 3.9 is able to inhibit endothelial cell migration in the VEGF-supplemented basal medium. This observation supports the notion that apple procyanidin fraction dp 3.9 inhibits endothelial cell migration by influencing the VEGF signalling pathway.

4.5.2 Apple procyanidin fraction dp 3.9 inhibits VEGF-induced tyrosine phosphorylation of VEGFR-2

The data from the migration wound healing assays indicates that HUVEC migration is stimulated by VEGF, and that the apple procyanidin fraction dp 3.9 effectively inhibits VEGF-induced HUVEC migration. This observation strongly suggests that the apple procyanidin fraction dp 3.9 is inhibiting VEGF signalling in HUVECs. Therefore the aim was to investigate the effects of apple procyanidin fraction dp 3.9 on VEGF signalling in HUVECs, specifically the phosphorylation of VEGFR-2.

The assays used to establish the phosphorylation of VEGFR-2 tyrosine 1175 site was analysed by Western blotting and/or by ELISA. HUVECs were treated with pre-incubated basal medium containing 10 ng/ml VEGF and either 1 µM or 8.9 µM apple procyanidin fraction dp 3.9 (Figure 4.10 and 4.11). In the absence of VEGF, phosphorylated VEGFR-2 could not be detected in HUVECs (Figure 4.11 A). However, when HUVECs were exposed to VEGF, VEGFR-2 was rapidly phosphorylated (by 1 min) and the intensity of the phosphorylated VEGFR-2 band peaked at 5 min (Figure 4.10). Subsequently, the intensity of the VEGFR-2 band declined between 30 min and 24 h. This is likely due to internalization and degradation of VEGFR-2, which has been reported elsewhere (Cebe-Suarez et al., 2006; Rahimi, 2006). VEGF-

induced phosphorylation of VEGFR-2 was completely inhibited within 5 min by 0.3 μM of apple procyanidin fraction dp 3.9 (VEGF concentration = 10 ng/ml VEGF) (Figure 4.11B). In a separate set of experiments it was shown that 0.5 μM apple procyanidin fraction dp 3.9 completed inhibited VEGF-induced VEGFR-2 phosphorylation (VEGF = 25 ng/ml) (Figure 4.11C).

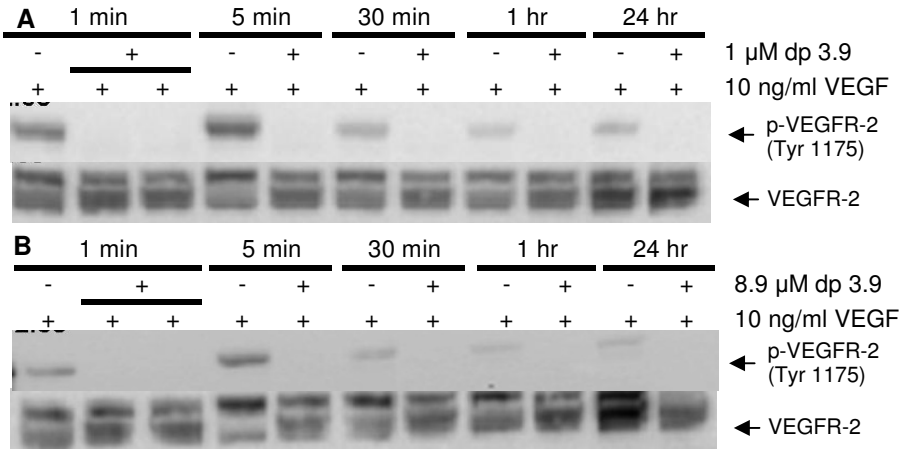


Figure 4.10: Apple procyanidin fraction dp 3.9 inhibits phosphorylation of VEGFR-2 at 1 min.

HUVECs were treated with pre-incubated basal medium containing 10 ng/ml of VEGF and 1 μM **(A)** or 8.9 μM **(B)** apple procyanidin fraction dp 3.9 for 1, 5, 30 min, 1 h and 24 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and the total VEGFR-2 levels. This data is representative of triplicate analysis of two independent experiments.

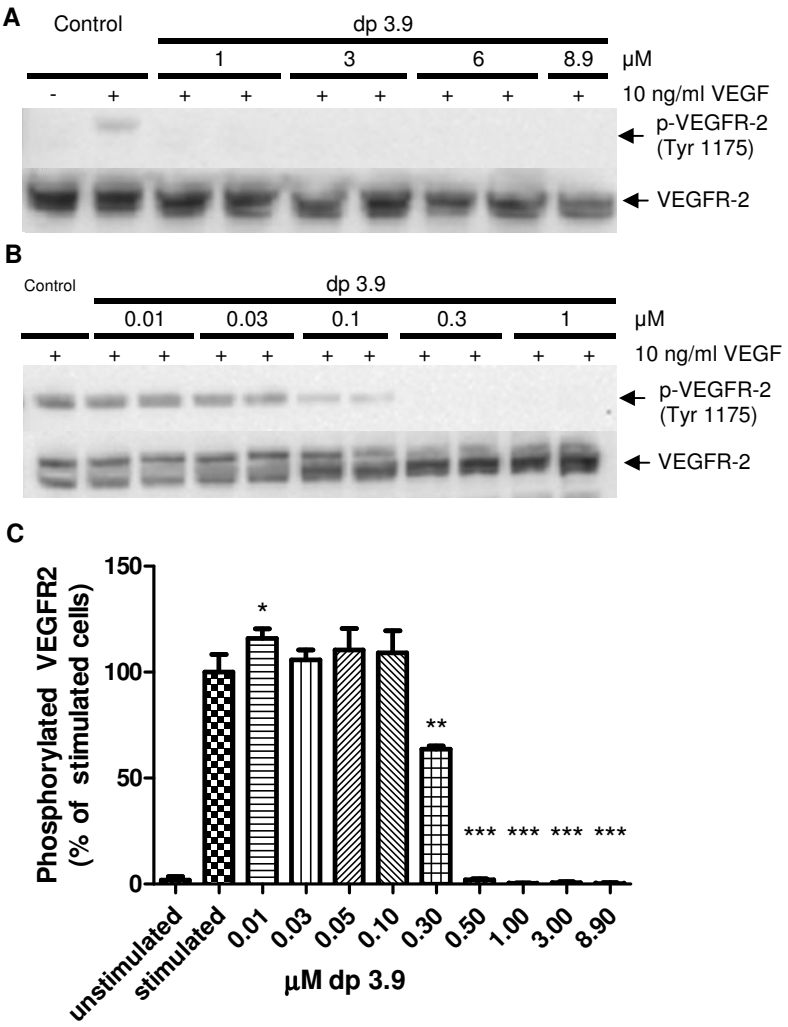


Figure 4.11: Apple procyanidin fraction dp 3.9 inhibits VEGFR-2 phosphorylation.

HUVECs were treated with pre-incubated basal medium containing 10 ng/ml **(A & B)** or 25 ng/ml **(C)** of VEGF and different concentrations of the apple procyanidin fraction dp 3.9 for 5 min. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 levels **(A & B)** or the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(C)**. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the stimulated cells. **A & B** is representative of triplicate analysis of two independent experiments and **C** is representation of duplicate analysis of one independent experiment.

4.5.3 Apple procyanidin fractions interact with the VEGF molecule and not the VEGF receptor-2

The data presented in Section 4.5.1 and 4.5.2 demonstrates that the apple procyanidin fraction dp 3.9 is able to inhibit endothelial cell migration as well as inhibit VEGF-

induced VEGFR-2 phosphorylation. However it is not known whether this inhibition occurs due to the apple procyanidin fraction interacting with the VEGF molecule or by interacting with the endothelial cells (e.g. binding to the VEGF receptors, interacting with the receptor, the cell membrane or interacting with intracellular kinases) to prevent intracellular signalling.

In the experiments described in Section 4.5.2, the polyphenol and VEGF were mixed together and subsequently added to the cells. Using this experimental design, it is not possible to determine whether polyphenols prevent VEGF-induced VEGFR-2 phosphorylation by interacting with VEGF or with the endothelial cells. In order to determine where the polyphenol is exerting its inhibitory effect, two further experimental designs were tested. In the first experimental design, confluent HUVEC monolayers were pre-treated with the apple procyanidin fraction for 4 h, then the medium was aspirated, the cells were washed with basal medium, and finally they were stimulated with VEGF (modification 1, Figure 4.3). In the second experimental design, confluent HUVEC monolayers were pre-stimulated with VEGF for 5 min and then the VEGF supplemented medium was treated with the apple procyanidin fraction (modification 2, Figure 4.3).

When the cells were pre-treated with the apple procyanidin fraction for 4 h, no inhibition in VEGF-induced VEGFR-2 phosphorylation was observed at any time point (Figure 4.12 A&B). When the cells were pre-stimulated with VEGF before the addition of the apple procyanidin fraction, an approximate 50 % reduction in VEGFR-2 phosphorylation was observed at 5 min and an approximate 70 % reduction in VEGFR-2 phosphorylation was observed from 30 min to 24 h (Figure 4.13 A&B). As described previously (Section 4.5.2), the peak expression of VEGF-induced VEGFR-2 phosphorylation occurs after 5 min and then declines between 15 min and 24 h (due to internalization and degradation of VEGFR-2). Therefore, for the purpose of quantifying

the effect of the apple procyanidins on VEGFR-2 phosphorylation, each treatment time point was compared to its respective stimulated cells control.

The data from the two different experimental designs applied, showed that the inhibitory effect of the apple procyanidin fraction is not due to the apple procyanidin fraction binding to the receptors and therefore preventing VEGF from binding to the cell surface but rather by binding to the VEGF molecule.

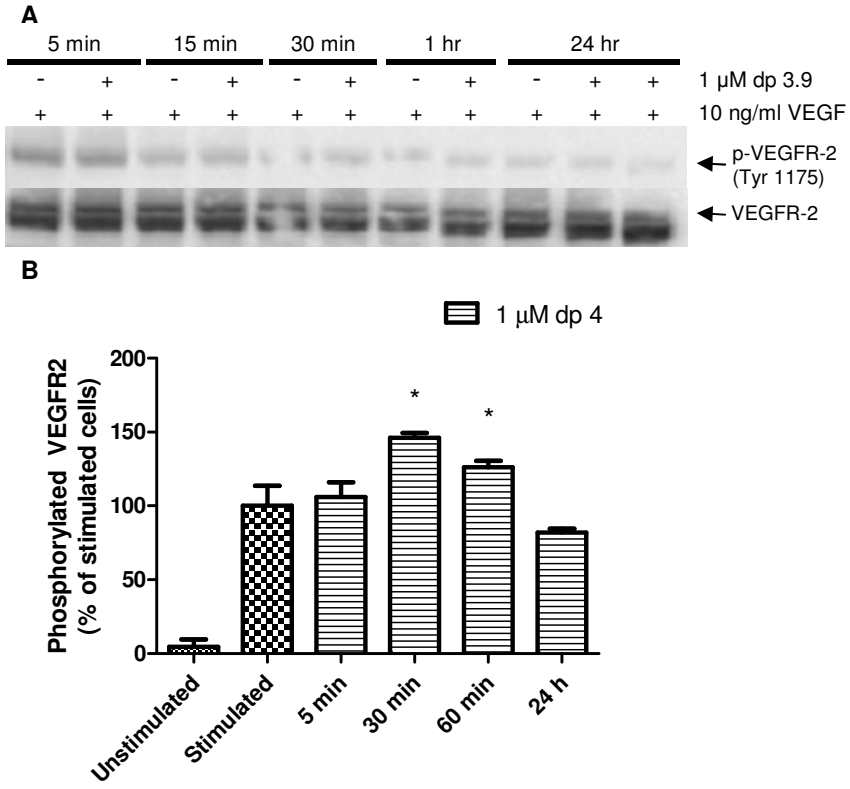


Figure 4.12: Apple procyanidin fraction dp 3.9 and dp 4 do not interact with the cell surface.

HUVECs were pre-treated with 1 µM apple procyanidin fraction dp 3.9 **(A)** or 1 µM apple procyanidin fraction dp 4 **(B)** for 4 h, washed with serum free medium and stimulated with 10 ng/ml **(A)** or 25 ng/ml **(B)** VEGF for various times. The cells were lysed and the proteins were either separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 **(A)** or the total amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(B)**. For ELISA data, values are expressed as a percentage of the stimulated cells from each time point **(B).** Bars represent means ± standard deviation. *$p < 0.05$, ** $p < 0.01$ compared to the stimulated cells. **(A)** is representative of triplicate analysis of two independent experiments and **(B)** is representative of duplicate analysis of one independent experiment.

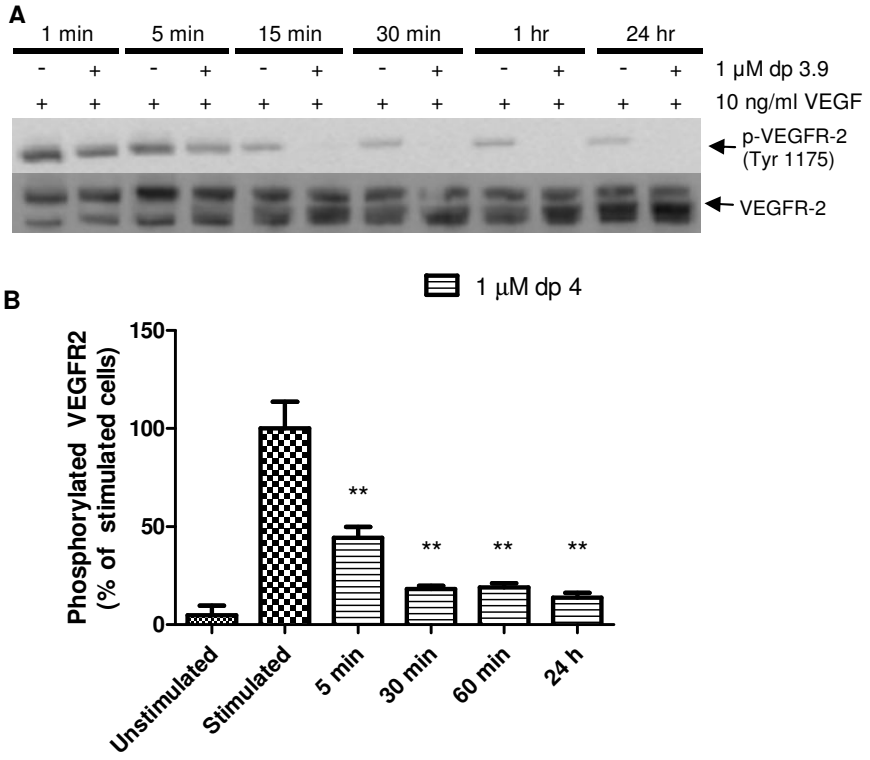


Figure 4.13: Apple procyanidin fraction dp 3.9 and dp 4 interacts with the VEGF molecule. HUVECs were pre-stimulated with 10 ng/ml **(A)** or 25 ng/ml **(B)** VEGF for 5 min and treated with 1 µM apple procyanidin fraction dp 3.9 **(A)** or 1 µM apple procyanidin fraction dp4 **(B)** for the times indicated. The cells were lysed and the proteins were either separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 **(A)** or the total amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(B)**. For ELISA data, values are expressed as a percentage of the stimulated cells from each time point **(B).** Bars represent means ± standard deviation. *$p < 0.05$, ** $p < 0.01$ compared to the stimulated cells. **(A)** is representative of triplicate analysis of two independent experiments and **(B)** is representative of duplicate analysis of one independent experiment.

4.5.4 The binding of the apple polyphenols to VEGF is not the consequence of non-specific protein binding

In the previous experiment it was shown that the apple procyanidin fraction inhibits VEGF-induced phosphorylation of VEGFR-2 by interacting with the VEGF molecule. However, procyanidins are well known for their affinity for proteins; protein binding by tannins (= procyanidins) is responsible for the oral astringency experienced when drinking a tannic red wine or eating dark chocolate, and is the basis for tanning leather.

This protein binding activity is generally non-specific as it is largely independent of the nature of the protein. Therefore, to determine whether the apple procyanidin fraction has a specific affinity for VEGF or if the binding to VEGF is non-specific, the ability of apple procyanidin fraction-treated VEGF to phosphorylate VEGFR2 was investigated in HUVECs in the presence and absence of high concentrations of serum proteins (Figure 4.14).

When incubated with the apple procyanidin fraction in EGM-2 medium (containing 2 % foetal calf serum as well as other serum proteins), VEGF-induced phosphorylation of VEGFR-2 was still inhibited. The intensity of the phosphorylated VEGFR-2 band was lower than in the basal medium and basal medium plus BSA samples because no additional VEGF was added to the medium. The EGM-2 medium contains between 0.8 and 2.8 ng/ml VEGF (Lonza). In the presence of basal medium plus BSA (1 mg/ml), the apple procyanidin fractions were able to inhibit the VEGF-induced phosphorylation of VEGFR-2, although it was slightly less potent than when the procyanidin fractions were applied in only VEGF-supplemented basal medium. A complete inhibition was observed with a treatment of 1 µM apple procyanidin fraction in VEGF-supplemented basal medium whereas an approximate 75 % inhibition was observed with the same treatment in BSA and VEGF-supplemented medium (Figure 4.14 B).

The concentration of VEGF in the treatments was either 10 ng/ml (0.26 nM) or 25 ng/ml (0.66 nM), whereas the concentration of BSA (1mg/ml or 15000 nM) was 4-5 orders of magnitude higher. These data show that the apple procyanidin fractions effectively inhibit the VEGF-induced phosphorylation of VEGFR-2 even in the presence of excess quantities of serum proteins. These observations indicate that the binding of apple procyanidins to VEGF or VEGFR-2 is specific, and not the result of non-specific protein binding.

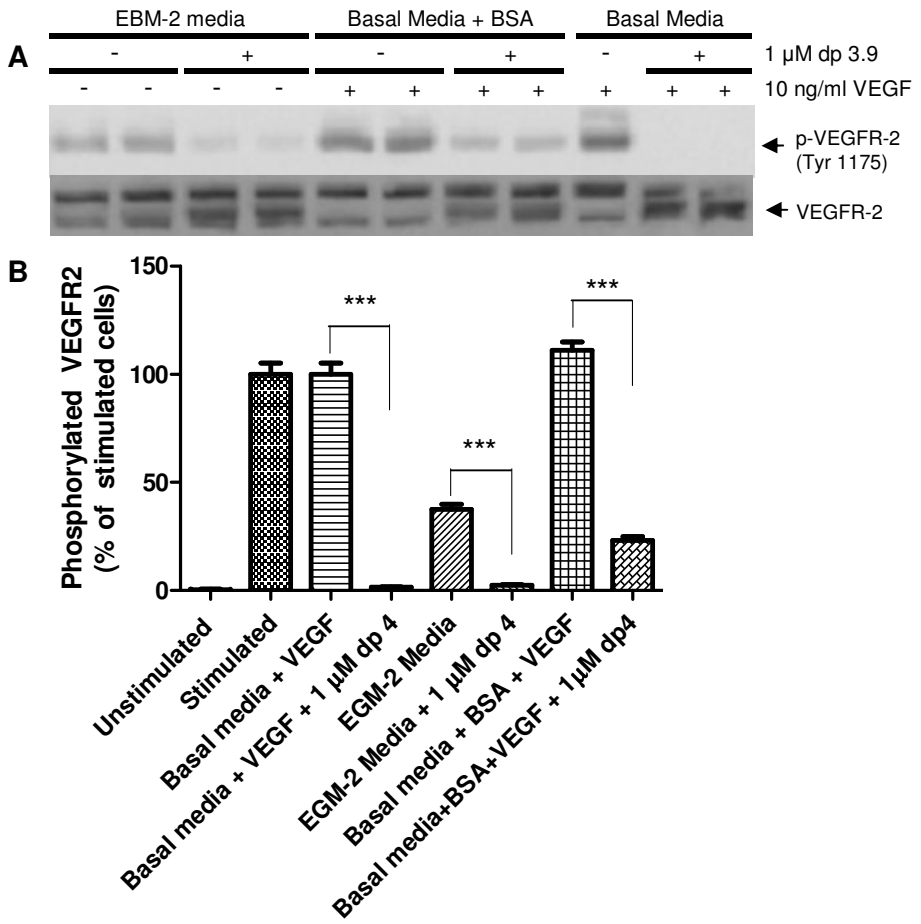


Figure 4.14: Apple procyanidin fractions are able to inhibit VEGFR-2 phosphorylation in the presence of other growth factors and in an excess of BSA.

(A) HUVECs were treated with either pre-incubated basal medium containing 10 ng/ml VEGF or BSA (1 mg/ml) plus 10 ng/ml VEGF in the presence or absence of 1 µM apple procyanidin fraction dp 3.9. **(B)** HUVECs were treated with either pre-incubated basal medium containing 25 ng/ml VEGF or BSA (1mg/ml) plus 25 ng/ml VEGF in the presence or absence of 1 µM apple procyanidin fraction dp 4. For the EGM-2 medium treatment, treatments were prepared in the same manner, but no additional VEGF was added. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 **(A)** or the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(B)**. Unstimulated = treatment with basal medium, stimulated = treatment with basal medium supplemented with 25 ng/ml VEGF. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. *** p <0.001 compared to the respective medium controls. Unstimulated = no VEGF, stimulated = 25 ng/ml VEGF. The data is representative of triplicate analysis of two independent experiments.

4.5.5 Apple procyanidin fraction inhibits phosphorylation of PLCγ1 but activates AKT

In Sections 4.5.1, 4.5.2 and 4.5.3 it was shown that apple procyanidin fractions are able to interact with VEGF and as a consequence inhibit endothelial cell migration and VEGF-induced VEGFR-2 phosphorylation. Phosphorylation of VEGFR-2 normally leads to intracellular signalling through the activation of other signalling molecules. In order to confirm that downstream signalling of VEGFR-2 is inhibited by the apple procyanidin fraction treatment two downstream signalling events were investigated. The downstream signalling events that were chosen were phosphorylation of phospholipase C gamma (PLCγ) and of serine/theronine kinases AKT/PKB (AKT).

PLCγ was selected because it is one of the first signalling proteins to become phosphorylated in response to VEGFR-2 phosphorylation. PLCγ binds to the phosphorylated Tyr 1175 site on the C-terminus and mediates the activation of the mitogen-activated protein kinase (MAPK) / extracellular signal regulated kinase 1/2 (ERK 1/2) cascade which drives proliferation of endothelial cells. The data presented in Figure 4.15 show that treatment of HUVECs with VEGF induced PLCγ1 phosphorylation. Peak expression of PLCγ1 phosphorylation occurred at 10 min and the levels of phosphorylated PLCγ1 slowly decreased at 30 min and 1 h. When exposed to the apple procyanidin fraction dp 3.9 treatment, VEGF-induced phosphorylation of PLCγ1 was significantly reduced at all time points. These data confirm that the apple polyphenol-mediated inhibition of the VEGF-induced activation of VEGFR-2 also prevents downstream signalling through PLCγ1.

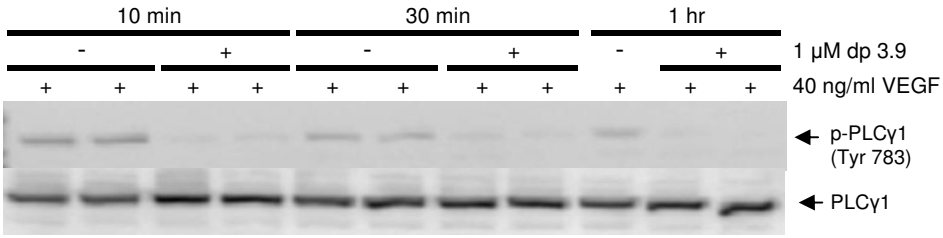


Figure 4.15: Apple procyanidin fraction dp 3.9 inhibits phosphorylation of PLCγ1

HUVECs were treated with pre-incubated basal medium containing 40 ng/ml of VEGF and 1 µM apple procyanidin dp 3.9 for 10, 30 min and 1 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated PLCγ1 at Tyr 783 and for total PLCγ1. The data is representative of triplicate analysis of three independent experiments.

The second point in the pathway that was investigated was AKT. AKT is activated downstream of phosphoinositide 3-kinase and mediates survival of endothelial cells as well as regulating nitric oxide production. AKT activation is further downstream in the VEGF signalling cascade and can also be activated through several other signalling pathways. The data presented show somewhat surprisingly that, VEGF stimulation did not cause AKT (Ser 473) to become phosphorylated (Figure 4.16). This was a repeatable observation (five independent experiments were conducted). However, AKT was phosphorylated when treated with apple procyanidin fraction dp 3.9, in the presence or absence of VEGF. The observation that AKT is not phosphorylated in the presence of VEGF is in disagreement with reports from the literature as it has been shown that VEGF causes the activation of AKT through VEGFR-2 phosphorylation (Zhang et al., 2003; Blanes et al., 2007).

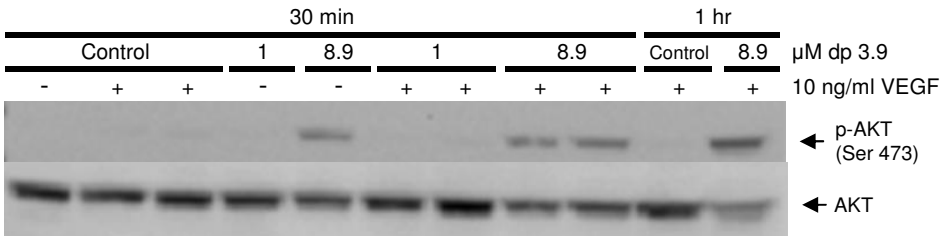


Figure 4.16: Apple procyanidin dp 3.9 promotes phosphorylation of AKT.

HUVECS were treated with pre-incubated basal medium containing 10 ng/ml of VEGF and 1 and 8.9 µM apple procyanidin fraction dp 3.9 for 30 min and 1 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated AKT at Ser 473 and total AKT. The data is representative of triplicate analysis of five independent experiments.

4.5.6 The ability of polyphenols to inhibit VEGFR-2 signalling is highly dependent on their structure

The apple procyanidin fraction dp 3.9, that was used to treat the cells, is not a pure fraction as it contains monomers (epicatechin and catechin) and oligomers (dimers to heptamers) (Figure 4.18). Therefore the effects of pure (-)-epicatechin, (+)-catechin and procyanidin dimer B2 on the VEGF signalling pathway were investigated. When HUVECS were treated with epicatechin, catechin or procyanidin dimer B2 there was no inhibition of VEGFR-2 phosphorylation at a concentration up to 20 µM, whereas apple procyanidin dp 3.9 completely inhibited VEGFR-2 phosphorylation at a concentration of 0.3 µM (Figure 4.17). This shows that flavanols need to be an oligomer and be at least a trimer in order to be able to inhibit the VEGF-induced phosphorylation of VEGFR-2.

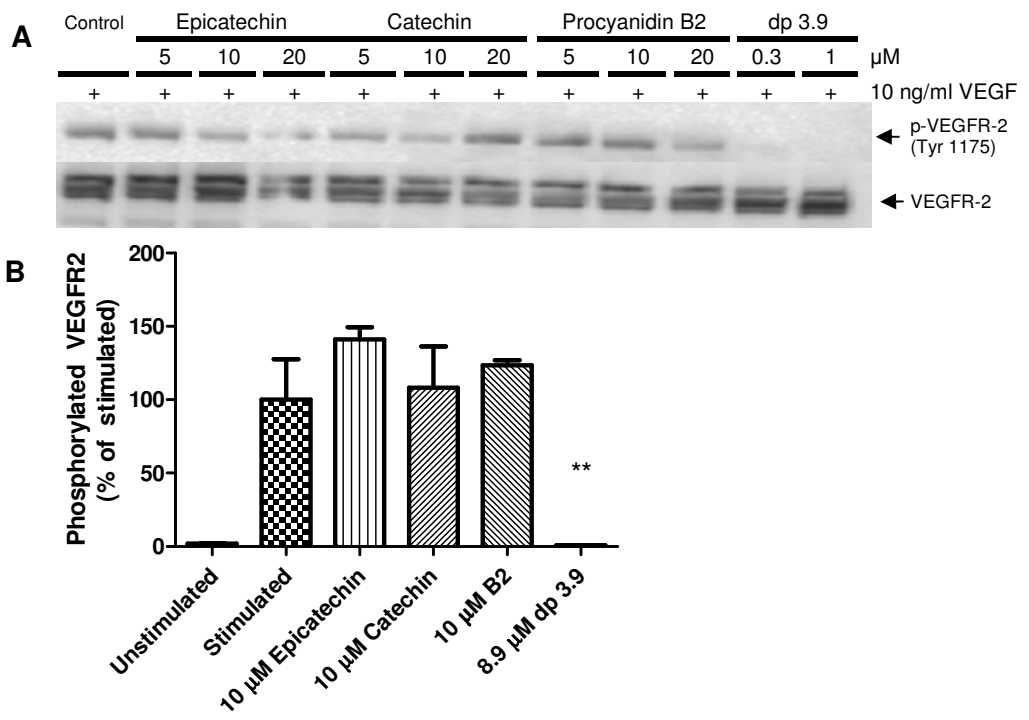


Figure 4.17: Apple procyanidin fraction dp 3.9, but not epicatechin, catechin, or procyanidin B2, inhibits VEGF-induced phosphorylation of VEGFR-2.

HUVECs were treated with pre-incubated basal medium containing 10 ng/ml **(A)** or 25 ng/ml **(B)** of VEGF and treated with either epicatechin, catechin, procyanidin dimer B2 or apple procyanidin fraction dp 3.9 for 5 min. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 **(A)** or the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(B)**. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. ** p <0.01 compared to the stimulated cells. The data is representative of triplicate analysis of two independent experiments.

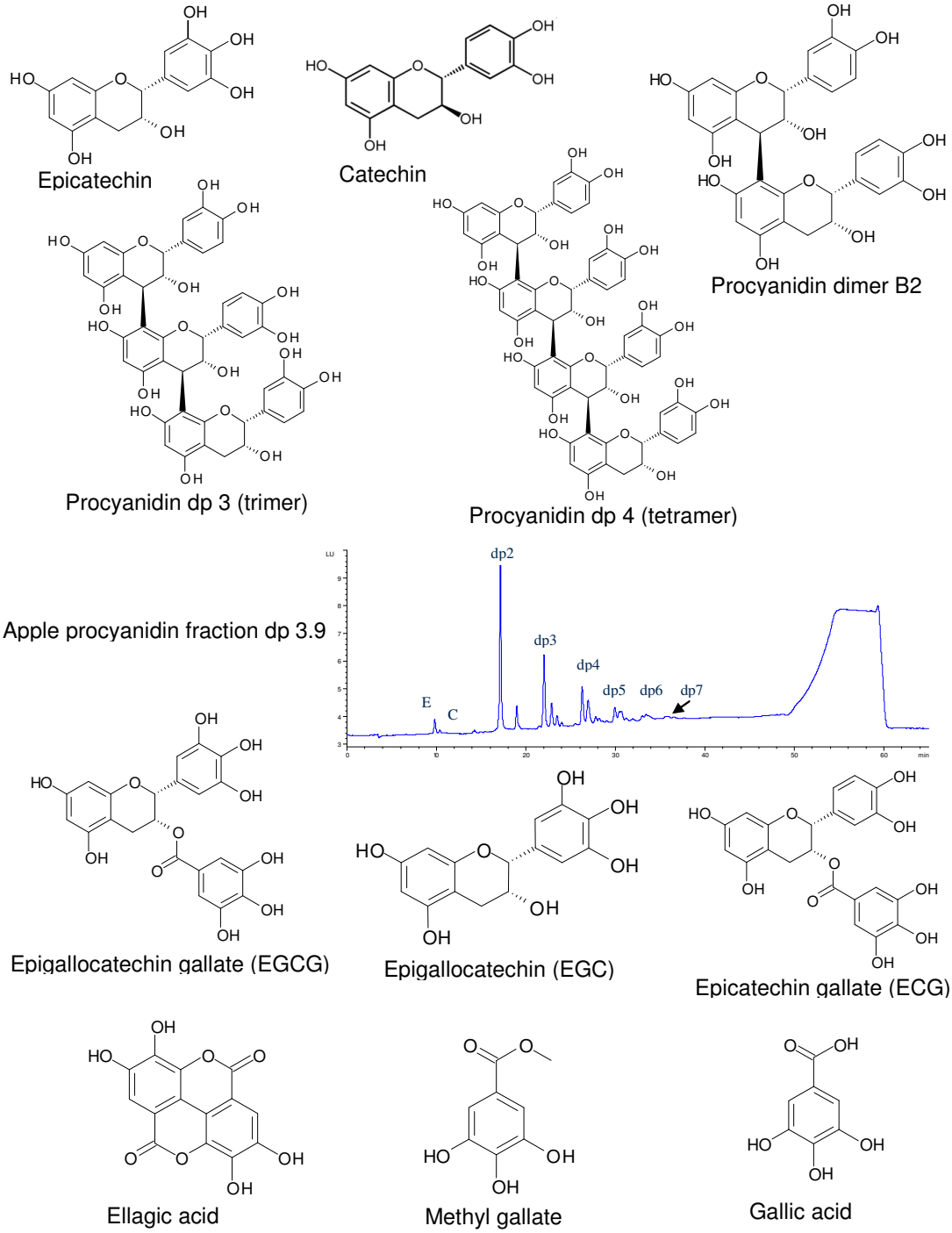


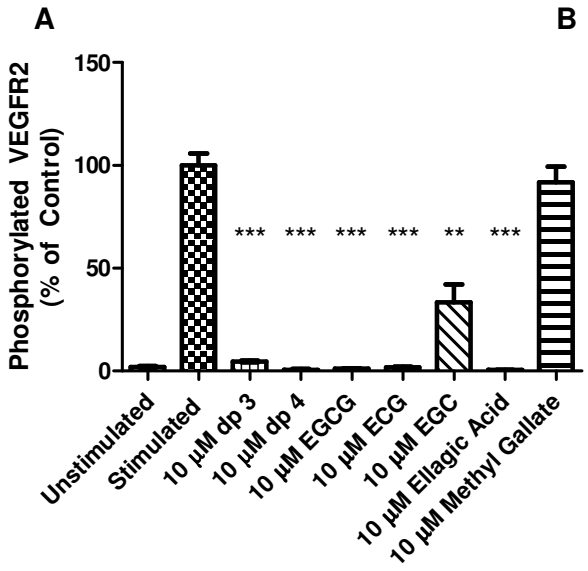
Figure 4.18: Polyphenol structures investigated.

Example of a normal-phase high-pressure liquid chromatography (HPLC) chromatogram of apple procyanidin fraction dp 3.9. Normal-phase HPLC is used for the quantification of polymeric flavanols. Flavanols were separated according to degree of polymerisation using a Luna silica column (250 x 4.6 mm^2, 5 µM particle size; Pheomenex, Macclesfield, UK) and a mobile phase consisting of a 65 min gradient (0 – 30 min, 14.0 – 28.4 % B; 30 – 45 min, 28.4 – 39.6 % B; 45 – 50 min, 39.6 – 86.0 % B; 50 – 55 min, 86.0 % B; 55 – 65 min, 14.0 % B; with a constant 4 % of C maintained throughout the gradient) where solvent A is dichloromethane, B is methanol and C is acetic acid/water (1:1, v/v). Flavanols were detected by fluorescence emission at 316 nm, following excitation at 276 nm. E: epicatechin; C: catechin; dp 2: dimers; dp 3: trimers, dp 4: tetramers; dp 5: pentamers; dp 6: hexamers; dp 7: heptamers.

In order to examine how polyphenol structures influences the inhibition of VEGF induced VEGFR-2 phosphorylation, apple procyanidin fractions dp 3 and dp 4, and certain green tea polyphenols were investigated (Figure 4.19). Apple procyanidin fraction dp 3 completely inhibited VEGF-induced VEGFR-2 phosphorylation at a concentration of 10 µM and with a half inhibitory concentration (IC_{50}) of 0.78 µM. Apple procyanidin fraction dp 4 was also able to completely inhibit VEGF-induced VEGFR-2 phosphorylation at a concentration of 10 µM. However, it was more potent than apple procyanidin fraction dp 3 as the IC_{50} of apple procyanidin fraction dp 4 was 0.28 µM. Therefore, indicating that the higher the degree of polymerisation, the greater the ability to inhibit VEGFR-2 signalling. The IC_{50} was determined by using a log (inhibitor) versus normalised response – variable slope analysis tool on GraphPad Prism software.

The ability of green tea polyphenols to inhibit VEGFR-2 phosphorylation is also dependent on key structural features (Figure 4.19). The green tea catechin, EGCG, which is galloylated at the 3-position and contains a trihydroxy B-ring, completely inhibited VEGFR-2 phosphorylation at a concentration of 1 µM (Figure 4.20) and the IC_{50} was estimated to be 0.09 µM (Figure 4.19). The phenolics ECG and ellagic acid were also able to completely inhibit VEGF-induced VEGFR-2 phosphorylation, while EGC and methyl gallate caused a 60 % and 10 % inhibition of VEGF-induced VEGFR-2 phosphorylation, respectively. The observations that EGC (IC_{50}: 42.9 µM) was a poor inhibitor, and ECG (IC_{50}: 0.16 µM) was a better inhibitor than ECG but not as effective as EGCG, indicates that both 3-galloylation and the trihydroxy B-ring are important for inhibitory activity. Methyl gallate (IC_{50}: >200 µM) did not inhibit phosphorylation, showing that a gallate ester is not sufficient for inhibitory activity. Gallic acid (IC_{50}: >200 µM) was a weak inhibitor while ellagic acid (a dimer of gallic acid) (IC_{50}: 0.23 µM) completely inhibited VEGF signalling. These data show that the ability to

inhibit VEGF signalling is highly sensitive to the size of the polyphenol and the number of hydroxyl groups.



Polyphenol	IC_{50} (µM)	Molecular Weight	No. of hydroxyl groups
EGCG	0.09	458	8
ECG	0.16	442	7
Ellagic acid	0.23	302	4
dp 4	0.28	1154	20
dp 3.9	0.32	1125	Mixture of compounds
dp 3	0.78	866	15
EGC	42.9	306	6
dp 2	52.6	578	10
Catechin	214.7	290	5
Epicatechin	> 200	290	5
Methyl Gallate	> 200	184	3
Gallic acid	> 200	170	4

Figure 4.19: Certain phenolics are able to inhibit VEGFR-2 phosphorylation at low concentrations.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml of VEGF and treated with either apple procyanidin fraction dp 3, apple procyanidin fraction dp 4, EGCG, ECG, EGC, ellagic acid or methyl gallate for 5 min. The cells were lysed and the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA **(A)**. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. ** p <0.01, *** p <0.001 compared to the stimulated cells. HUVECs were treated with pre-incubated basal medium containing 25 ng/ml of VEGF and different concentrations of polyphenols for 5 min. The cells were lysed and the total amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA and the half inhibitory concentrations (IC_{50}) were determined by using the log (inhibitor) versus normalised response – variable slope analysis tool on GraphPad Prism software **(B)**. The IC_{50} determination for EGCG, ECG, EGC, gallic acid, ellagic acid and methyl gallate was carried out by Mark Winterbone (IFR, Norwich). The data is representative of duplicate analysis of one independent experiment.

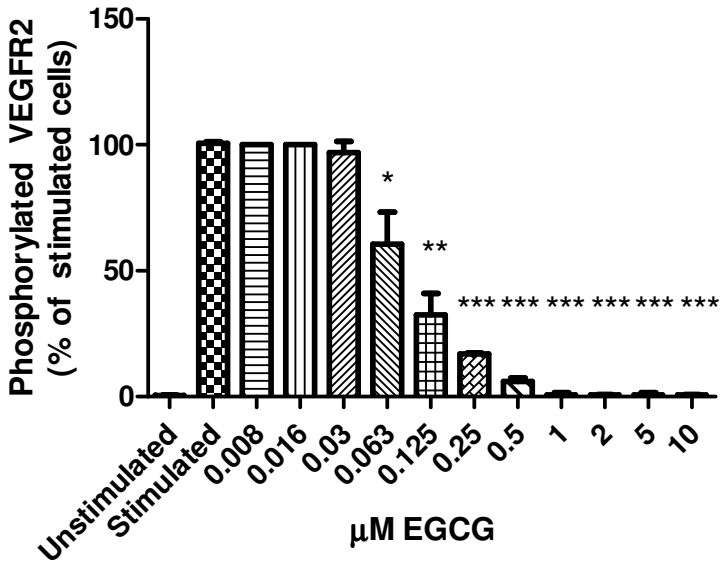


Figure 4.20: EGCG inhibits VEGFR-2 phosphorylation.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml VEGF and different concentrations of EGCG for 5 min. The cells were lysed and the amount of phosphorylated VEGFR-2 was quantified by phospho-VEGFR-2 (Tyr1175) ELISA. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ compared to the stimulated cells. This work was carried out by Mark Winterbone (IFR, Norwich).

4.6 Discussion

The data presented in this chapter show that: (1) certain green tea polyphenols are able to inhibit VEGF induced VEGFR-2 phosphorylation at dietary relevant concentrations; (2) the inhibitory effect of certain polyphenols is highly dependent on their structure; (3) the inhibitory action of these polyphenols is due to the polyphenols interacting with the VEGF molecule; and (4) the interaction between the polyphenol and VEGF molecule is specific as the polyphenol is still able to inhibit VEGF induced VEGFR-2 phosphorylation in the presence of FBS and other growth factors. This is the first time it has been shown that polyphenols inhibit VEGF induced VEGFR-2 phosphorylation by binding to the VEGF molecule.

Inhibition of the VEGF signalling pathway, and subsequently angiogenesis, is an effective strategy in the treatment of chronic diseases and is currently being used as a

therapeutic target in certain types of cancers. Anti-VEGF antibodies, aptamers and small molecule VEGFR tyrosine kinase inhibitors are available for the treatment of several forms of cancer (Giles, 2001; Ferrara and Kerbel, 2005). Certain polyphenols are also able to inhibit VEGF-induced VEGFR-2 phosphorylation (Kondo et al., 2002b; Lamy et al., 2002; Tang et al., 2003; Lamy et al., 2006; Mojzis et al., 2008; Wen et al., 2008; Lu et al., 2010), although the exact mechanism of this inhibition has not been fully investigated. In principle, polyphenols may inhibit VEGF induced VEGFR-2 phosphorylation in three ways; (1) by binding to the VEGF molecule and thus preventing VEGF from binding to its receptors; (2) by binding to the VEGF receptor(s) and thus preventing VEGF from binding to the receptor(s); or (3) by binding to different cellular compartments and thus preventing VEGF from binding to the receptor(s) or by inhibiting VEGF's kinase activity.

Inhibition of VEGF-induced VEGFR-2 phosphorylation may be due to the polyphenol binding to the VEGF protein and preventing VEGF from binding to its receptor. Kondo et al. (2002) investigated the possibility of catechins interfering with VEGF signalling by using an [^{125}I]-labelled VEGF binding assay and concluded that the polyphenol (EGCG) inhibited VEGF-induced VEGFR-2 phosphorylation by inhibiting the binding of VEGF to VEGFR-2. However the experimental technique preformed was to pre-treat HUVECs with the polyphenol for 30 min prior to 90 min exposure of VEGF. Therefore while the results show that the polyphenol prevents VEGF binding to its receptor by approximately 40 % it can not be deduced whether the inhibition is due to the polyphenol interacting with the VEGF molecule, the receptor, or the cellular membrane. Other researchers have proposed that polyphenols do not inhibit VEGFR-2 phosphorylation by interacting with the receptor as there was no reduction in VEGFR-2 expression in response to the polyphenol treatment (Tang et al., 2003; Rodriguez et al., 2006). Both of these researchers used the same method: treat the cells with polyphenol for 24 h, wash with medium and treat the cells with VEGF for either 30 min

or 24 h. Inhibition of intracellular signalling has also been demonstrated by polyphenol-protein binding with different proteins. For example, in the case of B-cell lymphoma 2 (Bcl-2) proteins, the polyphenol (EGCG) inhibits the anti-apoptotic function of Bcl-2 proteins by directly binding to the BH3 pocket of the Bcl-2 (Khan et al., 2006). Direct protein binding has also been observed with EGCG and PDGF (Weber et al., 2004).

On the other hand, polyphenols may exert their inhibitory effect by binding to the VEGFRs and thus preventing VEGF from binding to its receptor. Previously published data has shown that after extended incubations with polyphenols (EGCG and catechin-gallate or delphinidin), VEGF-induced VEGFR-2 phosphorylation is inhibited as a result of interactions between the polyphenol and the receptor (Lamy et al., 2002; Lamy et al., 2006). This conclusion was based on their experimental method: treating HUVECs with the polyphenol in serum free medium for 18 h, replacing the medium with fresh serum free medium and treating the cells with VEGF for 1 min. Therefore, concluding that with no direct competition of VEGF and polyphenol, the method of interaction must lie between the polyphenol and receptor. However a statistically significant difference was not observed in the current chapter after a 24 h incubation of apple procyanidin fraction dp 3.9 and dp 4 following the same experimental procedures.

Polyphenols may also exert their inhibitory effect by cellular incorporation and therefore cause a displacement of VEGF binding. Weber et al. (2004) demonstrated an inhibition in PDGF phosphorylation in response to EGCG treatment in vascular smooth muscle cells and after at [^{3}H]-labelled EGCG assay an incorporation of EGCG into different cellular compartments, including cell surface membranes, was observed. Therefore, it was concluded that the incorporation of EGCG into different cellular compartments leads to a non-displaceable binding of PDGF to non-receptor binding sites (Weber et al., 2004). The incorporation of anthocyanins into different cellular compartments in aortic endothelial cells has also been observed (Youdim et al., 2000).

The results presented in the literature have not determined the exact mechanism on how polyphenols inhibit VEGF-induced VEGFR-2 phosphorylation, but have presented theories based on the experimental design used. Therefore the data presented in this chapter has tried to determine the exact mechanism of inhibition. This was accomplished by using three different experimental designs in order to establish the method of inhibition: (1) pre-treatment of HUVECs with the polyphenol to establish whether the polyphenol is inhibiting VEGF-induced VEGFR-2 phosphorylation by interacting with the cell membrane; (2) pre-stimulation of HUVECs with VEGF to establish whether inhibition of VEGF-induced VEGFR-2 phosphorylation is dependent on the interaction between the polyphenol and the VEGF protein; and (3) a pre-incubation of polyphenol and VEGF protein to determine the length of time it takes to inhibit VEGF-induced VEGFR-2 phosphorylation. The data presented show that no inhibition of VEGF-induced VEGFR-2 phosphorylation is observed with pre-treatment of the cells with the polyphenol between 5 min and 24 h; time dependent inhibition of VEGFR-2 phosphorylation is observed with pre-stimulation of the cells with VEGF; and immediate inhibition of VEGFR-2 phosphorylation is observed with incubation of polyphenol and VEGF prior to cell treatment. These results establish that the polyphenol causes the inhibitory effect by interacting with the VEGF molecule rather than the cellular membrane. The array data presented by Liu et al. (2008) supports the notion that the inhibitory effect of polyphenols is due to the interaction with the VEGF molecule. Liu et al. (2008) reported gene expression data for HUVECs pre-treated with EGCG (20 µM) for 24 h followed by a 30 min stimulation of VEGF (50 ng/ml). Pre-treating the cells with EGCG followed a VEGF stimulation resulted in no differentially expressed genes identified when they were compared to VEGF-stimulated cells. However when the cells, which were pre-treated with EGCG followed by VEGF stimulation were compared to cells that were pre-treated with EGCG, 123 genes were reported to be differentially expressed (Liu et al., 2008). The results presented by Liu et

al. (2008) shows that pre-treatment of cells with EGCG followed by VEGF stimulation is unable to block genes differentially regulated by VEGF, which therefore supports the data presented in this chapter showing that polyphenols interact with the VEGF molecule. Since the data presented in this chapter shows that the inhibition of VEGF-induced VEGFR-2 signalling was instantaneous, incorporation of the polyphenol into different cellular compartments is unlikely.

Polyphenols are well known for their affinity for protein binding (Baxter et al., 1997; Jöbstl et al., 2004; Papadopoulou and Frazier, 2004; Papadopoulou et al., 2005) and this characteristic property of polyphenols is responsible for their astringency, enzyme inhibition and tanning ability (Ezaki-Furuichi et al., 1987; Luck et al., 1994; Murray et al., 1994; Baxter et al., 1997; Bacon and Rhodes, 2000; Jöbstl et al., 2004). The protein binding activity is generally non-specific as it is largely independent of the nature of the protein. Further experimental designs described here involving different types of medium has confirmed that the binding interaction between the polyphenol and VEGF molecule is specific as the polyphenol was still able to inhibit VEGF-induced phosphorylation of the receptor in the presence or absence of excess BSA, FBS and other cytokines and growth factors.

The inhibitory effect of certain polyphenols on VEGF-induced VEGFR-2 phosphorylation was achieved at physiological concentrations. The data from the literature range from very high and physiologically unrealistic doses (Kondo et al., 2002b; Kojima-Yuasa et al., 2003; Neuhaus et al., 2004; Wen et al., 2008; Lu et al., 2010) to physiologically relevant doses (Lamy et al., 2002; Rodriguez et al., 2006). In the case of procyanidins the physiological relevance is still unknown as an effective plasma analysis method for the detection of procyanidin oligomers has yet to be established. It has been stated that cocoa procyanidins are not degraded and can reach the intestine and that higher molecular weight procyanidins are poorly absorbed

by the intestinal lumen and therefore unlikely to reach the plasma (Rios et al., 2002a; Tsang et al., 2005; Appeldoorn et al., 2009b). However procyanidin trimers have been detected in rat plasma with a concentration of 4 µM after a high dose (1000 mg/kg of an apple procyanidin extract) (Shoji et al., 2006a). Procyanidin trimers have also been detected in rat plasma after the consumption of 300 mg/kg grape seed extract (amount not quantified) (Prasain et al., 2009) and after the consumption of 1 g/kg grape seed procyanidin extract (8.55 µM detected in plasma) (Serra et al., 2009). The detection of procyanidins indicated that they are not degraded into monomers (Prasain et al., 2009). Data presented here demonstrates that when exposed to VEGF prior to VEGF treatment of HUVECs, apple procyanidin trimers and tetramers inhibited VEGF-induced VEGFR-2 phosphorylation at low concentrations (IC_{50} of 0.78 µM and 0.28 µM, respectively). These concentrations are lower than the procyanidin concentrations described in the literature, 10 µg/ml (approximately 17 µM) (Wen et al., 2008) or 15 µg/ml (approximately 15 µM) (Lu et al., 2010), that were able to cause an inhibition of VEGF induced VEGFR-2 phosphorylation. EGCG has also been shown to inhibit VEGF induced VEGFR-2 phosphorylation at physiological concentrations ranging from 0.01 µM to 2 µM (Lamy et al., 2002; Rodriguez et al., 2006), while the data in this chapter has shown an IC_{50} of 0.09 µM for EGCG inhibition of VEGFR-2 phosphorylation. The concentrations at which these green tea catechins are able to inhibit VEGFR-2 signalling are physiologically relevant as the concentration of catechins in plasma, after the consumption of four cups of green tea, is between 0.2 µM and 1 µM (Lee et al., 1995; Yang et al., 1998b; Tang et al., 2003; Rodriguez et al., 2006).

Based on the investigation of different polyphenol structures, it was shown that the ability to inhibit VEGF signalling is related to the size of the polyphenol (epicatechin polymers with a degree of polymerisation greater than 3), the presence of a gallate group at the 3-position and the total number of hydroxyl groups. Therefore the

inhibitory effect of certain polyphenols is highly dependent on their structure. This is in agreement with what has been stated in the literature. Researchers have concluded that since EGCG was the most potent inhibitor, and ECG was the second most potent inhibitor, that the gallate group at the 3-position may be responsible for the observed inhibitory effect (Kondo et al., 2002b; Khan et al., 2006; Lorenz et al., 2009a). The ester bond present in EGCG, ECG, catechin-gallate has also been suggested to have a role in the inhibitory effect observed by these polyphenols (Lamy et al., 2002). In the case of delphinidin, the presence of three hydroxyl groups at the B-ring and a free hydroxyl group at the 3-position appear to be essential for its potency compared to other anthocyanidins (Lamy et al., 2006). Therefore, the specific structures of polyphenols are crucial for the inhibition of VEGF induced VEGFR-2 phosphorylation and the potency of that inhibition.

Confirmation of the inhibition of VEGF intracellular signalling in response to polyphenol treatment has been demonstrated by examining two downstream signalling pathways, PLCγ1 and AKT. PLCγ is an important protein as it mediates the activation of the MAPK/ERK 1/2 cascade and proliferation of endothelial cells. VEGF stimulation of cells has been shown to activate PLCγ1 phosphorylation (Xia et al., 1996; Cohen et al., 1999; Gille et al., 2001; Labrecque et al., 2005), whereas polyphenol treatment has inhibited either VEGF or PDGF induced PLCγ1 phosphorylation (Sachinidis et al., 2000; Weber et al., 2004; Labrecque et al., 2005). The results presented in this chapter are in agreement with the previously reported literature data.

However, in the case of AKT phosphorylation, the results presented in this chapter contradict those presented in the literature, that AKT phosphorylation was not activated by VEGF treatment. AKT is located several steps further downstream in the VEGF signalling pathway and it mediates survival of endothelial cells as well as regulating nitric oxide production. Others have shown that VEGF treatment of HUVECs induces

AKT phosphorylation (Chavakis et al., 2001; Tang et al., 2003; Riesterer et al., 2004; Rodriguez et al., 2006; Stangl et al., 2007) and that polyphenol treatment inhibited VEGF-induced AKT phosphorylation (Tang et al., 2003; Rodriguez et al., 2006; Stangl et al., 2007). However, the data presented in this chapter has shown that (1) VEGF alone (10 ng/ml or 25 ng/ml) did not cause an increase in AKT phosphorylation and (2) the polyphenol (apple procyanidin fraction dp 3.9), in the presence or absence of VEGF, induces AKT phosphorylation. No phosphorylation of AKT was observed when chronic lymphocytic leukaemia cells were treated with various doses of VEGF (Lee et al., 2004). Previous literature reports have shown that polyphenols are able to induce AKT phosphorylation (Wu et al., 2006; Kim et al., 2007; Stangl et al., 2007; Lorenz et al., 2009a; Montagut et al., 2010) and part of the cardio-protective effects of polyphenols has been attributed to the AKT activation-dependent increase in nitric oxide production; an increase of nitric oxide production results in vasorelaxation of the endothelium (Dimmeler et al., 1999; Anselm et al., 2007). Therefore, depending on the state of the cells (stimulated versus unstimulated), polyphenols can exert opposite effects on intracellular signalling (Stangl et al., 2007). However, this does not explain why, in the experiments described here, VEGF did not induce AKT phosphorylation.

Therefore based on the results presented in this chapter it can be concluded that certain polyphenols are able to inhibit VEGF-induced VEGFR-2 phosphorylation and intracellular signalling at physiological conditions and that the inhibition occurs through the interactions between the polyphenol and VEGF protein.

4.7 Conclusion

The results from this chapter have shown that VEGFR-2 phosphorylation is inhibited almost instantly once the polyphenol and VEGF are mixed together, and complete inhibition is observed after 5 min of incubation. The observed inhibitory effect is due to

the polyphenols interacting with the VEGF molecule. This is a specific interaction as the polyphenol was still able to inhibit VEGF-induced VEGFR-2 phosphorylation in the presence of FBS, cytokines, other growth factors and an excess of BSA. An investigation of polyphenol structures in relation to VEGF inhibitory activity has shown that the inhibition of phosphorylation is dependent on key structural features (epicatechin polymers with a degree of polymerisation greater than 3; 3-galloylation; and a trihydroxy B-ring). The most potent inhibitory polyphenols were able to inhibit VEGFR-2 phosphorylation at physiological concentrations. These findings are significant because they provide a plausible link between consumption of polyphenols and a reduced risk of CVD. More importantly this data shows a novel mechanism of how polyphenols inhibit VEGF induced VEGFR-2 phosphorylation. While this research has only shown an interaction with VEGF, it raises the possibility that polyphenols can affect other signalling pathways by interacting with other signalling peptides such as growth factors and hormones.

CHAPTER FIVE

BINDING INTERACTIONS BETWEEN POLYPHENOLS AND VEGF

Chapter 5 : Binding interactions between polyphenols and VEGF

5.1 Abstract

The data presented in Chapter 4 demonstrated that certain polyphenols potently inhibit VEGF-induced VEGFR-2 phosphorylation by interacting directly with VEGF and not with its receptors or the cell membrane. Interactions between proteins and ligands may be covalent (the result of a chemical reaction which would form a VEGF-polyphenol adduct) or non-covalent (e.g. through hydrogen binding, ionic binding or hydrophobic interactions which would result in a VEGF-polyphenol complex). The overall aim of the research presented in this chapter was to characterise the nature of the interaction between VEGF and inhibitory polyphenols. Extensive dialysis and concentration of VEGF-polyphenol adducts/complexes did not restore VEGF activity. This observation has shown that the interaction is not an easily reversible dynamic equilibrium between bound and unbound forms of VEGF and the observation also supports the formation of either a covalent adduct or a strong non-covalent complex. Polyphenol-treated VEGF exhibited exactly the same SDS-PAGE migration properties under reducing conditions as untreated VEGF which strongly suggests that polyphenol-mediated inhibition of VEGF activity is not due to the formation of VEGF-polyphenol adduct(s). LTQ Orbitrap mass spectrometry analysis of protease digested VEGF-polyphenol complexes identified peptides accounting for approximately 67 % of the full length of the VEGF protein, none of which had been covalently modified. Next, the kinetics of polyphenol-mediated inhibition of VEGF activity was investigated using apple procyanidin fraction dp 4. The data showed that the polyphenol-mediated inhibition of VEGF activity was time-dependent, providing further evidence that inhibition was not the result of the formation of a freely-reversible complex. The log-transformed kinetic data did not fit a

simple linear model, but did fit a two-stage model in which an initial rapid, linear phase of inhibition (rate constant = 1.29 x 10^{-3}) was followed by a slower linear phase of inhibition (rate constant = 0.18 x 10^{-3}). These data show that the inhibition of VEGF activity is the result of non-covalent slow-binding of one or more molecules of apple procyanidin fraction dp 4 or EGCG to the VEGF protein which results in the formation of a tightly bound VEGF-polyphenol complex.

5.2 Introduction

In Chapter 4 it was established that at low (physiological) concentrations, certain polyphenols can inhibit VEGF-induced VEGFR-2 phosphorylation by interacting with the VEGF molecule and not its receptors. Therefore, the aim of the research covered in this chapter was to determine the nature of the binding interaction(s) occurring between the polyphenols and VEGF.

Polyphenols are known for their tendency to bind to proteins (Baxter et al., 1997; Jöbstl et al., 2004; Papadopoulou and Frazier, 2004; Papadopoulou et al., 2005). This characteristic property of polyphenols is responsible for their astringency, enzyme inhibition and tanning ability (Ezaki-Furuichi et al., 1987; Luck et al., 1994; Murray et al., 1994; Baxter et al., 1997; Bacon and Rhodes, 2000; Jöbstl et al., 2004). The understanding of the nature of the interaction (specific versus non-specific, reversible versus irreversible, and covalent versus non-covalent) between polyphenols and proteins and whether the polyphenol induces conformational changes to the protein is important in the understanding of the mechanisms involved in polyphenol-protein binding.

5.2.1 Specific versus non-specific binding

The polyphenol may either bind to the protein specifically or non-specifically (Figure 5.1). Specific protein binding involves the interaction of the polyphenol to specific amino acid side chains on the protein. For example in the case of enzyme-substrate binding, the factors that determine substrate specificity for enzymes are conformational and chemical compatibility between the substrate and its binding site on the enzyme (Hrmova and Fincher, 2001). Non-specific binding involves the interaction of polyphenols to numerous hydrophobic side chains or hydrophobic regions on the

protein structure. The investigation of the specificity and thermodynamics of tannin-protein (BSA) binding was previously conducted and based on the general shape of the binding isotherms it was concluded that tannins interact non-specifically with BSA rather than binding to specific receptor sites on the protein while gelatine-tannin interactions involved a two stage binding process (Frazier et al., 2003). The first stage involves a specific binding interaction and the second stage involves non-specific interactions (Frazier et al., 2003). However, the majority of the literature has focused on the ability of polyphenols to bind non-specifically to proteins.

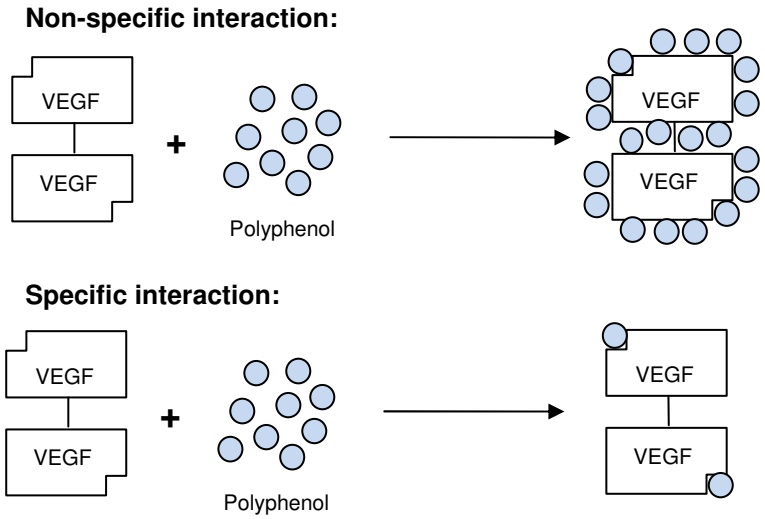


Figure 5.1: Schematic model of non-specific and specific protein-polyphenol interactions

5.2.2 Reversible versus irreversible binding

The specific or non-specific binding of a polyphenol to a protein may also be a reversible or irreversible interaction (Figure 5.2). An irreversible binding reaction between the polyphenol and protein usually involves the polyphenol covalently modifying the protein and the interaction cannot therefore be reversed. Irreversible interactions display time-dependent inhibition and their potency cannot be determined by an IC_{50} value. An irreversible binding reaction can also be the result of a very strong non-covalent interaction. In this case the polyphenol would bind rapidly to the protein

and then undergo a slower rearrangement involving a protein conformational change to result in a very tightly bound complex. Non-covalent interactions are associated with a reversible interaction and the bound polyphenol can be easily removed by dilution of dialysis under these conditions.

The mechanism of protein-polyphenol complexes has generally been regarded as reversible (McManus et al., 1985; Luck et al., 1994; Bennick, 2002; Charlton et al., 2002). The soluble complexes may reach a size where they start to precipitate, but these insoluble complexes are usually reversible (Siebert et al., 1996; Siebert, 2006). However oxidation, complex formation with metal ions, or changes in pH make the precipitation process irreversible (Luck et al., 1994).

The binding interaction between polyphenols and proteins can also involve a conformational change to the structure of the protein (Figure 5.2). The conformational change of the protein by the polyphenol will result in the loss of the activity of the protein. However, it is unlikely that protein conformation is changed by polyphenols. The addition of polyphenol (quercetin, rutin, epicatechin or catechin) did not result in a change of the conformation of BSA (Papadopoulou et al., 2005).

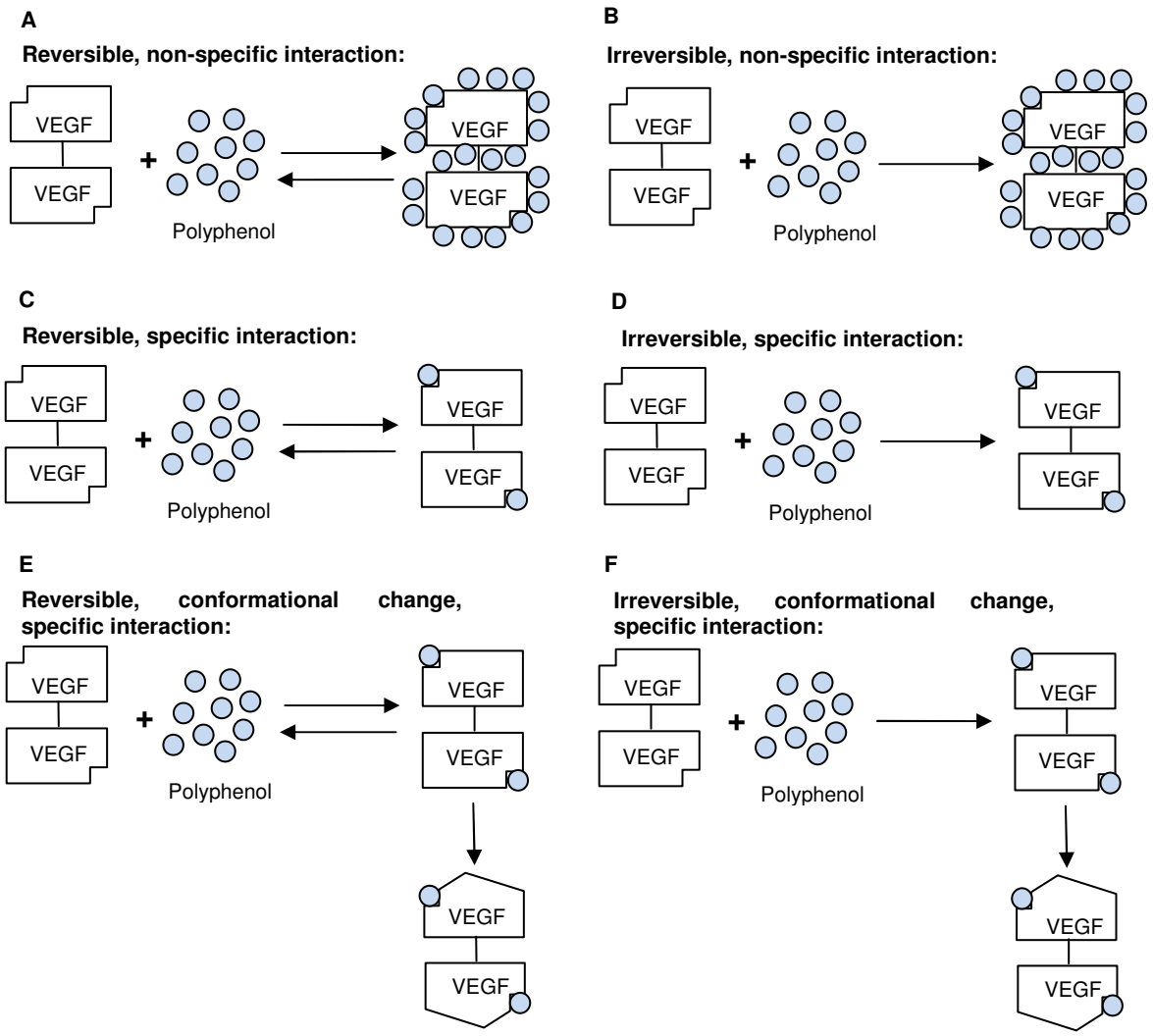


Figure 5.2: Schematic model of reversible, irreversible, conformational change, non-specific and specific protein-polyphenol interactions

5.2.3 Covalent versus non-covalent binding

Polyphenols may also bind to proteins non-covalently (involving hydrogen bonds, ionic bonds, van der Waals forces and hydrophobic interactions) or covalently (Loomis, 1974; Butler et al., 1984; Ezaki-Furuichi et al., 1987; Appel, 1993; Bennick, 2002). Hydrogen bonds are a weak to moderate intermolecular force (the forces of interactions between molecules) that occur between a hydrogen atom that is covalently bonded to a very electronegative atom, and a lone pair of electrons on another small electronegative atom such as nitrogen, oxygen or fluorine (Figure 5.3 A). For example,

hydrogen bonds occur between polyphenols and proteins when hydrogen bonds are formed between the hydroxyl groups of polyphenol 'donor' and amide carbonyl groups of the protein 'acceptor' (Loomis, 1974; Appel, 1993; Hagerman et al., 1998; Hofmann et al., 2006). Hydrogen bonds are stronger than most other intermolecular forces; however they are much weaker than both ionic bonds and covalent bonds. Ionic bonds are a chemical bond formed by electrostatic attraction between positive and negative ions (Figure 5.3 B), while hydrophobic interactions are the interactions driven by the exclusion of non-polar residues from water (Figure 5.3 C). Hydrophobic interactions are driven by a decrease in entropy that would result from water molecules being forced to become more ordered in order to accommodate the dispersed hydrophobic regions (Appel, 1993). Ionic bonds can be formed if the polyphenol is ionized to create a phenolate ion with a net negative charge, which can interact with the basic amino acid residues of proteins (Loomis, 1974; Appel, 1993). Hydrophobic bonds are formed by the attraction of the hydrophobic aromatic rings of the polyphenol and the hydrophobic regions of other compounds such as aliphatic and aromatic side chains of amino acids or proline residues in proteins (Loomis, 1974; Appel, 1993; Luck et al., 1994; Murray et al., 1994). Covalent binding occurs when two or more molecules share electrons between atoms, and it is the strongest form of binding (Figure 5.3 D).

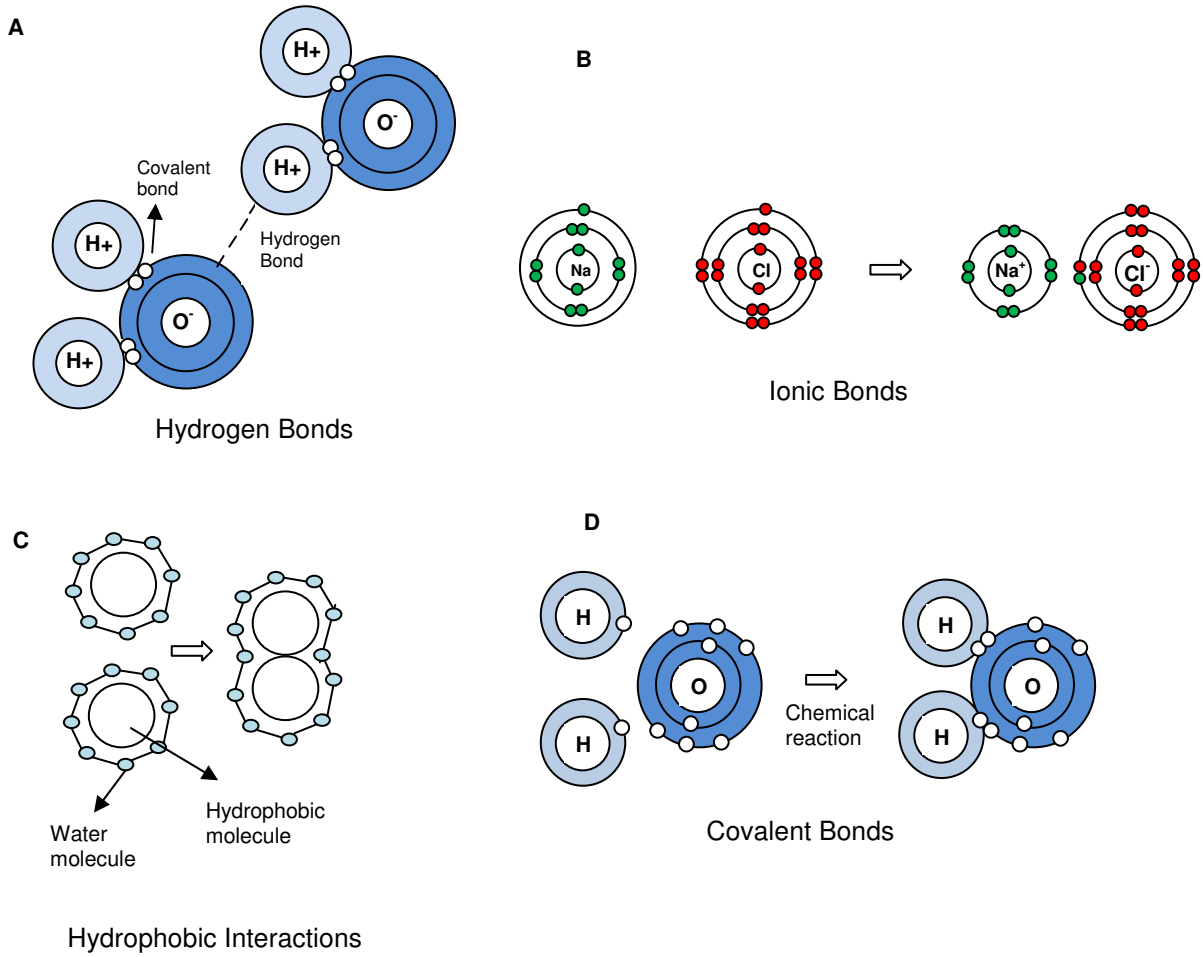


Figure 5.3: Examples of different types of intermolecular bonds.

(A) hydrogen binding; (B) ionic binding, (C) hydrophobic interactions, and (D) covalent binding.

It has been suggested that hydrogen binding and hydrophobic interactions are the major interactions between polyphenols and proteins (Butler et al., 1984), while covalent bonds and ionic bonds are less likely to occur (Bennick, 2002). Covalent bonds are most likely to occur under oxidizing conditions (Bennick, 2002). Ionic bonds are not likely to occur between polyphenols and proteins because it has been demonstrated that no interactions between polyphenols and proteins could be observed at pH values under which the phenolic hydroxyl groups would be ionized (Hagerman and Butler, 1978; McManus et al., 1985). Therefore the main types of interactions to occur between polyphenols and proteins are hydrogen bonds and hydrophobic interactions; although under certain circumstances covalent bonds can occur.

A number of reports have provided evidence that the major types of non-specific interactions to occur between polyphenols and proteins are hydrophobic interactions, and that hydrogen binding occurs as a minor interaction that provides strength and stability and reinforces the hydrophobic interactions (Oh et al., 1980; Hagerman and Butler, 1981; Luck et al., 1994; Murray et al., 1994; Hagerman et al., 1998; Vergé et al., 2002; Jöbstl et al., 2004; Papadopoulou et al., 2005; Richard et al., 2006; Diniz et al., 2008). Proteins or peptides with a high proline and/or arginine content (such as bradykinin [BDK] or salivary proline-rich proteins [PRPs]), are relatively large, are hydrophobic and possess conformationally open and flexible structures and therefore represent good candidates for polyphenol-protein interactions (Hagerman and Butler, 1981; Butler et al., 1984; Luck et al., 1994; Murray et al., 1994; Bacon and Rhodes, 2000; de Freitas and Mateus, 2002; Vergé et al., 2002; Jöbstl et al., 2004). In contrast, tightly coiled, globular proteins, such as ribonuclease A, cytochrome c, lysozyme and myoglobulin have a lower binding affinity for polyphenols (Hagerman and Butler, 1981; Luck et al., 1994). Hydrophobic interactions and hydrogen bonds are formed between polyphenols and proteins under non-oxidizing conditions (Hagerman et al., 1998; Diniz et al., 2008).

Under oxidizing conditions covalent bonds are more likely to occur between polyphenols and proteins (Diniz et al., 2008). In oxidizing conditions (for example in plasma samples) polyphenols react with reactive oxygen species, such as OH•, O_2^-• and ROO•, (Chen and Hagerman, 2004b) and the reaction products of polyphenols (semiquinone radicals or quinones) can form covalent bonds with nucleophiles, such as amino acids or thiol groups (Bennick, 2002; Chen and Hagerman, 2004b; Diniz et al., 2008). The covalent bonds formed between polyphenols and proteins, in oxidizing conditions, are resistant to disruption by protein denaturants (Hagerman et al., 1998;

Chen and Hagerman, 2004b). It has also been stated that covalent binding is favoured by a high pH or by the presence of polyphenol oxidase (Oh et al., 1980). However, the exact nature of the binding and the binding affinities between the polyphenol and protein is dependent on various parameters, including pH, temperature and structure (of the protein or polyphenol) (Luck et al., 1994; de Freitas and Mateus, 2001; Diniz et al., 2008).

5.2.4 Polyphenol-protein non-specific binding: the effect of pH

The pH of the solution may have an influence on the binding affinities between polyphenols and proteins. The affinities of proteins for polyphenols were shown to be highly dependent on the pH of the solution (Hagerman and Butler, 1981). It has been shown that the polyphenol-protein binding affinities are strongest when the pH is near the isolectric point (pI) of the protein (Hagerman and Butler, 1981; Ezaki-Furuichi et al., 1987; Hagerman et al., 1998; Riedl and Hagerman, 2001; Bennick, 2002). Therefore, proteins with an acidic pI have a greater affinity for polyphenols at a lower pH and proteins with a basic pI have a greater affinity for polyphenols at a higher pH. For example acidic proteins, such as bovine serum albumin (BSA) and PRPs, have a stronger affinity for polyphenols at pH 4.5 and between pH 3.5 and 5.0, respectively, while basic proteins, such as lysozyme have greater affinities at a higher pH (Hagerman and Butler, 1981; Ezaki-Furuichi et al., 1987; Hagerman et al., 1998; de Freitas and Mateus, 2001). However, different pH values (pH 5.0, 6.2 and 6.8) showed a slight upward trend in binding affinity between epicatechin and BSA, but the trend was not significant (Papadopoulou and Frazier, 2004). Charlton et al. (2002) also observed that binding affinities were unaffected by pH between 3.8 and 6.0.

The pH of the solution also determines the type of binding that occurs between polyphenols and proteins. Binding at a low pH is generally favoured by hydrogen

bonds, but hydrophobic interactions may also be involved (Barbeau and Kinsella, 1983). At pHs close to the pI of the protein, hydrophobic interactions are favoured due to the minimalisation of charge repulsion (Barbeau and Kinsella, 1983) and covalent bonds are favoured by a high pH (Oh et al., 1980).

5.2.5 Polyphenol-protein non-specific binding: the effect of temperature

The binding affinities between polyphenols and proteins have also been reported to be affected by temperature. A decrease in binding affinity between BDK and -1,2,3,4,6-penta-*O*-galloyl-D-glucose (PGG) and a basic PRP and EGCG or PGG has been observed with an increase in temperature (3 ºC to 55 ºC) however increases or decreases in binding affinities in response to temperature are dependent on the polyphenol or protein under investigation (Charlton et al., 2002). An increase in complex formation and precipitation between BSA and PGG and between protein isolated from spinach leaves and chlorogenic acid was observed when the temperature increased from 4 ºC to 40 ºC (Barbeau and Kinsella, 1983; Hagerman et al., 1998) while the complex formation and precipitation of BSA by epicatechin$_{16}$ (4→8) catechin (EC_{16}-C) was not affected by temperature (Hagerman et al., 1998; Hofmann et al., 2006). Butler et al. (1984) also observed that the complex formed between polyphenols and proteins was temperature dependent as well as dependent on the protein under investigation. It was observed that the complex between procyanidin-B2 with BSA or lysozyme was not affected by differences in temperature, however the complex between procyanidin-B2 and trypsin weakened in response to an increase in temperature (Butler et al., 1984). An increase in complex formation due to temperature differences may be due to temperature-induced conformational changes in the

structure of the protein as protein conformational changes are known to alter the binding affinity for small ligand molecules (Barbeau and Kinsella, 1983).

5.2.6 Polyphenol-protein non-specific binding: the effect of structure

Along with pH and temperature, the size and structure of the protein or the polyphenol play an important role in polyphenol-protein binding. Polyphenol-protein binding affinities are dependent on the size of the protein; proteins which have a molecular weight less than 20 kDa have lower polyphenol-protein binding affinities than proteins with a molecular weight greater than 20 kDa, with the exception of PRPs (Hagerman and Butler, 1981).

The ability of polyphenols to bind to proteins is also dependent on the molecular size of the polyphenol (Beart et al., 1985; McManus et al., 1985; Ezaki-Furuichi et al., 1987; Bacon and Rhodes, 2000; de Freitas and Mateus, 2001; Bennick, 2002; Brás et al., 2010). For example, the binding affinity of galloylated D-glucose compounds to BSA increases incrementally with the addition of each phenolic ester group and reaches a maximum binding strength with β-penta-*O*-galloyl-D-glucose (Haslam, 1974; Beart et al., 1985; McManus et al., 1985). The same was observed with flavan-3-ols; the binding affinity was low for the monomers (epicatechin and catechin) and increased with the degree of polymerisation for the procyanidins (Ezaki-Furuichi et al., 1987; de Freitas and Mateus, 2002; Brás et al., 2010). While an increase in molecular weight of the polyphenol results in greater polyphenol-protein binding affinities, the conformational mobility and flexibility of the polyphenols is also important in polyphenol-protein binding (Beart et al., 1985; de Freitas and Mateus, 2001; Bennick, 2002). For example, quercetin has a stronger binding affinity to BSA compared to rutin (a glycoside of quercetin with the addition of the disaccharide rutinose) (Papadopoulou et al., 2005). While the molecular size of rutin is greater than quercetin the addition of

rutinonse causes the structure to become less hydrophobic and less flexible than quercetin resulting in a lower binding affinity.

Therefore, according to available data in the literature, the interactions between polyphenols and proteins are likely to be non-specific and reversible and hydrophobic interactions are likely to be the main binding interaction involved between polyphenols and proteins with hydrogen binding supporting the hydrophobic interactions. The ability of polyphenol-protein binding affinity is also dependent on the pH, temperature, and the size and structure of the polyphenol and of the protein.

5.3 Objectives

In the previous chapter it was established that certain polyphenols inhibit VEGF-induced VEGFR-2 phosphorylation by interacting with the VEGF molecule. Polyphenols are known to interact with and bind to proteins either non-covalently or covalently. Therefore, the objectives of this chapter were to achieve an understanding of the nature of the interaction between certain polyphenols (apple procyanidin fraction dp 3.9 or dp4 and EGCG) and VEGF.

5.4 Materials and methods

5.4.1 Materials

Human recombinant $VEGF_{165}$ was obtained from R&D Systems Europe Ltd (Abingdon, UK). Purified epigallocatechin gallate (EGCG) was purchased from Extrasynthese (Genay, France) and electrophoresis reagents were purchased from Invitrogen. The VEGFR-2, phospho-VEGFR-2 (Tyr 1175), the anti-rabbit IgG, HRP-linked antibody and the PathScan phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit were from Cell Signaling and the VEGF rabbit polyclonal antibody was purchased from Thermo Scientific. Apple procyanidin fraction dp 4 was purified from the 2007 apples (Chapter 3) provided by Coressence ltd (Herefordshire, UK) as previously described (Yanagida et al., 1999; Shoji et al., 2005; Shoji et al., 2006b), with some modifications. A brief description of the method can be found in Chapter 4 or a more detailed description of the method is located in the Supplementary Information (Supplementary Information 1).

5.4.2 Cell culture and flavanol treatments

5.4.2.1 Cell culture

The culture of HUVECs is described in Chapter 4, Section 4.4.2.

5.4.2.2 Flavanol treatments

Flavanol treatments were prepared in Universal tubes prior to adding them to the confluent monolayers. Appropriate flavanol concentrations were added to a Universal tube containing basal medium and VEGF (25 ng/ml) and incubated at room temperature for 5 min. Control treatments were prepared in the same manner. HUVEC confluent monolayers were washed twice with warm phosphate buffered saline (PBS),

to remove any residual growth factors, and prepared treatments were added to the respective wells and incubated for 5 min at 37 ºC under a humidified atmosphere containing 5 % CO_2.

5.4.3 VEGFR-2 phosphorylation measurement

The extraction of protein was the same method as described in Chapter 4, Section 4.4.6 and the measurement of phosphorylated VEGFR-2 by ELISA and Western blot followed the same procedure as described in Chapter 4, Section 4.4.7.

5.4.4 Removal of unbound polyphenol from VEGF

5.4.4.1 Removal of unbound polyphenol from VEGF using dialysis

Unbound polyphenol was removed from VEGF using a Slide-A-Lyzer MINI Dialysis Unit (Thermo Scientific) with a molecular weight cut off (MWCO) of 3500 Da. The concentration used for dialysis was increased by a factor of 56 from the control samples (the concentration in the control samples were 25 ng/ml VEGF and 0.3 µM apple procyanidin fraction dp 3.9 or dp 4). Thus the starting concentration of VEGF was 1400 ng/ml and 16.8 µM for apple procyanidin fraction dp 3.9 or dp 4. The samples were prepared with distilled water and incubated for 5 min, at room temperature, in an eppendorf prior to transferring to the dialysis unit. The samples were dialysed, in one litre of distilled water, for 2 h at room temperature. The retentate was then diluted in 4 ml of basal medium to give a final concentration of VEGF of 25 ng/ml. The samples were used to treat HUVECs for the determination of phosphorylated VEGFR-2 as described in Section 5.4.2.

5.4.4.2 Removal of unbound polyphenol from VEGF using centrifugal concentration

Unbound polyphenol was removed from VEGF using a MICROCON spin column (MICROCON) with a MWCO of 3000 Da. The concentration used for the spin column experiment was increased by a factor of 20 compared to the control samples (the concentration in the control samples were 25 ng/ml VEGF and 0.3 µM apple procyanidin fraction dp 3.9 or dp 4). Thus the starting concentration of VEGF was 500 ng/ml and 6 µM for apple procyanidin fraction dp 3.9 or dp4. The samples were prepared in basal medium and were incubated for 5 min, at room temperature, prior to transferring to the spin column. The solution was concentrated 20 fold by centrifugation, the concentrate was then diluted with basal medium and concentration was repeated. This process was repeated for a total of three times. The concentrate was diluted to the starting volume and then diluted in 4 ml of medium to give a final concentration of VEGF of 25 ng/ml. The samples were used to treat HUVECs for the determination of phosphorylated VEGFR-2 as described in Section 5.4.2.

5.4.5 SDS-PAGE

For reducing conditions, sample preparation included incubating 10 ng VEGF (761.61 ng/ml) with 30.5 µM apple procyanidin fraction dp 4 or EGCG for 5 min at room temperature. NuPAGE LDS Sample Buffer (4x) and DTT were added and the samples were denatured at 70 ºC for 10 min. Electrophoresis was carried out in a NuPAGE Novex 10% Bis-Tris gel using NuPAGE MES SDS Running Buffer (20x) and NuPAGE antioxidant. Proteins were transferred on to a 0.45 µM nitrocellulose membrane. Membranes were blocked with 5 % skimmed milk in TBST for 1 h at room temperature. The membrane was washed with TBST and followed by an overnight incubation with the primary antibody (25 µl VEGF rabbit polyclonal antibody in 5 ml of 5% BSA in TBST) at 4 ºC. The membrane was washed with TBST and incubated with the

secondary antibody (10 µl anti-rabbit IgG, HRP-linked antibody in 10 ml of 5 % skimmed milk in TBST) for 1 h at room temperature. The membranes were developed using Pierce SuperSignal West Pico chemiluminescent substrate and the fluorescence intensity was measured using a BioRad Fluor-S MultiImager.

The same procedure was used for non-reducing conditions with the exception that no DTT or NuPAGE antioxidant were added. Also, 20 ng of VEGF (1273.8 ng/ml) and 51 µM of apple procyanidin fraction dp 4 or EGCG was used.

5.4.6 Native-PAGE

VEGF (100 ng or 7843 ng/ml) was incubated with 314 µM of either apple procyanidin fraction dp 4 or EGCG for 5 min before the addition of 10 % n-dodecyl-β-D-maltoside (DMM) (final concentration 0.001 %) and NativePAGE 5 % G-250 Sample Buffer (4x). Electrophoresis was carried out in a NativePAGE Novex 4-16 % Bis-Tris Gel using anode buffer (NativePAGE Running Buffer [20x] plus distilled water) and cathode buffer (NativePAGE Running Buffer [20x], NativePAGE cathode buffer [20x] and distilled water). Before protein transfer on to a polyvinylidene fluoride (PVDF) membrane, the gel was incubated in 0.1 % sodium dodecyl sulphate (SDS) in order to create enough charge so that the proteins will migrate towards the anode side, and will not denature the proteins. Proteins were then fixed on the PVDF membrane by incubating the membrane in 20 ml of 8 % acetic acid for 15 min. Membranes were blocked with 5 % skimmed milk in TBST for 1 h at room temperature. The membrane was washed with TBST and followed by an overnight incubation with the primary antibody (25 µl VEGF rabbit polyclonal antibody in 5 ml of 5 % BSA in TBST) at 4 ºC. The membrane was washed with TBST and incubated with the secondary antibody (10 µl anti-rabbit IgG, HRP-linked antibody in 10 ml of 5 % skimmed milk in TBST) for 1 h at room temperature. The membranes were developed using Pierce SuperSignal West Pico

chemiluminescent substrate and the fluorescence intensity was measured using a BioRad Fluor-S MultiImager.

5.4.7 Isoelectric focusing (IEF)

VEGF (100 ng or 6666.66 ng/ml) was incubated with 267 µM of either apple procyanidin fraction dp 4 or EGCG for 5 min before the addition of Novex IEF Sample Buffer pH 3-10 (2x). Electrophoresis was carried out in a Novex pH 3-10 IEF gel using Novex IEF Anode Buffer and Novex IEF Cathode Buffer pH 3-10 (10x). The gel was equilibrated in a chilled solution of 0.7 % acetic acid for 10 min and then transferred on to a PVDF membrane. Membranes were blocked with 5 % skimmed milk in TBST for 1 h at room temperature. The membrane was washed with TBST and followed by an overnight incubation with the primary antibody (25 µl VEGF rabbit polyclonal antibody in 5 ml of 5 % BSA in TBST) at 4 ºC. The membrane was washed with TBST and incubated with the secondary antibody (10 µl anti-rabbit IgG, HRP-linked antibody in 10 ml of 5 % skimmed milk in TBST) for 1 h at room temperature. The membranes were developed using Pierce SuperSignal West Pico chemiluminescent substrate and the fluorescence intensity was measured using a BioRad Fluor-S MultiImager.

5.4.8 Proteolysis and peptide sequencing by mass spectrometry

VEGF (10 µg) was diluted in 20 mM ammonium bicarbonate (pH 7.0) to give a final concentration of 1.4 µg/ml. The solution was then aliquoted into seven eppendorfs; two vials were treated as VEGF controls (samples 1 and 2), two were treated with 56 µM apple procyanidin fraction dp 4 (samples 3 and 4) and two were treated with 56 µM EGCG (samples 5 and 6). The concentration of VEGF used was 56 times greater than what was used for cell treatments (25 ng/ml). In order to maintain the VEGF to polyphenol concentration ratio, the concentrations of apple procyanidin fraction dp 4

and EGCG used to treat the VEGF samples were also increased by a factor of 56. The samples were incubated for 19 h at 4 ºC and then at room temperature for 2 h. After incubation, the samples were dialysed for 2 h, against one litre of 20 mM ammonium bicarbonate (pH 7.0), in a Slide-A-Lyzer MINI dialysis unit. A small portion of the sample was diluted in basal medium to give a final concentration of VEGF of 25 ng/ml. The samples were used to treat HUVECs for the determination of phosphorylated VEGFR-2 as described in Section 5.4.2. The remainder of the samples were freeze-dried.

Once the samples were freeze-dried they were given to the IFR Proteomics Platform for sample preparation for analysis by mass spectrometry using the LTG Orbitrap (Thermo). Briefly, samples were dissolved in protein digest buffer (8M urea, 100 mM tris buffer pH 8.0 and 5 mM DTT) and incubated at room temperature for 2 h. Alkylation buffer (150 mM iodoacetamide) was added to samples 2, 4 and 6 and distilled water was added to samples 1, 3 and 5 and incubated in the dark for 20 min. Enzyme buffer (10 ml of 50 mM NH_4HCO_3 pH 7.8 and 100 µl of 1M $CaCl_2$) was added to each sample to give a final urea concentration of 1 M. Trypsin or trypsin plus chymotrypsin were added to each sample and incubated at 28 ºC overnight. A zip tip was then used to clean up the peptides for mass spectrometry using the OMIX protocol (Varian). The samples were then centrifuged to dryness using the speed vacuum and subsequently diluted in 0.5 % formic acid and analysed on the LTQ Orbitrap. The data derived from the LTQ Orbitrap were analysed and interpreted by the IFR Proteomics Platform and the resulting sequence information was received.

5.5 Results

5.5.1 VEGF activity is not recovered from the VEGF-polyphenol complex following dialysis and dilution

The first question to be asked in the investigation of the nature of the interaction between VEGF and polyphenols was whether the polyphenol could be separated from the VEGF-polyphenol complex by dialysis or dilution. If the interaction between VEGF and the polyphenol is non-covalent and does not involve a change in protein conformation resulting in tight non-covalent binding, then the polyphenol should be removed by these methods and the VEGF activity recovered.

All experiments in which VEGF activity was being assessed included a no VEGF treatment (vehicle only) and a VEGF only treatment as controls. No phosphorylation of VEGFR-2 was observed in vehicle only treated cells while untreated VEGF was able to induced VEGFR-2 phosphorylation (Figure 5.4, 5.5 and 5.6). Incubation of apple procyanidin fraction dp 3.9 or dp 4 (0.3 µM) with VEGF reduced the ability of VEGF to induce VEGFR-2 phosphorylation compared to the VEGF control. An observed 30 % to 70 % inhibition of VEGF-induced VEGFR-2 phosphorylation was observed with the apple procyanidin fraction dp 3.9 and dp 4 treatments which is in keeping with the IC_{50} of apple procyanidin fraction dp 3.9 (0.32 µM) and apple procyanidin fraction dp 4 (0.3 µM) (Chapter 4, Figure 4.13).

After a 2.5 h incubation at room temperature (the period of time used for dialyzing and diluting) untreated VEGF still retained its VEGFR-2 activating activity, but 0.3 µM apple procyanidin dp 4-treated VEGF was now able to completely inhibit VEGFR-2 phosphorylation (Figure 5.6). Repeated concentration and dilution of VEGF with a spin column reduced the activity of VEGF by approximately 50 % relative to the control

(Figure 5.6). This loss of activity was most likely due to non-specific binding of VEGF to the spin column membrane, as VEGF still retained its activity after incubation at room temperature for 2.5 h. No reduction in VEGF activity was observed when VEGF was dialysed. Therefore, based on these results dialysis is a better method in retaining the VEGFR-2 activating activity of VEGF.

VEGF-induced VEGFR-2 phosphorylation was completely inhibited in apple procyanidin fraction dp 3.9-treated VEGF dialysed and concentrated samples (analysis by Western blot; Figure 5.4 and 5.5). Dialysis of apple procyanidin fraction dp 4-treated VEGF samples resulted in a 50 % inhibition of VEGF-induced VEGFR-2 phosphorylation, whereas concentrating the apple procyanidin fraction dp 4-treated VEGF samples completely inhibited VEGF-induced VEGFR-2 phosphorylation (analysis by ELISA, Figure 5.6). The difference in the amount of retained VEGF activity is due to the length of incubation of the polyphenol and protein during the experimental procedures. In the case of dialysis, the un-bound polyphenol would have been removed in the first 10 min of the dialysis and no further complex formation between the polyphenol and protein occurs. Therefore the apple procyanidin fraction dp 4-treated VEGF samples retained 50 % of the activity of VEGF because the IC_{50} concentration was used. One cycle of concentration of VEGF through the use of spin columns takes approximately 1 h. Therefore, the polyphenol and VEGF have a longer incubation period and further complex formation between the polyphenol and VEGF can occur during this time. Thus, VEGF-polyphenol complex formation is dependent on the length of incubation.

These results demonstrate that the interaction between VEGF and apple procyanidin fraction dp 3.9 and dp 4 is not a standard non-covalent interaction. The interaction involved between the VEGF-polyphenol complex may therefore be a covalent

interaction or a strong non-covalent interaction which results on the conformational change in the protein.

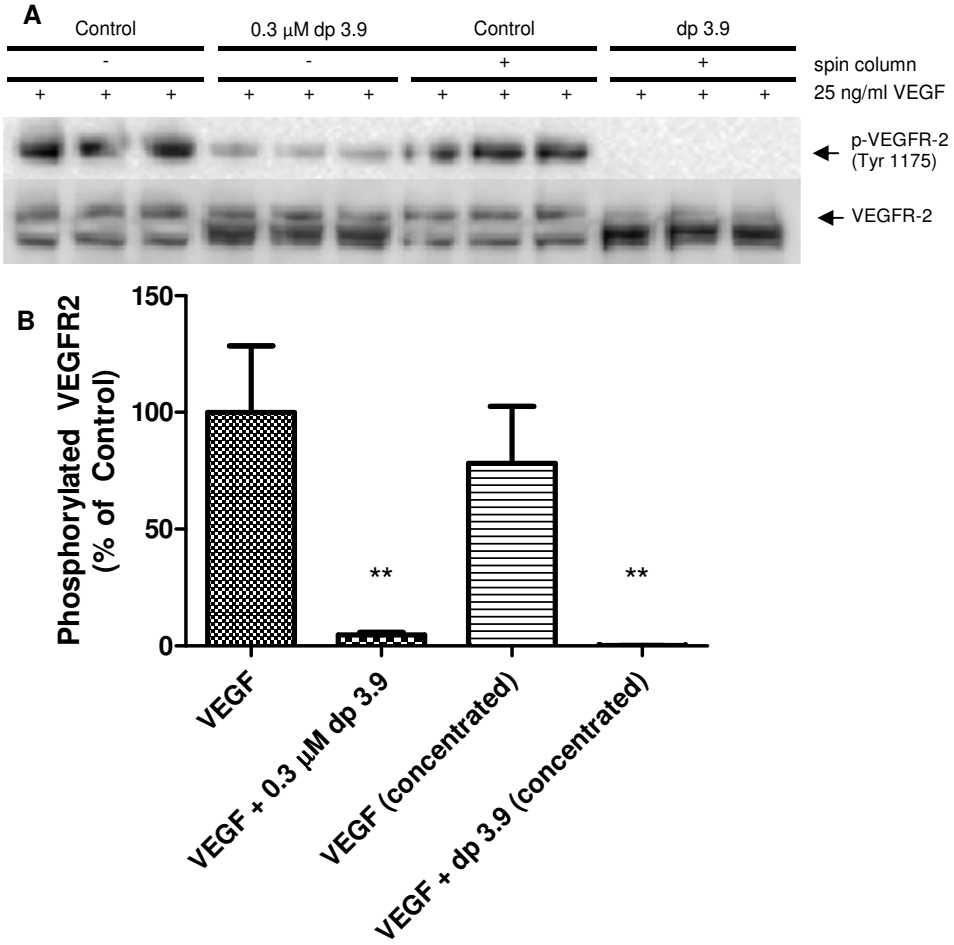


Figure 5.4: Inhibition of the VEGFR-2 activation activity of VEGF by apple procyanidin fraction dp 3.9 is retained after removal of unbound polyphenol using a centrifugal membrane concentrator device.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml VEGF and 0.3 µM apple procyanidin fraction dp 3.9 or samples prepared by concentrating by spin column for 5 min. For the spin column samples, VEGF (500 ng/ml) was incubated with or without apple procyanidin fraction dp 3.9 (6 µM) in basal medium for 5 min at room temperature. The solution was concentrated 20 fold by centrifugation using a MICROCON spin column. The concentration solution was diluted with basal medium and re-constituted. This process was repeated for a total of three times. Finally the concentrate was diluted to give a final concentration of VEGF of 25 ng/ml. The treatments were added to HUVECs and incubated for 5 min, the cells were then lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 levels. **(A)** Western blot data; **(B)** semi-quantitative data. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. ** p <0.01 compared to the stimulated cells (stimulated = 25 ng/ml VEGF). The data presented are representative of two independent experiments.

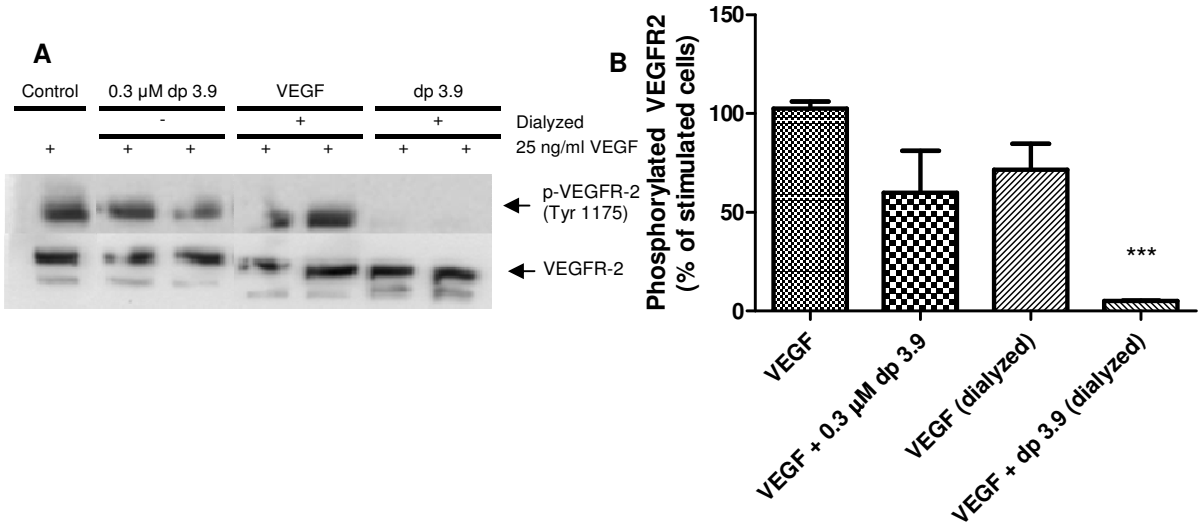


Figure 5.5: Inhibition of the VEGFR-2 activation activity of VEGF by apple procyanidin fraction dp 3.9 is retained after removal of unbound polyphenol using a dialysis membrane.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml VEGF and 0.3 µM apple procyanidin fraction dp 3.9 or samples prepared by concentrating by dialysis for 5 min. For the dialysed samples, VEGF (1400 ng/ml) was incubated with or without apple procyanidin fraction dp 3.9 (16.8 µM) in basal medium for 5 min at room temperature. The solution was dialysed using a Slide-A-Lyzer MINI dialysis unit. The retentate was diluted to give a final concentration of VEGF of 25 ng/ml. The treatments were added to HUVECs and incubated for 5 min, the cells were then lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 levels. (**A**) Western blot data; (**B**) semi-quantitative data. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. *** p <0.001 compared to the stimulated cells (stimulated = 25 ng/ml VEGF). The data presented are representative of two independent experiments.

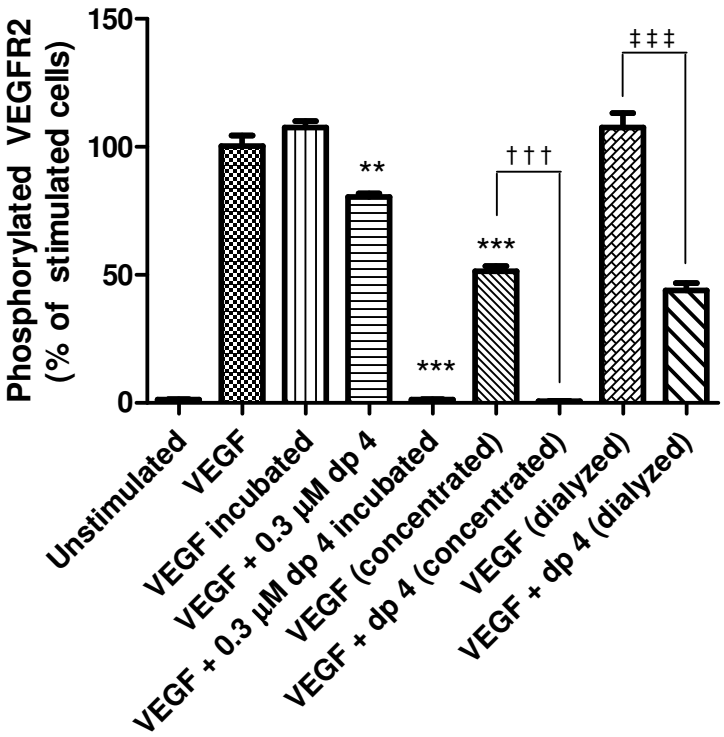


Figure 5.6: Inhibition of the VEGFR-2 activation activity of VEGF by apple procyanidin fraction dp 3.9 is retained after removal of unbound polyphenol using a centrifugal membrane concentrator device and a dialysis membrane.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml VEGF and 0.3 µM apple procyanidin fraction dp 4 or samples prepared by concentrating by spin column or dialysis for 5 min. For the spin column samples, VEGF (500 ng/ml) was incubated with or without apple procyanidin fraction dp 4 (6 µM) in basal medium for 5 min at room temperature. The solution was concentrated 20 fold by centrifugation using a MICROCON spin column. The concentration solution was diluted with basal medium and re-constituted. This process was repeated for a total of three times. Finally the concentrate was diluted to give a final concentration of VEGF of 25 ng/ml. For the dialysed samples, VEGF (1400 ng/ml) was incubated with or without apple procyanidin fraction dp 4 (16.8 µM) in basal medium for 5 min at room temperature. The solution was dialysed using a Slide-A-Lyzer MINI dialysis unit. The retentate was diluted to give a final concentration of VEGF of 25 ng/ml. The treatments were added to HUVECs and incubated for 5 min, the cells were then lysed and the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. ** p <0.01, *** p <0.001 compared to the stimulated cells; ††† p < 0.001 compared to VEGF (spin column); and ‡‡‡ p < 0.001 compared to VEGF (dialysis). Unstimulated = no VEGF, VEGF = 25 ng/ml VEGF, incubation = 2.5 h. The data presented are representative of duplicate biological and duplicate technical replicates of one independent experiment.

5.5.2 Possible precipitation of VEGF by polyphenols

The inhibition of VEGF-induced VEGFR-2 phosphorylation may be the result of the aggregation of the VEGF protein by the polyphenol. Therefore experiments were conducted in order to investigate the possible precipitation of the VEGF-polyphenol complex.

In order to assess whether precipitation occurs as a result of VEGF-polyphenol complex formation, 25 ng/ml of VEGF was incubated with either the vehicle, 1 µM apple procyanidin fraction dp 4, EGCG or epicatechin in a volume of 2 ml of basal medium for 5 min at room temperature. The solution was then centrifuged so that any insoluble protein would be located in the pellet and the soluble protein would be located in the supernatant. A pellet was not visible in any of the samples; therefore a small percentage (~ 100 µl) of the supernatant was retained in order to ensure the pellet was not removed. HUVECs were then treated with the supernatant and the reconstituted pellets for 5 min.

In the non-polyphenol treated VEGF samples, the majority of the VEGF was located in the supernatant and a small percentage was located in the pellet as observed by the darker band for VEGFR-2 phosphorylation in the supernatant samples (Figure 5.7). The phosphorylation of VEGFR-2 in the VEGF pellet sample was almost certainly due to the presence of the residual supernatant.

VEGF-induced VEGFR-2 phosphorylation was inhibited by apple procyanidin fraction dp 4 and EGCG in the supernatant and pellet samples (Figure 5.7). Epicatechin, a structurally similar polyphenol to apple procyanidin fraction dp 4 and EGCG, which was shown to be ineffective in inhibiting VEGF-induced VEGFR-2 phosphorylation

(Chapter 4, Section 4.5.3) did not cause VEGF to precipitate upon centrifugation. The majority of VEGF was located in the supernatant and a small percentage of VEGF was located in the pellet due to the presence of residual supernatant (Figure 5.7).

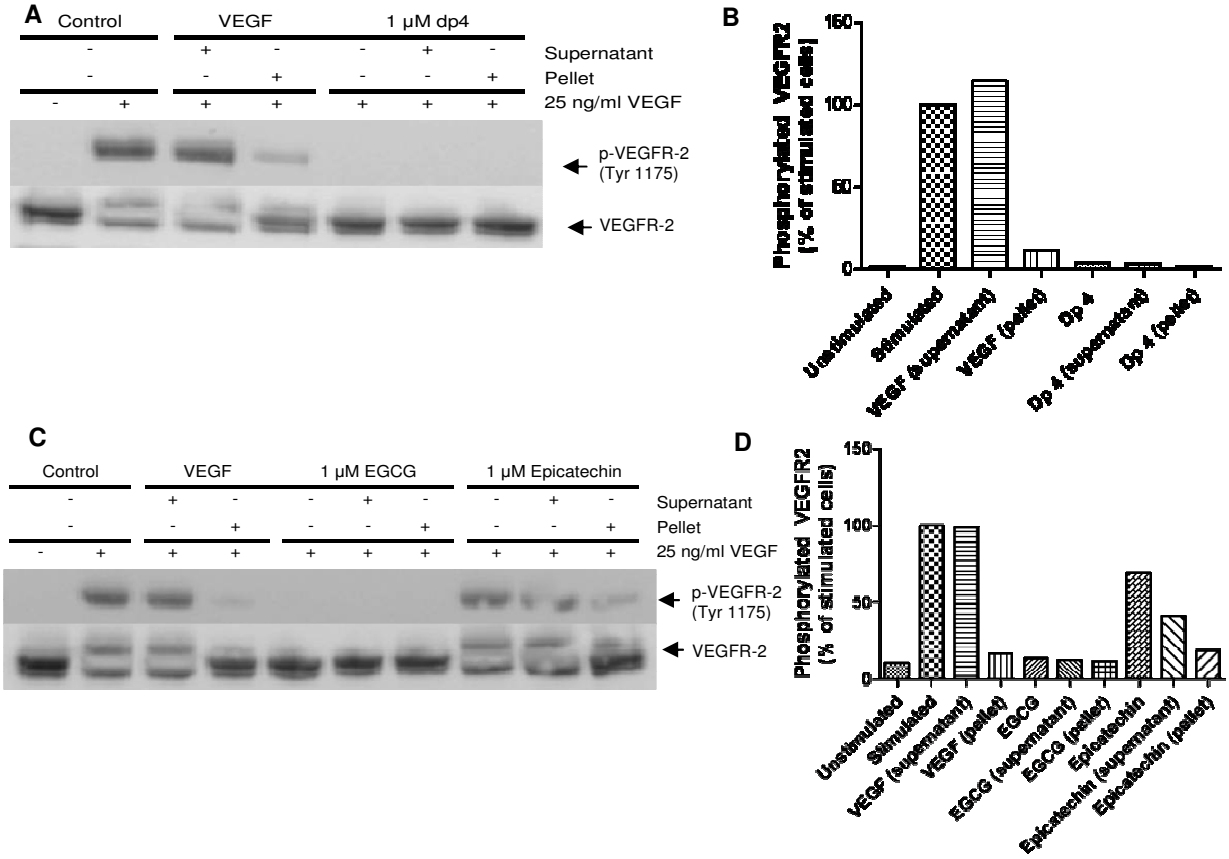


Figure 5.7: VEGF precipitation test of apple procyanidin fraction dp 4, EGCG and epicatechin.

HUVECs were treated with pre-incubated basal medium containing 25 ng/ml VEGF and 1 µM apple procyanidin fraction dp 4 **(A & B)**, EGCG **(C & D)** or epicatechin **(C & D)** for five min or with samples prepared for the precipitation test. For the precipitation test samples, VEGF (25 ng/ml) was incubated with or without apple procyanidin fraction dp 4 (1 µM), EGCG (1 µM), or epicatechin (1 µM) in basal medium for 5 min and centrifuged for 120 min at 5000 g. The supernatants were collected for the treatments and the pellets were re-suspended in 4 ml of basal medium. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2 levels. **(A & C)** Western blot images; **(B & D)** semi-quantitative data from Western blots. The data presented are representative of two independent experiments.

However as the cell assay used determines the activity of VEGF through the phosphorylation of VEGFR-2, it is unable to detect the location of VEGF in the apple procyanidin fraction dp 4 and EGCG treated VEGF samples as these treatments inhibit the activity of VEGF. Therefore in order to assess whether the VEGF was located in the supernatant or the pellet of the apple procyanidin fraction dp 4 or EGCG treated VEGF, the detection of VEGF protein was done using Western blotting (i.e. antibody detection of VEGF).

One hundred nanograms of VEGF protein was either incubated with the vehicle (0.1 % DMSO) or 350 µM apple procyanidin fraction dp 4 or EGCG for 5 min. The experiment was conducted in sterile water and the use of higher concentrations of VEGF and polyphenols were required for VEGF antibody detection. After incubation, the samples were centrifuged so that any insoluble protein would be located in the pellet and the soluble protein would be located in the supernatant. A pellet was not visible in any of the samples; therefore a small percentage of the supernatant was retained in order to ensure the pellet was not removed. The pellet was then reconstituted with water and the samples were run on a SDS-PAGE gel prior to antibody detection.

Under reducing conditions, VEGF and apple procyanidin fraction dp 4 treated VEGF samples have a molecular weight of approximately 21 kDA (Figure 5.8). After centrifugation of the VEGF sample, VEGF was located in the pellet and no VEGF was located in the supernatant. This same observation was seen in the apple procyanidin fraction dp 4 or EGCG treated VEGF samples. The location of VEGF in the pellet of the non-polyphenol-treated VEGF and the polyphenol-treated VEGF samples may be due to the high concentrations of VEGF that were used or the poor solubility of VEGF in sterile water. Therefore, these experiments have not demonstrated whether or not

precipitation of the VEGF-polyphenol complex occurs and further analysis using different buffers (e.g. ammonium bicarbonate) may provide evidence to show whether the complex is precipitable.

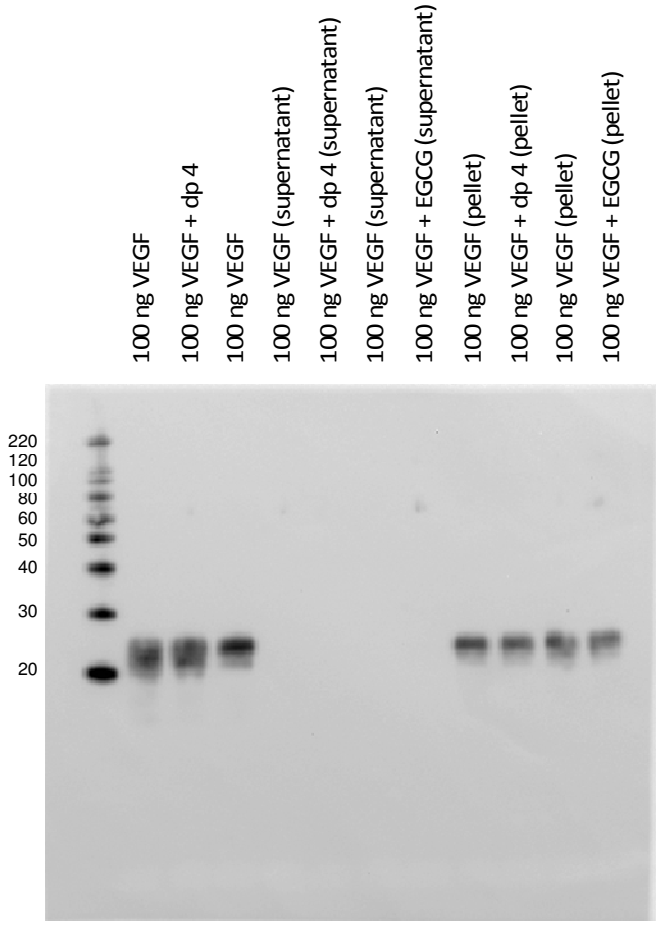


Figure 5.8: SDS-PAGE gel (reducing conditions) testing whether polyphenol (apple procyanidin fraction dp 4 or EGCG) treatments precipitate the protein (VEGF).

VEGF (100 ng) was incubated with or without 304.6 µM apple procyanidin fraction dp 4 or EGCG for 5 min and then either loaded onto the gel or centrifuged at 13000 x g for 30 min prior to the completion of the electrophoresis method described in the Section 5.4.5. This data is representative of two independent experiments.

5.5.3 Binding interactions between apple procyanidin fraction dp 4 and EGCG investigated by SDS-PAGE, native-PAGE and isoelectric focusing gels

The effect of incubating VEGF with polyphenols on VEGF gel electrophoretic properties was investigated. First, apple procyanidin fraction dp 4 and EGCG treated VEGF was analysed using sodium dodecylsufate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE gels separate proteins according to their molecular weight under denaturing conditions. SDS is the denaturing agent used and it denatures and unfolds the proteins by wrapping around the hydrophobic portions of the protein. The SDS-PAGE gels were run under both reducing and non-reducing conditions. Under reducing conditions, DTT is included which is a strong reducing agent that cleaves protein disulfide bonds (cysteine-cysteine bonds). DTT therefore will cleave disulfide bonds in the VEGF protein, and cause the reduction of VEGF from a dimer to a monomer.

Under reducing conditions, VEGF has an apparent molecular weight of 21 kDa (Figure 5.9A). In the apple procyanidin fraction dp 4 and EGCG treated VEGF samples, the apparent molecular weight of the samples was also 21 kDa. Under non-reducing conditions, VEGF has an apparent molecular weight of 40 kDa (Figure 5.9B). In the apple procyanidin fraction dp 4 and EGCG treated VEGF samples the apparent molecular weight of the samples was also 40 kDa. Therefore the polyphenol is not causing a modification of the VEGF protein mass under reducing or non-reducing conditions and the interaction between VEGF and the polyphenol is most likely not due to covalent binding.

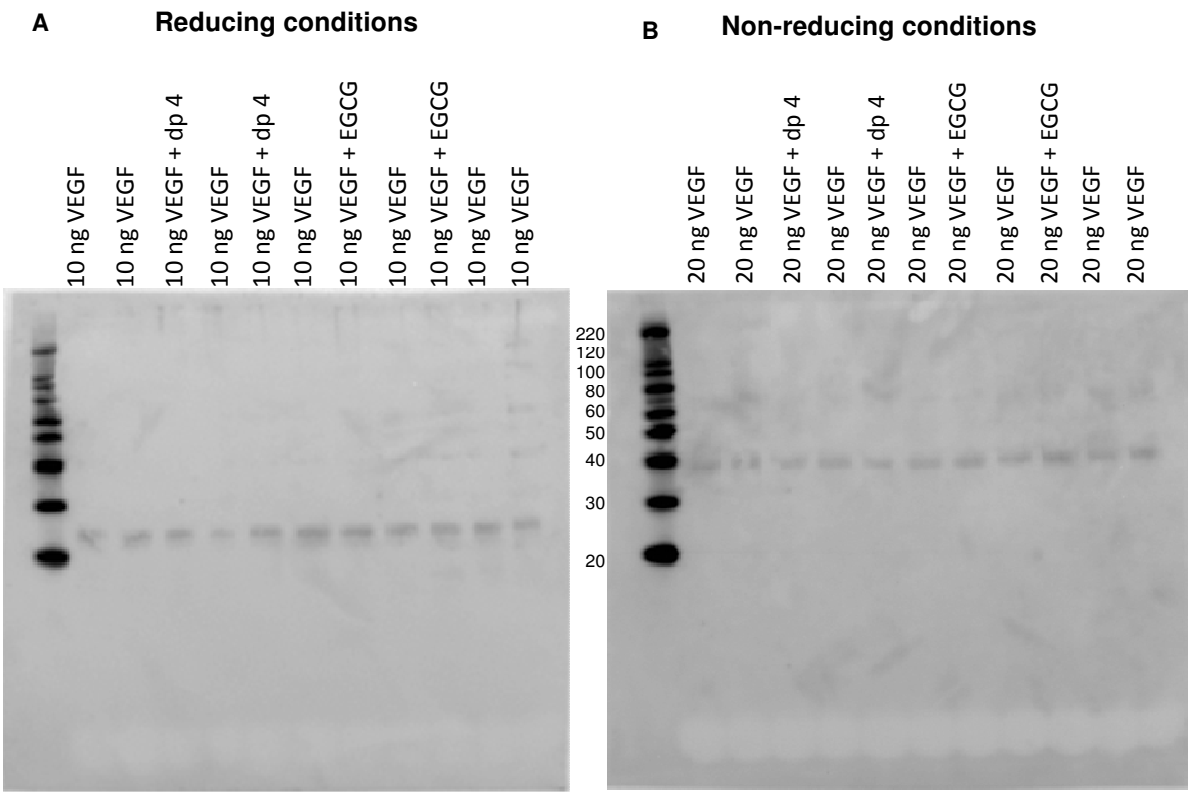


Figure 5.9: SDS-Page gels of control (VEGF) and treatments (VEGF and apple procyanidin fraction dp4 or EGCG).

VEGF (10 ng) was incubated for 5 min with or without 30.5 µM apple procyanidin fraction dp 4 or EGCG prior to reduction and electrophoresis procedure described in Section 5.4.5 (**A**); or VEGF (20 ng) was incubated with or without 51 µM apple procyanidin fraction dp 4 or EGCG for 5 min prior to the electrophoresis procedure described in Section 5.4.5 (**B**). This data is representative of three independent experiments.

Next, the effects of polyphenol treatments on VEGF were examined under native-PAGE conditions. Native-PAGE gels are preformed under native or non-denaturing conditions that maintain the native protein conformation, subunit interaction and biological activity. Proteins on native-PAGE gels are separated based on their charge to mass ratios. Native-PAGE gels are also run under milder conditions than SDS-PAGE gels and therefore strong non-covalent binding of the polyphenol to the protein may be retained during migration through the gel. The native-PAGE gel results showed diffuse bands covering a large molecular weight range that were not suitable for detecting small changes in the mass ratios (Figure 5.10).

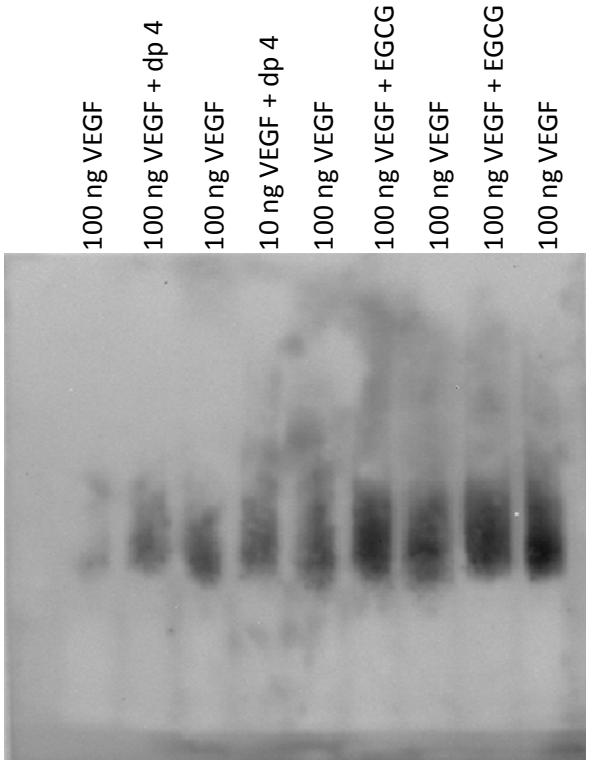


Figure 5.10: Native-PAGE gel of controls (VEGF) and treatments (VEGF and apple procyanidin fraction dp 4 or EGCG).

VEGF(100 ng) was incubated with or without 313.7 µM apple procyanidin fraction dp 4 or EGCG for 5 min prior to the electrophoresis procedure described in Section 5.4.6. This data is representative of three independent experiments.

The last method used to investigate the effect of polyphenol treatment on VEGF gel migration properties was isoelectric focussing (IEF). IEF, similar to native-PAGE, is run under milder conditions to SDS-PAGE gels but will detect changes in the pI of a protein. The pI is the pH at which a protein has no net charge and will not migrate further in an electric field. A dark band was detected in lane 3 (100 ng VEGF + dp 4) and faint bands were observed in lane 4 (100 ng VEGF), lane 5 (100 ng VEGF + dp 4) and lane 6 (100 ng VEGF, Figure 5.11). The pI range of the pH 3-10 IEF gels used was between 3.5 and 8.5 and the pI of VEGF has been reported to be between 8 and 8.5 (Ferrara et al., 1991; Patterson et al., 2010). Therefore VEGF is located at the upper range limit of the gel. Since the pI of VEGF is at the upper range limit of the gel, the majority of the VEGF appears to have migrated off the gel. Since a source of higher pH

range IEF gels could not be identified, it was not possible to establish whether or not the polyphenol treatment of VEGF affected the pI of VEGF.

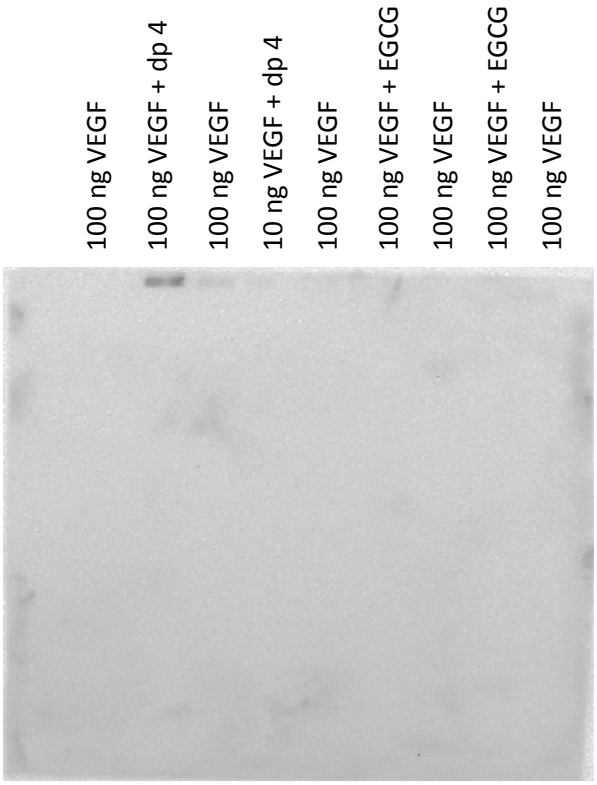


Figure 5.11: IEF gel of controls (VEGF) and treatments (VEGF and apple procyanidin fraction dp 4 or EGCG).

VEGF (100 ng) was incubated with or without 266.8 µM apple procyanidin fraction dp 4 or EGCG prior to the electrophoresis method described in Section 5.4.7. This data is representative of two independent experiments.

5.5.4 Pre-incubation with 0.2 µM apple procyanidin fraction dp 4 inhibits VEGF induced VEGFR-2 phosphorylation in a time dependent manner.

In Section 5.5.1 it was shown that incubating VEGF with 0.3 µM apple procyanidin fraction dp 4 (its IC_{50}) for 2.5 h resulted in complete inhibition of VEGF activity (no induction of VEGFR-2 phosphorylation observed in HUVECs). Since a 5 min incubation of the apple procyanidin fraction dp 4 with VEGF only resulted in an approximate 50 % inhibition of VEGF activity, it appeared that the inhibition of VEGF activity was time-dependent. In order to investigate the kinetics of the inhibition of VEGF activity by apple procyanidin fraction dp 4, a time course was conducted.

Apple procyanidin fraction dp 4 (0.2 µM) was incubated with 25 ng/ml VEGF in basal medium for 5, 10, 15, 30, 60 and 120 min prior to cell treatment. A concentration of 0.2 µM was used because it is below the IC_{50} of apple procyanidin fraction dp 4 and therefore changes in VEGF activity could be monitored over time. The treatment of VEGF with 0.2 µM apple procyanidin fraction dp 4 resulted in an inhibition of VEGF-induced VEGFR-2 phosphorylation in a time-dependent manner (Figure 5.12).

A 5 min pre-incubation of VEGF and apple procyanidin fraction dp 4 resulted in a 30 % inhibition of VEGF-induced VEGFR-2 phosphorylation and after a 10 min pre-incubation, no further inhibition of VEGFR-2 phosphorylation occurred. A pre-incubation period of 15 min resulted in a 70 % inhibition of VEGFR-2 phosphorylation and no further changes in the amount of phosphorylated VEGFR-2 occurred after a 30 min incubation. A 60 min pre-incubation reduced the amount of phosphorylated VEGFR-2 by 75 % and a 120 min pre-incubation resulted in a 90 % inhibition of VEGF-induced VEGFR-2 phosphorylation. A complete inhibition of VEGFR-2 phosphorylation was not achieved during this time course.

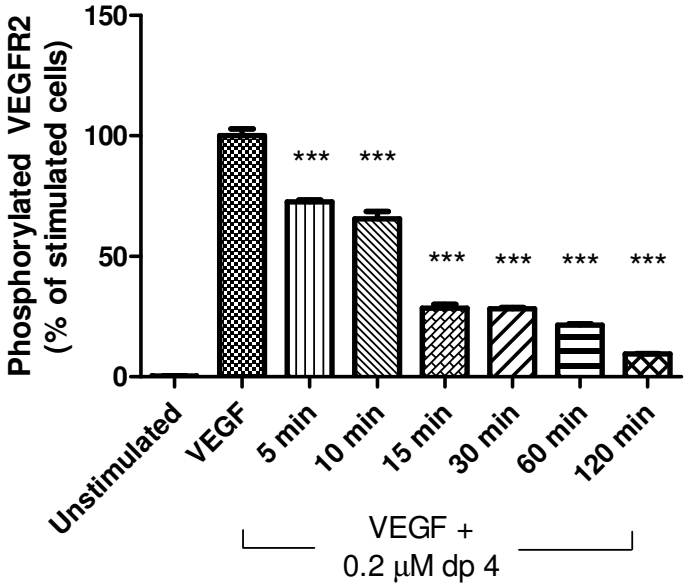


Figure 5.12: Apple procyanidin fraction dp 4 inhibits VEGF induced VEGFR-2 phosphorylation in a time dependent manner.

VEGF (25 ng/ml) and 0.2 µM apple procyanidin fraction dp 4 were pre-incubated in basal medium for 5, 10, 15, 30, 60 and 120 min. HUVECs were then treated for 5 min with the pre-incubated basal medium containing VEGF and apple procyanidin fraction dp 4. The cells were lysed and the amount of phosphorylated VEGFR-2 was quantified by a phospho-VEGFR-2 (Tyr1175) ELISA. Bars represent means ± standard deviation expressed as a percentage of the stimulated cells. *** p <0.001 compared to the stimulated cells. The data presented is representative of one independent experiment of biological duplicates and technical duplicates.

The data presented in Figure 5.12 appears to show that the inhibition of VEGF activity is not linear. Therefore the data were then presented as a scatter plot to determine whether the data fits a nonlinear regression model (Figure 5.13). However, the data presented in Figure 5.13 show that a nonlinear regression model is not a good fit of the data as the 10 min and 15 min time points do not fit the curve.

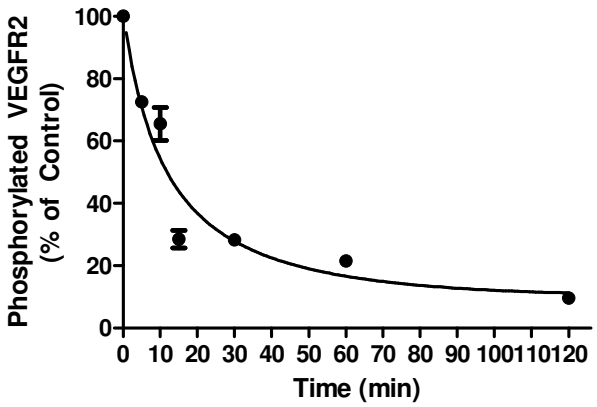


Figure 5.13: Apple procyanidin fraction dp 4 inhibits VEGF induced VEGFR-2 phosphorylation in a time dependent manner (nonlinear regression fit of the data from Figure 5.12).

Further evidence that the kinetic data does not fit a first order reaction model was obtained by log transforming the data (Figure 5.14A). By visual inspection, a linear model did not fit the data as the data were not normally distributed along the line. However, the transformation of the data indicated that a multi-stage binding process might occur between the polyphenol and VEGF. The first stage in the binding process appears to be rapid and occurs between 0 and 15 min where a 30 % reduction in VEGF activity is observed (Figure 5.14B). The second stage, which is slower, occurs between 15 min and 120 min where the VEGF activity is reduced from 70 % to 10 % (Figure 5.14B). This data suggest that the interaction between VEGF and apple procyanidin fraction dp 4 is not a simple process and may involve the conformational change of the VEGF protein by apple procyanidin fraction dp 4.

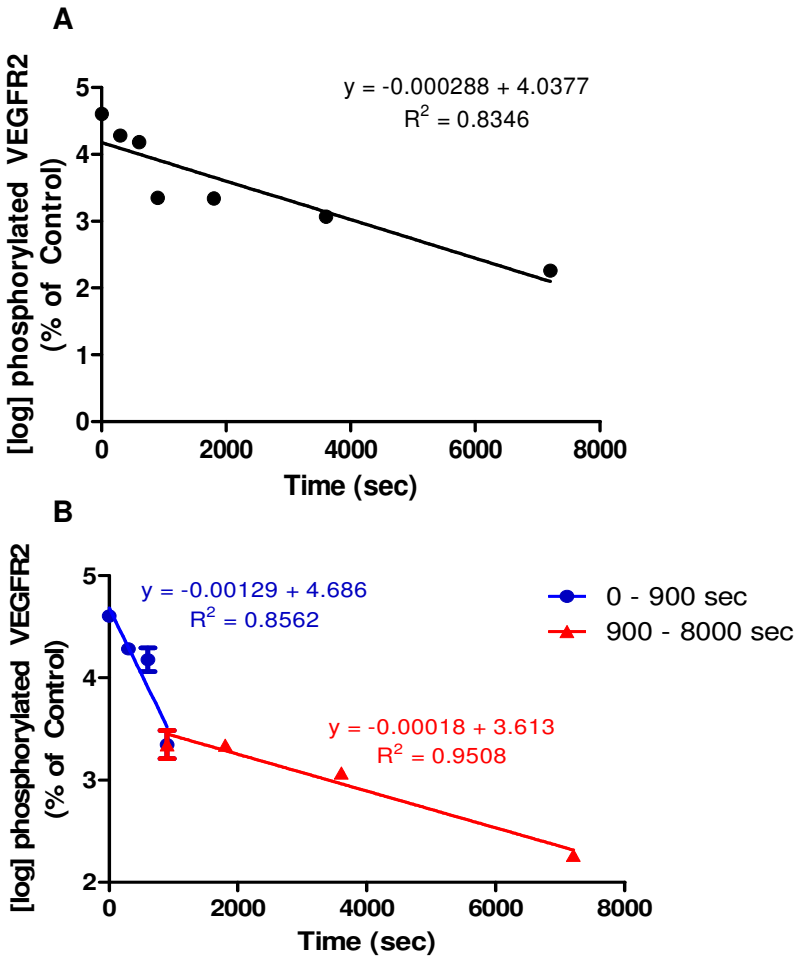


Figure 5.14: Apple procyanidin fraction dp 4 inhibits VEGF induced VEGFR-2 phosphorylation in a time dependent manner (log transform of the data from Figure 5.12).

From the log transform of the data, a rate constant can be determined. A rate constant describes the speed of which a reaction occurs. While the determination of the rate constant in this instance is only approximate due to the small data set, the reaction in the first stage of the binding process has a rate constant of 1.29 x 10^{-3} s^{-1} and has a half-life of 533 sec (8.9 min) (Figure 5.14B). The reaction in the second stage of the binding process has a rate constant of 0.18 x 10^{-3} s^{-1} and has a half-life of 3850 sec (64.2 min) (Figure 5.14B). This confirms that the first stage occurs at a quicker rate than the second stage of the binding process.

5.5.5 Analysis of VEGF-polyphenol complexes using mass spectrometry

The final approach used to assess the nature of the interaction between polyphenols (apple procyanidin fraction dp 4 or EGCG) and VEGF involved the use of mass spectrometry. Mass spectrometry was used to obtain sequence data of VEGF and to determine whether there were any modifications in the VEGF sequence after polyphenol treatment. VEGF (1.4 µg/ml) was incubated with 56 µM of either apple procyanidin fraction dp 4 or EGCG for 21 h and then dialysed for 2 h to remove any excess polyphenol. In order to confirm the VEGF activity status of the control and treated VEGF samples, VEGF-induced VEGFR-2 phosphorylation was assessed by cell culture. The un-treated VEGF samples induced VEGFR-2 phosphorylation but VEGF-induced VEGFR-2 phosphorylation was inhibited in the treatment samples (VEGF plus apple procyanidin dp 4 or EGCG) (Figure 5.16A&B and 5.17A&B). After confirmation of VEGF activity in the control and treatment samples, VEGF and VEGF plus apple procyanidin fraction dp 4 or EGCG samples were run on an Orbitrap mass spectrometer to determine whether any modification in the mass of VEGF could be detected in the polyphenol treated samples.

The VEGF protein was not detected on the Orbitrap mass spectrometer in its native state (data not shown), probably because the molecular weight of VEGF was too large to be detected. The enzymatic digestion of VEGF and the polyphenol-treated VEGF was therefore required to detect any differences in the sequence of VEGF by Orbitrap MS. In the first instance trypsin was used as the enzyme. Trypsin cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine except when either is followed by proline (Figure 5.15). Using trypsin as the enzyme resulted in an approximate 33 % coverage of the VEGF sequence (Figure 5.16C). Because only a small percentage of the sequence could be detected after trypsin digestion, it could not

be determined whether the polyphenol treatment modified the VEGF sequence. However, in the areas of the sequence covered, there was no difference between the VEGF-control and VEGF-polyphenol treated samples.

MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV
PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC
ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE
LNERTCRCDK PRR

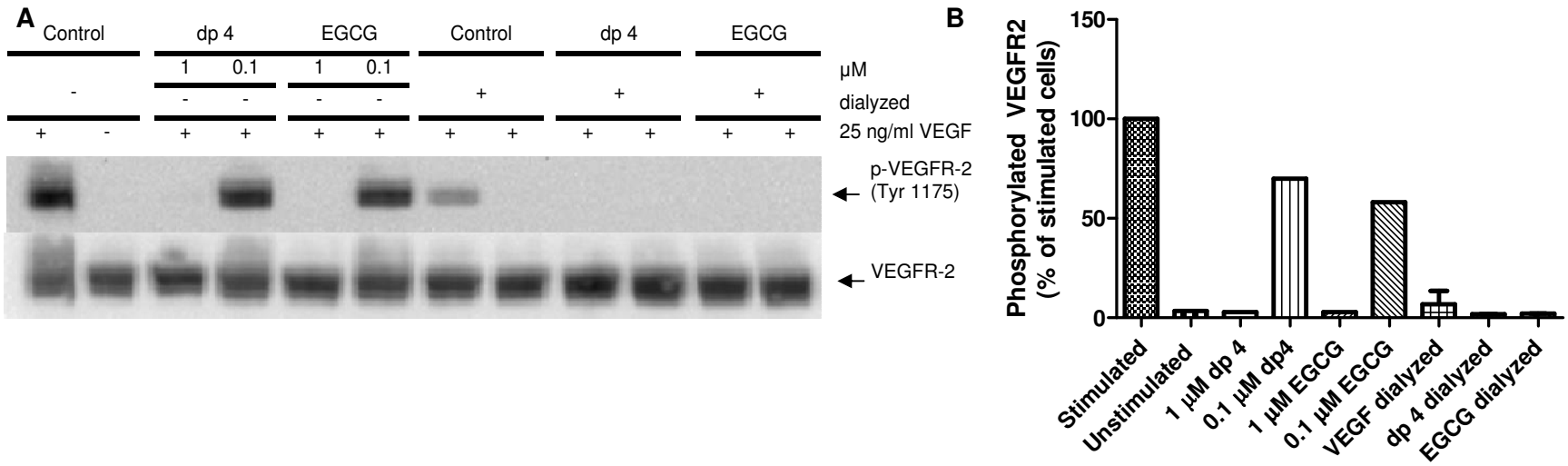
Figure 5.15: Amino acids on the VEGF sequence where trypsin and chymotrypsin are predicted to cleave the peptide chain.

The peptide chain cleavages occur on the carboxyl side (the right side) of the marked amino acids. Trypsin cleaves after amino acids lysine (K) and arginine (R) except when either is followed by proline (P) (cleavage sites marked in red). Chymotrypsin cleaves after amino acids tyrosine (Y), tryptophan (W) or phenylalanine (F) (cleavage sites are marked in green).

Therefore in order to get more coverage of the VEGF sequence two enzymes, trypsin and chymotrypsin, were used in the digestion. Trypsin cleaves amino acids lysine and arginine and chymotrypsin cleaves peptide chains at the carboxyl side of the amino acids tyrosine, tryptophan or phenylalanine (Figure 5.15). Using both trypsin and chymotrypsin in the digestion process resulted in a greater coverage (approximately 67 % coverage) than just using trypsin alone (Figure 5.17C). However there are still large parts in the VEGF sequence (amino acids 52 – 80, 135 – 147, and 155 – 163) for which there is a lack of sequence information. In order to be certain if any modifications in the VEGF sequence are occurring with the polyphenol treatment, full coverage of the VEGF sequence is required.

However, with the coverage achieved with the trypsin and chymotryspin digestion there are parts in the VEGF sequence that can be seen with the VEGF samples but not with the polyphenol treated samples. The amino acid region 95 – 105 in the un-treated VEGF samples are always detected, however in the polyphenol-treated VEGF

samples, sometimes the peptide is observed and sometimes it is not. There are inconsistencies in whether this region has been detected. In the apple procyanidin fraction dp 4-treated VEGF, the amino acid region was not detectable in the first set of data (VEGF + dp 4 [3.1] and [3.2]) and in one measurement of the second data set (VEGF + dp 4 [4.1]) while in the second measurement the amino acid regions 100 to 105 are detectable. In the EGCG-treated VEGF, the amino acid regions 95 – 105 are only detectable in one set of the measurements of each data set (VEGF + EGCG [5.1], [6.1]). These inconsistencies in the observation of the amino acid regions 95 – 105 are most likely due to a detection problem rather than do to a potential modification in the protein sequence because the duplicate biological replicates and the duplicate technical replicates are not consistent.



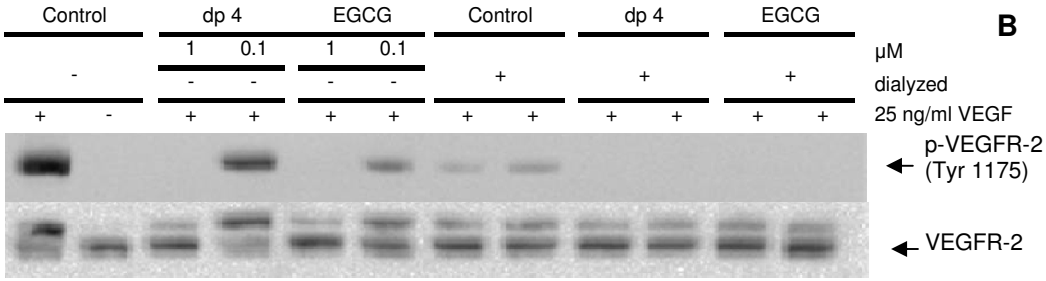
C

Treatment	VEGF Sequence
	10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 163
VEGF (1)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF (2)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (3)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (4)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (5)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (6)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR

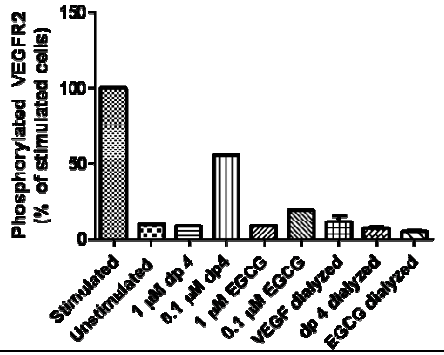
Figure 5.16: Partial coverage of the VEGF sequence is detected by LTQ Orbitrap using trypsin as the digestion enzyme.

VEGF (10 μg) was diluted in 20 mM ammonium bicarbonate (pH 7.0) to give a final concentration of 1.4 μg/ml. The solution was aliquoted into eppendorfs and treated with either 56 μM of apple procyanidin dp4 (VEGF + dp4 3 and 4) or EGCG (VEGF + EGCG 5 and 6). The solutions were incubated overnight and then subsequently dialyzed against ammonium bicarbonate for 2 h. A portion of the sample was diluted to give a final concentration of 25 ng/ml VEGF and applied to HUVECs for subsequent Western Blot determination of VEGFR-2 phosphorylation (**A**) and the semi-quantitative data (**B**). The remainder of the sample was freeze-dried and digested, alkylated, treated with trypsin and analyzed by LTQ Orbitrap (**C**). The sample preparation for the mass spectrometry work and the mass spectrometry was done by Fran Mulholland as part of the IFR Proteomics Platform. Detected regions are shown in red.

A



B



C

Treatment	VEGF Sequence
	10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 163
VEGF (1)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF (1)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF (2)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF (2)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (3)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (3)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (4)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + dp 4 (4)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (5)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (5)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (6)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR
VEGF + EGCG (6)	MAEGGGQNHH EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE IEYIFKPSCV PLMRCGGCCN DEGLECVPTE ESNITMQIMR IKPHQGQHIG EMSFLQHNKC ECRPKKDRAR QENPCGPCSE RRKHLFVQDP QTCKCSCKNT DSRCKARQLE LNERTCRCDK PRR

Figure 5.17: Two-thirds coverage of the VEGF sequence is obtained by LTQ Orbitrap using trypsin and chymotrypsin as the digestion enzymes.

VEGF (10 μg) was diluted in 20 mM ammonium bicarbondate (pH 7.0) to give a final concentration of 1.4 μg/ml. The solution was aliquoted into eppendorfs and treated with either 56 μM of apple procyanidin dp4 (VEGF + dp 4 3 and 4) or EGCG (VEGF + EGCG 5 and 6). The solutions were incubated overnight and then subsequently dialyzed against ammonium bicarbonate for 2 h. A portion of the sample was diluted to give a final concentration of 25 ng/ml VEGF and applied to HUVECs for subsequent Western Blot determination of VEGFR-2 phosphorylation (**A**). (**B**) is the semi-quantitative data from the Western Blot. The remainder of the sample was freeze-dried and digested, alkylated, treated with trypsin and chymotrypsin and analyzed by LTQ Orbitrap (**C**). The sample preparation for the mass spectrometry work and the mass spectrometry was done by Fran Mulholland as part of the IFR Proteomics Platform. Detected regions are shown in red.

5.6 Discussion

The investigation of the interaction between polyphenols and VEGF has shown that (1) the VEGF-polyphenol complex formation involves non-covalent interactions; (2) the interaction is not a freely reversible dynamic equilibrium; and (3) the kinetic analysis of the inhibition of VEGF activity by apple procyanidin fraction dp 4 appeared to show a two-phase binding process which is consistent with either the binding of polyphenol at more than one site on VEGF or slow tight binding which usually involves a change of protein conformation. The investigation of the VEGF-polyphenol complex formation was not able to determine (1) whether the polyphenol causes the precipitation of VEGF; and (2) whether the VEGF-polyphenol complex formation was due to specific or non-specific binding. The amount of available protein was the main limitation of the investigation, therefore the experimental techniques applied had to account for the limited amount of available protein.

Extensive dialysis and concentration of non-polyphenol treated and polyphenol-treated VEGF to remove unbound polyphenols has shown that the interaction between polyphenols and VEGF is essentially irreversible and either due to covalent binding (Figure 2.2B or D) or a strong non-covalent interaction which is typically the result of a protein conformational change (Figure 2.2F). If the interaction between polyphenols and VEGF was a simple reversible non-covalent interaction (Figure 2.2A or C), VEGF-induced VEGFR-2 phosphorylation would be observed in the polyphenol-treated VEGF samples that were dialysed or concentrated. A strong non-covalent interaction was also favoured by the kinetic analysis of the inhibition of VEGF activity by apple procyanidin fraction dp 4 as it suggested that the interaction between VEGF and the polyphenol involved a two-stage binding process that either involves the binding of the polyphenol to more than one site on the VEGF molecule or the slow tight binding which usually involves a change of protein conformation (Figure 5.14). The likelihood of a covalent

interaction between VEGF and the polyphenol was not favoured by SDS-PAGE gel work or mass spectrometry. The polyphenol-treated VEGF did not cause a change in the mass of VEGF under reducing or non-reducing conditions (SDS-PAGE, Figure 5.9) or cause any covalent modifications in the VEGF sequence (mass spectrometry, Figure 5.16).

Non-covalent interactions between polyphenols and proteins are also favoured by the literature (Hagerman et al., 1998; de Freitas and Mateus, 2001; Moini et al., 2001; Carvalho et al., 2004; Prigent et al., 2009; Cala et al., 2010). Procyanidins are believed to mainly bind to proteins via non-covalent interactions (Prigent et al., 2009). Procyanidins contain several groups (e.g. aromatic rings and the carbon-hydrogen skeleton of the pyranic ring) which provide numerous sites of potential hydrophobic regions to interact with proteins (de Freitas and Mateus, 2001). The aromatic rings of procyanidins are uncharged, have a flat surface, are hydrophobic in nature and will stack face-to-face with the hydrophobic regions of the exposed pyrrolidine rings of PRPs (Carvalho et al., 2004). Flavanols are also hydrophilic in nature due to the phenol groups present in the structure (Verstraeten et al., 2003; Hofmann et al., 2006) and may therefore also involve hydrophilic interactions. It was previously shown that the interaction between PGG and BSA is predominately characterised by hydrophobic interactions with weak hydrogen binding while hydrogen binding is the primary interaction between EC_{16}-C and BSA (Hagerman et al., 1998). Procyanidins, from a French maritime pine bark extract, bind non-covalently to milk xanthine oxidase and involves hydrophobic interactions (Moini et al., 2001). Recently, it was also shown that for procyanidin concentrations below the critical micelle concentration involved hydrophobic interactions between PRPs and procyanidins whereas procyanidin concentrations above the critical micelle concentration involved both hydrophobic and hydrophilic interactions (Cala et al., 2010).

While procyanidins bind to proteins non-covalently, EGCG can either bind covalently (Ishii et al., 2008; Cao et al., 2009) or non-covalently (Jöbstl et al., 2006; Hudson et al., 2009; Hasni et al., 2011; Kanakis et al., 2011) to proteins. It has been suggested that covalent binding is more likely to occur between EGCG and proteins as EGCG is prone to oxidation in cell culture and in physiological conditions (Cao et al., 2009). EGCG is easily oxidized to form a semiquinone radical that rearranges to an *o*-quinone at either the B ring (gallyl) or the D ring (gallate) and the *o*-quinone then can react with the nucleophilic thiol group of cysteine residues to form adducts (Ishii et al., 2008). Irreversible covalent interactions were detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) between EGCG and glyceraldehyde-3-phosphate dehydrogenase and it was shown that EGCG covalently binds to the cysteinyl thiol residues (Ishii et al., 2008). Irreversible covalent interactions were also detected by MALDI-TOF between EGCG quinine and arginine, cysteine, methionine and α-NH_2 of the N-terminus of peptides (Cao et al., 2009). EGCG is also known to bind strongly to arginine and aromatic residues (Williamson et al., 2006). Non-covalent interactions have also been shown to occur between EGCG and proteins; the EGCG-BSA and EGCG-reduced and carboxymethyalted κ-casein complex formation involves hydrophobic interactions (Hudson et al., 2009) and it has been suggested that the increase in entropy, as analysed by single molecule force microscopy, between EGCG and β-casein is due to hydrophobic interactions (Jöbstl et al., 2006). The interaction between EGCG and casein proteins (Hasni et al., 2011) and EGCG and β-lactoglobulin (Kanakis et al., 2011) involved both hydrophobic and hydrophilic interactions and the interaction between PGG and BSA was the result of a reversible non-covalent interaction (Chen and Hagerman, 2004a). Therefore under different circumstances, EGCG may bind covalently or non-covalently to proteins. In the case of VEGF-EGCG binding, the data presented in this chapter favours a non-covalent interaction.

The data presented in this chapter have shown that the interaction between VEGF and polyphenols is a non-covalent irreversible reaction. This is in contrast with the literature as the mechanism of reported non-covalent protein-polyphenol complexes has generally been regarded as reversible (McManus et al., 1985; Luck et al., 1994; Bennick, 2002; Charlton et al., 2002). However as the kinetics analysis of the VEGF activity appeared to show a two-phase binding process between VEGF and polyphenols, the polyphenol may cause a potential irreversible protein conformational change. It has been previously reported that polyphenol-protein binding occurs in three stages: (1) polyphenols bind to proteins on multiple sties which causes the physical size of the protein to decrease and become more spherical and compact; (2) as the polyphenol concentration increases, the polyphenols bound to proteins cross-link different protein molecules and dimerization and aggregation occurs; and (3) as more polyphenol is added to the solution dimers aggregate together and precipitate out of solution (Charlton et al., 2002; Jöbstl et al., 2004). This three-phase binding process was observed between EGCG and β-casein (Jöbstl et al., 2004) and EGCG and a salivary PRP (Pascal et al., 2008). Polyphenols have also been shown to induce conformational changes in proteins. The binding of green tea polyphenols to casein proteins resulted in the conformational change of the secondary structure which led to the unfolding of the protein (Hasni et al., 2011) and the binding of polyphenols (resveratrol, genistein and curcumin) to BSA also resulted in the conformational change to BSA and led to the partial unfolding of the protein (Bourassa et al., 2010). Whereas the secondary structure alteration of β-lactoglobulin by green tea polyphenols resulted in stabilization of the protein structure (Kanakis et al., 2011). The tertiary structure of elastase was also shown to undergo a moderate structural loosening upon procyanidin complexation (Brás et al., 2010). Therefore the multi-stage binding process between VEGF and polyphenols, which involves the conformational change of VEGF,

may be a consequence of the binding interaction. Further experimental investigation of this is required.

However, as proteins are prone to precipitation by polyphenols, it may be possible that the inhibition of VEGF activity is due to the precipitation of the protein. The cellular determination of the precipitation of VEGF in this chapter was not an appropriate method to detect precipitation because apple procyanidin fraction dp 4 and EGCG completely inhibit VEGF-induced VEGFR-2 phosphorylation. However, the treatment of VEGF with epicatechin did not lead to protein precipitation. Epicatechin is structurally related to apple procyanidin fraction dp 4 and EGCG but does not inhibit VEGF activity. The fact that epicatechin does not precipitate VEGF suggests that apple procyanidin fraction dp 4 or EGCG may not cause VEGF to precipitate. The detection of the location (in the pellet or supernatant) of VEGF and polyphenol-treated VEGF by Western blot showed that the VEGF was located in the pellet of the un-treated and polyphenol-treated VEGF samples. The location of VEGF in the pellets of the VEGF samples is either due to the high concentrations of proteins/polyphenols used or the use of sterile water instead of a buffer.

The polyphenol to protein ratio used for the detection of VEGF by Western blot was 1522:1. This is substantially greater than the polyphenol to protein ratios previously reported to cause precipitation of proteins. According to Jöbstl et al. (2004) the onset of aggregation occurs at a molar ratio of EGCG to β-casein of about 10:1; aggregation between EGCG or PGG and a basic PRP occurs at a molar ratio of 5.5:1 and 0.75:1, respectively (Charlton et al., 2002); aggregation between EGCG or condensed tannins and PRP occurs at a molar ratio of 80:1 and 0.3:1, respectively (Pascal et al., 2008) and aggregation between EC_{16}-C or PGG and BSA occurs at a molar ratio of 2.2:1 and 21.7:1, respectively (Hagerman et al., 1998). Protein aggregation occurs at low molar

ratios of polyphenol to protein because β-casein and basic PRPs have large numbers of polyphenol binding sites; β-casein has 35 proline residues and 14 aromatic rings (another major polyphenol binding site) while basic PRPs have high affinities for polyphenols due to their high proline content (Hagerman and Butler, 1981; Bennick, 1987; Charlton et al., 2002; Jöbstl et al., 2004).

Although the polyphenol to protein ratio used in this chapter was substantially greater than what was previously reported to precipitate proteins in the literature, it is possible that VEGF is not being precipitated by the polyphenol as polyphenols can bind strongly to certain proteins without causing them to precipitate (Bennick, 2002). Precipitation of proteins by polyphenols is also related to the proline content of the protein (Lopez and Edens, 2005) and VEGF is not a proline rich protein (contains only 11 proline residues). However, the investigation of whether polyphenols precipitate VEGF remains inconclusive and further experiments need to be conducted.

Therefore based on the data presented in this chapter, it was shown that VEGF-polyphenol interactions are a result of a potential irreversible non-covalent interaction which may involve a protein conformational change. The data were unable to establish whether the polyphenol causes the precipitation of VEGF or whether the interaction between VEGF and the polyphenol was due to specific or non-specific binding. Therefore, in order to gain a full understanding of the interaction involved between VEGF and polyphenols further research is required. Experimental techniques such as the titration of polyphenols into proteins as assessed by nuclear magnetic resonance can be used to characterise the nature of the interaction (e.g. polyphenol self-association, peptide/polyphenol self association and analysis of the polyphenol association state when bound to the peptide) of protein-polyphenol complexes (Baxter et al., 1997; Charlton et al., 2002; Cala et al., 2010). Capillary electrophoresis is

another method that can be used to characterise the equilibrium binding (association/dissociation) constants between polyphenols and proteins (Papadopoulou and Frazier, 2004; Diniz et al., 2008). Both the size and the total number of aggregates present in the solution can be detected by dynamic light scattering (Brás et al., 2010). The application of surface plasmon resonance can be used to determine whether the interaction between VEGF and polyphenols is the result of specific or non-specific binding (Douat-Casassus et al., 2009). Non-covalent interactions can also be characterised by MALDI-TOF mass spectrometry (Chen and Hagerman, 2004a). Fourier transform infrared (Bourassa et al., 2010), circular dichroism (Bourassa et al., 2010; Brás et al., 2010) and fluorescence quenching (Bourassa et al., 2010; Brás et al., 2010) spectroscopic methods can be used to analyse the polyphenol binding mode, the binding constant and the effects on complexation on protein stability and conformation. The potential binding sites can also be determined by applying molecular modelling (Bourassa et al., 2010; Brás et al., 2010; Cala et al., 2010).

5.7 Conclusions

The investigation of the interaction between polyphenols and VEGF has shown that (1) the VEGF-polyphenol complex formation involves non-covalent interactions; (2) the interaction is not a freely reversible dynamic equilibrium; and (3) the kinetic analysis of the inhibition of VEGF activity by apple procyanidin fraction dp 4 appeared to show a two-phase binding process which is consistent with either the binding of polyphenol at more than one site on the VEGF molecule or slow tight binding which usually involves a change of protein conformation. The amount of available protein was the main limitation of the investigation, therefore the experimental techniques applied had to account for the limited amount of available protein. Further investigation (with a larger supply of protein) is essential in gaining a full understanding of how polyphenols interact with VEGF and inhibit VEGF activity.

CHAPTER SIX

INVESTIGATION OF GENE EXPRESSION CHANGES INDUCED BY APPLE PROCYANIDIN FRACTION DP 4 AND VEGF TREATMENTS USING WHOLE GENOME ARRAYS

Chapter 6 : Investigation of gene expression changes induced by apple procyanidin fraction dp 4 and VEGF treatments using whole genome arrays

6.1 Abstract

Data presented in Chapter 4 has shown that apple procyanidin fraction dp 4 inhibits VEGF-induced VEGFR-2 phosphorylation at low concentrations. Because apple procyanidin fraction dp 4 treatments were shown to completely inhibit VEGF-induced activation of the VEGFR-2 receptor, and VEGFR-2 activation is thought to be responsible for the majority of VEGF-mediated effects in vascular endothelial cells, it was postulated that apple procyanidin fraction dp 4 treatment would block all VEGF-induced changes in vascular endothelial cells. However, the polyphenol is likely to also interact with other receptors, membranes and possibly other cell components such as intracellular enzymes. In order to (1) determine if apple procyanidin fraction dp 4 completely inhibits genes differentially expressed by VEGF and (2) establish whether apple procyanidin fraction dp 4 regulates additional genes that are independent of the inhibition of VEGF signalling, the investigation of gene expression changes by apple procyanidin fraction dp 4 and VEGF treatments using whole genome arrays were conducted. Analysis of the microarray data has shown, for the first time, that there were no significant differences in transcript profiles between apple procyanidin fraction dp 4 VEGF complex-treated HUVECs and apple procyanidin fraction dp 4-treated HUVECs. This result shows that the apple procyanidin fraction dp 4 treatment completely blocks VEGF-regulated genes. The microarray analysis has also shown that the apple procyanidin fraction dp 4 treatment regulates genes involved in angiogenesis, apoptosis, signalling and transcription that are independent of VEGF. One of the VEGF-independent genes regulated by apple procyanidin fraction dp 4 that was of

particular interest was Krüppel-like factor 2 as it is recognised to be an important transcription factor in the suppression of pro-inflammatory and pro-atherosclerotic genes. This finding is investigated further in the next chapter. The results from this microarray analysis show that apple procyanidin fraction dp 4 completely blocks the genes regulated by VEGF as well as regulating additional genes involved in angiogenesis and inflammation independently of VEGF. Therefore the complete inhibition of VEGF-mediated genes by apple procyanidin fraction dp 4 provides a plausible link between consumption of polyphenols and a reduced risk of CVD.

6.2 Introduction

Data presented in Chapter 4 has shown that at low (physiological) concentrations, apple procyanidin fraction dp 4 completely inhibits VEGF-induced VEGFR-2 phosphorylation. Because apple procyanidin fraction dp 4 treatments were shown to completely inhibit VEGF-induced activation of VEGFR-2, and VEGFR-2 activation is thought to be responsible for the majority of VEGF-mediated effects in vascular endothelial cells (Giles, 2001; Ferrara and Kerbel, 2005; Schweighofer et al., 2009), it was postulated that apple procyanidin fraction dp 4 treatments would block all VEGF-induced changes in vascular endothelial cells. However, apple procyanidin fraction dp 4 is likely to interact with other receptors, membranes and possibly other cell components as procyanidins have also been shown to induce biological functions in vascular endothelial cells that are not dependent on VEGF-induced signalling (Sen and Bagchi, 2001; Kenny et al., 2004a; Kenny et al., 2004b; Kaur et al., 2006; Engelbrecht et al., 2007; Terra et al., 2007; Miura et al., 2008; Hsu et al., 2009; Caton et al., 2010; Terra et al., 2011). Therefore, the aim of the research covered in this chapter was to determine whether apple procyanidin fraction dp 4 treatment completely blocks VEGF-induced changes in vascular endothelial cells and to establish whether apple procyanidin fraction dp 4 regulates additional genes that are independent of the inhibition of VEGF signalling by investigating the gene expression changes by apple procyanidin fraction dp 4 and VEGF treatment using whole genome arrays.

6.2.1 VEGF-regulated intracellular mechanisms

The binding of VEGF to its receptors leads to downstream signalling cascades involved in changes in endothelial cell migration, invasion, proliferation, permeability and tube formation *in-vivo* and *in-vitro* by inducing genes that regulate angiogenesis (Abe and Sato, 2001; Jih et al., 2001; Schweighofer et al., 2009). The previously reported

analysis of VEGF-regulated genes identified by microarray analysis showed that the angiogenesis related genes directly regulated by VEGF were: alpha-2-macroglobulin (A2M), angiotensin 1 converting enzyme (ACE), angiopoietin-2 (ANGPT2), bactericidal/permeability-increasing protein (BPI), CD34 molecule, cyclooxygenase 2 (COX2), cysteine-rich, angiogenic inducer 61 (CYR61), down syndrome critical region 1 (DSCR-1), early growth response 1 (EGR1), early growth response 3 (EGR3), heparin-binding epidermal growth factor (HB-EGF), integrin αv, integrin α5, integrin β1, integrin β3, thrombomodulin (THBD), urokinase plasminogen activator (PLAU), VEGF-C, and vascular cell adhesion molecule 1 (VCAM1) (Zhang et al., 1999; Abe and Sato, 2001; Weston et al., 2002; Yang et al., 2002; Wary et al., 2003; Minami et al., 2004; Schoenfeld et al., 2004).

In addition to inducing angiogenesis, VEGF regulates genes involved in endothelial cell survival (Gupta et al., 1999; Jih et al., 2001; Schoenfeld et al., 2004) and inflammation (Weston et al., 2002; Schweighofer et al., 2009). VEGF promotes endothelial cell survival by regulating anti-apoptotic genes like B-cell lymphoma 2 (Bcl-2), serine/theronine kinases (AKT), caspases and extracellular signal regulated kinase (ERK) as well as directly and selectively activating the MAPK/ERK pathway (Gupta et al., 1999; Jih et al., 2001). VEGF, in contrast to other growth factors, contains a significant inflammatory component and therefore may be possible to promote inflammation on its own (Schweighofer et al., 2009). Vascular cell adhesion molecule-1 (VCAM1), inflammatory cell adhesion molecule-1 (ICAM1), interleukin 8 (IL8) E-selectin (SELE) are some of the inflammatory genes which are regulated by VEGF (Weston et al., 2002; Schweighofer et al., 2009).

6.2.2 Procyanidin-regulated intracellular mechanisms

Procyanidins have been reported to induce numerous biological functions; however their most distinct functions include their anti-angiogenic (Kenny et al., 2004a; Kenny et al., 2004b; Kaur et al., 2006; Engelbrecht et al., 2007; Miura et al., 2008; Wen et al., 2008; Hsu et al., 2009; Caton et al., 2010; Lu et al., 2010) and anti-inflammatory properties (Sen and Bagchi, 2001; Terra et al., 2007; Terra et al., 2011). The anti-angiogenic properties of procyanidins include their ability to inhibit VEGF signalling (Chapter 4) (Wen et al., 2008; Lu et al., 2010), inhibit proliferation (Kaur et al., 2006; Miura et al., 2008; Hsu et al., 2009), induce apoptosis (Kaur et al., 2006; Engelbrecht et al., 2007; Miura et al., 2008; Hsu et al., 2009), decrease the expression of V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ErbB2) receptors (Kenny et al., 2004a; Kenny et al., 2004b), and induce KLF2 expression (Caton et al., 2010). The microarray analysis of an apple oligomeric procyanidin fraction showed that the oligomeric procyanidin fraction was able to significantly modulate the expression levels of several genes that are associated with key angiogenesis-related functions, such as cell proliferation and migration (García-Conesa et al., 2009). VEGF, as previously described in Chapter 4, is a potent pro-angiogenic growth factor involved in the activation of downstream signalling events related to endothelial cell migration, invasion, proliferation, permeability (Giles, 2001; Dulak, 2005; Cebe-Suarez et al., 2006). ErbB2 receptors are receptors that are important in angiogenesis regulation and over-expression of ErbB2 receptors results in the induction of VEGF (Kenny et al., 2004a; Kenny et al., 2004b). KLF2 is recognised to be an important transcription factor in the suppression of pro-inflammatory and pro-atherosclerotic genes such as VCAM1, SELE, endothelin-1 (EDN1) and tissue factor as well as inducing antithrombotic and thrombolytic genes (Bhattacharya et al., 2005; Dekker et al., 2006; Caton et al., 2010). Over-expression of

KLF2 has also been reported to potently abrogate VEGF-mediated angiogenesis (Hamik et al., 2006).

Procyanidins have also been reported to have anti-inflammatory properties. Procyanidin anti-inflammatory properties include their ability to reduce NF-κB activity (Terra et al., 2007; Terra et al., 2011), reduce markers of systemic inflammation (tumour necrosis factor-alpha [TNF-α] and C-reactive protein) (Terra et al., 2011), and down-regulate endothelial adhesion molecules (ICAM1 and VCAM1) (Sen and Bagchi, 2001; Garbacki et al., 2005). NF- κB is one of the most important regulators of pro-inflammatory gene expression (Tak and Firestein, 2001). Although procyanidins have been shown to be effective at preventing the onset of inflammation, it still remains unclear whether procyanidins exert anti-inflammatory effects once inflammation has been established (Terra et al., 2011)

Procyanidins have been reported to have anti-angiogenic and anti-inflammatory properties. VEGF, a potent pro-angiogenic growth factor which contains a significant inflammatory component, has been implicated as a key feature in procyanidins anti-angiogenic properties. In order to determine the extent of the interaction between procyanidins and VEGF the investigation of the gene expression changes by apple procyanidin fraction dp 4 and VEGF treatments were conducted using whole genome arrays. While an investigation of a procyanidin fraction has already been reported, this is the first time that the number of differentially expressed genes by a purified tetrameric procyanidin fraction (apple procyanidin fraction dp 4) has been described. This is important because the procyanidin fraction used in the previous study (García-Conesa et al., 2009) was a mixture of flavanols (contained flavanol monomers [epicatechin and catechin] and oliogmers [dimers to heptamers]) and it could not be determined whether a specific compound was responsible for the observed effects or if

it was a combined effect. This is also the first report describing differences in gene expression following the simultaneous co-treatment of a polyphenol-treated VEGF treatment.

6.3 Objectives

The main aim of the microarray experiment was to (1) determine if a potent VEGF inhibitor, apple procyanidin fraction dp 4, completely blocks VEGF-regulated genes; and (2) establish whether apple procyanidin fraction dp 4 regulates additional genes that are independent of inhibition of VEGF signalling and identify what these genes are.

6.4 Materials and methods

6.4.1 Materials

Human recombinant $VEGF_{165}$ and anti-human VEGF antibody were obtained from R&D Systems Europe Ltd (Abingdon, UK). Electrophoresis reagents were purchased from Invitrogen and the VEGFR-2, phospho-VEGFR-2 (Tyr 1175), the anti-rabbit IgG, HRP-linked antibodies were from Cell Signaling. All real time Reverse Transcription Polymerase Chain Reaction (RT-PCR) products were from Applied Biosystems. The apple procyanidin fraction dp 4 was purified from the 2007 apples (Chapter 3) provided by Coressence ltd (Herefordshire, UK) as previously described (Yanagida et al., 1999; Shoji et al., 2005; Shoji et al., 2006b), with some modifications. A brief description of the method can be found in Chapter 4 or a more detailed description of the method is located in Supplementary Information 1.

6.4.2 Cell culture and treatments

6.4.2.1 Cell culture

The culture of HUVECs is described in Chapter 4, Section 4.4.2.

6.4.2.2 Treatments

Treatments (control [treated with the vehicle DMSO], VEGF, anti-VEGF antibody, and apple procyanidin fraction dp 4) were prepared in Universal tubes and incubated at room temperature for 5 min prior to adding them to the confluent monolayers. HUVEC confluent monolayers were washed twice with PBS, to remove any residual growth factors, and prepared treatments were added to the respective wells and incubated at 37 ºC under a humidified atmosphere containing 5 % CO_2. Treatments were incubated for 6 h for the microarray experiment and a time course was conducted for the angiopoietin-2 (ANGPT2) gene assay determination.

6.4.3 VEGFR-2 phosphorylation measurement

The extraction of protein was the same method as described in Chapter 4, Section 4.4.6 and the measurement of phosphorylated VEGFR-2 by Western blot followed the same procedure as described in Chapter 4, Section 4.4.7.

6.4.4 RNA extraction

After treatment incubation, HUVECs were washed twice with warm PBS and the ribronucleic acid (RNA) was extracted using the RNeasy® Mini Kit (Qiagen Ltd, UK) according to the manufacturer's protocol. The homogenization step in the protocol was achieved by using a QIAshredder (Qiagen Ltd, UK) and the optional on-column DNase digestion was preformed. The quality and quantity of RNA present in the samples was

quantified using a Beckman DU-640 spectrometer prior to storage at -80 ºC. For a pure and good quality RNA sample the absorbance at 260/280 should be approximately 2; this was observed for all the samples.

The quality of the RNA, for the microarray samples, was also assessed by the Nottingham Arabidopsis Stock Centre (Nottingham, UK) using an RNA Nano LapChip kit and an Agilent 2100 Bioanalyzer. The quality of the RNA is measured by loading the samples onto the chip, which then pass through microchannels into the separation channel. The RNA fragments are then separated according to their size by means of molecular sieving and the fragments are detected by fluorescence at the detection point. High quality RNA electropherograms show the following characteristics: clear 28S and 18S peaks; and low noise between the peaks and minimal low molecular weight contamination. Marginally degraded RNA samples include the presence of low molecular weight species, noise between 28S and 18S peaks, and a smaller 28S peak than an 18S peak. Degraded RNA samples have a lack of 28S and 18S peaks and consist only of low molecular weight species.

All samples exhibited high quality RNA electropherograms. An example electropherogram of one of the microarray samples is shown in Figure 6.1.

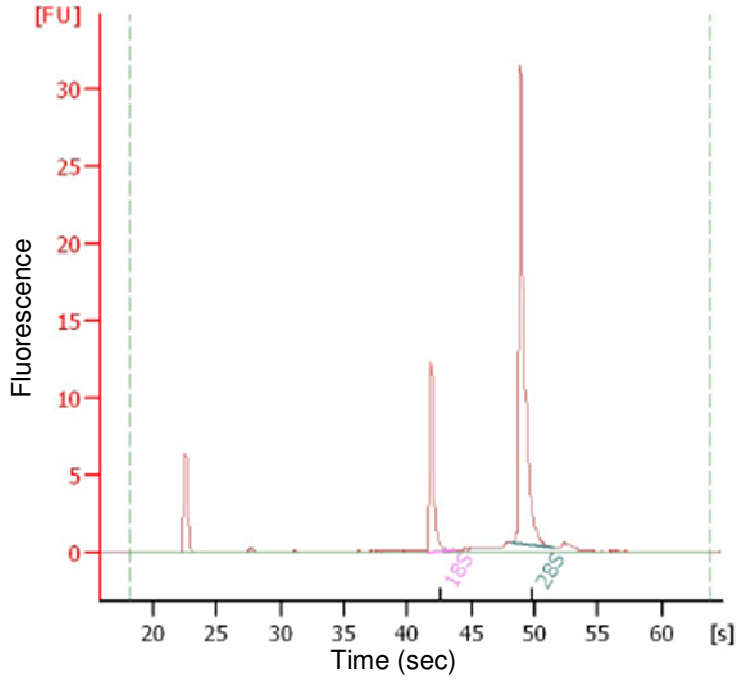


Figure 6.1: Example of an electropherogram showing a good quality RNA generated by the Agilent 2100 Bioanalyzer.

6.4.5 Real-time Reverse Transcription Polymerase Chain reaction

Pre-designed gene assays for Angiopoietin 2 (ANGPT2) was purchased from Applied Biosystems (Assay ID: Hs00169867_m1). Target gene mRNA levels were determined by real time RT-PCR using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) and normalised to the housekeeping gene 18S (Sigma). The real time RT-PCR reactions were carried out in a Microamp Optical 96-well plate in a total volume of 20 µl per well containing TaqMan® RNA-to-C_T^{TM} *1-Step* Kit, 20 ng total RNA and appropriate concentrations of primers and probes. Real time RT-PCR conditions were as follows: one cycle of 48 ºC for 30 min, one cycle of 95 ºC for 10 min followed by 40 cycles at 95 ºC for 15 sec and one cycle at 60 ºC for 1 min. The concentration of RNA was normalised to 18S and the data were analysed as mean ± standard deviation. Differences were considered significant at $p \leq 0.05$.

6.4.6 Affymetrix GeneChip® Human Exon 1.0 ST Array

RNA samples were analysed using the Affymetrix GeneChip® Human Exon 1.0 ST Array (Affymetrix, Santa Clara, CA). The exon array contains approximately 5.4 million features (probes) grouped into 1.4 million probe sets and which are further grouped into over 1 million exon clusters. Exon clusters are further grouped into Probe Selection Regions (PSRs). A PSR represents a region of the genome that is predicted to act as an integral coherent unit of transcriptional behaviour. In most cases, a PSR is an exon; however in certain cases, due to overlapping exon structures, several PSRs may form contiguous, non-overlapping subsets of a true biological exon. The exon clusters are further categorised into transcript clusters; exons which share splice sites, or were derived from overlapping exonic sequences, or were single exon clusters bounded on the genome by spliced content were annotated to belong to the same transcript cluster. A transcript cluster roughly corresponds to a gene.

The design of the Affymetrix GeneChip® Human Exon 1.0 ST Array allows for two levels of analysis: gene expression and alternative splicing. For the purposes of this chapter only gene expression analysis was investigated.

6.4.6.1 Sample processing, terminal labelling, hybridization and scanning

Sample processing and hybridization was preformed by the Nottingham Arabidopsis Stock Centre according to the Affymetrix protocol. Sample processing including generation of sense-strand cDNA from total RNA, fragmentation, labelling and hybridization was preformed. Briefly, sense-strand cDNA was synthesised from total RNA according to the Ambion WT Expression Kit protocol. After synthesizing sense-strand cDNA, the sample is fragmented and labelled. During fragmentation, Fragmentation Master Mix is added to the samples and incubated at 37 ºC for 60 min, 93 ºC for 2 min and 4 ºC for 2 min. Subsequently labelling reagents are added to the

fragmented single-strand DNA and incubated at 37 ºC for 60 min, 70 ºC for 10 min and 4 ºC for 2 min. The next step is hybridization; a hybridization cocktail and the fragmented and labelled single-strand DNA are prepared. It is then hybridised to the probe array for 17 h at 45 ºC in a hybridization oven. After hybridization the hybridization cocktail is removed and the probe array undergoes an automated washing and staining procedure with streptavidin phycoerythrin conjugate. The probe is then scanned using an Affymetrix GeneChip® Scanner 3000. The software defines the probe cells and computes intensities for each cell. Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension.

6.4.6.2 Array analysis

The data was analysed using R (R.Development.Core.Team, 2009) / Bioconductor (Gentleman et al., 2004) and the *aroma.affymetrix package* (Bengtsson et al., 2008b). R is a language and environment for statistical computing and graphing, Bioconductor provides tools for the analysis and comprehension of high throughput genomic data and is based on the R programming language, and *aroma.affymetrix* is an R package for analysing large Affymetrix data sets. All arrays in this study were background corrected and quantile normalised together using Robust Multiarray Average (RMA) to generate gene-level summaries. For gene annotation, the 20071112 custom chip definition file (CDF) file available at the *aroma.affymetrix* website containing the core probesets (18,708 transcript clusters and 284,258 probesets) was used. Subsequent statistical analysis to identify differentially expressed genes was preformed using the Linear Model for MicroArrays (*limma*) (Smyth, 2004). Genes were identified as differentially expressed at different Benjamini and Hochberg adjusted *p*-values. The Database for Annotation, Visualization and Integrated Discovery v6.7 (DAVID;

http://www.david.abcc.ncifcrf.gov/) was used to identify Gene Ontology (GO) categories associated with specific gene lists (Huang et al., 2008).

6.4.6.3 Comparison of array data with existing published data

Generated gene lists were compared with existing published data for VEGF microarray analyses as well as apple procyanidin fraction dp 3.9 microarray analyses using the data deposited in the NCBI Gene Expression Omnibus (GEO) profiles website (http://www.ncbhi.nlm.nih.gov/geo) and ArrayExpress (www.ebi.ac.uk/arrayexpress).

6.4.7 Statistical analysis

Where indicated, comparisons between mean values and treated samples were carried out using a two tailed t-test. A p value < 0.05 was considered significant.

6.5 Results

6.5.1 Determination of treatment parameters for microarray analysis

The concentration of apple procyanidin fraction dp 4 and VEGF used in the cell treatment for the microarray analysis was 1 µM and 10 ng/ml, respectively. The concentrations used were based on the results presented in Chapter 4. One micromolar apple procyanidin fraction dp 4 is the lowest concentration that completely inhibits VEGF-induced VEGFR-2 phosphorylation after a 5 min treatment and 10 ng/ml VEGF induces VEGFR-2 phosphorylation after a 5 min treatment. It was also shown that a 10 ng/ml VEGF treatment induced gene expression data in previous microarray analyses (Jih et al., 2001; Schoenfeld et al., 2004).

The aim of the microarray analysis was to identify the differentially expressed genes between treatments. Therefore in order to determine the optimum length of treatment, a gene expression assay was conducted for ANGPT2. ANGPT2 was picked as the gene of interest because it is a protein growth factor that promotes angiogenesis and has been shown to be induced by VEGF (Abe and Sato, 2001; Hiltunen et al., 2002; Yang et al., 2002).

HUVECs were treated with VEGF (10 ng/ml) and incubated for 0, 1, 2, 4, 6, 8 and 24 h prior to RNA extraction and real-time RT-PCR analysis. A significant increase in ANGPT2 expression, compared to the control was observed from 2 h to 24 h (Figure 6.2). The ANGPT2 quantity in the control samples also increased with time compared to the control at 0 h. Six hours was selected as the time point of interest because the increase in ANGPT2 was statistically significant ($p < 0.0001$) and the increase in ANGPT2 expression in the control ($p = 0.0043$) was not as great as compared to the 8 h ($p = 0.0012$) and 24 h ($p < 0.0001$) samples. Previously, the number of genes which were up-regulated in response to VEGF treatment was shown to reach a maximum at 6 h in both HUVECs and myometrial microvascular endothelial cells (Jih et al., 2001; Weston et al., 2002) and ANGPT2 was reported to be down-regulated by apple procyanidin fraction dp 3.9 after a 6 h incubation (García-Conesa et al., 2009). Therefore based on the results from the ANGPT2 gene expression assay and the information from the literature the time point selected for the microarray study was 6 h.

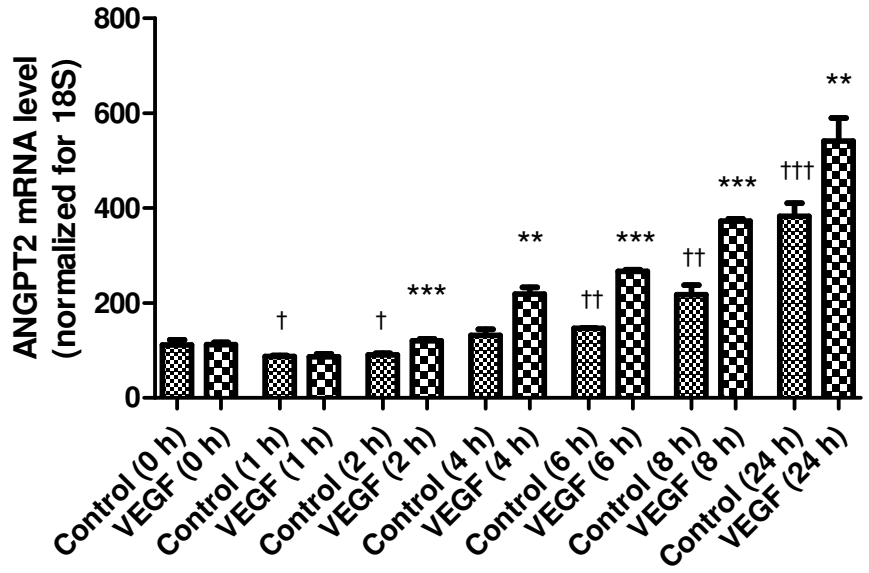


Figure 6.2: Angiopoietin-2 quantity in VEGF treated HUVECs.

HUVECs were treated with 10 ng/ml VEGF and incubated for 0, 1, 2, 4, 6, 8 and 24 h. RNA was extracted from the cells using the RNeasy® Mini Kit and the ANGPT-2 quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of ANGPT-2 normalised against 18S. $^{**}p < 0.01$, *** $p < 0.001$ compared to the control at each time point. † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ compared to the control at 0 h.

6.5.2 Confirmation of VEGF induced VEGFR-2 phosphorylation / inhibition

Confirmation of VEGF-induced VEGFR-2 phosphorylation and inhibition of VEGF-induced VEGFR-2 phosphorylation by apple procyanidin fraction dp 4 and anti-VEGF antibody was evaluated by Western blot prior to further processing of the microarray samples. Treatments were pre-incubated with the basal medium in a Universal tube for 5 min followed by a 6 h cellular incubation prior to protein extraction. The experiment was conducted in parallel with the microarray experiment to ensure the same conditions were applied to both experiments.

In the absence of VEGF, phosphorylation of VEGFR-2 could not be detected in HUVECs. However, when HUVECs are exposed to VEGF, VEGFR-2 phosphorylation

was induced. Apple procyanidin fraction dp 4 does not induce VEGFR-2 phosphorylation, but, when treated with 10 ng/ml VEGF, apple procyanidin fraction dp 4 completely inhibits VEGF-induced VEGFR-2 phosphorylation. The anti-VEGF antibody also completely inhibits VEGF-induced VEGFR-2 phosphorylation. These results confirmed that the treatments are working in the correct manner and further processing of the microarray samples was continued.

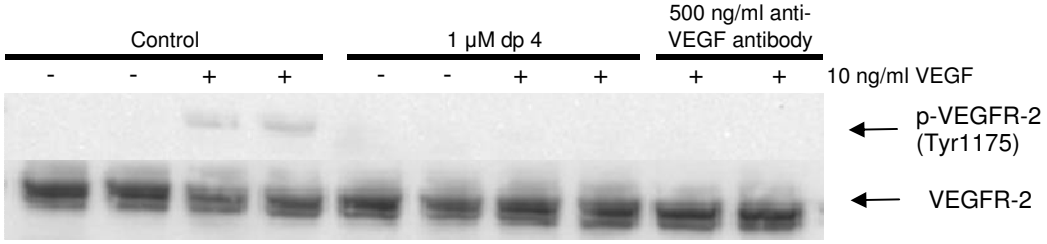


Figure 6.3: VEGFR-2 phosphorylation is induced by VEGF and inhibited by apple procyanidin fraction dp 4 and anti-VEGF antibody treatments.

HUVECs were treated with the vehicle (DMSO), 10 ng/ml VEGF, 1 µM apple procyanidin fraction dp 4, 1 µM apple-procyanidin fraction dp 4-treated VEGF (10ng/ml), or 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) for 6 h. The cells were lysed and the protein was separated on a NuPAGE gel and probed for the presence of phosphorylated VEGFR-2 at the tyrosine 1175 site and for total VEGFR-2.

6.5.3 Affymetrix GeneChip® Human Exon 1.0 ST Array data analysis

Gene expression levels of VEGF, apple procyanidin fraction dp 4, and anti-VEGF antibody were examined using Affymetrix GeneChip® Human Exon 1.0 ST Arrays. In total, 15 samples were hybridised to the chip from a single experiment during which HUVECs were treated, in triplicate biological replicates, with 5 min pre-incubated treatments in basal medium followed by a 6 h incubation prior to RNA extraction. There were 5 different cell treatments: 1) control (vehicle DMSO); 2) 10 ng/ml VEGF; 3) 1 µM apple procyanidin fraction dp 4; 4) 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml); and 5) 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml). All arrays were RMA-background corrected and quantile normalised (Bengtsson et al., 2008a). Potential array outliers were determined by performing a normalised unscaled standard

error (NUSE) plot (Figure 6.4A) and a relative log expression (RLE) plot (Figure 6.4B) on RMA and quantile normalised arrays. The NUSE plot identifies any arrays which have elevated standard errors relative to other arrays in the data set by standardising the standard error across the arrays to have a median of 1 for each gene. Deviating arrays are identified by an increased median and generally medians higher than 1.05 are considered alarming. RLE plots are constructed using log-scale estimates for the expression of each probe set on each array. For each probe set and each array, ratios are calculated between the expression of a probe set and the median expression of this probe set across all arrays of the experiment. It is assumed that, despite biological variability, the majority of genes do not change their expression across environmental conditions. RLE boxplots are therefore expected to be centred on zero and show a small interquartile range. An array with quality problems may result in a box that has a relatively greater spread or that is not centred near zero.

Treatments Negctrl_1, Negctrl_2, VEGFandDp4_2 and VEGFandDp4_3 showed a greater spread in the RLE plot than the other treatments. Based on the NUSE and RLE plots, no arrays were identified as outliers.

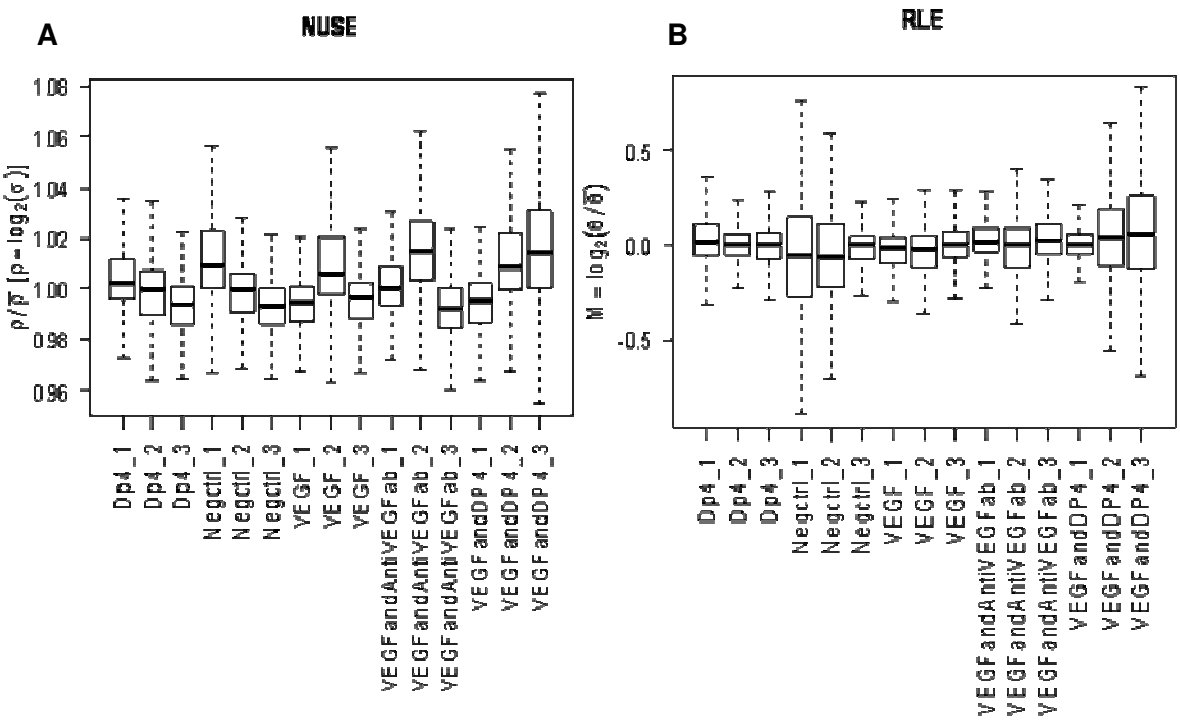


Figure 6.4: Quality control boxplots.

NUSE boxplot (A), RLE boxplot (B). Dp 4 = 1 µM apple procyanidin fraction dp 4; Negctrl = control (vehicle DMSO); VEGF = 10 ng/ml VEGF; VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody; VEGFandDp4 = 10 ng/ml VEGF and 1 µM apple procyanidin fraction dp 4.

The data was also subjected to hierarchical cluster analysis using MATLAB software to establish whether the triplicate biological samples were closely related to one another and to reveal any distinctions between the samples. Initially, probes were identified that had expression levels with a coefficient of variation (standard deviation / mean) between 0.1 and 1000 across all the samples. The probes that met these criteria were then used to generate a hierarchical cluster dendrogram using a centroid method of linkage. The hierarchical cluster dendrogram shows that for the control, VEGF and anti-VEGF antibody-treated VEGF treatments, the biological triplicates of each treatment are clustered together to form a separate group with a similar expression pattern (Figure 6.5). For the apple procyanidin fraction do 4 and apple procyanidin fraction dp 4-treated VEGF treatments, these two treatments are clustered together suggesting a similarity between the two treatments. This dendrogram demonstrates the

reproducibility of the array data and highlights the robustness of the biological triplicates.

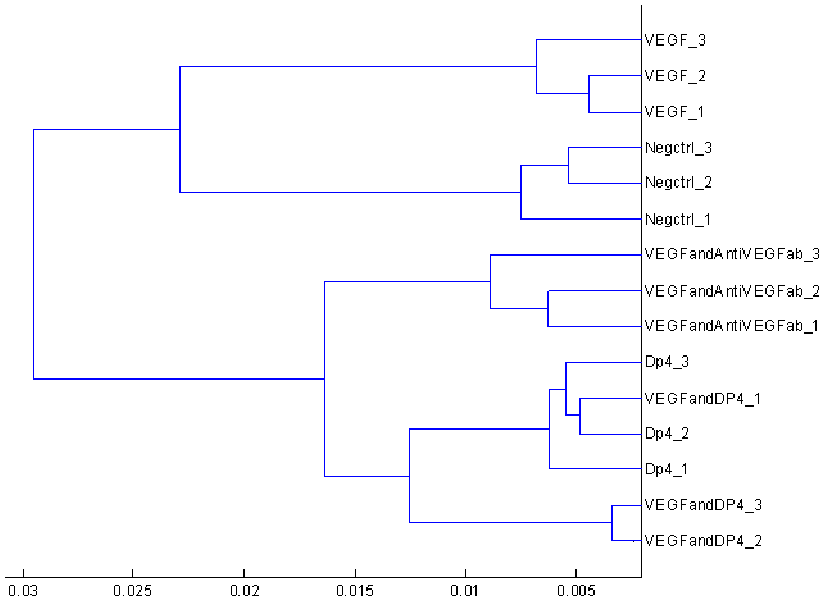


Figure 6.5: Hierarchical clustering dendrogram of HUVECs treated with vehicle (DMSO), VEGF, apple procyanidin fraction dp 4, apple procyanidin fraction dp-treated VEGF and anti-VEGF antibody-treated VEGF in three biological replicates for 6 hours.

Hierarchical clustering was preformed using MATLAB software. 10878 probes out of 18708 were identified to have expression levels with a coefficient of variation (standard deviation / mean) between 0.1 and 1000 across all the samples.

6.5.4 Identification of the number of differentially expressed genes and their biological processes between the treatments

To identify genes that were differentially expressed, pair-wise comparisons were preformed. In total 9 pair-wise comparisons were made between the means of the biological replicates:

1. VEGF versus control (vehicle DMSO)
2. Apple procyanidin fraction dp 4 versus control
3. Apple procyanidin fraction dp 4-treated VEGF versus control
4. Apple procyanidin fraction dp 4 versus VEGF

5. Apple procyanidin fraction dp 4-treated VEGF versus VEGF
6. Apple procyanidin fraction dp 4-treated VEGF versus apple procyanidin fraction dp 4
7. Anti-VEGF antibody-treated VEGF versus control
8. Anti-VEGF antibody-treated VEGF versus VEGF
9. Anti-VEGF antibody-treated VEGF versus apple procyanidin fraction dp 4

The number of differentially expressed genes identified by these pair-wise comparisons is described in Table 6.1. Genes were identified as differentially expressed at different Benjamini and Hochberg adjusted *p*-values.

Table 6.1: Identification of differentially expressed genes from pair-wise comparisons

Comparison	$p < 0.05$	$p < 0.01$	$p < 0.001$
1. VEGF v Negctrl	890 (350, 540)	337 (162, 175)	132 (74, 58)
2. Dp4 v Negctrl	2689 (1195, 1494)	446 (186, 260)	94 (37, 57)
3. VEGFandDp4 v Negctrl	11575 (7268, 4307)	6825 (4168, 2657)	511 (180, 331)
4. Dp4 v VEGF	1031 (454, 577)	524 (206, 318)	235 (83, 152)
5. VEGFandDp4 v VEGF	3426 (1675, 1751)	1159 (370, 789)	360 (95, 265)
6. VEGFandDp4 v Dp4	0	0	0
7. VEGFandAntiVEGFab v Negctrl	3688 (2075, 1613)	353 (170, 183)	0
8. VEGFandAntiVEGFab v VEGF	866 (450, 416)	333 (139, 194)	131 (45, 86)
9. VEGFandAntiVEGFab v Dp4	271 (146, 125)	100 (53, 47)	37 (21, 16)

The up-regulated and down-regulated genes are shown in brackets with the up-regulated gene preceding the down-regulated genes (e.g. up-regulated, down-regulated).

Dp 4 = 1 µM apple procyanidin fraction dp 4; Negctrl = control (vehicle DMSO); VEGF = 10 ng/ml VEGF; VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody; VEGFandDp4 = 10 ng/ml VEGF and 1 µM apple procyanidin fraction dp 4.

The apple procyanidin fraction dp 4-treated VEGF treatment when compared to the control (comparison 3) has the greatest number of genes that are differentially expressed. However when compared to the apple procyanidin fraction dp 4 treatment (comparison 6) there are no statistically significant genes altered at any *p*-value. It is expected that the number of differentially expressed genes altered by the anti-VEGF antibody-treated VEGF compared to the VEGF treatment (comparison 8) would be the same as the VEGF treatment compared to the control (comparison 1) because the antibody should prevent VEGF from binding to its receptors and therefore be the same as the control treatment. The treatments appear to be working correctly as the number of differentially expressed genes between comparison 1 and comparison 8 are approximately the same. For further analysis regarding the pair-wise comparisons refer to Section 6.5.5 for the apple procyanidin fraction dp 4 comparisons and Section 6.5.6 for the anti-VEGF antibody comparisons.

The GO categories associated with the specific gene lists were identified using the DAVID software. The gene list obtained using a p value of < 0.001 was used to produce a manageable set of genes to perform more detailed functional analysis. The top ten biological processes regulated by the treatments are described in Tables 6.2 - 6.8 and the full list of biological processes regulated by the treatments are described in the Supplementary Information (Supplementary Information 2: Biological processes regulated by the different treatment comparisons). Any biological process term which had three or fewer genes associated with that biological process or had a fold enrichment of less than 1.5 were not included in the analysis. Those biological processes that fit the exclusion criteria were excluded because fold enrichments of 1.5 and above are only considered as interesting and fold enrichments obtained from terms with a few number of genes (three or less) are not as reliable as those obtained from larger number of genes (Huang et al., 2008). Fold enrichment measures the magnitude

of enrichment and is defined as the ratio of two proportions. For example, if 40 out of 400 (i.e. 10 %) of the inputted genes are involved in “kinase activity” versus 300 out of 30000 genes (i.e. 1 %) in the human genome (population background) are associated with “kinase activity” then the fold enrichment is tenfold (i.e. 10 % / 1 % = 10) (Huang et al., 2008).

The main biological processes regulated by the VEGF treatment (compared to the control) are cell migration and angiogenesis (Table 6.2). The top biological processes regulated by the apple procyanidin fraction dp 4 treatment (compared to the control; comparison 2) are leukocyte and T-cell activation/proliferation (Table 6.3) however when it is compared to the VEGF treatment (comparison 4) the main biological processes regulated are development and signalling pathways (Table 6.4). When VEGF and apple procyanidin fraction dp 4 are treated in combination and compared to the control (comparison 3), cell migration and differentiation are the main biological process regulated by the treatment (Table 6.5). Whereas when the treatment is compared to the VEGF treatment (comparison 5) the main biological processes regulated include development and signalling pathways (Table 6.6) which is similar to comparison 2. The main biological processes regulated by anti-VEGF antibody-treated VEGF compared to the control are signalling pathways and enzyme activity (Table 6.7) and when the treatment is compared to the apple procyanidin fraction dp 4 treatment the main biological processes regulated are response to stimulus, development and angiogenesis (Table 6.8).

Table 6.2: Top ten biological processes out of 129 genes regulated by VEGF compared to the control.

The top ten biological processes, out of 132 biological processes, regulated by the VEGF treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 129	Fold Enrichment
Regulation of endothelial cell migration	4	20.2
Negative regulation of hydrolase activity	5	12.7
Regulation of angiogenesis	5	9.8
Positive regulation of cell migration	6	8.4
Regulation of caspase activity	5	7.8
Positive regulation of locomotion	6	7.6
Positive regulation of cell motion	6	7.6
Regulation of endopeptidase activity	5	7.5
Regulation of peptidase activity	5	7.1
Response to unfolded protein	4	7.1

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.3: Top ten biological processes out of 92 genes regulated by apple procyanidin fraction dp 4 compared to the control.

Top ten biological processes out of 92 genes regulated by the apple procyanidin fraction dp 4 treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 92	Fold Enrichment*
Negative regulation of MAP kinase activity	4	19.6
Negative regulation of leukocyte activation	5	14.7
Negative regulation of cell activation	5	13.9
Negative regulation of lymphocyte activation	4	12.4
Positive regulation of mononuclear cell proliferation	4	11.7
Positive regulation of leukocyte proliferation	4	11.7
Positive regulation of cell adhesion	4	11.3
Positive regulation of T cell activation	5	11.1
Regulation of lymphocyte differentiation	4	10.8
Regulation of T cell proliferation	4	10.6

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.4: Top ten biological processes out of 233 genes regulated by apple procyanidin fraction dp 4 compared to VEGF.

Top ten biological processes out of 233 genes regulated by the apple procyanidin fraction dp 4 treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 233	Fold Enrichment*
Response to retinoic acid	6	11.7
Cardiac cell differentiation	4	9.6
Negative regulation of MAP kinase activity	5	9.5
Ureteric bud development	5	9.2
Response to vitamin A	6	9.2
Negative regulation of defence response	4	7.4
Metanephros development	5	7.3
BMP signalling pathway	5	7.3
Cell-cell junction organization	4	7.2
ER-nuclear signalling pathway	4	7.2

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.5: Top ten biological processes out 502 genes regulated by apple procyanidin fraction dp 4-treated VEGF compared to the control.

Top ten biological processes out of 502 genes regulated by the apple procyanidin fraction dp 4-treated VEGF treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 502	Fold Enrichment*
Mismatch repair	5	7.2
Regulation of leukocyte migration	4	6.3
Regulation of striated muscle cell differentiation	6	6.3
Regulation of skeletal muscle fibre development	4	5.5
Positive regulation of anti-apoptosis	5	5.4
Regulation of endothelial cell migration	4	5.3
Regulation of muscle cell differentiation	6	5.3
Regulation of nitric oxide biosynthetic process	4	5.0
Positive regulation of alpha-beta T cell activation	4	5.0
Cardiac cell differentiation	4	4.9

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.6: Top ten biological processes out of 355 genes and regulated by apple procyanidin fraction dp 4-treated VEGF compared to VEGF.

Top ten biological processes out of genes regulated by the apple procyanidin fraction dp 4-treated VEGF treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 355	Fold Enrichment*
Response to retinoic acid	6	8.9
Ureteric bud development	6	8.4
Negative regulation of MAP kinase activity	6	8.2
Cardiac cell differentiation	4	7.0
Cell-cell junction organization	5	7.0
Response to vitamin A	6	7.0
Cell junction organization	8	6.9
Metanephros development	6	6.7
Calcium-mediated signalling	5	6.6
Odontogenesis of dentine-containing tooth	5	6.3

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.7: Top ten biological processes out of 129 genes regulated by anti-VEGF antibody-treated VEGF compared to VEGF.

Top ten biological processes out of 129 genes regulated by the anti-VEGF antibody-treated VEGF treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Top ten biological processes	No. of genes involved out of 129	Fold Enrichment*
ER-nuclear signalling pathway	4	13.5
Negative regulation of hydrolase activity	4	9.8
Response to mechanical stimulus	4	8.7
Response to unfolded protein	5	8.7
Regulation of caspase activity	5	7.6
Regulation of endopeptidase activity	5	7.3
Regulation of peptidase activity	5	6.9
Response to protein stimulus	6	6.8
Cartilage development	4	6.6
Regulation of inflammatory response	4	6.4

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

Table 6.8: Top biological processes and regulated by anti-VEGF antibody-treated VEGF compared to apple procyanidin fraction dp 4.

Top biological processes regulated by the anti-VEGF antibody-treated VEGF treatment versus the apple procyanidin fraction dp 4 treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The top biological processes were ranked according to the fold enrichment*.

Biological process	No. of genes involved out of 37	Fold Enrichment*
Response to nutrient	4	10.5
Angiogenesis	4	10.1
Anti-apoptosis	5	9.1
Blood vessel morphogenesis	5	8.8
Response to steroid hormone stimulus	4	7.8
Blood vessel development	5	7.6
Response to nutrient levels	4	7.4
Vasculature development	5	7.4
Tube development	4	6.8
Positive regulation of transport	4	6.7

*fold enrichment = measures the magnitude of enrichment of the gene members associated with each biological process term.

While the different comparisons regulate a varied list of biological processes there are some similarities between the biological processes regulated by the different comparisons. The similar biological processes include cell migration, differentiation, signalling pathways, development and angiogenesis. As VEGF is a pro-angiogenic growth factor it would be expected that these processes would be regulated by the VEGF treatment as they are the main processes involved in angiogenesis. It has also been reported that the main biological processes regulated by an apple procyanidin fraction dp 3.9 treatment were cell death, cell growth and proliferation, cell migration, cell signalling and cell development (García-Conesa et al., 2009). Therefore it would also be expected to observe these processes in the apple procyanidin fraction dp 4 treatments.

Lists of the specific genes that are altered in HUVECs in response to VEGF, apple procyanidin fraction dp 4 and anti-VEGF antibody treatments are listed in the Supplementary Information (Supplementary Information 3: Genes differentially expressed in response to treatments). The genes are ranked into gene ontology groups

according to the gene ontology database http://www.geneontology.org/. The genes that had more than one gene ontology grouping were ranked according to the amount of citations in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) for that particular function.

6.5.5 Pair-wise comparisons of apple procyanidin faction dp 4-treated VEGF treatments were conducted in order to establish whether apple procyanidin fraction dp 4 negates the effects of VEGF

Apple procyanidin fraction dp 4 has been shown to inhibit VEGF-induced VEGFR-2 phosphorylation (Figure 6.3 and Chapter 4), but it is not known whether the treatment negates the genes regulated by VEGF. In order to determine whether apple procyanidin fraction do 4 negates the effects of VEGF different pair-wise comparisons were conducted. In the first comparison, apple procyanidin fraction dp4-treated VEGF was compared to apple procyanidin fraction dp 4 (comparison 6). In this pair-wise comparison there were no statistically significant genes that were differentially expressed at any *p*-value (Table 6.1). This result shows that apple procyanidin fraction dp 4, when treated in combination with VEGF, completely inhibits VEGF-induced changes in gene expression.

In the second pair-wise comparison, apple procyanidin fraction dp 4-treated VEGF was compared to the control (comparison 3). In this comparison 511 genes were differentially expressed (using the most stringent *p*-value; $p < 0.001$; Table 6.1). It was expected that comparison 3 would have the same number of genes that were differentially expressed as the apple procyanidin fraction dp 4 versus control comparison (comparison 2) because if the apple procyanidin fraction dp 4 treatment was inhibiting VEGF-induced changes in gene expression, comparison 3 would be the

same as comparison 2 (i.e. apple procyanidin fraction dp 4-treated VEGF = apple procyanidin fraction dp 4). However, there were only 94 genes that were differentially expressed in comparison 2 (using the most stringent *p*-value; $p < 0.001$; Table 6.1). Using Venn diagram analysis it was shown that out of the differentially expressed genes in comparisons 2 and 3, 89 genes were common and positively correlated between the two groups (Figure 6.6). The majority of the genes regulated by comparison 2 are common with comparison 3. Therefore the apple procyanidin fraction dp 4-treated VEGF treatment is regulating additional genes than apple procyanidin fraction dp 4 alone.

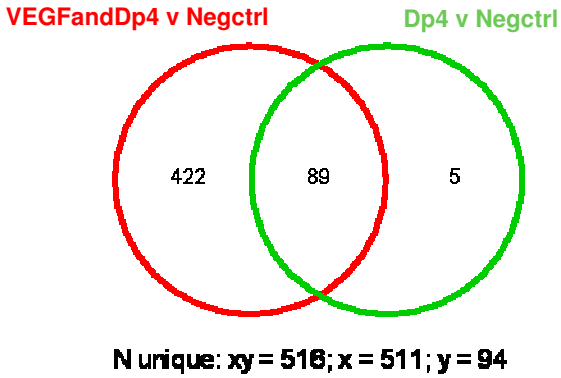


Figure 6.6: Comparison between genes differentially expressed by apple procyanidin fraction dp 4-treated VEGF versus control and apple procyanidin fraction dp 4 versus control ($p < 0.001$).

Overlap between genes differentially expressed by apple procyanidin fraction dp 4-treated VEGF when compared to the control and by apple procyanidin fraction dp 4 when compared to the control. If all gene changes were independent from each other, 3 genes would have been expected to be common between the two data sets as opposed to 89. (False discovery rate = number of genes differentially expressed by VEGF and Dp 4 v Negctrl divided by number of transcripts [18708] and then multiplied by the number of genes differentially regulated by Dp 4 v Negctrl.) Negctrl = control (vehicle DMSO); VEGFandDp4 = 10 ng/ml VEGF plus 1 µM apple procyanidin fraction dp 4; Dp4 = 1 µM apple procyanidin fraction dp 4.

The third, and final, pair-wise comparison was between apple procyanidin fraction dp 4-treated VEGF and VEGF (comparison 5). In this comparison 360 genes were differentially expressed (using the most stringent *p*-value; $p < 0.001$; Table 6.1). It was expected that comparison 5 would have the same number of genes that were

differentially expressed as the apple procyanidin fraction dp 4 versus VEGF comparison (comparison 4) because if the apple procyanidin fraction dp 4 treatment was inhibiting VEGF-induced changes in gene expression, comparison 5 would be the same as comparison 4 (i.e. apple procyanidin fraction dp 4-treated VEGF = apple procyanidin fraction dp 4). However, there were only 235 genes that were differentially expressed in comparison 4 (using the most stringent *p*-value; $p < 0.001$; Table 6.1). Using Venn diagram analysis it was shown that out of the differentially expressed genes in comparisons 4 and 5, 206 genes were common and positively correlated between the two comparisons (Figure 6.7). The majority of the genes regulated by comparison 4 are common with comparison 5. Therefore this also suggests that the apple procyanidin fraction dp 4-treated VEGF treatment is regulating additional genes than apple procyanidin fraction dp 4 alone.

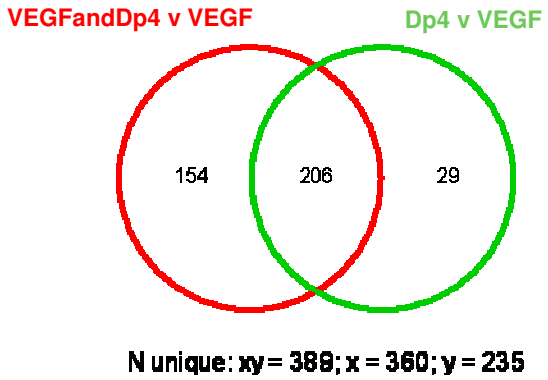


Figure 6.7: Comparison between genes differentially expressed by apple procyanidin fraction dp 4-treated VEGF versus VEGF and apple procyanidin fraction dp 4 versus VEGF ($p < 0.001$).

Overlap between genes differentially expressed by apple procyanidin fraction dp 4-treated VEGF when compared to VEGF and by apple procyanidin fraction dp 4 when compared to VEGF. If all gene changes were independent from each other, 5 genes would have been expected to be common between the two data sets as opposed to 206. VEGF = 10 ng/ml VEGF; VEGFandDp4 = 10 ng/ml VEGF plus 1 µM apple procyanidin fraction dp 4; Dp4 = 1 µM apple procyanidin fraction dp 4.

While the number of genes regulated by the apple procyanidin fraction dp 4-treated VEGF treatment versus the control or VEGF treatments are not similar to the number of genes regulated by the apple procyanidin fraction dp 4 treatment versus the control or VEGF treatments, there are no genes which are differentially regulated in the apple procyanidin fraction dp 4-treated VEGF treatment versus apple procyanidin fraction dp 4 treatment. This shows that apple procyanidin fraction dp 4 inhibits VEGF-regulated genes.

6.5.6 Pair-wise comparisons of anti-VEGF antibody-treated VEGF treatments were conducted in order to establish whether the anti-VEGF antibody could be used as a control for the apple procyanidin fraction dp 4 treatment

As apple procyanidin fraction dp 4 was shown to inhibit VEGF-induced VEGFR-2 phosphorylation (Figure 6.3 and Chapter 4) an anti-VEGF antibody treatment was included in the experimental design to serve as a positive control for the apple procyanidin fraction dp 4 treatment. The anti-VEGF antibody works by inhibiting VEGF from binding to its receptors. Inhibition of VEGF-induced VEGFR-2 phosphorylation was observed with a treatment of 500 ng/ml anti-VEGF antibody, therefore in the presence of the anti-VEGF antibody, VEGF is unable to induce VEGFR-2 phosphorylation (Figure 6.3). It is therefore expected that treating the cells with an anti-VEGF antibody treated-VEGF treatment would be the same as treating the cells with the control and no genes would be regulated by the treatment. However, the anti-VEGF antibody-treated VEGF treatment versus the control (comparison 7) had more than 3600 differentially expressed genes at a p value < 0.05 and 353 differentially expressed genes at a p value of < 0.01 but at a p value of <0.001 there were no differentially expressed genes which were statistically significant (Table 6.1). As the

anti-VEGF antibody-treated VEGF versus the control is regulating genes at the two higher stringent *p*-values, it suggests that the anti-VEGF antibody is regulating additional genes.

In order to confirm whether the anti-VEGF antibody-treated VEGF treatment was regulating additional genes independently of VEGF, a Venn diagram comparison was conducted between comparison 1 (VEGF versus control) and comparison 7 (anti-VEGF antibody-treated VEGF versus control). Comparison 1 differentially regulated 337 genes while comparison 7 differentially regulated 353 genes ($p < 0.01$; Table 6.1). As the anti-VEGF antibody-treated VEGF treatment should be the same as the control treatment, performing a Venn diagram between these two comparisons will provide an indication of the number of differentially expressed genes regulated by the anti-VEGF antibody treatment. Between the two comparisons only 29 genes were common (Figure 6.8), thus providing further evidence that the anti-VEGF antibody is regulating additional genes which are independent of its primary function (inhibition of VEGF activity).

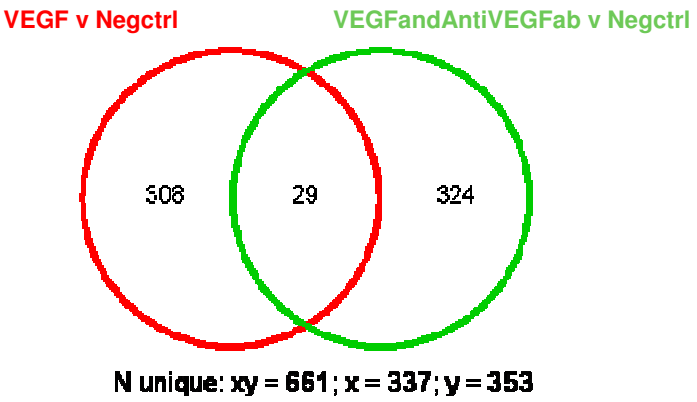


Figure 6.8: Comparison between the genes differentially expressed by VEGF compared to the control and by anti-VEGF antibody-treated VEGF compared to the control ($p < 0.01$).

Overlap between genes differentially expressed by VEGF when compared to the control and by anti-VEGF antibody-treated VEGF when compared to control. If all gene changes were independent from each other, 6 genes would have been expected to be common between the two data sets as opposed to 29. Negctrl = control (vehicle DMSO); VEGF = 10 ng/ml VEGF; VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody.

Further pair-wise comparisons were conducted in order to identify the effect of the anti-VEGF antibody treatment. In the first pair-wise comparison, the anti-VEGF antibody-treated VEGF was compared to the VEGF treatment (comparison 8) and 131 genes were differentially regulated by this comparison (using the most stringent *p*-value; $p < 0.001$; Table 6.1). It was expected that comparison 8 would have the same number of genes that were differentially expressed as the VEGF versus control comparison (comparison 1) because if the anti-VEGF antibody treatment was inhibiting VEGF-induced changes in gene expression comparison 8 would be the same as comparison 1 (i.e. anti-VEGF antibody-treated VEGF = control). Comparison 1 had 132 genes that were differentially expressed. Therefore the two comparisons have essentially the same number of genes being regulated, however only 82 of the genes were common between the two treatments (Figure 6.9). If the anti-VEGF antibody was working correctly, it would also be expected that the regulated genes from comparison 8 would be inversely correlated to comparison 1. The commonly regulated

genes between the two comparisons were all inversely correlated, thus indicating that the anti-VEGF antibody is negating some of the effects of VEGF, but is also regulating additional genes.

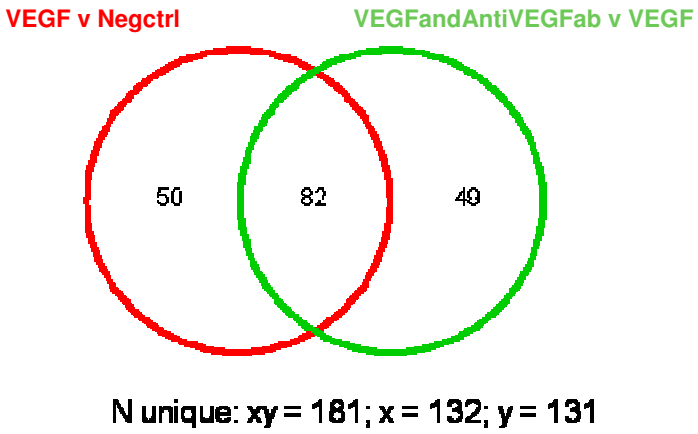


Figure 6.9: Comparison between genes differentially expressed by VEGF compared to the control or anti-VEGF antibody-treated VEGF treatments ($p < 0.001$).

Overlap between genes differentially expressed by VEGF when compared to the control and by anti-VEGF antibody-treated VEGF when compared to VEGF. If all gene changes were independent from each other, 1 gene would have been expected to be common between the two data sets as opposed to 82. Negctrl = control (vehicle DMSO); VEGF = 10 ng/ml VEGF; VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody.

The second, and final, pair-wise comparison was between anti-VEGF antibody-treated VEGF and apple procyanidin fraction dp 4 (comparison 9). In this comparison 37 genes were differentially expressed (using the most stringent *p*-value; $p < 0.001$; Table 6.1). It was expected that comparison 9 would have the same number of genes that were differentially expressed as the apple procyanidin fraction dp 4 versus control comparison (comparison 2) because if the anti-VEGF antibody was inhibiting VEGF-induced changes in gene expression comparison 9 would be the same as comparison 2 (i.e. anti-VEGF antibody-treated VEGF = control). However, there were 94 genes that were differentially expressed in comparison 2 (using the most stringent *p*-value; $p < 0.001$; Table 6.1). Using a Venn diagram analysis it was shown that out of

the differentially expressed genes in comparisons 2 and 9, 25 genes were common and inversely correlated between the two comparisons (Figure 6.10).

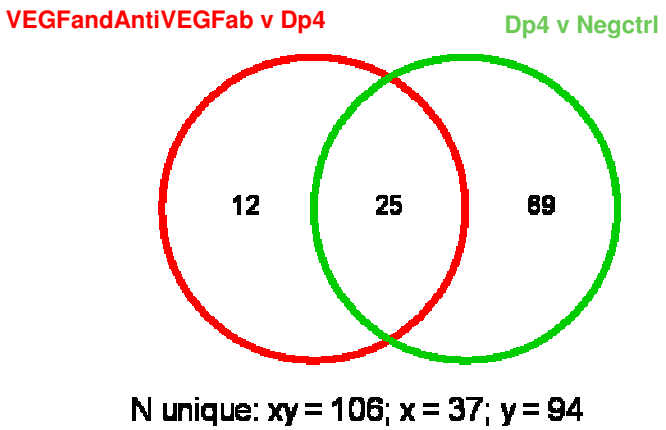


Figure 6.10: Comparison between genes differentially expresed by apple procyanidin fraction dp4 compared to the control or anti-VEGF antibody-treated VEGF treatments ($p < 0.001$).

Overlap between genes differentially expressed by anti-VEGF antibody-treated VEGF when compared to the apple procyanidin fraction dp 4 treatment and by apple procyanidin fraction dp 4 treatment to the control. If all gene changes were independent from each other, 1 gene would have been expected to be common between the two data sets as opposed to 25. Negctrl = control (vehicle DMSO); Dp4 = 1 µM apple procyanidin fraction dp4 VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody.

Based on these results, it is likely that the anti-VEGF antibody is inhibiting VEGF from binding to its receptors as well as regulating additional genes. The regulation of additional genes observed in the different pair-wise comparisons are likely due to the type of antibody used; the anti-VEGF antibody that was used was a polyclonal antibody. Polyclonal antibodies provide certain problems as they are capable of binding to several different epitopes on any given antigen (Lipman et al., 2005; Haurum, 2006) and therefore may exert additional effects beyond neutralization. Therefore the additional regulated genes observed by this treatment are most likely due to the use of a polyclonal antibody and the anti-VEGF antibody treatment can not be used as a positive control for the apple procyanidin fraction dp 4 treatment.

6.5.7 Identification of angiogenic genes regulated by VEGF confirms VEGF is working correctly

Previous microarray analyses have reported that VEGF induces the following genes: A2M, ACE, ANGPT2, BPI, COX2, DSCR-1, EGR1, EGR3, HB-EGF, THBD, PLAU and VCAM1 (Arkonac et al., 1998; Abe and Sato, 2001; Weston et al., 2002; Yang et al., 2002; Wary et al., 2003; Minami et al., 2004; Schoenfeld et al., 2004; Hamik et al., 2006; Suehiro et al., 2010). Of the angiogenic related genes which have been reported to be regulated by VEGF, the microarray analysis in this study has confirmed the up-regulation of the following genes by VEGF: A2M, ANGPT2, EGR3, PLAU, THBD, IL8 and VCAM1 (Table 6.9). It was not expected to observe gene expression data for COX2, EGR1, CYR61, HB-EGF, BPI and DSCR-1 because the peak expression of these genes occurs between 0.5 and 2 h (Abe and Sato, 2001; Minami et al., 2004; Suehiro et al., 2010) and the induction of ACE occurs at 12 h (Abe and Sato, 2001). Induction of 54 % of the angiogenic genes known to be induced by VEGF confirms that VEGF is working correctly in this experiment.

Table 6.9: Fold change of angiogenic, apoptotic, inflammatory, signalling and transcription genes differential expressed by the VEGF versus the control comparison.

	Microarray fold change
	VEGF versus control
A2M	2.18***
ANGPT2	1.81***
PLAU	1.25**
IL8	1.35*
THBD	2.43***
VCAM1	2.28***
VEGFC	1.41***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. p = adjusted p-value for multiple comparisons

6.5.8 Apple procyanidin fraction dp 4 regulates additional genes that are independent of the inhibition of VEGF signalling

In order to identify the genes which are differentially regulated by the apple procyanidin fraction dp 4 treatment that are independent of VEGF signalling a Venn diagram analysis was preformed between the VEGF versus control comparison and the apple procyanidin fraction dp 4 versus control comparison (Figure 6.11). There were 337 genes differentially regulated by the VEGF versus control comparison and 446 genes differentially regulated by the apple procyanidin fraction dp 4 comparison ($p < 0.01$, Table 6.1) and 75 genes were common between the two groups (Figure 6.11). Of those 75 genes, 66 were positively correlated and 9 were negatively correlated. Some of the positively correlated genes included VEGFC, PLAU, NF-κB2, ADAMTS1, THBD and DUSP5 and ANGPT2 were inversely correlated (Table 6.9).

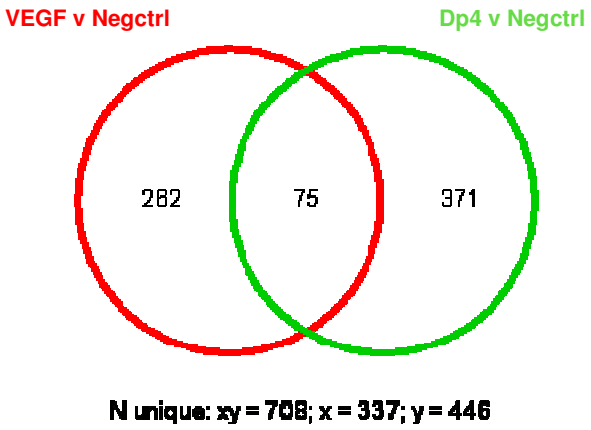


Figure 6.11: Comparison between genes differentially expressed by VEGF compared to the control and apple procyanidin fraction dp4 compared to the control ($p < 0.01$).
Overlap between genes differentially expressed by VEGF compared to the control and apple procyanidin fraction dp 4 compared to the control. If all gene changes were independent from each other, 1 gene would have been expected to be common between the two data sets as opposed to 75. Negctrl = control (vehicle DMSO); Dp4 = 1 µM apple procyanidin fraction dp4 VEGFandAntiVEGFab = 10 ng/ml VEGF plus 500 ng/ml anti-VEGF antibody.

Of the angiogenic, apoptotic, inflammatory, signalling and transcription genes known to be regulated by VEGF (Abe and Sato, 2001; Jih et al., 2001; Hiltunen et al., 2002; Schweighofer et al., 2009) and confirmed in this array (Table 6.9), the majority of those genes are positively regulated by apple procyanidin fraction dp 4, with the exception of IL8, ets variant 1 (ETV1) and ets variant 5 (ETV5) which are negatively regulated by apple procyanidin fraction dp 4 (Table 6.10).

Apple procyanidin fraction dp 4 also regulates additional genes that are independent of VEGF. These include: angiogenesis related genes: apelin (APLN), bone morphogenic protein 4B (BMP4) and angiotensin 1 converting enzyme (ACE); apoptosis related gene: caspase 10, apoptosis-related cysteine peptidease (CASP10); signalling molecules: connective tissue growth factor (CTGF), cysteine-rich, angiogenic inducer, 61 (CYR61) and EDN1; and transcription factors: krüppel-like factor 2 (lung) (KLF2) and krüppel-like factor 4 (gut) (KLF4) (Table 6.10). ADAMTS1 is a secreted matrix metalloproteinase that has been shown to inhibit endothelial cell proliferation. CASP10 is involved in apoptosis and CYR61 and CTGF are members of the CNN family of proteins and are involved in regulating endothelial cell function and promoting cell growth and migration. EDN1 is a potent vasoconstrictor that promotes proliferation and migration and the genes: bone morphogenetic protein 4 (BMP4), APLN, KIT ligand (KITLG), KLF2 and KLF4 have been shown to be involved in angiogenic processes and/or vascular function (García-Conesa et al., 2009).

The majority of the genes that are regulated by apple procyanidin fraction dp 4 in Table 6.10 are also regulated by the apple procyanidin fraction dp 4-treated VEGF treatment with the exception of APLN and Bcl2 which is only regulated by the apple procyanidin fraction dp 4 treatment. The genes CASP6 and caspase 8, apoptosis-related cysteine peptidase (CASP8) are induced by the apple procyanidin fraction dp 4-

treated VEGF treatment but not with apple procyanidin fraction dp 4 in the absence of VEGF. The anti-VEGF antibody treatment has reduced the induction of the genes alpha-2-macroglobulin (A2M) and period homolog 1 (Drosophila) (PER1) and has either induced or down-regulated the genes CASP10, intercellular adhesion molecule 1 (ICAM1), CYR61, dual specificity phosphatase 5 (DUSP5), KLF2, and KLF4 independent of VEGF.

Table 6.10: Fold change of angiogenic, apoptotic, inflammatory, signalling and transcription genes differential expressed by VEGF, apple procyanidin fraction dp 4, apple procyanidin fraction dp 4-treated VEGF and anti-VEGF antibody-treated VEGF.

	Microarray fold change			
	VEGF versus control	**dp 4 versus control**	**VEGF and dp 4 versus control**	**VEGF and anti-VEGF antibody versus control**
Angiogenesis				
A2M	2.18***	1.22*	1.22**	1.25*
ACE	--	1.24*	1.21***	--
ADAMTS1	1.57***	1.36**	1.20*	--
ANGPT2	1.81***	-1.71***	-1.61***	--
APLN	--	-1.37**	--	--
BMP4	--	-1.86***	-1.76***	--
PLAU	1.25**	1.32**	1.16**	--
THBD	2.43***	1.31**	1.34**	--
VEGFC	1.41***	1.44***	1.28***	--
Apoptosis				
Bcl2	1.25*	1.26*	--	1.21*
CASP6	-1.45**	--	-1.31**	--
CASP8	--	--	-1.24**	--
CASP10	--	-1.62***	-1.88***	-1.27*
Inflammatory				
SELE	1.65**	--	-1.34*	
ICAM1	1.45**	1.89***	1.78***	1.51**
IL8	1.35*	-1.31*	-1.34*	--
NF-κB2	1.30**	1.52***	1.53***	1.17*
VCAM1	2.28***	--	--	--
Signalling-related molecules				
CTGF	--	-1.31**	-1.27**	--
CYR61	--	-1.61***	-1.62***	-1.15*
DUSP5	1.24**	1.31**	1.32**	1.30**
EDN1	--	-1.36**	-1.35***	--
KITLG	1.66**	1.47*	--	--
Transcription factors				
ETV1	1.32*	-1.81***	-1.86**	--
ETV5	1.33*	-1.64***	-1.38**	--
EGR3	1.33*	1.26*	1.37**	--
KLF2	--	1.67***	1.75***	1.27**
KLF4	--	1.78***	1.84***	1.24***
HLX	1.30**	1.51***	1.52***	--
MEF2C	1.59***	1.39**	1.20*	--
NR4A2	1.65**	1.24*	1.21**	--
PER1	1.30**	1.22*	1.29**	1.17*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. p = adjusted p-value for multiple comparisons

6.6 Discussion

The data presented in Chapter 4 shows that apple procyanidin fraction dp 4 inhibits VEGF-induced VEGFR-2 activity at low (physiological concentrations). Procyanidins have also been shown to have anti-angiogenic and anti-inflammatory properties that are not necessarily dependent on VEGF-induced signalling. Therefore the aims of the research presented in this chapter were to determine whether apple procyanidin fraction dp 4 blocks VEGF-induced changes in vascular endothelial cells and to establish whether apple procyanidin fraction dp 4 regulates additional genes that are independent of the inhibition of VEGF signalling. The data presented in this chapter shows that apple procyanidin fraction dp 4 (1) completely inhibits VEGF-dependent gene expression; and (2) alters the expression of a large number of genes not dependent on VEGF, the majority relating to angiogenesis and inflammation which are independent of VEGF.

The pair-wise comparison of the apple procyanidin fraction dp 4-treated VEGF cells versus the apple procyanidin fraction dp 4-treated cells was conducted in order to determine whether apple procyanidin fraction dp 4 completely blocks VEGF-dependent gene expression. This pair-wise comparison showed that there were no additional genes that were being regulated by the apple procyanidin fraction dp 4-treated VEGF treatment compared to the apple procyanidin fraction dp 4 treatment (comparison 6, Table 6.1). This result confirms that the apple procyanidin fraction dp 4 treatment completely blocks all VEGF-mediated changes in gene expression.

This is the first time that the co-treatment of VEGF and a polyphenol has been shown to completely block VEGF-mediated changes in gene expression. After an extensive search of the literature, this is the first time that a polyphenol has also been reported to completely block a signalling molecule-mediated changes in gene expression. Liu et al.

(2008) have previously reported gene expression data for HUVECs pre-treated with EGCG (20 µM) for 24 h followed by a 30 min stimulation of VEGF (50 ng/ml). In this experimental design, it was shown that there were no differentially expressed genes when the cells which were pre-treated with EGCG for 24 h followed by a 30 min VEGF stimulation were compared to the cells stimulated with VEGF. However, when cells that were pre-treated with EGCG followed by VEGF stimulation were compared to the cells that were pre-treated with EGCG, 123 genes were reported to be differentially expressed (Liu et al., 2008). The results presented by Liu et al. (2008) shows that pre-treatment of cells with EGCG followed by VEGF stimulation is unable to block genes differentially regulated by VEGF. Therefore, the data presented in this chapter show, for the first time, that the co-treatment of a dietary polyphenol (at physiological concentrations) completely blocks VEGF-mediated changes in gene expression. The comparison of the results presented in this chapter and those by Liu et al. (2008) reiterate the point that the inhibition of VEGF signalling is due to the polyphenol interacting with the VEGF molecule and not the VEGF receptors (Chapter 4, Section 4.5.3).

Because VEGF-induced VEGFR-2 signalling and VEGF-mediated changes in gene expression are completely blocked with the treatment of apple procyanidin fraction dp 4, apple procyanidin fraction dp 4 treatment may have nutraceutical and pharmacological applications for the prevention and treatment of VEGF-induced angiogenesis. The current anti-VEGF drugs available have been reported to increase blood pressure after prolonged periods of use (Kappers et al., 2010) and the systematic reviews and meta-analyses of the anti-VEGF drugs bevacizumab, sorafenib and sunitinib have shown an increased risk of developing hypertension among users of these drugs (Zhu et al., 2007; Wu et al., 2008b; Zhu et al., 2009). The increased susceptibility of developing hypertension is the result of the inhibition of NO production.

VEGF has been reported to stimulate the production of NO through the phosphorylation of AKT (Chavakis et al., 2001; Tang et al., 2003; Riesterer et al., 2004; Rodriguez et al., 2006; Stangl et al., 2007) and the inhibition of VEGF signalling by anti-VEGF drugs would therefore be expected to decrease the production of NO (Wang et al., 2004). Bevacizumab, a monoclonal anti-VEGF antibody, was shown to completely inhibit VEGF-induced NO production in HUVECs (Wang et al., 2004). Apple procyanidin fraction dp 3.9, on the other hand, inhibits VEGF signalling but still induces NO bioavailability by inducing AKT phosphorylation at 1 h (Chapter 4, 4.5.5). Gene expression changes for AKT in the current array analysis was searched, however the expression of AKT was not significant in apple procyanidin fraction dp 4-treated cells compared to the control at the experimental time point of 6 h. As apple procyanidin fraction dp 3.9 and dp 4 are similar in nature, it is highly likely that apple procyanidin fraction dp 4 will also induce phosphorylation of AKT. Therefore the inhibition of VEGF signalling by apple procyanidin fraction dp 4 may not cause an increase in blood pressure or risk of hypertension, as observed for anti-VEGF antibodies, and may be more applicable as an anti-VEGF treatment than the current anti-VEGF drugs.

The anti-VEGF drug treatments may also regulate additional functions beyond their role in neutralizing the effect of VEGF. The mulit-analyte analysis of aqueous humor obtained from patients undergoing bevacizumab injection for VEGF-related ocular pathologies showed that the bevacizumab treatment affected the nature of the eye beyond what would be expected by neutralization of VEGF alone (Sharma et al., 2010). The treatment of rectal cancer cells and tumour-associated macrophages with bevacizumab was shown to block VEGF-induced genes but also significantly up-regulate the expression of genes involved in angiogenesis, independently of VEGF (Xu et al., 2009). The data presented in this chapter has also shown that treatment of HUVECs with an anti-VEGF polyclonal antibody differentially regulates gene

expression independently of its main function of neutralizing VEGF activity (Section 6.5.6). Therefore the use of polyclonal antibodies (shown in this chapter) and monoclonal antibodies (Xu et al., 2009; Sharma et al., 2010) has been shown to cause additional functions beyond the neutralization of VEGF activity. The evidence that antibodies exert additional effects beyond their primary function is a problem as the antibodies might exert detrimental effects; therefore further research regarding the implications of antibody treatments on gene expression and biological processes is required. Thus the potential use of procyanidins may be a better treatment therapy as they are known to have anti-angiogenic and anti-inflammatory properties.

The data presented in this chapter has shown that apple procyanidin fraction dp 4 deferentially regulates the expression of genes involved in angiogenesis and inflammation that are independent of VEGF induction. These genes include the up-regulation of KLF2 and KLF4 and the down-regulation of APLN, BMP4, CASP10, CTGF and EDN1. KLF2 and KLF4 belong to the krüppel-like family of transcriptional regulators that are involved in the regulation of cellular growth and differentiation (Bhattacharya et al., 2005). APLN and BMP4 are genes involved in angiogenesis (Kasai et al., 2004; Rothhammer et al., 2007); CTGF is involved in regulating endothelial cell function and promoting cell growth and migration (Brigstock, 2002) and EDN1 is a potent vasoconstrictor that promotes proliferation and migration (Salani et al., 2000).

Out of the VEGF-independent genes regulated by apple procyanidin fraction dp 4, KLF2 was considered particularly interesting because over-expression of KLF2 has been reported to potently abrogate VEGF-mediated angiogenesis (Hamik et al., 2006). KLF2 is recognised to be an important transcription factor in the suppression of pro-inflammatory and pro-atherosclerotic genes such as VCAM1, SELE, EDN1 and

tissue factor as well as inducing antithrombotic and thrombolytic genes (Bhattacharya et al., 2005; Dekker et al., 2006; Caton et al., 2010). KLF2 is also implicated in the regulation of cell processes including cell growth, development and differentiation (Bhattacharya et al., 2005; Suske et al., 2005; Hamik et al., 2006; Wu et al., 2008a). Therefore further investigation of the induction of KLF2 expression by apple procyanidin fraction dp 4 was conducted and is reported in Chapter 7.

6.7 Conclusion

The main aims of the current chapter were to determine if apple procyanidin fraction dp 4 completely inhibits genes differentially expressed by VEGF and to establish whether apple procyanidin fraction dp 4 regulates additional genes that are independent of the inhibition of VEGF signalling. The data presented in this chapter showed that apple procyanidin fraction dp 4 (1) completely inhibits VEGF-dependent gene expression; and (2) alters the expression of a large number of genes not dependent on VEGF, the majority relating to angiogenesis and inflammation which are independent of VEGF. The array results showed for the first time that apple procyanidin fraction dp 4 (at potentially physiological concentrations) completely blocked VEGF-mediated gene regulation. Therefore the complete inhibition of VEGF-mediated genes by apple procyanidin fraction dp 4 provides a plausible link between consumption of polyphenols and a reduced risk of CVD.

CHAPTER SEVEN

INDUCTION OF KLF2 MRNA EXPRESSION BY APPLE PROCYANIDIN FRACTION DP 4 IS INDEPENDENT OF PI3K/AKT ACTIVATION

Chapter 7 : Induction of KLF2 mRNA expression by apple procyanidin fraction dp 4 is independent of PI3K/AKT activation

7.1 Abstract

In the microarray experiment conducted in Chapter 6 it was found that apple procyanidin fraction dp 4 induces the transcription of genes related to angiogenesis and inflammation which were independent of VEGF. One of those genes that was found to be induced by apple procyanidin fraction dp 4 was KLF2. Reports have suggested that KLF2 expression is up-regulated in response to increased transcription of MEF2 through a PI3K/AKT signalling pathway and it was recently demonstrated that ANGPT1 induces KLF2 expression through this pathway (Sako et al., 2009). Therefore it was hypothesised that apple procyanidin fraction dp 4 induces KLF2 expression by increasing ANGPT1 protein secretion which in turn induces KLF2 expression through the stimulation of MEF2 transcription by PI3K/AKT activation. Hence the ability of apple procyanidin fraction dp 4 and ANGPT1 to induce AKT phosphorylation and KLF2, MEF2A and ANGPT1 transcription was investigated in the presence or absence of PI3K inhibitors. Apple procyanidin fraction dp 4 and ANGPT1 induced AKT phosphorylation and significantly increased both KLF2 and MEF2A mRNA levels in the absence of the PI3K inhibitors. However, apple procyanidin fraction dp 4 did not cause an increase in ANGPT1 mRNA levels or protein secretion. These data indicate that apple procyanidin fraction dp 4 induction of KLF2 mRNA expression is not dependent on the activation of the PI3K/AKT pathway by ANGPT1. In the presence of the PI3K inhibitors, apple procyanidin fraction dp 4 and ANGPT1-induced AKT phosphorylation was reduced. However, apple procyanidin fraction dp 4 was still able to significantly induce both KLF2 and MEF2A mRNA expression, but there was no significant increase after ANGPT1 treatment. Therefore, the induction of KLF2 mRNA expression by apple

procyanidin fraction dp 4 is independent of the PI3K/AKT signalling pathway and may be induced by another pathway such as the MEK5/ERK5, ERK1/2, JNK or p38 kinase signalling pathway. Induction of KLF2 expression by apple procyanidin fraction dp 4 may be an important mechanism explaining how certain polyphenols inhibit angiogenesis as well as reducing the risk of cardiovascular disease.

7.2 Introduction

Apple procyanidin fraction dp 4 inhibits the cellular effects exerted by VEGF as well as inducing genes related to angiogenesis and inflammation which are independent of VEGF, as described in Chapter 6. One of the genes that was found to be induced by apple procyanidin fraction dp 4 was Krüppel-like factor 2 (KLF2). KLF2 was identified as a gene of interest because it has been recognised as an important transcription factor in the suppression of pro-inflammatory and pro-atherosclerotic genes such as VCAM1, E-selectin, EDN1 and tissue factor as well as inducing antithrombotic and thrombolytic genes (Bhattacharya et al., 2005; Dekker et al., 2006; Caton et al., 2010).

7.2.1 Krüppel-like factor 2

KLF2 is a member of the Krüppel-like factor (KLF) family of transcription factors. KLFs are a subclass of the zinc-family of transcriptional regulators implicated in the regulation of cell processes including cell growth, development and differentiation (Bhattacharya et al., 2005; Suske et al., 2005; Hamik et al., 2006; Wu et al., 2008a). There are more than 15 known family members and the KLFs are named according to the tissues in which they were originally shown to be enriched (Bieker, 2001; Cullingford et al., 2008). For example, KLF1 was found to be enriched in the erythroid and is also known as erythroid KLF; KLF2 is lung KLF; KLF4 is gut KLF; KLF5 is intestinal KLF; and KLF15 is kidney KLF (Black et al., 2001; Cullingford et al., 2008).

Members of the KLF family bind to GC-rich or CACCC sequences in the promoter region of target genes to regulate transcription (Black et al., 2001; Kaczynski et al., 2003; Bhattacharya et al., 2005; Lin et al., 2005). Although the KLFs have similar DNA binding activities they vary widely in their regulation of transcription. KLFs can either function as transcriptional activators or repressors depending on the cellular context in

which they function and the promoters to which they bind (Turner and Crossley, 1999; Black et al., 2001; Kaczynski et al., 2003; Wu et al., 2008a). However KLFs are important transcription factors as expression and gene-knockout studies have shown that KLFs regulate fundamental cellular processes such as growth, development, apoptosis, angiogenesis, proliferation and differentiation (Black et al., 2001; Suske et al., 2005; Cullingford et al., 2008; Hart et al., 2011).

KLF2 has numerous functions in the suppression of pro-inflammatory and pro-atherosclerotic genes as well as inducing antithrombotic and thrombolytic genes and regulating angiogenesis. KLF2 is expressed at high levels in the lung but is also abundant in endothelial cells, T cells, preadipocytes and lymphocytes (Turner and Crossley, 1999; Bhattacharya et al., 2005; Dekker et al., 2005; Suske et al., 2005; Hamik et al., 2006). In the embryo, KLF2 expression is required for normal vessel function and vascular development. KLF2 deficient mice exhibit vascular defects despite showing normal angiogenesis and vasculogenesis (Suske et al., 2005; Dekker et al., 2006), however these mice die mid gestation due to haemorrhaging as a result of defective blood vessel morphology and destabilization of the vessel wall due to the lack of KLF2 (Kuo et al., 1997; Turner and Crossley, 1999; Dekker et al., 2005; Lin et al., 2005; Suske et al., 2005; Hamik et al., 2006). KLF2 has also been shown to suppress pro-inflammatory targets such as VCAM1, E-selectin, EDN1 and tissue factor (Bhattacharya et al., 2005; Dekker et al., 2005; Caton et al., 2010), induce anti-inflammatory and anti-thrombotic targets such as eNOS and THBD (Dekker et al., 2005; Lin et al., 2005; van Thienen et al., 2006), promote T-cell quiescence and survival (Turner and Crossley, 1999; Black et al., 2001; Hart et al., 2011), and regulate angiogenesis through the inhibition of VEGF mediated angiogenesis via reduction in VEGFR2 mRNA and protein expression (Bhattacharya et al., 2005; Dekker et al., 2006; Hamik et al., 2006) and the induction of the potent anti-migratory factor SEMA3F (Dekker et al., 2006).

7.2.2 Induction of Krüppel-like factor 2 expression

KLF2 mRNA expression has been shown to be induced by steady laminar shear stress (Dekker et al., 2002; Groenendijk et al., 2004; Suske et al., 2005; Hamik et al., 2006; van Thienen et al., 2006), angiopoietin 1 (ANGPT1) (Sako et al., 2009), EDN1 (Cullingford et al., 2008), statins (van Thienen et al., 2006) and by apple procyanidin extracts (García-Conesa et al., 2009; Caton et al., 2010). It has been reported that induction of KLF2 expression is via the activation of transcription factor myocyte enhancer factor 2 (MEF2) through the mitogen-activated protein kinase 5 (MEK5)/ERK5 (Sohn et al., 2005; Parmar et al., 2006; van Thienen et al., 2006; Sako et al., 2009; Young et al., 2009) or PI3K/AKT signalling pathway (Huddleson et al., 2005; van Thienen et al., 2006; Sako et al., 2009).

Recently it has also been demonstrated that phosphorylation of Tie2 by ANGPT1 stimulated the transcriptional activity of MEF2, through the PI3K/AKT pathway, to induce KLF2 expression (Sako et al., 2009). ANGPT1 is a protein that suppresses anti-inflammatory and anti-permeability factors and regulates endothelial survival (Stoeltzing et al., 2003) and MEF2 is a family of transcription factors, composed of four members (MEF2A, MEF2B, MEF2C and MEF2D), known for their regulation in vascular function (Ornatsky et al., 1999; van Thienen et al., 2006; Maiti et al., 2008).

In a microarray analysis, apple procyanidin fraction dp 4 was shown to up-regulate KLF2, MEF2A and MEF2C gene expression in HUVECs (Chapter 6). The regulation of ANGPT1 and AKT gene expression was not significant with the apple procyanidin fraction dp 4 at the experimental time point of 6 h (Chapter 6). It was therefore hypothesised that apple procyanidin fraction dp 4 induces KLF2 expression through an

increase in ANGPT1 protein secretion which in turn causes an increase in KLF2 expression through the P13K/AKT/MEF2 pathway (Figure 7.1).

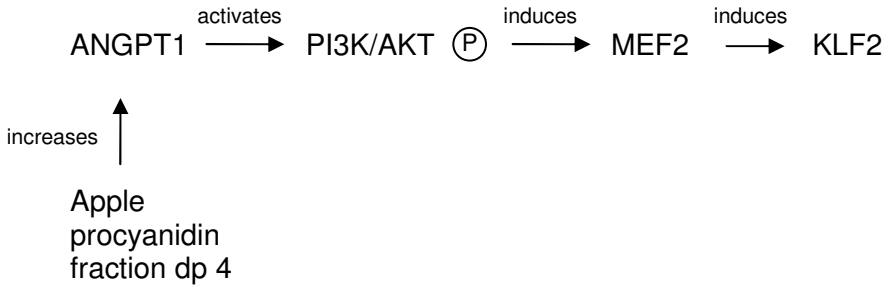


Figure 7.1: Proposed mechanism of KLF2 induction by apple procyanidin fraction dp 4.
It is hypothesised that apple procyanidin fraction dp 4 increases the expression of KLF2 through the increase in protein secretion of ANGPT1. ANGPT1/Tie2 signalling causes the activation and phosphorylation Ⓟ of PI3K/AKT which then induces MEF2 transcription which in turn induces KLF2 expression.

7.3 Objectives

The overall aim of the current study was to investigate whether apple procyanidin fraction dp 4 induces KLF2 expression by causing an increase in ANGPT1 protein secretion which in turn induces KLF2 expression through the stimulation of MEF2 transcription by PI3K/AKT phosphorylation. The specific aims of the chapter were to: 1) evaluate the ability of apple procyanidin fraction dp 4 and ANGPT1 to induce AKT phosphorylation; 2) measure the induction of MEF2 and KLF2 mRNA levels by apple procyanidin fraction dp 4 and ANGPT1 treatments; 3) determine whether apple procyanidin fraction dp 4 increases ANGPT1 mRNA expression and protein secretion; and 4) establish whether the increase in KLF2 mRNA expression is dependent on the PI3K/AKT pathway.

7.4 Materials and methods

7.4.1 Materials

Human recombinant angiopoietin-1 and human angiopoietin-1 Quantikine ELISA kit was obtained from R&D Systems Europe Ltd (Abingdon, UK). All RT-PCR products were from Applied Biosystems. Electrophoresis reagents were purchased from Invitrogen. The phospho-AKT (Ser 473) monoclonal antibody, the anti-rabbit IgG HRP-linked antibody, the PI3K inhibitors LY294002 and wortmannin were from Cell Signaling and the KLF2 antibody and bovine anti-goat IgG HRP-linked antibody was from Santa Cruz (CA, USA). The apple procyanidin fraction dp 4 was purified from the 2007 apples (Chapter 3) provided by Coressence ltd (Herefordshire, UK) as previously described (Yanagida et al., 1999; Shoji et al., 2005; Shoji et al., 2006b), with some modifications. A brief description of the method can be found in Chapter 4 or a more detailed description of the method is located in the Supplementary Information (Supplementary Information 1).

7.4.2 Cell culture and treatments

7.4.2.1 Cell culture

The culture of HUVECs is the same as described in Chapter 4, Section 4.4.2.

7.4.2.2 Treatments

HUVECs were treated in two different manners, with and without PI3K inhibitors. In the absence of PI3K inhibitors flavanol and growth factor treatments were prepared in Universal tubes prior to adding them to the confluent monolayers. Apple procyanidin fraction dp 4 (1 µM), ANGPT1 (200 ng/ml) or the control (vehicle: DMSO) were added to a Universal tube containing basal medium and incubated at room temperature for 5 min. HUVEC confluent monolayers were washed twice with warm phosphate buffered saline (PBS), to remove any residual growth factors, and prepared treatments were

added to the respective wells and incubated for 5 min, 1, 4, 6 and/or 24 h for Western and mRNA analysis at 37 ºC under a humidified atmosphere containing 5 % CO_2.

In the presence of PI3K inhibitors, HUVEC confluent monolayers were washed twice with warm phosphate buffered saline (PBS) and prepared basal medium containing the PI3K inhibitors were added to the cells for 1 h. The prepared PI3K inhibitor medium contained either 50 µM LY294002 or 1 µM wortmannin. After incubation either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) were added to the cells and incubated for 5 min, 1, 4, 6 and/or 24 h for Western and mRNA analysis at 37 ºC under a humidified atmosphere containing 5 % CO_2.

7.4.3 Protein determination

7.4.3.1 Protein extraction

The extraction of protein was the same method as described in Chapter 4, Section 4.4.6.

7.4.3.2 Western blot

Phosphorylated AKT expression levels and KLF2 protein levels were detected by Western blot. The method of preparing the protein and the gels and the blocking, probing, detection and re-probing of the gels was the same method as described in Chapter 4, Section 4.4.7.

For the detection of phosphorylated AKT, the primary antibody used was of phospho-AKT (Ser 473) antibody (5 µl antibody in 5 ml of 5 % BSA in TBST) and the secondary antibody was anti-rabbit IgG, HRP-linked antibody (10 µl in 10 ml of 5 % skimmed milk in TBST). For the detection of KLF2, the primary antibody used was KLF2 antibody (25 µl KLF2 antibody in 5 ml of 5 % skimmed milk in TBST) and the secondary

antibody was bovine anti-goat IgG HRP-linked antibody (5 µl in 10 ml of 5 % skimmed milk in TBST).

7.4.3.3 Angiopoietin-1 ELISA determination

ANGPT1 protein levels were measured by ELISA following the manufacturer's instructions using the cell medium to determine ANGPT1 secreted protein levels. After the incubation, washing and labelling steps, absorption was measured at 450 nm using a Dynex 96 well microplate reader.

7.4.4 RNA extraction

After treatment incubation, HUVECs were washed twice with warm PBS and the RNA was extracted using the RNeasy® Mini Kit (Qiagen Ltd, UK) according to the manufacturer's protocol. The homogenization step in the protocol was achieved by using a QIAshredder (Qiagen Ltd, UK). The quality and quantity of RNA present in the samples was quantified using a Beckman DU-640 spectrometer prior to storage at -80 ºC. For a pure and good quality RNA sample the absorbance at 260/280 should be approximately 2; this was observed for all the samples.

7.4.5 Real-time Reverse Transcription Polymerase Chain reaction

Pre-designed gene assays for KLF2 (Assay ID: Hs00360439_g1), MEF2A (Assay ID: Hs01050409_m1) and ANGPT1 (Assay ID: Hs00375822_m1) were purchased from Applied Biosystems. Target gene mRNA levels were determined by real time RT-PCR as described in Chapter 6, Section 6.4.5.

7.4.6 Statistical analysis

Where indicated, comparisons between mean values between control and treated samples were carried out using a two tailed t-test. A p value < 0.05 was considered significant.

7.5 Results

7.5.1 Apple procyanidin fraction dp 4 and angiopoietin-1 both induce KLF2 expression

The microarray analysis in Chapter 6 showed that apple procyanidin fraction dp 4 induced KLF2 expression (1.67 fold induction) at a p value of < 0.001. Therefore in order to test the hypothesis that apple procyanidin fraction dp 4 induces KLF2 expression through the ANGPT1 signalling pathway and confirm the results of the microarray analysis, the KLF2 transcript levels in apple procyanidin fraction dp 4 and ANGPT1 treated HUVECs was quantified.

HUVECs were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) for 5 min, 1, 4, 6 and 24 h and analysed for KLF2 mRNA transcript levels by RT-PCR. The data show that HUVECs treated with the vehicle exhibited an increase in KLF2 mRNA levels at 1 h (Figure 7.2). The expression of KLF2, in vehicle-treated cells, at 4 h and 6 h then decreased to below the level at 5 min and returned to baseline at 24 h. This shows that KLF2 mRNA levels fluctuate during a 24 h period.

When HUVECs were treated with apple procyanidin fraction dp 4, a statistically significant increase in KLF2 mRNA levels was observed at 4 h (p = 0.01) and 6 h (p = 0.01). A statistically significant increase in KLF2 mRNA levels was also observed

with ANGPT1 treatment at 1 h (p = 0.043), 4 h (p = 0.01), 6 h (p = 0.01) and 24 h (p = 0.008). Based on these results it can be concluded that apple procyanidin fraction dp 4 and ANGPT1 induce KLF2 gene expression and that ANGPT1 causes a more prolonged induction of KLF2 expression than apple procyanidin fraction dp 4. The increase of KLF2 expression by apple procyanidin fraction dp 4 is also consistent with the microarray analysis.

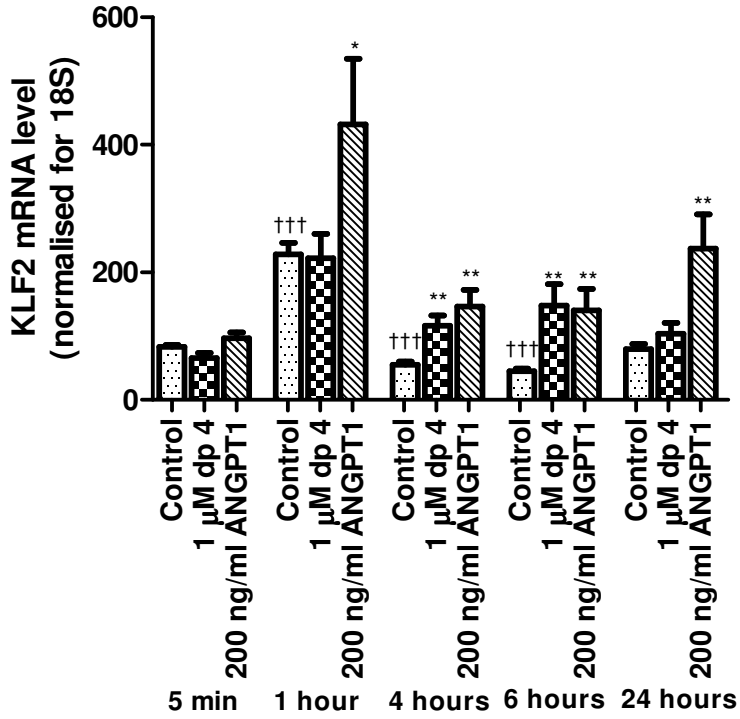


Figure 7.2: Apple procyanidin fraction dp 4 and ANGPT1 induce KLF2 mRNA transcription.

HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 5 min, 1, 2, 4, 6 and h. RNA was extracted from the cells using the RNeasy® Mini Kit and the KLF2 quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of KLF2 normalised against 18S. *p < 0.05, ** p < 0.01 compared to the control at each time point. ††† p < 0.001 compared to the control at 0 h. Data are representative of three independent experiments with two biological and two technical replicates.

KLF2 protein content was also determined by Western blot. HUVECs were treated with either 1 µM apple procyanidin fraction dp 4 or 200 ng/ml ANGPT1 for 5 min, 1, 4, 6 and 24 h. The data demonstrate that there is no change in protein expression in the vehicle, apple procyanidin fraction dp 4 or ANGPT1 treated cells (Figure 7.3).

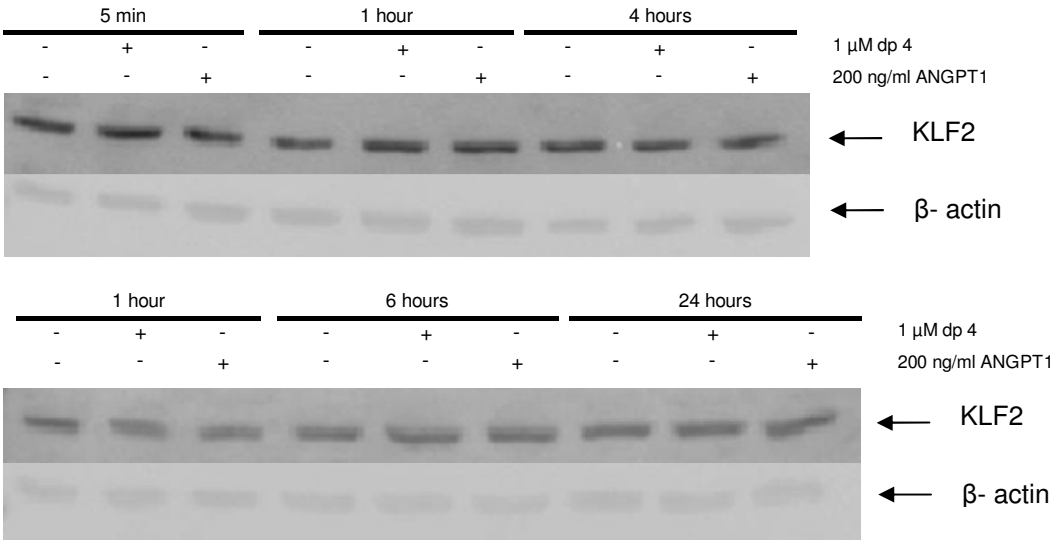


Figure 7.3: Apple procyanidin fraction dp 4 and ANGPT1 do not change the protein expression of KLF2.

HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) for 5 min, 1, 4, 6 or 24 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of KLF2 and β-actin (control protein). Data are representative of triplicate replicates of three independent experiments.

7.5.2 Apple procyanidin fraction dp 4 and angiopoietin-1 induce phosphorylation of AKT

It has previously been reported that the induction of KLF2 expression is dependent on the activation of the PI3K/AKT pathway (Huddleson et al., 2005; van Thienen et al., 2006; Cullingford et al., 2008; Sako et al., 2009). In order to confirm that the induction of KLF2 by apple procyanidin fraction dp 4 and ANGPT1 is dependent on the activation of AKT, the effects of these treatments on AKT phosphorylation were evaluated.

HUVECs were treated for 1 h with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) in order to confirm phosphorylation of AKT. The data presented show that HUVECs, with no added treatments, do not induce phosphorylation of AKT. However, when HUVECs were treated with either apple

procyanidin fraction dp 4 or ANGPT1, phosphorylation of AKT was induced (Figure 7.4).

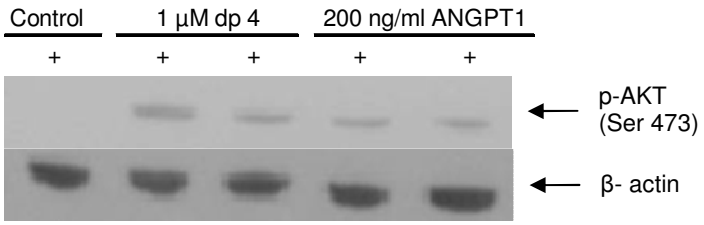


Figure 7.4: Apple procyanidin fraction dp 4 and ANGPT1 induce phosphorylation of AKT. HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) for 1 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated AKT at the serine 473 site and β-actin (control protein). Data are representative of triplicate replicates of six independent experiments.

7.5.3 Apple procyanidin fraction dp 4 and angiopoietin-1 induce MEF2A expression

After activation of the PI3K/AKT signalling pathway, the next step in the induction of KLF2 expression is the increase in MEF2 expression. In the microarray analysis from Chapter 6, apple procyanidin fraction dp 4 was shown to increase MEF2A (1.32 fold induction) and MEF2C (1.39 fold induction) expression after a 6 h incubation ($p < 0.01$). To confirm the results of the microarray analysis, the induction of MEF2A transcription was determined by RT-PCR.

HUVECs were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) for 5 min, 1, 4, 6 and 24 h. The quantity of MEF2A expression increased in the control samples from 80 to 110 ng during the time period 5 min to 24 h (Figure 7.5). This shows that MEF2A transcription increases over a 24 h period.

When treated with apple procyanidin fraction dp 4, a significant increase in MEF2A mRNA levels was observed at the treatment time points of 5 min ($p = 0.043$), 4 h

($p < 0.0001$) and 6 h ($p = 0.002$) when compared to the control at each time point. A significant increase in MEF2A mRNA expression was also observed with the treatment of ANGPT1. A significant increase was observed at 4 h ($p = 0.036$) and 6 h ($p = 0.031$) when compared to the control at each time point. Based on these results in can be concluded that apple procyanidin fraction dp 4 and ANGPT1 induce MEF2A transcription and that apple procyanidin fraction dp 4 causes a greater induction of MEF2A expression than ANGPT1. The increase of MEF2A expression by apple procyanidin fraction dp 4 is also consistent with the data from the microarray analysis.

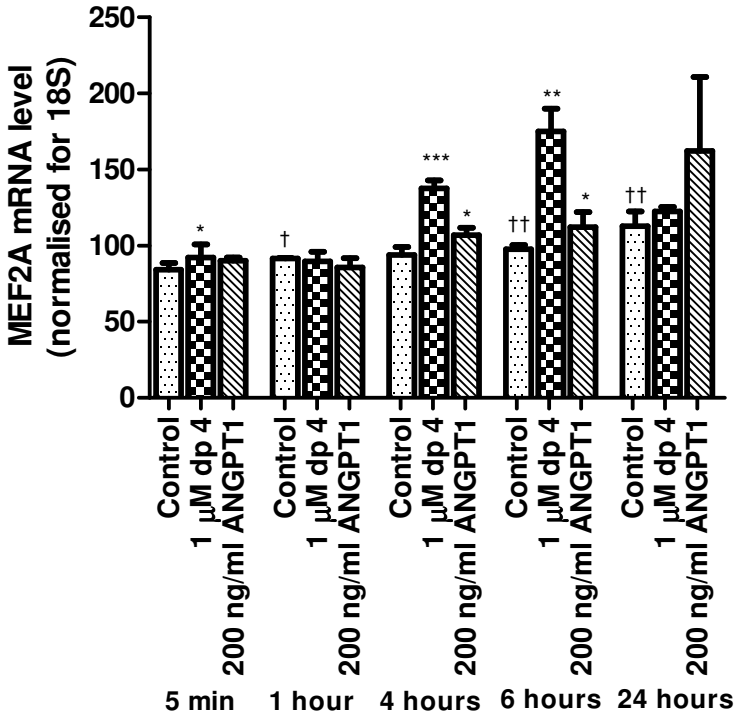


Figure 7.5: Apple procyanidin fraction dp 4 and ANGPT1 induce MEF2A mRNA transcription.

HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 5 min, 1, 2, 4, 6 and h. RNA was extracted from the cells using the RNeasy® Mini Kit and the MEF2A quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of MEF2A normalised against 18S. *$p < 0.05$, *** $p < 0.001$ compared to the control at each time point. † $p < 0.05$, †† $p < 0.01$ compared to the control at 0 h. Data are representative of three independent experiments with two biological and two technical replicates.

7.5.4 Apple procyanidin fraction dp 4 does not increase angiopoietin-1 protein secretion or induce angiopoietin-1 expression

In Sections 7.5.1-3 it was shown that apple procyanidin fraction dp 4 induces PI3K/AKT phosphorylation and induces MEF2A and KLF2 transcription in the same manner as ANGPT1. Therefore the induction of KLF2 by apple procyanidin fraction dp 4 may be dependent on apple procyanidin fraction dp 4 increasing the protein secretion of ANGPT1.

HUVECs were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or vehicle (DMSO, control) for 5 min, 1, 4, 6 and 24 h and ANGPT1 expression was either analysed by RT-PCR or ELISA. Analysis by RT-PCR measures the increase in ANGPT1 mRNA levels whereas analysis by ELISA measures the quantity of circulating ANGPT1 protein in the medium.

For RT-PCR analysis the data shows that there is an overall decrease in ANGPT1 mRNA expression in HUVECs treated with the vehicle. The decrease is statistically significant at 4 h ($p = 0.044$), 6 h ($p = 0.002$) and 24 h ($p < 0.0001$) when compared to the 5 min control time point (Figure 7.6). When HUVECs were treated with apple procyanidin fraction dp 4, a statistically significant decrease in ANGPT1 mRNA levels was also observed at 4 h ($p = 0.025$) and 6 h ($p < 0.0001$). Treating the cells with ANGPT1 did not further increase ANGPT1 transcription.

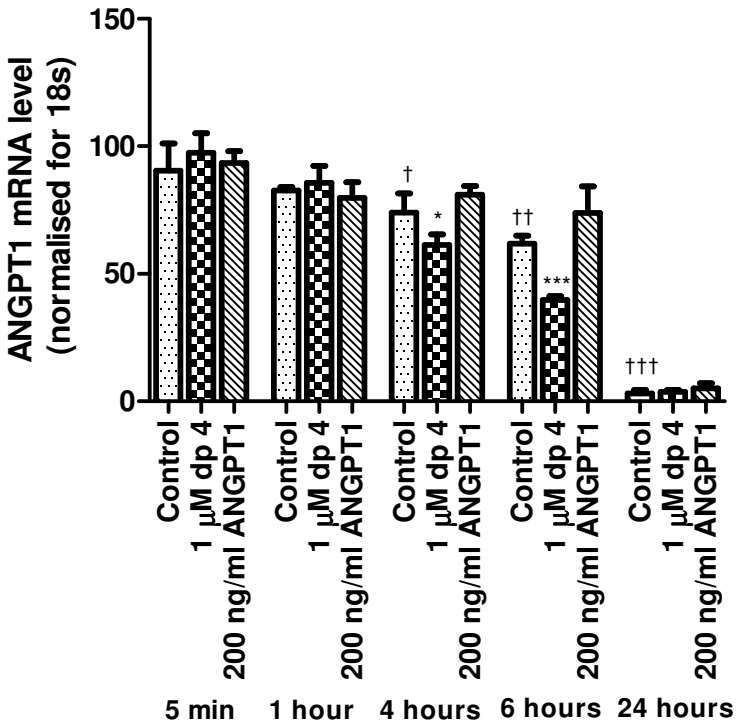


Figure 7.6: Apple procyanidin fraction dp 4 does not induce ANGPT1 mRNA transcription.

HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 5 min, 1, 2, 4, 6 and h. RNA was extracted from the cells using the RNeasy® Mini Kit and the ANGPT1 quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of ANGPT1 normalised against 18S. $^{**}p < 0.01$, *** $p < 0.001$ compared to the control at each time point. †† $p < 0.01$ compared to the control at 0 h. Data are representative of three independent experiments with two biological and two technical replicates.

The determination of the quantity of ANGPT1 protein circulating by ELISA showed that the vehicle and apple procyanidin fraction dp 4 treated HUVECs do not cause an increase in the amount of ANGPT1 protein secreted in the medium (Figure 7.7). The quantification of these two treatments by ELISA resulted in values below the plate blank measurement; therefore the quantities of ANGPT1 were not detectable. The amount of ANGPT1 in the medium of ANGPT1 treated HUVECs decreased from 37.75 ng/ml to 14.75 ng/ml from 5 min to 24 h. Since the HUVECs were treated with 200 ng/ml of ANGPT1, these data suggest that a large proportion of ANGPT1 is utilised by the cells in the first 5 min and this is followed by a slow reduction in ANGPT1 from 1 h to 24 h.

Based on these results it can be concluded that apple procyanidin fraction dp 4 does not induce ANGPT1 mRNA expression or increase ANGPT1 protein secretion. Therefore the induction of KLF2 by apple procyanidin fraction dp 4 is independent of ANGPT1.

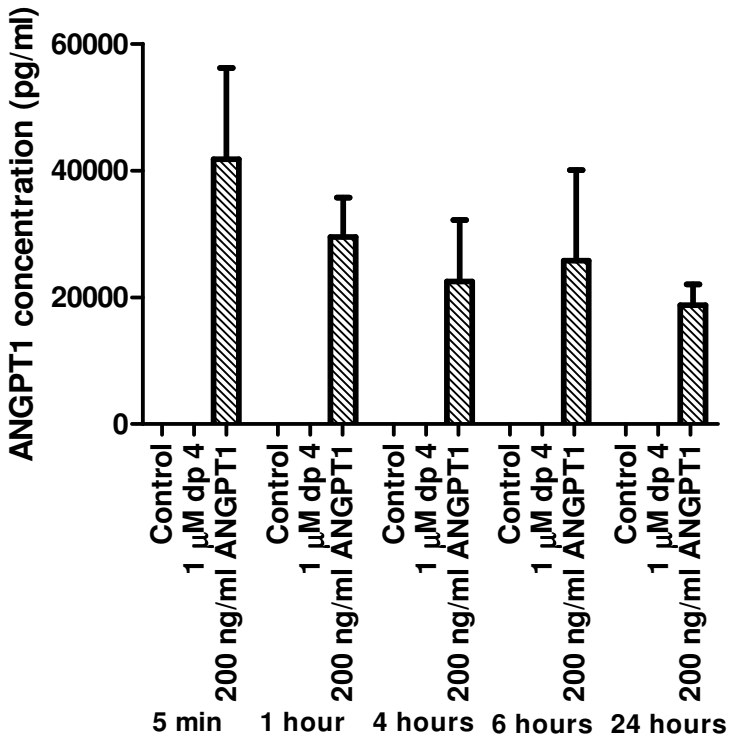


Figure 7.7: Apple procyanidin fraction dp 4 does not induce ANGPT1 protein secretion. HUVECs were treated with pre-incubated basal medium containing either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 5 min, 1, 2, 4, 6 and h. The medium was collected, centrifuged and the ANGPT1 protein content was determined by an Angiopoietin-1 Quantikine ELISA kit. ANGPT1 content could not be determined as it was below the plate bland measurement. Bars represent means ± standard deviation expressed as the quantity of ANGPT1 (pg/ml). Data are representative of one independent experiment with two biological and two technical replicates.

7.5.5 Apple procyanidin fraction dp 4 induced KLF2 mRNA expression is not dependent on AKT phosphorylation

Apple procyanidin fraction dp 4-induced KLF2 mRNA transcription has been shown to be similar to ANGPT1-induced KLF2 mRNA transcription; both compounds induce AKT

phosphorylation and MEF2A and KLF2 expression to a similar extent. However, the induction of AKT phosphorylation, and MEF2A and KLF2 mRNA transcription by apple procyanidin fraction dp 4 is not a result of increased ANGPT1/Tie2 signalling (Section 7.5.4). In order to test whether the induction of KLF2 by apple procyanidin fraction dp 4 is dependent on the activation of the PI3K/AKT signalling pathway, HUVECs were treated with PI3K inhibitors.

HUVECs were pre-treated with either 50 µM LY294002, 1 µM wortmannin or no inhibitors for 1 h to allow for the inhibition of PI3K. Pre-incubated HUVECs with or without inhibitors were then treated with either 1 µM apple procyanidin fraction dp 4 or 200 ng/ml or the vehicle (DMSO, control) for 1 h for Western blot analysis or for 6 h for mRNA expression analysis. The data in Figure 7.8 show that when HUVECs were treated with the vehicle, AKT phosphorylation is not increased, but when the cells are treated with either apple procyanidin fraction dp 4 or ANGPT1, phosphorylation of AKT increases substantially (Figure 7.8 A & B). When treated with the PI3K inhibitors LY294002 (Figure 7.8 A) or wortmannin (Figure 7.8 B) the induction of AKT phosphorylation is reduced compared to the inhibitor-free cell treatments.

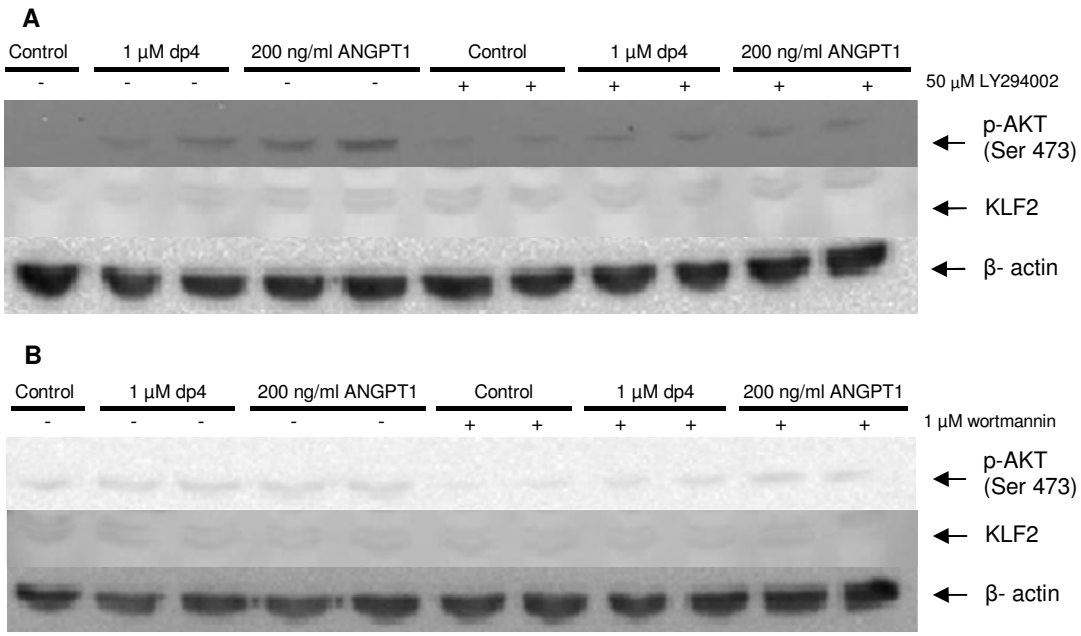


Figure 7.8: PI3K inhibitors LY294002 and wortmannin reduce apple procyanidin fraction dp 4 and ANGPT1-induced AKT phosphorylation.

HUVECs were treated with either basal medium or medium containing 50 µM LY294002 **(A)** or 1 µM wortmannin **(B)** for 1 h prior to the addition of treatments. Pre-incubated HUVECs with or without PI3K inhibitors were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 1 h. The cells were lysed and the proteins were separated on a NuPAGE gel and probed for the presence of phosphorylated AKT at the serine 473 site and β-actin (control protein). Data are representative of triplicate replicates of two independent experiments.

RT-PCR analysis of KLF2 shows that apple procyanidin fraction dp 4 and ANGPT1 treatments caused a significant increase of KLF2 mRNA levels in the absence of PI3K inhibitors (Figure 7.9). PI3K inhibitor LY294002 significantly increased ($p < 0.001$) KLF2 mRNA levels in vehicle treated HUVECs whereas wortmannin significantly decreased ($p < 0.001$) KLF2 mRNA transcription. Apple procyanidin fraction dp 4 was still able to induce KLF2 mRNA transcription in the presence of LY294002 ($p = 0.009$) and wortmannin ($p = 0.008$) while the increase in ANGPT1-induced KLF2 expression was not significant ($p > 0.05$). Western blot analysis of KLF2 protein expression shows no difference between treatments (Figure 7.8).

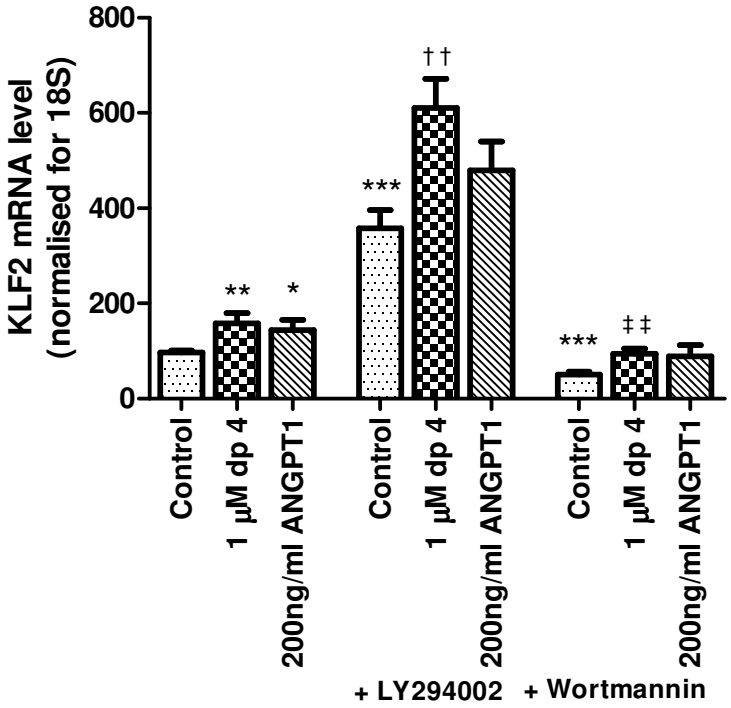


Figure 7.9: Apple procyanidin fraction dp 4 induces KLF2 mRNA transcription in the presence or absence of the PI3K inhibitors LY294002 and wortmannin.

HUVECs were treated with either basal medium or medium containing 50 µM LY294002 or 1 µM wortmannin for 1 h prior to the addition of treatments. Pre-incubated HUVECs with or without PI3K inhibitors were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 1 h. RNA was extracted from the cells using the RNeasy® Mini Kit and the KLF2 quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of KLF2 normalised against 18S. * $p < 0.05$, ** $p < 0.01$, compared to the control of each treatment condition. †† $p < 0.01$ compared to the control with LY294002. ‡ ‡ $p < 0.01$ compared to the control with wortmannin. Data are representative of two independent experiments with two biological and two technical replicates.

The change in MEF2A expression in response to apple procyanidin fraction dp 4 treatment follows a similar pattern to that of KLF2. PI3K inhibitor LY294002 causes a significant decrease ($p = 0.005$) in MEF2A mRNA levels in vehicle treated HUVECs while there is no change ($p > 0.05$) in MEF2A expression in wortmannin treated cells (Figure 7.10). Apple procyanidin fraction dp 4 treatment induces MEF2A expression in the absence ($p = 0.003$) and presence of PI3K inhibitors (LY294002 $p < 0.0001$; wortmannin $p = 0.007$). No significant increases ($p > 0.05$) in MEF2A mRNA levels in response to ANPGT1 were observed in any of the treatments. Based on this data it can

be concluded that the induction of KLF2 expression by apple procyanidin fraction dp 4 is independent of the activation of the PI3K/AKT signalling pathway. However, since ANGPT1 did not cause a significant increase in MEF2A or KLF2 mRNA expression in the presence of either of the PI3K inhibitors, it can be concluded that the activation of these two transcription factors by ANGPT1 is dependent on PI3K/AKT activation.

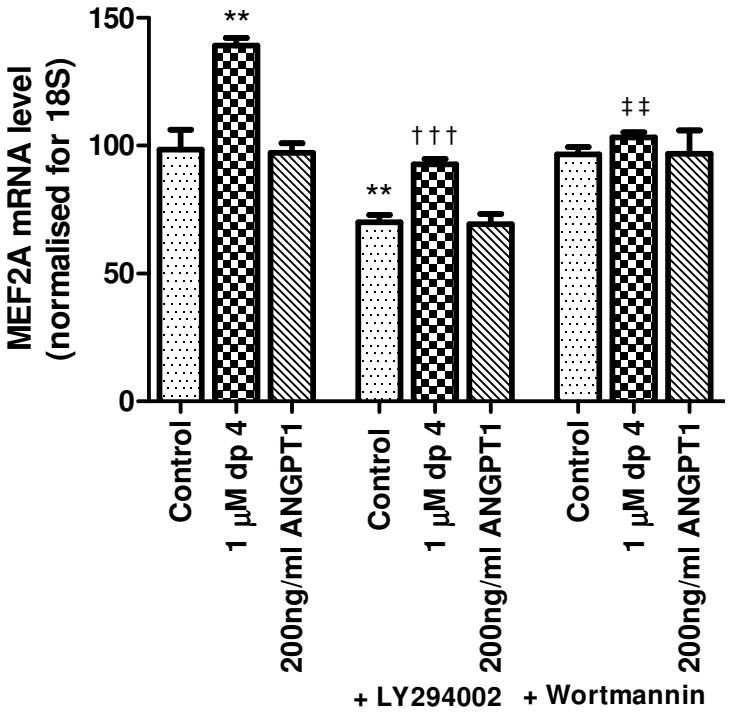


Figure 7.10: Apple procyanidin fraction dp 4 induces MEF2A mRNA transcription in the presence or absence of the PI3K inhibitors LY294002 and wortmannin.

HUVECs were treated with either basal medium or medium containing 50 µM LY294002 or 1 µM wortmannin for 1 h prior to the addition of treatments. Pre-incubated HUVECs with or without PI3K inhibitors were treated with either 1 µM apple procyanidin fraction dp 4, 200 ng/ml ANGPT1 or the vehicle (DMSO, control) for 1 h. RNA was extracted from the cells using the RNeasy® Mini Kit and the MEF2A quantity was determined by real time RT-PCR. Bars represent means ± standard deviation expressed as a quantity of MEF2A normalised against 18S. ** $p < 0.01$, ***$p < 0.001$, compared to the control. ††† $p < 0.001$ compared to the control with LY294002. ‡‡ $p < 0.01$ compared to the control with wortmannin. Data are representative of two independent experiments with two biological and two technical replicates.

7.6 Discussion

The data presented in this chapter show that: (1) apple procyanidin fraction dp 4 and ANGPT1 activate the PI3K/AKT pathway and induce MEF2A and KLF2 mRNA transcription; (2) the induction of KLF2 by apple procyanidin fraction dp 4 was independent of ANGPT1; and (3) the induction of KLF2 expression by apple procyanidin fraction dp 4 was not dependent on phosphorylation of AKT by PI3K whereas induction of KLF2 expression by ANGPT1 was dependent on the activation of AKT by PI3K.

KLF2 is an important transcription factor involved in the suppression of pro-inflammatory and pro-atherosclerotic genes as well as inducing anti-thrombotic genes. KLF2 mRNA has been shown to be induced by a number of factors including ANGPT1, apple procyanidin fraction dp 3.9 (García-Conesa et al., 2009) and tetrameric and pentameric apple procyanidin fractions (Caton et al., 2010). The induction of KLF2 expression has been shown to be dependent on the activation of MEF2 transcriptional activity (Huddleson et al., 2005; van Thienen et al., 2006; Sako et al., 2009). MEF2 transcription factors are a subfamily of the MADS box family of transcription factors and the MEF2 family members (MEF2A, MEF2B, MEF2C and MEF2D) are best known for their critical role in the morphogenesis and myogenesis of skeletal, cardiac and smooth muscle cells (Ornatsky et al., 1999; Kumar et al., 2005; Hamik et al., 2006; van Thienen et al., 2006; Maiti et al., 2008). Target gene knockdown of MEF2 has been shown to reduce KLF2 expression (Kumar et al., 2005; Sen-Banerjee et al., 2005; Sako et al., 2009), therefore creating a pivotal role of MEF2 in the induction of KLF2.

MEF2 factors have been implicated as targets for signalling cascades in response to serum stimulation and cellular stressors (Ornatsky et al., 1999) and it has been shown that the transcriptional activity of MEF2 is increased by the MEK5/ERK5 (Kato et al.,

1997; Yang et al., 1998a; Ornatsky et al., 1999; Sohn et al., 2005; Parmar et al., 2006; Young et al., 2009) or the PI3K/AKT signalling pathways (Huddleson et al., 2005; van Thienen et al., 2006; Sako et al., 2009; Zahlten et al., 2010). The present study investigated the role of the PI3K/AKT pathway in the induction of MEF2 and KLF2 expression, partly because AKT had already been shown to be induced by apple procyanidins (Chapter 4, Section 4.5.5). It was hypothesised that the induction of KLF2 expression may be dependent on apple procyanidin fraction dp 4 increasing ANGPT1 protein secretion. ANGPT1 is an important mediator of angiogenesis through its regulation of endothelial cell survival (Stoeltzing et al., 2003) and has been shown to induce KLF2 expression through the activation of the PI3K/AKT pathway (Sako et al., 2009; Fukuhara et al., 2010). ANGPT1 exerts its cellular effects by binding to the tyrosine receptor Tie2.

Treatment of HUVECs with apple procyanidin fraction dp 4 and ANGPT1 induced phosphorylation of AKT as well as increasing the transcription of MEF2A and KLF2. These observations are consistent with previously reported data showing induction of AKT phosphorylation by apple procyanidins (Chapter 4, Section 4.5.5) and ANGPT1 (Sako et al., 2009) as well as the increases in KLF2 expression induced by apple procyanidins (Chapter 6) (García-Conesa et al., 2009; Caton et al., 2010) and ANGPT1 (Sako et al., 2009). However apple procyanidin fraction dp 4 did not increase ANGPT1 mRNA transcription or protein secretion, therefore the activation of the P13K/AKT pathway by apple procyanidin fraction dp 4 was independent of ANGPT1/Tie2 signalling. In order to establish whether apple procyanidin fraction dp 4-induced KLF2 expression was dependent on the PI3K/AKT signalling pathway two chemically unrelated PI3K inhibitors were investigated. PI3K inhibitor LY294002 is a highly selective inhibitor of PI3K and blocks PI3K-dependent AKT kinase activity (Huddleson et al., 2006). Wortmannin is a specific and direct inhibitor of PI3K, and inhibition by wortmannin is irreversible and non-competitive (Dimmeler et al., 1998).

The induction of KLF2 expression has been shown to be either regulated by the PI3K/AKT pathway (Huddleson et al., 2005; Huddleson et al., 2006; Sako et al., 2009; Zahlten et al., 2010) or not regulated by the PI3K/AKT pathway (Cullingford et al., 2008; Sinclair et al., 2008; Young et al., 2009). Zahlten et al. (2010) demonstrated that the PI3K inhibitors LY294002 and wortmannin both reduced pneumococci-related KLF2 protein expression in human bronchial epithelial cells. LY294002 was also shown to reduce the activation of a KLF2 reporter gene in pneumococci-infected lung epithelial cells (Zahlten et al., 2010). In HUVECs, wortmannin reduced the basal expression of KLF2 as well as inhibiting ANGPT1 induced KLF2 expression (Sako et al., 2009). While Huddleson et al. (2005, 2006) showed that induction of KLF2 expression by shear stress was blocked when human or mouse hemangioendothelioma cells and HUVECs were treated with LY294002. In contrast it was shown that the PI3K inhibitor LY294002 increased both basal expression of KLF2 and promoted the increase in KLF2 expression induced by endothelian-1 or H_2O_2 in cardiac myocytes (Cullingford et al., 2008). In $CD8^+$T cells, the mRNA expression of KLF2 was significantly increased in the presence of the PI3K inhibitor LY294002 (Sinclair et al., 2008). Further, the inhibitors LY294002 and wortmannin had little effect on shear-induced increases in KLF2 expression in HUVECs (Young et al., 2009).

The present chapter showed that basal expression of KLF2 was reduced in HUVECs treated with wortmannin, but an increase in basal expression was observed with the PI3K inhibitor LY294002. ANGPT1-induced KLF2 expression was also reduced in response to wortmannin and LY294002 treatment, however the apple procyanidin fraction dp 4 was still able to induce KLF2 transcription in the presence of both PI3K inhibitors. The observed ANGPT1-mediated induction of KLF2 transcription and inhibition of KLF2 transcription in response to wortmannin are consistent with the data presented by Sako et al. (2009), and it has generally been shown that wortmannin

decreases KLF2 expression. However, LY294002 treatment has had opposing effects in different cells lines, and both induction and inhibition of KLF2 transcription have been reported. Huddleson et al. (2005, 2006) has shown that KLF2 transcription in HUVECs was inhibited with LY294002 while in the present study it was shown to be increased in response to LY294002. The reason for this discrepancy may be due to differences in cell culture/treatment conditions; Huddleson et al. (2005, 2006) used HUVECs exposed to shear stress while HUVECs in the present study were investigated in the static state. Therefore the induction of KLF2 expression via the PI3K/AKT pathway may be dependent on the cell line used and the treatments applied.

KLF2 transcription has been shown to be increased in response to other signalling pathways. In addition to the PI3K/AKT pathway, KLF2 has been reported to be increased in response to activation of the MEK5/ERK5 signalling pathway. For example, resveratrol has been reported to induce KLF2 expression through SIRT and a MEK/MEF2-dependent and ERK5-independent pathway (Gracia-Sancho et al., 2010) as well as inducing KLF4 expression via a MEK5/MEF2-dependent, ERK5-independent pathway (Villarreal et al., 2010). Apple procyanidin fraction dp 4 may induce KLF2 expression through this pathway, or through the other three families of MAP kinases: the extracellular signal-related protein kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinase (Ornatsky et al., 1999; Kato et al., 2000) or through another not yet identified signalling pathway. From the microarray data in Chapter 6, gene expression changes for MEK5, JNK, ERK1/2 and p38 kinase were investigated in the list of differentially expressed genes by apple procyanidin fraction dp 4 versus control. In this list, ERK1/2 was induced 1.12 fold (p = 0.047) and the expression changes of MEK5, JNK and p38 kinase were not significant (Chapter 6).

Apple procyanidin fractions have been reported to increase ERK1/2 and JNK expression in human colon cancer-derived metastatic SW620 cells (Gossé et al.,

2005). However, inhibition of ERK1/2 had no effect on KLF2 promoter activation by fluid flow (Huddleson et al., 2005) and the inhibition of p38 kinase, ERK1/2 or JNK had no effect on KLF2 expression in lung epithelial cells (Zahlten et al., 2010). Therefore the effects of these pathways on the induction of KLF2 expression by apple procyanidin fraction dp 4 in HUVECs needs to be investigated.

7.7 Conclusions

The induction of KLF2 expression by apple procyanidin fraction dp 4 is not dependent on PI3K/AKT activation of the transcription factor MEF2, in contrast to ANGPT1 induction of KLF2 transcription, which is dependent on the PI3K/AKT signalling pathway. It can be concluded that the induction of KLF2 transcription via the PI3K/AKT pathway is dependent on the cell line used and the treatment applied; different stimuli, such as ANGPT1 and apple procyanidin fraction dp 4, trigger different signalling pathways to stimulate an increase in KLF2 transcription. The induction of KLF2 transcription has also been shown to be induced via the MEK5/ERK5, ERK1/2, JNK and p38 kinase signalling pathway. Therefore apple procyanidin fraction dp 4 may induce KLF2 transcription through one of these pathways and further investigation on how apple procyanidin fraction dp 4 increases KLF2 transcription is required. The induction of KLF2 transcription by apple procyanidin fraction dp 4 may be an important mechanism explaining how certain polyphenols inhibit VEGF-induced angiogenesis as well as reducing the risk of cardiovascular disease.

CHAPTER EIGHT

GENERAL DISCUSSION AND RECOMMENDATIONS FOR FURTHER RESEARCH

Chapter 8 : General discussion and recommendations for further research

8.1 Summary of main findings

The overall aims of this thesis were to (1) assess the potential of apples as a rich source of extractable bioactive polyphenols and (2) investigate the bioactivity of apple polyphenols with a focus on understanding mechanisms of action in the vascular endothelium. The main findings presented in this thesis are as follows:

- It was shown that polyphenol concentrations are highest in small, young fruits but total polyphenol yield is highest in large mature fruits, and that polyphenol concentrations and yields were inversely associated with growing season temperatures and positively associated with growing season rainfall and sunlight. These observations provide new insights into the sources of the significant inter-seasonal variation in polyphenol content and composition of apples.

- Although the hypothesis that microwave heat treating of fresh apples prior to pressing would destroy browning enzymes and increase polyphenol yields during apple pressing was disproved, it was demonstrated that hot water extraction of apple pomace, the by-product of apple juice production, was a very effective method of extracting the polyphenols retained in the pomace and yielded a polyphenol-rich aqueous extract.

- Consumption of an apple polyphenol-rich extract supplemented apple juice by human subjects resulted in small but significant improvements in mean arterial

stiffness and vascular age, but not in reflective index, compared to a low polyphenol apple juice, demonstrating the potential for health benefits from consumption of apple polyphenols.

- A novel mechanism by which certain apple polyphenols can alter vascular endothelial cell function at physiological concentrations has been demonstrated. This involves binding of apple procyanidin fraction dp4 directly to the signalling peptide VEGF, which renders VEGF unable to induce signalling through its receptor VEGFR-2. Only a few of the polyphenols tested functioned in this way and it was shown that inhibition of VEGF function was not due to non-specific protein binding.

- Subsequently it was demonstrated that the inhibition of VEGF activity is apparently the result of non-covalent slow binding of one or more molecules of apple procyanidin fraction dp 4 to the VEGF protein which results in the formation of a tightly bound VEGF-polyphenol complex, and that VEGF-apple procyanidin fraction dp 4 complexes were unable to induce any of the multiple changes in gene transcription observed for untreated VEGF.

- The global analysis of genes altered by apple procyanidin fraction dp 4, VEGF or VEGF:apple procyanidin fraction dp 4 complex treatments also demonstrated that apple procyanidin fraction dp 4 regulates genes involved with angiogenesis and inflammation that are independent of VEGF, including induction of KLF2 expression. Finally it was demonstrated that the apple procyanidin fraction dp4-induced up-regulation of KLF2 transcription was not dependent on PI3K/AKT activation of the transcription MEF2, in contrast to ANGPT1-mediated induction of KLF2 transcription, which is dependent on the PI3K/AKT signalling pathway.

The following sections discuss the implications of the major findings of the research presented in the previous chapters in the context of the principal objective which was to investigate polyphenols in apples and their interactions with vascular endothelial cells.

8.1.1 Does the consumption of dietary polyphenols contribute to the health benefits associated with certain foods (e.g. fruits, vegetables and cocoa)?

The consumption of fruits and vegetables is associated with a reduced risk of chronic diseases, including cardiovascular disease as discussed in Chapter 1. The protective effects of fruits and vegetables are attributed, in part, to the polyphenols present in these foods. The intake of fruits, vegetables and cocoa products rich in epicatechin and other flavanols have been shown, *in vivo*, to (1) improve endothelial function of arterial vessels (Stein et al., 1999; Agewall et al., 2000; Chou et al., 2001; Duffy et al., 2001; Hodgson et al., 2002; Engler et al., 2004a; Grassi et al., 2005b; Schroeter et al., 2006); (2) lower blood pressure (Hollenberg et al., 1997; Taubert et al., 2003; Grassi et al., 2005a; Schroeter et al., 2006); and (3) reduce LDL cholesterol (Hooper et al., 2008; Jia et al., 2010). The improvements in endothelial function and blood pressure are due to the increase in the bioavailability and bioactivity of NO as a result of the consumption of flavanols (Heiss et al., 2003; Grassi et al., 2005b; Schroeter et al., 2006).

The majority of the dietary intervention trials have assessed the effects of soy- or soy-isoflavone-products or the effects of flavanols from tea, cocoa/chocolate or red wine/grapes on endothelial function. To date there has only been one dietary intervention trial assessing the effects of apple polyphenols on endothelial function (Auclair et al., 2010). The dietary intervention trial by Auclair et al. (2010) showed that there was no significant difference between the long-term consumption of a high-polyphenol or a low-polyphenol re-hydrated lyophilised apple sample in

hypercholesterolemic subjects. The human intervention trial presented in this thesis contributes to the existing data on the effects of flavanols on endothelial function and stiffness index as it shows that markers of vascular function (stiffness index and vascular age) are improved after the consumption of a polyphenol-rich apple extract supplemented drink (~78 mg epicatechin) in nine non-compromised male subjects. The consumption of 78 mg epicatechin is the equivalent of eating 6.5 dessert apples (average 8.33 mg epicatechin per 100 g), eating 2 cider apples (average 28.67 mg epicatechin per 100g), drinking 5 servings of pure apple juice (serving size is 200 ml; average 7.76 mg epicatechin per 100 ml) or drinking ~ 2000 servings of apple juice from concentrate (average 0.04 mg epicatechin per 100 ml; average epicatechin contents in apple products obtained from www.phenol-explorer.eu). The data from the human intervention trial in this thesis shows that high polyphenol apples can improve vascular function in the same manner as tea, cocoa/chocolate and red wine/grapes.

Flavanols have also been shown, *in vitro*, to induce numerous cellular effects but their ability to (1) increase NO bioavailability by inhibiting NADPH oxidase activity; (2) enhance endogenous antioxidant defences by increasing Nrf2 expression; and (3) inhibit VEGF-induced angiogenesis most strongly supports their putative role in promoting cardiovascular health. However, *in-vitro* studies do not always take into account the bioavailability and metabolism of dietary polyphenols. The majority of cell-protective research of flavanols has shown a protective effect of flavanols at supraphysiological concentrations (Williamson and Manach, 2005). For example, the NO-preserving effect of flavanol treatments on endothelial cells was achieved at concentrations of 10 µM to 40 µM (Steffen et al., 2005; Steffen et al., 2007). Flavanols were shown to inhibit NADPH oxidase activity at concentrations of 10 µM (Steffen et al., 2007) and the inhibition of VEGF-induced VEGFR-2 phosphorylation by flavanols in endothelial cells was shown to occur at concentrations ranging from 15 µM to 100 µM (Kondo et al., 2002b; Neuhaus et al., 2004; Wen et al., 2008; Lu et al., 2010). However,

the physiological concentrations of dietary flavanols do not exceed 10 µM in practice, and thus the effects of polyphenols *in-vitro* at concentrations > 10 µM are generally not valid (Williamson and Manach, 2005). For example, green tea polyphenols have been shown to have antioxidant effects *in-vitro* at high concentrations that are not achievable as plasma concentrations, but no effect on serum lipid concentrations or resistance of LDL to oxidation *ex-vivo* was observed after the daily consumption of 6 cups of green or back tea per day for four weeks (van het Hof et al., 1997). The majority of the data available from *in-vitro* studies are also based on the use of flavanol forms which are present in foods rather than how they would exist in circulation. As the majority of polyphenols are rapidly and extensively metabolised (glucuronidated, sulfated or methylated), the *in-vitro* studies are not representative of what occurs *in-vivo* (Kroon et al., 2004). Therefore it is important to take into account diet relevant protective mechanisms of polyphenols when researching their cell-protective effects.

Numerous reports have shown that certain polyphenols are able to inhibit VEGF-induced VEGFR-2 phosphorylation at high and physiologically unrealistic concentrations (15 µM to 100 µM) (Kondo et al., 2002b; Kojima-Yuasa et al., 2003; Neuhaus et al., 2004; Wen et al., 2008; Lu et al., 2010) and have not directly investigated the mechanisms of how polyphenols inhibit VEGFR-2 signalling. Some researchers have proposed that polyphenols do not inhibit VEGFR-2 signalling by interacting with the receptor because their was no reduction in VEGFR-2 expression following polyphenol treatment (Tang et al., 2003; Rodriguez et al., 2006), while other researchers stated that polyphenols may exert their inhibitory effect by binding to VEGFR-2 and thus prevent VEGF from binding to its receptor (Lamy et al., 2002; Lamy et al., 2006). These two theories were based on the same experimental design: pre-treat the cells with polyphenols, remove medium, and treat the cells with VEGF. Therefore the present literature indicates a lack of knowledge of the exact mechanism of how polyphenols inhibit VEGFR-2 signalling.

The data presented in this thesis have shown for the first time that an apple procyanidin fraction and EGCG were able to specifically inhibit VEGF-induced VEGFR-2 phosphorylation by binding directly and non-covalently to the VEGF molecule, and that the polyphenol-VEGF complex was important in respect of inhibiting VEGFR-2 phosphorylation. Further, this occurred at physiological concentrations (EGCG IC_{50} = 0.09 µM; apple procyanidin fraction dp 4 IC_{50} = 0.28 µM). The apple procyanidin fraction was also shown to completely negate the cellular effects of VEGF as well as regulate additional genes (e.g. KLF2) involved in angiogenesis and inflammation which are independent of VEGF as assessed by an Affymetrix oligo array. These findings are significant because they provide a plausible link between consumption of dietary polyphenols and a reduced risk of CVD. More importantly this data shows a novel mechanism of how polyphenols inhibit VEGF-induced VEGFR-2 phosphorylation. While this research has only shown an interaction with VEGF, it raises the possibility that polyphenols can affect other signalling pathways by interacting with other signalling peptides such as growth factors and hormones.

8.1.2 Potential application of apple polyphenols as nutraceuticals and functional foods

As polyphenols have been shown to have numerous health-related benefits, the enrichment of foods and beverages with polyphenols may prove to be beneficial for human health. Due to the health-related benefits of polyphenols several companies (e.g. Mars Incorporated, Nestlé, Kraft Foods, and Danisco) are currently developing and promoting polyphenol-rich products (Mars, www.marsbotanical.com; Nestlé, www.research.nestle.com; Kraft Foods, www.kraftbrands.com/kraftnutrition; Danisco, www.danisco.com/wps/wcm/connect/www/corporate/media/news/company_news/2008/october/pressrelease424_en.htm). Functional foods are foods with an additional or

enhanced physiological benefit beyond the traditional nutrients it contains (Buttriss, 2010) and nutraceuticals are any substance that is a food or a part of a food that provides health benefits including the prevention or treatment of a disease. Polyphenols may be regarded as nutraceuticals or functional foods based on their ability to protect lipids from oxidative damage, maintain normal blood pressure, improve endothelial function and/or inhibit VEGF-induced angiogenesis.

For example polyphenols could be used as nutraceulticals for the inhibition of VEGF-induced angiogenesis. The use of polyphenols in reducing VEGF-induced angiogenesis may prove to be more beneficial than the current anti-VEGF drugs available. Anti-VEGF drugs have been reported to increase blood pressure after prolonged periods of use (Kappers et al., 2010) and systematic reviews and meta-analyses of the anti-VEGF drugs bevacizumab, sorafenib and sunitinib have also shown an increased risk of developing hypertension among users of these drugs (Zhu et al., 2007; Wu et al., 2008b; Zhu et al., 2009). VEGF stimulates the production of NO through the phosphorylation of AKT (Chavakis et al., 2001; Tang et al., 2003; Riesterer et al., 2004; Rodriguez et al., 2006; Stangl et al., 2007) and the inhibition of VEGF signalling by anti-VEGF drugs would therefore be expected to decrease the production of NO (Wang et al., 2004). On the other hand, data presented here have demonstrated that certain polyphenols inhibit VEGF signalling but still induce NO bioavailability by increasing AKT phosphorylation. Therefore the inhibition of VEGF signalling by certain polyphenols may not cause an increase in blood pressure or risk of hypertension, as observed for anti-VEGF antibodies, and may be more applicable as an anti-VEGF treatment than the current anti-VEGF drugs.

Recently, health claims related to cocoa flavanols and their protection of lipids from oxidative damage and maintenance of normal blood pressure was considered by the European Food Safety Authority (EFSA) (EFSA Panel on Dietetic Products, 2010). On

the basis of the current published data available in supporting the claim for cocoa flavanols and their protection of lipids from oxidative damage and maintenance of normal blood pressure, EFSA concluded that a cause and effect relationship had not been established (EFSA Panel on Dietetic Products, 2010). Therefore the pursuit of polyphenol health claims still continues.

In order to use apple polyphenols as nutraceuticals and functional foods the full polyphenol profile of the apple fruit needs to be well defined and the extraction method needs to be efficient. When considering the use of apple fruit, harvesting for extraction can be based on the optimal yield of the individual polyphenol of interest, while the harvesting date of fruit for consumption can not be adjusted because of taste characteristics. The investigation of the effect of growth and maturity and seasonal variations on the concentration and content of polyphenols in apples has been previously reported (Lister et al., 1994; Mayr et al., 1995; van der Sluis et al., 2001; Lata et al., 2005; Takos et al., 2006; Lata and Tomala, 2007; Renard et al., 2007; Stracke et al., 2009). However the reports were lacking a full apple polyphenol profile analysis. The data presented here show, for the first time, a complete quantitative HPLC analysis of how all the polyphenols found in apples change during the growing season and between growing seasons, and it is the first time a direct effect of seasonal variations on individual apples trees has been reported. The results from this research show, for the first time, that mean temperature, during the growing season months, was negatively correlated with polyphenol content and concentration and that the total amount of rainfall was positively correlated with the concentration and content of polyphenols in apples. The research presented also show for the first time that the polyphenol content and concentration of apples and the percentage of individual polyphenols in the apples, from the same tree, is variable between growing seasons. Therefore the synthesis of polyphenols in apples is a complex process which is dependent on several variables. However the general trends of the changes in

concentration and content of polyphenols in apples remains the same between different growing seasons. As the taste characteristics of apples are not important when extracting polyphenols from apples, the harvesting of apples can therefore be based on the optimal yield of the individual polyphenol of interest.

The extraction of polyphenols from apples has been previously reported; however these extraction methods do not provide an overall high extraction yield or change the taste characteristics of the polyphenol-enriched juice (Spanos et al., 1990; Schols et al., 1991; Will et al., 2000; Gerard and Roberts, 2004; Mihalev et al., 2004; van der Sluis et al., 2004). The research presented in this thesis showed for the first time that the hot water extraction of apple pomace, a by-product of apple juice production, was a very efficient method in extracting polyphenols. However the initial pressing of the apples, the incubation and second pressing steps resulted in a loss of polyphenols, and further investigation is required to improve the recovery of polyphenols during these processes. Hot water extraction might be used with different fruits to increase the extraction yield of polyphenols. The extracted polyphenols can then either be supplemented back into the product (e.g. enriched juice), can be used as a polyphenol supplementation in other products or have pharmacological applications (e.g. inhibition of VEGF signalling).

8.2 Recommendations for further research

The data presented here show that apple polyphenols interact with vascular endothelial cells to improve vascular function and have shown the potential application of apple polyphenols as nutraceuticals and functional foods. Isolated apple polyphenols (procyanidins) were shown to have a nutraceutical and/or a pharmacological application in the inhibition of VEGF signalling. It was identified that the use of apple procyanidins may be more applicable than the current anti-VEGF drugs available as they may not cause an increase in blood pressure as reported, in the literature, with the prolonged use of anti-VEGF antibodies. In order to assess the possibility of pharmacological use of apple procyanidins further research is required. In particular further research in developing a more sensitive method in analysing the bioavailability of procyanidins in humans and determining whether procyanidins are able to reduce atherosclerotic plaque progression (for example in an apolipoprotein E/apolipoprotein mouse model of atherosclerosis) is required.

Apple polyphenols, through the consumption of an apple polyphenol-rich apple extract supplement drink, were also reported here to improve indices of vascular function (vascular stiffness index and vascular age). However as a recently published report showed no effect on endothelial function after the long-term consumption of lyophilised apple extract, further investigation of the effect of the consumption of apple polyphenols, from different food matrices, on endothelial function is required. The investigation of the acute consumption of apple polyphenol-rich foods in different food matrices (for example the consumption of a polyphenol-rich re-hydrated lyophilised apple sample compared to the consumption of a polyphenol-rich apple extract supplemented drink) will provide further evidence regarding apple polyphenols and their vasodilatory effects on endothelial function and the impact on food matrices on endothelial function.

In order to use apple polyphenols for a pharmacological product or as a supplement, they are required to be efficiently extracted from the apple fruit. Currently the extraction of polyphenols from apple pomace, a by-product of apple juice production, does not take into account the loss of polyphenols associated with juicing. The data presented here show that while a hot water extraction efficiently extracts the polyphenols from the apple pomace a large proportion of the polyphenols are lost during juicing. The loss is most likely due to polyphenol oxidases present in the apple fruit. Reducing the loss of polyphenols associated with fruit juicing will also ultimately result in an increase in the polyphenol content of the extracted juice. Food technologists therefore need to investigate how to reduce the loss of polyphenols involved in juicing. The investigation of alternative methods (for example juicing apples in a reduced oxygen controlled environment) which inactivates the polyphenol oxdiase enzymes present in apple fruits but also efficiently extracts the polyphenols will also be beneficial in the producing apple polyphenol extracts for use as nutraceuticals or functional foods.

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SUPPLEMENTARY INFORMATION 1

PURIFICATION OF APPLE PROCYANIDIN FRACTIONS DP 3 AND DP 4

Supplementary Information 1

Purification of apple procyanidin fractions dp 3 and dp 4

Step 1: Removal of flavonoids and phenolics

The apple procyanidin fractions dp 3 and dp 4 were purified from the 2007 apples (Chapter 2) provided by Coressence ltd (Herefordshire, UK). The purification method was based on previously published methods (Yanagida et al., 1999; Shoji et al., 2005; Shoji et al., 2006b), with some modifications.

Fifty grams of freeze-dried apple powder (Tree C, Week 6 from 2007) was extracted in 1250 ml of 0.1 % potassium disulphite, shaken and left to stand for 10 min. The phenolic profile of sample Tree C from Week 6 is described in Table S1.1. The sample was then centrifuged (J6-M1 centrifuge, Beckman Coulter) for 10 min at 4200 x rpm and filtered through a miracloth (Calbiochem). The sample was passed through a pre-wetted Sepabeads SP-850 (Sigma) column (355 mm x 25 mm). The Sepabeads SP-850 column was prepared by transferring the dry resin to a 500 ml glass beaker, adding sufficient methanol to cover the resin bed by 1 – 2 inches and stirring gently for approximately one 1 min to ensure complete mixing. The material was then allowed to stand for 15 min. The majority of the methanol was then carefully removed and distilled water was added and left to stand for 5 min. The Sepabeads SP-850 was then transferred to column. Samples of each fraction were collected and analysed using two different HPLC methods (reverse-phase HPLC analysis and normal-phase HPLC analysis) as described in Chapter 2, Section 2.4.5. Example chromatograms of the standards are shown in Figure S1.1.

Table S1.1: Polyphenol profile of freeze-dried apple sample Tree C (Week 6) from 2007

Polyphenol	mg/kg dry weight	Polyphenol	mg/kg dry weight
Epicatechin	4462.5	Chlorogenic acid	3988.4
Catechin	378.1	Phloridzin	1897.5
Dimer (dp 2)	3259.8	Rutin	88.2
Trimer (dp 3)	2974.6	Quercetin-3-galactoside	297.0
Tetramer (dp 4)	2507.1	Quercetin-3-glucoside	101.2
Pentamer (dp 5)	1766.9	Quercetin-3-arabinoside	41.0
Hexamer (dp 6)	1838.6	Quercetin-3-xyloside	100.1
Heptamer (dp 7)	1468.5	Quercetin-3-rhamnoside	110.1
Total	**25279.7 mg/kg (dry weight)**		

After adding the sample to the Sepabeads SP-850 column, the fraction eluted (Fraction: Speabeads filtrate) contained chlorogenic acid only in the sample (Figure S1.2). Fractions were eluted by gravitational force only; no vacuum pressure was applied. The column was washed with 300 ml of distilled water and the resulting fraction (Fraction: Sepabeads distilled water rinse) contained chlorogenic acid and epicatechin (Figure S1.3). The crude apple polyphenol fraction was eluted with 200 ml of 50 % (v/v) ethanol (Fraction 1). Fraction 1 contained epicatechin, catechin, procyanidins dp 2 – 7, chlorogenic acid, phloridzin as well as quercetin glycosides (Figure S1.4).

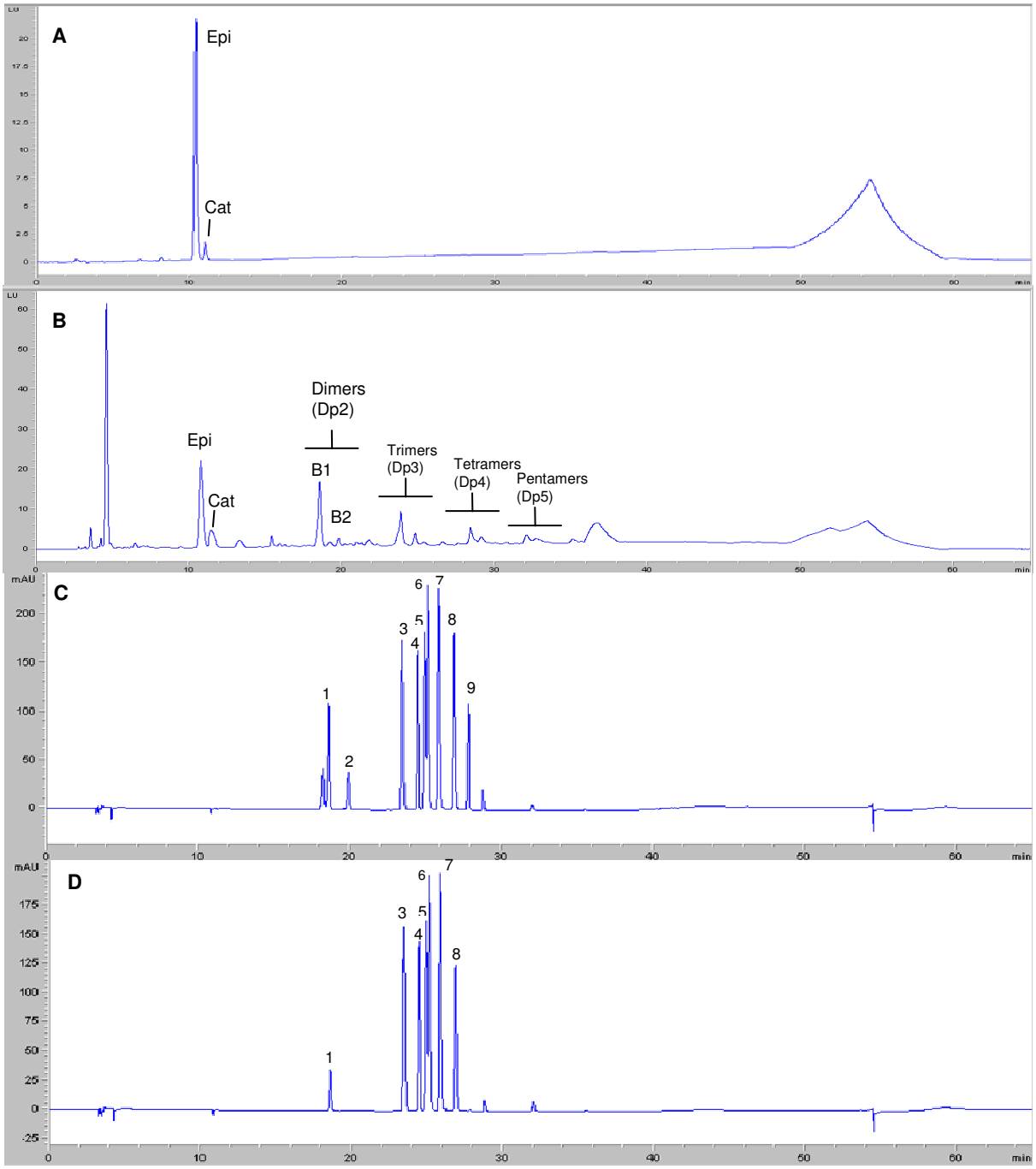

Figure S1.1: Example chromatograms for standards.

Example chromatogram of normal-phase HPLC analysis of an epicatechin standard **(A)** and the NIST chocolate standard **(B)**. Peak IDs: epi = epicatechin and cat = catechin. Example chromatogram of reverse-phase HPLC analysis of a mixed standard at 270 nm **(C)** and 370 nm **(D)**. Peak ID: 1= chlorogenic acid, 2 = epicatechin, 3 = quercetin-3-*O*-arabinose, 4 = quercetin-3-*O*-rutinoside (rutin), 5 = quercetin-3-*O*-galactoside, 6 = quercetin-3-*O*-glucoside, 7 = quercetin-3-*O*-xyloside, 8 = quercetin-3-*O*-rhamnoside.

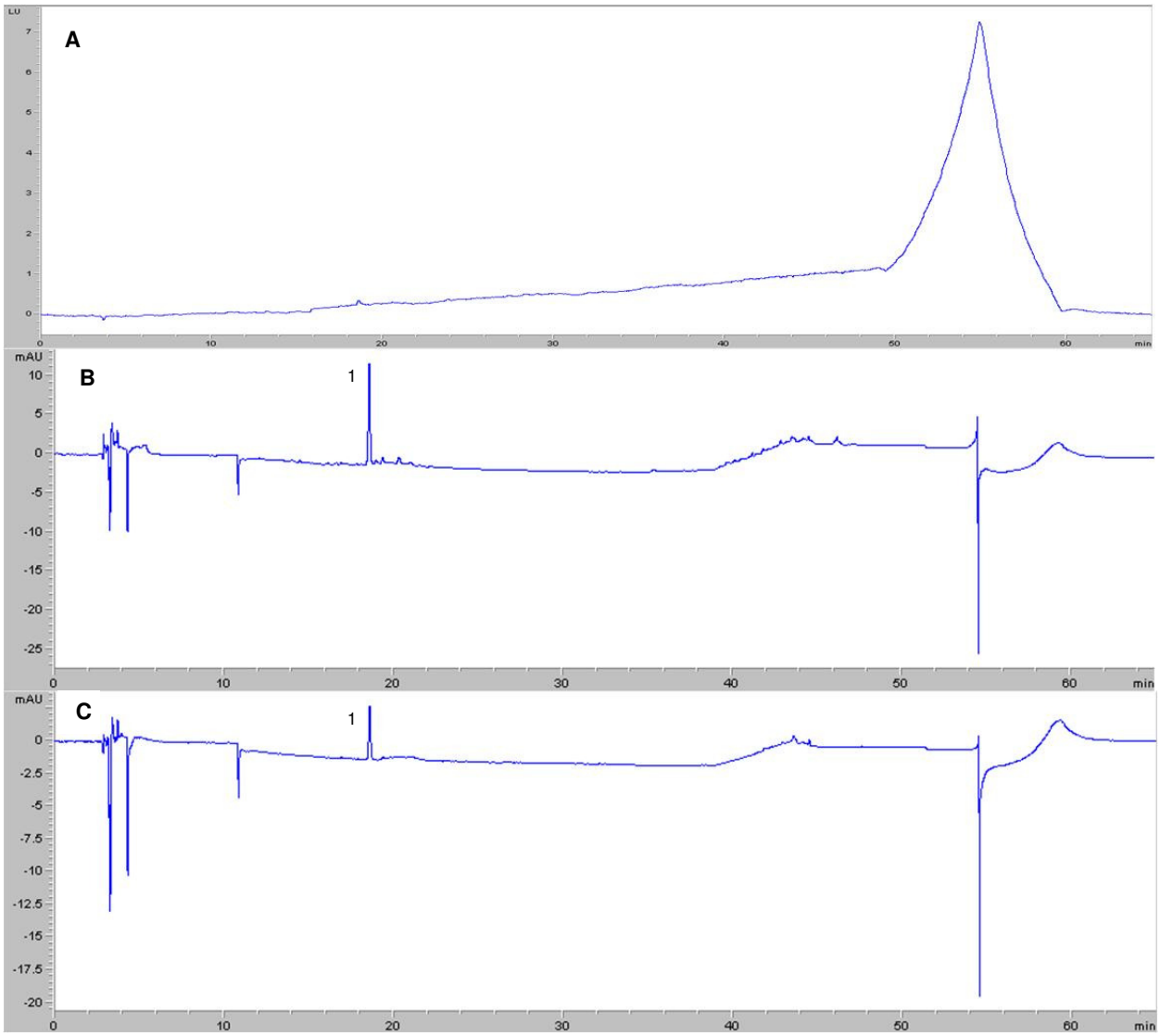

Figure S1.2: HPLC chromatograms of Sepabeads filtrate fraction.

Normal-phase HPLC analysis chromatogram of the sepabeads filtrate fraction **(A)**. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of the sepabeads filtrate fraction. Peak ID: 1= chlorogenic acid.

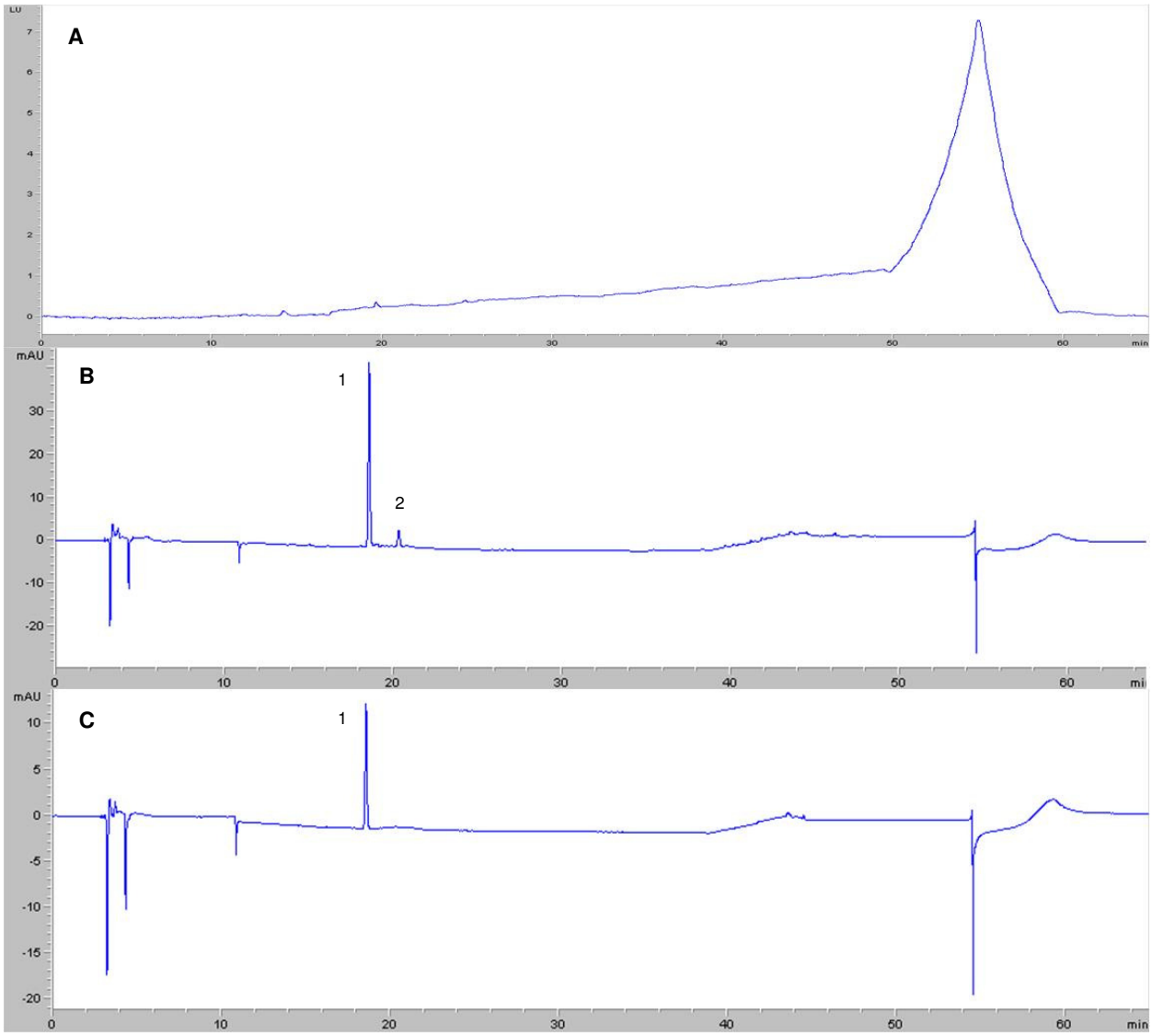

Figure S1.3: HPLC chromatograms of Sepabeads distilled water fraction.

Normal-phase HPLC analysis chromatogram of the sepabeads distilled water fraction **(A)**. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of the sepabeads distilled water fraction. Peak ID: 1= chlorogenic acid.

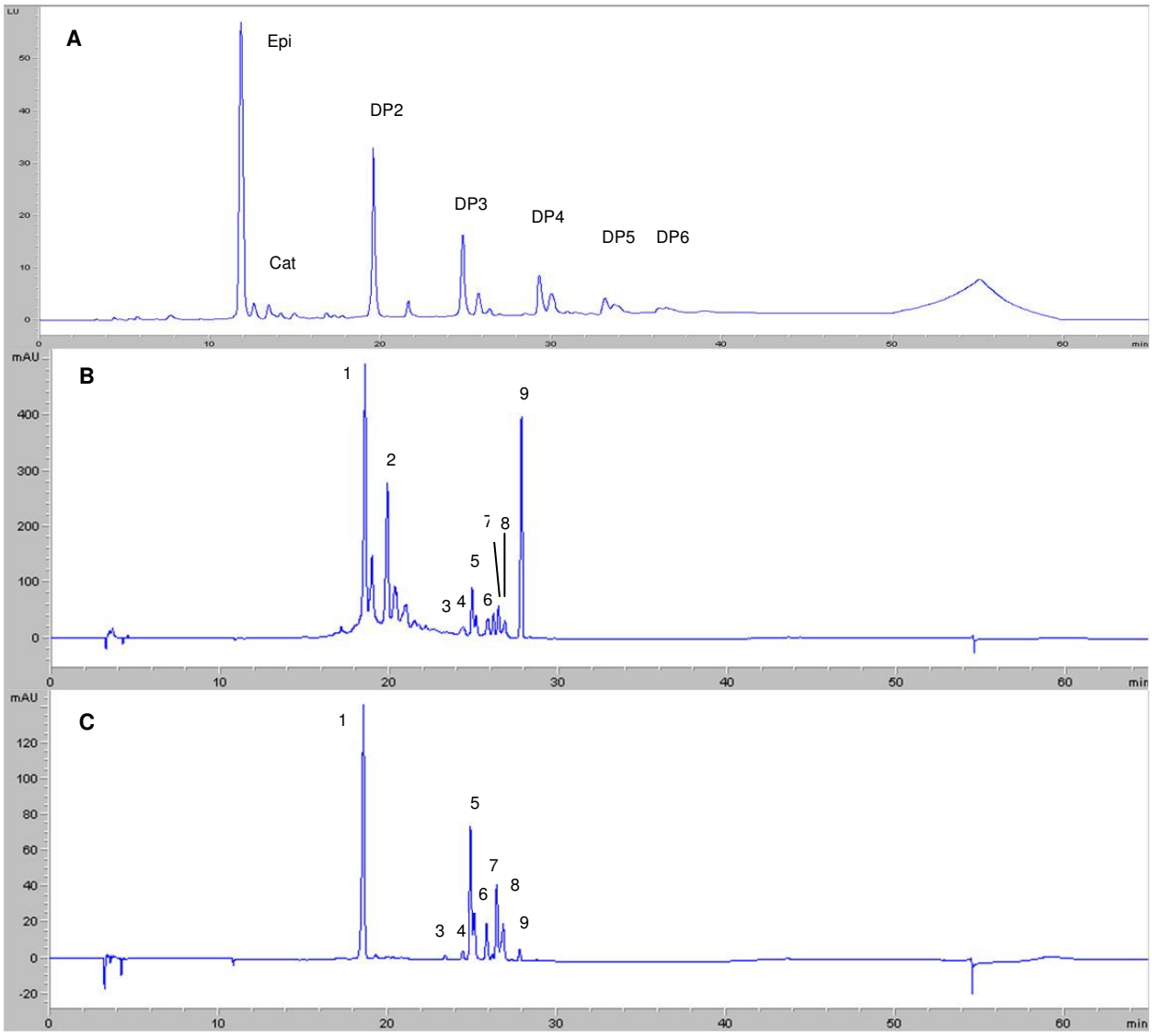

Figure S1.4: HPLC chromatograms of Fraction 1 (polyphenol fraction).

Normal-phase HPLC analysis chromatogram of Fraction 1 **(A)**. Peak ID: epi = epicatechin; cat = catechin; DP2 = dimers; DP3 = trimers; DP4 = tetramers; DP5 = pentamers; DP6 = hexamers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction 1. Peak ID: 1= chlorogenic acid, 2 = epicatechin, 3 = quercetin-3-*O*-arabinose, 4 = quercetin-3-*O*-rutinoside (rutin), 5 = quercetin-3-*O*-galactoside, 6 = quercetin-3-*O*-glucoside, 7 = quercetin-3-*O*-xyloside, 8 = quercetin-3-*O*-rhamnoside.

Fraction 1 was evaporated on the rotary evaporator at 45 °C to a volume of approximately 60 ml and the pH of Fraction 1 was adjusted to 6.5 with 1 N sodium hydroxide (starting pH was 3.2). Meanwhile, the Diaion HP-20ss (Sigma) column was prepared in the same manner as the Sepabeads SP-850 column. Fraction 1 was then added to the Diaion HP-20ss column (355 mm x 25 mm) and the non-absorbed fraction (Fraction 2) was collected. Fraction 2 contained small quantities of epicatechin,

catechin, procyanidins, chlorogenic acid, phloridzin and quercetin glycosides (Figure S1.5). The column was rinsed with 250 ml of distilled water and the resulting fraction (Fraction: Diaion distilled water rinse) contained chlorogenic acid and small quantities of epicatechin (Figure S1.6). The procyanidin fraction (Fraction 3) was eluted with 700 ml of 25 % (v/v) ethanol (Figure S1.7) and the other flavonoid fraction (Fraction 4) was eluted with 400 ml of 50% (v/v) ethanol (Figure S1.8).

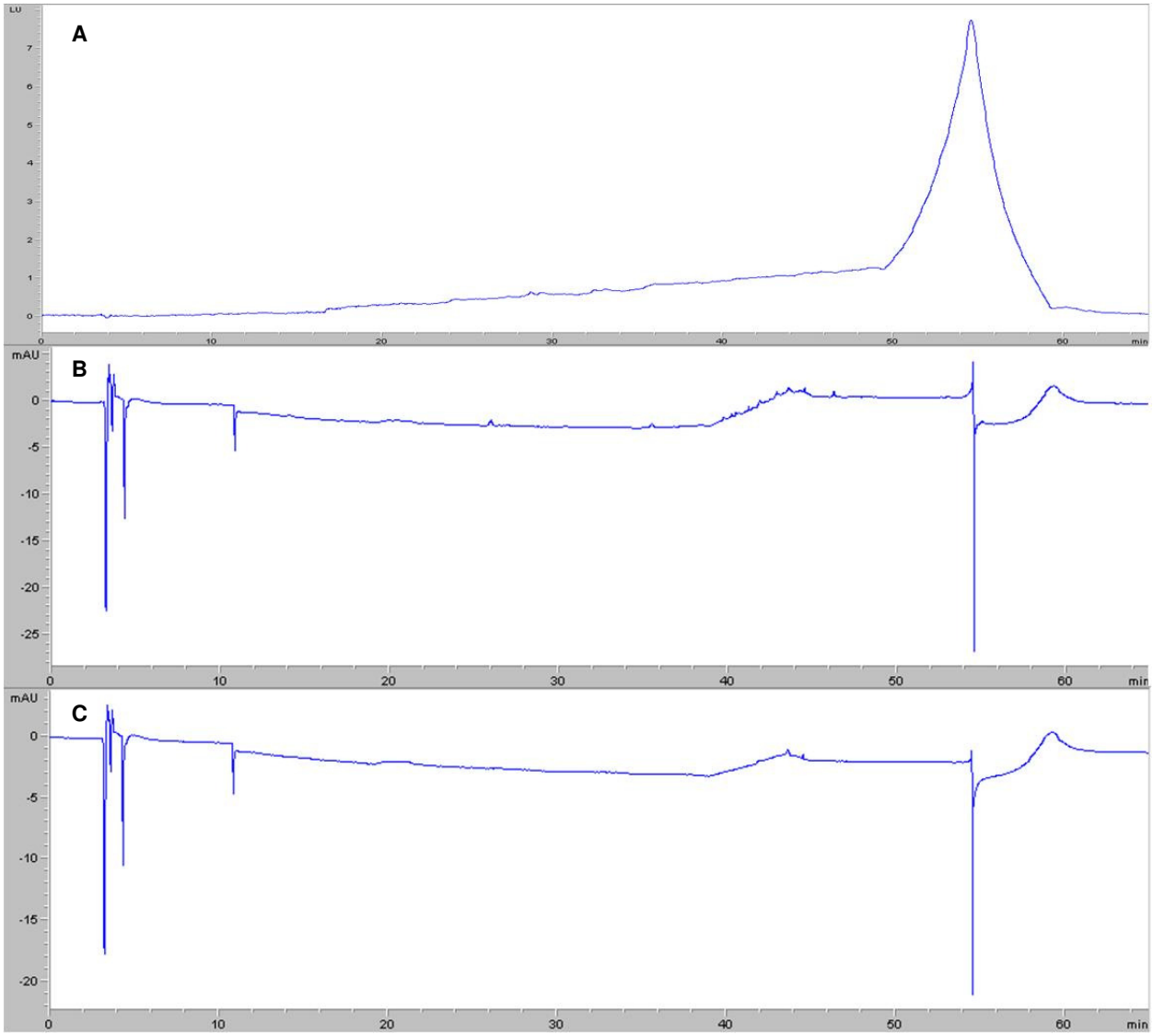

Figure S1.5: HPLC chromatograms of Fraction 2.

Normal-phase HPLC analysis chromatogram of Fraction 2 **(A)**. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction 2.

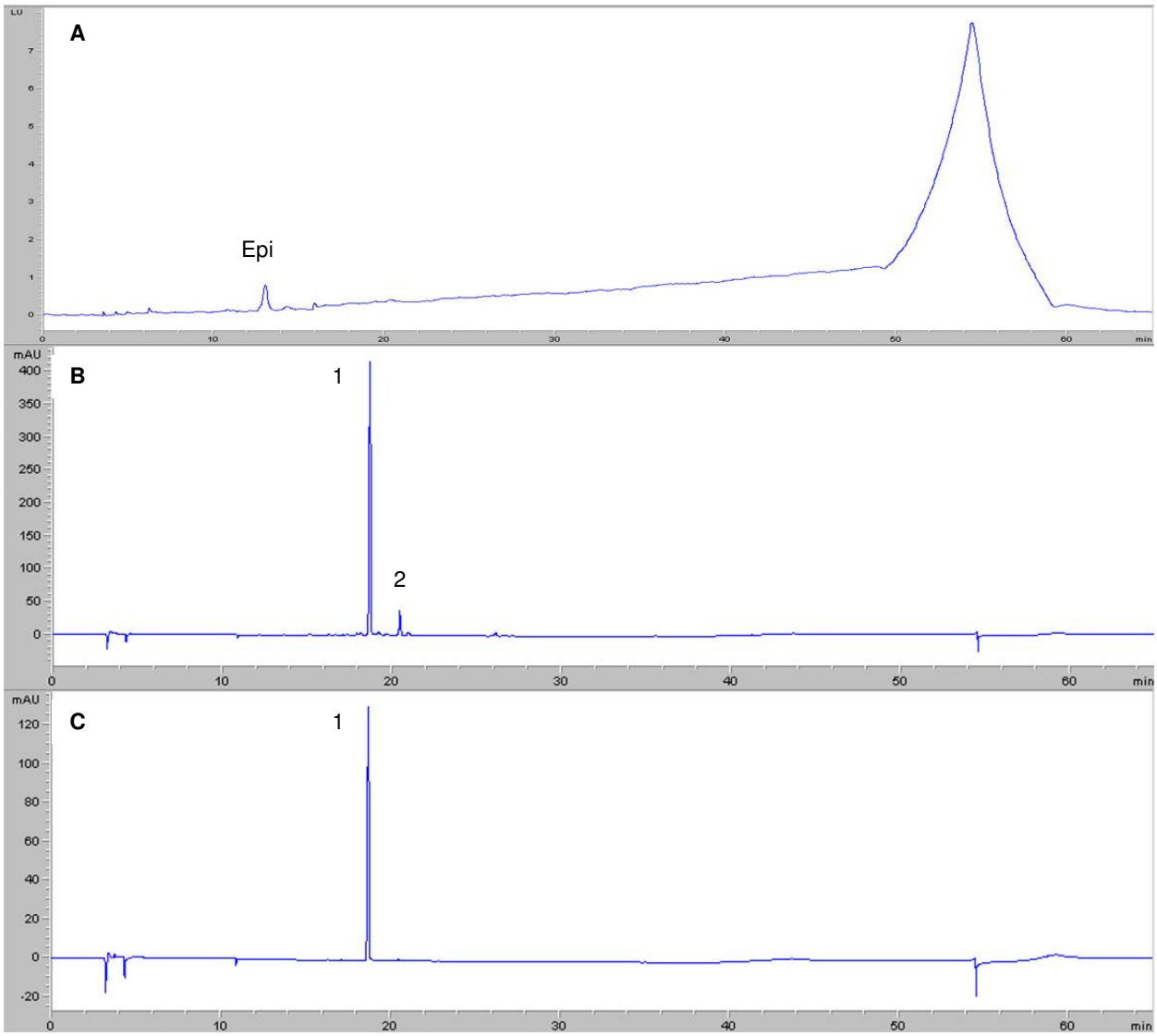

Figure S1.6: HPLC chromatograms of Diaion distilled water rinse.

Normal-phase HPLC analysis chromatogram of the Diaion distilled water rinse fraction **(A)**. Peak ID: epi = epicatechin. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of the Diaion distilled water rinse fraction. Peak ID: 1= chlorogenic acid, 2 = epicatechin.

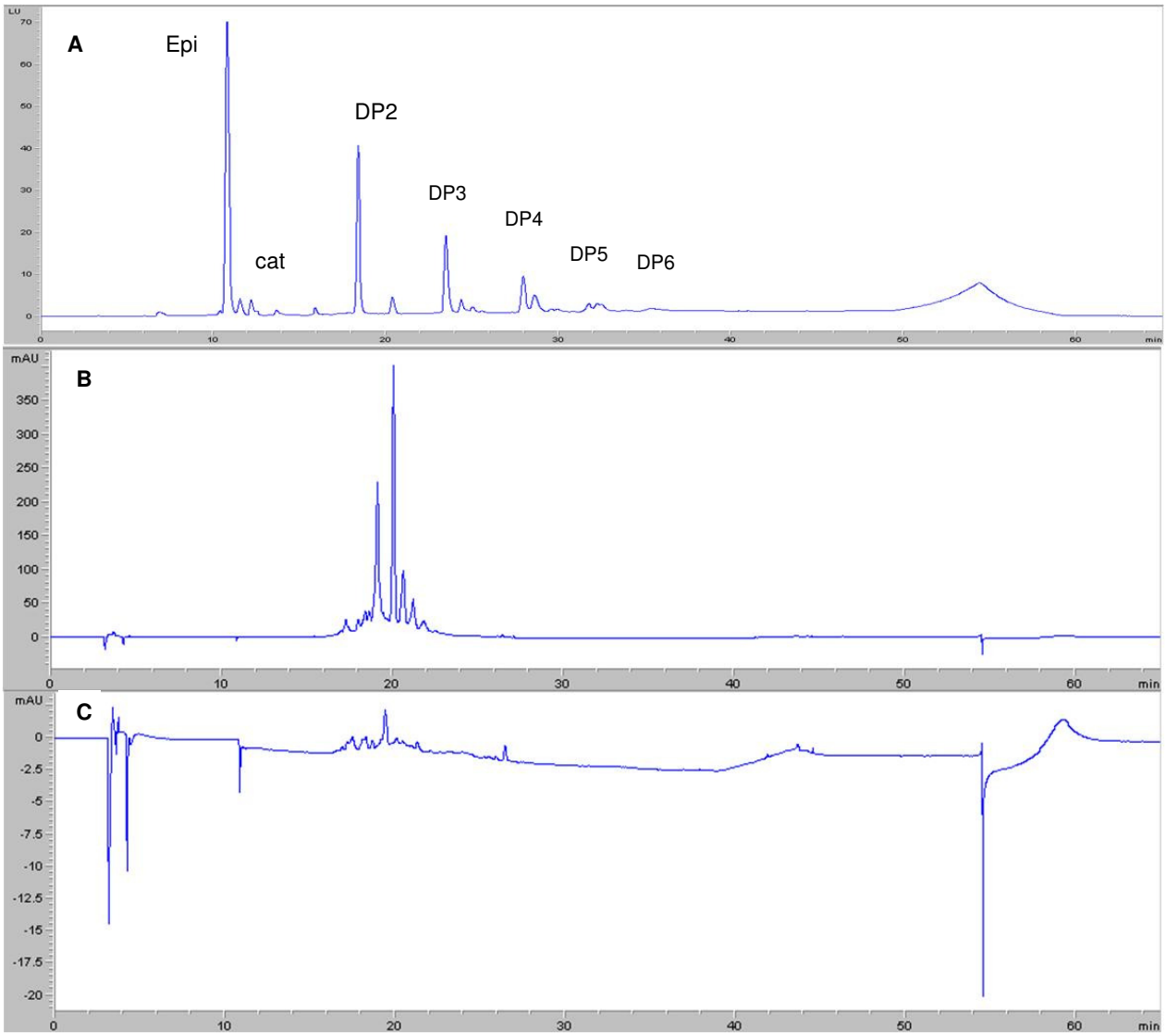

Figure S1.7: HPLC chromatograms of Fraction 3.

Normal-phase HPLC analysis chromatogram of Fraction 3 **(A)**. Peak ID: epi = epicatechin; cat = catechin; DP2 = dimers; DP3 = trimers; DP4 = tetramers; DP5 = pentamers; DP6 = hexamers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction 3.

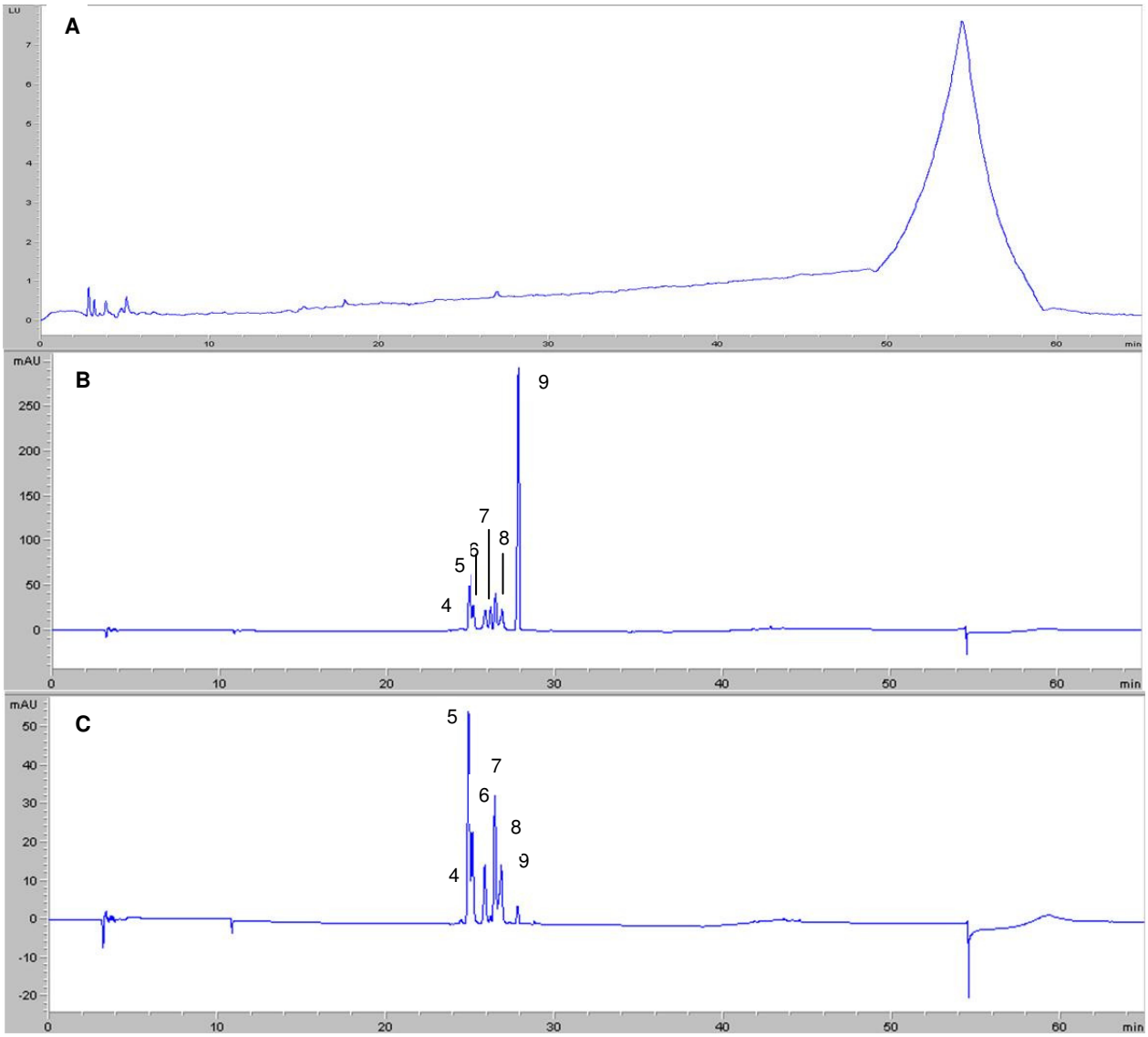

Figure S1.8: HPLC chromatograms of Fraction 4.

Normal-phase HPLC analysis chromatogram **(A)** of Fraction 4. Reverse-phase HPLC anslysis of chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction 4. Peak ID: 1= chlorogenic acid, 2 = epicatechin, 3 = quercetin-3-*O*-arabinose, 4 = quercetin-3-*O*-rutinoside (rutin), 5 = quercetin-3-*O*-galactoside, 6 = quercetin-3-*O*-glucoside, 7 = quercetin-3-*O*-xyloside, 8 = quercetin-3-*O*-rhamnoside.

From the HPLC data, it can be confirmed that the Fraction 3 (the procyanidin fraction) does not contain any chlorogenic acid, phloridzin and qurecetin glycosides. These compounds were eluted in Fraction 5.

Step 2: Removal of impurities

Before separating the procyanidins according to their degree of polymerisation on an Inertsil PREP-Sil (30 mm x 250 mm) column, a pre-cautionary column guard step was undertaken to make sure any impurities remaining in the fraction were removed so that any polar substances would not stick to the Inertsil PREP-Sil column (GL Sciences Inc). Fraction 3 was concentrated by rotary evaporator and the fraction was reconstituted in 3 ml of methanol. The reconstituted fraction was loaded on to a silica column insert and dried in the vacuum oven for 1 h. The silica column insert was inserted into a pre-wetted Biotage Snap FLASH Purification Cartridge HP-Sil (Biotage) and ran on the Medium Pressure Liquid Chromatography (MPLC) (Gilson). The column was pre-wetted with Solvent A. The flow rate was set to 45 ml/min and the method used a mobile consisting of 82 % dichloromethane, 14 % methanol and 4 % of 50 % (v/v) acetic acid (solvent A) and 96 % methanol and 4 % of 50 % (v/v) acetic acid (solvent B) with a 120 min gradient (0 – 10 min, 0 % B; 10 – 15 min, 18 % B; 15 - 18 min, 30 % B; 18 – 20 min 88 % B; 20 – 120 min, 0 % B) and was monitored at a wavelength of 280 nm with a DAD detector.

The fractions were collected for the following retention times (RT): Fraction A, 8 - 10 min; Fraction B, 12 – 14 min; Fraction C, 14 – 16 min; Fraction D, 16 – 17 min; Fraction E, 17 – 20 min; Fraction G, 20 – 28 min (Figure S1.9). A sample of each fraction was run on the HPLC using reverse-phase and normal-phase methods. Fraction A contained epicatechin and catechin (Figure S1.10); Fraction B consisted of dimmers (Figure S1.11) while Fraction C contained dimers and trimers (Figure S1.12). Fraction D had a combination of trimers, tetramers and pentamers (Figure S1.13) while Fraction E had a combination of tetramers, pentamers and hexamers (Figure S1.14).

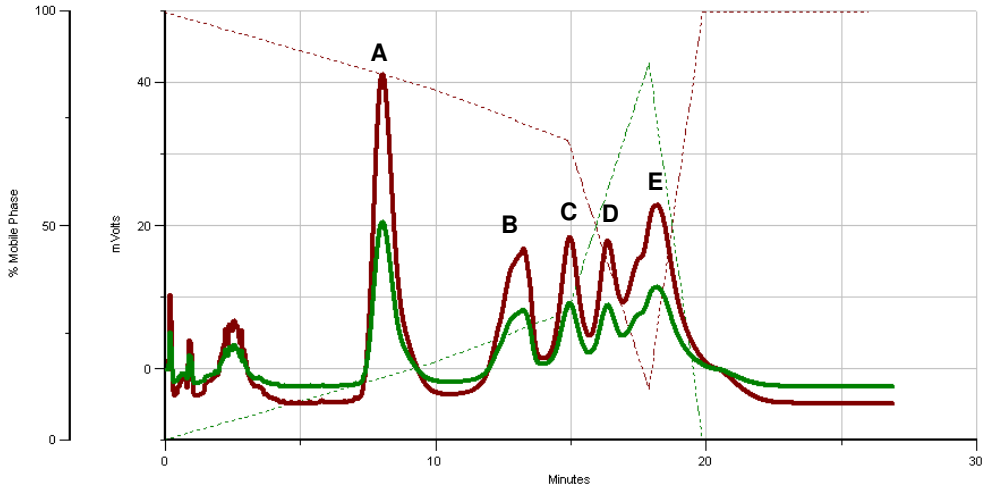

Figure S1.9: Chromatogram of fractions collected on the MPLC using a Biotage Snap FLASH Purification HP-Sil cartridge.

A = Fraction A, RT 8 – 10 min; B = Fraction B, RT: 12 – 14 min; C = Fraction C, RT = 14 – 16 min; D = Fraction D, RT 16 – 17 min; E = Fraction E, RT: 17 – 20 min.

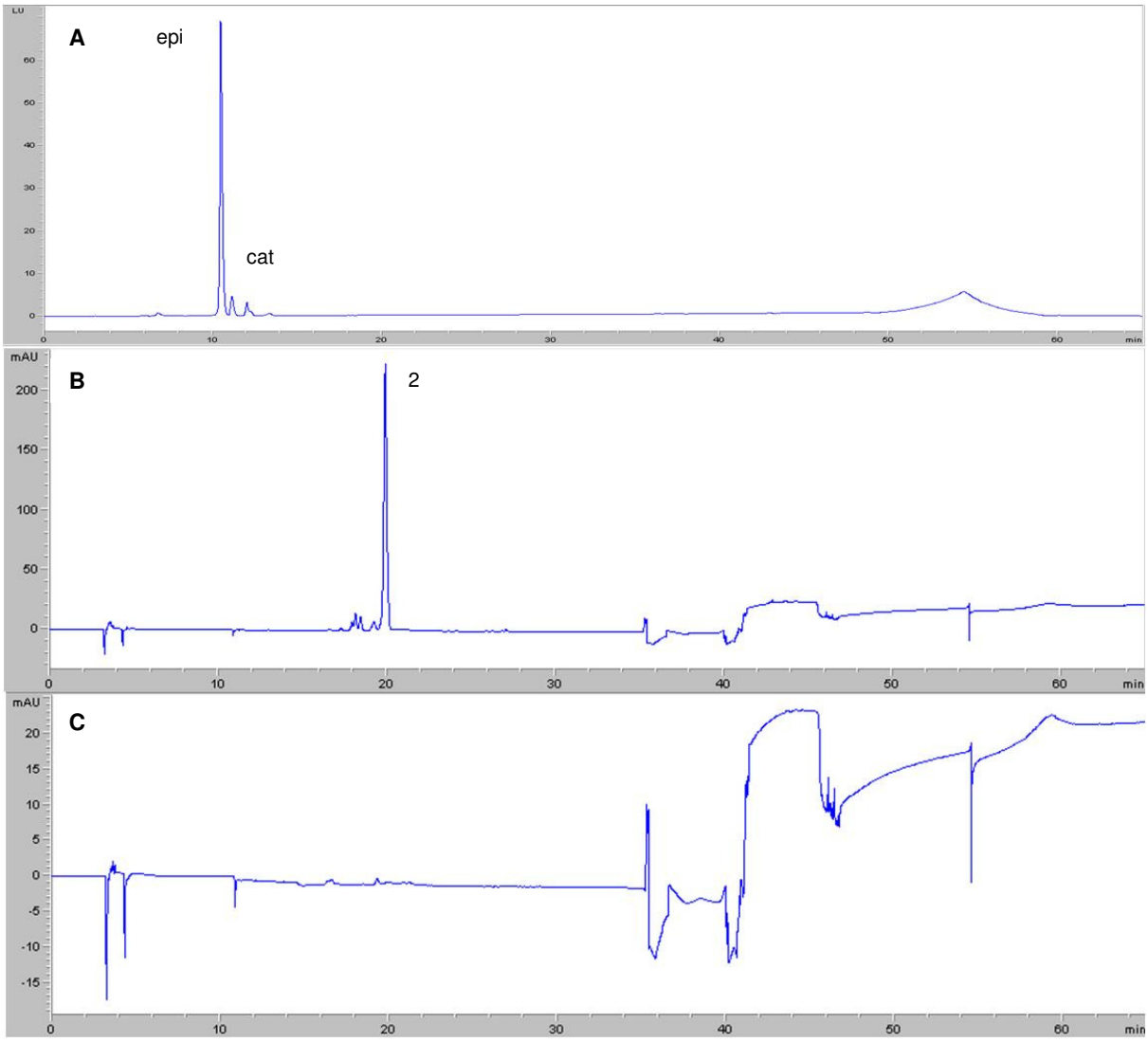

Figure S1.10: Chromatograms of Fraction A.

Normal-phase HPLC analysis chromatogram of Fraction A **(A)**. Peak ID: epi = epicatechin, cat = catechin. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction A. Peak ID: 2 = epicatechin.

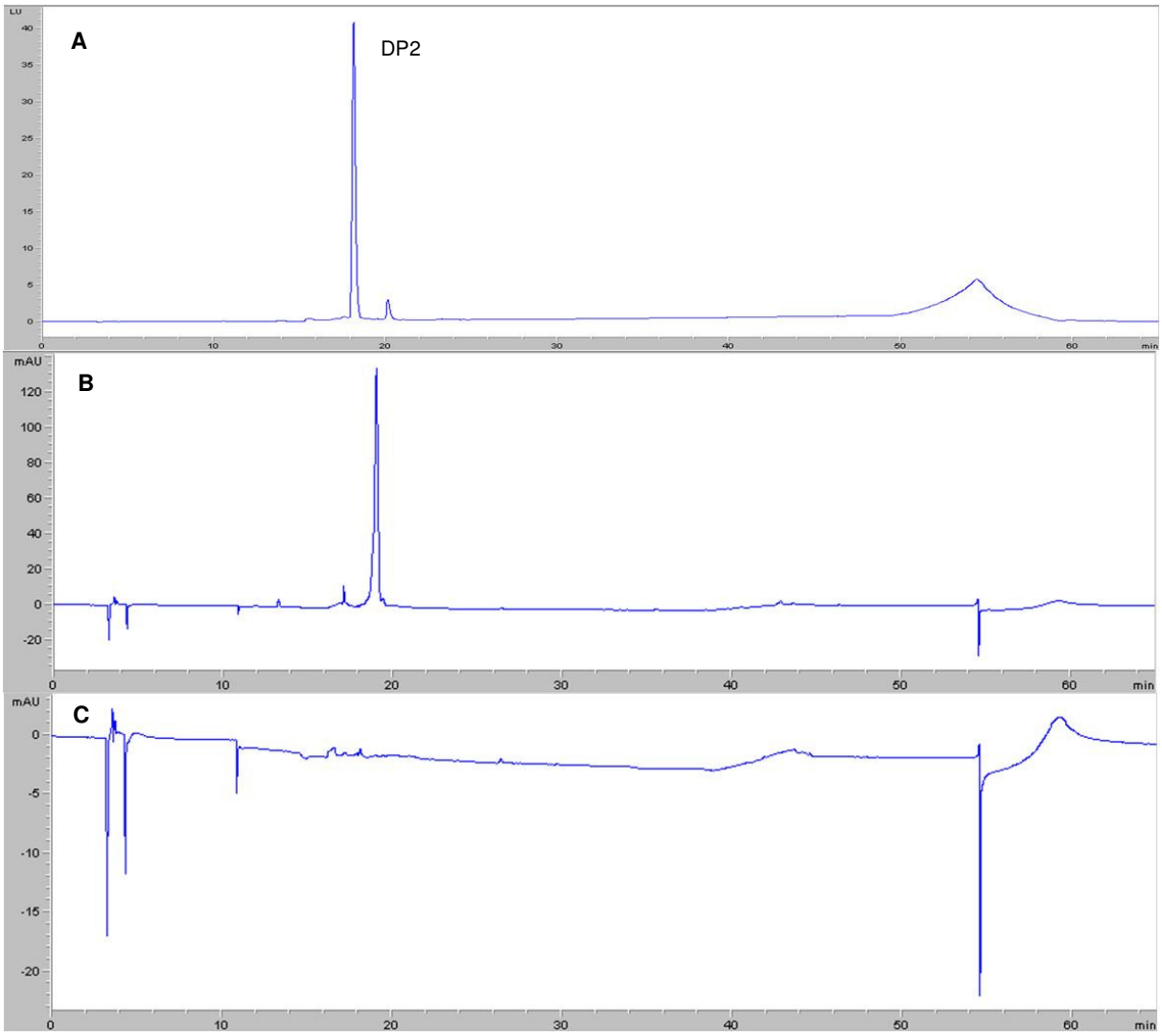

Figure S1.11: Chromatograms of Fraction B.

Normal-phase HPLC analysis chromatogram of Fraction B **(A)**. Peak ID: DP2 = dimers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction B.

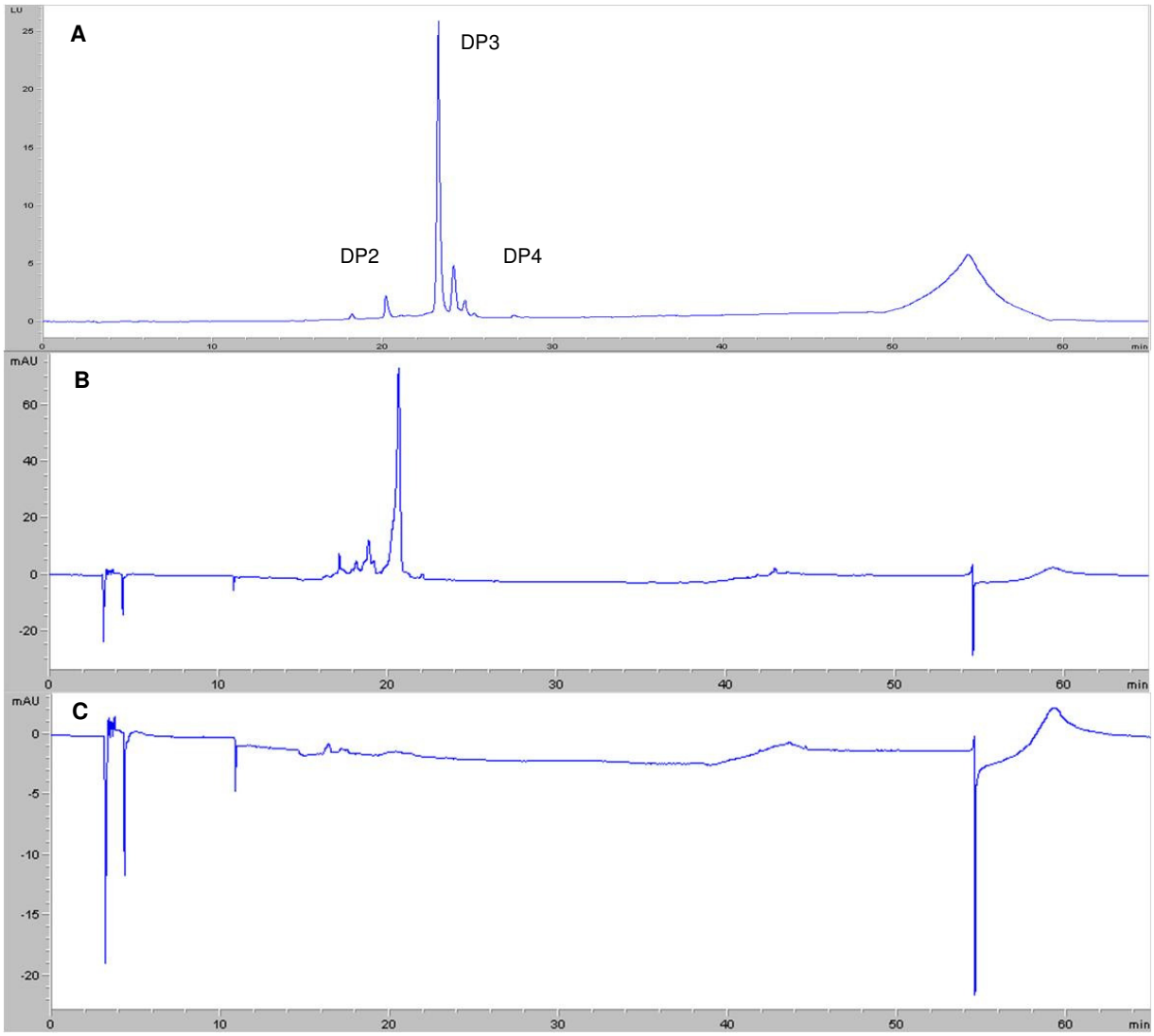

Figure S1.12: HPLC chromatograms of Fraction C.

Normal-phase HPLC analysis chromatogram of Fraction C **(A)**. Peak ID: DP2 = dimers; DP3 = trimers; DP4 = tetramers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction C.

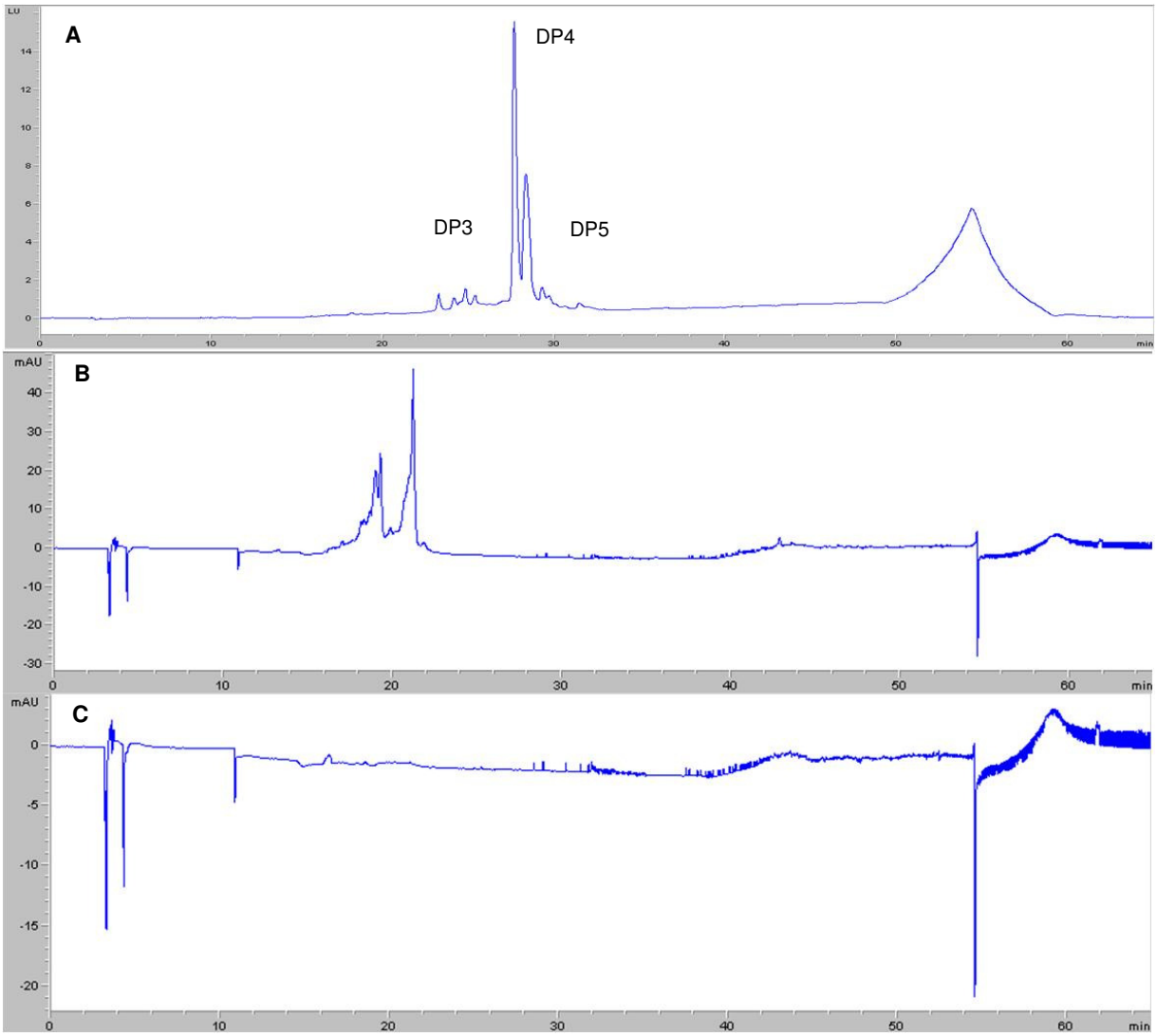

Figure S1.13: HPLC chromatograms of Fraction D.

Normal-phase HPLC analysis chromatogram of Fraction D **(A)**. Peak ID: DP3 = trimers; DP4 = tetramers; DP5 = pentamers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction D.

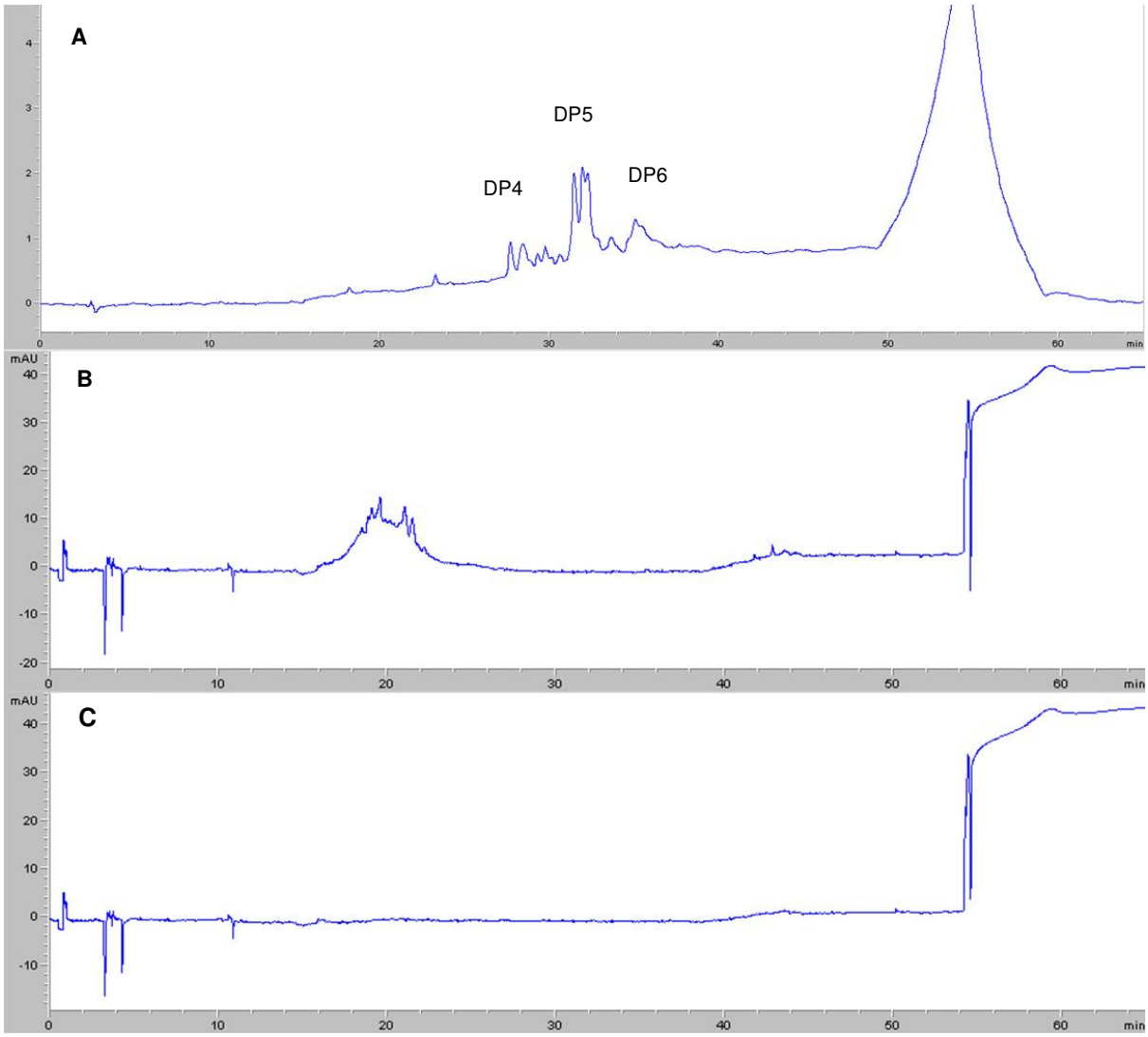

Figure S1.14: HPLC chromatograms of Fraction E.

Normal-phase HPLC analysis chromatogram of Fraction E **(A)**. Peak ID: DP4 = tetramers; DP5 = pentamers; DP6 = hexamers. Reverse-phase HPLC analysis chromatograms at 270 nm **(B)** and 370 nm **(C)** of Fraction E.

Fractions B, C, D and E (Combined Fraction) were combined and concentrated by rotary evaporator in preparation for the Step 3.

Step 3: Separation of apple procyanidins

The procyanidins in the Combined Fraction were separated according to the degree of polymerization by performing normal-phase chromatography using an Inertsil PREP-Sil (30 mm x 250 mm) (GL Sciences Inc) column on the preparatory-HPLC (Gilson). The combined Fraction was reconstituted in 2 ml of methanol and loaded onto the column. The flow rate was 24 ml/min and the method used a mobile phase containing hexane/methanol/ethyl acetate in a 8:3:1 ratio (solvent A) and hexane/methanol/ethyl acetate in a 2:3:1 ratio (solvent B) with a 185 min gradient (0 – 35 min, 0 % B; 35 - 185 min, 100 % B) and was monitored at 330 nm with a diode array detector (DAD).

The fractions were collected at the following RTs: Fraction I, 23 – 27 min; Fraction J, 44 – 54 min; Fraction K, 67 – 78 min; Fraction L, 87 – 98 min; Fraction M, 105 - 111 min; Fraction N, 111 – 115 min; and Fraction O, 119 – 124 min (Figure S1.15). A sample of each fraction was run on the HPLC using reverse-phase and normal-phase methods. Epicatechin and catechin were eluted in Fraction I, the dimers were eluted in Fraction J and the trimers and tetramers were eluted in Fractions K and L, respectively (Figure S1.16). No purified compounds were detected in Fractions M, N or O (Figure S1.17). Pentamers and hexamers were unable to be purified due to low starting material of these compounds. Each procyanidin fraction obtained was initially concentrated by rotary evaporator, reconstituted with 2 ml of methanol and the further concentrated using a Universal Vacuum System UVS800DDA (Thermo Savani) attached to a SPD Speed Vacuum (Thermo Electron Corporation).

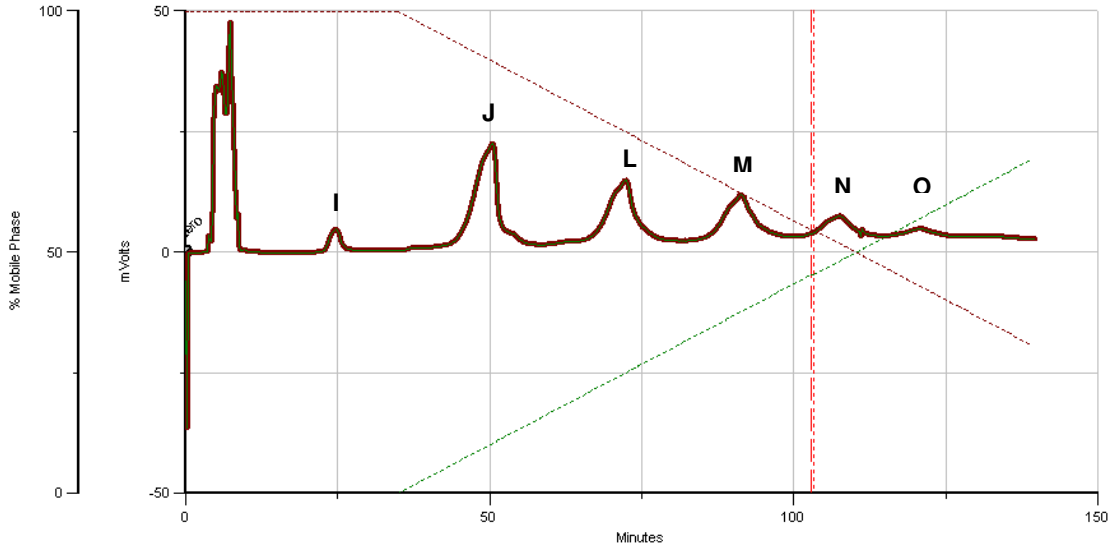

Figure S1.15: Chromatogram of fractions collected on the Prep-HPLC using the Intersil PREP Sil column.

DAD wavelength detection at 330 nm. Peak IDs: I = Fraction I, RT 23 – 27 min; J = Fraction J, RT: 44 - 54 min; K = Fraction K, RT = 67 – 78 min; L = Fraction L, RT 87 – 98 min; M = Fraction M, RT: 105 - 111 min; N = Fraction N, 111- 115 min; O = Fraction O, 119 – 124 min.

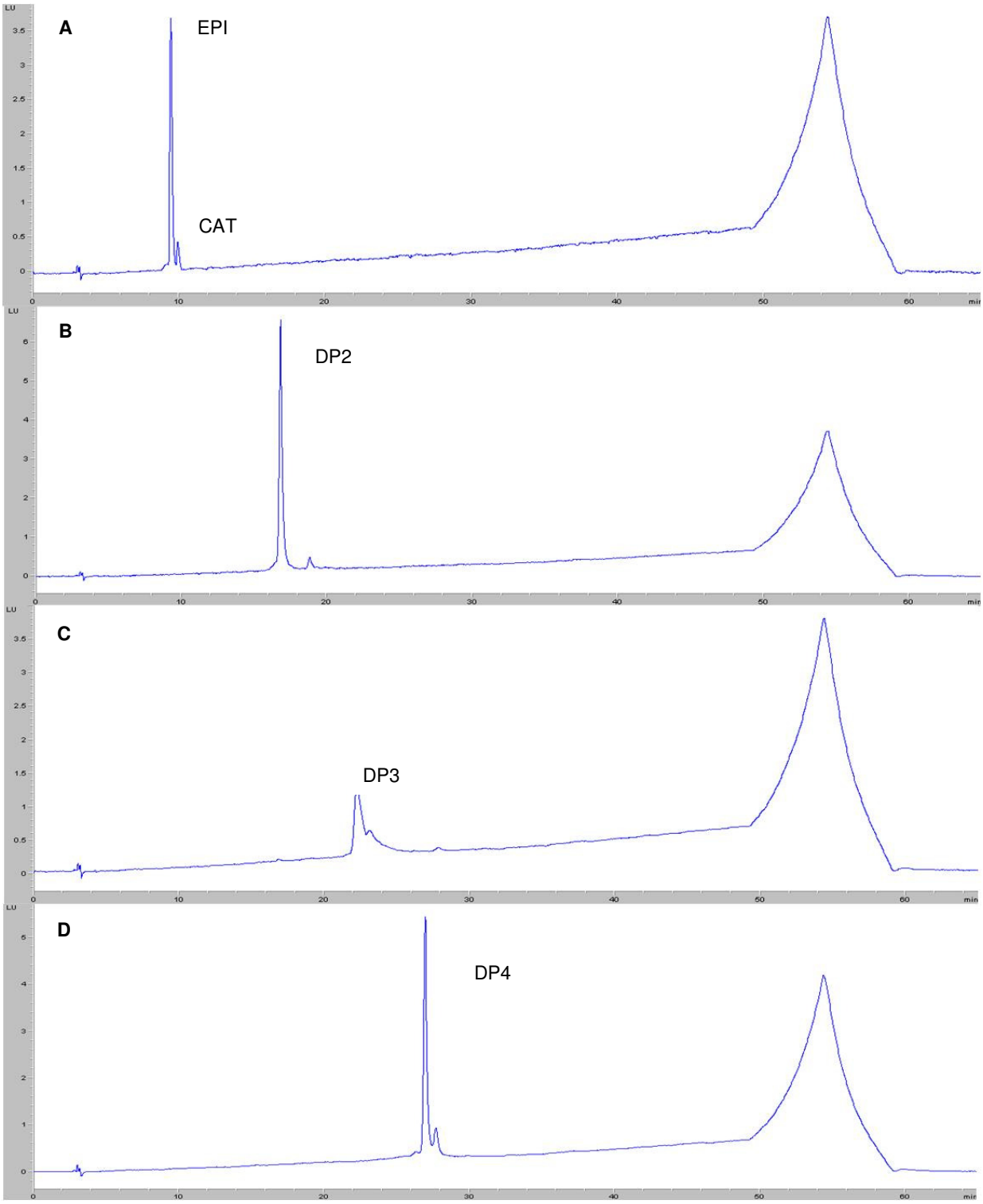

Figure S1.16: Normal phase chromatograms of purified fractions collected on the Intersil Prep Sil column.

(A) Fraction I; **(B)** Fraction J; **(C)** Fraction K; **(D)** Fraction L. Peak IDs: EPI = epicatechin; CAT = catechin, DP2 = dimers; DP3 = trimers; DP4 = tetramers.

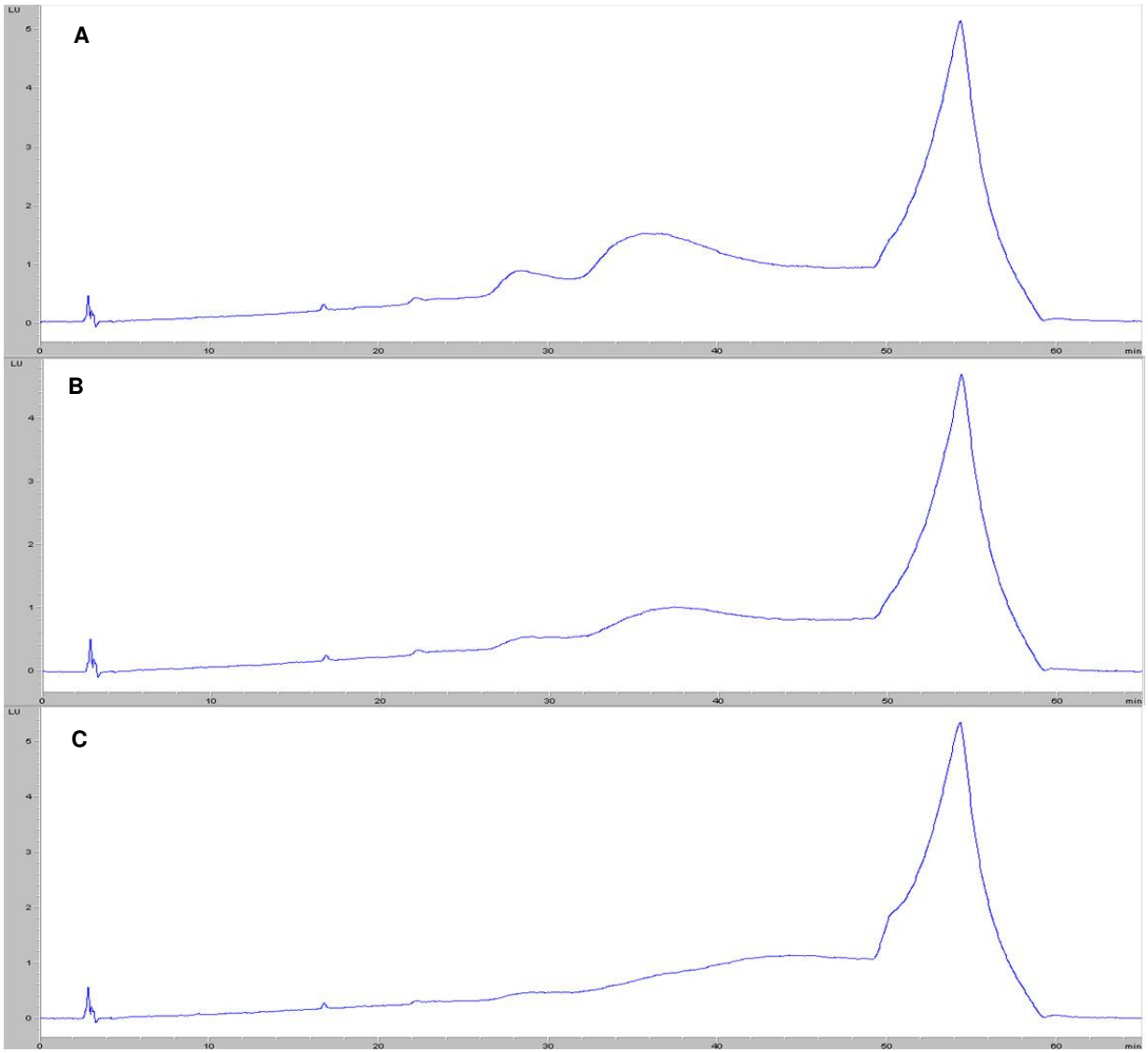

Figure S1.17: Normal-phase HPLC chromatograms of purified fractions collected on the Intersil Prep Sil column.

(A) Fraction M; **(B)** Fraction N; **(C)** Fraction O; **(D)** Fraction L.

The trimer (Fraction K) and tetramer (Fraction L) fractions were checked for purity by mass spectrometry analysis. The mass spectrometry work was preformed and interpreted by Mark Philo. Briefly the samples were infused directly from the vial in positive electron mode. For the trimer fraction no peak was detected at a mass of 867 Da (molecular weight of trimers plus a proton), however the sodium adduct at 889 Da was detected (Figure S1.18). A peak was detected at a mass of 1155 Da (molecular weight of tetramers plus a proton) and the sodium adduct at 1177 Da for the tetramer fraction (Figure S1.19).

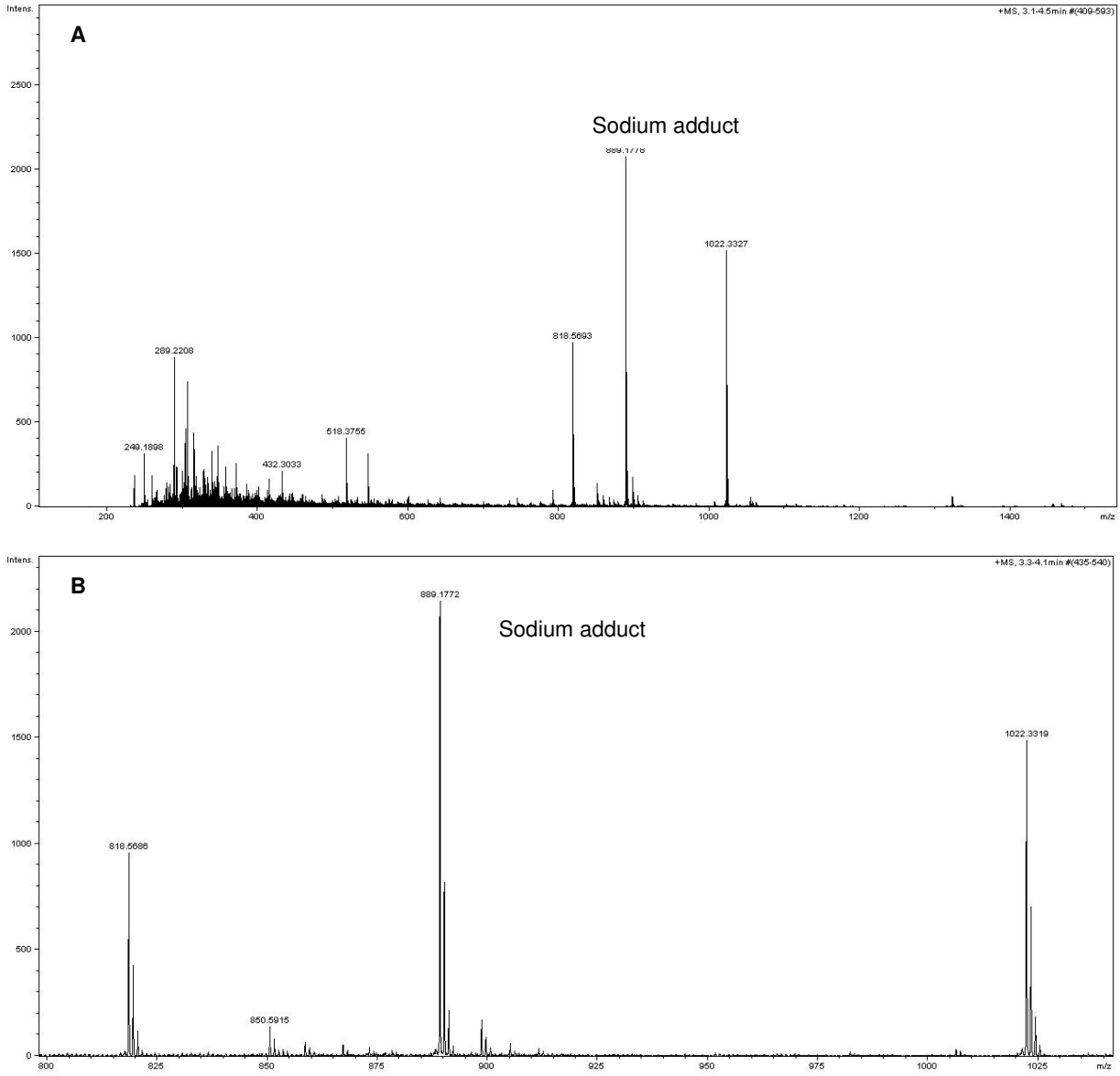

Figure S1.18: Mass spectrometry analysis of the trimer fraction (Fraction K).

(A) Full scan; **(B)** area zoomed in of mass of interest.

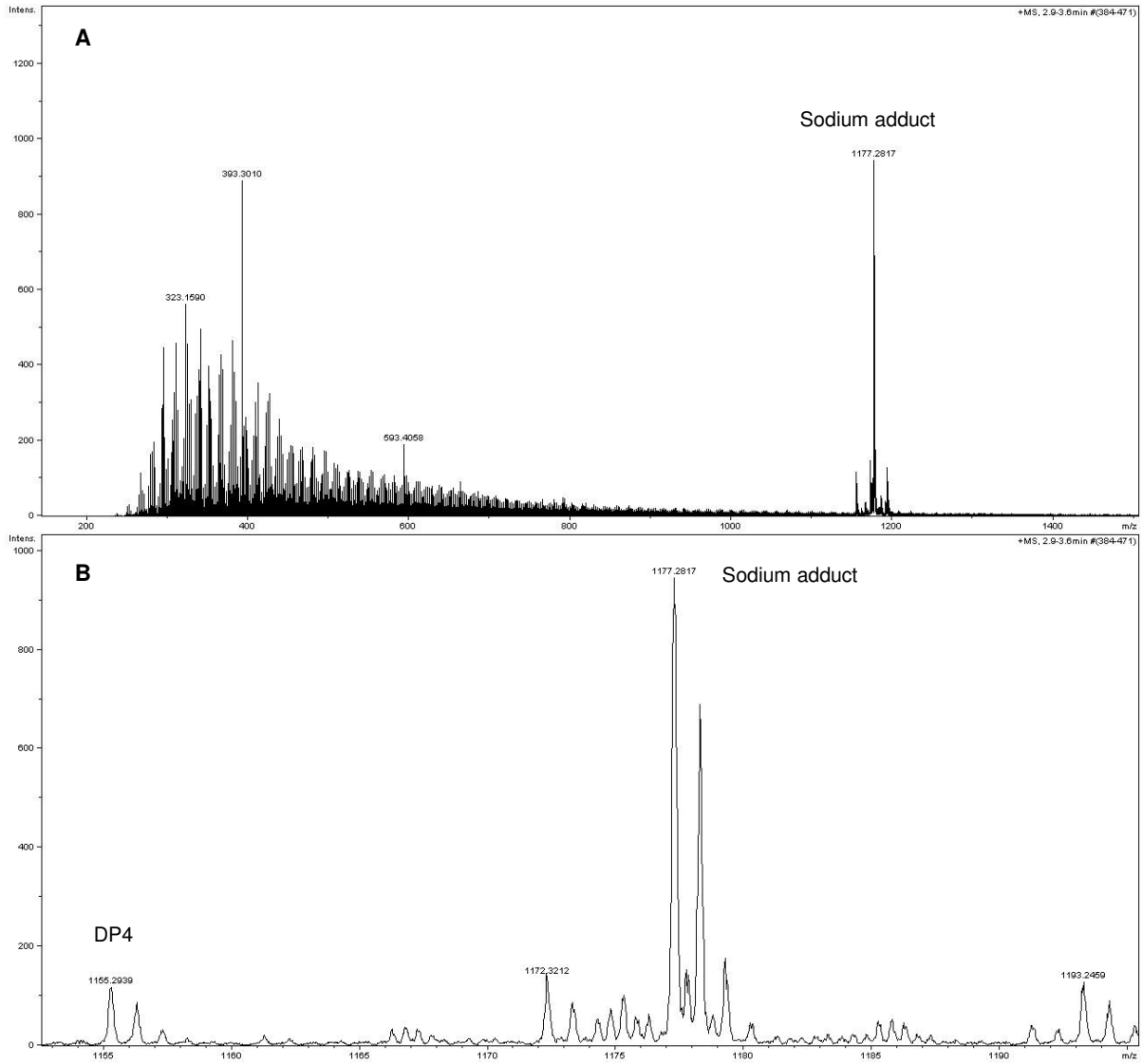

Figure S1.19: Mass spectrometry analysis of the tetramer fraction (Fraction L).

(A) Full scan; **(B)** area zoomed in of mass of interest.

SUPPLEMENTARY INFORMATION 2

BIOLOGICAL PROCESSES REGULATED BY THE DIFFERENT TREATMENT COMPARISONS

Supplementary Information 2

Biological processes regulated by the different treatment comparisons

The GO categories associated with the specific gene lists were identified using the DAVID software. The gene list obtained using a *p* value of < 0.001 was used to produce a manageable set of genes to perform more detailed functional analysis. Any biological process term which had three or fewer genes associated with that biological process or had a fold enrichment of less than 1.5 were not included in the analysis. Those biological processes that fit the exclusion criteria were excluded because fold enrichments of 1.5 and above are only considered as interesting and fold enrichments obtained from terms with a few number of genes (three or less) are not as reliable as those obtained from larger number of genes (Huang et al., 2008). Fold enrichment measures the magnitude of enrichment and is defined as the ratio of two proportions. For example, if 40 out of 400 (i.e. 10 %) of the inputted genes are involved in “kinase activity” versus 300 out of 30000 genes (i.e. 1 %) in the human genome (population background) are associated with “kinase activity” then the fold enrichment is tenfold (i.e. 10 % / 1 % = 10) (Huang et al., 2008).

The biological processes regulated by VEGF versus the control are described in Table S2.1. Tables S2.2 and S2.3 describe the biological processes regulated by apple procyanidin faction dp 4 versus the control and apple procyanidin fraction dp 4 versus VEGF, respectively. The biological processes regulated by apple procyanidin fraction dp 4-treated VEGF versus the control and apple procyanidin fraction dp 4-treated VEGF versus VEGF are described in Tables S2.4 and S2.5, respectively. Tables S2.6 and S2.7 describe the biological processes regulated by anti-VEGF antibody-treated

VEGF versus the control and anti-VEGF antibody-treated VEGF versus apple procyanidin fraction dp 4, respectively.

Table S2.1: Biological processes regulated by VEGF compared to the control.

Biological processes, regulated by the VEGF treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 129	Fold Enrichment
Regulation of endothelial cell migration	4	20.2
Negative regulation of hydrolase activity	5	12.7
Regulation of angiogenesis	5	9.8
Positive regulation of cell migration	6	8.4
Regulation of caspase activity	5	7.8
Positive regulation of locomotion	6	7.6
Positive regulation of cell motion	6	7.6
Regulation of endopeptidase activity	5	7.5
Regulation of peptidase activity	5	7.1
Response to unfolded protein	4	7.1
Regulation of cell migration	8	6.0
Response to protein stimulus	5	5.8
Regulation of locomotion	8	5.2
Regulation of cell motion	8	5.2
Transmembrane receptor protein serine/threonine kinase signaling pathway	4	4.9
Regulation of gene-specific transcription	5	4.7
Response to estrogen stimulus	4	4.7
Response to nutrient	5	4.4
Ossification	4	4.3
Cell migration	9	4.2
Reproductive structure development	4	4.1
Bone development	4	4.0
Wound healing	6	4.0
Regulation of response to external stimulus	5	4.0
Response to extracellular stimulus	7	3.9
Response to metal ion	4	3.9
Response to inorganic substance	6	3.8
Localization of cell	9	3.8
Cell motility	9	3.8
Leukocyte differentiation	4	3.8
Response to nutrient levels	6	3.8
Response to hypoxia	4	3.7
Negative regulation of transport	4	3.7
Positive regulation of developmental process	8	3.6
Di-, tri-valent inorganic cation transport	5	3.6
Response to oxygen levels	4	3.6
Response to wounding	15	3.6
Tube development	6	3.5
Negative regulation of molecular function	9	3.4
Enzyme linked receptor protein signaling pathway	9	3.3
Response to steroid hormone stimulus	5	3.3
Negative regulation of catalytic activity	7	3.2
Regulation of cell development	5	3.1
Response to endogenous stimulus	10	3.1

Table S2.1: Biological processes regulated by VEGF compared to the control continued

Biological process	No. of genes involved out of 129	Fold Enrichment
Inflammatory response	8	3.1
Regulation of hydrolase activity	8	3.1
Response to hormone stimulus	9	3.1
Blood vessel development	6	3.1
Anti-apoptosis	5	3.1
Vasculature development	6	3.0
Response to organic substance	17	3.0
Negative regulation of programmed cell death	8	2.8
Negative regulation of cell death	8	2.8
Positive regulation of transcription	12	2.7
Cell motion	10	2.7
Positive regulation of transcription, DNA-dependent	10	2.7
Positive regulation of RNA metabolic process	10	2.7
Negative regulation of apoptosis	7	2.5
Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	12	2.5
Positive regulation of cell proliferation	8	2.4
Response to abiotic stimulus	7	2.4
Regulation of cell proliferation	15	2.4
Positive regulation of nitrogen compound metabolic process	12	2.4
Positive regulation of macromolecule biosynthetic process	12	2.3
Regulation of transcription from RNA polymerase II promoter	13	2.3
Positive regulation of cellular biosynthetic process	12	2.2
Positive regulation of macromolecule metabolic process	15	2.2
Positive regulation of biosynthetic process	12	2.2
Regulation of programmed cell death	14	2.2
Regulation of cell death	14	2.2
Negative regulation of cellular biosynthetic process	9	2.0
Regulation of apoptosis	13	2.0
Negative regulation of biosynthetic process	9	2.0
Ddefense response	9	1.9
Cell surface receptor linked signal transduction	22	1.6
Regulation of RNA metabolic process	20	1.5
Regulation of transcription, DNA-dependent	19	1.5

Table S2.2: Biological processes regulated by apple procyanidin fraction dp 4 compared to the control.

Biological processes regulated by the apple procyanidin fraction dp 4 treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 92	Fold Enrichment
Negative regulation of MAP kinase activity	4	19.6
Negative regulation of leukocyte activation	5	14.7
Negative regulation of cell activation	5	13.9
Negative regulation of lymphocyte activation	4	12.4
Positive regulation of mononuclear cell proliferation	4	11.7
Positive regulation of leukocyte proliferation	4	11.7
Positive regulation of cell adhesion	4	11.3
Positive regulation of T cell activation	5	11.1
Regulation of lymphocyte differentiation	4	10.8
Regulation of T cell proliferation	4	10.6
Positive regulation of protein transport	4	10.1
Negative regulation of immune system process	5	10.0
Response to vitamin	4	9.9
Branching morphogenesis of a tube	4	9.9
Homeostasis of number of cells	6	9.9
Regulation of mononuclear cell proliferation	5	9.7
Regulation of leukocyte proliferation	5	9.7
Morphogenesis of a branching structure	4	8.8
Regulation of T cell activation	6	8.6
Positive regulation of lymphocyte activation	5	8.6
Regulation of leukocyte activation	8	8.1
Regulation of lymphocyte activation	7	8.0
Regulation of lymphocyte proliferation	4	7.9
Negative regulation of protein kinase activity	4	7.9
Positive regulation of leukocyte activation	5	7.8
Response to corticosteroid stimulus	4	7.8
Regulation of cell activation	8	7.7
Negative regulation of kinase activity	4	7.6
Positive regulation of cell activation	5	7.5
Positive regulation of cell migration	4	7.4
Regulation of MAP kinase activity	6	7.1
Negative regulation of multicellular organismal process	7	7.1
Myeloid cell differentiation	4	7.1
Negative regulation of transferase activity	4	7.1
Angiogenesis	6	6.8
Positive regulation of cell motion	4	6.7
Positive regulation of locomotion	4	6.7
Regulation of immune effector process	4	6.7
Positive regulation of cell differentiation	9	6.5
Regulation of cell adhesion	5	6.2
Positive regulation of secretion	4	6.2
Response to nutrient	5	5.9
Regulation of protein transport	4	5.8
Regulation of secretion	7	5.8
Striated muscle tissue development	4	5.6
Positive regulation of multicellular organismal process	8	5.6
Response to organic cyclic substance	4	5.5
Blood vessel morphogenesis	7	5.5
Regulation of establishment of protein localization	4	5.5
Positive regulation of developmental process	9	5.3

Table S2.2: Biological processes regulated by apple procyanidin fraction dp 4 compared to the control continued

Biological process	No. of genes involved out of 92	Fold Enrichment
Positive regulation of transport	7	5.2
Rube morphogenesis	4	5.2
Response to hypoxia	4	5.0
Regulation of synaptic transmission	4	5.0
Regulation of cell migration	5	5.0
Negative regulation of transport	4	4.9
Cell fate commitment	4	4.8
Regulation of protein localization	4	4.8
Response to oxygen levels	4	4.7
Blood vessel development	7	4.7
Regulation of cellular localization	7	4.7
Vasculature development	7	4.6
MAPKKK cascade	5	4.6
Regulation of cytokine production	5	4.6
Regulation of transmission of nerve impulse	4	4.6
Wound healing	5	4.5
Regulation of neurological system process	4	4.4
Regulation of locomotion	5	4.3
Response to steroid hormone stimulus	5	4.3
Regulation of cell motion	5	4.3
Regulation of phosphorylation	12	4.3
Regulation of binding	4	4.3
Positive regulation of immune system process	6	4.3
Response to nutrient levels	5	4.2
Response to oxidative stress	4	4.1
Regulation of phosphorus metabolic process	12	4.1
Regulation of phosphate metabolic process	12	4.1
Negative regulation of cell communication	6	4.1
Positive regulation of cell proliferation	10	4.1
Regulation of protein kinase activity	8	3.9
Regulation of protein amino acid phosphorylation	4	3.8
Hemopoietic or lymphoid organ development	6	3.8
Endocytosis	5	3.8
Rube development	5	3.8
Membrane invagination	5	3.8
Sensory organ development	5	3.8
Regulation of kinase activity	8	3.7
Response to extracellular stimulus	5	3.7
Negative regulation of cell proliferation	8	3.7
Blood circulation	4	3.6
Circulatory system process	4	3.6
Response to hormone stimulus	8	3.6
Immune system development	6	3.6
Regulation of transferase activity	8	3.6
Hemopoiesis	5	3.5
Cell activation	6	3.5
Positive regulation of cell death	9	3.5
Embryonic morphogenesis	6	3.3
Response to endogenous stimulus	8	3.3
Regulation of system process	6	3.3
Regulation of cell proliferation	15	3.2
Protein kinase cascade	7	3.2
Induction of apoptosis	6	3.1
Induction of programmed cell death	6	3.1

Table S2.2: Biological processes regulated by apple procyanidin fraction dp 4 compared to the control continued

Biological process	No. of genes involved out of 92	Fold Enrichment
Positive regulation of apoptosis	8	3.1
Positive regulation of programmed cell death	8	3.1
Membrane organization	7	3.1
Regulation of cell death	15	3.1
Cell migration	5	3.1
Cell-cell signaling	11	3.1
Cell proliferation	8	3.0
Regulation of growth	6	3.0
Enzyme linked receptor protein signaling pathway	6	2.9
Regulation of apoptosis	14	2.9
Regulation of programmed cell death	14	2.9
Response to organic substance	12	2.8
Response to wounding	8	2.5
Cell motion	7	2.5
Apoptosis	8	2.3
Programmed cell death	8	2.2
Positive regulation of cellular biosynthetic process	9	2.2
Positive regulation of biosynthetic process	9	2.2
Cell death	9	2.1
Death	9	2.1
Positive regulation of nitrogen compound metabolic process	8	2.1
Regulation of transcription, DNA-dependent	16	1.6
Regulation of RNA metabolic process	16	1.6

Table S2.3: Biological processes regulated by apple procyanidin fraction dp 4 compared to VEGF.

Biological processes, regulated by the apple procyanidin fraction dp 4 treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 233	Fold Enrichment
Response to retinoic acid	6	11.7
Cardiac cell differentiation	4	9.6
Negative regulation of MAP kinase activity	5	9.5
Ureteric bud development	5	9.2
Response to vitamin A	6	9.2
Negative regulation of defense response	4	7.4
Metanephros development	5	7.3
BMP signaling pathway	5	7.3
Cell-cell junction organization	4	7.2
ER-nuclear signaling pathway	4	7.2
Odontogenesis	6	7.1
Regulation of angiogenesis	7	7.1
Calcium-mediated signaling	4	7.0
Response to vitamin	7	6.7
Negative regulation of hydrolase activity	5	6.5
Odontogenesis of dentine-containing tooth	4	6.4
Monocarboxylic acid transport	5	6.4
Mesenchymal cell differentiation	5	6.3
Mesenchymal cell development	5	6.3
Ovulation cycle	6	6.2
Mesenchyme development	5	6.1
Neuron fate commitment	4	6.1
Regulation of inflammatory response	7	5.9
Regulation of smooth muscle cell proliferation	4	5.8
Ovulation cycle process	5	5.6
Response to unfolded protein	6	5.5
Cell junction organization	5	5.5
Positive regulation of cell adhesion	5	5.5
Female gonad development	5	5.4
Regulation of striated muscle tissue development	4	5.2
Regulation of muscle development	4	5.1
Female sex differentiation	5	5.0
Development of primary female sexual characteristics	5	5.0
Regulation of cell migration	13	5.0
Response to estrogen stimulus	8	4.9
Response to estradiol stimulus	4	4.8
Regulation of locomotion	14	4.7
Response to mechanical stimulus	4	4.6
Negative regulation of secretion	4	4.6
Cardiac muscle tissue development	4	4.6
Leukocyte migration	4	4.6
Response to nutrient	10	4.6
Regulation of response to external stimulus	11	4.5
Striated muscle tissue development	8	4.4
Regulation of cell motion	13	4.4
Cartilage development	5	4.3
Response to hypoxia	9	4.3
Negative regulation of cell activation	4	4.3
Response to protein stimulus	7	4.2
Cell fate commitment	9	4.2

Table S2.3: Biological processes regulated by apple procyanidin fraction dp 4 compared to VEGF continued.

Biological process	No. of genes involved out of 233	Fold Enrichment
Response to lipopolysaccharide	5	4.2
Response to oxygen levels	9	4.1
Muscle tissue development	8	4.1
Response to glucocorticoid stimulus	5	4.1
Regulation of caspase activity	5	4.0
Angiogenesis	9	3.9
Positive regulation of protein transport	4	3.9
Positive regulation of response to external stimulus	4	3.9
Positive regulation of locomotion	6	3.9
Negative regulation of immune system process	5	3.9
Regulation of endopeptidase activity	5	3.9
Skeletal muscle organ development	4	3.9
Skeletal muscle tissue development	4	3.9
Branching morphogenesis of a tube	4	3.9
Regulation of cell adhesion	8	3.9
Muscle cell differentiation	7	3.8
Negative regulation of protein kinase activity	5	3.8
Wound healing	11	3.8
Striated muscle cell differentiation	5	3.8
Response to corticosteroid stimulus	5	3.8
Rransmembrane receptor protein serine/threonine kinase signaling pathway	6	3.8
Response to molecule of bacterial origin	5	3.7
Cell cycle arrest	6	3.7
Reproductive structure development	7	3.7
Response to steroid hormone stimulus	11	3.7
Negative regulation of kinase activity	5	3.7
Regulation of peptidase activity	5	3.7
Rhythmic process	7	3.6
Gonad development	6	3.6
Positive regulation of cell migration	5	3.6
Regulation of I-kappaB kinase/NF-kappaB cascade	6	3.6
Regulation of Rho protein signal transduction	5	3.6
Response to nutrient levels	11	3.5
Negative regulation of transferase activity	5	3.4
Organelle localization	5	3.4
Kidney development	5	3.4
Ossification	6	3.4
Gland development	7	3.3
Negative regulation of transport	7	3.3
Positive regulation of I-kappaB kinase/NF-kappaB cascade	5	3.3
Blood coagulation	5	3.3
Negative regulation of response to stimulus	5	3.3
Coagulation	5	3.3
Positive regulation of cell motion	5	3.3
Positive regulation of multicellular organismal process	12	3.2
Lipid transport	7	3.2
Regulation of MAP kinase activity	7	3.2
Negative regulation of cell proliferation	18	3.2
Response to organic cyclic substance	6	3.2
Development of primary sexual characteristics	6	3.2
Response to extracellular stimulus	11	3.2
Appendage development	5	3.2
Limb development	5	3.2

Table S2.3: Biological processes regulated by apple procyanidin fraction dp 4 compared to VEGF continued.

Biological process	No. of genes involved out of 233	Fold Enrichment
Carboxylic acid transport	7	3.2
Negative regulation of multicellular organismal process	8	3.1
Anti-apoptosis	10	3.1
Bone development	6	3.1
Organic acid transport	7	3.1
Cell migration	13	3.1
Hemostasis	5	3.1
Response to wounding	25	3.1
Blood vessel morphogenesis	10	3.1
Enzyme linked receptor protein signaling pathway	16	3.0
Response to hormone stimulus	17	3.0
Chemotaxis	7	3.0
Lipid localization	7	3.0
Raxis	7	3.0
Regulation of GTPase activity	5	3.0
Urogenital system development	5	3.0
Response to peptide hormone stimulus	7	2.9
Blood vessel development	11	2.9
Rransmembrane receptor protein tyrosine kinase signaling pathway	10	2.9
Positive regulation of signal transduction	13	2.8
Muscle organ development	9	2.8
Vasculature development	11	2.8
Growth	8	2.8
Response to oxidative stress	7	2.8
Localization of cell	13	2.8
Cell motility	13	2.8
Reproductive developmental process	11	2.8
Cell motion	20	2.8
Inflammatory response	14	2.8
Regulation of hydrolase activity	14	2.8
Positive regulation of cell communication	14	2.7
Response to endogenous stimulus	17	2.7
Response to organic substance	30	2.7
Positive regulation of protein kinase cascade	7	2.7
Tube development	9	2.7
Sex differentiation	6	2.7
Regulation of cell activation	7	2.6
Regulation of secretion	8	2.6
Epidermis development	7	2.6
Positive regulation of developmental process	11	2.5
Positive regulation of hydrolase activity	7	2.5
Skeletal system development	12	2.4
Regulation of phosphate metabolic process	18	2.4
Regulation of phosphorus metabolic process	18	2.4
Negative regulation of cell differentiation	8	2.4
Ectoderm development	7	2.4
Regulation of Ras protein signal transduction	7	2.4
Regulation of phosphorylation	17	2.4
Regulation of cellular localization	9	2.3
Sensory organ development	8	2.3
Negative regulation of catalytic activity	10	2.3
Regulation of system process	11	2.3
Negative regulation of molecular function	12	2.3

Table S2.3: Biological processes regulated by apple procyanidin fraction dp 4 compared to VEGF continued.

Biological process	No. of genes involved out of 233	Fold Enrichment
Regulation of protein kinase cascade	9	2.3
Regulation of cell proliferation	28	2.3
Cell-cell signaling	21	2.3
Regulation of small GTPase mediated signal transduction	8	2.3
Pattern specification process	9	2.3
Regulation of protein kinase activity	12	2.2
Positive regulation of cell differentiation	8	2.2
Locomotory behavior	9	2.2
Regulation of kinase activity	12	2.2
Regulation of cell death	27	2.2
Cell morphogenesis involved in differentiation	8	2.1
Regulation of apoptosis	26	2.1
Positive regulation of cell death	14	2.1
Regulation of programmed cell death	26	2.1
Regulation of transferase activity	12	2.1
Cell morphogenesis	11	2.0
Negative regulation of apoptosis	11	2.0
Negative regulation of programmed cell death	11	2.0
Negative regulation of cell death	11	2.0
Positive regulation of apoptosis	13	2.0
Positive regulation of programmed cell death	13	2.0
Regulation of cell cycle	10	1.9
Embryonic morphogenesis	9	1.9
Cell proliferation	13	1.9
Regulation of growth	10	1.9
Cell adhesion	19	1.9
Biological adhesion	19	1.9
Cellular component morphogenesis	11	1.8
Reproductive process in a multicellular organism	13	1.8
Multicellular organism reproduction	13	1.8
Defense response	16	1.8
Positive regulation of gene expression	15	1.7
Positive regulation of biosynthetic process	17	1.6
Positive regulation of macromolecule biosynthetic process	16	1.6
Protein amino acid phosphorylation	16	1.6
Cell death	17	1.6
Death	17	1.6
Positive regulation of macromolecule metabolic process	20	1.5

Table S2.4: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to the control.

Biological processes, regulated by the apple procyanidin fraction dp 4-treated VEGF treatment versus the control from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 502	Fold Enrichment
Mismatch repair	5	7.2
Regulation of leukocyte migration	4	6.3
Regulation of striated muscle cell differentiation	6	6.3
Regulation of skeletal muscle fiber development	4	5.5
Positive regulation of anti-apoptosis	5	5.4
Regulation of endothelial cell migration	4	5.3
Regulation of muscle cell differentiation	6	5.3
Regulation of nitric oxide biosynthetic process	4	5.0
Positive regulation of alpha-beta T cell activation	4	5.0
Cardiac cell differentiation	4	4.9
Negative regulation of MAP kinase activity	5	4.8
Positive regulation of cell-substrate adhesion	4	4.7
Regulation of skeletal muscle tissue development	4	4.7
Regulation of interferon-gamma production	4	4.5
Regulation of JUN kinase activity	5	4.4
Regulation of anti-apoptosis	5	4.3
Positive regulation of T cell proliferation	5	4.2
Positive regulation of JUN kinase activity	4	4.1
Regulation of striated muscle tissue development	6	3.9
DNA-dependent DNA replication	7	3.9
Response to retinoic acid	4	3.9
Response to UV	7	3.9
Positive regulation of cell adhesion	7	3.9
Regulation of muscle development	6	3.9
DNA damage response, signal transduction resulting in induction of apoptosis	4	3.8
Mesenchymal cell differentiation	6	3.8
Mesenchymal cell development	6	3.8
Regulation of stress-activated protein kinase signaling pathway	8	3.8
Regulation of osteoblast differentiation	5	3.8
Regulation of alpha-beta T cell activation	4	3.7
Muscle fiber development	4	3.7
Mesenchyme development	6	3.7
Positive regulation of cell migration	10	3.6
Odontogenesis	6	3.6
Peptidyl-tyrosine phosphorylation	5	3.5
Regulation of cell-substrate adhesion	5	3.5
Regulation of JNK cascade	7	3.5
Negative regulation of leukocyte activation	6	3.4
Positive regulation of mononuclear cell proliferation	6	3.4
Positive regulation of leukocyte proliferation	6	3.4
Peptidyl-tyrosine modification	5	3.4
Erythrocyte homeostasis	5	3.4
Positive regulation of cell motion	10	3.3
Positive regulation of locomotion	10	3.3
Negative regulation of cell activation	6	3.3
Striated muscle cell development	5	3.2
Regulation of T cell proliferation	6	3.1
Regulation of cell migration	16	3.1
Regulation of angiogenesis	6	3.1

Table S2.4: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to the control continued.

Biological process	No. of genes involved out of 502	Fold Enrichment
Negative regulation of lymphocyte activation	5	3.0
Positive regulation of T cell activation	7	3.0
Muscle cell development	5	3.0
Blood coagulation	9	3.0
Coagulation	9	3.0
Leukocyte migration	5	2.9
Cardiac muscle tissue development	5	2.9
Positive regulation of lymphocyte proliferation	5	2.9
Branching morphogenesis of a tube	6	2.9
B cell activation	7	2.9
Homeostasis of number of cells	9	2.9
Response to cytokine stimulus	7	2.9
Regulation of ossification	7	2.9
Cellular response to insulin stimulus	6	2.8
Protein amino acid autophosphorylation	7	2.8
Cytokine-mediated signaling pathway	6	2.8
Hemostasis	9	2.8
Regulation of MAP kinase activity	12	2.8
Muscle cell differentiation	10	2.8
Striated muscle tissue development	10	2.7
Negative regulation of immune system process	7	2.7
Regulation of locomotion	16	2.7
Regulation of cell motion	16	2.7
Negative regulation of protein kinase activity	7	2.7
Regulation of MAPKKK cascade	9	2.7
Response to hypoxia	11	2.7
Regulation of leukocyte proliferation	7	2.7
Striated muscle cell differentiation	7	2.7
Regulation of mononuclear cell proliferation	7	2.7
Angiogenesis	12	2.6
Muscle tissue development	10	2.6
Negative regulation of kinase activity	7	2.6
Regulation of cytokine biosynthetic process	6	2.6
Morphogenesis of a branching structure	6	2.6
Activation of protein kinase activity	9	2.6
Response to oxygen levels	11	2.6
Regulation of cellular response to stress	8	2.5
DNA replication	14	2.5
Negative regulation of transferase activity	7	2.4
Myeloid cell differentiation	7	2.4
Regulation of body fluid levels	10	2.4
Positive regulation of lymphocyte activation	7	2.3
Response to insulin stimulus	7	2.3
Regulation of T cell activation	8	2.3
Immune system development	19	2.2
Positive regulation of MAP kinase activity	7	2.2
Regulation of cell adhesion	9	2.2
Response to light stimulus	9	2.2
Cell activation	19	2.2
Heart development	14	2.2
Blood vessel morphogenesis	14	2.2
Blood vessel development	16	2.1
Negative regulation of cell differentiation	14	2.1
Wound healing	12	2.1

Table S2.4: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to the control continued.

Biological process	No. of genes involved out of 502	Fold Enrichment
Response to peptide hormone stimulus	10	2.1
Regulation of protein kinase activity	22	2.1
Vasculature development	16	2.1
Hemopoiesis	15	2.1
Positive regulation of protein kinase activity	14	2.0
Regulation of kinase activity	22	2.0
Hemopoietic or lymphoid organ development	16	2.0
Regulation of lymphocyte activation	9	2.0
Regulation of leukocyte activation	10	2.0
Positive regulation of kinase activity	14	2.0
Response to hormone stimulus	22	1.9
Regulation of protein kinase cascade	15	1.9
Forebrain development	9	1.9
Response to endogenous stimulus	24	1.9
Regulation of transferase activity	22	1.9
Cell migration	16	1.9
Regulation of cell activation	10	1.9
Leukocyte activation	14	1.9
Positive regulation of transferase activity	14	1.9
Regulation of protein amino acid phosphorylation	10	1.9
DNA repair	16	1.9
Response to DNA damage stimulus	21	1.9
Response to steroid hormone stimulus	11	1.9
Localization of cell	17	1.8
Cell motility	17	1.8
Regulation of phosphorylation	26	1.8
Positive regulation of cell proliferation	23	1.8
Lymphocyte activation	11	1.8
Tube development	12	1.8
Cell proliferation	24	1.8
Positive regulation of multicellular organismal process	13	1.8
Regulation of phosphorus metabolic process	26	1.8
Regulation of phosphate metabolic process	26	1.8
Positive regulation of developmental process	15	1.7
DNA metabolic process	26	1.7
Negative regulation of cell proliferation	19	1.7
Positive regulation of cell differentiation	12	1.7
Protein kinase cascade	19	1.7
Response to organic substance	37	1.7
Positive regulation of cell death	22	1.7
Protein amino acid phosphorylation	33	1.6
Cell motion	23	1.6
Response to wounding	26	1.6
Inflammatory response	16	1.6
Positive regulation of apoptosis	21	1.6
Positive regulation of programmed cell death	21	1.6
Regulation of cell proliferation	38	1.6
Ion homeostasis	19	1.5
Cellular response to stress	26	1.5
Cell-cell signaling	27	1.5
Phosphate metabolic process	43	1.5
Phosphorus metabolic process	43	1.5

Table S2.5: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to VEGF.

Biological processes regulated by the apple procyanidin fraction dp 4-treated VEGF treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 355	Fold Enrichment
Response to retinoic acid	6	8.9
Ureteric bud development	6	8.4
Negative regulation of MAP kinase activity	6	8.2
Cardiac cell differentiation	4	7.0
Cell-cell junction organization	5	7.0
Response to vitamin A	6	7.0
Cell junction organization	8	6.9
Metanephros development	6	6.7
Calcium-mediated signaling	5	6.6
Odontogenesis of dentine-containing tooth	5	6.3
Regulation of endothelial cell proliferation	4	6.1
Cell junction assembly	5	6.0
Phospholipid transport	4	6.0
Cell fate determination	4	6.0
Establishment of vesicle localization	4	6.0
ER-nuclear signaling pathway	4	5.6
Negative regulation of defense response	4	5.5
Odontogenesis	6	5.5
Regulation of angiogenesis	7	5.5
Negative regulation of secretion	6	5.4
Vesicle localization	4	5.3
Negative regulation of hydrolase activity	5	5.1
Positive regulation of cell division	4	5.0
Response to unfolded protein	7	4.8
Mesenchymal cell differentiation	5	4.8
Mesenchymal cell development	5	4.8
Mesenchyme development	5	4.7
Neuron fate commitment	4	4.7
Regulation of inflammatory response	7	4.5
Negative regulation of protein kinase activity	8	4.5
BMP signaling pathway	4	4.5
Response to vitamin	6	4.5
Ovulation cycle	6	4.4
Response to mechanical stimulus	5	4.4
Negative regulation of kinase activity	8	4.4
Leukocyte migration	5	4.3
Cytokine production	4	4.3
Regulation of cell division	4	4.2
Positive regulation of cell adhesion	5	4.1
Negative regulation of transferase activity	8	4.1
Negative regulation of cell activation	5	4.0
Angiogenesis	12	4.0
Ovulation cycle process	5	4.0
Regulation of striated muscle tissue development	4	3.9
Monocarboxylic acid transport	4	3.9
Regulation of muscle development	4	3.9
Female gonad development	5	3.8
Positive regulation of response to external stimulus	5	3.8
Striated muscle cell development	4	3.8
Branching morphogenesis of a tube	5	3.8

Table S2.5: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to VEGF continued.

Biological process	No. of genes involved out of 355	Fold Enrichment
Response to estrogen stimulus	8	3.7
Organelle localization	7	3.7
Regulation of intracellular protein transport	4	3.7
Regulation of I-kappaB kinase/NF-kappaB cascade	8	3.7
Response to protein stimulus	8	3.7
Positive regulation of protein transport	5	3.7
Response to hypoxia	10	3.7
Muscle cell differentiation	9	3.7
Female sex differentiation	5	3.6
Development of primary female sexual characteristics	5	3.6
Negative regulation of immune system process	6	3.6
Positive regulation of I-kappaB kinase/NF-kappaB cascade	7	3.5
Cell fate commitment	10	3.5
Regulation of cell migration	12	3.5
Response to oxygen levels	10	3.5
Regulation of response to external stimulus	11	3.4
Lipid transport	10	3.4
Striated muscle cell differentiation	6	3.4
Regulation of locomotion	13	3.3
Morphogenesis of a branching structure	5	3.3
Cartilage development	5	3.3
Striated muscle tissue development	8	3.3
Amino acid transport	6	3.3
Negative regulation of transport	9	3.3
Blood vessel morphogenesis	14	3.3
Cell migration	18	3.2
Dephosphorylation	10	3.2
Response to nutrient	9	3.2
Response to glucocorticoid stimulus	5	3.2
Muscle tissue development	8	3.1
Regulation of MAP kinase activity	9	3.1
Lipid localization	10	3.1
Regulation of caspase activity	5	3.1
Wound healing	12	3.1
Kidney development	6	3.1
Response to steroid hormone stimulus	12	3.1
Regulation of cell motion	12	3.1
Negative regulation of cell cycle	5	3.0
Regulation of protein transport	7	3.0
Acute inflammatory response	6	3.0
Blood vessel development	15	3.0
Regulation of endopeptidase activity	5	3.0
Regulation of Rho protein signal transduction	6	3.0
Protein amino acid dephosphorylation	8	3.0
Positive regulation of protein kinase cascade	10	2.9
Vasculature development	15	2.9
Response to corticosteroid stimulus	5	2.9
Coagulation	6	2.9
Blood coagulation	6	2.9
Localization of cell	18	2.9
Cell motility	18	2.9
Regulation of cell adhesion	8	2.9
Cell cycle arrest	6	2.9

Table S2.5: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to VEGF continued.

Biological process	No. of genes involved out of 355	Fold Enrichment
Transmembrane receptor protein serine/threonine kinase signaling pathway	6	2.9
Regulation of peptidase activity	5	2.9
Regulation of establishment of protein localization	7	2.8
ATP metabolic process	6	2.8
Carbohydrate biosynthetic process	6	2.8
Hemostasis	6	2.7
Reproductive structure development	7	2.7
Positive regulation of secretion	6	2.7
Response to wounding	29	2.7
Rhythmic process	7	2.7
Urogenital system development	6	2.7
Carboxylic acid transport	8	2.7
Organic acid transport	8	2.7
Gonad development	6	2.6
Cell motion	25	2.6
Inflammatory response	17	2.6
Regulation of protein kinase activity	18	2.6
Purine ribonucleoside triphosphate metabolic process	6	2.5
Ribonucleoside triphosphate metabolic process	6	2.5
Amine transport	6	2.5
Positive regulation of signal transduction	15	2.5
Response to nutrient levels	10	2.5
Purine ribonucleotide metabolic process	7	2.5
Regulation of protein localization	7	2.5
Regulation of kinase activity	18	2.5
Tube development	11	2.5
Response to extracellular stimulus	11	2.5
Negative regulation of cell proliferation	18	2.5
Regulation of secretion	10	2.4
Regulation of phosphorus metabolic process	24	2.4
Regulation of phosphate metabolic process	24	2.4
Regulation of phosphorylation	23	2.4
Response to hormone stimulus	18	2.4
Anti-apoptosis	10	2.4
Regulation of cellular localization	12	2.4
Regulation of transferase activity	18	2.4
Regulation of protein kinase cascade	12	2.4
Negative regulation of molecular function	16	2.4
Ribonucleotide metabolic process	7	2.3
Negative regulation of catalytic activity	13	2.3
Cell proliferation	20	2.3
Regulation of cell activation	8	2.2
Positive regulation of cell communication	15	2.2
Positive regulation of transport	10	2.2
Glycoprotein metabolic process	9	2.2
Tissue morphogenesis	8	2.2
Response to endogenous stimulus	18	2.2
Enzyme linked receptor protein signaling pathway	15	2.2
Response to organic substance	31	2.1
Muscle organ development	9	2.1
Regulation of hydrolase activity	14	2.0
Positive regulation of cell death	18	2.0
Positive regulation of multicellular organismal process	10	2.0

Table S2.5: Biological processes regulated by apple procyanidin fraction dp 4-treated VEGF compared to VEGF continued.

Biological process	No. of genes involved out of 355	Fold Enrichment
Transmembrane receptor protein tyrosine kinase signaling pathway	9	2.0
Positive regulation of developmental process	11	1.9
Positive regulation of apoptosis	17	1.9
Positive regulation of programmed cell death	17	1.9
Cell-cell signaling	23	1.9
Reproductive developmental process	10	1.9
Regulation of cell proliferation	30	1.9
Induction of apoptosis	12	1.8
Induction of programmed cell death	12	1.8
Regulation of cell death	30	1.8
Regulation of cell cycle	12	1.8
Regulation of apoptosis	29	1.8
Regulation of programmed cell death	29	1.8
Regulation of system process	11	1.8
Defense response	20	1.6
Negative regulation of macromolecule biosynthetic process	17	1.5

Table S2.6: Biological processes regulated by anti-VEGF antibody-treated VEGF compared to VEGF.

Biological processes regulated by anti-VEGF antibody-treated VEGF treatment versus the VEGF treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 129	Fold Enrichment
ER-nuclear signaling pathway	4	13.5
Negative regulation of hydrolase activity	4	9.8
Response to mechanical stimulus	4	8.7
Response to unfolded protein	5	8.7
Regulation of caspase activity	5	7.6
Regulation of endopeptidase activity	5	7.3
Regulation of peptidase activity	5	6.9
Response to protein stimulus	6	6.8
Cartilage development	4	6.6
Regulation of inflammatory response	4	6.4
Response to lipopolysaccharide	4	6.3
Response to estrogen stimulus	5	5.7
Response to molecule of bacterial origin	4	5.6
Positive regulation of cell migration	4	5.4
Positive regulation of cell motion	4	4.9
Positive regulation of locomotion	4	4.9
Transmembrane receptor protein tyrosine kinase signaling pathway	9	4.9
Regulation of response to external stimulus	6	4.6
Regulation of gene-specific transcription	5	4.6
Carbohydrate biosynthetic process	4	4.5
Regulation of cell migration	6	4.3
Ossification	4	4.2
Bone development	4	3.9
Enzyme linked receptor protein signaling pathway	11	3.9
Rhythmic process	4	3.9
Response to metal ion	4	3.8
Response to steroid hormone stimulus	6	3.8
Regulation of locomotion	6	3.8
Regulation of cell motion	6	3.8
Golgi vesicle transport	4	3.7
Response to inorganic substance	6	3.7
Response to hypoxia	4	3.6
Negative regulation of transport	4	3.6
Cell migration	8	3.6
Response to extracellular stimulus	6	3.3
Localization of cell	8	3.3
Cell motility	8	3.3
Regulation of nervous system development	5	3.2
Response to organic substance	19	3.2
Regulation of system process	8	3.2
Response to nutrient levels	5	3.0
Response to abiotic stimulus	9	3.0
Response to wounding	13	3.0
Inflammatory response	8	3.0
Regulation of hydrolase activity	8	3.0
Blood vessel development	6	3.0
Anti-apoptosis	5	3.0
Vasculature development	6	2.9
Response to endogenous stimulus	9	2.7

Table S2.6: Biological processes regulated by anti-VEGF antibody-treated VEGF compared to VEGF continued.

Biological process	No. of genes involved out of 129	Fold Enrichment
Response to hormone stimulus	8	2.7
Positive regulation of developmental process	6	2.6
Negative regulation of transcription, DNA-dependent	7	2.4
Negative regulation of programmed cell death	7	2.4
Negative regulation of cell death	7	2.4
Negative regulation of RNA metabolic process	7	2.4
Negative regulation of macromolecule biosynthetic process	10	2.3
Negative regulation of cellular biosynthetic process	10	2.2
Negative regulation of transcription	8	2.2
Negative regulation of biosynthetic process	10	2.2
Cell motion	8	2.1
Cation transport	9	2.0
Regulation of phosphorus metabolic process	8	2.0
Regulation of phosphate metabolic process	8	2.0
Regulation of programmed cell death	13	2.0
Regulation of cell death	13	2.0
Cell surface receptor linked signal transduction	26	1.9
Regulation of cell proliferation	12	1.9
Negative regulation of macromolecule metabolic process	11	1.8
Regulation of apoptosis	12	1.8

Table S2.7: Biological processes regulated by anti-VEGF antibody-treated VEGF compared to apple procyanidin fraction dp 4.

Biological processes regulated by anti-VEGF antibody-treated VEGF treatment versus the apple procyanidin fraction dp 4 treatment from the $p < 0.001$ gene list from Table 6.1 were identified using the DAVID software. The biological processes were ranked according to the fold enrichment score.

Biological process	No. of genes involved out of 37	Fold Enrichment
Response to nutrient	4	10.5
Angiogenesis	4	10.1
Anti-apoptosis	5	9.1
Blood vessel morphogenesis	5	8.8
Response to steroid hormone stimulus	4	7.8
Blood vessel development	5	7.6
Response to nutrient levels	4	7.4
Vasculature development	5	7.4
Tube development	4	6.8
Positive regulation of transport	4	6.7
Response to extracellular stimulus	4	6.7
Positive regulation of multicellular organismal process	4	6.2
Negative regulation of apoptosis	5	5.3
Negative regulation of programmed cell death	5	5.2
Negative regulation of cell death	5	5.2
Response to hormone stimulus	5	5.1
Embryonic morphogenesis	4	5.0
Response to endogenous stimulus	5	4.6
Positive regulation of cell proliferation	5	4.5
Positive regulation of cell communication	4	4.5
Enzyme linked receptor protein signaling pathway	4	4.3
Positive regulation of transcription from RNA polymerase II promoter	4	4.1
Regulation of phosphorylation	5	4.0
Multicellular organism reproduction	5	4.0
Reproductive process in a multicellular organism	5	4.0
Regulation of phosphorus metabolic process	5	3.9
Regulation of phosphate metabolic process	5	3.9
Regulation of cell death	7	3.2
Defense response	5	3.2
Regulation of transcription from RNA polymerase II promoter	6	3.1
Cell-cell signaling	5	3.1
Response to organic substance	6	3.1
Immune response	5	3.0
Positive regulation of nitrogen compound metabolic process	5	3.0
Regulation of apoptosis	6	2.8
Positive regulation of cellular biosynthetic process	5	2.8
Regulation of programmed cell death	6	2.8
Regulation of transcription, DNA-dependent	12	2.7
Positive regulation of biosynthetic process	5	2.7
Regulation of RNA metabolic process	12	2.7
Intracellular signaling cascade	7	2.1
Regulation of transcription	12	1.8

SUPPLEMENTARY INFORMATION 3

GENES DIFFERENTIALLY EXPRESSED IN RESPONSE TO TREATMENTS

Supplementary Information 3

Genes differentially expressed in response to treatments

The genes that are altered in HUVECs in response to VEGF, apple procyanidin fraction dp 4 and anti-VEGF antibody treatments are listed in Tables S2.1-7. The gene list that represents a *p* value of <0.001 was used to produce a manageable set of probes to perform more detailed functional analysis. The probes are ranked into gene ontology groups according to the gene ontology database http://www.geneontology.org/. Those probes that had more than one gene ontology grouping were ranked according to the amount of citations in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) for that particular function.

Table S3.1: List of differentially expressed genes in HUVECs after a six hour treatment of 10 ng/ml VEGF compared to the control (*p* < 0.001; 132 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	1.81
NM_005429	VEGFC	vascular endothelial growth factor C	1.41
Apoptosis			
NM_002135	NR4A1	nuclear receptor subfamily 4, group A, member 1	1.70
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	-1.64
NM_003810	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	-2.22
NM_018003	UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	1.53
Binding / Cell Adhesion			
NM_001130861	CLDN5	claudin 5	-1.56
NM_001850	COL8A1	collagen, type VIII, alpha 1	-1.44
NM_024785	FAM124B	family with sequence similarity 124B	-1.83
NM_024582	FAT4	FAT tumor suppressor homolog 4 (Drosophila)	-1.66
NM_016946	F11R	F11 receptor	-1.36
NM_130759	GIMAP1	GTPase, IMAP family member 1	-1.68
NM_015660	GIMAP2	GTPase, IMAP family member 2	-1.83
NM_018326	GIMAP4	GTPase, IMAP family member 4	-1.61
NM_024711	GIMAP6	GTPase, IMAP family member 6	-1.44
NM_175571	GIMAP8	GTPase, IMAP family member 8	-1.71
NM_022143	LRRC4	leucine rich repeat containing 4	-1.41
NM_022750	PARP12	poly (ADP-ribose) polymerase family, member 12	-1.30
NM_001040429	PCDH17	protocadherin 17	1.61
NM_024787	RNF122	ring finger protein 122	1.39

Table S3.1: List of differentially expressed genes in HUVECs after a six hour treatment of 10 ng/ml VEGF compared to the control ($p < 0.001$; 132 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Cell communication			
NM_001142481	C5orf13	chromosome 5 open reading frame 13	-1.46
NM_014421	DKK2	dickkopf homolog 2 (Xenopus laevis)	1.86
NM_018431	DOK5	docking protein 5	1.84
NM_012302	LPHN2	latrophilin 2	1.70
NM_005065	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	1.48
NM_015144	ZCCHC14	zinc finger, CCHC domain containing 14	1.37
Cell Cycle / Cell Proliferation			
NM_006988	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.57
NM_015147	CEP68	centrosomal protein 68kDa	-1.62
NM_001946	DUSP6	dual specificity phosphatase 6	1.39
NM_001085377	MCC	mutated in colorectal cancers	-1.37
NM_006472	TXNIP	thioredoxin interacting protein	-1.67
NM_002688	SEPT5	septin 5	1.67
NM_153618	SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	3.90
NM_145728	SYNM	synemin, intermediate filament protein	1.53
NM_014909	VASH1	vasohibin 1	1.47
NM_000222	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-2.03
Development			
NM_003633	ENC1	ectodermal-neural cortex (with BTB-like domain)	-1.74
NM_006350	FST	follistatin	1.84
NM_002060	GJA4	gap junction protein, alpha 4, 37kDa	-2.55
NM_005266	GJA5	gap junction protein, alpha 5, 40kDa	-2.12
NM_003250	THRA	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)	-1.35
NM_005450	NOG	noggin	2.06
NM_002360	MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)	1.38
DNA Binding / Replication / Repair / Transcription			
NM_014945	ABLIM3	actin binding LIM protein family, member 3	-1.31
NM_005935	AFF1	AF4/FMR2 family, member 1	-1.36
NM_001986	ETV4	ets variant 4	1.51
NM_001134673	NFIA	nuclear factor I/A // 1p31.3-p31.2	-1.39
NM_005378	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	1.63
Immune Response			
NM_007074	CORO1A	coronin, actin binding protein, 1A	1.55
NM_005080	XBP1	X-box binding protein 1	1.53
Metabolic / Biosynthetic Process			
NM_022060	ABHD4	abhydrolase domain containing 4	1.44
NM_003654	CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	-1.42
NM_015892	CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	-1.41
NM_015892	CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	-1.33

Table S3.1: List of differentially expressed genes in HUVECs after a six hour treatment of 10 ng/ml VEGF compared to the control ($p < 0.001$; 132 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process continued			
NM_032385	C5orf4	chromosome 5 open reading frame 4	-1.57
NM_000499	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	-2.10
NM_012328	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.16
NM_006260	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	1.78
NM_014674	EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.50
NM_019891	ERO1LB	ERO1-like beta (S. cerevisiae)	2.19
NM_007076	FICD	FIC domain containing	1.81
NM_002153	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	1.60
NM_018192	LEPREL1	leprecan-like 1	1.42
NM_001144967	NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	1.62
NM_004414	RCAN1	regulator of calcineurin 1	2.26
NM_014746	RNF144A	ring finger protein 144A	-1.75
NM_006588	SULT1C4	sulfotransferase family, cytosolic, 1C, member 4	1.96
NM_032431	SYVN1	synovial apoptosis inhibitor 1, synoviolin	1.69
Response to stimulus			
NM_000014	A2M	alpha-2-macroglobulin	2.18
NM_005409	CXCL11	chemokine (C-X-C motif) ligand 11	-3.69
NM_001993	F3	coagulation factor III (thromboplastin, tissue factor)	1.59
NM_005347	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	1.45
NM_014685	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	1.90
NM_006389	HYOU1	hypoxia up-regulated 1	1.42
NM_006010	MANF	mesencephalic astrocyte-derived neurotrophic factor	1.49
NM_000603	NOS3	nitric oxide synthase 3 (endothelial cell)	-1.42
NM_003155	STC1	stanniocalcin 1	3.76
NM_000361	THBD	thrombomodulin	2.43
NM_006528	TFPI2	tissue factor pathway inhibitor 2	1.81
NM_001078	VCAM1	vascular cell adhesion molecule 1	2.28
Signal Transduction			
NM_000020	ACVRL1	activin A receptor type II-like 1	-1.41
NM_006449	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	-1.44
NM_173515	CNKSR3	CNKSR family member 3	2.03
NM_138445	GPR146	G protein-coupled receptor 146	-1.74
NM_001945	HBEGF	heparin-binding EGF-like growth factor	1.67
NM_005204	MAP3K8	mitogen-activated protein kinase kinase kinase 8	1.53
NM_006186	NR4A2	nuclear receptor subfamily 4, group A, member 2	1.65
NM_002646	PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	-1.46
NM_007211	RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	1.51
NM_016084	RASD1	RAS, dexamethasone-induced 1	1.83
NM_001010000	ARHGAP28	Rho GTPase activating protein 28	-1.79
NM_001128615	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	-1.46
NM_030964	SPRY4	sprouty homolog 4 (Drosophila)	-1.82
NM_015028	TNIK	TRAF2 and NCK interacting kinase	-1.48

Table S3.1: List of differentially expressed genes in HUVECs after a six hour treatment of 10 ng/ml VEGF compared to the control ($p < 0.001$; 132 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transcription			
NM_001200	BMP2	bone morphogenetic protein 2	1.97
NM_178423	HDAC9	histone deacetylase 9	1.52
NM_030915	LBH	limb bud and heart development homolog (mouse)	1.97
NM_014368	LHX6	LIM homeobox 6	-1.44
NM_018717	MAML3	mastermind-like 3 (Drosophila)	-1.34
NM_002397	MEF2C	myocyte enhancer factor 2C	1.59
NM_205860	NR5A2	nuclear receptor subfamily 5, group A, member 2	1.53
NM_001198	PRDM1	PR domain containing 1, with ZNF domain	2.74
NM_005686	SOX13	SRY (sex determining region Y)-box 13	-1.45
Transport			
NM_001001396	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	-1.56
NM_004827	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-1.91
NM_198098	AQP1	aquaporin 1	-2.16
NM_022113	KIF13A	kinesin family member 13A	1.42
NM_138444	KCTD12	potassium channel tetramerisation domain containing 12	-1.66
NM_152999	STEAP2	six transmembrane epithelial antigen of the prostate 2	-1.46
NM_003043	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	-1.50
NM_003045	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.61
NM_001128431	SLC39A14	solute carrier family 39 (zinc transporter), member 14	2.07
Miscellaneous / Other			
NM_020437	ASPHD2	aspartate beta-hydroxylase domain containing 2	-1.52
NM_032866	CGNL1	cingulin-like 1	-1.60
NM_005851	CDK2AP2	cyclin-dependent kinase 2 associated protein 2	1.55
NM_001135101	CRELD2	cysteine-rich with EGF-like domains 2	1.57
NM_014600	EHD3	EH-domain containing 3	3.21
NM_001130025	FAM115C	family with sequence similarity 115, member C	1.37
NM_173511	FAM117B	family with sequence similarity 117, member B	-1.32
NM_015687	FILIP1	filamin A interacting protein 1	-1.58
NM_007223	GPR176	G protein-coupled receptor 176	1.50
NM_001134476	LRRC8B	leucine rich repeat containing 8 family, member B	1.57
NM_173198	NR4A3	nuclear receptor subfamily 4, group A, member 3	1.38
NM_015993	PLLP	plasma membrane proteolipid (plasmolipin)	-1.70
NM_015157	PHLDB1	pleckstrin homology-like domain, family B, member 1	1.39
NM_001104587	SLFN11	schlafen family member 11	1.75
NM_022044	SDF2L1	stromal cell-derived factor 2-like 1	1.87
NM_003898	SYNJ2	synaptojanin 2	-1.37
NM_005723	TSPAN5	tetraspanin 5	-1.41
NR_002816	THSD1P	thrombospondin, type I, domain containing 1 pseudogene	-1.52
NM_013390	TMEM2	transmembrane protein 2	1.61
NM_006134	TMEM50B	transmembrane protein 50B	1.96

Table S3.2: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to the control ($p < 0.001$; 94 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	-1.71
NM_001202	BMP4	bone morphogenetic protein 4	-1.86
NM_001554	CYR61	cysteine-rich, angiogenic inducer, 61	-1.61
NM_002133	HMOX1	heme oxygenase (decycling) 1	1.59
NM_005924	MEOX2	mesenchyme homeobox 2	-1.66
NM_005429	VEGFC	vascular endothelial growth factor C	1.44
Apoptosis			
NM_001003940	BMF	Bcl2 modifying factor	1.46
NM_032977	CASP10	caspase 10, apoptosis-related cysteine peptidase	-1.62
NM_019058	DDIT4	DNA-damage-inducible transcript 4	1.60
NM_182757	RNF144B	ring finger protein 144B	-1.91
NM_020870	SH3RF1	SH3 domain containing ring finger 1	-1.37
NM_005092	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	-2.44
Binding / Cell adhesion			
NM_001850	COL8A1	collagen, type VIII, alpha 1	-1.64
NM_001338	CXADR	coxsackie virus and adenovirus receptor	-1.62
NM_130759	GIMAP1	GTPase, IMAP family member 1	-1.40
NM_175571	GIMAP8	GTPase, IMAP family member 8	-1.61
NM_024787	RNF122	ring finger protein 122	1.74
Cell Communication			
NM_000863	HTR1B	5-hydroxytryptamine (serotonin) receptor 1B	-1.61
NM_002758	MAP2K6	mitogen-activated protein kinase kinase 6	-1.61
NM_005923	MAP3K5	mitogen-activated protein kinase kinase kinase 5	-1.57
NM_024870	PREX2	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	-1.66
NM_032784	RSPO3	R-spondin 3 homolog (Xenopus laevis)	-1.57
NM_015191	SIK2	salt-inducible kinase 2	1.48
Cell Cycle / Cell Proliferation			
NM_000757	CSF1	colony stimulating factor 1 (macrophage)	1.43
NM_001946	DUSP6	dual specificity phosphatase 6	-1.49
NM_004429	EFNB1	ephrin-B1	1.52
NM_002192	INHBA	inhibin, beta A	-1.46
Development			
NM_012242	DKK1	dickkopf homolog 1 (Xenopus laevis)	-2.03
NM_182964	NAV2	neuron navigator 2	1.69
NM_013960	NRG1	neuregulin 1	-1.44
NM_001025252	TPD52	tumor protein D52	-1.43
NM_005239	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.45
DNA Binding / Replication / Repair / Transcription			
NM_004956	ETV1	ets variant 1	-1.81
NM_001515	GTF2H2	general transcription factor IIH, polypeptide 2, 44kDa	-1.70
NM_000270	NP	nucleoside phosphorylase	1.42
NM_005378	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	2.01
NR_027302	XPA	xeroderma pigmentosum, complementation group A	-1.63

Table S3.2: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to the control (*p* < 0.001*;* 94 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Inflammatory Response			
NM_000201	ICAM1	intercellular adhesion molecule 1	1.89
NM_000600	IL6	interleukin 6 (interferon, beta 2)	-1.92
NM_002852	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	-2.21
Immune Response			
NM_014143	CD274	CD274 molecule	-2.09
NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	1.59
Metabolic / Biosynthetic Process			
NM_005099	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	1.68
NM_174858	AK5	adenylate kinase 5	-1.96
NM_007034	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	-1.80
NM_024090	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	-2.00
NM_001097634	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	-1.67
NM_023112	OTUB2	OTU domain, ubiquitin aldehyde binding 2	1.52
NM_001159323	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.76
NM_000930	PLAT	plasminogen activator, tissue	1.48
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.55
NM_001143676	SGK1	serum/glucocorticoid regulated kinase 1	-1.62
NM_001130012	SLC9A3R2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	1.52
NM_015028	TNIK	TRAF2 and NCK interacting kinase	-1.43
Response to Stimulus			
NM_004827	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-1.94
NM_018948	ERRFI1	ERBB receptor feedback inhibitor 1	-1.51
NM_002229	JUNB	jun B proto-oncogene	1.71
NM_001128304	PLSCR4	phospholipid scramblase 4	-1.92
NM_003234	TFRC	transferrin receptor (p90, CD71)	-2.08
Signal Transduction			
NM_014391	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	-1.53
NM_004864	GDF15	growth differentiation factor 15	1.50
NM_001077494	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	1.52
NM_015368	PANX1	pannexin 1	1.37
NM_001102445	RGS4	regulator of G-protein signalling 4	-1.61
NM_005842	SPRY2	sprouty homolog 2 (Drosophila)	-1.61
NM_005841	SPRY1	sprouty homolog 1, antagonist of FGF signalling (Drosophila)	-1.57
NM_005658	TRAF1	TNF receptor-associated factor 1	1.90
Transcription			
NM_004454	ETV5	ets variant 5	-1.64
NM_021958	HLX	H2.0-like homeobox	1.51

Table S3.2: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to the control (*p* < 0.001*;* 94 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transcription continued			
NM_003884	KAT2B	K(lysine) acetyltransferase 2B	-1.48
NM_016270	KLF2	Krüppel-like factor 2 (lung)	1.67
NM_004235	KLF4	Krüppel-like factor 4 (gut)	1.78
NM_002398	MEIS1	Meis homeobox 1	-1.55
NM_032246	MEX3B	mex-3 homolog B (C. elegans)	1.41
Transport			
NM_152999	STEAP2	six transmembrane epithelial antigen of the prostate 2	-1.64
NM_145176	SLC2A12	solute carrier family 2 (facilitated glucose transporter), member 12	-1.59
NR_024194	TOM1	target of myb1 (chicken)	1.43
Miscellaneous / Other			
NM_003670	BHLHE40	basic helix-loop-helix family, member e40	1.44
NM_198545	C1orf187	chromosome 1 open reading frame 187	1.50
NM_178496	C3orf59	chromosome 3 open reading frame 59	-1.63
NM_032623	C4orf49	chromosome 4 open reading frame 49	-1.67
NM_001035254	FAM102A	family with sequence similarity 102, member A	1.48
NM_020116	FSTL5	follistatin-like 5	-1.84
ENST00000423909	FLJ33996	hypothetical protein FLJ33996	-1.56
BC004406	KIAA1539	KIAA1539	1.44
NM_001040078	LGALS9C	lectin, galactoside-binding, soluble, 9C	1.45
NM_001113546	LIMA1	LIM domain and actin binding 1	-1.45
AY176665	NSAP11	nervous system abundant protein 11	-1.73
NM_015157	PHLDB1	pleckstrin homology-like domain, family B, member 1	1.36
NM_001134438	PHLDB2	pleckstrin homology-like domain, family B, member 2	-1.49
NM_015150	RFTN1	raftlin, lipid raft linker 1	-1.42
NM_004657	SDPR	serum deprivation response (phosphatidylserine binding protein)	-1.55
NM_005723	TSPAN5	tetraspanin 5	-1.51
NM_021202	TP53INP2	tumor protein p53 inducible nuclear protein 2	1.39

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control ($p < 0.001$; 511 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	-1.61
NM_001202	BMP4	bone morphogenetic protein 4	-1.76
NM_001554	CYR61	cysteine-rich, angiogenic inducer, 61	-1.62
NM_002133	HMOX1	heme oxygenase (decycling) 1	1.55
NM_005924	MEOX2	mesenchyme homeobox 2	-1.55
NM_194358	RNF41	ring finger protein 41	-1.38
NM_005429	VEGFC	vascular endothelial growth factor C	1.28
Apoptosis			
NM_001003940	BMF	Bcl2 modifying factor	1.46
NM_032977	CASP10	caspase 10, apoptosis-related cysteine peptidase	-1.88
NM_019058	DDIT4	DNA-damage-inducible transcript 4	1.44
NM_021960	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	-1.46
NM_002135	NR4A1	nuclear receptor subfamily 4, group A, member 1	1.60
NM_182757	RNF144B	ring finger protein 144B	-1.83
NM_004760	STK17A	serine/threonine kinase 17a	-1.64
NM_020870	SH3RF1	SH3 domain containing ring finger 1	-1.43
NM_001561	TNFRSF9	tumor necrosis factor receptor superfamily, member 9	1.38
NM_005092	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	-2.77
NM_003841	TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	-1.82
Binding / Cell Adhesion			
NM_078481	CD97	CD97 molecule	1.30
NM_001850	COL8A1	collagen, type VIII, alpha 1	-1.79
NM_004370	COL12A1	collagen, type XII, alpha 1	-1.63
NM_001338	CXADR	coxsackie virus and adenovirus receptor	-1.72
NM_024582	FAT4	FAT tumor suppressor homolog 4 (Drosophila)	-1.54
NM_000173	GP1BA	glycoprotein Ib (platelet), alpha polypeptide	1.62
NM_130759	GIMAP1	GTPase, IMAP family member 1	-1.47
NM_015660	GIMAP2	GTPase, IMAP family member 2	-1.55
NM_175571	GIMAP8	GTPase, IMAP family member 8	-1.67
NM_152722	HEPACAM	hepatocyte cell adhesion molecule	1.44
NM_000210	ITGA6	integrin, alpha 6	-1.43
NM_000212	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	-1.46
NM_015520	MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	-1.46
NM_015480	PVRL3	poliovirus receptor-related 3	-1.65
NM_024787	RNF122	ring finger protein 122	1.52
NM_014000	VCL	vinculin	-1.36
Cell Communication / Signalling			
NM_001025105	CSNK1A1	casein kinase 1, alpha 1	-1.56
NM_001233	CAV2	caveolin 2	-1.37

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Cell Communication / Signalling continued			
NM_003607	CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	-1.51
NM_005283	XCR1	chemokine (C motif) receptor 1	1.68
NM_001135597	CCDC88A	coiled-coil domain containing 88A	-1.51
NM_001258	CDK3	cyclin-dependent kinase 3	1.29
NM_001259	CDK6	cyclin-dependent kinase 6	-1.44
NM_004090	DUSP3	dual specificity phosphatase 3	1.30
NM_002759	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-1.79
NM_002037	FYN	FYN oncogene related to SRC, FGR, YES	-1.49
NM_020660	GJD2	gap junction protein, delta 2, 36kDa	1.77
NM_153442	GPR26	G protein-coupled receptor 26	1.47
NM_015234	GPR116	G protein-coupled receptor 116	-1.72
NM_020455	GPR126	G protein-coupled receptor 126	-1.60
NM_002072	GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	-1.34
NM_000863	HTR1B	5-hydroxytryptamine (serotonin) receptor 1B	-1.56
NM_012302	LPHN2	latrophilin 2	-1.42
NM_002310	LIFR	leukemia inhibitory factor receptor alpha	-1.84
NM_002758	MAP2K6	mitogen-activated protein kinase kinase 6	-1.65
NM_002419	MAP3K11	mitogen-activated protein kinase kinase kinase 11	1.34
NM_153240	NPHP3	nephronophthisis 3 (adolescent)	-1.39
NM_133494	NEK7	NIMA (never in mitosis gene a)-related kinase 7	-1.69
NM_001014831	PAK4	p21 protein (Cdc42/Rac)-activated kinase 4	1.36
NM_024870	PREX2	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	-1.94
NM_017934	PHIP	pleckstrin homology domain interacting protein	-1.54
NM_206907	PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.38
NM_182948	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	-1.45
NM_014906	PPM1E	protein phosphatase 1E (PP2C domain containing)	1.43
NM_000944	PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	-1.55
NM_080683	PTPN13	protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	-1.36
NM_152573	RASEF	RAS and EF-hand domain containing	1.35
NM_032784	RSPO3	R-spondin 3 homolog (Xenopus laevis)	-1.62
NM_015191	SIK2	salt-inducible kinase 2	1.37
NM_080489	SDCBP2	syndecan binding protein (syntenin) 2	1.45
NM_001057	TACR2	tachykinin receptor 2	1.47
NM_003243	TGFBR3	transforming growth factor, beta receptor III	-1.43
Cell Cycle / Cell Proliferation			
NM_025009	CEP135	centrosomal protein 135kDa	-1.62
NM_000757	CSF1	colony stimulating factor 1 (macrophage)	1.33
NM_057749	CCNE2	cyclin E2	-1.86

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Cell Cycle / Cell Proliferation continued			
NM_001946	DUSP6	dual specificity phosphatase 6	-1.47
NM_004429	EFNB1	ephrin-B1	1.45
NM_032410	HOOK3	hook homolog 3 (Drosophila)	-1.53
NM_002192	INHBA	inhibin, beta A	-1.58
NM_033402	LRRCC1	leucine rich repeat and coiled-coil domain containing 1	-1.72
NM_020310	MNT	MAX binding protein	1.45
NM_001105078	MECOM	MDS1 and EVI1 complex locus	-1.44
NM_002393	MDM4	Mdm4 p53 binding protein homolog (mouse)	-1.65
NM_032485	MCM8	minichromosome maintenance complex component 8	-1.48
NM_005923	MAP3K5	mitogen-activated protein kinase kinase kinase 5	-1.68
NM_001085377	MCC	mutated in colorectal cancers	-1.47
NM_012224	NEK1	NIMA (never in mitosis gene a)-related kinase 1	-1.51
NM_002498	NEK3	NIMA (never in mitosis gene a)-related kinase 3	-1.57
NM_003999	OSMR	oncostatin M receptor	-1.57
NM_018151	RIF1	RAP1 interacting factor homolog (yeast)	-1.79
NM_018243	SEP11	septin 11	-1.27
NM_001060	TBXA2R	thromboxane A2 receptor	1.43
NM_006322	TUBGCP3	tubulin, gamma complex associated protein 3	-1.34
NM_145314	UCMA	upper zone of growth plate and cartilage matrix associated	1.42
NM_000222	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-1.51
NM_006646	WASF3	WAS protein family, member 3	-1.33
NM_033131	WNT3A	wingless-type MMTV integration site family, member 3A	1.51
Cell Migration			
NM_033138	CALD1	caldesmon 1	-1.37
Development			
NM_002313	ABLIM1	actin binding LIM protein 1	-1.34
NM_016441	CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	-1.31
NM_012242	DKK1	dickkopf homolog 1 (Xenopus laevis)	-1.94
NM_001405	EFNA2	ephrin-A2	1.46
NM_004425	ECM1	extracellular matrix protein 1	1.36
NM_001145772	GPR56	G protein-coupled receptor 56	1.37
NM_019594	LRRC8A	leucine rich repeat containing 8 family, member A	1.31
NM_018174	MAP1S	microtubule-associated protein 1S	1.61
NM_013960	NRG1	neuregulin 1	-1.38
NM_182964	NAV2	neuron navigator 2	1.49
NM_152754	SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	-1.59
NM_001135599	TGFB2	transforming growth factor, beta 2	-1.48
NM_020127	TUFT1	tuftelin 1	-1.36

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Development continued			
NM_001025252	TPD52	tumor protein D52	-1.37
NM_007124	UTRN	utrophin	-1.48
NM_005239	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.37
NM_017953	ZNHIT6	zinc finger, HIT type 6	-1.81
NM_005095	ZMYM4	zinc finger, MYM-type 4	-1.46
DNA Binding / Replication / Repair / Transcription			
NM_005224	ARID3A	AT rich interactive domain 3A (BRIGHT-like)	1.36
NM_004956	ETV1	ets variant 1	-1.86
NM_144571	CNOT6L	CCR4-NOT transcription complex, subunit 6-like	-1.50
NM_015891	CDC40	cell division cycle 40 homolog (S. cerevisiae)	-1.42
NM_016448	DTL	denticleless homolog (Drosophila)	-1.54
NM_001159936	EBNA1BP2	EBNA1 binding protein 2	-1.55
NM_000124	ERCC6	excision repair cross-complementing rodent repair deficiency, complementation group 6	-1.58
NM_130398	EXO1	exonuclease 1	-1.44
NM_014285	EXOSC2	exosome component 2	-1.35
NM_001515	GTF2H2	general transcription factor IIH, polypeptide 2, 44kDa	-1.81
NM_018078	LARP1B	La ribonucleoprotein domain family, member 1B	-1.57
NM_020961	METTL14	methyltransferase like 14	-1.50
NM_002388	MCM3	minichromosome maintenance complex component 3	-1.44
NM_000179	MSH6	mutS homolog 6 (E. coli)	-1.46
NM_015934	NOP58	NOP58 ribonucleoprotein homolog (yeast)	-1.61
NM_007362	NCBP2	nuclear cap binding protein subunit 2, 20kDa	-1.68
NM_001134673	NFIA	nuclear factor I/A	-1.34
NM_005596	NFIB	nuclear factor I/B	-1.33
NM_000901	NR3C2	nuclear receptor subfamily 3, group C, member 2	-1.46
NM_000270	NP	nucleoside phosphorylase	1.32
NM_002526	NT5E	5'-nucleotidase, ecto (CD73)	-1.44
NM_015342	PPWD1	peptidylprolyl isomerase domain and WD repeat containing 1	-1.74
NM_000534	PMS1	PMS1 postmeiotic segregation increased 1 (S. cerevisiae)	-1.41
NR_003085	PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	-1.56
NM_016937	POLA1	polymerase (DNA directed), alpha 1, catalytic subunit	-1.51
NM_007195	POLI	polymerase (DNA directed) iota	-1.52
NM_019014	POLR1B	polymerase (RNA) I polypeptide B, 128kDa	-1.60
NM_033109	PNPT1	polyribonucleotide nucleotidyltransferase 1	-1.72
NR_027775	PMS2L5	postmeiotic segregation increased 2-like 5	-1.48
NM_020165	RAD18	RAD18 homolog (S. cerevisiae)	-1.44
NM_016316	REV1	REV1 homolog (S. cerevisiae)	-1.48

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
DNA Binding / Replication / Repair / Transcription continued			
NM_024945	RMI1	RMI1, RecQ mediated genome instability 1, homolog (S. cerevisiae)	-1.50
NM_003800	RNGTT	RNA guanylyltransferase and 5'-phosphatase	-1.56
NM_006938	SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	-1.49
NM_003096	SNRPG	small nuclear ribonucleoprotein polypeptide G	-2.00
NM_001142286	SMC6	structural maintenance of chromosomes 6	-1.73
NM_018419	SOX18	SRY (sex determining region Y)-box 18	1.38
NM_007114	TMF1	TATA element modulatory factor 1	-1.64
NM_003598	TEAD2	TEA domain family member 2	1.41
NM_013293	TRA2A	transformer 2 alpha homolog (Drosophila)	-1.41
NM_020810	TRMT5	TRM5 tRNA methyltransferase 5 homolog (S. cerevisiae)	-1.36
NR_027302	XPA	xeroderma pigmentosum, complementation group A	-1.69
NM_005378	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	1.99
NM_000553	WRN	Werner syndrome, RecQ helicase-like	-1.65
NM_003432	ZNF131	zinc finger protein 131	-1.39
NM_032819	ZNF341	zinc finger protein 341	1.45
NM_152320	ZNF641	zinc finger protein 641	-1.39
Inflammatory Response			
NM_000201	ICAM1	intercellular adhesion molecule 1	1.78
NM_000575	IL1A	interleukin 1, alpha	-1.64
NM_000600	IL6	interleukin 6 (interferon, beta 2)	-1.94
NM_002349	LY75	lymphocyte antigen 75	-1.49
NM_002852	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	-2.23
NM_002965	S100A9	S100 calcium binding protein A9	1.63
NM_003265	TLR3	toll-like receptor 3	-1.49
Immune Response			
NM_014143	CD274	CD274 molecule	-2.37
NM_001115152	CD300L	CD300 molecule-like family member d	1.56
NM_005755	EBI3	Epstein-Barr virus induced 3	1.49
NM_002053	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	-1.54
NM_001083539	KIR3DS1	killer cell immunoglobulin-like receptor, three domains, short cytoplasmic tail, 1	1.61
NM_012312	KIR2DS2	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2	1.43
NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	1.59
NM_025239	PDCD1LG2	programmed cell death 1 ligand 2	-1.57
NM_004155	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	-1.53

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process			
NM_020186	ACN9	ACN9 homolog (S. cerevisiae)	-1.84
NM_005099	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	1.60
NM_014272	ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	1.37
NM_182920	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	-1.37
NM_199355	ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	-1.46
NM_174858	AK5	adenylate kinase 5	-2.22
NM_001126	ADSS	adenylosuccinate synthase	-1.64
NM_000689	ALDH1A1	aldehyde dehydrogenase 1 family, member A1	-1.37
NM_003659	AGPS	alkylglycerone phosphate synthase	-1.52
NM_015328	AHCYL2	adenosylhomocysteinase-like 2	-1.37
NM_001145815	AMDHD2	amidohydrolase domain containing 2	1.40
NM_005763	AASS	aminoadipate-semialdehyde synthase	-1.47
NM_016201	AMOTL2	angiomotin like 2	-1.42
NR_027833	APOL3	apolipoprotein L, 3	1.26
NM_030643	APOL4	apolipoprotein L, 4	1.39
NM_030817	APOLD1	apolipoprotein L domain containing 1	1.46
NM_012287	ACAP2	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2	-1.54
NM_001088	AANAT	arylalkylamine N-acetyltransferase	1.45
NM_024590	ARSJ	arylsulfatase family, member J	-1.65
NM_004674	ASH2L	ash2 (absent, small, or homeotic)-like (Drosophila)	-1.42
NM_014616	ATP11B	ATPase, class VI, type 11B	-1.65
NM_018371	CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	-1.42
NM_001823	CKB	creatine kinase, brain	1.30
NM_004074	COX8A	cytochrome c oxidase subunit 8A (ubiquitous)	1.44
NM_000772	CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18	1.28
NM_000774	CYP2F1	cytochrome P450, family 2, subfamily F, polypeptide 1	1.76
NM_007034	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	-1.99
NM_001031723	DNAJB14	DnaJ (Hsp40) homolog, subfamily B, member 14	-1.47
NM_194283	DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	-1.65
NM_018370	DRAM1	DNA-damage regulated autophagy modulator 1	1.35
NM_024090	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	-2.05
NM_001442	FABP4	fatty acid binding protein 4, adipocyte	-1.75
NM_001042762	FIGNL1	fidgetin-like 1	-1.67

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process			
NM_001097634	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	-1.71
NM_015554	GLCE	glucuronic acid epimerase	-1.29
NM_004446	EPRS	glutamyl-prolyl-tRNA synthetase	-1.51
NM_194247	HNRNPA3	heterogeneous nuclear ribonucleoprotein A3	-1.68
NM_012267	HSPBP1	HSPA (heat shock 70kDa) binding protein, cytoplasmic cochaperone 1	1.28
NM_005575	LNPEP	leucyl/cystinyl aminopeptidase	-1.45
NM_002422	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	1.37
NM_005940	MMP11	matrix metallopeptidase 11 (stromelysin 3)	1.36
NM_004995	MMP14	matrix metallopeptidase 14 (membrane-inserted)	1.33
NM_014632	MICAL2	microtubule associated monoxygenase, calponin and LIM domain containing 2	-1.30
NM_007208	MRPL3	mitochondrial ribosomal protein L3	-1.49
NM_015971	MRPS7	mitochondrial ribosomal protein S7	-1.69
NM_022100	MRPS14	mitochondrial ribosomal protein S14	-1.36
NM_017971	MRPL20	mitochondrial ribosomal protein L20	-1.44
NM_017971	MRPL20	mitochondrial ribosomal protein L20	-1.50
NM_015514	NGDN	neuroguidin, EIF4E binding protein	-1.50
NM_023112	OTUB2	OTU domain, ubiquitin aldehyde binding 2	1.56
NM_017784	OSBPL10	oxysterol binding protein-like 10	-1.43
NM_003712	PPAP2C	phosphatidic acid phosphatase type 2C	1.54
NM_003713	PPAP2B	phosphatidic acid phosphatase type 2B	1.32
NM_018290	PGM2	phosphoglucomutase 2	-1.38
NM_024664	PPCS	phosphopantothenoylcysteine synthetase	-1.39
NM_001159323	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.49
NM_002662	PLD1	phospholipase D1, phosphatidylcholine-specific	-1.72
NM_016518	PIPOX	pipecolic acid oxidase	1.40
NM_000930	PLAT	plasminogen activator, tissue	1.45
NM_000961	PTGIS	prostaglandin I2 (prostacyclin) synthase	1.32
NM_006252	PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	-1.70
NM_024854	PYROXD1	pyridine nucleotide-disulphide oxidoreductase domain 1	-1.42
NM_017712	PGPEP1	pyroglutamyl-peptidase I	1.44
NM_002913	RFC1	replication factor C (activator 1) 1, 145kDa	-1.62
NM_024583	SCRN3	secernin 3	-1.65
NM_001142298	SQSTM1	sequestosome 1	1.32
NM_001143676	SGK1	serum/glucocorticoid regulated kinase 1	-1.62
NM_022733	SMAP2	small ArfGAP2	-1.34
NM_001130012	SLC9A3R2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	1.69
NM_001128205	SULF1	sulfatase 1	-1.37
NM_014465	SULT1B1	sulfotransferase family, cytosolic, 1B, member 1	-1.64

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process continued			
NM_015028	TNIK	TRAF2 and NCK interacting kinase	-1.61
NM_015017	USP33	ubiquitin specific peptidase 33	-1.56
NM_032236	USP48	ubiquitin specific peptidase 48	-1.59
NM_145236	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	1.42
NM_003358	UGCG	UDP-glucose ceramide glucosyltransferase	-1.55
NM_003115	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	-1.57
NM_207309	UAP1L1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1	1.58
NM_018359	UFSP2	UFM1-specific peptidase 2	-1.70
NM_139281	WDR36	WD repeat domain 36	-1.52
Response to Stimulus			
NM_182680	AMELX	amelogenin (amelogenesis imperfecta 1, X-linked)	1.35
NM_002940	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	-1.68
NM_004827	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-1.84
NM_001219	CALU	calumenin	-1.44
NM_004101	F2RL2	coagulation factor II (thrombin) receptor-like 2	-1.56
NM_004417	DUSP1	dual specificity phosphatase 1	-1.43
NM_001955	EDN1	endothelin 1	-1.35
NM_018948	ERRFI1	ERBB receptor feedback inhibitor 1	-1.66
NM_006732	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1.56
NM_014905	GLS	glutaminase	-1.52
NM_002229	JUNB	jun B proto-oncogene	1.77
NM_002276	KRT19	keratin 19	1.51
NM_006469	IVNS1ABP	influenza virus NS1A binding protein	-1.67
NM_000877	IL1R1	interleukin 1 receptor, type I	-1.75
NM_004210	NEURL	neuralized homolog (Drosophila)	1.51
NM_006179	NTF4	neurotrophin 4	1.65
NM_014582	OBP2A	odorant binding protein 2A	1.64
NM_001004450	OR1B1	olfactory receptor, family 1, subfamily B, member 1	1.69
NM_030883	OR2H1	olfactory receptor, family 2, subfamily H, member 1	1.46
NM_001004687	OR2L3	olfactory receptor, family 2, subfamily L, member 3	2.00
NM_001004717	OR4L1	olfactory receptor, family 4, subfamily L, member 1	2.20
NM_001005203	OR8S1	olfactory receptor, family 8, subfamily S, member 1	1.46
NM_001004760	OR51V1	olfactory receptor, family 51, subfamily V, member 1	1.91
NM_021105	PLSCR1	phospholipid scramblase 1	-1.62
NM_001128304	PLSCR4	phospholipid scramblase 4	-1.95
NM_000965	RARB	retinoic acid receptor, beta	-1.55

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control ($p < 0.001$; 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Response to Stimulus continued			
NM_003155	STC1	stanniocalcin 1	1.62
NM_012397	SERPINB13	serpin peptidase inhibitor, clade B (ovalbumin), member 13	1.36
NM_003234	TFRC	transferrin receptor (p90, CD71)	-2.48
Signal Transduction			
NM_005737	ARL4C	ADP-ribosylation factor-like 4C	1.40
NM_014391	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	-1.49
NM_025045	BAIAP2L2	BAI1-associated protein 2-like 2	1.52
NM_000710	BDKRB1	bradykinin receptor B1	-1.97
NM_001014796	DDR2	discoidin domain receptor tyrosine kinase 2	-1.35
NM_015221	DNMBP	dynamin binding protein	-1.40
NM_020996	FGF6	fibroblast growth factor 6	1.47
NM_004864	GDF15	growth differentiation factor 15	1.49
NM_005456	MAPK8IP1	mitogen-activated protein kinase 8 interacting protein 1	1.38
NM_018945	PDE7B	phosphodiesterase 7B	-1.54
NM_006255	PRKCH	protein kinase C, eta	-1.39
NM_006243	PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha isoform	-1.62
NM_001077494	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	1.53
NM_152663	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2	-1.86
NM_001161616	RGL3	ral guanine nucleotide dissociation stimulator-like 3	1.40
NM_016084	RASD1	RAS, dexamethasone-induced 1	1.47
NM_006506	RASA2	RAS p21 protein activator 2	-1.75
NM_005052	RAC3	ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	1.38
NM_001102445	RGS4	regulator of G-protein signalling 4	-1.81
NM_005168	RND3	Rho family GTPase 3	-1.61
NM_004815	ARHGAP29	Rho GTPase activating protein 29	-1.54
NM_001080479	RGNEF	Rho-guanine nucleotide exchange factor	-1.34
NM_007315	STAT1	signal transducer and activator of transcription 1, 91kDa	-1.50
NM_005841	SPRY1	sprouty homolog 1, antagonist of FGF signalling (Drosophila)	-1.56
NM_005842	SPRY2	sprouty homolog 2 (Drosophila)	-1.68
NM_015595	SGEF	Src homology 3 domain-containing guanine nucleotide exchange factor	-1.36
NM_015077	SARM1	sterile alpha and TIR motif containing 1	1.29
NM_012454	TIAM2	T-cell lymphoma invasion and metastasis 2	-1.40
NM_005658	TRAF1	TNF receptor-associated factor 1	1.73
NM_003565	ULK1	unc-51-like kinase 1 (C. elegans)	1.41
NM_007314	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	-1.33

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transcription			
NM_014109	ATAD2	ATPase family, AAA domain containing 2	-1.58
NM_015446	AHCTF1	AT hook containing transcription factor 1	-1.52
NM_014739	BCLAF1	BCL2-associated transcription factor 1	-1.52
NM_018429	BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	-1.46
NM_001142568	BBX	bobby sox homolog (Drosophila)	-1.60
NM_015125	CIC	capicua homolog (Drosophila)	1.37
NM_001127192	CNBP	CCHC-type zinc finger, nucleic acid binding protein	-1.53
NM_014597	DNTTIP2	deoxynucleotidyltransferase, terminal, interacting protein 2	-1.54
NM_006494	ERF	Ets2 repressor factor	1.36
NM_015030	FRYL	FRY-like	-1.65
NM_032575	GLIS2	GLIS family zinc finger 2	1.55
NM_006582	GMEB1	glucocorticoid modulatory element binding protein 1	-1.42
NM_021958	HLX	H2.0-like homeobox	1.52
NM_003071	HLTF	helicase-like transcription factor	-1.64
NM_005474	HDAC5	histone deacetylase 5	1.31
NM_178423	HDAC9	histone deacetylase 9	1.37
NM_014212	HOXC11	homeobox C11	1.44
NM_005354	JUND	jun D proto-oncogene	1.45
NM_003884	KAT2B	K(lysine) acetyltransferase 2B	-1.52
NM_016270	KLF2	Kruppel-like factor 2 (lung)	1.75
NM_004235	KLF4	Kruppel-like factor 4 (gut)	1.84
NM_005583	LYL1	lymphoblastic leukemia derived sequence 1	1.45
NM_002398	MEIS1	Meis homeobox 1	-1.72
NM_003926	MBD3	methyl-CpG binding domain protein 3	1.33
NM_032246	MEX3B	mex-3 homolog B (C. elegans)	1.42
NM_001042539	MAZ	MYC-associated zinc finger protein (purine-binding transcription factor)	1.32
NM_016132	MYEF2	myelin expression factor 2	-1.39
NM_001146312	MYOCD	myocardin	1.40
NM_152995	NFXL1	nuclear transcription factor, X-box binding-like 1	-1.53
NM_018137	PRMT6	protein arginine methyltransferase 6	-1.70
NM_012446	SSBP2	single-stranded DNA binding protein 2	-1.43
NM_003082	SNAPC1	small nuclear RNA activating complex, polypeptide 1, 43kDa	-1.66
NM_013306	SNX15	sorting nexin 15	1.32
NM_005642	TAF7	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	-1.59
NM_003201	TFAM	transcription factor A, mitochondrial	-1.45
NM_016075	VPS36	vacuolar protein sorting 36 homolog (S. cerevisiae)	-1.42
NM_153028	ZNF75A	zinc finger protein 75a	-1.38
NM_001008727	ZNF121	zinc finger protein 121	-1.64
NM_003441	ZNF141	zinc finger protein 141	-1.42

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Gene ID	Gene Symbol	Gene Name	Fold Change
Transcription continued			
NM_001146175	ZNF414	zinc finger protein 414	1.34
NM_181489	ZNF445	zinc finger protein 445	-1.41
NM_001136156	ZNF507	zinc finger protein 507	-1.38
NR_026746	ZNF619	zinc finger protein 619	-1.48
NM_018293	ZNF654	zinc finger protein 654	-1.55
NM_133474	ZNF721	zinc finger protein 721	-1.60
NM_015534	ZZZ3	zinc finger, ZZ-type containing 3	-1.52
Transport			
NM_152565	ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	1.30
NM_002858	ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	-1.67
NM_000722	CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	-1.59
NM_173872	CLCN3	chloride channel 3	-1.49
NM_001830	CLCN4	chloride channel 4	-1.32
NM_000744	CHRNA4	cholinergic receptor, nicotinic, alpha 4	1.50
NM_015018	DOPEY1	dopey family member 1	-1.50
NM_000810	GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	0.69
NM_016072	GOLT1B	golgi transport 1 homolog B (S. cerevisiae)	-1.57
NR_027850	MTX2	metaxin 2	-1.78
NM_004542	NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	1.43
NM_015938	NMD3	NMD3 homolog (S. cerevisiae)	-1.56
NM_032523	OSBPL6	oxysterol binding protein-like 6	-1.36
NM_000466	PEX1	peroxisomal biogenesis factor 1	-1.50
NM_002234	KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5	1.41
NR_024194	TOM1	target of myb1 (chicken)	1.39
NM_012288	TRAM2	translocation associated membrane protein 2	-1.30
NM_079834	SCAMP4	secretory carrier membrane protein 4	0.70
NM_022754	SFXN1	sideroflexin 1	-1.41
NM_152999	STEAP2	six transmembrane epithelial antigen of the prostate 2	-1.68
NM_014191	SCN8A	sodium channel, voltage gated, type VIII, alpha subunit	-1.47
NM_017585	SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6	1.27
NM_145176	SLC2A12	solute carrier family 2 (facilitated glucose transporter), member 12	-1.53
NM_014037	SLC6A16	solute carrier family 6, member 16	1.38
NM_177550	SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	1.45
NM_021194	SLC30A1	solute carrier family 30 (zinc transporter), member 1	-1.37
NM_017964	SLC30A6	solute carrier family 30 (zinc transporter), member 6	-1.54

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transport continued			
NM_181785	SLC46A3	solute carrier family 46, member 3	-1.39
NM_003715	USO1	USO1 homolog, vesicle docking protein (yeast)	-1.50
NM_021083	XK	X-linked Kx blood group (McLeod syndrome)	1.54
Miscellaneous / Other			
NM_152326	ANKRD9	ankyrin repeat domain 9	1.39
NM_025190	ANKRD36B	ankyrin repeat domain 36B	-1.48
NM_020337	ANKRD50	ankyrin repeat domain 50	-1.40
NM_025190	ANKRD36B	ankyrin repeat domain 36B	-1.51
NM_025190	ANKRD36B	ankyrin repeat domain 36B	-1.56
NM_015379	BRI3	brain protein I3	1.33
NM_014603	CDR2L	cerebellar degeneration-related protein 2-like	1.44
NM_018372	C1orf103	chromosome 1 open reading frame 103	-1.84
NM_178550	C1orf110	chromosome 1 open reading frame 110	-1.64
NM_198545	C1orf187	chromosome 1 open reading frame 187	1.54
BC034598	C1orf212	chromosome 1 open reading frame 212	-1.50
NM_178496	C3orf59	chromosome 3 open reading frame 59	-1.84
NM_001112736	C3orf63	chromosome 3 open reading frame 63	-1.64
NM_018352	C4orf43	chromosome 4 open reading frame 43	-1.84
NM_032623	C4orf49	chromosome 4 open reading frame 49	-1.56
NM_001085411	C5orf33	chromosome 5 open reading frame 33	-1.70
AK290939	C6orf130	chromosome 6 open reading frame 130	-1.60
NM_198468	C6orf167	chromosome 6 open reading frame 167	-1.68
NM_017586	C9orf7	chromosome 9 open reading frame 7	1.39
NM_153256	C10orf47	chromosome 10 open reading frame 47	1.47
NM_152261	C12orf23	chromosome 12 open reading frame 23	-1.43
NM_013300	C12orf24	chromosome 12 open reading frame 24	-1.43
NM_032366	C16orf13	chromosome 16 open reading frame 13	1.33
NM_174983	C19orf28	chromosome 19 open reading frame 28	1.35
NM_033520	C19orf33	chromosome 19 open reading frame 33	1.43
NM_080608	C20orf165	chromosome 20 open reading frame 165	1.40
NM_144492	CLDN14	claudin 14	1.39
NM_003950	F2RL3	coagulation factor II (thrombin) receptor-like 3	1.72
NM_206833	CTXN1	cortexin 1	1.38
NM_145056	DACT3	dapper, antagonist of beta-catenin, homolog 3 (Xenopus laevis)	1.51
NM_006773	DDX18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	-1.71
NM_001007533	DYSFIP1	dysferlin interacting protein 1	1.73
NM_015252	EHBP1	EH domain binding protein 1	-1.50
NM_022140	EPB41L4A	erythrocyte membrane protein band 4.1 like 4A	-1.54
NM_001035254	FAM102A	family with sequence similarity 102, member A	1.48
NM_198947	FAM111B	family with sequence similarity 111, member B	-1.61
NM_001130025	FAM115C	family with sequence similarity 115, member C	-1.32
NM_001105079	FBRS	fibrosin	1.43
BC151150	FLJ25996	FLJ25996 protein	1.51
NM_001001685	FLJ45079	FLJ45079 protein	1.65
NM_020116	FSTL5	follistatin-like 5	-1.83
NM_014170	GTPBP8	GTP-binding protein 8 (putative)	-1.52

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Miscellaneous / Other continued			
NM_015473	HEATR5A	HEAT repeat containing 5A	-2.12
NR_026929	DKFZp434L192	hypothetical protein DKFZp434L192	1.89
ENST00000370520	RP11-93B14.6	hypothetical protein FLJ32154	1.70
ENST00000423909	FLJ33996	hypothetical protein FLJ33996	-1.44
NM_178511	LOC255783	hypothetical protein LOC255783	1.52
ENST00000320322	LOC554207	hypothetical LOC554207	1.49
NM_001014380	KATNAL1	katanin p60 subunit A-like 1	-1.46
NM_020803	KLHL8	kelch-like 8 (Drosophila)	-1.63
NM_213656	KRT39	keratin 39	1.36
NM_001004325	KRTAP5-2	keratin associated protein 5-2	1.53
NM_014431	KIAA1274	KIAA1274	1.69
NM_001142749	KIAA1324L	KIAA1324-like	-1.57
BC004406	KIAA1539	KIAA1539	1.47
NM_004907	IER2	immediate early response 2	1.44
NM_022347	IFRG15	interferon responsive gene 15	-1.74
NM_009587	LGALS9	lectin, galactoside-binding, soluble, 9	1.49
NM_001040078	LGALS9C	lectin, galactoside-binding, soluble, 9C	1.54
NM_001134476	LRRC8B	leucine rich repeat containing 8 family, member B	-1.66
NM_198565	LRRC33	leucine rich repeat containing 33	1.54
NM_001113546	LIMA1	LIM domain and actin binding 1	-1.57
NM_006726	LRBA	LPS-responsive vesicle trafficking, beach and anchor containing	-1.55
NM_001017967	MARVELD3	MARVEL domain containing 3	1.57
NM_012216	MID2	midline 2	-1.35
NM_018728	MYO5C	myosin VC	-1.58
NM_018177	N4BP2	NEDD4 binding protein 2	-1.58
AY176665	NSAP11	nervous system abundant protein 11	-1.64
NM_183372	RP11-94I2.2	neuroblastoma breakpoint family, member 11-like	-1.34
NM_144573	NEXN	nexilin (F actin binding protein)	-2.09
NM_198514	NHLRC2	NHL repeat containing 2	-1.48
NM_173198	NR4A3	nuclear receptor subfamily 4, group A, member 3	1.33
NM_016167	NOL7	nucleolar protein 7, 27kDa	-1.51
NM_178507	OAF	OAF homolog (Drosophila)	1.44
NM_024576	OGFRL1	opioid growth factor receptor-like 1	-1.57
NM_016023	OTUD6B	OTU domain containing 6B	-1.50
NM_017734	PALMD	palmdelphin	-1.53
NR_002181	PPY2	pancreatic polypeptide 2	1.52
NM_006457	PDLIM5	PDZ and LIM domain 5	-1.38
NM_020651	PELI1	pellino homolog 1 (Drosophila)	-1.72
NM_032728	PPAPDC3	phosphatidic acid phosphatase type 2 domain containing 3	1.41
NM_001134438	PHLDB2	pleckstrin homology-like domain, family B, member 2	-1.51
NM_198859	PRICKLE2	prickle homolog 2 (Drosophila)	-1.42

Table S3.3: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to the control (*p* < 0.001*;* 511 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Miscellaneous / Other continued			
NM_017607	PPP1R12C	protein phosphatase 1, regulatory (inhibitor) subunit 12C	1.38
NM_020700	PPM1H	protein phosphatase 1H (PP2C domain containing)	1.56
NM_015150	RFTN1	raftlin, lipid raft linker 1	-1.50
NM_001003793	RBMS3	RNA binding motif, single stranded interacting protein	-1.56
NM_175636	RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	-1.55
NM_002962	S100A5	S100 calcium binding protein A5	1.86
NM_014016	SACM1L	SAC1 suppressor of actin mutations 1-like (yeast)	-1.50
NM_004657	SDPR	serum deprivation response (phosphatidylserine binding protein)	-1.55
NM_174919	SH3D20	SH3 domain containing 20	1.41
NM_138402	SP140L	SP140 nuclear body protein-like	-1.50
NM_181727	SPATA12	spermatogenesis associated 12	1.56
NM_152703	SAMD9L	sterile alpha motif domain containing 9-like	-1.67
NM_139215	TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	-1.46
NM_005723	TSPAN5	tetraspanin 5	-1.59
NM_024753	TTC21B	tetratricopeptide repeat domain 21B	-1.68
NM_144721	THAP6	THAP domain containing 6	-1.75
NM_172000	TEDDM1	transmembrane epididymal protein 1	1.37
NM_032824	TMEM87B	transmembrane protein 87B	-1.52
NM_022484	TMEM168	transmembrane protein 168	-1.49
NM_018241	TMEM184C	transmembrane protein 184C	-1.50
NM_003449	TRIM26	tripartite motif-containing 26	-1.40
NM_033452	TRIM47	tripartite motif-containing 47	1.45
NM_145034	TOR1AIP2	torsin A interacting protein 2	-1.58
NM_207381	TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3	1.47
NM_021202	TP53INP2	tumor protein p53 inducible nuclear protein 2	1.38
NM_175748	UBR7	ubiquitin protein ligase E3 component n-recognin 7 (putative)	-1.62
NM_014991	WDFY3	WD repeat and FYVE domain containing 3	-1.53
NM_007086	WDHD1	WD repeat and HMG-box DNA binding protein 1	-1.48
NM_022828	YTHDC2	YTH domain containing 2	-1.70
NM_198581	ZC3H6	zinc finger CCCH-type containing 6	-1.57
AK056855	---		1.69
BC033184			1.46
NA			-1.35
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Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to VEGF (10 ng/ml) (p < 0.001; 235 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	-3.09
NM_001202	BMP4	bone morphogenetic protein 4	-2.00
NM_001554	CYR61	cysteine-rich, angiogenic inducer, 61	-1.71
NM_002133	HMOX1	heme oxygenase (decycling) 1	1.93
NM_005924	MEOX2	mesenchyme homeobox 2	-1.75
NM_194358	RNF41	ring finger protein 41	-1.34
NM_000459	TEK	TEK tyrosine kinase, endothelial	1.47
Apoptosis			
NM_019058	DDIT4	DNA-damage-inducible transcript 4	2.02
NM_182757	RNF144B	ring finger protein 144B	-1.51
NM_005460	SNCAIP	synuclein, alpha interacting protein	-2.21
NM_198057	TSC22D3	TSC22 domain family, member 3	1.54
NM_003810	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1.84
NM_005092	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	-3.03
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	1.44
NM_033285	TP53INP1	tumor protein p53 inducible nuclear protein 1	1.34
NM_018003	UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	-1.39
Binding / Cell Adhesion			
NM_001143668	AMIGO2	adhesion molecule with Ig-like domain 2	-1.43
NM_078481	CD97	CD97 molecule	1.38
NM_001130861	CLDN5	claudin 5	1.66
NM_001338	CXADR	coxsackie virus and adenovirus receptor	-1.69
NM_004370	COL12A1	collagen, type XII, alpha 1	-1.64
NM_001776	ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1	-1.59
NM_016946	F11R	F11 receptor	1.33
NM_032876	JUB	jub, ajuba homolog (Xenopus laevis)	-1.41
NM_022143	LRRC4	leucine rich repeat containing 4	1.62
NM_001143995	LPXN	leupaxin	1.44
NM_032420	PCDH1	protocadherin 1	1.49
NM_001040429	PCDH17	protocadherin 17	-1.40
Cell Communication / Signalling			
NM_017413	APLN	apelin	-1.66
NM_001233	CAV2	caveolin 2	-1.45
NM_014421	DKK2	dickkopf homolog 2 (Xenopus laevis)	-1.48
NM_004438	EPHA4	EPH receptor A4	1.54
NM_004444	EPHB4	EPH receptor B4	1.41
NM_000824	GLRB	glycine receptor, beta	-1.64
NM_207123	GAB1	GRB2-associated binding protein 1	-1.49
NM_002226	JAG2	jagged 2	1.44
NM_012302	LPHN2	latrophilin 2	-2.24
NM_002755	MAP2K1	mitogen-activated protein kinase kinase 1	-1.39
NM_021724	NR1D1	nuclear receptor subfamily 1, group D, member 1	1.53

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Cell Communication / Signalling continued			
NM_003629	PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	1.59
NM_020182	PMEPA1	prostate transmembrane protein, androgen induced 1	1.43
NM_015191	SIK2	salt-inducible kinase 2	1.58
NM_005065	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	-1.63
Cell Cycle / Cell Proliferation			
NM_001130914	BTG3	BTG family, member 3	-1.35
NM_015147	CEP68	centrosomal protein 68kDa	1.64
NM_001254	CDC6	cell division cycle 6 homolog (S. cerevisiae)	-2.39
NM_000757	CSF1	colony stimulating factor 1 (macrophage)	1.31
NM_057749	CCNE2	cyclin E2	-1.95
NM_015097	CLASP2	cytoplasmic linker associated protein 2	-1.42
NM_001946	DUSP6	dual specificity phosphatase 6	-2.08
NM_004429	EFNB1	ephrin-B1	1.59
NM_001001557	GDF6	growth differentiation factor 6	-1.83
NM_003483	HMGA2	high mobility group AT-hook 2	-1.44
NM_002192	INHBA	inhibin, beta A	-1.44
NM_024039	MIS12	MIS12, MIND kinetochore complex component, homolog (S. pombe)	-1.63
NM_018116	MSTO1	misato homolog 1 (Drosophila)	-1.52
NM_153618	SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	-3.46
NM_002688	SEPT5	septin 5	-1.65
NM_014909	VASH1	vasohibin 1	-1.62
Development			
NM_006079	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	-1.65
NM_012242	DKK1	dickkopf homolog 1 (Xenopus laevis)	-1.89
NM_006350	FST	follistatin	-1.86
NM_002060	GJA4	gap junction protein, alpha 4, 37kDa	2.93
NM_001040167	LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	2.04
NM_013960	NRG1	neuregulin 1	-1.40
NM_182964	NAV2	neuron navigator 2	1.71
NM_005450	NOG	noggin	-1.78
NM_001135599	TGFB2	transforming growth factor, beta 2	-1.44
NM_020163	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	1.50
NM_001649	SHROOM2	shroom family member 2	1.38
NM_005239	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.36
DNA Binding / Replication / Repair / Transcription			
NM_194071	CREB3L2	cAMP responsive element binding protein 3-like 2	-1.37
NM_004956	ETV1	ets variant 1	-2.40
NM_001986	ETV4	ets variant 4	-1.66
NM_025081	KIAA1305	KIAA1305	1.39

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
DNA Binding / Replication / Repair / Transcription continued			
NM_021038	MBNL1	muscleblind-like (Drosophila)	-1.35
NM_002526	NT5E	5'-nucleotidase, ecto (CD73)	-1.65
NM_001135215	ZNF323	zinc finger protein 323	1.47
NM_153695	ZNF367	zinc finger protein 367	-1.34
NM_175872	ZNF792	zinc finger protein 792	1.40
Inflammatory Response			
NM_000584	IL8	interleukin 8	-1.77
NM_000450	SELE	selectin E	-2.05
Immune Response			
NM_014143	CD274	CD274 molecule	-1.83
NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	1.43
NM_005080	XBP1	X-box binding protein 1	-1.66
Metabolic / Biosynthetic Process			
NM_005099	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	1.70
NM_022060	ABHD4	abhydrolase domain containing 4	-1.37
NM_174858	AK5	adenylate kinase 5	-2.24
NR_027833	APOL3	apolipoprotein L, 3	1.55
NM_030643	APOL4	apolipoprotein L, 4	1.50
NM_030817	APOLD1	apolipoprotein L domain containing 1	1.54
NM_024590	ARSJ	arylsulfatase family, member J	-1.51
NM_018371	CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	-1.58
NM_032385	C5orf4	chromosome 5 open reading frame 4	1.42
NM_012328	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	-2.09
NM_016306	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	-1.78
NM_006260	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	-1.86
NM_019891	ERO1LB	ERO1-like beta (S. cerevisiae)	-2.51
NM_014674	EDEM1	ER degradation enhancer, mannosidase alpha-like 1	-1.59
NM_004836	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	-1.67
NM_004102	FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	1.43
NM_007076	FICD	FIC domain containing	-1.71
NM_002056	GFPT1	glutamine-fructose-6-phosphate transaminase 1	-1.71
NM_002153	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	-1.86
NM_018192	LEPREL1	leprecan-like 1	-1.69
NR_027405	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	-1.99
NM_023112	OTUB2	OTU domain, ubiquitin aldehyde binding 2	1.47
NM_015288	PHF15	PHD finger protein 15	1.47
NM_001159323	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.55
NM_000303	PMM2	phosphomannomutase 2	-1.42
NM_058179	PSAT1	phosphoserine aminotransferase 1	-1.66

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process continued			
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.50
NM_004414	RCAN1	regulator of calcineurin 1	-2.41
NM_000965	RARB	retinoic acid receptor, beta	-1.86
NM_014746	RNF144A	ring finger protein 144A	1.65
NM_001143676	SGK1	serum/glucocorticoid regulated kinase 1	-1.61
NM_001142298	SQSTM1	sequestosome 1	1.49
NM_152996	ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	-1.56
NM_006588	SULT1C4	sulfotransferase family, cytosolic, 1C, member 4	-1.96
NM_032431	SYVN1	synovial apoptosis inhibitor 1, synoviolin	-1.47
NM_017414	USP18	ubiquitin specific peptidase 18	-1.49
NM_003358	UGCG	UDP-glucose ceramide glucosyltransferase	-1.79
Response to Stimulus			
NM_000014	A2M	alpha-2-macroglobulin	-1.79
NM_005409	CXCL11	chemokine (C-X-C motif) ligand 11	3.16
NM_144601	CMTM3	CKLF-like MARVEL transmembrane domain containing 3	1.30
NM_138410	CMTM7	CKLF-like MARVEL transmembrane domain containing 7	1.43
NM_001993	F3	coagulation factor III (thromboplastin, tissue factor)	-1.65
NM_004417	DUSP1	dual specificity phosphatase 1	-1.60
NM_005347	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.62
NM_014685	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-1.85
NM_006389	HYOU1	hypoxia up-regulated 1	-1.46
NM_002229	JUNB	jun B proto-oncogene	1.52
NM_006010	MANF	mesencephalic astrocyte-derived neurotrophic factor	-1.54
NM_001130487	NDRG4	NDRG family member 4	1.55
NM_000603	NOS3	nitric oxide synthase 3 (endothelial cell)	1.42
NM_001122	PLIN2	perilipin 2	1.41
NM_001128304	PLSCR4	phospholipid scramblase 4	-1.69
NM_003155	STC1	stanniocalcin 1	-2.70
NM_000361	THBD	thrombomodulin	-1.85
NM_006528	TFPI2	tissue factor pathway inhibitor 2	-1.99
NR_024168	TLR4	toll-like receptor 4	-1.47
NM_003234	TFRC	transferrin receptor (p90, CD71)	-1.97
NM_001078	VCAM1	vascular cell adhesion molecule 1	-2.08
Signal Transduction			
NM_014391	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	-1.62
NM_173515	CNKSR3	CNKSR family member 3	-2.32
NM_001014796	DDR2	discoidin domain receptor tyrosine kinase 2	-1.61
NM_015221	DNMBP	dynamin binding protein	-1.38
NM_014800	ELMO1	engulfment and cell motility 1	-1.45
NM_138445	GPR146	G protein-coupled receptor 146	1.67

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Signal Transduction continued			
NM_001945	HBEGF	heparin-binding EGF-like growth factor	-1.95
NM_003866	INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa	-1.41
NM_015368	PANX1	pannexin 1	1.36
NM_006255	PRKCH	protein kinase C, eta	-1.42
NM_015149	RGL1	ral guanine nucleotide dissociation stimulator-like 1	-1.61
NM_007211	RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	-1.69
NM_016084	RASD1	RAS, dexamethasone-induced 1	-1.38
NM_001102445	RGS4	regulator of G-protein signalling 4	-1.62
NM_199282	ARHGAP27	Rho GTPase activating protein 27	1.47
NM_001128615	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	1.72
NM_181784	SPRED2	sprouty-related, EVH1 domain containing 2	-1.40
NM_005658	TRAF1	TNF receptor-associated factor 1	1.67
NM_005078	TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	1.36
NM_005841	SPRY1	sprouty homolog 1, antagonist of FGF signalling (Drosophila)	-2.35
NM_005842	SPRY2	sprouty homolog 2 (Drosophila)	-1.79
Transcription			
NM_024997	ATF7IP2	activating transcription factor 7 interacting protein 2	-1.61
NM_014109	ATAD2	ATPase family, AAA domain containing 2	-1.76
NM_001200	BMP2	bone morphogenetic protein 2	-2.17
NM_006763	BTG2	BTG family, member 2	1.51
NM_004454	ETV5	ets variant 5	-2.18
NM_080759	DACH1	dachshund homolog 1 (Drosophila)	-1.64
NM_016270	KLF2	Kruppel-like factor 2 (lung)	1.50
NM_004235	KLF4	Kruppel-like factor 4 (gut)	1.54
NM_030915	LBH	limb bud and heart development homolog (mouse)	-1.66
NM_014368	LHX6	LIM homeobox 6	1.52
NM_032246	MEX3B	mex-3 homolog B (C. elegans)	1.35
NM_205860	NR5A2	nuclear receptor subfamily 5, group A, member 2	-1.70
NM_001080951	PLAGL1	pleiomorphic adenoma gene-like 1	-1.43
NM_001198	PRDM1	PR domain containing 1, with ZNF domain	-2.74
NM_005686	SOX13	SRY (sex determining region Y)-box 13	1.66
Transport			
NM_022735	ACBD3	acyl-Coenzyme A binding domain containing 3	1.34
NM_170665	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-1.59
NM_001001396	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	1.38
NM_207628	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.49
NM_198098	AQP1	aquaporin 1 (Colton blood group)	2.06
NM_022113	KIF13A	kinesin family member 13A	-1.42
NM_001025579	NDEL1	nudE nuclear distribution gene E homolog (A. nidulans)-like 1	-1.38

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transport continued			
NM_138444	KCTD12	potassium channel tetramerisation domain containing 12	1.43
NM_006363	SEC23B	Sec23 homolog B (S. cerevisiae)	-1.60
NM_014822	SEC24D	SEC24 family, member D (S. cerevisiae)	-1.65
NM_003038	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	-1.45
NM_006931	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	1.45
NM_003043	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	1.90
NM_003045	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	-1.70
NM_003982	SLC7A7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	-1.46
NM_004733	SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1	-1.57
NM_001001479	SLC35E4	solute carrier family 35, member E4	1.47
NM_001128431	SLC39A14	solute carrier family 39 (zinc transporter), member 14	-2.35
NM_005630	SLCO2A1	solute carrier organic anion transporter family, member 2A1	1.50
Miscellaneous / Other			
NM_014600	EHD3	EH-domain containing 3	-3.54
NM_001839	CNN3	calponin 3, acidic	-1.41
NM_001802	CDR2	cerebellar degeneration-related protein 2, 62kDa	-1.43
NM_198545	C1orf187	chromosome 1 open reading frame 187	1.63
NM_020130	C8orf4	chromosome 8 open reading frame 4	1.73
NM_017787	C10orf26	chromosome 10 open reading frame 26	1.39
NM_032933	C18orf45	chromosome 18 open reading frame 45	-1.55
NM_020939	CPNE5	copine V	1.43
NM_001135101	CRELD2	cysteine-rich with EGF-like domains 2	1.42
NM_178470	DCAF12L1	DDB1 and CUL4 associated factor 12-like 1	-1.45
NM_014787	DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6	-1.63
AF151906	FAM18B	family with sequence similarity 18, member B	-1.54
NM_153711	FAM26E	family with sequence similarity 26, member E	1.61
NM_001130025	FAM115C	family with sequence similarity 115, member C	-1.78
NM_173511	FAM117B	family with sequence similarity 117, member B	1.33
NM_024785	FAM124B	family with sequence similarity 124B	1.61
NM_006948	HSPA13	heat shock protein 70kDa family, member 13	-1.46
NM_009587	LGALS9	lectin, galactoside-binding, soluble, 9	1.61
NM_001040078	LGALS9C	lectin, galactoside-binding, soluble, 9C	1.49
NM_001134476	LRRC8B	leucine rich repeat containing 8 family, member B	-2.37
NM_015116	LRCH1	leucine-rich repeats and calponin homology (CH) domain containing 1	-1.67
NM_017762	MTMR10	myotubularin related protein 10	-1.64
AY176665	NSAP11	nervous system abundant protein 11	-1.55
NM_144573	NEXN	nexilin (F actin binding protein)	-2.00

Table S3.4: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4 compared to 10 ng/ml VEGF (*p* < 0.001*;* 235 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Miscellaneous / Other continued			
NM_001031701	NT5DC3	5'-nucleotidase domain containing 3	-1.43
NM_021255	PELI2	pellino homolog 2 (Drosophila)	1.44
NM_001100164	PHACTR2	phosphatase and actin regulator 2	-1.64
NM_015993	PLLP	plasma membrane proteolipid (plasmolipin)	2.17
NM_001104587	SLFN11	schlafen family member 11	-2.06
NM_004657	SDPR	serum deprivation response (phosphatidylserine binding protein)	-1.33
NR_002816	THSD1P	thrombospondin, type I, domain containing 1 pseudogene	1.48
NM_175861	TMTC1	transmembrane and tetratricopeptide repeat containing 1	-1.52
NM_013390	TMEM2	transmembrane protein 2	-1.68
NM_031442	TMEM47	transmembrane protein 47	-1.35
NM_006134	TMEM50B	transmembrane protein 50B	-2.29
NM_080660	ZC3HAV1L	zinc finger CCCH-type, antiviral 1-like	-1.35
NM_194250	ZNF804A	zinc finger protein 804A	-1.40

Table S3.5: List of differentially expressed genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) ($p < 0.001$; 360 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_000014	A2M	alpha-2-macroglobulin	-1.79
NM_001147	ANGPT2	angiopoietin 2	-2.93
NM_001202	BMP4	bone morphogenetic protein 4	-1.89
NM_001554	CYR61	cysteine-rich, angiogenic inducer, 61	-1.71
NM_002133	HMOX1	heme oxygenase (decycling) 1	1.88
NM_002632	PGF	placental growth factor	1.42
NM_025208	PDGFD	platelet derived growth factor D	-1.47
NM_005924	MEOX2	mesenchyme homeobox 2	-1.64
NM_194358	RNF41	ring finger protein 41	-1.40
NM_006291	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.38
Apoptosis			
NM_032977	CASP10	caspase 10, apoptosis-related cysteine peptidase	-1.60
NM_019058	DDIT4	DNA-damage-inducible transcript 4	1.82
NM_016315	GULP1	GULP, engulfment adaptor PTB domain containing 1	-1.77
NM_003024	ITSN1	intersectin 1 (SH3 domain protein)	-1.34
NM_002583	PAWR	PRKC, apoptosis, WT1, regulator	-1.41
NM_182757	RNF144B	ring finger protein 144B	-1.44
NM_005460	SNCAIP	synuclein, alpha interacting protein	-2.16
NM_003810	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1.64
NM_005092	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	-3.43
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	1.52
NM_033285	TP53INP1	tumor protein p53 inducible nuclear protein 1	1.33
NM_018003	UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	-1.53
Binding / Cell Adhesion			
NM_001712	CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	1.36
NM_078481	CD97	CD97 molecule	1.38
NM_001130861	CLDN5	claudin 5	1.82
NM_004370	COL12A1	collagen, type XII, alpha 1	-1.83
NM_001338	CXADR	coxsackie virus and adenovirus receptor	-1.80
NM_001776	ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1	-1.74
NM_000210	ITGA6	integrin, alpha 6	-1.52
NM_032876	JUB	jub, ajuba homolog (Xenopus laevis)	-1.38
NM_022143	LRRC4	leucine rich repeat containing 4	1.60
NM_032420	PCDH1	protocadherin 1	1.54
NM_014000	VCL	vinculin	-1.37
Cell Communication / Signalling			
NM_001233	CAV2	caveolin 2	-1.50
NM_014421	DKK2	dickkopf homolog 2 (Xenopus laevis)	-1.44
NM_004438	EPHA4	EPH receptor A4	1.47
NM_004444	EPHB4	EPH receptor B4	1.39
NM_000824	GLRB	glycine receptor, beta	-1.66
NM_004838	HOMER3	homer homolog 3 (Drosophila)	1.32
NM_002226	JAG2	jagged 2	1.39
NM_012302	LPHN2	latrophilin 2	-2.40

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Gene ID	Gene Symbol	Gene Name	Fold Change
Cell Communication / Signalling continued			
NM_002755	MAP2K1	mitogen-activated protein kinase kinase 1	-1.38
NM_024717	MCTP1	multiple C2 domains, transmembrane 1	-1.48
NM_004687	MTMR4	myotubularin related protein 4	-1.37
NM_016231	NLK	nemo-like kinase	-1.35
NM_014840	NUAK1	NUAK family, SNF1-like kinase, 1	-1.42
NM_021724	NR1D1	nuclear receptor subfamily 1, group D, member 1	1.59
NM_002742	PRKD1	protein kinase D1	-1.41
NM_181805	PKIG	protein kinase (cAMP-dependent, catalytic) inhibitor gamma	1.54
NM_002834	PTPN11	protein tyrosine phosphatase, non-receptor type 11	-1.45
NM_177968	PPM1B	protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform	-1.40
NM_015191	SIK2	salt-inducible kinase 2	1.46
Cell Cycle / Cell Proliferation			
NM_001130914	BTG3	BTG family, member 3	-1.35
NM_003672	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	-1.61
NM_001254	CDC6	cell division cycle 6 homolog (S. cerevisiae)	-2.21
NM_021873	CDC25B	cell division cycle 25 homolog B (S. pombe)	1.32
NM_015147	CEP68	centrosomal protein 68kDa	1.62
NM_057749	CCNE2	cyclin E2	-2.10
NM_015097	CLASP2	cytoplasmic linker associated protein 2	-1.65
NM_001946	DUSP6	dual specificity phosphatase 6	-2.05
NM_007036	ESM1	endothelial cell-specific molecule 1	-1.63
NM_004429	EFNB1	ephrin-B1	1.51
NM_207123	GAB1	GRB2-associated binding protein 1	-1.65
NM_002192	INHBA	inhibin, beta A	-1.55
NM_002357	MXD1	MAX dimerization protein 1	-1.58
NM_004991	MECOM	MDS1 and EVI1 complex locus	-1.33
NM_024039	MIS12	MIS12, MIND kinetochore complex component, homolog (S. pombe)	-2.03
NM_018116	MSTO1	misato homolog 1 (Drosophila)	-1.57
NM_005065	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	-1.81
NM_153618	SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	-4.23
NM_002688	SEPT5	septin 5	-1.55
NM_001048201	UHRF1	ubiquitin-like with PHD and ring finger domains 1	-1.49
NM_014909	VASH1	vasohibin 1	-1.57
NM_006887	ZFP36L2	zinc finger protein 36, C3H type-like 2	1.36
Cell Migration			
NM_006505	PVR	poliovirus receptor	-1.32
Development			
NM_006079	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	-1.54
NM_012242	DKK1	dickkopf homolog 1 (Xenopus laevis)	-1.80
NM_004425	ECM1	extracellular matrix protein 1	1.42
NM_014888	FAM3C	family with sequence similarity 3, member C	-1.43

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Gene ID	Gene Symbol	Gene Name	Fold Change
Development continued			
NM_006350	FST	follistatin	-1.87
NM_002060	GJA4	gap junction protein, alpha 4, 37kDa	3.18
NM_001040167	LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	1.94
NM_013960	NRG1	neuregulin 1	-1.35
NM_182964	NAV2	neuron navigator 2	1.51
NM_005450	NOG	noggin	-1.72
NM_000297	PKD2	polycystic kidney disease 2 (autosomal dominant)	-1.55
NM_152754	SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	-1.58
NM_020163	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	1.48
NM_001649	SHROOM2	shroom family member 2	1.39
NM_005668	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	-1.48
NM_001135599	TGFB2	transforming growth factor, beta 2	-1.57
DNA Binding / Replication / Repair / Transcription			
NM_194071	CREB3L2	cAMP responsive element binding protein 3-like 2	-1.34
NM_016448	DTL	denticleless homolog (Drosophila)	-1.56
NM_004956	ETV1	ets variant 1	-2.46
NM_001986	ETV4	ets variant 4	-1.55
NM_025081	KIAA1305	KIAA1305	1.41
NM_018078	LARP1B	La ribonucleoprotein domain family, member 1B	-1.62
NM_014793	LCMT2	leucine carboxyl methyltransferase 2	-1.38
NM_021038	MBNL1	muscleblind-like (Drosophila)	-1.42
NM_144778	MBNL2	muscleblind-like 2 (Drosophila)	-1.30
NM_002526	NT5E	5'-nucleotidase, ecto (CD73)	-1.72
NM_017793	RPP25	ribonuclease P/MRP 25kDa subunit	1.49
NM_001039697	SNAPC3	small nuclear RNA activating complex, polypeptide 3, 50kDa	-1.41
NM_007114	TMF1	TATA element modulatory factor 1	-1.72
NM_003598	TEAD2	TEA domain family member 2	1.39
NR_027302	XPA	xeroderma pigmentosum, complementation group A	-1.54
NM_004926	ZFP36L1	zinc finger protein 36, C3H type-like 1	1.33
NM_001135215	ZNF323	zinc finger protein 323	1.40
NM_032819	ZNF341	zinc finger protein 341	1.36
NM_152320	ZNF641	zinc finger protein 641	-1.38
Inflammatory Response			
NM_000575	IL1A	interleukin 1, alpha	-1.59
NM_000584	IL8	interleukin 8	-1.81
NM_002852	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	-1.50
NM_000450	SELE	selectin E	-2.22
Immune Response			
NM_014143	CD274	CD274 molecule	-2.07
NM_001114752	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	-1.45

Table S3.5: List of differentially regulated genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 360 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Immune Response continued			
NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	1.43
NM_003326	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4	-1.54
NM_005080	XBP1	X-box binding protein 1	-1.50
Metabolic / Biosynthetic Process			
NM_022060	ABHD4	abhydrolase domain containing 4	-1.36
NM_005099	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	1.62
NM_014272	ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	1.34
NM_182920	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	-1.38
NM_174858	AK5	adenylate kinase 5	-2.53
NM_002860	ALDH18A1	aldehyde dehydrogenase 18 family, member A1	-1.44
NR_027833	APOL3	apolipoprotein L, 3	1.52
NM_030643	APOL4	apolipoprotein L, 4	1.49
NM_030817	APOLD1	apolipoprotein L domain containing 1	1.60
NM_024590	ARSJ	arylsulfatase family, member J	-1.64
NM_005603	ATP8B1	ATPase, class I, type 8B, member 1	-1.41
NM_014616	ATP11B	ATPase, class VI, type 11B	-1.68
NM_003654	CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	1.37
NM_018371	CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	-1.55
NM_032385	C5orf4	chromosome 5 open reading frame 4	1.41
NM_000774	CYP2F1	cytochrome P450, family 2, subfamily F, polypeptide 1	1.78
NM_013352	DSE	dermatan sulfate epimerase	-1.43
NM_003647	DGKE	diacylglycerol kinase, epsilon 64kDa	-1.61
NM_007034	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	-1.49
NM_012328	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	-2.27
NM_016306	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	-1.74
NM_006260	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	-2.17
NM_024090	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	-1.79
NM_014674	EDEM1	ER degradation enhancer, mannosidase alpha-like 1	-1.63
NM_019891	ERO1LB	ERO1-like beta (S. cerevisiae)	-2.81
NM_004836	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	-1.87
NM_001442	FABP4	fatty acid binding protein 4, adipocyte	-1.82
NM_017946	FKBP14	FK506 binding protein 14, 22 kDa	-1.84
NM_007076	FICD	FIC domain containing	-1.78
NM_002056	GFPT1	glutamine-fructose-6-phosphate transaminase 1	-1.87
NM_002153	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	1.66
NM_005530	IDH3A	isocitrate dehydrogenase 3 (NAD+) alpha	-1.41
NM_018192	LEPREL1	leprecan-like 1	-1.74
NM_032121	MAGT1	magnesium transporter 1	-1.45
NM_014730	MLEC	malectin	-1.32
NR_027405	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	-2.02
NM_000240	MAOA	monoamine oxidase A	-1.40

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Gene ID	Gene Symbol	Gene Name	Fold Change
Metabolic / Biosynthetic Process continued			
NM_018946	NANS	N-acetylneuraminic acid synthase	-1.52
NM_001146276	NCEH1	neutral cholesterol ester hydrolase 1	-1.41
NM_012229	NT5C2	5'-nucleotidase, cytosolic II	-1.41
NM_023112	OTUB2	OTU domain, ubiquitin aldehyde binding 2	1.50
NM_018233	OGFOD1	2-oxoglutarate and iron-dependent oxygenase domain containing 1	-1.38
NM_015288	PHF15	PHD finger protein 15	1.45
NM_003712	PPAP2C	phosphatidic acid phosphatase type 2C	1.49
NM_000303	PMM2	phosphomannomutase 2	-1.51
NM_058179	PSAT1	phosphoserine aminotransferase 1	-1.72
NM_014819	PJA2	praja ring finger 2	-1.58
NM_004414	RCAN1	regulator of calcineurin 1	-2.39
NM_014746	RNF144A	ring finger protein 144A	1.73
NM_001143676	SGK1	serum/glucocorticoid regulated kinase 1	-1.61
NM_001142298	SQSTM1	sequestosome 1	1.46
NM_001136258	SGMS2	sphingomyelin synthase 2	-1.69
NM_152996	ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	-1.59
NM_015589	SAMD4A	sterile alpha motif domain containing 4A	-1.37
NM_001128205	SULF1	sulfatase 1	-1.45
NM_006588	SULT1C4	sulfotransferase family, cytosolic, 1C, member 4	-2.31
NM_032431	SYVN1	synovial apoptosis inhibitor 1, synoviolin	-1.40
NM_014305	TGDS	TDP-glucose 4,6-dehydratase	-1.50
NM_017414	USP18	ubiquitin specific peptidase 18	-1.65
NM_003358	UGCG	UDP-glucose ceramide glucosyltransferase	-1.92
NM_003115	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	-1.63
NM_207309	UAP1L1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1	1.49
Response to Stimulus			
NM_005409	CXCL11	chemokine (C-X-C motif) ligand 11	2.45
NM_138410	CMTM7	CKLF-like MARVEL transmembrane domain containing 7	1.45
NM_001993	F3	coagulation factor III (thromboplastin, tissue factor)	-1.41
NM_004417	DUSP1	dual specificity phosphatase 1	-1.70
NM_018948	ERRFI1	ERBB receptor feedback inhibitor 1	-1.51
NM_005347	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.65
NM_014685	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-1.97
NM_006389	HYOU1	hypoxia up-regulated 1	-1.56
NM_001144878	IMPA1	inositol(myo)-1(or 4)-monophosphatase 1	-1.52
NM_002229	JUNB	jun B proto-oncogene	1.57
NM_002276	KRT19	keratin 19	1.46
NM_006010	MANF	mesencephalic astrocyte-derived neurotrophic factor	-1.55
NM_001130487	NDRG4	NDRG family member 4	1.62
NM_000603	NOS3	nitric oxide synthase 3 (endothelial cell)	1.53
NM_001122	PLIN2	perilipin 2	1.35

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Gene ID	Gene Symbol	Gene Name	Fold Change
Response to Stimulus continued			
NM_001128304	PLSCR4	phospholipid scramblase 4	-1.72
NM_000965	RARB	retinoic acid receptor, beta	-2.06
NM_003155	STC1	stanniocalcin 1	-2.33
NM_006528	TFPI2	tissue factor pathway inhibitor 2	-2.12
NM_000361	THBD	thrombomodulin	-1.81
NR_024168	TLR4	toll-like receptor 4	-1.57
NM_003234	TFRC	transferrin receptor (p90, CD71)	-2.34
NM_001078	VCAM1	vascular cell adhesion molecule 1	-2.55
Signal Transduction			
NM_014391	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	-1.58
NM_003567	BCAR3	breast cancer anti-estrogen resistance 3	1.34
NM_173515	CNKSR3	CNKSR family member 3	-2.62
NM_001014796	DDR2	discoidin domain receptor tyrosine kinase 2	-1.82
NM_015221	DNMBP	dynamin binding protein	-1.46
NM_014800	ELMO1	engulfment and cell motility 1	-1.52
NM_138445	GPR146	G protein-coupled receptor 146	1.64
NM_001945	HBEGF	heparin-binding EGF-like growth factor	-1.86
NM_003866	INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa	-1.40
NM_018945	PDE7B	phosphodiesterase 7B	-1.57
NM_025179	PLXNA2	plexin A2	-1.74
NM_006255	PRKCH	protein kinase C, eta	-1.52
NM_006266	RALGDS	ral guanine nucleotide dissociation stimulator	1.37
NM_015149	RGL1	ral guanine nucleotide dissociation stimulator-like 1	-1.79
NM_007211	RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	-1.76
NM_001102445	RGS4	regulator of G-protein signalling 4	-1.82
NM_024605	ARHGAP10	Rho GTPase activating protein 10	-1.52
NM_199282	ARHGAP27	Rho GTPase activating protein 27	1.49
NM_001128615	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	1.56
NM_005841	SPRY1	sprouty homolog 1, antagonist of FGF signalling (Drosophila)	-2.34
NM_005842	SPRY2	sprouty homolog 2 (Drosophila)	-1.87
NM_030964	SPRY4	sprouty homolog 4 (Drosophila)	-1.56
NM_181784	SPRED2	sprouty-related, EVH1 domain containing 2	-1.42
NM_005078	TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	1.36
Transcription			
NM_024997	ATF7IP2	activating transcription factor 7 interacting protein 2	-1.81
NM_014109	ATAD2	ATPase family, AAA domain containing 2	-1.94
NM_001200	BMP2	bone morphogenetic protein 2	-2.03
NM_006763	BTG2	BTG family, member 2	1.49
NM_080759	DACH1	dachshund homolog 1 (Drosophila)	-1.67
NM_004454	ETV5	ets variant 5	-1.83
NM_016270	KLF2	Kruppel-like factor 2 (lung)	1.58
NM_004235	KLF4	Kruppel-like factor 4 (gut)	1.59
NM_014368	LHX6	LIM homeobox 6	1.58

Table S3.5: List of differentially regulated genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 360 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transcription continued			
NM_030915	LBH	limb bud and heart development homolog (mouse)	-1.58
NM_005583	LYL1	lymphoblastic leukemia derived sequence 1	1.46
NM_032246	MEX3B	mex-3 homolog B (C. elegans)	1.36
NM_016132	MYEF2	myelin expression factor 2	-1.39
NM_205860	NR5A2	nuclear receptor subfamily 5, group A, member 2	-1.87
NM_001080951	PLAGL1	pleiomorphic adenoma gene-like 1	-1.50
NM_001198	PRDM1	PR domain containing 1, with ZNF domain	-2.53
NM_003082	SNAPC1	small nuclear RNA activating complex, polypeptide 1, 43kDa	-1.77
NM_005686	SOX13	SRY (sex determining region Y)-box 13	1.66
NM_022117	TSPYL2	TSPY-like 2	-1.42
NM_016206	VGLL3	vestigial like 3 (Drosophila)	-1.61
Transport			
NM_022735	ACBD3	acyl-Coenzyme A binding domain containing 3	-1.42
NM_014570	ARFGAP3	ADP-ribosylation factor GTPase activating protein 3	-1.47
NM_001113738	ARL17P1	ADP-ribosylation factor-like 17 pseudogene 1	-1.80
NM_170665	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-1.75
NM_020453	ATP10D	ATPase, class V, type 10D	-1.58
NM_207628	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.45
NM_198098	AQP1	aquaporin 1 (Colton blood group)	1.98
NR_026745	COG6	component of oligomeric golgi complex 6	-1.68
NM_007235	XPOT	exportin, tRNA (nuclear export receptor for tRNAs)	-1.42
NM_016072	GOLT1B	golgi transport 1 homolog B (S. cerevisiae)	-2.02
NM_006855	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	-1.42
NM_022113	KIF13A	kinesin family member 13A	-1.59
NM_003826	NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	-1.48
NM_001025579	NDEL1	nudE nuclear distribution gene E homolog (A. nidulans)-like 1	-1.46
NM_138444	KCTD12	potassium channel tetramerisation domain containing 12	1.48
NM_198686	RAB15	RAB15, member RAS onocogene family	-1.34
NM_004580	RAB27A	RAB27A, member RAS oncogene family	-1.34
NM_006363	SEC23B	Sec23 homolog B (S. cerevisiae)	-1.75
NM_014822	SEC24D	SEC24 family, member D (S. cerevisiae)	-1.72
NM_004170	SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	-1.64
NM_003038	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	-1.50
NM_003043	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	1.64
NM_003045	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	-1.70

Table S3.5: List of differentially regulated genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 360 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Transport continued			
NM_003982	SLC7A7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	-1.51
NM_001029998	SLC10A7	solute carrier family 10 (sodium/bile acid cotransporter family), member 7	-1.37
NM_001046	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	-1.45
NM_030780	SLC25A32	solute carrier family 25, member 32	-1.45
NM_004733	SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1	-1.78
NM_005827	SLC35B1	solute carrier family 35, member B1	-1.45
NM_001001479	SLC35E4	solute carrier family 35, member E4	1.43
NM_001128431	SLC39A14	solute carrier family 39 (zinc transporter), member 14	-2.54
NM_013306	SNX15	sorting nexin 15	1.30
NM_139164	STARD4	StAR-related lipid transfer (START) domain containing 4	-1.62
Miscellaneous / Other			
NM_015379	BRI3	brain protein I3	1.41
NM_015916	CALHM2	calcium homeostasis modulator 2	1.37
NM_001839	CNN3	calponin 3, acidic	-1.44
NM_001802	CDR2	cerebellar degeneration-related protein 2, 62kDa	-1.45
NM_198545	C1orf187	chromosome 1 open reading frame 187	1.68
NM_173552	C3orf58	chromosome 3 open reading frame 58	-1.34
NM_024315	C7orf23	chromosome 7 open reading frame 23	-1.55
NM_020130	C8orf4	chromosome 8 open reading frame 4	1.56
NM_017782	C10orf18	chromosome 10 open reading frame 18	-1.47
NM_017787	C10orf26	chromosome 10 open reading frame 26	1.33
NM_024942	C10orf88	chromosome 10 open reading frame 88	-1.61
NM_152261	C12orf23	chromosome 12 open reading frame 23	-1.56
NM_020456	C13orf1	chromosome 13 open reading frame 1	-1.40
NM_032933	C18orf45	chromosome 18 open reading frame 45	-1.67
NM_144492	CLDN14	claudin 14	1.42
NM_138357	CCDC109A	coiled-coil domain containing 109A	-1.44
NM_030627	CPEB4	cytoplasmic polyadenylation element binding protein 4	-1.56
NM_020939	CPNE5	copine V	1.38
NM_178470	DCAF12L1	DDB1 and CUL4 associated factor 12-like 1	-1.42
NM_006773	DDX18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	-1.54
NM_014787	DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6	-1.62
NM_020354	ENTPD7	ectonucleoside triphosphate diphosphohydrolase 7	-1.56
NM_014600	EHD3	EH-domain containing 3	-3.84
NM_198947	FAM111B	family with sequence similarity 111, member B	-1.70
NM_001130025	FAM115C	family with sequence similarity 115, member C	-1.81
NM_024785	FAM124B	family with sequence similarity 124B	1.58
AF151906	FAM18B	family with sequence similarity 18, member B	-1.76
NM_153711	FAM26E	family with sequence similarity 26, member E	-1.71
NM_033387	FAM78A	family with sequence similarity 78, member A	1.34
NM_144963	FAM91A1	family with sequence similarity 91, member A1	-1.46
NM_144963	FAM91A1	family with sequence similarity 91, member A1	-1.59

Table S3.5: List of differentially regulated genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 360 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Miscellaneous / Other continued			
NM_058229	FBXO32	F-box protein 32	-1.98
NM_032892	FRMD5	FERM domain containing 5	-1.39
NM_180989	GPR180	G protein-coupled receptor 180	-1.57
NM_006948	HSPA13	heat shock protein 70kDa family, member 13	-1.73
NM_152230	IPMK	inositol polyphosphate multikinase	-1.52
NM_022347	IFRG15	interferon responsive gene 15	-1.62
NM_020888	KIAA1522	KIAA1522	1.39
NM_009587	LGALS9	lectin, galactoside-binding, soluble, 9	1.65
NM_001040078	LGALS9C	lectin, galactoside-binding, soluble, 9C	1.59
NM_015116	LRCH1	leucine-rich repeats and calponin homology (CH) domain containing 1	-1.90
NM_001134476	LRRC8B	leucine rich repeat containing 8 family, member B	-2.61
NM_198565	LRRC33	leucine rich repeat containing 33	1.56
NM_019556	MOSPD1	motile sperm domain containing 1	-1.45
NM_017762	MTMR10	myotubularin related protein 10	-1.66
NM_018177	N4BP2	NEDD4 binding protein 2	-1.67
NM_144573	NEXN	nexilin (F actin binding protein)	-2.31
NM_198514	NHLRC2	NHL repeat containing 2	-1.56
NM_001031701	NT5DC3	5'-nucleotidase domain containing 3	-1.62
NM_005013	NUCB2	nucleobindin 2	-1.64
NM_024576	OGFRL1	opioid growth factor receptor-like 1	-1.57
NM_020651	PELI1	pellino homolog 1 (Drosophila)	-1.75
NM_021255	PELI2	pellino homolog 2 (Drosophila)	1.37
NM_001100164	PHACTR2	phosphatase and actin regulator 2	-1.73
NM_015993	PLLP	plasma membrane proteolipid (plasmolipin)	2.20
NM_153026	PRICKLE1	prickle homolog 1 (Drosophila)	-1.38
NM_130809	PRRC1	proline-rich coiled-coil 1	-1.35
NM_153020	RBM24	RNA binding motif protein 24	1.50
NM_014016	SACM1L	SAC1 suppressor of actin mutations 1-like (yeast)	-1.53
NM_001104587	SLFN11	schlafen family member 11	-2.24
NM_144682	SLFN13	schlafen family member 13	-1.46
NM_004657	SDPR	serum deprivation response (phosphatidylserine binding protein)	-1.33
NM_024745	SHCBP1	SHC SH2-domain binding protein 1	-1.47
NM_022648	TNS1	tensin 1	1.29
NM_015914	TXNDC11	thioredoxin domain containing 11	-1.30
NR_002816	THSD1P	thrombospondin, type I, domain containing 1 pseudogene	1.45
NM_138786	TM4SF18	transmembrane 4 L six family member 18	-1.37
NM_175861	TMTC1	transmembrane and tetratricopeptide repeat containing 1	-1.66
NM_152588	TMTC2	transmembrane and tetratricopeptide repeat containing 2	-1.46
NM_013390	TMEM2	transmembrane protein 2	-1.84
NM_018266	TMEM39A	transmembrane protein 39A	-1.55
NM_031442	TMEM47	transmembrane protein 47	-1.38
NM_006134	TMEM50B	transmembrane protein 50B	-2.53

Table S3.5: List of differentially regulated genes in HUVECs after a six hour treatment of 1 µM apple procyanidin fraction dp 4-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 360 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Miscellaneous / Other continued			
NM_145041	TMEM106A	transmembrane protein 106A	1.40
NM_175748	UBR7	ubiquitin protein ligase E3 component n-recognin 7 (putative)	1.55
NM_173834	YIPF6	Yip1 domain family, member 6	-1.49
NM_001005404	YPEL2	yippee-like 2 (Drosophila)	-1.46
NM_080660	ZC3HAV1L	zinc finger CCCH-type, antiviral 1-like	-1.45
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Table S3.6: List of differentially expressed genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) ($p < 0.001$; 131 genes).

Gene ID	Gene Abbreviation	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	-1.82
NM_005429	VEGFC	vascular endothelial growth factor C	-1.36
Apoptosis			
NM_005460	SNCAIP	synuclein, alpha interacting protein	-1.63
NM_003810	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.09
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	1.57
NM_018003	UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	-1.56
Binding / Cell Adhesion			
NM_001130861	CLDN5	claudin 5	1.76
NM_024711	GIMAP6	GTPase, IMAP family member 6	1.45
NM_022143	LRRC4	leucine rich repeat containing 4	1.38
Cell Communication			
NM_019074	DLL4	delta-like 4 (Drosophila)	1.55
NM_018431	DOK5	docking protein 5	-1.77
NM_014421	DKK2	dickkopf homolog 2 (Xenopus laevis)	-1.56
NM_207123	GAB1	GRB2-associated binding protein 1	-1.59
NM_012302	LPHN2	latrophilin 2	-2.05
NM_021724	NR1D1	nuclear receptor subfamily 1, group D, member 1	1.61
NM_005065	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	-1.71
Cell Cycle / Cell Proliferation			
NM_006988	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-1.56
NM_003672	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	-1.55
NM_015147	CEP68	centrosomal protein 68kDa	1.71
NM_015097	CLASP2	cytoplasmic linker associated protein 2	-1.70
NM_001946	DUSP6	dual specificity phosphatase 6	-1.44
NM_005524	HES1	hairy and enhancer of split 1, (Drosophila)	1.64
NM_018116	MSTO1	misato homolog 1 (Drosophila)	-1.51
NM_001142393	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	1.39
NM_153618	SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	-3.64
NM_002688	SEPT5	septin 5	-1.46
NM_145728	SYNM	synemin, intermediate filament protein	-1.49
NM_006472	TXNIP	thioredoxin interacting protein	1.56
NM_000222	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	1.65
Development			
NM_014888	FAM3C	family with sequence similarity 3, member C	-1.66
NM_006350	FST	follistatin	-2.10
NM_005266	GJA5	gap junction protein, alpha 5, 40kDa	2.64
NM_002060	GJA4	gap junction protein, alpha 4, 37kDa	3.87
NM_001002295	GATA3	GATA binding protein 3	1.42

Table S3.6: List of differentially expressed genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 131 genes).

Gene ID	Gene Abbreviation	Gene Name	Fold Change
Development continued			
NM_003250	THRA	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)	1.41
DNA Binding / Replication / Repair / Transcription			
NM_006734	HIVEP2	human immunodeficiency virus type I enhancer binding protein 2	-1.74
NM_021038	MBNL1	muscleblind-like (Drosophila)	-1.39
NM_025081	KIAA1305	KIAA1305	1.45
Immune Response			
NM_005080	XBP1	X-box binding protein 1	-1.41
Metabolic / Biosynthetic Process			
NM_022060	ABHD4	abhydrolase domain containing 4	-1.35
NM_003654	CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	1.53
NM_015892	CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	1.57
NM_015892	CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	1.66
NM_032385	C5orf4	chromosome 5 open reading frame 4	1.53
NM_000499	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	2.03
NM_013352	DSE	dermatan sulfate epimerase	-1.50
NM_152879	DGKD	diacylglycerol kinase, delta 130kDa	-1.62
NM_012328	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	-2.34
NM_006260	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	-1.91
NM_014674	EDEM1	ER degradation enhancer, mannosidase alpha-like 1	-1.79
NM_019891	ERO1LB	ERO1-like beta (S. cerevisiae)	-2.46
NM_004836	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	-1.76
NM_007076	FICD	FIC domain containing	-1.59
NM_000158	GBE1	glucan (1,4-alpha-), branching enzyme 1	-1.54
NM_002056	GFPT1	glutamine-fructose-6-phosphate transaminase 1	-1.82
NM_030792	GDPD5	glycerophosphodiester phosphodiesterase domain containing 5	1.41
NM_018192	LEPREL1	leprecan-like 1	-1.71
NM_001080424	KDM6B	lysine (K)-specific demethylase 6B	1.60
NM_001144967	NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	-1.44
NM_004414	RCAN1	regulator of calcineurin 1	-2.25
NM_014746	RNF144A	ring finger protein 144A	1.76
NM_006588	SULT1C4	sulfotransferase family, cytosolic, 1C, member 4	-2.16
NM_032431	SYVN1	synovial apoptosis inhibitor 1, synoviolin	-1.36
Response to Stimulus			
NM_000014	A2M	alpha-2-macroglobulin	-1.75
NM_005409	CXCL11	chemokine (C-X-C motif) ligand 11	3.22
NM_001993	F3	coagulation factor III (thromboplastin, tissue factor)	-1.49
NM_005347	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.51
NM_014685	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-1.85

Table S3.6: List of differentially expressed genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 131 genes).

Gene ID	Gene Abbreviation	Gene Name	Fold Change
Response to Stimulus continued			
NM_006389	HYOU1	hypoxia up-regulated 1	-1.48
NM_006010	MANF	mesencephalic astrocyte-derived neurotrophic factor	-1.65
NM_000603	NOS3	nitric oxide synthase 3 (endothelial cell)	1.36
NM_001122	PLIN2	perilipin 2	1.40
NM_003155	STC1	stanniocalcin 1	-3.05
NM_000361	THBD	thrombomodulin	-2.11
NM_006528	TFPI2	tissue factor pathway inhibitor 2	-1.97
NR_024168	TLR4	toll-like receptor 4	-1.36
NM_001078	VCAM1	vascular cell adhesion molecule 1	-2.13
Signal Transduction			
NM_004041	ARRB1	arrestin, beta 1	1.42
NM_173515	CNKSR3	CNKSR family member 3	-2.16
NM_001014796	DDR2	discoidin domain receptor tyrosine kinase 2	-1.72
NM_138445	GPR146	G protein-coupled receptor 146	1.89
NM_032553	GPR174	G protein-coupled receptor 174	1.37
NM_001945	HBEGF	heparin-binding EGF-like growth factor	-1.71
NM_006186	NR4A2	nuclear receptor subfamily 4, group A, member 2	-1.53
NM_006266	RALGDS	ral guanine nucleotide dissociation stimulator	1.44
NM_007211	RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	-1.64
NM_016084	RASD1	RAS, dexamethasone-induced 1	-1.44
NM_024605	ARHGAP10	Rho GTPase activating protein 10	-1.59
NM_001010000	ARHGAP28	Rho GTPase activating protein 28	1.71
NM_015149	RGL1	ral guanine nucleotide dissociation stimulator-like 1	-1.64
Transcription			
NM_001200	BMP2	bone morphogenetic protein 2	-1.78
NM_014368	LHX6	LIM homeobox 6	1.49
NM_030915	LBH	limb bud and heart development homolog (mouse)	-1.80
NM_002397	MEF2C	myocyte enhancer factor 2C	-1.79
NM_205860	NR5A2	nuclear receptor subfamily 5, group A, member 2	-1.83
NM_001198	PRDM1	PR domain containing 1, with ZNF domain	-2.38
Transport			
NM_170665	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-1.56
NM_020453	ATP10D	ATPase, class V, type 10D	-1.75
NM_198098	AQP1	aquaporin 1 (Colton blood group)	2.19
NM_022113	KIF13A	kinesin family member 13A	-1.73
NM_138444	KCTD12	potassium channel tetramerisation domain containing 12	1.69
NM_014822	SEC24D	SEC24 family, member D (S. cerevisiae)	-1.63
NM_006363	SEC23B	Sec23 homolog B (S. cerevisiae)	-1.47
NM_003045	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	-1.53
NM_001046	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	-1.70
NM_004733	SLC33A1	solute carrier family 33 (acetyl-CoA transporter), member 1	-1.72

Table S3.6: List of differentially expressed genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to VEGF (10 ng/ml) (*p* < 0.001*;* 131 genes).

Gene ID	Gene Abbreviation	Gene Name	Fold Change
Transport continued			
NM_001128431	SLC39A14	solute carrier family 39 (zinc transporter), member 14	-2.47
NM_013306	SNX15	sorting nexin 15	1.34
Miscellaneous / Other			
NM_001613	ACTA2	actin, alpha 2, smooth muscle, aorta	1.59
NM_032933	C18orf45	chromosome 18 open reading frame 45	-1.51
NM_058187	C21orf63	chromosome 21 open reading frame 63	1.42
NM_020939	CPNE5	copine V	1.41
NM_030627	CPEB4	cytoplasmic polyadenylation element binding protein 4	-1.62
NM_014787	DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6	-1.53
NM_020354	ENTPD7	ectonucleoside triphosphate diphosphohydrolase 7	-1.54
NM_014600	EHD3	EH-domain containing 3	-3.28
NM_033387	FAM78A	family with sequence similarity 78, member A	1.50
NM_001130025	FAM115C	family with sequence similarity 115, member C	-1.64
NM_024785	FAM124B	family with sequence similarity 124B	1.57
NM_015687	FILIP1	filamin A interacting protein 1	1.51
NR_024042	GUCY2E	guanylate cyclase 2E	1.63
NM_006948	HSPA13	heat shock protein 70kDa family, member 13	-1.54
NM_001134476	LRRC8B	leucine rich repeat containing 8 family, member B	-2.01
NM_015993	PLLP	plasma membrane proteolipid (plasmolipin)	2.14
NM_001104587	SLFN11	schlafen family member 11	-2.00
NM_144682	SLFN13	schlafen family member 13	-1.46
NR_002816	THSD1P	thrombospondin, type I, domain containing 1 pseudogene	1.48
NM_013390	TMEM2	transmembrane protein 2	-1.83
NM_006134	TMEM50B	transmembrane protein 50B	-1.88
NM_015271	TRIM2	tripartite motif-containing 2	-1.48

Table S3.7: List of differentially expressed genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to apple procyanidin fraction dp 4 (1 µM) ($p < 0.001$; 37 genes).

Gene ID	Gene Symbol	Gene Name	Fold Change
Angiogenesis			
NM_001147	ANGPT2	angiopoietin 2	1.70
NM_001202	BMP4	bone morphogenetic protein 4	1.81
NM_005924	MEOX2	mesenchyme homeobox 2	1.64
NM_005429	VEGFC	vascular endothelial growth factor C	-1.38
Apoptosis			
NM_182757	RNF144B	ring finger protein 144B	1.63
NM_005092	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	1.95
Binding / Cell Adhesion			
NM_002982	CCL2	chemokine (C-C motif) ligand 2	1.43
NM_024787	RNF122	ring finger protein 122	-1.49
Cell Communication			
NM_017413	APLN	apelin	1.63
NM_015191	SIK2	salt-inducible kinase 2	-1.43
Cell Cycle / Cell Proliferation			
NM_001946	DUSP6	dual specificity phosphatase 6	1.44
Development			
NM_006079	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	1.42
NM_012242	DKK1	dickkopf homolog 1 (Xenopus laevis)	2.05
NM_001002295	GATA3	GATA binding protein 3	1.51
NM_182964	NAV2	neuron navigator 2	-1.69
DNA Binding / Replication / Repair / Transcription			
NM_004956	ETV1	ets variant 1	1.65
NM_005378	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-1.64
Inflammatory Response			
NM_002852	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	1.67
Immune Response			
NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	-1.39
Metabolic / Biosynthetic Process			
NM_005099	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	-1.58
NM_018371	CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	1.50
NM_001097634	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	1.64
NM_001159323	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	-1.51
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-1.79
NM_001142298	SQSTM1	sequestosome 1	-1.41
Signal Transduction			
NM_014391	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	1.51
NM_015368	PANX1	pannexin 1	-1.42
NM_005842	SPRY2	sprouty homolog 2 (Drosophila)	1.60

Table S3.7: List of differentially regulated genes in HUVECs after a six hour treatment of 500 ng/ml anti-VEGF antibody-treated VEGF (10 ng/ml) compared to apple procyanidin fraction dp 4 (1 µM) (*p* < 0.001*;* 37 genes) continued.

Gene ID	Gene Symbol	Gene Name	Fold Change
Signal Transduction continued			
NM_005841	SPRY1	sprouty homolog 1, antagonist of FGF signalling (Drosophila)	1.72
Transcription			
NM_004454	ETV5	ets variant 5	1.90
NM_002397	MEF2C	myocyte enhancer factor 2C	-1.56
NM_022157	RRAGC	Ras-related GTP binding C	-1.47
NM_020245	TULP4	tubby like protein 4	-1.37
NM_152320	ZNF641	zinc finger protein 641	1.53
Transport			
NM_003043	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	-1.70
NM_003046	SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	-1.78
Miscellaneous / Other			
NM_004657	SDPR	serum deprivation response (phosphatidylserine binding protein)	1.35